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The G Protein-Coupled Receptor GPR30 Mediates the Proliferative Effects Induced by 17β-Estradiol and Hydroxytamoxifen in Endometrial Cancer Cells

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The growth of both normal and transformed epithelial cells of the female reproductive system is stimulated by estrogens, mainly through the activation of estrogen receptor α (ERα), which is a ligand-regulated transcription factor. The selective ER modulator tamoxifen (TAM) has been widely used as an ER antagonist in breast tumor; however, long-term treatment is associated with an increased risk of endometrial cancer. To provide new insights into the potential mechanisms involved in the agonistic activity exerted by TAM in the uterus, we evaluated the potential of 4-hydroxytamoxifen (OHT), the active metabolite of TAM, to transactivate wild-type ERα and its splice variant expressed in Ishikawa and HEC1A endometrial tumor cells, respectively. OHT was able to antagonize only the activation of ERα by 17β-estradiol (E2) in Ishikawa cells, whereas it up-regulated c-*fos* expression in a rapid manner similar to E2 and independently of ERα in both cell lines. This stimulation occurred through the G protein-coupled receptor named GPR30 and required Src-related and epidermal growth factor receptor tyrosine kinase activities, along with the activation of both ERK1/2 and phosphatidylinositol 3-kinase/AKT pathways. Most importantly, OHT, like E2, stimulated the proliferation of Ishikawa as well as HEC1A cells. Transfecting a GPR30 antisense expression vector in both endometrial cancer cell lines, OHT was no longer able to induce growth effects, whereas the proliferative response to E2 was completely abrogated only in HEC1A cells. Furthermore, in the presence of the inhibitors of MAPK and phosphatidylinositol 3-kinase pathways, PD 98059 and wortmannin, respectively, E2 and OHT did not elicit growth stimulation. Our data demonstrate a new mode of action of E2 and OHT in endometrial cancer cells, contributing to a better understanding of the molecular mechanisms involved in their uterine agonistic activity. (*Molecular Endocrinology* 20: 631–646, 2006)

ESTROGENS ARE PLEIOTROPIC hormones that regulate the growth and differentiation of many tissues. Acting as mitogens, they also promote the development of breast and endometrial tumors, which cumulatively account for about 40% of cancer incidence among women (1). The classical mechanism of action of 17β-estradiol (E2) (1) involves binding to estrogen receptors (ER) α and β, which interact with the estrogen response elements (EREs) located in the regulatory region of target genes (2, 3). It is well known that the ERs contain two main transcription activation functions (AF): the N-terminal AF1 and the C-terminal AF2, which is associated with the ligand-binding domain responsible for hormone-dependent transactivation (4, 5). The involvement of both AF1 and AF2 in ER-mediated transcriptional activity depends on the cell and promoter examined, because in a certain context an individual domain functions as the only determinant, whereas in other cases AF2 acting in combination with AF1 elicits the maximal potency in the ER response (4, 6, 7).

Approximately 50% of all breast cancers express elevated levels of ERα (8), and prolonged exposure to E2 is a major risk factor for breast tumor progression (9). Consequently, ERα antagonists such as tamoxifen (TAM), ICI 182,780 (ICI), and raloxifene have been used in the front-line pharmacological management of ERα-positive breast cancers to inhibit the mitogenic action of E2 (10–14). Although originally considered an antiestrogen, TAM is now classified as a selective ER modulator because in the breast it functions as an

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Abbreviations: AF, Activation function; AP1, activating protein 1; AS-ODN, antisense oligonucleotide; Cx, cycloheximide; DEX, dexamethasone; DHT, 5α-dihydrotestosterone; DN, dominant negative; E2, 17β-estradiol; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERE, estrogen-responsive element; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; ICI, ICI 182,780; OHT, hydroxytamoxifen; PD, PD 98059; PI3K, phosphatidylinositol 3-kinase; PR, progesterone receptor; PRG, progesterone; PT, pertussis toxin; SRE, serum response element; TAM, tamoxifen; WM, wortmannin.

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antagonist, whereas in the uterus, bone, and the cardiovascular system it displays agonistic activity (15, 16). Furthermore, TAM can induce uterine cell growth *in vivo* in animal models (17) as well as in humans (18, 19). Also, the active metabolite of TAM, 4-hydroxytamoxifen (OHT), stimulates the proliferation of both human endometria (20, 21) and cultured human endometrial carcinoma cells (21, 22). Many molecular mechanisms have been proposed to explain this phenomenon; however, it still remains to be completely elucidated.

In normal and neoplastic uterine tissue, the expression of full-length ERa mRNA is generally greater than the expression of a given splice variant (23, 24). Occasionally, ERα mRNA splice variants are translated into proteins that may act either as dominant-positive or dominant-negative (DN) *vs.* the wild-type ERα (25). In addition, an altered expression of ER coregulators has been associated with the progression of breast cancers toward a more malignant and TAM-resistant phenotype (26). The involvement of coactivators to promote TAM agonism may represent a relevant hypothesis; however, alteration of their expression patterns did not parallel the development of endometrial malignancy (27). The tissue-specific effects of TAM may follow the activation of cellular signaling pathways, including ERα phosphorylation at both serine 118 and 167, as well as the MAPK kinase kinase 1-dependent increase in TAM agonistic activity (28).

A large number of studies have also been performed toward the identification of membrane-associated estrogen signals that may alter gene expression independently of the nuclear ERs. Intriguingly, recent reports, including our own (29–31), have described the estrogen-binding and activation properties of a G protein-coupled receptor (GPCR) named GPR30, which has therefore been proposed as a candidate for triggering a broad range of rapid E2 activity at the plasma membrane level. In this respect, we have demonstrated that in breast cancer cells lacking ERs, GPR30 mediates up-regulation of the c-*fos* protooncogene by either E2 or estrogen-like compounds, such as the phytoestrogens genistein and quercetin (29). c-*fos* is a classical early gene whose expression is rapidly and transiently induced by different extracellular stimuli, including mitogens and hormones (32–38). The nuclear protein encoded by c-*fos* interacts with c-*jun* family members to form the heterodimeric activating protein 1 (AP1) transcription factor (39–42), which regulates a series of genes as well as interacting proteins (43–48). The transcription of c-*fos* is controlled by multiple *cis* elements, such as the cAMP response element (49), the signal transducers and activators of transcription group of transcription factors (50), and the serum response element (SRE), which recruits the ternary complex factors, including Elk-1 and serum-response factor accessory proteins 1 and 2 (51–55). As it concerns ERα, binding to the imperfect palindromic ERE sequence within the c-*fos* promoter is not sufficient for transactivation because it requires interaction of ERα with a downstream Sp1 site (56). Besides, it has been reported that E2 signaling may converge on the SRE target sequence through a nongenomic ERα pathway leading to MAPK-dependent phosphorylation and binding of Elk-1 to the SRE sequence (57).

In the present study we show that E2 and OHT up-regulate c-*fos* expression in a rapid manner through the membrane-associated GPR30 protein in Ishikawa and HEC1A human endometrial cancer cells. Our results provide new insights into the molecular mechanisms by which OHT exerts a growth stimulatory action in these uterine tumor cells.

RESULTS

E2, But Not OHT, Transactivates and Down-Regulates ERα in Ishikawa Cells

Considering the controversies regarding the ability of E2 and OHT to activate the endogenous and/or overexpressed ERα in Ishikawa cells (25, 58–60), we first examined whether a transiently transfected ER reporter gene responds to both ER ligands. E2 induced a dose-dependent luciferase expression, whereas OHT as well as ICI were not able to activate ERα in the concentration range used (Fig. 1A). However, 10 μM of both ER antagonists repressed the luciferase activity elicited by 100 nM E2 (Fig. 1B). An additional hallmark of ERα activation by an agonist is represented by reduction of its expression consequent to the high turnover of the E2-activated receptor protein and the transcription inhibition of its own gene (61). Hence, we investigated whether the levels of ERα contained in Ishikawa cells could be regulated by E2 and OHT. Western analysis of ERα performed with an anti-ERα antibody raised against the C-terminus region recognized a protein corresponding to the size of wild-type ERα (66 kDa; Fig. 1, C and E). In addition, the ERα antibody recognized a protein with a smaller size ($<$48 kDa; Fig. 1, C and E), which was no longer detected using an anti-ERα antibody raised against the N-terminus domain (data not shown). Short treatments did not modify the levels of ERα and its variant (Fig. 1, C and D), whereas a 12-h exposure to E2 markedly reduced the content of wild-type ERα (Fig. 1, E and F). As it concerns ERβ expression, no changes were observed in either of the ligands used (Fig. 1, C and D, and E and F). Overall, these findings indicate that in Ishikawa cells the transcriptional activation of endogenous ERα gene is dependent on E2, whereas OHT exerts an antagonist activity.

