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## G protein-coupled estrogen receptor in colon function, immune regulation and carcinogenesis

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### Abstract

Estrogens play important roles in the development and progression of multiple tumor types. Accumulating evidence points to the significance of estrogen action not only in tumors of hormonally regulated tissues such as the breast, endometrium and ovary, but also in the development of colorectal cancer (CRC). The effects of estrogens in physiological and pathophysiological conditions are mediated by the nuclear estrogen receptors  $\alpha$  and  $\beta$ , as well as the membrane-bound G protein-coupled estrogen receptor (GPER). The roles of GPER in CRC development and progression, however, remain poorly understood. Studies on the functions of GPER in the colon have shown that this estrogen receptor regulates colonic motility as well as immune responses in CRC-associated diseases, such as Crohn's disease and ulcerative colitis. GPER is also involved in cell cycle regulation, endoplasmic reticulum stress, proliferation, apoptosis, vascularization, cell migration, and the regulation of fatty acid and estrogen metabolism in CRC cells. Thus, multiple lines of evidence suggest that GPER may play an important role in colorectal carcinogenesis. In this review, we present the current state of knowledge regarding the contribution of GPER to colon function and CRC.

**Key words:** G protein-coupled estrogen receptor; Colorectal cancer; Proliferation; Migration; Colonic motility; Inflammatory bowel disease

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**Core tip:** G protein-coupled estrogen receptor (GPER) is a membrane-bound estrogen receptor that participates in the rapid non-genomic actions of estrogens involving numerous downstream signaling pathways. GPER is expressed in the gastrointestinal tract and is engaged in physiological and pathophysiological processes in the colon. This review aims to assess the significance of GPER expression and estrogenic signaling in colorectal carcinogenesis.

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## INTRODUCTION

The roles of estrogen (17 $\beta$ -estradiol) and its receptors in gastrointestinal (GI) diseases are complex. The lower incidence rate of colon cancer in women compared to men suggests a protective role for the female sex hormone estrogen<sup>[1,2]</sup>. However, because estrogen has at least three receptors, contradictory pro- and anti-tumorigenic mechanisms have been observed depending on the receptor-mediated mechanisms examined. The effects of estrogens are mediated by receptors including the nuclear estrogen receptors (*i.e.*, ER $\alpha$  and ER $\beta$ )<sup>[3]</sup> and the G protein-coupled estrogen receptor (GPER, previously known as GPR30)<sup>[4]</sup>. GPER is a seven-transmembrane receptor cloned from the ER-positive MCF-7 cell line<sup>[5]</sup>, among other sources<sup>[6-11]</sup>. As opposed to nuclear estrogen receptors that are primarily responsible for the genomic actions of estrogens, GPER initiates many rapid non-genomic actions of estrogen involving secondary messengers, which can also ultimately lead to secondary gene expression changes<sup>[4,12,13]</sup>. GPER activity is not only stimulated by 17 $\beta$ -estradiol, but also by numerous xeno- and phyto-estrogens (*e.g.*, bisphenols and genistein), clinically relevant anti-hormonal therapeutic agents (*e.g.*, tamoxifen and fulvestrant) and synthetic GPER-selective ligands (*e.g.*, G-1, G15 and G36) as summarized in Table 1<sup>[4]</sup>. GPER expression and activity, the latter often defined employing GPER-selective ligands, have been linked to many aspects of normal physiology and pathophysiology<sup>[14-16]</sup>.

Experimental evidence strongly suggests that estrogen, and therefore its receptors, plays important roles in neoplastic transformation of the colon<sup>[17,18]</sup>. One challenge in understanding these roles is the often-contradictory results regarding estrogen receptors and their roles in GI diseases. Although estrogen is generally thought to be anti-inflammatory, and inflammation typically contributes to carcinogenesis, the roles of individual estrogen receptors are complex, which may result in conflicting observations. Among the various estrogen receptors, ER $\beta$ , in particular, has been suggested to act as a tumor suppressor in colorectal cancer (CRC) and serves as a prognostic factor for CRC progression. Stevanato Filho *et al*<sup>[18]</sup> observed significantly lower ER $\beta$  levels in CRC patients with clinical stage III and IV disease compared to patients with stage I and II disease. The absence of ER $\beta$  in CRC is thus a poor prognostic factor associated with higher mortality [hazard ratio = 3.0, 95% confidence interval (CI) = 1.2-7.5]. However, in contrast to its established tumor-protective role, Cho *et al*<sup>[17]</sup> used ER-deficient mice to show that, in addition to ER $\beta$ , ER $\alpha$  is also crucial for enterocyte growth and differentiation. Both nuclear estrogen receptors appear to be important modulators of colon neoplastic transformation as ER $\beta$  or ER $\alpha$  deficiency was associated with tumor progression and abnormal mucosal histology in an APC-dependent tumorigenesis model employing C57BL/6-Min/+ mice<sup>[17]</sup>. However, in addition to the nuclear estrogen receptors, accumulating evidence suggests that GPER is also involved in many aspects of CRC cell pathophysiology. In this review, we summarize the evidence for GPER expression and function in the colon and in colorectal carcinogenesis.

