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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 de-

velopment. 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

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, cyclohexximide; 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-4H-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 × 10⁵) 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 MgCl₂, 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′-GGAGACATGAGAGCT-GCCA-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′-C-ACTGACATGGAAGACAGGAACCT-3′ (cyclin A reverse); 5′-TCTA-

AGATGAAGGAGACCATC-3′ (cyclin D1 forward) and 5′-GCGGTA-GTAGGACAGGAAGTTGTT-3′ (cyclin D1 reverse); 5′-TTCTATCCT-AATACCATCGACG-3′ (pS2 forward) and 5′-TTTGAGTAGTCAAA-GTCAGAGC-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′-GATCT-CAGCACGGCAAAT-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
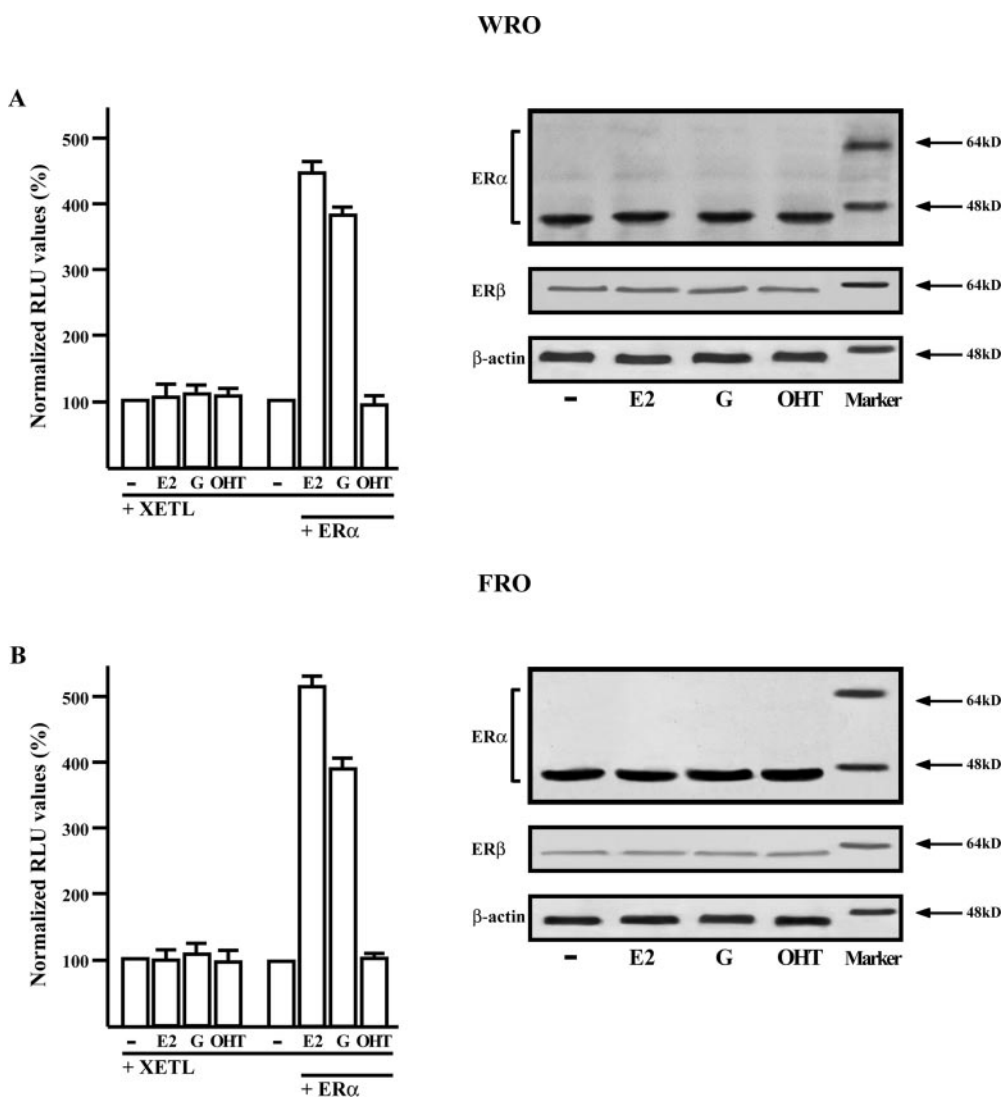
WRO

