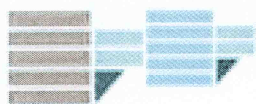

Università della Calabria

DOTTORATO DI RICERCA IN BIOCHIMICA CELLULARE ED ATTIVITA' DEI FARMACI IN ONCOLOGIA (XXV ciclo)

Tesi di Dottorato in Patologia Generale
Settore scientifico disciplinare: MED/04

GPER mediates the up-regulation of Fatty Acid Synthase (FASN) induced by 17β-estradiol in cancer cells and Cancer-Associated Fibroblasts (CAFs)

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Abstract

Activation of lipid metabolism is an early event in carcinogenesis and a central hallmark of many tumors. Fatty acid synthase (FASN) is a key lipogenic enzyme catalyzing the terminal steps in the de novo biogenesis of fatty acids. In cancer cells, FASN may act as a metabolic oncogene given that it confers growth and survival advantages to these cells, whereas its inhibition effectively and selectively kills tumor cells. Hormones like estrogens and growth factors contribute to the transcriptional regulation of FASN expression also through the activation of downstream signaling and a crosstalk among diverse transduction pathways. In this study, we demonstrate for the first time that 17β-estradiol (E2) and the selective GPER ligand G-1 regulate FASN expression and activity through the GPER-mediated signaling which involved the EGFR/ERK/c-fos/AP1 transduction pathway, as ascertained by using specific pharmacological inhibitors, performing gene-silencing experiments and ChiP assays in breast SkBr3, colorectal LoVo, hepatocarcinoma HepG2 cancer cells and breast cancer-associated fibroblasts (CAFs). In addition, the proliferative effects induced by E2 and G-1 in these cells involved FASN as the inhibitor of its activity, named cerulenin, abolished the growth response to both ligands. Our data suggest that GPER may be included among the transduction mediators involved by estrogens in regulating FASN expression and activity in cancer cells and CAFs that strongly contribute to cancer progression.

Chapter I

Introduction

1.1 Breast, colorectal and hepatocellular cancer

Breast cancer

Breast cancer is the most common malignancy and the leading cause of cancer-related death in women worldwide. Whereas localized disease is largely curable, metastatic or recurrent disease carries a unfavorable 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 (2).

The management of breast cancer has changed considerably in the last two decades with improvements in systemic therapy and advances in surgical techniques (3). There are two main types of breast cancer:

- Ductal carcinoma starts in the ducts that move milk from the breast to the nipple. Most breast cancers are of this type.
- Lobular carcinoma starts in the parts of the breast, called lobules 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."

- 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.
- Lobular carcinoma in situ (LCIS) is a marker for an increased risk of invasive cancer in the same or both breasts (Fig. 1.1).

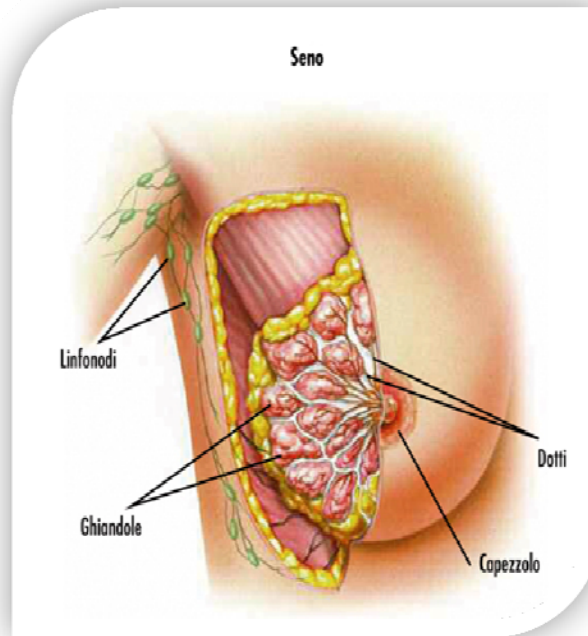

FIGURE 1.1 *Breast anatomy.*

Many breast cancers are sensitive to the estrogens. This means that estrogens cause the breast cancer tumor to grow. Over the course of a lifetime, 1 in 8 women will be diagnosed with breast cancer. There are many risk factors:

- *Age and gender*. The risk of developing breast cancer increases with age. Most advanced breast cancer cases are found in women over age 50 (4). Women are 100 times more likely to get breast cancer than men.
- *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 (5).
- *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 (6).
- *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 (7).

Other risk factors include:

- *Alcohol use.* Drinking more than 1-2 glasses of alcohol a day may increase your risk for breast cancer (8).
- *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 (9).
- *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 (10).
- *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 (10).
- *Radiation.* The radiation therapy to treat cancer of the chest area, increase higher risk to develop breast cancer (11).

Treatment is based on many factors, including: type and stage of the cancer, whether the 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 lymph node 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 of time. All women will continue to have blood tests, mammograms, and other tests

after treatment. Women who have had a mastectomy may have reconstructive breast surgery, either at the same time as the mastectomy or later.

Colorectal cancer

Colon, or colorectal, cancer starts in the large intestine (colon) or the rectum (end of the colon). According to the American Cancer Society, colorectal cancer is one of the leading causes of cancer-related deaths in the United States. However, early diagnosis can often lead to a complete cure. Almost all colon cancer starts in glands in the lining of the colon and rectum. There is no single cause of colon cancer. Nearly all colon cancers begin as benign polyps, which slowly develop into cancer (12) (Fig.1.2).

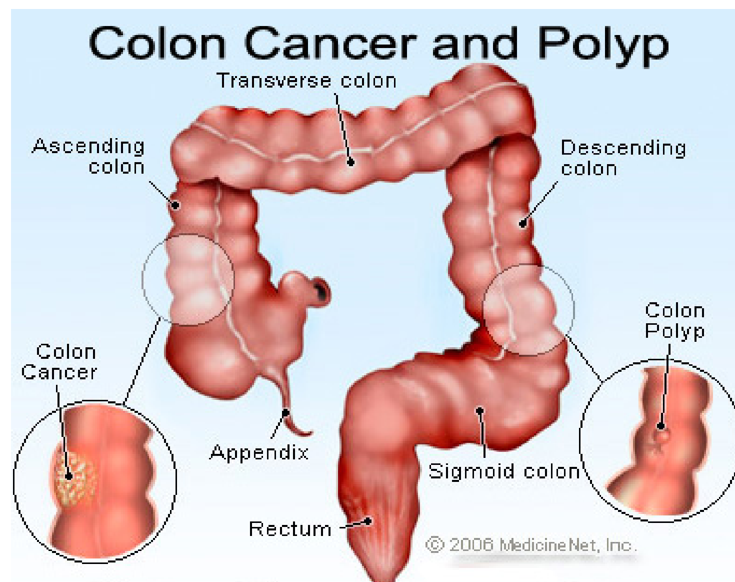

FIGURE 1.2 *Picture of colon (colorectal) cancer.*

There is a higher risk for colon cancer if you:

- Are older than 60
- Are African American of eastern European descent
- Eat a diet high in red or processed meats
- Have cancer elsewhere in the body
- Have colorectal polyps
- Have inflammatory bowel disease (Crohn's disease or ulcerative colitis)
- Have a family history of colon cancer

- Have a personal history of breast cancer

Certain genetic syndromes also increase the risk of developing colon cancer. Two of the most common are:

- Familial adenomatous polyposis (FAP)
- Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome

Food may play a role in your risk of colon cancer. Colon cancer may be associated with a high-fat, low-fiber diet and red meat. However, some studies have found that the risk does not drop if you switch to a high-fiber diet, so this link is not yet clear. Smoking cigarettes and drinking alcohol are other risk factors for colorectal cancer (13).

Many cases of colon cancer have no symptoms. The following symptoms, however, may indicate colon cancer:

- Abdominal pain and tenderness in the lower abdomen
- Blood in the stools
- Diarrhea, constipation, or other change in bowel habits
- Narrow stools
- Weight loss with no known reason

Stages of colon cancer are:

Stage 0: Very early cancer on the innermost layer of the intestine

Stage I: Cancer is in the inner layers of the colon

Stage II: Cancer has spread through the muscle wall of the colon

Stage III: Cancer has spread to the lymph nodes

Stage IV: Cancer has spread to other organs

Treatment depends on many things, including the stage of the cancer. In general, treatments may include: surgery (most often a colectomy) to remove cancer cells; chemotherapy to kill cancer cells; radiation therapy to destroy cancerous tissue. The death rate for colon cancer has dropped in the last 15 years. This may be due to increased awareness and screening by colonoscopy. Colon cancer can almost always be caught by colonoscopy in its earliest and most curable stages. Almost all men and women age 50 and older should have a colon cancer screening. Patients at risk may need earlier screening. Colon cancer screening can often find polyps before they become cancerous. Removing these polyps may prevent colon cancer. Changing your diet and lifestyle is important. Some evidence suggests that low-fat and high-fiber diets may reduce your risk of colon cancer (14).

Hepatocellular carcinoma

Hepatocellular carcinoma accounts for most liver cancers. This type of cancer occurs more often in men than women. It is usually seen in people age 50 or older. However, the age varies in different parts of the world (Fig.1.3).

The disease is more common in parts of Africa and Asia than in North or South America and Europe (15). In most cases, the cause of liver cancer is usually scarring of the liver (cirrhosis). Cirrhosis may be caused by:

- Alcohol abuse (the most common cause in the United States)
- Autoimmune diseases of the liver
- Hepatitis B or C virus infection
- Inflammation of the liver that is long-term (chronic)
- Iron overload in the body (hemochromatosis)

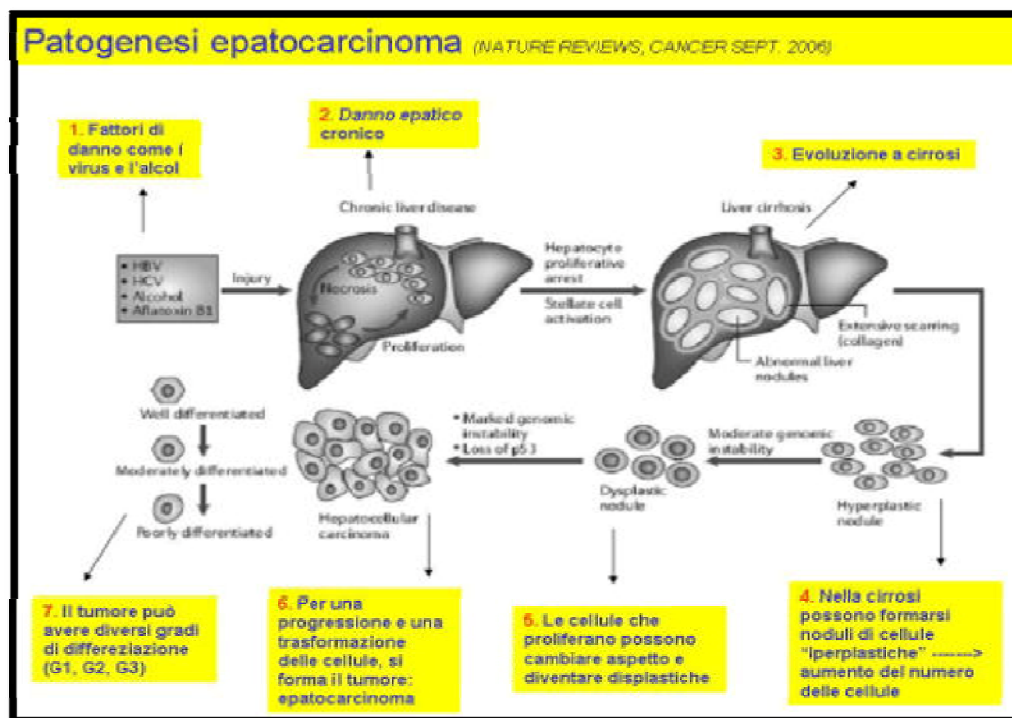

FIGURE 1.3 *Pathogenesis of hepatocarcinoma.*

Patients with hepatitis B or C are at risk for liver cancer, even if they have not developed cirrhosis (16). Aggressive surgery or a liver transplant can successfully treat small or slow growing tumors if they are diagnosed early. However, few patients are diagnosed early.

Chemotherapy delivered straight into the liver with a catheter can help, but it will not cure the disease. Radiation treatments in the area of the cancer may also be helpful. However, many patients have liver cirrhosis or other liver diseases that make these treatments more difficult. The usual outcome is poor, because only 10-20% of hepatocellular carcinomas can be removed completely using surgery. If the cancer cannot be completely removed, the disease is usually fatal within 3-6 months. However, survival can vary, and occasionally people will survive much longer than 6 months.

Complication:

- Gastrointestinal bleeding
- Liver failure
- Spread (metastasis) of the cancer

Preventing and treating viral hepatitis may help reduce your risk. Childhood vaccination against hepatitis B may reduce the risk of liver cancer in the future.

Avoid drinking excessive amounts of alcohol. Certain patients may benefit from screening for hemochromatosis. If you have chronic hepatitis or known cirrhosis, periodic screening with liver ultrasound or measurement of blood alpha fetoprotein levels may help detect this cancer early (17).

1.2 Tumor microenvironment

Cancer has long been regarded a disease consisting of a group of transformed cells which have acquired proliferative and invasive capacities. Accordingly, therapeutic anti-cancer therapies have been concentrated to targeting tumor cells alone. In order for cancer to be effectively controlled, carcinogenesis and tumor progression needs to be viewed involving complex interactions with its environment; *the tumor microenvironment* (18). Currently, more data indicate that we need to revise our ideas on carcinogenesis and carcinomas and regard these as phenomena that occur in tissues, not just in cancer cells. The development of a tumor occurs in an environment that consists of a complex system containing many different cell types. The tumor microenvironment contains endothelial cells and their precursors, pericytes, smooth muscle cells, fibroblasts of various phenotypes, myofibroblasts, neutrophils and other granulocytes (eosinophils and basophils), mast cells (MCs), T, B, and natural killer lymphocytes and antigen presenting cells such as macrophages and dendritic cells. All these cells can in one way or another participate in tumor progression. The presence of leucocytes in tumor tissues was until late thought to be an attempt of the immune system to eradicate the tumor. It has been shown that leukocyte cells such as macrophages, granulocytes and mast

cells all have been associated in one way or another with promotion of malignancy. Tumor-associated leucocytes are variably loaded with an assorted array of cytokines, cytotoxic mediators as well as proteolytic enzymes that promote all the steps associated with malignancy within tumors (19-21). The role of granulocytes has been extensively studied with contradictory results. It has been shown that circulating neutrophilic polymorphonuclear cells (PMNs) isolated from tumor bearing animals reduce the number of metastatic foci in the lungs (22). On the other hand, in vitro studies reveal that PMNs stimulate tumor cell attachment to endothelial monolayers, a relevant step for tumor migration (23,24). Next to this, neutrophylic granulocytes have also been shown to promote the migratory capacity in breast cancer cells. Other authors have shown that tumor-associated PMNs were involved in tumor angiogenesis by the production of vascular endothelial growth factor (VEGF) and Interleukin (IL-8) and in tumor invasion by the release of matrix metalloproteinases (MMPs) and elastase (25-26) (Fig.1.4).

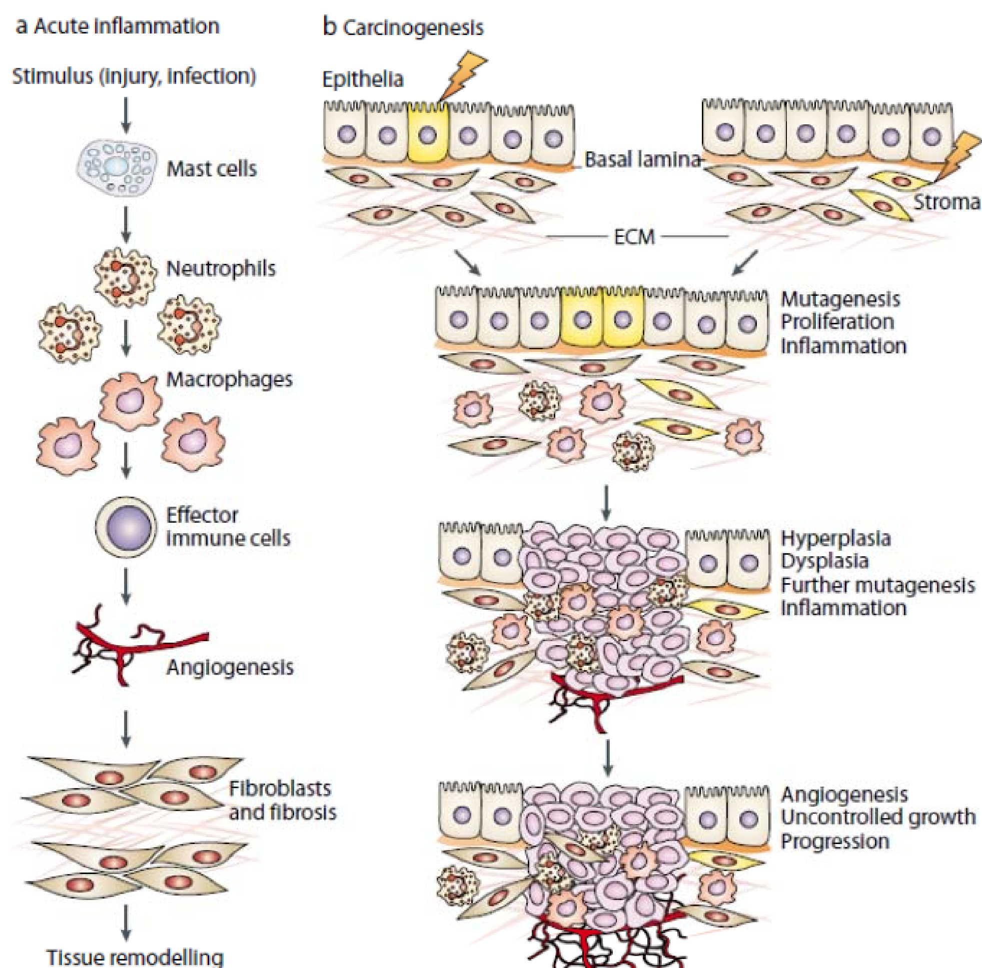

FIGURE 1.4 *Illustrates the sequence of events and involvement of the tumor microenvironment during carcinogenesis.*

A current concept of tumor progression and interaction with the microenvironment is that it roughly resembles an inflammatory process. The transformation taking place during tumorigenesis may lead to disrupted proliferation. This disruption is regarded as cell damage and causes an inflammatory reaction, in order to repair and reconstruct the damaged lesion. This inflammatory reaction includes leukocyte infiltration and stromal and endothelial cell activation. This alteration of tissue homeostasis further promotes tumor progression, which in turn further activates the surrounding stroma, eventually also leading to neovacularization, or tumor angiogenesis, which is a critical step in the further progression, invasion and metastasis of a tumor. As such, in fact, a reaction to restore the damage caused by the tissue transformation, paradoxically results in further promoting the progression, survival and replication of the dysfunctional epithelial cells. As the microenvironment has such a crucial role in carcinogenesis and metastasis, it represents a crucial target not only for cancer therapy but also for chemopreventive strategies as further elaborated on above. There is already a large amount of information about specific cells and molecules in the tumor microenvironment that are targets for cancer therapy at present (27,28). The supporting players in the tumor microenvironment include stromal fibroblasts, infiltrating immune cells, the blood and lymphatic vascular networks, and the extracellular matrix. There is abundant evidence that an abnormal stromal context contributes to, or is even required for, tumor formation and progression. 'Normalization' of the stromal environment should therefore be able to slow or even reverse tumor progression (29). The potential of a normal context to suppress a tumorigenic phenotype has been shown in different experimental settings. For example it has been demonstrated that the presence of a reconstituted physiological basement membrane induces pre-malignant breast epithelial cells to undergo growth arrest and form polarized alveolar structures, as normal epithelial cells would (30). This normalization is in part mediated by integrins, as blockade of signaling by β1-integrin reverted tumorigenesis despite maintained genetic abnormalities in the epithelial cells (31). Potential therapeutic target components of the tumor microenvironment include stromal cells such as endothelial cells, tumor associated fibroblasts, macrophages, extracellular matrix (ECM) molecules such as thrombospondin and fibronectin (FN), matrix-degrading proteases and inhibitors such as matrix metalloproteinases (MMPs) and tissue metalloproteinase inhibitors (TIMPs) and regulatory molecules such as integrins, growth factors and chemokines (32). These agents may provide an interesting alternative to traditional tumor cell-directed therapy. Because of the complexity of the tumor milieu, the most beneficial therapy will likely involve the combination of one or more agents directed at this new target.

1.2.1 Cancer-associated fibroblasts (CAFs)

Cancer associated fibroblasts (CAFs) is one of the most crucial components of the tumor microenvironment which promotes the growth and invasion of cancer cells by various mechanisms. CAFs demonstrate a high degree of heterogeneity due to their various origins; however, many distinct morphological features and physiological functions of CAFs have been identified. It is becoming clear that the crosstalk between the cancer cells and the CAFs plays a key role in the progression of cancer, and understanding this mutual relationship would eventually enable us to treat cancer patients by targeting CAFs. In this review, we will discuss the latest findings on the role of CAFs in tumorigenesis and metastasis as well as potential therapeutic implication of CAFs. The role of CAFs in tumor progression is multifaceted. Similarly to immune cells, which initially repress malignant growth, CAFs inhibit early stages of tumor progression, mainly through the formation of gap junctions between activated fibroblasts (33,34) (Fig. 1.5).

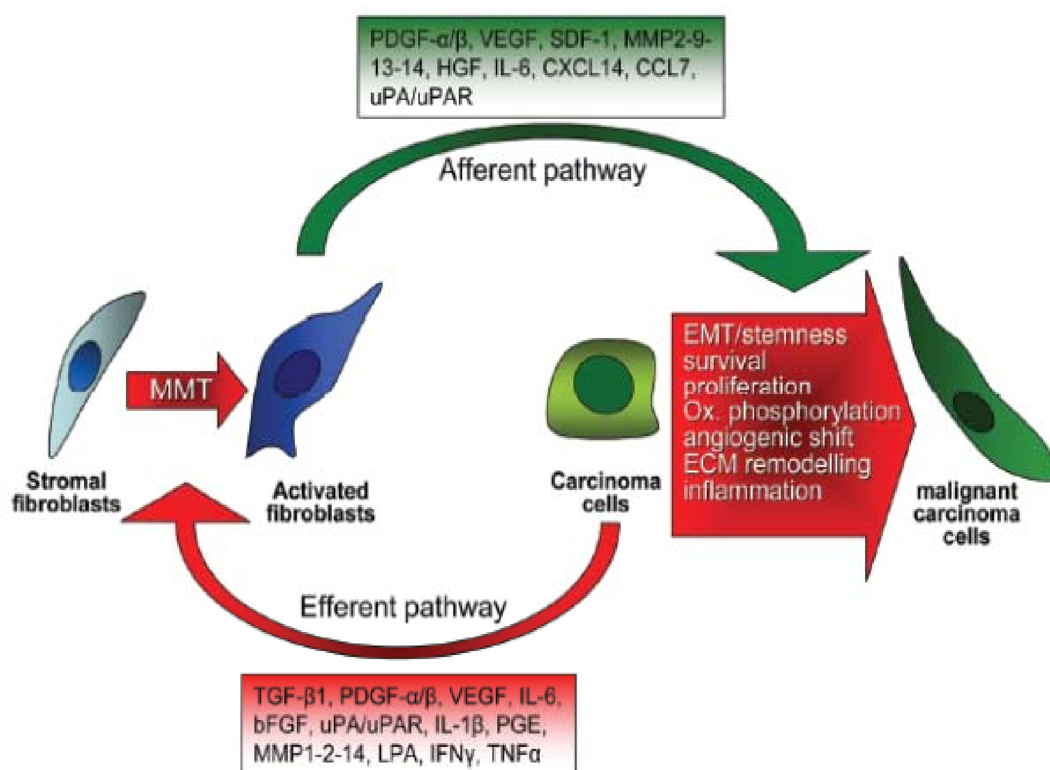

FIGURE 1.5 *Interplay between CAFs and tumor cells. Tumor progression needs a positive and reciprocal feedback between CAFs and cancer cells. Cancer cells induce and maintain the fibroblasts activated phenotype which, in turn, produce a series of growth factors and cytokines that sustain tumor progression by promoting ECM remodelling, cell proliferation, angiogenesis and EMT.*

Conversely, later on CAFs become activated by several tumor secreted factors and promote both tumor growth and progression. Two closely interactive pathways are established in the crosstalk between cancer and stromal cells: a) in the “efferent” pathway, cancer cells trigger a reactive response in the stroma, and b) in the “afferent” pathway, the modified stromal cells in the surrounding microenvironment affect cancer cell responses (35) (Figure a). The trans-differentiation of CAFs, a process commonly called mesenchymal-mesenchymal transition (MMT), is currently poorly understood. TGF- β1 has been largely acknowledged to be one of the major tumor-cell derived factors affecting CAF activation (36). Nevertheless other profibrotic factors can be released by cancer cells and act on CAFs inducing their activation, including PDGF-α/β (37), basic fibroblast growth factor (b-FGF) (38) or interleukin (IL)-6. Several data indicate that activation of CAFs is under a clear redox control. Tumor growth factor (TGF)-β1 causes an increase in reactive oxygen species (ROS) in CAFs, which is responsible for downregulation of gap junctions between CAFs, for their achievement of MFphenotype, as well as for their tumor promoting activity in skin tumors (39,40). Antioxidant treatments, or the micronutrient selenite, prevent CAF activation and their enhancement of tumor invasion (39). In keeping, the activation of prostate CAFs by tumor-secreted IL-6 is again redox-dependent (41), and the oxidative stress due to JunD genetic inactivation promotes myofibroblast differentiation and tumour spreading in breast adenocarcinoma (42). Again antioxidant treatments blocks secretion by CAFs of matrix metalloproteases (MMPs) or stromal-derived factor (SDF)-1, thereby affecting the CAF “efferent” pathway. In resident human mammary fibroblasts progressively converting into CAFs, SDF-1 and TGF-β1 have been involved in the acquisition of two autocrine signaling loops, which initiate and maintain the differentiation of fibroblasts into myofibroblasts and the concurrent tumor-promoting phenotype (43). A key unsolved question on CAFs is their possible multiple origin. It is becoming evident that CAFs origin can vary both between different tumor hystotypes and within different areas of individual tumors. In keeping with the idea to develop an effective therapeutic stromal strategy (see below), extensive information about the taxonomy of CAFs in different tumor is mandatory. We can roughly classify the line of evidence about CAFs origin in: i) resident; ii) mesenchymal stem cell (MSC)-derived; iii) mutational (Fig.1.6).

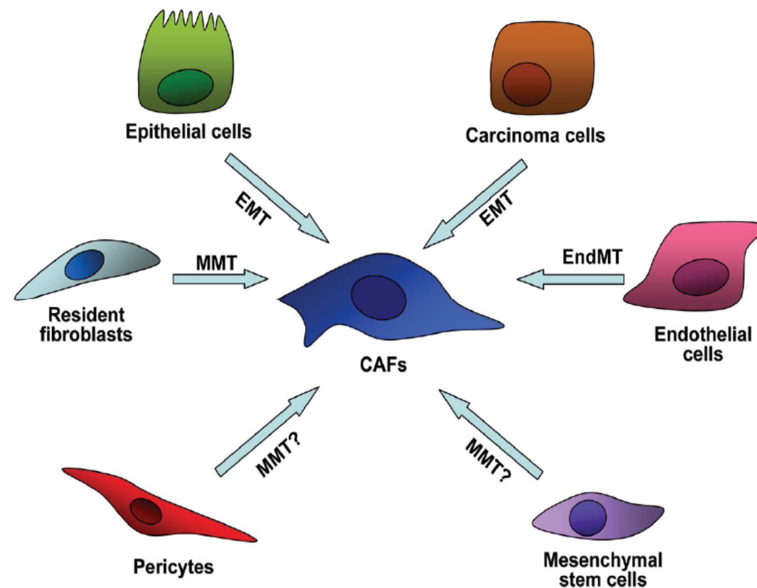

FIGURE 1.6. *Multiple origins of CAFs within tumor microenvironment. CAFs can stem from trans-differentiation of resting resident fibroblasts or pericytes within tumor microenvironment, through mesenchymal mesenchymal transition (MMT). Alternatively CAFs could derive from bone marrow mesenchymal stem cells (MSCs), or from epithelial normal or transformed cells via epithelial to mesenchymal trasnsition (EMT), or finally from endothelial cells via endothelial to mesenchymal transition (EndMT).*

Beside growth factors, pro-inflammatory cytokines, such as interleukins, interferons and members of the tumor necrosis factor family, are produced both by stromal and cancer cells, and exert tumor-modulating effects (44). Expression by CAFs of cytokines and chemokines leads to immune cell infiltration that in turn promotes angiogenesis and metastasis (45). Fibroblastderived SDF-1 enhanced invasiveness of pancreatic cancer cells, showing a synergy with IL-8 in the promotion of a complete angiogenic response in recruited endothelial cells (46). SDF-1 secreted by breast cancer CAFs has been involved in mobilization of endothelial precursor cells from bone marrow, thereby inducing de novo angiogenesis, as well as in tumor growth through a paracrine effect on CXCR4 expressing cancer cells (47). In addition, increased secretion of CXCL14 chemokine by CAFs has been reported in prostate cancer stromal fibroblasts. CXCL14 increases both growth and migration of fibroblasts, which in turn increased their activity on tumor cells affecting their growth, angiogenesis and macrophage infiltration (48). A recent paper demonstrated that CAFs associated to incipient neoplasia exhibit a proinflammatory signature, leading them to mainly overexpress SDF-1, IL-6 and IL-1β, as well as to recruit proangiogenic macrophages and promote tumor growth. This gene set is under the transcriptional control of nuclear factor-κB (NF-κB) and cyclooxygenase 2 (COX-2), thereby strengthening the link between CAFs and inflammatory mediators in tumor progression (49). Furthermore, in breast adenocarcinoma CAFs have been

found affected by oxidative stress-mediated activation of hypoxia-inducible factor-1 (HIF-1), which in turn activates the secretion of SDF-1.Interestingly we have recently reported that in prostate carcinoma CAF contact leads cancer cells to activate the same pro-inflammatory gene signature (NF-κB, COX-2 and HIF-1), leading them to achieve a motile phenotype, and confirming that stromal and tumor cells share common key pathways during tumor progression (50).

1.3 Estrogens

Estrogens are physiologically important hormones in both females and males. Several different types of estrogens are synthesized throughout life: the three major naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the predominant estrogen during reproductive years both in terms of absolute serum levels as well as in terms of estrogenic activity. In post-menopausal women estrone becomes the primary form of estrogen in the body, it is generally considered as the most important and potent estrogen and during pregnancy estriol is the predominant circulating estrogen in terms of serum levels. Although estriol is the most abundant of the three estrogens it is also the weakest, whereas estradiol is the strongest. The main source of estrogens is the ovarian follicles of the fertile woman, while in post-menopausal, the synthesis occurs also in extra-ovarian tissues (bone, skin, placenta, adipose tissue, fibroblasts, hypothalamic neurons). The synthesis takes places from cholesterol, which is converted in androstenedione. Androstenedione is a substance of weak androgenic activity which serves predominantly as a precursor for more potent androgens such as testosterone as well as estrogen. The conversion of androstenedione to testosterone is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD), whereas the conversion of androstenedione and testosterone into estrone and estradiol, respectively is catalyzed by aromatase, a member of the cytochrome P450 super-family, which is represent an important target of hormone responsive tumors (51) (Fig. 1.7).

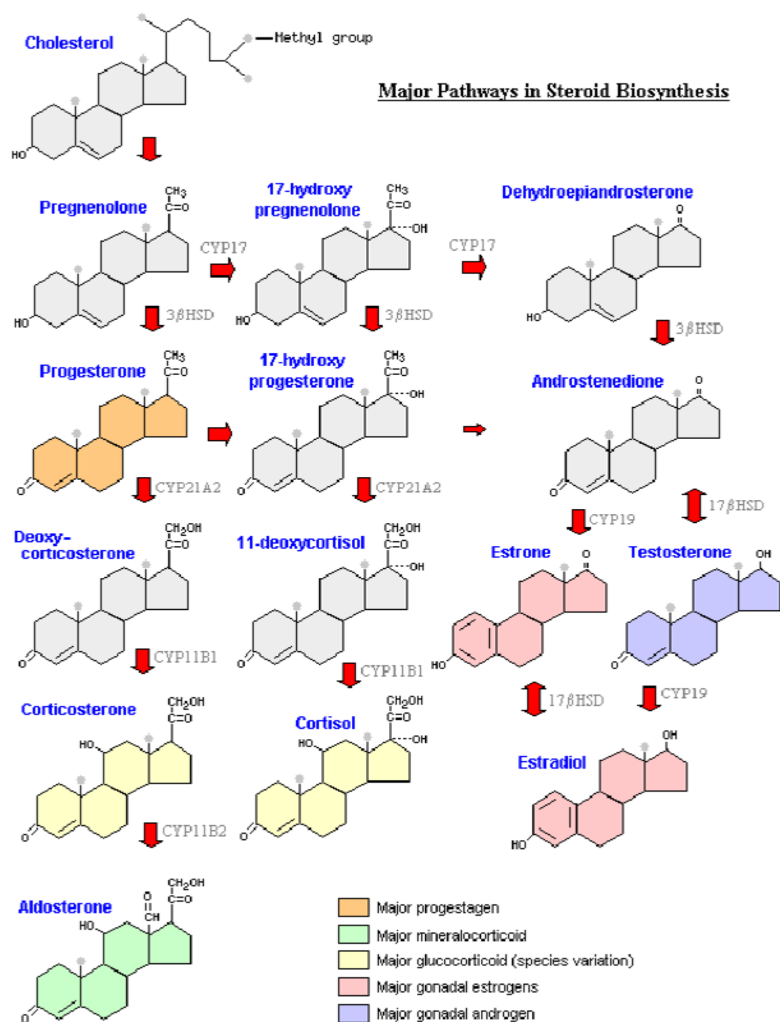

FIGURE 1.7 *Pathway of estrogens biosynthesis.*

Estrogen is primarily a sexual growth hormone. As such, estrogen influences the health and tissue growth of the vagina, fallopian tubes and the oocytes in the ovaries. Estrogen also plays an important role in sexual maturation and development when facilitating the growth of secondary female sexual characteristics, such as the breasts. Estrogen also plays a part in placenta production during fetal growth as well as the regulation of the endometrium walls. (52).

Estrogens production affects the liver. With the help of estrogen, the liver is able to produce important proteins, including those used for blood clotting as well as lipoproteins. Lipoproteins are the vessels that carry specific fats throughout your blood stream. Low-density lipoproteins are responsible for carrying fats to the cells that need it. High-density lipoproteins are responsible for removing fat from the blood stream and sending it back to the liver so that it is unable to calcify into arterial plaque. In the cardiovascular system, it has

been reported that estrogens may exert a protective action against ischemia (53,54) and related diseases which occur in premenopausal women. Estrogen is also an important part of keeping the bones healthy, especially in women; as menopause occurs and estrogen levels decrease, women have a much greater chance of developing bone diseases such as osteoporosis. Although not produced in as great quantities as in women, estrogen also is produced and plays an important role for men as well. Produced in small amounts by the Sertoli cells of the testicle, estrogen helps with sperm health and maturity. Moreover, a wide number of studies have demonstrated that estrogens play also a key role in the development and progression of cancer (55).

The main representative of this class compounds is 17β-estradiol (E2). After production and secretion into the circulation, the vast majority of E2 is transported in the blood bound to serum albumin and sex-hormone binding globulin (SHBG). E2 reaches its target tissues via the blood circulation, and enters the cells by dissociation across the cell membranes.

1.4 Estrogen receptors

1.4.1 ERα and ERβ

The biological effects elicited by these hormones are mainly mediated by the classical estrogen receptor (ER) α and ERβ, which act as ligand-activated transcription factors binding to specific DNA sequences (estrogen responsive element, ERE) located within the promoter region of target genes (56).

The two receptors are frequently distributed in different cells and organs and mediate opposite effects (57,58). ERα is mainly expressed in breast, endometrium, uterus and hypothalamus. The expression of the ERβ protein is found in ovarian stroma cells, kidney, brain, bone, heart, lungs, intestinal mucosa, prostate. The two receptors are encoded by different genes and are located on different chromosomes. ERα is encoded at 6q25.1 and ERβ at 14 q23.2 (59,60). However, structurally the two receptors have much in common. They consist of several individual domains, each with important functions. Close to the COOH-terminus (called the F domain) is the ligand binding domain (LBD-domain) or E-domain with 59% homology between the two receptor subtypes. This region allows the receptors to dimerize and form functional homo- or heterodimers (61). Furthermore, it contains one of the two transcriptional activating domains of the receptors, the activator function 2 (AF-2). The AF-2 domain induces ligand-dependent activation of promoter elements. Differences in the LBD-domain

and hence, the ligand binding pocket, generate ER subtype specificity for both natural and synthetic ligands. The D-domain joins the E-domain to the C-domain which comprises the DNA binding domain (DBD-domain). The NH_2-terminal A/Bdomain contains the AF-1 domain, which is constitutively active and mediates transcriptional activation/inactivation independent of ligand binding. However, this area has only 16 % sequence homology between the receptor subtypes and the AF-1 domain of ERβ has a weaker function than that of ERα (62). Consequently, ERα and ERβ bind to estrogen response elements with similar affinity (Fig.1.8).

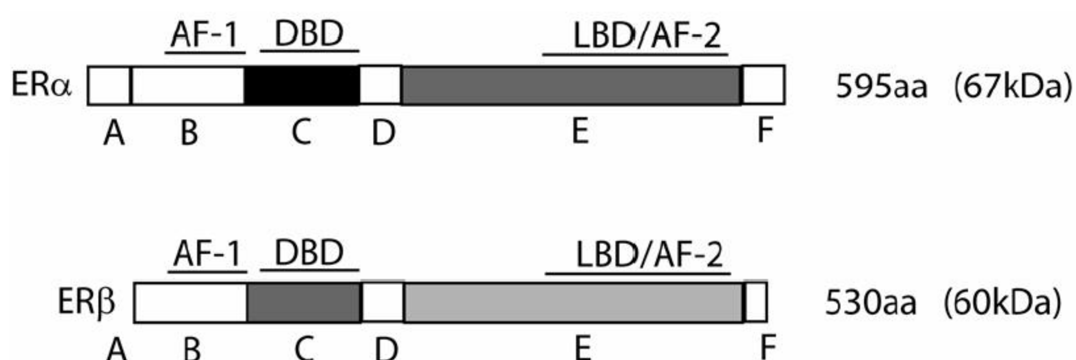

FIGURE 1.8 *Schematic representation of the ERα and ERβ structures. The domains of the receptors include the DBD, LBD and the two transcriptional activator domains AF-1 and AF-2. Full-length ERα is 595 amino acids long whereas ERβ is 530 amino acids long*

In their unloaded state, ERs are associated with inhibitory protein complexes containing Heat Shock Proteins (Hsp90, Hsp70, Hsp56) in the cytosolic or nuclear compartments. Upon ligand activation, the receptors dissociate, change conformation and form functional dimers at certain DNA-elements (52). Depending on the presence of ERα and ERβ or both in a specific cell, the receptors form functional homo or heterodimers on the promoter elements. The classical pathway involves binding of ER-dimers to an estrogen response element (ERE), a palindrome with the sequence GGTCAnnnTGACC, where “n” can be any nucleotide. In addition, ER can bind to DNA directly or indirectly through alternative elements. ER-binding at Activator Protein 1 (AP1) responsive elements involves indirect binding through Jun/Fos-proteins (63). In addition, ER can activate transcription through Specificity Protein 1 (SP1) (64). When the receptors bind to DNA-response elements, transcription is affected through recruitment of co-regulatory proteins. Depending on the promoter context, type of ligand and

receptor subtype, these co-regulatory proteins can be co-activators and co-repressors (65). Co-activators modify the chromatin to facilitate recruitment of RNA-polymerase II, with subsequent transcription. Two ER-associated co-activators, the SRC-family and p300/CBP-associated factor have intrinsic histone acetylase (HAT) activity. In contrast, co-repressors decrease acetylations in the chromatin, resulting in inhibition of the transcription machinery. ER-associated co-repressors such as SMRT and N-CoR recruit histone deacetylases (HDACs) which block the recruitment of the RNApolymerase machinery to the promoter (Fig.1.9).

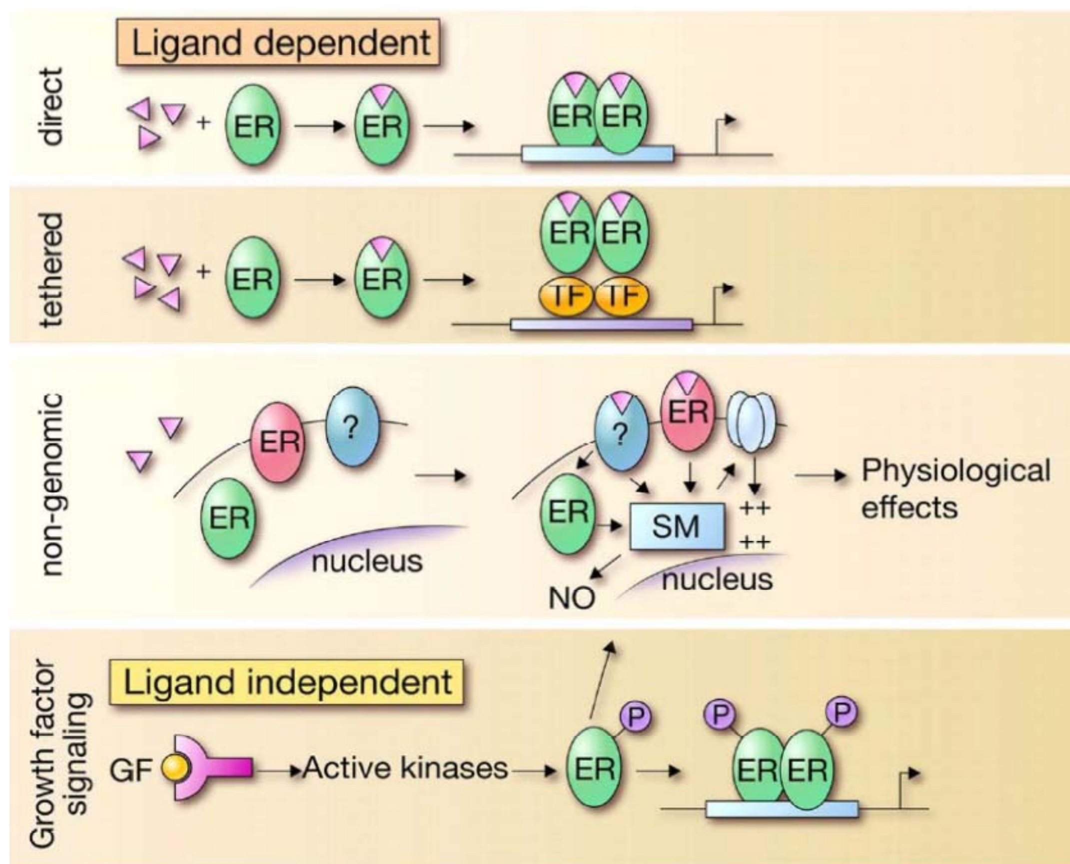

FIGURE 1.9. *Four different pathways of ER action: The classical (direct) pathway includes ligand activation and a direct DNA binding to estrogen response elements (ERE). The tethered pathway involves protein-protein interaction of ERs with other transcription factors and indirect DNA binding. The non-genomic pathway involves rapid estrogenic effects, which have been observed in some cells. ER-activity can also be regulated through a ligand-independent pathway by growth factor signaling. ER; estrogen receptor, GF; growth factor, P; phosphate, SM; second messager and TF; transcription factor.*

1.4.2 G protein-coupled estrogen receptor (GPER)

In the last years, numerous studies have suggested that a member of the 7-transmembrane G protein-coupled receptor family, the G protein-coupled estrogen receptor-1 (GPER, formerly called GPR30), mediates estrogen signals in a wide number of normal and cancer cells (66). In this regard, it has been largely reported that the G protein-coupled receptors (GPCRs) transducer extracellular signals into intracellular effector pathways through the activation of heterotrimeric G proteins which lead to cancer initiation and progression (67). Moreover, multilayered cross-talk between GPCRs and growth factor receptors has an instrumental role in orchestrating downstream signaling molecules that are implicated in cancer development, angiogenesis and metastasis. On the basis of these findings, various GPCRs and their targets represent promising therapeutic targets in drug discovery towards innovative anti-cancer strategies. As it concerns GPER, increasing evidence has indicated that it can be considered as an intriguing signaling molecule involved in complex pathways through which estrogens regulate diverse physiopathological processes (68). In particular, GPER mediates rapid signals induced by estrogens and even antiestrogens like tamoxifen, prompting major biological responses such as gene expression, proliferation and migration in cancer cells (69). In this regard, it has been shown that the ligand binding to GPER triggers the release of the membrane-tethered heparan-bound epidermal growth factors (HB-EGF) which bind to and activate the Epidermal Growth Factor Receptor (EGFR) (70). Then, the EGFR transactivation mediated by GPER stimulates a signaling network which includes calcium mobilization, MAPK and PI3-K activation in a variety of cell types (68). Notably, the expression of GPER has been demonstrated in a wide number of tumors and associated with negative clinical features and poor survival rates in patients with breast, endometrial and ovarian carcinomas, suggesting that GPER may be considered a predictor of an aggressive disease (71,72).

In this context, our previous studies have shown that ligand-activated growth factor receptors up-regulate GPER expression in diverse types of cancer cells (73-75). In particular, EGF and IGF-I were able to transactivate the promoter of GPER and to induce its expression at both mRNA and protein levels, hence highlighting the functional cross-talk which may occur between GPER, EGFR and IGF-IR signaling in estrogen-sensitive tumors. As EGFR and IGF-IR activity has been associated with tamoxifen resistance in breast cancer (76,77), the up-regulation of GPER mediated by these receptors may be included among the mechanisms involved in the failure of tamoxifen therapy in this malignancy (Fig.1.10).

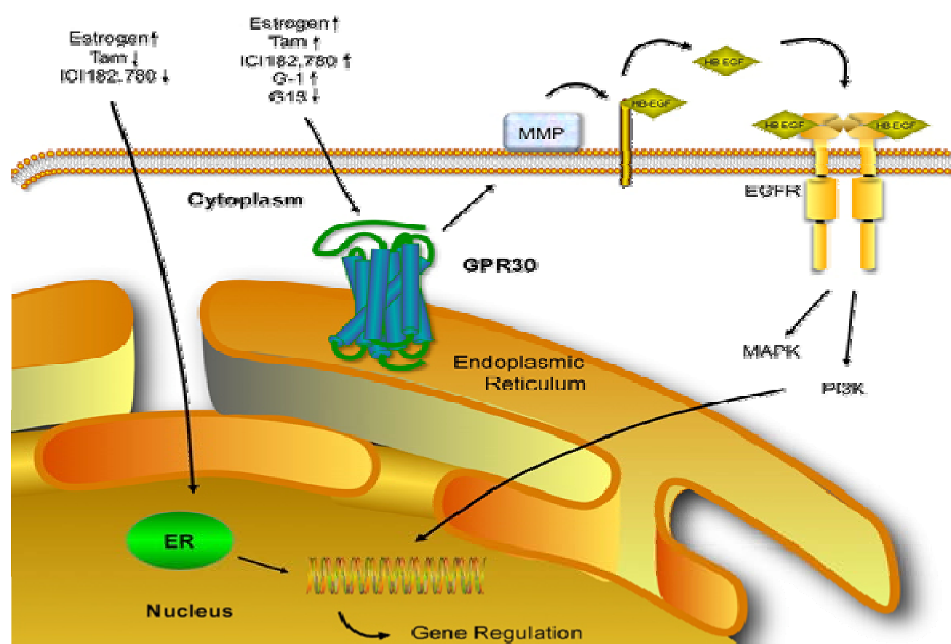

FIGURE 1.10 *Molecular pathways triggering by estrogens through GPER also called GPR30.*

Accordingly, in breast cancer patients treated with tamoxifen alone GPER expression was found increased and associated with reduced survival rates (78). Hence, the promiscuous and opposite action elicited by GPER and ERα ligands has broad implications in the estrogen receptors-mediated activation of signaling pathways and gene transcription. Indeed, a variety of compounds interacts with both the classical ERs and GPER, including estrogens, phyto- and xenoestrogens as well as antiestrogens (66). Consequently, considerable efforts have been made in order to develop selective ligands and antagonist compounds for both GPER and ERα. The identification of the selective GPER agonists G-1 (79), GPER-L1 and GPER-L2 (80) and GPER antagonists G-15 and G-36 (81,82) contributed to better characterize the function role exerted by GPER in diverse model systems, including cancer. Taking into account the main role exerted by both ERs and GPER in hormone-dependent tumors and the mixed agonist/antagonist activity exerted by antiestrogens (i.e. tamoxifen), the identification of the first compound (named MIBE) which is able to bind to and inhibit the transduction signaling mediated by both receptors, could represent a promising pharmacological approach in order to obtain major therapeutic benefits respect to the use of the current selective antagonists (83). Recently, the activation of GPER signaling has been found to occur also following hypoxic conditions, that play a fundamental role in the tumor microenvironment

toward cancer progression (84). Interestingly, the main hypoxia responsive gene HIF-1α mediated GPER expression and function in cancer cells and also in cardiomyocytes, suggesting that GPER may be engaged in the complex adaptive cell responses to low oxygen tension. In the tumor microenvironment, further important players are cancer-associated fibroblasts (CAFs), which strongly cooperate toward tumor growth and invasiveness (85). Interestingly, estrogenic GPER signaling has been involved in gene expression as well as proliferation and migration in CAFs derived from breast cancer malignancies (86). In particular, estrogens induced a cross-talk between GPER and activated EGFR and their recruitment to the cyclin D1 promoter sequence, suggesting that GPER might act also as a transcription factor in CAFs.

1.5 Metabolism in cancer cells

Altered metabolism in human cancers has long been recognized. The first observation of increased anaerobic glycolysis in cancer cells was made by Otto Warburg, the so called "Warburg effect" (87). The "Warburg effect" has now become a hallmark of the transformed phenotype of cancer cells, and is thought to provide growth advantages to these cells (88,89). Multiple molecular mechanisms, both intrinsic and extrinsic, converge to alter core cellular metabolism and provide support for the three basic needs of dividing cells: rapid ATP generation to maintain energy status; increased biosynthesis of macromolecules; and tightened maintenance of appropriate cellular redox status. Metabolic changes are a common feature of cancerous tissues, although it is unclear to what extent these metabolic changes are important in low-grade slow growing tumors. It is becoming clear that alterations to metabolism balance the need of the cell for energy with its equally important need for macromolecular building blocks and maintenance of redox balance. To this end, a key molecule produced as a result of altered cancer metabolism is reduced nicotinamide adenine dinucleotide phosphate (NADPH), which functions as a cofactor and provides reducing power in many enzymatic reactions that are crucial for macromolecular biosynthesis. NADPH is also an antioxidant and forms part of the defence against reactive oxygen species (ROS) that are produced during rapid proliferation. High levels of ROS can cause damage to macromolecules, which can induce senescence and apoptosis. Cells work against the negative effects of ROS by producing antioxidant molecules, such as reduced glutathione (GSH) and thioredoxin (TRX). Several of these antioxidant systems, including GSH and TRX, rely on the reducing power of NADPH to maintain their activities (90,91) (Fig.1.11).