## GPER MODULATES COLONIC MOTILITY

Colonic motility regulates the frequency and timing of defecation as well as the consistency of stools, with the main symptoms of colon movement disorders being

**Table 1 G protein-coupled estrogen receptor ligands**

Agonist	Type
Estrogen (17 $\beta$ -estradiol)	Natural steroid
Bisphenol A	Synthetic xenoestrogen
Genistein	Natural phytoestrogen
Tamoxifen	Synthetic therapeutic
Fulvestrant (ICI 182,780)	Synthetic therapeutic
G-1	Synthetic selective ligand
Antagonist	Type
G15	Synthetic selective ligand
G36	Synthetic selective ligand

constipation and diarrhea. A large retrospective study demonstrated a positive association between chronic constipation and a higher prevalence, as well as risk, of benign neoplasm and CRC<sup>[19]</sup>. Moreover, incidence rate ratio analyses have shown that an increased risk of both benign and malignant colorectal neoplasms appears to be directly related to the severity of constipation. Additional evidence linked constipation, defined as fewer than three bowel movements per week, with an overall 2.4-fold increase in CRC development<sup>[20]</sup>. Interestingly, although women overall have a slightly lower risk of developing CRC than men, women with constipation appear to be more prone to CRC development (odds ratio = 2.7, 95% CI = 1.5-5.0) compared to men with constipation (odds ratio = 1.7, 95% CI = 0.6-4.9). Mechanistically, although colon motor dysfunction may increase exposure of the GI tract to carcinogens, evidence suggests that colonic transit time also influences bacterial metabolism and mucosal turnover, both of which may affect CRC development<sup>[21]</sup>.

Two independent studies have revealed that GPER activity influences colonic motility *in vivo*<sup>[22,23]</sup>. Li *et al*<sup>[22]</sup> demonstrated that GPER activity affects colonic motility in multiple phases of the estrous cycle in female mice. Colonic transit time, which was significantly longer during the proestrus and estrus phases (*vs* diestrus phase), was reduced by selective GPER inhibition with the GPER antagonist G15<sup>[24,25]</sup>. Similarly, in ovariectomized mice, acute estrogen treatment increased colonic transit time, and was reversed by co-administration of G15. *Ex vivo*, GPER activation with the GPER-selective agonist G-1<sup>[26]</sup> inhibited circular muscle strip contraction in a nitric oxide (NO)-dependent manner, and stimulated NO production in cultured myenteric nitrergic neurons, which together act to reduce colonic motor function. Further supporting this mechanism, Zielińska *et al*<sup>[23]</sup> and Li *et al*<sup>[22]</sup>, demonstrated, using the colonic bead expulsion test, that GPER stimulation by G-1 or estrogen treatment prolongs colonic transit time in both male and female mice. The inhibitory effect of GPER activation on the number of fecal pellets excreted was further confirmed *in vivo* employing a mouse model of hypermotility. The potential mechanism by which GPER affects colonic motility appears to involve inhibition of muscle contractility, as determined by electrical field- and bethanechol-stimulated longitudinal smooth muscle contractions<sup>[23]</sup>.

Further indirect evidence highlighting the importance of GPER in the regulation of colonic motor function is derived from studies of irritable bowel syndrome (IBS) patients<sup>[27,28]</sup>. Alterations in GPER mRNA and protein expression in the colonic mucosa, as well as serum estrogen levels, were reported in samples from IBS patients with constipation (IBS-C) or diarrhea (IBS-D)<sup>[27]</sup>. An increased number of GPER-positive colonic mast cells in IBS-D patients compared to either healthy control or IBS-C patients has also been observed<sup>[28]</sup>. GPER-positive cell staining in the colonic mucosa also correlated with increased abdominal pain severity in IBS-D patients and increased expression of GPER in the cytoplasm of mast cells, which are associated with abdominal bloating frequency and dysmotility-like dyspeptic symptom severity and frequency in IBS patients. These results suggest a possible role for GPER in mast cells and immune function at large, which play an important role in intestinal function and disease<sup>[29]</sup>.

