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Mechanisms of estrogen signaling via GPR30

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Abstract

The effects of estrogen are widespread throughout the body. Although the classical nuclear estrogen receptors have been known for many years to decades and their primary modes of action as transcriptional regulators is well understood, certain aspects of estrogen biology remain inconsistent with the mechanisms of action of these receptor. More recently, the G protein-coupled receptor, GPR30/GPER, has been suggested to contribute to some of the cellular and physiological effects of estrogen. Not only does GPR30 mediate some of the rapid signal transduction events following cell stimulation, such as calcium mobilization and kinase activation, it also appears to regulate rapid transcriptional activation of genes such as *c-fos*. Since many cells and tissues co-express classical estrogen receptors and GPR30, there exists great diversity in the possible avenues of synergism and antagonism. In this review, we will provide an overview of GPR30 function, focusing on the rapid signaling events that culminate in the transcriptional activation of certain genes.

Keywords

Estrogen; Estrogen Receptor; GPR30; GPER; Transcription

1. Introduction

Estrogen is an important hormone in mammalian biology, regulating numerous processes in many if not most or all tissues throughout the body. It is the most-studied steroid hormone, in part due to the early discovery of a soluble receptor (Jensen et al., 1967) and the discovery of the importance of this hormone in breast carcinogenesis (Beatson, 1896; MacGregor and Jordan, 1998; Moore et al., 1967). Estrogen is synthesized predominantly in the ovaries although localized synthesis has also been reported (Baquedano et al., 2007). As an uncharged steroid molecule, it passes freely through cellular membranes by passive diffusion (Muller et al., 1979). Estrogen-based steroids such as estriol, estrone and estrogen/estrone sulfate also play critical roles in the biological functions of this hormone pathway (Pasqualini et al., 1989). Furthermore, estrogenic activity is found in a large variety of natural sources (phytoestrogens, such as soy-based products) as well as man-made materials (xenoestrogens, also known as environmental estrogens or endocrine disruptors, such as pesticides, herbicides, polychlorinated biphenyls and plasticizers) (Jacobs and Lewis, 2002; Starek, 2003).

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Physiological responses to estrogen are initiated by cellular receptors that alter conformation upon ligand binding, resulting in altered protein-protein interactions and the activation of cell signaling pathways (Edwards, 2005). Although these responses are often categorized either as rapid/non-genomic, occurring within minutes of cell stimulation, or genomic, characterized by changes in gene transcription occurring on the time frame of hours, such distinctions are largely subjective. Traditionally, rapid cellular responses are often associated with cell surface receptors including growth factor receptors and G protein-coupled receptors and are linked to downstream pathways including calcium mobilization, kinase activation and nitric oxide production (Lefkowitz, 2004), whereas genomic responses to steroids are associated with ligand-activated transcription factors, such as the classical steroid receptors (Edwards, 2005). In reality, most hormones initiate both rapid signaling events and transcriptional responses (DeWire et al., 2007; Lange et al., 2007). Nongenomic signaling events can occur with a slower time course, whereas rapid signaling events can stimulate pathways that directly or indirectly modulate gene expression (Fu and Simoncini, 2008; Ma and Pei, 2007).

Both genomic and rapid signaling events initiated by estrogen have traditionally been solely attributed to the classical estrogen receptors (ER α and ER β). Whereas the transcriptional actions of classical ERs have been thoroughly investigated, the mechanisms by which rapid signaling occur have been described in less detail, although reports have described many varied mechanisms (Moriarty et al., 2006). In the last few years, a member of the 7-transmembrane G protein-coupled receptor family, GPR30, has been implicated in mediating both rapid and transcriptional events in response to estrogen under certain circumstances (Filardo and Thomas, 2005; Prossnitz et al., 2008a; Prossnitz et al., 2008b). This review summarizes the history of GPR30 and focuses on mechanisms of signal transduction as well as transcriptional activation in response to estrogen and estrogenic compounds, both natural and man-made.

2. Characterization of GPR30-dependent cellular functions

GPR30 was first identified as an orphan member of the 7-transmembrane receptor family by multiple groups in the late 1990's (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). However, little could be gleaned regarding its possible function from either the predicted amino acid sequence of the gene or from its tissue distribution (based on RNA expression). Based on amino acid sequence homology, GPR30 bore the most similarity to the chemokine subfamily of GPCRs, although the highest homology (with the IL8 receptors) was only about 30% (Owman et al., 1996). Interestingly, one of the cloning approaches utilized differential cDNA library screening between ER-positive MCF7 and ER-negative MDA-MB-231 breast cancer cells lines (Carmeci et al., 1997). Comparing expression of GPR30 in a number of ER-positive and -negative cells lines and tissue revealed a strong positive correlation between ER and GPR30 expression, suggesting a possible link to physiologic responses in estrogen-responsive tissues and cancers (Carmeci et al., 1997).