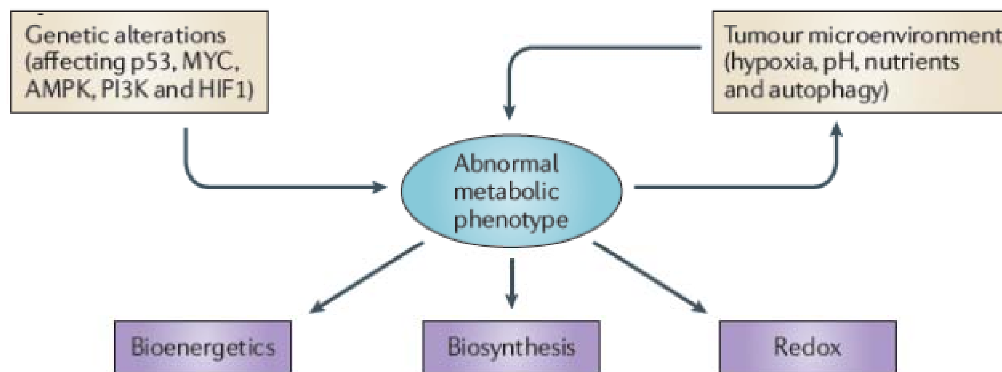

FIGURE 1.11 *Determinants of the tumour metabolic phenotype. The metabolic phenotype of tumour cells is controlled by intrinsic genetic mutations and external responses to the tumour microenvironment. Oncogenic signalling pathways controlling growth and survival are often activated by the loss of tumour suppressors (such as p53) or the activation of oncoproteins (such as PI3-K). The resulting altered signalling modifies cellular metabolism to match the requirements of cell division. Abnormal microenvironmental conditions such as hypoxia, low pH and/or nutrient deprivation elicit responses from tumour cells, including autophagy, which further affect metabolic activity. These adaptations optimize tumour cell metabolism for proliferation by providing appropriate levels of energy in the form of ATP, biosynthetic capacity and the maintenance of balanced redox status. AMPK, AMP-activated protein kinase; HIF1, hypoxia-inducible factor 1.*

In addition to the genetic changes that alter tumor cell metabolism, the abnormal tumor microenvironment such as hypoxia, pH and low glucose concentrations have a major role in determining the metabolic phenotype of cancer cells (92-94). Mutations in oncogenes and tumor suppressor genes cause alterations to multiple intracellular signalling pathways that affect tumor.

1.5.1 Fatty acid synthase (FASN)

One of the metabolic changes in cancer is the altered lipogenic pathway with increased de novo fatty acid synthesis (95). Fatty acids serve as important substrates of metabolism for energy, essential building blocks of cellular membranes, intracellular second messengers, and anchorage for membrane proteins. Fatty acids exist either as components of triacylglycerol, phospholipids and cholesterol or in free forms. Free fatty acids include dietary ones and the ones derived from de novo synthesis catalyzed by fatty acid synthase (FASN) in lipogenic tissues such as liver, adipose tissue, lactating breast and cycling endometrium. However, the altered lipogenic pathway in cancers did not become a focus of interest until 1994, when Kuhjada and colleagues identified the oncogenic antigen-519 (OA-519), a molecule found in tumor cells from breast cancer patients with markedly worsened prognosis, as fatty acid synthase (FASN) (96). Human FASN is a 270kDa cytosolic enzyme that is responsible for 16

carbon fatty acid palmitate synthesis in cells, by condensation of acetyl-CoA and malonyl-CoA using NADPH as cofactor (Fig.1.12).

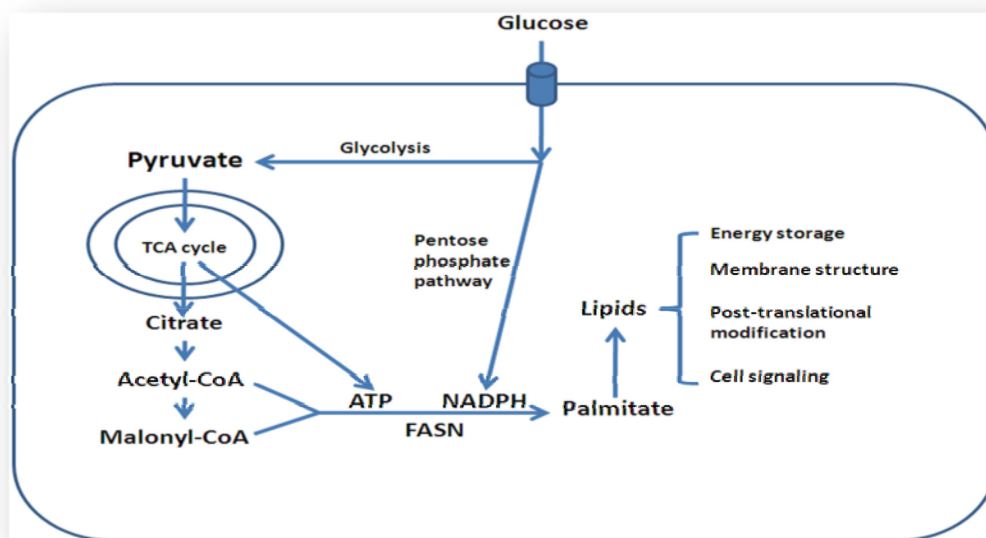

FIGURE 1.12 *Role of FASN in lipid metabolism.*

It is also referred as the cytosolic type I FASN complex while type II fatty acid synthesis system exists in mammalian mitochondria, which resembles the prokaryotic type II FASN. It is believed that the type II system produces fatty acids that play important roles in the mitochondrial function (97). The type I FASN has recently been shown to have oncogenic activity (98,99) and its inhibition has been shown to effectively and selectively kill cancer cells, with minimal side effects to normal cells (100-102). Thus, targeting type I FASN opens a new window of opportunity for metabolically combating cancers. In this review, we will focus on the cytosolic type I FASN protein and perform a critical review on the recent progresses in understanding the structure, function, and the role of FASN in cancers and pharmacological targeting FASN for human cancer treatment. In normal adults, FASN is primarily expressed in hormone-sensitive cells and cells with high lipid metabolisms (103). FASN expression in normal liver and adipose tissues is controlled mainly by nutritional signals. In a well-nourished individual, normal cells are rarely needed and the FASN protein level is low. Carbohydrate ingestion, thyroid hormone, insulin, and glucocorticoid coordinately up-regulate while unsaturated fatty acids, cyclic-AMP, and glucagon down-regulate FASN expression (104). In cycling endometrium, FASN expression is high in the proliferative phase and decreases in the secretory differentiation phase. Proliferative gland

and stroma cells have high levels of FASN, as well as high levels of estrogen and progesterone receptors, indicating that FASN expression may be under the control by hormone and associate with proliferation (105). In lactating breast tissues, FASN expression is up-regulated to produce milk fat (106). In cancer cells and pre-neoplastic lesions, the expression of FASN has been found to be up-regulated (105,107-113). Because of FASN up-regulation, over 90% of the triacylglycerol in cancer cells are synthesized de novo despite the presence of high levels of circulating free fatty acids. Cancer cells are so dependent on de novo fatty acid synthesis that inhibition of lipogenesis targeting FASN induces apoptosis selectively in human cancer cells both in vitro and in vivo (114-116), with minimal effect on normal cells (102,117). FASN expression in cancer cells is no longer responsive to the nutritional signals and its expression is regulated at multiple steps including gene amplification, transcription, translation and post-translational modifications. The increased FASN gene copy number has been found in prostate cancer cell line PC-3 and LNCaP, as well as in prostate adenocarcinoma and metastatic cancers (118). The increased FASN staining in tumor tissues correlates with a 25% increase in gene copy number, whereas in benign tissues, only 1% of the cells with high FASN staining showed increased gene copy number. Thus, gene amplification in cancer cells may partly contribute to the increased FASN expression in prostate cancers. Transcriptional regulation of FASN expression has been well-studied and is considered the major contributor to the increased FASN expression in cancer cells. Growth factors, hormones and their receptors have been shown to be the main factors that cause up-regulation of FASN transcription in cancer cells. Epidermal growth factor (EGF) can stimulate FASN expression through EGF receptor ERBB1 and ERBB2 (119). In breast and prostate cancer cells that have functional hormone receptors, FASN expression has been shown to be up-regulated at transcriptional level upon hormone treatment (52,120). The effect of growth factors or hormones and their receptors on FASN expression involves complicated downstream signaling and crosstalk between multiple signal transduction pathways. The two well-studied major pathways that are possibly involved in regulating FASN expression are the mitogen-activated protein kinase (MAPK) and PI3-K/AKT pathways. The major transcription factor that is involved in regulating FASN transcription is sterol regulatory element binding protein 1 (SREBP-1). SREBP-1 is one of the two SREBP membrane bound transcription factors of the basic-helix-loop-helix-leucine zipper family that regulate fatty acid and cholesterol synthesis (121). The membrane-bound SREBPs are activated and released from membranes by protease cleavage in response to fatty acid and cholesterol depletion. The active SREBPs then translocate into nucleus and activate gene

transcription. It has been suggested that SREBP-1 is important in regulating fatty acid synthesis while SREBP-2 is for cholesterol synthesis (122) (Fig.1.13).

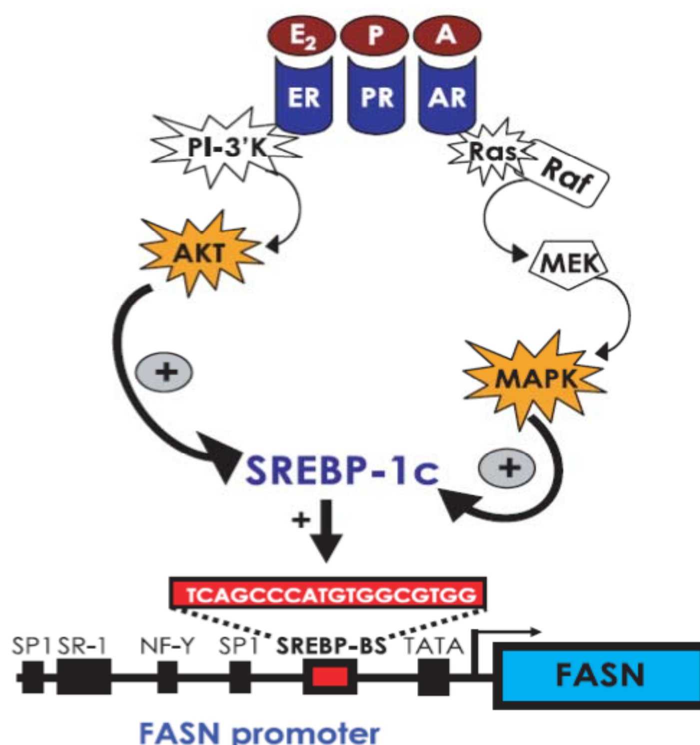

FIGURE 1.13 *Hormonal regulation of FASN gene expression in cancer cells. FASN gene regulation in hormone-sensitive neoplastic cells seems to occur through modulation of the transcription factor SREBP-1c, a critical intermediate in the pro- and antilipogenic actions of several hormones and nutrients that binds to sterol regulatory elements (SREBP-BS) in the promoter region of the target gene FASN. SREBP-1c up-regulation and nuclear maturation appears to be driven by the activation of MEK1/MEK2 3 ERK1/2 MAPK, and PI3-K AKT signaling cascades that occurs in response to the specific binding of SHs such as androgens (A), progestins (P), and E2 to their receptors (AR, PR and ER, respectively).*

Regulation of FASN expression at its post-translational stability/degradation step has also been suggested. In prostate cancer cells, FASN protein stability has been shown to be regulated by an ubiquitin-specific protease, USP2a (123). Knockdown of USP2a reduced FASN expression. Microarray analysis from human prostate cancers has revealed a significant association between the genes in fatty acid metabolism and high USP2a expression (124). As discussed above, FASN was initially identified as an independent prognostic molecule in breast cancer cells from patients with markedly worsened prognosis (96,125). Breast cancers with high level of FASN staining were 4 times more likely to recur and metastasize than the ones with no staining (125). Further studies of breast cancer samples indicated that patients with high FASN expression showed significantly shorter disease free survival and overall survival, even in patients with very early stage of breast cancer (97). It is now clear that

increased FASN expression associates with cancer progression, higher risk of recurrence and shorter survival in many other types of cancers including prostate cancer (126), renal cell carcinoma (127), endometrium carcinoma (128), colorectal carcinoma (129) ovarian neoplasms (130), squamous cell carcinoma of lung (131), melanoma (132), nephroblastoma (133), soft tissue sarcoma (134), head and neck squamous cell carcinoma (112), pancreatic carcinoma (111) and squamous cell carcinoma of the tongue (113). As discussed above, several signal transduction pathways may mediate the function of FASN in tumorigenesis and resistance to drug treatments. Although the detailed mechanism of FASN action in signal transduction pathways remains to be determined, various hypotheses have been proposed. Fatty acids synthesized by FASN in cancer cells are not only used for cellular membrane construction, but also involved in the production of lipid signaling molecules, anchorage of membrane proteins, and modulate cellular responses to anticancer drugs.

Chapter II

Materials and Methods

2.1 Materials

17β-Estradiol (E2) and cerulenin were purchased from Sigma-Aldrich Srl. (Milan, Italy). Tyrphostin AG1478 (AG) was purchased from Biomol Research Laboratories, Inc (Milan, Italy). PD98059 (PD) was obtained from Calbiochem (Milan, Italy). 1-[4-(-6-Bromobenzol [1, 3] diodo-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c-] quinolin8yl] ethanone (G-1) was purchased from Merck KGaA (Frankfurt, Germany). All compounds were dissolved in DMSO, except for cerulenin which was solubilized in ethanol.

2.2 Cell cultures

The SkBr3 breast cancer cells were maintained in RPMI-1640 (Invitrogen, Gibco, Milan, Italy) without phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin. The LoVo colorectal adenocarcinoma cells and the LNCaP prostate cancer cells were maintained in RPMI-1640 with phenol red, supplemented with 10% fetal bovine serum (FBS) and 100μg/ml penicillin/streptomycin. The hepatocarcinoma cells HepG2 and the MCF-7 breast cancer cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin. All cell lines were grown in a 37°C incubator with 5% CO2. Cancer-associated fibroblasts (CAFs) were extracted as previously described (135) and maintained in a mixture of MEDIUM 199 and HAM'S F-12 (1:1) supplemented with 10% FBS. Briefly, breast cancer specimens were collected from primary tumors of patients who had undergone surgery. Tissues from tumors were cut into smaller pieces (1-2 mm 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% CO2. Primary cells cultures of breast fibroblasts 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 assess fibroblasts activation, we used anti-fibroblast activated protein α (FAPα) antibody (H-56; Santa Cruz Biotechnology DBA) (Fig. 2.1).

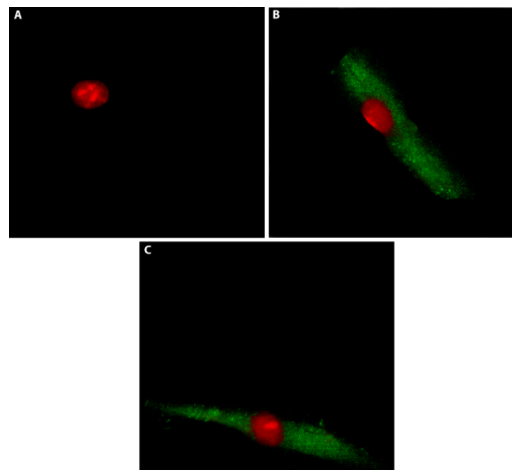

FIGURE 2.1 *Characterization of CAFs. CAFs were immunostained by anti-cytokeratin 14 (A), anti-vimentin (B) and anti FAPα (C) antibody.*

2.3 Gene expression studies

Total RNA was extracted using Trizol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gels stained with ethidium bromide. Only samples that were not degraded and showed clear 18S and 28S bands under ultraviolet light were used for real-time PCR. Total cDNA was synthesized from the RNA by reverse transcription using the murine leukaemia virus reverse transcriptase (Invitrogen, Milan, Italy) following the protocol provided by the manufacturer. The expression of selected gene was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc., Milano, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc., Milano, Italy) and are as follows: FASN and the ribosomal protein 18S, which was used as a control gene to obtain normalized values: FASN (Human) Fwd: 5'-CATCCAGATAGGCCTCATAGAC-3' and Rev: 5'-CTCCATGAAGTAGGAGTGGAAG-3'; 18S (human, mouse) Fwd: 5'-GGCGTCCCCCAACTTCTTA-3' and Rev: 5'-

GGGCATCACAGACCTGTTATT-3'. Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression. For all experiments, cells were switched to medium without serum 24h before treatments. FASN expression was evaluated also using semiquantitative RT-PCR, as previously described (data not shown) (136).

2.4 Western blot analysis

SkBr3, LoVo, HepG2 cells and CAFs were grown in 10-cm dishes and exposed to drugs for the appropriate time, then washed twice with ice-cold PBS and solubilized with 50 mM Hepes buffered solution, pH 7.5, containing 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Protein concentration in the supernatant was determined according to the Bradford method. Equal amounts (10-50µg) of the whole cell lysate was electrophoresed through a reducing SDS/8% (w/v) polyacrylamide gel and electroblotted onto a nitrocellulose membrane which was probed with primary antibodies against FASN (A-5), c-Fos (H-125), phosphorylated ERK1/2 (E-4), ERK2 (C-14), GPER (N-15), EGFR (1005), p-EGFR Tyr 1173 (sc-12351) and β-actin (C2), all purchased from Santa Cruz Biotechnology, Inc. The levels of proteins and phosphoproteins were detected, after incubation with the horseradish peroxidase-linked secondary antibodies, by the ECL® (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy).

2.5 Gene silencing experiments and plasmids

Cells were plated onto 10-cm dishes, maintained in serum-free medium for 24 h and then transfected for additional 24h or 48h before treatments with a control vector or an independent shRNA sequence for each target gene using Fugene6 (Roche Molecular Biochemicals, Milan, Italy). The shRNA plasmid for EGFR was purchased from SABioscience Corporation (Frederick, MD, USA). Short hairpin constructs against human GPER (shGPER) were generated and used as previously described (69). The plasmid DN/*c-fos*, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA binding (137), was a kind gift from Dr. C. Vinson (NIH, Bethesda, MD, USA). The expression vector for Flag-tagged human GPER has been described (73). It was used to generate the GPER rescue vector containing silent mutations in the shRNA targeted sequence: codons 293-297 were changed to CCG TGTAAA CAAAGT. The expression

vector for human FASN was a kind gift from Dr. M. Loda (Dana-Farber Cancer Institute, Boston, MA, 02115).

2.6 Immunostaining assay

Fifty percent confluent cultured SkBr3, LoVo, HepG2 cells and CAFs grown on cover slips were serum deprived for 24h and treated for 18h with 1nM E2. Then cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS and incubated overnight with a mouse primary antibody against FASN (1:500). After incubation, the slides were extensively washed with PBS and incubated with propidium iodide (1:1000 Sigma-Aldrich) for cell nuclei detection and donkey anti-mouse IgG-FITC (1:250; purchased from Santa Cruz Biotechnology). Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS) were used for experiment evaluation.

2.7 Chromatin immunoprecipitation assay (ChIP)

SkBr3, LoVo, HepG2 cells and CAFs were grown in 10-cm dishes to 70-80% confluence, shifted to serum free medium for 24h and then treated with vehicle, 1nM E2, and G-1 for 3h. Thereafter, cells were cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with the anti-FASN antibody or non specific IgG (Santa Cruz Biotechnology, DBA, Milan, Italy). Pellets were washed, eluted with a buffer consisting of 1% SDS and 0.1 mol/L $NaHCO_3$, and digested with proteinase K. DNA was obtained by phenol/chloroform extraction and precipitated with ethanol. A 4μl volume of each sample was used as template to amplify an AP1 containing region corresponding to -1606/-1596 located in the 5'-flanking region of FASN gene by real-time PCR (Applied Biosystems, Milan, Italy). The primers used were: Fwd 5'-CTGGCAGCCAGGGCCA-3' and Rev 5'-GCTGTGGTTGACGCACGG-3'. To verify the specificity of c-Fos recruitment at the AP1 site, we also performed ChIP assay using the following primers: Fwd 5'-ACGCTCATTGGCCTGGG-3' and Rev 5'-TGGCTCCCTCTAGGCCGG-3' which amplify the estrogen target gene SREBP-1c containing region corresponding to -189/-171 located in the 5'-flanking region of FASN gene (138). In particular, it was shown that the binding to the SREBP-1c site occurs in an ER-dependent manner upon estrogen stimulation (138). Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA

quantification was performed by using 4µl of the template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as percent differences with respect to the relative inputs.

2.7 Proliferation assay

For quantitative proliferation assays $1x10^4$ SkBr3, LoVo, HepG2 cells and CAFs were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and further incubated in a medium supplemented with 2.5% charcoal-treated FBS. Ligands were added at this point; medium was changed every day (with ligands and cerulenin were applicable). On day 6 (after 5 days of treatment), cells were trypsinized and counted using Countess™ automated cell counter (purchased from Invitrogen Milan, Italy).

2.8 Migration assay

Migration assays were performed using Boyden chambers (Costar Transwell, 8mm polycarbonate membrane). Cells were seeded in the upper chambers. E2 and G-1 alone or in combination with cerulenin were added to the medium without serum in the bottom wells. After 24h, cells on the bottom side of the membrane were fixed and counted.

2.9 FASN enzymatic activity assay

FASN activity in whole cells was measured by the incorporation of [1, 2 ^{14}C] acetate (Perkin-Elmer Milan, Italy) into fatty acids. Cells were plated in 6 well plates at $3x10^5$ cells per dish and incubated overnight. The next day after 12 hours of starvation, cells were treated with vehicle, 1nM E2, 1µM cerulenin and 1nM E2 + 1µM cerulenin overnight and then incubated with 0,5µCi /ml [1, 2 ^{14}C] acetate for 8 hours. Cells were washed and harvested in 1x phosphate-buffered saline (1x PBS) and [1, 2 ^{14}C] incorporated lipids were extracted with chloroform/methanol (1:4). After centrifugation at 12000×g for 10 minutes, the lower phase containing radiolabeled lipids was counted by scintillation counter. FASN activity was calculated as nmol/mg total protein/min and variations were reported as fold respect to the vehicle-treated cells. Each experiment was repeated at least in triplicate.

2.10 Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman-Keuls' testing to determine differences in means. $P<0.05$ was considered as statistically significant.

Chapter III

Results

3.1 E2 and G-1 induce FASN expression in ER-negative cancer cells.

In order to provide novel insights into the mechanisms by which estrogens may regulate FASN gene in cancer cells, we began the present study evaluating FASN expression upon exposure to E2 and the GPER ligand G-1 in breast SkBr3, colorectal LoVo, hepatocarcinoma HepG2 tumor cells and in CAFs which lack the classical ERs but express GPER (Figure 3.1).

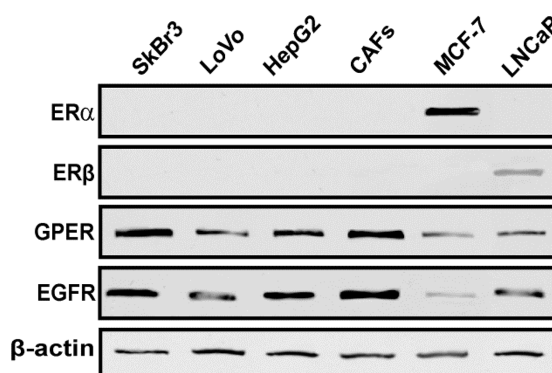

FIGURE 3.1 *Immunoblotting of ERα, ERβ, GPER, EGFR in SkBr3, LoVo, HepG2 cells and CAFs. MCF-7 breast and LNCaP prostate cancer cells were used as control for the expression of ERα and ERβ, respectively. β-actin was used as loading control.*

In time course experiments, E2 and G-1 showed the ability to increase the mRNA expression of FASN, as evaluated by real-time PCR (Figure 3.2 A-D). The up-regulation of FASN mRNA was paralleled by increased FASN protein levels upon exposure to E2 and G-1 (Figure 3.2 E-L), as also evaluated by immunofluorescence studies (Figure 3.3).

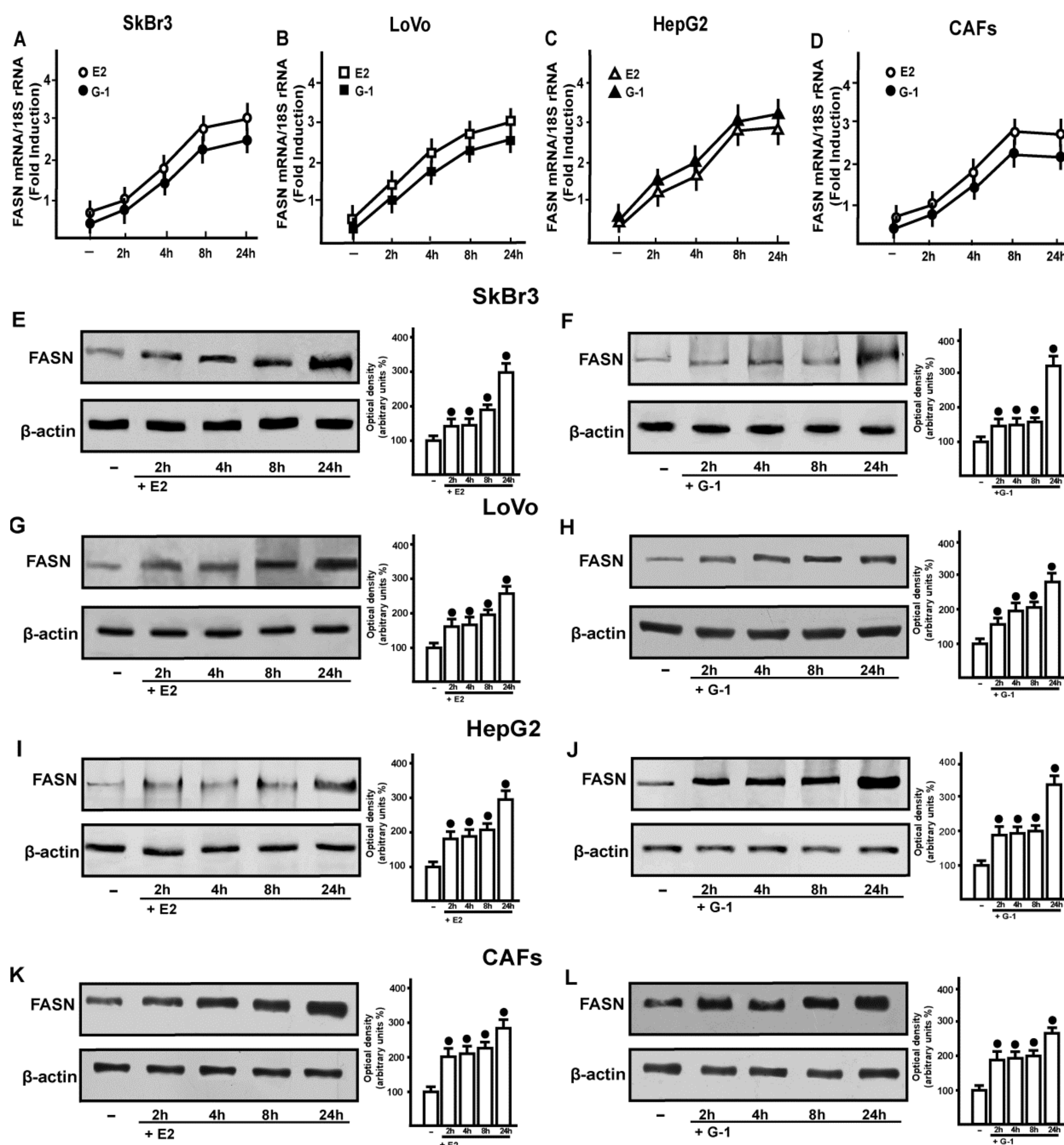

FIGURE 3.2 *E2 and G-1 induce FASN expression in SkBr3, LoVo, HepG2 cells and CAFs. 1nM E2 and 1µM G-1 up-regulate FASN expression at both mRNA (A-D) and protein level (E-L), as evaluated by real-time PCR and immunoblotting, respectively. In RNA experiments, gene expression was normalized to 18S expression and results are shown as fold changes of mRNA expression compared to cells treated with vehicle (-). Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. (●) indicate $p < 0.05$ for cells receiving vehicle (-) versus treatments.*

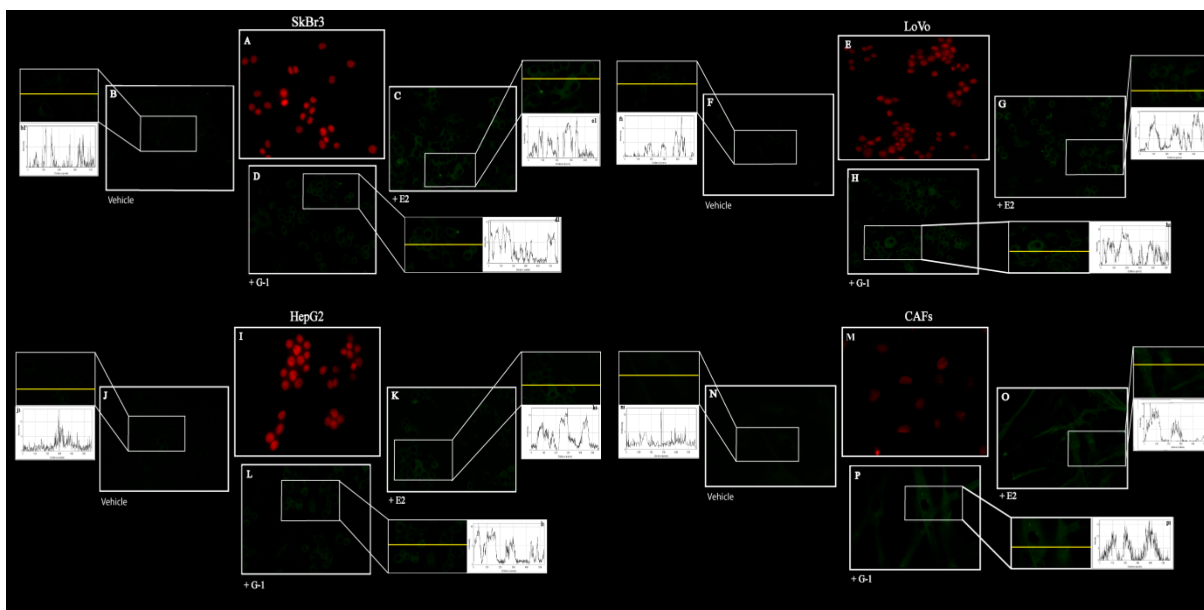

FIGURE 3.3 *Representative fluorescence images of FASN immunolabelling. SkBr3, LoVo, HepG2 cells and CAFs were fixed, permeabilized, and stained with anti-FASN antibody. (A, E, I, M), nuclei (in red) were stained by propidium iodide. Cells were treated for 24h with vehicle (B, F, J, N), 1nM E2 (C, G, K, O) and 1µM G-1 (D, H, L, P) and FASN accumulation is evidenced by the green signal. For descriptive purposes, panels b1, c1, d1, f1, g1, h1, j1, k1, l1, n1, m1, p1 show the plot profiles obtained at the level of the yellow line of the corresponding inset using the program WCIF Image J for Windows. Note the higher values indicating zones of intense labeling. Each experiment shown is representative of 10 random fields. Data are representative of three independent experiments.*

3.2 GPER/EGFR/ERK/c-Fos/AP1 signaling mediates FASN expression induced by estrogens.

These results prompted us to evaluate the role exerted by GPER signaling in the up-regulation of FASN by E2. Silencing GPER in all cells used, E2 and G-1 did not induce FASN expression suggesting that GPER mediates this effect. The requirement for GPER and the specificity of the GPER knockdown were further emphasized by the fact that the co-transfection of a shRNA-resistant version of GPER ('GPER rescue') restored the response. (Figure 3.4 A,C,E,G).

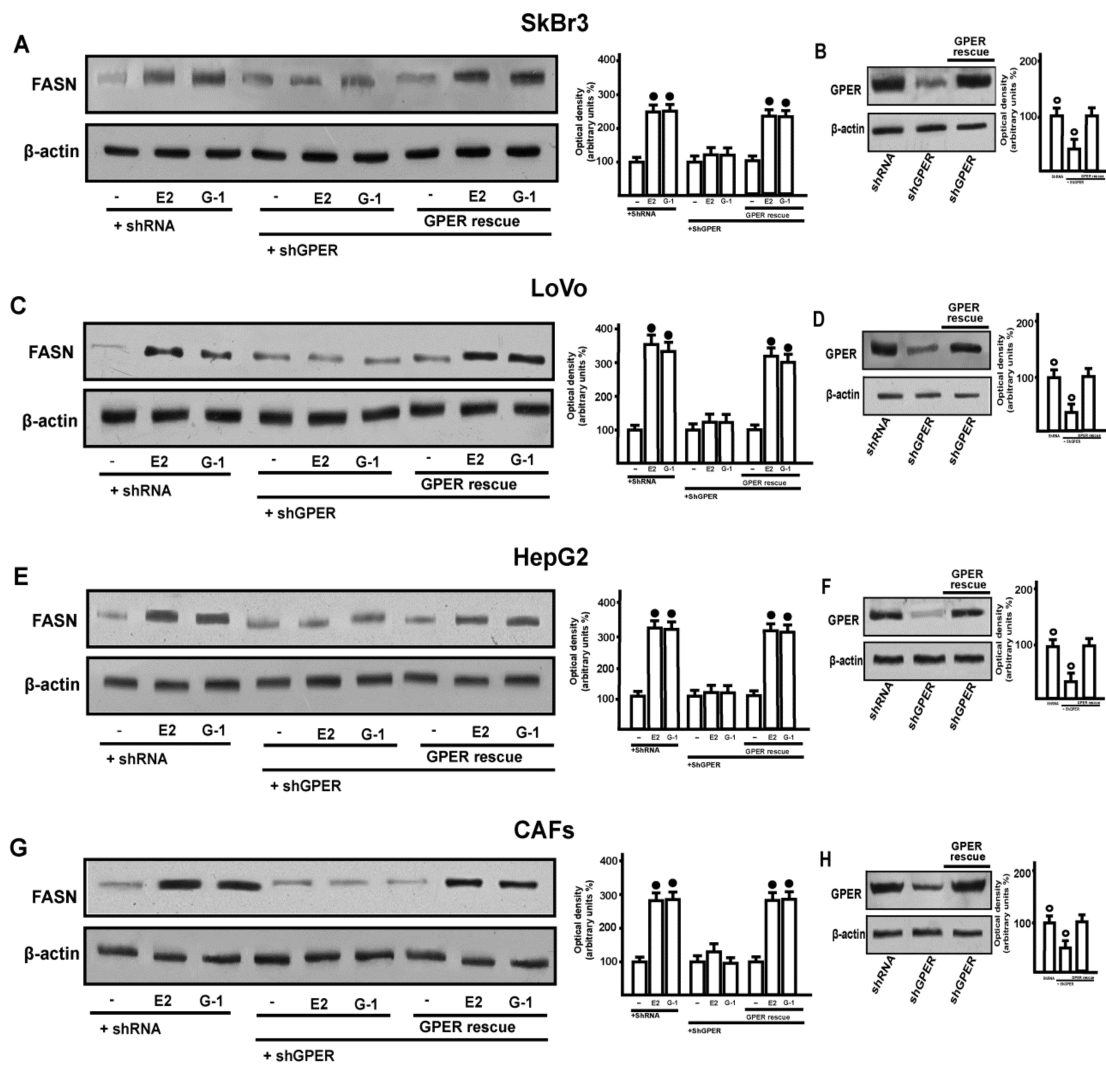

FIGURE 3.4 *GPER mediates the up-regulation of FASN protein levels by E2 and G-1 in SkBr3, LoVo HepG2 cells and CAFs. (A, C, E, G) the up-regulation of FASN by 1nM E2 or 1μM G-1 is abolished transfecting cells with shGPER and restored co-transfecting a resistant version of GPER named "GPR30 rescue". Side panels show densitometric analyses of the blots normalized to β-actin. (B, D, F, H) efficacy of GPER silencing and the restored GPER protein with GPER rescue. Each data point represents the mean ± SD of three independent experiments. (●, ○) indicate p< 0.05 for cells receiving vehicle (-) versus treatments.*

As GPER activation triggers EGFR signaling (139,73), we next demonstrated that the increase of FASN protein levels induced by E2 and G-1 requires EGFR as determined through gene silencing experiment (Figure 3.5 A,C,E,G).

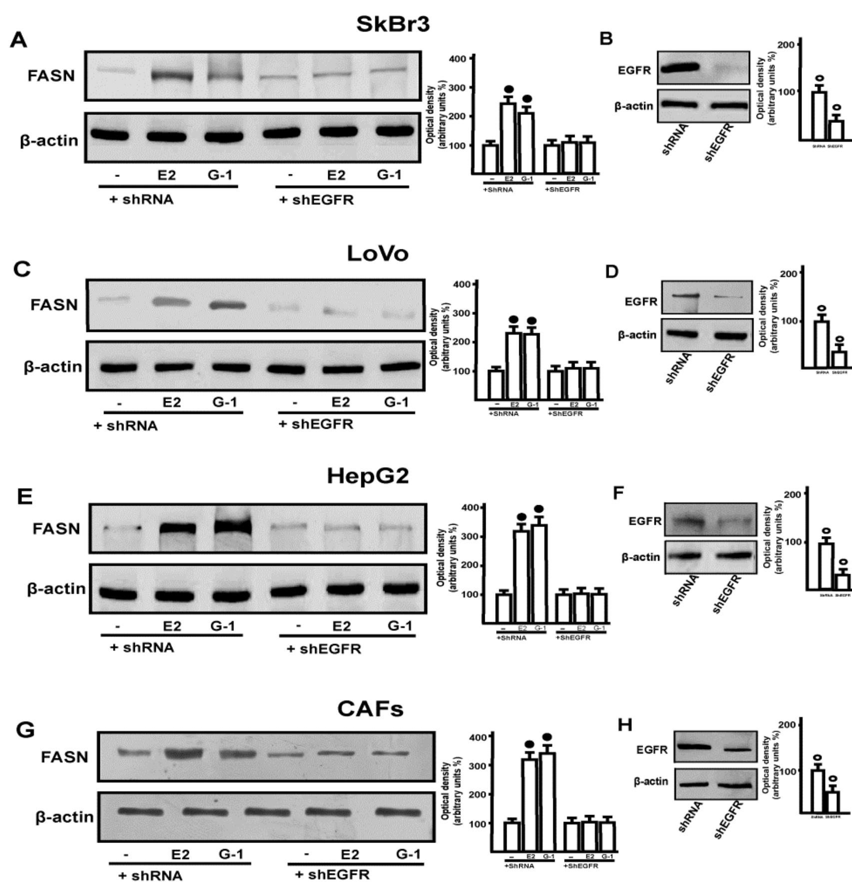

FIGURE 3.5 *EGFR is required for the up-regulation of FASN protein levels by E2 and G-1 in SkBr3, LoVo, HepG2 cells and CAFs. (A, C, E, G) cells were transfected with shRNA or shEFGR for 24h and then treated with 1nM E2 or 1μM G-1 for 24h. Side panels show densitometric analyses of blot normalized to β-actin. (B, D, F, H), efficacy of EGFR silencing. Each data point represents the mean ± SD of three independent experiments. (●, ○) indicate p<0.05 for cells treated with vehicle (-) versus treatments.*

Confirming this observation, E2 and G-1 lost the ability to up-regulate FASN protein expression using the EGFR inhibitor AG1478 (AG) as well as the MEK inhibitor PD98059 (PD) (Figure 3.6 A-D).

The latter finding was nicely supported by EGFR activation and the rapid ERK phosphorylation induced by E2 and G-1 in SkBr3, LoVo, HepG2 cancer cells and in CAFs (Figure 3.6 E-H).

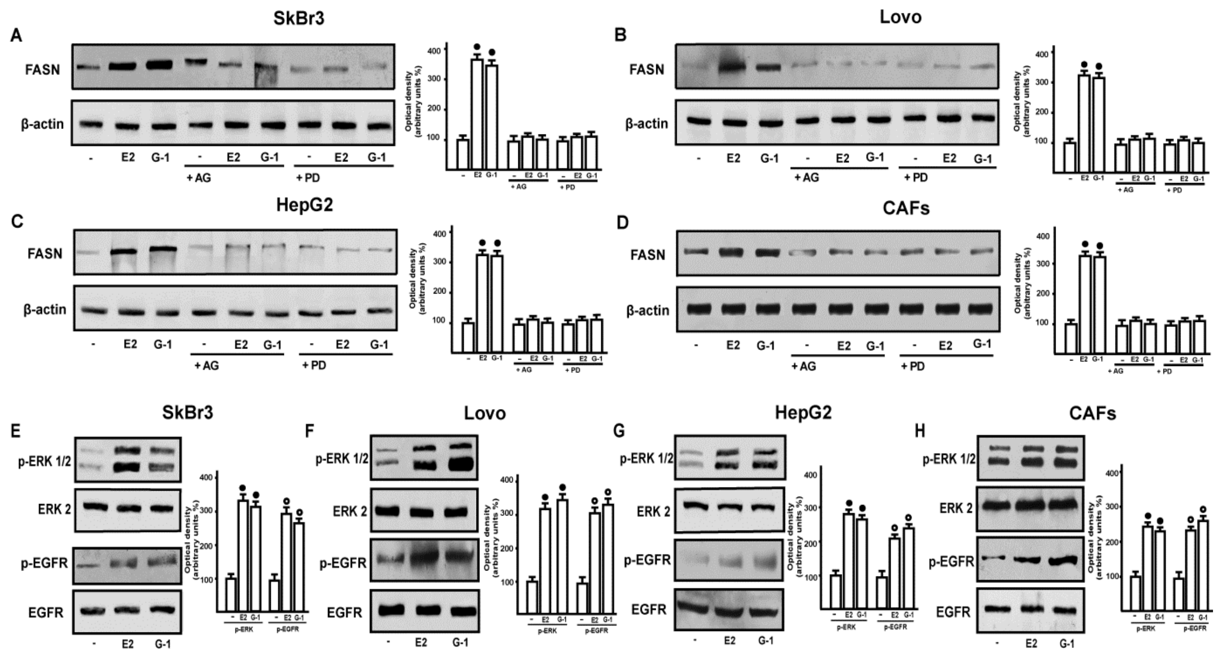

FIGURE 3.6 *The EGFR/ERK signaling mediates the up-regulation of FASN induced by E2 and G-1 in SkBr3, LoVo, HepG2 cells and CAFs. (A-D), cells were treated for 24h with vehicle (-), 1nM E2 and 1µM G-1 alone and in combination with 10µM EGFR inhibitor AG1478 (AG), 10µM MEK inhibitor PD98089 (PD). (E-H) ERK1/2 activation and $EGFR^{Tyr1173}$ phosphorylation in SkBr3, LoVo, HepG2 cells and CAFs treated with vehicle (-), 1nM E2 and 1µM G-1 for 15 min. Side panels show densitometric analyses of the blots normalized to β-actin (in the case of FASN expression), ERK2 (in the case of p-ERK1/2), EGFR (in the case of p-EGFR). Each data point represents the mean ± SD of three independent experiments. (●, ○) indicate $p<0.05$ for cells receiving vehicle (-) versus treatments.*

As the GPER/EGFR/ERK transduction signaling triggers c-Fos expression (73,67,83), we first ascertained this response to E2 and G-1 (Figure 3.7 A-H) and then we determined that c-Fos is recruited to the AP1 site located within the promoter sequence of FASN (Figure 3.7 I-L). Amplifying a FASN promoter region containing the SREBP-1c site which is an ER-mediated estrogen target gene (138), we did not observe the recruitment of c-Fos (data not shown) hence indicating the specificity of its binding to the AP1 site.

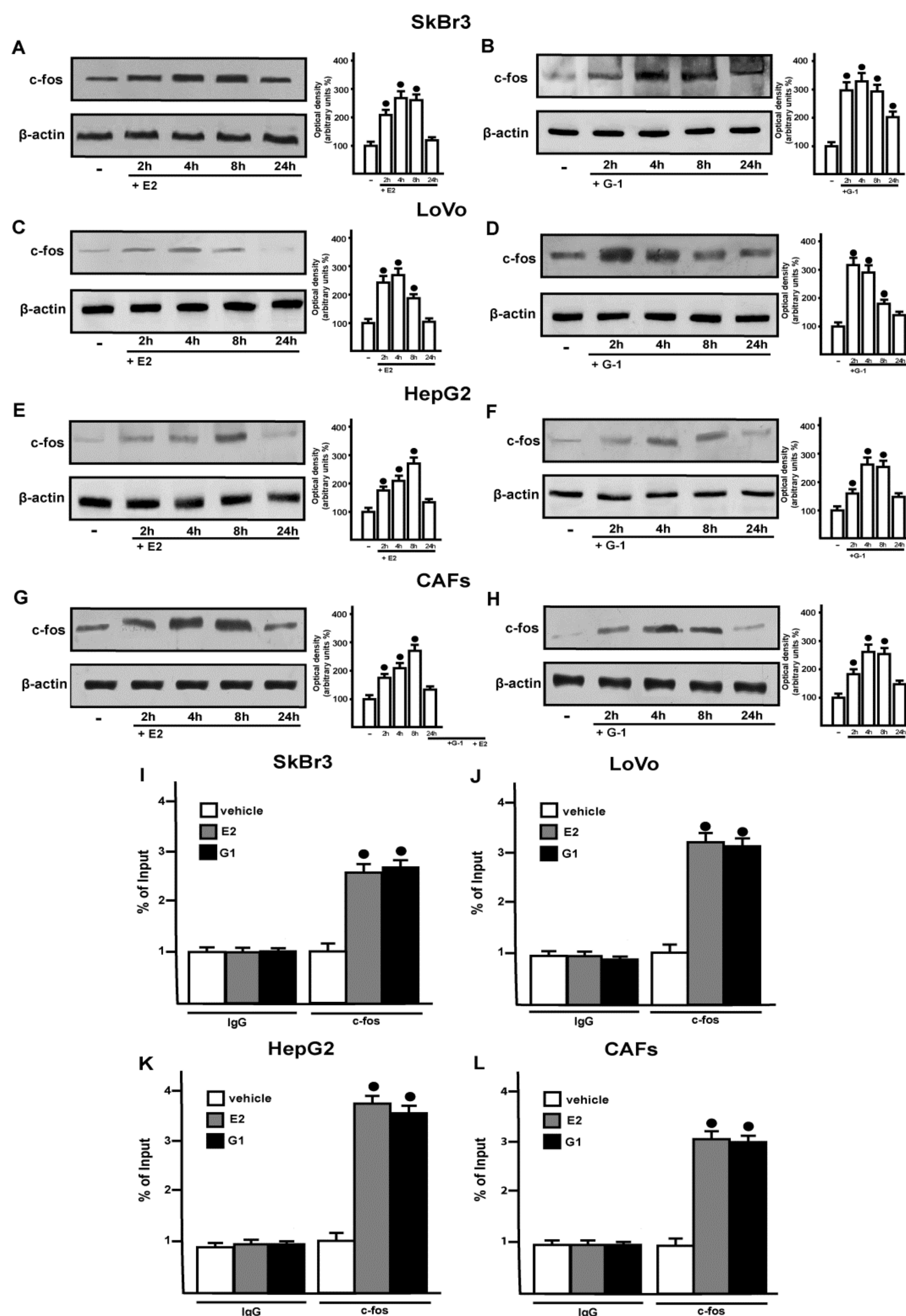

FIGURE 3.7 *Immunoblots of c-fos protein expression in SkBr3, LoVo, HepG2 cells and CAFs treated with vehicle (-), 1nM E2 and 1µM G-1 for the indicated times (A-H). E2 and G-1 induce the recruitment of c-fos to the AP1 site located within the FASN 5'-flanking region in SkBr3, LoVo HepG2 cells and CAFs (I-L). Cells were treated for 3h with vehicle, 1nM E2 and 1µM G-1, therefore the chromatin immunoprecipitation procedure was performed by using anti-c-fos or non-specific anti-IgG antibodies. The amplified sequences were evaluated by real-time PCR. Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. (●) indicate $p<0.05$ for cells receiving vehicle (-) versus treatments.*

Moreover, using a dominant-negative variant of c-*fos* the induction of FASN by E2 and G-1 was no longer evident (Figure 3.8 A-D), further confirming the role played by c-Fos in this biological response. Taken together, these findings indicate that the GPER/EGFR/ERK/c-Fos/AP1 transduction pathway mediates the transcription of FASN induced by E2 and G-1 in our model system.

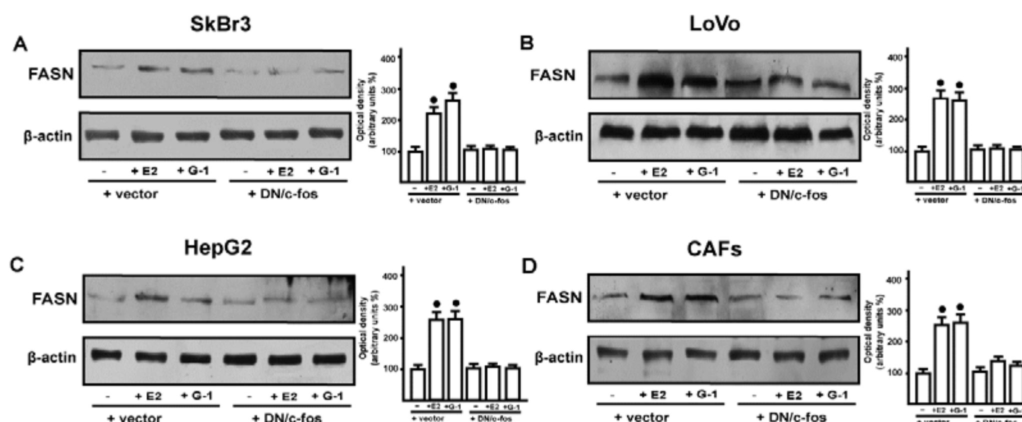

FIGURE 3.8 *(A-D) an expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the up-regulation of FASN protein levels by E2 and G-1. Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. (●) indicate $p<0.05$ for cells receiving vehicle (-) versus treatments.*

3.3 FASN is involved in the proliferation and migration induced by E2 and G-1.

In diverse cancer cell types, FASN activity stimulates the synthesis of lipids which are necessary for the initiation of signaling pathways involved in cell proliferation and migration (95,120,138). Performing proliferation assays in SkBr3, LoVo, HepG2 cells and CAFs, the increased cell growth observed upon exposure to E2 and G-1 was abolished using the inhibitor of the FASN activity named cerulenin [(2S,3R)-2,3-epoxi-4-oxo-7,10-dodecadienoxylamide] (Figure 3.9 A-D), which was previously shown to repress cancer cell growth by inhibiting fatty acid synthesis (100,140-145). Proliferation assays were also performed using cerulenin in cells transfected with an expression vector of FASN (Figure 3.9 A-D).

