GPER AND HUMAN PATHOLOGIES

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GPER AND HUMAN PATHOLOGIES

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EDITORIAL
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Editorial: GPER and Human Pathologies

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Editorial on the Research Topic

GPER and Human Pathologies

More than 40% of commercialized drugs exert their action through G protein-coupled receptors (GPCRs), indicating that the modulation of these hepta-transmembrane proteins is important for the control of downstream signaling pathways and related diseases. Twenty-five years ago, a new orphan GPCR initially named CMKRL2 was discovered by Owman et al. (1). This receptor was named CMKRL2 as it was shown high degree of identity and consensus features with chemoattractant receptors, in activated B cells isolated from Burkitt's lymphoma (1). Later, it was characterized by Carmeci et al. in breast cancer cells and claimed to participate in hormonal response (2). Thus, it was renamed GPR30 and, later, GPER (for G protein-coupled estrogen receptor), as it was demonstrated to bind estradiol. Since then, its role in different physiological systems and diseases has been revealed, particularly in metabolic disorders, cancer, immunity and inflammation, cardiovascular function as well as the brain. Its role in metabolic disease and cancer is highlighted by Rouhimoghadam et al. In connection with the second point, implications of GPER for anti-estrogen therapy are indisputable. In addition, epidemiologic studies indicate its potential as a valuable prognostic factor, particularly in the context of cancer. For example, high expression of GPER and DKK2 correlates with survival in epithelial ovarian cancer, as demonstrated by Fraungruber et al. The fact that the first peptidic GPER modulator ERα17p, an inverse agonist which has been designed from estrogen receptor α (3), exerts both anti-proliferative (4) and anti-nociceptive actions at similar *in vivo* doses, reflects not only the multifaceted character of GPER but also the possibility to simultaneously treat through a same target two or more pathologies (Mallet et al.).

In the light of the above considerations, Kim and Jung propose that interfering with GPER could open new avenues for the development of original therapeutic approaches devoted to the control of tumor growth. These authors have demonstrated that chrysin-nanoparticles inhibit the growth of triple negative breast tumors and associated metastasis in xenografted mice. Even if GPER signaling is predominantly estradiol-dependent, its control could be extended also to males with testicular germ cell cancers, as discussed by Chevalier et al. In fact, GPER is expressed in most malignant diseases such as melanoma, breast, pancreatic, prostate, colorectal and hepatocellular cancers. It shares also important functions in immune responses, as clearly stated by Notas et al., with a role in autoimmune pathologies such as multiple sclerosis, Parkinson's disease and atherosclerosis-related inflammation. Hernández-Silva et al. demonstrate that GPER interferes in the development and

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immune response in female reproductive cancers. In this regard, it should be stressed that the GPER inverse agonist ERα17p shares, additionally to its antiproliferative and anti-nociceptive profiles, anti-inflammatory action (see Mallet et al.). These observations are closely related to the expression of GPER in B and T lymphocytes, monocytes, eosinophils and neutrophils. In the cardiovascular compartment, chronic activation of GPER protects against oxidative stress-induced cardiomyoblast death (Imam Aliagan et al.). Also present in the digestive system, GPER could be of interest in the control of the gallstone formation, a major hepatobiliary disease with a higher prevalence in women than in men, or of irritable bowel syndrome, inflammatory bowel diseases and colorectal cancer (DeLeon et al. and Jacenik and Krajewska). The issues addressed in this Research Topic reveal a key role for GPER in a panel of pathologies. Even if the number of GPER modulators is limited, its control could undoubtedly open new exciting medical approaches.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Owman C, Blay P, Nilsson C, Lolait SJ. Cloning of Human cDNA Encoding a Novel Heptahelix Receptor Expressed in Burkitt's Lymphoma and Widely Distributed in Brain and Peripheral Tissues. *Biochem Biophys Res Commun* (1996) 228:285–92. doi: 10.1006/bbrc.1996.1654
2. Carmeci C, Thompson DA, Ring HZ, Francke U, Weigel RJ. Identification of a Gene (GPR30) with Homology to the G-Protein-Coupled Receptor Superfamily Associated with Estrogen Receptor Expression in Breast Cancer. *Genomics* (1997) 45:607–17.
3. Jacquot Y, Gallo D, Leclercq G. Estrogen Receptor Alpha - Identification by a Modelling Approach of a Potential Polyproline II Recognizing Domain Within the AF-2 Region of the Receptor That Would Play a Role of Prime Importance in Its Mechanism of Action. *J Steroid Biochem Mol Biol* (2007) 104:1–10. doi: 10.1016/j.jsbmb.2006.10.008
4. Pelekanou V, Kampa M, Gallo D, Notas G, Troullinaki M, Duvillier H, et al. The Estrogen Receptor Alpha-Derived Peptide ERα17p (P(295)-T(311)) Exerts Pro-Apoptotic Actions in Breast Cancer Cells *In Vitro* and *In Vivo*, Independently From Their ERα Status. *Mol Oncol* (2011) 5:36–47. doi: 10.1016/j.molonc.2010.11.001

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MINI REVIEW
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Significance of G Protein-Coupled Estrogen Receptor in the Pathophysiology of Irritable Bowel Syndrome, Inflammatory Bowel Diseases and Colorectal Cancer

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The regulatory role of estrogens and nuclear estrogen receptors, i. e., estrogen receptor α and β has been reported in gastrointestinal diseases. However, the contribution of G protein-coupled estrogen receptor, the membrane-bound estrogen receptor, is still poorly understood. Unlike nuclear estrogen receptors, which are responsible for the genomic activity of estrogens, the G protein-coupled estrogen receptor affects the "rapid" non-genomic activity of estrogens, leading to modulation of many signaling pathways and ultimately changing gene expression. Recently, the crucial role of G protein-coupled estrogen receptor in intestinal pathogenesis has been documented. It has been shown that the G protein-coupled estrogen receptor can modulate the progression of irritable bowel syndrome, inflammatory bowel diseases such as Crohn's disease and ulcerative colitis as well as colorectal cancer. The G protein-coupled estrogen receptor appears to be a potent factor regulating abdominal sensitivity and pain, intestinal peristalsis, colitis development, proliferation and migration potential of colorectal cancer cells and seems to be a useful target in gastrointestinal diseases. In this review, we present the current state of knowledge about the contribution of the G protein-coupled estrogen receptor to irritable bowel syndrome, inflammatory bowel diseases and colorectal cancer.

Keywords: G protein-coupled estrogen receptor, irritable bowel syndrome, inflammatory bowel diseases, Crohn's disease, ulcerative colitis, colorectal cancer

INTRODUCTION

The G protein-coupled estrogen receptor (GPER, previously known as GPR30) is a seven-transmembrane receptor discovered, among others, in breast cancer tissue and estrogen receptor-positive MCF-7 cell line (1–7). In addition to acting *via* nuclear estrogen receptors (ERs), i.e., ERα and ERβ, estrogens have been reported to induce ligand-dependent signaling by the membrane-bound estrogen receptor named GPER. In contrast to nuclear ERs, which predominantly regulate expression of target genes through direct interaction with estrogen response element or indirectly through transcription factors, GPER is responsible for "rapid" non-genomic activity of estrogens, leading to modulation of many signaling pathways and ultimately gene expression. Studies of the mechanisms underlying the effects of GPER under physiological and pathological conditions

have shown that activation of GPER leads to stimulation of signaling pathways dependent on both $G\alpha_s$ and $G\beta\gamma$ proteins. Epidermal growth factor receptor (EGFR), mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC) and adenylyl cyclase (AC) are the main GPER-dependent pathways regulated by the action of G proteins (8–12). It was also found that GPER is capable to affect nuclear factor-κB (NF-κB) and Notch as well as Hippo signaling, where the membrane-bound estrogen receptor regulates phosphorylation of crucial proteins through $G\alpha_{q-11}$ action, enhancing the proliferation and migration potential of breast cancer cells (11, 13, 14). GPER action appears unrelated to G protein-independent signals such as β-arrestin recruitment (15, 16). Beyond 17β-estradiol, which is a natural agonist of GPER, various ligands have an affinity for this estrogen receptor (**Figure 1**). Among them there are therapeutic agents that belong to the classes of selective nuclear ER modulators (e.g., tamoxifen), selective nuclear ER down-regulators (e.g., fulvestrant), and xenoestrogens (e.g., atrazine, bisphenol A, genistein and quarcetin) as well as synthetic GPER selective agonist (i.e., G-1) and antagonists (i.e., G15 and G36) (17). All synthetic GPER selective ligands are based on the tetrahydro-3H-cyclopenta[c]quinoline scaffold, but the GPER antagonist G15 is characterized by the absence of ethanone moiety compared to the GPER agonist G-1, while the second GPER antagonist, i.e., G36, has an isopropyl moiety in the place of the ethanone moiety at position C8.

GPER expression is not restricted to estrogen responsive tissues. GPER is widely expressed in human tissues, including breast, ovaries, uterus, placenta, testis, prostate, bone marrow, thymus, bones, smooth and skeletal muscles, brain, blood vessels, heart, lung, liver and intestine (18). Considerable evidence suggests that GPER is essential in cardiovascular diseases, obesity, diabetes, immune disorders and infectious diseases as well as in neoplastic transformation and tumor progression (17, 19, 20). In this review, we summarize the evidence for GPER expression and function in the pathophysiology of intestinal diseases, i.e., irritable bowel syndrome, inflammatory bowel diseases and colorectal cancer.

IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) is a common, functional gastrointestinal disorder that is manifested by abdominal pain and bowel habit disturbance. From a clinical point of view, IBS is divided into subtypes related to changes in gastrointestinal motility, i.e., diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C) and IBS with altered bowel habits (IBS-A or IBS-M). The pathophysiology of IBS is still elusive, but several factors such as microbiota, environmental and genetic variations, seem to be responsible for the development of IBS.

The first evidence suggesting the importance of GPER in IBS was revealed by Qin et al. (21) who evaluated the expression of estrogen receptor genes in the intestine of IBS patients. Higher expression of GPER at mRNA level in the intestine of IBS-D patients in relation to IBS-C patients and healthy subjects was documented. Jacenik et al. (22) also observed overexpression of GPER at the level of mRNA in colonic tissue, but in both IBS-C and IBS-D patients. However, after taking into account the sex and age of the patients, statistically significant overexpression of GPER at mRNA level was observed only in men with IBS-D, suggesting a gander-specific role of estrogen signaling through this estrogen receptor in the progression of functional bowel diseases.

There are several hypotheses regarding the mechanisms by which GPER may be engaged in the progression of IBS, one of which is related with mast cell regulation. Mast cells are one of the main cell types involved in the activation of immune response in the gastrointestinal tract. Stimulated mast cells degranulate and release a wide spectrum of mediators such as amines, proteoglycans, proteases and lysosomal enzymes as well as cytokines, which leads to modulation of permeability and regulation of smooth muscle contraction (23). In IBS, alteration of mast cells number or density in the intestine was documented in several independent studies and seems to be associated with IBS pathogenesis (24–26). Although the results of Sundin et al. (27) indicate that there is no change in the infiltration and localization of mast cells in the colonic mucosa of IBS patients compared to healthy individuals, they report evidence indicating a relationship between abdominal sensitivity and mast cell number. GPER seems to be expressed in the tryptase$^+$ mast cells and the expression of GPER in the cytoplasm of mast cells and GPER$^+$ cells was found to be significantly higher in the colonic mucosa of IBS-D patients compared to IBS-C patients and healthy subjects. It should be noted that Qin et al. (21) found a positive correlation between the number of GPER$^+$ cells and the severity of abdominal pain and not the duration of symptoms in the IBS-D patients. The importance of GPER activity modulation on abdominal pain was confirmed in a mouse model. Zielinska et al. (28) found that treatment with GPER agonists, i.e., 17β-estradiol or G-1, is associated with lower pain-induced behaviors in mice treated with mustard oil. In contrast, GPER selective antagonist G15 reduces the positive effect of GPER agonists on pain-induced behaviors.

In vivo studies in which Xu et al. (29) used control, stressed, and ovariectomized (OVX) rats revealed that estrogen receptor ligands acting through GPER are able to regulate visceral hypersensitivity, mast cell degranulation and mast cell tryptase expression as well as histamine levels in rat intestine. It was estimated that rats subjected to wrap partial restraint stress were characterized by increased visceromotor response. In the intestine of stressed rats a higher number of mast cells and up-regulated level of histamine were found. Both effects were reduced when OVX rats were used compared to control rats. Xu et al. (29) observed that the administration of 17β-estradiol led to an increase of visceromotor response, but pre-treatment with the GPER selective antagonist G15 counteracted the enhancing effects in OVX rats. Consistent with this, OVX rats treated with GPER selective agonist G-1 manifested dose-dependent up-regulated visceromotor response levels. It should be noted that in addition to modulating visceral motor response, GPER ligands also affect the expression level of tryptase in mast cells and histamine in the rat intestines, indicating the important role of

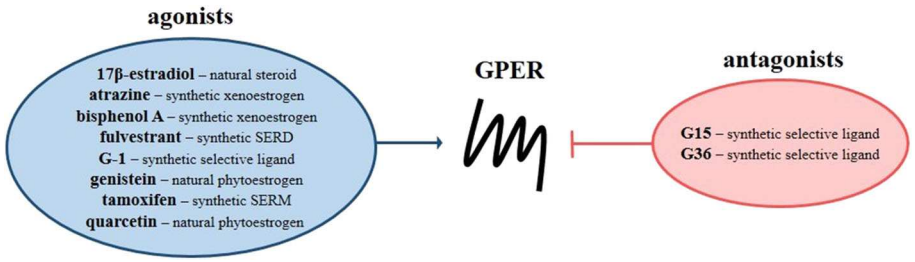

FIGURE 1 | G protein-coupled estrogen receptor ligands. Scheme presents natural and synthetic agonists (blue circle) and antagonists (red circle) of GPER. SERD, selective nuclear estrogen receptor down-regulators; SERM, selective nuclear estrogen receptor modulators.

GPER and mast cells in colon hypersensitivity in the female IBS rat model (29).

Mast cells appear not to be the only cell type involved IBS development. Enteric neurons and enteric glial cells play an essential role in the regulation of gastrointestinal motility, and motility impairment is the main hallmark of functional gastrointestinal diseases, including IBS. It has to be noted that changes in gastrointestinal motility are primarily driven by alteration of enteric nervous system, i.e., complex network of enteric neurons and glia which regulate for instance fluid exchange across the mucosa, blood flow in the intestine and gastrointestinal motility (30, 31). GPER has been shown to be expressed in the neuronal population of cells in the human and mouse intestines (29, 32, 33). Liu et al. (33) by immunofluorescent staining documented that GPER is present in the cytoplasm of enteric neurons and glial cells of the stomach, duodenum, jejunum, ileum and colon of male and female mice.

Two independent studies indicate that modulation of GPER activity affects colonic motility involving both neuronal cells and circular muscle strip contraction (28, 32). It was found that GPER inhibition with the GPER selective antagonist G15 decreases colonic transit time in the proestrus and estrus phases but not diestrus stage compared to untreated phase-matched female mice. It has been proven that 17β-estradiol administration prolonged the colonic transit time while G15 treatment reduced the effect of exogenous estrogen on colonic transit time in OVX mice. *Ex vivo* analysis using colonic circular muscle strips confirmed that GPER activation with the GPER selective agonist G-1 reduced the contractile response of the muscle strips to carbachol and this phenomenon was abolished by tetrodotoxin, suggesting that GPER may act through a neurogenic mechanism. In fact, it has been documented that GPER activation stimulated the release of nitric oxide in myenteric neurons, which was decreased by the nitric oxide synthase inhibitor (32). *In vivo* experiments using colonic bead expulsion test and mouse model of hypermotility carried out by Li et al. (32) and Zielinska et al. (28) have documented that GPER activation prolongs colonic transit time and is associated with lower number of fecal pellets.

Potential mechanisms by which GPER can modulate progression of irritable bowel syndrome are summarized in **Figure 2**.

INFLAMMATORY BOWEL DISEASES

Crohn's disease (CD) and ulcerative colitis (UC) are the major types of inflammatory bowel disease (IBDs) that manifest themselves as chronic intestine inflammation. The most evidence emphasizing estrogen significance in IBDs comes from clinical observations that take into account hormone fluctuations in pre-menopausal and post-menopausal women in the prevalence and symptoms of IBDs (34–36). On the other hand, many reports indicate that estrogen receptors regulate the immune response affecting not only intestinal cells, but also immune cells (37–41). However, the importance of G protein-coupled estrogen receptor in IBDs is still poorly understood, but available data indicate that GPER may be involved in the pathways responsible for progression of IBDs.

It was documented that patients with CD and UC are characterized by a lack of changes, both in the level of circulating 17β-estradiol and enzymes involved in estrogen metabolism in relation to reference values and control group. Nevertheless, alterations in estrogen receptor expression, including GPER were found in the intestine of patients with IBDs (42–44). Significantly higher levels of GPER in the intestine have been demonstrated in both CD and UC patients compared to healthy controls. Higher expression of GPER at the mRNA level was also observed in an independent cohort using dataset provided by Gene Expression Omnibus. Nevertheless, when sex and age of women were taken into consideration, higher expression of GPER at the protein level was found in the intestine of men with both types of IBDs in relation to sex-matched controls. In the case of women, up-regulation of GPER expression in the intestine of women with UC under the age of 50 compared to sex and age-matched controls was noted (43). Interesting evidence was also provided by Włodarczyk et al. (42) who documented differences in GPER expression in non-inflamed and inflamed intestine obtained from patients with CD, but not in patients with UC. Alterations in GPER expression in IBDs suggest that GPER may be involved in the immune response in the progression of colitis in patients with CD and UC.

The functional significance of GPER in CD was provided by *in vivo* studies using trinitrobenzene sulfonic acid (TNBS)-induced CD model in mice (44, 45). It was demonstrated that modulation of GPER activity using estrogen receptor agonists and antagonists affects the development of colitis.

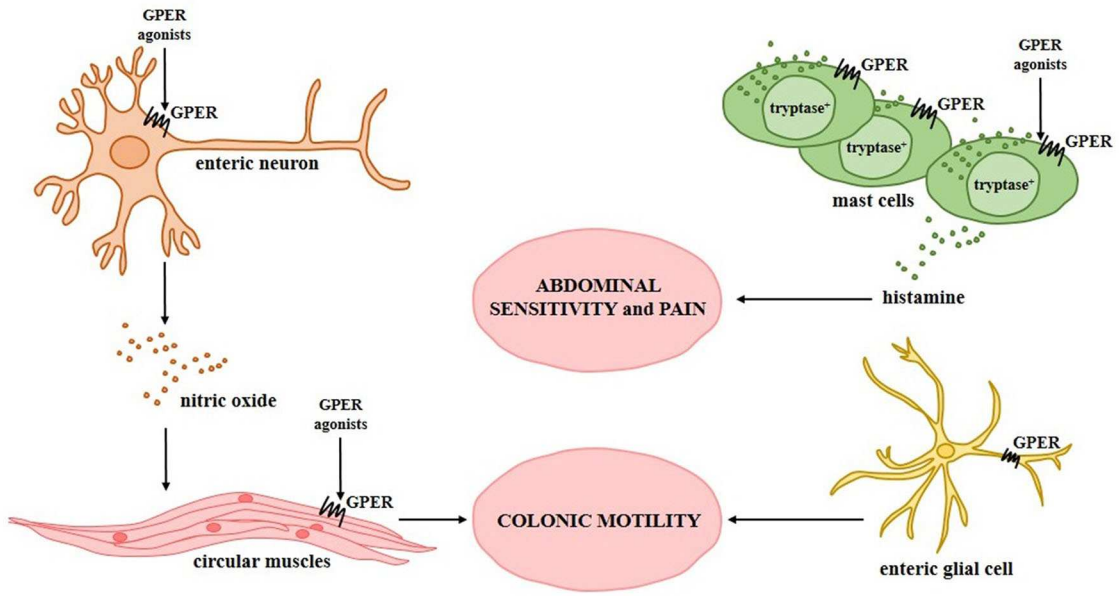

FIGURE 2 | Relationship between G protein-coupled estrogen receptor and irritable bowel syndrome. Experimentally confirmed GPER activity in specific types of intestinal cells during the progression of irritable bowel syndrome and its association with the symptoms observed in patients with irritable bowel syndrome is shown.

GPER activation has been shown to improve macroscopic and microscopic scores as well as reduces the mortality of mice with CD in relation to untreated mice with CD. In contrast, inhibition of GPER by its selective antagonist G15, was found to be not associated with an improvement of the above mentioned parameters and mortality in the murine model of CD. It is worth noting that GPER is overexpressed in the intestine of male mice with induced CD, as in the intestine of men with CD (43, 44). Interestingly, GPER agonists and antagonists affect not only GPER but also nuclear ERs expression and localization. Immunohistochemical analysis revealed that ERα is localized in the cytoplasm of goblet cells in the intestine of control and TNBS-treated mice supplemented with G-1 and 17β-estradiol. In contrast, the lack of ERα expression in the cytoplasm of goblet cells has been documented in the intestine of TNBS-treated and TNBS-treated mice supplemented with GPER selective antagonist. Accumulating body of evidence suggests that estrogen signaling is strictly synchronized and all estrogen receptors play a crucial role in the regulation of several major cellular signaling pathways (20, 46–52). In the intestine cross-talk between estrogen receptors seems to be important of patients with CD and the value of ERα/ERβ ratio in the serum may be useful to predict endoscopic activity in CD patients (53). At the molecular level GPER appears to be engaged in the modulation of signaling pathway of extracellular signal-regulated kinases (ERKs), which leads to changes in expression of immune-related genes which was documented in the intestine of IBDs patients and murine model. It was shown that intestine of mice with CD is characterized by higher expression of immunomodulatory genes and GPER activation significantly reduces the levels of immune-related genes (44). The major signaling pathways induced by GPER in bowel diseases are summarized in **Figure 3**.

COLORECTAL CANCER

Current evidence based on experimental studies and available datasets provided by Gene Expression Omnibus and Oncomine documented lower expression of GPER at the mRNA and protein levels in the intestine of colorectal cancer (CRC) patients compared to adjacent control tissue (11). Moreover, a gradual decrease of GPER expression appears to be associated with CRC stage and lymph node metastasis in CRC patients (11). Liu et al. (11) provided evidence indicating that promoter methylation and histone H3 deacetylation represent mechanisms responsible for regulation of GPER expression in CRC. Conflicting results regarding the clinical relevance of GPER expression in CRC patients are from Kaplan-Meier analysis (11, 54, 55). Lower expression of GPER in the intestine of CRC patients seems to be associated with poorer survival rate in relation to CRC patients with high intestinal expression of GPER (11). On the other hand, Bustos et al. (54) reported that the survival is affected by the sexual dimorphism of CRC patients. Higher expression of GPER has been shown to be associated with poor relapse-free survival in women with stage 3 and 4 but not stage 1 and 2 CRC or men regardless of stage.

The first studies highlighting the role of estrogen signaling through GPER in CRC were conducted by Santolla et al. (56) who found a link between GPER and fatty acid synthase (FASN) in the neoplastic transformation of colon. FASN is a key

lipogenic enzyme which is able to act as a metabolic oncogene in several types of cancer, such as breast and colorectal cancer (57–59). Functional cross-talk between GPER activation, FASN expression and CRC cells proliferation and migration has been documented. *In vitro* studies using GPER agonists and LoVo cells have revealed that GPER by affecting the EGFR/ERK/c-Fos/AP1 signaling pathway is responsible for regulation of FASN expression and modulates the potential for CRC cells proliferation and migration (56). In studies covering a wide spectrum of potential mechanisms that may be involved in CRC progression, Liu et al. (11) found that GPER participates in numerous processes and pathways affecting proliferation of CRC cells. Both *in vitro* and *in vivo* studies revealed that GPER is engaged in cell cycle, endoplasmic reticulum stress and modulation of apoptosis which are crucial in the regulation of proliferation, migration and invasion of CRC cells. Liu et al. (11) reported that CRC cells treated with GPER selective agonist G-1 have a higher proportion of cells in the apoptotic phase and their mitochondria are characterized by lower membrane polarity. In line, protein expression analysis showed that treatment of CRC cells with G-1 is related with up-regulation of pro-apoptotic while down-regulation of anti-apoptotic proteins. Activation of reactive oxygen species, ERKs signal and NF-κB suppression appear to be involved in the inhibition of GPER-mediated CRC cell growth. Two-way action of GPER depending on the oxygen level in CRC cells was suggested by Bustos et al. (54). GPER has been shown to be able to modulate two major angiogenic factors, i.e., hypoxia inducible factor (HIF) and vascular endothelial growth factor (VEGF), which are related to the progression of many cancer types. It was found that, under normoxic condition GPER mediates inhibition whereas under hypoxic conditions GPER enhances HIF-1α and VEGFA expression in CRC cells. Beyond regulation of hypoxia-related genes, estrogens acting through GPER seem to potentiate hypoxia-induced proliferation and migration of CRC cells, while under normoxic condition they suppress cell proliferation and migration of CRC cells (54).

Studies conducted by Gilligan et al. (55, 60) clarify the impact of local concentrations of active estrogens and subsequent action on the development and progression of CRC. Clinical observations have indicated that local steroid sulfatase (STS) and 17β-hydroxysteroid dehydrogenase (HSD17) B2, B7, and B12 activity and expression in the intestine of CRC patients are disturbed, favoring 17β-estradiol synthesis in the intestine of CRC patients. STS is an enzyme which converts circulating sulfated estrogen into active form, while HSD17B7 and HSD17B12 catalyze the conversion of estrone to 17β-estradiol and HSD17B2 catalyzes the conversion of 17β-estradiol to estrone (61). Both 17β-estradiol administration and STS overexpression are related with increased CRC cell proliferation, which has been confirmed in *in vitro* and *in vivo* models (55, 60). Interestingly, estrogens increase proliferation through

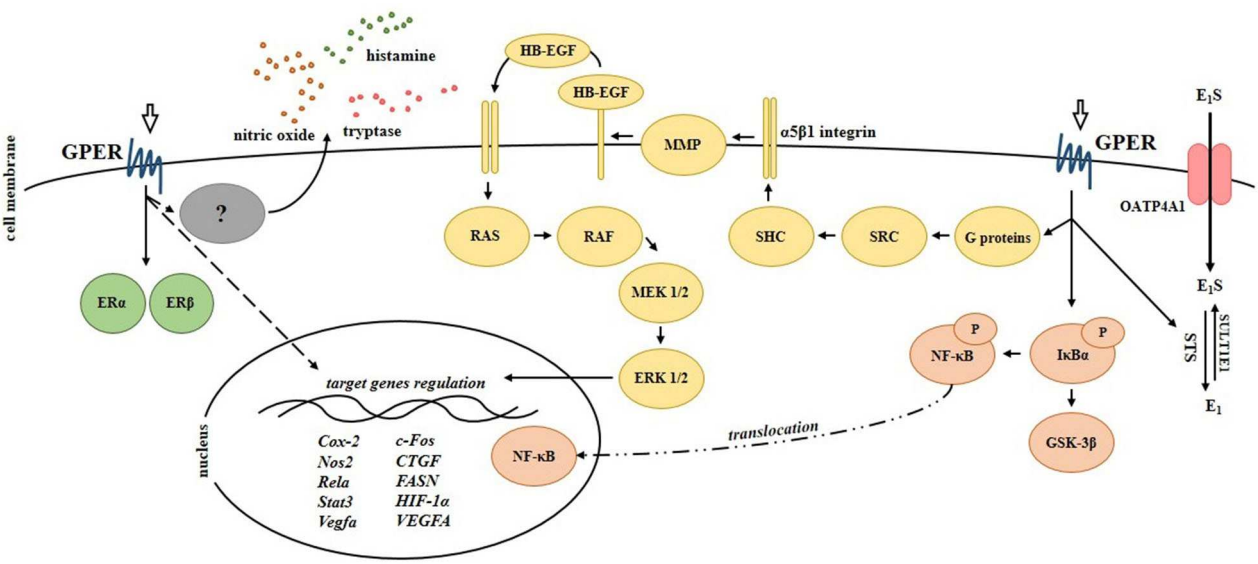

FIGURE 3 | Signaling pathways regulated by G protein-coupled estrogen receptor in the intestinal cells. Scheme shows experimentally proven signals mediated by GPER in bowel diseases. Cox-2, cycloxygenase-2; CTGF, connective tissue growth factor; E1, estrone; E_1S, estrone sulfate; ERα, estrogen receptor α; ERβ, estrogen receptor β; EGFR, epidermal growth factor receptor; ERK 1/2, extracellular signal-regulated kinase 1/2; FASN, fatty acid synthase; c-Fos, FBJ osteosarcoma (subunit of AP1 transcription factor); GSK-3β, glycogen synthase kinase-3β; HB-EGF, heparin-binding epidermal growth factor; HIF-1α, hypoxia-inducible factor-1α; IκBα, NF-κB inhibitor α; MEK 1/2, mitogen-activated protein kinase kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; Nos2, nitric oxide synthase 2; OATP4A1, organic anion transporter polypeptide 4A1; P, phosphorylation; RAF, rapidly accelerated fibrosarcoma (serine-threonine kinase); RAS, rat sarcoma (small GTPase); Rela, nuclear factor NF-κB subunit; SHC, adapter protein containing SRC homology 2 domain; SRC, non-receptor tyrosine kinase; Stat3, signal transducer and activator of transcription 3; STS, steroid sulfatase; SULT1E1, sulfotransferase family 1E member 1; Vegfa/VEGFA, vascular endothelial growth factor A.

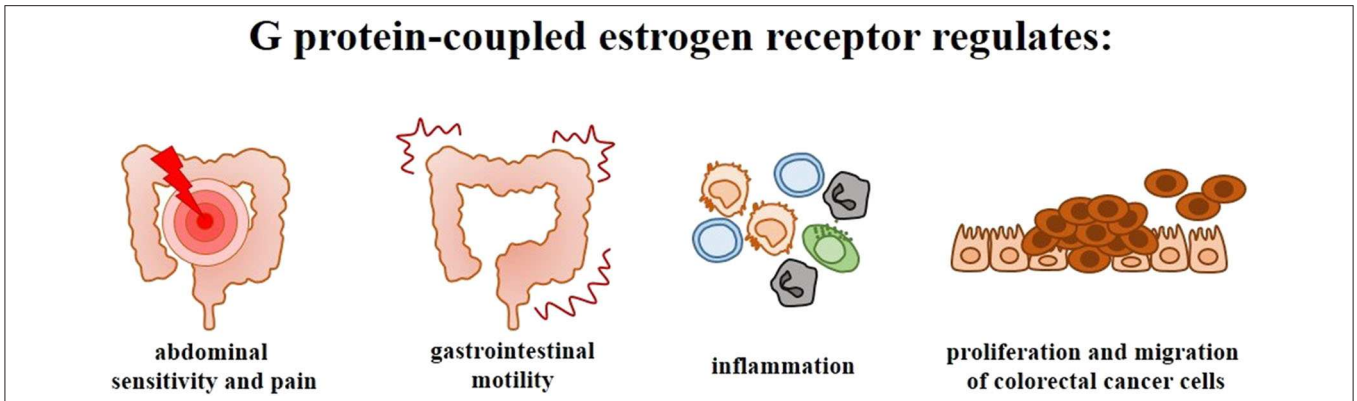

FIGURE 4 | Significance of G protein-coupled estrogen receptor in the intestinal diseases. Scheme illustrating the main symptoms and phenomena regulated by GPER in irritable bowel syndrome, inflammatory bowel diseases and colorectal cancer.

GPER signaling affecting expression of connective tissue growth factor (CTGF), which is crucial factor related to the proliferation, survival, and migration of cancer cells (55). On the other hand, modulation of GPER activity affects STS activity and may act as an estrogenic positive feedback loop leading to the development and progression of CRC (60).

Despite the inconclusive results regarding the level of GPER expression and activity in CRC, the membrane-bound estrogen receptor seems to be an important factor responsible for the modulation of multiple processes leading to the development and progression of CRC. The reasons for these discrepancies may result, for example, from differences in study groups in terms of gender and age. In above mentioned studies, it was proved that the patient's sex seems to be critical in assessing the role of the G protein-coupled estrogen receptor in intestinal diseases. Additionally, the hormonal status of women (pre- and post-menopausal period) is not insignificant and should be taken into consideration in studies on estrogen signaling in intestinal diseases. Different effects of GPER may also be the result of specific cellular context. As demonstrated by Bustos et al. (54) in order to fully understand an estrogenic response it is essential to appreciate not only the estrogen receptor status of the tumor cells but also the hypoxic conditions of the local tumor microenvironment. However, further analysis using clinical material and *in vivo* models are needed to determine significance of GPER in CRC.

CONCLUSIONS

Clinical and experimental studies indicate that nuclear ERs, especially ERβ which is mainly expressed in the intestine, play an important role in the proliferation and differentiation of intestine cells as well as in the organization and maintenance of intestine architecture. Nevertheless, the available findings support the thesis, that in addition to nuclear ERs, GPER expression and activity is responsible for the development and progression of intestinal diseases, i.e., irritable bowel syndrome, inflammatory bowel diseases and colorectal cancer. GPER has been shown to be involved in abdominal sensitivity and pain, intestinal motility, and colitis as well as proliferation and migration of colorectal cancer cells (**Figure 4**). However, further studies are needed to determine clinical and therapeutic potential of GPER in bowel diseases.

AUTHOR CONTRIBUTIONS

DJ and WK contributed to writing, critical revisions, editing and approval of the submitted version of manuscript.

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REFERENCES

1. Owman C, Blay P, Nilsson C, Lolait SJ. Cloning of human cDNA encoding a novel heptahelix receptor expressed in burkitt's lymphoma and widely distributed in brain and peripheral tissues. *Biochem Biophys Res Commun.* (1996) 228:285–92. doi: 10.1006/bbrc.1996.1654
2. Carmeci C, Thompson DA, Ring HZ, Francke U, Weigel RJ. Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics.* (1997) 45:607–17. doi: 10.1006/geno.1997.4972
3. Feng Y, Gregor P. Cloning of a novel member of the G protein-coupled receptor family related to peptide receptors. *Biochem Biophys Res Commun.* (1997) 231:651–4. doi: 10.1006/bbrc.1997.6161
4. Kvingedal AM, Smeland, E.B. A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue. *FEBS Lett.* (1997) 407:59–62. doi: 10.1016/S0014-5793(97)00278-0
5. O'Dowd BF, Nguyen T, Marchese A, Cheng R, Lynch KR, Heng, et al. Discovery of three novel G-protein-coupled receptor genes. *Genomics.* (1998) 47:310–3. doi: 10.1006/geno.1998.5095
6. Takada Y, Kato C, Kondo S, Korenaga R, Ando J. Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells

exposed to fluid shear stress. *Biochem Biophys Res Commun.* (1997) 240:737–41. doi: 10.1006/bbrc.1997.7734
7. Barton M, Filardo EJ, Lolait SJ, Thomas P, Maggiolini M, Prossnitz ER. Twenty years of the G protein-coupled estrogen receptor GPER: historical and personal perspectives. *J Steroid Biochem Mol Biol.* (2018) 176:4–15. doi: 10.1016/j.jsbmb.2017.03.021
8. Filardo EJ, Quinn JA, Bland KI, Frackelton, Jr AR. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol.* (2000) 14:1649–60. doi: 10.1210/mend.14.10.0532
9. Yang WR, Zhu FW, Zhang JJ, Wang Y, Zhang JH, Lu C, et al. PI3K/Akt activated by GPR30 and Src regulates 17β-estradiol-induced cultured immature boar sertoli cells proliferation. *Reprod Sci.* (2017) 24:57–66. doi: 10.1177/1933719116649696
10. Peixoto P, Aires RD, Lemos VS, Bissoli NS, Santos R. GPER agonist dilates mesenteric arteries via PI3K-Akt-eNOS and potassium channels in both sexes. *Life Sci.* (2017) 183:21–7. doi: 10.1016/j.lfs.2017.06.020
11. Liu Q, Chen Z, Jiang G, Zhou Y, Yang X, Huang H, et al. Epigenetic down regulation of G protein-coupled estrogen receptor (GPER) functions as a tumor suppressor in colorectal cancer. *Mol Cancer.* (2017) 16:87. doi: 10.1186/s12943-017-0654-3
12. Yu X, Stallone JN, Heaps CL, Han G. The activation of G protein-coupled estrogen receptor induces relaxation via cAMP as well as potentiates contraction via EGFR transactivation in porcine coronary arteries. *PloS ONE.* (2018) 13:e0191418. doi: 10.1371/journal.pone.0191418
13. Pupo M, Pisano A, Abonante S, Maggiolini M, Musti AM. GPER activates notch signaling in breast cancer cells and cancer-associated fibroblasts (CAFs). *Int J Biochem Cell Biol.* (2014) 46:56–67. doi: 10.1016/j.biocel.2013.11.011
14. Zhou X, Wang S, Wang Z, Feng X, Liu P, Lv XB, et al. Estrogen regulates hippo signaling via GPER in breast cancer. *J Clin Invest.* (2015) 125:2123–35 doi: 10.1172/JCI79573
15. Cheng, S-B, Quinn JA, Graeber CT, Filardo EJ. Down-modulation of the G protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. *J Biol Chem.* (2011) 286:22441–55. doi: 10.1074/jbc.M111.224071
16. Gonzalez de Valdivia E, Broselid S, Kahn R, Olde B, Leeb-Lundberg LMF. G protein-coupled estrogen receptor 1 (GPER1)/GPR30 increases ERK1/2 activity through PDZ motif-dependent and –independent mechanisms. *J Biol Chem.* (2017) 292:9932–43. doi: 10.1074/jbc.M116.765875
17. Prossnitz ER, Arterburn JB. International union of basic and clinical pharmacology XCVII G protein-coupled estrogen receptor and its pharmacologic modulators. *Pharmacol Rev.* (2015) 67:505–40. doi: 10.1124/pr.114.009712
18. Olde B, Leeb-Lundberg LM. GPR30/GPER1: searching for a role in estrogen physiology. *Trends Endocrinol Metab.* (2009) 20:409–16. doi: 10.1016/j.tem.2009.04.006
19. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol.* (2011) 7:715–26. doi: 10.1038/nrendo.2011.122
20. Jacenik D, Cygankiewicz AI, Krajewska WM. The G protein-coupled estrogen receptor as a modulator of neoplastic transformation. *Mol Cell Endocrinol.* (2016) 429:10–8. doi: 10.1016/j.mce.2016.04.011
21. Qin B, Dong L, Guo X, Jiang J, He Y, Wang X, et al. Expression of G protein-coupled estrogen receptor in irritable bowel syndrome and its clinical significance. *Int J Clin Exp Pathol.* (2014) 7:2238–46.
22. Jacenik D, Cygankiewicz AI, Fichna J, Mokrowiecka A, Małecka-Panas E, Krajewska WM. Estrogen signaling deregulation related with local immune response modulation in irritable bowel syndrome. *Mol Cell Endocrinol.* (2018) 471:89–96. doi: 10.1016/j.mce.2017.07.036
23. Moon TC, Befus AD, Kulka M. Mast cell mediators: their differential release and the secretory pathways involved. *Front Immunol.* (2014) 5:569. doi: 10.3389/fimmu.2014.00569
24. Katinios G, Casado-Bedmar M, Walter SA, Vicario M, González-Castro AM, Bednarska O, et al. Increased colonic epithelial permeability and mucosal eosinophilia in ulcerative colitis in remission compared with irritable bowel syndrome and health. *Inflamm Bowel Dis.* (2020) 16:izz328. doi: 10.1093/ibd/izz328
25. Robles A, Perez Ingles D, Myneedu K, Deoker A, Sarosiek I, Zuckerman MJ, et al. Mast cells are increased in the small intestinal mucosa of patients with irritable bowel syndrome: a systematic review and meta-analysis. *Neurogastroenterol Motil.* (2019) 31:e13718. doi: 10.1111/nmo.13718
26. Bashashati M, Moossavi S, Cremon C, Barbaro MR, Moraveji S, Talmon G, et al. Colonic immune cells in irritable bowel syndrome: a systematic review and meta-analysis. *Neurogastroenterol Motil.* (2018) 30:e13192. doi: 10.1111/nmo.13192
27. Sundin J, Nordlander S, Eutamene H, Alquier-Bacquie V, Cartier C, Theodorou V, et al. Colonic mast cell numbers, symptom profile, and mucosal expression of elements of the epithelial barrier in irritable bowel syndrome. *Neurogastroenterol Motil.* (2019) 31:e13701. doi: 10.1111/nmo.13701
28. Zielinska M, Fichna J, Bashashati M, Habibi S, Sibaev A, Timmermans JP, et al. G protein-coupled estrogen receptor and estrogen receptor ligands regulate colonic motility and visceral pain. *Neurogastroenterol Motil.* (2017) 29:e13025. doi: 10.1111/nmo.13025
29. Xu S, Wang X, Zhao J, Yang S, Dong L, Qin B. GPER-mediated, oestrogen-dependent visceral hypersensitivity in stressed rats is associated with mast cell tryptase and histamine expression. *Fundam Clin Pharmacol.* (2020) doi: 10.1111/fcp.12537. [Epub ahead of print].
30. Furness JB. The enteric nervous system: normal functions and enteric neuropathies. *Neurogastroenterol Motil.* (2008) 20:32–8. doi: 10.1111/j.1365-2982.2008.01094.x
31. Wood JD. Enteric nervous system: neuropathic gastrointestinal motility. *Dig Dis Sci.* (2016) 61:1803–16. doi: 10.1007/s10620-016-4183-5
32. Li Y, Xu J, Jiang F, Jiang Z, Liu C, Li L, et al. G protein-coupled estrogen receptor is involved in modulating colonic motor function via nitric oxide release in C57BL/6 female mice. *Neurogastroenterol Motil.* (2016) 28:432–42. doi: 10.1111/nmo.12743
33. Liu J, Lin G, Fang M, Rudd JA. Localization of estrogen receptor ERα, ERβ and GPR30 on myenteric neurons of the gastrointestinal tract and their role in motility. *Gen Comp Endocrinol.* (2019) 272:63–75. doi: 10.1016/j.ygcen.2018.11.016
34. Kane SV, Sable K, Hanauer SB. The menstrual cycle and its effect on inflammatory bowel disease and irritable bowel syndrome: a prevalence study. *Am J Gastroenterol.* (1998) 93:1867–72. doi: 10.1111/j.1572-0241.1998.540_i.x
35. Bharadwaj S, Kulkarni G, Shen B. Menstrual cycle, sex hormones in female inflammatory bowel disease patients with and without surgery. *J Dig Dis.* (2015) 16:245–55. doi: 10.1111/1751-2980.12247
36. Rolston VS, Boroujerdi L, Long MD, McGovern D, Chen W, Martin CF, et al. The influence of hormonal fluctuation on inflammatory bowel disease symptom severity-a cross-sectional cohort study. *Inflamm Bowel Dis.* (2018) 24:387–93. doi: 10.1093/ibd/izx004
37. Harnish DC, Albert LM, Leathurby Y, Eckert AM, Ciarletta A, Kasaian M, et al. Beneficial effects of estrogen treatment in the HLA-B27 transgenic rat model of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol.* (2004) 286:118–25. doi: 10.1152/ajpgi.00024.2003
38. Bábíčková J, Tóthová L, Lengyelová E, Bartonová A, Hodosy J, Gardlík R, et al. Sex differences in experimentally induced colitis in mice: a role for estrogens *Inflammation.* (2015) 38:1996–2006. doi: 10.1007/s10753-015-0180-7
39. Pierdominici M, Maselli A, Varano B, Barbati C, Cesaro P, Spada C, et al. Linking estrogen receptor β expression with inflammatory bowel disease activity. *Oncotarget.* (2015) 6:40443–51. doi: 10.18632/oncotarget.6217
40. Armstrong CM, Allred KF, Weeks BR, Chapkin RS, Allred CD. Estradiol has differential effects on acute colonic inflammation in the presence and absence of estrogen receptor β expression. *Dig Dis Sci.* (2017) 62:1977–84. doi: 10.1007/s10620-017-4631-x
41. Mohammad I, Starskaia I, Nagy T, Guo J, Yatkin E, Väänänen K, et al. Estrogen receptor α contributes to T cell-mediated autoimmune inflammation by promoting T cell activation and proliferation. *Sci Signal.* (2018) 11:eaap9415. doi: 10.1126/scisignal.aap9415
42. Włodarczyk M, Sobolewska-Włodarczyk A, Cygankiewicz AI, Jacenik D, Piechota-Polanczyk A, Stec-Michalska K, et al. G protein-coupled receptor 30 (gpr30) expression pattern in inflammatory bowel disease patients suggests its key role in the inflammatory process. A preliminary study. *J Gastrointestin Liver Dis.* (2017) 26:29–35. doi: 10.15403/jgld.2014.1121.261.gpr
43. Jacenik D, Cygankiewicz AI, Mokrowiecka A, Małecka-Panas E, Fichna J, Krajewska WM. Sex- and age-related estrogen signaling alteration in

inflammatory bowel diseases: modulatory role of estrogen receptors. *Int J Mol Sci.* (2019) 20:3175. doi: 10.3390/ijms20133175
44. Jacenik D, Zielinska M, Mokrowiecka A, Michlewska S, Małecka-Panas E, Kordek R, et al. G protein-coupled estrogen receptor mediates anti-inflammatory action in Crohn's disease. *Sci Rep.* (2019) 9:6749. doi: 10.1038/s41598-019-43233-3
45. Jacenik D, Zielinska M, Michlewska S, Fichna J, Krajewska WM. Visualization of estrogen receptors in colons of mice with TNBS-induced Crohn's disease using immunofluorescence. *J Vis Exp.* (2020) 157:e60813. doi: 10.3791/60813
46. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Andò S, et al. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17β-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol.* (2006) 20:631–46. doi: 10.1210/me.2005-0280
47. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, et al. G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res.* (2007) 67:1859–66. doi: 10.1158/0008-5472.CAN-06-2909
48. Kang L, Wang ZY. Breast cancer cell growth inhibition by phenethyl isothiocyanate is associated with down-regulation of oestrogen receptor-α36. *J Cell Mol Med.* (2010) 14:1485–93. doi: 10.1111/j.1582-4934.2009.00877.x
49. Kang L, Zhang X, Xie Y, Tu Y, Wang D, Liu Z, et al. Involvement of estrogen receptor variant ER-α36, not GPR30, in nongenomic estrogen signaling. *Mol Endocrinol.* (2010) 24:709–21. doi: 10.1210/me.2009-0317
50. Huang GS, Gunter MJ, Arend RC, Li M, Arias-Pulido H, Prossnitz ER, et al. Co-expression of GPR30 and ERβ and their association with disease progression in uterine carcinosarcoma. *Am J Abstetr Gynecol.* (2010) 203:242.e1–e5. doi: 10.1016/j.ajog.2010.04.046
51. Gao F, Ma X, Ostmann AB, Das SK. GPR30 activation opposes estrogen-dependent uterine growth via inhibition of stromal ERK1/2 and estrogen receptor alpha (ERα) phosphorylation signals. *Endocrinology.* (2011) 152:1434–47. doi: 10.1210/en.2010-1368
52. Wallacides A, Chesnel A, Ajj H, Chillet M, Flament S, Dumond H. Estrogens promote proliferation of the seminoma-like TCam-2 cell line through a GPER-dependent ERα36 induction. *Mol Cell Endocrinol.* (2012) 350:61–71. doi: 10.1016/j.mce.2011.11.021
53. Linares PM, Algaba A, Urzainqui A, Guijarro-Rojas M, González-Tajuelo R, Garrido J, et al. Ratio of circulating estrogen receptors beta and alpha (ERβ/ERα) indicates endoscopic activity in patients with Crohn's disease *Dig Dis Sci.* (2017) 62:2744–54. doi: 10.1007/s10620-017-4717-5
54. Bustos V, Nolan ÁM, Nijhuis A, Harvey H, Parker A, Poulsom R, et al. GPER mediates differential effects of estrogen on colon cancer cell proliferation and migration under normoxic and hypoxic conditions. *Oncotarget.* (2017) 8:84258–75. doi: 10.18632/oncotarget.20653
55. Gilligan LC, Rahman HP, Hewitt AM, Sitch AJ, Gondal A, Arvaniti A, et al. Estrogen activation by steroid sulfatase increases colorectal cancer proliferation via GPER. *J Clin Endocrinol Metab.* (2017) 102:4435–47. doi: 10.1210/jc.2016-3716
56. Santolla MF, Lappano R, De Marco P, Pupo M, Vivacqua A, Sisci D, et al. G protein-coupled estrogen receptor mediates the up-regulation of fatty acid synthase induced by 17β-estradiol in cancer cells and cancer-associated fibroblasts. *J Biol Chem.* (2012) 287:43234–45. doi: 10.1074/jbc.M112.417303
57. Yoon S, Lee MY, Park SW, Moon JS, Koh YK, Ahn YH, et al. Up-regulation of acetyl-CoA carboxylase alpha and fatty acid synthase by human epidermal growth factor receptor 2 at the translational level in breast cancer cells. *J Biol Chem.* (2007) 282:26122–31. doi: 10.1074/jbc.M702854200
58. Zhou L, Jiang S, Fu Q, Smith K, Tu K, Li H, et al. FASN, ErbB2-mediated glycolysis is required for breast cancer cell migration. *Oncol Rep.* (2016) 35:2715–22. doi: 10.3892/or.2016.4627
59. Wang H, Xi Q, Wu G. Fatty acid synthase regulates invasion and metastasis of colorectal cancer via Wnt signaling pathway. *Cancer Med.* (2016) 5:1599–606. doi: 10.1002/cam4.711
60. Gilligan LC, Gondal A, Tang V, Hussain MT, Arvaniti A, Hewitt AM, et al. Estrone sulfate transport and steroid sulfatase activity in colorectal cancer: implications for hormone replacement therapy. *Front Pharmacol.* (2017) 8:103. doi: 10.3389/fphar.2017.00103
61. RiŽner TL. The important roles of steroid sulfatase and sulfotransferases in gynecological diseases. *Front Pharmacol.* (2016) 7:30. doi: 10.3389/fphar.2016.00030

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REVIEW
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Expression and Role of the G Protein-Coupled Estrogen Receptor (GPR30/GPER) in the Development and Immune Response in Female Reproductive Cancers

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Cancer is a major public health issue and represents the second leading cause of death in women worldwide, as female reproductive-related neoplasms are the main cause of incidence and mortality. Female reproductive cancers have a close relationship to estrogens, the principal female sex steroid hormones. Estrogens exert their actions by the nuclear estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα, and ERβ act as transcription factors mediating genomic effects. Besides, the G protein-coupled estrogen receptor (GPER, formerly known as GPR30) was recently described as a seven-transmembrane receptor that mediates non-genomic estrogenic signaling, including calcium mobilization, cAMP synthesis, cleavage of matrix metalloproteinases, transactivation of epidermal growth factor receptor (EGFR), and the subsequent activation of PI3K and MAPK signaling pathways, which are the reasons why it is related to cellular processes, such as cell-cycle progression, cellular proliferation, differentiation, apoptosis, migration, and invasion. Since its discovery, selective agonists and antagonists have been found and developed. GPER has been implicated in a variety of hormone-responsiveness tumors, such as breast, endometrial, ovarian, cervical, prostate, and testicular cancer as well as lung, hepatic, thyroid, colorectal, and adrenocortical cancers. Nevertheless, GPER actions in cancer are still debatable due to the conflicting information that has been reported to date, since many reports indicate that activation of this receptor can modulate carcinogenesis. In contrast, many others show that its activation inhibits tumor activity. Besides, estrogens play an essential role in the regulation of the immune system, but little information exists about the role of GPER activation on its modulation within cancer context. This review focuses on the role that the stimulation of GPER plays in female reproductive neoplasms, specifically breast, endometrial, ovarian, and cervical cancers, in its tumor activity and immune response regulation.

Keywords: female reproductive cancers, estrogen receptor, G protein-coupled estrogen receptor, GPER, GPR30, estrogen, hormone

INTRODUCTION

Incidence, Mortality, and Prevalence of Gynecological Cancer in the World

Cancer is a leading public health problem in the world (1). It is estimated that incidence and mortality rates will increase by 63.4 and 75.1% by 2040, respectively, due to the growth and aging of the world population (2). Among females, gynecological cancer (including reproductive organs and breast) is the neoplasm with the highest incidence and mortality being the second leading cause of death worldwide (3, 4) (**Table 1**).

Breast cancer has the highest incidence, and it is the leading cause of cancer-related death among women worldwide, with 2,088,849 new cases and 626,679 deaths in 2018. Breast cancer represents 30.12% of the prevalent cases of cancer in women in the last 5 years and 15.03% of cancer-related deaths (3, 4).

Cervical cancer is the fourth most incident cancer and the fourth leading cause of cancer-related death worldwide, with 569,847 new cases and 311,365 deaths in 2018. It represents 6.46% of the prevalent cases of cancer in the last 5 years and 7.47% of cancer-related death. In Latin America and the Caribbean, it ranks third in incidence and prevalence rates only after breast and colorectal cancer (4).

In 2018, there were 382,069 new cases of uterine corpus cancer, also known as endometrial cancer, and caused 89,929 deaths, thus being the sixth most frequently diagnosed cancer and fourteenth cancer with more deaths worldwide, representing 5.62% of the prevalent cases of cancer in the last 5 years and 2.16% of cancer-related deaths (4).

In 2018, ovarian cancer was the eighth-most diagnosed cancer with 295,414 new cases and eighth cancer that generated the most deaths, 184,799 to be exact. It is important to mention that, despite having the lowest incidence rate among all gynecological cancers, ovarian cancer reports the highest mortality/incidence ratio (0.63), making it the most lethal (4).

Overall, gynecological cancer had 38.69% of incidence, 29.09% of mortality, and 45.54% of prevalence (5 years) of all cancers in the world in 2018, demonstrating its relevance as a public health problem (4) (**Table 1**).

Estrogen and Their Receptors

Estrogen is a steroid hormone associated with the female reproductive organs and is responsible for the development of female sexual characteristics. There are 3 types of natural estrogens: estrone, 17β-estradiol (E2) and estriol. From the previously mentioned forms of estrogen, estradiol is the most common form of estrogenic hormone in the treatment of menopause symptoms as hormone replacement therapy (HRT) (5).

In women, estrogens are synthesized mostly in the ovaries and adrenal glands. Estrogens exert their function primarily through cytosolic estrogen receptors (ERs) existing in target tissues (6). Estrogen is essential for the physiological functions of several organs in the human body. **Table 2** summarizes the main effects exerted by this steroid hormone on various organs of the female body.

Canonically, it has been described that estrogen exerts its action at tissues through binding to estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Nevertheless, another receptor has recently been described: the G protein-coupled estrogen receptor (GPER, formerly called GPR30) (7, 8).

The genomic estrogen receptors ERα and ERβ belong to the nuclear receptor family and act as transcriptional factors in palindromic sequences called estrogen response elements. They can also interact with other transcriptional factors such as Sp-1, AP-1, and NF-κB to promote the expression of several genes related to cellular processes as proliferation, differentiation, and survival (7, 9).

GPER Ligands and Signaling Pathways

Studies show that the three physiological form of estrogen (estrone, E2, and estriol) can bind to GPER. Estrone and E2 have been described as agonists for GPER, while estriol acts as an antagonist. Other molecules that have been found to act as agonists for GPER include therapeutic agents such as diethylstilbestrol, tamoxifen and its metabolite 4-hydroxytamoxifen, ethynylestradiol, raloxifene, and fulvestrant, as well as xenoestrogens such as bisphenol A and phytoestrogens like resveratrol, zearalenone, and genistein (8, 10, 11).

TABLE 1 | Incidence, mortality, and prevalence of gynecological cancers (including breast) worldwide in 2018.

Cancer	Incidence (total new cases of all cancers: 8,622,539)			Mortality (total deaths of all cancers: 4,169,387)			Prevalence, 5 years (total cases of all cancers: 22,826,472)			Proportion mortality/incidence
	Cancer most frequently diagnosed	Number of cases	(%)	Cause of cancer-related death	Number of deaths	(%)	Most prevalent cancer	Number of cases	(%)	
Breast	1st	2,088,849	24.23	1st	626,679	15.03	1st	6,875,099	30.12	0.30
Cervical	4th	569,847	6.61	4th	311,365	7.47	4th	1,474,265	6.46	0.55
Uterine corpus	6th	382,069	4.43	14th	89,929	2.16	5th	1,283,348	5.62	0.24
Ovarian	8th	295 414	3.42	8th	184,799	4.43	7th	762,663	3.34	0.63
Total	N/A	3,336,179	38.69	N/A	1,212,772	29.09	N/A	10,395,375	45.54	N/A

Information collected from data published at https://gco.iarc.fr/today, accessed [2019 August 19]. N/A, not applicable.

TABLE 2 | Physiological effects of estrogen in various female organs.

Female organs	Physiological effects of estrogen
Breast	Estrogen is responsible for the development of mammary gland tissue and parenchymal and stromal changes in breast tissue at puberty in females. Estrogen is also responsible for the development of mammary ducts during puberty and, during pregnancy, functions to secrete breast milk in postpartum lactation.
Uterus	In the uterus, estrogen helps to proliferate endometrial cells in the follicular phase of the menstrual cycle, thickening the endometrial lining in preparation for pregnancy.
Vagina	Estrogen supports the proliferation of epithelial mucosa cells of the vagina and the vulva. In the absence of estrogen, the vaginal and vulvar mucosal epithelium becomes thin and presents with symptoms of dryness known as vulvovaginal atrophy.

Additionally, G-1, a selective agonist (12) and two selective antagonists, G15 (13) and G36 (14), have been synthesized and used in numerous studies.

GPER/GPR30 has been classically described as a non-genomic receptor, which exerts rapid signaling actions. GPER belongs to the GPCR family, and it is canonically classified as a membrane-bound protein. Still, there is controversy over the fact that its expression has been found not only at the plasma membrane but also at the endoplasmic reticulum and the nucleus in some cases. GPER activation leads to cAMP production and PKA activation. Furthermore, such activation promotes the mobilization of calcium from the endoplasmic reticulum through PLC. In addition, it activates Src proteins and promotes the activation of MMP-2/9, resulting in EGFR transactivation. It is also able to consecutively activate MAPK and PI3K/Akt to promote the expression of several genes associated with cell survival, proliferation, differentiation, migration, and invasion (**Figure 1**) (11, 15, 16).

GPER Activation in Female Reproductive Cancers

Numerous studies have shown that GPER activation has a positive modulating effect on the molecular mechanisms that determine carcinogenesis. This protumor action has been reported in different female reproductive cancers. However, some reports in the literature suggest that GPER activation has an antitumor role (**Figure 1**). This review focuses on varied findings regarding the role of GPER in female reproductive cancers.

Ovarian Cancer

Ovarian cancer tumors are classified according to their histopathology and biological behavior, dividing them into epithelial, germ cell, and sexual cord stromal tumors (17).

In ovarian tissue samples, GPER is broadly expressed in high-risk ovarian cancer, associated with lower 5-year survival rates (18). Also, it has been found that this receptor is expressed in both benign and malignant tumors, and nearly one-third of malignant tumors overexpressed mRNA of GPER (19). Besides, its co-expression, along with EGFR, was associated with more reduced progression-free survival in ovarian cancer patients (20). Furthermore, it was shown that GPER is overexpressed in granulosa cell tumors, predicting a poor outcome in newly diagnosed patients (21).

On the opposite side, low expression of GPER in ovarian cancer tissue samples compared with benign and low malignant potential tumors was shown. A decrease in GPER expression was also reported during disease progression, which correlated with disease-free survival (22).

Regarding the receptor GPER localization, it was overexpressed in the nucleus and cytoplasm of serous and mucinous ovarian adenocarcinoma biopsies; its expression was higher in both advanced stages and patients with recurrence. Besides, its nuclear expression was predictive of poor overall survival and poor 5-year progression-free survival. GPER is also expressed in SKOV-3, OVCAR-3, and OVCAR5 cell lines and is abundantly co-expressed with ERα and ERβ in the SKOV3 cell line (22–24).

Studies in cell line models have been performed to determine the effect of GPER stimulation in ovarian cancer, and some of these studies have concluded that GPER activation promotes pro-oncogenic activities. For example, in the BG-1 cell line, G-1 induced the expression of pS2, cyclin A, cyclin D1, and cyclin E, while E2 promoted the expression of c-Fos and PR. Also, E2 and G-1 promoted cellular proliferation through pERK1/2 (25).

In OVCAR5 cells, E2 and G-1 agonists promote cell proliferation, increase the number of cells in the S phase of the cell cycle, increase the level of cyclin D1 and c-Fos, and reduce the level of active caspase 3 (26). Another study showed that both E2 and G-1 induce the migration and invasion in OVCAR5 cells, as well as the expression of MMP-9 and its proteolytic activity (27).

In the SKOV3 cell line, it was determined that GPER promotes cell proliferation by upregulation of c-Fos and cyclin D1 in a ligand-independent manner. Besides, it promoted migration and invasion, as well as MMP-2 and MMP-9 expression and their proteolytic activities (24).

Bisphenol A and tetrabromobisphenol A, two exogenous GPER ligands, have been reported to stimulate proliferation of OVCAR-3 and KGN cells (28). In the Caov-3 cell line, G-1 promotes this same cellular event through activation of the GPER/EGFR/Akt signaling pathway (20).

Some other studies conducted in cellular models have determined that the GPER stimulation is the opposite: the promotion of anticancer effects. For example, in SKOV-3 and OVCAR-3 cell lines, the specific stimulation of GPER with G-1 inhibited cell proliferation by induction of cell-cycle arrest in the sub-G1 phase as well as apoptosis (22).

Another study proved that G-1 alters morphology, decreases viability, suppresses proliferation, and induces apoptosis in IGROV-1 and SKOV-3 cell lines. Among the mechanisms involved in these processes, increased expression of the cell-cycle inhibitor P21CIP1 was found, as well as DNA fragmentation, decrease in the levels of the anti-apoptotic protein BCL-2, significant increase in cleaved PARP and fodrin, and microtubule assembly blockage during cell-cycle progression. In the SKOV-3 cell line, an increase was found in caspase 3/7 activity, while no significant changes were found in IGROV-1 cells. In

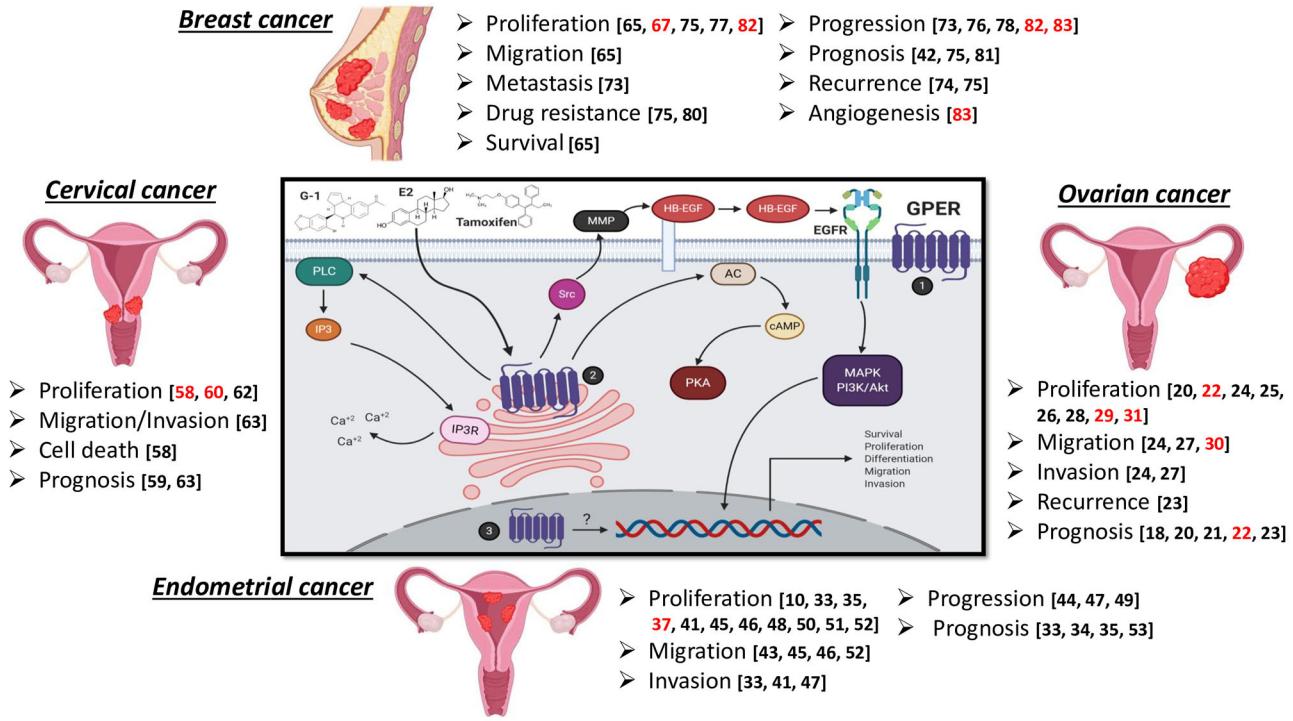

FIGURE 1 | GPER signaling pathway and effect of its stimulation in female reproductive cancers. Subcellular location of GPER has been found at the plasma membrane (1), endoplasmic reticulum (2), and the nucleus (3). Black references refer to promotion of disease progression, such as cell proliferation, survival, migration, invasion, metastasis, poor prognosis, drug resistance, and increased recurrence, while the references in red suggest an anti-tumor role. Created with BioRender.

addition, G-1 did not increase the expression of apoptosis-inducing factor (AIF) in IGROV-1 cells, but its translocation from the mitochondria to the nuclear area was observed (29).

E2 was reported to reduce the migration of OVCAR-3, SKOV3-IP, HEY, and TOV-112D cell lines by interfering with the expression of uPAR (30).

In KGN cells, G-1 alters cell morphology and suppresses proliferation independently of GPER, by increasing caspase 3/7 activity and arresting cell cycle in the G2/M phase (31).

Endometrial Cancer

Endometrial cancer (EC) is classified in types I and II, according to the histological grade and myometrial invasion. Overweight, obesity, familiar history of EC, diabetes, nulliparity, and race are between the leading risk factor for the development of EC; however, one of the most important is the continuous exposure to endogenous or exogenous estrogenic stimulation, which is considered to be the cause of ∼90% of the cases and represents the majority of type I EC, while type II is not related to estrogen (32).

Tamoxifen, a genomic estrogen receptor (ER) antagonist used in the treatment of breast cancer, is also considered an important risk factor for the development of EC. Endometrial tumors are heterogeneous with respect to ER expression. The fact that tamoxifen can act as GPER agonist was recently discovered (10); thus, the likely protumor effect is considered, which is the reason why performing further comprehensive research about the effects of GPER stimulation on the carcinogenesis of endometrial cancer, as well as the underlying mechanisms, is imperative.

GPER is overexpressed in EC biopsies and correlates with advanced stages of the disease, as well as high histopathological grade, aggressive subtypes, deep myometrial invasion, and poor overall survival, and it is similarly expressed between types I and II EC, with no differences between menopausal status. In addition, it is expressed in the luminal and basal surface of the EC epithelium. Furthermore, GPER was expressed higher in patients receiving tamoxifen treatments (33–36). Contrarily, a loss in GPER expression in EC tumors compared to the normal endometrium was observed (37), and this was correlated with a high FIGO stage, high histological grade, non-endometrioid histology, aneuploidy, low ERα expression, and disease progression (38). On the other hand, in cell lines derived from endometrial tumors, the expression of GPER is also varied, in RL95-2 it is high, in HEC-1A moderate, and in HEC-1B absent (37). Regarding its location, GPER was found at the cytoplasm and plasma membrane of the KLE and RL95-2 cell lines, and in normal endometrium, the location was predominantly intracellular (33, 35).

GPER mRNA expression has also been reported in the normal endometrium, finding higher levels in proliferative phases compared to secretory ones within the menstrual cycle. Additionally, epithelial cells contained a higher expression of

GPER mRNA than stroma cells. At the protein level, the expression is present at both endometrial and decidua tissue in glandular, luminal, and stromal cells (39).

It has also been described that E2, G-1, OHT, IGF-1, and insulin favor the increased expression of GPER at mRNA and protein levels (35, 40–42). Several studies have reported that direct stimulation with E2, G-1, and OHT increases the carcinogenic characteristics of EC cell lines, such as proliferation, migration, and invasion, involving mechanisms such as activation of signaling pathways like MAPK and PI3K, calcium influx via Cav1.3 and DAKα, phosphorylation of FAK, and increased production of MMP-2/9, c-Fos, and cyclin D1 (33, 35, 40, 41, 43–50). However, only one report showed the inhibition of proliferation in RL95-2 and HEC-1A cell lines stimulated with G-1 in a dose-dependent manner (37). Also, miR-195 controls GPER expression *in vitro* in AN3-CA and HEC-1A cells (44).

Other signaling pathways have been found to interact with GPER to induce a plethora of effects in EC cell lines. IGF-1 triggers the expression of cyclin D1 and CTGF, promoting proliferation and migration of Ishikawa cells (42). Gankyrin decreases PTEN activity, leading to the activation of the GPER/PI3K/Akt pathway due to the stimulation with E2, which promotes the expression of cyclin D1 and, consequently, RL95-2 cell proliferation. This finding supports the fact that gankyrin and PTEN are inversely correlated in endometrial cancer biopsies (51). Another study found that the stimulation of GPER promotes the activation of the EGFR/MAPK pathway and induces the expression and recruitment of Egr-1 to the promoter region of CTGF and cyclin D1 genes in Ishikawa cells and CAFs treated with E2, G-1, and OHT (52). GPER was highly expressed in endometrial cancer biopsies that exhibited insulin resistance and positively correlated with TET1, a protein that regulates hydroxymethylation of DNA at the GPER promoter region, increasing its expression via the PI3K/Akt signaling pathway (40). The autocrine motility factor (AMF) interacts with GPER activating the PI3K/Akt pathway, promoting proliferation, and regulating apoptosis in SPEC-2 cells. In EC tissue, a high expression of both GPER and AMF has been correlated with poor prognosis in patients (53).

The capacity of GPER stimulation to induce tumors was studied in animal models. One of these studies found that the receptor activation promotes the ability of the RL95-2 cell line to produce a solid tumor in a xenograft model of athymic mice (47). Besides, GPER blockade inhibited growth tumor in an athymic mouse model based in HEC-1A cell line xenograft (49).

Cervical Cancer

High-risk human papillomavirus (HPV) is necessary for the development of cervical cancer (CC) but requires the participation of other factors as estrogen signaling (54). Its signaling discovered the effects of estrogen on cervical carcinogenesis through nuclear receptors, mainly ERα, in murine models (55, 56). Besides, a study reported that both ERα and ERβ increase their expression as the disease progresses from cervical intraepithelial neoplasia (CIN) to CC (57). Another study showed the immunopositivity to GPER in CC, and its expression in CIN and normal cervical epithelium. A predominant localization at the cytoplasm and nucleus of the cervical tissues was found, and GPER expression in CIN samples was statistically significantly lower than in normal epithelium and CC samples (58). The expression of this receptor was highly found in CC tissue samples, and its localization was reported to be cytoplasmic and at the plasma membrane. In addition, a positive correlation between cytoplasmic GPER expression and the tumor suppressor proteins p16 and p53 was found; its expression was associated with a favorable prognosis of the disease (59).

GPER expression has been detected in CC-derived cell lines HeLa, SiHa, C-33A, and Caski, and its stimulation with G-1 reduces the viability of HeLa and SiHa cell lines through the activation of ERK1/2 by dysregulating cyclin B and inducing cell-cycle arrest in G2/M phase (60). In another report, stimulation of GPER with G-1 was shown to inhibit cell proliferation of HeLa, SiHa, and C-33A cell lines by inducing processes such as apoptosis, necrosis, and senescence (58). Besides, it was demonstrated in a transduced non-tumorigenic keratinocyte model that E6 and E7 oncogenes from HPV 16 and HPV 18 increase GPER expression at both mRNA and protein levels and that E7 oncogene modulates GPER localization in the nucleus (61).

Mono-ethylhexyl phthalate (MEHP), an environmental estrogenic chemical, can promote the proliferation of HeLa and SiHa cervical cancer cells through the GPER/PI3K/Akt pathway but have no effect over invasion and expression of MMPs (62).

A high GPER expression has been reported in cervical adenocarcinoma cell lines HeLa229, OMC4, HCA1, CAC-1, and TMCC1 and in tissue samples of cervical adenocarcinoma collected from patients. E2 and G-1 stimulation increases claudin-1 expression, contributing to malignant potential by increasing proliferation of CAC-1 and HCA1 cell lines through MAPK/ERK and PI3K/Akt. Furthermore, it was also observed that knocking out for claudin-1 expression in the TMCC1 cell line reduces its capability for proliferation, invasion, and migration. In a mouse xenograft model, tumors derived from GPER-knockout cells were smaller and grew slower than those derived from the control cells. Finally, this report found a positive correlation between GPER and claudin-1 co-expression and lower overall survival in cervical adenocarcinoma patients (63).

Breast Cancer

Prolonged exposure to estrogen is the leading risk factor for breast cancer progression. In breast cancer cells, E2 enhances the stimulatory effects by binding to estrogen nuclear receptors, which regulate the expression of genes that contribute to the proliferation, migration, and survival of cancer cells (64, 65).

The aggressiveness of breast cancer tumors is related to the presence or absence of estrogen receptors, being classified into positive (RE+) and negative (RE–) tumors for the estrogen receptor (66), with RE– being intrinsically more aggressive due to the lack of effectiveness of treatments based on tamoxifen and aromatase inhibitors (67).

There is controversy about the location of GPER. Although GPER belongs to a family of surface receptors, which conventionally mediate transmembrane signaling of cell

membrane-impermeable ligands, numerous studies have shown that GPER is detectable both in the plasma membrane and intracellularly in breast cancer cells (68–70).

As in other neoplasms, in breast cancer, the function of GPER is not clear yet. GPER has been proposed as a mediator of the estrogen action in breast malignancies, regulating critical biological responses to estrogens, such as changes in gene transcription, proliferation, and cell migration within the tumor microenvironment (70–72). Besides, it has been associated with increased tumor size, high risk of metastatic disease, recurrence, and reduced survival rates in patients with breast cancer (10, 73–75) favoring disease progression (76).

The proliferative effect generated by GPER has not only been studied in breast tumor tissues. In explants of non-tumor breast tissues, it was observed that G-1, the selective agonist of GPER, was able to stimulate the proliferation of tissues in culture. In contrast, G36, a GPER antagonist, blocked G-1-induced proliferation in non-cancerous human breast tissues (77).

An essential event for the spread and progression of carcinomas is the mesenchymal–epithelial transition (MET) in which GPER participates directly. This was demonstrated in breast cancer cell lines incubated with G15, a GPER antagonist. It was observed that G15 prevents breast cancer cells from undergoing mesenchymal–epithelial transition via GPER inhibition, and a synergistic effect was observed when incubating cells with doxorubicin and G15, causing increased sensitivity to this drug by the breast cancer cell lines (78).

GPER plays an important role in the development of resistance to treatment in breast cancer because RE antagonists such as tamoxifen and fulvestrant act as GPER agonists, stimulating proliferation and cell growth. Cellular treatment with tamoxifen for prolonged periods increases the overregulation of GPER stimulated by E2 and its relocation of the endoplasmic reticulum to the cell membrane (75).

Tamoxifen acts as a growth factor due to its ability to transactivate EGFR via GPER; this is the mechanism by which the MCF-7 cell line develops resistance to endocrine therapy (79). A significant correlation between GPER expression and the expression of EGFR and HER-2 has been observed. GPER-positive tumors are often less responsive to tamoxifen therapy due to the resistance generated by GPER (75).

Cytoplasmic GPER enhances the GPER/cAMP/PKA signaling pathway in breast cancer-associated fibroblasts, generating high tumor metabolic activity and resistance to tamoxifen, herceptin-2, and epirubicin treatment. It also promotes the transfer of high levels of energy between stromal cells and cancer cells (80).

For these and other studies, GPER has been proposed as a biomarker predictive of biologically aggressive phenotypes associating it with adverse results and poor survival of breast cancer patients. That is why GPER could be a therapeutic target (42, 81).

Controversially, some studies assign an antitumor function to the GPER. It was observed that estrogens significantly suppress breast cancer growth, inducing cell-cycle arrest in the G1 phase during hypoxia through GPER activation. This is due to low expression levels of ERα and the enhanced activation of GPER by estrogen. In addition, a positive correlation has been determined between GPER expression and adverse clinical outcomes in patients with breast and ovarian cancer (82).

Another study showed that GPER activation could significantly inhibit the cellular proliferation of ER- breast cancer. They observed that, in cultured breast cancer cells, treatment with G-1 decreased the expression of cyclin B, induced the arrest of the cell cycle in the G2/M phase, and caused apoptosis related to mitochondria, concluding that the activation of GPER can inhibit proliferation *in vitro* and *in vivo* through the generation of reactive oxygen species (ROS), apoptosis through the caspase pathway, and the decrease in cyclin B expression (67).

Additionally, in cell lines and in murine models of triple-negative breast cancer (TNBC), it has been observed that the activation of GPER by its G-1 agonist significantly inhibits the expression of IL-6 and VEGF-A and is also capable of suppressing the angiogenesis and progression of TNBC (83).

The effects of G-1 on proliferation and survival are highly controversial. Different studies suggest that the concentration of G-1 in the tumor microenvironment defines its function; some reports have shown that G-1 stimulates proliferation of breast cancer cells in a dose-dependent manner, in the range of 10 nM to 1 μM (52, 65, 77, 84), while micromolar levels potentially suppress the growth of breast cancer cells (65, 67, 85, 86).

There is evidence that G-1 can suppress the proliferation of breast cancer cells and induce cell apoptosis independently of GPER. However, the intracellular target and the mechanisms of inhibition of cell proliferation and induction of tumor cell apoptosis carried out by G-1 are unknown, demonstrating the need to deepen the study about the existence of other receptors for G-1 and the functions they exert after forming the receptor–ligand complex (31).

The expression pattern of GPER and its subcellular location is still a debate because it has been found at different sites within the cell. Using murine knockout models for GPER, it has been shown that its overexpression and its location in the plasma membrane are important events for breast cancer progression (87), while the absence of GPER in the plasma membrane has an excellent long-term prognosis in patients with ERalpha + breast cancer treated with tamoxifen (88, 89).

On the other hand, the expression of cytoplasmic GPER in breast carcinomas is associated with low tumor stages, better histological differentiation, and a better overall clinical outcome; the expression of nuclear GPER is associated with less favorable tumor properties. This indicates that GPER can have different cellular functions depending on its subcellular location and influence the development and prognosis of the disease (90, 91).

