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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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Running title: GPR30 function in thyroid cancer cells

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Nonstandard abbreviations:

TC, thyroid cancer; GPR30, G protein couplet receptor 30; G, genistein;

OHT, 4-hydroxytamoxifen; DEX, dexamethasone; PRG, progesterone

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ABSTRACT

The higher incidence of thyroid carcinoma (TC) in women during reproductive years compared to men as well as 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 have evaluated the potential of 17β-estradiol (E2), genistein (G) and 4-hydroxytamoxifen (OHT) to regulate the expression of diverse estrogen target genes as well as 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 ERs, whereas both WRO and FRO cells express a single variant of ERα which 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 that lacking the estrogen responsive elements (EREs), or to increase *c-fos*, cyclin A and D1 expression. Notably, we have demonstrated that the G protein-coupled receptor GPR30 and the MAPK pathway mediate both the up-regulation of *c-fos* and the growth response to E2, G and OHT in TC cells studied, since these stimulatory effects were prevented silencing GPR30 as well as using the MAPK inhibitor PD 98059. Our findings provide new insight into the molecular mechanisms through which estrogens may induce the progression of TC.

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INTRODUCTION

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 three 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 world-wide (Waterhouse et al., 1982) together with the increased risk in women taking estrogens for gynaecological disorders (Ron et al., 1987; Persson et al., 1996), have suggested that these steroids may influence the progression of TC as largely demonstrated for breast and endometrial carcinoma (reviewed in reference 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) as well as 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 classical mechanism of E2 action involves the binding to ERα and ERβ that contain two main transcription activation functions (AF): the N-terminal AF1 and the C-terminal AF2 which is associated with the ligand-binding domain (LBD) 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). Interestingly, previous studies including our own (Maggiolini et al., 2004; Revankar et al., 2005; Thomas et al., 2005; Vivacqua et al., 2006) have demonstrated that

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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 and references therein). The transcription of *c-fos* is regulated by multiple *cis* elements including the serum response element (SRE) which recruits the ternary complex factors Elk-1 and the serum response factor (SRF) 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 SRE 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 have evaluated the potential of E2, genistein (G) and 4-hydroxytamoxifen (OHT) to regulate the expression of diverse estrogen target genes as well as 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.

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MATERIALS AND METHODS

Reagents. E2, G, OHT, cycloheximide (Cx), wortmannin (WM), LY, pertussis toxin (PT), PD 98059 (PD), dexamethasone (DEX), progesterone (PRG) and 5α-dihydrotestosterone (DHT) were purchased from Sigma-Aldrich Corp. (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 (DBA, Milan, Italy), H-89 and PP2 were obtained from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in dimethyl sulfoxide (DMSO), except 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 (DMEM) 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 estrogen response element (ERE) from the Xenopus vitellogenin A2 gene (nucleotides -334 to -289), the herpes simplex virus thymidine kinase (tk) 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, USA) and M. Cobb (University of Texas, Dallas, USA),

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respectively. The *Renilla* luciferase expression vector pRL-TK (Promega, Milan, Italy) was used as a transfection standard.

Transfections and Luciferase Assays. WRO and FRO cells ($1x10^5$) were plated into 24-well dishes with 500 µl of regular growth medium/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 about 12 h. Luciferase activity was measured with 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 (RLU) 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 MgCl2, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, 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, Milan, Italy). Equal amounts of whole protein extract were resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences), 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, PR, β-actin (all purchased from Santa Cruz Biotechnology, DBA, Milan, Italy), ERβ (Serotec, Milan, Italy), pERK1/2, ERK2 (Cell Signaling Technology, Inc, Celbio, Milan, Italy) and then revealed using the ECL system (Amersham Biosciences, Milan, Italy). 5 µg DN/ERK and

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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 we have previously described (Maggiolini et al., 1999). For ERα, c-*fos*, cyclin A, cyclin D1, pS2, progesterone receptor (PR) and the acidic ribosomal phosphoprotein P0 (36B4) which was used as a control gene, the primers were: 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'-TCTAAGATGAAGGAGACCATC-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); 5'-CTCAACATCTCCCCCTTCTC-3' (36B4 forward) and 5'-CAAATCCCATATCCTCGTCC-3' (36B4 reverse), to yield products respectively of 438, 420, 354, 354, 210, 239 and 408 bp, 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 previously described (Kanda and Watanabe, 2003). The oligonucleotides used were: 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 of the indicated oligonucleotides was transfected using FuGENE 6 reagent as recommended by the manufacturer for 4 h, before treatment with ligands.