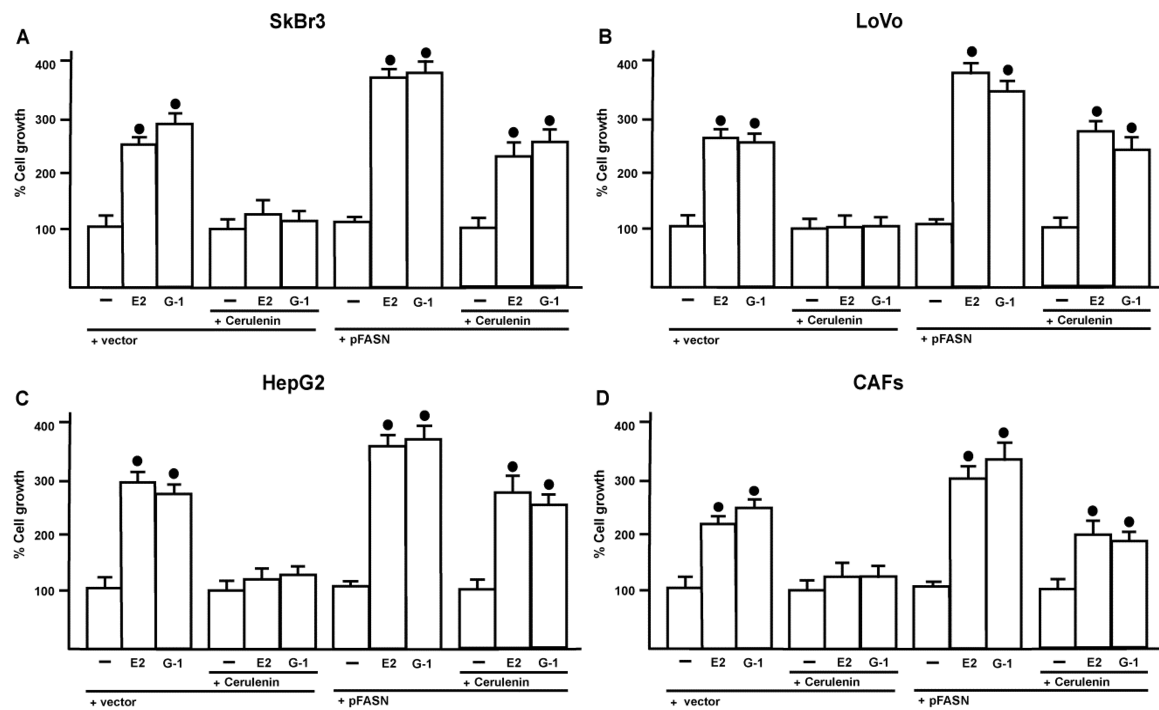

FIGURE 3.9 *E2 and G-1 induce proliferative effects in SkBr3, LoVo, HepG2 cells and CAFs. In the proliferation assay, cells were transfected with an empty vector (vector) or an expression vector of FASN (pFASN) every two days, cells were treated with vehicle (-), 1nM E2, 100nM G-1 alone and in combination with 1µM cerulenin every day for 5 days and then counted on day 6 (A-D). Values shown are mean ± SD of three independent experiments. (●) indicate p<0.05 for cells receiving vehicle (-) versus treatments.*

The over-expression of FASN (Figure 3.10) restored the growth effects induced by E2 and G-1, hence confirming that FASN contribute to this biological response.

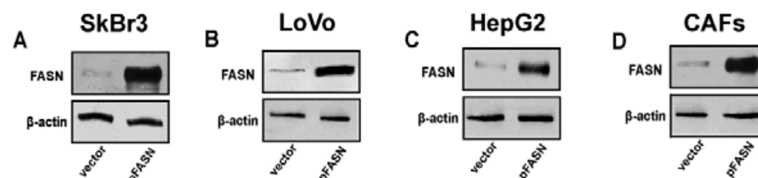

FIGURE 3.10 *Efficacy of FASN over-expression obtained transfecting SkBr3, LoVo, HepG2 cells and CAFs with a FASN expression vector (pFASN) (A-D). β-actin was used as loading control.*

Next, the migration of all cell types promoted by E2 and G-1 was abolished in presence of cerulenin (Figure 3.11), further corroborating the aforementioned results.

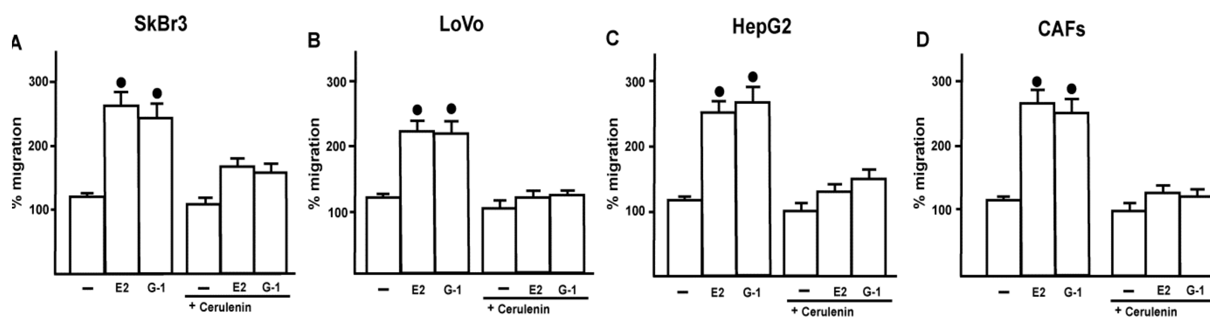

FIGURE 3.11. *The migration induced by 1nM E2 and 100nM G-1 in SkBr3, LoVo, HepG2 cells and CAFs is abolished using 1µM cerulenin. Values shown are mean ± SD of three independent experiments. (●) indicate p<0.05 for cells receiving vehicle (-) versus treatments.*

In order to assess the efficacy of cerulenin, we evaluated FASN enzymatic activity by measuring the incorporation of [1,2 ^{14}C] acetate into fatty acids. As shown in figure 3.12 (panels A-D), cerulenin inhibited FASN activity induced by E2 and G-1. Thereafter, transfecting cells with the shGPER the induction of FASN activity by E2 and G-1 was no longer evident (Figure 3.12 E-H), suggesting that GPER mediates FASN expression and activity by E2 and G-1 in cancer cells and CAFs.

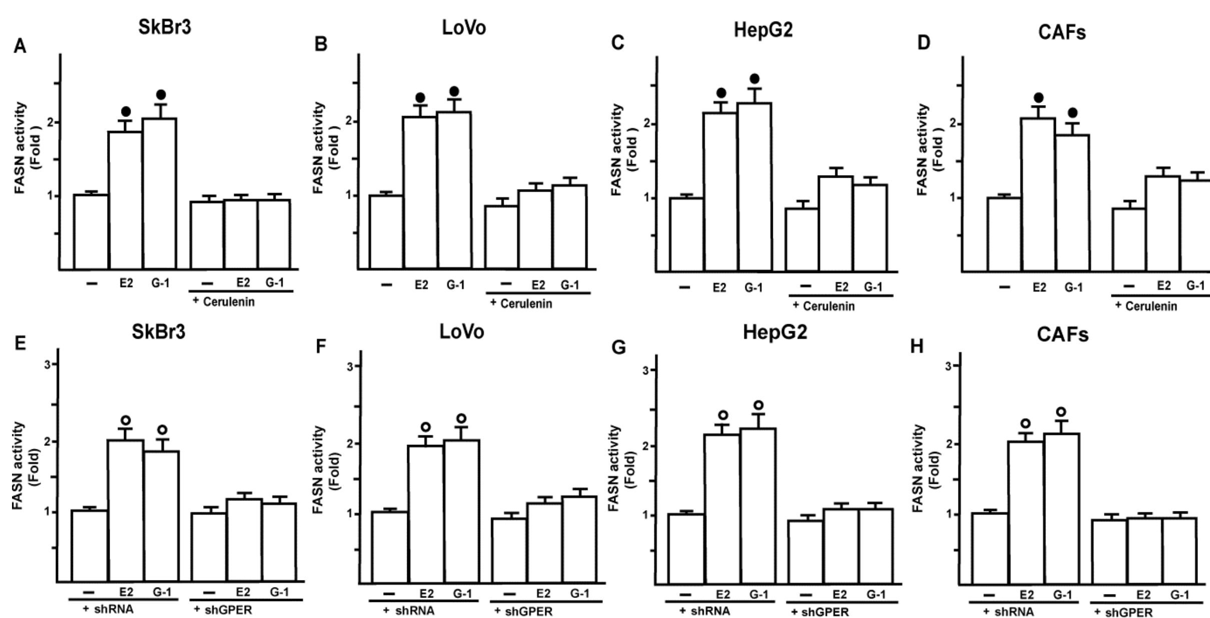

FIGURE 3.12 *(A-D), in SkBr3, LoVo, HepG2 cells and CAFs 1nM E2 and 1µM G-1 induce an increase of FASN enzymatic activity as evaluated by measuring the incorporation of [1, 2 ^{14}C] acetate into fatty acids. FASN activity induced by E2 and G-1 was abolished using 1µM cerulenin for 24h (A-D). FASN activity induced by E2 and G-1 was prevented transfecting cells with shGPER (E-H). Each column represents the mean ± SD of three separated experiments. (●, ○) indicate p<0.05 for cells receiving vehicle versus treatments.*

Chapter IV

Discussion

4.1 Discussion

FASN is a key lipogenic enzyme which plays a relevant role in cancer pathogenesis and development (146). Accordingly, FASN expression has been found elevated in numerous types of cancer (147-149) and detected in a most intense manner in carcinomas with higher risk of recurrence and death (95), hence delineating its functional nature of a metabolic oncogene. As it concerns the regulation of FASN levels, steroid hormones, growth factors (for example EGFR and ERBB2) and the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways were shown to modulate FASN expression (105,150-152). However, how FASN is up-regulated in the first place in normal or preneoplastic cells to prime tumorigenesis is currently unclear and the specific cytotoxicity of FASN inhibition in cancer cells as well as its role in chemotherapeutic resistance remains to be clarified. Overall, the aforementioned data regarding the FASN-dependent fatty acid synthesis in cancer cells make this enzyme as a suitable target for cancer treatment, mainly considering that the silencing of FASN expression inhibits the proliferation and induces apoptosis in cancer cells (140,141). In this regard, it is worth nothing that the pharmacologic inhibitor of FASN activity, cerulenin, induced a selective cytotoxicity in cancer cells by decreasing fatty acid synthesis which delayed the progression of breast, ovarian, and prostate human cancer xenografts and suppressed liver metastasis in a colon cancer xenograft model (100,145).

Steroid hormones may have a role in the regulation of FASN expression in hormone-responsive tumors. For example, FASN expression was shown to contribute to the estrogen-driven response which stimulated the proliferation in hormone-dependent endometrial cells (153). In MCF-7 breast cancer cells, FASN expression was influenced by E2 and progestins through the sterol receptor element binding protein 1 (SREBP-1) pathway as also observed in prostate cancer cells by androgens (154). In these studies, the activation of steroid receptors mediated the up-regulation of FASN as the antiandrogen bicalutamide, the antiprogestin mifepristone (RU486) and the antiestrogens 4-hydroxytamoxifen and faslodex (ICI 182,780)

inhibited the FASN response to the cognate ligands of hormone receptors (120,138,153-155). Nevertheless, the inhibition of MAPK and phosphatidylinositol 3-kinase (PI3K) signaling pathways abolished the FASN induction by steroids (156-158), suggesting that complex transduction mechanisms may contribute to the regulation of FASN expression.

In the context of these findings, our current results provide evidence regarding a new mechanism by which FASN may be regulated in a variety of tumor cells. We demonstrate that E2 and G-1 induce FASN expression and activity through the GPER-mediated signaling which involves the EGFR/ERK/c-Fos/AP1 transduction pathway. In particular, we show that the induction of FASN by E2 and G-1 is mediated by sequential events such as the rapid activation of ERK1/2 and the stimulation of c-Fos, which is then recruited to an AP1 site located within the FASN promoter sequence. Worthy, FASN was required for important biological responses to E2 and G-1 like cell proliferation and migration in cancer cells and CAFs lacking the classical ERs but expressing GPER. Tumor progression is not achieved solely by cancer cells, but neoplastic epithelial cells coexist in carcinomas with several types of stromal cells that generate the microenvironment of the cancer cells (159). Among the stromal components, the most important type of cells recruited into the tumor mass are represented by fibroblasts, which acquiring an activated phenotype act as important regulators of the paracrine signals between stromal and cancer cells (85). In particular, the specialized group of fibroblasts, referred to as CAFs, actively contribute to the growth and invasion of tumor cells by providing a unique tumor microenvironment (160). In this regard, it has been reported that CAFs express a wide number of growth factors and extracellular matrix remodeling enzymes that promote the proliferation and invasion of tumor cells as well as angiogenesis and chemo-resistance (161,162). In breast carcinoma approximately 80% of stromal fibroblasts exhibit the activated phenotype which induces the proliferation of cancer cells at the metastatic sites, stimulating the tumor growth like to the primary tumor (47). In addition, stromal fibroblasts may promote the local production of estrogens, which largely contribute to the progression of breast carcinomas through a signal cross-talk with many transduction pathways activated by growth factors (163). CAFs may trigger tumour progression also through further mechanisms as they facilitate the invasiveness of otherwise non-invasive cancer cells when co-injected into mice (164). Altogether, the aforementioned information do not recapitulate the complex interactions between the tumour epithelium and stromal cells as the intricated pathways leading to cancer progression still remain to be fully dissected. Interestingly, the present study demonstrates that GPER mediates the up-regulation of FASN by E2 and G-1 also in CAFs. In addition, using cerulenin we demonstrated that the

estrogen-induced proliferation and migration of CAFs involves FASN activity. These findings together with our previous data showing that GPER is required for the migration of CAFs induced by E2 (86), further highlight the potential of estrogens to stimulate tumor progression through the GPER-mediated FASN expression and activity.

The present investigation provides novel insights into the molecular mechanisms by which the endogenous lipogenesis may exert an oncogenic role in the development of estrogen sensitive tumors. In this regard, the lipogenic features of cancer cells through GPER may offer new avenues in order to identify and develop innovative therapeutic agents capable of successfully interfering with the initiation and progression of both primary and metastatic hormone-responsive tumors.

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PUBLICATIONS

1. GPER mediates the up-regulation of fatty acid synthase (FASN) induced by 17β-estradiol in cancer cells and cancer-associated fibroblasts (CAFs). **Santolla MF**, Lappano R, De Marco P, Pupo M, Vivacqua A, Sisci D, Abonante S, Iacopetta D, Cappello AR, Dolce V, Maggiolini M. J Biol Chem. 2012 Nov 7. [Epub ahead of print]

2. Recent advances in the rationale design of GPER ligands. Rosano C, Lappano R, **Santolla MF**, Ponassi M, Donadini A, Maggiolini M. Curr Med Chem. 2012 Oct 31. [Epub ahead of print]

3.Two novel GPER agonist induce gene expression changes and growth effects in cancer cells. Lappano R, Rosano C, **Santolla MF**, Pupo M, De Francesco EM, De Marco P, Ponassi M, Spallarossa A, Ranise A, Maggiolini M. Current Cancer Drug target. 2012 Jun;12(5):531-42

4. Bisphenol A induces gene expression changes and proliferative effects through GPER in breast cancer cells and cancer-associated fibroblasts. Pupo M, Pisano A, Lappano R, **Santolla MF**, De Francesco EM, Rosano C, Maggiolini M. Environmental Health Perspectives, 2012 Aug; 120 (8):1177-82. Epub 2012 May 2

5. Insulin-like growth factor-I regulates GPER expression and function in cancer cells De Marco P, Bartella V, Vivacqua A, Lappano R, **Santolla MF**, Morcavallo A, Pezzi V. Belfiore A, Maggiolini M. Oncogene, 2012 Mar 19. doi: 10.1038/onc.2012.97. [Epub ahead of print]

6. MIBE acts as antagonist ligand of both estrogen receptor alpha and GPER in breast cancer cells. Lappano R, **Santolla MF**, Pupo M, Sinicropi MS, Caruso A, Rosano C, Maggiolini M. Breast Cancer Research 2012 Jan 17; 14 (1):R12

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GPER MEDIATES THE UP-REGULATION OF FATTY ACID SYNTHASE (FASN) INDUCED BY 17β-ESTRADIOL IN CANCER CELLS AND CANCER-ASSOCIATED FIBROBLASTS (CAFs)

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Running title: *GPER regulates fatty acid synthase expression*

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Keywords: cancer cells; GPER; FASN; cancer-associated fibroblasts; estrogens

Background: Fatty acid synthase (FASN) is a key lipogenic enzyme regulated by various factors including estrogens.
Results: GPER mediates FASN expression and activity induced by estrogens in cancer cells.
Conclusion: Fatty acid biogenesis is regulated by estrogens through GPER.
Significance: GPER may be included among the transduction mediators involved by estrogens in regulating FASN expression and activity.

SUMMARY

Activation of lipid metabolism is an early event in carcinogenesis and a central hallmark of many tumors. Fatty acid synthase (FASN) is a key lipogenic enzyme catalyzing the terminal steps in the *de novo* biogenesis of fatty acids. In cancer cells, FASN may act as a metabolic oncogene given that it confers growth and survival advantages to these cells, whereas its inhibition effectively and selectively kills tumor cells. Hormones like estrogens and growth factors contribute to the transcriptional regulation of FASN expression also through the activation of downstream signaling and a crosstalk among diverse transduction pathways. In this study, we demonstrate for the first time that 17β-estradiol (E2) and the selective GPER ligand G-1 regulate FASN expression and activity through the GPER-mediated signaling which involved the EGFR/ERK/c-fos/AP1 transduction pathway, as ascertained by using specific pharmacological inhibitors, performing gene-silencing experiments and ChiP assays in breast SkBr3, colorectal LoVo, hepatocarcinoma HepG2 cancer cells and breast cancer-associated fibroblasts (CAFs). In addition, the proliferative effects induced by E2 and G-1 in these cells involved FASN as the inhibitor of its activity, named cerulenin, abolished the growth response to both ligands. Our data suggest that GPER may be included among the transduction mediators involved by estrogens in regulating FASN expression and activity in cancer cells and CAFs that strongly contribute to cancer progression.

Estrogens trigger multiple biological responses mainly through the estrogen receptor (ER)α and ERβ (1,2) which act as ligand-activated transcription factors binding to the estrogen responsive elements located within the promoter of target genes (3-5). In addition, an increasing number of evidence has recently demonstrated that the G protein-coupled receptor, named GPER, functions as an estrogen receptor in normal and cancer cells (6-9). Indeed, GPER is widely distributed in neural, placental, hearth,

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prostate, hepatic, bone, vascular epithelial, lymphoid and reproductive tissues as well as in breast, endometrial, ovarian and thyroid carcinomas (10-14). Several studies including our own (15-21) have shown that GPER mediates estrogen (17β-estradiol) signals activating the epidermal growth factor receptor (EGFR)/ERK/AP1 transduction pathway (22-28). In this context, it has been reported that GPER stimulates through $G\alpha_s$ the cAMP pathway and through Gβγ the Src activity, which leads to the shedding of heparin binding-EGF and the activation of EGFR (12). As a consequence, several signaling cascades like ERK, PI3K and phospholipase C are engaged in the stimulation of downstream biological responses including gene expression changes, cell proliferation and migration (6,14). One main metabolic change in cancer cells is represented by an altered lipogenic pathway such as an increased synthesis of fatty acids, that are important substrates in the energy production, building blocks of cellular membranes, intracellular second messengers and anchorage for membrane proteins (29). Free fatty acids derive from both the diet and *de novo* synthesis, which is catalyzed in lipogenic tissues by fatty acid synthase (FASN) that is able to generate palmitate from malonyl-CoA and acetil-CoA in presence of NADPH (29,30). In normal cells, FASN expression is relatively low and occurs in liver and adipose tissues mainly through nutritional signals; conversely in cancer cells FASN levels are elevated and independent of nutritional signals (31). FASN has been strongly associated with cell proliferation, aggressiveness and metastasis in different types of tumors and considered predictive of poor prognosis in diverse malignancies (32). Although the mechanisms involved in the up-regulation of FASN in tumor cells remain to be completely understood, an intricate interplay between estrogen signaling and FASN function has been found in breast tumors (33). In the present study, we demonstrate for the first time that E2 regulates FASN expression and function through GPER in different types of cancer cells which do not express ERs. On the basis of our results, GPER signaling may be included among the transduction pathways by which E2 triggers fatty acid biogenesis which strongly contributes to the development and aggressive features of diverse tumors.

EXPERIMENTAL PROCEDURES

Materials- 17β-Estradiol (E2) and cerulenin were purchased from Sigma-Aldrich Srl. (Milan, Italy). Tyrphostin AG1478 (AG) was purchased from Biomol Research Laboratories, Inc (Milan, Italy). PD98059 (PD) was obtained from Calbiochem (Milan, Italy). 1-[4-(-6-Bromobenzol [1, 3] diodo-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c-] quinolin8yl] ethanone (G-1) was purchased from Merck KGaA (Frankfurt, Germany). All compounds were dissolved in DMSO, except for cerulenin which was solubilized in ethanol.

Cell Cultures- The SkBr3 breast cancer cells were maintained in RPMI-1640 (Invitrogen, Gibco, Milan, Italy) without phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin/streptomycin. The LoVo colorectal adenocarcinoma cells and the LNCaP prostate cancer cells were maintained in RPMI-1640 with phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin/streptomycin. The hepatocarcinoma cells HepG2 and the MCF-7 breast cancer cells were cultured in DMEM (Dulbecco's modified Eagle's medium) with phenol red, supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin/streptomycin. All cell lines were grown in a 37° C incubator with 5% CO2. Cancer-associated fibroblasts (CAFs) were extracted as previously described (25) and maintained in a mixture of MEDIUM 199 and HAM'S F-12 (1:1) supplemented with 10% FBS. Primary cells cultures of breast fibroblasts were characterized by immunofluorescence. Briefly cells were incubated with human anti-vimentin (V9) and human anti-cytokeratin 14 (LL001) all antibodies from Santa Cruz Biotechnology, DBA (Milan, Italy). In addition, we used antifibroblast activated protein α (FAPα) antibody (H-56), also purchased from Santa Cruz Biotechnology, DBA (Milan, Italy), for fibroblasts activation characterization (data not shown).

Gene expression studies- Total RNA was extracted using Trizol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gels

stained with ethidium bromide. Only samples that were not degraded and showed clear 18S and 28S bands under ultraviolet light were used for real-time PCR.

Total cDNA was synthesized from the RNA by reverse transcription using the murine leukaemia virus reverse transcriptase (Invitrogen, Milan, Italy) following the protocol provided by the manufacturer. The expression of selected gene was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc., Milano, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc., Milano, Italy) and are as follows: FASN and the ribosomal protein 18S, which was used as a control gene to obtain normalized values: FASN (Human) Fwd: 5'-CATCCAGATAGGCCTCATAGAC-3' and Rev: 5'-CTCCATGAAGTAGGAGTGGAAG-3'; 18S (human, mouse) Fwd: 5'-GGCGTCCCCCAACTTCTTA-3' and Rev: 5'-GGGCATCACAGACCTGTTATT-3'. Assays were performed in triplicate and the results were normalized for 18S expression and then calculated as fold induction of RNA expression. For all experiments, cells were switched to medium without serum 24 h before treatments. FASN expression was evaluated also using semiquantitative RT-PCR, as previously described (34).

Western Blot Analysis- SkBr3, LoVo, HepG2 cells and CAFs were grown in 10-cm dishes and exposed to drugs for the appropriate time, then washed twice with ice-cold PBS and solubilized with 50 mM Hepes buffered solution, pH 7.5, containing 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Protein concentration in the supernatant was determined according to the Bradford method. Equal amounts (10–50 µg) of the whole cell lysate was electrophoresed through a reducing SDS/8% (w/v) polyacrylamide gel and electroblotted onto a nitrocellulose membrane which was probed with primary antibodies against FASN (A-5), c-fos (H-125), phosphorylated ERK1/2 (E-4), ERK2 (C-14), GPER (N-15), EGFR (1005), p-EGFR Tyr 1173 (sc-12351) and β-actin (C2), all purchased from Santa Cruz Biotechnology, Inc. The levels of proteins and phosphoproteins were detected, after incubation with the horseradish peroxidase-linked secondary antibodies, by the ECL® (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy).

Gene Silencing Experiments and plasmids- Cells were plated onto 10-cm dishes, maintained in serum-free medium for 24 h and then transfected for additional 24 h or 48 h before treatments with a control vector or an independent shRNA sequence for each target gene using Fugene6 (Roche Molecular Biochemicals, Milan, Italy). The shRNA plasmid for EGFR was purchased from SABioscience Corporation (Frederick, MD, USA). Short hairpin constructs against human GPER (shGPER) were generated and used as previously described (7). The plasmid DN/c-fos, which encodes a c-fos mutant that heterodimerizes with c-fos dimerization partners but does not allow DNA binding (35), was a kind gift from Dr. C. Vinson (NIH, Bethesda, MD, USA). The expression vector for Flag-tagged human GPER has been described (15). It was used to generate the GPER rescue vector containing silent mutations in the shRNA targeted sequence: codons 293–297 were changed to CCG TGTAAA CAAAGT. The expression vector for human FASN was a kind gift from Dr. M. Loda (Dana-Farber Cancer Institute, D1536, 44 Binney Street, Boston, MA, 02115).

Immunostaining assay- Fifty percent confluent cultured SkBr3, LoVo, HepG2 cells and CAFs grown on cover slips were serum deprived for 24 h and treated for 18 h with 1nM E2. Then cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS and incubated overnight with a mouse primary antibody against FASN (1:500). After incubation, the slides were extensively washed with PBS and incubated with propidium iodide (1:1000 Sigma-Aldrich) for cell nuclei detection and donkey anti-mouse IgG-FITC (1:250; purchased from Santa Cruz Biotechnology). Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS) were used for experiment evaluation.

Chromatin immunoprecipitation (ChiP) assay- SkBr3, LoVo, HepG2 cells and CAFs

were grown in 10-cm dishes to 70-80% confluence, shifted to serum free medium for 24h and then treated with vehicle, 1nM E2, and G-1 for 3h. Thereafter, cells were cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with sonicated salmon DNA/protein A agarose (Upstate Biotechnology, Inc., Lake Placid, NY) and immunoprecipitated with the anti-FASN antibody or non specific IgG (Santa Cruz Biotechnology, DBA, Milan, Italy). Pellets were washed, eluted with a buffer consisting of 1% SDS and 0.1 mol/L $NaHCO_3$, and digested with proteinase K. DNA was obtained by phenol/chloroform extraction and precipitated with ethanol. A 4 µl volume of each sample was used as template to amplify an AP1 containing region corresponding to -1606/-1596 located in the 5'-flanking region of FASN gene by real-time PCR (Applied Biosystems, Milan, Italy). The primers used were: Fwd 5'-CTGGCAGCCAGGGCCA-3' and Rev 5'-GCTGTGGTTGACGCACGG-3'. To verify the specificity of c-fos recruitment at the AP1 site, we also performed ChiP assay using the following primers: Fwd 5'-ACGCTCATTGGCCTGGG-3' and Rev 5'-TGGCTCCCTCTAGGCCGG-3' which amplify the estrogen target gene SREBP-1c containing region corresponding to -189/-171 located in the 5'-flanking region of FASN gene (36). In particular, it was shown that the binding to the SREBP-1c site occurs in an ER-dependent manner upon estrogen stimulation (36). Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 4µl of the template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as percent differences with respect to the relative inputs.

Proliferation assays- For quantitative proliferation assays $1x10^4$ SkBr3, LoVo, HepG2 cells and CAFs were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and further incubated in a medium supplemented with 2.5 % charcoal-treated FBS. Ligands were added at this point; medium was changed every day (with ligands and cerulenin were applicable). On day 6 (after 5 days of treatment), cells were trypsinized and counted using Countess™ automated cell counter (purchased from Invitrogen Milan, Italy).

Migration assay- Migration assays were performed using Boyden chambers (Costar Transwell, 8mm polycarbonate membrane). Cells were seeded in the upper chambers. E2 and G-1 alone or in combination with cerulenin were added to the medium without serum in the bottom wells. After 24 h, cells on the bottom side of the membrane were fixed and counted.

FASN enzymatic activity assay- FASN activity in whole cells was measured by the incorporation of [1, 2 ^{14}C] acetate (Perkin-Elmer Milan, Italy) into fatty acids. Cells were plated in 6 well plates at $3x10^5$ cells per dish and incubated overnight. The next day after 12 hours of starvation, cells were treated with vehicle, 1nM E2, 1µM cerulenin and 1nM E2 + 1µM cerulenin overnight and then incubated with 0, 5 µCi /ml [1, 2 ^{14}C] acetate for 8 hours. Cells were washed and harvested in 1x phosphate-buffered saline (1x PBS) and [1, 2 ^{14}C] incorporated lipids were extracted with chloroform/methanol (1:4). After centrifugation at 12000×g for 10 minutes, the lower phase containing radiolabeled lipids was counted by scintillation counter. FASN activity was calculated as nmol/mg total protein/min and variations were reported as fold respect to the vehicle-treated cells. Each experiment was repeated at least in triplicate.

Statistical analysis- Statistical analysis was performed using ANOVA followed by Newman-Keuls' testing to determine differences in means. $P<0.05$ was considered as statistically significant.

RESULTS

E2 and G-1 induce FASN expression in ER-negative cancer cells. In order to provide novel insights into the mechanisms by which estrogens may regulate FASN gene in cancer cells, we began the present study evaluating FASN expression upon exposure to E2 and the GPER ligand G-1 in breast SkBr3, colorectal LoVo, hepatocarcinoma HepG2 tumor cells and in CAFs which lack the classical ERs but express GPER (Suppl. fig. 1). In time course experiments, E2 and G-1 showed the ability to increase the mRNA expression of FASN, as evaluated by real-time PCR (Fig. 1A-D) and using a semiquantitative PCR (data not shown) (34). The up-regulation of FASN mRNA was

paralleled by increased FASN protein levels upon exposure to E2 and G-1 (Fig. 1E-L), as also evaluated by immunofluorescence studies (Fig. 2).

GPER/EGFR/ERK/c-fos/AP1 signaling mediates FASN expression induced by estrogens. These results prompted us to evaluate the role exerted by GPER signaling in the up-regulation of FASN by E2. Silencing GPER in all cells used, E2 and G-1 did not induce FASN expression suggesting that GPER mediates this effect. The requirement for GPER and the specificity of the GPER knockdown were further emphasized by the fact that the co-transfection of a shRNA-resistant version of GPER ('GPER rescue') restored the response. (Fig. 3A,C,E,G). As GPER activation triggers EGFR signaling (14,23), we next demonstrated that the increase of FASN protein levels induced by E2 and G-1 requires EGFR as determined through gene silencing experiment (Fig. 4A,C,E,G). Confirming this observation, E2 and G-1 lost the ability to up-regulate FASN protein expression using the EGFR inhibitor AG1478 (AG) as well as the MEK inhibitor PD98059 (PD) (Fig. 5A-D). The latter finding was nicely supported by EGFR activation and the rapid ERK phosphorylation induced by E2 and G-1 in SkBr3, LoVo, HepG2 cancer cells and in CAFs (Fig. 5E-H). As the GPER/EGFR/ERK transduction signaling triggers c-fos expression (15-22), we first ascertained this response to E2 and G-1 (Fig. 6A-H) and then we determined that c-fos is recruited to the AP1 site located within the promoter sequence of FASN (Fig. 6I-L). Amplifying a FASN promoter region containing the SREBP-1c site which is an ER-mediated estrogen target gene (36), we did not observe the recruitment of c-fos (data not shown) hence indicating the specificity of its binding to the AP1 site. Moreover, using a dominant-negative variant of c-fos the induction of FASN by E2 and G-1 was no longer evident (Fig. 6M-P), further confirming the role played by c-fos in this biological response. Taken together, these findings indicate that the GPER/EGFR/ERK/c-fos/AP1 transduction pathway mediates the transcription of FASN induced by E2 and G-1 in our model system.

FASN is involved in the proliferation and migration induced by E2 and G-1. In diverse cancer cell types, FASN activity stimulates the synthesis of lipids which are necessary for the initiation of signaling pathways involved in cell proliferation and migration (30,32,36). Performing proliferation assays in SkBr3, LoVo, HepG2 cells and CAFs, the increased cell growth observed upon exposure to E2 and G-1 was abolished using the inhibitor of the FASN activity named cerulenin [(2S,3R)-2,3-epoxi-4-oxo-7,10-dodecadienoxylamide] (Fig. 7A-D), which was previously shown to repress cancer cell growth by inhibiting fatty acid synthesis (37-43). Proliferation assays were also performed using cerulenin in cells transfected with an expression vector of FASN (Fig. 7A-D). The over-expression of FASN (Suppl. Fig. 2) restored the growth effects induced by E2 and G-1, hence confirming that FASN contribute to this biological response. Next, the migration of all cell types promoted by E2 and G-1 was abolished in presence of cerulenin (Suppl. fig. 3), further corroborating the aforementioned results. In order to assess the efficacy of cerulenin, we evaluated FASN enzymatic activity by measuring the incorporation of [1, 2 ^{14}C] acetate into fatty acids. As shown in figure 8 (panels A-D), cerulenin inhibited FASN activity induced by E2 and G-1. Thereafter, transfecting cells with the shGPER the induction of FASN activity by E2 and G-1 was no longer evident (Fig. 8 E-H), suggesting that GPER mediates FASN expression and activity by E2 and G-1 in cancer cells and CAFs.

DISCUSSION

FASN is a key lipogenic enzyme which plays a relevant role in cancer pathogenesis and development (33). Accordingly, FASN expression has been found elevated in numerous types of cancer (44-46) and detected in a most intense manner in carcinomas with higher risk of recurrence and death (29), hence delineating its functional nature of a metabolic oncogene. As it concerns the regulation of FASN levels, steroid hormones, growth factors (for example EGFR and ERBB2) and the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways were shown to modulate FASN expression (31,47-50). However, how FASN is up-regulated in the first place in normal or preneoplastic cells to prime tumorigenesis is currently unclear and the specific cytotoxicity of FASN inhibition in cancer cells as well as its role in

chemotherapeutic resistance remain to be clarified. Overall, the aforementioned data regarding the FASN-dependent fatty acid synthesis in cancer cells make this enzyme as a suitable target for cancer treatment, mainly considering that the silencing of FASN expression inhibits the proliferation and induces apoptosis in cancer cells (37,38). In this regard, it is worth nothing that the pharmacologic inibitor of FASN activity, cerulenin, induced a selective cytotoxicity in cancer cells by decreasing fatty acid synthesis which delayed the progression of breast, ovarian, and prostate human cancer xenografts and suppressed liver metastasis in a colon cancer xenograft model (39-43).

Steroid hormones may have a role in the regulation of FASN expression in hormone-responsive tumors. For example, FASN expression was shown to contribute to the estrogen-driven response which stimulated the proliferation in hormone-dependent endometrial cells (51). In MCF-7 breast cancer cells, FASN expression was influenced by E2 and progestins through the sterol receptor element binding protein 1 (SREBP-1) pathway as also observed in prostate cancer cells by androgens (52). In these studies, the activation of steroid receptors mediated the up-regulation of FASN as the antiandrogen bicalutamide, the antiprogestin mifepristone (RU486) and the antiestrogens 4-hydroxytamoxifen and faslodex (ICI 182,780) inhibited the FASN response to the cognate ligands of hormone receptors (30,36,53-56). Nevertheless, the inhibition of MAPK and phosphatidylinositol 3-kinase (PI3K) signaling pathways abolished the FASN induction by steroids (32,51), suggesting that complex transduction mechanisms may contribute to the regulation of FASN expression.

In the context of these findings, our current results provide evidence regarding a new mechanism by which FASN may be regulated in a variety of tumor cells. We demonstrate that E2 and G-1 induce FASN expression and activity through the GPER-mediated signaling which involves the EGFR/ERK/c-fos/AP1 transduction pathway. In particular, we show that the induction of FASN by E2 and G-1 is mediated by sequential events such as the rapid activation of ERK1/2 and the stimulation of c-fos, which is then recruited to an AP1 site located within the FASN promoter sequence. Worthy, FASN was required for important biological responses to E2 and G-1 like cell proliferation and migration in cancer cells and CAFs lacking the classical ERs but expressing GPER.

Tumor progression is not achieved solely by cancer cells, but neoplastic epithelial cells coexist in carcinomas with several types of stromal cells that generate the microenvironment of the cancer cells (57). Among the stromal components, the most important type of cells recruited into the tumor mass are represented by fibroblasts, which acquiring an activated phenotype act as important regulators of the paracrine signals between stromal and cancer cells (58). In particular, the specialized group of fibroblasts, referred to as CAFs, actively contribute to the growth and invasion of tumor cells by providing an unique tumor microenvironment (59). In this regard, it has been reported that CAFs express a wide number of growth factors and extracellular matrix remodeling enzymes that promote the proliferation and invasion of tumor cells as well as angiogenesis and chemoresistance (60,61). In breast carcinoma approximately 80% of stromal fibroblasts exhibit the activated phenotype which induces the proliferation of cancer cells at the metastatic sites, stimulating the tumor growth like to the primary tumor (62). In addition, stromal fibroblasts may promote the local production of estrogens, which largely contribute to the progression of breast carcinomas through a signal cross-talk with many transduction pathways activated by growth factors (63). CAFs may trigger tumour progression also through further mechanisms as they facilitate the invasiveness of otherwise non-invasive cancer cells when co-injected into mice (64). Altogether, the aforementioned information do not recapitulate the complex interactions between the tumour epithelium and stromal cells as the intricated pathways leading to cancer progression still remain to be fully dissected. Interestingly, the present study demonstrates that GPER mediates the up-regulation of FASN by E2 and G-1 also in CAFs. In addition, using cerulenin we demonstrated that the estrogen-induced proliferation and migration of CAFs involves FASN activity. These findings together with our previous data showing that GPER is required for the migration of CAFs induced by E2 (65), further highlight the potential of

estrogens to stimulate tumor progression through the GPER-mediated FASN expression and activity.

The present investigation provides novel insights into the molecular mechanisms by which the endogenous lipogenesis may exert an oncogenic role in the development of estrogen sensitive tumors. In this regard, the lipogenic features of cancer cells through GPER may offer new avenues in order to identify and develop innovative therapeutic agents capable of successfully interfering with the initiation and progression of both primary and metastatic hormone-responsive tumors.

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FOOTNOTES

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[1]The abbreviations used are: FASN, fatty acid synthase; ER, Estrogen Receptor; GPER, G-Protein Coupled Estrogen Receptor; EGFR, Epidermal Growth Factor Receptor; MAPK, Mitogen Activated Protein Kinases.

FIGURE LEGENDS

FIGURE 1. E2 and G-1 induce FASN expression in SkBr3, LoVo, HepG2 cells and CAFs. 1nM E2 and 1µM G-1 up-regulate FASN expression at both mRNA (A-D) and protein level (E-L), as evaluated by real-time PCR and immunoblotting, respectively. In RNA experiments, gene expression was normalized to 18S expression and results are shown as fold changes of mRNA expression compared to cells treated with vehicle (-). Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. (●) indicate p <0.05 for cells receiving vehicle (-) versus treatments.

FIGURE 2. Representative fluorescence images of FASN immunolabelling. SkBr3, LoVo, HepG2 cells and CAFs were fixed, permeabilized, and stained with anti-FASN antibody. (A, E, I, M), nuclei (in red) were stained by propidium iodide. Cells were treated for 24h with vehicle (B, F, J, N), 1nM E2 (C, G, K, O) and 1µM G-1 (D, H, L, P) and FASN accumulation is evidenced by the green signal. For descriptive purposes, panels b1, c1, d1, f1, g1, h1, j1, k1, l1, n1, m1, p1 show the plot profiles obtained at the level of the yellow line of the corresponding inset using the program WCIF Image J for Windows. Note the higher values indicating zones of intense labeling. Each experiment shown is representative of 10 random fields. Data are representative of three independent experiments.

FIGURE 3. GPER mediates the up-regulation of FASN protein levels by E2 and G-1 in SkBr3, LoVo HepG2 cells and CAFs. (A, C, E, G) the up-regulation of FASN by 1nM E2 or 1µM G-1 is abolished transfecting cells with shGPER and restored co-transfecting a resistant version of GPER named "GPR30 rescue". Side panels show densitometric analyses of the blots normalized to β-actin. (B, D, F, H) efficacy of GPER silencing and the restored GPER protein with GPER rescue. Each data point represents the mean ± SD of three independent experiments. (●, ○) indicate p< 0.05 for cells receiving vehicle (-) versus treatments.

FIGURE 4. EGFR is required for the up-regulation of FASN protein levels by E2 and G-1 in SkBr3, LoVo, HepG2 cells and CAFs. (A, C, E, G) cells were transfected with shRNA or shEFGR for 24h and then treated with 1nM E2 or 1µM G-1 for 24h. Side panels show densitometric analyses of blot normalized to β-actin. (B, D, F, H), efficacy of EGFR silencing. Each data point represents the mean ± SD of three independent experiments. (●, ○) indicate p<0.05 for cells treated with vehicle (-) versus treatments.

FIGURE 5. The EGFR/ERK signaling mediates the up-regulation of FASN induced by E2 and G-1 in SkBr3, LoVo, HepG2 cells and CAFs. (A-D), cells were treated for 24h with vehicle (-), 1nM E2 and 1µM G-1 alone and in combination with 10µM EGFR inhibitor AG1478 (AG), 10µM MEK inhibitor PD98089 (PD). (E-H) ERK1/2 activation and $EGFR^{Tyr1173}$ phosphorylation in SkBr3, LoVo, HepG2 cells and CAFs treated with vehicle (-), 1nM E2 and 1µM G-1 for 15 min. Side panels show densitometric analyses of the blots normalized to β-actin (in the case of FASN expression), ERK2 (in

the case of p-ERK1/2), EGFR (in the case of p-EGFR). Each data point represents the mean ± SD of three independent experiments. (●, ○) indicate $p<0.05$ for cells receiving vehicle (-) versus treatments.

FIGURE 6. Immunoblots of c-fos protein expression in SkBr3, LoVo, HepG2 cells and CAFs treated with vehicle (-), 1nM E2 and 1μM G-1 for the indicated times (A-H). E2 and G-1 induce the recruitment of c-fos to the AP1 site located within the FASN 5'-flanking region in SkBr3, LoVo HepG2 cells and CAFs (I-L). Cells were treated for 3h with vehicle, 1nM E2 and 1μM G-1, therefore the chromatin immunoprecipitation procedure was performed by using anti-c-fos or non-specific anti-IgG antibodies. The amplified sequences were evaluated by real-time PCR. (M-P) an expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the up-regulation of FASN protein levels by E2 and G-1. Side panels show densitometric analyses of the blots normalized to β-actin. Each data point represents the mean ± SD of three independent experiments. (●) indicate $p<0.05$ for cells receiving vehicle (-) versus treatments.

FIGURE 7. E2 and G-1 induce proliferative effects in SkBr3, LoVo, HepG2 cells and CAFs. In the proliferation assay, cells were transfected with an empty vector (vector) or an expression vector of FASN (pFASN) every two days, cells were treated with vehicle (-), 1nM E2, 100nM G-1 alone and in combination with 1μM cerulenin every day for 5 days and then counted on day 6 (A-D). Values shown are mean ± SD of three independent experiments. (●) indicate $p<0.05$ for cells receiving vehicle (-) versus treatments.

FIGURE 8. (A-D), in SkBr3, LoVo, HepG2 cells and CAFs 1nM E2 and 1μM G-1 induce an increase of FASN enzymatic activity as evaluated by measuring the incorporation of [1, 2 ^{14}C] acetate into fatty acids. FASN activity induced by E2 and G-1 was abolished using 1μM cerulenin for 24h (A-D). FASN activity induced by E2 and G-1 was prevented transfecting cells with shGPER (E-H). Each column represents the mean ± SD of three separated experiments. (●, ○) indicate $p<0.05$ for cells receiving vehicle versus treatments.