GPER Modulates the Intracellular Calcium Flow in Breast Cancer

The positive and negative effects that GPER has on the proliferation of various cell lines have been attributed to its modulating effect on intracellular calcium flow. GPER is capable of coupling to different Ca^{2+} channels such as IP3R (inositol triphosphate receptors) in ER-positive MCF-7 cells and ryanodine receptors (RyR) in ER-negative SKBr3 cells. Sustained abnormal increases in intracellular levels of Ca^{2+} may lead to inhibition of proliferation and induce apoptosis. Partial

inhibition of the plasma membrane Ca^{2+}-ATPase in MCF-7 cells causes a moderate increase in intracellular levels of Ca^{2+}, leading to inhibition of proliferation altering the cell-cycle kinetics (92).

Relationship Between GPER and Immune Response

Estrogen is extensively related to the modulation of cellular responses of the immune system and is largely studied within the pathology of inflammatory and autoimmune diseases context, mainly due to its actions through ERα/β (93). However, its role in cancer immunology is poorly understood.

GPER has been found to be expressed in adaptive and innate immune response cells such as circulating B and T lymphocytes and monocytes (94), macrophages (95, 96), neutrophils (97–99), eosinophils (100), and dendritic cells (98).

GPER is necessary for the apoptosis of double-positive thymocytes and contributes to thymic atrophy (101) as well as the survival of naïve T cells in mice (102). GPER-specific stimulation with G-1 promoted the production of IL-10 in $CD4^+$ T cells cultured *ex vivo*, under Th17-polarizing conditions, similar to several autoimmune diseases via MAPK/ERK (103) and induced FOXP3 expression in regulatory T cells (104).

G-1 is capable of inhibiting the production of IL-6, TNF-α, IL-12 (p40), and CCL5 in human macrophages treated with LPS. Besides, G-1 reduced disease severity in an EAE multiple-sclerosis model (105).

In human neutrophils obtained from healthy donors, GPER activation increased the respiratory burst, cell viability, and expression of CD11b and CD62L, two markers of neutrophil activation. Likewise, G-1 promoted the expression of *IL1B*, *CXCL2*, *COX2*, *SOCS3*, *GCSF*, and *IL1RA* genes and increased the production of CXCL8 protein. These effects occurred through the cAMP/PKA/CREB, p38 MAPK, and ERK signaling pathways (98).

G-1 magnified the effect of eotaxin on eosinophil chemotaxis and inhibited spontaneous apoptosis of eosinophils by reducing caspase-3 activity, but it had the opposite effect on eosinophils previously stimulated with IL-5, a cytokine that promotes their survival in eosinophilic inflammation, inducing apoptosis by increasing caspase-3 activity. Nevertheless, GPER activation had no effect on eosinophil degranulation (100).

All these data suggest that estrogen signaling through GPER may have a modulatory effect on the immune system by decreasing important features for inflammation. This could lead to a protective role for autoimmune diseases, as occurs in multiple sclerosis studies; and therefore, immune system modulation by GPER stimulation could be investigated as a potential clinical target in inflammatory diseases.

What Effects Does GPER Have on the Expression of Cytokines Found in Cancer?

Little is known about the role that GPER activation could play over the induction of immune system response or other important components, as cytokine expression, on cancer. Here we reviewed the information published to date.

In endometrial cancer, GPER stimulation with both E2 and G-1 increased IL-6 expression through the MAPK pathway in KLE and RL95-2 cell lines (33). In addition, GM-CSF, VEGF, and IL-8 levels are increased and associated with high GPER expression in primary cultures from endometrial cancer tumors (106).

It has been observed that GPER activation reduces TNFα-induced IL-6 expression in the SKBR3 cell line and reduces IL-6 and VEGF-A levels in triple-negative breast cancer cell lines MDA-MB-231 and BT-549 through inhibition of NF-κB transcriptional activity (83, 107). Likewise, E2 prevented the action of TGF-β in the migration of MCF-7 and MDA-MB-231 breast cancer cell lines via GPER/ERK1/2, resulting in an inhibitory effect of Smad signaling (108).

Additionally, E2 and G-1 induced IL-1β expression in CAFs, while in MCF-7 and SKBR3 breast cancer cell lines they promoted IL1R1 expression, stimulating migration and invasion of breast cancer cells (109). Interestingly, it was observed that the lack of N-glycosylation in the N-terminal portion of GPER drives this receptor to the nucleus, becoming a transcription-like factor that promotes the expression of CTFG in CAFs and in the SKBR3 cell line. CTFG is a cytokine that increases MDA-MB-231 cell line migration capacity (110).

Tumor-promoting inflammation and immune system modulation by cancer cells are well-recognized as hallmarks for cancer progression (111). The evidence mentioned above suggests that GPER signaling is involved in the expression of cytokines by neoplastic cells, which are related to migration and angiogenesis. Nevertheless, there is a lack of information demonstrating the effect of GPER stimulation over the immune system's cells, as well as their interaction in the tumor microenvironment. Therefore, the elucidation of these mechanisms is necessary for a better understanding of the effect of GPER in carcinogenesis and tumor progression, making the estrogen signaling a potential therapeutic target in female reproductive cancers.

CONCLUSION

Exposure to estrogen for long periods represents the main factor in the development of several cancers, including ovary, endometrial, cervical, and breast cancer. The main effects of estrogen through their receptors are related to cell survival, growth, and proliferation. Nevertheless, little is known about the effect of estrogen signaling through the G protein-coupled estrogen receptor in female reproductive cancers, as well as in the modulation of the immune system in these cancers. In general, it has been related to protumor processes such as increased cell survival, proliferation, migration, metastasis, and tumor growth, as well as the production of cytokines, which promote these effects. However, it is important to mention that there are some reports that show complete opposite results than the aforementioned studies, suggesting a possible antitumor role. This makes it difficult to propose a specific conclusion and creates the need for additional thorough research in this field.

AUTHOR CONTRIBUTIONS

CH-S, JV-P, and AP-S planned, wrote, and contributed to the critical review of the manuscript. In addition to these activities, AP-S directed this work. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin.* (2019) 69:7–34. doi: 10.3322/caac.21551
2. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M. *Global Cancer Observatory: Cancer Tomorrow. International Agency for Research on Cancer.* (2018). Available online at: https://gco.iarc.fr/tomorrow (accessed August 19, 2019).
3. Torre LA, Islami F, Siegel RL, Ward EM, Jemal A. Global cancer in women: burden and trends. *Cancer Epidemiol Biomarkers Prev.* (2017) 26:444–57. doi: 10.1158/1055-9965.EPI-16-0858
4. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M. Global cancer observatory: cancer today. *International Agency for Research on Cancer.* (2018). Available online at: https://gco.iarc.fr/today (accessed August 19, 2019).
5. Delgado BJ, Lopez-Ojeda W. *Estrogen. StatPearls.* (2019). Available online at: https://www.ncbi.nlm.nih.gov/books/NBK538260/ (accessed October 10, 2019).
6. Amenyogbe E, Chen G, Wang Z, Lu X, Lin M, Lin AY. A review on sex steroid hormone estrogen receptors in mammals and fish. *Int J Endocrinol.* (2020) 2020:1–9. doi: 10.1155/2020/5386193
7. Eyster KM. Estrogen receptors methods and protocols methods in molecular biology 1366. In: *Methods in Molecular Biology.* (2016). p. 18–19. Available online at: http://www.springer.com/series/7651
8. Prossnitz ER, Arterburn JB. International union of basic and clinical pharmacology. XCVII. G protein–coupled estrogen receptor and its pharmacologic modulators. *Pharmacol Rev.* (2015) 67:505–40. doi: 10.1124/pr.114.009712
9. Pakdel F. Molecular pathways of estrogen receptor action. *Int J Mol Sci.* (2018) 19:2591. doi: 10.3390/ijms19092591
10. Prossnitz ER, Barton M. Estrogen biology: new insights into GPER function and clinical opportunities. *Mol Cell Endocrinol.* (2014) 389:71–83. doi: 10.1016/j.mce.2014.02.002
11. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol.* (2011) 7:715–26. doi: 10.1038/nrendo.2011.122
12. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol.* (2006) 2:207–12. doi: 10.1038/nchembio775
13. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, et al. *In vivo* effects of a GPR30 antagonist. *Nat Chem Biol.* (2009) 5:421–7. doi: 10.1038/nchembio.168
14. Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologa CG, et al. Identification of a GPER/GPR30 antagonist with improved estrogen receptor counterselectivity. *J Steroid Biochem Mol Biol.* (2011) 127:358–66. doi: 10.1016/j.jsbmb.2011.07.002
15. Qian H, Xuan J, Liu Y, Shi G. Function of G-protein-coupled estrogen receptor-1 in reproductive system tumors. *J Immunol Res.* (2016) 2016:6. doi: 10.1155/2016/7128702
16. Xu S, Yu S, Dong D, Lee LTO. G Protein-coupled estrogen receptor: a potential therapeutic target in cancer. *Front Endocrinol.* (2019) 10:1–12. doi: 10.3389/fendo.2019.00725
17. Vanderhyden BC, Dorward AM. Ovarian cancer and the environment: rodent models. Third *Edit*. In: *Comprehensive Toxicology: Third Edition*. Oxford: Elsevier. (2018). p. 362–380.
18. Smith HO, Arias-Pulido H, Kuo DY, Howard T, Qualls CR, Lee SJ, et al. GPR30 predicts poor survival for ovarian cancer. *Gynecol Oncol.* (2009) 114:465–71. doi: 10.1016/j.ygyno.2009.05.015
19. Kolkova Z, Casslén V, Henic E, Ahmadi S, Ehinger A, Jirström K, et al. The G protein-coupled estrogen receptor 1 (GPER/GPR30) does not predict survival in patients with ovarian cancer. *J Ovarian Res.* (2012) 5:9. doi: 10.1186/1757-2215-5-9
20. Fujiwara S, Terai Y, Kawaguchi H, Takai M, Yoo S, Tanaka Y, et al. GPR30 regulates the EGFR-Akt cascade and predicts lower survival in patients with ovarian cancer. *J Ovarian Res.* (2012) 5:1. doi: 10.1186/1757-2215-5-35
21. Heublein S, Mayr D, Friese K, Jarrin-Franco MC, Lenhard M, Mayerhofer A, et al. The g-protein-coupled estrogen receptor (GPER/GPR30) in ovarian granulosa cell tumors. *Int J Mol Sci.* (2014) 15:15161–72. doi: 10.3390/ijms150915161
22. Ignatov T, Modl S, Thulig M, Weißenborn C, Treeck O, Ortmann O, et al. GPER-1 acts as a tumor suppressor in ovarian cancer. *J Ovarian Res.* (2013) 6:1–10. doi: 10.1186/1757-2215-6-51
23. Zhu CX, Xiong W, Wang ML, Yang J, Shi HJ, Chen HQ, et al. Nuclear G protein-coupled oestrogen receptor (GPR30) predicts poor survival in patients with ovarian cancer. *J Int Med Res.* (2018) 46:723–31. doi: 10.1177/0300060517717625
24. Yan Y, Jiang X, Zhao Y, Wen H, Liu G. Role of GPER on proliferation, migration and invasion in ligand-independent manner in human ovarian cancer cell line SKOV3. *Cell Biochem Funct.* (2015) 33:552–9. doi: 10.1002/cbf.3154
25. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, et al. G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17β-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res.* (2007) 67:1859–66. doi: 10.1158/0008-5472.CAN-06-2909
26. Liu H, Yan Y, Wen H, Jiang X, Cao X, Zhang G, et al. A novel estrogen receptor GPER mediates proliferation induced by 17b-estradiol and selective GPER agonist G-1 in estrogen receptor α (ERα)-negative ovarian cancer cells. *Cell Biol Int.* (2014) 38:631–8. doi: 10.1002/cbin.10243
27. Yan Y, Liu H, Wen H, Jiang X, Cao X, Zhang G, et al. The novel estrogen receptor GPER regulates the migration and invasion of ovarian cancer cells. *Mol Cell Biochem.* (2013) 378:1–7. doi: 10.1007/s11010-013-1579-9
28. Hoffmann M, Gogola J, Kotula-Balak M, Ptak A. Stimulation of ovarian cell proliferation by tetrabromobisphenol A but not tetrachlorobisphenol A through G protein-coupled receptor 30. *Toxicol Vitr.* (2017) 45:54–9. doi: 10.1016/j.tiv.2017.08.009
29. Wang C, Lv X, He C, Hua G, Tsai MY, Davis JS. The G-protein-coupled estrogen receptor agonist G-1 suppresses proliferation of ovarian cancer cells by blocking tubulin polymerization. *Cell Death Dis.* (2013) 4:1–11. doi: 10.1038/cddis.2013.397
30. Henic E, Noskova V, Høyer-Hansen G, Hansson S, Casslén B. Estradiol attenuates EGF-induced rapid uPAR mobilization and cell migration via the G-protein-coupled receptor 30 in ovarian cancer cells. *Int J Gynecol Cancer.* (2009) 19:214–22. doi: 10.1111/IGC.0b013e31819bcb75
31. Wang C, Lv X, Jiang C, Davis JS. The putative G-protein coupled estrogen receptor agonist G-1 suppresses proliferation of ovarian and breast cancer cells in a GPER-independent manner. *Am J Transl Res.* (2012) 4:390–402. doi: 10.1158/1538-7445.AM2012-3920
32. Sorosky JI. Endometrial cancer. *Obstet Gynecol.* (2012) 120:383–97. doi: 10.1097/AOG.0b013e3182605bf1
33. He YY, Cai B, Yang YX, Liu XL, Wan XP. Estrogenic G protein-coupled receptor 30 signaling is involved in regulation of endometrial carcinoma by promoting proliferation, invasion potential, and interleukin-6 secretion via the MEK/ERK mitogen-activated protein kinase pathway. *Cancer Sci.* (2009) 100:1051–61. doi: 10.1111/j.1349-7006.2009.01148.x

34. Smith HO, Leslie KK, Singh M, Qualls CR, Revankar CM, Joste NE, et al. GPR30: a novel indicator of poor survival for endometrial carcinoma. *Am J Obstet Gynecol.* (2007) 196:386.e1–386.e11. doi: 10.1016/j.ajog.2007.01.004
35. Zhang L, Li Y, Lan L, Liu R, Wu Y, Qu Q, et al. Tamoxifen has a proliferative effect in endometrial carcinoma mediated via the GPER/EGFR/ERK/cyclin D1 pathway: a retrospective study and an *in vitro* study. *Mol Cell Endocrinol.* (2016) 437:51–61. doi: 10.1016/j.mce.2016.08.011
36. Wan J, Yin Y, Zhao M, Shen F, Chen M, Chen Q. The positivity of G-protein-coupled receptor-30 (GPR 30), an alternative estrogen receptor is not different between type 1 and type 2 endometrial cancer. *Oncotarget.* (2017) 8:90897–904. doi: 10.18632/oncotarget.18545
37. Skrzypczak M, Schüler S, Lattrich C, Ignatov A, Ortmann O, Treeck O. G protein-coupled estrogen receptor (GPER) expression in endometrial adenocarcinoma and effect of agonist G-1 on growth of endometrial adenocarcinoma cell lines. *Steroids.* (2013) 78:1087–91. doi: 10.1016/j.steroids.2013.07.007
38. Krakstad C, Trovik J, Wik E, Engelsen IB, Werner HMJ, Birkeland E, et al. Loss of GPER identifies new targets for therapy among a subgroup of ERα-positive endometrial cancer patients with poor outcome. *Br J Cancer.* (2012) 106:1682–8. doi: 10.1038/bjc.2012.91
39. Kolkova Z, Noskova V, Ehinger A, Hansson S, Casslén B. G protein-coupled estrogen receptor 1 (GPER, GPR 30) in normal human endometrium and early pregnancy decidua. *Mol Hum Reprod.* (2010) 16:743–51. doi: 10.1093/molehr/gaq043
40. Lv QY, Xie BY, Yang BY, Ning CC, Shan WW, Gu C, et al. Increased TET1 expression in inflammatory microenvironment of hyperinsulinemia enhances the response of endometrial cancer to estrogen by epigenetic modulation of GPER. *J Cancer.* (2017) 8:894–902. doi: 10.7150/jca.17064
41. Du GQ, Zhou L, Chen XY, Wan XP, He YY. The G protein-coupled receptor GPR30 mediates the proliferative and invasive effects induced by hydroxytamoxifen in endometrial cancer cells. *Biochem Biophys Res Commun.* (2012) 420:343–9. doi: 10.1016/j.bbrc.2012.02.161
42. De Marco P, Bartella V, Vivacqua A, Lappano R, Santolla MF, Morcavallo A, et al. Insulin-like growth factor-I regulates GPER expression and function in cancer cells. *Oncogene.* (2013) 32:678–88. doi: 10.1038/onc.2012.97
43. Tsai CL, Wu HM, Lin CY, Lin YJ, Chao A, Wang TH, et al. Estradiol and tamoxifen induce cell migration through GPR30 and activation of focal adhesion kinase (FAK) in endometrial cancers with low or without nuclear estrogen receptor α (ERα). *PLoS ONE.* (2013) 8:e72999. doi: 10.1371/journal.pone.0072999
44. Deng J, Wang W, Yu G, Ma X. MicroRNA-195 inhibits epithelial-mesenchymal transition by targeting G protein-coupled estrogen receptor 1 in endometrial carcinoma. *Mol Med Rep.* (2019) 20:4023–32. doi: 10.3892/mmr.2019.10652
45. Hao J, Bao X, Jin B, Wang X, Mao Z, Li X, et al. Ca2+ channel subunit a 1D promotes proliferation and migration of endometrial cancer cells mediated by 17β-estradiol via the G protein-coupled estrogen receptor. *FASEB J.* (2015) 29:2883–93. doi: 10.1096/fj.14-265603
46. Filigheddu N, Sampietro S, Chianale F, Porporato PE, Gaggianesi M, Gregnanin I, et al. Diacylglycerol kinase α mediatses 17-β-estradiol-induced proliferation, motility, and anchorage-independent growth of Hec-1A endometrial cancer cell line through the G protein-coupled estrogen receptor GPR30. *Cell Signal.* (2011) 23:1988–96. doi: 10.1016/j.cellsig.2011.07.009
47. He YY, Du GQ, Cai B, Yan Q, Zhou L, Chen XY, et al. Estrogenic transmembrane receptor of GPR30 mediates invasion and carcinogenesis by endometrial cancer cell line RL95-2. *J Cancer Res Clin Oncol.* (2012) 138:775–83. doi: 10.1007/s00432-011-1133-7
48. Wei Y, Zhang Z, Liao H, Wu L, Wu X, Zhou D, et al. Nuclear estrogen receptor-mediated Notch signaling and GPR30-mediated PI3K/AKT signaling in the regulation of endometrial cancer cell proliferation. *Oncol Rep.* (2012) 27:504–10. doi: 10.3892/or.2011.1536
49. Ge X, Guo R, Qiao Y, Zhang Y, Lei J, Wang X, et al. The G protein-coupled receptor GPR30 mediates the nontranscriptional effect of estrogen on the activation of PI3K/Akt pathway in endometrial cancer cells. *Int J Gynecol Cancer.* (2013) 23:52–9. doi: 10.1097/IGC.0b013e31827912b8
50. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Andò S, et al. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17β-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol.* (2006) 20:631–46. doi: 10.1210/me.2005-0280
51. Zhang J, Yang Y, Zhang Z, He Y, Liu Z, Yu Y, et al. Gankyrin plays an essential role in estrogen-driven and GPR30-mediated endometrial carcinoma cell proliferation via the PTEN/PI3K/AKT signaling pathway. *Cancer Lett.* (2013) 339:279–87. doi: 10.1016/j.canlet.2012.10.037
52. Vivacqua A, Romeo E, De Marco P, De Francesco EM, Abonante S, Maggiolini M. GPER mediates the Egr-1 expression induced by 17β-estradiol and 4-hydroxitamoxifen in breast and endometrial cancer cells. *Breast Cancer Res Treat.* (2012) 133:1025–35. doi: 10.1007/s10549-011-1901-8
53. Li Y, Jia Y, Bian Y, Tong H, Qu J, Wang K, et al. Autocrine motility factor promotes endometrial cancer progression by targeting GPER-1. *Cell Commun Signal.* (2019) 17:1–14. doi: 10.1186/s12964-019-0336-4
54. Bronowicka-Kłys DE, Lianeri M, Jagodzinski PP. The role and impact of estrogens and xenoestrogen on the development of cervical cancer. *Biomed Pharmacother.* (2016) 84:1945–53. doi: 10.1016/j.biopha.2016.11.007
55. Brake T, Lambert PF. Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model. *Proc Natl Acad Sci USA.* (2005) 102:2490–5. doi: 10.1073/pnas.0409883102
56. Chung SH, Wiedmeyer K, Shai A, Korach KS, Lambert PF. Requirement for estrogen receptor α in a mouse model for human papillomavirus-associated cervical cancer. *Cancer Res.* (2008) 68:9928–34. doi: 10.1158/0008-5472.CAN-08-2051
57. Riera-Leal A, De Arellano AR, Ramírez-López IG, Lopez-Pulido EI, Rodríguez JRD, Macías-Barragan JG, et al. Effects of 60 kDa prolactin and estradiol on metabolism and cell survival in cervical cancer: co-expression of their hormonal receptors during cancer progression. *Oncol Rep.* (2018) 40:3781–93. doi: 10.3892/or.2018.6743
58. Hernandez-Silva CD, Riera-Leal A, Ortiz-Lazareno PC, Jave-Suárez LF, Arellano AR De, Lopez-Pulido EI, et al. GPER overexpression in cervical cancer versus premalignant lesions: its activation induces different forms of cell death. *Anticancer Agents Med Chem.* (2019) 19:783–91. doi: 10.2174/1871520619666190206171509
59. Friese K, Kost B, Vattai A, Marmé F, Kuhn C, Mahner S, et al. The G protein-coupled estrogen receptor (GPER/GPR30) may serve as a prognostic marker in early-stage cervical cancer. *J Cancer Res Clin Oncol.* (2018) 144:13–9. doi: 10.1007/s00432-017-2510-7
60. Zhang Q, Wu YZ, Zhang YM, Ji XH, Hao Q. Activation of G-protein coupled estrogen receptor inhibits the proliferation of cervical cancer cells via sustained activation of ERK1/2. *Cell Biochem Funct.* (2015) 33:134–42. doi: 10.1002/cbf.3097
61. Ramírez-López IG, Ramírez De Arellano A, Jave-Suárez LF, Hernández-Silva CD, García-Chagollan M, Hernández-Bello J, et al. Interaction between 17β-estradiol, prolactin and human papillomavirus induce E6/E7 transcript and modulate the expression and localization of hormonal receptors. *Cancer Cell Int.* (2019) 19:1–10. doi: 10.1186/s12935-019-0935-6
62. Yang W, Tan W, Zheng J, Zhang B, Li H, Li X. MEHP promotes the proliferation of cervical cancer via GPER mediated activation of Akt. *Eur J Pharmacol.* (2018) 824:11–6. doi: 10.1016/j.ejphar.2018.01.040
63. Akimoto T, Takasawa A, Takasawa K, Aoyama T, Murata M, Osanai M, et al. Estrogen/GPR30 signaling contributes to the malignant potentials of ER-negative cervical adenocarcinoma via regulation of claudin-1 expression. *Neoplasia.* (2018) 20:1083–93. doi: 10.1016/j.neo.2018.08.010
64. Germain D. Estrogen carcinogenesis in breast cancer. *Endocrinol Metab Clin North Am.* (2011) 40:473–84. doi: 10.1016/j.ecl.2011.05.009
65. Lv X, He C, Huang C, Hua G, Wang Z, Remmenga SW, et al. G-1 inhibits breast cancer cell growth via targeting colchicine-binding site of tubulin to interfere with microtubule assembly. *Mol Cancer Ther.* (2017) 16:1080–91. doi: 10.1158/1535-7163.MCT-16-0626
66. Deblois G, Giguère V. Oestrogen-related receptors in breast cancer: control of cellular metabolism and beyond. *Nat Rev Cancer.* (2013) 13:27–36. doi: 10.1038/nrc3396
67. Wei W, Chen ZJ, Zhang KS, Yang XL, Wu YM, Chen XH, et al. The activation of G protein-coupled receptor 30 (GPR30) inhibits proliferation of estrogen receptornegative breast cancer cells *in vitro* and *in vivo*. *Cell Death Dis.* (2014) 5:e1428. doi: 10.1038/cddis.2014.398

68. Petrie WK, Dennis MK, Hu C, Dai D, Arterburn JB, Smith HO, et al. G protein-coupled estrogen receptor-selective ligands modulate endometrial tumor growth. *Obstet Gynecol Int.* (2013) 2013:1–17. doi: 10.1155/2013/472720
69. Sandén C, Broselid S, Cornmark L, Andersson K, Daszkiewicz-Nilsson J, Mårtensson UEA, et al. G protein-coupled estrogen receptor 1/G protein-coupled receptor 30 localizes in the plasma membrane and traffics intracellularly on cytokeratin intermediate filaments. *Mol Pharmacol.* (2011) 79:400–10. doi: 10.1124/mol.110.069500
70. Lappano R, Pisano A, Maggiolini M. GPER function in breast cancer: An overview. *Front Endocrinol.* (2014) 5:1–6. doi: 10.3389/fendo.2014.00066
71. Girgert R, Emons G, Gründker C. Inactivation of GPR30 reduces growth of triple-negative breast cancer cells: possible application in targeted therapy. *Breast Cancer Res Treat.* (2012) 134:199–205. doi: 10.1007/s10549-012-1968-x
72. Mo Z, Liu M, Yang F, Luo H, Li Z, Tu G, et al. GPR30 as an initiator of tamoxifen resistance in hormone-dependent breast cancer. *Breast Cancer Res.* (2013) 15:R114. doi: 10.1186/bcr3581
73. Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, et al. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res.* (2006) 12:6359–66. doi: 10.1158/1078-0432.CCR-06-0860
74. Liu Q, Li JG, Zheng XY, Jin F, Dong HT. Expression of CD133, PAX2, ESA, and GPR30 in invasive ductal breast carcinomas. *Chin Med J.* (2009) 122:2763–9.
75. Ignatov A, Ignatov T, Weienborn C, Eggemann H, Bischoff J, Semczuk A, et al. G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer. *Breast Cancer Res Treat.* (2011) 128:457–66. doi: 10.1007/s10549-011-1584-1
76. Lappano R, Maggiolini M. GPER is involved in the functional liaison between breast tumor cells and cancer-associated fibroblasts (CAFs). *J Steroid Biochem Mol Biol.* (2018) 176:49–56. doi: 10.1016/j.jsbmb.2017.02.019
77. Scaling AL, Prossnitz ER, Hathaway HJ. GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast. *Horm Cancer.* (2014) 5:146–60. doi: 10.1007/s12672-014-0174-1
78. Liu Y, Du FY, Chen W, Fu PF, Yao MY, Zheng S-S. G15 sensitizes epithelial breast cancer cells to doxorubicin by preventing epithelial-mesenchymal transition through inhibition of GPR30. *Am J Transl Res.* (2015) 7:967–75.
79. Ignatov A, Ignatov T, Roessner A, Costa SD, Kalinski T. Role of GPR30 in the mechanisms of tamoxifen resistance in breast cancer MCF-7 cells. *Breast Cancer Res Treat.* (2010) 123:87–96. doi: 10.1007/s10549-009-0624-6
80. Yu T, Yang G, Hou Y, Tang X, Wu C, Wu XA, et al. Cytoplasmic GPER translocation in cancer-associated fibroblasts mediates cAMP/PKA/CREB/glycolytic axis to confer tumor cells with multidrug resistance. *Oncogene.* (2017) 36:2131–45. doi: 10.1038/onc.2016.370
81. Ignatov T, Claus M, Nass N, Haybaeck J, Seifert B, Kalinski T, et al. G-protein-coupled estrogen receptor GPER-1 expression in hormone receptor-positive breast cancer is associated with poor benefit of tamoxifen. *Breast Cancer Res Treat.* (2019) 174:121–7. doi: 10.1007/s10549-018-5064-8
82. Sathya S, Sudhagar S, Lakshmi BS. Estrogen suppresses breast cancer proliferation through GPER / p38 MAPK axis during hypoxia. *Mol Cell Endocrinol.* (2015) 417:200–10. doi: 10.1016/j.mce.2015.09.032
83. Liang S, Chen Z, Jiang G, Zhou Y, Liu Q, Su Q, et al. Activation of GPER suppresses migration and angiogenesis of triple negative breast cancer via inhibition of NF-κB/IL-6 signals. *Cancer Lett.* (2017) 386:12–23. doi: 10.1016/j.canlet.2016.11.003
84. Santolla MF, Avino S, Pellegrino M, De Francesco EM, De Marco P, Lappano R, et al. SIRT1 is involved in oncogenic signaling mediated by GPER in breast cancer. *Cell Death Dis.* (2015) 6:e1834–12. doi: 10.1038/cddis.2015.201
85. Lubig J, Lattrich C, Springwald A, Häring J, Schüler S, Ortmann O, et al. Effects of a combined treatment with GPR30 agonist G-1 and herceptin on growth and gene expression of human breast cancer cell lines. *Cancer Invest.* (2012) 30:372–9. doi: 10.3109/07357907.2012.666690
86. Broselid S, Cheng B, Sjöström M, Lövgren K, Klug-De Santiago HLP, Belting M, et al. G protein-coupled estrogen receptor is apoptotic and correlates with increased distant disease-free survival of estrogen receptor-positive breast cancer patients. *Clin Cancer Res.* (2013) 19:1681–92. doi: 10.1158/1078-0432.CCR-12-2376
87. Molina L, Figueroa CD, Bhoola KD, Ehrenfeld P. GPER-1/GPR30 a novel estrogen receptor sited in the cell membrane: therapeutic coupling to breast cancer. *Expert Opin Ther Targets.* (2017) 21:755–66. doi: 10.1080/14728222.2017.1350264
88. Sjöström M, Hartman L, Grabau D, Fornander T, Malmström P, Nordenskjöld B, et al. Lack of G protein-coupled estrogen receptor (GPER) in the plasma membrane is associated with excellent long-term prognosis in breast cancer. *Breast Cancer Res Treat.* (2014) 145:61–71. doi: 10.1007/s10549-014-2936-4
89. De Marco P, Cirillo F, Vivacqua A, Malaguarnera R, Belfiore A, Maggiolini M. Novel aspects concerning the functional cross-talk between the insulin/IGF-I system and estrogen signaling in cancer cells. *Front Endocrinol.* (2015) 6:10–2. doi: 10.3389/fendo.2015.00030
90. Cheng S Bin, Graeber CT, Quinn JA, Filardo EJ. Retrograde transport of the transmembrane estrogen receptor, G-protein-coupled-receptor-30 (GPR30/GPER) from the plasma membrane towards the nucleus. *Steroids.* (2011) 76:892–6. doi: 10.1016/j.steroids.2011.02.018
91. Samartzis EP, Noske A, Meisel A, Varga Z, Fink D, Imesch P. The G protein-coupled estrogen receptor (GPER) is expressed in two different subcellular localizations reflecting distinct tumor properties in breast cancer. *PLoS ONE.* (2014) 9:e83296. doi: 10.1371/journal.pone.0083296
92. Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, et al. The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res.* (2010) 70:1184–94. doi: 10.1158/0008-5472.CAN-09-3068
93. Khan D, Ansar Ahmed S. The immune system is a natural target for estrogen action: opposing effects of estrogen in two prototypical autoimmune diseases. *Front Immunol.* (2016) 6:635. doi: 10.3389/fimmu.2015.00635
94. Kvingedal AM, Smeland EB. A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue. *FEBS Lett.* (1997) 407:59–62. doi: 10.1016/S0014-5793(97)00278-0
95. Kanda N, Watanabe S. 17β-estradiol enhances the production of nerve growth factor in THP-1-derived macrophages or peripheral blood monocyte-derived macrophages. *J Invest Dermatol.* (2003) 121:771–80. doi: 10.1046/j.1523-1747.2003.12487.x
96. Rettew JA, McCall SH, Marriott I. GPR30/GPER-1 mediates rapid decreases in TLR4 expression on murine macrophages. *Mol Cell Endocrinol.* (2010) 328:87–92. doi: 10.1016/j.mce.2010.07.017
97. Cabas I, Rodenas MC, Abellán E, Meseguer J, Mulero V, García-Ayala A. Estrogen signaling through the G protein–coupled estrogen receptor regulates granulocyte activation in fish. *J Immunol.* (2013) 191:4628–39. doi: 10.4049/jimmunol.1301613
98. Rodenas MC, Tamassia N, Cabas I, Calzetti F, Meseguer J, Cassatella MA, et al. G protein-coupled estrogen receptor 1 regulates human neutrophil functions. *Biomed Hub.* (2017) 2:1–13. doi: 10.1159/000454981
99. Blesson CS, Sahlin L. Expression pattern and signalling pathways in neutrophil like HL-60 cells after treatment with estrogen receptor selective ligands. *Mol Cell Endocrinol.* (2012) 361:179–90. doi: 10.1016/j.mce.2012.04.006
100. Tamaki M, Konno Y, Kobayashi Y, Takeda M, Itoga M, Moritoki Y, et al. Expression and functional roles of G-protein-coupled estrogen receptor (GPER) in human eosinophils. *Immunol Lett.* (2014) 160:72–8. doi: 10.1016/j.imlet.2014.03.012
101. Wang C, Dehghani B, Magrisso IJ, Rick EA, Bonhomme E, Cody DB, et al. GPR30 contributes to estrogen-induced thymic atrophy. *Mol Endocrinol.* (2008) 22:636–48. doi: 10.1210/me.2007-0359
102. Isensee J, Meoli L, Zazzu V, Nabzdyk C, Witt H, Soewarto D, et al. Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology.* (2009) 150:1722–3. doi: 10.1210/en.2008-1488
103. Brunsing RL, Prossnitz ER. Induction of interleukin-10 in the T helper type 17 effector population by the G protein coupled estrogen receptor (GPER) agonist G-1. *Immunology.* (2011) 134:93–106. doi: 10.1111/j.1365-2567.2011.03471.x
104. Brunsing RL, Owens KS, Prossnitz ER. The G protein-coupled estrogen receptor (GPER) agonist G-1 expands the regulatory T-cell

population under TH17-polarizing conditions. *J Immunother.* (2013) 36:190–6. doi: 10.1097/CJI.0b013e31828d8e3b

105. Blasko E, Haskell CA, Leung S, Gualtieri G, Halks-Miller M, Mahmoudi M, et al. Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *J Neuroimmunol.* (2009) 214:67–77. doi: 10.1016/j.jneuroim.2009.06.023
106. Smith HO, Stephens ND, Qualls CR, Fligelman T, Wang T, Lin CY, et al. The clinical significance of inflammatory cytokines in primary cell culture in endometrial carcinoma. *Mol Oncol.* (2013) 7:41–54. doi: 10.1016/j.molonc.2012.07.002
107. Okamoto M, Mizukami Y. GPER negatively regulates TNFα-induced IL-6 production in human breast cancer cells via NF-κB pathway. *Endocr J.* (2016) 63:485–93. doi: 10.1507/endocrj.EJ15-0571
108. Kleuser B, Malek D, Gust R, Pertz HH, Potteck H. 17-β-estradiol inhibits transforming growth factor-β signaling and function in breast cancer cells via activation of extracellular signal-regulated kinase through the G protein-coupled receptor 30. *Mol Pharmacol.* (2008). 74:1533–43. doi: 10.1124/mol.108.046854
109. De Marco P, Lappano R, Francesco EM De, Cirillo F, Pupo M, Avino S, et al. GPER signalling in both cancer-associated fibroblasts and breast cancer cells mediates a feedforward IL1β/IL1R1 response. *Sci Rep.* (2016) 6:24354. doi: 10.1038/srep24354
110. Pupo M, Bodmer A, Berto M, Maggiolini M, Dietrich PY, Picard D. A genetic polymorphism repurposes the G-protein coupled and membrane-associated estrogen receptor GPER to a transcription factor-like molecule promoting paracrine signaling between stroma and breast carcinoma cells. *Oncotarget.* (2017) 8:46728–44. doi: 10.18632/oncotarget.18156
111. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell.* (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013

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Upregulation of G Protein-Coupled Estrogen Receptor by Chrysin-Nanoparticles Inhibits Tumor Proliferation and Metastasis in Triple Negative Breast Cancer Xenograft Model

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Triple-negative breast cancer (TNBC) is associated with a high mortality rate among women globally. TNBC shows a high rate of recurrence and distant metastasis. Particularly, the chemotherapy is limited because hormone therapy of breast cancer is ineffective. Thus, an effective chemotherapeutic agent is needed for tumor suppression. Chrysin-nanoparticles (chrysin-NPs) were investigated for their inhibitory effect on a MDA-MB-231-derived xenograft model. To gain insight into the underlying mechanisms, we conducted human matrix metalloproteinase (MMP) array, western blot, and immunohistochemistry analysis. Furthermore, *in vivo* imaging was used to monitor the chemotherapeutic efficacy of chrysin-NPs in a metastasis mouse model. Chrysin-NPs significantly inhibited the proliferation of MDA-MB-231 cells *via* the PI3K/JNK pathway and induced cell death through the p53-apoptosis pathway, leading to delayed MDA-MB-231-derived tumor growth. Interestingly, chrysin-NPs significantly induced G protein-coupled estrogen receptor (GPER) expression, which suppresses MMPs and NF-κB expression. Chrysin-NPs acted as effective metastasis inhibitors. Our results suggest that chrysin-NPs may be used as an effective adjuvant formulation to inhibit TNBC progression.

Keywords: chrysin-nanoparticle, triple-negative breast cancer, metastasis, tumor progression, G protein-coupled estrogen receptor

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INTRODUCTION

Breast cancer is a leading cause of deaths in woman worldwide (1, 2). Breast cancer is divided into four different subtypes; luminal A (estrogen receptor (ER) and progesterone receptor (PR) positive, human epidermal receptor 2 (HER2) negative and low Ki-67 level), luminal B (ER and PR positive, HER2 positive or negative and high Ki-67 level), HER2 enrich (ER and PR negative), and triple negative (ER, PR, and HER2 negative) (3, 4). The therapeutic modalities for triple-negative breast cancer (TNBC) are limited to surgery or conventional chemotherapy as TNBC patients will not respond to endocrine therapy or receptor targeting treatments (5). TNBC accounts for 15% of all cases of breast carcinoma (6), has the poorest overall survival of all breast cancer subtypes (7), and

has the highest rates of epithelial-to-mesenchymal transition (EMT) metastasis, possibly resulting from the remarkable phenotypic similarity between TNBC cells and mammary stem cells (8).

Therefore, development of targeted therapies for TNBC is urgently needed. The course of tumor metastasis entails a series of stages that lead to the formation of secondary tumors in distant organs (9). The process of invasion is instigated as the original tumor cells pass through the basement membrane and extracellular matrix, journey through the circulatory system, and attach at a new location to proliferate and produce secondary tumors (10, 11). Resultantly, research efforts focused on identifying and understanding the mechanisms concerned in tumor cell invasion may lead to the development of novel approaches to inhibit tumor progression in TNBC patients. The key enzymes responsible for ECM breakdown are matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endopeptidases involved in the regulation of cell growth, migration, angiogenesis and invasion (11–13). The role of MMPs in a variety of cancers has been reviewed elsewhere (14, 15). In breast cancer, the expression levels of MMPs were reported to be higher than in normal breast tissues (16, 17). For instance, MMP-1,−2,−7,−9,−10,−11,−13,−14, and−15 were documented for their contribution to breast cancer proliferation and metastasis (18–21). Previous studies have reported that the G protein-coupled estrogen receptor-1 (GPER, formerly known as GPR30) was associated with disease progression in cancer patients (22). A wide number of natural and synthetic compounds, including estrogens and anti-estrogens, elicit stimulatory effects in breast cancer through GPER upregulation and activation (23). Estrogen signaling and ERα are well-documented for their contribution to the progression of ER-positive breast cancers (24). In TNBC patients who lack the expression of ER, stimulation by estrogen or/and anti-estrogen is mediated via GPER (24–26), supporting the contributory role of GPER in TNBC disease progression (26). Other authors have also reported that GPER is involved in the development and/or proliferation of renal (27), endometrial (28, 29), and ovarian cancers (30, 31).

Chrysin, also called 5,7-dihydroxyflavone, is a flavone found in the clock flower and in honeycomb (32). It has various physiological activities including anti-inflammatory, antioxidant, hypoglycemic and anti-aromatase activity (32). The compound was reported to inhibit the proliferation of non-small cell lung cancer cells (33, 34). In another study, it was confirmed that invasion and migration of TNBC MDA-MB-231 cells were reduced in the presence of low concentrations of chrysin (7). However, chrysin is difficult to apply to *in vivo* system because it is insoluble in water. Thus, chrysin is effective, but its reports are limited (35, 36). In order to overcome the disadvantages (solubility and degradation), many researchers are studying to improve the efficacy and effectiveness of the drug by using a drug delivery system (37, 38). Among many drug delivery systems, polymers that synthesize biodegradable polyesters have been applied for many years (39–41), nanoparticles composed of hydrophobic poly (ε-caprolactone) (PCL) and hydrophilic poly(ethylene glycol) (PEG) among several polymers (42, 43) have been reported to be good potential carriers for anti-cancer agents (39, 44). PCL is a biodegradable, biocompatible, hydrophobic and non-toxic thermoplastic polyester (45). PEG is a common constituent for the hydrophilic outer shell and is known to reduce the adhesion of plasma proteins, solubility in water and organic solvents, stabilization of particles, and lack of toxicity (46, 47). In addition, it has been reported that polymeric nanoparticles with hydrophilic PEG outer shell can increase the circulation time of the hydrophobic anticancer agents in the body and prevent recognition by macrophages of the reticuloendothelial system (RES) after intravenous administration (47, 48). Additionally, PCL-PEG nanoparticles (without drug) were non-toxic in the liver and kidney of mice (49). Several researches reported that polymer could improve the bioavailability of chrysin (33, 50).

The aim of our study was to determine whether chrysin-nanoparticles (chrysin-NPs) may be used as an effective adjuvant formulation to inhibit the progression and metastasis of TNBC using a xenograft model.

MATERIALS AND METHODS

Preparation of Chrysin-NPs

Chrysin-NPs were produced as previously described, with minor modifications (**Figure 1**) (33). In brief, chrysin and polyethylene glycol-β-polycaprolactone copolymer (mPEG-PCL, PolySciTech, West Lafayette, IN, USA) were mixed at a ratio 1: 10 (w/w) in dichloromethane (DCM): methanol (1.5:1 v/v, Duksan reagent, Gyeonggi-do, Korea) solution. The mixture was then homogenized for 1 min and a solution of 1% polyvinyl alcohol was added to create an emulsion. Chrysin-NPs micelles were obtained upon evaporation. Chrysin-loading efficiencies were calculated as a previous report (33). Chrysin-NPs were prepared when it used. The average of encapsulated efficiency was 61.1% in chrysin-NPs.

Cell Culture

MDA-MB-231 and MDA-MB-231_luc cells (luciferase-expressing cells, kindly provided from Prof. Moon, Duksung Women's University) were maintained in RPMI-1640 medium (GenDEPOT, Barker, TX, USA) containing 10% fetal bovine serum (YOUNGINFRONTIER, Seoul, Korea) and 1% penicillin/streptomycin (GenDEPOT) in a 5% CO_2 humidified atmosphere at 37°C.

MTT Assay

MDA-MB-231 cells (5,000 cells/well) were seeded in 96-well plates and incubated for 24 h. Chrysin (Sigma-Aldrich, St Louis, MO, USA), NP or chrysin-NPs were then added for a 48 h-incubation period. G-1 and G-15 (Cayman Chemical, Michigan, USA) were added for a 24 and 48 h-incubation period. MTT (Sigma-Aldrich) was added to the media for a further 3 h, and the supernatant was gently removed and discarded. DMSO (Sigma-Aldrich) was added and absorbance (560 nm) was determined using a microplate reader (Infinite M200 PRO; Tecan Inc., Grödig, Austria). The data were represented a mean ± standard deviation (*SD*, $n = 4$).

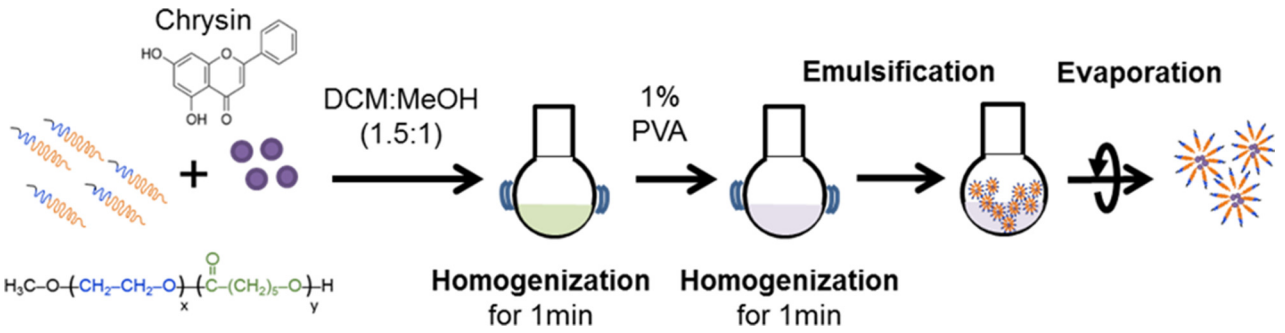

FIGURE 1 | Production flowchart of chrysin-loaded nanoparticles.

Experimental *in vivo* Study

All animal experiments were approved by the Institutional Animal Care and Use Committee of Duksung Women's University in accordance with the guidelines for the care and use of laboratory animals. Five-weeks-old female Balb/c nude mice were obtained from JUNGAH BIO (Gyeonggi, Korea). Healthy mice were left to acclimatize for 1 week prior to any procedural work. The conditions in the laboratory were 20°C, 50% humidity, and a 12/12-h light/dark cycle. Diet was provided with drinking water *ad-libitum*.

In vivo Tumor Growth Monitoring

MDA-MB-231 cells (5×10^6 cells/mouse) were orthotopically implanted into the mammary fat pads of mice. When MDA-MB-231-derived tumor reached at volume of 150–200 mm^3, mice were randomly divided into 2 groups ($n = 6$/group). Tumor sizes were measured three times per week. Tumor volumes were calculated using the following equation:

$$\text{Tumor volume } (mm^3) = (\text{Length} \times \text{Width}^2) \times 0.5$$

The data were represented as mean ± standard deviation (*SD*).

Luminescence Measurement Using *in vivo* Imaging

MDA-MB-231_luc cells (1×10^5 cells/mice) were intravenously injected into the tail vein of NGRA mice (5-weeks-old, female). To track tumor cell movement, 100 μL of D-luciferin (XenoLightTM D-luciferin potassium salt, PerkinElmer, EU) was intraperitoneally injected two times per week to NGRA mice bearing MDA-MB-231_luc cells. Mice were anesthetized with isoflurane (TerrellTM, Piramal, PA, USA) and their luminescence was quantified within 10 min of the injection using an *in vivo* imaging system (VISQUETM *in vivo* Elite, Vieworks, Gyeonggi-do, Korea). When luminescence was detected, mice were randomly divided into two groups ($n = 3$/group).

Administration of Chrysin-NPs

The mouse control group was injected with saline and the treated group was intravenously injected with chrysin-NPs (10 mg/kg) three times per week for 20 days. Mouse body weights were recorded three times per week.

Western Blot Analysis

Tumor tissues were homogenized in a radioimmunoprecipitation assay (RIPA) buffer (GenDEPOT) containing a protease and phosphatase inhibitor cocktail (GenDEPOT). The extracted proteins were quantified using a bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA, USA). Proteins were then separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). Membranes were blocked with 5% skimmed milk in tris-buffered saline supplemented with polysorbate 20 (TBST) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween20), washed and incubated with anti-p-JNK antibody (sc-12882, Santa Cruz Biotechnology, Texas, USA, 1:1,000), anti-JNK antibody (sc-571, Santa Cruz Biotechnology, 1:1,000), anti-Akt antibody (H-136, sc-8312, Santa Cruz Biotechnology, 1:1000), anti-p-Akt (C400, Cell signaling technology, MA, USA, 1:1000), anti-GSK-3α/β antibody (sc-7291, Santa Cruz Biotechnology, 1:1000), anti-NK-κB antibody (MAB3026, Millipore, Darmstadt, Germany, 1:1000), anti-MMP-10 antibody (sc-80197, Santa Cruz Biotechnology, 1:1,000), anti-MMP-2 antibody (MAB13405, Millipore, 1:1000), anti-PI3K antibody (C73F8, Cell signaling technology, 1:1000), anti-GPER antibody (ab188999, Abcam, USA, 1:1000) or anti-β-actin antibody (A4331, Sigma Aldrich, 1:5,000) at 4°C, overnight. The membrane was incubated with a secondary antibody (1:3,000) at room temperature for 3 h. Blots were visualized using enhanced chemiluminescent (ECL) solution and observed using the ChemiDocTM imager (FluorChemE, Germany). Images of western blots were quantified using Image J software.

Human Mmatrix Metalloproteinase Antibody Array

MMP-related proteins were detected using a human MMP array kit (RayBiotech, USA). The extracted tumor tissues were lysed using a RIPA buffer (GenDEPOT) containing a protease and phosphatase inhibitor cocktail (GenDEPOT). The resulting lysate was applied to the membrane array kit and incubated overnight at 4°C. After several washings, the membrane was incubated with HRP-Streptavidin for 2 h at room temperature, washed and further incubated with the kit detection buffer for

2 min at room temperature. MMP-related protein expression was observed using the ChemiDocTM imager.

Tissue Preparation

Tumor tissues were isolated and embedded into optical cutting temperature (OCT) compound (Leica, Nussloch, Germany) or paraffin. Frozen or paraffin blocks were sectioned into 5 μm slices.

TUNEL Assay

TUNEL assay was performed as previously described (33). Sections were hydrated with 100, 90, and 70% ethanol, bathed in 3% H_2O_2/distilled water (DW), washed with DW, and then incubated in DW at 60°C for 1 h. The sections were subsequently cooled to room temperature for 1 h, treated with terminal deoxynucleotidyl transferase (TdT) labeling buffer, and then incubated in TdT (Sigma)/biotinylated deoxyuridine (Roche Diagnostics, Mannheim, Germany) for 1 h at 37°C in a humidified chamber. The reaction was stopped using terminating buffer, and slides were washed with DW. The tissue sections were then blocked with 2% bovine serum albumin (BSA; bioWORLD, Dublin, OH, USA) in PBS, washed and incubated with the ABC complex also diluted in PBS. A final wash was carried out using 0.05 M Tris buffer, and color development with DAB substrate (Vector laboratories, Inc., Burlingame, CA, USA). Results were examined under a microscope (Leica). Images of tissues ($n = 5$, each group) were quantified using Image J software and plotted as percent of stained area.

Immunohistochemistry

Immunohistochemical staining was performed as previously described (33). Tissue sections were incubated with anti-Ki-67 antibody (ab16667, Abcam, 1:100) and the expression of Ki-67 was visualized using a DAB peroxidase substrate kit (Vector laboratories). Cell nuclei were visualized using hematoxylin-based counterstain solution (Sigma-Aldrich).

Statistical Analysis

All data were analyzed using Prism7 (GraphPad Software Inc., San Diego, CA, USA) using Student's *t*-test or ANOVA test. Differences were considered statistically significant when *p*-value was inferior to 0.05.

RESULTS

Chrysin-NPs Inhibit Metastasis-Related Signaling and Induce Cell Death in MDA-MB-231 Cells

The anti-cancer properties of chrysin-NPs were investigated in MDA-MB-231 TNBC-like cells using an MTT assay (**Figure 2A**). Our results showed that chrysin-NPs inhibited cell viability in a dose-dependent manner, in a similar manner to chrysin itself. No change in cell viability was detected in the NP-vehicle control wells. These observations suggest that, when delivered in a nanoparticulate format, chrysin maintained its anti-cancer properties *in vitro*. Upon western blot analysis of the expression levels of molecules known to be involved in cancer progression (**Figure 2B**), we found that PI3K and NF-κB levels were found to be lower upon treatment with chrysin-NPs. Similarly, the expression levels of MMP-10 and MMP-2, which are known to play a role in invasion and metastasis, were also in decline.

Chrysin-NPs Delay Tumor Growth Through Apoptosis

To evaluate the chemotherapeutic efficacy of chrysin-NPs, tumor growth was compared between control and chrysin-NP-treated mice. A chrysin only treated group was omitted in this study on the basis that in a previous study, chrysin-NPs were shown to be injectable and effective at preserving its biological activities *in vivo* (33). Upon repeated administration of chrysin-NPs, a significant growth delay was observed in MDA-MB-231-derived tumors (**Figure 3A**). Additionally, a decrease in tumor weight was detected in mice treated with chrysin-NPs, but the data were not significantly different due to the large difference in individual data (**Figure 3B**). Similarly, the expression level of Ki-67, a proliferation marker, declined in mice that were administered with chrysin -NPs compared to control mice (**Figure 3C**). The tumor tissues treated with chrysin-NPs presented the morphological hallmarks of apoptosis (**Figure 3D**, black arrow). Apoptotic cells (stained area) were quantified (**Figure 3D**, graph). The tumor tissues treated with chrysin-NPs significantly increased apoptosis. Altogether, our results indicate that chrysin-NPs significantly repressed TNBC-derived tumor growth through inhibition of proliferation and induction of apoptosis.

Chrysin-NPs Suppress Metastatic Signaling in MDA-MB-231-Derived Tumors

We previously showed (**Figure 2**) that chrysin-NPs exerted inhibitory effects on cancer progression *in vitro*. Here we sought to determine whether chrysin-NPs could exert any inhibition on the invasion mechanisms *in vivo*. The expression levels of MMPs were measured to evaluate invasion and migration in MDA-MB-231-derived tumor tissues. As shown in **Figure 4A**, the expression levels of MMP-1, 2, 3, 9, 10, and 13 declined in the tumor tissues isolated from mice treated with chrysin-NPs when compared with the control mice. On the other hand, the expression levels of MMP-8 (neutrophil collagenase) were on the rise upon treatment with chrysin-NPs, while expression of tissue inhibitor of metalloprotease (TIMP)-1 decreased. To gain insight into the mechanism of action of chrysin-NPs, we investigated the expression levels of markers of the PI3K/Akt pathway and GPER pathway on the basis of their reported contribution to MMPs expression, invasion, migration and metastasis (**Figure 4B**). Our results indicated a rise in the expression of PI3K and phospho-Akt (p-Akt), and a decrease in the expression of GSK-3β and NF-κB in tumors collected from mice treated with chrysin-NPs.

Chrysin-NPs Inhibit Metastasis

We next sought to investigate the effect of chrysin-NPs on TNBC-derived metastasis *in vivo*. Chrysin-NPs treated mice were observed using an *in vivo* imaging system. While control mice showed a time-dependent increase in luminescence hence metastasis, the mice treated with chrysin-NPs showed

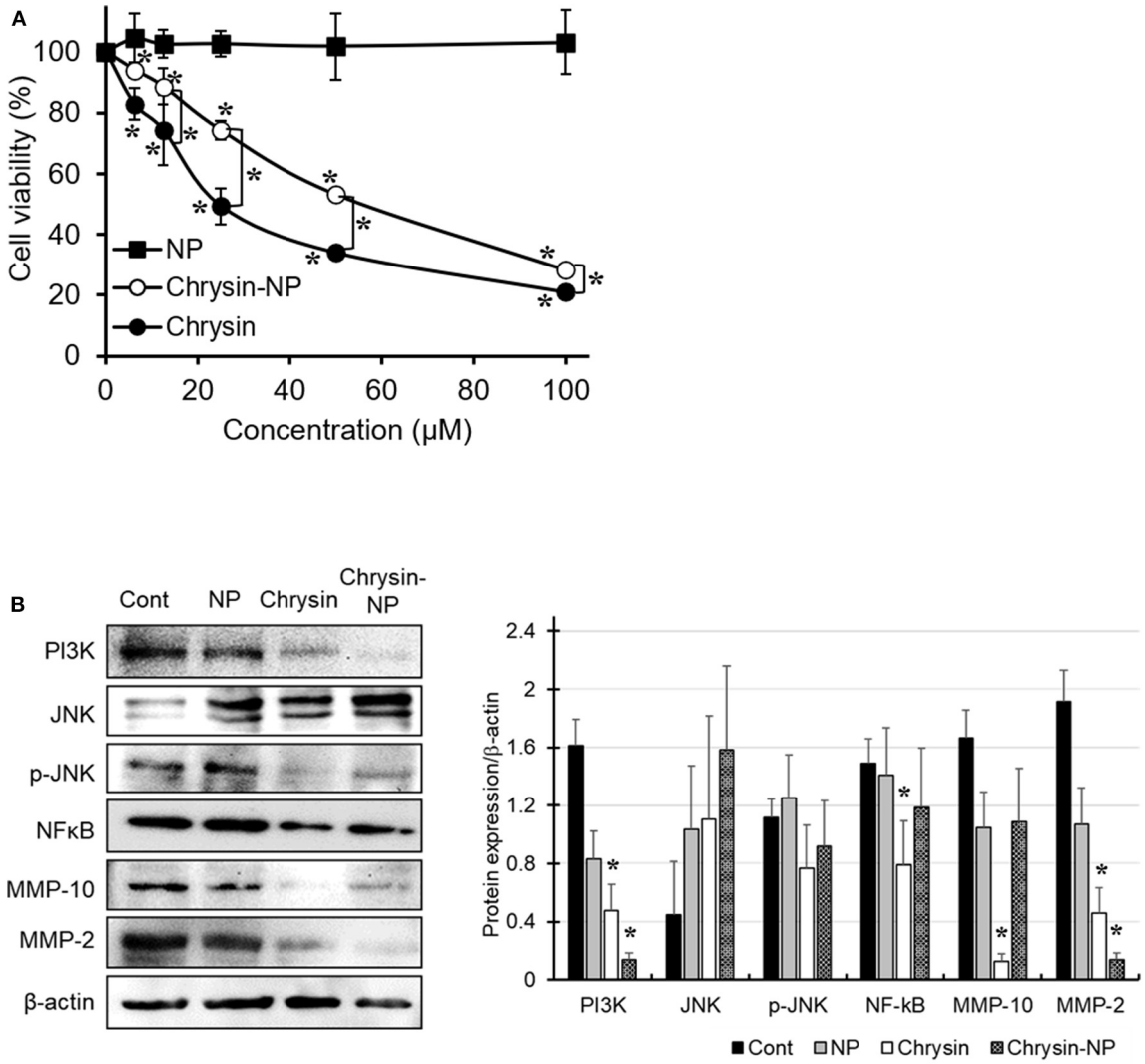

FIGURE 2 | Chrysin-NPs inhibit cell viability and PI3K/JNK signaling pathway. MDA-MB-231 cells were treated with chrysin or chrysin-NPs for 48 h. **(A)** Cell viability was assessed by MTT assay. Data were represented as mean ± S.D. ($n = 6$). *$p < 0.05$ (ANOVA). **(B)** The expression levels of PI3K/JNK signaling proteins were detected by western blot analysis. Data were represented as mean ± S.D. ($n = 3$). *$p < 0.05$ (ANOVA).

a significant lower incidence of metastasis (**Figures 5A,B**). In particular, the number and density of metastatic spots (**Figure 5C**, black arrows) observed in the liver tissues of control mice were higher than those observed in the liver tissues of chrysin-NPs-treated mice. The liver tissues of control mice showed tumor nests and loose blood vessels, while the liver tissues of chrysin-NPs treated mice didn't (**Figure 5D**).

DISCUSSION

The aim of our study was to investigate the anti-cancer properties of chrysin encapsulated into mPEG-PCL nanoparticles. Our results showed that chrysin-NPs suppressed TNBC progression via activation of the GPER signaling pathway *in vivo*. Chrysin-NPs induced apoptosis in MDA-MB-231-derived tumors (**Figure 3D**) and inhibited tumor growth in a xenograft model (**Figure 3A**). Furthermore, our data indicate that chrysin-NPs suppressed metastasis (**Figure 5**). Using these results, the proposed model was described for the inhibitory mechanism of tumor progression by chrysin-NPs in xenograft model (**Figure 6**).

In a previous study, we found that both chrysin-NPs and chrysin could delay tumor growth in a lung cancer xenograft model (33). Here, we showed that chrysin-NPs could inhibit the early stages of TNBC disease progression. Furthermore, chrysin-NPs lowered the expression levels of MMPs (**Figure 4A**). Several studies have reported that MMPs played an important role in cancer proliferation, angiogenesis, and metastasis in various

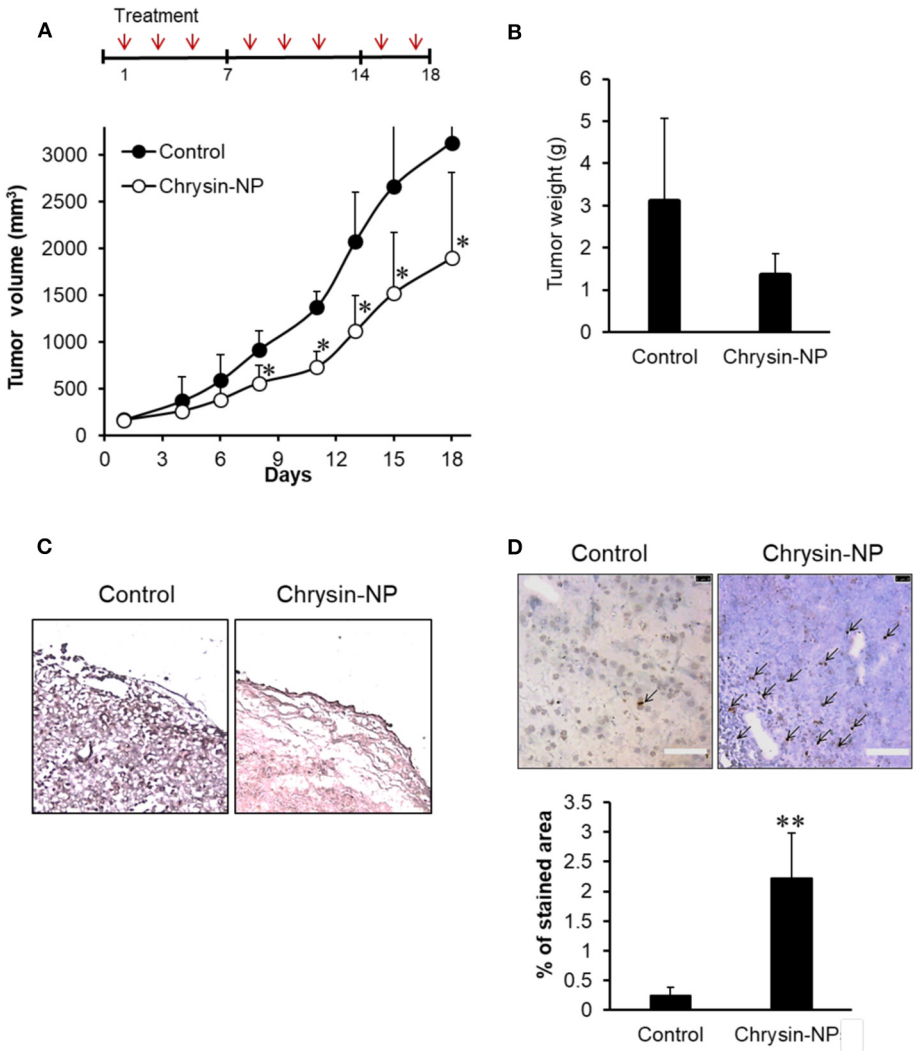

FIGURE 3 | Chrysin-NPs suppress tumor growth in MDA-MB-231-derived xenograft models. **(A)** Administration schedule of chrysin-NPs in mice. Chrysin-NPs (10 mg/kg) were administrated intravenously every other day, three times per week for 18 days. Growth curve of tumors in mice treated with chrysin-NPs. Data are represented as mean ± S.D. ($n = 6$). *$p < 0.05$ (Student's *t*-test). **(B)** Comparison of tumor weights between sham-treated control and chrysin-NP treated group. Data are represented as mean ± S.D. ($n = 4$). **(C)** Expression of Ki-67 in MDA-MB-231-derived tumor tissues visualized by immunohistochemistry. Scale bar, 100 μm. **(D)** The brown spots (black arrows) denotes TUNEL-positive apoptotic cells. Scale bar, 100 μm. Apoptotic cells are quantified. Data are represented as mean ± S.D. ($n = 5$). **$p < 0.005$ (Student's *t*-test).

cancers. Here chrysin-NPs inhibited the expression of MMP-1,−2,−3,−9,−10, and−13, while the expression of MMP-8 was higher in our MDA-MB-231-derived xenograft model than in the control group (**Figure 4A**). In particular, the expression levels of MMP-2 and−10 were consistently lower upon treatment with chrysin-NPs both *in vitro* and *in vivo* (**Figures 2**, **4**). These results are in line with previous studies (7). Down regulation of MMP-1,−2,−3,−9,−10, and−13 was associated with cancer progression and poor prognosis in breast cancer patients (16, 17, 51, 52). On the other hand, MMP-8 was shown to exert anti-proliferative and inhibitory activities on the spread of cancer cells to tissues, with a net inhibitory effect on metastasis (53). TIMP-1 was described for its inhibitory activity on metalloproteinase, but there have been conflicting reports on its anti-apoptotic activity and its role in stimulating cell proliferation in breast cancer (10, 54). In the present study, the decreased expression of TIMP-1 upon treatment with chrysin-NPs was consistent with its anti-apoptotic activities. These results reinforce our observation that chrysin-NPs exert multiple suppressive effects on cancer progression *in vivo* through inhibition of TIMP-1, cell proliferation, and metastasis via downregulation of MMPs.

We next sought to elucidate the molecular mechanisms involved in MMPs signaling upon chrysin-NP treatment. The JNK signaling pathway is well-documented for its role in cancer progression and development. Activation of the JNK pathway was confirmed by western blot analysis of tumor tissue

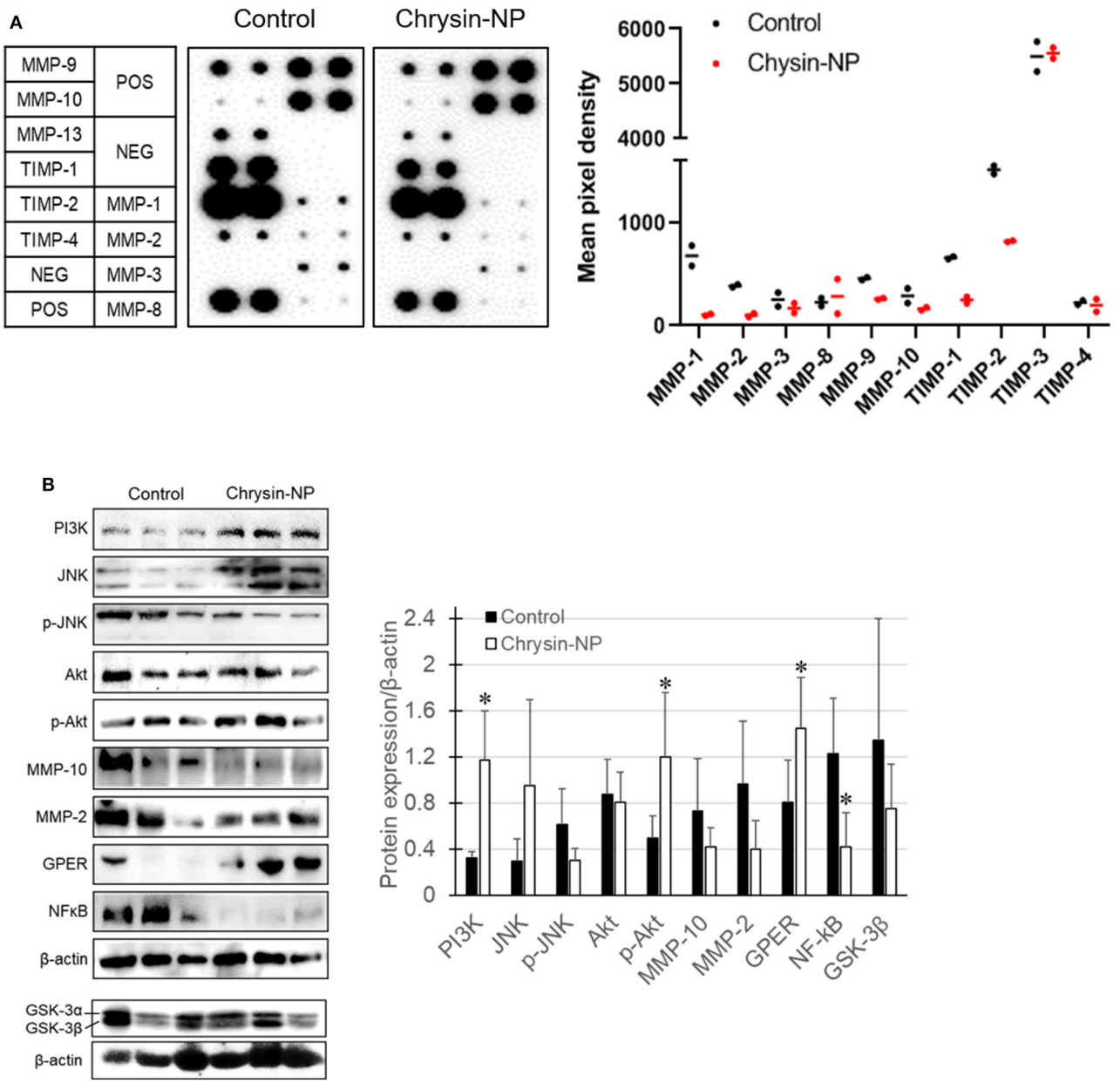

FIGURE 4 | Chrysin-NPs inhibit metastatic signaling pathways *via* GPER. **(A)** Map of MMP and TIMP arrays and their respective expression level. Relative expression of MMPs and TIMPs upon treatment with chrysin-NPs. **(B)** Metastasis-related protein levels upon incubation with chrysin-NPs. The quantification was performed through the Imaga J (■, control group; □, chrysin-NP group, left panel). Relative expression level of proteins are represented as mean ± S.D. ($n = 6$). *$p < 0.05$ (Student's *t*-test).

homogenates: expression of total-JNK was not significant change upon treatment with chrysin-NPs while the expression of p-JNK was lower. Furthermore, expression levels of MMP-2, MMP-10, and NF-kB were lower in the chrysin-NPs-treated group as compared with the control group. Previous authors have reported that MMP-2 was regulated *via* PI3K and NF-kB pathway in breast cancer (55) and the inhibition of MMP-9 was associated with the inhibition of p-JNK (56). Our results also showed that the reduction of PI3K, p-JNK, and NF-kB by chrysin occurred the inhibition of MMPs in MDA-MB-231 cells. However, chrysin-NPs induced PI3K/p-Akt expression level in MDA-MB-231-derived tumor tissues (**Figure 4B**). The result might be to cell-cell interaction, and crosstalk of cancer cell signaling and cells of tumor microenvironment (immune cells, fibroblast, stem cells). Chrysin-driven inhibition of MMP-10 was reported to be associated with the inhibition of p-Akt in breast cell lines (7). Our results also highlighted that chrysin-NPs induced higher *in vivo* activation of the PI3K/Akt signaling pathway compared

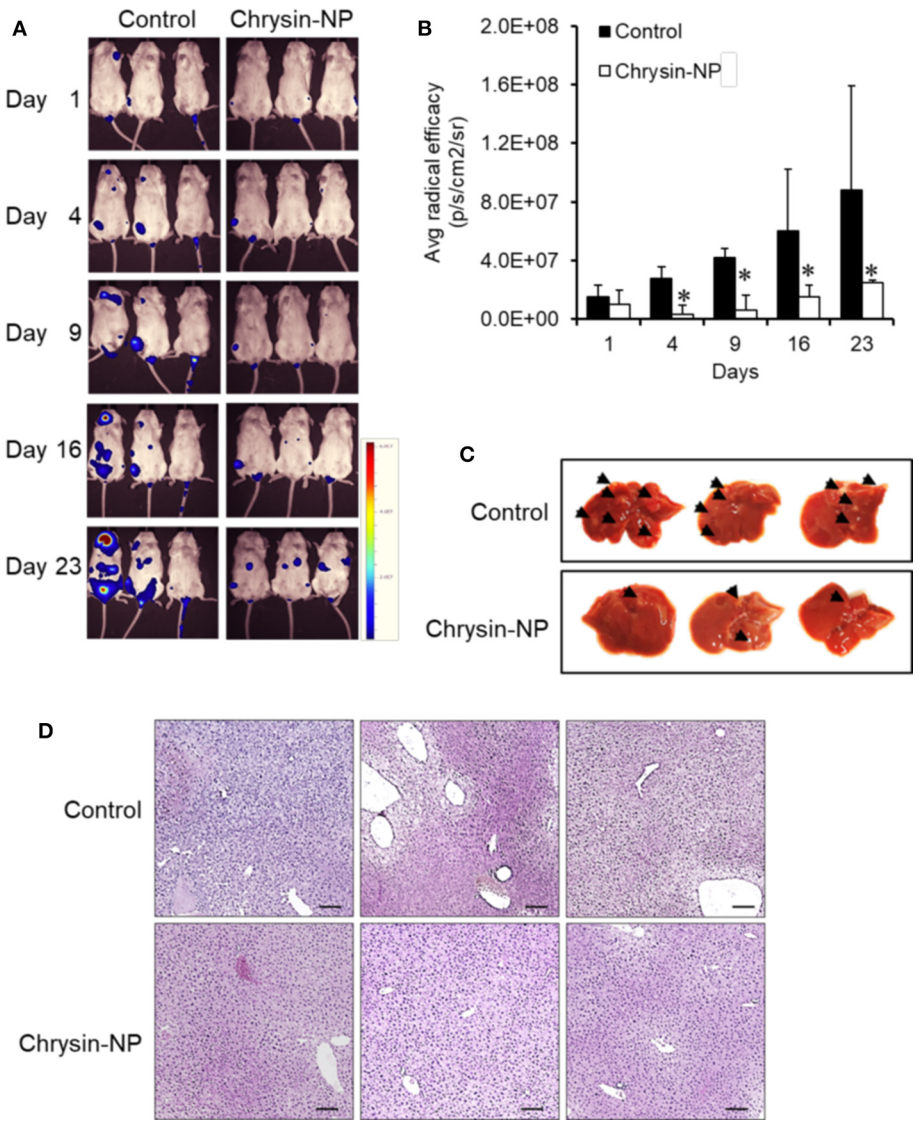

FIGURE 5 | Chrysin-NPs suppress TNBC-related metastasis. **(A)** *In vivo* imaging of luminescence in mice administered with MDA-MB-231_luc cells. **(B)** Quantitation of luminescence ($n = 3$/group). Data are represented as mean ± S.D. $^*P < 0.05$ (Student's *t*-test). **(C)** Photograph of liver **(D)** Histological analysis of liver tissues stained with H& E. Scale bar, 100 μm.

to chrysin alone (**Figure 4B**). Altogether, our results concur with the observation that chrysin-NPs may be used as an effective suppressor of cancer progression.

Next, we investigated the anti-metastatic efficacy of chrysin-NPs *in vivo*. As shown in **Figure 5**, the administration of chrysin-NPs led to significant inhibition of tumor cell metastasis. Downregulation of MMPs and TIMP-1 expressions upon treatment with chrysin-NPs was associated with lower levels of NF-kB. We also investigated the changes in expression of GPER upon treatment with chrysin-NPs. GPER is an alternate estrogen receptor with a structure distinct from the two canonical estrogen receptors, ERα and ERβ, and present multiple functions in a variety of tissues. Estrogen and/or anti-estrogen-induced effects mediated via GPER have been previously reported in TNBC. GPER is also known to regulate estrogen signaling during the progression of TNBC, but the exact mechanisms remain unclear. Previous authors have reported that GPER activation by G-1 resulted in inhibition of metastasis and EMT via NF-κB (57), and suppression of tumor proliferation in breast cancer (29, 58, 59). GPER activation is also known to inhibit metastasis and proliferation in endometrial, ovarian, liver and adrenocortical cancers (28, 31, 60, 61). The activation of GPER, another target receptor in TNBC, was shown to inhibit cell migration and invasion (22). In contrast, other authors have reported that GPER activation could induce invasion and migration in kidney cancer, with a poor prognosis for the patients (24). Here, we showed that the administration of chrysin-NPs led to higher GPER expression levels in tumor tissues (**Figure 4B**), and lower expression levels of

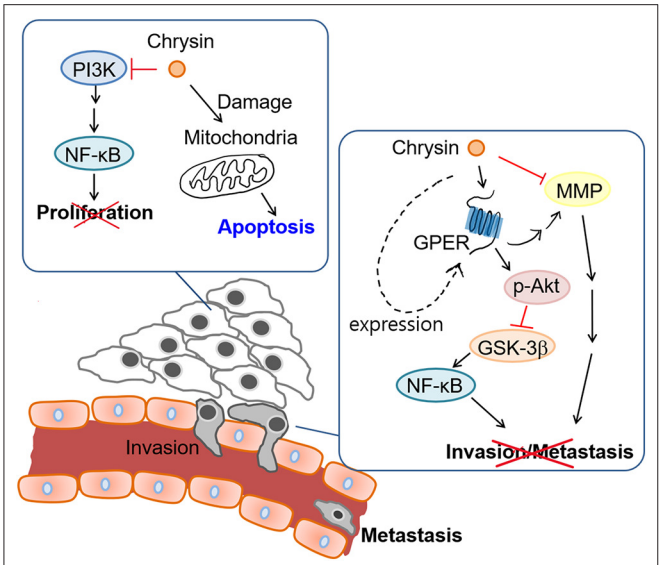

FIGURE 6 | Proposed model for the mechanism of tumor suppression by Chrysin-NPs in TNBC cells.

NF-κB, a signaling molecule downstream of GPER. As shown in **Figure 6** and **Supplementary Data**, PI3K/Akt signaling activated by GPER inhibited GSK-3β, an inhibitor of NK-kB (57, 62). The result suggests that chrysin could induce the GPER expression and suppress the metastasis of TNBC cells as a GPER agonist.

Our results showed that the loading of chrysin onto mPEG-PCL nanoparticles resulted in the enhancement of its anti-cancer properties. Most importantly, we found that chrysin-NPs activated a GPER-mediated NF-κB signaling pathway. These results strongly support that chrysin-NPs exert inhibitory effect on tumor growth and prevent metastasis. Hence our study supports the use of chrysin-NPs as a novel chemo-adjuvant for the treatment of TNBC patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Duksung Women's University.

