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Dottorato di Ricerca in Bio-Patologia Molecolare

Tesi di Dottorato

The G protein-coupled receptor GPR30 mediates estrogen signaling in cancer cells

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To my scientific lighthouses
Anna Maria Musti
and
Marcello Maggiolini

"When the going gets tough
The tough get going….."

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List of Abbreviations

17β-Estradiol (E2)

2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine (Atrazine)

(1-[4-(6-bromobenzo[1,3]diodo-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin -8-yl]-ethanone) (G-1)

5α-Dihydrotestosterone (DHT)

4-Hydroxytamoxifen (OHT)

Activating Protein-1 (AP-1)

Activation Function (AF2)

AG1478 (AG)

Dexamethasone (DEX)

DNA-Binding Domain (DBD);

Dominant Negative (DN)

Epidermal Growth Factor (EGF)

Epidermal Growth Factor Receptor (EGFR)

Estrogen Receptor (ER)

Estrogen Response Element (ERE)

Extracellular Signal-Regulated protein Kinase (ERK)

G protein coupled receptor (GPCR)

Genistein (G)

GF109203X (GFX)

Heat shock protein (hsp)

Hormone Response Elements (HRE)

ICI 182,780 (ICI)

Insulin-Like Growth Factor-1 (IGF-1)

Ligand-Binding Domain (LBD)

LY 294,002 (LY)

Mitogen Activated Protein Kinase (MAPK)

PD98059 (PD)

Phosphatidylinositol 3-kinase (PI3K)

Progesterone Receptor (PR)

Progesterone (PRG)

Pertussis Toxin (PT)

Serum Response Factor (SRF)

Tamoxifen (TAM)

Ternary Complex Factor (TCF)

Transactivation Domain (AF-1)

Wortmannin (WM)

SOMMARIO

Gli estrogeni (E2) regolano la crescita e la differenziazione di molti tessuti. Agendo da mitogeni sono inoltre in grado di promuovere lo sviluppo di tumori estrogeno-sensibili come il tumore mammario ed ovarico. Gli effetti biologici degli estrogeni sono mediati dal Recettore Estrogenico (ER) α e β, che agendo da fattori di trascrizione, legano le Sequenze Responsive agli Estrogeni (ERE) presenti nelle regioni promoter di geni target. Diversi studi hanno dimostrato che segnali estrogenici possono alterare l'espressione genica attraverso meccanismi indipendenti dai classici ERs. In particolare, è stato visto che gli estrogeni possono indurre importanti effetti biologici attraverso il recettore di membrana GPR30 che è in grado di attivare la via trasduzionale del Recettore dell' Epidermal Growth Factor (EGFR). Se gli estrogeni agiscono sul pathway trasduzionale EGFR/ERK solo mediante il legame con GPR30 o attivando anche ERα è poco chiaro, poichè gli estrogeni sono in grado di legare entrambi i recettori. La recente identificazione di un ligando selettivo per GPR30, G-1, ha fornito nuove possibilità per differenziare le funzioni di ERs e GPR30. Per valutare i meccanismi molecolari coinvolti nell'azione proliferativa degli estrogeni attraverso GPR30, abbiamo analizzato la capacità di G-1 di indurre effetti proliferativi in cellule tumorali ovariche estrogeno-sensibili, esprimenti ERα e in cellule tumorali mammarie estrogeno-sensibili ma non esprimenti ERα. Abbiamo inoltre valutato gli effetti di G-1 sull'attivazione delle ERK e sull'induzione di c-fos e altri geni coinvolti nella progressione del ciclo cellulare. Abbiamo dimostrato che G-1 induce la proliferazione di entrambe le linee tumorali, indipendentemente dalla presenza di ERα. Considerato che in cellule tumorali ovariche l'espressione di ERα è richiesta sia per la proliferazione cellulare che per l'induzione di c-fos e l'attivazione delle ERK, la capacità di GPR30 di mediare segnali estrogenici indipendentemente da ERα potrebbe costituire una condizione di adattamento funzionale in cellule tumorali ERα negative.

In un ulteriore studio abbiamo valutato la regolazione dell'espressione di GPR30 in cellule tumorali mammarie ER-negative SKBR3. Trasfezioni transienti effettuate con un plasmide codificante per la regione promotore di GPR30 hanno mostrato che un sito AP-1 situato nella regione è necessario per l'attivazione del promoter di GPR30 in risposta al trattamento con EGF. EGF è inoltre in grado di up-regolare i livelli proteici di GPR30 che si accumulano prevalentemente nel compartimento intracellulare. Questo effetto stimolatorio indotto dall'EGF sull'espressione di GPR30 è innescato attraverso la rapida fosforilazione delle ERK e l'induzione di c-fos. Inoltre sia l'abrogazione dell'espressione di GPR30 che un dominante negativo di c-fos hanno ridotto la proliferazione cellulare indotta dall'E2 nelle cellule SKBR3 e BT20.

Abbiamo infine dimostrato che GPR30 è coinvolto nell'induzione degli effetti stimolatori esercitati dall'atrazina in cellule tumorali ovariche. L'atrazina è il più comune contaminante di falde acquifere e di acque di superficie. Fra gli effetti sul sistema endocrino, è stato descritto come l'atrazina interferisca nei processi mediati da androgeni ed estrogeni. Studi di binding hanno dimostrato che tali fenomeni sono indotti dall'atrazina in assenza di un diretto agonismo o antagonismo sui recettori steroidei. Utilizzando come sistema modello la linea di tumore ovarico BG-1 abbiamo dimostrato che l'atrazina non è in grado di indurre alcun effetto diretto attraverso ERα. ma stimola la fosforilazione delle ERK e l'espressione di c-fos, fenomeni aboliti dall'antagonista di ERs ICI 182,780, dall'inibitore delle MAPK, PD98059 e dall'inibitore del recettore dell'EGF AG1478. Silenziando l'espressione di ERα e di GPR30 la fosforilazione delle ERK e l'induzione di c-fos venivano notevolmente ridotte così come la proliferazione cellulare indotta dall'atrazina.

I nostri studi su GPR30 hanno consentito nuove conoscenze sui meccanismi molecolari coinvolti dagli estrogeni nella progressione di tumori ormono-sensibili.

SUMMARY

Estrogens are pleiotropic hormones that regulate the growth and differentiation of many tissues. By acting as mitogens they also promote the development of breast and ovarian tumors. The biological effects of estrogens are classically mediated by the estrogen receptor (ER)s α and β which function as hormone-inducible transcription factors binding to the estrogen-responsive element (ERE) located within the promoter region of target genes. Several studies have demonstrated that membrane-associated estrogen signals may alter gene expression through non-genomic mechanisms that are independent of nuclear ERs. In particular, it has been shown that estrogens can signal through the membrane G-protein coupled receptor 30 (GPR30). GPR30 mediates non genomic signaling of E2 in a variety of estrogen-sensitive cancer cells through activation of the Epidermal Growth Factor Receptor (EGFR) pathway.

Whether E2 acts on the EGFR/ERK transduction pathway only through GPR30 binding or also through ERα binding is less clear, since E2 binds to both receptors although with different affinity. G-1 is the first well-known GPR30-selective ligand and its recent identification has provided new opportunities to differentiate between ERs and GPR30 function. To better understand the molecular mechanisms involved in the proliferative action of E2-GPR30 signaling, we evaluated the ability of G-1 to induce cell growth of E2-responsive ovarian cancer cells expressing ERα as well as of E2-responsive breast cancer cells not expressing ERα. We have also investigated the effect of G-1 on ERK activation and on induction of c-fos and other genes involved in the progression of the cell cycle. We found that G-1 induces the proliferation of both positive and negative ERα cancer cells. However, in ovarian tumor cells, ERα expression was required for cell proliferation as well as for c-fos stimulation and ERK activation, suggesting that the capacity of GPR30 to signal independently of ERα is a specific feature of ERα negative tumors. Next, we investigated the molecular mechanism involved in GPR30 expression. To this end we assessed GPR30 expression and promoter activity in SkBr3 and BT20 breast cancer cells

(lacking the classical ERs), by either E2, or G-1, or Insulin like Growth Factor-I (IGF-I), or Epidermal Growth Factor (EGF). Transient transfections with an expression plasmid encoding a short 5'-flanking sequence of the GPR30 gene revealed that an AP-1 site located within this region is required for the GPR30 promoter activity in response to EGF. Accordingly, EGF up-regulated GPR30 protein levels, which accumulated predominantly in the intracellular compartment. The stimulatory role elicited by EGF on GPR30 expression was triggered through rapid ERK phosphorylation and c-fos induction which was strongly recruited to the AP-1 site found in the short 5'-flanking sequence of the GPR30 gene. Furthermore, either the abrogation of GPR30 expression or the expression of Dominant Negative DN/c-fos reduced E2-dependent proliferation of SkBr3 and BT20 cancer cells.

After having investigated the molecular mechanisms linking E2/GPR30 signaling to cancer cell proliferation, we examined whether such a pathway is also involved in the cancerogenic effect of the synthetic compound atrazine. Atrazine is the most common pesticide contaminant of groundwater and surface water. Among the endocrine-disrupting effects, atrazine interferes with androgen- and estrogen-mediated processes. Based on binding affinity studies, this occurs without direct agonism or antagonism of the cognate receptors for these steroids. Epidemiologic studies have associated long-term exposure to triazine herbicides with an increased risk of ovarian cancer in female farm workers in Italy.

We used BG-1 ovarian cancer cells as a model system and found that ERα was modulated at both mRNA and protein levels by E2, whereas atrazine did not produce any effect and did not show any binding affinity for ERα. Furthermore, atrazine did not stimulate aromatase activity in BG-1 cells, but was able to induce ERK phosphorylation (which was abolished by the ER antagonist ICI182,780, PD98059 and AG1478) and c-fos expression. Both ERK and c-fos stimulation induced by atrazine were abolished knocking-down ERα and GPR30 in BG-1 cancer cells. Furthermore, we found that atrazine induced ovarian cancer cell proliferation, which was inhibited by silencing the

expression of either GPR30 or ERα.

Our results have contributed to provide new insights into the molecular mechanisms implicated in tumor progression.

INTRODUCTION

Estrogens (E2) are important hormones in mammalian physiology, regulating the development and homeostasis of many organs. Estrogen is the best characterized member of the family of steroid hormones that includes progesterone, testosterone, glucocorticoids, and mineralocorticoids. The highly hydrophobic nature of steroid ligands allows them to pass through cellular membranes by passive diffusion. Estrogen action is required for normal development and growth of female reproductive tissue (Couse and Korach 1999), but also to regulate bone integrity (Termine and Wong 1998), cardiovascular function (Guzzo 2000), the central nervous system (Hurn and Macrae 2000) and the immune system (Kovacs et al., 2002).

The major estrogen-producing organ is the ovary but recent studies have revealed the synthesis of estrogen at multiple discrete sites where it may have highly localized effects (Baquedano et al,. 2007). Plasma concentrations of estrogen in women are commonly in the 1 nM range, although the normal concentration in breast tissue of postmenopausal women, has been reported to be 10-20-fold higher than serum concentration, suggesting local production or concentration of the hormone (Geisler 2003). The biological effects of estrogens are mediated by a specific nuclear receptor (ER) that recognizes and binds the hormone, transmitting this information to downstream effectors. The first described ER, ERα, was characterized in 1973 on the basis of specific binding activity in rat uterus/vagina extracts (Jensen and Desombre 1973). Its DNA sequence was determined in 1986 (Greene et al., 1986) and the first crystal structure of an ER ligand-binding domain was described in 1997 (Brzozowski et

al., 1997). A second related ER, ERβ was identified in 1996 (Kuiper et al. 1997). The ERs are coded from two separate genes: ERα is located at chromosomal locus 6q25.1 (Menasce et al., 1993), and encodes a 66kDa protein of 595 amino acids, whereas ERβ is found at position 14q22-24 (Enmark et al., 1997) encoding a 54kDa protein of 485 aminoacids.

As for the other members of the steroid/thyroid hormone superfamily of nuclear receptors, ERα and ERβ are composed of three independent but interacting functional domains: the NH2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (Nilsson et al, 2001). Binding of a ligand to ER triggers conformational changes in the receptor and this leads to changes in the rate of transcription of estrogen-regulated genes. These events include receptor dimerization, receptor-DNA interaction, recruitment of and interaction with co-activators and other transcription factors, and formation of a pre-initiation complex (Nilsson, 2001). The N-terminal domain of nuclear receptors encodes a ligand-independent activation function (AF1) involved in protein-protein interactions, and transcriptional activation of target-gene expression. Comparison of the AF1 domains of the two ERs has revealed that, in ERα, this domain is very active in stimulation of reporter-gene expression from a variety of estrogen response element (ERE)-reporter constructs, in different cell lines (Cowley and Parker, 1999). Differences in the NH2-terminal regions of ERα and ERβ may explain the differences between the two receptors in their response to various ligands. In ERα, two distinct parts of AF1 are required for agonism of E2 and the partial agonism of tamoxifen,

respectively (McDonnel et al, 1995) whereas in ERβ, this dual function of AF1 is missing (McInerney et al, 1998). The DBD contains a two zinc finger structure, important in receptor dimerization and in binding of receptors to specific DNA sequences (Nilsson et al, 2001). The DBDs of ERα and ERβ are highly homologous (Nilsson et al, 2001). In particular, the P box sequence, critical for target-DNA recognition and specificity, is identical in the two receptors. Thus ERα and ERβ can be expected to bind to various EREs with similar specificity and affinity.

The COOH-terminal, E/F-, or LBD mediates ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene expression (Nilsson et al, 2001).

The ligand binding domains (LBD) of ERα and ERβ share a high degree of homology in their primary amino acid sequence and are also very similar in their tertiary architecture. Many compounds tested so far bind to ERα and ERβ with similar affinities and similar potencies in activation of ERE-mediated reporter gene expression (Kuiper et al, 1998). ERβ shares considerable homology in the DNA binding region (97%) with ERα, while this homology is markedly lower (55%) in the LBD, but the trans-activation mode of action of both ERs, is similar (Petterson et al., 1997). In the absence of its cognate ligand, ERs are recovered in the cytosolic fraction of target cell homogenates in inactive untransformed hetero-oligomeric complexes which contain one steroid-binding subunit and a non steroid, non-DNA-binding component, identified as a heat shock protein (hsp90). An important physiologic role for hsp90 is that of

maintaining the receptor in a non functional state: interaction of hsp90 and LBD of the receptor, would interfere with several LBD and DNA binding domain (DBD) functions, resulting in the repression of the transcriptional activity of ER (Picard 1990 and 2002; Pratt 2003). Another essential characteristic of hsp90 is to mediate receptor trafficking from the cytoplasmatic fraction to the nucleus, through a microtubule dependent mechanism (Pratt and Toft 1990).

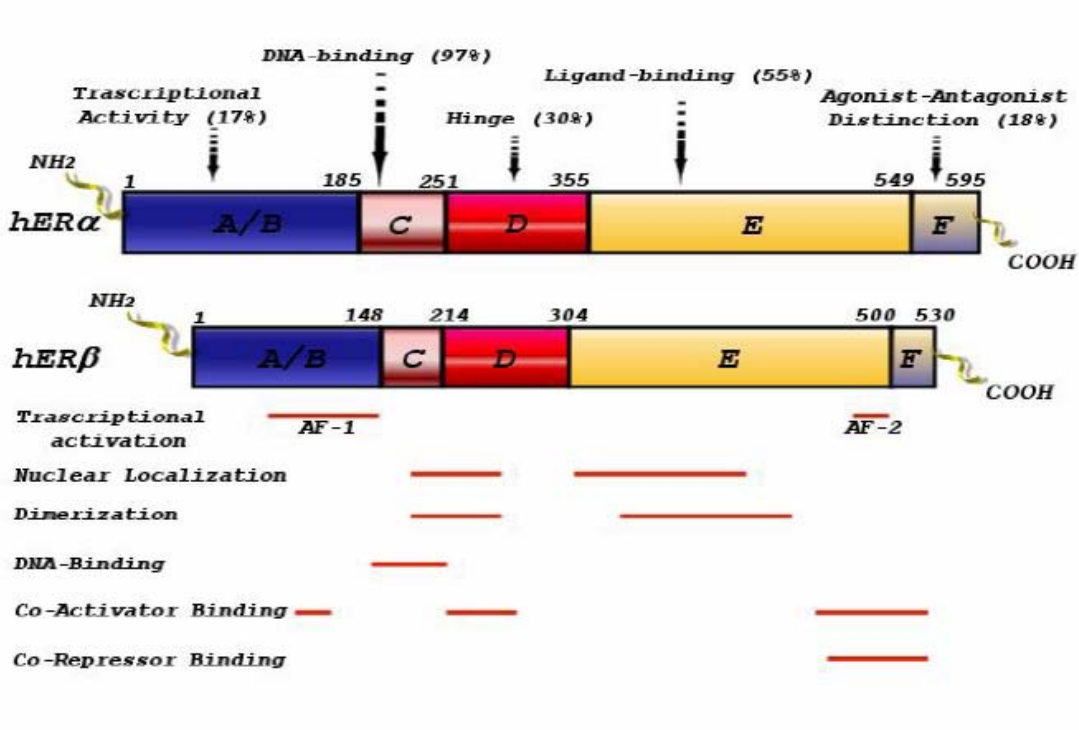


Fig. 1: *ERα and ERβ functional domains.*

Both ERs are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues (Petterson and Gustafsson 2001). ERα is expressed primarily in the uterus, liver, kidney and heart, whereas ERβ is expressed principally in the ovary, prostate, lung, gastrointestinal tract, bladder and hematopoietic and central nervous systems. ERs are, however, co-expressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone and certain regions of the brain (Matthews and Gustafsson 2003). Cellular responses to estrogens are often divided into two broad categories: Genomic and Non-Genomic Responses. Genomic responses are characterized by gene transcription changes and occur in the time frame of hours to days, while non-genomic responses are generally rapid signaling events. Classical ERs, mediate their primary effects at the genomic level, but in recent years, it has become clear that not all effects of estrogens and compounds with estrogen-like activity can be explained by the classic genomic mechanism. In addition, the growth of estrogen-dependent tumors may also have an important non-genomic component (Singleton et al. 2003). It has been shown that estrogens act rapidly by activating membrane receptors coupled to G proteins (GPCRs) (Kelly et al., 2001; Acconcia et al. 2004; Li et al. 2003; Razandi et al. 2004). These receptors are able to mediate estrogen function including transcriptional signaling as well as non-genomic or rapid signaling (Govind and Thampan 2003). Some reports described estrogen binding sites on intracellular membrane (Evans and Muldoon 1991), other reports suggest that palmitoylation (Acconcia et al. 2004; Li et al.2003) or

phosphorylation (Balasenthil et al., 2004) may transfer ERs to the cytoplasmic face of the plasma membrane. Also adaptor proteins, such as Shc (Evinger and Levin 2005) and NMAR, (Boonyaratanakornkit and Edwards 2004) can recruit ERα to the plasma membrane. Classical steroids receptors, bind DNA after ligand stimulation, but they can also act in the presence or absence of ligand (Lu et al., 2006), independently of direct DNA binding to scaffold transcription factors, like AP1 (Barkhem et al., 2004; Kushner et al., 2000), or induce the activation of kinases, like MAPKs, phosphatidylinositol 3-kinase (PI3K), Src or lead to phosphorylation and transcriptional events through transcription factors like Elk-1 (Duan et al., 2001) and serum response factor (SRF) (Duan et al., 2002). Therefore, in addition to transcriptional regulation estrogens can also mediate cellular effects including the generation of the second messengers like Ca^{2+}, cAMP and NO, as well as activation of receptor tyrosine kinases, EGFR and IGF-1R and protein/lipid kinases (Hall et al., 2001; Ho and Liao 2002; Kelly and Levin 2001; Levin 2001-2002; Razandi et al., 2003). The AP-1 transcription factor participates in the control of cellular responses to stimuli that regulate proliferation, differentiation, immune responses, cell death and the response to genotoxic agents or stress (Angel 1991). AP-1 is composed of Jun family members (c-Jun, JunB and JunD) that can form either homo- or hetero-dimers among themselves. Jun proteins also dimerize with fos family members (c-fos, fosB, Fra1 and Fra2) (Curran and Franza 1988) and with members of the Activating Transcription Factor (ATF) family of proteins (Karin 1994). These proteins are characterized by a highly charged, basic DBD, immediately

adjacent to an amphipathic dimerization domain, referred to as the "leucine zipper". The composition of the subunit is determined by the nature of the extracellular stimulus and the MAPK signaling pathway that is activated: the expression and activity of c-Jun and c-fos are tightly regulated by members of the mitogen-activated protein kinase (MAPK) family, including c-Jun N-terminal kinases (JNKs), extracellular signal-regulated protein kinase 5 (ERK5), and p38MAPK kinases and by acting on transcription factors of the TCF family such as Elk-1, can cause induction of the c-fos gene. Upon stimulation, the regulation of AP-1 activity occurs by activating the transcription of these genes as well as by phosphorylation of existing Jun and Fos proteins at specific serine and threonine sites (Vinciguerra et al., 2008; Shaulian and Karin 2001). AP-1 activity is regulated by a broad range of physiological and pathological stimuli, including cytokines, growth factors, stress signals and infections, as well as by oncogenic stimuli (Karin and Shaulian 2001; Shaulian and Karin 2001). The proto-oncogene c-fos plays a relevant role in the regulation of normal cell growth, differentiation, and cellular transforming processes (Curran 1988). In particular, c-fos is classified as a prototypical "immediate early gene" since its expression is rapidly induced by numerous extracellular stimuli, including hormones and mitogens (Weisz and Bresciani 1993; Ginty et al, 1994; Hill and Treisman 1995; Bonapace et al, 1996).

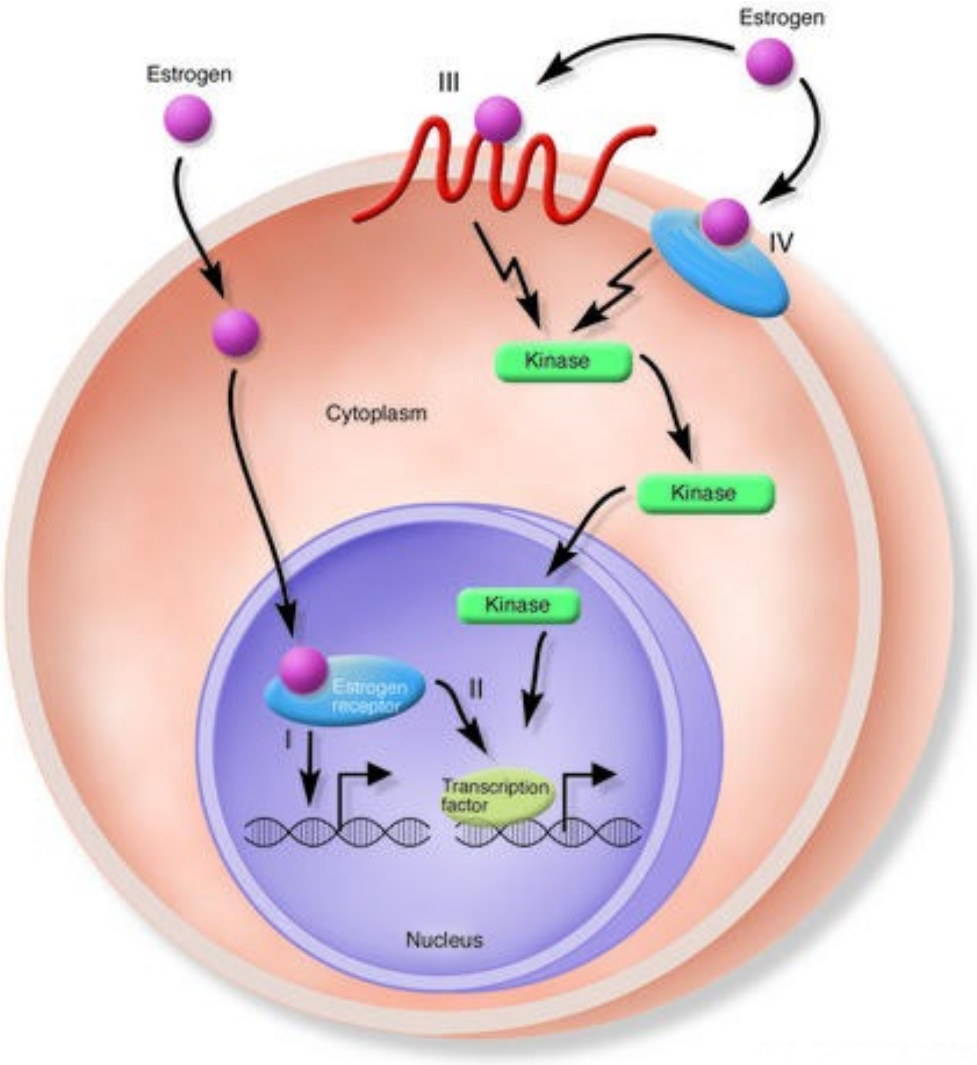


Fig. 2: *Genomic (I and II) and nongenomic (III and IV) actions of estrogens*

G protein–coupled receptors (GPCRs) represent the largest class of cell surface signaling molecules in the human genome (Venter et al., 2001). GPCRs are coupled to a heterotrimeric signal-transducing guanine nucleotide-binding proteins (G proteins). Ligand binding (Gether and Kobilka, 1998) to these receptors activates their downstream regulatory proteins (Prossnitz et al., 2004) and an effector enzyme to generate an intracellular second messenger. All G protein−coupled receptors (GPCRs) contain seven membrane-spanning regions with their N-terminal segment on the exoplasmic face and their C-terminal segment on the cytosolic face of the plasma membrane. One such receptor, GPR30, was cloned by different groups using highly disparate approaches (Carmeci et al. 1997; O'Dowd et al. 1998; Owman et al., 1996; Takada et al., 1997) in the late 1990s. It was not until 2000 that a possible function for this GPR30 was identified from experiments demonstrating MAP kinase (ERK1/2) activation by estrogen, as well as the pure ER antagonists ICI182,780 and Tamoxifen, which mimics estrogen function in certain tissues but acts as an antagonist in other tissues and are collectively known as SERMs (Selective Estrogen Receptor Modulator). Responses were demonstrated in breast cancer cell lines expressing GPR30 but not in cell lines lacking GPR30 (Filardo et al., 2000). Signaling in response to estrogen could be restored in the latter cell lines by expressing GPR30. They found that estrogen-dependent signaling acted through a pertussis toxin-sensitive pathway: indicating the involvement of G proteins (Filardo et al., 2000).

Cellular activation by GPR30 occurred through a mechanism involving transactivation of the epidermal growth factor receptor (EGFR) via a G protein–dependent pathway (Filardo et al., 2000-2002-2008; Maggiolini et al., 2004; Vivacqua et al., 2006a-2006b;). At that time such transactivation pathways from GPCRs to EGFR were still a relatively new concept yet were known to involve metalloproteinase cleavage of proheparin-binding (-bound) epidermal growth factor–like growth factor (pro-HB-EGF) (Daub et al.,1996; Prenzel et al., 1999). A follow-up report described GPR30-mediated elevation of cAMP by estrogen as a mechanism to restore EGF activated ERK1/2 to basal levels through protein kinase A (PKA)-dependent inhibition of Raf-1 activity (Filardo et al., 2002). Furthermore, GPR30-mediated up-regulation of nerve growth factor production in macrophages by induction of c-fos expression has also been demonstrated (Kanda and Watanabe, 2003). The up-regulation of c-fos by estrogen and phytoestrogens has also been shown in breast cancer cells (Maggiolini et al., 2004).

The majority of GPCRs are expressed in the plasma membrane, but some GPCRs may be functionally expressed at intracellular sites (Gobeil et al., 2006). This is particularly true of GPCRs with lipophilic ligands. Where is GPR30 localized? This question is still open, because using subcellular markers, one team showed that GPR30 is expressed in an intracellular compartment, the endoplasmic reticulum but also in the Golgi apparatus and nuclear membrane. In addition, they were unable to detect transfected or endogenously expressed GPR30 on the plasma membrane (Revankar et al., 2005; Revankar et al., 2007).

Recently, other two teams reported expression of GPR30 in the plasma membrane (Thomas et al., 2005; Funakoshi et al., 2006). The proposed role of GPR30 in cellular estrogen responsiveness was, until recently, based on the correlation of receptor expression with estrogen-mediated signaling (Filardo et al., 2000; Kanda and Watanabe, 2003a; Kanda and Watanabe, 2003b; Kanda and Watanabe, 2004; Ylikomi et al., 2004). The affinity of E2 for GPR30 was demonstrated using tritiated estrogen fluorescent E2 derivates (Revankar et al., 2005; Revankar et al., 2007; Thomas et al., 2005). The ER antagonists ICI182,780 and tamoxifen, were also shown to bind GPR30 (Thomas at el., 2005) which is consistent with previous studies showing that these same compounds were agonists for GPR30 (Filardo et al., 2000).

Furthermore it was demonstrated that Tamoxifen activates PI3K through GPR30 but not ERα, suggesting a possible involvement in tamoxifen-resistant breast cancers and/or the increased incidence and severity of endometrial cancers in women treated with tamoxifen. GPR30 has been demonstrated to mediate the proliferative effects of both estrogen and tamoxifen in endometrial cancer cells (Vivacqua et al., 2005).

