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The Novel Estrogen Receptor, G Protein-Coupled Receptor 30, Mediates the Proliferative Effects Induced by 17β-Estradiol on Mouse Spermatogonial GC-1 Cell Line

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Many studies have indicated that estrogens could have a role in the regulation of testicular function. However, it remains uncertain whether estrogens are able to directly activate signaling pathways in male germ cells. Estrogens are synthesized by the enzyme aromatase and classically act by binding to estrogen receptors (ERs)-α and ERβ. Knockout mice for both receptor isoforms exhibit a testicular phenotype that is less severe than aromatase knockout mice, suggesting the existence of an estrogen-binding receptor that may compensate for the lack of ERs. Recently studies using estrogen-sensitive tumor cell lines have demonstrated that the G-protein-coupled receptor (GPR)-30 binds and mediates estrogen action through the activation of the epidermal growth factor receptor (EGFR)/ERK/fos transduction pathway. The present study investigated the ability of 17β-estradiol (E2) to activate this pathway in the mouse spermatogonial cell line (GC-1). Using the GC-1 cell line as a model system, we demonstrated that GC-1 cells express GPR30 and ERα but not ERβ. E2, the selective GPR30 agonist G1, and the selective ERα agonist 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol activated the rapid ERK1/2-fos signaling cascade. This response was abrogated by the EGFR inhibitor AG1478, ERK inhibitor PD98059 and ER inhibitor ICI 182780, or by silencing GPR30 expression. Moreover, E2 and G1 up-regulated cyclin D1 expression and GC-1 cell proliferation. Our results indicate for the first time that estrogens, through a cross talk between GPR30 and ERα, activate the rapid EGFR/ERK/fos pathway, which in turn stimulate mouse GC-1 cell proliferation. Further studies to elucidate the involvement of rapid estrogen signaling pathways in the regulation of male fertility are warranted. (*Endocrinology* 149: 5043–5051, 2008)

ESTROGEN IS THOUGHT to have a regulatory role in the testis because the absence of estrogen receptors (ERs) causes adverse effects on spermatogenesis and steroidogenesis (1–3). Moreover, several chemicals present in the environment have been designated xenoestrogens due to their ability to bind and activate ERs and have also been shown to affect testicular gene expression (1–4). However, the mechanisms by which estrogens influence male fertility and spermatogenesis remain uncertain.

The biological effects of estrogens are mediated by ERα and ERβ, which are widely distributed in the male reproductive tract (5–8). ERα immunostaining has been detected in Leydig and peritubular myoid cells but not in Sertoli or germ cells (3, 5, 7, 8). However, other studies have reported different results (10–12). ERβ expression has been detected in multiple cell types, including Sertoli cells, and in some but not all germ cells (13, 14). Whereas it is generally agreed that both ER isoforms are expressed by the epithelial cells of the efferent ductules and epididymis, there are conflicting reports in the literature regarding their localization in the testis possibly due to the specificity of the antisera used in immunocytochemistry experiments (15).

Information on estrogen effects on testis physiology can be derived from mice with single and combined deletion of ERα and ERβ genes (αERKO, βERKO, and αβERKO) (16) or from the aromatase-deficient mouse (ArKO) (17). ERα −/− animals have reduced fertility because of abnormal fluid reabsorption in the efferent ductules (2), whereas spermatogenesis, steroidogenesis and fertility are not affected in βERKO animals (16). On the other hand, ArKO mice show an age-dependent disruption of spermatogenesis, a significant reduction in testis weight and compromised fertility (18, 19). The late onset of the altered phenotype in male ArKO mice may be attributable to the action of estrogenic compounds that are present in the diet and capable of agonistic effects on spermatogenesis (20). It has also been reported that estrogen influences Sertoli cell proliferation and suppress differentiation (15); however, no alterations were observed in the number of Sertoli cells and spermatogonia in the αERKO mouse (16). Moreover, ERβ inactivation increases the number of spermatogonia by more than 50% in neonatal mice (21).

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Abbreviations: AP-1, Activator protein-1; ArKO, aromatase-deficient mouse; Ct, cycle threshold; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E2, 17β-estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERKO, mice with deletion of ER; FBS, fetal bovine serum; GPR-30, G-protein-coupled receptor 30; ICI, ICI 182780; pen/strep, penicillin/streptomycin; PPT, 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol.

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Experiments using spermatogonia from αERKO mice transplanted into wild-type animals demonstrated that Sertoli cells, but not germ cells, require ERα to sustain the process of spermatogenesis (22, 23). Interestingly, studies using an organ culture system have revealed that Sertoli cells do not mediate the effects of estrogens on rat gonocyte development (24), thus supporting the hypothesis of a direct action of estrogens on germ cells.

Given the amount of data reported in ERKO mice, it is still debatable whether the potential effects of estrogen on spermatogenesis may be mediated by alternative receptors. This observation is supported by the fact that aromatase knockout mice show a more severe testicular phenotype than the ER knockouts (17). In this regard, a large body of evidence has demonstrated that estrogens can function not only through the classic genomic mechanism mediated by ERα and ERβ but also can trigger rapid responses that involve transduction pathways different from those activated by ERs (25). Recently our and other studies identified a transmembrane estrogen-binding protein, the G protein-coupled receptor-30 (GPR-30), which is able to mediate estrogen action (26–29). The discovery of GPR30 has generated a great deal of interest toward the identification of unknown functions and mechanisms triggered by estrogen outside the nucleus. GPR30 is a possible candidate for rapid estrogen signaling based on the observations that it mediates ERK activation and c-*fos* expression in an ER-independent manner (28–30). Recently GPR30 has been identified in a variety of human and rodent estrogen target tissues (31–34) suggesting the potential of an ER-independent mechanisms in 17β-estradiol (E2) action in these contexts.

Several studies suggested that estrogens induce spermatogonial proliferation through ERK1/2 and c-*fos* protein activation (35–37). The molecular mechanisms regulating these processes in rodent spermatogonia are not well defined. c-*fos* could play an important role in regulating genes involved in germ cell progression because homozygous mice with c-*fos* null mutation show delayed or absent gametogenesis (38).

