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Thesis

Molecular mechanisms involved in the biological responses to estrogens and antiestrogens in cancer cells

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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. Many studies have identified membrane-associated estrogen signals which may alter gene expression independently of the nuclear ERs. Previously, we suggested that evaluating the levels of G protein-coupled receptor 30 (GPR30) in combination with those of a set of GPR30 target genes might be more informative to assess the outcome in certain types of cancer. We recently assessed that GPR30 triggers proliferative stimuli of several natural compounds (phytoestrogens) as well as synthetic compounds (xenoestrogens) in a variety of estrogen-sensitive cancer cells. Moreover, we demonstrated that 4-hydroxytamoxifen (OHT) like estrogens is able to induce cell proliferation through GPR30 by induction of c-Fos and other target genes. On the other hand, we have also examined, in specific cellular contexts, the molecular mechanisms of OHT-induced apoptosis. In this regard, our studies were focused on the activation of c-Jun, a major component of the AP-1 transcription factor, which represents a paradigm for the transcriptional response to stress. Transactivation of c-Jun is regulated by Jun-N-terminal kinases (JNKs) through phosphorylation at serine 63 and 73 (S63/S73), as well as at threonine 91 and 93 (T91/T93). We show that following a short exposure to the DNA-damaging compound etoposide, c-Jun phosphorylation is restricted to S63/S73. In contrast, JNK-dependent phosphorylation of T91/T93 requires continuous exposure to the drug and is impaired by caffeine treatment or alanine substitution of the adjacent threonine 95

(T95). Hence, our study suggests that c-Jun may sense the strength of genotoxic stress, in apoptosis cell death, through DNA-damage dependent phosphorylation of T95, which in turn augments c-Jun transactivation by JNKs. Finally, we analysed whether c-Jun, the major nuclear target of JNK, has a role in OHT-induced apoptosis of SkBr3 breast cancer cells. We show that before DNA fragmentation and caspase 3/7 activation, cytotoxic concentrations of OHT induce JNK-dependent phosphorylation of c-Jun at JNK sites earlier shown to regulate c-Jun-mediated apoptosis. In addition, OHT induced ERK-dependent expression of c-Fos and transactivation of an AP-1-responsive promoter. In particular, the ectopic expression of dominant-negative constructs blocking either AP-1 activity or c-Jun N-terminal phosphorylation prevented DNA fragmentation after OHT treatment. Furthermore, both c-Fos expression and c-Jun N-terminal phosphorylation preceded OHT-dependent activation of caspase 3-7 in different types of tamoxifen-sensitive cancer cells, but not in OHT-resistant LNCaP prostate cancer cells. Taken together, our results indicate that the c-Jun/c-Fos AP-1 complex has a pro-apoptotic role in OHT-treated cancer cells and suggest that pharmacological boosts of c-Jun activation may be useful in a combination therapy setting to sensitize cancer cells to tamoxifen-mediated cell death.

Our data contribute to better understand the molecular mechanisms involved in biological effects elicited in different cancer cell types by estrogens and antiestrogens, providing new insights for the comprehension of apoptotic mechanisms induced by antiestrogenic therapy in cancer.

Gli estrogeni sono ormoni pleitropici che 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 dei geni target. Numerosi studi hanno identificato segnali estrogenici associati alla membrana che modificherebbero l'espressione genica indipendentemente da ERs nucleari. Negli ultimi anni, attraverso lo studio dei livelli di espressione di un recettore accoppiato a proteine G (GPR30) e dei geni target da esso regolati, il nostro gruppo di lavoro ha potuto fornire ulteriori importanti informazioni riguardo alcuni tipi di tumore.

Recentemente, abbiamo dimostrato che GPR30 media gli effetti proliferativi di alcuni composti naturali (fitoestrogeni), così come di composti sintetici (xenoestrogeni) in diverse linee cellulari tumorali sensibili all'azione degli estrogeni. Successivamente abbiamo dimostrato che il 4-hydroxytamoxifen (OHT), al pari degli estrogeni, è capace di indurre proliferazione cellulare attraverso GPR30. Abbiamo inoltre valutato, in specifici contesti cellulari, i meccanismi molecolari dell'apoptosi indotta da OHT. A tal proposito, abbiamo focalizzato i nostri studi sull'attivazione di c-Jun, il principale componente del fattore di trascrizione AP-1. La transattivazione di c-Jun, fondamentale nella risposta trascrizionale allo stress cellulare, è regolata dalla fosforilazione da parte di una classe di chinasi denominate Jun-N-terminal kinases (JNKs) delle serine 63 e 73 (S63/73), così come delle treonine 91 e 93 (T91/T93) situate nella regione N-terminale di c-Jun. Abbiamo dimostrato che dopo una breve esposizione all' etoposide, composto in grado di danneggiare il DNA, la fosforilazione di c-Jun è

ristretta alle S63/73. Al contrario, la fosforilazione di T91/T93 richiede esposizioni prolungate al trattamento ed è abolita dall'esposizione alla caffeina o dalla sostituzione del sito di fosforilazione adiacente, treonina 95 (T95), con un'alanina. Pertanto, i nostri studi indicano che c-Jun potrebbe comportarsi da sensore di intensità dello stress genotossico capace di innescare la morte cellulare per apoptosi, attraverso la fosforilazione di T95 in seguito a danno al DNA. Infine, abbiamo valutato il ruolo di c-Jun, principale target delle JNK, nell'apoptosi indotta da OHT in cellule di tumore mammario SkBr3. Abbiamo dimostrato che prima della frammentazione del DNA e dell'attivazione delle caspasi 3/7, concentrazioni citotossiche di OHT sono in grado di indurre la fosforilazione di c-Jun a livello dei siti target delle JNK, coinvolte, come dimostrato in precedenza, nella regolazione dell'apoptosi mediata da c-Jun. Inoltre, OHT è in grado di aumentare l'espressione di c-Fos e la transattivazione di AP-1 in maniera dipendente dalle ERK. In particolare, l'espressione ectopica di costrutti in grado di bloccare sia l'attività di AP-1 che la fosforilazione di specifici siti a livello N-terminale di c-Jun, è stata in grado di prevenire il danno al DNA dopo trattamento con OHT. Inoltre, l'aumento dei livelli proteici di c-Fos e della fosforilazione di c-Jun indotti da OHT, precedono l'attivazione delle caspasi 3-7 in diversi tipi di cellule tumorali sensibili al trattamento con OHT, ma non nella linea cellulare di tumore prostatico LNCaP, resistente a OHT. I nostri risultati dimostrano che il complesso AP-1, formato da c-Jun e c-Fos, svolge un ruolo pro-apoptotico nelle cellule tumorali trattate con OHT e individuano l'attivazione di c-Jun come bersaglio farmacologico da utilizzare in una terapia combinata che sensibilizzi le cellule tumorali alla morte cellulare indotta dal tamoxifene.

I nostri dati contribuiscono ad una migliore conoscenza dei meccanismi molecolari coinvolti negli effetti biologici esercitati dagli estrogeni e dagli antiestrogeni in diverse tipologie di cellule tumorali suggerendo, nuovi campi di studio per la comprensione del meccanismo apoptotico indotto dagli antiestrogeni nella terapia antitumorale attualmente in uso.

INTRODUCTION

Hormonal Risk And Protective Factors for Breast Cancer

Worldwide, breast cancer is by far the most common cancer amongst women, with an incidence rate more than twice that of colorectal cancer and cervical cancer and about three times that of lung cancer. However breast cancer mortality worldwide is only 25% greater than that of lung cancer in women (*World Health Organization International Agency for Research on Cancer, 2003*). In 2004, breast cancer caused 519,000 deaths worldwide (7% of cancer deaths; almost 1% of all deaths) (*World Health Organization, 2006*).

The number of cases worldwide has significantly increased since the 1970s, a phenomenon partly blamed on modern lifestyles in the Western world (*Laurance, 2006*) (Fig. 1).

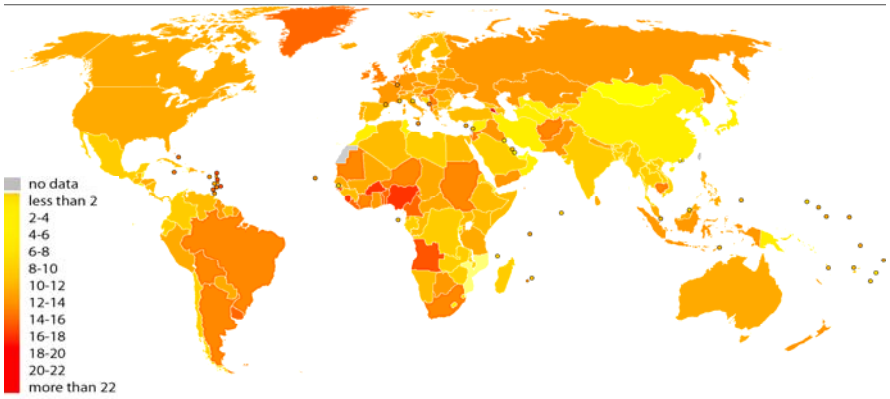
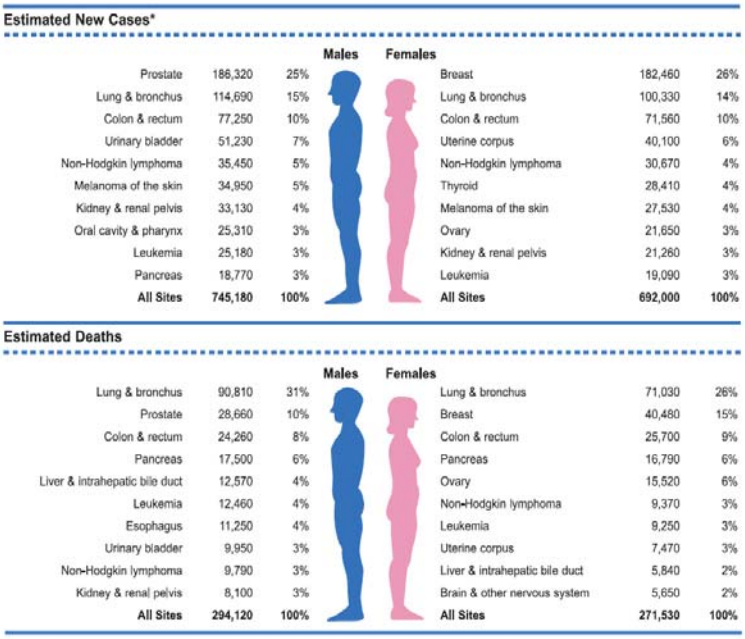


Fig. 1. *Age-standardised death rates from Breast cancer by country (per 100,000 habitants)*

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*) (Fig. 2).



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

There has been an evident decline in breast cancer mortality since 1997, most likely the result of therapy with tamoxifen and perhaps other forms of chemotherapy (*McKean-Cowdin et al., 2000*). Existing evidence regarding the hormonal etiology of breast cancer supports the hypothesis that estrogen is the primary stimulant for breast cell proliferation (*Henderson and Feigelson, 2000*).

Hormonally related risk factors for female breast cancer equate with a greater cumulative lifetime exposure to estrogen and include early age at menarche, late age at menopause, null parity or late age at first full-term pregnancy, and obesity (Table 1). Protective factors include higher parity, long lactation, and bilateral ovariectomy (*Davis et al., 1997; Kreiger et al., 1999; Parazzini et al., 1997*). Reproductive risk factors are associated with exposure to estradiol, progesterone, and other hormones; and reproductive hormones are also believed to underlie increased risk associated with alcohol consumption, lack of physical activity, higher body mass index and weight gain after menopause, and low premenopausal body mass index (*Bernstein et al., 2002*). Much attention has been focused on dietary differences, particularly fat consumption, to explain both the international pattern of breast cancer occurrence and changes in rates of breast cancer following migration to high-risk, usually Western nations from low-risk countries (*Armstrong and Doll, 1975*). Diet seems very likely to affect breast cancer risk, as it does in animals, but epidemiologic studies have failed to identify specific dietary constituents that increase or decrease risk. Effects of fat and fruits and vegetables have been extensively studied, so far providing no consistent evidence of dietary risk factors (*Gandini et al., 2000; Holmes et al., 1999; Hunter and Willett., 1996; Michels., 2002; Smith-Warner et al., 2001; Willett, 1999*). High soy intake in Asia has been proposed as a factor in reduced breast cancer rates there, although epidemiologic studies so far provide limited evidence of a protective effect (*Adlercreutz, 2002; Hilakivi-Clarke et al., 2001; Trock et al., 2000*). Pharmaceutical hormones similarly affect risk. Both estrogen-only and estrogen-progesterone hormone replacement therapy (HRT) and oral contraceptives (OCs) are the exogenous counterparts to endogenous

hormonal exposures experienced by women and therefore are of concern as potential contributors to breast cancer risk. Other more common allelic variations in estrogen metabolism genes (e.g. CYP17, CYP19, HSD17B1) in breast cancer risk are also under study (Fig. 3; Table 1-2) (*Henderson and Feigelson, 2000*). Sequence variants in CYP17 and HSD17B1 have been reported to individually, and in an additive manner; increase the risk of advanced breast cancer (*Feigelson et al., 2001*). Genes in the growth factor pathway, as well as genes encoding proteins in intracellular pathways, steroid receptor transactivation, and DNA repair, are additional important sources of such candidate genes.

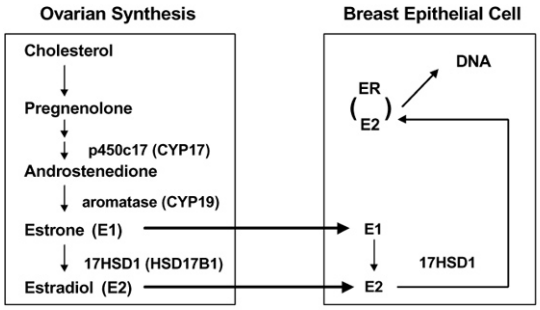


Fig. 3. *Schematic presentation of estrogen metabolism in the ovaries and breast epithelium and four candidate genes that may play a role in breast cancer etiology. The genes of interest are the cytochrome P450c17a (CYP17) gene, the aromatase cytochrome P450 (CYP19) gene, the 17β-hydroxysteroid dehydrogenase 1 (HSD17B1) gene, and the estrogen receptor (ER) gene.*

Table 1 . A Summary of Established Hormonal Risk And Protective Factors for Breast Cancer

Risk factors (increased exposure to estrogen and/or progesterone)
• Early menarche
• Late menopause
• Obesity (postmenopausal women)
• Hormone replacement therapy
Protective factors (reduced exposure to estrogen and/or progesterone)
• Lactation
• Early age at full-term pregnancy
• Physical activity (exercise)

Table 2. Candidate Genes in Hormone-Related Cancers

Cancer Site	Proliferative Hormone	Relevant Genes
Breast	Estrogen	CYP17, CYP19, HSD17B1, ER
	Progesterone	CYP17, PR
Endometrium	Estrogen	CYP17, HSD17B1, HSD17B2, ER, PR
Ovary	FSH, Progesterone	FSH, FSHR, HSD17B1, PR, INHB
Prostate	Testosterone (dihydrotestosterone)	CYP17, HSD17B3, SR05AL, AR

Endocrine disruptors in Breast Cancer

A class of hormonally active chemicals, referred to as *endocrine disruptors*, may affect breast cancer primarily at the phase of tumor promotion. They may also affect mammary gland development and responsiveness to other carcinogens. It has been suggested that exposure to endocrine disruptors, including chemicals that mimic estrogens or *xenoestrogens*, might play an important role in breast cancer risk (*Davis et al., 1993*). More than 500 chemicals have been found to be weakly estrogenic in various assays, including many chemicals in common use, such as constituents of detergents, pesticides, and plastics (*Jobling et al., 1998; Nishihara et al., 2000; Soto et al., 1995*) (Table 3). In a variety of short term *in vitro* assays, many of these chemicals are able to bind the ER, initiate transcription of estrogen-regulated genes, and stimulate breast cancer cells *in*

vitro to proliferate (*Korach and McLachlan, 1995; Shelby et al., 1996; Soto et al., 1995*). Short-term *in vivo* assays, such as increase in uterine weight in rodents, are also used to demonstrate estrogenic activity (*O'Connor et al., 1996*). Furthermore, the effects of these compounds have been frequently observed in wildlife in a more natural context. Sexual disruption of wild fish has been reported in rivers receiving wastewater effluent from industries containing mixtures of endogenous and pharmaceutical estrogens and industrial chemical endocrine disruptors (*Jobling et al., 1998*). The identification of estrogenic compounds in the environment has raised significant issues regarding the relevance of the potential adverse health effects (*Rudel, 1997*). Some researchers maintain that the potency of many of these endocrine-disrupting pollutants is typically much lower than the potency of endogenous estrogens, and so their effects will likely be insignificant (*Safe, 1995*). Others are concerned about the exposure to endocrine-disrupting chemicals levels of endogenous hormones are very low, such as *in utero* or during prepubertal, or postmenopausal time periods. Furthermore, it must be taken into consideration that additive effects of low-level estrogenic pollutants can act together even when each individual component of the mixture is present below a threshold for effect (*Silva et al., 2002*). Finally, the *in vivo* estrogenic effects of a range of compounds demonstrate that estrogenic compounds can exhibit different mechanisms and effects (*Gould et al., 1998; Rudel, 1997*). This diversity is attributed, at least in part, to the fact that the shape of the ER ligand (either E2 or an endocrine disruptor) affects the binding of the ER-ligand complex to DNA sequences and subsequent gene expression. Current research into selective estrogen response modifiers (SERMs, like tamoxifen) for menopause and breast cancer prevention

is a result of this phenomenon (*Emmen and Korach, 2001*). Endocrine disruptors can also act indirectly, for example, by up- or down-regulating the enzymes that metabolize endogenous estrogens or by affecting synthesis of endogenous hormones (*NRC, 1999*). Although research in this area focuses on measuring circulating serum or urinary levels of endogenous hormones, it is important to note that human breast tissue, both normal and tumour tissue can metabolize hormones and create its own local hormonal environment independent of circulating levels (*Adams, 1991; Adams et al., 1992*). Thus, effects of chemicals on the local hormone environment in the breast may be more relevant than effects on circulating hormone levels. 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*).

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*). 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*) (Tab. 3). 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*).

Table 3. Selected endocrine-disrupting chemicals

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	
Amsonic 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

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. (1995), and SRI International (1995).

Endocrine Therapy: Estrogens and Antiestrogens

It was first shown over 100 years ago (*Powles, 2002*) that approximately one-third of premenopausal women with advanced breast cancer will respond to oophorectomy or removal of the ovaries. Since advances in the understanding of reproductive endocrinology and steroid biochemistry permitted the development of specific strategies to restrict the availability of estrogen, the hormone was widely believed to be responsible for the development of breast carcinoma (*Henderson et al., 1988*). As early as 1936, researchers predicted that a therapeutic agent might be found that could block the stimulatory effects of estrogen in breast tissue (*Lacassagne, 1936*). The first non-steroidal antiestrogens synthesized produced toxic side effects, but only tamoxifen (ICI 46474, Nolvadex), a synthetic triphenylethylene agent demonstrated efficacy and a low incidence of side effects (Fig. 4). Furthermore aromatase inhibitors, such as letrozole, which inhibit the synthesis of estrogens (*Powles, 2002*), have been successfully developed for the treament of advanced breast cancer.

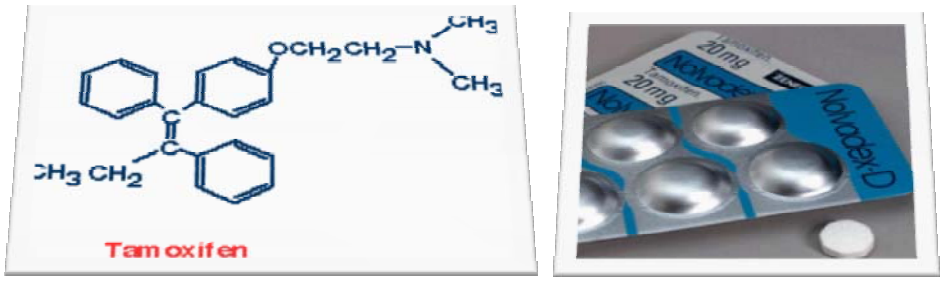


Fig. 4. *Structure and pharmaceutical/commercial form of tamoxifen.*

Tamoxifen Mode of Action in Breast Cancer

Tamoxifen is a competitive inhibitor of estradiol binding to the ER and can reversibly prevent estrogen-stimulated growth in vitro. Similarly, tamoxifen will prevent estrogen-stimulated growth of ER-positive breast cancer cells transplanted into immune deficient (athymic) mice (*Nilsen et al., 2000*). Estrogens are believed to modulate cell growth by causing an increase in stimulatory growth factors (e.g. TGF-α) and a decrease in inhibitory growth factors (e.g. TGF-β) (*Dickson and Lippman, 1987*). These growth factors are thought to influence the cell cycle by interaction with their respective membrane receptors. The regulatory mechanism functions as an autocrine loop, however also paracrine (cell-cell) influences of growth factors (e.g. IGF-1) can play a role in modulating the replication of epithelial cells. Antiestrogens interfere with the stimulatory effects of estrogen by blocking the ER (Fig. 5), causing the cell to be held at the G1 phase of the replicative cycle (*Osborne, 1983*).

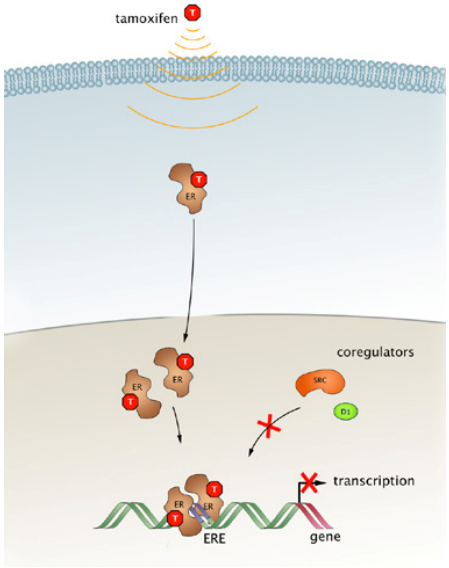


Figure 5. *Antiestrogenic activity of tamoxifen.*

Initially, experimental studies indicated that tamoxifen acted as an anti-estrogenic competititor by preferentially binding to the ER and denying access to estrogen. Since then it has become apparent that tamoxifen can initiate both estrogenic and antiestrogenic events (*Powles, 2002*) by binding to the ER. Tamoxifen binds to the LBD pocket in the same way as other ligands, such as estradiol to form transcriptional complexes to activate or switch off genome transcription. Tamoxifen is rapidly absorbed and is extensively metabolized (Fig. 6) to *N*-desmethyltamoxifen and 4-hydroxytamoxifen, the latter has a high affinity for the ER (*Jordan et al., 1977*) and may play a significant role in the antitumor actions of tamoxifen (*Etienne et al., 1989*).

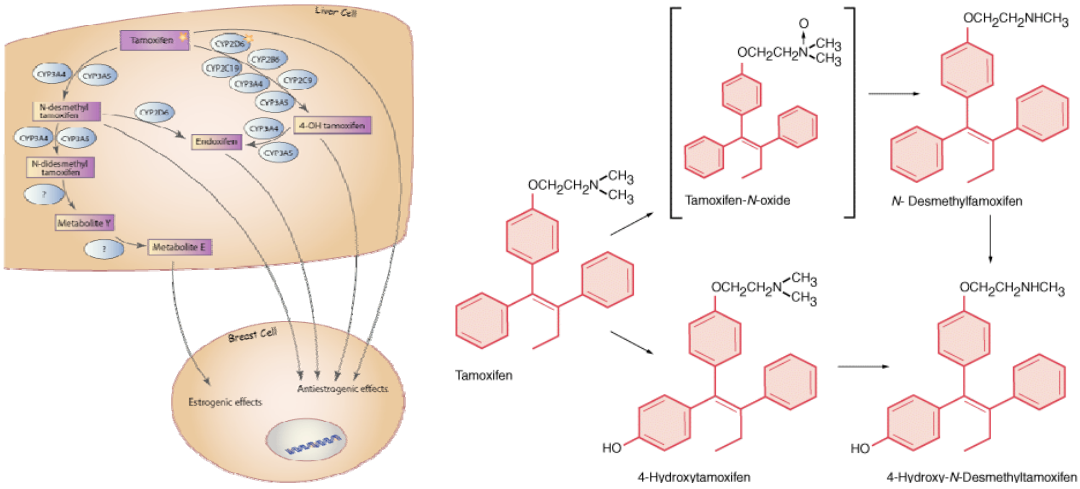


Figure 6. *Metabolism and metabolites of tamoxifen.*

Tamoxifen Use in Advanced Breast Cancer

Tamoxifen is indicated for treatment of metastatic disease in postmenopausal patients (*Furr and Jordan, 1984*). Approximately, one-third of patients respond and especially those with ER-positive disease are more likely to benefit from tamoxifen therapy. Tamoxifen is considered to be the first-line endocrine

therapy due to reduced incidence of side effects and time to progression. The third generation peripheral aromatase inhibitors, such as *letrozole* are powerful inhibitors of estrogen synthesis and produce undetectable levels of circulating estrogen (*Powles, 2002*), although side effects are more serious since long-term medication might give rise to adverse effects (*Smith et al., 1981*). Tamoxifen is also effective in the treatment of advanced disease in premenopausal women; however, serious concerns have been raised about the biologic consequences of long-term tamoxifen treatment for women with breast cancer. One concern in premenopausal women is the rise in circulating estrogen levels (*Smith et al., 1981*). Although tamoxifen has an appropriate level of estrogenic activity in some tissues, it may be deleterious for others (Fig. 7). The administration of estrogen to women increases the risk of thrombosis.

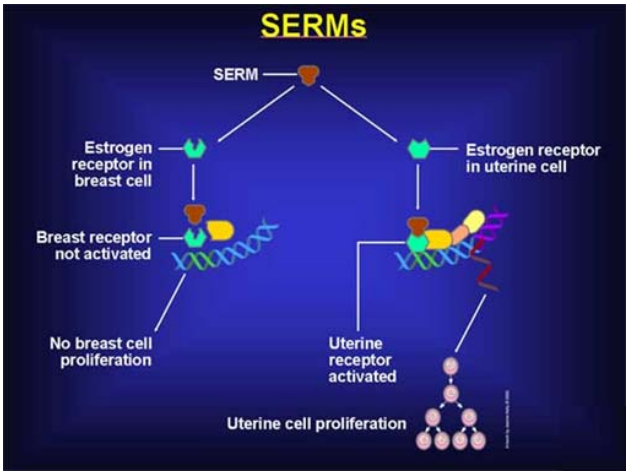


Figure 7. ***The effects of tamoxifen on human tissues.*** *Tamoxifen have both good and bad effects on specific human tissues. Development of new SERMs should maximise the good effects and minimise the bad effects.*

Tamoxifen is known to produce some estrogen-like effects in postmenopausal women (*Furr and Jordan, 1984*) with increased endometrial thickening, hyperplasia, and fibroids following prolonged tamoxifen therapy (*Kedar, 1994*). Endometrial carcinoma has been reported to occur in patients receiving adjuvant tamoxifen therapy (*Killackey, 1985*). There is a modest increase in endometrial carcinoma associated with tamoxifen, but only in postmenopausal women. Premenopausal women are not at risk for an increase in endometrial cancer (*Bernstein et al., 1999*).

Estrogen Receptor-dependent and Estrogen Receptor-independent Pathways for tamoxifen-induced apoptosis

Estrogens, acting through estrogen receptors (ERs), regulate the growth and differentiation of cells of the reproductive system. Binding of 17β-estradiol (E2) to the ER induces a conformational change that enables the ER to recruit transcriptional co-activators and to induce expression of estrogen-regulated genes. Several estrogen-inducible genes, including c-*myc*, TGF-β, and cathepsin D, are implicated in malignant transformation or tumor metastases (*Dubik et al., 1992; Bates et al., 1988; Rochefort et al., 1990; Spyratos et al., 1989*). Tamoxifen and its active metabolite, 4-hydroxytamoxifen (OHT), are nonsteroidal selective estrogen receptor modulators (SERMs) that compete with E2 and other estrogens for binding to the ER. Structural studies and chromatin immunoprecipitations show that OHT·ER induces an ER conformation that does not recruit coactivators to target genes and in many cell and promoter contexts recruits co-repressors (*Shang et al., 2000; Shang and Brown, 2002*). The therapeutic effectiveness of tamoxifen in treatment of hormone-dependent

cancers and in preventing breast cancer in high risk women is thought to arise primarily from its ability to compete with estrogens for binding to the ER. It is thought that tamoxifen·ER and OHT·ER are unable to effectively activate transcription of genes important for the growth and development of estrogen-dependent tumors. However, several often-conflicting studies show that tamoxifen and OHT can actively induce programmed cell death of cancer cells (*Mandlekar and Kong, 2001*). The mechanism(s) by which tamoxifen and OHT induce programmed cell death have been quite controversial, with even the identity of the toxic agents in dispute. One group reported that high concentrations of tamoxifen, but not of OHT, induce cell death (*Dietze et al., 2001*). Others indicated that both tamoxifen and OHT induce cell death (*Mandlekar et al., 2000*). Although a recent report was consistent with a role for ER in OHT-induced apoptosis (*Zhang and Shapiro, 2000*), other workers suggest a number of different mechanisms for tamoxifen-induced apoptosis. The effects of tamoxifen might be mediated through an ER-independent increase in reactive oxygen species, resulting in caspase activation (*Mandlekar et al., 2000a; Mandlekar et al., 2000b*), or through an influx of extracellular calcium (*Kim et al., 1999; Zhang et al., 2000*). In addition, effects of tamoxifen on the levels of proteins important in cell growth including protein kinase C (*O'Brian et al., 1986; Gundimeda et al., 1996*), TGF-β (*Chen et al., 1996; Colletta et al., 1990*), and c-Myc (*Kang et al., 1996; Leng et al., 2000*) have been reported. Resolution of the role of ER in tamoxifen- and OHT-induced apoptosis is complicated by the fact that available ER-positive and ER-negative breast cancer cell lines are derived from independent tumors. Many compounds that are known to induce programmed cell death (PCD) work via pathways that involve mitochondria. The presence of

an apoptotic stimulus triggers a rapid increase in mitochondrial permeability, leading to mitochondrial dysfunction. One of the causes of the mitochondrial permeability transition is the translocation of the proapoptotic Bax protein from the cytosol to the mitochondria, where it forms selective channels in the outer mitochondrial membrane and facilitates the release into the cytosol of cytochrome *c* (*Shimizu et al., 1999; Shiraishi et al., 2001*). In the classic apoptotic pathway, this cytosolic cytochrome *c* forms a complex with procaspase 9 and Apaf-1 called the apoptosome, which leads to the ATP-dependent cleavage and activation of pro-caspase 9, the initiator caspase in mitochondrial apoptosis. Activation of pro-caspase 9 results in activation of downstream executioner caspases, such as caspase 3 (*Leist and Jaattela, 2001; Strasser et al., 2000; Mattson, 2000*). Moreover, OHT is able to induce two independent pathways of PCD. An ER-independent pathway kills ER-negative HeLa cells, requires 10–20 μM tamoxifen or OHT. In contrast, submicromolar amounts of tamoxifen and OHT trigger cell death only in ER-positive HeLa cells (*Obrero et al., 2002*). This effect is blocked by pre-treatment with E2, RAL, and ICI 182,780, demonstrating that binding of tamoxifen and OHT to the ER is required for this pathway of PCD. The ER-dependent and ER-independent pathways both trigger a mitochondrial permeability transition and share other features of mitochondrial apoptosis, such as translocation of the proapoptotic Bax protein from the cytosol into the mitochondria and the release of cytochrome *c* into the cytosol (*Obrero et al., 2002*). However, in contrast to ER-independent PCD, which displays typical apoptotic markers such as PARP cleavage, chromatin condensation, and DNA laddering, a different cell morphology, as well as the absence of those markers, indicates that the ER-dependent pathway does not involve caspase activation.

The ER-dependent pathway does not result in the cleavage and activation of pro-caspase 9, the initiator caspase in mitochondrial apoptosis. The OHT·ER-mediated PCD pathway resembles the caspase-independent pathway referred to as necrosis-like PCD (*Obrero et al., 2002*) (Fig. 8).

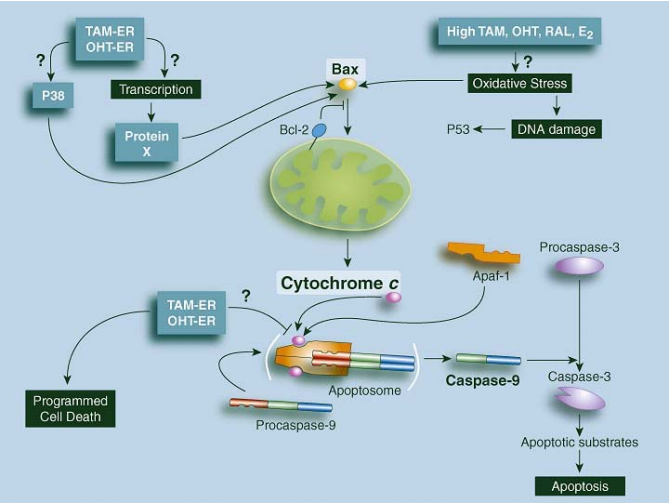


Figure 8. *ER-independent apoptotic mechanisms of tamoxifen*

Hormone induced activation of cell-surface receptors: signaling from plasma membrane to nucleus

Estrogens 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 17β-estradiol (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*) (Fig. 9). 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 and Toft, 2003*). Another essential characteristic of hsp90 is to mediate receptor trafficking from the cytoplasmatic fraction to the nucleus, through a microtubule dependent mechanism (*Pratt, 1990*).

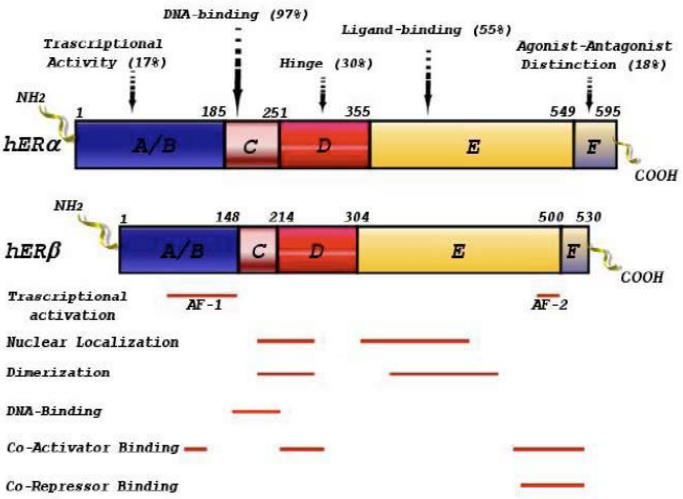


Fig. 9. ERα and ERβ functional domains.

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 (Fig. 10).

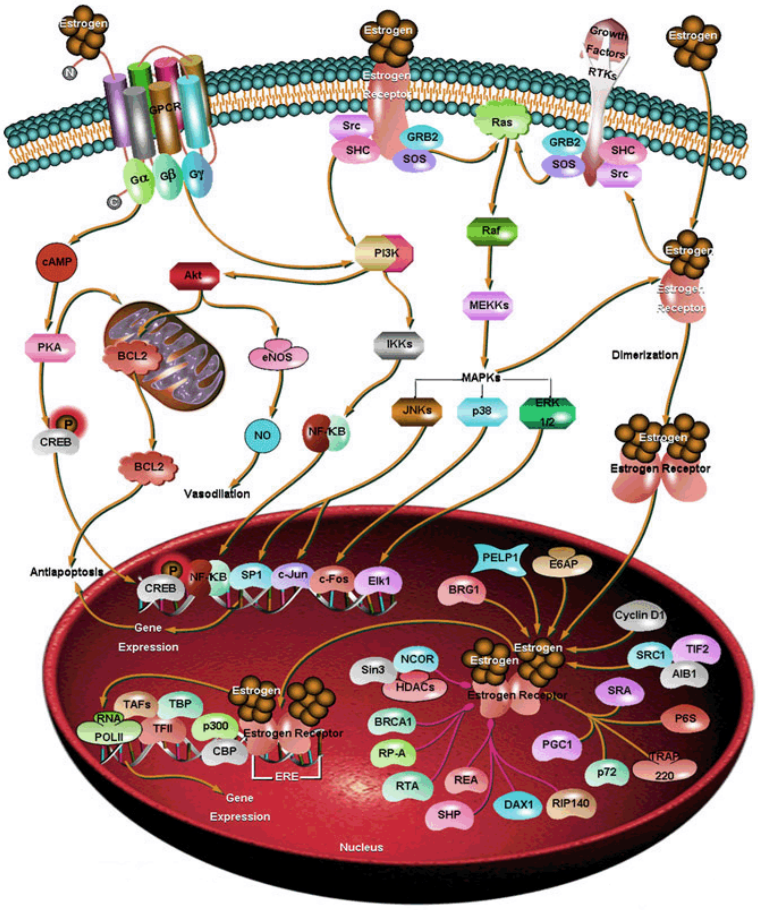


Fig. 10. *Genomic and non-genomic actions of estrogens*

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. 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 ERα and ERβ , influencing breast cancer cell proliferation in a dose-dependent manner (*Maggiolini et al., 2004*). In addition, the structure–activity relationships of resveratrol (RSV), which is classified as a phytoestrogen due to its ability to interact with estrogen receptors, and the analogs 4,49-dihydroxystilbene (4,49-DHS), 3,5-dihydroxystilbene (3,5-DHS), 3,49-dihydroxystilbene (3,49-DHS), 4-hydroxystilbene (4-HS) have been assessed using as model systems the ERα-positive and negative MCF7 and SkBr3SkBr3 breast cancer cells, respectively. In binding assays and transfection experiments RSV and the analogs showed the following order of agonism for ERα: 3,49-DHS > 4,49-DHS > 4-HS > RSV, while 3,5-DHS did not elicit any ligand properties. Hence, subtle changes in the structure of the RSV derivatives may be responsible for the different ERα-

mediated biological responses observed in estrogen-sensitive cancer cells (*Lappano et al., 2009*).

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 and Levin, 2001; Acconcia et al., 2004; Li et al., 2003; Razandi et al., 2003*). 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 the 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 AP-1 (*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 and 2002; Razandi et al., 2003*).

The membrane receptor GPR30

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. The GPCR, transduces signals through heterotrimeric G proteins (α,β,γ), which after stimulation by ligand, dissociates into the protein subunit Gα and the protein complex Gβγ (Fig. 11).

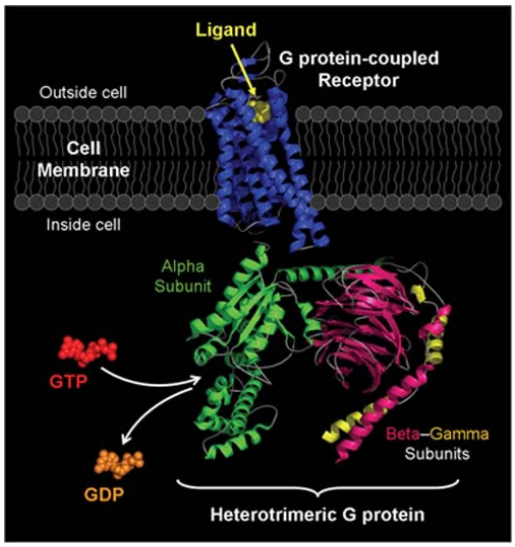


Fig. 11. *Protein structure of G protein–coupled receptors (GPCRs)*

One such receptor, GPR30, was cloned by different groups using highly disparate approaches in the late 1990 (*Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997*). 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 ICI 182,780 and tamoxifen, which mimics estrogen function in certain tissues but acts as an antagonist in other tissues and are collectively known as SERMs. 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 EGFR via a G protein-dependent pathway (Fig. 12) (*Filardo et al., 2000-2002-2008; Maggiolini et al., 2004; Vivacqua et al., 2006a-2006b*).

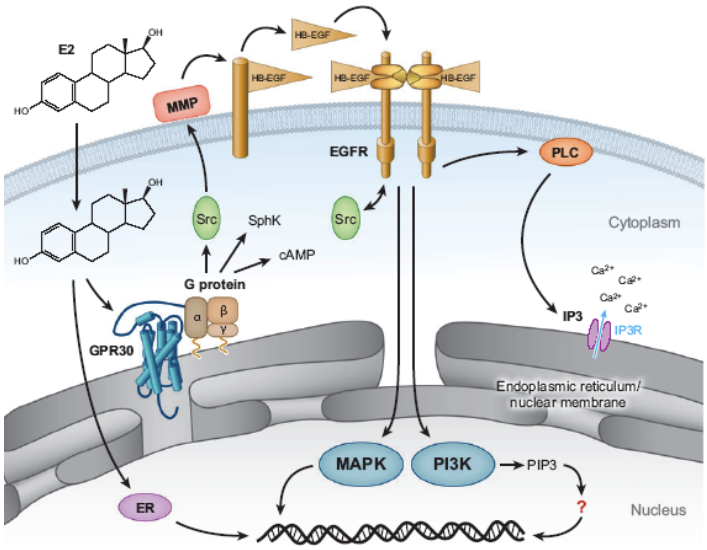


Figure 12. *Mechanisms of estrogen-mediated signalling through GPR30. Estrogen is freely permeable gaining access to intracellular estrogen receptors, ER alpha and GPR30.*

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, 2003a*). 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 ICI 182,780 and tamoxifen, were also shown to bind GPR30 (*Thomas et al., 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., 2006b*). 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. The selective GPR30 ligand G-1 permitted the evaluation of GPR30 to mediate proliferative effects in ovarian cancer cells expressing both ERα and GPR30. Hence, it was demonstrated that a cross-talk between ERα and GPR30 could mediate proliferative effects induced by E2 and G-1 in ovarian cancer cells (*Albanito et al., 2007*). Moreover, it was evaluated whether GPR30 could also be implicated in the growth effects 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 (*Albanito et al., 2008a*). Furthermore, it was found that EGF modulates GPR30 expression through the MAPK pathway (*Albanito et al., 2008b*).

The steroid hormone estrogen can signal through several receptors and pathways. Although the transcriptional responses mediated by the nuclear ERs have been extensively characterized, the changes in gene expression elicited by signalling through the membrane-associated ER GPR30 have not been studied. In

ER-negative human breast cancer cells the activation of GPR30 signaling by estrogen or by 4-hydroxytamoxifen (OHT), an ER antagonist but GPR30 agonist, induces a transcription factor network, which resembles that induced by serum in fibroblasts (*Pandey et al., 2009*). The most strongly induced gene, CTGF, appears to be a target of these transcription factors. The secreted factor connective tissue growth factor (CTGF) not only contributes to promote proliferation but also mediates the GPR30-induced stimulation of cell migration (*Pandey et al., 2009*). In these studies we provided a framework for understanding the physiological and pathological functions of GPR30. As the activation of GPR30 by OHT also induces CTGF in fibroblasts from breast tumor biopsies, these pathways may be involved in promoting aggressive behavior of breast tumors in response to endogenous estrogens or to OHT being used for endocrine therapy (*Pandey et al., 2009*). GPR30 transcripts have been 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. Recent data evidenced a gene expression pattern of GPR30 in postnatal 7 (young) and 60 (adult) days of age hamsters as shown by its heterogeneous mRNA distribution in hypothalamic, amygdalar and cerebellar areas of both sex. (*Canonaco et al., 2008*). 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 breast cancer cells express all three-estrogen receptors (ERα, ERβ, and GPR30) whereas SkBr3 breast cancer 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 (Fig. 14) (*Filardo et al., 2008*). Furthermore, 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 (Prossnitz and Maggiolini, 2009; Maggiolini and Picard, 2009).

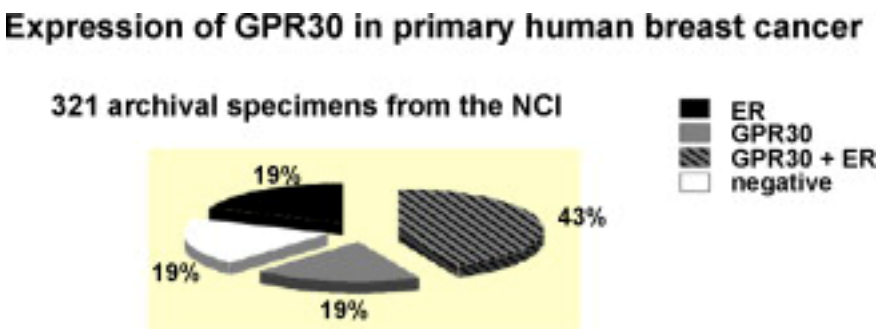


Figure 14. *Co-expression of GPR30 and ER in primary human breast tumors (Filardo et al., 2008).*

Activation Protein 1 (AP-1)

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 and Karin, 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" (Fig. 13).

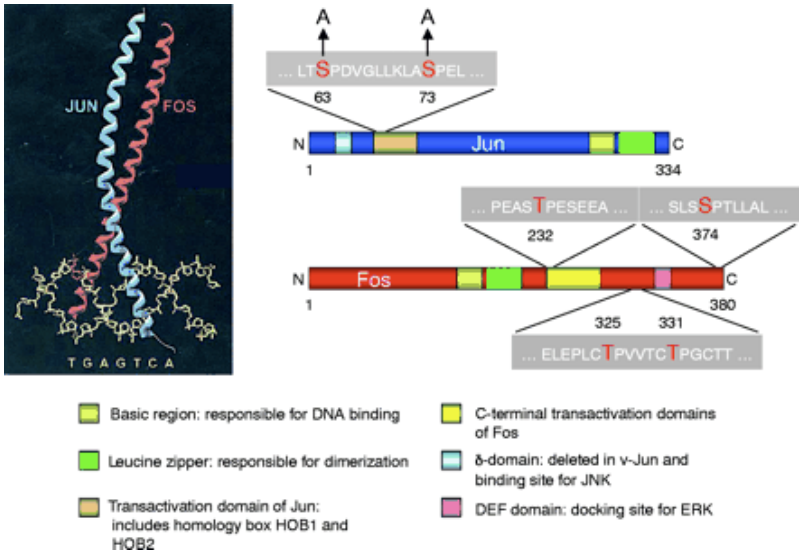


Figure 13. *The Jun-Fos heterodimer. The bZIP domains of Jun and Fos form an X-shaped a-helical structure, which binds to the palindromic AP-1 site (TGAGTCA). The bZIP domain of Jun is shown in blue and the bZIP domain of Fos in red. The DNA backbone is shown in yellow. The Jun and Fos proteins exhibit several domains, including the bZIP domain (leucine zipper plus basic domain), transactivation domains and docking sites for several kinases, such as JNK or ERK. These kinases phosphorylate two serine and threonine residues and thereby modulate the activity of both proteins. JNK specifically phosphorylates serine residues within the transactivation domain of Jun at position 63 and 73 and thereby regulates its transactivation activity. Mutation of serine to alanine generates a Jun mutant (Jun-AA) that cannot be activated by JNKs. Jun is also phosphorylated by casein kinase II, GSK-3ß and ERK, which is not depicted in this scheme. ERK phosphorylates threonine residues at positions 325 and 331 and a serine residue at position 374 of Fos. Additionally, a Fos-related kinase phosphorylates a threonine residue at position 232 of Fos.*

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,

and 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 and Franza, 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*).

The transcription of c-*fos* is regulated by different cis-elements present in the promoter region of the gene: the Ca^{2+}-cAMP response element (Ca^{++}/CRE); or the serum response element (SRE; the sis-inducible element (SIE)) (*Sheng et al., 1991; Renquin, 2001*) (see Fig. 13). Serum or growth factors rapidly induce the expression of c-*fos* through Ras-MAPK and the SRF (serum responsive factor), and also through the ternary complex factor composed of Elk-1, Sap-1 and Sap-2 (SRF accessory proteins) which then regulate the expression of target genes involved in cell proliferation (*Hill and Treisman, 1995*). Many studies have also demonstrated that E2 is able to induce the expression of c-*fos* in breast cancer cells through ERα (*Weisz and Bresciani, 1993; Bonapace et al., 1996*). The promoter region of c-*fos* contains an imperfect ERE palidrome sequence, which is unable to transactivate c-*fos*, however ERα interacts with the Sp1 region at GC

rich sites downstream from the imperfect ERE palindrome (*Duan et al., 1998*). Later it was also demonstrated that E2 is able to activate a non-genomic pathway independently of ERα, which may involve Elk-1 phosphorylation in breast cancer (*Duan et al., 2001*). This was also observed in endometrial cancer cells, whereby c-fos was induced by E2 and tamoxifen through ERK1/2 activation and involvement of SRE (*Singleton et al., 2003*).

c-Jun is the name of a gene and protein which, in combination with c-Fos, forms the AP-1 early response transcription factor (see Fig. 13). It was first identified as the Fos-binding protein p39 and only later rediscovered as the product of the c-Jun gene. It is activated through double phosphorylation by the JNK pathway but has also a phosphorylation-independent function. c-Jun knockout is lethal, but transgenic animals with a mutated c-Jun that cannot be phosphorylated (termed c-JunAA) can survive. This gene is the putative transforming gene of avian sarcoma virus 17. It encodes a protein, which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression. This gene is intronless and is mapped to 1p32-p31, a chromosomal region involved in both translocations and deletions in human malignancies.

Genetic and biochemical studies have shown that the biological activity of c-Jun mainly relays on JNK-dependent phosphorylation of its N-terminal domain at S63/S73 (*Eferl and Wagner, 2003; Shaulian and Karin, 2001*). Phosphorylation of S63/S73 is required for c-Jun-mediated cellular transformation, tumor progression and cancer development (Behrens et al., 2000; Nateri et al., 2005; Smeal et al., 1991). Abrogation of S63/S73 phosphorylation reduces fibroblast survival in response to UV radiation (*Wisdom et al., 1999*) and leads to

chemoresistance acquisition in various tumor cells to DNA-damaging drugs (*Potapova et al., 2001*). These studies suggest that when phosphorylated at S63/S73, c-Jun augments DNA repair, as also evidenced by the accumulation of spontaneous DNA damage occurring in presence of the genetic ablation of c-Jun (*MacLaren et al., 2004*). Moreover, several studies have shown that c-Jun phosphorylation at S63/S73 is required for neuronal apoptosis (*Dunn et al., 2002; Raivich and Behrens, 2006; Shaulian & Karin, 2001*). Subsequent investigations have revealed that c-Jun is phosphorylated by a variety of stress and pro-inflammatory signals on a second group of MAPK sites at T91/T93 (*Morton et al., 2003; Papavassiliou et al., 1995*) involved in c-Jun transactivation (*Weiss et al., 2003*). In this context, genetic analyses have shown that both groups of MAPK sites require JNK expression in order to be phosphorylated in response to stress or pro-inflammatory stimuli (*Morton et al., 2003*). Conversely, in absence of JNK expression, EGF can induce phosphorylation at S63/S73 but not at T91/T93 (Morton et al., 2003), indicating that T91/T93 phosphorylation is specifically up regulated by stress and pro-inflammatory signals. In line, multisite phosphorylation of c-Jun on all four MAPK sites has been detected during chronic hypoxia (*Laderoute et al., 2002*), neuronal apoptosis by tropic factor deprivation (*Hongisto et al., 2003*) and UV-induced apoptosis of different types of mammalian cells (*Hamdi et al., 2005; Ui et al., 1998*). Altogether, these studies suggest that different physiological contexts may elicit distinct patterns of c-Jun N-terminal phosphorylation, which in turn correlate with diverse biological output of c-Jun activation.

Hence, to assess this hypothesis recent studies have investigated whether different grades of genotoxic stress could induce distinct N-terminal

phosphorylation of c-Jun. As an experimental model system, a differential exposure of human embryonic kidney 293 (HEK-293) cells to etoposide (*Vinciguerra et al., 2008*), a DNA toposoimerase II inhibitor responsible for both double and single DNA strand breaks were used (*Cliby et al., 2002; Costanzo et al., 2003*). By damaging DNA, etoposide, a DNA toposoimerase II inhibitor responsible for both double and single DNA strand breaks (*Clibyet al., 2002; Costanzo et al., 2003*), triggers the cellular response to genotoxic stress consisting in a complex network of signaling pathways designed to induce the onset of cell cycle delay and DNA repair (*Sancar et al., 2004*). Activation of IP3 kinase family members ATM and ATR is essential for initiating DNA damage pathways in response to almost all genotoxic stress including etoposide. Upon genotoxic stress, ATM and ATR activate a network of damage-response leading to activation of the checkpoint kinases, Chk1 and Chk2, and DNA repair proteins (*Sancar et al., 2004*). If DNA damage persists and unrepaired DNA accumulates, the same network may lead to the induction of apoptotic programs (*Roos and Kaina, 2006*). Etoposide-mediated activation of the DNA damage response can be abrogated by caffeine (*Clifford et al., 2003; Costanzo et al., 2003*), an inhibitor of ATM and ATR kinases (*Sarkaria et al., 1999*), able to reverse cell cycle checkpoint function in response to a wide number of genotoxic stress signals (*reviewed in Kaufmann et al., 2003*). Hence, c-Jun is promptly phosphorylated on S63/S73 following a short exposure to etoposide. In contrast, a continuous exposure to the drug induces c-Jun phosphorylation at T91/T93, an event that is caffeine-sensitive and linked to an intact threonine 95 (T95). Furthermore, the stress-induced biological activity of c-Jun is impaired by alanine substitution of T95,

pointing to T95 as a novel regulatory site integrating the strength of genotoxic stress with the output of c-Jun activity (*Vinciguerra et al., 2008*).

In the present thesis, the molecular mechanisms involved in OHT-induced apoptosis were evaluated using as a model system the ER-negative SkBr3 breast cancer cells. In particular, the study demonstrates (i) the specific phosphorylation of c-Jun and (ii) the up-regulation of c-Fos as separate ways contributing to AP-1-mediated apoptosis in response to OHT exposure. The work provides new insights in order to sensitize cancer cells to tamoxifen-induced cell death.