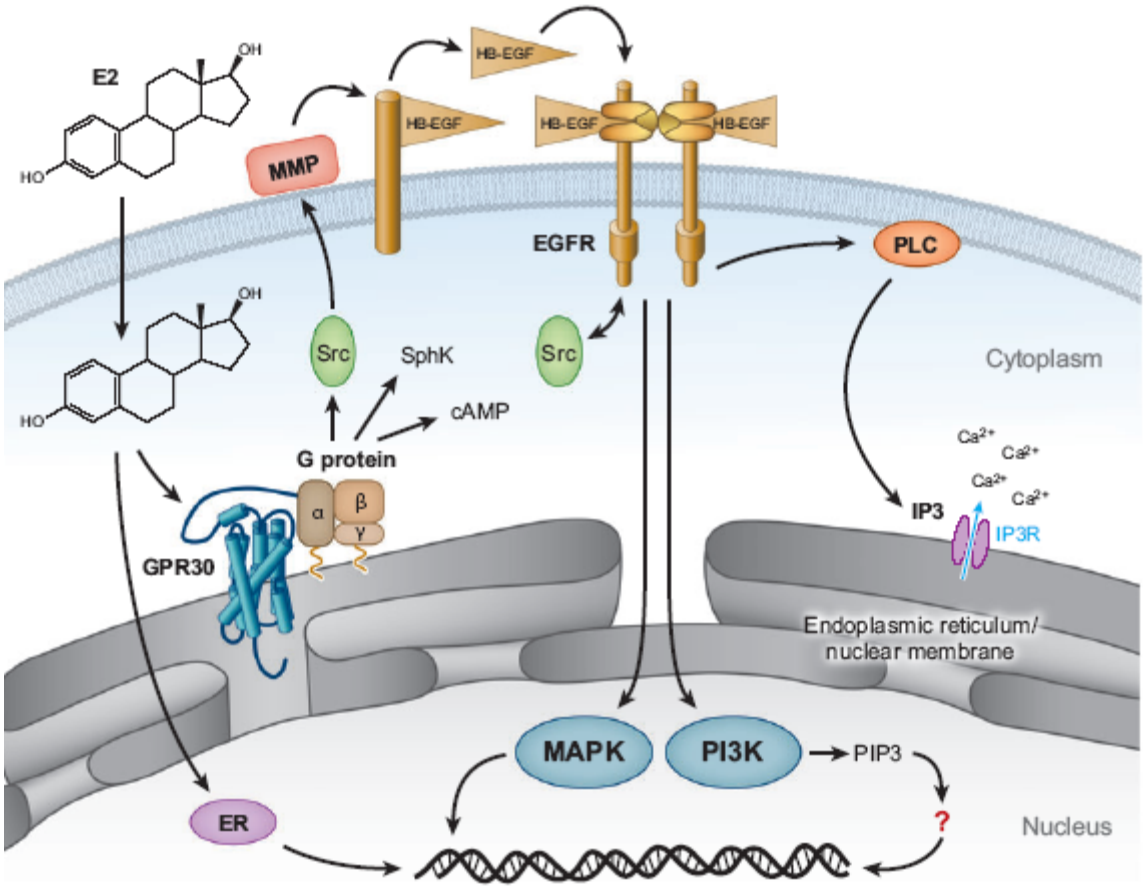


Fig. 3: *Mechanisms of estrogen-mediated signalling through GPR30. Estrogen is freely permeable gaining access to intracellular estrogen receptors, ER alpha and GPR30.*

GPR30 transcripts were reported to be widely distributed in normal and malignant human tissues, with high levels of expression found in the heart, lung, liver, intestine, ovary, and brain (O'Dowd et al., 1998), although there were discrepancies in the reported expression levels in some tissues (i.e., the placenta, lung, and liver) (Owman et al., 1996; Takada et al., 1997). Several primary breast cancers (Camerci et al., 1997) and lymphomas (Owman et al., 1996) also expressed GPR30 transcripts, although many others were negative.

A critical question regarding the expression pattern of GPR30 in tumors centers on its co-expression with classical ERs and whether the two receptor types are expressed in an overlapping or an exclusive pattern. That MCF-7 cells express all three estrogen receptors (ERα, ERβ, and GPR30) whereas SKBR3 cells express only GPR30 suggested that all combinations of receptor expression patterns would likely be possible. Approximately two-thirds of all breast carcinomas express ERα. Whereas in these patients ERα antagonists such as Tamoxifen and Raloxifene have represented front-line endocrine therapy, aromatase inhibitors are now expanding in use. Nevertheless, approximately 25% of patients with ER-positive breast carcinomas do not respond to Tamoxifen therapy (EBCTCG 2005).

An analysis of 321 cases of primary breast cancer showed that approximately 60% of the breast tumor cases expressed levels of GPR30 similar to that normal breast cancer, while 40% of the breast cancer cases expressed low or undetectable levels of GPR30 protein. Codependency for GPR30 and ER was observed, as roughly 40% of the cases co-expressed each receptor type. Twenty percent of the tumors were doubly negative, failing to express GPR30 and ER, with the remaining 40% expressing either one receptor or the other. Interestingly, half of the 122 ER-negative tumors, scored positively for GPR30, possibly suggesting that an ER-negative tumor that retains GPR30 may remain estrogen responsive by signaling through EGFRs (Filardo et al., 2008).

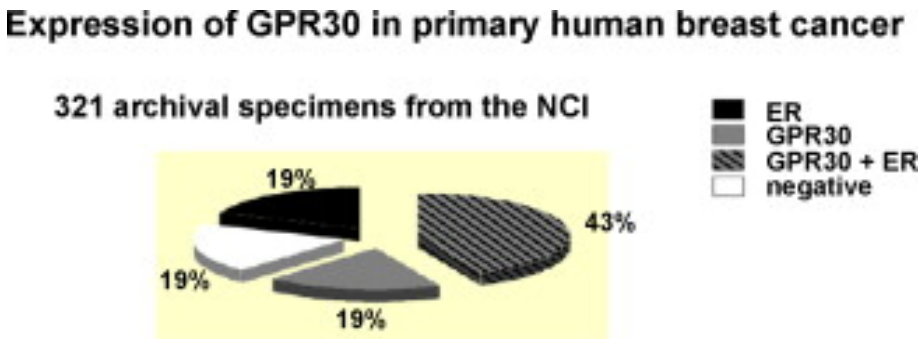


Fig. 4: *Co-expression of GPR30 and ER in primary human breast tumors. (Filardo et al., 2008)*

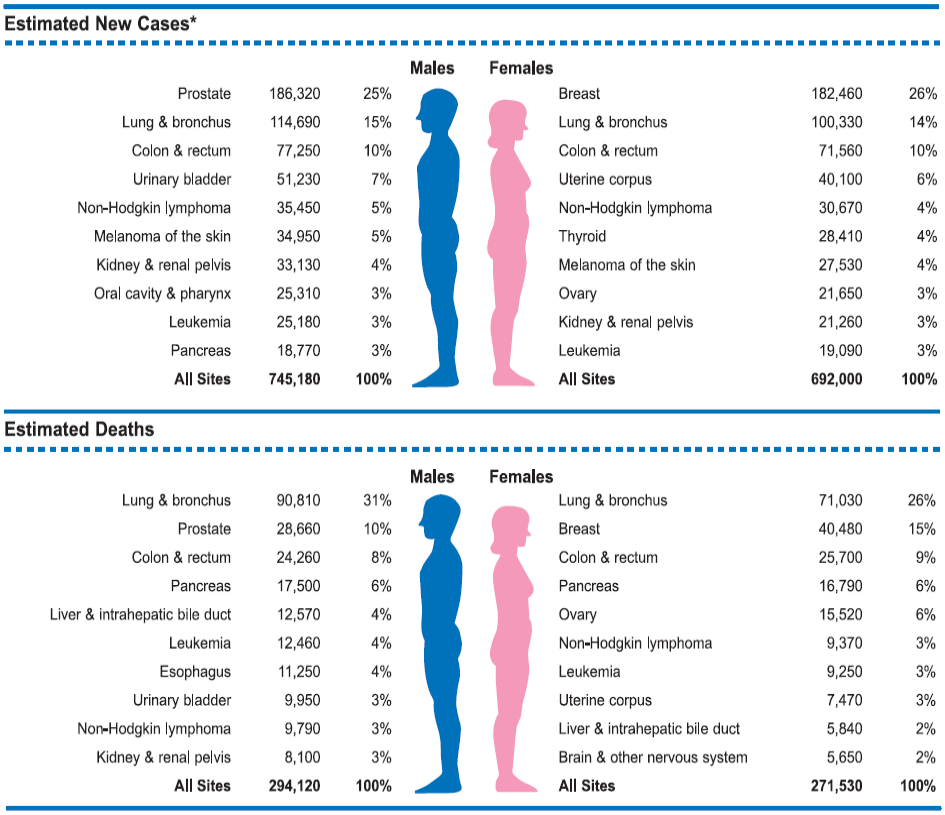
Therefore, the recent identification of the first GPR30-selective ligand G-1 (Bologa et al., 2007) has provided new opportunities to further differentiate between the functions of the ER family member and GPR30 in mediating the multifaceted mechanisms of estrogen action.

A large and compelling body of epidemiologic and experimental data implicates estrogens in the etiology of neoplasias such breast and ovarian carcinoma (Eisen et al., 1998; Barkhem et al., 1998; Williams et al., 1999; Chen et al., 1999; Bai et al., 2000; Rodriguez et al., 2001; Riman et al., 2002; Lacey et al., 2002).

Estrogens can enhance the development of breast cancer by stimulating cell proliferation rate and thereby increasing the number of errors occurring during DNA replication (epigenetic effects), as well as by causing DNA damage via their genotoxic metabolites produced during oxidation reactions (genotoxic effects) (Gadducci et al., 2005).

Breast cancer is the most common cancer in women and is estimated to have accounted for 182,460 new cancer diagnoses and 40,480 deaths in 2008 (Jemal et al., 2008). The incidence is highest in highly industrialized countries like North

America, Northern Europe, and Australia, where age-adjusted rates are 75-92 per 100,000 women (standardized to year 2000 world population), and lowest in Asia and Africa, where incidence is less than 22 per 100,000 (Parkin et al., 2001). Ovarian cancer is the fourth leading cause of tumor death in Western countries representing the most fatal gynecologic malignancy with the overall 5-year survival rate about 10% to 20% (Boete et al., 1993) and is also estimated to have accounted for 21,650 new cases and 15,520 deaths in 2008 (Jemal et al, 2008).



Estimated New Cases*

Males			Females		
Prostate	186,320	25%	Breast	182,460	26%
Lung & bronchus	114,690	15%	Lung & bronchus	100,330	14%
Colon & rectum	77,250	10%	Colon & rectum	71,560	10%
Urinary bladder	51,230	7%	Uterine corpus	40,100	6%
Non-Hodgkin lymphoma	35,450	5%	Non-Hodgkin lymphoma	30,670	4%
Melanoma of the skin	34,950	5%	Thyroid	28,410	4%
Kidney & renal pelvis	33,130	4%	Melanoma of the skin	27,530	4%
Oral cavity & pharynx	25,310	3%	Ovary	21,650	3%
Leukemia	25,180	3%	Kidney & renal pelvis	21,260	3%
Pancreas	18,770	3%	Leukemia	19,090	3%
All Sites	**745,180**	**100%**	**All Sites**	**692,000**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	90,810	31%	Lung & bronchus	71,030	26%
Prostate	28,660	10%	Breast	40,480	15%
Colon & rectum	24,260	8%	Colon & rectum	25,700	9%
Pancreas	17,500	6%	Pancreas	16,790	6%
Liver & intrahepatic bile duct	12,570	4%	Ovary	15,520	6%
Leukemia	12,460	4%	Non-Hodgkin lymphoma	9,370	3%
Esophagus	11,250	4%	Leukemia	9,250	3%
Urinary bladder	9,950	3%	Uterine corpus	7,470	3%
Non-Hodgkin lymphoma	9,790	3%	Liver & intrahepatic bile duct	5,840	2%
Kidney & renal pelvis	8,100	3%	Brain & other nervous system	5,650	2%
All Sites	**294,120**	**100%**	**All Sites**	**271,530**	**100%**

Fig. 5: *Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths, by Sex, United States, 2008.(Jemal et al., 2008).*

Breast and ovarian cancer are common in western countries: environmental factors may play an essential role in hormone-dependent tumor etiology. In fact, estrogenic activity can be found in a large variety of natural and man-made compounds.

Phytoestrogens are natural substances derived from sources such as plants or fungi: they are typically flavonoids or isoflavonoids. For example the phytoestrogens Genistein and Quercetin, copiously present in soyabeans, vegetables and fruits, exert estrogenic activity through direct binding and activation of the estrogen receptor alpha and beta, influencing breast cancer cell proliferation in a dose-dependent manner (Maggiolini et al., 2004). Synthetic estrogenic compounds, called xenoestrogens, environmental estrogens or disruptors, include a variety of pesticides, polychlorinated biphenyls and plasticizers and are almost ubiquitous in our society (Starek 2003; Jacobs and Lewis 2002).

Compound	Exposures/uses
Pesticides	
Atrazine	Selective herbicide
Chlordane	Insecticide, acaricide, veterinary pharmaceutical
Chlorpyrifos	Insecticide, acaricide
Cypermethrin	Insecticide
2,4-Dichlorophenoxyacetic acid	Herbicide
DDT (and associated compounds)	Contact insecticide
Dieldrin, aldrin, endrin	Formerly as insecticide
Lindane	Insecticide
Malathion	Insecticide
Methoxychlor	Insecticide, veterinary pharmaceutical
Pentachlorophenol	Insecticide for termite control, wood preservative
Permethrin, sumithrin	Insecticide
Toxaphene	Insecticide
Tributyl tin (chloride)	Biocide, rodent repellent
Vinclozolin	Agricultural fungicide
Persistent nonpesticide OCs and PAHs	
PAHs	Compounds present in industrial air pollutants, smoke from coal or coke-burners, tobacco tar, some foods
Polybrominated biphenyls	Formerly as flame retardant
Polybrominated diphenyl ethers	Flame retardants
PCBs (Aroclor 1254)	No longer produced commercially—since 1974, in closed electrical capacitors and transformers; before 1972, in transformers and other electrical equipment, carbonless copy paper
Dioxins and furans	Produced during incineration, paper manufacturing, and production of chlorine aromatics; impurity in some herbicides
Phenols and alkylphenols	
Bisphenol A	Polycarbonate and polyester–styrene resins
4-*tert*-Butylphenol	Intermediate in the manufacturing of varnish and lacquer resins, soap antioxidant
Nonylphenol polyethoxylate, 4-nonylphenol, 4-octylphenol	Surfactant, detergent, defoaming agent, some pesticide formulations, degradation product of alkylphenol ethoxylated antioxidant in some plastics
o-Phenylphenol	Disinfectant fungicide, in the rubber industry
Phthalates	
bis(2-Ethylhexyl) phthalate, butyl benzyl phthalate	Commonly used plasticizer for polyvinyl chloride polymers
Di-*n*-butyl phthalate, diethyl phthalate	Personal care products such as nail polish, perfume, hair spray, plasticizers, inks, adhesives, other uses
Parabens	
Butyl, ethyl, methyl, propyl paraben	Pharmaceutical aid (antifungal), preservative in foods; in creams, lotions, ointments, other cosmetics
Other organics	
Arsonic acid	In manufacturing of dyes, bleaching agents, optical brighteners or fluorescent whitening agents
Styrene	Manufacturing plastics, synthetic rubber, resins; insulator
Vinyl acetate	Used in the production of a wide range of polymers, including polyvinyl acetate, polyvinyl alcohol; widely used in production of adhesives, paints, food packaging
Metals	
Cadmium, lead	Batteries, plastic stabilizers, pigments
Mercury	Thermometers, dentistry, pharmaceuticals, agricultural chemicals, antifouling paints, many other uses
Phytoestrogens	
Genistein, coumestrol, zearalenone	Soy, grains, grain molds

Fig. 5: *Data from Budavari (1996),Harris et al. (1997),IARC (1998),Illinois Environmental protection agency(1997), Routledge et al. (1998) Smith and Quinn (1992), Soto et al., and SRI International (1995).*

Atrazine, belongs to the 2-chloro-*s*-triazine family of herbicides and is the most common pesticide contaminant of groundwater and surface water (Fenelon and Moore 1998; Kolpin et al., 1998; Miller et al., 2000). Atrazine is able to interfere with androgen- and estrogen-mediated processes (Cooper et al. 1999, 2000, 2007; Cummings et al., 2000; Friedmann 2002; Narotsky et al., 2001; Stoker et al., 2000). This action occurs without direct agonism or antagonism of the ER or Androgen Receptor (AR) (Roberge et al. 2004). Previous studies have shown

that atrazine reduces androgen synthesis (Babic-Gojmerac et al. 1989; Kniewald et al., 1995) as well as stimulates estrogen production (Heneweer et al., 2004; Keller and McClellan-Green 2004; Sanderson et al., 2002). Epidemiologic studies, also have related long-term exposure to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (Donna et al. 1989) and breast cancer in the general population of Kentucky in the United States (Kettles et al. 1997).

Whether E2 acts on the EGFR/ERK transduction pathway only through GPR30 binding or also through ERα binding is less clear, since E2 binds to both receptors although with different affinity. Using the selective GPR30 ligand G-1 in the first study our research group evaluated the ability of GPR30 to mediate proliferative effects in ovarian cancer cells expressing both ERα and GPR30. We demonstrated a cross-talk between the ERα and GPR30 to induce proliferative effects induced by E2 and G-1 in ovarian cancer cells.

In our second study we evaluated whether GPR30 is also implicated in the growth effect induced by the pesticide Atrazine in ovarian cancer cells and also in this case we found that GPR30 and ERα are both involved in this response. Our third question was about the regulation of GPR30 expression. We found that GPR30 expression is modulated by EGF through the MAPK pathway.

Our results contribute to provide new insight on the role of GPR30 in transducing estrogenic mechanisms implicated in tumor progression.

RESULTS

G Protein–Coupled Receptor 30 (GPR30) Mediates Gene Expression Changes and Growth Response to 17β-Estradiol and Selective GPR30 Ligand G-1 in Ovarian Cancer Cells

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INTRODUCTION

Ovarian cancer is the fourth leading cause of tumor death in Western countries (Greenle et al., 2000). ERα mediates the mitogenic action of estrogens in breast cancer by inducing a variety of genes involved in cell proliferation. A large body of evidence has identified different mechanisms whereby membrane-initiated signaling involving growth factor receptor or membrane ERs mimics or contributes to the function of nuclear ERs (Levin et al., 2005; Deroo and Korach, 2006). Recent studies have shown that GPR30, mediates the non-genomic signaling of E2 in a variety of estrogen-responsive cancer cells through activation of the EGFR transduction pathway (Bologa et al., 2006; Maggiolini et al., 2004; Revankar et al., 2005; Thomas et al., 2005; Filardo et al., 2002; Vivacqua et al., 2006). Considering that GPR30 binds most ER ligands (Thomas et al., 2005), it remains unclear how ERα contributes to GPR30/EGFR signaling in cancer cells. Therefore, the recent identification of the first GPR30-selective

ligand G-1 (Bologa et al., 2006) has provided new opportunities to further differentiate between the functions of the ER family member and GPR30 in mediating mechanisms of estrogen action. In the present study, we have ascertained the ability of G-1 to induce cell growth of estrogen-sensitive ovarian cancer cells expressing ERα as well as breast cancer cells not expressing ERα. We used BG-1 cancer cells as a model, which derived from a solid tumor tissue of a patient with stage III ovarian adenocarcinoma and express clinically relevant levels of ERα but lack ERβ (Bardin et al., 2004).

RESULTS

G-1 does not active ERα but induces the transcription of c-fos promoter constructs.

We first transiently transfected an ER reporter gene in BG-1 cancer cells: the exposure to 100nM E2 induced a strong ERα transactivation which was no longer observed in presence of the ER antagonist ICI 182,780. In contrast, treatments with 100nM G-1 failed to induce luciferase expression or to block that observed on addition of E2 (Fig. 1A). Considering that the down-regulation of ERα induced by an agonist has been considered an additional hallmark of receptor activation (Santagati et al. 1997), we analyzed ERα protein levels. As documented by Western Blotting analysis, the levels of ERα were robustly down-regulated only in presence of E2 (Fig. 1B). To confirm the aforementioned observation we did an immunocytochemical staining: the expression of ERα was reduced only by E2 (Fig. 1C). In order to evaluate the role of GPR30 we

evaluated if its specific ligand could activate a transiently transfected full-length human c-fos promoter (-2.2kb) and a c-fos mutant lacking the ERE sequences (-1.172bp). As can be seen in fig. 1D, G1 transactivated c-fos similar to E2.

The ternary complex factor member Elk1 is crucial for the ERK-dependent activation of the promoter of the c-fos gene (Karin 1994). G-1 and E2 activated Elk1 in the context of a Gal4 fusion protein; however, the transcriptional response was not substantially enhanced by E2 in combination with G-1 (Fig. 1D), suggesting that E2 and G-1 act through the same signal transduction pathway.

G-1 and E2 induce the mRNA expression of c-fos and other estrogen target genes.

To evaluate whether G-1 and E2 could up-regulate c-fos along with other well-known estrogen target genes in BG-1 cells, we performed semi-quantitative RT-PCR experiments comparing mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. Of note, a short treatment (1 h) with 100 nM of E2 and G-1 enhanced c-fos levels, which were still evident after a 24-h exposure to E2 (Fig. 2A and B). The expression of pS2, cyclin A, and cyclin E was stimulated by both E2 and G-1 after 24 h of treatment (Fig. 2A and B), whereas the levels of cyclin D1 increased at both short and prolonged exposure to both compounds (Fig. 2A and B). In contrast, the expression of PR was up-regulated only by E2 at both times of observation, indicating that an E2-activated ERα-dependent mechanism is involved in the regulation of this gene. To further support this finding, we turned to the SKBR3

cells, which do not express detectable amounts of ERs. As shown in Fig. 2C and D, E2 failed to regulate PR, whereas both E2 and G-1 retained the ability to induce c-fos expression, which we previously showed to be dependent on GPR30 expression.

Transduction pathways involved in the up-regulation of c-fos protein levels exerted by G-1 and E2.

Does G-1–dependent activation of c-fos require ERα- and/or GPR30-mediated signaling? As shown in Fig. 3A, either the ER antagonist ICI 182,780 or the GPCR inhibitor pertussis toxin reduced the induction of c-fos obtained after treatment with 100 nmol/L of E2 and G-1 for 2 h, suggesting that both receptors are implicated in the effect of each ligand. Furthermore, the stimulation of c-fos by both G-1 and E2 was equally abrogated by the EGFR kinase inhibitor tyrphostin AG 1478, the mitogen-activated protein kinase (MAPK) inhibitor PD 98059, and the Src family tyrosine kinase inhibitor PP2 but not using the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (Fig. 3B and C), suggesting that both ligands signal through the EGFR/ERK signaling pathway. Moreover, steroids, such as DHT, progesterone, and dexamethasone, did not increase c-fos protein levels (Fig. 3D), revealing that a ligand specificity is required for the regulation of c-fos in ovarian cancer cells. To further assess the role of ERα and GPR30 we analyzed the response of c-fos to both compounds in the absence of either ERα or GPR3O expression. As shown in Fig. 4A, both antisense oligonucleotides turned down the c-fos induction by E2 and G-1,

although each oligonucleotide selectively silenced only the expression of the specific oligonucleotide target sequence (Fig. 4B and C). Moreover, the rapid activation of ERK1/2 on addition of 100 nmol/L of E2 and G-1 was abrogated by both antisense oligonucleotides (Fig. 5A). The inhibitors of EGFR signaling, such as AG 1478, PD 98059, and PP2, prevented ERK1/2 activation by E2 and G-1 (Fig. 5B), thus eliciting a repressive action similar to that observed for c-fos up-regulation by ligands.

G-1 and E2 stimulate the proliferation of the ovarian BG-1 and 2008 tumor cells and breast SKBR3 carcinoma cells.

In BG-1 cells, the growth-stimulatory effects induced by G-1 and E2 were abolished by the EGFR inhibitor AG 1478, the MAPK inhibitor PD 98059, and the Src kinase inhibitor PP2 (Fig. 6A), establishing that the EGFR/ERK signaling pathway mediates the stimulatory action of both ligands. Moreover, the abrogation of ERα or GPR30 expression also abrogated the E2-stimulated and G-1–stimulated cell growth (Fig. 6A). Similar results were also obtained using a different ovarian cancer cell line, named 2008 (Fig. 6B), which expresses the same receptor pattern of BG-1 cells (Safei et al., 2005). However, the results shown in Fig. 2C and D show that G-1 is able to up-regulate c-fos in ER-negative SKBR3 cells. It has been previously reported that E2 induces ERK activation in SKBR3 cells (Maggiolini et al., 2004). Therefore, we investigated the ability of ligands to stimulate SKBR3 cell proliferation. As shown in Fig. 6C, 100 nmol/L of E2 and G-1 promoted SKBR3 cell growth, which was abolished by AG 1478, PD 98059, and PP2 or by abrogation of GPR30 expression (Fig. 6C).

To rule out the possibility that SKBR3 cells expressed detectable levels of ERs sufficient to signal cell growth, we assessed ligand-induced proliferation in the presence of ERα and ERβ antisense oligonucleotides. As evidenced in Fig. 6D, the transfection of either ERα and ERβ antisense oligonucleotides at a concentration able to abrogate the target receptor expression respectively in MCF7 and MDA-MB-231 breast cancer cells had no effect on SKBR3 cell growth, establishing in this cellular context that GPR30 is sufficient to signal E2-induced proliferation. Cumulatively, these data indicate that, although ERα is required for the G-1/GPR30 signaling pathway in ovarian cancer cells, GPR30 may induce cell growth independently of ERα expression depending on the tumor type.

Taken together, these findings suggest that both ERα and GPR30 are required for proliferation of ovarian cancer cells in response to either E2 or G-1. Because the effect of both ligands on cell growth as well as on c-fos induction was abrogated by inhibition of EGFR kinase activity or its downstream effectors, our results also indicate that both ERα and GPR30 are both necessary to signal proliferation of ovarian cancer cells through the EGFR/ERK transduction pathway.

G Protein-Coupled Receptor 30 and Estrogen Receptor α are involved in the proliferative effects induced by Atrazine in ovarian cancer cells.

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INTRODUCTION

Atrazine, one of the most common pesticide contaminants, has been shown to up-regulate aromatase activity in certain estrogen-sensitive tumors without binding or activating the ER (Roberge et al. 2004; Tennant et al. 1994a, 1994b). GPR30 which is structurally unrelated to the ER, mediates rapid actions of E2 and environmental estrogens and is able to mediate rapid E2-dependent responses such as gene expression and cancer cell proliferation (Revankar et al., 2005; Thomas et al., 2005; Maggiolini et al., 2004; Vivacqua et al., 2006a; Vivacqua et al., 2006b). Given the ability of atrazine to exert estrogen-like activity in cancer cells, we evaluated whether atrazine could signal through GPR30 in stimulating biological responses in cancer cells.

RESULTS

Atrazine does not activate ERα in cancer cells.

Does atrazine trans-activate ERs? To investigate the potential capability of atrazine to act through the ERs and considering that atrazine increases the

incidence of estrogen-sensitive tumors in different experimental models (Cooper et al. 2007), we transiently transfected an ER reporter gene in estrogen-sensitive ovarian (BG-1), breast (MCF-7) and endometrial (Ishikawa) cancer cells. The exposure to E2 provoked a strong ERα transactivation which was absent in the presence of the ER antagonist ICI in all cell lines used (Fig. 2A, Fig. 2B and Fig. 2C). In contrast, the treatment with atrazine did not stimulate luciferase expression and did not block the induction observed upon addition of E2 (Fig. 2A, Fig. 2B and Fig. 2C). Atrazine is also unable to active an expression vector encoding ERα transiently transfected in ER-negative SKBR3 breast cancer cells (Fig 2D). We then used a heterologous system formed by chimeric proteins consisting of the DNA binding domain (DBD) of the yeast transcription factor Gal4 and the ERα or ERβ hormone binding domain (HBD) which were transiently transfected in SKBR3 cells. Gal4 ERα ERβ were strongly induced by E2 but not upon atrazine treatment (Fig. 2E and Fig 2F).

Atrazine neither regulates ERα expression nor competes with estrogen binding to ERα.

Then we investigated whether atrazine could modulate ERα expression in BG-1 cells. The ERα expression was down-regulated at both mRNA and protein levels by 100 nM E2, whereas 1 μM atrazine did not produce any modulatory effect (Fig 3A and Fig 3B). In agreement with these results atrazine did not show any binding capacity for ERα (Fig. 3C) as already reported (Cooper et al. 2007). Altogether, our findings rule out that the estrogenic action of atrazine occurs through binding and direct activation of ERα.

Aromatase activity is not induced by atrazine.

Precedent studies demonstrated that atrazine is able to up-regulate aromatase expression in different cell contexts (Cooper et al. 2007; Fan et al. 2007a, 2007b; Roberge et al. 2004; Sanderson et al. 2001, 2002).We analyzed aromatase activity through tritiated water release assays in BG-1 cells. 1 μM atrazine did not stimulate aromatase activity in BG-1 cancer cells but in contrast strongly induced in human H295R adrenocorticocarcinoma cells previously used as a model system to assess aromatase catalytic activity (Heneweer et al. 2004; Sanderson et al. 2001). Atrazine resulted neither an ERα activator nor an aromatase regulator in estrogen-sensitive ovarian cancer cells.

ERK phosphorylation is stimulated by atrazine.

In order to evaluate if the potential estrogenic activity of atrazine is exerted through a rapid cellular response, we evaluated its ability to induce ERK phosphorylation in BG-1 cells. Atrazine and E2 stimulated ERK phosphorylation (Fig. 5A; Fig 5B and Fig. 6A). The ERK activation was observed also in 2008 ovarian cancer cells which present a receptor expression pattern similar to BG-1 cells (Safei et al. 2005) (Fig. 6D). Which transduction pathway is required in this activity induced by atrazine? We then investigated ERK phosphorylation co-treating the cells with specific inhibitors. The ER antagonist ICI, the EGFR and ERK inhibitors AG and PD, respectively, prevented ERK activation induced by both E2 and atrazine, whereas GFX, H89 and WM, inhibitors of protein kinase C (PKC), protein kinase A (PKA) and PI3K, respectively, did not (Fig. 6B; Fig. 6C; Fig. 6E and Fig. 6F). A previous study observed that ICI is able to trigger ERK phosphorylation (Filardo et al.

2000). We treated the SKBR3 breast cancer cells to increasing concentrations of ICI but we did not observe any ERK activation.

Atrazine up-regulates the mRNA expression of estrogen target genes.

After having determined that atrazine induces a rapid ERK activation, we evaluated in BG-1 cells its ability to modulate the expression of the early gene c-fos along with other estrogen target genes. We performed semi-quantitative RT-PCR assays comparing mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. A 1h treatment with 1 µM atrazine enhanced c-fos and cyclin A levels although to a lesser extent than 100 nM E2, which also stimulated PR, pS2 and cyclin D1 expression (table 1). After a 24 h treatment, atrazine increased PR, pS2 and cyclin A levels while E2 additionally induced the expression of c-fos, cathepsin D, cyclins D1 and E (Table 1).