In the present study, we show that mouse spermatogonia and GC-1 cells express GPR30. In GC-1 cells both E2 and the specific GPR30 agonist G1 trigger ERK activation, c-*fos* stimulation, and the transcription of the cell cycle regulatory gene cyclin D1. Our data demonstrate for the first time that GPR30 mediates the physiological action of estrogens in the mouse spermatogonia GC-1 cell line and represent a step toward the comprehension of the role of E2 in spermatogenesis.

Materials and Methods

Cell cultures and treatments

GC-1 cells (mouse spermatogonia type B cell line; American Type Culture Collection, Manassas, VA) were cultured in DMEM/F-12 growth medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin (pen/strep) (Invitrogen, S.R.L., San Giuliano Milanese, Italy). Cells were maintained in growth medium for 48 h and then starved for 24 h using DMEM/F-12 medium supplemented 1% pen/strep before being treated. For RNA and protein extraction cells were cultured in 60-mm dishes (1×10^6 cells) and for cell proliferation in 12-well plates (2×10^5 cells). TM3 cells (immature mouse Leydig cell lines; American Type Culture Collection) were cultured in DMEM/Ham's F12 (Invitrogen) supplemented with 5% horse serum, 2.5% FBS, 1% glutamine, 1% pen/strep (Collaborative Biomedical, Bedford, MA), and antibiotics in a 24-well plate. Treatments were performed at different times using: ICI 182780 (ICI; Tocris Bioscience, Ellisville, MO), PD98059 (Sigma, St. Louis, MO), AG1478 (Sigma), E2 (Sigma), G1 (Merck KGaA, Frankfurt, Germany), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) (Tocris Bioscience), and 4,4′,4″-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol (PPT) (Tocris Bioscience).

Plasmid preparation and cell transfection

The mouse GPR30 coding sequence was amplified by RT-PCR. Gene-specific primers, based on the published GPR30 sequence (GenBank accession no. NM_029771) were: forward, 5′-GGA GCT GTC ACA TAA AAC AGC-3′ and reverse, 5′-GCT AGG TGT GTG CAA GTC CT-3′. PCR conditions were: 1 min at 94 C, 1 min at 52 C, and 2 min at 72 C for 30 cycles. The expected 1246-bp fragment was first cloned into pCRII-TOPO TA cloning vector (Invitrogen) and then excised using *Hin*dIII and *Xho*I and cloned into *Xho*I/*Hin*dIII sites of pcDNA3.1/zeo (Invitrogen). The correct orientation of the insert was confirmed by sequencing. HEK293 cells (American Type Culture Collection) were maintained in DMEM/F-12 containing 5% FBS and 1% pen/strep. Cells were transfected with 1 μg of GPR30 expression vector using Lipofectamine LTX (Invitrogen) in a 1:3 ratio according to the manufacturer's instructions. Cells were lysed 24 h after transfection for protein extraction and used for Western blot analysis.

Immunohistochemical analysis

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded testes dissected from C57BL/6 mice at 12 wk of age. Testes were surgically removed by qualified, specialized animal care staff in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health) and used for experiments. Tissues were heated for antigen retrieval. Paraffin-embedded sections, 5 μm thick, were mounted on slides precoated with poly lysine and then deparaffinized and dehydrated (seven to eight serial sections). After antigen retrieval and endogenous peroxidase removal, using deparaffinized Biotin blocking system (Dako Corp., Carpinteria CA), endogenous biotin was blocked by adding avidin solution. Immunodetection was carried out using anti-GPR30 (1:50) primary antibody (MBL International Corp., Woburn, MA) at 4 C overnight. GPR30 was visualized with streptavidin-biotin-peroxidase complex (LSAB+ kit peroxidase; Dako) to reveal antibody-antigen reactions. All slides were counterstained with hematoxylin. The primary antibody was replaced by BSA in negative control sections.

Western blot analysis

After treatments GC-1 cells were lysed in ice-cold lysis buffer containing protease inhibitors (20 mM Tris, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, and 10 μM leupeptin) for protein extraction. Lysates were sonicated for 2 min with an ultrasonic bath in iced water (Progen Scientific Ltd., Heidelberg, Germany) and then centrifuged at 13,000 rpm for 10 min at 4 C. The protein content was determined by the Bradford method. The proteins were separated on 11% sodium dodecyl sulfate/polyacrylamide gels and then electroblotted onto a nitrocellulose membrane. Blots were incubated overnight at 4 C with the following: 1) anti-GPR30 polyclonal antibody (1:1000) (MBL International); 2) anti-ERα (F-10) antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA); 3) anti-ERβ antibody (1:500) (Serotec, Oxford, UK) and anti-ERβ (H-150) antibody (1:1000) (Santa Cruz Biotechnology); 4) anti-ERK antibody (1:1000) (Cell Signaling Technology, Celbio, Milan, Italy); 5) anti-phosphorylated ERK antibody (1:1000) (Cell Signaling Technology); 6) anti-cyclin D1 (M-20) antibody (1:1000) (Santa Cruz Biotechnology); and 7) anti-β-tubulin antibody (1:1000) (Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ), and immunoreactive bands were visualized with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences, Cologno Monzese, Italy). To assure equal loading of proteins, membranes were stripped and incubated overnight with β-tubulin antibody.

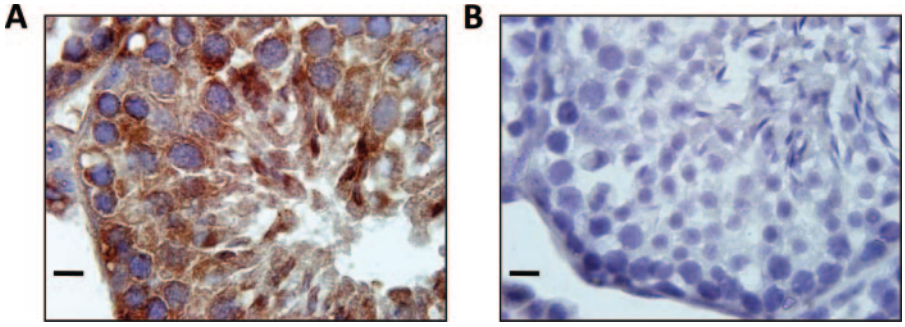

FIG. 1. Immunolocalization of GPR30 in the mouse testis. A, Immunodetection of GPR30. B, Negative control. *Scale bars*, 5 μm.