RESULTS

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c-Jun activation is required for 4-hydroxytamoxifen-induced cell death in breast cancer cells

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Introduction

Tamoxifen is largely used in the first-line treatment of estrogen receptor (ER)-positive breast cancer (*Love, 1989*). By binding to ER, tamoxifen antagonizes estrogen activity by recruiting co-repressors of transcription to ER target genes (*Smith et al., 1997*). However, in vitro studies have shown that at micromolar concentrations tamoxifen exerts anticancer activity by inducing apoptosis of tumor cells regardless of ER expression (*Mandlekar and Kong, 2001*). Apoptotic cell death is characterized by the activation of a cascade of intracellular cysteine proteases culminating with the activation of downstream caspases 3, 6 and 7. These effector-caspases execute the terminal phases of apoptosis by cleaving a variety of substrates including those triggering DNA fragmentation (*Porter and Janicke, 1999*). Several transduction pathways have been shown to mediate tamoxifen-induced apoptosis, including activation of PKC, intracellular Ca^{2+} and TGF-β (*Mandlekar and Kong, 2001*). It has been shown that tamoxifen-dependent elevation of intracellular Ca^{2+} triggers apoptosis of human hepatoblastoma HepG2 cancer cells through the accumulation of reactive oxygen species (ROS) obtained by non-phagocytic activation of NADPH oxidase (*Lee et al., 2000*). ROS-induced apoptosis requires the participation of further cell death signaling pathways, including the JNK family of stress-responsive MAPKs (*Shen et al., 1996*). In particular, it has been shown that prevention of ROS accumulation by antioxidants inhibits both JNK activation and apoptosis in tamoxifen-treated cancer cells (*Mandlekar et al., 2000b; Mabuchi et al., 2004*). These studies pointed to ROS-activated JNK as a crucial effector of tamoxifen-induced apoptosis. However, downstream targets of the JNK pathway involved in tamoxifen-induced apoptosis have not been identified. It has been largely

shown that c-Jun represents the principal transcription factor mediating gene regulation by JNK (*Davis, 2000; Dunn et al., 2002*). JNK induces c-Jun transactivation by phosphorylating the c-Jun N-terminal domain at serines 63/73 and threonines 91/93 (*Bannister et al., 1995; Papavassiliou et al., 1995; Morton et al., 2003; Weiss et al., 2003; Nateri et al., 2005; Vinciguerra et al., 2008*). By forming stable homodimeric or heterodimeric complexes with Fos or ATF family members, c-Jun constitutes the AP-1 transcription factor. Both genetic and biochemical studies indicate that AP-1 is involved in different cellular processes including proliferation, differentiation and apoptosis (*Shaulian and Karin, 2001; Eferl and Wagner, 2003; Hess et al., 2004*). However, several studies have shown that the functional output of AP-1 depends on the levels of expression and activation of the individual AP-1 members that make up the AP-1 dimers, as well as the type of stimulus and the intracellular environment (*Eferl and Wagner, 2003; Hess et al., 2004*). It has been widely shown that the JNK c-Jun pathway contributes to stress-induced apoptosis in different cell types, such as neurons, fibroblasts or DNA-damaged cancer cells (*Behrens et al., 1999; Dunn et al., 2002; Raivich, 2008*). In contrast, c-Jun phosphorylation at serine 63 and 73 (S63/S73) is also important for cell cycle progression or intestinal tumor progression (*Eferl and Wagner, 2003; Wada et al., 2004; Nateri et al., 2005*), suggesting that additional phosphorylation of c-Jun at threonine 91 and 93 (T91/T93) might be critical for c-Jun pro-apoptotic functions. In line with this suggestion, we have recently shown that phosphorylation of c-Jun at all four JNK sites is crucial for c-Jun pro-apoptotic function in response to DNA damage (*Vinciguerra et al., 2008*). Furthermore, JNK-specific phosphorylation of c-Jun at T91/T93, but not at S63/S73, requires a priming phosphorylation at T95 by a

yet to be identified stress-induced kinase (*Vinciguerra et al., 2008*). These observations suggest that depending on the type of stimulus c-Jun may or may not be phosphorylated at all four terminal sites by JNK and correspondingly it may result in different cellular consequences. Therefore, the analysis of N-terminal phosphorylation of c-Jun at both S63/73 and T91/T93 is essential for associating c-Jun activation to either prosurvival or pro-apoptotic pathways. To date, c-Jun phosphorylation in response to tamoxifen has been analysed only in tamoxifen-resistant MCF-7-derived xenografts and limited to the S63 site (*Schiff et al., 2000*).

c-Fos, the main heterodimeric partner of c-Jun, has also been shown to be involved in both proliferative and apoptotic pathways (*Hess et al., 2004*). c-Fos expression can be induced by a variety of stimuli, each acting on one or more multiple cis-elements contained within the promoter of c-Fos (*Treisman, 1995*). In this regard, it has been reported that the serum-responsive element recruits ERK-activated Elk-1 as well as the serum responsive factor accessory proteins 1 and 2 to the c-Fos promoter sequence (*Price et al., 1995*). Accordingly, in our previous studies both estrogen and 4-hydroxytamoxifen (OHT) induced c-Fos expression by ERK-dependent activation of Elk-1 (*Maggiolini et al., 2004; Vivacqua et al., 2006a-b*). These studies, as well as other reports (*Reviewed in Prossnitz and Maggiolini, 2009*), indicate that the aforementioned stimulations may occur through the GPR30 in conjunction with activated EGFR and relayed on a pertussis toxinsensitive pathway, confirming the involvement of Gi/o heterotrimeric G proteins. In this study, we have assessed the role of the c-Jun/c-Fos AP-1 complex in OHT-induced apoptosis. To this aim, we have examined JNK-dependent phosphorylation of c-Jun at both S73 and T91/T93

sites as well as the induction of both c-Fos expression and AP-1 transactivation in response to OHT. Our results indicate that both JNK-dependent activation of c-Jun and ERK-mediated expression of c-Fos are crucial for OHT-induced apoptosis in ER-negative breast cancer cells.

JNK-specific phosphorylation of c-Jun precedes apoptosis in OHT-treated SkBr3 cells

Micromolar concentrations of OHT have been shown to induce apoptotic cell death in ER-negative breast cancer cells through caspase 3 and JNK pathways (*Mandlekar et al., 2000a*). We used the ER-negative breast cancer-derived cell line SkBr3 as a model system to analyse the role of JNK-activated c-Jun in the OHT-dependent apoptosis. First, we examined whether micromolar concentrations of OHT, which were earlier reported to be cytotoxic in SkBr3 cells (*Basu et al., 2004*), could induce hallmarks of apoptotic cell death such as DNA fragmentation and caspase 3/7 activation. As shown by flow cytometric analysis, DNA fragmentation was induced by 10 μM OHT and was further increased by 25 μM OHT, whereas neither concentration had any influence on cell cycle phases (Figures 1a and b). The effect of 10 μM OHT on DNA fragmentation was further confirmed by TdT-mediated dUTP nick end labeling (TUNEL) staining and cell viability assay (Figures 1c and d). Furthermore, OHT induced caspase 3/7 activities (Figure 1e), suggesting that DNA fragmentation results from apoptosis. Next, we analysed whether JNK-activated c-Jun precedes OHT-dependent apoptosis in SkBr3 cells. To this end, we examined the ability of OHT to induce c-Jun N-terminal phosphorylation at either (S73, a hallmark of c-Jun activation by JNK (*Dunn et al., 2002*), or at T91/T93, which are stringently correlated with c-Jun pro-apoptotic activity (*Vinciguerra et al., 2008*). Phosphorylation of both S73 and T91/T93 sites were induced following a 2 h exposure to OHT and no longer detected in presence of the JNK-specific inhibitor SP 600125 (SP) (Figures 2a and b), indicating that JNK-specific activation of c-Jun precedes OHT-induced apoptosis. In contrast, neither the

EGFR inhibitor AG 1478 (AG), nor the MEK/ERK inhibitor PD 98059 (PD) and the ER pure antiestrogen ICI 182 780 (ICI) prevented c-Jun phosphorylation by OHT. As OHT has been shown to induce JNK activation through generation of oxidative stress (*Lee et al., 2000; Mandlekar and Kong, 2001; Mabuchi et al., 2004*), we next analysed the effect of the antioxidant N-acetyl-L-cysteine (NAC) on the JNK/c-Jun pathway. As shown in Figure 2c, both activation of JNK and c-Jun phosphorylation at T91/T93 sites were prevented by NAC treatment. In line with previous studies, the exposure to NAC prevented also DNA fragmentation by OHT (Figure 2d). These results indicate that c-Jun pro-apoptotic phosphorylation by OHT is triggered by oxidative stress and is associated with DNA fragmentation.

OHT induces c-Fos expression in SkBr3 cells

In addition to c-Jun activation, we analysed whether OHT induces c-Fos expression in SkBr3 cells, at both messenger RNA and protein levels. As shown by either reverse transcription-PCR analysis or western blots, the expression of c-Fos was rapidly induced by OHT (Figures 3a and b). In agreement with our previous studies performed in different cancer cell types (*Vivacqua et al., 2006a-b*), c-Fos induction by OHT was sensitive to AG and PD but not to SP or ICI (Figure 3b). It is noteworthy that the effect of OHT on c-Fos expression was also abrogated silencing GPR30 expression (Figures 3c and d), which we have shown earlier to mediate c-Fos induction by estrogens through ERK activation (*Maggiolini et al., 2004; Vivacqua et al., 2006a-b*). Accordingly, OHT-dependent activation of ERK was sensitive to either PD or abrogation of GPR30 expression (Figures 3e and f). In contrast, NAC treatment had no effect on either ERK

activation or c-Fos expression, indicating that OHT induces the ERK/c-Fos pathway independently from its ability to trigger oxidative stress (Supplementary Figure 1). Taken together, these results suggest that OHT induces c-Fos expression through the GPR30–EGFR–ERK transduction pathway in SkBr3 cells.

OHT transactivates an AP-1-responsive promoter

Next, we evaluated whether OHT-dependent induction of c-Jun N-terminal phosphorylation and c-Fos expression could lead to AP-1 transactivation. To this end, we tested the ability of OHT to activate the AP-1-responsive collagenase promoter fused to a luciferase reporter gene (AP-1-luc). As shown in Figure 4a, OHT induced AP-1-luc transactivation, which was sensitive to SP, AG or PD, but not to ICI. Furthermore, only AG and PD inhibited the transactivation of the c-Fos promoter by OHT (Figure 4b), hence corroborating the specific role exerted by ERK activation in OHT-induced c-Fos expression. Overall, these findings suggest that both JNK-specific activation of c-Jun and ERK-dependent induction of c-Fos expression contribute to AP-1 activation by OHT.

Abrogation of either c-Jun phosphorylation or AP-1 transactivation protects SkBr3 cells from OHT-induced apoptosis

We then analysed the specific involvement of the c-Jun/c-Fos AP-1 complex in OHT-induced apoptosis. To this aim, we performed TUNEL assays in OHT-treated cells ectopically expressing a truncated form of c-Fos (A-Fos), which was shown earlier to efficiently knockdown c-Jun/c-Fos AP-1 heterodimers (*Olive et*

al., 1997). As shown in Figures 5a–f, approximately 60% of SkBr3 cells transfected with an empty vector resulted positive for TUNEL staining after exposure to OHT. In contrast, only 11% of A-Fos-transfected SkBr3 cells were TUNEL positive (Figures 5g–l), indicating that the c-Jun/c-Fos AP-1 complex is involved in OHT-induced apoptosis. As shown in Figure 5s, the constitutive expression of c-Fos slightly decreased the percentage of TUNEL-positive cells (Figures 5m–r), suggesting that c-Fos alone cannot sensitize SkBr3 cells to OHT-induced apoptosis. To further analyse the direct role of c-Jun activation in OHT-mediated apoptosis, we ectopically expressed in SkBr3 cells either a HA-tagged c-Jun-wt protein or a HA-tagged c-Jun mutant bearing alanine substitutions of S63/S73 and T91/T93 JNK sites (c-Jun-PanAla), which was shown to impair c-Jun activation as well as c-Jun-mediated apoptosis (*Papavassiliou et al., 1995; Watson et al., 1998; Dunn et al., 2002*). Expression of HA-tagged c-Jun-PanAla protected SkBr3 cells from OHT-induced apoptosis, as only 6% of HA-positive cells were also positive for TUNEL staining, while 58% of HA-negative cells were TUNEL positive (Figures 6i–p). In contrast, ectopic expression of c-Jun-wt had no effect on DNA fragmentation (Figures 6a–h), indicating the abrogation of JNK-specific phosphorylation of c-Jun is essential to protect SkBr3 cells from OHT-induced apoptosis. Furthermore, a c-Jun mutant that cannot be specifically phosphorylated at the T91/T93 JNK sites (*Vinciguerra et al., 2008*) was as efficient as c-Jun-PanAla to protect SkBr3 cells from OHT-dependent apoptosis (Figures 6q–x), showing that c-Jun N-terminal phosphorylation at T91/T93 site has a pivotal role in OHT-dependent apoptosis.

OHT induces c-Jun activation and c-Fos expression in different cancer cell types

To validate the role of AP-1 activation in OHT-induced apoptosis, we examined whether OHT couples the induction of both c-Fos expression and c-Jun phosphorylation to caspase 3/7 activation in different cancer cell types. As shown in Figures 7a and b, OHT stimulated both c-Fos expression and c-Jun phosphorylation in ER-negative BT20 and ER-positive MCF-7 breast cancer cells, HepG2 hepatocarcinoma cells, LoVo colorectal adenocarcinoma cells and SCLC-R1 lung cancer cells. As observed for SkBr3 cells (Figure 3), OHT-dependent induction of c-Fos was specifically abolished by AG and PD (Figure 7a), as well as by silencing GPR30 expression (Supplementary Figure 2). Furthermore, in all cell lines OHT-stimulated c-Jun N-terminal phosphorylation in a JNK-specific manner, because only SP abrogated c-Jun phosphorylation (Figure 7b). Similarly, OHT induced caspase3/7 activity (Figure 7c) and DNA fragmentation (Supplementary Figure 3) in all cancer cell types, except for the OHT-resistant LNCaP prostate cancer cells. Remarkably, OHT also failed to induce either c-Fos expression or c-Jun phosphorylation in LNCaP cells (Figures 7d and e), corroborating the functional link between AP-1 activation and OHT-induced apoptosis. As our results indicate that activation of the JNK/c-Jun pathway by OHT is triggered by oxidative stress, we asked whether the resistance of LNCaP cells to OHT-induced apoptosis correlated with a deficiency in ROS production and consequential lack of activation of the JNK/c-Jun pathway. To this end, we examined the ability of OHT to generate ROS in either SkBr3 or LNCaP cells by DCFH fluorescence (*Shen et al., 1996*). As show in Figure 8, OHT increased the basal level of ROS in SkBr3 cells, but not in LNCaP

cells, suggesting that the failure to generate ROS may contribute to protect LNCaP cells from OHT-dependent apoptosis.

DISCUSSION

The effects of estrogens are versatile and fundamental throughout the body. Estrogens exert diverse biological effects in animals and humans, and many of these effects result from a direct interaction of estrogen with an intracellular receptor that activates the expression of genes encoding proteins with important biological functions (*Kumar et al., 1987; Evans, 1988; Weisz et al., 1990; Parker, 1991*). 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. Recently, the 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., 2006b*) and HEC50 (*Revankar et al., 2005*) endometrial cancer, JEG choriocarcinoma (*Revankar et al., 2005*), BG-1 ovarian cancer, and thyroid carcinoma (*Vivacqua et*

al., 2006a) as well as in some brain areas of the hamster (*Canonaco et al., 2008*). Our previous reports demonstrated estrogen-mediated cell proliferation to be dependent on GPR30 (*Maggiolini et al., 2004; Vivacqua et al., 2006a-b*). Our data suggested that the c-Fos/AP-1 complex is implicated in tumor progression elicited by estrogens, phytoestrogens, xenoestrogens and antiestrogens through GPR30, in different cancer cell types (*Albanito et al., 2007; Albanito et al., 2008a;.Pandey et al., 2009; Albanito et al., 2008b*)

Furthermore, we studied the role of c-Jun activation in cell death induced by 4-hydroxytamoxifen (OHT) in breast cancer cells and in diverse cancer cell types. Accumulating evidence suggests that tamoxifen-induced apoptosis may consist in the generation of oxidative stress and subsequent activation of the JNK family of stress-responsive MAPKs (*Lee et al., 2000; Mandlekar and Kong, 2001; Mabuchi et al., 2004*). c-Jun mediates proapoptotic functions of JNK in different types of cells including neurons, fibroblasts and cancer cells (*Dunn et al., 2002; Hess et al., 2004; Raivich, 2008*). However, c-Jun does not mediate all paradigms of JNK-dependent apoptosis and its pro-apoptotic function depends on the type of stimulus as well as on the heterodimeric partner forming the AP-1 transcription factor (*Hess et al., 2004; Raivich et al., 2006*). In this study, we analysed whether JNK-dependent activation of c-Jun has a role in OHT-induced cell death of ER-negative SkBr3 breast cancer cells. To this end, we first established by fluorescence-activated cell sorting (FACS) analyses the range of micromolar OHT concentrations required to induce DNA fragmentation, as also confirmed by TUNEL and cell viability assays. Importantly, we found that OHT triggers caspase 3/7 activity, suggesting that DNA fragmentation by OHT is a secondary consequence of apoptosis rather than necrosis. Moreover, OHT had no effects on cell cycle progression, excluding the

possibility that OHT-dependent cytotoxicity was due to cell growth arrest. Next, we analysed the ability of OHT to induce JNK-dependent phosphorylation of c-Jun at the S73 site, which is considered a hallmark of c-Jun activation in several paradigms of stress signals (*Dunn et al., 2002*). We detected both JNK activation and c-Jun phosphorylation at S73 on a 2-h exposure to OHT. Remarkably, OHT also induced phosphorylation of c-Jun at T91/T93 sites, which was shown to be crucial for c-Jun-mediated cell death in response to either ultraviolet or etoposide exposure (*Vinciguerra et al., 2008*). Moreover, both S73 and T91/T93 phosphorylations by OHT were specifically sensitive to SP, indicating that JNK-specific activation of c-Jun precedes apoptosis in SkBr3 cells. Interestingly, OHT-induced c-Jun N-terminal phosphorylation without alteration of c-Jun expression, suggesting that activation of c-Jun by OHT is limited to pre-existing c-Jun proteins. Accordingly, our (*unpublished observations*) and other studies (*Nephew et al., 1993*) have indicate that c-Jun expression is not regulated by OHT treatment. Importantly, DNA degradation as well as JNK activation and N-terminal phosphorylation of c-Jun, were abrogated by antioxidant treatments. These results suggest that generation of oxidative stress, presumably by NADPH activation (*Lee et al., 2000*), could be the initial OHT-dependent signal leading to activation of the JNK/c-Jun pathway and thereafter to apoptosis. We then asked whether c-Jun activation by OHT was coupled to induction of c-Fos expression. Transactivation assays of the c-Fos promoter combined with the analysis of steady-state levels of both c-Fos protein and messenger RNA indicated that OHT leads to transcriptional induction of c-Fos expression. Interestingly, both the transactivation of the c-Fos promoter and the induction of c-Fos protein levels were sensitive to AG or PD, but not to SP or ICI, indicating that OHT induces c-Fos

expression independently of its ability to induce JNK activity but rather in an EGFR/ERK-mediated manner. Accordingly, OHT-dependent activation of ERK was abrogated by PD but not by SP, confirming that JNK activity is not required for the signaling pathway upstream c-Fos expression. Moreover, both c-Fos expression and ERK activation by OHT were sensitive to GPR30 silencing, in accordance with our previous studies performed in different cancer cells (*Maggiolini et al., 2004; Vivacqua et al., 2006a-b; Albanito et al., 2007*). It is noteworthy that the ERK/c-Fos pathway was not altered by antioxidant treatment, thus corroborating the fact that OHT initiates two distinct signaling pathways converging to activation of the c-Jun/c-Fos AP-1 complex. Induction of c-Fos expression coupled to c-Jun N-terminal phosphorylation is the most frequent encountered paradigm of AP-1 activation in mammalian cells. Indeed, we found that OHT-induced AP-1 transactivation in an ERK and JNK-dependent manner, suggesting that the heterodimeric c-Jun/c-Fos AP-1 complex functions in OHT-induced apoptosis of cancer cells. Alternatively, because of the crucial role of AP-1 in a variety of cellular events, activation of c-Jun/c-Fos AP-1 complex by OHT may reflect a pro-survival response.

To evaluate the specific involvement of c-Jun/c-Fos in OHT-dependent apoptosis, we abrogated c-Jun/c-Fos AP-1 activity in SkBr3 cells by two independent experimental approaches: (i) ectopic expression of A-Fos, a truncated form of c-Fos that has been shown to specifically knock-down c-Jun/c-Fos AP-1 heterodimers (*Olive et al., 1997*); and (ii) ectopic expression of c-Jun-PanAla, a c-Jun mutant bearing alanine substitution of all four JNK phosphorylation sites S63/S73 and T91/T93, which has been shown to act as a c-Jun dominant negative in several cell lines (*Papavassiliou et al., 1995; Watson et al., 1998; Dunn et al.,*

2002). A-Fos overexpression protected cells from OHT-induced apoptosis, indicating that c-Jun/c-Fos heterodimers are directly involved in apoptosis. This finding was further corroborated by the evidence that AP-1 transactivation by OHT was dependent on both ERK and JNK activity. Similarly, overexpression of c-Jun-PanAla resulted in the protection of SkBr3 cells from OHT-induced apoptosis. As Jun-wt had no effect on DNA degradation, the protective effect of c-Jun-PanAla presumably results from its ability to act as dominant-negative, indicating that JNK-activated c-Jun is necessary to trigger OHT-induced apoptosis. Moreover, a c-Jun mutant that specifically cannot be phosphorylated at the T91/T93 JNK sites was as efficient as c-Jun-PanAla in protecting SkBr3 cells from OHT-dependent apoptosis, suggesting that phosphorylation of c-Jun at S63/S73 is not sufficient to trigger c-Jun-dependent apoptosis. It is noteworthy that ectopic expression of c-Jun could not significantly increase the number of TUNEL-positive cells, indicating that activation of pre-existing c-Jun proteins by OHT is sufficient to trigger apoptosis. Our data show that OHT induces both ERK/c-Fos and JNK/c-Jun pathways in various OHT-sensitive cancer cells, pointing to c-Jun/c-Fos AP-1 activation as a general mechanism mediating OHT-dependent apoptosis, rather than a peculiar response of SkBr3 cells. Accordingly, OHT-induced caspase 3/7 activity and DNA degradation in all cancer cell types used, except in OHT-resistant LNCaP prostate cancer cells. It is noteworthy that OHT failed to induce both c-Fos expression and c-Jun N-terminal phosphorylation in LNCaP cells, validating that the up-regulation of c-Jun/c-Fos AP-1 by cytotoxic concentrations of OHT is linked to caspase 3/7 activation and in turn to cell death. How c-Jun/AP-1 drives cell death in OHT-treated cancer cells remains to be elucidated. The JNK/c-Jun pathway has been shown to regulate the release of cytochrome c

from mitochondria (*Whitfield et al., 2001*). In line with this observation, active c-Jun is required for the expression of genes that increase mitochondrial outer membrane permeability, such as Bim and Dp5, which are members of the proapoptotic BH3-only subfamily of Bcl-2 proteins (*Ham et al., 2005*). A crucial role of mitochondria has been also shown in OHT-induced apoptosis, as it can elicit the release of mitochondrial cytochrome c altering the expression of Bcl-2 proteins (*Mandlekar and Kong, 2001; Kallio et al., 2005; Lagadec et al., 2008*). Therefore, downregulation of the antiapoptotic Bcl-2 protein together with upregulation of one or more proapototic Bcl-2 proteins may constitute a critical mechanism promoting mitochondrial dysfunction by OHT. Presumably, the JNK/c-Jun pathway may contribute to this intrinsic proapoptotic mechanism by upregulating one or more proapototic BH3-only proteins. In contrast, as c-Jun/AP-1 controls tumor necrosis factor-α expression (*Liu et al., 2000; Pope et al., 2000*), we cannot exclude a putative role of c-Jun in a cross talk between the death receptor and mitochondrial pathways.

Our previous studies have shown that one micromolar OHT induces cell proliferation in thyroid and endometrial cancer cells by activating the EGFR/GPR30/ERK pathway and in turn c-Fos expression (*Vivacqua et al., 2006a-b*), whereas it has no effects on the JNK/c-Jun pathway (*Madeo A, unpublished observations*). These observations together with this study lead to the hypothesis that the functional output of c-Fos induction by OHT may depend on whether or not there is concomitant c-Jun activation by JNK signaling. As antioxidant pathways are often enhanced in cancer cells (*Friesen et al., 2004; Young et al., 2004; Recktenwald et al., 2008; Ballatori et al., 2009*), the resistance of certain cancer cells to tamoxifen may depend on their ability to prevent ROS

accumulation and in turn JNK-specific activation of c-Jun. In line with this hypothesis we found that OHT-induced ROS accumulation in SkBr3 cells but not in LNCaP cells (*Madeo et al., 2009*).

In conclusion, our data have contributed to better understand the activation of estrogenic pathways by a variety of natural and synthetic compounds in biological effects elicited in different cancer cell types, providing new insights for the knowledge of the molecular mechanisms implicated in cancer progression but also in programmed cell death induced by antiestrogens. In particular, we have demonstrated the role of the c-Jun/c-Fos AP-1 complex in OHT-induced apoptosis providing that pharmacological boosts of c-Jun N-terminal phosphorylation may be useful to sensitize cancer cells to OHT-mediated cell death.

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ORIGINAL ARTICLE

c-Jun activation is required for 4-hydroxytamoxifen-induced cell death in breast cancer cells

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The c-Jun N-terminal kinase (JNK) has been shown to mediate tamoxifen-induced apoptosis in breast cancer cells. However, the downstream mediators of the JNK pathway linking tamoxifen to effectors of apoptosis have yet to be identified. In this study, we analysed whether c-Jun, the major nuclear target of JNK, has a role in tamoxifen-induced apoptosis of SkBr3 breast cancer cells. We show that before DNA fragmentation and caspase 3/7 activation, cytotoxic concentrations of 4-hydroxytamoxifen (OHT) induced JNK-dependent phosphorylation of c-Jun at JNK sites earlier shown to regulate c-Jun-mediated apoptosis. In addition, OHT induced ERK-dependent expression of c-Fos and transactivation of an AP-1-responsive promoter. In particular, the ectopic expression of dominant-negative constructs blocking either AP-1 activity or c-Jun N-terminal phosphorylation prevented DNA fragmentation after OHT treatment. Furthermore, both c-Fos expression and c-Jun N-terminal phosphorylation preceded OHT-dependent activation of caspase 3–7 in different types of tamoxifen-sensitive cancer cells, but not in OHT-resistant LNCaP prostate cancer cells. Taken together, our results indicate that the c-Jun/c-Fos AP-1 complex has a pro-apoptotic role in OHT-treated cancer cells and suggest that pharmacological boosts of c-Jun activation may be useful in a combination therapy setting to sensitize cancer cells to tamoxifen-mediated cell death.
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Keywords: c-Jun; c-Fos; AP-1; JNK; apoptosis; 4-hydroxytamoxifen

Introduction

Tamoxifen is largely used in the first-line treatment of estrogen receptor (ER)-positive breast cancer (Love, 1989). By binding to ER, tamoxifen antagonizes estrogen activity by recruiting co-repressors of transcription to ER target genes (Smith *et al*., 1997). However, *in vitro* studies have shown that at micromolar concentrations tamoxifen exerts anticancer activity by inducing apoptosis of tumor cells regardless of ER expression (Mandlekar and Kong, 2001). Apoptotic cell death is characterized by the activation of a cascade of intracellular cysteine proteases culminating with the activation of downstream caspases 3, 6 and 7. These effector-caspases execute the terminal phases of apoptosis by cleaving a variety of substrates including those triggering DNA fragmentation (Porter and Janicke, 1999). Several transduction pathways have been shown to mediate tamoxifen-induced apoptosis, including activation of PKC, intracellular Ca^{2+} and TGF-β (Mandlekar and Kong, 2001). It has been shown that tamoxifen-dependent elevation of intracellular Ca^{2+} triggers apoptosis of human hepatoblastoma HepG2 cancer cells through the accumulation of reactive oxygen species (ROS) obtained by not-phagocytic activation of NADPH oxidase (Lee *et al*., 2000). ROS-induced apoptosis requires the participation of further cell death signaling pathways, including the c-Jun N-terminal kinase (JNK) family of stress-responsive MAPKs (Shen *et al*., 1996). In particular, it has been shown that prevention of ROS accumulation by antioxidants inhibits both JNK activation and apoptosis in tamoxifen-treated cancer cells (Mandlekar *et al*., 2000; Mabuchi *et al*., 2004). These studies pointed to ROS-activated JNK as a crucial effector of tamoxifen-induced apoptosis. However, downstream targets of the JNK pathway involved in tamoxifen-induced apoptosis have not been identified.

It has been largely shown that c-Jun represents the principal transcription factor mediating gene regulation by JNK (Davis, 2000; Dunn *et al*., 2002). JNK induces c-Jun transactivation by phosphorylating the c-Jun N-terminal domain at serines 63/73 and threonines 91/93 (Bannister *et al*., 1995; Papavassiliou *et al*., 1995; Morton *et al*., 2003; Weiss *et al*., 2003; Nateri *et al*., 2005; Vinciguerra *et al*., 2008). By forming stable homodimeric or heterodimeric complexes with Fos or ATF family members, c-Jun constitutes the inducible activator protein-1 (AP-1) transcription factor. Both

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genetic and biochemical studies indicate that AP-1 is involved in different cellular processes including proliferation, differentiation and apoptosis (Shaulian and Karin, 2001; Eferl and Wagner, 2003; Hess *et al*., 2004). However, several studies have shown that the functional output of AP-1 depends on the levels of expression and activation of the individual AP-1 members that make up the AP-1 dimers, as well as the type of stimulus and the intracellular environment (Eferl and Wagner, 2003; Hess *et al*., 2004). It has been widely shown that the JNK-c-Jun pathway contributes to stress-induced apoptosis in different cell types, such as neurons, fibroblasts or DNA-damaged cancer cells (Behrens *et al*., 1999; Dunn *et al*., 2002; Raivich, 2008). In contrast, c-Jun phosphorylation at serine 63 and 73 (S63/S73) is also important for cell cycle progression or intestinal tumor progression (Eferl and Wagner, 2003; Wada *et al*., 2004; Nateri *et al*., 2005), suggesting that additional phosphorylation of c-Jun at threonine 91 and 93 (T91/T93) might be critical for c-Jun pro-apoptotic functions. In line with this suggestion, we have recently shown that phosphorylation of c-Jun at all four JNK sites is crucial for c-Jun pro-apoptotic function in response to DNA damage (Vinciguerra *et al*., 2008). Furthermore, JNK-specific phosphorylation of c-Jun at T91/T93, but not at S63/S73, requires a priming phosphorylation at T95 by a yet to be identified stress-induced kinase (Vinciguerra *et al*., 2008). These observations suggest that depending on the type of stimulus c-Jun may or may not be phosphorylated at all four terminal sites by JNK and correspondingly it may result in different cellular consequences. Therefore, the analysis of N-terminal phosphorylation of c-Jun at both S63/73 and T91/T93 is essential for associating c-Jun activation to either pro-survival or pro-apoptotic pathways. To date, c-Jun phosphorylation in response to tamoxifen has been analysed only in tamoxifen-resistant MCF-7-derived xenografts and limited to the S63 site (Schiff *et al*., 2000).

c-Fos, the main heterodimeric partner of c-Jun, has also been shown to be involved in both proliferative and apoptotic pathways (Hess *et al*., 2004). c-Fos expression can be induced by a variety of stimuli, each acting on one or more multiple *cis*-elements contained within the promoter of c-Fos (Treisman, 1995). In this regard, it has been reported that the serum-responsive element recruits ERK-activated Elk-1 as well as the serum-responsive factor accessory proteins 1 and 2 to the c-Fos promoter sequence (Price *et al*., 1995). Accordingly, in our previous studies both estrogen and 4-hydroxytamoxifen (OHT)-induced c-Fos expression by ERK-dependent activation of Elk-1 (Maggiolini *et al*., 2004; Vivacqua *et al*., 2006a, b). These studies, as well as other reports (Prossnitz and Maggiolini, 2009), indicate that the aforementioned stimulations may occur through the G protein-coupled receptor (GPR)30 in conjunction

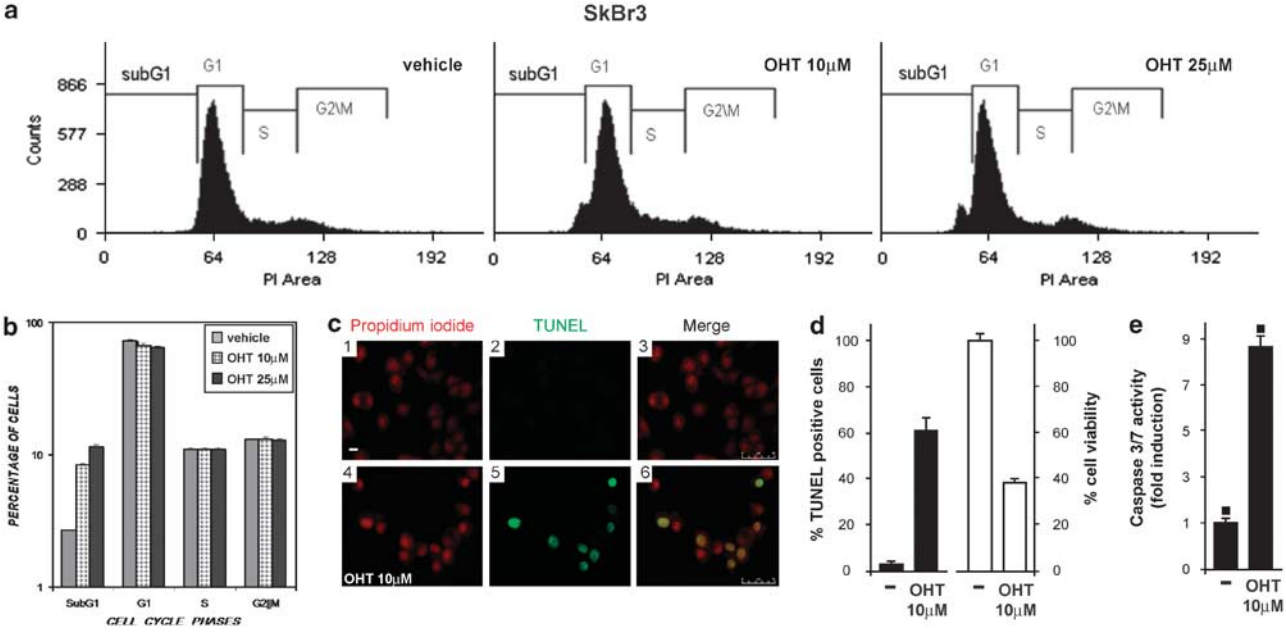


Figure 1 4-hydroxytamoxifen (OHT) induces apoptotic cell death in SkBr3 cells. (**a**) Cells were treated for 18 h with or without the indicated OHT concentrations. The cell-cycle distribution was measured by flow cytometry as described in materials and methods section. The region to the left of the G1 peak, designated sub-G1, was defined as cells undergoing apoptosis-associated DNA degradation. (**b**) In bar graphs the data represent the mean values of three independent experiments with bars indicating s.d. (**c**) TdT-mediated dUTP nick end labeling (TUNEL) staining of SkBr3 cells treated with or without 10 μM OHT for 18 h. Magnification is indicated by bars (50 μm). Each experiment shown is representative of 20 random fields observed (**d**) Bar graphs represent the percentage of either TUNEL-positive cells (left bar graph) or viable cells (right bar graph) in OHT-treated versus cells treated with vehicle. Values are the mean of three independent experiments with bars indicating s.d. (**e**) Caspase 3/7 activity in OHT-treated cancer cells. Bar graph shows a representative experiment with means of triplicate samples. Caspase activity was normalized to the control, values of cells receiving vehicle (−) were set as onefold induction on which the activity induced by OHT was calculated. Error bars show ± s.d. ■ indicates $P<0.05$ for cells receiving vehicle (−) versus treatment.

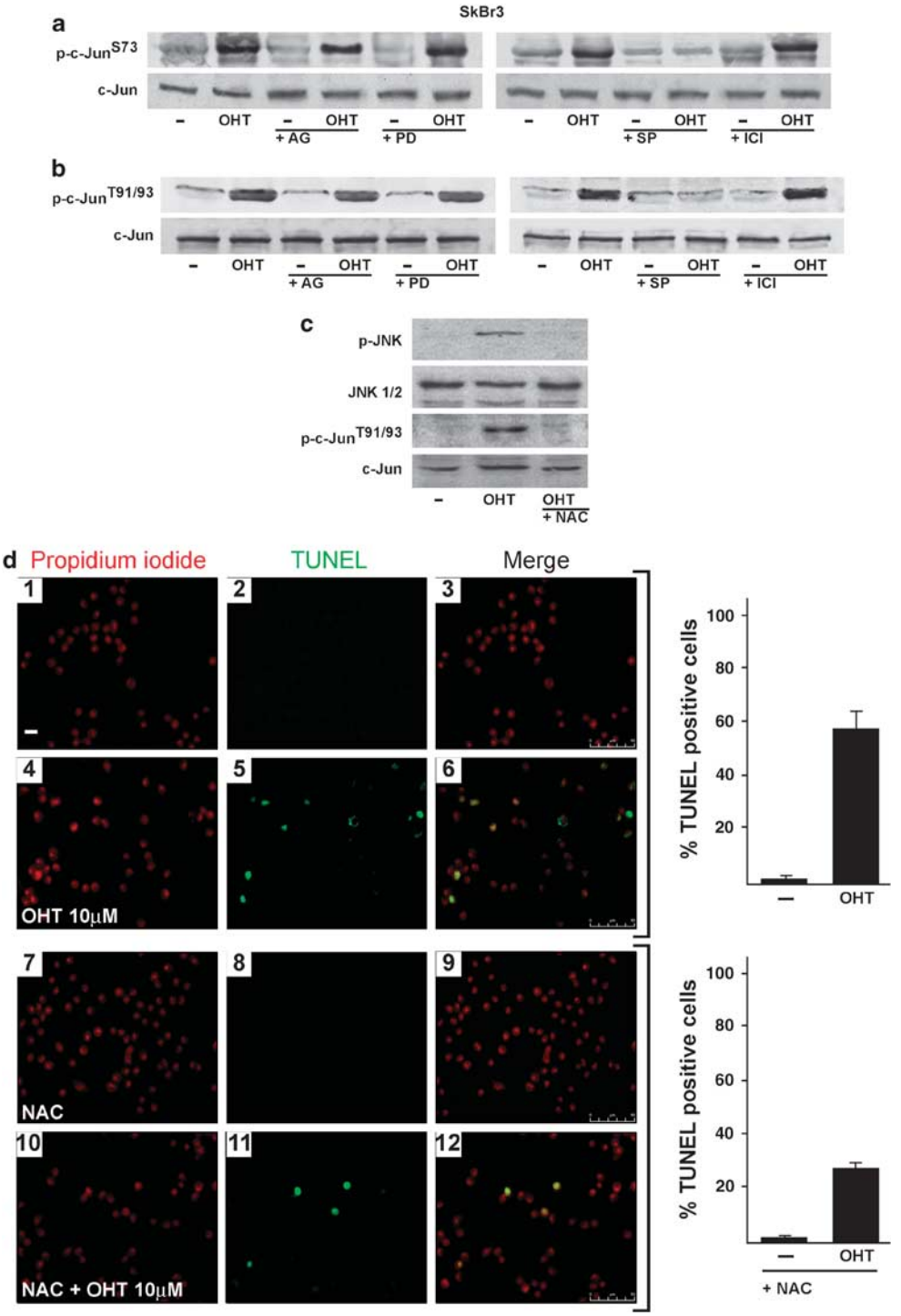


Figure 2 c-Jun N-terminal kinase (JNK)-dependent phosphorylation of c-Jun precedes 4-hydroxytamoxifen (OHT)-induced apoptosis in SkBr3 cells. (**a** and **b**) SkBr3 cells were treated with vehicle (−) or with 10 μM OHT either alone or in combination with the EGFR inhibitor tyrphostin AG 1478 (AG), MEK inhibitor PD 98059 (PD), the JNK-specific inhibitor SP 600125 (SP) or the pure antiestrogen ICI 182 780, as indicated. Western blot analyses were performed using phospho-specific antibodies for p-c-Jun^{S73} and p-c-Jun$^{T91/T93}$ as indicated. Expression levels of c-Jun were detected using the non-phospho-specic antibodies, indicated as c-Jun. (**c**) SkBr3 cells were treated with vehicle (−) or with 10 μM OHT, either alone or in combination with 10 mM *N*-acetyl-L-cysteine (NAC) as indicated. JNK activation and c-Jun phosphorylation at threonine 91 and 93 (T91/T93) were detected by immunoblotting using phospho-specific antibodies for p-JNK (T183/Y185) or p-c-Jun$^{T91/T93}$, respectively. Expression levels of JNK and c-Jun proteins were detected using the non-phospho-specic antibodies, as indicated. (**d**) TdT-mediated dUTP nick end labeling (TUNEL) staining of SkBr3 cells treated for 18 h with vehicle (1–3), 10 μM OHT (4–6), 10 mM NAC (7–9), 10 mM NAC in combination with 10 μM OHT (10–12). Magnification is indicated by bars (50 μm). Each experiment shown is representative of 20 random fields observed. Bar graphs show the percentage of TUNEL-positive cells and represent the mean of three independent experiments with bars indicating s.d.

with activated EGFR and relayed on a pertussis toxin-sensitive pathway, confirming the involvement of Gi/o heterotrimeric G proteins.

In this study, we have assessed the role of the c-Jun/c-Fos AP-1 complex in OHT-induced apoptosis. To this aim, we have examined JNK-dependent phosphorylation of c-Jun at both S73 and T91/T93 sites as well as the induction of both c-Fos expression and AP-1 transactivation in response to OHT. Our results indicate that both JNK-dependent activation of c-Jun and ERK-mediated expression of c-Fos are crucial for OHT-induced apoptosis in ER-negative breast cancer cells.

Results

JNK-specific phosphorylation of c-Jun precedes apoptosis in OHT-treated SkBr3 cells

Micromolar concentrations of OHT have been shown to induce apoptotic cell death in ER-negative breast cancer

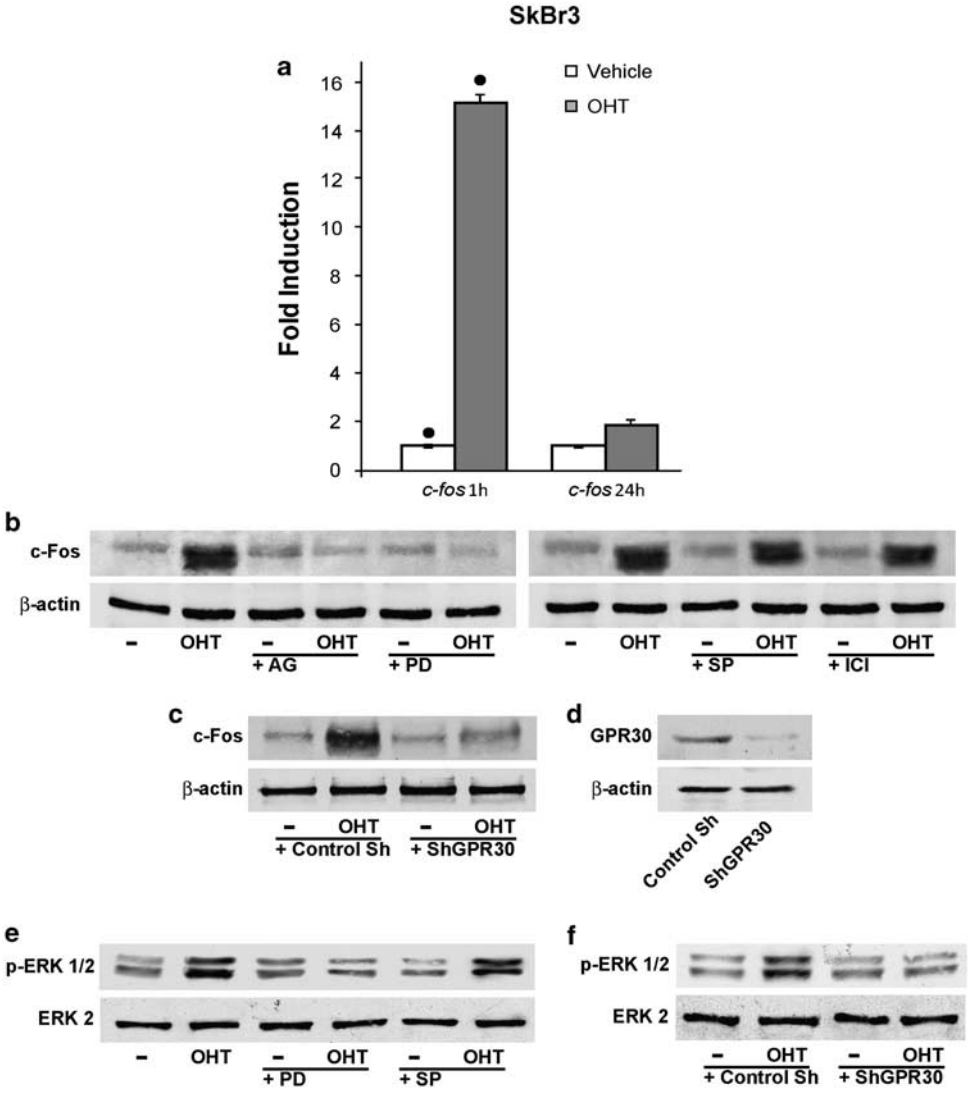


Figure 3 4-hydroxytamoxifen (OHT) induces c-Fos expression and ERK-activation in a GPR30-dependent manner. (**a**) Messenger RNA (mRNA) expression of c-Fos was evaluated by real-time PCR in SkBr3 cells treated for 1 and 24 h with vehicle (−) or 10 μM OHT, as indicated. 'Fold induction' represents the values of c-Fos mRNA expression after normalization to the expression level of 18S mRNA. Error bars indicate s.d. of measurements of triplicate samples (± s.d.). ● indicates $P<0.05$ for cells receiving vehicle (−) versus treatment. (**b**) SkBr3 cells were treated either with vehicle (−) or 10 μM OHT, either alone or in combination with AG 1478 (AG), or PD 98059 (PD), or SP 600125 (SP) or ICI 182 780 (ICI), as indicated. Western blots were performed by using anti-c-Fos antibodies to detect c-Fos protein levels, or with anti β-actin antibodies as loading control. (**c**) SkBr3 cells were transfected either with control short hairpin RNA (shRNA) (Control Sh) or with a shRNA specific for GPR30 (ShGPR30), then cells were treated or not with 10 μM OHT, as indicated. c-Fos protein levels were detected by western blot analysis as described in panel **b**. (**d**) Western blot analyses of G protein-coupled receptor 30 (GPR30) levels in SkBr3 cells earlier transfected with either Control Sh or with ShGPR30. The β-actin antibody was used as loading control. (**e**) SkBr3 cells were treated or not for 5 min with 10 μM OHT alone or in combination with PD or SP, as indicated. Western blots were performed by using anti-phospho-specific p-ERK 1/2 antibodies, or anti-ERK 2 antibodies as indicated. (**f**) SkBr3 cells were transfected as described in panel (**c**) and then treated or not with 10 μM OHT for 5 min, as indicated. Detection of ERK phosphorylation was performed as described in panel (**e**).

cells through caspase 3 and JNK pathways (Mandlekar *et al*., 2000). We used the ER-negative breast cancer-derived cell line SkBr3 as a model system to analyse the role of JNK-activated c-Jun in the OHT-dependent apoptosis. First, we examined whether micromolar concentrations of OHT, which were earlier reported to be cytotoxic in SkBr3 cells (Basu *et al*., 2004), could induce hallmarks of apoptotic cell death such as DNA fragmentation and caspase 3/7 activation. As shown by flow cytometric analysis, DNA fragmentation was induced by 10 μM OHT and was further increased by 25 μM OHT, whereas neither concentration had any influence on cell cycle phases (Figures 1a and b). The effect of 10 μM OHT on DNA fragmentation was further confirmed by TdT-mediated dUTP nick end labeling (TUNEL) staining and cell viability assay (Figures 1c and d). Furthermore, OHT-induced caspase 3/7 activities (Figure 1e), suggesting that DNA fragmentation results from apoptosis. Next, we analysed whether JNK-activated c-Jun precedes OHT-dependent apoptosis in SkBr3 cells. To this end, we examined the ability of OHT to induce c-Jun N-terminal phosphorylation at either S73, a hallmark of c-Jun activation by JNK (Dunn *et al*., 2002), or at T91/T93, which are stringently correlated with c-Jun pro-apoptotic activity (Vinciguerra *et al*., 2008). Phosphorylation of both S73 and T91/T93 sites was induced on a 2 h exposure to OHT and no longer detected in presence of the JNK-specific inhibitor SP (Figures 2a and b), indicating that JNK-specific activation of c-Jun precedes OHT-induced apoptosis. In contrast, neither the EGFR inhibitor AG, nor the MEK/ERK inhibitor PD and the ER pure antiestrogen ICI prevented c-Jun phosphorylation by OHT. As OHT has been shown to induce JNK activation through generation of oxidative stress (Lee *et al*., 2000; Mandlekar and Kong, 2001; Mabuchi *et al*., 2004), we next analysed the effect of the antioxidant *N*-acetyl-L-cysteine (NAC) on the JNK/c-Jun pathway. As shown in Figure 2c, both activation of JNK and c-Jun phosphorylation at T91/T93 sites were prevented by NAC treatment. In line with previous studies, the exposure to NAC prevented also DNA fragmentation by OHT (Figure 2d). These results indicate that c-Jun pro-apoptotic phosphorylation by OHT is triggered by oxidative stress and is associated with DNA fragmentation.

OHT induces c-Fos expression in SkBr3 cells

In addition to c-Jun activation, we analysed whether OHT induces c-Fos expression in SkBr3 cells, at both messenger RNA and protein levels. As shown by either reverse transcription–PCR analysis or western blots, the expression of c-Fos was rapidly induced by OHT (Figures 3a and b). In agreement with our previous studies performed in different cancer cell types (Vivacqua *et al*., 2006a, b), c-Fos induction by OHT was sensitive to AG and PD but not to SP or ICI (Figure 3b). It is noteworthy that the effect of OHT on c-Fos expression was also abrogated silencing GPR30 expression (Figures 3c and d), which we have shown earlier to mediate c-Fos

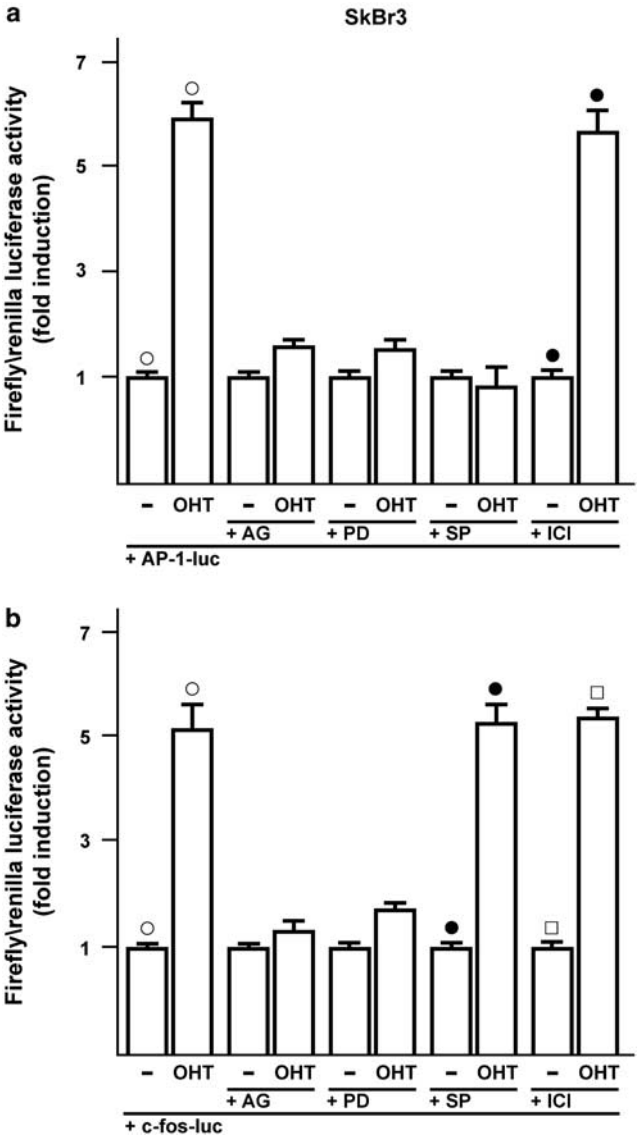


Figure 4 Both the activator protein-1 (AP-1)-responsive collagenase promoter and the c-Fos promoter are transactivated by 4-hydroxytamoxifen (OHT). (**a**) SkBr3 cells were transfected with the proximal AP-1-responsive collagenase promoter fused to a luciferase reporter gene and treated or not with OHT alone or in combination with AG 1478 (AG), or PD 98059 (PD), or SP 600125 (SP) or ICI 182 780 (ICI), as indicated. Renilla luciferase expression vector (pRL-TK) was used as a transfection control. The luciferase activities were normalized to the internal transfection control and values of cells receiving vehicle (−) were set as onefold induction on which the activity induced by 10 μM OHT was calculated. Error bars indicate s.d. of normalized luciferase activities of three independent experiments each performed in triplicates (±s.d.). ○, ●, □, $P<0.05$, for cells receiving vehicle (−) versus OHT. (**b**) SkBr3 cells were transfected with the luciferase reporter plasmid c-Fos-luc containing a 2.2-kb long upstream region of human *c-fos* gene together with Renilla luciferase expression vector. After transfection, cells were treated with vehicle (−) or 10 μM OHT, either alone or in combination with AG, PD, SP or ICI, as indicated. Luciferase activities were determined as described in panel (**a**). Error bars indicate s.d. of normalized luciferase activities of three independent experiments each performed in triplicates (±s.d.). ○, ●, □, $P<0.05$, for cells receiving vehicle (−) versus treatment.

induction by estrogens through ERK activation (Maggiolini *et al*., 2004; Vivacqua *et al*., 2006a, b). Accordingly, OHT-dependent activation of ERK was sensitive to either PD or abrogation of GPR30 expression (Figures 3e and f). In contrast, NAC treatment had no effect on either ERK activation or c-Fos expression, indicating that OHT induces the ERK/c-Fos pathway independently from its ability to trigger oxidative stress (Supplementary Figure 1). Taken together, these results suggest that OHT induces c-Fos expression through the GPR30–EGFR–ERK transduction pathway in SkBr3 cells.

OHT transactivates an AP-1-responsive promoter

Next, we evaluated whether OHT-dependent induction of c-Jun N-terminal phosphorylation and c-Fos expression lead to AP-1 transactivation. To this end, we tested the ability of OHT to activate the AP-1-responsive collagenase promoter fused to a luciferase reporter gene (AP-1-luc). As shown in Figure 4a, OHT-induced AP-1-luc transactivation, which was sensitive to either SP, AG or PD, but not to ICI. Furthermore, only AG and PD inhibited the transactivation of the c-Fos promoter by OHT (Figure 4b), hence corroborating the specific role exerted by ERK activation in OHT-induced c-Fos expression. Overall, these findings suggest that both JNK-specific activation of c-Jun and ERK-dependent induction of c-Fos expression contribute to AP-1 activation by OHT.