Transduction pathways involved by atrazine in the up-regulation of c-fos protein levels.

The protein expression of c-fos was used as a molecular sensor of atrazine action at the genomic level. After a short treatment (2h) atrazine and E2 induced up-regulation of c-fos in BG-1 and 2008 cells (Fig. 8). The induction of c-fos level was abolished by the ER antagonist ICI and the EGFR and ERK inhibitors, AG and PD respectively (Fig. 8). GFX, H89 and WM, inhibitors of PKC, PKA and PI3K, respectively, did not interfere with c-fos stimulation (Fig. 8). Thus, in ovarian cancer cells atrazine involves ERα and the EGFR-MAPK pathway to trigger c-fos protein increase. Our previous results demonstrate that c-fos stimulation by E2 occurs through GPR30 and requires ERα and EGFR-mediated signaling in cancer cells expressing both receptors (Albanito et al. 2007;

Maggiolini et al. 2004; Vivacqua et al. 2006a, 2006b). Could atrazine act in a similar manner? The transactivation of c-fos induced by both E2 and atrazine, were no longer observed silencing either ERα or GPR30 in BG-1 and 2008 cells (Fig. 9). Furthermore, could atrazine induce a rapid response in a cell context expressing only GPR30? Using ER-negative SKBR3 breast cancer cells we analyzed ERK phosphorylation and c-fos expression after treatment with atrazine. As shown in (Fig. 10), atrazine was able to induce gene expression which was abolished knocking-down GPR30.

The proliferation of ovarian cancer cells induced by atrazine occurs through GPR30 and requires both ERα and EGFR-MAPK-mediated signaling.

We observed that both E2 and atrazine induced the proliferation of BG-1 and 2008 cells in a concentration-dependent manner (Fig. 11A and Fig. 11E). The proliferative effects elicited by E2 and atrazine were abolished in presence of AG and PD (Fig. 11B and Fig. 11F) or after knocking-down the expression of either GPR30 or ERα (Fig. 11C; Fig11D; Fig. 11G and Fig. 11H). Both receptors and the EGFR/MAPK transduction pathway are involved in the growth effects induced by atrazine in ovarian cancer cells.

Epidermal Growth Factor Induces G Protein-Coupled Receptor 30 Expression in Estrogen Receptor-Negative Breast Cancer cells.

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INTRODUCTION

Numerous data have suggested that the interaction of EGFR with estrogenic pathways can occur at different levels. E2 can couple various G proteins, thereby triggering nongenotropic effects through the trans-activation of the EGFR (Levin 2003; Keen and Davidson 2003; Roskoski 2004; Razandi et al., 2004). It has been shown that GPR30 is implicated in EGFR transactivation by E2 (Filardo et al., 2000). E2 induces also EGF-like activity in female reproductive tissue (DiAugustine et al., 1988; Nelson et al., 1991) and similarly activates biochemical signals associated with the EGFR transduction pathway (Migliaccio et al., 1996; Martin et al., 2000). GPR30 can act independently from ERs in inducing estrogen-dependent EGFR action. How is GPR30 expression regulated? In this study we used SKBR3 and BT20 breast cancer cells lacking ERs, to evaluate the regulation of GPR30 expression by E2, G-1, IGF-1 and EGF.

RESULTS

EGF transactivates the 5'flanking region of GPR30 through an AP1 site in ER-negative breast cancer cells

To analyze GPR30 expression we cloned a vector coding a 648-bp fragment located at the 5' flanking region of the human GPR30 gene containing different transcription factor binding sites, such as those for the AP1 and SP1 activating proteins (Fig. 1). Then we transiently transfected the construct in SKBR3 and BT20 breast cancer cells. We evaluated its response induced by E2 and G-1 and the growth factors EGF and IGF-I, both involved in tumor development and progression. Only EGF was able to trans-activate the cloned GPR30 5'flanking region (Fig 2A and Fig 2B). The luciferase induction stimulated by EGF was not observed co-treating the cells with the EGFR and ERK inhibitors AG and PD, whereas the response to EGF was not abrogated by PP2, H89, or LY, inhibitors of the Src family tyrosine kinase, the protein kinase A (PKA), and phosphatidylinositol 3-kinase (PI3K) transduction pathways, respectively (Fig. 2B, Fig 2D). We also cloned two expression vectors mutated in AP1 and SP1 sites which are potentially involved in the responsiveness to EGF (Fig. 3A). In both SKBR3 and BT20 cells, the construct mutated in the AP1 site, -477 to -471 region (GPR30AP1mut) did not respond to EGF, whereas the construct mutated in the SP1 site, -138 to -133 region (GPR30SP1mut) still maintained the EGF responsiveness (Fig. 3, B and C).

EGF up-regulates GPR30 expression

To evaluate GPR30 expression and the transduction pathways involved in its regulation, we performed a semiquantitative RT-PCR assay. A short EGF

treatment (1h) in SKBR3 cells increased GPR30 mRNA expression, and while AG and PD prevented this response, PP2, H89, and LY did not evidence any inhibitory effect (Fig. 4A and Fig 4B). The protein levels of GPR30 were evaluated after a 2h EGF exposure and the regulation was similar to the mRNA regulation (Fig. 4C). Using a different approach, we evaluated the cellular localization of GPR30 after EGF stimulation by confocal microscopy in SKBR3 cells. The treatment with EGF (2h) induced an intracellular GPR30 accumulation, which was no longer observed in presence of AG or PD (Fig. 5A). The specificity of detection in SKBR3 cells was verified by neutralizing the GPR30 antibody by 10-fold molar excess of the antigen peptide (Fig. 5B). The GPR30-negative HEK-293 cells showed no immunodetection of GPR30 (Fig. 5C, upper panels), whereas the nuclei were stained with propidium iodide (Fig. 5C, lower panels).

The EGFR-ERK transduction pathway mediates GPR30 induction by EGF

In SKBR3 cells a rapid ERK1/2 phosphorylation induced by EGF was no longer evident in presence of AG and PD but still persisted using PP2, H89, and LY in combination with EGF (Fig. 6A). In a variety of hormone-sensitive tumor cells (Maggiolini et al.,2004; Vivacqua et al., 2006a; 2006b), EGFR/ERK-mediated pathways led to early induction of c-fos. In line with the results obtained on ERK activation, EGF induced a strong c-fos increase, which was abrogated by AG and PD but by PP2, H89, or LY (Fig. 6B). Is the c-fos up-regulation induced by EGF involved in GPR30 expression? To answer this question we performed a ChIP analysis immunoprecipitating cell chromatin with an anti-c-fos antibody and amplifying the AP1 site located within the GPR30-5'flanking region. The

treatment with EGF strongly recruited c-fos to the AP1 site, which was dependent on EGFR/ERK signaling. In fact, AG and PD inhibited this association whereas PP2, H89, and LY did not elicit any inhibitory activity (Fig. 6C). Using a primer DNA control that does not contain the AP1 site we did not visualize any ethidium bromide staining (Fig. 6C)

The up-regulation of GPR30 by EGF engages E2 to boost the proliferation of breast cancer cells

In SKBR3 and BT20 cells, the growth effects, induced by E2 and EGF alone, further increased in presence of both substances (Fig. 7A and Fig 7E). How does GPR30 contribute to this biological effect? The growth effects of E2 alone or in combination with EGF were prevented transfecting cells with shGPR30, which knocked down GPR30 expression (Fig. 7B and Fig. 7F). We also, transfected the cells with a DN/c-fos expression plasmid, which blocked the AP1-mediated transcriptional activity (Fig. 7C and Fig. 7G), and we did not observe the proliferative effects induced by either mitogen (Fig. 7D and Fig. 7H). Consequently, c-fos/AP1 signaling exerts a key role in the growth stimulation of both mitogens in SKBR3 and BT20 cells. Taken together, the up-regulation of GPR30 after exposure to EGF may represent a molecular mechanism through which EGF engages E2 to boost the proliferative effects elicited in these ER-negative breast cancer cells.

Materials and Methods

Reagents

17β-estradiol (E2), Epidermal Growth Factor (EGF), Insulin-like Growth Factor-1 (IGF-1), 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine, (Atrazine), Genistein (G), 4-hydroxytamoxifen (OHT), Cycloheximide (Cx), Pertussis Toxin (PT), 5α-Dihydrotestosterone (DHT), Progesterone (PRG), Dexamethasone (DEX), H89, LY 294,002 (LY), Wortmannin (WM), and PD98059 (PD) were purchased from Sigma-Aldrich (Milan, Italy). (1-[4-(6-bromobenzo[1,3]diodo-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin -8-yl]-ethanone) (G-1) was kindly provided by ChemDiv,Inc. (San Diego, CA). AG1478 (AG) and AG490 was purchased from Biomol Research Laboratories (DBA, Milan, Italy). ICI 182,780 (ICI) was obtained from Tocris Chemicals (Bristol, United Kingdom) and GF109203X (GFX) from Calbiochem, (VWR International, Milan, Italy). All compounds were solubilized in DMSO, except E2 and PD which were dissolved in ethanol.

Cell Culture

All cell lines were grown in Dulbecco's Minimal essential medium (DMEM) or in RPMI 1640 medium without phenol red supplemented with L-glutamine (2mM), Penicillin (100U/ml), streptomycin (100U/ml), and 10% foetal bovine serum (FBS). Cells were switched to medium without serum 24hs before transfections or confocal staining and immunocytochemical staining, 48hs before RT-PCR or immunoblot, and 72hs before the evaluation of ERK1/ERK2 phosphorylation.

Plasmids.

The firefly luciferase reporter plasmids used were:

- XETL, for ERα and ERβ carrying the firefly luciferase sequences under the control of an estrogen-response element upstream of the thymidine kinase promoter;
- HEGO used for the expression of ERα;
- GK1, gene reporter for all Gal4 fusion proteins;
- Gal4 chimeras Gal-ERα and Gal-ERβ fusion protein;
- c-fos and the deletion mutant c-fosΔERE (which lacks the ERE sequence), encoding -2.2-kb and -1172 bp 5′ upstream fragments of the human c-fos respectively;
- Gal4-Elk1 fusion protein;
- DN/c-fos;
- GPR30/AS expression plasmid.

Transfection and Luciferase assays.

Cells were plated into 24-well dishes with 500 ml of regular growth medium per well the day before transfection. The medium was replaced with that lacking serum on the day of transfection , which was performed using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.3 μg of reporter plasmid and 3ng of pRL-TK. Ligands were added to the cells after 8-9hs and incubated for 8-24hs, depending on the different assays. Luciferase activity was measured with the Dual Luciferase kit (Promega, Milan, Italy) according to the manufacturer's

recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the *Renilla* luciferase activity. The normalized relative light unit values (RLU) obtained from untreated cells were set as 1-fold induction on which the activity induced by treatments was calculated.

RT-PCR.

The evaluation of gene expression was done by semiquantitative RT-PCR by using the direct incorporation of digossigenin-11-dUTP(DIG-dUTP) during the amplification of cDNA. After the extraction from cells, RNA was converted in cDNA by the reverse transcriptase enzyme and the genes of interest were amplified by PCR. PCR products were then separated on 1.2% agarose gel and transferred on a nylon membrane, probed with the antibody against digossigenin conjugated to peroxidase and revealed using the ECL system (GE Healthcare, Italy).

The primers used were:

36B4 forward 5´-CTCAACATCTCCCCCTTCTC-3´

36B4 reverse 5´-CAAATCCCATATCCTCGTCC-3´

GPR30 forward 5'CTGGGGAGTTTCCTGCTGA-3'

GPR30 reverse 5'-GCTTGGGAAGTCACACCAT-3'

ERα forward 5'-AATTCAGATAATCGACGCCAG-3'

ERα reverse5'-GTGTTTCAACATTCTCCCTCCTC-3'

c-fos forward 5´-AGAAAAGGAGAATCCGAAGGGAAA-3´

c-fos reverse 5´-ATGATGCTGGGACAGGAAGTC-3´

PR forward 5´-ACACCTTGCCTGAAGTTTCG-3´

PR reverse 5´-CTGTCCTTTTCTGGGGGACT-3´

pS2 forward 5´-TTCTATCCTAATACCATCGACG-3´

pS2 reverse 5´-TTTGAGTAGTCAAAGTCAGAGC-3´

Cyclin A forward 5´-GCCATTAGTTTACCTGGACCCAGA-3´

Cyclin A reverse 5´-CACTGACATGGAAGACAGGAACCT-3´

Cyclin D1 forward 5´-TCTAAGATGAAGGAGACCATC-3´

Cyclin D1 reverse 5´-GCGGTAGTAGGACAGGAAGTTGTT-3´

Cyclin E forward 5´-CCTGACTATTGTGTCCTGGC-3´

Cyclin E reverse 5´-CCCGCTGCTCTGCTTCTTAC-3´

Western Blotting.

Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 ml of 50mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, a mixture of protease inhibitors containing 1mmol/L aprotinin, 20mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. Protein concentration was determined using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich). Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel electrophoresis in glycine buffer (0.02mM Tris, 0.2mM glycine, 1% SDS). Proteins were transferred to a nitrocellulose membrane (GE Healthcare, Italy) using the above buffer with the addition of 20% methanol, and probed overnight at 4°C with the specific antibodies and then revealed using the ECL system (GE Healthcare, Italy). All the antibodies were

purchased from Santa Cruz Biotechnology, DBA, Milan, Italy. Polyclonal GPR30 was purchased from MBL-Eppendorf, Milan, Italy.

Aromatase assay.

The aromatase activity in subconfluent cell culture medium was measured by tritiated water-release assay using 0.5 μM [1ß-^{3}H(N)]androst-4-ene-3,17-dione (25.3 Ci/mmol; DuPont NEN, Boston, MA, USA) as a substrate. The cells were treated in a 6-well dish in culture medium in the presence of Atrazine or DMSO for 40 hs and then incubated with [1 ß -^{3}H(N)]androst-4-ene-3,17-dione. Incubations were performed at 37 °C for 6 h under a 95%:5% air/CO_2 atmosphere. The results obtained were calculated as pmol/h, and normalized to milligram of protein (pmol/h per mg protein) and expressed as percentages of untreated cells (100%).

ER binding assay.

BG-1 cells were stripped of any estrogen by keeping them in medium without serum for 2 days. Cells were incubated with 1 nM [2,4,6,7-^{3}H]E2 (89 Ci/ mmol; Amersham Bioscience) and increasing concentrations of nonlabeled E2 or Atrazine for 1 h at 37° C in a humidified atmosphere of 95% air/ 5% CO_2. After removal of the medium, cells were washed with ice-cold PBS/0.1% methylcellulose twice, harvested by scraping and centrifugation, and lysed with 100% ethanol, 500 μl per 60 mm dish, for 10 min at room temperature (Lee et al. 1996). The radioactivity of extracts was measured by liquid scintillation counting.

Chromatin immunoprecipitation (ChIP).

Cells grown in 10 cm plates were shifted for 24 hs to medium lacking serum and then treated for 2 hs with vehicle or 50 ng/ml EGF. The immuno-cleared chromatin was precipitated with anti-c-fos antibody. A 4 μl volume of each sample was used as template to amplify by PCR two fragments located next to the GPR30-5′ flanking region: one fragment of 261 bp containing the AP1 site and the second fragment of 364 bp (from -937 to -1301) not containing the AP1 site. The primer pairs used to amplify the first fragment were: 5′-CGTGCCCATACCTTCATTGCTTCC-3′ (forward) and 5′-CCTGGCCGGGTGTCTGTAG-3′ (reverse) while the primer pairs used to amplify the second fragment were: 5'-CCGTGGCCCGCTGCATAGAGAAC-3' (forward) and 5'-GAGAGGGAGAAGTGGGCTGTC-3' (reverse). The PCR conditions were 45 seconds (s) at 94°C, 40 s at 58°C, and 90 s at 72°C. The amplification products obtained in 25 cycles were analysed in a 2% agarose gel and visualized by ethidium bromide staining. 3 μl of the initial preparations of soluble chromatin were amplified to control input DNA before precipitation.

GPR30 plasmid constructs and GPR30 short hairpin RNA (shGPR30).

To generate the luciferase expression vector for the GPR30-5′flanking region (GPR30), a 641 bp fragment next to the 5′-flanking region of the GPR30 gene was amplified by PCR using the following primer pairs 5′-AACACTGGCTTTCCCTTCCTATCT-3′ (forward) and 5′-CTTGAAGTGAGCCTGGCATTTGTC-3′ (reverse) from genomic DNA which

was extracted from SkBr3 cells by Trizol reagent as suggested by the manufacturer (Invitrogen, Milan, Italy). PCR primer pairs were selected analyzing the 5′-flanking region of GPR30 gene in chromosome 7, location 7p22.3. The PCR amplification was performed using 1.25U GoTaq DNA polymerase according to the manufacturer instructions (Promega, Milan, Italy). PCR conditions were 5 min 95°C, followed by 1 min 94°C, 1 min at 58°C, and 1 min at 72°C for 30 cycles. The fragment was then inserted in the pCR 2.1 plasmid using the TA cloning kit (Invitrogen, Milan, Italy), sequenced and cut with Hind III and Xho I. The insert was cloned in the pGL3 basic vector (Promega, Milan, Italy). Analyses of GPR30-5′ flanking region revealed an AP1 (-471 to -477) and an SP1 (-133 to -138) consensus binding sites. Mutations from position -471 to -477 in the GPR30-5′ flanking sequence corresponding to an AP1 motif and from position -133 to -138 corresponding to the SP1 binding site (Fig 2 A), were generated using QuikChange XL Site-Directed Mutagenesis kit (Stratagene, Milan, Italy). The following pairs of primers were used to generate the AP1 and Sp1 mutants: GPR30AP1mut (forward) 5′-CCCTGCCTGTGGGAGACGCCCACGTCCAGCCTCC-3′ and (reverse) 5′-GGAGGCTGGACGTGGGCGTCTCCCACAGGCAGGG-3′; GPR30SP1mut (forward) 5′-GGACGAGCACGCGGAGATCACTCGCCTCCACGG-3′ and (reverse) 5′-CCGTGGAGGCGAGGTGATCTCCGCGTGCTCGTCC-3′. All plasmids were sequenced before use. Plasmid 3x-FLAG-hGPR30 was constructed using the *HindIII/BamHI* sites in pCMV10.3x-ratFLAG. hGPR30 was amplified with

primers CCCCAAGCTTatggatgtgacttcccaag and CAGCGGATCCctacacggcactgctgaac (restriction sites are underlined). Reference plasmid was Prl-3x-FLAG expressesing an unrelated 26 kDa protein. Short hairpin (sh)RNA constructs against human GPR30 were bought from Openbiosystems (Biocat.de, Heidelberg, Germany) with catalog number RHS4533-M001505. The targeting strand generated from the shRNA vectors sh1, sh2, sh3, sh4 and unrelated control are respectively: CGAGTTAAAGAGGAGAAGGAA, CTCCCTCATTGAGGTGTTCAA, CGCTCCCTGCAAGCAGTCTTT, GCAGTACGTGATCGGCCTGTT, CGACATGAAACCGTCCATGTT. In order to evaluate the effectiveness of the different shRNA constructs, HEK-293 cells were seeded at about 50% confluency in 6 cm plates. 6-8 hs later, cells were transfected using the calcium-phosphate method with 1 µg of 3x-FLAG-hGPR30, 10 µg of shRNA construct and 2 µg of Prl-3x-FLAG. Prl-3xFLAG was used as a transfection control. 40 hs after transfection, cells were harvested and lysed with 20 mM Tris-HCl [pH 8], 100 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM monovanadate, 1 mM DTT, and protease inhibitors. DNA was sheared by several passages through a 25-gauge needle. Lysates were cleared by centrifugation, and protein concentrations were determined by the Bradford method. 30 µg of lysates were subjected to western blot analysis with the FLAG antibody M2 (Sigma, Lausanne Switzerland). With a 74% knock-down of 3x-FLAG-hGPR30 expression the shRNA construct sh3 showed the highest efficacy. Hereafter, sh3 is referred to as shGPR30.

Immunocytochemical staining.

Cells were treated as indicated and then fixed in fresh paraformaldehyde (2%for 30 min). After paraformaldehyde removal, hydrogen peroxide (3% in methanol for 30 min) was used to inhibit endogenous peroxidase activity. Cells were then incubated normal horse serum (10% for 30 min)to block the nonspecific binding sites. Immunocytochemical staining was done using as the primary antibody a mouse monoclonal immunoglobulin G (IgG)gener ated against ERα (overnight at 4°C). A biotinylated horse anti-mouse IgG
(1:600 for 60 min at room temperature)was applied as the secondary antibody (Vector Laboratories, Burlingame, CA). Subsequently, the amplification of avidin-biotin-horseradish peroxidase complex (1:100 for 30 min at room temperature; Vector Laboratories)was carried out and 3,3¶-diaminobenzidine tetrachloride dihydrate (Vector Laboratories)was used as a detection system. Cells were rinsed after each step with TBS [0.05 mol/L Tris-HCl plus 0.15 mol/L NaCl (pH 7.6)] containing 0.05% Triton X-100. In control experiments, cells were processed replacing the primary antibody with mouse serum (Dako S.p.A., Milan, Italy)or using a primary antibody preabsorbed (48 h at 4jC)with an excess of purified ERα protein (M-Medical).

Confocal microscopy.

50% confluent cultured cells grown on coverslips were serum deprived for 24hs and then treated as indicated. Then, cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton 100X, washed three times with PBS and

incubated for 1 h with 2 mg/ml primary antibody against GPR30. After incubation with the GPR30 antibody, the slides were washed three times with PBS and incubated with 1 mg/ml rhodamine-conjugated donkey anti-rabbit IgG (Calbiochem, Milan, Italy). The cellular expression and localization of GPR30 was evaluated by confocal microscope with 1000x magnification. The optical sections were taken at the central plane.

Proliferation assay.

For quantitative proliferation assay, 10 000 cells 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) and cells were trypsinized and counted in a haemocytometer on day 6. A concentration of 200 ng/L of the indicated shRNA, or GPR30/AS (200 ng) or 200 ng of empty vector was transfected using Fugene 6 Reagent as recommended by the manufacturer the day before treatments, and then renewed every 2 days before counting.

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.

DISCUSSION and CONCLUSIONS

The effects of estrogens E2 are versatile and fundamental throughout the body. Although some of the earliest effects of estrogen were shown to be rapid in nature, the majority of studies following the discovery of the classical ER focused on long-term transcriptional activities of the hormone. More recently rapid signaling events mediated by E2 have been the object of increasing interest. The transcriptional mechanisms of ER function through ERE sequences have been investigated but gene regulation by estrogen at promoters lacking EREs require further investigation (Carroll and Brown, 2006). E2 is able to start phosphorylation events through MAPKs and PI3Ks thus modulating gene expression via alternate promoter sites such as serum response elements through the actions of Elk-1 and SRF (Duan et al., 2001; Duan et al. 2002). The activation of these pathways has been typically associated with growth factor receptors and GPCRs. With the recent identification of GPR30, as an estrogen-responsive GPCR, our understanding of the mechanisms of estrogen action has been broadened. GPR30 activates rapid kinase signaling pathways and also mediates transcriptional regulation of genes previously associated with estrogen action through ERs. GPR30 protein expression had been observed in many cancer cell lines: MCF-7 and SKBR3 breast cancer (Carmerci et al., 1997; Filardo et al., 2000; Thomas et al., 2005; Revankar et al., 2005), HEC1A (Vivacqua et al., 2006a) and HEC50 (Revankar et al., 2005) endometrial cancer, JEG choriocarcinoma (Revankar et al., 2005), BG-1 ovarian cancer, and thyroid carcinoma (Vivacqua et al., 2006b). Our previous reports demonstrated estrogen-mediated cell proliferation to be dependent on GPR30 (Maggiolini et

al., 2004; Vivacqua et al., 2006a; Vivacqua et al., 2006b). How does GPR30 act in cancer progression? A critical question regarding the expression pattern of GPR30 in tumors tissues is its co-expression with classical ERs. For example, MCF-7 cells express ERα, ERβ, and GPR30 whereas SKBR3 cells express only GPR30 thus suggesting that all combinations of receptor expression patterns would likely be possible. In our first study (Albanito et al., 2007) we utilized the GPR30 specific agonist G-1 (Bologa et al., 2006) to examine the contribution of GPR30 to estrogen signaling and transcriptional activation. The G-1 specificity against classical ERs was confirmed in BG-1 ovarian cancer cells that express ERα and GPR30, but not ERβ (Bardin et al., 2004): E2, but not G-1, was able to stimulate the transcription of an ERE luciferase reporter and to induce the down-regulation of ERα. Our group previously demonstrated that c-fos expression was an early response to estrogen stimulation in MCF-7 and SKBR3 by GPR30 through the use of GPR30 knockdown (Maggiolini et al.,2004; Vivacqua et al.,2006a; Vivacqua et al., 2006b). In BG-1 ovarian cells, both E2 and G-1-mediated transcription of a luciferase reporter containing a 2.2-kb promoter for human c-fos and the transcription of a c-fos reporter gene lacking the ERE sequences. This transcriptional activation indicated that both compounds could initiate transcription of c-fos independently of the ERE. We show that both ligands induce the mRNA of well known estrogen-responsive genes such as c-fos and those encoding PR, pS2, and cyclins A, D1, and E. Whereas E2 induced all genes at 24 h, G-1 induced all genes except PR. In SKBR3 cells, which lack classical ERs but express GPR30, and E2 and G-1 activated c-fos but not PR

mRNA. These results together suggest that estrogen mediated activation of PR mRNA occurs exclusively through ERα, whereas transcriptional activation of the c-fos, pS2, and cyclins A, D1, and E genes can occur through GPR30 and as well as ERα. Interestingly, knockdown of either ERα or GPR30 in BG-1 cells significantly reduced c-fos induction. Specific knockdown of GPR30 almost completely blocked G-1 induction of c-fos, while the cells retained a low level of activity in response to E2. Upon ERα knockdown, both responses were substantially but not completely blocked. These results suggest that ERα and GPR30 both have to be present in BG-1 cells to obtain the maximal induction of c-fos. A similar result was obtained with the knockdown of either ERα or GPR30 in BG-1 examining cell proliferation. Both E2 and G-1 induced cell growth, however depletion of either ERα or GPR30 completely deleted the response to both ligands. The same results were obtained in 2008 ovarian cancer cells which share the same receptor pattern as BG-1 cells. Because GPR30 was able to induce c-fos in ERα negative and GPR30 positive SKBR3 cells, we evaluated the growth properties of SKBR3 cells induced by the ligands. E2 and G-1 stimulated growth, and GPR30 knockdown prevented it. Although ERα and ERβ are not detectable in SKBR3 cells, we treated these cells with antisense oligonucleotides for ERα and ERβ, but no reduction of proliferation was observed. In conclusion our results suggest that when both receptors are present, an interplay may exist between the systems whereas when only GPR30 is present it can signal in the absence of ERs.

A number of pesticides, industrial by-products, and manufactured products such as plastics, and natural chemicals have been shown to disrupt the endocrine system. The man-made compounds, named xenoestrogens, are referred to as endocrine-disrupting chemicals (EDCs). Recently these chemicals have received growing attention because endocrine disruption is being considered an relevant aspect of risk assessment. EDCs interfere with the synthesis, secretion, transport, action or elimination of natural hormones which sustain development, reproduction and behavior (Kavlock et al. 1996; Welshons et al, 2003). After ingestion and/or absorption, EDCs can alter endocrine functions through a variety of mechanisms including nuclear steroid receptor binding and activation (Cooper and Kavlock, 1997; Masuyama et al, 2000). Estrogenic EDCs, chemicals that act as hormone mimics via ER mechanisms, are currently the largest group of known endocrine disruptors. Estrogenic EDCs have the potential to be biologically active at low, environmentally relevant doses. Atrazine is the most common pesticide contaminant of groundwater and surface water (Fenelon and Moore 1998; Miller et al., 2000). Previous studies have reported that atrazine exerts estrogen activity by modulating the aromatase enzyme in cancer cells showing high levels of this enzyme (Fan et al., 2007a; Fan et al., 2007b; Heneewer et al., 2004) but not through binding or activation of ERα (Connor et al., 1996; Roberge et al.,2004). In our second report (Albanito et al., 2008b) we first verified that atrazine is unable to bind or activate ERα or to induce aromatase activity in BG-1 ovarian cancer cells. A variety of environmental contaminants show similar binding affinities for

GPR30 and agonistic activities as for ERs (Thomas and Dong 2006). We demonstrated that GPR30 and ERα in through the EGFR/MAPK pathway are implicated in the biological response to atrazine in ovarian cancer cells and its action is similar to the selective GPR30 ligand G-1 that acts without binding or activating ERα (Albanito et al., 2007). In BG-1 ovarian cancer cells E2 modulated target genes directly activated by ERα while the GPR30/EGFR pathway is involved in atrazine biological activity as demonstrated using GPR30 knock-down in proliferation assay and using specific pharmacologic inhibitors. As previously reported only for nuclear receptors (Migliaccio et al., 2006), we demonstrated that a complex interplay between GPR30 and ERα with growth factor receptors exists knocking-down the two receptors in BG-1 ovarian cancer cells. We analyzed ERK phosphorylation in presence of inhibitors like ICI, AG and PD which blocked the activation induced by atrazine, suggesting that multiple transduction pathways contributed to this response. Atrazine is able to stimulate gene expression and proliferation effects in estrogen-sensitive ovarian cancer cells through GPR30 and involving ERα.

What about GPR30 expression? What is the pathway involved in its regulation? The EGFR transduction pathway is involved in estrogen action (Cuhna et al., 2000) it has been shown that intrauterine E2 administration augments EGF concentrations (DiAugustine et al., 1988) and EGFR autophosphorylation (Mukku and Stancel, 1985). Furthermore experiments conducted in vitro, demonstrated that E2 stimulates numerous EGFR associated cascades such as MAPKs (Razandi et al., 2003; Filardo et al.,2000; Filardo et al, 2002). To trigger

MAPK activity GPR30 couples membrane-associated enzymes along with a familiar regulatory circuit controlled by independent G protein signaling pathways.