RNA extraction and cDNA synthesis

Before experiments, cells were maintained overnight in serum-free medium without phenol red. Cells were then treated for 1 or 24 h, and RNA was extracted using the TRizol RNA isolation system (Invitrogen). Each RNA sample was treated with DNase I (Ambion, Austin, TX), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One microgram of total RNA was reverse transcribed in a final volume of 30 μl using the ImProm-II reverse transcriptase system kit (Promega Italia S.R.L., Milano, Italy). cDNAs were directly used for PCR or diluted 1:3 in nuclease-free water to be used for real-time PCR. Samples were aliquoted and stored at −20 C.

PCR and real-time PCR

Two microliters of cDNAs were used for PCR using gene-specific primers. For ERα, the PCR conditions were 1 min at 94 C, 1 min at 52 C, and 2 min at 72 C for 35 cycles using the following primers: forward, 5′-AAT TCT GAC AAT CGA CGC CAG-3′ and reverse, 5′-GTG CTT CAA CAT TCT CCC TCC TC-3′ (GenBank accession no. NM_007956) generating a 344-bp product. For ERβ, the PCR conditions were 1 min at 94 C, 1 min at 56 C, and 2 min at 72 C for 35 cycles using the following primers: forward, 5′-GAG GGG AAG TGC GTG GAA GG-3′ and reverse, 5′-CCC GAG ATT GAG GAC TTG TAC CC-3′ (GenBank accession no. NM_207707), generating a 430-bp product. For GPR30, the PCR conditions were 1 min at 94 C, 1 min at 52 C, and 2 min at 72 C for 35 cycles using the following primers: forward, 5′-TGG TGG TGA ACA TCA GCT TC-3′ and reverse, 5′-AAG CTC ATC CAG GTG AGG AA-3′ (GenBank accession no. NM_029771), generating a 217-bp product. For glyceraldehyde-3-phosphate dehydrogenase, the PCR conditions were 1 min at 94 C, 1 min at 55 C, and 2 min at 72 C for 20 cycles using the following primers: forward, 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse, 5′-TCC ACC ACC CTG TTG CTG TA-3′ (GenBank accession no. NM_008084), generating a 452-bp product. PCR products were analyzed on a 1% agarose gel and visualized by ethidium bromide staining.

For real-time RT-PCR, primers for the amplification were based on published sequences for the mouse c-*fos* and cyclin D1 genes. The nucleotide sequences of the primers for fos were: forward, 5′-GAG GAG GGA GCT GAC AGA TAC ACT-3′ and reverse, 5′-GAT TGG CAA TCT CAG TCT GCA A-3′. For cyclinD1 amplification, the following primers were used: forward, 5′-TCC GCA AGC ATG CAC AGA-3′ and reverse, 5′-GGT GGG TTG GAA ATG AAC TTC A-3′. PCRs were performed in the iCycler iQ detection system (Bio-Rad, Hercules, CA), using 0.1 μM of each primer, in a total volume of 30 μl following the manufacturer's recommendations. SYBR Green universal PCR master mix (Bio-Rad) with the dissociation protocol was used for gene amplification, and negative controls contained water instead of first-strand cDNA. Each sample was normalized to its 18S rRNA content. The 18S quantification was performed using a TaqMan rRNA reagent kit (Applied Biosystems, Applera Italia, Monza, Milano, Italy) following the method provided in the TaqMan rRNA control reagent kit (Applied Biosystems). The relative gene expression levels were normalized to a calibrator that was chosen to be the basal, untreated sample. Final results were expressed as n-fold differences in gene expression relative to 18S rRNA and calibrator, calculated following the ΔΔCt method as follows: n-fold = $2^{-(\Delta Ctsample - \Delta Ctcalibrator)}$. The ΔCt values of the sample and calibrator were determined by subtracting the average cycle threshold (Ct) value of the 18S rRNA reference gene from the average Ct value of the different genes analyzed.

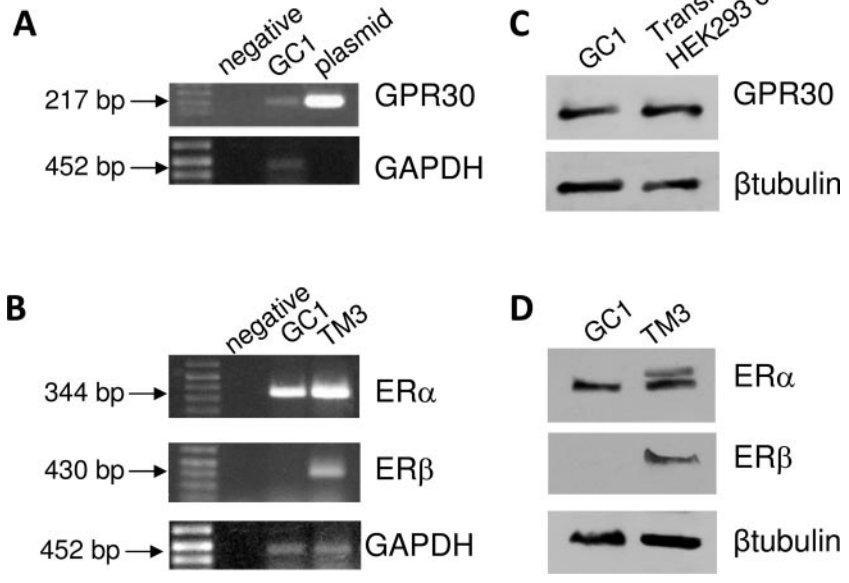

FIG. 2. Expression of GPR30 and ERs in GC-1 cells. A, RT-PCR analysis of GPR30 in GC-1 cells. A plasmid containing the mouse GPR30 coding sequence was used as positive control in the PCR. B, RT-PCR analysis of mRNA levels of ERα and ERβ in mouse spermatogonia GC-1 cells (GC-1). TM3 mouse Leydig cells were used as positive control (TM3). Negative control was obtained by using water instead of cDNA in the PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The size of the amplified fragments are indicated. C and D, Fifty micrograms of total protein were used for Western blot analyses. C, GPR30 protein expression was analyzed in GC-1 cells and HEK293 cells transfected with a GPR30 expression vector used as positive control. D, ERα and ERβ protein expression was analyzed in GC-1 cells using TM3 cells as positive control. β-Tubulin was used as a loading control. RT-PCR and blots are representative of three independent experiments with similar results.