Abrogation of either c-Jun phosphorylation or AP-1 transactivation protects SkBr3 cells from OHT-induced apoptosis

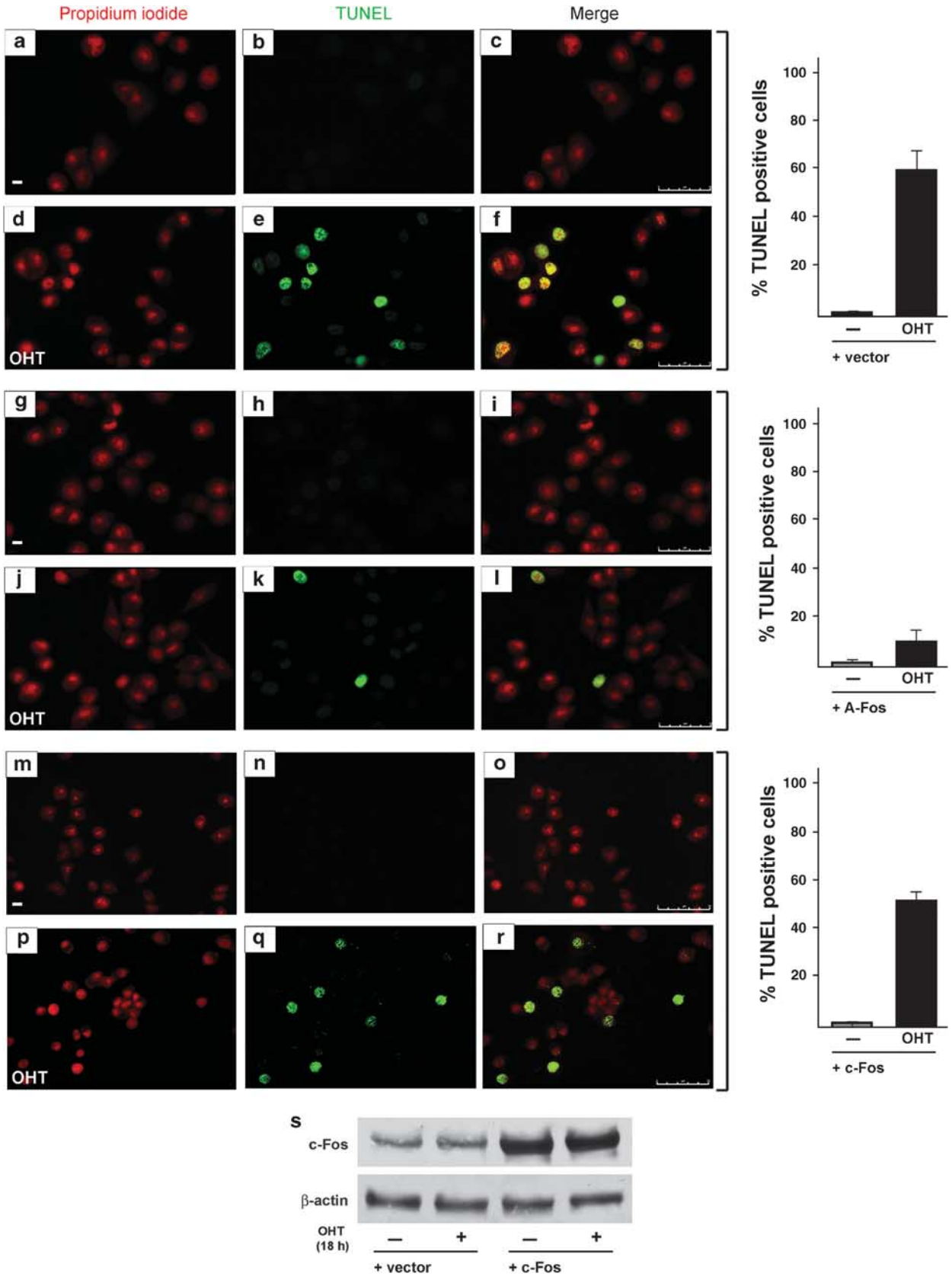
We then analysed the specific involvement of c-Jun/c-Fos AP-1 complex in OHT-induced apoptosis. To this aim, we performed TUNEL assays in OHT-treated cells ectopically expressing a truncated form of c-Fos (A-Fos), which was shown earlier to efficiently knockdown c-Jun/c-Fos AP-1 heterodimers (Olive *et al*., 1997). As shown in Figures 5a–f, approximately 60% of SkBr3 cells transfected with an empty vector resulted positive for TUNEL staining after exposure to OHT. In contrast, only 11% of A-Fos-transfected SkBr3 cells were TUNEL positive (Figures 5g–l), indicating that the c-Jun/c-Fos AP-1 complex is involved in OHT-induced apoptosis. As shown in Figure 5s, the constitutive expression of c-Fos slightly decreased the percentage of TUNEL-positive cells (Figures 5m–r), suggesting that c-Fos alone cannot sensitize SkBr3 cells to OHT-induced apoptosis. To further analyse the direct role of c-Jun activation in OHT-mediated apoptosis, we ectopically expressed in SkBr3 cells either a HA-tagged c-Jun-wt protein or a HA-tagged c-Jun mutant bearing alanine substitutions of S63/S73 and T91/T93 JNK sites (c-Jun-PanAla), which was shown to impair c-Jun activation as well as c-Jun-mediated apoptosis (Papavassiliou *et al*., 1995; Watson *et al*., 1998; Dunn *et al*., 2002). Expression of HA-tagged c-Jun-PanAla protected SkBr3 cells from OHT-induced apoptosis, as only 6% of HA-positive cells were also positive for TUNEL staining, while 58% of HA-negative cells were TUNEL positive (Figures 6i–p). In contrast, ectopic expression of c-Jun-wt had no effect on DNA fragmentation (Figures 6a–h), indicating the abrogation of JNK-specific phosphorylation of c-Jun is essential to protect SkBr3 cells from OHT-induced apoptosis. Furthermore, a c-Jun mutant that cannot be specifically phosphorylated at the T91/T93 JNK sites (Vinciguerra *et al*., 2008) was as efficient as c-Jun-PanAla to protect SkBr3 cells from OHT-dependent apoptosis (Figures 6q–x), showing that c-Jun N-terminal phosphorylation at T91/T93 site has a pivotal role in OHT-dependent apoptosis.

OHT induces c-Jun activation and c-Fos expression in different cancer cell types

To validate the role of AP-1 activation in OHT-induced apoptosis, we examined whether OHT couples the induction of both c-Fos expression and c-Jun phosphorylation to caspase 3/7 activation in different cancer cell types. As shown in Figures 7a and b, OHT stimulated both c-Fos expression and c-Jun phosphorylation in ER-negative BT20 and ER-positive MCF-7 breast cancer cells, HepG2 hepatocarcinoma cells, LoVo colorectal adenocarcinoma cells and SCLC-R1 lung cancer cells. As observed for SkBr3 cells (Figure 3), OHT-dependent induction of c-Fos was specifically abolished by AG and PD (Figure 7a), as well as by silencing GPR30 expression (Supplementary Figure 2). Furthermore, in all cell lines OHT-stimulated c-Jun N-terminal phosphorylation in a JNK-specific manner, because only SP abrogated c-Jun phosphorylation (Figure 7b). Similarly, OHT-induced caspase3/7 activity (Figure 7c) and DNA fragmentation (Supplementary Figure 3) in all cancer cell types, except for the OHT-resistant LNCaP prostate cancer cells. Remarkably, OHT also failed to induce either c-Fos expression or c-Jun phosphorylation in LNCaP cells (Figures 7d and e), corroborating the functional link between AP-1 activation and OHT-induced apoptosis. As our results indicate that activation of the JNK/c-Jun pathway by OHT is triggered by oxidative stress, we asked whether

Figure 5 c-Fos/activator protein-1 (AP-1) is required for 4-hydroxytamoxifen (OHT)-induced apoptosis in SkBr3 cells. (**a**–**r**) Representative images of TdT-mediated dUTP nick end labeling (TUNEL) staining of SkBr3 cells transfected with either an empty plasmid vector (**a**–**f**) or with a plasmid vector expressing the A-Fos mutant (**g**–**l**), or a plasmid vector expressing the c-Fos (**m**–**r**). After transfection, cells were treated or not with 10 μM OHT for 18 h as indicated. Nuclei were staining by Propidium iodide (red), or TUNEL (green) as indicated. Magnification is indicated by bars (50 μm). Each experiment shown is representative of 20 random fields observed. Bar graphs show the percentage of TUNEL-positive cells and represent the mean of three independent experiments with bars indicating s.d. (**s**) c-Fos ectopic expression in SkBr3-transfected cells corresponding to panel m-r was examined by western blot analysis by using a c-Fos-specific antibody, as indicated. A β-actin-specific antibody was used as a loading control.

the resistance of LNCaP cells to OHT-induced apoptosis correlated with a deficiency in ROS production and consequential lack of activation of the JNK/c-Jun pathway. To this end, we examined the ability of OHT to generate ROS in either SkBr3 or LNCaP cells by DCFH fluorescence (Shen *et al*., 1996). As show in



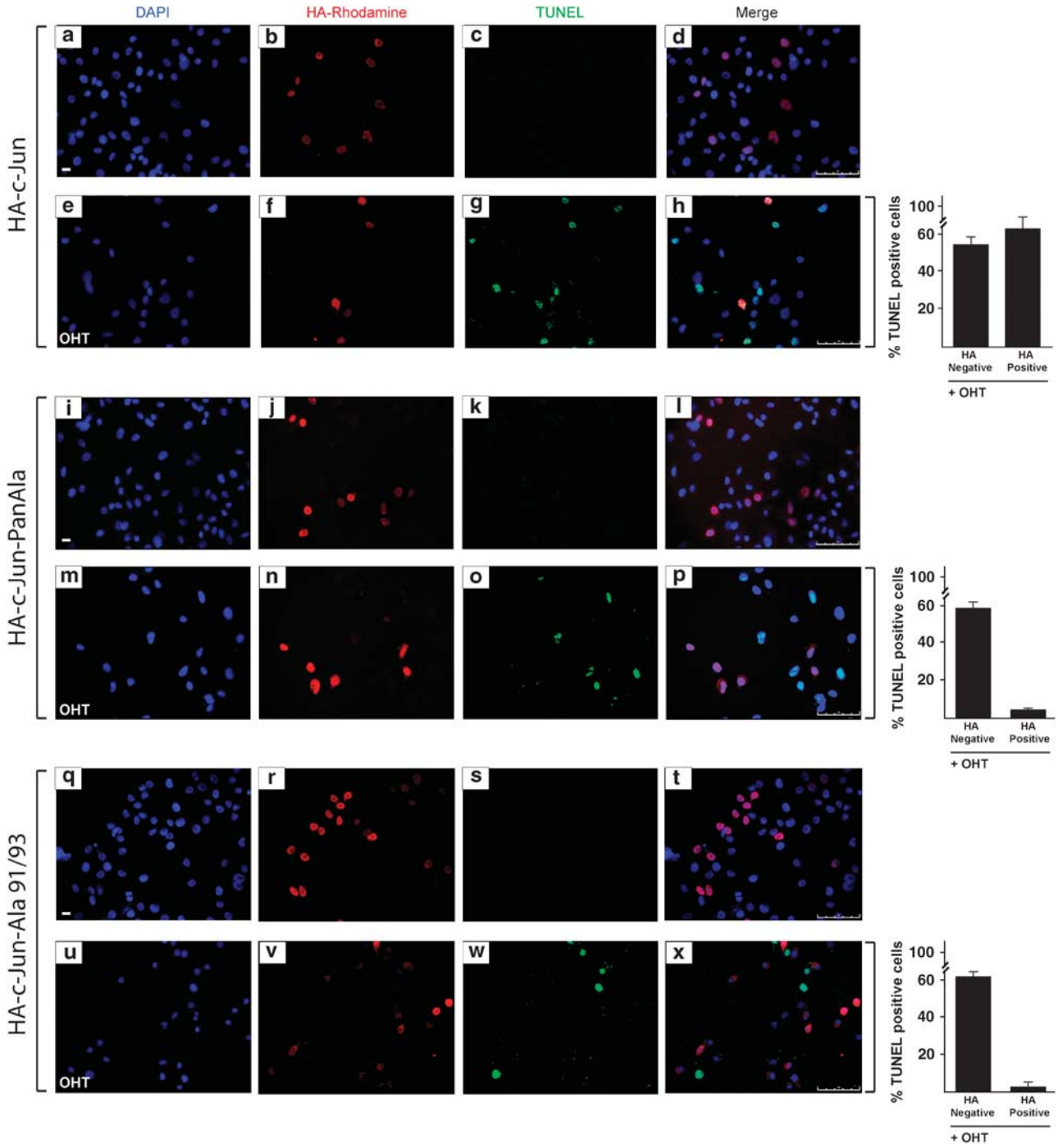


Figure 6 Lack of c-Jun N-terminal phosphorylation protects SkBr3 cells from 4-hydroxytamoxifen (OHT)-induced apoptosis. Representative images of TdT-mediated dUTP nick end labeling (TUNEL) and HA co-staining of SkBr3 cells ectopically expressing HA-c-Jun-wt (**a**–**h**), or HA-c-Jun-Pan-Ala (**i**-**p**), or Ha-c-Jun-Ala/91/93 (**q**–**x**). Transfected cells were treated with either vehicle or 10 μM OHT for 18 h, as indicated. Nuclei were staining by DAPI (blue), or HA (red), or TUNEL (green), as indicated. Magnification are indicated by the bars (50 μm). Each experiment shown is representative of 20 random fields observed. Bar graphs represent the percentage of TUNEL-positive cells in either HA-negative or HA-positive cells treated with OHT. Values represent the mean of three independent experiments with bars indicating s.d.

Figure 8, OHT increased the basal level of ROS in SkBr3 cells, but not in LNCaP cells, suggesting that the failure to generate ROS may contribute to protect LNCaP cells from OHT-dependent apoptosis.

Discussion

Accumulating evidence suggests that tamoxifen-induced apoptosis may consist in the generation of oxidative

stress and subsequent activation of the JNK family of stress-responsive MAPKs (Lee *et al*., 2000; Mandlekar and Kong, 2001; Mabuchi *et al*., 2004). c-Jun mediates proapoptotic functions of JNK in different types of cells including neurons, fibroblasts and cancer cells (Dunn *et al*., 2002; Hess *et al*., 2004; Raivich, 2008). However, c-Jun does not mediate all paradigms of JNK-dependent apoptosis and its pro-apoptotic function depends on the type of stimulus as well as on the heterodimeric partner forming the AP-1 transcription factor (Hess *et al*., 2004; Raivich and Behrens, 2006). In this study, we analysed whether JNK-dependent activation of c-Jun has a role in OHT-induced cell death of ER-negative SkBr3 breast cancer cells. To this end, we first established by fluorescence-activated cell sorting analyses the range of micromolar OHT concentrations required to induce DNA fragmentation (Figure 1), as also confirmed by TUNEL and cell viability assays (Figure 1). Importantly, we found that OHT triggers caspase 3/7 activity (Figure 1), suggesting that DNA fragmentation by OHT is a secondary consequence of apoptosis rather than necrosis. Moreover, OHT had no effects on cell cycle progression (Figure 1), excluding the possibility that OHT-dependent cytotoxicity was due to cell growth arrest.

Next, we analysed the ability of OHT to induce JNK-dependent phosphorylation of c-Jun at the S73 site, which is considered a hallmark of c-Jun activation in several paradigms of stress signals (Dunn *et al*., 2002). We detected both JNK activation (Figure 2) and c-Jun phosphorylation at S73 on a 2 h exposure to OHT (Figure 2). Remarkably, OHT also induced phosphorylation of c-Jun at T91/T93 sites (Figure 2), which was shown to be crucial for c-Jun-mediated cell death in response to either ultraviolet or etoposide (Vinciguerra *et al*., 2008). Moreover, both S73 and T91/T93 phosphorylations by OHT were specifically sensitive to SP, indicating that JNK-specific activation of c-Jun precedes apoptosis in SkBr3 cells. Interestingly, OHT-induced c-Jun N-terminal phosphorylation without alteration of c-Jun expression (Figures 2 and 7), suggesting that activation of c-Jun by OHT is limited to pre-existing c-Jun proteins. Accordingly, our (unpublished observations) and other studies (Nephew *et al*., 1993), have indicate that c-Jun expression is not regulated by OHT treatment. Importantly, DNA degradation as well as JNK activation and N-terminal phosphorylation of c-Jun, were abrogated by antioxidant treatments (Figure 2). These results suggest that generation of oxidative stress, presumably by NADPH activation (Lee *et al*., 2000), could be the initial OHT-dependent signal leading to activation of the JNK/c-Jun pathway and thereafter to apoptosis.

We then asked whether c-Jun activation by OHT was coupled to induction of c-Fos expression. Transactivation assays of the c-Fos promoter (Figure 4) combined with the analysis of steady-state levels of both c-Fos protein and messenger RNA (Figure 3) indicated that OHT leads to transcriptional induction of c-Fos expression. Interestingly, both the transactivation of the c-Fos promoter and the induction of c-Fos protein levels were sensitive to AG or PD, but not to SP or ICI, indicating that OHT induces c-Fos expression independently of its ability to induce JNK activity but rather in an EGFR/ERK-mediated manner. Accordingly, OHT-dependent activation of ERK was abrogated by PD but not by SP (Figure 3), confirming that JNK activity is not required for the signaling pathway upstream c-Fos expression. Moreover, both c-Fos expression (Figure 3) and ERK activation (Figure 3) by OHT were sensitive to GPR30 silencing, in accordance with our previous studies performed in different cancer cells (Maggiolini *et al*., 2004; Vivacqua *et al*., 2006a, b; Albanito *et al*., 2007). It is noteworthy that the ERK/c-Fos pathway was not altered by antioxidant treatment (Supplementary Figure 1), thus corroborating the fact that OHT initiates two distinct signaling pathways converging to activation of the c-Jun/c-Fos AP-1 complex.

Induction of c-Fos expression coupled to c-Jun N-terminal phosphorylation is the most frequent encountered paradigm of AP-1 activation in mammalian cells. Indeed, we found that OHT-induced AP-1 transactivation in an ERK and JNK-dependent manner (Figure 4), suggesting that the heterodimeric c-Jun/c-Fos AP-1 complex functions in OHT-induced apoptosis of cancer cells. Alternatively, because of the crucial role of AP-1 in a variety of cellular events, activation of c-Jun/c-Fos AP-1 complex by OHT may reflect a pro-survival response. To evaluate the specific involvement of c-Jun/c-Fos in OHT-dependent apoptosis, we abrogated c-Jun/c-Fos AP-1 activity in SkBr3 cells by two independent experimental approaches: (i) ectopic expression of A-Fos, a truncated form of c-Fos that has been shown to specifically knock-down c-Jun/c-Fos AP-1 heterodimers (Olive *et al*., 1997); and (ii) ectopic expression of c-Jun-PanAla, a c-Jun mutant bearing alanine substitution of all four JNK phosphorylation sites S63/S73 and T91/T93, which has been shown to act as a c-Jun dominant negative in several cell lines (Papavassiliou *et al*., 1995; Watson *et al*., 1998; Dunn *et al*., 2002). A-Fos overexpression protected cells from OHT-induced apoptosis, indicating that c-Jun/c-Fos heterodimers are directly involved in apoptosis. This finding was further corroborated by the evidence that AP-1 transactivation by OHT was dependent on both ERK and JNK activity (Figure 4). Similarly, overexpression of c-Jun-PanAla resulted in the protection of SkBr3 cells from OHT-induced apoptosis (Figure 6). As Jun-wt had no effect on DNA degradation (Figure 6), the protective effect of c-Jun-PanAla presumably results from its ability to act as dominant-negative, indicating that JNK-activated c-Jun is necessary to trigger OHT-induced apoptosis. Moreover, a c-Jun mutant that specifically cannot be phosphorylated at the T91/T93 JNK sites was as efficient as c-Jun-PanAla in protecting SkBr3 cells from OHT-dependent apoptosis, suggesting that phosphorylation of c-Jun at S63/S73 is not sufficient to trigger c-Jun-dependent apoptosis. It is noteworthy that ectopic expression of c-Jun could not significantly increase the number of TUNEL-positive cells (Figure 6), indicating that activation of pre-existing c-Jun proteins by OHT is sufficient to trigger apoptosis.

Our data show that OHT induces both ERK/c-Fos and JNK/c-Jun pathways in various OHT-sensitive cancer cells (Figure 7), pointing to c-Jun/c-Fos AP-1 activation as a general mechanism mediating OHT-dependent apoptosis, rather than a peculiar response of SkBr3 cells. Accordingly, OHT-induced caspase 3/7 activity and DNA degradation in all cancer cell types used, except in OHT-resistant LNCaP prostate cancer cells (Figure 7). It is noteworthy that OHT failed to induce both c-Fos expression and c-Jun N-terminal phosphorylation in LNCaP cells (Figure 7), validating that the upregulation of c-Jun/c-Fos AP-1 by cytotoxic concentrations of OHT is linked to caspase 3/7 activation and in turn to cell death.

How c-Jun/AP-1 drives cell death in OHT-treated cancer cells remains to be elucidated. The JNK/c-Jun pathway has been shown to regulate the release of cytochrome c from mitochondria (Whitfield *et al*., 2001). In line with this observation, active c-Jun is required for the expression of genes that increase mitochondrial outer membrane permeability, such as Bim and Dp5, which are members of the proapoptotic BH3-only subfamily of Bcl-2 proteins (Ham *et al*., 2005). A crucial role of mitochondria has been also shown in OHT-induced apoptosis, as it can elicit the release of mitochondrial cytochrome c altering the expression of Bcl-2 proteins (Mandlekar and Kong, 2001; Kallio *et al*., 2005; Lagadec *et al*., 2008). Therefore, down-regulation of the antiapoptotic Bcl-2 protein together with upregulation of one or more proapototic Bcl-2 proteins may constitute a critical mechanism promoting mitochondrial dysfunction by OHT. Presumably, the JNK/c-Jun pathway may contribute to this intrinsic proapoptotic mechanism by upregulating one or more proapototic BH3-only proteins. In contrast, as c-Jun/AP-1 controls tumor necrosis factor-α expression (Liu *et al*., 2000; Pope *et al*., 2000), we cannot exclude a putative role of c-Jun in a cross talk between the death receptor and mitochondrial pathways.

Our previous studies have shown that one micromolar OHT induces cell proliferation in thyroid and endometrial cancer cells by activating the EGFR/GPR30/ERK pathway and in turn c-Fos expression (Vivacqua *et al*., 2006a, 2006b), whereas it has no effects on the JNK/c-Jun pathway (Madeo A, unpublished observations). These observations together with this study lead to the hypothesis that the functional output of c-Fos induction by OHT may depend on whether or not there is a concomitant c-Jun activation by JNK signaling. As antioxidant pathways are often enhanced in cancer cells (Friesen *et al*., 2004; Young *et al*., 2004; Recktenwald *et al*., 2008; Ballatori *et al*., 2009), the resistance of certain cancer cells to tamoxifen may depend on their ability to prevent ROS accumulation and in turn JNK-specific activation of c-Jun. In line with this hypothesis we found that OHT induced ROS accumulation in SkBr3 cells but not in LNCaP cells (Figure 8). Hence, pharmacological boosts of c-Jun N-terminal phosphorylation may be useful to sensitize cancer cells to OHT-mediated cell death.

Materials and methods

Cell culture

SkBr3 breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% fetal bovine serum (FBS). MCF-7 and BT20 breast cancer cells were cultured respectively in DMEM-F12 or MEM with phenol red supplemented with 10% FBS. HePG2 hepatocarcinoma cells, LoVo colorectal adenocarcinoma cells and LNCaP prostate cancer cells were maintained in RPMI 1640 with phenol red and supplemented with 10% FBS. SCLC-R1 lung cancer cells were cultured in RPMI 1640 with phenol red, one $\times$ non-essential amino acids, one $\times$ sodium pyruvate and 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots and reverse transcription–PCR.

Drugs and chemicals

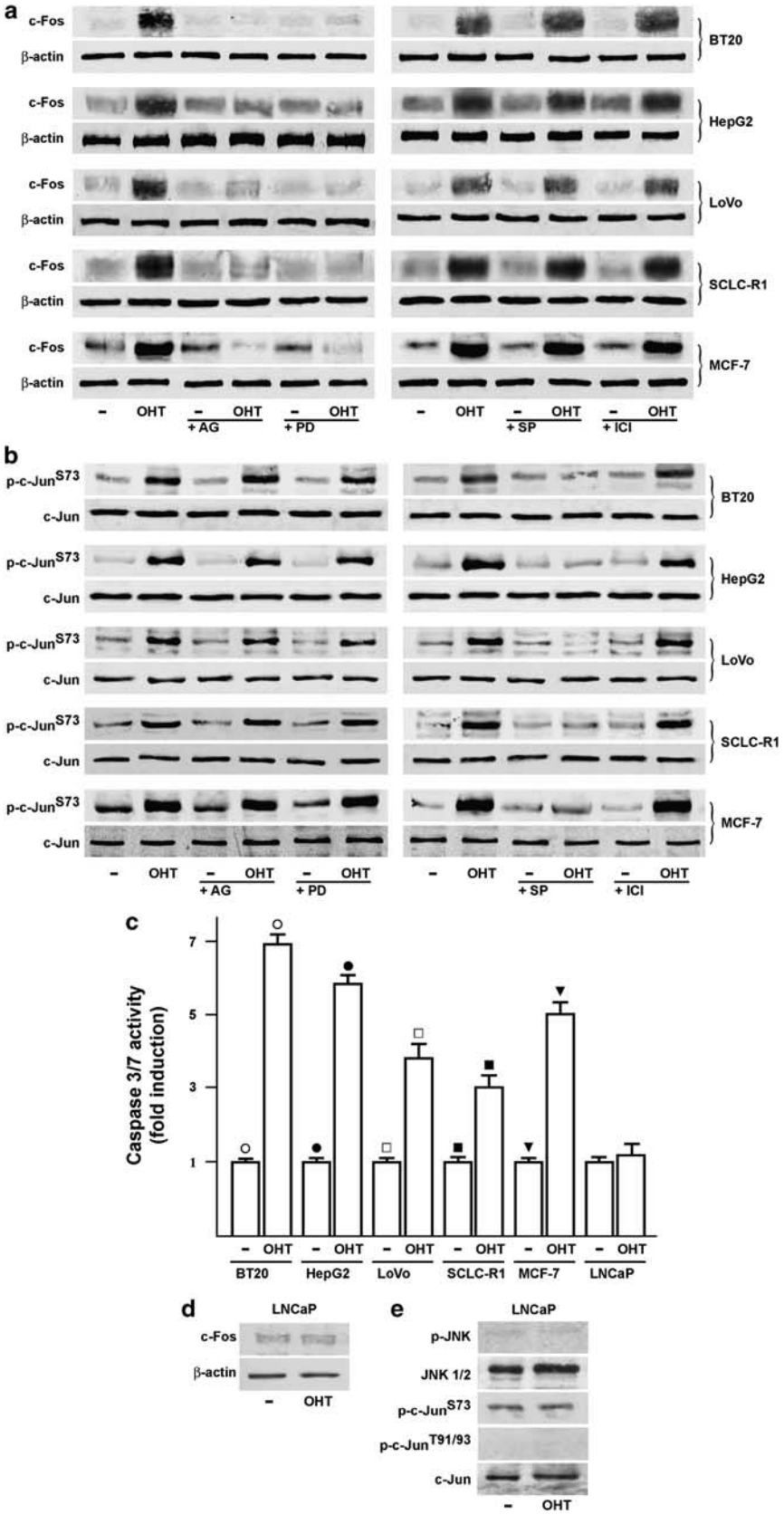
4-hydroxytamoxifen, PD 98059 (PD), SP 600125 (SP), NAC were purchased from Sigma-Aldrich Corporation (Milan, Italy). ICI 182 780 (ICI) was obtained from Tocris Chemicals (Bristol, UK), AG 1478 (AG) was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). All compounds were solubilized in dimethyl sulfoxide (DMSO), except for OHT that was dissolved in ethanol (EtOH) and N-acetyl-L-cysteine (NAC) that was dissolved in H_2O. For TUNEL assays and immunofluorescent detection experiments propidium iodide and DAPI were purchased from Sigma-Aldrich Corporation.

Plasmids

The short hairpin RNA construct to knock-down the expression of GPR30 and the unrelated short hairpin RNA control construct have been described earlier (Albanito *et al*.,

Figure 7 Inducton of c-Jun phosphorylation and c-Fos expression by 4-hydroxytamoxifen (OHT) is associated with caspase 3/7 activity in different cancer cell types. (**a**) BT-20, HepG2, LoVo, SCLC-R1 and MCF-7 cells were treated with vehicle (−) or 10 μM OHT for 2 h, either alone or in combination with AG 1478 (AG), or PD 98059 (PD), or SP 600125 (SP) or ICI 182 780 (ICI), as indicated. Western blots were performed by using anti-c-Fos antibodies to detect c-Fos protein levels, or with anti β-actin antibodies as loading control. (**b**) c-Jun phosphorylation (p-c-Jun^{S73}) in the various cell lines and treatments described in panel (**a**). Western blot analyses were performed as described in Figure 2a. (**c**) Caspase 3/7 activity in OHT-treated cancer cell lines. Bar graph shows a representative experiment with means of triplicate samples. Caspase activity was normalized to the control and values of cells receiving vehicle (−) were set as onefold induction on which the activity induced by OHT was calculated. Error bars show ± s.d. ○, ●, □, ■, ▼, $P<0.05$, for cells receiving vehicle (−) versus OHT. (**d**) LNCaP cells were treated either with vehicle or 10 μM OHT for 2 h, then c-Fos expression was detected by western blot by using anti-c-Fos antibodies, or β-actin antibodies as a loading control. (**e**) LNCaP cells were treated with vehicle or 10 μM OHT for 2 h, c-Jun N-terminal kinase (JNK) activation and c-Jun phosphorylation at threonine 91 and 93 (T91/T93) and serine 73 (S73) were detected by western blot analysis using phospho-specific antibodies for p-JNK (T183/Y185) or for p-c-Jun$^{T91/T93}$, or p-c-Jun^{S73}, respectively. Expression levels of JNK and c-Jun proteins were detected using the non-phospho-specic antibodies, as indicated.

2008). The expression vector encoding for c-Fos protein was described earlier (Papavassiliou *et al*., 1992). The plasmid A-Fos was a kind gift from Dr C Vinson (NIH, Bethesda, MD, USA). The expression vector encoding for c-Jun protein (HA-c-Jun) and a non-functional c-Jun protein mutated in phosphorylation sites (HA-c-Jun-PanAla and HA-c-Jun-Ala



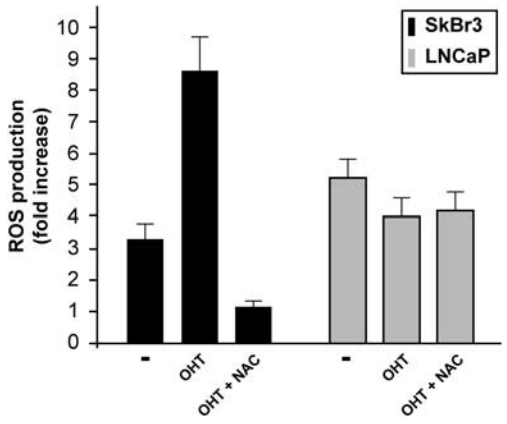


Figure 8 4-hydroxytamoxifen (OHT) generates reactive oxygen species (ROS) in SkBr3 cells but not in LNCaP cells. Cells were treated for 2 h with either vehicle, or 10 μM OHT, or 10 μM OHT and 10 mM *N*-acetyl-L-cysteine (NAC), as indicated. ROS were measured by flow cytometry as described in materials and methods. Results are expressed as fold increase compared with the initial DCF fluorescence intensity. Values represent the mean of three independent experiments with bars indicating s.d.

91/93) have been described earlier (Musti *et al*., 1997). The luciferase reporter plasmid for c-fos encoding a −2.2 kb 5′ upstream fragment of human c-fos was a kind gift from K Nose, Showa University, Tokyo, Japan. The luciferase reporter plasmid for 4 × AP-1-responsive collagen promoter was a kind gift from H van Dam (Department of Molecular Cell Biology, Leiden University, Leiden, The Netherlands).

Flow cytometry
Evaluation of cell-cycle distribution and subG1 peaks by flow cytometric analysis was performed as follows: floating and adherent cells were collected by trypsin/ethylene-diaminetetraacetic acid, washed in cold phosphate-buffered saline (PBS) and fixed with 70% cold ethanol for 1 h. After removing ethanol by PBS washes, cells were incubated in 10 mg/ml deoxyribonuclease-free ribonuclease A for 30 min at 37 °C and then in 50 mg/ml Propidium iodide for 20 min at 4 °C. Cells were then analysed by flow cytometry using a FacScan Flow Cytometer Apparatus (488 nm argon laser-CyAn ADP, Dako, Milan, Italy). Acquisition and analysis was performed using Summit software. The analysis on dot-plot of FL2 area versus FL2 width gating was performed to exclude doublets from G2M region. For each sample, 30 000 events were analysed.

Transfection and luciferase assay
SkBr3 cells (1×10^5) were plated in 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 500 ng of AP-1-luc or c-fos-luc promoter plasmids and 1 ng of pRL–cytomegalovirus. After 6 h, 500 μl of normal growth medium, and treatments were added to wells and then cells were incubated for an additional 18 h. Luciferase activity was measured using the Dual Luciferase Kit (Promega, Milan, Italy) 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 onefold induction on which the activity induced by treatments was calculated.

Immunoblotting
Cells were grown in 10-cm dishes, exposed to treatments and then lysed in 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0,1% odium dodecyl sulfate, and a mixture of protease inhibitors (Boeringher Ingelheim, Milan, Italy). Equal amounts of whole protein extract were resolved on a 10% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Amersham Biosciences, Arlington Heights, IL, USA), probed overnight at 4 °C with the antibodies against the indicated proteins and then revealed using the enhanced chemiluminescence system (Amersham Biosciences). For GPR30 silencing, cells were plated into 10-cm dishes, maintained in serum-free medium for 24 h, and then transiently transfected for an additional 24 h before treatments with 5 μg/plate short hairpin GPR30 or with the control empty vector. Transfections were performed using Fugene 6 reagent. For c-Fos overexpression, cells were plated into 10-cm dishes, maintained in normal growth medium and then transiently transfected in FBS 5% for 6 h before treatment with 10 μM OHT for additional 18 h. Transfections were performed using Fugene 6 reagent. GPR30 was revealed using the rabbit polyclonal antiserum LS-A4271 (MBL-Eppendorf, Milan, Italy). c-Jun, p-c-Jun^{S73}, c-Fos, p-ERK 1/2, ERK 2, β-actin (all purchased from Santa Cruz Biotechnology, Milan, Italy), p-c-Jun$^{T91/T93}$ (Abcam, Milan, Italy), p-JNK, JNK 1/2 (Cell Signaling Technology, Milan, Italy) were revealed by immunoblotting with the appropriate mouse monoclonal antibody sc-8432 or the rabbit polyclonal antibody sc-2004 (Santa Cruz Biotechnology).

Reverse transcription and real-time PCR
Total RNA was extracted using the TRIzol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol and reversed messenger RNA was quantified spectrophotometrically. Total complementary DNA was synthesized from the RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Invitrogen) following the protocol provided by the manufacturer. The expression of *c-fos* was quantified by real-time PCR using StepOne sequence detection system (Applied Biosystems Inc., Milan, Italy) following the manufacturer's instructions. Assays were performed in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc.). For *c-fos* and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5′-CGAGCCCTTTGATGACTTCCT-3′ (*c-fos* forward); 5′-GGAGCGGGCTGTCTCAGA-3′ (*c-fos* reverse); 5′-GGCGTCCCCCAACTTCTTA-3′ (*18S* forward) and 5′-GGGCATCACAGACCTGTTAT-3′ (*18S* reverse).

Immunofluorescence and TdT-mediated dUTP nick end labeling assays
Cells were seeded in two-well Lab-Tek II chamber slides at a density of 1×10^5/well and incubated for 24 h in the corresponding maintenance media. Using Fugene 6 reagent as recommended by the manufacturer, cells were transiently transfected in FBS 5% for 6 h with an empty vector, the c-Fos, A-Fos and the HA-tagged c-Jun, c-Jun-PanAla (bearing alanine substitutions of all JNK sites) and c-Jun-Ala 91/93

(c-Jun mutant in T91/T93) expression vectors. Cells were then treated for 18 h with vehicle or 10 μM OHT.

For immunofluorescence staining, cultured SkBr3 cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% TWEEN three times for 5 min, then were blocked for 1 h at room temperature with PBS containing 15% normal donkey serum (Santa Cruz Biotechnology, DBA, Milan, Italy), 0.1% Triton X-100 and 0.05% TWEEN. After incubations with a primary antibody against HA (HA-probe F-7 purchased from Santa Cruz Biotechnology, DBA, Milan, Italy) in PBS containing 15% normal donkey serum, 0.1% Triton X-100 and 0.05% TWEEN, slides were washed three times with PBS and incubated with the donkey secondary antibody against mouse IgG conjugated to rhodamine (1:300) (Santa Cruz Biotechnology, DBA).

For the detection of DNA fragmentation at the cellular level, cells were stained using DeadEnd Fluorometric TUNEL System (Promega) following the manufacturer's instructions. Nuclei of cells were stained with propidium iodide for the TUNEL assay or DAPI for immunofluorescent detection in combined TUNEL assay. Leica AF6000 Advanced Fluorescence Imaging System supported by quantification and image processing software Leica Application Suite Advanced Fluorescence (Leica Microsystems CMS, GbH Mannheim, Germany) were used for experiment evaluation.

Determination of caspase 3/7

Caspase 3/7 activation was determined using the Caspase-Glo 3/7 Assay (Promega) following the protocol provided by the manufacturer. Cells were plated in regular growth medium. Cancer cells were treated in FBS 5% with 10 μM OHT for 18 h and then incubated with Caspase-Glo 3/7 Reagent for 3 h. Thereafter, the caspase activity was determined by luminometer (Lumat LB 9507 Berthold, Pforzheim, Germany).

Dichlorofluorescein assay

DCFH–DA was dissolved in dimethyl sulfoxide as stock solution and kept frozen in 20 °C. For loading the cells with DCFH, DCFH–DA from stock solution was mixed with loading medium (99% RPMI 1640 and 1% FBS) to a final concentration of 100 M. Adherent cells were collected by trypsin/ethylene-diaminetetraacetic acid, washed for two times in cold PBS and then incubated in 1-ml solution containing PBS and DCF for 30 min at 37 °C in the dark. After removing DCF by PBS wash, cells were incubated in 1 ml PBS solution, in ice, and then analysed by flow cytometry using a FacScan Flow Cytometer Apparatus (Dako). Acquisition and analysis was performed using SummitTM software. For each sample, 30 000 events were analysed.

Cell viability

For cell viability assay, cells were seeded (2×10^4) in 24-well plates in normal growth medium supplemented with 10% FBS. Cells were washed extensively once then had attached and treated with 10 μM OHT for 18 h in the medium supplemented with 5% FBS. Then, cells were trypsinized and the numbers of Trypan blue excluding viable cells were counted in a haemocytometer (Strober, 2001).

Statistical analysis

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

Conflict of interest

The authors declare no conflict of interest.

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Negative charged threonine 95 of c-Jun is essential for c-Jun N-terminal kinase-dependent phosphorylation of threonine 91/93 and stress-induced c-Jun biological activity

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Abstract

Activation of c-Jun, a major component of the AP-1 transcription factor, represents a paradigm for transcriptional response to stress. Transactivation of c-Jun is regulated by Jun-N-terminal kinases (JNKs) through phosphorylation at serine 63 and 73 (S63/S73), as well as at threonine 91 and 93 (T91/T93). How these two groups of phosphoacceptor sites respond to different grades of genotoxic stress and whether DNA-damage pathways influence the extent of their JNK-dependent phosphorylations remain to be elucidated. Here, we show that following a short exposure to the DNA-damaging compound etoposide, c-Jun phosphorylation is restricted to S63/S73. In contrast, JNK-dependent phosphorylation of T91/T93 requires continuous exposure to the drug and is impaired by caffeine treatment or alanine substitution of the adjacent threonine 95 (T95). Conversely, c-Jun mutations switching the T95/Q96 site into a canonical site for mitogen activated protein kinase (MAPK) phosphorylation (T95/P96) rescues T91/T93 phosphorylation in presence of caffeine, suggesting that a preceding phosphorylation at T95 exposes T91/T93 to JNK-dependent phosphorylation. Moreover, we show that alanine substitution at T95 impairs c-Jun transactivation and c-Jun-mediated cell death, indicating that negatively charged T95 is a general constraint for c-Jun activation. Hence, our study suggests that c-Jun may sense the strength of genotoxic stress through DNA-damage dependent phosphorylation of T95, which in turn augments c-Jun transactivation by JNKs.

Keywords: c-Jun; JNK; DNA damage; Phosphorylation; Etoposide

Abbreviations: AP-1, activator protein 1; MAPK, mitogenic activated protein kinase; EGF, epidermal growth factor; IP3, inositol-1,4,5-trisphosphate; PIKK, phosphatidylinositol kinase-related protein kinase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; Chk1, checkpoint kinase 1; Chkk2, checkpoint kinase 2; HA, hemagglutinin; MKK7, MAPK kinase 7; MAPKK, MAPK kinase

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1. Introduction

c-Jun is the prototypical member of the Jun family of nuclear factors. By forming stable homodimer or heterodimer complexes with Fos or ATF family members it constitutes the AP-1 transcription factor (Shaulian & Karin, 2001). Genetic and biochemical studies have shown that the biological activity of c-Jun mainly relays on JNK-dependent phosphorylation of its N-terminal domain at S63/S73 (Eferl & Wagner, 2003; Shaulian & Karin, 2001). Phosphorylation of S63/S73 is required for c-Jun-mediated cellular transformation, tumor progression and cancer development (Behrens, Jochum, Sibilia, & Wagner, 2000; Nateri, Spencer-Dene, & Behrens, 2005; Smeal, Binetruy, Mercola, Birrer, & Karin, 1991). Abrogation of S63/S73 phosphorylation reduces fibroblast survival in response to UV radiation (Wisdom, Johnson, & Moore, 1999) and leads to chemoresistance acquisition in various tumor cells to DNA-damaging drugs (Potapova, Basu, Mercola, & Holbrook, 2001). These studies suggest that when phosphorylated at S63/S73, c-Jun augments DNA repair, as also evidenced by the accumulation of spontaneous DNA damage occurring in presence of the genetic ablation of c-Jun (MacLaren, Black, Clark, & Gillespie, 2004). Moreover, several studies have shown that c-Jun phosphorylation at S63/S73 is required for neuronal apoptosis (Dunn, Wiltshire, MacLaren, & Gillespie, 2002; Raivich & Behrens, 2006; Shaulian & Karin, 2001). Subsequent investigations have revealed that c-Jun is phosphorylated by a variety of stress and pro-inflammatory signals on a second group of MAPK sites at T91/T93 (Morton, Davis, McLaren, & Cohen, 2003; Papavassiliou, Treier, & Bohmann, 1995) involved in c-Jun transactivation (Weiss et al., 2003). In this context, genetic analyses have shown that both groups of MAPK sites require JNK expression in order to be phosphorylated in response to stress or pro-inflammatory stimuli (Morton et al., 2003). Conversely, in absence of JNK expression, EGF can induce phosphorylation at S63/S73 but not at T91/T93 (Morton et al., 2003), indicating that T91/T93 phosphorylation is specifically up-regulated by stress and pro-inflammatory signals. In line, multisite phosphorylation of c-Jun on all four MAPK sites has been detected during chronic hypoxia (Laderoute et al., 2002), neuronal apoptosis by tropic factor deprivation (Hongisto et al., 2003) and UV-induced apoptosis of different types of mammalian cells (Hamdi et al., 2005; Ui et al., 1998). Altogether, these studies suggest that different physiological contexts may elicit distinct patterns of c-Jun N-terminal phosphorylation, which in turn correlate with diverse biological output of c-Jun activation.

In the present study we assessed this hypothesis by investigating whether different grades of genotoxic stress could induce distinct N-terminal phosphorylation of c-Jun. As a experimental model system, we used a differential exposure of human embryonic kidney 293 (HEK-293) cells to etoposide, a DNA toposoimerase II inhibitor responsible for both double and single DNA strand breaks (Cliby, Lewis, Lilly, & Kaufmann, 2002; Costanzo et al., 2003). By damaging DNA, etoposide triggers the cellular response to genotoxic stress consisting in a complex network of signaling pathways designed to induce the onset of cell cycle delay and DNA repair (Sancar, Lindsey-Boltz, Unsal-Kacmaz, & Linn, 2004). Activation of IP3 kinase family members ATM and ATR is essential for initiating DNA damage pathways in response to almost all genotoxic stress including etoposide. Upon genotoxic stress, ATM and ATR activate a network of damage-response leading to activation of the checkpoint kinases, Chk1 and Chk2, and DNA repair proteins (Sancar et al., 2004). If DNA damage persists and unrepaired DNA accumulates, the same network may lead to the induction of apoptotic programs (Roos & Kaina, 2006). Etoposide-mediated activation of the DNA damage response can be abrogated by caffeine (Clifford, Beljin, Stark, & Taylor, 2003; Costanzo et al., 2003), an inhibitor of ATM and ATR kinases (Sarkaria et al., 1999), able to reverse cell cycle checkpoint function in response to a wide number of genotoxic stress signals (reviewed in Kaufmann et al., 2003).

In the present study, we found that c-Jun is promptly phosphorylated on S63/S73 following a short exposure to etoposide. In contrast, a continuous exposure to the drug induces c-Jun phosphorylation at T91/T93, an event that is caffeine-sensitive and linked to an intact threonine 95 (T95). Furthermore, the stress-induced biological activity of c-Jun is impaired by alanine substitution of T95, pointing to T95 as a novel regulatory site integrating the strength of genotoxic stress with the output of c-Jun activity.

2. Materials and methods

2.1. Cell culture and transfections

Human embryonic kidney cells (H-293) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) (Life Technology). All media were supplemented with 2mM glutamine, 100 IU/ml penicillin-G and 100 μg/ml of streptomycin (Life Technology). For transfections, HEK-293 cells were seeded at 8×10^5 cells/100 mm plate and transfected with the indicated plasmids DNA

on the following day either by Lipofectamine (Invitrogen) or Fugene-6 (Roche-Diagnostics) reagents as recommended by manufacturers. Transfected cells were harvested 36–48 h after transfection.

2.2. Plasmids

The expression vectors for Ha-c-Jun, c-Jun–wt/Gal4 and c-Jun-PanAla/Gal4 have been previously described (Vinciguerra et al., 2004). Briefly, the expression plasmid for the Gal4-cJun was generated by subcloning a DNA fragment corresponding to a Zip-truncated form of human c-Jun (amino acid residues 1–266) into a mammal expression vector in frame with an Ubiquitin-promoter driven Gal4 DNA binding domain. HA-tagged c-Jun mutants, as well as Gal4-cJun mutants were generated by PCR-based oligonucleotide-directed mutagenesis system as previously described (Vinciguerra et al., 2004). EGFP expressing plasmid used in cell death assays was purchased by Quiagen.

2.3. Luciferase assays

Expression plasmids expressing Gal4-fused protein were cotransfected with the reporter plasmid Gal4-luciferase, and a *Renilla* luciferase expression vector pRL-CMV (Promega) in H-293 cells. Transient transfections were performed using Fugene-6 reagent as recommended by the manufacturer (Roche-Diagnostics). After transfection, cells were incubated for 16 h and luciferase activity was measured by using a Dual Luciferase Kit (Promega) according to the recommendations of the manufacturer. Firefly luciferase activity was normalized to the internal transfection control provided by the *Renilla* luciferase signal.

2.4. Immunoblotting

Cell extracts from HEK 293 cells were prepared in a buffer containing 20 mM Tris HCl (pH 8.0), 150 mM NaCl, 10mM NaF, 1 μM Na_3VO_4, 0.1% SDS and Protease inhibitors (Boehringer Mannheim). Samples were then boiled in SDS-sample buffer for 5 min, and the proteins were separated on 10% SDS-PAGE gels and detected by western-blot as previously described (Vinciguerra et al., 2004). HA-tagged proteins were detected by using an anti-Ha mouse monoclonal antibody (Santa Cruz). Multisite phosphorylation of c-Jun was detected using phospho-specific (P)-c-Jun antibodies that specifically recognise c-Jun when it is phosphorylated at serine 63 (Cell Signal), or serine 73 (Cell Signal), or threonine 91–93 (Abcam). P-anti-JNK1–2, P-anti-ATM (S1891), P-anti-Chk1 (S345), P-anti Chk2 (T65) were all purchased from Cell Signal. Antibodies recognizing total JNK1–2 and ATM were purchased from Santa Cruz. Antibodies recognizing total Chk1 and Chk2 were purchased from Cell Signal. All antibodies were used according to the manufacturer's recommendations. Immunocomplexes were detected using horseradish peroxidase-conjugated secondary antibody followed by ECL (Amersham).

2.5. Cell death assay

Evaluation of subG1 peaks by flow cytometry analysis was performed as follows: floating and adherent cells were collected by trypsin/EDTA, washed in cold PBS and fixed with 70% cold ethanol for 30 min. Ethanol was removed by PBS washes and cells were incubated in 50 mg/ml propidium iodide, 10 mg/ml deoxyribonuclease-free ribonuclease A for 2 h at 4 °C. Cells were then analysed by flow cytometry using a FacScan Flow Cytometer Apparatus (488 nm argon laser-Becton Dickinson, Mountain View, CA, U.S.A.). Acquisition and analysis was performed with CellQuest (version 3.3 Becton Dickinson) software on MacIntosh G3 workstation. The percent of sub-diploid cells was calculated by dividing the number of cells displaying a red fluorescence FL2 area (used band pass filter 585 nm was used to collect the signal of fluorescence emitted by propidium iodide) on dot histogram smaller than the G0–G1 diploid peak, by the total number of collected cells × 100. The analysis on dot-plot of FL2 area versus FL2 width gating was performed to exclude doublets from G2M region. For each sample, 20,000 events were analysed. For experiments shown in Fig. 1B, after treatments, cell were tripsinized and collected by centrifugation, washed twice with PBS/pH7.4 and then incubated in PBS containing 50 mg/ml Propidium iodide (PI) at 37 °C for 30 min. Cell death was measured by counting PI stained cells by flow cytometric analysis on a Becton Dickinson Facscan, Montain View, CA).

3. Results

3.1. c-Jun N-terminal domain is differentially phosphorylated by genotoxic stress

To investigate whether c-Jun N-terminal domain is differentially phosphorylated by different grades of genotoxic stress, we firstly characterized the range of cellular stress generated by either short or long exposure of HEK-293 cells to etoposide. As shown by the levels of Chk1 and Chk2 phosphorylation (Fig. 1A, line

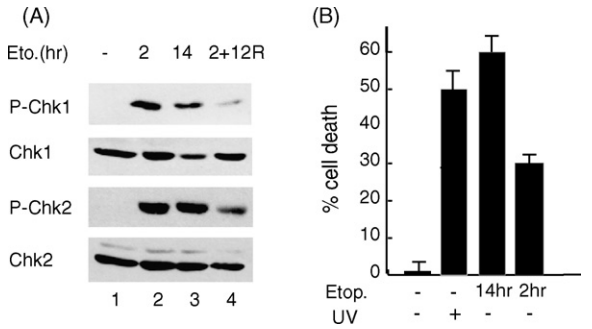

Fig. 1. Checkpoint signaling and cell death by etoposide treatments. (A) HEK-293 cells were treated with vehicle (line 1), treated with 5 μM etoposide for either 2 h (line 2) or 14 h (line 3), treated for 2 h and then incubated for further 12 h after removal of the drug (line 4). Western blot analysis was performed by using phospho-antibodies specific for Chk1 (S345) and Chk2 (T65), and with non-phospho-specific antibodies, as indicated. (B) HEK-293 cells were treated with UV (50 J/m^2), with etoposide (5 μM) for either 2 or 14 h, as indicated; after treatments cells were incubated up to 72 h in absence of etoposide or UV radiation. Cell death was measured by flow cytometric analysis of propidium iodide stained cells. Error bars indicate S.D. of three independent experiments.

3), prolonged exposure to etoposide led to sustained activation of DNA-damage signaling and induced cell death as effectively as UV radiation (Fig. 1B). Conversely, removal of etoposide turned down both Chk1 and Chk2 phosphorylation (Fig. 2A, line 4) and reduced the percent of cell death (Fig. 1B). Next, we analysed site-specific phosphorylation of c-Jun by using phospho-specific antibodies for S63/S73 and T91/T93. As show in Fig. 2A (line 3), prolonged exposure to etoposide led to JNK activation and c-Jun phosphorylation on all four S63/S73 and T91/T93 phosphoacceptor sites. Interestingly, c-Jun phosphorylation was restricted to S63/S73 following removal of etoposide, although JNK phosphorylation remained elevated (Fig. 2A, line 4). Notably, phospho-specific antibodies for either c-Jun-S63 or c-Jun-S73 detected a shifted phosphorylated form of c-Jun, previously shown to be phosphorylated at all four JNK sites (Ui et al., 1998), only after prolonged treatment with etoposide (Fig. 2A, line 3). These results suggest that an additional signal, triggered by persisting genotoxic stress, is necessary for T91/T93 phosphorylation. To assess this hypothesis and whether a kinase different than JNK may lead to T91/T93 phosphorylation, we treated cells either with the JNK-dependent inhibitor SP600125 or caffeine, which has been previously shown to block etoposide-mediated activation of checkpoint signaling pathways (Clifford et al., 2003; Costanzo et al., 2003). As shown in Fig. 2B, T91/T93 phosphorylation resulted sensitive to either drug, indicating that persisting DNA damage triggers a further signal required for JNK-dependent phosphorylation of T91/T93. Notably, caffeine inhibited ATM autophosphorylation, known as

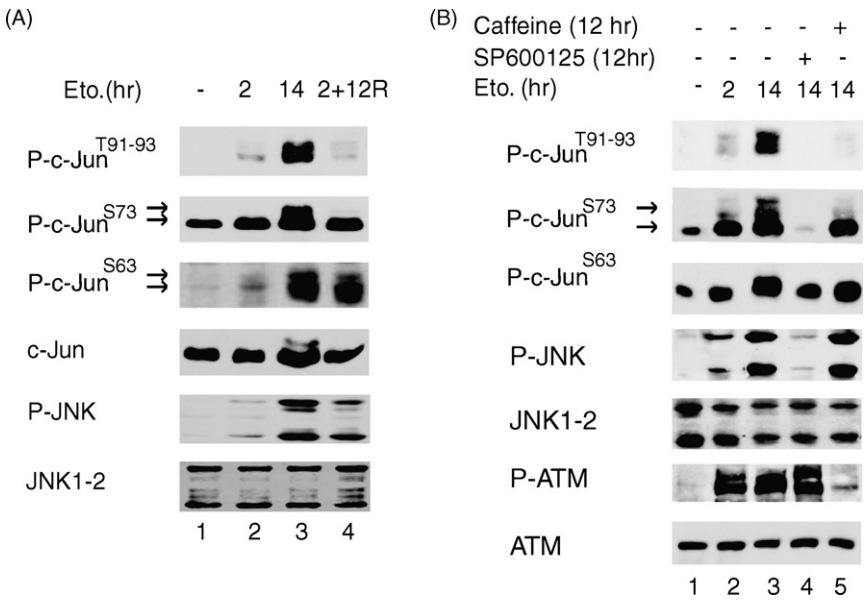

Fig. 2. Differential phosphorylation of c-Jun N-terminal domain by genotoxic stress. (A) HEK-293 cells were treated as described in Fig. 1A and western blot analysis was performed using phospho-specific antibodies for P-c-Jun (T91/T93), P-c-Jun (S63), P-c-Jun (S73) and P-JNK (T183/Y185), as indicated. Expression levels of either c-Jun or JNK proteins were detected using their correspondent non-phospho-specific antibodies, indicated as c-Jun and JNK1–2. (B) HEK-293 cells were treated for 2 h with 5 μM etoposide and then incubated for additional 12 h either in presence or in absence of caffeine (5 mM) or SP600125 (20 μM), as indicated. Western blot analysis was performed using phospho-specific antibodies for P-c-Jun, P-JNK and P-ATM (S1891), or with non-phospho-specific antibodies, as indicated.

a hallmark of its activation (Bakkenist & Kastan, 2003), without affecting either JNK or S73 phosphorylation (Fig. 2B), thus excluding that the inhibition of T91/T93 phosphorylation results from JNK inactivation. These results suggest that JNK activity is essential for c-Jun phosphorylation at all four sites, however a further signal triggered by active DNA-damage pathways is necessary for T91/T93 phosphorylation.