Based on such correlations, Filardo *et al.* (2000) investigated the functional role of GPR30 in the rapid activation of MAPKs by estrogen in breast cancer cells. Estrogen-mediated activation of Erk1/2 in ER-negative SKBr3 cells as well as GPR30-transfected ER-negative MDA-MB-231 cells demonstrated that expression of GPR30 correlated with the functional response to estrogen. Interestingly, in addition to estrogen, the ER antagonist, ICI 182,780, also stimulated MAPK activation via GPR30. Furthermore, signaling occurred via the transactivation of EGFRs, following matrix metalloproteinase activation and release of HB-EGF. Overall, the results suggested that ER-negative cells could maintain responsiveness to estrogen through the expression of GPR30 (Filardo, 2002).

Over the next few years, a small number of reports related to GPR30 were published. It was reported that progestin upregulates GPR30 expression in MCF-7 cells (Ahola et al., 2002c)

and that this expression is essential for progestin-mediated growth inhibition (Ahola et al., 2002b), which occurs at least in part as a result of Erk inactivation (Ahola et al., 2002a). A follow-up report by Filardo *et al.* described a second phase of GPR30-dependent signaling via adenylyl cyclase that led to the gradual attenuation of Erk activation (Filardo et al., 2002). Subsequently, in keratinocytes, GPR30 was suggested to promote estrogen-mediated inhibition of oxidative stress-induced apoptosis by promoting Bcl-2 expression (Kanda and Watanabe, 2003b) and to promote cell growth by stimulation of cyclin D expression (Kanda and Watanabe, 2004). Furthermore, upregulation of nerve growth factor production in macrophages through *c-fos* induction was demonstrated (Kanda and Watanabe, 2003a). The upregulation of *c-fos* by estrogen and phytoestrogens was also shown in breast cancer cells (Maggiolini et al., 2004). Throughout these studies, however, there was no demonstration that GPR30 directly initiated the observed effects, only that GPR30 expression correlated with responsiveness to estrogen.

In early 2005, two reports provided evidence that GPR30 likely binds estrogen. Thomas *et al.* described the saturable binding of tritiated estrogen ($K_d \sim 3$ nM) to membranes of SKBr3 cells (reduced by siRNA treatment targeting GPR30) and GPR30-transfected HEK cells (Thomas et al., 2005). Estrogen treatment of GPR30-transfected cell membranes also led to the activation of GTP-binding proteins and the production of cAMP. At the same time, Revankar *et al.* described the binding ($K_d \sim 6$ nM) and colocalization of a fluorescent estrogen to GPR30 in both GPR30-transfected cells as well as endogenously expressing cells (Revankar et al., 2005). The binding affinity for GPR30 represented a ten-fold higher value than that determined for ER α ($K_d \sim 0.5$ nM). Interestingly, expression of a GFP-tagged GPR30 as well as antibody staining of endogenously expressed GPR30 revealed that the vast majority, if not essentially all, of GPR30 was localized to intracellular membranes, predominantly the endoplasmic reticulum (and not the plasma membrane), suggesting a novel site of action for GPR30 function. Examples of signaling from intracellular GPCRs (particularly with membrane permeable ligands) are being more routinely described and therefore this paradigm is slowly gaining acceptance, although it is not clear how signaling from an internal membrane compartment would differ from that initiated by a plasma membrane-localized receptor (Boivin et al., 2008). The ability of this pool of GPR30 to bind estrogen was confirmed by staining with the fluorescent estrogen, suggesting that this pool might be functionally active. Additional studies demonstrated that GPR30, but not ER α , was activated by tamoxifen to stimulate PI3K activity and that in SKBr3 breast cancer cells, PI3K activation by estrogen depended on the presence of GPR30. Finally, although estrogen-mediated activation of PI3K could also be mediated by ER α , this mechanism did not involve EGFR transactivation, which was required for GPR30. Thus, although both ER and GPR30 are both capable of activating PI3K in response to estrogen treatment, the two receptors utilize distinct signaling pathways and respond differentially to tamoxifen (Revankar et al., 2005).