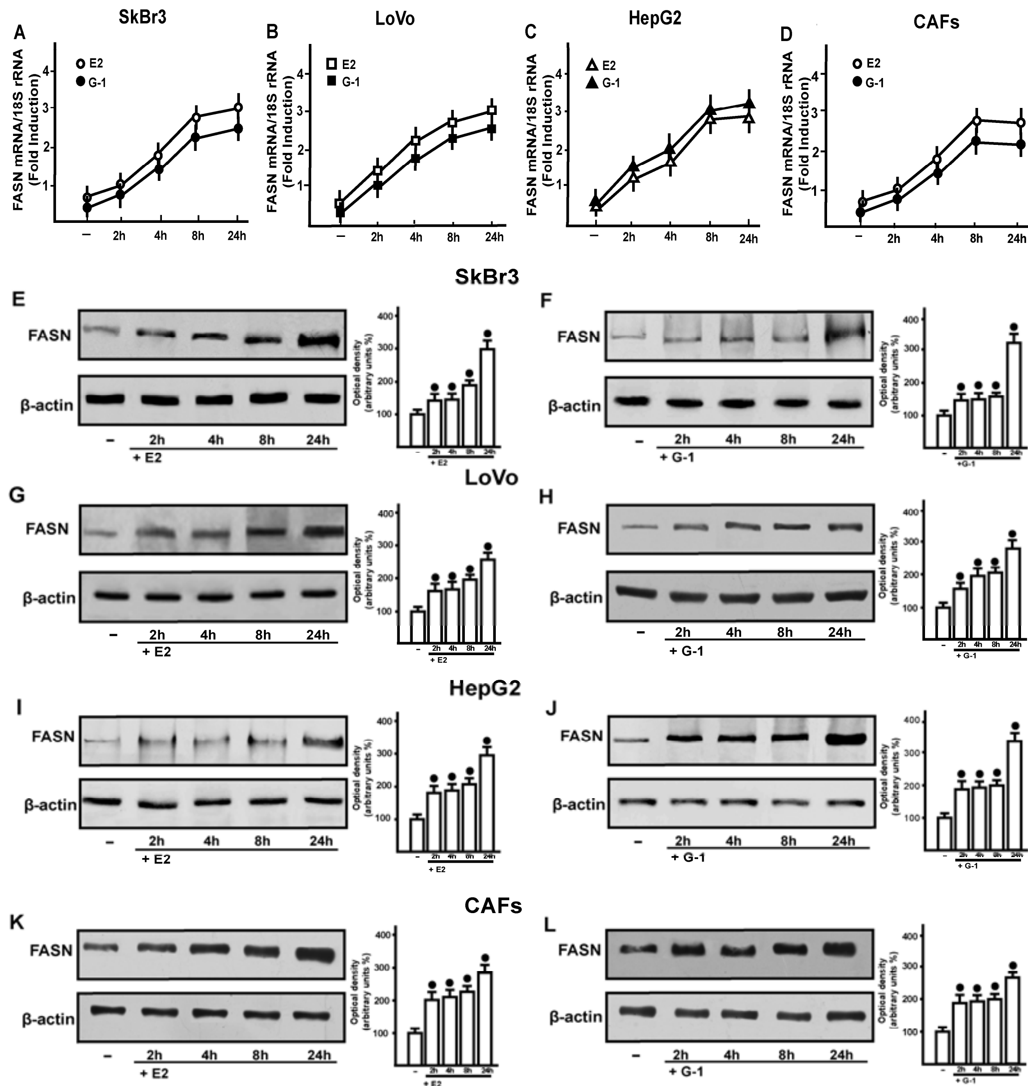

Fig.1

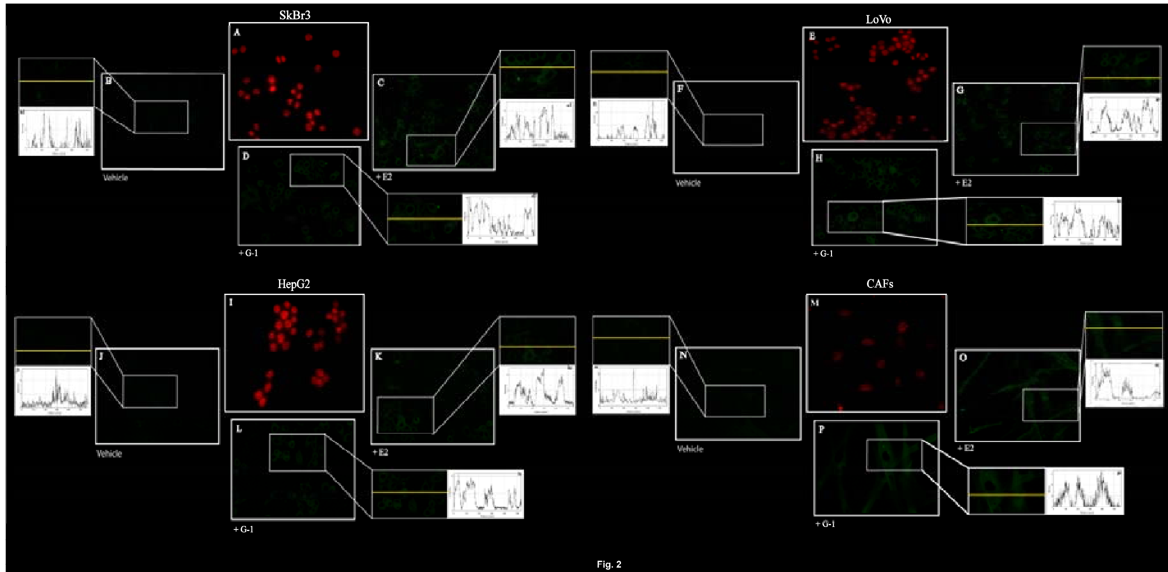
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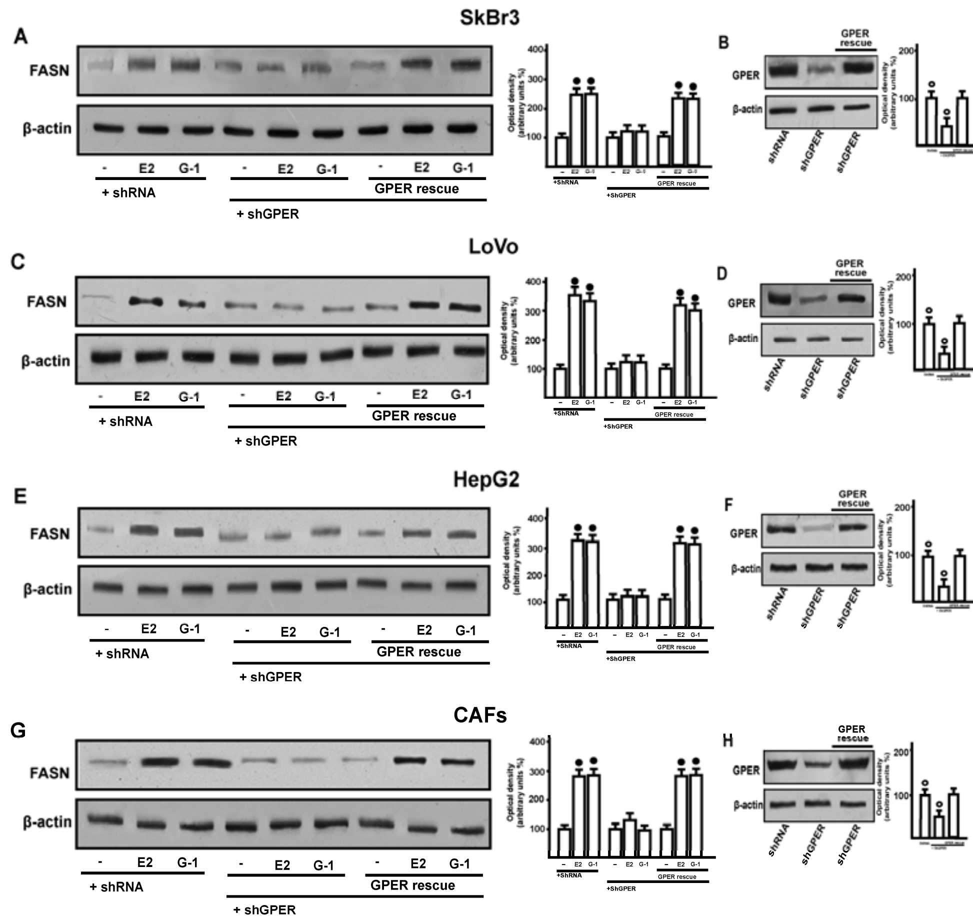
Fig. 2



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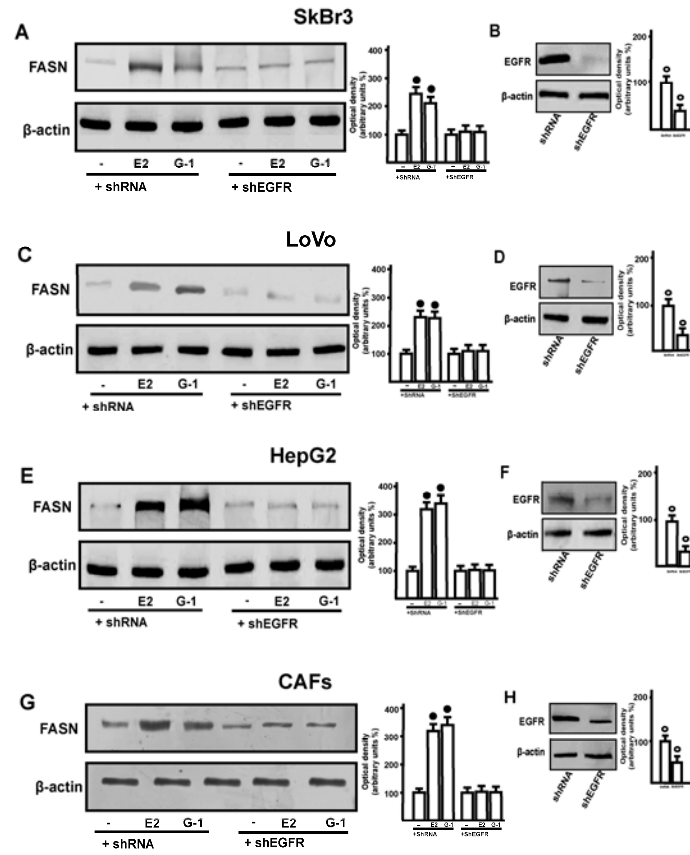

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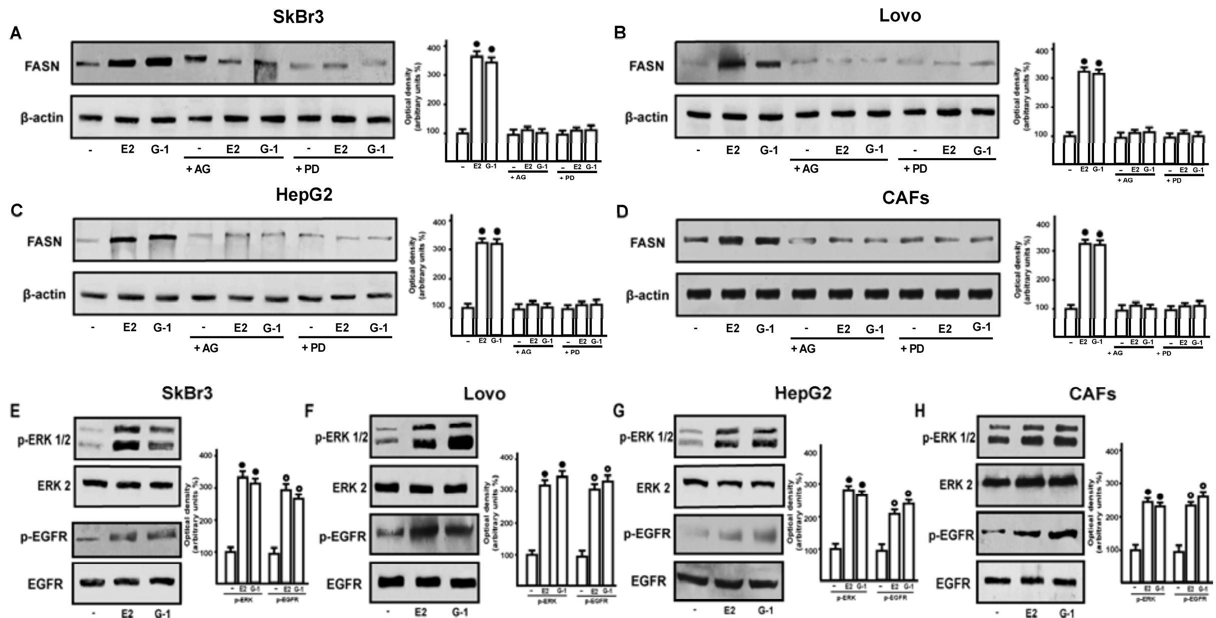

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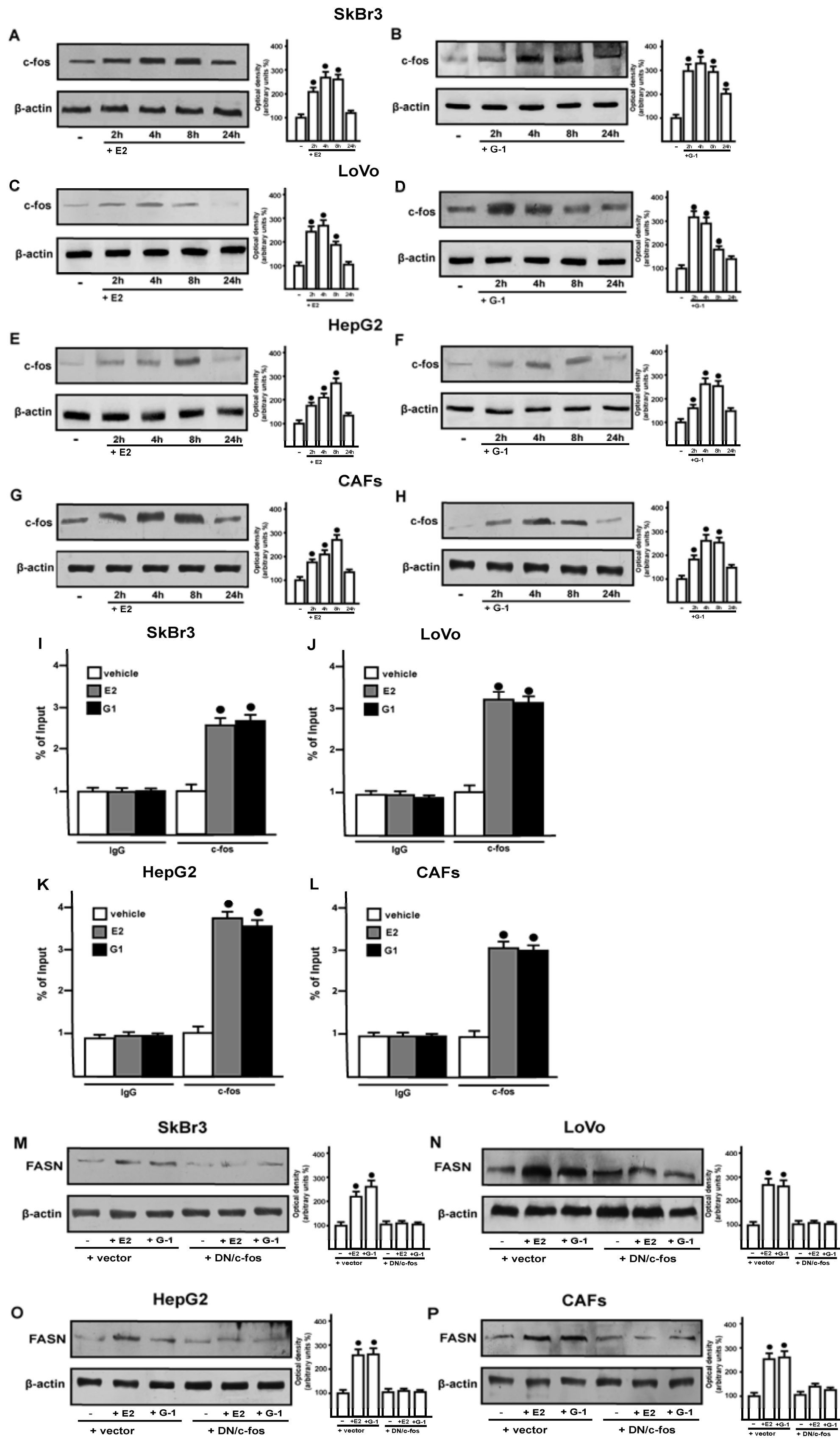

Fig. 6

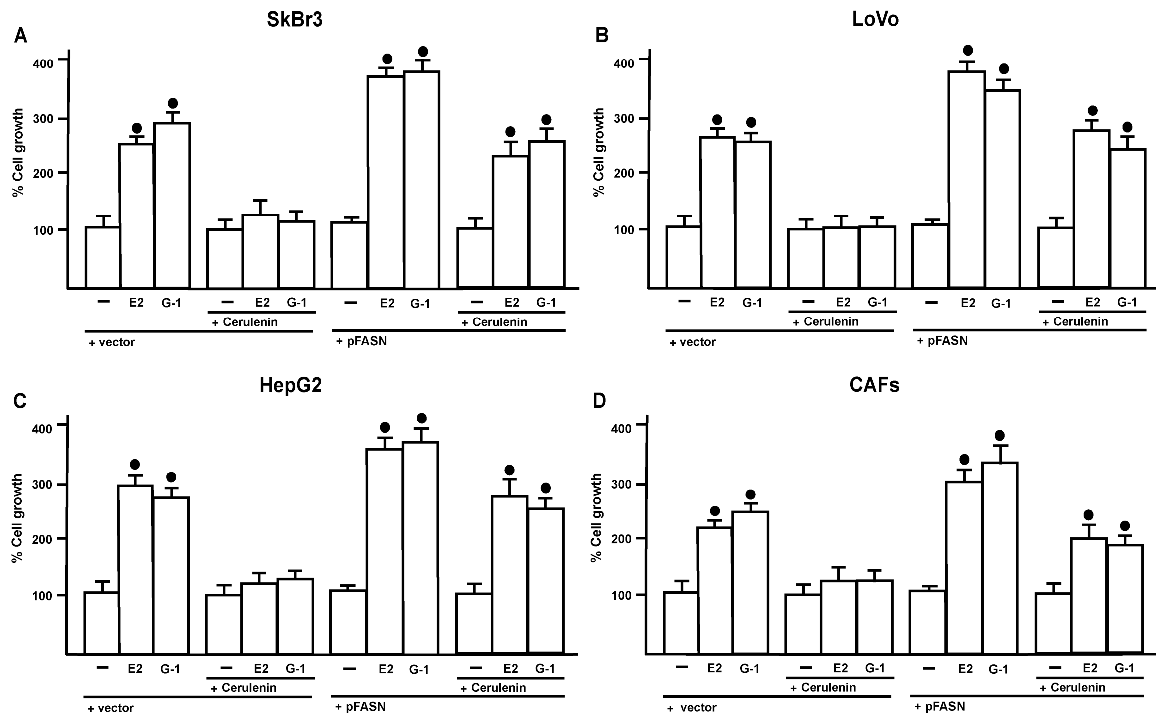

Fig. 7

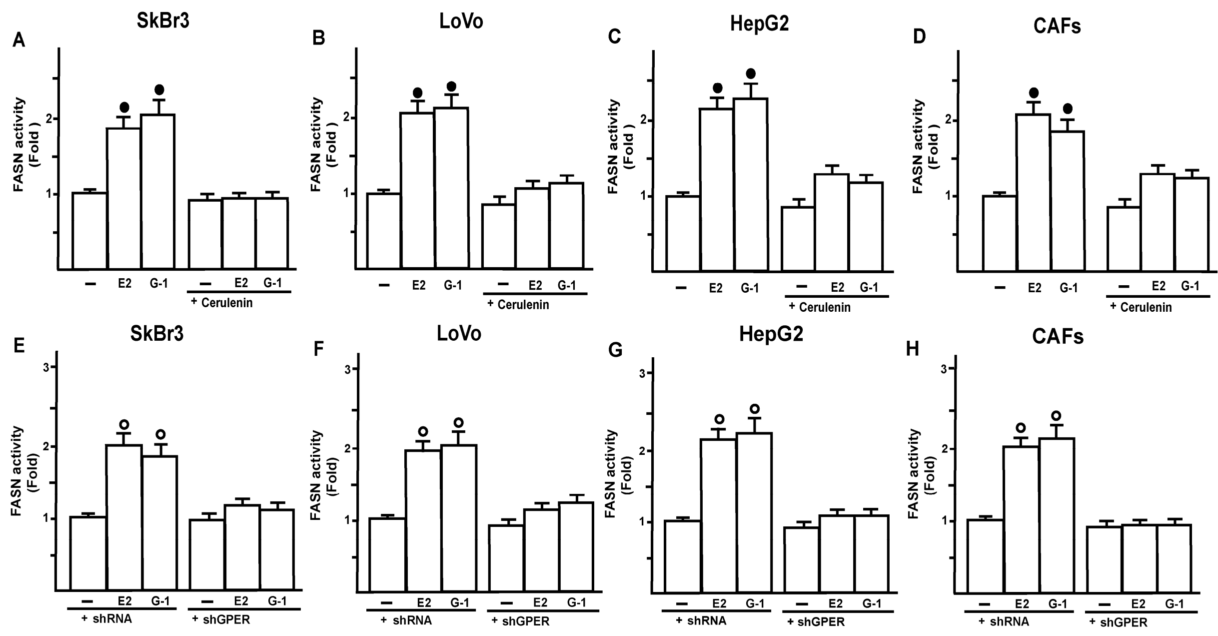

Fig. 8

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Recent Advances in the Rationale Design of GPER Ligands

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Abstract: G-Protein Coupled Receptor (GPCR) superfamily, which comprises approximately 900 members, is the largest family of protein targets with proven therapeutic value. Although at least 500 GPCRs have been identified as therapeutically relevant, only thirteen GPCRs have been structurally characterized in apo-form or in complex with ligands. GPCRs share relatively low sequence similarity making hard the process of homology modelling, nevertheless some successful hits have been determined. Recently, the G-protein-coupled estrogen receptor 1 (GPER, formerly known as GPR30) has attracted increasing interest due to its ability in mediating estrogen signaling in different normal and cancer tissues. In this regard, the identification of selective GPER ligands has provided valuable tools in order to differentiate the specific functions elicited by this novel estrogen receptor respect to those exerted by the classical estrogen receptors (ERs). In this review, we focus on GPER examining "in silico" docking simulations and evaluating the different binding modes of diverse natural and synthetic ligands.

Keywords: GPR30/GPER, estrogens, antiestrogens, GPCRs, receptor, agonists/antagonists, atomic structures, "in silico" docking simulations, homology modelling, small molecules ligands, virtual screening, binding modes.

INTRODUCTION

GPCRs, also known as seven-transmembrane domain, heptahelical, serpentine or G protein-linked receptors (GPLR), belong to the largest family of cell-surface molecules which represent the targets of approximately 40% of current medicinal drugs [1]. GPCRs are ubiquitous in mammalian [2], regulate several physiological processes and play an important role in multiple diseases ranging from cardiovascular dysfunction, depression, pain, obesity to cancer [3, 4]. One member of this superfamily, named GPR30/GPER, mediates estrogen signaling in different cell contexts independently or cooperating with the classical estrogen receptor (ER)α [5-14], leading to gene expression changes and relevant biological responses [15]. GPER acts by transactivating the Epidermal Growth Factor Receptor (EGFR), which thereafter induces the increase of intracellular cyclic AMP (cAMP), calcium mobilization and the activation of the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinases (MAPKs) [15]. Moreover, the rapid responses to estrogenic signals mediated by GPER regulate the expression of a typical gene signature, including c-fos and the connective tissue growth factor (CTGF), which are involved in the proliferation and migration of diverse cell types [15-20]. In addition to the physiological responses mediated by GPER in the reproductive, nervous, endocrine, immune and cardiovascular systems [21], its role in cancer has been supported by increasing evidence based on different tumor models [15]. Accordingly, GPER has been associated with high-grade endometrial tumors, clinical and pathological biomarkers of poor outcome in breast cancer and poor prognosis in ovarian cancer [22-24]. As EGFR and Insulin-like Growth Factor (IGF) signaling regulate GPER expression and function in diverse cancer cell types, the functional cross-talks between GPER and growth factor transduction pathways may be an additional factor contributing to the aggressive progression of estrogen-sensitive tumors [12, 25, 26].

As it concerns the ligands of GPER, many ER agonists and antagonists like estrogens, phyto-xenoestrogens and the antiestrogens 4-hydroxytamoxifen (OHT) and fulvestrant (ICI 182 780) bind to this receptor, although exhibiting an opposite action in some cases [15]. For instance, unlike the antagonistic properties displayed by OHT and ICI 182 780 with respect to the classical ERs, both compounds act as GPER agonists [15]. Conversely, the well known ER agonist estriol exerts inhibitory effects on GPER-mediated signaling [16]. In recent years, a series of selective GPER ligands acting either as agonists or antagonists have been identified providing a useful tool to differentiate the specific functions mediated by this novel estrogen receptor respect to those exerted by ERs [15-21]. In this regard, "in silico" prediction of the possible binding modes of GPER with ligands would be of particular interest for the discovery of novel drugs as well as the elucidation of the biological processes mediated by this receptor in a selective manner. Only thirteen atomic structures of GPCRs are currently known (Table **I**), making the homology modelling as a daunting task. In the last years, the structural works on GPCRs has provided a better understanding on the mechanisms by which ligands can bind to and modulate the activity of these receptors [27, 28]. In order to outflank the gap of experimental knowledge about the atomic structures of GPCRs, previous studies including our owns [16-18, 29] have been carried out by using the bovine rhodopsin 3D structure as a template model.

Table I. Identified Atomic Structures of GPCRs

Protein Name	PDB ID	Reference
Bovine Rhodopsin	1F88	[40]
Turkey β_1-adrenergic receptor	2VT4	[73]
Human β_2-adrenergic receptor	2R4R	[41]
Human A2a adenosine receptor	3EML	[74]
Human CXCR4 Chemokine Receptor	3ODU	[75]
Human D(3) dopamine receptor	3PBL	[76]
Human Histamine H1 receptor	3RZE	[77]
Human Sphingosine 1-phosphate receptor 1 (SIP1)	3V2W	[78]
Human muscarinic acetylcholine receptor	3UON	[79]
Murine δ-opioid receptor	4EJ4	[80]
Human κ-opioid receptor	4DJH	[81]
Murine μ-opioid receptor	4DKL	[82]
Human Nociceptin/orphanin FQ receptor	4EA3	[83]

HOMOLOGY MODELLING AND GPER

The basic requirement for the rational drug design is the availability of the target three dimensional atomic coordinates that are

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mostly provided by X-ray crystal diffraction data but can be also furnished by NMR and other techniques. In this field, a huge amount of studies has been performed on the most different biomolecules considering targets with well defined three dimensional structure like tubulin [30-32] and integrin receptors [33] or dealing with target structures modelled by homology such as the phosphodiesterase 9A [34], the DNA Gyrase B [35] and other GPCRs [36, 37] eventually targeting complexes such as the association between hexokinase I and the mitochondrial porine VDAC1 [38]. Lacking the target structure, a three dimensional model can be built considering that homologous proteins with similar sequences must exhibit similar structures [39]. The value of a model depends on the quality of the sequence alignment between the query protein and the template as well as on the presence of alignment gaps. Usually, a similarity between the two sequences over 50% allows a very good accuracy of the model, while the confidence is weak if the identity is lower than 25%. However, the recent advances in the field of computational biology applied to fold recognition allow to build structural models with a good grade of accuracy even in presence of a low sequence identity. As it concerns GPER, a good grade of confidence can be obtained in building the transmembrane regions while loops modelling is more susceptible to inaccuracy.

GPER STRUCTURE

GPCRs are divided into the following 6 classes in accordance with the structural homology and functional similarity: Class A (Rhodopsin-like receptors), Class B (Secretin receptors), Class C (Metabotropic glutamate/pheromone receptors), Class D (Fungal mating pheromone receptors), Class E (Cyclic AMP receptors), Class F (Frizzled/Smoothened receptors). GPER belongs to the Rhodopsin-like subfamily and shares a sequence identity of about 24.6% (297 residues) with bovine Rhodopsin, which is the first GPCR atomic structure solved (PDB code 1F88) [40]. Bovine Rhodopsin has been the only available GPCR structure for a long time, thereafter the human β2-adrenergic receptor structure was determined in 2007 [41]. Therefore, "in silico" design of GPER ligands has been initially performed by using bovine Rhodopsin as X-ray template in order to build GPER homology modelling. Alignment errors and the low sequence similarity between these two GPCRs allowed a trustable model exclusively of the seven helices of the GPER transmembrane region. The remaining portions of the protein including the cytosolic loops have been modelled "ab-initio" using the programs Robetta [42] and Modeller [43]. Despite the low degree of identity, the initial GPER model has been validated by different "*in vitro*" tests [16-18]. The final model of GPER includes 375 aminoacids and a disulphide bond between the Cys130 and Cys207 residues. The global fold, common to all GPCRs, is composed by 7 transmembrane helices forming a helical bundle, a N-terminal region (Met1- Phe60) and a C-terminal portion (Leu328 - Val375). Helices TM-I, TM-V, TM-VI, TM-VII display a kink induced by a proline residue Fig. (**1**). These kinks, which are well conserved among GPCRs, are supposed to enable the structural rearrangements needed for the activation of the G protein effectors [44]. Moreover, the C-terminal region seems to be structured with helices VIII (Thr330 - Lys342) and IX (Leu345 - Ile360). While helix VIII is present in all Rhodopsin-like GPCRs, helix IX is unexpectedly predicted by the computational secondary structure analysis. Electrostatic charge distribution is calculated using the program DelPhi [45] and mapped onto the GPER surface Fig. (**2**).

GPER AND SMALL MOLECULES LIGANDS

The process of modern drug design can be pursued following two different approaches: the first based on the small molecule (ligand-based) and the second based on the protein target (protein-based). This last method led to the discovery of the most successful drugs currently employed in cancer therapy (i.e. Gleevec, Iressa and Tarceva), making highly desirable the availability of an experimental three dimensional model of the biological targets [46-48]. However, GPCRs are membrane proteins particularly refractory to the "classical" protein crystallography pipeline as it is often difficult their overexpression and solubility and consequently the crystallization process. Fortunately, computer based methods have been increasing successful in identifying the atomic structure of a biological target on the basis of its primary structure [49, 50], in particular for GPCRs [51, 52]. To date, the availability of a GPER 3D model allowed us to pursue a "protein-based" approach in order to characterize the interaction of different ligands with this receptor [16-18]. For instance, in Fig. (**3**) is shown one of the many possible routes to be followed in drug design.

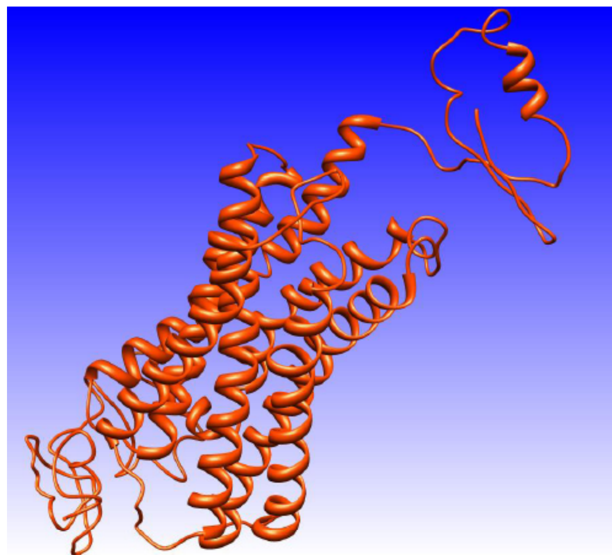

Fig. (1). Molecular model of the three-dimensional structure of GPER.

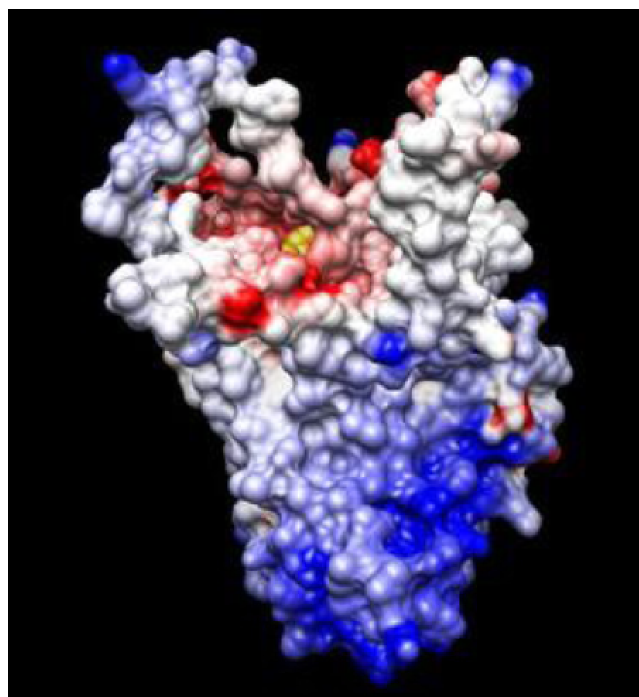

Fig. (2). Negative (red) and positive (blue) electrostatic potential of the GPER protein surface.

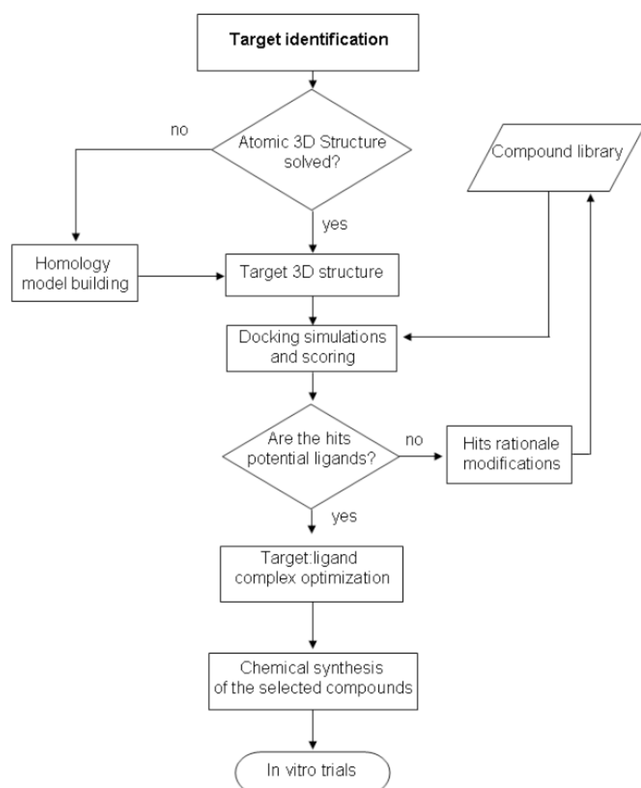

Fig. (3). One of the many possible routes showing the drug design stages.

By using ligand-based as well as mixed biomolecular and virtual screening, several GPER natural and synthetic ligands (acting as agonists or antagonists) have been identified by our and other groups Fig. (**4**) [8, 10, 11, 53-58], as discussed below. In this regard, the binding modes of some GPER ligands are shown in Fig. (**5**). The first approach to the design of GPER ligands was the evaluation of the binding modes of two major estrogens such as 17β-estradiol (E2) and estriol (E3). Estrogens are steroid hormones which play a critical role in multiple physiological and pathological processes [59]. The action of estrogens are mainly mediated by ERs [60, 61], however increasing evidence has recently demonstrated that these steroids trigger rapid responses also through the GPER transduction signaling in normal and cancer cells [5, 7, 21, 62-64]. Unlike to the antagonistic properties displayed by the antiestrogens OHT and ICI with respect to ERs, these compounds act as GPER agonists [5, 6, 8, 10, 11, 19]. Moreover, differently to E2 which binds to and activates both ERs and GPER, the ER agonist E3 acts as an antagonist ligand of GPER [16]. In particular, in silico docking simulations showed that E3 binding pocket is located in a deep cleft of GPER, where ten hydrophobic residues (V116, M133, L137, F206, F208, F278, I279, I308, V309 and F314) together with four polar aminoacids (Y123, Q138, D210 and E275) contribute to stabilize the ligand [16]. Of note, competitive assays performed in ER-negative and GPER-positive SkBr3 breast cancer cells corroborated the results obtained by molecular modelling [16]. Cumulatively, these data suggest that estrogenic/antiestrogenic agents may elicit opposite functions through ERs and GPER. A second step in the design of GPER ligands was a mixed approach based on virtual and biomolecular screening techniques. This method allowed the identification of a synthetic GPER ligand, named G-1 [8], which provided new opportunities towards the characterization of GPER-mediated signaling and functions. In this context, other moieties

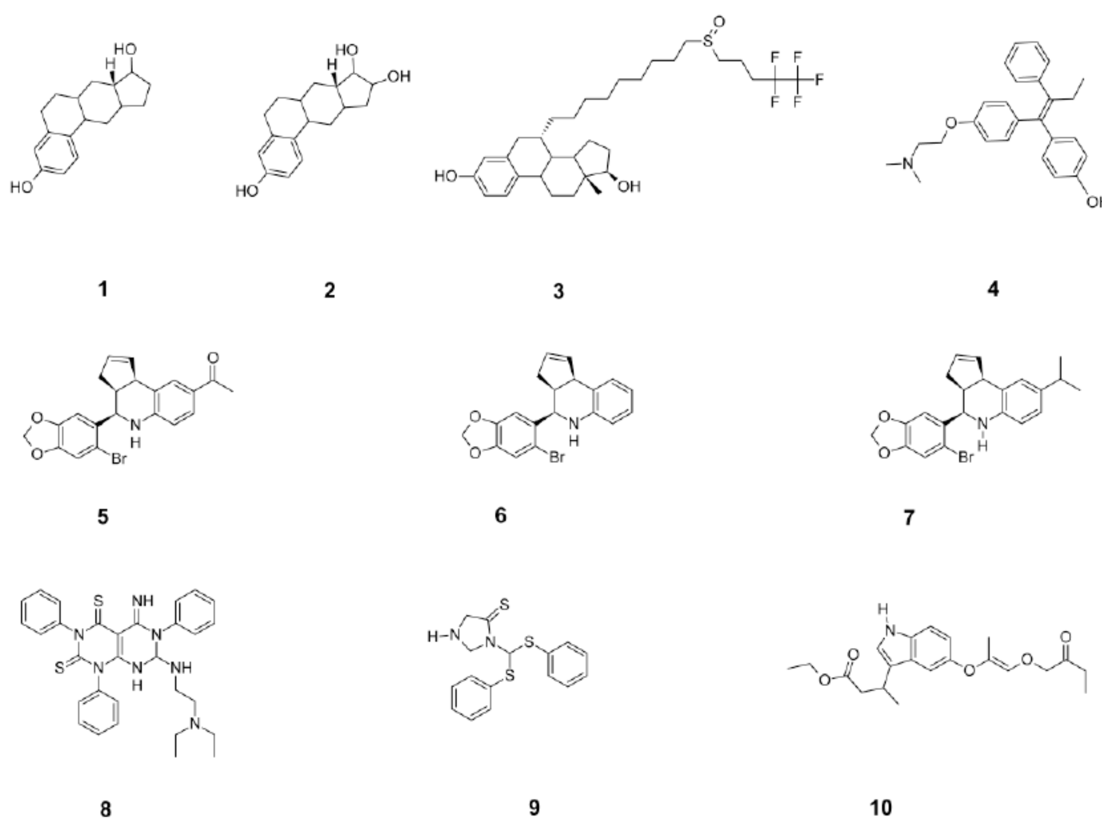

Fig. (4). Structures of some GPER ligands: (1) 17-β estradiol, (2) estriol, (3) ICI 182 780, (4) 4-hydroxytamoxifen, (5) G-1, (6) G-15, (7) G-36, (8) GPER-L1, (9) GPER-L2, (10) MIBE.

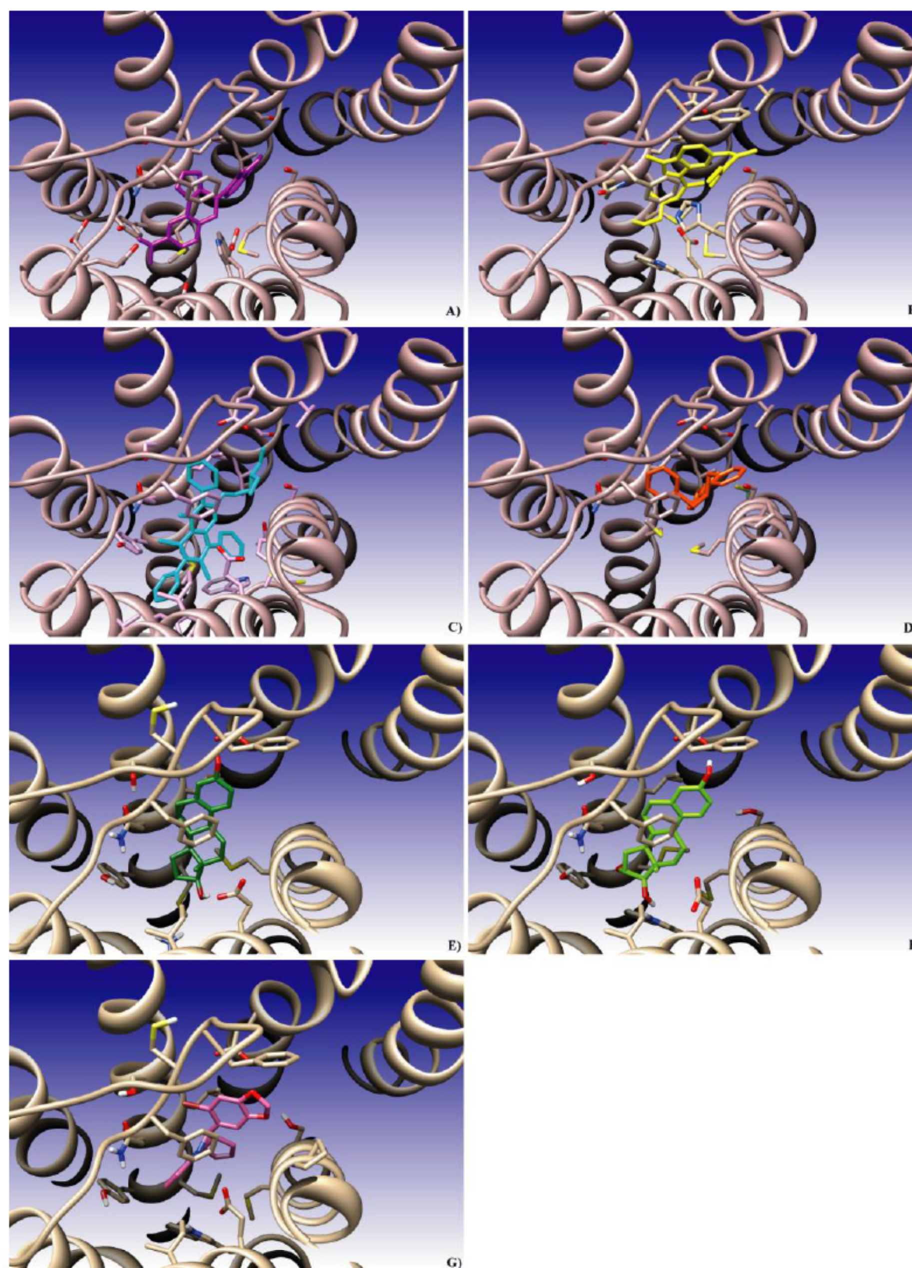

Fig. (5). GPER binding site. Protein TM helices are represented as solid ribbons while ligands are reported as sticks. Panel A, G-1 in purple sticks; panel B, MIBE in yellow; panel C, GPER-L1 in cyan; panel D, GPER-L2 in orange; panel E, 17-β estradiol in dark green; panel F, estriol in light green; panel G, G-15 in pink.

based on the same cyclopental[c]quinoline scaffold were described [54, 58]. Competition assays demonstrated that G-1 does not bind to ERs, while a K_i of 11nM was found in the case of GPER. G-1 displayed also the ability to activate multiple signaling pathways via GPER, such as calcium mobilization, PI3K and MAPK activation, that can lead to gene regulation and cell proliferation [8, 13, 16]. In addition, G-1 has been used in order to evaluate the role of GPER *in vivo*, including thymic atrophy, experimental autoimmune encephalomyelitis and vascular regulation [14, 62]. On the other hand, the discovery of G-1 paved the way to the synthesis of further GPER ligands closely related to its structure as several iodo-substituted tetrahydro-3H-cyclopenta[c]quinolines and the two GPER antagonists G-15 and G-36 [54, 57, 58]. In particular, the ethanone moiety within the G-1 molecule was involved in the activation of GPER through the formation of hydrogen bonds, which are lacking in the case of G-15 as it does not present an ethanone group. Accordingly, G-15 prevented biological responses mediated by GPER in cancer cells and *in vivo* like epithelial uterine cell proliferation and anti-depressive effects which were induced by using G-1 and estrogens [54]. The further GPER antagonist G-36 was generated replacing the reactive ethanone moiety of G-1 with a hydrophobic isopropyl group [58]. Docking analysis with G-36 yielded a score comparable to that of G-1 with a similar steric clash of the isopropyl group with Arg 394. Similar to G-15, G-36 inhibited calcium mobilization, PI3K and MAPK activation mediated by GPER as well as the proliferation *in vivo* of uterine epithelial cells [58]. Recently, two novel selective ligands of GPER were identified Fig. (**4**) [17]. Docking simulations were carried out using as targets the atomic structures of ERs (PDB codes 1G50 and 3ERT) and the molecular model of GPER. In detail, 7-({[2-(diethylamino)ethyl]

amino}methyl)-5-imino-1,3,6-triphenyl-5,6-dihydropyrimido[4,5-d]pyrimidine-2,4(1H,3H)-dithione and 1-[bis(phenylthio)methyl] imidazolidine-2-thione (referred to as GPER-L1 and GPER-L2, respectively), exhibited a very poor binding affinity for the atomic structures of ERs neither in the open nor in the closed form, whilst both compounds showed a good binding affinity for GPER. Using [3H]E2 in SkBr3 cells, the GPER binding properties of GPER-L1 and GPER-L2 were characterized by performing competitive assays [16]. GPER-L1 and GPER-L2 displaced the radioligand with a higher affinity respect to G-1 and E2, thus confirming the results obtained by "in silico" docking simulations. GPER-L1 and GPER-L2 also induced gene expression changes and growth effects mediated by GPER in breast cancer cells, while both chemicals failed to bind to and activate ER-mediated signaling. Fig. (**6**) shows a scheme reporting the steps followed in designing two of the above described moieties: GPER-L1 and G1. In developing GPER-L1 Fig. (**6**, compound n. **8**) from E2 Fig. (**6**, compound n. **1**), it was conserved the decahydronaphthalene core building the final molecule in analogy to merbarone **(**5-(N-Phenylcarbamoyl)-2-thiobarbituric acid**),** a well-known catalytic inhibitor of topoisomerase II [17]. In the case of G1 Fig. (**6**, compound n. **5**), it was developed the final ligand by screening the chemical space of the possible derivatives of 4-methyl-2,3,3a,4,5,9b-hexahydro-1*H*-cyclopenta[*c*]quinoline [8].

The exclusive property to bind to and inhibit both GPER and ERα-dependent pathways was exhibited by a further agent, the ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1Hindol -3-yl]but-2-enoate, which was referred to as MIBE Fig. (**4**) [18]. This molecule was first docked to the ligand binding pocket of ERα in both the closed and open conformations [18]. A better affinity for the last conformation was evidenced together with a binding mode similar to that adopted by the ER antagonist OHT in the crystallographic structure complex (PDB code 3ERT) [65]. Using GPER as target of the docking simulations, MIBE showed a binding mode similar to that of G-1 as the methylindole of MIBE superposed with the tetrahydro-3H-cyclopental[c]quinoline scaffold of G-1, while the propyl propanoate moiety of MIBE overlapped to the 5-bromo-2H-1,3-benzodioxole moiety of G-1. Unlike to G-1, MIBE does not present an ethanone group and lacks the ability to form hydrogen bonds with the helix TM III through the Asn 138 residue. Moreover, a methyl group of MIBE generates a short contact with TMVII through His 307 side chain. Ligand binding studies and functional assays validated the results obtained by molecular modelling and docking simulations, as MIBE exhibited a good ligand affinity for GPER and ERα and displayed the ability to inhibit the transduction signaling mediated by these receptors in breast cancer cells. Hence, the antagonistic action exerted by MIBE on both receptors may guarantee major therapeutic benefits respect to the current antiestrogens in hormone-dependent tumors like breast cancer. The methodology used to perform docking and ligand chemical synthesis are reported in the original works [8, 16-18, 54, 58]. In particular, three dimensional protein visualization and manipulation were carried out using the program COOT [66], docking simulations were performed using the softwares Autodock [67] and GOLD [68], figures were drawn with the program Chimera [69].

CONCLUSIONS

Since the early 1980s, docking of small molecules to protein binding sites has been heralded as a solution to face the problems of the pharmaceutical industry [70]. Currently, it is well accepted that the knowledge of the three dimensional structure of a biological

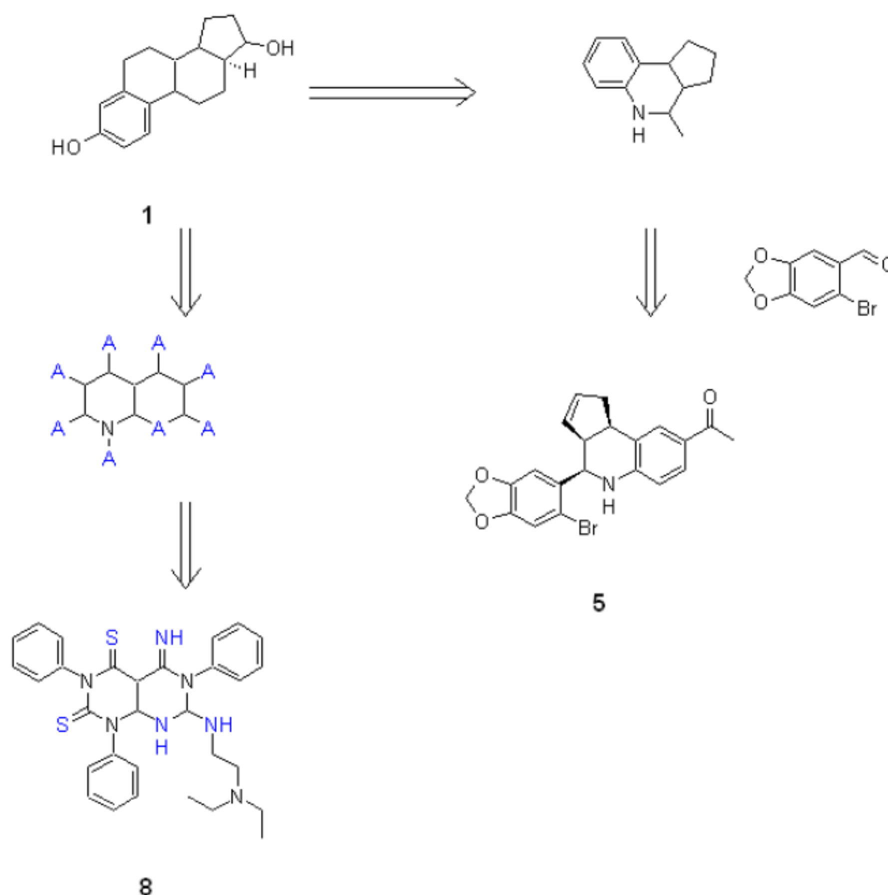

Fig. (6). Scheme of the steps followed in designing some ligands of GPER: 17-β estradiol (1), G-1 (5) and GPER-L1 (8).

target may provide essential information in order to understand its functions and important hints for rational drug design. However, the progress in determining the atomic structure of GPCRs is very slow [71], being the bovine Rhodopsin the first GPCR structure solved in 2000 [40]. Technical advances are now promising toward the determination of further structures of GPCRs as only 13 non redundant configurations have been determined up today (Table **I**). Nevertheless, the possibility that a number of important drug targets will be solved in the near future is still low making the search on GPCR structure a kind of "holy grail" of Structure Based Drug Design. This situation limits the drug discovery process restricting the research to the ligand binding and GPCR activation through biomolecular techniques [72]. Fortunately, in the last years it has been observed an increasing success of computer-based methods for the prediction of tertiary protein structures, especially in the field of GPCRs.

As it concerns GPER, an increasing number of studies has demonstrated its ability to mediate biological responses to estrogenic compounds in different cell contexts. As GPER is expressed in multiple tumor cells including breast, endometrial, ovarian and thyroid carcinomas as well as in cancer-associated fibroblasts [14, 20], its potential to contribute to tumor progression induced by estrogens should be taken into account particularly in the aforementioned malignancies [15]. Accordingly, GPER expression was associated with negative clinical features and poor survival rates in patients with breast, endometrial and ovarian carcinomas, suggesting that GPER may be a predictor of aggressive diseases [22-24]. The discovery of novel molecules targeting GPER is of outstanding interest in order to further clarify its biological functions as well as to develop novel tools for a more comprehensive treatment of estrogen-dependent tumors.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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ABBREVIATIONS

CTGF	=	Connective Tissue Growth Factor
E2	=	17β-estradiol
E3	=	Estriol
EGFR	=	Epidermal Growth Factor Receptor
ER	=	Estrogen Receptor
ERK	=	Extracellular Signal-Regulated Kinase
G-1	=	1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c]quinolin-8-yl]-Ethanone
G-15	=	4-(6-Bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c]quinoline
G-36	=	(4-(6-bromo-benzo[1,3]dioxol-5-yl)-8-isopropyl-3a,4,5,9btetrahydro-3H-cyclopenta[c]quinoline
GPCR	=	G-Protein Coupled Receptor
GPER	=	G Protein-Coupled Estrogen Receptor
GPER-L1	=	7-({[2-(diethylamino)ethyl]amino}methyl)-5-imino-1,3,6-triphenyl-5,6-dihydropyrimido[4,5-d]pyrimidine-2,4(1H,3H)-dithione
GPER-L2	=	1-[bis(phenylthio)methyl]imidazolidine-2-thione
ICI 182 780	=	Fulvestrant
IGF	=	Insulin-Like Growth Factor
MAPK	=	Mitogen-Activated Protein Kinase
MIBE	=	Ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1Hindol-3-yl]but-2-enoate
OHT	=	4-hydroxytamoxifen

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Two Novel GPER Agonists Induce Gene Expression Changes and Growth Effects in Cancer Cells

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Abstract: Although the action of estrogens has been traditionally explained by the binding to and transactivation of the nuclear estrogen receptor (ER)α and ERβ, recently the G protein-coupled receptor GPR30/GPER has been involved in the rapid estrogen signaling. We investigated the ability of two original molecules, which were named GPER-L1 and GPER-L2, to bind to and activate the GPER transduction pathway in cancer cells. Competition assays, docking simulations, transfection experiments, real-time PCR, immunoblotting, gene silencing technology and growth assays were performed to ascertain the selective action of GPER-L1 and GPER-L2 in activating the GPER-mediated signaling. Both compounds, which did not show any ability to bind to and activate the classical ERs, were able to bind to GPER and to trigger the rapid activation of the GPER/EGFR/ERK transduction pathway which led to the up-regulation of GPER-target genes. Notably, GPER-L1 and GPER-L2 induced the proliferation of SkBr3 breast and Ishikawa endometrial cancer cells at nM concentrations through GPER, hence providing further evidence on their capability to elicit relevant biological responses mediated by GPER. The identification and characterization of these novel compounds as selective GPER agonists represent a valuable tool to further dissect the pharmacology of this novel estrogen receptor and to better differentiate the specific functions elicited by each estrogen receptor subtype in cancer cells.

Keywords: Breast cancer cells, endometrial cancer cells, estrogen receptors, GPER-L1, GPER-L2, GPR30/GPER.

INTRODUCTION

G protein–coupled receptors (GPCRs) are cell-surface signaling proteins involved in multiple physiological functions as well as in human disease including cancer [1]. Recently, the seven transmembrane receptor GPR30/GPER has been identified as a novel membrane estrogen receptor able to mediate rapid estrogen signaling [2]. Even though GPER functions are distinct from those of the classical nuclear estrogen receptor (ER)α and ERβ, several studies have demonstrated that these receptors cooperate in mediating relevant biological actions in different cell contexts [3-6]. GPER was involved in a number of estrogen-induced transduction events, such as Epidermal Growth Factor Receptor (EGFR) transactivation, increasing of intracellular cyclic AMP (cAMP), calcium mobilization and activation of the mitogen-activated protein kinases (MAPKs) cascade [7]. Moreover, GPER was implicated in a broad range of physiological functions in regards to the reproduction, the metabolism, the bone, the cardiovascular, the nervous and immune systems [8]. Likewise, the potential of GPER to elicit stimulatory effects in numerous types of tumors has been largely demonstrated [2]. In this regard, previous studies have shown that GPER, through the EGFR/MAPK transduction pathway, mediates gene transcription and growth responses induced by both estrogen and antiestrogen in breast, endometrial, ovarian and thyroid cancer cells [3, 9-12]. Of note, ligand-activated EGFR lead to the up-regulation of GPER expression in both ER-negative and positive cancer cells, further extending the functional cross-talk between these receptors [6, 13]. As it concerns clinical findings, GPER overexpression was associated with lower survival rates in endometrial and ovarian cancer patients [14-15] and with a higher risk of developing metastatic disease in breast cancer patients [16].

