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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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Abstract

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. In this study we evaluated the transduction pathways through which BPA influences cell proliferation and migration in human breast cancer cells and cancer-associated fibroblasts (CAFs), that lack the classical ERs. Specific pharmacological inhibitors and gene-silencing procedures showed that BPA induces the expression of the GPER target genes c-FOS, EGR-1, and CTGF through the GPER/EGFR/ERK transduction signaling 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. Our 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.

Chapter 1

Introduction

1.1 Introduction

Breast cancer is the most frequent malignancy and the leading cause of cancer death among females (Jemal A. *et al.* 2011). The elevated incidence of breast cancer in women has been associated with prolonged exposure to high levels of estrogens (Kovalchuk O. *et al.* 2007) and environmental contaminants (Brody J.G. *and* Rudel R.A. 2003). Since 1950's a plethora of synthetic chemicals, including pesticides and industrial chemicals, have been introduced into the environment under the premise that they would improve standards of living without any negative consequences. However, starting from 1990's it has been demonstrated that these environmental chemicals had hormone-like effects and the term endocrine disruption was coined upon to describe a class of chemicals including those that act as agonists and antagonists of the estrogen receptors (ERs),androgen receptor, thyroid hormone receptor, and others (Soto A.M. *and* Sonnenschein C. 2010). Moreover, it has been suggested that some endocrine disrupters may contribute to the development of hormone-dependent cancers, such as breast and endometrial cancers (Jobling S. *et al.*

1995; Sonnenschein C. *and* Soto A.M 1998). The xenoestrogen Bisphenol A is one of the endocrine disruptors that has been most thoroughly studied. BPA has been widely used since the 1950s as a monomer that is polymerized to manufacture polycarbonate plastic and epoxy resins (Fernandez S.V. *and* Russo J. 2010). Moreover, BPA also is used as an additive in many other products, with global capacity at more than six billion pounds per year (Vandenberg L.N. *et al.* 2009). Human exposure occurs when BPA is released from common items such as plastic-lined food and beverage cans, as well as and from some dental sealants (Brotons J.A. *et al.* 1995, Olea N. *et al.* 1996) and exposure to BPA has been correlated with the incidence of diverse types of tumors (Ho S.M. *et al.* 2006, Keri R.A. *et al.* 2007, Maffini M.V. *et al.* 2006). BPA has estrogenic activity both in vivo and in vitro and is thought to be an environmental estrogen (Welshons W.V. *et al.* 2006). Previous investigations (reviewed by Vandenberg L.N. 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 J.C. *et al.* 1998, Kuiper G.G. *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 L. *et al.* 2007, Maggiolini M. *et al.* 2004, Prossnitz E.R. *and* Maggiolini M. 2009, Vivacqua A. *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 D.P. *et al.* 2009). BPA is one of several environmental estrogens that have exhibited the ability to bind to GPER (Thomas P. *and* Dong J. 2006) and to activate transduction pathways (Dong S. *et al.* 2011) involved in the biological responses of both normal and neoplastic cells.

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 N.A. *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 N.A. *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).

1.2 Breast cancer

The breast lies between the second and sixth ribs, from the sternal edge to the edge of the axilla, and against the pectoralis muscle on the chest wall. Breast tissue also projects into the axilla as the tail of Spence (Hassiotou F. *and* Geddes D. 2012). The breast is composed of 15-20 lobes that radiate from the nipple. Each lobe arises from multiple lobules, which connect to a common terminal interlobular duct. These ducts then continue to their outlet at the nipple (Bertos N.R. *and* Park M. 2011) (Figure 1.1).

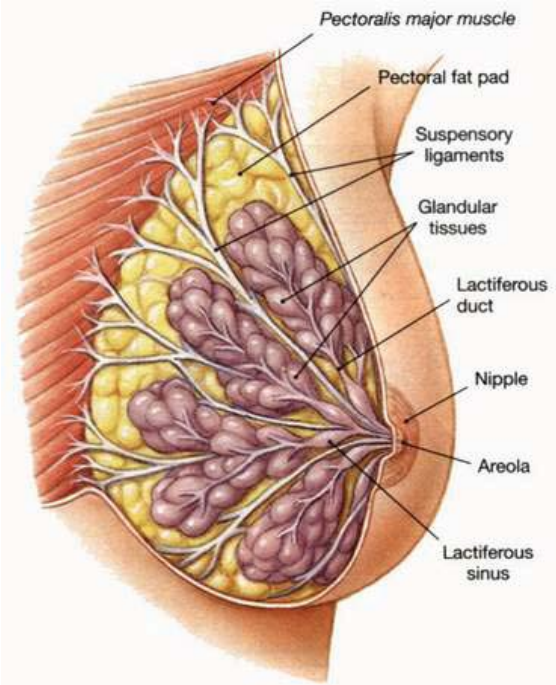

Figure 1.1 Representation of the anatomy of the breast

Histologically, lobules and ducts are lined by a single layer of luminal epithelial cells, surrounded by transversely oriented myoepithelial cells. These structures are separated from the surrounding tissue, or stroma, by a basement membrane, the breach of which distinguishes invasive carcinoma from carcinoma in situ (Pinder S.E. *and* Ellis I.O. 2003). The surrounding stroma comprises ECM, discrete cells (e.g., fibroblasts, immune cells, and adipocytes), and organized structures (e.g., blood vessels), each of which contributes to the overall configuration of the local microenvironment (Bertos N.R. *and* Park M. 2011) (Figure 1.2).

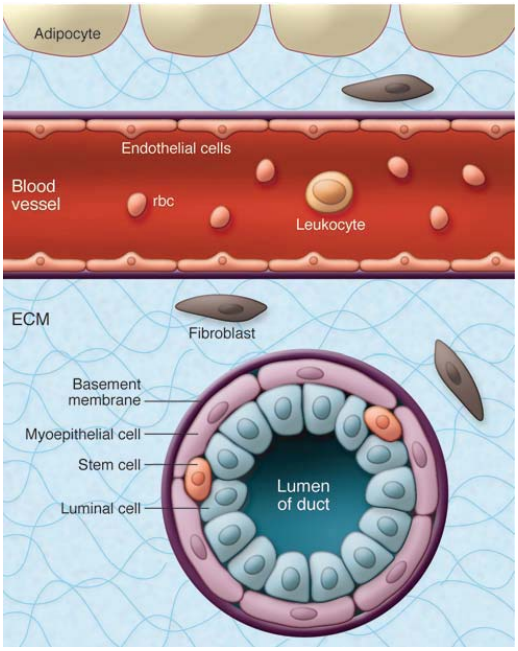

Figure 1.2 Representation of the structure of human breast at histological level

Breast cancer affects millions of women per year and has become the second leading cause of death among women. The most common type of breast cancer is a carcinoma that originates in epithelial cells. About 85% of breast carcinomas originate within the cells of the ducts (ductal carcinoma); the remaining 15% begin in the cells that line the lobules (lobular carcinoma). Non-carcinomatous breast cancers are rare and originate in the connective tissues of the breast (Li C.I. *et al.* 2005, Weigelt B. *et al.* 2010). Almost 1.4 million women were diagnosed with breast cancer worldwide in 2008 and approximately 459,000 deaths were recorded. Incidence rates were much higher in more developed countries compared to less developed countries (71.7/100,000 and 29.3/100,000 respectively), whereas the corresponding mortality rates were 17.1/100,000 and 11.8/100,000 (Youlden D.R. *et al.* 2012) (Figure 1.3).

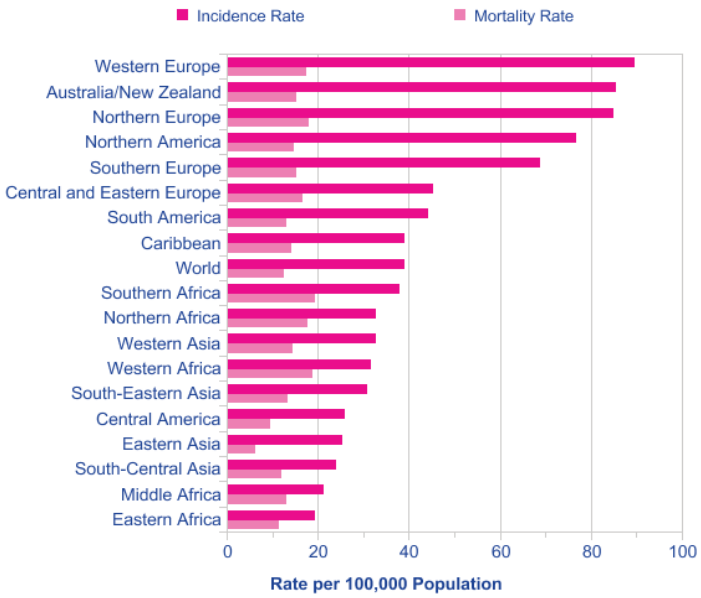

Figure 1.3 Incidence and mortality of breast cancer worldwide

The elevated incidence of breast cancer in women has been associated with prolonged exposure to high levels of estrogens. The relationship between estrogen and breast cancer is supported by epidemiological data that demonstrated that women who receive hormone replacement therapy (HRT) are more likely to develop breast cancer than those who have never used HRT (Bakken K. *et al.* 2004, Beral V. 2003, Chlebowski R.T. *et al.* 2003, Colditz G.A. 2005, Li C.I. *et al.* 2003, Rossouw J.E. *et al.* 2002). Other factors involved in the development of breast cancer incidence include the socioeconomic status, some food additives, some antibiotics, radiation, mutations at genes BRCA1, BRCA2, metabolizing enzyme polymorphisms, epidermal growth factor and its receptor (HER), androgen levels, and insulin-like growth factor (Bernstein J.L. *et al.* 2004, Zhang Y. *et al.* 2004). Age, alcohol consumption, diet and smocking represent further risk factors for breast cancer

development (Brody J.G. *et al.* 2007, Coutelle C. *et al.* 2004, Egan K.M. *et al.* 2002). Along with other etiological factors, there is growing scientific evidence that exposure to chemicals, is associated with increased incidence of breast cancer among women (Fucic A. *et al.* 2012).

Although it has been predicted that the international incidence of female breast cancer will reach approximately 3.2 million new cases per year by 2050 (Hortobagyi G.N. *et al.* 2005), over recent decades an improvement in breast cancer survival has been observed in more developed parts of the world and has been attributed to the introduction of population-based screening using mammography and the systemic use of adjuvant therapies (Youlden D.R. *et al.* 2012). On a more positive note, the gains achieved in the treatment of breast cancer over the previous 20-30 years appear set to continue, led by the prospect of better tailoring therapies to individual patients through molecular profiling (Harbeck N. *et al.* 2010, Viale G. 2009)

1.3 Tumor microenvironment

Despite efforts to uncover new targeted therapies, a vast number of women die due to refractory or recurrent breast tumors. Most breast cancer studies have focused on the intrinsic characteristics of breast tumor cells, including altered growth, proliferation, and metabolism. However, emerging research suggests that the tumor microenvironment can substantially affect relapse rates and therapeutic responses. The “seed and soil” hypothesis postulates that an appropriate host microenvironment (the soil) is needed for the optimal growth of tumor cells (the seed) (Paget S. 1989). Indeed, evidences indicate that tumors are

composed of parenchyma and stroma, two discrete but interactive parts that cross-talk to promote tumor growth. Recently, many investigations support the notion that tumor stromal cells play important roles in tumor initiation, progression, and metastasis (Y. Mao *et al.* 2012). Tumour microenvironmental elements include structural components such as the extracellular matrix or hypoxia as well as stromal cells, either resident cells or recruited from circulating precursors, as macrophages and other inflammatory cells, endothelial cells, adipose tissue and cancer-associated fibroblasts (CAFs). All these elements synergistically play a specific role in cancer progression (Cirri P. *and* Chiarurgi P. 2012) (Figure 1.4).

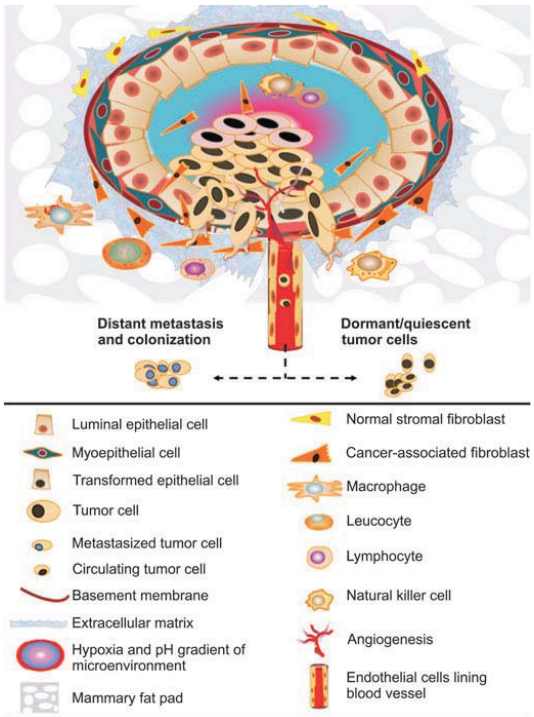

Figure 1.4 The stromal context of tumors. Invading tumour cells secrete growth factors that stimulate angiogenesis and inflammation through the recruitment and activation of several other cells, including endothelial precursor cells or tumour-associated neuthrophils or macrophages. These activated stromal cells, together with tumour cells themselves, secrete several ECM-degrading enzymes, whose collective activity promotes tumour cell invasion

Tumor associated macrophages (TAMs) secretes growth factors that promote angiogenesis (Dirkx A.E. et al. 2006, Roland C.L. *et al.* 2009), growth (Lewis C.E. and Pollard J.W. 2006), invasion, migration (Lin EY, *et al.* 2001)., metastatic spread (Oosterling, S.J. *et al.* 2005), and immunosuppression. In breast cancer, infiltrating TAMs correlate with poor prognostic features (DeNardo D.G. *et al.* 2011), higher tumor grade (Lee A.H. *et al.* 1997), high vascular grade, increased necrosis (Leek R.D. *et al.* 1996), and decreased disease-free survival and overall survival (Campbell M.J. *et al.* 2011). Due to the fact that macrophages are derived from the same cell lineage as osteoclasts, the major target of bisphosphonates (BPs), which also increase apoptosis and decrease proliferation, migration, and invasion in breast cancer cell lines and mice models. Therefore, targeting TAMs by BPs is a potential choice, and it also has been used to good effect in vitro and in mouse models (Coscia M. *et al.* 2010, Zeisberger S.M. *et al.* 2006). Notably, not only macrophages but also other kinds of infiltrating leukocytes promote breast cancer progression. One study showed that more infiltrating leukocytes were found in DCIS with focal myoepithelial cell layer disruptions (Man Y.G. *and* Sang Q.X. 2004), which indicated that leukocytes may promote breast cancer invasive progress. In a spontaneous mouse model of breast cancer, CD4+ Treg lymphocytes were found increasingly infiltrated in tumor, and depletion of these T cells by IL-2 immunotoxin fusion protein can inhibit tumor growth (Knutson K.L. *et al.* 2006). Another study showed that the metastatic spread of ErbB-2-transformed carcinoma cells required CD4+CD25+ T cells which secrete RANKL and implicate into the metastatic process (Tan W. *et al.* 2011).. Moreover, the cells which secrete RANKL also have a high expression of forkhead box P3 (FOXP3), a transcription factor produced by regulatory T cells, so the CD4+CD25+FOXP3+ Treg cells can stimulate the metastatic progression by

RANKL in the RANK-expressing breast/mammary carcinoma cells. This indicates that anti-RANKL-RANK maybe an effective strategy to prevent breast cancer metastasis. Interestingly, recent findings also suggest that infiltrating number of CD8+ T lymphocytes positively correlate with patient survival (Mahmoud S.M., *et al.* 2011) and that high CD8 and low FOXP3 cells infiltrating after neoadjuvant chemotherapy were significantly related to improved recurrence-free survival and overall survival (OS). Based on these findings, targeting immune cells may be an emerging strategy for cancer treatment. Endothelial cells also play important roles in cancer growth and invasion. Human umbilical endothelial cells (HUVECs) induced the higher proliferation of preneoplastic MCF10AT1-EIII8 (referred as EIII8) in EIII8–fibroblasts– HUVEC tricultures than EIII8–fibroblast co-cultures (Shekhar M.P. *et al.* 2001). This finding suggests that endothelial cells can help breast cancer initiation. Moreover, TNF-α production by endothelial and other stromal cells induced by chemotherapeutic agents increases the CXCL1/2 expression in cancer cells via NF-κB and then CXCL1/2 attract CD11b+ Gr1+ myeloid cells into the tumor, which produce chemokines including S100A8/9 that enhance cancer cell survival, thus amplifying the CXCL1/2- S100A8/9 loop and causing chemoresistance. This network of endothelial–carcinoma–myeloid signaling interactions provides a mechanism linking chemoresistance and metastasis, with opportunities for intervention by a CXCR2 blocker (Acharyya S. *et al.* 2011).. Adipose tissue, consisting of mainly mature adipocytes and progenitors (preadipocytes and adipose-derived stem cells, ADSCs), is the most abundant component surrounding breast cancer cells. There is cumulative evidence supporting that cancer-associated adipose (CAA) tissue is a key component of breast cancer progression and carcinogenesis. It has been shown that collagen VI (COLVI) is abundantly expressed

in CAAs and involved in mammary tumor progression in vivo (Iyengar P. *et al.* 2005, Iyengar, P. *et al.* 2003). Moreover, IL-6 plays a role in CAA–cancer cell interaction and promotes an aggressive phenotype in prostate cancer (Finley D.S. *et al.* 2009). There is also evidence that ADSCs promote the growth and survival of breast cancer cells as well as their migratory and invasive capacities in vitro and in vivo by secreting cytokines (IL-6, IL8, CCL-5, and CXCL12/SDF-1), the expansion of cancer stem cells, and inducing EMT in the cancer cells in a PDGF-dependent manner (Devarajan E. *et al.* 2011, Walter M. *et al.* 2009, Welte G. *et al.* 2012) (Figure 1.5).

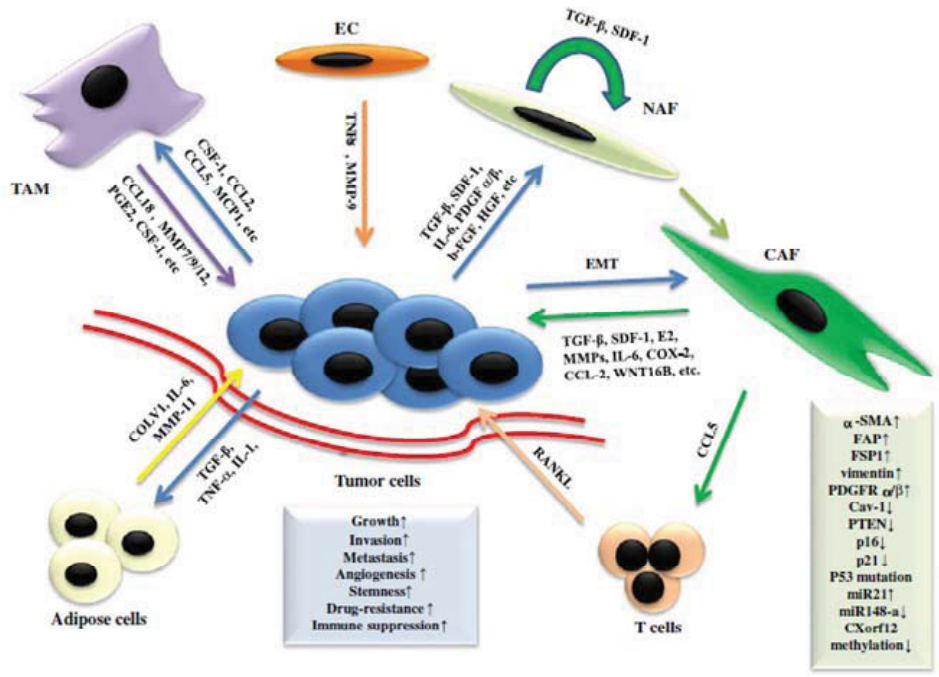

Figure 1.5 Schematic representation showing the role of stromal cells in microenvironment and breast cancer progression. Stromal cells promote tumor growth, invasion, and metastasis through secreting multiple cytokines, chemokines, and other growth factors. Moreover, tumor cells also affect the phenotype of stromal cells. Therefore, tumor and stromal cell interactions are truly reciprocal; while stromal cells may support tumors, tumor cells in turn modulate the microenvironments within which they inside.

Cancer-associated fibroblasts (CAFs) are the most frequent component of tumor stroma, especially in breast and pancreatic cancers (Kalluri R. *and* Zeisberg M. 2006, Ostman A. *and* Augsten M. 2009).

1.3.1 Cancer-associated fibroblasts (CAFs)

Fibroblasts are non-vascular, non-epithelial and non-inflammatory cells that form the basic cellular component of connective tissue and contribute to its structural integrity (Tarin D., 1969). They play important roles in wound healing, regulation of epithelial differentiation and inflammation (Tomasek J.J. *et al.* 2002). In healthy organs, fibroblasts have a low proliferation index and minimum metabolic capacity. By contrast, during wound healing fibroblasts were found to be activated with increasing expression of alpha smooth muscle actin (α-SMA) and the ED-A splice of fibronectin (Gabbiani G. *et al.* 1971). Currently, in agreement with the concept that tumors are similar to a chronic non-healing wound (Dvorak H.F. 1986), fibroblasts have been found to be activated in cancer. These activated fibroblasts, termed cancer-associated fibroblasts (CAFs), share many similarities with activated fibroblasts found in wounds and inflammatory sites (Polyak K. *and* Kalluri R., 2010). These cells present plump spindle-shaped mesenchymal structure with indented nuclei, peripheral myofilaments and fibronexus junctions (De Wever O. *et al.* 2008). CAFs are also positive for vimentin and desmin, but do not express cytokeratin, CD31 and smooth muscle myosin. These markers are often used to distinguish between myofibroblasts and normal fibroblast, epithelial, endothelial or smooth muscle cells

(Beacham D.A. *and* Cukierman E. 2005). In breast tumors 80% of fibroblasts are in active form (Aboussekhra A. 2011). 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. However, some evidence suggests that the origins of CAFs (Figure 1.6) may be:

- activated resident fibroblasts. There is evidence suggesting that the activation of resident fibroblasts is induced by many cancer-secreted factors, such as TGF-β and CXCL12/ SDF-1 (Kojima Y. *et al.* 2010), or by losing suppressor genes, such as PTEN, CAV- 1, p53, and p21 (Kiaris H. *et al.* 2005, Moskovits N. *et al.* 2006, Trimboli A.J. *et al.* 2009, Trimis G. *et al.* 2008, Trimmer C. *et al.* 2011);

- bone-marrow derived mesenchymal stem cells (MSCs). It has been demonstrated that in vivo, labeled MSCs have been found localized within tumor mass and differentiated into CAFs and pericytes with high expressions of α-SMA, FAP, tenascin-C, etc. (Spaeth E.L. *et al.* 2009);

- malignant tumor cells that undergo EMT changes (Kalluri R. *and* Zeisberg M 2006, Spaeth E.L. et al. 2009). Malignant epithelial cancer cells can obtain high invasive and metastatic characteristics by exposure to many factors (i.e., PDGF, TGF-β, EGF, etc.).