## GPER IN IMMUNE REGULATION AND COLONIC INFLAMMATION

Crohn's disease (CD) and ulcerative colitis (UC), the two most commonly diagnosed

types of inflammatory bowel disease (IBD), are characterized by chronic colon inflammation and are associated with a higher risk of CRC development<sup>[27]</sup>. A meta-analysis performed by Flores *et al.* reported that patients with histologic inflammation are more prone to colorectal dysplasia and/or cancer development (odds ratio = 2.6, 95% CI = 1.5-4.5) compared to patients without mucosal inflammation<sup>[30]</sup>. For UC patients, the increased risk of CRC development appears to be related to disease duration<sup>[31]</sup>. A meta-analysis by von Roon *et al.*<sup>[32]</sup> further revealed that patients with CD experience a higher relative risk of CRC development, related to both the anatomic localization of disease and patient age. CD patients with disease affecting ileocolic and colon segments experience relative risks of 4.6 (95% CI = 2.1-10.3) and 13.4 (95% CI = 5.7-13.2) for the development of ileocolic and colorectal cancer, respectively. Furthermore, population-based studies have shown that the presence of CD before the age of 25 and 30 correlates with very high relative risks of CRC development (21.5, 95% CI = 11.4-40.4 and 9.5, 95% CI = 3.1-23.2, before the age of 25 and 30, respectively)<sup>[32]</sup>.

Immunomodulatory roles for estrogen and its receptors in the pathogenesis of IBD have been documented<sup>[17,33-36]</sup>, but the specific mechanisms and roles of GPER in modulating immune responses remain unclear. In addition to the nuclear estrogen receptors, GPER is expressed at both the mRNA and protein levels in intestinal samples obtained from both IBD patient subtypes (*i.e.*, CD and UC)<sup>[37]</sup>. In patients with CD, GPER protein content was increased in non-inflamed colon areas compared to the control group but lower in inflamed colon tissue compared to non-inflamed colon, suggesting the possibility of a complex protective role for GPER in colitis<sup>[37]</sup>. To date, although there is no direct evidence showing GPER regulates immune responses in CRC or the CRC microenvironment, several studies show that GPER is indeed expressed in multiple immune cells, including monocytes/macrophages, neutrophils, B and T cells<sup>[38-44]</sup>. Accumulating evidence suggests that GPER modulates cytokine and cytokine receptor expression in immune cells, cancer cells and cancer-associated fibroblasts (CAFs). In breast cancer, estrogen signaling through GPER regulates CAF-mediated progression of breast cancer and is associated with several signaling pathways leading to the modulation of gene expression<sup>[45-48]</sup>. Pro-tumorigenic effects of GPER activation result from receptor tyrosine kinase [*e.g.*, insulin-like growth factor receptor I and epidermal growth factor receptor (EGFR)] modulation and downstream effector proteins such as AKT and extracellular signal-regulated kinase (ERK)<sup>[47]</sup>. GPER-mediated ERK activation in breast CAFs leads to changes in the expression of the proto-oncogenes *c-Fos*, cyclin D1 and connective tissue growth factor (CTGF)<sup>[45,46,48]</sup>, which play an important role in many cancers including CRC<sup>[49]</sup>.

GPER also plays an important role in the regulation of interleukin (IL)-1 $\beta$  and IL-1R expression in CAFs and breast cancer cells, leading to a gene expression profile associated with cancer cell invasiveness<sup>[50]</sup>. GPER's immunomodulatory activity occurs not only in CAFs, but also in several immune cell types. In lipopolysaccharide (LPS)-stimulated primary human macrophages, GPER activation leads to inhibition of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-6, IL-12 and C-C motif ligand (CCL) 5 secretion<sup>[38]</sup>. A similar effect was observed in the murine macrophage cell line RAW 264.7, where G-1 treatment inhibited TNF- $\alpha$  secretion from LPS-stimulated cells. The anti-inflammatory properties of GPER activation were confirmed in an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis), where G-1 therapy reduced the severity of symptoms and the number of nervous system-infiltrating macrophages<sup>[38]</sup>. The ability of GPER to inhibit LPS-induced IL-6 expression in murine macrophages was shown to occur *via* NF- $\kappa$ B signaling<sup>[51]</sup>. Together, these data suggest a potentially central role for GPER in regulating inflammation during IBD.