It was also demonstrated that GPR30 can act in an ER-independent manner in mediating estrogen action. In particular, the rapid E2 effect is not correlated with ER expression because it happens in human ER-negative cancer cells as shown in our and other studies (Maggiolini et al., 2004; Vivacqua et al., 2006a; 2006b; Filardo et al., 2000; 2002; Filardo 2002; Filardo and Thomas 2005). ER antagonists such as the anti-estrogen tamoxifen, support estrogen action in breast cancer cells that present GPR30 independently of whether they express ERs (Filardo et al., 2000; Filardo et al., 2002). These conclusions suggest that ERs are not necessary for GPR30-dependent EGFR activation however, as already discussed above, GPR30 and ERα collaborate in mediating the E2 and G-1 effects in ovarian cancer cells (Albanito et al., 2007). Our third study (Albanito et al., 2008a) describes GPR30 regulation and its activity. We established that EGF is able to transactivate the GPR30-5′flanking region in SKBR3 and BT20 breast cancer cells through the EGFR pathway. Furthermore EGF up-regulates the expression of GPR30 protein, localized in the cytoplasmic compartment as shown by confocal microscopy. Previous studies have demonstrated that GPR30 is predominantly expressed in the endoplasmic reticulum (Revankar et al.,2005; Revankar et al., 2007;). These data raise the question of how ligand binding to a GPCR within cells could initiate signaling events, involving transactivation of EGFR (Levin 2005). Although the transduction cascade

initiated by GPR30 still remains to be elucidated, often GPCR-mediated transactivation of EGFR occurs through shuttle cytosolic molecules, which activate metalloproteinases leading, sequentially, to the release of EGF-like ligands (Hasbi et al., 2005). We describe the molecular mechanisms that EGF engages in this pathway: it induces direct actions such as rapid ERK activation and induction of c-fos, which in turn is recruited to an AP1 site located next to the GPR30-5'flanking region. The growth induction stimulated by E2 and EGF alone in SKBR3 and BT20 breast cancer cells was boosted by the exposure to the two compounds in combination. Furthermore, knocking-down GPR30 expression abrogated the proliferation stimulated by E2 alone and that additionally induced by E2 used in combination with EGF. Our results indicate that EGF engages a positive feedback loop through GPR30- mediated signals, such as those elicited by E2, to enhance the potential of the EGFR signaling system.

In conclusion, the biological effects of estrogen are mediated through the combined actions of the classical nuclear ERs and GPR30, and both can mediate rapid signaling events and modulate transcriptional activity.

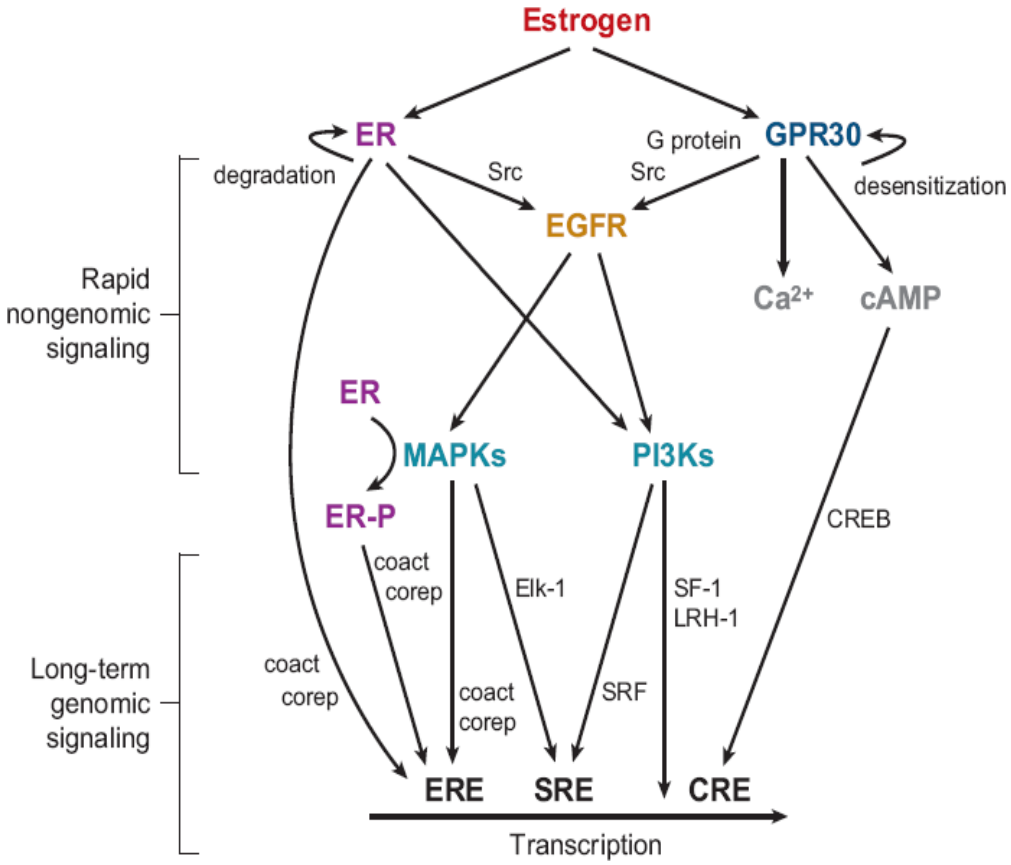

Fig. 6: *Crosstalk between rapid and genomic signaling mediated by E2 through ERs and GPR30. (Prossnitz et al., 2008)*

The ER and GPR30 downstream signaling pathways include MAPKs which modulate nuclear transcriptional events, cell proliferation induced by E2 and compounds such as the pesticide atrazine, but also regulate the expression of GPR30. Our studies also contribute to understand the role of GPR30 in the stimulatory effects induced by estrogen in ER-lacking cancer cells, and indicate that a combinatorial approach which blocks both receptors with selective agents is warranted in order to provide new treatment options for cancer diseases.

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PUBBLICATIONS

G Protein–Coupled Receptor 30 (GPR30) Mediates Gene Expression Changes and Growth Response to 17β-Estradiol and Selective GPR30 Ligand G-1 in Ovarian Cancer Cells

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Abstract

Estrogens play a crucial role in the development of ovarian tumors; however, the signal transduction pathways involved in hormone action are still poorly defined. The orphan G protein–coupled receptor 30 (GPR30) mediates the nongenomic signaling of 17β-estradiol (E2) in a variety of estrogen-sensitive cancer cells through activation of the epidermal growth factor receptor (EGFR) pathway. Whether estrogen receptor α (ERα) also contributes to GPR30/EGFR signaling is less understood. Here, we show that, in ERα-positive BG-1 ovarian cancer cells, both E2 and the GPR30-selective ligand G-1 induced *c-fos* expression and estrogen-responsive element (ERE)-independent activity of a *c-fos* reporter gene, whereas only E2 stimulated an ERE-responsive reporter gene, indicating that GPR30 signaling does not activate ERα-mediated transcription. Similarly, both ligands up-regulated cyclin D1, cyclin E, and cyclin A, whereas only E2 enhanced progesterone receptor expression. Moreover, both GPR30 and ERα expression are required for *c-fos* stimulation and extracellular signal-regulated kinase (ERK) activation in response to either E2 or G-1. Inhibition of the EGFR transduction pathway inhibited *c-fos* stimulation and ERK activation by either ligand, suggesting that in ovarian cancer cells GPR30/EGFR signaling relays on ERα expression. Interestingly, we show that both GPR30 and ERα expression along with active EGFR signaling are required for E2-stimulated and G-1–stimulated proliferation of ovarian cancer cells. Because G-1 was able to induce both *c-fos* expression and proliferation in the ERα-negative/GPR30-positive SKBR3 breast cancer cells, the requirement for ERα expression in GPR30/EGFR signaling may depend on the specific cellular context of different tumor types. [Cancer Res 2007;67(4):1859–66]

Introduction

Ovarian cancer is the fourth leading cause of tumor death in Western countries and represents the most fatal gynecologic malignancy because the overall 5-year survival rate is only 10% to 20% (1, 2). Consequently, the identification of molecular targets would be useful to define pharmacologic interventions toward an improved outcome of patients with ovarian carcinoma.

Estrogens are major regulators of growth and differentiation in normal ovaries and also play an important role in the progression of ovarian cancer (reviewed in ref. 3 and references therein). Likewise, a marked proliferative response to estrogens was shown in ovarian surface epithelial cells representing the site of 90% of malignancies (4) and an increased risk of ovarian tumor was observed in postmenopausal women receiving estrogen replacement therapy (5–7). In line with the aforementioned observations, antiestrogenic treatments repressed the growth of ovarian carcinoma both *in vitro* and *in vivo* (8–11), and the aromatase inhibitor letrozole, which depletes the bioavailability of estrogens at tissue levels, showed clinical benefit in a subgroup of ovarian cancer patients (12, 13).

The biological effects of estrogens are classically mediated by the estrogen receptor (ER) α and ERβ, which function as hormone-inducible transcription factors binding to the estrogen-responsive element (ERE) located within the promoter region of target genes (14). In the normal ovary, the levels of ERβ are high and predominate over ERα, whereas an opposite pattern characterizes the development of ovarian cancer (reviewed in ref. 15 and references therein), which often expresses ERα levels similar to those found in breast carcinoma (16). It has been largely reported that ERα mediates the mitogenic action of estrogens in breast cancer by inducing a variety of genes involved in cell proliferation. In this respect, the estrogen-regulated proteins, such as pS2, progesterone receptor (PR), *c-fos*, and different cyclins (17, 18), have been identified as useful prognostic markers for predicting the responsiveness to antiestrogen therapy (16–18). As it concerns the ovary, the estrogen-driven growth of epithelial carcinoma tissues has been linked to ERα-mediated regulation of a plethora of genes involved in crucial cell function as recently shown by using microarray technology (19). Moreover, it should be taken into account that the levels of 17β-estradiol (E2) usually present at picomolar-nanomolar concentrations reach micromolar levels in the dominant follicle selected to ovulate (20–23). Consequently, the ovarian surface epithelium surrounding the ovary is exposed to elevated E2 levels that have been largely overlooked.

A large body of evidence has identified different mechanisms whereby membrane-initiated signaling involving growth factor receptor or membrane ERs mimics and/or contributes to the function of nuclear ERs (reviewed in refs. 24, 25 and references therein). Recent studies, including our own (26–32), have shown that the G protein–coupled receptor (GPCR), named GPR30, mediates the nongenomic signaling of E2 in a variety of estrogen-responsive

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cancer cells through activation of the epidermal growth factor receptor (EGFR) transduction pathway. On the other hand, considering that GPR30 binds most of ER ligands (29), thus far the contribution of ERα to GPR30/EGFR signaling in cancer cells has not been clearly assessed. Therefore, the recent identification of the first GPR30-selective ligand G-1 (26) has provided new opportunities to further differentiate between the functions of the ER family member and GPR30 in mediating the multifaceted mechanisms of estrogen action.

In the present study, we have ascertained the ability of G-1 to induce cell growth of E2-responsive ovarian cancer cells expressing ERα as well as of E2-responsive breast cancer cells not expressing ERα. We have also investigated the effect of G-1 on the induction of *c-fos* and other genes involved in the progression of cell cycle. We have found that G-1 induces the proliferation of both positive and negative ERα cancer cells. However, in ovarian tumor cells, ERα expression was required for cell proliferation as well as for *c-fos* stimulation and ERK activation, suggesting that the capacity of GPR30 to signal independently of ERα is a specific feature of ERα-negative tumors. By differentiating between the functions of ERα and GPR30, our data provide new insight into the mechanisms facilitating nongenomic estrogen signaling in different types of E2-responsive cancer cells.

Materials and Methods

Reagents. G-1 (1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone) was kindly provided by ChemDiv, Inc. (San Diego, CA). E2, cycloheximide, wortmannin, LY 294,002, pertussis toxin, PD 98059, dexamethasone, progesterone, and 5α-dihydrotestosterone (DHT) were purchased from Sigma-Aldrich Corp. (Milan, Italy). ICI 182,780 was obtained from Tocris Chemicals (Bristol, United Kingdom), AG 1478 and AG 490 were purchased from Biomol Research Laboratories, Inc. (DBA, Milan, Italy), and PP2 was obtained from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in DMSO, except E2, PD 98059, and wortmannin, which were dissolved in ethanol.

Cell culture. Human BG-1 and 2008 ovarian cancer cells, MCF7, and MDA-MB-231 breast cancer cells were all maintained in DMEM without phenol red supplemented with 10% fetal bovine serum (FBS). SKBR3 breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots, reverse transcription-PCR (RT-PCR), and immunocytochemical staining.

Plasmids. The firefly luciferase reporter plasmid for ERα was XETL, which contains the ERE from the *Xenopus* vitellogenin A2 gene (nucleotides −334 to −289), the herpes simplex virus thymidine kinase promoter region (nucleotides −109 to +52), the firefly luciferase coding sequence, and the SV40 splice and polyadenylation sites from plasmid pSV232A/L-AA5′. Reporter plasmids for c-*fos* and its deletion mutant c-*fos*ΔERE (which lacks the ERE sequence) encode −2.2 and −1,172 kb 5′ upstream fragments of human c-*fos*, respectively (gifts from K. Nose, Showa University, Tokyo, Japan). The reporter plasmid Gal4-luc was described together with the expression vectors for Gal4-Elk1 in our previous study (33). The *Renilla* luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard.

Transfections and luciferase assays. Cells (1×10^5) were plated into 24-well dishes with 500 μL of regular growth medium per well the day before transfection. The medium was replaced with that lacking serum on the day of transfection, which was done using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 0.5 μg of reporter plasmid, 0.1 μg of effector plasmid (where applicable), and 5 ng of pRL-TK. After 4 h, the serum-free medium containing the indicated treatment was renewed and then cells were incubated for ~18 h. Luciferase activity was measured with the Dual Luciferase kit (Promega) according to the manufacturer's recommendations. Firefly luciferase values were normalized to the internal transfection control provided by the *Renilla* luciferase activity. The normalized relative light unit values obtained from untreated cells were set as 1-fold induction on which the activity induced by treatments was calculated.

Western blotting. Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 μL of 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, 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). 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 the antibody against ERα (F-10),

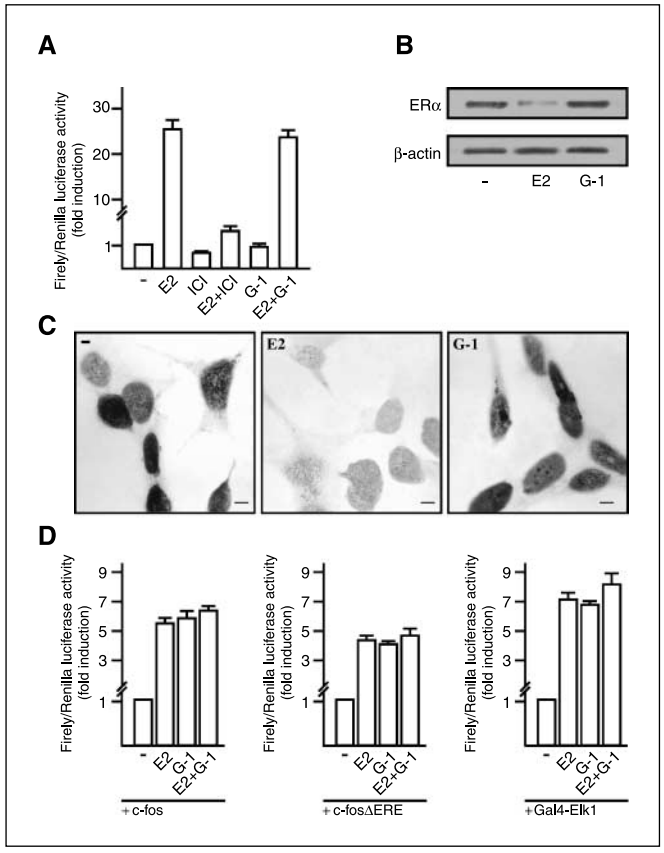

Figure 1. The specific GPR30 ligand G-1 does not activate ERα but induces the transcription of *c-fos* promoter constructs. *A,* BG-1 cells were transfected with the ER luciferase reporter plasmid XETL and treated with 100 nmol/L of E2 and G-1 and 10 μmol/L of the ER antagonist ICI 182,780 (*ICI*). The luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (−) were set as 1-fold induction on which the activity induced by treatments was calculated. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD. *B,* immunoblots of ERα from BG-1 cells. Cells were treated with vehicle (−) or 100 nmol/L of E2 and G-1 for 24 h. β-Actin serves as loading control. *C,* ERα immunodetection in BG-1 cells. Cells were treated with vehicle (−) or 100 nmol/L of E2 and G-1 for 2 h. Each experiment is representative of at least 10 tests. Bar, 5 μm. *D,* transcriptional activation of *c-fos* promoter constructs and Gal4-Elk1 by E2 and G-1 in BG-1 cells. The luciferase reporter plasmid c-fos encoding a −2.2-kb-long upstream region of human *c-fos*, the deletion mutant c-*fos*ΔERE lacking the ERE sequence and encoding a −1,172 bp upstream fragment of human *c-fos*, and the luciferase reporter plasmid for the fusion protein consisting of Elk1 and the Gal4 DNA-binding domain were transfected in BG-1 cells treated with vehicle (−) or 100 nmol/L of E2 and G-1. The luciferase values were standardized to the internal transfection control, and values of cells receiving vehicle were set as 1-fold induction on which the activity induced by treatments was calculated. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD.

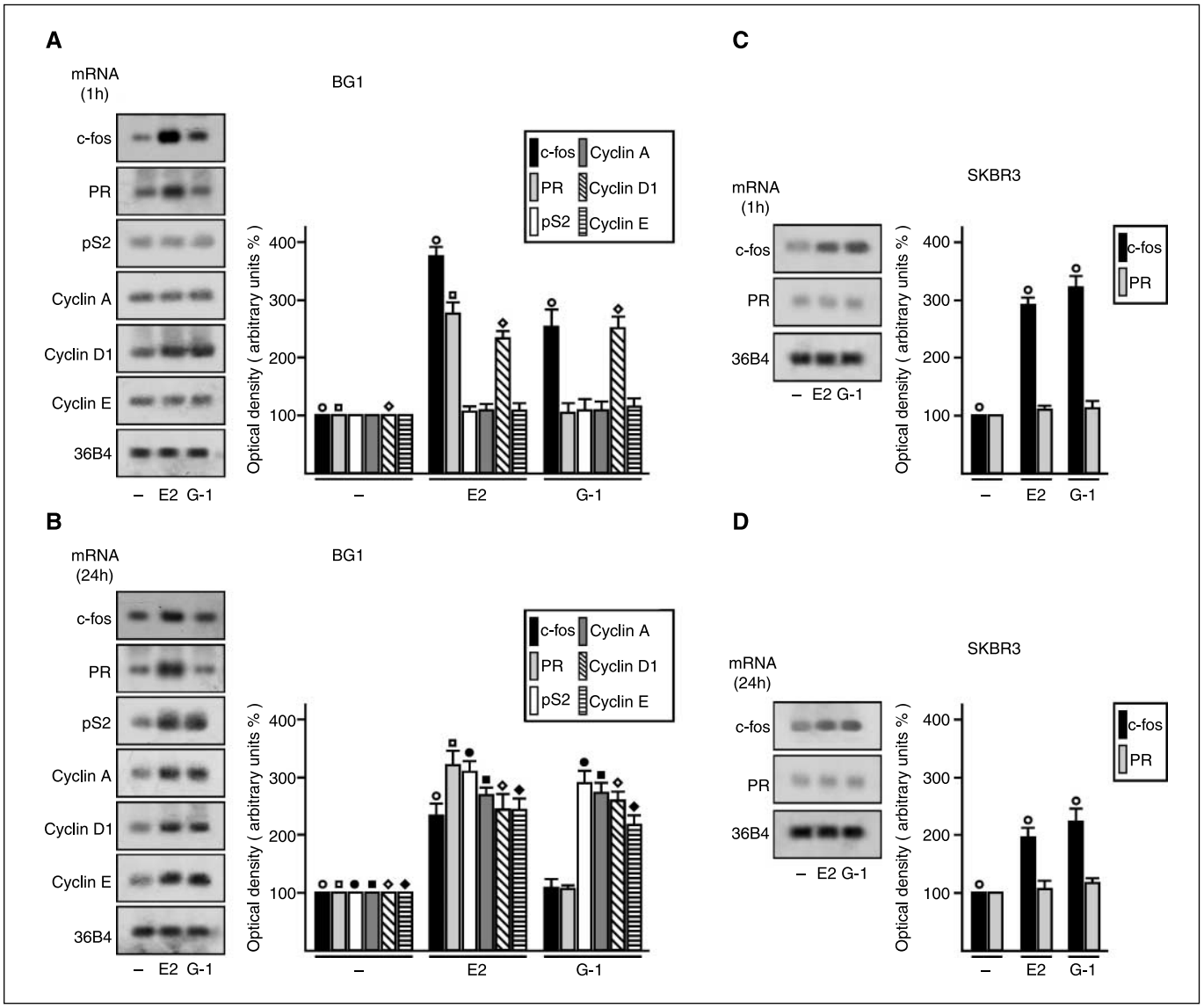

Figure 2. mRNA expression of estrogen target genes in BG-1 and SKBR3 cells. The expression of *c-fos*, PR, pS2, cyclin A, cyclin D1, and cyclin E was evaluated as indicated by semiquantitative RT-PCR in cells treated for 1 h (*A* and *C*) and 24 h (*B* and *D*) with vehicle (–) or 100 nmol/L of E2 and G-1; the housekeeping gene *36B4* was determined as a control. *Columns,* mean of three independent experiments after densitometry and correction for 36B4 expression; *bars,* SD. ○, □, ●, ■, ◇, and ♦, $P < 0.05$, for cells receiving vehicle (–) versus treatments.

c-*fos*, GPR30, β-actin (all purchased from Santa Cruz Biotechnology, DBA, Milan, Italy), ERβ (Serotec, Oxford, United Kingdom) phosphorylated ERK1/2, and ERK2 (Cell Signaling Technology, Inc., Celbio, Milan, Italy), and then revealed using the enhanced chemiluminescence system (Amersham Biosciences).

Reverse transcription-PCR. The evaluation of gene expression was done by semiquantitative RT-PCR as we have described previously (34). For c-*fos*, cyclin A, cyclin D1, pS2, PR, and the *acidic ribosomal phosphoprotein P0* (*36B4*), which was used as a control gene, the primers were 5′-AGAAAAGGAGAATCCGAAGGGAAA-3′ (c-*fos* forward) and 5′-ATGATGCTGGGACAGGAAGTC-3′ (c-*fos* reverse), 5′-ACACCTTGCCTGAAGTTTCG-3′ (PR forward) and 5′-CTGTCCTTTTCTGGGGGACT-3′ (PR reverse), 5′-TTCTATCCTAATACCATCGACG-3′ (pS2 forward) and 5′-TTTGAGTAGTCAAAGTCAGAGC-3′ (pS2 reverse), 5′-GCCATTAGTTTACCTGGACCCAGA-3′ (cyclin A forward) and 5′-CACTGACATGGAAGACAGGAACCT-3′ (cyclin A reverse), 5′-TCTAAGATGAAGGAGACCATC-3′ (cyclin D1 forward) and 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (cyclin D1 reverse), 5′-CCTGACTATTGTGTCCTGGC-3′ (cyclin E forward) and 5′-CCCGCTGCTCTGCTTCTTAC-3′ (cyclin E reverse), and 5′-CTCAACATCTCCCCCTTCTC-3′ (36B4 forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse) to yield products respectively of 420, 196, 210, 354, 354, 488, and 408 bp, with 20 PCR cycles for c-*fos*, PR, pS2, cyclin A, and cyclin E and 15 PCR cycles for both cyclin D1 and 36B4.

Antisense oligodeoxynucleotide experiments. Antisense oligodeoxynucleotides were purchased from MWG/M-Medical (Milan, Italy) and synthesized as described previously (35). The oligonucleotides used were 5′-TTGGGAAGTCACATCCAT-3′ for GPR30, 5′-GACCATGACCATGACCCT-3′ for ERα, 5′-CATCACAGCAGGGCTATA-3′ for ERβ, and 5′-GATCTCAGCACGGCAAAT-3′ for the scrambled control. For antisense experiments, a concentration of 200 nmol/L of the indicated oligonucleotides was transfected using Fugene 6 reagent as recommended by the manufacturer for 6 to 8 h before treatment with ligands.

Immunocytochemical staining. Cells were treated as indicated and then fixed in fresh paraformaldehyde (2% for 30 min). After paraformaldehyde removal, hydrogen peroxide (3% in methanol for 30 min) was used to inhibit endogenous peroxidase activity. Cells were then incubated with

normal horse serum (10% for 30 min) to block the nonspecific binding sites. Immunocytochemical staining was done using as the primary antibody a mouse monoclonal immunoglobulin G (IgG) generated against ERα (F-10; 1:50 overnight at 4°C). A biotinylated horse anti-mouse IgG (1:600 for 60 min at room temperature) was applied as the secondary antibody (Vector Laboratories, Burlingame, CA). Subsequently, the amplification of avidin-biotin-horseradish peroxidase complex (1:100 for 30 min at room temperature; Vector Laboratories) was carried out and 3,3′-diaminobenzidine tetrachloride dihydrate (Vector Laboratories) was used as a detection system. Cells were rinsed after each step with TBS [0.05 mol/L Tris-HCl plus 0.15 mol/L NaCl (pH 7.6)] containing 0.05% Triton X-100. In control experiments, cells were processed replacing the primary antibody with mouse serum (Dako S.p.A., Milan, Italy) or using a primary antibody preabsorbed (48 h at 4°C) with an excess of purified ERα protein (M-Medical).

Proliferation assays. For quantitative proliferation assays, 10,000 cells 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) and cells were trypsinized and counted in a hemocytometer on day 6. A concentration of 200 nmol/L of the indicated antisense oligodeoxynucleotides was transfected using Fugene 6 reagent as recommended by the manufacturer for 6 h before treatments, transfections, and treatments were renewed every 2 days.

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

G-1 does not activate ERα but induces the transcription of c-fos promoter constructs. BG-1 cells derived from a solid tumor tissue of a patient with stage III ovarian adenocarcinoma express clinically relevant levels of ERα but lack ERβ, consistent with the well-known receptor expression patterns found in primary ovarian tumors (15, 36). To better understand the molecular mechanisms involved in the proliferative action of estrogens in the ovary, we first evaluated the ability of E2 and G-1 to activate a transiently transfected ER reporter gene in BG-1 cells, which were used as a model for ovarian cancer. The exposure to 100 nmol/L E2 induced a strong ERα transactivation, which was no longer observed in the presence of 10 μmol/L of the ER antagonist ICI 182,780 (Fig. 1*A*). In contrast, treatments with 100 nmol/L G-1 and even concentrations ranging from 1 nmol/L to 10 μmol/L (data not shown) failed to stimulate luciferase expression or to block that observed on addition of E2 (Fig. 1*A*), consistent with the recent observation that G-1 is neither an agonist nor an antagonist for ERα (26). Considering that the down-regulation of ERα protein levels induced by an agonist has been considered an additional hallmark of receptor activation (37), we investigated whether the expression of ERα could be modulated by E2 and G-1 in BG-1 cells. As documented by Western blot analysis, the levels of ERα were robustly down-regulated in the presence of 100 nmol/L E2, whereas the same amount of G-1 did not show any modulatory effect on ERα protein content (Fig. 1*B*). To confirm the aforementioned observation using a different assay, we did an immunocytochemical study treating BG-1 cells with 100 nmol/L of E2 and G-1. The expression of ERα was again substantially reduced only by E2 (Fig. 1*C*), further ruling out the potential of G-1 to trigger ERα-mediated activity. Because our report and previous reports by others (26–32) have shown that GPR30 participates in biochemical as well as in biological responses elicited by estrogens in hormone-sensitive tumor cells, we evaluated whether its specific ligand G-1 could activate a transiently transfected full-length human c-fos promoter (−2.2 kb), which contains several target sequences responding to a variety of extracellular signals (38). Interestingly, G-1 transactivated *c-fos* similar to E2 and the same transcriptional response was still observed using a *c-fos* mutant lacking the ERE sequences (−1,172 bp; Fig. 1*D*). Nevertheless, we did not observe any synergism between E2 and G-1 on the *c-fos* promoter constructs used (Fig. 1*D*). As the ternary complex factor member Elk1 is crucial for the ERK-dependent activation of the *c-fos* gene promoter (39), we investigated whether G-1 and E2 could induce the Elk1-mediated transcriptional activity in BG-1 cells. Each compound activated Elk1 in the context of a Gal4 fusion protein; however, the transcriptional response was not substantially enhanced by E2 in combination with G-1 (Fig. 1*D*), suggesting that E2 and G-1 act through the same signal transduction pathway.

G-1 and E2 induce the mRNA expression of c-fos and other estrogen target genes. It has been widely shown that the expression of the *c-fos* gene is rapidly induced by a variety of extracellular stimuli (27, 31, 40, 41). To evaluate whether G-1 and E2 could up-regulate *c-fos* along with other well-known estrogen target genes in BG-1 cells, we did semiquantitative RT-PCR experiments comparing mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. Of note, a short treatment (1 h) with 100 nmol/L of E2 and G-1 enhanced *c-fos* levels, which were still evident after a 24-h exposure to E2 (Fig. 2*A* and *B*). The expression of pS2, cyclin A, and cyclin E was stimulated by both E2 and G-1 after 24 h of treatment (Fig. 2*A* and *B*), whereas the levels of cyclin D1 increased either at short or prolonged exposure to both compounds (Fig. 2*A* and *B*). In contrast, the expression of PR was up-regulated only by E2 at both

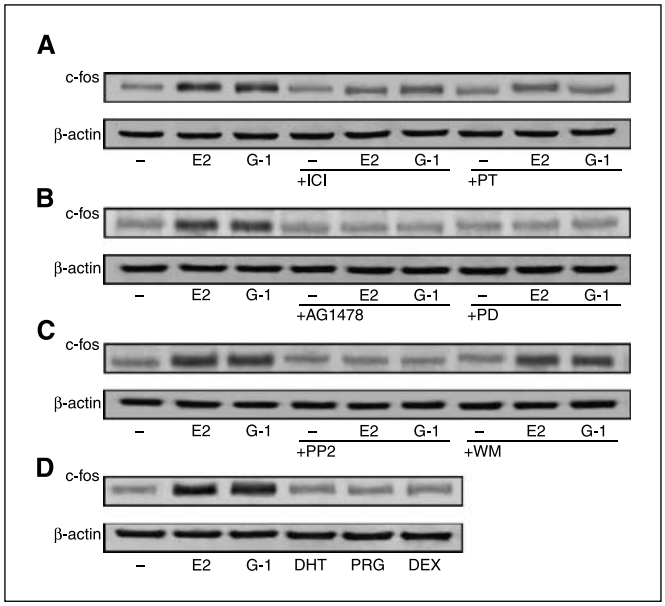

Figure 3. Immunoblots of *c-fos* from BG-1 cells. *A,* cells were treated for 2 h with vehicle (–) or 100 nmol/L of E2 and G-1 and in combination with 10 μmol/L ICI 182,780 or 100 ng/mL of G protein inhibitor pertussis toxin (*PT*). *B,* cells were treated for 2 h with vehicle (–) or 100 nmol/L of E2 and G-1 and in combination with 10 μmol/L of EGFR kinase inhibitor tyrphostin AG 1478 or 10 μmol/L of MAPK/ERK kinase (MEK) inhibitor PD 98059 (*PD*). *C,* cells were treated for 2 h with vehicle (–) or 100 nmol/L of E2 and G-1 and in combination with 10 μmol/L of Src family tyrosine kinase inhibitor PP2 or 10 μmol/L of PI3K inhibitor wortmannin (*WM*). *D,* cells were treated for 2 h with vehicle (–) or 100 nmol/L of E2, G-1, DHT, progesterone (*PRG*), and dexamethasone (*DEX*). β-Actin serves as a loading control.