E2 and OHT Neither Transactivate Nor Regulate the ERα Splice Variant Expressed in HEC1A

Next, we evaluated the potential transcriptional effects of E2 and OHT in HEC1A human endometrial cancer cells, because ER expression and E2 responsiveness

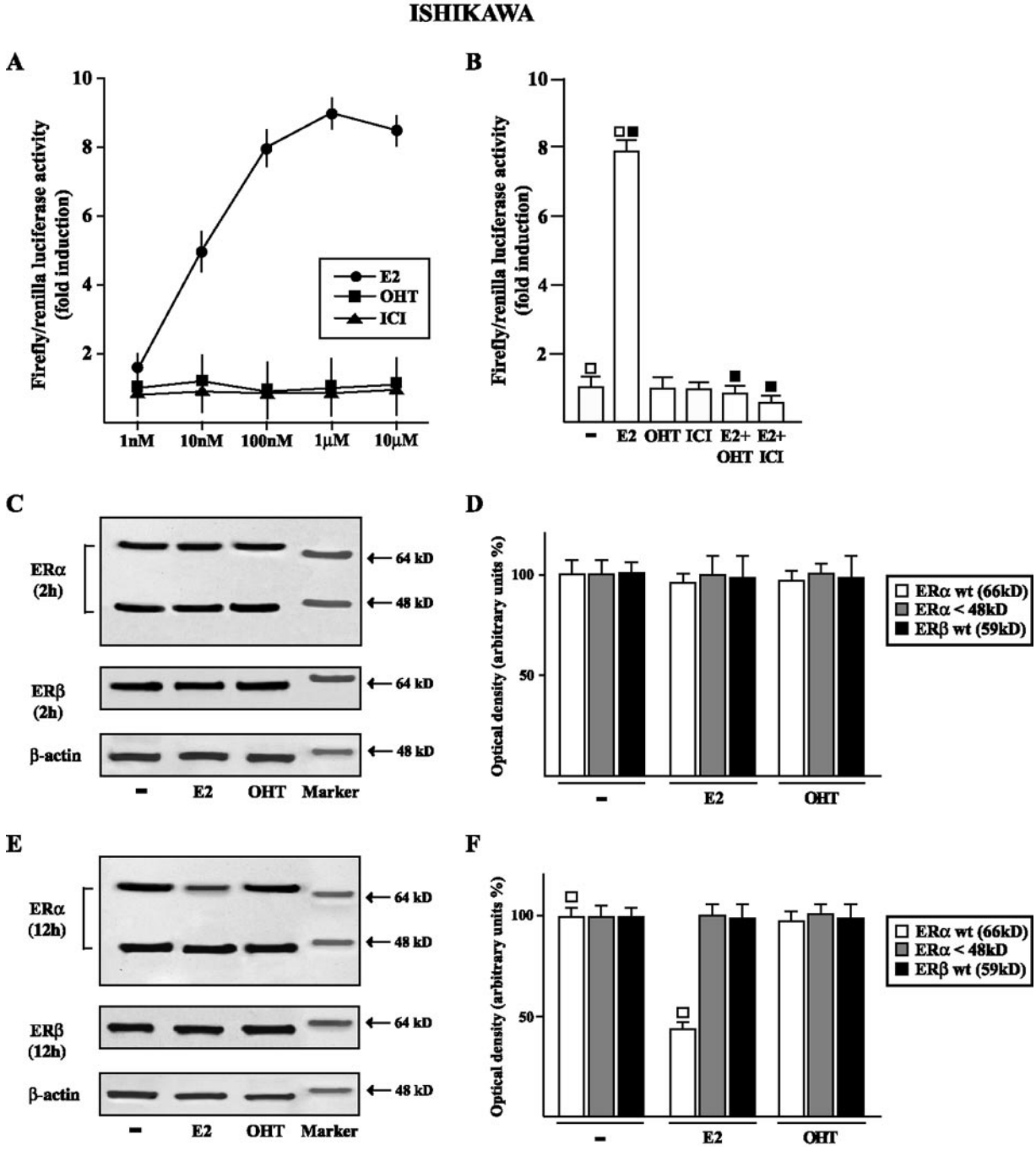

Fig. 1. E2, But Not OHT, Activates and Down-Regulates Wild-Type ERα in Ishikawa Cells

Ishikawa cells were transfected with the luciferase reporter plasmid XETL and treated with increasing concentrations of E2, OHT, and ICI (A) or with 100 nM E2, 10 μM OHT, and ICI alone or in combination as indicated (B). The luciferase activities were normalized to the internal transfection control, and values of untreated cells (−) 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 untreated (−) *vs.* E2-treated cells; ■, $P < 0.05$ for E2-treated cells *vs.* those treated with E2 and OHT or ICI. C and E, Immunoblots of ERα and ERβ from Ishikawa cells treated with 1 μM E2 or OHT for the indicated times. β-Actin serves as a loading control. D and F, Quantitative representations of data (mean ± SD) from three independent experiments including those in C and E, respectively. □, $P < 0.05$ for untreated (−) *vs.* E2-treated cells.

are still controversial in these cells (62–67). In agreement with previous reports (63, 65, 67), we did not detect any ERE-mediated transcriptional activity on E2, OHT, or ICI in the concentration range used (Fig. 2A). Furthermore, E2 and OHT had no effect on the protein levels of ERβ or the ERα splice variant con-