- endothelial cells by the endothelial-to mesenchymal transition with CD31 loss and higher expression of α-SMA and fibroblast-specific protein (FSP)-1 (Zeisberg E.M. *et al.* 2007).

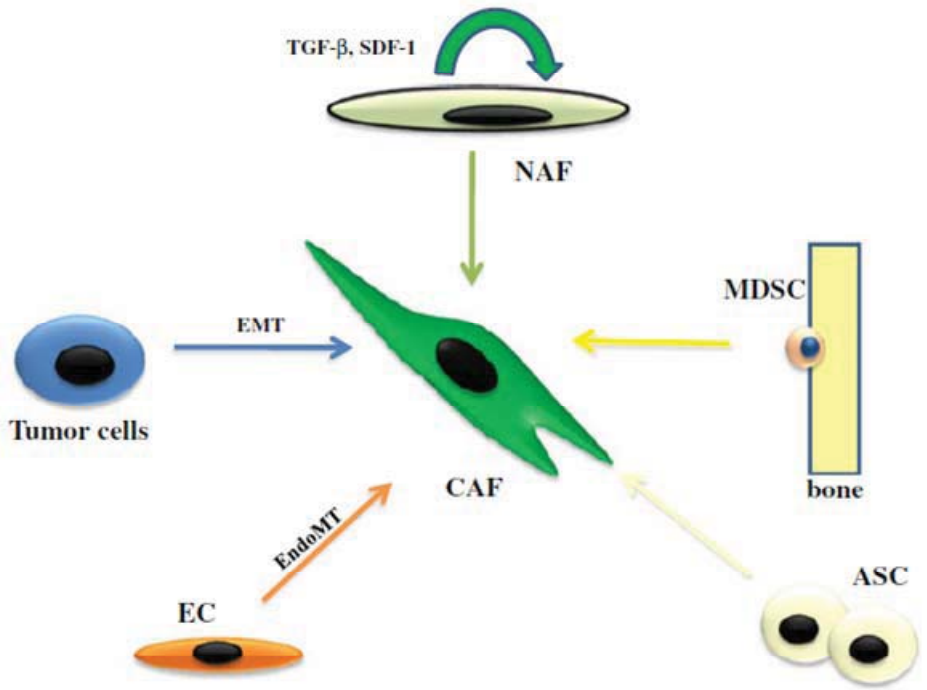

Figure 1.6 Origin of CAFs. Schematic of cells that may transit to (arrows) CAFs

CAFs promote breast tumor onset and progression in different ways (Hasebe T. *et al.* 2001, Orimo A. *et al.* 2005, Saito R.A. *et al.* 2010, Zhang C. *et al.* 2009) such as affecting estradiol (E2) levels; secreting many kinds of factors (HGF,TGF-β,SDF-1,VEGF, IL-6, etc.) and matrix metalloproteinases (MMPs); and inducing stemness, epigenetic changes and EMT. CAFs not only induce mammary carcinogenesis but also promote invasion and metastasis in breast cancer (Hasebe T. *et al.* 2001, Hu M. *et al.* 2009, Orimo A. *et al.* 2005, Shekhar M.P. *et al.* 2001, Stuelten C.H. *et al*. 2010). The transition from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is a good example to understand the process of tumor invasion. It was found that CAFs induced the invasive ability of DCIS

epithelial cells both in vitro and in vivo (Hu M. *et al.* 2008, Hu M. *et al.* 2009). CAFs achieved this induction of invasion through increasing MMP14 expression and MMP9 activity. Cancer metastasis is a complicated process that requires multiple events including EMT of the epithelial cancer cells, induction of angiogenesis, intravasation and extravasation of cancer cells, EMT cells regaining epithelial traits (mesenchymal-to-epithelial transition, MET), and finally forming a new colony in the appropriate distant microenvironment. In this process, CAFs work together with other stromal cells to complete the organ-specific metastasis (Figure 1.7).

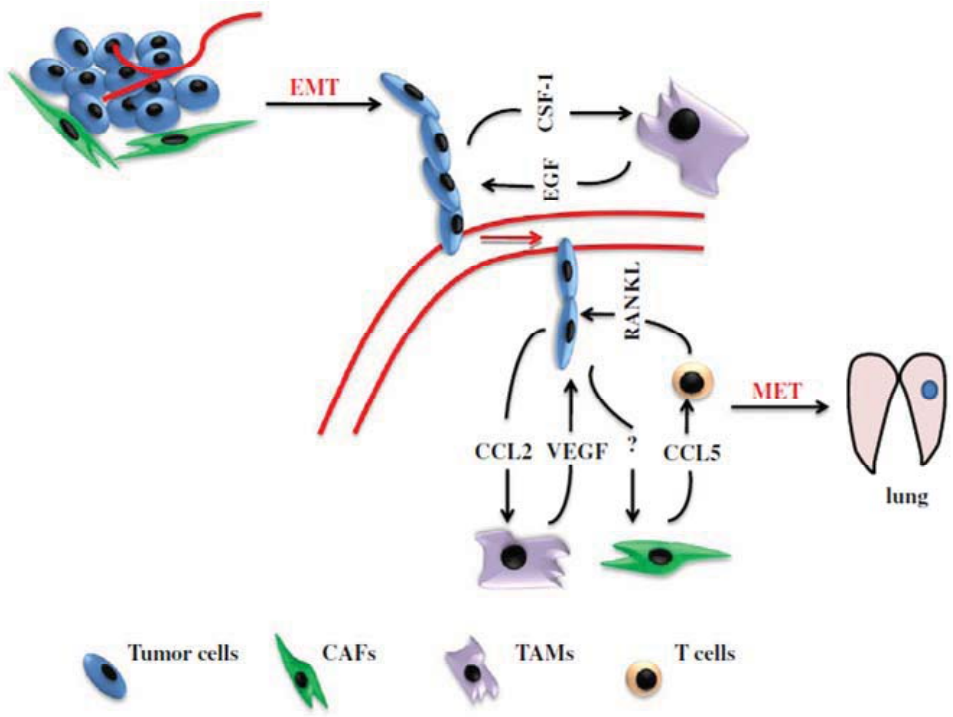

Figure 1.7 Diagram showing the role of breast cancer stromal cells in lung metastasis. The breast cancer cells homed to the lung may initially present mesenchymal characteristics by EMT changes. With the help of multiple types of stromal cells, tumor cells had epithelial characteristics, again by MET changes, finally forming secondary tumors.

Moreover, there are growing evidences supporting a role for genetic changes in breast cancer stroma as contributing to cancer progression (Campbell I. *et al.* 2009, Hu M. *et al.* 2005). Serial analyses demonstrated that epigenetic changes in breast cancer cells can foster tumor malignancy; however, there are also dramatic and consistent modifications in gene expression within the fibroblasts from primary human breast tumors (Allinen M. *et al.* 2004). These changes include histone modifications and alterations in the expression of DNA methyltransferases, chromatin modifying factors, and microRNAs (Enkelmann A. *et al.* 2011, Fiegl H. *et al.* 2006, Hu M. *et al.* 2005,). Recently, increasing evidence shows that CAFs can induce endocrine/ chemotherapy and target therapeutic resistances in breast cancer treatment (Loeffler M. *et al.* 2006, Martinez-Outschoorn U.E. *et al.* 2011, Sun Y. *et al.* 2012).

In summary, it has been largely demonstrated that CAFs play a key role in promoting the onset, progression, and chemoresistance of tumors through the activation of a series of intracellular pathways (Xing *et al.* 2010). In breast cancer some of these pathways may be activated by estrogen and molecules that have estrogen-like activity, such as xenoestrogens.

1.4 Estrogens

Estrogens are sex steroid hormones which exhibit a broad spectrum of physiological functions ranging from regulation of the menstrual cycle and reproduction to modulation of bone density, brain function, and cholesterol mobilization (Koos R.D. 2011, Shang Y. 006). Despite the normal and beneficial physiological actions of endogenous estrogen in

women, abnormally high estrogen levels are associated with the increased incidence of certain types of cancer, especially those of the breast and endometrium. The predominant intracellular estrogen is 17β-estradiol (E2). Other types of estrogen include estrone (E1) and estriol (E3) (Figure 1.8). In premenopausal women, E1 and E2 are secreted primarily by the ovaries during the menstrual cycle, with minor levels derived from adipose tissue and the adrenal glands. The placenta also produces E3 during pregnancy (Liang J. *and* Shang Y. 2012).

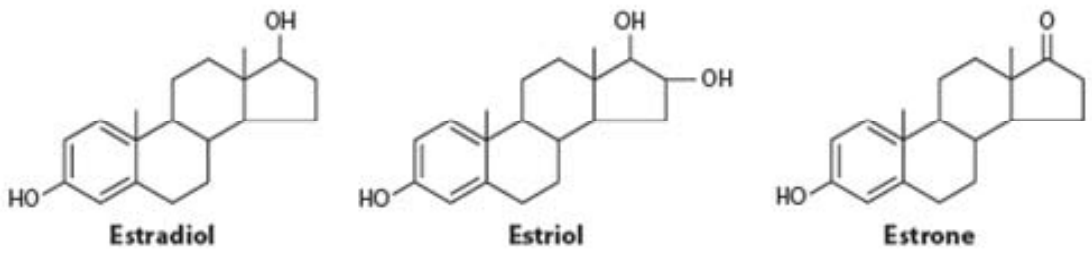

Figure 1.8 Chemical structures of estrogens

In the ovaries, granulosa cells synthesize estrogen from androgen (Auchus M.L. *and* Auchus R.J. 2012). Ovarian production of estrogen is regulated by the hypothalamic-pituitary-ovarian (HPO) axis and begins by anterior pituitary release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in response to the hypothalamic peptide gonadotropin-releasing hormone (GnRH). Acting in concert, LH stimulates androgen production, whereas FSH up-regulates aromatase, which catalyzes the rate-limiting and final step of estrogen biosynthesis: the aromatization of androgen to estrogen (Figure 1.9)

During ovulation, E2 production rises dramatically by eight- to ten-fold. High levels of estrogen in turn act via negative feedback to dampen estrogen production to inhibit the release of GnRH, LH, and FSH (Miller W.L. *and* Auchus R.J. 2011.). The primary mediator of estrogen biosynthesis in postmenopausal women is aromatase, which is found in adipose tissue as well as in the ovaries, placenta, bone, skin, and brain (Chumsri S. *et al* 2011). After menopause, ovarian estrogen biosynthesis is minimal, and circulating estrogen is derived principally from peripheral aromatization of adrenal androgen. As such, for obese postmenopausal women, adipose tissue becomes the main source of estrogen biosynthesis; this biosynthetic route is far less significant for non-obese postmenopausal women (Cleary M.P. *and* Grossmann M.E. 2009.).

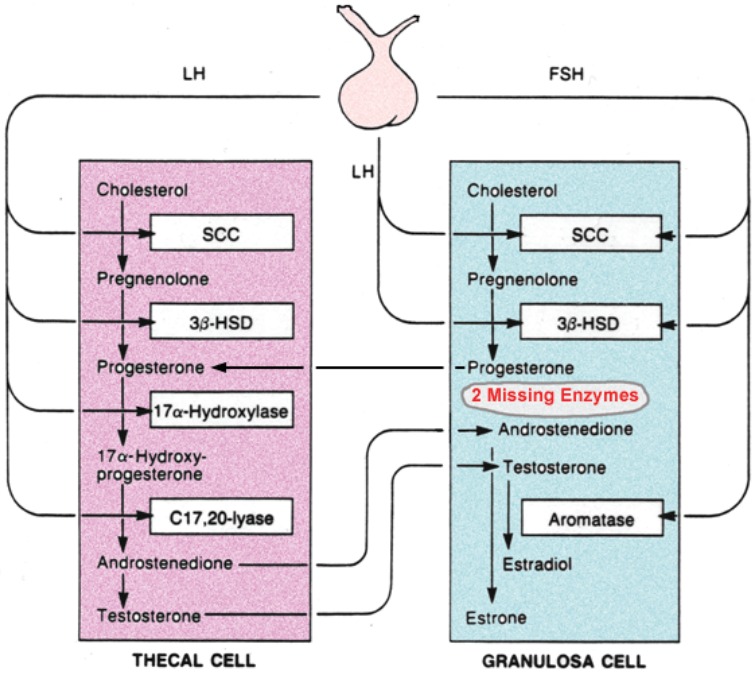

Figure 1.9 Ovarian production of estrogens

Numerous studies have demonstrated the association of estrogen with the development and/or progression of various types of cancer, including cancers of the breast, endometrium, ovary, prostate, lung, and colon (Folkerd E.J. and Dowsett M. 2010, Shang Y. 2007,).

1.5 Estrogen receptors (ERs)

1.5.1 ER α and ER β

Estrogen mediates its biological effects in target tissues primarily by binding to specific intracellular receptors, estrogen receptor (ER)α and ERβ. These receptors are encoded by ESR1 and ESR2, respectively; each gene is located on a different chromosome (Marino M. *et al.* 2006). Like all other members of the nuclear receptors super-family, human ERα and ERβ, are ligand-activated receptors with high degree of sequence homology and similar three-dimensional structure. ERα contains 595 amino acid and has a molecular weight of 67 kDa whereas ERβ is smaller in size, possesses 530 amino acids and of 59 kDa (Green S. *et al.* 1986, Ogawa S. *et al.* 1998). In particular, the ERs are modular proteins composed of four functional domains (Figure 1.10):

- the N-terminal transactivation domain which is involved in protein–protein interactions and in transcriptional activation of target-gene expression (Nilsson S. *et al.* 2001). Activation function-1 (AF-1) domain is present in this region and is able to regulate ERE-based gene transcription, even in the absence of ligand (Kumar R. *and* Thompson E.B. 2003; Acconcia F. *and* Marino M. 2011).

Figure 1.10 Schematic diagram showing the domain organization of human ERα and ERβ.

- the DNA binding domain (DBD) which plays the most important role in receptor dimerization and in the binding of specific DNA sequences (i.e., EREs) (Nilsson S. *et al.* 2001). This domain is folded into a globular shape, containing two α-helices perpendicular to one another, forming the base of the hydrophobic core, here amino acid sequences of the two α-helices is low (Ruff M. *et al.* 2000). Although the ERα and ERβ DBD contains zinc atom at core of four conserved Cys residues, in two groups forming the tetrahedral co-ordination, they do not form the three-dimensional structure seen in classic zinc finger proteins (Green S. *et al.* 1986). They can be considered as two interdependent sub-domains, differing both structurally and functionally. The first sub-domain is the proximal box or P-box helps in DNA recognition and the second sub-domain, the distal box or D-box involved in receptor dimerization (Green S. *et al.* 1986; Nilsson S. *et al.* 2001; Ascenzi P. *et al*. 2006) (Figure 1.11).

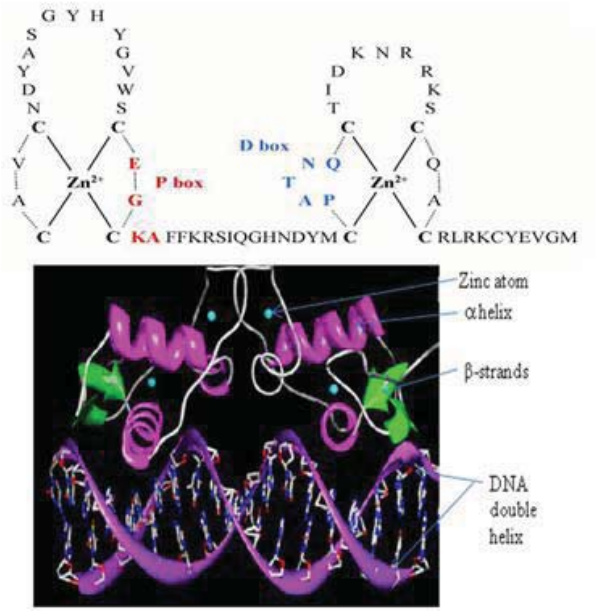

Figure 1. 11 Upper: Schematic representation of DBD. Amino acid residues forming the P and D box are shown in red and blue, respectively. Lower: DBD forms 'head to head' dimer and complexed with DNA.

- The hinge region which is the most variable region within ERs. The structure–function relationship of this domain is not very clear. This region contains both parts of the nuclear localization signal NLS of ERs as well as different sites for post-translational modifications, such as acetylation and sumoylation (Ruff M. *et al*. 2000; Ascenzi P. *et al.* 2006).

- the C-terminal E/F region encompassing the LBD, the AF-2 domain, the homo- and/or hetero-dimerization domain, and part of the nuclear localization region. The E/F region is also involved in the binding of chaperone proteins, such as heat-shock proteins (Hsp) 70 and 90 in the absence of ligands (Ruff M. *et al.* 2000; Nilsson S. *et al.* 2001).

It has been demonstrated that ERα and ERβ act by multiple mechanisms. In classical genomic mechanism, ligand-activated ERs dimerize and translocate in the nucleus where they recognize specific estrogen response elements (ERE) located in the promoter region of DNA of the target genes (Tsai M.J. *and* O'Malley B.W. 1994) (Figure 1.12).

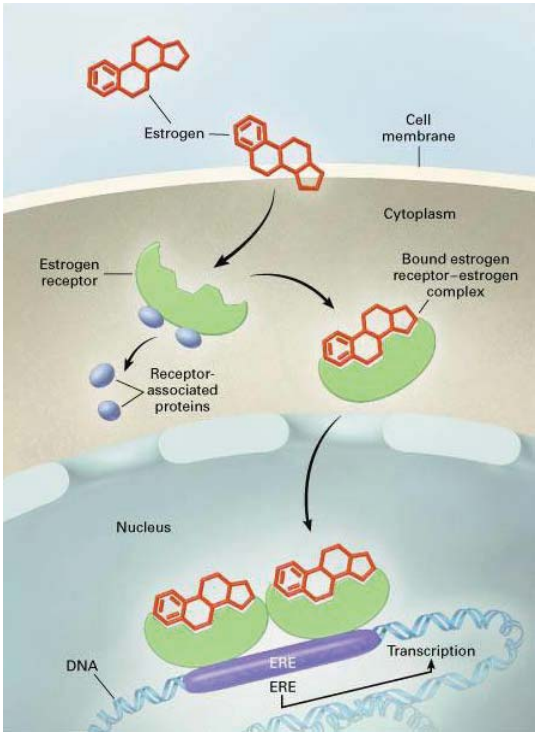

Figure 1.12 Illustration of the classic genomic mechanism by which estrogens activate gene transcription

Besides, E2 can also modulate gene expression by a second indirect mechanism involving the interaction of ER with other transcription factors such as the activator protein (AP)-1, nuclear factor-kB (NF-kB), stimulating protein-1 (Sp-1) which, in turn, binds their specific DNA elements (O'Lone R. *et al.* 2004, Kalaitzidis D. *and* Gilmore T.D. 2005) (Figure 1.13).

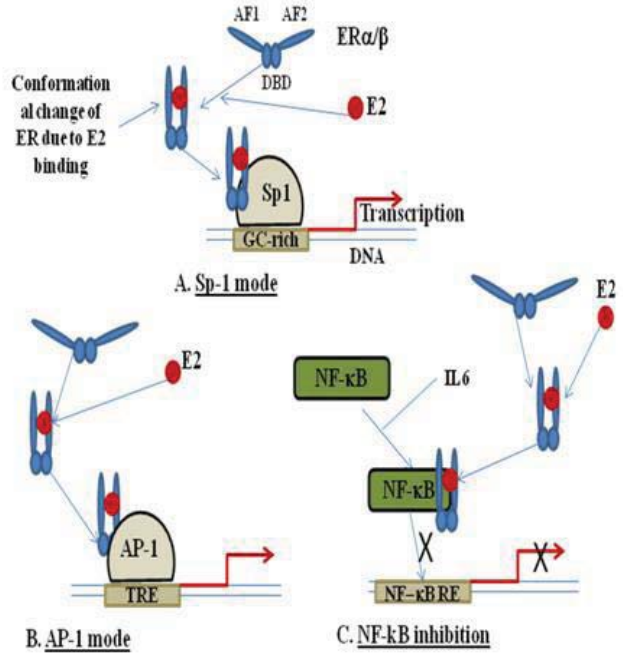

Figure 1.13 Model representing the various modes through which estrogen receptors (ERs) can modulate transcription of genes. ERs-DNA indirect association occurs through protein–protein interactions with the Sp1 (A), AP-1 (B), and NFκB (C) proteins.

In addition to the classical mechanism of estrogen signal transduction, which implies the binding of the receptor to DNA, there are a number of non-genomic signaling through which estrogen may exert their biological effects (Figure 1.14). Indeed, it is now well accepted that ER function can be modulated by extra-cellular signals even in the absence of E2. These findings focus primarily on the ability of polypeptide growth factors such as epidermal growth factors (EGF) and insulin like growth factor-1/2 (IGF-1/2) to activate ER and increase the expression of E2 target genes (Hall J.M. *et al*. 2001). Moreover, E2 exerts its non-genomic actions, that are too rapid to be accounted for by the activation of RNA and protein synthesis, through the activation of four main signaling cascade: phospholipase

C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidyl inositol 3 kinase (PI3K)/AKT, and cAMP/ protein kinase A (PKA) (Marino M. *et al.* 2006). A rapid activation of the cAMP/PKA pathway has been demonstrated in many different cell types (Ascenzi P. *et al.* 2006). Phospholipase C (PLC) dependent IP3 production, calcium influx, and PKC activation have also been reported in many different cultured cell types. Moreover, E2 rapidly stimulates the activation of MAPK pathways in MCF-7 cell-line, endothelial, bone and HepG2 cells. E2 can also down regulates MAPK phosphatase-1 activity, leading to the up regulation of extracellular regulated kinase (ERK) activity in breast cancer cells (Acconcia F. *an*d Kumar R. 2005, Levin E.R. 2005, Björnström L. *and* Sjöberg M. 2005).