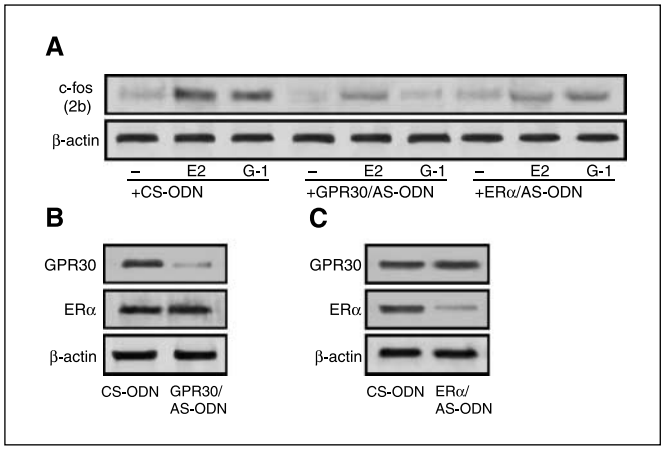

Figure 4. GPR30 and ERα antisense oligonucleotides abrogate the up-regulation of *c-fos* induced by E2 and G-1 in BG-1 cells. *A,* cells transfected with control scrambled (*CS-ODN*), GPR30 (*GPR30/AS-ODN*), or ERα (*ERα/AS-ODN*) antisense oligonucleotides were treated with 100 nmol/L of E2 and G-1. *B,* immunoblots showing GPR30 and ERα expression from cells transfected with control scrambled or GPR30 antisense oligonucleotides. *C,* immunoblots showing GPR30 and ERα expression from cells transfected with control scrambled or ERα antisense oligonucleotides. β-Actin serves as a loading control.

times of observation, indicating that an E2-activated ERα-dependent mechanism is involved in the regulation of this gene. To further support this finding, we turned to the SKBR3 cells, which do not express detectable amounts of ERs (27). As shown in Fig. 2*C* and *D*, E2 failed to regulate PR, whereas both E2 and G-1 retained the ability to induce *c-fos* expression, which we previously showed to be dependent on GPR30 expression (27). Next, no synergism between E2 and G-1 was observed in the regulation of any of the genes studied in BG-1 or SKBR3 cells (data not shown).

Transduction pathways involved in the up-regulation of c-fos protein levels exerted by G-1 and E2. We have previously shown that, in hormone-sensitive tumor cells, the E2-dependent induction of *c-fos* requires GPR30 expression and activity as well as EGFR-mediated signaling (27, 31, 32). Therefore, we asked whether G-1–dependent activation of *c-fos* requires both ERα- and GPR30-mediated signaling. As shown in Fig. 3*A*, either the ER antagonist ICI 182,780 or the GPCR inhibitor pertussis toxin reduced the induction of *c-fos* obtained on addition of 100 nmol/L of E2 and G-1 for 2 h, suggesting that both (receptor mediated) transduction mechanisms are required for the effect of each ligand. However, pertussis toxin lowered the induction of *c-fos* on G-1 more effectively than on E2, indicating that E2 may also lead to *c-fos* up-regulation through a GPR30-independent pathway as previously proposed (27). Furthermore, the stimulation of *c-fos* by both G-1 and E2 was equally abrogated by the EGFR kinase inhibitor tyrphostin AG 1478, the mitogen-activated protein kinase (MAPK) inhibitor PD 98059, and the Src family tyrosine kinase inhibitor PP2 but not using the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (Fig. 3*B* and *C*), suggesting that both ligands signal through the EGFR/ERK signaling pathway. Moreover, steroids, such as DHT, progesterone, and dexamethasone, did not increase *c-fos* protein levels (Fig. 3*D*), revealing that a ligand specificity is required for the regulation of *c-fos* in ovarian cancer cells. To further assess the role of ERα and GPR30 on the action elicited by E2 and G-1, we analyzed the response of *c-fos* to both compounds in the absence of either ERα or GPR3O expression. As shown in Fig. 4*A*, both antisense oligonucleotides for ERα (ERα/AS-ODN) and GPR30 (GPR30/AS-ODN) turned down the increase of *c-fos* induced by E2 and G-1, although each oligonucleotide selectively silenced only the expression of the specific oligonucleotide target sequence (Fig. 4*B* and *C*). These observations corroborate the results shown in Fig. 3*A* and indicate that ERα and GPR30 are mutually required for the enhancement of *c-fos* induced by cognate ligands. Moreover, the rapid activation of ERK1/2 on addition of 100 nmol/L of E2 and G-1 was abrogated by both antisense oligonucleotides (Fig. 5*A*), in line with recent results obtained using only E2 (42) but in contrast to other findings (43). Next, the inhibitors of EGFR signaling, such as AG 1478, PD 98059, and PP2, prevented ERK1/2 activation by E2 and G-1 (Fig. 5*B*), thus eliciting a repressive action similar to that observed for *c-fos* up-regulation by ligands. Together with results evidenced in Fig. 3*A* and *B*, these observations suggest that, in ovarian cancer cells, both ERα and GPR30 are necessary for activating EGFR/ERK signaling and the subsequent induction of *c-fos* in response to E2 and G-1.

G-1 and E2 stimulate the proliferation of the ovarian BG-1 and 2008 tumor cells and the breast SKBR3 carcinoma cells. The aforementioned findings were recapitulated in a more complex physiologic response, such as cell proliferation. In BG-1 cells, the growth-stimulatory effects induced by G-1 and E2 were abolished by the EGFR inhibitor AG 1478, the MAPK inhibitor PD 98059, and the Src kinase inhibitor PP2 (Fig. 6*A*), establishing that the EGFR/ERK signaling pathway mediates the stimulatory action of both ligands. Moreover, the abrogation of ERα or GPR30 expression also abrogated the E2-stimulated and G-1–stimulated cell growth (Fig. 6*A*). Similar results were also obtained using a different ovarian cancer cell line, named 2008 (Fig. 6*B*), which expresses the same receptor pattern of BG-1 cells (44). Altogether, these findings suggest that both ERα and GPR30 are required for proliferation of ovarian cancer cells in response to either E2 or G-1. Because the effect of both ligands on cell growth as well as on *c-fos* induction was abrogated by inhibition of EGFR kinase activity or its downstream effectors, our results also indicate that both ERα and GPR30 are mutually necessary to signal proliferation of ovarian cancer cells through the EGFR/ERK transduction pathway. However, the results shown in Fig. 2*C* and *D* show that G-1 is able to up-regulate *c-fos* in ER-negative SKBR3 cells. Besides, it has been previously reported that E2 does induce ERK activation in

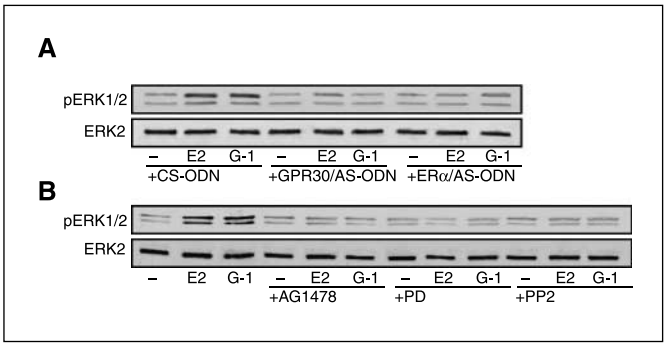

Figure 5. ERK1/2 phosphorylation in BG-1 cells. *A,* cells transfected with control scrambled (*CS-ODN*), GPR30 (*GPR30/AS-ODN*), or ERα (*ERα/AS-ODN*) antisense oligonucleotides were treated for 5 min with vehicle (–) or 100 nmol/L of E2 and G-1. *B,* cells were treated for 5 min with vehicle (–) or 100 nmol/L of E2 and G-1 and in combination with 10 μmol/L of EGFR kinase inhibitor tyrphostin AG 1478, 10 μmol/L of MEK inhibitor PD 98059, or 10 μmol/L of Src family tyrosine kinase inhibitor PP2.

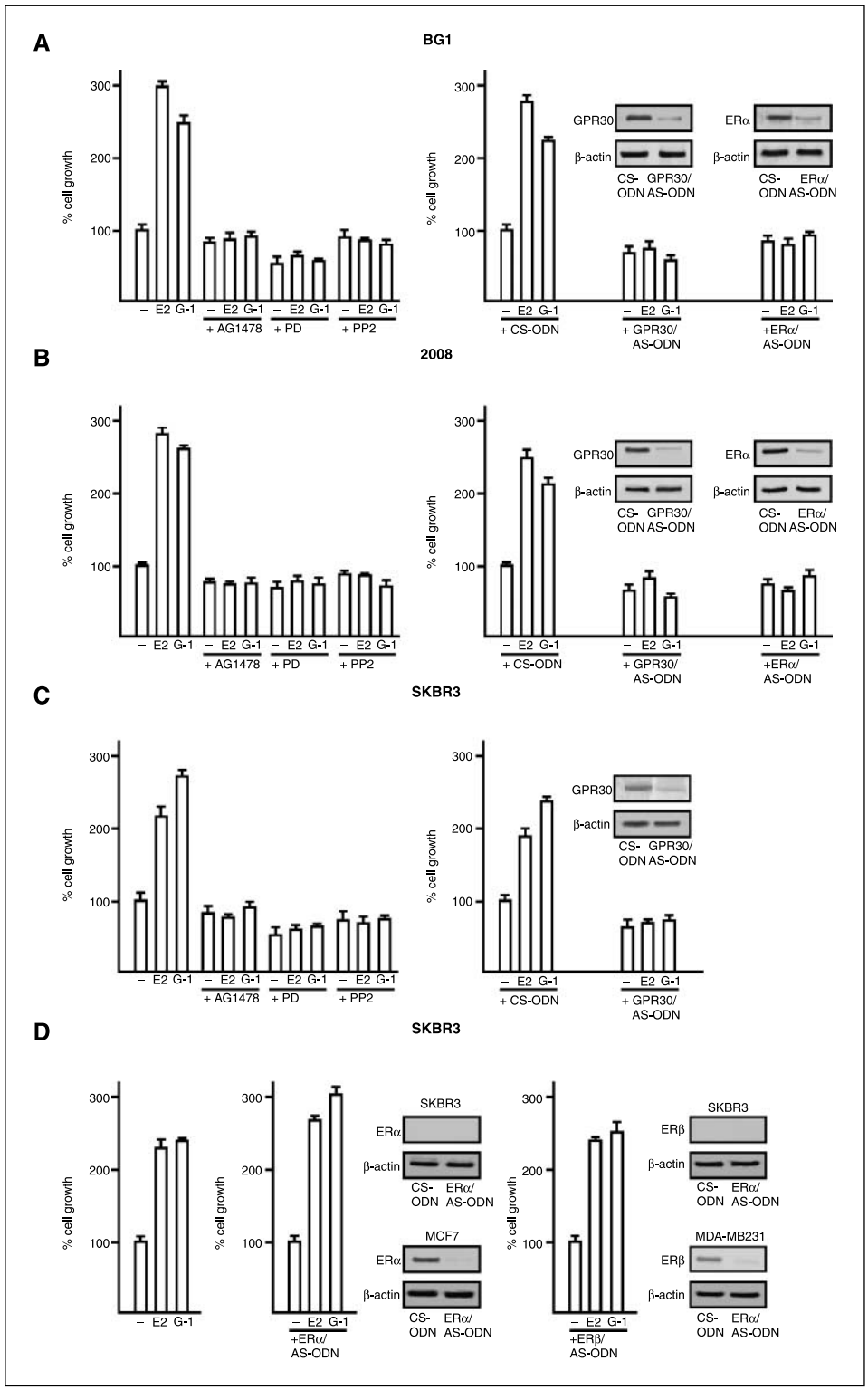

Figure 6. E2 and G-1 stimulate the proliferation of the ovarian BG-1 and 2008 tumor cells and the breast SKBR3 carcinoma cells. BG-1 cells (*A*), 2008 cells (*B*), and SKBR3 cells (*C*) were treated with vehicle (−) or 100 nmol/L of E2 and G-1 in medium containing 2.5% charcoal-stripped FBS (medium was refreshed and treatments were renewed every 2 d) and then counted on day 6. Cells cultured in the above experimental conditions were also treated with vehicle or 100 nmol/L of E2 and G-1 in combination with 10 μmol/L of EGFR kinase inhibitor tyrphostin AG 1478, 10 μmol/L of MEK inhibitor PD 98059, and 10 μmol/L of Src family tyrosine kinase inhibitor PP2 and counted on day 6. Proliferation of cells receiving vehicle was set as 100% on which cell growth induced by treatments was calculated. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD. BG-1 cells (*A*), 2008 cells (*B*), and SKBR3 cells (*C* and *D*) were transfected as indicated with control scrambled (*CS-ODN*), GPR30 (*GPR30/AS-ODN*), ERα (*ERα/AS-ODN*), or ERβ (*ERβ/AS-ODN*) antisense oligonucleotides and treated with vehicle (−) or 100 nmol/L of E2 and G-1. Transfection and treatments were renewed every 2 d and then cells were counted on day 6. Proliferation of cells receiving vehicle was set as 100% on which cell growth induced by treatments was calculated. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD. Efficacy of oligonucleotide silencing was ascertained by specific immunoblots also using MCF7 and MDA-MB-231 breast cancer cells.

SKBR3 cells (27). Therefore, we investigated the ability of ligands to stimulate SKBR3 cell proliferation. As shown in Fig. 6*C*, 100 nmol/L of E2 and G-1 promoted SKBR3 cell growth, which was abolished by AG 1478, PD 98059, and PP2 or by abrogation of GPR30 expression (Fig. 6*C*). To rule out the possibility that SKBR3 cells expressed undetectable levels of ERs sufficient to signal cell growth, we assessed ligand-induced proliferation in the presence of ERα and ERβ antisense oligonucleotides. As evidenced in Fig. 6*D*, the transfection of either ERα and ERβ antisense oligonucleotides at a concentration able to abrogate the target receptor expression respectively in MCF7 and MDA-MB-231 breast cancer cells had no effect on SKBR3 cell growth, establishing in this cellular context that GPR30 is sufficient to signal E2-induced proliferation. Cumulatively, these data indicate that, although ERα is required

for the G-1/GPR30 signaling pathway in ovarian cancer cells, GPR30 may induce cell growth independently of ERα expression depending on the tumor type.

Discussion

Ovarian surface epithelial cells, which represent the site of 90% of malignancies, show a striking proliferative response to estrogens (4). As it concerns ERα, its expression in ovary tumors has been associated with an increased rate of cell proliferation and a less favorable prognosis (45) contrary to that reported in breast cancer (46).

A wide number of studies have established that estrogens drive cancer cell growth not only by activating the transcriptional function of ERs but also by initiating nongenomic EGFR-dependent signaling pathways consisting in either ERK or AKT activation (14, 24). Recent studies have shown that, in breast cancer cells (27) as well as in endometrial cancer cells (31) and even in thyroid carcinoma cells (32), the nongenomic signaling triggered by E2 relays on expression and activity of GPR30, which in turn activates the EGFR signaling pathway (30). Whether E2 acts on the EGFR/ERK transduction pathway only through GPR30 binding or also through ERα binding is less clear because E2 binds to both receptors although with different affinity (28, 29). In the present article, by using either E2 or the selective GPR30 ligand G-1, we have determined the specificity of each signaling receptor in mediating E2 responsiveness of ovarian cancer cells. First, we show that both ligands induce the expression of *c-fos*, pS2, and cyclins A, D1, and E, whereas only E2 modulates ERα-dependent transcription and PR expression (Figs. 1 and 2). In addition, both ligands stimulate the proliferation of two different ovarian cancer cell lines in an EGFR-dependent fashion, suggesting that, as in breast cancer cells, GPR30 is part of the E2 nongenomic signaling pathway. Furthermore, two lines of evidence indicate that both ERα and GPR30 are mutually required for E2 and G-1 pleiotropic effects: (*a*) *c-fos* up-regulation by each ligand is sensitive to both ICI 182,780 and pertussis toxin (Fig. 3) and (*b*) abrogation of ERα and GPR30 expression by specific antisense oligonucleotides abolishes *c-fos* stimulation as well as ERK activation (Figs. 4 and 5) and cell proliferation (Fig. 6) induced by both ligands. Because the effect of E2 and G-1 on *c-fos* promoter activity or on Elk1 transactivation is not synergic (Fig. 1), we conclude that GPR30 and ERα act on the same signaling pathway respectively upstream and downstream of EGFR. In line with this model, it has been shown that GPR30 in response to E2 induces the release of surface-bound proHB-EGF (30), whereas ERα tyrosine phosphorylation by EGFR activation plays a key role in the steroid receptor interaction with Src and the regulation of Src-associated ERK kinase activity, which in turn stimulates a mitogenic signaling network known to be engaged by growth factors (reviewed in ref. 47 and references therein). Besides, it is well known that the EGFR-mediated transcriptional activation of unliganded ERα can also occur through the critical serine residue at position 118, which is the major phosphorylation site resulting from activation of the MAPK pathway (48–50).

It will be certainly interesting to define to what extent the cross-talk between ERα and GPR30 may influence the development of estrogen-sensitive tumors and/or the failure of endocrine therapeutic agents.

On the other hand, in the present and a previous study (27), we have shown that, in the ERα-negative SKBR3 breast cancer cells, GPR30 is able to elicit ERK activation and *c-fos* induction through EGFR signaling pathway. Interestingly, we now also show that both E2 and G-1 are able to induce SKBR3 cell proliferation, which relays on the activity of EGFR and its downstream effectors regardless of transfection of antisense oligonucleotides abrogating ERα and ERβ expression. In contrast, a GPR30 antisense oligonucleotide abolished both E2-mediated or G-1–mediated proliferation of SKBR3 cells, suggesting that the relevance of a functional interaction between ERα and GPR30 depends on the specific cellular context and type of tumor. It remains as an intriguing open question if and how other endogenous factors cross-interact with GPR30 in mediating E2-dependent proliferation of ERα-negative cancer cells.

In conclusion, the present study provides new insight toward the design of pharmacologic molecules targeting crucial metabolic cascades and genes directly involved in cell proliferation of ovarian cancer cells. Furthermore, it represents the first example of how the selective GPR30 ligand G-1 can provide a useful experimental model to screen for estrogen-like properties exerted through GPR30 in estrogen-sensitive tumors.

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G Protein-Coupled Receptor 30 and Estrogen Receptor α Are Involved in the Proliferative Effects Induced by Atrazine in Ovarian Cancer Cells

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Outline of section headers:

Abstract

Background: Atrazine, one of the most common pesticide contaminants, has been shown to up-regulate aromatase activity in certain estrogen-sensitive tumors without binding or activating the estrogen receptor (ER). Recent investigations have demonstrated that the orphan G-protein coupled receptor 30 (GPR30), which is structurally unrelated to the ER, mediates rapid actions of 17β-estradiol (E2) and environmental estrogens.

Objectives: Given the ability of atrazine to exert estrogen-like activity in cancer cells, we evaluated the potential of atrazine to signal through GPR30 in stimulating biological responses in cancer cells.

Methods and Results: Atrazine did not transactivate the endogenous ERα in different cancer cell contexts or chimeric proteins encoding the ERα and ERβ hormone binding domain in gene reporter assays. Moreover, atrazine neither regulated the expression of ERα, nor stimulated aromatase activity. Interestingly, atrazine induced ERK phosphorylation and the expression of estrogen target genes. Using specific signaling inhibitors and gene silencing, we demonstrated that atrazine stimulated the proliferation of ovarian cancer cells through the GPR30-EGFR transduction pathway and the involvement of ERα.

Conclusions: Our results indicate a novel mechanism through which atrazine may exert relevant biological effects in cancer cells. On the basis of the present data atrazine should be included among the environmental contaminants potentially able to signal via GPR30 in eliciting estrogenic action.

Introduction

Atrazine belongs to the 2-chloro-*s*-triazine family of herbicides (Figure 1) and is the most common pesticide contaminant of groundwater and surface water (Fenelon and Moore 1998; Kolpin et al. 1998; Lode et al. 1995; Miller et al. 2000; Müller et al. 1997; Solomon et al. 1996; Thurman and Cromwell 2000). Among the endocrine-disrupting effects, atrazine interferes with androgen- and estrogen-mediated processes (Babic-Gojmerac et al. 1989; Cooper et al. 1999, 2000; Cummings et al. 2000; Friedmann 2002; Kniewald et al. 1979, 1995; Narotsky et al. 2001; Shafer et al. 1999; Simic et al. 1991; Stoker et al. 1999, 2000). Based on binding affinity studies, this occurs without direct agonism or antagonism of the cognate receptors for these steroids (Roberge et al. 2004; Tennant et al. 1994a, 1994b). In this respect, previous investigations have suggested that atrazine reduces androgen synthesis and action (Babic-Gojmerac et al. 1989; Kniewald et al. 1979, 1980, 1995; Simic et al. 1991) as well as stimulates estrogen production (Crain et al. 1997; Heneweer et al. 2004; Keller and McClellan-Green 2004; Sanderson et al. 2000, 2001, 2002; Spano et al. 2004). The latter ability is exerted through at least two mechanisms which converge on increasing aromatase expression and activity. First, inhibiting phosphodiesterase, atrazine up-regulates cAMP which induces the expression of SF-1, an important regulator of the PII promoter of aromatase gene *CYP19*. The enhanced transcription of the aromatase gene increases both enzymatic activity of aromatase and estrogen production (Heneweer et al. 2004; Lehmann et al. 2005; Morinaga et al. 2004; Roberge et al. 2004; Sanderson et al. 2000, 2001). Next, atrazine binds to SF-1 and facilitates the recruitment of this factor to the PII promoter of the aromatase gene further stimulating the biological effects described above (Fan et al. 2007a, 2007b).

Epidemiologic studies have associated long-term exposure to triazine herbicides with increased risk of ovarian cancer in female farm workers in Italy (Donna et al.1989) and breast cancer in the general population of Kentucky in the United States (Kettles et al. 1997). In addition, atrazine leads to tumor development in the mammary gland and reproductive organs of female F344 rats (Pinter et

al. 1990), while in Sprague-Dawley rats it causes an earlier onset of mammary and pituitary tumors (Wetzel et al. 1994), a typical response to exogenously administered estrogens (Brawer et al. 1975). Given the potential ability of atrazine to interfere with reproduction and to cause cancer, the European Union banned its use. On the contrary, the U.S. Environmental Protection Agency (EPA) has approved the use of atrazine due to the lack of a clear association between the levels of exposure and cancer incidence in pesticide applicators (Sass and Colangelo 2006; Rusiecki et al. 2004; Gammon et al. 2005; Young et al. 2005; McElroy et al. 2007).

Regarding the apparent estrogenic effects of atrazine, previous studies have demonstrated that triazine herbicides do not bind or activate the classical estrogen receptor (ER) (Connor et al. 1996; Tennant et al. 1994a, 1994b). In recent years, increasing evidence has demonstrated in different experimental models that steroid hormones, including estrogens, can exert rapid actions interacting with receptors located within/near the cell membrane (Falkenstein et al. 2000; Norman et al. 2004; Revelli et al. 1998). The importance of this signaling mechanism is becoming more widely recognized as steroid membrane receptors have been implicated in a large number of physiological functions. Moreover, it has been suggested that nongenomic estrogen actions, like genomic ones, are susceptible to interference from environmental estrogens (Thomas 2000). Of note, these compounds compete with [3H]E2 for binding to estrogen membrane receptors (Loomis and Thomas 2000) and exert agonist effects on nongenomic transduction pathways in different cell contexts (Loomis and Thomas 2000; Nadal et al. 2000; Ruehlmann et al. 1988; Watson et al. 1999). However, the precise identity and function of many steroid membrane receptors is still controversial in terms of their specific molecular interactions with endogenous and environmental estrogens.

A seven-transmembrane receptor named GPR30, which is structurally unrelated to the nuclear ER, has been recently shown to mediate rapid actions of estrogens (Filardo et al. 2002; Revankar et al. 2005). Recombinant GPR30 protein, produced in ER-negative HEK-293 cells, exhibited all the steroid binding and signaling characteristics of a functional estrogen membrane receptor (Thomas et al. 2005; Thomas and Dong 2006). Our and other studies have also demonstrated that GPR30

mediates the rapid response to E2 in a variety of estrogen-responsive cancer cells by activating the epidermal growth factor receptor (EGFR)-MAPK transduction pathway (Albanito et al. 2007; Bologa et al. 2006; Filardo et al. 2000; Maggiolini et al. 2004; Revankar et al. 2005; Thomas et al. 2005; Vivacqua et al. 2006a, 2006b).

In the present study, for the first time we demonstrate that atrazine stimulates gene expression and growth effects in estrogen-sensitive ovarian cancer cells through GPR30 and the involvement of ERα. Moreover, we show that GPR30 mediates the stimulatory effects of atrazine in ER-negative SkBr3 breast cancer cells.

Materials and Methods

Reagents. Atrazine, 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine, 17-β-estradiol (E2), H89, Wortmannin (WM), and PD98059 (PD) were purchased from Sigma-Aldrich (Milan, Italy). AG1478 (AG) was purchased from Biomol Reaserch Laboratories (DBA, Milan, Italy). ICI 182,780 (ICI) was obtained from Tocris Chemicals (Bristol, United Kingdom) and GF109203X (GFX) from Calbiochem, (VWR International, Milan, Italy). All compounds were solubilized in DMSO, except E2 and PD which were dissolved in ethanol.

Cell Culture. Human BG-1 and 2008 ovarian cancer cells as well as human Ishikawa endometrial cancer cells were maintained in DMEM without phenol red supplemented with 10% FBS. H295R adrenal carcinoma cells were cultured in DMEM/F12 1:1 supplemented with 1% ITS Liquid Media Supplement (100x; Sigma), 10% calf serum and antibiotics. Human MCF-7 breast cancer cells were maintained in DMEM with phenol red supplemented with 10% FBS and human SkBr3 breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots and reverse transcription-PCR (RT-PCR).

Plasmids. Firefly luciferase reporter plasmids used were XETL for ERα (Bunone et al. 1996) and GK1 for the Gal4 fusion proteins (Webb et al. 1998). XETL contains the ERE from the Xenopus vitellogenin A2 gene (nucleotides -334 to -289), the herpes simplex virus thymidine kinase promoter region (nucleotides -109 to +52), the firefly luciferase coding sequence, and the SV40 splice and polyadenylation sites from plasmid pSV232A/L-AA5. Gal4 chimeras Gal-ERα and Gal-ERβ were expressed from plasmids GAL93.ER(G) and GAL.ERβ, respectively. They were constructed by transferring the coding sequences for the hormone binding domain (HBD) of ERα (amino acids 282–595) from HEG0 (Bunone et al. 1996) and for the ERβ HBD (C-terminal 287 amino acids) from plasmid pCMV5-hERβ into the mammalian expression vector pSCTEVGal93 (Seipel et al. 1992). The renilla luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard.

Transfection and Luciferase assays. BG-1, MCF-7, Ishikawa and SkBr3 cells ($1x10^5$) were plated into 24-well dishes with 500 µl per well of DMEM (BG-1, MCF-7, Ishikawa cells) or RPMI 1640 (SkBr3 cells) containing 10% FBS the day before transfection. The medium was replaced with DMEM or RPMI 1640 both supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red on the day of transfection. Transfections were performed using the Fugene6 Reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany) with a mixture containing 0.3 µg of reporter plasmid, 1 ng of pRL-TK and 0.1 µg of effector plasmid where applicable. After 5 to 6 h, the medium was replaced again with serum-free DMEM lacking phenol red and supplemented with 1% CS-FBS, ligands were added at this point and cells were incubated for 16 to 18 h. Luciferase activity was measured with the Dual Luciferase kit (Promega) according to the manufacturer's recommendations. Firefly luciferase values were 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 1-fold induction upon which the activity induced by treatments was calculated.

