

## REVIEW

# The unfolding stories of GPR30, a new membrane-bound estrogen receptor

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## Abstract

Steroid hormones such as estrogens are known to signal through ligand-regulated transcription factors of the nuclear receptor superfamily. In addition, they elicit rapid nongenomic responses from membrane-associated receptors. One of these receptors belongs to an entirely different family of proteins. The G protein-coupled and seven-transmembrane receptor, GPR30, is now widely recognized as an estrogen receptor (ER), hence its official new acronym

GPER. It appears to mediate a wide range of responses to estrogen in a large variety of cell types. Its functions are clearly distinct from those of the classical nuclear ERs, although these pathways may overlap and interact in some cases. Here, we review the history of the discovery of this new ER, the evidence for the claim that it is an ER, its signal transduction, and its potential functions in physiology and disease.

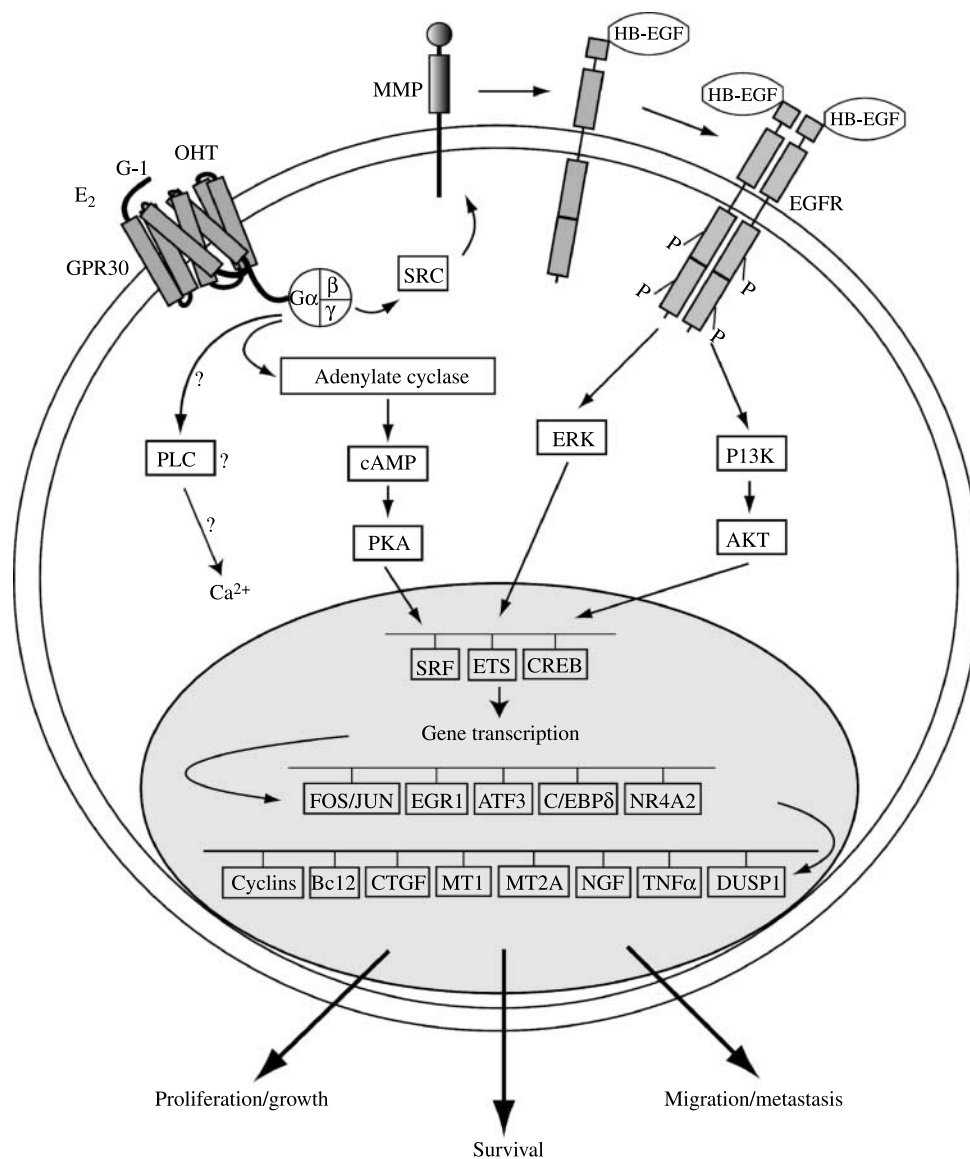
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## Discovery and history of GPR30