It is worth to note that although SP600125 inhibits JNK activity by blocking ATP binding, we found that it also inhibited JNK phosphorylation (Fig. 2B). Such a peculiar finding has been previously shown by other studies reporting negative effects of SP600125 on JNK phosphorylation by DNA-damaging drugs or LPS (Lee et al., 2006; Yang et al., 2007). Presumably, in vivo treatment of SP600125 may activate secondary pathways leading to unbalanced regulation of JNK specific phosphatases.

3.2. T95 is required for DNA-damage dependent phosphorylation of T91/T93

Based on the observations shown above, we considered that caffeine might interfere with T91/T93 phosphorylation by inhibiting an intramolecular mechanism involving c-Jun phosphorylation at one, or more, of the three ATM/ATR consensus sites (Kim, Lim, Canman, & Kastan, 1999) present within the c-Jun primary structure (Fig. 3A). To assess this hypothesis, we generated HA-tagged c-Jun mutant proteins bearing single alanine substitutions at specific sites (c-Jun/A95, or c-Jun/A249, or c-Jun/A325), or at all three sites (c-Jun/3xAQ). HA-Jun proteins were transiently expressed in HEK-293 cells and c-Jun phosphorylation was analysed using phospho-specific antibodies as described in the Materials and Methods section. As shown in Fig. 3B, etoposide-dependent phosphorylation of c-Jun at T91/T93 was strongly reduced exclusively in c-Jun/3xAQ and c-Jun/A95 mutants. Conversely, the ratio of S73 phosphorylation to T91/T93 phosphorylation was appreciably higher in c-Jun/3xAQ or c-Jun/A95 compared to c-Jun-wt (or c-JunA249 and c-JunA325). These results indicate that etoposide-dependent phosphorylation of c-Jun on T91/T93 requires an intact T95, unless anti-phospho T91/T93 antibodies (P-cJun$^{T91/T93}$) cross-react predominantly with phosphorylated T95 rather than phosphorylated T91/T93. To evaluate this possibility we examined the ability of P-cJun$^{T91/T93}$ antibodies to immunoreact with a c-Jun mutant bearing an alanine substitution at T91/T93 (c-JunA91–93), or a c-Jun mutant containing alanine substitution at all MAPK sites S63/S73 and T91/T93–95 (c-Jun-pan ala). As shown in Fig. 3C, P-cJun$^{T91/T93}$ immunoreacted only with c-Jun-wt, while the anti-phospho S73 antibody recognized both c-Jun-wt and c-Jun-A91–93. These observations imply that the P-cJun$^{T91/T93}$ antibody is predominantly reactive towards phosphorylated T91/T93. However, we cannot exclude the possibility that T95 is not phosphorylated in

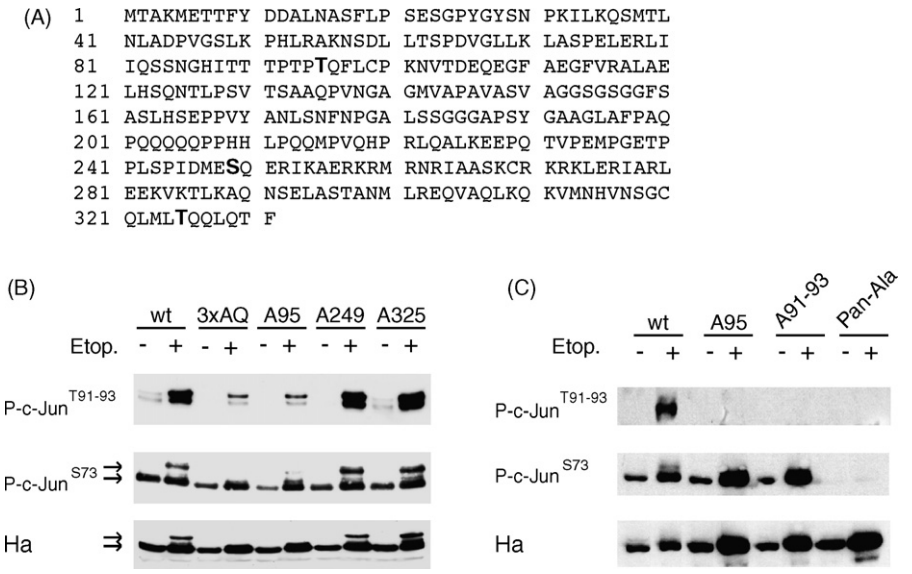

Fig. 3. Abrogation of T95 affects JNK-dependent phosphorylation of T91/T93. (A) Primary sequence of human c-Jun. ATM/ATR consensus-sites are in "Helvetica bold". (B and C) HEK-293 cells were transfected with HA-c-Jun-wt or HA-c-Jun mutants, as indicated. After 24 h, transfected cells were treated with etoposide (5 μM) for 16 h and then harvested for western analysis of c-Jun phosphorylation using the indicated phospho-specific antibodies. The expression levels of HA-c-Jun proteins were normalized using HA antibodies.

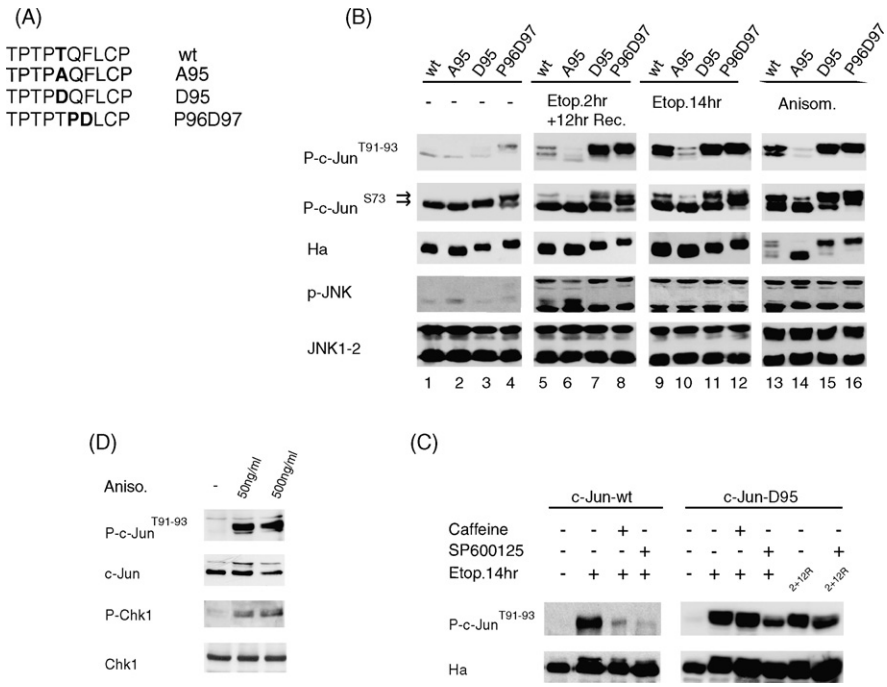

Fig. 4. Phosphorylated T95 is required for T91–93 phosphorylation. (A) Schematic configuration of HA-c-Jun mutants used in panel (B). (B) HEK-293 cells were transfected with HA-c-Jun-wt or HA-c-Jun mutants, as indicated. After 24 h, transfected cells were treated with etoposide for 2 h and then left in culture for additional 12 h either in absence (lines 5–8) or in presence of the drug (lines 9–12), or with anisomycin (500 ng/ml) for 30 min (lines 13–16). Western blot analysis was performed with the indicated phospho-specific antibodies, or with HA and non-phospho-specific JNK1–2 antibodies, as indicated. (C) HEK-293 cells were transfected with HA-c-Jun-wt or HA-c-JunD95, as indicated. After transfection, cells were treated with etoposide either in presence or absence of inhibitors, as indicated. 2 + 12R (last two lines) indicate cells treated for 2 h with etoposide and then left in culture for additional 12 h in absence of the drug. Western blot analysis was performed with the indicated antibodies. (D) HEK-293 cells were treated with anisomycin for 30 min and western blot analysis was performed using the indicated antibodies.

the A91–93 mutant and that the P-cJunT91/T93 antibody cross-reacted, although to a lesser extent, with phosphorylated T95.

3.3. Aspartic acid substitution of T95 triggers JNK-dependent phosphorylation of T91/T93 in absence of persisting DNA damage

Our data suggest that the lack of negative charge on position 95 might be the missing signal impairing T91/T93 phosphorylation in response to transient exposure to etoposide. To test this hypothesis we analysed c-Jun mutant proteins presenting substitution of T95 with negatively charged aspartic acid (c-Jun/D95), or proline/aspartate substitution of glutamine96/phenylalanine97 (c-Jun/P96D97), therefore converting the TQ site in a canonical (TPD) MAPK-phosphorylation site (Fig. 4A). A transient exposure to etoposide led to strong phosphorylation of T91/T93 in both c-Jun/D95 and c-Jun/P96D97 proteins (Fig. 4B, lines 7–8) as compared to c-Jun-wt (Fig. 4B, line 5). Conversely, the level of JNK activation or S73 phosphorylation was similar in all c-Jun variants, although the c-Jun/P96D97 mutant showed the highest rate of shifted protein, presumably mirroring c-Jun phosphorylation on multiple sites. Note that this mutant form of c-Jun showed an appreciable basal level of shifted protein (Fig. 4B, line 4), suggesting that such a conformation of the MAPK site may permit the cluster of T91/T93/T5 phosphoacceptor sites to be phosphorylated in absence of stress-activated JNKs, as for S73. However, as discussed below, upon etoposide treatment, T91/T93 phosphorylation was abolished by SP600125 preincubations. As expected, a prolonged exposure to etoposide induced a similar level of T91/T93 phosphorylation in all c-Jun variants except for c-Jun/A95. Notably, SP600125 prevented T91/T93 phosphorylation of c-Jun/D95 by a transient exposure to etoposide (Fig. 4C), excluding that aspartate substitution of T95 rescued the dependency of T91/T93 phosphorylation from JNK activity. Similar results were obtained for c-Jun/P96D97 mutant (data not shown). These results indicate that a negative charge on position T95 provided either by MAPK-dependent phosphorylation or by a negatively charged amino acid

exerts a crucial role in the JNK-dependent phosphorylation of T91/T93. Based on these observations, we considered that the effect of caffeine on T91/T93 phosphorylation (Fig. 2B) might reflect inhibition of T95 negative charging. To evaluate this aspect, we investigated whether aspartate substitution of T95 would rescue T91/T93 phosphorylation in presence of caffeine. As shown in Fig. 4C, T91/T93 phosphorylation of transfected c-Jun/wt, as for endogenous c-Jun (Fig. 2B), resulted to be sensitive to either caffeine or SP600125. Differently, T91/T93 phosphorylation of c-Jun/D95 was resistant to caffeine but sensitive to SP600125 (Fig. 4C), suggesting that the inhibition of the DNA damage pathways prevents T95 phosphorylation.

As the kinetics of JNK activation by etoposide is slow, we asked whether anisomycin, a ribotoxic drug inducing rapid and strong activation of JNKs, would lead to T91/T93 phosphorylation in a T95-independent fashion. To this end, we analysed the pattern of c-Jun N-terminal phosphorylation in the c-Jun mutants described above. As shown by the ratio of c-Jun proteins detected with c-Jun$^{T91/T93}$ phospho-specific antibodies to c-Jun proteins detected with HA antibodies, T91/T93 phosphorylation resulted quite similar between c-Jun/wt and c-Jun/D95, or c-Jun-P96/D97 (Fig. 4B, lines 13, 15 and 16). In contrast anisomycin-induced phosphorylation of T91/T93 was strongly diminished in c-Jun/A95, while the phosphorylation status of S73 and JNK was not altered (Fig. 4B, line 14). These results demonstrate that an intact T95 is essential for T91/T93 phosphorylation independently of the kinetics of JNK activation. Interestingly, anisomycin led to Chk1 phosphorylation (Fig. 4D), therefore providing a further link between T91/T93 phosphorylation and DNA-damage pathways.

3.4. Negatively charged T95 is required for c-Jun biological activity

c-Jun expression and phosphorylation has been shown to play a crucial role in several paradigms of cell death, among which genotoxic-stress induced apoptosis (reviewed in Waetzig, Zhao, & Herdegen, 2006). Therefore we asked whether alanine substitution of T95 affected c-Jun-mediated enhancement of cell death in response to UV radiation or etoposide. To this end, sets of tissue culture plates containing the same number of adherent HEK-293 cells were transiently transfected with plasmids expressing either empty vectors, or HA-c-Jun-wt, or HA-c-Jun-A95, together with an expression vector encoding the enhanced green fluorescent protein (EGFP) (Cormack, Valdivia, & Falkow, 1996). After 24 h, percentages of transfected cells were monitored by fluorescence microscopy. Sets of HEK-293 transfected cells showing even efficiency of EGFP transfection (about 40% of total cells) were either treated with 5 μM etoposide for 18 h, or irradiated with UV light (50 J/m^2) and incubated for further 6 h. After genotoxic treatments cells were collected and analysed for cell death by flow cytometric analysis of sub-2n DNA content (sub G1pick) (Fig. 5A), as described in the Materials and Methods section. The expression levels of Ha-c-Jun protein and the relative extent of T91/T93 phosphorylation were examined by western blots, as shown in Fig. 5D. As expected, c-Jun-wt overexpression increased the rate of cell death in response to either etoposide or UV. In contrast, expression of HA-c-Jun-A95 did not further enhance the basal levels of cell death (Fig. 5A), indicating that an intact T95 is essential for c-Jun-mediated enhancement of cell death. Although the expression levels of Ha-c-Jun-wt and HA-c-Jun-A95 were comparable in the different conditions (Fig. 5D), T91/T93 phosphorylation was detected only in HA-c-Jun-wt after UV or etoposide treatments (Fig. 5D), suggesting that the effect of HA-c-Jun proteins on cell death was strictly associated with the extent of T91–93 phosphorylation. Furthermore, the evidence that UV-induced phosphorylation of T91/T93 is strongly reduced in c-Jun-A95 (Fig. 5D) corroborates the essential role elicited by T95 in controlling T91/T93 phosphorylation in response to DNA damage.

Recent studies have shown that JNK-inducible transactivation of c-Jun requires all four intact S63/S73 and T91/T93 phosphorylation sites (Weiss et al., 2003). Therefore, we assessed the effect of A/T95 substitution on c-Jun transactivation by JNKs. To this end, we used fusion proteins that replaced the basic region/leucine region (bZip) domain of c-Jun with the DNA binding domain of the yeast transcription factor Gal4 (c-Jun/Gal4) (Vinciguerra et al., 2004). As previously reported (Vinciguerra et al., 2004), overexpression of MKK7, a MAPKK specifically activating JNKs, increased the activity of c-Jun-wt/Gal4 (Fig. 5B). In contrast, MKK7 had no effect on the activity of c-Jun/Gal4 fusion protein bearing alanine substitution of S63/S73, T91/T93 and T95, indicating that MKK7 overexpression stimulates c-Jun transactivation in a phosphorylation dependent fashion. Similarly, c-Jun/Gal4 mutants bearing alanine substitution at T91/T93 (c-Jun-A91–93/Gal4), was no longer stimulated by MKK7 overexpression. Notably, alanine substitution of the single T95 was sufficient to abrogate MKK7-dependent stimulation of c-Jun transactivation, indicating that T95 is essential for c-Jun-dependent transcription, presumably by controlling the levels of

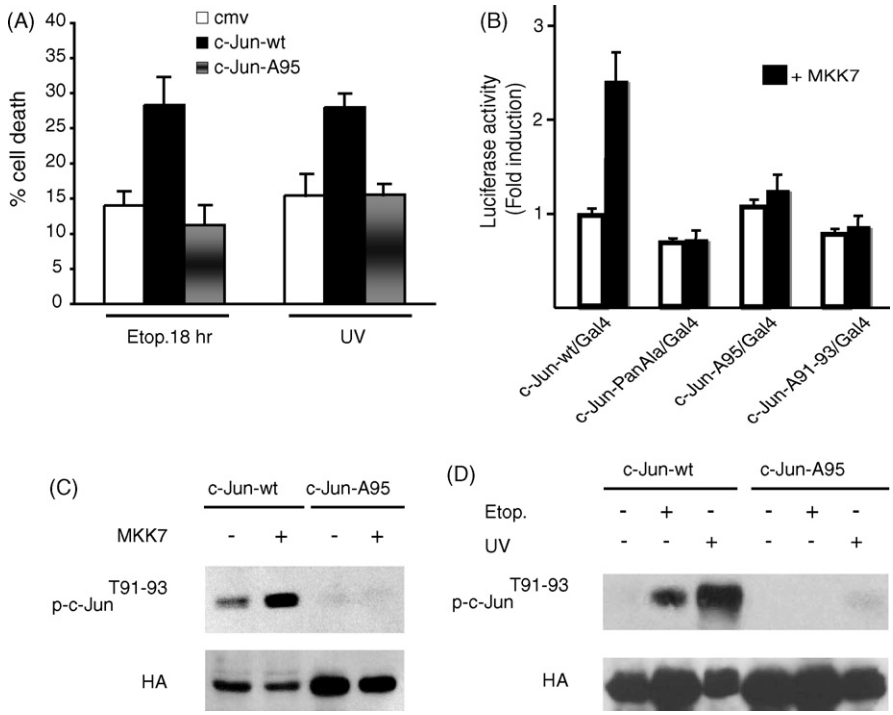

Fig. 5. Abrogation of T95 affects the biological activity of c-Jun. (A) HEK-293 cells were transfected with CMV-EGFP expressing plasmid together with HA-c-Jun-wt, HA-cJun-A95, or with CMV-empty vector, as indicated. After 24 h, cells were monitored for even transfection efficiency by fluorescence microscopy and then treated either with 5 μM etoposide for 18 h or with UV (50 J/m^2), and incubated for further 6 h. After treatments, cells were collected and cell death was evaluated by measuring sub-G1 peaks by flow cytometric analysis. The levels of HA-c-Jun protein expression were evaluated by western blot analysis (panel D). Error bars indicate S.D. of four independent experiments. (B) HEK 293 cells were transfected with plasmids expressing the c-Jun/Gal4 fusion proteins, the reporter plasmid Gal4-luciferase and a *Renilla* luciferase expression vector (pRL-TK), which was used as a transfection control. Gal4/cJun-proteins were co-expressed with MKK7 expression plasmid where indicated. Firefly luciferase activity was normalized to the internal transfection control provided by the *Renilla* luciferase. Luciferase activity of cells expressing cJun-wt/Gal4 and treated with vehicle was arbitrarily set to 1. Error-bars indicate S.D. of three independent experiments, each performed in triplicate. (C) HEK 293 cells were transfected with plasmids expressing HA-tagged c-Jun proteins. c-Jun proteins were co-expressed with MKK7 expression plasmid, where indicated. After 24 h, transfected cells were harvested and western blot analysis of cellular extracts was performed with the indicated antibodies. (D) Cellular extracts of transfected cells described in (A) were analysed by Western blot using the indicated antibodies.

T91/T93 phosphorylation. In line, we observed that MKK7 overexpression induced T91/T93 phosphorylation of c-Jun-wt protein, but not that of the c-Jun-A95 mutant (Fig. 5C).

4. Discussion

In response to various stress signals, JNK triggers c-Jun biological activity by inducing phosphorylation of the c-Jun N-terminal domain at positions S63/S73 and T91/T93, which in turn regulates c-Jun-dependent transcription. The present study reveals that JNK activity, *per se*, is necessary but not sufficient to induce c-Jun biological activity in response to genotoxic stress. The presence of a negative charge on T95, presumably provided by a yet unknown stress induced-kinase, is essential to expose sites T91/T93 of c-Jun to JNK-dependent phosphorylation and subsequently to induce c-Jun transcriptional activity.

Two lines of evidence indicate that an additional stress-induced signal, besides JNK activation, is required for T91/T93 phosphorylation: firstly, despite activation by JNK, c-Jun N-terminal phosphorylation in response to transient exposure to etoposide is restricted to S63/S73 (Fig. 2A); secondly, caffeine prevents T91/T93 phosphorylation in response to prolonged exposure to etoposide without affecting JNK activation or S63/S73 phosphorylation (Fig. 2C). The latter observation excludes that genotoxic stress might regulate T91/T93 phosphorylation by merely inducing JNK activation. On the other hand, the indication that T91/T93 phosphorylation is prevented by both caffeine and SP600125 rules out that the activity of a kinase other than JNK may induce T91/T93 phosphorylation. Accordingly, in absence of

JNK expression, T91/T93 are not phosphorylated by either stress or pro-inflammatory stimuli (Morton et al., 2003). However, whether T91/T93 phosphorylation is directly driven by JNKs, or by a JNK-regulated kinase, remains to be determined.

Interestingly, the inhibitory effect of A/T95 substitution on T91/T93 phosphorylation (Figs. 3 and 4) suggests that a negative charge on T95 might represent the putative modification required for JNK-dependent phosphorylation of T91/T93. In accordance with this hypothesis, we show that T95 replacement with negatively charged aspartic acid leads to JNK-dependent phosphorylation of T91/T93 in presence of caffeine, or in absence of persisting DNA damage (Fig. 4). Since negative charge of threonine/serine are mostly provided by kinase-dependent phosphorylation, our study points to T95 as a putative DNA damage dependent-phosphorylation site essential for JNK-dependent phosphorylation of T91/T93. In line with this hypothesis, replacement of the TQ acceptor site with a canonical MAPK phosphorylation site leads to T91/T93 phosphorylation in absence of persisting DNA damage, excluding that the negative charged aspartic acid substitution of T95 might mimic a structural modification other than phosphorylation (Fig. 4B). Furthermore, the observation that alanine substitution of T95 prevents T91/T93 phosphorylation in response to various types of genotoxic stress, such as anisomycin and UV radiation (Figs. 4B, 5D), suggests that phosphorylation of T95 is a general prerequisite for stress-induced activation of c-Jun, rather than specific to etoposide-mediated phosphorylation of c-Jun.

A wide number of studies have pointed to up-regulation of c-Jun expression coupled to JNK-dependent phosphorylation of the c-Jun N-terminal domain as a crucial mechanism leading to stress-induced apoptosis (Waetzig et al., 2006). Remarkably, we found that c-Jun-A95 is not able to enhance either UV or etoposide-induced cell death (Fig. 5A), pointing to T95 phosphorylation as an essential post-transcriptional modification required for the induction of c-Jun biological activity.

c-Jun mostly acts by forming either homodimers or heterodimers with other members of the AP-1 family of transcription factors. Accordingly, most mutations modulating c-Jun biological output affect its transcriptional activity. Phosphorylation of both groups of MAPK sites (S63/S73 and T91/T93) is required for signal-induced transcriptional activity of c-Jun (Weiss et al., 2003). The evidence that alanine substitution of T95 affects both c-Jun transactivation and T91/T93 phosphorylation (Fig. 5C) suggests that an uncharged alanine at position T95 affects c-Jun-mediated apoptosis by impairing its transactivation potential.

Depending on the environmental and genetic background, activation of c-Jun by JNKs can elicit quite diverse biological programs including cell survival or apoptosis. Presumably, according to the physiological condition, active c-Jun may influence different cellular responses by shifting the balance of the expression pattern of distinct AP-1 responsive genes. The present findings suggest that c-Jun may perceive the strength of genotoxic stress through phosphorylation of T95 which in turn allowing JNK-dependent phosphorylation of T91/T93, drives c-Jun transcriptional activity and the expression pattern of distinct c-Jun target genes.

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Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF

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The steroid hormone oestrogen can signal through several receptors and pathways. Although the transcriptional responses mediated by the nuclear oestrogen receptors (ER) have been extensively characterized, the changes in gene expression elicited by signalling through the membrane-associated ER GPR30 have not been studied. We show here for ER-negative human breast cancer cells that the activation of GPR30 signalling by oestrogen or by hydroxytamoxifen (OHT), an ER antagonist but GPR30 agonist, induces a transcription factor network, which resembles that induced by serum in fibroblasts. The most strongly induced gene, *CTGF*, appears to be a target of these transcription factors. We found that the secreted factor connective tissue growth factor (CTGF) not only contributes to promote proliferation but also mediates the GPR30-induced stimulation of cell migration. These results provide a framework for understanding the physiological and pathological functions of GPR30. As the activation of GPR30 by OHT also induces *CTGF* in fibroblasts from breast tumour biopsies, these pathways may be involved in promoting aggressive behaviour of breast tumours in response to endogenous oestrogens or to OHT being used for endocrine therapy.

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Introduction

The steroid hormone oestrogen binds and activates the oestrogen receptors (ER) α and β, two members of the nuclear receptor superfamily. Activated ERs regulate the transcription of target genes by binding either directly to specific DNA sequences or by tethering to other DNA-bound transcription factors. ERs have been extensively studied at the molecular, cellular, physiological and pathological levels (reviewed by Dahlman-Wright *et al*, 2006; Deroo and Korach, 2006; Heldring *et al*, 2007). Tamoxifen and its hydroxylated active form hydroxytamoxifen (OHT) are synthetic ER ligands that compete with the physiological oestrogen 17β-estradiol (E2) for binding. Depending on promoter, cell and signalling context, OHT functions either as a partial agonist or as a partial antagonist. The latter mode has led to its use for endocrine therapy of ERα-positive breast tumours, the proliferation of which can be stimulated by E2 (reviewed by Jordan, 2004).

The early discovery of Filardo *et al* (2000) that the presence of the completely unrelated transmembrane receptor GPR30 can mediate oestrogen responsiveness of ER-negative breast cancer cells came as a big surprise. GPR30 was later shown to be a genuine ER (Revankar *et al*, 2005; Thomas *et al*, 2005). In addition to E2, OHT also functions as a GPR30 agonist (Revankar *et al*, 2005; Vivacqua *et al*, 2006a, b). The GPR30 signalling pathway has been studied in a variety of cell lines. GPR30 couples to a trimeric G protein, stimulating the cAMP pathway most likely through a $G_{\alpha s}$ (Thomas *et al*, 2005) and Src through Gβγ (Filardo, 2002). Subsequently, Src promotes the shedding of heparin-binding EGF-like growth factor and activation of the EGF receptor (Filardo *et al*, 2000). This in turn activates a whole series of intracellular signalling events, most notably the activation of mitogen-activated protein kinases (MAPK) Erk1/2, PI3 kinase and phospholipase C (reviewed by Prossnitz *et al*, 2008). Further cellular responses lie downstream of these signals, including the activation of the gene *FOS* (Maggiolini *et al*, 2004).

It is unlikely that the activation of *FOS* can account for all of the biological effects of GPR30 signalling that have been reported. For example, E2 is able to stimulate the proliferation of breast, thyroid and ovarian carcinomas through GPR30 (Vivacqua *et al*, 2006b; Albanito *et al*, 2007, 2008). This effect is clearly independent of ERs and can also be observed with a GPR30-specific ligand, but how GPR30 signalling stimulates proliferation remains unclear. Although the genomic effects of ERα have been extensively studied, and in particular in breast cancer cells (see Carroll and Brown, 2006; Dudek and Picard, 2008 and references therein), the global changes in gene expression triggered by GPR30 signalling are not known. Unlike a transcription factor such as ERα, GPR30 would have to effect these changes indirectly. Nevertheless, GPR30-mediated changes in gene expression patterns have to be considered a specific output of this signal-transduction pathway. Here, we report the transcriptional consequences of GPR30 signalling in human breast cancer cells. The most strongly induced gene suggested a new function of GPR30 signalling in cell migration and proved to be functionally relevant for our understanding of the biological effects of GPR30 signalling.

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Results

Gene expression profiling of GPR30 signalling

To determine the changes in gene expression that GPR30 signalling elicits, we chose human SKBr3 breast cancer cells as our model system. These cells lack both ERα and ERβ but express GPR30 and display GPR30 signalling (Filardo *et al*, 2000; Maggiolini *et al*, 2004). Despite the absence of other known ERs in SKBr3 cells, we knocked down *GPR30* expression with an antisense strategy (Revankar *et al*, 2005; Vivacqua *et al*, 2006b) (Supplementary Figure 1A) to ascertain that any observed ligand-induced changes in gene expression are mediated by GPR30. Serum-deprived cells were treated for only 1 h with E2 or OHT to capture the primary responses. It should be pointed out here, that the OHT concentration (10 μM) used for induction is comparable to the micromolar OHT concentrations that are reached in breast tissue of patients undergoing tamoxifen therapy (Kisanga *et al*, 2004).

The mRNA levels of a total of 175 genes were induced by at least 1.3-fold by one of the treatments by comparison with uninduced control cells (Supplementary Figure 1B). At this point, we decided that we would only consider those genes as potential GPR30 target genes that fulfilled the following stringent criteria: at least 1.3-fold induction by both E2 and OHT, and at least a 1.3-fold reduction of the OHT response by antisense-mediated *GPR30* knockdown. These criteria defined 36 genes as GPR30 target genes (Figure 1; Supplementary Table 1). In total, 19 of these 36 genes were induced by more than two-fold by OHT. Within the short time frame of the treatment, no gene was significantly repressed according to the same criteria (data not shown).

We then undertook a Q-PCR experiment with the same RNA samples for a panel of genes to validate the microarray results and to obtain more quantitative data. Qualitatively, GPR30-mediated induction could be confirmed for all of them, although, not surprisingly, larger quantitative differences were obtained by Q-PCR (Figure 2). The gene encoding the connective tissue growth factor (CTGF, also known as CCN2) proved to be induced 15- to 16-fold by OHT and E2. It is a technical limitation of microarray analyses that some genes with a relatively modest induction fall through the cracks. This is the case, for example, for *JUN*. Our short list of 36 genes contains the genes *FOS* and *FOSB* (Supplementary Table 1). These encode components of the heterodimeric transcription factor AP1. Surprisingly, our gene list contains none of the genes, such as *JUN*, that encode heterodimeric partner proteins of Fos proteins. Although *JUN* did not pass the third stringent criterion (reduction by at least 1.3-fold in the *GPR30* knockdown sample) in the microarray analysis, it easily passed all criteria for a GPR30 target gene in the Q-PCR experiment, including a two-fold induction by both ligands (Figure 2). We therefore include *JUN* as a GPR30 target gene and consider it very likely that there are other false negatives in the microarray data.

CTGF is a GPR30 target gene

CTGF is by far the gene most strongly induced by E2 or OHT. We performed an immunoblot analysis to determine whether the dramatic induction seen at the mRNA level leads to increased CTGF protein expression in SKBr3 cells.

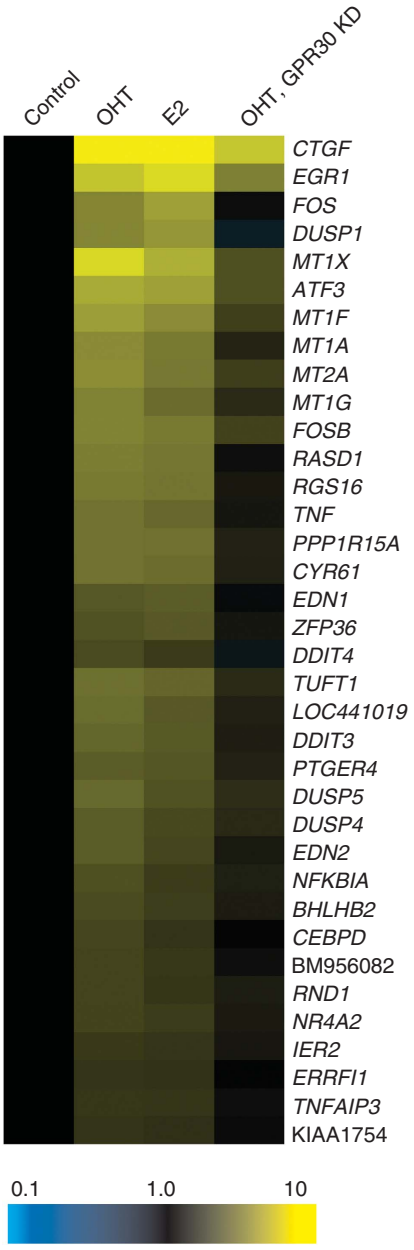

Figure 1 Colour-coded map of hierarchically clustered gene expression profiles. For each gene and condition, the colour indicates the ratio of the values obtained for the treated and untreated samples (as listed in Supplementary Table 1). GPR30 KD, *GPR30* knockdown.

Figure 3A shows that this is the case and that this increase can be blunted by an shRNA-mediated knock down of *GPR30*. The requirement for GPR30 and the specificity of the GPR30 knockdown are further emphasized by the fact that the co-transfection of an shRNA-resistant version of *GPR30* ('GPR30 rescue') restores the response. The increase at the protein level might seem modest, but note that only cell-associated proteins, and not proteins already released into the medium, were immunoblotted. We further explored the generality of this response with other cell lines and the GPR30-specific ligand G-1 (Bologa *et al*, 2006) (see Figure 3B and C). *CTGF* is induced by OHT in the human breast cancer cell lines MCF7 and BT-20, which are ERα positive and negative, respectively.

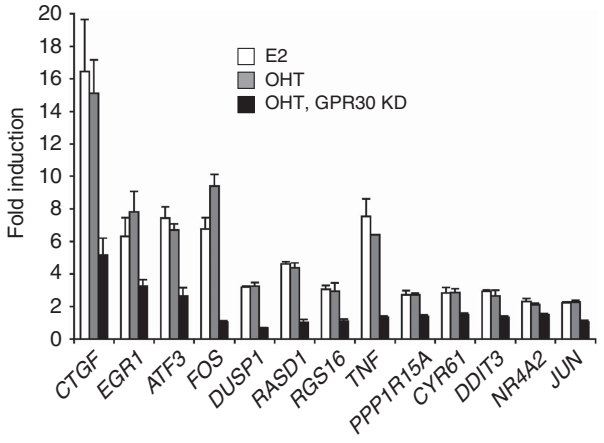

Figure 2 Q-PCR validation of a subset of GPR30-regulated genes. 'Fold induction' denotes the ratio of the values obtained for the treated and untreated samples. Error bars indicate standard deviations of measurements of triplicate samples. GPR30 KD, *GPR30* knockdown.

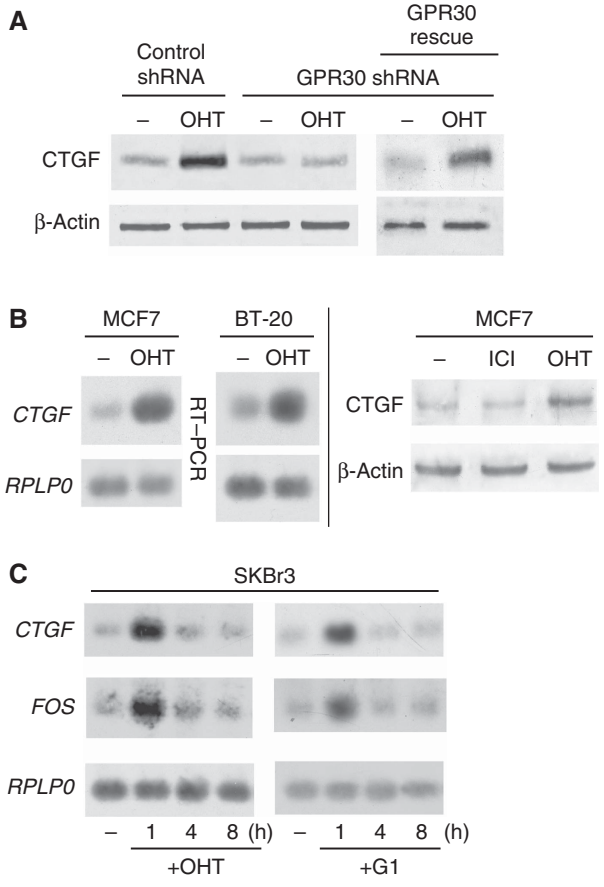

Figure 3 Induction of *CTGF* mRNA and protein in a variety of breast cancer cell lines. (**A**) Immunoblot analysis of CTGF expressed by SKBr3 cells. Cells were transfected with shRNA constructs and the GPR30 rescue vector, and treated with OHT as indicated. (**B**) Immunoblots of semiquantitative RT–PCR products ('RT–PCR') and CTGF protein (rightmost panel). (**C**) Time course of *CTGF* and *FOS* induction; semiquantitative RT–PCR analysis of SKBr3 cells treated with OHT or G1. β-Actin and the *RPLP0* mRNA served as internal standards for the immunoblot and RT–PCR experiments, respectively.

The induction is seen both at the mRNA and protein levels, and it is not elicited by the antioestrogen ICI 182′780 (ICI) (Figure 3B), at least not in MCF7 cells under our experimental conditions. Importantly, the OHT induction of *CTGF* in MCF7 cells is independent of ERα as it can still be observed when ERα is knocked down (Supplementary Figure 2). The time course experiment confirms the activation of the *CTGF* and *FOS* genes by OHT, and shows an identical activation by G-1. Induction at the mRNA level is transient in that it is clearly observed after 1 h but has subsided 3 h later. Note that the microarray analysis was performed with RNA from cells treated for 1 h.

Mediators of the transcriptional response to GPR30 signalling

The GPR30-mediated activation of target genes must be indirect. Previous analyses had indicated that GPR30 leads to the activation of Erk1/2 (Filardo *et al*, 2000; Maggiolini *et al*, 2004). MAPK can activate transcription factors such as the serum response factor (SRF) and members of the ETS family by direct phosphorylation (see for example, Posern and Treisman, 2006; Gutierrez-Hartmann *et al*, 2007). Moreover, it has been pointed out that the increase in cAMP elicited by GPR30 signalling could be expected to activate CREB (Prossnitz *et al*, 2008). These factors in turn activate the expression of the second tier of transcription factors such as c-Fos, FosB, c-Jun, EGR1, ATF3, C/EBPδ and NR4A2 (Nurr1). In addition to *FOS*, which we already knew to be activated by GPR30 signalling (Maggiolini *et al*, 2004), the genes for the aforementioned second tier transcription factors are all in our list of GPR30-induced genes (complemented with *JUN* from the Q-PCR experiment).

As a first step towards elucidating the signalling and transcription factor network that might underlie the transcriptional response to GPR30 signalling, we downloaded 5 kb of upstream sequences (relative to the start sites of transcription) for 34 of the 36 target genes of Supplementary Table 1. We scanned them for the presence of common sequence motifs and compared those with the known DNA-binding sequences of the TRANSFAC database to identify the corresponding transcription factors. The results of these analyses are displayed in Figure 4A for *CTGF* and for the complete set of target genes in Supplementary Table 2. SRF is by far the most over-represented transcription factor with EGR2, CREB and ATF among the runners up. Overall, the results of this bioinformatic analysis are entirely compatible with the aforementioned activation scheme.

Binding sites for AP1, of which c-Fos can be a component, are also highly represented, although not over-represented by more than two-fold, in promoters of GPR30 target genes (data not shown). For *CTGF* (Figure 4A), whose upstream sequences contain AP1 sites, we experimentally verified the role of c-Fos. The expression of a dominant-negative variant of c-Fos in SKBr3 cells abolishes the induction of *CTGF* by OHT or E2 (Figure 4B). To assess whether 5′ flanking sequences of the *CTGF* gene would be sufficient to mediate the GPR30 response, we used a reporter gene containing a 2 kb *CTGF* promoter fragment upstream of the luciferase-coding region (Chaqour *et al*, 2006; see Figure 4A). Upon transfection into SKBr3 cells, this reporter gene could be induced more than two-fold with E2 in a GPR30-dependent manner (Figure 4C). The response to OHT, which appears to

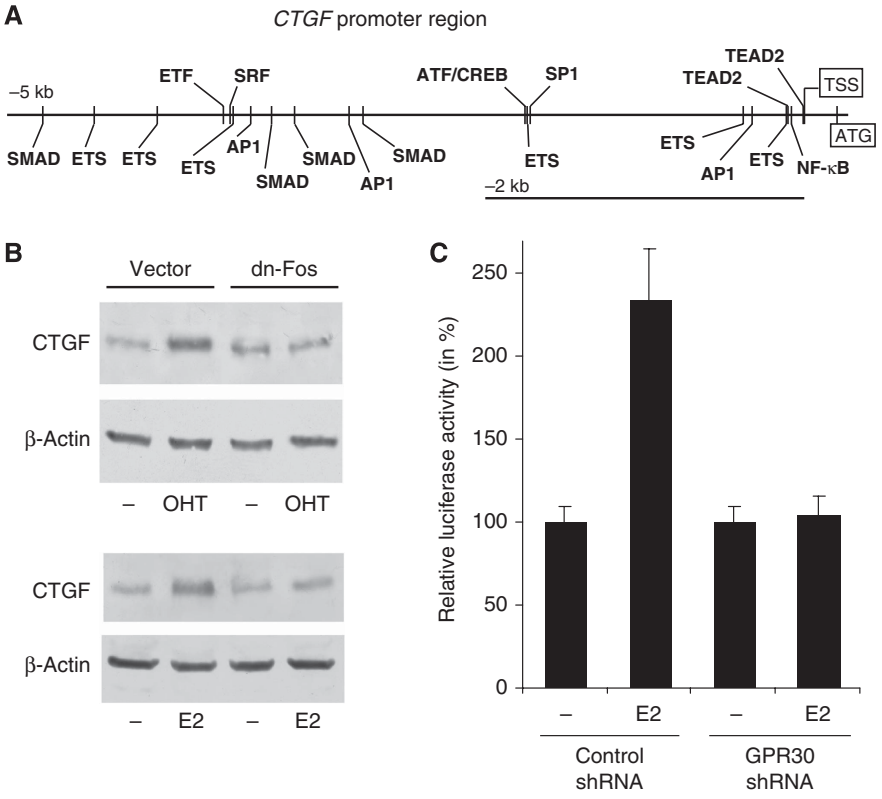

Figure 4 Transcriptional control of *CTGF* induction. (**A**) Transcription factor map of *CTGF* promoter region. Sites with factors indicated above the promoter line are over-represented at least two-fold in all GPR30 target genes (see Supplementary Table 2). AP1 and some additional factors reported in the literature are indicated below the line (see Leask and Abraham, 2006). The shorter line indicates the 2-kb fragment present in the *CTGF* luciferase reporter construct. The start sites of transcription (TSS) and translation (ATG) are indicated. (**B**) Immunoblot analysis of CTGF from SKBr3 cells expressing a dominant-negative version of c-Fos (dn-Fos). (**C**) E2 activation of a *CTGF* luciferase reporter gene co-transfected with shRNA constructs into SKBr3 cells as indicated. For each pair of samples, the values of the uninduced one were set to 100%. Error bars indicate standard deviations of normalized luciferase activities of triplicate samples.

be a stronger inducer of GPR30 signalling, could not be determined. The prolonged exposure of the cells to OHT, which this transactivation assay requires, turned out to be too toxic for SKBr3 cells. As observed for the induction of endogenous CTGF protein, AP1 turned out to be important for induction of the *CTGF* reporter gene, and this observation could be extended to ETS and SRF (Supplementary Figure 3A). A preliminary survey of signalling mediators that are required for the induction of *CTGF* highlights the role of the EGF receptor (see also Filardo *et al*, 2000) and the MAPK signalling cascade (Supplementary Figure 3B), mirroring our previous findings related to the induction of *FOS* (Albanito *et al*, 2008). In contrast to *FOS*, the induction of *CTGF* further depends on actin dynamics, a well-known regulator of SRF activity (Sotiropoulos *et al*, 1999). These results are compatible with the notion that these signalling pathways mediate the GPR30-induced activation of these transcription factors leading to the activation of *CTGF* and possibly other target genes.

GPR30 signalling promotes migration through CTGF induction

We next wondered what the biological significance of the potent induction of CTGF by GPR30 signalling might be. As CTGF had already been reported to be both sufficient and necessary for the stimulation of migration of other breast cancer cell lines (Chen *et al*, 2007), we considered the possibility that GPR30 signalling might promote migration through the induction of CTGF. The migration of SKBr3 cells was analysed with a Boyden chamber migration assay. With this assay, the number of SKBr3 cells that are able to migrate through a polycarbonate filter during a 3-h treatment period is counted. Figure 5A demonstrates that both OHT and CTGF stimulate the migration of SKBr3 cells more than two-fold. In the following experiment, we determined whether the stimulation of migration by OHT is indeed mediated by GPR30 signalling and the induction of CTGF. The transient knockdown of *GPR30* or *CTGF* expression using transfected shRNA constructs completely abolishes the stimulation of migration by OHT (Figure 5B). The addition of CTGF to the medium of cells, in which *GPR30* or *CTGF* are knocked down, rescues their migration defect. This result along with an immunoblot confirming the knockdown of *CTGF* at the protein level (Figure 5C) attests to the specificity of the RNA interference experiment. Thus, OHT stimulates the migration of SKBr3 cells through GPR30, and the GPR30-dependent induction of *CTGF* expression is necessary for this stimulatory effect. The fact that added CTGF stimulates migration to a similar extent as OHT, both in control and *GPR30* knockdown cells, indicates that the GPR30-mediated induction of *CTGF* is also sufficient for this stimulation.

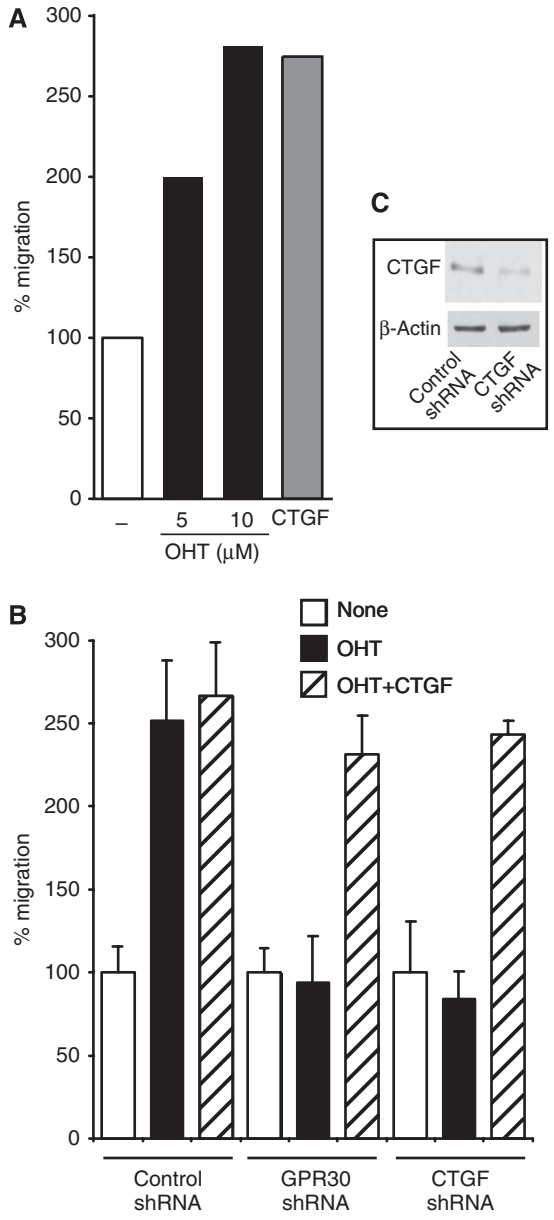

Figure 5 CTGF-dependent stimulation of cell migration by GPR30 signalling. (**A**) OHT and CTGF added to the medium induce the migration of SKBr3 cells. Bar graph shows a representative experiment with means of duplicate samples, standardized to the untreated control set to 100%. (**B**) Transient knockdown of *GPR30* or *CTGF* in SKBr3 cells blocks OHT stimulation of migration, and CTGF added to the medium restores it. Bar graph shows a representative experiment with means of triplicate samples, standardized to the respective untreated controls set to 100%. Error bars show standard deviations. (**C**) Immunoblot illustrating the extent of CTGF knockdown of a typical experiment.

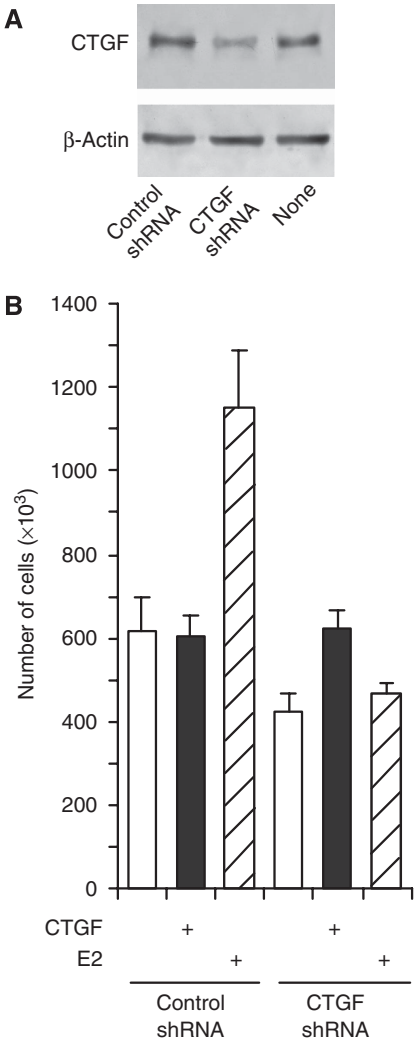

Figure 6 CTGF is required for the proliferative stimulation of SKBr3 by E2. (**A**) CTGF immunoblot of stable *CTGF* knockdown SKBr3 cells. They are compared with SKBr3 cells stably transduced with an unrelated control shRNA and untransfected wild-type SKBr3 cells. (**B**) Proliferation assay with SKBr3 cells stably transduced with shRNA constructs and treated with CTGF or E2 as indicated. Bar graph shows means of triplicate samples with standard deviations.

A possible role for CTGF in promoting proliferation

We have previously reported that GPR30 signalling stimulates the proliferation of cell lines, including SKBr3 cells, representing a variety of different carcinomas (Vivacqua *et al*, 2006b; Albanito *et al*, 2007, 2008) and mouse spermatogonia (Sirianni *et al*, 2008). It is very likely that the activation of growth-related transcription factors, such as the ones mentioned above, constitutes a cell-autonomous proliferative stimulus. In addition, primary or secondary GPR30 target genes that encode secreted factors such as CTGF might stimulate proliferation in an autocrine or paracrine manner. Indeed, there is evidence in the literature for a proliferative effect of CTGF on a variety of cell types (Rachfal and Brigstock, 2005; Leask and Abraham, 2006; De Winter *et al*, 2008), but its effects on normal or cancerous breast epithelial cells are less clear and possibly more complex (see below and Discussion). Pilot experiments indicated that CTGF added to the medium does not stimulate the proliferation of SKBr3 cells (data not shown; see also Figure 6). Although this finding is compatible with the observation that CTGF does not stimulate the proliferation of MCF7 cells (Chen *et al*, 2007), we noticed that SKBr3 cells grow poorly when *CTGF* is stably knocked-down below the basal level by a virally transduced shRNA construct (Figure 6). Remarkably, this defect can be corrected by adding CTGF to the medium. In contrast, unlike wild-type SKBr3 cells (Albanito *et al*, 2008), *CTGF* knockdown cells are resistant to the proliferative stimulus of E2. These results indicate that CTGF can have a

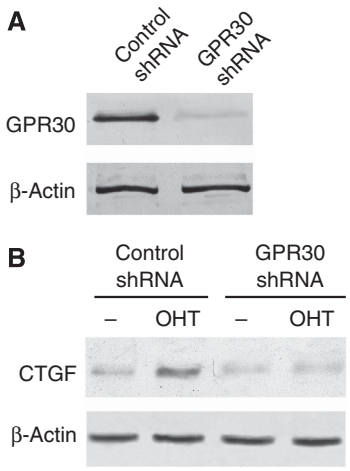

Figure 7 GPR30 expression and signalling in fibroblasts from breast tumours. The data shown are from a representative experiment of three experiments performed with samples from different patients. (**A**) Immunoblot analysis showing expression and knock down of GPR30 in transfected fibroblasts. (**B**) Immunoblot analysis of CTGF expressed by fibroblasts. Cells were transfected with indicated shRNA constructs and treated with OHT as indicated.

proliferative effect on such breast carcinoma cells under certain conditions, and that CTGF is required for the proliferative response to E2.

Contribution to GPR30 signalling from the stroma

Given that secreted factors regulating proliferation and migration need not only be made by the breast carcinoma cells themselves, we examined the expression and signalling of GPR30 in fibroblasts obtained from biopsies of primary breast tumours. GPR30 is clearly expressed in these fibroblasts, and its activation by OHT stimulates the expression of CTGF (Figure 7).

Discussion

In the process of characterizing the transcriptional response to GPR30 signalling, we have found a set of target genes that contribute to mediating the proliferative stimulus of GPR30 activators for carcinoma cell lines. Moreover, the prominent induction of *CTGF* by both E2 and OHT led us to the discovery that GPR30 signalling stimulates cell migration through CTGF. As these responses may occur in breast cancer cells independently of their ERα status as well as in the surrounding stromal cells, these findings may have important implications for our understanding of endocrine resistance in breast cancer.

GPR30 signalling activates a transcription factor network

Our microarray analysis indicated that GPR30 signalling triggers the activation of a network of transcription factors. A first tier, including SRF, members of the ETS family and CREB, is directly activated through post-translational modifications by kinases that are activated by GPR30 signalling. These transcription factors are the ones that transcriptionally activate the expression of a second tier of transcription factors (for example, Fos and Jun proteins, EGR1, ATF3, C/EBPδ and NR4A2). The response is most likely further amplified by GPR30-stimulated post-translational modifications of these proteins and positive feedback loops between many of these transcription factors. GPR30 itself may be part of a positive reinforcement loop as we have found that its expression is induced through MAPK and c-Fos in response to the growth factor EGF (Albanito *et al*, 2008). It remains to be seen whether these mechanisms, possibly involving other transcription factors mentioned above, also operate in response to GRP30 signalling, which incidentally depends on the EGF receptor (Filardo *et al*, 2000).

Considering the GPR30 signalling pathway, it is not surprising that the GPR30 response resembles the well-characterized transcriptional 'immediate early response' of fibroblasts to serum (Iyer *et al*, 1999). The strong induction of *CTGF* is consistent with the following: (i) *CTGF* is known to be activated by a panel of different extracellular signals and its promoter contains binding sites for transcription factors of the 'immediate early response', notably SRF, ETS, ATF, AP1, and TEAD2/ETF (Leask and Abraham, 2006; Cooper *et al*, 2007; Figure 4A); (ii) the inhibitory effect of a dominant-negative Fos points to a role for AP1, and additional preliminary functional results suggest that ETS and SRF are also required; and (iii) the activation of MAPK and actin dynamics are essential for the activation of both the full complement of these transcription factors and *CTGF*.