RT-PCR. The evaluation of gene expression was done by semiquantitative RT-PCR as we have described previously (Maggiolini M. et al. 1999). For ERα, c-fos, PR, pS2, CathepsinD, Cyclin A, Cyclin D1, Cyclin E and the acid phosphoprotein P0 (36B4), which was used as a control gene, the primers were: 5'-AATTCAGATAATCGACGCCAG-3' (ERα forward) and 5'-GTGTTTCAACATTCTCCCTCCTC-3' (ERα reverse), 5′-AGAAAAGGAGAATCCGAAGGGAAA-3′ (c-fos forward) and 5′-ATGATGCTGGGACAGGAAGTC-3′ (c-fos reverse), 5′-ACACCTTGCCTGAAGTTTCG-3′ (PR forward) and 5′-CTGTCCTTTTCTGGGGGACT-3′ (PR reverse), 5′-TTCTATCCTAATACCATCGACG-3′ (pS2 forward) and 5′-TTTGAGTAGTCAAAGTCAGAGC-3′(pS2 reverse), 5′-AACAACAGGGTGGGCTTC-3′ (CathepsinD forward), and 5′-ATGCACGAAACAGATCTGTGCT-3′ (CathepsinD Reverse) 5′-GCCATTAGTTTACCTGGACCCAGA-3′ (cyclinA forward) and 5′-

CACTGACATGGAAGACAGGAACCT-3′ (cyclinA reverse), 5′-TCTAAGATGAAGGAGACCATC-3′, (cyclinD1 forward) and 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (cyclin D1 reverse), 5′-CCTGACTATTGTGTCCTGGC-3′ (cyclin E forward) and 5′-CCCGCTGCTCTGCTTCTTAC-3′ (cyclin E reverse), and 5′-CTCAACATCTCCCCCTTCTC-3′ (36B4 forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse) to yield products of 345, 420, 196, 210, 303, 354, 354, 488, and 408 bp, respectively with 20 PCR cycles for ERα, c-fos, PR, pS2, cathepsin D, cyclin A, cyclin E and with 15 PCR cycles for both cyclin D1 and 36B4.

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 $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, a mixture of protease inhibitors containing 1mmol/L aprotinin, 20mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. Samples were then diluted 10 times and protein concentration was determined using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich). 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 the antibody against ERα (F−10), c-fos (H-125), β-actin (C-2), phosphorylated ERK1/2 (E-4) and ERK2 (C-14) (all purchased from Santa Cruz Biotechnology, DBA, Milan, Italy), human P450 aromatase (MCA 2077S purchased from Serotec, Milan, Italy) and then revealed using the ECL Western Blotting Analysis System (GE Healthcare, Milan, Italy).

ER binding assay. BG-1 cells were stripped of any estrogen by keeping them in medium without serum for 2 days. Cells were incubated with 1 nM [2,4,6,7-^{3}H]E2 (89 Ci/ mmol; Amersham Bioscience) and increasing concentrations of nonlabeled E2 or Atrazine for 1 h at 37° C in a humidified atmosphere of 95% air/ 5% CO_2. After removal of the medium, cells were washed with ice-cold PBS/ 0.1% methylcellulose twice, harvested by scraping and centrifugation, and lysed with

100% ethanol, 500 µl per 60 mm dish, for 10 min at room temperature (Lee et al. 1996). The radioactivity of extracts was measured by liquid scintillation counting.

Aromatase assay. In subconfluent BG-1 or H295R cells, aromatase activity was measured in the cell culture medium by tritiated water-release using 0.5 µM [1ß-^{3}H(N)]androst-4-ene-3,17-dione (25.3 Ci/mmol; DuPont NEN, Boston, MA, USA) as a substrate (Lephart and Simpson 1991). The cells were treated in a 6-well dish in culture medium in the presence of Atrazine or DMSO for 40 hs and then incubated with [1 ß -^{3}H(N)]androst-4-ene-3,17-dione. Incubations were performed at 37 °C for 6 h under a 95%:5% air/CO_2 atmosphere. The results obtained were calculated as pmol/h, and normalized to milligram of protein (pmol/h per mg protein) and expressed as percentages of untreated cells (100%).

GPR30 and ERα silencing experiments. Cells were plated onto 10-cm dishes, maintained in antibiotic-free medium for 24 h and then transfected for additional 24 h before treatments with a mixture containing Opti-MEM, 8 µl/well LipofectAMINE 2000 (Invitrogen, Milan, Italy) and 0.5 µg/well vector or shGPR30 (Albanito et al. 2008), control siRNA or ERα siRNA (Sigma-Aldrich, Milan, Italy).

Proliferation assay. For quantitative proliferation assay, 10,000 cells 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) and cells were trypsinized and counted in a haemocytometer on day 6. A concentration of 200 ng/L of the indicated shRNA was transfected using Fugene 6 Reagent as recommended by the manufacturer the day before treatments, and then renewed every 2 days before counting.

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

Atrazine does not activate ERα in cancer cells. Based on the evidence that atrazine produces early onset and increased incidence of estrogen-sensitive tumors in different experimental models (Cooper et al. 2007), we first evaluated whether atrazine could activate a transiently transfected ER reporter gene in estrogen-sensitive ovarian (BG-1), breast (MCF-7) and endometrial (Ishikawa) cancer cells. The exposure to 100 nM of E2 induced a strong ERα transactivation which was absent in the presence of 10 μM of the ER antagonist ICI in all cell contexts evaluated (Figure 2 A-C). In contrast, treatments with 1 μM atrazine and even concentrations ranging from 1 nM to 10 μM (data not shown) failed to stimulate luciferase expression or to block that observed upon addition of E2 (Figure 2 A-C). Moreover, atrazine did not activate an expression vector encoding ERα transiently transfected in the ER-negative SkBr3 breast cancer cells (Figure 2 D). To confirm that atrazine is not an ERα agonist and to examine whether ERβ could respond to atrazine, we turned to a completely heterologous system. Chimeric proteins consisting of the DNA binding domain (DBD) of the yeast transcription factor Gal4 and the ERα or ERβ hormone binding domain (HBD) transiently transfected in SkBr3 cells were strongly activated by E2 but not upon atrazine treatment (Figure 2 E-F), further corroborating the aforementioned results.

Atrazine neither regulates ERα expression nor competes with estrogen binding to ERα. Considering that the down-regulation of ERα induced by an agonist has been considered an additional hallmark of receptor activation (Santagati et al. 1997), we further investigated whether atrazine could modulate ERα expression in BG-1 cells, which lack ERβ (data not shown) according to previous investigations and receptor expression patterns found in primary ovarian tumors (Bardin et al. 2004; Geisinger et al. 1989). As shown in Figure 3 (panels A and B), ERα was down-regulated at both mRNA and protein levels by 100 nM E2, whereas 1 μM atrazine did not produce any modulatory effect. In agreement with these results and those obtained in transfection experiments, atrazine did not show any binding capacity for ERα (Figure 3 C) as already reported

(Cooper et al. 2007). Altogether, our findings rule out that the estrogen action of atrazine occurs through binding and direct activation of ERα.

Aromatase activity is not induced by atrazine. Given that atrazine is able to up-regulate aromatase expression and function in different cell contexts (Cooper et al. 2007; Fan et al. 2007a, 2007b; Roberge et al. 2004; Sanderson et al. 2000, 2001), we then determined aromatase activity by tritiated water release assays in BG-1 cells. As shown in Figure 4, 1 μM atrazine did not stimulate aromatase activity, which in contrast was strongly induced in human H295R adrenocorticocarcinoma cells previously used as a model system to assess aromatase catalytic activity (Heneweer et al. 2004; Sanderson et al. 2001). In addition, the low aromatase protein expression detected in BG-1 cells did not increase upon exposure to 1 μM atrazine (data not shown). Hence, atrazine is neither an ERα activator nor an aromatase regulator in estrogen-sensitive ovarian cancer cells.

ERK phosphorylation is stimulated by atrazine. In recent years, numerous reports have demonstrated that estrogens and xenoestrogens can generate rapid signaling via second messenger systems such as Ca^{2+}, cAMP, nitric oxide and G proteins, which in turn lead to activation of different downstream kinases (Bulayeva and Watson 2004; Watson et al. 2007).

In order to evaluate whether the potential estrogenic activity of atrazine is exerted through a rapid cellular response, we investigated its ability to produce ERK phosphorylation in BG-1 cells. Interestingly, atrazine stimulated ERK phosphorylation, although a higher concentration as well as a prolonged time were required to trigger this biochemical response when compared to E2 (Figure 5 A-B; Figure 6 A). The ERK activation was delayed in presence of 1 μM atrazine compared to 100 nM E2 also in 2008 ovarian cancer cells (Figure 6 D) which present a similar receptor expression as BG-1 cells (Safei et al. 2005). In order to determine the transduction pathways involved in ERK activation by atrazine, cells were exposed to 100 nM E2 and 1 μM atrazine along with specific inhibitors widely used to pinpoint the mechanisms contributing to ERK phosphorylation (Bulayeva and Watson 2004). Of note, the ER antagonist ICI, the EGFR and ERK inhibitors AG and PD,

respectively, prevented ERK activation induced by both E2 and atrazine, whereas GFX, H89 and WM, inhibitors of protein kinase C (PKC), protein kinase A (PKA) and PI3K, respectively, did not (Figure 6 B-C and Figure 6 E-F). Considering that in a previous study ICI was able to trigger ERK phosphorylation (Filardo et al. 2000), we exposed SkBr3 cells to increasing concentrations of ICI. Neither after 5 min (data not shown) nor after 20 min of treatments (Figure 7) did we observed ERK activation. Hence, in our experimental conditions ICI showed only an ERK inhibitor activity.

Atrazine up-regulates the mRNA expression of estrogen target genes. Having determined that atrazine signals through a rapid ERK activation, we evaluated in BG-1 cells its ability to regulate the expression of c-fos, an early gene which responds to a variety of extracellular stimuli including estrogens (Maggiolini et al. 2004; Nephew et al. 1993; Singleton et al. 2003; Vivacqua et al. 2006a), along with other estrogen target genes. To this end, we performed semiquantitative RT-PCR experiments comparing mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. A short treatment (1 h) with 1 μM of atrazine enhanced c-fos and cyclin A levels although to a lesser extent than 100 nM of E2, which also stimulated PR, pS2 and cyclin D1 expression (table 1). After a 24 h treatment, atrazine increased PR, pS2 and cyclin A levels while E2 additionally induced the expression of c-fos, cathepsin D, cyclins D1 and E (table 1). Results similar to those described above were obtained in 2008 cells (data not shown). Hence, atrazine is able to stimulate the expression of diverse estrogen target genes without an apparent activation of ERα (see discussion).

Transduction pathways involved by atrazine in the up-regulation of c-fos protein levels.

Using c-fos expression as a molecular sensor of atrazine action at the genomic level, we sought to determine whether c-fos protein levels are also regulated by atrazine in a rapid manner and the transduction pathways involved in this response (Figure 8). Interestingly, the up-regulation of c-fos observed in BG-1 and 2008 cells after a short treatment (2 h) was abolished either by the ER antagonist ICI or the EGFR and ERK inhibitors, AG and PD respectively (Figure 8). On the contrary, GFX, H89 and WM, inhibitors of PKC, PKA and PI3K, respectively, did not interfere

with c-fos stimulation (Figure 8). Thus, in ovarian cancer cells atrazine involves ERα and the EGFR-MAPK pathway to trigger c-fos protein increase. On the basis of these and our previous results showing that c-fos stimulation by E2 occurs through GPR30 and requires ERα and EGFR-mediated signaling in cancer cells expressing both receptors (Albanito et al. 2007; Maggiolini et al. 2004; Vivacqua et al. 2006a, 2006b), we examined whether atrazine could act in a similar manner. Interestingly, both E2 and atrazine were no longer able to induce c-fos expression silencing either ERα or GPR30 in BG-1 and 2008 cells (Figure 9). In order to evaluate whether atrazine could induce a rapid response in a cell context expressing GPR30 alone, we turned to the ER-negative SkBr3 breast cancer cells. Notably, both ERK phosphorylation and c-fos induction stimulated by atrazine were abolished knocking-down GPR30 (Figure 10), indicating that the response to atrazine is differentially regulated according to the cancer cell types.

The proliferation of ovarian cancer cells induced by atrazine occurs through GPR30 and requires both ERα and EGFR-MAPK-mediated signaling. The aforementioned results were recapitulated in a more complex physiological assay such as cell growth. We observed that both E2 and atrazine induced the proliferation of BG-1 and 2008 cells in a concentration-dependent manner (Figure 11 A and Figure 11 E). Moreover, the growth effects elicited by E2 and atrazine were no longer evident in presence of AG and PD (Figure 11 B and Figure 11 F) or after silencing the expression of either GPR30 or ERα (Figure 11 C-D and Figure 11 G-H), indicating that both receptors along with the EGFR/MAPK transduction pathway are involved in the growth effects as well as in the c-fos expression profile showed above.

Discussion

In the present study we have demonstrated for the first time that atrazine exerted an estrogen-like activity in ovarian and breast cancer cells through GPR30, which is recently receiving growing interest due to its ability to mediate rapid estrogen signals (Albanito et al. 2007; Albanito et al. 2008; Filardo et al. 2006, Filardo et al. 2007; Revankar et al. 2005, Revankar et al. 2007). Previous studies have demonstrated that atrazine elicits estrogen action by up-regulating aromatase activity in certain cancer cells with elevated aromatase levels (Fan et al. 2007a, 2007b; Heneweer et al. 2004; Sanderson et al. 2000, 2001), but neither by binding to nor activating ERα (Connor et al. 1996; Roberge et al. 2004; Tennant 1994a). Using different tumor cells and reporter genes we confirmed that atrazine did not interact directly with ERα, yet it did not stimulate aromatase activity in our model system likely as a consequence of a very low aromatase expression. Nevertheless, atrazine induced the expression of diverse estrogen target genes recalling previous studies which demonstrated the recruitment of ERα by distinct compounds and growth factors to gene promoter sequences different from the classical estrogen response element (reviewed in Dudek and Picard 2008).

Interestingly, we demonstrated that GPR30 and ERα together with the EGFR/MAPK pathway are involved in the biological response to atrazine in ovarian cancer cells, which is in accordance with our recent investigation showing that the selective GPR30 ligand G-1 exerts biological activity similar to that of atrazine without binding or activating ERα (Albanito et al. 2007). Hence, our data argue that a complex interplay between different ERs and transduction pathways contribute to atrazine activity, which nevertheless is still noticeable in presence of GPR30 alone as demonstrated in SkBr3 breast cancer cells. It is worth noting that although E2 exhibited an exclusive up-regulation of target genes through direct activation of ERα, the GPR30-EGFR transduction pathway was involved in estrogen-induced proliferation of ovarian tumor cells, as evidenced silencing GPR30 and using specific pharmacological inhibitors.

A variety of environmental contaminants exhibit similar binding affinities for GPR30 and agonist activities as for ERs (Thomas and Dong 2006). Of interest, in the current study atrazine triggered rapid biological responses through GPR30 in both ovarian and breast cancer cells irrespective of ERα expression and despite a low binding affinity for GPR30 ectopically expressed in HEK293 cells (Thomas and Dong 2006). In line with these findings, an efficient competitor of E2 for the endogenous GPR30 in SKBR3 cells, such as ortho, para-DDE derivative, was ineffective in binding to recombinant GPR30 (Thomas et al. 2005; Thomas and Dong 2006). Likely, the interaction of atrazine with GPR30 is facilitated by the relative abundance of this membrane receptor in cancer cells respect to cells engineered to express recombinant GPR30 and/or yet unknown factors may contribute to the binding to GPR30 by these contaminants.

As it concerns the role of ERα, we proved a complex interplay with GPR30 exists as previously reported with some growth factor receptors (Migliaccio et al. 2006), however the molecular mechanisms involved remain to be elucidated. Our and previous investigations indicate that environmental estrogens exert pleiotropic actions by directly binding to ERα as well as through GPR30-EGFR signaling, which can engage ERα depending on the receptor expression pattern present in different cell types. This mode of action of xenoestrogens fits well with the results obtained knocking-down GPR30 or ERα expression in ovarian cancer cells, since silencing each gene prevented the growth response to atrazine.

Our data recall the results of previous studies showing that xenoestrogens mimic rapid estrogen action in several animal and cell models (Bulayeva and Watson 2004; Loomis and Thomas 2000; Nadal et al. 2000; Ruehlmann et al. 1988; Watson et al. 1999, 2007). Particularly, in GH3/B6/F10 pituitary tumor cells diverse xenoestrogens induced ERK phosphorylation with a temporally distinct activation pattern compared to E2 (Bulayeva et al. 2004). Different inhibitors such as ICI, AG and a membrane disrupting agent blocked ERK activation suggesting that multiple transduction pathways contributed to this rapid cell response. On the basis of the inhibitory activity exerted by ICI, it was hypothesized that an ER localized to the plasma membrane could mediate

ERK phosphorylation response by xenoestrogens depending on the different ER binding affinities. Moreover, it was argued that the wide diversity in signaling cascades leading to ERK activation may be explained by the nature of membrane ERs and the need of their interactions with various signaling partners. Interestingly, our findings have provided evidence that ERα may be involved by xenoestrogens without a direct binding activity and produce relevant responses such as ERK phosphorylation, gene expression and cell growth.

A subset of estrogen-sensitive cell tumors can proliferate independently from ER expression (i.e. ER-negative cells). In this condition, well represented by the SkBr3 breast cancer cells, GPR30-EGFR signaling may still allow for environmental estrogen activity as we have shown in the present and a previous study (Maggiolini et al. 2004). Hence, multiple transduction pathways triggered simultaneously at the membrane level as well as within each cell type may contribute to the nature and magnitude of biological responses to distinct estrogenic compounds. These consequently should be examined individually for their complex mechanistic and functional outcomes which result from interaction with a different repertoire of receptor proteins.

Atrazine is the most common pesticide contaminant of groundwater and surface water and a potent endocrine disruptor. Here, we have provided novel insight regarding the potential role of GPR30 in mediating the action of atrazine in endocrine-related diseases, such as estrogen-sensitive tumors.

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Table 1: mRNA expression (mean+/-SD) induced by 100 nM E2 and 1 μM atrazine (Atr) in BG-1 cells. The values calculated by optical density in cells treated with vehicle were set as 100% upon which the expression induced by treatments was expressed as % variation.

Gene	E2 (1h)	E2 (24h)	Atr (1h)	Atr (24h)
c-fos	423 ± 28*	239 ± 17*	269 ± 21*	120 ± 9
PR	228 ± 18*	298 ± 18*	122 ± 18	180 ± 11*
pS2	175 ± 17*	270 ± 21*	99 ± 19	187 ± 20*
CathepsinD	106 ± 9	217 ± 16*	102 ± 5	109 ± 6
CyclinA	262 ± 22*	293 ± 23*	220 ± 20*	190 ± 22*
CyclinD1	258 ± 19*	242 ± 19*	107 ± 4	118 ± 8
CyclinE	120 ± 11	343 ± 21*	118 ± 8	119 ± 10

* indicates significant difference ($P<0.05$) induced by treatments respect to vehicle.

Figure Legends

Figure 1. Structure of 17β-estradiol and atrazine.

Figure 2. ERα transactivation in the indicated cell lines. BG-1 (A), MCF-7 (B), Ishikawa (C) cells were transfected with the ER luciferase reporter plasmid XETL and treated with 100nmol/L E2, 1μmol/L atrazine and 10 μmol/L ER antagonist ICI 182,780 (ICI). The luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (-) were set as 1-fold induction, upon which the activity induced by treatments was calculated. SkBr3 cells were transfected with ER luciferase reporter gene XETL and ERα expression plasmid (D), with Gal4 reporter gene (GK1) and the Gal4 fusion proteins encoding the hormone binding domain of ERα (E) and ERβ (F) and treated with 100nmol/L E2, 1μmol/L atrazine and 10 μmol/L ER antagonist ICI 182,780 (ICI). Columns: mean of three independent experiments performed in triplicate; bars: SD. ○, $P<0.05$, for cells receiving vehicle (-) versus treatment.

Figure 3. (A) mRNA expression of ERα in BG-1 cells. The mRNA expression of ERα was evaluated by semiquantitative RT-PCR in cells treated for 24 h with vehicle (-) or 100nmol/L E2 and 1μmol/L atrazine. The housekeeping gene 36B4 was determined as a control. The result shown is representative of 3 independent experiments. (B) immunoblot of ERα from BG-1 cells. Cells were treated for 24 h with vehicle (-) or 100nmol/L E2 and 1μmol/L atrazine. β-actin serves as a loading control. The results shown in (A) and (B) are representative of 3 independent experiments. (C) ERα binding assay using increasing concentrations of atrazine.

Figure 4. Aromatase activity in BG-1 and H295R cells. Aromatase activity was assessed by tritiated water-release in BG-1 and H295R cells treated with vehicle (-) or 1μmol/L atrazine. The results obtained were calculated as pmol/h, normalized to protein (pmol/h per mg protein) and expressed as percentages of untreated cells (100%). Columns: mean of three independent experiments each performed in triplicate; bars: SD. ○, $P<0.05$, for cells receiving vehicle (-) versus treatment.

Figure 5. ERK1/2 phosphorylation in BG-1 cells exposed to increasing concentrations of E2 and atrazine.

Figure 6. (A) and (D), BG-1 and 2008 cells were treated with vehicle (-) or 100 nmol/L E2 and 1μmol/L atrazine for 5, 10, 20 and 30 min. (B-C) and (E-F), BG-1 and 2008 cells were treated for 20 min with vehicle (-) or 100 nmol/L E2 and 1μmol/L atrazine in combination with 10 μmol/L o ICI 182,780 (ICI), AG1478 (AG), PD98059 (PD), GF109203X (GFX), H89 and Wortmannin (WM), inhibitors of ER, EGFR, MEK, PKC, PKA and PI3K, respectively.

Figure 7. ERK1/2 phosphorylation in SkBr3 cells treated with increasing concentrations of ICI.

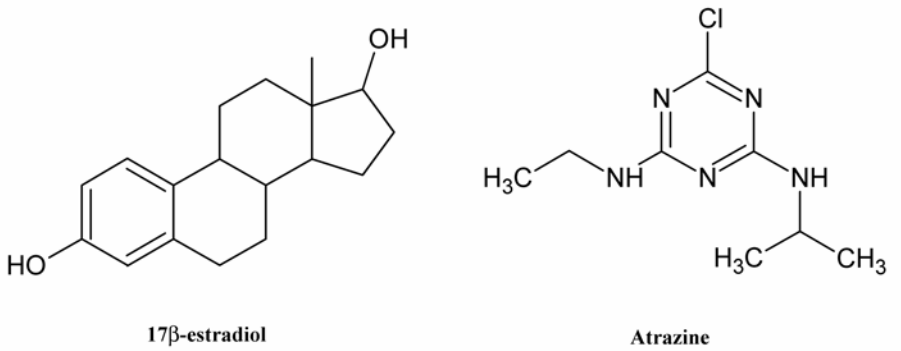
Figure 8. Immunoblots of c-fos from BG-1 and 2008 cells. BG-1 (A-B) and 2008 (C-D) cells were treated for 2 h with vehicle (-) or 100 nmol/L E2 and 1 μmol/L atrazine in combination with 10 μmol/L ICI 182,780 (ICI), AG1478 (AG), PD98059 (PD), GF109203X (GFX), H89 and Wortmannin (WM), inhibitors of ER, EGFR, MEK, PKC, PKA and PI3K, respectively. β-actin served as a loading control.

Figure 9. Immunoblots of c-fos from BG-1 and 2008 cells knocking-down ERα and GPR30 expression. BG-1 (A-B) and 2008 (C-D) cells were transfected with control siRNA or siRNA-ERα and with vector or shGPR30 and treated for 2 h with vehicle (-) or 100 nmol/L E2 and 1 μmol/L atrazine. Efficacy of ERα and GPR30 silencing was ascertained by immunoblots as shown in side panels. β-actin served as a loading control.

Figure 10. ERK1/2 phosphorylation (A) and c-fos expression (B) knocking-down GPR30 in SkBr3 cells treated with vehicle (-) or 1 μmol/L atrazine. (C), efficacy of GPR30 silencing was ascertained by immunoblots. β-actin served as a loading control.

Figure 11. (A and E), proliferation of BG-1 and 2008 cells exposed to increasing concentrations of E2 and atrazine. (B and F), BG-1 and 2008 cells were treated with vehicle (-), 100nmol/L E2 and 1 μmol/L atrazine in medium containing 2.5% charcoal-stripped FBS and then counted on day 6 (medium was refreshed and treatments were renewed every 2 days). Cells cultured in the above

experimental conditions were also treated with vehicle or 100nmol/l E2 and 1μmol/L atrazine in combination with 10 μmol/L AG1478 (AG) and PD98059 (PD), EGFR and MEK inhibitors, respectively, and counted on day 6. (C and G), BG-1 and 2008 cells were transfected with vector or shGPR30 as well as with control siRNA or siRNA-ERα (D and H) and treated with vehicle (-), 100nmol/L E2 and 1 μmol/L atrazine. Transfections and treatments were renewed every 2 days and then cells were counted on day 6. Proliferation of cells receiving vehicle was set as 100%, upon which cell growth induced by treatments was calculated. Columns: mean of three independent experiments performed in triplicate; bars, SD. ○, □, $P<0.05$, for cells receiving vehicle (-) versus treatment. Efficacy of ERα and GPR30 silencing was ascertained by immunoblots (see Figure 9).



17β-estradiol **Atrazine**

Figure 1

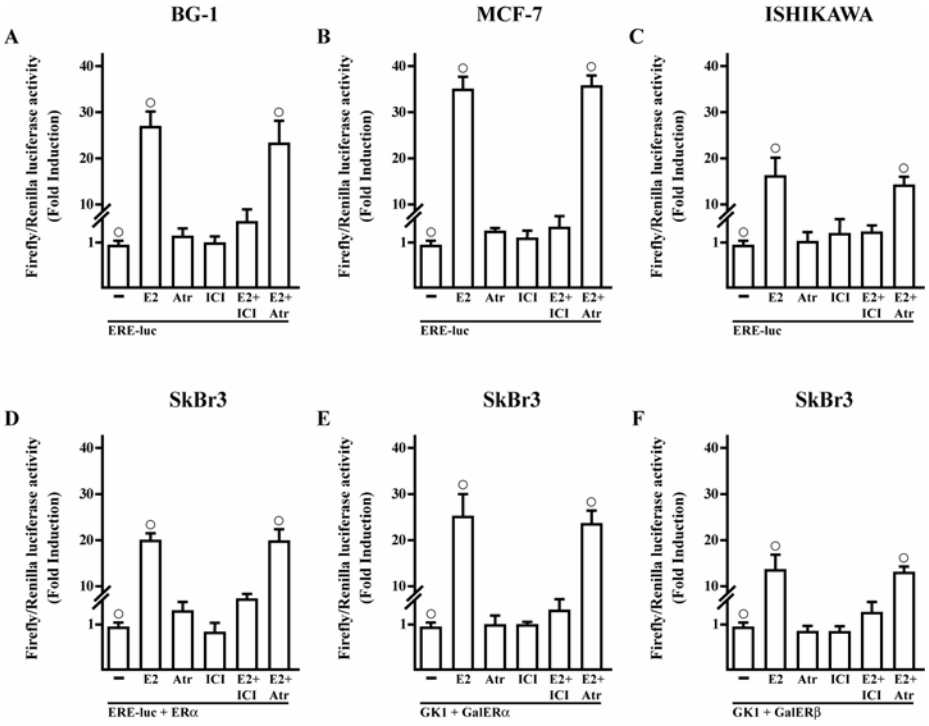

A
BG-1
B
MCF-7
C
ISHIKAWA
D
SkBr3
E
SkBr3
F
SkBr3
Firefly/Renilla luciferase activity
(Fold Induction)
40
30
20
10
1
–
E2
Atr
ICI
E2+ ICI
E2+ Atr
ERE-luc
ERE-luc + ERα
GK1 + GalERα
GK1 + GalERβ

Figure 2

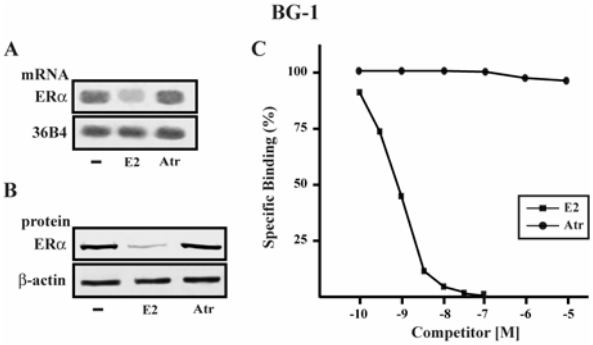

BG-1
A
mRNA
ERα
36B4
–
E2
Atr
B
protein
ERα
β-actin
–
E2
Atr
C
100
75
50
25
Specific Binding (%)
E2
Atr
-10
-9
-8
-7
-6
-5
Competitor [M]

Figure 3

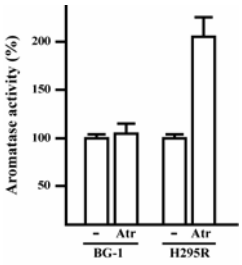


Figure 4

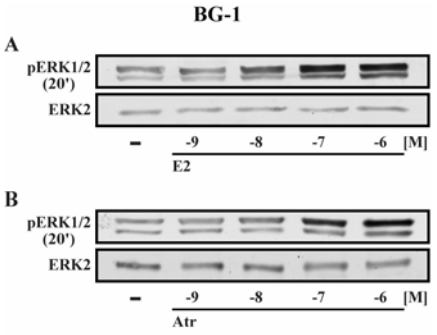


Figure 5

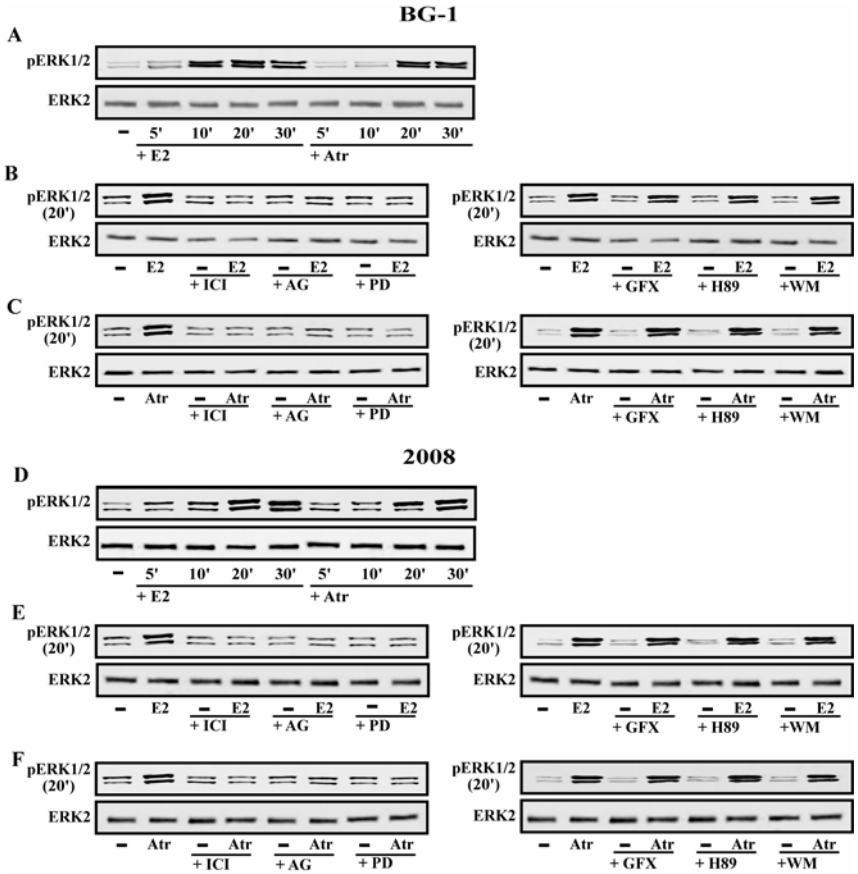

BG-1
A
pERK1/2
ERK2
– 5' 10' 20' 30' 5' 10' 20' 30'
+ E2
+ Atr
B
pERK1/2 (20')
ERK2
– E2 – E2 + ICI – E2 + AG – E2 + PD
– E2 – E2 + GFX – E2 + H89 – E2 +WM
C
pERK1/2 (20')
ERK2
– Atr – Atr + ICI – Atr + AG – Atr + PD
– Atr – Atr + GFX – Atr + H89 – Atr +WM
2008
D
pERK1/2
ERK2
– 5' 10' 20' 30' 5' 10' 20' 30'
+ E2
+ Atr
E
pERK1/2 (20')
ERK2
– E2 – E2 + ICI – E2 + AG – E2 + PD
– E2 – E2 + GFX – E2 + H89 – E2 +WM
F
pERK1/2 (20')
ERK2
– Atr – Atr + ICI – Atr + AG – Atr + PD
– Atr – Atr + GFX – Atr + H89 – Atr +WM