In contrast to what some text books might have advocated, for a lot of hormones and receptors, there is no one-to-one relationship. The steroid hormone estrogen, represented by the most potent physiological estrogen 17 $\beta$ -estradiol (E<sub>2</sub>), is no exception. Estrogens have a multitude of different molecular targets, including two well-characterized members of the nuclear receptor superfamily, the estrogen receptors (ER)  $\alpha$  and  $\beta$  (listed as ESR1 and ESR2 in the HUGO & MGI Databases) (Dahlman-Wright *et al.* 2006, Heldring *et al.* 2007). In turn, the latter and other molecular targets also respond to a more or less broad range of physiological and synthetic compounds. When the discovery of a second nuclear ER, ER $\beta$ , was reported in 1996 (Kuiper *et al.* 1996), it did come as a big surprise because it was felt that there was no 'need' for yet another ER. The only known ER at the time, ER $\alpha$ , was then shown to mediate rapid nongenomic signaling responses as well (Migliaccio *et al.* 1996). With this, it seemed that all estrogen responses were covered. This might explain why the more recent discovery of GPR30 as a membrane-associated ER was (and to some extent still is) met with a lot of skepticism. The field has come a long way since its discovery (Carmeci *et al.* 1997), and there are several recent and authoritative reviews on it (Filardo *et al.* 2008, Prossnitz

*et al.* 2008, Prossnitz & Maggiolini 2009). The goal of this review is to present the recent advances of GPR30 research, while highlighting its diversity and complexity and discussing remaining controversies and knowledge gaps.

The history of the discovery of GPR30 is unusual. In hindsight, one of the first hints came from the observation that estrogen could stimulate adenylate cyclase activity and thus the production of cAMP in human MCF7 breast cancer cells (Aronica *et al.* 1994; Fig. 1). The authors concluded from the fact that ER $\alpha$ -negative MDA-MB-231 breast cancer cells did not display this response that ER( $\alpha$ ) was the most likely mediator. What they could not yet know is that MCF7 cells express GPR30, whereas MDA-MB-231 cells do not. Indeed, GPR30 was finally cloned a few years later as a cDNA of a gene that is differentially expressed in MCF7 compared with MDA-MB-231 cells (Carmeci *et al.* 1997). Considering that the expression profiles of the two cell lines must differ by a large number of mRNAs, it is amazing that the GPR30 cDNA came out of this screen as the only novel sequence. Be it as it may, the same authors, based on a survey of a limited number of breast carcinoma cell lines and biopsies, concluded that GPR30 expression correlates with ER( $\alpha$ ) expression. More recent analyses with a larger number of samples clearly challenge this conclusion (Filardo *et al.* 2006, 2008). Again a few years later, Filardo *et al.* (2000)



**Figure 1** Schematic representation of the GPR30 signaling network. Abbreviations not mentioned in the text are: MMP, matrix metalloprotease; PLC, phospholipase C; PKA, protein kinase A. As pointed out in the text, the connections to an increase in calcium and those between the trimeric G-proteins and the PI3K-Akt pathway remain unclear. Note that we put GPR30 somewhat arbitrarily at the plasma membrane, but signaling would presumably largely work the same way with GPR30 at the membrane of the endoplasmic reticulum. Most target genes shown at the bottom are from [Pandey \*et al.\* \(2009\)](#).

showed that estrogen activates the mitogen-activated kinases (MAPK) Erk1/2 even in ER-negative SKBr3 cells. Unlike MDA-MB-231 cells, these were found to be GPR30 positive. A first convincing genetic case for GPR30 as a novel ER was made by the demonstration that rapid estrogen responses could be transferred into nonresponsive MDA-MB-231 by transfection with a GPR30 expression vector ([Filardo \*et al.\* 2002](#)). Further genetic evidence came from experiments with keratinocytes in which GPR30 expression was blocked

with antisense oligonucleotides ([Kanda & Watanabe 2004](#)). These are some of the pioneering publications that ended up launching an entirely new field.

Upon the initial cloning of GPR30, the sequence immediately revealed that it is part of the huge family of G-protein coupled receptors spanning the membrane seven times (7TM-GPCRs). Within this family that comprises hundreds of members, it belongs to the class A rhodopsin-like ones and in there, it constitutes the chemokine receptor-like 2

subfamily. With 28% sequence identity, it is most closely related to GPCRs such as the angiotensin II 1A receptor and the interleukin 8A receptor (28% identity) (Carmeci *et al.* 1997, Feng & Gregor 1997). These ‘family ties’ with GPCRs that bind peptide or protein ligands raises the intriguing question of whether GPR30 might also have such ligands, perhaps in addition to estrogens. A final word here about nomenclature: the protein that we will continue to refer to as GPR30 in this review was cloned in parallel by several different groups and hence, got started with multiple different names, all in the same year (for example Bonini *et al.* 1997, Carmeci *et al.* 1997, Feng & Gregor 1997, Takada *et al.* 1997). It should be noted that the rat ortholog was originally named GPR41 (Bonini *et al.* 1997, Kimura *et al.* 2001), an unfortunate choice in view of the fact that there is an unrelated homonymous human GPCR. A decade later, now that GPR30 has been recognized as a membrane-bound ER, it has received a new official name: G-protein coupled ER1 (GPER).