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Immunocytochemical staining. Cells were maintained in medium lacking serum for 3 days, treated for 1 h and then fixed in fresh paraformaldehyde (PFA 2% for 30 min). After PFA 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 non-specific 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, Santa Cruz Biotechnology; 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, USA). Subsequently, the amplification of avidin-biotin-horseradish peroxidase complex (ABC complex/HRP; Vector Laboratories; 1:100 for 30 min at room temperature) 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-X100 (TBS-T). In control experiments cells were processed replacing the primary antibody with mouse serum (Dako S.p.A., Milan, Italy) or using a primary antibody pre-absorbed (48 h at 4 °C) with an excess of purified ERα protein (M-Medical, Milan, Italy).

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 incubated in medium containing 5% charcoal stripped-FBS with the indicated treatments; medium was renewed every two days (with treatments) and cells were trypsinized and counted in a hemocytometer. 200 ng GPR30/AS or 200 ng empty vector were transfected using FuGENE 6 reagent as recommended by the manufacturer every two days.

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

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RESULTS

E2, G, and OHT neither transactivate nor regulate the expression of the ERα variant and ERβ expressed in TC cells. In order 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 panels A of figure 1, both cell lines did not show any evidence of ERE-dependent functional response to 100 nM of each compound as also observed upon concentrations ranging from 1 nM to 10 µM (data not shown). However, co-transfecting an expression vector encoding ERα (HEG0), only E2 and G induced the luciferase activity (panels A in fig.1). 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 kD) with respect to ERα wild-type (66 kD) (panels B in fig. 1). This variant of ERα was not modified upon exposure to 100 nM 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 (panels B in fig. 1). Taken together, these results indicate that the isoform of ERα expressed in WRO and FRO cells does not mediate the ERE-dependent transcriptional effects.

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 (Tora et al., 1989; Kumar and Chambon, 1988). As further evidence that the variant of ERα found in TC cells is not able to trigger classical genomic actions, we performed an immunocytochemical assay in order to evaluate its subcellular distribution after treatment with ligands. To verify the specificity of the ERα antibodies used and the localization of ERα in presence of E2, we transfected ER-negative HeLa cells with an expression

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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 (panels 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 (panels 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 classical 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) which contains diverse sequences responding to a variety of extracellular stimuli (Hill and Treisman, 1995 and references therein). Interestingly, the *c-fos* promoter construct was transactivated by all treatments which were no longer able to elicit such effect in 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). Of note, E2, G and OHT induced the Elk-1-mediated transcription that was abrogated by PD and WM (Fig. 3C). Altogether, these results indicate that the regulation of *c-fos* promoter by estrogens may occur through diverse mechanisms independently of ERα.

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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 progesterone receptor (PR), pS2 (Cavailles et al., 1989), 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 figure 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, while pS2 and PR did not display any change (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. 5A-B). The ER antagonist ICI was neither able 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), the PI3K inhibitor WM (fig. 5K). However, the protein kinase A (PKA) inhibitor H-89 was not able to block the up-regulation of *c-fos* upon 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). Collectively, these findings suggest that the regulation of *c-fos* involves

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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. Interestingly, 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), while 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. 5E-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 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 (panels A in fig. 8). 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). Interestingly, the growth effects observed upon addition of ligands were abrogated either transfecting a GPR30 antisense expression vector (GPR30/AS) (panels B in fig. 8) or in presence of the MEK, PI3K and EGFR inhibitors PD, WM and AG1478, respectively (panels C in fig. 8). As confirmed by the growth assay, the stimulatory action of ligands in TC cells involves GPR30 along with distinct transduction pathways.