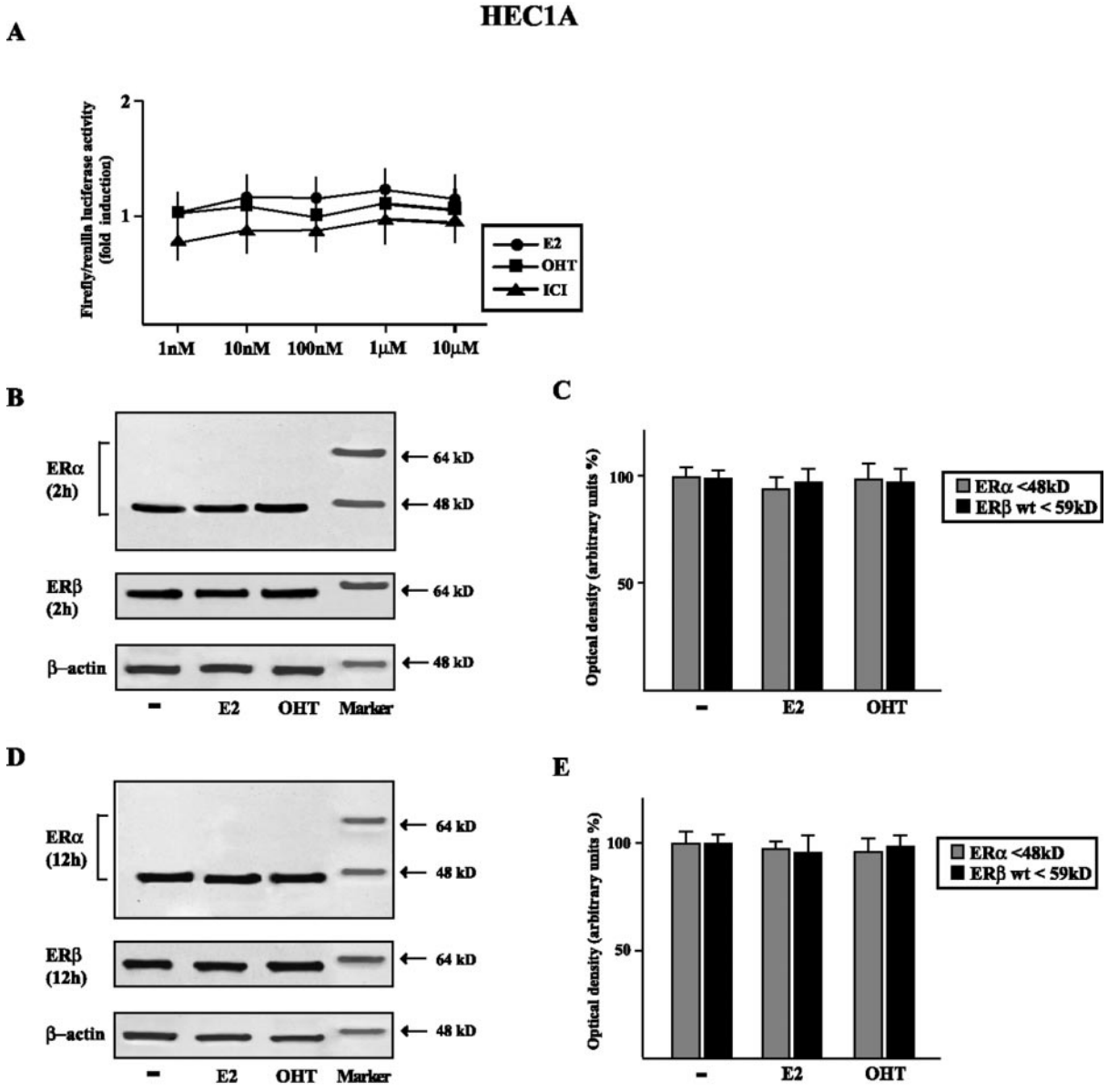

Fig. 2. The Splice Variant of ERα Expressed in HEC1A Cells Is Neither Activated Nor Modulated by E2 and OHT

A, HEC1A cells were transfected with the luciferase reporter plasmid XETL and treated with increasing concentrations of E2, OHT, and ICI. The luciferase activities were normalized to the internal transfection control, and values of untreated cells were set at 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. B and D, Immunoblots of ERα and ERβ from HEC1A cells treated with 1 μM E2 or OHT for the indicated times. β-Actin served as a loading control. C and E, Quantitative representations of data (mean ± SD) from three independent experiments including those in B and D, respectively.

tained in HEC1A cells (Fig. 2, B and C, and D and E). Collectively, these results suggest that the ERα splice variant expressed in the endometrial cancer cells used is not able to induce ERE-mediated transcriptional activity or to act as a DN form of wild-type ERα.

E2 and OHT Transactivate c-fos Promoter Constructs in Ishikawa Cells

Having established that OHT acts as an antagonist of the classical ERα-ERE pathway in Ishikawa cancer cells, we examined its ability to activate a transiently transfected full-length human c-*fos* promoter (−2.2 kb), which contains several target sequences responding to a variety of extracellular stimuli (32–38). Interestingly, E2 and OHT, but not ICI, were able to transactivate c-*fos* in an ERE-independent manner, because an ERE deletion mutant (−1172 bp) still elicited the transcriptional response (Fig. 3A). The ternary complex factor member Elk-1 is crucial for the ERK-dependent activation of the c-*fos* gene promoter (52);

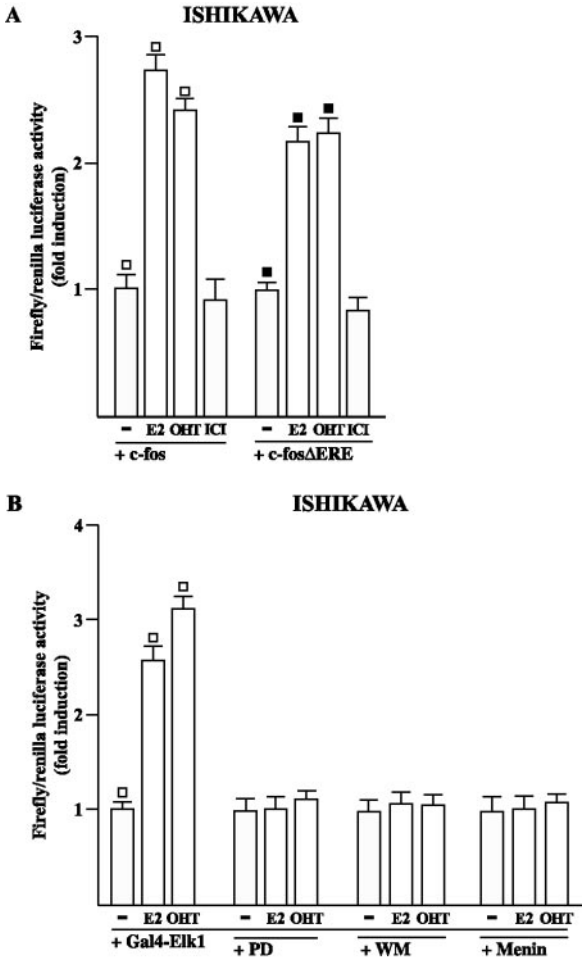

Fig. 3. Transcriptional Activation of c-*fos* and Gal4-Elk1 Reporters by E2 and OHT in Ishikawa Cells

A, The luciferase reporter plasmid c-*fos* encoding a −2.2-kb-long upstream region of human c-*fos* as well as the deletion mutant c-*fos*ΔERE lacking the ERE sequence and encoding a −1172-bp upstream fragment of human c-*fos* are activated by 1 μM E2 and OHT in Ishikawa cells. B, The luciferase reporter plasmid for the fusion protein consisting of Elk1 and the Gal4 DNA binding domain is activated by 1 μM E2 and OHT in Ishikawa cells. A concentration of 10 μM PD, 10 μM WM, and the expression vector for the menin gene reversed the response. The luciferase activities were standardized to the internal transfection control, and values of untreated cells (−) were set at 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. □ and ■, $P < 0.05$ for untreated cells (−) *vs.* treatments.

therefore, we investigated the effects of E2 and OHT on Elk-1-mediated transcriptional activity. As shown in Fig. 3B, both E2 and OHT induced Elk-1 transactivation, which was abrogated by the MAPK kinase-specific inhibitor PD 98059 (PD) and or the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (WM). In addition, Elk1 activity was no longer noticeably overexpressing the oncosuppressor protein menin, previously shown by us to inhibit the ERK-dependent activation of Elk-1 (68). Hence, distinct signal transduction pathways converge to regulate c-*fos* expression, which could be considered an additional sensor of estrogen signaling.

E2 and OHT Rapidly Induce c-fos mRNA Expression in Ishikawa Cells

It is well established that c-*fos* gene expression is promptly stimulated by a variety of extracellular signals, including E2 (29, 60, 69). To investigate whether OHT is also able to induce in a rapid manner the expression of c-*fos* or two estrogen target genes such as the well known progesterone (PRG) receptor (PR) and pS2 (70), we performed semiquantitative RT-PCR experiments comparing the mRNA levels after standardization on a housekeeping gene encoding the ribosomal protein 36B4. A short exposure (1 h) of Ishikawa cells to E2 and OHT up-regulated c-*fos* expression (Fig. 4, A and B), which was no longer noticeable after 12 h of treatment (Fig. 4, C and D). The

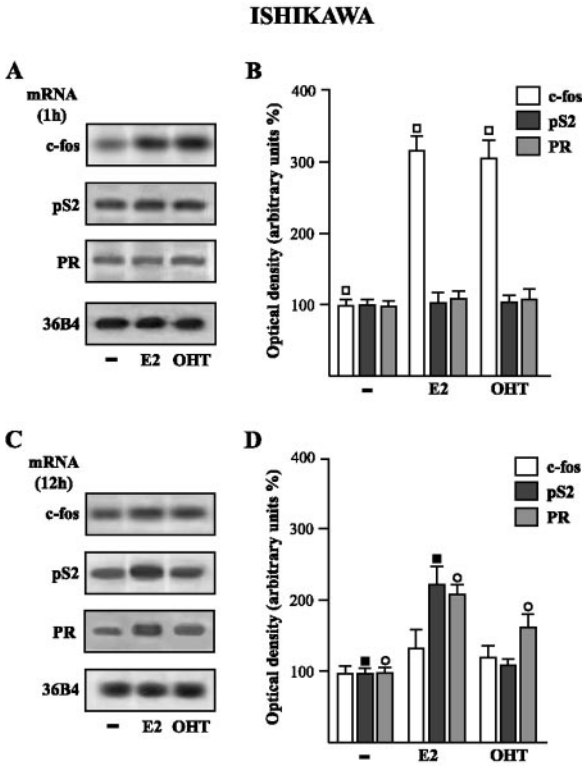

Fig. 4. Rapid Induction of c-*fos* mRNA by E2 and OHT in Ishikawa Cells

A and C, c-*fos*, pS2, and PR semiquantitative RT-PCR from Ishikawa cells treated with 1 μM E2 and OHT for the indicated times; 36B4 mRNA levels were determined as a control. B and D, Quantitative representation of data (mean ± SD) from three independent experiments after densitometry and correction for 36B4 expression including those of A and C, respectively. □, ■, and ○, $P < 0.05$ for untreated cells (−) *vs.* treatments.

latter time was also required for E2 to increase the mRNA levels of pS2 and PR and for OHT to enhance PR levels (Fig. 4, C and D). These results indicate that in Ishikawa cells, exposure time and ligand specificity regulate the expression of estrogen target genes.