3. Identification and characterization of GPR30-selective ligands

Since estrogen is capable of binding to and activating classical estrogen receptors as well as GPR30, pharmacological studies are at best inconclusive due to the lack of specificity of this ligand. Furthermore, the ER antagonists/SERMs tamoxifen and ICI182,780 have been shown to act as GPR30 agonists (Filardo et al., 2000; Revankar et al., 2005) for an in depth review of GPR30 ligand binding properties, see (Prossnitz et al., 2008b). Thus, in an effort to identify receptor-specific ligands, Bologna, Revankar *et al.* (Bologna et al., 2006) screened a library of approximately 10,000 compounds for chemical similarity to estrogen and the top 100 compounds were tested for GPR30 activity. Of these, one displayed activity against GPR30, serving as an agonist of the receptor. In addition, this compound, termed G-1, was inactive against classical estrogen receptors and thus represented the first selective GPR30 ligand (Bologna et al., 2006).

Functional studies revealed that G-1 was capable of eliciting calcium mobilization as well as PI3K activation in cells expressing GPR30 but not in cells expressing either ER α or ER β , where estrogen itself elicited signaling through all three receptors (Bologa et al., 2006). Furthermore, G-1, like estrogen, mediated an inhibition of chemotaxis towards EGF/serum in both MCF-7, which express classical ERs and GPR30, and SKBr3 cells, which express only GPR30 (Bologa et al., 2006). Subsequent works by other groups have utilized G-1 to examine the role of GPR30 in multiple systems. Albanito *et al.* demonstrated that G-1, through GPR30, induced gene expression of *c-fos* in an ERE-independent manner (see below) but ERK- and ER-dependent manner in ovarian cancer cells (Albanito et al., 2007). Interestingly, both GPR30 and ER α along with active EGFR signaling are required for estrogen-stimulated proliferation (Albanito et al., 2007). However, in ER-negative SKBr3 cells, GPR30 alone was sufficient induce transcription and proliferation. These results suggest that in different cellular contexts, the pathways utilized by estrogen may vary depending on the complement of receptors expressed.

Recently, Pang *et al.* have cloned GPR30 from the Atlantic croaker (Pang et al., 2008). Treatment of croaker and zebrafish oocytes *in vitro* with estrogen as well as G-1 reduced both spontaneous and progestin-induced oocyte maturation. Furthermore, injection of GPR30 antisense oligonucleotides in zebrafish oocytes blocked the inhibitory effects of estrogen on oocyte maturation, confirming a role for GPR30 in the control of meiotic arrest. A role for GPR30 has also been documented in the estrogen-mediated stimulation of primordial follicle formation in the hamster ovary (Wang et al., 2008b), where GPR30 is expressed in both granulosa and theca cells and its expression is regulated by gonadotropins (Wang et al., 2007).

A study by Teng *et al.* examined the role of GPR30 in urothelial cell proliferation, where estrogen is known to stimulate cell proliferation mainly through the classical estrogen receptors, although the response is reduced at high estrogen concentrations (Teng et al., 2008). However, urothelial cells were also shown to express high levels of GPR30, raising the question as to the specific roles of individual estrogen receptors in these cells. In urothelial cells, G-1 stimulation inhibited cell proliferation, in contrast to the effects of estrogen. Furthermore, overexpression of GPR30 inhibited, whereas siRNA targeting GPR30 increased, estrogen-induced cell proliferation suggesting that the inhibitory effects of estrogen on cell proliferation correlate with GPR30 expression. Furthermore, G-1 failed to induce *c-fos*, *c-jun* or cyclin D1 expression, and GPR30 overexpression abolished estrogen-induced *c-fos*, *c-jun* or cyclin D1 expression whereas GPR30 downregulation enhanced expression of the same genes. In all, these results suggest that in urothelial cells the effects of estrogen on proliferation are complex, with the classical estrogen receptors stimulating proliferation and GPR30 serving to inhibit proliferation via downregulation of the AP-1 components *c-fos* and *c-jun* with commensurate decreases in cyclin D1 expression.