GPR30 expression and signalling in carcinomas

Activation of GPR30 by oestrogens elicits proliferative responses of breast and other carcinomas (Vivacqua *et al*, 2006b; Albanito *et al*, 2007, 2008). Although GPR30 is also expressed in normal breast epithelium, a survey of 321 primary breast tumours showed that 60% maintain GPR30 expression, including half of all ER-negative tumours (Filardo *et al*, 2006, 2008). Unlike ERα, the expression of which correlates with good prognosis, GPR30 expression was found to correlate very strongly with tumour size, HER-2 expression and distant metastasis. Remarkably, another study on breast cancer failed to find a similar correlation (Kuo *et al*, 2007), but methodological differences and a smaller cohort compared with the aforementioned studies call for more investigations. In the meantime, it is noteworthy that GPR30 expression was also associated with poor prognosis in a survey of endometrial carcinomas (Smith *et al*, 2007). Even in GPR30-positive cells, GPR30 is not expressed abundantly at the mRNA and protein levels (data not shown). Rather than monitoring only the expression of GPR30 itself, it might be more informative to combine it with measurements of the expression of a set of GPR30 target genes. Hence, our results highlight a set of GPR30 target genes that might both provide a signature for GPR30 signalling and a mechanistic underpinning of the aforementioned phenomena.

GPR30 target genes and cancer

Our experiments with SKBr3 cells demonstrate that CTGF is necessary for the stimulation of proliferation and migration by GPR30 signalling. Although CTGF is sufficient to stimulate migration, steady-state levels of CTGF might normally be sufficient to sustain a basal level of proliferation. The stimulation of proliferation by GPR30 signalling might arise from the combined induction of *CTGF* with multiple other GPR30 target genes. How the highly transient activation of GPR30 target genes that we have observed at the mRNA level is

converted into this long-term response remains to be further analysed. Perhaps not a single, but repeated pulses of GPR30 signalling may be sufficient to produce relatively persistent higher levels of key proteins such as CTGF.

CTGF defines the cystein knot family of proteins along with Cyr61 (also known as CCN1) and Nov. Note that *CYR61* is also induced by GPR30 signalling in SKBr3 cells, albeit much less than *CTGF* (Figures 1 and 2; Supplementary Table 1). Cyr61 and CTGF bind integrins and heparan sulphate-containing proteoglycans. CTGF also binds receptors or co-receptors for other signalling molecules such as Wnt, and CCN proteins even bind growth factors and cytokines themselves. These findings have led to the notion that CCN proteins exert their biological functions by modifying the action of other signals and the interactions with the extracellular matrix (reviewed by Rachfal and Brigstock, 2005; Leask and Abraham, 2006; De Winter *et al*, 2008; Holbourn *et al*, 2008). This may explain why the effects of CCN proteins are exquisitely dependent on cell type and experimental conditions, and why they may at times be seemingly contradictory. For example, low concentrations of CTGF can promote angiogenesis, but high concentrations inhibit the angiogenic effects of VEGF (Inoki *et al*, 2002). The evidence for an implication of CTGF in breast cancer is equally confusing. Although we have observed stimulatory effects of CTGF with SKBr3 cells, CTGF was shown to mediate TGFβ-induced apoptosis of MCF7 cells (Hishikawa *et al*, 1999). Nevertheless, *CTGF* was found to be part of a gene expression signature of osteolytic metastatic variants of the ER-negative breast cancer cell line MDA-MB-231, and to contribute to metastasis upon overexpression (Kang *et al*, 2003). A recent study confirmed this association and notably demonstrated that the overexpression of the genome organizer protein SATB1, which is overexpressed by aggressive breast cancer cells, induces CTGF expression (Han *et al*, 2008). In contrast, in a survey of 122 human breast tumours, Cyr61 and CTGF were found to be oppositely correlated with poor outcome, being high and low, respectively (Jiang *et al*, 2004). Elevated Cyr61 and CTGF levels have been reported to be characteristic of a number of other carcinomas (Bleau *et al*, 2005; Rachfal and Brigstock, 2005; Deng *et al*, 2007; Liu *et al*, 2008; Mullis *et al*, 2008). Whether the ERα status of breast tumours and cell lines may influence the effects of CCN proteins and account for some of the apparent discrepancies remains to be investigated.

There is yet another set of GPR30 target genes, the expression of which may contribute to breast cancer progression. Of the 11 most strongly induced genes, 5 are metallothionein genes. These include four members of the *MT1* gene cluster and one, *MT2A*, from another chromosome. Interestingly, high expression of metallothioneins in breast tumours correlates with poor outcome, perhaps owing to their protective and proliferative functions (Bay *et al*, 2006).

Relevance to tamoxifen resistance of breast cancer and to physiology

Tamoxifen is used clinically for the endocrine treatment of ERα-positive breast cancer. How and why many of these tumours ultimately become resistant to this therapy remain poorly understood (reviewed by Herynk and Fuqua, 2007; Riggins *et al*, 2007). On the basis of our findings, we would suggest that GPR30 continues to mediate stimulatory oestrogen signals for proliferation and migration even in tumours that lose ERα expression. Indeed, GPR30 may become a risk factor in patients that are treated with tamoxifen as OHT is a potent GPR30 agonist. Thus, for a subset of breast tumours as well as other carcinomas, perhaps independently of ERα status, activation of GPR30 might actually promote poor outcome by stimulating tissue invasion and remodelling through CTGF. GPR30 antagonists, which are not yet available, could be of great therapeutic value. As our results with primary fibroblasts indicate, the stroma could contribute to relaying stimulatory signals of oestrogens or OHT to breast carcinoma cells, again independently of ERα status. It has been increasingly recognized that there is a decisive interplay between cancer cells and stroma (Bhowmick and Moses, 2005; Finak *et al*, 2008). Future studies should therefore include the evaluation of GPR30 levels in the cancer-associated stroma.

Although our transcriptome analysis was carried out with a breast cancer cell line, its results, which we have partially confirmed with primary fibroblasts, may be of heuristic value for understanding other biological GPR30 functions. The induction of a network of transcription factors and target genes such as *CTGF*, *CYR61* and metallothioneins may be relevant to the wide range of GPR30 functions currently being unravelled in thymic atrophy (Wang *et al*, 2008a), mechanical hyperalgesia (Kuhn *et al*, 2008), liver injury (Hsieh *et al*, 2007), in the hypothalamus (Qiu *et al*, 2006; Brailoiu *et al*, 2007; Canonaco *et al*, 2008), for primordial follicle formation (Wang *et al*, 2008b), and for the proliferation of urothelial cells (Teng *et al*, 2008) and osteoblasts (Teplyuk *et al*, 2008).

Materials and methods

Cell culture

SKBr3 breast cancer cells were maintained in RPMI-1640 without phenol red supplemented with 10% fetal bovine serum (FBS). MCF7 and BT-20 breast cancer cells were cultured in Dulbecco's modified Eagle's medium and MEM, respectively, supplemented with 10% FBS. At 24 h prior to induction experiments, MCF7 and BT-20 cells were switched to a medium without serum and without phenol red. Lentiviruses were produced by transient co-transfection of lentiviral shRNA constructs with the VSV-G envelope plasmid pMD2G and the packaging plasmid psPAX2 (gifts from Dr D Trono; see http://tronolab.epfl.ch) into 293T cells. SKBr3 cells with a stable knockdown of *CTGF* were generated by lentiviral transduction of an shRNA construct (see below). In parallel, we obtained cells stably transduced with an unrelated control shRNA. To this end, cells were consecutively infected twice overnight, washed twice and subjected to a selection with 3 μg/ml puromycin for at least 24 h to eliminate the small fraction of non-infected cells.

Generation of primary fibroblast cells from breast cancer tissues

Breast cancer specimens were collected from primary tumours of patients who signed informed consent and underwent surgery before any pharmacological treatment. Following tumour excision, small pieces (1–2 mm diameter) were placed in a digestion solution (400 IU collagenase, 100 IU hyalurodinase and 20% FBS in Hank's balanced salt solution; all components from Sigma) containing antibiotics and antimycotics (Sigma) and incubated at 37°C with 5% CO_2 for 5–6 h. After centrifugation at 90 *g* for 2 min, the supernatant containing fibroblast cells was centrifuged at 500 *g* for 8 min, then resuspended and cultured in RPMI-1640 medium supplemented with 20% FBS, antibiotics and antimycotics.

Plasmids

The *GPR30* knockdown for the microarray analysis was carried out with the plasmid GPR30/AS (Revankar *et al*, 2005) using the related empty expression vector pRK5 (Schall *et al*, 1990) as a negative control. The *CTGF* luciferase reporter plasmid p(−1999/+36)-luc

(Chaqour *et al*, 2006), which is based on the backbone of vector pGL3-basic (Promega), was a gift from Dr B Chaqour. The plasmid A-FOS, which encodes a c-Fos mutant that heterodimerizes with c-Fos dimerization partners but does not allow DNA binding (Gerdes *et al*, 2006), was obtained from Dr C Vinson. We have previously reported the characteristics and the evaluation of an shRNA construct to knock down the expression of *GPR30* and of an unrelated shRNA control construct (Albanito *et al*, 2008). The shRNA construct for *CTGF* was obtained from the same supplier (Open Biosystems; www.Biocat.de). It has clone ID TRCN0000061950 and is based on the same lentiviral expression vector pLKO.1 as the other shRNA constructs. The targeting strand generated from the *CTGF* shRNA construct is TAGTACAGCGATT CAAAGATG. The expression vector for Flag-tagged human GPR30 has been described (Albanito *et al*, 2008). It was used to generate the GPR30 rescue vector containing silent mutations in the shRNA-targeted sequence: codons 293–297 were changed to CCG TGT AAA CAA AGT (changes underlined).

Microarray analysis

SKBr3 cells were transiently transfected either with the empty expression vector pRK5 or with the plasmid GPR30/AS using the FuGENE 6 transfection reagent (Roche) at 3 μl/μg DNA (5 μg DNA per 10 cm dish) in a medium with only 1% FBS. At 36 h after transfection, cells were switched to serum-free medium for 12 h before induction with 100 nM E2 or 10 μM OHT for 1 h. Total RNA from triplicate samples was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. RNA quality was assessed with a BioAnalyzer (Bio-Rad). All RNA samples were stored at −80°C until required for further processing. Expression profiles were determined with HumanWG-6 v2.0 BeadChip microarrays (Illumina) at the genomics platform of the University of Geneva. The data have been submitted to GEO (access code GSE11567).

Promoter sequence analysis

Sequence retrieval: 5000 nucleotide sequences from upstream of the transcription start sites of the 34 genes in Supplementary Table 1 with a RefSeq accession number were retrieved from the UCSC genome browser (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/bigZips/upstream5000.fa.gz). Similarly, a large set of promoter sequences, to be used as a background/control sequences, was retrieved from http://ani.embl.de/trawler. Transcription factor identification: TRANSFAC Professional 11.4 was downloaded from Biobase (http://www.biobase-international.com) and installed locally on a computer running Ubuntu. The set of all vertebrate transcription factor-binding sites was retrieved from matrix.dat in TRANSFAC. For these factors, a number of profiles were generated with matrix similarity ranging from 0.8 to 1.00 and core similarity ranging from 0.8 to 1.00. The profile minFP to minimize false positives was generated from MATCH on the website. MATCH was used locally to identify transcription factor-binding sites in the set of promoter sequences with the profile minFP. Thereafter, F-MATCH was used with a cutoff *P*-value of 0.001 to identify over-represented transcription factor-binding sites. These transcription factor-binding sites were sorted by their relative over-representation in the target sequence set. To retrieve the predicted sites for 5000 bp of *CTGF* upstream sequences, the profile with 0.95/0.95 matrix/core similarity was used.

PCR analyses

Quantitative real-time PCR analysis was performed for the triplicate RNA samples used for the microarray experiment. To identify appropriate control genes for standardization, the analysis was performed with three candidates that had proved useful in many other gene expression analyses (*TFRC*, *ALAS1* and *TBP*). As *TFRC* and *ALAS1* varied the least across all sample, all measurements were normalized to the geometric mean of these two control genes. Semiquantitative RT–PCR was carried out as described (Maggiolini *et al*, 1999) with total RNA from cells induced for 1 h. *FOS*, *CTGF* and the internal control *RPLP0* (also known as 36B4) cDNAs yielded bands of 420, 392 and 408 bp with 20, 20 and 10 PCR cycles, respectively. The primers are listed in Supplementary Table 3.

Immunoblotting

Total protein extracts were prepared from cells after a 2-h induction. For inhibition experiments with shRNAs or the dominant-negative c-Fos variant, cells were transiently transfected in a medium without serum and induced 24 h later. CTGF, β-actin and GPR30 were revealed by immunoblotting with the goat polyclonal antiserum sc-14939, the mouse monoclonal antibody sc-8432 (Santa Cruz Biotechnology) and the rabbit polyclonal antiserum LS-A1183 (MBL-Eppendorf), respectively.

Reporter gene assay

The *CTGF* luciferase construct was co-transfected with a *Renilla* luciferase expression plasmid as an internal transfection control into SKBr3 cells as described above for the microarray analysis. Induction with 100 nM E2 was carried out overnight. Luciferase activities of triplicate samples were measured with the Dual-Luciferase Reporter Assay system (Promega).

Migration and proliferation assays

Migration assays were performed with SKBr3 cells in triplicate using Boyden chambers (Costar Transwell, 8 μm polycarbonate membrane). For knockdown experiments, cells were transfected with shRNA constructs directed against GPR30 or *CTGF* or with an unrelated shRNA construct (3 μg DNA with FuGENE 6 at a 3 μl to 1 μg DNA ratio in 60 mm plates) in standard growth medium. After 24 h, they were seeded in the upper chambers. OHT or CTGF was added to the medium without serum in the bottom wells. After 3 h, cells on the bottom side of the membrane were fixed and counted. Human CTGF was purchased from MBL and added at 100 ng/ml. For proliferation assays, SKBr3 cells with a stable *CTGF* (or control) knockdown were cultured in a medium supplemented with 5% charcoal-treated FBS. Where indicated, CTGF was added each day. Cells were counted at day 6.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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A sexually dimorphic distribution pattern of the novel estrogen receptor G-protein-coupled receptor 30 in some brain areas of the hamster

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Abstract

The isolation of the G-protein-coupled receptor 30 (GPR30), an orphan membrane receptor unrelated to nuclear estrogen receptors (ERs), has become a key factor towards the unraveling of rapid estrogen action. This membrane receptor together with cellular signaling intermediaries, i.e., extracellular signal-dependent kinases 1 and 2, may promote neuronal proliferation and differentiation activities. In the present study, an evident gene expression pattern of GPR30 characterized postnatal 7 (young) and 60 (adult) days of age hamsters as shown by its heterogeneous mRNA distribution in hypothalamic, amygdalar and cerebellar areas of both sexes. In particular, most of the brain areas considered in the adult hamster plus only the amygdala and cerebellum of young animals behaved in a sexually dimorphic fashion. This similar pattern was also detected for the ERα and β, as shown by the latter receptor prevailing in young and adult females, while the former predominated in young females. Even for the two kinases, a sexually dimorphic distribution was featured above all for young hamsters. Overall, the findings of the present study established a distinct expression pattern of the novel ER (GPR30) that may operate differently in some brain areas of the hamster and this may provide interesting insights regarding its probable neuroprotective role during the execution of some hibernating states, which are typical of our rodent model.
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Introduction

A plethora of evidence has demonstrated that estrogens are involved in many physiological processes in mammals, including developmental features, cellular homeostasis and neurobiological activities (Kow *et al.* 2005, Simpson *et al.* 2005) as well as pathological conditions (Maggiolini *et al.* 2004). Recently, in addition to the classical genomic mechanisms of action of intracellular estrogen receptor (ER) α and β, some studies have begun to emphasize the importance of a rapid non-genomic steroid action by the binding of 17β-estradiol (E_2) to a G-protein-coupled receptor 30 named GPR30. Although it is often straightforward to link the physiological effects of E_2 to a genomic model, considerable controversy still exists on its ability to elicit transcriptional responses independently of the classical nuclear receptor isoforms. On the basis of such observations, growing attention is beginning to be focused on some major health conditions such as tumor formations in which estrogen-dependent activities in the absence of ERs seem to predominately operate through GPR30 (Filardo & Thomas 2005). At the brain level, an elevated number of estrogen-dependent neuronal actions in areas such as hypothalamus (HTH), which is noted for its estrogen-enriched properties (McEwen 1991), seem to underlie GPR30 as a key factor for rapid cerebral estrogen actions (Canonaco *et al.* 2002). Recently, studies have pointed to the specific activity of GPR30 being tightly linked to some cellular signaling intermediaries, such as extracellular signal-related kinase (ERK) 1/2, since estrogens are able to induce their activities even in the absence of ERs (Sweatt 2004) and the inhibition of these factors by the rapid signaling cascades seems to decrease the potentiation of neuronal transcriptional activities (Vasudevan *et al.* 2005). Moreover, the aforementioned signaling intermediaries that seem to overlap the expression pattern of GPR30 throughout the brain have been reported to regulate neuronal proliferation, differentiation and postsynaptic processing. In this context, estrogen-induced neuronal signals seem to facilitate early ERK-dependent migration of brain elements and namely neurons and glia (Zsarnovszky & Belcher 2004).

On the basis of these neuronal characteristics, it was our intention to evaluate the sexually dimorphic distribution pattern of GPR30 in some brain areas of young postnatal day 7 (PND7) and 60-day-old (PND60, adults) golden hamster (*Mesocricetus auratus*), a hibernating rodent. In addition, this pattern was correlated to that

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of both ERα and ERβ as well as ERK1/2 considering the protective roles exerted by these factors against excitotoxicity events in cortical and hippocampal neurons (Nilsen & Diaz 2003). Overall, the resulting sexually dimorphic distribution pattern of GPR30 expressing neurons could nicely represent a starting point regarding its role not only on estrogen functions in the absence of ERs, but also on ischemic-like events that are evoked in hibernators during arousal (Canonaco *et al.* 2005).

Materials and Methods

Animals and dissections of brain regions

Sexually mature female ($n=6$) and male ($n=6$) golden hamsters (*M. auratus*) at PND60 (adults) plus female ($n=10$) and male ($n=10$) pups at PND7 were purchased from Charles River (Como, Italy). They were housed in the stabularium of the Cellular Biology Department (University of Calabria, Cosenza, Italy) and maintained at a 14 h light:10 h darkness schedule. Animal maintenance and experimental procedures were in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals. Efforts were made to minimize animal suffering and reduce the number of specimens used. The brain areas, such as amygdala (AMY), hypothalamus (HTH), thalamus (TH), and cerebellum (Cb), of both sexes at PND60, and at PND7 ages were dissected from the brain of anesthetized decapitated hamsters. For this part, a dissecting microscope was used in order to carefully isolate and remove these specific brain areas that by placing the brain dorsal side up on filter paper moistened with ice-cold RNAase-free PBS it was possible to view them. All operations were handled with sterilized surgical instruments that were cleaned with RNAaseZap to assure no contamination of these brain areas. The Cb was the first area to be removed because by making a coronal cut along the mesencephalic portion behind the inferior colliculi, it was possible to easily remove this rhombencephalic area according to its anatomical position reported in the stereotaxic atlas of Paxinos & Watson (1982). Maintaining the brain in the same position and using this atlas, two coronal cuts were made just behind the rostral and caudal limits of the diencephalic area to assure the removal of HTH and TH, while the AMY was removed by making cuts along the lateral portions of both right and left hemispheres of the rostral–caudal block. Once these areas were removed, they were quickly frozen at −80 °C.

Selection of primers

Primers used for RT-PCR were designed on the basis of rat gene sequences: ERα (accession AF181077) forward 5′-CGCGCCTACGAGTTCAAC-3′ and reverse 5′-CTCTTAAAGAAAGCCTTGCAGCC-3′; ERβ (accession AB076607) forward 5′-CTATGCAGAACCTCAAAAGAGT CC-3′ and reverse 5′-TTCGTGGCTGGACAGATATAATC-3′; GPR30 (accession DQ237895) forward 5′-GTGGCCGACTCCCTGA TCG-3′ and reverse 5′-CGG GCATGGTGCTTGGTGC-3′; ERK1 (accession S46779) forward 5′-CCTCCAATCTGCTTATCA AC-3′ and reverse 5′-GGGCCTTCATGTTAATGATA-3′; ERK2 (accession M64300) forward 5′-TGGTACAGAGCTCCAGAAAT-3′ and reverse 5′-GAGCCTGTTCAACTTCAATC-3′; the housekeeping gene 36B4 forward 5′-CTCA ACATCTCCCCCTTCTC-3′ and reverse 5′-CAAATCCCATATCCTCGTCC-3′.

RNA extraction and RT-PCR

Total RNA was extracted from the above-mentioned dissected brain areas of every animal using Triazol reagent as suggested by the manufacturer (Invitrogen). The purity and the integrity of RNA were checked by gel electrophoresis before carrying out any analytical procedure. To determine even low gene expression, PCR was performed for 40 cycles using the above primers. The cycles were for ERα/β: 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, for GPR30: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, for ERK1/2: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, for the housekeeping gene 36B4: 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, as illustrated in all figure panels of representative analyses. To check for the presence of DNA contamination, a RT-PCR was performed using 1 μg total RNA without Moloney murine leukemia virus reverse transcriptase (negative control). The PCR products were analyzed on 1·5% agarose gel and stained with ethidium bromide. At first, all fragments of DNA amplified were cloned and sequenced to verify their exact corresponding sequences, which showed a high homology (>80%) between the rat sequence and the hamster for all genes considered in this study (data not shown).

Statistical analysis

DNA quantity in each lane was analyzed by scanning densitometry and optical density (OD; ±S.E.M.) were evaluated via computer-assisted image analyzer system (National Institutes of Health-Scion Image 2.0). The different expression levels were compared between the two animal sexes of the same PND age using one-way ANOVA plus *post hoc* Student's *t*-test, ⋆$P<0{\cdot}05$ (moderately significant); ⋆⋆$P<0{\cdot}01$ (significant); ⋆⋆⋆$P<0{\cdot}001$ (very robust).

Results

Distribution of ERα/β and GPR30

The application of primers for ERα/β and GPR30 supplied an age-dependent sexually dimorphic pattern of their gene expression. In particular, the notable OD for both ERs (Fig. 1a–d) when compared with housekeeping gene (Fig. 1e) allowed us to show that an early (PND7) moderately significant ($P<0{\cdot}05$) expression capacity characterized both

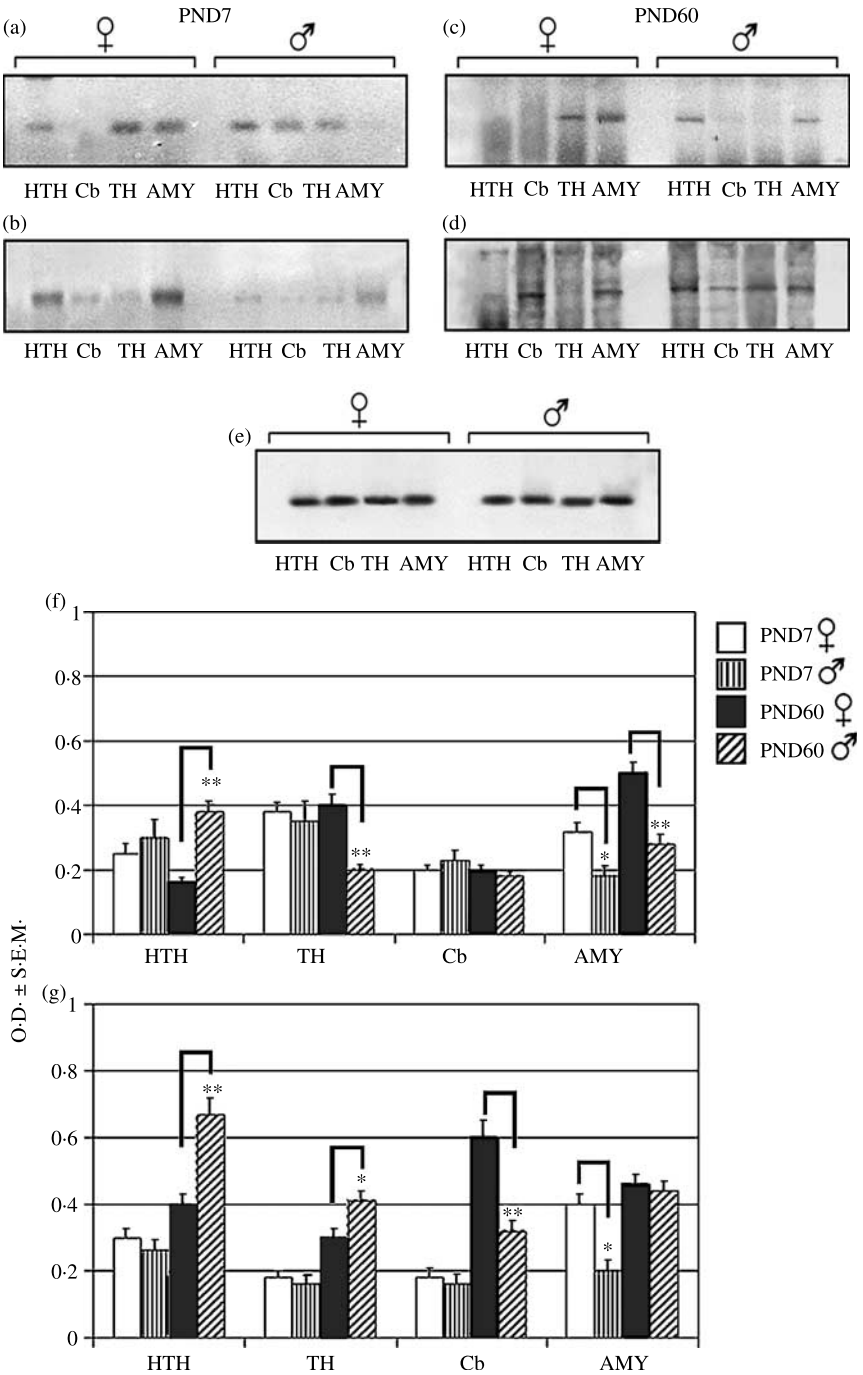

Figure 1 Sexually dimorphic gene expression of (a and c) ERβ and (b and d) ERα was performed in some brain areas of (a and b) PND7 and (c and d) PND60 *Mesocricetus auratus* by RT-PCR. The control (e) for this and other signaling factors was determined by the application of the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (f) ERβ and (g) ERα in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of *Mesocricetus auratus*. Data obtained by RT-PCR were calculated as optical density (O.D. ± S.E.M.) via computer-assisted image analyzer system (National Institutes of Health – Scion Image 2.0). Statistical analysis: ANOVA plus *post hoc* test (Neuman–Keul's multiple range test) were used, $*P<0{\cdot}05$; $**P<0{\cdot}01$; $***P<0{\cdot}001$.

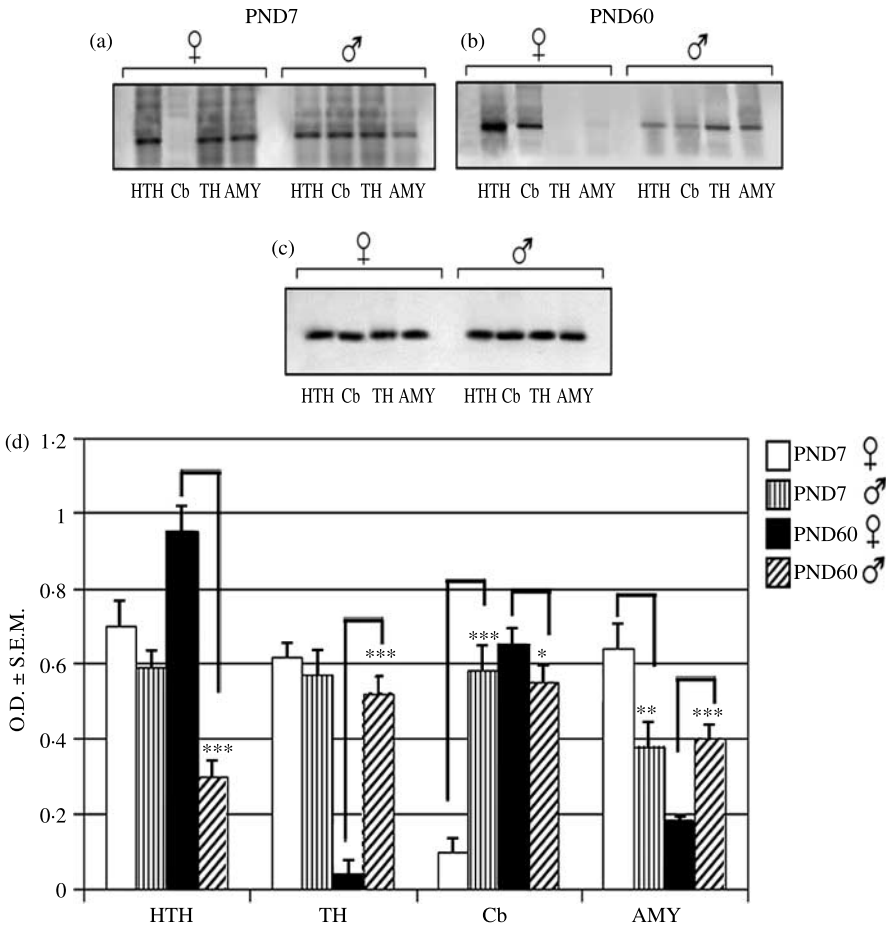

Figure 2 Sexually dimorphic gene expression of GPR30 was investigated in the same brain areas as for the ER study in (a) PND7 and (b) PND60 *Mesocricetus auratus* by RT-PCR. Similarly the control (c) for this receptor was determined by applying the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (d) GPR30 in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of *Mesocricetus auratus*. Data obtained by RT-PCR were calculated as O.D. (±S.E.M.) via computer-assisted image analyzer system. Statistical analysis: ANOVA plus *post hoc* test (Neuman–Keul's multiple range test) were applied, $*P<0{\cdot}05$; $**P<0{\cdot}01$; $***P<0{\cdot}001$.

ERβ (Fig. 1f) and α (Fig. 1g) of the female AMY with respect to that of the male. In adult males, a significant ($P<0{\cdot}01$) expression capacity was detected in HTH for both ERs, apart moderately significant expression levels of ERα in TH, while significant expression levels were typical of ERβ and ERα in the female AMY and Cb respectively.

Interestingly enough, even GPR30 supplied a heterogeneous expression pattern in the different hamster brain areas (Fig. 2a and b), as reported for PND7 male which exhibited a very robust ($P<0{\cdot}001$) GPR30 expression level in Cb, while a significant expression signal was detected in AMY of the female hamster (Fig. 2d). In line with the expression pattern of the two ERs, also the greater expression differences of GPR30 appeared to preferentially occur in adult hamsters as shown by the very robust and moderately significant mRNA levels of this membrane receptor in HTH and Cb of the female respectively. On the other hand, a very robust expression signal was instead obtained in both TH and AMY of male adults (Fig. 2d).

When the fitting of GPR30 and the two ERs were handled on representative schemes of medial-posterior telencephalic and diencephalic areas (Fig. 3), it was possible to observe a sexually dimorphic distribution pattern of these two classes of receptors as early as the developmental stages. In a first case, overlapping dense quantities of GPR30 plus ERα and β were demonstrated in AMY and HTH of both animal sex at PND7, aside the lack of ERα in the latter brain area of the male hamster (Fig. 3a and b). However, an evident sexually dimorphic pattern was mostly typical of adults. Indeed, GPR30 was densely located in the female HTH, while moderate and low levels were instead observed in the male AMY and HTH respectively (Fig. 3c and d). Concerning the ERs, the α isoform seemed to be densely located in the different brain areas of the male adult hamster, despite

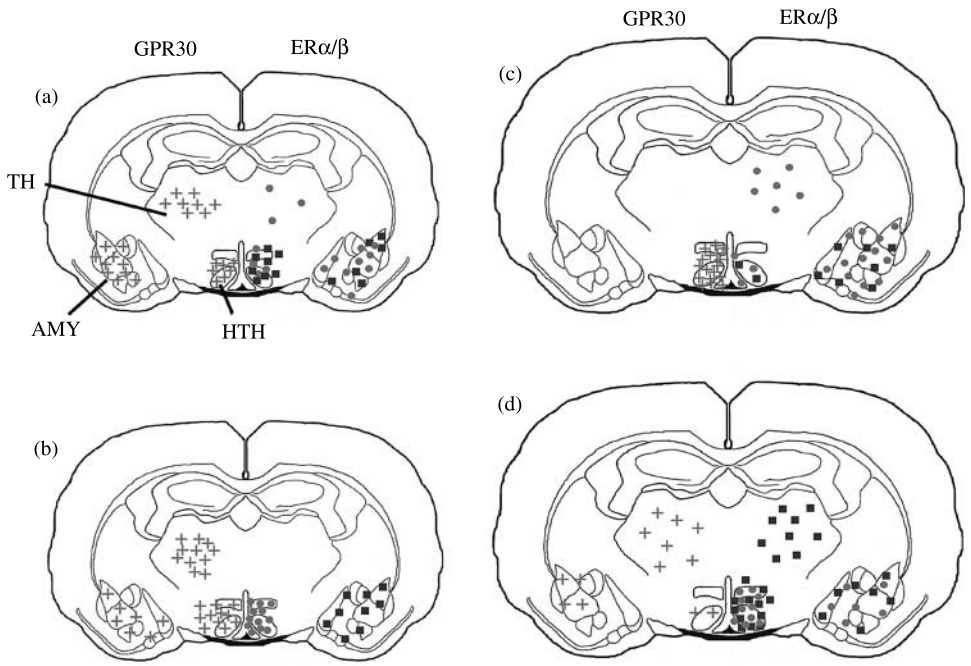

Figure 3 Sexually dimorphic distribution patterns of ERα (square), ERβ (circle), and GPR30 (cross) were illustrated in representative schemes in (a and c) female and (b and d) male medial-posterior hamster brain sections for (a and b) PND7 and (c and d) PND60. The expression signals were categorized as follows: dense O.D. (>100%)=>8 symbols; moderate O.D. (>60<100%)=>4<8 symbols; low O.D. to no signal (>0<60%)=>0<4 symbols.

the moderate and low levels being typical of the female AMY and HTH respectively. In this same animal sex, dense ERβ levels are favorably located in TH and AMY.

Distribution of ERK1/2

Given that GPR30 functions are tightly linked to ERK1/2, the distribution pattern of these two factors was also taken into consideration. Overall, a heterogeneous pattern was detected in both developmental stages. In particular, elevated ERK1/2 signals were shown in male and female PND7 animals, while lower levels characterized both adult animal sexes (Fig. 4a–d). In spite of the very robust signal registered for ERK1 in the PND7 male Cb with respect to the same brain areas of the female (Fig. 4f), it was the diencephalic areas such as HTH and TH of PND7 female that supplied very robust and moderately significant expression levels respectively. This feature was still maintained in the adult female hamster, as demonstrated by very robust expression levels in HTH and TH of this sex with respect to the same male brain areas (Fig. 4f). In a comparable manner, also the expression of the other signal factor ERK2 exhibited an evident sexually dimorphic pattern in PND7 animals, as shown by very robust levels in the male HTH and AMY (Fig. 4g). On the other hand, very robust and moderately significant signals were typical of female TH and Cb respectively. Nevertheless, a consistent sexually dimorphic expression pattern of ERK2 like ERK1 was not a very common feature of adults since a very robust signal was only observed in TH of male with respect to that of the female (Fig. 4g).

Discussion

This first comprehensive study of GPR30, ERα/β, and ERK1/2 in some brain areas of young and adult hamsters displayed an evident sexually dimorphic expression pattern, suggesting that hibernators could very well be a useful model for unveiling the sexually differentiated neurobiological role of the classical action of estrogens, i.e., classical versus rapid non-genomic actions. At present, mechanisms dealing with estrogen-mediated cellular responses are becoming increasing complex, especially if we consider the recent involvement of GPR30 activity in human cancer cells of reproductive organs (Maggiolini *et al.* 2004, Vivacqua *et al.* 2006, Albanito *et al.* 2007). The functional role of GPR30 has recently been also extended to a wide range of neuronal functions, namely gene expression, differentiation, neuroprotection plus neuroendocrine secretion (Qiu *et al.* 2006). In line with these observations, GPR30 may be considered a novel ER, which could readily elicit responses to estrogens during ischemia crisis observed during neurodegenerative syndromes.

Indeed, the results of the current study highlighting a differentiated distribution pattern of this orphan membrane

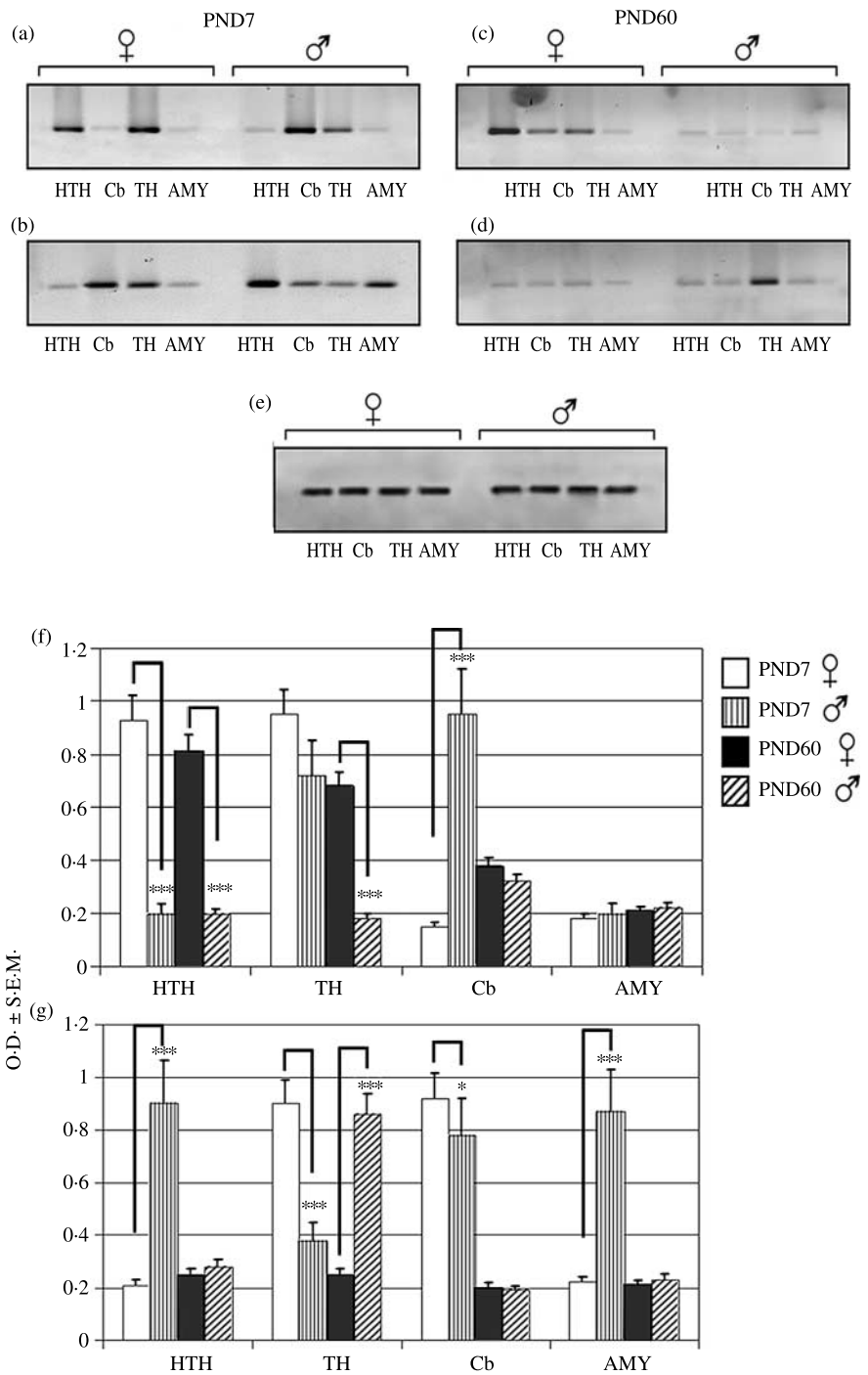

Figure 4 RT-PCR was also used to evaluate the sexually dimorphic gene expression of (a and b) ERK1 and (c and d) ERK2 in the same brain areas as for the ER study of (a and c) PND7 and (b and d) adult *Mesocricetus auratus*. The control (e) for these kinases was determined by the application of the housekeeping gene encoding the ribosomal protein 36B4. The expression levels of (f) ERK1 and (g) ERK2 in some brain regions (HTH, Cb, TH, and AMY) of PND7 and PND60 in female and male of *Mesocricetus auratus*. Data obtained by RT-PCR were expressed as O.D. (±S.E.M.) and elaborated via computer-assisted image analyzer system. Statistical analysis: ANOVA plus *post hoc* test (Neuman–Keul's multiple range test) were used, $*P<0{\cdot}05$; $**P<0{\cdot}01$; $***P<0{\cdot}001$.

receptor in estrogen-dense brain sites and areas lacking ERs (Simerly *et al.* 1990) such as HTH and AMY (McEwen 1991) of young hamsters suggest an early non-genomic type of estrogen effects occurring via GPR30 sites. It is not surprising that GPR30 is operating during developmental stages because recently some authors have begun to link its activity to stressful conditions which may lead to eventual neurogenic and apoptotic effects at an early biological stage (Filardo & Thomas 2005). The notable expression levels occurring in HTH and AMY is in a good agreement with the dense levels observed in the former brain area of male and female rats (Brailoiu *et al.* 2007) and this could begin to point to GPR30 as an alternative mediator of ERα/β-dependent biological functions (Perèz *et al.* 2003) plus its involvement on the regulation of energy homeostasis at the HTH (Qiu *et al.* 2006) of hibernators. Moreover, the response of some major hypothalamic nuclei, namely the ventromedial hypothalamic nucleus to rapid estrogen signals via the activation of GPR30 might be responsible for the triggering of specific cell factors (kinases) which in turn decreases the potentiation of transcriptional activities thus accounting for altered lordosis behavior (Vasudevan *et al.* 2005). In the case of AMY, a great density of GPR30 expressing neurons was detected in young female hamsters, while this effect subsequently predominated in male adults. The fact that this limbic area displays such sexually dimorphic differences in an age-dependent manner tends to support amygdalar neurosteroidal mechanisms of GPR30 provoking greater mood disorders in females (Walf *et al.* 2006).

It is worthy to note that an overlapping distribution pattern of ERK1/2 to that of GPR30 occurred in most brain areas of our hibernating model. This relationship should not be considered an astonishing phenomenon especially since these two kinases mediate signals in mostly all estrogenic functions (Zsarnovszky & Belcher 2004), such as the remodeling and protection of vascular cells (Chambliss *et al.* 2005) and during anti-apoptotic processes in neuronal cells (Koriyama *et al.* 2003). A very great expression for both kinases in HTH was typical of AMY and Cb of young animals and appeared to be markedly lower in adults, in agreement with the early ERKs high embryonic levels reported in rat Cb (Simerly *et al.* 1990). On the other hand, elevated levels of the two kinases in adult HTH and TH seem to fit well with their role on endocrine-related activities in hamsters such as satiety and on visual functions respectively (Coogan & Piggins 2005).

In a comparable fashion, even the distribution of the classical ERs exhibited an evident sexually dimorphic pattern for both developmental periods. Interestingly, the prevalence of ERβ in most female brain areas, while α predominates in males are, to a large extent, supported by the pattern obtained for the rat (Perèz *et al.* 2003). However, despite the different developmental periods in which these isoforms prevail, they definitely seem to have a great bearing on brain morpho-functional features as suggested by ERα promoting a numerically elevated quantity of dendritic spines and axospinous synapses (Simerly *et al.* 1990). AMY is the other brain area that demonstrated a sexually dimorphic pattern, i.e., the prevalence of ERβ in young females, which is an overlapping condition of high levels detected in the rat lateral amygdalar area (Zhang *et al.* 2002). It is very likely that the distribution discrepancy of these two subtypes could be of a species-specificity nature as supported by male ewe hypothalamic areas containing greater densities of α isoform (Scott *et al.* 2000). However, independently of the different developmental periods in which the two ERs interact, they seem to play a key role on brain morpho-functional features as indicated by ERα being responsible for the elevated number and density of dendritic spines and axospinous synapses (Adams *et al.* 2002).

Overall, the sexually dimorphic distribution of GPR30 in some hamster limbic areas proposes this receptor as an important mediator of estrogen-dependent biological events. This feature is sustained by the expression of GPR30 not only in ER-enriched areas and so tends to further corroborate its critical role in the estrogen actions above all in facultative hibernators. Studies in our animal model are only at the beginning and further interests especially during the different hibernating states could prove to be useful for the unveiling of pathological processes such as ischemia that occurs during the arousal phase (Canonaco *et al.* 2005) of this particular physiological condition.

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Disclosure

The authors declare that no conflict of interest that would prejudice its impartiality in part or as whole.

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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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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 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 extracellular signal-regulated kinase (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–epidermal growth factor receptor 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.

Key words: 17β-estradiol, atrazine, estrogen receptor, GPR30, ovarian cancer cells. *Environ Health Perspect* 116:1648–1655 (2008). doi:10.1289/ehp.11297 available via *http://dx.doi.org/* [Online 22 July 2008]

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). The interference of atrazine with androgen and estrogen action does not occur by direct agonism or antagonism of cognate receptors for these steroids as shown by binding affinity studies (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) and 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 that 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 (Pintér et al. 1990), whereas 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 and Sonnenschein 1975).

Given the potential ability of atrazine to interfere with reproduction and to cause cancer, the European Union banned its use. However, the U.S. Environmental Protection Agency has approved the use of atrazine because of the lack of a clear association between the levels of exposure and cancer incidence in pesticide applicators (Gammon et al. 2005; McElroy et al. 2007; Rusiecki et al. 2004; Sass and Colangelo 2006; Young et al. 2005).

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 or 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 physiologic 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 [^{3}H]17β-estradiol ([^{3}H]E_2) 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 are still controversial in terms of their specific molecular interactions with endogenous and environmental estrogens.

A seven-transmembrane receptor, G-protein–coupled receptor 30 (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

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characteristics of a functional estrogen membrane receptor (Thomas et al. 2005; Thomas and Dong 2006). Our studies and others have also demonstrated that GPR30 mediates the rapid response to E_2 in a variety of estrogen-responsive cancer cells by activating the epidermal growth factor receptor (EGFR)–mitogen-activated protein kinase (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 have demonstrated 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. We purchased atrazine [2-chloro-4-(ethylamine)-6-(isopropylamine)-*s*-triazine], 17β-estradiol (E_2), *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89), wortmannin (WM), and PD98059 (PD) from Sigma-Aldrich (Milan, Italy); AG1478 (AG) from Biomol Research Laboratories (DBA, Milan, Italy); ICI 182,780 (ICI) from Tocris Chemicals (Bristol, UK); and GF109203X (GFX) from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in dimethyl sulfoxide (DMSO), except E_2 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 phenol red–free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). H295R adrenal carcinoma cells were cultured in DMEM/F12 1:1 supplemented with 1% ITS Liquid Media Supplement (Sigma-Aldrich), 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 phenol red–free RPMI 1640 supplemented with 10% FBS. Cells were switched to medium without serum the day before experiments for immunoblots and reverse transcription-polymerase chain reaction (RT-PCR).

Plasmids. Firefly luciferase reporter plasmids used were XETL for ERα (Bunone et al. 1996) and GK1 for yeast transcription factor Gal4 fusion proteins (Webb et al. 1998). XETL contains the estrogen response element (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). We used the *Renilla* luciferase expression vector pRL-TK (Promega, Milan, Italy) as a transfection standard.

Transfection and luciferase assays. BG-1, MCF-7, Ishikawa, and SkBr3 cells (1×10^5) were plated into 24-well dishes with 500 µL/well DMEM (BG-1, MCF-7, and Ishikawa cells) or RPMI 1640 (SkBr3 cells) containing 10% FBS the day before transfection. We replaced the medium with phenol red–free DMEM or RPMI 1640, both supplemented with 1% charcoal-stripped (CS) FBS, on the day of transfection. Transfections were performed using FuGENE 6 Reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany) with a mixture containing 0.3 µg of reporter plasmid, 1 ng pRL-TK, and 0.1 µg effector plasmid where applicable. After 5–6 hr, 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 16–18 hr. We measured luciferase activity with the Dual Luciferase Kit (Promega) according to the manufacturer's recommendations. Firefly luciferase values were normalized to the internal transfection control provided by *Renilla* luciferase activity. The normalized relative light unit values obtained from cells treated with vehicle were set as 1-fold induction, from which the activity induced by treatments was calculated.

RT-PCR. Using semiquantitative RT-PCR as described previously (Maggiolini et al. 1999), we evaluated gene expression for *ERα* [GenBank accession no. NM 000125 (National Center for Biotechnology Information 2008)], c-*fos* (NM 005252), progesterone receptor (*PR;* NM 000926), *pS2* (NM 003225), cathepsin D (NM 001909), cyclin A (NM 001237), cyclin D1 (NM 053056), cyclin E (NM 001238), and the acid phosphoprotein *P0* (*36B4*) (NM 001002) used as a control gene. We used the primers 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′ (cathepsin D forward), and 5′-ATGCACGAAACAGATCTGTGCT-3′ (cathepsin D 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 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, and cyclin E and 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% sodium dodecyl sulfate (SDS), and a mixture of protease inhibitors containing 1 mmol/L aprotinin, 20 mmol/L phenylmethylsulfonyl fluoride, and 200 mmol/L sodium orthovanadate. We then diluted samples 10 times and determined protein concentration 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 and transferred to a nitrocellulose membrane (Amersham Biosciences, Milan, Italy). Membranes were probed overnight at 4°C with the antibody against ERα (F-10), c-fos (H-125), β-actin (C-2), phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2; E-4), and ERK2 (C-14), all purchased from Santa Cruz Biotechnology, DBA (Milan, Italy), and human P450 aromatase (MCA 2077S; 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

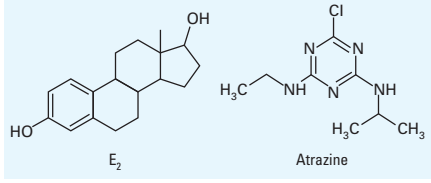

Figure 1. Structures of E_2 and atrazine.

with 1 nM [2,4,6,7-^{3}H]E2 (89 Ci/mmol; Amersham Biosciences) and increasing concentrations of nonlabeled E_2 or atrazine for 1 hr at 37°C in a humidified atmosphere of 95% air/5% CO_2. After removal of the medium, cells were washed with ice-cold phosphate-buffered saline/0.1% methylcellulose twice, harvested by scraping and centrifugation, and lysed with 100% ethanol, 500 µL/60-mm dish, for 10 min at room temperature (Lee and Gorski 1996). We measured the radioactivity of extracts by liquid scintillation counting.

Aromatase assay. In subconfluent BG-1 or H295R cells, we measured aromatase activity in the cell culture medium by tritiated water release using 0.5 µM [1β-^{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 six-well dish in culture medium in the presence of atrazine or DMSO for 40 hr and then incubated with [1β-^{3}H(*N*)]androst-4-ene-3,17-dione. Incubations were performed at 37°C for 6 hr under a 95%/5% air/CO_2 atmosphere. The results were calculated as picomoles per hour, normalized to milligrams of protein (pmol/hr per 1 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 hr, and then transfected for additional 24 hr before treatments with a mixture containing Opti-MEM, 8 µL/well LipofectAMINE 2000 (Invitrogen, Milan, Italy), and 0.5 µg/well vector or short hairpin GPR30 (shGPR30) (Albanito et al. 2008), control small interfering RNA (siRNA), or ERα siRNA (Sigma-Aldrich).

Proliferation assay. For the quantitative proliferation assay, we seeded 10,000 cells in 24-well plates in regular growth medium. Cells were washed once they had attached and then incubated in medium containing 2.5% CS-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. The day before treatments, 200 ng/L of the indicated short hairpin RNA was transfected using FuGENE 6 Reagent as recommended by the manufacturer, and then renewed every 2 days before counting.

Statistical analysis. Statistical analysis was performed using analysis of variance followed by Newman-Keuls testing to determine differences in means. *p*-Values < 0.05 are considered statistically significant.

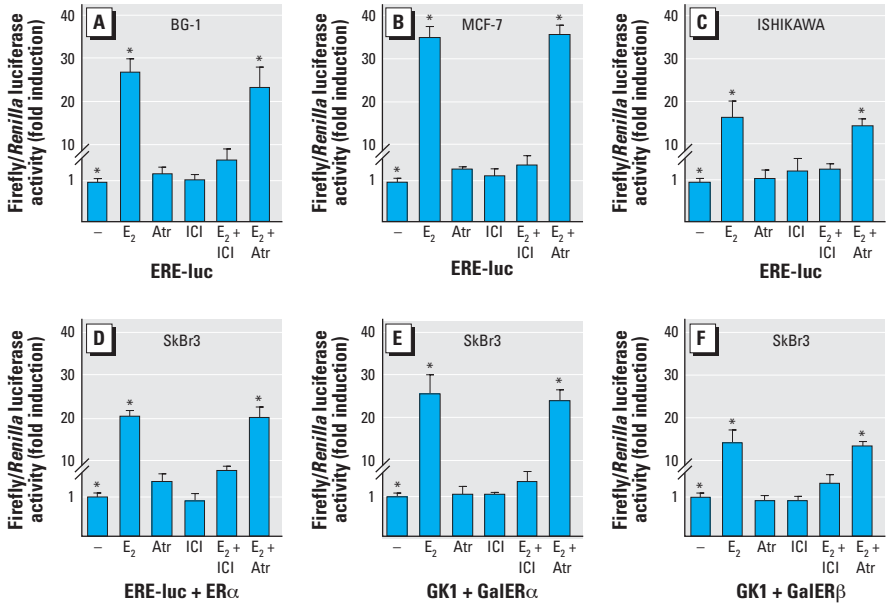

Figure 2. ERα transactivation in BG-1 (*A*), MCF-7 (*B*), and Ishikawa (*C*) cells transfected with the ER luciferase reporter plasmid XETL (ERE-luc) and treated with 100 nmol/L E_2 or 1 µmol/L atrazine (Atr), with and without 10 µmol/L ER antagonist ICI. Luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (–) were set as 1-fold induction, from which the activity induced by treatments was calculated. (*D–F*) SkBr3 cells were transfected with ER luciferase reporter gene *XETL* and ERα expression plasmid (*D*) and with Gal4 reporter gene (*GK1*) and the Gal4 fusion proteins encoding the HBD of ERα (GalERα; *E*) and or ERβ (GalERβ; *F*) and treated with 100 nmol/L E_2 or 1 µmol/L atrazine, with and without 10 µmol/L ICI. Values shown are mean ± SD of three independent experiments performed in triplicate.
*$p < 0.05$ compared with vehicle.