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DISCUSSION

It has been previously demonstrated that the human thyroid gland exhibits the potential for both E2 synthesis and intracrine or paracrine estrogen responsiveness which may increase following 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 anti-apoptotic Bcl-xL without involvement of ERα (Lee et al., 2005).

In order 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 which 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 ability of E2, G, and OHT to regulate other genes known to be estrogen-sensitive. Interestingly, all compounds induced the expression of *c-fos*, cyclins A and D1, while 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 previously documented (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

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genes involved in cycle progression as well as 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, since specific inhibitors of EGFR, Src and PI3K signaling were each able to abrogate *c-fos* induction and the proliferative effects obtained upon addition of ligands. It is worth to note 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 and references therein). 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 reentry 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).

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The above evidence has provided a nice scenario for our data regarding the effects triggered by ligands in thyroid tumor cells: i) the rapid MAPK activation, ii) the induction of *c-fos* and cyclins A and D1, iii) the growth response to E2, G and OHT. Moreover, using specific inhibitors we have documented that GPR30 along with 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 (reviewed in 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 have suggested novel biological targets which could be taken into account for pharmacologic 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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REFERENCES

Albanese C, D'Amico M, Reutens AT, Fu M, Watanabe G, Lee RJ, Kitsis RN, Henglein B, Avantaggiati M, Somasundaram B, Thimmapaya B and Pestell RJ (1999) Activation of the cyclin D1 gene by the E1A-associated protein p300 through AP-1 inhibits cellular apoptosis. *J Biol Chem* **270**:34186-34195

Butt AJ, McNeil CM, Musgrove EA and Sutherland RL (2005) Downstream targets of growth factor and oestrogen signalling and endocrine resistance: the potential roles of c-Myc, cyclin D1 and cyclin E. *Endocr Relat Cancer* **12** Suppl 1:S47-59

Castoria G, Migliaccio A, Bilancio A, Di Domenico M, De Falco A, Lombardi M, Fiorentino R, Varricchio L, Barone MV and Auricchio F (2001) PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J* **20**:6050-6059

Cavailles V, Garcia M and Rochefort H (1989) Regulation of cathepsin-D and pS2 gene expression by growth factors in MCF7 human breast cancer cells. *Mol Endocrinol* **3**:552-558

Cosma MP (2002) Ordered recruitment: gene-specific mechanism of transcription activation. *Mol Cell* **10**:227-236

Dalla Valle L, Ramina A, Vianello S, Fassina A, Belvedere P and Colombo L (1998) Potential for estrogen synthesis and action in human normal and neoplastic thyroid tissues. *J Clin Endocrinol Metab* **83**:3702-3709

Duan R, Porter W and Safe S (1998) Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* **139**:1981-1990

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Duan R, Xie W, Burghardt RC and Safe S (2001) Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. *J Biol Chem* **276**:11590-11598

Filardo EJ, Quinn JA, Bland KI and Frackelton AR Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* **10**:1649-1660

Furlanetto TW, Nguyen LQ and Jameson JL (1999) Estradiol increases proliferation and down-regulates the sodium/iodide symporter gene in FRTL-5 cells. *Endocrinology* **140**:5705-5711

Gallo A, Cuozzo C, Esposito I, Maggiolini M, Bonofiglio D, Vivacqua A, Garriamone M, Weiss C, Bohmann D and Musti AM (2002) Menin uncouples Elk-1, JunD and c-Jun phosphorylation from MAP kinase activation. *Oncogene* **21**:6434-6445

Henderson BE, Ross RK, Pike MC and Casagrande JT (1982) Endogenous hormones as a major factor in human cancer. *Cancer Res* **42**:3232-3239

Hill CS and Treisman R (1995) Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J* **14**:5037-5047

Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ and Thun MJ American Cancer Society 2004 Cancer Statistics. *CA Cancer J Clin* **54**:8-29

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Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.026344
This article has not been copyedited and formatted. The final version may differ from this version.