GPER activity in macrophages may also be important in tumor development and progression within the GI tract. The importance of tumor-associated macrophages (TAMs), specifically in pancreatic cancer has been recently highlighted<sup>[41]</sup>. In a murine model of pancreatic cancer, treatment with tamoxifen, which acts as a GPER agonist (see Table 1), reduced the percentage of tissue macrophages as well as the polarization of TAMs to the M2 phenotype. In macrophage-like murine RAW 264.7 cells, GPER regulated focal adhesions, cell-extracellular matrix attachment and invasion<sup>[41]</sup>. Macrophages are associated with the development and progression of colitis and neoplastic transformation of the colon. Whereas M1 macrophages are elevated in intestinal samples from IBD patients where they promote inflammation, during CRC progression, increased M2/M1 ratios appear to correlate with increased liver metastasis<sup>[52-54]</sup>. Overall, these complex actions of GPER suggest a "protective" role in CRC development and progression.

IL-6 is an important mediator of cancer cell function and tumor development; however, conflicting data exist as to the role of GPER in its regulation. Bisphenol A (BPA), a non-selective ER/GPER agonist (Table 1) induces proliferation, migration and invasion of laryngeal cancer cells, as a result of increased IL-6 mRNA expression,



potentially *via* GPER activation<sup>[55]</sup>. Furthermore, treatment with the GPER-selective inhibitor G15 and/or siRNA targeting of GPER attenuated cell proliferation, migration and IL-6 expression of laryngeal cancer cells. These processes involved signal transducer and activator of transcription 3 (STAT3) activation by GPER/IL-6 signaling as assessed by siRNA targeting of IL-6 or GPER<sup>[55]</sup>. As STAT3 is known to regulate both pro- and anti-inflammatory cytokine production, the exact role of GPER in these complex processes needs to be further investigated. It should be noted that BPA action may also depend on both the cell type and the nuclear estrogen receptor status of cells<sup>[56]</sup>. In contrast to these results, in a more “typical” anti-inflammatory estrogen response, GPER inhibited IL-6 expression *via* NF- $\kappa$ B inhibition, leading to reduced migration of triple negative MDA-MB-231 breast cancer cells<sup>[57]</sup>. As a result, in MDA-MB-231 xenograft tumors, GPER activation inhibited both angiogenesis and metastasis. GPER-mediated inhibition of IL-6 production was also demonstrated in TNF $\alpha$ -stimulated breast cancer cells and osteosarcoma cells where GPER inhibited IL-6 expression, suppressing migration and invasion<sup>[58,59]</sup>. Overall, these results suggest the effects of GPER expression and activity may be tumor type- and context-specific.

Accumulating evidence reveals that GPER is a crucial immunoregulatory factor not only in macrophages, but also in granulocytes, which significantly impact IBD progression. GPER modulates multiple mediators of the immune response in fish acidophilic granulocytes<sup>[40]</sup>, including *IL-1 $\beta$*  and *IL-10*, as well as prostaglandin-endoperoxide synthase 2 and prostaglandin D2 synthase expression, through activation of the cAMP/PKA/CREB signaling pathway. These mechanisms are important in IBD<sup>[60]</sup> and thus should be further examined. In a murine model of castration-resistant prostate cancer, tumor neutrophil influx was observed following G-1 therapy and was associated with extensive tumor necrosis, suggesting a previously unrecognized role for GPER in men<sup>[61]</sup>.