E2 and OHT Up-Regulate c-fos Protein Levels in Ishikawa and HEC1A Cells

To ascertain the transduction pathways involved in c-*fos* regulation by E2 and OHT, we first investigated whether c-*fos* protein expression mimics the mRNA accumulation upon these ligands. In Ishikawa cells, a 2-h exposure to E2 and OHT up-regulated c-*fos* expression, which was no longer noticeable after 12 h of treatment (Fig. 5, A and B). Next, we analyzed c-*fos* protein levels after treating cells for 2 h with E2 and OHT in the presence of specific inhibitors of distinct metabolic cascades. The protein synthesis inhibitor cycloheximide (Cx) abolished the effects of both ligands (Fig. 5C), indicating that the up-regulation of c-*fos* is strictly dependent on new protein synthesis. ICI slightly inhibited the increase in c-*fos* after E2 and OHT treatment (Fig. 5D), whereas the MAPK inhibitor PD (Fig. 5E) and the ectopically expressed DN form of the ERK protein (Fig. 5F) completely blocked c-*fos* induction. The latter effect was also obtained using the Src family tyrosine kinase inhibitor PP2 (AG 1879) (Fig. 5G), the epidermal growth factor (EGF) receptor (EGFR) kinase inhibitor tyrphostin AG 1478 (Fig. 5H), the PI3K inhibitor WM (Fig. 5I), and the GPCR inhibitor pertussis toxin (PT; Fig. 5J). On the contrary, the protein kinase A inhibitor H-89 did not repress the c-*fos* response to E2 and OHT at either the mRNA or protein level (data not shown).

To evaluate these results in a different cellular context, we analyzed HEC1A endometrial cancer cells, which contain only an ERα splice variant, as documented in the present study (Fig. 2, B and D). Also in HEC1A cells, E2 and OHT rapidly (2 h) up-regulated c-*fos* protein levels (Fig. 6, A and B), which were dependent on new protein synthesis (Fig. 6C) and were not altered in presence of ICI (Fig. 6D). The c-*fos* response displayed the same sensitivity to inhibitors used in Ishikawa cells (Fig. 6, E–J). The steroids dexamethasone (DEX), PRG, and 5α-dihydrotestosterone (DHT) did not enhance c-*fos* content (Fig. 6K), as also observed after 17α-estradiol treatment (data not shown), indicating that a ligand specificity is required for the rapid response observed. Cumulatively, our data suggest that diverse transduction pathways are triggered by E2 and OHT to regulate c-*fos* expression in endometrial cancer cells.

GPR30 and MAPK Activation Mediate the Up-Regulation of c-fos by E2 and OHT in Ishikawa and HEC1A Cells

On the basis of the results described above and considering that diverse extracellular factors signal through GPCRs and result in ERK1/2 activation (71–73), we investigated the involvement of this transduction pathway in c-*fos* regulation by E2 and OHT in Ishikawa and HEC1A cells. As shown in Fig. 7, A and B, and E and F, in both endometrial cancer cell lines a specific GPR30 antisense oligonucleotide (GPR30/AS-ODN) abrogated the response to the ligands used, whereas a control scrambled oligonucleotide had no effect. GPR30/AS-ODN efficiently silenced GPR30 protein expression (Fig. 7, C and G), whereas ERα levels (Fig. 7, D and H) as well as those of PR (data not shown) were not altered. Interestingly, in human cervical HeLa cancer cells, which express very low levels of GPR30 (Fig. 8C), both ligands failed to up-regulate c-*fos* expression. However, c-*fos* was induced by E2- and OHT-transfecting cells with a plasmid encoding GPR30, but not in control cells transfected with an empty vector (Fig. 8). Moreover, HeLa cells engineered to overexpress GPR30 were growth stimulated by the treatments, although not in a significant manner compared to control cells (data not shown).

Because GPCR signals result in ERK1/2 activation (71–73), and in the present study both PD and DN/ERK inhibited c-*fos* induction (Figs. 5 and 6), we evaluated MAPK activation in Ishikawa and HEC1A cells. A 5-min treatment with E2 and OHT induced ERK1/2 phosphorylation without changes in total ERK2 (Fig. 9), indicating that both ligands signal through the MAPK pathway in the endometrial cancer cells used.

E2 and OHT Stimulate the Proliferation of Ishikawa and HEC1A Cells

These findings were also evaluated in a more complex physiological response such as cell proliferation. Of note, either E2 or OHT induced growth effects in Ishikawa and HEC1A cells (Fig. 10A). However, transfecting a GPR30/AS expression vector, the stimulatory action of OHT was no longer noticeable in either cell line, whereas the proliferation produced by E2 was abrogated completely in HEC1A cells and partially in Ishikawa cells (Fig. 10, B–D). Finally, the proliferative effects elicited by E2 or OHT were abrogated in both endometrial cancer cell lines in presence of the MAPK inhibitor PD and the PI3K inhibitor WM (Fig. 10E). Hence, cell growth assays confirmed the involvement of GPR30 and the transduction pathways studied in mediating the stimulatory action of both ligands in Ishikawa and HEC1A cells.

DISCUSSION

It has been largely documented that treatment with TAM, widely used in women to prevent breast cancer recurrence, is associated with an increased incidence of proliferative and neoplastic endometrial changes (74). In this regard, previous studies have demonstrated that TAM is converted into reactive species