Kanda N and Watanabe S (2003) 17beta-estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bcl-2 expression. *J Invest Dermatol* **121**:771-780

Kraus S, Benard O, Naor Z and Seger R (2003) c-Src is activated by the epidermal growth factor receptor in a pathway that mediates JNK and ERK activation by gonadotropin-releasing hormone in COS7 cells. *J Biol Chem* **278**:32618-32630

Kumar V and Chambon P (1988) The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. *Cell* **55**:145-156

Kushner PJ, Agard D, Feng WJ, Lopez G, Schiau A, Uht R, Webb P and Greene G (2000) Oestrogen receptor function at classical and alternative response elements. *Novartis Found Symp* **230**:20-26

Lee ML, Chen GG, Vlantis AC, Tse GM, Leung BC and van Hasselt CA (2005) Induction of thyroid papillary carcinoma cell proliferation by estrogen is associated with an altered expression of Bcl-xL. *Cancer J* **11**:113-121

Levin ER (2005) Integration of the extranuclear and nuclear actions of estrogen *Mol Endocrinol* **19**:1951-1959

Luttrell LM, van Biessen T, Hawes BE, Koch WJ, Krueger KM, Touhara K and Lefkowitz RJ (1997) G-protein-coupled receptors and their regulation: activation of the MAP kinase signaling pathway by G-protein-coupled receptors. *Adv Second Messenger Phosphoprotein Res* **31**:263-277

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Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.026344
This article has not been copyedited and formatted. The final version may differ from this version.

Maggiolini M, Donzè O and Picard D (1999) A non-radioactive method for inexpensive quantitative RT-PCR. *Biol Chem* **380**:695-697

Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D and Andò S (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* **279**:27008-27016

Manole D, Schildknecht B, Gosnell B, Adams E and Derwahl M (2001) Estrogen promotes growth of human thyroid tumor cells by different molecular mechanisms. *J Clin Endocrinol Metab* **86**:1072-1077

Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM, Salvatore G, Caiazzo F, Basolo F, Giannini R, Kruhoffer M, Orntoft T, Fusco A and Santoro M (2005) The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. *J Clin Invest* **115**:1068-1081

Murphy LO, Smith S, Chen RH, Fingar DC and Blenis J (2002) Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol* **4**:556-564

Persson I, Yuen J, Bergkvist L and Schairer C (1996) Cancer incidence and mortality in women receiving estrogen and estrogen-progestin replacement therapy long term follow-up of a Swedish cohort. *Int J Cancer* **67**:327-332

Pike MC, Pearce CL and Wu AH (2004) Prevention of cancers of the breast, endometrium and ovary. *Oncogene* **23**:6379-6391

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 23, 2024

Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.026344
This article has not been copyedited and formatted. The final version may differ from this version.

Ron E, Kleinerman RA, Boice Jr JD, Livolsi VA, Flannery JT and Fraumeni Jr JF (1987) A population-based case-control study of thyroid cancer. *J Natl Cancer Inst* **79**:1-12

Santagati S, Gianazza E, Agrati P, Vegeto E, Patrone C, Pollio G and Maggi A (1997) Oligonucleotide squelching reveals the mechanism of estrogen receptor autologous down-regulation. *Mol Endocrinol* **11**:938-949

Sunters A, Thomas DP, Yeudall WA and Grigoriadis AE (2004) Accelerated cell cycle progression in osteoblasts overexpressing the c-fos proto-oncogene: induction of cyclin A and enhanced CDK2 activity. *J Biol Chem* **279**:9882-9891

Thomas P, Pang Y, Filardo EJ and Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **146**:624-632

Tora L, White J, Brou C, Tasset D, Webster N, Scheer E and Chambon P (1989) The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59**:477–487

Treisman R (1995) Journey to the surface of the cell: Fos regulation and the SRE. *EMBO J* **14**:4905-4913

Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Andò S and Maggiolini M (2006) The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol* **20**:631-646

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Molecular Pharmacology Fast Forward. Published on July 11, 2006 as DOI: 10.1124/mol.106.026344
This article has not been copyedited and formatted. The final version may differ from this version.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**:1625-1630