FRO

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 tran-
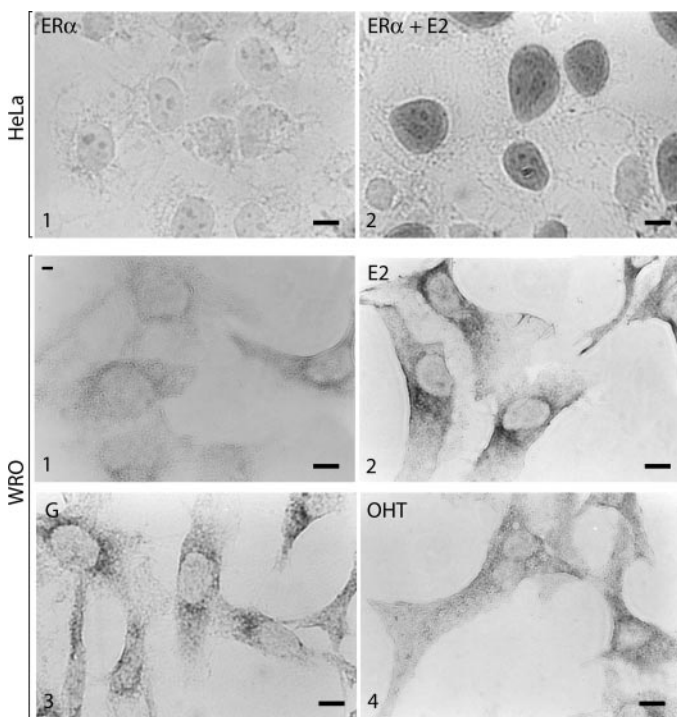

Fig. 2. Immunocytochemical detection of the ERα variant expressed in WRO cells. HeLa, ER-negative HeLa cells were transfected for 24-h in serum-deprived conditions with 0.5 μg of an expression plasmid encoding ERα. 1, cells receiving vehicle; 2, cells treated with 100 nM E2 for 2 h. WRO, cells were serum-deprived 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.

siently 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.

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
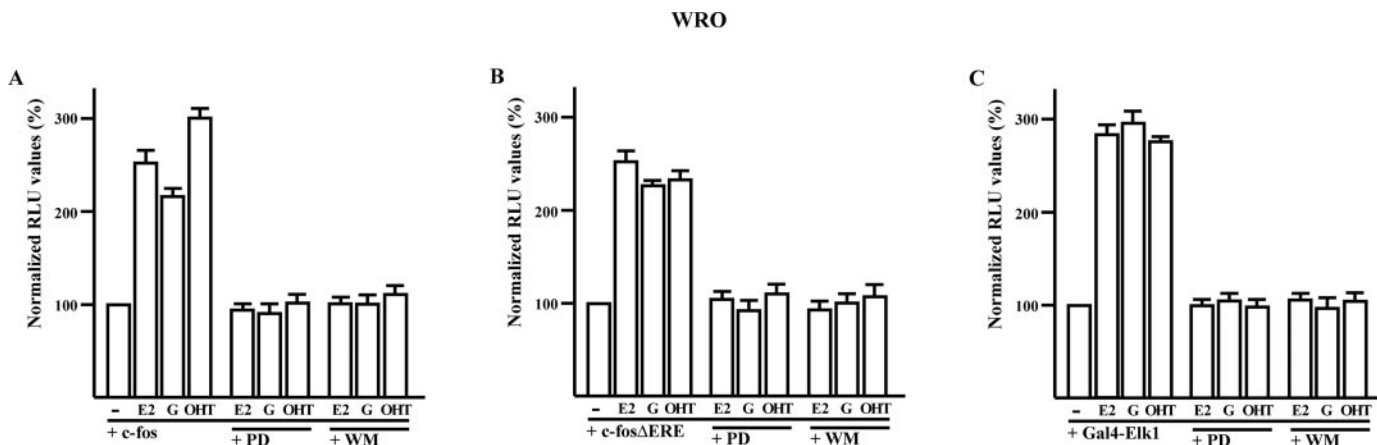
WRO

