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FUNCTIONAL COOPERATION BETWEEN GPER AND AHR TOWARD BREAST CANCER PROGRESSION

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INDEX

Abstract

The cytochrome P450 1B1 (CYP1B1) is a heme-thiolate monooxygenase involved in both estrogen and environmental contaminants metabolism. For instance, CYP1B1 catalyzes the hydroxylation of 17-β estradiol (E2) leading to the production of 4-hydroxyestradiol that may act as a potent carcinogenic agent. In addition, CYP1B1 is overexpressed in different tumors including breast cancer. In this scenario, it is worth mentioning that CYP1B1 expression is triggered by estrogens through the estrogen receptor (ER)α in breast cancer cells. In the present study, we evaluated whether the G protein estrogen receptor namely GPER may provide an alternate route toward the expression and function of CYP1B1 in ER-negative breast cancer cells, in cancer-associated fibroblasts (CAFs) obtained from breast cancer patients, in CAFs derived from a cutaneous metastasis of an invasive mammary ductal carcinoma and in breast tumor xenografts. Our results show that GPER along with the EGFR/ERK/c-Fos transduction pathway can lead to CYP1B1 regulation through the involvement of a half-ERE sequence located within the CYP1B1 promoter region. As a biological counterpart, we found that both GPER and CYP1B1 mediate growth effects *in vitro* and *in vivo*. Altogether, these data suggest that estrogens in ER-negative cell contexts may engage the alternate GPER signaling toward CYP1B1 regulation. CYP1B1 is a well-known target gene of the aryl hydrocarbon receptor (AHR) that may be activated by the carcinogenic pollutant 3-methylcholanthrene (3MC). Hence, we aimed to provide novel insights into the molecular mechanisms by which 3MC and E2 may activate a cross talk between AHR and GPER transduction pathways leading to the stimulation of breast cancer cells and CAFs. In particular, our results demonstrate that 3MC and E2 trigger the EGFR/ERK/c-Fos signalling through both AHR and GPER toward the up-regulation of CYP1B1 and cyclin D1 as well as the stimulation of growth responses. Altogether, the present findings suggest that a functional interaction between AHR and GPER may occur toward breast cancer progression.

Chapter 1
Introduction

1.1 Introduction

Breast cancer is the most frequent malignancy and the second leading cause of cancer death among females [1]. The elevated incidence of breast cancer in women has been associated with prolonged exposure to high levels of estrogens [2] and environmental contaminants [3]. Estrogens act mainly through the classical estrogen receptor (ER)α and ERβ [4], however, the identification of GPER as a further estrogen receptor has suggested new possibilities by which estrogenic compounds might cause biological effects in different normal and cancer cell types [5-6]. There is increasing alertness that estrogens may regulate certain cell functions through a network of signaling pathways. Beyond estrogens, certain metabolites of either 17β-estradiol (E2) or other compounds as dioxin, benzo(a)pyrene (BaP) and polycyclic aromatic hydrocarbons (PAHs) may influence the development of breast malignancy. Therefore, a great attention has been addressed to the mechanisms involved in the metabolism and the biological functions of both estrogens and xenobiotics [7-8]. For instance, it has been reported that diverse cytochrome P450 enzymes (CYP) contribute to key processes leading to the metabolism of pro-carcinogen compounds [9]. In particular, CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), which is a heme-thiolate monooxygenase mainly expressed in endocrine-regulated tissues like breast, uterus and ovary, has been indicated as a primary enzyme involved in estrogen and xenobiotics metabolism [10]. In addition, CYP1B1 has been suggested to play an essential role in the development of various hormone-dependent tumors, including breast cancer, through the biotransformation of endogenous estrogens and environmental carcinogens [7, 11-14]. In this context, CYP1B1 is involved in the metabolism of E2 into 4-hydroxyestradiol (4OHE2) that forms DNA adducts and generates free radicals leading to DNA damage and tumorigenesis in different tissues like breast [2, 15-16]. Several compounds as dioxin, (BaP) and PAHs stimulate the transcription of CYP1B1 [8, 17] as wells as its metabolic activity [2]. It is worth noting that estrogens generate a feed-forward

loop triggering the transcription of CYP1B1, which in turn is primarily involved in the metabolic conversion of these steroids [17-19]. For instance, the transcription of CYP1B1 was induced in breast and endometrial cancer cells by E2 through the activation of ERα and its binding to an estrogen responsive element (ERE) located within the CYP1B1 promoter sequence [18]. These findings may underline the physiological relevance of CYP1B1 regulation by estrogens in the landscape of the estrogen homeostasis and action, in particular in hormone-sensitive tissues [2, 18-19]. The transcription of CYP1B1 is mainly regulated by the aryl hydrocarbon receptor (AHR), which acts as ligand-activated transcription factor and it is known for mediating the toxicity and tumor-promoting properties of diverse environmental contaminants [20-23]. High levels of AHR and its constitutive nuclear localization have been found in aggressive tumors and tumor cell lines, suggesting that the AHR is chronically activated in tumors and facilitates their progression [22]. The biological responses induced by environmental pollutants such as 3-methylcholanthrene (3MC) involve AHR and its functional interactions with diverse signal molecules, contributing to the development and progression of diverse types of tumours [24-26].

1.2 Breast tumors

Breast cancer is the most common malignancy and the second leading cause of cancer-related death in women worldwide. Whereas localized disease is largely curable, metastatic or recurrent disease carries an unfavourable prognosis [1]. As a greater percentage of breast cancers are being diagnosed at an earlier stage, the medical community has been challenged to develop diagnostic and treatment modalities that maximize benefit from therapy while reducing the morbidity associated with treatment [27]. The management of breast cancer has changed considerably in the last two decades with improvements in systemic therapy and advances in surgical techniques [28]. There are two main types of breast cancer:

1. Ductal carcinoma starts in the ducts that move milk from the breast to the nipple.

2. Lobular carcinoma starts in the lobules of the breast that produce milk. In rare cases, breast cancer can start in other areas of the breast.

Breast cancer may be invasive or non-invasive. Non-invasive breast cancer is also called "in situ."

3. Ductal carcinoma in situ (DCIS), or intraductal carcinoma, is breast cancer in the lining of the milk ducts that has not yet invaded nearby tissues. It may progress to invasive cancer if untreated.

4. Lobular carcinoma in situ (LCIS) is a marker for an increased risk of invasive cancer in the same or both breasts (Figure 1.2.1).

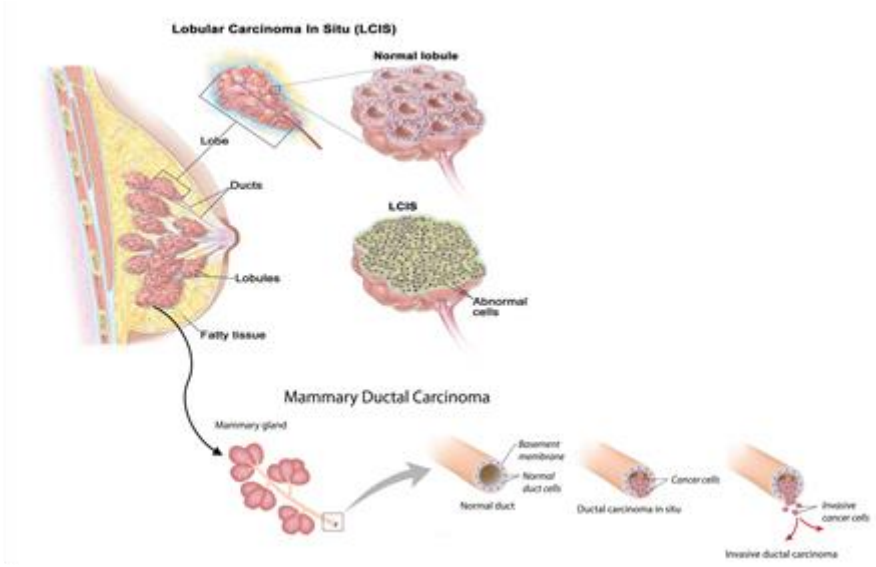


Fig. 1.2.1 Representation of the anatomy of the Lobular Carcinoma and Mammary Ductal Carcinoma

Many risk factors lead to breast malignancy development:

- Age and gender. The risk of developing breast cancer increases with age. Most advanced breast cancer cases are found in women over age 50 [29]. Women are 100 times more likely to get breast cancer than men are.
- Family history of breast cancer. You may also have a higher risk for breast cancer if you have a close relative who has had breast, uterine, ovarian, or colon cancer. About 20-30% of women with breast cancer have a family history of the disease.
- Genes. Some people have genes that make them more likely to develop breast cancer. The most common gene defects are found in the BRCA1 and BRCA2 genes. These genes normally produce proteins that protect you from cancer. If a parent passes you a defective gene, you have an increased

risk for breast cancer. Women with one of these defects have up to an 80% chance of getting breast cancer sometime during their life [30].

- Menstrual cycle. Women who got their periods early (before age 12) or went through menopause late (after age 55) have an increased risk for breast cancer [31].

Other risk factors include:

- Alcohol use. Drinking more than 1-2 glasses of alcohol a day may increase your risk for breast cancer [32].
- Childbirth. Women who have never had children or who had them only after age 30 have an increased risk for breast cancer. Being pregnant more than once or becoming pregnant at an early age reduces your risk of breast cancer [33].
- Hormone replacement therapy (HRT). You have a higher risk for breast cancer if you have received hormone replacement therapy with estrogen for several years or more [34].
- Obesity. Obesity has been linked to breast cancer, although this link is controversial. The theory is that obese women produce more estrogen, which can fuel the development of breast cancer [35].
- Radiation. The radiation therapy to treat cancer of the chest area, increase higher risk to develop breast cancer [36].

Treatment is based on many factors, including: type and stage of the cancer, whether them cancer is sensitive to certain hormones, whether the cancer over-expresses a gene called HER2/neu. In general, cancer treatments may include chemotherapy medicines to kill cancer cells, radiation therapy to destroy cancerous tissue, surgery to remove cancerous tissue, lumpectomy removes the breast lump; mastectomy removes all or part of the breast; hormonal therapy. Most women receive a combination of treatments. For women with stage I, II, or III breast cancer, the main aim is to treat the cancer and prevent it from returning. For women with stage IV cancer, the objective is to improve symptoms and help them live longer. In most cases, stage IV breast cancer cannot be cured.

- Stage 0 and DCIS. Lumpectomy plus radiation or mastectomy is the standard treatment. There is some controversy on how best to treat DCIS.
- Stage I and II. Lumpectomy plus radiation or mastectomy with some sort of lymphnode removal is the standard treatment. Hormone therapy, chemotherapy, and biologic therapy may also be recommended following surgery.
- Stage III. Treatment involves surgery, possibly followed by chemotherapy, hormone therapy, and biologic therapy.
- Stage IV. Treatment may involve surgery, radiation, chemotherapy, hormonal therapy or a combination of these treatments.

After treatment, some women will continue to take medications such as tamoxifen for a period. All women will continue to have blood tests, mammograms, and other tests after treatment. Women who had a mastectomy may have reconstructive breast surgery, either at the same time as the mastectomy or later.

1.3 Estrogens and the classical estrogen receptor (ER)

Estrogens are sex steroid hormones, which exhibit a broad spectrum of physiological functions ranging from regulation of the menstrual cycle and reproduction to modulation of bone density, brain function, and cholesterol mobilization [37]. Despite the normal and beneficial, physiological actions of endogenous estrogen in women, abnormally high estrogen levels are associated with the increased incidence of certain types of cancer, in particular breast and endometrial cancer. The predominant intracellular estrogen is 17β-estradiol (E2). Other types of estrogen include estrone (E1) and estriol (E3) (Figure 1.3.1). In premenopausal women, primarily the ovaries secrete E1 and E2 during the menstrual cycle, with minor levels derived from adipose tissue and the adrenal glands. The placenta also produces E3 during pregnancy [38].

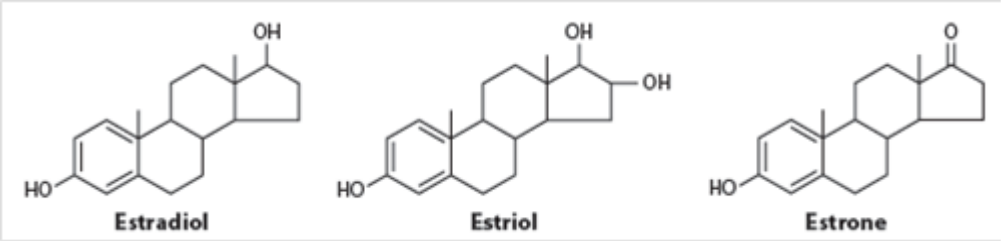


Fig. 1.3.1 Chemical structures of estrogens

In the ovary, granulosa cells synthesize estrogen from androgen [39]. Ovarian production of estrogen is regulated by the hypothalamic-pituitary-ovarian (HPO) axis and begins by anterior pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in response to the hypothalamic peptide gonadotropin-releasing hormone (GnRH). Acting in concert, LH stimulates androgen production, whereas FSH up-regulates aromatase, which catalyzes the rate-limiting and final step of estrogen biosynthesis: the aromatization of androgen to estrogen (Figure 1.3.2). During ovulation, E2 production rises dramatically by eight- to ten-fold. High levels of estrogen in turn act via negative feedback to dampen estrogen production to inhibit the release of GnRH, LH, and FSH [40]. The primary mediator of estrogen biosynthesis in postmenopausal women is aromatase, which is found in adipose tissue as well as in the ovaries, placenta, bone, skin, and brain [41]. After menopause, ovarian estrogen biosynthesis is minimal, and circulating estrogen is derived principally from peripheral aromatization of adrenal androgen. As such, for obese postmenopausal women, adipose tissue becomes the main source of estrogen biosynthesis; this biosynthetic route is far less significant for non-obese postmenopausal women [42].

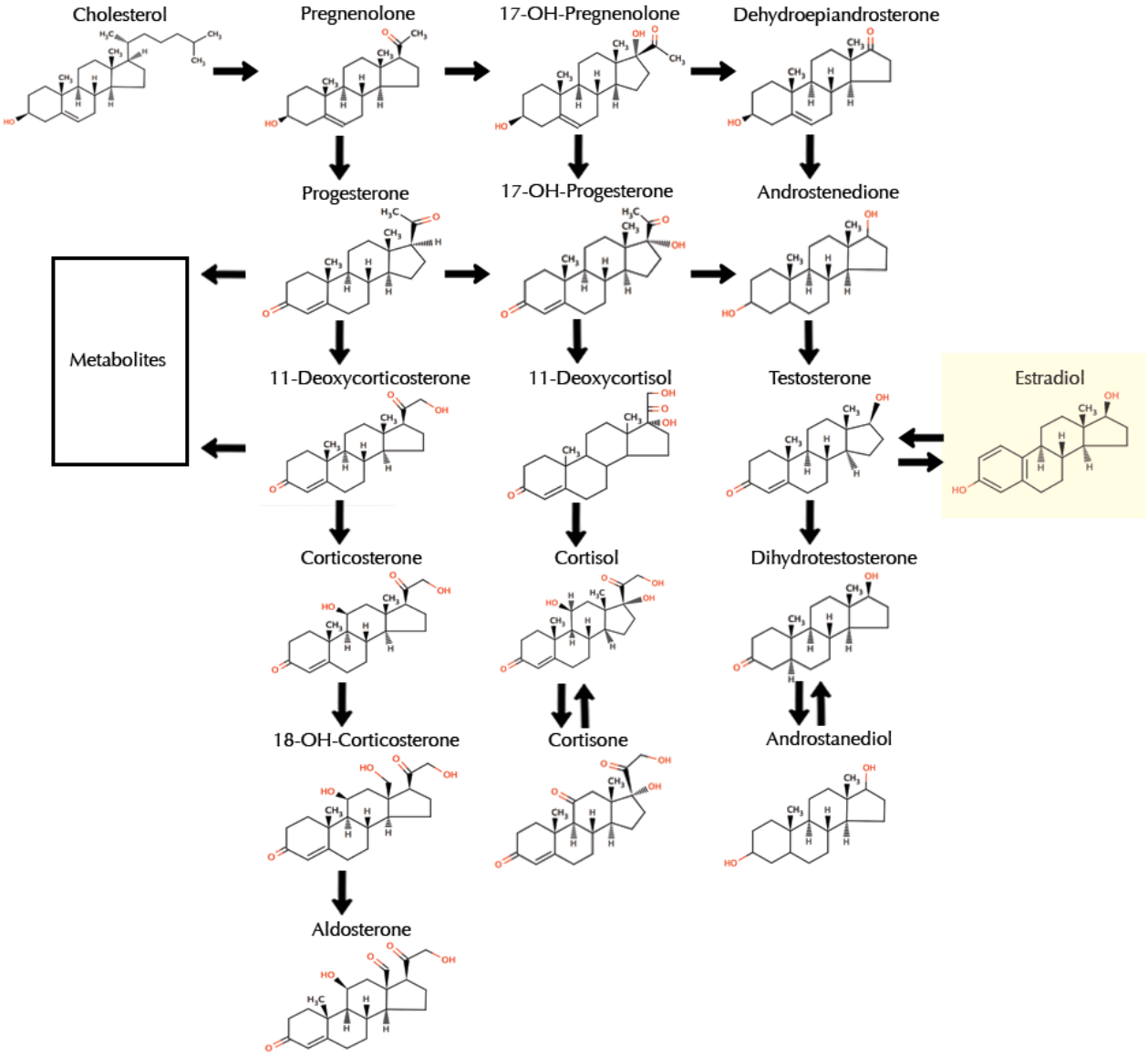


Fig. 1.3.2 Biosynthesis of Estrogens

Numerous studies have demonstrated the association of estrogen with the development and/or progression of various types of cancer, including breast, endometrium, ovary, prostate, lung, and colon cancer [43-44]. Estrogen mediates its biological effects in target tissues primarily by binding to specific intracellular receptors named ERα and ERβ (Fig.1.3.3). These receptors are encoded respectively by ESR1 and ESR2 which are located on different chromosomes. Like all other members of the nuclear receptors super-family, human ERα and ERβ, are ligand-activated receptors with high degree of sequence homology and similar three-dimensional structure. The ERs are modular proteins composed of four functional domains (Fig.1.3.3):

- The N-terminal transactivation domain, which is involved in protein protein interactions and in transcriptional activation of target-gene expression.

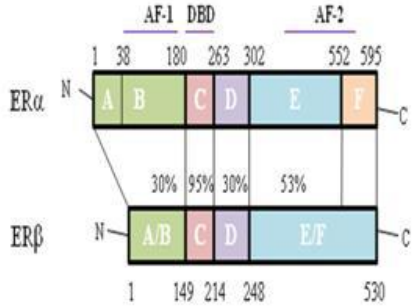


Fig.1.3.3. Schematic diagram showing the domain organization of human ERα and ERβ

- The DNA binding domain (DBD), which plays the most important role in receptor dimerization and in the binding of specific DNA sequences (i.e.EREs).
- The hinge region which is the most variable region within ERs.
- The C-terminal E/F region encompassing the LBD, the AF-2 domain, the homo- and/or hetero-dimerization domain, and part of the nuclear localization region.

It has been demonstrated that ERα acts by multiple mechanisms. In classical genomic mechanism, ligand-activated ERs dimerize and translocate in the nucleus where they recognize specific estrogen response elements (ERE) located in the promoter region of DNA of the target genes.

Besides, E2 can also modulate gene expression by a second indirect mechanism involving the interaction of ER with other transcription factors such as the activator protein (AP)-1, nuclear factor-kB (NF-kB), specificity protein 1 (SP1) which, in turn, binds their specific DNA elements. In addition to the classical mechanism of estrogen signal transduction, which implies the binding of the receptor to DNA, there are a number of non-genomic signaling through which estrogen may exert their biological effects. Indeed, it is now well accepted that ER function can be modulated by extra-cellular signals even in the absence of E2. These findings focus primarily on the ability of polypeptide growth factors such as epidermal growth factors (EGF) and insulin like growth factor-I/II (IGF-I/II) to activate ER and increase the expression of E2 target genes. Moreover, E2 exerts its non-genomic actions, which are too rapid to be accounted for by the activation of RNA and protein synthesis, through the activation of four main signaling cascade: phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/AKT, and cAMP/ protein kinase A (PKA). A rapid activation of the cAMP/PKA

pathway has been demonstrated in many different cell types. Phospholipase C (PLC) dependent IP3 production, calcium influx, and PKC activation have also been reported in many different cultured cell types. Moreover, E2 rapidly stimulates the activation of MAPK pathways in MCF-7 cell-line, endothelial, bone and HepG2 cells. E2 can also down regulates MAPK phosphatase-1 activity, leading to the up regulation of extracellular regulated kinase (ERK) activity in breast cancer cells (Figure 1.3.4).

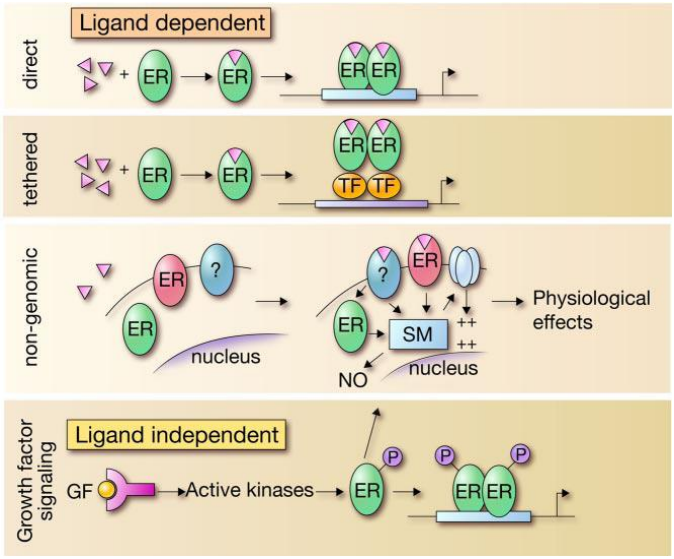


Fig.1.3.4. Representation that summarizes the possible mechanisms of estrogen signal transduction

The E_2-induced rapid signals indicate its localization at the plasma membrane. Some authors have suggested that the non genomic actions of estrogen are mediated through a subpopulation of ERα and ERβ located to the plasma membrane. However, in the last few years, a member of the 7-transmembrane G protein-coupled receptor family, GPR30/GPER, has been implicated in mediating both rapid and transcriptional events in response to estrogen under certain circumstances.

1.4 The G protein-coupled estrogen receptor (GPER)

Estrogens regulate different physiological function such as development, reproduction and homeostasis; however, they are involved in the progression of different tumours. The biological actions of estrogens are traditionally mediated by the activation of classical estrogenic receptors ERα and ERβ, which act as transcriptional factors binding specific DNA region, named estrogen responsive elements (EREs) located within the promoter sequence of target genes. Estrogenic receptors are considered as nuclear receptors;

therefore, the estrogenic non-genomic actions are mediated by receptors located on the plasma membrane. In recent years, the identification of GPER as an estrogen receptor has opened a new scenario regarding a further mechanism trough which estrogenic compounds can trigger relevant biological actions in diverse cell contexts. GPER was first identified as an orphan member of the 7-transmembrane receptor family in the late 1990s [47-48]. GPER belongs to the rhodopsin-like receptor superfamily [47] and its gene is mapped to chromosome 7p22.3 [49]. Several studies have reported GPER expression on the plasma membrane, in the endoplasmic reticulum and in the Golgi apparatus as well as in the nucleus of CAFs extracted from mammary biopsies [50-52]. As it concerns signalling pathways, it has been demonstrated that GPER ligands may bind the receptor and activate heterotrimeric G proteins, which then activate Src and adenylyl cyclase (AC) resulting in intracellular cAMP production. Src is involved in matrix metalloproteinases (MMP) activation, which cleave pro-heparan-bound epidermal growth factor (pro-HB-EGF) and release free HB-EGF. The latter activates EGF receptor (EGFR), leading to multiple downstream events; for example, activation of phospholipase C (PLC), PI3K, and MAPK [53]. Activated PLC produces inositol triphosphate (IP3), which further binds to IP3 receptor and leads to intracellular calcium mobilization [54]. The downstream signal of PI3K is AKT transduction pathway. Main biological consequence of AKT activation is closely related to cancer cell growth, catalogued loosely into three aspects: survival, proliferation and growth [55]. The MAPK and PI3K cascade results in activation of numerous cytosolic pathways and nuclear proteins, which further regulate transcription factors such as SRF, CREB, and members of the E26 transformation specific (ETS) family by direct phosphorylation [4,56]. This promotes the expression of a second wave of transcription factors such as Fos, JUN, EGR1, ATF3, C/EBPδ, and NR4A2. Cells are then reprogrammed under the effect of this network of transcription factors and a series of GPER target genes, like CTGF, cyclins, EGR-1, HIF-1, VEGF, are up-regulated [4] (Figure 1.4.1). Superimposed on these responses, there may be a variety of signalling crosstalk pathways and both negative and positive feedback loops. For example, it has been demonstrated that EGF up-regulates GPER 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 GPER promoter [49]. Considering that GPER signalling uses the EGFR/MAPK pathway, a positive feedback loop is conceivable. This mechanism is also operational for EGF and the related growth factor

TGFα in ERα-positive breast cancer cells [57]. GPER gene expression has been detected in at least four kinds of human tumour specimens or cell lines, including breast cancer [4, 58-61], endometrial cancer [62-65], ovarian cancer [49, 66], thyroid cancer [67], and a rat pheochromocytoma cell line PC-12 [68]. In addition, there is a growing body of evidence supporting that GPER is strongly associated with cancer proliferation [49, 62, 65, 67,69-72], migration [4, 73], invasion [62], metastasis [60-61], differentiation [62], and drug resistance [74-75]. Indeed, as estrogen stimulates the progression of breast cancer in approximately two-thirds of patients who are ER + [76-77], some selective estrogen receptor modulators (SERMs), such as tamoxifen, have been clinically used to antagonize the binding of estrogen to its classic ERs, which is an effective therapeutic strategy in attenuating the growth of ER+ breast cancers. However, there are around 25% of ER + breast cancer patients that do not respond to anti-estrogen therapy (Early Breast Cancer Trialists Collaborative Group 2005). It implies that blockade of classic ERs alone may not be enough to abolish estrogen-induced breast cancer cell growth, since estrogen may promote cell growth through other receptor besides classic ERs. Such hypothesis is further supported by the discovery of GPER as the third specific ER with different structure and function to ERα and ERβ. GPER has a high binding affinity to not only estrogen, but also some SERMs, such as tamoxifen and ICI 182,780. Estrogen and SERMs stimulate GPER action without any antagonist effects [59]. These important findings provide a new possible mechanism for the progression of estrogen-related cancers, and raise a new potential target for anti-estrogen therapy. As it concerns clinical findings, GPER overexpression was associated with lower survival rates in endometrial and ovarian cancer patients [64] as well as with a higher risk of developing metastatic disease in breast cancer patients [60]. Moreover, in a previous extensive survey, GPER was found to be highly expressed and significantly associated with tumour size (>2 cm), with the presence of distant metastases and increased human EGFR-2 (HER-2)/neu expression [60]. Likewise, in a recent study performed in the aggressive inflammatory breast cancer, the majority of tumours were GPER positive [78], suggesting that GPER expression may be considered a predictor of an aggressive disease. In addition to the aforementioned studies on the potential functions of GPER in cancer and possibly other pathological conditions, this receptor was implicated in a broad range of physiological functions regarding the reproduction, the metabolism, the bone, the cardiovascular, the nervous and immune systems [79]. Estrogen binds to GPER with a high affinity of a reported Kd 2.7 nM [59] or

6 nM [6], through which alternative estrogen signalling pathways are activated. Moreover, two different synthetic compounds, G-1 [80] and G15 [81], which were identified using virtual and bio-molecular screening, are respectively a specific agonist and antagonist of GPER. In addition, different studies show that ICI 182,780 [58-59], tamoxifen [58], and 4-hydroxytamoxifen (OHT) [4, 65-67] are also able to bind GPER and mimic estrogen effects. It has been reported that a variety of xenoestrogens, including bisphenol A, can bind and activate GPER leading to important biological responses [82].