Multiple reports have highlighted that GPER also stimulates anti-inflammatory immune responses through the modulation of T cell function and cytokine expression, representing a potential therapeutic target for certain autoimmune diseases. In one study of autoimmune encephalomyelitis, splenocytes cultured from G-1-treated mice and stimulated with antigen, yielded lower IFN- $\gamma$ , TNF- $\alpha$ , IL-17, CCL4 and CCL5 protein levels in supernatants compared to splenocytes from vehicle-treated mice<sup>[38]</sup>. In other studies, GPER activation increased production of IL-10 (an anti-inflammatory cytokine that inhibits several immune cell types) in CD4<sup>+</sup> T cells under Th17 polarizing conditions, generating hybrid autoregulatory T cell populations<sup>[39]</sup>. This effect was abolished by GPER and ERK inhibitors, but not by p38 or Jun N-terminal kinase inhibitors, suggesting that GPER regulates IL-10 production through ERK signaling. GPER’s anti-inflammatory action may be mediated through specific immune cell types as a higher frequency of IL-10-producing CD4<sup>+</sup> (but not CD8<sup>+</sup>) T cells is observed in mouse splenocytes following GPER activation<sup>[62]</sup>. Moreover, G-1-treated mice showed a significantly higher population of IL-10-producing GPER<sup>+</sup> CD4<sup>+</sup> T cells, consistent with the ability of GPER to regulate IL-10 production.

In additional T cell studies employing a mouse model of asthma, G-1 reduced the level of Th2 cytokines, such as IL-5 and IL-13, in bronchoalveolar lavage fluid, suggesting negative regulation of acute asthma through IL-10-producing T cells<sup>[62]</sup>. In IBD, several intestinal T cell populations are dysregulated, with the extent of dysregulation correlating to disease severity. In clinical samples obtained from IBD patients with active disease, higher and lower levels of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, respectively, are present compared to healthy controls and IBD patients with inactive disease<sup>[63]</sup>. However, in CRC tumors, only an increased percentage of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells was observed<sup>[64]</sup>. The significance of regulatory T cells in inflammation and CRC progression, together with the anti-inflammatory properties of GPER, indicates that GPER activation in CD4<sup>+</sup> T cells may be a promising target to modulate colon immune responses. However, although GPER activity in T cells generally appears to promote anti-inflammatory responses, which would have a positive impact in inflammatory diseases, such responses may have a potentially negative impact in cancer, where immune monitoring is critical. Taken together, these results suggest that as an important regulator of colon inflammation and immune responses in CRC, GPER action may also be important in the context of long-term immune response deregulation, which is critical in CRC development associated with IBD.

## THE ROLES OF GPER IN CRC CELL PROLIFERATION AND APOPTOSIS

Accumulating evidence indicates important roles for estrogen and its receptors,

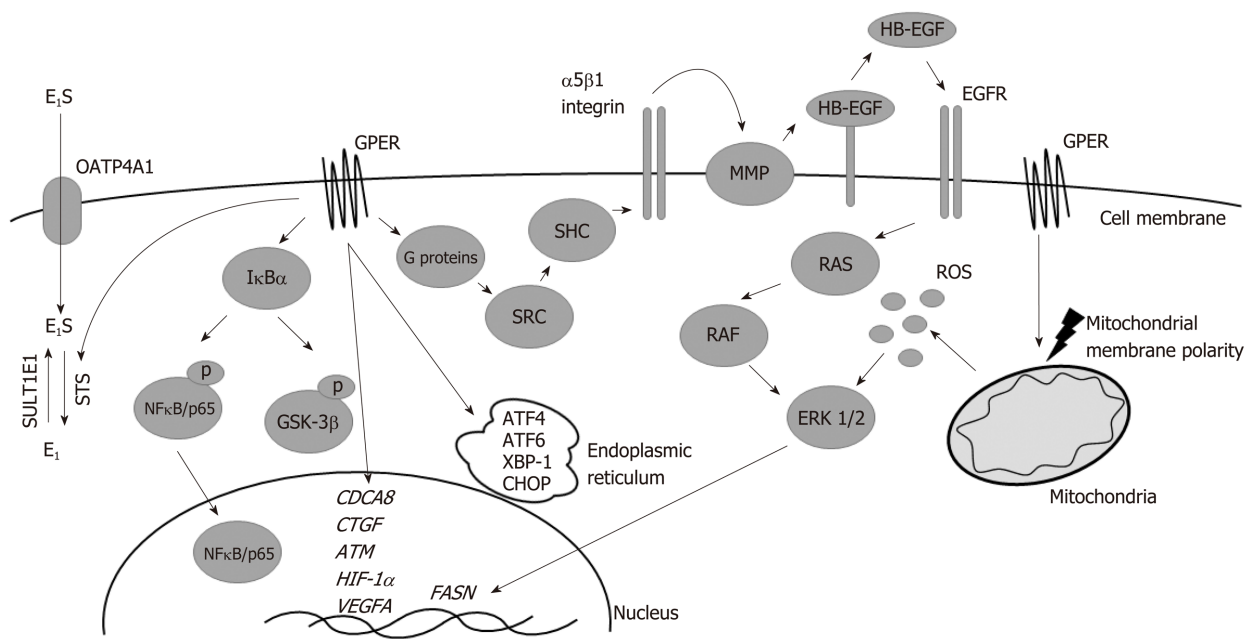
including GPER, as revealed by selective ligands such as G-1, in the regulation of cancer cell growth, survival and function<sup>[65]</sup>. GPER appears to regulate cancer cell proliferation and survival not only in estrogen-associated cancers such as breast<sup>[66]</sup>, ovarian<sup>[67]</sup> or endometrial<sup>[68,69]</sup>, but also in other cancer types not traditionally associated with estrogen (e.g., lung and CRC)<sup>[47,70-77]</sup>. Several cellular mechanisms are regulated by GPER in cancer cells, including cell cycle, endoplasmic reticulum stress and apoptosis. In both HCT-116 and SW-480 CRC cells, GPER activation by G-1 led to cell cycle arrest and inhibition of proliferation<sup>[74]</sup>. The higher proportion of HCT-116 cells in the apoptotic sub-G1 phase, as well as lower mitochondrial membrane polarity, following GPER activation, suggests that GPER activation promotes apoptosis in CRC cells. Consistent with this, G-1 treatment induced up-regulation of pro-apoptotic factors such as Bcl-2-associated X protein, cyclin-dependent kinase inhibitor 1 (p21), and cleaved caspase-3, with down-regulation of anti-apoptotic factors, such as B-cell lymphoma 2 (Bcl-2) and procaspase-3.