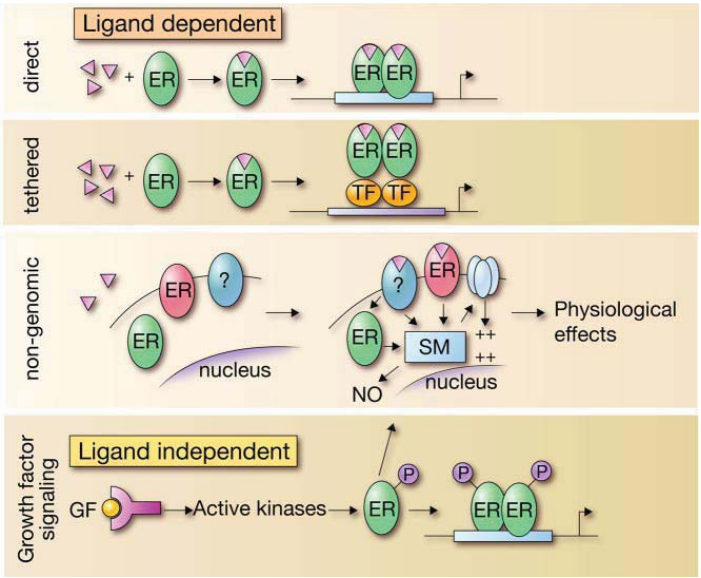

Figure 1.14 Representation which summarizes the possible mechanisms of estrogen signal transduction.

The E2-induced rapid signals indicate its localization at the plasma membrane. Some authors have suggested that the nongenomic actions of estrogen are mediated through a subpopulation of the classical ERs, ERα and ERβ, that is located and/or transported to the plasma membrane (Razandi M. *et al.* 2000; Acconcia F. *and* Kumar R 2005). However, in the last few years, a member of the 7-transmembrane G protein-coupled receptor family, GPR30/GPER, has been implicated in mediating both rapid and transcriptional events in response to estrogen under certain circumstances (Maggiolini M. *and* Picard D. 2010).

1.5.2 The G protein-coupled estrogen receptor (GPER)

In recent years, the identification of GPER as a novel estrogen receptor has opened a new scenario regarding a further mechanism trough which estrogenic compounds can trigger relevant biological actions in different cell contexts. GPER was first identified as an orphan member of the 7-transmembrane receptor family by multiple groups in the late 1990s (Carmeci C. *et al,* 1997, O'Dowd B.F. *et al.* 1998, Owman C. *et al.* 1996, Takada Y. *et al.* 1997). GPER belongs to the rhodopsin-like receptor superfamily (Carmeci C. et al, 1997) and its gene is mapped to chromosome 7p22.3 (Albanito L. *et al.* 2008b). There are four alternate transcriptional splicing variants encoding the same protein which is comprised of 375 amino acids, and contains seven transmembrane spanning segments (Wang C. *et al.* 2007). Although GPER is a seven-transmembrane GPCR, its subcellular localization remains to be fully elucidated. Indeed, several studies have reported the presence of GPER at the plasma membrane, in the endoplasmic reticulum and in the Golgi apparatum as well as in the nucleus of CAFs extracted from mammary biopsies (Filardo

E.J. *et al.* 2007, Madeo A. *and* Maggiolini M. 2010, Thomas P. *et al.* 2005, Revankar C.M. *et al.* 2007). As it concerns signalling pathways, it has been demonstrated that GPER ligands may bind to the receptor and activate heterotrimeric G proteins, which then activate Src and adenylyl cyclase (AC) resulting in intracellular cAMP production. Src is involved in matrix metalloproteinases (MMP) activation, which cleave pro-heparan-bound epidermal growth factor (pro-HB-EGF) and release free HB-EGF. The latter activates EGF receptor (EGFR), leading to multiple downstream events; for example, activation of phospholipase C (PLC), PI3K, and MAPK (Maggiolini M. *and* Picard D. 2010). Activated PLC produces inositol triphosphate (IP3), which further binds to IP3 receptor and leads to intracellular calcium mobilization (Filardo E.J. *and* Thomas P. 2012). The downstream signal of PI3K is AKT pathway. Main biological consequence of AKT activation is closely related to cancer cell growth; catalogued loosely into three aspects: survival, proliferation and growth (Vivanco I. *et al.* 2002). The activation of MAPK and PI3K results in activation of numerous cytosolic pathways and nuclear proteins, which further regulate transcription factors such as SRF, CREB, and members of the E26 transformation specific (ETS) family by direct phosphorylation (Pandey D.P. *et al.* 2009, Posern G. *and* Treisman R. 2006). These promotes the expression of a second wave of transcription factors such as FOS, JUN, EGR1, ATF3, C/EBPδ, and NR4A2. Cells are literally reprogrammed under the effect of this network of transcription factors and a series of GPER target genes such as CTGF are up-regulated (Pandey D.P. *et al.* 2009) (Figure 1.15).

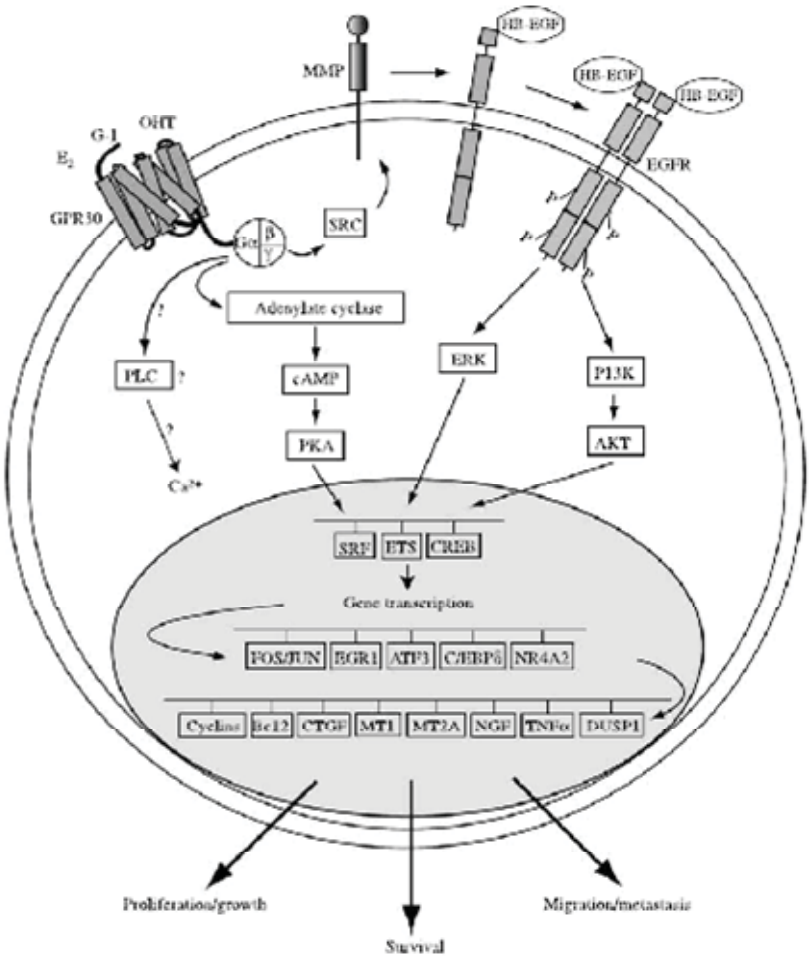

Figure 1.15 Schematic representation of the GPER signaling network.

Superimposed on these responses, there may be a variety of signaling crosstalk pathways and both negative and positive feedback loops. For example, it has been demonstrated that EGF up-regulates GPER expression through the EGFR/MAPK pathway in ER-negative breast cancer cells, most likely by promoting the recruitment of the c-FOS-containing transcription factor AP-1 to the GPER promoter (Albanito L. *et al.* 2008b). Considering that GPER signaling uses the EGFR/MAPK pathway, a positive feedback loop is

conceivable. This mechanism is also operational for EGF and the related growth factor TGFα in ERα-positive breast cancer cells (Vivacqua A. *et al.* 2009). GPER gene expression has been detected in at least four kinds of human tumor specimens or cell lines, including breast cancer (Albanito L. *et al.* 2008b, Carmeci C. *et al.* 1997, Filardo E.J. 2002, Filardo E.J. *et al*. 2008 Filardo E.J. *et al.* 2000, Filardo E.J. *et al.* 2006, Kuo W.H. *et al.* 2007, Pandey D.P. *et al.* 2009, Thomas P. *et al.* 2005), endometrial cancer (He Y.Y. *et al.* 2009, Leblanc K. *et al.* 2007, Smith H.O. *et al.* 2007, Vivacqua A. *et al.* 2006b), ovarian cancer (Albanito L. *et al.* 2008a, Albanito L. *et al.* 2007, Henic E. *et al.* 2009), thyroid cancer (Vivacqua A. *et al*. 2006a), and a rat pheochromocytoma cell line PC-12. (Alyea R.A *et al.* 2008). In addition, there is a growing body of evidence supporting that GPER is strongly associated with cancer proliferation (Albanito L. *et al.* 2008b, Albanito L. *et al.* 2007, Filardo E.J. *et al.* 2000, He Y.Y. *et al.* 2009, Kang K. *et al.* 2009, Liu Z. *et al.* 2008, Maggiolini M. *et al.* 2004, Vivacqua A. *et al.* 2006a, 2006b), migration (Henic E. *et al*. 2009, Pandey D.P. *et al.* 2009), invasion (He Y.Y. *et al.* 2009), metastasis (Filardo E.J. *et al*. 2006, Filardo E.J. *et al.* 2008), differentiation (He Y.Y. *et al.* 2009), and drug resistance (Kleuser B. *et al.* 2008, Lapensee E.W. *et al.* 2009). Indeed, as estrogen stimulates the progression of breast cancer in approximately two-thirds of patients who are ER + (Ali S. *and* Coombes R.C. 2000, Hanstein B. *et al.* 2004), some selective estrogen receptor modulators (SERMs), such as tamoxifen, have been clinically used to antagonize the binding of estrogen to its classic ERs, which is an effective therapeutic strategy in attenuating the growth of ER+ breast cancers. However, there are around 25% of ER + breast cancer patients who do not respond to anti-estrogen therapy (Early Breast Cancer Trialists' Collaborative Group 2005). It implies that blockade of classic ERs alone may not

be enough to completely abolish estrogen-induced breast cancer cell growth, since estrogen may promote cell growth through other receptor besides classic ERs. Such hypothesis is further supported by the discovery of GPER as the third specific ER with different structure and function to ERα and ERβ. GPER has a high binding affinity to not only estrogen, but also some SERMs, such as tamoxifen and ICI 182,780. Estrogen and SERMs stimulate GPER action without any antagonist effects (Thomas P. *et al.* 2005). These important findings provide a new possible mechanism for the progression of estrogen-related cancers, and raise a new potential target for anti-estrogen therapy. As it concerns clinical findings, GPER overexpression was associated with lower survival rates in endometrial and ovarian cancer patients (Smith H.O. *et al.* 2007, Smith H.O. *et al.* 2009) as well as with a higher risk of developing metastatic disease in breast cancer patients (Filardo E.J *et al.* 2006). Moreover, in a previous extensive survey, GPER was found to be highly expressed and significantly associated with tumor size (>2 cm), with the presence of distant metastases and increased human EGFR-2 (HER-2)/neu expression (Filardo E.J. *et al.* 2006). Likewise, in a recent study performed in the aggressive inflammatory breast cancer, the majority of tumors were GPER positive (Arias-Pulido H. *et al.* 2010), suggesting that GPER expression may be considered a predictor of an aggressive disease. In addition to the aforementioned studies on the potential functions of GPER in cancer and possibly other pathological conditions, this receptors was implicated in a broad range of physiological functions regarding the reproduction, the metabolism, the bone, the cardiovascular, the nervous and immune systems (Olde B. *and* Leeb-Lundberg L.M. 2009). Estrogen binds to GPER with a high affinity of a reported Kd 2.7 nM (Thomas P. *et al.* 2005) or 6 nM (Prossnitz E.R. *et al.* 2008), through which alternative estrogen

signaling pathways are activated. Moreover, two different synthetic compounds,G-1 (Bologa C.G. *et al.* 2006) and G-15 (Dennis M.K. *et al*. 2011), which were identified using virtual and bio-molecular screening, are respectively a specific agonist and antagonist of GPER. Recently, a novel inhibitors of both GPER and ERα has been identified and named MIBE (Lappano R. *et al.* 2012). In addition, different studies shows that ICI 182,780 (Filardo E.J. *et al.* 2002, Thomas P. et al. 2005), tamoxifen (Filardo E.J. *et al*. 2002), and 4-hydroxytamoxifen (OHT) (Pandey D.P. *et al.* 2009, Vivacqua A. *et al.* 2006a, 2006b) are also able to bind GPER and mimic estrogen effects. It has been reported that a variety of xenoestrogens, including bisphenol A, can bind and activate GPER leading to important biological responses (Thomas P. *and* Dong J. 2006).

1.6 Xenoestrogens

Xenoestrogens encompass a variety of chemicals that have estrogen-like effects. Most frequently, xenoestrogens are agriculture chemicals such as pesticides and industrial by-products (certain plastics or detergents) widely spread in the environment, compounds from plants (phytoestrogens) such as isoflavones from soy (geniestein, daidzein), or coumesterol from red clover. In addition, there are synthetic drugs like DES, a potent synthetic estrogen that was widely prescribed to pregnant women from the 1940s through the 1970s in the mistaken belief that it could prevent threatened miscarriages (Alonso-Magdalena P. *et al.* 2012*)*. Over the 1990s, the appearance of adverse reproductive effects in aquatic and wildlife species living within or near contaminated areas was reported (Colborn T. *et al,* 1993; Sonnenschein C. *and* Soto A.M. 1998; Guillette L.J. *et al.* 1994;

Sumpter J.P *and* Jobling S. 1993). In parallel, the estrogenic activity of some of these compounds such as octylphenol and bisphenol-A was accidentally discovered in the laboratory, because they disrupted experiments that studied the effects of natural estrogens (Soto A.M. *et al.* 1991; Krishnan A.V. *et al.* 1993). Throughout the years, substantial evidence has pointed to the fact that these chemicals can mimic the action of the natural hormone E2, although they do not exhibit a similar structure to estrogens (Jobling S. *et al.* 1995). Moreover, it has been suggested that some endocrine disrupters may contribute to the development of hormone-dependent cancers, such as breast cancer (Fernandez S.V. *and* Russo J. 2010). Between xenoestrogens, bisphenol A (BPA) is one of the endocrine disrupting chemicals that has been most thoroughly studied.

1.6.1 Bisphenol A

Bisphenol-A (BPA) (Figure 1.16) was first synthesized by Dianin in 1891 and reported to be a synthetic estrogen in the 1930s (Dodds E.C. *and* Lawson W.1936). In the 1950s, BPA was rediscovered as a compound that could be polymerized to make polycarbonate plastic, and from that moment on until now, it has been commonly used in the plastic industry.

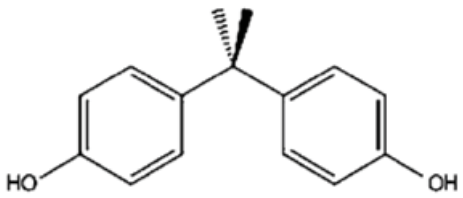

Figure 1.16 Chemical structure of Bisphenol A

BPA is one of the highest volume chemicals produced worldwide, with over 6 billion pounds produced each year and over 100 tons released into the atmosphere by the yearly production (Vandenberg L.N. *et al.* 2009). It is used as the base compound in the manufacture of polycarbonate plastic and the resin lining of food and beverage cans, and as an additive in other widely used plastics such as polyvinyl chloride and polyethylene terephthalate (Welshons W.V. *et al.* 2006). It is present not only in food and beverage containers, but also in some dental material (Olea N. *et al.* 1996). Numerous studies have found that BPA can leach from polycarbonate containers; heat and either acidic or basic conditions accelerate the hydrolysis of the ester bond linking BPA monomers, leading to a release of BPA with the concomitant potential human exposure (Kang J.H. *et al.* 2006, Richter C.A. *et al.* 2007). Indeed, the potential for BPA exposure has already been demonstrated since BPA was detected in 95% of the urine samples in the USA (Calafat A.M. *et al.* 2005). Moreover, it has been detected in amniotic fluid, neonatal blood, placenta, cord blood and human breast milk (Richter C.A. *et al.* 2007). Concerning the potential risk of this compound, in the 1980s the lowest-observable-adverse effect-level (LOAEL) for BPA was determined at 50 mg/kgbw/day, and the Environmental Protection Agency (EPA) calculated a ‘‘reference dose’’ or safe dose of 50 lg/kgbw/day in a series of studies in which the changes of body weight in animals fed diets containing BPA were analyzed (http:// www.epa.gov/iris/subst/0356.htm, U.E.P.A.). However, since that time, numerous scientific evidence supports that BPA can interfere with the endocrine signaling pathways at doses below the calculated safe dose, particularly after fetal, neonatal or perinatal exposure, but also after adult exposure. In this regard, fetal and perinatal exposures to BPA in rodents were shown to affect the brain, mammary gland and

reproductive tract as well as to stimulate the development of hormone-dependent tumors (Durando M. *et al.* 2007, Ho S.M. *et al.* 2006; Maffini M.V. *et al.* 2006, Munoz-de-Toro M. *et al.* 2005). Although since its discovery has been highlighted as BPA could have estrogen like activity, this was rediscovered in 1993 and confirmed by performing different assays such as: competitive binding to ER, proliferation of MCF-7 breast cancer cells, induction of progesterone receptors, and reversal estrogen action by tamoxifen (Krishnan A.V. *et al.* 1993). Indeed, BPA, with its two benzene rings and two (4, 4')-OH substituents, fits in the ER binding pocket (Gould J.C. *et al.* 1998) (Figure 1.17).

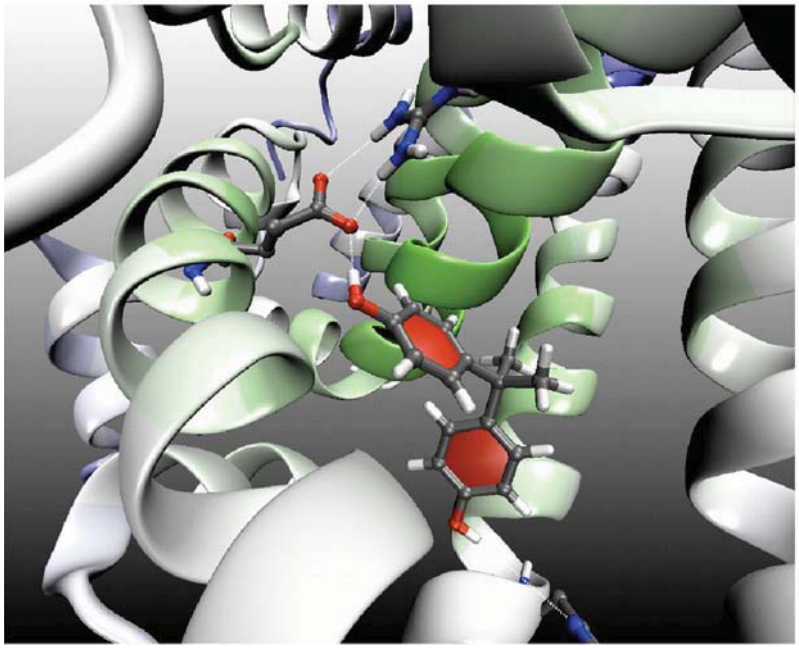

Figure 1.17 Binding of Bisphenol A to the estrogen receptor α

Biochemical assays have examined the kinetics of BPA binding to ER and have determined that BPA binds both ERα and ERβ, with approximately 10-fold higher affinity to ERβ (Kuiper *et al.* 1997, Matthews *et al.* 2001, Routledge *et al.* 2000). However, the affinity of BPA for the ERs is approximately 10,000-fold weaker than that of estradiol (Andersen H.R. *et al.* 1999, Kuiper G.G. *et al.* 1998, Fang H. *et al*. 2000). Therefore, the low dose effects exerted by BPA in different cell types, can be explained at least partially because this endocrine disruptors may bind differently than E2 within the ligand domain of estrogen receptors (ERs) (Gould J.C. *et al.* 1998). There are also differences in the BPA co-activator recruitment, as is indicated by the fact that the BPA/ERβ complex showed over a 500-fold greater potency than BPA/ERα in recruiting the coactivator TIF2. This is a reflection of the more efficient capacity that ERβ has to potentiate receptor gene activity in some cell types (Routledge E.J. *et al.* 2000, Safe S.H. et al. 2002). In addition, it has been largely demonstrated that BPA elicits rapid responses via non-classical estrogen triggered pathways (Nadal A. *et al.* 2000, Quesada I. *et al.* 2002; Watson C.S. *et al.* 2005). BPA has been shown to bind to both the membrane-bound form of ER (mER) and GPER in different cellular contexts (Wetherill Y.B. *et al.* 2007) (Figure 1.18). In particular, it has been evidenced that BPA, upon binding to GPER, activates intracellular pathways that may be involved in the biological responses of normal and neoplastic cells (Bouskine A. *et al.* 2009, Dong S. *et al.* 2011, Sheng Z.G. *and* Zhu B.Z. 2011).