A major challenge in dissecting estrogen signaling is the identification of novel compounds able to differentiate the pharmacology of the novel GPER over that of the classical ERs by targeting each receptor subtype in a selective manner. In this respect, 17β-estradiol (E_2) and both the antiestrogens 4-hydroxytamoxifen (OHT) and ICI 182,780 were demonstrated to exert stimulatory effects as GPER ligands in different cell contexts [9-12, 17-19], whereas estriol was shown to act as a GPER antagonist in ER-negative breast cancer cells [20]. The identification of G-1 [21] and G-15 [22], which act as a selective GPER agonist and antagonist, respectively, provided new opportunities towards the characterization of GPER signaling as well as the evaluation of both common and distinct estrogen receptors-mediated functions. Moreover, iodo-substituted quinoline derivatives have been described as valuable GPER-selective radiotracers, which may be useful for the characterization of the receptor binding properties [23]. Recently, we recently identified the first ligand of ERα and GPER, named MIBE, which displayed the unique property to inhibit GPER- and ERα-mediated signaling in breast cancer cells [24]. The exclusive antagonistic activity exerted by this compound on both ERα and GPER-mediated signaling could represent a promising pharmacological approach toward a more comprehensive treatment in breast cancer patients.

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Here, we demonstrate that two novel compounds, which were named GPER-L1 and GPER-L2 (Fig. **1**), selectively bind to and activate GPER signaling at nM concentrations in cancer cells. The ligand specificity of both compounds for GPER may represent a helpful tool to further dissect the pharmacology of this novel estrogen receptor.

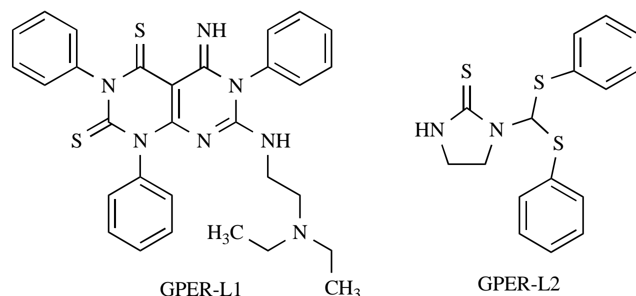

Fig. (1). Chemical structures of GPER-L1 and GPER-L2.

MATERIAL AND METHODS

Chemistry

All building blocks used were purchased by Chiminord and Aldrich Chemical (Milan, Italy). Solvents were reagent grade. DMF was dried on molecular sieves (5Å 1/16" inch pellets). Unless otherwise stated, all commercial reagents were used without further purification. Organic solutions were dried over anhydrous sodium sulphate. Thin layer chromatography (TLC) system for routine monitoring the course of reactions and confirming the purity of analytical samples employed aluminium-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}): $CHCl_3$ was used as developing solvent and detection of spots was made by UV light and/or by iodine vapours. Yields were not optimized. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer 398 spectrometer as KBr discs. ^{1}H NMR spectra (200 MHz) were recorded in $CDCl_3$ on a Varian Gemini 200 instrument. Chemical shifts were reported in δ (ppm) units relative to the internal standard tetramethylsilane, and the splitting patterns were described as follows: bs (broad singlet), s (singlet), t (triplet) and m (multiplet). The first order values reported for coupling constants J were given in Hz. Elemental analyses were performed by an EA1110 Elemental Analyser (Fison-Instruments, Milan); all compounds were analyzed for C, H, N and S and the analytical results were within ±0.4% of the theoretical values.

Synthesis of 7-({[2-(diethylamino)ethyl]amino}methyl)-5-imino-1,3,6-triphenyl-5,6-dihydropyrimido[4,5-d]pyrimidine-2,4(1H,3H)-dithione (GPER-L1)

GPER-L1 synthesis was accomplished through a modification of a previously published protocol [25]. Briefly, the condensation of malononitrile with phenylisothiocyanate (3 equivalents) in the presence of sodium hydride led to the pyrimido-pyrimidine scaffold which was subsequently S-methylated at position 7. The nucleophilic displacement of the thiomethyl group with N,N-diethylethylenediamine led to the desired compound in good yields.

Mp 153-155 ^{0}C. Yield: 54%. IR (KBr) cm^{-1} 3279; 1627; 1574. ^{1}H NMR ($CDCl_3$) δ 0.65 (t, J = 7.2 Hz, 6H, 2 CH_3); 1.96-2.36 (m, 6H, 3 CH_2N); 2.58-2.88 (m, 2H, CH_2NH); 6.68-7.72 (m, 15H, arom H); 11.43 (bs, 1H, NH imide, exghangeable). Anal. Calcd. for $C_{28}H_{27}N_7S_2$: C:63.97; H: 5.18; N: 18.65; S: 12.20. Found: C: 63.99; H: 5.27; N: 18.68; S: 12.05

Synthesis of 1-[bis(phenylthio)methyl]imidazolidine-2-thione (GPER-L2)

GPER-L2 was prepared by an highly convergent one pot procedure. As previously described [26], the condensation of 2-imidazolidinethione with benzoyl chloride in DMF lead to the formation of N-methyleniminium salt. The in situ reaction of this intermediate with 2 equivalents of thiophenol allowed the isolation of the desired dithioketal compound [Palenzona MG, 1999, unpublished observations].

Mp: 92-94 °C. Yield: 19. %. IR (KBr) cm^{-1} 3455, 1466. ^{1}H NMR ($CDCl_3$) δ 3.10-3.60 (m, 2H, CH_2); 3.65-4.12 (m, 2H, CH_2); 6.80 (bs, 1H, NH exchangeable); 7.12-7.80(m, 11, arom H + CH). Anal. Calcd. for $C_{16}H_{16}N_2S_3$: C: 57.80; H: 4.85; N: 8.42; S: 28.93. Found: C: 57.62; H: 4.69; N: 8.51; S: 29.12

Reagents

17β-estradiol (E_2) and 4-hydroxytamoxifen (OHT) were purchased from Sigma-Aldrich Corp. (Milan, Italy). G-1 (1-[4-(-6-bromobenzol[1,3]diodo-5-yl)-3a,4,5,9b-tetrahidro3H5 cyclopenta[c]quinolin-8yl]-ethanone) was bought from Merck KGaA (Frankfurt, Germany). AG1478 (AG) and PD98059 (PD) were obtained from Biomol Reaserch Laboratories, Inc. (DBA, Milan, Italy). G-15 was kindly provided by Dr Eric R. Prossnitz (University of New Mexico Health Sciences Center, Albuquerque). All compounds were solubilized in dimethyl sulfoxide (DMSO), except E_2 and PD, which were dissolved in ethanol.

Cell Culture

SkBr3 breast cancer cells and Ishikawa endometrial cancer cells were maintained in RPMI 1640 and DMEM respectively, without phenol red supplemented with 10% FBS. MCF7 breast cancer cells were maintained in DMEM with phenol red supplemented with 10% FBS. The day before experiments for immunoblots and real-time PCR assays cells were switched to medium without serum, thereafter cells were treated as indicated.

Transfection, Luciferase Assays and Gene Silencing Experiments

Plasmids and Luciferase Assays were previously described [27-31]. In particular, the luciferase reporter plasmid for 4 X AP-1-responsive collagen promoter was a kind gift from H van Dam (Department of Molecular Cell Biology, Leiden University, Leiden, The Netherlands). As an internal transfection control, we cotransfected the plasmid pRL-TK (Promega, Milan, Italy). Short hairpin RNA construct against human GPER (previously called shGPR30) and the unrelated shRNA control construct have been previously described [13]. Briefly, short hairpin (sh)RNA

constructs against human GPER were bought from Openbiosystems (Biocat.de, Heidelberg, Germany) with catalog no. RHS4533-M001505. The targeting strands generated from the shRNA vectors sh1, sh2, sh3, sh4, and unrelated control are complementary to the following sequences, respectively: CGAGTTAAAGAGGAGAAGGAA, CTCCCTCATTGAGGTGTTCAA, CGCTCCCTGCAAGCAGTCTTT, GCAGTACGTGATCGGCCTGTT, and CGACATGAAACCGTCCATGTT. On the basis of the major silencing efficacy, sh3 was used and referred to as shGPR30/shGPER.

Cells were plated into 24-well plates with 500 µl of regular growth medium/well the day before transfection. Cell medium was replaced with medium supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red on the day of transfection, which was performed using the Fugene6 Reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5 µg of reporter plasmid and 2 ng of pRL-TK. After 6 h, the medium was replaced again with serum-free DMEM lacking phenol red and supplemented with 1% CS-FBS, treatments were added at this point and cells were incubated for additional 18 h. Luciferase activity was then measured using 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 set as one-fold induction on which the activity induced by treatments was calculated. For the gene silencing experiments, cells were plated into 10-cm dishes, maintained in serum-free medium for 24 h and then transfected for additional 24 h before treatments using Fugene6 and control vector (shRNA) or shGPER.

Ligand Binding Assays

In ligand binding assay for GPER, SkBr3 cells were grown in 10-cm cell culture dishes, washed two times and incubated with 1 nM $[^3H]E_2$ (89 Ci/ mmol; Ge Healthcare, Milan, Italy) in the presence or absence of increasing concentration of nonlabeled competitors (G-1, GPER-L1 and GPER-L2, E_2 and G-15). Then, cells were incubated for 2 hours at 37°C and washed three times with ice-cold PBS; the radioactivity collected by 100% ethanol extraction was measured by liquid scintillation counting. Competitor binding was expressed as a percentage of maximal specific binding. Each point is the mean of three observations. In ligand binding assay for ERα, the ability of GPER-L1 and GPER-L2 to compete with $[^3H]E_2$ was also evaluated and compared with that of E_2. Two picomoles of purified recombinant human ERα protein purchased from PanVera, Invitrogen S.r.l. (Milan, Italy) was incubated with 1nM $[^3H]E_2$ (89 Ci/mmol; Ge Healthcare, Milan, Italy) and increasing concentrations of nonlabeled E_2 or GPER-L1 and GPER-L2 for 2 hours at 37° C in a humidified atmosphere of 95% air/5% CO2. Bound and free radioligands were separated on Sephadex G-25 PD-10 columns. The amount of receptor-bound $[^3H]E_2$ was determined by liquid scintillation counting.

Reverse Transcription and Real-Time PCR

Cells were grown in 10-cm dishes in regular growth medium and then switched to medium lacking serum for 24 h. Thereafter, treatments were added for the times indicated and cells were processed for mRNA extraction using Trizol reagent (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically and its quality was checked by electrophoresis through agarose gel stained with ethidium bromide. Only samples that were not degraded and showed clear 18S and 28S bands under ultraviolet light were used for real-time PCR. Total cDNA was synthesized from RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Invitrogen, Milan, Italy) following the protocol provided by the manufacturer. The expression of selected genes was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc, Milan, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc, Milano, Italy). Assays were performed in triplicate and the mean values were used to calculate expression levels, using the relative standard curve method. For c-fos, ATF3, CTGF, Cyr61, EGR1, MT1X, MT2A and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5'-CGAGCCCTTTGATGACTTCCT-3' (c-fos forward), 5'-GGAGCGGGCTGTCTCAGA-3' (c-fos reverse); 5′-AAGTGAGTGCTTCTGCCATC-3′ (ATF3 forward) and 5′-TTTCTTTCTCGTCGCCTCTTTT-3′ (ATF3 reverse); 5'-ACCTGTGGGATGGGCATCT-3' (CTGF forward), 5'-CAGGCGGCTCTGCTTCTCTA-3' (CTGF reverse); 5′-GAGTGGGTCTGTGACGAGGAT-3′ (Cyr61 forward) and 5′-GGTTGTATAGGATGCGAGGCT-3′ (Cyr61 reverse); 5'-GCCTGCGACATCTGTGGAA-3' (EGR1 forward), 5'-CGCAAGTGGATCTTGGTATGC-3' (EGR1 reverse); 5'-TGTCCCGCTGCGTGTTT-3' (MT1X forward) and 5'-TTCGAGGCAAGGAGAAGCA-3' (MT1X reverse); 5'-CCCGCTCCCAGATGTAAAGA-3' (MT2A forward) and 5'-GGTCACGGTCAGGGTTGTACATA-3' (MT2A reverse) and 5'- GGCGTCCCCCAACTTCTTA -3' (18S forward) and 5'- GGGCATCACAGACCTGTTATT -3' (18S reverse), respectively.

Western Blotting

Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 µL of 50 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% sodium dodecyl sulfate (SDS), and a mixture of protease inhibitors containing 1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. 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, Milan, Italy), probed overnight at 4°C with antibodies against GPER (N-15), c-fos (H-125), Cyclin D1 (M-20), β-actin (C-2), phosphorylated ERK1/2 (E-4), ERK2 (C-14), all purchased from Santa Cruz Biotechnology (DBA, Milan, Italy), and then revealed using

the ECL™ Western Blotting Analysis System (GE Healthcare, Milan, Italy).

Proliferation Assay

For quantitative proliferation assay, cells (1 x 10^5) were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% charcoal-stripped FBS with the indicated treatments; medium was renewed every 2 days (with treatments) before dimethylthiazoldiphenyltetrazoliumbromide (MTT, Sigma-Aldrich, Milan, Italy) assays (performed according to the manufacturer's protocol). A concentration of 250ng/L of the control shRNA or shGPER plasmids was transfected using Fugene 6 Reagent the day before treatments and then renewed every 2 days before counting.

Molecular Modelling and Docking Simulations

All molecular structures of the ligands screened "*in silico*" were built and energy minimized with the programs InsightII and Discover3 (Biosym/MSI, San Diego, CA, USA). To analyse the receptor-ligand complexes, we built an homology model of GPER using, as a template, the atomic coordinates of the bovine Rhodopsin (PDB code 1U19) [32] which shares 40% amino acid sequence similarity to our target, and the program MODELLER [33]. The derived model was then energy minimized using the program DISCOVER3 from the InsightII suite (Accelrys, Inc., San Diego - CA - USA) by the ESFF force field and stereochemistry was further optimized by the program REFMAC5 [34]. In order to investigate the binding mode of different ligands to GPER and to evaluate the binding energies of the resulting complexes, we used a combination of the computer programs AutoDock 3.05/ADT [35-36] and GOLD v.5.0.1. For each ligand tested, we initially performed a "blind docking": the docking of small molecules to their targets was done without *a priori* knowledge of the location of the binding site by the system. A preliminary global docking was performed with AutoDock using a grid encompassing the whole protein surface. The docking experiment consisted of 100 Lamarckian Genetic Algorithm runs. The generated docking poses were ranked in order of increasing docking energy values and clustered on the basis of a RMSD cut-off value of 0.5 Å. From the structural analysis of the best solutions (lowest energy) of each cluster, we could highlight the protein binding site. A second docking was performed using the program GOLD v5.0.1. The following residues on GPER model were defined with flexible side chains: Tyr123, Gln138, Phe206, Phe208, Glu275, Phe278 and His282, allowing a free rotation of their side chains. The binding cavity was defined as centred around the O atom of Phe208, with a radius of 20 Å and a total of 100,000 genetic algorithm (GA) operations were performed on each moiety tested. The results obtained by this second simulations allowed us to define the binding modes of the ligand tested with precision. All figures were drawn with the program Chimera [37].

Statistical Analysis

Statistical analysis was done using ANOVA followed by Newman-Keuls' testing to determine differences in means. $P<0.05$ was considered as statistically significant.

RESULTS

GPER-L1 and GPER-L2 Docking Simulations

The GPER homology model was preliminary used to simulate the binding of the selective GPER ligand G-1. Visual inspection demonstrated that the ligand binding pocket of the protein consists of a deep cleft where ten hydrophobic residues (Val116, Met133, Leu137, Phe206, Phe208, Phe278, Ile279, Ile308, Val309 and Phe314) and five polar aminoacids (Tyr123, Gln138, Asp210, Glu275 and His282) contribute to stabilize the ligand binding through Van der Waals interactions and hydrogen bonds, respectively. The predicted affinity of G-1 for the protein was in keeping with literature data [21] thus providing indirect validation on the quality of both the protein model and the docking protocol. On the basis of this evidence, we performed in silico screening of an in house chemical library composed by more than 300 original compounds. Out of the evaluated compounds, GPER-L1 and GPER-L2 demonstrated an excellent affinity for GPER (Fig. **2**) and were therefore proposed for experimental evaluation. Despite the structural differences, these two molecules share some features as the ability to interact through a π - π stacking with the residues Phe206 and Phe208. In addition, GPER-L1 and GPER-L2 expose a phenyl ring in a highly hydrophobic

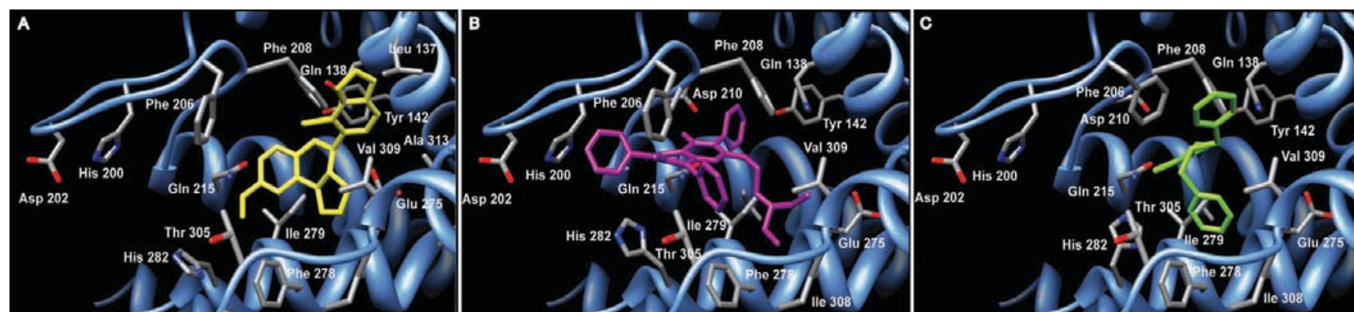

Fig. (2). The three dimensional model of GPER is schematically reported as a light blue ribbon cartoon. The binding modes of the different ligands tested are as following: G-1 in yellow (panel **A**), GPER-L1 in purple (panel **B**), GPER-L2 in light green (panel **C**). Residue positioning is not conserved among the panels due to the flexible docking approach used in the simulations.

pocket formed by the protein residues Phe278, Ile279, Ile308 and Val309, hence both displaying the potential to bind to GPER.

GPER-L1 and GPER-L2 Selectively Bind to GPER

In order to further characterize the potential binding properties of GPER-L1 and GPER-L2, we performed a whole cell binding assay by using $[^3H]E_2$ in ER-negative SkBr3 breast cancer cells, as previously reported [20]. In our experimental conditions, GPER-L1 and GPER-L2 displaced the radioligand with an affinity corresponding to approximately 100 nM, which was higher respect to the GPER ligands G-1, E_2 and G-15 (Fig. **3**). In further competitive binding experiments performed *in vitro* using the purified human ERα protein, E_2 displaced the $[^3H]E_2$ whereas GPER-L1, GPER-L2 and G-1 did not show any binding ability for ERα (Supplementary Fig. **1**). Next, to verify whether GPER-L1 and GPER-L2 might be able to activate the classical ERs in a ligand-independent manner, we transiently transfected the ER reported gene in both MCF7 and SkBr3 breast cancer cells. Only E_2 transactivated the endogenous ERα in MCF7 cells as well as chimeric ERα and ERβ proteins (consisting of the DNA binding domain of the yeast transcription factor Gal4 and the ligand binding domain of ERα and ERβ) which were transfected in SkBr3 cells (Supplementary Fig. **2**). Taken together, these results demonstrate that GPER-L1 and GPER-L2 are selective GPER ligands, since they do not exhibit binding and activating properties for ERα.

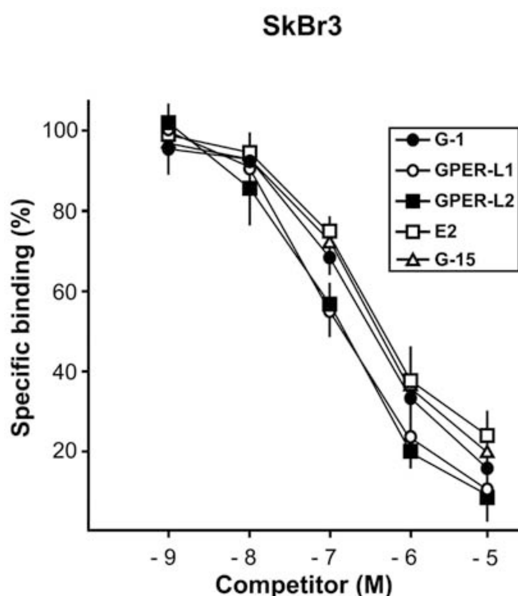

Fig. (3). Binding assay of GPER-L1 and GPER-L2 in SkBr3 cells. Competition curves of increasing concentration of G-1, GPER-L1, GPER-L2, E_2 and G-15 expressed as a percentage of maximum specific $[^3H]E_2$ binding. Each data point represents the mean of three separate experiments performed in triplicate in SkBr3 cells.

GPER-L1 and GPER-L2 Induce GPER-Mediated Gene Expression

To verify whether the binding properties of GPER-L1 and GPER-L2 for GPER trigger intracellular molecular signaling, such as ERK phosphorylation which is known to characterize the ligand activation of this receptor [2], we used both ER-negative breast (SkBr3) and ER-positive endometrial (Ishikawa) cancer cells as model systems. In dose-response studies, both compounds induced ERK phosphorylation starting from the nanomolar concentration range (Fig. **4A-D**). Notably, ERK activation upon exposure to 100 nM GPER-L1 and GPER-L2 was prevented either in SkBr3 or Ishikawa cells silencing GPER expression (Fig. **4E-F** and Supplementary Fig. **3**), suggesting that this receptor mediates the response to both ligands. In addition, using the EGFR inhibitor AG and the ERK inhibitor PD we determined that the EGFR/ERK transduction pathway is involved in ERK activation by both compounds in SkBr3 (Fig. **4G-H**) as well as in Ishikawa cells (data not shown).

Considering that GPER-mediated signaling has been shown to regulate the transcription of several genes [2, 10], we assessed that GPER-L1 and GPER-L2 also stimulate in a time-dependent manner the mRNA expression of well known GPER target genes, as evaluated by real-time PCR (Fig. **5A-B**). The GPER agonists G-1 and E_2 also up-regulated the expression of the GPER target genes although with a lower efficacy compared to GPER-L1 and GPER-L2, whereas the GPER antagonist G-15 did not exhibit any stimulatory property (Fig. **5A-B**). As the GPER/EGFR/ERK-activated transduction pathway has been largely shown to up-regulate the expression of the oncogene c-fos [38], we also determined that GPER-L1 and GPER-L2 (Fig. **6A-B**) as well as G-1 and E_2 treatments (Supplementary Fig. **4**) stimulate c-fos protein expression paralleling the mRNA induction. As it concerns the GPER antagonist G-15, it did not exhibit any ability to induce c-fos protein levels (Supplementary Fig. **4**) as observed at the mRNA levels. The EGFR and ERK inhibitors, AG and PD respectively (Fig. **6C-D**) abolished the c-fos protein increase confirming that the EGFR/ERK transduction pathway is involved in this response. Importantly, the induction of c-fos by GPER-L1 and GPER-L2 was blocked in both SkBr3 (Fig. **6E** and Supplementary Fig. **5**) and Ishikawa cells (data not shown) silencing GPER expression. Members of the Fos family interact with Jun proteins to form the activator protein-1 (AP-1) complex, which has been largely implicated in relevant biological processes, including invasion and metastasis, proliferation, differentiation, survival and angiogenesis [39]. Therefore, we asked whether GPER-L1 and GPER-L2-dependent c-fos induction could induce the response of a luciferase reporter gene encoding for a responsive collagenase promoter sequence (AP-1-luc). As shown in Fig. **6** (panel **F**), GPER-L1 and GPER-L2 transactivated the AP-1-luc reporter plasmid suggesting that both compounds trigger the c-fos/AP-1 mediated signaling.

GPER-L1 and GPER-L2 Stimulate the Proliferation of Cancer Cells

As a biological counterpart of the aforementioned results, we evaluated the potential of GPER-L1 and GPER-L2 to regulate a relevant response such as cell proliferation. Both ligands induced growth effects in SkBr3 and Ishikawa cells with a maximum stimulation corresponding to a concentration of 10 nM after 5 days of treatment (Fig. **7A,C**).

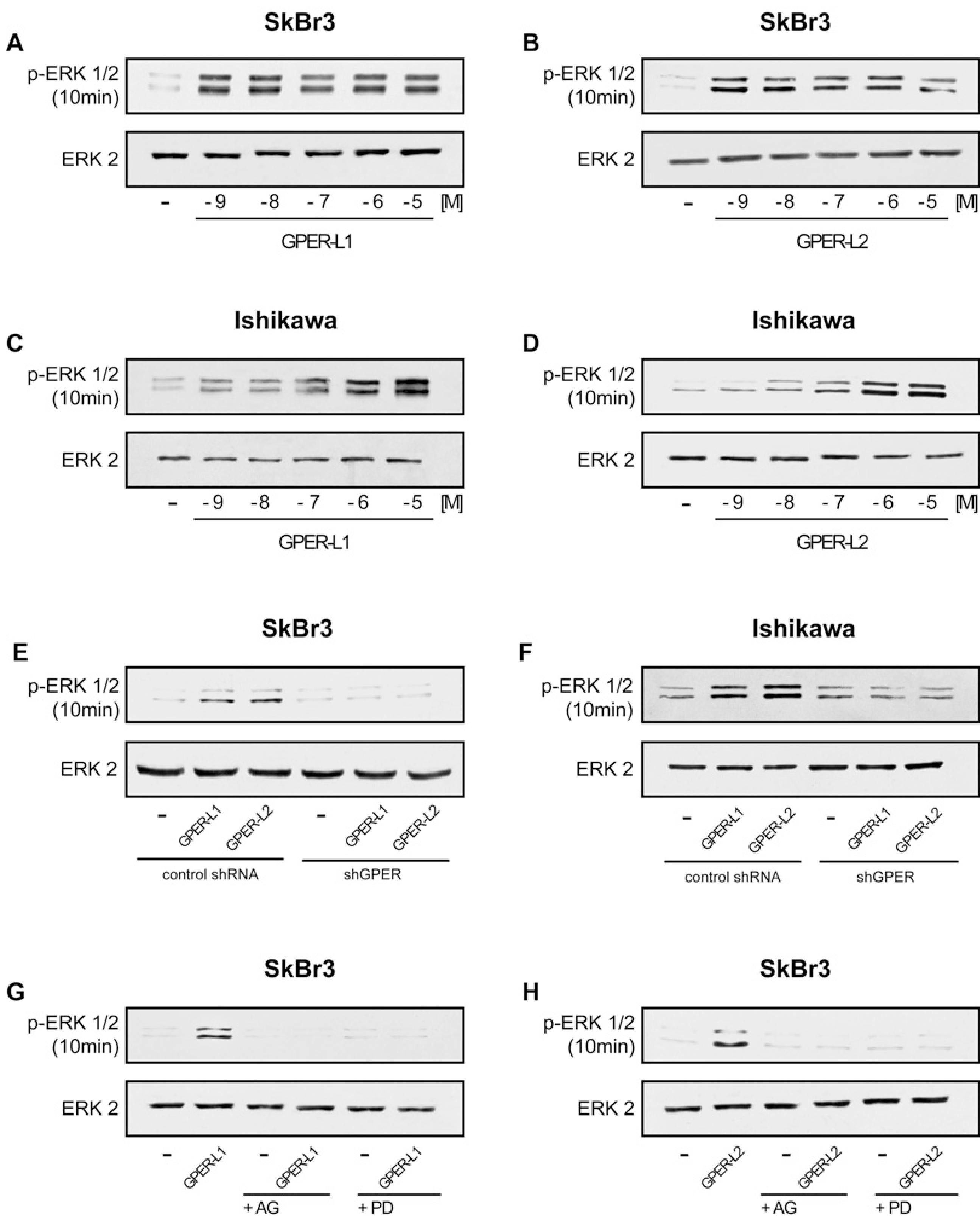

Fig. (4). GPER-L1 and GPER-L2 activate ERK1/2 in a GPER-dependent manner. ERK1/2 phosphorylation (p-ERK1/2) in SkBr3 (**A-B**) and Ishikawa (**C-D**) cells exposed to increasing concentrations of GPER-L1 or GPER-L2 for 10 min. ERK1/2 phosphorylation in SkBr3 (**E**) and Ishikawa (**F**) cells silencing GPER expression. Cells were transfected with control shRNA or shGPER and treated for 10 min with vehicle (–) or 100 nM GPER-L1 or GPER-L2. (**G-H**) ERK1/2 phosphorylation in SkBr3 cells treated with vehicle (–) or 100 nM GPER-L1 or GPER-L2 in combination with 10 µM inhibitors of EGFR and MAPK, AG or PD respectively. ERK2 serves as a loading control. Data shown are representative of three independent experiments.

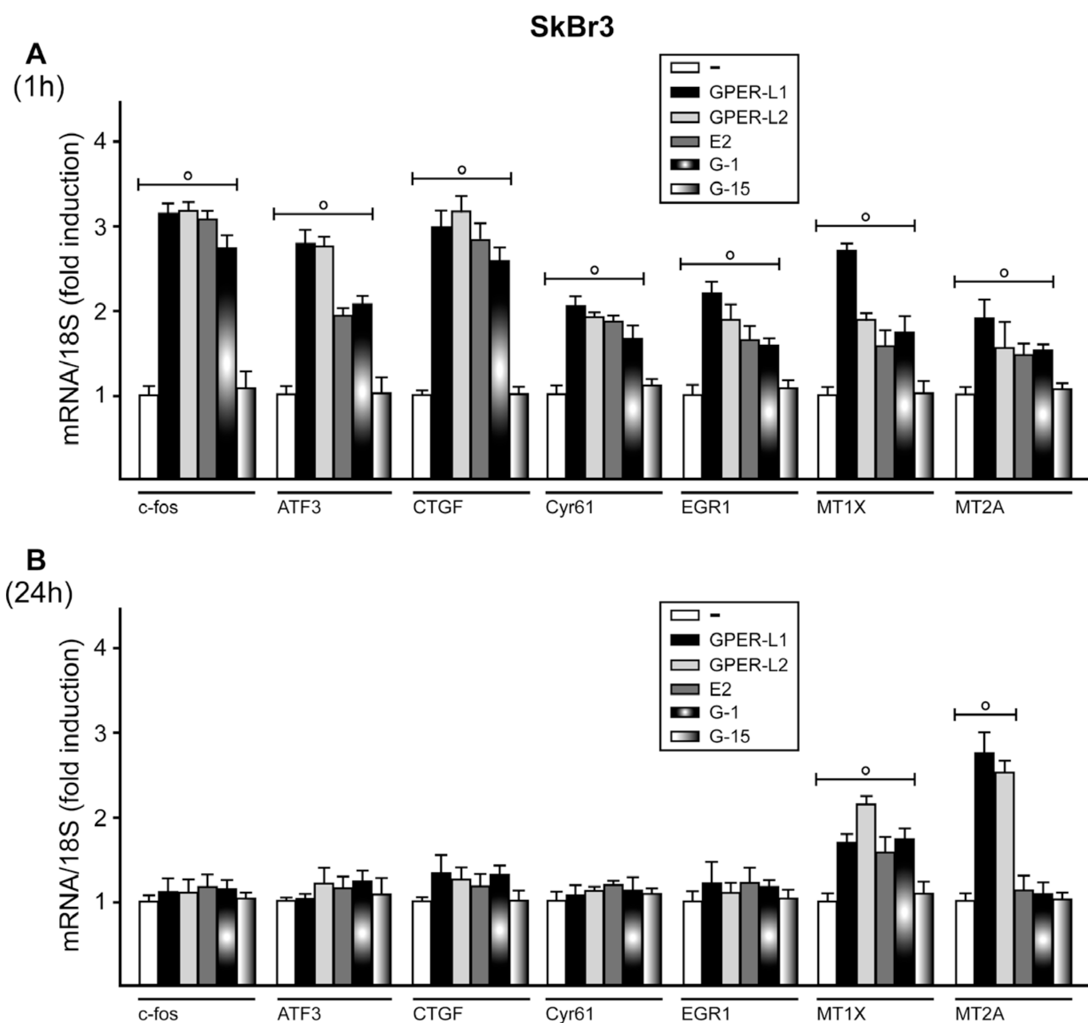

Fig. (5). mRNA expression of GPER target genes evaluated by real-time PCR. SkBr3 cells were treated with vehicle or 100 nM GPER-L1, GPER-L2, E_2, G-1 and G-15 for 1 h (**A**) and 24 h (**B**), as indicated. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. (◦) indicates $p < 0.05$ for cells receiving vehicle (–) versus treatments.

Notably, the growth stimulation induced by GPER-L1 and GPER-L2 was higher respect to that observed using OHT (Supplementary Fig. **6**), which is known to elicit proliferative effects in endometrial cancer cells. Next, the proliferative responses to 10 nM GPER-L1 and GPER-L2 were no longer evident silencing GPER expression (Fig. **7B,D**), indicating that GPER mediates the growth stimulation induced by both ligands. In order to further evaluate these data and taken into account our previous results [3, 5, 10, 40], we sought to determine whether the expression of cyclin D1 is regulated by GPER-L1 and GPER-L2 through the GPER/EGFR/ERK transduction pathway. Dose-response assays demonstrated that the up-regulation of cyclin D1 protein levels induced in SkBr3 cells by both compounds (Fig. **8A-B**) were abrogated using the EGFR inhibitor AG and the ERK inhibitor PD (Fig. **8C-D**). Moreover, the cyclin D1 protein induction by GPER-L1 and GPER-L2 was abrogated in both SkBr3 and Ishikawa cells silencing GPER expression (Fig. **8E-F**; Supplementary Fig. **7**). Cumulatively, these findings suggest that GPER-L1 and GPER-L2 as GPER ligands induce growth stimulatory effects in different cancer cell contexts through the GPER/EGFR/ERK signaling.

DISCUSSION

In the present study, we provide evidence regarding the ability of two novel compounds, named GPER-L1 and GPER-L2, to bind to and activate GPER signaling but not ER-mediated effects. By performing different functional assays, we have demonstrated that these ligands act as GPER agonists being able to induce stimulatory actions in cancer cells. In particular, GPER-L1 and GPER-L2 promoted rapid ERK phosphorylation and the up-regulation of a number of GPER target genes through the activation of the GPER/EGFR/ERK transduction pathway. Of note, both compounds stimulated proliferative effects in a GPER-dependent fashion, as ascertained in SkBr3 breast and Ishikawa endometrial cancer cells, which express GPER but are ER-negative (SkBr3) and ER-positive (Ishikawa), respectively.

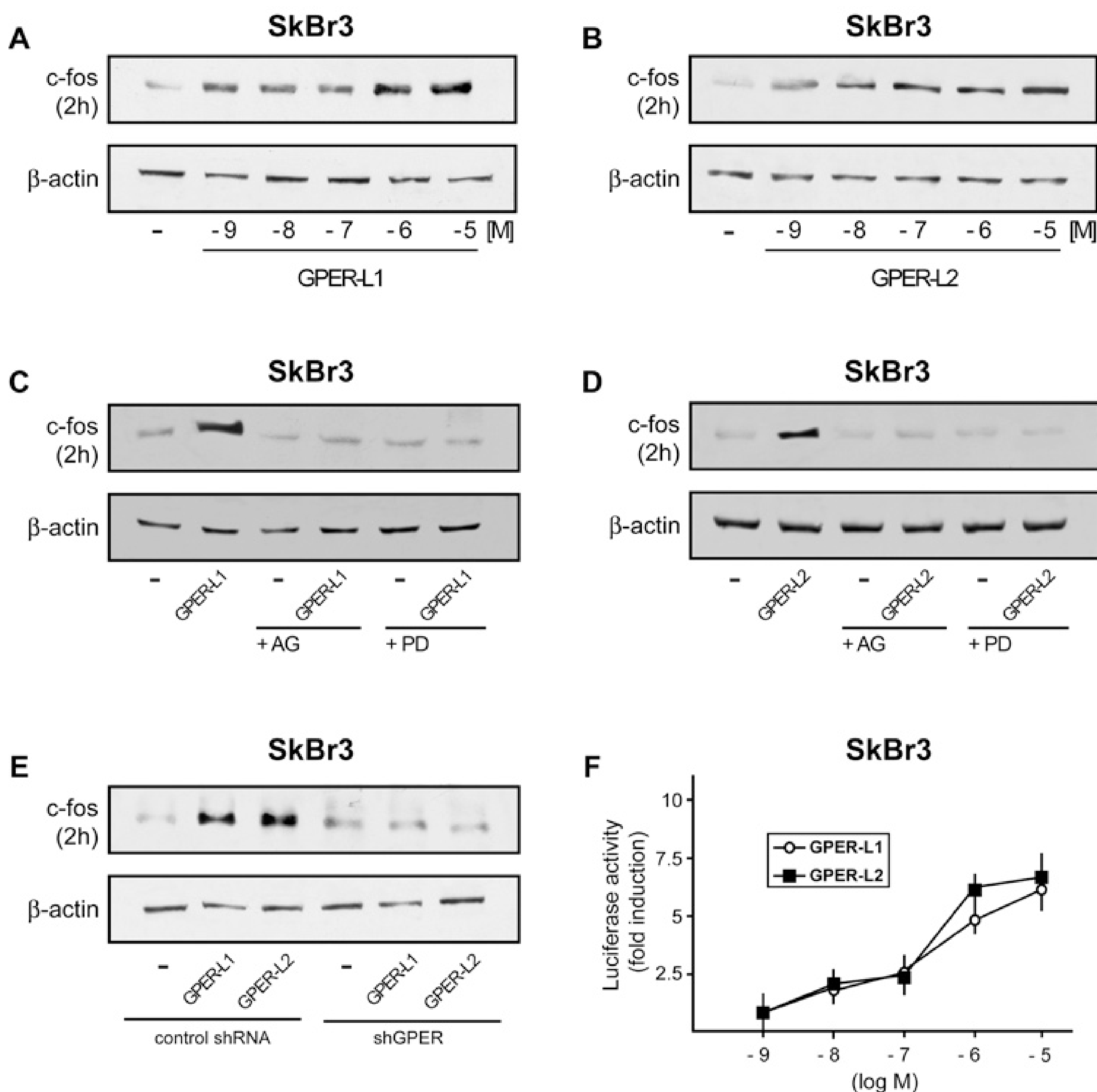

Fig. (6). GPER-L1 and GPER-L2 up-regulate c-fos protein levels in a GPER-dependent manner. (**A-B**) c-fos expression in SkBr3 cells exposed to increasing concentrations of GPER-L1 or GPER-L2 for 2 h. (**C-D**) SkBr3 cells were treated for 2 h with vehicle (–) or 100 nM GPER-L1 or GPER-L2 in combination with 10 μM AG and PD, inhibitors of EGFR and MAPK, respectively. (**E**) Immunoblot of c-fos from SkBr3 cells after silencing GPER expression. Cells were transfected with control shRNA or shGPER and treated for 2 h with vehicle (–) or 100 nM GPER-L1 or GPER-L2. β-actin serves as a loading control. Data shown are representative of three independent experiments. (**F**) GPER-L1 and GPER-L2 transactivate the activator protein-1 (AP-1)-responsive collagenase promoter fused to a luciferase reporter gene (AP-1-luc) in SkBr3 cells. Cells were transfected with AP-1-luc and treated with vehicle (–) and increasing concentrations of GPER-L1 or GPER-L2, as indicated. Renilla luciferase expression vector (pRL-TK) was used as a transfection control. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (–) were set as one fold induction on which the activity induced by treatments was calculated. Values shown are mean ± SD of three independent experiments performed in triplicate.

GPER has by now imposed oneself as an intriguing signaling molecule involved in complex pathways through which estrogens regulate diverse physiological processes. The ligand binding to GPER triggers the release of the membrane-tethered HB-EGF which binds to unoccupied EGFR, resulting in its activation [17]. GPER, *via* the EGFR transactivation, generates numerous cell signaling pathways like intracellular calcium mobilization, MAPK and PI3-K activation in a variety of cell types. In particular, GPER mediates rapid estrogen and antiestrogen-dependent signals prompting major biological responses such as gene expression and cancer cell proliferation and migration [2].

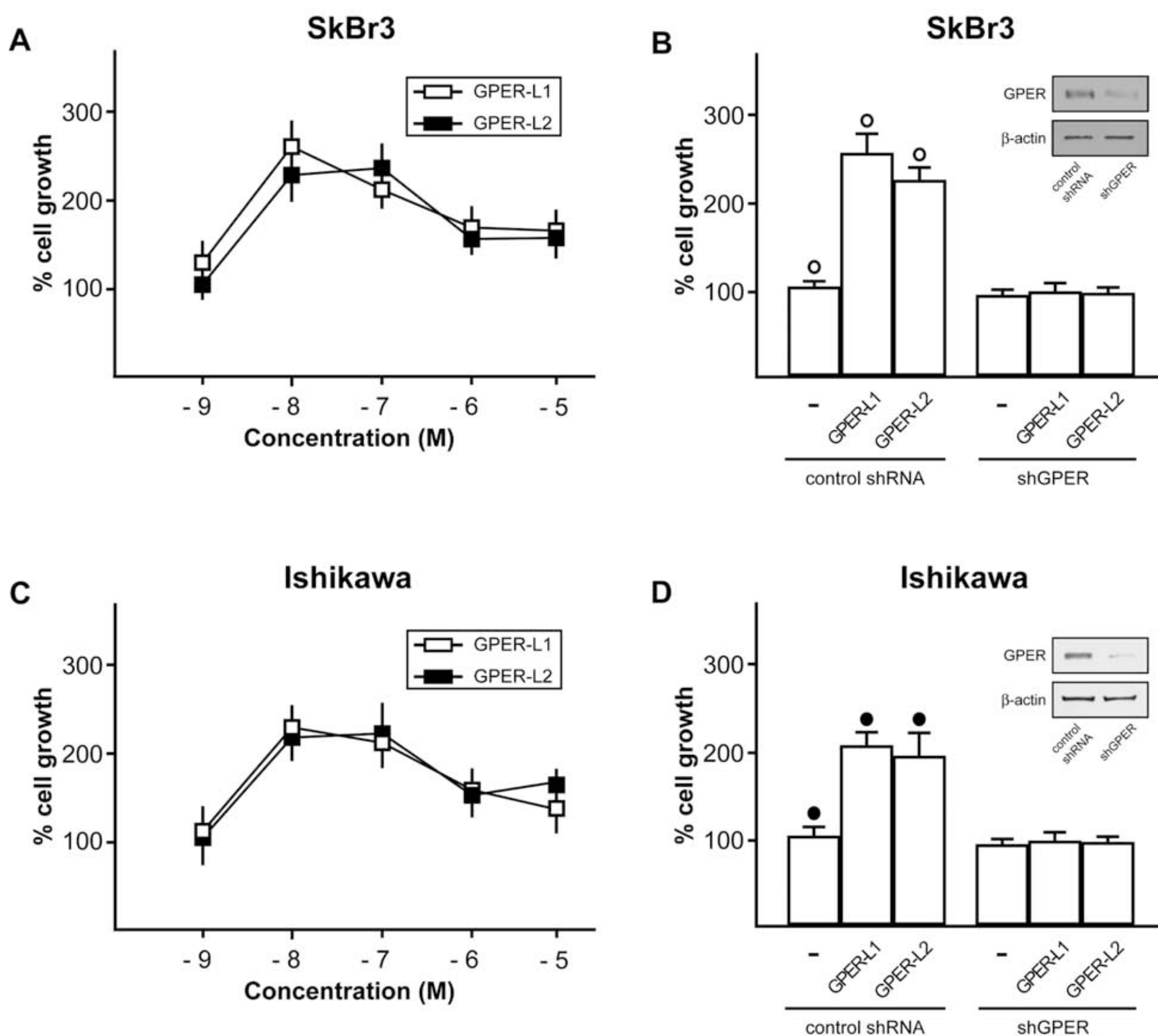

Fig. (7). GPER-L1 and GPER-L2 induce the proliferation of SkBr3 and Ishikawa cells. (**A**,**C**) Cells were treated for 5 days with increasing concentrations of GPER-L1 or GPER-L2 and counted on day 6. Cell proliferation induced by 10 nM GPER-L1 or GPER-L2 was prevented silencing GPER expression with a specific shRNA in SkBr3 (**B**) and Ishikawa (**D**) cells. Values shown are mean ± SD of three independent experiments performed in triplicate. (◦), (•) indicate p <0.05 for cells receiving vehicle (–) versus treatments. The efficacy of GPER silencing was evaluated by immunoblots as shown. β-actin serves as a loading control.

As previously reported [7], GPER is expressed in a wide number of tumors including breast, endometrial, ovarian and thyroid carcinomas. In cells derived from these types of cancer, estrogens can stimulate proliferative responses through GPER, which consequently contributes to tumor progression [2]. Further supporting the role exerted by GPER in hormone-sensitive tumors, its expression was associated with negative clinical features and poor survival rates in patients with breast, endometrial and ovarian carcinomas, suggesting that GPER may be considered a predictor of an aggressive disease [14-16].

Diverse molecules, including E_2, bind to and activate the classical estrogen receptors as well as GPER [7]. However, estriol which is an ER agonist acts as GPER antagonist, as it inhibits GPER-mediated responses like gene transcription and the proliferation of ER-negative breast cancer cells [20]. As it concerns the selective estrogen receptor modulator (SERM) tamoxifen and the pure ER antagonist ICI 182,780, both compounds as GPER ligands trigger the signaling mediated by this receptor [9-12, 17, 19]. Hence, the GPER agonism elicited by tamoxifen suggests that conventional anti-estrogenic therapies might stimulate rather than inhibit a number of tamoxifen-resistant tumors. Accordingly, the GPER/EGFR transduction pathway was involved in the development of tamoxifen resistance in breast cancer as well as in the endometrial abnormalities associated with tamoxifen treatment [41-42]. The phytoestrogen genistein and the environmental contaminant bisphenol A, which are known to mimic the biological effects of estrogen by binding to the ERs, demonstrated stimulatory activities also through the binding to GPER [9, 43]. Of note, a combination of virtual and biomolecular screening targeting GPER allowed the identification of the first selective GPER ligand named

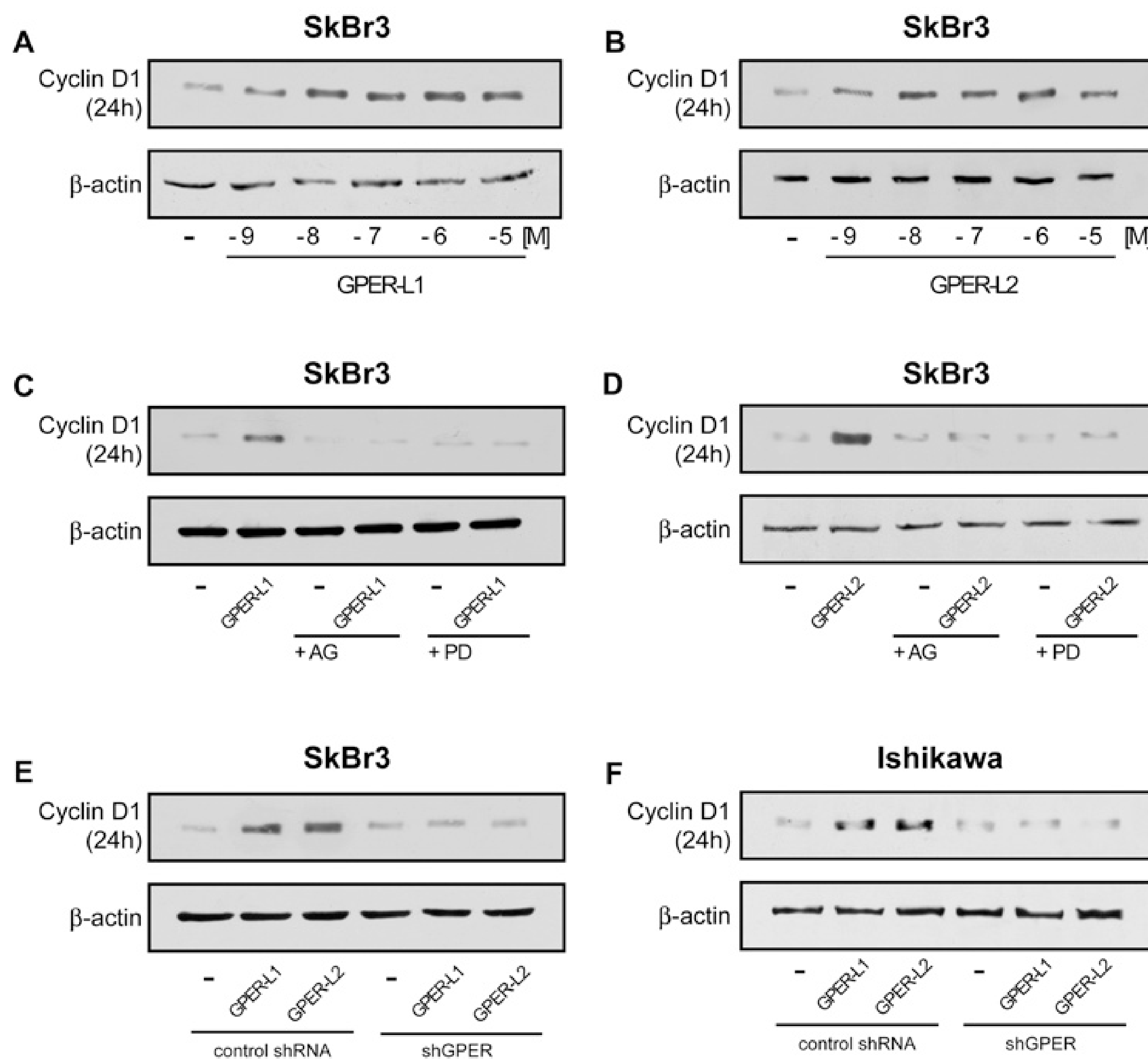

Fig. (8). GPER-L1 and GPER-L2 up-regulate cyclin D1 protein expression in a GPER-dependent manner. (**A-B**) Cyclin D1 expression in SkBr3 cells exposed to increasing concentrations of GPER-L1 or GPER-L2 for 24 h. (**C-D**) SkBr3 cells were treated for 24 h with vehicle (–) or 10 nM GPER-L1 or GPER-L2 in combination with 10 μM AG and PD, inhibitors of EGFR and MAPK, respectively. (**E-F**) Immunoblots of cyclin D1 from SkBr3 (**E**) and Ishikawa (**F**) cells after silencing GPER expression. Cells were transfected with control shRNA or shGPER and then treated for 24 h with vehicle (–) or 10 nM GPER-L1 or GPER-L2. β-actin serves as a loading control. Data shown are representative of three independent experiments.