### Signal transduction pathways

Some of the very first reports already established that the GPCR GPR30 does indeed couple to G-proteins in breast cancer cells. Inhibitor studies and measurements of GTP recruitment to membranes revealed that both G $\beta\gamma$  (Filardo *et al.* 2000) and a G $\alpha_s$  (Thomas *et al.* 2005) may play a role in signaling. Downstream of G-proteins, E<sub>2</sub>-induction leads to activation of a SRC-like tyrosine kinase, phosphorylation of the adaptor protein SHC, and, presumably through the activation of a metalloprotease, the extracellular release of heparan-bound epidermal growth factor (HB-EGF). A very recent publication indicates that integrin  $\alpha_5\beta_1$  acts as a conduit between SRC and metalloproteases (Quinn *et al.* 2009). Activation of SRC promotes the formation of SHC-integrin complexes and through these a fibronectin matrix assembly and metalloprotease activation. The release of HB-EGF allows it to activate the EGF receptor (EGFR) resulting in the induction of the MAPK pathway (Filardo *et al.* 2000; Fig. 1). The fact that E<sub>2</sub> induces the production of cAMP has already been mentioned above. It could be linked to a GPR30 response using membrane preparations of ER-negative SKBr3 cells as well as in whole cells transfected with GPR30 (Filardo *et al.* 2002, 2007, Thomas *et al.* 2005), and by a GPR30 antisense approach in keratinocytes and macrophages (Kanda & Watanabe 2003a,b). It appears to be the G $\alpha_s$  leg of G-protein signaling that is responsible for the E<sub>2</sub> stimulation of adenylate cyclase and the ensuing increase in cAMP in breast cancer cells (Thomas *et al.* 2005). Similarly, a variety of phyto- and xenoestrogens are also able to stimulate cAMP production through GPR30 (Thomas *et al.* 2005, Thomas & Dong 2006). Somewhat paradoxically, the generation of cAMP attenuates MAPK activity via the inhibitory activity of protein kinase A on RAF1 (Filardo *et al.* 2002). It is likely that the exact balance between inhibition and stimulation of the MAPK pathway will depend on timing and cell type.

There are yet other early events. E<sub>2</sub> activates phosphoinositide 3-kinases (PI3K) through GPR30. As a result of the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), the anti-apoptotic and proliferative kinase AKT is activated. This follows its recruitment to membranes via its pleckstrin homology domain, a phenomenon that has been used as an elegant read-out of GPR30 signaling (Revankar *et al.* 2005). PI3K activation was further shown to be sensitive to the EGFR inhibitor AG1478 but insensitive to pertussis toxin. The latter suggests that the activation of PI3K, unlike that of a SRC-like kinase, is not downstream of G $\beta\gamma$ . Using new GPR30-specific pharmacological tools, this was recently further substantiated (Dennis *et al.* 2009). The specific GPR30 antagonist G15 (see also below) inhibited the stimulation of PI3K by both E<sub>2</sub> and the specific GPR30 agonist G-1 (see below) in SKBr3 breast cancer cells, which endogenously express GPR30. The stimulation of PIP3 production by E<sub>2</sub> and G-1, and their inhibition by G15, could also be demonstrated with GPR30 exogenously expressed in ER-negative COS7 cells. An increase in intracellular Ca<sup>2+</sup> was shown to be a very rapid response to GPR30 activation in different cell contexts (Revankar *et al.* 2005, Bologna *et al.* 2006, Filardo *et al.* 2007, Dennis *et al.* 2009). Similarly to other downstream events, this E<sub>2</sub> response could be inhibited with the antagonist G15 (Dennis *et al.* 2009). Formally, it has not been determined what triggers the increase in calcium and where it comes from. Both a release from intracellular calcium stores and an influx from outside are possible. Phospholipase C appears not to be involved (Revankar *et al.* 2005), but this may depend on cell type or indicate that some other phospholipase is (Fig. 1). The details of some of the connections need to be further investigated.

While all of these signaling events happen within seconds and minutes, there is accumulating evidence that GPR30 signaling triggers more long-term transcriptional responses (Prossnitz & Maggiolini 2009). Kanda & Watanabe established this link with macrophages and keratinocytes. The elevated levels of cAMP that GPR30 signaling triggers lead to the induced expression of *c-fos* in macrophages (Kanda & Watanabe 2003a) and activation of the transcription factor CREB in keratinocytes (Kanda & Watanabe 2003b, 2004). These in turn activate the expression of target genes such as nerve growth factor in macrophages and cyclin D2 and BCL2 in keratinocytes. The anti-apoptotic effects of E<sub>2</sub>-induced BCL2 upregulation are also involved in attenuating hepatic injury caused by trauma-hemorrhage (Hsieh *et al.* 2007). In breast cancer cells, other signaling pathways may predominate to induce some of the same factors and genes. In ER-negative SKBr3 breast cancer cells, E<sub>2</sub> induces *c-fos* expression through the GPR30/EGFR/MAPK signaling cascade (Maggiolini *et al.* 2004). E<sub>2</sub> and the partial ER antagonist but GPR30 agonist (see below) hydroxytamoxifen (OHT) both stimulate the expression of *c-fos* and elicit proliferative effects through GPR30 in thyroid and endometrial cancer cells (Vivacqua *et al.* 2006a,b). In ovarian cancer cells, both E<sub>2</sub> and G-1 upregulate numerous estrogen-responsive genes including