Waterhouse J, Muir C, Shanmugaratnam K, Powel J (1982) Cancer incidence in five continents. pp 185-198, IARC, Lyons, France

Whitmarsh AJ, Shore P, Sharrocks AD and Davis RJ (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**:403-407

Yane K, Kitahori Y, Konishi N, Okaichi K, Ohnishi T, Miyahara H, Matsunaga T, Lin JC and Hiasa Y (1994) Expression of the estrogen receptor in human thyroid neoplasms. *Cancer Lett* **84**:59-66

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FOOTNOTES

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FIGURE LEGENDS

Fig.1. The isoform of ERα expressed in WRO and FRO cells is neither activated nor modulated by E2, G and OHT. Panels A, cells were transfected with the ER luciferase reporter plasmid XETL and the wild-type ERα where applicable and treated with 100 nM 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. Panels B, immunoblots of ERα and ERβ from cells treated with 100 nM ligands for 12-h. β-actin serves as loading control.

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. In panel 1 are shown cells receiving vehicle and in panel 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 (panel 1) or 100 nM E2 (panel 2), G (panel 3), OHT (panel 4). Each experiment is representative of at least 10 tests. Bar, 5 μM.

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-fosΔERE 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 98059 (PD) or 10 μM wortmannin (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

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activity induced by treatments was calculated. Each data point represents the mean ± S.D. of three independent experiments performed in triplicate.

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

Fig.5. Immunoblots of *c-fos* from WRO cells. WRO cells were treated for the indicated times with 100 nM E2, G, OHT (Panels A and B). Cells were exposed to 100 nM E2, G, OHT in combination with 10 µM ICI (Panel C), 50 µM protein synthesis inhibitor cycloheximide (Cx) (Panel D), 10 µM MEK inhibitor PD 98059 (PD) (Panel E), the expression vector for the dominant negative ERK2 (DN/ERK) (Panel F), 10 µM EGF receptor kinase inhibitor tyrphostin AG 1478 (Panel G), 10 µM JAK2 and JAK3 activity inhibitor AG 490 (Panel H), 100 ng/ml of G protein inhibitor pertussis toxin (PT) (Panel I), 10 µM Src family tyrosine kinase inhibitor PP2 (Panel J), 10 µM PI3K inhibitor wortmannin (WM) (Panel K), 10 µM protein kinase A (PKA) inhibitor H-89 (Panel L). Furthermore, cells were also treated with 100 nM dexamethasone (DEX), 100 nM progesterone (PRG) and 100 nM dihydrotestosterone (DHT) (Panel M). β-actin serves as a loading control.

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

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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.** Panels 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 two 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. Panels B, cells were transfected with an empty vector (v) or an expression vector for GPR30 antisense (GPR30/AS), treated as in panels 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. Panels C, cells were treated with 100 nM E2, G and OHT or in combination with 10 μM MEK inhibitor PD 98059 (PD), 10 μM PI3K inhibitor wortmannin (WM) or 10 μM EGF receptor 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.

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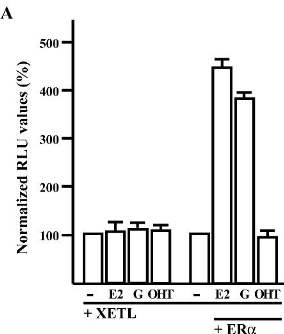
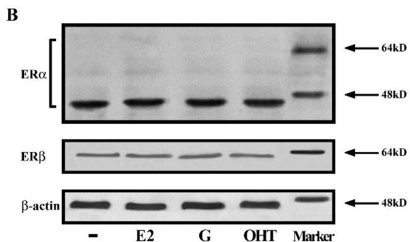
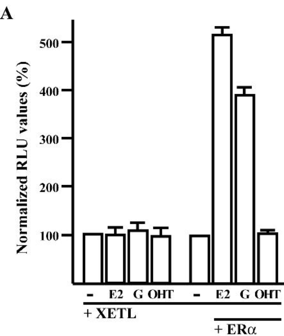
WRO