Cell proliferation

For the determination of cell proliferation treatments were performed for 48 h in serum-free medium without phenol red. Control cells were treated with the same amount of vehicle alone (dimethylsulfoxide), which never exceeded the concentration of 0.01% (vol/vol). [^{3}H]thymidine incorporation was evaluated after a 6-h incubation period with 1 μCi [^{3}H]thymidine per well (PerkinElmer Life Sciences, Boston, MA). Cells were washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and lysed in 1 ml 0.1 M NaOH at 37 C for 30 min. The total suspension was added to 10 ml optifluor fluid and was counted in a scintillation counter.

Antisense oligodeoxynucleotide experiments

Antisense oligonucleotides were purchased from MWG (Florence, Italy). The oligonucleotides used were: 5′-TGG AGT AGT CGC ATC CAT-3′ for GPR30 and 5′-GAT CTC AGC ACG GCA AAT-3′ for the scrambled control (26). A concentration of 200 nM of the indicated oligonucleotides was transfected using Lipofectamine LTX (Invitrogen) in a 1:3 ratio according to manufacturer's instructions. Cells were maintained in medium containing the transfection mix for 36 h before being treated for 5 min and then lysed and used for Western analysis. For proliferation experiments cells were maintained in medium containing the transfection mix for 24 h; medium was then replaced and cells treated for 48 h before being incubated with [^{3}H]thymidine and assayed for cell proliferation.

Data analysis and statistical methods

Results from triplicate experiments were analyzed by Student *t* tests or one-way ANOVA followed by Student-Newman-Keuls *post hoc* test as appropriate, using SigmaStat version 3.0 (SPSS, Chicago, IL). Specifically, Student's *t* test was used for comparisons between two groups, giving *t* and *P* values; ANOVA was used for comparisons between more than two groups and gave *F* and *P* values.

Results

Immunolocalization of GPR30 in mouse testis

Immunohistochemical analyses revealed a strong GPR30 immunoreactivity in the cytoplasm of germ cells in the mouse testes (Fig. 1A), whereas negative control testes showed immunonegative reaction for GPR30 (Fig. 1B).

Expression of GPR30 and ERs in GC-1 cells

We used RT-PCR and Western analysis to investigate the expression of GPR30 as well as the ERs in the GC-1 mouse germ cell line. Analysis of mRNA revealed that GC1 cells express GPR30 (Fig. 2A) and ERα but not ERβ (Fig. 2B). We then confirmed these results at the protein level. Western analyses revealed the expected 42-kDa band for GPR30 protein (Fig. 2C) and a 67-kDa band for ERα (Fig. 2D), whereas no signals were detected using different specific antibodies against the ERβ isoform (Fig. 2D).

E2 and the specific GPR30 agonist G1 induce c-fos mRNA expression in mouse GC-1 cells

GPR30 mediates rapid estrogen signaling leading to the induction of the activator protein-1 (AP-1) family member c-*fos*. For this reason, we investigated the effects of E2 and selective activators of ERα (PPT), ERβ (DPN) and GPR30 (G1) on c-*fos* mRNA expression using quantitative real-time RT-PCR. A 1-h treatment produced a significant induction in c-*fos* mRNA expression in the presence of E2 (100 nM) ($t_{10} = 19.012$, $P < 0.001$), PPT (1 μM) ($t_{10} = 57.625$, $P < 0.001$), and G1 (1 μM) ($t_{10} = 16.725$, $P < 0.001$) (Fig. 3A). As expected, DPN (1 μM) did not induce