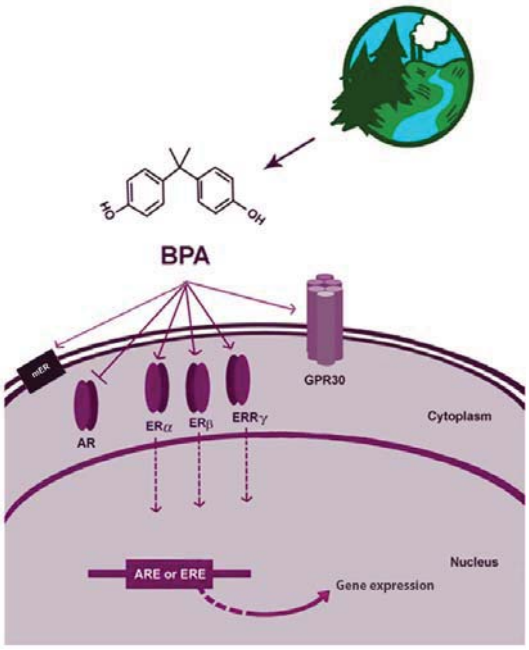

Figure 1.18 A model of BPA's potential manner of action. Environmental exposure to BPA affects the developing brain and behavior. Acting as an endocrine activating compound, BPA can weakly activate several estrogen (ER) and estrogen-related receptors such as ERα, ERβ, ERR-γ, membrane ER (mER) and GPR30 and/or antagonize the androgen receptor (AR).

1.7 Aim of the study

The aim of this study was to demonstrate that BPA induces gene expression changes and proliferative responses through GPER in breast cancer cells and CAFs obtained from breast cancer patients, both of which lack the classical estrogen receptors ERα and ERβ. In addition, we wanted to show that GPER mediates the migration of CAFs, induced by BPA, and also promotes migratory effects in breast cancer cells upon stimulation of CAFs with BPA, further highlighting the ability of this endocrine disruptor to promote cancer progression also through these important players of the tumor microenvironment.

Chapter 2

Materials and Methods

2.1 Reagents

Bisphenol A (BPA), N-[2-(p-bromocinnamylamino)ethyl]-5-soquinolinesulfonamide dihydrochloride (H89), PD98059 (PD) and arsenic trioxide (As2O3) were purchased from Sigma-Aldrich (Milan, Italy); AG1478 (AG) was bought 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). As2O3 was dissolved in phosphate-buffered saline,; BPA and PD were dissolved in ethanol; AG1478, H89, and G‑1 were solubilized in dimethyl sulfoxide (DMSO).

2.2 Cell cultures

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).

2.3 Isolation, cultivation, and characterization of CAFs

CAFs were extracted as previously described (Madeo A. *and* Maggiolini M. 2010). 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) (Figure 2.1).

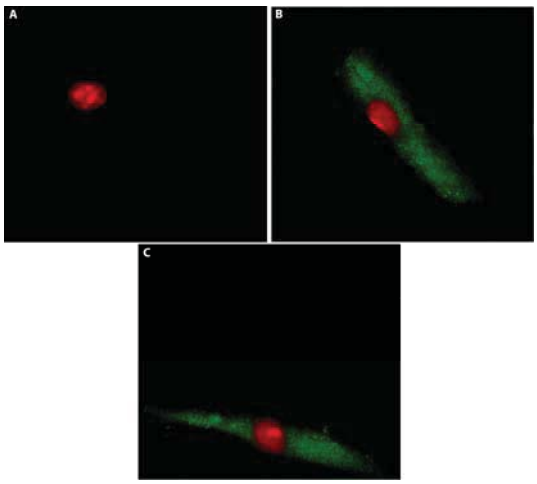

Figure 2.1 **Characterization of CAFs.** CAFs were immunostained by anti-cytokeratin 14 (A), anti-vimentin (B) and anti FAPα (C)antibody.

2.4 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 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 dodecylsulfate (SDS), and a mixture of protease inhibitors containing 1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. Then, we diluted samples 10 times and determined protein concentration using Bradford reagent (Sigma-Aldrich, Milan, Italy). The method of Bradford is based on the observation that the absorbance maximum of a solution of Coomassie Brillant Blue G250 shifts from 465 to 595 nm upon binding with proteins. The amount of bound colorant depends on the content of basic amino acids in proteins in solution. Equal amounts of whole protein extract were resolved on a 10% SDS polyacrylamide. After electrophoresis, the proteins were transferred from the gel onto a nitrocellulose membrane by electroblotting in buffer with low salt content (25 mM TRIS, 192 mM Glycine pH 8.3, 0.1% SDS, 20% Methanol). Then, the membrane was placed in a solution of no-fat milk at 5% in 1X TBST (Tris HCl 100 mM pH 7.5, 1M NaCl, 1% Tween 20) for one hour at room temperature in order to block all sites of non-specific hydrophobic interactions. Afterward, nitrocellulose filter were probed overnight at 4°C with the antibody 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). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology,

DBA Milan, Italy) and immunoreactive bands were visualized with the ECL western blotting detection system (GE Healthcare, Milan, Italy). Results of densitometric analyses of Western blots were obtained using ImageJ software (Abramoff M.D. *et al.* 2004).

2.5 Transient transfections

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

efficiency, low cytotoxicity, quick and simple protocol for usage and some can be used also in the presence of serum

.

2.5.1 Plasmids

The CTGF luciferase reporter plasmid p(-1999/+36)-luc (Chaqour B. *et al.* 2006), 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 (Kaneyama J.K. *et al.* 2002) 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 (Chen C.C. *et al.* 2004), 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). The short hairpin (sh) RNA constructs to knock down the expression of GPER and CTGF and the unrelated shRNA control construct were obtained and used as previously described (Pandey D.P. *et al.* 2009).

2.5.2 Gene silencing experiments

To perform the silencing of GPER and CTGF we used the technique of shRNA (short hairpin RNA). These are small molecules of double-stranded RNA hairpin-shaped (hairpin) which are

processed by a specific enzyme called DICER. This enzyme cuts the double-stranded RNA and form fragments of double-stranded RNA of about 19-25 nucleotides. Whereafter, the enzyme complex RISC separates the two strands and transfers one near the mRNA containing the sequence of gene which should be to be silenced. This filament will bind with the complementary sequence at the mRNA level and causes the block of translation or degradation of the mRNA itself. For 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 hours before treatments with shRNA, shGPER or ShCTGF using Fugene6.

2.5.3 Luciferase assays

To perform the luciferase assay two "reporter" enzymes are simultaneously expressed in a single system and their activities are measured. The activity of the experimental reporter is correlated to the specific conditions of treatment, while the basal cell activity is compared to that of the co-transfected control reporter (pRL-CMV). Comparing the activity of the experimental and control reporters, it is possible to normalize experimental variability which generally is caused by the differences between the number of cells and effectiveness of the transfection. In this assay in one sample are measured sequentially the activities of two luciferase: the firefly or firely luciferase (Photius pyralis) and the Renilla luciferase (Renilla reniformis). These enzymes have different structures and requires different substrates, so that it is possible to discriminate selectively the respective bioluminescent reactions. The activity of firefly luciferase is measured initially adding the LAR II (Luciferase Assay Reagent II) to the cell lysate. This generates a light signal that is appropriately quantified using a

luminometer (Lumat model LB 9507, Berthold Technology). Then, adding in the same tube the Stop & Glo reagent, the first enzymatic reaction is stopped and and simultaneously start the second reaction catalyzed by Renilla which also generates a light signal. Finally, the values of the Luciferase activity are compared with the corresponding values of Renilla and expressed as " relative Luciferase units ". In this study for the luciferase assays, cells 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 hoursr, ligands were 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.

2.6 RT-PCR and real-time PCR

Total RNA was extracted from cells manteined for 24 hours in medium without serum and treated with ligand for indicated times using Trizol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and cDNA was synthesized from the RNA by reverse transcription using murine leukemia virus

reverse transcriptase (Invitrogen). We quantified the expression of selected genes by real-time PCR. This method is based on the use of intercalating agents which bind to double stranded DNA. These molecules, when excited by laser beams, emit fluorescence and allow to follow in real-time the progress of the reaction and the increase of the amount of nucleic acid. In this study we used SYBR Green as the detection method and the Step One sequence detection system (Applied Biosystems Inc., Milan, Italy). Specific primers for the genes c-FOS, CTGF, EGR-1 and the control gene 18S 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. The sequences of primer used are listed in Table 2.1.

Gene	Primer Fw	Primer Rv
c-FOS	5'-CGAGCCCTTTGATGACTTCCT-3'	5'-GGAGCGGGCTGTCTCAGA-3'
CTGF	5'-ACCTGTGGGATGGGCATCT-3'	5'-CAGGCGGCTCTGCTTCTCTA-3'
EGR-1	5'-GCCTGCGACATCTGTGGAA-3'	5'-.CGCAAGTGGATCTTGGTATGC-3'
18S	5'-GCGTCCCCCAACTTCTTA-3'	5'-GGGCATCACAGACCTGTTATT-3'

Table 2.1 Sequences of primers used

2.7 Wound-healing assays

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.

2.8 Proliferation assays

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.

2.9 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.

Chapter 3

Results

3.1 BPA induces ERK1/2 activation through GPER

To evaluate the ability of BPA to induce ERK 1/2 activation through GPER we used the SKBR3 cells and CAFs which both express GPER and lack ERs (Figure 3.1)

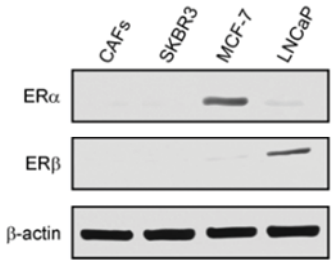

Figure 3.1 CAFs and SKBR3 cells are ERα and ERβ negative. Western blot analyses of ERα and ERβ protein expression in CAFs, SKBR3 and MCF-7 breast cancer cells and LNCaP prostate cancer cells. β-actin antibody was used as a loading control.

Considering that in our (Maggiolini M. *and* Picard D. 2010) and other (Dong S. *et al.* 2011) previous investigations ERK 1/2 phosphorylation was found as a downstream signaling induced by the ligand activation of GPER, we began the present study evaluating the ERK 1/2 activation by increasing concentrations of BPA and the selective GPER agonist G-1 in

SKBR3 cells and CAFs. BPA (Figure 3.2 A,C) and G‑1 (Figure 3.2 B,D) induced ERK1/2 phosphorylation in both cell types in a dose-dependent manner.

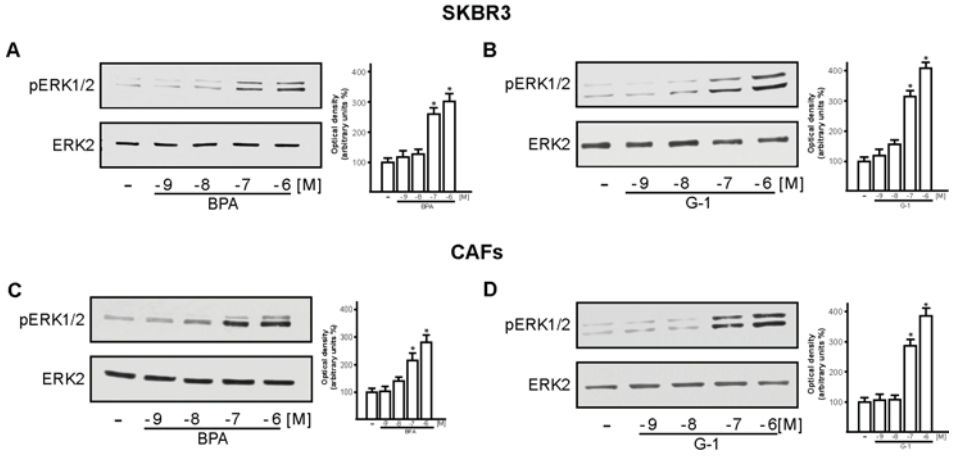

Figure 3.2. Induction of ERK1/2 phosphorylation (pERK1/2) by BPA and G‑1 through GPER in SKBR3 cells and CAFs. (A,B) SKBR3 cells were treated for 30 min with vehicle (–) or increasing concentrations of BPA (A) or G‑1 (B). (C,D) CAFs were treated for 30 min with vehicle (–) or increasing concentrations of BPA (c) or G‑1 (d). Graphs show densitometric analyses of the blots normalized to ERK2; values shown represent the mean ± SD of three independent experiments. *p < 0.05 compared with vehicle.

Moreover, to elucidate the intracellular pathway trough which BPA induces ERK 1/2 activation in SKBR3 cells, we evaluate ERK 1/2 phosphorytion in presence of the inhibitors of EGFR (AG), ERK (PD) and PKA (H-89). As shown in Figure 3.3 panel A, when AG or PD was added, ERK1/2 activation was not evident, but it was present when H89 was added. Interestingly, ERK1/2 phosphorylation by BPA was abolished by silencing GPER expression in both SKBR3 and CAFs (Figure 3.3 B,D), 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 3.3 panels C and E, respectively.

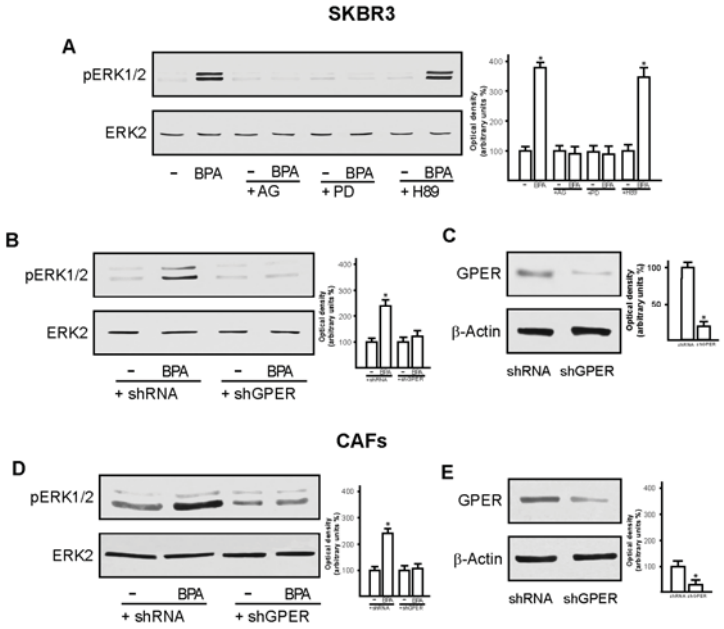

Figure 3.3 BPA induces ERK1/2 phosphorylation (pERK1/2) in SKBR3 cells and CAFs through the GPER/EGFR/ERK signaling pathway. (A) ERK1/2 phosphorylation in SKBR3 cells treated for 30 min with vehicle or 1 μM BPA alone or in combination with 10 μM AG1478, PD, or H89 (inhibitors of EGFR, MEK, and PKA, respectively). (B) ERK1/2 phosphorylation in SKBR3 cells transfected with shRNA or shGPER and treated with vehicle or 1 μM BPA for 30 min. (C) Efficacy of GPER silencing. (D) ERK1/2 phosphorylation in CAFs transfected with shRNA or shGPER and treated with vehicle or 1 μM BPA for 30 min. (E) Efficacy of GPER silencing in CAFs. Graphs show densitometric analyses of the blots normalized to ERK 2 (A,B,D) or β-Actin (C,E); values shown represent the mean ± SD of three independent experiments.*$p < 0.05$ compared with vehicle.

Moreover, to demonstrate the specificity of BPA action, we used the environmental contaminant arsenic (Nordstrom D.K. 2002), which elicits the ability of breast cancer cells to activate ERK1/2 (Ye J. *et al.* 2005). We observed that ERK1/2 phosphorylation induced by 10 μM As2O3 was still present in SKBR3 cells transfected with shGPER (data not shown).

3.2 BPA stimulates the expression of GPER target genes

GPER-mediated signaling regulates the transcription of diverse target genes (Pandey D.P *et*

al. 2009). In the present study, BPA transactivated the promoter sequence of c-FOS, EGR‑1, and CTGF (Figure 3.4 A) in SKBR3 cells, and accordingly stimulated mRNA expression of these genes in both SKBR3 cells and CAFs (Figures 3.4 B,C).

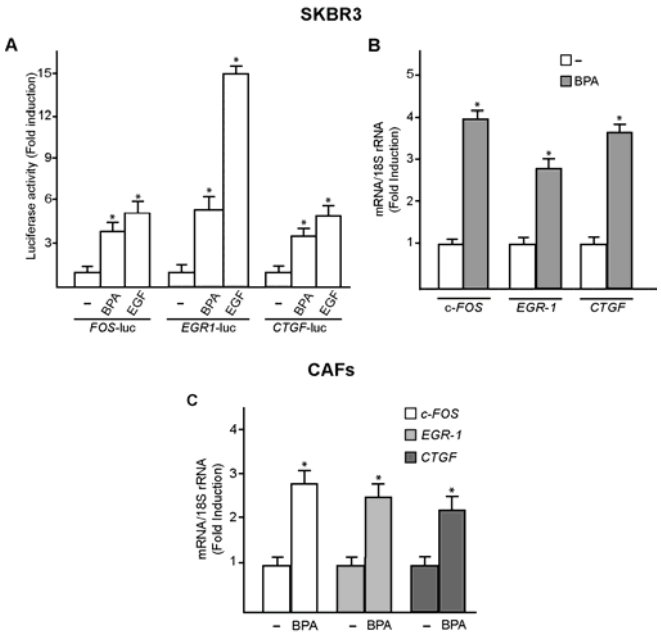

Figure 3.4 Expression of GPER target genes (c‑FOS, EGR‑1, and CTGF) in SKBR3 cells and CAFs in response to BPA treatment. (a) Evaluation of c-FOS, EGR‑1, and CTGF luciferase reporter genes in transfected SKBR3 cells treated with vehicle (–), 1 µM BPA, or EGF (50 ng/mL; positive control). Luciferase activity 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,C) Evaluation of c-FOS, EGR‑1, and CTGF mRNA expression by real-time PCR in SKBR3 cells (B) and CAFs (C) 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. *$p < 0.05$ compared with vehicle.

In accordance with these findings, BPA induced the protein levels of c-FOS, EGR‑1, and CTGF (Figure 3.5 A). 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 in SKBR3 cells (Figure 3.5 A). Notably, the c-FOS, EGR-1, and CTGF

protein increases after exposure to BPA were abrogated by silencing GPER in both SKBR3cells and CAFs (Figures 3.5 B,D). The efficacy of GPER silencing was ascertained by immunoblotting experiments in SKBR3 cells and CAFs as shown in Figure 3.5 panels C and E, respectively.

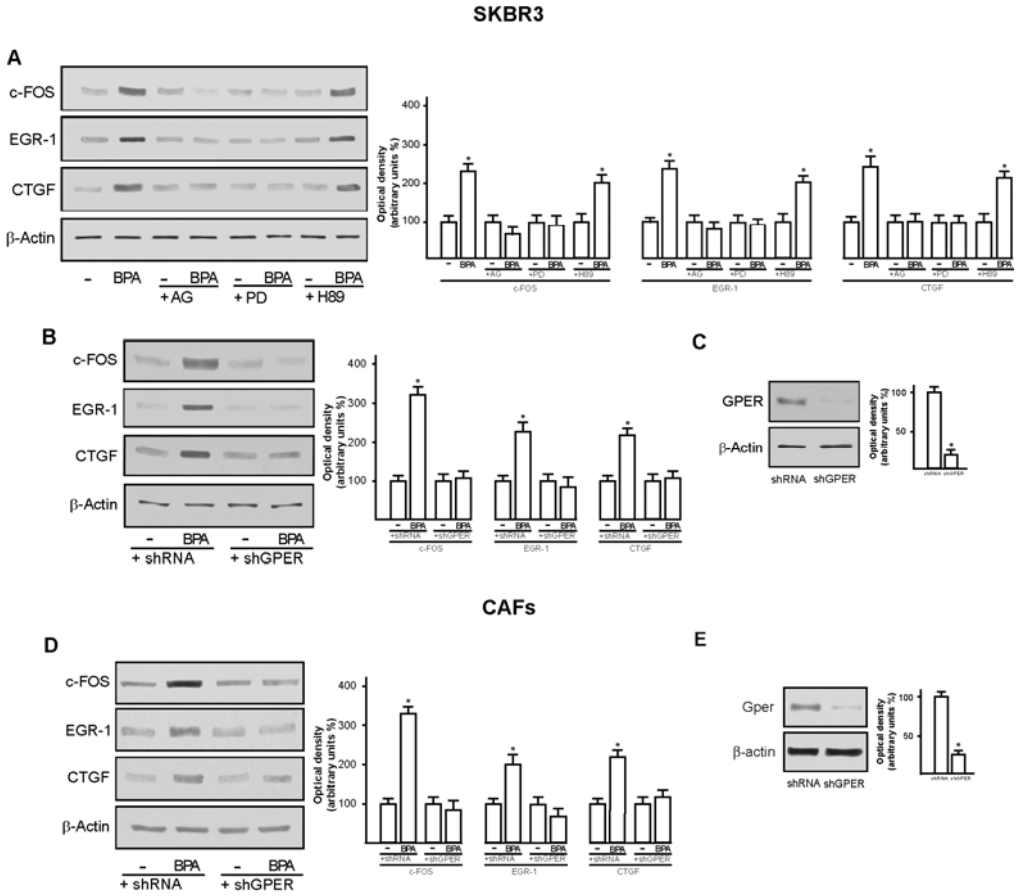

Figure 3.5 BPA induces protein levels of GPER target genes through the GPER/EGFR/ERK transduction pathway. (A) Immunoblots showing c-FOS, EGR-1, and CTGF protein expression in SKBR3 cells treated with vehicle or 1 μM BPA alone or in combination with 10 μM AG1478, PD, or H89 (inhibitors of EGFR, MEK, and PKA respectively). (B) 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. (C) Efficacy of GPER silencing in SKBR3 cells. (D) 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. (E) Efficacy of GPER silencing in CAFs. 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.

3.3 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 3.6 A,B).

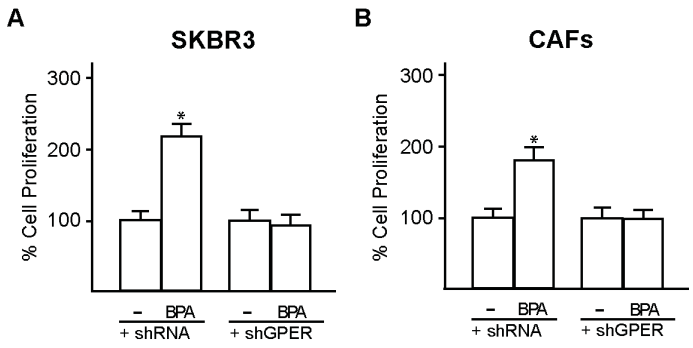

Figure 3.6 Induction of proliferation in SKBR3 cells and CAFs. Proliferation in SKBR3 cells (A) and CAFs (B) treated with vehicle (–) or 1 μM BPA for 5 days after silencing GPER expression. Values shown represent the mean ± SD percent of vehicle control of three independent experiments, each performed in triplicate. $p<0.05$ compared with vehicle.