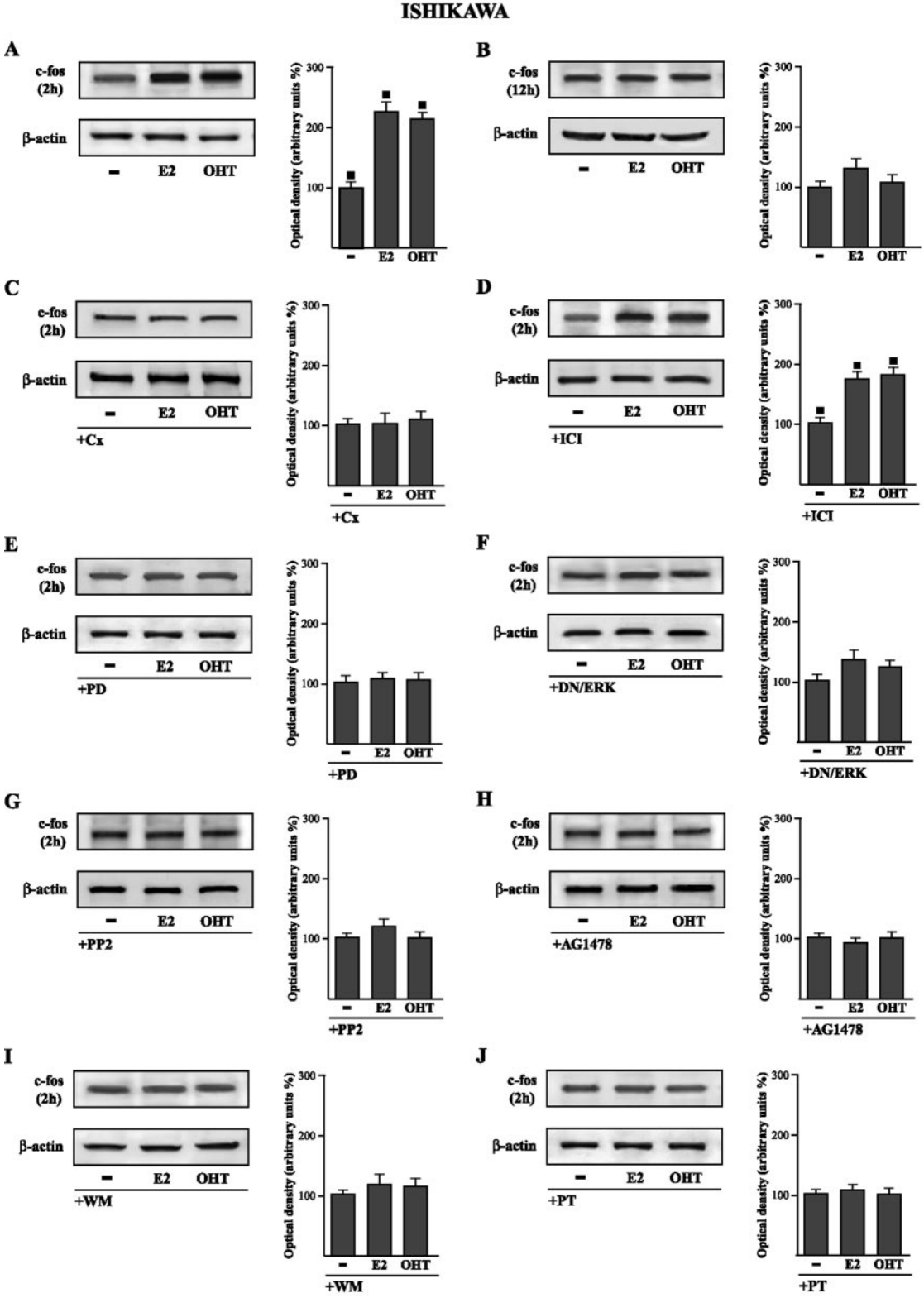

Fig. 5. Immunoblots of c-*fos* from Ishikawa Cells

Ishikawa cells were treated for the indicated times with 1 mM E2 and OHT (A and B) and in combination with 50 μM protein synthesis inhibitor Cx (C), 1 mM ICI (D), 10 μM of the MAPK inhibitor PD (E), the expression vector for the DN/ERK2 (F), 10 μM of the Src family tyrosine kinase inhibitor PP2 (G), 10 μM of the EGFR kinase inhibitor tyrphostin AG 1478 (H), 10 μM of the PI3K inhibitor WM (I), and 100 ng/ml of the G protein inhibitor PT (J). β-Actin was used as a loading control. The side panels show the quantitative representations of data (mean ± SD) from three independent experiments performed for each condition. ■, $P < 0.05$ for untreated cells (−) *vs.* treatments.

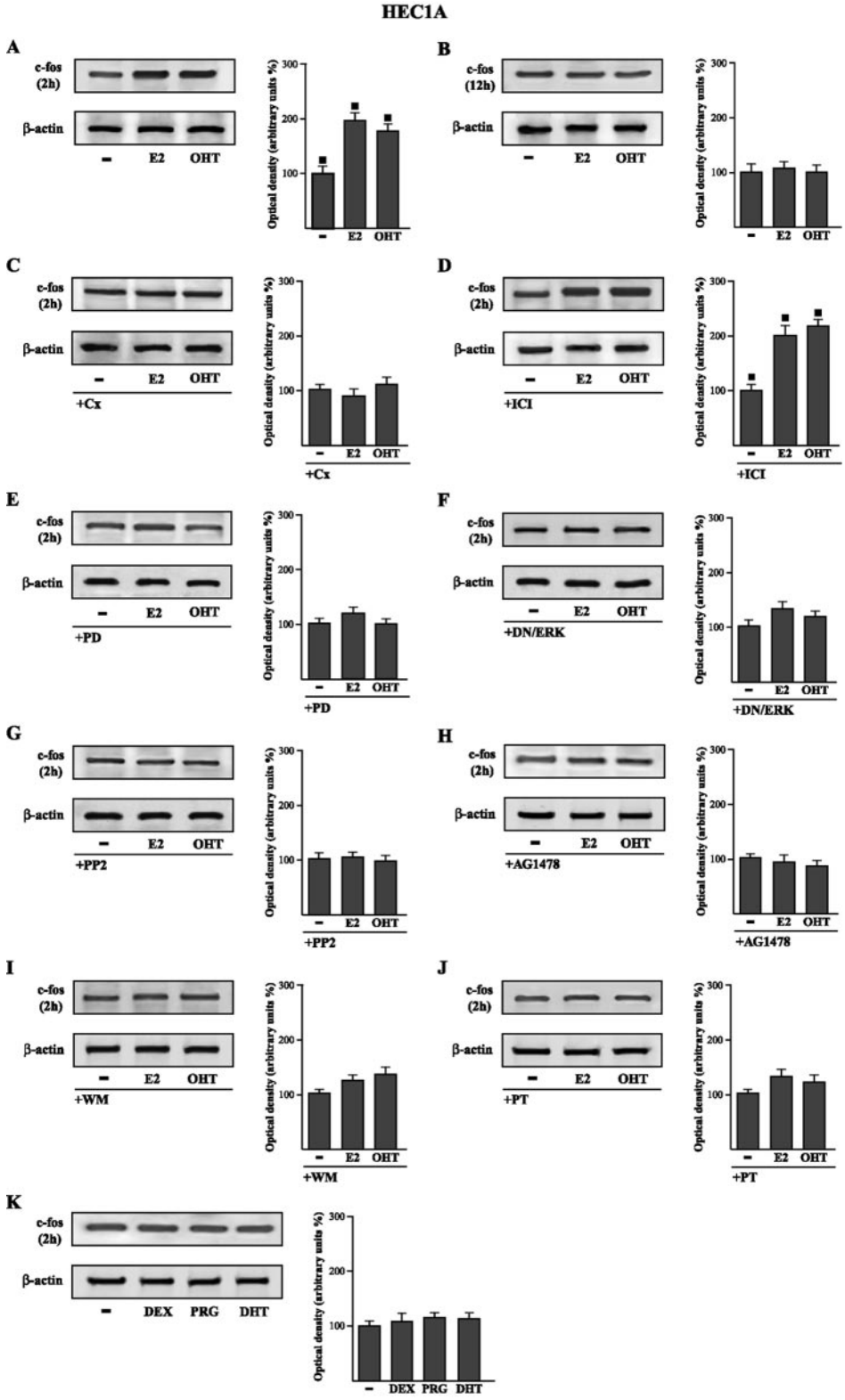

Fig. 6. Immunoblots of c-*fos* from HEC1A Cells

HEC1A cells were treated for the indicated times with 1 μM E2 and OHT (A and B) and in combination with 50 μM of the protein synthesis inhibitor Cx (C), 1 mM ICI (D), 10 μM of the MAPK inhibitor PD (E), the expression vector for the DN/ERK2 (F), 10 μM of the Src family tyrosine kinase inhibitor PP2 (G), 10 μM of the EGFR kinase inhibitor tyrphostin AG 1478 (H), 10 μM of the PI3K inhibitor WM (I), and 100 ng/ml of the G protein inhibitor PT (J). Besides, cells were treated with 1 μM DEX, 1 μM PRG and 1 μM DHT (K). β-actin was used as a loading control. The side panels show the quantitative representations of data (mean $\pm$ SD) from three independent experiments performed for each condition. ■, $P < 0.05$ for untreated cells (−) *vs.* treatments.