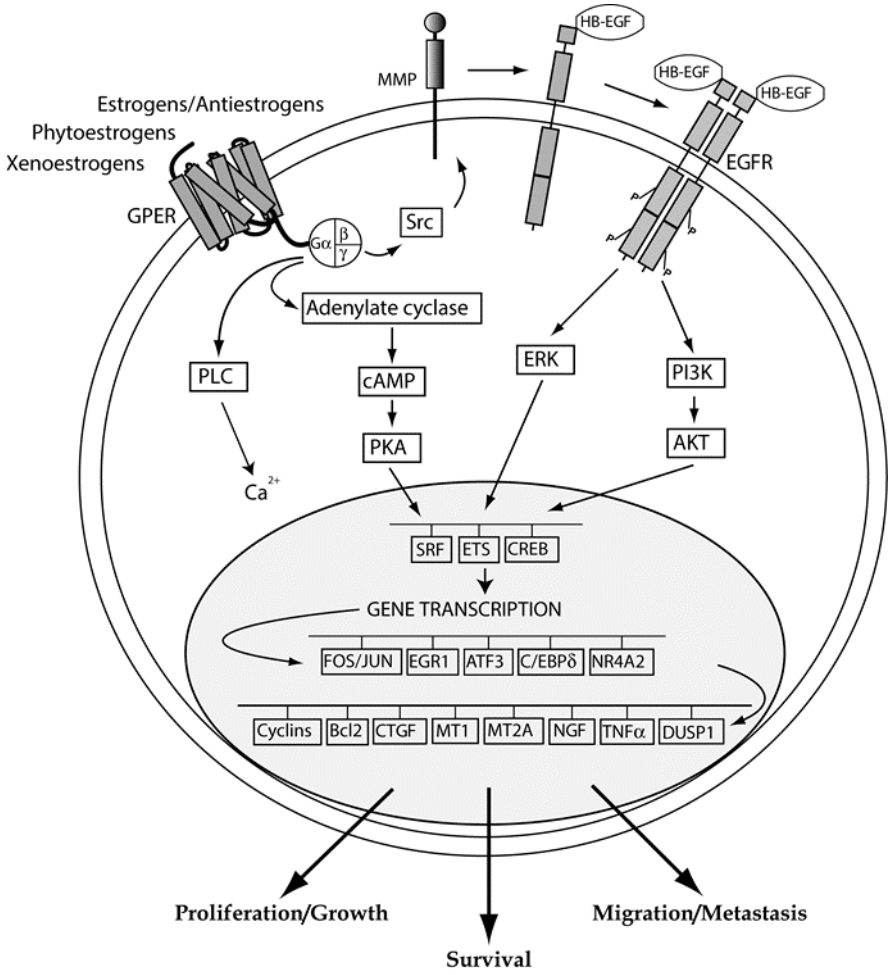


Fig. 1.4.1 GPER-mediated transduction pathways

GPER was also demonstrated to mediate the stimulatory action of estrogens in cancer-associated fibroblasts (CAFs), indicating its potential to contribute to cancer progression also through these important players of the tumour microenvironment [51, 83].

1.5 Tumor Microenvironment

The breast cancer microenvironment is a complex combination of several different cell types and molecules and is a key contributor to malignant progression [84]. The role of tumor microenvironment is becoming more and more important in breast cancer. Several stromal cell types are implicated in promoting the 'hallmarks' of cancer cells [85]. Tumor microenvironment includes fibroblasts, macrophages, immune cells, adipocytes, endothelial cells, and antigenic vascular cells (Figure 1.5.1). Stromal cells surround and interact with tumor cells. Over the last years, a robust body of evidence has highlighted the importance of the crosstalk between tumor and stoma. Tumor microenvironment has been shown to play a crucial role in tumorigenesis, from initiation to progression. Stromal cells promote cancer growth and invasion through the chemokine–chemokine receptor axis [86-87]. Infiltrating immune cells energize the immune effectors and vascular cells permit nutrients and oxygen uptake by tumors. In a normal mammary duct, there are luminal epithelial cells internally and epithelial cells externally delimited by a basement membrane, which maintains the luminal cell polarity [88]. The extracellular matrix (ECM) allows communication with the surrounding stroma. Genetic and epigenetic alterations lead to luminal cell proliferation, loss of epithelial polarity and decrease of myoepithelial cells, and changes in the ECM/basal membrane, finally resulting in mammary tumor development [89].

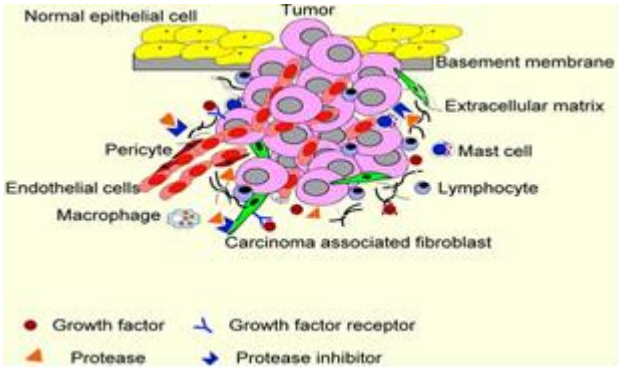


Fig. 1.5.1 Tumour Microenvironment

Among the components of the tumour microenvironment, the cancer-associated fibroblasts (CAFs) represent a particularly important cell type, which stimulate cancer progression towards an aggressive phenotype [86-87]. Numerous studies on the origin of CAFs (Figure

1.5.2) have reported that the precursors could be resident tissue fibroblasts, bone marrow-derived cells (BMDCS), hematopoietic stem cells, epithelial cells (epithelial-mesenchymal transition, EMT), endothelial cells, (endothelial-mesenchymal transition; EndMT) and cells derived from adipose tissue [90-91]. During the last few years, many studies have highlighted the importance of the cross talk between the tumour and the stroma. As opposed to normal fibroblasts, CAFs [92] improve tumor growth and metastasis by producing growth factors and ECM proteins, as well as by modulating immune polarization [93]. In addition, the number of CAFs is increased during tumor progression [94]. Accordingly, growth factors, cytokines, chemokines, and matrix metalloproteinases secreted by stromal cells lead to the recruitment of macrophages, endothelial precursor cells, and regulatory lymphocytes, which sustain tumor progression [95]. It is worth noting that stroma has been correlated with clinical outcomes and response to therapy in breast cancer [96]. The expression of ECM genes, uniformly expressed in both neoplastic and adjacent stromal cells, may divide breast cancers into different subgroups with different clinical outcomes [97-98]. A study performing hierarchical clustering of the gene-expression profile of ECM-related genes classified breast cancer samples into four groups associated with different clinical outcomes [99]. Stromal signatures are highly informative for patients with breast cancer. A serum-activated gene-expression signature from activated fibroblasts was identified as a negative prognostic factor in patients with breast cancer [100]. In addition, a 26-gene signature called the stroma-derived prognostic predictor was generated by tumor-associated stroma and matched normal stroma from breast cancer samples [101]. This signature was found to be an independent prognostic factor [102]. So tumor microenvironment influences patient outcomes and stromal gene expression signatures represent a strong prognostic value recapitulating the immune, angiogenic, and hypoxic responses [100]. The stromal cells can be divided into three general classes:

- Infiltrating immune cells
- Angiogenic vascular cells
- Cancer-associated fibroblastic cells

In the process of tumor formation, the normal microenvironment ‘niche’ changes to an altered (ie, reactive or desmoplastic) stroma which is composed of non-malignant supporting cells (ie, blood vessels, infiltrating inflammatory cells and blast-like cells) [101-102]. This altered microenvironment acts as a collaborative partner in the process of

tumourigenesis by influencing the homeostasis of cancer cells via paracrine regulators (e.g., growth factors, cytokines and chemokines) and exosomes containing nucleic acids [101, 103-105]. CAFs are α-smooth muscle actin positive, spindle-shaped, blast-like cells. Differentiation of CAFs from other cell types, such as local fibroblasts, hepatic stellate cells, mesenchymal stem cells, endothelial and epithelial cells, is mainly mediated by transforming growth factor-β1 (TGF-β1), but other factors, such as growth hormones (ie, epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF)), chemokines, epigenetic regulators and oxidative stress also may play a role in CAFs differentiation [104, 106-107] (Figure 1.5.2). CAFs, phenotypically, closely resemble normal myofibroblasts, but they express specific markers (ie, fibroblast activation protein (FAP), fibroblast-specific protein 1, neuronglial antigen-2, vimentin, Thy-1, tenascin (TN)-C, periostin (POSTN), palladin or podoplanin (PDPN)) and display an increased proliferation and migratory behaviour in vitro [108-109]. CAFs produce and secrete various extracellular matrix (ECM) proteins (ie, collagens I, III, IV), proteoglycans (ie, fibronectin, laminin, TN), chemokines (eg, CXCL and CCL), cytokines (eg, interleukin (IL)-6 and IL-8) and other tumour-promoting factors which affect vascularization (ie, PDGF, vascular endothelial growth factor (VEGF), stromal-derived factor-1 (SDF-1), matrix metalloproteinase (MMPs)), proliferation capacity, tumour cell invasiveness and survival (ie, TGF-β, EGF, hepatocyte growth factor (HGF) or FGF) [101, 110-112]. Regarding anticancer therapy, the frequency of genetic mutations in CAFs is one of the most important issues. Cells with genetic stability may be less prone to escape or resistance to chemotherapy than those with genomic instability [113]. Several studies demonstrated that high percentage of CAFs undergo genetic alterations, such as loss of heterozygosis or mutation of tumour suppressor genes (i.e., phosphatase and tensin homolog and P53) [114-117]. The theory of genetic coevolution of CAFs and the neighbouring cells (i.e., random mutation of CAFs generated independently from neoplastic epithelial cells that may support tumour progression) is under debate due to the potential artefacts caused by the analytical methods used for the identification of these genetic alterations [118]. Other groups described that the somatic mutations of CAFs are found to be extremely rare and are unlikely to be responsible for their stable cancer-promoting attributes [119]. Interestingly, CAFs derived proteins, which may have an important role in the development of environment-mediated drug resistance [101-102], may act as powerful prognostic markers and [101] may be promising targets of anticancer therapy [118].

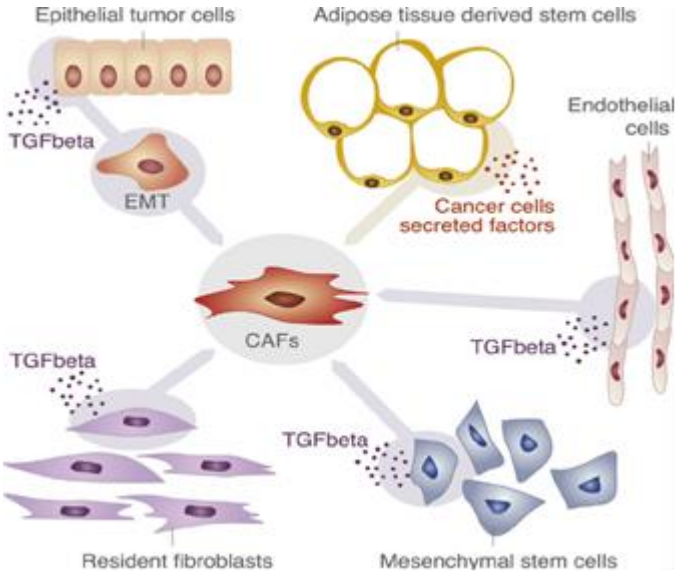


Fig. 1.5.2 CAFs origin

1.6 Environmental contaminants

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds comprised of two or more fused benzene rings, belonging to this group: 2-aminoanthracene, benz[a]anthracene (B[a]A), benzo[a]pyrene (B[a]P), B[a]P-4,5-dihydroepoxide (BPE), B[a]P diol epoxide (BPDE), benzo[e]pyrene (B[e]P), benz[j]aceanthrylene (B[j]A), benzo[k]fluoranthene (B[k]F), benz[l]aceanthrylene (B[l]A), cyclopenta[c,d] pyrene (CPP), dibenzo[a,l]pyrene (DB[al]P), 7,12-dimethylbenz[a] anthracene (DMBA), DMBA-3,4-diol, fluoranthene, fluorene, indeno [1,2,3-c,d]pyrene (IND), 3-methylcholanthrene, 1-methylpyrene, 1- nitropyrene, perylene, phenanthrene, and pyrene [120] (Figure 1.6.1). They generally originate from combustion processes and are widely distributed in the natural environment as a result of atmospheric transportation, wet and dry deposition, and surface-to-air exchange processes [121-122]. Sixteen PAHs are included among the 129 priority pollutants announced by the U.S. Environmental Protection Agency [123], some of which are carcinogenic [124-125]. ePAHs and the constitutent PAHs may be taken up by living organisms and accumulate via the food chain, and are exposed to humans via inhalation and the ingestion of food; therefore, they pose potential threats to the ecosystem and human health [126-127]. ePAHs can be found in various sources, such as air, dust, smoke, sediment, water, soil and oil in the environment

and/or pollutants, as forms such as particulate matter and materials in oven, fuel and tar. PAHs in the air mainly originate from the incomplete combustion of carbonaceous materials such as fossil fuel and biomass including emissions from coke and coal burning in occupational settings and forest fire, the exhaust fumes of motor vehicles, flues of biomass burning, for cooking and heating in rural areas, and tobacco smoke [121, 128-130].

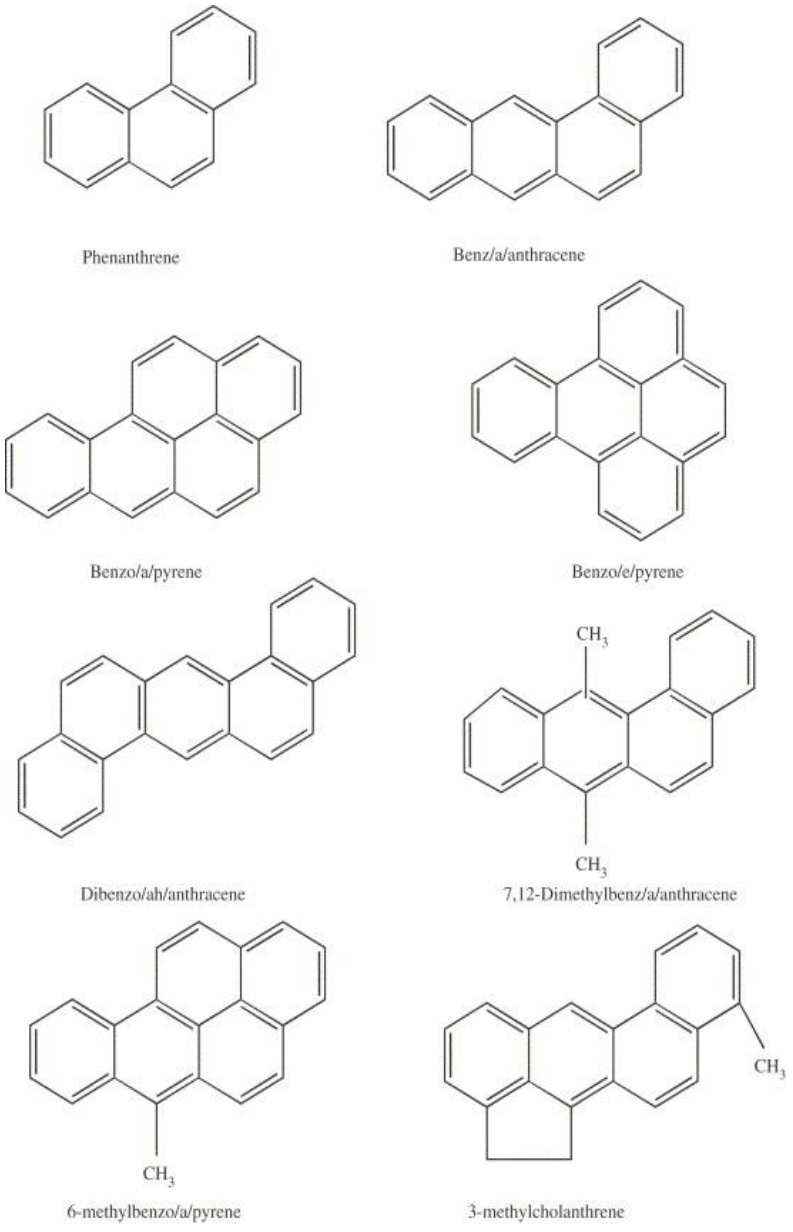


Fig. 1.6.1 chemical structures of polycyclic aromatic hydrocarbons (PAHs)

These chemical carcinogens in the environment are chemically inert in themselves and require metabolic activation by cytochrome P450 (CYP) enzymes to more reactive metabolites in order to exhibit carcinogenicity in experimental animals and humans. Of the

17 families of human CYPs identified to date, the CYP1, 2, and 3 family members play major roles in the metabolic activation of a variety of environmental carcinogens (Figure 1.6.2).

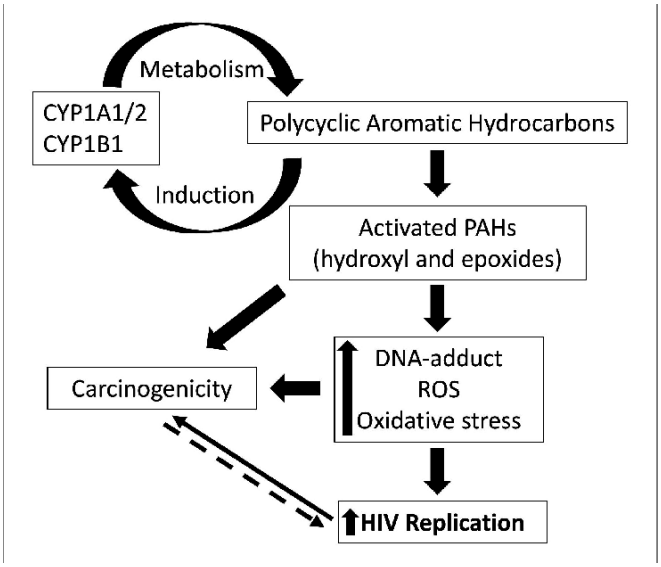


Fig. 1.6.2 Schematic representation of the cytochrome P450-CYP-mediated effects of PAHs

It has been suggested that CYP1A1 and CYP1B1 are responsible for the activation of most carcinogenic PAHs to epoxide intermediates, which are further converted to more reactive diol-epoxides with the aid of epoxide hydrolase. Many studies have reported correlations between the induction of cytochrome P450 (CYP), elevated 7-ethoxyresorufin-O-deethylase (EROD) activities, lysosomal membrane destabilization, DNA damage, and endocrine and reproductive effects in fish and invertebrates with PAH contamination in the water and sediments they inhabit. Humans may be exposed to environmental PAHs via the intake of PAH-contaminated food as well as the unintentional ingestion of soil and dust via hand-to-mouth behaviors, inhalation, and dermal contact [127, 130-133]. Epidemic studies have demonstrated that heavy exposure to PAHs from occupational environments increases the risk of developing various cancers including lung, skin, bladder, and larynx cancers [126, 134-136]. PAHs are known to stimulate AHR mediated cell signalling, and their mechanisms of actions have been examined extensively [137]. AHR is a ligand-activated transcription factor that binds to planar aromatic hydrocarbons, such as flavonoids, polyphenols, indoles, and the polyaromatic and halogenated aromatic hydrocarbons of natural and industrial origins. Previous studies described the association or crosstalk between AHR and various cell signaling pathways, such as mitogen-activated protein kinase (MAPK), NF-kB, and cell cycle/ apoptosis-related pathways [138-141]. Since AHR

is not a direct regulator of hormonal actions, the mechanisms underlying the endocrine-disrupting effects of AHR and its ligands require the involvement of hormone receptors and/or regulators. Since estrogen and estrogenic chemicals are an important category of endocrine modulators and sometimes act as endocrine disruptors, the involvement of estrogen signalling associated with AHR-mediated cell signalling stimulated with PAHs has been investigated in an attempt to elucidate the mechanisms responsible for their biological effects [21,142-143]. The biological activities of PAHs are categorized by specific signalling pathways (MAPK and other signalling pathways), regulatory mechanisms (chromatin/epigenetic regulation, cell cycle/DNA damage control, and cytoskeletal/adhesion regulation), and cell functions (apoptosis, autophagy, immune responses/inflammation, neurological responses, and development/differentiation), receptor-mediated pathways, growth factor/cytokine-signalling pathways, and key-regulator/modulator-dependent pathways. 3-Methylcholanthrene is involved in signalling pathways, cell functions, such as chromatin condensation in apoptosis and cell adhesion regulation during epithelial-mesenchymal transition [144] and in diseases, such as rheumatoid arthritis [145]. Many naturally occurring and anthropogenic compounds exhibit estrogenic properties in a variety of in vivo and in vitro animal bioassay systems. PAHs act in the same manner as hormonal estrogens, duplicating their physiologic effects, directly compete with and inhibit the effects of hormonal estrogens and act indirectly to modify estrogen availability or expression of hormonal effects in target tissues. Benzo[a]pyrene (BaP), is a mammary carcinogen, as are several other members of the PAH class [146-150]. For many years, 7,12-dimethylbenz[a]anthracene (DMBA) and 3-methylcholanthrene (3MC) have been used as model compounds for studying the molecular mechanisms of mammary carcinogenesis in the Sprague-Dawley rat Mammary. Tumours induced by PAH are predominantly estrogen-dependent, either directly or indirectly via release of pituitary prolactin [151-155]. Only a few carcinogenic PAHs and their hydroxyl derivatives have been studied for ER binding properties. The results of ER binding studies with DMBA in calf uterine cytosol and rat mammary and uterine cytosol were conflicting [156-158]. The binding affinity of 3MC for rat uterine ER was reported to be similar to that of tamoxifen [159], although in other studies [160] 3MC binding was observed only to a non-ER cytosol protein. Neither 3MC, dibenz[a,h]anthracene, or dibenz[a,c]anthracene interfered with estradiol binding in calf uterine cytosol [161]. By altering the regulatory activity of fibroblasts, PAH may conceivably affect the estrogenic

control of mammary growth promotion and differentiation, and ultimately cell transformation. Since DMBA and other carcinogenic PAH, are genotoxic per se following metabolic bioactivation, it is not currently possible to assess the relative contribution, if any, of altered peripheral estrogen metabolism to the process of PAH-induced neoplastic transformation in estrogen-responsive tissues. Carcinogenic PAHs extensively localize in breast tissue of rats [162-163], are metabolized by human mammary cells to reactive products that bind to DNA [164], and elicit genotoxic effects in human and rat mammary epithelial cells [165]. In a recent clinical report, BaP-like DNA adducts were detected at significant levels in the breast tissues of 41% of a group of breast cancer patients, and were absent in all noncancerous controls [166]. These findings lend strong support to the possible involvement of carcinogenic PAHs in the etiology of human breast cancer, although they suggest no a prior reason why estrogen interactions should participate. Nevertheless, the currently available scientific literature for DMBA and 3MC provides persuasive evidence for the hypothesis that certain carcinogenic PAHs produce a unique duality of pathologic effects encompassing both genotoxic and non-genotoxic components. The genotoxic component may cause the activation of oncogenes, inactivation of tumour-suppressor genes, or amplification of growth factor genes, while the non-genotoxic component results in increased cell proliferation. A fundamental assumption in carcinogenesis is that irreversible fixation of genetic damage can only occur in replicating cells, and the greater the rate of cell division the greater will be the probability for initiated cells to progress to a malignant tumour [167]. Carcinogenic PAH such as DMBA and 3MC possess not only the capability for producing direct DNA damage. This dynamic relationship between genotoxic and mitogenic stimuli, in conjunction with dose and various host factors (e.g., age, genetic predisposition, high fat diet), may be a critical determinant of cancer risk for exposure to all carcinogenic PAHs having steroidal activity.