Moreover, in wound-healing assays in CAFs, migration induced by BPA was abolished by knocking down GPER expression (Figure 3.7).

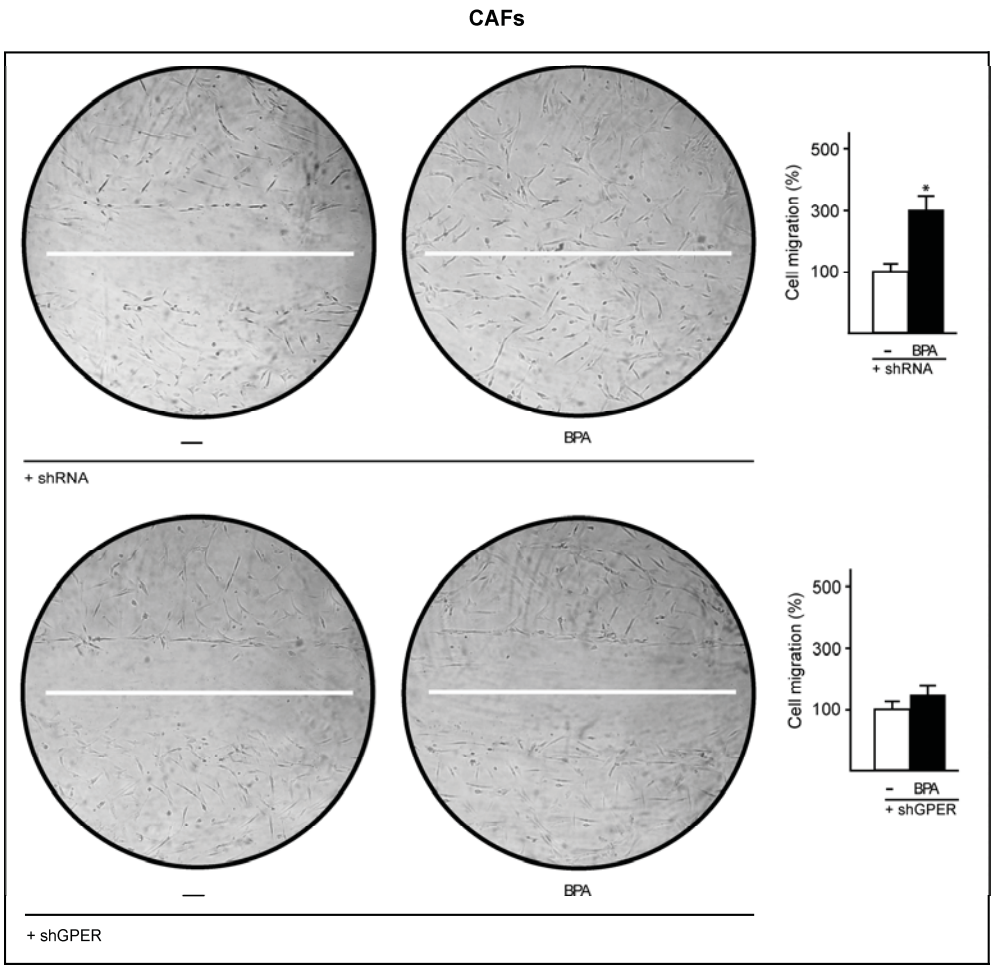

Figure 3.7 BPA induces migration of CAFs. Migration in CAFs treated with vehicle or 1 μM BPA for 48 hr after silencing GPER expression. Values shown represent the mean ± SD percent of vehicle control of three independent experiments, each performed in triplicate. *p < 0.05 compared with vehicle.

In addition, 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 3.8). Overall, these findings demonstrate that BPA induces stimulatory effects as a GPER agonist in both ER-negative SKBR3 breast cancer cells and CAFs.

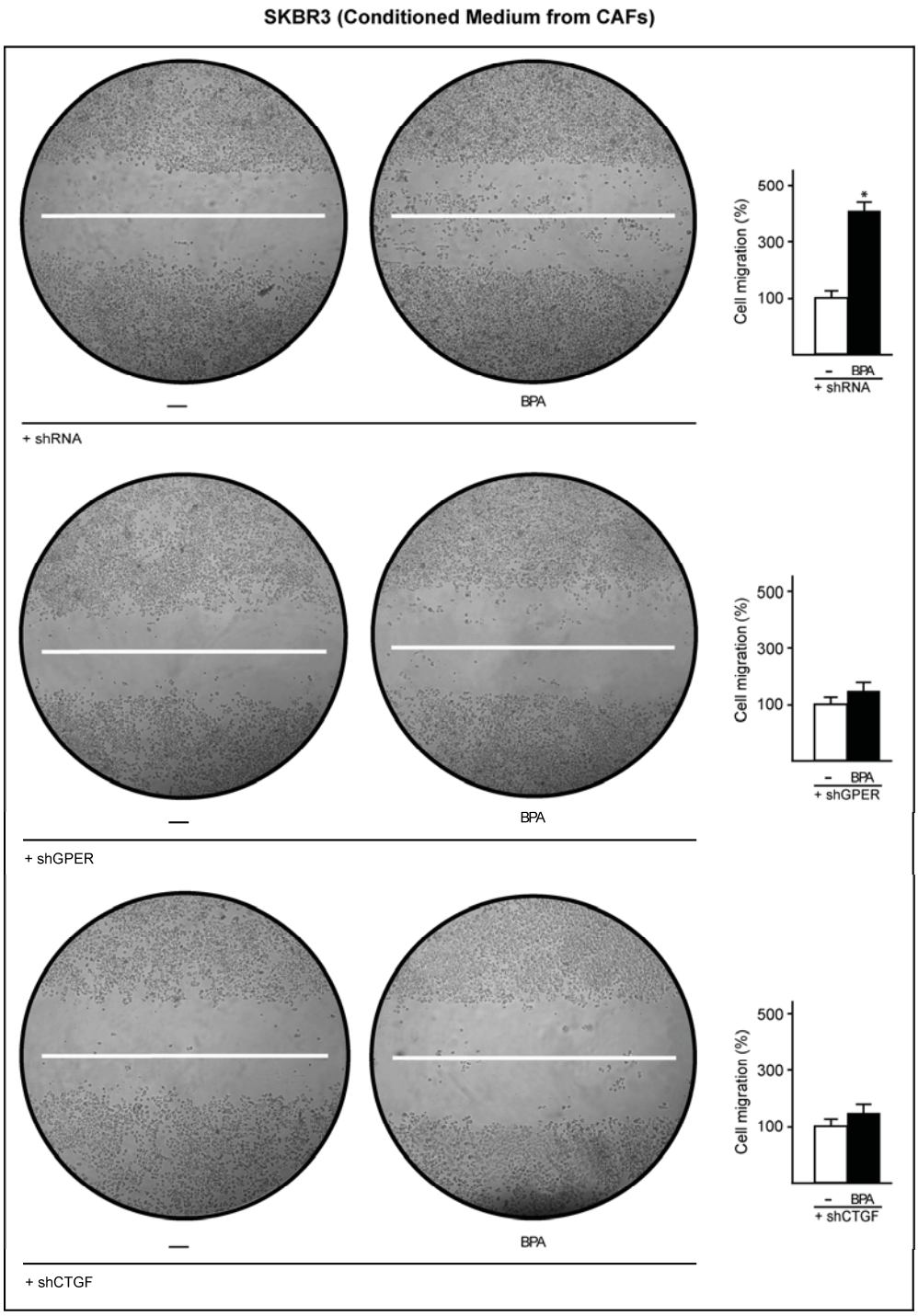

Figure 3.8 Induction of migration of SKBR3 cells through the stimulation of CAFs with BPA. Migration in SKBR3 cells cultured in conditioned medium from CAFs with silenced expression of GPER and CTGF. Values shown represent the mean ± SD percent of vehicle control of three independent experiments, each performed in triplicate. *$p < 0.05$ compared with vehicle

Chapter 4

Discussion

There has been increased interest in understanding the molecular mechanisms involved in the endocrine-disrupting effects of BPA (Vandenberg L.N. *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 M. *et al.* 2007, Munoz-de-Toro M. *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 C.M. *et al.* 2001). Analogously, BPA induced the proliferation of uterine and vaginal epithelial cells in ovariectomized rats (Steinmetz R. *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 J.C. *et al.* 1998, Kuiper G.G. *et al* 1998, Vivacqua A. *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 breast

cancer cells (Alonso-Magdalena P. *et al.* 2005, Noguchi S. *et al.* 2002, Watson C.S. *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 S. *et al.* 2011) and the proliferation of both human seminoma cells (Bouskine A. *et al.* 2009) and mouse spermatogonial cells (Sheng Z.G. *and* Zhu B.Z. 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 D.P. *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 N.A. *and* Moses H.L. 2005).

The present findings are relevant to the results obtained in a previous study (Albanito *et al.* 2008a) 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 L. *et al.* 2008a) 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 W.V. *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 L.N. *et al.* 2009) have estimated 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 L.N. *et al.* 2009). Hence, future studies should include mathematical models of potential exposures, particularly because many sources of BPA exposure have been identified (Vandenberg L.N. *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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Publications

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

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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).

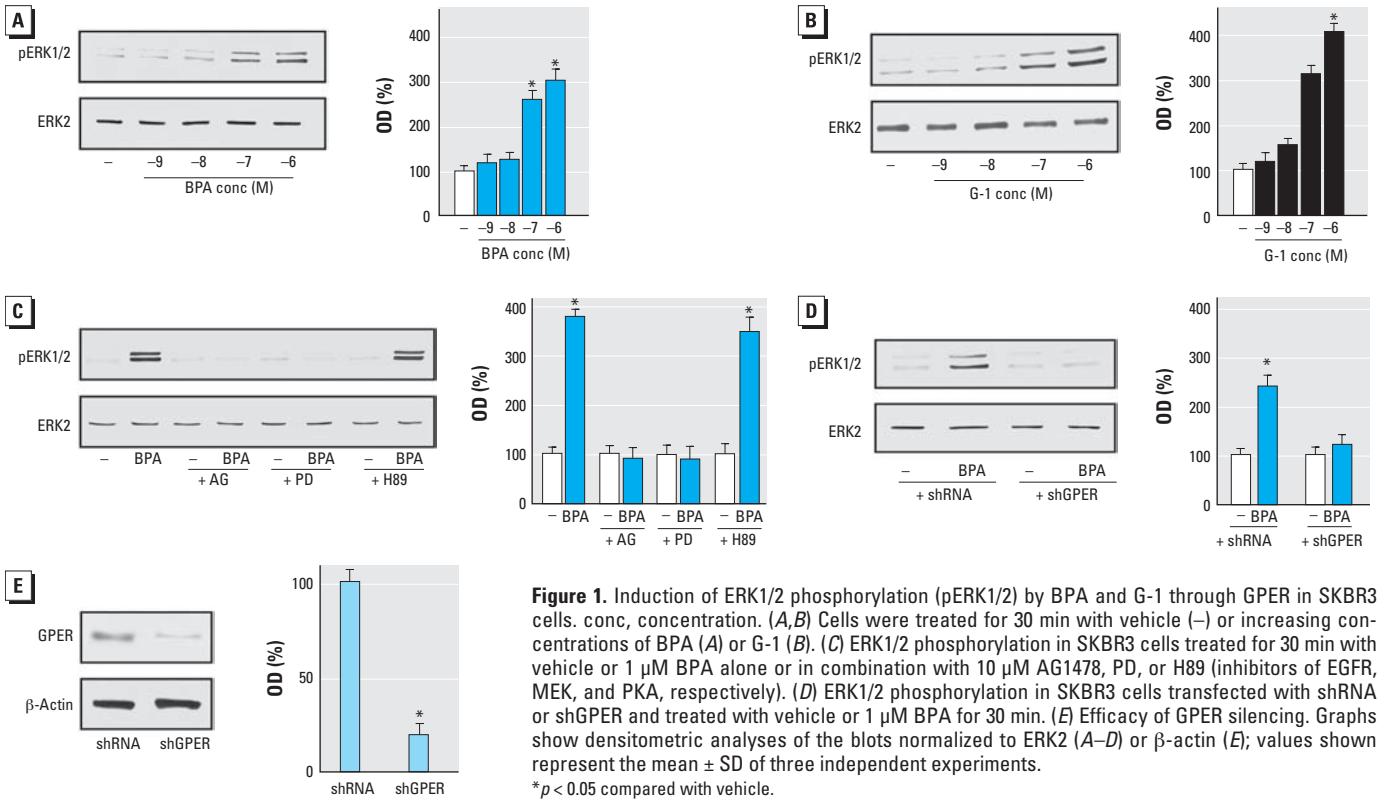

Figure 1. Induction of ERK1/2 phosphorylation (pERK1/2) by BPA and G-1 through GPER in SKBR3 cells. conc, concentration. (*A*,*B*) Cells were treated for 30 min with vehicle (–) or increasing concentrations of BPA (*A*) or G-1 (*B*). (*C*) ERK1/2 phosphorylation in SKBR3 cells treated for 30 min with 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

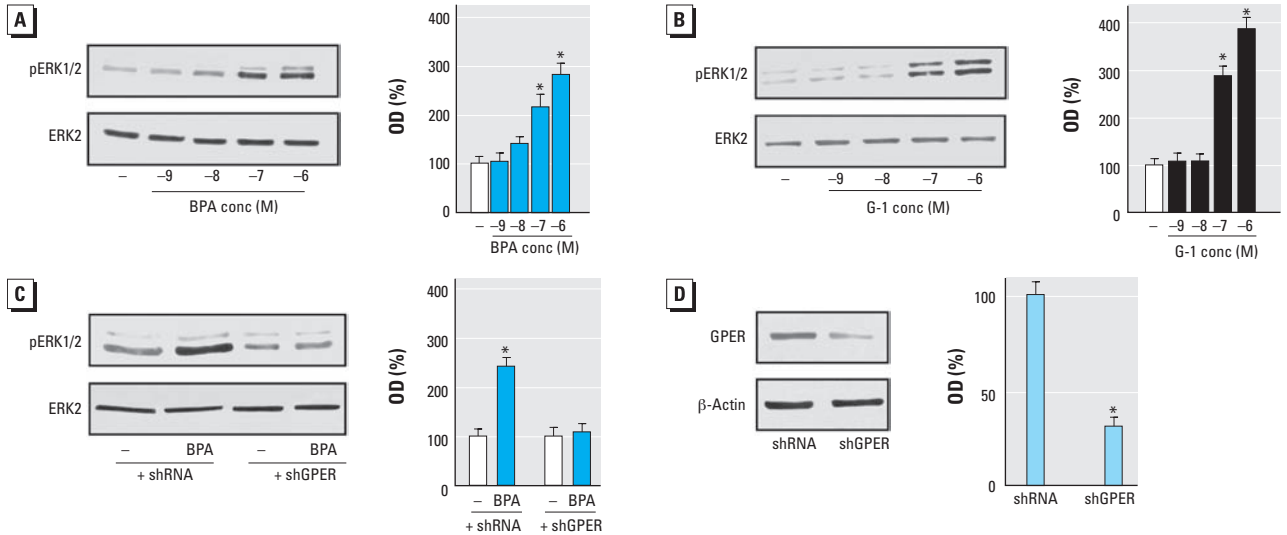

Figure 2. Induction of ERK1/2 phosphorylation (pERK1/2) by BPA and G-1 through GPER in CAFs. conc, concentration. (*A*,*B*) CAFs were treated for 30 min with vehicle (–) or increasing concentrations of BPA (*A*) or G-1 (*B*). (*C*) ERK1/2 phosphorylation in CAFs transfected with shRNA or shGPER and treated 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

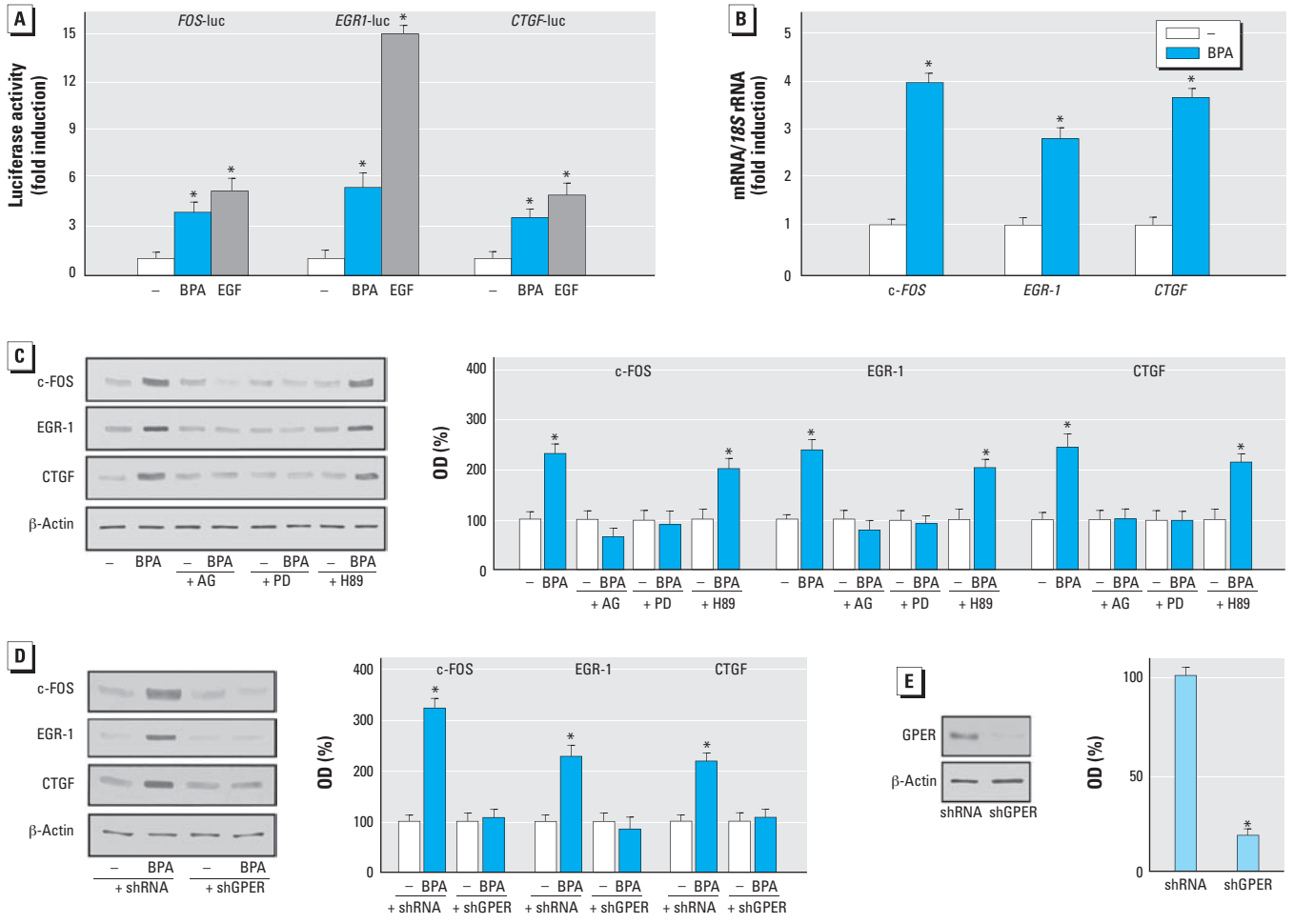

Figure 3. Expression of GPER target genes (*c-FOS*, *EGR-1*, and *CTGF*) in SKBR3 cells in response to BPA treatment. (*A*) Evaluation of *c-FOS*, *EGR-1*, and *CTGF* luciferase reporter genes in transfected SKBR3 cells treated with vehicle (–), 1 μM BPA, or EGF (50 ng/mL; positive control). Luciferase 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

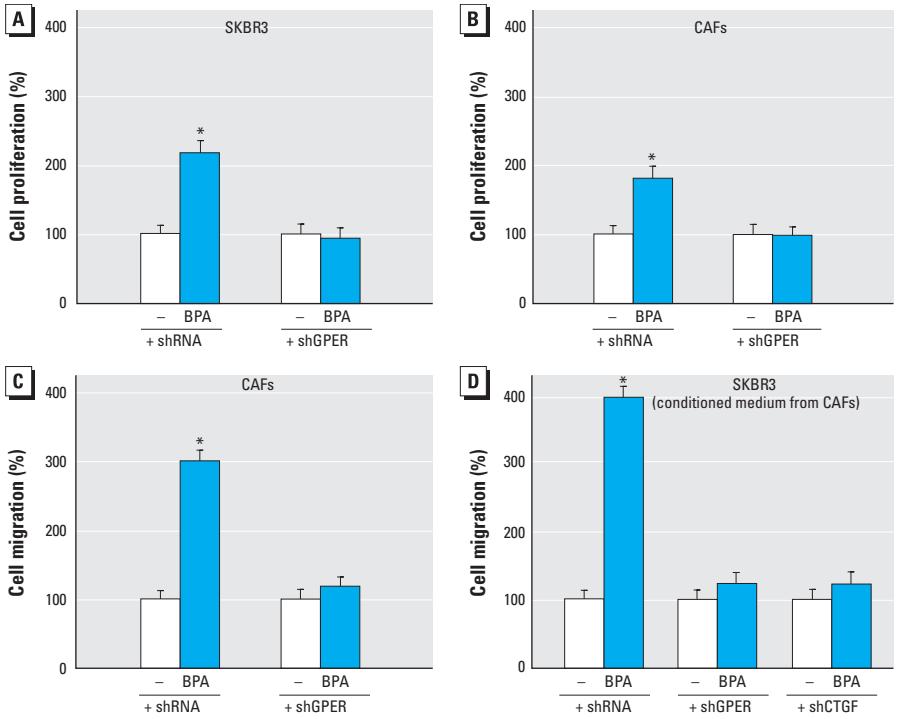

Figure 5. Induction of proliferation and migration in SKBR3 cells and CAFs. (*A*,*B*) Proliferation in SKBR3 cells (*A*) and CAFs (*B*) treated with vehicle (–) or 1 μM BPA for 5 days after silencing GPER expression. (*C*) Migration in CAFs treated with vehicle or 1 μM BPA for 48 hr after silencing GPER 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.