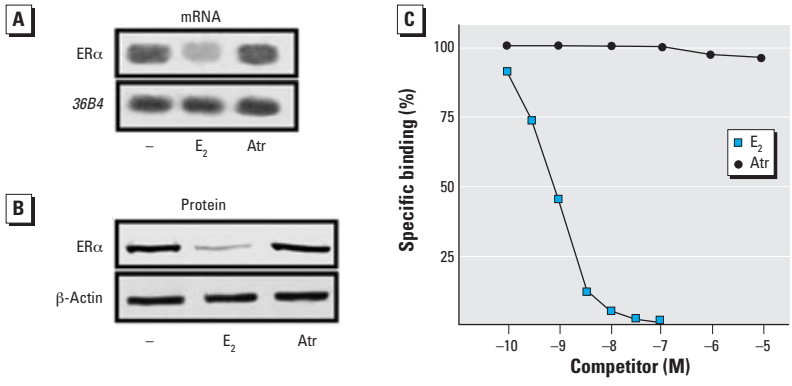

Figure 3. mRNA expression and binding of ERα in BG-1 cells treated for 24 hr with vehicle (–), 100 nmol/L E_2, or 1 µmol/L atrazine (Atr). (*A*) mRNA expression of ERα was evaluated by semiquantitative RT-PCR; the values of housekeeping gene *36B4* were determined as a control. (*B*) Immunoblot of ERα from BG-1 cells, with 100 nmol β-actin serving as a loading control. Results in (*A*) and (*B*) are representative of three independent experiments. (*C*) ERα binding assay using increasing concentrations of atrazine.

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. Exposure to 100 nM E_2 induced a strong ERα transactivation that was absent in the presence of 10 µM of the ER antagonist ICI in all cell contexts evaluated (Figure 2A–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 E_2 (Figure 2A–C). Moreover, atrazine did not activate an expression vector encoding ERα transiently transfected in ER-negative SkBr3 breast cancer cells (Figure 2D). 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 of the yeast transcription factor Gal4 and the ERα or ERβ HBD transiently transfected in SkBr3

cells were strongly activated by E_2 but not upon atrazine treatment (Figure 2E,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), and express a receptor expression pattern similar to that found in primary ovarian tumors (Bardin et al. 2004; Geisinger et al. 1989). As shown in Figure 3A,B, 100 nM E_2 down-regulated ERα at both mRNA and protein levels, whereas 1 μM atrazine did not produce any modulatory effect. In agreement with these results and those obtained in transfection experiments, atrazine showed no binding capacity for ERα (Figure 3C), as previously 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 leads to activation of different downstream kinases (Bulayeva and Watson 2004; Watson et al. 2007).

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 and prolonged time period were required to trigger this biochemical response compared with E_2 (Figures 5A,B, 6A). ERK activation was also delayed in the presence of 1 μM atrazine compared with 100 nM E_2 in 2008 ovarian cancer cells (Figure 6D), which present a receptor expression similar to that of BG-1 cells (Safei et al. 2005). To determine the transduction pathways involved in ERK activation by atrazine, cells were exposed to 100 nM E_2 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 inhibitor AG and the ERK inhibitor PD prevented ERK activation induced by both E_2 and atrazine, whereas GFX, H89, and WM, inhibitors of protein kinase C (PKC), protein kinase A (PKA), and phosphoinositide 3-kinase (PI3K), respectively, did not (Figure 6B,C,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. We observed no ERK activation after

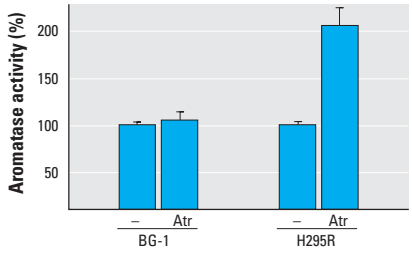

Figure 4. Aromatase activity assessed by tritiated water release in BG-1 and H295R cells treated with vehicle (–) or 1 μmol/L atrazine (Atr). Results are expressed as percentages of untreated cells (100%). Values are mean ± SD of three independent experiments, each performed in triplicate.
*$p < 0.05$ compared with vehicle.

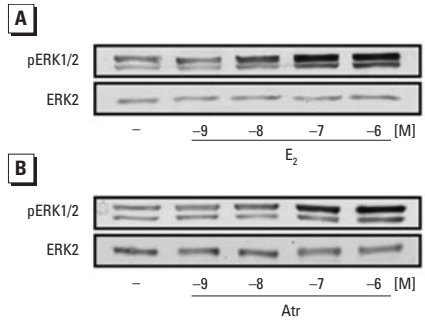

Figure 5. ERK1/2 phosphorylation (pERK1/2) in BG-1 cells exposed to increasing concentrations of E2 or atrazine (Atr) for 20 min.

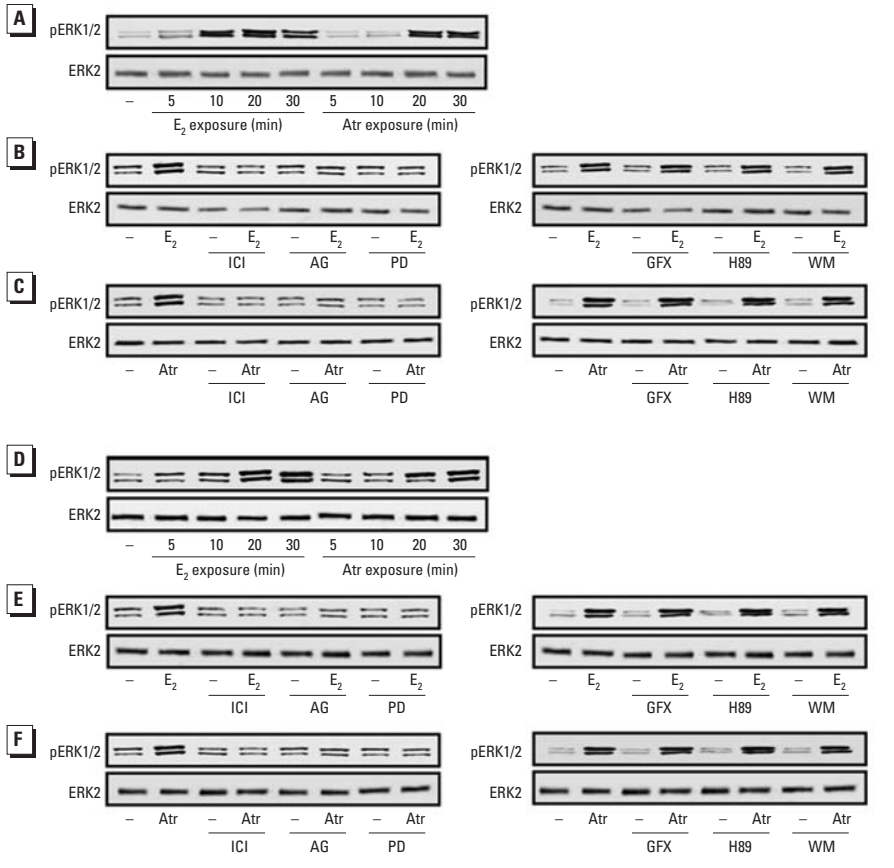

Figure 6. BG-1 (*A–C*) and 2008 (*D–F*) cells treated with vehicle (–) or 100 nmol/L E_2 with or without 1 μmol/L atrazine (Atr) for 5, 10, 20, or 30 min (*A,D*), or for 20 min with vehicle E_2 (*B* and *E*), or 1 μmol Atr in combination with 10 μmol/L ICI, AG, PD, GFX, H89, or WM, inhibitors of ER, EGFR, MEK (MAP/ERK kinase), PKC (protein kinase C), PKA (protein kinase A), and PI3K (phosphoinositide 3-kinase), respectively. pERK1/2, phosphorylated ERK 1/2.

either 5 min (data not shown) or 20 min of treatment (Figure 7). Hence, in our experimental conditions, ICI showed only 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 that responds to a variety of extracellular stimuli, including estrogens (Maggiolini et al. 2004; Nephew et al. 1993; Singleton et al. 2003; Vivacqua et al. 2006ab), 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 hr) with 1 μM atrazine enhanced c-*fos* and cyclin A levels, although to a lesser extent than 100 nM E_2, which also stimulated PR, pS2, and cyclin D1 expression (Table 1). After a 24-hr treatment, atrazine increased PR, pS2, and cyclin A levels, whereas E_2 additionally induced the expression of c-*fos*, cathepsin D, cyclin D1, and cyclin E (Table 1). We obtained results similar to those described above 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.

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 hr) was abolished by the ER antagonist ICI, the EGFR inhibitor AG, or the ERK inhibitor PD (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 E_2 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 E_2 and atrazine were no longer able to induce c-*fos*

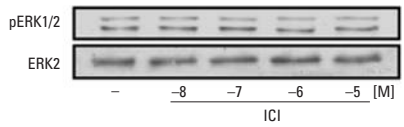

Figure 7. ERK1/2 phosphorylation (pERK1/2) in SkBr3 cells treated for 20 min with vehicle (–) or increasing concentrations of ICI.

Table 1. mRNA expression (mean percent variation ± SD) induced by 100 nM E_2 and 1 μM atrazine in BG-1 cells.

	E_2		Atrazine	
Gene	1 hr	24 hr	1 hr	24 hr
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*
Cathepsin D	106 ± 9	217 ± 16*	102 ± 5	109 ± 6
Cyclin A	262 ± 22*	293 ± 23*	220 ± 20*	190 ± 22*
Cyclin D1	258 ± 19*	242 ± 19*	107 ± 4	118 ± 8
Cyclin E	120 ± 11	343 ± 21*	118 ± 8	119 ± 10

The values calculated by optical density in cells treated with vehicle were set at 100%, and the expression induced by treatments is presented as percent variation.
*$p < 0.05$ compared with vehicle.

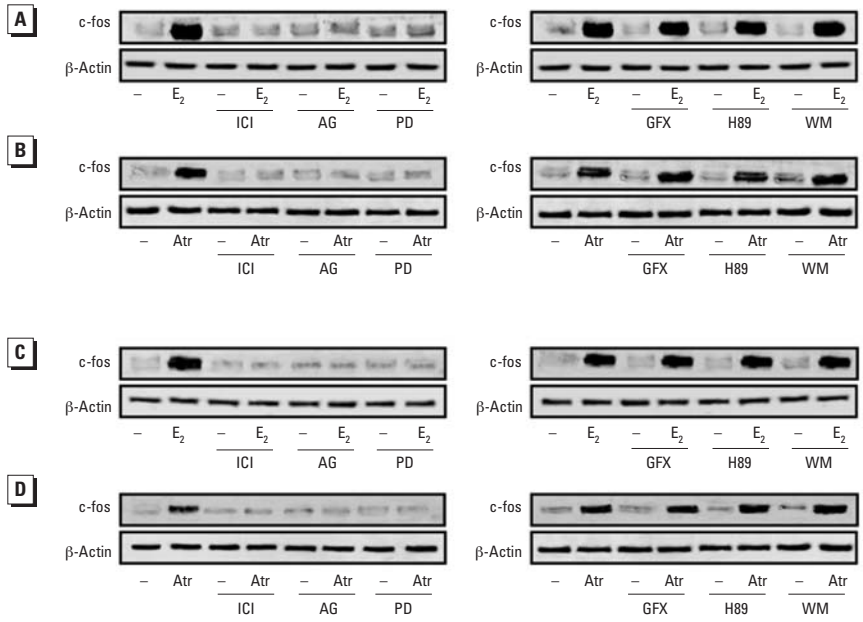

Figure 8. Immunoblots of c-fos from BG-1 (*A,B*) and 2008 (*C,D*) cells treated for 2 hr with vehicle (–), 100 nmol/L E_2, or 1 μmol/L atrazine (Atr) in combination with 10 μmol/L ICI, AG, PD, GFX, H89, or WM, inhibitors of ER, EGFR, MEK, PKC, PKA, and PI3K, respectively. β-Actin served as a loading control.

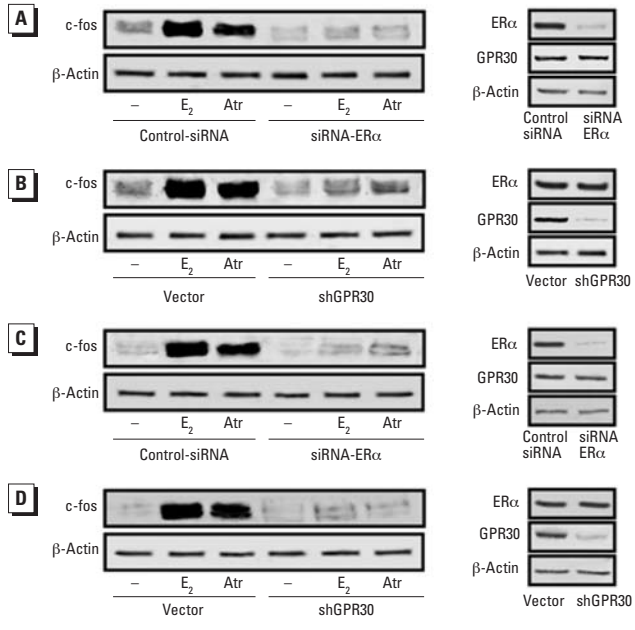

Figure 9. Immunoblots of c-fos from BG-1 (*A,B*) and 2008 (*C,D*) cells after silencing ERα and GPR30 expression. Cells were transfected with control siRNA or siRNA-ERα (*A,C*) or with vector or shGPR30 (*B,D*) and treated for 2 hr with vehicle (–) or 100 nmol/L E_2 or 1 μmol/L atrazine (Atr). Efficacy of ERα and GPR30 silencing was ascertained by immunoblots, as shown in side panels. β-Actin served as a loading control.

expression after silencing either ERα or GPR30 in BG-1 and 2008 cells (Figure 9). To evaluate whether atrazine could induce a rapid response in a cell context expressing GPR30 alone, we turned to ER-negative SkBr3 breast cancer cells. Notably, both ERK phosphorylation and c-*fos* induction stimulated by atrazine were abolished after silencing GPR30 (Figure 10), indicating that the response to atrazine is differentially regulated according to cancer cell type.

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 physiologic assay such as cell growth. We observed that both E_2 and atrazine induced the proliferation of BG-1 and 2008 cells in a concentration-dependent manner (Figure 11A,E). Moreover, the growth effects elicited by E_2 and atrazine were no longer evident in the presence of AG and PD (Figure 11B,F) or after silencing the expression of either GPR30 or ERα (Figure 11C,D,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 described above.

Discussion

In the present study, we demonstrated for the first time that atrazine exerts an estrogen-like activity in ovarian and breast cancer cells through GPR30, which is recently of interest because of its ability to mediate rapid estrogen signals (Albanito et al. 2007, 2008; Filardo et al. 2006, 2007; Revankar et al. 2005, 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 not by binding to or 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 that demonstrated the recruitment of ERα by distinct compounds and growth factors to gene promoter sequences different from the classical estrogen response element (reviewed by Dudek and Picard 2008).

Interestingly, we showed 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 indicate that a complex interplay between different ERs and transduction pathways contributes to atrazine activity, which nevertheless is still noticeable in the presence of GPR30 alone, as demonstrated in SkBr3 breast cancer cells. Although E_2 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 by silencing GPR30 and using specific pharmacologic inhibitors.

A variety of environmental contaminants exhibit binding affinities for GPR30 and agonist activities similar to those for ERs (Thomas and Dong 2006). In the present 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 HEK-293 cells (Thomas and Dong 2006). In line with these findings, an efficient competitor of E_2 for endogenous GPR30 in SkBr3 cells, such as an *ortho*,*para*-dichlorodiphenyldichloroethylene (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 with respect to cells engineered to express recombinant GPR30, and/or yet unknown factors may contribute to the binding to GPR30 by these contaminants.

Regarding the role of ERα, we proved that a complex interplay with GPR30 exists, as previously reported with some growth factor receptors (Migliaccio et al. 2006), but the

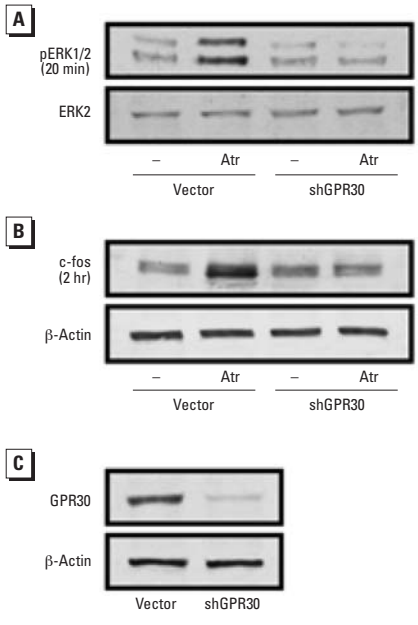

Figure 10. ERK1/2 phosphorylation (*A*) and c-fos expression (*B*) after silencing GPR30 in SkBr3 cells treated with vehicle (–) or 1 μmol/L atrazine (Atr). (*C*) The efficacy of GPR30 silencing was ascertained by immunoblots. β-Actin served as a loading control.

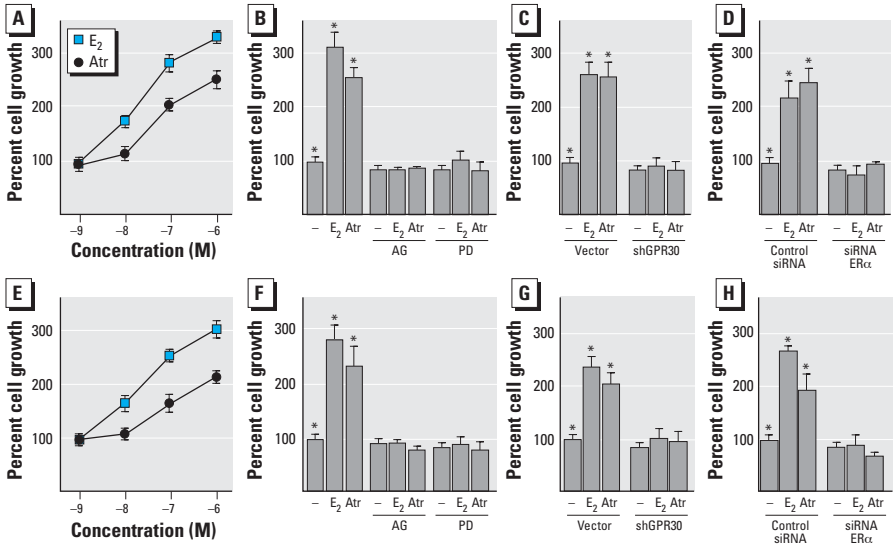

Figure 11. Proliferation of BG-1 (*A–D*) and 2008 (*E–H*) cells exposed to E_2 or atrazine (Atr). (*A,D*) Proliferation of cells in response to increasing concentrations of E_2 or Atr. (*B–H*) Proliferation of cells treated with vehicle (–), 100 nmol/L E_2, or 1 μmol/L Atr with or without 10 μmol/L AG or PD (*B,F*) (*C,D, G, H*) or transfected with vector or shGPR30 (*C,G*) or with control siRNA or siRNA-ERα (*D,H*). See "Materials and Methods" for details of experiments. Proliferation of cells receiving vehicle was set as 100%, and the cell growth induced by treatments was calculated. Values shown are mean ± SD of three independent experiments performed in triplicate; Efficacy of ERα and GPR30 silencing was ascertained by immunoblots (Figure 9).
*$p < 0.05$ compared with treated cells.

molecular mechanisms involved remain to be elucidated. Our study 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 after silencing GPR30 or ERα expression in ovarian cancer cells, because 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 with E_2 (Bulayeva and Watson 2004). In the latter study, on the basis of the inhibitory activity exerted by ICI, the authors hypothesized that an ER localized to the plasma membrane could mediate the ERK phosphorylation response by xenoestrogens, depending on their different ER binding affinities. Moreover, the authors suggested that the signaling cascades leading to ERK activation may involve the nature of membrane ERs and their ability to interact with various signaling partners (Bulayeva and Watson 2004). 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 SkBr3 breast cancer cells, GPR30–EGFR signaling may still allow for environmental estrogen activity as we have shown in the present study as well as in 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 that result from interaction with a different repertoire of receptor proteins.

Atrazine, a potent endocrine disruptor, is the most common pesticide contaminant of groundwater and surface water. 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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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

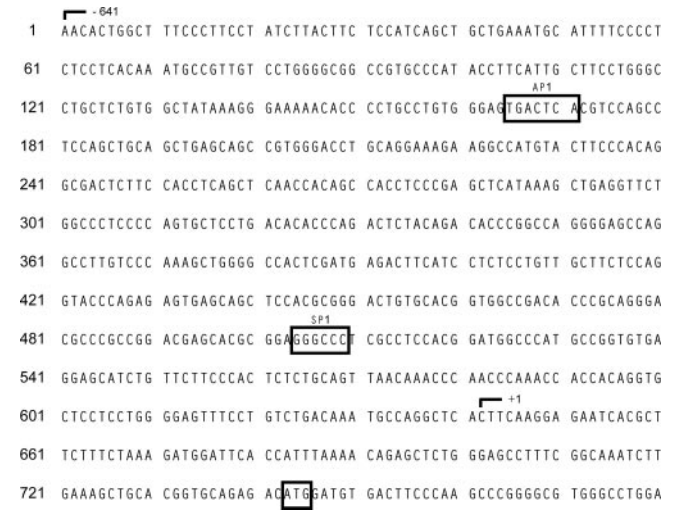

FIG. 1. Sequence of the GPR30–5′-flanking region used to generate luciferase reporter constructs.

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

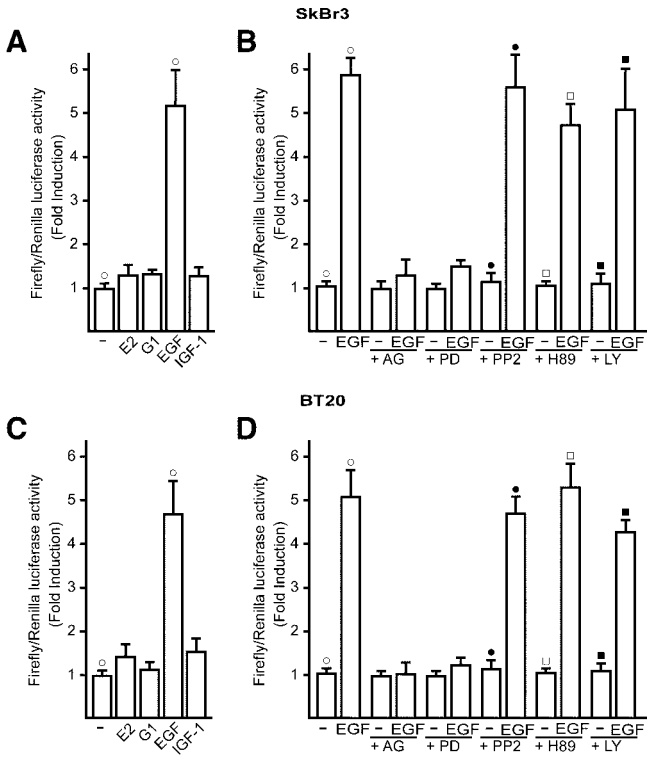

FIG. 2. The GPR30–5′-flanking region is transactivated by EGF in SkBr3 and BT20 breast cancer cells. A–D, Cells were transfected with a reporter plasmid encoding the GPR30–5′-flanking region and treated with 100 nM E2, 1 μM G-1, 50 ng/ml IGF-I, or 50 ng/ml EGF, and 10 μM EGFR inhibitor tyrphostin AG 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

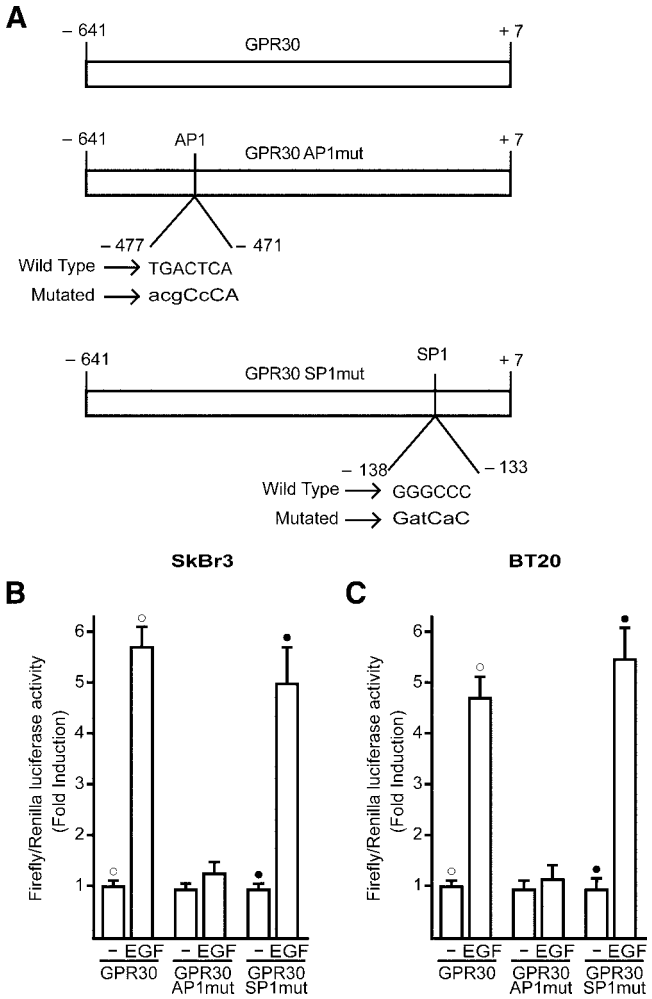

FIG. 3. An AP1 site is responsible for transactivation of the GPR30–5′-flanking region induced by EGF in SkBr3 and BT20 breast cancer cells. A, AP1 (GPR30AP1mut) and SP1 (GPR30SP1mut) mutations generated within the GPR30–5′-flanking region. B and C, Cells were transfected with the reporter plasmids described 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.

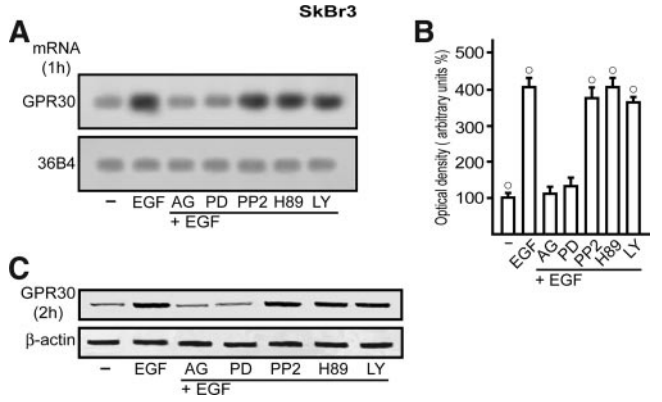

FIG. 4. EGF up-regulates GPR30 expression in SkBr3 cells. A, The expression of GPR30 was evaluated by semiquantitative RT-PCR in cells treated for 1 h with vehicle (–) or 50 ng/ml EGF alone and in combination with 10 μM EGFR inhibitor tyrphostin AG, 10 μM MEK inhibitor PD, 10 μM Src family tyrosine kinase 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

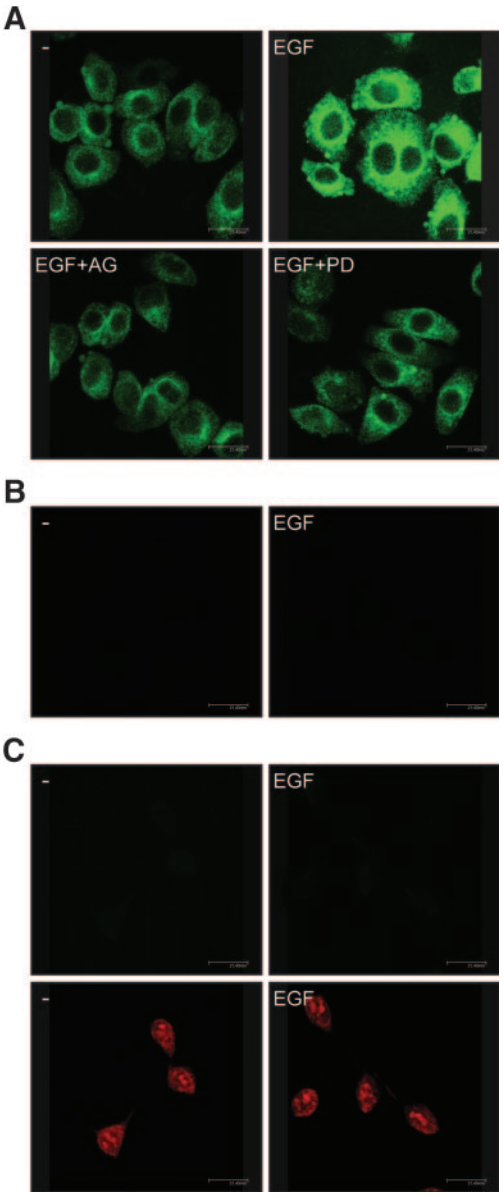

FIG. 5. GPR30 localization in SkBr3 cells. A, GPR30 evaluation by confocal microscopy in SkBr3 cells fixed, permeabilized, and stained with anti-GPR30 antibody. Cells were treated for 2 h with vehicle (−) or 50 ng/ml EGF alone and in combination with EGFR inhibitor tyrphostin AG, 10 μM MEK inhibitor 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.

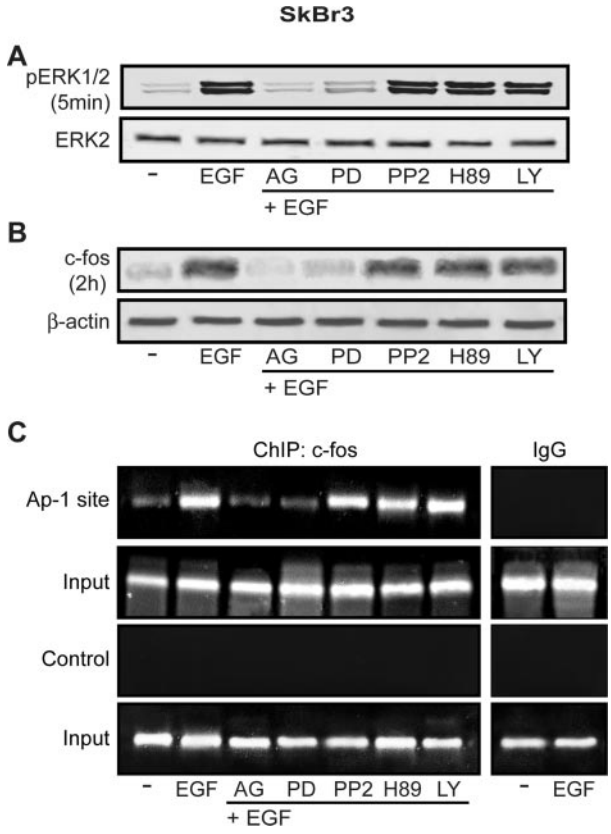

FIG. 6. EGF induces ERK1/2 phosphorylation and c-*fos* expression, which is recruited at the AP1 site located in the GPR30–5′-flanking region. A, The rapid ERK1/2 phosphorylation induced by 50 ng/ml EGF in SkBr3 cells is abrogated in presence of 10 μM EGFR inhibitor tyrphostin AG and 10 μM MEK inhibitor 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

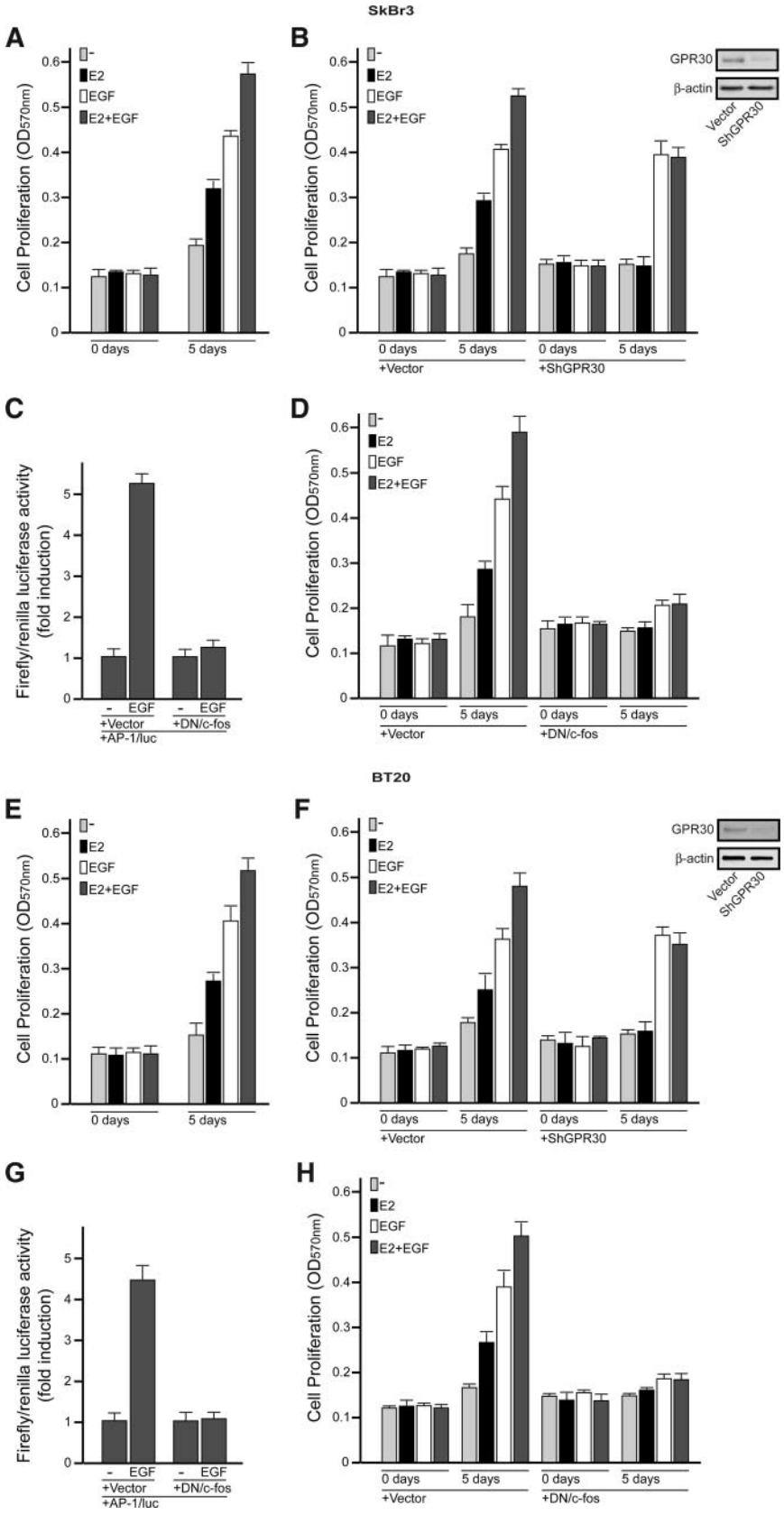

FIG. 7. In SkBr3 and BT20 breast cancer cells, EGF engages E2 through GPR30 to boost the growth effects, which were monitored by MTT assay. A and E, The combination of E2 and EGF treatments enhances the proliferation of SkBr3 and BT20 cells stimulated by each mitogen used alone. Cells were treated with 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.

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Research Article

Structure–activity relationships of resveratrol and derivatives in breast cancer cells

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Resveratrol (RSV) is classified as a phytoestrogen due to its ability to interact with estrogen receptors (ERs). We assessed structure–activity relationships of RSV and the analogs 4,4′-dihydroxystilbene (4,4′-DHS), 3,5-dihydroxystilbene (3,5-DHS), 3,4′-dihydroxystilbene (3,4′-DHS), 4-hydroxystilbene (4-HS) using as model systems the ERα-positive and negative MCF7 and SkBr3 breast cancer cells, respectively. In binding assays and transfection experiments RSV and the analogs showed the following order of agonism for ERα: 3,4′-DHS > 4,4′-DHS > 4-HS > RSV, while 3,5-DHS did not elicit any ligand properties. Computational docking analysis and real-time PCR revealed for each analog a distinct ERα binding orientation and estrogen target gene expression profile. Interestingly, the aforementioned order of ligand activity was confirmed in proliferation assays which also showed the lack of growth stimulation by 3,5-DHS. Our data suggest that subtle changes in the structure of the RSV derivatives examined may be responsible for the different ERα-mediated biological responses observed in estrogen-sensitive cancer cells.

Keywords: Breast cancer / Docking analysis / Estrogen receptor / Hydroxystilbenes / Resveratrol

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1 Introduction

Breast cancer is the most frequent tumor and the major cause of death among women in the United States [1]. The proliferation of many breast tumor cells is stimulated by the binding of 17β-estradiol (E2) to the estrogen receptor (ER) isoforms, ERα and ERβ, which belong to the nuclear receptor super-family. The transcriptional activity of the ERs is mediated by both an N-terminal ligand-independent activation function (AF-1) and a ligand-dependent activation function (AF-2) located in the ligand binding domain (LBD) [2]. The LBD contains a molecular switch helix–helix 12-which regulates the communication between ligand- and coactivator-binding sites [2, 3]. In ERα, as in other steroid hormone receptors, the ligand-binding pocket is a compact ellipsoid cavity, closely resembling the surface of the steroid ligands [4]. In the presence of agonist ligands, helix 12 is stabilized in a conformation that allows it to form one side of the coactivator-binding site and to completely encapsulate the ligand in the pocket. Antagonist ligands, such as the selective ER modulator (SERM) tamoxifen, resemble agonist ligands but contain an additional extended group [2–4]. The bulky side chain on tamoxifen protrudes between helices 3 and 11, and physically obstructs helix 12 from adopting the agonist conformation, thus preventing coactivator recruitment to the LBD [3]. The binding of E2 to the LBD of ERα induces conformational changes allowing receptor–chromatin interaction and the transcriptional regulation of target genes [5, 6]. A large body of data indicates that dietary factors play a sig-

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Abbreviations: 4-HS, 4-hydroxystilbene; **CS,** charcoal-stripped; **DHS,** dihydroxystilbene; **E2,** 17β-estradiol; **ER,** estrogen receptor; **ICI,** ICI 182,780; **LBD,** ligand binding domain; **PR,** progesterone receptor; **RSV,** resveratrol

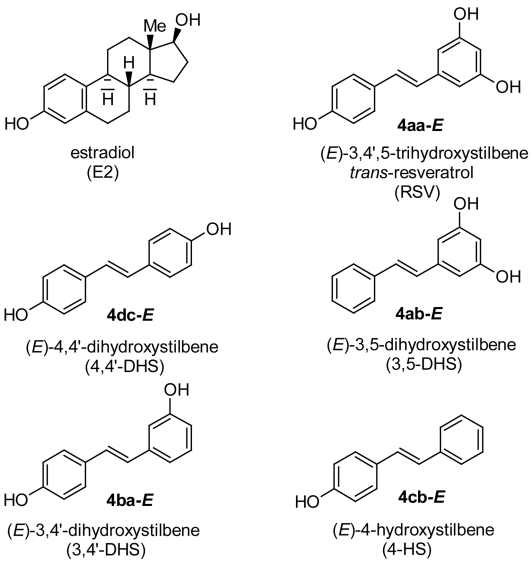


Figure 1. Chemical structures of E2, 3,4′,5-trihydroxystilbene (RSV), 4,4′-dihydroxystilbene (4,4′-DHS), 3,5-dihydroxystilbene (3,5-DHS), 3,4′-dihydroxystilbene (3,4′-DHS), and 4-HS.

nificant role in breast tumor development [7, 8]. For example, the low incidence of breast cancer in Asian women, who consume a high soy diet containing a significant amount of phytoestrogens [9], has been associated with the possibility that phytoestrogens may antagonize the effects of E2 [10]. However, there is no conclusive evidence that phytoestrogens act as antiestrogens [11–13]. Another possibility is that phytoestrogens exert antiproliferative and cancer-protective effects through an ER-independent pathway [14, 15].

Resveratrol (RSV) (Fig. 1) is a phytoalexin predominantly ingested with red grapes, peanuts, and berries [16–18]. RSV has been classified as a phytoestrogen due to its ability to interact with ERα and ERβ [6, 19–21]. On the basis of the estrogenic and antiestrogenic properties, RSV has also been considered a selective ER-modulator [19, 22–24]. It has been shown that high RSV levels may exert an antiproliferative effect in breast cancer cells regardless their ER status [25–27]. However, as reported for other phytoestrogens [6, 21, 28, 29], a biphasic response to RSV on cell growth has been reported depending on the concentration of RSV exposure. To date, the chemopreventive and cardioprotective effects of RSV have been attributed to its antioxidant and anticoagulant properties [30, 31].

Despite our detailed knowledge on the activities elicited by RSV through ERα, much less is known regarding whether different analogs of RSV could be accommodated in the receptor binding cavity and consequently induce the expression of genes such as pS2, Cathepsin D, progesterone receptor (PR), c-*fos*, Cyclin A, Cyclin D1, largely involved in the biological response to estrogens [32–34].

Using an *in vitro* screening model based on ERα-positive MCF7 and ER-negative SkBr3 breast cancer cells, we have investigated the structure–activity relationships of RSV and four hydroxystilbene analogs (Fig. 1), which were synthesized to assess the influence of the number and position of the hydroxyl groups present in the aromatic ring(s).

2 Materials and methods

2.1 Chemistry

Melting points were determined with a Reichert Thermovar apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 25°C on a Bruker DPX Avance 300 or on a Bruker DPX Avance 500 spectrometer in $CDCl_3$ or DMSO-d_6 solutions at 300 or 500 and 75 or 126 MHz, respectively, with Me_4Si as internal standard. Chemical shifts (δ) and coupling constants (*J*) are given in ppm and in Hz, respectively. IR spectra were taken with a Perkin-Elmer Paragon 1000 PC FT-IR spectrometer. Mass spectra were obtained using a Shimadzu QP-2010 GC-MS apparatus at 70 eV ionization voltage. Microanalyses were carried out with a Carlo Erba Elemental Analyzer Mod. 1106. All reactions were analyzed by TLC on silica gel 60 F_{254} and by GLC using a Shimadzu GC-2010 gas chromatograph and capillary columns with polymethylsilicone + 5% phenylsilicone as the stationary phase (HP-5). Column chromatography was performed on silica gel 60 (Merck, 70–230 mesh). Evaporation refers to the removal of solvent under reduced pressure.

2.1.1 General procedure for the preparation of hydroxystilbenes 4aa-*E* (RSV), 4ab-*E*, 4ba-*E*, and 4cb-*E*

First step: Wittig reaction followed by isomerization to give methoxystilbenes 3-E. To a solution of 2 (4.4 mmol) and *t*-BuOK (494 mg, 4.4 mmol) in anhydrous THF (50 mL) maintained under nitrogen at –10°C with stirring was added 1 (dropwise in the case of 1b and 1c, in small portions in the case of 1a) (3.67 mmol). After additional stirring at –10°C under nitrogen for 1 h, the mixture was poured into water (*ca*. 100 mL). The resulting mixture was neutralized with 1 N HCl and then extracted with diethyl ether (3 × 20 mL). The collected organic phases were dried over Na_2SO_4. After filtration, the solvent was evaporated and the residue purified by column chromatography on silica gel, using 8:2 hexane–Et_2O (3aa, 3ba) or 9:1 hexane–Et_2O (3ab, 3cb) as eluent, to give 3 as an *E* + *Z* mixture. To a solution of 3 (*Z* + *E* mixture) in THF (50 mL) maintained under nitrogen with stirring was added PhSSPh (120 mg, 0.55 mmol in the case of 3aa; 143 mg, 0.65 mmol in the case of 3ab; 156 mg, 0.71 mmol in the case of 3ba; 68 mg, 0.31 mmol in the case of 3cb) and the resulting mixture was allowed to reflux for 1 h (in the case of 3aa), 2 h (in the case of 3ba), 4 h (in the case of 3ab), 6 h (in the case of 3cb). The solvent was evaporated and the residue was purified by column chromatography on silica gel using 8:2 hexane–Et_2O

(3aa, 3ba) or 9:1 hexane – Et_2O (3ab, 3cb) as eluent, to give pure isomers 3-*E*. The yields obtained in each case (based on starting 1) together with the characterization data for compounds 3-*E* are given below.

Second step: Deprotection of methoxystilbenes 3-E to give hydroxystilbenes 4-E. To a solution of 3-*E* (1.43 mmol) in anhydrous CH_2Cl_2 (30 mL) maintained under nitrogen at –20°C with stirring was added BBr_3 dropwise (2.51 g, 10.0 mmol in the case of 3aa-*E*, 3ab-*E*, and 3ba-*E*; 1.07 g, 4.27 mmol in the case of 3cb-*E*). The mixture, maintained under nitrogen with stirring, was allowed to warm up to room temperature (*ca*. 4 – 5 h), and then it was poured into water (*ca*. 100 mL) and extracted with AcOEt (3 × 20 mL). The collected organic phases were washed with brine (20 mL) and then dried over Na_2SO_4. After filtration, the solvent was evaporated and the residue purified by column chromatography on silica gel using 6:4 hexane – acetone (4aa-*E*), 7:3 hexane – AcOEt (4ab-*E*, 4ba-*E*), 8:2 hexane – AcOEt (4cb-*E*) as eluent, to give pure 4-*E*. The yields obtained in each case (based on 3-*E*) together with the characterization data for compounds 4-*E* are given below.

2.1.2 Preparation of (*E*)-4,4′-dihydroxystilbene 4cd-*E* (stilbestrol)

A mixture of 4-hydroxybenzaldehyde 1d′ (1.04 g, 8.52 mmol), 4-hydroxyphenylacetic acid 2c′ (1.96 g, 12.88 mmol), Ac_2O (15 mL), and Et_3N (3 mL) was heated at 180°C under nitrogen for 6 h. After cooling to room temperature, 5% HCl (30 mL) and AcOEt (30 mL) were added to the mixture. Phases were separated, and the aqueous layer was extracted with AcOEt (3 × 30 mL). The collected organic phases were washed with brine (50 mL) and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure to obtain diacetylated α-(*p*-hydroxyphenyl)-*p*-hydroxycinnamic acid, which was then deacetylated at room temperature with K_2CO_3 in MeOH. After removal of the solvent under reduced pressure, the residue was diluted with 5% HCl (30 mL) and AcOEt (30 mL). Phases were separated, and the aqueous layer was extracted with AcOEt (3 × 30 mL). The collected organic phases were washed with brine (50 mL) and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure to obtain crude α-(*p*-hydroxyphenyl)-*p*-hydroxycinnamic acid. Quinoline (12 mL) and $CuCrO_3$ (500 mg) were added to crude α-(*p*-hydroxyphenyl)-*p*-hydroxycinnamic acid (2.5 g), and the reaction flask was heated at 240°C under nitrogen for 5 h. After cooling, the mixture was filtered through celite and washed with AcOEt (2 × 30 mL). The filtrate was washed with 5% HCl (2′ × 30 mL) and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The black oil thus obtained was purified by chromatography on silica gel using petroleum ether – AcOEt from 8:2 to 6:4 as the eluent, to give pure (*E*)-4,4′-dihydroxystilbene 4dc-*E* as an off-white solid (430 mg, 24% based on starting 1d′).

(E)-3,4′,5-Trimethoxystilbene (3aa-E). Yield: 735 mg, starting from 610 mg of 3,5-dimethoxybenzaldehyde (74%). Colorless solid, mp 56°C, lit. [35] 55 – 56°C. IR (KBr): ν = 3074 (w), 2991 (m), 2934 (m), 2834 (m), 1592 (s), 1511 (m), 1460 (m), 1279 (m), 1251 (m), 1071 (m), 1033 (m), 955 (m), 859 (m), 773 (m) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.42 – 7.38 (m, 2 H, H-2′ + H-6′), 7.01 (distorted d, *J* = 16.5, 1 H, C*H*=CH), 6.87 (distorted d, *J* = 16.5, 1 H, CH=C*H*), 6.87 – 6.83 (m, 2 H, H-3′ + H-5′), 6.63 (d, *J* = 2.2, 2 H, H-2 + H-6), 6.36 (t, *J* = 2.2, 1 H, H-4), 3.77 (s, 6 H, 2 OCH_3), 3.75 (s, 3 H, OCH_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 161.0, 159.4, 139.7, 129.9, 128.7, 127.8, 126.5, 114.1, 104.3, 99.6, 55.25, 55.20; GC-MS: *m/z* = 270 (100) [M^+], 269 (17), 255 (6), 239 (14), 227 (6), 224 (10), 212 (8), 197 (6), 196 (10), 195 (9), 181 (7), 169 (8), 165 (8), 153 (11), 152 (14), 141 (8), 115 (8); anal. calcd for $C_{17}H_{18}O_3$ (270.32): C, 75.53; H, 6.71; found C, 75.62; H, 6.69.

(E)-3,5-Dimethoxystilbene (3ab-E). Yield: 764 mg, starting from 610 mg of 3,5-dimethoxybenzaldehyde (87%). Colorless solid, mp 56°C, lit. [35] 55°C. IR (KBr): ν = 3074 (w), 3027 (w), 3001 (w), 2967 (m), 2941 (w), 2842 (w), 1592 (m), 1515 (s), 1464 (m), 1419 (m), 1313 (w), 1268 (s), 1226 (m), 1155 (m), 1140 (m), 1027 (m), 959 (m), 861 (w), 749 (m) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.49 – 7.46 (m, 2 H, H-2′+ H-6′), 7.35 – 7.30 (m, 2 H, H-3′ + H-5′), 7.26 – 7.21 (m, 1 H, H-4′), 7.06 (distorted d, *J* = 16.5, 1 H, C*H*=CH), 7.01 (distorted d, *J* = 16.5, 1 H, CH=C*H*), 6.66 (d, *J* = 2.2, 2 H, H-2 + H-6), 6.38 (t, *J* = 2.2, 1 H, H-4), 3.78 (s, 6 H, 2 OCH_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 161.0, 139.3, 137.1, 129.2, 128.7, 127.7, 126.6, 104.6, 100.0, 55.3; GC-MS: *m/z* = 240 (100) [M^+], 239 (37), 225 (11), 224 (9), 209 (19), 208 (11), 194 (14), 178 (9), 166 (10), 165 (34), 153 (12), 152 (13); anal. calcd for $C_{16}H_{16}O_2$ (240.30): C, 79.97; H, 6.71; found C, 80.03; H, 7.70.

(E)-3,4′-Dimethoxystilbene (3ba-E). Yield: 847 mg, starting from 500 mg of 3-methoxybenzaldehyde (96%). Colorless solid, mp 109 – 110°C, lit. [35] 108 – 109°C. IR (KBr): ν = 3074 (w), 3027 (w), 3001 (w), 2967 (m), 2941 (w), 2842 (w), 1592 (s), 1515 (s), 1464 (m), 1419 (w), 1312 (w), 1268 (s), 1226 (m), 1155 (w), 1140 (m), 1027 (m), 959 (m), 861 (w), 810 (m), 749 (m) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.43 – 7.39 (m, 2 H, H-2′ + H-6′), 7.25 – 7.21 (m, 1 H, H-5), 7.08 – 6.99 (m, 3 H, H-2 + H-6 + C*H*=CH), 6.92 (distorted d, *J* = 16.5, 1 H, CH=C*H*), 6.88 – 6.84 (m, 2 H, H-3′ + H-5′), 6.77 (dd, *J* = 8.2, 2.2, 1 H, H-4), 3.79 (s, 3 H, OCH_3), 3.76 (s, 3 H, OCH_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 159.9, 159.3, 139.1, 130.0, 129.6, 128.5, 127.8, 126.5, 119.0, 114.1, 112.9, 111.5, 55.23, 55.16; GC-MS: *m/z* = 240 (100) [M^+], 239 (10), 210 (7), 209 (16), 197 (9), 194 (10), 182 (13), 166 (11), 165 (30), 153 (15), 152 (12); anal. calcd for $C_{16}H_{16}O_2$ (240.30): C, 79.97; H, 6.71; found C, 79.89; H, 6.73.

(E)-4-Methoxystilbene (3cb-E). Yield: 623 mg, starting from 500 mg of 4-methoxybenzaldehyde (81%). Colorless

solid, mp 134–136°C, lit. [35] 134–134.5°C. IR (KBr): ν = 3023 (w), 3004 (w), 2965 (w), 2838 (w), 1603 (m), 1513 (s), 1447 (m), 1297 (w), 1252 (s), 1180 (m), 1031 (s), 968 (m), 860 (w), 813 (m), 750 (s), 689 (m) cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.48–7.45 (m, 2 H, H-2′ + H-6′), 7.44–7.41 (m, 2 H, H-2 + H-6), 7.34–7.30 (m, 2 H, H-3′ + H-5′), 7.23–7.19 (m, 1 H, H-4′), 7.05 (distorted d, J = 16.5, 1 H, C*H*=CH), 6.95 (distorted d, J = 16.5, 1 H, CH=C*H*), 6.89–6.84 (m, 2 H, H-3 + H-5), 3.78 (s, 3 H, OCH_3); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 159.3, 137.7, 130.1, 128.6, 128.2, 127.7, 127.2, 126.6, 126.3, 114.1, 55.3; GC-MS: m/z = 210 (100) [M^+], 209 (17), 195 (20), 194 (7), 179 (13), 178 (8), 177 (6), 167 (39), 166 (17), 165 (53), 152 (29), 139 (10), 115 (11), 89 (9), 76 (5), 63 (12); anal. calcd for $C_{15}H_{14}O$ (210.27): C, 85.68; H, 6.71; found C, 85.59; H, 6.72.

(E)-3,4′,5-Trihydroxystilbene (4aa-E, trans-RSV). Yield: 293 mg, starting from 386 mg of (*E*)-3,4′,5-trimethoxystilbene (90%). Colorless solid, mp 255–257°C, lit. [35] 256–257°C. IR (KBr): ν = 3289 (s, br), 1603 (m), 1588 (s), 1510 (m), 1456 (w), 1324 (w), 1252 (m), 1154 (m), 965 (m), 830 (m) cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): δ = 9.57 (s, br, 1 H, OH), 9.23 (s, br, 2 H, 2 OH), 7.43–7.39 (m, 2 H, H-2′ + H-6′), 6.96 (distorted d, J = 16.5, 1 H, C*H*=CH), 6.84 (distorted d, J = 16.5, 1 H, CH=C*H*), 6.80–6.76 (m, 2 H, H-3′ + H-5′), 6.43 (d, J = 2.2, 2 H, H-2 + H-6), 6.16 (t, J = 2.2, 1 H, H-4); ^{13}C NMR (126 MHz, DMSO-d_6): δ = 158.4, 157.1, 139.2, 128.0, 127.81, 127.77, 125.6, 115.5, 104.3, 101.7; MS (direct injection): m/z = 228 (100) [M^+], 227 (18), 213 (4), 211 (10), 210 (5), 199 (5), 182 (4), 181 (14), 165 (4), 157 (4), 152 (4); anal. calcd for $C_{14}H_{12}O_3$ (228.24): C, 73.67; H, 5.30; found C, 73.75; H, 5.28.