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,
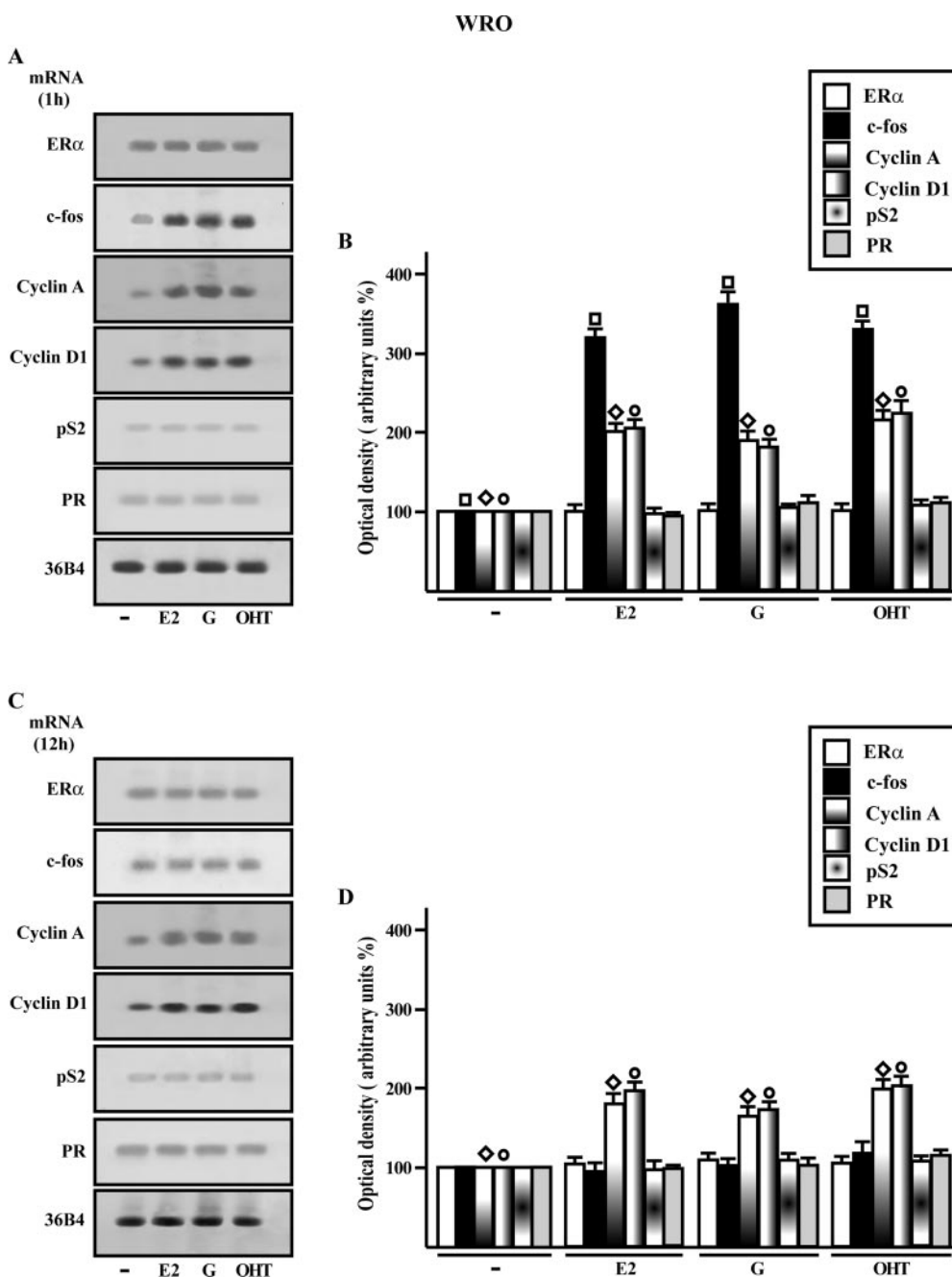
WRO