Estrogen is also known to have multiple neurological effects. In this regard, Kuhn *et al.* have utilized G-1 to examine the signaling pathway by which estrogen acts peripherally to induce mechanical hyperalgesia through PKC ϵ activation (Kuhn et al., 2008). They demonstrated that G-1 but not classical ER agonists activated PKC ϵ in neurons of dissociated dorsal root ganglia. Furthermore, ICI 182,780, an ER antagonist, but a GPR30 agonist, was able to mimic the mechanical hyperalgesia of G-1 when injected into the paws of adult rats, further substantiating a role for GPR30 in this pathway. In another study, Alyea *et al.* have investigated the contribution of membrane estrogen receptors in the estrogen-mediated modulation of dopamine transporters in nerve growth factor differentiated PC12 pheochromocytoma cells (Alyea et al., 2008). A brief exposure to estrogen or EDC initiated the efflux of preloaded dopamine. Knockdown studies using siRNA revealed the ER α depletion blocked estrogen-mediated efflux, whereas GPR30 depletion increased efflux (with ER β depletion having no effect). Interestingly, treatment with G-1 alone had no effect on efflux; however, co-

administration of estrogen and G-1 resulted in substantial inhibition of the estrogen response, suggesting that in this system GPR30 serves to antagonize the stimulatory effect of ER α .

In contrast to the above reports, G-1 has also been used to discount GPR30 involvement in certain systems. One of the major feedback targets for estrogen in the brain is the gonadotropin-releasing hormone (GnRH) neurons, which regulate gonadal function and fertility in mammals. Romano *et al.* determined that G-1 (at 100 nM) had no effect on the calcium dynamics of GnRH neurons, where estrogen and ER α -selective agonists displayed activity (Romano *et al.*, 2008). In the reproductive biology area, Otto *et al.* have performed *in vivo* experiments with G-1 to examine the effect on mammary and uterine tissue (Otto *et al.*, 2008b). In their study, estrogen, but not G-1, regulated expression of Wnt-4, Frizzled-2, IGF-1 or cyclin E1. In addition, a proliferative response to estrogen, but not G-1, was observed in both the endometrium (at 18 hours) and the mammary gland (at 3 weeks). Furthermore, G-1 failed to induce ductal growth or endbud formation in the mammary gland. Unfortunately, it is unclear from such studies whether the appropriate conditions were employed as GPR30 may exhibit altered kinetics or responses from those primarily evoked by classical estrogen receptors. Two recent characterizations of GPR30 knockout mice in one case revealed no obvious defects in reproductive organs (Otto *et al.*, 2008a) but in the other revealed alterations in glucose tolerance, bone growth, blood pressure and serum insulin-like growth factor-I levels (Martensson *et al.*, 2008). In this latter study, aged female GPR30 knockout mice were hyperglycemic with impaired glucose tolerance, which was associated with decreased insulin expression and release, both *in vivo* and in isolated pancreatic islets. Nevertheless, still more recent publications described the ability of G-1 to induce vasorelaxation with resulting decreases in blood pressure (Haas *et al.*, 2009) as well as a role for G-1 in ameliorating the effects of multiple sclerosis in an animal model of autoimmune encephalomyelitis (Wang *et al.*, 2009). In both studies, G-1 activity was absent in GPR30 knockout mice, confirming the physiological activity of G-1 through GPR30.

It is however possible that for some of these estrogen-mediated activities, GPR30 and ERs work in concert, as recently illustrated in estrogen-induced thymic atrophy. Using a GPR30 knockout mouse as well as the GPR30-selective ligand G-1, Wang *et al.* reported that GPR30 contributes, along with ER α , to estrogen-induced thymic atrophy (Wang *et al.*, 2008a). This study revealed that, whereas ER α exclusively mediated the early developmental blockage of thymocyte development, GPR30 was indispensable for thymocyte apoptosis, occurring preferentially in T cell receptor β chain^{-/low} double-positive thymocytes. In a study to examine the mechanisms involved in the estrogen-mediated stimulation of IGF-I mRNA and muscle growth, Kamanga-Sollo *et al.* demonstrated using bovine muscle satellite cell cultures, that although G-1 stimulated the induction of IGF-I mRNA (as did ICI 182,780), it did not stimulate cell proliferation (Kamanga-Sollo *et al.*, 2008). They concluded that, whereas GPR30 mediates the estrogen-stimulated increase in IGF-I mRNA, ER α mediates the proliferative effect. Thus, the interaction of GPR30 and ERs appears to be complex and the elucidation of GPR30 function in physiology will likely require prolonged and in depth analyses.