Cancer cell growth arrest and apoptosis are also regulated by endoplasmic reticulum stress signals (reviewed by Sano and Reed<sup>[78]</sup>). Among several factors modulating endoplasmic reticulum stress, protein expression of ATF4 and 6, XBP-1 and CHOP increased upon G-1 treatment of HCT-116 cells<sup>[74]</sup> (Figure 1). Elevated reactive oxygen species (ROS) and ERK1/2 phosphorylation, resulting from GPER-mediated signaling, also contributed to growth arrest of HCT-116 cells<sup>[74]</sup>. These pathways are particularly important in chronic inflammation, where immune cell-released ROS and cytokines activate the NF- $\kappa$ B pathway. GPER mediates inhibition of I $\kappa$ B $\alpha$ , which leads to phosphorylation of NF $\kappa$ B/p65 and nuclear translocation, as well as GSK-3 $\beta$  phosphorylation (Figure 1). Constitutive NF- $\kappa$ B activity in cancer cells drives neoplastic transformation and tumor progression, affecting cancer cell proliferation and survival<sup>[79-81]</sup> as well as tumor angiogenesis<sup>[82]</sup>, metabolism<sup>[83,84]</sup>, immune response<sup>[85]</sup> and metastatic potential of cancer cells<sup>[82]</sup>.

GPER may also be a regulator of genetic transmission during cell division in CRC cells. In HT-29 cells, BPA up-regulated mRNA levels of CDCA8 (also known as Borealin), a crucial member of the chromosomal passenger complex that mediates several events during mitosis<sup>[86]</sup> (Figure 1). Although BPA is classically thought to function through the nuclear estrogen receptors, particularly ER $\alpha$ , it also binds and activates GPER (Table 1)<sup>[4]</sup>. Since HT-29 cells express only GPER and ER $\beta$ , the effects of BPA on CDCA8 expression are likely mediated, at least in part, by GPER<sup>[86]</sup>. GPER also regulates ataxia telangiectasia mutated (ATM), an important protein in carcinogenesis, through regulation of the cell cycle and DNA repair (reviewed by Branzei and Foiani<sup>[87]</sup>). In a human breast cancer study, ATM phosphorylation was positively correlated with lymph node metastasis<sup>[88]</sup>. However, in CRC and colon adenomas, increased ATM promoter methylation was observed compared to control tissue<sup>[89]</sup>. Estrogen, acting through GPER, represses ATM expression under both normoxic and hypoxic conditions in HT-29 cells<sup>[70]</sup>, with the effect of estrogen on ATM expression being stronger under hypoxic conditions, suggesting an effect of oxygen levels on estrogen's effects.