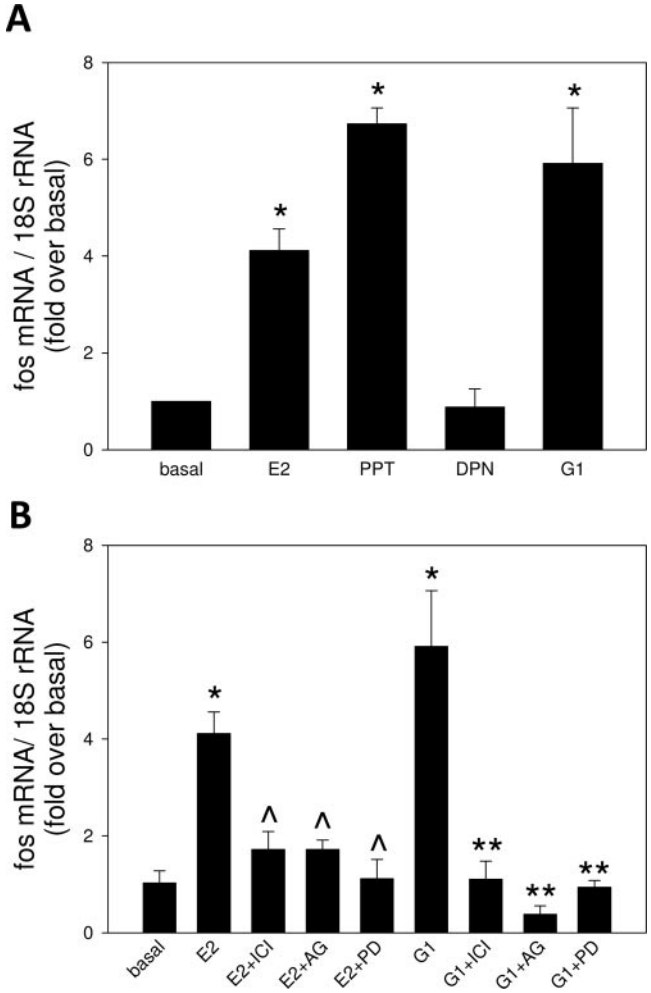

FIG. 3. Effects of ERs and GPR30 activators and inhibitors on c-*fos* mRNA. Cells were treated in serum-free medium for 1 h with E2 (100 nM) PPT (1 μM), DPN (1 μM), or G1 (1 μM) (A) or with E2 (100 nM) or G1 (1 μM) combined with ICI (10 μM), AG1478 (AG) (10 μM), or PD98059 (PD) (10 μM) (B). Total RNA was extracted, and real-time RT-PCR was used to analyze c-*fos* mRNA levels. *Columns*, mean of values from three separate RNA samples; *bars*, SE. Each sample was normalized to its 18S rRNA content. *, $P < 0.01$, compared with basal; ^, $P < 0.001$, compared with E2; **, $P < 0.001$, compared with G1.

c-*fos* expression, confirming that in GC-1 cells, ERβ is not active ($t_{10} = 0.786$, $P = 0.45$).

c-*fos* induction via GPR30 occurs after activation of epidermal growth factor receptor (EGFR) and MAPKs. Therefore, we tested the effects of inhibitors for EGFR (AG1478) and mitogen-activated ERK-activating protein kinase (MEK) (PD98059) on G1- and E2-dependent c-*fos* activation (Fig. 3B). To clarify the role of ERα, we pretreated GC-1 cells with the pure ER antagonist ICI before exposing cells to estradiol or G1. All of the tested inhibitors were able to significantly inhibit E2 [F (3, 20) = 94.05, $P < 0.001$] and G1 [F (3, 20) = 393.55, $P < 0.001$] induction of c-*fos* (Fig. 3B).

Rapid activation of ERK1/2 by E2 and G1 in mouse GC-1 cells

Because PD98059, an inhibitor of MAPK, was able to prevent c-*fos* induction by both E2 and G1, we investigated the