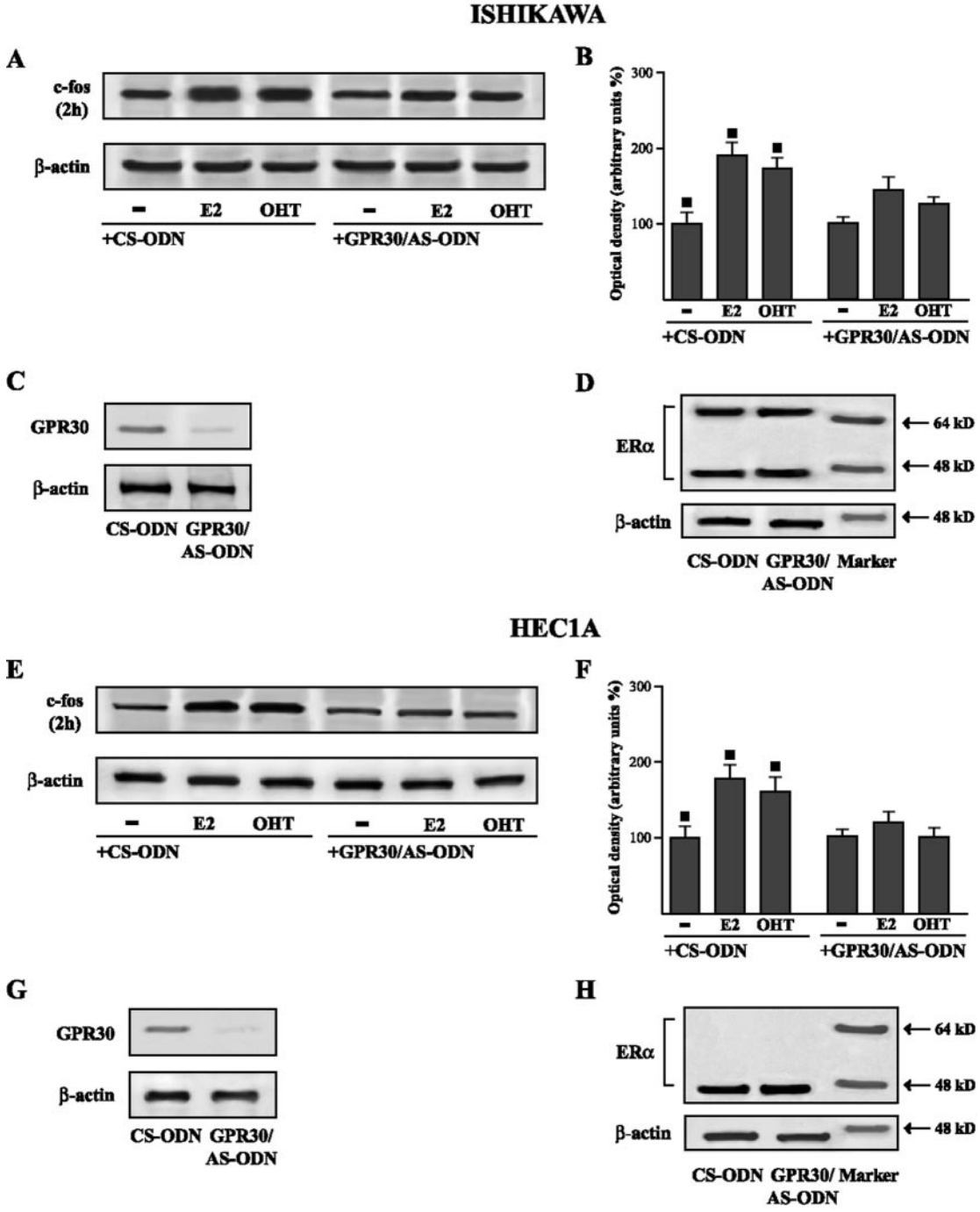

Fig. 7. c-*fos* Induction by E2 and OHT Is Abrogated by Transfecting Endometrial Cancer Cells with a GPR30 AS-ODN Oligonucleotide

Ishikawa (A) and HEC1A (E) cells transfected with control scrambled (CS-ODN) or GPR30/AS-ODN oligonucleotides were treated with 1 μM E2 and OHT. B and F, Quantitative representation of data (mean $\pm$ SD) from three independent experiments including those in A and E, respectively. GPR30 and ERα protein expression in Ishikawa (C and D, respectively) and in HEC1A (G and H, respectively) cells transfected with CS-ODN or GPR30/AS-ODN. β-Actin was used as a loading control. ■. $P < 0.05$ for untreated cells (−) *vs.* treatments.

forming DNA adducts, leading, in turn, to endometrial carcinogenesis (75–78). As it concerns the mechanism through which TAM acts as an estrogen agonist in the uterus, it has been suggested that both the AF1 domain of ERα (62) as well as cell- and promoter-specific factors may be involved (28, 79, 80). Furthermore, it has been shown that coregulator proteins facilitating ERα interaction with the general transcriptional ma-

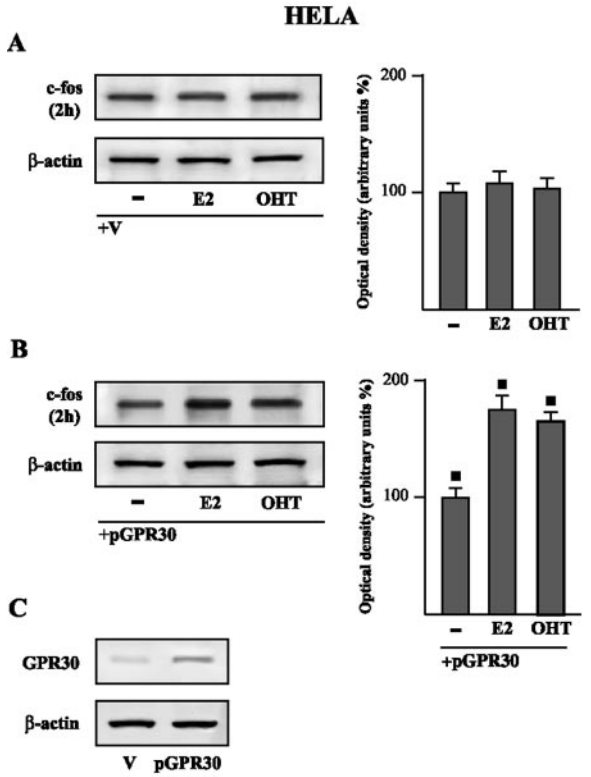

Fig. 8. c-*fos* Is Up-Regulated by E2 and OHT in HeLa Cells Transfected with a Plasmid Encoding GPR30

HeLa cells were transfected in 10-cm dishes with 5 μg empty vector (v; A) or 5 μg expression plasmid encoding GPCR30 (pGPR30; B) and were treated with 1 μM E2 and OHT. The *side panels* are the quantitative representation of data (mean ± SD) from three independent experiments including those in A and B, respectively. C, GPR30 protein expression in HeLa cells transfected with an empty vector (v) or pGPR30. β-Actin was used as a loading control. ■, $P < 0.05$ for untreated cells (-) *vs.* treatments.

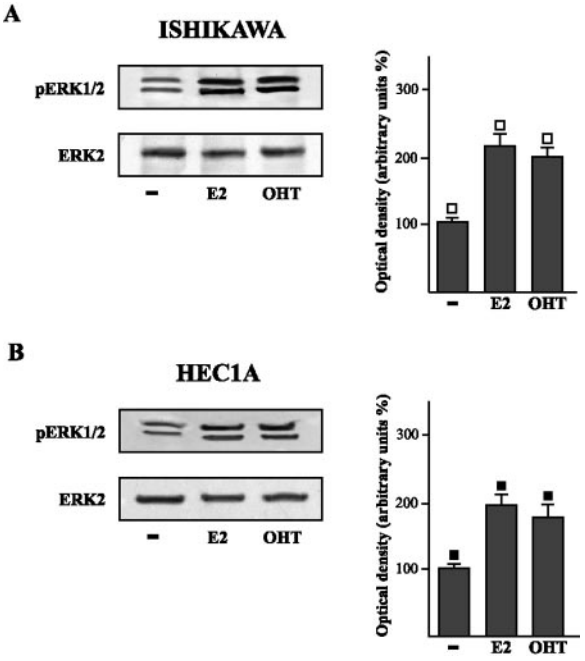

Fig. 9. ERK1/2 Phosphorylation in Endometrial Cancer Cells

Ishikawa (A) and HEC1A (B) cells were treated for 5 min with 1 μM E2 and OHT. Total ERK2 proteins were used to normalize ERK1/2 expression. The *side panels* show the quantitative representations of data (mean ± SD) from three independent experiments including those in A and B, respectively. □ and ■, $P < 0.05$ for untreated cells (−) *vs.* treatments.

chinery and chromatin could be responsible for the differential ability of partial agonists/antagonists to modify gene expression (81, 82).