Figure 6

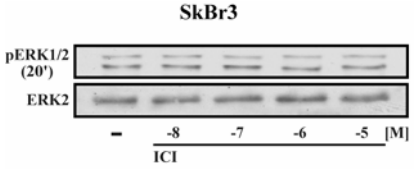


Figure 7

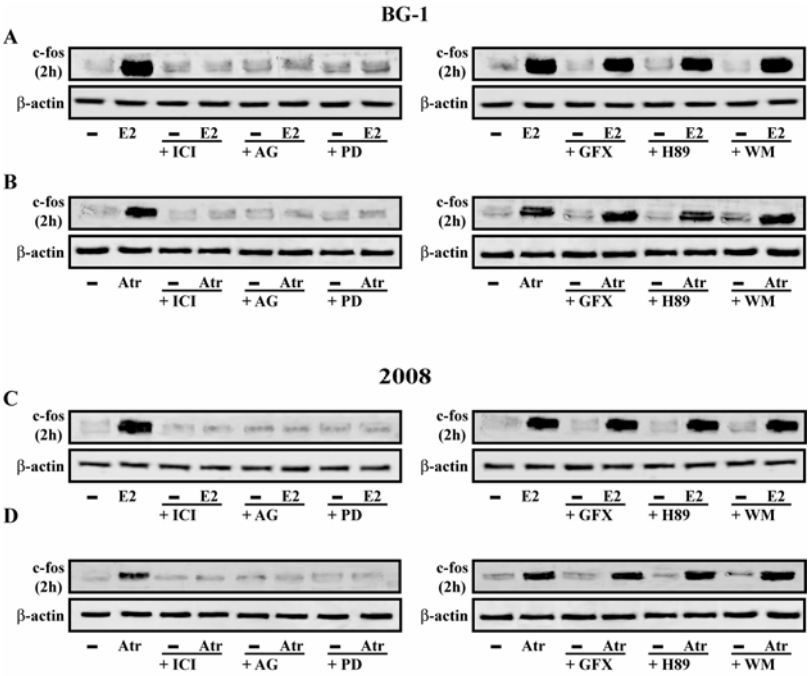

BG-1
A
c-fos (2h)
β-actin
– E2
– E2 + ICI
– E2 + AG
– E2 + PD
– E2 + GFX
– E2 + H89
– E2 + WM
B
– Atr
– Atr + ICI
– Atr + AG
– Atr + PD
– Atr + GFX
– Atr + H89
– Atr + WM
2008
C
D

Figure 8

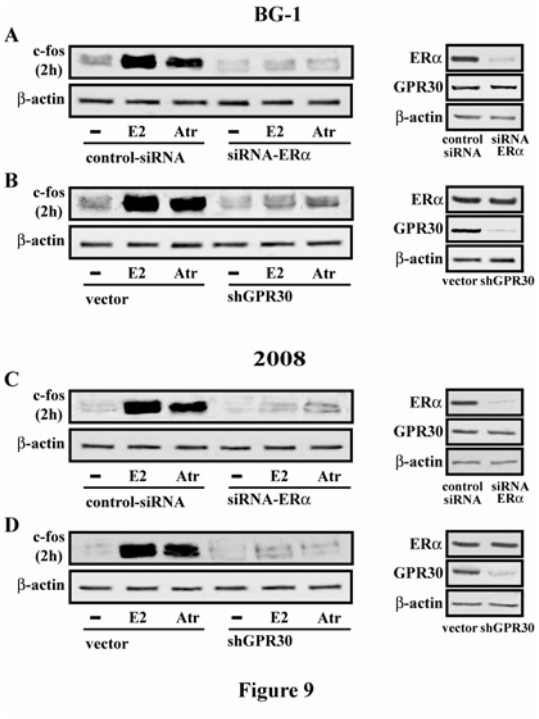

BG-1
A
c-fos (2h)
β-actin
– E2 Atr – E2 Atr
control-siRNA
siRNA-ERα
ERα
GPR30
β-actin
control siRNA
siRNA ERα
B
c-fos (2h)
β-actin
– E2 Atr – E2 Atr
vector
shGPR30
ERα
GPR30
β-actin
vector shGPR30
2008
C
c-fos (2h)
β-actin
– E2 Atr – E2 Atr
control-siRNA
siRNA-ERα
ERα
GPR30
β-actin
control siRNA
siRNA ERα
D
c-fos (2h)
β-actin
– E2 Atr – E2 Atr
vector
shGPR30
ERα
GPR30
β-actin
vector shGPR30

Figure 9

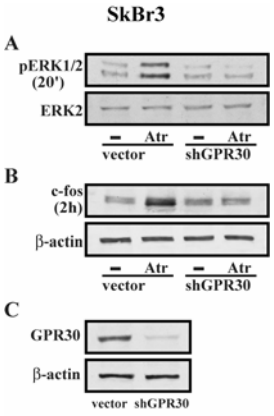

SkBr3
A
pERK1/2
(20')
ERK2
–
Atr
–
Atr
vector
shGPR30
B
c-fos
(2h)
β-actin
–
Atr
–
Atr
vector
shGPR30
C
GPR30
β-actin
vector
shGPR30

Figure 10

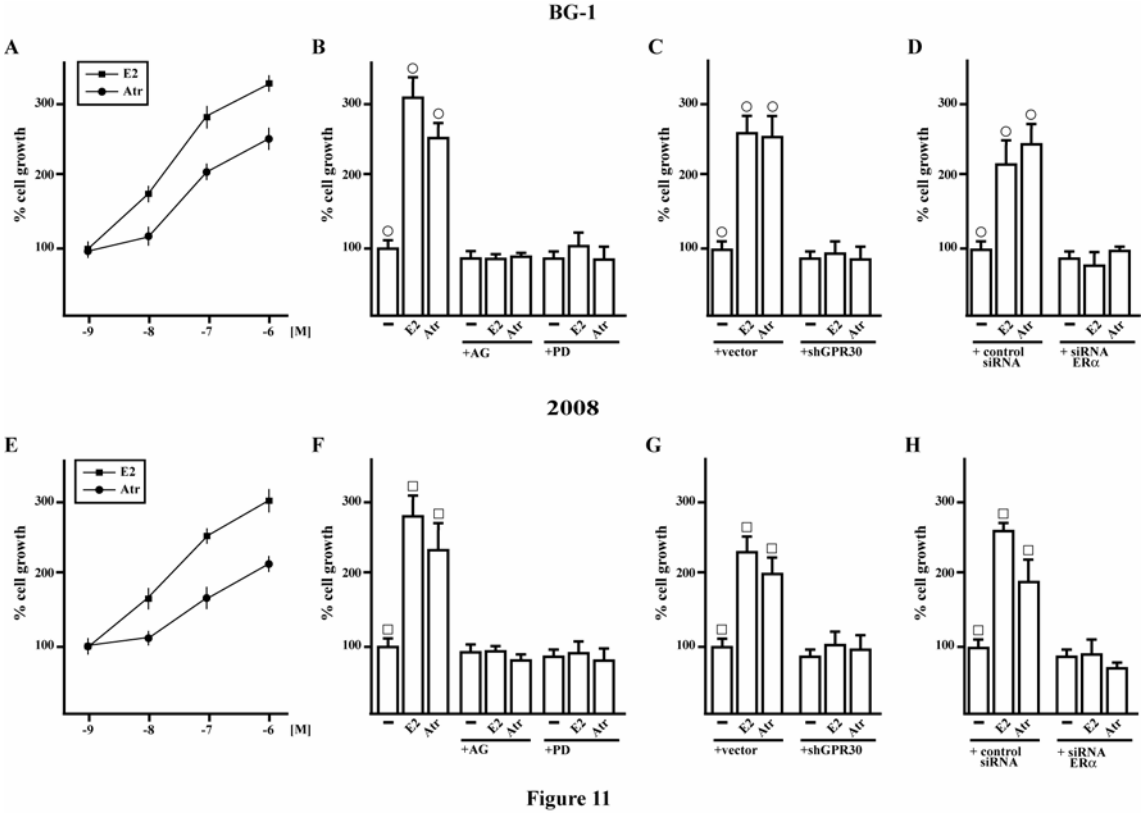

BG-1
A
B
C
D
E2
Atr
% cell growth
[M]
+AG
+PD
+vector
+shGPR30
+ control siRNA
+ siRNA ERα
2008
E
F
G
H

Figure 11

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Epidermal Growth Factor Induces G Protein-Coupled Receptor 30 Expression in Estrogen Receptor-Negative Breast Cancer Cells

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Different cellular receptors mediate the biological effects induced by estrogens. In addition to the classical nuclear estrogen receptors (ERs)-α and -β, estrogen also signals through the seven-transmembrane G-protein-coupled receptor (GPR)-30. Using as a model system SkBr3 and BT20 breast cancer cells lacking the classical ER, the regulation of GPR30 expression by 17β-estradiol, the selective GPR30 ligand G-1, IGF-I, and epidermal growth factor (EGF) was evaluated. Transient transfections with an expression plasmid encoding a short 5′-flanking sequence of the GPR30 gene revealed that an activator protein-1 site located within this region is required for the activating potential exhibited only by EGF. Accordingly, EGF up-regulated GPR30 protein levels, which accumulated predominantly in the intracellular compartment. The stimulatory role elicited by EGF on GPR30 expression was triggered through rapid ERK phosphorylation and c-*fos* induction, which was strongly recruited to the activator protein-1 site found in the short 5′-flanking sequence of the GPR30 gene. Of note, EGF activating the EGF receptor-MAPK transduction pathway stimulated a regulatory loop that subsequently engaged estrogen through GPR30 to boost the proliferation of SkBr3 and BT20 breast tumor cells. The up-regulation of GPR30 by ligand-activated EGF receptor-MAPK signaling provides new insight into the well-known estrogen and EGF cross talk, which, as largely reported, contributes to breast cancer progression. On the basis of our results, the action of EGF may include the up-regulation of GPR30 in facilitating a stimulatory role of estrogen, even in ER-negative breast tumor cells. (*Endocrinology* 149: 3799–3808, 2008)

GIVEN THE ARRAY of extracellular cues to which they are exposed, cells have developed complex machinery for the reception and interpretation of external stimuli. Multiple intracellular signaling pathways are activated by these signals, which are then translated into changes of cellular functions. A common theme in the arrangement of these pathways is the integration and cross talk between contiguous cascades to fine-tune biological outcomes as diverse as cell proliferation, differentiation, and migration. The transactivation of receptor tyrosine kinases by G protein-coupled receptors (GPCRs) is a nice example of communication and cooperation between different signaling networks. In this regard, agonist binding to GPCRs results in transactivation of the epidermal growth factor (EGF) receptor (EGFR) and activation of the ERK/MAPK cascade in a variety of cellular contexts. Traditionally, the EGF network has been viewed as a direct orchestrator of cell replication under physiological and pathological conditions; nevertheless, the involvement of cross-signaling with steroid hormones such as estrogens has largely been demonstrated, particularly in the regulation of normal mammary development and breast cancer progression (1). Indeed, aberrant expression and activation of EGFR is frequently observed in estrogen-sensitive tumors like breast and ovary, in which it correlates with a poorer patient prognosis (2, 3). In addition, up-regulation of EGFR signaling is thought to be an important mechanism, conferring antiestrogen resistance of breast cancer resulting in the failure of endocrine therapy (4).

Several lines of evidence have suggested that the interaction of EGFR with estrogen signaling can occur at different levels. The major estrogen, 17β-estradiol (E2), primarily acts through cognate nuclear receptors [estrogen receptors (ERs)], leading to regulation of gene expression, which has traditionally been deemed as genotropic estrogen activity. Many E2-responsive genes are indeed key signaling molecules that participate in EGFR signaling (1). Alternatively, a cell membrane-associated form of ER has been reported to couple with and activate various G proteins, thereby triggering nongenotropic effects through the transactivation of the EGFR (1, 5). More recently our and other studies have shown that an orphan GPCR, named GPR30, is able to me-

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Abbreviations: AG, AG1478; AP1, activator protein-1; ChIP, chromatin immunoprecipitation; DN, dominant negative; E2, 17β-estradiol; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; FBS, fetal bovine serum; G-1, 1-[4-(-6-bromobenzol[1, 3]diodo-5-yl)-3a,4,5,9b-tetrahidro-3H-cyclopenta[c]quinolin-8yl]ethanone; GPCR, G protein-coupled receptor; LY, LY 294,002; MTT, dimethylthiazoldiphenyltetra-zoliumbromide; PD, PD98059; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine; sh, short hairpin; SP1, specificity protein-1.

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diate rapid E2-dependent signals prompting major biological responses such as gene expression and cancer cell proliferation (6–11). Interestingly, it has been shown that GPR30 is involved in the EGFR transactivation by E2 (12) as exhibited by other GPCR ligands (13–17). In addition, different studies including our own have demonstrated that E2 and the mixed ER agonist/antagonist 4-hydroxytamoxifen can signal through GPR30 to activate the EGFR-MAPK cascade, even in cancer cells lacking ERs (8, 18).

From all these studies, it is possible to assume that E2 can initiate rapid MAPK signaling in an ER-dependent and ER-independent manner. First, E2 can bind a membrane ER, quite similar or identical with the nuclear receptor, and subsequently activate G proteins; second, E2 can directly activate GPCR at the membrane/intracellular level (see below) in an ER-independent manner, thereby signaling to G protein activation. However, because GPR30 was found to be localized close to the endoplasmic reticulum (6), whether this intracellular receptor coupled with G proteins can directly transactivate EGFR as well as its physiological function(s) remains to be fully understood. ER inhibition has proven to be an effective means of blocking the growth of breast tumors expressing ER, and this modality of treatment still remains the standard endocrine therapy for ER+ tumors. Although there is general concordance between ER expression and responsiveness to ER antagonism, as indicated by greater disease-free survival at 5-yr follow-up for postmenopausal patients with ER+ tumors receiving tamoxifen (19), roughly one in four patients do not respond to tamoxifen therapy from the onset, and after a few years in most patients, treatment with this antiestrogen produces agonist effects.

A variety of explanations have been offered to account for unresponsiveness to ER antagonism, including: 1) intratumoral heterogeneity in ER expression, 2) evolution of mutant ERs with reduced affinity for ER antagonists, 3) drug resistance, 4) partial receptor antagonism, and 5) the presence or absence of trans-acting factors that influence ER functionality. These interpretations have prompted strategies better designed to assess ER activity and have served as the rationale for the discovery and use of new endocrine agents with more complete ER antagonist activity. In this concern, the existence of an alternative ER, such as GPR30, which is potentially stimulated by ER antagonists, may provide a further possible explanation for the 4-hydroxytamoxifen failure. To date, studies in animal and cell models have long indicated that estrogens manifest physiologic actions and biochemical effects inconsistent with its classical genomic mechanism of action (20). For instance, estrogen induces EGF-like activity in female reproductive tissue (21, 22) and likewise activates biochemical signals typically associated with the EGFR transduction pathway (23, 24). In this regard, it should be noted that GPR30 can act independently from ERs in triggering estrogen-dependent EGFR action. Indeed, GPR30 may play an important role in breast cancer biology because it provides a mechanism through which estrogens promote EGF-like effects. According to this model, ER-negative breast tumors also may remain estrogen responsive through GPR30. This concept should be taken into account, particularly in those patients receiving endocrine therapy because OHT behaves similarly to estradiol, being capable to elicit EGFR activation in breast cancer cells (9, 10, 18).

Given that GPR30 involves the EGFR pathway in mediating the estrogen signals, in the present study, we evaluated the regulation of GPR30 expression and demonstrate for the first time that EGF is able to induce GPR30 protein levels that accumulate in the intracellular compartment. Consequently, EGF generates a regulatory loop engaging E2 to boost the proliferation of ER-negative breast cancer cells.

Materials and Methods

Reagents

E2, EGF, IGF-I, H89, LY 294,002 (LY), and PD98059 (PD) were purchased from Sigma-Aldrich Corp. (Milan, Italy). AG1478 (AG) was purchased from Biomol Research Laboratories, Inc. (DBA, Milan, Italy), and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d] pyrimidine (PP2) was obtained from Calbiochem (VWR International, Milan, Italy). 1-[4-(-6-bromobenzol[1, 3]diodo-5-yl)-3a,4,5,9b-tetrahidro-3H-cyclopenta[c-]quinolin-8yl]ethanone (G-1) was purchased from Merck KGaA (Frankfurt, Germany). All compounds were solubilized in dimethylsulfoxide, except E2 and PD, which were dissolved in ethanol.

Cell culture

SkBr3 breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% fetal bovine serum (FBS). BT20 breast cancer cells and HEK-293 embryonal kidney cells were cultured in MEM and DMEM, respectively, with phenol red supplemented with 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots, RT-PCR, and confocal microscopy assessment.

Plasmids

To generate the luciferase expression vector for the GPR30–5′flanking region (GPR30), a 641-bp fragment next to the 5′-flanking region of the GPR30 gene was amplified by PCR using the following primer pairs: 5′-AACACTGGCTTTCCCTTCCTATCT-3′ (forward) and 5′-CTTGAAGTGAGCCTGGCATTTGTC-3′ (reverse) from genomic DNA, which was extracted from SkBr3 cells by Trizol reagent as suggested by the manufacturer (Invitrogen, Milan, Italy). PCR primer pairs were selected analyzing the 5′-flanking region of GPR30 gene in chromosome 7, location 7p22.3. The PCR amplification was performed using 1.25 U GoTaq DNA polymerase according to the manufacturer's instructions (Promega, Milan, Italy). PCR conditions were 5 min at 95 C followed by 1 min at 94 C, 1 min at 58 C, and 1 min at 72 C for 30 cycles. The fragment was then inserted in the pCR 2.1 plasmid using the TA cloning kit (Invitrogen), sequenced, and cut with *Hin*dIII and *Xho*I. The insert was cloned in the pGL3 basic vector (Promega). Analyses of GPR30–5′ flanking region revealed an activator protein-1 (AP1; −471 to −477) and an specificity protein-1 (SP1; −133 to −138) consensus binding sites. Mutations from position −471 to −477 in the GPR30–5′ flanking sequence corresponding to an AP1 motif and from position −133 to −138 corresponding to the SP1 binding site (Fig. 2A) were generated using QuikChange XL site-directed mutagenesis kit (Stratagene, Milan, Italy).

The following pairs of primers were used to generate the AP1 and Sp1mutants: GPR30AP1mut (forward), 5′-CCCTGCCTGTGGGAGACGCCCACGTCCAGCCTCC-3′ and (reverse) 5′-GGAGGCTGGACGTGGGCGTCTCCCACAGGCAGGG-3′; GPR30SP1mut (forward), 5′-GGACGAGCACGCGGAGATCACTCGCCTCCACGG-3′ and (reverse) 5′-CCGTGGAGGCGAGGTGATCTCCGCGTGCTCGTCC-3′. All plasmids were sequenced before use. Plasmid 3x-FLAG-hGPR30 was constructed using the *Hin*dIII/*Bam*HI sites in pCMV10.3x-ratFLAG (25). hGPR30 was amplified with primers CCCCAAGCTTatggatgtgacttcccaag and CAGCGGATCCctacacggcactgctgaac (restriction sites are *underlined*). Reference plasmid Prl-3x-FLAG is a gift from K. Strub (Department of Cell Biology, University of Genève, Genève, Switzerland), and it expresses an unrelated 26-kDa protein. Short hairpin (sh)RNA constructs against human GPR30 were bought from Openbiosystems (Biocat.de, Heidelberg, Germany) with catalog no. RHS4533-M001505. The targeting strands gen-

erated from the shRNA vectors sh1, sh2, sh3, sh4, and unrelated control are complementary to the following sequences, respectively: CGAGTTAAAGAGGAGAAGGAA, CTCCCTCATTGAGGTGTTCAA, CGCTCCCTGCAAGCAGTCTTT, GCAGTACGTGATCGGCCTGTT, and CGACATGAAACCGTCCATGTT.

To evaluate the effectiveness of the different shRNA constructs, HEK-293 cells were seeded at about 50% confluency in 6-cm plates. Six to 8 h later, cells were transfected using the calcium-phosphate method with 1 μg of 3x-FLAG-hGPR30, 10 μg of shRNA construct, and 2 μg of Prl-3x-FLAG. Prl-3xFLAG was used as a transfection control. Forty hours after transfection, cells were harvested and lysed with 20 mM Tris-HCl (pH 8), 100 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM monovanadate, 1 mM dithiothreitol, and protease inhibitors. DNA was sheared by several passages through a 25-gauge needle. Lysates were cleared by centrifugation, and protein concentrations were determined by the Bradford method. Thirty micrograms of lysates were subjected to Western blot analysis with the FLAG antibody M2 (Sigma, Lausanne Switzerland). With a 74% knockdown of 3x-FLAG-hGPR30 expression shRNA construct, sh3 showed the highest efficacy. Hereafter sh3 is referred to as shGPR30. The dominant-negative (DN)/c-*fos* plasmid, a gift from C. Vinson (National Institutes of Health, Bethesda, MD), consists of an acidic amphipathic protein sequence appended onto the N terminus of the fos leucine zipper, replacing the normal basic region critical for DNA binding. The reporter plasmid for 4xAP1-responsive collagen promoter, a gift from H. van Dam (Department of Molecular Cell Biology, Leiden University, Leiden, The Netherlands), contains four AP1 binding sequences (TGAC/GTCA) inserted into a luciferase construct with the minimal promoter sequences from the albumin gene. The GPR30 expression vector was kindly provided by R. Weigel (Department of Surgery, Thomas Jefferson University, Philadelphia, PA) (8).

Transfection and luciferase assays

SkBr3 and BT20 cells (1×10^5) were plated into 24-well dishes with 500 μl of regular growth medium per well the day before transfection. The medium was replaced with that lacking serum on the day of transfection performed using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Milan, Italy) with a mixture containing 300 ng of GPR30 expression vector and 3 ng of pRL-TK. After 5 h, the serum-free medium containing the indicated treatments was renewed, and then cells were incubated for 18 h. Luciferase activity was measured with the dual luciferase kit (Promega) according to the manufacturer's recommendations. Firefly luciferase values were 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 1-fold induction upon which the activity induced by treatments was calculated.

Western blotting

SkBr3 cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 μl of 50 mmol/liter NaCl; 1.5 mmol/liter $MgCl_2$; 1 mmol/liter EGTA; 10% glycerol; 1% Triton X-100; 1% sodium dodecyl sulfate; a mixture of protease inhibitors containing 1 mmol/liter aprotinin, 20 mmol/liter phenylmethylsulfonyl fluoride, and 200 mmol/liter sodium orthovanadate. Protein concentration was determined using Bradford reagent according to the manufacturer recommendations (Sigma-Aldrich). Equal amounts of whole protein extract were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel; transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy); probed overnight at 4 C with the antibody against GPR30 (MBL-Eppendorf, Milan, Italy), c-*fos*, β-actin, phosphorylated ERK1/2, and ERK2 (all purchased from Santa Cruz Biotechnology, DBA, Milan, Italy); and then revealed using the ECL Western blotting analysis system (Amersham Biosciences).

RT-PCR

SkBr3 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 1 h, and cells were processed for mRNA extraction using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. The mRNA expression was evaluated by semiquantitative RT-PCR as previously described (26). For GPR30 and the acid phosphoprotein P0 (36B4), which was used as a control gene, the primers were 5′-CTGGGGAGTTTCCTGCTGA-3′ (GPR30 forward) and 5′-GCTTGGGAAGTCACACCAT-3′ (GPR30 reverse) and 5′-CTCAACATCTCCCCCTTCTC-3′ (36B4 forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse) to yield products, respectively, of 155 and 408 bp, with 15 PCR cycles for both genes.

Confocal microscopy

Fifty percent confluent cultured SkBr3 and HEK-293 cells grown on coverslips were serum deprived for 24 h and then treated for 2 h with 50 ng/ml EGF and 10 μM AG and PD as indicated. Then cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS, and incubated for 1 h with 2 mg/ml primary antibody against GPR30. After incubation with the GPR30 antibody, the slides were washed three times with PBS and incubated with 1 mg/ml rhodamine-conjugated donkey antirabbit IgG (Calbiochem). HEK-293 cells were also stained with propidium iodide. The cellular expression and localization of GPR30 was evaluated by confocal microscope with $\times$1000 magnification. The optical sections were taken at the central plane. The specificity of the detection was verified by neutralizing the GPR30 antibody with the antigen peptide, which was produced by the TNT quick coupled transcription/translation systems (Promega).

Chromatin immunoprecipitation (ChIP)

Cells grown in 10-cm plates were shifted for 24 h to medium lacking serum and then treated for 2 h with vehicle or 50 ng/ml EGF. ChIP assay was performed as previously described (27). The immuno-cleared chromatin was precipitated with anti-c-*fos* antibody or nonspecific IgG (Santa Cruz Biotechnology, DBA). A 4-μl volume of each sample was used as template to amplify by PCR two fragments located next the GPR30–5′ flanking region: one fragment of 261 bp containing the AP1 site and the second fragment of 364 bp (from -937 to -1301) not containing the AP1 site. The primer pairs used to amplify the first fragment were: 5′-CGTGCCCATACCTTCATTGCTTCC-3′ (forward) and 5′-CCTGGCCGGGTGTCTGTAG-3′ (reverse), whereas the primer pairs used to amplify the second fragment were: 5′-CCGTGGCCCGCTGCATAGAGAAC-3′ (forward) and 5′-GAGAGGGAGAAGTGGGCTGTC-3′ (reverse). The PCR conditions were 45 sec at 94 C, 40 sec at 58 C, and 90 sec at 72 C. The amplification products obtained in 25 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. Three microliters of the initial preparations of soluble chromatin were amplified to control input DNA before precipitation.

Cell proliferation assay

Cells (10,000) 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 d (with treatments), and cell growth was monitored by dimethylthiazoldiphenyltetra-zoliumbromide (MTT) assay according to the manufacturer's protocol (Sigma). A concentration of 200 ng/liter of the shGPR30 or DN/c-*fos* was transfected using Fugene 6 reagent (Roche Diagnostics) as recommended by the manufacturer the day before treatments and then renewed every 2 d before MTT assay.

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

EGF transactivates the 5′ flanking region of GPR30 through an AP1 site in ER-negative breast cancer cells

In our previous studies (8–11), we demonstrated that GPR30 mediates the stimulatory effects elicited by E2 and other agonists, including the selective GPR30 ligand G-1, in a variety of tumor cells expressing or lacking ERs. The GPR30

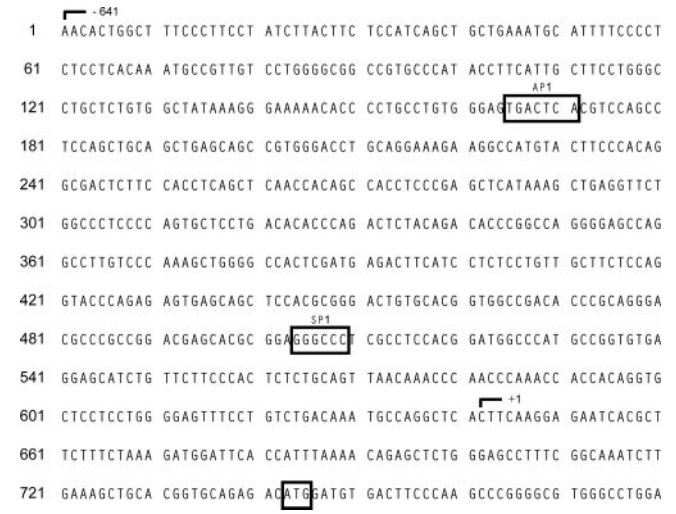

FIG. 1. Sequence of the GPR30–5′-flanking region used to generate luciferase reporter constructs.

activity was clearly coupled to the EGFR-MAPK transduction pathway, which in turn promoted gene expression changes and cell proliferation. Given that no data are currently available regarding the molecular mechanisms involved in the regulation of the GPR30 promoter sequence, we first cloned and characterized the functional response of a 648-bp fragment located at the 5′ flanking region of the human GPR30 gene containing different transcription factor binding sites, such as those for the AP1 and SP1 activating proteins (Fig. 1). Thereafter, we transiently transfected the above construct in ER-negative SkBr3 and BT20 breast cancer cells to evaluate its response to E2 and G-1 as well as the growth factors EGF and IGF-I largely involved in cancer development and progression. As shown in Fig. 2 (A and C), only EGF was able to transactivate the GPR30–5′ flanking region cloned, whereas the other ligands did not exhibit stimulatory activity. Next, the luciferase expression triggered by EGF was no longer observed in presence of the EGFR and ERK inhibitors AG and PD, respectively, whereas the response to EGF was not altered by PP2, H89, and LY, inhibitors of the Src family tyrosine kinase, the protein kinase A (PKA), and phosphatidylinositol 3-kinase (PI3K) transduction pathways, respectively (Fig. 2, B and D). To further assess the activity of the GPR30–5′ flanking region described above, we also cloned two expression vectors mutated in AP1 and SP1 sites, which are potentially involved in the responsiveness to EGF (Fig. 3A). Interestingly, transfection analysis showed that EGF stimulation differentially transactivated these mutants (Fig. 3, B and C). In both SkBr3 and BT20 cells, the construct mutated in the −477 to −471 region (GPR30AP1mut) did not respond to EGF, whereas the construct mutated in the −138 to −133 region (GPR30SP1mut) still maintained the EGF responsiveness. Thus, the AP1 site spanning from −477 to −471 bp within the GPR30–5′ flanking region is required for the transactivation induced by EGF.