(E)-3,5-Dihydroxystilbene (4ab-E). Yield: 242 mg, starting from 343 mg of (*E*)-3,5-dimethoxystilbene (80%). Colorless solid, mp 154–155°C, lit. [35] 157–158°C. IR (KBr): ν = 3289 (s), 1617 (m), 1599 (s), 1588 (s), 1501 (w), 1472 (m), 1358 (w), 1252 (m), 1169 (m), 1148 (m), 1008 (m), 963 (m), 840 (w), 689 (m) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 9.34 (s, 2 H, 2 OH), 7.62–7.56 (m, 2 H, H-2′ + H-6′), 7.41–7.32 (m, 2 H, H-3′ + H-5′), 7.30–7.22 (m, 1 H, H-4′), 7.15–7.03 (AB system, J = 16.9, 2 H, CH=CH), 6.53 (d, J = 2.1, 2 H, H-2 + H-6), 6.26 (t, J = 2.1, 1 H, H-4); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 158.6, 138.8, 137.1, 129.1, 128.6, 127.9, 127.4, 126.4, 104.9, 102.6; MS (direct injection): m/z = 212 (100) [M^+], 211 (36), 197 (10), 195 (7), 194 (6), 193 (5), 183 (4), 181 (2), 165 (12), 153 (2), 141 (2); anal. calcd for $C_{14}H_{12}O_2$ (212.24): C, 79.22; H, 5.70; found C, 79.34; H, 5.68.

(E)-3,4′-Dihydroxystilbene (4ba-E). Yield: 197 mg, starting from 343 mg of (*E*)-3,4′-dimethoxystilbene (65%). Colorless solid, mp 216–218°C, lit. [35] 216–218°C. IR (KBr): ν = 3290 (s), 1616 (m), 1598 (s), 1588 (s), 1501 (w), 1472 (m), 1358 (w), 1252 (m), 1169 (m), 1149 (m), 1008 (m), 963 (m), 840 (w), 689 (m) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 9.58 (s, br, 1 H, OH), 9.39 (s, br, 1 H, OH), 7.45–7.38 (m, 2 H, H-2′ + H-6′), 7.18–6.88 (m, 5 H, H-2 + H-5 + H-6 + CH=CH), 6.80–6.74 (m, 2 H, H-3′ + H-5′), 6.64 (dd, J = 8.1, 2.1, 1 H, H-4); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.6, 157.3, 138.9, 129.4, 128.3, 127.7, 125.5, 117.1, 115.6, 114.3, 112.7; MS (direct injection): m/z = 212 (100) [M^+], 211 (25), 197 (10), 195 (7), 195 (13), 194 (8), 193 (11), 183 (10), 181 (11), 177 (8), 167 (6), 166 (7), 165 (33), 153 (8), 63 (5); anal. calcd for $C_{14}H_{12}O_2$ (212.24): C, 79.22; H, 5.70; found C, 79.15; H, 5.72.

(E)-4-Hydroxystilbene (4cb-E). Yield: 168.2 mg, starting from 300 mg of (*E*)-4-methoxystilbene (60%). Colorless solid, mp 196–197°C, lit. [35] 195–196°C. IR (KBr): ν = 3409 (s, br), 1633 (w), 1455 (w), 1372 (m), 1327 (w), 1207 (w), 1075 (m), 929 (m), 903 (m) cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ = 9.61 (s, br, 1 H, OH), 7.57–7.51 (m, 2 H, H-2′ + H-6′), 7.47–7.40 (m, 2 H, H-2 + H-6), 7.38–7.31 (m, 2 H, H-3′ + H-5′), 7.26–7.18 (m, 1 H, H-4′), 7.16 (distorted d, J = 16.4, 1 H, C*H*=CH), 7.02 (distorted d, J = 16.4, 1 H, CH=C*H*), 6.82–6.75 (m, 2 H, H-3 + H-5); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.3, 137.6, 128.5, 128.1, 127.8, 126.9, 126.0, 125.2, 115.6; GC-MS: m/z = 196 (100) [M^+], 195 (37), 181 (19), 179 (11), 178 (12), 177 (28), 176 (7), 167 (20), 166 (8), 165 (25), 152 (16), 115 (6), 89 (9), 77 (7), 76 (8), 63 (7); anal. calcd for $C_{14}H_{12}O$ (196.24): C, 85.68; H, 6.16; found C, 85.76; H, 6.15.

(E)-4,4′-Dihydroxystilbene (4dc-E). Yield: 430 mg, starting from 1.04 g of 4-hydroxybenzaldehyde (24%). Off-white solid, mp 297–300°C, lit. [36] 291–292°C. ^1H NMR (300 MHz, DMSO-d_6): δ (ppm) = 9.41 (s, 2 H, 2 OH), 7.33 (BB′ multiplet, 4 H, H-2 + H-6 + H-2′ + H-6′), 6.88 (s, 2 H, HC=CH), 6.72 (AA′ multiplet, 4 H, H-3 + H-5 + H-3′ + H-5′); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) = 157.2, 129.1, 127.8, 125.7, 115.9; GC-MS: m/z = 212 (100) [M^+], 197 (10), 165 (22), 141 (2), 115 (4), 77 (6); anal. calcd for $C_{14}H_{12}O_2$ (212.26): C, 79.22; H, 5.70; found: C, 79.29; H, 5.78.

2.2 Biological assays and molecular modeling

2.2.1 Reagents

E2 was purchased from Sigma–Aldrich and ICI 182,780 (ICI) was obtained from Tocris Chemicals.

2.2.2 Plasmids

Firefly luciferase reporter plasmids used were XETL [37] for ERα and GK1 [38] for the Gal4 fusion proteins. XETL contains the estrogen response element (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, Gal-ER(G521R), Gal-ER(L525A), and Gal-ER(M543A/

L544A) were expressed from plasmids GAL93.ER(G), GAL93.ER(G521R), GAL93.ER(L525A), and GAL93.ER (M543A/L544A), respectively. They were constructed by transferring the coding sequences for the hormone binding domain (HBD) of ERα (amino acids 282 – 595) from HEG0 [37], pCMVhERG521R [39], pCMVhERL525A [39], and a PCR270 mutagenized intermediate with the point mutations M543A-L544A, respectively, into the mammalian expression vector pSCTEVGal93 [40]. The *Renilla reniformis* luciferase expression vector pRL-CMV (Promega) was used as a transfection standard.

2.2.3 Cell culture

Human breast cancer MCF7 cells were maintained in DMEM with phenol red supplemented with 10% FBS. ER-negative SkBr3 human breast cancer cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS. MCF7 cells to be processed for immunoblot and RT-PCR assays were switched to medium without serum and phenol red 24 h before treatments.

2.2.4 ER binding assay

MCF-7 cells were stripped of any estrogen by keeping them in medium without serum for 2 days. Cells were incubated with 1 nM [2,4,6,7-3H]E2 (89 Ci/mmol; Amersham Bioscience) and increasing concentrations of nonlabeled E2, RSV or each derivative for 1 h at 37°C in a humidified atmosphere of 95% air/5% CO2. 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 [41]. The radioactivity of extracts was measured by liquid scintillation counting.

2.2.5 Transfections and luciferase assays

Cells were transferred into 24-well plates with 500 µL of regular growth medium/well the day before transfection. MCF7 cell medium was replaced with DMEM supplemented with 1% charcoal-stripped (CS) FBS lacking phenol red and serum on the day of transfection, which was performed using the Fugene6 Reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany) with a mixture containing 0.2 µg of reporter plasmid and 1 ng of pRL-CMV. After 5 – 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 – 18 h. SkBr3 cell medium was replaced with RPMI without phenol red and serum on the day of transfection, which was performed using the Fugene6 Reagent with a mixture containing 0.5 µg of reporter plasmid, 1 ng of pRL-CMV, and 0.1 µg of effector plasmid where applicable. After 5 – 6 h ligands were added and cells were incubated for 16 – 18 h. Luciferase activity was then measured with the Dual Luciferase kit (Promega) according to the manufacturer's recommendations. Firefly luciferase activity was normalized to the internal transfection control provided by the *Renilla* luciferase activity.

2.2.6 Reverse transcription and real-time PCR

Total RNA was extracted using Trizol commercial kit (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gels stained with ethidium bromide. Only samples that were not degraded and showed clear 18S and 28S bands under UV light were used for RT-PCR. Total cDNA was synthesized from the RNA by reverse transcription using the murine leukemia virus reverse transcriptase (Invitrogen) following the protocol provided by the manufacturer. The expression of selected genes was quantified by real-time PCR using Step One (TM) sequence detection system (Applied Biosystems, Milano, Italy), following the manufacturer's instructions. Gene-specific primers were designed using Primer Express version 2.0 software (Applied Biosystems). Assays were performed in triplicate, and the mean values were used to calculate expression levels, using the relative standard curve method. For pS2, Cathepsin D, PR, c-*fos*, Cyclin A, Cyclin D1, and the ribosomal protein 18S, which was used as a control gene to obtain normalized values, the primers were: 5′-GCCCCCCGTGAAAGAC-3k (pS2 forward) and 5′-CGTCGAAACAGCAGCCCTTA-3′ (pS2 reverse); 5′-CTGGATCCACCACAAGTACAACA-3′ (Cathepsin D forward), 5′-CGAGCCATAGTGGATGTCAAAC-3′ (Cathepsin D reverse); 5′-GAGTTGTGAGAGCACTGGATGCT-3′ (PR forward) and 5′-CAACTGTATGTCTTGACCTGGTGAA-3′ (PR reverse); 5′-CGAGCCCTTTGATGACTTCCT-3′ (c-*fos* forward), 5′-GGAGCGGGCTGTCTCAGA-3′ (c-*fos* reverse); 5′-TGCACCCCTTAAGGATCTTCCT-3′ (Cyclin A forward), 5′-GTGAACGCAGGCTGTTTACTGT-3′ (Cyclin A reverse); 5′-GTCTGTGCATTTCTGGTTGCA-3′ (Cyclin D1 forward), 5′-GCTGGAAACATGCCGGTTA-3′ (Cyclin D1 reverse); and 5′-GGCGTCCCCCAACTTCTTA-3′ (18S forward); 5′-GGGCATCACAGACCTGTTATT-3′ (18S reverse).

2.2.7 Immunoblotting

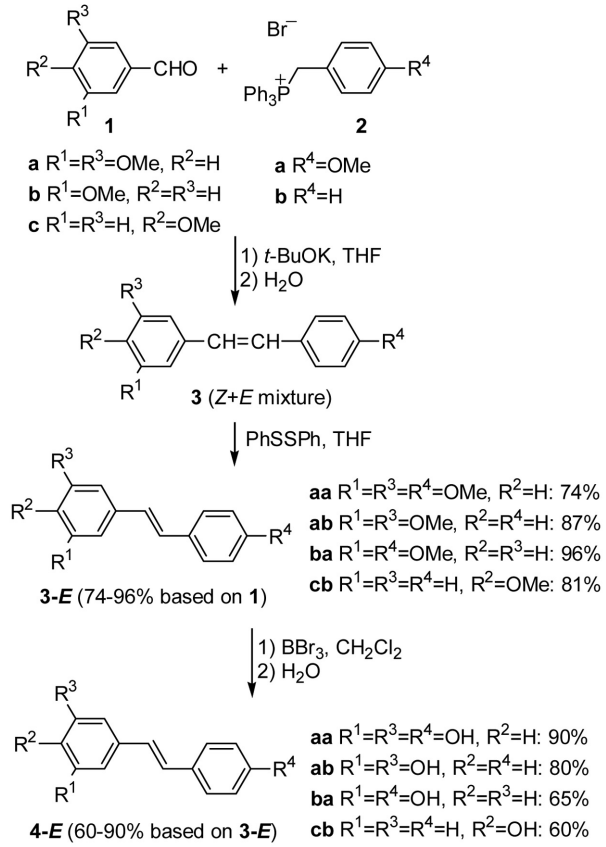
MCF7 cells were grown in 10 cm dishes and exposed to ligands for 18 h before lysis in 500 µL of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF), and Na-orthovanadate. Equal amounts of total protein were resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with the antibodies F-10 against ERα and β-actin (Santa Cruz Biotechnology), and revealed using the ECL Western Blotting Detection Reagents (GE Healthcare, Amersham).

2.2.8 Proliferation assays

For quantitative proliferation assays 1×10^4 MCF7 cells were seeded in 24-well plates in regular growth medium. Cells were washed once they had attached and further incubated in medium without serum for 24 h. Thereafter, the medium of MCF7 cells was changed and supplemented with 5% CS-FBS. Ligands were added at this point; medium was changed every day (with ligands). On day 6 (after 5 days of treatment), cells were trypsinized and counted using a hemocytometer.

2.2.9 Molecular modeling

(i) preparation of ligands and receptor molecules for docking. Molecular structures of E2, RSV, 4,4′-DHS, 3,5-DHS, 3,4′-DHS, and 4-hydroxystilbene (4-HS) were built and energy minimized with the programs InsightII and Discover3 (Biosym/MSI, San Diego, CA, USA). To model the receptor–ligand complexes, the coordinates of ERα (PDB:1G50) receptor in complex with the corresponding steroid hormone were used. The receptor and the ligands were prepared for docking using ADT, the AutoDock tool graphical interface [42]. For each receptor structure polar hydrogens were added, Kollman charges were assigned and atomic solvatation parameters were added. Polar hydrogen charges of the Gasteiger-type were assigned and the nonpolar hydrogens were merged with the carbons, the internal degrees of freedom and torsions were set for all the designed small molecules. Protein mutations were introduced into the crystallographic structure of ERα using the program O [43]. The resulting atomic models were then submitted to different cycles of molecular dynamics followed by energy minimization using the programs Insight II and Discover3. The resulting models were used as targets for molecular docking simulations using the program AutoDock 3.05. (ii) Docking simulations. In a first phase, each moiety was docked into the active site using the program AutoDock 3.05 [44] with the macromolecule considered as a rigid body and the ligands being flexible. The search was extended over the whole receptor protein. Affinity maps for all the atom types present, as well as an electrostatic map, were computed with a grid spacing of 0.375 Å. The search was carried out with the Lamarckian Genetic Algorithm: populations of 256 individuals with a mutation rate of 0.02 were evolved for 100 generations. Evaluation of the results was performed by sorting the different complexes with respect to the predicted binding energy. A cluster analysis based on root mean square deviation values, with reference to the starting geometry, was subsequently performed and the lowest energy conformation of the more populated cluster was considered as the most promising bioactive candidate. When clusters of molecules were almost equipopulated, and their energy distribution is spread, the corresponding moiety was not considered a good ligand.



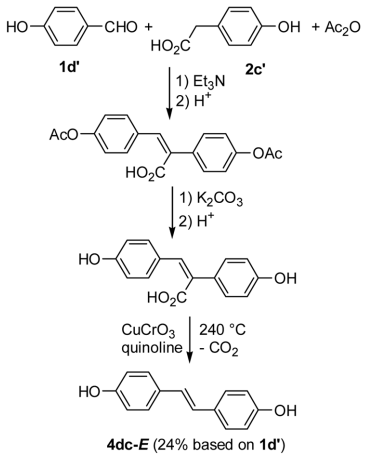
Scheme 1.

All calculations were performed on a Linux workstations equipped with 2 XEON processors, figures were realized with CCP4MG [45].

3 Results and discussion

3.1 Synthesis of Hydroxystilbenes 4aa-*E* (RSV), 4ab-*E* (3,5-DHS), 4ba-*E* (3,4′-DHS), 4cb-*E* (4-HS), 4dc-*E* (4,4′-DHS)

Hydroxystilbenes 4aa-*E*, 4ab-*E*, 4ba-*E*, and 4cb-*E* were prepared by Wittig reaction between the appropriate methoxybenzaldehyde 1a, 1b, or 1c and the appropriate benzyltriphenylphosphonium bromide 2a or 2b followed by isomerization and deprotection, according to Scheme 1. (*E*)-4,4′-dihydroxystilbene 4dc (stilbestrol) was prepared by condensation between 4-hydroxybenzaldehyde 1d′ and 4-hydroxyphenylacetic acid 2c′ followed by decarboxylation, according to Scheme 2. RSV and the analogs were synthesized to evaluate the role of the number of the hydroxyl groups present in the aromatic ring(s) as well as the influence of their relative position on the biological activity exerted by each compound.



Scheme 2.

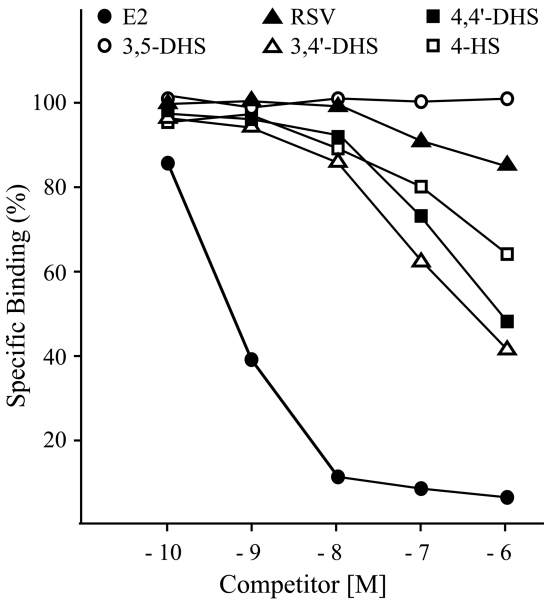


Figure 2. ERα binding assay using increasing concentrations of RSV and analogs. Each point represents the mean of three separate experiments performed in triplicate.

3.2 ERα binding assay

We began our study evaluating whether RSV and derivatives might interact with ERα in extracts of MCF7 cells, which do not express ERβ as judged by RT-PCR (data not shown). Competition binding studies confirmed that RSV is a weak ligand for ERα [20, 21, 46], while all derivatives displayed a higher affinity respect to RSV except 3,5-DHS which did not bind ERα even at the highest concentration used (Fig. 2).

RSV and analogs activate but do not down-regulate endogenous ERα. We then examined whether RSV and the four analogs activate a transiently transfected ER reporter gene (XETL) in MCF7 cells. The reporter plasmid XETL

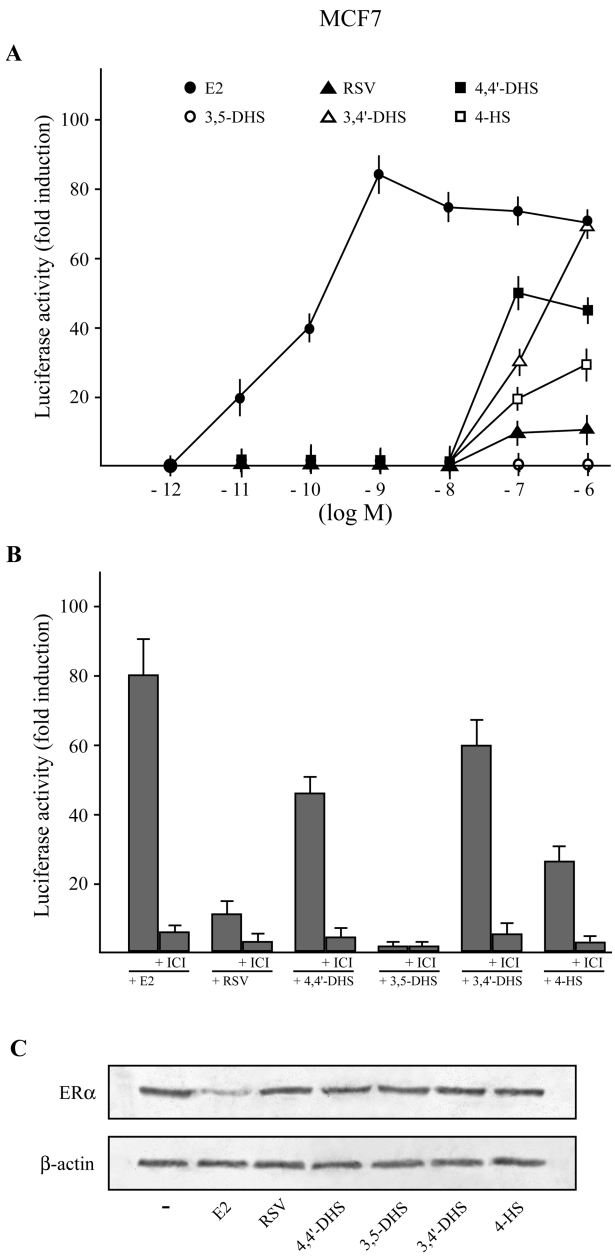


Figure 3. RSV and analogs activate endogenous ERα. (A) MCF7 breast cancer cells were transfected with the luciferase reporter plasmid XETL and treated with increasing concentrations (logarithmic scale) of E2, RSV, and analogs. Luciferase activities were standardized to the internal transfection control and expressed as the ratio of induced activity in absence of ligand. (B) Activation by E2, RSV, and analogs is mediated by ERα. MCF7 cells transfected with the reporter plasmid XETL were treated with 1 nM E2 or 1 μM RSV and analogs with and without 10 μM ER-antagonist ICI. Each data point represents the mean of three experiments performed in triplicate. (C) Immunoblot of ERα from MCF7 cells treated for 24 h with 1 nM E2 or 1 μM RSV and analogs. The results shown are representative of three independent experiments. β-actin serves as loading control.

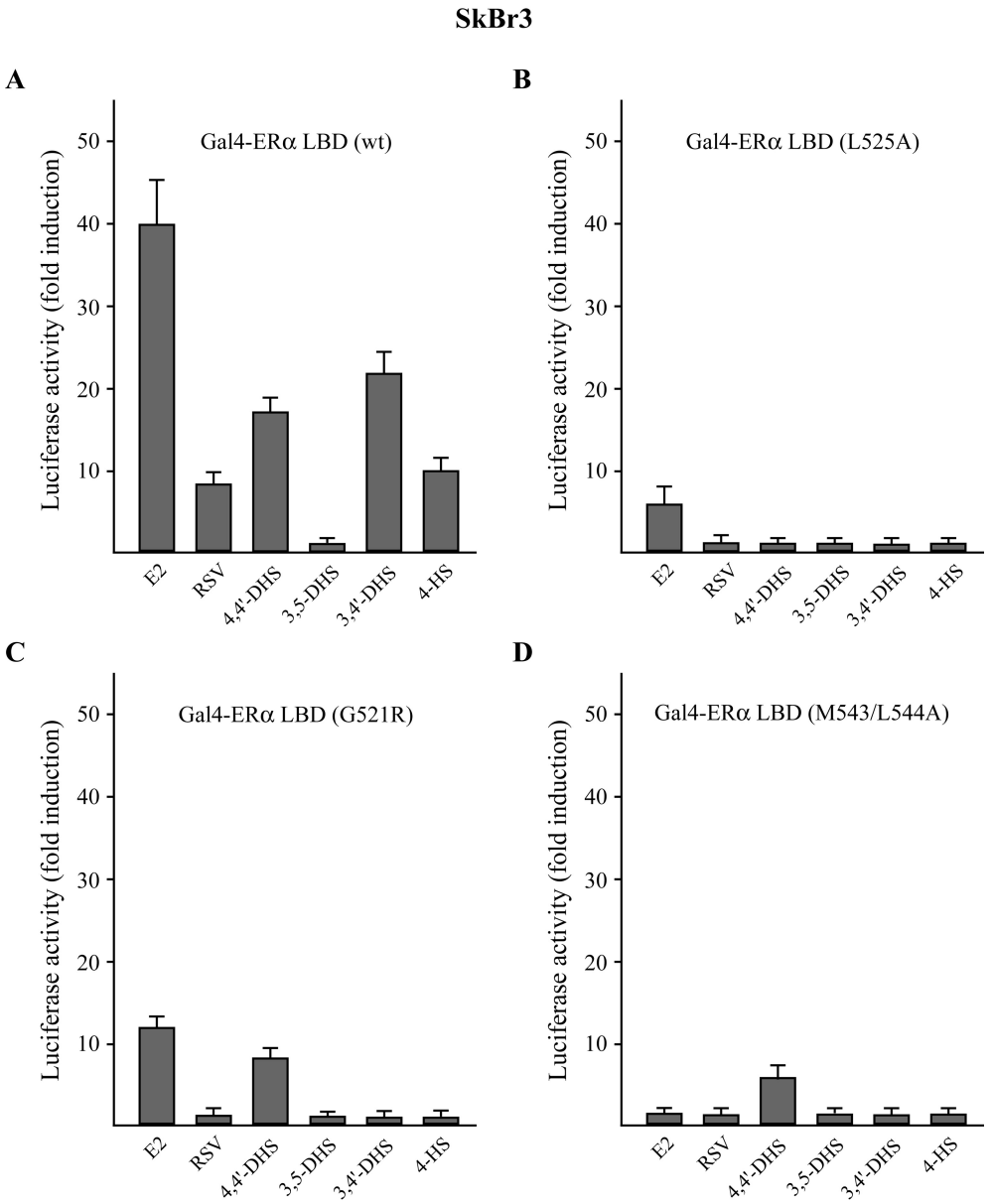


Figure 4. RSV and analogs are ERα agonists in a heterologous system. (A) Fusion proteins consisting of the Gal4 DNA-binding domain and the ERα-LBD are activated by RSV and analogs in transfected SkBr3 breast cancer cells. (B – D) Transactivating response of fusion proteins consisting of the Gal4 DNA-binding domain and ERα-LBD point mutations as indicated. Each data point represents the mean of three experiments performed in triplicate.

carries firefly luciferase sequences under the control of an ERE upstream of the thymidine kinase promoter. As an internal transfection control, we cotransfected a plasmid expressing *Renilla* luciferase, which is enzimatically distinguishable from firefly luciferase, from the strong cytomegalovirus enhancer/promoter. The highest luciferase activity was observed in the presence of 1 nM E2 and 100 pM treatment produced a half maximal stimulation (Fig. 3A). RSV and all the analogs, except 3,5-DHS, were also able to induce luciferase expression, although with lower efficacies compared to E2 even at the highest concentration used, according to the results obtained in competition experiments. Moreover, the transcriptional activity induced by the compounds tested was mediated by ERα since it was no longer evident in the presence of the pure ER antagonist ICI (Fig. 3B). In breast cancer cells, an additional hallmark of full ERα activation by agonists including E2 is represented by the down-regulation of receptor levels through an increased turnover of the E2-activated ERα protein and a reduced transcription of its own gene [47]. Accordingly, a 24 h treatment with 1 nM E2 strongly reduced ERα protein expression in MCF7 cells. RSV and analogs did not show

any modulatory ability at a concentration of 1 μM (Fig. 3C), as recently reported using RSV [48]. Altogether, our results show that, although RSV and three analogs are able to transactivate ERα, they do not trigger all mechanisms involved in ERα down-regulation induced by the cognate ligand E2, likely due to their weak binding affinity for ERα.

3.3 Transcriptional activation of ERα by RSV and analogs in a heterologous system

To examine whether RSV and the analogs transactivate ERα directly, we turned to a completely heterologous system. A chimeric protein consisting of the DNA binding domain (DBD) of the yeast transcription factor Gal4 and the ERα LBD was activated by RSV and all the compounds synthesized, except 3,5-DHS (Fig. 4A) in ER-negative SkBr3 cells. These data demonstrate that the LBD of ERα is sufficient for the transcriptional response and that these compounds act as agonists for the AF-2 domain. We also assessed the response of ERα LBD mutants using Gal4 fusion proteins. The two point mutants L525A and G521R, which require a considerably higher E2 concentration for activation [39], failed to respond to RSV and the analogs, with the exception of the G521R mutant which was sensitive to 4,4′-DHS (Fig. 4B–D). The latter compound also activated the M543/L544A mutant which did not respond to any of the other compounds including E2. These findings suggest that an intact hormone binding pocket of ERα is required for the transactivation elicited by 4,4′-DHS, 3,4′-DHS, and 4-HS whereas in the presence of LBD mutations each compound assumes a particular orientation which may lead to the activation of ERα (see later).

3.4 Docking simulations of wild-type and mutated ERα

The crystal structure of human ERα LBD in complex with its natural ligand E2 (PDB Code 1G50) [49] was used as target for all our docking simulations. Visual inspection of the three dimensional structure of the protein showed that the hormone binding pocket is a mainly hydrophobic buried pocket in which two hydrophilic residues (Arg 394 and His 524) contribute to stabilize the ligand through hydrogen bonds with their hydroxyl groups. The natural ligand E2, RSV and a set of four RSV analogs (Fig. 5) were docked into the hormone binding pocket of ERα to studying the binding modes at a molecular level. In order to investigate the binding of ligands to ERα and evaluate the binding energies of the resulting complexes, we used the computer program AutoDock 3.05/ADT [42, 44]. For each ligand tested, we performed a "blind docking": the docking of small molecules to their targets was done without *a priori* knowledge of the location of the binding site by the system. A preliminary global docking of the natural ligand E2, obtained from the crystal structure of wild-type ERα (PDB code 1G50)

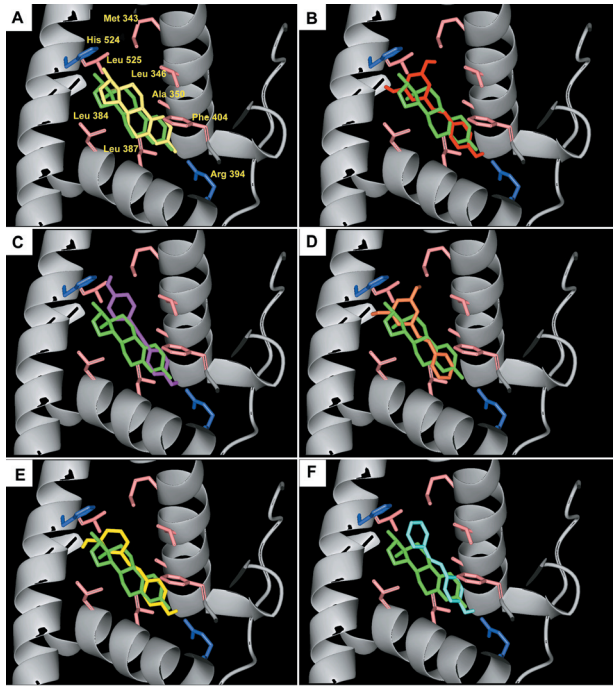


Figure 5. Representation of the binding modes between the tested ligands and ERα, as derived from docking simulations. In all the panels, residues Arg 394 and His 524 involved in hydrogen bond formation with the –OH groups of the ligands, are drawn in blue while hydrophobic residues delimiting the pocket are reported in pink. (A) E2 bound to the ERα, as derived from crystallographic structure (lemon, PDB code:1G50) superposed to the best result of docking simulations (green). (B) RSV (red) superposed to E2 (green). (C) 4,4′-DHS (purple) superposed to E2 (green). (D) 3,5-DHS (tan) superposed to E2 (green). (E) 3,4′-DHS (yellow) superposed to E2 (green). (F) 4-HS (cyan) superposed to E2 (green).

[49], was performed with AutoDock using a grid encompassing the whole protein surface. The docking experiment consisted of 100 simulations which were ranked in order of increasing docking energy values and grouped in clusters with similar conformations (RMSD 0.5 Å). The resulting lowest energy solution was superposed over the crystallographic structure, displaying an RMDS value of 0.112 Å, thus confirming the computational procedure applied with X-ray experiment results. For each compound tested, the solution of docking simulations was grouped by AutoDock in several clusters with comparable binding energies. From the structural analysis of the best solutions (lowest energy) of each cluster, we could highlight differences in the binding orientation. Although the results from AutoDock simulations did not support the hypothesis of a single binding mode, we were able to assess the quality of docked ligands by using, as choice criteria, the evaluation of the hydrophobic contributions as well as visual inspection and comparison of complexes to the crystallographic complex ERα:E2. The final results of docking simulations using the wild-type

Table 1. Hydrogen-bonds and hydrophobic interactions between ligands and the ERα wild-type docking site

	Hydrogen bonds length (Å)			Hydrophobic contacts
	Arg 394	His 524	Leu 525 N-pept.	
E2	2.73	2.71	=	Leu387, Met388, Phe404, Met421, Ile424, Leu525
RSV	2.64	=	=	Leu384, Leu387, Leu391, Phe404, Leu525
4,4′-DHS	2.69	2.78	=	Met343, Leu346, Thr347, Ala350, Leu387, Phe404, Leu525
3,5-DHS	=	=	=	Met343, Leu387, Met388, Leu391, Phe404, Leu525
3,4′-DHS	2.74	3.08	2.97	Met343, Leu387, Phe404, Leu525
4-HS	2.74	=	=	Met343, Thr347, Ala350, Leu387, Phe404, Leu525

Table 2. Affinity constants Ki as calculated by the program AutoDock 3.05

	Wt ERα	G521R	L525A	M543A/L544A
E2	7.7×10^{-9} M	NB	5.2×10^{-8}	8.2×10^{-9}
RSV	3.4×10^{-6} M	NB	NB	3.6×10^{-6}
4,4′-DHS	2.1×10^{-6} M	6.7×10^{-6}	1.7×10^{-5}	2.7×10^{-6}
3,5-DHS	NB	NB	NB	NB
3,4′-DHS	2.1×10^{-6} M	NB	NB	2.8×10^{-6}
4-HS	3.7×10^{-6} M	NB	NB	3.5×10^{-6}

Ki = exp ($\Delta G \times 1000$)/(Rcal × TK) ΔG representing docking energy, Rcal = 1.98719 and TK = 298.15 K, NB = not a ligand.

protein as target, were in good agreement with experimental data, confirming a strong affinity of the protein for E2, a good binding capacity for 4,4′-DHS and 3,4′-DHS ligands, low affinity for RSV and 4-HS, and no affinity for 3,5-DHS (Table 1).

In order to study the mode of binding of RSV analogs, the best docked complexes were subjected to LIGPLOT [50] analysis which allowed us to identify and specify the ligand-protein contacts. Residues involved in ligand binding are reported in Table 1. As expected, the type of interaction is mainly hydrophobic, due to the characteristics of the tested molecules. Two hydrophilic residues (Arg 394 and His 524) are involved in a H-bond through the hydroxyl groups of the bound moieties. The wild-type ERα:E2 complex, as derived from docking simulations, is represented in Fig. 5A; docked E2 (green), resulting from the simulations, is superposed to the crystallographic moiety (purple) for comparison. Panels B-F report the most probable binding mode of the tested ligands (different colors) superposed to the docked E2 (green). Remarkably, some of the residues involved in ligand binding are common for all the tested molecules. The absence of a OH group in one of the two aromatic rings of some moieties, makes the interaction with the target less favored. Being highly hydrophobic and smaller than hormones, these ligands can be accommodated with multiple conformations in the wide binding cavity, mainly lined by hydrophobic residues. Because of these characteristics, the specificity of binding is lower than that for the natural ligand E2.

After performing the simulation using the wild-type protein as target, we attempted to dock the same six ligands on three protein mutants: G521R, L525A, and M543A/L544A, whose structures were built by performing mutations on the coordinates of the wild-type moiety. Table 2 summarizes the results obtained with these new calculations. In particular, the G521R mutation involves the substitution of a residue with a very small side chain (hydrogen atom) with a branched, long and positively charged residue. The cavity hosting the ligand binding site in the wild-type protein is partially occupied by the arginine side chain and therefore the docking simulation indicated only a small affinity for 4,4′-DHS. Docking of E2 on this mutant was not successful, even though we observed a small increase in transcriptional activity. This may be explained considering that in our simulations the protein target is considered as a rigid body whereas real proteins are flexible molecules. The substituted residue R521 could be somehow displaced by the incoming ligand, accommodating some room for binding. Leu525 is important for binding with all the ligands tested here, as reported in Table 1, facilitating hydrophobic contacts between the protein and the skeleton of the ligands thus acting as a "driving force" for the docking. Also in this case, the simulations are in agreement with the *in vitro* tests, displaying a small residual affinity only for E2 to the protein. Different is the case concerning the double mutant M534A/L544A. Although docking simulation reported the same magnitude of activity for the ligands as for the wild-type protein, almost no fold induction was detected *in vitro*. The two mutations, in fact, occur in helix 12, which is not very close to the binding site, however still distant enough to be considered by the docking software as important. At the same time it is known that helix 12 is essential for ER

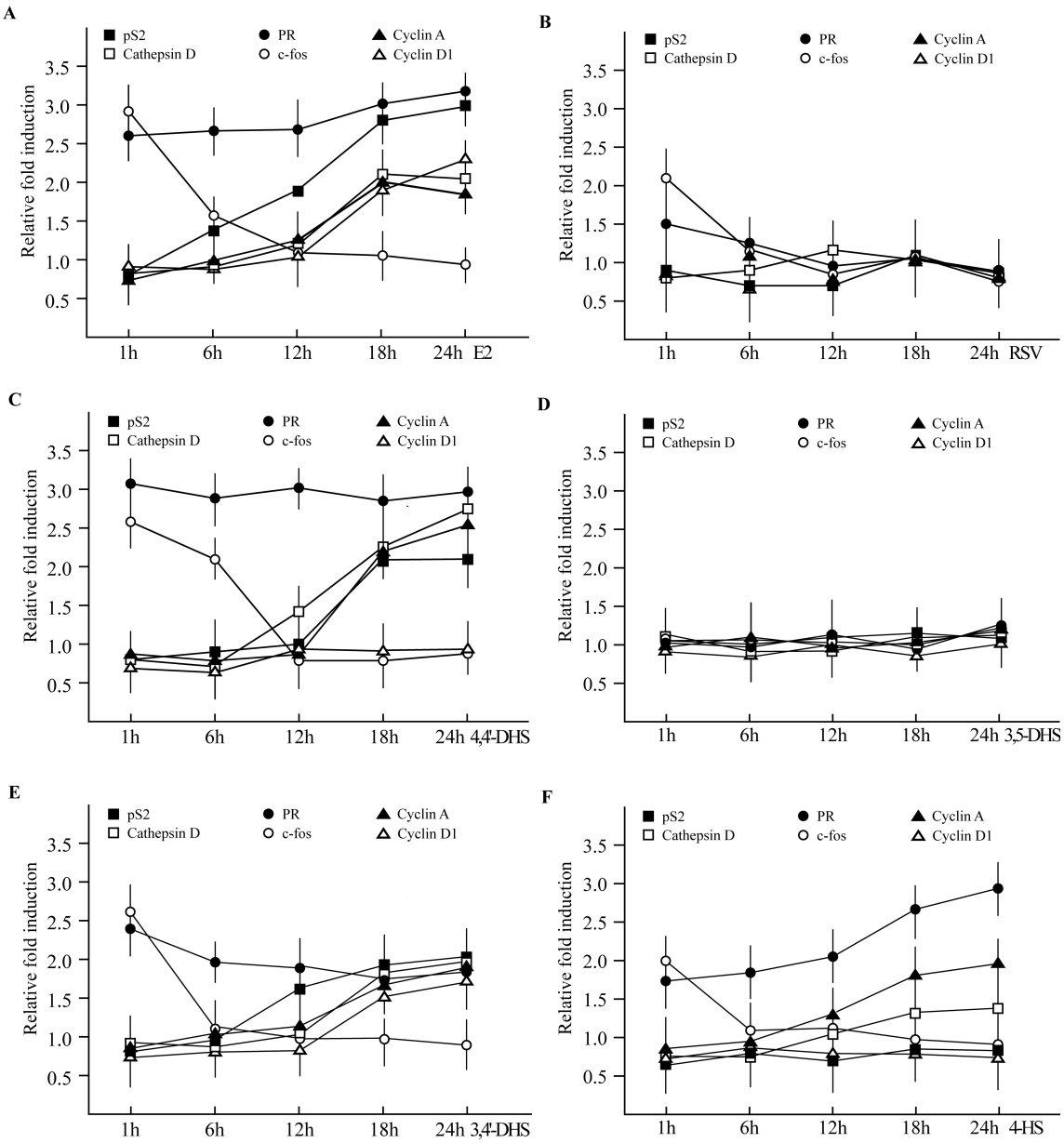


Figure 6. mRNA expression of pS2, Cathepsin D, PR, c-*fos*, Cyclin A and Cyclin D1 evaluated by real-time RT-PCR. MCF-7 cells were treated with vehicle (–), 100 nM E2 (A), 1 μM RSV (B), 4,4′-DHS (C), 3,5-DHS (D), 3,4′-DHS (E), or 4-HS (F) for the indicated times. Results obtained from experiments performed in triplicate were normalized for 18S expression and shown as fold change of RNA expression compared to cells treated with vehicle.

transcriptional activation function. In fact, when an agonist is bound to the ER, helix 12 is oriented anti-parallel to helix 11, capping the ligand binding pocket. This leaves a hydrophobic groove exposed for the binding of coregulator proteins. When an antagonist is bound, helix 12 is displaced via an extended side chain. Helix 12 moves outward, rotates, and packs into the hydrophobic groove between helices 3, 4, and 5. As a result, coactivators needed for transcription cannot bind. What may happen is that this double mutation does not prevent ligand binding, but can perturb the correct displacement of helix 12, thus preventing the binding of cofactors needed for transcription.

3.5 Gene expression changes elicited by RSV and analogs

Having determined that RSV and analogs activate ERα, we evaluated by real-time RT-PCR the potential of each compound to regulate the expression of genes known to be involved in the biological response to estrogens, such as

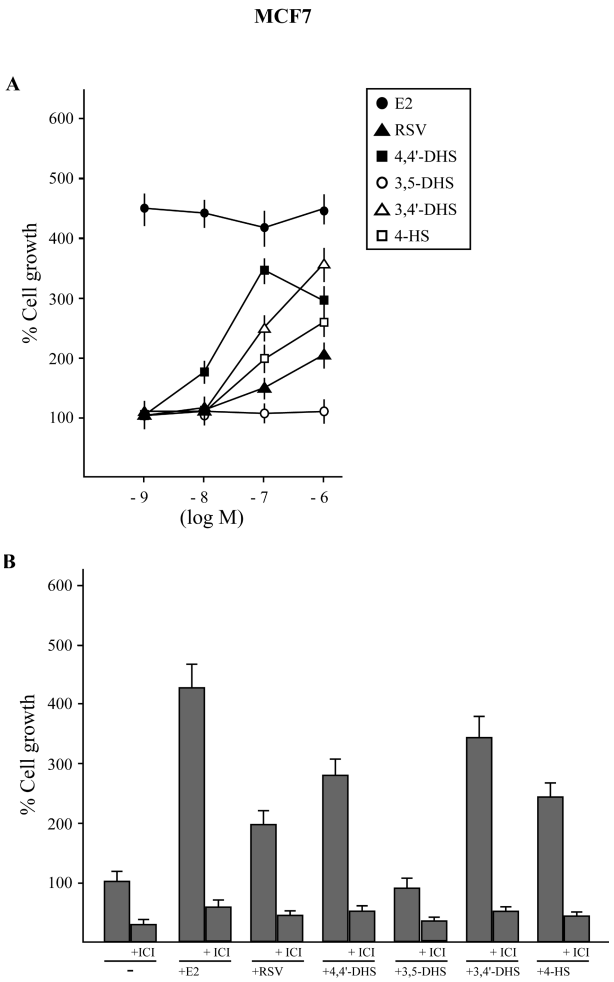


Figure 7. RSV and analogs stimulate proliferative effects in MCF7 breast cancer cells. (A) Cells were treated for 5 days with increasing concentrations (logarithmic scale) of E2, RSV and analogs and counted on day 6. (B) The proliferative effects induced by RSV and analogs are ERα mediated. Cells were treated with 1 nM E2 or 1 μM RSV and analogs with and without 10 μM ER-antagonist ICI. Proliferation of cells receiving vehicle (–) was set as 100% upon which cell growth induced by treatments was calculated. Each data point is the average of three independent experiments.

pS2, Cathepsin D, PR, c-*fos*, Cyclin A, and Cyclin D1 [32–34]. All compounds, except the 3,5-DHS, rapidly (1-h) up-regulated the mRNA expression of PR and c-*fos* (Fig. 6). The expression levels of Cathepsin D, PR, and Cyclin A increased following longer exposures to 4,4′-DHS, 3,4′-DHS, and 4-HS, while pS2 was up-regulated only by 4,4′-DHS and 3,4′-DHS and Cyclin D1 by 3,4′-DHS (Fig. 6). These results suggest that RSV and the analogs studied may have overlapping and yet distinct abilities to regulate the expression of estrogen target genes. Accordingly, it has been reported that different ligands promote distinct molecular conformations and positioning of helix-12 when bound to the ER [32, and references therein]. Interestingly, the latter findings demonstrate that the conformation of the ligand–receptor complex and its ensuing biological activity are tightly connected to the subtle changes in the structure of ligands and not only to dramatic alterations.

3.6 RSV and analogs induce proliferative effects in MCF7 breast cancer cells

On the basis of the above results, we aimed to evaluate a more complex physiological response. We analyzed the effects of RSV and analogs on the proliferation of MCF7 breast cancer cells. Cells were treated for 5 days with increasing concentrations of E2, RSV, and analogs and counted on day 6. Figure 7A shows the results obtained with treatments compared to cells treated with vehicle. RSV and analogs, with the exception of 3,5-DHS, were all able to stimulate the proliferation of MCF7 cells in line with the ability exhibited in transfection experiments. The growth effects elicited by the compounds tested were no longer evident in the presence of the ER antagonist ICI (Fig. 7B), suggesting that ERα mediates cell proliferation in this cell context.

4 Concluding remarks

In the present study, using cell based assays we demonstrated that RSV and analogs exhibit the following order of agonism for ERα: 3,4′-DHS > 4,4′-DHS > 4-HS > RSV. Moreover, the 3,5-DHS derivative did not elicit any ligand activity. Computer modeling indicated that subtle changes in the structure of the compounds examined lead to different binding positions and ERα-LBD conformations which may be responsible for selective gene regulation and growth stimulation in estrogen-sensitive cancer cells.

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The authors have declared no conflict of interest.

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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),

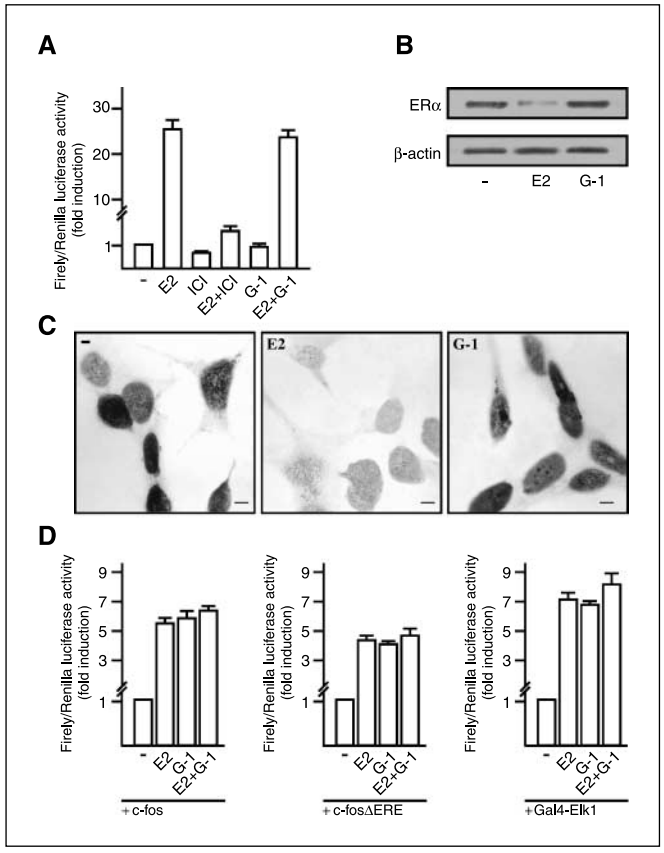

Figure 1. The specific GPR30 ligand G-1 does not activate ERα but induces the transcription of *c-fos* promoter constructs. *A,* BG-1 cells were transfected with the ER luciferase reporter plasmid XETL and treated with 100 nmol/L of E2 and G-1 and 10 μmol/L of the ER antagonist ICI 182,780 (*ICI*). The 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.

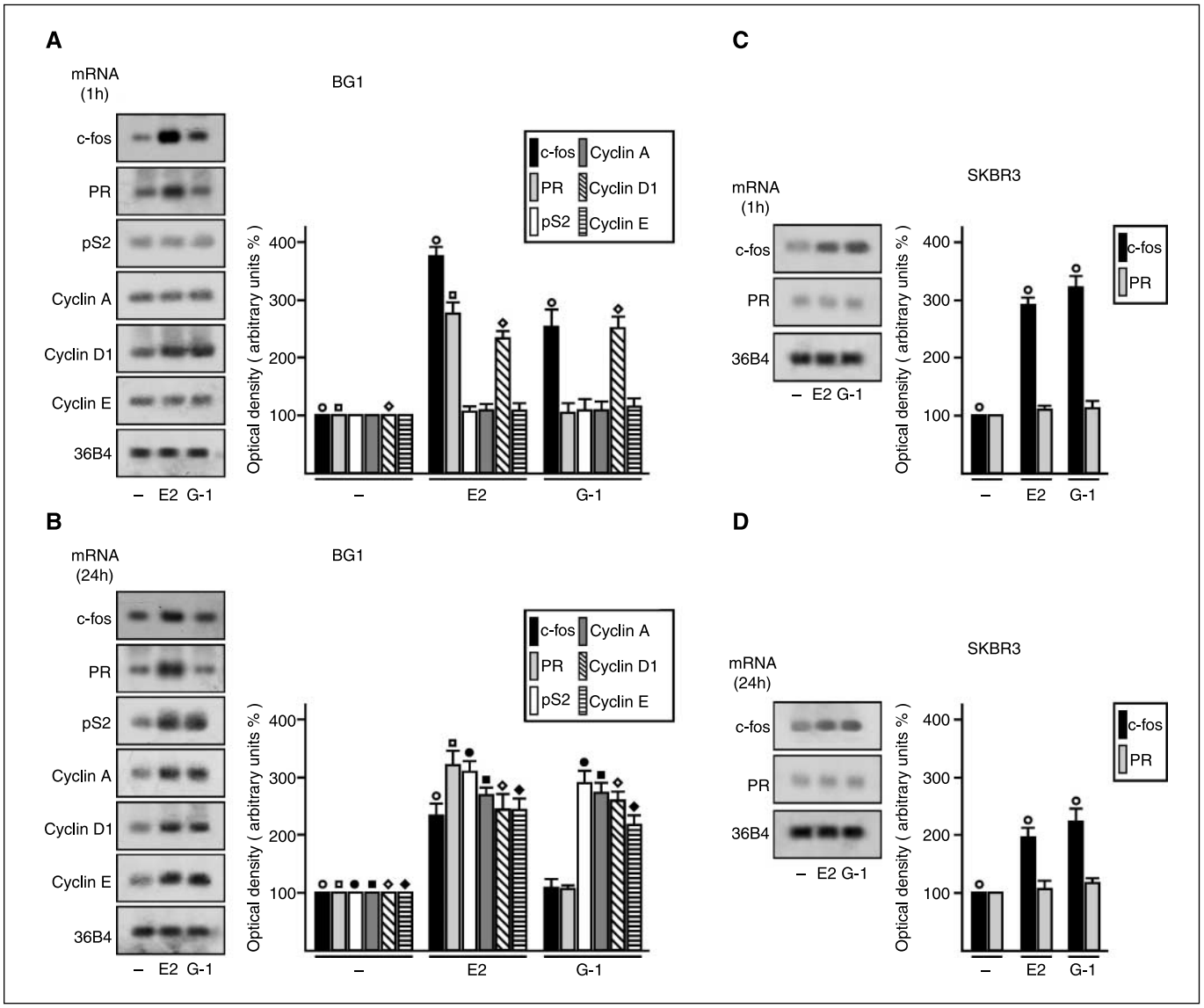

Figure 2. mRNA expression of estrogen target genes in BG-1 and SKBR3 cells. The expression of *c-fos*, PR, pS2, cyclin A, cyclin D1, and cyclin E was evaluated as indicated by semiquantitative RT-PCR in cells treated for 1 h (*A* and *C*) and 24 h (*B* and *D*) with vehicle (–) or 100 nmol/L of E2 and 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

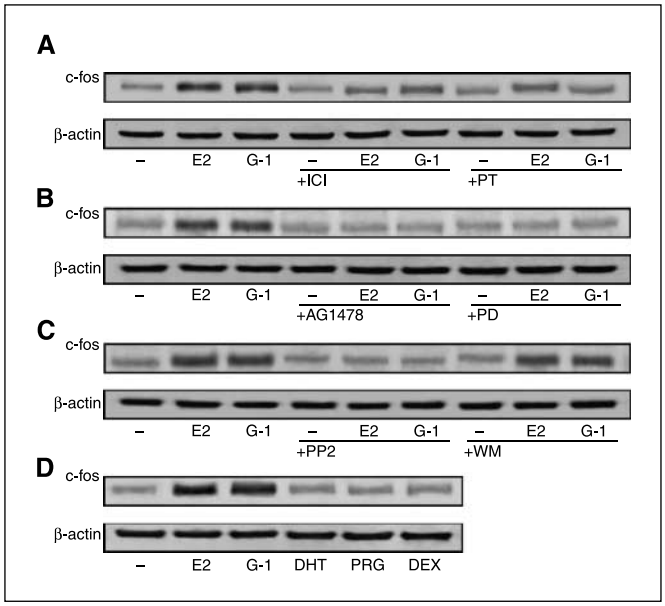

Figure 3. Immunoblots of *c-fos* from BG-1 cells. *A,* cells were treated for 2 h with vehicle (–) or 100 nmol/L of E2 and G-1 and in combination with 10 μmol/L ICI 182,780 or 100 ng/mL of G protein inhibitor pertussis toxin (*PT*). *B,* cells were treated for 2 h with vehicle (–) or 100 nmol/L of E2 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.

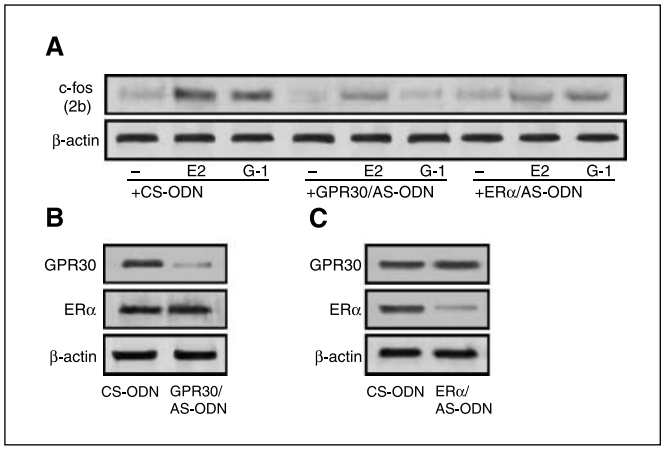

Figure 4. GPR30 and ERα antisense oligonucleotides abrogate the up-regulation of *c-fos* induced by E2 and G-1 in BG-1 cells. *A,* cells transfected with control scrambled (*CS-ODN*), GPR30 (*GPR30/AS-ODN*), or ERα (*ERα/AS-ODN*) antisense oligonucleotides were treated with 100 nmol/L of E2 and G-1. *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

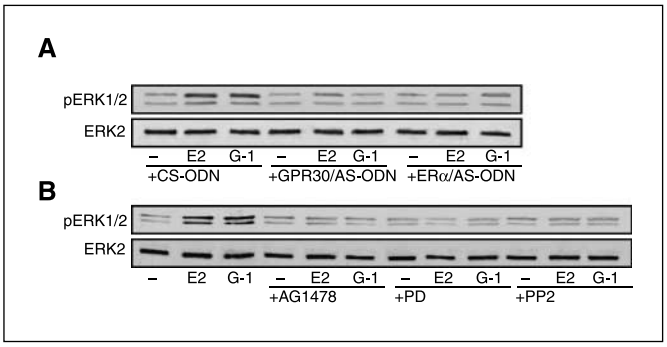

Figure 5. ERK1/2 phosphorylation in BG-1 cells. *A,* cells transfected with control scrambled (*CS-ODN*), GPR30 (*GPR30/AS-ODN*), or ERα (*ERα/AS-ODN*) antisense oligonucleotides were treated for 5 min with vehicle (–) or 100 nmol/L of E2 and G-1. *B,* cells were treated for 5 min with vehicle (–) or 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.