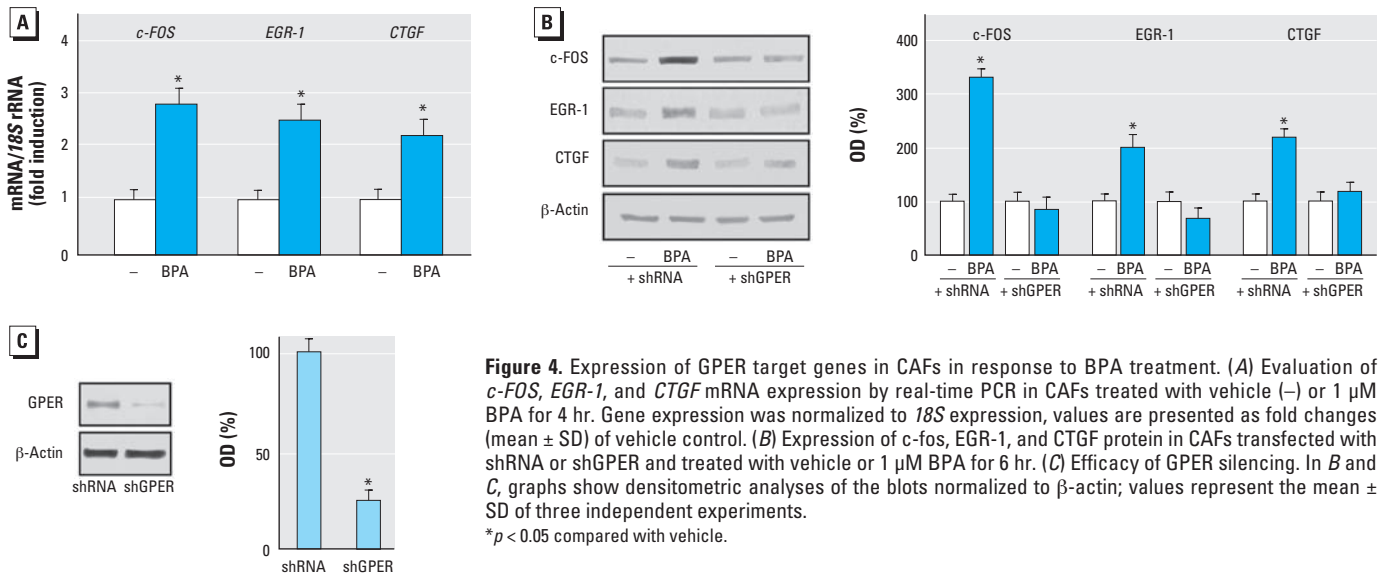

Figure 4. Expression of GPER target genes in CAFs in response to BPA treatment. (*A*) Evaluation of *c-FOS*, *EGR-1*, and *CTGF* mRNA expression by real-time PCR in CAFs treated with vehicle (–) or 1 μM BPA for 4 hr. Gene expression was normalized to *18S* expression, values are presented as fold changes (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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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,

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.

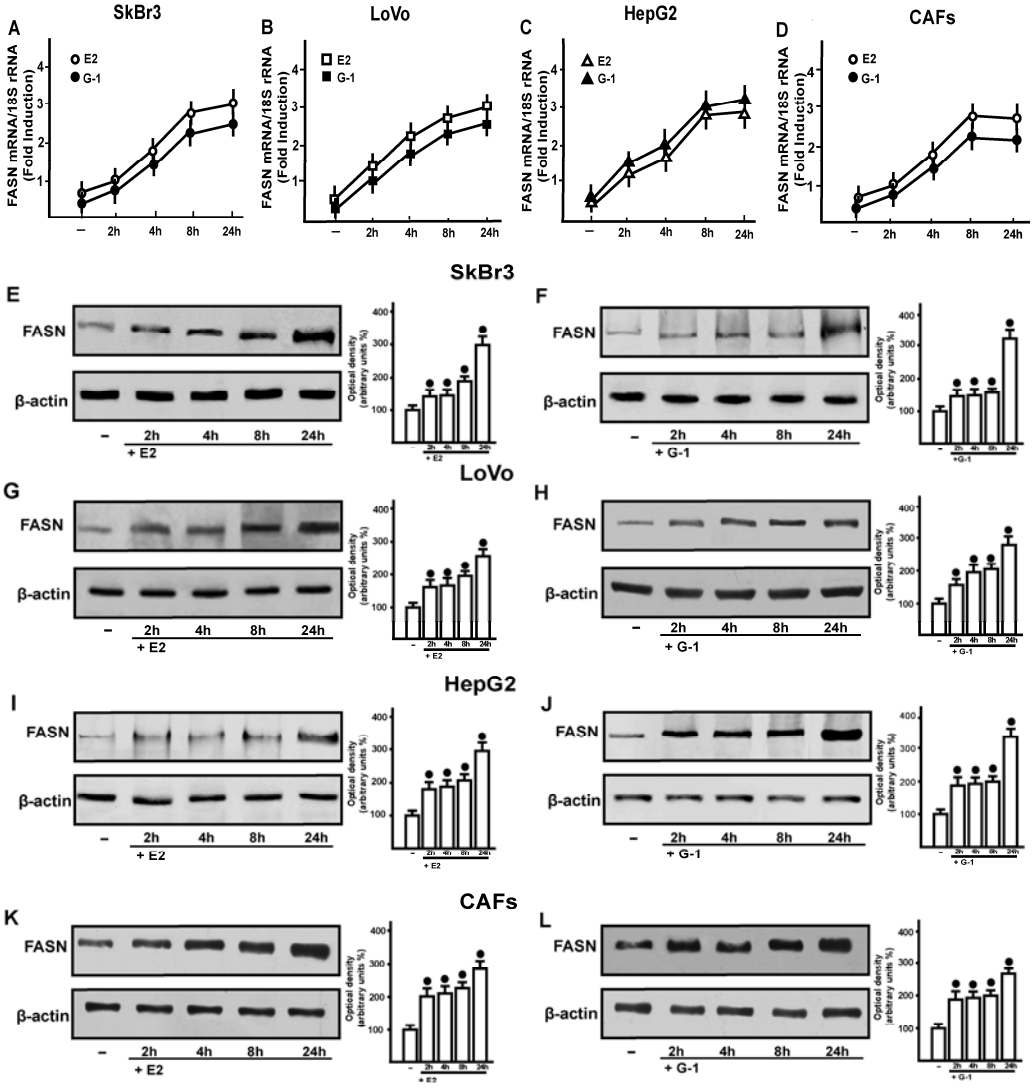

Fig.1

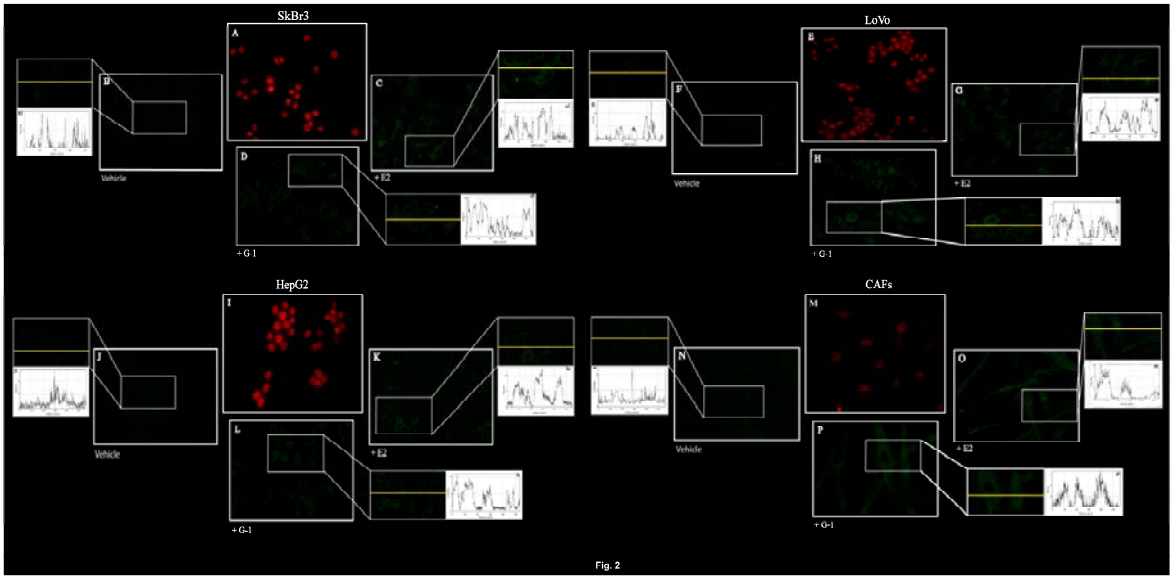

Fig. 2

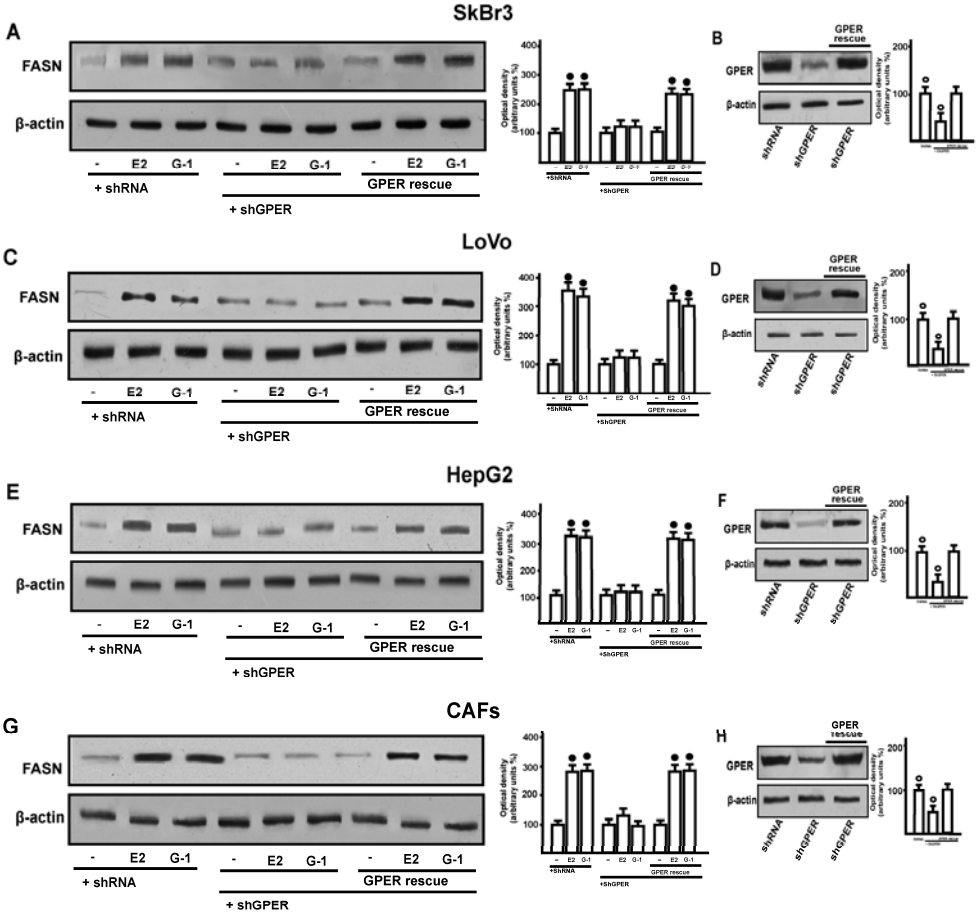

Fig.3

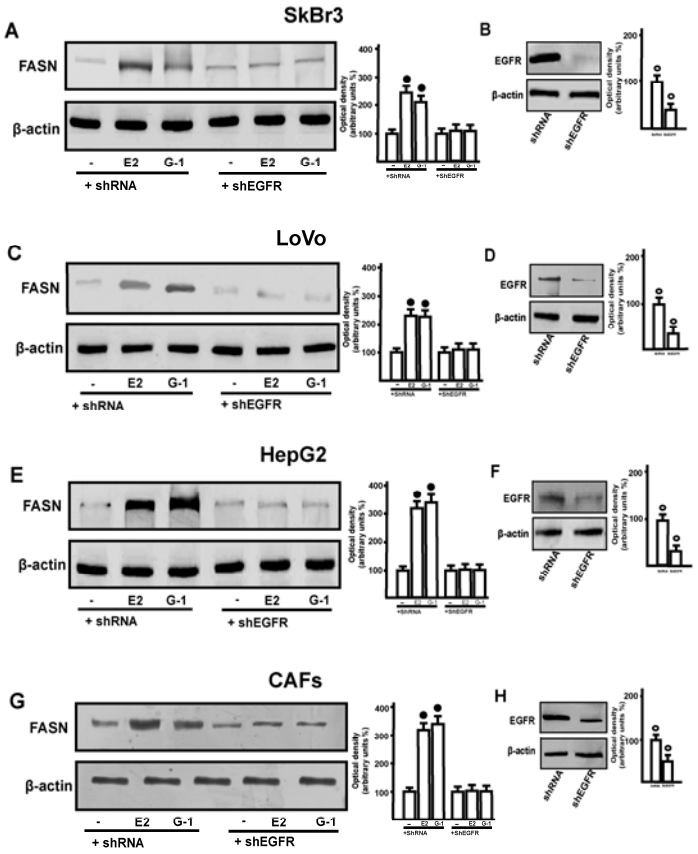

Fig. 4

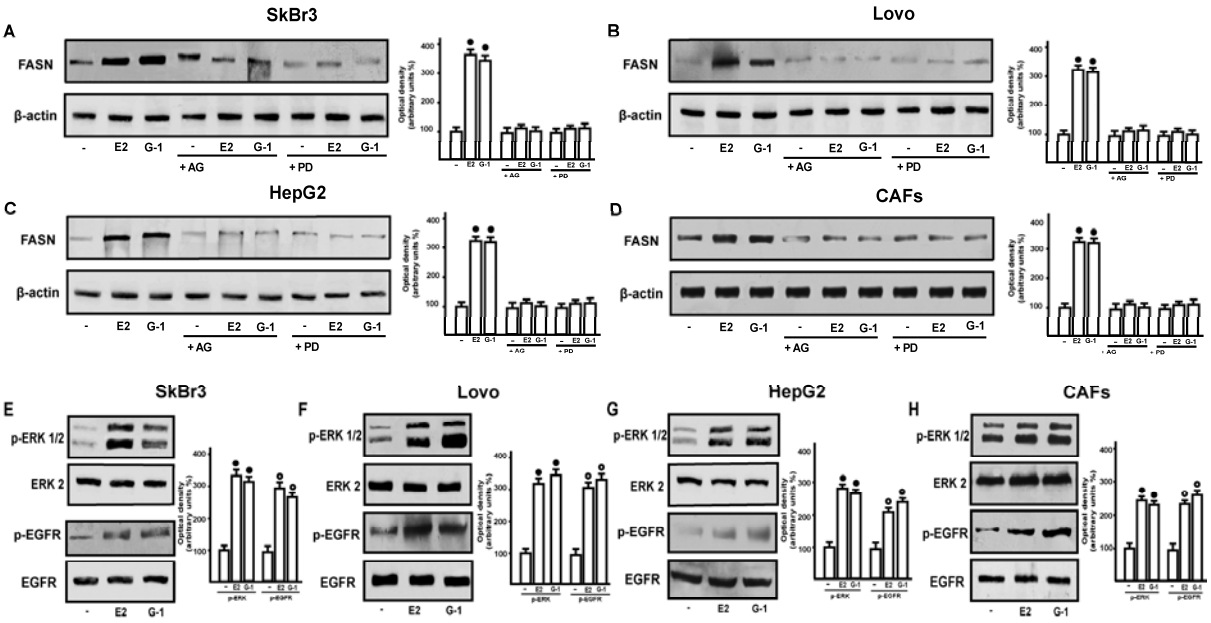

Fig. 5

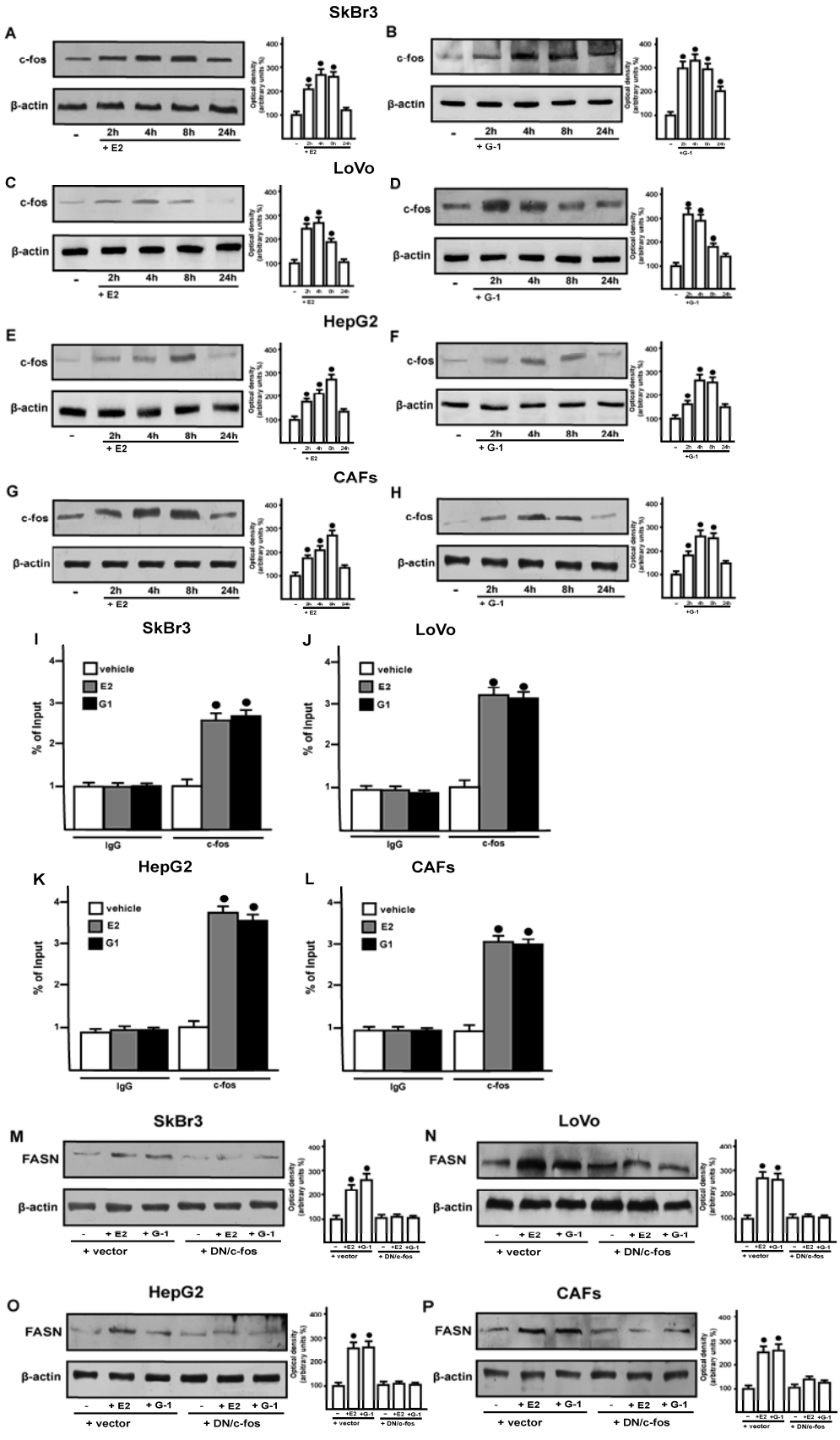

Fig. 6

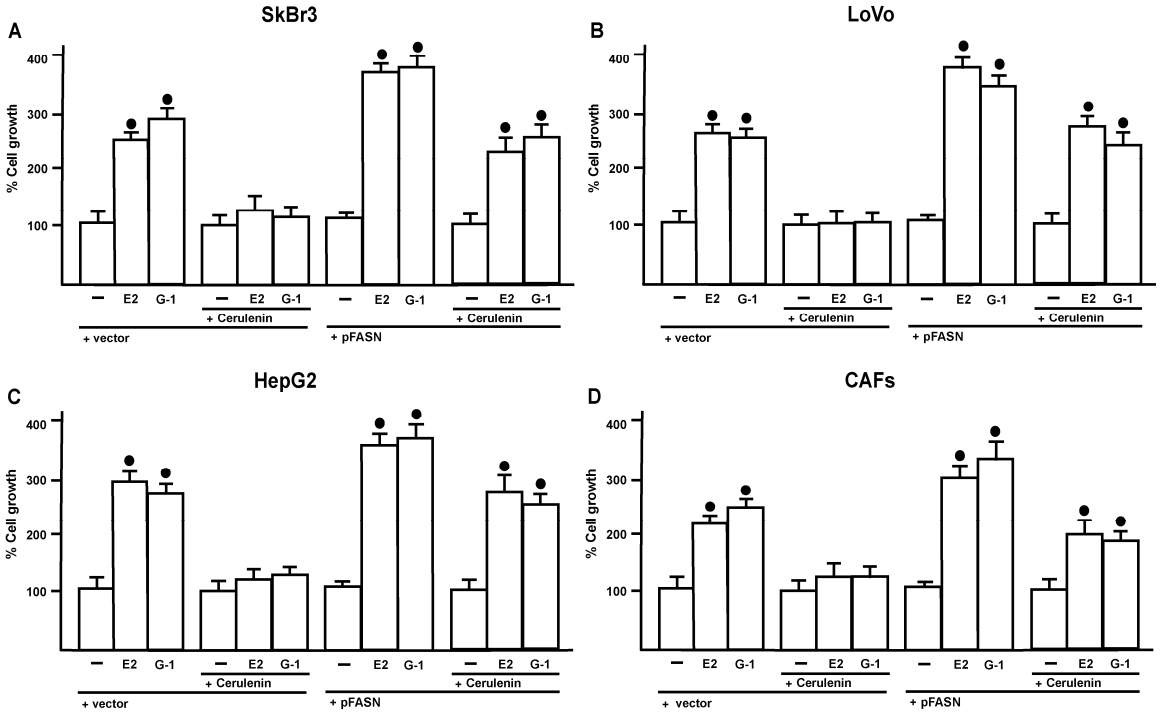

Fig. 7

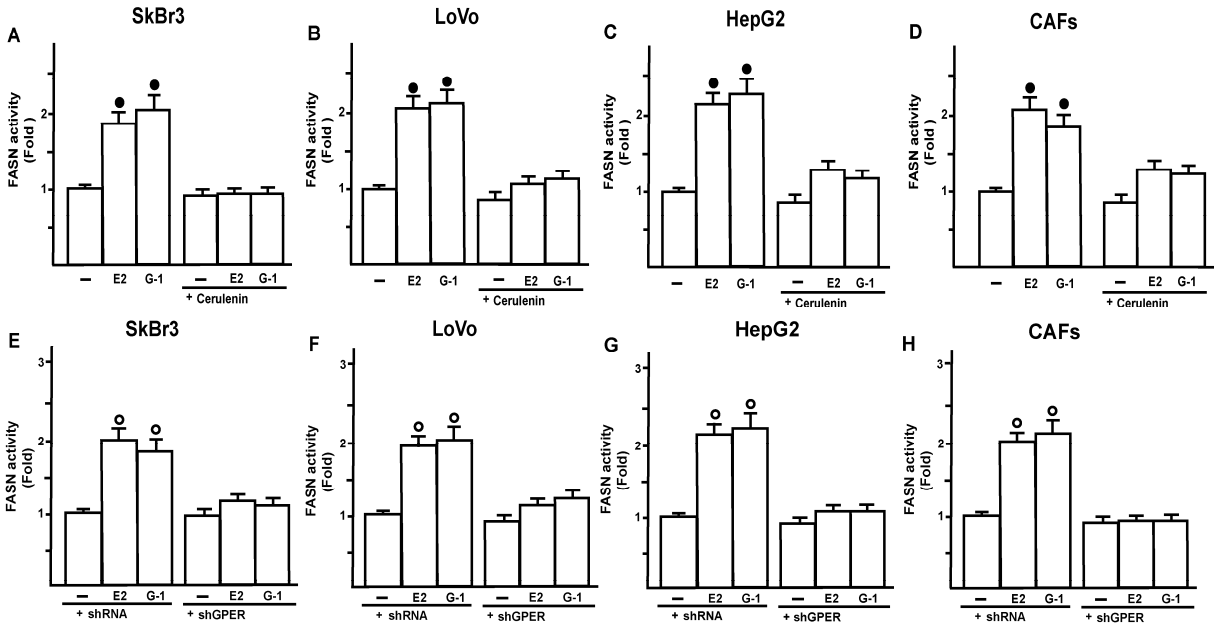

Fig. 8

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.