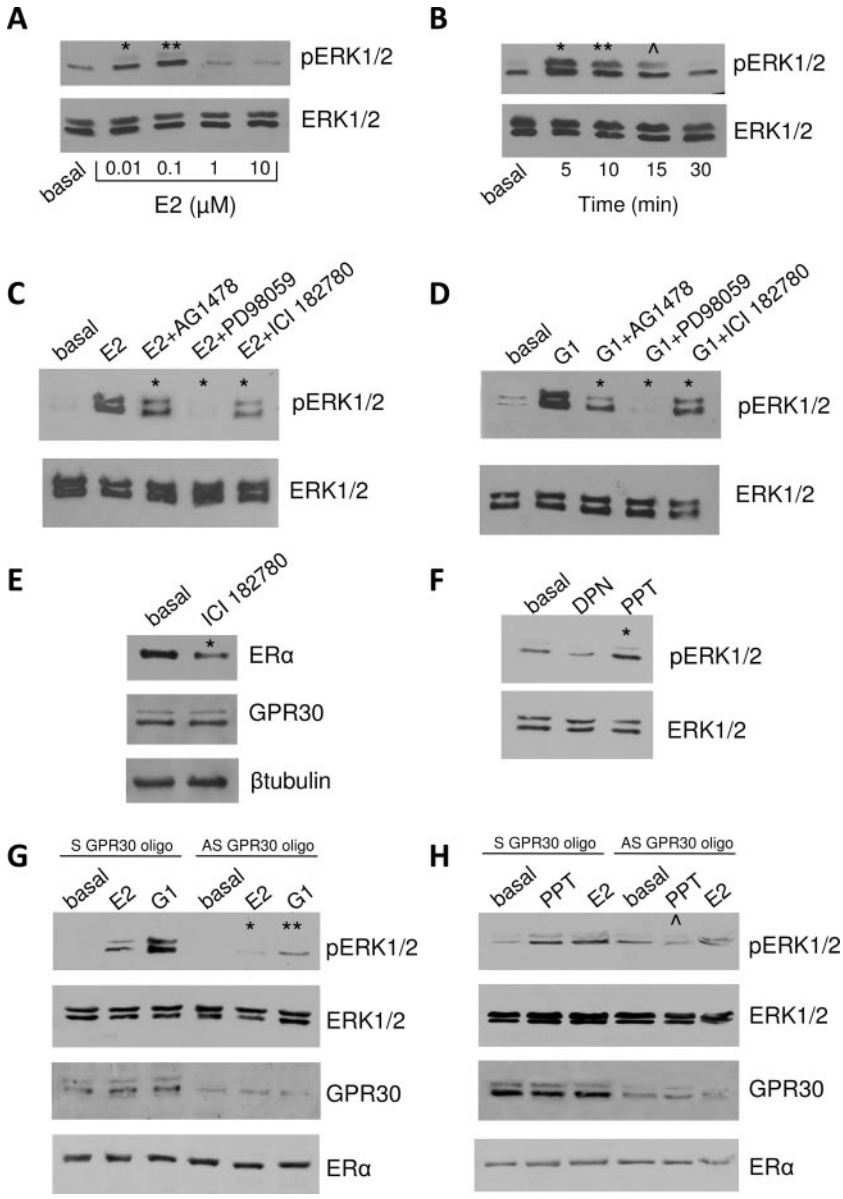

FIG. 4. Effects of ERs and GPR30 activators and inhibitors on ERK activation. A, Cells were treated for 15 min with the indicated concentrations of E2. *, $P < 0.001$ ($t_4 = 20.889$); **, $P < 0.001$ ($t_4 = 13.665$), compared with basal. B, Cells were treated with the indicated times with 100 nM of E2. *, $P < 0.001$ ($t_4 = 38.265$); **, $P < 0.001$ ($t_4 = 29.289$); ^, $P < 0.001$ ($t_4 = 12.512$), compared with basal. C, Cells were incubated for 30 min with AG1478 (10 μM) or PD98059 (10 μM) or ICI (10 μM) before being stimulated for 5 min with E2 (100 nM). *, $P < 0.01$ [F (3, 8)= 116.52], compared with E2. D, Cells were incubated for 30 min with AG1478 (10 μM) or PD98059 (10 μM) or ICI (10 μM) before being stimulated for 5 min with G1(1 μM). *, $P < 0.01$ [F (3, 8) = 288.02], compared with G1. E, Cells were untreated or treated for 30 min with ICI (10 μM). *, $P < 0.001$ ($t_4 = 9.798$). F, Cells were treated for 5 min with DPN (1 μM) or PPT (1 μM). *, $P < 0.001$ ($t_4 = 12.082$). G and H, Cells were transfected for 36 h with either scrambled (S) or antisense (AS) GPR30 oligos before being stimulated for 5 min with E2 (100 nM), G1 (1 μM), or PPT (1 μM). Antisense oligonucleotide blocked E2 effects [*, $P < 0.001$ ($t_4 = 20.208$)]; G1 [**, $P < 0.001$ ($t_4 = 29.918$)]; and PPT [^, $P < 0.001$ ($t_4 = 25.628$)]. Western blot analyses were performed on 50 μg of total proteins extracted from GC-1 cells untreated (basal) or treated as indicated. Blots are representative of three independent experiments with similar results. Statistical analysis was performed on densitometric values obtained from the three blots.

ability of these agonists to activate MAPKs p42/44 MAPK, also called ERK1/2, by evaluating the phosphorylation status. Using increasing doses of E2 (0.01–10 μM) for 15 min, we observed that only low doses of E2, with 100 nM being the most effective, were able to induce ERK phosphorylation (Fig. 4A). We then investigated the kinetics of ERK activation by E2 (100 nM) and found that induction occurs rapidly with a peak at 5 min (Fig. 4B). E2-induced activation was completely abolished in the presence of PD98059, whereas AG1478 and ICI reduced but did not abolish this activation (Fig. 4C). A very similar activation and inhibition pattern was observed after 5 min treatment with G1 (1 μM), used either alone or in combination with inhibitors (Fig. 4D). Importantly, all the effects seen in the presence of ICI occurred as a consequence of a reduction in ERα protein levels, without influencing GPR30 expression (Fig. 4E); exposure to ICI for 30 min led to a significant reduction in ERα expression but not GPR30 (Fig. 4E). DPN (1 μM) did not affect ERK activation status, whereas PPT (1 μM) induced ERK phosphorylation (Fig. 4F).

The role of GPR30 in mediating ERK activation was investigated by reducing GPR30 expression using an antisense oligonucleotide (Fig. 4, G and H). Whereas cells transfected with a scrambled oligo still responded to E2, G1, and PPT by inducing ERK phosphorylation, the antisense oligonucleotide blocked the effects of these agonists. The specificity of the antisense oligonucleotide effect was investigated by analyzing the expression of GPR30 and ERα on the same blots. As shown in Fig. 4, G an and H, the GPR30 protein signals disappeared, whereas there was no change in ERα expression levels.

E2 and G1 induce the expression of cyclin D1 and mouse GC-1 cell proliferation

To determine the physiological role of estrogen-dependent GPR30 activation, we evaluated whether the E2-dependent