EGF up-regulates GPR30 expression

On the basis of the results obtained in transfection experiments, we asked whether EGF regulates GPR30 expression

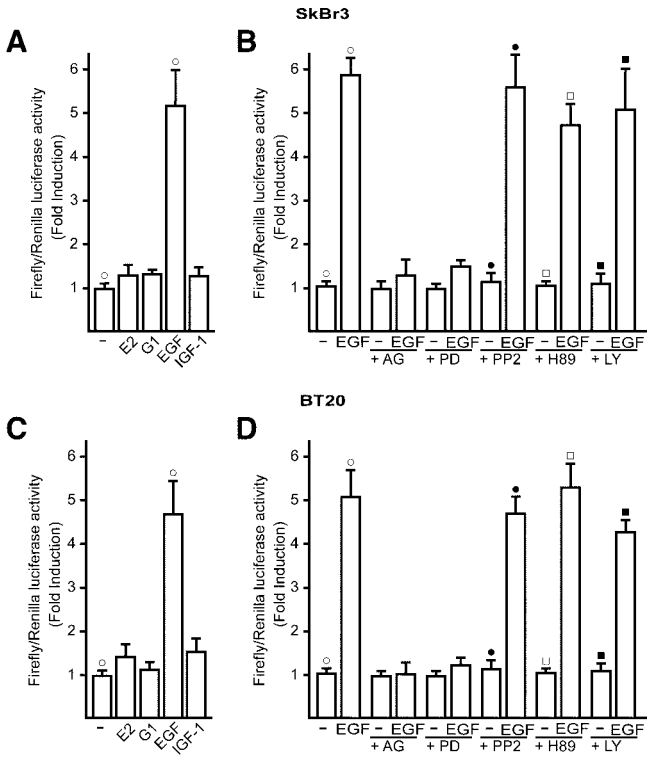

FIG. 2. The GPR30–5′-flanking region is transactivated by EGF in SkBr3 and BT20 breast cancer cells. A–D, Cells were transfected with a reporter plasmid encoding the GPR30–5′-flanking region and treated with 100 nM E2, 1 μM G-1, 50 ng/ml IGF-I, or 50 ng/ml EGF, and 10 μM EGFR inhibitor tyrphostin AG 1478 (AG), 10 μM MEK inhibitor PD, 10 μM Src family tyrosine kinase inhibitor PP2, 10 μM PKA inhibitor H89, 10 μM PI3K inhibitor LY, as indicated. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as 1-fold induction upon which the activity induced by treatments was calculated. Each data point represents the mean ± SD of three independent experiments performed in triplicate. ○, ●, □, ■, $P < 0.05$, for cells receiving vehicle (−) *vs.* treatment.

and what transduction pathway(s) could be involved in such ability. To this end, we first performed semiquantitative RT-PCR assays comparing mRNA levels after standardization with a housekeeping gene encoding the ribosomal protein 36B4. Interestingly, a short EGF treatment (1 h) in SkBr3 cells increased GPR30 mRNA expression, yet AG and PD prevented such response, whereas PP2, H89, and LY did not evidence any inhibitory effect (Fig. 4, A and B). The GPR30 protein levels evaluated on a 2-h EGF exposure paralleled the mRNA increase showing a similar signaling regulation (Fig. 4C). To confirm with a different approach the aforementioned findings and evaluate the localization of GPR30 after EGF stimulation, we assessed GPR30 expression by confocal microscopy in SkBr3 cells. GPR30-negative HEK-293 cells were used as controls. Notably, the treatment with EGF (2 h) induced an intracellular GPR30 accumulation, which was no longer evident in presence of AG or PD (Fig. 5A). The specificity of detection in SkBr3 cells was verified by neutralizing the GPR30 antibody by 10-fold molar excess of the antigen peptide (Fig. 5B). Moreover, the GPR30-negative HEK-293 cells showed no immunodetection of GPR30 (Fig. 5C, *upper panels*), whereas the nuclei were stained with propidium

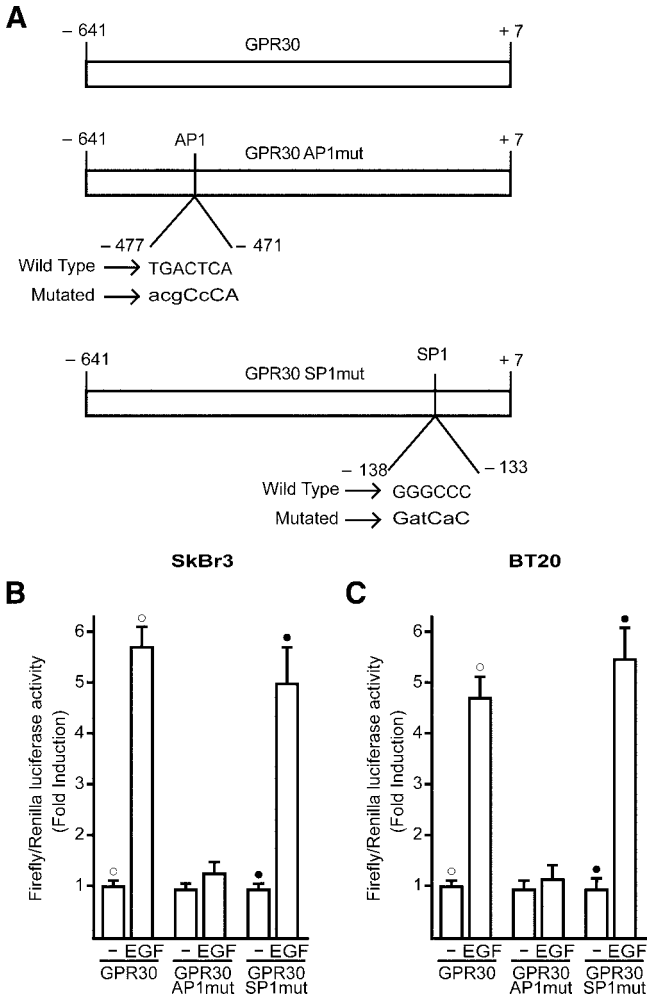

FIG. 3. An AP1 site is responsible for transactivation of the GPR30–5′-flanking region induced by EGF in SkBr3 and BT20 breast cancer cells. A, AP1 (GPR30AP1mut) and SP1 (GPR30SP1mut) mutations generated within the GPR30–5′-flanking region. B and C, Cells were transfected with the reporter plasmids described in A and treated with 50 ng/ml EGF. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (–) were set as 1-fold induction upon which the activity induced by treatments was calculated. Each data point represents the mean ± SD of three independent experiments performed in triplicate. ○, ●, $P < 0.05$, for cells receiving vehicle (–) *vs.* treatment.

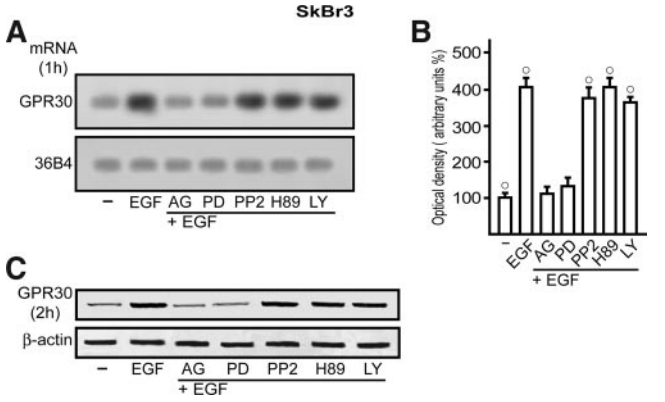

FIG. 4. EGF up-regulates GPR30 expression in SkBr3 cells. A, The expression of GPR30 was evaluated by semiquantitative RT-PCR in cells treated for 1 h with vehicle (–) or 50 ng/ml EGF alone and in combination with 10 μM EGFR inhibitor tyrphostin AG, 10 μM MEK inhibitor PD, 10 μM Src family tyrosine kinase inhibitor PP2, 10 μM PKA inhibitor H89, 10 μM PI3K inhibitor LY, as indicated. The housekeeping gene 36B4 was determined as a control. B, Quantitative representation of GPR30 mRNA expression (mean ± SD) of three independent experiments after densitometry and correction for 36B4 expression. ○, $P < 0.05$, for cells receiving vehicle (–) *vs.* treatment. C, Immunoblot of GPR30 from SkBr3 cells treated for 2 h with vehicle (–) or 50 ng/ml EGF alone and in combination with 10 μM AG, 10 μM PD, 10 μM PP2, 10 μM H89, and 10 μM LY, as indicated. β-Actin served as a loading control.

iodide (Fig. 5C, *lower panels*). Hence, in SkBr3 cells, EGF stimulation triggers GPR30 accumulation at the intracellular level (see *Discussion*).

The EGFR-ERK transduction pathway mediates GPR30 induction by EGF

Next, we ascertained that in SkBr3 cells a rapid ERK1/2 phosphorylation induced by EGF is no longer evident in presence of AG and PD but still persists using PP2, H89, and LY at the same time of EGF (Fig. 6A). Given the potential involvement of the PKA transduction pathway in ERK signaling, cells were treated with H89 3, 6, and 12 h before EGF stimulation. Even in these conditions, H89 did not modify the EGF-stimulated ERK1/2 phosphorylation (data not shown), suggesting that PKA does not influence ERK activation in our experimental model. We previously reported (8–11) that in a variety of hormone-sensitive tumor cells, EGFR/ERK-mediated signals lead to early induction of c-*fos*, which plays a relevant role in normal cell growth and cellular transformation mainly interacting with diverse members of c-jun family (28). The fos-jun heterodimers form the AP1 transcription factor complex, which binds cognate sites located within promoters of target genes (28). In line with the results obtained on ERK activation, EGF induced a strong c-*fos* increase, which was abrogated by AG and PD but not in presence of PP2, H89, and LY (Fig. 6B), suggesting that the EGFR-ERK signaling is the key pathway involved in the regulation of c-*fos* in SkBr3 cells. To evaluate whether the EGF-induced up-regulation of c-*fos* is involved in GPR30 expression, we performed ChIP analysis immunoprecipitating cell chromatin with an anti-c-*fos* antibody and amplifying the AP1 site located within the GPR30-5′ flanking region. As shown in Fig. 6C, EGF strongly recruited c-*fos* at the AP1 site, which was dependent on EGFR-ERK signaling because AG and PD abrogated this association whereas PP2, H89, and LY did not elicit inhibitory activity. Using primer pairs amplifying a control DNA sequence that does not contain the AP1 site, we did not visualize any ethidium bromide staining (Fig. 6C).

The up-regulation of GPR30 by EGF engages E2 to boost the proliferation of breast cancer cells

The biological counterpart of the aforementioned findings was ascertained evaluating cell proliferation by MTT assay. In SkBr3 and BT20 cells, the growth effects, stimulated by E2 and EGF alone, further increased in presence of both mitogens (Fig. 7, A and E). The role of GPR30 in the biological activity elicited by E2 was clearly evidenced silencing GPR30 in both breast cancer cells. As shown in Fig. 7 (B and F), the

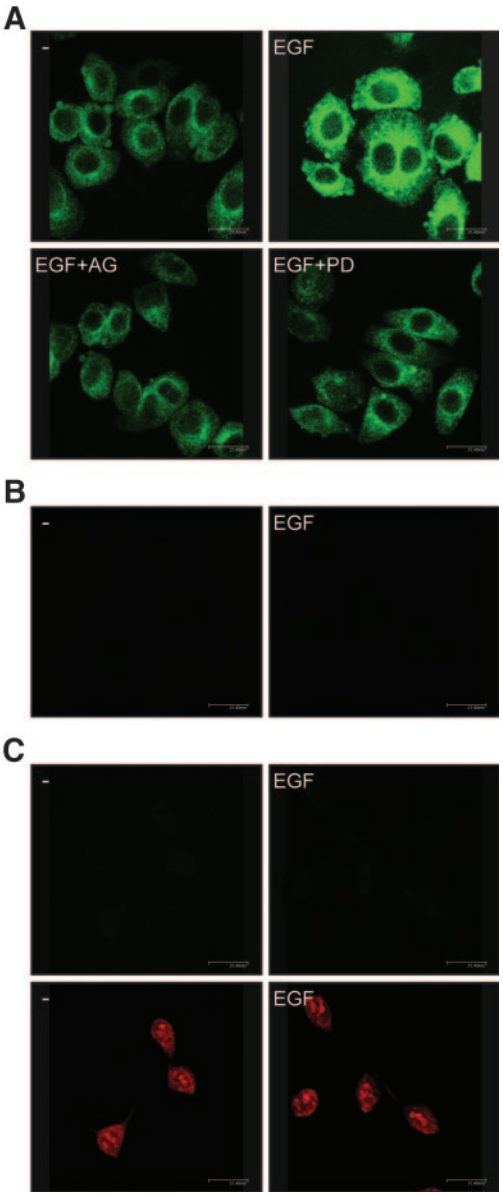

FIG. 5. GPR30 localization in SkBr3 cells. A, GPR30 evaluation by confocal microscopy in SkBr3 cells fixed, permeabilized, and stained with anti-GPR30 antibody. Cells were treated for 2 h with vehicle (−) or 50 ng/ml EGF alone and in combination with EGFR inhibitor tyrphostin AG, 10 μM MEK inhibitor PD, as indicated. B, SkBr3 cells were treated for 2 h with vehicle (−) or 50 ng/ml EGF and stained with GPR30 antibody, which was preneutralized with the antigen peptide. C, HEK-293 cells were treated for 2 h with vehicle (−) or 50 ng/ml EGF and stained with GPR30 antibody (*upper panels*) or propidium iodide (*lower panels*). The *white bars* denote 21,43 μm. Data are representative of three independent experiments.

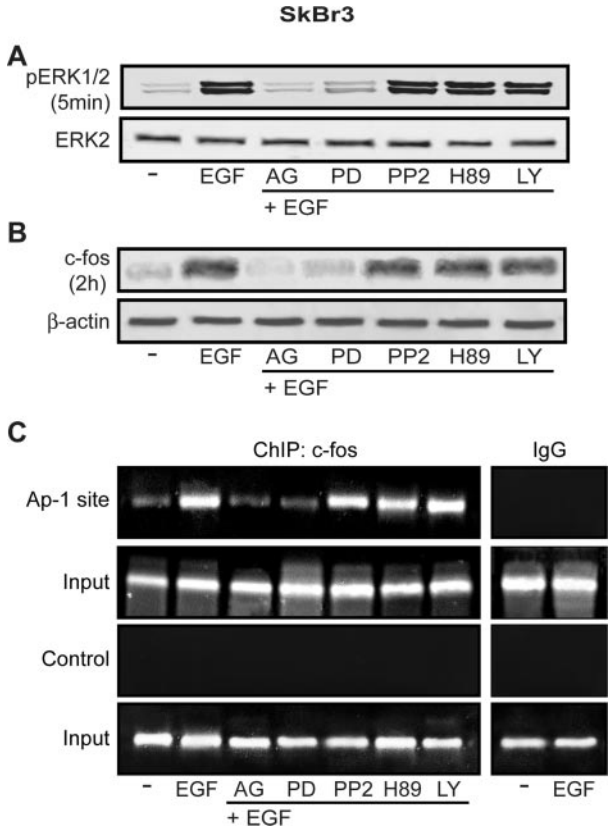

FIG. 6. EGF induces ERK1/2 phosphorylation and c-*fos* expression, which is recruited at the AP1 site located in the GPR30–5′-flanking region. A, The rapid ERK1/2 phosphorylation induced by 50 ng/ml EGF in SkBr3 cells is abrogated in presence of 10 μM EGFR inhibitor tyrphostin AG and 10 μM MEK inhibitor PD but not in presence of 10 μM Src family tyrosine kinase inhibitor PP2, 10 μM PKA inhibitor H89, or 10 μM PI3K inhibitor LY. B, The up-regulation of c-*fos* induced by 50 ng/ml EGF in SkBr3 cells is abrogated in presence of 10 μM AG and 10 μM PD but not in presence of 10 μM PP2, 10 μM H89, or 10 μM LY. C, EGF treatment (50 ng/ml) induces in SkBr3 cells the recruitment of c-*fos* at the AP1 site located in the GPR30–5′-flanking region. This recruitment is abrogated by 10 μM AG or 10 μM PD but persists in presence of 10 μM PP2, 10 μM H89, or 10 μM LY. The amplification of a region lacking the AP1 site (control) does not show the recruitment following the same experimental conditions described above. In control samples, nonspecific IgG was used instead of the primary antibody.

growth effects of E2 alone or in combination with EGF were prevented transfecting cells with shGPR30, which knocked down GPR30 expression. Engineering cells to express the DN/c-*fos*, which effectively blocked the AP1-mediated transcriptional activity (Fig. 7, C and G), we did not observe the proliferative effects induced by either E2 or those triggered by EGF (Fig. 7, D and H). Hence, the c-*fos*/AP1 signaling exerts a key role in the growth stimulation of both mitogens in SkBr3 and BT20 cells. Taken together, the up-regulation of GPR30 after exposure to EGF may represent a molecular mechanism through which EGF engages E2 to boost the proliferative effects elicited in these ER-negative breast cancer cells.

Discussion

Positive feedback loops enhance the amplitude and prolong the active state of transduction pathways to convey robustness in the face of variable inputs (29). In the case of EGFR, the output of the main switch, as can occur through receptor activation by ligand binding, is fine-tuned by the MAPK pathway involved in the expression of GPCRs, which in turn is coupled to EGFR signaling in diverse cell types (5, 30). In this regard, agonist-stimulated GPCRs lead to intracellular activation of diverse metalloproteinases, release of

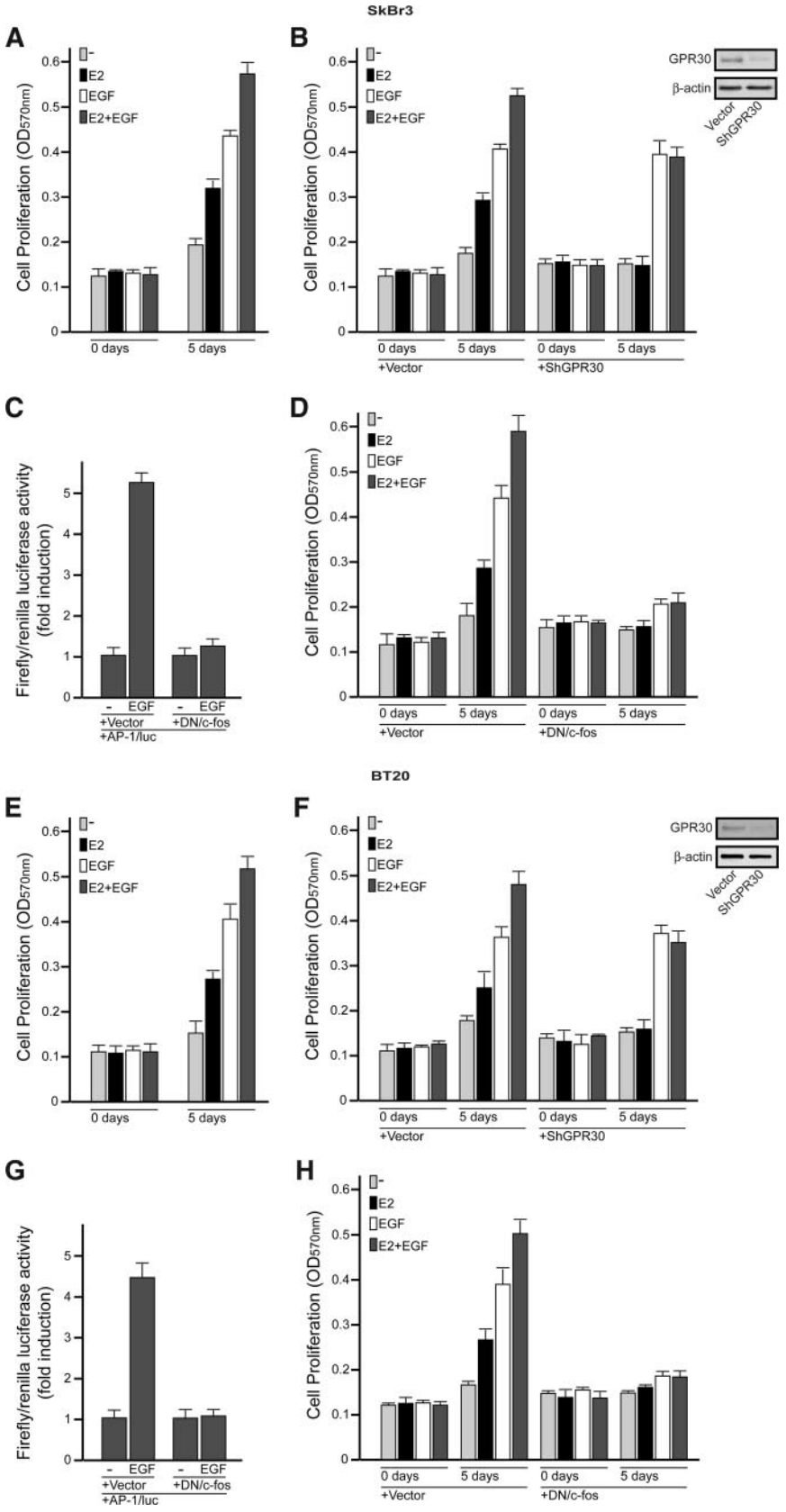

FIG. 7. In SkBr3 and BT20 breast cancer cells, EGF engages E2 through GPR30 to boost the growth effects, which were monitored by MTT assay. A and E, The combination of E2 and EGF treatments enhances the proliferation of SkBr3 and BT20 cells stimulated by each mitogen used alone. Cells were treated with vehicle or 100 nM E2 and/or 50 ng/ml EGF in medium containing 2.5% charcoal-stripped FBS (medium was refreshed and treatments were renewed every 2 d). B and F, The growth effects induced by E2 alone or in combination with EGF were abolished by GPR30 silencing in both SkBr3 and BT20 cells. Cells were transfected with an empty vector or shGPR30 and the next day were treated with vehicle (−), 100 nM E2, and/or 50 ng/ml EGF. Transfections and treatments were renewed every 2 d. Efficacy of GPR30 silencing was evaluated by immunoblots, as indicated. C and G, The DN/c-*fos* construct effectively blocked the AP1 mediated transcriptional activity in SkBr3 and BT20 cells. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as 1-fold induction upon which the activity induced by 50 ng/ml EGF was calculated. D and H, The growth effects induced by E2 and EGF used alone or in combination were abolished transfecting the SkBr3 and BT20 cells with the DN/c-*fos* expression vector. Cells were transfected with an empty vector or the DN/c-*fos* construct and the next day were treated with vehicle (−), 100 nM E2, and/or 50 ng/ml EGF. Each data point is the mean ± SD of three independent experiments performed in triplicate.

EGFR ligands at the cell surface, and subsequent activation of EGFR-MAPK signaling (5, 30).

The EGFR transduction pathway has been implicated in estrogen action (31). Intrauterine E2 administration increased EGF concentrations (21) and EGFR autophosphorylation (32), whereas neutralizing antibodies against EGF inhibited estrogen-induced uterine growth (22). In addition, *in vitro* experiments proved that E2 stimulates various EGFR-associated cascades, including MAPK activation, dependent on rapid release of heparin-binding EGF and activation of matrix metalloproteinases-2 and -9 (5, 12, 18, 20, 33).

It is worth noting that GPR30-mediated, estrogen-induced ERK activation occurs via G$\beta\gamma$-subunit protein signaling and downstream Src involvement because pertussis toxin and Src inhibitors blunt ERK activity by E2 but not EGF. Moreover, E2-induced ERK signaling is prevented by: 1) specific inhibitors of EGFR tyrosine kinase; 2) neutralizing heparin-binding EGF antibodies; and 3) down-regulation of pro-heparin-binding EGF by a diphtheria toxin mutant (12). Therefore, to trigger MAPK activity, GPR30 couples membrane-associated enzymes along with a familiar regulatory circuit controlled by independent G protein signaling pathways.

In this concern, one important aspect regarding GPR30-mediated signals is that ER antagonists binding GPR30 may behave as estrogen agonists in stimulating HB-EGF release from tumor cells (18, 20). This observation has relevant implications on the possible role elicited by GPR30 in human cancer biology including the potential agonist activity exerted by ER antagonists in tumor progression (8–11, 20, 33).

Indeed, evidence that GPR30 can act in an ER-independent manner in mediating estrogen action is provided by diverse experimental observations. First, rapid E2 stimulation does not correlate with ER expression because it occurs in human ER-negative cancer cells as shown in our and other studies (8–12, 18, 20, 33). Second, ER antagonists promote rapid estrogen action in breast cancer cells expressing GPR30 independently of whether they express estrogen receptor-α gene or estrogen receptor-β gene, the genes encoding ERα and ERβ, respectively (12, 18). These findings suggest that ER or ER-related proteins are not required in GPR30-dependent EGFR activation; however, it should be noted that GPR30 and ERα cooperate in mediating the effects of E2 and even those exerted by the selective GPR30 ligand G-1, as we recently demonstrated in ovarian cancer cells (11).

The present study provides novel evidence regarding the regulation and activity of GPR30 in ER-negative SkBr3 and BT20 breast cancer cells. For the first time, we have demonstrated that EGF through the EGFR transduction pathway transactivates the GPR30-5′flanking region and up-regulates the expression of GPR30 protein, which localizes intracellularly as demonstrated by confocal microscopy.

In accordance with our results, GPR30 was visualized predominantly in the endoplasmic reticulum (6, 34), and even functional GPCRs (35–37) and receptor tyrosine kinases like EGFR (38) were found in the intracellular compartment. These observations raise the question of how ligand binding to a GPCR within cells could initiate signaling events, particularly those involving transactivation of EGFR. Given that G protein $\beta\gamma$-subunits are initially targeted to the endoplasmic reticulum in which they subsequently associate with G protein α-subunits (39), the basic machinery for a GPCR to initiate signaling may also be located close to the endoplasmic reticulum. Although the transduction cascade initiated by GPR30 remains to be completely elucidated, often the GPCR-mediated transactivation of EGFR occurs through shuttle cytosolic molecules, which activate metalloproteinases leading, in turn, to the release of EGF-like ligands (40). Of note, recent investigations in endometrial and breast tumors (41, 42) corroborate the aforementioned results because GPR30 staining yielded uniform density throughout the cell, consistent with a primarily intracellular location. A similar expression pattern of GPR30 has been observed in neurons (43), although contradictory results have also been reported (25, 44). In this regard, the regulation and activity of a distinct subcellular distribution of GPR30 in both normal and cancer cell contexts is still an open question.

The c-*fos* represents a prototypical early gene because its expression is rapidly induced by different extracellular stimuli including mitogens and hormones (45). The nuclear protein encoded by c-*fos* interacting with c-jun family members form the heterodimeric AP1 transcription factor complex (28), which regulates the expression of genes involved in proliferation, invasion, differentiation, and cell survival (46). The transcription of c-*fos* is controlled by multiple *cis*-elements such as the serum-response element mediating growth factor-induced c-*fos* expression, which leads to the activation of the MAPK transduction pathway (47).

Several studies have shown that ERα is also involved in the regulation of c-*fos* (48), although E2 and other compounds stimulate c-*fos* expression and cell proliferation through GPR30-EGFR-MAPK signaling in ER-negative breast tumor cells as we previously demonstrated (8, 11). Here we provide novel insight into the molecular mechanisms by which EGF can further convey robustness to this pathway because it induces consecutive events such as rapid ERK activation and induction of c-*fos*, which in turn is recruited to an AP1 site located next to the GPR30-5′ flanking region. Interestingly, the biological action exerted by E2 and EGF recapitulated the aforementioned effects in ER-negative breast cancer cells. Indeed, the growth stimulation induced by each compound was boosted by the exposure to E2 in combination with EGF, whereas GPR30 silencing abrogated the proliferation stimulated by E2 alone and that additionally induced by E2 used in combination with EGF. Hence, the present data suggest that EGF triggers a positive feedback loop engaging GPR30-mediated signals, such as those elicited by E2, to enhance the potential of the EGFR signaling network.

The possible binding and activation of GPR30 by ER antagonists should be taken into account when considering either the failure of their inhibitory activity in breast cancer or the agonist effects observed in other tissues like the endometrium. Thus, our findings point toward the need of new endocrine agents able to block widespread estrogen action without exerting any stimulatory outcome through transduction pathways shared by the steroid and growth factor signaling network. From the data currently available, the potential of GPR30-mediated signals should

be considered in estrogen-sensitive tumors to discover innovative antiestrogens. GPR30 overexpression was recently associated with lower survival rates in endometrial cancer patients (41) and higher risk of developing metastatic disease in patients with breast tumor (42). Therefore, the expression levels of GPR30 may characterize not only estrogen sensitivity and the potential response to endocrine pharmacological intervention in these tumors but could also be predictive of biologically aggressive phenotypes consistent with adverse outcome and survival. In addition, the up-regulation of GPR30 expression by the ligand-activated EGFR further extends our knowledge regarding the cross talk between EGF and E2 signaling in breast cancer progression. Likewise, our results indicate that the action of EGF may include the up-regulation of GPR30 in facilitating a stimulatory role of estrogen even in ER-negative breast tumor cells.

Acknowledgments

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