*c-fos*, pS2, and cyclins A, D1 and E, while other direct ER $\alpha$  target genes such as the progesterone receptor (PR) gene only respond to E<sub>2</sub> (Albanito *et al.* 2007). In these ovarian cancer cells, the knockdown of GPR30 or ER $\alpha$  revealed an interesting codependence between these receptors for the induction of *c-fos* by G-1 and E<sub>2</sub>. In contrast, as pointed out above, the upregulation of *c-fos* and, importantly, cell proliferation in response to E<sub>2</sub> is solely GPR30 dependent in a cell context such as the SKBr3 breast cancer cells lacking nuclear ERs (Albanito *et al.* 2007). The dual dependence on ER $\alpha$  and GPR30 in the case of ovarian cancer cells was further substantiated and extended with the demonstration that the environmental contaminant atrazine stimulates the activation of estrogen target genes and cell proliferation through the GPR30/EGFR/MAPK signal transduction pathway. Although atrazine does not bind to nor activate ER $\alpha$ , this receptor along with GPR30 is required to elicit the estrogen-like effects (Albanito *et al.* 2008a). The mouse spermatogonial cell line, GC-1, represents yet another case where both the induction of proliferation and of expression of *c-fos* and cyclin D1 by either E<sub>2</sub> or G-1 depend on both ER $\alpha$  and GPR30 (Sirianni *et al.* 2008). Whereas it is easy to see how E<sub>2</sub> signaling could depend on a dual input through both types of receptors, this remains unclear for the GPR30-specific agonist G-1. The presence of ER $\alpha$ , even in its unliganded form, might somehow set the stage for a GPR30-mediated G-1 response.

Most recently, we undertook a systematic characterization of the genomic responses to GPR30 signaling in breast cancer cells (Pandey *et al.* 2009). We determined the changes in the gene expression profile of SKBr3 cells elicited by GPR30 signaling. We discovered that this involves a whole network of transcription factors. Rapid posttranslational activation of a first tier of transcription factors including *SRE*, *CREB*, and members of the ETS family promotes the expression of a second wave of transcription factors such as *FOS*, *JUN*, *EGR1*, *ATF3*, *C/EBP $\delta$* , and *NR4A2*. The activities of many of these are further augmented at the protein level under the effects of GPR30 signaling, for example by phosphorylation by MAPK. Cells are literally reprogrammed under the effect of this network of transcription factors. Superimposed on these responses, there may be a variety of signaling crosstalk pathways and both negative and positive feedback loops. For example, we found that EGF upregulates GPR30 expression through the EGFR/MAPK pathway in ER-negative breast cancer cells, most likely by promoting the recruitment of the c-FOS-containing transcription factor *AP-1* to the GPR30 promoter (Albanito *et al.* 2008b). Considering that GPR30 signaling uses the EGFR/MAPK pathway, a positive feedback loop is conceivable. This mechanism is also operational for EGF and the related growth factor TGF $\alpha$  in ER $\alpha$ -positive breast cancer cells (Vivacqua *et al.* 2009). The concomitant presence of ER $\alpha$  and GPR30 even allows for a ligand-modulated physical interaction, which provides additional, but still unexplored opportunities for crosstalk. Another intriguing example is that of endometrial and

choriocarcinoma cells, where the GPR30-induced production of PIP3 stimulates the activity of the nuclear receptor SF-1 (NR5A1 as listed in the HUGO Database), which in turn enhances the expression of aromatase and thus the production of estrogen and proliferation (Lin *et al.* 2009). In some cases, the presence of ER $\alpha$  might complicate matters as it has been shown to mediate the repression of GPR30 expression (Lupien *et al.* 2009). Clearly, there is a lot of room for cell specificity modulating GPR30 signaling.