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
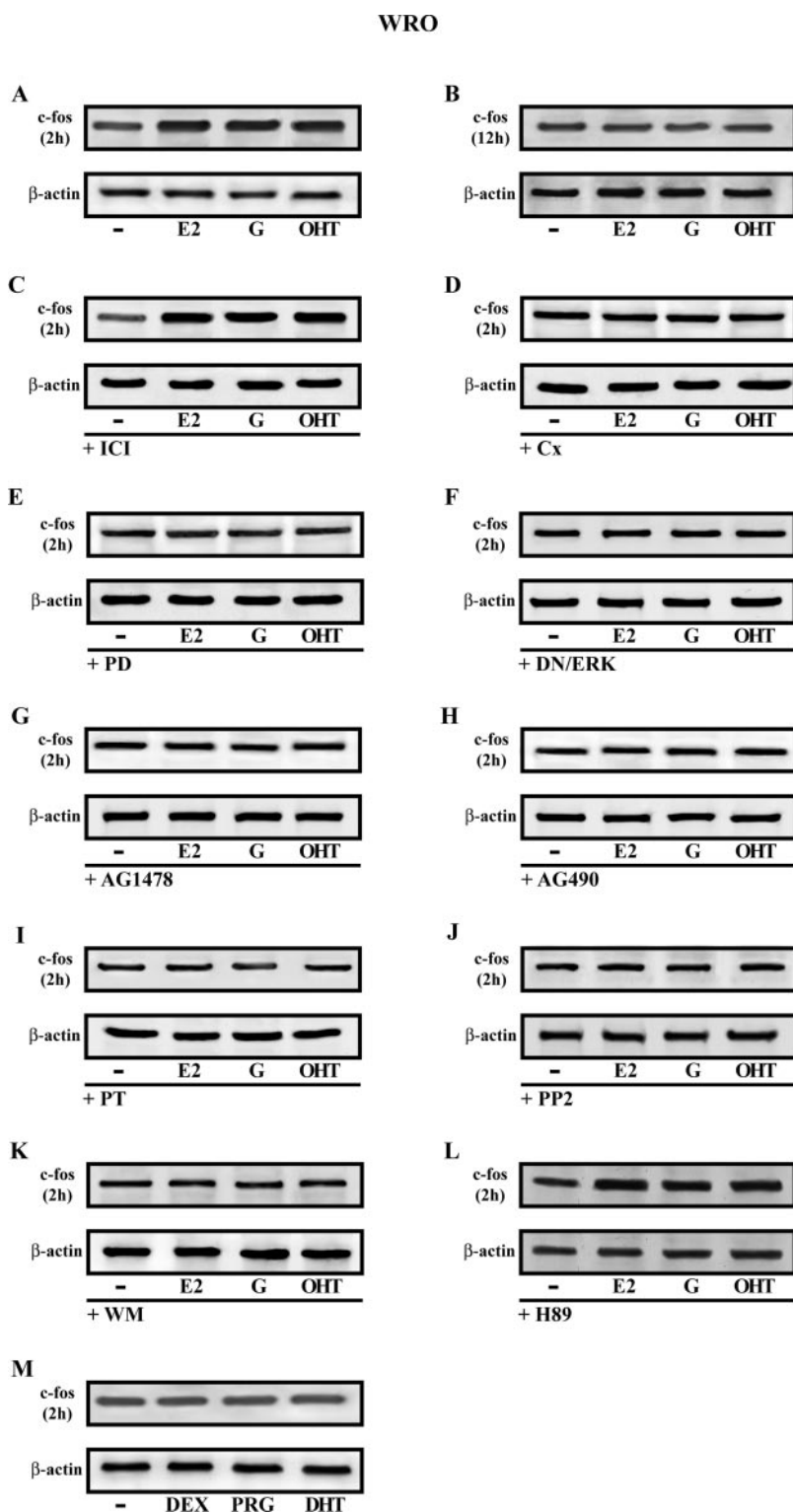

Fig. 5. Immunoblots of c-*fos* from WRO cells. WRO cells were treated for the indicated times with 100 nM E2, G, and OHT (A and B). Cells were exposed to 100 nM E2, G, and OHT in combination with 10 μM ICI (C), 50 μM protein synthesis inhibitor Cx (D), 10 μM MEK inhibitor PD (E), the expression vector 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.

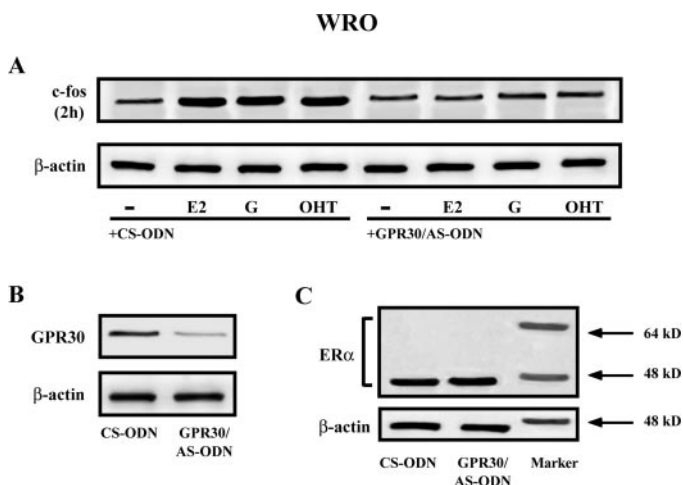On the basis of these observations, we then verified the