AUTHOR CONTRIBUTIONS

JJ: conceptualization, formal analysis, data curation, writing review, editing, visualization, supervision, project administration, and funding acquisition. KK: methodology, validation, and investigation. KK and JJ: writing original draft preparation. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.560605/full#supplementary-material

REFERENCES

1. Hong S, Won YJ, Park YR, Jung KW, Kong HJ, Lee ES, et al. Cancer statistics in korea: incidence, mortality, survival, and prevalence in 2017. *Cancer Res Treat.* (2020) 52:335–50. doi: 10.4143/crt.2020.206
2. Ghoncheh M, Pournamdar Z, Salehiniya H. Incidence and mortality and epidemiology of breast cancer in the world. *Asian Pac J Cancer Prev.* (2016) 17(Suppl.3):43–6. doi: 10.7314/APJCP.2016.17.S3.43
3. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res.* (2007) 13:4429–34. doi: 10.1158/1078-0432.CCR-06-3045
4. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA.* (2003) 100:8418–23. doi: 10.1073/pnas.0932692100
5. Aydiner A, Derin D, Topuz E, Guney N, Saip P, Eralp Y, et al. MAPK overexpression is associated with anthracycline resistance and increased risk for recurrence in patients with triple-negative breast cancer. *Ann Oncol.* (2007) 19:669–74. doi: 10.1093/annonc/mdm522
6. deSantis C, Siegel R, Bandi P, Jemal A. Breast cancer statistics, 2011. *CA Cancer J Clin.* (2011) 61:409–18. doi: 10.3322/caac.20134
7. Yang B, Huang J, Xiang T, Yin X, Luo X, Huang J, et al. Chrysin inhibits metastatic potential of human triple-negative breast cancer cells by modulating matrix metalloproteinase-10, epithelial to mesenchymal transition, and PI3K/Akt signaling pathway. *J Appl Toxicol.* (2014) 34:105–12. doi: 10.1002/jat.2941
8. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med.* (2010) 363:1938–48. doi: 10.1056/NEJMra1001389
9. Lirdprapamongkol K, Sakurai H, Abdelhamed S, Yokoyama S, Maruyama T, Athikomkulchai S, et al. A flavonoid chrysin suppresses hypoxic survival and metastatic growth of mouse breast cancer cells. *Oncol Rep.* (2013) 30:2357–64. doi: 10.3892/or.2013.2667
10. Park SY, Kim YH, Kim Y, Lee SJ. Frondoside A has an anti-invasive effect by inhibiting TPA-induced MMP-9 activation via NF-kappaB and AP-1 signaling in human breast cancer cells. *Int J Oncol.* (2012) 41:933–40. doi: 10.3892/ijo.2012.1518
11. Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. *Semin Cancer Biol.* (2000) 10:415–33. doi: 10.1006/scbi.2000.0379
12. Bergers G, Coussens LM. Extrinsic regulators of epithelial tumor progression: metalloproteinases. *Curr Opin Genet Dev.* (2000) 10:120–7. doi: 10.1016/S0959-437X(99)00043-X

13. Tester AM, Waltham M, Oh SJ, Bae SN, Bills MM, Walker EC, et al. Pro-matrix metalloproteinase-2 transfection increases orthotopic primary growth and experimental metastasis of MDA-MB-231 human breast cancer cells in nude mice. *Cancer Res.* (2004) 64:652–8. doi: 10.1158/0008-5472.CAN-0384-2
14. Du X, Lin BC, Wang QR, Li H, Ingalla E, Tien J, et al. MMP-1 and Pro-MMP-10 as potential urinary pharmacodynamic biomarkers of FGFR3-targeted therapy in patients with bladder cancer. *Clin Cancer Res.* (2014) 20:6324–35. doi: 10.1158/1078-0432.CCR-13-3336
15. Zhang X, Yin P, Di D, Luo G, Zheng L, Wei J, et al. IL-6 regulates MMP-10 expression via JAK2/STAT3 signaling pathway in a human lung adenocarcinoma cell line. *Anticancer Res.* (2009) 29:4497–501.
16. Hegedus L, Cho H, Xie X, Eliceiri GL. Additional MDA-MB-231 breast cancer cell matrix metalloproteinases promote invasiveness. *J Cell Physiol.* (2008) 216:480–5. doi: 10.1002/jcp.21417
17. Benson CS, Babu SD, Radhakrishna S, Selvamurugan N, Ravi Sankar B. Expression of matrix metalloproteinases in human breast cancer tissues. *Dis Markers.* (2013) 34:395–405. doi: 10.1155/2013/420914
18. Pivetta E, Scapolan M, Pecolo M, Wassermann B, Abu-Rumeileh I, Balestreri L, et al. MMP-13 stimulates osteoclast differentiation and activation in tumour breast bone metastases. *Breast Cancer Res.* (2011) 13:R105. doi: 10.1186/bcr3047
19. Basset P, Bellocq JP, Wolf C, Stoll I, Hutin P, Limacher JM, et al. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature.* (1990) 348:699–704. doi: 10.1038/348699a0
20. Sternlicht MD, Bergers G. Matrix metalloproteinases as emerging targets in anticancer therapy: status and prospects. *Emerg Therapeutic Targets.* (2000) 4:609–33. doi: 10.1517/14728222.4.5.609
21. Cathcart J, Pulkoski-Gross A, Cao J. Targeting matrix metalloproteinases in cancer: bringing new life to old ideas. *Genes Dis.* (2015) 2:26–34. doi: 10.1016/j.gendis.2014.12.002
22. Jung J. Role of G protein-coupled estrogen receptor in cancer progression. *Toxicol Res.* (2019) 35: 209–14. doi: 10.5487/TR.2019.35.3.209
23. Lappano R, Maggiolini M. G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov.* (2011) 10:47–60. doi: 10.1038/nrd3320
24. Liang S, Chen Z, Jiang G, Zhou Y, Liu Q, Su Q, et al. Activation of GPER suppresses migration and angiogenesis of triple negative breast cancer via inhibition of NF-kappaB/IL-6 signals. *Cancer Lett.* (2017) 386:12–23. doi: 10.1016/j.canlet.2016.11.003
25. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol.* (2011) 7:715–26. doi: 10.1038/nrendo.2011.122
26. Marjon NA, Hu C, Hathaway HJ, Prossnitz ER. G protein-coupled estrogen receptor regulates mammary tumorigenesis and metastasis. *Mol Cancer Res.* (2014) 12:1644–54. doi: 10.1158/1541-7786.MCR-14-0128-T
27. Liang S, Chen Z, Jiang G, Zhou Y, Liu Q, Su Q, et al. Corrigendum to "Activation of GPER suppresses migration and angiogenesis of triple negative breast cancer via inhibition of NF-kappaB/IL-6 signals" [Cancer Lett. 386 (2017) 12-23]. *Cancer Lett.* (2018) 414:310. doi: 10.1016/j.canlet.2017.10.025
28. Du GQ, Zhou L, Chen XY, Wan XP, He YY. The G protein-coupled receptor GPR30 mediates the proliferative and invasive effects induced by hydroxytamoxifen in endometrial cancer cells. *Biochem Biophys Res Commun.* (2012) 420:343–9. doi: 10.1016/j.bbrc.2012.02.161
29. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Ando S, et al. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol.* (2006) 20:631–46. doi: 10.1210/me.2005-0280
30. Ignatov T, Modl S, Thulig M, Weißenborn C, Treeck O, Ortmann O, et al. GPER-1 acts as a tumor suppressor in ovarian cancer. *J Ovarian Res.* (2013) 6:51. doi: 10.1186/1757-2215-6-51
31. Albanito L, Madeo A, Lappano R, Vivacqua A, Rago V, Carpino A, et al. G protein-coupled receptor 30 (GPR30) mediates gene expression changes and growth response to 17beta-estradiol and selective GPR30 ligand G-1 in ovarian cancer cells. *Cancer Res.* (2007) 67:1859–66. doi: 10.1158/0008-5472.CAN-06-2909
32. Jung J. Emerging utilization of chrysin using nanoscale modification. *J Nanomater.* (2016) 7:2016. doi: 10.1155/2016/2894089
33. Kim KM, Lim HK, Shim SH, Jung J. Improved chemotherapeutic efficacy of injectable chrysin encapsulated by copolymer nanoparticles. *Int J Nanomed.* (2017) 12:1917–25. doi: 10.2147/IJN.S132043
34. Lim HK, Kim KM, Jeong SY, Choi EK, Jung J. Chrysin increases the therapeutic efficacy of docetaxel and mitigates docetaxel-induced edema. *Integr Cancer Ther.* (2017) 16:496–504. doi: 10.1177/1534735416645184
35. Eatemadi A, Daraee H, Aiyelabegan HT, Negahdari B, Rajeian B, Zarghami N. Synthesis and characterization of chrysin-loaded PCL-PEG-PCL nanoparticle and its effect on breast cancer cell line. *Biomed Pharmacother.* (2016) 84:1915–22. doi: 10.1016/j.biopha.2016.10.095
36. Anari E, Akbarzadeh A, Zarghami N. Chrysin-loaded PLGA-PEG nanoparticles designed for enhanced effect on the breast cancer cell line. *Artif Cells Nanomed Biotechnol.* (2016) 44:1410–6. doi: 10.3109/21691401.2015.1029633
37. Rosen H, Abribat T. The rise and rise of drug delivery. *Nat Rev Drug Discov.* (2005) 4:381–5. doi: 10.1038/nrd1721
38. Danafar H. Applications of copolymeric nanoparticles in drug delivery systems. *Drug Res.* (2016) 66:506–19. doi: 10.1055/s-0042-109865
39. Danafar H, Rostamizadeh K, Davaran S, Hamidi M. Drug-conjugated PLA-PEG-PLA copolymers: a novel approach for controlled delivery of hydrophilic drugs by micelle formation. *Pharm Dev Technol.* (2017) 22:947–57. doi: 10.3109/10837450.2015.1125920
40. Duncan R, Ringsdorf H, Satchi-Fainaro R. Polymer therapeutics–polymers as drugs, drug and protein conjugates and gene delivery systems: past, present and future opportunities. *J Drug Target.* (2006) 14:337–41. doi: 10.1080/10611860600833856
41. Ting JM, Porter WW 3rd, Mecca JM, Bates FS, Reineke TM. Advances in polymer design for enhancing oral drug solubility and delivery. *Bioconjug Chem.* (2018) 29:939–52. doi: 10.1021/acs.bioconjchem.7b00646
42. Grossen P, Witzigmann D, Sieber S, Huwyler J. PEG-PCL-based nanomedicines: a biodegradable drug delivery system and its application. *J Control Release.* (2017) 260:46–60. doi: 10.1016/j.jconrel.2017.05.028
43. Zou T, Dembele F, Beugnet A, Sengmanivong L, de Marco A, Li MH. Nanobody-functionalized polymersomes. *J Control Release.* (2015) 213:e79–80. doi: 10.1016/j.jconrel.2015.05.132
44. Li R, Yan J, Xie L, Zhang Y, Gao J, Liu Q, et al. Facile optimization and evaluation of PEG-PCL block copolymeric nanoparticles for anticancer drug delivery using copolymer hybrids and histoculture drug response assays. *J Biomed Nanotechnol.* (2018) 14:321–30. doi: 10.1166/jbn.2018.2485
45. Sinha VR, Bansal K, Kaushik R, Kumria R, Trehan A. Poly-epsilon-caprolactone microspheres and nanospheres: an overview. *Int J Pharm.* (2004) 278:1–23. doi: 10.1016/j.ijpharm.2004.01.044
46. Cuong NV, Hsieh MF. Recent advances in pharmacokinetics of polymeric excipients used in nanosized anti-cancer drugs. *Curr Drug Metab.* (2009) 10:842–50. doi: 10.2174/138920009790274586
47. Otsuka H, Nagasaki Y, Kataoka K. PEGylated nanoparticles for biological and pharmaceutical applications. *Adv Drug Deliv Rev.* (2003) 55:403–19. doi: 10.1016/S0169-409X(02)00226-0
48. Zahr AS, Davis CA, Pishko MV. Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol). *Langmuir.* (2006) 22:8178–85. doi: 10.1021/la060951b
49. Cuong NV, Hsieh MF, Chen YT, Liau I. Doxorubicin-loaded nanosized micelles of a star-shaped poly (epsilon-caprolactone) -polyphosphoester block co-polymer for treatment of human breast cancer. *J Biomater Sci Polym Ed.* (2011) 22:1409–26. doi: 10.1163/092050610X510533
50. Baidya D, Kushwaha J, Mahadik K, Patil S. Chrysin-loaded folate conjugated PF127-F68 mixed micelles with enhanced oral bioavailability and anticancer activity against human breast cancer cells. *Drug Dev Ind Pharm.* (2019) 45:852–60. doi: 10.1080/03639045.2019.1576726
51. Sarkar S, Nuttall RK, Liu S, Edwards DR, Yong VW. Tenascin-C stimulates glioma cell invasion through matrix metalloproteinase-12. *Cancer Res.* (2006) 66:11771–80. doi: 10.1158/0008-5472.CAN-05-0470
52. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol.* (2001) 17:463–516. doi: 10.1146/annurev.cellbio.17.1.463
53. Juurikka K, Butler GS, Salo T, Nyberg P, Astrom P. The role of MMP8 in cancer: a systematic review. *Int J Mol Sci.* (2019) 20:4506. doi: 10.3390/ijms20184506

54. Cheng G, Fan X, Hao M, Wang J, Zhou X, Sun X. Higher levels of TIMP-1 expression are associated with a poor prognosis in triple-negative breast cancer. *Mol Cancer.* (2016) 15:30. doi: 10.1186/s12943-016-0515-5
55. Das K, Prasad R, Ansari SA, Roy A, Mukherjee A, Sen P. Matrix metalloproteinase-2: a key regulator in coagulation proteases mediated human breast cancer progression through autocrine signaling. *Biomed Pharmacother.* (2018) 105:395–406. doi: 10.1016/j.biopha.2018.05.155
56. Xia Y, Lian S, Khoi PN, Yoon HJ, Joo YE, Chay KO, et al. Chrysin inhibits tumor promoter-induced MMP-9 expression by blocking AP-1 via suppression of ERK and JNK pathways in gastric cancer cells. *PLoS ONE.* (2015) 10:e0124007. doi: 10.1371/journal.pone.0124007
57. Chen ZJ, Wei W, Jiang GM, Liu H, Wei WD, Yang X, et al. Activation of GPER suppresses epithelial mesenchymal transition of triple negative breast cancer cells via NF-kappaB signals. *Mol Oncol.* (2016) 10:775–88. doi: 10.1016/j.molonc.2016.01.002
58. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J.* (2009) 28:523–32. doi: 10.1038/emboj.2008.304
59. Vivacqua A, Romeo E, De Marco P, De Francesco EM, Abonante S, Maggiolini M. GPER mediates the Egr-1 expression induced by 17β-estradiol and 4-hydroxitamoxifen in breast and endometrial cancer cells. *Breast Cancer Res Treat.* (2012) 133:1025–35. doi: 10.1007/s10549-011-1901-8
60. Wei T, Chen W, Wen L, Zhang J, Zhang Q, Yang J, et al. G protein-coupled estrogen receptor deficiency accelerates liver tumorigenesis by enhancing inflammation and fibrosis. *Cancer Lett.* (2016) 382:195–202. doi: 10.1016/j.canlet.2016.08.012
61. Chimento A, Sirianni R, Casaburi I, Zolea F, Rizza P, Avena P, et al. GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth *in vitro* and *in vivo*. *Oncotarget.* (2015) 6:19190–203. doi: 10.18632/oncotarget.4241
62. Medunjanin S, Schleithoff L, Fiegehenn C, Weinert S, Zuschratter W, Braun-Dullaeus RC. GSK-3beta controls NF-kappaB activity via IKKgamma/NEMO. *Sci Rep.* (2016) 6:38553. doi: 10.1038/srep38553

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REVIEW
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G Protein-Coupled Estrogen Receptor in Immune Cells and Its Role in Immune-Related Diseases

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G protein-coupled estrogen receptor 1 (GPER1), is a functional estrogen receptor involved in estrogen related actions on several systems including processes of the nervous, reproductive, metabolic, cardiovascular, and immune system. Regarding the latter, GPER is expressed in peripheral B and T lymphocytes as well as in monocytes, eosinophils, and neutrophils. Several studies have implicated GPER in immune-mediated diseases like multiple sclerosis, Parkinson's disease, and atherosclerosis-related inflammation, while a recent report suggests that its deletion could be responsible for a form of familial immunodeficiency. It has also been suggested that it is a key regulator of immune-mediated events in breast, pancreatic, prostate, and hepatocellular cancer as well as in melanoma. GPER has been also reported to interact with classic ER-alpha or its splice variants in order to modify immune functions. This review aims to present current knowledge relating GPER to immune functions, the cellular and signaling pathways involved, as well as the potential clinical implications of GPER modulation in immune-related diseases.

Keywords: GPER1, estrogen receptor, immune cells, inflammation, TLR4

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INTRODUCTION

Our perception of the mechanisms involved in estrogen (patho)physiological effects has progressed significantly in the last fifteen years, with the discovery of G protein-coupled estrogen receptor 1 (GPER1, previously known as G protein-coupled receptor 30 or GPR30). GPER1 is a seven transmembrane-domain G protein-coupled receptor (GPCR) that in 2005 was reported independently by two research groups to bind 17β-estradiol (E2) with high affinity and to induce unique and specific signaling, upon its activation by this ligand (1, 2). GPER1 was at the time the answer for the rapid estrogen actions pointing out the need for a paradigm shift in the field. However, parallel reports for membrane anchoring of classic ERα and ERβ *via* palmitoylation, tethered actions, and role of specific ER splice variants, further added to the complexity of rapid, extranuclear steroid signaling (3). Hundreds of studies further explored the role of GPER1 in cellular physiology and the regulation of rapid steroid actions. As a result, the role of GPER1 in estrogen actions in several systems is now well accepted, even though not fully elucidated yet.

Most of the GPER1 actions are mediated through the rapid activation of G proteins, Adenylyl cyclase/PKA, tyrosine kinases, the membrane-associated guanylate kinase (MAGUK) family of PDZ domain proteins, MAP kinases, and PI3K (4–6). Moreover, several lines of evidence also suggest that, upon

GPER1 activation, specific actions on gene expression can be modified (7). Apart from estradiol, a variety of drugs, phytoestrogens, and xenoestrogens were found to exert actions *via* GPER1, while potent synthetic agonists and antagonists have been synthesized and utilized to understand GPER1-dependent actions and specific estradiol biologic effects (8). Multiple research groups have identified specific GPER1 actions in distinct cell types, organs, and systems. The research produced in this field in the last 15 years is now reaching maturity and GPER1 targeting is now also studied as a novel therapeutic approach in cancer, cerebrovascular, metabolic, and neurodegenerative diseases (9–11). Indeed, GPER1 has been found to regulate estrogenic effects on specific immune functions, not only in humans but also in various other species (12–16). Therefore, research regarding the role of this membrane receptor in diseases characterized by sexual dimorphism, like atherosclerosis, some types of cancer, and several autoimmune conditions, could provide further insight into the pathophysiology of these diseases and create opportunities for novel therapies.

The role of GPER1 in the immune system is another field of potential GPER1 actions. The immunomodulatory effect of GPER1 has also been implicated in cancer immune tolerance, although data for potential therapeutic implications in this field are limited (17, 18). In this review, we will analyze the current knowledge regarding the expression of GPER1 in the immune system and will review the diseases, or disease-models, where this receptor might play an important pathophysiological role. Additionally, the signaling mechanisms involved and the interaction of GPER1 with critical molecules regulating major immune functions will also be discussed.

TABLE 1 | Actions attributed to GPER1 in each cell type of the immune system.

Cell type	Action	Reference
T-lymphocytes	• Induces thymic atrophy (controversial)	(21–25)
	• Induces IL-10 in CD4+ T cells	(26, 27)
	• Enhances CD4+ T cells Foxp3 expression and Foxp3 positive T-cells	(28)
	• Increases T-cell proliferation (fish)	(29, 30)
B-lymphocytes	• Decreases activation-induced B cell proliferation	(29)
	• Increases IgG (memory)	
	• Inhibits proliferation (fish)	(30)
	• Enhances natural antibody production (mice)	(30).
Monocytes/ macrophages	• Decreases TLR4 expression,	(31)
	• Blocks the inflammatory response to LPS and expression of PGE2, IL-6, and TNFα	
	• Mediates E2 anti-inflammatory action on LPS activated human monocytes and *in vitro* differentiated macrophages *via* interaction with ERα36 and NFκB and blocks IL-6 and TNFα release	(32)
	• Inhibits hepatocarcinogenesis in the DEN induced HCC model by inhibiting IL-6 expression by Kupffer cells	(33)
Eosinophils	• Increases CCL11 induced chemotaxis	(34)
	• Blocks caspase-3 dependent spontaneous apoptosis in resting eosinophils	
	• Increases apoptosis in IL-5 stimulated eosinophils.	
	• Suppresses airway inflammation (mouse asthma model)	(35)
Neutrophils	• Increases IL1b, CXCL8, and COX2 expression	(36)
	• Enhances respiratory burst	
	• Increases life span	
	• Has anti-inflammatory effects in the equivalent of human neutrophils in fish	(13, 37, 38)

EXPRESSION AND FUNCTION OF GPER1 BY CELLS OF THE IMMUNE SYSTEM

GPER1 mRNA is expressed in CD34+, CD38+ hemopoietic cells, and mature cells of the immune system. Its appearance in the early stages of immune cell development suggests its role in their maturation and function (19, 20). The functional role of GPER1 in each population of immune cells has been explored in several published works. However, the depth of our knowledge is still limited and several controversies have arisen from conflicting results, as presented in **Table 1**. Below, we summarize the findings of GPER1 detection and actions in specific immune cell populations:

Lymphocytes

GPER1 is expressed in bone marrow B lineage CD19+ IgM- cells (pro- and pre-B cells), in peripheral B cells, and in circulating T cells (19, 21, 29). Peripheral T and B cells express GPER1, showing a distinct subcellular distribution, different from the classical ERα and ERβ. These cells also exhibit membrane binding sites for estrogen that are attributed to GPER1 and membrane-bound forms of the classic estrogen receptors, which display significant ligand-dependent internalization and recycling (29).

T Lymphocytes

In T-cells, GPER1 expression has been specifically reported in CD4+ $CD44^{lo}CD62L^{hi}Foxp3^{-}$ naive T-cell and CD4+ Foxp3+ T-reg cells (28). GPER1 has been found to affect cytokine expression, lineage progression, and proliferation. Although the mechanisms involved are not fully elucidated, early GPER1 expression in adaptive immunity-related cells could affect their maturation. Early reports disclosed that estrogen mediates thymic atrophy *via* the classic estrogen receptor ERα (39). Interestingly, after the discovery of GPER1, it was found that its loss in mice leads to partial resistance to E2-induced thymic atrophy *via* thymocyte/naive T-cell apoptosis. In contrast, in wild-type mice, the GPER1 agonist G-1 effects on thymic atrophy were equivalent to that of estrogen (19, 21). This finding, however, could not be reproduced in three other early studies (22, 23), using a different KO mouse strain.

The role of GPER1 in the induction of IL-10 expression by CD4+ T cells and especially in the T helper 17 (Th17) subpopulation, has been studied more thoroughly; GPER1 specific agonist G1 increases IL-10 expression in these cells, activating the ERK kinase pathway, a well-established signaling pathway of this receptor (26, 27). Increased IL-10 expression was

also observed *in vivo*, in splenocytes isolated from G1-treated male mice (26). G-1 also enhances Foxp3 expression in CD4+ T cells and increases the number of Foxp3+ T-cells, when they are polarized *in vitro* toward the Th17 lineage (28). In the same study, it is reported that G1 also induces small increases in the expression of PD-1 and CTLA-4. It is to note that prior investigations have attributed this estrogen-elicited action to an effect of the classical estrogen receptors (40, 41).

Finally, although estrogen has been reported to decrease activation-induced T cell proliferation, E2-BSA, acting exclusively on membrane estrogen receptors, enhanced cell growth. In addition, in fish, estrogen increases the proliferation of T-cells specifically *via* GPER1 (29, 30).

B-Lymphocytes

Similarly to T-cells, activation-induced B cell proliferation is decreased by estrogen, while membrane-only acting estrogen enhances it. The effect of membrane acting estrogen, potentially *via* GPER1, also increases IgG production in mice, but only when immune memory has been established (29). This suggests that GPER1 may affect the function of memory B-lymphocytes or plasmacytes, a finding that should be further studied, since it may explain recorded gender-specific differences in adaptive immunity. In fish, however, GPER1 mediates estrogen-dependent inhibition of B-cell proliferation GPER1 (30), while both estrogen and G1 also raise natural antibody production in mice *via* GPER1 (30).

Monocytes/Macrophages

Several research groups have reported that GPER1 is expressed in monocytic cell lines, CD14+ monocytes, in *in vitro* differentiated macrophages and in tissue-resident macrophages (19, 31, 32, 42).

It has been repeatedly shown that estrogen can inhibit monocyte/macrophages activation and this was attributed to the classical estrogen receptors (43). This estrogenic action could be crucial for diseases that display sexual dimorphism, like atherogenesis, asthma, and some types of cancer. It was later found that GPER1 mediates the anti-inflammatory effect of estrogen in the monocyte/macrophages population through multiple mechanisms. Both 17beta-estradiol and G1 decrease TLR4 expression in RAW 264.7 cells and primary mouse peritoneal macrophages and this effect is abolished in GPER1 knockdown cells. Treatment of RAW 264.7 cells with G1 leads to a diminished inflammatory response to LPS and decreased expression of PGE2, IL-6, and TNFα (31). Our group has reported that GPER1 is also crucial for E2 anti-inflammatory action in LPS activated primary human monocytes and *in vitro* differentiated human macrophages: GPER1 mediated this effect *via* its direct physical interaction with the 36-kDa ERα splice variant, called ERα36, and the p65 subunit of NFκB. The formation of this hetero-protein complex led to a reduced capacity of NFκB to activate the expression of key molecules like IL-6 and TNFα (32). This is physiologically relevant since we also found expression and co-localization of ERα36 and GPER1 in macrophage cells infiltrating coronary artery atherosclerosis plaques from coronary heart disease patients.

In an elegant set of experiments focusing on liver tumorigenesis, Wei et al. found that GPER1 knockout mice display accelerated hepatocarcinogenesis in the diethylnitrosamine (DEN) hepatocellular carcinoma (HCC) model. This was attributed to increased local inflammation and fibrosis, accompanied by elevated IL-6. Since the major source of IL-6 in the liver are Kupffer cells (liver resident macrophages), they isolated bone marrow mononuclear cells from wild type and knockout mice and found that LPS induced IL-6 production is blocked in these cells in a GPER1-dependent manner (33). These findings are also relevant for human HCC where they found decreased GPER1 expression in tumors versus adjacent non-tumor tissue.

Eosinophils

Although it has been reported that CD15+ cells do not express GPER1 (19) another group reported GPER1 expression both at the mRNA and protein level in highly purified eosinophils (34). In their study, Tamaki et al. found that G-1 does not provoke eosinophil degranulation or chemotaxis, but increased CCL11-induced chemotaxis. GPER1 effect on eosinophil apoptosis is dependent on their activation status. G1 blocked caspase-3 dependent spontaneous apoptosis of resting eosinophils but had an opposite effect on IL-5 stimulated cells. These findings suggest that low estrogen levels may lead to worsening of eosinophil-dependent conditions *via* loss of GPER1 dependent control. Interestingly, the decline of estrogen levels during the premenstrual period is believed to worsen medical conditions like asthma, a well-known eosinophil-dependent condition (44). Although data in humans are lacking, it was found that GPER1 suppressed airway inflammation in a mouse model of asthma (35). GPER1 could, therefore, be part of the pathophysiology of several eosinophil related diseases that display sexual dimorphism or perimenstrual variation.

Neutrophils

The expression of GPER1 on the surface of human neutrophils was only recently reported, related to a significantly modified gene expression profile. Previous reports have shown that polymorphonuclear cells express the classical ERα and ERβ estrogen receptors, which were believed to have mostly anti-inflammatory actions (45, 46). Contrary to these reports regarding the effects of estrogen on neutrophils, G1 activation of GPER1 triggered a proinflammatory reaction with increased cytokine (IL1b, CXCL8) and COX2 expression, enhanced respiratory burst and increased life span (36). This finding supports a differential role of GPER1, compared to classical estrogen receptors, in regulating inflammation in these cells. However, it seems that several key pieces of the puzzle are still missing.

Significantly more data regarding the expression and the role of GPER1 in neutrophils come from studies in fish. The research groups involved, however, have reported that in fish G1 has mostly anti-inflammatory effects, *via* changes in the expression

profile of acidophilic granulocytes (the equivalent of human neutrophils) (13, 37, 38).

MECHANISMS RELATED TO GPER1 ACTIONS AND INTERACTIONS WITH OTHER MOLECULES

Most studies on immune-related action of GPER1 have focused on phenotypic events and less is known regarding the underlying signaling mechanisms. In the few studies that included intracellular signaling, the major GPER1-related pathways involved extracellular signal-regulated kinase 1/2 (ERK1/2), phosphoinositide 3-kinase (PI3K), and NFκB (26, 29, 32). In human neutrophils, the major pathways also involved cAMP/protein kinase A/cAMP-response element-binding protein, p38 mitogen-activated protein kinase, and ERK (36).

We have shown that GPER1 physically interacts with ERα36 and the p65 subunit of NFκB. This complex is found both in the cytoplasm and the nucleus, and is related to the estrogen inhibitory NFκB-mediated expression of IL-6 and TNFα (32). Furthermore, other groups have reported functional crosstalk between GPER and other nuclear steroid receptors including the vitamin D receptor (VDR) (47), the glucocorticoid receptor (GR) (48), and the mineralocorticoid receptor (MR) (49, 50) although the latter has been strongly questioned due to lack of proof for aldosterone binding to GPER1 (51). Furthermore, Vivaqua et al. have reported functional and physical interactions between GPR30, activated EGFR and ERα-alpha that may set off complex signaling cascades in hormone-sensitive cancer cells (52). This is an interesting mechanism since in the same study GPER1 was also found to be upregulated by EGF and TGF alpha in endometrial and tamoxifen-resistant breast cancer cells *via* the EGFR/ERK transduction pathway and c-fos (52).

Another controversial finding, related to the effects of steroids *via* GPER1, has to do with the effect of dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, DHEA), a molecule with a significant functional role in human immunity, to act *via* GPER1 [reviewed in (53)]. It has been reported that rapid DHEA-induced miR-21 transcription involves GPER1, estrogen receptor α-36 (ERα36), EGFR signaling, and activation of c-Src, ERK1/2, and PI3K (54). Although the results of this study have not been followed-up, the interaction of GPER1 with ERα36, also reported by our group, points out that such an interaction might be a more general model of GPER1 action.

GPER1 INVOLVEMENT IN IMMUNE-RELATED HUMAN DISEASES

As GPER1 is expressed in different human immune cells (presented above) regulating their life span and/or activation, a crucial role of GPER1 in a wide range of immune-related disorders has been suggested. These include chronic inflammatory and autoimmune diseases as well as immunodeficiencies [recently GPER1 deletion has been reported to be central for a case of familial immunodeficiency (55)]. For the scope of this review, we will concentrate on GPER1 involvement in inflammation-associated disorders.

Neuroinflammatory Disorders

Estrogen was known for many years to be active in the central nervous system (CNS) [see (56, 57) for reviews]. They arrive at their target cells either through the general circulation (by crossing the blood-brain barrier-BBB-) or through local production by neurons or astrocytes (58, 59). Several studies, including ours, reported estrogen to possess antiapoptotic and antioxidant activities [reviewed in (60)], which position them as anti-oxidant and anti-inflammatory agents, in the CNS. These beneficial effects of estrogen have resulted in the investigational use of estrogen in many clinical trials for inflammatory CNS conditions, presented in **Table 2**. The main targets of all these trials were intracellular ERα or ERβ, which are present in astrocytes or glial cells [excellently reviewed in (57)]. However, in our study, before the identification of GPER1 (1, 2), we reported that, in PC12 cells, BSA-bound estrogen mediates anti-apoptotic effects through membrane binding, mobilization of intracellular Ca2+ and activation of specific intracellular kinases pathways, independently from the activation of ERα/β (61). In addition, membrane estrogen binding sites, lately associated with GPER1, were identified in preparations of rat brain tissue. Later on (62), using the same model (PC12 cells), we have reported a detailed intracellular pathway. It includes NOS activation, CREB's, and NFκB nuclear translocation, leading to a pro-survival effect of estrogen *via* the BCL2-family of anti-apoptotic proteins.

The discovery of GPER1 shed a new light on the effect of estrogen in neuro-inflammation. Indeed, GPER1 was found, in addition to neuronal cells (61), also on microglial cells and astrocytes (63–67). Anti-inflammatory effects were attributed

TABLE 2 | Clinical trials using estrogen agonists or antagonists in inflammatory CNS conditions.

Condition	Estrogen compounds	
	Agonists	Antagonists
Traumatic Brain Injury	NCT00973674	NCT00065767 (Raloxifene)
Stroke	NCT00026039	NCT00368459 (Raloxifene)
	NCT01040182	
	NCT00005466	
Alzheimer's Disease	NCT00018343	
	NCT00006399	
	NCT00000176	
	NCT00000177	
	NCT00066157	
	NCT03718494	
	NCT03101085	
	NCT02142777	
	NCT01982578 (Genistein)	
Parkinson's Disease	NCT00234674	
ALS		NCT02166944 (Tamoxifen)
		NCT01257581 (Tamoxifen)
		NCT00214110 (Tamoxifen)

Source: www.clinicaltrials.gov accessed on May 18, 2020.

to GPER1 in several systems, including cells of the CNS (1, 8, 27, 61, 62, 66–71).

Although no clinical trials are available for the time being, due to the absence of a clinically available specific GPER1 agonist or antagonist, there are compelling preclinical indications about a specific involvement of this receptor in neuro-inflammatory diseases. Indeed, the GPER1 specific agonist G1 was found to be beneficial in an animal model of experimental encephalomyelitis and multiple sclerosis (68), by reducing the severity of the disease and reducing the level of pro-inflammatory cytokines. This effect was also reported by other groups (24, 41) and was attributed to the anti-inflammatory effect of GPER1, mediated by PD1 inhibition (24) [an element which was exploited also in the case of melanoma therapeutic manipulation (72)], or inhibition of pro-inflammatory cytokines (41). G1 also has a protective effect in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse Parkinson's Disease model. G1 is directly neuroprotective, but most importantly it has an indirect effect through an anti-inflammatory action on immune cells (macrophages, lymphocytes) (69, 73). Finally, GPER1 reduced neural injury and improved neural damage in a mouse model of ischemic brain injury, through inhibition of the TLR4-mediated inflammatory process (66).

Other Inflammatory Diseases

The anti-inflammatory effect of GPER1 has been investigated in several diseases and conditions, outside the CNS. The main organ that has been investigated is the vascular endothelium. Indeed, many reports (15, 16, 32, 74–77) investigated the anti-inflammatory effects of GPER1-mediated E2 effect in normal and atherosclerotic vessels. In mice with pronounced atherosclerosis, GPER1 deficiency was an aggravating factor, linked to disease progression. The effect of GPER1 was mediated by infiltrating immune cells (macrophages, lymphocytes) and was mediated by the GPER1-induced prostanoid production by the vascular endothelium (78). TNF-induced vascular inflammation (a condition which mimics the cellular stimuli induced by infiltrating immune cells), could also be attenuated by activation of GPER1, and enhanced by GPER1 antagonists, or activation of ERα, suggesting an opposing role of nuclear and extranuclear estrogen actions in the vascular endothelium (79). This finding led the authors to propose specific pharmacological options for GPER1 activation in vascular inflammation and derived atherosclerosis (80) and a specific role of this receptor in the maintenance of heart health (75).

Interestingly, GPER1 seems to play a significant role in large bowel physiology and disease [see (12) for a review]. More specifically, GPER1 seems to be downregulated in Inflammatory Bowel Disease and especially Crohn's disease, as compared to the normal tissue, suggestive of a protective role of the receptor in bowel inflammation (81). Although the data are not conclusive, the fact that GPER1 is expressed preferentially in normal tissue (81), together with its anti-inflammatory effect on different lineages of circulating or tissue-resident immune cells, as discussed above (28, 31–33, 35, 44), suggest a potential role of this receptor in bowel inflammation, a condition that when is present for prolonged periods of time (chronic colonic inflammation) is a risk factor for colon carcinogenesis (12). For more details on this topic please refer to the specific review in this special issue.

As described previously, GPER1 has been also implicated in liver inflammation, liver fibrosis, and hepatocarcinogenesis (33). In the absence of GPER1 the latter is increased and is accompanied by enhanced immune cell infiltration and production of inflammatory mediators like interleukin-6 (IL-6), through action on stellate cells rather than on hepatocytes, an effect reported previously (82). Therefore, GPER1 may prevent hepatocarcinogenesis *via* its anti-inflammatory effects.

Another condition characterized by a low degree of chronic inflammation is obesity, resulting in the emergence of Type II diabetes (15). In this condition, an underlying low-grade chronic inflammation is considered an important factor leading to insulin resistance. The anti-inflammatory effect of GPER1, documented by the administration of G-1 in experimental animals, verified the importance of this receptor in reducing vascular inflammation in adipose tissue, liver, and pancreas (33, 83–87). Interestingly, another mechanism GPER1 affects diabetes and hypercholesterolemia, is a direct action on lipid metabolism (84) and insulin signaling (84, 85). These effects have a direct impact on the generation and aggravation of type II diabetes, as discussed in detail in another review in the context of this thematic issue.

Finally, by modulating tissue and infiltrating immune cell-regulated inflammation, a role of GPER1 was reported in the regulation of endometriosis (88).

Cancer and Tissue Micro-Environment Inflammation

GPER1 has also been implicated in cancer and stroma-related inflammation, a hot topic in cancer research, and a preferential therapeutic target in cancer treatment. (The role of GPER1 in cancer is the object of a specific review, in this special issue.)

As discussed above, GPER1 activation inhibits PD1 production and action of pro-inflammatory cytokines, positioning this receptor as an interesting player for the modulation of the tumor microenvironment (24, 41). This element has been exploited in in melanoma (72). Furthermore, GPER1 stimulation by tamoxifen [acting as an agonist on this receptor (2)] inhibits the myofibroblastic differentiation of pancreatic stellate cells in the tumor microenvironment of pancreatic tumors, hampering their ability to remodel the extracellular matrix and to promote cancer cell invasion. GPER1 activation reduces the recruitment and polarization of the M2 phenotype of tumor-associated macrophages, inhibiting tumor inflammation, and immune suppression (87). However, GPER activation by either E2 or G-1 has been found to induce IL1β expression in cancer associated fibroblasts, and IL-1R1 in breast cancer cells, leading to a more aggressive phenotype (89). Furthermore, T-lymphocytes-related apoptosis induction by GPER1 (90, 91), leads to an inability of the major immune cells infiltrating breast stroma, in primary or metastatic breast cancer to support tumor expansion (92, 93). Overall, this positions GPER1 as a good prognostic and/or therapeutic target in several cancers, where the tumor microenvironment is critical for tumor expansion.

In addition to the modulation of the tumor microenvironment, GPER1 activation has a direct immunomodulatory effect on the tumor tissue, *per se*. Indeed, GPER1 was found to be an androgen-repressed gene and is therefore highly expressed in castration-resistant but not in androgen-responsive prostate cancer (94). Through a thorough analysis of xenografted prostate tumors in mice, the authors report that GPER1 up-regulation (and its activation by G1) results in an increased expression of genes related to the interplay between innate and adaptive immunity. Furthermore, they report substantial necrosis of xenografted tumors through increased production of neutrophil attracting cytokines. Therefore, GPER1 is a pro-inflammatory mediator in castration-resistant prostate cancer involved in neutrophil movement, accumulation, adhesion, activation, and phagocytic respiratory burst. Interestingly, a similar E2-induction of the mammary gland with a resulting inflammation was also reported during mammary gland involution (95), although the authors do not specifically investigate the implication of GPER1.

GPER1, through modification of local inflammation and the corresponding immune response, has been reported to play a role in inflammatory breast cancer (74). Specifically, if GPER1 is co-expressed with ERα, it is a good prognostic marker, related to improved overall survival and disease-free survival. GPER1 also increases miR-148a, which in turn induces HLA-G, in both ER+ and triple-negative breast cancer cells (96). The expression of the latter molecule impairs the immune evasion of breast cancer, again suggesting that GPER1 is a good prognostic indicator in breast cancer.

Finally, as discussed above, GPER1 has an indirect impact in colon carcinogenesis through modulation of immune responses (12), while in a thorough investigation, Wei et al. propose that the effect of GPER1 on liver tumorigenesis might be attributed to the anti-inflammatory effect of the agent rather than to a direct action on cancer cells (33).

FUTURE PERSPECTIVES IN GPER1 IMMUNITY-RELATED RESEARCH

GPER1 actions on immune functions seem to be abundant and could be critically important, especially in neuro-inflammation and in inflammatory processes related to atherosclerosis. A universal finding across systems and cell types seems to be GPER1 dependent modulation of TLR4 mediated events, with hints that this could be a mechanism affecting several other fundamental pathways exploiting NFκB to lead to inflammation. Current studies have given us just a glimpse of the potential of this molecule and more studies are needed in this area.

The field of GPER1 research has been, however, obscured by "availability bias" characterized by the narrow focus on single molecules and mechanisms. The complexity of estrogen-mediated anti-inflammatory actions may include interactions between GPER1 and classical estrogen receptors or their isoforms, as well as interactions of GPER1 with other nuclear receptors with the capacity to regulate the immune system. The latter is a critical issue for the explanation of the diverse actions of estrogen and G1 on the same mechanism, seen not only in GPER1 action on immune functions but also in other systems. Since the physiologically relevant molecule is estrogen, our current knowledge regarding the effects of G1 suggests that GPER1 could have a more universal role as a central rheostat for diverse intracellular mechanisms related to inflammation. Therefore, future studies on the role of GPER1 on immune functions should focus on a thorough analysis of all the potential molecular interactions and intracellular mechanisms in each cell type and each disease model.

Another important issue that should be further evaluated is the potential role of GPER1 in the mediation of sexual dimorphism in human diseases. It would be interesting to clarify if there exist hormone independent differences in immune cell GPER1 levels between males and females, or if sex-dependent differences in estrogen levels are critical, although both phenomena could be important in different clinical conditions. Sex related differences in the expression of several other GPCRs have already been described and have been related to sex dimorphism in cardiovascular diseases and stress responses (97, 98).

Finally, the site where GPER1 resides is also an interesting research subject. Membrane, endoplasmic reticulum, Golgi apparatus, and nuclear localization of GPER1 (as observed in immune cells), suggest molecular modifications that could also affect its function. Deciphering GPER1 cellular trafficking could also help us find ways to exploit its immune-modulating capacity.

CONCLUSIONS

GPER1 is a fascinating molecule that continuous to surprise us with its diverse functions in the immune system. Since its discovery, it has caused a paradigm shift in the way we understand estrogen actions and the gender-dimorphism of several pathologies. Its role in the immune system only now starts to unravel and initial data are promising. Moreover, the role of GPER1 does not seem to be related only to estrogen. GPER1 seems to have a more universal role in regulating the function of almost all immune cells and several pro-inflammatory mechanisms. Although there are still a lot of uncharted territories to cover, the GPCR nature of GPER1 and the existence of specific agonists and antagonists make it a convenient therapeutic target for the immune system. Hopefully, the best is yet to come.

AUTHOR CONTRIBUTIONS

All authors agreed on the initial draft of the review. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* (2005) 307:1625–30. doi: 10.1126/science.1106943
2. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* (2005) 146:624–32. doi: 10.1210/en.2004-1064
3. Kampa M, Pelekanou V, Notas G, Stathopoulos EN, Castanas E. The estrogen receptor: two or more molecules, multiple variants, diverse localizations, signaling and functions. Are we undergoing a paradigm-shift as regards their significance in breast cancer? *Hormones (Athens)* (2013) 12:69–85. doi: 10.1007/BF03401288
4. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* (2008) 70:165–90. doi: 10.1146/annurev.physiol.70.113006.100518
5. Broselid S, Berg KA, Chavera TA, Kahn R, Clarke WP, Olde B, et al. G protein-coupled receptor 30 (GPR30) forms a plasma membrane complex with membrane-associated guanylate kinases (MAGUKs) and protein kinase A-anchoring protein 5 (AKAP5) that constitutively inhibits cAMP production. *J Biol Chem* (2014) 289:22117–27. doi: 10.1074/jbc.M114.566893
6. Fuentes N, Silveyra P. Estrogen receptor signaling mechanisms. *Adv Protein Chem Struct Biol* (2019) 116:135–70. doi: 10.1016/bs.apcsb.2019.01.001
7. Notas G, Kampa M, Pelekanou V, Castanas E. Interplay of estrogen receptors and GPR30 for the regulation of early membrane initiated transcriptional effects: A pharmacological approach. *Steroids* (2012) 77:943–50. doi: 10.1016/j.steroids.2011.11.005
8. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol* (2011) 7:715–26. doi: 10.1038/nrendo.2011.122
9. Bourque M, Dluzen DE, Di Paolo T. Signaling pathways mediating the neuroprotective effects of sex steroids and SERMs in Parkinson's disease. *Front Neuroendocrinol* (2012) 33:169–78. doi: 10.1016/j.yfrne.2012.02.003
10. Feldman RD. Heart Disease in Women: Unappreciated Challenges, GPER as a New Target. *Int J Mol Sci* (2016) 17. doi: 10.3390/ijms17050760
11. Hsu LH, Chu NM, Lin YF, Kao SH. G-Protein Coupled Estrogen Receptor in Breast Cancer. *Int J Mol Sci* (2019) 20. doi: 10.3390/ijms20020306
12. Jacenik D, Beswick EJ, Krajewska WM, Prossnitz ER. G protein-coupled estrogen receptor in colon function, immune regulation and carcinogenesis. *World J Gastroenterol* (2019) 25:4092–104. doi: 10.3748/wjg.v25.i30.4092
13. Cabas I, Chaves-Pozo E, Mulero V, Garcia-Ayala A. Role of estrogens in fish immunity with special emphasis on GPER1. *Dev Comp Immunol* (2018) 89:102–10. doi: 10.1016/j.dci.2018.08.001
14. Feldman RD, Limbird LE. GPER (GPR30): A Nongenomic Receptor (GPCR) for Steroid Hormones with Implications for Cardiovascular Disease and Cancer. *Annu Rev Pharmacol Toxicol* (2017) 57:567–84. doi: 10.1146/annurev-pharmtox-010716-104651
15. Barton M, Prossnitz ER. Emerging roles of GPER in diabetes and atherosclerosis. *Trends Endocrinol Metab* (2015) 26:185–92. doi: 10.1016/j.tem.2015.02.003
16. Filardo EJ, Thomas P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology* (2012) 153:2953–62. doi: 10.1210/en.2012-1061
17. Bouman A, Heineman MJ, Faas MM. Sex hormones and the immune response in humans. *Hum Reprod Update* (2005) 11:411–23. doi: 10.1093/humupd/dmi008
18. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev* (2007) 28:521–74. doi: 10.1210/er.2007-0001
19. Wang C, Dehghani B, Magrisso IJ, Rick EA, Bonhomme E, Cody DB, et al. GPR30 contributes to estrogen-induced thymic atrophy. *Mol Endocrinol* (2008) 22:636–48. doi: 10.1210/me.2007-0359
20. Olsen NJ, Kovacs WJ. Gonadal steroids and immunity. *Endocr Rev* (1996) 17:369–84. doi: 10.1210/edrv-17-4-369
21. Isensee J, Meoli L, Zazzu V, Nabzdyk C, Witt H, Soewarto D, et al. Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology* (2009) 150:1722–30. doi: 10.1210/en.2008-1488
22. Otto C, Rohde-Schulz B, Schwarz G, Fuchs I, Klewer M, Brittain D, et al. G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. *Endocrinology* (2008) 149:4846–56. doi: 10.1210/en.2008-0269
23. Windahl SH, Andersson N, Chagin AS, Martensson UE, Carlsten H, Olde B, et al. The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice. *Am J Physiol Endocrinol Metab* (2009) 296:E490–496. doi: 10.1152/ajpendo.90691.2008
24. Wang C, Dehghani B, Li Y, Kaler LJ, Proctor T, Vandenbark AA, et al. Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. *J Immunol* (2009) 182:3294–303. doi: 10.4049/jimmunol.0803205
25. Martensson UE, Salehi SA, Windahl S, Gomez MF, Sward K, Daszkiewicz-Nilsson J, et al. Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* (2009) 150:687–98. doi: 10.1210/en.2008-0623
26. Brunsing RL, Prossnitz ER. Induction of interleukin-10 in the T helper type 17 effector population by the G protein coupled estrogen receptor (GPER) agonist G-1. *Immunology* (2011) 134:93–106. doi: 10.1111/j.1365-2567.2011.03471.x
27. Filardo EJ, Quinn JA, Bland KI, Frackelton ARJr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* (2000) 14:1649–60. doi: 10.1210/mend.14.10.0532
28. Brunsing RL, Owens KS, Prossnitz ER. The G protein-coupled estrogen receptor (GPER) agonist G-1 expands the regulatory T-cell population under TH17-polarizing conditions. *J Immunother* (2013) 36:190–6. doi: 10.1097/CJI.0b013e31828d8e3b
29. Schneider AE, Karpati E, Schuszter K, Toth EA, Kiss E, Kulcsar M, et al. A dynamic network of estrogen receptors in murine lymphocytes: fine-tuning the immune response. *J Leukoc Biol* (2014) 96:857–72. doi: 10.1189/jlb.2A0214-080RR
30. Rodenas MC, Cabas I, Gomez-Gonzalez NE, Arizcun M, Meseguer J, Mulero V, et al. Estrogens Promote the Production of Natural Neutralizing Antibodies in Fish through G Protein-Coupled Estrogen Receptor 1. *Front Immunol* (2017) 8:736. doi: 10.3389/fimmu.2017.00736
31. Rettew JA, Mccall SHT, Marriott I. GPR30/GPER-1 mediates rapid decreases in TLR4 expression on murine macrophages. *Mol Cell Endocrinol* (2010) 328:87–92. doi: 10.1016/j.mce.2010.07.017
32. Pelekanou V, Kampa M, Kiagiadaki F, Deli A, Theodoropoulos P, Agrogiannis G, et al. Estrogen anti-inflammatory activity on human monocytes is mediated through cross-talk between estrogen receptor ERalpha36 and GPR30/GPER1. *J Leukoc Biol* (2016) 99:333–47. doi: 10.1189/jlb.3A0914-430RR
33. Wei T, Chen W, Wen L, Zhang J, Zhang Q, Yang J, et al. G protein-coupled estrogen receptor deficiency accelerates liver tumorigenesis by enhancing inflammation and fibrosis. *Cancer Lett* (2016) 382:195–202. doi: 10.1016/j.canlet.2016.08.012
34. Tamaki M, Konno Y, Kobayashi Y, Takeda M, Itoga M, Moritoki Y, et al. Expression and functional roles of G-protein-coupled estrogen receptor (GPER) in human eosinophils. *Immunol Lett* (2014) 160:72–8. doi: 10.1016/j.imlet.2014.03.012
35. Itoga M, Konno Y, Moritoki Y, Saito Y, Ito W, Tamaki M, et al. G-protein-coupled estrogen receptor agonist suppresses airway inflammation in a mouse model of asthma through IL-10. *PloS One* (2015) 10:e0123210. doi: 10.1371/journal.pone.0123210
36. Rodenas MC, Tamassia N, Cabas I, Calzetti F, Meseguer J, Cassatella MA, et al. G Protein-Coupled Estrogen Receptor 1 Regulates Human Neutrophil Functions. *BioMed Hub* (2017) 2:1–13. doi: 10.1159/000454981
37. Szwejser E, Maciuszek M, Casanova-Nakayama A, Segner H, Verburg-Van Kemenade BML, Chadzinska M. A role for multiple estrogen receptors in immune regulation of common carp. *Dev Comp Immunol* (2017) 66:61–72. doi: 10.1016/j.dci.2016.04.003
38. Cabas I, Rodenas MC, Abellan E, Meseguer J, Mulero V, Garcia-Ayala A. Estrogen signaling through the G protein-coupled estrogen receptor regulates granulocyte activation in fish. *J Immunol* (2013) 191:4628–39. doi: 10.4049/jimmunol.1301613

39. Erlandsson MC, Ohlsson C, Gustafsson JA, Carlsten H. Role of oestrogen receptors alpha and beta in immune organ development and in oestrogen-mediated effects on thymus. *Immunology* (2001) 103:17–25. doi: 10.1046/j.1365-2567.2001.01212.x
40. Polanczyk MJ, Hopke C, Huan J, Vandenbark AA, Offner H. Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *J Neuroimmunol* (2005) 170:85–92. doi: 10.1016/j.jneuroim.2005.08.023
41. Yates MA, Li Y, Chlebeck PJ, Offner H. GPR30, but not estrogen receptor-alpha, is crucial in the treatment of experimental autoimmune encephalomyelitis by oral ethinyl estradiol. *BMC Immunol* (2010) 11:20. doi: 10.1186/1471-2172-11-20
42. Liarte S, Chaves-Pozo E, Abellan E, Meseguer J, Mulero V, Garcia-Ayala A. 17beta-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation. *Dev Comp Immunol* (2011) 35:19–27. doi: 10.1016/j.dci.2010.07.007
43. Calippe B, Douin-Echinard V, Laffargue M, Laurell H, Rana-Poussine V, Pipy B, et al. Chronic estradiol administration in vivo promotes the proinflammatory response of macrophages to TLR4 activation: involvement of the phosphatidylinositol 3-kinase pathway. *J Immunol* (2008) 180:7980–8. doi: 10.4049/jimmunol.180.12.7980
44. Ensom MH, Chong E, Carter D. Premenstrual symptoms in women with premenstrual asthma. *Pharmacotherapy* (1999) 19:374–82. doi: 10.1592/phco.19.6.374.31050
45. Stygar D, Westlund P, Eriksson H, Sahlin L. Identification of wild type and variants of oestrogen receptors in polymorphonuclear and mononuclear leucocytes. *Clin Endocrinol (Oxf)* (2006) 64:74–81. doi: 10.1111/j.1365-2265.2005.02420.x
46. Cassatella MA, Guasparri I, Ceska M, Bazzoni F, Rossi F. Interferon-gamma inhibits interleukin-8 production by human polymorphonuclear leucocytes. *Immunology* (1993) 78:177–84.
47. Subramanian S, Miller LM, Grafe MR, Vandenbark AA, Offner H. Contribution of GPR30 for 1,25 dihydroxyvitamin D(3) protection in EAE. *Metab Brain Dis* (2012) 27:29–35. doi: 10.1007/s11011-011-9266-6
48. Ylikomi T, Vienonen A, Ahola TM. G protein-coupled receptor 30 down-regulates cofactor expression and interferes with the transcriptional activity of glucocorticoid. *Eur J Biochem* (2004) 271:4159–68. doi: 10.1111/j.1432-1033.2004.04353.x
49. Gros R, Ding Q, Sklar LA, Prossnitz EE, Arterburn JB, Chorazyczewski J, et al. GPR30 expression is required for the mineralocorticoid receptor-independent rapid vascular effects of aldosterone. *Hypertension* (2011) 57:442–51. doi: 10.1161/HYPERTENSIONAHA.110.161653
50. Rigiracciolo DC, Scarpelli A, Lappano R, Pisano A, Santolla MF, Avino S, et al. GPER is involved in the stimulatory effects of aldosterone in breast cancer cells and breast tumor-derived endothelial cells. *Oncotarget* (2016) 7:94–111. doi: 10.18632/oncotarget.6475
51. Wendler A, Wehling M. Is GPR30 the membrane aldosterone receptor postulated 20 years ago? *Hypertension* (2011) 57:e16; author reply e17. doi: 10.1161/HYPERTENSIONAHA.111.170977
52. Vivacqua A, Lappano R, De Marco P, Sisci D, Aquila S, De Amicis F, et al. G protein-coupled receptor 30 expression is up-regulated by EGF and TGF alpha in estrogen receptor alpha-positive cancer cells. *Mol Endocrinol* (2009) 23:1815–26. doi: 10.1210/me.2009-0120
53. Prall SP, Muehlenbein MP. DHEA Modulates Immune Function: A Review of Evidence. *Vitam Horm* (2018) 108:125–44. doi: 10.1016/bs.vh.2018.01.023
54. Teng Y, Radde BN, Litchfield LM, Ivanova MM, Prough RA, Clark BJ, et al. Dehydroepiandrosterone Activation of G-protein-coupled Estrogen Receptor Rapidly Stimulates MicroRNA-21 Transcription in Human Hepatocellular Carcinoma Cells. *J Biol Chem* (2015) 290:15799–811. doi: 10.1074/jbc.M115.641167
55. Sloboda N, Sorlin A, Valduga M, Beri-Dexheimer M, Bilbault C, Fouyssac F, et al. Deletion of chr7p22 and chr15q11: Two Familial Cases of Immune Deficiency: Extending the Phenotype Toward Dysimmunity. *Front Immunol* (2019) 10:1871. doi: 10.3389/fimmu.2019.01871
56. Compagnone NA, Mellon SH. Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* (2000) 21:1–56. doi: 10.1006/frne.1999.0188
57. Yilmaz C, Karali K, Fodelianaki G, Gravanis A, Chavakis T, Charalampopoulos I, et al. Neurosteroids as regulators of neuroinflammation. *Front Neuroendocrinol* (2019) 55:100788. doi: 10.1016/j.yfrne.2019.100788
58. Mellon SH, Griffin LD. Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol Metab* (2002) 13:35–43. doi: 10.1016/S1043-2760(01)00503-3
59. Zwain IH, Yen SS. Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. *Endocrinology* (1999) 140:3843–52. doi: 10.1210/endo.140.8.6907
60. Garcia-Segura LM, Azcoitia I, Doncarlos LL. Neuroprotection by estradiol. *Prog Neurobiol* (2001) 63:29–60. doi: 10.1016/S0301-0082(00)00025-3
61. Alexaki VI, Charalampopoulos I, Kampa M, Vassalou H, Theodoropoulos P, Stathopoulos EN, et al. Estrogen exerts neuroprotective effects via membrane estrogen receptors and rapid Akt/NOS activation. *FASEB J* (2004) 18:1594–6. doi: 10.1096/fj.04-1495fje
62. Alexaki VI, Charalampopoulos I, Kampa M, Nifli AP, Hatzoglou A, Gravanis A, et al. Activation of membrane estrogen receptors induce pro-survival kinases. *J Steroid Biochem Mol Biol* (2006) 98:97–110. doi: 10.1016/j.jsbmb.2005.08.017
63. Kuo J, Hamid N, Bondar G, Prossnitz ER, Micevych P. Membrane estrogen receptors stimulate intracellular calcium release and progesterone synthesis in hypothalamic astrocytes. *J Neurosci* (2010) 30:12950–7. doi: 10.1523/JNEUROSCI.1158-10.2010
64. Pawlak J, Karolczak M, Krust A, Chambon P, Beyer C. Estrogen receptor-alpha is associated with the plasma membrane of astrocytes and coupled to the MAP/Src-kinase pathway. *Glia* (2005) 50:270–5. doi: 10.1002/glia.20162
65. Sierra A, Gottfried-Blackmore A, Milner TA, Mcewen BS, Bulloch K. Steroid hormone receptor expression and function in microglia. *Glia* (2008) 56:659–74. doi: 10.1002/glia.20644
66. Zhang Z, Qin P, Deng Y, Ma Z, Guo H, Guo H, et al. The novel estrogenic receptor GPR30 alleviates ischemic injury by inhibiting TLR4-mediated microglial inflammation. *J Neuroinflam* (2018) 15:206. doi: 10.1186/s12974-018-1246-x
67. Schaufelberger SA, Rosselli M, Barchiesi F, Gillespie DG, Jackson EK, Dubey RK. 2-Methoxyestradiol, an endogenous 17beta-estradiol metabolite, inhibits microglial proliferation and activation via an estrogen receptor-independent mechanism. *Am J Physiol Endocrinol Metab* (2016) 310:E313–322. doi: 10.1152/ajpendo.00418.2015
68. Blasko E, Haskell CA, Leung S, Gualtieri G, Halks-Miller M, Mahmoudi M, et al. Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *J Neuroimmunol* (2009) 214:67–77. doi: 10.1016/j.jneuroim.2009.06.023
69. Guan J, Yang B, Fan Y, Zhang J. GPER Agonist G1 Attenuates Neuroinflammation and Dopaminergic Neurodegeneration in Parkinson Disease. *Neuroimmunomodulation* (2017) 24:60–6. doi: 10.1159/000478908
70. Prossnitz ER, Barton M. Estrogen biology: new insights into GPER function and clinical opportunities. *Mol Cell Endocrinol* (2014) 389:71–83. doi: 10.1016/j.mce.2014.02.002
71. Liu L, Zhao Y, Xie K, Sun X, Gao Y, Wang Z. Estrogen-induced nongenomic calcium signaling inhibits lipopolysaccharide-stimulated tumor necrosis factor alpha production in macrophages. *PloS One* (2013) 8:e83072. doi: 10.1371/journal.pone.0083072
72. Natale CA, Li J, Zhang J, Dahal A, Dentchev T, Stanger BZ, et al. Activation of G protein-coupled estrogen receptor signaling inhibits melanoma and improves response to immune checkpoint blockade. *Elife* (2018) 7. doi: 10.7554/eLife.31770
73. Cote M, Bourque M, Poirier AA, Aube B, Morissette M, Di Paolo T, et al. GPER1-mediated immunomodulation and neuroprotection in the myenteric plexus of a mouse model of Parkinson's disease. *Neurobiol Dis* (2015) 82:99–113. doi: 10.1016/j.nbd.2015.05.017
74. Arias-Pulido H, Royce M, Gong Y, Joste N, Lomo L, Lee SJ, et al. GPR30 and estrogen receptor expression: new insights into hormone dependence of inflammatory breast cancer. *Breast Cancer Res Treat* (2010) 123:51–8. doi: 10.1007/s10549-009-0631-7
75. Groban L, Tran QK, Ferrario CM, Sun X, Cheng CP, Kitzman DW, et al. Female Heart Health: Is GPER the Missing Link? *Front Endocrinol (Lausanne)* (2019) 10:919. doi: 10.3389/fendo.2019.00919

76. Reslan OM, Khalil RA. Vascular effects of estrogenic menopausal hormone therapy. *Rev Recent Clin Trials* (2012) 7:47–70. doi: 10.2174/157488712799363253
77. Kong BS, Cho YH, Lee EJ. G protein-coupled estrogen receptor-1 is involved in the protective effect of protocatechuic aldehyde against endothelial dysfunction. *PloS One* (2014) 9:e113242. doi: 10.1371/journal.pone.0113242
78. Meyer MR, Fredette NC, Barton M, Prossnitz ER. G protein-coupled estrogen receptor inhibits vascular prostanoid production and activity. *J Endocrinol* (2015) 227:61–9. doi: 10.1530/JOE-15-0257
79. Chakrabarti S, Davidge ST. G-protein coupled receptor 30 (GPR30): a novel regulator of endothelial inflammation. *PloS One* (2012) 7:e52357. doi: 10.1371/journal.pone.0052357
80. Chakrabarti S, Davidge ST. Analysis of G-Protein Coupled Receptor 30 (GPR30) on Endothelial Inflammation. *Methods Mol Biol* (2016) 1366:503–16. doi: 10.1007/978-1-4939-3127-9_39
81. Wlodarczyk M, Sobolewska-Wlodarczyk A, Cygankiewicz AI, Jacenik D, Piechota-Polanczyk A, Stec-Michalska K, et al. G Protein-Coupled Receptor 30 (GPR30) Expression Pattern in Inflammatory Bowel Disease Patients Suggests its Key Role in the Inflammatory Process. A Preliminary Study. *J Gastrointest Liver Dis* (2017) 26:29–35. doi: 10.15403/jgld.2014.1121.261.gpr
82. Cortes E, Lachowski D, Rice A, Thorpe SD, Robinson B, Yeldag G, et al. Tamoxifen mechanically deactivates hepatic stellate cells via the G protein-coupled estrogen receptor. *Oncogene* (2019) 38:2910–22. doi: 10.1038/s41388-018-0631-3
83. Meyer MR, Clegg DJ, Prossnitz ER, Barton M. Obesity, insulin resistance and diabetes: sex differences and role of oestrogen receptors. *Acta Physiol (Oxf)* (2011) 203:259–69. doi: 10.1111/j.1748-1716.2010.02237.x
84. Sharma G, Hu C, Brigman JL, Zhu G, Hathaway HJ, Prossnitz ER. GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. *Endocrinology* (2013) 154:4136–45. doi: 10.1210/en.2013-1357
85. Sharma G, Hu C, Staquicini DI, Brigman JL, Liu M, Mauvais-Jarvis F, et al. Preclinical efficacy of the GPER-selective agonist G-1 in mouse models of obesity and diabetes. *Sci Transl Med* (2020) 12. doi: 10.1126/scitranslmed.aau5956
86. Sharma G, Prossnitz ER. G-Protein-Coupled Estrogen Receptor (GPER) and Sex-Specific Metabolic Homeostasis. *Adv Exp Med Biol* (2017) 1043:427–53. doi: 10.1007/978-3-319-70178-3_20
87. Cortes E, Sarper M, Robinson B, Lachowski D, Chronopoulos A, Thorpe SD, et al. GPER is a mechanoregulator of pancreatic stellate cells and the tumor microenvironment. *EMBO Rep* (2019) 20. doi: 10.15252/embr.201846556
88. Castro J, Araya G, Inostroza P, Hidalgo P, Gonzalez-Ramos R, Sovino H, et al. Differential expression of upstream stimulatory factor (USF) 2 variants in eutopic endometria from women with endometriosis: estradiol regulation. *Biol Res* (2015) 48:56. doi: 10.1186/s40659-015-0047-2
89. De Marco P, Lappano R, De Francesco EM, Cirillo F, Pupo M, Avino S, et al. GPER signalling in both cancer-associated fibroblasts and breast cancer cells mediates a feedforward IL1beta/IL1R1 response. *Sci Rep* (2016) 6:24354. doi: 10.1038/srep24354
90. Barton M. Not lost in translation: Emerging clinical importance of the G protein-coupled estrogen receptor GPER. *Steroids* (2016) 111:37–45. doi: 10.1016/j.steroids.2016.02.016
91. Molina L, Figueroa CD, Bhoola KD, Ehrenfeld P. GPER-1/GPR30 a novel estrogen receptor sited in the cell membrane: therapeutic coupling to breast cancer. *Expert Opin Ther Targets* (2017) 21:755–66. doi: 10.1080/14728222.2017.1350264
92. Szekely B, Bossuyt V, Li X, Wali VB, Patwardhan GA, Frederick C, et al. Immunological differences between primary and metastatic breast cancer. *Ann Oncol* (2018) 29:2232–9. doi: 10.1093/annonc/mdy399
93. Pelekanou V, Barlow WE, Nahleh ZA, Wasserman B, Lo YC, Von Wahlde MK, et al. Tumor-Infiltrating Lymphocytes and PD-L1 Expression in Pre- and Posttreatment Breast Cancers in the SWOG S0800 Phase II Neoadjuvant Chemotherapy Trial. *Mol Cancer Ther* (2018) 17:1324–31.
94. Lam HM, Ouyang B, Chen J, Ying J, Wang J, Wu CL, et al. Targeting GPR30 with G-1: a new therapeutic target for castration-resistant prostate cancer. *Endocr Relat Cancer* (2014) 21:903–14.
95. Chung HH, Or YZ, Shrestha S, Loh JT, Lim CL, Ong Z, et al. Estrogen reprograms the activity of neutrophils to foster protumoral microenvironment during mammary involution. *Sci Rep* (2017) 7:46485.
96. Tao S, He H, Chen Q, Yue W. GPER mediated estradiol reduces miR-148a to promote HLA-G expression in breast cancer. *Biochem Biophys Res Commun* (2014) 451:74–8. doi: 10.1016/j.bbrc.2014.07.073
97. Mouat MA, Coleman JLJ, Smith NJ. GPCRs in context: sexual dimorphism in the cardiovascular system. *Br J Pharmacol* (2018) 175:4047–59. doi: 10.1111/bph.14160
98. Brunton PJ, Donadio MV, Russell JA. Sex differences in prenatally programmed anxiety behaviour in rats: differential corticotropin-releasing hormone receptor mRNA expression in the amygdaloid complex. *Stress* (2011) 14:634–43. doi: 10.3109/10253890.2011.604750

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Chronic GPER1 Activation Protects Against Oxidative Stress-Induced Cardiomyoblast Death via Preservation of Mitochondrial Integrity and Deactivation of Mammalian Sterile-20-Like Kinase/Yes-Associated Protein Pathway

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Introduction: Estrogen (17β-estradiol, E2) is well-known to induce cardioprotective effects against ischemia/reperfusion (I/R) injury. We recently reported that acute application of E2 at the onset of reperfusion *in vivo* induces cardioprotective effects against I/R injury *via* activation of its non-steroidal receptor, G protein-coupled estrogen receptor 1 (GPER1). Here, we investigated the impact and mechanism underlying chronic GPER1 activation in cultured H9c2 rat cardiomyoblasts.

Methods: H9c2 rat cardiomyoblasts were cultured and pretreated with the cytotoxic agent H_2O_2 for 24 h and incubated in the presence of vehicle (control), GPER1 agonists E2 and G1, or GPER1 agonists supplemented with G15 (GPER1 antagonist) for 48 or 96 h. After treatment, cells were collected to measure the rate of cell death and viability using flow cytometry and Calcein AM assay or MTT assay, respectively. The resistance to opening of the mitochondrial permeability transition pore (mPTP), the mitochondrial membrane potential, and ATP production was assessed using fluorescence microscopy, and the mitochondrial structural integrity was observed with electron microscopy. The levels of the phosphorylation of mammalian sterile-20-like kinase (MST1) and yes-associated protein (YAP) were assessed by Western blot analysis in whole-cell lysate, while the expression levels of mitochondrial biogenesis genes, YAP target genes, and proapoptotic genes were measured by qRT-PCR.

Results: We found that after H_2O_2 treatment, chronic E2/G1 treatment decreased cell death effect was associated with the prevention of the S phase of the cell cycle arrest compared to control. In the mitochondria, chronic E2/G1 activation treatment preserved the cristae morphology, and increased resistance to opening of mPTP, but

with little change to mitochondrial fusion/fission. Additionally, chronic E2/G1 treatment predominantly reduced phosphorylation of MST1 and YAP, as well as increased MST1 and YAP protein levels. E2 treatment also upregulated the expression levels of TGF-β and PGC-1α mRNAs and downregulated PUMA and Bim mRNAs. Except for ATP production, all the E2 or G1 effects were prevented by the cotreatment with the GPER1 antagonist, G15.

Conclusion: Together, these results indicate that chronic GPER1 activation with its agonists E2 or G1 treatment protects H9c2 cardiomyoblasts against oxidative stress-induced cell death and increases cell viability by preserving mitochondrial structure and function as well as delaying the opening of mPTP. These chronic GPER1 effects are associated with the deactivation of the non-canonical MST1/YAP mechanism that leads to genetic upregulation of cell growth genes (CTGF, CYR61, PGC-1α, and ANKRD1), and downregulation of proapoptotic genes (PUMA and Bim).

Keywords: G protein-coupled estrogen receptor 1 (GPER1), rat cardiomyoblasts, mitochondrial function, MST1/YAP pathway, mPTP opening, mitochondrial dynamics, cell cycle

INTRODUCTION

Estrogen (17β-estradiol, E2) has been shown to exert protective effects against various deleterious conditions. Studies in gerbils (1), mice (2), and rats (3) revealed that female animals exhibited smaller cerebral infarct sizes than their male counterparts following carotid artery occlusion or middle cerebral artery occlusion, suggesting a neuroprotective role for estrogen. In the cardiovascular system, others and our group have provided evidence that acute pre- or post-E2 treatment can induce cardioprotective effects against ischemia/reperfusion (I/R) injury, cardiac hypertrophy, cardiac remodeling, and heart failure (4–8). Further, studies in mice revealed that E2 treatment confers renoprotective effects and ameliorates acute kidney outcomes following severe cardiac arrest (9, 10).

E2 has been shown to act via its three known estrogen receptors (ER): ERα (11), ERβ (12), or the G-protein coupled estrogen receptor 1 (GPER1) (13). Estrogen is the most abundant female sex hormone, which activates complex pathways involving genomic targets mediated through the classical receptors, ERα and ERβ (11, 12). The classical genomic pathways involve transcription of prosurvival genes facilitated by estrogen response elements (EREs) (14) and super-enhancers (15). Another genomic signaling is controlled by an indirect nuclear ER binding to DNA that is mediated by cofactors like NF-κB or AP-1 and SP-1 to exert their transcription regulation (16, 17). Classic ERs at the plasma membrane and cytosol can also mediate E2 action via non-genomic signaling, including activating kinases or binding to scaffold proteins to modulate multiple prosurvival pathways (17–19). Besides these effects mediated through its two steroidal ERs, a complementary but separate mode of rapid E2 actions have been reported that depend on agonist activation of the membrane-bound GPER1. GPER1 is highly expressed in almost all the organs, including the myocardium (13, 20), brain (21), kidney (22), and myometrium (23). Although E2's genomic mechanisms via the nuclear ERs are now well-characterized, those predominantly mediated by GPER1 activation still need to be explored. More recently, there has been a new wave of research focusing on GPER1's mechanisms of mediating estrogenic responsive effects. In fact, others and we have provided evidence that GPER1 activation with its specific agonist, G1, confers cardioprotective effects against I/R injury (20, 24, 25) via MAPK, PI-3K/Akt, and MEK/ERK/GSK3β pathways. In isolated rat hearts subjected to I/R, GPER1 activation was shown to improve functional recovery and reduce myocardial infarct size (24). Further, using isolated perfused hearts from male GPER1 knockout mice, we showed that GPER1, but not the classical ERs, plays a key role in mediating acute pre-ischemic E2-induced cardioprotection against I/R injury (4). We also showed that acute GPER1 stimulation during reperfusion elicits cardioprotective effects involving the delay of mitochondrial permeability transition pore (mPTP) opening, reduction of mitochondrial dysfunction, and mitophagy (5, 20). In intestinal crypt cells, pre-ischemic GPER1 activation has been suggested to alleviate the injury induced by I/R and improve proliferative ability of crypt stem cell by inhibiting iNOS expression (26).

In cells, an increase in the production of reactive oxygen species (ROS) is responsible for the induction of oxidative stress that is involved in the development of several diseases, including liver diseases (27, 28). Oxidative stress, such as an increase in H_2O_2 levels, results from an imbalance between the systemic production of ROS and a biological system's ability to clean the reactive intermediates. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA, subsequently leading to cell apoptosis (29). The mechanism of this H_2O_2-induced apoptosis involves the inhibition of Bcl-2 family proteins and caspases. Pronsato's group has reported that 17β-estradiol (E2) can protect C2C12 skeletal muscle cells from H_2O_2-induced apoptosis by reverting PKCδ, JNK, and p66Shc activation and exerting a beneficial action over mitochondria (30, 31). Using

the same model of C2C12 cell line treated with H_2O_2, Boland's group found that the inhibition of the antiapoptotic action of E2 was more pronounced when ER-beta was immunoneutralized or suppressed by mRNA silencing. In fact, Vasconsuelo et al. has shown that transfection of C2C12 cells with either ER-alpha siRNA or ER-beta siRNA blocked the activation of Akt by E2, and suggested differential involvement of ER isoforms depending on the step of the apoptotic/survival pathway (32). On the other hand, in cultured adipose tissue, islet, neuronal cells, and cardiomyocytes, E2 actions via GPER1 have been found to protect against H_2O_2-induced oxidative stress and toxicity (33). Further, E2 cytoprotection in these cells occurs independently of nuclear events or de novo protein synthesis and is mediated by rapid subcellular mechanisms, suggesting a classical and nuclear ER-independent mechanism (33). In this study, we will define the mechanism by which chronic E2-GPER1 activation induces cytoprotective effects against H_2O_2 deleterious effects, which are not fully understood yet.

The classical and nuclear ER, ERα, and ERβ, have been shown to localize in the mitochondria of cardiac cells (34, 35). Hence, their role in modulating mitochondrial structure and function in both normal and pathological conditions is not surprising. However, GPER1 has not been found to localize in mitochondria; nonetheless, studies have confirmed a role for GPER activation in the preservation of mitochondrial structural integrity and maintenance of function after I/R (36, 37). In fact, DNA microarray and gene set enrichment analysis (GSEA) in GPER1 KO mice revealed that GPER1's cardioprotective effects both in physiological and pathological conditions might be related to enhancements in mitochondrial function (37). Our group has also reported that acute pre- and post-ischemic GPER1 activation induces cardioprotective effects against I/R injury by protecting mitochondrial integrity and function, and reducing mitophagy (4, 5), hence, providing a premise for GPER1-induced mitochondrial protection. However, whether chronic GPER1 actions impact the mitochondria still needs to be studied. Also, acute GPER1's effects and signaling is starting to be well-documented; the mechanisms underlying chronic GPER1 actions still need further clarification. In fact, few studies have investigated the mechanisms involved in chronic GPER1 actions. Using cardiac arrest-induced global ischemia, chronic pretreatment with G1 *in vivo* has been shown to induce cardioprotective effects against I/R injury (38). Also, chronic activation of GPER1 using G1 has been shown to protect hippocampal and striatal neurons from injury following cardiac arrest and cardiopulmonary resuscitation (CA/CPR)-induced cerebral ischemia (38, 39).

Recently, accumulating literature suggests a strong cross-talk between the genomic and non-genomic GPER1's downstream pathways. GPER1 and the plasma membrane-associated estrogen receptors (mERs), mERα, and mERβ have been reported to mediate both genomic and non-genomic effects (40, 41). In breast cancer cells, GPER1 actions have been found to stimulate key regulators of the evolutionarily conserved Hippo pathway that involves the yes-associated protein 1 (YAP) and transcriptional coactivator with a PDZ-binding domain (TAZ), which are homologous transcription coactivators (40, 42). Moreover, GPER1 activation in the same cancer cell line has been shown to mediate the expression of an array of genes, including CTGF, CYR61, EDN1, and EGR1 (43–45), which are well-established YAP/TAZ target genes. This suggests that the Hippo/YAP/TAZ pathway might be a key downstream signaling pathway of GPER1 long-term actions, especially in breast cancer tumorigenesis (40). The Hippo pathway plays a critical role in cardiac development, regeneration, and disease (46, 47). Dysregulation of the Hippo pathway *in utero* can lead to various congenital cardiac abnormalities (46, 48, 49). Cardiac-specific deletion of the Hippo pathway components and overexpression of activated YAP in mouse embryos resulted in increased cardiomyocyte proliferation leading to cardiomegaly and enlarged hearts in embryos (48, 50). On the other hand, the ablation of YAP in cardiac tissue led to cardiac hypoplasia and lethality (48, 49). In fact, a new study suggests that YAP activation induces proliferation (cardiogenesis) in adult cardiomyocytes by partially reprograming them to a more fetal and proliferative state through enhancing chromatin accessibility (51). Activation of YAP, or deficiency of the Hippo pathway, has also been shown to improve cardiac tissue survival and function after myocardial infarction (46, 52, 53). However, whether GPRI1 activation induces protection against cell death via deactivation still needs further investigations.