1.7 Aryl hydrocarbon receptor (AHR)

AHR is a cytosolic ligand-activated transcription factor, which belongs to the member of bHLH/PAS (basic helix–loop–helix/period [Per]-aryl hydrocarbon receptor nuclear translocator [ARNT]-single-minded [SIM]) family of heterodimeric transcriptional regulators [168-170]. The bHLH motif located in the amino (N)-terminal of AHR protein, has two functionally distinctive and highly conserved domains, which together make up a region of approximately 60 amino-acid residues. At the N-terminal end of this motif is the

basic domain, which binds AHR to DNA at the consensus regulatory sequences (5′-T/GCGTG-3′) termed AHREs (aryl hydrocarbon response elements), also XREs (xenobiotic response elements) or DREs (dioxin response elements), located in the promoter region of its target genes. At the carboxyl (C)-terminal end of this motif is the helix–loop–helix (HLH) domain, which facilitates protein–protein interactions to form heterodimeric complexes with its partner protein, the ARNT. The PAS domains, including PAS-A and PAS-B, support specific secondary interaction with the ARNT, so that the heterozygous protein complex can form. The ligand-binding site of AHR is contained within the PAS-B domain and contains several conserved residues critical for ligand binding. Finally, a Q-rich domain is located in the C-terminal region of the protein and is involved in co-activator recruitment and transactivation (Figure 1.7.1). In the absence of ligand, AHR exists as part of a cytosolic protein complex, which contains two molecules of the 90 kDa molecular chaperone heat shock protein 90 (HSP90), the HSP90-interacting protein p23 and the immunophilin-like protein XAP2 (also AIP or ARA9). HSP90 interacts with AHR via both the bHLH region and PAS B, which contains the ligand region, and this association is essential for AHR signalling.

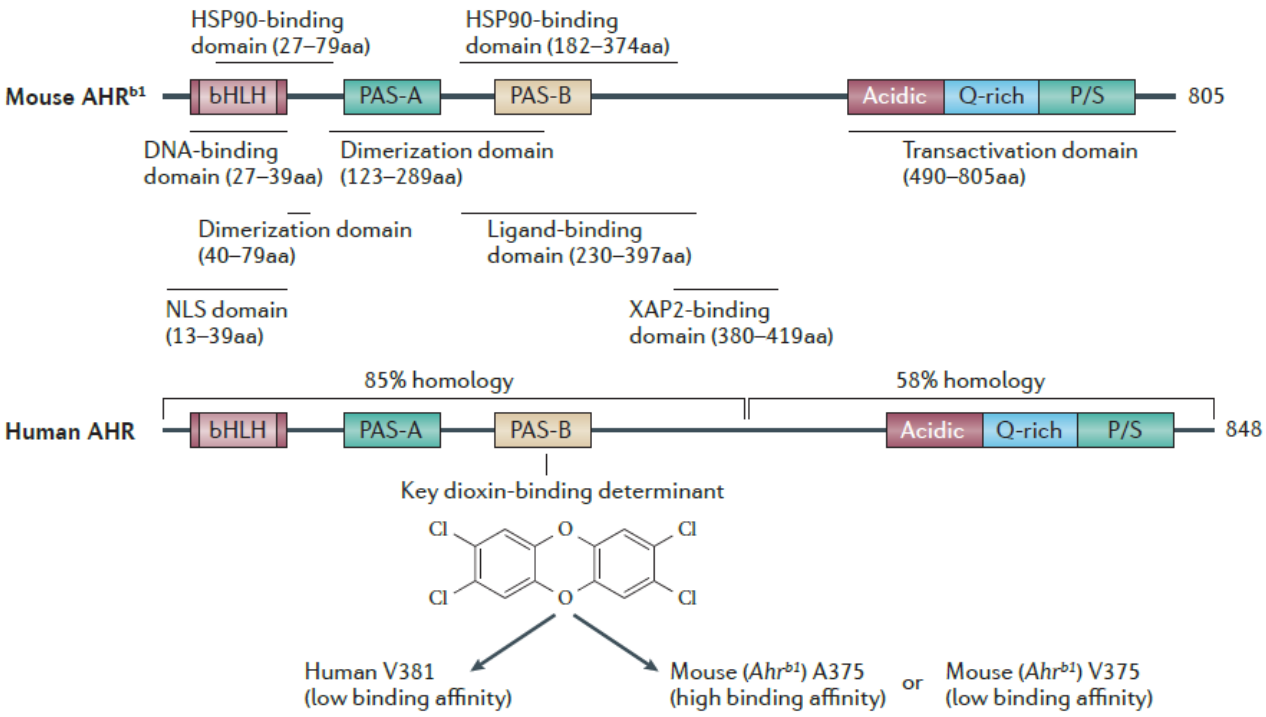


Fig. 1.7.1 Functional Domains of Mouse and Human AHR

Following activation by agonist (such as 2,3,7,8- tetrachlorodibenzodioxin, TCDD) binding, AHR changes its conformation that exposes the nuclear localization sequence (NLS) through alteration of XAP2 binding, and its N-terminal nuclear localization signal is activated, directing it to translocate into the nucleus, where it releases its chaperone proteins and dimerizes with its partner protein, the ARNT. This AHR/ARNT heterodimer interacts with several histone acetyltransferases, chromatin remodeling factors, and a partially characterized set of co-activators and/or co-repressors, and the resulting multiprotein complex binds to the XREs and simultaneously bridges the enhancer and the TATA box in the promoter region of its target genes, and recruits RNA polymerase II to initiate transcription [171]. Once transcriptional regulation has occurred, AHR is quickly exported to the cytosol by chromosome region maintenance 1 (CRM1), where it is degraded by the 26S ubiquitin–proteasome, hence preventing constitutive receptor activity [172]. A picture as to how the AHR/ARNT heterodimer actually mediates gene transcription is starting to emerge. The AHR/ARNT complex can alter transcription both by binding to its cognate response element and through tethering to other transcription factors [173]. In addition, AHR activity in the cell is negatively regulated by the presence of the AHR repressor protein (AHRR), whose expression is regulated by AHR. The AHRR is also an AHR-related bHLH/PAS transcription factor, which may dimerize with the ARNT and compete with AHR to bind the XREs. The result is a negative feedback mechanism that involves a down regulation of all genes regulated by AHR [174]. Furthermore, many factors are necessary for AHR-mediated modulation of target gene transcription [173, 175-181]. As a consequence of AHR activation, many detoxification genes are transcriptionally induced, including those coding for the phase I drug/xenobiotic-metabolizing cytochrome P450 enzymes (such as CYP1A1, CYP1A2, CYP1B1, and CYP2B1), the phase II enzymes (e.g. UDPglucuronosyl transferase (UGT1A6), NAD(P)H dependent quinone oxydoreductase-1 (NQO1), the aldehyde dehydrogenase (ALDH3A1), and several glutathione-S-transferases), and the phase III transporters (for example, P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and organic anion transporting polypeptide 2 (OATP2)), which are expressed in many tissues, such as liver, intestine, kidney and brain, and play crucial roles in drug absorption, distribution and excretion. This drug-metabolizing enzyme system plays central roles in the metabolism, elimination and detoxification (or activation) of xenobiotics and drugs introduced into the human body [182]. In addition to the genomic pathway, several non-genomic pathways

have been identified recently. For instance, following exposure to TCDD and other AHR ligands, there is a rapid increase in intracellular calcium concentration (from both extracellular and endoplasmic reticulum sources). TCDD also leads to the functional activation of the tyrosine kinase Src by releasing it from the AHR complex [183]. This could be accompanied by the activation of the Focal Adhesion Kinase (FAK) and by the modification of the adhesion properties of the cell through disruption of focal adhesion points [183-184]. Src activation could be accompanied also very rapidly by the activation of MAP kinases, ERK1 and ERK2. All these processes may converge to regulate pathophysiological processes such as inflammation. Indeed, the calcium influx causes the activation of protein kinase C (PKCa) which phosphorylates a serine residue of a cytosolic enzyme, phospholipase A2 (cPLA2) with the subsequent production of arachidonic acid. The parallel activation of MAP kinases by Src leads to the transcription of cyclooxygenase 2 (COX2) which uses arachidonic acid to produce prostaglandins that can cause inflammation. Thus, these two signalling pathways, which were initially activated by AHR ligands, converge towards the stimulation of inflammation [185]. Moreover, the AHR interacts with Wnt/b-catenin, ER-alpha or NF-kB and strongly modulates their actions [186-189]. On the other hand, these transcription factors also affect AHR signalling. For example, b-catenin has been indicated as a coactivator of this receptor [190]. DNA microarray studies have established that AHR either directly or indirectly regulates a myriad of genes involved in a wide variety of biochemical pathways, including energy metabolism, lipid and cholesterol synthesis, xenobiotic metabolism and various transportation pathways. AHR, it may be considered as an important intracellular chemosensor responsive to both natural and man-made environmental compounds, it is widely expressed in a variety of animal species and humans [191-192]. For several decades, AHR has been studied largely because of its critical role in xenobiotic-induced toxicity and carcinogenesis [193-194]. This is an increasing area that covers multiple aspects of physiology, such as cell proliferation and differentiation, endogenous mechanisms of activation, gene regulation, tumour development, cell motility and migration, and others (Figure 1.7.2) [193].

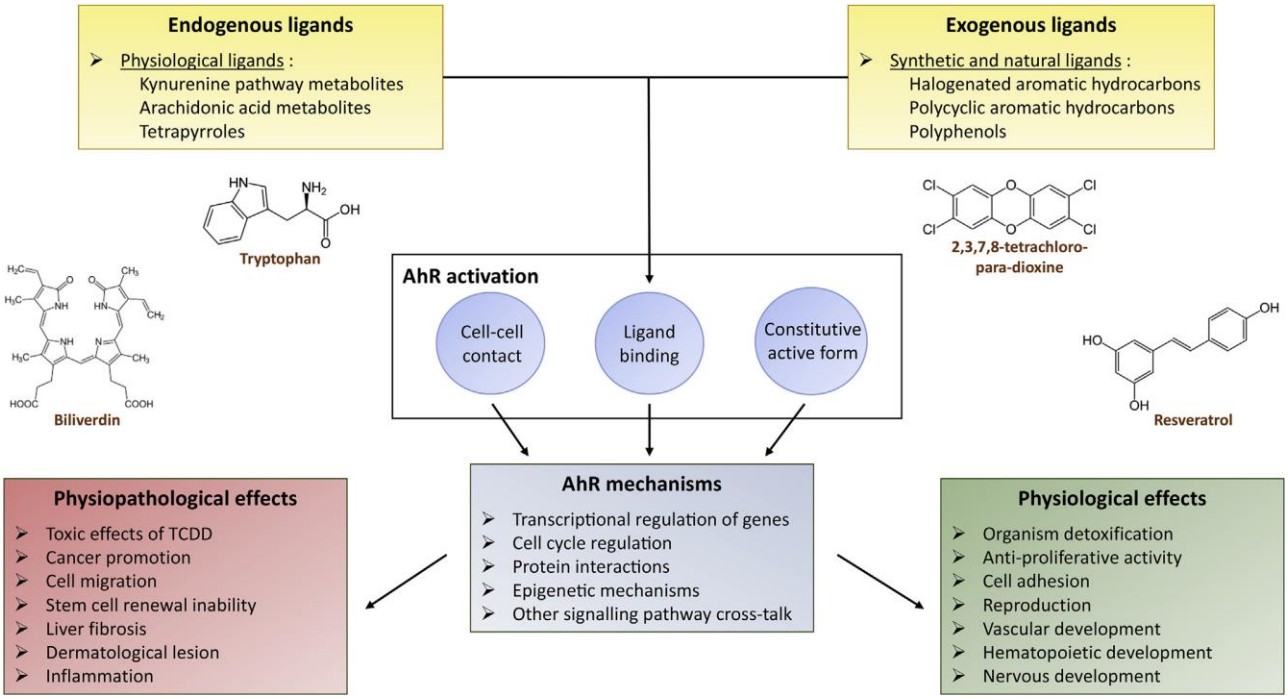


Fig. 1.7.2. The functional relationship between the AHR ligands and the regulatory roles of this receptor in physiology and pathophysiology

1.7.1 Cytochrome P450 1B1 (CYP1B1)

Numerous studies have indicated that some 17β-estradiol (E2) metabolites and environmental contaminants may influence the development of breast cancer; therefore, great attention has been addressed to a better understanding of the biological effects induced by estrogenic and xenobiotics metabolites and the processes of their biosynthesis [7, 195]. Furthermore, it has been demonstrated that various cytochrome P450 enzymes (CYPs) participate actively in the key processes involved in the metabolism of E2 [9]. In particular, CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), a heme-thiolate monooxygenase mainly expressed in hormone-sensitive tissues such as breast, uterus and ovary, has been indicated as a primary enzyme involved in estrogen and environmental carcinogens metabolism [10]. The gene encoding for CYP1B1 is placed on chromosome 2, at the level of region 2p21-22 [196-197]; the gene contains three exons (371, 1044 and 3707 bp) and two introns (390 and 3032 bp) [196, 198-199]. Although mainly located at the nuclear level, CYP1B1 was also found in the cytoplasmic compartment [200]. CYP1B1 expression has been reported increased in tumor tissues and in tumor microenvironment compared to the normal counterpart [200-203] (Figure

1.7.1.1). CYP1B1 over-expression is a prognostic factor in neoplastic progression and tumour metabolism and although it has been observed in diverse cancer cells (colon, pulmonary, renal, bladder and glaucoma), it appears particularly high in hormone-responsive carcinomas (prostatic, mammary, endometrial, and ovarian) [198, 202, 204].

Cell type	Role of CYP1B1	CYP1B1 inhibition
 Cancer cells	Bioactivation of xenobiotics Steroid hormones metabolism Production of pro-inflammatory cytokines	Chemoprevention
	Production of pro-angiogenic factors	Angioprevention
 Fibroblasts	Bioactivation of xenobiotics Production of pro-inflammatory cytokines	Chemoprevention
	Production of pro-angiogenic factors	Angioprevention
 Endothelial cells	Production of pro-inflammatory cytokines	Chemoprevention
	Angiogenesis Production of pro-angiogenic factors	Angioprevention
 Pericytes	Production of pro-angiogenic factors	Angioprevention
 Immune cells	Immunosuppression Production of pro-inflammatory cytokines	Chemoprevention
	Production of pro-angiogenic factors	Angioprevention

Fig. 1.7.1.1 Role of CYP1B1 in cancer cells and cancer microenvironment

Therefore, the induction of CYP1B1 and the biotransformation of endogenous estrogens and environmental carcinogens, represents an important element in determining what is the risk associated with hormone-dependent tumours, including breast cancer [7, 11, 14]. The metabolic activity of CYP1B1 is a key to better understand the role of environmental compounds in cancer initiation and progression. Many factors are involved in genomic mutation accumulation and in cancer development, in this context CYP1B1 activates polycyclic aromatic hydrocarbons, aromatic and heterocyclic amines, aflatoxin B1 in pro-carcinogenic compounds (Figure 1.7.1.2) [205-207].

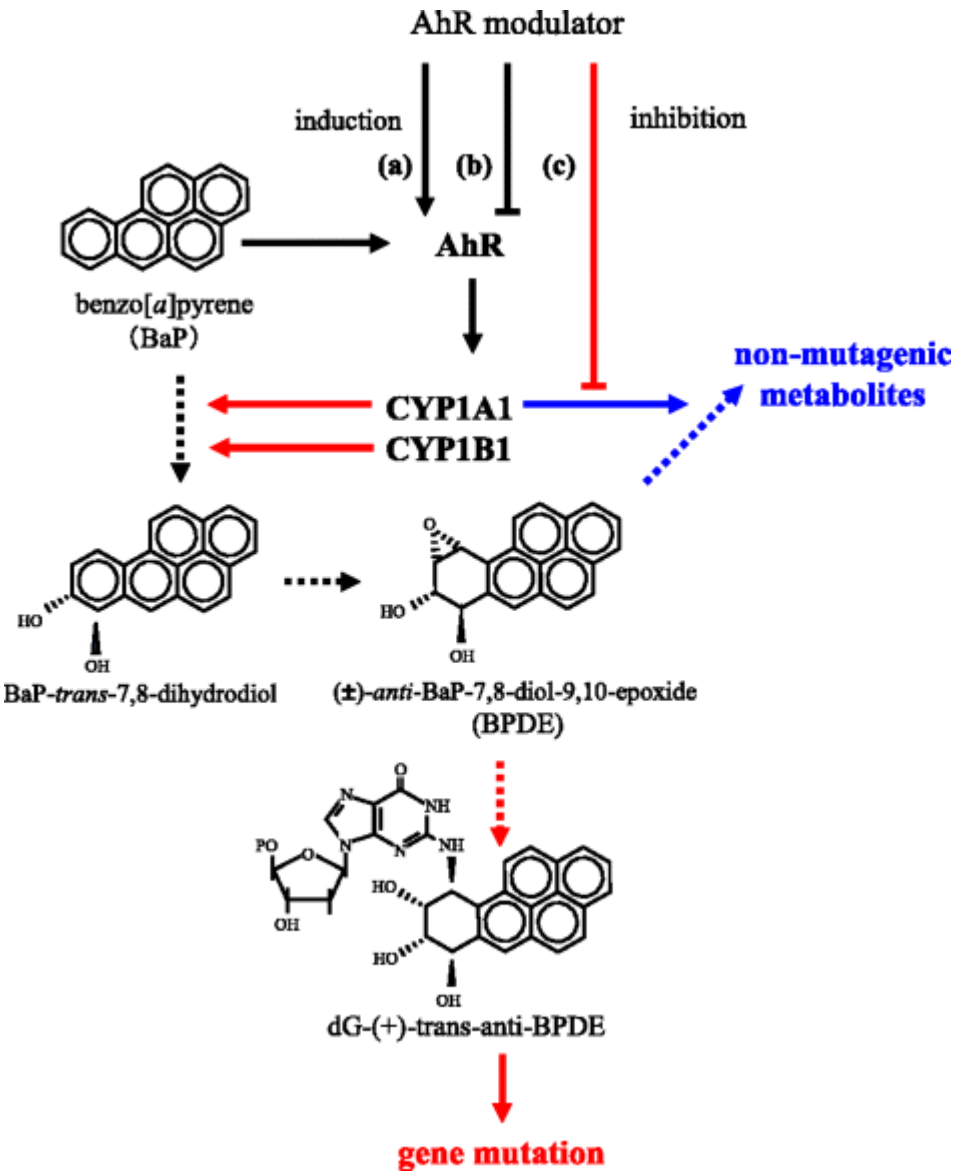


Fig 1.7.1.2 CYP1B1-mediated PAHs metabolism

In addition, CYP1B1 catalyzes the hydroxylation of E2, leading to the formation of 4OHE2 [196] and subsequently of oestradiol-3,4-quinone, a potent carcinogen metabolite which, by binding to the N-7 position of the guanine, leads the destabilization of the glycosidic bond and subsequent DNA purification and mutagenesis [2, 8, 19, 209] (Figure 1.7.1.3). Considering that 4OHE2 levels in hormone-sensitive tumours, such as breast cancer, are higher than normal tissues [8, 14, 202], CYP1B1 is considered a valid pharmacological target in the therapeutic treatment of hormone-dependent cancer [210].

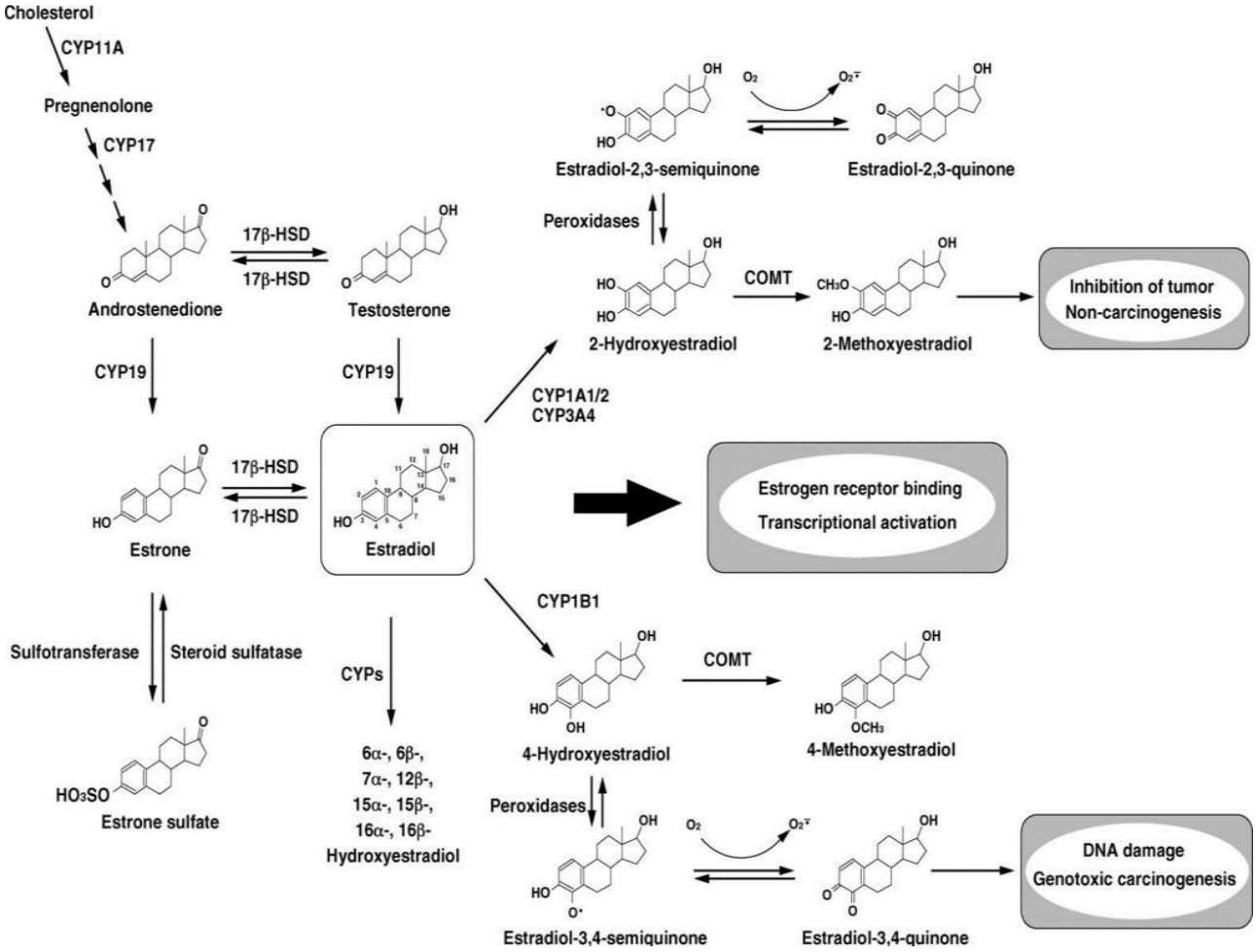


Fig. 1.7.1.3. CYP1B1-mediated estrogen metabolism

The transcription of CYP1B1 is mainly regulated by AHR, which acts as a transcription factor activated by specific ligands, such as dioxin, halogenated aromatic hydrocarbons, BaP and PAHs [8, 211]. In particular, following agonist binding, the cytoplasmic complex consisting of AHR, the heat shock protein-90 and the XAP2 and p23 proteins, translocate at the nuclear level, with consequent dissociation of the AHR complex and formation of the AHR/ARNT heterodimer. This heterodimer binds specific DNA region, named dioxin-responsive-elements (DRE), located in the promoter sequences of target genes including CYP1B1 [212-214] (Figure 1.7.1.4). Growing body of evidence have demonstrated that estrogens generate a feed-forward loop triggering the transcription of CYP1B1, which in turn is primarily involved in the metabolic conversion of these steroids [17-19]. For instance, the transcription of CYP1B1 was induced in breast and endometrial cancer cells by E2 through the activation of ERα and its binding to an estrogen responsive element (ERE) located within the CYP1B1 promoter sequence [18]. These findings may underline

the physiological relevance of CYP1B1 regulation by estrogens in the landscape of the estrogen homeostasis and action, in particular in hormone-sensitive tissues [2, 18- 19].

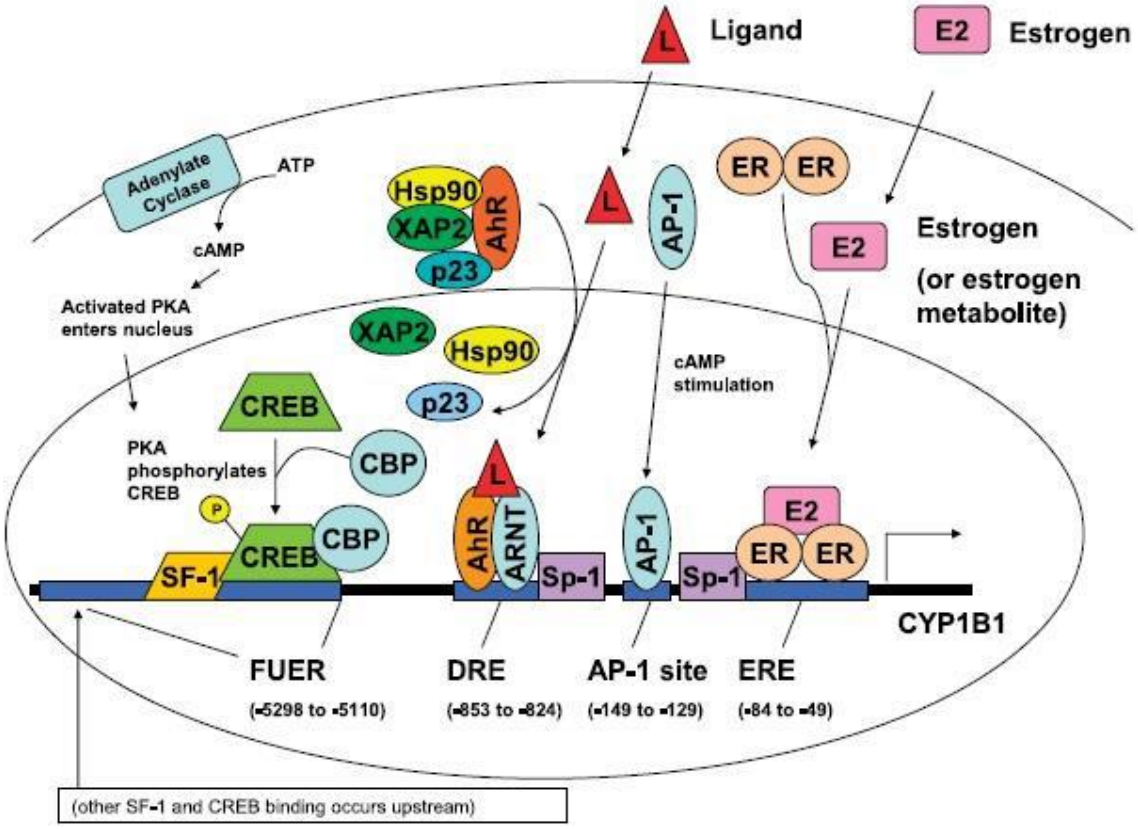


Fig 1.7.1.4. Transcriptional regulation of CYP1B1

1.8 Aim of the study

The aim of this study was to ascertain the potential role elicited by estrogens on CYP1B1 expression and metabolic activity. In particular, we aimed to establish whether estrogenic GPER signalling regulates CYP1B1 expression in ER-negative and GPER-positive breast cancer cells, CAFs obtained from breast cancer patients and CAFs derived from a cutaneous metastasis of an invasive mammary ductal carcinoma (met-CAFs). Moreover, we aimed to evaluate the possible functional cross-talk between GPER and AHR in breast cancer cells and CAFs, which may affect gene expression changes and biological effects upon exposure to the cognate ligands, estrogens and 3MC, respectively. Our results further extend the molecular mechanisms by which 3MC and estrogens could affect breast cancer progression, hence providing novel biological targets for innovative treatments of breast tumor.