### Role of GPR30 in carcinomas

As already alluded to above, GPR30 is widely expressed in carcinoma cell lines including ER-positive MCF7 and ER-negative SKBr3 breast cancer cells (Carneci *et al.* 1997, Filardo *et al.* 2000), Hec1A (Vivacqua *et al.* 2006b) and Hec50 (Revankar *et al.* 2005) endometrial cancer cells, JEG choriocarcinoma cells (Revankar *et al.* 2005), BG-1 ovarian cancer cells (Albanito *et al.* 2007), and thyroid carcinoma cell lines (Vivacqua *et al.* 2006a). We have shown for many of these that estrogens can stimulate their proliferation through GPR30 (Vivacqua *et al.* 2006a,b, Albanito *et al.* 2007; see also Lin *et al.* 2009), supporting the notion that GPR30 might contribute to carcinogenesis. Endocrine therapy is often the treatment of choice for breast cancer, including in advanced cases as long as they remain estrogen dependent (Ariazi *et al.* 2006). Approximately two-thirds of all breast carcinomas express ER $\alpha$ , and yet, 25% of all patients do not respond to tamoxifen therapy (Early Breast Cancer Trialists' Collaborative Group, 2005; see <http://www.ctsu.ox.ac.uk/projects/ebctcg>). Even when they initially do, a large fraction of patients eventually develop hormone-refractory cancer lesions, which are characterized by their rapid growth and invasiveness. What exactly promotes this transition remains a matter of intense debate and investigation. The reasons are probably multifactorial, but the discovery of GPR30 as a new ER puts yet another potential player on the map. The fact that the partial ER antagonist OHT behaves as an agonist for GPR30 (Maggiolini *et al.* 2004, Thomas *et al.* 2005, Vivacqua *et al.* 2006a,b, Henic *et al.* 2009, Pandey *et al.* 2009) suggests that conventional anti-estrogenic therapies might in fact stimulate rather than inhibit a subset of tamoxifen-resistant tumors. Whether the activation of GPR30 by estrogens or anti-estrogens indeed contributes to the switch or progression from hormone sensitive to pharmacologically hormone-insensitive breast cancer still needs to be experimentally corroborated. In this context, an interesting paradox to resolve in future studies is that the GPR30 gene has not emerged in gene expression signatures for aggressive breast cancer (Miller & Liu 2007) nor has it scored as a hit in functional screens for genes contributing to tamoxifen resistance in breast cancer cells (Meijer *et al.* 2006, van Agthoven *et al.* 2009). Given the complexity of these cancers, it is likely that GPR30 will prove to be but one contributing factor and only for a subset of cancers.

The most extensive survey to date has been performed by Filardo *et al.* (2006). They examined the presence of ER $\alpha$ , PR, and GPR30 by immunohistochemistry in 361 breast carcinomas (321 invasive and 40 intraductal tumors) and 12 controls obtained from breast reduction surgery. Normal breast tissue was positive for ER $\alpha$ , PR, and GPR30. Among the *in situ* cases, 42% were GPR30 positive, 63% ER $\alpha$  positive, and 45% PR positive. Among invasive cases, 62% were GPR30 positive, 62% ER $\alpha$  positive, and 40% PR positive. In 19% of all cases, neither ER $\alpha$  nor GPR30 were detected, whereas in 43% of all cases, there was coexpression of both receptors, indicating a significant but incomplete association between ER $\alpha$  and GPR30 expression. A similar association between GPR30 and PR was not detected. It is noteworthy that the overexpression of GPR30 was significantly associated with tumor size (> 2 cm), the presence of distant metastases, and increased HER-2/neu expression. In contrast, high levels of ER $\alpha$  were inversely correlated with HER-2/neu and tumor size. Thus, these data provide the first evidence regarding the role of GPR30 in human breast carcinoma and suggest that GPR30 overexpression may be a predictor of an aggressive disease.

We recently suggested that evaluating the levels of GPR30 in combination with those of a set of GPR30 target genes might be more informative to assess the outcome in certain types of cancer (Pandey *et al.* 2009). We showed that the gene for the connective tissue growth factor (CTGF) is the most strongly induced GPR30 target gene in ER-negative SKBr3 breast cancer cells and that CTGF is necessary for the GPR30-mediated stimulation of proliferation and migration. Whereas CTGF overexpression has been correlated with aggressive behavior of breast cancer cell lines (Kang *et al.* 2003, Han *et al.* 2008), CTGF was found to be oppositely correlated with poor outcome in a survey of 122 human breast tumors (Jiang *et al.* 2004). Yet, elevated CTGF levels have been reported to characterize a number of other types of carcinomas (Bleau *et al.* 2005, Deng *et al.* 2007, Liu *et al.* 2008, Mullis *et al.* 2008). Hence, further work on this issue and on the question whether the ER $\alpha$  status may influence the effects of CTGF and other target proteins of GPR30 in breast cancer and other malignancy is clearly warranted.