In this study, using H9c2 rat cardiomyoblasts treated with a cytotoxic agent, H_2O_2, we investigated whether chronic GPER1 activation protects against H9c2 cell death by preserving mitochondrial integrity and deactivating the Hippo/YAP pathway.

MATERIALS AND METHODS

Experimental Protocols

All protocols followed the Guide for the Care and Use of Laboratory Animals (US Department of Health, NIH) and received the UT Health Science Center at San Antonio Institutional Animal Care and Use Committee (IACUC) institutional approval.

Animals

Adult male Sprague–Dawley rats (4–6 months old, $n = 4$) were obtained from Charles River Laboratories. The animals were housed in the animal-specific pathogen-free facility at UTHSCSA's main campus in cages with standard wood bedding and space for two rats. The animals had free access to food and drinking water *ad libitum* and a 12-h shift between light and darkness. The animals were selected randomly, and the data analysis was performed by a blinded investigator.

Cell Culture and Treatments

H9c2 rat cardiomyoblast cell line was purchased from the American Type Culture Collection (ATCC Cat# CRL-1446, RRID: CVCL_0286). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS; GIBCO-BRL, Grand Island, NY, USA), 100 U/mL penicillin–streptomycin and grown in an atmosphere of 5% CO_2-95% humidified air at

37°C. The culture medium was changed every second day. Cells between passages 4 and 7 were seeded at a density of 0.5×10^6 in six-well plates, or a density of 0.7×10^6 in T-25 flasks and were used for experiments at 80–90% confluence ($\sim 1.5 \times 10^6$ for six-well plates and $\sim 2.8 \times 10^6$ for T-25 flasks. Cells were regularly tested for mycoplasma using the Lookout Mycoplasma PCR detection kit (MP0035, Sigma). The cultured cells were exposed to H_2O_2 (Sigma, H1009) at a concentration of 100 μM for 24 h at 37°C. Cells were immediately washed three times with cold PBS, and the media was replaced with either serum-free DMEM plus 100 U/mL penicillin–streptomycin and 0.01% DMSO (Control, vehicle), or with 40 nM E2 (E8875, Sigma), or 1 μM G1 (Cayman, 10008933), or E2 (40 nM)/G1 (1 μM) + G15 (1 μM, Cayman, 14673), for 48 and 96 h. The cells were selected randomly, and the data analysis was performed by a blinded investigator.

Cell Viability

Cell viability was assessed spectrofluorometrically using either a Calcein AM assay (ThermoFisher Scientific, catalog no. C3100MP) according to the manufacturer's instructions or 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by following standard protocols. Briefly, cells were cultured in a 96-well plate and treated with 100 μM H_2O_2 for 24 h, followed by the incubation in a media supplemented with the different drugs for 48 or 96 h as described above. Media containing the drug treatments were carefully aspirated, and cells were used for MTT assay or Calcein AM assay.

Flow Cytometry Analysis

Percent live and dead cells were determined using Annexin V-PE/7-AAD Apoptosis Detection kit (BD Bioscience, BD PharMingen, catalog no. 556547) according to the manufacturer's instructions with Annexin V PE replaced with Annexin V APC. Cells treated with H_2O_2 were washed twice in PBS and incubated in the presence of the different drugs, as described above. After 48 h of drug treatment, cells were resuspended in 400 μL of 1x binding buffer and stained with 5 μL of APC-conjugated Annexin V (BD PharMingen, catalog no. 550475) and 5 μL 7-aminoactinomycin-D (7-AAD; BD PharMingen, catalog no. 559763) and analyzed using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA, UTHSCSA flow cytometry core). Cells considered viable possess intact membranes and excluded both dyes (Annexin V and 7-AAD negative); cells that are in early apoptosis are Annexin V positive and PI or 7-AAD negative, and cells that are in late apoptosis or already dead are both Annexin V and PI or 7-AAD positive.

BrdU Labeling and Cell Cycle Analysis

For cell cycle progression analysis, cells were cultured and treated as described above. Cells were labeled with propidium iodide BrdU and analyzed by flow cytometry using the FITC BrdU Flow kit obtained from BD Biosciences following the manufacturer's instructions. Briefly, cells were pulse-labeled with 10 μM BrdU in culture medium for 30 min, trypsinized washed with PBS, fixed, and permeabilized with triton (0.25%). Incorporated BrdU in cells was exposed by DNase treatment and stained by an FITC-conjugated anti-BrdU antibody. Total DNA was stained by 7-AAD (7-amino-actinomycin D). Data were collected on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA, UTHSCSA flow cytometry core) and analyzed with CellQuest Pro software.

Mitochondrial Structural Integrity

H9c2 cells pretreated with H_2O_2 and incubated in media containing different drugs, as described above, were processed for electron microscopy imaging to observe mitochondrial quality and morphology as described previously (54). Cells were fixed in 2.5% (wt/vol) glutaraldehyde (Fluka), at 4°C overnight. Cells were then washed with PBS and fixed in 2% (wt/vol) osmium tetroxide for 2 h at room temperature. The fixed cells were dehydrated in a graded alcohol series and embedded in Eponate 12 medium, and the blocks were cured at 60°C for 48 h. Sections (70 nm) were cut with an RMC ultramicrotome and mounted on Formvar-coated grids. The sections were double-stained with uranyl acetate and lead citrate, and finally examined and imaged with a 100CX JEOL transmission electron microscope.

Mitochondrial Membrane Potential Measurement

MMP was assessed fluorometrically using MitoTracker Red CMXROS assay kit (ThermoFisher Scientific, catalog no. M7512) according to manufacturer's protocol. H9c2 cells were plated on coverslips for labeling and allowed to reach 70–80% confluence, after which cells were treated with or without H_2O_2 for 24 h and subjected to different treatments (control, G1, G1 + G15) for 48 h. Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 10 μM was used as positive control). Cells were incubated with 150 nM MitoTracker Red for 1 h at 37°C. The fluorescence intensity was measured using the ImageJ program. Data plotted were normalized on cell number.

Detection of mPTP Opening

H9c2 cells were pretreated with H_2O_2 for 24 h and incubated in a media containing control [vehicle, DMSO (0.01%)], G1, or G1 + G15 for 48 h. The opening of the mPTP was assessed using a Transition Pore Assay kit (MitoProbe; Life Technologies) according to the manufacturer's instructions. Briefly, cells were incubated with 2 μM Calcein and 1 mM $CoCl_2$ in Hank's Balanced Salt Solution (HBSS)/Ca^{2+} at 37°C for 15 min while protected from light. Calcein diffuses into cells passively and accumulates into the cytosol and mitochondria to liberate the highly polar fluorescent dye Calcein. $CoCl_2$ can quench the cytosolic fluorescence, while mitochondrial fluorescence is maintained. Opening of mPTP instigates the release of Calcein from the mitochondria into the cytosol, which results in a reduction in fluorescence. After two washes with HBSS/Ca^{2+}, the calcein fluorescence intensity of cells ($\sim$30,000 for each experiment) was detected by high-content screening at 488/530 nm using an LSR II flow cytometer (BD Biosciences).

ATP Assay

Intracellular ATP levels in cells treated with H_2O_2 (24 h), followed by 48 h treatment with control (vehicle), G1, or G1

+ G15 were quantified using an ATP Bioluminescence Assay kit (Roche Applied Science, catalog no. A22066) according to the manufacturer's protocol. The luminescence of the cells was measured using a plate reader. The concentration of ATP in each group was obtained using an ATP standard curve and normalized to the protein concentrations of the samples, which were determined using the BCA assay.

Western Blot Analysis

H9c2 cells were lysed in a buffer containing (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 10 mM Hepes, 0.1% octylphenyl-polyethylene glycol (IGEPAL CA-630), 0.25% sodium deoxycholate, pH 7.4, supplemented with Complete Protease Inhibitor Cocktail Tablets. Whole-cell lysates were centrifuged at 13,000 × g, for 10 min at 4°C. Equal amounts (40 μg) of proteins were loaded in each well of 4–20% Tris-glycine gels (Bio-Rad) and subjected to electrophoresis for 90 min at 125 V of constant voltage as described previously (55). Gels were transferred onto a nitrocellulose membrane by electrophoretic transfer at 90 V of constant voltage for 90 min. After transfer, the membrane was washed, blocked with 5% blocking solution and probed with the following antibodies anti-phospho-YAP (Ser127) (Cell Signaling Technology Cat# 4911, RRID:AB_2218913) 2 μg/mL, anti-YAP (Cell Signaling Technology Cat# 14074, RRID:AB_2650491) 1 μg/mL, anti-phospho-Mst1/2 (pThr183), (Sigma-Aldrich Cat# SAB4504042, RRID:AB_2665403) 2 μg/mL, anti-Mst1/2 (Cell Signaling Technology Cat# 14946, RRID:AB_2798654) 1 μg/mL, anti-Drp1 (Cell Signaling Technology Cat# 8570, RRID:AB_10950498) 1 μg/mL, anti-Mfn1 (Cell Signaling Technology Cat# 14739, RRID:AB_2744531) 1 μg/mL, anti-phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology Cat# 9251, RRID:AB_331659) 2 μg/mL, anti-SAPK/JNK (Cell Signaling Technology Cat# 9252, RRID:AB_2250373) 1 μg/mL, and anti-GAPDH (Cell Signaling Technology Cat# 2118, RRID:AB_561053) 1 μg/mL, were incubated at 4°C overnight. The immunoreactive bands were visualized using secondary Li-Cor antibodies (LI-COR Biotechnologies, Lincoln, NE): IRE 800CW goat anti-rabbit antibody (LI-COR Biosciences Cat# 926-32211, RRID:AB_621843) 0.1 μg/mL, and IRE Dye 680RD goat anti-mouse antibody, LI-COR Biosciences Cat# 926-68070, RRID:AB_10956588) 0.1 μg/mL. The band intensity was quantified using Li-Cor Odyssey® CLx Imaging System.

Transfection

pLKO1-shYAP1 and pCMV-flag S127A YAP were gifts from Kunliang Guan (Addgene plasmid # 27368; RRID:Addgene_27368) (44), (Addgene plasmid # 27370; RRID:Addgene_27370) (56). pcDNA-Flag Yap1 was a gift from Yosef Shaul (Addgene plasmid # 18881; RRID:Addgene_18881) (57). H9c2 cells passage 4–7, at 70–80% confluence, were transfected with either pLKO1-shYAP1, or pCMV-flag-S127A YAP or pcDNA-Flag Yap1 using Lipofectamine 3000 (ThermoFisher Scientific, catalog no. L3000015) according to manufacturer's protocols.

Isolation of Adult Cardiomyocytes

Cardiomyocytes from adult 4–6 months old (minimum 300 g) rats were isolated following the procedures described in Ref. (58). Briefly, animals were injected intraperitoneally with heparin (200 IU/kg), and 20 min later, they were anesthetized with ketamine (80 mg·kg^{-1} i.p.) and xylazine (8 mg·kg^{-1} i.p.). Hearts were then harvested and instantaneously arrested in ice-cold PBS (KCl 2 mM, KH_2PO_4 1.5 mM, NaCl 138 mM, Na_2HPO_4 8.1 mM) to remove excess blood. Hearts were transferred to ice-cold Tyrode's solution [NaCl 130 mM, KCl 5.4 mM, $MgCl_2$ 1 mM, Na2HPO4 0.6 mM, Glucose 10 mM, Taurine 5 mM, 2,3-butanedione monoxime 10 mM, and Hepes 10 mM, pH 7.4, oxygenated with 95% (vol/vol) O2–5% (vol/vol) CO_2], and mounted on a modified Langendorff apparatus at a constant pressure of 80 cm H_2O. After 5 min of perfusion at 37°C with Tyrode's solution, the heart was perfused for 10 min with Tyrode's solution containing 186 U/mL Collagenase Type-2 and 0.5 U/mL Protease Type-XIV, and then washed for 5 min with a high K + buffer (KB) [KCl 25 mM, KH_2PO_4 10 mM, $MgSO_4$ 2 mM, glucose 20 mM, Taurine 20 mM, Creatine 5 mM, K-Glutamate 100 mM, aspartic acid 10 mM, EGTA 0.5 mM, Hepes 5 mM, and 1% (wt/vol) BSA, pH 7.2 oxygenated with 95% O_2-5% (vol/vol) CO_2]. After washing, the left ventricle was cut into pieces in KB solution to dissociate cells. Isolated cardiomyocytes were filtered (100-μm strainer) and centrifuged for 2 min at 1,000 × g for further use. Cardiomyocytes from each heart were divided into four groups (sham, control, G1, and G1 + G36) triplicate and cultured in 24-well plate.

Hypoxia and Reoxygenation of Isolated Cardiomyocytes

To simulate hypoxia, substrate (glucose/serum) and oxygen deprivation, freshly isolated cardiomyocytes were resuspended in serum-free/glucose-free HEPES-buffered medium [mmol/L: NaCl 113, KCl 4.7, HEPES 10, $MgSO_4$ 1.2, Taurine 30, calcium chloride ($CaCl_2$) 1, pH 7.4, and 37°C]. Cardiomyocytes were incubated in a controlled hypoxic chamber, O_2/CO_2 incubator containing a humidified atmosphere of 1% O_2, 5% CO_2, and 94% N_2 at 37°C. After hypoxia, the cells were reoxygenated in a normoxic incubator at 37°C by replacing the hypoxic media with DMEM supplemented with 10% FBS. For untreated (sham) conditions, freshly isolated cardiomyocytes were washed twice with a HEPES-buffered medium (mmol/L: NaCl 113; KCl 4.7, HEPES 10, $MgSO_4$ 1.2, Taurine 30, $CaCl_2$ 1, pH 7.4, and 37°C) and incubated with HEPES-buffered medium supplemented with bovine calf serum (5%) and glucose (5.5 mmol/L).

Immunofluorescence Staining

For immunofluorescence staining, cells cultured on coverslips were pretreated with H_2O_2 and incubated in the different milieu as described above. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25% Triton X-100. After blocking in 3% BSA for 30 min, slides were incubated with the first antibody diluted in 1% BSA overnight. After washing with PBS, coverslips were incubated overnight with YAP antibodies (Novus Cat# NB110-58358, RRID:AB_922796) and with the secondary antibodies Alexa Fluor 488 Goat anti-rabbit

(Abcam Cat# ab150077, RRID:AB_2630356). Images were taken on a Zeiss Axiovert 200M inverted motorized fluorescence microscope (Carl Zeiss Microscope, Jena, Germany).

RNA Extraction, cDNA Synthesis, and Real-Time PCR Amplification

Total RNA was extracted from H9c2 cells using Trizol reagent (Invitrogen) followed by DNase digestion for 10 min at room temperature with RNase-Free DNAse Set (Qiagen), and cleaned up with RNeasy Mini Kit (Qiagen). The quality of the RNA was determined by electrophoresis through agarose gels; only RNA samples with 28S:18S, rRNA ratio $\geq$ 2were used. Oligo-dT primer was used to target mRNAs present in the total RNA samples for conversion into cDNAs by reverse transcriptase (RT). Cleaned-up total RNA (2 μg) was reverse transcribed in a final volume of 20 μL containing 1x RT buffer, 0.5 mM dNTP Mix, 10 units of RNasin RNase inhibitor (Promega), 4 units of Omniscript RT (Qiagen), and 1 μM oligo-dT primer. Samples were incubated at 37°C for 60 min, followed by RT inactivation at 95°C for 5 min. Real-time PCR and gene-specific primers were used for quantification of TGF-β, PGC1-α, Nrf1, and YAP-responsive genes and FOXO3 responsive genes using Fast SYBR™ Green Master Mix (ThermoFisher, catalog number 4385614). The specificity of the reaction was verified by melt curve analysis. The relative quantification in gene expression was determined using the 2–ΔΔCt method (59). Using this method, we obtained the fold changes in gene expression normalized to internal control genes (β-actin, and/or GAPDH). The following primers were used for amplification

Gene	Forward primer (5′-3′)	Reverse primer(5′-3′)
TGF-β	GACCGCAACAACGCAATCTA	AGGTGTTGAGCCCTTTCCA
PGC1-α	GATGCCAACAAGAACAAAGGT	TCTGGGGTCAGAGGAAGAGA
Nrf1	CCAAACCCACAGAGAACAGAA	TCCATGCATGAACTCCATCT
CTGF	CAAGCTGCCCGGGAAAT	CGGTCCTTGGGCTCATCA
CYR61	GTGCCGCCTGGTGAAAGAGA	GCTGCATTTCTTGCCCTTTTTTAG
ANKRD1	ATCCATGATGGTTTTTCGAGTAGAGG	GGCCTCGAGTCAGAACGTAGCTATGCGC
Bim	GCCCCTACCTCCCTACAGAC	CCTTATGGAAGCCATTGCAC
PUMA	AGTGCGCCTTCACTTTGG	CAGGAGGCTAGTGGTCAGGT
GAPDH	GCAAGTTCAATGGCACAG	CATTTGATGTTAGCGGGAT
β-actin	ATCTGGCACCACACCTTC	AGCCAGGTCCAGACGCA

Statistical Analysis

Data presented in bar graphs are expressed as means, and error bars are the standard errors of the mean (± SEMs) for a minimum of three independent trials ($n \geq 3$). Comparisons were conducted using the one-way ANOVA with Tukey's corrections for multiple comparisons, where appropriate, using Prism 8 (Graphpad Software). A difference of $P < 0.05$ was considered to be statistically significant.

RESULTS

Chronic GPER1 Activation Induces Cytoprotective Effects Against H_2O_2-Induced H9c2 Cell Death

E2 has been shown to act via its three known estrogen receptors (ERs): ERα, ERβ, or GPER1. We determined the influence of each ER against H_2O_2-induced cytotoxic effects using cultured H9c2 cardiomyoblasts. We found that E2 as well as all the ER-specific agonists, PPT (for ERα), DPN (for ERβ), and G1 (for GPER1) increased the level of live cells compared to control (**Figure 1A**). However, the level of live cells in the E2-treated group was significantly higher than that of PPT and DPN groups, but E2 effects were similar to G1, suggesting that the E2-induced increase in cell survival might be mainly mediated through GPER1. We, therefore, evaluated the cytoprotective effects of GPER1 activation in chronic E2 treatment against the harmful effects of oxidative stress on H9c2 cardiomyoblasts. To this end, H_2O_2 pretreated cells were incubated in the presence of vehicle (control), E2, or E2 + G15 (G15 is a GPER1 selective antagonist) after 48 and 96 h. We found that chronic E2 treatment increased cell viability measured by Calcein AM assay compared to control (**Figure 1B**). Since these chronic effects of E2 were similar after 48 and 96 h, we chose a 48-h time point for further experiments. We also found that E2 treatment reduced cell death induced by H_2O_2 treatment compared to control. In fact, the rate of cell death that was 49 ± 2% in the control group (vehicle) was decreased to 25 ± 1.5% in E2-treated group (**Figure 1C**). Similar results were obtained with cell viability (**Figure 1D**) in which the level of live cell was increased in the E2-treated group (75 ± 4%) vs. control (50 ± 2%). However, all these E2 protective effects were antagonized by cotreatment with G15. In fact, when E2 was cotreated with G15 (E2 + G15 group), the levels of cell death were increased to 36 ± 2%, similar to the control group (49 ± 2%), and the cell viability was decreased from the E2 group (75 ± 4%) compared to the E2 + G15 group (47.5 ± 3%).

Together, these results suggest that chronic E2 treatment induces cytoprotective effects against H_2O_2-induced H9c2 cell death through GPER1 activation.

Chronic GPER1 Activation Protects Against H_2O_2-Induced Mitochondrial Dysfunction in H9c2 Cells

The cytotoxic agent H_2O_2 treatment has been shown to induce mitochondrial depolarization, increase mitochondrial calcium overload, alter ATP synthase activity, and affect mitochondrial dynamics (60, 61). Therefore, we tested whether chronic GPER1 actions can restore mitochondrial function after H_2O_2 insult. To this end, we measured MMP and unveiled the mitochondrial structural integrity in H9c2 cells pretreated with H_2O_2 followed by treatment with vehicle (control), E2, or E2 + G15. We found that chronic E2 treatment prevented H_2O_2-induced dissipation of MMP. In fact, the control mitochondria displayed a reduction

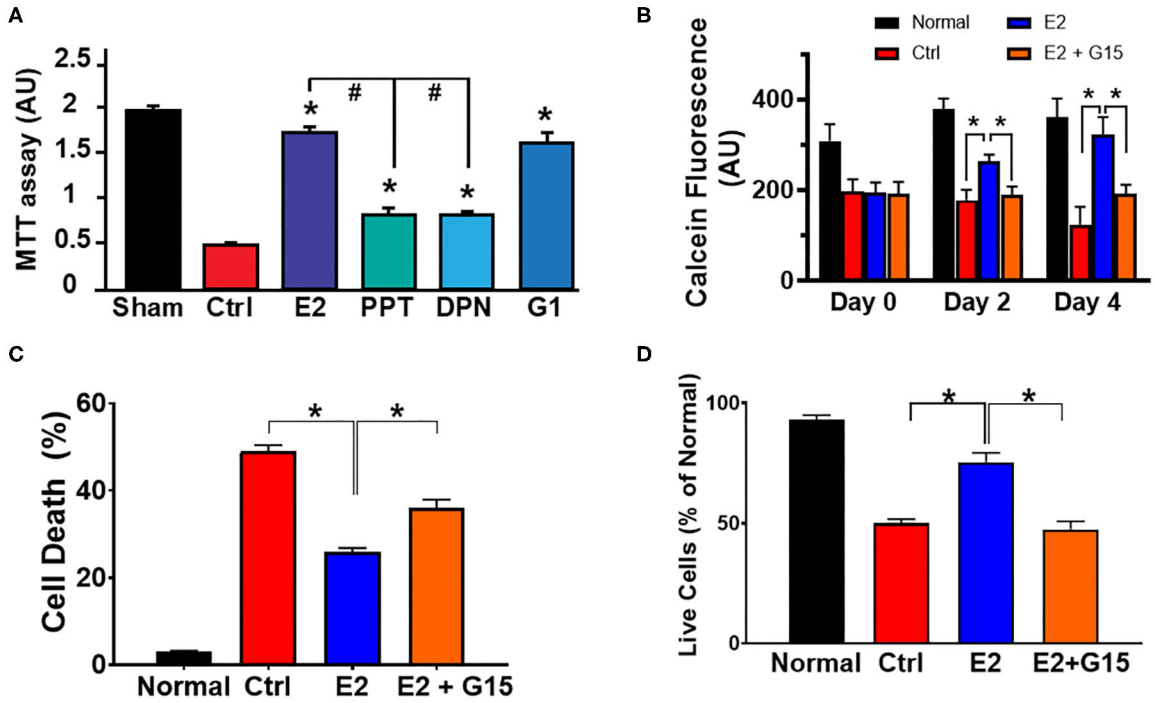

FIGURE 1 | Chronic G protein-coupled estrogen receptor 1 (GPER1) activation induces cytoprotective effects in H9c2 cells. **(A)** Bar graph showing the cell viability of H9c2 cardiomyoblasts after H_2O_2 insult, and treatment with estrogen (17β-estradiol; E2) and specific agonists for estrogen receptor (ER)α (PPT), ERβ (DPN), and GPER1 (G1). Note that G1 cytoprotective effect is similar to E2. **(B)** Bar graph showing the Calcein AM viability assay on normal H9c2 cells at 24 h post H_2O_2 treatment (day 0) and after chronic treatment with either control media (Ctrl; red bars), E2 (40 nM, blue bars), or E2 (40 nM) + GPER1 antagonist (G15) (1 μM) (E2 + G15; orange bars) for 48 h (day 2) or 96 h (day 4). Values are expressed as means ± standard error of the mean (SEM) of five independent experiments $^*P < 0.001$, $n = 5$/group, nine wells for each. Note that E2 treatment for 48 or 96 h increases in the fluorescence intensity compared to the control and E2 + G15 indicating an increase in cell viability of cells treated chronically with E2. **(C)** Bar graph showing a reduction in cell death by apoptosis (Annexin V+/PI+) in E2-treated cells compared to control (Ctrl, vehicle) and E2 + G15-treated cells. Values are expressed as means ± SEM $^*P < 0.05$. **(D)** Bar graph showing an increase in cell viability [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] in the chronic E2 group compared to the control and E2 + G15. Values are expressed as means ± SEM of five independent experiments $^*P < 0.05$, $n = 5$/group, nine wells for each. * Compared to control, # compared to E2-treated group.

in fluorescence intensity of MitoTracker Red compared to H_2O_2-untreated cells (141,173 ± 26,198 vs. 286,448 ± 31,520). However, chronic E2-treated mitochondria exhibited higher fluorescence intensity, almost similar to the H_2O_2-untreated cell mitochondria (250,912 ± 45,145) (**Figure 2A**). This E2-induced higher fluorescence intensity was prevented by cotreatment with G15. These results suggest that chronic E2 treatment preservation of the MMP is mediated through GPER1 activation.

We also defined the effect of chronic GPER1 activation on the mitochondrial structure of H9c2 cells treated with H_2O_2. Observation of electron microscopy images of treated cells in each group showed that after H_2O_2, 84 ± 5% of the mitochondria from control-treated cells were damaged and characterized with smaller, ruptured, and fragmented cristae morphology compared to H_2O_2-untreated in which mitochondria cristae were mostly normal and not disrupted (**Figure 2B**). However, after H_2O_2 treatment, cells incubated in the presence of E2 had only displayed 28 ± 3% damaged mitochondria. In comparison, cell cotreatment with E2 + G15 exhibited 73 ± 6% damaged mitochondria. This data suggest that selective inhibition of GPER1 abridges the preservation mitochondrial integrity and function effects induced by E2 treatment.

We finally determined whether chronic activation of GPER1 preservation of mitochondria integrity and function was related to increased fission and fusion. We, therefore, measured the levels of fission proteins, dynamin-related protein 1 (Drp1), and Mitofusin 2 (Mfn2), which regulates fusion. We observed no changes in both Drp1 and Mfn2 protein levels (**Figure 2C**) in the E2-treated group compared to the control (vehicle), suggesting that chronic E2 preservation of mitochondrial structure and function is not via increased mitochondrial dynamics.

Together, these results indicate that chronic E2 treatment preserves against H_2O_2 insult increase in mitochondrial cristae damage, which presumably is responsible for the increase in mitochondrial membrane potential via GPER1 activation.

Chronic GPER1 Activation Inhibits mPTP Opening After H_2O_2 Insult

We further assessed the impact of the regulation of the mitochondrial permeability transition pore (mPTP) opening in

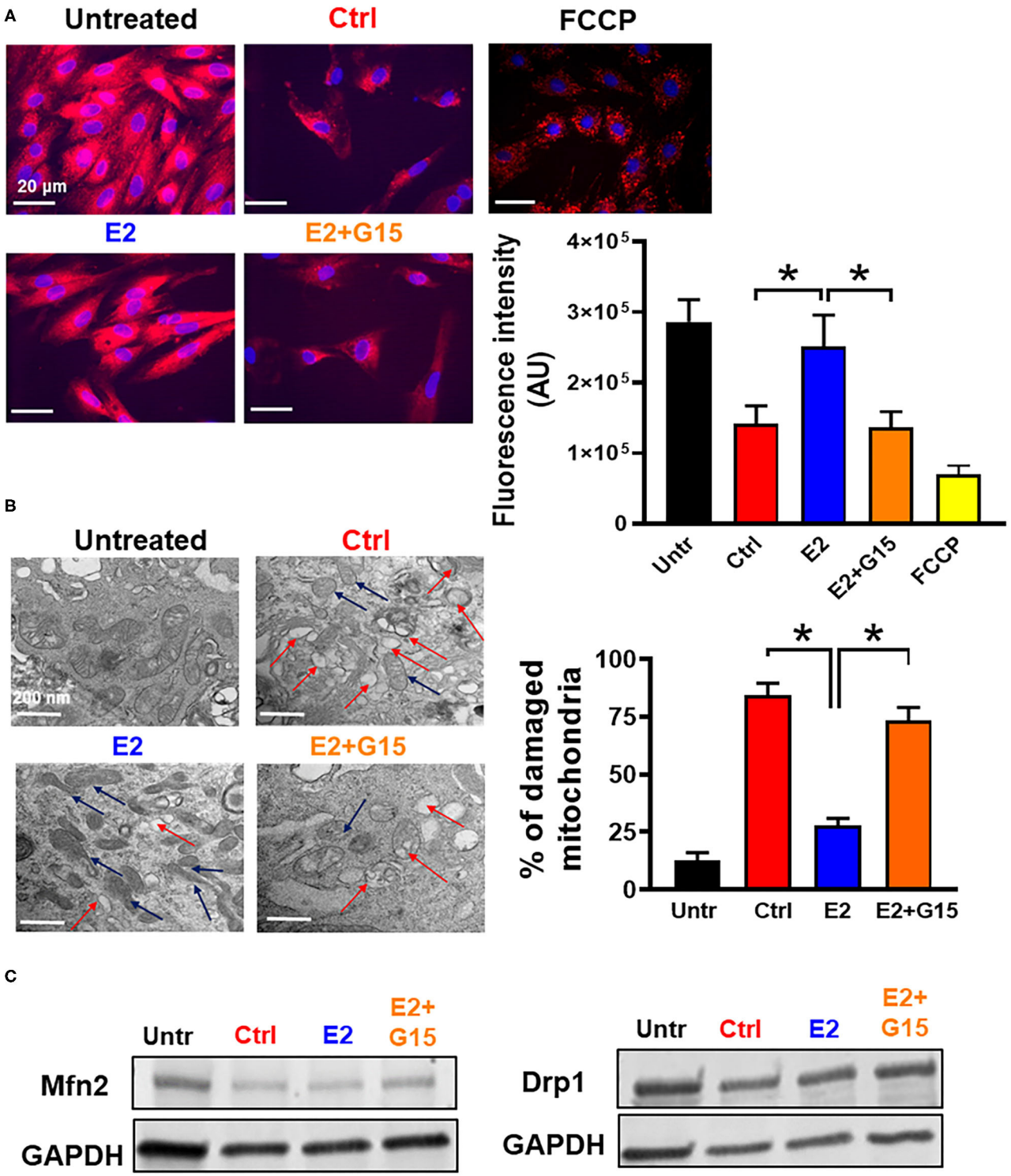

FIGURE 2 | Chronic GPER1 activation preserves mitochondrial structure and function. **(A)** *Left*: Representative fluorescent microscope images used to measure mitochondrial membrane potential using the MitoTracker Red dye in H9c2 cells treated with H_2O_2 followed by incubation in control (Ctrl), E2 (blue), and E2 + G15 (orange), $n = 5$/group, nine wells for each. Red color denotes MitoTracker Red staining. FCCP (yellow) was used as positive control. *Right*: The relative fluorescence intensities of MitoTracker Red quantified on a per-cell basis in H9c2 cells treated as described above. Values are expressed as means ± SEM of five independent experiments $^*P < 0.001$. **(B)** *Left*: Microscopy images of H9c2 cells showing damaged mitochondria cristae in cells treated with Ctrl, and E2 + G15 compared to E2-treated cell mitochondria. *Right*: Bar graph showing percentage of damaged mitochondria in each group. Fragmented or disrupted cristae with empty spaces (in the matrix) were considered damaged mitochondria, while mitochondria with dense continuous cristae were considered as good or undamaged. A minimum of 100

(Continued)

FIGURE 2 | mitochondria were counted in each group. Values are expressed as means ± SEM of five independent experiments *$P < 0.05$, $n = 5$/group **(C)** Representative immunoblots showing no change in the levels of mitochondrial fission protein (Drp1) and mitochondrial fusion protein (Mfn2) in all the three groups Control (vehicle), E2, and E2 + G15.

the mechanism of chronic GPER1 activation. The mPTP opening is a well-known effector that mediates cell death by apoptosis and necrosis (62, 63). H9c2 cells were pretreated with H_2O_2 and incubated in the presence of control (vehicle), E2, E2 + G15, or Cyclosporine A (CsA). The mPTP opening was measured using the Calcein-$CoCl_2$ assay. To confirm the specificity of this assay to the mPTP opening, we measured the calcein fluorescence intensity (CFI) in H_2O_2-treated H9c2 cells in the presence of CsA, a known inhibitor of the mPTP opening. We found that CsA treatment dramatically increased the CFI when compared to control. We observed that the CFI was drastically reduced in the vehicle-treated cells (Control) compared to H_2O_2-untreated cells, which indicate that mPTP opening was more active after H_2O_2 treatment vs. the untreated (**Figures 3B,C**). However, the CFI was much higher in chronic E2-treated cells compared to the control (**Figures 3B,C**). In addition, cotreatment with E2 + G15 displayed a much-reduced CFI compared to E2-treated cells and similar to the control group. These results indicate that chronic E2 treatment induces inhibition of mPTP opening via activation of GPER1.

In yeast, H_2O_2 treatment resulted in a decrease in ATP production in response to oxidative stress (64). We, therefore, sought to determine whether chronic E2 treatment following H_2O_2 insult in H9c2 cells enhances mitochondrial ATP production. We observed that after H_2O_2 treatment, control (vehicle)-treated cells decreased the levels of ATP production when compared with H_2O_2-untreated cells (32.6 ± 1.43 nmol/mg^{-1} in untreated compared to 20.102 ± 2.135 nmol/mg^{-1} in the Control group) (**Figure 3C**). Consistently, after H_2O_2 treatment, chronic E2 treatment increased the levels of ATP production compared to the control (28.986 ± 3.06 nmol/mg^{-1} vs. 20.102 ± 2.135 nmol/mg^{-1}). However, cotreatment with E2 + G15 did not significantly change the levels of ATP production (27.14 ± 2.168 nmol/mg^{-1}) vs. the E2-treated group (**Figure 3C**) suggesting that E2 induces preservation of the mitochondrial structure via GPER1 activation, and this effect is not associated with the increase in the rate of ATP production indicating improvement of mitochondrial function.

Together, these results indicate that chronic GPER1 activation-induced preservation of the mitochondrial integrity is associated with the inhibition of mPTP opening.

Chronic GPER1 Activation After H_2O_2 Treatment Does Not Induce Mitochondrial Biogenesis

Since we established that most of the chronic cytoprotective effects of E2 were mediated by GPER1 activation, we, therefore, proceeded to use the selective GPER1 agonist, G1, for our other experiments. We found that chronic GPER1 activation reduces mitochondrial dysfunction caused by H_2O_2 treatment. We determined whether chronic GPER1 activation protects the mitochondrial structural integrity by favoring mitochondrial biogenesis. To this end, pretreated cells with H_2O_2 were incubated in a no-serum media in the presence of vehicle (control), G1, or G1 + G15. We measured the mRNA levels of peroxisome-proliferator-activated receptor γ coactivator-1α (PGC-1α), which is a master regulator of mitochondrial biogenesis (65) and TGF-β1 that are negatively regulated by PGC-1α (66), as well as nuclear respiratory factor 1 (Nrf1), which is known to activate mitochondrial transcription factor A (TFAM) (67). We observed that the mRNA levels of PGC-1α and TGF-β1 were increased in G1-treated cells compared to the control, and cotreatment of G1 with G15 prevented these G1 effects (**Figure 4A**). However, we did not observe any differences in the mRNA levels of Nrf1 or TFAM in all the groups (**Figure 4A**). We further measured the levels of OXPHOS proteins (CII-SDHB, CIII-UQCR2, and CV-ATP5A), known as transcriptional targets of PGC-1α. Consistently, we did not observe any differences in the levels of OXPHOS proteins among the three different treatment groups (**Figure 4B**). However, this surprising result was not in conformity with the increase in the TGF-β1 mRNA levels observed in the G1-treated group compared to the control, and the G1 + G15 groups (**Figure 4A**). We, therefore, conclude that chronic GPER1 activation following H_2O_2 insult does not impact the mitochondrial biogenesis process.

Chronic GPER1 Activation Negatively Regulates the Hippo/YAP Pathway

The Hippo pathway can be regulated by hormonal signals that act through G-protein-coupled receptors (68), e.g., estrogen through GPER1 (40). Therefore, we tested whether the increase in the cells' survival induced by chronic treatment with the GPER1 agonists is mediated through the Hippo/YAP pathway. To this end, we compared the levels of phosphorylated MST1 (p-MST1) and YAP (p-YAP) in the control, G1, or G1 + G15 cells pretreated with an H_2O_2 agent. Western blot analysis revealed that the levels of p-MST1 and p-YAP were reduced in the G1-treated group compared to the control, and this G1 effect was abolished by the addition of G15 (**Figure 4C**). We also conversely found that the protein levels of both MST1 and YAP were increased in G1-treated cells compared to the control. Here, also the G1 effect was prevented by G15. Phosphorylation of YAP results in YAP−14–3–3 binding and cytoplasmic retention (69). In the contrary, non-phosphorylated YAP translocates to the nucleus, where it binds to transcriptional enhanced associate domain (TEAD) protein family to stimulate expression of cell growth and survival genes (44). We, therefore, determined whether G1 treatment increases YAP nuclear translocation. Using the immunocytochemistry approach, we observed that YAP expression was dispersed in the cytoplasm of control-treated cells (**Figure 4D**), while upon G1 treatment, YAP signal increasingly colocalized with DAPI (a dye

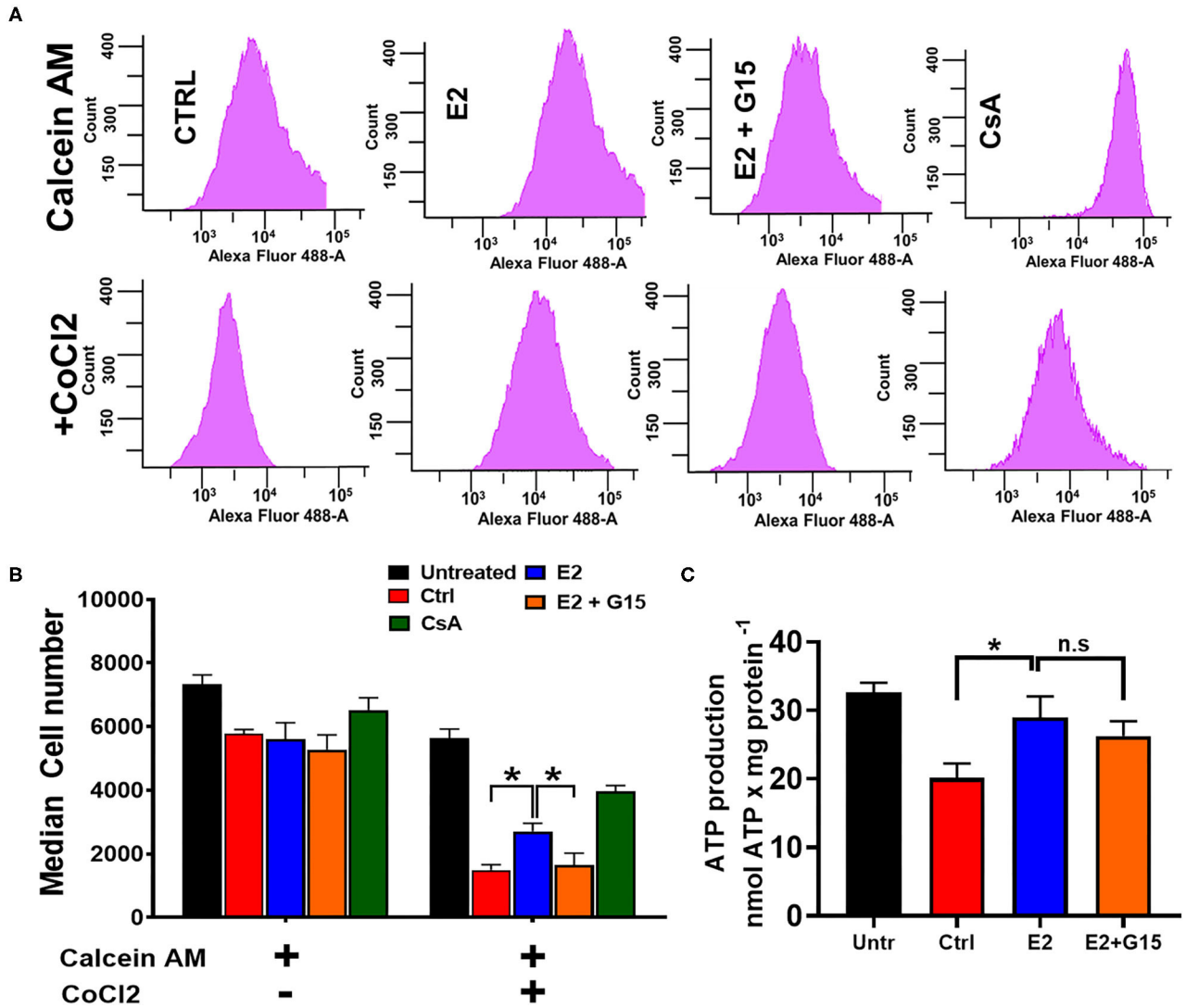

FIGURE 3 | Chronic GPER1 protects against H_2O_2-induced mitochondrial permeability transition pore (mPTP) opening. **(A)** Representative flow cytometry histograms of calcein fluorescence in the absence of $CoCl_2$ (top) and in the presence of $CoCl_2$. **(B)** Graph showing calcein fluorescence intensity in H9c2 cells treated with calcein/cobalt indicating the degree of mPTP activation. The graph shows no difference in fluorescence in all groups when treated with calcein only. However, the calcein fluorescence intensity is decreased in cells treated with control (red bars) and E2 + G15 (orange bars) compared to E2 group (blue bars) after calcein + $CoCl_2$ treatment. Cyclosporine A (1 μM) (green bars) was used as a positive control. Values are expressed as means ± SEM $^*P < 0.05$, $n = 6$/group. **(C)** Bar graph showing an increase in luminescence (ATP production) in cells treated with E2 and E2 + G15 compared with the control. Note that there is no difference in ATP production in the E2 and E2 + G15 groups, suggesting a mechanism independent of GPER1. Values are expressed as means ± SEM of five independent experiments $^*P < 0.05$, $n = 5$/group.

that stains nucleic acids). Cotreatment of G1 with G15 prevented the nuclear translocation of YAP and enhanced its cytoplasmic retention (**Figure 4D**). Further, we measured the mRNA levels of YAP-responsive genes (CTGF, CYR61, and ANKRD1), and we found that chronic G1 treatment increased the transcription of CTGF, CYR61, and ANKRD1 (**Figures 5A,B**) using β-actin (**Figure 5A**) or GAPDH (**Figure 5B**) as an internal control. These results indicate that chronic GPER1 activation activates YAP-mediated transcription of prosurvival genes. Since MST1 has been shown to trigger FOXO3 nuclear translocation leading to apoptosis (70), we measured the mRNA levels of PUMA and Bim, which are FOXO3-responsive genes after chronic G1 treatment. Our results show that both PUMA and Bim mRNAs were significantly increased in the control group of cells pretreated with H_2O_2 and that chronic G1 treatment decreased the levels of these two proapoptotic genes (**Figures 5A,B**).

To confirm the involvement of YAP in the mechanism of chronic GPER1 action, we determined whether knockdown of YAP in cells affects the G1-induced cytoprotective effects. To this end, H9c2 cells were transfected with a plasmid containing

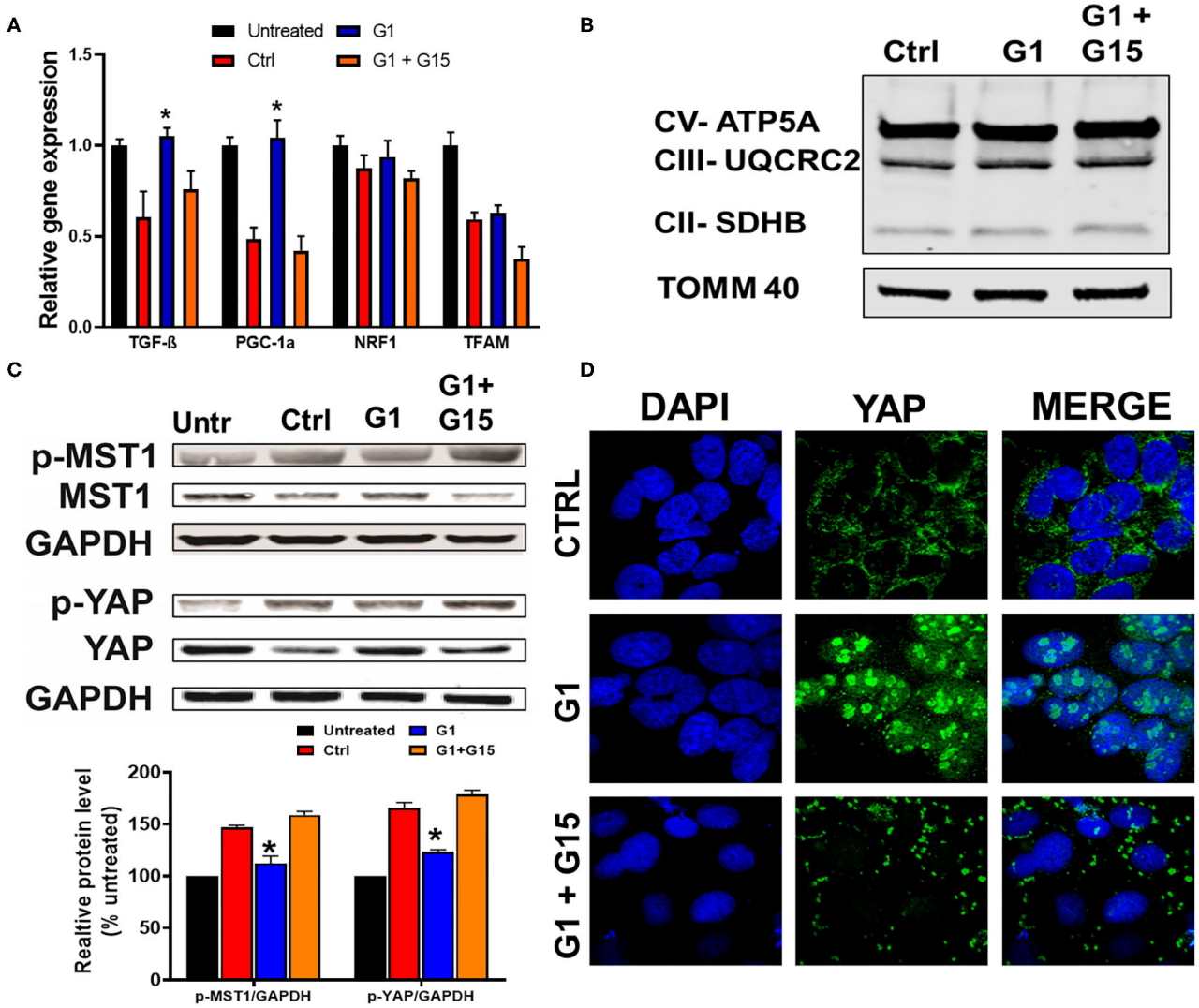

FIGURE 4 | Chronic GPER1 does not impact mitobiogenesis, but deactivates the Hippo/YAP pathway. **(A)** qRT-PCR analysis of mitochondrial biogenesis genes (PGC1-α and TGF-β) in H9c2 cells after chronic treatment with control (Ctrl), G1, and G1 + G15. Error bars indicate SEM. *$P < 0.05$, one-way ANOVA with Tukey posttest, $n = 6$ in each group. β-Actin was used as an internal control. Note an increase in both PGC1-α and TGF-β mRNA expression levels, and no change in mitochondrial transcription factor A (TFAM) and nuclear respiratory factor 1 (Nrf1) expression levels in the G1-treated group compared to the control and G1 + G15. **(B)** Immunoblots of mitochondrial fractions showing no changes in the protein levels involved in the mitochondrial oxidative phosphorylation after G1 treatment compared to the control and G1 + G15. **(C)** *Top*: Immunoblots showing a reduction in phosphorylated mammalian sterile-20-like kinase (p-MST1) and phosphorylated yes-associated protein (p-YAP) levels in the G1-treated cells compared to the Ctrl and G1 + G15 cells. *Bottom*: Bar graph (normalized to untreated) showing a reduction in p-MST1 and p-YAP in the G1-treated cells. Values are expressed as means ± SEM of five independent experiments *$P < 0.05$, $n = 5$/group. **(D)** Confocal microscopy images of H9c2 cells treated with control (Ctrl), G1, or G1 + G15, for 48 h and labeled with DAPI (blue) and anti-YAP (green) as well as the overlay of both signals. Note the increase in YAP–DAPI colocalization in G1-treated cells compared to Ctrl and G1 + G15.

shRNA against YAP1 (pLKO1-shYAP1). The knockdown efficiency was determined by Western blot analysis (**Figure 5C**). We observed that knockdown of YAP increased cell death in H9c2 cells (4.7 ± 0.53% in control plasmid vs. 12.63 ± 1.46% in pLKO1-shYAP1 transfected cells) (**Figure 5C**). However, the level of cell death in control plasmid treated cells was increased compared to YAP knockdown cells after treatment with control (vehicle) (48.83 ± 2.26% vs. 61.94 ± 4.82%), and in G1 + G15 (52.36 ± 3.2% vs. 61.23 ± 2.15%), in control-treated vs. pLKO1-shYAP1 treated, respectively. We also found that the rate of cell death in G1-treated cells was increased in YAP knocked down cells compared to the control (26.7 ± 4.43% vs. 47.67 ± 3.82%, respectively). This result suggests that the deletion of YAP increases cell's susceptibility to H_2O_2-induced cell death. We also determined whether the overexpression of YAP alone or the S127A mutant YAP, which constitutively remains in the nucleus and is transcriptionally more active (71), protects H9c2 cells from H_2O_2-induced cell death. We found

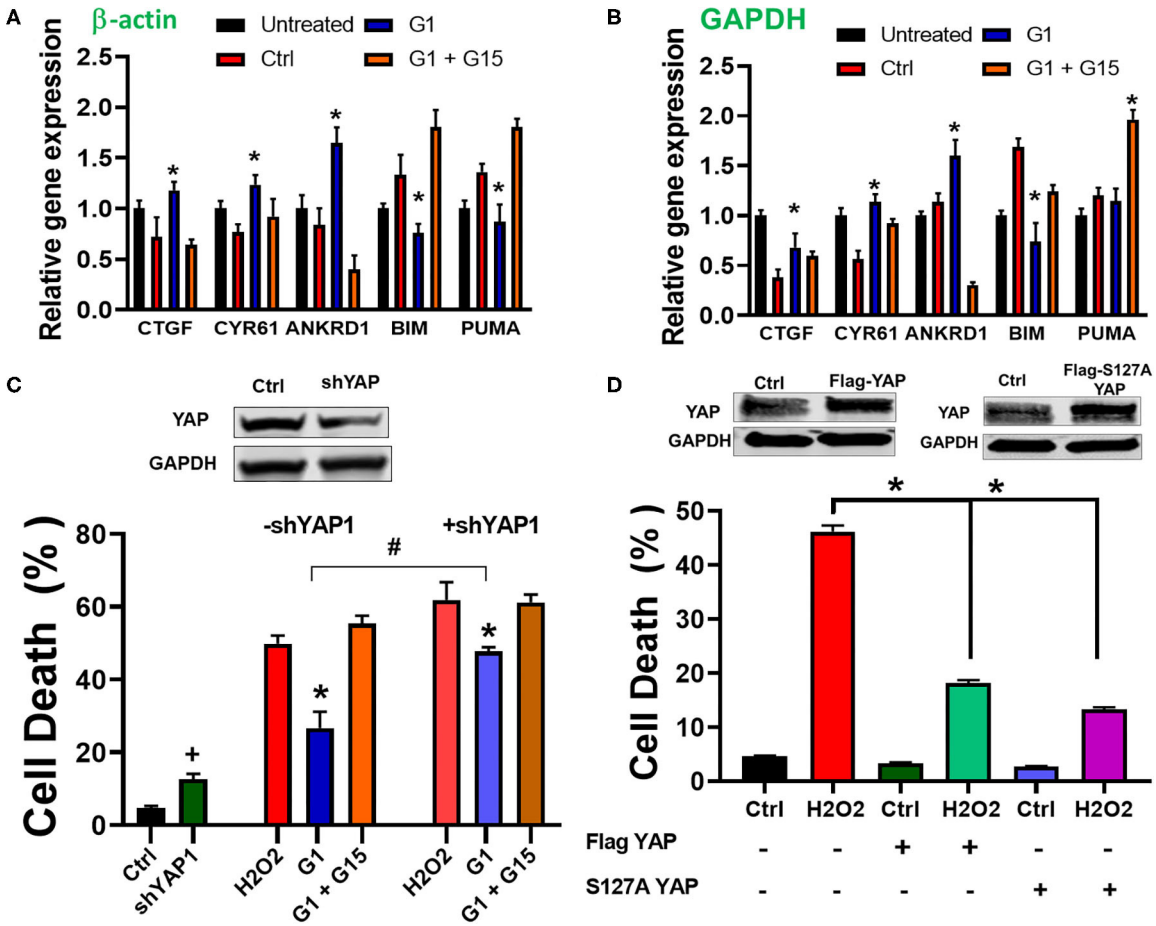

FIGURE 5 | Chronic GPER1 activation increases expression of YAP target genes. **(A)** qRT-PCR analysis of YAP-responsive genes in H_2O_2-treated H9c2 cells after incubation with control (Ctrl, vehicle), G1, or G1 + G15 using β-actin as an internal control. **(B)** Using GAPDH as an internal control. Note that G1 treatment increases the expression levels of CYR61, CTGF, and ANKRD1 mRNA, but reduces the expression of FOXO3-responsive genes (Bim and PUMA). $N = 6$/group. Error bars indicate SEM. $^*P < 0.05$, one-way ANOVA with Tukey post-test. **(C)** Top: immunoblot showing a reduction in YAP protein levels following knockdown by shRNA. Bottom: Bar graph showing an increase in cell death by apoptosis (Annexin V+/PI+) in YAP knockdown cells and after H_2O_2 treatment, followed by the incubation in the three different conditions. Note that the rate of cell death in YAP knockdown + E2 treatment was increased compared to E2 only. Values are expressed as means ± SEM $^*P < 0.001$, $n = 6$/group, $^{\#}P < 0.001$, $n = 6$/group vs. E2, and $^{+}P < 0.05$ vs. control plasmid $n = 6$/group. **(D)** Top: Immunoblots showing an increase in YAP protein levels following overexpression of Flag-YAP or S127A Flag-YAP. *Bottom*: Bar graph showing a decrease in cell death by apoptosis (Annex in V +/PI +) in YAP-overexpressed group compared to control. Note that after H_2O_2 treatment, the rate of cell death is reduced in YAP-overexpressing cells compared to PCMV6 plasmid (control). Values are expressed as means ± SEM $^*P < 0.001$ $n = 6$/group.

that cells overexpressing either YAP or S127A YAP were resistant to H_2O_2-induced cell death (**Figure 5C**). In fact, while cells transfected with control (PCMV6) plasmid and treated with H_2O_2 resulted in 48.4 ± 3.3% cell death, those overexpressing YAP or S127A YAP resulted only in 17.2 ± 2.5% and 13.4 ± 1.6% cell death, respectively (**Figure 5D**). This result indicates that the regulation of YAP influences H9c2 cell death induced by H_2O_2 insult.

Together, these results indicate that chronic GPER1 actions are mediated by regulation of the downregulation of the Hippo/YAP pathway that includes the cytosolic accumulation of YAP and its translocation in the nucleus where it promotes the upregulation of prosurvival genes and the downregulation of proapoptotic genes.

Chronic GPER1 Activation Reduces MST1–JNK Signaling

Activation of the MST1–JNK pathway has been described to promote cell death (72). We, thus, studied whether chronic GPER1 actions deactivate the MST1–JNK axis in H9c2 cardiomyoblasts treated with H_2O_2 agent. Western blot analysis performed in whole-cell lysate after treatment with the control, G1, and G1 + G15 revealed that the levels of phosphorylated JNK (p-JNK) were drastically increased in control (vehicle) vs. H_2O_2-untreated cells (**Figure 6A**). However, the p-JNK levels were reduced in chronic G1-treated cells when compared to the control (**Figure 6A**). Here also, G1 effects were prevented by G15 (**Figure 6A**) as the levels of p-JNK in the G1 + G15 group were similar to those in the control. This result suggests that the

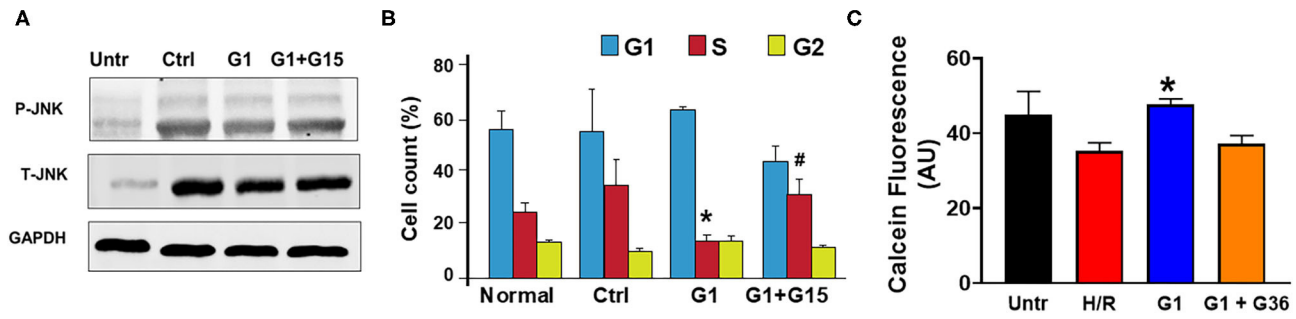

FIGURE 6 | Chronic GPER1 activation protects isolated cardiomyocytes from hypoxia/reoxygenation-induced cell death. **(A)** Representative immunoblots showing a reduction in the expression levels of p-JNK1 and T-JNK1 (total) in the G1-treated cells compared to the Ctrl and G1 + G15. **(B)** Bar graph showing the cell cycle progression analysis assessed by monitoring DNA content with flow cytometry. Relative cell number in each phase was quantified and mean percentage of cells at G1, S, and G2 phases are represented. Note that E2-treated group has fewer cells in the S phase compared to the Ctrl and G1 + G15 groups. Values are expressed as means ± SEM $^{*}P < 0.05$ vs. Ctrl, $^{\#}P < 0.05$ vs. Ctrl, $n = 6$/group. **(C)** Cell viability assay (calcein fluorescence) on isolated adult cardiomyocytes subjected to 6-h hypoxia followed by 48-h reoxygenation (H/R) in media supplemented with no serum (control), G1, or G1 + G36. Note the increase in the fluorescence intensity in G1-treated cells compared to control, and G1 + G36, indicating an increase in cell viability.

mechanism of chronic GPER1 actions in H9c2 cells treated with the H_2O_2 agent involves deactivation of the MST1–JNK axis.

Chronic GPER1 Activation Rescues H9c2 Myoblasts From H_2O_2-Induced S phase Arrest

We further determined the impact of chronic GPER1 activation on cell cycle progression. We found that G1 treatment prevented H_2O_2-induced inhibition of cell proliferation. In fact, our results indicate an accumulation of cells in the S phase following H_2O_2 treatment in the control group (35 ± 3.45%) compared to H_2O_2-untreated cells (25 ± 0.35%). We observed a slight depletion of cells from the G2/M phase, suggesting that H_2O_2 treatment leads to S phase cell cycle arrest and a corresponding decreased cell entry into the G2-M stage (**Figure 6B**). However, this S phase cell cycle arrest was abrogated by G1 treatment. While control-treated cells had 35% of the cells trapped in the S phase, chronic G1 treatment reduced that level of S phase of cells to 14 ± 2.5% (**Figure 6B**). However, cotreatment of cells with G1 and G15 failed to rescue cells from the S phase cell cycle arrest (32.44 ± 6). These results suggest that chronic GPER1 actions protect against H_2O_2-induced S phase arrest of cell cycle.

Chronic GPER1 Protects Adult Cardiomyocytes Against Hypoxia Reoxygenation Injury

We, thereafter, determined whether our findings obtained using H9c2 cardiomyoblasts are transposable to other cardiac cell types. To this end, we isolated adult cardiomyocytes from 4- to 6-month-old male Sprague–Dawley rats and subjected them to 6-h hypoxia followed by 48-h reoxygenation. G1 (1 μm), G1 + G36 (100 μm), or vehicle (control) were supplemented in the culture medium at the onset of reoxygenation. The hypoxia/reoxygenation was substituted to H_2O_2 treatment for these experiments as this model mimics the *in vivo* ischemia/reperfusion-induced increase in reactive oxygen species (ROS) that is known to increase H_2O_2 production by the mitochondria. The cell viability was determined using Calcein assay. We found that G1-treated cardiomyocytes exhibited higher calcein fluorescence intensity compared to the control (vehicle). Here also, supplementation of G36 (another GPER1 antagonist) (73) prevented G1-induced increase in cardiomyocyte viability (**Figure 6C**). These results indicate that chronic GPER1 activation improves cardiomyocyte survival and viability against hypoxia/reoxygenation-induced cell death.

DISCUSSION

In this study, we report that chronic GPER1 activation induces cytoprotective effects against H9c2 rat cardiomyoblasts subjected to a cytotoxic H_2O_2 agent treatment by preventing the S phase cell cycle arrest, reducing mitochondrial dysfunction, delaying the mPTP opening, and deactivating the MST1/YAP and MST1/JNK pathways.

GPER1 activation is now well-known for inducing protective effects in several disease models, including I/R injury, hypertension (4–6), Parkinson's disease (23), retinal ganglion degeneration (74), and breast cancer (75). In fact, GPER1 activation has been reported to exert protective effects against harmful effects in several other organs, including the heart (4–6), brain (21, 76), muscle (77), testes (77), intestine (26), and kidney (78). Using isolated perfused heart model, others and we have reported that acute (∼1 h) pre-ischemic E2 treatment induces cardioprotective effects against I/R injury through GPER1 activation (4–8). Recently, using animals genetically modified subjected to I/R, we have revealed that pre-ischemic E2 treatment induces cardioprotective effects essentially through GPER1 activation and that ERα (Esr1) and ERβ (Esr2) are not needed for this effect (4). We further demonstrated in intact rats (*in vivo*), subjected to LAD artery occlusion followed by reperfusion, that the acute post-ischemic E2 administration induced reduction in myocardial infarct size compared to vehicle effects were abolished by the GPER1-selective antagonist, G15

(5). The acute effects of E2 have been extensively studied more so than the chronic effects. Indeed, chronic activation of E2 has also been shown to improve cell survival after injury (38, 39, 79), but the mechanism by which chronic E2 administration post-stress elicits protective effects remain elusive. In this study, using both H9c2 cardiomyoblasts and adult cardiomyocytes, we found that chronic treatment with the GPER1 agonists G1 or E2 reduces H_2O_2- and hypoxia/reoxygenation-induced cell death and reduction in viability compared to vehicle. We reveal that these G1/E2 effects were prevented by the GPER1 antagonists, G15 or G36, suggesting that chronic GPER1 activation induces cytoprotective effects against oxidative stress-induced cell death. This observation is in the line of many studies showing that *in vitro* activation of GPER1 induces cytoprotective effects in different models (80, 81). However, the mechanisms by which chronic GPER1 activation induces cytoprotection is being elucidated because of the assumption that GPER1 activation mediates mostly the rapid action of estrogen.

Lozano and Elledge have reported that cell cycle arrest in response to DNA damage from a variety of stimuli allows time for repair or direct cell apoptosis (82). After DNA damage, the cell cycle is arrested at the transition from G_1 to S phase or from G_2 to M phase of the cell cycle. We recently observed that H9c2 cells treated with Mitofilin siRNA have condensed and fragmented nuclei, and this effect was associated with a prolonged S phase of the cell cycle that promotes cell apoptosis (83). We, therefore, postulated that the cytoprotective effect of chronic GPER1 activation could result from its ability to prevent the H_2O_2-induced cell cycle arrest. In fact, we found that after H_2O_2 treatment, in control-treated cells, 35% of the cells were trapped in the S phase much more than untreated cells indicating that H_2O_2 treatment causes S phase cell cycle arrest that decreases cell entry into the G2-M stage. However, after chronic G1 treatment, the level at S phase was reduced to 14% only (**Figure 6A**). Since the cotreatment of cells with G1 supplemented with G15 failed to rescue cells from the S phase cell cycle arrest, it suggests a mechanism via GPER1 activation.

The role of mitochondria in the generation of cellular energy in normal physiological functions is well-known. Mitochondria are organelles that provide a lot of energy to support cardiac contraction in cardiomyocytes, whereas cardiomyocyte damage can arise as a result of mitochondrial dysfunction (84, 85). Hence, despite their crucial role in cellular function, the mitochondria have also been implicated in the process of cell death (86). We studied whether chronic GPER1 activation might induce cytoprotective effects against oxidative stress-induced cell death by preserving mitochondrial structural integrity and function. Our results indicate that after H_2O_2 treatment, chronic incubation of cells with G1/E2 preserves the mitochondrial structure compared to the control. As shown in **Figure 2B**, the mitochondria from cells treated with E2 display a normal morphology having regular cristae similar to H_2O_2-untreated compared to untreated mitochondria in which the cristae were mostly disrupted. We found that chronic E2 treatment preserves the mitochondrial membrane potential from dissipation (MMP) caused by H_2O_2 treatment compared to the control, suggesting that chronic G1/E2 treatment protects the mitochondria structure intensity, therefore improving their function (**Figure 2A**). Opening of the mitochondrial permeability transition pore (mPTP) in cardiac I/R injury because of Ca^{2+} overload and/or increased mitochondrial reactive oxygen species production causes cell death (4). The mPTP opening is currently considered as a crucial event in the mechanism of cell death after I/R (87). We, therefore, determined whether preservation of the mitochondrial structure by chronic G1/E2 treatment leads to the deactivation of the mPTP opening. Our result indicates that chronic E2 treatment delays the opening of the mPTP compared to the vehicle (**Figures 3A,B**). This result suggests that chronic G1/E2 actions on the mitochondria result in the delay of the mPTP opening, which is consistent with our previous report that acute E2 treatment exerts cardioprotective effects against I/R injury via inhibition of the mPTP opening (4). Because all these G1/E2 effects are prevented by cotreatment with the GPER1 antagonist, G15, suggesting a GPER1-dependent mechanism, our findings, therefore, indicate that chronic GPER1 actions protect H9c2 myoblasts against death by preserving mitochondrial structure and function as well as inhibiting the mPTP opening (**Figure 3C**).

Mitochondrial quality control depends upon a balance between biogenesis and autophagic destruction (88). Mitochondria are now well-recognized to be able to modulate their morphology by fission and fusion events (89) and that different morphological states are related to multiple physiological and pathophysiological conditions (7). Mitochondrial fragmentation is often linked to mitochondrial dysfunction as this morphological state predominates during elevated stress levels and cell death (8). Mitochondrial fission requires the cytosolic dynamin-related protein 1 (Drp1) (90), while the outer membrane mitofusin (Mfn) 1 and 2 mediate mitochondrial fusion (91). We determined whether the preservation of mitochondrial structure and function was linked to mitochondria dynamics. We found that the levels of both Drp1 and Mfn2 proteins were not changed in the E2-treated group compared to the control (vehicle) (**Figure 2C**), suggesting that chronic E2 rescue of mitochondrial integrity is not related to mitochondrial dynamics. Because GPER1 has not been found to localize in the mitochondria, its actions on the mitochondria can only be facilitated by subcellular signaling that we will define in future studies.

GPER1 actions have been shown to involve multiple signaling related to cell survival and proliferation such as MEK/ERK, PI3K/Akt, mTOR, and Hippo/YAP pathways (4, 6, 40, 47, 92). In breast cancer cells, the Hippo/YAP/TAZ pathway has been reported as a key downstream signaling branch of GPER1 actions and plays a critical role in breast tumorigenesis (40). The Hippo/YAP pathway has been previously described to play a key role in cardiac development and regeneration (46, 47). YAP is a key effector of the Hippo pathway. Inhibition of YAP phosphorylation is believed to promote YAP nuclear accumulation, where it upregulates its downstream genes. In an *in vitro* model, treatment with bisphenol, which can promote the migration, but not the proliferation of triple-negative breast cancer cells, has been found to activate YAP, and the inhibition of GPER1 attenuated the effects of BPS-induced YAP

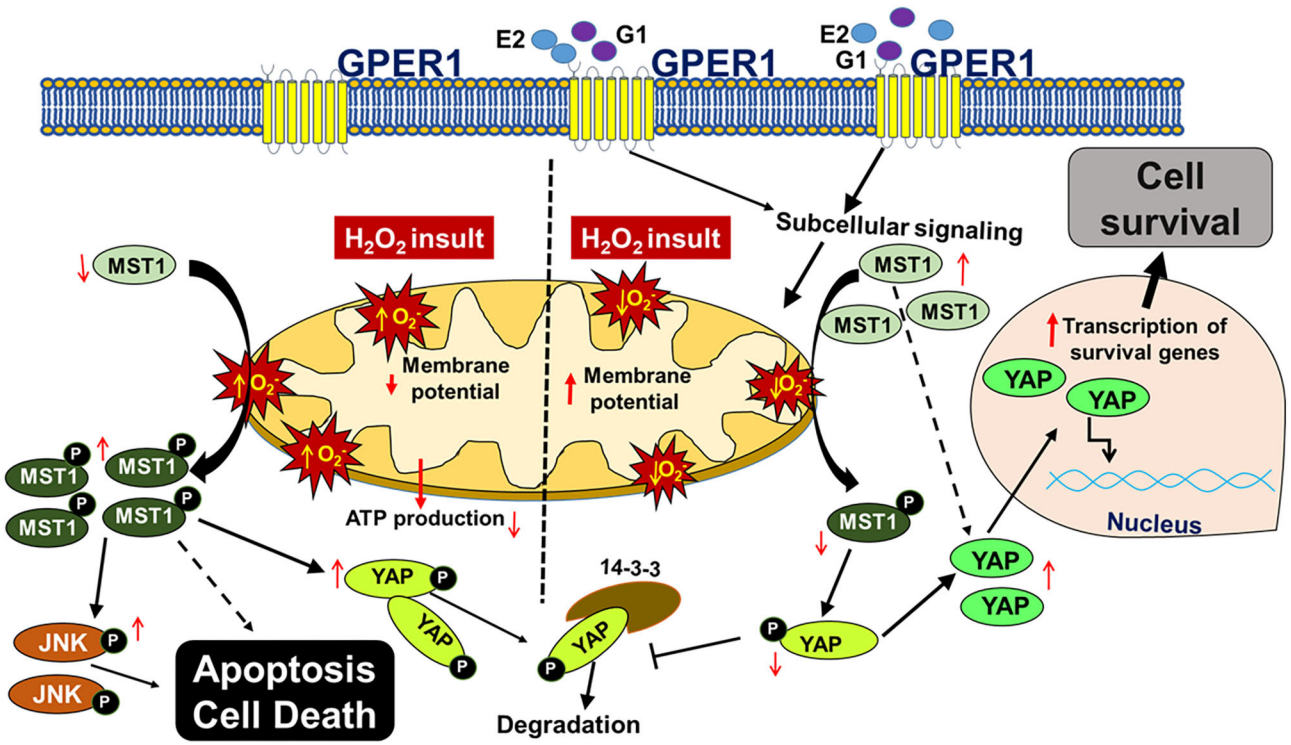

FIGURE 7 | Scheme depicting the mechanism underlying chronic G1-induced cytoprotective effects against H_2O_2-induced cell death. In cultured H9c2 myoblasts, treatment with the cytotoxic agent, H_2O_2 increases the reactive oxygen species (ROS, O_2) production, which promotes the phosphorylation of MST1 (p-MST1). p-MST1 initiates activation of cascades that lead to phosphorylation of YAP (p-YAP). p-YAP is sequestered in the cytosol and subsequently degraded. In addition, increase in p-MST1 promotes the phosphorylation of JNK that upregulates proapoptotic genes. On the other hand, chronic GPER1 activation with its agonists E2 or G1 stimulates subcellular signaling that preserves mitochondrial structure and polarization leading to reduced ROS production and delayed mPTP opening. Reduction in ROS production is postulated to mitigate the phosphorylation of MST1, which increases the non-phosphorylated YAP. Thereafter, YAP translocates into the nucleus to upregulate prosurvival genes that, therefore, promote cell survival.

dephosphorylation (93). Also, the mammalian Ste20-like kinase-1 (MST1), which has been shown to mediate H_2O_2-induced cell death (94), is a central player in the Hippo/YAP pathway. Phosphorylation of MST1 in response to harmful stimuli promotes phosphorylation YAP, and its subsequent degradation by 14-3-3, therefore, favoring upregulation of proapoptotic genes. On the contrary, an increase in the cytosolic MST1 levels due to the decrease in its phosphorylation promotes dephosphorylation of YAP, and increases the levels of YAP, facilitating its translocation in the nucleus where its activation production of genes takes place. We, thus, investigated whether chronic GPER1 actions involve regulation of the MST1/YAP axis. We found that G1 treatment reduces the phosphorylation of both MST1 and YAP, resulting in accumulation and translocation of YAP in the nucleus (**Figures 4C,D**). The involvement of YAP in the mechanism of G1 is confirmed by the significant increase in cell death in the G1 group when YAP was pre-deleted by shYAP treatment (**Figure 5C**). We also found a G1-induced decrease in YAP phosphorylation that increases the levels of non-phosphorylated YAP, which is associated with the upregulation of prosurvival genes, including CTGF, CYR61, and ANKRD1 as well as the downregulation of genes involved in apoptosis such as PUMA and Bim. We postulate that the deactivation of the MST1/YAP pathway by the reduction of mitochondria dysfunction that might result in less production of free radicals plays a critical role in the mechanism of chronic GPER1 activation-induced cytoprotective effects. However, further investigations are needed to clearly determine the mitochondria-dependent signaling that inhibits the Hippo/YAP pathway. Nevertheless, we favor the opinion that the reduction in the production of mitochondrial ROS is one of the key reasons for MST1 dephosphorylation.

In this paper, we abundantly refer to GPER1 activation effects as "cytoprotective," which should logically be used if GPER1 activation was performed before the oxidative stress. In our protocol, GPER1 was activated after the oxidative stress insult; therefore, these GPER1 effects would be better qualified as rescue or restorative effects.

CONCLUSION

In this study, we report that chronic activation of GPER1 in H9c2 cardiomyoblasts pretreated with the cytotoxic agent, H_2O_2,

reduces cell death and enhances cell viability by preserving mitochondrial structural integrity and function that result in the high MMP, the delay of the mPTP opening. Together, these chronic GPER1 activation effects on the mitochondria promote the deactivation of the Hippo/YAP pathway, resulting in the translocation of YAP in the nucleus, where it promotes the upregulation of prosurvival genes and downregulation of proapoptotic genes (**Figure 7**).

DATA AVAILABILITY STATEMENT

All datasets generated for this study and included in the article are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by UT Health Science Center at San Antonio Institutional Animal Care and Use Committee (IACUC) institutional.