Chapter 2

Materials and Methods

2.1 Reagents

17β-Estradiol (E2), salicylamide (2-hydroxybenzamide), resorufin (7-hydroxy-3H-phenoxazin-3-one), resorufin ethyl ether (7-ethoxy-3H-phenoxazin-3-one), 3-methylcholanthrene (3MC), CH223191 1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl-1H-pyrazole-5-carboxamide, were purchased from Sigma-Aldrich (Milan, Italy). G-1 (1-[4-(-6-bromobenzol [1,3]diodo-5-yl)-3a,4,5,9b-tetrahidro3H5cyclopenta[c]quinolin-8yl]-ethanone), G-15 (3aS,4R,9bR)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone and TMS 1-[2,(3,5-dimethoxyphenyl)ethenyl]-2,4-dimethoxybenzene were obtained from Tocris Bioscience (Space, Milan, Italy). Tyrphostin AG1478 (AG) and PD98059 (PD) were obtained from Calbiochem (DBA, Milan, Italy). Mithramycin A (MTMA) was purchase from Abcam (Euroclone, Milan, Italy). All the aforementioned compounds were dissolved in dimethyl sulfoxide (DMSO), except for salicylamide that was dissolved in methanol, 3MC that was dissolved in toluene and mithramycin A that was dissolved in ethanol.

2.2 Cell cultures

SkBr3 and MDA-MB-231 breast cancer cells were obtained by ATCC (Manassas, VA, USA), used less than 6 months after resuscitation, routinely tested, and authenticated according to the ATCC suggestions. SkBr3 cells were maintained in RPMI-1640 (Life Technologies, Milan, Italy) without phenol red, supplemented with 10% fetal bovine serum (FBS) and 100μg/ml penicillin/streptomycin (Life Technologies, Milan, Italy). MDA-MB-231 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) (Life Technologies, Milan, Italy) with phenol red, with a supplement of 5% FBS and 100 μg/ml of penicillin/streptomycin. CAFs obtained from breast malignancies and met-CAFs obtained from biopsy of cutaneous metastasis in a patient with a primary invasive mammary ductal carcinoma, who previously had undergone surgery, were characterized

and maintained as we have previously described [51, 215]. Briefly, specimens were cut into smaller pieces (1–2mm diameter), placed in digestion solution (400 IU collagenase, 100 IU hyaluronidase, and 10% serum, containing antibiotic and antimycotic solution) and incubated overnight at 37 °C. The cells were then separated by differential centrifugation at 90× g for 2 min. Supernatant containing fibroblasts was centrifuged at 485× g for 8 min; the pellet obtained was suspended in fibroblasts growth medium (Medium 199 and Ham's F12 mixed 1:1 and supplemented with 10% FBS) and cultured at 37°C in 5% CO_2. Primary cells cultures of metastasis-derived fibroblasts and CAFs were characterized by immunofluorescence. Briefly, cells were incubated with human anti-vimentin (V9) and human anti-cytokeratin 14 (LL001), both from Santa Cruz Biotechnology (DBA, Milan, Italy). To characterize fibroblasts activation, we used anti-fibroblast activated protein α (FAPα) antibody (H-56; Santa Cruz Biotechnology, DBA, Milan, Italy) (data not shown). CAFs and metastasis-derived CAFs were maintained in Medium 199 and Ham's F12 (mixed 1:1) supplemented with 10% FBS and 100μg/ml penicillin/streptomycin. All cell lines were grown in a 37°C incubator with 5% CO_2. All cell lines to be processed for immunoblot and RT-PCR assays were switched to medium without serum and phenol red the day before treatments. Signed informed consent from all the patients was obtained and all samples were collected, identified and used in accordance with approval by the Institutional Ethical Committee Board (Regional Hospital of Cosenza, Italy).

2.3 Real-time PCR

Total RNA was extracted from cells maintained for 24 hours in medium without serum and treated with ligand for indicated times, using Trizol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and cDNA was synthesized from the RNA by reverse transcription using murine leukemia virus reverse transcriptase (Invitrogen). We quantified the expression of selected genes by real-time PCR. This method is based on the use of intercalating agents, which bind to double stranded DNA. These molecules, when excited by laser beams, emit fluorescence and allow to follow in real-time the progress of the reaction and the increase of the amount of nucleic acid. In this study, we used SYBR Green as the detection method and platform Quant Studio7 Flex Real-Time PCR (Life Technologies). Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems). For CYP1B1, c-Fos, cyclin D1, cyclin E, cyclin A and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the

primers were: 5′-TGTGCCTGTCACTATTCCTCATG-3′ (CYP1B1 forward) and 5′-GGGAATGTGGTAGCCCAAGA-3′ (CYP1B1 reverse); 5′-CGAGCCCTTTGATGACTTCCT-3′ (c-Fos forward) and 5′-GGAGCGGGCTGTCTCAGA-3′ (c-Fos reverse); 5′-GTCTGTGCATTTCTGGTTGCA-3′ (cyclin D1 forward) and 5′-GCTGGAAACATGCCGGTTA-3′ (cyclin D1 reverse); 5′-GCATGTCACCGTTCCTCCTTG-3′ (cyclin A forward) and 5′-GGGCATCTTCACGCTCTATTTT-3′ (cyclin A reverse); 5′-GATGACCGGGTTTACCCAAAC-3′ (cyclin E forward) and 5′-GAGCCTCTGGATGGTGCAA-3′ (cyclin E reverse); 5′-GGCGTCCCCCAACTTCTTA-3′ (18S forward) and 5′-GGGCATCACAGACCTGTTATT-3′ (18S reverse). Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression.

2.4 Western blotting

Cells were grown in 10-cm dishes, exposed to treatments and then lysed in 500 μL of 50 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), and a mixture of protease inhibitors containing 1mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride and 200 mmol/L sodium orthovanadate. Protein lysates from tumour homogenates obtained from nude mice were processed as previously described [217]. Protein concentration was determined using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich, Milan, Italy). Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences, Sigma-Adrich, Milan, Italy), probed overnight at 4 °C with antibodies against CYP1B1 (TA339934), cyclin D1 (TA801655), cyclin E (TA590076), cyclin A (TA890057) (OriGene Technologies, DBA, Milan, Italy), AHR (Cell Signalling), GPER (AB137479) (ABCAM, Euroclone, Milan, Italy), c-Fos (E8), pEGFR Tyr 1173 (sc-12351), EGFR (1005), phosphorylated ERK1/2 (E-4), ERK2 (C-14), and β-actin (C-2) (Santa Cruz Biotechnology, DBA). Proteins were detected by horseradish peroxidase-linked secondary antibodies (Santa Cruz Biotechnology, DBA) and then revealed using the chemiluminescent substrate for western blotting Westar Nova 2.0 (Cyanagen, Biogenerica, Catania, Italy).

2.5 Transient transfections

The transfections allow inserting exogenous biological material, such as nucleic acids, into the eukaryotic cell. The transfection is defined "transient" when the inserted genetic material remains in the cell as an extrachromosomal fragment and does not integrate into the cellular genome; in this case, the features induced by transfection persist for a short time, usually disappear prior to 72 hours. The main problem in the transfer of nucleic acids is provided by the presence of negative charges, due to phosphate groups, in the skeleton of the molecules. Because of these charges, the exogenous material is not able to overcome the cell membrane, as electrostatic forces of repulsion occur. One of the methods of transfection more employed to mask the anionic groups of the DNA is represented by the use of cationic lipids. This method is included in the field of chemical techniques of transfection and requires the use of amphipathic lipid molecules, which associate to form liposomes. These, being constituted by amphipathic lipids, in contact with the aqueous environment form a phospholipid bilayer very similar to cell membranes. Moreover, the liposomes may contain within them charged molecules, such as DNA, as their polar heads are turned towards the inner of the vesicle. This complex lipid/DNA can fuse with the plasma membrane and carry the exogenous material within the cell. The cationic lipids most commonly used have characteristics such as high efficiency; low cytotoxicity, quick and simple protocol for usage and some can be used also in the presence of serum.

2.5.1 Plasmids

The plasmid DN/c-Fos, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA binding, was a kind gift from Dr C. Vinson (NIH, Bethesda, MD, USA). pGL3-promoter plasmid containing the 5’-flanking region from -2299 to +25 respect to the transcription initiation site (TIS) [195] of the CYP1B1 gene and CYP1B1 promoter deletion constructs containing fragments -1652 to +25, -1243 to +25, -1022 to +25, -988 to +25, -910 to +25 respect to TIS were generated as previous described [217].

2.5.2 Gene silencing experiments

Cells were plated into 10-cm dishes and transfected using X-treme GENE 9 DNA Transfection Reagent (Roche Diagnostics, Sigma-Adrich, Milan, Italy) for 24 h before treatments with control shRNA, shRNA for GPER (shGPER) or shRNA for CYP1B1 (shCYP1B1, Santa Cruz Biotechnology, DBA, Milan, Italy). The silencing of GPER

expression was obtained by using the constructs, which we have previously described, and used [218].

2.5.3 Luciferase assays

To perform the luciferase assay two "reporter" enzymes are simultaneously expressed in a single system and their activities are measured. The activity of the experimental reporter is correlated to the specific conditions of treatment, while the basal cell activity is compared to that of the co-transfected control reporter (pRL-CMV). Comparing the activity of the experimental and control reporters, it is possible to normalize experimental variability that generally is caused by the differences between the number of cells and effectiveness of the transfection. In this assay in one sample are measured sequentially the activities of two luciferase: the firefly or firefly luciferase (Photius pyralis) and the Renilla luciferase (Renilla reniformis). These enzymes have different structures and requires different substrates, so that it is possible to discriminate selectively the respective bioluminescent reactions. The activity of firefly luciferase is measured initially adding the LAR II (Luciferase Assay Reagent II) to the cell lysate. This generates a light signal that is appropriately quantified using a luminometer (Lumat model LB 9507, Berthold Technology). Then, adding in the same tube the Stop & Glo reagent, the first enzymatic reaction is stopped and simultaneously start the second reaction catalyzed by Renilla, which also generates a light signal. Finally, the values of the Luciferase activity are compared with the corresponding values of Renilla and expressed as “Relative Luciferase Units”. In this study for the luciferase assays, Cells (1×10^5) were plated into 24-well dishes with 500 µl/well of regular growth medium the day before transfection. Growth medium was replaced with medium lacking serum on the day of transfection, which was performed using X-tremeGene9 reagent, as recommended by the manufacturer (Roche Diagnostics), with a mixture containing 0.5 µg of each reporter plasmid and 1 ng of pRL-TK. After 8 h, the medium was replaced with fresh medium lacking serum and the cells were incubated for 18 h with treatments. Luciferase activity was then measured with the Dual Luciferase Kit (Promega, Milan, Italy) according to the manufacturer’s recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase activity. The normalized relative light unit values obtained from cells treated with vehicle (−) were defined as one fold induction, relative to which the activity induced by treatments was calculated.

2.6 Site-directed mutagenesis

Site-directed mutagenesis is a molecular biology method that is used to make specific and intentional changes to the DNA sequence of a gene and any gene products. Also called site-specific mutagenesis or oligonucleotide-directed mutagenesis, it is used for investigating the structure and biological activity of DNA, RNA, and protein molecules, and for protein engineering. The basic procedure requires the synthesis of a short DNA primer. This synthetic primer contains the desired mutation and is complementary to the template DNA around the mutation site so it can hybridize with the DNA in the gene of interest. The mutation may be a single base change (a point mutation), multiple base changes, deletion, or insertion. The single-strand primer is then extended using a DNA polymerase, which copies the rest of the gene. The gene thus copied contains the mutated site, and is then introduced into a host cell as a vector and cloned. Finally, mutants are selected by DNA sequencing to check that they contain the desired mutation. The p-GL3-promoter plasmid containing the 5'-flanking region from -1652 to +25 respect to TIS of the CYP1B1 gene was used as template to generate as previously described [219] the DNA fragment from -513 to -95 respect to TIS containing a half-ERE site (see results section), which was amplified by PCR using the following primers: sense 5'-CGAGGTACCCTGATCTCGCCGCAAGAACT-3' and anti-sense 5'-GTCGCTAGCGCCGCACACCAGGCC-3'. The CYP1B1 deletion construct from -513 to -95 lacking the half-ERE site (see results section) was amplified by PCR using the following primers: sense 5'-CGAGGTACCCTGATCTCGCCGCAAGAACT-3' and anti-sense 5'-GTCGCTAGCGCCGCACACCAGGCCGACTCCCGTCCAGG-3'. The amplified DNA fragments were digested with KpnI and NheI and cloned into the pGL3-promoter plasmid (Promega, Milan, Italy). The sequence of each construct was verified by nucleotide sequence analysis.

2.7 Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) is a type of immunoprecipitation experimental technique used to investigate the interaction between proteins and DNA in cells. It aims to determine whether specific proteins are associated with specific genomic regions, such as transcription factors on promoter sequences or other DNA binding sites, and possibly defining cistromes. ChIP also aims to determine the specific location in the genome that various histone modifications are associated with, indicating the target of the histone

modifiers [220]. Briefly, the conventional method is as follows: DNA and associated proteins on chromatin in living cells or tissues are crosslinked. The DNA-protein complexes (chromatin-protein) are then sheared into ~500 bp DNA fragments by sonication or nuclease digestion. Cross-linked DNA fragments associated with the protein(s) of interest are selectively immunoprecipitated from the cell debris using an appropriate protein-specific antibody. The associated DNA fragments are purified and their sequence is determined. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with in vivo. In this study, the cells grown on 10-cm plates were shifted for 24 h in a medium lacking serum and then exposed to treatments for 3 h. Thereafter, cells were cross-linked with 1% formaldehyde and sonicated. Supernatants were immuno-cleared with salmon DNA/protein A-agarose (Upstate Biotechnology, Inc., Lake Placid, NY, USA) and immunoprecipitated with anti c-Fos or nonspecific IgG (Santa Cruz Biotechnology, DBA). Pellets were washed, eluted with a buffer consisting of 1% SDS and 0.1 mol/L NaHCO3 and digested with proteinase K. DNA was obtained by phenol/chloroform extractions and precipitated with ethanol. A 4µl volume of each immunoprecipitated DNA sample and input were used as a template to amplify by PCR the region containing a half-ERE site located in the CYP1B1 promoter region. The primers used to amplify this fragment were as follows: 5'-CTGCTGGTAGAGCTCCGAGG-3' (forward) and 5'-CCCGCTGCTCTGCTTCTTAC-3' (reverse). Data were normalized to the input for the immunoprecipitation.

2.8 Ethoxyresorufin-O-deethylase activity assay

The ethoxyresorufin-O-deethylase (EROD) assay monitors the activity of enzymes belonging to the cytochrome P-450 family (CYP1A1, CYP1A2, CYP1B1). It is a simple, sensitive and accurate assay. Cytochrome P450 monooxygenase (CYP1B1) is induced by a series of toxic compounds and endogenous chemicals, it is expressed mainly in the liver, but it can also be detected in other organs including: kidneys, skin, lungs, adrenal gland, gonads and brain. Evaluation of CYP1B1 activity is a recent technique. As with most enzyme assays, EROD activity can be normalized to total protein values. The cells (7×10^4 cells/ml) were grown in 24-well plates for 48 h, then were shifted for 24 h in a medium lacking serum and then treated for 18 h. The cells were washed with PBS, and fresh medium containing salicylamide to inhibit conjugating enzymes (1.5 mM) was added to the wells. The plate was incubated at 37°C for 5 min, then 7-ethoxyresorufin was added (final concentration of 5 µM) and the reaction was carried out for 1 hour at 37°C with

gentle stirring of the plate every 5 min. Aliquots of cell suspensions (200 µL) were transferred to tubes and the reaction was terminated by the addition of an equal volume of ice-cold methanol, which resulted in immediate cell lysis. Then, samples were centrifuged at 3,000 rpm for 10 min and the supernatants transferred to an opaque 96-well plate and the fluorescence was read using Gene5 2.01 Software in Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, AHSI, Milan Italy) with excitation and emission at 530 and 590 nm, respectively. Standard curves for resorufin formation were also performed. Data were normalized to total protein content, which was determined using the Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich, Milan, Italy).

2.9 Immunofluorescence

SkBr3 cells grown on a cover slip were serum deprived for 18 h and treated for 4 h with vehicle (−), 1 µM 3MC, 10 nM E2 and 100 nM G-1 alone or in combination with 1 µM AHR inhibitor CH223191 and 100 nM GPER antagonist G15. Cells were then fixed in ice-cold methanol at room temperature for 10 min, permeabilized with 0.2 % Triton X-100, washed three times with PBS, and incubated with 1% BSA in PBS at room temperature for 1 h. After washing with PBS, the cells were incubated with a rabbit primary antibody against AHR (Cell Signalling) (diluted in 1% BSA/PBS) at 4 °C for 18 h. After incubation, cells were washed three times with PBS and incubated with an Alexa fluor 594-conjugated secondary antibody (Thermofisher scientific, Milan, Italy) for 1 h at room temperature. Finally, the cells were washed three times with PBS, incubated with DAPI (40, 6-diamidino-2-phenylindole) (1:1000) for 3 min and, after washing, immunofluorescence images were obtained by Cytation 3 Cell Imaging Multimode reader (BioTek) and analysed using the software Gen5.

2.10 Co-immunoprecipitation assay

After stimulation with 1 µM 3MC, 10 nM E2 and 100 nM G-1, SkBr3 breast cancer cells were washed with PBS and lysed using 500 µl RIPA buffer with a mixture of protease inhibitors containing 1.7 mg/ml aprotinin, 1 mg/ml leupeptin, 200 mmol/liter phenylmethylsulfonyl fluoride, 200 mmol/liter sodium orthovanadate, and 100 mmol/liter sodium fluoride. Samples were then centrifuged at 13,000 rpm for 10 min, and protein concentrations were determined using Bradford reagent. Protein (250 µg) was then incubated for 2 hours with 900 µl of immunoprecipitation buffer with inhibitors, 2 µg of GPER or AHR antibody and 20 µl of Protein A/G agarose immunoprecipitation reagent

(DBA, Milan, Italy). Samples were then centrifuged at 13,000 rpm for 5 min at 4° C to pellet beads. Pellets were washed four times with 500 µl of PBS and centrifuged at 13,000 rpm for 5 min at 4° C. Supernatants were collected, resuspended in 20 µl RIPA buffer with protease inhibitors, 2X SDS sample buffer (40 mM Tris-HCl; 4% glycerol; 2% SDS) and β-mercaptoethanol and heated to 95° C for 5 min. Samples were then run on 10% SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-GPER or rabbit anti-AHR antibody. Western blot analysis and ECL detection were performed as described above.

2.11 Molecular docking

The structure of GPER was built by using GPCR-I-TASSER, which is an algorithm specifically designed to model G protein-coupled receptors [221]. The resulting conformation was a seven-helix structure, in agreement with previous predictions [222-224], with the exception of the first 50 amino acid residues in the N-terminal regions that did not make part of the helix bundle core and therefore not included in the transmembrane region. The protein conformation was refined through molecular dynamics (MD) simulations performed with the GROMACS package [225]. 3MC is a benz[a]antracene derivative characterized by a rigid polycondensed cyclic structure, which lacks any flexibility due to the absence of rotatable bonds involving non-hydrogen atoms. Classical molecular docking subsumes apolar hydrogens into the carbon atoms they are attached to, and adapts the ligand to the receptor through a search consisting in rotations around chemical bonds. As this technique may only provide a poor prediction of the binding to GPER, it was only applied to obtain starting models of the ligand/protein complex that was subsequently refined by further MD simulations. In particular, the same protocol was applied to test the binding of 3MC to GPER along with three known ligands: the agonists 17β-estradiol (E2) and G-1, the antagonist G15. AutoDock Vina [226] was used to predict the initial position of 3MC bound to GPER. Both the ligand and the protein were prepared through AutoDock Tools [227] in order to convert the structures and merge apolar hydrogens. A volume of 32 Å x 44 Å x 36 Å was identified within GPER, including any potential cavity for the ligand binding. Besides, very high exhaustiveness was employed in the roto-translation of 3MC. The best ten docking poses were clustered to reduce the number of similar binding modes (within a cut-off distance < 3 Å), resulting in four 3MC distinct initial locations. MD simulations of the molecular complexes were carried out for each starting pose by using AMBER ff99SB-ILDN force field [228] for the protein and

GAFF [229] for the ligand. Each run was carried out for 10 ns in the isobaric-isothermal ensemble in explicit water, with Cl–counterions added to obtain an overall neutral system. Other simulation conditions were as previously described for similar protein-ligand complexes [230-231]. The system was equilibrated for 2.5 ns and structures were sampled every 0.5 ns to evaluate the binding energy and the ligand location. The affinity was assessed by using the AutoDock Vina scoring function [226] without any further search (i.e. using a score-only evaluation).

2.12 Proliferation assay

Cells (1×10^5) were seeded in 24-well plates in regular growth medium, washed once they had attached and then incubated in medium containing 2.5% charcoal-stripped FBS, transfected for 24 h and then exposed to treatments. Transfection were renewed every 2 days and treatments every days. Cells were counted on day 5 using the Countess Automated Cell Counter, as recommended by the manufacturer's protocol (Life Technologies, Milan, Italy).

2.13 *In vivo* studies

Female 45-day-old athymic nude mice (nu/nu Swiss; Envigo Laboratories) were maintained in a sterile environment. At day 0, exponentially growing MDA-MB-231 cells ($2.5x10^6$ per mouse) were inoculated in mammary fat pad in 0.1 mL of Matrigel (Cultrex; Trevigen Inc.). When the tumors reached average ~0.15 cm^3 (i.e., in about 1 week), mice were randomized and divided into four groups, according to treatments administered by intramuscular (G-1) and/or subcutaneous (TMS) injection for 21 days. The first group of mice (n = 7) was treated daily with vehicle (0.9% NaCl with 0.1% albumin and 0.1% Tween-20; Sigma-Aldrich), the second group of mice (n = 7) was treated daily with G-1 (0.5 mg/kg/die), the third group of mice (n = 7) was treated daily with TMS (0.3 mg/kg/die), and the fourth group of mice (n = 7) was treated daily with G-1 in combination with TMS (the concentrations were similar to those described above). G-1 and TMS were dissolved in DMSO at 1 mg/mL. MDA-MB-231 xenograft tumor growth was evaluated twice a week by caliper measurements, along two orthogonal axes: length (L) and width (W). Tumor volumes (in cubic centimeters) were estimated by the following formula: TV=L × (W2)/2. After 21 days of treatment, the animals were killed following the standard protocols and tumors were dissected from the neighboring connective tissue. Specimens of tumors were frozen in nitrogen and stored at –80 °C; the remaining tumor tissues of each

sample were fixed in 4% paraformaldehyde and embedded in paraffin for the histologic analyses. Animal experiments were conducted according to Italian law (D.L. 26/2014), the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (2011), and the Directive 2010/63/EU of the European Parliament on the protection of animals used for Scientific research. The Italian Ministry of Health, Rome (authorization n. 199/2015-PR), approved the animal research project.

2.14 Immunohistochemistry

Paraffin embedded sections, 5 μm thick, were mounted on slides precoated with poly-lysine, and then they were deparaffinized and dehydrated (7-8 serial sections). Immunohistochemical experiments were performed after heat-mediated antigen retrieval. Hydrogen peroxide (3% in distilled water) was used, for 30 min, to inhibit endogenous peroxidase activity while normal goat serum (10%) was utilized, for 30 min, to block the non-specific binding sites. Immunodetection was carried out using anti-Ki67 and cyclin D1 (1:100) (DAKO, Denmark), cyclin E (1:200) (Bethyl Laboratories, Texas, USA) and cyclin A (1:50) (Abcam, Euroclone, Milan, Italy) primary antibodies at 4°C overnight. Then, a universal biotinylated IgG was applied (1:600) for 1 hour at RT, followed by ABC/HRP. Immunoreactivity was visualized by using DAB. The negative controls were made with DAKO mouse IGg1 (cod.X0931) for Ki67, DAKO immunoglobulin fraction (cod.X0936) for cyclin D1 at the same concentration of primary antibodies, rabbit serum at 5% for cyclin E and cyclin A. Sections were also counterstained with hematoxylin. Six-seven serial sections were processed for each sample from two independent operators.