In contrast to the breast where tamoxifen is usually antiproliferative, epithelial cells of the female reproductive system proliferate upon exposure to either estrogen or tamoxifen (Gottardis *et al.* 1988). Furthermore, women treated with tamoxifen against breast cancer display an increased incidence of endometrial cancer. While this is consistent with the fact that OHT is an ER $\alpha$  agonist in this tissue (Shang & Brown 2002, Shang 2006), ER $\alpha$ -independent mechanisms might also be at play. This is supported by the above-mentioned demonstration that GPR30-mediated proliferative stimuli are effective in endometrial and other cancer cell lines. To examine the potential role of GPR30 in endometrial cancers, Smith *et al.* (2007) performed an immunohistochemical analysis of ER $\alpha$ , PR, GPR30, EGFR, and the proliferation marker

Ki-67 on tumors from patients with endometrial adenocarcinoma. GPR30 was positively correlated with EGFR but negatively with PR expression. There was a strong positive correlation between ER $\alpha$  and PR expression, whereas GPR30 overexpression occurred more frequently in endometrial carcinomas exhibiting deep myometrial invasion, high-grade, biologically aggressive histological subtypes, and advanced stage. In patients with GPR30 overexpression, the overall survival rate was significantly worse than that of patients with low GPR30 expression (65.2 and 100% respectively). Although the sample size was too small for a multivariate analysis, in patients with stage I disease, GPR30 overexpression was associated with poorer survival rates compared with patients with low GPR30 expression (80 and 100% respectively). A very recent study by the same authors indicates a similar correlation in ovarian cancer (Smith *et al.* 2009). Together, these reports support the hypothesis that GPR30 represents an estrogen-responsive receptor that is overexpressed and functionally relevant in high-risk breast, endometrial, and ovarian carcinomas.

### Physiological functions

In addition to the afore-mentioned studies on the potential functions of GPR30 in cancer and possibly other pathological conditions, the last few years have seen an explosion of studies aimed at uncovering its physiological functions. These advances have been dramatically aided by new genetic and pharmacological tools. Specifically, several mouse knockouts have been reported (Wang *et al.* 2008a, Haas *et al.* 2009, Isensee *et al.* 2009, Mårtensson *et al.* 2009, Otto *et al.* 2009), and both a synthetic agonist (Bologa *et al.* 2006) and antagonist (Dennis *et al.* 2009) have been identified. The specific agonist G-1 as well as RNA interference to knockdown GPR30 expression has already been widely used in cell- or tissue-based systems (see below).

Initially, with a sex steroid like estrogen, the reproductive tissues naturally attracted a lot of attention. As discussed above, GPR30 does play a role in a subset of carcinomas. Moreover, using the GPR30-specific agonist G-1 and antagonist G15, Prossnitz *et al.* found that GPR30 mediates part of the proliferative responses of the uterine epithelium to estrogen (Dennis *et al.* 2009). However, most of the available evidence points to a large variety of functions of GPR30 in other tissues, which should not come as a surprise in view of the complexity of estrogen functions, even those mediated by the nuclear ERs (Dahlman-Wright *et al.* 2006, Heldring *et al.* 2007). At this point, a fixation on reproductive functions (Otto *et al.* 2008, 2009, Levin 2009) can only hinder progress.

One might have expected that the deletion of the GPR30 gene in the mouse would lead to unambiguous insights. This is unfortunately not yet the case. The first mouse knockout report by Wang *et al.* provided evidence for a role of GPR30 in the sexual dimorphism of immune responses. GPR30 knockout mice displayed an impaired estrogen-induced

thymic atrophy and, as a pharmacological correlate, G-1 could induce this response (Wang *et al.* 2008a). Strangely, the Otto & Ruiz Noppinger groups could not even detect the expression of GPR30 or of an integrated GPR30-lacZ reporter in the thymus (Isensee *et al.* 2009, Otto *et al.* 2009), but did identify some changes in the T-cell compartment (Otto *et al.* 2009). The GPR30-dependence of these changes was however questioned since they were not sexually dimorphic as one would have expected for a process regulated by a sex steroid. Another study also failed to identify any GPR30-dependence of estrogenic effects on the thymus (Windahl *et al.* 2009). Whatever the role of GPR30 in the thymus may be, a recent publication adds credence to a link with immunity in demonstrating that GPR30 is required to mediate protective effects of estrogen (and G-1) against an experimental autoimmune encephalomyelitis (Wang *et al.* 2009). A similar controversy appears to pertain to another phenotype. The Olde & Leeb-Lundberg groups found that GPR30-deficient female mice had a number of metabolic issues including hyperglycemia, impaired glucose tolerance, reduced bone growth and increased blood pressure, and linked some of these to a role of GPR30 in E<sub>2</sub>-induced release of insulin by  $\beta$ -cells (Mårtensson *et al.* 2009). A follow-up analysis of the potential role of GPR30 for longitudinal bone growth showed that GPR30 is not required for the bone-sparing effects of estrogen but for basal and estrogenic responses of growth plates (Windahl *et al.* 2009). In contrast to what was reported from studying this mouse model, no changes in body growth or glucose tolerance were revealed with the GPR30-lacZ mouse, another GPR30-deficient mouse line (Isensee *et al.* 2009). Yet another independent study seems to confirm that there may at least be metabolic problems in the absence of GPR30. Barton *et al.* observed an increased body weight and visceral adiposity in GPR30-deficient mice of both sexes, and a GPR30-dependent vascular dilation in response to G-1 (Haas *et al.* 2009). While these authors logically detected GPR30 expression in fat tissue, others failed to do so and also failed to see differences in obesity and the increased body weight despite the fact that other phenotypic changes were seen in that case (Mårtensson *et al.* 2009). In appreciating this bewildering complexity, it might be important to take into account differences in genetic background and experimental protocol. It will be critical to control the estrogen environment carefully and therefore to compare sham-operated and ovariectomized animals of the relevant genotypes with and without E<sub>2</sub> complementation.