G-1 [21]. This non-steroidal chemical has been shown to induce several GPER-dependent responses such as calcium mobilization, PI3-K and MAPK activation, gene expression changes and cell proliferation [7], thus contributing to better understand the physiological role elicited by GPER in diverse systems as well as in cancer [44]. Thereafter, it was identified G-15 which acts as a GPER antagonist although it exhibits a chemical structure close related to that of G-1 [22]. The recent development of iodo-substituted quinoline derivatives showing the ability to bind to GPER could further contribute to characterize the receptor binding properties as well as to develop new imaging applications. However, the high lipophilicity of these radioligands may limit their *in vivo* biodistribution and clearance [23]. In addition, the recent identification of MIBE which displayed the exclusive antagonistic action on both ERα and GPER in breast cancer cells, could represent a novel promising tool for a more comprehensive pharmacological approach in estrogen-dependent tumors expressing both receptors [24].

On the basis of our results, the novel GPER agonists, GPER-L1 and GPER-L2, could represent further valuable experimental tools towards a better characterization of the transcriptional response mediated by GPER. Indeed, the selective binding properties for GPER exhibited by GPER-L1 and GPER-L2 would contribute to further dissect the distinct functions mediated by the classical and novel estrogen receptors.

CONFLICT OF INTEREST

Declared none.

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ABBREVIATIONS

AP-1	=	activating protein-1
E_2	=	17β-estradiol
EGFR	=	Epidermal Growth Factor Receptor
ER	=	Estrogen Receptor
ERK	=	extracellular signal-regulated kinase
G-1	=	1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone
G-15	=	4-(6-Bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline
GPER	=	G protein-coupled estrogen receptor
MAPK	=	mitogen-activated protein kinase
PI3K	=	phophatidylinositol 3-kninase

SUPPLEMENTARY MATERIALS

Supplementary material is available on the publishers web site along with the published article.

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Bisphenol A Induces Gene Expression Changes and Proliferative Effects through GPER in Breast Cancer Cells and Cancer-Associated Fibroblasts

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Background: Bisphenol A (BPA) is the principal constituent of baby bottles, reusable water bottles, metal cans, and plastic food containers. BPA exerts estrogen-like activity by interacting with the classical estrogen receptors (ERα and ERβ) and through the G protein-coupled receptor (GPR30/GPER). In this regard, recent studies have shown that GPER was involved in the proliferative effects induced by BPA in both normal and tumor cells.

Objectives: We studied the transduction signaling pathways through which BPA influences cell proliferation and migration in human breast cancer cells and cancer-associated fibroblasts (CAFs).

Methods and results: We used as a model system SKBR3 breast cancer cells and CAFs that lack the classical ERs. Specific pharmacological inhibitors and gene-silencing procedures were used to show that BPA induces the expression of the GPER target genes c-*FOS*, *EGR-1*, and *CTGF* through the GPER/EGFR/ERK transduction pathway in SKBR3 breast cancer cells and CAFs. Moreover, we observed that GPER is required for growth effects and migration stimulated by BPA in both cell types.

Conclusions: Results indicate that GPER is involved in the biological action elicited by BPA in breast cancer cells and CAFs. Hence, GPER-mediated signaling should be included among the transduction mechanisms through which BPA may stimulate cancer progression.

Key words: bisphenol A, breast cancer cells, cancer-associated fibroblasts, GPR30/GPER, tumor microenvironment. *Environ Health Perspect* 120:1177–1182 (2012). http://dx.doi.org/10.1289/ehp.1104526 [Online 2 May 2012]

Bisphenol A (BPA), used largely in the manufacture of polycarbonate plastics, is the constituent of a wide array of consumer products, including plastic food containers, baby bottles, and the lining of metal food cans (Welshons et al. 2006). Humans are exposed to BPA mainly at the time of consumption of water and foods through the materials used for containers and packages (Vandenberg et al. 2009).

BPA is able to accelerate growth and puberty, alter the ovarian cycle in females (Mlynarcíková et al. 2005; Rasier et al. 2006), interfere with embryonic development, and to induce aneuploidy (Takai et al. 2000). Moreover, a relationship between BPA blood levels, obesity, polycystic ovary syndrome, repeated miscarriage, and endometrial hyperplasia has been found in women, suggesting that it may act as an endocrine disruptor (Welshons et al. 2006). Exposure to BPA has also been correlated with the incidence of diverse types of tumors (Ho et al. 2006; Keri et al. 2007; Maffini et al. 2006).

BPA has estrogenic activity both *in vivo* and *in vitro* and is thought to be an environmental estrogen (Welshons et al. 2006). Previous investigations (reviewed by Vandenberg et al. 2009) have demonstrated that BPA binds to and activates the estrogen receptor (ERα and ERβ), although the affinity of BPA for these receptors was approximately 10,000-fold weaker with respect to estradiol (Gould et al. 1998; Kuiper et al. 1998). In recent years, the identification of G protein-coupled receptor (GPER) as a novel estrogen receptor has suggested new possibilities by which estrogenic compounds might cause biological effects in different cell types (Albanito et al. 2007; Maggiolini et al. 2004; Prossnitz and Maggiolini 2009; Vivacqua et al. 2006a, 2006b). In this regard, we reported a characteristic signature elicited by estrogenic GPER signaling in SKBR3 breast cancer cells and we identified a network of transcription factors, such as *c-FOS*, early growth response protein 1 (*EGR-1*), and connective tissue growth factor (*CTGF*), that may be involved in important biological functions (Pandey et al. 2009).

BPA is one of several environmental estrogens that have exhibited the ability to bind to GPER (Thomas and Dong 2006) and to activate transduction pathways (Dong et al. 2011) involved in the biological responses of both normal and neoplastic cells. For example, BPA stimulated the proliferation of mouse spermatogonial cells (Sheng and Zhu 2011) and human seminoma cells (Bouskine et al. 2009) and induced chemoresistance in breast cancer cells (Lapensee et al. 2009) through activation of GPER.

The contribution of the stromal microenvironment to the development of a wide variety of tumors has been highlighted by clinical evidence and the use of mouse models (Bhowmick et al. 2004a). A growing body of data has also suggested that tumor cells actively recruit cancer-associated fibroblasts (CAFs), which remain activated and play a prominent role in cancer progression (Bhowmick et al. 2004b). In breast carcinoma approximately 80% of stromal fibroblasts may acquire the activated phenotype that promotes the proliferation of cancer cells at metastatic sites, stimulating tumor growth such as for the primary tumor (Kalluri and Zeisberg 2006).

In this study, we demonstrate that BPA exerts a stimulatory action through GPER in breast cancer cells and CAFs.

Materials and Methods

Reagents. We purchased bisphenol A (BPA), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-soquinolinesulfonamide dihydrochloride (H89), PD98059 (PD), and arsenic trioxide (As_2O_3) from Sigma-Aldrich (Milan, Italy); AG1478 (AG) from Biomol Research Laboratories (DBA, Milan, Italy), and 1-(4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[*c*]quinolin-8-yl)-ethanone (G-1) from Calbiochem (Merck KGaA, Frankfurt, Germany). As_2O_3 was dissolved in phosphate-buffered saline, and BPA and PD were dissolved in ethanol; AG1478, H89, and G-1 were solubilized in dimethyl sulfoxide (DMSO).

Cell culture. **SKBR3 cells**. SKBR3 human breast cancer cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were changed to medium without serum the day before experiments for immunoblotting and reverse-transcription polymerase chain reaction (RT-PCR).

CAFs. CAFs were extracted as previously described (Madeo and Maggiolini 2010). Briefly, breast cancer specimens were collected from primary tumors of patients who had undergone surgery. Signed informed consent

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was obtained from all the patients and from the institutional review board(s) of the Regional Hospital of Cosenza. Tissues from tumors were cut into smaller pieces (1–2 mm 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 breast fibroblasts 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 assess fibroblasts activation, we used anti-fibroblast activated protein α (FAPα) antibody (H-56; Santa Cruz Biotechnology DBA) (data not shown).

Western blotting. SKBR3 cells and CAFs were grown in 10-cm dishes, exposed to treatments or ethanol (or DMSO), which was used as the vehicle, and then lysed as described previously (Pandey et al. 2009). Protein concentrations were determined using Bradford reagent (Sigma-Aldrich) according to the manufacturer's recommendations. Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy). Membranes were probed overnight at 4°C with antibodies against c-Fos (H-125), β-actin (C-2), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2; E-4), Egr-1 (588), CTGF (L-20), ERK2 (C-14), ERα (F-10), or GPR30 (N-15), all from Santa Cruz Biotechnology, DBA (Milan, Italy), or ERβ from Serotec (Space Import Export, Milan, Italy). Results of densitometric analyses of Western blots, obtained using ImageJ software (Abramoff et al. 2004), are presented as optical density (OD; expressed in arbitrary units) relative to the control (ERK2 or β-actin).

Plasmids and luciferase assays. The *Ctgf* luciferase reporter plasmid p(-1999/+36)-luc, which is based on the backbone of vector pGL3-basic (Promega), was a gift from B. Chaqour (Department of Anatomy and Cell Biology, State University of New York Downstate Medical Center, Brooklyn, NY, USA). The luciferase reporter plasmid for *c-FOS* encoding a –2.2-kb 5′ upstream fragment of human *c-FOS* was a gift from K. Nose (Department of Microbiology, Showa University School of Pharmaceutical Sciences, Hatanodai, Shinagawa-ku, Tokyo, Japan). The *EGR-1* luciferase reporter plasmid pEgr-1A, which contains the –600 to +12 5′-flanking sequence from the human *EGR-1* gene was a gift from S. Safe (Department of Veterinary Physiology and Pharmacology, Texas A&M University, Houston, TX, USA). For the luciferase assays, cells were transferred into 24-well plates containing 500 µL of regular growth medium per well the day before transfection. On the day of transfection, SKBR3 cell medium was replaced with RPMI without phenol red and serum, and transfection was performed using Fugene6 Reagent (Roche Molecular Biochemicals, Milan, Italy) and a mixture containing 0.5 µg of each reporter plasmid. Renilla luciferase (pRL-CMV; 1 ng) was used as a transfection control. After 5–6 hr, ligand was added and cells were incubated for 16–18 hr. We measured luciferase activity using the Dual Luciferase Kit (Promega, Milan, Italy) according to the manufacturer's recommendations. Firefly luciferase values generated by the reporter plasmid were normalized to Renilla luciferase values. Normalized values obtained from cells treated with ethanol vehicle were set as 1-fold induction, and the activity induced by treatments was calculated based on this value.

RT-PCR and real-time PCR. Total RNA was extracted 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 using SYBR Green as the detection method and the Step One sequence detection system (Applied Biosystems Inc., Milan, Italy).

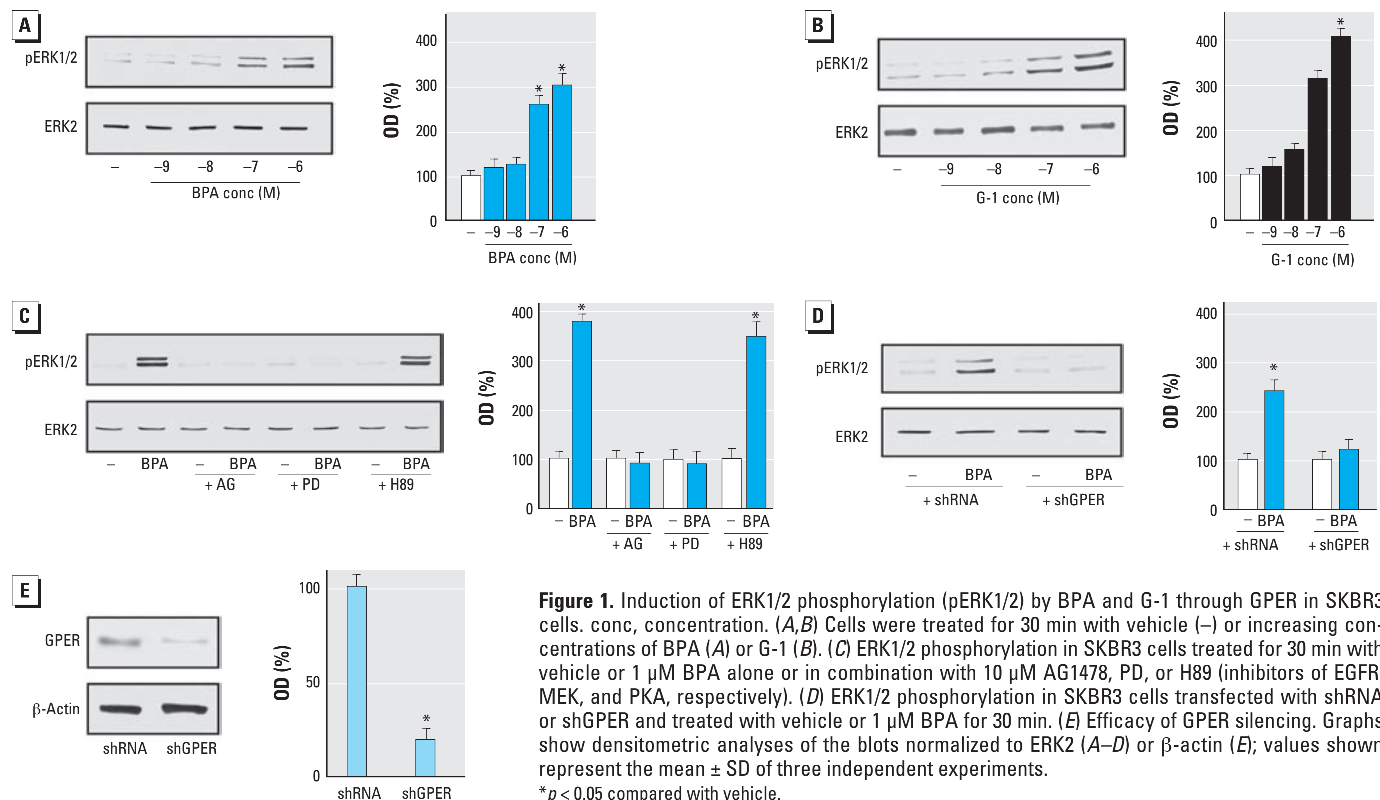

Figure 1. Induction of ERK1/2 phosphorylation (pERK1/2) by BPA and G-1 through GPER in SKBR3 cells. conc, concentration. (*A*,*B*) Cells were treated for 30 min with vehicle (–) or increasing concentrations of BPA (*A*) or G-1 (*B*). (*C*) ERK1/2 phosphorylation in SKBR3 cells treated for 30 min with vehicle or 1 µM BPA alone or in combination with 10 µM AG1478, PD, or H89 (inhibitors of EGFR, MEK, and PKA, respectively). (*D*) ERK1/2 phosphorylation in SKBR3 cells transfected with shRNA or shGPER and treated with vehicle or 1 µM BPA for 30 min. (*E*) Efficacy of GPER silencing. Graphs show densitometric analyses of the blots normalized to ERK2 (*A*–*D*) or β-actin (*E*); values shown represent the mean ± SD of three independent experiments.
*$p < 0.05$ compared with vehicle.

Gene-specific primers were designed using Primer Express software (version 2.0; Applied Biosystems Inc.). Assays were performed in triplicate. We used mean values to calculate expression levels by the relative standard curve method. For the sequences of primer used, see Supplemental Material, Table S1(http://dx.doi.org/10.1289/ehp.1104526).

Gene silencing experiments. Cells were plated onto 10-cm dishes, maintained in serum-free medium for 24 hr, and then transfected for an additional 24 hr before treatments using Fugene6. The short hairpin (sh) RNA constructs to knock down the expression of *GPER* and *CTGF* and the unrelated shRNA control construct have been described previously (Pandey et al. 2009).

Wound-healing assay. CAFs were seeded into 12-well plates in regular growth medium. When at 70% to 80% confluency, the cells were transfected with shGPER using Fugene6 reagent for 24 hr. Transfected cells were washed once, medium was replaced with 2.5% charcoal-stripped FBS, and cells were treated. We then used a p200 pipette tip to scratch the cell monolayer. In experiments performed using conditioned medium, CAFs were plated into 12-well plates and transfected with 500 ng shRNA control plasmid or shGPER or shCTGF plasmids using Fugene6, as recommended by the manufacturer. After 24 hr, CAFs were treated with 1 µM BPA, and the conditioned medium was collected and filtered through a sterile nonpyrogenic 0.2 µm filter. The conditioned medium obtained was added to subconfluent SKBR3 cells, and a series of scratches were made using a p200 pipette tip. We evaluated cell migration in three independent experiments after 48 hr of treatment; data are expressed as a percentage of cells in the wound area upon treatment compared with cells receiving vehicle.

Proliferation assay. SKBR3 cells and CAFs were seeded in 24-well plates in regular growth medium. After cells attached, they were washed, incubated in medium containing 2.5% charcoal-stripped FBS, and transfected with 500 ng shGPER or control shRNA plasmids using Fugene6 reagent. After 24 hr, cells were treated with 1 µM BPA, and the transfection and treatment were renewed every 2 days. We counted the cells using the COUNTESS automated cell counter (Invitrogen) following the manufacturer's recommendations.

Statistical analysis. For statistical analysis, we used analysis of variance followed by Newman-Keuls testing to determine differences in means. p-Values < 0.05 are considered statistically significant.

Results

BPA induces ERK1/2 activation through GPER. Using SKBR3 cells and CAFs, which both express GPER and lack ERs [see Supplemental Material, Figure S1 (http://dx.doi.org/10.1289/ehp.1104526)], we evaluated ERK1/2 activation by increasing concentrations of BPA and the GPER ligand G-1, as GPER activation leads to ERK1/2 phosphorylation (Dong et al. 2011; Maggiolini and Picard 2010). BPA and G-1 induced ERK1/2 phosphorylation in both cell types in a dose-dependent manner (Figures 1A,B and 2A,B). When the epidermal growth factor receptor (EGFR) inhibitor AG1478 or the mitogen-activated protein kinase kinase MEK inhibitor PD was added, ERK1/2 activation was not evident, but it was present when the protein kinase A (PKA) inhibitor H89 was added (Figure 1C). Interestingly, ERK1/2 phosphorylation by BPA was abolished by silencing GPER expression (Figures 1D, 2C), suggesting that GPER is required for ERK1/2 activation after exposure to BPA. We ascertained the efficacy of GPER silencing using immunoblots in SKBR3 cells and CAFs as shown in Figures 1E and 2D, respectively. Moreover, to demonstrate the specificity of BPA action, we used the environmental contaminant arsenic (Nordstrom 2002), which elicits the ability of breast cancer cells to activate ERK1/2 (Ye et al. 2005). We observed that ERK1/2 phosphorylation induced by 10 µM As_2O_3 was still present in SKBR3 cells transfected with shGPER (data not shown).

BPA stimulates the expression of GPER target genes. GPER-mediated signaling regulates the transcription of diverse target genes (Pandey et al. 2009). In the present study, BPA transactivated the promoter sequence of *c-FOS*, *EGR-1*, and *CTGF* (Figure 3A), and accordingly stimulated mRNA expression of these genes (Figures 3B, 4A). In accordance with these findings, BPA induced the protein levels of c-FOS, EGR-1, and CTGF (Figure 3C). As observed with ERK1/2 activation, the EGFR inhibitor AG1478 and the ERK inhibitor PD, but not the PKA inhibitor H89, repressed the up-regulation of these proteins by BPA (Figure 3C). Notably, the c-FOS, EGR-1, and CTGF protein increases after exposure to BPA were abrogated by silencing GPER in both SKBR3 cells and CAFs (Figures 3D, 4B). The efficacy of GPER silencing was ascertained by immunoblotting experiments in SKBR3 cells

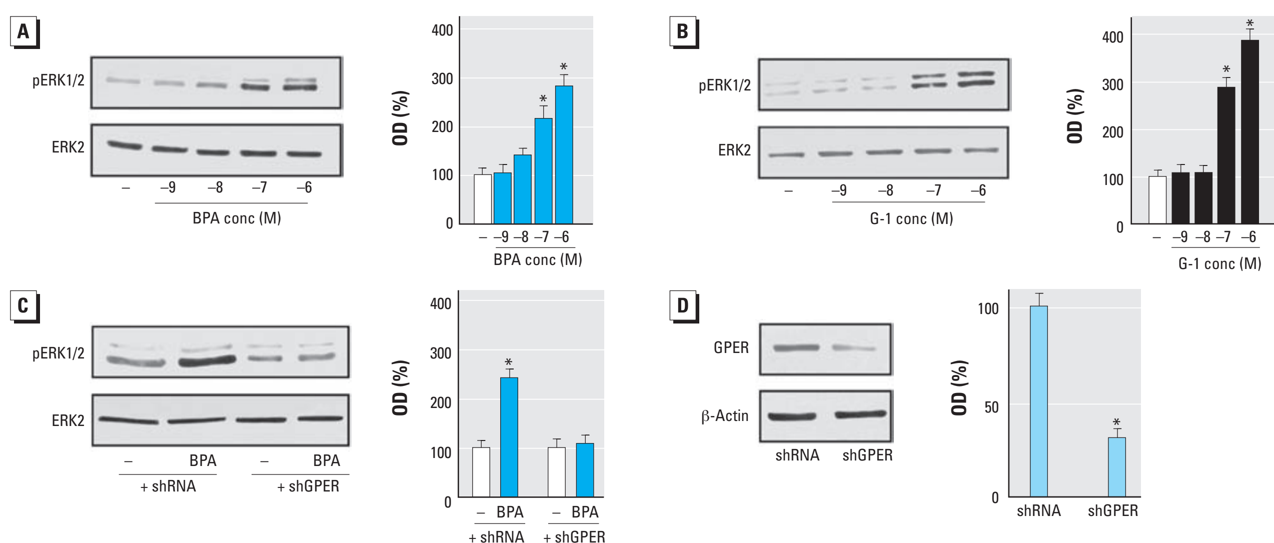

Figure 2. Induction of ERK1/2 phosphorylation (pERK1/2) by BPA and G-1 through GPER in CAFs. conc, concentration. (*A*,*B*) CAFs were treated for 30 min with vehicle (–) or increasing concentrations of BPA (*A*) or G-1 (*B*). (*C*) ERK1/2 phosphorylation in CAFs transfected with shRNA or shGPER and treated with vehicle or 1 µM BPA for 30 min. (*D*) Efficacy of GPER silencing in CAFs. Graphs show densitometric analyses of the blots normalized to ERK2 (*A*–*C*) or β-actin (*D*); values shown represent the mean ± SD of three independent experiments.
*p < 0.05 compared with vehicle.

and CAFs as shown in Figures 3E and 4C, respectively. Taken together, these results demonstrate that BPA regulates the expression of *c-FOS*, *EGR-1*, and *CTGF* through the GPER/EGFR/ERK transduction pathway.

BPA induces cell proliferation and migration through GPER. The aforementioned results were recapitulated in the complex physiologic responses such as cell proliferation and migration. The proliferative effects observed in both SKBR3 cells and CAFs after 5-day treatment with BPA were cancelled when GPER expression was silenced by shGPER (Figure 5A,B). Moreover, in wound-healing assays in CAFs, migration induced by BPA was abolished by knocking down GPER expression (Figure 5C). To evaluate whether the treatment of CAFs with BPA could induce the migration of tumor cells through secreted factor(s), we performed wound-healing assays in SKBR3 cells cultured with conditioned medium from CAFs. Interestingly, the migration of SKBR3 cells was not evident after silencing GPER or CTGF expression in CAFs (Figure 5D). Overall, these findings demonstrate that BPA induces stimulatory effects as a GPER agonist in both ER-negative SKBR3 breast cancer cells and CAFs.

Discussion

There has been increased interest in understanding the molecular mechanisms involved in the endocrine-disrupting effects of BPA (Vandenberg et al. 2009). In this regard, fetal and perinatal exposures to BPA in rodents have been shown to affect the brain, mammary gland, and reproductive tract, as well as to stimulate the development of hormone-dependent tumors (Durando et al. 2007; Munoz-de-Toro et al. 2005). Moreover, the estrogenic actions of BPA, including increased uterine wet weight, luminal epithelial height, and increased expression of the estrogen-inducible protein lactoferrin, were reported in prepubescent CD-1 mice (Markey et al. 2001). Analogously, BPA induced the proliferation of uterine and vaginal epithelial cells in ovariectomized rats (Steinmetz et al. 1998). In regard to the mechanisms by which BPA can exert estrogen-like effects, it has been reported that BPA's two benzene rings and two (4,4′)-OH substituents fitting in the ER binding pocket allow the binding to and activation of both ERα and ERβ, which in turn mediate the transcriptional responses to BPA (Gould et al. 1998; Kuiper et al 1998; Vivacqua et al 2003). In addition, rapid nongenomic effects involving diverse transduction pathways were observed upon exposure to BPA in pancreatic islet, endothelial, and hypophysial cells and in

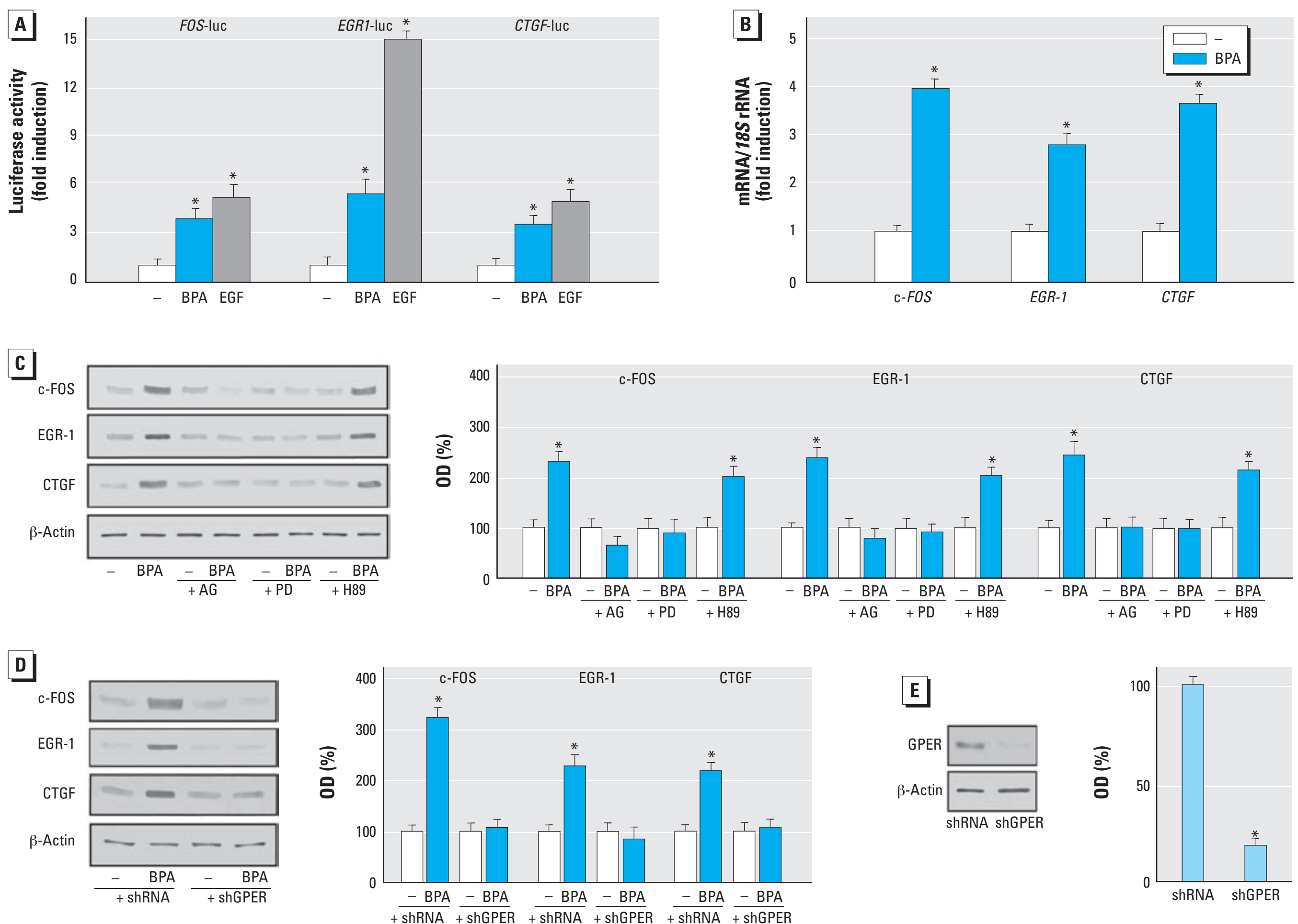

Figure 3. Expression of GPER target genes (*c-FOS*, *EGR-1*, and *CTGF*) in SKBR3 cells in response to BPA treatment. (*A*) Evaluation of *c-FOS*, *EGR-1*, and *CTGF* luciferase reporter genes in transfected SKBR3 cells treated with vehicle (–), 1 μM BPA, or EGF (50 ng/mL; positive control). Luciferase activity was normalized to the internal transfection control; values are presented as fold change (mean ± SD) of vehicle control and represent three independent experiments, each performed in triplicate. (*B*) Evaluation of *c-FOS*, *EGR-1*, and *CTGF* mRNA expression by real-time PCR in cells treated with 1 μM BPA for 4 hr. Gene expression was normalized to *18S* expression, and values are presented as fold change (mean ± SD) of vehicle control. (*C*) Immunoblots showing c-FOS, EGR-1, and CTGF protein expression in SKBR3 cells treated with vehicle or 1 μM BPA alone or in combination with 10 μM AG1478, PD, or H89 (inhibitors of EGFR, MEK, and PKA respectively). (*D*) Protein levels of c-FOS, EGR-1, and CTGF in SKBR3 cells transfected with shRNA or shGPER and treated with vehicle or 1 μM BPA for 6 hr. (*E*) Efficacy of GPER silencing. Graphs show densitometric analyses of the blots normalized to β-actin; values represent the mean ± SD of three independent experiments.
*$p < 0.05$ compared with vehicle.

breast cancer cells (Alonso-Magdalena et al. 2005; Noguchi et al. 2002; Watson et al. 2007). In this context, the novel estrogen receptor GPER was recently shown to mediate the BPA-dependent rapid activation of intracellular signaling (Dong et al. 2011) and the proliferation of both human seminoma cells (Bouskine et al. 2009) and mouse spermatogonial cells (Sheng and Zhu 2011).

To investigate the potential of GPER to mediate estrogenic signals such as those elicited by BPA, we used SKBR3 breast cancer cells and CAFs, both of which express GPER and lack ERs. Interestingly, we found that in both cell types BPA triggers rapid ERK activation through the GPER/EGFR transduction pathway and induces the expression of genes that characterize estrogenic GPER-mediated signaling (Pandey et al. 2009). In addition, we determined that BPA stimulates the proliferation and migration of SKBR3 cells and CAFs through GPER. Of note, conditioned medium from BPA-treated CAFs induced the migration of SKBR3 cells, suggesting that BPA may also promote a functional crosstalk between cancer cells and CAFs. These data regarding CAFs are particularly intriguing given that these cells actively contribute to cancer growth and progression even at metastatic sites (Bhowmick and Moses 2005).

The present findings are relevant to the results obtained in a previous study (Albanito et al. 2008) in which we found that atrazine, another environmental contaminant, triggered estrogen-like activity through the GPER/EGFR/ERK transduction pathway in hormone-sensitive ovarian cancer cells. Moreover, in that study (Albanito et al. 2008) we observed that atrazine induced functional crosstalk between GPER and ERα in accordance with the results of Sheng and Zhu (2011) who demonstrated a similar interaction in mouse spermatogonial cells after exposure to BPA. Overall, these findings, together with results of the present study, contribute to a better understanding on the multifaceted mechanisms by which environmental estrogens may act as endocrine stimulators in hormone-dependent malignancies.

BPA is consistently detected in almost all individuals in developed nations (Welshons et al. 2006), suggesting that humans are exposed to BPA continuously. In addition, the rapid metabolic clearance of BPA and its detectable levels in human blood and urine suggest that the intake of BPA may be higher than indicated by diverse studies and that long-term daily intake may lead to its bioaccumulation. In this regard, previous studies (Vandenberg et al. 2009) have estimated

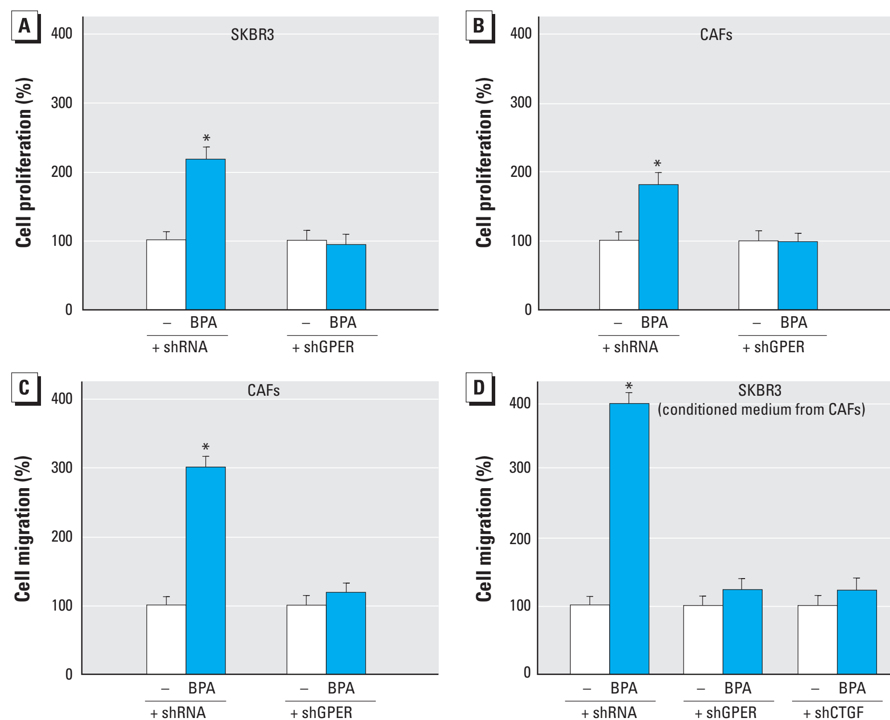

Figure 5. Induction of proliferation and migration in SKBR3 cells and CAFs. (*A*,*B*) Proliferation in SKBR3 cells (*A*) and CAFs (*B*) treated with vehicle (–) or 1 µM BPA for 5 days after silencing GPER expression. (*C*) Migration in CAFs treated with vehicle or 1 µM BPA for 48 hr after silencing GPER expression. (*D*) Migration in SKBR3 cells cultured in conditioned medium from CAFs with silenced expression of *GPER* and *CTGF*. Values shown represent the mean ± SD percent of vehicle control of three independent experiments, each performed in triplicate.
*$p < 0.05$ compared with vehicle.

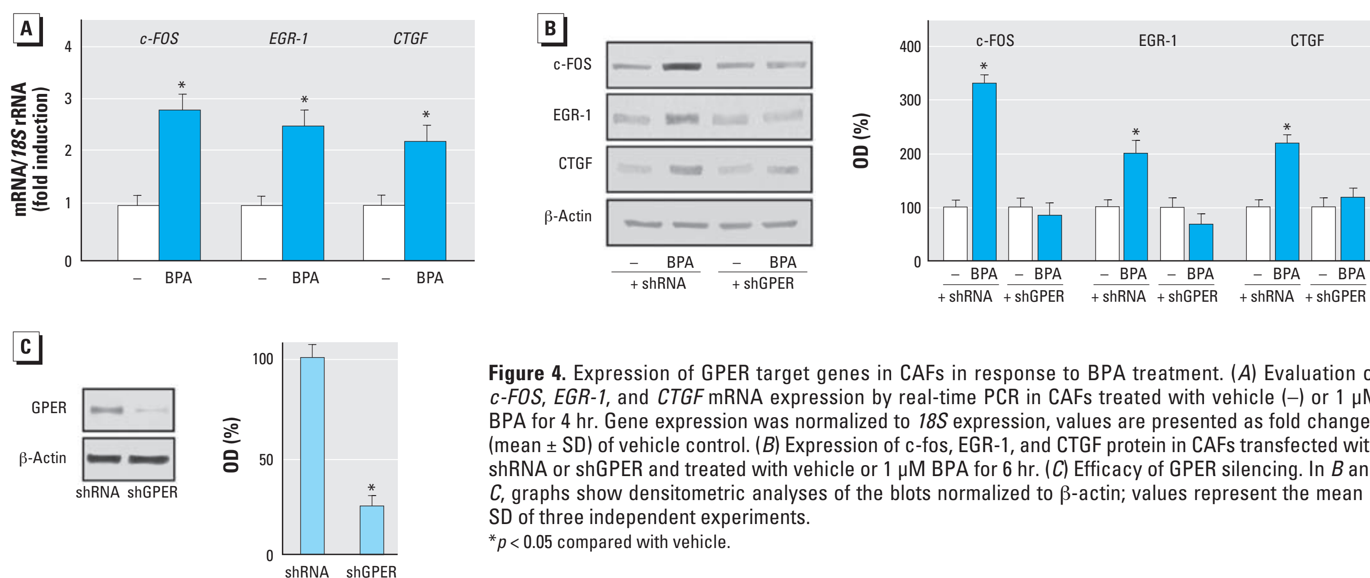

Figure 4. Expression of GPER target genes in CAFs in response to BPA treatment. (*A*) Evaluation of *c-FOS*, *EGR-1*, and *CTGF* mRNA expression by real-time PCR in CAFs treated with vehicle (–) or 1 µM BPA for 4 hr. Gene expression was normalized to *18S* expression, values are presented as fold changes (mean ± SD) of vehicle control. (*B*) Expression of c-fos, EGR-1, and CTGF protein in CAFs transfected with shRNA or shGPER and treated with vehicle or 1 µM BPA for 6 hr. (*C*) Efficacy of GPER silencing. In *B* and *C*, graphs show densitometric analyses of the blots normalized to β-actin; values represent the mean ± SD of three independent experiments.
*$p < 0.05$ compared with vehicle.

that human exposure ranges from < 1 µg/kg/day to almost 5 µg/kg/day (0.325 mg/day/adult). However, pharmacokinetic modeling data have shown that oral intakes up to 100 mg/day/adult would be required to explain the reported human circulating levels (Vandenberg et al. 2009). Hence, future studies should include mathematical models of potential exposures, particularly because many sources of BPA exposure have been identified (Vandenberg et al. 2009). These observations suggest that the BPA concentration used in the present study is achievable in humans. In the present study, we found that BPA is able to trigger GPER-mediated signaling in breast cancer cells and CAFs, which contributes to tumor progression. Thus, GPER may a potential mediator of the estrogen-like activity of BPA, as well as a further biological target in estrogen-sensitive tumors.

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ORIGINAL ARTICLE

Insulin-like growth factor-I regulates GPER expression and function in cancer cells

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Functional cross talk between insulin-like growth factor-I (IGF-I) system and estrogen signaling has been largely reported, although the underlying molecular mechanisms remain to be fully elucidated. As GPR30/GPER mediates rapid cell responses to estrogens, we evaluated the potential of IGF-I to regulate GPER expression and function in estrogen receptor (ER)α-positive breast (MCF-7) and endometrial (Ishikawa) cancer cells. We found that IGF-I transactivates the GPER promoter sequence and upregulates GPER mRNA and protein levels in both cells types. Similar data were found, at least in part, in carcinoma-associated fibroblasts. The upregulation of GPER expression by IGF-I involved the IGF-IR/PKCδ/ERK/c-fos/AP1 transduction pathway and required ERα, as ascertained by specific pharmacological inhibitors and gene-silencing. In both MCF-7 and Ishikawa cancer cells, the IGF-I-dependent cell migration required GPER and its main target gene *CTGF*, whereas the IGF-I-induced proliferation required both GPER and cyclin D1. Our data demonstrate that the IGF-I system regulates GPER expression and function, triggering the activation of a signaling network that leads to the migration and proliferation of cancer cells.

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Keywords: cancer cells; ERα; GPER; IGF-I; signal transduction

INTRODUCTION

Estrogens regulate the growth and differentiation of many normal and neoplastic tissues including breast, endometrial and ovarian tumors.[1,2] Although the biological responses to estrogens are mainly mediated by estrogen receptors (ER)α and ERβ,[3,4] the G protein-coupled receptor GPR30/GPER has been recently shown to also mediate estrogen signaling in a variety of normal and cancer cell types.[5-8] In particular, GPER has been involved in rapid events induced by estrogens, including the transactivation of epidermal growth factor receptor, the activation of the mitogen-activated protein kinase and phosphoinositide3-kinase (PI3K) transduction pathways, the stimulation of adenylylcyclase and the mobilization of intracellular calcium (Ca^{2+}).[7,9-11] Accumulating evidence has also indicated that GPER signaling triggers a typical gene expression profile, as evidenced by diverse reports including microarray analysis.[11-13] As far as the transcriptional regulation of GPER expression is concerned, our previous studies have demonstrated that EGFR ligands are able to transactivate the promoter of *GPER* and upregulate its mRNA and protein levels in cancer cells.[14,15] Of note, the upregulation of GPER was associated with higher risk of developing metastatic disease in patients with breast cancer[16] and lower survival rates in endometrial cancer patients.[17]

Insulin-like growth factor-I (IGF-I) belongs to a group of cellular and secreted factors (IGFs) that exert important roles in multiple biological systems.[18-21] The IGF-I action is mediated by IGF-IR, a transmembrane heterotetramer, whose cytoplasmic tyrosine kinase domain is linked to diverse transduction cascades.[22,23] Moreover, IGF-I is known to signal through AP-1 in breast cancer cells[24] and to stimulate the expression of genes involved in cell cycle progression, such as *cyclin D1*, by cross talking with estrogen-dependent pathways.[25]

The interaction between IGF-I and ERα in regulating breast development and carcinogenesis has been well established.[26,27] For instance, several studies have demonstrated that IGF-I induces the activation of ERα in a ligand-independent manner through the involvement of the mitogen-activated protein kinase signaling.[26,28] Recently, increasing evidences have also extended our knowledge regarding the functional cross talk between growth factors transduction pathways and GPCR-activated signaling.[29] In this regard, we have previously shown that the EGFR system is involved in the GPER expression and function.[14,15] In addition, a strict dependence of the IGF-I signaling on GPCRs was reported in many physiological functions[30] as well as in the development of diverse malignancies.[31]

In the present study, we evaluated the ability of IGF-I to regulate GPER expression and function in ER-positive breast (MCF-7) and endometrial (Ishikawa) cancer cells. Our results demonstrate that the upregulation of GPER by IGF-I is mediated by the IGF-IR/PKCδ/ERK/c-fos/AP1 transduction pathway and involves ERα. As GPER was required for the IGF-I-induced cell migration and proliferation, our findings contribute to better understand the functional cross talk between these two important factors toward innovative therapeutic intervention in estrogen-sensitive tumors.

RESULTS

IGF-I induces GPER expression

In order to provide novel insights into the cross talk between IGF-I system and estrogen signaling, we sought to evaluate the ability of IGF-I to regulate GPER expression in breast MCF-7 and endometrial Ishikawa cancer cells. Interestingly, we found that IGF-I upregulates the mRNA and protein levels of GPER in both cell types (Figures 1a – d). Next, we determined that the GPER protein induction is abrogated in the presence of the IGF-IR inhibitor AG1024 (AG), the MEK inhibitor PD98059 (PD), the PKC inhibitor GF109203X (GF) and the PKCδ inhibitor Rottlerin (Rot), but it still

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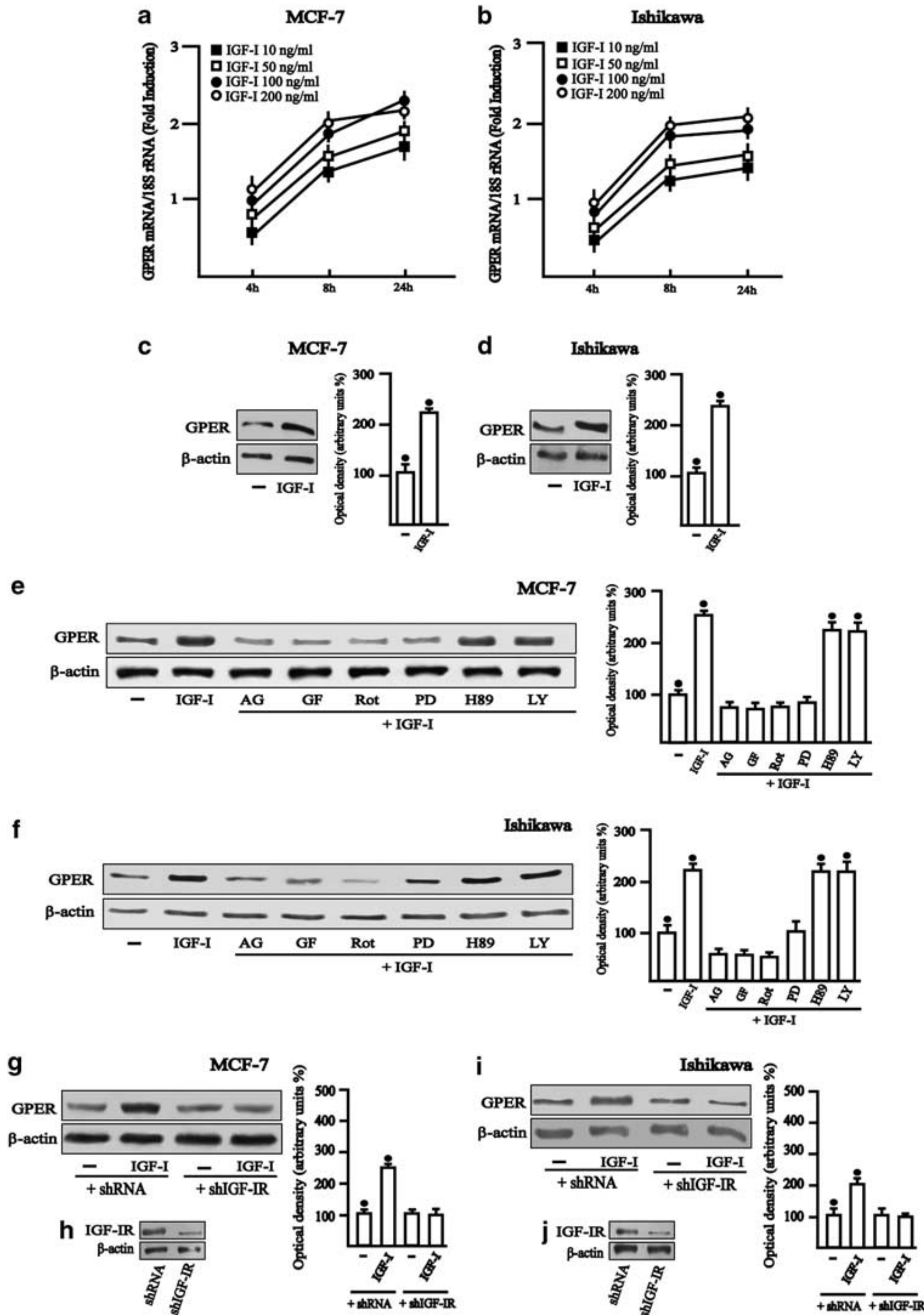

Figure 1. IGF-I upregulates GPER expression in MCF-7 and Ishikawa cells. (**a**, **b**) IGF-I induces GPER mRNA expression, as evaluated by real-time PCR. The mRNA expression of *GPER* was normalized to 18S expression. Results are shown as fold changes of mRNA expression upon treatment compared to cells treated with vehicle (−). (**c**, **d**) GPER protein levels were evaluated by immunoblotting in cells treated for 24 h with 100 ng/ml IGF-I. (**e**, **f**) GPER protein expression was evaluated by immunoblotting in cells treated for 24 h with vehicle (−) or 100 ng/ml IGF-I alone and in combination with 10 μM IGF-IR inhibitor tyrphostin AG1024 (AG), 10 μM PKC inhibitor GF109203X (GF), 10 μM PKCδ inhibitor Rottlerin (Rot), 10 μM MEK inhibitor PD98059 (PD), 10 μM PKA inhibitor H89, 10 μM PI3K inhibitor LY294,002 (LY), as indicated. (**g**–**j**) The upregulation of GPER protein levels by 100 ng/ml IGF-I was abrogated in the presence of shIGF-IR. Side panels show densitometric analysis of the blots normalized to β-actin. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. ○, ●, ■ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments.

persists using the PKA and phosphoinositide3-kinase inhibitors, H89 and LY294,002 (LY), respectively (Figures 1e–f). Corroborating the aforementioned findings, the upregulation of GPER was also prevented by silencing IGF-IR expression (Figures 1g–j). In immunofluorescence studies performed in MCF-7 cells, IGF-I further confirmed the ability to upregulate GPER expression (Figure 2), an effect that was no longer observed transfecting cells with shGPER (Figure 2). According to these observations, IGF-I

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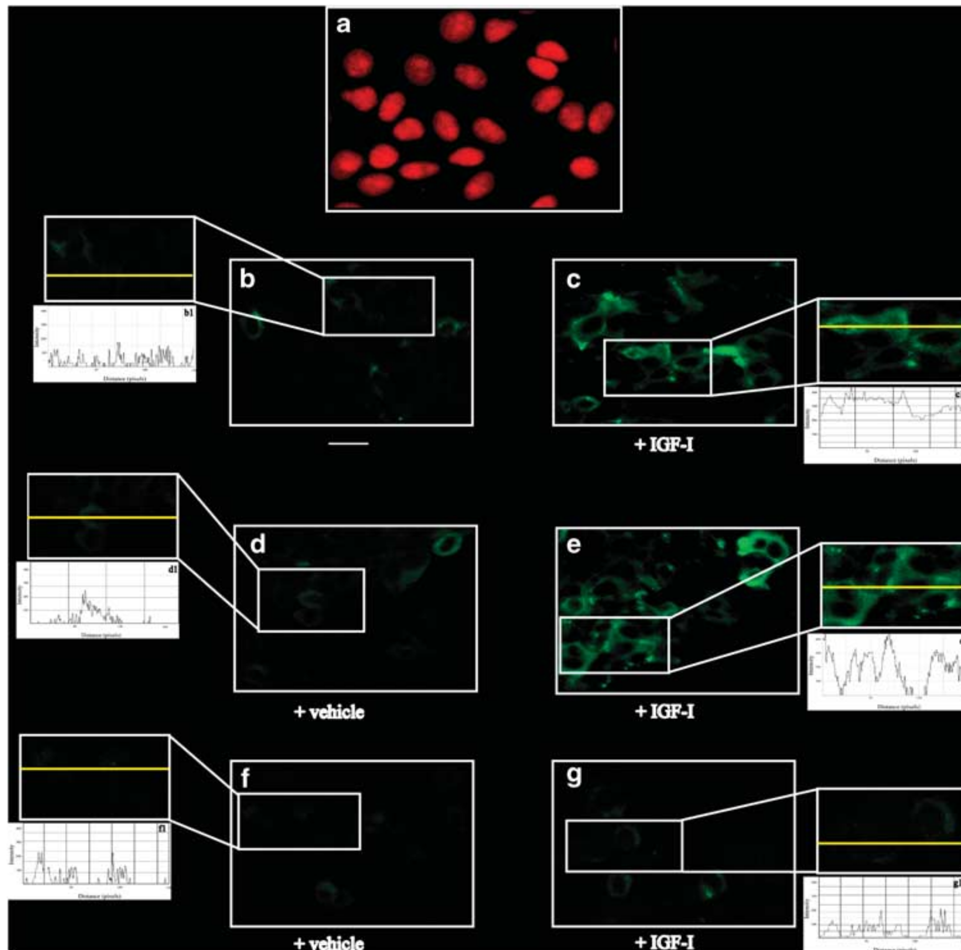

Figure 2. Representative fluorescence images of GPER immunolabelling. MCF-7 cells were fixed, permeabilized and stained with anti-GPER antibody. (**a**) Nuclei (red) were stained by propidium iodide. (**b**, **c**) Cells were treated for 24 h with vehicle (−) or 100 ng/ml IGF-I (as indicated), GPER accumulation was evidenced by the green signal. MCF-7 cells were transfected with a control shRNA (**d**, **e**) or with a shGPER (**f**, **g**) and treated as described above, then stained with GPER antibody. For descriptive purposes, Figures 1b–g, show the plot profiles obtained at the level of the yellow line of the corresponding inset using the program WCIF Image J for Windows. Note the higher values indicating zones of intense labeling. Each experiment shown is representative of 10 random fields. Data are representative of three independent experiments.

transactivated the *GPER* promoter construct transiently transfected in MCF-7 cells (Supplementary Figure 1).