2.15 Imaging

Tissue samples were visualized using an OLYMPUS BX41 microscope (Olympus Europa, Germany) and the images were taken with CSV1.14 software using a CAM XC-30 for images acquisition.

2.16 3D Cell growth assay

The SkBr3 cells were plated in single-cell suspension in 2% agar–coated plates. To generate three-dimensional spheroids, the plates were rotated for 4 h at 37 °C. Cells were treated and medium partially renewed every 2 days. After 3 weeks, spherical formation containing >10 cells was scored under a light microscope. The experiments were replicated

2 times, and a representative set of data was photographed. Cell number was determined, after trypsinization of spheroids, by direct cell counting at 20 days of treatment.

2.17 Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman-Keuls' testing to determine differences in means. Statistical comparisons for in vivo studies were made using the Wilcoxon–Mann–Whitney test. $P < 0.05$ was considered statistically significant.

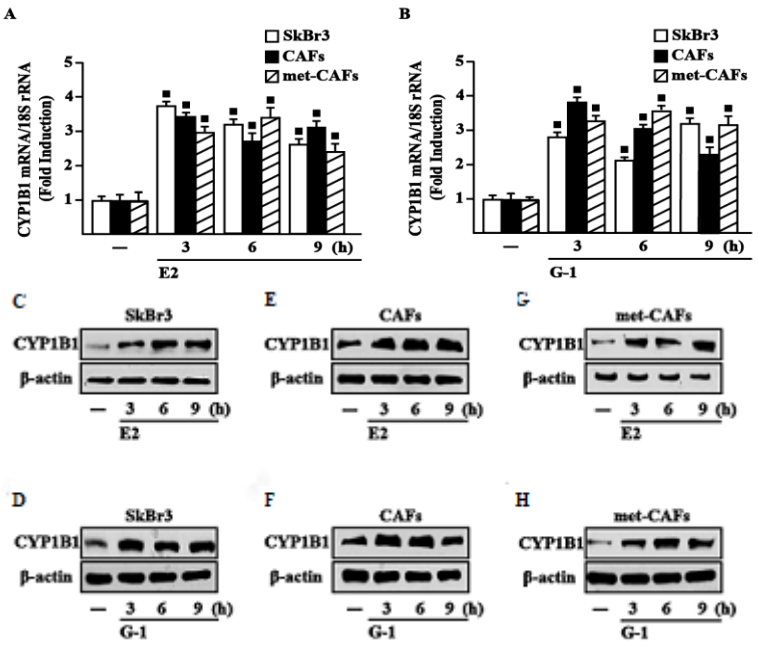
Chapter 3

Results

Results

3.1 E2 and G-1 induce CYP1B1 expression through GPER-mediated signalling.

Previous studies ascertained that estrogens up-regulate CYP1B1 levels through ERα in diverse cancer cells [19], therefore, we asked whether estrogens might trigger CYP1B1 expression through GPER in an ER-independent manner. Of note, E2 and the selective GPER agonist G-1 induced CYP1B1 mRNA (Fig. 1A-B) and protein levels (Fig. 1C-H) in cell contexts lacking ER but expressing GPER as SkBr3 breast cancer cells, CAFs and met-CAFs. Next, the silencing of GPER expression abrogated the CYP1B1 protein induction by E2 and G-1 in SkBr3 cells (Fig. 1I-J), CAFs (Fig. 1M-N) and met-CAFs (Fig. 1Q-R). In addition, we found that the EGFR inhibitor AG1478 (AG) and the MEK inhibitor PD98059 (PD) abrogate the increased expression of CYP1B1 upon E2 and G-1 treatments in SkBr3 cells (Fig. 1K-L), CAFs (Fig. 1O-P) and met-CAFs (Fig. 1S-T). Taken together, these findings suggest that the GPER/EGFR/ERK transduction pathway is involved in CYP1B1 expression upon exposure to E2 and G-1 in our model systems.



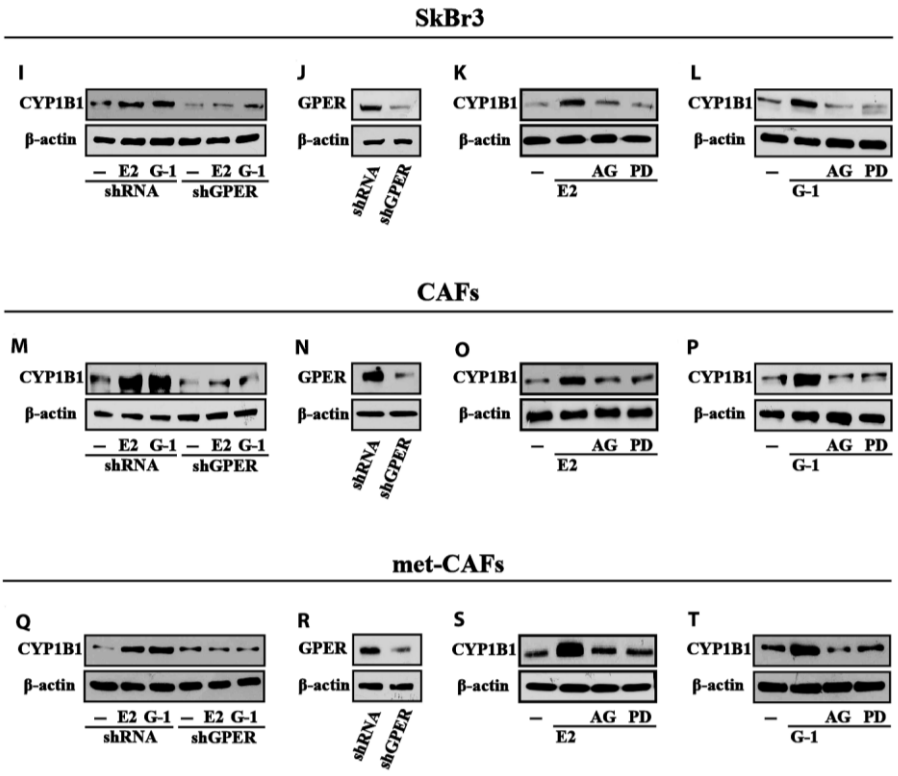
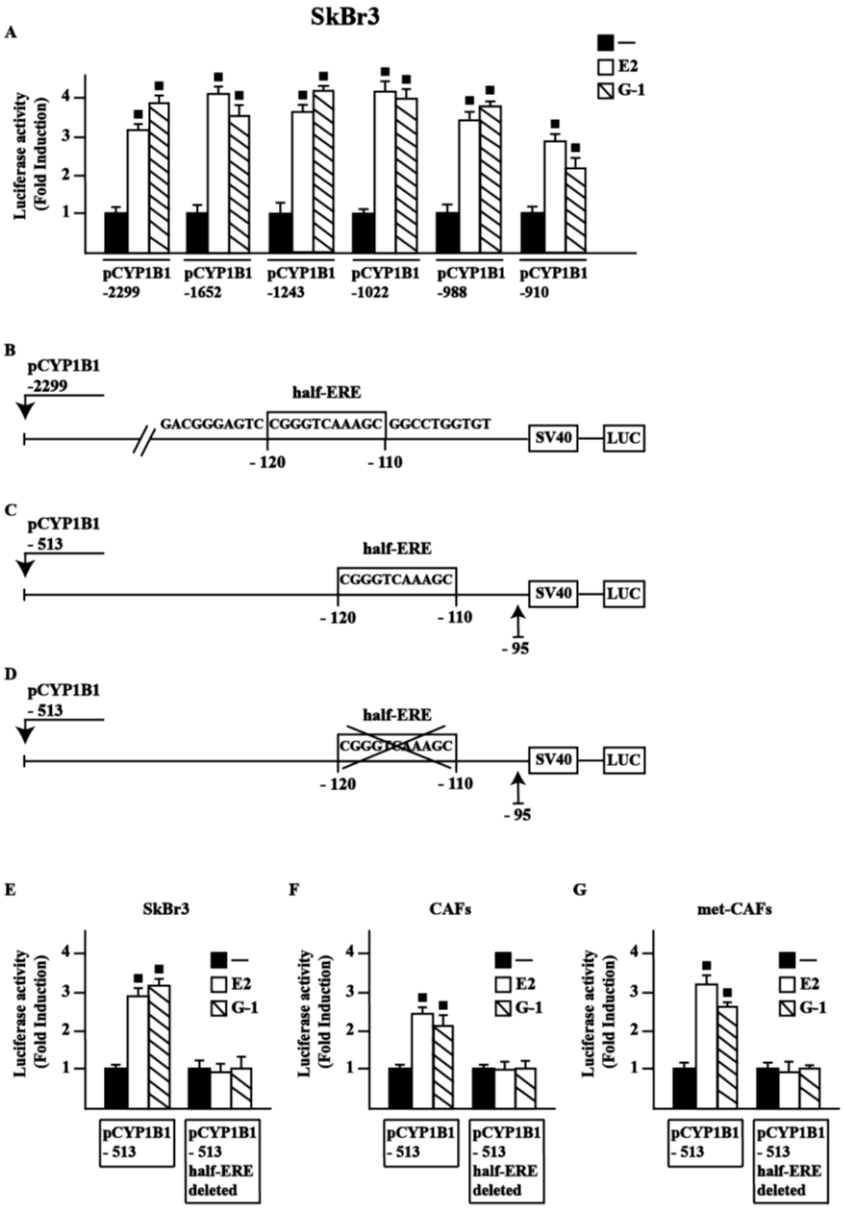


Figure 1. ***GPER mediates CYP1B1 induction by E2 and G-1 in SkBr3 cells, CAFs and met-CAFs.*** E2 (10 nM) (A) and G-1 (100 nM) (B) induce the mRNA expression of CYP1B1, as indicated. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments with E2 and G-1 respect to cells treated with vehicle (−). (■) $P < 0.05$ for cells receiving treatments versus vehicle. Evaluation of CYP1B1 protein levels in SkBr3 cells, CAFs and met-CAFs treated with 10 nM E2 (C, E, G) and 100 nM G-1 (D, F, H), as indicated. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. The up-regulation of CYP1B1 protein levels induced by 10 nM E2 and 100 nM G-1 is abrogated in SkBr3 cells (I), CAFs (M) and met-CAFs (Q) transfected for 24 h with shRNA or shGPER and then treated for 6 h with vehicle (−), 10 nM E2 and 100 nM G-1. (J, N, R) Efficacy of GPER silencing. Evaluation of CYP1B1 protein levels in SkBR3 cells (K-L), CAFs (O-P) and met-CAFs (S-T) upon treatment for 6 h with vehicle, 10 nM E2 and 100 nM G-1 alone or in combination with 1 μM EGFR inhibitor AG1478 (AG) or 10 μM MEK inhibitor PD98059 (PD). β-actin serves as a loading control. Results shown are representative of at least two independent experiments

3.2 A half-ERE site is required for CYP1B1 transcription by E2 and G-1.

In order to provide novel insights into the transcriptional activation of CYP1B1 by E2 and G-1, we first ascertained that E2 and G-1 stimulate the luciferase activity of diverse CYP1B1 promoter deletion constructs in SkBr3 cells (Fig. 2A), CAFs and met-CAFs (data not shown). Among other sequences, we focused on a half-ERE site [232-233] located from -120 to -110 respect to the transcription initiation site (TIS) of the CYP1B1 promoter (Fig. 2B). By site-directed mutagenesis, we generated two further deleted CYP1B1 promoter constructs containing (Fig. 2C) or lacking (Fig. 2D) the half-ERE site. Worthy, E2 and G-1 stimulated the luciferase activity only transfecting in SkBr3 cells (Fig. 2E), CAFs (Fig. 2F) and met-CAFs (Fig. 2G) the plasmid containing the half-ERE site, hence suggesting that this site is involved in CYP1B1 transcription upon treatment with ligands used (see below). Thereafter, the luciferase activity of representative CYP1B1 promoter constructs induced by E2 and G-1 was no longer evident silencing GPER expression or in the presence of the EGFR inhibitor AG1478 (AG) and the MEK inhibitor PD98059 (PD) in SkBr3 cells (Fig. 2H-I), both CAFs and met-CAFs (data not shown), in accordance with the results shown in figure 1. Collectively, these results indicate that E2 and G-1 regulate CYP1B1 transcription through the GPER/EGFR/ERK transduction pathway.


SkBr3
A
—
E2
G-1
Luciferase activity
(Fold Induction)
pCYP1B1
-2299
pCYP1B1
-1652
pCYP1B1
-1243
pCYP1B1
-1022
pCYP1B1
-988
pCYP1B1
-910
B
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-2299
half-ERE
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- 110
C
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LUC
- 120
- 110
- 95
D
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SV40
LUC
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- 110
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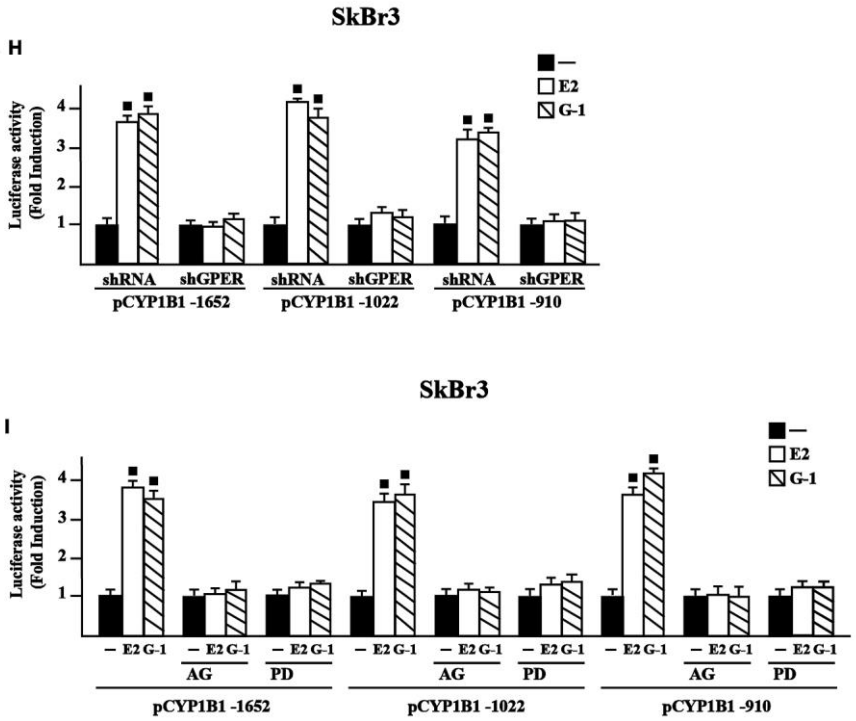
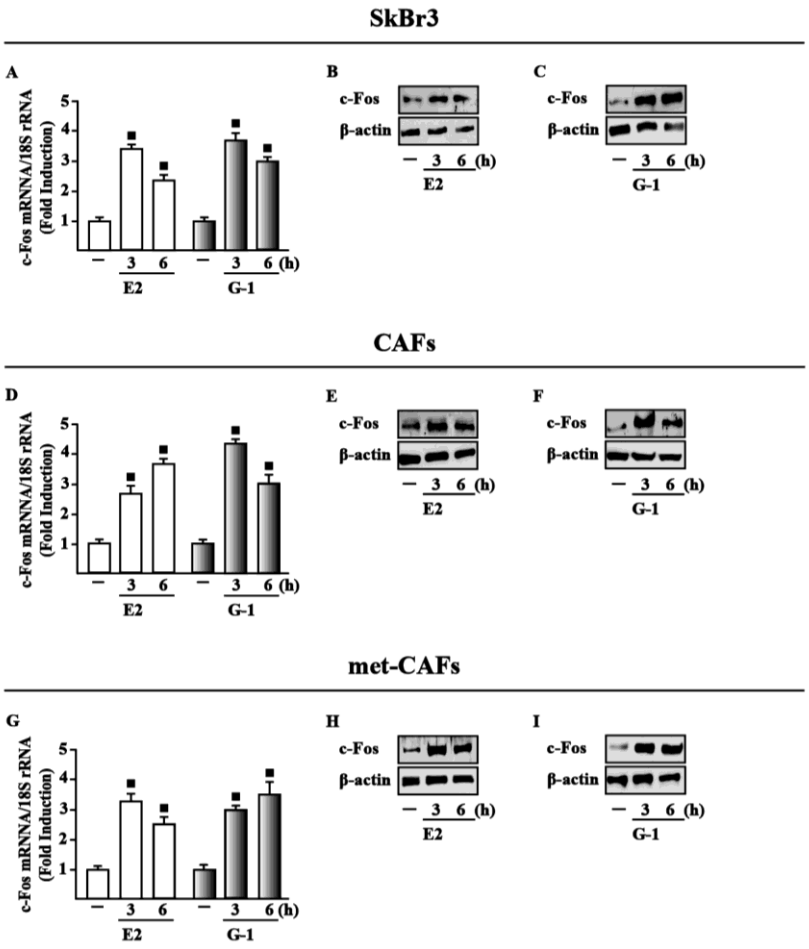


Figure 2. ***E2 and G-1-stimulate the transcriptional activation of CYP1B1 promoter constructs.*** (A) SkBr3 cells were transiently transfected for 8 h with the indicated CYP1B1 promoter constructs, then cells were treated for 18 h with vehicle (−), 10 nM E2 or 100 nM G-1. Schematic representation of the CYP1B1 5'-flanking region containing a half-ERE binding motif (B), a deletion construct containing a half-ERE binding motif (C) and a deletion construct lacking a half-ERE binding motif (D), as indicated. SkBr3 cells (E), CAFs (F) and met-CAFs (G) were transiently transfected for 8 h with the deleted CYP1B1 promoter constructs shown in panels C and D, then treated for 18 h with vehicle, 10 nM E2 and 100 nM G-1, as indicated. (H) SkBr3 cells were transfected for 24 h with shRNA or shGPER, then transfected for 8 h with CYP1B1 promoter constructs and thereafter treated for 18 h with vehicle (−), 10 nM E2 and 100 nM G-1, as indicated. (I) Luciferase activities of CYP1B1 promoter constructs in SkBr3 cells treated for 18 h with vehicle, 10 nM E2, 100 nM G-1 alone or in combination with 10 μM EGFR inhibitor AG1478 (AG) and 10 μM MEK inhibitor PD98059 (PD), as indicated. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold induction upon which the activities induced by treatments were calculated. Each column represents the mean ± SD for three independent experiments, each performed in triplicate. (■) indicates $P < 0.05$ for cells receiving treatments versus vehicle.

3.3 c-Fos is involved in CYP1B1 expression by E2 and G-1.

In order to further assess the transduction mechanisms leading to the CYP1B1 expression, we ascertained that E2 and G-1 trigger c-Fos expression at both mRNA and protein levels in SkBr3 cells (Fig. 3A-C), CAFs (Fig. 3D-F) and met-CAFs (Fig. 3G-I), according to our previous studies [234]. Considering that a half-ERE sequence may differ in only one nucleotide from a canonical AP1 binding site [232-233], we then established that E2 and

G-1 trigger the recruitment of c-Fos to the half-ERE site located within the CYP1B1 promoter in SkBr3 cells (Fig. 3J), CAFs and met-CAFs (data not shown), however this response was no longer evident transfecting the DN/c-Fos construct in SkBr3 cells (Fig. 3K), CAFs and met-CAFs (data not shown). Further supporting these findings, the up-regulation of CYP1B1 protein levels and the transactivation of a representative CYP1B1 construct induced by E2 and G-1 was prevented transfecting SkBr3 cells, CAFs and met-CAFs with the DN/c-Fos (Fig. 3L-Q). Taken together, these data indicate that c-Fos is involved in the regulation of CYP1B1 by E2 and G-1.



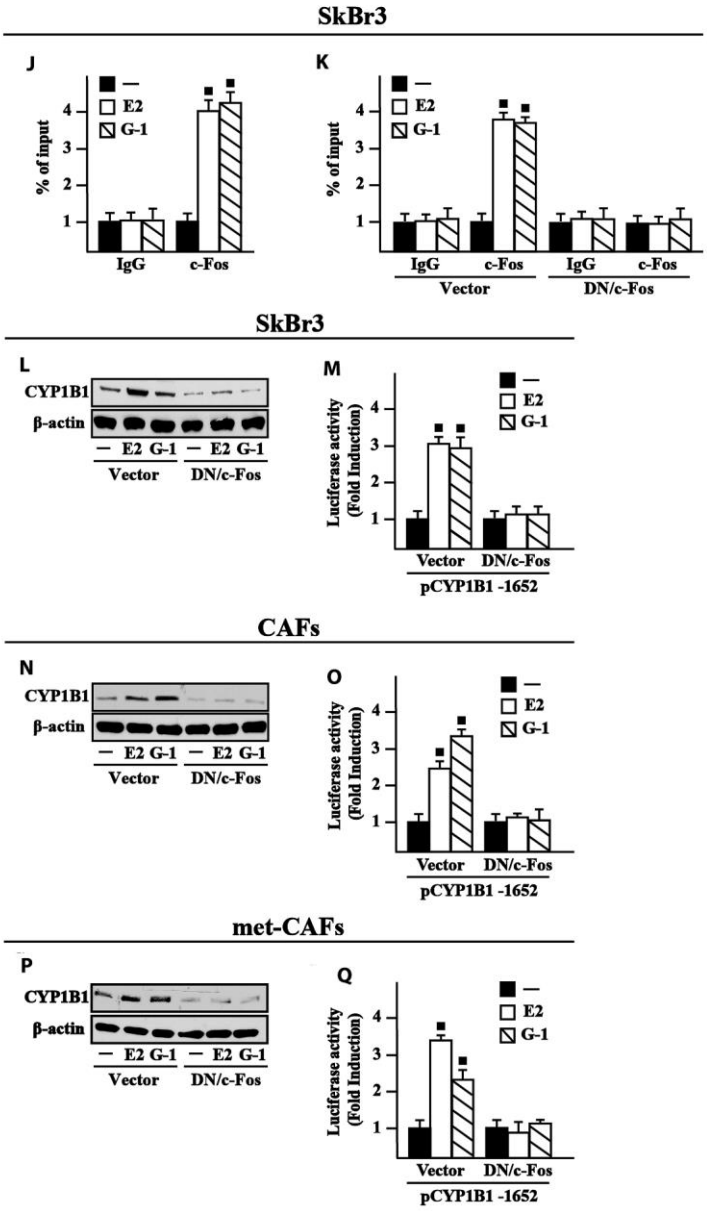


Figure 3. ***c-Fos is involved in the up-regulation of CYP1B1 by E2 and G-1 in SkBr3 cells, CAFs and met-CAFs.*** c-Fos mRNA expression by real-time PCR in SkBr3 cells (A**)**, CAFs (D) and met-CAFs (G) treated with vehicle (−), 10 nM E2 and 100 nM G-1, as indicated. Data obtained in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon E2 and G-1 treatments respect to cells treated with vehicle. (■) $P < 0.05$ for cells receiving treatments versus vehicle. c-Fos protein levels in SkBr3 cells (B-C), CAFs (E-F), met-CAFs (H-I) treated with vehicle, 10 nM E2 and 100 nM G-1, as indicated. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (J) Recruitment of c-Fos induced by 10 nM E2 and 100 nM G-1 to the half-ERE site located within the CYP1B1 promoter sequence in SkBr3 cells. In control samples non-specific IgG was used instead of the primary antibody. (K) SkBr3 cells were transfected for 18 h with a vector or a construct encoding for a dominant negative form of c-Fos (DN/c-Fos), then treated for 3 h with vehicle (−), 10 nM E2 and 100 nM G-1 and thereafter submitted to the chromatin immunoprecipitation

procedure using anti-c-Fos or nonspecific anti-IgG antibodies. The amplified sequences were evaluated by real-time PCR. CYP1B1 protein levels in SkBr3 cells (L), CAFs (N) and met-CAFs (P) transfected for 18 h with a vector or DN/c-Fos and then treated for 6 h with vehicle, 10 nM E2 and 100 nM G-1, as indicated. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. SkBr3 cells (M), CAFs (O) and met- CAFs (Q) were transfected for 18 h with a CYP1B1construct, a vector or DN/c-Fos and then treated for 18 h with vehicle, 10 nM E2 and 100 nM G-1. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold induction upon which the activities induced by treatments were calculated.

3.4 CYP1B1 activity is stimulated by E2 and G-1.

Previous investigations have suggested that an increased expression of CYP1B1 leads to its enhanced enzymatic activity in cancer cells [12, 235-236]. Therefore, we assessed that a treatment for 18 h with E2 and G-1 stimulate CYP1B1 activity in SkBr3 cells (Fig. 4A), CAFs (Fig. 4B) and met-CAFs (Fig. 4C), as evaluated by EROD assay. Accordingly, we found that the selective CYP1B1 inhibitor named tetramethoxystilbene (TMS) abolishes the CYP1B1 enzymatic activity induced by E2 and G-1 (Fig. 4A-C), thus suggesting its usefulness toward the evaluation of CYP1B1 involvement in certain biological responses (see below).

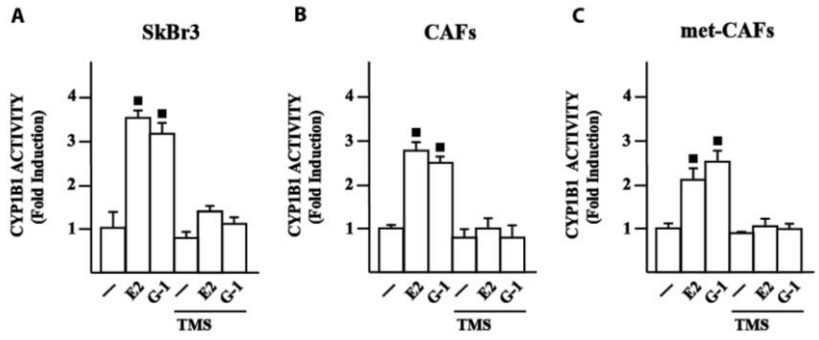


Figure 4. ***E2 and G-1 stimulate CYP1B1 activity in SkBr3 cells, CAFs and met-CAFs.*** CYP1B1 activity evaluated by EROD assay in SkBr3 cells (A), CAFs (B) and met-CAFs (C) treated for 18 h with vehicle (−), 10 nM E2 and 100 nM G-1 alone or in combination with 5 μM CYP1B1 inhibitor TMS. Fluorescence values of cells receiving vehicle were set as 1-fold induction upon which values induced by treatments were calculated. Each column represents the mean ± SD for three independent experiments, each performed in triplicate. (■) indicates $P < 0.05$ for cells receiving treatments versus vehicle (−).