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.
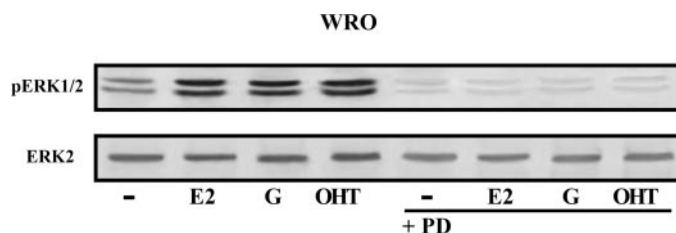
Fig. 7. ERK1/2 phosphorylation in WRO cells. WRO cells were treated for 5 min with 100 nM E2, G, OHT, and 10 μM PD as indicated. Total ERK2 proteins were used to normalize ERK1/2 expression.
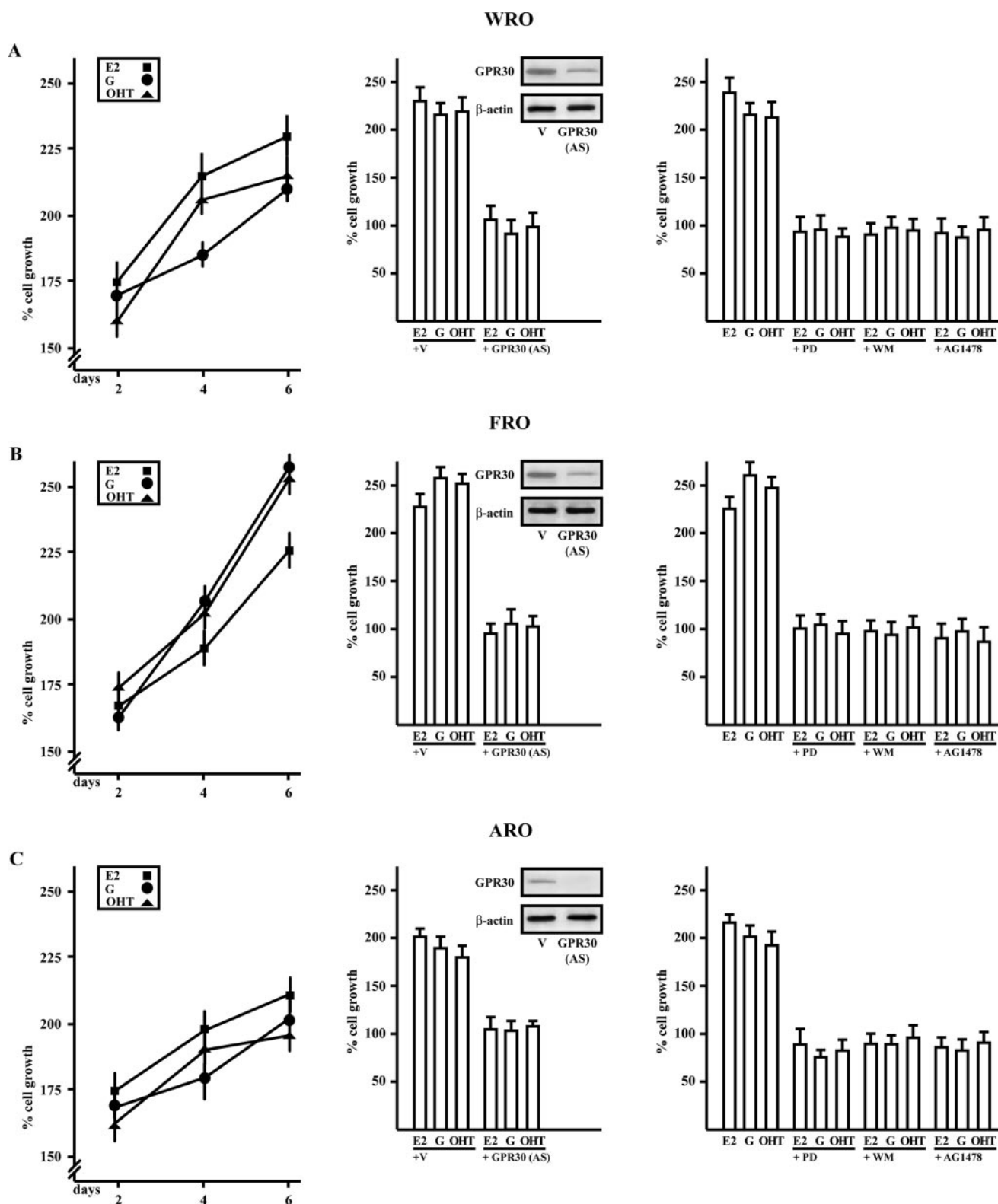
Fig. 8. E2, G, and OHT stimulate the proliferation of WRO, FRO, and ARO cells. A, cells were treated with 100 nM E2, G, and OHT in medium containing 5% charcoal-stripped FBS (medium was refreshed and treatments were renewed every 2 days) and then counted on the indicated days. Proliferation of cells 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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