FRO



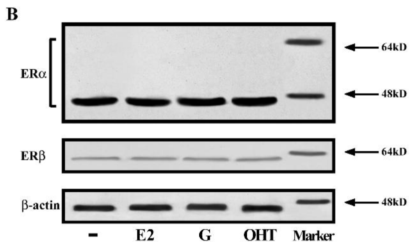


Fig. 1

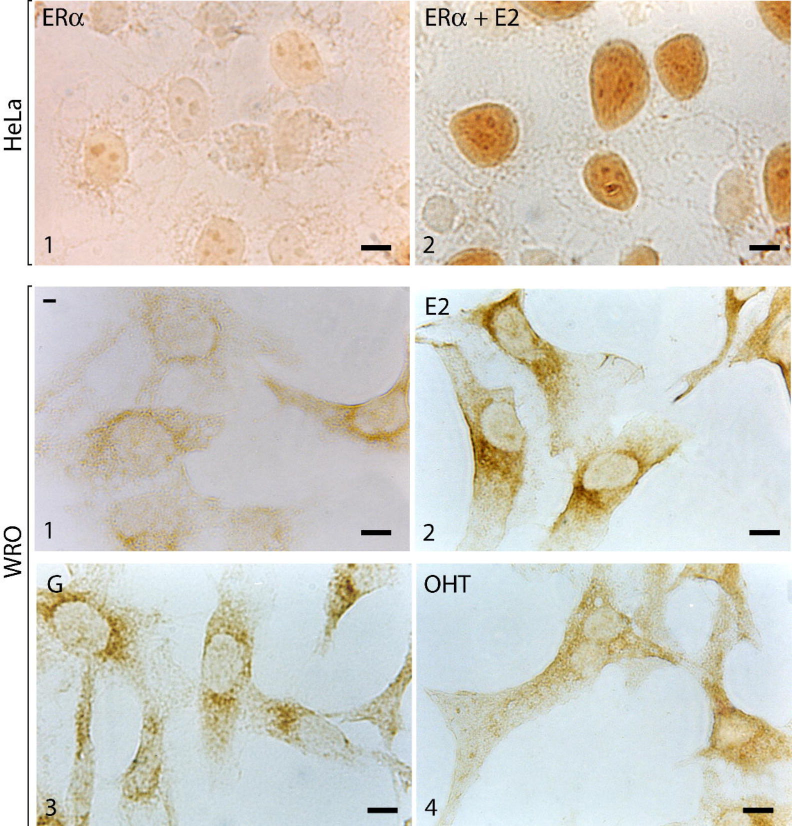
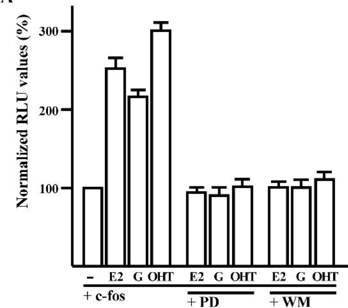
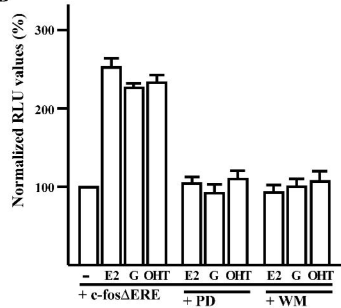