3.5 GPER and CYP1B1 are involved in the up-regulation of growth regulatory genes by E2 and G-1.

Estrogenic GPER signalling has been shown to trigger relevant effects in cancer cells as well as in CAFs through the induction of growth regulators like cyclins [51, 237-238]. Accordingly, we found that E2 and G-1 stimulate the expression of cyclin D1, cyclin E and cyclin A at both mRNA and protein levels in SkBr3 cells, CAFs and met-CAFs, however these responses were abrogated using the GPER antagonist G15 as well as in the presence of the CYP1B1 inhibitor TMS (Fig. 5). Nicely fitting with these findings, the proliferative effects elicited by E2 and G-1 in SkBr3 cells, CAFs and met-CAFs were prevented silencing GPER or CYP1B1 as well as in the presence of the GPER and CYP1B1 inhibitors, G15 and TMS, respectively (Fig. 6). Taken together, these results suggest that both GPER and CYP1B1 contribute to the growth responses prompted by E2 and G-1 in our model systems.

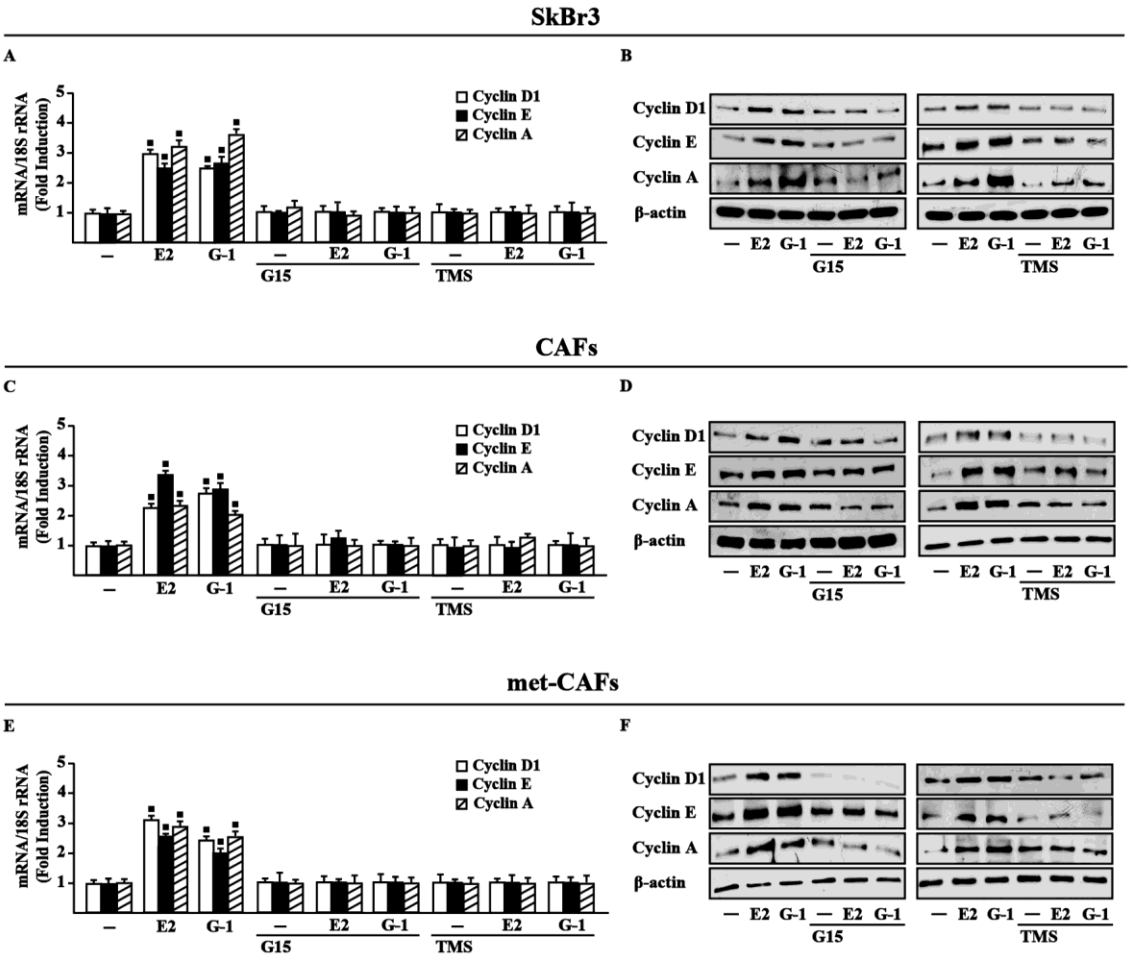


Figure 5. ***GPER and CYP1B1 mediate the up-regulation of cyclin D1, cyclin E and cyclin A by E2 and G-1 in SkBr3 cells, CAFs and met-CAFs.*** Cyclin D1, cyclin E and cyclin A mRNA expression in SkBr3 cells (A), CAFs (C) and met-CAFs (E) treated for 18 h with vehicle (−), E2 (10 nM) and G-1 (100 nM) alone or in combination with 100 nM GPER antagonist G15 and 5 μM CYP1B1 inhibitor TMS, as evaluated by real-time PCR. Data obtained in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes upon E2 and G-1 treatments respect to cells treated with vehicle. (■) $P < 0.05$ for cells receiving treatments versus vehicle. Cyclin D1, cyclin E and cyclin A protein levels in SkBr3 cells (B), CAFs (D) and met-CAFs (F) upon treatments for 18 h with vehicle (−), E2 (10 nM) and G-1 (100 nM) alone or in combination with 100 nM GPER antagonist G15 and 5 μM CYP1B1 inhibitor TMS. β-actin serves as a loading control. Results shown are representative of at least two independent experiments.

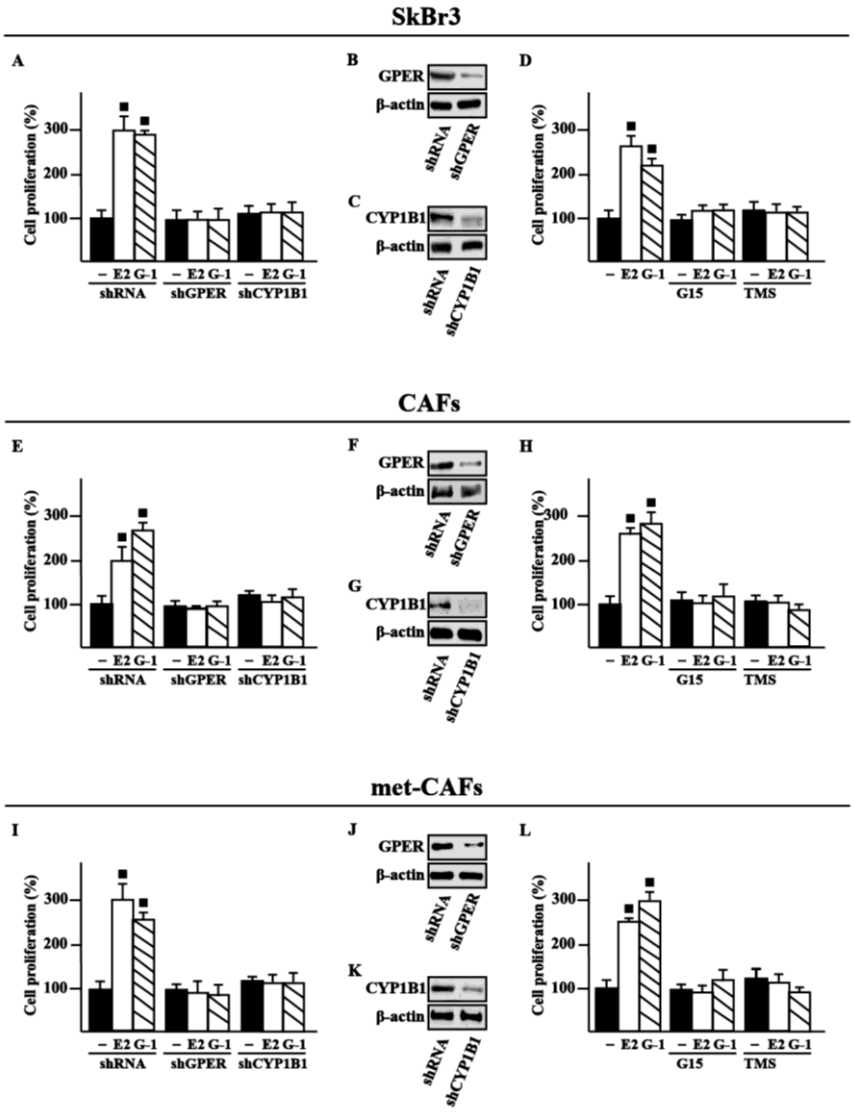


Figure 6. ***GPER and CYP1B1 are involved in the proliferative effects induced by E2 and G1 in SkBr3 cells, CAFs and met-CAFs.*** The proliferation of SkBr3 cells (A), CAFs (E) and met-CAFs (I) induced by 10 nM E2 or 100 nM G-1 is prevented silencing GPER or CYP1B1 expression. Cells were transfected every 2 days with shRNA, shGPER or shCYP1B1, treated every day with ligands and then counted on day 5. Efficacy of GPER (B, F, J) and CYP1B1 (C, G, K) silencing. β-actin serves as a loading control. The proliferation of SkBr3 cells (D), CAFs (H) and met-CAFs (L) induced by 10 nM E2 or 100 nM G-1 is prevented by 100 nM GPER antagonist G15 and 1 μM CYP1B1 inhibitor TMS. Proliferation of cells treated with vehicle (−) was set as 100% upon which cell growth induced by treatments was calculated. Each data point is the mean ± SD of three independent experiments performed in triplicate. (■) $P < 0.05$ for cells receiving treatments versus vehicle.

3.6 GPER and CYP1B1 are involved in the growth effects triggered by E2 and G-1 in breast cancer xenografts.

In order to strengthen the aforementioned observations we turned to the high metastatic and invasive MDA-MB-231 breast cancer cells [239] that were used both *in vitro* and *in vivo* studies. First, we determined that E2 and G-1 induce CYP1B1 expression at both mRNA (Fig. 7A) and protein levels though GPER (Fig. 7B-E) also in these cells. Corroborating the results obtained in SkBr3 cells, CAFs and met-CAFs, we thereafter ascertained that E2 and G-1 stimulate the luciferase activity of diverse CYP1B1 promoter constructs (Fig. 7F) except for the half-ERE deleted plasmid (Fig. 7G). Likewise, we found that E2 and G-1 up-regulate the expression of cyclin D1, cyclin E and cyclin A in MDA-MB-231 cells, however these responses were no longer evident silencing GPER (Fig. 7H-I) or using the GPER antagonist G15 (Fig. 7J) and the CYP1B1 inhibitor TMS (Fig. 7K). Recapitulating the abovementioned findings, E2 and G-1 promoted the proliferation of MDA-MB-231 cells through GPER and CYP1B1, as ascertained silencing their expression (Fig. 7L-N) and using G15 or TMS (Fig. 7O). Then, in order to evaluate the role of CYP1B1 on tumor growth *in vivo*, 45-day-old female nude mice were injected with MDA-MB-231 cells into the mammary fat pad region and treated with vehicle, G-1 and TMS alone or in combination. These treatments were well tolerated as no change in body weight and in food or water consumption were observed together with no evidence of reduced motor function. Among the different groups of mice, no significant difference was assessed after the sacrifice in the mean weights or histologic features of the major organs (liver, lung, spleen and kidney), thus indicating a lack of toxic effects. Of note, TMS treatment prevented the tumor growth induced by G-1 (Fig. 8A-B) and the up-regulation of cyclin protein levels in tumor homogenates (Fig. 8C). In addition, an increased expression of the proliferative marker Ki67, together with that of cyclin D1, cyclin E and cyclin A was found in tumor tissue sections obtained from G-1 treated mice with respect to those treated with vehicle (Fig. 8D). Worthy, these effects were prevented in the group of animals receiving G-1 in combination with TMS (Fig. 8D). Overall, these data suggest that GPER and CYP1B1 are involved in the stimulatory effects exerted by E2 and G-1 in MDA-MB-231 breast cancer cells both *in vitro* and *in vivo*.

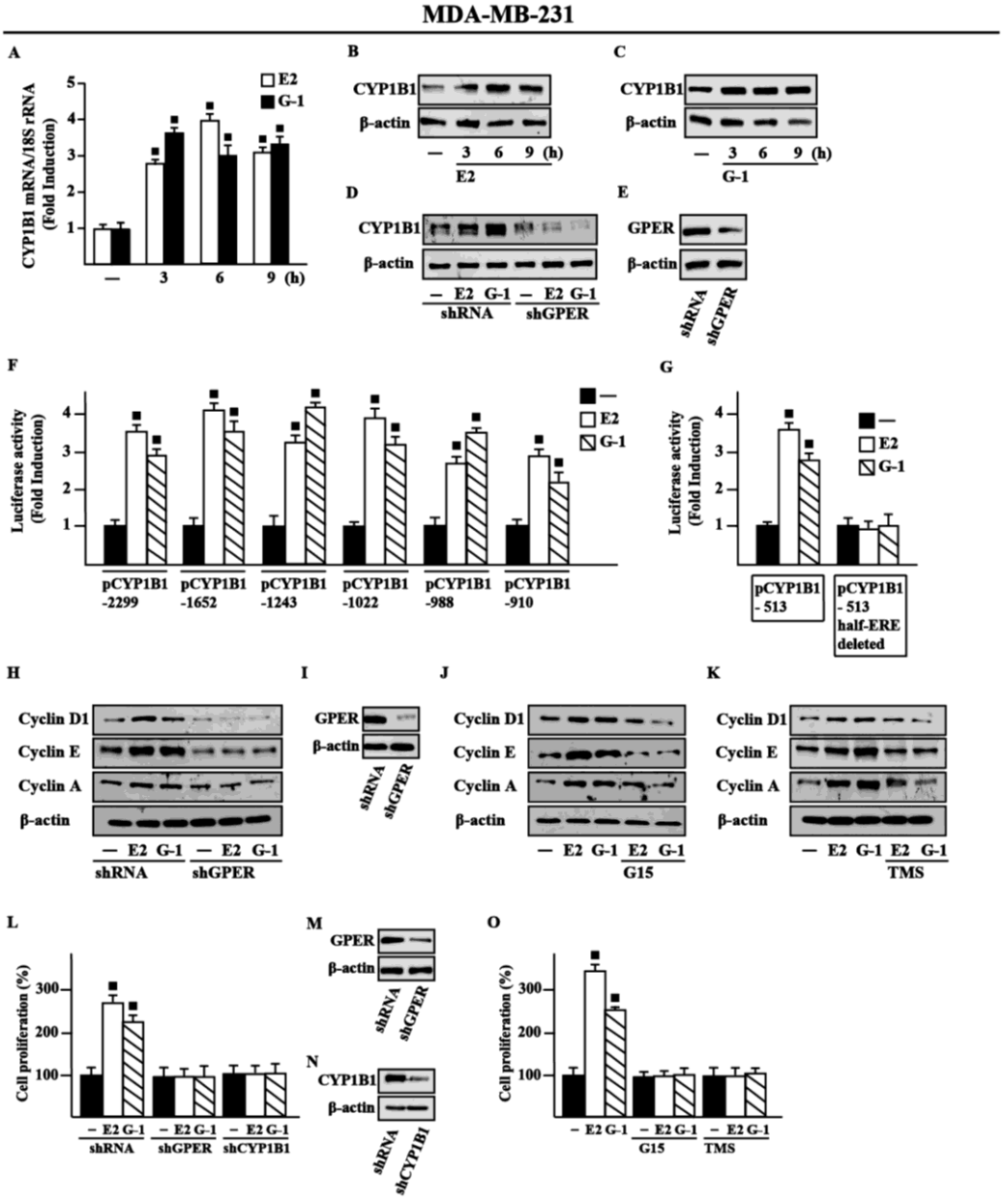


Figure 7. ***E2 and G-1 induce CYP1B1 expression through GPER in MDA-MB-231 breast cancer cells.*** (A) E2 (10 nM) and G-1 (100 nM) induce CYP1B1 mRNA expression in MDA-MB-231 cells, as evaluated by real-time PCR. Data obtained in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes upon E2 and G-1 treatments respect to cells exposed to vehicle (−). (B-C) CYP1B1 protein levels in MDA-MB-231 cells treated with 10 nM E2 and 100 nM G-1, as indicated. (D) CYP1B1 protein levels upon treatments with 10 nM E2 and 100 nM G-1 in cells transfected with shRNA or shGPER. (E) Efficacy of GPER silencing. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (F) Cells were transiently transfected for 8 h with the indicated CYP1B1 promoter constructs, then cells were treated for 18 h with vehicle (−), 10 nM E2 or 100 nM G-1. (G) Cells were transiently transfected for 8 h with the deleted CYP1B1 promoter constructs shown in figure 2C and 2D, and then treated for 18 h with vehicle, 10 nM E2 and 100 nM G-1, as indicated. The luciferase activities were normalized to the internal transfection control and values of cells receiving

vehicle were set as 1-fold induction upon which the activities induced by treatments were calculated. Each column represents the mean ± SD for three independent experiments, each performed in triplicate. (H) Cyclin D1, cyclin E and cyclin A protein levels in cells transiently transfected with shRNA or shGPER for 24 h, then treated for 18 h with vehicle, 10 nM E2 or 100 nM G-1. (I) Efficacy of GPER silencing. Cyclin D1, cyclin E and cyclin A protein levels in cells treated for 18 h with vehicle, 10 nM E2 and 100 nM G-1 alone or in combination with 100 nM GPER antagonist G15 (J) and 5 μM CYP1B1 inhibitor TMS (K). β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (L) Cell proliferation induced by 10 nM E2 or 100 nM G-1 is prevented silencing GPER or CYP1B1 expression. Cells were transfected every 2 days with shRNA, shGPER or shCYP1B1, treated every day with ligands and then counted on day 5. Efficacy of GPER (M) and CYP1B1 (N) silencing. β-actin serves as a loading control. (O) Cell proliferation induced by 10 nM E2 or 100 nM G-1 is prevented by 100 nM GPER antagonist G15 and 1 μM CYP1B1 inhibitor TMS. Proliferation of cells treated with vehicle was set as 100% upon which cell growth induced by treatments was calculated. Each data point is the mean ± SD of three independent experiments performed in triplicate. (■) $P < 0.05$ for cells receiving treatments versus vehicle.

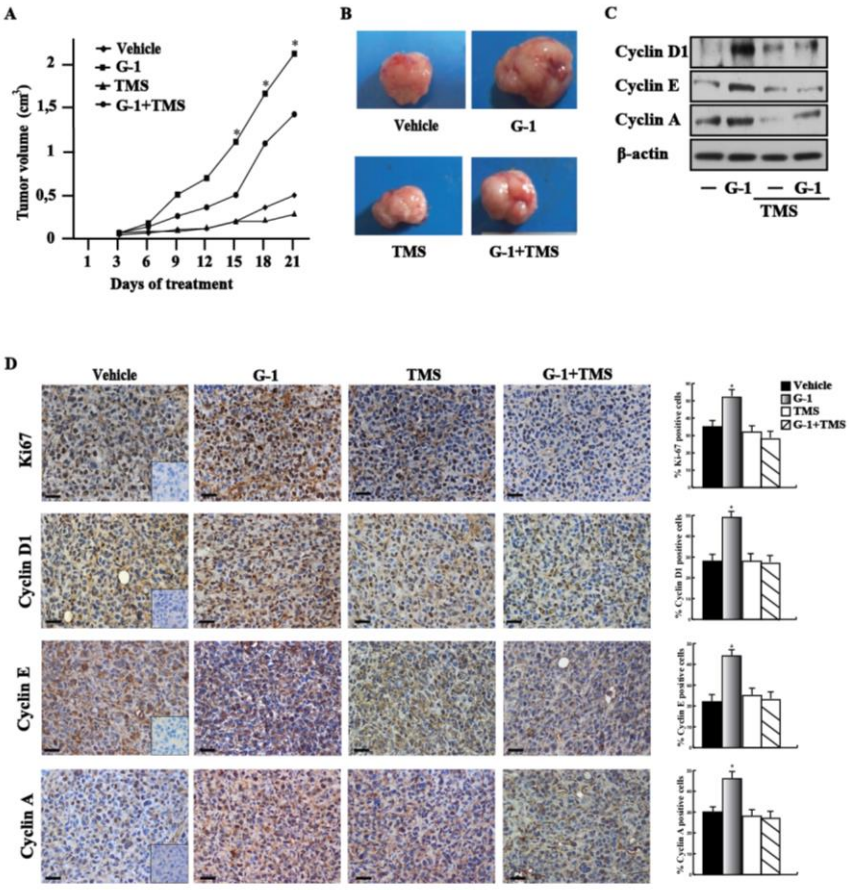


Figure 8. ***CYP1B1 is involved in the growth of MDA-MB-231 xenografts.*** (A) Tumor volume from MDA-MB-231 xenografts implanted in female athymic nude mice treated for 21 days with vehicle, G-1, TMS or both compounds, as indicated. (*) indicates $P < 0.05$ for animals treated with G-1 *versus* animals treated with

vehicle. (B) Representative images of explanted tumors at day 21, scale bar, 0.3 cm. (C) Cyclin D1, cyclin E, cyclin A protein levels in tumor homogenates from MDA-MB-231 xenografts treated as reported above. β-actin serves as loading control. Results shown are representative of two independent experiments. (D) Ki67, cyclin D1, cyclin E and cyclin A immunodetection in paraffin embedded sections of explanted tumors from breast cancer xenografts treated with vehicle, G-1 and TMS alone or in combination, as indicated. Scale bar: 25 μm. Insert negative control. Histograms represent the percentage (± SD) of immunostained positive cells treated with G-1 and TMS alone or in combination versus vehicle treated cells. (*) indicates $P < 0.05$.

3.7 3MC induces CYP1B1 expression through both AHR and GPER.

On the basis of the results described above and considering previous studies showing that 3MC binds to ERα and induces CYP1B1 expression in cancer cells through AHR [8, 18, 171, 240-241], we first evaluated whether this chemical may stimulate CYP1B1 expression also through GPER. Worthy, 3MC induced the mRNA (Fig. 9A) and protein levels (Fig. 9B-G) of CYP1B1 in cell contexts lacking ER but expressing GPER as SkBr3 breast cancer cells and CAFs. Moreover, CYP1B1 protein expression induced by 3MC was abolished using the selective inhibitors of AHR and GPER namely CH223191 and G15, respectively (Fig. 9B, E), as well as silencing GPER expression (Fig. 9C-D, F-G). Likewise, these inhibitors prevented the transactivation of three distinct CYP1B1 promoter deletion constructs triggered by 3MC in SkBr3 cells and CAFs (Fig. 9H). Overall, these data suggest that both AHR and GPER mediate the up-regulation of CYP1B1 upon 3MC exposure in breast cancer cells and CAFs.

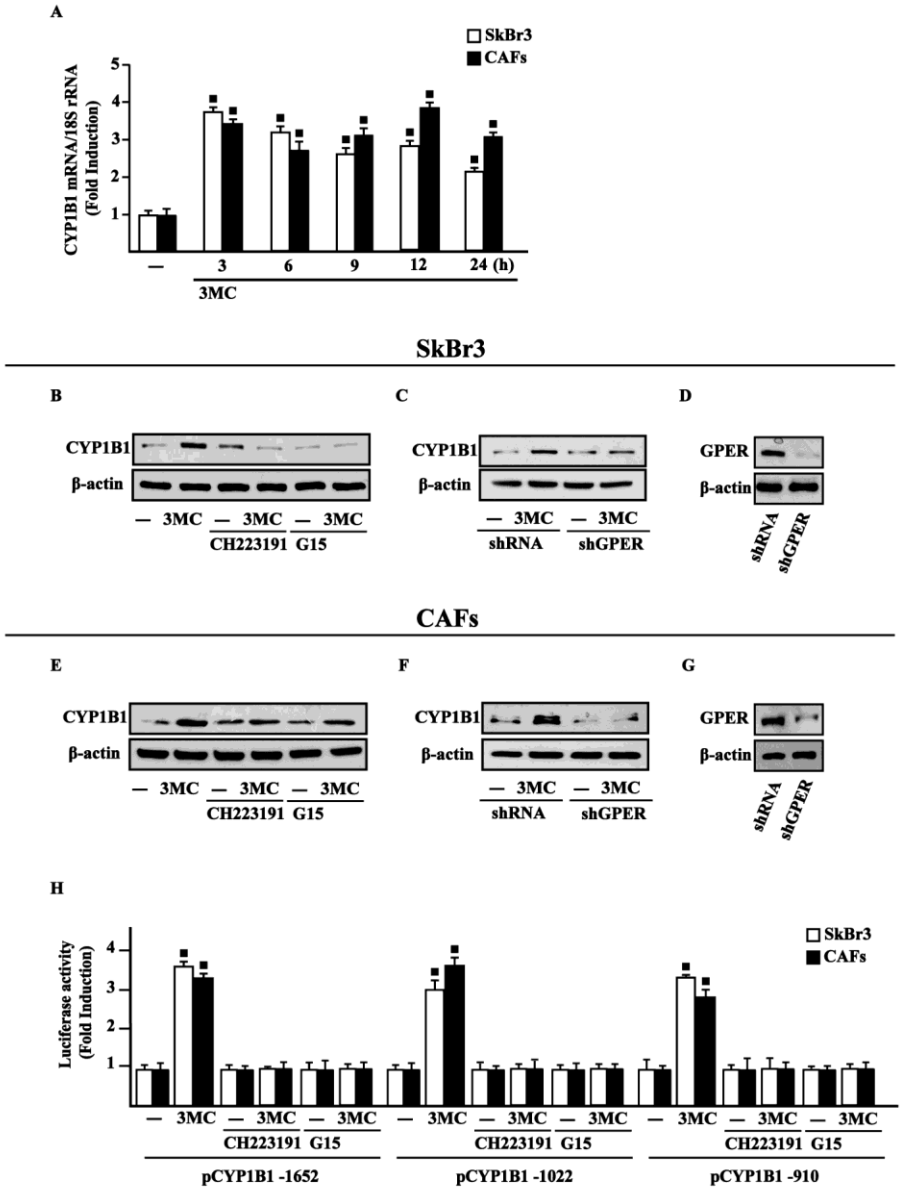


Figure 9. ***AHR and GPER are involved in CYP1B1 induction by 3MC in SkBr3 cells and CAFs.*** (A) 3MC (1μM) induce the mRNA expression of CYP1B1 in SkBr3 cells and CAFs, as indicated. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments with 3MC respect to cells treated with vehicle (−). Evaluation of CYP1B1 protein levels in SkBr3 cells (B) and CAFs (E) upon treatment for 6 h with vehicle (−), 1 μM 3MC alone and in combination with 1 μM AHR inhibitor CH223191 or 100 nM GPER antagonist G15. The up-regulation of CYP1B1 protein levels induced by 3MC is abrogated in SkBr3 cells (C) and CAFs (F) transfected for 24 h with shGPER and then treated for 6 h with vehicle and 1 μM 3MC. (D, G) Efficacy of GPER silencing. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (H) Luciferase activities of CYP1B1 promoter constructs in SkBr3 cells and CAFs treated for 18 h with vehicle and 1 μM 3MC alone and in combination with 1 μM AHR inhibitor CH223191 or 100 nM GPER antagonist G15, as indicated. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold

induction upon which the activities induced by treatments were calculated. Each column represents the mean ± SD of three independent experiments, each performed in triplicate. (■) indicates $P < 0.05$ for cells receiving treatments versus vehicle.