Hypoxia plays an important role in tumor progression, affecting tumor vascularization, epithelial-mesenchymal transition and metastasis as well as chemo- and radio-resistance<sup>[90]</sup>. Under hypoxic conditions, the pathways activated by hypoxia-inducible factor (HIF) control numerous cellular proteins, including vascular endothelial growth factor (VEGF), driving tumor growth. GPER mediates estrogen's suppression or enhancement of HIF-1 $\alpha$  and VEGFA expression under normoxic and hypoxic conditions, respectively<sup>[70]</sup>. Furthermore, GPER mediates opposing functions in HT-29 and DLD1 CRC cells, dependent on oxygen levels, with GPER agonists suppressing proliferation under normoxic conditions, but increasing proliferation under hypoxic conditions.

The production and presence of local estrogens may be important in the development and progression of CRC. The ability of GPER to regulate CRC cell proliferation may thus represent a mechanism through which the known effects of estrogen on CRC are mediated. *In vitro* and *in vivo* studies have shown that estrogen promotes CRC in part through the dysregulation of enzymes involved in estrogen metabolism. Specifically, in post-menopausal women and men with CRC, compared to age-matched control groups, higher colonic activity of the enzyme steroid sulfatase (STS), which converts circulating sulfated estrogens to the active forms, has been observed<sup>[91]</sup>. Furthermore, increased protein and mRNA levels of 17 $\beta$ -hydroxysteroid dehydrogenase (HSD17) B7 and B12 (responsible for the conversion of estrone to estrogen) are present in human CRC tissue samples, whereas mRNA and protein levels of the enzyme catalyzing conversion of estrogen to estrone (*i.e.*, HSD17B2) are decreased. Experimental evidence strongly suggests that supplementation with estrogen or STS overexpression increases CRC cell proliferation *in vitro* and *in vivo*. The latter was demonstrated using a murine CRC xenograft model with STS-



**Figure 1 Signaling pathways modulated by G protein-coupled estrogen receptor in colorectal cancer cells.** ATF4: Activating transcription factor 4; ATF6: Activating transcription factor 6; ATM: Ataxia telangiectasia mutated; CDCA8: Cell division cycle A8; CHOP: C/EBP-homologous protein; CTGF: Connective tissue growth factor; E<sub>1</sub>: Estrone; E<sub>1</sub>S: Estrone sulfate; EGFR: Epithelial growth factor receptor; ERK 1/2: Extracellular signal-regulated kinase 1/2; FASN: Fatty acid synthase; GPER: G protein-coupled estrogen receptor; HB-EGF: Heparin-binding epidermal growth factor; HIF-1α: Hypoxia-inducible factor-1α; IκBα: NFκB inhibitor α; MMP: Matrix metalloproteinase; NFκB/p65: Nuclear factor κ-light-chain-enhancer of activated B cells; OATP4A1: Organic anion transporter polypeptide 4A1; P: Phosphorylation; RAF: Rapidly accelerated fibrosarcoma, serine-threonine kinase; RAS: Rat sarcoma, small GTPase; ROS: Reactive oxygen species; SHC: Adapter protein containing SRC homology 2 domain; SRC: Non-receptor tyrosine kinase; STS: Steroid sulfatase; SULF1E1: Sulfotransferase family 1E member 1; VEGFA: Vascular endothelial growth factor A; XBP-1: X-box binding protein 1.

overexpressing cells<sup>[91]</sup>. Activation of GPER by estrogen derived from these multiple sources results in the up-regulation of CTGF, EGR1 and ATF3, with CTGF up-regulation being required for enhanced cell proliferation, with estrone sulfate transport and GPER-stimulated STS activity producing a novel estrogen-generating positive feedback loop (Figure 1) that may play an important role in CRC progression<sup>[92]</sup>. Interestingly, in addition to estrogen and G-1, the breast cancer therapeutic agents, tamoxifen and fulvestrant (ICI 182.780), also increased STS activity (in a GPER-dependent manner), suggesting these drugs could have a negative impact on CRC development and progression.