Fig. 2

WRO





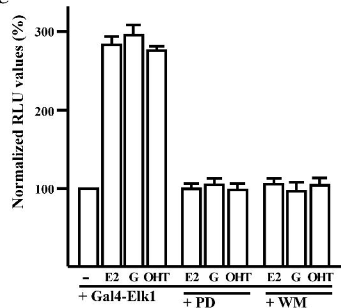
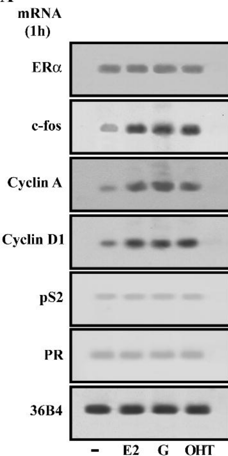
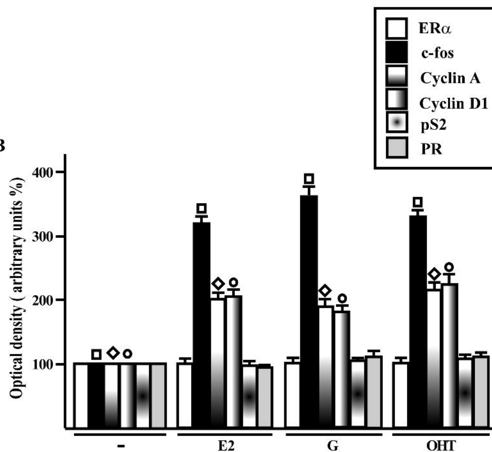
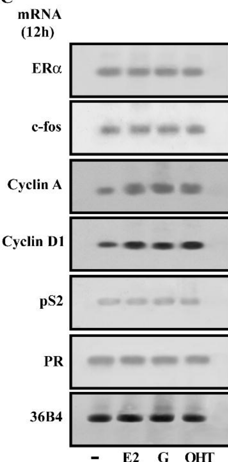


Fig. 3

WRO







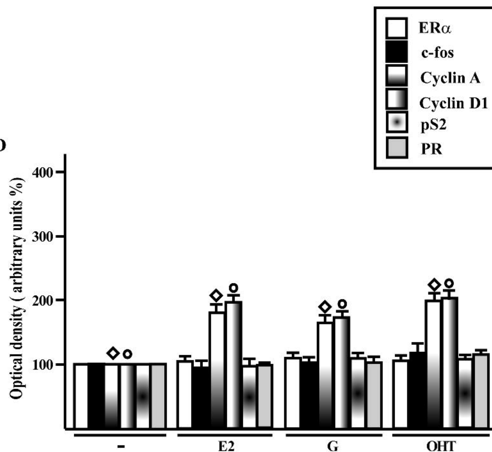


Fig. 4

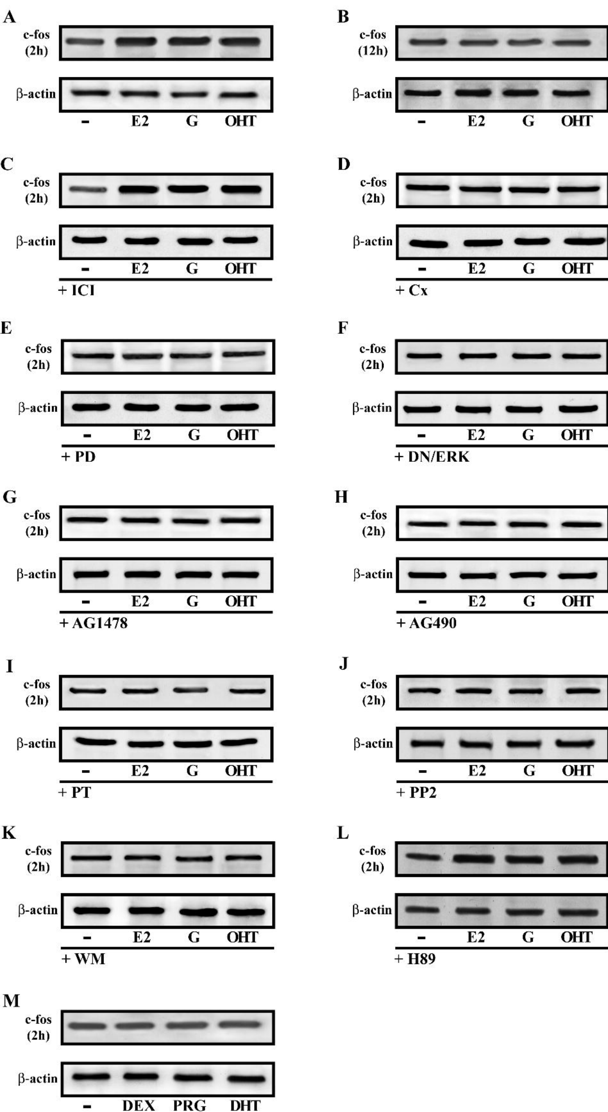
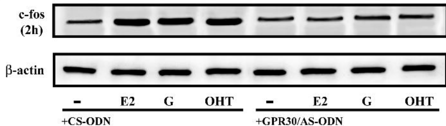
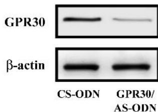