Molecular mechanisms involved in the upregulation of GPER by IGF-I

As the inhibitors of PKC and MEK transduction pathways prevented the upregulation of GPER induced by IGF-I, we then evaluated the activation of PKC and ERK in MCF-7 cells. IGF-I promoted rapid PKCδ phosphorylation, which was no longer evident in the presence of the PKC inhibitors GF and Rot, while it was still present using the MEK inhibitor PD (Figure 3a). Moreover, IGF-I also induced a rapid phosphorylation of ERK1/2, which was abolished by PD as well as by the PKC inhibitors GF and Rot (Figure 3b). Taken together, these data suggest that ERK1/2 activation is downstream PKCδ in MCF-7 cells treated with IGF-I.

It has been previously shown that the activation of the ERK transduction pathway leads to a rapid upregulation of c-fos,[11,13,32,33] which has a relevant role in the growth stimulation of normal and cancer cells mainly forming the AP1 transcription complex with jun family members.[34] Accordingly, the ERK activation upon exposure to IGF-I was paralleled by the induction of c-fos (Figure 3c). Of note, this response was abrogated using AG, GF, Rot and PD, suggesting that the IGF-IR/PKCδ/ERK signaling mediates the regulation of c-fos induced by IGF-I in MCF-7 cells (Figure 3c). Confirming the aforementioned data, IGF-I transactivated an AP1-luc-responsive collagenase promoter construct that was transiently transfected in MCF-7 cells, while the luciferase activity was abrogated in presence of AG, GF, Rot, PD (Figure 3d) or co-transfecting a dominant-negative form of c-fos (DN/c-fos) (Figure 3e). Of note, performing chromatin immunoprecipitation assay in MCF-7 cells, we ascertained that IGF-I induces the recruitment of c-fos to the AP1 site located within the promoter sequence of *GPER* (Figure 3f). In accordance with these results, the transactivation of the *GPER* promoter construct by IGF-I was abolished co-transfecting the DN/c-fos (Figure 3g) and the upregulation of the GPER protein levels was prevented in the presence of the DN/c-fos (Figure 3h). Taken together, these findings indicate that the IGF-IR/PKCδ/ERK/c-fos-AP1 transduction pathway mediates the transcription of GPER induced by IGF-I.

GPER is involved in the migration and proliferation promoted by IGF-I

As *CTGF* is a major gene target of GPER activity,[12] we asked whether CTGF responds to IGF-I through GPER. In MCF-7 cells, IGF-I transactivated the *CTGF* promoter construct (Figure 4a) and this effect was prevented silencing *GPER* expression. Likewise, the

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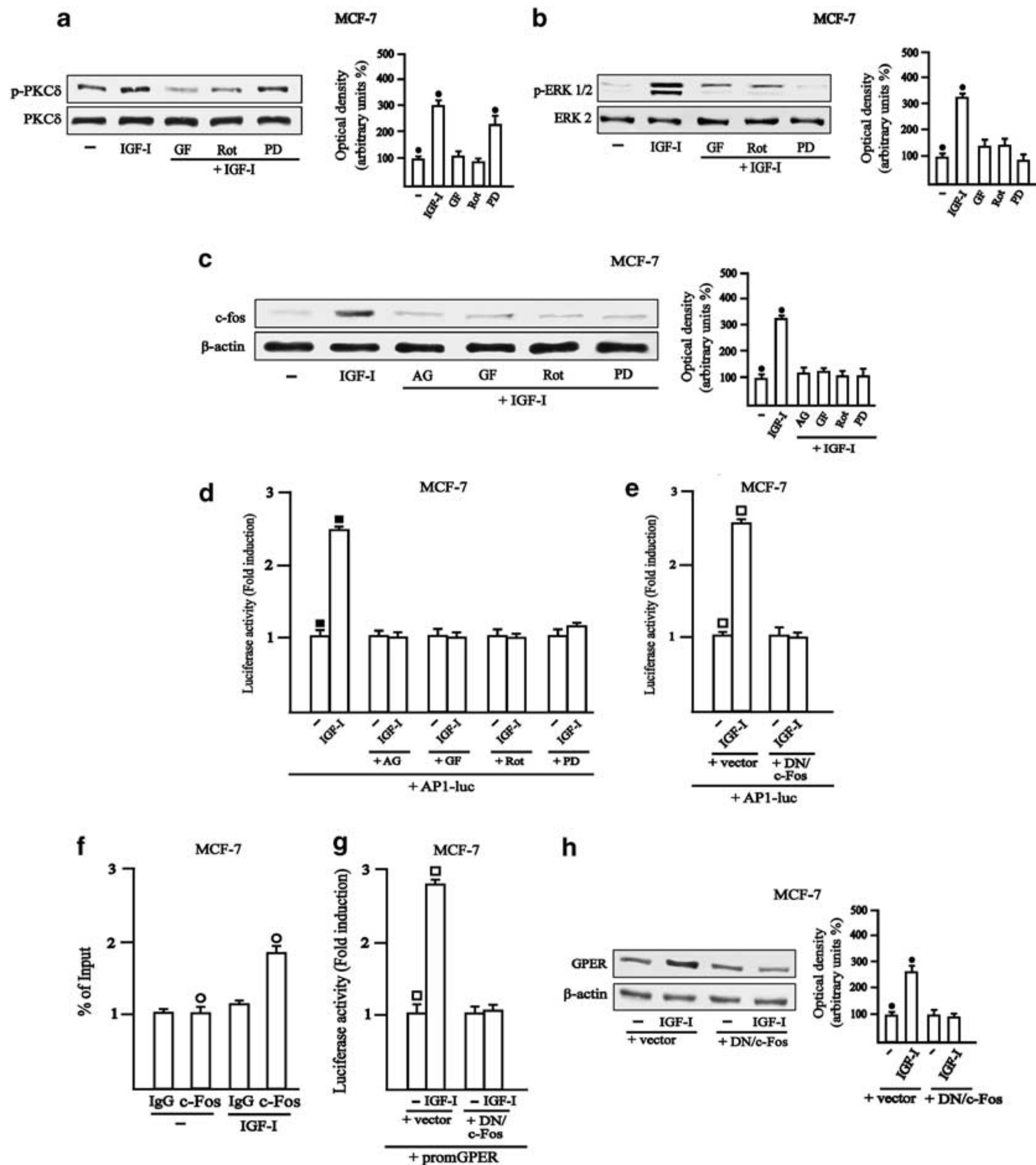

Figure 3. Transduction pathways mediating GPER upregulation by IGF-I in MCF-7 cells. Immunoblots of p-PKCδ (**a**) and p-ERK1/2 (**b**) from MCF-7 treated for 15 min with vehicle (−) or 100 ng/ml IGF-I alone and in combination with 10 μM PKC inhibitor GF109203X (GF), 10 μM PKCδ inhibitor Rottlerin (Rot), 10 μM MEK inhibitor PD98059 (PD). Immunoblots shown are representative of experiments performed in triplicate. Side panels show densitometric analysis of the blots normalized to total ERK2 and PKCδ. (**c**) Immunoblotting of c-fos from MCF-7 cells treated for 3 h with vehicle (−) or 100 ng/ml IGF-I alone and in combination with 10 μM IGF-IR inhibitor tyrphostin AG1024 (AG), 10 μM PKC inhibitor GF109203X (GF), 10 μM PKCδ inhibitor Rottlerin (Rot), 10 μM MEK inhibitor PD98059 (PD). (**d**) Cells were transfected with AP1-luc-responsive collagenase promoter and treated with 100 ng/ml IGF-I alone and in combination with AG, GF, Rot or PD, as indicated. (**e**) The expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the transactivation of AP1-luc by 100 ng/ml IGF-I. (**f**) A 100-ng/ml IGF-I induces the recruitment of c-fos to the AP1 site located within the *GPER* promoter sequence. In control samples, non-specific IgG was used instead of the primary antibody. (**g**) The expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the transactivation of the GPER promoter construct. (**h**) The expression vector encoding for a dominant negative form of c-fos (DN/c-fos) blocked the upregulation of GPER protein levels by 100 ng/ml IGF-I. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction upon which the activities induced by 100 ng/ml IGF-I were calculated. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. ●, ■, □, ○ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments. Side panels in (**c**) and (**h**) show densitometric analysis of the blots normalized to β-actin.

induction of the CTGF protein levels by IGF-I in both MCF-7 and Ishikawa cells was no longer evident, abrogating the expression of *GPER* (Figures 4b and d). As a biological counterpart, the migration stimulated by IGF-I after both 6 h (data not shown) and 24 h of treatment (Figure 5) was abolished silencing either *GPER* or *CTGF* expression by transfecting MCF-7 and Ishikawa cells for 24 h with specific shGPER and shCTGF constructs (Supplementary Figure 2). Considering that both cell types used express ERα, we next determined that its expression is also required for the migration induced by IGF-I after both 6 h (data not shown) and 24 h of treatment (Figure 5 and Supplementary Figure 2) (see Discussion). On the basis of previous investigations showing that IGF-I

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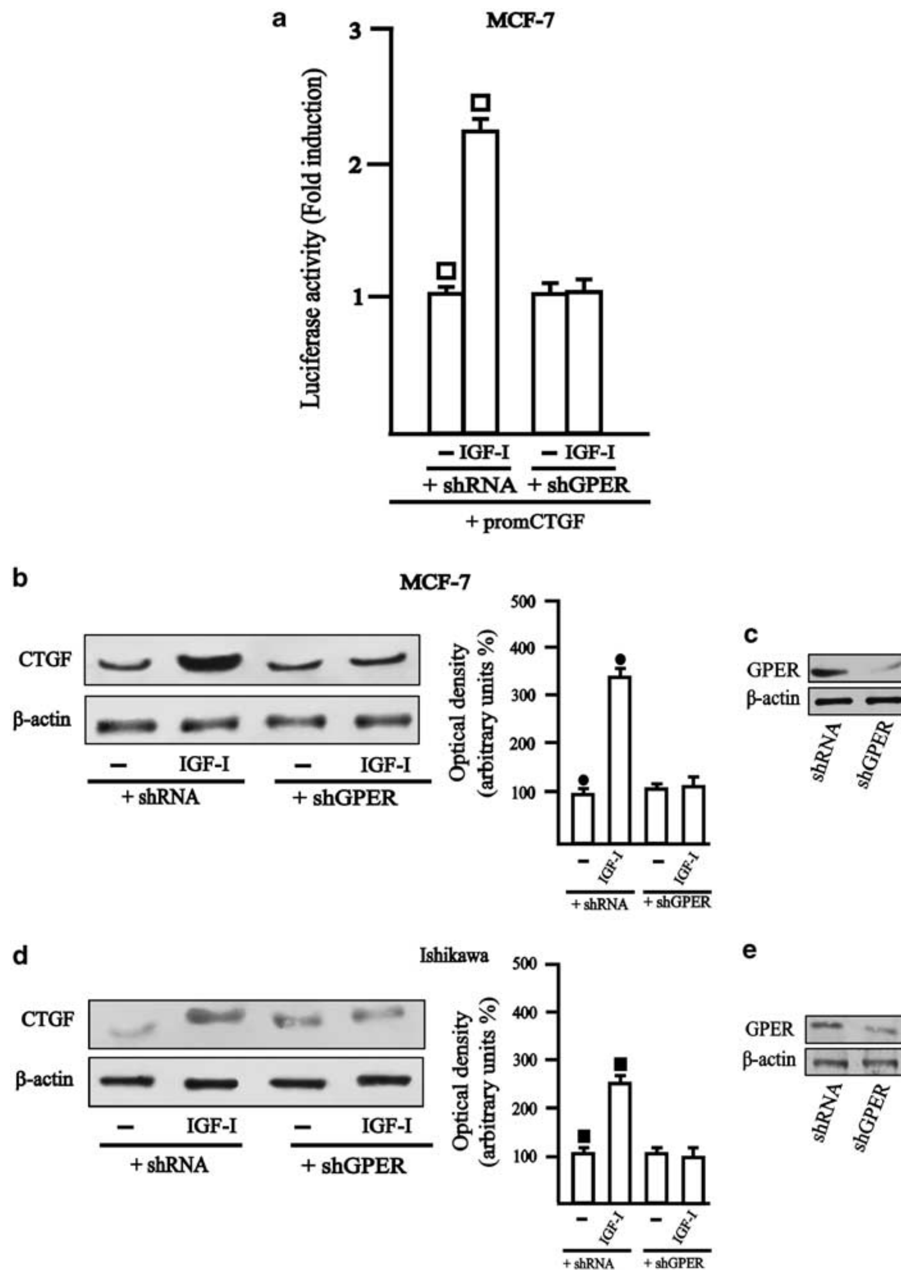

Figure 4. IGF-I transactivates the *CTGF* promoter construct and upregulates CTGF protein levels. (**a**) The IGF-I (100 ng/ml) induced transactivation of *CTGF* promoter construct is abrogated in presence of shGPER in MCF-7 cells. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction upon which the activities induced by 100 ng/ml IGF-I were calculated. (**b**, **d**) In MCF-7 and Ishikawa cells treated for 3 h with 100 ng/ml IGF-I, the upregulation of CTGF protein levels was abrogated in presence of shGPER. Side panels show densitometric analysis of the immunoblots normalized to β-actin. (**c**, **e**) Efficacy of *GPER* silencing by shGPER. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. □, ●, ■ Indicate $P < 0.05$ for cells receiving vehicle (−) versus treatments.

promotes the proliferation of cancer cells,[25,26] we therefore asked whether a functional cross talk between IGF-I and GPER is involved in the growth responses to IGF-I. Notably, the proliferation induced by IGF-I in MCF-7 and Ishikawa cells was abolished by silencing *GPER* expression (Figures 6a and c). In accordance with these observations, the upregulation of cyclin D1 induced by IGF-I in MCF-7 cells was abrogated knocking down *GPER* expression (Figure 6e) or transfecting the DN/c-fos construct (Figure 6g). In addition, a direct interaction between GPER and cyclin D1 was found upon exposure to IGF-I in MCF-7 cells (Figures 6h–i). Collectively, these results suggest that GPER is involved in a signaling network that mediates the migration and proliferation induced by IGF-I in MCF-7 and Ishikawa cells.

ERα is involved in the regulation of GPER by IGF-I

Considering the well known cross talk between IGF-I system and ERα in cancer cells,[27] we aimed at evaluating whether ERα is involved in the upregulation of GPER expression induced by IGF-I. Interestingly, the transactivation of the *GPER* promoter construct by IGF-I was prevented using the ERα inhibitor ICI 182,780 (ICI) (Figure 7a). Accordingly, the increase of GPER protein levels by

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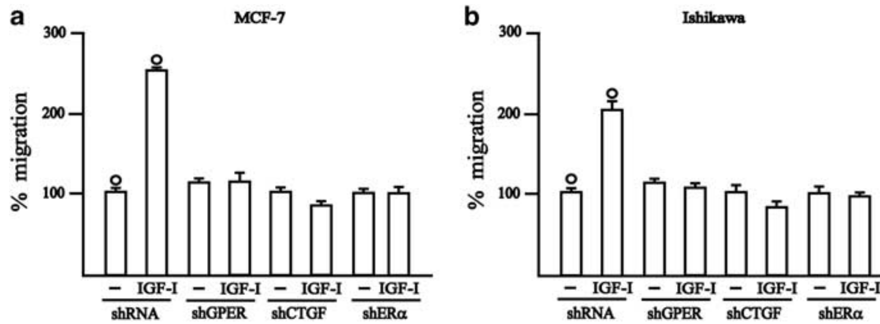

Figure 5. GPER, CTGF and ERα are involved in the migration of (**a**) MCF-7 and (**b**) Ishikawa cells induced by IGF-I. Cell migration promoted by IGF-I was abolished silencing *GPER*, *CTGF* or *ERα* expression. Bar graph shows a representative experiment with means of triplicate samples, standardized to the respective untreated controls set to100%. Error bars show standard deviations. ○ Indicates $P<0.05$ for cells receiving vehicle (−) versus treatments.

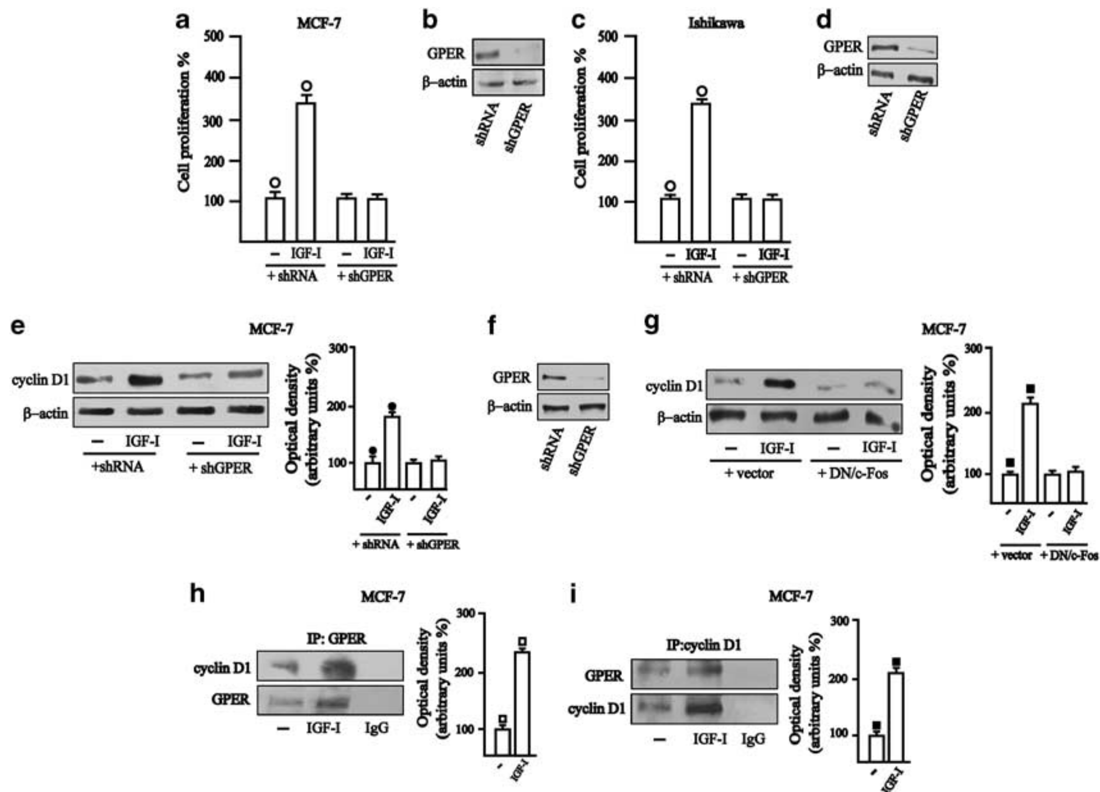

Figure 6. GPER is required for proliferation of MCF-7 and Ishikawa cells induced by IGF-I. (**a**, **c**) Cell proliferation induced by 100 ng/ml IGF-I was abrogated by silencing GPER expression. (**e**, **g**) The upregulation of cyclin D1 protein by 100 ng/ml IGF-I was abolished in the presence of shGPER and DN/c-fos. Side panels show densitometric analysis of the blots normalized to β-actin. (**b**, **d**, **f**) Efficacy of *GPER* silencing. (**h**, **i**) The treatment for 24 h with 100 ng/ml IGF-I strongly increases the coimmunoprecipitation of GPER with cyclin D1 in MCF-7 cells, as indicated. In control samples, non-specific IgG was used instead of the primary antibody. Each column represents the mean ± s.d. of three independent experiments performed in triplicate. ○, ●, ■, □ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments.

IGF-I was abolished in the presence of ICI or silencing *ERα* expression (Figures 7b–d). Further corroborating these results, in MCF-7 cells the recruitment of p-ERα^{Ser118} to an AP1 site located within the GPER promoter sequence induced by IGF-I (Figure 7e) was no longer evident transfecting cells with the DN/c-fos construct (Figure 7f).

Using carcinoma-associated fibroblasts obtained from breast tumors,[35] we further confirmed the ability of IGF-I to upregulate the expression of GPER and its target gene *CTGF*[12] (Supplementary Figure 3). Hence, the property of IGF-I to regulate GPER expression can be extended to these cancer surrounding cells, which mainly contribute to the malignant tumor features.[36]

DISCUSSION

Cross talk between estrogen and IGF-I signaling has been involved in several biological functions in breast cancer cells, including proliferation, survival, transformation, migration, adhesion and invasion.[37,38] For instance, IGF-I transactivates ERα in a ligand-independent manner,[28] and activated IGF-IR interacts with ERα

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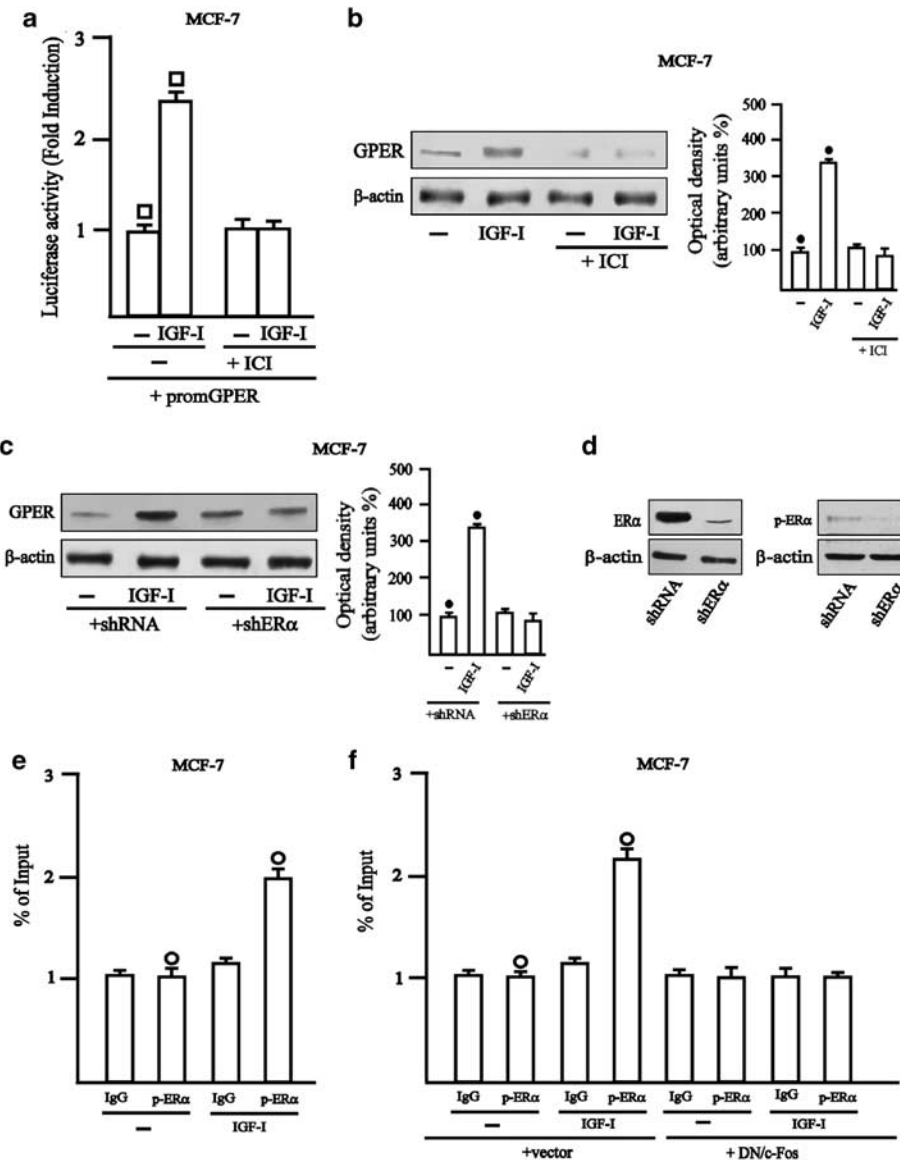

Figure 7. ERα is involved in the GPER transcription by IGF-I. (**a**) The transactivation of *GPER* promoter construct induced by 100 ng/ml of IGF-I is abrogated in presence of 10 μM ICI 182,780 (ICI). The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction upon which the activity induced by treatments was calculated. (**b**, **c**) The IGF-I induced upregulation of GPER protein levels was abolished by 10 μM ICI and by silencing ERα expression. (**d**) Efficacy of *ERα* and p-$ERα^{Ser118}$ silencing. (**e**, **f**) The recruitment of p-$ERα^{Ser118}$ induced by 100 ng/ml IGF-I to the AP1 site located within the *GPER* promoter sequence is abolished in presence of an expression vector encoding a dominant negative form of c-fos (DN/c-fos). In control samples non-specific IgG was used instead of the primary antibody. Each column represents the mean ± s.d. of three independent experiments. □, ●, ○ Indicate $P<0.05$ for cells receiving vehicle (−) versus treatments.

contributing to breast cancer development and progression.[39,40] In addition, IGF-IR has been shown to be involved in non-genomic, membrane-associated ERα activity,[37] although many aspects remain to be further elucidated. In this regard, previous studies on the functional interaction between the IGF-I system and estrogens have focused only on estrogen signaling mediated by ERα. In recent years, the identification of GPER as a novel ER has opened a new scenario, which is still under investigation for the assessment of its full impact on the action of estrogens. Considering the functional cross talk between IGF-I and the estrogen-dependent pathways as well as the lack of data on the ability of IGF-I system to engage GPER in triggering biological responses, in the present study, we aimed to evaluate whether IGF-I could regulate GPER expression and function in MCF-7 and Ishikawa cancer cells. These cells express relatively low amounts of insulin receptors (IR) that may contribute to the formation of IR/IGF-IR hybrid receptors, which also bind to IGF-I with a slightly lower affinity than homotypic IGF-IR.[41-43] Therefore, both MCF-7 and Ishikawa cells have low IR/IGF-IR content;[42,43] hence, we believe that most of the IGF-I effects on GPER signaling are mediated by the homotypic IGF-IR.

Our results demonstrate that IGF-I transactivates the promoter of *GPER* and upregulates its expression at both mRNA and protein levels in MCF-7 and Ishikawa cancer cells. In particular, we show that the induction of GPER by IGF-I is mediated by sequential events such as the rapid activation of PKCδ and ERK1/2 and the stimulation of c-fos, which is then recruited to an AP1 site located within the *GPER* promoter sequence. The functional role elicited

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by the AP1 complex is essential, as the transactivation of the *GPER* promoter sequence and the upregulation of GPER expression were abrogated by using a construct encoding for a dominant negative form of c-fos. Most importantly, GPER and its major target gene *CTGF* were required for cell migration induced by IGF-I. Given that CTGF primarily modulates and coordinates cell responses through the involvement of components of the extracellular matrix,[44,45] the GPER/CTGF signaling activated by IGF-I might contribute to cell motility and invasion during cancer progression. Likewise, we also show that cell proliferation in response to IGF-I, requires a functional cross talk between GPER and the main cell cycle regulator cyclin D1. On the basis of the aforementioned observations, GPER may contribute to the intricate transduction network engaged by IGF-I in triggering important biological effects in cancer cells.

It has been largely reported that ERα mediates biological responses leading to the progression of estrogen-sensitive tumors.[46] In the classical model of action, ERα bound to the estrogen-responsive elements located within the promoters of target genes recruits an array of co-factors involved in the regulation of the transcriptional machinery.[24,47] In addition, ERα modulates gene expression by a functional interaction with transcription factors, like AP1,[47] as well as with GPER as demonstrated in our previous studies.[11,17] In this regard, the present data reveal that ERα is also involved in the IGF-I-dependent regulation of GPER expression and therefore to GPER-mediated action. Hence, extending the current knowledge on the cross talk between ERα and GPER, our results indicate that these different ERs cooperate in mediating various extracellular stimuli leading to gene regulation and growth effects in cancer cells. Different forms of interaction between GPCRs and growth factor receptors have become increasingly evident in different cellular functions,[29] including cancer growth, angiogenesis and metastasis.[48] In particular, IGF-IR requires GPCRs in regulating many physiological functions.[49] Indeed, the cross talk between IGF-I and GPCR-dependent pathways occurs at multiple levels in a variety of malignancies.[50] For instance, a physical association between IGF-IR and the G protein subunits, Gα and Gβγ, has been involved in cell migration in breast cancer cells.[51] In addition, IGF-I cooperated with agonists of GPCRs in promoting cell responses such as DNA synthesis, proliferation and anchorage-independent growth in pancreatic cancer cells.[31] The identification of GPER has expanded our understanding on the regulation and action of GPCRs. In this vein, it is worth to note that EGFR ligands transactivated the promoter of *GPER* and upregulated its expression in cancer cells.[14,15] In addition, we have recently demonstrated that hypoxia, through the HIF-1α-mediated transduction pathway, induces GPER expression, hence suggesting a new mechanism by which estrogens may exert relevant biological effects under hypoxic conditions.[52] We have also found that GPER interacting with EGFR acts as a transcription factor in carcinoma-associated fibroblasts,[35] indicating the potential of GPER to affect cancer progression through the stimulation of cells surrounding tumors. Similarly, the present findings that GPER expression and function are regulated by IGF-I in cancer cells as well as in carcinoma-associated fibroblasts, shed new light on a novel mechanism by which the IGF-I system and estrogen signaling cooperate toward cancer development and progression. Of note, previous reports have shown that higher GPER levels were associated with worse clinical pathological features and lower survival rates in estrogen-sensitive tumors.[16,17] Therefore, the expression levels of GPER may characterize not only the estrogen sensitivity and the response to endocrine pharmacological intervention in these tumors but could also be predictive of biologically aggressive phenotypes consistent with adverse outcome and survival. Thus, a better understanding on the GPER regulation and function represents a new challenge for a more comprehensive therapeutic approach in estrogen-responsive malignancies including breast and endometrial tumors. In this regard, our results further indicate that GPER is involved in an intricate tumorigenic transduction network, which should be considered as a promising therapeutic target toward innovative intervention.

MATERIALS AND METHODS

Reagents

IGF-I, H89, LY294,002 (LY) and PD98059 (PD) were purchased from Sigma-Aldrich Corp. (Milan, Italy). Bisindolylmaleimide I (GF109203X) was bought from Santa Cruz Biotechnology (DBA, Milan, Italy), 3-bromo-5-t-butyl-4-hydroxybenzylidenemalonitrile (AG1024) and Rottlerin from Calbiochem (Milan, Italy). ICI 182,780 (ICI) was obtained from Tocris Chemicals (Bristol, UK). All compounds were solubilized in dimethylsulfoxide, except PD and IGF-I, which were dissolved in ethanol and in water, respectively.

Cell culture

MCF-7 breast cancer cells were maintained in DMEM/F-12 (Invitrogen, Gibco, Milan, Italy) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma, Milan, Italy). Ishikawa endometrial cancer cells were maintained in DMEM (Invitrogen, Gibco) without phenol red supplemented with 10% fetal bovine serum 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma). Cells were switched to medium without serum the day before experiments; thereafter cells were treated as indicated. Primary fibroblast cells from breast cancer tissues were obtained as we have previously described[35] and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum, 100 μg/ml penicillin/streptomycin and 2 mM L-glutamine (Sigma).

Plasmids

The GPER luciferase expression vector promGPER was previously described.[52] The CTGF luciferase reporter plasmid promCTGF (-1999/ + 36)-luc was a gift from Dr B Chaqour.[53] The luciferase reporter plasmid for AP-1-responsive collagen promoter was a kind gift from H van Dam (Department of Molecular Cell Biology, Leiden University, Leiden, Netherlands). As an internal transfection control, we cotransfected the plasmid pRL-TK (Promega, Milan, Italy) that expresses RenillaLuciferase. The plasmid DN/c-fos, which encodes a c-fos mutant that heterodimerizes with c-fos dimerization partners but does not allow DNA biding,[54] was a kind gift from Dr C Vinson (NIH, Bethesda, MD, USA). The Sure Silencing (sh) IGF-IR, (sh) ERα and the respective control plasmids (shRNA), generated in pGeneClip Puromycin Vector, were purchased from SA Bioscience Corp. (Frederick, MD, USA) and used according to the manufacturer's recommendations (more information is available at www.sabiosciences.com). Short hairpin constructs against human GPER (shGPER) and CTGF (shCTGF) were obtained and used as previously described.[12,14] In brief, they were generated in lentiviral expression vector pLKO.1 purchased by Euroclone, Milan, Italy. The targeting strand generated from the GPER shRNA construct is 5′-CGCTCCCTGCAAGCAGTCTTT-3′. The targeting strand generated from the CTGF shRNA construct is 5′-TAGTACAGCGATTCAAAGATG-3′.

Transfections and luciferase assays

MCF-7 cells (1×10^5) were plated into 24-well dishes with 500 μl/well of regular growth medium the day before transfection. The medium was replaced with DMEM lacking serum and phenol red on the day of transfection, which was performed using FuGene6 reagent, as recommended by the manufacturer (Roche Diagnostics, Milan, Italy), with a mixture containing 0.5 μg of reporter plasmid and 2 ng of pRL-TK. After 6 h the medium was replaced again with DMEM lacking serum and phenol red, treatments were added and cells were incubated for an additional 24 h. Luciferase activity was then measured with the Dual Luciferase Kit (Promega Italia, 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 set

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as onefold induction upon which the activity induced by treatments was calculated.

Reverse transcription and real-time PCR

Total RNA was extracted using Trizol commercial kit (Invitrogen) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gel stained with ethidium bromide. Total cDNA was synthesized from RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Invitrogen) following the protocol provided by the manufacturer. The expression of selected genes was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems Inc, Milano, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc.). Assays were performed in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. For GPER and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5′-ACACACC TGGGTGGACACAA-3′ (GPER forward); 5′-GGAGCCAGAAGCCACATCTG-3′ (GPER reverse); 5′-GGCGTCCCCCAACTTCTTA-3′ (18S forward); and 5′-GGG CATCACAGACCTGTTATT-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.

Western blotting

Cells were grown in a 10-cm dishes and exposed to ligands before lysis in 500 μl of lysis buffer containing: 50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% TritonX-100, 1% sodium dodecyl sulfate (SDS), a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Protein concentrations were determined according to the Bradford method (Sigma-Aldrich). Equal amount of whole protein extracts were electrophoreted through a reducing SDS/10% (w/v) polyacrilamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy). Membranes were blocked and probed with primary antibodies against GPER (N-15), CTGF (L-20), c-Fos (H-125), phosphorylated ERK $\frac{1}{2}$ (E-4), and ERK2 (C-14), phosphorylated PKCδ (Thr 507), PKCδ (C-20), cyclin D1 (M-20), ERα (F-10), IGF-IR (7G11), and β-actin (C2) purchased from Santa Cruz Biotechnology (DBA), and p-$ER\alpha^{Ser118}$(16J4) purchased from Cell Signaling Technology. The levels of protein and phospho proteins were detected with appropriate secondary HRP-conjugated antibodies and the ECL (enhanced chemiluminescence) System (GE Healthcare, Milan, Italy). All experiments were performed in triplicate and blots shown are representative.

Co-immunoprecipitation

For co-immunoprecipitation of GPER/cyclin D1 complex, cells were lysed using 500 μl of RIPA buffer (Sigma) with a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). A total of 125 μg proteins were incubated overnight with 2 μg of GPER (N-15) or cyclin D1 (M-20) antibody and 20 μl of protein A/G agarose immunopreciptation reagent (Santa Cruz Biotechnology, Inc.). After four washes in PBS, samples were resuspended in 20 μl of SDS-sample buffer (0.5 M Tris–HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 4% bromophenol blue). Western Blot analysis was performed as described above.

Chromatin immunoprecipitation

Cells grown in 10 cm plates were shifted for 24 h to medium lacking serum and then treated with vehicle or 100 ng/ml IGF-I for 3 h. Chip assay was performed as we have previously described.[52] The immune cleared chromatin was immunoprecipitated with anti c-fos (H-125) and anti ERα (F-10) antibodies or non-specific IgG (Santa Cruz Biotechnology, DBA).

A 4-μl volume of each immunoprecipitated DNA sample was used as template to amplify, by RT–PCR, one fragment of 261 bp, containing an AP-1 site, located into the GPER promoter region. The primer pairs used to amplify this fragment were: 5′-CGTGCCCATACCTTCATTGCTTCC-3′ (forward) and 5′-CCTGGCCGGGTGTCTGTG-3′(reverse). Real-time PCR data were normalized with respect to unprocessed lysates (input DNA). Inputs DNA quantification was performed by using 4 μl of the template DNA. The relative antibody-bound fractions were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as percent differences with respect to the relative inputs.

Immunostaining assay

Fifty percent confluent cultured MCF-7 cells grown on coverslips were serum deprived and transfected for 12 h with a control shRNA or a shRNA specific for GPER (shGPR30), using Fugene 6 reagent, as recommended by the manufacturer, and then treated for 24 h with vehicle or 100 ng/ml IGF-I. Thereafter cells were fixed in 4% paraformaldehyde; permeabilized with 0.2% TritonX-100, washed three times with PBS, blocked and incubated overnight with a rabbit primary antibody GPR30 (N-15) (1:500; purchased from Santa Cruz Biotechnology). After incubation, the slides were extensively washed with PBS and incubated with donkey anti-rabbit IgG-FITC (1:500, from Santa Cruz Biotechnology) and propidium iodide (1:1000, Sigma-Aldrich). Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS, GbH Mannheim, Germany) were used for experiments evaluation.

Proliferation assay

For quantitative proliferation assay, cells (1×10^5) were seeded in 12-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% charcoal-stripped fetal bovine serum; when used, 500 ng of the indicated shRNA were added to cells using Fugene 6 reagent as recommended by the manufacturer and then renewed every 2 days before counting; treatments were added every day. Evaluation of cell growth was performed after 4 days using automatic counter (Countess - Invitrogen). Data shown are representative of three independent experiments performed in triplicate.

Migration assay

Migration assays were performed using Boyden chambers (Costar Transwell, 8 mm polycarbonate membrane). For knockdown experiments, cells were transfected with shRNA constructs directed against GPER or CTGF or ERα with an unrelated shRNA construct (3 μg DNA/well transfected with FuGene6 reagent in medium without serum). After 24 h, cells were seeded in the upper chambers. IGF-I was added to the medium without serum in the bottom wells. After 24 h, cells on the bottom side of the membrane were fixed and counted.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls' testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Breast Cancer RESEARCH

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MIBE acts as antagonist ligand of both estrogen receptor α and GPER in breast cancer cells

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Abstract

Introduction: The multiple biological responses to estrogens are mainly mediated by the classical estrogen receptors ERα and ERβ, which act as ligand-activated transcription factors. ERα exerts a main role in the development of breast cancer; therefore, the ER antagonist tamoxifen has been widely used although its effectiveness is limited by *de novo* and acquired resistance. Recently, GPR30/GPER, a member of the seven-transmembrane G protein-coupled receptor family, has been implicated in mediating the effects of estrogens in various normal and cancer cells. In particular, GPER triggered gene expression and proliferative responses induced by estrogens and even ER antagonists in hormone-sensitive tumor cells. Likewise, additional ER ligands showed the ability to bind to GPER eliciting promiscuous and, in some cases, opposite actions through the two receptors. We synthesized a novel compound (ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate), referred to as MIBE, and investigated its properties elicited through ERα and GPER in breast cancer cells.

Methods: Molecular modeling, binding experiments and functional assays were performed in order to evaluate the biological action exerted by MIBE through ERα and GPER in MCF7 and SkBr3 breast cancer cells.

Results: MIBE displayed the ability to act as an antagonist ligand for ERα and GPER as it elicited inhibitory effects on gene transcription and growth effects by binding to both receptors in breast cancer cells. Moreover, GPER was required for epidermal growth factor receptor (EGFR) and ERK activation by EGF as ascertained by using MIBE and performing gene silencing experiments.

Conclusions: Our findings provide novel insights on the functional cross-talk between GPER and EGFR signaling. Furthermore, the exclusive antagonistic activity exerted by MIBE on ERα and GPER could represent an innovative pharmacological approach targeting breast carcinomas which express one or both receptors at the beginning and/or during tumor progression. Hence, the simultaneous inhibition of both ERα and GPER may guarantee major therapeutic benefits in respect to the use of a selective estrogen receptor antagonist.

Introduction

Estrogens regulate many aspects of human physiology and influence diverse pathological processes, including the development of hormone-dependent tumors [1]. The biological actions of estrogens are mainly mediated by the estrogen receptor (ER)α and ERβ, which belong to the nuclear receptor superfamily [1]. Acting as ligand-activated transcription factors, ERs regulate gene expression by binding to responsive elements (ERE) located within the promoter region of estrogen target genes [1]. In addition, gene regulation can occur in response to estrogens through plasma membrane receptors, such as growth factor receptors or G protein-coupled receptors, and by protein kinase signaling cascades [2].

Prolonged exposure to estrogens represents a major risk factor for the progression of breast cancer [3], which expresses elevated levels of ERα in approximately 70% of cases [4]. Consequently, ERα antagonists like tamoxifen and raloxifene are currently used as frontline pharmacological interventions in ERα-positive breast cancer in order to inhibit the mitogenic stimulation of estrogens [5]. Although there is general concordance between ERα expression and responsiveness to ER-

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targeted agents, as indicated by a greater five-year disease-free survival for ERα-positive patients receiving tamoxifen, one in four patients does not respond to treatment from the onset and in most patients tamoxifen produces agonist effects after a few years [6].

In order to further characterize the molecular mechanisms involved in the action of estrogens, recent studies have demonstrated that the G protein-coupled receptor, named GPR30/GPER, mediates rapid biological responses to estrogens in diverse normal, as well as transformed, cell types [7]. The potential role of GPER in cancer was supported by numerous investigations performed in different tumor cells, including breast [8-10], endometrial [11], ovarian [12], thyroid [13], prostate [14] and testicular germ cells [15]. In accordance with these findings, GPER has been associated with aggressive features of breast cancer [16], high-grade endometrial tumors [17] and poor prognosis in ovarian cancer [18]. Since its identification to date, the transduction signaling and gene expression profile triggered by GPER have been extensively characterized. The early discovery [8] of a transmembrane receptor able to mediate estrogen responsiveness in ER-negative breast cancer cells was then confirmed by several reports by which GPER was considered as a genuine ER [10,19]. Indeed, a whole series of intracellular events, such as the rapid phosphorylation of mitogen-activated protein kinases (MAPK) ERK1/2, the activation of PI3-kinase (PI3K) and phospholipase C (PLC), the increase in cAMP concentrations and the intracellular calcium mobilization, was shown to follow GPER activation by both estrogens and anti-estrogens [20]. In particular, it was demonstrated that GPER-dependent ERK activation occurs via the transactivation of the epidermal growth factor receptor (EGFR) through matrix metalloproteinase activity and integrin α5β1, which trigger the extracellular release of heparan-bound epidermal growth factor (HB-EGF) [8,21]. Interestingly, a physical and functional cross-talk between GPER and EGFR contributes to the intricate signaling network involved in the stimulation of hormone-sensitive tumors [22,23].

The rapid responses to estrogenic signals mediated by GPER regulate a typical gene signature, as revealed in previous studies, including a microarray analysis [7,24]. Of note, GPER target genes were shown to contribute to the proliferation and migration in diverse cancer cell types [9,11-13,22,24,25] as well as in cancer associated fibroblasts (CAFs) [26].

GPER exhibits many of the expected characteristics of an estrogen receptor, including the capability to bind to estrogens, phyto- and xenoestrogens and even the ER antagonists 4-hydroxytamoxifen (OHT) and fulvestrant (ICI 182 780) [10,19,27,28]. Surprisingly, unlike the antagonistic properties displayed by these anti-estrogens with respect to the classical ERs, both compounds act as GPER agonists [8,11,19,24]. Conversely, the well known ER agonist estriol exerts inhibitory effects on GPER-mediated signaling [28], confirming the potential opposite functions elicited by estrogenic/anti-estrogenic agents through each type of estrogen receptor. In addition to the selective GPER agonist G-1 [29], GPER ligands showing antagonistic properties have been identified [30,31]. Recently, a GPER antagonist showed at high concentrations limited binding properties and stimulatory activity on ER-mediated transcription [30]. The use of these compounds has greatly advanced our understanding of the role of GPER in numerous biological systems as well as in cancer.

On the basis of the aforementioned findings, GPER may be considered as an additional therapeutic target in estrogen-sensitive tumors, such as breast cancer. In this regard, the opposite functional activity elicited by anti-estrogens through the classical ERs and GPER as stated above, could represent a therapeutic concern toward the pharmacological inhibition of all types of estrogen receptor.

We discovered a novel compound, ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl] but-2-enoate (referred to as MIBE) (Figure 1), which displays the unique property to bind to and inhibit GPER- and ERα-mediated signaling in breast cancer cells. The antagonistic action exerted by MIBE on both estrogen receptor types could represent a novel, promising tool for a more comprehensive pharmacological approach to estrogen-dependent tumors such as breast cancer.

Materials and methods

Molecular modelling and docking simulations

For docking simulations we used as targets the crystallographic coordinates of ERα in complex with E2 (closed-conformation) as well as with OHT (open conformation) and a GPER molecular model built by homology as described elsewhere (PDB code 1G50; PDB code 3ERT) [28,32,33]. Docking studies were performed by GOLD 5.0.1 (the Cambridge Crystallographic Data Center, UK), a program using a genetic algorithm useful to investigate the full range of ligand conformational flexibility and a partial protein side chain flexibility. As active sites of ERα, we identified those atoms that are within 20 Å distance from each atom of the ligand experimental position. Regarding GPER, we identified the O atom of Phe 208 as the protein active site centre on the basis of our previous docking simulations [28]. In this case, the active site atoms were considered those located within 20 Å from the centre. For each structure, 10 docking solutions were generated allowing an early termination of the

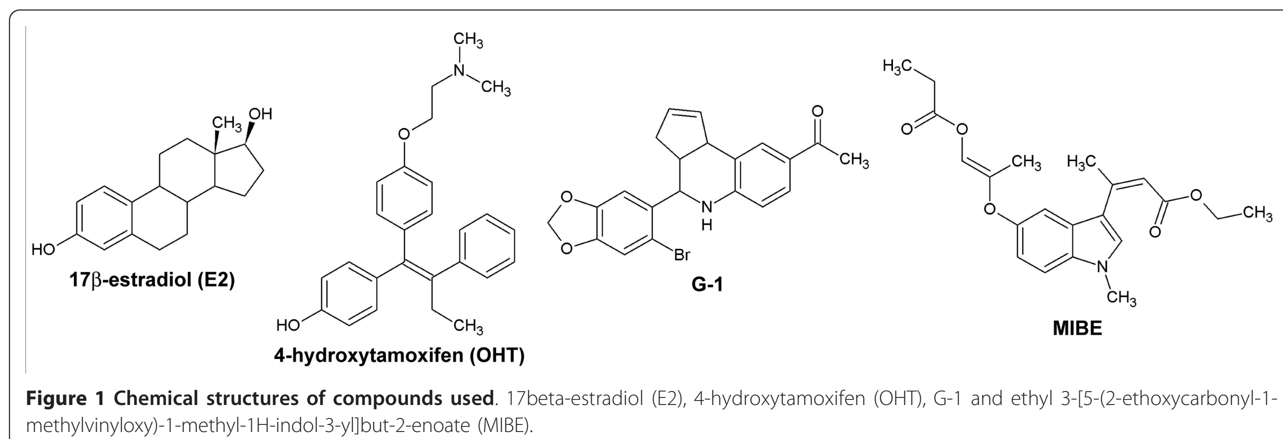

Figure 1 Chemical structures of compounds used. 17beta-estradiol (E2), 4-hydroxytamoxifen (OHT), G-1 and ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate (MIBE).

process, if the respective RMSDs of the three highest ranked docking solutions were within 1.5 Å of each other. The default GOLD settings were used for running the simulations. ERα protein side chains Met342, Glu353, Trp383, Met388, Arg394, Phe404, His524 and Leu525 were considered as flexible, while in the GPER model the residues Tyr123, Gln138, Phe206, Phe208, Glu275, Phe278 and His282 were defined flexible side chains allowing their free rotation. The molecular structures of the ligands screened *in silico* were built and energy minimized with the programs Insight II and Discover3 (Biosym/MSI, San Diego, CA, USA). All the figures were drawn with the program Chimera (UCSF, San Francisco, CA, USA) [34].