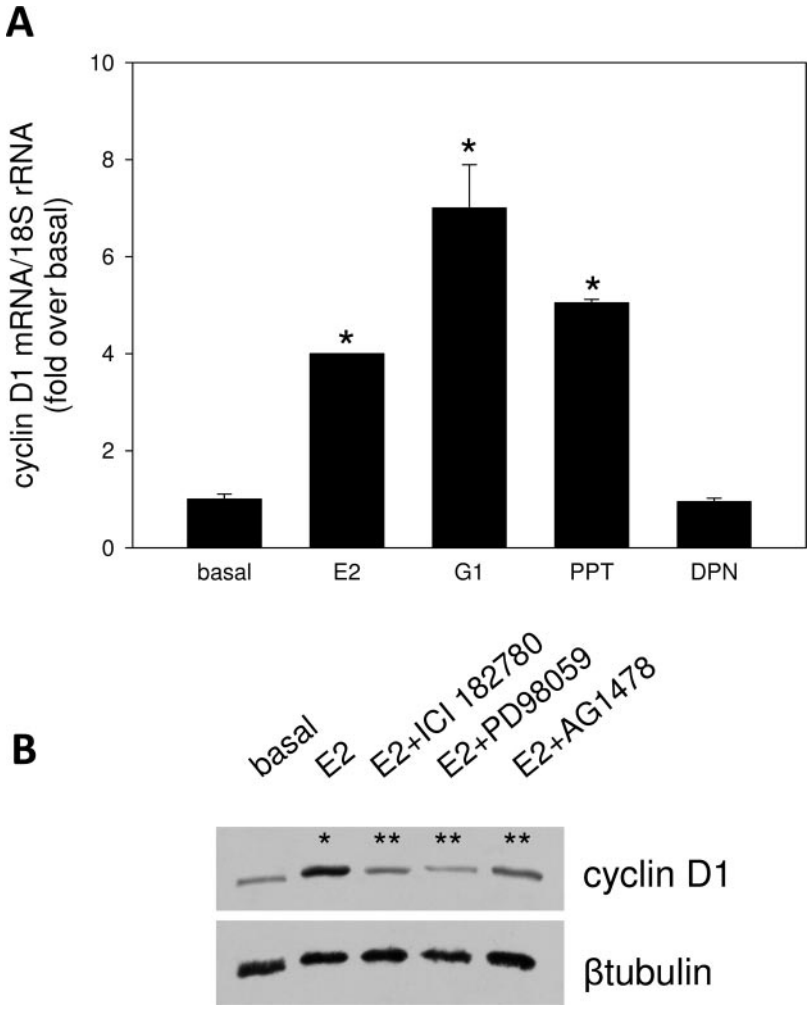

FIG. 5. Activation of ERα and GPR30 induces cell regulatory cyclin D1 expression in GC-1 cells. A, Cells were treated in serum-free medium for 24 h with E2 (100 nM), G1 (1 μM), PPT (1 μM), or DPN (1 μM). Total RNA was extracted, and real-time RT-PCR was used to analyze cyclin D1 mRNA levels. *Columns*, mean of values from three separate RNA samples; *bars*, SE. Each sample was normalized to its 18S rRNA content. *, $P < 0.001$, compared with basal. B, Western blot analyses were performed on 50 μg of total proteins extracted from GC-1 cells untreated (basal) or treated for 24 h with E2 (100 nM) alone or combined with ICI (10 μM), AG1478 (10 μM), or PD98059 (10 μM). Blots are representative of three independent experiments with similar results. β-Tubulin was used as a loading control. *, $P < 0.001$ ($t_4 = 8.660$), compared with basal; **, $P < 0.001$ [F (3, 8) = 55.89], compared with E2.

effects observed in GC-1 cells could lead to a change in the ability of cells to proliferate. We first examined changes in E2-regulated cell cycle genes, such as cyclin D1, by quantifying both mRNA and protein levels. Cells were treated for 24 h with E2; G1; and the selective ERα and ERβ agonists, PPT and DPN. All of these activators except DPN induced a significant increase in cyclin D1 mRNA (E2: $t_{10} = 73.632$, $P < 0.001$; G1: $t_{10} = 23.738$, $P < 0.001$; PPT: $t_{10} = 44.062$, $P < 0.001$; DPN: $t_{10} = 1.266$, $P = 0.234$) (Fig. 5A). When cells were treated for 24 h with E2 alone or combined with the inhibitors ICI, AG1478, and PD98059, we found that E2 induced both cyclin D1 mRNA and protein levels ($t_4 = 8.660$, $P < 0.001$). All the tested inhibitors reduced the E2 effects [F (3, 8) = 55.89 $P < 0.001$] (Fig. 5B).

Based on these observations, we directly evaluated the ability of GC-1 cells to proliferate in response to ERs and GPR30 activation using the thymidine incorporation method. E2 and G1 significantly induced GC-1 proliferation (E2: $t_{10} = 14.478$, $P < 0.001$; G1: $t_{10} = 59.063$, $P < 0.001$). The inhibitors ICI, AG1478, and PD98059 reduced basal GC-1 proliferation (ICI: $t_{10} = 29.122$, $P < 0.001$; AG: $t_{10} = 28.814$, $P < 0.001$; PD: $t_{10} = 28.814$, $P < 0.001$) and also decreased, E2- [F (3, 20) = 66.89, $P < 0.001$] and G1-induced proliferation [F (3, 20) = 1177.33, $P < 0.001$] (Fig. 6A). In addition, when GPR30 expression was blocked by a GPR30 antisense oligonucleotide, E2 no longer induced proliferation (scrambled GPR30+E2 *vs.* scrambled GPR30: $t_{16} = 29.447$, $P < 0.001$; antisense GPR30 + E2 *vs.* antisense GPR30: $t_{16} = 0.212$, $P = 0.63$) (Fig. 6B).

Discussion

In the present study, we investigated the ability of E2 to activate rapid responses leading to mouse GC-1 cell proliferation through the novel estrogen receptor GPR30. Immunohistochemistry in adult mouse testes clearly showed GPR30 expression in germ cells. Because primary cultures of pure adult mouse spermatogonia are particularly difficult to obtain, we used the GC-1 cell line as an experimental model (40).

GC-1 cells display specific features common to type B spermatogonia and early spermatocytes. These include: 1) nuclei that contain large clumps of heterochromatin, similar in appearance to type B spermatogonia and leptotene spermatocytes (41); 2) a prominent Golgi apparatus similar to that in the preleptotene spermatocytes (42); 3) occasional appearance of vacuolated mitochondria, typical of early to late pachytene spermatocytes and early spermatids (42, 43); and