3.8 Interaction between GPER and 3MC.

On the basis of the aforementioned findings regarding the regulation of CYP1B1 expression by 3MC via GPER and considering the ability of 3MC to bind to ERα [240-241], we aimed to provide insights into the potential of this chemical to interact also with GPER. Docking calculations to predict the 3MC-GPER complex evidenced binding modes with affinity scores ranging between -7.8 and -6.9 kcal/mol. Four distinct poses were obtained after clustering the docking poses (Figure 10), which were simulated in distinct molecular dynamics (MD) runs. The binding affinity was estimated on the equilibrated system in order to allow the ligand to adapt within the protein cavity emerging from the transmembrane region. Sampling of the complex led to the evaluation of the binding energy for 16 structures of the 3MC/GPER complex for each run. A number of observations can be drawn from these data. First, there is no clear correlation between the binding scores obtained in the docking calculations and the ones estimated from the MD simulations. This indicates that the sole use of molecular docking to assess the binding energy of 3MC to GPER gives poor predictions of the affinity, as it could be expected for such a rigid ligand. Second, the most favorable binding modes appraised during the MD simulations show in general a higher affinity toward the receptor compared to the corresponding docking poses (with the exception of simulation S-3; see also below). This observation further suggests to take into account the dynamics of the protein matrix to provide the ligand accommodation. The average value obtained in simulation provides the most accurate prediction of the binding affinity of 3MC towards GPER. The scores calculated are consistent with the binding of 3MC in the pocket of GPER with good affinities (up to -8.3 ± 1.0 kcal/mol), which would correspond to dissociation constants in the low micromolar range. The only exception was obtained in the simulation S-3, which reproduced at most a weak binding location of 3MC. Standard deviations from the average values of the binding affinities were in all cases ≤ 1 kcal/mol, consistent with the variations that could be expected due to thermal agitation of the ligand within the binding pocket. Visual inspections of the structures of complex sampled in the MD simulations gave further details on the binding locations of 3MC. As shown in figure 10 (B), the Tyr55 and His52 are key residues within the GPER site for the 3MC binding, allowing the

accommodation of the ligand through interactions with their side chain ring and backbone group, respectively. In the binding position, 3MC may promote local deformations of the protein structure through two distinct mechanisms. One consists in bringing closer the β-hairpin between the helices H4 and H5, hence forming further interactions with it (simulations S-4 and S-1, Figure 10 A). Alternatively, 3MC encourages distortion in the central region of the GPER N-terminal α-helix (i.e., H1) and inserts between it and the adjacent helix H2 (simulation S-2, Figure 10 B). To further support the aforementioned results, docking simulations were also performed with three known ligands of GPER: the agonists E-2 and G-1 and the antagonist G15. As shown in Figure 10 (C), all ligands (including 3MC) occupy the same binding pocket within GPER and differ only for details in their binding modes. The anchoring locations are in agreement with previous studies [222-224] that identified key GPER residues involved in the ligand association. For instance, Ile279 (Figure 10 (C) panel (II)) was already reported as a residue crucial for the binding of E2 [222, 224] and Phe206/His307 (Figure 10 (C) panel (III)) were found to facilitate the binding of G-1 [222-223]. The binding energies of E-2, G-1 and G15 varied in the range between -8.7 and -7.8 kcal/mol, suggesting that 3MC may mimic these ligands to bind GPER, although with a slightly lower specificity and affinity. On the basis of our MD results, 3MC may act as a ligand of GPER occupying at least in two binding modes the same pocket identified in previous computational studies. Association of 3MC is predicted to have a good affinity (-8.3 kcal/mol) comparable to the binding energies obtained for other known ligands of GPER, although the variability in the anchoring location lead to a lower specificity.

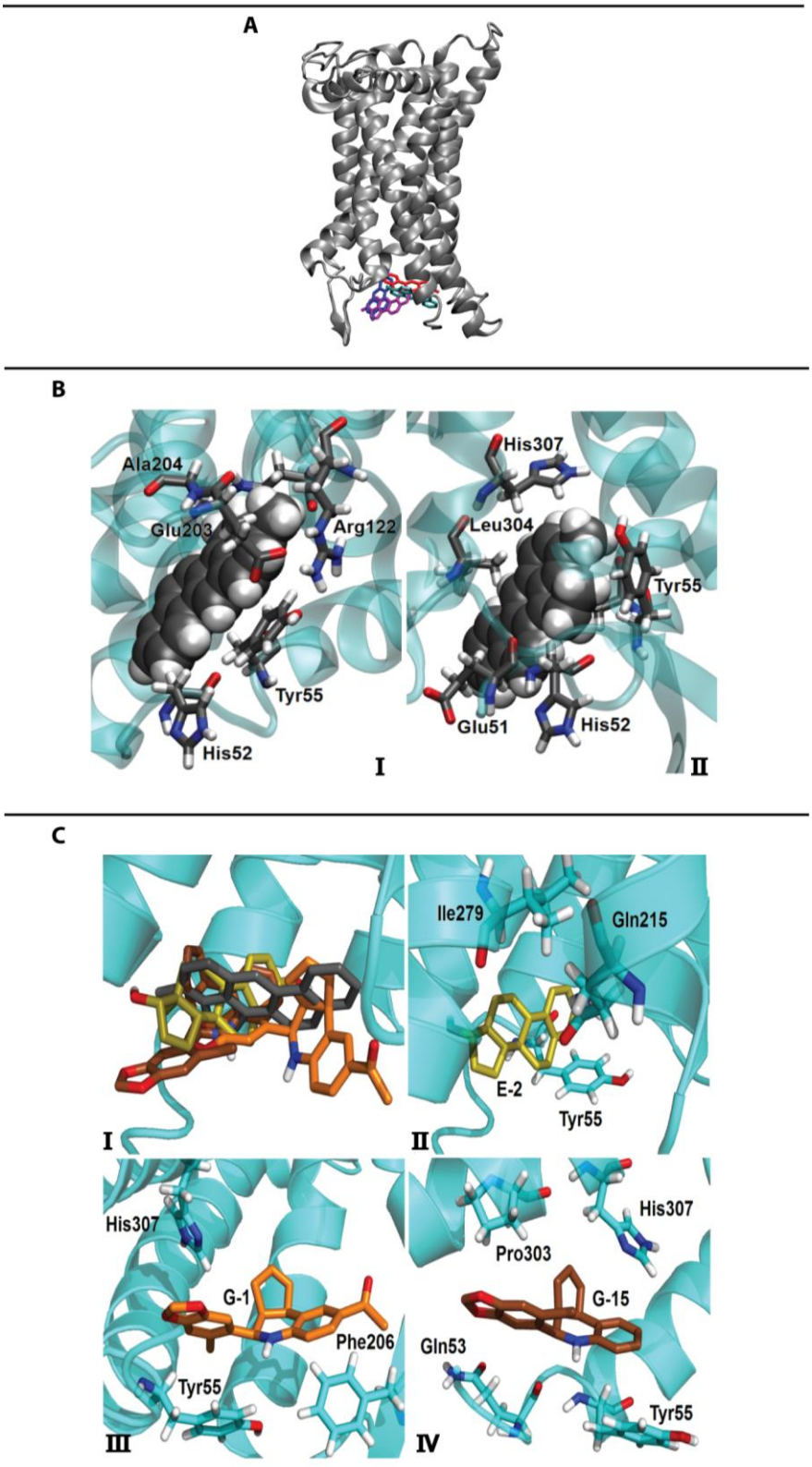


Figure 10. ***Docking poses of 3MC bound to GPER.*** (A) Four best poses selected by clustering the binding modes obtained in the docking simulations are shown. The binding affinity decreases in the following order: magenta → cyan → blue → red. The mobile N-terminal region of GPER (first 50 amino acids residues) is not presented. (B) Two representative binding modes of 3MC (in van der Waals representation) to GPER

obtained in distinct MD runs are shown: (I) simulation S-4 and S-1, and (II) simulation S-2. Binding energies, as estimated with the scoring function of AutoDock Vina [226], are similar in the two cases: -8.3 ± 1.0 and -8.2 ± 0.6 kcal/mol, respectively. Key protein residues in the binding pockets are also evidenced. (C) Binding modes of 3MC (gray), E2 (yellow), G-1 (orange) and G15 (brown) are indicated cumulatively in I. Details of the protein residues anchoring the ligands to GPER are shown for E2 (II), G-1 (III) and G15 (IV).

3.9 3MC, E2 and G-1 induce AHR nuclear translocation and CYP1B1 expression through AHR and GPER- mediated signalling.

As previous studies have demonstrated that AHR upon 3MC binding mainly localizes within the nuclear compartment [24, 144, 173], we next determined that the GPER agonists E2 and G-1 are able as 3MC to induce the nuclear translocation of AHR in SkBr3 cells (Figure 11) and CAFs (data not shown), however this effect was no longer evident using the AHR inhibitor CH223191 and the GPER antagonist G15 (Fig. 11).

On the basis of our data showing that E2 and G-1 induce the expression of the AHR target gene CYP1B1 via GPER in ER-negative breast cancer cells and CAFs, we then ascertained that the up-regulation of CYP1B1 mRNA and protein levels by E2 and G-1 (Fig. 12A-C) is abolished in the presence of the AHR inhibitor CH223191 (Fig. 12B, C). Similar results were obtained evaluating the transcriptional activation of the CYP1B1 promoter constructs transfected in SkBr3 cells and CAFs (Fig. 12D). Further corroborating these findings, we also assessed that 3MC, E2 and G-1 prompt a physical interaction of GPER with AHR, as evidenced by co-immunoprecipitation studies performed in SkBr3 cells (Fig. 12E-F) and CAFs (data not shown).

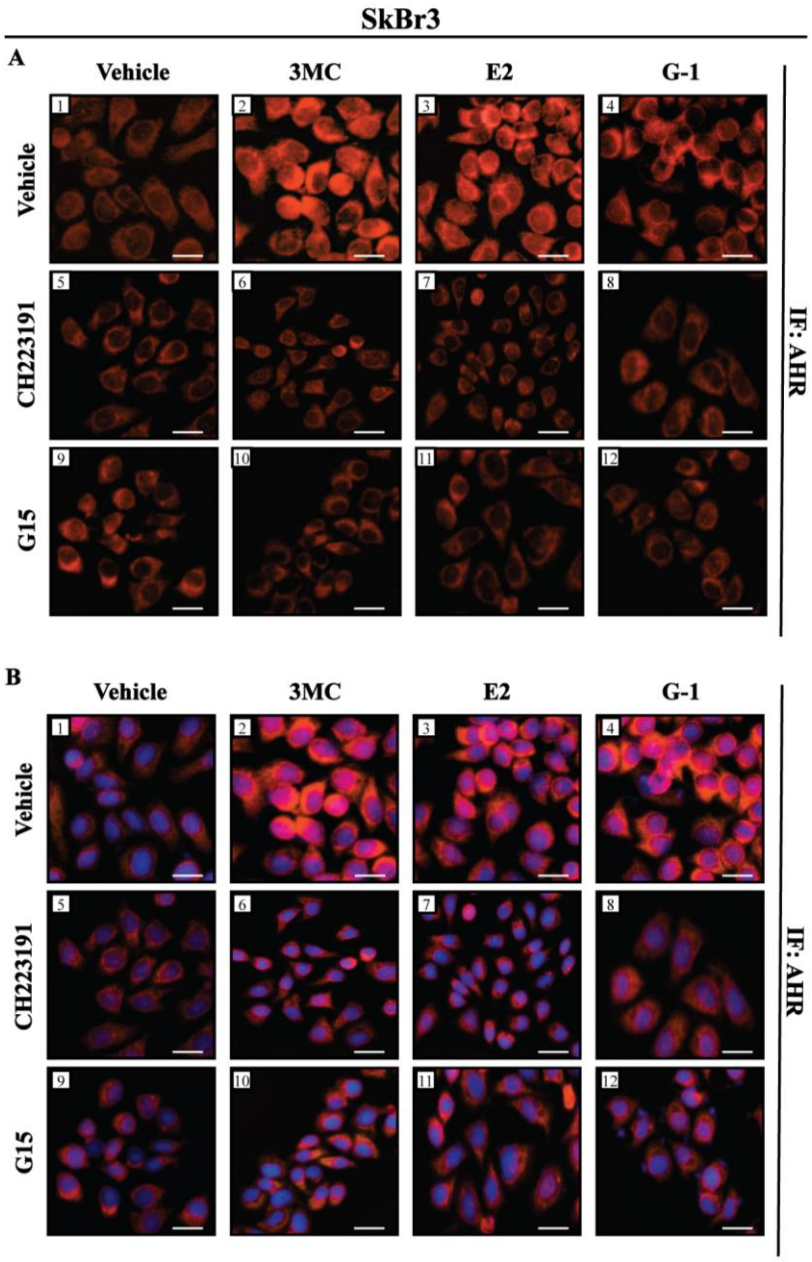


Figure 11. ***GPER in involved in the nuclear translocation of AHR triggered by 3MC, E2 and G-1.*** **(**A) Effects of a 4 h treatment with 3MC (1 μM), E2 (10 nM) and G-1 (100 nM) alone or in combination with 1 μM AHR inhibitor CH223191 or 100 nM GPER antagonist G15 on AHR nuclear translocation in SkBr3 cells. (B) Merge panels depicting the overlap of the two fluorophores used to assess AHR nuclear traslocation. Red signal: AHR. Blue signal: Nuclei. Images shown are representative of ten random fields from three independent experiments. Scale bar: 100 μM.

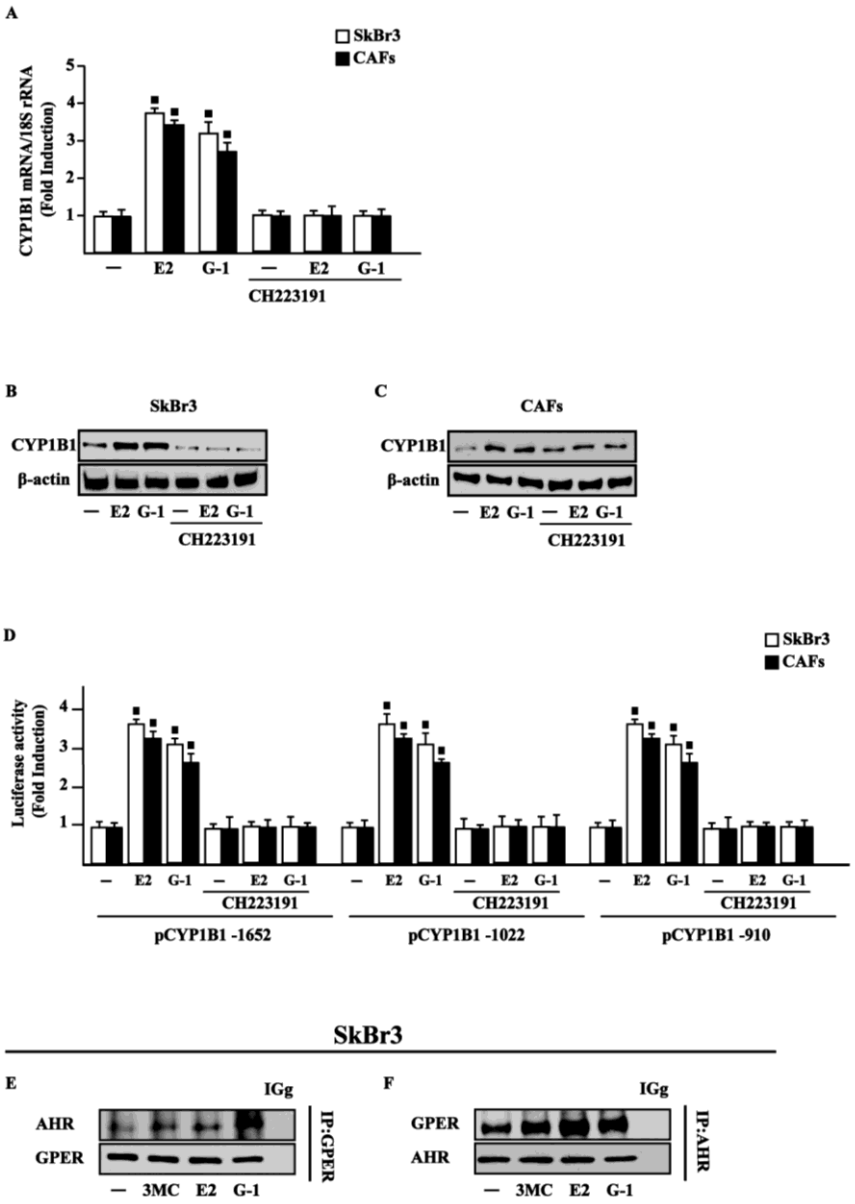


Figure 12. ***E2 and G-1 in SkBr3 cells and CAFs involve AHR in the CYP1B1 induction.*** **(**A) CYP1B1 mRNA expression evaluated in SkBr3 cells and CAFs treated for 6 h with vehicle (−), E2 (10 nM) and G-1 (100 nM) alone or in combination with 1 μM AHR inhibitor CH223191. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments with E2 and G-1 respect to cells treated with vehicle (−). Evaluation of CYP1B1 protein levels in SkBr3 cells (B) and CAFs (C) upon treatment for 6 h with vehicle (−), E2 (10 nM) and G-1 (100 nM) alone or in combination with 1 μM AHR inhibitor CH223191. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (D) Luciferase activities of CYP1B1 promoter constructs in SkBr3 cells and CAFs treated for 18 h with vehicle (−), E2 (10 nM) and G-1 (100 nM) alone or in combination with 1 μM AHR inhibitor CH223191, as indicated. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold induction upon which the activities induced by treatments were calculated. Each column represents the mean ± SD of three independent experiments, each performed in

triplicate. (■) indicates $P < 0.05$ for cells receiving treatments versus vehicle. (E, F) Co-immunoprecipitation studies performed in SkBr3 cells treated with 3MC (1 μM), E2 (10 nM) and G-1 (100 nM) for 4 h, as indicated. In control samples, nonspecific IgG was used instead of the primary antibody.

3.10 3MC, E2 and G-1 activate the EGFR/ERK/c-Fos transduction pathway toward CYP1B1 regulation.

Numerous studies have shown that in cancer cells AHR interacts with multiple growth factor-driven signalling, including the EGFR transduction pathway [25, 242]. In particular, ligands of AHR as 3MC and dioxin may stimulate gene expression changes and growth responses in cancer cells through a Src-mediated cross-talk involving AHR and EGFR [242-243]. On the basis of these data and given that GPER activation triggers the EGFR/ERK signaling in cancer cells [244] we sought to evaluate the role played by AHR in EGFR and ERK phosphorylation upon exposure to ligands of both AHR and GPER. Worthy, in SkBr3 cells (Figure 13A-F) and CAFs (data not shown) the rapid EGFR and ERK activation induced by 3MC, E2 and G-1 was prevented either by the AHR inhibitor CH223191 or by the GPER antagonist G15. In agreement with findings showing that GPER along with the EGFR/ERK/c-Fos transduction pathway lead to the regulation of CYP1B1 expression and reminiscing the 3MC capability to stimulate c-Fos expression [140, 245-249], we established that 3MC, E2 and G-1 up-regulate c-Fos mRNA (Fig. 13G, J) and protein levels (Fig. 13H-I, K-L) through both AHR and GPER in SkBr3 cells and CAFs, as ascertained using specific inhibitors of these receptors. Then, we assessed that c-Fos is involved in the transactivation of CYP1B1 promoter deletion constructs by 3MC, as this response was no longer evident transfecting the DN/c-Fos expression vector in both SkBr3 cells and CAFs (Figure 13M). Further supporting these findings, the CYP1B1 protein induction by 3MC was abolished transfecting cells with the DN/c-Fos construct (Figure 13N-O). Taken together, these data indicate that 3MC regulates the transcription of CYP1B1 through AHR and the GPER/EGFR/ERK/c-Fos transduction pathway.

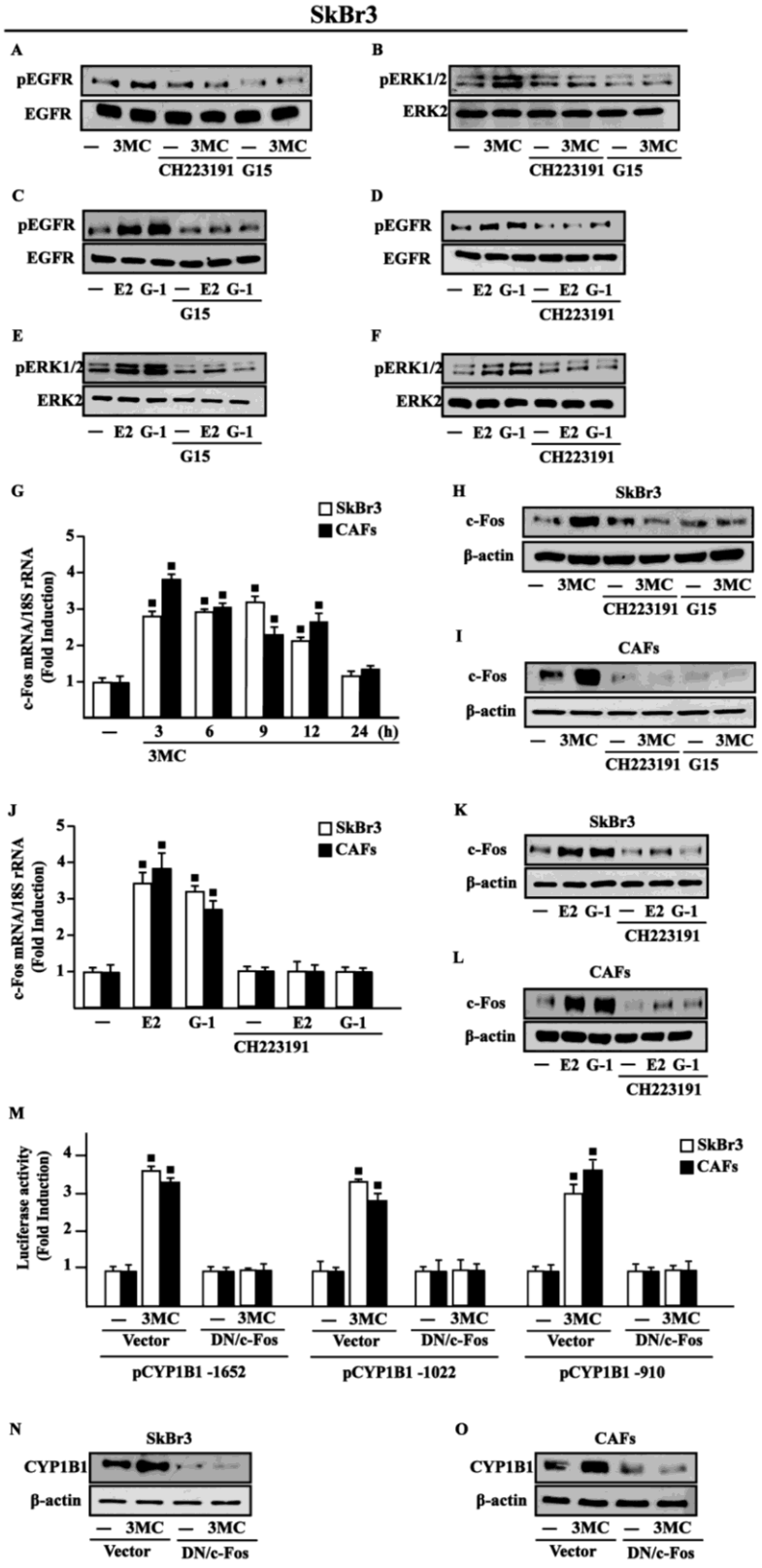


Figure 13. ***GPER and AHR are involved in the activation of the EGFR/ERK1/2/c-Fos transduction signaling by 3MC, E2, G-1.*** Phosphorylation of EGFR (A), ERK1/2 (B) in SkBr3 cells treated for 15 min with vehicle (–) or 1 μM 3MC alone or in combination with 1 μM AHR inhibitor CH223191 and 100 nM GPER antagonist G15, as indicated. Phosphorylation of EGFR and ERK1/2 in SkBr3 cells treated for 15 min

with E2 (10 nM) and G-1 (100 nM) alone or in combination with 100 nM GPER antagonist G15 (C, E) or 1 μM AHR inhibitor CH223191 (D, F). β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (G) 3MC (1 μM) induces the mRNA expression of c-Fos in SkBr3 cells and CAFs, as indicated. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments with 3MC respect to cells treated with vehicle (−). Evaluation of c-Fos protein levels in SkBr3 cells (H) and CAFs (I) upon a 6 h treatment with vehicle (−) and 3MC (1 μM) alone or in combination with 1 μM AHR inhibitor CH223191 and 100 nM GPER antagonist G15. (J) mRNA expression of c-Fos in SkBr3 cells and CAFs upon a 6 h treatment with E2 (10 nM) and G-1 (100 nM) alone or in combination with 1 μM AHR inhibitor CH223191. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments with 3MC respect to cells treated with vehicle (−). Evaluation of c-Fos protein levels in SkBr3 cells (K) and CAFs (L) upon a 6 h treatment with vehicle (−), E2 (10 nM) and G-1 (100 nM) alone or in combination with 1 μM AHR inhibitor CH223191. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. (M) Luciferase activities of CYP1B1 promoter constructs in SkBr3 cells and CAFs transfected for 8 h with CYP1B1 constructs, a vector or DN/c-Fos and then treated for 18 h with vehicle and 1 μM 3MC. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle were set as 1-fold induction upon which the activities induced by treatments were calculated. Each column represents the mean ± SD of three independent experiments, each performed in triplicate. (■) indicates $P < 0.05$ for cells receiving treatments versus vehicle. CYP1B1 protein levels in SkBr3 cells (N) and CAFs (O) transfected for 18 h with a vector or DN/c-Fos and then treated for 6 h with vehicle and 1 μM 3MC, as indicated. β-actin serves as a loading control. Results shown are representative of at least two independent experiments.