4. GPR30-mediated transcriptional activation

Although it is often, but not always, straightforward to link the transcriptional response of estrogens with the ligand-dependent genomic model of ER activity, the observation that the rapid signaling events mediated by GPR30 can also lead to the activation of transcriptional machinery has provided further insight into the complexity of estrogen function regardless of ER expression. In this vein, Kanda and Watanabe demonstrated that E2 through GPR30 upregulates nerve growth factor production by inducing *c-fos* expression via cAMP in macrophages (Kanda and Watanabe, 2003a). The same authors demonstrated that E2 induces cyclin D2 and Bcl-2 expression via protein kinase A-mediated CREB phosphorylation in

keratinocytes (Kanda and Watanabe, 2003b; Kanda and Watanabe, 2004). In a similar vein, Hsieh *et al.* recently showed that E2 attenuates hepatic injury after trauma-hemorrhage by upregulating Bcl-2 expression through a GPR30 and PKA-dependent pathway (Hsieh *et al.*, 2007).

The expression of the oncogene *c-fos*, used as an early molecular sensor of estrogen action, provided further evidence of GPR30-dependent transcriptional activation by E2 in ER-positive MCF7 and more importantly, ER-negative SKBr3 breast cancer cells (Maggiolini *et al.*, 2004). This study proved that GPR30 signaling requires EGFR and occurs through rapid ERK1/2 phosphorylation in triggering the genomic response to estrogen notably in tumor cells devoid of ERs (Maggiolini *et al.*, 2004). Further extending these results, E2, the phytoestrogen genistein and the 4-hydroxylated metabolite of the SERM tamoxifen (OHT) induced the expression of *c-fos* through the GPR30/EGFR/ERK signaling pathway and above all, also induced proliferation of thyroid tumor cells lacking ER (ARO cells) or cells expressing a non-transcriptionally active variant of ER α (FRO and WRO cells) (Vivacqua *et al.*, 2006a). Taking into account that thyroid cancer is three times more frequent in women than in men from the onset of puberty until menopause, when this ratio declines progressively (Henderson *et al.*, 1982), the gender-dependent difference observed worldwide (Waterhouse *et al.*, 1982) and the increased risk in women taking estrogens for gynecological disorders (Persson *et al.*, 1996), the GPR30 pathway may represent a new window to circumvent the classical ER-mediated biological thyroid cell response.

The agonist activity of E2 and OHT elicited through the GPR30/EGFR/ERK signaling pathway was also shown in endometrial cancer cells harboring WT ER α (Ishikawa) or its splice variant (Hec1A) (Vivacqua *et al.*, 2006b). In these cell contexts, OHT still retained the antagonist property on ER α activation by E2, yet mediated induction of *c-fos* and cell proliferation in a GPR30-dependent fashion similar to E2 (Vivacqua *et al.*, 2006). These findings provided further insight into the molecular mechanisms potentially involved in the increased incidence of endometrial cancer in women treated with tamoxifen for breast tumors (van Leeuwen *et al.*, 1994). Interestingly, in patients with endometrial carcinoma, GPR30 overexpression positively correlated with EGFR levels, occurred more frequently in high-grade, biologically aggressive histological subtypes and was associated with poorer survival rates (Smith *et al.*, 2007).

The recent availability of the GPR30-specific agonist G-1 (Bologa *et al.*, 2006) represented a key experimental tool towards a precise demonstration of the estrogen-induced and GPR30-mediated transcriptional activation events involving cross-talk with ER α (Albanito *et al.*, 2007). Taking advantage of the lack of any detectable activity of G-1 on the classical ER and using as model systems ovarian cancer cells expressing both ER α and GPR30, it was observed that G-1, like E2, up-regulated diverse estrogen-responsive genes including *c-fos*, pS2 and cyclins A, D1 and E; however, it failed to increase the ER α -target gene PR, which only responded to E2 treatment (Albanito *et al.*, 2007). These data were further corroborated using ER-negative and GPR30-positive SKBr3 cells, where G-1 like E2 stimulated *c-fos* expression, but had no effect on PR expression (Albanito *et al.*, 2007). Together, these results suggested that estrogen-activated PR expression occurs specifically through ER α , while GPR30, possibly together with ER α (see below), mediates the transcriptional activation of the other genes (Fig. 1). In addition, E2 and G-1 used in combination in ovarian cancer cells did not show any further increase in the transcriptional activation of *c-fos* compared to either compound alone, suggesting that a common pathway mediates the genomic response. Of note, knocking down GPR30 or ER α revealed a cross-talk between these estrogen receptors in the stimulation of *c-fos* by G-1 and E2. The above-mentioned regulation of *c-fos* was predictive of the ovarian cell growth observed silencing ER α or GPR30 expression, which interestingly prevented the proliferative effects induced by either ligand (Albanito *et al.*, 2007). In SKBr3 cells, which express GPR30 but lack ER, the knock-down of GPR30 was sufficient to block the growth

stimulation by G-1 and E2. Overall, the findings indicate that cooperation between ER α and GPR30 may take place when both receptors are co-expressed, as also suggested by Sukocheva et al. (Sukocheva et al., 2006).