While mouse knockout studies were producing a wealth of (confusing) data, many interesting insights have come from cell or organ culture experiments. RNA interference experiments have been used to demonstrate that GPR30 is involved in mediating estrogen stimulation of primordial follicle formation in the hamster (Wang *et al.* 2008b). Although GPR30 can stimulate the proliferation and migration of a subset of carcinomas (see above), the proliferation of osteoblast progenitors (Teplyuk *et al.* 2008) and a spermatogonial cell line (Sirianni *et al.* 2008), it is not

always stimulatory. For example, the proliferation of human primary bladder cells is inhibited by GPR30 signaling (Teng *et al.* 2008) as is the migration of ovarian cancer cells (Henic *et al.* 2009). In a model for trauma-hemorrhage of the liver, GPR30 was shown to mediate protective effects of E<sub>2</sub> (Hsieh *et al.* 2007). In fish, GPR30 has been found to mediate the E<sub>2</sub>-induced meiotic arrest of oocytes (Pang *et al.* 2008, Pang & Thomas 2009).

A wide range of effects of estrogens in neuronal cells appear to involve GPR30. These include roles in inhibiting dopamine efflux in PC-12 cells (Alyea *et al.* 2008) and serotonin signaling in the hypothalamus (Xu *et al.* 2009), in promoting the depolarization of spinal neurons (Dun *et al.* 2009), Ca<sup>2+</sup> oscillations and release of LHRH from primate neurons (Noel *et al.* 2009), and mechanical hyperalgesia in nociceptive neurons of rat dorsal root ganglia (Kuhn *et al.* 2008). Furthermore, there is evidence for additional functions in the hypothalamus as the control of energy homeostasis may involve GPR30 (Qiu *et al.* 2006), in accordance with the finding that G-1 causes Ca<sup>2+</sup> spikes in cultured rat hypothalamic neurons (Brailoiu *et al.* 2007), and that GPR30 is expressed there and elsewhere in the brain (see, for example Brailoiu *et al.* 2007, Canonaco *et al.* 2008, Hazell *et al.* 2009). This further agrees with a function of GPR30 in the hypothalamic-pituitary axis in regulating the E<sub>2</sub>-induced prolactin secretion (Lebesgue *et al.* 2009). An exciting new neurological role of GPR30 is indicated by a first series of experiments with the GPR30 antagonist G15. G15 blocked the antidepressive effects of both E<sub>2</sub> and G-1 in a mouse model (Dennis *et al.* 2009).

## Controversies

Perhaps the most fundamental controversy concerns the question whether GPR30 is an ER at all. This continues to be seriously disputed (Otto *et al.* 2008, 2009, Levin 2009). While dissent is part of and a driver of the scientific process, one should not forget that positive evidence remains a stronger argument than negative evidence. When some fail to see evidence for GPR30 signaling in ER-negative breast cancer cells (Pedram *et al.* 2006), to mention just that biological system, the positive evidence reported by others for breast cancer cells that are devoid of both ER $\alpha$  and ER $\beta$  (for example, Filardo *et al.* 2000, Maggiolini *et al.* 2004, Pandey *et al.* 2009) cannot be ignored. Several groups have shown that cells and/or isolated membranes with GPR30 bind E<sub>2</sub>, whereas cells/membranes without GPR30 do not (Revankar *et al.* 2005, Thomas *et al.* 2005, Thomas & Dong 2006, Filardo *et al.* 2007, Pang *et al.* 2008), unless of course they contain membrane-associated nuclear ERs. Similar data have been published for the agonist G-1 (Bologa *et al.* 2006) and the antagonist G15 (Dennis *et al.* 2009). Considering all of these and the other data discussed in this review, it seems difficult to argue that GPR30 is not an ER or that it is a mere 'collaborator' (Levin 2009). The ultimate proof might have to

come from a structural analysis of E<sub>2</sub>-bound GPR30, but the first structures of any liganded 7TM-GPCRs have only very recently been solved (Rosenbaum *et al.* 2009).