Considering that recent studies have revealed a functional interaction between either TAM or E2 with GPR30 in triggering rapid cellular responses to estrogen signals at the plasma membrane level (29–31), in the present study we have evaluated and demonstrated that in endometrial cancer cells, TAM retains the ability to antagonize ERα-dependent signaling, yet acquires that to induce c-*fos* expression and cell proliferation in a GPR30-mediated fashion. Agents able to block G protein and GPR30 signaling, such as PT and the GPR30/AS-ODN, both prevented the enhancement of c-*fos* in a similar manner as the specific inhibitors of the EGFR, Src, PI3K, and MAPK pathways (Figs. 5 and 6). Given that EGFR tyrosine kinase activity is required for GPR30-dependent ERK1/2 activation (29, 71), our results suggest novel molecular mechanisms that may serve as a unique signaling prototype leading to gene expression in various cell types. It is worth noting that our findings recall previous observations indicating the existence of sequential and even reciprocal cross-talk among the above-mentioned transduction pathways in diverse cellular contexts (83–85). For instance, in hepatocyte growth factor-treated myogenic cells, the specific PI3K inhibitor WM abolished MAPK/ERK and Elk-1 phosphorylations, which, in turn, stimulated cell proliferation (86).

The mechanistic differences among antiestrogens have already revealed the complex molecular pharmacology of ERα, which is now paralleled by the membrane estrogen-binding protein GPR30. Although E2 and the ER antagonists TAM and ICI bind to GPR30 directly (31), in our previous investigation we demonstrated that GPR30 mediates c-*fos* expression only after E2 treatment in breast cancer cells (29) and also after OHT treatment in endometrial tumor cells as shown in the present study. Collectively, our observations suggest that GPR30 function may be dependent on the cellular context and the specific binder used. In contrast, TAM acts differentially on GPR30- and ERα-mediated signals, and it even activates GPR30 in a spatially different manner from E2 (31). Interestingly, the above-mentioned findings were recapitulated in a complex physiological response such as proliferation of Ishikawa and HEC1A cells engineered to abolish GPR30 expression. E2 can induce growth effects through at least two diverse mechanisms: one involv-

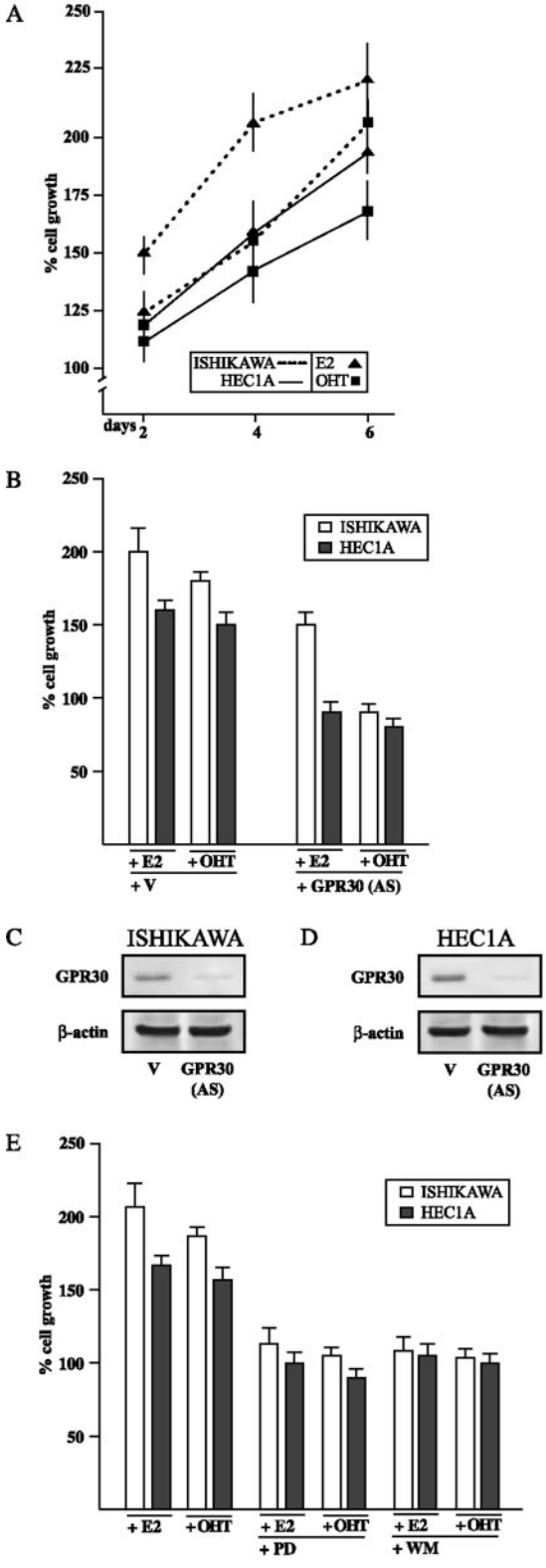

Fig. 10. E2 and OHT Stimulate the Proliferation of Endometrial Cancer Cells

A, Ishikawa and HEC1A cells were treated with 1 μM E2 and OHT in medium containing 5% charcoal-stripped FBS (medium was refreshed, and treatments were renewed every 2 d), then counted on the indicated day. Proliferation of untreated cells was set at 100% upon which cell growth induced by treatments was calculated. Each data point is the mean ± SD of three independent experiments performed in triplicate. B, Ishikawa and HEC1A cells were transfected with an empty vector (v) or an expression vector for GPR30/AS (see *Materials and Methods*), treated as described in A, then counted on d 6. Proliferation of untransfected and untreated cells was set at 100% upon which cell growth induced by treatments in presence of the empty vector or the expression vector for GPR30/AS was calculated. Each data point is the mean ± SD of three independent experiments performed in triplicate. GPR30 protein expression was determined in Ishikawa (C) and HEC1A (D) cells transfected with an empty vector (v) or GPR30/AS. β-Actin was used as a loading control. E, Ishikawa and HEC1A cells were treated with 1 μM E2 and OHT or in combination with 10 μM of the MAPK inhibitor PD and 10 μM of the PI3K inhibitor WM and counted on d 6. Proliferation of untreated cells was set at 100% upon which cell growth induced by treatments was calculated. Each data point is the mean ± SD of three independent experiments performed in triplicate.

ing the wild-type ERα and a second involving the GPR30-MAPK pathway, which are crucial for the OHT-induced effects or those exerted by E2 in an ERα-independent manner.

To provide new criteria for the selection of therapeutic interventions in endocrine-related disorders, the potential of distinct selective ER modulators to signal through GPR30 should be investigated at the level of distinct tissues. For instance, the widespread use of raloxifene in the prevention-therapy of osteoporosis in postmenopausal women should be carefully considered, because it could act to prevent or promote the development of estrogen-sensitive tumors. This scenario is particularly poignant, because the beneficial effects of raloxifene on osteoporosis requires continuous treatment for decades. In this context, it should be taken into account that abundant phytoestrogens, such as genistein and quercetin, induce gene expression by both ERα as well as GPR30 (29, 87). The environmental contaminant ortho,*para*-dichlorodiphenyldichoroethylene activates ERα (88) and binds to GPR30 (31). Hence, estrogen signaling has become increasingly complex, showing multifaceted biological actions that may have profound implications in the pathophysiology of breast, endometrial, ovarian, placenta, prostate, neural, and vascular tissues.

As mentioned above, controversial results have been reported on the ability of antiestrogens to stimulate transcription in endometrial cancer cells through promoters containing EREs or AP1-binding sites (12-*O*-tetradecanoyl phorbol 13-acetate response elements) regardless of ER content and the expression of splice variants (24–25, 58–60, 64, 89). Using two human-derived endometrial tumor cells that present important differences in ERα expression, we ascertained the ability of E2 and OHT to transactivate distinct reporter constructs as well as to induce gene expres-

sion. In line with the findings of others (60, 89, 90), prolonged exposure to these treatments was required to up-regulate pS2 and PR, whereas c-*fos* was rapidly enhanced, suggesting that independent mechanisms are involved in such genomic responses.