Chemistry

5-Hydroxy-1-methylindole was allowed to react with an excess of ethyl acetoacetate using Indium(III) chloride as a catalyst. The derivative ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl] but-2-enoate (MIBE) was obtained in good yield [35,36]. Melting points were determined on a Kofler melting point apparatus. IR spectra were taken with a Perkin Elmer BX FT-IR (Corporate Headquarters, Waltham, Massachusetts, USA). Mass spectra were taken on a JEOL JMS GCMate spectrometer at ionising potential of 70 eV (EI). ^{1}H-NMR (400 MHz) was recorded on a JEOL Lambda 400 Spectrometer (JEOL Ltd., Tokyo, Japan). Chemical shifts are expressed in parts per million downfield from tetramethylsilane as an internal standard. Thin layer chromatography (TLC) was performed on silica gel 60F-264 (Merck, Frankfurt, Germany). Commercial reagents were purchased from Aldrich Chemical (Milan, Italy), Acros Organics (Carlo Erba Reagenti S.p.A., Rodano, Milan, Italy) and Alfa Aesar (Karlsruhe, Germany). Unless otherwise stated, all commercial reagents were used without further purification.

Procedure for the preparation of MIBE was as follows. Indium (III) chloride (10 mol%) was added under nitrogen to a mixture of 5-hydroxy-1-methyl-1H-indole and ethyl acetoacetate. The reaction mixture was heated under reflux for two hours, and then it was left to cool to room temperature. Ice water was added and then the reaction mixture was extracted by ethyl acetate. The organic layers were collected and washed with brine, dried over $MgSO_4$ and evaporated under reduced pressure. The solid residue was washed with Et_2O, to give the pure compound MIBE a pink solid, yield of 65%, mp = 180°C; IR (KBr): 3412, 2984, 1705, 1622, 1473, 1373, 1168, 1088, 1027, 805 cm^{-1}. ^{1}H-NMR (d_6-DMSO) δ 8.94 (s, 1H, Ar); 7.32 (d, 1H, Ar, $J_{7,6}$ = 8.8 Hz); 6.87 (s, 1H, Ar); 7.32 (d, 1H, Ar, $J_{6,7}$ = 8.8 Hz); 6.04-6.01 (m, 2H, C = CH); 4.11-4.09 (q, 2H, CH_2); 3.90-3.88 (q, 2H, CH_2); 3.76 (s, 3H, NCH_3); 1.42 (s, 6H, C-CH_3); 1.24-1.20 (t, 3H, CH_3); 0.95-0.92 (t, 3H, CH_3). MS (EI) m/z: 371 (M^+, 14).

Reagents

17β-estradiol (E2), 4-hydroxytamoxifen (OHT) and 5α-dihydrotestosterone (DHT) were purchased from Sigma-Aldrich (Milan, Italy). G-1 (1-[4-(-6-bromobenzol [1,3] diodo-5-yl)-3a,4,5,9b-tetrahidro3H5 cyclopenta[c]quinolin-8yl]-ethanone) was bought from Calbiochem (Merck KGaA, Frankfurt, Germany). All compounds were solubilized in ethanol, except G-1 and MIBE which were dissolved in dimethyl sulfoxide (DMSO).

Cell culture

MCF7 breast cancer cells and human embryonal kidney Hek293 cells were maintained in DMEM with phenol red supplemented with 10% FBS. SkBr3 breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS. 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.

The experiments performed in this study do not require Institute Ethics Board approval, because only commercially available cell lines were used.

Plasmids

Firefly luciferase reporter plasmids used were ERE-luc for ERα [37], ARE-luc for the Androgen Receptor (AR) [38] and GK1 [37] for the Gal4 fusion proteins Gal-ERα and Gal-ERβ, which were expressed from plasmids GAL93.ER(G) and GAL93.ERβ, respectively, as previously described [37]. The full length AR expression plasmid (AR) was previously described [39]. As the internal transfection control, we co-transfected the plasmid pRL-TK (Promega, Milan, Italy) that expresses *Renilla* Luciferase. Short hairpin RNA construct against human GPER (shGPR30/shGPER) and the unrelated shRNA control construct were previously described [22].

Transfection, Luciferase assays and gene silencing experiments

Cells were plated into 24-well plates with 500 μl of regular growth medium/well the day before transfection. Cell medium was replaced with medium supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red and serum on the day of transfection, which was performed using the Fugene 6 Reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5 μg of reporter plasmid, 2 ng of pRL-TK, 0.1 μg of effector plasmid and 0.1 μg of full length AR expression plasmid where applicable. After 6 h, the medium was replaced again with serum-free medium lacking phenol red and supplemented with 1% CS-FBS, treatments were added at this point and cells were incubated for an additional 18 h. Luciferase activity was then measured using 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 set as one-fold induction upon which the activity induced by treatments was calculated.

For the gene silencing experiments, cells were plated into 10-cm dishes, maintained in serum-free medium for 24 h and then transfected for an additional 48 h before treatments using Fugene 6 (according to the manufacturer's recommendations) and control vector (shRNA) or shGPER.

Ligand binding assays

In ligand binding assay for ERα, the ability of MIBE to compete with [3H]E2 was evaluated and compared with that of E2. Two picomoles of purified recombinant human ERα protein purchased from PanVera, Invitrogen S.r.l. (Milan, Italy), each in 20 mM HEPES, pH 7.4, 1.5 mM EDTA, 0.5 mM dithiothreitol, and 10% (v/v) glycerol, was incubated with 1 nM [2,4,6,7-3H]E2 (89 Ci/mmol; Ge Healthcare, Milan, Italy) and increasing concentrations of nonlabeled E2 or MIBE for two hours at 37°C in a humidified atmosphere of 95% air/5% CO2. Bound and free radioligands were separated on Sephadex G-25 PD-10 columns. The amount of receptor-bound [3H]E2 was determined by liquid scintillation counting.

In ligand binding assay for GPER, SkBr3 cells were grown in 10-cm cell culture dishes, washed two times and incubated with 1 nM [2,4,6,7-3H]E2 (89 Ci/mmol; Ge Healthcare, Milan, Italy) in the presence or absence of an increasing concentration of nonlabeled competitors (E2, G-1, OHT and MIBE). Then, cells were incubated for two hours at 37°C and washed three times with ice-cold PBS; the radioactivity collected by 100% ethanol extraction was measured by liquid scintillation counting. Competitor binding was expressed as a percentage of maximal specific binding. Each point is the mean of three observations.

Reverse transcription and real-time PCR

Gene expression was evaluated by real-time PCR as we previously described [37]. For Cyclin D1, IRS-1, PR, pS2, c-fos, CTGF, Cyr61, EGR1, and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5'-GTCTGTGCAT TTCTGGTTGCA-3' (Cyclin D1 forward) and 5'-GCTGGAAACATGCCGGTTA-3' (Cyclin D1 reverse); 5'-GCCCGTGTTACTGTTCATTCAG-3' (IRS-1 forward) and 5'-AATAACGGACACTGCACAACAGTCT-3' (IRS-1 reverse); 5'-GAGTTGTGAGAGCACTG-GATGCT-3' (PR forward) and 5'-CAACTGTAT GTCTTGACCTGGTGAA-3' (PR reverse); 5'-GCCCCC CGTGAAAGAC-3' (pS2 forward) and 5'-CGTCGAAA-CAGCAGCCCTTA-3' (pS2 reverse); 5'-CGAGCCCT TTGATGACTTCCT-3' (c-fos forward), 5'-GGAGCGG GCTGTCTCAGA-3' (c-fos reverse); 5'-ACCTGTG GGATGGGCATCT-3' (CTGF forward), 5'-CAGGC GGCTCTGCTTCTCTA-3' (CTGF reverse); 5'-GAGT GGGTCTGTGACGAGGAT-3' (Cyr61 forward) and 5'-GGTTGTATAGGATGCGAGGCT-3' (Cyr61 reverse); 5'-GCCTGCGACATCTGTGGAA-3' (EGR1 forward), 5'-CGCAAGTGGATCTTGGTATGC-3' (EGR1 reverse); and 5'- GGCGTCCCCCAACTTCTTA -3' (18S forward) and 5'- GGGCATCACAGACCTGTTATT -3' (18S reverse), respectively.

Western blotting

Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 μL of 50 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 10% glycerol, 1%

Triton X-100, 1% sodium dodecyl sulfate (SDS), and a mixture of protease inhibitors containing 1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride and 200 mmol/L sodium orthovanadate. 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 (GE Healthcare, Milan, Italy), probed overnight at 4°C with antibodies against Cyclin D1 (M-20), IRS-1 (A-19), c-fos (H-125), CTGF (L-20), GPER (N-15), pEGFR Tyr 1173 (sc-12351), β-actin (C-2), phosphorylated ERK1/2 (E-4) and ERK2 (C-14) (all purchased from Santa Cruz Biotechnology, DBA, Milan, Italy), and then revealed using the ECL™ Western Blotting Analysis System (GE Healthcare, Milan, Italy).

Proliferation assay

For quantitative proliferation assay, cells (1×10^5) were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and then were incubated in medium containing 2.5% charcoal-stripped FBS with the indicated treatments; medium was renewed every two days (with treatments) before counting, using the Countess Automated Cell Counter, as recommended by the manufacturer's protocol (Invitrogen S.r.l., Milan, Italy).

Statistical analysis

Statistical analysis was done using ANOVA followed by Newman-Keuls' testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

Results

Molecular modeling and binding assays demonstrate that MIBE is a ligand of both ERα and GPER

On the basis of the results obtained in docking simulations as described in the Materials and methods section, we evaluated the affinity of MIBE for the ligand binding pockets of both ERα and GPER with respect to E2 and G-1, respectively (Figure 2). Docking E2 to the hormone binding pocket of a closed conformation of ERα (Figure 2a), we observed a binding mode similar to that reported in the experimental crystallographic complex (superposition of the solution provided by GOLD to the crystallographic structure led to a RMSD of 0.092Å) [32]. Docking MIBE to the same pocket using ERα in both the closed and open conformation, we evidenced a better affinity for the last conformation (Figure 2b) and a binding mode similar to that adopted by the ER antagonist OHT in the crystallographic structure (PDB code 3ERT) [33].

As it concerns the GPER ligand binding pocket, visual inspection showed that it lies within a deep cleft in where 10 hydrophobic residues (V116, Met133, Leu137, Phe206, Phe208, Phe 278, Ile279, Ile308, Val309 and Phe314) and 5 polar amino acids (Tyr123, Gln138, Asp210, Glu275 and His282) contribute to stabilize the ligands through Van der Waals interactions and hydrogen bonds, respectively. Using GPER as a target, docking simulations confirmed a good affinity of the protein for the agonist G-1 (Figure 2c) as previously demonstrated both *in silico* and *in vitro* [29]. Next, we docked MIBE to GPER using the same settings and parameters as for G-1. MIBE, which was positioned within the GPER binding site (Figure 2d), displayed a high affinity for GPER, even better than that exhibited by G-1. In particular, MIBE binds to GPER forming hydrogen bonds with the hydroxyl groups located on its branched arms, on one side with Y123 OH, on the other with Q215 NE2 and H282 ND1 atoms. MIBE is also stabilized in the protein binding pocket by Van der Waals interactions of its methyl groups with residues F208, I279, T305 and I308, while a π-π stacking interaction is formed by the aromatic rings of F208 and the indole ring of MIBE. Starting from the aforementioned observations, we performed diverse assays to fully evaluate the ligand binding properties and the potential agonist/antagonist activity of MIBE exerted through ERα and GPER.

In order to confirm whether MIBE is a ligand of ERα, we performed competitive binding experiments by using the recombinant ERα protein. MIBE displaced the radiolabeled E2 in a dose-dependent manner (Figure 3a) indubitably demonstrating its capability to bind to ERα in a direct fashion, although with a lower binding affinity in respect to E2 and OHT as 10 μM MIBE induced approximately 40% displacement of [3H]E2. On the basis of the ability of MIBE to interact with GPER in docking simulations, we also performed ligand binding studies using radiolabeled E2 as a tracer in ER-negative but GPER-positive SkBr3 breast cancer cells, as previously reported [28]. Hence, we performed binding experiments using cold E2, MIBE, the selective GPER ligand G-1 and OHT, which has been largely reported to act as a GPER agonist [7]. Interestingly, MIBE showed the capability to displace [3H]E2 (Figure 3b) in accordance with the results obtained in docking simulations. E2, G-1 and OHT confirmed the ability to compete with [3H]E2 as previously shown [28]. Collectively, our findings demonstrate that MIBE is a ligand of both ERα and GPER.

MIBE inhibits both ER transactivation and gene expression induced by E2

On the basis of these results, we aimed to ascertain whether MIBE could function as an agonist or

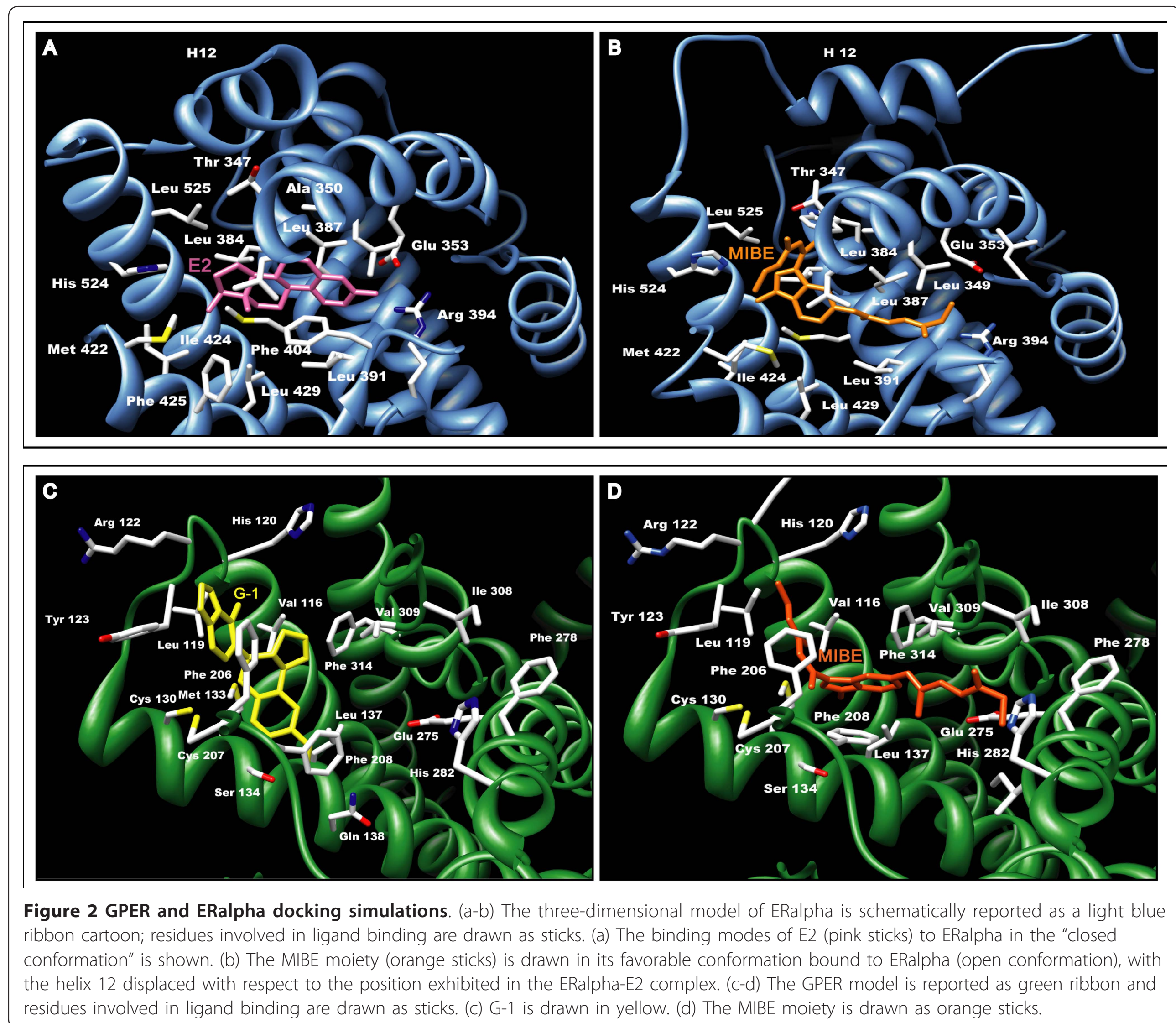

Figure 2 GPER and ERalpha docking simulations. (a-b) The three-dimensional model of ERalpha is schematically reported as a light blue ribbon cartoon; residues involved in ligand binding are drawn as sticks. (a) The binding modes of E2 (pink sticks) to ERalpha in the "closed conformation" is shown. (b) The MIBE moiety (orange sticks) is drawn in its favorable conformation bound to ERalpha (open conformation), with the helix 12 displaced with respect to the position exhibited in the ERalpha-E2 complex. (c-d) The GPER model is reported as green ribbon and residues involved in ligand binding are drawn as sticks. (c) G-1 is drawn in yellow. (d) The MIBE moiety is drawn as orange sticks.

antagonist for ERα and GPER. Initially, we evaluated the potential of MIBE in activating or inhibiting the ERα-mediated signaling. Hence, we transiently transfected an ER-reported gene in MCF7 breast cancer cells, which express ERα but not ERβ as judged by RT-PCR (data not shown). The reporter plasmid used carries firefly luciferase sequences under the control of an ERE upstream of the thymidine kinase promoter. As an internal transfection control, we co-transfected a plasmid expressing renilla luciferase which is enzymatically distinguishable from firefly luciferase by the strong cytomegalovirus enhancer/promoter. MIBE did not show any capability to transactivate ERα; however, it abrogated the luciferase activity induced by E2 like the ER antagonist OHT (Figure 4a, b). To confirm these data and to examine the response of ERβ, we transiently transfected the ER-negative Hek293 cells with chimeric proteins consisting of the DNA binding domain (DBD) of the yeast transcription factor Gal4 and the ligand binding domain (LBD) of ERα (GalERα) or ERβ (GalERβ), respectively. MIBE did not activate GalERα and GalERβ (Figure 4c, d), but prevented the transactivation of these chimeric proteins by E2 mimicking the inhibitory activity of OHT (Figure 4e, f). In order to evaluate whether MIBE acts through a further member of the steroid receptor superfamily as the AR, we transiently transfected the ER-negative Hek293 cells with an AR reporter gene along with the expression vector encoding AR. DHT transactivated the AR reporter gene, whereas MIBE neither activated AR nor prevented the DHT-induced activation of AR (Additional file 1). Together, these results provide

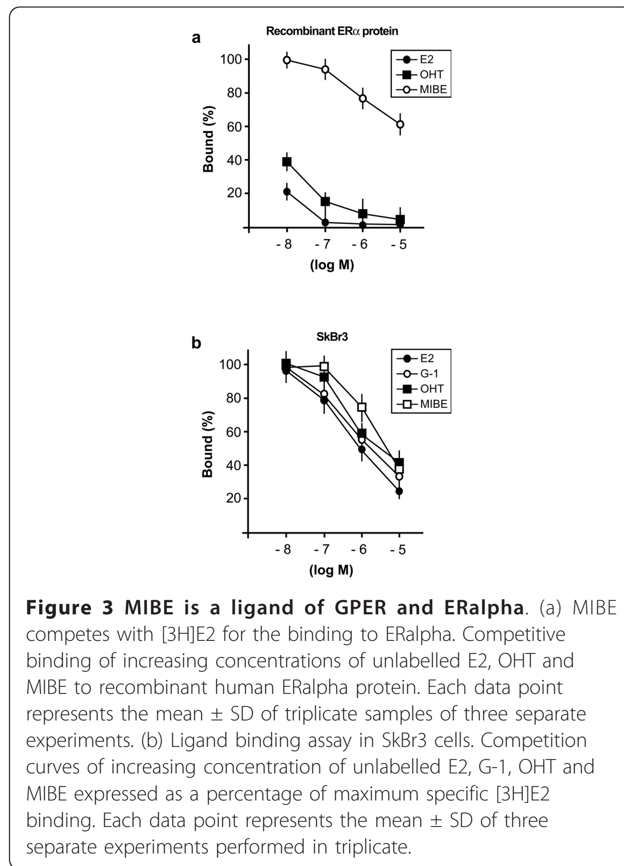

Figure 3 MIBE is a ligand of GPER and ERalpha. (a) MIBE competes with [3H]E2 for the binding to ERalpha. Competitive binding of increasing concentrations of unlabelled E2, OHT and MIBE to recombinant human ERalpha protein. Each data point represents the mean ± SD of triplicate samples of three separate experiments. (b) Ligand binding assay in SkBr3 cells. Competition curves of increasing concentration of unlabelled E2, G-1, OHT and MIBE expressed as a percentage of maximum specific [3H]E2 binding. Each data point represents the mean ± SD of three separate experiments performed in triplicate.

evidence regarding the specific action of MIBE on ER-mediated signaling.

In order to further demonstrate that MIBE acts as an ERα antagonist, we evaluated its ability to repress in MCF7 cells the mRNA expression of well known E2 target genes like pS2, Cyclin D1, PR and IRS-1. As determined by real-time PCR, the E2-dependent increase of all genes examined was prevented by MIBE as obtained using OHT (Figure 5a). Similarly, the protein expression of cyclin D1 and IRS-1 induced by E2 in MCF7 cells was inhibited by MIBE (and OHT) (Figure 5b, c).

MIBE prevents the proliferative effects triggered by E2

Considering that the regulation of estrogen target genes connects the signaling of E2 with the proliferation of breast cancer cells [40,41], we wanted to determine the biological significance of the antagonist action elicited by MIBE through ERα. MIBE as OHT did not stimulate growth effects used alone (Figure 5d); however, both compounds abolished the proliferation of MCF7 cells induced by E2 (Figure 5e). Hence, MIBE can be considered as an ER antagonist on the basis of its full inhibitory activity elicited on ER-mediated signaling.

MIBE prevents the GPER-mediated EGFR and ERK activation

Having established that MIBE is an inhibitor of ERα, we aimed to determine its functional activity on the GPER-mediated transduction pathway. Previous studies have indicated that GPER activation triggers the EGFR-dependent signaling in cancer cells, even involving a functional cross-talk between these receptors [8,9,23]. Then, we sought to evaluate the role played by GPER in EGFR phosphorylation upon exposure to its cognate ligand. Notably, in SkBr3 cells the EGFR activation induced by EGF was prevented by knocking down GPER expression (Figure 6a-d) as observed in the presence of MIBE (Figure 6e, f), which further demonstrated that it acts as an inhibitor of GPER-mediated function. Accordingly, the activation of EGFR triggered by G-1 was abolished in the presence of MIBE, hence confirming its inhibitory activity on GPER-mediated signaling (Additional file 2). Corroborating the aforementioned findings, MIBE showed the capability to inhibit the ERK activation upon EGF exposure (Figure 6g, h) as well as by the GPER activators E2, G-1 and OHT (Figure 6i-l). Overall, these results suggest that MIBE acting as an inhibitor of GPER blocks the EGFR activation and the ERK phosphorylation induced by EGF and the ligands of GPER, thus preventing the functional cross-talk between GPER and EGFR.

MIBE inhibits gene transcription and cell proliferation mediated by GPER

The characterization of the transcriptional response to GPER signaling has recently identified a set of target genes that mediate the stimulatory effects triggered by GPER activation in cancer cells [24]. Hence, we performed real-time PCR experiments to evaluate the potential of MIBE in regulating the expression of GPER-dependent genes. Of note, the up-regulation of c-fos, CTGF, Cyr61 and EGR1 induced by the GPER agonists E2, G-1 and OHT in SKBr3 cells was abolished in the presence of MIBE (Figure 7a). In accordance with these results, MIBE also prevented the increase of both c-fos and CTGF at the protein level (Figure 7b, c). Next, we wondered what might be the biological significance of the inhibitory action of MIBE through GPER signaling. As shown in panel d of Figure 7, the proliferative effects elicited by E2, G-1 and OHT in SKBr3 cells were inhibited by MIBE. Altogether, these findings demonstrate that MIBE acts as an antagonist of both ERα and GPER in breast cancer cells.

Discussion

In the present study, we identified the first ligand of ERα and GPER, referred to as MIBE, which acts as an

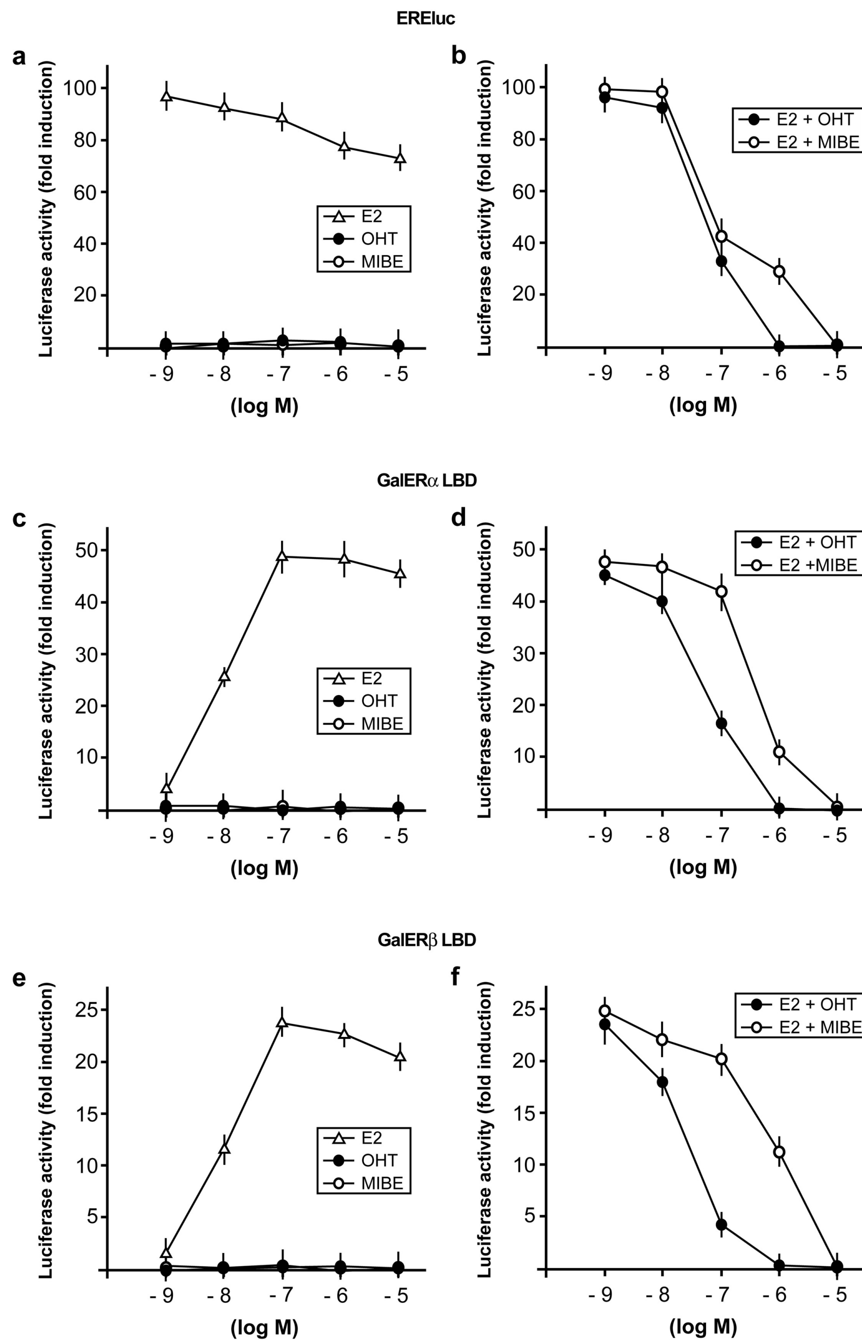

Figure 4 MIBE inhibits the transactivation of ERalpha induced by E2. (a) MCF7 cells were transfected with the ER luciferase reporter gene (EREluc) along with the internal transfection control Renilla Luciferase and treated with increasing concentrations (logarithmic scale) of E2, the ER antagonist OHT and MIBE. (b) MCF7 cells were transfected with the ER reporter gene and the internal transfection control Renilla Luciferase and treated with 10 nM E2 in combination with increasing concentration of OHT or MIBE, as indicated. (c, e) Hek293 cells were transfected with Gal4 reporter gene GK1, the Gal4 fusion proteins encoding the Ligand Binding Domain (LBD) of ERα (GalERalpha) or ERbeta (GalERbeta) and the internal transfection control Renilla Luciferase and treated with increasing concentrations (logarithmic scale) of E2, OHT and MIBE. (d, f) Hek293 cells were transfected with the Gal4 reporter gene GK1, the Gal4 fusion proteins GalERalpha or GalERbeta and the internal transfection control Renilla Luciferase and treated with 100 nM E2 in combination with increasing concentrations of OHT or MIBE, as indicated. Each data point represents the mean ± SD of three experiments performed in triplicate.

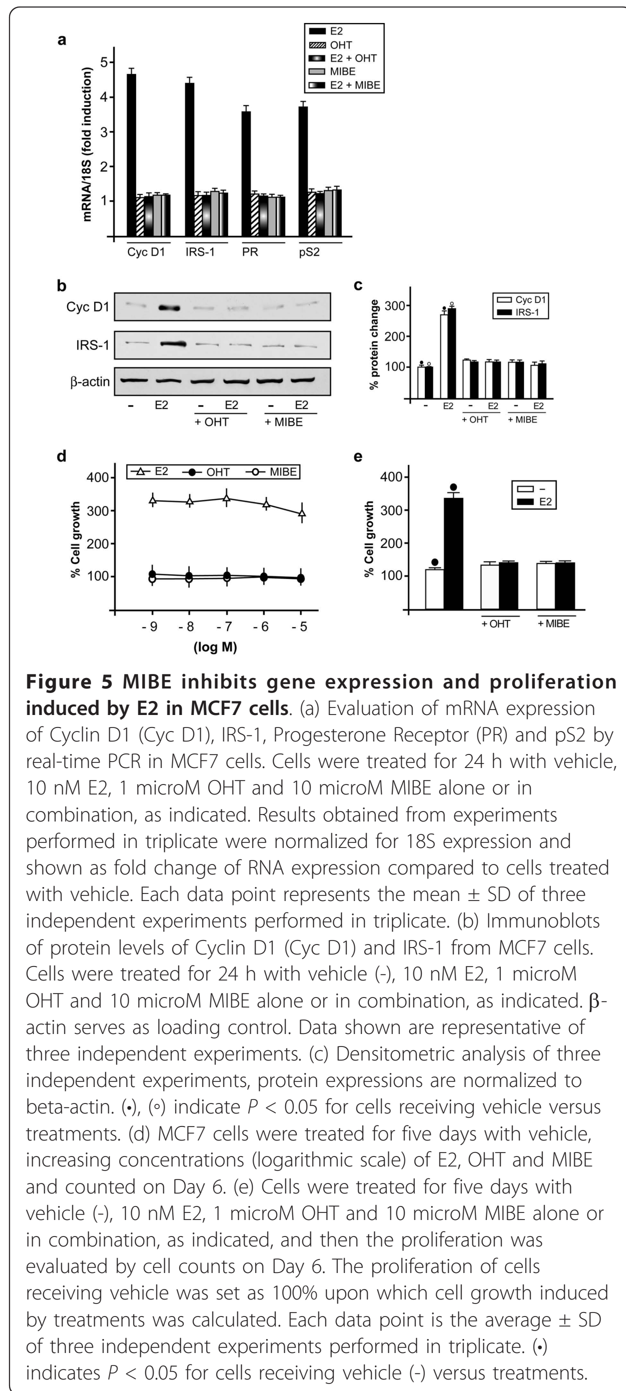

Figure 5 MIBE inhibits gene expression and proliferation induced by E2 in MCF7 cells. (a) Evaluation of mRNA expression of Cyclin D1 (Cyc D1), IRS-1, Progesterone Receptor (PR) and pS2 by real-time PCR in MCF7 cells. Cells were treated for 24 h with vehicle, 10 nM E2, 1 microM OHT and 10 microM MIBE alone or in combination, as indicated. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. Each data point represents the mean ± SD of three independent experiments performed in triplicate. (b) Immunoblots of protein levels of Cyclin D1 (Cyc D1) and IRS-1 from MCF7 cells. Cells were treated for 24 h with vehicle (-), 10 nM E2, 1 microM OHT and 10 microM MIBE alone or in combination, as indicated. β-actin serves as loading control. Data shown are representative of three independent experiments. (c) Densitometric analysis of three independent experiments, protein expressions are normalized to beta-actin. (•), (°) indicate $P < 0.05$ for cells receiving vehicle versus treatments. (d) MCF7 cells were treated for five days with vehicle, increasing concentrations (logarithmic scale) of E2, OHT and MIBE and counted on Day 6. (e) Cells were treated for five days with vehicle (-), 10 nM E2, 1 microM OHT and 10 microM MIBE alone or in combination, as indicated, and then the proliferation was evaluated by cell counts on Day 6. The proliferation of cells receiving vehicle was set as 100% upon which cell growth induced by treatments was calculated. Each data point is the average ± SD of three independent experiments performed in triplicate. (•) indicates $P < 0.05$ for cells receiving vehicle (-) versus treatments.

antagonist of both receptors in breast cancer cells. By molecular modeling and binding experiments we demonstrated that MIBE binds to both receptors, while through functional assays we showed that MIBE inhibits the ERα- and GPER-mediated signaling. In particular, using the ER-positive MCF7 and ER-negative SkBr3 breast cancer cells as a model system, we characterized the biological properties of MIBE. We found that in MCF7 cells MIBE blocks the ER

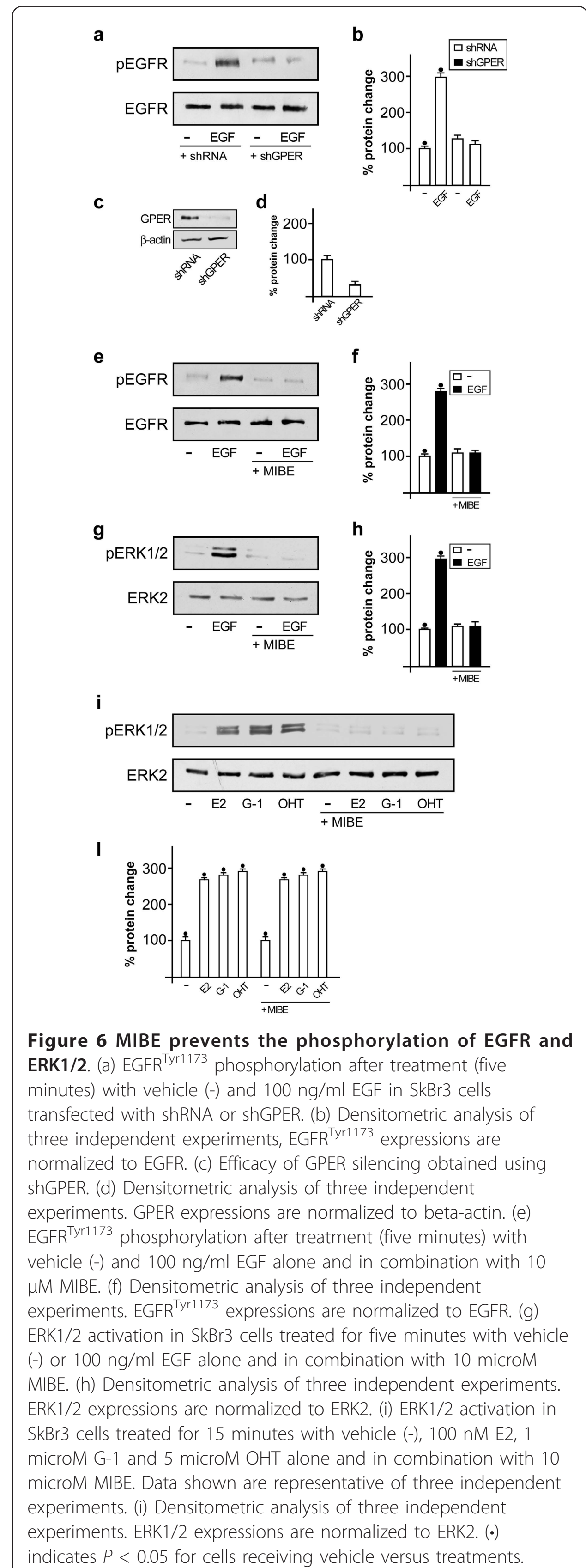

Figure 6 MIBE prevents the phosphorylation of EGFR and ERK1/2. (a) $EGFR^{Tyr1173}$ phosphorylation after treatment (five minutes) with vehicle (-) and 100 ng/ml EGF in SkBr3 cells transfected with shRNA or shGPER. (b) Densitometric analysis of three independent experiments, $EGFR^{Tyr1173}$ expressions are normalized to EGFR. (c) Efficacy of GPER silencing obtained using shGPER. (d) Densitometric analysis of three independent experiments. GPER expressions are normalized to beta-actin. (e) $EGFR^{Tyr1173}$ phosphorylation after treatment (five minutes) with vehicle (-) and 100 ng/ml EGF alone and in combination with 10 μM MIBE. (f) Densitometric analysis of three independent experiments. $EGFR^{Tyr1173}$ expressions are normalized to EGFR. (g) ERK1/2 activation in SkBr3 cells treated for five minutes with vehicle (-) or 100 ng/ml EGF alone and in combination with 10 microM MIBE. (h) Densitometric analysis of three independent experiments. ERK1/2 expressions are normalized to ERK2. (i) ERK1/2 activation in SkBr3 cells treated for 15 minutes with vehicle (-), 100 nM E2, 1 microM G-1 and 5 microM OHT alone and in combination with 10 microM MIBE. Data shown are representative of three independent experiments. (i) Densitometric analysis of three independent experiments. ERK1/2 expressions are normalized to ERK2. (•) indicates $P < 0.05$ for cells receiving vehicle versus treatments.

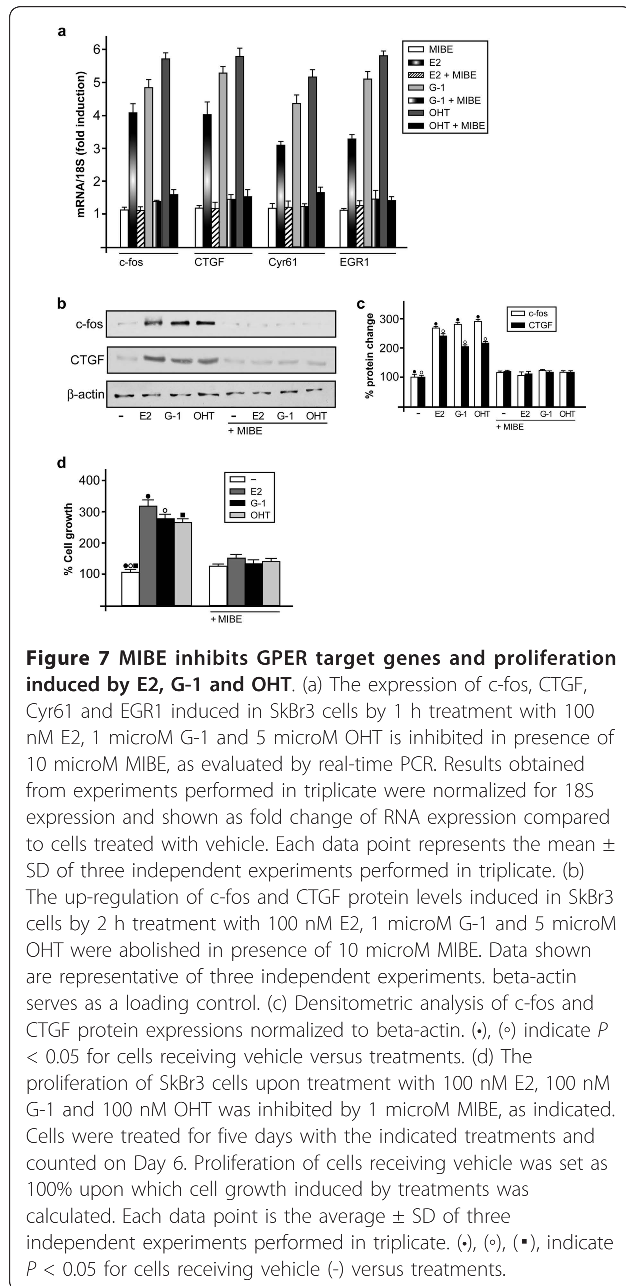

Figure 7 MIBE inhibits GPER target genes and proliferation induced by E2, G-1 and OHT. (a) The expression of c-fos, CTGF, Cyr61 and EGR1 induced in SkBr3 cells by 1 h treatment with 100 nM E2, 1 microM G-1 and 5 microM OHT is inhibited in presence of 10 microM MIBE, as evaluated by real-time PCR. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle. Each data point represents the mean ± SD of three independent experiments performed in triplicate. (b) The up-regulation of c-fos and CTGF protein levels induced in SkBr3 cells by 2 h treatment with 100 nM E2, 1 microM G-1 and 5 microM OHT were abolished in presence of 10 microM MIBE. Data shown are representative of three independent experiments. beta-actin serves as a loading control. (c) Densitometric analysis of c-fos and CTGF protein expressions normalized to beta-actin. (•), (∘) indicate $P < 0.05$ for cells receiving vehicle versus treatments. (d) The proliferation of SkBr3 cells upon treatment with 100 nM E2, 100 nM G-1 and 100 nM OHT was inhibited by 1 microM MIBE, as indicated. Cells were treated for five days with the indicated treatments and counted on Day 6. Proliferation of cells receiving vehicle was set as 100% upon which cell growth induced by treatments was calculated. Each data point is the average ± SD of three independent experiments performed in triplicate. (•), (∘), (▪), indicate $P < 0.05$ for cells receiving vehicle (-) versus treatments.

transactivation induced by E2 as well as the ER-mediated gene regulation and cell proliferation. In addition, in SkBr3 cells MIBE prevented the GPER-dependent responses, such as rapid ERK phosphorylation, gene transcription and growth effects induced by the GPER agonists E2, OHT and G-1. The exclusive antagonistic action exerted by MIBE on both ERα and GPER could represent a novel promising tool for a more comprehensive pharmacological approach in estrogen-dependent tumors like breast cancer, which express one or both receptors from the onset or following tumor progression.

Breast cancer is the most commonly diagnosed invasive malignancy and the second leading cause of cancer death in women [42]. Endocrine treatment along with surgery, chemotherapy, radiotherapy and targeted therapy are fundamental modalities for the therapeutic management of breast cancer. The expression of ERα in breast carcinomas correlates with the beneficial response to anti-estrogens [43], whereas the lacking of ERα is coupled to a worse prognosis and to short disease-free survival rates [44]. On the basis of the main role exerted by ERα in the development and progression of breast cancer and considering that this receptor is expressed in approximately 70% of breast tumors, the ER antagonist tamoxifen has been widely used, although its effectiveness is limited by *de novo* and acquired resistance [45]. In accordance with these data, comparative clinical studies have indicated that aromatase inhibitors blocking estrogen biosynthesis may provide major benefits in respect to ERα antagonists in breast cancer patients [46]. Among the various mechanisms involved in the resistance to endocrine treatment, the activation of transduction pathways different from those mediated by ERα has been proposed. For instance, an increased expression and/or activation of growth factor receptors, such as EGFR/HER2, have been associated with the failure of endocrine therapy in breast tumors [47]. Moreover, the existence of alternative ERs able to mediate estrogen signaling without exhibiting any sensitivity to the repressive action of the ER antagonists could be also involved in the resistance to endocrine agents. In this scenario, it has been recently demonstrated that GPER acts as an additional receptor mediating the effects of estrogens in a wide number of cell types, such as breast, endometrial and ovarian cancer cells [7]. Of note, diverse studies have shown that E2 as well as the anti-estrogens tamoxifen and ICI bind to and activate GPER signaling, including ERK phosphorylation and gene transcription, which in turn lead to cancer cell proliferation and migration [7].

The activation of the GPER transduction pathway requires the EGFR transactivation [8], in accordance with evidence showing that the agonist stimulation of diverse G-protein coupled receptors (GPCRs) triggers the transactivation of EGFR through the release of EGF-like ligands tethered at the cell surface and the subsequent generation of intracellular signaling [48]. In addition, the functional crosstalk which occurs between members of GPCR and growth factor receptor families contributes to the progression of different tumors [8,48]. In this regard, we have previously reported that GPER and EGFR physically and functionally interact in both ER-negative and ER-positive cancer cells [22,23]. Recently, it has also been found that a crosstalk among EGFR, the nerve growth factor (NGF) receptor TrkA

and the GPCR Formyl Peptide Receptor (FPR) occurs in monocytes [49]. In particular, the inhibition of EGFR prevented the ligand-dependent responses mediated by the other two receptors, while the inhibition of FPR abolished the EGFR and TrkA phosphorylation induced by EGF and NGF, respectively. Accordingly, the silencing of each receptor suppressed the capability of the other receptors to mediate the ligand-induced actions like ERK phosphorylation [49]. In line with these findings, our current results provide novel insight into the functional crosstalk between GPER and EGFR in cancer cells. Notably, we show for the first time that the activation of EGFR induced by its cognate ligand EGF is abolished by knocking down GPER expression or in the presence of MIBE, which is an inhibitor of GPER as ascertained in the present study. Nevertheless, further studies are needed to better understand the role played by GPER in the activation of EGFR by its cognate ligand EGF and to appreciate the potential of MIBE in preventing the crosstalk between GPER and EGFR which was previously well described [23].

On the basis of these remarks, it remains to be evaluated that the potential of MIBE to interfere with the functional crosstalk between EGFR and ERα, toward a better characterization of its inhibitory activity elicited in cell contexts expressing both receptors. In particular, considering that a physical and functional interaction between EGFR and ER leads to the activation of multiple intracellular cascades, including MAPK, phosphoinositide 3-kinase (PI3K) and other protein kinases [50-53], it would be interesting to ascertain whether MIBE could alter these transduction signals that have been involved in the proliferation of cancer cells [50,54-58].

In 2005, two reports provided evidence on the capability of estrogens and anti-estrogens to bind to GPER [10,19]. In particular, the ER antagonists tamoxifen and ICI displayed a high binding affinity for GPER, as assessed in competition assays. Surprisingly, unlike the antagonistic properties exhibited by these agents on the classical ER-mediated pathways, both tamoxifen and ICI act as GPER agonists [8,9,19]. In the following years, further ER ligands and activators showed the ability to bind to GPER eliciting promiscuous actions through the two receptors. For instance, the phytoestrogen genistein and the xenoestrogen bisphenol A, which exert estrogen-like activities binding to and activating ERα [9,59], displayed the ability to bind to and activate GPER signaling [9,27,60]. As it concerns the pesticide atrazine, it exerted estrogenic effects without binding to ERs [61] and exhibiting the capability to activate the GPER-mediated pathway despite a low binding affinity for this receptor [25,27]. Unlike E2 which exhibited ERα and GPER agonism in several investigations [7], the well known ERα ligand and activator estriol showed antagonistic properties for GPER-mediated signaling [28]. Besides, G-1 [29] and G-15, along with its derivatives [30,31] as ligands activated or inhibited, respectively, the GPER-mediated signaling, while some GPER antagonists triggered at high concentrations ER-dependent transcriptional responses [30].

GPER expression was indicated as a potential predictor of biological aggressive features in breast carcinomas [16]. Although a significant association between ERα and GPER was observed, approximately 50% of ERα-negative breast tumors retained GPER suggesting that the expression of these receptors may not be interdependent [16]. On the basis of these and the aforementioned findings, tumor cells that express GPER but lack ERα may be stimulated by estrogens and even by antiestrogens, such as tamoxifen. In this regard, it should be noted that the stimulatory effects on cancer progression elicited by estrogens via both ERα and GPER and by ERα antagonists through GPER address the need to discover novel drugs targeting simultaneously both receptors, in order to obtain major therapeutic benefits in respect to the use of the current selective antagonists.

Conclusions

The exclusive antagonistic activity exerted by MIBE on ERα- and GPER-mediated signaling as shown in the present study (Figure 8), could represent a promising pharmacological approach either at the beginning or during the progression of breast tumors which express one or both receptors. In this respect, further studies are needed to examine whether MIBE could be considered a useful tool towards a more comprehensive treatment in breast cancer.

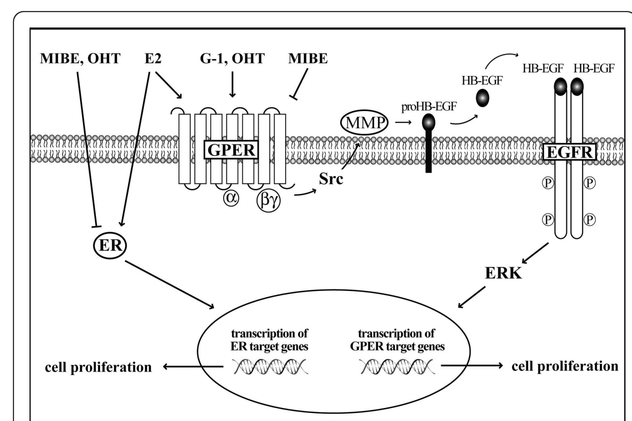

Figure 8 Schematic representation of the inhibitory activity exerted by MIBE on GPER- and ER-mediated signaling.

Additional material

Additional file 1: MIBE does not activate AR. Hek293 cells were transfected with AR luciferase reporter gene (ARE-luc) and AR expression plasmid along with the internal transfection control Renilla Luciferase, and treated with 10 nM DHT alone and in combination with 10 μM MIBE, as indicated. The normalized luciferase activities of cells treated with vehicle (-) were set as one-fold induction, upon which the activities induced by treatments were calculated. Each data point represents the mean ± SD of three experiments performed in triplicate.

Additional file 2: MIBE prevents the phosphorylation of EGFR induced by G-1. **(a)** $EGFR^{Tyr1173}$ phosphorylation after treatment (30 minutes) with vehicle (-) and 1 μM G-1 alone and in combination with 10 μM MIBE. **(b)** Densitometric analysis of three independent experiments, $EGFR^{Tyr1173}$ expressions are normalized to EGFR.

Abbreviations

AR: androgen receptor; CAFs: cancer associated fibroblasts; CS: charcoal-stripped; DBD: DNA binding domain; DHT: 5α-dihydrotestosterone; DMSO: dimethyl sulfoxide; E2: 17β-estradiol; EGFR: epidermal growth factor receptor; ER: estrogen receptor; ERK: extracellular signal-regulated kinase; FPR: formyl peptide receptor; G-1: 1-[4-(6-bromobenzo[1:3]dioxol-5-yl)-3a:4:5:9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone; G-15: 4-(6-Bromobenzo [1:3]dioxol-5-yl)-3a:4:5:9b-tetrahydro-3H-cyclopenta[c]quinoline; GPCRs: G-protein coupled receptors; GPER: G protein-coupled estrogen receptor; HB-EGF: heparan-bound epidermal growth factor; LBD: ligand binding domain; MAPK: mitogen-activated protein kinase; MIBE: ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate; NGF: nerve growth factor; OHT: 4-hydroxytamoxifen; PI3K: phophatidylinositol 3-kninase; PLC: phospholipase C; SDS: sodium dodecyl sulfate; TLC: thin layer chromatography.

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Authors' contributions

RL designed and performed the experiments, and wrote the paper. MFS and MP performed the experiments. MSS and AC synthesized MIBE. CR performed docking simulations. MM analyzed data and wrote the paper. All authors read and approved the final manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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