Nevertheless, GPR30 can be sufficient to signal alone in absence of ER as in SKBr3 breast cancer cells. In these latter cells, a recent study (Albanito et al., 2008a) showed that the widespread environmental contaminant and endocrine-disruptor, atrazine, activates GPR30-dependent signaling, although in ovarian cancer cells, both GPR30 and ER α were required to induce *c-fos* expression and cell proliferation in line with the aforementioned results obtained using E2 and G-1. Previous studies have shown that atrazine may exhibit an estrogen-like action increasing aromatase expression and activity without any direct agonism or antagonism of the classical ERs (Fan et al., 2007a; Fan et al., 2007b; Heneweer et al., 2004; Roberge et al., 2004; Sanderson et al., 2001; Tennant et al., 1994). In the ovarian cancer cells used by Albanito et al. (Albanito et al., 2008a), atrazine neither showed an ability to interact with ER α nor stimulated aromatase activity. Interestingly, atrazine acted through both GPR30 and ER α via the EGFR/MAPK signaling pathway to trigger transcriptional activation and cell proliferation. The authors concluded that a complex interplay between GPR30 and ER α contributes to atrazine activity, which nevertheless is still elicited in presence of GPR30 alone as demonstrated in ER α -negative SkBr3 breast cancer cells. From these data it is reasonable to argue that the evaluation of estrogenic activity of phyto- and xenoestrogens should be extended to their potential ability to activate GPR30 signaling alongside the well-known agonist effects exerted through the classical ER-mediated genomic response.

In order to examine the effects of extranuclear estrogen-mediated pathways on global gene expression, Madak-Erdogan *et al.* recently evaluated the action of E2 and estrogen-dendrimer conjugates (EDCs), which are unable to cross the nuclear membrane (Harrington et al., 2006), in a genome-wide cDNA microarray analysis of MCF-7 breast cancer cells (Madak-Erdogan et al., 2008). Approximately 25% of all E2-regulated genes responded to a 4 h treatment with EDC, independently of the recruitment of ER α to ERE. Furthermore, antiestrogens or ER α knockdown, as well as MAPK and c-Src kinase inhibitors, abolished the up-regulation of EDC-sensitive genes. Interestingly, the authors suggested that EDC signaling triggers transcriptional activation through rapid kinase activity via ER α without its recruitment to EREs. On the contrary, the authors failed to reveal any potential role for GPR30, possibly due to the experimental design and model system used.

A physiological role for GPR30-mediated transcriptional responses through cross-talk with ER α has recently been found in mouse spermatogonia GC-1 cells, which served to investigate the estrogen-mediated regulation of testicular function (Sirianni et al., 2008). On the basis of an altered testicular phenotype in ER α knockout mice (Das et al., 2000; Eddy et al., 1996), which is less severe compared to aromatase knockout mice, Sirianni *et al.* investigated the potential involvement of an estrogen-binding receptor different than the well-known ERs, such as GPR30, in estrogen signaling (Sirianni et al., 2008). The authors demonstrated that E2 and G-1 activate the EGFR/ERK pathway leading to the stimulation of *c-fos* and cyclin D1 expression as well as GC-1 cell growth. Interestingly, the proliferative effects induced by E2 and G-1 were abrogated using either the ER α antagonist ICI 182780 or silencing GPR30 expression. The results obtained are consistent with data recently reported by Bouskine *et al.*, demonstrating that E2, through the activity of a Gi protein, could induce rapid activation of ERK1/2 and PKA signaling pathways, which in turn are involved in the proliferation of human germ cell tumors (Bouskine et al., 2008).