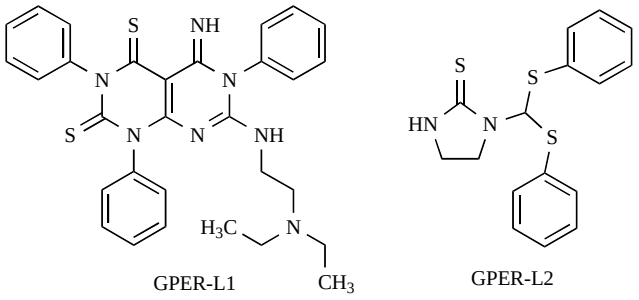

Fig. (1). Chemical structures of GPER-L1 and GPER-L2.

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: CGAGTTAAAGAGGAGAAGG AA, CTCCCTCATTGAGGTGTTCAA, CGCTCCCTGCA AGCAGTCTTT, 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 [^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]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

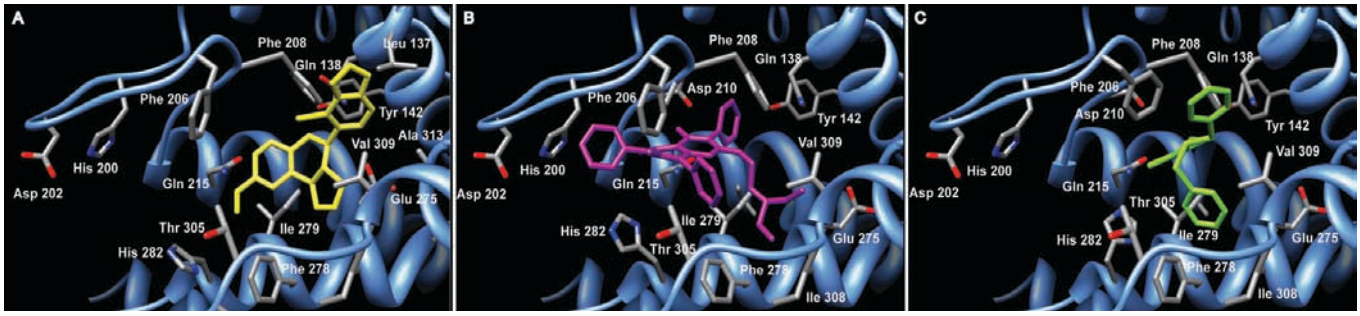

Fig. (2). The three dimensional model of GPER is schematically reported as a light blue ribbon cartoon. The binding modes of the different ligands tested are as following: G-1 in yellow (panel **A**), GPER-L1 in purple (panel **B**), GPER-L2 in light green (panel **C**). Residue positioning is not conserved 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 [^{3}H]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 [^{3}H]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.

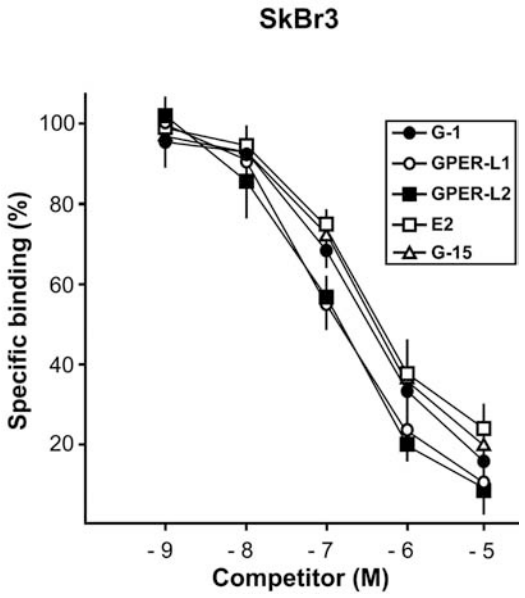

Fig. (3). Binding assay of GPER-L1 and GPER-L2 in SkBr3 cells. Competition curves of increasing concentration of G-1, GPER-L1, GPER-L2, E_2 and G-15 expressed as a percentage of maximum specific [^{3}H]E_2 binding. Each data point represents the mean of three separate experiments performed in triplicate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

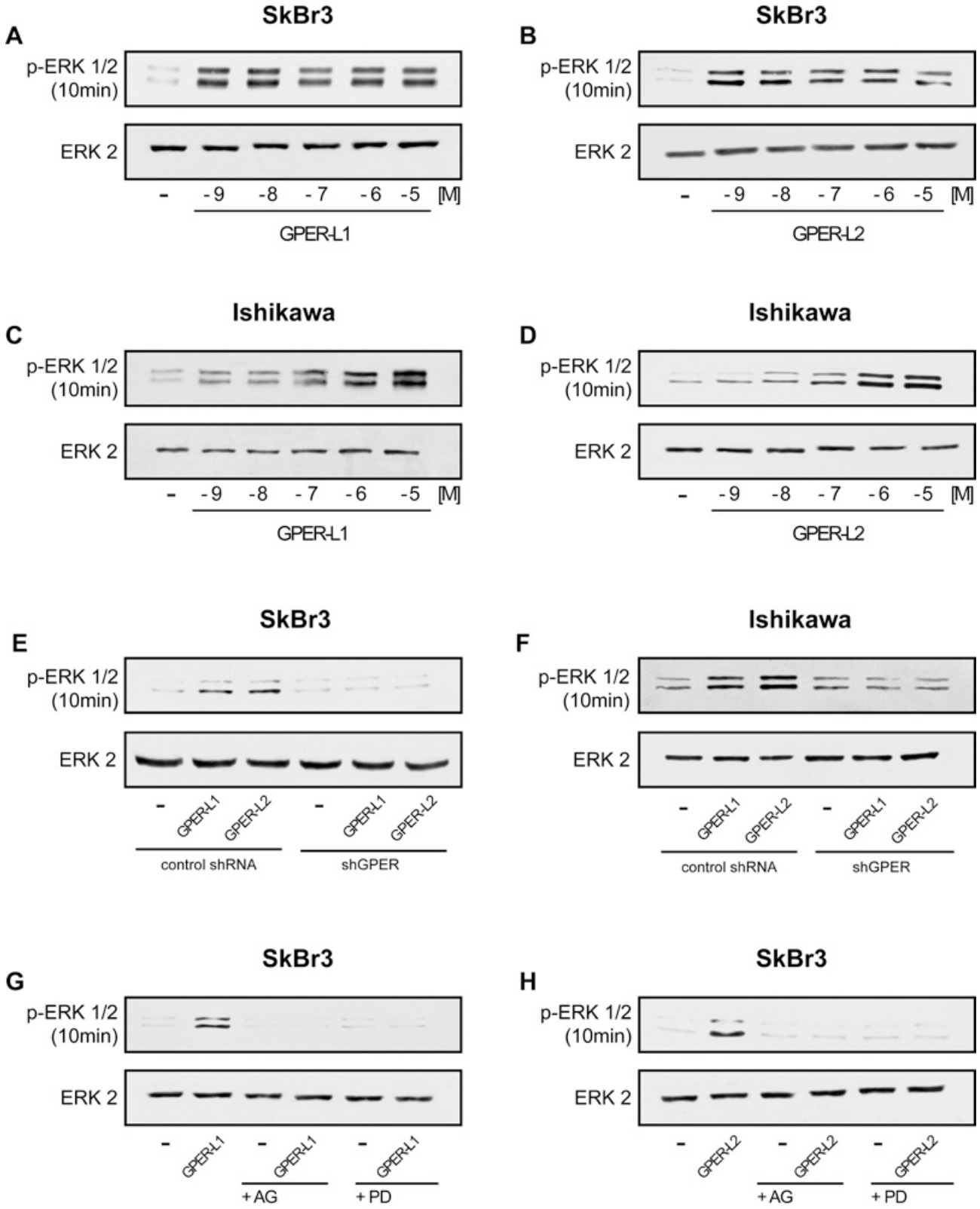

Fig. (4). GPER-L1 and GPER-L2 activate ERK1/2 in a GPER-dependent manner. ERK1/2 phosphorylation (p-ERK1/2) in SkBr3 (**A-B**) and Ishikawa (**C-D**) cells exposed to increasing concentrations of GPER-L1 or GPER-L2 for 10 min. ERK1/2 phosphorylation in SkBr3 (**E**) and Ishikawa (**F**) cells silencing 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.

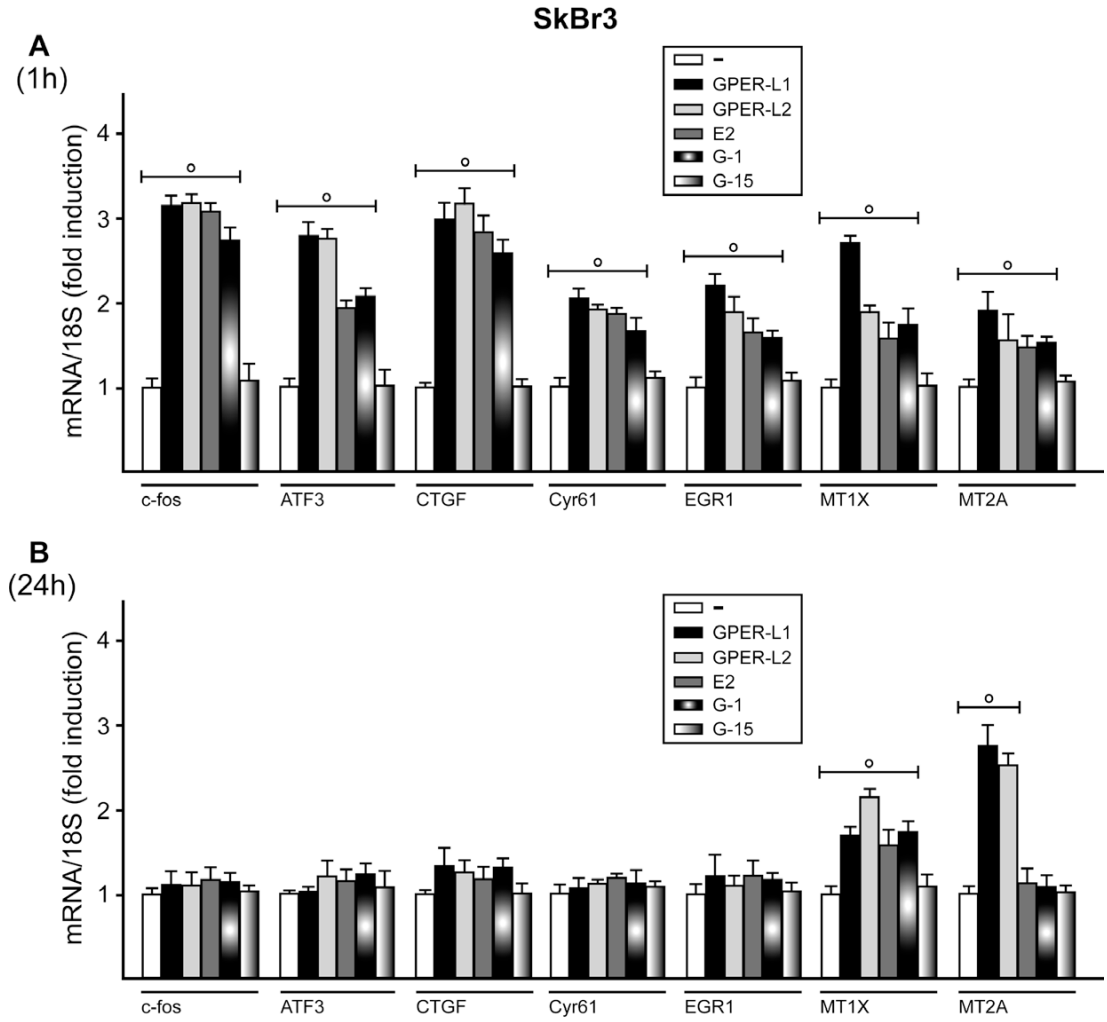

Fig. (5). mRNA expression of GPER target genes evaluated by real-time PCR. SkBr3 cells were treated with vehicle or 100 nM GPER-L1, GPER-L2, E_2, G-1 and G-15 for 1 h (**A**) and 24 h (**B**), as indicated. Results obtained from experiments performed in triplicate were normalized for 18S expression and 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.

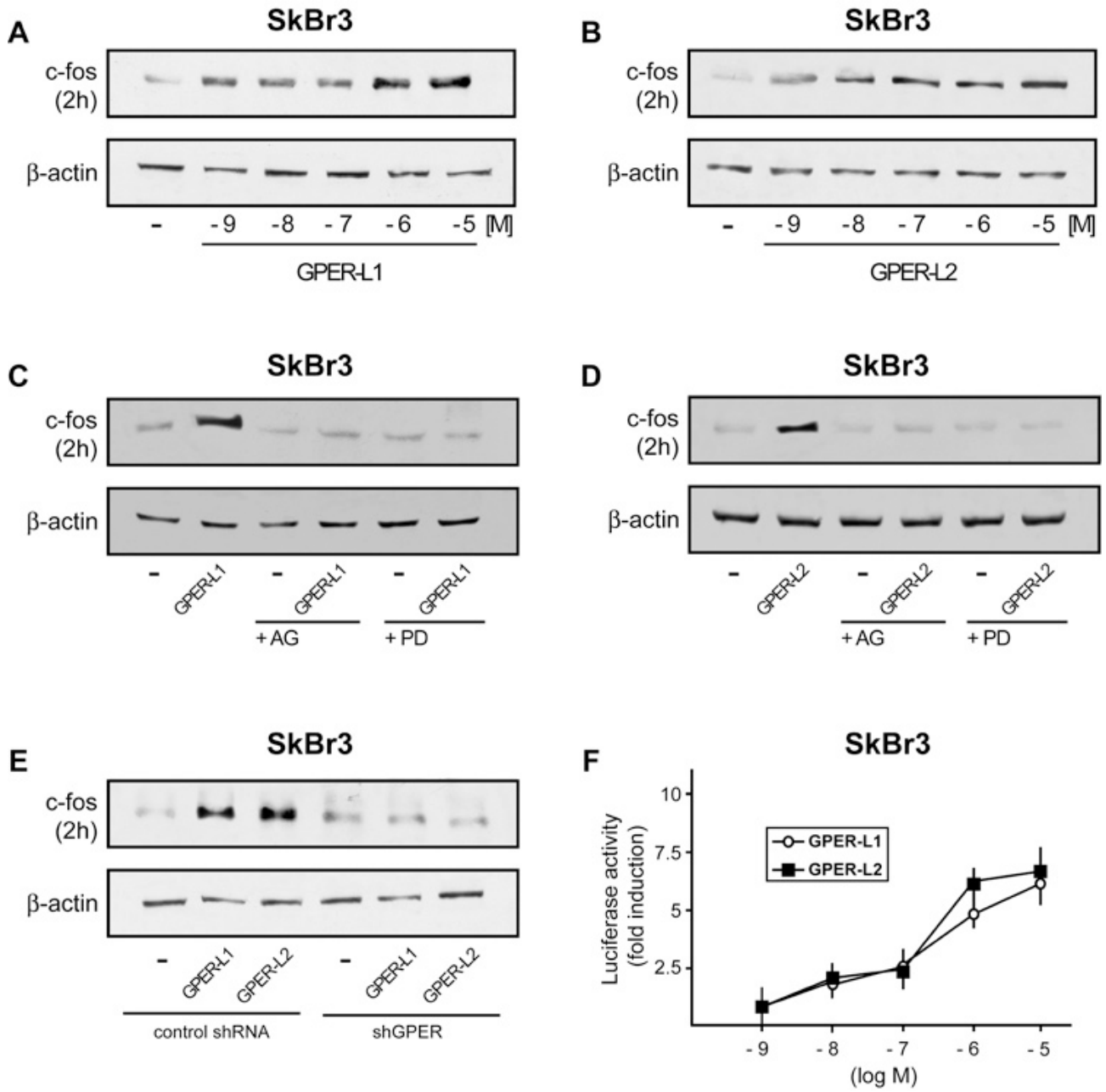

Fig. (6). GPER-L1 and GPER-L2 up-regulate c-fos protein levels in a GPER-dependent manner. (**A-B**) c-fos expression in SkBr3 cells exposed to increasing concentrations of GPER-L1 or GPER-L2 for 2 h. (**C-D**) SkBr3 cells were treated for 2 h with vehicle (–) or 100 nM GPER-L1 or GPER-L2 in combination 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].