AUTHOR CONTRIBUTIONS

NM, AI, and JB conceived and designed the research. NM, AI, and NT performed the experiments. AI, YF, and JB analyzed the data. AI, NT, YF, and JB interpreted the results of the experiments and edited and revised the manuscript. AI and JB drafted the manuscript. AI, NT, and JB prepared the figures. JB approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hall ED, Pazara KE, Linseman KL. Sex differences in postischemic neuronal necrosis in gerbils. *J Cereb Blood Flow Metab*. (1991) 11:292–8. doi: 10.1038/jcbfm.1991.61
2. Park EM, Cho S, Frys KA, Glickstein SB, Zhou P, Anrather J, Ross ME, Iadecola C. Inducible nitric oxide synthase contributes to gender differences in ischemic brain injury. *J Cereb Blood Flow Metab*. (2006) 26:392–401. doi: 10.1038/sj.jcbfm.9600194
3. Alkayed NJ, Harukuni I, Kimes AS, London ED, Traystman RJ, Hurn PD. Gender-linked brain injury in experimental stroke. *Stroke*. (1998) 29:159–65; discussion 166. doi: 10.1161/01.STR.29.1.159
4. Kabir ME, Singh H, Lu R, Olde B, Leeb-Lundberg LM, Bopassa JC. G protein-coupled estrogen receptor 1 mediates acute estrogen-induced cardioprotection via MEK/ERK/GSK-3beta pathway after Ischemia/reperfusion. *PLoS ONE*. (2015) 10:e0135988. doi: 10.1371/journal.pone.0135988
5. Feng Y, Madungwe NB, da Cruz Junho CV, Bopassa JC. Activation of G protein-coupled oestrogen receptor 1 at the onset of reperfusion protects the myocardium against ischemia/reperfusion injury by reducing mitochondrial dysfunction and mitophagy. *Br J Pharmacol*. (2017) 174:4329–44. doi: 10.1111/bph.14033
6. Pei H, Wang W, Zhao D, Su H, Su G, Zhao Z. G protein-coupled estrogen receptor 1 inhibits angiotensin II-induced cardiomyocyte hypertrophy via the regulation of PI3K-Akt-mTOR signalling and autophagy. *Int J Biol Sci*. (2019) 15:81–92. doi: 10.7150/ijbs.28304
7. Menazza S, Sun J, Appachi S, Chambliss KL, Kim SH, Aponte A, et al. Non-nuclear estrogen receptor alpha activation in endothelium reduces cardiac ischemia-reperfusion injury in mice. *J Mol Cell Cardiol*. (2017) 107:41–51. doi: 10.1016/j.yjmcc.2017.04.004
8. Luo T, Liu H, Kim JK. Estrogen protects the female heart from ischemia/reperfusion injury through manganese superoxide dismutase phosphorylation by mitochondrial p38beta at threonine 79 and serine 106. *PLoS ONE*. (2016) 11:e0167761. doi: 10.1371/journal.pone.0167761
9. Hutchens MP, Kosaka Y, Zhang W, Fujiyoshi T, Murphy S, Alkayed N, et al. Estrogen-mediated renoprotection following cardiac arrest and cardiopulmonary resuscitation is robust to GPR30 gene deletion. *PLoS ONE*. (2014) 9:e99910. doi: 10.1371/journal.pone.0099910
10. Ikeda M, Swide T, Vayl A, Lahm T, Anderson S, Hutchens MP. Estrogen administered after cardiac arrest and cardiopulmonary resuscitation ameliorates acute kidney injury in a sex- and age-specific manner. *Crit Care*. (2015) 19:332. doi: 10.1186/s13054-015-1049-8
11. Klinge CM. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Res*. (2001) 29:2905–19. doi: 10.1093/nar/29.14.2905
12. Subbiah MT. Mechanisms of cardioprotection by estrogens. *Proc Soc Exp Biol Med*. (1998) 217:23–9. doi: 10.3181/00379727-217-44201
13. Windahl SH, Andersson N, Chagin AS, Martensson UE, Carlsten H, Olde B, et al. The role of the G protein-coupled receptor GPR 30 in the effects of estrogen in ovariectomized mice. *Am J Physiol Endocrinol Metab*. (2008) 296:E490-6. doi: 10.1152/ajpendo.90691.2008
14. Walker P, Germond JE, Brown-Luedi M, Givel F, Wahli W. Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDLII genes. *Nucleic Acids Res*. (1984) 12:8611–26. doi: 10.1093/nar/12.22.8611
15. Hewitt SC, Grimm SA, Wu SP, DeMayo FJ, Korach KS. Estrogen receptor alpha (ERalpha)-binding super-enhancers drive key mediators that control uterine estrogen responses in mice. *J Biol Chem*. (2020) 295:8387–400. doi: 10.1074/jbc.RA120.013666
16. Yasar P, Ayaz G, User SD, Gupur G, Muyan M. Molecular mechanism of estrogen–estrogen receptor signaling. *Reprod Med Biol*. (2017) 16:4–20. doi: 10.1002/rmb2.12006
17. Puglisi R, Mattia G, Care A, Marano G, Malorni W, Matarrese P. Non-genomic effects of estrogen on cell homeostasis and remodeling with special focus on cardiac ischemia/reperfusion injury. *Front Endocrinol*. (2019) 10:733. doi: 10.3389/fendo.2019.00733
18. Acconcia F, Kumar R. Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Lett*. (2006) 238:1–14. doi: 10.1016/j.canlet.2005.06.018
19. Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, Iann MC, et al. Effect of estradiol on estrogen receptor-alpha gene expression and activity can be modulated by the ErbB2/PI 3-K/Akt pathway. *Oncogene*. (2003) 22:7998–8011. doi: 10.1038/sj.onc.1206769

20. Bopassa JC, Eghbali M, Toro L, Stefani E. A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. (2010) 298:H16–23. doi: 10.1152/ajpheart.00588.2009
21. Vajaria R, Vasudevan N. Is the membrane estrogen receptor, GPER1, a promiscuous receptor that modulates nuclear estrogen receptor-mediated functions in the brain? *Horm Behav*. (2018) 104:165–72. doi: 10.1016/j.yhbeh.2018.06.012
22. Cheng SB, Dong J, Pang Y, LaRocca J, Hixon M, Thomas P, et al. Anatomical location and redistribution of G protein-coupled estrogen receptor-1 during the estrus cycle in mouse kidney and specific binding to estrogens but not aldosterone. *Mol Cell Endocrinol*. (2014) 382:950–9. doi: 10.1016/j.mce.2013.11.005
23. Li JJ, Duan H, Wang S, Sun FQ, Gan L, Tang YQ, et al. Expression pattern of G-protein-coupled estrogen receptor in myometrium of uteri with and without adenomyosis. *Biomed Res Int*. (2017) 2017:5974693. doi: 10.1155/2017/5974693
24. Deschamps AM, Murphy E. Activation of a novel estrogen receptor, GPER, is cardioprotective in male and female rats. *Am J Physiol Heart Circ Physiol*. (2009) doi: 10.1152/ajpheart.00283.2009
25. De Francesco EM, Angelone T, Pasqua T, Pupo M, Cerra MC, Maggiolini M. GPER mediates cardiotropic effects in spontaneously hypertensive rat hearts. *PLoS ONE*. (2013) 8:e69322. doi: 10.1371/journal.pone.0069322
26. Chai S, Liu K, Feng W, Liu T, Wang Q, Zhou R, et al. Activation of G protein-coupled estrogen receptor protects intestine from ischemia/reperfusion injury in mice by protecting the crypt cell proliferation. *Clin Sci (Lond)*. (2019) 133:449–64. doi: 10.1042/CS20180919
27. Leung TM, Nieto N. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *J Hepatol*. (2013) 58:395–8. doi: 10.1016/j.jhep.2012.08.018
28. Jiang J, Yu S, Jiang Z, Liang C, Yu W, Li J, et al. N-acetyl-serotonin protects HepG2 cells from oxidative stress injury induced by hydrogen peroxide. *Oxid Med Cell Longev*. (2014) 2014:310504. doi: 10.1155/2014/310504
29. Li RG, Li TT, Hao L, Xu X, Na J. Hydrogen peroxide reduces lead-induced oxidative stress to mouse brain and liver. *Bull Environ Contam Toxicol*. (2009) 82:419–22. doi: 10.1007/s00128-008-9599-y
30. La Colla A, Vasconsuelo A, Milanesi L, Pronsato L. 17beta-estradiol protects skeletal myoblasts from apoptosis through p53, Bcl-2, and FoxO families. *J Cell Biochem*. (2017) 118:104–15. doi: 10.1002/jcb.25616
31. La Colla A, Boland R, Vasconsuelo A. 17beta-estradiol abrogates apoptosis inhibiting PKCdelta, JNK, and p66Shc activation in C2C12 Cells. *J Cell Biochem*. (2015) 116:1454–65. doi: 10.1002/jcb.25107
32. Vasconsuelo A, Milanesi L, Boland R. 17Beta-estradiol abrogates apoptosis in murine skeletal muscle cells through estrogen receptors: role of the phosphatidylinositol 3-kinase/Akt pathway. *J Endocrinol*. (2008) 196:385–97. doi: 10.1677/JOE-07-0250
33. Liu S, Mauvais-Jarvis F. Rapid, nongenomic estrogen actions protect pancreatic islet survival. *Islets*. (2009) 1:273–5. doi: 10.4161/isl.1.3.9781
34. Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM Jr, Valencia T, et al. Mitochondrial localization of estrogen receptor beta. *Proc Natl Acad Sci USA*. (2004) 101:4130–5. doi: 10.1073/pnas.0306948101
35. Jazbutyte V, Kehl F, Neyses L, Pelzer T. Estrogen receptor alpha interacts with 17beta-hydroxysteroid dehydrogenase type 10 in mitochondria. *Biochem Biophys Res Commun*. (2009) 384:450–4. doi: 10.1016/j.bbrc.2009.04.139
36. Mahmoodzadeh S, Dworatzek E. The role of 17beta-estradiol and estrogen receptors in regulation of Ca(2+) channels and mitochondrial function in cardiomyocytes. *Front Endocrinol*. (2019) 10:310. doi: 10.3389/fendo.2019.00310
37. Wang H, Sun X, Chou J, Lin M, Ferrario CM, Zapata-Sudo G, et al. Cardiomyocyte-specific deletion of the G protein-coupled estrogen receptor (GPER) leads to left ventricular dysfunction and adverse remodeling: a sex-specific gene profiling analysis. *Biochim Biophys Acta Mol Basis Dis*. (2017) 1863:1870–82. doi: 10.1016/j.bbadis.2016.10.003
38. Kosaka Y, Quillinan N, Bond C, Traystman R, Hurn P, Herson P. GPER1/GPR30 activation improves neuronal survival following global cerebral ischemia induced by cardiac arrest in mice. *Transl Stroke Res*. (2012) 3:500–7. doi: 10.1007/s12975-012-0211-8
39. Tang H, Zhang Q, Yang L, Dong Y, Khan M, Yang F, Brann DW, Wang R. GPR30 mediates estrogen rapid signaling and neuroprotection. *Mol Cell Endocrinol*. (2014) 387:52–8. doi: 10.1016/j.mce.2014.01.024
40. Zhou X, Wang S, Wang Z, Feng X, Liu P, Lv XB, et al. Estrogen regulates Hippo signaling via GPER in breast cancer. *J Clin Invest*. (2015) 125:2123–35. doi: 10.1172/JCI79573
41. Meyer MR, Haas E, Prossnitz ER, Barton M. Non-genomic regulation of vascular cell function and growth by estrogen. *Mol Cell Endocrinol*. (2009) 308:9–16. doi: 10.1016/j.mce.2009.03.009
42. Zhao B, Li L, Lei Q, Guan KL. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. *Genes Dev*. (2010) 24:862–74. doi: 10.1101/gad.1909210
43. Shimomura T, Miyamura N, Hata S, Miura R, Hirayama J, Nishina H. The PDZ-binding motif of Yes-associated protein is required for its co-activation of TEAD-mediated CTGF transcription and oncogenic cell transforming activity. *Biochem Biophys Res Commun*. (2014) 443:917–23. doi: 10.1016/j.bbrc.2013.12.100
44. Zhao B, Ye X, Yu J, Li L, Li W, Li S, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev*. (2008) 22:1962–71. doi: 10.1101/gad.1664408
45. Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, Picard D. Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J*. (2009) 28:523–32. doi: 10.1038/emboj.2008.304
46. Mia MM, Singh MK. The hippo signaling pathway in cardiac development and diseases. *Front Cell Dev Biol*. (2019) 7:211. doi: 10.3389/fcell.2019.00211
47. Zhou Q, Li L, Zhao B, Guan KL. The hippo pathway in heart development, regeneration, and diseases. *Circ Res*. (2015) 116:1431–47. doi: 10.1161/CIRCRESAHA.116.303311
48. Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, et al. Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci Signal*. (2011) 4:ra70. doi: 10.1126/scisignal.2002278
49. von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, et al. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc Natl Acad Sci USA*. (2012) 109:2394–9. doi: 10.1073/pnas.1116136109
50. Heallen T, Zhang M, Wang J, Bonilla-Claudio M, Klysik E, Johnson RL, et al. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science*. (2011) 332:458–61. doi: 10.1126/science.1199010
51. Monroe TO, Hill MC, Morikawa Y, Leach JP, Heallen T, Cao S, et al. YAP partially reprograms chromatin accessibility to directly induce adult cardiogenesis *in vivo*. *Dev Cell*. (2019) 48:765–79.e767. doi: 10.1016/j.devcel.2019.01.017
52. Lin Z, von Gise A, Zhou P, Gu F, Ma Q, Jiang J, et al. Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ Res*. (2014) 115:354–63. doi: 10.1161/CIRCRESAHA.115.303632
53. Leach JP, Heallen T, Zhang M, Rahmani M, Morikawa Y, Hill MC, et al. Hippo pathway deficiency reverses systolic heart failure after infarction. *Nature*. (2017) 550:260–4. doi: 10.1038/nature24045
54. Madungwe NB, Feng Y, Imam Aliagan A, Tombo N, Kaya F, Bopassa JC. Inner mitochondrial membrane protein MPV17 mutant mice display increased myocardial injury after ischemia/reperfusion. *Am J Transl Res*. (2020) 12:3412–28. Available online at: www.ajtr.org/ISSN:1943-8141/AJTR0109
55. Feng Y, Madungwe NB, Imam Aliagan AD, Tombo N, Bopassa JC. Liproxstatin-1 protects the mouse myocardium against ischemia/reperfusion injury by decreasing VDAC1 levels and restoring GPX4 levels. *Biochem Biophys Res Commun*. (2019) 520:606–11. doi: 10.1016/j.bbrc.2019.10.006
56. Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, et al. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev*. (2007) 21:2747–61. doi: 10.1101/gad.1602907

57. Levy D, Adamovich Y, Reuven N, Shaul Y. Yap1 phosphorylation by c-Abl is a critical step in selective activation of pro-apoptotic genes in response to DNA damage. *Mol Cell*. (2008) 29:350–61. doi: 10.1016/j.molcel.2007.12.022
58. Tombo N, Imam Aliagan AD, Feng Y, Singh H, Bopassa JC. Cardiac ischemia/reperfusion stress reduces inner mitochondrial membrane protein (mitofilin) levels during early reperfusion. *Free Radic Biol Med*. (2020) 158:181–94. doi: 10.1016/j.freeradbiomed.2020.06.039
59. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262
60. Gonzalez A, Granados MP, Salido GM, Pariente JA. H_2O_2-induced changes in mitochondrial activity in isolated mouse pancreatic acinar cells. *Mol Cell Biochem*. (2005) 269:165–73. doi: 10.1007/s11010-005-3457-6
61. Tatsumi T, Kako KJ. Effects of hydrogen peroxide on mitochondrial enzyme function studied *in situ* in rat heart myocytes. *Basic Res Cardiol*. (1993) 88:199–211.
62. Bopassa JC, Ferrera R, Gateau-Roesch O, Couture-Lepetit E, Ovize M. PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. *Cardiovasc Res*. (2006) 69:178–85. doi: 10.1016/j.cardiores.2005.07.014
63. Bopassa JC, Michel P, Gateau-Roesch O, Ovize M, Ferrera R. Low-pressure reperfusion alters mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol*. (2005) 288:H2750–5. doi: 10.1152/ajpheart.01081.2004
64. Osorio H, Carvalho E, del Valle M, Gunther Sillero MA, Moradas-Ferreira P, Sillero A. H_2O_2, but not menadione, provokes a decrease in the ATP and an increase in the inosine levels in *Saccharomyces cerevisiae*. An experimental and theoretical approach. *Eur J Biochem*. (2003) 270:1578–89. doi: 10.1046/j.1432-1033.2003.03529.x
65. Guazzoni G, Montorsi F, Colombo R, Di Girolamo V, Da Pozzo L, Rigatti P. Long term experience with the prostatic spiral for urinary retention due to benign prostatic hyperplasia. *Scand J Urol Nephrol*. (1991) 25:21–4. doi: 10.3109/00365599109024523
66. Choi HI, Park JS, Kim DH, Kim CS, Bae EH, Ma SK, Kim SW. PGC-1alpha suppresses the activation of TGF-beta/Smad signaling via targeting TGFbetaRI downregulation by let-7b/c upregulation. *Int J Mol Sci*. (2019) 20:5084. doi: 10.3390/ijms20205084
67. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. *Essays Biochem*. (2010) 47:69–84. doi: 10.1042/bse0470069
68. Meng Z, Moroishi T, Guan KL. Mechanisms of Hippo pathway regulation. *Genes Dev*. (2016) 30:1–17. doi: 10.1101/gad.274027.115
69. Zhao B, Li L, Tumaneng K, Wang CY, Guan KL. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev*. (2010) 24:72–85. doi: 10.1101/gad.1843810
70. Jang SW, Yang SJ, Srinivasan S, Ye K. Akt phosphorylates MstI and prevents its proteolytic activation, blocking FOXO3 phosphorylation and nuclear translocation. *J Biol Chem*. (2007) 282:30836–44. doi: 10.1074/jbc.M704542200
71. Tsujiura M, Mazack V, Sudol M, Kaspar HG, Nash J, Carey DJ, et al. Yes-associated protein (YAP) modulates oncogenic features and radiation sensitivity in endometrial cancer. *PLoS ONE*. (2014) 9:e100974. doi: 10.1371/journal.pone.0100974
72. Khan M, Rutten BPF, Kim MO. MST1 regulates neuronal cell death via JNK/Casp3 signaling pathway in HFD mouse brain and HT22 cells. *Int J Mol Sci*. (2019) 20:2504. doi: 10.3390/ijms20102504
73. Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologa CG, et al. Identification of a GPER/GPR30 antagonist with improved estrogen receptor counterselectivity. *J Steroid Biochem Mol Biol*. (2011) 127:358–66. doi: 10.1016/j.jsbmb.2011.07.002
74. Jiang M, Ma X, Zhao Q, Li Y, Xing Y, Deng Q, et al. The neuroprotective effects of novel estrogen receptor GPER1 in mouse retinal ganglion cell degeneration. *Exp Eye Res*. (2019) 189:107826. doi: 10.1016/j.exer.2019.107826
75. Martinez-Munoz A, Prestegui-Martel B, Mendez-Luna D, Fragoso-Vazquez MJ, Garcia-Sanchez JR, Bello M, et al. Selection of a GPER1 ligand via ligand-based virtual screening coupled to molecular dynamics simulations and its anti-proliferative effects on breast cancer cells. *Anticancer Agents Med Chem*. (2018) 18:1629–38. doi: 10.2174/1871520618666180510121431
76. Bourque M, Morissette M, Di Paolo T. Neuroprotection in Parkinsonian-treated mice via estrogen receptor alpha activation requires G protein-coupled estrogen receptor 1. *Neuropharmacology*. (2015) 95:343–52. doi: 10.1016/j.neuropharm.2015.04.006
77. Nishie T, Kobayashi Y, Kimura K, Okuda K. Acute stimulation of a smooth muscle constrictor by oestradiol-17beta via GPER1 in bovine oviducts. *Reprod Domest Anim*. (2018) 53:326–32. doi: 10.1111/rda.13108
78. Hutchens MP, Nakano T, Kosaka Y, Dunlap J, Zhang W, Herson PS, et al. Estrogen is renoprotective via a nonreceptor-dependent mechanism after cardiac arrest *in vivo*. *Anesthesiology*. (2010) 112:395–405. doi: 10.1097/ALN.0b013e3181c98da9
79. De Butte-Smith M, Gulinello M, Zukin RS, Etgen AM. Chronic estradiol treatment increases CA1 cell survival but does not improve visual or spatial recognition memory after global ischemia in middle-aged female rats. *Horm Behav*. (2009) 55:442–53. doi: 10.1016/j.yhbeh.2008.11.011
80. Nilsson BO, Olde B, Leeb-Lundberg LM. G protein-coupled oestrogen receptor 1 (GPER1)/GPR30: a new player in cardiovascular and metabolic oestrogenic signalling. *Br J Pharmacol*. (2011) 163:1131–9. doi: 10.1111/j.1476-5381.2011.01235.x
81. Kong BS, Cho YH, Lee EJ. G protein-coupled estrogen receptor-1 is involved in the protective effect of protocatechuic aldehyde against endothelial dysfunction. *PLoS ONE*. (2014) 9:e113242. doi: 10.1371/journal.pone.0113242
82. Lozano G, Elledge SJ. p53 sends nucleotides to repair DNA. *Nature*. (2000) 404:24–5. doi: 10.1038/35003670
83. Madungwe NB, Feng Y, Lie M, Tombo N, Liu L, Kaya F, et al. mitochondrial inner membrane protein (Mitofilin) knockdown induces cell death by apoptosis via an AIF-PARP-dependent mechanism and cell cycle arrest. *Am J Physiol Cell Physiol*. (2018) 315:C28–43. doi: 10.1152/ajpcell.00230.2017
84. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia—reperfusion, aging, and heart failure. *J Mol Cell Cardiol*. (2001) 33:1065–89. doi: 10.1006/jmcc.2001.1378
85. Gustafsson AB, Gottlieb RA. Heart mitochondria: gates of life and death. *Cardiovasc Res*. (2008) 77:334–43. doi: 10.1093/cvr/cvm005
86. Hockenbery DM, Giedt CD, O'Neill JW, Manion MK, Banker DE. Mitochondria and apoptosis: new therapeutic targets. *Adv Cancer Res*. (2002) 85:203–42. doi: 10.1016/S0065-230X(02)85007-2
87. Gateau-Roesch O, Argaud L, Ovize M. Mitochondrial permeability transition pore and postconditioning. *Cardiovasc Res*. (2006) 70:264–73. doi: 10.1016/j.cardiores.2006.02.024
88. Gottlieb RA, Gustafsson AB. Mitochondrial turnover in the heart. *Biochim Biophys Acta*. (2011) 1813:1295–301. doi: 10.1016/j.bbamcr.2010.11.017
89. Tilokani L, Nagashima S, Paupe V, Prudent J. Mitochondrial dynamics: overview of molecular mechanisms. *Essays Biochem*. (2018) 62:341–60. doi: 10.1042/EBC20170104
90. Ferreira-da-Silva A, Valacca C, Rios E, Populo H, Soares P, Sobrinho-Simoes M, et al. Mitochondrial dynamics protein Drp1 is overexpressed in oncocytic thyroid tumors and regulates cancer cell migration. *PLoS ONE*. (2015) 10:e0122308. doi: 10.1371/journal.pone.0122308
91. Westermann B. Mitochondrial fusion and fission in cell life and death. *Nat Rev Mol Cell Biol*. (2010) 11:872–84. doi: 10.1038/nrm3013
92. Chaturantabut S, Shwartz A, Evason KJ, Cox AG, Labella K, Schepers AG, et al. Estrogen activation of G-protein-coupled estrogen receptor 1 regulates phosphoinositide 3-kinase and mTOR signaling to promote

liver growth in zebrafish and proliferation of human hepatocytes. *Gastroenterology*. (2019) 156:1788–804.e1713. doi: 10.1053/j.gastro.2019.01.010

93. Deng Q, Jiang G, Wu Y, Li J, Liang W, Chen L, et al. GPER/Hippo-YAP signal is involved in Bisphenol S induced migration of triple negative breast cancer (TNBC) cells. *J Hazard Mater*. (2018) 355:1–9. doi: 10.1016/j.jhazmat.2018.05.013
94. Morinaka A, Funato Y, Uesugi K, Miki H. Oligomeric peroxiredoxin-I is an essential intermediate for p53 to activate MST1 kinase and apoptosis. *Oncogene*. (2011) 30:4208–18. doi: 10.1038/onc.2011.139

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G Protein-Coupled Estrogen Receptor, GPER1, Offers a Novel Target for the Treatment of Digestive Diseases

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There are gender differences between men and women in many physiological functions and diseases, which indicates that female sex hormones may be important. Traditionally, estrogen exerts its biological activities by activating two classical nuclear estrogen receptors, ESR1 and ESR2. However, the roles of estrogen in the regulation of physiological functions and the pathogenesis of diseases become more complicated with the identification of the G protein-coupled estrogen receptor (GPER1). Although many GPER1-specific ligands have been developed, the therapeutic mechanisms of exclusively targeting GPER1 are not yet well understood. Translational applications and clinical trial efforts for the identified GPER1 ligands have been focused primarily on the reproductive, cardiovascular, nervous, endocrine, and immune systems. More recently, research found that GPER1 may play an important role in regulating the digestive system. Cholesterol gallstone disease, a major biliary disease, has a higher prevalence in women than in men worldwide. Emerging evidence implies that GPER1 could play an important role, independent of the classical ESR1, in the pathophysiology of cholesterol gallstones in women. This review discusses the complex signaling pathways of three estrogen receptors, highlights the development of GPER1-specific ligands, and summarizes the latest advances in the role of GPER1 in the pathogenesis of gallstone formation.

Keywords: biliary sludge, bile salts, cholesterol gallstone disease, estrogen, estrogen receptors, gallbladder hypomotility, GPER1, GPER1 antagonists

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INTRODUCTION

The prevalence of digestive disease ranges from 10 to 27.8% in the United States (1, 2). Some common chronic digestive diseases include gallstone disease, nonalcoholic fatty liver disease, alcoholic liver disease, gastroesophageal reflux disease, irritable bowel syndrome, inflammatory bowel disease, gastric cancer, pancreatic cancer, and colon cancer. Many digestive disorders exhibit

Abbreviations: E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ESR1, estrogen receptor α; ESR2, estrogen receptor β; 2-ME, 2-methoxy-estradiol; GPER1, G protein-coupled estrogen receptor; QTL, quantitative trait locus.

a distinct gender difference in prevalence between women and men (3–5), suggesting that sex hormones are important. Over the past decades, many basic research and clinical investigations have been focused largely on the roles of estrogen, through two classical nuclear estrogen receptors, ESR1 and ESR2 (also called ERα and ERβ), in the regulation of physiological functions and the pathophysiology of diseases such as cardiovascular, kidney, nervous, reproductive, endocrine, and gastrointestinal disorders. However, the discovery of a new estrogen receptor called the G protein-coupled estrogen receptor (GPER1) has made it more complicated to investigate the roles of estrogen in the pathogenesis of numerous diseases because estrogen can produce its biological activities through one of the three nuclear receptor signaling pathways, or a combination of two, or all three. This review discusses the latest advances in the signaling pathways of three estrogen receptors, the development of GPER1-specific ligands, and the roles of GPER1 and its ligands in the pathogenesis of cholesterol gallstone disease.

COMPLEX SIGNALING PATHWAYS OF THREE ESTROGEN RECEPTORS

The identification of three estrogen receptors has implied that estrogen-stimulated receptor signaling is more complex than initially realized (**Figure 1**). The naturally occurring estrogens are 17β-estradiol (E2), estrone, and estriol, and all of them are C_{18} steroids. Cellular response to E2 can occur through the activation of the nuclear estrogen receptors, ESR1, and ESR2. The classical ER signaling through the ERs involves the binding of estrogen, receptor dimerization, and subsequent association of coactivator proteins that guide the dimerized ER subunit to

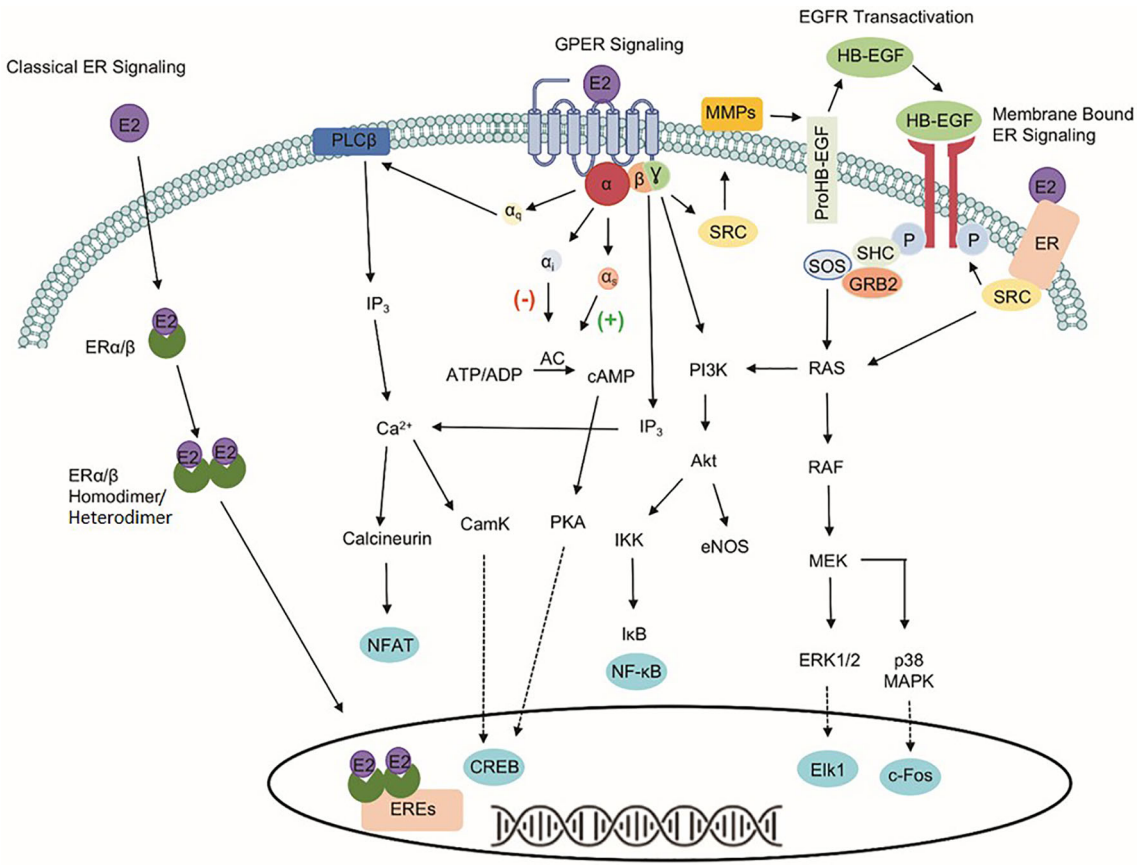

FIGURE 1 | Signaling pathways of three estrogen receptors. The classical estrogen receptors, ESR1 and ESR2, primarily exist within the cytoplasm and nucleus, as well as interact with estrogen response elements (EREs) after dimerization to drive genomic signaling. Unlike the nuclear estrogen receptors, GPER1 signaling pathway occurs through various second messengers. Phospholipase C Beta (PLCβ), inositol triphosphate (IP_3), nuclear factor of activated T-cells (NFAT), calcium/calmodulin-dependent protein kinase (CamK), cAMP response element-binding protein (CREB), adenylate cyclase (AC), protein kinase A (PKA), phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), IκB kinase (IKK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), endothelial nitric oxide synthase (eNOS), non-receptor tyrosine kinase (SRC), matrix metallopeptidases (MMPs), heparin-binding EGF-like growth factor (HB-EGF), son of sevenless (SOS), Src homology 2 domain-containing transforming protein (SHC), growth factor receptor-bound protein 2 (GRB2), RAS protein (RAS), RAF kinase (RAF), mitogen-activated protein kinase kinase (MEK), extracellular signal-regulated kinases 1/2 (ERK 1/2), Elk-1 transcription factor (Elk1), p38 mitogen-activated protein kinase (p38 MAPK), and c-Fos transcription factor (c-Fos).

estrogen response elements (EREs) that drive transcriptional activity (6, 7). In addition, variants or single nucleotide polymorphisms (SNPs) in the *ESR1* and the *ESR2* genes increase the complexity and diversity of E2-mediated signaling transduction.

The identification of GPER1, a 375-amino acid protein known previously as GPR30, makes the well-known ER signaling pathways more complicated. Unlike the classical nuclear estrogen receptors, GPER1 signaling occurs through various second messengers (8–12). Specifically, GPER1 has been shown to activate ERK1/2 phosphorylation through $G_{\beta,\gamma}$-dependent transactivation of epidermal growth factor receptor (EGFR), cAMP, calcium mobilization, and protein/lipid kinases (i.e., PKC and PKA) (8, 13–17). Interaction with these signaling pathways influences protein expression, apoptosis, cell proliferation, cell migration, and growth. Despite differences in signaling capabilities, ESR1, ESR2, and GPER1 are expressed ubiquitously throughout the human body and the variability of response in different tissues highlights the importance of understanding the druggability of each target separately due to the downstream signaling events that differ between the proteins (18).

CURRENT LIGANDS FOR THE MODULATION OF GPER1 SIGNALING

The effects of preferentially targeting GPER1 are not fully understood; therefore, there has been an increased research effort into the development of novel ligands to modulate GPER1 activity. Before the identification of GPER1-specific ligands, the antiestrogens, tamoxifen and fulvestrant, were shown to interact with GPER1 (19). While tamoxifen and fulvestrant block the ability of E2 to signal through ESR1 or ESR2, they also possess the ability to activate the GPER1 signaling pathway similarly to E2. The activity of antiestrogens at GPER1 highlights the cross-reactivity of estrogenic ligands and the difficulty in developing GPER1-specific ligands. In addition to antiestrogens, various non-selective GPER1 agonists have been identified: these include natural products like hydroxytyrosol and oleuropein, as well as phytoestrogens, such as coumestrol, and the endocrine-disrupting compounds Bisphenol A (BPA) (**Figure 2**) (20–22). Additional studies have identified synthetic polybrominated diphenyl ethers (PBDEs) and hydroxylated PBDEs as potential GPER1 ligands; however, these compounds likely exhibit no selective activity (23).

A hallmark challenge in the identification and discovery of GPER1-specific ligands has been the difficulty in achieving a crystalized structure of the receptor. Presently, a crystallized structure of GPER1 does not exist. For this reason, the identification and optimization of ligands has relied upon large-library virtual screening techniques and homology modeling (24–27). Due to the cross-reactivity of estrogenic ligands, a limited number of GPER1-specific ligands have been identified. The current benchmark for GPER1-specific ligands were identified through virtual screening of 10,000 into a model of GPER1 based on 2D- and 3D-similarity approaches and GPER-privileged substructures (24). From the screening, a substituted dihydroquinoline was identified and named GPR30-specific compound 1, G-1 (24). Binding studies revealed no appreciable binding to ESR1 or ESR2 below 100 nM (24). Subsequent functional bioassays with GPER1-transfected COS-7 cell and G-1 showed that E2 and G-1 exhibit an increase in calcium mobilization at 1 nM; however, a closer analysis of the data suggests that the kinetic profile of the calcium mobilization differs between the compounds such that G-1 exhibits slow receptor occupancy and an asymptotic curve and E2 exhibits fast receptor occupancy with a quick peak in calcium release (24). Medicinal chemistry approaches to modify the dihydroquinoline of G-1 altered the pharmacological activity of the scaffold from an agonist to an antagonist (28). While the identified antagonist, G-15, inhibited G-1 activity at GPER1, off-target binding to ESR1 and activation of EREs persisted (28). The reduction of binding and ERE activation was accomplished with the addition of an isopropyl group to the scaffold to make G-36 (29). While the G-series has become the standard for GPER1 agonists and antagonists, the success with the compounds has been variable and may be related to the tissue-specific signaling events of GPER1 (30, 31).

Since the development of the G-series of ligands, a limited number of groups have published data on synthesized ligands for GPER1. Lappano et al. proposed two tricyclic tetrahydroquinolines, GPER-L1 and GPER-L2 (32). These compounds were shown to bind exclusively to GPER1 without significant ESR1 binding above 100 μM (**Figure 2**) (33). Previously, we identified a series of N-thiazol-2-yl-1H-indole-2-carboxamide derivatives as GPER1 agonists (30). These compounds exhibited a similar effect on breast cancer proliferation as reported in the literature in response to the GPER1-selective agonist, G-1 (**Figure 2**) (30). Based on that work and further computational modeling, we have since reported the first structure-activity relationship for GPER1 antagonists and discovered CIMBA (2-cyclohexyl-4-isopropyl-N-(4-methoxybenzyl)aniline) (34). In addition to our group, Maggiolini et al. developed two selective GPER1 antagonists (PBX1 and PBX2) based on a benzo[b]pyrrolo[1,2-d][1,4]oxazin-4-one scaffold (**Figure 2**) (35). Both PBX1 and PBX2 effectively blocked agonist-induced GPER1 activity without transcriptional activation of the classical ERs. Additional non-selective GPER1 ligands have also been described in the literature. Unlike the GPER1 antagonists identified by DeLeon et al. and Maggioloini et al., Lappano et al. identified MIBE (ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate) and demonstrated that MIBE blocks agonist activity at both GPER1 and ESR1 (36, 37). In addition to MIBE novel ligands, such as calixpyrrole derivatives that include a cyclic structure and resemble a porphyrin ring system, have been proposed as GPER1 antagonists (**Figure 2**) (36, 37).

The limited number of available GPER1-specific ligands may be attributed to a lack of clarity in the localization as well as the complex pharmacology associated with GPER1. The localization and expression of GPER1 has been long debated. Numerous studies have shown that GPER1 is expressed both along the cell membrane surface as well as intracellularly within the endoplasmic reticulum and Golgi apparatus (38–40). After several decades, it is now recognized that even though GPER1 expression exists within the cell membrane, the expression level is substantially less than the

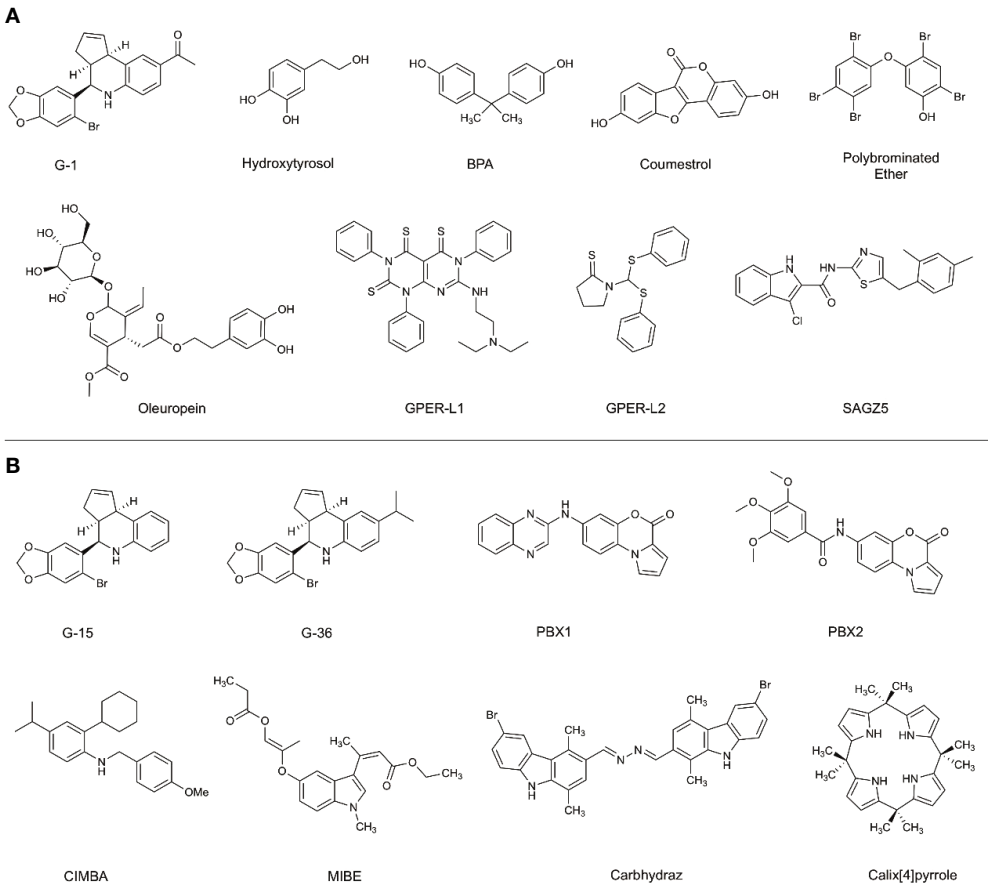

FIGURE 2 | Selective and non-selective GPER1 agonists **(A)** and antagonists **(B)**. **(A)** Various GPER1 agonists have been identified. This includes non-selective natural products like hydroxytyrosol and oleuropein, phytoestrogens such as coumestrol, as well as endocrine-disrupting compounds like BPA. Various synthetic GPER1 agonists have been identified. A series of polybrominated ethers have been identified; however, these compounds likely do not exhibit specificity for GPER1. Several GPER1-specific agonists have been identified. These compounds include G-1, GPER-L1, GPER-L2, and SAGZ5. **(B)** Currently, there are no known naturally occurring GPER1 antagonists. Modifications were made to the tetrahydroquinoline scaffold of G-1 to create G-15 and G-36. These alterations modified the activity of the compounds to antagonists. Since the identification of G-15 and G-36, there have been a limited number of GPER1-specific antagonists identified. These include PBX1, PBX2, CIMBA, carbhydraz, and calix[4]pyrrole. MIBE has been identified as an antagonist for GPER1 and ESR1. In certain circumstances, there may be a therapeutic benefit in jointly targeting GPER1 and ESR1.

subcellular expression (40). This has important implications for drug discovery in that GPER1 ligands may need to be lipophilic and able to cross the cell membrane to access the receptor. The data achieved relating to the pharmacology of the G-series has varied among groups and has posed challenges to defining G protein coupling (31, 40). Together, the localization and varied success with currently available probes substantiate the need for novel GPER1-specific ligands to better understand the pharmacology associated with GPER1 and the clinical implications for the receptor.

ROLE OF GPER1 IN CHOLESTEROL GALLSTONE DISEASE

Cholesterol gallstone disease is one of the most prevalent and costly digestive diseases in the United States, with at least 20 million Americans (12% of adults) being affected (41). Clinical and epidemiological investigations have demonstrated that women are twice as likely as men to form cholesterol gallstones in every population that has been studied (42). Oral contraceptives and conjugated estrogens significantly increase gallstone prevalence in premenopausal and postmenopausal women (43–53). Similar lithogenic effects are also found in men with prostate cancer during postoperative estrogen therapy (54–56). All these studies show that E2 is a critical risk factor for gallstone disease and a high predisposition to gallstones in women than in men is related to differences in how the liver metabolizes cholesterol in response to E2 (57). Although both ESR1 and ESR2 are expressed in the liver of mice and humans, ESR1 expression is approximately 50-fold higher compared to ESR2 expression (58). Despite these observations, the mechanism by which ESR1 plays a key role in mediating E2-

induced lithogenic actions at a cellular and molecular level is not yet fully understood. Exciting results show that E2 enhances cholelithogenesis by increasing hepatic expression of ESR1 but not ESR2, and the lithogenic actions of E2 can be blocked completely by the antiestrogenic agent, ICI 182,780 (58). Furthermore, the ESR1-selective agonist propylpyrazole, but not the ESR2-selective agonist diarylpropionitrile, promotes hepatic cholesterol output, leading to cholesterol-supersaturated bile and gallstones (58). Similar to E2 treatment, tamoxifen significantly increased biliary cholesterol secretion and gallstone prevalence (58, 59). These results indicate that the hepatic ESR1, but not ESR2, plays a critical role in E2-induced gallstones in female mice. More importantly, ESR1 stimulated by E2 dramatically increases hepatic expression of sterol regulatory element-binding protein-2 (SREBP-2), activating SREBP-2-responsive genes in the cholesterol biosynthetic pathway (60). Thus, the E2-treated mice continue to synthesize cholesterol despite its excess availability from high dietary cholesterol, which reflects a loss in controlling the negative feedback regulation of cholesterol synthesis. As a result, more newly synthesized cholesterol determined by the estrogen-ESR1-SREBP-2 pathway is secreted into bile, leading to biliary cholesterol hypersecretion and enhancing the lithogenicity of bile (60).

More interestingly, the deletion of *Esr1* diminishes susceptibility to E2-induced gallstones by reducing hepatic cholesterol secretion and desaturating gallbladder bile; however, this cannot completely protect against gallstone formation in mice treated with high doses of E2 and fed the lithogenic diet (61). As found by a powerful genetic quantitative trait locus (QTL) analysis, *Gper1* is a new gallstone gene, *Lith18*, on chromosome 5 in mice (62–66). GPER1 activated by its agonist, G-1, enhances cholelithogenesis by deterring expression of cholesterol 7α-hydroxylase, the rate-limiting enzyme for the classical pathway of bile salt synthesis (67). These metabolic abnormalities greatly increase biliary cholesterol concentrations in company with hepatic hyposecretion of biliary bile salts, leading to cholesterol-supersaturated gallbladder bile and accelerating cholesterol crystallization (68). Moreover, E2 activates GPER1 and ESR1 toproduce liquid crystalline versus anhydrous crystalline metastable intermediates evolving to cholesterol monohydrate crystals from supersaturated bile (69). However, cholesterol crystallization is drastically retarded in *Gper1*/*Esr1* double knockout mice. This indicates that GPER1 produces a synergistic lithogenic action with ESR1 to enhance E2-induced gallstone formation.

Impaired gallbladder motility is often a distinctive clinical feature of pregnant women and subjects received high doses of E2, which promotes the formation of biliary sludge, the precursor of gallstones (70–75). Immunohistochemical studies find that GPER1 is expressed predominately in the epithelial cells of the gallbladder (69). By contrast, ESR1 is expressed mainly in the smooth muscle of the gallbladder (69). This suggests that GPER1 could impair gallbladder motility, working independently of ESR1, as both can cause sluggish gallbladder contractility from different mechanisms. Indeed, G-1 impairs gallbladder emptying through the GPER1 pathway in mice, leading to sluggish gallbladder motility and accelerating the development of biliary sludge in the early stage of gallstone formation (67).

More recently, exciting evidence shows that a novel, potent GPER1-selective antagonist, CIMBA, reduces the prevalence of E2-induced gallstones in a dose-dependent manner by impeding the GPER1 signaling pathway in female wild-type mice (76). However, gallstones can be completely prevented in E2-treated ESR1 knockout mice even on the lithogenic diet (76). These results are consistent with the findings that the deletion of either *Esr1* or *Gper1* significantly reduces the prevalence of E2-induced gallstones but could not abolish it completely.

Overall, these studies have established a novel concept that GPER1 is involved in E2-dependent lithogenic actions, working independently of ESR1, as both GPER1 and ESR1 can promote the formation of E2-induced gallstones through different pathways. Thus, both GPER1 and ESR1 are potential therapeutic targets for cholesterol gallstone disease, particularly in women and patients exposed to high levels of E2 (77).

CONCLUSIONS AND FUTURE DIRECTIONS

The similarity between estrogenic compounds poses significant challenges in the design of new, selective ligands due to the promiscuous binding of estrogenic compounds to different types of ERs and is a particular challenge for designing new compounds. While estrogen binding is frequently associated with the nuclear ERs, GPER1 has been recognized as a new ER. A frequently neglected aspect of ER signaling is the ability of E2 and estrogenic compounds to directly activate calcium channels, specifically L-type calcium channels and calcium-activated BK (big potassium) channels (78, 79). The activation of ion channels by estrogenic compounds adds another level of complexity to studying ER signaling pathways and the design of GPER1-specific compounds.

The signaling pathways of ERs are complex and multifaceted. For this reason, studies that aim to examine a singular ER signaling pathway should not neglect existence of the three ERs. The therapeutic implications of targeting multiple ER signaling pathways are not well understood; however, evidence exists that cross-reactivity may severely limit the application of certain therapeutics. For instance, even though selective estrogen receptor modulators (SERMs) exhibit antiestrogen effects at the classical ERs, the cross-reactivity and activation of GPER1 may contribute to therapeutic resistance, which renders the therapeutic ineffective (80, 81). This limitation has been observed with tamoxifen. Alternatively, there may be some therapeutic opportunities for cross-reactivity, specifically in the gallbladder. In this circumstance, previous evidence has shown that inhibition of ESR1 or GPER1 alone is not sufficient to completely prevent gallstone formation (49). In this instance, the cross-reactivity of a compound, such as MIBE (**Figure 2**), which acts as an antiestrogen at both ESR1 and GPER1 may be a useful tool. The identification of new agonists has largely occurred in breast cancer cell lines that endogenously express GPER1. The

pharmacology associated with GPER1 may be tissue-specific since GPER1 is expressed ubiquitously throughout the body. The use of additional cell lines may lead to a greater number of potent and efficacious ligands.

In most areas of the digestive system, there are still opportunities for further understanding the impact of exclusively targeting GPER1 and understanding the potential pharmacological implications of targeting multiple ERs. While G-1 has served as a valuable tool for understanding the role of GPER1 in health and disease associated with the digestive system in animals, and development of further GPER1 agonists and antagonists will lead to potential therapeutics with greater activity, specificity, and solubility in water or oil. The role of GPER1 in cholesterol gallstone disease presented in this review highlights the potential importance of GPER1 in hepatobiliary diseases. Overall, the prevention of lithogensis *via* a GPER1 antagonist represents a novel treatment option for high-risk populations and may prove to be an adjunct therapy to nonsurgical gallstone treatments.

AUTHOR CONTRIBUTIONS

All authors equally contributed to writing this perspective. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. El-Serag HB, Sweet S, Winchester CC, Dent J. Update on the epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut* (2014) 63:871–80. doi: 10.1136/gutjnl-2012-304269
2. Saito YA, Schoenfeld P, Locke RG. III, The Epidemiology of Irritable Bowel Syndrome in North America: A Systematic Review. *Am J Gastroenterol* (2002) 97:1910–5. doi: 10.1016/S0002-9270(02)04270-3
3. Kim YS, Kim N, Kim GH. Sex and Gender Differences in Gastroesophageal Reflux Disease. *J Neurogastroenterol Motil* (2016) 22:575–88. doi: 10.5056/jnm16138
4. Kim YS, Kim N. Sex-Gender Differences in Irritable Bowel Syndrome. *J Neurogastroenterol Motil* (2018) 24:544–58. doi: 10.5056/jnm18082
5. Stinton LM, Shaffer EA. Epidemiology of Gallbladder Disease: Cholelithiasis and Cancer. *Gut Liver* (2012) 6:172–87. doi: 10.5009/gnl.2012.6.2.172
6. Nilsson S, Koehler KF. Oestrogen receptors and selective oestrogen receptor modulators: molecular and cellular pharmacology. *Basic Clin Pharmacol Toxicol* (2005) 96:15–25. doi: 10.1111/j.1742-7843.2005.pto960103.x
7. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* (1997) 389:753–8. doi: 10.1038/39645
8. Filardo EJ, Quinn JA, Bland KI, Frackelton ARJ. Estrogen-Induced Activation of Erk-1 and Erk-2 Requires the G Protein-Coupled Receptor Homolog, GPR30, and Occurs via Trans-Activation of the Epidermal Growth Factor Receptor through Release of HB-EGF. *Mol Cell Endocrinol* (2000) 14:1649–60. doi: 10.1210/mend.14.10.0532
9. Filardo EJ, Quinn JA, Frackelton A, Bland KI. Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* (2002) 16:70–84. doi: 10.1210/mend.16.1.0758
10. Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, et al. The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* (2004) 279:27008–16. doi: 10.1074/jbc.M403588200
11. Filardo EJ, Thomas P. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends Endocrinol Metab* (2005) 16:362–7. doi: 10.1016/j.tem.2005.08.005
12. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* (2005) 307:1625–30. doi: 10.1126/science.1106943
13. Filardo EJ, Thomas P. Minireview: G Protein-Coupled Estrogen Receptor-1, GPER-1: Its Mechanism of Action and Role in Female Reproductive Cancer, Renal and Vascular Physiology. *Endocrinology* (2012) 153:2953–62. doi: 10.1210/en.2012-1061
14. Björnström L, Sjöberg M. Mechanisms of Estrogen Receptor Signaling: Convergence of Genomic and Nongenomic Actions on Target Genes. *Mol Endocrinol* (2005) 19:833–42. doi: 10.1210/me.2004-0486
15. Levin ER. Invited Review: Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol* (2001) 91:1860. doi: 10.1152/jappl.2001.91.4.1860
16. Teskarik J, Mendoza C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *J Clin Endocrinol Metab* (1995) 80:1438–43. doi: 10.1210/jc.80.4.1438
17. Kelly MJ, Wagner EJ. Estrogen Modulation of G-protein-coupled Receptors. *Trends Endocrinol Metab* (1999) 10:369–74. doi: 10.1016/S1043-2760(99)00190-3
18. Nilsson S, Gustafsson JÅ. Estrogen Receptors: Therapies Targeted to Receptor Subtypes. *Clin Pharmacol Ther* (2011) 89:44–55. doi: 10.1038/clpt.2010.226
19. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* (2005) 146:624–32. doi: 10.1210/en.2004-1064
20. Xu F, Wang X, Wu N, He S, Yi W, Xiang S, et al. Bisphenol A induces proliferative effects on both breast cancer cells and vascular endothelial cells through a shared GPER-dependent pathway in hypoxia. *Environ Pollut* (2017) 231:1609–20. doi: 10.1016/j.envpol.2017.09.069
21. Chimento A, Casaburi I, Rosano C, Avena P, De Luca A, Campana C, et al. Oleuropein and hydroxytyrosol activate GPER/ GPR30-dependent pathways leading to apoptosis of ER-negative SKBR3 breast cancer cells. *Mol Nutr Food Res* (2014) 58:478–89. doi: 10.1002/mnfr.201300323
22. Molina L, Bustamante FA, Bhoola KD, Figueroa CD, Ehrenfeld P. Possible role of phytoestrogens in breast cancer via GPER-1/GPR30 signaling. *Clin Sci* (2018) 132:2583–98. doi: 10.1042/CS20180885
23. Cao L-Y, Ren X-M, Yang Y, Wan B, Guo L-H, Chen D, et al. Hydroxylated Polybrominated Diphenyl Ethers Exert Estrogenic Effects via Non-Genomic G Protein-Coupled Estrogen Receptor Mediated Pathways. *Environ Health Perspect* (2018) 126:057005–5. doi: 10.1289/EHP2387
24. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* (2006) 2:207–12. doi: 10.1038/nchembio775
25. Arnatt CK, Zhang Y. G Protein-Coupled Estrogen Receptor (GPER) Agonist Dual Binding Mode Analyses Toward Understanding of Its Activation Mechanism: A Comparative Homology Modeling Approach. *Mol Inf* (2013) 32:647–58. doi: 10.1002/minf.201200136
26. Bruno A, Aiello F, Costantino G, Radi M. Homology Modeling, Validation and Dynamics of the G Protein-coupled Estrogen Receptor 1 (GPER-1). *Mol Inf* (2016) 35:333–9. doi: 10.1002/minf.201501024
27. Méndez-Luna D, Martínez-Archundia M, Maroun RC, Ceballos-Reyes G, Fragoso-Vázquez MJ, González-Juárez DE, et al. Deciphering the GPER/GPR30-agonist and antagonists interactions using molecular modeling

studies, molecular dynamics, and docking simulations. *J Biomol Struct Dynamics* (2015) 33:2161–72. doi: 10.1080/07391102.2014.994102
28. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, et al. In vivo Effects of a GPR30 Antagonist. *Nat Chem Biol* (2009) 5:421–7. doi: 10.1038/nchembio.168
29. Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologa CG, et al. Identification of a GPER/GPR30 Antagonist with Improved Estrogen Receptor Counterselectivity. *J Steroid Biochem Mol Biol* (2011) 127:358–66. doi: 10.1016/j.jsbmb.2011.07.002
30. O'Dea A, Sondergard C, Sweeney P, Arnatt CK. A Series of Indole-Thiazole Derivatives Act as GPER Agonists and Inhibit Breast Cancer Cell Growth. *ACS Med Chem Lett* (2018) 9:901–6. doi: 10.1021/acsmedchemlett.8b00212
31. Feldman RD, Limbird LE. GPER (GPR30): A Nongenomic Receptor (GPCR) for Steroid Hormones with Implications for Cardiovascular Disease and Cancer. *Annu Rev Pharmacol Toxicol* (2017) 57:567–84. doi: 10.1146/annurev-pharmtox-010716-104651
32. Lappano R, Rosano C, Santolla MF, Pupo M, De Francesco EM, De Marco P, et al. Two novel GPER agonists induce gene expression changes and growth effects in cancer cells. *Curr Cancer Drug Targets* (2012) 12:531–42. doi: 10.2174/156800912800673284
33. Cerra B, Mostarda S, Custodi C, Macchiarulo A, Gioiello A. Integrating multicomponent flow synthesis and computational approaches for the generation of a tetrahydroquinoline compound based library. *Med Chem Comm 7* (2016) 7:439–46. doi: 10.1039/C5MD00455A
34. DeLeon C, Wang HH, Gunn J, Wilhelm M, Cole A, Arnett S, et al. Novel GPER Antagonist Protects Against the Formation of Estrogen-Induced Cholesterol Gallstones in Female Mice. *J Lipid Res* (2020) 61:767–77. doi: 10.1194/jlr.RA119000592
35. Maggiolini M, Santolla MF, Avino S, Aiello F, Rosano C, Garofalo A, et al. Identification of two benzopyrroloxazines acting as selective GPER antagonists in breast cancer cells and cancer-associated fibroblasts. *Future Med Chem* (2015) 7:437–48. doi: 10.4155/fmc.15.3
36. Lappano R, Rosano C, Pisano A, Santolla MF, De Francesco EM, De Marco P, et al. A calixpyrrole derivative acts as an antagonist to GPER, a G-protein coupled receptor: mechanisms and models. *Dis Models Mech* (2015) 8:1237. doi: 10.1242/dmm.021071
37. Lappano R, Santolla MF, Pupo M, Sinicropi MS, Caruso A, Rosano C, et al. MIBE acts as antagonist ligand of both estrogen receptor α and GPER in breast cancer cells. *Breast Cancer Res* (2012) 14:R12. doi: 10.1186/bcr3096
38. Zimmerman MA, Budish RA, Kashyap S, Lindsey SH. GPER-novel membrane oestrogen receptor. *Clin Sci (Lond)* (2016) 130:1005–16. doi: 10.1042/CS20160114
39. Cheng S-B, Quinn JA, Graeber CT, Filardo EJ. Down-modulation of the G-protein-coupled estrogen receptor, GPER, from the cell surface occurs via a trans-Golgi-proteasome pathway. *J Biol Chem* (2011) 286:22441–55. doi: 10.1074/jbc.M111.224071
40. Gaudet HM, Cheng SB, Christensen EM, Filardo EJ. The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. *Mol Cell Endocrinol* (2015) 418 Pt 3:207–19. doi: 10.1016/j.mce.2015.07.016
41. Wang DQ, Portincasa P. *Gallstones: Recent advances in epidemiology, pathogenesis, diagnosis and management*. New York, NY: Nova Science Publishers (2017).
42. Wang DQ, Portincasa P, Wang HH. "Bile formation and pathophysiology of gallstones". In: EJ Kuipers, editor. *Encyclopedia of Gastroenterology*. New York, NY: Elsevier (2020). p. 287–306.
43. Bennion LJ, Ginsberg RL, Gernick MB, Bennett PH. Effects of oral contraceptives on the gallbladder bile of normal women. *N Engl J Med* (1976) 294:189–92. doi: 10.1056/NEJM197601222940403
44. Grodstein F, Colditz GA, Hunter DJ, Manson JE, Willett WC, Stampfer MJ. A prospective study of symptomatic gallstones in women: relation with oral contraceptives and other risk factors. *Obstetr Gynecol* (1994) 84:207–14. doi: 10.1093/jnci/86.19.1466
45. Grodstein F, Colditz GA, Stampfer MJ. Postmenopausal hormone use and cholecystectomy in a large prospective study. *Obstetr Gynecol* (1994) 83:5–11. doi: 10.7326/0003-4819-128-9-199805010-00001
46. Uhler ML, Marks JW, Judd HL. Estrogen replacement therapy and gallbladder disease in postmenopausal women. *Menopause* (2000) 7:162–7. doi: 10.1097/00042192-200007030-00006
47. Simon JA, Hunninghake DB, Agarwal SK, Lin F, Cauley JA, Ireland CC, et al. Effect of estrogen plus progestin on risk for biliary tract surgery in postmenopausal women with coronary artery disease. The Heart and Estrogen/progestin Replacement Study. *Ann Internal Med* (2001) 135:493–501. doi: 10.7326/0003-4819-135-7-200110020-00008
48. Hart AR, Luben R, Welch A, Bingham S, Khaw KT. Hormone replacement therapy and symptomatic gallstones - a prospective population study in the EPIC-Norfolk cohort. *Digestion* (2008) 77:4–9. doi: 10.1159/000113897
49. Everson GT, McKinley C, Kern FJr. Mechanisms of gallstone formation in women. Effects of exogenous estrogen (Premarin) and dietary cholesterol on hepatic lipid metabolism. *J Clin Invest* (1991) 87:237–46. doi: 10.1172/JCI114977
50. Petitti DB. Estrogen use and gallstone disease. *Am J Public Health* (1988) 78:1365. doi: 10.2105/AJPH.78.10.1365
51. Dhiman RK, Chawla YK. Is there a link between oestrogen therapy and gallbladder disease? *Expert Opin Drug Saf* (2006) 5:117–29. doi: 10.1517/14740338.5.1.117
52. Cirillo DJ, Wallace RB, Rodabough RJ, Greenland P, LaCroix AZ, Limacher MC, et al. Effect of estrogen therapy on gallbladder disease. *J Am Med Assoc* (2005) 293:330–9. doi: 10.1001/jama.293.3.330
53. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *J Am Med Assoc* (2002) 288:321–33. doi: 10.1001/jama.288.3.321
54. Angelin B, Olivecrona H, Reihner E, Rudling M, Stahlberg D, Eriksson M, et al. Hepatic cholesterol metabolism in estrogen-treated men. *Gastroenterology* (1992) 103:1657–63. doi: 10.1016/0016-5085(92)91192-7
55. Henriksson P, Einarsson K, Eriksson A, Kelter U, Angelin B. Estrogen-induced gallstone formation in males. Relation to changes in serum and biliary lipids during hormonal treatment of prostatic carcinoma. *J Clin Invest* (1989) 84:811–6. doi: 10.1172/JCI114240
56. Sweeting J. Estrogen-induced gallstones in males. *Gastroenterology* (1990) 98:800–1. doi: 10.1016/0016-5085(90)90309-O
57. Wang HH, Liu M, Clegg DJ, Portincasa P, Wang DQ. New insights into the molecular mechanisms underlying effects of estrogen on cholesterol gallstone formation. *Biochim Biophys Acta* (2009) 1791:1037–47. doi: 10.1016/j.bbalip.2009.06.006
58. Wang HH, Afdhal NH, Wang DQ. Estrogen receptor alpha, but not beta, plays a major role in 17beta-estradiol-induced murine cholesterol gallstones. *Gastroenterology* (2004) 127:239–49. doi: 10.1053/j.gastro.2004.03.059
59. Akin ML, Uluutku H, Erenoglu C, Karadag A, Gulluoglu BM, Sakar B, et al. Tamoxifen and gallstone formation in postmenopausal breast cancer patients: retrospective cohort study. *World J Surg* (2003) 27:395–9. doi: 10.1007/s00268-002-6468-4
60. Wang HH, Afdhal NH, Wang DQ. Overexpression of estrogen receptor alpha increases hepatic cholesterogenesis, leading to biliary hypersecretion in mice. *J Lipid Res* (2006) 47:778–86. doi: 10.1194/jlr.M500454-JLR200
61. de Bari O, Wang HH, Portincasa P, Liu M, Wang DQ. The deletion of the estrogen receptor alpha gene reduces susceptibility to estrogen-induced cholesterol cholelithiasis in female mice. *Biochim Biophys Acta* (2015) 1852:2161–9. doi: 10.1016/j.bbadis.2015.07.020
62. Lyons MA, Korstanje R, Li R, Sheehan SM, Walsh KA, Rollins JA, et al. Single and interacting QTLs for cholesterol gallstones revealed in an intercross between mouse strains NZB and SM. *Mamm Genome* (2005) 16:152–63. doi: 10.1007/s00335-004-2446-5
63. Lyons MA, Wittenburg H. Cholesterol gallstone susceptibility loci: a mouse map, candidate gene evaluation, and guide to human LITH genes. *Gastroenterology* (2006) 131:1943–70. doi: 10.1053/j.gastro.2006.10.024
64. Wang HH, Portincasa P, Afdhal NH, Wang DQ. Lith genes and genetic analysis of cholesterol gallstone formation. *Gastroenterol Clinics North Am* (2010) 39:185–207. doi: 10.1016/j.gtc.2010.02.007
65. Krawczyk M, Wang DQ, Portincasa P, Lammert F. Dissecting the genetic heterogeneity of gallbladder stone formation. *Semin Liver Dis* (2011) 31:157–72. doi: 10.1055/s-0031-1276645
66. Wang TY, Portincasa P, Liu M, Tso P, Wang DQ. Mouse models of gallstone disease. *Curr Opin Gastroenterol* (2018) 34:59–70. doi: 10.1097/MOG.0000000000000417

67. Wang HH, de Bari O, Arnatt CK, Liu M, Portincasa P, Wang DQ. Activation of a novel estrogen receptor GPR30 enhances cholesterol cholelithogenesis in female mice. *Hepatology* (2020) (in press). doi: 10.1002/hep.31212
68. Di Ciaula A, Wang DQ, Portincasa P. An update on the pathogenesis of cholesterol gallstone disease. *Curr Opin Gastroenterol* (2018) 34:71–80. doi: 10.1097/MOG.0000000000000423
69. de Bari O, Wang TY, Liu M, Portincasa P, Wang DQ. Estrogen induces two distinct cholesterol crystallization pathways by activating ERalpha and GPR30 in female mice. *J Lipid Res* (2015) 56:1691–700. doi: 10.1194/jlr.M059121
70. de Bari O, Wang TY, Liu M, Paik CN, Portincasa P, Wang DQ. Cholesterol cholelithiasis in pregnant women: pathogenesis, prevention and treatment. *Ann Hepatol* (2014) 13:728–45. doi: 10.1016/S1665-2681(19)30975-5
71. Lammert F, Gurusamy K, Ko CW, Miquel JF, Mendez-Sanchez N, Portincasa P, et al. Gallstones. *Nat Rev Dis Primers* (2016) 2:16024. doi: 10.1038/nrdp.2016.24
72. Portincasa P, Di Ciaula A, Wang HH, Palasciano G, van Erpecum KJ, Moschetta A, et al. Coordinate regulation of gallbladder motor function in the gut-liver axis. *Hepatology* (2008) 47:2112–26. doi: 10.1002/hep.22204
73. Ko CW, Beresford SA, Schulte SJ, Matsumoto AM, Lee SP. Incidence, natural history, and risk factors for biliary sludge and stones during pregnancy. *Hepatology* (2005) 41:359–65. doi: 10.1002/hep.20534
74. Ko CW. Risk factors for gallstone-related hospitalization during pregnancy and the postpartum. *Am J Gastroenterol* (2006) 101:2263–8. doi: 10.1111/j.1572-0241.2006.00730.x
75. Ko CW, Sekijima JH, Lee SP. Biliary sludge. *Ann Internal Med* (1999) 130:301–11. doi: 10.7326/0003-4819-130-4-199902160-00016
76. DeLeon C, Wang HH, Gunn J, Wilhelm M, Cole A, Arnett S, et al. A novel GPER antagonist protects against the formation of estrogen-induced cholesterol gallstones in female mice. *J Lipid Res* (2020) 61:767–77. doi: 10.1194/jlr.RA119000592
77. Wang DQ, Afdhal NH, Brandt L. Gallstone Disease. In: Feldman M, Friedman LS, editors. *Sleisenger and Fordtrans Gastrointestinal and Liver Disease*. Philadelphia: Elsevier Saunders (2020). p. 1016–46.
78. Vega-Vela NE, Osorio D, Avila-Rodriguez M, Gonzalez J, García-Segura LM, Echeverria V, et al. L-Type Calcium Channels Modulation by Estradiol. *Mol Neurobiol* (2017) 54:4996–5007. doi: 10.1007/s12035-016-0045-6
79. Kow L-M, Pfaff DW. Rapid estrogen actions on ion channels: A survey in search for mechanisms. *Steroids* (2016) 111:46–53. doi: 10.1016/j.steroids.2016.02.018
80. Ignatov T, Claus M, Nass N, Haybaeck J, Seifert B, Kalinski T, et al. G-protein-coupled estrogen receptor GPER-1 expression in hormone receptor-positive breast cancer is associated with poor benefit of tamoxifen. *Breast Cancer Res Treat* (2019) 174:121–7. doi: 10.1007/s10549-018-5064-8
81. Ignatov A, Ignatov T, Roessner A, Costa SD, Kalinski T. Role of GPR30 in the mechanisms of tamoxifen resistance in breast cancer MCF-7 cells. *Breast Cancer Res Treat* (2010) 123:87–96. doi: 10.1007/s10549-009-0624-6

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REVIEW
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Therapeutic Perspectives on the Modulation of G-Protein Coupled Estrogen Receptor, GPER, Function

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Estrogens exert their physiological and pathophysiological effects *via* cellular receptors, named ERα, ERβ, and G-protein coupled estrogen receptor (GPER). Estrogen-regulated physiology is tightly controlled by factors that regulate estrogen bioavailability and receptor sensitivity, while disruption of these control mechanisms can result in loss of reproductive function, cancer, cardiovascular and neurodegenerative disease, obesity, insulin resistance, endometriosis, and systemic lupus erythematosus. Restoration of estrogen physiology by modulating estrogen bioavailability or receptor activity is an effective approach for treating these pathological conditions. Therapeutic interventions that block estrogen action are employed effectively for the treatment of breast and prostate cancer as well as for precocious puberty and anovulatory infertility. Theoretically, treatments that block estrogen biosynthesis should prevent estrogen action at ERs and GPER, although drug resistance and ligand-independent receptor activation may still occur. In addition, blockade of estrogen biosynthesis does not prevent activation of estrogen receptors by naturally occurring or man-made exogenous estrogens. A more complicated scenario is provided by anti-estrogen drugs that antagonize ERs since these drugs function as GPER agonists. Based upon its association with metabolic dysregulation and advanced cancer, GPER represents a therapeutic target with promise for the treatment of several critical health concerns facing Western society. Selective ligands that specifically target GPER have been developed and may soon serve as pharmacological agents for treating human disease. Here, we review current forms of estrogen therapy and the implications that GPER holds for these therapies. We also discuss existing GPER targeted drugs, additional approaches towards developing GPER-targeted therapies and how these therapies may complement existing modalities of estrogen-targeted therapy.

Keywords: GPER, estrogen receptors, therapeutics, anti-estrogens, cancer

INTRODUCTION

This review is organized in three general sections. First, we review basic information regarding estrogen bioavailability and its receptors. Second, we discuss the impact that GPER has upon our understanding of the influence of estrogen on human disease, and its implications for anti-estrogen therapy. Finally, we review existing pharmacological compounds that selectively target GPER and outline future potential approaches for targeting GPER.

ESTROGEN AND ITS RECEPTORS

Estrogens are gonadocorticoids and the primary female sex hormones. Their actions promote the development of female reproductive tissue and secondary sexual characteristics, and they influence all phases of reproduction including conception, fetal development, parturition, and nursing. Hence, estrogens exert their effects not only on reproductive tissue but on a wide range of physiological systems, including integumentary, central nervous, cardiovascular, skeletal, immune, metabolic, and excretory systems (1, 2). In humans, three forms of estrogen are synthesized. They are defined by their common 18 carbon (C-18) estrane ring structure and are numbered E1- E3 to reflect the number of hydroxyl groups linked to the estrane ring (**Figure 1**). Accordingly, they are named estrone (E1), estradiol (E2), and estriol (E3). Each of these endogenous estrogens is lipophilic and is presumed to exit and enter cells through their ability to freely diffuse across the plasma membrane. All endogenous estrogens are synthesized in the smooth endoplasmic reticulum in a shared pathway of steroidogenesis from cholesterol (C-27) (**Figure 2**). In this pathway, cholesterol is metabolized through a variety of enzymatic steps into (C-21) progestogens and (C-19) androgens that serve as the immediate steroid intermediate for estrogens. E1 and E2 are primarily secreted by ovarian granulosa cells in response to stimulation by neuroendocrine glycoprotein hormones, including luteinizing releasing hormone (LHRH), luteinizing hormone (LH), and follicle stimulating hormone (FSH), which are released from the hypothalamus and pituitary (3). During reproductive years, E1 and E2 are the two most common circulating estrogens found in plasma, with scant amounts of E3 measured. Estrogens can also be synthesized in a variety of non-ovarian tissues, including, adrenal gland, fat, brain, bone, skin, vascular smooth muscle and intestine (2). However, in these tissues, estrogens must be directly synthesized from androgens, as these tissues lack the necessary enzymatic machinery to synthesize C-19 androgens. E3 is synthesized at low levels in the liver and intestine by 16α-hydroxylation of E1 or E2 by cytochrome P450 enzymes, such as CYP3A4 (4). During pregnancy, E3 becomes the primary estrogen as it is synthesized at high levels by the placenta, far exceeding that of E1 or E2 in plasma. While its role in fetal development is not clear, low levels of E3 in maternal serum or urine is prognostic of poor perinatal health and congenital anomalies (5, 6).

The process by which estrogens are transported throughout the body and exert their biologic effects in target tissues is not completely understood. The vast majority of synthesized estrogen circulates in the plasma bound to either serum albumin or sex hormone binding globulin (SHBG) (7, 8). Only a small fraction (~ 1 to 2%) is unbound or "free" and available to bind to its receptors (9). E1 and E3 each bind SHBG with much

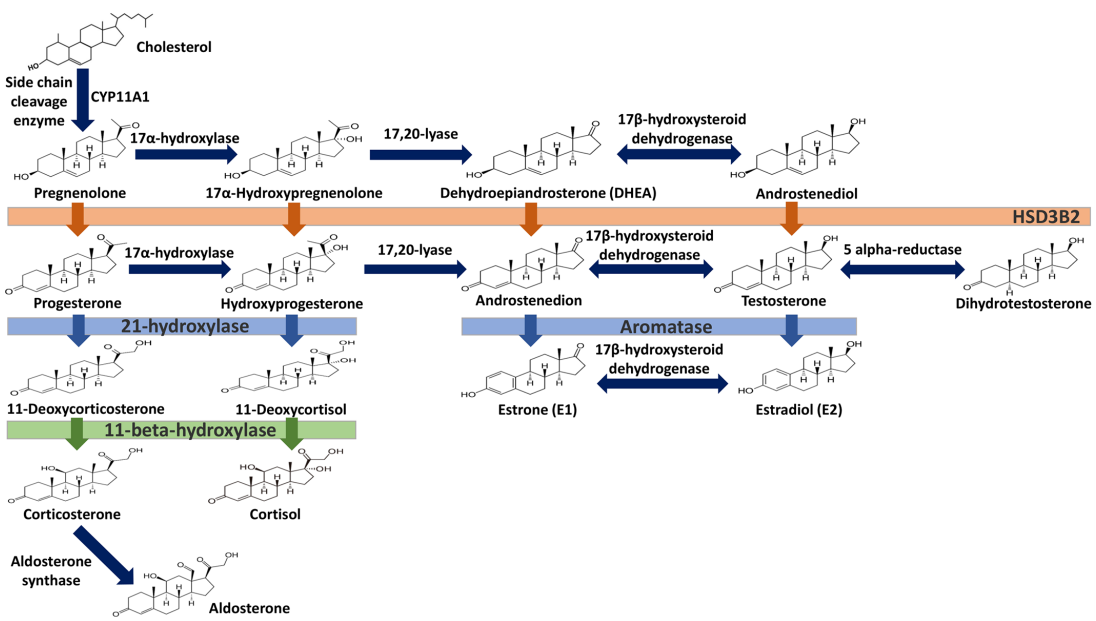


FIGURE 1 | Steroid hormone synthesis and metabolism. The diagram designates key enzymatic steps in steroidogenesis.

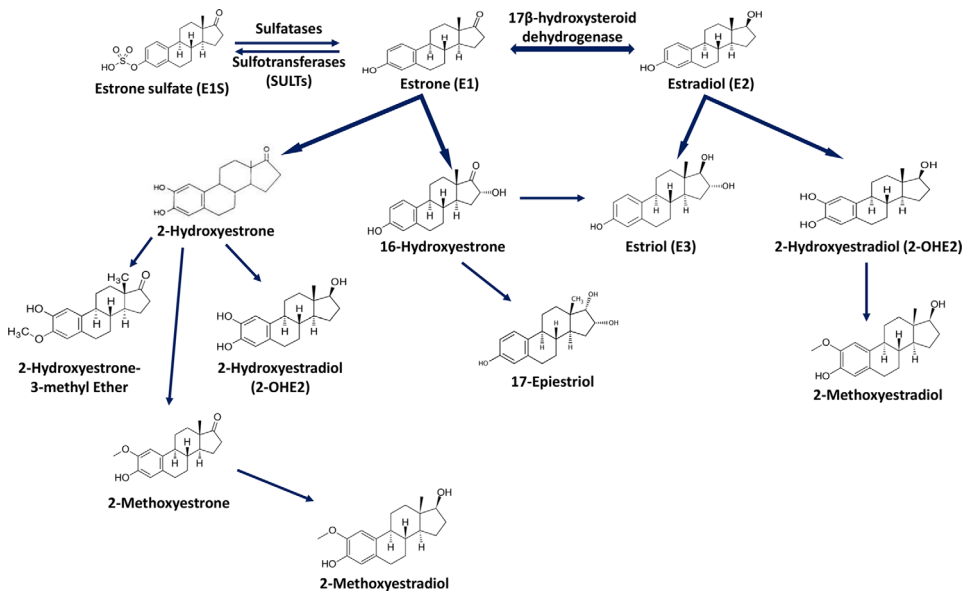


FIGURE 2 | Estrogen metabolism. This schematic identifies key intermediates in the metabolism of estrone and estradiol.

lower affinity than E2 and likewise each of these estrogens also shows a much lower affinity and potency for its receptors than E2 (10). SHBG also binds dihydrotestosterone (DHT) and testosterone (T) but with relative binding affinities that are 20- and 5-fold higher than for E2 (11, 12). In premenopausal women, SHBG levels are twice as high as in men and this has been suggested to limit their androgen and estrogen exposure (9, 13). SHBG concentrations decrease following menopause but increase during the sixth decade of life (14), and low serum levels of SHBG have been associated with hyperandrogenism and endometrial cancer (13). Ultimately, estrogens are eliminated from the body following their metabolic conversion to inactive metabolites, which poorly bind SHBG, and are excreted in urine and feces. Metabolic conversion occurs primarily in the liver but also in other tissues, and involves their biotransformation *via* enzyme-mediated conjugation to glucuronide, glutathione, methyl, and/or sulfate moieties, modifications which enhance their solubility in plasma and enhance its absorbability by tissues (15) (**Figure 2**). Among these estrogen conjugates, estrone sulfate (E1-S) is the most predominant in plasma, and its reclamation by steroid sulfatase is yet another route by which estrogen biosynthesis may occur in extragonadal tissue (16).