To this point, the aforementioned studies have broadened the molecular mechanisms through which estrogen signaling may involve the EGFR signaling pathway in modulating gene expression. In this regard, the genotropic activity of estrogen has been largely recognized to

be mediated by the ERs, although many E2-responsive genes are key molecules participating in EGFR signaling (Levin, 2003). Interestingly, the cell membrane-associated form of ER has been reported to couple with and activate diverse G proteins, thereby triggering biological responses via EGFR transactivation (Levin, 2003; Razandi et al., 2003). Moreover, E2 activation of GPR30 activates the EGFR signaling cascade (Filardo et al., 2000) similar to other GPCR ligands (Bhola and Grandis, 2008; Rozengurt, 2007). Recently, Albanito et al. provided evidence in ER-negative breast cancer cells that EGF, by up-regulating GPR30 expression, engages E2 to potentiate the biological response to EGFR signaling (Albanito et al., 2008b). This positive feedback loop between EGFR and GPR30 activity represents a further demonstration that signaling pathways may be able to auto-regulate the amplitude of their own activation to modulate signaling robustness in the face of variable inputs (Freeman, 2000). The results obtained by Albanito et al. also demonstrated that the classical ERs may not be required for GPR30/EGFR cross-talk; however, GPR30 and ER α can cooperate in mediating the action of E2 as observed in ER-positive, GPR30-positive ovarian cancer cells (Albanito et al., 2007). Finally, the clinical observation that GPR30 overexpression is associated with lower survival rates in endometrial cancer patients (Smith et al., 2007) and higher risk of developing metastatic disease in patients with breast tumor (Filardo et al., 2006) suggests an important involvement in carcinogenesis. Therefore, the expression levels of GPR30, which are regulated by EGF/EGFR signaling may characterize the estrogen sensitivity of these tumors in addition to predisposing tumors to an altered responsiveness to endocrine therapy.

Very recently, a set of genes has been identified that may contribute to the proliferative activities of GPR30 (Pandey et al., 2009). Of particular interest was the prominent induction of connective tissue growth factor (CTGF) by both E2 and OHT through GPR30 signaling, which enhanced breast cancer cell migration. As CTGF was also induced by OHT in fibroblasts from breast tumor biopsies, this points to the potential involvement of GPR30 pathways in the aggressive behavior of breast tumors, particularly in response to endogenous estrogens or OHT used in (chemo)therapeutic interventions.

5. Future Directions

As time progresses, the cellular and physiological roles of GPR30 are becoming better defined. As discussed in this article, an increasing number of laboratories are identifying very specific estrogen-mediated functions for GPR30, in addition to estrogen-mediated functions that are not mediated by GPR30. Overall, it appears that in some situations, GPR30 and ERs are both required, whereas in others, GPR30 can act in the absence of ERs to mediate estrogen-dependent effects. In yet other circumstances, GPR30 seems to antagonize the effects of ER α . In addition, not only does GPR30 mediate some of the rapid signaling events that follow estrogen administration, it also activates transcription of numerous genes, but as might be expected, not genes that are strongly under the regulation of classical EREs. With the use of selective ligands (including the recent identification of the first GPR30 antagonist (Dennis et al., 2009)), siRNA approaches and knockout animals, the future will continue to bring an enhanced understanding of the functions of GPR30.

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Abbreviations

cAMP cyclic adenosine monophosphate

EGF(R)	epidermal growth factor (receptor)
ER(s)	estrogen receptor(s)
ERE	estrogen response element
GPCR	G protein-coupled receptor
MAPK	mitogen-activated protein kinase
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
SERM	selective estrogen receptor modulator

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Figure 1. Signaling Pathways activated by GPR30 and Classical Estrogen Receptor

Estrogen, tamoxifen, G-1 and related compound are cell permeable and thus able to gain access to intracellular estrogen receptors (ER and/or GPR30). Ligand binding to classical estrogen receptors leads to direct transcriptional activation as well as rapid signaling (latter not shown). Stimulation of GPR30 activates heterotrimeric G proteins, which trigger multiple effectors resulting in camp production as well as Src and sphingosine kinase (SphK) activation. Matrix metalloproteinase (MMPs) activation leads to HB-EGF release that can transactivate epidermal growth factor receptors (EGFRs). EGFR activation leads to multiple downstream events including activation of MAP kinases (MAPKs) and PI3 kinases (PI3Ks), which result in expression of transcription factors such as *c-fos*. As a result, estrogen stimulation can lead to the expression of target genes whose promoters do not contain steroid response elements (EREs). The combined effects of these signaling and transcriptional events often lead to cell cycle progression and cell proliferation.