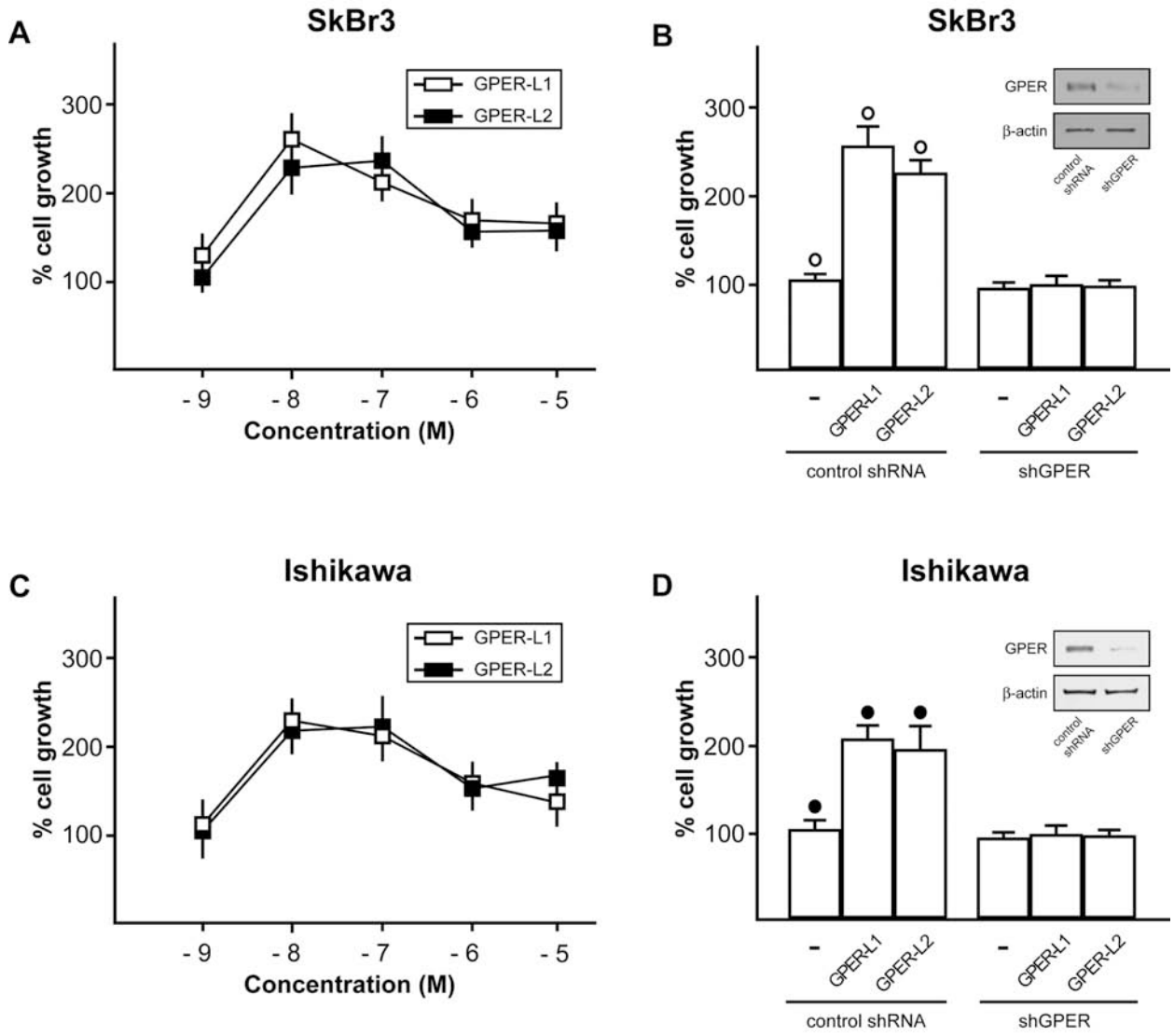

Fig. (7). GPER-L1 and GPER-L2 induce the proliferation of SkBr3 and Ishikawa cells. (**A**,**C**) Cells were treated for 5 days with increasing concentrations of GPER-L1 or GPER-L2 and counted on day 6. Cell proliferation induced by 10 nM GPER-L1 or GPER-L2 was prevented silencing GPER expression with 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

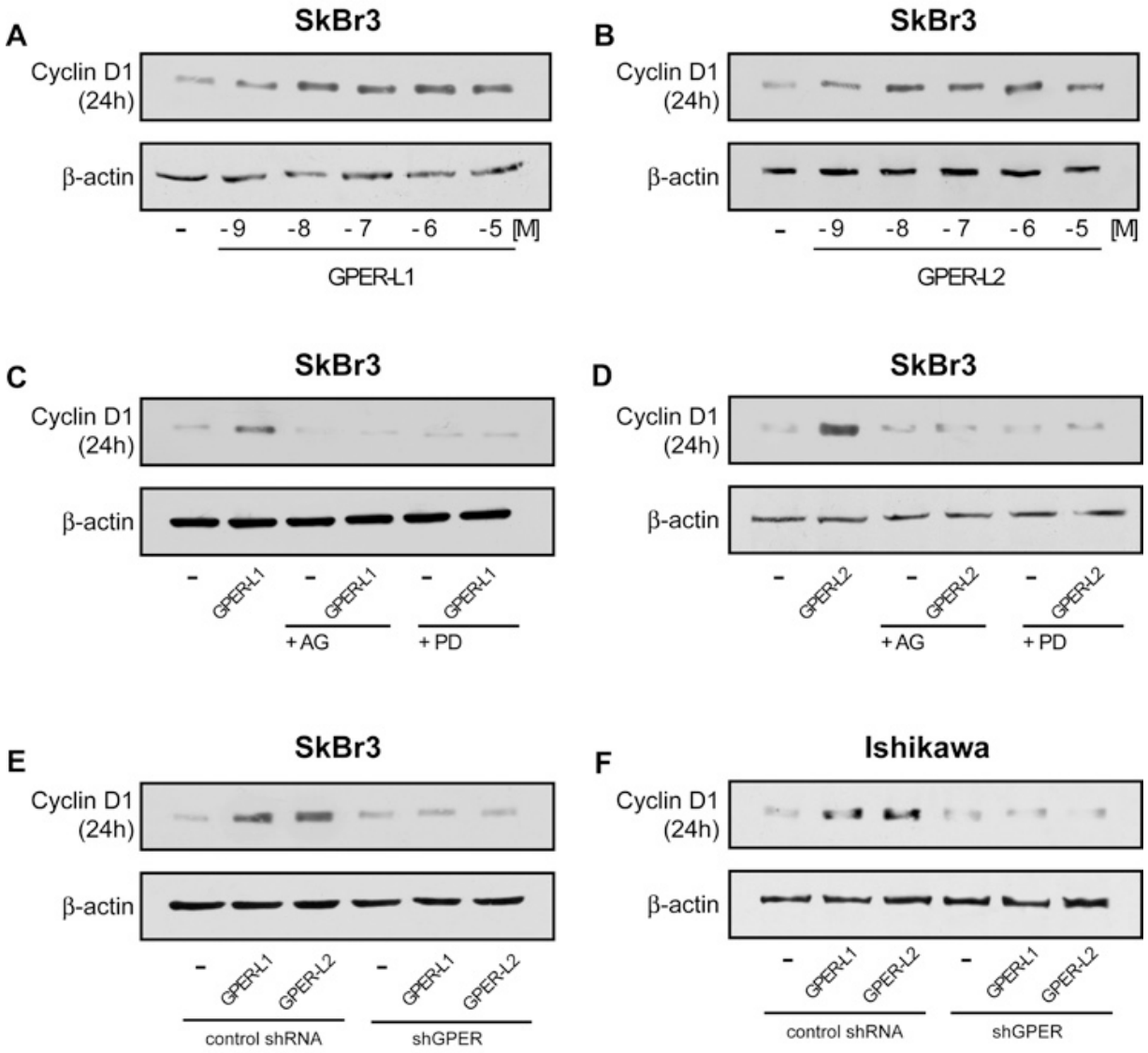

Fig. (8). GPER-L1 and GPER-L2 up-regulate cyclin D1 protein expression in a GPER-dependent manner. (**A**-**B**) Cyclin D1 expression in SkBr3 cells exposed to increasing concentrations of GPER-L1 or GPER-L2 for 24 h. (**C**-**D**) SkBr3 cells were treated for 24 h with vehicle (–) or 10 nM GPER-L1 or 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.

ACKNOWLEDGEMENTS

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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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Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

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

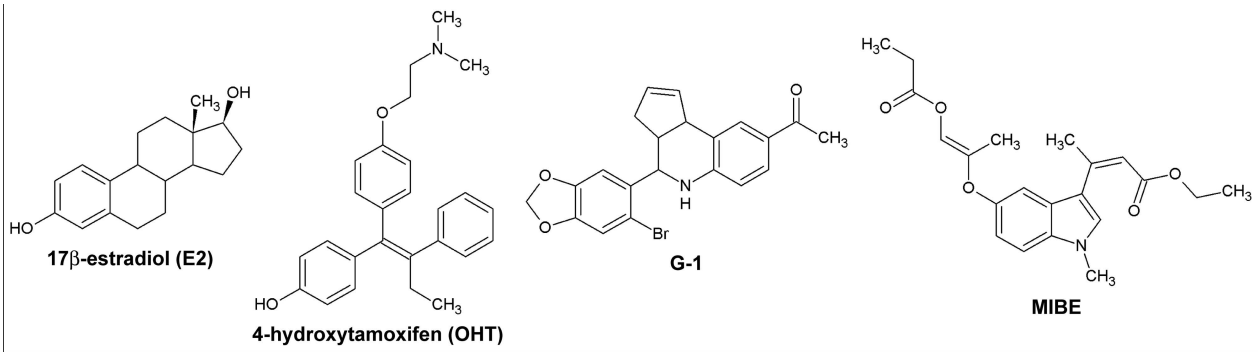

Figure 1 Chemical structures of compounds used. 17beta-estradiol (E2), 4-hydroxytamoxifen (OHT), G-1 and ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate (MIBE).

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

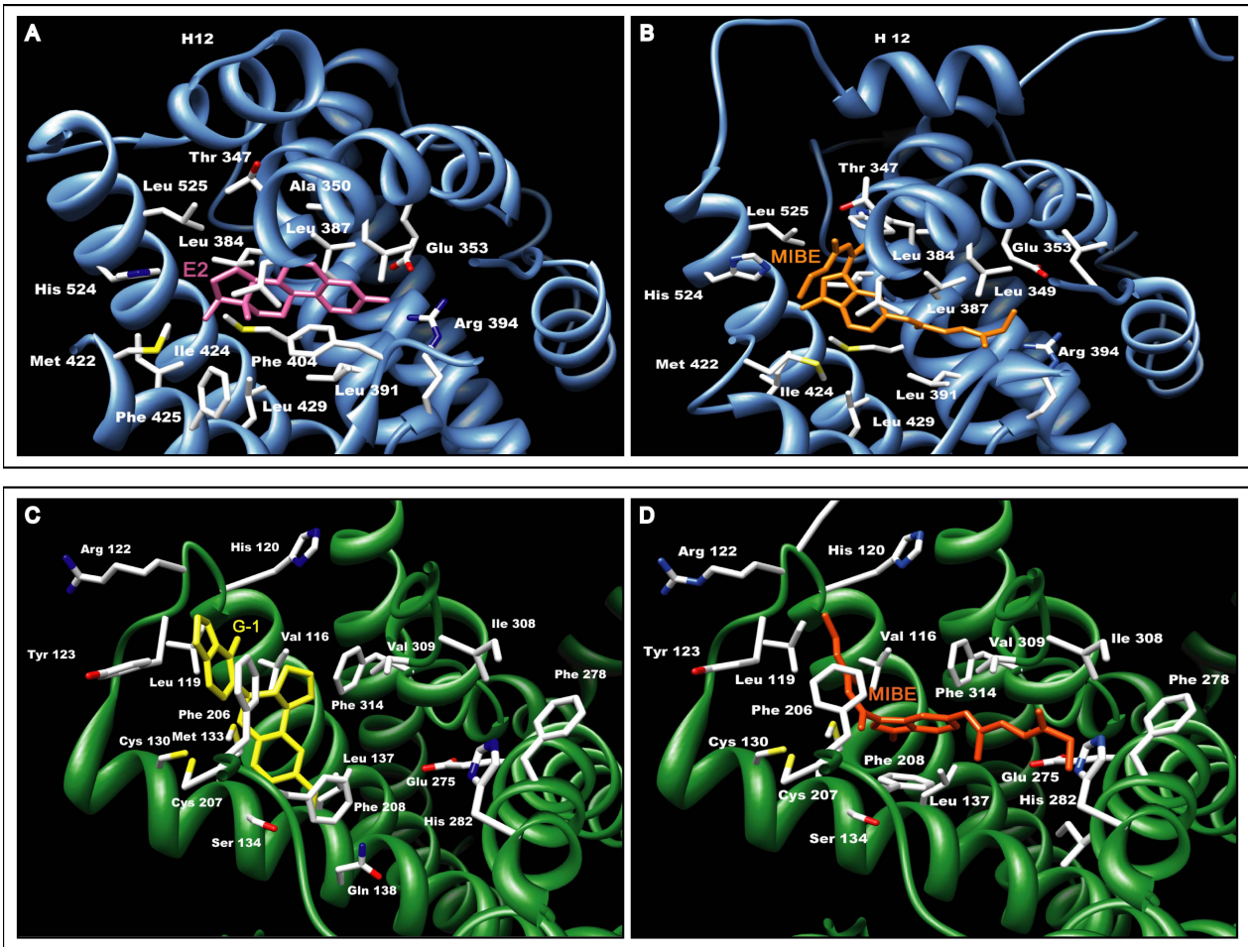

Figure 2 GPER and ERalpha docking simulations. (a-b) The three-dimensional model of ERalpha is schematically reported as a light blue ribbon cartoon; residues involved in ligand binding are drawn as sticks. (a) The binding modes of E2 (pink sticks) to ERalpha in the "closed conformation" is shown. (b) 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

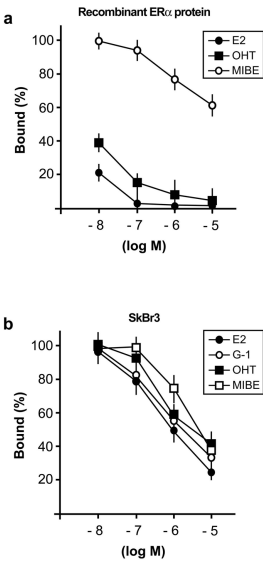

Figure 3 MIBE is a ligand of GPER and ERalpha. (a) MIBE competes with [3H]E2 for the binding to ERalpha. Competitive binding of increasing concentrations of unlabelled E2, OHT and MIBE to recombinant human ERalpha protein. Each data point represents the mean ± SD of triplicate samples of three separate 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

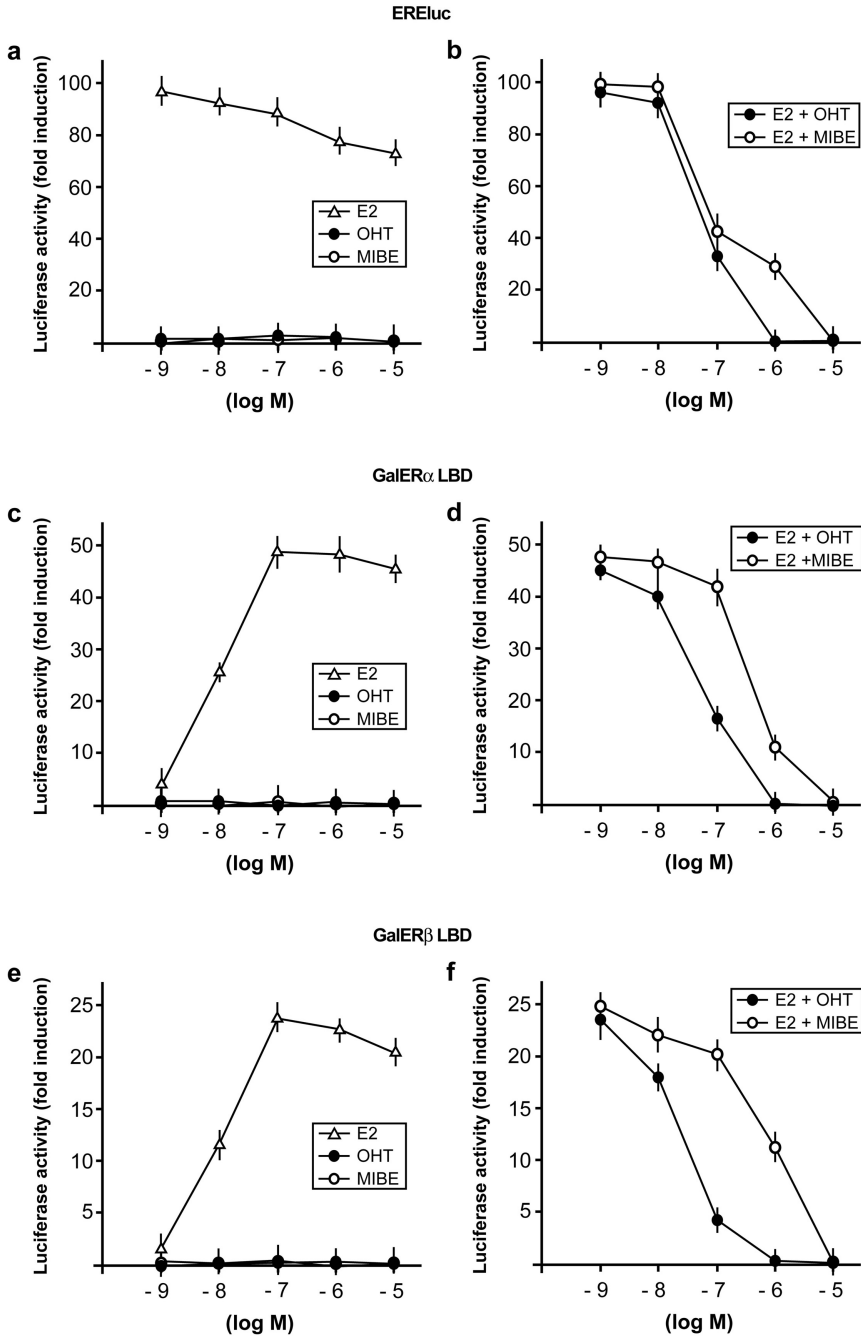

Figure 4 MIBE inhibits the transactivation of ERalpha induced by E2. (a) MCF7 cells were transfected with the ER luciferase reporter gene (EREluc) along with the internal transfection control Renilla Luciferase and treated with increasing concentrations (logarithmic scale) of E2, the ER antagonist OHT 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.

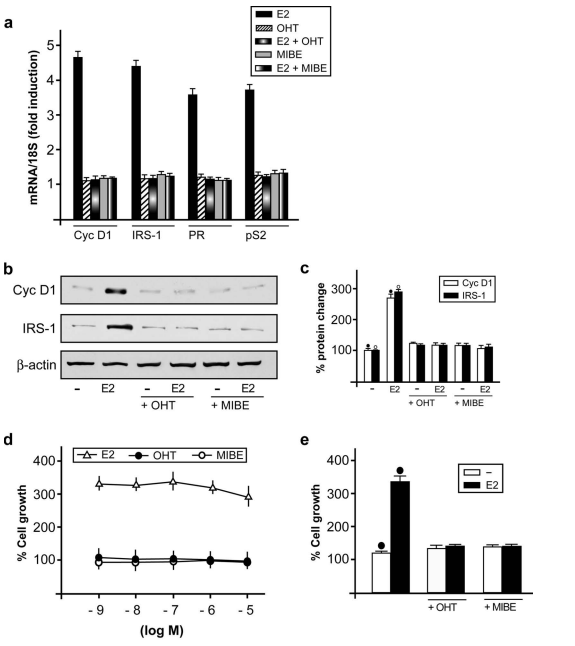

Figure 5 MIBE inhibits gene expression and proliferation induced by E2 in MCF7 cells. (a) Evaluation of mRNA expression of Cyclin D1 (Cyc D1), IRS-1, Progesterone Receptor (PR) and pS2 by real-time PCR in MCF7 cells. Cells were treated for 24 h with vehicle, 10 nM E2, 1 microM OHT and 10 microM MIBE 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

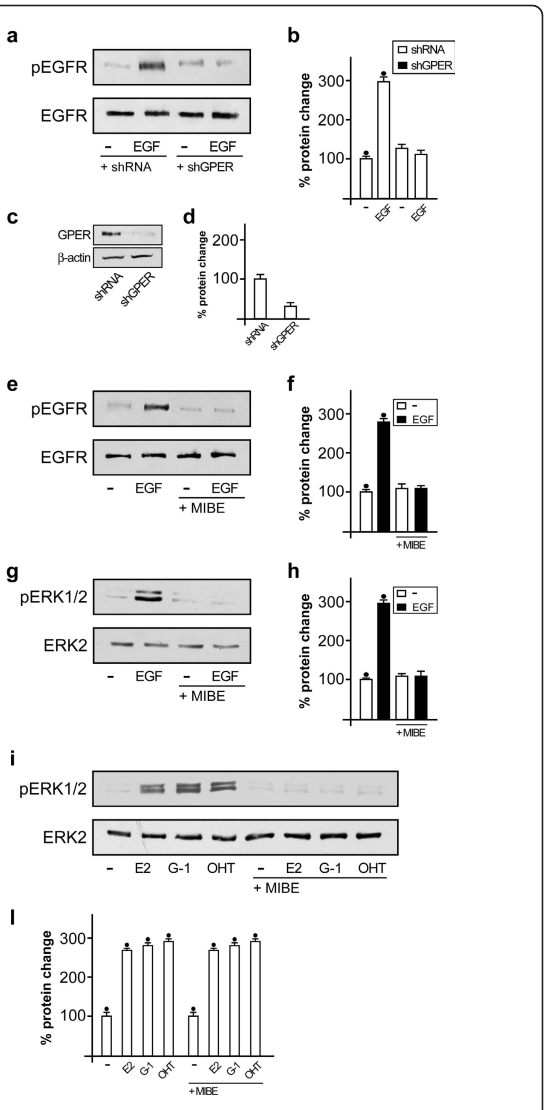

Figure 6 MIBE prevents the phosphorylation of EGFR and ERK1/2. (a) $EGFR^{Tyr1173}$ phosphorylation after treatment (five minutes) with vehicle (-) and 100 ng/ml EGF in SkBr3 cells transfected with shRNA or shGPER. (b) Densitometric analysis of three independent experiments, $EGFR^{Tyr1173}$ expressions 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. (l) Densitometric analysis of three independent experiments. ERK1/2 expressions are normalized to ERK2. (•) indicates $P < 0.05$ for cells receiving vehicle versus treatments.

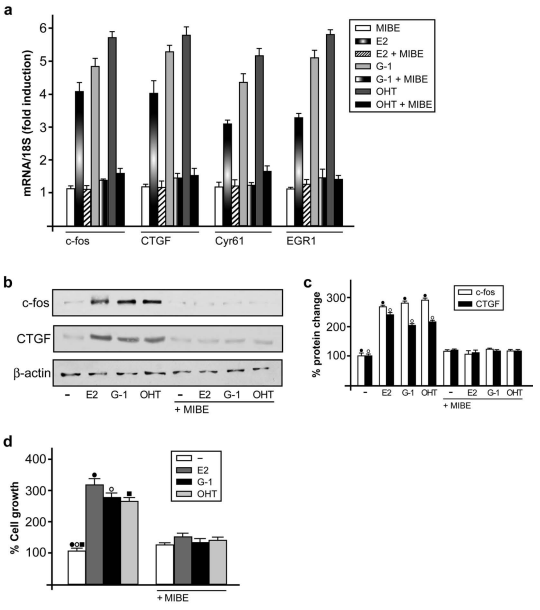

Figure 7 MIBE inhibits GPER target genes and proliferation induced by E2, G-1 and OHT. (a) The expression of c-fos, CTGF, Cyr61 and EGR1 induced in SkBr3 cells by 1 h treatment with 100 nM E2, 1 microM G-1 and 5 microM OHT is inhibited in presence of 10 microM MIBE, as evaluated by real-time PCR. Results 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 anti-estrogens, 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.

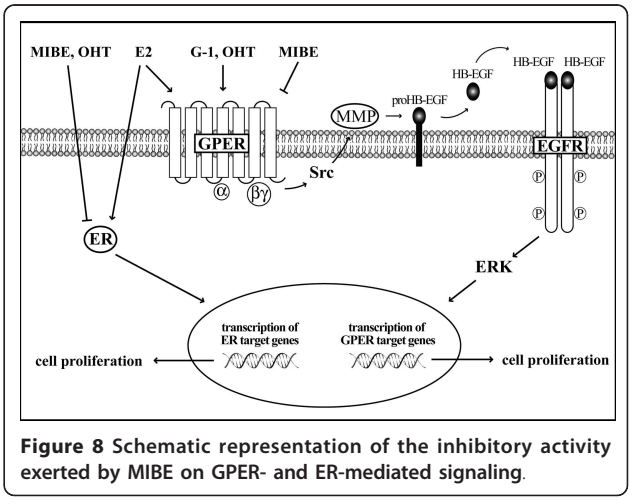

Figure 8 Schematic representation of the inhibitory activity exerted by MIBE on GPER- and ER-mediated signaling.

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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