The physiological effects of estrogen are manifested through the integrated action of cellular receptors that belong to the nuclear steroid hormone receptor (SHR) and G-protein coupled receptor (GPCR) superfamilies. This paradigm of coordinated signaling by estrogen through SHRs and GPCRs is evolutionarily conserved (17) and is also employed by progestogens (18, 19) and androgens (20). ER and GPER transmit intracellular signals *via* fundamentally distinct mechanisms that occur with distinct kinetics and involve unique signaling effectors (21) (**Figure 3**). In general, ERs are localized intracellularly and function as estrogen-inducible transcription factors, while GPER exhibits all the hallmarks of a plasma membrane receptor that manifests its actions through heterotrimeric G-proteins, which in turn transactivate plasma membrane receptors and enzymes (22). Evidence also exists that ERs may function similarly to GPER, and this has been reviewed elsewhere (23). Despite their differences in cellular location and mechanism of action, SHRs and GPCRs each undergo allosteric modulation in response to binding their cognate ligands, with signaling activity of SHRs and GPCRs enhanced by the physical interaction of their cognate ligands at specific receptor contact sites. The estrogen binding characteristics of GPER and ER are distinct, and they demonstrate a different dissociation constant, K_d, in radiotracer assays using ^{3}H-estradiol (**Table 1**). As discussed in detail (30), it is important to recognize that the relative binding affinities (RBAs) of ERα, ERβ and GPER cannot be readily compared due to the fact that ERs and GPER are expressed at different levels and they exist in different physicochemical environments; ER isolated in detergent-free cytosolic homogenates versus GPER enriched in lipid-rich plasma membrane preparations. Thus, the lower K_d that is measured for E2 in ER binding assays relative to GPER binding assays does not suggest that E2 has a higher affinity for ER relative to GPER. Because SHRs are readily isolated from the soluble fraction of cellular homogenates, crystallization and identification of physical ligand contact sites encoded with the structure of SHRs has been achieved (31–33). Crystal structures at resolutions of 2.6 angstroms for ER liganded to E2 or the ER antagonist, raloxifene (RAL), have been determined (34). These results show that E2 and RAL share contact sites with different binding modes and that each induces distinct conformations within the ER transactivation domain. The findings from these studies illustrate that the principal ligand contact sites of ER are defined within a hydrophobic cavity consisting of twelve helices

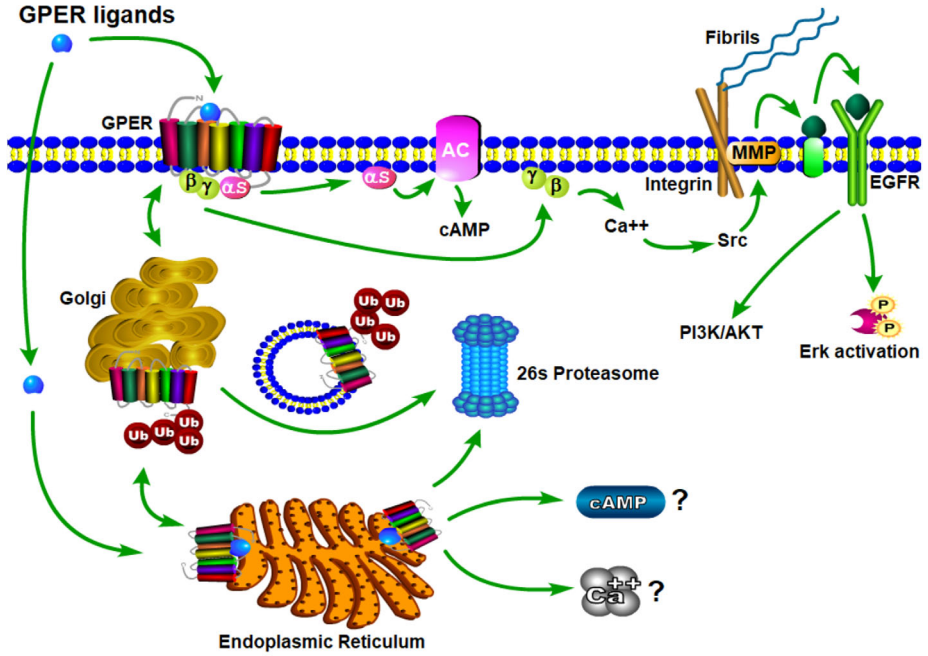
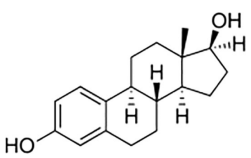
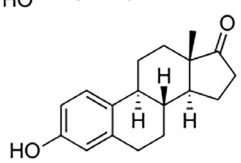
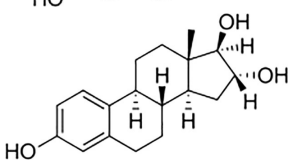


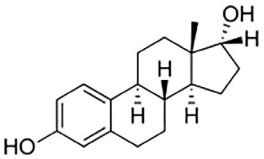
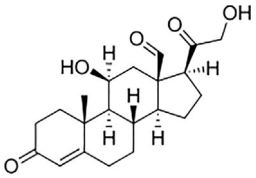
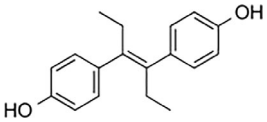
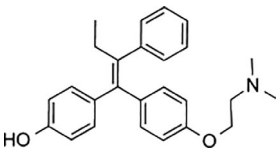
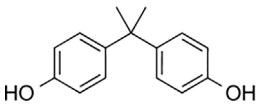
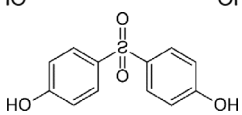
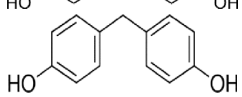
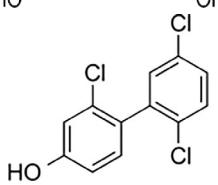
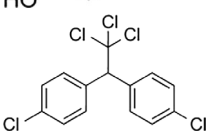
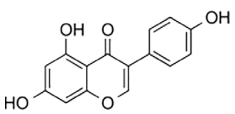
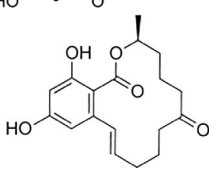
FIGURE 3 | Schematic model of GPER trafficking and signaling. Nascent GPER is biosynthesized in the endoplasmic reticulum (ER) where it undergoes carbohydrate addition, editing and dimerization prior to forward trafficking through the Golgi apparatus during its transport to the plasma membrane. Misfolded GPER is polyubiquitinated and degraded at the 26S-proteasome. At the plasma membrane GPER exists as a high affinity GDP-coupled Gαβγ heterotrimer. Upon engagement of estrogenic ligands, GPER assumes an activated confirmation resulting in the dissociation of Gαs and Gβγ subunit proteins, which in turn, stimulate adenylyl cyclase and integrin-dependent release of membrane-tethered EGF-ligands, respectively. Independent studies evaluating retrograde trafficking of GPER suggest that it undergoes constitutive endocytosis and degradation *via* a ubiquitin-transGolgi-proteasome pathway. It is not yet clear whether sustained GPER signaling is observed from intracellular receptor (question marks).

TABLE 1 | Relative binding affinities of estrogenic ligands to estrogen receptors.

Ligand	Structure	Relative Binding Affinity (RBA)		
		ERα	ERβ	GPER
Steroids				
17β-estradiol (E2)		100	100	100
Estrone (E1)		60	37	<0.04
Estriol (E3)		14	21	<0.4

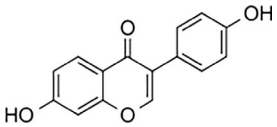
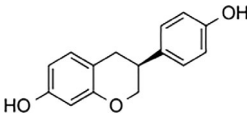
(Continued)

TABLE 1 | Continued

Ligand	Structure	Relative Binding Affinity (RBA)		
		ERα	ERβ	GPER
17α- estradiol		7	2	<0.04
Aldosterone		<0.0001	<0.0001 a	<0.00001
Diethylstilbestrol		236	221	<0.4
4-OH-tamoxifen		257	232	<4
Man-made estrogens				
Bisphenol A		0.01	0.01	1.1^
Bisphenol S		0.001	–	0.6^
Bisphenol F		0.001	–	ND^
OH-PCB-4		0.01	<0.01	0.1
p,p′-DDT		<0.01	<0.01	0.14
Dietary estrogens				
Genistein		0.7	13	3
Zearalenone		10	18	0.5

(Continued)

TABLE 1 | Continued

Ligand	Structure	Relative Binding Affinity (RBA)		
		ERα	ERβ	GPER
Ligand	**Structure**	**EC_{50} (nM)#**		
Daidzein		250	100	<1
Equol		200	74	100

RBAs for ERα and ERβ are based on reports from multiple sources (24–28). RBA determined from solubilized receptor competition experiments. ^Data are based on fluorescence competitive binding assay. RBA for GPER are based on values taken from (29). #EC_{50} is calculated based on functional assays.

(H1-12). Recognition of E2 within the ligand binding domain is achieved through a combination of hydrogen bond formation by the phenolic hydroxyls with polar residues contained within H3. H6 and H11, as well as alignment of the nonpolar character of estrane ring with hydrophobic residues that comprise these helices. As GPCRs are integral membrane proteins, purification is more challenging, and crystallization of GPER has yet to be achieved. In some regards, the hydrophobic environment provided within the closely aligned seven transmembrane helices of GPER is somewhat similar to the structure of the ER ligand binding domain. Several studies relying upon *in silico* molecular docking simulations have calculated principal binding interactions within the exoplasmic and/or transmembrane of GPER (35, 36). However, the role of these predicted ligand contact sites still needs to be evaluated by genetic studies which examine the influence of amino acid substitutions on GPER binding and signaling activity.

GPER IN METABOLIC DISEASE AND CANCER

Studies using knockout mice indicate that ER and GPER play different roles in estrogen physiology, with ER or GPER null mice primarily exhibiting reproductive (37, 38) and metabolic (39) deficits, respectively. This simple dichotomous description clearly oversimplifies the influence of each receptor type on estrogen physiology. However, collectively, the phenotypes of ER-null (40) and GPER-null (41) mice reflect the loss of reproductive function and metabolic homeostasis that is attributed to decreased ovarian estrogen biosynthesis accompanying menopause. While it is well appreciated that the metabolic effects of estrogen are manifested through ERs (42) and GPER (43), preclinical results published earlier this year in a study led by Sharma and Prossnitz, showed that chronic administration of the synthetic GPER selective agonist, G-1/Tespria, could restore fat, glucose and lipid homeostasis (44). This result indicates that targeting GPER may be an effective means for treating diabetes and obesity, and extends prior work that showed G-1 can ameliorate atherosclerosis in mice (45). The observation that chronic GPER signaling may alter metabolic activity has potential significance regarding a role for GPER in cancer as prolonged, uninterrupted estrogen exposure (46) and metabolic syndrome (47) are independent risk factors for cancer. Thus, GPER may serve as a centrally positioned factor that drives estrogen-induced carcinogenesis through chronic signaling that promotes metabolic disorder. In support of this concept, studies have linked GPER expression to clinical indices that predict advanced disease in breast cancer including increased tumor size, the presence of distant metastases, and tamoxifen-resistance (48–51). Similar results have been obtained in ovarian (52), endometrial (53), and testicular cancers (54) with GPER directly linked to poor survival. However, other reports suggest an inverse relationship between GPER and cancer progression (similar to that demonstrated by ER) (55, 56). The most likely explanation for the differences observed in the analysis of human cancer and GPER resides in the lack of a standardized procedure for its immunohistochemical detection and quantification in tumor biopsy specimens. For instance, some studies have set an absolute threshold for GPER expression among tumors, while others have focused on the relative difference between GPER in tumors and adjacent normal tissue in individual patients (55, 57, 58). Neither have laboratory studies resolved whether GPER is pro-oncogenic. Several observations strongly support that it is. First, GPER is required for the survival of xenograft-derived cancer stem cells and metastatic disease (59). Second, in breast cancer cells, GPER integrates assembly of the fibronectin matrix (60) with the release of EGF (61); thus satisfying two basic requirements or cellular survival: attachment to the extracellular matrix and responsiveness to growth factors. Third, in a preclinical model, breast cancer is less aggressive when GPER is genetically inactivated (62). Finally, the GPER selective antagonist, G36, delays the growth of type II endometrial cancer in mice (63). Nevertheless, other studies have suggested that GPER is tumor suppressive (64, 65). Specifically, stimulation with GPER-selective agonist, G-1 leads to pro-apoptotic signaling, as well as decreased proliferation and migration by cancer cells. Limitations of the latter studies are that G-1 was used at a 100-fold higher concentration than its reported K_i or EC_{50} (65) and receptor knockdown strategies were not used to test for off-target effects. In addition, these studies did not determine whether the G-1 responses also occur when the endogenous estrogen, E2 is applied, or for effects of selective GPER antagonists (G15 or G36). The latter point is particularly relevant because studies reporting GPER as tumor suppressive measured

inhibitory biological responses. Other studies have reported that the GPER promoter is methylated in a small percentage of cancer biopsies (66). Then again, genetic silencing is observed for many genes in cancer specimens, and this could be explained by genomic instability. Indeed, promoter methylation of ESR-1 (ERα) is common in breast cancer (67, 68). Notably, epigenetic silencing of GPER as an anti-cancer mechanism is at odds with data in public repositories, showing that GPER is widely expressed, and rarely mutated, in solid or hematopoietic cancers and in cancer cell lines. Thus, the conclusion that GPER is "tumor suppressive" is inconsistent with the widely accepted concept that a tumor suppressor gene requires genetic inactivation or epigenetic silencing. Furthermore, the idea that GPER is anti-oncogenic does not fit well with findings which suggest an active role for GPER in cancer progression in the tumor microenvironment (21). Specifically, the hypoxic environment created by proliferating cancer cells favors increased expression of GPER and local estrogen production. Breast cancer cells and cancer-associated fibroblasts (CAFs) upregulate GPER expression *via* hypoxia-inducing factor-1α (HIF-1α)-regulated transcriptional control (69). Increased AP-1 mediated aromatase transcription and activity is measured in breast cancer cells following estradiol or tamoxifen-mediated stimulation of GPER (70). Nor does an anti-oncogenic role for GPER reconcile with bioinformatic analyses that show that its expression correlates directly with pro-metastatic signaling pathways in estrogen receptor negative breast cancer (71). Nevertheless, the discrepancy between the pro-oncogenic and tumor suppressive activities of GPER has been discussed (72) and underscores the need to define the mechanisms that drive GPER activity and their relationship to oncogenesis.

IMPLICATIONS OF GPER FOR ANTI-ESTROGEN THERAPY

ERs and GPER act independently but coordinately to maintain homeostasis of estrogen-responsive tissue. Thus, it is likely that neoplasms that arise from these tissues may either continue to direct estrogen action through both receptor types or lose control of one or both receptor mechanisms during their evolution. In fact, this is the pattern that is observed in breast cancer with treatment-naive tumors containing both receptors, one or the other receptor, or neither receptor (73). From a clinical perspective, GPER disrupts the ER-centric, binary rubric which categorizes breast cancer as either estrogen responsive or nonresponsive, with nearly, 20% of all breast cancers expressing GPER in the absence of ER. Interestingly, a preponderance of these ER-GPER+ tumors are triple negative breast cancers that lack ER, progesterone receptor (PR) and her2/neu (74).

Therapeutic interventions that reduce bioavailable estrogen should be an effective means to prevent the biological action of ERα, ERβ, and GPER. At present, three common methods are employed for reducing bioavailable estrogen: i) ovarian ablation by ovariectomy or radiation, ii) ovarian suppression by bolus administration of a gonadotrophin releasing hormone (GnRH) superagonist, such as goserelin or leuprolide, or iii) chemical inhibition by administration of aromatase inhibitors (AIs), such as exemestane, letrozole or anastrazole. Each of these three treatment interventions are used for the treatment of breast cancer. However, no single method for reducing estrogen is failproof and each of these approaches induces premature menopause, which is associated with long-term mortality risks, including increased risk of cardiovascular disease (75) and loss of bone density (76), as well as menopausal symptoms that can impact on quality of life (77). Elimination of ovarian function, either permanently by ablation or temporarily by interrupting the neuroendocrine circuit of estrogen biosynthesis, does not interfere with nonovarian biosynthesis. AIs are effective in this manner in that their effects prevent estrogen biosynthesis independent of tissue origin. While AIs effectively delay breast cancer progression in approximately 50% of breast cancer patients, their beneficial value in the remaining patients is offset by their high rate of acquired and *de novo* resistance (78). In evaluating the efficacy of blockade of estrogen biosynthesis in the context of either GPER (or ER), it is important to point out that nuclear steroid hormone receptors (SHRs) and G-protein coupled receptors (GPCRs) are allosterically regulated receptors that are capable of ligand-independent action (79, 80). Thus, inhibition of estrogen biosynthesis may not be effective for patients whose tumors contain mutant receptors that lose ligand binding activity but retain constitutive signaling. Although ligand binding mutants have not yet been defined for GPER, they have been identified for other GPCRs (81) and for ER (82).

An important concern regarding therapies that block estrogen biosynthesis is that theoretically they should effectively increase the ability of exogenous estrogens to interact with their cellular receptors. Albeit, it is not known whether or not AIs alter the interaction of exogenous estrogens with either GPER or ER, as this has not yet been tested experimentally. This idea is particularly interesting in light of the fact that although xenoestrogens show low binding affinities relative to 17β-estradiol for ER. The same is not true for GPER, as xenoestrogens show much higher relative binding affinities for GPER (**Table 1**). In order to illustrate their potential effect on anti-estrogen therapy in the context of GPER, a few of the more abundant exogenous estrogens that are relevant for this discussion are mentioned here. For example, in independent assays, the dietary soy isoflavone, daidzein (DZN) exhibits a high relative potency for GPER relative to ER, with an EC_{50} in the subnanomolar range compared with an EC50 that is more than 100- to 200-fold higher for nuclear ERs. Dietary exposure to soy is not trivial, in fact, measurements of postprandial serum concentrations of DZN can exceed preovulatory levels of E2 by 10-fold (83). Adding further complexity to the influence of phytoestrogens on breast cancer is the popular belief that a soy-rich diet is breast cancer protective (84). Epidemiological studies have placed emphasis on whether metabolism of DZN to S-equol, which is exclusively mediated by the gut microbiome is a critical factor in influencing estrogen physiology and ER-targeted therapy (85). This concept is interesting in light of the finding

that Eastern women, whom show a two-fold reduced risk for developing breast cancer relative to Western women are twice as likely to harbor gut bacteria that metabolize DZN to S-equol (86). However, the oncogenic activity of DZN and S-equol is unclear as DZN exerts pro- and anti-oncogenic activity in mice, while other studies suggest that S-equol is anti-oncogenic (84). The influence of dietary estrogens on estrogen-targeted therapies is controversial (87). A recent guidance statement from the American Association of Clinical Endocrinologists (AACE) suggests that a soy-rich diet may be used as an alternative approach for estrogen replacement therapy (88) indicating that endogenous estrogens and phytoestrogens are biologically equivalent. Yet, an oft quoted study of 524 postmenopausal Chinese women with breast cancer showed improved survival and less recurrence in patients with the highest quartile of soy intake relative to counterparts in the lowest quartile of soy consumption (89). Significantly, this study showed a significant risk increase for patients receiving tamoxifen compared to those that received anastrozole. These data have been interpreted to indicate that soy may act competitively to block binding of tamoxifen to ER. Alternatively, these findings may suggest that the poorer survival observed in the tamoxifen arm of the study may be due to the fact that tamoxifen and soy isoflavones function as GPER agonists. Moreover, the Kang study did not control for obesity nor bacterial metabolism of DZN. Nonetheless, in humans avoidance of dietary soy or ingestion of DZN supplements by breast cancer patients receiving estrogen targeted therapy is encouraged (90) despite the fact that the RBA of DZN is 0.003% for ERα and 0.05% for Erβ (91). The question of whether soy isoflavones show enhanced carcinogenicity in the absence of endogenous estrogen has not yet been carefully addressed. Human and mouse studies which control for phytoestrogen intake, gut metabolome, and obesity in the presence or absence of AIs are necessary to evaluate the carcinogenicity of soy isoflavones in the face of AI therapy.

GPER also provides similar concerns regarding the carcinogenicity of the plasticizer, bisphenol A (BPA), the highest volume chemical produced world-wide (92). Human exposure to BPA is significant as >90% of the US population contains measurable amounts of BPA, with highest levels in children (93). BPA exhibits an RBA for GPER that is 100-fold greater than that measured for nuclear ERs (**Table 1**). In vitro studies indicate that BPA potency for GPER is high, with biological effects measured in the low nanomolar range in breast cancer cells and breast cancer-associated fibroblasts (60, 94) and in human seminoma and testicular cancer cells (95). Exposure to BPA is associated with many human diseases, including obesity, diabetes and cancer, and is able to induce toxicological effects in tissues and cultured cells (96). The Environmental Protection Agency and the Food and Drug Administration agree upon a safe reference dose (RfD) for BPA in humans at 50 μg/kg/day that was scaled from toxicology studies in rodents (97). Carcinogenicity testing at doses below and above the RfD in mice has yielded mixed results. While BPA is not considered a robust carcinogen, early life exposures in rodents at the RfD is associated with prostate and breast cancer (98). These authors duly underscore that the most vexing variable in the analysis of BPA carcinogenicity is the acknowledged error of scaling RfD between man and rodent due to the fact that BPA exhibits nonmonotonic dose responses in many biochemical and biological assays (99). Even more significant with regards to GPER, urinary concentrations of BPA in participants in the National Health and Nutrition Examination Survey (NHANES) demonstrated a positive association with metabolic syndrome (100). Moreover, exposure to BPA correlates with an increase in serum SHBG, even though BPA shows poor binding affinity for SHBG (12). Thus, theoretically, for a patient receiving AIs, BPA is a particularly potent GPER agonist. However, this has yet-to-be addressed in studies in which dietary estrogen intake, obesity, and gut metabolome are carefully controlled. Nonetheless, BPA is a particularly troubling environmental estrogen due to the fact that it is a malleable chemical structure that has been manipulated by chemists to produced more than 40 analogues. Many of these BPA similar are detected in humans at even higher concentrations than BPA (93, 101), and at least seven BPA analogues exhibit similar RBAs and relative potencies for GPER in breast cancer cells (35).

ER antagonism, using a selective estrogen receptor modulator (SERM), such as tamoxifen or a selective estrogen receptor degrader (SERD), such as fulvestrant, is yet another form of anti-estrogen therapy that is widely effective in the treatment of breast cancer, providing greater than 10 year survival in postmenopausal women with early stage, ER-positive cancer (102). Still, not all of these patients respond to ER antagonists, as *de novo* resistance occurs, and this may be due to many reasons, including: i) the presence of constitutively active ER mutants, ii) hyperactive growth factor signaling, or iii) the presence of an alternative estrogen receptor, i.e. GPER (103). GPER adds further complexity to anti-estrogen therapy in that ER antagonists, including tamoxifen, faslodex and raloxifene function as GPER agonists (21, 29). Furthermore, ER antagonism or AIs are not effective for postmenopausal women with late stage disease or for premenopausal women (104). Consistent with this idea, results from the SOFT (Suppression of Ovarian Function Trial) suggest that even further supplementation of estrogen-targeted therapy (Tamoxifen or AI) by adding ovarian suppression for premenopausal ER-positive breast cancer, while effective in reducing serum estrogen and disease relapse had no effect on overall survival (78). In this study, patients were not further stratified by whether their tumors expressed GPER. However, an argument could be made that patients whose tumors lacked GPER [approximately one-third of ER+ tumors (73)] may be more likely to respond to ER antagonism plus ovarian suppression. Further confusion regarding the role of estrogen and its receptors in female reproductive cancer comes from the disconnect between menopausal status and proliferative index, as measured by Ki-67 in tumor biopsy tissue. Breast tumors from patients with intact ovaries, show high mitotic indices, while postmenopausal women with ER-positive breast cancer are assigned either anti-estrogen therapy regardless of Ki-67 index (105). Chemotherapeutic agents, which are toxic but target rapidly proliferating cells are layered on top of anti-estrogen therapy for patients with aggressive estrogen-dependent cancers

(106), without consideration of their GPER status, which has been tied to chemotherapeutic resistance *via* its capacity to trigger EGFR transactivation (107). Recent results from the PALOMA-III trials, further showed that addition of palbociclib, which targets cyclin-dependent kinases, CDK4 and CDK6, to ER-targeted therapy (fulvestrant) provides increased overall survival for patients with advanced ER-positive breast cancer (108). Early results achieved with palbociclib in metastatic breast cancer are encouraging. Yet they do not resolve whether palbociclib selectively targets proliferation in fulvestrant-resistant, ER-positive breast cancer cells, or whether its actions directly influence GPER-dependent cellular responses associated with tumor cell metastasis and disease progression. Collectively, these examples indicate that definition of GPER status for patients with breast cancer may help to select patient populations which are best able to respond to existing anti-estrogen therapies, either ovarian suppression, ER antagonism or aromatase inhibitor.

EXISTING AND FUTURE PHARMACOLOGICAL COMPOUNDS THAT TARGET GPER

For all of the above reasons, therapeutic approaches that block GPER action hold great promise for the treatment of cancer. After all, nearly one-third of all FDA-approved drugs target GPCRs (109). While GPCR targeted drugs have been predominately used for the treatment of cardiovascular disease and diabetes, the concept of developing GPCR targeted cancer therapeutics has gained traction over the past decade (110). This is largely due to preclinical studies which link GPCRs to cancer growth and metastasis, often in a scenario where the GPCR involved is chronically exposed to local or circulating agonist. Examples of this include, the bioactive lipid, lysophosphatidic acid, and its receptor, LPAR-1 in breast cancer (111), chemokines, CXCL8/IL8 and CXCR1 and CXCR2 in melanoma, pancreatic cancer and gastric tumors (112) and CXCL12 and CXCR4 in multiple cancers (113). Consistent with the notion that chronic estrogen exposure may drive GPER oncogenesis, breast tumors with increased GPER plasma membrane density show poor prognosis (51). This may be consistent with the concept that GPCRs often demonstrate a hyperbolic relationship between ligand occupancy and receptor response (114). This is widely described as "fractional occupancy" and suggests that a small change in GPER plasma receptor density could result in a more than linear increase in GPER activity. It is also important to consider that GPER shows specific binding activity to estrogenic ligands, natural or synthetic, which are hydrophobic and/or lipophilic and easily diffuse through or insert themselves into a lipid bilayer. In fact, it has previously published that crude membrane fractions exhibit specific GPER binding activity (Thomas et al, 2005). Whether intracellular interaction between GPER and its ligands allows for sustained intracellular signaling or plays a role in the proper folding and transport of GPER to the plasma membrane has not yet been determined. In this regard, it is important to recognize that an intracellular staining pattern is observed in most, but not all, cell types (115). However, a plasma membrane staining pattern by immunohistochemical (IHC) analysis of microtome-sectioned, archival paraffin-embedded tissue is not easily detected unless the majority of the receptor is at the plasma membrane, and little is detected intracellularly. With this in mind, slight differences in GPER ligand sensitivity would be difficult to detect by IHC, however, measurement of GPER plasma membrane density by flow cytometric analysis of intact breast cancer cells (116) may provide a better handle as whether to apply anti-estrogen therapy in the context of GPER-targeted therapies described below.

Small Molecule GPER Antagonists

Several GPER antagonists have been developed (**Table 2**). While many of these first-generation drugs hold promise, we review below two GPER antagonists with half-maximal inhibitory concentration (IC_{50}) within the nanomolar range. The first GPER antagonist in this class, named G15, was developed by Prossnitz and colleagues using a combination of virtual and biomolecular screening steps (117). First these authors used a software-assisted virtual screen of the NIH Molecular Libraries Small Molecule Repository (MLSMR) of 144, 457 molecules. From this primary screen 57 compounds were isolated that were similar in structure to the GPER selective agonist, G-1, a substituted dihydroquinolone (24). These compounds were tested subsequently for their capacity to inhibit E2-mediated calcium mobilization in human SKBR3 breast cancer cells that express endogenous GPER but lack ERα and ERβ. G15 emerged from this screen based on its: i) structure and presumed ability to interact competitively with E2, ii) ability to block E2-dependent calcium signaling, and iii) measured binding affinity (K_d = 20 nM) for GPER, which was assessed using I^{125}-labelled G-1 as radiotracer. G-15 displays relatively low binding affinity for ERα and ERβ as measured in a competition assay employing an Alexa 633-estradiol conjugate as fluorotracer (K_i > 10 nM). *In vivo* testing has shown that G15 blocks a proliferative response in uterine epithelial cells (117). A G15 derivative, named G36 was subsequently synthesized by Dennis and Prossnitz, with even

TABLE 2 | IC50 for GPER antagonists.

Ligand	Affinity	Reference
	IC_{50} (nM)	
G15	[a]190	(117)
	[b]185	
G36	[a]112	(118)
	[b]165	
CIMBA	[c]60-90	(119)
MIBE	[c]1,750	(120)
PBX1	[c]250	(121)
PBX2	[c]300	(121)
C4PY	[c]900	(122)
CPT	5,000	(123)

[a,b,c] *IC_{50} was measured by competition binding assay to GPER between antagonist and fluorescent estrogen, iodinated G1 analog and [^{3}H] E_2, respectively.*

lower affinity interactions with ERα (118). G36 inhibits E2 and G-1-dependent calcium mobilization as well as erk-1/2 activation in SKBR3 cells (IC_{50} = 200 nM) and blocks the growth of transplanted estrogen-dependent type II endometrial cancer cells (63). Recently, Chris Arnatt and David Wang have collaborated to report a new GPER antagonist that protects ovariectomized ERα null mice from estrogen-induced cholesterol gallstones (119). Using a receptor-ligand interaction computational screen, a novel series of GPER-selective antagonists were generated, including one new compound, 2-cyclohexyl-4-isopropyl-N-(4-methoxybenzyl) aniline (CIMBA). that shows strong antagonism with selectivity for GPER. Specifically, CIMBA inhibits G-1 dependent calcium mobilization in HL60 cells (IC_{50} = 75 nM), with a binding activity for ERα or ERβ <10 μM in fluorescence polarization assays. Some differences were noted by Arnatt and colleagues with regards to the efficacy of G15, G36 and CIMBA to inhibit calcium mobilization, although all three GPER antagonists each showed inhibitory capacity for G-1 induced cAMP accumulation by homogenous time resolved fluorescence (HTRF). Thus, while the computational algorithms that yielded the G-series based and methoxybenzyl aniline based GPER antagonists were inherently distinct, both show similar capacity to inhibit G-1 induced GPER signaling, with each showing efficacy for reducing estrogen-induced pathology in mice.

Targeting G Proteins

An alternative approach to developing selective agents that block GPER action is to employ pharmaceutical compounds that directly target G proteins (124, 125). This strategy has the added benefit that although GPER is a driving force in the genesis of metabolic disorder and cancer, these are complex diseases in which multiple GPCRs are involved. Primary examples include chemokine receptors (CXCR1, CXCR2, CXCR4, CCR5, CCR7) that drive chronic inflammatory responses common to both obesity and cancer. The premise by which G protein blockade is effective as a therapeutic is the ability of these agents to preferentially inhibit signaling pathways shared by more than one GPCR. Towards this end, cell permeant pharmacological agents have been developed that interfere with conformational activation of the GPCR-Gαβγ complex following ligand binding. To date, pharmacological compounds that specifically inhibit Gα-GTPase have been limited to the Gαq proteins and include YM-254890 (126) and FR900359 (127). Gαq inhibitors show good preclinical success in thrombosis (128), asthma (129) and melanoma (130). In contrast, Gβγ inhibitors, which were initially based upon the carboxyl terminal domain structure of G-protein receptor kinase 2 but now also include M119 and gallein, show efficacy in preclinical models of opioid analgesia, chronic inflammatory disease, heart failure (124). Blockade of GPER-dependent EGFR transactivation in breast cancer cells is effective using a Gβγ-sequestrant peptide (131), and further study is needed to evaluate whether Gβγ-inhibitors are effective in mouse models of metabolic disorder and cancer.

"Biased" agonists that stabilize a GPCR conformation that preferentially activates one signaling pathway over another (132) represents a related approach towards selective inhibition of G protein dependent signaling. Oliceridine, a biased agonist for μ-opioid receptor was developed to favor Gαi-inhibition of adenylyl cyclase over Gβγ-dependent activation of β-arrestin (133) and has been evaluated in clinical trials for chronic pain. Although recent reports indicate that low agonist efficacy, rather than receptor bias, may explain the low side effect profile of oliceridine (134). Similarly, biased agonists have been developed and characterized for angiotensin I receptors that preferentially recruit β-arrestin for their potential use in reducing hypertension (135). Biased agonists have yet to make their way into the clinic. However, it is unclear at the moment whether the biased agonist conformation is unique to certain GPCRs or whether it has broad application. Still, our environment is replete with compounds that function as estrogen mimetics, and it may be possible by high throughput analysis of synthetic and nutraceutical compounds to identify biased GPER agonists that may have therapeutic value.

Targeting Downstream Signaling Effectors of GPER

Via GPER, estrogens trigger an epidermal growth factor (EGF)-autocrine loop (22) that holds significance for breast carcinoma, and potentially other malignancies that arise from epithelial tissue. In breast cancer this holds particular significance due to the reciprocal relationship that is often observed between ER and epidermal growth factor receptors (EGFRs) in primary tumors. This relationship has fostered the dichotomous categorization of breast cancers as either estrogen responsive or growth factor responsive. While GPER disrupts this simple binary scheme, GPER holds potential diagnostic value in selecting patients that may best benefit from either erbB1 or erbB2/her2/neu targeted therapy, particularly among premenopausal women. Assessment of GPER expression also may suggest the appropriate combinatorial assignment of AI or GPER antagonist with EGFR targeted antibody treatment. As discussed in section 3, GPER is expressed in a majority of TNBC, an aggressive subtype of breast cancer with no known molecular targets. erbB1/EGFR is also commonly overexpressed in TNBC, although results from numerous clinical trials reveal low response rates to anti-EGFR therapy for patients with TNBC (136). However, some patients do respond well, which may suggest a need to stratify patients for EGFR responsiveness and to develop combinatorial therapies. In both regards, GPER may have value. First, as a theranostic index. Second, GPER targeted therapeutics may fit well as part of a combinatorial anti-EGFR therapy for patients with erbB1 overexpressing TNBC.

Phosphoinositide 3 (PI3) kinase/AKT signaling lies downstream of erbB1/erbB2, and is activated following GPER stimulation (137). Activation of PI3K/AKT signaling occurs commonly in breast cancer and is associated with endocrine resistance and worse prognosis (138). Pan-PI3K inhibitors have fared poorly in clinical trials due to their toxicity, while the isoform-specific PI3K inhibitor, alpelisib, has been approved by the FDA as co-therapy with fulvestrant for patients with ER-positive, PI3Kalpha mutated advanced breast cancer (139). FDA

approval of alpelisib with fulveestrant followed the results of the SOLAR-1 trial that showed that patients receiving alpelisib with fulvestrant showed a median increase of 6 months of progression free survival. Future studies that include a more comprehensive view of patients which are estrogen responsive by including analysis of GPER, may lead to similarly designed clinical trials that combine either AIs or GPER targeted therapy with alpelisib.

Antibodies

Traditionally, small molecules have dominated as the preferred means to target GPCRs but recent pharmaceutical trends that favor immunotherapeutic approaches have led to the development of GPCR-targeted antibodies for clinical use (**Table 3**). The most significant progress has been made in the development of antibodies that block the binding of chemokines to their cognate GPCRs in cancer and inflammatory disease (144, 145). Notably, mogamulizumab/Poteligeo, an anti-CCR4 targeted therapy for refractory adult T cell leukemia and mycosis fungoides has received FDA approval (146). Likewise, the FDA has also approved erenumab/Aimovig, targeting Calcitonin Gene-Related Peptide Receptor (CGRPR) as a prophylactic treatment for migraine headaches (141). In addition, the angiogenesis/tumor metastasis-associated receptor, CXCR4, targeted by ulocuplumab (Bristol Myers Squibb), a fully humanized antibody that blocks binding of stromal-derived factor 1 (SDF-1) in adult myeloid leukemia has entered phase II trials (143). The CCR5-targeted antibody, leronlimab is currently under phase III investigation as an HIV therapy and has entered phase II testing to relieve chronic lung inflammation that accompanies COVID 19 infection (142). CCR2 targeted mAB, MLN1202/plozalizumab (Millenium/Takeda Oncology) has been evaluated in multiple clinical trials for cancer and other indications (147).

Once considered difficult to target *via* antibody-based approaches, the combined use of lipid-enriched GPCR preparations and the development of recombinant phage display technology has allowed for the rapid growth and development of antibodies that target GPCRs. The fact that GPCR heterodimerization is a widely accepted paradigm that adds diversity and complexity to GPCR functionality is an additional reason why antibody-based therapeutic approaches have gained traction relative to small molecule antagonists.

Antibodies that target GPCRs could also be used to deliver anti-cancer agents by conjugating the antibodies to nanoparticles (148). Such nanoparticles can be formed from biodegradable polymers and can physically entrap the anti-cancer agent throughout the nanoparticle (149). Through diffusion and degradation of the polymer, the drug can be released in a controlled manner to the target cancer (149). Polymers used to prepare these particles include but are not limited to poly lactic-co-glycolic acid (149), polysulfenamides (150), and polyanhydrides (151). Agents that can be loaded into the particles include proteins such as cancer antigens (152), nucleic acid based molecules like plasmid DNA (153) and CpG (154) and small molecule drugs like paclitaxel and doxorubicin (149, 155).

CONCLUSIONS

Anti-estrogen therapies are successfully employed for the treatment of breast cancer and anovulatory infertility. Still, at present, decisions regarding the appropriate assignment of anti-estrogen therapy in breast cancer are limited strictly upon the detection of ER in tumor biopsy specimens. This ER-centric perspective ignores the fact that 20% of breast cancers express GPER and in the absence of ER (73), and that GPER is expressed in a majority of TNBCs (74). Despite the relative success of ER antagonists, aromatase inhibitors and ovarian ablation/suppression strategies for postmenopausal women with early stage ER- positive cancer, resistance occurs. A further confounding variable for the assignment of anti-estrogen therapy is the fact that ER antagonists (both SERMS and SERDs) function as GPER agonists, which aligns with the finding that GPER is associated with tamoxifen resistance in breast cancer patients (103). The realization that daidzein (156) and environmental bisphenols (35) potently activate GPER further alters our perspective regarding the appropriate assignment of anti-estrogen therapy. In addition, recent clinical trials evaluating AI or TAM with ovarian suppression have shown a median increase in progression free survival suggesting that some patients may respond favorably to tandem anti-estrogen blockade. However, these studies did not include patients whose tumors are GPER- positive and ER-negative. Our current perspective for determining which patients may respond to anti-estrogen therapy is evolving, and is bolstered by findings that show that GPER associates with cancer progression variables (48, 52, 53), activates cellular receptors that facilitate cancer cell survival (54), promotes the survival of patient-derived breast cancer stem cells (59), and acts in the tumor microenvironment to drive cancer metastasis (62).

The development of GPER targeted therapies holds the promise of expanding our existing arsenal of estrogen-targeted

TABLE 3 | Status of GPCR therapeutic antibodies.

GPCR	Drug name	Brand name	Status	Indication	References
CCR4	mogamulizumab	Poteligeo	Approved, 2018	mycosis fungoides Sezary syndrome	(140)
CGRPR	erenumab	Aimovig	Approved, 2018	migraine prophylaxis	(141)
CCR5	leronlimab		Phase III Phase II	HIV COVID-19	(142)
CXCR4	ulocuplumab		Phase II	multiple myeloma	(143)
CCR2	plozalizumab		Investigational	diabetic nephropathy	

therapies. GPER is a therapeutic target that holds particular promise for the treatment of several critical health concerns facing Western society, including obesity, diabetes, vascular pathology and advanced cancer. In the preclinical setting, chronic administration of G1/Tespria restores fat, lipid, and glucose homeostasis in obese and diabetic mice without uterotropic effects (44). Analysis of human cancer and in mice suggest that GPER is linked to advanced cancer, and chronic estrogen exposure and metabolic syndrome are independent risk factors for many cancers. Thus, GPER provides a likely mechanism by which metabolic disorder may be part of the landscape for estrogen-driven malignancies. The selective GPER antagonists, G15 delays the growth of endometrial cancer (63) and exciting new data indicates that a new GPER antagonist, CIMBA, can prevent estrogen-induced gallstones (119). Additional methodologies for targeting GPER may also include direct blockade of G-proteins, the development of biased agonists and therapeutic antibodies. Collectively, these approaches may complement existing anti-estrogen therapies and improve our approach towards treating patients suffering from estrogen-driven malignancies and disease.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Carreau S, Silandre D, Bourguiba S, Hamden K, Said L, Lambard S, et al. Estrogens and male reproduction: a new concept. *Braz J Med Biol Res* (2007) 40(6):761–8. doi: 10.1590/S0100-879X2007000600003
2. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during aging: from periphery to brain. *Trends Mol Med* (2013) 19(3):197–209. doi: 10.1016/j.molmed.2012.12.007
3. Farzaneh S, Zarghi A. Estrogen Receptor Ligands: A Review (2013-2015). *Sci Pharm* (2016) 84(3):409–27. doi: 10.3390/scipharm84030409
4. Lee AJ, Cai MX, Thomas PE, Conney AH, Zhu BT. Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome P450 isoforms. *Endocrinology* (2003) 144 (8):3382–98. doi: 10.1210/en.2003-0192
5. Chatuphonprasert W, Jarukamjorn K, Ellinger I. Physiology and Pathophysiology of Steroid Biosynthesis, Transport and Metabolism in the Human Placenta. *Front Pharmacol* (2018) 9:1027–55. doi: 10.3389/fphar.2018.01027
6. Berkane N, Liere P, Oudinet J-P, Hertig A, Lefèvre G, Pluchino N, et al. From pregnancy to preeclampsia: a key role for estrogens. *Endocr Rev* (2017) 38(2):123–44. doi: 10.1210/er.2016-1065
7. Knochenhauer ES, Boots LR, Potter HD, Azziz R. Differential binding of estradiol and testosterone to SHBG. Relation to circulating estradiol levels. *J Reprod Med* (1998) 43(8):665–70.
8. Balogh A, Karpati E, Schneider AE, Hetey S, Szilagyi A, Juhasz K, et al. Sex hormone-binding globulin provides a novel entry pathway for estradiol and influences subsequent signaling in lymphocytes via membrane receptor. *Sci Rep* (2019) 9(1):4. doi: 10.1038/s41598-018-36882-3
9. Hammond GL. Diverse roles for sex hormone-binding globulin in reproduction. *Biol Reprod* (2011) 85(3):431–41. doi: 10.1095/biolreprod.111.092593
10. Smiley DA, Khalil RA. Estrogenic compounds, estrogen receptors and vascular cell signaling in the aging blood vessels. *Curr Med Chem* (2009) 16(15):1863–87. doi: 10.2174/092986709788186093
11. Round P, Das S, Wu T-S, Wähälä K, Van Petegem F, Hammond GL. Molecular interactions between sex hormone–binding globulin and nonsteroidal ligands that enhance androgen activity. *J Biol Chem* (2020) 295(5):1202–11. doi: 10.1074/jbc.RA119.011051
12. Xiong Q, Liu X, Shen Y, Yu P, Chen S, Hu J, et al. Elevated serum Bisphenol A level in patients with dilated cardiomyopathy. *Int J Environ Res Public Health* (2015) 12(5):5329–37. doi: 10.3390/ijerph120505329
13. Saez-Lopez C, Brianso-Llort L, Torres-Torronteras J, Simo R, Hammond GL, Selva DM. Resveratrol Increases Hepatic SHBG Expression through Human Constitutive Androstane Receptor: a new Contribution to the French Paradox. *Sci Rep* (2017) 7(1):12284. doi: 10.1038/s41598-017-12509-x
14. Maggio M, Lauretani F, Basaria S, Ceda GP, Bandinelli S, Metter EJ, et al. Sex hormone binding globulin levels across the adult lifespan in women–the role of body mass index and fasting insulin. *J Endocrinol Invest* (2008) 31(7):597–601. doi: 10.1007/BF03345608
15. Samavat H, Kurzer MS. Estrogen metabolism and breast cancer. *Cancer Lett* (2015) 356(2 Pt A):231–43. doi: 10.1016/j.canlet.2014.04.018
16. Reed M, Purohit A, Woo LL, Newman SP, Potter BV. Steroid sulfatase: molecular biology, regulation, and inhibition. *Endocr Rev* (2005) 26(2):171–202. doi: 10.1210/er.2004-0003
17. Norman AW, Mizwicki MT, Norman DP. Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model. *Nat Rev Drug Discov* (2004) 3(1):27–41. doi: 10.1038/nrd1283
18. Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A* (2003) 100(5):2231–6. doi: 10.1073/pnas.0336132100
19. Zhu Y, Bond J, Thomas P. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proc Natl Acad Sci U S A* (2003) 100 (5):2237–42. doi: 10.1073/pnas.0436133100
20. Heinlein CA, Chang C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Mol Endocrinol* (2002) 16 (10):2181–7. doi: 10.1210/me.2002-0070
21. Filardo EJ. A role for G-protein coupled estrogen receptor (GPER) in estrogen-induced carcinogenesis: Dysregulated glandular homeostasis, survival and metastasis. *J Steroid Biochem Mol Biol* (2018) 176:38–48. doi: 10.1016/j.jsbmb.2017.05.005
22. Filardo EJ, Thomas P. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends Endocrinol Metab* (2005) 16 (8):362–7. doi: 10.1016/j.tem.2005.08.005
23. Levin ER, Hammes SR. Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors. *Nat Rev Mol Cell Biol* (2016) 17(12):783–97. doi: 10.1038/nrm.2016.122
24. Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, et al. Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* (2006) 2(4):207–12. doi: 10.1038/nchembio775

25. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* (1997) 138(3):863–70. doi: 10.1210/endo.138.3.4979
26. Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* (1998) 139(10):4252–63. doi: 10.1210/endo.139.10.6216
27. Muthyala RS, Ju YH, Sheng S, Williams LD, Doerge DR, Katzenellenbogen BS, et al. Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. *Bioorg Med Chem* (2004) 12(6):1559–67. doi: 10.1016/j.bmc.2003.11.035
28. Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, et al. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol Sci* (2000) 54 (1):138–53. doi: 10.1093/toxsci/54.1.138
29. Prossnitz ER, Arterburn JB. International Union of Basic and Clinical Pharmacology. XCVII. G Protein-Coupled Estrogen Receptor and Its Pharmacologic Modulators. *Pharmacol Rev* (2015) 67(3):505–40. doi: 10.1124/pr.114.009712
30. Filardo EJ, Thomas P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal and vascular physiology. *Endocrinology* (2012) 153(7):2953–62. doi: 10.1210/en.2012-1061
31. Matias PM, Donner P, Coelho R, Thomaz M, Peixoto C, Macedo S, et al. Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *J Biol Chem* (2000) 275(34):26164–71. doi: 10.1074/jbc.M004571200
32. Tanenbaum DM, Wang Y, Williams SP, Sigler PB. Crystallographic comparison of the estrogen and progesterone receptor's ligand binding domains. *Proc Natl Acad Sci U S A* (1998) 95(11):5998–6003. doi: 10.1073/pnas.95.11.5998
33. Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, et al. Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* (1990) 249(4965):157–60. doi: 10.1126/science.2115209
34. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* (1997) 389(6652):753–8. doi: 10.1038/39645
35. Cao LY, Ren XM, Li CH, Zhang J, Qin WP, Yang Y, et al. and Bisphenol B Exert Higher Estrogenic Effects than Bisphenol A via G Protein-Coupled Estrogen Receptor Pathway. *Environ Sci Technol* (2017) 51(19):11423–30. doi: 10.1021/acs.est.7b03336
36. Mendez-Luna D, Martinez-Archundia M, Maroun RC, Ceballos-Reyes G, Fragoso-Vazquez MJ, Gonzalez-Juarez DE, et al. Deciphering the GPER/GPR30-agonist and antagonists interactions using molecular modeling studies, molecular dynamics, and docking simulations. *J Biomol Struct Dyn* (2015) 33(10):2161–72. doi: 10.1080/07391102.2014.994102
37. Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic animals. *Annu Rev Physiol* (2005) 67:285–308. doi: 10.1146/annurev.physiol.67.040403.115914
38. Hewitt SC, Korach KS. Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction* (2003) 125(2):143–9. doi: 10.1530/rep.0.1250143
39. Sharma G, Hu C, Brigman JL, Zhu G, Hathaway HJ, Prossnitz ER. GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. *Endocrinology* (2013) 154(11):4136–45. doi: 10.1210/en.2013-1357
40. Walker VR, Korach KS. Estrogen receptor knockout mice as a model for endocrine research. *ILAR J* (2004) 45(4):455–61. doi: 10.1093/ilar.45.4.455
41. Prossnitz ER, Hathaway HJ. What have we learned about GPER function in physiology and disease from knockout mice? *J Steroid Biochem Mol Biol* (2015) 153:114–26. doi: 10.1016/j.jsbmb.2015.06.014
42. Hevener AL, Clegg DJ, Mauvais-Jarvis F. Impaired estrogen receptor action in the pathogenesis of the metabolic syndrome. *Mol Cell Endocrinol* (2015) 418 Pt 3:306–21. doi: 10.1016/j.mce.2015.05.020
43. Sharma G, Mauvais-Jarvis F, Prossnitz ER. Roles of G protein-coupled estrogen receptor GPER in metabolic regulation. *J Steroid Biochem Mol Biol* (2018) 176:31–7. doi: 10.1016/j.jsbmb.2017.02.012
44. Sharma G, Hu C, Staquicini DI, Brigman JL, Liu M, Mauvais-Jarvis F, et al. Preclinical efficacy of the GPER-selective agonist G-1 in mouse models of obesity and diabetes. *Sci Transl Med* (2020) 12:528–41. doi: 10.1126/scitranslmed.aau5956
45. Meyer MR, Fredette NC, Howard TA, Hu C, Ramesh C, Daniel C, et al. G protein-coupled estrogen receptor protects from atherosclerosis. *Sci Rep* (2014) 4:7564. doi: 10.1038/srep07564
46. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol* (2011) 7(12):715–26. doi: 10.1038/nrendo.2011.122
47. Sharma G, Prossnitz ER. G-Protein-Coupled Estrogen Receptor (GPER) and Sex-Specific Metabolic Homeostasis. *Adv Exp Med Biol* (2017) 1043:427–53. doi: 10.1007/978-3-319-70178-3_20
48. Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, et al. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* (2006) 12(21):6359–66. doi: 10.1158/1078-0432.CCR-06-0860
49. Arias-Pulido H, Royce M, Gong Y, Joste N, Lomo L, Lee SJ, et al. GPR30 and estrogen receptor expression: new insights into hormone dependence of inflammatory breast cancer. *Breast Cancer Res Treat* (2010) 123(1):51–8. doi: 10.1007/s10549-009-0631-7
50. Ignatov T, Claus M, Nass N, Haybaeck J, Seifert B, Kalinski T, et al. G-protein-coupled estrogen receptor GPER-1 expression in hormone receptor-positive breast cancer is associated with poor benefit of tamoxifen. *Breast Cancer Res Treat* (2019) 174(1):121–7. doi: 10.1007/s10549-018-5064-8
51. Sjostrom M, Hartman L, Grabau D, Fornander T, Malmstrom P, Nordenskjold B, et al. Lack of G protein-coupled estrogen receptor (GPER) in the plasma membrane is associated with excellent long-term prognosis in breast cancer. *Breast Cancer Res Treat* (2014) 145(1):61–71. doi: 10.1007/s10549-014-2936-4
52. Smith HO, Arias-Pulido H, Kuo DY, Howard T, Qualls CR, Lee SJ, et al. GPR30 predicts poor survival for ovarian cancer. *Gynecol Oncol* (2009) 114 (3):465–71. doi: 10.1016/j.ygyno.2009.05.015
53. Smith HO, Leslie KK, Singh M, Qualls CR, Revankar CM, Joste NE, et al. GPR30: a novel indicator of poor survival for endometrial carcinoma. *Am J Obstet Gynecol* (2007) 196(4):386 e1–9; discussion e9-11. doi: 10.1016/j.ajog.2007.01.004
54. Rago V, Romeo F, Giordano F, Maggiolini M, Carpino A. Identification of the estrogen receptor GPER in neoplastic and non-neoplastic human testes. *Reprod Biol Endocrinol* (2011) 9:135. doi: 10.1186/1477-7827-9-135
55. Chen ZJ, Wei W, Jiang GM, Liu H, Wei WD, Yang X, et al. Activation of GPER suppresses epithelial mesenchymal transition of triple negative breast cancer cells via NF-kappaB signals. *Mol Oncol* (2016) 10(6):775–88. doi: 10.1016/j.molonc.2016.01.002
56. Rochefort H, Platet N, Hayashido Y, Derocq D, Lucas A, Cunat S, et al. Estrogen receptor mediated inhibition of cancer cell invasion and motility: an overview. *J Steroid Biochem Mol Biol* (1998) 65(1–6):163–8. doi: 10.1016/s0960-0760(98)00010-7
57. Liang S, Chen Z, Jiang G, Zhou Y, Liu Q, Su Q, et al. Activation of GPER suppresses migration and angiogenesis of triple negative breast cancer via inhibition of NF-kappaB/IL-6 signals. *Cancer Lett* (2017) 386:12–23. doi: 10.1016/j.canlet.2016.11.003
58. Luo H, Yang G, Yu T, Luo S, Wu C, Sun Y, et al. GPER-mediated proliferation and estradiol production in breast cancer-associated fibroblasts. *Endocr Relat Cancer* (2014) 21(2):355–69. doi: 10.1530/ERC-13-0237
59. Chan YT, Lai AC, Lin RJ, Wang YH, Wang YT, Chang WW, et al. GPER-induced signaling is essential for the survival of breast cancer stem cells. *Int J Cancer* (2020) 146(6):1674–85. doi: 10.1002/ijc.32588
60. Magruder HT, Quinn JA, Schwartzbauer JE, Reichner J, Huang A, Filardo EJ. The G protein-coupled estrogen receptor-1, GPER-1, promotes fibrillogenesis via a Shc-dependent pathway resulting in anchorage-independent growth. *Horm Cancer* (2014) 5(6):390–404. doi: 10.1007/s12672-014-0195-9

61. Quinn JA, Graeber CT, Frackelton ARJr., Kim M, Schwarzbauer JE, Filardo EJ. Coordinate regulation of estrogen-mediated fibronectin matrix assembly and epidermal growth factor receptor transactivation by the G protein-coupled receptor, GPR30. *Mol Endocrinol* (2009) 23(7):1052–64. doi: 10.1210/me.2008-0262
62. Marjon NA, Hu C, Hathaway HJ, Prossnitz ER. G protein-coupled estrogen receptor regulates mammary tumorigenesis and metastasis. *Mol Cancer Res* (2014) 12(11):1644–54. doi: 10.1158/1541-7786.MCR-14-0128-T
63. Petrie WK, Dennis MK, Hu C, Dai D, Arterburn JB, Smith HO, et al. G protein-coupled estrogen receptor-selective ligands modulate endometrial tumor growth. *Obstet Gynecol Int* (2013) 2013:472720. doi: 10.1155/2013/472720
64. Weissenborn C, Ignatov T, Ochel HJ, Costa SD, Zenclussen AC, Ignatova Z, et al. GPER functions as a tumor suppressor in triple-negative breast cancer cells. *J Cancer Res Clin Oncol* (2014) 140(5):713–23. doi: 10.1007/s00432-014-1620-8
65. Ignatov T, Modl S, Thulig M, Weissenborn C, Treeck O, Ortmann O, et al. GPER-1 acts as a tumor suppressor in ovarian cancer. *J Ovarian Res* (2013) 6 (1):51. doi: 10.1186/1757-2215-6-51
66. Weissenborn C, Ignatov T, Nass N, Kalinski T, Dan Costa S, Zenclussen AC, et al. GPER Promoter Methylation Controls GPER Expression in Breast Cancer Patients. *Cancer Invest* (2017) 35(2):100–7. doi: 10.1080/07357907.2016.1271886
67. Gaudet MM, Campan M, Figueroa JD, Yang XR, Lissowska J, Peplonska B, et al. DNA hypermethylation of ESR1 and PGR in breast cancer: pathologic and epidemiologic associations. *Cancer Epidemiol Biomarkers Prev* (2009) 18 (11):3036–43. doi: 10.1158/1055-9965.EPI-09-0678
68. Stone A, Zotenko E, Locke WJ, Korbie D, Millar EK, Pidsley R, et al. DNA methylation of oestrogen-regulated enhancers defines endocrine sensitivity in breast cancer. *Nat Commun* (2015) 6:7758. doi: 10.1038/ncomms8758
69. De Francesco EM, Pellegrino M, Santolla MF, Lappano R, Ricchio E, Abonante S, et al. GPER mediates activation of HIF1alpha/VEGF signaling by estrogens. *Cancer Res* (2014) 74(15):4053–64. doi: 10.1158/0008-5472.CAN-13-3590
70. Catalano S, Giordano C, Panza S, Chemi F, Bonofiglio D, Lanzino M, et al. Tamoxifen through GPER upregulates aromatase expression: a novel mechanism sustaining tamoxifen-resistant breast cancer cell growth. *Breast Cancer Res Treat* (2014) 146(2):273–85. doi: 10.1007/s10549-014-3017-4
71. Talia M, De Francesco EM, Rigiracciolo DC, Muoio MG, Muglia L, Belfiore A, et al. The G Protein-Coupled Estrogen Receptor (GPER) Expression Correlates with Pro-Metastatic Pathways in ER-Negative Breast Cancer: A Bioinformatics Analysis. *Cells* (2020) 9(3):622–34. doi: 10.3390/cells9030622
72. Hsu LH, Chu NM, Lin YF, Kao SH. G-Protein Coupled Estrogen Receptor in Breast Cancer. *Int J Mol Sci* (2019) 20(2):306–321. doi: 10.3390/ijms20020306
73. Filardo EJ, Quinn JA, Sabo E. Association of the membrane estrogen receptor, GPR30, with breast tumor metastasis and transactivation of the epidermal growth factor receptor. *Steroids* (2008) 73(9-10):870–3. doi: 10.1016/j.steroids.2007.12.025
74. Phadke S, Mott S, Bashir A, Bellizzi A, Resnick M, Sturtevant A, et al. Distribution of G-protein coupled estrogen receptor in treatment-naive triple negative breast cancer and association with clinicopathologic characteristics. *Cancer Res* (2020) 80(4):P2-11-16. Proceedings of the 2019 San Antonio Breast Cancer Symposium; 2019 Dec 10-14; San Antonio, TX. Philadelphia (PA). doi: 10.1158/1538-7445.SABCS19-P2-11-16
75. Iorga A, Cunningham CM, Moazeni S, Ruffenach G, Umar S, Eghbali M. The protective role of estrogen and estrogen receptors in cardiovascular disease and the controversial use of estrogen therapy. *Biol Sex Differ* (2017) 8 (1):33. doi: 10.1186/s13293-017-0152-8
76. Weitzmann MN, Pacifici R. Estrogen deficiency and bone loss: an inflammatory tale. *J Clin Invest* (2006) 116(5):1186–94. doi: 10.1172/JCI28550
77. Whiteley J, DiBonaventura M, Wagner JS, Alvir J, Shah S. The impact of menopausal symptoms on quality of life, productivity, and economic outcomes. *J Womens Health (Larchmt)* (2013) 22(11):983–90. doi: 10.1089/jwh.2012.3719
78. Nourmoussavi M, Pansegrau G, Popesku J, Hammond GL, Kwon JS, Carey MS. Ovarian ablation for premenopausal breast cancer: A review of treatment considerations and the impact of premature menopause. *Cancer Treat Rev* (2017) 55:26–35. doi: 10.1016/j.ctrv.2017.02.005
79. Weigel NL, Zhang Y. Ligand-independent activation of steroid hormone receptors. *J Mol Med (Berl)* (1998) 76(7):469–79. doi: 10.1007/s001090050241
80. Levoye A, Dam J, Ayoub MA, Guillaume JL, Jockers R. Do orphan G-protein-coupled receptors have ligand-independent functions? New insights from receptor heterodimers. *EMBO Rep* (2006) 7(11):1094–8. doi: 10.1038/sj.embor.7400838
81. Stoy H, Gurevich VV. How genetic errors in GPCRs affect their function: Possible therapeutic strategies. *Genes Dis* (2015) 2(2):108–32. doi: 10.1016/j.gendis.2015.02.005
82. Toy W, Weir H, Razavi P, Lawson M, Goeppert AU, Mazzola AM, et al. Activating ESR1 Mutations Differentially Affect the Efficacy of ER Antagonists. *Cancer Discov* (2017) 7(3):277–87. doi: 10.1158/2159-8290.CD-15-1523
83. King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am J Clin Nutr* (1998) 67 (5):867–72. doi: 10.1093/ajcn/67.5.867
84. Rice S, Whitehead SA. Phytoestrogens and breast cancer–promoters or protectors? *Endocr Relat Cancer* (2006) 13(4):995–1015. doi: 10.1677/erc.1.01159
85. Lampe JW. Emerging research on equol and cancer. *J Nutr* (2010) 140 (7):1369S–72S. doi: 10.3945/jn.109.118323
86. He F-J, Chen J-Q. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between Chinese women and women in Western countries and possible mechanisms. *Food Sci Hum Wellness* (2013) 2(3-4):146–61. doi: 10.1016/j.fshw.2013.08.002
87. Messina MJ, Wood CE. Soy isoflavones, estrogen therapy, and breast cancer risk: analysis and commentary. *Nutr J* (2008) 7:17. doi: 10.1186/1475-2891-7-17
88. Randel A. AACE releases guidelines for menopausal hormone therapy. *Am Family Physician* (2012) 86(9):864–8.
89. Kang X, Zhang Q, Wang S, Huang X, Jin S. Effect of soy isoflavones on breast cancer recurrence and death for patients receiving adjuvant endocrine therapy. *CMAJ Can Med Assoc J* (2010) 182(17):1857–62. doi: 10.1503/cmaj.091298
90. Hilakivi-Clarke L, Andrade JE, Helferich W. Is soy consumption good or bad for the breast? *J Nutr* (2010) 140(12):2326S–34S. doi: 10.3945/jn.110.124230
91. Jiang Y, Gong P, Madak-Erdogan Z, Martin T, Jeyakumar M, Carlson K, et al. Mechanisms enforcing the estrogen receptor beta selectivity of botanical estrogens. *FASEB J* (2013) 27(11):4406–18. doi: 10.1096/fj.13-234617
92. Björnsdotter MK, de Boer J, Ballesteros-Gómez A, Bisphenol A. and replacements in thermal paper: A review. *Chemosphere* (2017) 182:691–706. doi: 10.1016/j.chemosphere.2017.05.070
93. Lehmler HJ, Liu B, Gadogbe M, Bao W. Exposure to Bisphenol A, Bisphenol F, and Bisphenol S in U.S. Adults and Children: The National Health and Nutrition Examination Survey 2013-2014. *ACS Omega* (2018) 3(6):6523–32. doi: 10.1021/acsomega.8b00824
94. Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, et al. Bisphenol A induces gene expression changes and proliferative effects through GPER in breast cancer cells and cancer-associated fibroblasts. *Environ Health Perspect* (2012) 120(8):1177–82. doi: 10.1289/ehp.1104526
95. Bouskine A, Nebout M, Brucker-Davis F, Benahmed M, Fenichel P. Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. *Environ Health Perspect* (2009) 117(7):1053–8. doi: 10.1289/ehp.0800367
96. Rezg R, El-Fazaa S, Gharbi N, Mornagui B. Bisphenol A and human chronic diseases: current evidences, possible mechanisms, and future perspectives. *Environ Int* (2014) 64:83–90. doi: 10.1016/j.envint.2013.12.007
97. NationalToxicologyProgram. Carcinogenesis Bioassay of Bisphenol A (CAS No. 80-05-7) in F344 Rats and B6C3F1 Mice (Feed Study). *Natl Toxicol Program Tech Rep Ser* (1982) 215:1.

98. Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, Keri RA. A review of the carcinogenic potential of bisphenol A. *Reprod Toxicol* (2016) 59:167–82. doi: 10.1016/j.reprotox.2015.09.006
99. Hill CE, Myers JP, Vandenberg LN. Nonmonotonic Dose-Response Curves Occur in Dose Ranges That Are Relevant to Regulatory Decision-Making. *Dose Response* (2018) 16(3). doi: 10.1177/1559325818798282 1559325818798282.
100. Teppala S, Madhavan S, Shankar A. Bisphenol A and Metabolic Syndrome: Results from NHANES. *Int J Endocrinol* (2012) 2012:598180. doi: 10.1155/2012/598180
101. Chen D, Kannan K, Tan H, Zheng Z, Feng YL, Wu Y, et al. Bisphenol Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and Toxicity-A Review. *Environ Sci Technol* (2016) 50(11):5438–53. doi: 10.1021/acs.est.5b05387
102. Patel HK, Bihani T. Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. *Pharmacol Ther* (2018) 186:1–24. doi: 10.1016/j.pharmthera.2017.12.012
103. Ignatov A, Ignatov T, Weissenborn C, Eggemann H, Bischoff J, Semczuk A, et al. G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer. *Breast Cancer Res Treat* (2011) 128(2):457–66. doi: 10.1007/s10549-011-1584-1
104. Group EBCTC. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* (2005) 365(9472):1687–717. doi: 10.1016/S0140-6736(05)66544-0
105. Nishimura R, Osako T, Okumura Y, Hayashi M, Toyozumi Y, Arima N. Ki-67 as a prognostic marker according to breast cancer subtype and a predictor of recurrence time in primary breast cancer. *Exp Ther Med* (2010) 1(5):747–54. doi: 10.3892/etm.2010.133
106. Lumachi F, Santeufemia DA, Basso SM. Current medical treatment of estrogen receptor-positive breast cancer. *World J Biol Chem* (2015) 6(3):231–9. doi: 10.4331/wjbc.v6.i3.231
107. Yin H, Zhu Q, Liu M, Tu G, Li Q, Yuan J, et al. GPER promotes tamoxifen-resistance in ER+ breast cancer cells by reduced Bim proteins through MAPK/Erk-TRIM2 signaling axis. *Int J Oncol* (2017) 51(4):1191–8. doi: 10.3892/ijo.2017.4117
108. Turner NC, Slamon DJ, Ro J, Bondarenko I, Im SA, Masuda N, et al. Overall Survival with Palbociclib and Fulvestrant in Advanced Breast Cancer. *N Engl J Med* (2018) 379(20):1926–36. doi: 10.1056/NEJMoa1810527
109. Rask-Andersen M, Almen MS, Schioth HB. Trends in the exploitation of novel drug targets. *Nat Rev Drug Discov* (2011) 10(8):579–90. doi: 10.1038/nrd3478
110. Nieto Gutierrez A, McDonald PH. GPCRs: Emerging anti-cancer drug targets. *Cell Signal* (2018) 41:65–74. doi: 10.1016/j.cellsig.2017.09.005
111. Panupinthu N, Lee HY, Mills GB. Lysophosphatidic acid production and action: critical new players in breast cancer initiation and progression. *Br J Cancer* (2010) 102(6):941–6. doi: 10.1038/sj.bjc.6605588
112. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. *Theranostics* (2017) 7(6):1543–88. doi: 10.7150/thno.15625
113. Sun X, Cheng G, Hao M, Zheng J, Zhou X, Zhang J, et al. CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression. *Cancer Metastasis Rev* (2010) 29(4):709–22. doi: 10.1007/s10555-010-9256-x
114. Salahudeen MS, Nishtala PS. An overview of pharmacodynamic modelling, ligand-binding approach and its application in clinical practice. *Saudi Pharm J* (2017) 25(2):165–75. doi: 10.1016/j.jsps.2016.07.002
115. Gaudet H, Cheng S, Christensen E, Filardo E. The G-protein coupled estrogen receptor, GPER: The inside and inside-out story. *Mol Cell Endocrinol* (2015) 418:207–19. doi: 10.1016/j.mce.2015.07.016
116. Pillai V, Dorfman DM. Flow Cytometry of Nonhematopoietic Neoplasms. *Acta Cytol* (2016) 60(4):336–43. doi: 10.1159/000448371
117. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, et al. In vivo effects of a GPR30 antagonist. *Nat Chem Biol* (2009) 5(6):421–7. doi: 10.1038/nchembio.168
118. Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologa CG, et al. Identification of a GPER/GPR30 antagonist with improved estrogen receptor counterselectivity. *J Steroid Biochem Mol Biol* (2011) 127(3-5):358–66. doi: 10.1016/j.jsbmb.2011.07.002
119. DeLeon C, Wang HH, Gunn J, Wilhelm M, Cole A, Arnett S, et al. A novel GPER antagonist protects against the formation of estrogen-induced cholesterol gallstones in female mice. *J Lipid Res* (2020) 61(5):767–77. doi: 10.1194/jlr.RA119000592
120. Lappano R, Santolla MF, Pupo M, Sinicropi MS, Caruso A, Rosano C, et al. MIBE acts as antagonist ligand of both estrogen receptor alpha and GPER in breast cancer cells. *Breast Cancer Res* (2012) 14(1):R12. doi: 10.1186/bcr3096
121. Maggiolini M, Santolla MF, Avino S, Aiello F, Rosano C, Garofalo A, et al. Identification of two benzopyrroloxazines acting as selective GPER antagonists in breast cancer cells and cancer-associated fibroblasts. *Future Med Chem* (2015) 7(4):437–48. doi: 10.4155/fmc.15.3
122. Lappano R, Rosano C, Pisano A, Santolla MF, De Francesco EM, De Marco P, et al. A calixpyrrole derivative acts as an antagonist to GPER, a G-protein coupled receptor: mechanisms and models. *Dis Model Mech* (2015) 8(10):1237–46. doi: 10.1242/dmm.021071
123. Shi D, Zhao P, Cui L, Li H, Sun L, Niu J, et al. Inhibition of PI3K/AKT molecular pathway mediated by membrane estrogen receptor GPER accounts for cryptotanshinone induced antiproliferative effect on breast cancer SKBR-3 cells. *BMC Pharmacol Toxicol* (2020) 21(1):32. doi: 10.1186/s40360-020-00410-9
124. Campbell AP, Smrcka AV. Targeting G protein-coupled receptor signalling by blocking G proteins. *Nat Rev Drug Discov* (2018) 17(11):789–803. doi: 10.1038/nrd.2018.135
125. Ghanemi A. Targeting G protein coupled receptor-related pathways as emerging molecular therapies. *Saudi Pharm J* (2015) 23(2):115–29. doi: 10.1016/j.jsps.2013.07.007
126. Zhang H, Xiong XF, Boesgaard MW, Underwood CR, Brauner-Osborne H, Stromgaard K. Structure-Activity Relationship Studies of the Cyclic Depsipeptide Natural Product YM-254890, Targeting the Gq Protein. *ChemMedChem* (2017) 12(11):830–4. doi: 10.1002/cmdc.201700155
127. Schrage R, Schmitz AL, Gaffal E, Annala S, Kehraus S, Wenzel D, et al. The experimental power of FR900359 to study Gq-regulated biological processes. *Nat Commun* (2015) 6:10156. doi: 10.1038/ncomms10156
128. Kawasaki T, Taniguchi M, Moritani Y, Hayashi K, Saito T, Takasaki J, et al. Antithrombotic and thrombolytic efficacy of YM-254890, a G q/11 inhibitor, in a rat model of arterial thrombosis. *Thromb Haemost* (2003) 90(3):406–13. doi: 10.1160/TH03-02-0115
129. Matthey M, Roberts R, Seidinger A, Simon A, Schroder R, Kuschak M, et al. Targeted inhibition of Gq signaling induces airway relaxation in mouse models of asthma. *Sci Transl Med* (2017) 9(407):2288–98. doi: 10.1126/scitranslmed.aag2288
130. Onken MD, Makepeace CM, Wang S, Kaltenbronn KM, Kanai SM, Broekelmann TJ, et al. Pharmacologic targeting of Gq reveals new pathways in uveal melanoma. *Cancer Res* (2018) 78(13 Suppl): Abstract nr 4363. Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA). doi: 10.1158/1538-7445.AM2018-4363
131. Filardo EJ, Quinn JA, Bland KI, Frackelton ARJr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* (2000) 14(10):1649–60. doi: 10.1210/mend.14.10.0532
132. Michel MC, Charlton SJ. Biased Agonism in Drug Discovery-Is It Too Soon to Choose a Path? *Mol Pharmacol* (2018) 93(4):259–65. doi: 10.1124/mol.117.110890
133. DeWire SM, Yamashita DS, Rominger DH, Liu G, Cowan CL, Graczyk TM, et al. A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* (2013) 344(3):708–17. doi: 10.1124/jpet.112.201616
134. Gillis A, Gondin AB, Kliewer A, Sanchez J, Lim HD, Alamein C, et al. Low intrinsic efficacy for G protein activation can explain the improved side effect profiles of new opioid agonists. *Sci Signal* (2020) 13(625):3140–57. doi: 10.1126/scisignal.aaz3140
135. Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther* (2010) 335(3):572–9. doi: 10.1124/jpet.110.173005

136. Nakai K, Hung MC, Yamaguchi H. A perspective on anti-EGFR therapies targeting triple-negative breast cancer. *Am J Cancer Res* (2016) 6(8):1609–23.
137. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* (5715) 2005) 307:1625–30. doi: 10.1126/science.1106943
138. Verret B, Cortes J, Bachelot T, Andre F, Arnedos M. Efficacy of PI3K inhibitors in advanced breast cancer. *Ann Oncol* (2019) 30 Suppl 10:x12–20. doi: 10.1093/annonc/mdz381
139. Andre F, Ciruelos E, Rubovszky G, Campone M, Loibl S, Rugo HS, et al. Alpelisib for PIK3CA-Mutated, Hormone Receptor-Positive Advanced Breast Cancer. *N Engl J Med* (2019) 380(20):1929–40. doi: 10.1056/NEJMoa1813904
140. Ollila TA, Sahin I, Olszewski AJ. Mogamulizumab: a new tool for management of cutaneous T-cell lymphoma. *Onco Targets Ther* (2019) 12:1085–94. doi: 10.2147/OTT.S165615
141. Zhu C, Guan J, Xiao H, Luo W, Tong R. Erenumab safety and efficacy in migraine: A systematic review and meta-analysis of randomized clinical trials. *Med (Baltimore)* (2019) 98(52):e18483. doi: 10.1097/MD.0000000000018483
142. Kaplon H, Reichert JM. Antibodies to watch in 2019. *MAbs* (2019) 11 (2):219–38. doi: 10.1080/19420862.2018.1556465
143. Ghobrial IM, Liu CJ, Redd RA, Perez RP, Baz R, Zavidij O, et al. A Phase Ib/II Trial of the First-in-Class Anti-CXCR4 Antibody Ulocuplumab in Combination with Lenalidomide or Bortezomib Plus Dexamethasone in Relapsed Multiple Myeloma. *Clin Cancer Res* (2020) 26(2):344–53. doi: 10.1158/1078-0432.CCR-19-0647
144. Hutchings CJ, Koglin M, Olson WC, Marshall FH. Opportunities for therapeutic antibodies directed at G-protein-coupled receptors. *Nat Rev Drug Discov* (2017) 16(9):787–810. doi: 10.1038/nrd.2017.91
145. Vela M, Aris M, Llorente M, Garcia-Sanz JA, Kremer L. Chemokine receptor-specific antibodies in cancer immunotherapy: achievements and challenges. *Front Immunol* (2015) 6:12. doi: 10.3389/fimmu.2015.00012
146. Subramaniam JM, Whiteside G, McKeage K, Croxtall JC. Mogamulizumab: first global approval. *Drugs* (2012) 72(9):1293–8. doi: 10.2165/11631090-000000000-00000
147. Vergunst CE, Gerlag DM, Lopatinskaya L, Klareskog L, Smith MD, van den Bosch F, et al. Modulation of CCR2 in rheumatoid arthritis: a double-blind, randomized, placebo-controlled clinical trial. *Arthritis Rheum* (2008) 58 (7):1931–9. doi: 10.1002/art.23591
148. Jin H, Pi J, Zhao Y, Jiang J, Li T, Zeng X, et al. EGFR-targeting PLGA-PEG nanoparticles as a curcumin delivery system for breast cancer therapy. *Nanoscale* (2017) 9(42):16365–74. doi: 10.1039/c7nr06898k
149. Ebeid K, Meng X, Thiel KW, Do AV, Geary SM, Morris AS, et al. Synthetically lethal nanoparticles for treatment of endometrial cancer. *Nat Nanotechnol* (2018) 13(1):72–81. doi: 10.1038/s41565-017-0009-7
150. Geary SM, Hu Q, Joshi VB, Bowden NB, Salem AK. Diaminosulfide based polymer microparticles as cancer vaccine delivery systems. *J Control Release* (2015) 220(Pt B):682–90. doi: 10.1016/j.jconrel.2015.09.002
151. Wafa EI, Geary SM, Ross KA, Goodman JT, Narasimhan B, Salem AK. Single Dose of a Polyanhydride Particle-Based Vaccine Generates Potent Antigen-Specific Antitumor Immune Responses. *J Pharmacol Exp Ther* (2019) 370 (3):855–63. doi: 10.1124/jpet.118.252809
152. Joshi VB, Geary SM, Salem AK. Biodegradable particles as vaccine delivery systems: size matters. *AAPS J* (2013) 15(1):85–94. doi: 10.1208/s12248-012-9418-6
153. Abbas AO, Donovan MD, Salem AK. Formulating poly(lactide-co-glycolide) particles for plasmid DNA delivery. *J Pharm Sci* (2008) 97(7):2448–61. doi: 10.1002/jps.21215
154. Makkouk A, Joshi VB, Wongrakpanich A, Lemke CD, Gross BP, Salem AK, et al. Biodegradable microparticles loaded with doxorubicin and CpG ODN for in situ immunization against cancer. *AAPS J* (2015) 17(1):184–93. doi: 10.1208/s12248-014-9676-6
155. Chitphet K, Geary SM, Chan CH, Simons AL, Weiner GJ, Salem AK. Combining Doxorubicin-Loaded PEGylated Poly (Lactide-co-glycolide) Nanoparticles with Checkpoint Inhibition Safely Enhances Therapeutic Efficacy in a Melanoma Model. *ACS Biomater Sci Eng* (2019) 6(5):2659–67. doi: 10.1021/acsbiomaterials.9b01108
156. Kajta M, Rzemieniec J, Litwa E, Lason W, Lenartowicz M, Krzeptowski W, et al. The key involvement of estrogen receptor beta and G-protein-coupled receptor 30 in the neuroprotective action of daidzein. *Neuroscience* (2013) 238:345–60. doi: 10.1016/j.neuroscience.2013.02.005

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GPER and Testicular Germ Cell Cancer

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The G protein-coupled estrogen receptor (GPER), also known as GPR30, is a widely conserved 7-transmembrane-domain protein which has been identified as a novel 17β-estradiol-binding protein that is structurally distinct from the classic oestrogen receptors (ERα and ERβ). There are still conflicting data regarding the exact role and the natural ligand of GPER/GPR30 in reproductive tracts as both male and female knock-out mice are fertile and have no abnormalities of reproductive organs. Testicular germ cell cancers (TGCCs) are the most common malignancy in young males and the most frequent cause of death from solid tumors in this age group. Clinical and experimental studies suggested that estrogens participate in the physiological and pathological control of male germ cell proliferation. In human seminoma cell line, while 17β-estradiol (E2) inhibits *in vitro* cell proliferation through an ERβ-dependent mechanism, an impermeable E2 conjugate (E2 coupled to BSA), *in vitro* cell proliferation is stimulated by activating ERK1/2 and protein kinase A through a membrane GPCR that we further identified as GPER/GPR30. The same effect was observed with low but environmentally relevant doses of BPA, an estrogenic endocrine disrupting compound. Furthermore, GPER/GPR30 is specifically overexpressed in seminomas but not in non-seminomas and this overexpression is correlated with an ERβ-downregulation. This GPER/GPR30 overexpression could be linked to some genetic variations, as single nucleotide polymorphisms, which was also reported in other hormone-dependent cancers. We will review here the implication of GPER/GPR30 in TGCCs pathophysiology and the arguments to consider GPER/GPR30 as a potential therapeutic target in humans.

Keywords: testicular germ cell cancer, estrogen receptors, GPR30/GPER, endocrine disrupting compounds, fetal exposure, bisphenol A

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INTRODUCTION

Although relatively rare, testicular germ cell cancers (TGCC) are the most frequent solid cancer in young people (1, 2). Seminomas represent the most frequent histological form, occurring alone or associated with non-seminoma forms in 50-75% of cases (1, 2). Incidence rates of TGCC have been increasing worldwide for several decades (3, 4).

Risk factors for TGCC are described in **Table 1** and are mainly genetic. Indeed, incidence of TGCC is significantly increased in brothers and sons of TGCC patients (5, 7). Consistent with many epidemiological studies, gene variants that might predispose an individual to TGCC were identified

by genome-wide association studies (GWAS) (8, 9). These variants included common variations on 12q22 in the KITLG gene, but also on PDE11A, BAK1, SPRY, DMRT1, DAZL, and PRDM14 [reviewed in (10)]. Other classical risk factors are cryptorchidism (or undescended testis), inguinal hernia, and all sexual differentiation disorders (6, 11) (**Table 1**).

TGCC are considered to derive from a precursor lesion named "carcinoma *in situ* of the testis" or "germ cell neoplasia *in situ*" (GCNIS) (12). This lesion is present before birth, arising from the fetal germ cells (*i.e.* the gonocytes), and is reactivated after puberty under physiological hormonal stimulation (13). Epidemiological and clinical data have suggested that the increase of TGCC incidence could be related to environmental factors such as fetal exposure to endocrine disruptors (EDCs) with anti-androgenic and/or estrogenic effects (14, 15). However, this hypothesis supposes that TGCCs are estrogen-dependent tumors. In this review, we analyze the implication of classical and non-classical (GPER/GPR30) estrogen receptors in normal and malignant germ cells and the regulation of cell proliferation by xeno-estrogens and discuss how GPER/GPR30 could be considered as a potential therapeutic target in humans.