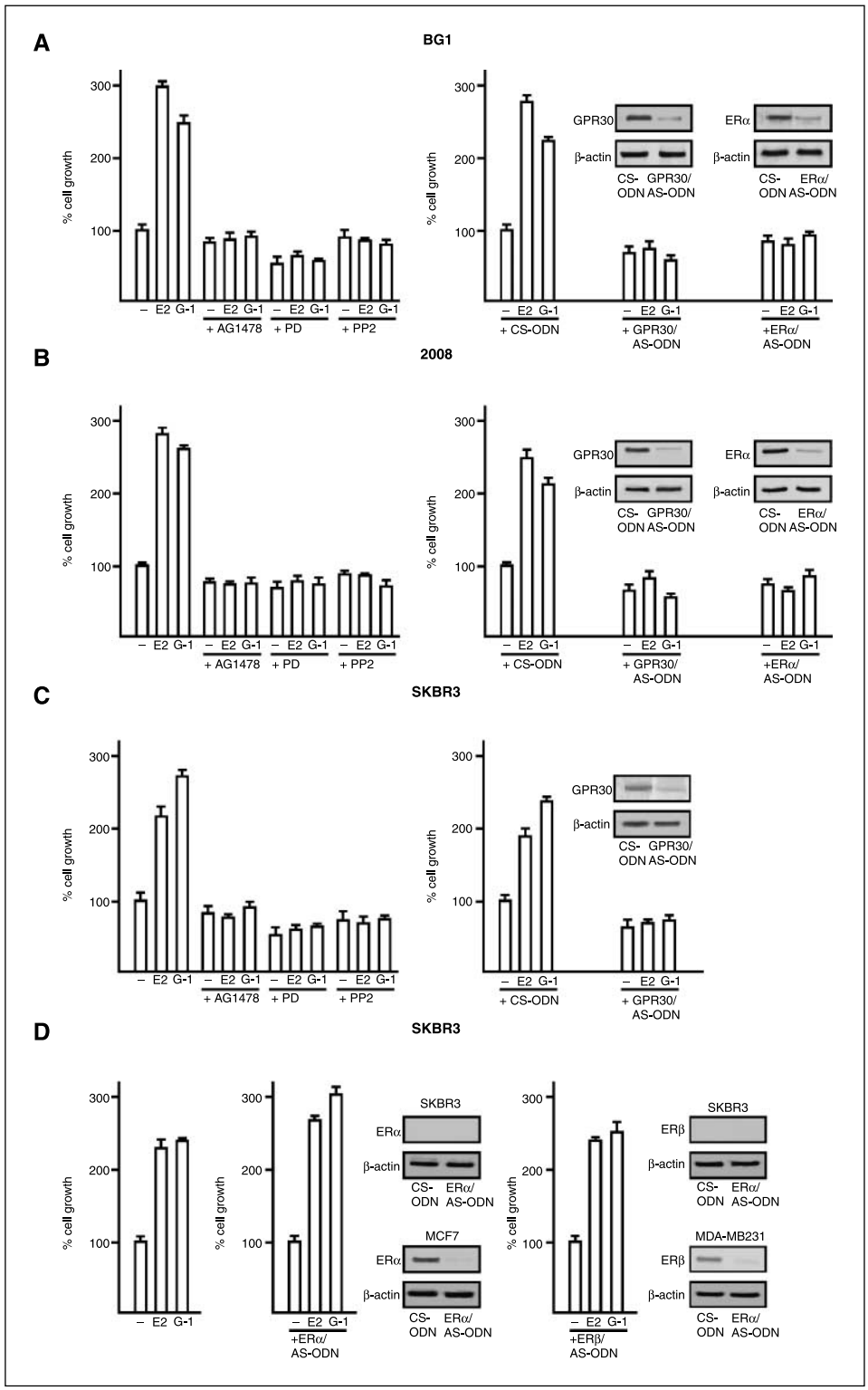

Figure 6. E2 and G-1 stimulate the proliferation of the ovarian BG-1 and 2008 tumor cells and the breast SKBR3 carcinoma cells. BG-1 cells (*A*), 2008 cells (*B*), and SKBR3 cells (*C*) were treated with vehicle (−) or 100 nmol/L of E2 and G-1 in medium containing 2.5% charcoal-stripped FBS (medium was 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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17β-Estradiol, Genistein, and 4-Hydroxytamoxifen Induce the Proliferation of Thyroid Cancer Cells through the G Protein-Coupled Receptor GPR30

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ABSTRACT

The higher incidence of thyroid carcinoma (TC) in women during reproductive years compared with men and the increased risk associated with the therapeutic use of estrogens have suggested a pathogenetic role exerted by these steroids in the development of TC. In the present study, we evaluated the potential of 17β-estradiol (E2), genistein (G), and 4-hydroxytamoxifen (OHT) to regulate the expression of diverse estrogen target genes and the proliferation of human WRO, FRO, and ARO thyroid carcinoma cells, which were used as a model system. We have ascertained that ARO cells are devoid of estrogen receptors (ERs), whereas both WRO and FRO cells express a single variant of ERα that was neither transactivated, modulated, nor translocated into the nucleus upon treatment with ligands. However, E2, G, and OHT were able either to induce the transcriptional activity of c-*fos* promoter constructs, including those lacking the estrogen-responsive elements, or to increase c-*fos*, cyclin A, and D1 expression. It is noteworthy that we have demonstrated that the G protein-coupled receptor 30 (GPR30) and the mitogen-activated protein kinase (MAPK) pathway mediate both the up-regulation of c-*fos* and the growth response to E2, G, and OHT in TC cells studied, because these stimulatory effects were prevented by silencing GPR30 and using the MEK inhibitor 2′-amino-3′-methoxyflavone (PD 98059). Our findings provide new insight into the molecular mechanisms through which estrogens may induce the progression of TC.

Thyroid carcinoma (TC) is the most common malignancy affecting the endocrine glands (Jemal et al., 2004). The conspicuous increased incidence during recent years as observed in the decade between 1994 and 2004 in the United States (>80%) (Jemal et al., 2004) has prompted investigators to better define the molecular mechanisms involved in TC development. Several lines of evidence have documented that mutations of genes such as BRAF, RET, and RAS coding for effectors along the MAPK pathway exert a crucial role in thyroid transformation (Melillo et al., 2005). However, the consistent demographic observation that TC is 3 times more frequent in women than in men from the onset of puberty until menopause when this ratio declines progressively (Henderson et al., 1982) still remains to be elucidated. The gender-dependent difference observed worldwide (Waterhouse et al., 1982) and the increased risk in women taking estrogens for gynecological disorders (Ron et al., 1987; Persson et al., 1996) have suggested that these steroids may influence the

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ABBREVIATIONS: TC, thyroid carcinoma; GPR30, G protein couplet receptor 30; G, genistein; OHT, 4-hydroxytamoxifen; DEX, dexamethasone; PRG, progesterone; PR, progesterone receptor; PD 98059 and PD, 2′-amino-3′-methoxyflavone; ER, estrogen receptor; E2, 17β-estradiol; ERE, estrogen response element; MAPK, mitogen-activated protein kinase; AF, activation function; GPCR, G protein-coupled receptor; Cx, cycloheximide; WM, wortmannin; PT, pertussis toxin; DHT, 5α-dihydrotestosterone; ICI 182,780 and ICI, faslodex; AG 1478, 4-(3′-chloroanilino)-6,7-dimethoxy-quinazoline; AG 490, α-cyano-(3,4-dihydroxy)-*N*-benzylcinnamide; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4,*d*]pyrimidine; FBS, fetal bovine serum; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; bp, base pair(s); kb, kilobase(s); ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; PI3K, phosphoinositide-3 kinase; EGFR, epidermal growth factor receptor; AP-1, activator protein 1; JAK, Janus tyrosine kinase; AS-ODN, antisense oligodeoxynucleotide; LY 294,002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one.

progression of TC, as largely demonstrated for breast and endometrial carcinoma (Pike et al., 2004). On the other hand, the ability of 17β-estradiol (E2) to elicit proliferative effects in human thyroid cancer cells (Manole et al., 2001; Lee et al., 2005) and in FRTL-5 rat thyroid cells (Furlanetto et al., 1999) and the expression of estrogen receptors (ERs) in normal and neoplastic thyroid tissues (Yane et al., 1994; Dalla Valle et al., 1998) have provided further evidence on the potential role exerted by E2 in the progression of TC.

The classic mechanism of E2 action involves the binding to ERα and ERβ that contain two main transcription activation functions (AFs): the N-terminal AF1, and the C-terminal AF2, which is associated with the ligand binding domain responsible for hormone-dependent transactivation (Tora et al., 1989). Both ERs after ligand activation and nuclear localization interact with the estrogen response elements (EREs) located within the regulatory region of target genes (Kumar and Chambon, 1988). It is interesting that previous studies, including our own (Maggiolini et al., 2004; Revankar et al., 2005; Thomas et al., 2005; Vivacqua et al., 2006), have demonstrated that estrogens are also able to activate a G protein-coupled receptor (GPCR) named GPR30, which mediates the transcription of genes like c-*fos* involved in the cycle progression of different tumor cells. c-*fos* is rapidly and transiently induced by different extracellular stimuli, such as mitogens and hormones (Hill and Treisman, 1995). The transcription of c-*fos* is regulated by multiple *cis*-elements, including the serum-response element, which recruits the ternary complex factors Elk-1 and the serum response factor accessory proteins 1 and 2 (Treisman, 1995). Moreover, E2 signaling may trigger a nongenomic ERα pathway, leading to MAPK-dependent phosphorylation and binding of Elk-1 to the serum-response element sequence (Duan et al., 2001). As it concerns the imperfect palindromic ERE sequence contained within the c-*fos* promoter, binding to ERα alone is not sufficient for transactivation but instead requires the receptor interaction with a downstream Sp1 site (Duan et al., 1998).

In the present study, we evaluated the potential of E2, genistein (G), and 4-hydroxytamoxifen (OHT) to regulate the expression of diverse estrogen target genes and the proliferation of human follicular WRO and both anaplastic FRO and ARO thyroid cancer cells used as a model system. Our data demonstrate for the first time to our knowledge that a GPR30-dependent mechanism is involved in both gene transcription and growth effects elicited by E2, G, and OHT in thyroid cancer cells.

Materials and Methods

Reagents. E2, G, OHT, cycloheximide (Cx), wortmannin (WM), LY 294,002, pertussis toxin (PT), PD 98059 (PD), dexamethasone (DEX), progesterone (PRG), and 5α-dihydrotestosterone (DHT) were purchased from Sigma-Aldrich Corporation (Milan, Italy). ICI 182,780 (ICI) was obtained from Tocris Chemicals (Bristol, UK), AG 1478 and AG 490 were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and H-89 and PP2 were obtained from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in dimethyl sulfoxide, except for E2, OHT, PD, and WM, which were dissolved in ethanol.

Cell Culture. Human follicular WRO and anaplastic FRO and ARO thyroid tumor cells (gifts from F. Arturi and A. Belfiore, University of Magna Grecia, Catanzaro, Italy), and HeLa cells were maintained in Dulbecco's modified Eagle's medium without phenol red supplemented with 10% fetal bovine serum (FBS) and Glutamax (Invitrogen, Milan, Italy). Cells were switched to medium without serum the day before RT-PCR, immunoblots, and the evaluation of ERK1/2 phosphorylation.

Plasmids. The firefly luciferase reporter plasmid for ERs was XETL, which contains the ERE from the *Xenopus laevis* 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 kb 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 (Gallo et al., 2002). The plasmid HEG0 was used to express ERα. The plasmids encoding antisense GPR30 (GPR30/AS) and dominant-negative ERK2 (DN/ERK) were kindly provided by E. R. Prossnitz (University of New Mexico, Albuquerque, NM) and M. Cobb (University of Texas, Dallas, TX), respectively. The *Renilla reniformis* luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard.

Transfections and Luciferase Assays. WRO and FRO 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 performed 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 approximately 12 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 *R. reniformis* luciferase activity. The normalized relative light unit values obtained from untreated cells were set as 100% activity, upon 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 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, and a mixture of protease inhibitors containing 1 mM aprotinin, 20 mM phenylmethylsulfonyl fluoride, and 200 mM sodium orthovanadate. Protein concentration was determined by Bradford reagent according to the manufacturer's recommendations (Sigma). Equal amounts of whole-protein extract were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (GE Healthcare, Milan, Italy), probed overnight at 4°C with the antibodies against the C- and N-terminal domains of ERα (F-10 and D-12, respectively), c-*fos*, GPR30, progesterone receptor (PR), β-actin (all purchased from Santa Cruz Biotechnology, DBA, Milan, Italy), ERβ (Serotec, Milan, Italy), pERK1/2, and ERK2 (both from Cell Signaling Technology, Inc., Celbio, Milan, Italy), and then revealed using the ECL system (GE Healthcare). Five micrograms of DN/ERK and GPR30/AS expression plasmid was transfected using FuGENE 6 reagent as recommended by the manufacturer for 24 h before treatments.

RT-PCR. The evaluation of gene expression was performed by semiquantitative RT-PCR as described previously (Maggiolini et al., 1999). For ERα, 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 the following: 5′-GGAGACATGAGAGCTGCCA-3′ (ERα forward) and 5′-CCAGCAGCATGTCGAAGATC-3′ (ERα reverse); 5′-AGAAAAGGAGAATCCGAAGGGAAA-3′ (c-*fos* forward) and 5′-ATGATGCTGGGACAGGAAGTC-3′ (c-*fos* reverse); 5′-GCCATTAGTTTACCTGGACCCAGA-3′ (cyclin A forward) and 5′-CACTGACATGGAAGACAGGAACCT-3′ (cyclin A reverse); 5′-TCTA-

AGATGAAGGAGACCATC-3′ (cyclin D1 forward) and 5′-GCGGTAGTAGGACAGGAAGTTGTT-3′ (cyclin D1 reverse); 5′-TTCTATCCTAATACCATCGACG-3′ (pS2 forward) and 5′-TTTGAGTAGTCAAAGTCAGAGC-3′ (pS2 reverse); 5′-CCTCGGACACCTTGCCTGAA-3′ (PR forward) and 5′-CGCCAACAGAGTGTCCAAGAC-3′ (PR reverse); and 5′-CTCAACATCTCCCCCTTCTC-3′ (36B4 forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse), to yield products of 438, 420, 354, 354, 210, 239, and 408 bp, respectively, with 20 PCR cycles for ERα, c-*fos,* cyclin A, cyclin D1, pS2; 25 PCR cycles for PR; and 15 PCR cycles for 36B4.

Antisense Oligodeoxynucleotide Experiments. AS-ODNs were purchased from M-Medical (Milan, Italy) and synthesized as described previously (Kanda and Watanabe, 2003). The oligonucleotides used were the following: 5′-TTGGGAAGTCACATCCAT-3′ for GPR30, 5′-GACCATGACCATGACCCT-3′ for ERα, and 5′-GATCTCAGCACGGCAAAT-3′ for the scrambled control. For antisense experiments, a concentration of 200 nM concentrations of the indicated oligonucleotides was transfected using FuGENE 6 reagent as recommended by the manufacturer for 4 h before treatment with ligands.

Immunocytochemical Staining. Cells were maintained in medium lacking serum for 3 days, treated for 1 h, 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 performed using as the primary antibody a mouse monoclonal immunoglobulin (Ig) G generated against the human C-terminal of ERα (F-10, 1:50 overnight at 4°C; Santa Cruz Biotechnology). A biotinylated horse-antimouse IgG (1:600 for 60 min at room temperature) was applied as the secondary antibody (Vector Laboratories, Burlingame, CA). Thereafter, 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 Tris-buffered saline (0,05 M Tris-HCl plus 0.15 M 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 extensively once they had attached and then were incubated in medium containing 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. GPR30/AS (200 ng) or 200 ng of empty vector was transfected using FuGENE 6 reagent as recommended by the manufacturer every 2 days.

Statistical Analysis. Statistical analysis was performed using analysis of variance followed by Newman-Keuls testing to determine

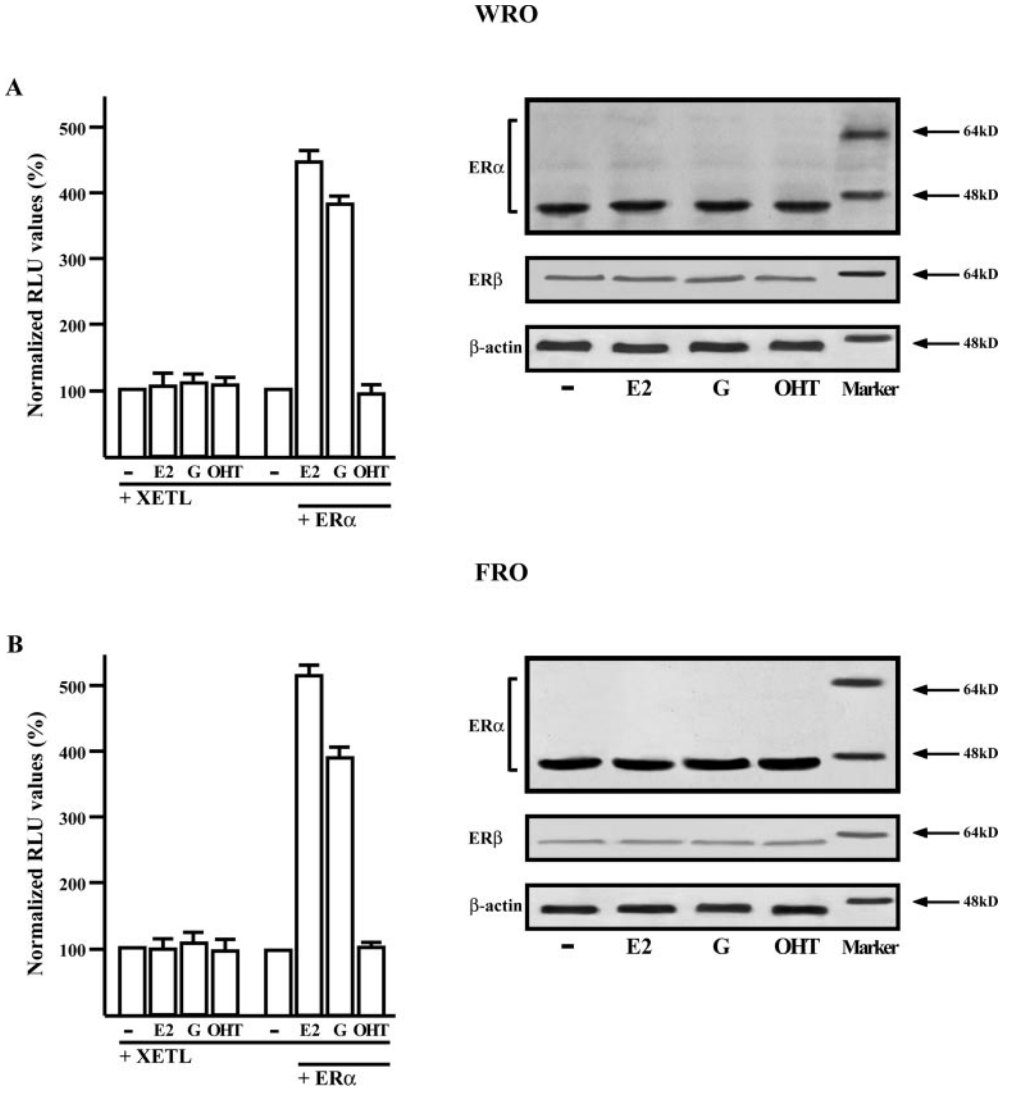

Fig. 1. The isoform of ERα expressed in WRO and FRO cells is neither activated nor modulated by E2, G, and OHT. A, cells were transfected with the ER luciferase reporter plasmid XETL and the wild-type ERα where applicable and treated with 100 nM concentration of ligands. The luciferase activities were normalized to the internal transfection control, and values of cells receiving vehicle (−) were set as 100%, upon which the activity induced by treatments was calculated. Each data point represents the mean ± S.D. of three independent experiments performed in triplicate. B, immunoblots of ERα and ERβ from cells treated with 100 nM ligands for 12 h. β-Actin serves as loading control.

differences in means. $p < 0.05$ was considered statistically significant.

Results

E2, G, and OHT neither Transactivate nor Regulate the Expression of the ERα Variant and ERβ Expressed in TC Cells. To evaluate the potential ability of E2, G, and OHT to elicit ER-mediated transcriptional effects, we transiently transfected the ER reporter gene XETL in WRO and FRO thyroid cancer cells. As shown in Fig. 1 A, neither cell line showed any evidence of ERE-dependent functional response to 100 nM concentrations of each compound, as also observed upon concentrations ranging from 1 nM to 10 μM (data not shown). However, cotransfecting an expression vector encoding ERα (HEG0), only E2 and G induced the luciferase activity (Fig. 1A). Given that decreased levels of ERα by agonists have been reported as an additional hallmark of receptor activation (Santagati et al., 1997), we ascertained the expression and potential regulation of ERα by treatments in WRO and FRO cells. Using an anti-ERα antibody raised against the C-terminal domain (F-10), we detected a single ERα isoform with a smaller size (less than 48 kDa) with respect to ERα wild type (66 kDa) (Fig. 1B). This variant of ERα was not modified upon exposure to a 100 nM concentration of ligands for 12 h and was no longer detected using an anti-ERα antibody raised against the N-terminal region (D-12) (data not shown). As it concerns the expression of ERβ, we did not observe any change in the aforementioned experimental conditions (Fig. 1B). Taken together, these results indicate that the isoform of ERα expressed in WRO and FRO cells does not mediate the ERE-dependent transcriptional effects.

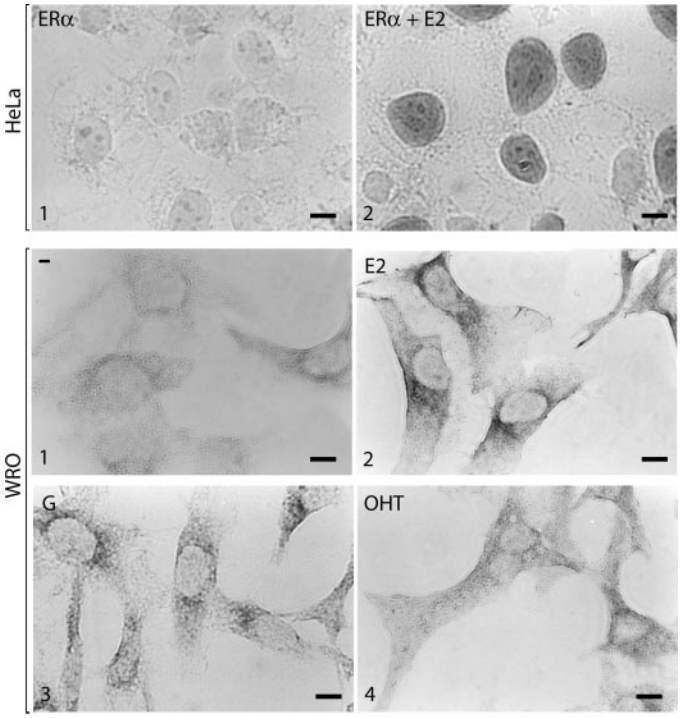

Fig. 2. Immunocytochemical detection of the ERα variant expressed in WRO cells. HeLa, ER-negative HeLa cells were transfected for 24-h in serum-deprived conditions with 0.5 μg of an expression plasmid encoding ERα. 1, cells receiving vehicle; 2, cells treated with 100 nM E2 for 2 h. WRO, cells were serum-deprived for 24 h and then treated for 2 h with vehicle (1) or 100 nM E2 (2), G (3), or OHT (4). Each experiment is representative of at least 10 tests. Bar, 5 μM.

Subcellular Localization of the ERα Variant Expressed in TC Cells. The ligand binding of ERα leads to conformational changes that result in receptor activation, nuclear localization, and transcriptional regulation of target genes (Kumar and Chambon, 1988; Tora et al., 1989). As further evidence that the variant of ERα found in TC cells is not able to trigger classic genomic actions, we performed an immunocytochemical assay to evaluate its subcellular distribution after treatment with ligands. To verify the specificity of the ERα antibodies used and the localization of ERα in the presence of E2, we transfected ER-negative HeLa cells with an expression plasmid encoding ERα. Using the antibody raised against the C-terminal domain of ERα (F-10), the weak immunodetection observed in cells treated with vehicle became strongly evident in the nuclear compartment after a brief exposure (2 h) to E2

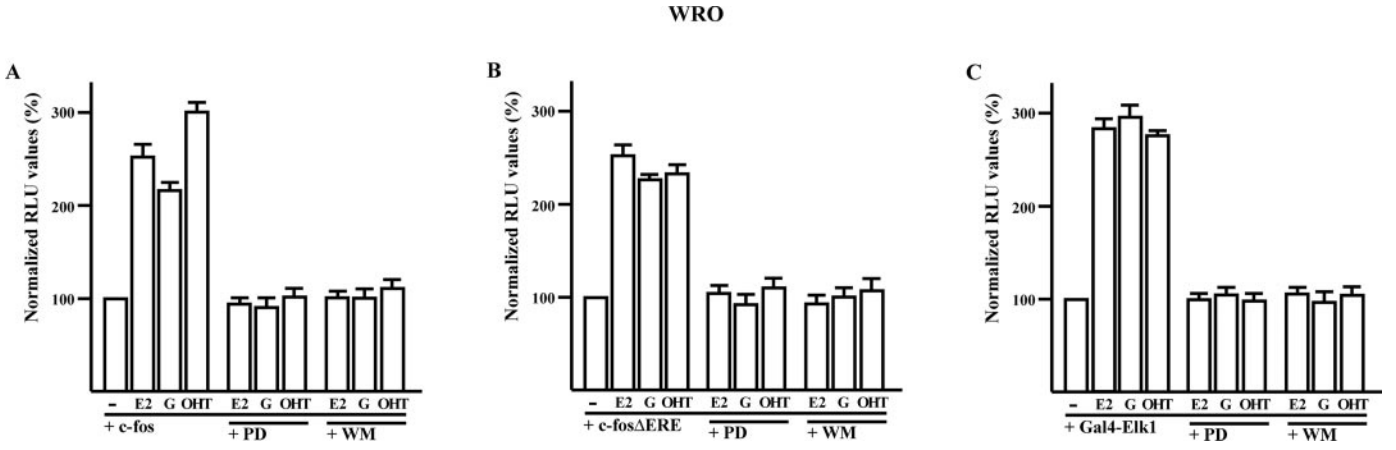

Fig. 3. Transcriptional activation of c-*fos* promoters and Gal4-Elk1 by E2, G, and OHT in WRO cells. A, the luciferase reporter plasmid c-*fos* encoding a −2.2-kb-long upstream region of human c-*fos,* and B, the deletion mutant c-*fos*ΔERE lacking the ERE sequence and encoding a −1172 bp upstream fragment of human c-*fos* were activated by 100 nM E2, G, and OHT. The transcriptional activity of both c-*fos* promoter constructs was abrogated by 10 μM PD or 10 μM WM. C, the luciferase reporter plasmid for the fusion protein consisting of Elk1 and the Gal4 DNA binding domain is activated by 100 nM E2, G, and OHT; Elk1 transcription was reversed by 10 μM PD or 10 μM WM. The luciferase values were standardized to the internal transfection control, and values of cells receiving vehicle (−) were set as 100%, upon which the activity induced by treatments was calculated. Each data point represents the mean ± S.D. of three independent experiments performed in triplicate.

(panel of HeLa cells in Fig. 2). Results similar to those described above were obtained in HeLa cells with the antibody raised against the N-terminal domain of ERα (D-12) (data not shown). Using the anti-ERα antibody F-10 in WRO cells, the immunoreactivity was displayed exclusively in the cytoplasm also in presence of ligands (panel of WRO cells in Fig. 2). In contrast, the anti-ERα antibody D-12 was not able to evidence any signal in WRO cells (data not shown). On the basis of these observations, we can further argue that the variant of ERα found in TC cells does not mediate transcriptional effects according to the classic mechanism of action of ERα.

E2, G, and OHT Transactivate c-*fos* Promoter Constructs. Considering the results described above and our previous investigations (Maggiolini et al., 2004; Vivacqua et al., 2006), we aimed to evaluate whether E2, G, and OHT could activate a full-length c-*fos* promoter (−2.2 kb) that contains diverse sequences responding to a variety of extracellular stimuli (Hill and Treisman, 1995). It is interesting that the c-*fos* promoter construct was transactivated by all treatments that were no longer able to elicit such an effect in the presence of the MEK and PI3K inhibitors PD and WM, respectively (Fig. 3A). The transactivation of c-*fos* by ligands occurred in an ERE-independent manner, given that an expression vector encoding a c-*fos* promoter lacking the ERE sequences (−1172 bp) induced the transcriptional activity, which was repressed again by PD and WM (Fig. 3B). It has been largely reported that the ternary complex factor member Elk-1 is crucial for the ERK-dependent activation of the c-*fos* promoter gene (Treisman, 1995). It is noteworthy that E2, G, and OHT induced the Elk-1-mediated transcription that was abrogated by PD and WM (Fig. 3C). Altogether,

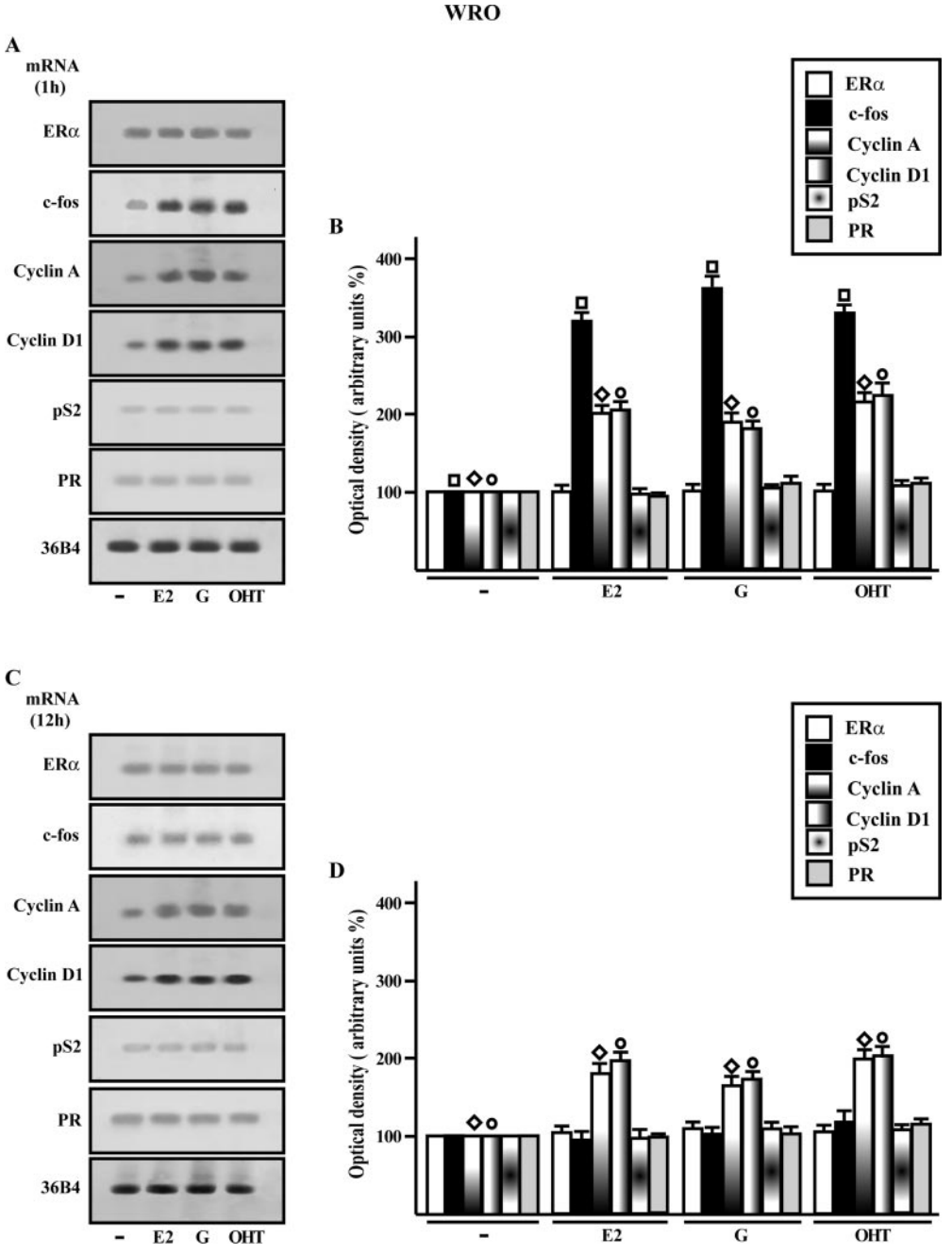

Fig. 4. The mRNA of c-*fos* and cyclins A and D1 are up-regulated by E2, G, and OHT in WRO cells. A and C, the expression of ERα, c-*fos*, cyclins A and D1, pS2, and PR were evaluated by semiquantitative RT-PCR in WRO cells treated with 100 nM E2, G, and OHT for the indicated times; 36B4 levels were determined as a control. B and D, the quantitative representation of data (mean ± S.D.) of three independent experiments after densitometry and correction for 36B4 expression. □, ◇, and ○ indicate $p < 0.05$ for cells receiving vehicle (−) versus treatments.

these results indicate that the regulation of c-*fos* promoter by estrogens may occur through diverse mechanisms independently of ERα.

The mRNA Expression of c-*fos*, Cyclin A, and Cyclin D1 Is Up-Regulated by E2, G, and OHT. Having established that the c-*fos* promoter is transactivated by the ligands used, we evaluated whether E2, G, and OHT stimulate the expression of c-*fos* along with other estrogen target genes such as the well-known PR, pS2 (Cavailles et al., 1989) and cyclins A and D1 (Butt et al., 2005). To this end, we performed semiquantitative RT-PCR experiments comparing the mRNA levels after standardization on a housekeeping gene encoding the ribosomal protein 36B4. As shown in Fig. 4, a short exposure (1 h) of WRO cells to ligands induced an up-regulation of c-*fos,* which was no longer observed after 12 h of treatment. Moreover, E2, G, and OHT were able to enhance at both times of observation the expression of cyclins A and D1, whereas pS2 and PR did not display any change

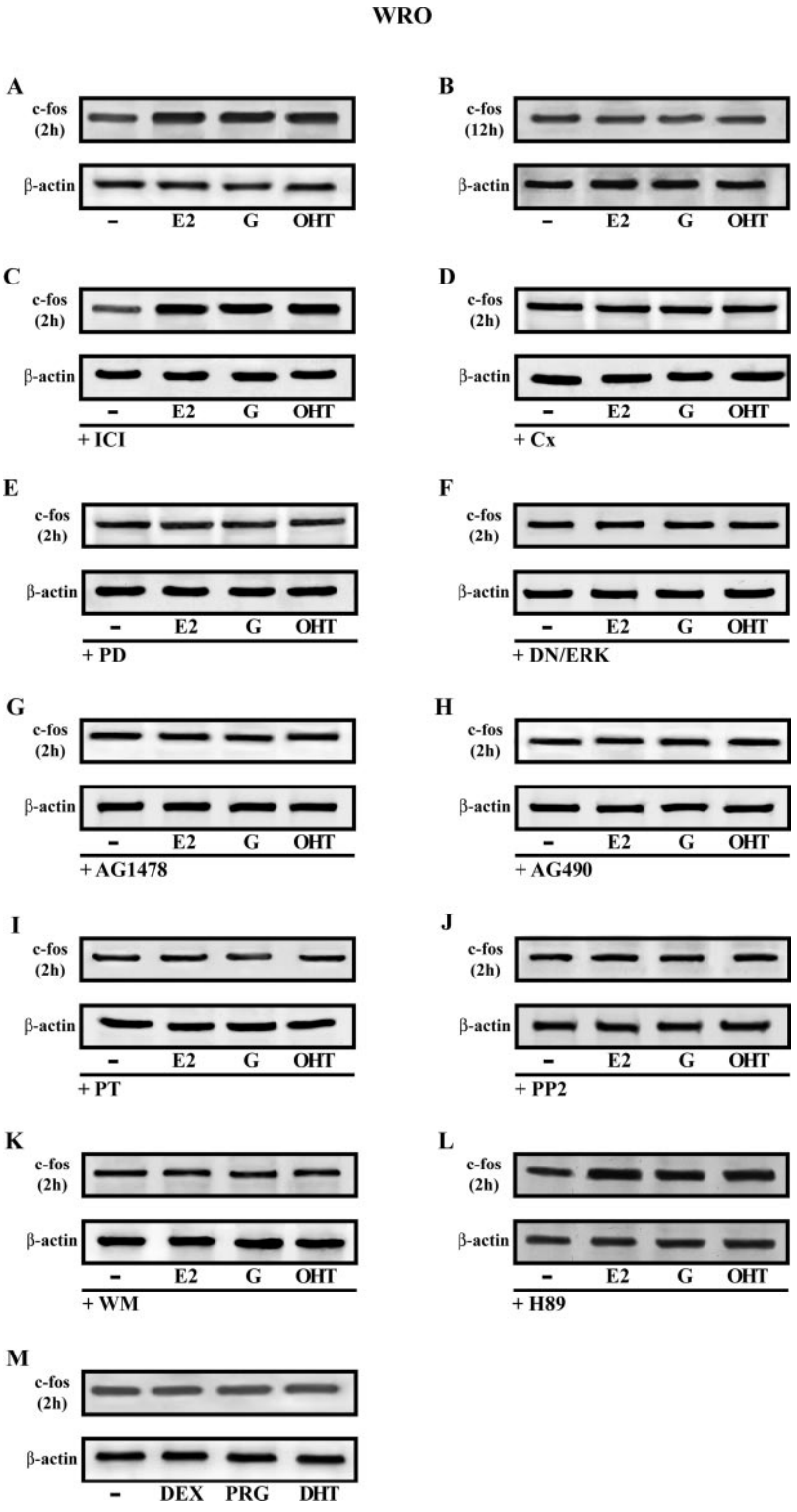

Fig. 5. Immunoblots of c-*fos* from WRO cells. WRO cells were treated for the indicated times with 100 nM E2, G, and OHT (A and B). Cells were exposed to 100 nM E2, G, and OHT in combination with 10 μM ICI (C), 50 μM protein synthesis inhibitor Cx (D), 10 μM MEK inhibitor PD (E), the expression vector for the dominant-negative ERK2 (DN/ERK) (F), 10 μM EGFR kinase inhibitor tyrphostin AG 1478 (G), 10 μM JAK2 and JAK3 activity inhibitor AG 490 (H), 100 ng/ml G protein inhibitor PT (I), 10 μM Src family tyrosine kinase inhibitor PP2 (J), 10 μM PI3K inhibitor WM (K), and 10 μM protein kinase A inhibitor H-89 (L). Furthermore, cells were also treated with 100 nM DEX, 100 nM PRG, and 100 nM DHT (M). β-Actin serves as a loading control.

(Fig. 4). Hence, genes known to be estrogen-sensitive are modulated in a distinct fashion in the TC cells studied.

Transduction Pathways Involved in the Regulation of c-*fos* by E2, G, and OHT. On the basis of the aforementioned results, we wanted to determine whether the expression of c-*fos* at the protein level mimics the mRNA response to ligands. In WRO cells, a short exposure (2 h) to E2, G, and OHT increased c-*fos* protein content, which was no longer notable after 12 h of treatment (Fig. 5, A and B). The ER antagonist ICI was able neither to modify c-*fos* expression (data not shown) nor to abrogate the increase observed upon exposure to the other compounds (Fig. 5C). On the contrary, Cx prevented the enhancement of c-*fos* by ligands, suggesting that a new protein synthesis regulates this process (Fig. 5D). Next, the induction of c-*fos* was abrogated using the MEK inhibitor PD (Fig. 5E), the ectopically expressed dominant-negative form of the ERK protein (Fig. 5F), the EGFR kinase inhibitor tyrphostin AG 1478 (Fig. 5G), the JAK2 and JAK3 activity inhibitor AG 490 (Fig. 5H), the GPCR inhibitor PT (Fig. 5I), the Src family tyrosine kinase inhibitor PP2 (Fig. 5J), and the PI3K inhibitor WM (Fig. 5K). However, the protein kinase A inhibitor H-89 was not able to block the up-regulation of c-*fos* upon the addition of ligands (Fig. 5L). As it concerns DEX, PRG, and DHT, none of these compounds stimulated c-*fos* expression (Fig. 5M), as also observed using 17α-estradiol (data not shown). Together, these findings suggest that the regulation of c-*fos* involves diverse transduction pathways in TC cells, as we have previously demonstrated in different cellular contexts (Maggiolini et al., 2004; Vivacqua et al., 2006).

GPR30 and MAPK Activation Mediate c-*fos* Stimulation by E2, G, and OHT. Given that distinct signals trigger ERK1/2 activation through GPCRs (Filardo et al., 2000), we analyzed the role exerted by GPR30 in the up-regulation of c-*fos* induced by E2, G, and OHT. It is interesting that in WRO cells transfected with a specific GPR30 antisense oligonucleotide (GPR30/AS-ODN), the response of c-*fos* to ligands was inhibited, whereas a control scrambled oligonucleotide had no effect (Fig. 6A). GPR30/AS-ODN silenced the expression of GPR30 (Fig. 6B) although it did not interfere with that of the ERα variant (Fig. 6C).

Having demonstrated that both PD and the DN/ERK prevent the increase of c-*fos* (Fig. 5, E and F), we evaluated the activation of MAPK in WRO cells. A very brief exposure (5 min) to treatments induced the ERK1/2 phosphorylation which was no longer notable in the presence of PD (Fig. 7). Taken together, these data indicate that E2, G, and OHT signal via GPR30 and the MAPK pathway in TC cells.

E2, G, and OHT Stimulate the Proliferation of WRO, FRO, and ARO Cells. As a biological counterpart of the aforementioned results obtained, E2, G, and OHT stimulated the proliferation of WRO, FRO, and ARO cells (Fig. 8A). It should be pointed out that the latter cell line does not express ERs, as we ascertained by RT-PCR and Western blotting (data not shown). It is interesting that the growth effects observed upon the addition of ligands were abrogated either transfecting a GPR30 antisense expression vector (GPR30/AS) (Fig. 8B) or in the presence of the MEK, PI3K, and EGFR inhibitors PD, WM, and AG1478, respectively (Fig. 8C). As confirmed by the growth assay, the stimulatory action of ligands in TC cells involves GPR30 along with distinct transduction pathways.

Discussion

It has been demonstrated previously that the human thyroid gland exhibits the potential for both E2 synthesis and intracrine or paracrine estrogen responsiveness, which may increase after the process of tumorigenesis (Yane et al., 1994; Dalla Valle et al., 1998). Besides, functional ERs were evidenced in FRTL-5 rat thyroid cells that displayed growth stimulatory effects upon exposure to E2 (Furlanetto et al., 1999). As it concerns the mechanisms of estrogen action, the E2-enhanced rates of cell proliferation were associated with either an up-regulation of ERα and cyclin D1 (Manole et al., 2001) or an increase of the antiapoptotic Bcl-xL without involvement of ERα (Lee et al., 2005).

To evaluate the activity exerted by ERα in the human follicular WRO and anaplastic FRO thyroid tumor cells, we first determined that both cell lines express only a single isoform of ERα that was unable to trigger any ERE-mediated transcriptional activity. Given the potential of this variant of ERα to interact with other DNA-responsive elements, such as activator protein 1 (AP-1) or Sp1 (Kushner et al., 2000), we performed an immunocytochemical study to detect its subcellular distribution. Even in presence of ligands, the ERα isoform was localized in the cytoplasmic compartment, ruling out its direct involvement in DNA binding and gene transcription.

On the basis of these observations, we then verified the

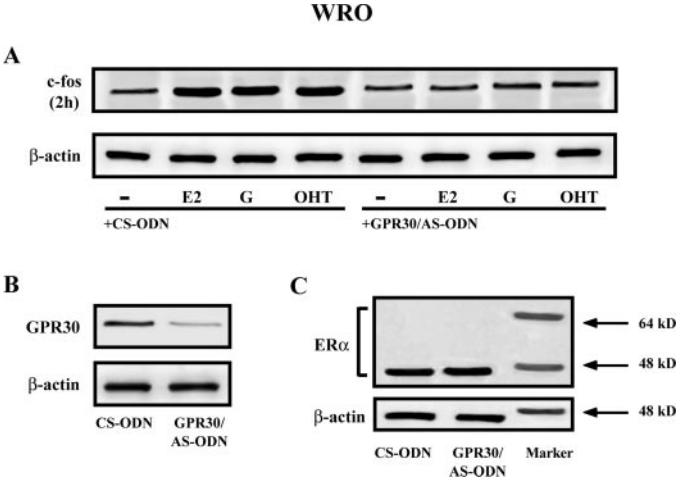

Fig. 6. A GPR30 antisense oligonucleotide abrogates c-*fos* up-regulation by E2, G, and OHT in WRO cells. A, WRO cells transfected with control-scrambled (CS-ODN) or GPR30 antisense (GPR30/AS-ODN) oligonucleotides were treated with 100 nM E2, G, and OHT. Immunoblots of GPR30 (B) and ERα (C) from WRO cells transfected with CS-ODN or GPR30/AS-ODN oligonucleotides. β-Actin serves as a loading control.

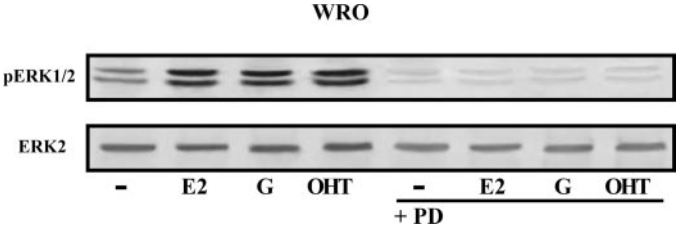

Fig. 7. ERK1/2 phosphorylation in WRO cells. WRO cells were treated for 5 min with 100 nM E2, G, OHT, and 10 μM PD as indicated. Total ERK2 proteins were used to normalize ERK1/2 expression.

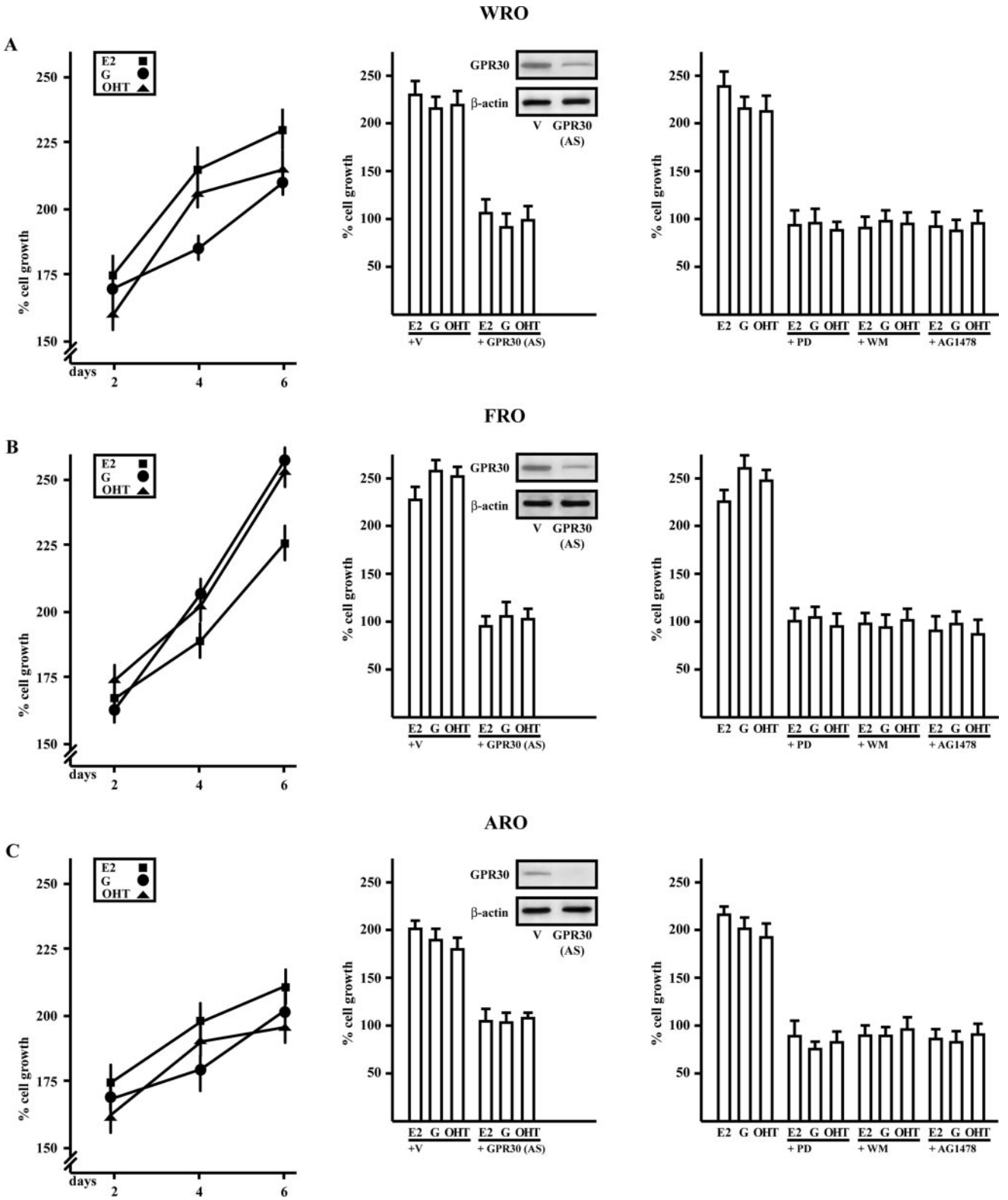

Fig. 8. E2, G, and OHT stimulate the proliferation of WRO, FRO, and ARO cells. A, cells were treated with 100 nM E2, G, and OHT in medium containing 5% charcoal-stripped FBS (medium was refreshed and treatments were renewed every 2 days) and then counted on the indicated days. Proliferation of cells receiving vehicle was set as 100%, upon which cell growth induced by treatments was calculated. Each data point is the mean ± S.D. of three independent experiments performed in triplicate. B, cells were transfected with an empty vector (v) or an expression vector for GPR30 antisense (GPR30/AS), treated as in A, and then counted on day 6. Proliferation of cells receiving vehicle was set as 100%, upon which cell growth induced by treatments was calculated. Each data point is the mean ± S.D. of three independent experiments performed in triplicate. GPR30 protein expression in cells transfected with an empty vector (v) or GPR30/AS on day 6. β-Actin serves as a loading control. C, cells were treated with 100 nM E2, G, and OHT or in combination with 10 μM MEK inhibitor PD, 10 μM PI3K inhibitor WM, or 10 μM EGFR kinase inhibitor tyrphostin AG 1478 and counted on day 6. Proliferation of cells receiving vehicle was set as 100%, upon which cell growth induced by treatments was calculated. Each data point is the mean ± S.D. of three independent experiments performed in triplicate.

ability of E2, G, and OHT to regulate other genes known to be estrogen-sensitive. It is interesting that all of the compounds induced the expression of c-*fos* and cyclins A and D1, whereas pS2 and PR did not show alterations. This result would not be surprising because the complex transcription machinery requires a cell-specific recruitment of a plethora of cofactors and the displacement of corepressors on the promoter sites of distinct genes, as documented previously (Cosma, 2002). The rapid c-*fos* response to ligands along with their ability to transactivate an expression vector encoding the promoter of c-*fos* lacking ERE sequences prompted us to identify the transduction pathways involved in these stimulatory effects elicited by E2, G, and OHT in TC cells. We have shown for the first time that the expression of genes involved in cycle progression and the proliferation of thyroid tumor cells are mediated by GPR30 and the MAPK pathway, which was confirmed by the growth assay performed in ER-negative ARO cells. Furthermore, we have demonstrated that a complex interplay among distinct intracellular pathways contributes to these biological features, because specific inhibitors of EGFR, Src, and PI3K signaling were each able to abrogate c-*fos* induction and the proliferative effects obtained upon the addition of ligands. It is worth noting that our findings are in agreement with previous data suggesting the existence of a functional network among diverse metabolic cascades, which may allow an identical molecular cross-talk in different cellular contexts (Castoria et al., 2001; Kraus et al., 2003). Besides, the present results recall our recent data regarding the involvement of the aforementioned transduction pathways in gene transcription and proliferation of breast (SKBR3) and endometrial (HEC1A) cancer cells completely devoid of ERs or expressing an ERα variant, respectively (Maggiolini et al., 2004; Vivacqua et al., 2006).

Fos family proteins, including c-*fos,* interact with members of the Jun family to form heterodimers that bind to the AP-1 sites located within the regulatory region of genes critical for cell cycle progression (Hill and Treisman, 1995). In this context, it has been suggested that c-*fos* through AP-1 acts as a transcriptional regulator of the cyclin D1 gene, linking mitogenic signaling to cycle re-entry in many cell types (Albanese et al., 1999). Moreover, in a recent study (Sunters et al., 2004), c-*fos* overexpression resulted in cyclin D1 increase and cyclin A-dependent accelerated progression of the cell cycle. In line with these findings, we have associated the response of c-*fos* to E2, G, and OHT with the transcription regulation of cyclins A and D1 in TC cells. As it concerns the mechanisms by which diverse extracellular stimulations initiate the rapid transcription of mitogenic genes, the MAPK cascade was recognized to exert a crucial role triggering the rapid up-regulation of c-*fos* (Whitmarsh et al., 1995) and a series of phosphorylation events that further increase its transcriptional potential and the biological outcome (Murphy et al., 2002).

The above evidence has provided a nice scenario for our data regarding the effects triggered by ligands in thyroid tumor cells: 1) the rapid MAPK activation; 2) the induction of c-*fos* and cyclins A and D1; and 3) the growth response to E2, G, and OHT. Moreover, using specific inhibitors, we have documented that GPR30 and diverse transduction pathways mediate these ligand-induced stimulations. Our findings have also demonstrated that ERα is not required for the complex biological activity exerted by treatments.

The well-established mode of action of estrogens, such as binding to ERs which in turn activate gene transcription through an interaction with ERE and other DNA sequences (Levin, 2005), should be extended to GPR30, which mediates estrogenic signals in breast, endometrial, and thyroid tumor cells, as we have ascertained in the present and previous studies (Maggiolini et al., 2004; Vivacqua et al., 2006). Our data suggest novel biological targets that could be taken into account for pharmacological interventions with molecular inhibitors in TC. Given the stimulatory effects elicited by E2 in neoplastic thyroid cells as evidenced by our and other reports (Yane et al., 1994; Dalla Valle et al., 1998; Manole et al., 2001; Lee et al., 2005), medications containing estrogens or compounds able to mimic the estrogen activity should be carefully considered at least in women who had TC or are suspected of occult thyroid malignancy.

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