Intuitively, a 7TM-GPCR like GPR30 might be expected to be localized at the plasma membrane. It turns out that for GPR30 not even the subcellular localization is clear. Prossnitz *et al.* provided seemingly compelling evidence for a localization of both tagged exogenous and endogenous GPR30 at the endoplasmic reticulum (Revankar *et al.* 2005). This is supported by a very recent report from another group demonstrating the colocalization of GPR30 with calnexin (Lin *et al.* 2009). According to others, GPR30, at least in oxytocin neurons, is mainly localized in the Golgi (Sakamoto *et al.* 2007). Other authors found it on the plasma membrane of primary neurons but intracellularly in some transfection experiments (Funakoshi *et al.* 2006). Filardo & Thomas and their colleagues carefully revisited this issue with a variety of staining and biochemical approaches. They also showed activation of GPR30 signaling with E<sub>2</sub> coupled to carrier proteins such as BSA that should have prevented access to intracellular GPR30 (Filardo *et al.* 2007; see also Thomas *et al.* 2005, Pang *et al.* 2008). Likewise, the afore-mentioned LHRH release can also be stimulated with a cell-impermeable estrogen dendrimer conjugate (Noel *et al.* 2009). Whether the demonstration that FITC-labeled E<sub>2</sub>-BSA rapidly gains access to the membrane of the endoplasmic reticulum in a GPR30-dependent manner suggesting shuttling of GPR30 (Wang *et al.* 2008b) tilts the balance one way or another is highly unlikely. It cannot be excluded that the fluorochrome FITC altered the membrane tropism of E<sub>2</sub>-BSA. At the very least, all of these groups agree that GPR30 is a membrane-bound ER. It seems that novel tools or approaches might need to be developed to sort out where the bulk of GPR30 resides and where a possibly smaller subset of functional molecules act. It is of course not impossible that the answer may depend on cell type, and even for a given cell type, on additional intra- and extracellular factors.

The potential role of GPR30 in the reproductive system has been construed as a problem or controversy. As discussed above, while GPR30 may play some role in primary follicle formation, oocyte maturation, the proliferation of the uterine epithelium, and pathologically in some carcinomas of the breast, endometrium, and ovary, its main functions might lie outside of the reproductive system. Some of these are likely to be linked to sex-specific functions of estrogen, whereas others will not. Some will be complementary to those of ER $\alpha$  and ER $\beta$ , others will not.

Another 'issue' is the pharmacology of GPR30. It is fair to say that it remains extremely poorly understood. E<sub>2</sub> and the specific ligand G-1 are undoubtedly agonists. So are some environmental and plant-derived estrogens (Maggiolini *et al.* 2004, Thomas & Dong 2006, Vivacqua *et al.* 2006a), the partial ER antagonist OHT (see above), and the anti-estrogen ICI 182 780 in some settings (Filardo *et al.* 2000, Thomas *et al.* 2005, Henic *et al.* 2009, Pang & Thomas 2009, Quinn *et al.* 2009) but not all (Pandey *et al.* 2009). A related issue is that of

the concentrations required to obtain a full response. While we have typically used  $\mu$ M concentrations with carcinoma cells and argued for OHT that this is in a therapeutically meaningful range (see Pandey *et al.* 2009), those working with E<sub>2</sub> and G-1 in other biological systems have observed binding affinities and half-maximal responses in the nanomolar or subnanomolar range (Revankar *et al.* 2005, Thomas *et al.* 2005, Thomas & Dong 2006, Filardo *et al.* 2007, Pang *et al.* 2008). To what extent cell-type differences exist and whether the type of assay, for example the induction of a Ca<sup>2+</sup>-influx within seconds to minutes or of gene expression changes within hours, influences the dose-response for different ligands remains to be more carefully explored.

## Future

With the discovery of GPR30 as a new ER, the estrogen field has opened a Pandora's box whose full impact has yet to be appreciated. We are really only at the beginning of deciphering the contributions of GPR30 to physiological and pathological estrogen responses. As argued above, its main function may not be in the reproductive system but in some of those other instances where the sex steroid estrogen conditions cells and tissues. The biological functions of GPR30 will eventually emerge most clearly from studies that use a combination of genetic (for example, mouse knockouts in controlled genetic backgrounds, RNA interference, gene transfer), pharmacological (synthetic agonists and antagonists) and biochemical (highly specific and validated antibodies and other types of probes) tools. A connection of GPR30 to diseases such as cancer has already tentatively emerged, but larger sample sizes with more measured parameters and patient follow-up are needed to clarify it.

Considering the available biological and pharmacological data, E<sub>2</sub> is a very likely physiological ligand, but the establishment of a more comprehensive pharmacological spectrum may reveal others. It is even conceivable that E<sub>2</sub> is not the main physiological ligand. Indeed, in view of the conceptual and practical difficulties of determining what the physiological ligand for any GPCR is, one might be well advised to keep an open mind about it. Moreover, it appears that GPR30 is a target for estrogenic endocrine disruptors. The consequences of this will also need to be evaluated as the biological functions of GPR30 are filled in.

Finally, much of the cell and molecular biology of GPR30 remains uncharted territory. How does it go to the membrane (and which membrane after all)? What defines GPR30 as an ER at the molecular level? What are its structural and functional relationships to its next kins in the GPCR family? To this date, no structure-function studies have been done at all. Although human and mouse GPR30 are 87% identical, the remaining 13% could potentially generate considerable species differences in GPR30 function and pharmacology. This should be taken into account in interpreting results from animal models.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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