COULD TGCC BE A HORMONE-DEPENDENT CANCER?

Environmental Features

Several studies have reported abnormalities of male genital tracts in animals that were accidently exposed to endocrine disruptors, such as hypospadias and cryptorchidism in alligators (16) or panthers (17), especially in the case of exposition to the organochloride dichlorodiphenyltrichloroethane (DDT) or its metabolites (DDE, DDD), which exhibit estrogenic properties. However, there is actually no animal model of TGCC, except for transgenic mice with targeted overexpression of GDNF in spermatogonia (18).

In humans, early fetal exposure to diethylstilbestrol (DES), a synthetic estrogen used during the 1960's, was responsible for an increased incidence rate of cryptorchidism and hypofertility by impairment of sperm quality in sons and in grandsons (19, 20). Such an exposure was also suggested to be responsible for the occurrence of TGCC in the offspring of two meta-analysis (21, 22). In past studies, the association between occupational exposure and risk to develop TGCC (23–25) was well-documented and offered suggestive or strong arguments. However, more recent epidemiological case-control studies reported conflicting data for fetal exposure to p,p′-DDT (estrogenic compound) or to p,p′-DDE (a stable metabolite of DDT with antiandrogenic properties) (26–31).

TABLE 1 | Usual risk factors of testicular germ cell cancers.

Risk Factor	Risk estimate or range Odd Ratio (95% CI)
Low birth weight (versus normal)	1.34 (1.08 – 1.67)
Low gestational age (versus not low)	1.31 (1.07 – 1.59)
Cryptorchidism	4.30 (3.62 – 5.11)
Inguinal hernia	1.63 (1.37 – 1.94)
Twinning	1.22 (1.03 – 1.44)
Prior TGCC	12.4 (11.0 – 13.9)
Father with TGCC	3.78 (1.94 – 6.63)
Brother with TGCC	12.74 (6.38 – 22.64)
Adult height (per 5 cm increase)	1.13 (1.07 – 1.19)

TGCC, testicular germ cell cancers.
Adapted from Cook MB. et al. (5). and Mc Glynn KA. et al. (6).

Estrogens and Normal Germ Cells

Testicular concentrations of 17β-estradiol (E2) are 10 to 100 times higher than those measured in blood (32). E2 is produced after testosterone conversion by aromatase in all mammalian testes, including humans (33). Estrogens are essential for spermatogenesis control but the type of estrogen receptors involved and the molecular mechanisms by which estrogens may precisely act during spermatogenesis still remain incompletely understood (34).

Expression of classical and non-classical estrogen receptors expression in mammalian testes is well-established. It exhibits some species specificity and some controversial results, especially in humans [reviewed in (35)]. Indeed, in humans, the classical nuclear estrogen receptor ERβ has been clearly identified in most germ cells, including fetal gonocytes (36), neonatal, prepubertal (37), and adult spermatogonia (38), while ERα is not expressed in human gonocytes (36) or neonatal or prepubertal spermatogonia (37). However, data concerning the expression of ERα by male germ cells are inconsistent, as some authors reported an expression in elongated spermatids and mature spermatozoa (39) and others did not find any expression of ERα at all (38, 40). In fact, these inconsistent observations could be due to the existence of a truncated isoform of ERα lacking exon 1, called ERα46, which has been identified in human adult spermatozoa (41). This isoform could participate in non-genomic membrane signaling. Indeed, one reported case of a man with an inactivating mutation of ERα gene was associated with a normal sperm count but with completely abnormal motility (42).

GPER/GPR30 and Testis

GPR30 is a widely conserved orphan GPCR, which has been renamed as G protein-coupled estrogen receptor (GPER) (HUGO & MGI Databases). It is a seven-transmembrane domain protein, identified for the first time in a triple-negative breast cancer cell line, that can bind E2 and other estrogenic compounds independently of the classic estrogen receptors (ERα and ERβ). The precise subcellular localization of GPER/GPR30 is still a matter of debate as it has been detected at the plasma membrane but also in the endoplasmic reticulum and Golgi apparatus (43).

GPER/GPR30 has been identified in numerous rodents and human estrogen targets normal or malignant tissues where it can mediate rapid E2-induced non genomic signaling events (43). GPER/GPR30 can activate cell proliferation through several signaling pathways involving MAP kinases, ERK1/2, and PI3K pathways (44, 45) but also microRNA regulation (46–48), EGFR transactivation (49, 50), HIF induced pathway (51, 52), IGF-R

pathway (53, 54), NF-kB pathway (55, 56), and crosstalk with other receptors (classical or truncated estrogen receptors, or other steroids receptors) (57–59). Within those pathways, the activation of ERK1/2 is undoubtedly the most consistent pathway across cell types and is usually considered as a key factor in cancer prognosis.

Analyzing normal human testes from a fertile man, we previously reported that GPER/GPR30 was expressed by both somatic (Sertoli and Leydig cells) and germ cells (60). Amazingly, Rago et al. (61). reported a negative staining in adult germ cells, probably due to the use of abnormal granulomatous testes. As expression of GPER/GPR30 in human fetal gonocytes has not yet been studied; it could be possible that only immature germ cells and gonocytes express GPER/GPR30, explaining these inconsistent data [reviewed in (62)].

ESTROGENS, GERM CELLS PROLIFERATION, AND TGCC

Estrogen Receptors and Malignant Germ Cells

Estrogen receptor expression is a well-recognized prognosis factor of estrogen-dependent cancers, especially in the case of breast cancer (63–65). Several teams have suggested that TGCCs could be estrogen-dependent cancers as they express both ERβ and GPER/GPR30 (66–70). We previously reported in a large cohort of TGCCs that GPER/GPR30 was overexpressed only in seminoma but not in non-seminoma tumors (60) and promoted seminoma cell proliferation (71). Pais et al. (72) reported that expression of ERβ was decreased in seminoma but remained high in teratomas. In the same way, Boscia et al. (69) showed that ERβ was downregulated in seminomas and reported a negative association between the expression of ERβ and GPER/GPR30 protein. This inverse receptor expression pattern could reflect a switch in estrogen responsiveness from a suppressive (66) to a promoting profile (60, 67), as it has also been observed in other estrogen-dependent cancers and was correlated to a poorer prognosis (63–65).

Genetic factors could of course explain this specific profile of expression. Variants of ERβ were explored but studies reported inconsistent data. Ferlin et al. (73) reported a weak but not significant association between one variant for ERβ and an increase risk of TGCC in Italian men, while Brokken et al. (74) described exactly the opposite in a cohort of 367 Nordic patients with TGCC and two other variants of the ERβ. In our large cohort of 169 TGCCs, we were able to describe that seminomas were characterized by a loss of homozygous ancestral genotype concerning two polymorphisms located in the promoter region of GPER/GPR30 (75). We assumed that this genotype could explain a part of GPER/GPR30 overexpression in seminomas. This expression profile could also be determined by epigenetic modulation of ERβ and GPER/GPR30 genes (low expression of ERβ due to an hypermethylation of its promoter and high expression of GPER/GPR30 gene due to an hypomethylation of its promoter). Indeed, fetal exposure to EDCs is supposed to induce such epigenetic modulation as reported, for example, by Zama et al. (76) who reported that fetal and neonatal exposure to the endocrine disruptor methoxychlor was responsible for a down regulation of ovarian ERβ gene expression.

Anway et al. (77) were the first to observe and to report several epigenetic modifications in rodent DNA male germ cells after gestational exposure to vinclozolin (antiandrogenic compound) or methoxychlor (estrogenic compound). These data have been recently confirmed by Dumasia et al. (78) for xenoestrogens signaling through ERβ. Since this first publication of Anway et al. (77) DNA methylation (hyper- and hypo-) (79, 80), onco-miRNAs expression (miR 371-373) (81, 82), or chromatin modifications have been reported in TGCC (83). However, even if experimental data in rodents suggested that these epigenetic modifications might be induced by fetal exposure to EDCs, it remains to be proven that such epigenetic modifications exist in humans and can be induced by fetal exposure to EDCs.

Putative Role of GPER/GPR30 in Malignant Germ Cells

JKT-1 cell line is derived from a human testicular seminoma (84), which expressed functional aromatase (66) and is able to convert testosterone into E2 and as well as ERβ, but not ERα. At physiological concentrations (10^{-7} to 10^{-9} M), we previously reported that E2 was able to inhibit *in vitro* JKT-1 cell proliferation involving an ERβ pathway (66). We conjugated E2 to bovine serum albumin (E2-BSA) for the purpose that E2 cannot cross the plasma membrane and then cannot link to its canonical receptor ERβ. In this condition, E2-BSA at the same concentrations (10^{-7} to 10^{-9} M) stimulated *in vitro* JKT-1 cell proliferation by activating the ERK1/2 and PKA pathways. E2-BSA is responsible for a rapid (15 min) phosphorylation of CREB. This effect was not inhibited by ICI-182,780, an antagonist of ERβ, but by *Pertussis toxin*, suggestive of the involvement of a membrane G-protein-coupled receptor (GPCR). Similar results were obtained with bisphenol A (BPA) at low and very low (nM to pM) concentrations (85), the levels already found in male cord blood and in more than 95% of the worldwide population (86, 87).

Among EDCs, BPA is especially a matter of concern as populations exhibit worldwide with detectable blood and/or urine levels of BPA (86), and so it is used as a monomer to manufacture a wide range of objects containing polycarbonate plastic and resins. BPA is considered an estrogenic EDC and is recognized as a substance of very high concern (SVHC) by the European Chemicals Agency (ECHA) because several experimentations and data reported that it is involved in developmental, reproductive, and malignant diseases by mimicking the natural hormone E2 and by interfering with endogenous pathways at selective periods, especially during fetal life (88). However, BPA exhibits a weak affinity for the classical ERs, which is 1,000–2,000 times lower than E2. Thus, it has been suggested that BPA could act through other receptors than classical ERs, for example GPER/GPR30, PPARγ gamma, or ERRγ gamma (88).

In our JKT-1 seminoma cells model, we were able to identify the GPCR involved in the promoting action of E2-BSA and BPA as GPER/GPR30 (71). Indeed, the BPA-induced promotive effect was mimicked by G1 alone, a specific agonist of GPER/GPR30, while it was totally inhibited by G15, a partial antagonist of GPER/GPR30, as well as a selective anti-GPER/GPR30 siRNA (**Figure 1A**) (60, 71). This GPER/GPR30-mediated signaling of BPA was also reported in other hormone-dependent tumors. For example, Pupo et al. (90) reported that BPA could increase the proliferation of SKBr3 breast cancer cells, which lack the classical ERs, through a GPER/GPR30-EGFR/ERK transduction pathway.

Interestingly, the dose-response curve that we obtained for BPA in our model was non-monotonic and showed an inverted U-shape curve (**Figure 1B**). Non-monotonic dose response curves (NMDRC) have already been reported and well-documented for natural hormones. NMDRC have also been suggested for EDCs, especially in the case of BPA, but there are few consistent data available in the literature (91). Most authors explained that these atypical dose-response curves resulted from the complex interactions between the ligand (*i.e.*, the natural hormone or an EDC) and a hormone receptor. In our model, it could, for example, be explained by the resultant of the double opposite effect of BPA on ERβ and GPER/GPR30 (60, 85). Indeed, at low doses (nM or pM), BPA acts only through GPER/GPR30 by a promotive effect while it acts also through ERβ at higher dose (mM), which counteracted the promotive GPER/GPR30-mediated effect (66). In order to confirm this hypothesis, we exposed JKT-1 cells to variable doses of BPA together with a fixed dose of E2. The BPA dose-response curve that we obtained kept its inverted U-shape aspect but was down-translated, confirming that BPA can act either through ERβ or GPER/GPR30 depending on the other estrogenic compounds that are present in the cell environment. This parameter is particularly important to consider since in most cases we are exposed to EDC mixtures.

Furthermore, in the same cellular model, the effects of several EDCs on *in vitro* proliferation were totally different and dependent on the resultant of the two expressed receptors, ERβ and GPER/GPR30. For example, atrazine, another estrogenic pesticide, induced a suppressive effect on seminoma cell proliferation *in vitro* involving a GPER/GPR30-dependent pathway (92). In the same way, an alkylphenol mix promoted seminoma cell proliferation through a GPER/GPR30-dependent pathway (93). However, in this case, the promoting effect is also mediated through ERα36, which is a truncated form of the canonical ERα66 (without both transcriptional activation domains (AF-1 and AF-2)) and was first described first by Wang et al. (94) in 2005. It seems to participate in non-genomic estrogen signaling concurrently to and/or associated with GPER/GPR30, as demonstrated in breast cancer cell lines (94) and in seminoma-like TCam-2 cell line (95). Thus, the presence of ERα36 in tumors is an important parameter to consider before considering selective antagonists of GPER/GPR30 as a therapeutic target in TGCC or other estrogen-dependent cancers.

The crosstalk among GPER/GPR30 signaling, classical estrogen receptors, and other nuclear receptors involved in testis physiology regulation is also important to consider (96). Through such interactions, GPER/GPR30 could probably modulate the tumor microenvironment and through this mediate TGCC progression and aggressiveness, especially by inducing epithelial-mesenchymal-transition (97, 98), as has been reported in breast cancer (98, 99) and in pancreatic adenocarcinoma (100).

COULD GPER/GPR30 CONSTITUTE A POTENTIAL THERAPEUTIC TARGET FOR TGCC?

Accumulating evidence supports the role of GPER/GPR30 in cancer progression and metastasis in estrogen-dependent cancers (especially in breast cancer), even though GPER/GPR30 signaling can differently affect the development of cancer depending on the type of tissue, but also in the same tissue depending on the type of ligand (92). A better comprehension of the molecular pathways involved in TGCC development, in particular the role of GPER/GPR30 in tumor progression, points out new tools like agonists or antagonists of GPER/GPR30, which could be used going forward by clinicians to target cancer cells and improve the patient's chance of survival (68, 101).

Three pharmacological GPER/GPR30-ligands were routinely available to study GPER/GPR30 functions. The first one, G-1, was identified by Bologa in 2006 and is a specific agonist of GPER/GPR30, while G-15 and G-36, identified respectively in 2009 and 2011 by Dennis, are GPER/GPR30 antagonists. However, G-15 exhibits a partial cross-reactivity with ERα explaining why G-36 is mainly used in the study of GPER/GPR30 (102). Other pharmacological ligands were synthetized (GPER/GPR30-L1 and GPER/GPR30-L2) (102, 103) but they exhibit variable affinities for GPER/GPR30 and potential cross-reactivity with classical ERs, explaining why they cannot be considered as therapeutic tools at this time (104). These small molecules were used especially *in vitro*, as we did with seminoma cells; in our model, G-1 was able to mimic the proliferative effect of BPA while G-15 neutralized this effect and reduced cell proliferation in the presence of BPA (71). Thus, G-15 may be a helpful adjuvant in the treatment of TGCC. Nevertheless, to date, no studies have reported the use of GPER/GPR30 antagonists in this way.

However, agonists and antagonists of GPER/GPR30 were tested in the treatment of other tumors. For example, as we observed *in vitro* in seminoma cells, G-15 was also able to decrease the *in vitro* proliferation of non-small cell lung cancer (105) while G-1 was reported to induce malignant cell proliferation, invasion, and migration in primary cultured lung cancer cells (106) and in ER-negative breast cancer cells (107, 108) involving SIRT1 (108). At the opposite end, G-1 was able to decrease *in vivo* the tumor volume of pancreatic ductal adenocarcinoma in mice (109) and of adrenocortical carcinoma in a xenograft model (110, 111).

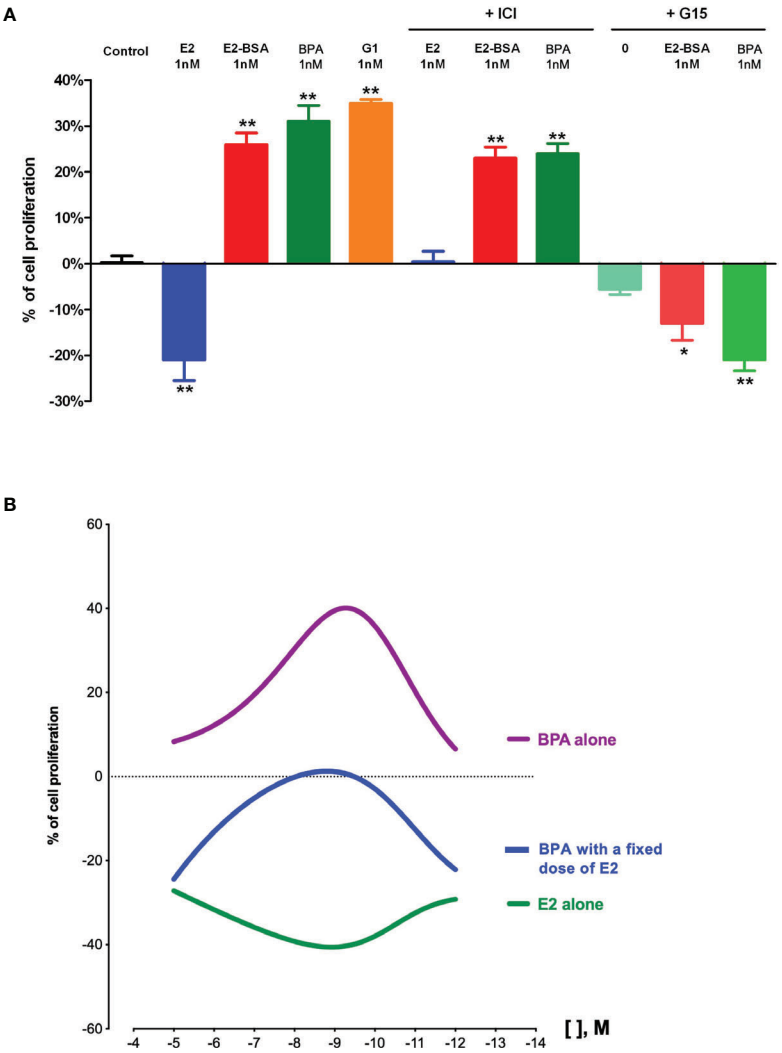

FIGURE 1 | Effects of estrogens and bisphenol A on human testicular seminoma cell (JKT-1) proliferation *in vitro*. **(A)** Analysis of JKT-1 cells proliferation *in vitro*, adapted from Chevalier et al. (71) JKT-1 cells were seeded in six-well plates (0.6×10^6 cells/well). After 48 h, the JKT-1 cells were washed and estrogen starved overnight in phenol red-free DMEM (Dulbeccos's Modified Eagle Medium) supplemented with 1% charcoal-stripped fetal bovine serum. Serum-deprived JKT-1 cells were then incubated for 24 hours with 17β-estradiol (E2; 1 nM), E2-BSA (1 nM), or bisphenol A (BPA; 1 nM), after a pre-treatment with G15 (1 nM) or ICI-182,780 (1 μM). G1 (1 nM) was used as a positive control. Values shown are expressed in percent change in cell number compared to control (steroid-free medium containing DMSO for bisphenol A or medium containing ethanol for estrogens, G1, and G15) given as the mean ± SE of at least three independent experiments. Cell counting was performed using a Malassez hemocytometer and confirmed using Vi-CELL automate (Beckman Coulter, Fullerton, CA). *$p < 0.05$; **$p < 0.001$. **(B)** Dose-response curves obtained with 17β-estradiol (E2) and bisphenol A (BPA) in JKT-1 cells *in vitro*, adapted from Fenichel et al. (89). and Bouskine et al. (85). JKT-1 cells were seeded in six-well plates (0.6×10^6 cells/well). After 48 h, the JKT-1 cells were washed and estrogen starved overnight in phenol red-free DMEM (Dulbeccos's Modified Eagle Medium) supplemented with 1% charcoal-stripped fetal bovine serum. Serum-deprived JKT-1 cells were then incubated for 24 hours with 17β-estradiol (E2) alone or bisphenol A (BPA) alone at variable doses from 10^{-5} M to 10^{-12} M obtained by serial dilutions, or with a fixed dose of E2 (10^{-9} M) and BPA at variable doses (same range, from 10^{-5} M to 10^{-12} M). Values shown are expressed in percent change in cell number compared to control (steroid-free medium containing DMSO for bisphenol A or medium containing ethanol for estrogens) given as the mean ± SE of nine independent experiments for each condition. Cell counting was performed using a Malassez hemocytometer and confirmed using Vi-CELL automate (Beckman Coulter, Fullerton, CA). Modeling of dose-response curves were performed using GraphPad Prism version 8.4.3 for Mac OS X, GraphPad Software, San Diego, California USA, www.graphpad.com.

Interestingly, G-1 was also able to reduce the side effects of chemotherapy, as, for example, the cardiac toxicity of doxorubicin is usually used as an adjuvant therapy in breast cancer (112). This beneficial effect is related to the well-documented GPER/GPR30 actions on the vascular system, involving in this specific case the Nox1 pathway, which could constitute new therapeutic tools (113, 114).

Actually, only one clinical study is registered in Clinical Trials involving a GPER/GPR30 agonist. The NCT04130516 is a phase 1, first-in-human, open-label, multicenter study (up to six study

sites in the United States) designed to characterize the safety, tolerability, and antitumor effects of LNS8801 administered orally in patients with advanced cancer (solid tumor or lymphoma). The recruitment is still on-going, and the estimated primary completion date is the end of 2021.

Finally, even though GPER/GPR30 modulation represents a potential novel strategy in cancer therapy, there remains a lack of solid clinical evidence supporting the specificity of GPER/GPR30 antagonists, especially in TGCC.

When compared with normal tissues, GPER/GPR30 is highly expressed in breast cancer and its high expression at the plasma membrane is strongly correlated with a poor prognosis, especially in triple negative tumors (115). This overexpression of GPER/GPR30 was also related to tamoxifen resistance (116, 117). Thus, GPER/GPR30 could be considered as a potential therapeutic target in such estrogen-dependent cancers.

CONCLUSION

Since its discovery in breast cancer, the role of GPER/GPR30 in estrogen-dependent malignancies has been of great interest. TGCC, the most common solid cancer in young men, expresses classical estrogen receptors (ERβ) but also GPER/GPR30. While E2 is responsible for a suppressive effect through an ERβ-dependent pathway, EDCs like BPA could induce *in vitro* seminoma cell proliferation by binding to GPER/GPR30. Furthermore, GPER/GPR30 is overexpressed in seminoma, probably due to genetic and/or epigenetic modulations that could be induced by fetal exposure to some EDCs. As proposed by Skakkebaek (4), an estrogenic environment might impair normal differentiation and proliferation of normal fetal, perinatal, and peripubertal germ stem cells, and then predispose an individual to TGCC, meaning it may be considered as an estrogen-dependent cancer. In our model, we have showed that G-15, a partial antagonist of GPER/GPR30, was able to reduce *in vitro* the BPA-induced cell proliferation (71) and may constitute a potential adjuvant in the treatment of TGCC. However, there remains a lack of solid clinical evidence to consider its clinical use. Direct regulation of GPER/GPR30 expression by siRNA silencing and/or nanotechnology could offer, at last, another tool to target GPER/GPR30 in cancer therapy.

AUTHOR CONTRIBUTIONS

NC and PF designed the study and contributed to the discussions and manuscript. NC and RP-B researched and interpreted data. SC and CH contributed to discussions and manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Bosl GJ, Motzer RJ. Testicular germ-cell cancer. *New Engl J Med* (1997) 337(4):242–53. doi: 10.1056/NEJM199707243370406
2. Bray F, Richiardi L, Ekbom A, Pukkala E, Cuninkova M, Moller H. Trends in testicular cancer incidence and mortality in 22 European countries: continuing increases in incidence and declines in mortality. *Int J Cancer* (2006) 118(12):3099–111. doi: 10.1002/ijc.21747
3. Le Cornet C, Lortet-Tieulent J, Forman D, Beranger R, Flechon A, Fervers B, et al. Testicular cancer incidence to rise by 25% by 2025 in Europe? Model-based predictions in 40 countries using population-based registry data. *Eur J Cancer* (2014) 50(4):831–9. doi: 10.1016/j.ejca.2013.11.035
4. Skakkebaek NE. A Brief Review of the Link between Environment and Male Reproductive Health: Lessons from Studies of Testicular Germ Cell Cancer. *Hormone Res Paediatrics* (2016) 86(4):240–6. doi: 10.1159/000443400
5. Cook MB, Akre O, Forman D, Madigan MP, Richiardi L, McGlynn KA. A systematic review and meta-analysis of perinatal variables in relation to the risk of testicular cancer–experiences of the son. *Int J Epidemiol* (2010) 39 (6):1605–18. doi: 10.1093/ije/dyq120
6. McGlynn KA, Trabert B. Adolescent and adult risk factors for testicular cancer. *Nat Rev Urol* (2012) 9(6):339–49. doi: 10.1038/nrurol.2012.61
7. Hemminki K, Chen B. Familial risks in testicular cancer as aetiological clues. *Int J Androl* (2006) 29(1):205–10. doi: 10.1111/j.1365-2605.2005.00599.x
8. Rapley EA, Turnbull C, Al Olama AA, Dermitzakis ET, Linger R, Huddart RA, et al. A genome-wide association study of testicular germ cell tumor. *Nat Genet* (2009) 41(7):807–10. doi: 10.1038/ng.394
9. Ferlin A, Pengo M, Pizzol D, Carraro U, Frigo AC, Foresta C. Variants in KITLG predispose to testicular germ cell cancer independently from spermatogenic function. *Endocrine-Related Cancer* (2012) 19(1):101–8. doi: 10.1530/ERC-11-0340
10. Xing JS, Bai ZM. Is testicular dysgenesis syndrome a genetic, endocrine, or environmental disease, or an unexplained reproductive disorder? *Life Sci* (2018) 194:120–9. doi: 10.1016/j.lfs.2017.11.039
11. Cook MB, Akre O, Forman D, Madigan MP, Richiardi L, McGlynn KA. A systematic review and meta-analysis of perinatal variables in relation to the risk of testicular cancer–experiences of the mother. *Int J Epidemiol* (2009) 38(6):1532–42. doi: 10.1093/ije/dyp287
12. Skakkebaek NE. Possible carcinoma-in-situ of the testis. *Lancet* (1972) 2 (7776):516–7. doi: 10.1016/s0140-6736(72)91909-5
13. Sonne SB, Almstrup K, Dalgaard M, Juncker AS, Edsgard D, Ruban L, et al. Analysis of gene expression profiles of microdissected cell populations indicates that testicular carcinoma in situ is an arrested gonocyte. *Cancer Res* (2009) 69(12):5241–50. doi: 10.1158/0008-5472.CAN-08-4554
14. Olesen IA, Sonne SB, Hoei-Hansen CE, Rajpert-De Meyts E, Skakkebaek NE. Environment, testicular dysgenesis and carcinoma in situ testis. *Best Pract Res Clin Endocrinol Metab* (2007) 21(3):462–78. doi: 10.1016/j.beem.2007.04.002

15. Lymperi S, Giwercman A. Endocrine disruptors and testicular function. *Metabol: Clin Exp* (2018) 86:79–90. doi: 10.1016/j.metabol.2018.03.022
16. Guillette LJ Jr., Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect* (1994) 102(8):680–8. doi: 10.1289/ehp.94102680
17. Facemire CF, Gross TS, Guillette LJJr. Reproductive impairment in the Florida panther: nature or nurture? *Environ Health Perspect* (1995) 103 (Suppl 4):79–86. doi: 10.1289/ehp.103-1519283
18. Meng X, de Rooij DG, Westerdahl K, Saarma M, Sariola H. Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. *Cancer Res* (2001) 61(8):3267–71.
19. Palmer JR, Herbst AL, Noller KL, Boggs DA, Troisi R, Titus-Ernstoff L, et al. Urogenital abnormalities in men exposed to diethylstilbestrol in utero: a cohort study. *Environ Health Global Access Sci Source* (2009) 8:37. doi: 10.1186/1476-069X-8-37
20. Fenichel P, Brucker-Davis F, Chevalier N. The history of Distilbene(R) (Diethylstilbestrol) told to grandchildren–the transgenerational effect. *Annal D'endocrinol* (2015) 76(3):253–9. doi: 10.1016/j.ando.2015.03.008
21. Strohsnitter WC, Noller KL, Hoover RN, Robboy SJ, Palmer JR, Titus-Ernstoff L, et al. Cancer risk in men exposed in utero to diethylstilbestrol. *J Natl Cancer Institute* (2001) 93(7):545–51. doi: 10.1093/jnci/93.7.545
22. Martin OV, Shialis T, Lester JN, Scrimshaw MD, Boobis AR, Voulvoulis N. Testicular dysgenesis syndrome and the estrogen hypothesis: a quantitative meta-analysis. *Environ Health Perspect* (2008) 116(2):149–57. doi: 10.1289/ehp.10545
23. Ryder SJ, Crawford PI, Pethybridge RJ. Is testicular cancer an occupational disease? A case-control study of Royal Naval personnel. *J R Naval Med Service* (1997) 83(3):130–46.
24. Hardell L, Malmqvist N, Ohlson CG, Westberg H, Eriksson M. Testicular cancer and occupational exposure to polyvinyl chloride plastics: a case-control study. *Int J Cancer* (2004) 109(3):425–9. doi: 10.1002/ijc.11709
25. Knight JA, Marrett LD, Weir HK. Occupation and risk of germ cell testicular cancer by histologic type in Ontario. *J Occup Environ Med* (1996) 38(9):884–90. doi: 10.1097/00043764-199609000-00010
26. Giannandrea F, Paoli D, Figa-Talamanca I, Lombardo F, Lenzi A, Gandini L. Effect of endogenous and exogenous hormones on testicular cancer: the epidemiological evidence. *Int J Dev Biol* (2013) 57(2-4):255–63. doi: 10.1387/ijdb.130015fg
27. Hardell L, van Bavel B, Lindstrom G, Carlberg M, Dreifaldt AC, Wijkstrom H, et al. Increased concentrations of polychlorinated biphenyls, hexachlorobenzene, and chlordanes in mothers of men with testicular cancer. *Environ Health Perspect* (2003) 111(7):930–4. doi: 10.1289/ehp.5816
28. Purdue MP, Engel LS, Langseth H, Needham LL, Andersen A, Barr DB, et al. Prediagnostic serum concentrations of organochlorine compounds and risk of testicular germ cell tumors. *Environ Health Perspect* (2009) 117(10):1514–9. doi: 10.1289/ehp.0800359
29. McGlynn KA, Quraishi SM, Graubard BI, Weber JP, Rubertone MV, Erickson RL. Persistent organochlorine pesticides and risk of testicular germ cell tumors. *J Natl Cancer Institute* (2008) 100(9):663–71. doi: 10.1093/jnci/djn101
30. Bonde JP, Flachs EM, Rimborg S, Glazer CH, Giwercman A, Ramlau-Hansen CH, et al. The epidemiologic evidence linking prenatal and postnatal exposure to endocrine disrupting chemicals with male reproductive disorders: a systematic review and meta-analysis. *Hum Reprod Update* (2016) 23(1):104–25. doi: 10.1093/humupd/dmw036
31. Cohn BA, Cirillo PM, Christianson RE. Prenatal DDT exposure and testicular cancer: a nested case-control study. *Arch Environ Occup Health* (2010) 65(3):127–34. doi: 10.1080/19338241003730887
32. Carreau S, Bourguiba S, Lambard S, Galeraud-Denis I, Genissel C, Levallet J. Reproductive system: aromatase and estrogens. *Mol Cell Endocrinol* (2002) 193(1-2):137–43. doi: 10.1016/S0303-7207(02)00107-7
33. Hess RA. Estrogen in the adult male reproductive tract: a review. *Reprod Biol Endocrinol RB&E* (2003) 1:52. doi: 10.1186/1477-7827-1-52
34. Carreau S, Bouraima-Lelong H, Delalande C. Role of estrogens in spermatogenesis. *Front Biosci* (2012) 4:1–11. doi: 10.2741/e356
35. Carreau S, Hess RA. Oestrogens and spermatogenesis. *Philos Trans R Soc London Ser B Biol Sci* (1546) 2010) 365:1517–35. doi: 10.1098/rstb.2009.0235
36. Boukari K, Ciampi ML, Guiochon-Mantel A, Young J, Lombes M, Meduri G. Human fetal testis: source of estrogen and target of estrogen action. *Hum Reprod* (2007) 22(7):1885–92. doi: 10.1093/humrep/dem091
37. Berensztein EB, Baquedano MS, Gonzalez CR, Saraco NI, Rodriguez J, Ponzio R, et al. Expression of aromatase, estrogen receptor alpha and beta, androgen receptor, and cytochrome P-450scc in the human early prepubertal testis. *Pediatr Res* (2006) 60(6):740–4. doi: 10.1203/01.pdr.0000246072.04663.bb
38. Makinen S, Makela S, Weihua Z, Warner M, Rosenlund B, Salmi S, et al. Localization of oestrogen receptors alpha and beta in human testis. *Mol Hum Reprod* (2001) 7(6):497–503. doi: 10.1093/molehr/7.6.497
39. Cavaco JE, Laurentino SS, Barros A, Sousa M, Socorro S. Estrogen receptors alpha and beta in human testis: both isoforms are expressed. *Syst Biol Reprod Med* (2009) 55(4):137–44. doi: 10.3109/19396360902855733
40. Saunders PT, Millar MR, Macpherson S, Irvine DS, Groome NP, Evans LR, et al. ERbeta1 and the ERbeta2 splice variant (ERbetacx/beta2) are expressed in distinct cell populations in the adult human testis. *J Clin Endocrinol Metab* (2002) 87(6):2706–15. doi: 10.1210/jcem.87.6.8619
41. Lambard S, Galeraud-Denis I, Saunders PT, Carreau S. Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors. *J Mol Endocrinol* (2004) 32(1):279–89. doi: 10.1677/jme.0.0320279
42. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, et al. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *New Engl J Med* (1994) 331(16):1056–61. doi: 10.1056/NEJM199410203311604
43. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* (2005) 307(5715):1625–30. doi: 10.1126/science.1106943
44. Jacenik D, Cygankiewicz AI, Krajewska WM. The G protein-coupled estrogen receptor as a modulator of neoplastic transformation. *Mol Cell Endocrinol* (2016) 429:10–8. doi: 10.1016/j.mce.2016.04.011
45. Xu S, Yu S, Dong D, Lee LTO. G Protein-Coupled Estrogen Receptor: A Potential Therapeutic Target in Cancer. *Front Endocrinol* (2019) 10:725:725. doi: 10.3389/fendo.2019.00725
46. Tao S, He H, Chen Q, Yue W. GPER mediated estradiol reduces miR-148a to promote HLA-G expression in breast cancer. *Biochem Biophys Res Commun* (2014) 451(1):74–8. doi: 10.1016/j.bbrc.2014.07.073
47. Teng Y, Radde BN, Litchfield LM, Ivanova MM, Prough RA, Clark BJ, et al. Dehydroepiandrosterone Activation of G-protein-coupled Estrogen Receptor Rapidly Stimulates MicroRNA-21 Transcription in Human Hepatocellular Carcinoma Cells. *J Biol Chem* (2015) 290(25):15799–811. doi: 10.1074/jbc.M115.641167
48. Vivacqua A, De Marco P, Santolla MF, Cirillo F, Pellegrino M, Panno ML, et al. Estrogenic gper signaling regulates mir144 expression in cancer cells and cancer-associated fibroblasts (cafs). *Oncotarget* (2015) 6(18):16573–87. doi: 10.18632/oncotarget.4117
49. Filardo EJ, Quinn JA, Bland KI, Frackelton AR Jr. Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* (2000) 14 (10):1649–60. doi: 10.1210/mend.14.10.0532
50. Filardo EJ. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *J Steroid Biochem Mol Biol* (2002) 80(2):231–8. doi: 10.1016/s0960-0760(01)00190-x
51. Recchia AG, De Francesco EM, Vivacqua A, Sisci D, Panno ML, Ando S, et al. The G protein-coupled receptor 30 is up-regulated by hypoxia-inducible factor-1alpha (HIF-1alpha) in breast cancer cells and cardiomyocytes. *J Biol Chem* (2011) 286(12):10773–82. doi: 10.1074/jbc.M110.172247
52. De Francesco EM, Lappano R, Santolla MF, Marsico S, Caruso A, Maggiolini M. HIF-1alpha/GPER signaling mediates the expression of VEGF induced by hypoxia in breast cancer associated fibroblasts (CAFs). *Breast Cancer Res* (2013) 15(4):R64. doi: 10.1186/bcr3458
53. De Marco P, Bartella V, Vivacqua A, Lappano R, Santolla MF, Morcavallo A, et al. Insulin-like growth factor-I regulates GPER expression and function in cancer cells. *Oncogene* (2013) 32(6):678–88. doi: 10.1038/onc.2012.97

54. Avino S, De Marco P, Cirillo F, Santolla MF, De Francesco EM, Perri MG, et al. Stimulatory actions of IGF-I are mediated by IGF-IR cross-talk with GPER and DDR1 in mesothelioma and lung cancer cells. *Oncotarget* (2016) 7(33):52710–28. doi: 10.18632/oncotarget.10348
55. Okamoto M, Mizukami Y. GPER negatively regulates TNFalpha-induced IL-6 production in human breast cancer cells via NF-kappaB pathway. *Endocr J* (2016) 63(5):485–93. doi: 10.1507/endocrj.EJ15-0571
56. Zhu P, Liao LY, Zhao TT, Mo XM, Chen GG, Liu ZM. GPER/ERK&AKT/NF-kappaB pathway is involved in cadmium-induced proliferation, invasion and migration of GPER-positive thyroid cancer cells. *Mol Cell Endocrinol* (2017) 442:68–80. doi: 10.1016/j.mce.2016.12.007
57. Feldman RD, Ding Q, Hussain Y, Limbird LE, Pickering JG, Gros R. Aldosterone mediates metastatic spread of renal cancer via the G protein-coupled estrogen receptor (GPER). *FASEB J* (2016) 30(6):2086–96. doi: 10.1096/fj.15-275552
58. Rigiracciolo DC, Scarpelli A, Lappano R, Pisano A, Santolla MF, Avino S, et al. GPER is involved in the stimulatory effects of aldosterone in breast cancer cells and breast tumor-derived endothelial cells. *Oncotarget* (2016) 7 (1):94–111. doi: 10.18632/oncotarget.6475
59. Parker BM, Wertz SL, Pollard CM, Desimine VL, Maning J, McCrink KA, et al. Novel Insights into the Crosstalk between Mineralocorticoid Receptor and G Protein-Coupled Receptors in Heart Adverse Remodeling and Disease. *Int J Mol Sci* (2018) 19(12):3764. doi: 10.3390/ijms19123764
60. Chevalier N, Vega A, Bouskine A, Siddeek B, Michiels JF, Chevallier D, et al. GPR30, the non-classical membrane G protein related estrogen receptor, is overexpressed in human seminoma and promotes seminoma cell proliferation. *PloS One* (2012) 7(4):e34672. doi: 10.1371/journal.pone.0034672
61. Rago V, Giordano F, Brunelli E, Zito D, Aquila S, Carpino A. Identification of G protein-coupled estrogen receptor in human and pig spermatozoa. *J Anat* (2014) 224(6):732–6. doi: 10.1111/joa.12183
62. Fenichel P, Chevalier N. Is Testicular Germ Cell Cancer Estrogen Dependent? The Role of Endocrine Disrupting Chemicals. *Endocrinology* (2019) 160(12):2981–9. doi: 10.1210/en.2019-00486
63. Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, et al. Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* (2006) 12(21):6359–66. doi: 10.1158/1078-0432.CCR-06-0860
64. Smith HO, Arias-Pulido H, Kuo DY, Howard T, Qualls CR, Lee SJ, et al. GPR30 predicts poor survival for ovarian cancer. *Gynecol Oncol* (2009) 114 (3):465–71. doi: 10.1016/j.ygyno.2009.05.015
65. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* (2008) 70:165–90. doi: 10.1146/annurev.physiol.70.113006.100518
66. Roger C, Lambard S, Bouskine A, Mograbi B, Chevallier D, Nebout M, et al. Estrogen-induced growth inhibition of human seminoma cells expressing estrogen receptor beta and aromatase. *J Mol Endocrinol* (2005) 35(1):191–9. doi: 10.1677/jme.1.01704
67. Bouskine A, Nebout M, Mograbi B, Brucker-Davis F, Roger C, Fenichel P. Estrogens promote human testicular germ cell cancer through a membrane-mediated activation of extracellular regulated kinase and protein kinase A. *Endocrinology* (2008) 149(2):565–73. doi: 10.1210/en.2007-1318
68. Franco R, Boscia F, Gigantino V, Marra L, Esposito F, Ferrara D, et al. GPR30 is overexpressed in post-puberal testicular germ cell tumors. *Cancer Biol Ther* (2011) 11(6):609–13. doi: 10.4161/cbt.11.6.14672
69. Boscia F, Passaro C, Gigantino V, Perdona S, Franco R, Portella G, et al. High levels of GPR30 protein in human testicular carcinoma in situ and seminomas correlate with low levels of estrogen receptor-beta and indicate a switch in estrogen responsiveness. *J Cell Physiol* (2015) 230(6):1290–7. doi: 10.1002/jcp.24864
70. Rago V, Romeo F, Giordano F, Maggiolini M, Carpino A. Identification of the estrogen receptor GPER in neoplastic and non-neoplastic human testes. *Reprod Biol Endocrinol RB&E* (2011) 9:135. doi: 10.1186/1477-7827-9-135
71. Chevalier N, Bouskine A, Fenichel P. Bisphenol A promotes testicular seminoma cell proliferation through GPER/GPR30. *Int J Cancer* (2012) 130(1):241–2. doi: 10.1002/ijc.25972
72. Pais V, Leav I, Lau KM, Jiang Z, Ho SM. Estrogen receptor-beta expression in human testicular germ cell tumors. *Clin Cancer Res* (2003) 9(12):4475–82.
73. Ferlin A, Ganz F, Pengo M, Selice R, Frigo AC, Foresta C. Association of testicular germ cell tumor with polymorphisms in estrogen receptor and steroid metabolism genes. *Endocrine-Related Cancer* (2010) 17(1):17–25. doi: 10.1677/ERC-09-0176
74. Brokken LJ, Lundberg-Giwercman Y, Rajpert De-Meyts E, Eberhard J, Stahl O, Cohn-Cedermark G, et al. Association of polymorphisms in genes encoding hormone receptors ESR1, ESR2 and LHCGR with the risk and clinical features of testicular germ cell cancer. *Mol Cell Endocrinol* (2012) 351 (2):279–85. doi: 10.1016/j.mce.2011.12.018
75. Chevalier N, Paul-Bellon R, Camparo P, Michiels JF, Chevallier D, Fenichel P. Genetic variants of GPER/GPR30, a novel estrogen-related G protein receptor, are associated with human seminoma. *Int J Mol Sci* (2014) 15 (1):1574–89. doi: 10.3390/ijms15011574
76. Zama AM, Uzumcu M. Fetal and neonatal exposure to the endocrine disruptor methoxychlor causes epigenetic alterations in adult ovarian genes. *Endocrinology* (2009) 150(10):4681–91. doi: 10.1210/en.2009-0499
77. Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* (2005) 308 (5727):1466–9. doi: 10.1126/science.1108190
78. Dumasia K, Kumar A, Deshpande S, Balasinor NH. Estrogen signaling, through estrogen receptor beta, regulates DNA methylation and its machinery in male germ line in adult rats. *Epigenetics* (2017) 12(6):476–83. doi: 10.1080/15592294.2017.1309489
79. Markulin D, Vojta A, Samarzija I, Gamulin M, Beceheli I, Jukic I, et al. Association Between RASSF1A Promoter Methylation and Testicular Germ Cell Tumor: A Meta-analysis and a Cohort Study. *Cancer Genomics Proteomics* (2017) 14(5):363–72. doi: 10.21873/cgp.20046
80. Killian JK, Dorssers LC, Trabert B, Gillis AJ, Cook MB, Wang Y, et al. Imprints and DPPA3 are bypassed during pluripotency- and differentiation-coupled methylation reprogramming in testicular germ cell tumors. *Genome Res* (2016) 26(11):1490–504. doi: 10.1101/gr.201293.115
81. Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, et al. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* (2006) 124(6):1169–81. doi: 10.1016/j.cell.2006.02.037
82. Lobo J, Gillis AJM, Jeronimo C, Henrique R, Looijenga LHJ. Human Germ Cell Tumors are Developmental Cancers: Impact of Epigenetics on Pathobiology and Clinic. *Int J Mol Sci* (2019) 20(2):258. doi: 10.3390/ijms20020258
83. Almstrup K, Nielsen JE, Mlynarska O, Jansen MT, Jorgensen A, Skakkebaek NE, et al. Carcinoma in situ testis displays permissive chromatin modifications similar to immature foetal germ cells. *Br J Cancer* (2010) 103 (8):1269–76. doi: 10.1038/sj.bjc.6605880
84. Bouskine A, Vega A, Nebout M, Benahmed M, Fenichel P. Expression of embryonic stem cell markers in cultured JKT-1, a cell line derived from a human seminoma. *Int J Androl* (2010) 33(1):54–63. doi: 10.1111/j.1365-2605.2009.00950.x
85. Bouskine A, Nebout M, Brucker-Davis F, Benahmed M, Fenichel P. Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. *Environ Health Perspect* (2009) 117(7):1053–8. doi: 10.1289/ehp.0800367
86. Chevalier N, Fenichel P. Endocrine disruptors: new players in the pathophysiology of type 2 diabetes? *Diabetes Metab* (2015) 41(2):107–15. doi: 10.1016/j.diabet.2014.09.005
87. Fenichel P, Dechaux H, Harthe C, Gal J, Ferrari P, Pacini P, et al. Unconjugated bisphenol A cord blood levels in boys with descended or undescended testes. *Hum Reprod* (2012) 27(4):983–90. doi: 10.1093/humrep/der451
88. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocrine Rev* (2009) 30(1):75–95. doi: 10.1210/er.2008-0021
89. Fenichel P, Chevalier N, Brucker-Davis F. Bisphenol A: an endocrine and metabolic disruptor. *Annal D'endocrinol* (2013) 74(3):211–20. doi: 10.1016/j.ando.2013.04.002
90. Pupo M, Pisano A, Lappano R, Santolla MF, De Francesco EM, Abonante S, et al. Bisphenol A induces gene expression changes and proliferative effects

through GPER in breast cancer cells and cancer-associated fibroblasts. *Environ Health Perspect* (2012) 120(8):1177–82. doi: 10.1289/ehp.1104526
91. Vandenberg LN. Non-monotonic dose responses in studies of endocrine disrupting chemicals: bisphenol a as a case study. *Dose Response* (2014) 12 (2):259–76. doi: 10.2203/dose-response.13-020.Vandenberg
92. Chevalier N, Paul-Bellon R, Fenichel P. Comment on "Effects of Atrazine on Estrogen Receptor alpha- and G Protein-Coupled Receptor 30-Mediated Signaling and Proliferation in Cancer Cells and Cancer-Associated Fibroblasts". *Environ Health Perspect* (2016) 124(4):A64–5. doi: 10.1289/ehp.1510927
93. Ajj H, Chesnel A, Pinel S, Plenat F, Flament S, Dumond H. An alkylphenol mix promotes seminoma derived cell proliferation through an ERalpha36-mediated mechanism. *PloS One* (2013) 8(4):e61758. doi: 10.1371/journal.pone.0061758
94. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF. Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. *Biochem Biophys Res Commun* (2005) 336(4):1023–7. doi: 10.1016/j.bbrc.2005.08.226
95. Wallacides A, Chesnel A, Ajj H, Chillet M, Flament S, Dumond H. Estrogens promote proliferation of the seminoma-like TCam-2 cell line through a GPER-dependent ERalpha36 induction. *Mol Cell Endocrinol* (2012) 350 (1):61–71. doi: 10.1016/j.mce.2011.11.021
96. Chimento A, De Luca A, Nocito MC, Avena P, La Padula D, Zavaglia L, et al. Role of GPER-Mediated Signaling in Testicular Functions and Tumorigenesis. *Cells* (2020) 9(9):2115. doi: 10.3390/cells9092115
97. Filardo EJ. A role for G-protein coupled estrogen receptor (GPER) in estrogen-induced carcinogenesis: Dysregulated glandular homeostasis, survival and metastasis. *J Steroid Biochem Mol Biol* (2018) 176:38–48. doi: 10.1016/j.jsbmb.2017.05.005
98. De Francesco EM, Maggiolini M, Musti AM. Crosstalk between Notch, HIF-1alpha and GPER in Breast Cancer EMT. *Int J Mol Sci* (2018) 19(7):2011. doi: 10.3390/ijms19072011
99. De Francesco EM, Sims AH, Maggiolini M, Sotgia F, Lisanti MP, Clarke RB. GPER mediates the angiocrine actions induced by IGF1 through the HIF-1alpha/VEGF pathway in the breast tumor microenvironment. *Breast Cancer Res* (2017) 19(1):129. doi: 10.1186/s13058-017-0923-5
100. Rice A, Cortes E, Lachowski D, Oertle P, Matellan C, Thorpe SD, et al. GPER Activation Inhibits Cancer Cell Mechanotransduction and Basement Membrane Invasion via RhoA. *Cancers (Basel)* (2020) 12(2):289. doi: 10.3390/cancers12020289
101. Chieffi P. An up-date on novel molecular targets in testicular germ cell tumors subtypes. *Intractable Rare Dis Res* (2019) 8(2):161–4. doi: 10.5582/irdr.2019.01055
102. Prossnitz ER, Arterburn JB. International Union of Basic and Clinical Pharmacology. XCVII. G Protein-Coupled Estrogen Receptor and Its Pharmacologic Modulators. *Pharmacol Rev* (2015) 67(3):505–40. doi: 10.1124/pr.114.009712
103. Zacarias-Lara OJ, Mendez-Luna D, Martinez-Ruiz G, Garcia-Sanchez JR, Fragoso-Vazquez MJ, Bello M, et al. Synthesis and In Vitro Evaluation of Tetrahydroquinoline Derivatives as Antiproliferative Compounds of Breast Cancer via Targeting the GPER. *Anticancer Agents Med Chem* (2019) 19 (6):760–71. doi: 10.2174/1871520618666181119094144
104. DeLeon C, Wang HH, Gunn J, Wilhelm M, Cole A, Arnett S, et al. A novel GPER antagonist protects against the formation of estrogen-induced cholesterol gallstones in female mice. *J Lipid Res* (2020) 61(5):767–77. doi: 10.1194/jlr.RA119000592
105. Liu C, Liao Y, Fan S, Fu X, Xiong J, Zhou S, et al. G-Protein-Coupled Estrogen Receptor Antagonist G15 Decreases Estrogen-Induced Development of Non-Small Cell Lung Cancer. *Oncol Res* (2019) 27 (3):283–92. doi: 10.3727/096504017X15035795904677
106. Liu C, Liao Y, Fan S, Tang H, Jiang Z, Zhou B, et al. G protein-coupled estrogen receptor (GPER) mediates NSCLC progression induced by 17beta-estradiol (E2) and selective agonist G1. *Med Oncol* (2015) 32(4):104. doi: 10.1007/s12032-015-0558-2
107. De Francesco EM, Pellegrino M, Santolla MF, Lappano R, Ricchio E, Abonante S, et al. GPER mediates activation of HIF1alpha/VEGF signaling by estrogens. *Cancer Res* (2014) 74(15):4053–64. doi: 10.1158/0008-5472.CAN-13-3590
108. Santolla MF, Avino S, Pellegrino M, De Francesco EM, De Marco P, Lappano R, et al. SIRT1 is involved in oncogenic signaling mediated by GPER in breast cancer. *Cell Death Dis* (2015) 6:e1834. doi: 10.1038/cddis.2015.201
109. Natale CA, Li J, Pitarresi JR, Norgard RJ, Dentchev T, Capell BC, et al. Pharmacologic Activation of the G Protein-Coupled Estrogen Receptor Inhibits Pancreatic Ductal Adenocarcinoma. *Cell Mol Gastroenterol Hepatol* (2020). 10(4):868–80.e1. doi: 10.1016/j.jcmgh.2020.04.016
110. Chimento A, Sirianni R, Casaburi I, Zolea F, Rizza P, Avena P, et al. GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth in vitro and in vivo. *Oncotarget* (2015) 6(22):19190–203. doi: 10.18632/oncotarget.4241
111. Casaburi I, Avena P, De Luca A, Sirianni R, Rago V, Chimento A, et al. GPER-independent inhibition of adrenocortical cancer growth by G-1 involves ROS/Egr-1/BAX pathway. *Oncotarget* (2017) 8(70):115609–19. doi: 10.18632/oncotarget.23314
112. De Francesco EM, Rocca C, Scavello F, Amelio D, Pasqua T, Rigiracciolo DC, et al. Protective Role of GPER Agonist G-1 on Cardiotoxicity Induced by Doxorubicin. *J Cell Physiol* (2017) 232(7):1640–9. doi: 10.1002/jcp.25585
113. da Silva JS, Sun X, Ahmad S, Wang H, Sudo RT, Varagic J, et al. G-Protein-Coupled Estrogen Receptor Agonist G1 Improves Diastolic Function and Attenuates Cardiac Renin-Angiotensin System Activation in Estrogen-Deficient Hypertensive Rats. *J Cardiovasc Pharmacol* (2019) 74(5):443–52. doi: 10.1097/FJC.0000000000000721
114. Barton M, Meyer MR, Prossnitz ER. Nox1 downregulators: A new class of therapeutics. *Steroids* (2019) 152:108494. doi: 10.1016/j.steroids.2019.108494
115. Tutzauer J, Sjostrom M, Bendahl PO, Ryden L, Ferno M, Leeb-Lundberg LMF, et al. Plasma membrane expression of G protein-coupled estrogen receptor (GPER)/G protein-coupled receptor 30 (GPR30) is associated with worse outcome in metachronous contralateral breast cancer. *PloS One* (2020) 15(4):e0231786. doi: 10.1371/journal.pone.0231786
116. Sjostrom M, Hartman L, Grabau D, Fornander T, Malmstrom P, Nordenskjold B, et al. Lack of G protein-coupled estrogen receptor (GPER) in the plasma membrane is associated with excellent long-term prognosis in breast cancer. *Breast Cancer Res Treat* (2014) 145(1):61–71. doi: 10.1007/s10549-014-2936-4
117. Ignatov A, Ignatov T, Weissenborn C, Eggemann H, Bischoff J, Semczuk A, et al. G-protein-coupled estrogen receptor GPR30 and tamoxifen resistance in breast cancer. *Breast Cancer Res Treat* (2011) 128(2):457–66. doi: 10.1007/s10549-011-1584-1

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G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients

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Purpose: Wnt pathway modulator Dickkopf 2 (Dkk2) and signaling of the G protein-coupled estrogen receptor (GPER) seem to have essential functions in numerous cancer types. For epithelial ovarian cancer (EOC), it has not been proven if either Dkk2 or the GPER on its own have an independent impact on overall survival (OS). So far, the correlation of both factors and their clinical significance has not systematically been investigated before.

Methods: Expression levels of Dkk2 were immunohistochemically analyzed in 156 patient samples from different histologic subtypes of EOC applying the immune-reactivity score (IRS). Expression analyses were correlated with clinical and pathological parameters to assess for prognostic relevance. Data analysis was performed using Spearman's correlations, Kruskal-Wallis-test and Kaplan-Meier estimates.

Results: Highest Dkk2 expression of all subtypes was observed in clear cell carcinoma. In addition, Dkk2 expression differed significantly ($p<0.001$) between low and high grade serous ovarian cancer. A significant correlation of Dkk2 with the cytoplasmic GPER expression was noted ($p=0.001$) but not for the nuclear estrogen receptor alpha (ERα) or beta (ERβ). Patients exhibiting both, high expression Dkk2 (IRS>4) and GPER (IRS>8), had a significantly better overall survival compared to patients with low expression (61 months vs. 33 months; $p=0.024$).

Conclusion: Dkk2 and GPER expression correlates in EOC and combined expression of both is associated with improved OS. These findings underline the clinical significance of both pathways and indicate a possible prognostic impact as well as a potential for treatment strategies addressing interactions between estrogen and Wnt signaling in ovarian cancer.

Keywords: Dickkopf 2, G protein-coupled estrogen receptor, Wnt signaling, estrogen, epithelial ovarian cancer

INTRODUCTION

Epithelial ovarian cancer (EOC) causes most deaths of gynecological malignancies (1) with a relative 5-year survival of almost 45% (2). The need to identify suitable screening methods, prognostic markers and efficient therapies is crucial. So far, standard treatment for primary disease consists of debulking surgery and a platinum-based chemotherapy with antiangiogenics and/or Poly-ADP-Ribose-Polymerase (PARP) inhibitors (3). Apart from clinicopathological aspects such as the stage in the system of the International Federation of Gynecology and Obstetrics (FIGO), volume of residual disease after debulking surgery, patients' age, and histological subtype (4–7), there are no reliable prognostic factors to predict the clinical course. With regards to the molecular background and specific gene mutations, EOC is histologically separated into clear cell, endometrioid, mucinous, and serous carcinoma of low or high grade (LGSC/HGSC) (8).

Revealing molecular events that cause ovarian cancer and are responsible for its progression represent a major challenge for translational research. One approach is to understand the importance and complexity of the Wnt signaling pathway and its regulation (9–11). Secreted Wnt glycoproteins translate their function *via* binding to Frizzled receptors and co-receptors such as low-density-lipoprotein-related protein 5/6 (LRP5/6) (11). Subsequently, Wnt proteins exhibit their effects on several cellular processes by activating either the canonical Wnt/β-catenin or at least two non-canonical β-catenin-independent pathways (12). Alterations in Wnt signaling components, such as APC (adenomatous polyposis coli) protein, AXIN and β-catenin and downregulation of modulatory Wnt antagonists have been described to be involved in the onset of several cancer types (10, 13, 14). As a consequence, modulators of the Wnt pathway like members of the Dickkopf family (Dkk1-4) may play an essential role during development (15, 16) and tumorigenesis (17, 18). Dkks bind to LRP5/6 with higher affinity than Wnt (19). Dkk2 seems able to act as agonist as well as antagonist for Wnt/LRP6 signaling depending on the cellular context and therefore co-factors such as krm2 (18–20). In EOC Zhu et al. suggest that Dkk2 may functions as a Wnt pathway inhibitor (13).

Estrogen (E2, 17β-estradiol) has numerous cellular functions in the human body including gynecologic cancer biology (21) and interactions between estrogen and Wnt signaling have been described (22–25). In this context an interplay of Dkk2 and estrogen receptors (ER) could link these two mechanisms and classical nuclear ERα or ERβ as well as the G protein-coupled estrogen receptor (GPER) could be involved in this process.

GPER is a transmembrane receptor with intracellular domains binding E2 (26), which mediates rapid non-genomic estrogen signaling (27). Its activation *via* agonists like G1 or E2 (28) leads to cAMP production (29), activation of extracellular signal-related kinase 1 and 2 (Erk1/2) (28), mobilization of intracellular Ca^{2+}, phosphatidylinositol 3-kinase (PI3K) activation (26) and the induction of metalloproteinases which then transactivates the epidermal growth factor receptor (30). GPER can also indirectly impact gene transcription (31). Since its role in ovarian cancer has been conflicting so far (32–34) this analysis focused on the correlation of Dkk2 with GPER to identify a possible link between Wnt and estrogen and investigating their potential prognostic significance.

METHODS

Patients

In this study 156 formalin-fixated and paraffin-embedded tissue specimens of epithelial ovarian cancer from patients who had been treated in the Department of Obstetrics and Gynecology at Ludwig-Maximilians-University of Munich between 1990 and 2002 were analyzed. Numerous markers were already examined in this collective in preceding studies (35–37). Clinical data was collected from the patient's charts and information about the follow up was acquired from the Munich Cancer Registry.

Only patients with malignant, non-borderline tumors were included in the study. Seventy-three patients (46.8%) were older or age 60 years at the initial diagnosis and 83 patients (53.2%) were younger than 60 years. There were no data available about estrogen replacement therapy in postmenopausal women. Pathologists categorized the histological subtypes of the samples: LGSC (n=24), HGSC (n=80), endometrioid (n=21), clear cell (n=12), mucinous (n=13). According to the updated FIGO classification from 2014, specimens of serous ovarian cancer were re-evaluated and attributed to low-grade (G1) and high-grade (G3) histology. Endometrioid and mucinous ovarian cancer samples were related to G1, G2, and G3. Clear cell cancer was always categorized as G3 (38). Staging was done following the FIGO classification: I (n=35), II (n=10), III (n=103), IV (n=3) (**Table 1**).

Sampling and Microarray Construction

Three core biopsies for each EOC patient were taken from paraffin-embedded and formalin-fixed tumor blocks in our archive. The biopsies were assembled in tissue microarrays (TMA) paraffin blocks. Those TMA paraffin blocks were cut into serial sections at 2 μm and fixed on slides. A pathologist verified that representative areas of the tumor were aligned on the slides.

Immunohistochemistry

Immunohistochemical staining of paraffin-embedded and formalin-fixed tissue micro arrays of ovarian cancer specimens for Dkk2 was performed as previously described (39). The TMA slides were dewaxed in Roticlear (Carl Roth Karlsruhe,

Abbreviations: Dkk2, Dickkopf2; EOC, epithelial ovarian cancer; E2, estrogen, 17β-estradiol; ERα, nuclear estrogen receptor alpha; ERβ, nuclear estrogen receptor alpha beta; Erk1, extracellular signal-related kinase 1; Erk2, extracellular signal-related kinase 2; GPER, G protein-coupled estrogen receptor; HGSC, high-grade serous carcinoma; IRS, immune-reactivity score; LGSC, low-grade serous carcinoma; LRP5, low-density-lipoprotein-related protein 5; LRP6, low-density-lipoprotein-related protein 6; OS, overall survival; PI3K, phosphatidylinositol 3-kinase; ROC curve, receiver operating characteristics curve; TCF, T-cell factor; TMA, tissue microarrays.

TABLE 1 | Correlation between Dkk2 expression and clinicopathologic characteristics of ovarian cancer patients.

Characteristics		Total	Dkk2 low expression	Dkk2 high expression	P value
		Number of cases(%)	Number of cases	Number of cases	
Age(y)					
	≥60y	73 (46.8)	28	21	**<0.001**
	<60y	83 (53.2)	17	38	
Tumor histology					
	LGSC	24 (15.4)	2	17	**0.001**
	HGSC	80 (51.3)	28	27	
	Clear cell	12 (7.7)	0	6	
	Endometrioid	21 (13.5)	7	4	
	Mucinous	13 (8.3)	6	2	
	Missing	6 (3.8)			
FIGO					
	I	35 (22.4)	8	14	0.615
	II	10 (6,4)	2	4	
	III	103 (66.0)	33	39	
	IV	3 (1.9)	1	0	
	Missing	5 (3.2)			
Expression of GPER					
low expression (IRS ≤ 8)		83 (53.2)	33	28	**0.005**
high expression (IRS>8)		70 (44.9)	11	31	

Dkk2, Dickkopf2; GPER, G protein-coupled estrogen receptor; HGSC, high-grade serous carcinoma; LGSC, low-grade serous carcinoma; FIGO, International Federation of Gynecology and Obstetrics.
Bold numbers represent p-values < 0.05.

Germany) for 20 min. The endogenous peroxidase was suppressed with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol (20 min). The specimens were rehydrated in a descending alcohol series (100%, 70%, 50% ethanol). The epitopes were retrieved by putting the slides in a pressure cooker with sodium citrate buffer (pH 6.0) for 5 min. After cooling to room temperature, the slides were washed in in distilled water and phosphate-buffered saline (PBS). To evade unspecific staining reagent 1 of the polymer kit (ZytoChem Plus HRP Polymer System, Berlin, Germany) was administered for 5 min. Next the slides incubated at +4°C for 16 h with the primary anti-body Anti-Dkk2 polyclonal rabbit IgG (ProteinTech, Manchester, UK). As negative controls the primary antibody was replaced by normal rabbit immunoglobulin G([IgG] supersensitive rabbit negative control; BioGenex, Fremont, California). Washing in PBS and the application of reagents 2 (20 min) and 3 (30 min) of the polymer kit anticipated the substrate-staining with chromogen diaminobenzidine (Dako, Hamburg, Germany). Counterstaining in Mayer acidic hematoxylin (Waldeck-Chroma, Münster, Germany) and dehydration in an ascending series of alcohol followed by Roticlear was performed. Cervical tissue was served as positive control.

Using a microscope (Leitz, Wetzlar, Germany) the immune-reactivity score (IRS) was applied to assess the immunostaining extent semi-quantitatively by two blinded examiners. The IRS is composed of the staining intensity (0=negative, 1=low, 2=moderate, 3=strong) multiplied with the percentage of stained cells (0=no staining, 1%≤10% positive cells, 2 = 11%–50% positive cells, 3 = 51%–80% positive cells, 4%≥81% positive cells). The immunoreactivity score ranges from 0 to 2: negative, 3 to 4: weak, 6 to 8: moderate, and 9 to 12: strong (40). Formerly published staining results of GPER in this panel recorded in the archive of the laboratory were recaptured (36).

Staining Evaluation

In order to define reliable cut-off points for the IRS of the Dkk2 staining the receiver operating characteristics (ROC) curve was used. ROC curve illustrates sensitivity on the y-axis plotted against (1-specificity) on x-axis (41). With Youden Index (42) the optimal cut-off was defined with highest possible values for sensitivity and specificity. For the Dkk2 staining IRS 0-4 was considered as weak and IRS 6-12 as high. Regarding its components, the IRS can never have a value of 5. GPER expression was divided into low (IRS ≤ 8) vs. high (IRS>8) according to the median (36).

Statistical Analysis

Statistical analysis was operated with SPSS 25 (IBM, Chicago, IL, USA). With the Kruskal-Wallis analysis the null hypothesis was tested against its opposite. Further Spearman's correlation analysis and Kruskal-Wallis analysis was applied for testing correlation of Dkk2 and GPER scores. The Kaplan-Meier estimate was used for analyzing times to event variables. Correlations between mean Dkk2 expression and clinicopathologic characteristics were assessed with Chi-Square tests (**Table 1**, crosstab). For all tests p-values ≤ 0.05 were considered as statistically significant. Figures were designed with SPSS 25 and Microsoft Power Point 2016 (Microsoft, Redmond, WA, USA).

RESULTS

Correlations between Dkk2 expression and clinicopathologic characteristics of EOC patients are displayed in **Table 1**. A median IRS of 6 for anti-Dkk2 staining was observed in the 131 of 152 cases (86%) with adequate staining. Applying ROC curve analysis, an IRS>4 was selected as cut-off.

Dkk2 expression differed significantly between the histological cancer subtypes (**Figures 1A–F**) with clear cell carcinomas showing the highest median IRS of 12 compared to the other subtypes (range: 9–12; p<0.001). While endometrioid and mucinous EOCs exhibited both a median IRS of 4, the overall cohort of serous EOCs had moderate staining extent at IRS of 6 which subdivided into high-grade serous histology with an IRS of 4 (range 0–12) and significantly higher for low-grade serous histology with an IRS of 6 (range 4–12; p<0.001).

Performing correlation analysis of Dkk2 expression and clinicopathological parameters such as distant metastasis, affected lymph nodes, FIGO classification, and grading, no significant results were found. In addition, Dkk2 expression was examined in comparison to other potentially pathological

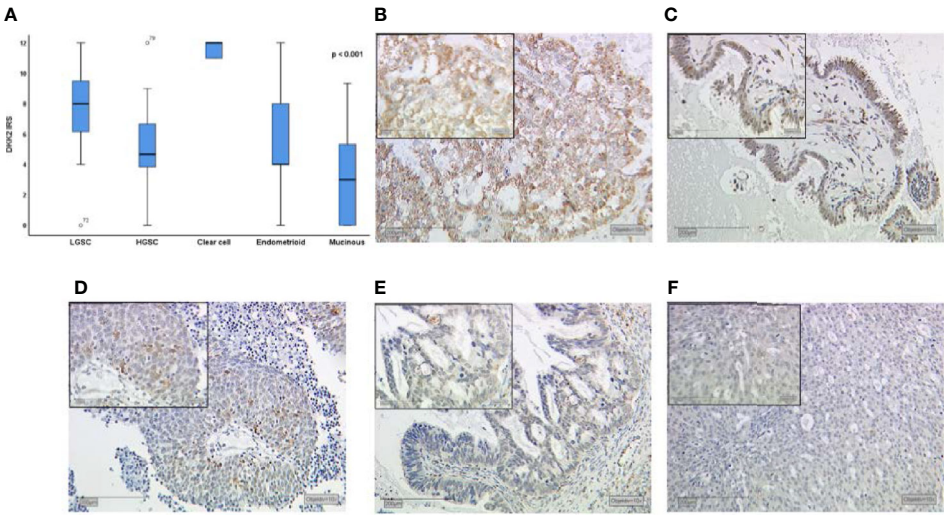

FIGURE 1 | Dkk2 expression patterns in different histological subtypes of EOC after immunohistochemical staining was performed as shown in a Kruskal-Wallis analysis for histological subtypes **(A)**. Clear cell carcinomas **(B)** presented the strongest staining patterns. Low-grade serous carcinomas (LGSC; **C**) had shown moderate Dkk2 expression. For endometrioid **(D)**, mucinous **(E)** and high-grade serous carcinomas (HGSC; **F**) the median IRS was lower. Scale bares equal 200 µm.

markers with a possible impact on the prognosis of EOC. Cytoplasmatic Dkk2 was observed to correlate significantly with cytoplasmic GPER expression (cc=0.304, p=0.001). Further analysis revealed that high Dkk2 expression is correlated to high GPER expression (**Figure 2**). In contrast, Dkk2 did not correlate with either ERα or ERβ expression (**Table 2**).

Patients with high Dkk2 expression (IRS>4) exhibited longer OS with a median of 65 months compared to 35 months in Kaplan-Meier analysis, although this difference was not

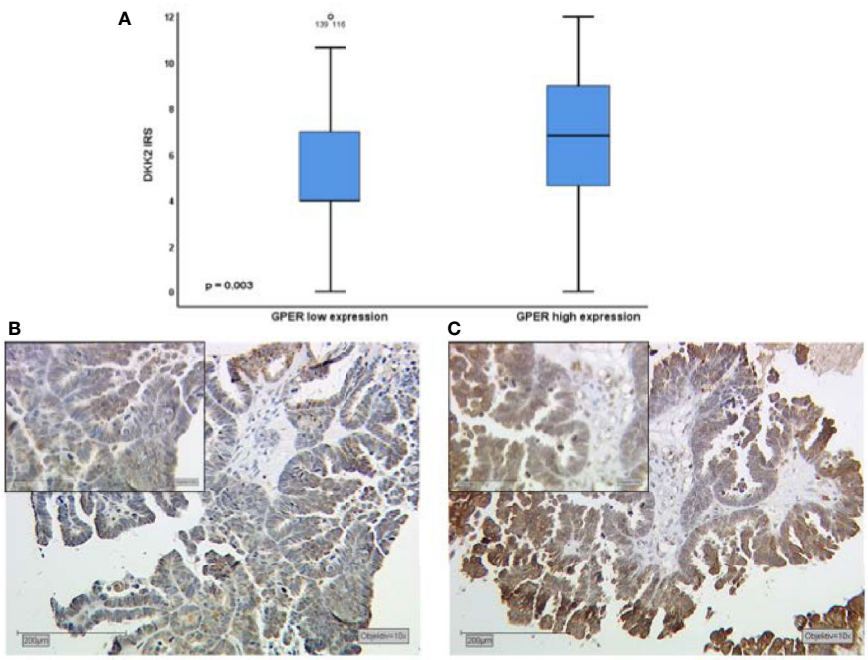

FIGURE 2 | Kruskal-Wallis analysis for correlation of Dkk2 and GPER expression **(A)**. High expression of Dkk2 **(B)** correlates with high GPER expression **(C)** in tissue samples of the same patient. Scale bares equal 200 µm.

TABLE 2 | Results of Spearman's correlation analysis of Dkk2 with the different estrogen receptors (GPER, ERα, ERβ).

Staining	DKK2	GPER	*ERα*	ERβ
DKK2				
cc	1.000	**0.304**	0.092	-0.080
p	.	**0.001**	0.298	0.366
n	125	124	131	128

Dkk2, Dickkopf 2; GPER, G protein-coupled estrogen receptor; cc, correlation coefficient; p, two-tailed significance; n, number of patients.
Bold numbers represent p-values < 0.05.

statistically significant (p=0.207; **Figure 3A**). The same trend was observed for GPER expression as published before with longer OS for patients with high expression but without statistical significance (36). When the expression analyses of the two markers were combined, patients with high Dkk2 (IRS>4) as well as high GPER (IRS>8) expression had a significantly longer OS with 61 months compared to 33 months in patients with low expression of both influenced OS (p=0.024; **Figure 3B**).

DISCUSSION

Dkk2 as a Wnt/β-catenin antagonist may play an important role in ovarian cancer (13, 18, 43). In this analysis, we investigated the expression of Dkk2 in the different histological subtypes of epithelial ovarian cancer, its relation to clinicopathological aspects and its impact on OS. Clear cell carcinoma exhibited

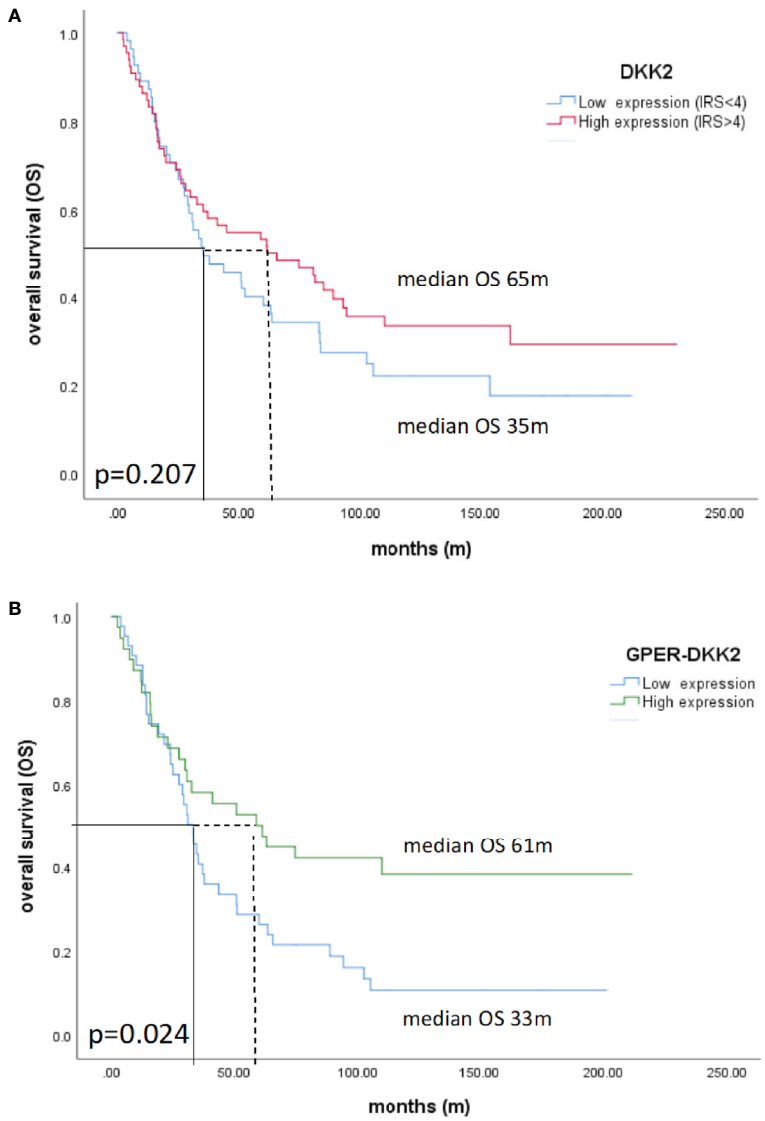

FIGURE 3 | Kaplan-Meier estimates of Dkk2 **(A)** and Dkk2 combined with GPER expression **(B)** were analyzed. Though not statistically significant, high cytoplasmic Dkk2 **(A)** and GPER (36) expression was connoted with a longer OS. Patients with carcinomas highly expressing both Dkk2 and GPER in the cytoplasm compared to patients with carcinomas lowly expressing Dkk2 and GPER showed significantly (61 months vs. 33 months, p=0.024) increased OS **(B)**.

the highest Dkk2 expression at all and LGSC showed significantly higher expression compared to the other histologies, which could reflect the different pathogenesis and origins of the histological subtypes (44).

In a previous study from Zhu et al. it has been shown that Dkk2 is frequently methylated and therefore epigenetically silenced in ovarian cancer. Lower Dkk2 expression levels correlated with tumor progression and advanced tumor stages (FIGO III-IV). By treating mice with the DNA methyltransferase inhibitor 5- aza-2′-deoxycitidine (decitabine) in order to re-establish Dkk2 expression in mice tumor growth was impaired (13). This is in accordance with our findings, suggesting an impact of Dkk2 on OS although this was not significant. Seemingly aberrant DNA methylation patterns also play a major role in platinum resistance, therefore the potential of epigenetic modulator decitabine to restore sensitivity towards platinum has been successfully tested in a phase II clinical trial (45). So far, agents for epigenetic therapy may cause severe adverse effects, in particular when they are administered in combination with chemotherapy. This underscores the necessity of more selective epigenetic modulators (46).

The impact of GPER on the OS of ovarian cancer patients has been controversially discussed so far (32–34). The conflicting results in these studies may arise from application of different concentrations for the agonists E2 and G1 and the investigation in different cancer cell lines. Accounting for these and the current results, GPER may not be sufficient to predict OS on its own. However, in combination with other factors like Luteinizing Hormone/Choriogonadotropin Receptor and Follicle Stimulating Hormone Receptor (36) or Dkk2, it could serve as a positive prognostic factor for patients suffering from epithelial ovarian cancer.

As previous studies elucidated a possible connection between estrogen and Wnt signaling (22–25), we investigated the relationship of Dkk2 with estrogen receptors. Subcellular localization of the DKK2 staining pattern was noted which has been previously attributed to the Golgi apparatus (www.proteinatlas.org). Unlike other studies in breast cancer which have shown an association between plasma membrane expression and outcome, plasma membrane expression of GPER was not detected in the ovarian cancer samples evaluated here (47). We could demonstrate a strong correlation of high cytoplasmic Dkk2 and high cytoplasmic GPER expression levels in EOC samples. In contrast, no correlation of Dkk2 with the traditional estrogen receptors ERα or ERβ was noted. To the best of our knowledge, a possible connection of GPER and Dkk2 has not yet been investigated. The described association of higher Dkk2 expression in younger patients may be reflected by more patients in premenopausal status and therefore relate to the estrogen levels in these patients.