## GPER REGULATES CRC CELL MIGRATION

An increasing number of studies describes a modulatory effect of GPER activity on cancer cell migration and invasion in multiple cancer types, including breast (including triple-negative), endometrial, ovarian, lung, thyroid, kidney, and granulosa cell<sup>[57,71,77,93-105]</sup>. In previous work that demonstrated the dual role of GPER activation on CRC cell proliferation (see above), oxygen-dependent GPER-mediated effects on the migration of HT-29 and DLD-1 cells was also observed<sup>[70]</sup>. Scratch wound and Boyden chamber assays demonstrated that both G-1 and estrogen inhibited migration under normoxic conditions, whereas GPER stimulation enhanced migration of CRC cells under hypoxic conditions. Fatty acid synthase (FASN), a key lipogenic enzyme that affects neoplastic transformation of the breast, colon and liver, has been described as a metabolic oncogene<sup>[106-108]</sup> and is regulated by many factors, including estrogens<sup>[108,109]</sup>. GPER stimulation by G-1 or estrogen increases expression and activity of FASN *via* EGFR/ERK/c-Fos/AP-1 signaling, resulting in increased growth and migration of CRC cells, which was in turn decreased by a FASN inhibitor<sup>[108]</sup>. Thus, GPER activity regulates cell motility through multiple complex mechanisms (Figure 1).

## CLINICAL SIGNIFICANCE OF GPER IN CRC

There is conflicting evidence that GPER may act as a tumor suppressor as well as a

tumor promoter in CRC. A seven-fold down-regulation of GPER mRNA in CRC samples compared to adjacent control tissue has been observed<sup>[74]</sup>. Immunohistochemical analysis confirmed a decrease in GPER protein expression in CRC patients that was associated with decreased survival. Lower GPER expression also correlated with tumor progression and lymph node metastasis. Bioinformatic analyses of datasets (accession numbers: GSE2091 and GSE871) confirmed these results and showed statistically significant lower GPER mRNA levels in both colon and rectal adenoma samples compared to control samples. *In vitro* evidence further demonstrated that decreased GPER expression in HTC-8 and SW-480 CRC cells correlated with higher promoter methylation of the GPER gene as compared to LS147T CRC cells that exhibit high GPER expression. Beyond promoter methylation, histone H3 acetylation may represent another mechanism regulating GPER expression in CRC cell lines and human tissues as down-regulation of GPER expression in HCT-8 and SW-480 cells was associated with decreased histone H3 acetylation. In contrast to the above results, based on Kaplan-Meier analyses of public data (accession number: GSE39582), high GPER expression was associated with poor relapse-free survival in women with stage 3/4 (but not stage 1/2) CRC, but not in men with disease of any stage<sup>[70]</sup>. Overall, these results suggest that GPER plays a complex role in colorectal carcinogenesis that is further complicated by sexual dimorphism, potentially as a result of estrogen-dependent signaling in CRC.

## CONCLUSION

Estrogen signaling modulates cancer cell proliferation, invasion and migration, acting not only through the nuclear estrogen receptors  $\alpha$  and  $\beta$ , but also through the GPER. GPER is without a doubt an important mediator of colorectal neoplastic transformation and progression. Clinical and experimental data are however, not unambiguous, which may result from the use of CRC cell lines with different GPER expression levels or GPER-mediated signaling pathways. On the other hand, recent studies have revealed the dual role of GPER in CRC development, potentially related to the modulation of both anti-tumorigenic and pro-tumorigenic effects, depending in part on oxygen levels in cancer cells. At the molecular level, GPER-mediated signaling in CRC cells regulates endoplasmic reticulum and mitochondrial functions, the metabolism of fatty acids and estrogens and the expression of genes directly involved in cell proliferation and survival (summarized in Table 2). In conclusion, GPER appears to be an important mediator of estrogenic actions in both neoplastic transformation of the colon and tumor progression, effects that need to be considered in the application and development of therapeutic strategies.

**Table 2 Processes modulated by G protein-coupled estrogen receptor and their importance in neoplastic transformation of the colon**

Features of cancer cells	Process	Anti-tumorigenic	Pro-tumorigenic	Ref.
Proliferation and tumor growth	Apoptosis	Yes	No	[73]
	Cell cycle	Yes	No	[73]
	DNA repair	No	Yes	[64]
	Endoplasmic reticulum stress	Yes	No	[73]
	Estrogen metabolism	No	Yes	[90,91]
	Mitochondrial membrane polarity	Yes	No	[73]
	Oxygen level	Yes under normoxia	Yes under hypoxia	[64]
Migration	Fatty acid metabolism	No	Yes	[107]
	Oxygen level	Yes under normoxia	Yes under hypoxia	[64]

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