3.11 Cyclin D1 and CYP1B1 are involved in the growth effects triggered by 3MC, E2 and G-1 through AHR and GPER.

In accordance with our previous studies showing that estrogenic GPER signaling triggers relevant effects in cancer cells and CAFs through growth regulatory genes like cyclins [51; 237-238), we determined that 3MC stimulates the expression of cyclin D1 at both mRNA and protein level in SkBr3 cells (Fig. 14A-C) and CAFs (Fig. 14A, D-E) through AHR and GPER, as assessed using the inhibitors of these receptors, respectively CH223191 and G15. Similar results were obtained in SkBr3 cells (Fig. 14F-G) and CAFs (Fig. 14H-I) treated with E2 and G1 in combination with the AHR inhibitor CH223191, suggesting that both AHR and GPER are involved in the up-regulation of cyclin D1 by 3MC, E2 and G-1.

On the basis of our data indicating that GPER and CYP1B1 are involved in the induction of cyclins by E2 and G-1, we then found that the CYP1B1 inhibitor TMS as well as the silencing of CYP1B1 expression abolish the up-regulation of cyclin D1 by 3MC in SkBr3 cells (Fig. 15A-C) and CAFs (Fig. 15F-H).

The Specificity Protein 1 (SP1) transcription factor has been shown to contribute to the CYP1B1 mediated increase of growth regulatory genes like cyclin D1 as well as the proliferation, migration and invasion of cancer cells [14, 250-259]. Considering these findings together with the role elicited by SP1 toward the oncogenic transformation prompted by CYP1B1 [14], we next established that cyclin D1 protein induction by 3MC, E2 and G-1 is prevented using the SP1 inhibitor mithramycin A (MTM A) in SkBr3 cells and CAFs (Fig. 15D-E, I-J). Nicely recapitulating the aforementioned data, the growth of two-dimensionally (2D)-cultured SkBr3 cells induced by 3MC, E2 and G-1 was abolished either in the presence of CH223191, G15, TMS and MTM A, inhibitors of AHR, GPER, CYP1B1 and SP1, respectively, or silencing CYP1B1 and GPER expression (Fig. 16A-E). Similar results were also obtained in a three-dimensional (3D)-culture system (Fig. 16F-G). Collectively, these results suggest that AHR and GPER are involved in the CYP1B1 and cyclin D1 induction upon 3MC, E2 and G-1 exposure toward the growth responses observed in breast cancer cells.

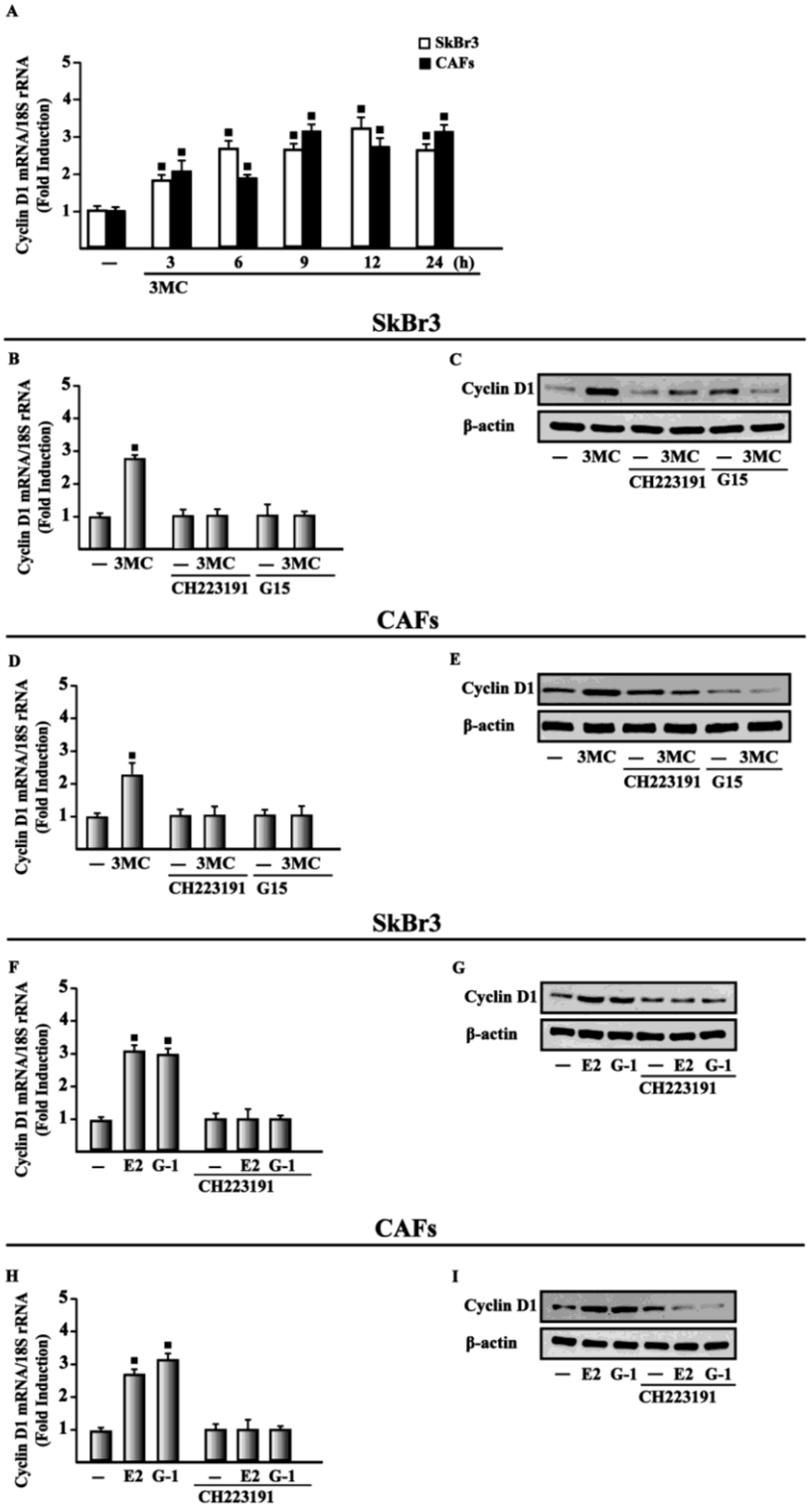


Figure 14. ***GPER and AHR are involved in the up-regulation of cyclin D1 by 3MC, E2 and G-1 in SkBr3 cells and CAFs.*** (A) Cyclin D1 mRNA expression in SkBr3 cells and CAFs treated with 1 μM 3MC, as indicated. mRNA expression of cyclin D1 in SkBr3 cells (B) and CAFs (D) upon treatments for 18 h with 1 μM 3MC alone and in combination with 1 μM AHR inhibitor CH223191 or 100 nM GPER antagonist G15. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments with 3MC respect to cells

treated with vehicle (−). Cyclin D1 protein levels in SkBr3 cells (C) and CAFs (E) upon treatments for 18 h with vehicle (−) and 1 μM 3MC alone or in combination with 1 μM AHR inhibitor CH223191 and 100 nM GPER antagonist G15. β-actin serves as a loading control. Results shown are representative of at least two independent experiments. Cyclin D1 mRNA expression in SkBr3 cells (F) and CAFs (H) treated for 18 h with vehicle (−), 10 nM E2 and 100 nM G-1 alone or in combination with 1 μM AHR inhibitor CH223191. Data obtained by real-time PCR in three independent experiments performed in triplicate were normalized to 18S expression and shown as fold changes of CYP1B1 expression upon treatments respect to cells treated with vehicle (−). (■) $P < 0.05$ for cells receiving treatments versus vehicle. Cyclin D1 protein levels in SkBr3 cells (G) and CAFs (I) upon treatments for 18 h with vehicle (−), 10 nM E2 and 100 nM G-1 alone or in combination with 1 μM AHR inhibitor CH223191. β-actin serves as a loading control. Results shown are representative of at least two independent experiments.

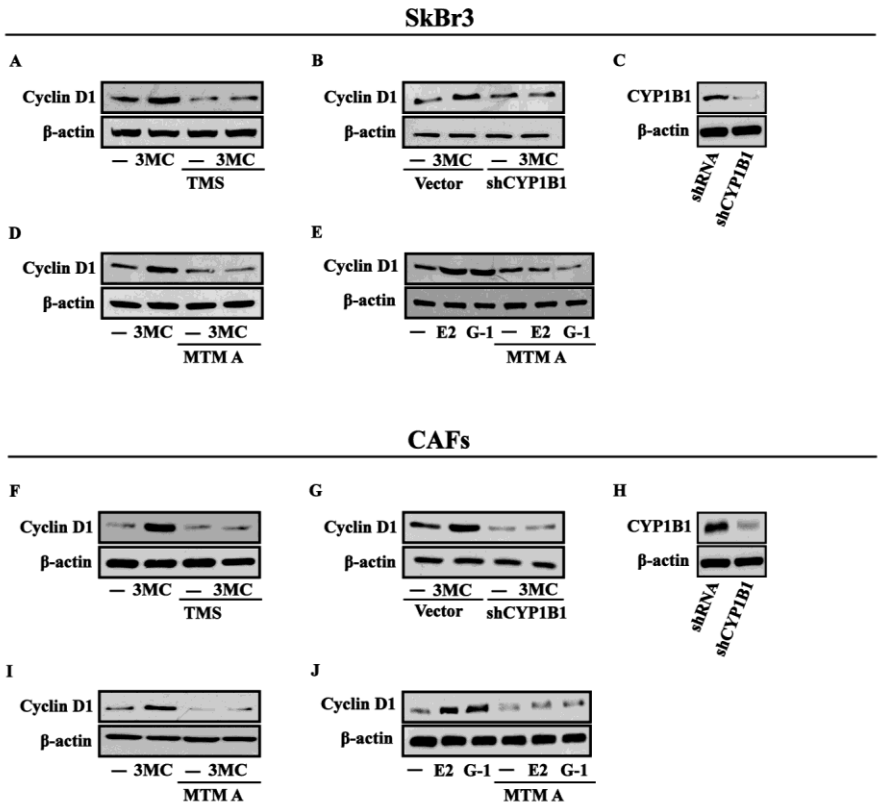


Figure 15. ***The up-regulation of cyclin D1 by 3MC, E2 and G-1 involves the SP1 transcription factor.*** Cyclin D1 protein levels in SkBr3 cells (A) and CAFs (F) upon treatments for 18 h with vehicle (−) and 1 μM 3MC alone or in combination with 5 μM CYP1B1 activity inhibitor TMS. Cyclin D1 protein expression in SkBr3 cells (B) and CAFs (G) transiently transfected with a shRNA or shCYP1B1 for 24 h, then treated for 18 h with vehicle and 1 μM 3MC. (C, H) Efficacy of CYP1B1 silencing. Cyclin D1 protein levels in SkBr3 cells (D, E) and CAFs (I, J) treated for 18 h with vehicle and 1 μM 3MC, 10 nM E2 and 100 nM G-1 alone or in combination with 100 nM SP1 inhibitor Mithramycin A. β-actin serves as a loading control. Results shown are representative of at least two independent experiments

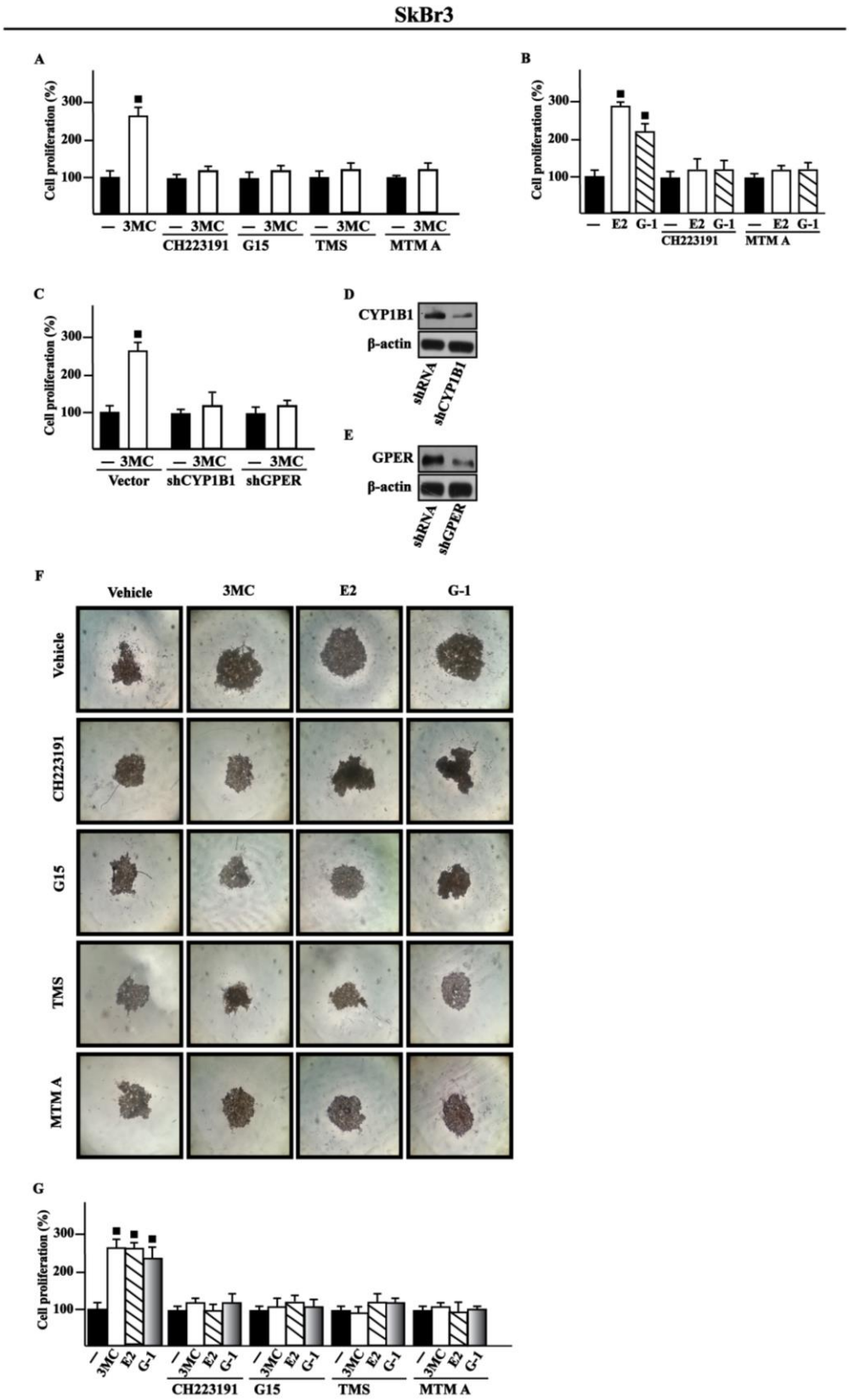


Figure 16. ***Transduction signalling involved in the proliferative effects triggered by 3MC, E2 and G1 in SkBr3 cells.*** (A) The proliferation of SkBr3 cells induced by 100 nM 3MC is prevented by 100 nM AHR inhibitor CH223191, 100 nM GPER antagonist G15, 1 μM CYP1B1 inhibitor TMS and 10 nM SP1

antagonist mithramycin A. (B) The proliferation of SkBr3 cells induced by 10 nM E2 and 100 nM G-1 is prevented by 100 nM AHR inhibitor CH223191 or 10 nM SP1 antagonist Mithramycin A. The proliferation of SkBr3 cells (C) induced by 100 nM 3MC is prevented silencing CYP1B1 or GPER expression. Cells were transfected every 2 days with shRNA, shCYP1B1 or shGPER, treated every day with ligands and then counted on day 5. Efficacy of CYP1B1 (D) and GPER (E) silencing. β-actin serves as a loading control. Proliferation of cells treated with vehicle (−) was set as 100% upon which cell growth induced by treatments was calculated. Each data point is the mean ± SD of three independent experiments performed in triplicate. (F) SkBr3 cells, seeded in 2% agar-coated plates and cultured as three-dimensional spheroids, were treated for 20 days with 100 nM 3MC, 10 nM E2 and 100 nM G-1 alone or in combination with 100 nM AHR inhibitor CH223191, 100 nM GPER antagonist G15, 1 μM CYP1B1 inhibitor TMS and 10 nM SP1 antagonist mithramycin A, as indicated. (G) Graphic representation of cell number after 20 days of exposure to treatments. The number of cells treated with vehicle (−) was set as 100% upon which cell growth induced by treatments was calculated. (■) $P < 0.05$ for cells receiving treatments versus vehicle.

Chapter 4

Discussion

In the present study we provided novel insights on the stimulatory action exerted by 3MC and estrogens through AHR and GPER in breast cancer. In particular, we demonstrated that these ligands require both receptors to trigger CYP1B1-mediated growth effects in breast cancer cells and the surrounding microenvironment. In particular, we have ascertained that estrogens and 3MC through AHR and GPER/EGFR/ERK transduction pathways regulate CYP1B1 expression and function in ER-negative breast cancer cells and CAFs obtained from breast cancer patients. Biologically, we have demonstrated that GPER is involved in CYP1B1-mediated proliferative effects exerted by estrogens in breast tumor cells as well as in tumor xenograft models of breast cancer. Moreover, our data provide evidence that the biological response induced in breast cancer cells by 3MC and estrogens may involve a functional interaction between AHR and GPER.

Estrogens are involved in important physiological functions as the maintenance of the female reproductive system; however, these steroids may also contribute to the development of breast malignancies [260]. Estrogen mainly act through the classical ER, nevertheless several studies have demonstrated that GPER can mediate the stimulatory effects of estrogens in both normal and malignant tissues, including breast cancer [4, 51, 53, 261]. For instance, ligand-activated GPER triggers a network of transduction pathways such as EGFR, intracellular cyclic AMP, calcium mobilization, MAPK and PI3K, thus leading to the induction of genes involved in the proliferation, migration and invasion of cancer cells including breast tumor cells [53]. Likewise, a clinical correlation between

GPER expression and increased tumor size, distant metastasis and recurrence has been found in human breast tumor specimens, suggesting that GPER levels may be predictive of aggressive breast malignancies [59, 262]. Various studies have also revealed that certain GPER-mediated responses to estrogens target important components of the tumor microenvironment driving cancer progression as CAFs [5]. In particular, GPER has been involved in the transcription of genes toward the proliferation, migration and adhesion/spreading of CAFs derived from breast tumor patients [5]. Noteworthy, we have ascertained that GPER mediates the stimulatory action of estrogens not only in CAFs obtained from primary breast malignancies but also in CAFs derived from a cutaneous metastasis of an invasive mammary ductal carcinoma. In this regard, it is worthy mentioning that metastasis-associated CAFs may elicit stimulatory effects in metastatic cancer cells similar to those triggered by CAFs at primary tumor sites [263]. Indeed, it is now unquestioned that both tumor growth and the essential steps of the metastatic process are not only dependent on cancer cells, but rather involve a promiscuous interaction between tumor cells and components of the tumor microenvironment as CAFs [264-267]. Likewise, recent observations have indicated that cancer cells might carry CAFs during their migration to metastatic sites, in such way these co-traveling cells may facilitate tumor development in further tissues [268].

Several studies have suggested that estrogens may play a role in the development of hormone-sensitive tumors also via oxidative estrogen metabolism [17]. CYP1B1 is a major E2 hydroxylase involved in estrogen biosynthesis and metabolism, generation of DNA damaging pro-carcinogens and resistance to anti-hormone therapies [12]. For instance, CYP1B1 catalyzes the hydroxylation of E2 leading to the formation of 4OHE2 [193], which may trigger the induction of estradiol-3,4-quinone, the strongest ultimate carcinogenic estrogen metabolite that, binding to the N-7 position of guanine, leads to the

destabilization of the glycosidic bond and the subsequent DNA depurination and mutagenesis [2, 8, 19, 209]. Considering that CYP1B1 expression increases in tumor tissues compared to the normal counterpart [14, 202] and given that the levels of 4OHE2 is higher in hormone-sensitive tumors like breast cancer respect to normal tissues [8], this cytochrome has attracted increasing interest as potential target in novel anticancer strategies, especially in the treatment of hormone-related tumors [210].

The transcription of CYP1B1 is mainly regulated by the aryl hydrocarbon receptor (AHR) that acts as a ligand-activated transcription factor [211]. Xenobiotics like dioxin, halogenated aromatic hydrocarbons, BaP and PAHs are AHR activators of CYP1B1 transcription [8, 210]. In accordance with these findings, it has been recently reported that the GPER agonist G-1 is also able to up-regulate the expression of both AHR and CYP1B1 in ER-positive breast cancer cells, although the molecular mechanisms involved remain to be elucidated [269]. Furthermore, CYP1B1 can be regulated by other transcription factors as SP1, cAMP–response element binding protein (CREB) and ER [19]. In this context, it is worth noting that CYP1B1 may be induced by its own substrates [2]. For instance, E2-activated ERα triggers the transcription of CYP1B1 through an estrogen responsive element (ERE) located within the CYP1B1 promoter sequence in breast tumor cells [18]. These findings may indicate that the regulation of CYP1B1 expression and activity by its own substrates like estrogens and environmental contaminants would be pathologically important for their metabolism also in hormone-responsive tissues. Moreover, previous studies proposed the CYP1B1 inhibitor namely TMS as a potential chemopreventive agent in hormone-sensitive tumors as it prevented the formation of the carcinogenic estrogen metabolite 4OHE2, induced apoptotic cell death selectively in cancer cells and reduced tumor volume of tamoxifen-resistant breast cancer xenografts [9, 11, 13, 270-272]. In this scenario, our data provide novel insights into the current knowledge regarding the

regulation of CYP1B1 expression and function by estrogens and 3MC. In particular, our findings suggest that GPER and AHR may be included among the transduction mediators involved by both estrogens and 3MC in the regulation of CYP1B1 toward the growth of breast cancer.

On the basis of the role elicited by GPER in tumor progression, a number of studies has been performed in order to identify compounds that acting as agonists of GPER, may promote relevant activities in tumor cells [57, 60, 83, 237, 273-276]. For instance, the environmental contaminant bisphenol A [277] and the pesticide atrazine [274] were shown to trigger stimulatory effects through GPER in breast cancer cells. Further searching for ligand-activated GPER signaling, in the current study we have ascertained that 3MC may engage GPER toward kinase activation and gene transcription. The environmental pollutant 3MC, which is present in cigarette smoke and mainly formed by incomplete combustion processes, may exert carcinogenic effects through both AHR and the classical ER [143, 171, 240, 272, 278-281]. As 3MC, several contaminants like dioxin, BaP and 7,12-dimethylbenz[a]anthracene (DMBA) trigger the transcription of pro-carcinogenic genes, including cytochrome P450 enzymes, by binding to and activating AHR, which is involved in cancer cell proliferation, invasion and drug resistance [22-23, 139, 171, 241, 280, 282]. In particular, the ligand binding to AHR promotes the receptor translocation within the nucleus and its interaction with specific response elements located in the promoter regions of target genes as CYP1B1 [22, 25, 209, 283-284]. In addition, AHR ligands may induce their own metabolism regulating the expression and the activity of cytochrome P450 enzymes like CYP1B1 [171, 283, 285]. Of note, high levels of CYP1B1 may lead to an increased expression and constitutive activation of AHR, hence facilitating a feed-forward loop involved in tumor progression [286]. The biological responses induced in cancer cells by ligands of AHR, such as 3MC, may involve a functional interaction of

AHR with the EGFR transduction pathway [25] and diverse signaling molecules like transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) [287]. This cooperative action may have important implications toward the development of various malignancies including breast cancer [25-26, 139, 288-289]. For instance, a Src-mediated crosstalk between AHR and EGFR triggers the ERK1/2 activation and the stimulation of cancer cell growth [25, 290]. Further extending these data, we have ascertained that both AHR and GPER are involved in the activation of the EGFR/ERK/c-Fos transduction signaling by ligands of these receptors as 3MC, E2, G-1, which also stimulate the interaction of AHR with GPER in breast cancer cells and CAFs. This novel signaling pathway may represent a further target in setting innovative strategies in breast tumor patients.

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