In our study, a high Dkk2 expression in combination with a high cytoplasmic level of GPER had a significant prognostic impact on OS which might help to find new approaches for possible treatment strategies accounting for the correlation of estrogen and Wnt signaling pathways. As Dkk2 is a modulator of the Wnt pathway, therapeutics addressing this cascade could be combined with agents modulating GPER. Although promising in early stage development, previous strategies targeting Wnt proteins like tumor associated MUC1 (TA-MUC1) inhibitor gatipotuzumab and others have not led to durable responses and not reached clinical significance so far (48). Very recently, a Wnt modulator of Dkk1 (DKN-01) has shown interesting activity and is currently in a phase 2 basket trial which still supports the rationale for this approach (49).

In renal cancer cells, the selective estrogen receptor modulator genistein reportedly abolished miR-1260b, which is able to suppress Wnt signaling modulators like Dkk2, and therefore preserved levels of these proteins (24). Genistein is not exclusively binding to GPER though, it also inflects ERα and/or ERβ (50). In hepatocytes administering the GPER antagonist G15 attenuated β-cat Ser675 phosphorylation and T-cell factor (TCF) expression suggesting an involvement of GPER in β-cat/TCF activities (51). Beside cell culture experiments, analyzing methylation patterns with methylation-specific polymerase chain reaction could help to further investigate the suggested interactions of GPER and Dkk2. Implementing TCF/LEF (lymphoid enhancing factor) reporter assays, could be assessed to evaluate possible effects of GPER agonists or antagonists on the Wnt signaling pathway.

There are some factors limiting our study. First of all, it is retrospective based on a single dataset with a relatively low sample size which may not be sufficient to elucidate all subtype-specific differences in an heterogenous tumor like ovarian cancer (44). Additional specific information of patient characteristics like an history of hormonal replacement therapy could enrich the investigation how estrogen levels interact with Dkk2 and better account for possible environmental toxicants. In Kaplan-Meier analysis, subtype-specific evaluation did not reveal significant differences regarding OS between patients with high and low Dkk2 expression so that results can be considered as a base for further research in ovarian cancer. Further methods will be necessary capture the extensive complexity of GPER and Wnt signaling pathways with their possible interaction as indicated.

However, aside from these limitations our data is in accordance with previous findings in EOC literature (13, 33, 36, 45, 52) and elucidate that targeting the GPER receptor as well as the Wnt pathway could represent promising therapeutic strategy in ovarian cancer. The study might provide an impetus to further investigate the crosstalk between estrogen and Wnt signaling in regard to the therapeutic potential in EOC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ludwig-Maximilians-University, Munich,

Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

PF, UJ, SH, AB, SM, and FT contributed conception and design of the study. DM performed histological examinations on the patient tumour tissue. PF, CK, UJ, and PR did the laboratory work. PF, TK, UJ, and PR performed the statistical analysis. PF and UJ wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* (2019) 69: (1):7–34. doi: 10.3322/caac.21551
2. Baldwin LA, Huang B, Miller RW, Tucker T, Goodrich ST, Podzielinski I, et al. Ten-year relative survival for epithelial ovarian cancer. *Obstet Gynecol* (2012) 120(3):612–8. doi: 10.1097/AOG.0b013e318264f794
3. Kim JY, Cho CH, Song HS. Targeted therapy of ovarian cancer including immune check point inhibitor. *Korean J Intern Med* (2017) 32(5):798–804. doi: 10.3904/kjim.2017.008
4. du Bois A, Reuss A, Pujade-Lauraine E, Harter P, Ray-Coquard I, Pfisterer J. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO). *Cancer* (2009) 115(6):1234–44. doi: 10.1002/cncr.24149
5. Aletti GD, Gostout BS, Podratz KC, Cliby WA. Ovarian cancer surgical resectability: relative impact of disease, patient status, and surgeon. *Gynecol Oncol* (2006) 100(1):33–7. doi: 10.1016/j.ygyno.2005.07.123
6. Vergote I, De Brabanter J, Fyles A, Bertelsen K, Einhorn N, Sevelda P, et al. Prognostic importance of degree of differentiation and cyst rupture in stage I invasive epithelial ovarian carcinoma. *Lancet (Lond Engl)* (2001) 357 (9251):176–82. doi: 10.1016/s0140-6736(00)03590-x
7. Dembo AJ, Davy M, Stenwig AE, Berle EJ, Bush RS, Kjorstad K. Prognostic factors in patients with stage I epithelial ovarian cancer. *Obstet Gynecol* (1990) 75(2):263–73.
8. Duska LR, Kohn EC. The new classifications of ovarian, fallopian tube, and primary peritoneal cancer and their clinical implications. *Ann Oncol* (2017) 28(suppl_8):viii8–viii12. doi: 10.1093/annonc/mdx445
9. Ricken A, Lochhead P, Kontogiannea M, Farookhi R. Wnt signaling in the ovary: identification and compartmentalized expression of wnt-2, wnt-2b, and frizzled-4 mRNAs. *Endocrinology* (2002) 143(7):2741–9. doi: 10.1210/endo.143.7.8908
10. Ying Y, Tao Q. Epigenetic disruption of the WNT/beta-catenin signaling pathway in human cancers. *Epigenetics* (2009) 4(5):307–12.
11. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell* (2012) 149 (6):1192–205. doi: 10.1016/j.cell.2012.05.012
12. Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* (2008) 4(2):68–75. doi: 10.4161/org.4.2.5851
13. Zhu J, Zhang S, Gu L, Di W. Epigenetic silencing of DKK2 and Wnt signal pathway components in human ovarian carcinoma. *Carcinogenesis* (2012) 33 (12):2334–43. doi: 10.1093/carcin/bgs278
14. Martin-Orozco E, Sanchez-Fernandez A, Ortiz-Parra I, Ayala-San Nicolas M. WNT Signaling in Tumors: The Way to Evade Drugs and Immunity. *Front Immunol* (2019) 10:2854(2854). doi: 10.3389/fimmu.2019.02854
15. Devotta A, Hong CS, Saint-Jeannet JP. Dkk2 promotes neural crest specification by activating Wnt/beta-catenin signaling in a GSK3beta independent manner. *Elife* (2018) 7:e3440. doi: 10.7554/eLife.34404
16. Hassler C, Cruciat CM, Huang YL, Kuriyama S, Mayor R, Niehrs C. Kremen is required for neural crest induction in Xenopus and promotes LRP6-mediated Wnt signaling. *Development* (2007) 134(23):4255–63. doi: 10.1242/dev.005942
17. Park H, Jung HY, Choi HJ, Kim DY, Yoo JY, Yun CO, et al. Distinct roles of DKK1 and DKK2 in tumor angiogenesis. *Angiogenesis* (2014) 17(1):221–34. doi: 10.1007/s10456-013-9390-5
18. Shao YC, Wei Y, Liu JF, Xu XY. The role of Dickkopf family in cancers: from Bench to Bedside. *Am J Cancer Res* (2017) 7(9):1754–68.
19. Niehrs C. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* (2006) 25(57):7469–81. doi: 10.1038/sj.onc.1210054
20. Mao B, Niehrs C. Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene* (2003) 302(1-2):179–83. doi: 10.1016/s0378-1119(02)01106-x
21. Hamilton KJ, Hewitt SC, Arao Y, Korach KS. Estrogen Hormone Biology. *Curr Top Dev Biol* (2017) 125:109–46. doi: 10.1016/bs.ctdb.2016.12.005
22. Shen HH, Yang CY, Kung CW, Chen SY, Wu HM, Cheng PY, et al. Raloxifene inhibits adipose tissue inflammation and adipogenesis through Wnt regulation in ovariectomized rats and 3 T3-L1 cells. *J BioMed Sci* (2019) 26 (1):62. doi: 10.1186/s12929-019-0556-3
23. Bhukhai K, Suksen K, Bhummaphan N, Janjorn K, Thongon N, Tantikanlayaporn D, et al. A phytoestrogen diarylheptanoid mediates estrogen receptor/Akt/glycogen synthase kinase 3beta protein-dependent activation of the Wnt/beta-catenin signaling pathway. *J Biol Chem* (2012) 287(43):36168–78. doi: 10.1074/jbc.M112.344747
24. Hirata H, Ueno K, Nakajima K, Tabatabai ZL, Hinoda Y, Ishii N, et al. Genistein downregulates onco-miR-1260b and inhibits Wnt-signalling in renal cancer cells. *Br J Cancer* (2013) 108(10):2070–8. doi: 10.1038/bjc.2013.173
25. Scott EL, Brann DW. Estrogen regulation of Dkk1 and Wnt/beta-Catenin signaling in neurodegenerative disease. *Brain Res* (2013) 1514:63–74. doi: 10.1016/j.brainres.2012.12.015
26. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* (2005) 307(5715):1625–30. doi: 10.1126/science.1106943
27. Albanito L, Lappano R, Madeo A, Chimento A, Prossnitz ER, Cappello AR, et al. G-protein-coupled receptor 30 and estrogen receptor-alpha are involved in the proliferative effects induced by atrazine in ovarian cancer cells. *Environ Health Perspect* (2008) 116(12):1648–55. doi: 10.1289/ehp.11297
28. Barton M. Not lost in translation: Emerging clinical importance of the G protein-coupled estrogen receptor GPER. *Steroids* (2016) 111:37–45. doi: 10.1016/j.steroids.2016.02.016
29. Aronica SM, Kraus WL, Katzenellenbogen BS. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc Natl Acad Sci USA* (1994) 91(18):8517–21. doi: 10.1073/pnas.91.18.8517
30. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* (1999) 402(6764):884–8. doi: 10.1038/47260
31. Filardo EJ, Thomas P. Minireview: G protein-coupled estrogen receptor-1, GPER-1: its mechanism of action and role in female reproductive cancer, renal

and vascular physiology. *Endocrinology* (2012) 153(7):2953–62. doi: 10.1210/en.2012-1061
32. Fujiwara S, Terai Y, Kawaguchi H, Takai M, Yoo S, Tanaka Y, et al. GPR30 regulates the EGFR-Akt cascade and predicts lower survival in patients with ovarian cancer. *J Ovarian Res* (2012) 5(1):35. doi: 10.1186/1757-2215-5-35
33. Ignatov T, Modl S, Thulig M, Weissenborn C, Treeck O, Ortmann O, et al. GPER-1 acts as a tumor suppressor in ovarian cancer. *J Ovarian Res* (2013) 6 (1):51. doi: 10.1186/1757-2215-6-51
34. Kolkova Z, Casslen V, Henic E, Ahmadi S, Ehinger A, Jirstrom K, et al. The G protein-coupled estrogen receptor 1 (GPER/GPR30) does not predict survival in patients with ovarian cancer. *J Ovarian Res* (2012) 5:9. doi: 10.1186/1757-2215-5-9
35. Czogalla B, Kuhn C, Heublein S, Schmockel E, Mayr D, Kolben T, et al. EP3 receptor is a prognostic factor in TA-MUC1-negative ovarian cancer. *J Cancer Res Clin Oncol* (2019) 145(10):2519–27. doi: 10.1007/s00432-019-03017-8
36. Heublein S, Mayr D, Vrekoussis T, Friese K, Hofmann SS, Jeschke U, et al. The G-protein coupled estrogen receptor (GPER/GPR30) is a gonadotropin receptor dependent positive prognosticator in ovarian carcinoma patients. *PLoS One* (2013) 8(8):e71791. doi: 10.1371/journal.pone.0071791
37. Deuster E, Mayr D, Hester A, Kolben T, Zeder-Goss C, Burges A, et al. Correlation of the Aryl Hydrocarbon Receptor with FSHR in Ovarian Cancer Patients. *Int J Mol Sci* (2019) 20(12):2862. doi: 10.3390/ijms20122862
38. Meinhold-Heerlein I, Fotopoulou C, Harter P, Kurzeder C, Mustea A, Wimberger P, et al. The new WHO classification of ovarian, fallopian tube, and primary peritoneal cancer and its clinical implications. *Arch Gynecol Obstet* (2016) 293(4):695–700. doi: 10.1007/s00404-016-4035-8
39. Heidegger H, Dietlmeier S, Ye Y, Kuhn C, Vattai A, Aberl C, et al. The Prostaglandin EP3 Receptor Is an Independent Negative Prognostic Factor for Cervical Cancer Patients. *Int J Mol Sci* (2017) 18(7):1571. doi: 10.3390/ijms18071571
40. Remmele W, Hildebrand U, Hienz HA, Klein PJ, Vierbuchen M, Behnken LJ, et al. Comparative histological, histochemical, immunohistochemical and biochemical studies on oestrogen receptors, lectin receptors, and Barr bodies in human breast cancer. *Virchows Arch A Pathol Anat Histopathol* (1986) 409(2):127–47. doi: 10.1007/bf00708323
41. Hoo ZH, Candlish J, Teare D. What is an ROC curve? *Emerg Med J* (2017) 34 (6):357–9. doi: 10.1136/emermed-2017-206735
42. Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. *Biom J* (2005) 47(4):458–72. doi: 10.1002/bimj.200410135
43. Wu W, Glinka A, Delius H, Niehrs C. Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling. *Curr Biol* (2000) 10 (24):1611–4. doi: 10.1016/s0960-9822(00)00868-x
44. Kurman RJ, Shih Ie M. Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer–shifting the paradigm. *Hum Pathol* (2011) 42 (7):918–31. doi: 10.1016/j.humpath.2011.03.003
45. Matei D, Fang F, Shen C, Schilder J, Arnold A, Zeng Y, et al. Epigenetic resensitization to platinum in ovarian cancer. *Cancer Res* (2012) 72(9):2197–205. doi: 10.1158/0008-5472.Can-11-3909
46. Moufarrij S, Dandapani M, Arthofer E, Gomez S, Srivastava A, Lopez-Acevedo M, et al. Epigenetic therapy for ovarian cancer: promise and progress. *Clin Epigenet* (2019) 11(1):7. doi: 10.1186/s13148-018-0602-0
47. Sjostrom M, Hartman L, Grabau D, Fornander T, Malmstrom P, Nordenskjold B, et al. Lack of G protein-coupled estrogen receptor (GPER) in the plasma membrane is associated with excellent long-term prognosis in breast cancer. *Breast Cancer Res Treat* (2014) 145(1):61–71. doi: 10.1007/s10549-014-2936-4
48. Jung YS, Park JI. Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond beta-catenin and the destruction complex. *Exp Mol Med* (2020) 52(2):183–91. doi: 10.1038/s12276-020-0380-6
49. ClinicalTrials. A Study of DKN-01 as a Monotherapy or in Combination With Paclitaxel in Patients With Recurrent Epithelial Endometrial or Epithelial Ovarian Cancer or Carcinosarcoma (P204). (2020). https://clinicaltrials.gov/ct2/show/NCT03395080.
50. Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. *Nat Rev Endocrinol* (2011) 7(12):715–26. doi: 10.1038/nrendo.2011.122
51. Tian L, Shao W, Ip W, Song Z, Badakhshi Y, Jin T. The developmental Wnt signaling pathway effector beta-catenin/TCF mediates hepatic functions of the sex hormone estradiol in regulating lipid metabolism. *PLoS Biol* (2019) 17(10): e3000444. doi: 10.1371/journal.pbio.3000444
52. Wang C, Lv X, He C, Hua G, Tsai MY, Davis JS. The G-protein-coupled estrogen receptor agonist G-1 suppresses proliferation of ovarian cancer cells by blocking tubulin polymerization. *Cell Death Dis* (2013) 4:e869. doi: 10.1038/cddis.2013.397

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ORIGINAL RESEARCH
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The Antitumor Peptide ERα17p Exerts Anti-Hyperalgesic and Anti-Inflammatory Actions Through GPER in Mice

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Persistent inflammation and persistent pain are major medical, social and economic burdens. As such, related pharmacotherapy needs to be continuously improved. The peptide ERα17p, which originates from a part of the hinge region/AF2 domain of the human estrogen receptor α (ERα), exerts anti-proliferative effects in breast cancer cells through a mechanism involving the hepta-transmembrane G protein-coupled estrogen receptor (GPER). It is able to decrease the size of xenografted human breast tumors, in mice. As GPER has been reported to participate in pain and inflammation, we were interested in exploring the potential of ERα17p in this respect. We observed that the peptide promoted anti-hyperalgesic effects from 2.5 mg/kg in a chronic mice model of paw inflammation induced by the pro-inflammatory complete Freund's adjuvant (CFA). This action was abrogated by the specific GPER antagonist G-15, leading to the conclusion that a GPER-dependent mechanism was involved. A systemic administration of a Cy5-labeled version of the peptide allowed its detection in both, the spinal cord and brain. However, ERα17p-induced anti-hyperalgesia was detected at the supraspinal level, exclusively. In the second part of the study, we have assessed the anti-inflammatory action of ERα17p in mice using a carrageenan-evoked hind-paw inflammation model. A systemic administration of ERα17p at a dose of 2.5 mg/kg was responsible for reduced paw swelling. Overall, our work strongly suggests that GPER inverse agonists, including ERα17p, could be used to control hyperalgesia and inflammation.

Keywords: GPER, ERα17p, pain, hyperalgesia, inflammation

INTRODUCTION

Estrogens and their classical receptors, *i.e.* ERα and β, interfere with pain pathways, through specific proteins and different molecular mechanisms (1). For example, 17β-estradiol (E_2) facilitates heterodimerization of κ and μ opioid receptors *via* a membrane estrogen receptor (ER)-dependent process (1, 2). Opioid peptides exert antiestrogenic effects by interfering with AP-1-driven

transcription (3). Thus, ERα and β could explain, at least in part, sex differences in pain sensitivity (1).

The newly discovered G protein-coupled estrogen receptor (GPER) is expressed, inter alia, in different regions of the central nervous system (CNS) such as the hippocampus and the hypothalamus, brain stem, the spinal cord, and autonomic and sensory ganglia (4–7), where it participates in a panel of neurophysiological events including pain. These effects are mediated through mechanisms involving an increase in the concentration of intracellular calcium and the accumulation of reactive oxygen species (ROS) (8–10). Likewise, the selective GPER agonist G-1 induces the depolarization of ventral and dorsal horn and cultured spinal neurons to mediate nociception, two events that are abolished by the specific GPER antagonist G-15 (5). Tamoxifen and fulvestrant, which also behave as GPER agonists, induce hyperalgesia (11–13). Hence, GPER has an indisputable role in nociception *via* rapid steroid hormone signaling pathways.

The 17-mer GPER-interacting peptide ERα17p (sequence: H_2N-PLMIKRSKKNSLALSLT-COOH) was designed from the human ERα hinge and ligand-binding domains (residues 295-311) (14, 15). It corresponds to a surface-exposed polyproline II (PPII) region, which is composed of amino acids belonging to the C-terminus of the hinge region (D domain) and to the N-terminus of the AF2 transactivation function (E/F domains) (14). In the context of the whole protein, this fragment is in charge of the recruitment of transcription regulatory partners such as Ca^{2+}-calmodulin (16) and Hsp70 (17). It is also subjected to post-translational modifications such as acetylation, phosphorylation, and SUMOylation [see (18) and references herein]. The KRSKK motif (residues 299–303), which is targeted by proteolytic enzymes (19), corresponds to the third ERα nuclear localization sequence (20). Hence, this part of the receptor appears crucial for the control of the turnover of ERα, its translocation and associated transcription.

In the light of the above observations, we have extensively studied the peptide ERα17p, notably in ERα-positive and -negative human breast cancer cells where it has been shown to exert a panel of activities. In steroid-deprived conditions, it promotes ER-dependent transcription and the proliferation exclusively of ERα-positive breast cancer cells through the activation of genes that are also activated by E_2 (21–23). Thus, ERα17p can be seen as an estrogen-like molecule in these atypical experimental conditions. In breast cancer cells incubated in complete (physiological) culture medium, *i.e.*, in medium containing steroids and growth factors, it induces apoptosis (24). Since these effects are observed in both ERα-positive and -negative breast cancer cells with, however, a preference for ERα-positive cell lines, it is likely that a mechanism depending partially on ERα is involved (24). A decrease in the migration of breast cancer cells through actin cytoskeleton rearrangements is also observed (25). Accordingly, ERα17p decreases the size of tumors xenografted in mice by about 50%, at low dose (1.5 mg/kg) and over a short period (three times a week for 4 weeks) (24). These observations highlight the amazing pharmacological plasticity of G protein-coupled receptors (GPCRs) (26) and show the putative biased agonist character of ERα17p. ERα17p also induces the proteasome-dependent degradation of GPER and inhibits the activation of the epidermal growth factor receptor (EGFR) and of the extracellular signal-regulated kinase (ERK1/2). It also decreases the level of the protein c-fos (15). In combination with its GPER interaction, ERα17p interacts with artificial and breast cancer cell membranes (27, 28).

Because of the role of GPER in nociception (8, 29–33) and inflammation (34–42), it was decided to study the action of ERα17p on inflammation-induced hyperalgesia and edema, by using complete Freund's adjuvant (CFA) and carrageenan mice models, respectively. The involvement of ERα17p in hyperalgesia and inflammation was evaluated by testing its action *in vivo*, in the presence and in the absence of G-15, a selective GPER antagonist. Strikingly, systemically administered ERα17p supports anti-nociception between 2.5 and 10 mg/kg, a dose range for which an antitumor activity, against ERα-negative breast tumors, has previously been observed, *in vivo* (24). Thus, targeting the GPER could be a promising approach not only to fight cancer, but also to control inflammation and related pain. Therefore, ERα17p could be proposed as a lead compound for the synthesis of new a generation of polymodal (antitumor, analgesic, and anti-inflammatory) drugs.

MATERIAL AND METHODS

Animals

Male mice CD1 (20–22 g, Janvier, France) were acclimatized for a week before testing. They were housed under controlled environmental conditions (21–22°C; 55% humidity, 12 h light/dark cycles, food and water *ad libitum*). Male and female mice CD1 have been used for the fluorecent imaging experiment.

Ethics

The studies involving animals were reviewed and approved by the Auvergne Animal Experiment Ethics Committee, CE2A, and by the French Ministry of Higher Education and Innovation (authorization N° 18022) and performed according to European legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes, and complied with the recommendations of the International Association for the Study of Pain (IASP).

Chemicals

The selective GPER antagonist G-15 [(3a*S**,4*R**,9b*R**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline] was purchased from Tocris Bio-Techne SAS (Noyal-Châtillon-sur-Seiche, France). Morphine and λ-carrageenan were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). We used the Fmoc strategy to synthesize the peptide ERα17p (sequence: H_2N-PLMIKRSKKNSLALSLT-COOH) and its Cy5-labeled analogue [sequence: H_2N-ERα17p-Pra(Cy5)-COOH], as previously described (15, 27). Briefly, the Cy5-labeled peptide was obtained by adding a propargylglycine

(Pra) in the C-terminus of ERα17p and then, the Cy5 fluorescent probe on the propargyl moiety by using the click chemistry strategy (15). Then, the peptides were purified by reverse phase HPLC and identified by MALDI-TOF mass spectrometry (15).

All tested molecules were dissolved in saline solution except for G-15 which was dissolved in saline with 5% Tween80 and 5% DMSO. Drug solutions were prepared extemporaneously before use.

Complete Freund's adjuvant (CFA), which was administered by periarticular injection, consists of *Mycobacterium butyricum* (Ref DF0640-33-7, Difco Laboratories, Detroit, USA) dissolved in paraffin oil and aqueous saline solution (0.9% NaCl) prior to an autoclave sterilization for 20 min at 120°C.

Intracerebroventricular and Intrathecal Injections

Injections were carried out in mice anaesthetized with isoflurane (1–2%). Intracerebroventricular (i.c.v.) injections were made at the bregma level with a syringe and a calibrated needle with a guide so that the needle length was 4 mm (43). The injected volume was 2 µl *per* mouse. For intrathecal (i.t.) injections, the anesthetized mouse was held in one hand by the pelvic girdle and a 25-gauge × 1-inch needle connected to a 25 µl Hamilton syringe was inserted into the subarachnoid space between lumbar vertebrae 5 and 6 until a tail flick was elicited (44). The syringe was held in position for a few seconds after the injection of a volume of 2 µl *per* mouse.

Monoarthritic Model

A persistent inflammatory pain model was produced by injection, under brief anesthesia (2.5% isoflurane inhalation), of 5 µl of CFA on either side of the left ankle joint of male mice (45). Behaviors tests were performed before and 7 days after CFA injection.

Von Frey Test

Mice were acclimatized to the testing environment before baseline testing. The experimenter was blinded to the mice treatments. On the behavior testing day (7 days after CFA injection), mice were placed individually in Plexiglas compartments 8 cm (L) × 3.5 cm (W) × 8 cm (D), on an elevated wire mesh platform to afford access to the ventral surface of the hindpaws and were allowed to acclimatize for 1 h before testing. Von Frey filaments ranging from 0.02 to 1.4 g were applied perpendicularly to the plantar surface of the paw. Paw withdrawal or licking was considered as a positive response. Fifty percent paw withdrawal threshold (PWT) in grams was determined with a modified version of the Dixon up–down method, as previously described (46).

Carrageenan Model and Edema Measurement

Paw edema was induced in male mice by an intraplantar (left hindpaw) subcutaneous injection of 20 µl of 3% λ-carrageenan with a 50 µl Hamilton syringe and a 26-gauge needle (43). Paw edema was measured before induction of inflammation and the effects of the drugs were assessed 4 h after carrageenan injection with a caliper.

Ex Vivo Fluorescence Imaging

Ex vivo fluorescence imaging was performed with the IVIS Spectrum system (Perkin Elmer, Waltham, MA, USA) and a Cy5 filter set (excitation wavelength: 640 nm; emission wavelength: 680 nm). The peptide H_2N-ERα17p-Pra(Cy5)-COOH (2 mg/kg) was injected intraperitoneally to female and male mice that were sacrificed 30 min post-injection. The brain and spinal cord were then removed to perform *ex vivo* fluorescence imaging of isolated organs. All images were acquired and analyzed with Living Image 4.7.2 software (PerkinElmer, Waltham, MA, USA). Experiments were performed on the IVIA multimodal imaging platform (Clermont-Ferrand, France).

Experimental Protocol

The design, analysis and reporting of the research were carried out in accordance with the ARRIVE guidelines (47). Treatments were administered according to the method of equal blocks, in order to assess the effect of the different treatments over the same time interval, thereby avoiding unverifiable and time-variable environmental influences. All behavioral tests were performed in a quiet room by the same blinded experimenter. To ensure the methodological quality of the study, we followed the recommendations of Rice et al (48). Intraperitoneal (i.p.) administrations of ERα17p (1.25, 2.5, and 10 mg/kg), morphine (1 mg/kg), H_2N-ERα17p-Pra(Cy5)-COOH (2 mg/kg), and G-15 (0.3 mg/kg) were performed with a constant volume of 10 ml/kg. To investigate the influence of GPER in the response to ERα17p, the selective GPER antagonist G-15 was administrated either i.p. (0.3 mg/kg, 10 ml/kg), i.c.v. (5 µg/mouse in 2 µl), or i.t. (5 µg/mouse in 2 µl) 20 min before ERα17p. The local anti-inflammatory effect of the peptide was investigated by an intraplantar (i.pl.) injection of ERα17p (20 µg in 10 µl).

Statistical Analysis

Results were expressed as mean ± SEM and were recorded with Prism 7 (GraphPad™ Software Inc., San Diego, CA, USA). Data were tested for normality (Shapiro-Wilk test) and for equal variance (Fisher test). Multiple measurements were compared with two-way ANOVA. For kinetic data, the *post hoc* comparisons were performed by the Sidak test (number of groups = 2) or by the Dunnett test (number of groups > 2). The Kruskal-Wallis *post hoc* test was performed to have a mean comparison of the area under the time-course curves (AUC). Values of $p < 0.05$ were considered statistically significant. The AUC (0–180 min.) of 50% mechanical threshold (individual values) were calculated by the trapezoidal rule taking in reference the PWT baseline after CFA (threshold at time T_0). The AUC of individual values is the sum of each area between experimental times from 0 to 180 min. calculated as: (time T − time before time T) × [(threshold at time T − threshold at time T_0) + (thresholds obtained at time T_0 or at time before time T − threshold at time T_0)/2]. AUC was expressed as mean ± SEM (in g × min.).

RESULTS

ERα17p Reduces Hyperalgesia

To explore the action of ERα17p on hyperalgesia, we used the von Frey test in a complete Freund's adjuvant (CFA) model (arthritis model). A decrease in the mechanical paw withdrawal threshold (PWT) was observed from 0.66 ± 0.05 g to 0.20 ± 0.04 g (n = 42, $p < 0.001$, *t*-test) for all mice, 7 days after CFA injection (**Figure 1A**). A PWT value of ~0.20 g was recorded with the vehicle (control, saline solution at 10 ml/kg) throughout the experiment. At 30 min and at a dose of 1.25 mg/kg i.p., the peptide induced a transitory anti-hyperalgesic effect. A marked decrease in hyperalgesia was observed at higher doses, *i.e.*, between 2.5 and 10 mg/kg i.p., from 30 to 90 min (**Figure 1A**). The values obtained for 60 min were: 0.64 ± 0.10 g for ERα17p at 2.5 mg/kg and 0.60 ± 0.12 g for ERα17p at 10 mg/kg (control: 0.18 ± 0.05 g, $p = 0.04$, Dunnett *post-hoc* test). These results were confirmed by calculation of the area under the curve (AUC, in g.min.), where a significant difference was observed between ERα17p (2.5 and 10 mg/kg) or morphine (1 mg/kg, i.p., used as positive control) treated mice and vehicle-treated mice. The AUC values recorded for 2.5, 10 mg/kg ERα17p and morphine were 74.2 ± 15.3 g.min. ($p = 0.006$), 64.6 ± 19.8 g.min. ($p = 0.046$), and 86.6 ± 33.7 g.min. ($p = 0.003$), respectively (Kruskal-Wallis test; AUC vehicle: −0.38 ± 9.95 g.min., **Figure 1B**).

These results were confirmed in a standard screening test used for analgesic candidates with acetic acid-induced inflammation. The two previous most active doses of ERα17p were tested in mice after an intraperitoneal injection of acetic acid 0.6% i.p. At the doses of 2.5 and 10 mg/kg, a significant decrease in the number of abdominal writhings was observed (2.86 ± 2.32, $p < 0.001$ and 8.13 ± 4.62, $p = 0.017$, respectively; vehicle: 30.63 ± 4.22, Kruskal-Wallis test, **Supplementary Figure 1A**). ERα17p at a dose >10 mg/kg failed to modify spontaneous locomotor activity (**Supplementary Figure 1B**).

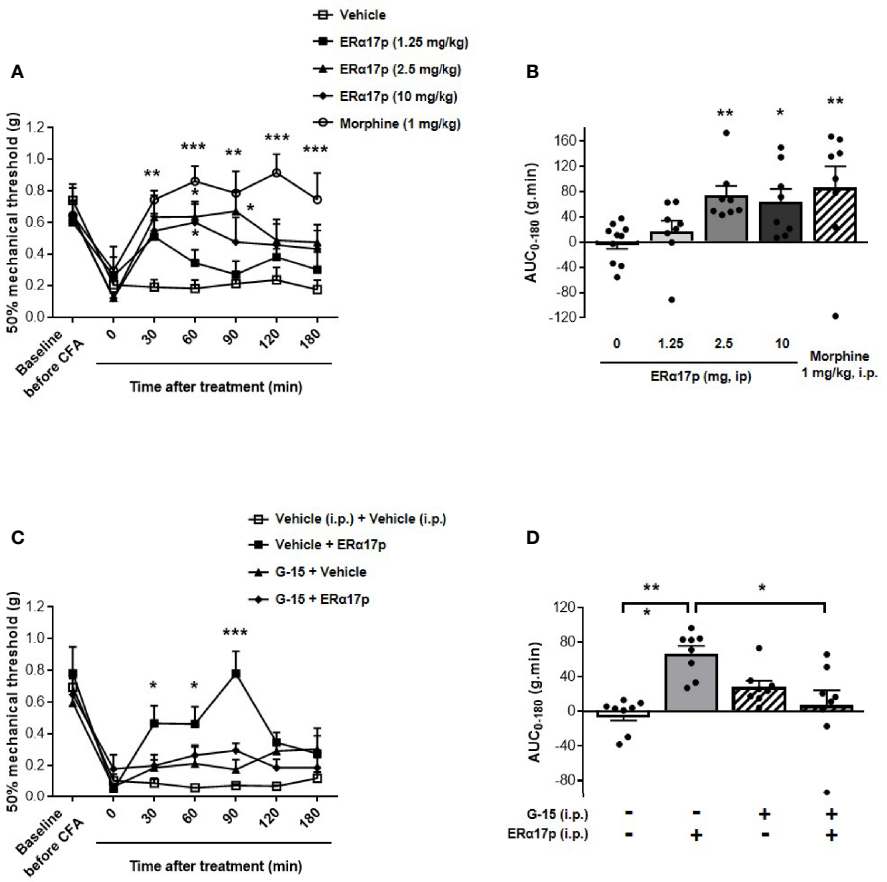

FIGURE 1 | GPER-dependent action of ERα17p in tactile hypersensitivity in a CFA model. The Von Frey test was performed to assess the impact of ERα17p on CFA-induced mechanical hypersensitivity in inflammatory pain. The 50% paw withdrawal threshold (PWT) was determined with a modified version of the Dixon up–down method. **(A)** The anti-hyperalgesic action of ERα17p was determined by measuring dose-dependent effects. The Von Frey test was assessed before injection of CFA (baseline) and after that of vehicle (saline solution) or ERα17p (1.25, 2.5, and 10 mg/kg, i.p.) 7 days after CFA injection. **(C)** Involvement of GPER was determined using ERα17p with or without G-15. Mice were i.p. pre-treated with vehicle (5% DMSO, 5% Tween80 in saline solution, reference) or G-15 (0.3 mg/kg) 15 min before administration of vehicle (saline) or ERα17p (2.5 mg/kg, i.p.). **(B, D)** Area under the time-course AUC (0–180 min) of PWT variations obtained from **(A, C)**, respectively. Data are expressed as mean ± SEM (n = 8–10 *per* group). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, when compared to the vehicle group (or G-15+ ERα17p group, as mentioned in **D**); two-way ANOVA followed by Dunnett *post hoc* test for time comparison or Kruskal-Wallis test for AUC mean comparison.

The Anti-Hyperalgesic Action of ERα17p Is GPER-Dependent

In the second part of this work, we studied the involvement of GPER in the anti-hyperalgesic action of ERα17p. The peptide used at 2.5 mg/kg i.p. was administered to CFA mice 30 min after an injection of the specific GPER antagonist G-15 (0.3 mg/kg i.p.) (49). As previously observed, ERα17p significantly increased PWT at 30 min (ERα17p: 0.47 ± 0.11 g; vehicle: 0.09 ± 0.03 g, p = 0.04, Dunnett *post-hoc* test), 60 min (ERα17p: 0.46 ± 0.11 g; vehicle: 0.06 ± 0.01 g, p = 0.02, Dunnett *post-hoc* test), and 90 min (ERα17p: 0.78 ± 0.11 g; vehicle: 0.08 ± 0.01 g, $p < 0.001$, Dunnett *post-hoc* test). G-15, inactive by itself, abolished the anti-hyperalgesic action of ERα17p (**Figure 1C**). These results were confirmed by AUC values over 180 min (ERα17p: 66.9 ± 9.0 g.min. *vs* ERα17p + G-15: 7.2 ± 17.2 g.min., p = 0.018 and AUC vehicle: −3.7 ± 6.7 g.min. *vs* ERα17p + G15, $p < 0.9$, Kruskal-Wallis test), as shown in the **Figure 1D**.

ERα17p Diffuses Into the Brain and Spinal Cord

The i.p. injected ERα17p distribution in the CNS was determined by using a Cy5-labeled version of the peptide [*i.e.*, H_2N-ERα17p-Pra(Cy5)-COOH], which we used in a previous work (15). *Ex vivo* fluorescence staining showed an important diffusion of the Cys-5-labeled peptide in the supra-spinal (**Figure 2A**) and spinal (**Figure 2B**) compartments.

Only the Supraspinal Pool of GPER Is Involved in the Anti-Hyperalgesic Action of ERα17p

To determine the site of the central action of ERα17p, two series of experiments were performed: assessment of its effect 1) after its central injections and 2) after its systemic injection, following a central administration of the GPER antagonist G-15.

An intracerebroventricular injection of 1 μg/mouse of the peptide failed to induce a significant increase of thresholds. In contrast, a significant enhancement of PWT was observed 15 and 30 min after an i.c.v. injection of 2.5 μg/mouse of ERα17p (p = 0.04 and p = 0.01, respectively, Dunnett *post-hoc* test, **Figure 3A**). With 5 μg/mouse, a more robust anti-hyperalgesic effect was detected from 15 to 90 min, with a maximum at 60 min (**Figure 3A**). Assessment of AUC confirmed this dose-dependent effect: a dose of 1 μg/mouse failed to induce any change in PWT (AUC ERα17p 1 μg/mouse: 25.38 ± 11.7 g.min.; AUC vehicle: 12.47 ± 7.66 g.min., $p > 0.9$, Kruskal-Wallis test, **Figure 3B**). Significant effects were recorded with 2.5 μg/mouse ERα17p (AUC: 76.45 ± 17.79 g.min., p = 0.011, Kruskal-Wallis test) and 5 μg/mouse (AUC: 79.49 ± 22.98 g.min., p = 0.012, Kruskal-Wallis test, **Figure 3B**), when compared to the vehicle.

To assess the involvement of the supraspinal pool of GPER in the action of ERα17p, 5 μg/mouse of G-15 were injected i.c.v., 20 min before a systemic injection of the peptide (2.5 mg/kg, i.p.) or of the vehicle (10 ml/kg). Except for an isolated peak at 45 min, the scores obtained in animals treated with ERα17p and pre-treated with G-15 were not different from those of the vehicle group throughout the experiment (**Figure 3C**). PWT AUC values confirmed the anti-hyperalgesic properties of the peptide (AUC ERα17p alone: 81.29 ± 8.44 g.min.; AUC vehicle: 7.51 ± 5.35 g.min., $p > 0.001$, Kruskal-Wallis test) and the marked decrease in its effect by G-15 (**Figure 3D**), revealing, thereby, that the anti-hyperalgesic effect of systemic ERα17p involves supraspinal GPER.

The effects of the peptide at the spinal level were extensively studied with the same strategy (**Figure 4**). Intrathecally administered ERα17p at doses of 1, 2.5 and 5 μg/mouse induced a significant anti-hyperalgesic effect compared to vehicle, only at the dose of 5 μg/mouse at times 30 min (ERα17p: 0.81 ± 0.08 g; vehicle: 0.40 ± 0.05 g, $p < 0.001$, Dunnett *post-hoc* test), 45 min (ERα17p: 0.94 ± 0.10 g; vehicle: 0.45 ± 0.05 g, $p < 0.001$, Dunnett *post-hoc* test) and 60 min (ERα17p: 0.87 ± 0.10 g; vehicle: 0.50 ± 0.14 g, p = 0.004, Dunnett *post-hoc* test) (**Figure 4A**). This observation was confirmed by AUC (**Figure 4B**). The anti-hyperalgesic effect of 5 μg/mouse of ERα17p (i.t.) was reduced by G-15 (5 μg) co-administered 20 min before by the same route (**Figures 4C, D**). Thus, the anti-hyperalgesic effect of ERα17p directly administered in the spinal cord is mediated by GPER.

The fact that a drug involves a local target when injected locally does not mean that it is the case when it is systemically

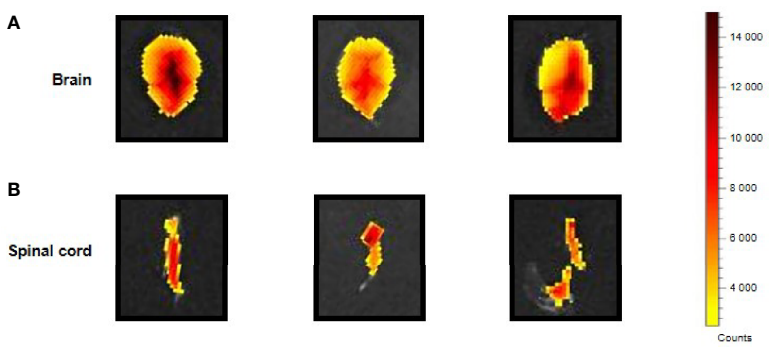

FIGURE 2 | CNS distribution of the Cy5-labeled ERα17p peptide. Upper views of brain **(A)** and spinal cord **(B)** sampled from three mice 30 min after an i.p. injection of H_2N-ERα17p-Pra(Cy5)-COOH (2 mg/kg).

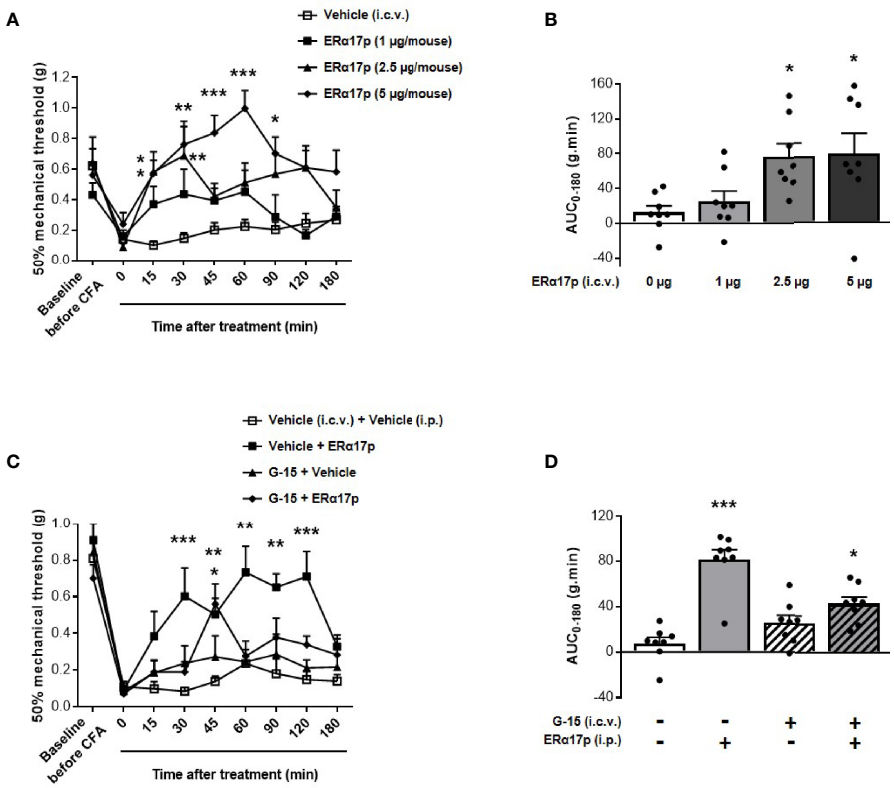

FIGURE 3 | Involvement of supraspinal GPER in ERα17p action in the CFA model. **(A, C)** Area under the time-course AUC (0–180 min) of PWT variations from **(B, D)**, respectively. **(B)** Time-course effect of an i.c.v. administration of vehicle (saline solution, 2 µl/mice) or ERα17p (1, 2.5 and 5 µg/mice) on mechanical hypersensitivity in CFA mice model. **(D)** Involvement of supraspinal GPER in the action of ERα17p with or without G-15 i.c.v. Mice were i.c.v. pre-treated with vehicle (5% DMSO, 5% Tween80 in saline solution, 2 µl/mice) or G-15 (5 µg/mice) 20 min before administration of vehicle (saline solution, reference) or ERα17p (2.5 mg/kg, i.p.). Data are expressed as mean ± SEM (n = 8–9 *per* group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the vehicle group; two-way ANOVA followed by Dunnett *post hoc* test for time comparison or Kruskal-Wallis test for AUC mean comparison.

administered. We therefore investigated the involvement of spinal GPER after an intraperitoneal injection of ERα17p. When intrathecally injected 20 min before a systemic injection of ERα17p (2.5 mg/kg, i.p.), G-15 (5 µg/mouse) failed to modify the anti-hyperalgesic action of the peptide (**Figure 4E**), as confirmed by AUCs. Indeed, both AUC of ERα17p (40.73 ± 10.0 g.min.) and AUC of ERα17p+G15 (46.55 ± 7.12 g.min.) were significantly increased compared to AUC of vehicle (−4.63 ± 7.08 g.min., $p = 0.014$ and $p = 0.001$, Kruskal-Wallis test, respectively) but not statistically different between them $p > 0.999$, Kruskal-Wallis test (**Figure 4F**). This result indicates that the anti-hyperalgesic effect of systemic ERα17p is not mediated by spinal GPER.

ERα17p Exerts GPER-Dependent Anti-Inflammatory Effects

The anti-inflammatory action of ERα17p was explored by measuring its impact on carrageenan-induced edema. Four hours after an intraplantar (i.pl.) carrageenan injection, the diameter of the paw significantly increased from 2.10 ± 0.03 cm to 3.24 ± 0.05 cm (n = 43, $p < 0.001$, *t*-test; **Figure 5A**). After a systemic administration of ERα17p (2.5 mg/kg, i.p.), the time-course of the ankle diameter showed reduced edema from 30 min (ERα17p: 2.78 ± 0.9 cm; vehicle: 3.24 ± 0.09 cm, $p = 0.03$, Dunnett *post-hoc* test, **Figure 5A**) to 60 min (ERα17p: 2.61 ± 0.14 cm; vehicle: 3.16 ± 0.11 cm, $p = 0.03$, Dunnett *post-hoc* test). A pre-treatment with G-15 (0.3 mg/kg, i.p.) 15 min before the injection of ERα17p (2.5 mg/kg, i.p.), abolished the previously observed anti-inflammatory action of the peptide (**Figure 5A**).

In the last part of this work, we investigated a potential local anti-inflammatory action of ERα17p. The peptide was directly administered in the paw at a concentration close to the highest soluble dose (*i.e.*, 20 µg in 10 µl *per* mouse, i.pl.). We observed a significant decrease in carrageenan-induced edema (*i.e.*, ankle diameter) at 15, 30, and 90 min (**Figure 5B**). The maximum effect was observed 30 min after the injection (ERα17p: 2.84 ± 0.09 cm; vehicle: 3.39 ± 0.14 cm, $p = 0.002$, Sidak test, **Figure 5B**).

DISCUSSION

Several studies that have outlined the involvement of the hepta-transmembrane estrogen receptor GPER in pain (8, 29–33) have

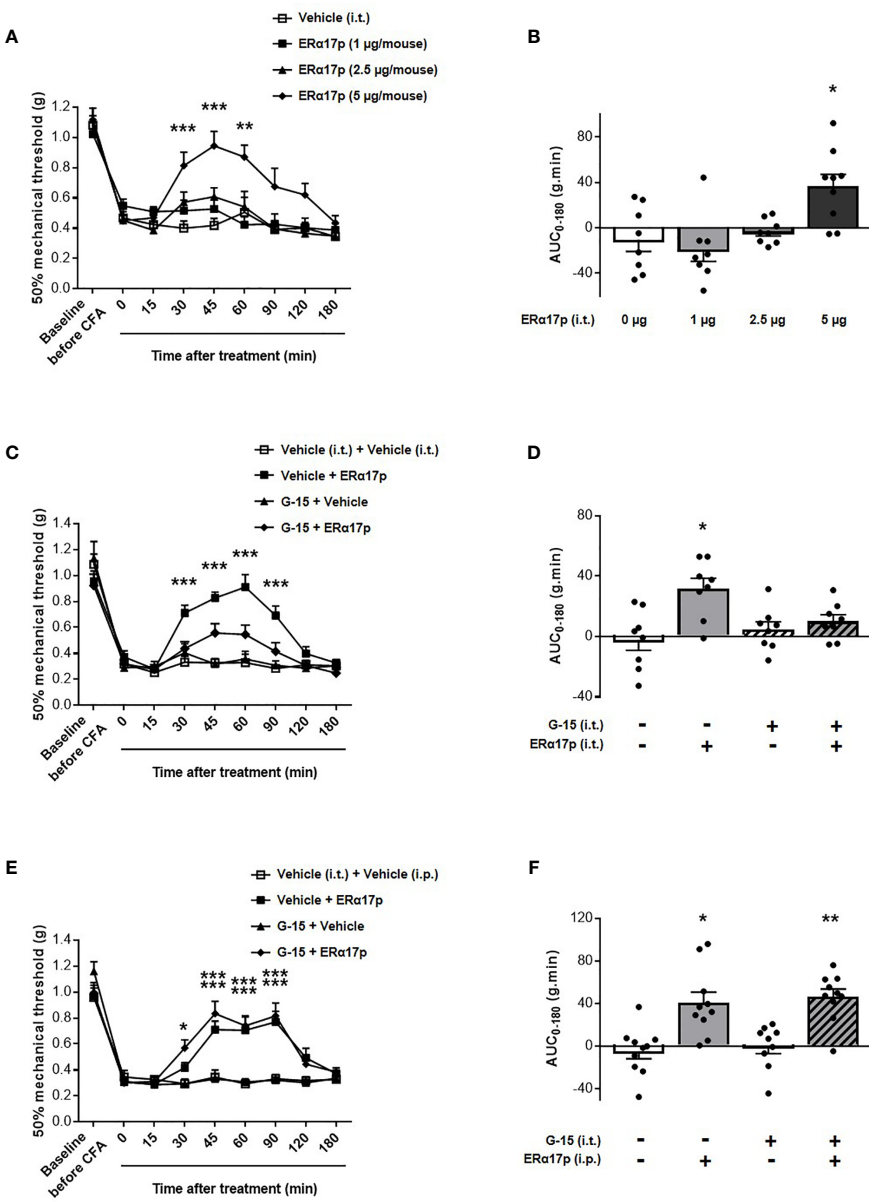

FIGURE 4 | Spinal GPER is not involved in the action of ERα17p in the CFA model. **(A)** Time-course effect of the intrathecal administration of vehicle (saline solution, reference, 2 µl), ERα17p (1, 2.5 and 5 µg/mice) on mechanical hypersensitivity in CFA mice. **(C)** Evaluation of the effect of intrathecally administered ERα17p (5 µg/mice) or vehicle 20 min after G-15 (5 µg/mice, i.t.) or vehicle administration. **(E)** The involvement of spinal GPER in the mechanism of action of systemic ERα17p is investigated by testing ERα17p i.p. with or without G-15 i.t. Mice were i.t. pre-treated with vehicle (saline solution, 2 µl/mice, reference) or G-15 (5 µg/mice) 20 min before an administration of vehicle (saline solution, reference) or ERα17p (2.5 mg/kg, i.p.). **(B, D, F)** Area under the time-course (AUC, 0–180 min) of PWT variations from **(A, C, E)**, respectively. Data are expressed as mean ± SEM (n = 8–9 *per* group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with the vehicle group; two-way ANOVA followed by Dunnett *post hoc* test for time comparison or Kruskal-Wallis test for AUC mean comparison.

prompted our interest in studying the influence of the GPER inverse agonist ERα17p (15) on hyperalgesia. We were all the more interested in this approach that a number of ER ligands have been shown to be involved in nociceptive responses including those responses resulting from rheumatoid arthritis (50–52).

In the present study, we have shown that CFA-induced hypersensitivity was markedly reduced by one i.p. injection of ERα17p at a concentration of 2.5 mg/kg, which is the concentration required to achieve maximum effect. The results obtained with 2.5 and 10 mg/kg ERα17p are similar , suggesting a saturation of the signaling cascade or the formation of pharmacologically inert peptide aggregates. Indeed, it has been shown that ERα17p was prone to form amyloid-like fibrils and aggregates *in vitro* (53, 28). Although internalized in vacuoles, these fibrils and aggregates are devoid of cytotoxicity (28).

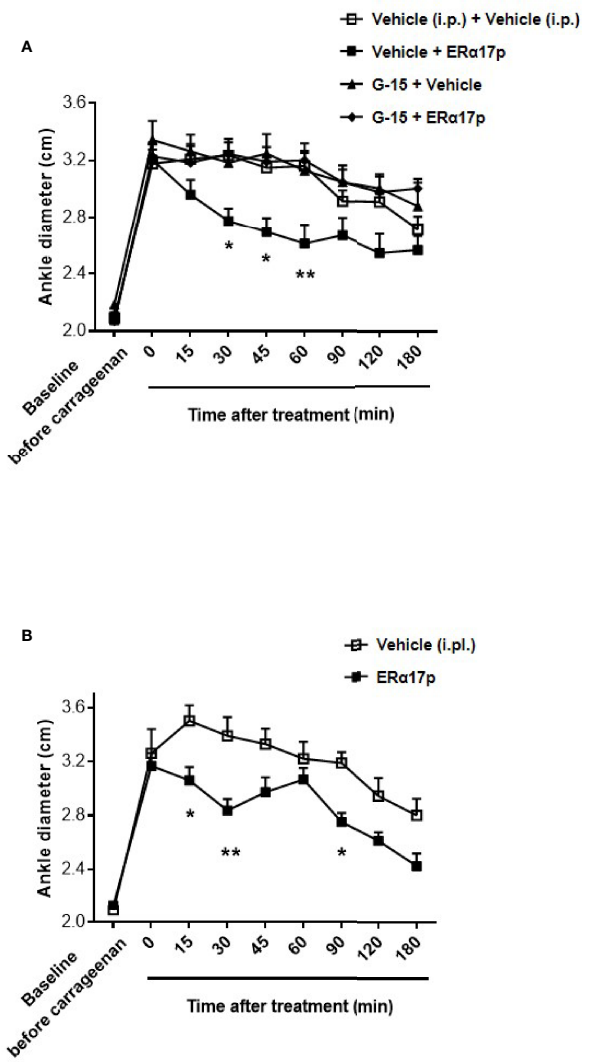

FIGURE 5 | GPER involvement in the anti-inflammatory action of ERα17p in the carrageenan model. **(A)** Ankle diameter of mice was measured before (baseline) and 4 h after carrageenan injection. The involvement of GPER in the mechanism of action of ERα17p was investigated with or without G-15. Mice were i.p. pretreated with vehicle (5% DMSO, 5% Tween80 in saline solution, 10 ml/kg, reference) or G-15 (0.3 mg/kg), 20 min before the administration of vehicle (saline solution, reference) or ERα17p (2.5 mg/kg, 10 ml/kg, i.p.). **(B)** Effect of an intra-plantar (i.pl.) injection of vehicle (saline solution, 10 µl, reference) or of ERα17p (20 µg) on edema measured by ankle diameter (in cm) induced by carrageenan. Data are expressed as mean ± SEM (n = 10–12 *per* group). Two-way ANOVA followed by Dunnett *post hoc* test **(A)** or Sidak *post hoc* test **(B)**. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the vehicle group.

We then sought to identify the receptor by which ERα17p could exert supraspinal analgesia. GPER is expressed all along pain pathways (4, 6, 7, 54) and is involved in pain modulation (5). Accordingly, G-1, a specific GPER agonist belonging to the family of the cyclopentyl[c]quinolines, induces nociception when systemically (33, 55) or locally (8, 9, 29) administered. Likewise, tamoxifen and fulvestrant, two GPER agonists, induce painful symptoms (56–60). Fulvestrant induces painful disorders such as headache and joint and musculoskeletal pain (61, 62).

Since we have previously demonstrated that the anti-proliferative activity of ERα17p was mediated through the GPER (15), we have hypothesized that this membrane receptor could constitute the keystone of the anti-hyperalgesic action of the peptide. Accordingly, we have observed that the anti-hyperalgesic action of ERα17p was abrogated by the GPER antagonist G-15, highlighting a GPER-dependent mechanism. As reported by others, G-15 fails to influence by itself pain threshold (PWT) when systemically administered in inflammatory and neuropathic models (63), or when intrathecally injected in a neuropathic model (64). The absence of G-15-mediated analgesic effects, whereas the GPER inverse agonist ERα17p is active, reinforces the concept of an intrinsic/constitutive physiological pro-nociceptive profile of GPER.

We then assessed the ability of ERα17p to cross the blood brain barrier. Using a Cy5-labeled (fluorescent) version of the peptide, we observed a strong fluorescence signal at the spinal cord and in the brain. Since cyanines, *per se*, do not diffuse in the CNS (65, 66), we assume that the brain and spinal cord staining detected with H_2N-ERα17p-Pra(Cy5)-COOH would be exclusively due to the peptide, which consequently is able to cross the blood-brain barrier. The mechanism by which ERα17p reaches the CNS will be subject to future investigations.

Analgesic activity was observed following direct injection of the peptide into the brain. Although this suggests that the brain could be the site of action of the peptide, it does not necessarily imply a direct involvement of a supraspinal GPER population. Thus, we administered ERα17p intraperitoneally and G-15 *via* the intracerebroventricular route. In these experimental conditions, G-15 did not affect pain threshold on its own but decreased ERα17p-induced analgesia, thus definitively confirming the involvement of a supraspinal pool of GPER.

An anti-hyperalgesic effect GPER-dependent was also observed when the peptide was injected intrathecally. Opinion differs greatly on the involvement of the spinal cord pool of GPER in nociception, with some authors providing evidence of the nociceptive effects of G-1 when intrathecally injected (9) and others failing to detect any effect (32, 64, 67). Despite its ability to diffuse into the spinal cord, analgesic effects resulting from a systemic administration of ERα17p were not abolished by an intrathecal injection of G-15. Thus, a spinal action of the systemically administered peptide in the spinal cord seems unlikely. This apparent discrepancy could be due to the fact that the spinal concentration of the peptide after its systemic administration of the would be peptide, too small to induce analgesic effect.

Finally, we observed that ERα17p possessed an anti-inflammatory effect at the dose of 2.5 mg/kg. This effect being fully abolished by G-15, a GPER-induced pro-inflammatory constitutive activity is likely (8, 29–33). Some studies show no evidence of the beneficial effects of pure GPER agonists on inflammation (63, 68, 69) while others have concluded to their anti-inflammatory action (34, 38, 39). Although further investigations are required the concomitant anti-hyperalgesic

and anti-inflammatory effects displayed by ERα17p strongly suggest that this peptide could be of clinical interest in the management of inflammatory pain.

CONCLUSION

By using mouse models of mechanical hypersensitivity and inflammation, we have shown that the GPER inverse agonist and antitumor compound ERα17p was active *in vivo* on pain and inflammation. These effects were observed at the dose of 2.5 mg/kg and upward, i.e., at doses for which antitumor activity is also observed. These beneficial effects were abolished by the specific GPER antagonist G-15, leading to the conclusion that is involved. We have also evidenced that the anti-hyperalgesic action of ERα17p occurred at the supraspinal level. The mechanism by which the peptide cross the blood brain barrier remains to be determined. Finally, our results suggest that peptides resulting from the proteasome-dependent ERα turnover could play a pivotal role in some physiological and pathological processes through the GPER membrane protein (70).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving animals were reviewed and approved by the Auvergne Animal Experiment Ethics Committee, CE2A and by the French Ministry of Higher Education and Innovation (authorization N° 18022) and performed according to European legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes, and complied with the recommendations of the International Association for the Study of Pain.

AUTHOR CONTRIBUTIONS

YJ, AE, and CM conceived the design of this study. LB, SL, CC, and CM performed experiments. LB, CC, CM, AE, and YJ analyzed and interpreted data. YJ, CM, and AE wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.578250/full#supplementary-material

REFERENCES

1. Lee CW-S, Ho I-K. Sex differences in opioid analgesia and addiction: interactions among opioid receptors and estrogen receptors. *Mol Pain* (2013) 9:45. doi: 10.1186/1744-8069-9-45
2. Liu N-J, Chakrabarti S, Schnell S, Wessendorf M, Gintzler AR. Spinal synthesis of estrogen and concomitant signaling by membrane estrogen receptors regulate spinal κ- and μ-opioid receptor heterodimerization and female-specific spinal morphine antinociception. *J Neurosci* (2011) 31:11836–45. doi: 10.1523/JNEUROSCI.1901-11.2011
3. Oszter A, Vértes Z, Töröcsik B, Környei JL, Kovács KA, Vértes M. Antiestrogenic effect of opioid peptides in rat uterus. *J Steroid Biochem Mol Biol* (2000) 74:25–32. doi: 10.1016/s0960-0760(00)00085-6
4. Brailoiu E, Dun SL, Brailoiu GC, Mizuo K, Sklar LA, Oprea TI, et al. Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J Endocrinol* (2007) 193:311–21. doi: 10.1677/JOE-07-0017
5. Dun SL, Brailoiu GC, Gao X, Brailoiu E, Arterburn JB, Prossnitz ER, et al. Expression of estrogen receptor GPR30 in the rat spinal cord and in autonomic and sensory ganglia. *J Neurosci Res* (2009) 87:1610–9. doi: 10.1002/jnr.21980
6. Takanami K, Sakamoto H, Matsuda K-I, Hosokawa K, Nishi M, Prossnitz ER, et al. Expression of G protein-coupled receptor 30 in the spinal somatosensory system. *Brain Res* (2010) 1310:17–28. doi: 10.1016/j.brainres.2009.11.004
7. Tian Z, Wang Y, Zhang N, Guo Y-Y, Feng B, Liu S-B, et al. Estrogen receptor GPR30 exerts anxiolytic effects by maintaining the balance between GABAergic and glutamatergic transmission in the basolateral amygdala of ovariectomized mice after stress. *Psychoneuroendocrinology* (2013) 38:2218–33. doi: 10.1016/j.psyneuen.2013.04.011
8. Liverman CS, Brown JW, Sandhir R, McCarson KE, Berman NEJ. Role of the oestrogen receptors GPR30 and ERalpha in peripheral sensitization: relevance to trigeminal pain disorders in women. *Cephalalgia* (2009) 29:729–41. doi: 10.1111/j.1468-2982.2008.01789.x
9. Deliu E, Brailoiu GC, Arterburn JB, Oprea TI, Benamar K, Dun NJ, et al. Mechanisms of G protein-coupled estrogen receptor-mediated spinal nociception. *J Pain* (2012) 13:742–54. doi: 10.1016/j.jpain.2012.05.011
10. Lu C-L, Herndon C. New roles for neuronal estrogen receptors. *Neurogastroenterol Motil* (2017) 29:e13121. doi: 10.1111/nmo.13121
11. Kuhn J, Dina OA, Goswami C, Suckow V, Levine JD, Hucho T. GPR30 estrogen receptor agonists induce mechanical hyperalgesia in the rat. *Eur J Neurosci* (2008) 27:1700–9. doi: 10.1111/j.1460-9568.2008.06131.x

12. Lu C-L, Hsieh J-C, Dun NJ, Oprea TI, Wang PS, Luo J-C, et al. Estrogen rapidly modulates 5-hydroxytrytophan-induced visceral hypersensitivity *via* GPR30 in rats. *Gastroenterology* (2009) 137:1040–50. doi: 10.1053/j.gastro.2009.03.047
13. Fehrenbacher JC, Loverme J, Clarke W, Hargreaves KM, Piomelli D, Taylor BK. Rapid pain modulation with nuclear receptor ligands. *Brain Res Rev* (2009) 60:114–24. doi: 10.1016/j.brainresrev.2008.12.019
14. Jacquot Y, Gallo D, Leclercq G. Estrogen receptor alpha–identification by a modeling approach of a potential polyproline II recognizing domain within the AF-2 region of the receptor that would play a role of prime importance in its mechanism of action. *J Steroid Biochem Mol Biol* (2007) 104:1–10. doi: 10.1016/j.jsbmb.2006.10.008
15. Lappano R, Mallet C, Rizzuti B, Grande F, Galli GR, Byrne C, et al. The Peptide ERα17p Is a GPER Inverse Agonist that Exerts Antiproliferative Effects in Breast Cancer Cells. *Cells* (2019) 8:590. doi: 10.3390/cells8060590
16. Bouhoute A, Leclercq G. Modulation of estradiol and DNA binding to estrogen receptor upon association with calmodulin. *Biochem Biophys Res Commun* (1995) 208:748–55. doi: 10.1006/bbrc.1995.1401
17. Gallo D, Haddad I, Duvillier H, Jacquemotte F, Laïos I, Laurent G, et al. Trophic effect in MCF-7 cells of ERalpha17p, a peptide corresponding to a platform regulatory motif of the estrogen receptor alpha–underlying mechanisms. *J Steroid Biochem Mol Biol* (2008) 109:138–49. doi: 10.1016/j.jsbmb.2007.12.012
18. Gallo D, Leclercq G, Jacquot Y. The N-terminal part of the ligand-binding domain of the human estrogen receptor α: A new target for estrogen disruptors. In: . *Medicinal Chemistry Research Progress*. New York, NY, USA: Nova. (2009) 207–24.
19. Seielstad DA, Carlson KE, Kushner PJ, Greene GL, Katzenellenbogen JA. Analysis of the structural core of the human estrogen receptor ligand binding domain by selective proteolysis/mass spectrometric analysis. *Biochemistry* (1995) 34:12605–15. doi: 10.1021/bi00039a016
20. Ylikomi T, Bocquel MT, Berry M, Gronemeyer H, Chambon P. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J* (1992) 11:3681–94. doi: 10.1002/j.1460-2075.1992.tb05453.x
21. Gallo D, Jacquemotte F, Cleeren A, Laïos I, Hadiy S, Rowlands MG, et al. Calmodulin-independent, agonistic properties of a peptide containing the calmodulin binding site of estrogen receptor alpha. *Mol Cell Endocrinol* (2007) 268:37–49. doi: 10.1016/j.mce.2007.01.012
22. Gallo D, Jacquot Y, Cleeren A, Jacquemotte F, Ioanna L, Laurent G, et al. Molecular basis of agonistic activity of ERalpha17p, a synthetic peptide corresponding to a sequence located at the N-terminal part of the estrogen receptor alpha ligand binding domain. *Lett Drug Design Discovery* (2007) 4:346–55. doi: 10.2174/157018007780867807
23. Notas G, Kampa M, Pelekanou V, Troullinaki M, Jacquot Y, Leclercq G, et al. Whole transcriptome analysis of the ERα synthetic fragment P295-T311 (ERα17p) identifies specific ERα-isoform (ERα, ERα36)-dependent and -independent actions in breast cancer cells. *Mol Oncol* (2013) 7:595–610. doi: 10.1016/j.molonc.2013.02.012
24. Pelekanou V, Kampa M, Gallo D, Notas G, Troullinaki M, Duvillier H, et al. The estrogen receptor alpha-derived peptide ERα17p (P(295)-T(311)) exerts pro-apoptotic actions in breast cancer cells *in vitro* and *in vivo*, independently from their ERα status. *Mol Oncol* (2011) 5:36–47. doi: 10.1016/j.molonc.2010.11.001
25. Kampa M, Pelekanou V, Gallo D, Notas G, Troullinaki M, Pediaditakis I, et al. ERα17p, an ERα P295 -T311 fragment, modifies the migration of breast cancer cells, through actin cytoskeleton rearrangements. *J Cell Biochem* (2011) 112:3786–96. doi: 10.1002/jcb.23309
26. Flordellis CS. The plasticity of the 7TMR signaling machinery and the search for pharmacological selectivity. *Curr Pharm Des* (2012) 18:145–60. doi: 10.2174/138161212799040556
27. Byrne C, Khemtémourian L, Pelekanou V, Kampa M, Leclercq G, Sagan S, et al. ERα17p, a peptide reproducing the hinge region of the estrogen receptor α associates to biological membranes: A biophysical approach. *Steroids* (2012) 77:979–87. doi: 10.1016/j.steroids.2012.02.022
28. Trichet M, Lappano R, Belnou M, Salazar Vazquez LS, Alves I, Ravault D, et al. Interaction of the Anti-Proliferative GPER Inverse Agonist ERα17p with the Breast Cancer Cell Plasma Membrane: From Biophysics to Biology. *Cells* (2020) 9:447. doi: 10.3390/cells9020447
29. Alvarez P, Bogen O, Levine JD. Role of nociceptor estrogen receptor GPR30 in a rat model of endometriosis pain. *Pain* (2014) 155:2680–6. doi: 10.1016/j.pain.2014.09.035
30. Luo J, Huang X, Li Y, Li Y, Xu X, Gao Y, et al. GPR30 disrupts the balance of GABAergic and glutamatergic transmission in the spinal cord driving to the development of bone cancer pain. *Oncotarget* (2016) 7:73462–72. doi: 10.18632/oncotarget.11867
31. Bi R-Y, Meng Z, Zhang P, Wang X-D, Ding Y, Gan Y-H. Estradiol upregulates voltage-gated sodium channel 1.7 in trigeminal ganglion contributing to hyperalgesia of inflamed TMJ. *PloS One* (2017) 12:e0178589. doi: 10.1371/journal.pone.0178589
32. Jiang M, Liu Y, Wu H, Ma Z, Gu X. High Estrogen Level Modifies Postoperative Hyperalgesia *via* GPR30 and MMP-9 in Dorsal Root Ganglia Neurons. *Neurochem Res* (2020) 45:1661–73. doi: 10.1007/s11064-020-03032-z
33. Xu S, Wang X, Zhao J, Yang S, Dong L, Qin B. GPER-mediated, oestrogen-dependent visceral hypersensitivity in stressed rats is associated with mast cell tryptase and histamine expression. *Fundam Clin Pharmacol* (2020) 34:433–43. doi: 10.1111/fcp.12537
34. Blasko E, Haskell CA, Leung S, Gualtieri G, Halks-Miller M, Mahmoudi M, et al. Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *J Neuroimmunol* (2009) 214:67–77. doi: 10.1016/j.jneuroim.2009.06.023
35. Wang C, Dehghani B, Li Y, Kaler LJ, Proctor T, Vandenbark AA, et al. Membrane estrogen receptor regulates experimental autoimmune encephalomyelitis through up-regulation of programmed death 1. *J Immunol* (2009) 182:3294–303. doi: 10.4049/jimmunol.0803205
36. Yates MA, Li Y, Chlebeck PJ, Offner H. GPR30, but not estrogen receptor-alpha, is crucial in the treatment of experimental autoimmune encephalomyelitis by oral ethinyl estradiol. *BMC Immunol* (2010) 11:20. doi: 10.1186/1471-2172-11-20
37. Chakrabarti S. Davidge ST. G-protein coupled receptor 30 (GPR30): a novel regulator of endothelial inflammation. *PloS One* (2012) 7:e52357. doi: 10.1371/journal.pone.0052357
38. Meyer MR, Fredette NC, Howard TA, Hu C, Ramesh C, Daniel C, et al. Prossnitz ER. G protein-coupled estrogen receptor protects from atherosclerosis. *Sci Rep* (2014) 4:7564. doi: 10.1038/srep07564
39. Meyer MR, Fredette NC, Barton M. Prossnitz ER. G protein-coupled estrogen receptor inhibits vascular prostanoid production and activity. *J Endocrinol* (2015) 227:61–9. doi: 10.1530/JOE-15-0257
40. Zhao Z, Wang H, Lin M, Groban L. GPR30 decreases cardiac chymase/angiotensin II by inhibiting local mast cell number. *Biochem Biophys Res Commun* (2015) 459:131–6. doi: 10.1016/j.bbrc.2015.02.082
41. Sharma G, Mauvais-Jarvis F, Prossnitz ER. Roles of G protein-coupled estrogen receptor GPER in metabolic regulation. *J Steroid Biochem Mol Biol* (2018) 176:31–7. doi: 10.1016/j.jsbmb.2017.02.012
42. Jacenik D, Zielińska M, Mokrowiecka A, Michlewska S, Małecka-Panas E, Kordek R, et al. Krajewska WM. G protein-coupled estrogen receptor mediates anti-inflammatory action in Crohn's disease. *Sci Rep* (2019) 9:6749. doi: 10.1038/s41598-019-43233-3
43. Dalmann R, Daulhac L, Antri M, Eschalier A, Mallet C. Supra-spinal FAAH is required for the analgesic action of paracetamol in an inflammatory context. *Neuropharmacology* (2015) 91:63–70. doi: 10.1016/j.neuropharm.2014.11.006
44. Kerckhove N, Mallet C, François A, Boudes M, Chemin J, Voets T, et al. Ca(v) 3.2 calcium channels: the key protagonist in the supraspinal effect of paracetamol. *Pain* (2014) 155:764–72. doi: 10.1016/j.pain.2014.01.015
45. Kerckhove N, Boudieu L, Ourties G, Bourdier J, Daulhac L, Eschalier A, et al. Ethosuximide improves chronic pain-induced anxiety- and depression-like behaviors. *Eur Neuropsychopharmacol* (2019) 29:1419–32. doi: 10.1016/j.euroneuro.2019.10.012
46. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* (1994) 53:55–63. doi: 10.1016/0165-0270(94)90144-9
47. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. NC3Rs Reporting Guidelines Working Group. Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. *J Gene Med* (2010) 12:561–3. doi: 10.1002/jgm.1473
48. Rice ASC, Cimino-Brown D, Eisenach JC, Kontinen VK, Lacroix-Fralish ML, Machin I, et al. Animal models and the prediction of efficacy in clinical trials

of analgesic drugs: a critical appraisal and call for uniform reporting standards. *Pain* (2008) 139:243–7. doi: 10.1016/j.pain.2008.08.017
49. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, et al. In vivo effects of a GPR30 antagonist. *Nat Chem Biol* (2009) 5:421–7. doi: 10.1038/nchembio.168
50. Roman-Blas JA, Castañeda S, Largo R, Herrero-Beaumont G. Osteoarthritis associated with estrogen deficiency. *Arthritis Res Ther* (2009) 11:241. doi: 10.1186/ar2791
51. Islander U, Jochems C, Lagerquist MK, Forsblad-d'Elia H, Carlsten H. Estrogens in rheumatoid arthritis; the immune system and bone. *Mol Cell Endocrinol* (2011) 335:14–29. doi: 10.1016/j.mce.2010.05.018
52. Martín-Millán M, Castañeda S. Estrogens, osteoarthritis and inflammation. *Joint Bone Spine* (2013) 80:368–73. doi: 10.1016/j.jbspin.2012.11.008
53. Ruggeri FS, Byrne C, Khemtemourian L, Ducouret G, Dietler G, Jacquot Y. Concentration-dependent and surface-assisted self-assembly properties of a bioactive estrogen receptor α-derived peptide. *J Pept Sci* (2015) 21:95–104. doi: 10.1002/psc.2730
54. Nourbakhsh F, Atabaki R, Roohbakhsh A. The role of orphan G protein-coupled receptors in the modulation of pain: A review. *Life Sci* (2018) 212:59–69. doi: 10.1016/j.lfs.2018.09.028
55. An G, Li W, Yan T, Li S. Estrogen rapidly enhances incisional pain of ovariectomized rats primarily through the G protein-coupled estrogen receptor. *Int J Mol Sci* (2014) 15:10479–91. doi: 10.3390/ijms150610479
56. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Andò S, et al. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol* (2006) 20:631–46. doi: 10.1210/me.2005-0280
57. Maggiolini M, Picard D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J Endocrinol* (2010) 204:105–14. doi: 10.1677/JOE-09-0242
58. Noda-Seino H, Sawada K, Hayakawa J, Ohyagi-Hara C, Mabuchi S, Takahashi K, et al. Estradiol and raloxifene induce the proliferation of osteoblasts through G-protein-coupled receptor GPR30. *J Endocrinol Invest* (2013) 36:21–7. doi: 10.3275/8301
59. Rosano C, Lappano R, Santolla MF, Ponassi M, Donadini A, Maggiolini M. Recent advances in the rationale design of GPER ligands. *Curr Med Chem* (2012) 19:6199–206. doi: 10.2174/092986731209066199
60. Rosano C, Ponassi M, Santolla MF, Pisano A, Felli L, Vivacqua A, et al. Macromolecular Modelling and Docking Simulations for the Discovery of Selective GPER Ligands. *AAPS J* (2016) 18:41–6. doi: 10.1208/s12248-015-9844-3
61. Di Leo A, Jerusalem G, Petruzelka L, Torres R, Bondarenko IN, Khasanov R, et al. Final overall survival: fulvestrant 500 mg vs 250 mg in the randomized CONFIRM trial. *J Natl Cancer Inst* (2014) 106:djt337. doi: 10.1093/jnci/djt337
62. Robertson JFR, Bondarenko IM, Trishkina E, Dvorkin M, Panasci L, Manikhas A, et al. Fulvestrant 500 mg versus anastrozole 1 mg for hormone receptor-positive advanced breast cancer (FALCON): an international, randomised, double-blind, phase 3 trial. *Lancet* (2016) 388:2997–3005. doi: 10.1016/S0140-6736(16)32389-3
63. Liu S, Tian Z, Guo Y, Zhang N, Feng B, Zhao M. Activation of GPR30 attenuates chronic pain-related anxiety in ovariectomized mice. *Psychoneuroendocrinology* (2015) 53:94–107. doi: 10.1016/j.psyneuen.2014.12.021
64. Wright DM, Small KM, Nag S, Mokha SS. Activation of Membrane Estrogen Receptors Attenuates NOP-Mediated Tactile Antihypersensitivity in a Rodent Model of Neuropathic Pain. *Brain Sci* (2019) 9:14. doi: 10.3390/brainsci9060147
65. Spinelli A, Girelli M, Arosio D, Polito L, Podini P, Martino G, et al. Intracisternal delivery of PEG-coated gold nanoparticles results in high brain penetrance and long-lasting stability. *J Nanobiotechnol* (2019) 17:49. doi: 10.1186/s12951-019-0481-3
66. Lesniak WG, Mishra MK, Jyoti A, Balakrishnan B, Zhang F, Nance E, et al. Biodistribution of fluorescently labeled PAMAM dendrimers in neonatal rabbits: effect of neuroinflammation. *Mol Pharm* (2013) 10:4560–71. doi: 10.1021/mp400371r
67. Small KM, Nag S, Mokha SS. Activation of membrane estrogen receptors attenuates opioid receptor-like1 receptor-mediated antinociception *via an* ERK-dependent non-genomic mechanism. *Neuroscience* (2013) 255:177–90. doi: 10.1016/j.neuroscience.2013.10.034
68. Pelekanou V, Kampa M, Kiagiadaki F, Deli A, Theodoropoulos P, Agrogiannis G, et al. Estrogen anti-inflammatory activity on human monocytes is mediated through cross-talk between estrogen receptor ERα36 and GPR30/GPER1. *J Leukoc Biol* (2016) 99:333–47. doi: 10.1189/jlb.3A0914-430RR
69. Engdahl C, Jochems C, Windahl SH, Börjesson AE, Ohlsson C, Carlsten H, et al. Amelioration of collagen-induced arthritis and immune-associated bone loss through signaling *via* estrogen receptor alpha, and not estrogen receptor beta or G protein-coupled receptor 30. *Arthritis Rheum* (2010) 62:524–33. doi: 10.1002/art.25055
70. Gallo D, Haddad I, Laurent G, Vinh J, Jacquemotte F, Jacquot Y, et al. Regulatory function of the P295-T311 motif of the estrogen receptor alpha - does proteasomal degradation of the receptor induce emergence of peptides implicated in estrogenic responses? *Nucl Recept Signal* (2008) 6:e007. doi: 10.1621/nrs.06007

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