Fig. 5

WRO





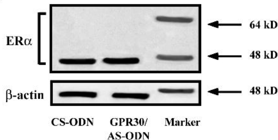


Fig. 6

Fig. 7

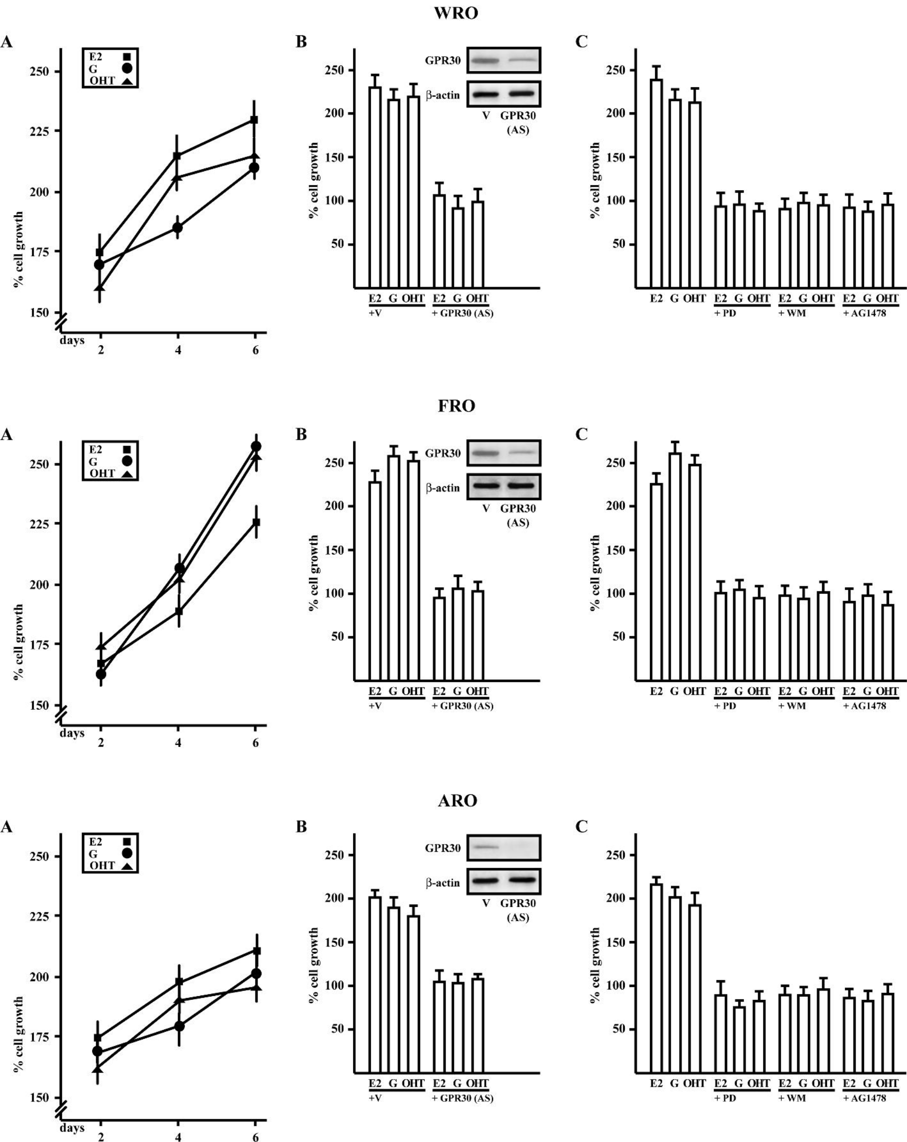


Fig. 8