Notably, the data we report suggest that c-*fos* induction is a GPR30-ERK1/2-dependent transcriptional effect that involves Elk-1 signaling to c-*fos* promoter, as also demonstrated in breast cancer cells (29, 57). Indeed, our model system, designed to highlight the transduction pathways contributing to such molecular events in endometrial cancer cells, has outlined the complex cascade described in Fig. 11.

c-*fos* belongs to a family of transcription factors that, alone and/or after heterodimerization with *jun* family members generates the AP1 transcription factor, binds to cognate sites at the regulatory sequences of genes involved in relevant cellular functions such as proliferation (32–48). Interestingly, c-*fos* was shown to be a transcriptional regulator of cyclin D1 (91), which translocates from the cytoplasm to the nucleus in the mouse uterine epithelium undergoing OHT-induced cell cycle progression (92). Hence, the up-regulation of c-*fos* by extracellular mitogens and growth factors could represent a unique early molecular sensor to evaluate the genomic responses leading to growth stimulation in endometrial cancer cells.

One major concern with TAM therapy for breast cancer is the tumorigenic action in the endometrium. The present study provides new insights into the molecular mechanisms by which TAM may elicit undesired side effects that if selectively abrogated could endorse the benefits of long-term treatment alongside its prophylactic use in breast tumor disease.

MATERIALS AND METHODS

Reagents

E2, OHT, Cx, WM, LY 294002, PT, PD, DEX, PRG, and DHT were purchased from Sigma-Aldrich Corp. (Italy). ICI was obtained from Tocris Chemicals (Bristol, UK), tyrphostin AG 1478 (AG 1478) was purchased from Biomol Research Laboratories, Inc. (Milan, Italy), and H-89 and PP2 were obtained from Calbiochem (VWR International, Milan, Italy). All compounds were solubilized in dimethylsulfoxide, except E2, OHT, PD, and WM, which were dissolved in ethanol.

Plasmids

The firefly luciferase reporter plasmid for ERs was EREtkLuc (also called XETL) (29), which presents the following features: 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 simian virus 40 splice and polyadenylation sites from plasmid pSV232A/L-AA5′. Reporter plasmids for c-*fos* and its deletion mutant c-*fos*DERE (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) (93). The reporter plasmid Gal4-luc was described together with the expression vectors for Gal4-Elk1 and menin in our previous study (68). The plasmids encoding GPR30 (pGPR30),GPR30/AS, and DN ERK2 were provided by R. Weigel (Thomas Jefferson University, Philadelphia, PA), E. R. Prossnitz (University of Albuquerque, Las Cruces, NM), and M. Cobb (University of Texas, Dallas, TX), respectively. The *Renilla* luciferase expression vector pRL-thymidine kinase (Promega Corp., Milan, Italy) was used as a transfection standard.

Cell Culture

Ishikawa human endometrial cancer cells were obtained from D. Picard (University of Geneva, Geneva, Switzerland) and HEC1A human endometrial cancer cells were a gift from A. Peri (University of Florence, Florence, Italy), respectively. HeLa human cervical cancer cells were purchased from American Type Culture Collection (Manassas, VA). Ishikawa and HeLa cells were maintained in DMEM without phenol red supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Inc., Milan, Italy), whereas HEC1-A cells were maintained in RPMI 1640 without phenol red supplemented with 10% FBS (Invitrogen Life Technologies, Inc.). Cells were switched to medium without serum 48 h before RT-PCR, immunoblots, and evaluation of ERK1/2 phosphorylation.

Transfections and Luciferase Assays

A total of 100,000 Ishikawa or HEC1A cells were plated into 24-well dishes with 500 μl 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, Indianapolis, IN) with a mixture containing 0.5 μg reporter plasmid, 0.1 μg effector plasmid where applicable, and 2 ng pRL-thymidine kinase. After 4 h, we renewed the serum-free medium containing the indicated treatment, and then cells were incubated for about 12 h.

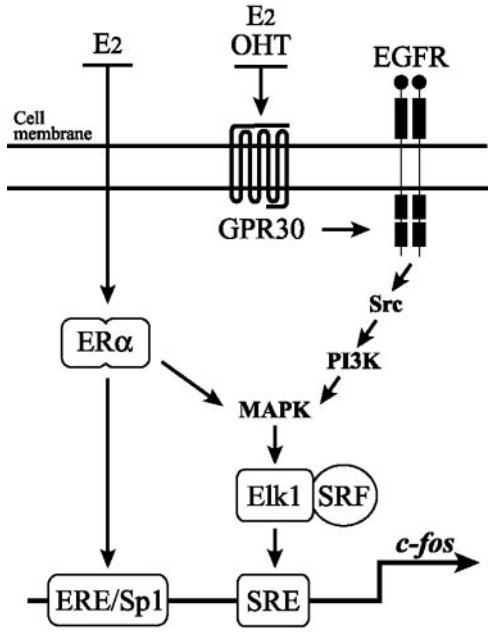

Fig. 11. Model of the Potential Signal Transduction Pathways Triggered by E2 and OHT for c-*fos* Expression in Endometrial Cancer Cells

SRF, Serum-response factor.

Luciferase activity was measured with the dual luciferase kit (Promega Corp.) 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 at 1-fold induction, upon which the activity induced by treatments was calculated.

RT-PCR

The evaluation of gene expression was performed by semiquantitative RT-PCR as we have previously described (94). For c-*fos*, pS2, PR, and the acidic ribosomal phosphoprotein P0 (36B4), which was used as a control gene, the primers were, respectively, 5′-AGAAAAGGAGAATCCGAAGGGAAA-3′ (c-*fos* forward), 5′-ATGATGCTGGGACAGGAAGTC-3′ (c-*fos* reverse), 5′-TTCTATCCTAATACCATCGACG-3′ (pS2 forward), 5′-TTTGAGTAGTCAAAGTCAGAGC-3′ (pS2 reverse), 5′-TAGTGAGGGGGCAGTGGAAC-3′ (PR forward), 5′-AGGAGGGGGTTTCGGGAATA-3′ (PR reverse), 5′-CTCAACATCTCCCCCTTCTC-3′ (36B4 forward), and 5′-CAAATCCCATATCCTCGTCC-3′ (36B4 reverse), to yield products of 420, 210, 442, and 408 bp, respectively, with 20, 15, 20, and 15 PCR cycles.

Western Blotting

Cells were grown in 10-cm dishes, exposed to ligands, and then lysed in 500 μl 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% sodium dodecyl sulfate, and a mixture of protease inhibitors containing 1 mM aprotinin, 20 mM phenylmethylsulfonylfluoride, and 200 mM sodium orthovanadate. The protein concentration was determined using Bradford reagent according to the manufacturer's recommendations (Sigma-Aldrich Corp.). Equal amounts of whole protein extract were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences, Arlington Heights, IL), probed overnight at 4 C with the antibodies against ERα (F-10 and D-12), c-*fos* (D-1), PR (B-30), β-actin (all purchased from Santa Cruz Biotechnology, Inc., Milan, Italy), ERβ and GPR30 (Serotec and Lifespan Biosciences, respectively, Milan, Italy), pERK1/2 and ERK2 (Cell Signaling Technology, Inc., and Cellbio, Milan, Italy) and then revealed using the enhanced chemiluminescence system (Amersham Biosciences). Five micrograms of DN/ERK expression plasmid was transfected using FuGene 6 reagent as recommended by the manufacturer (Roche) for 24 h before treatments.

AS-ODN Experiments

AS-ODNs were purchased from MWG (Florence, Italy) and synthesized as previously described (95, 96). The ODNs used were 5′-TTGGGAAGTCACATCCAT-3′ for GPR30 and 5′-GATCTCAGCACGGCAAAT-3′ for the scrambled control. For AS experiments, a concentration of 200 nM of the indicated ODNs was transfected using FuGene 6 reagent as recommended by the manufacturer (Roche) for 4 h, before treatment with ligands.

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 d (with treatments), and cells were trypsinized and counted in a hemocytometer. Two hundred nanograms of GPR30/AS or 200 ng empty vector was transfected using FuGene 6 reagent as recommended by the manufacturer (Roche) every 2 d.

Statistical Analysis

Statistical analysis was performed using ANOVA, followed by Newman-Keuls testing to determine differences in means. $P < 0.05$ was considered statistically significant.

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