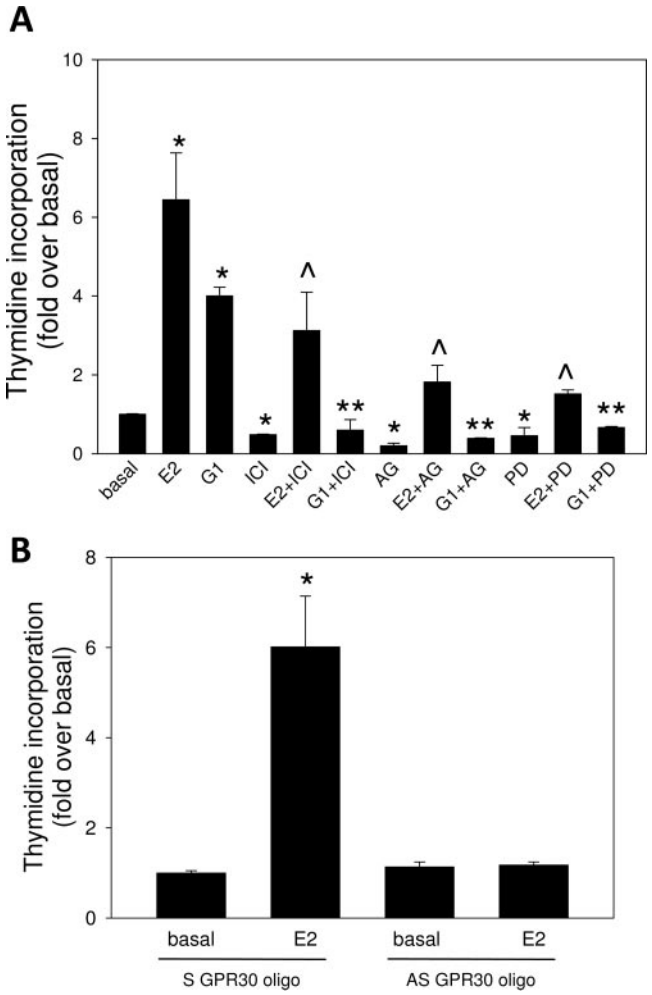

FIG. 6. Effects of ERα and GPR30 activation or inhibition on GC-1 cell proliferation. A, Cells were maintained for 24 h in serum-free medium and then treated for 48 h as indicated. Where antagonists were used in combination, they were added 30 min before stimuli: E2 100 nM, G1 1 μM, ICI 10 μM, AG1478 (AG) 10 μM, PD98059 (PD) 10 μM. B, Cells were transfected with either scrambled (S) or antisense (AS) GPR30 oligos (200 nM) in medium only; the following day without removing the medium, cells were stimulated with 100 nM E2, and treatment was left for 48 h. Proliferation was evaluated by [^{3}H]thymidine incorporation analysis. *Columns*, Mean of fold over untreated (basal) cells, are the average of three independent experiments each done in triplicate; *bars*, SE. *, $P < 0.001$, compared with basal; ^, $P < 0.001$, compared with E2; **, $P < 0.001$, compared with G1.

4) no expression of phosphoglycerate kinase, normally expressed at the secondary spermatocytes stage of differentiation (40). In addition, GC-1 cells contain features common to early spermatogonia such as multiple nucleoli and mitochondria with parallel cristae perpendicular to the long axis (41, 44). These characteristics correspond to those of the predominant germ cell types in the 10-d-old prepuberal mouse testis (41) used as a source of primary cells for immortalization. The use of the GC-1 cell line expressing GPR30 is a valid first approach to investigate the role played by GPR30 in a rapid estrogen-activated pathway in premeiotic germ cells among which spermatogonia represent the most important cell population. It remains to be determined whether estrogens can play a role in spermatogonia proliferation through a rapidly activated pathway.

Previous reports have shown that E2 is able to induce spermatogonia proliferation in *Rana esculenta* and *Podarcis sicula* through ERK activation (31, 33). In addition, in *R esculenta* E2 induces fos protein activation, which determines spermatogonia proliferation (32). The involvement of AP-1 family members in germ cell development has also been demonstrated in the mouse. In particular, the protooncogenes c-*fos*, c-jun, and c-*myc* are highly expressed in type B spermatogonia (45). Homozygous mice with a c-*fos* null mutation, among other abnormalities, show delayed or absent gametogenesis, confirming the important role exerted by c-*fos* in regulating spermatogenesis (38).

Despite data from ERKO and ArKO mice, the potential effects of estrogen on spermatogenesis mediated through alternative receptors is still unknown.

The discovery of a novel receptor mediating estrogen-dependent pathways in germ cells, potentially able to compensate for the lack of ERs, may provide an explanation for the differences between the more severe testicular phenotype observed in ArKO mice, compared with ERKO mice. These findings open new perspectives toward the evaluation of the role of estrogens in testis development and function.

For the first time, we have demonstrated that mouse testis express GPR30. Our data support the hypothesis that GPR30 could represent the alternative receptor through which estrogens sustain spermatogenesis in ERKO mice.

Using the specific agonist G1, we showed that GPR30 activates G protein-dependent pathways in GC-1 cells. In contrast with a previous report (46), we did not detect any ERβ mRNA or protein expression in GC-1 cells, although we did observe both ERα mRNA and protein expression, which were not investigated by the same authors (46). Moreover, using the selective ERβ agonist DPN (47), we did not obtain any response in GC-1 cells, whereas the specific ERα agonist PPT, which was unable to activate either ERβ (48) or GPR30 (our unpublished observations), activated rapid responses in GC-1 cells. On the basis of these findings, the previously reported (46) transcriptional response to E2 in GC-1 cells transfected with an expression plasmid containing an estrogen-responsive element could be mediated by ERα.

Our data indicate that both ERα and GPR30 are required for E2, PPT, and G-1 pleiotropic effects because c-*fos* upregulation and ERK1/2 activation by each ligand were sensitive to the pure ERα antagonist ICI, which reduces ERα expression but does not change GPR30 protein levels and because silencing of GPR30 expression by specific antisense oligonucleotides abolished ERK activation and cell proliferation induced by all ligands without altering ERα expression.

In addition, given the comparable effects of E2, PPT, and G-1 on c-*fos* expression, we propose that GPR30 and ERα could act through the same signaling pathway downstream of EGFR. In line with this model, it has been shown that GPR30 in response to E2 induces the release of surface-bound membrane-anchored heparin-binding EGF-like growth factor (proHB-EGF) (49), whereas ERα tyrosine phosphorylation by EGFR activation plays a key role in ERK phosphorylation, leading to the stimulation of a mitogenic signaling network (50–53).

One mechanism through which c-*fos* stimulates cell proliferation is by the regulation of cell cycle genes, such as cyclin D1, which contains AP-1sites within the promoter region (54–56). Cyclin D1 has been shown to play a specific role during the G1/S phase transition in adult mouse spermatogonia cells (9). In our GC-1 cells E2, G1, and PPT were able to induce cyclin D1 expression as well as cell proliferation. The inhibitors ICI, AG1478, and PD98059 prevented cyclin D1 expression and agonist-induced cell proliferation. In addition, when GPR30 expression was blocked using specific GPR30 antisense oligonucleotides, E2 was no longer able to induce proliferation, suggesting that GPR30 is involved in the signaling pathway triggered by E2 in GC-1 cell growth. A recent study in a human tumor germ cell line clearly demonstrated that estrogens, through a nonclassical ER associated with a G protein, induce cell proliferation via rapid activation of ERK1/2 and protein kinase A signaling (39). Thus, GPR30 may mediate estrogen-dependent germ cell proliferation also in humans.

In summary, the present study demonstrates for the first time that estrogens, through a GPR30 and ERα cross talk, activate the rapid EGFR/ERK/c-*fos* signaling cascade, which in turn induces mouse GC-1 cell proliferation. The findings that GC-1 cells display several features common to spermatogonia, that GPR30 is expressed in mouse germ cells, and that E2 induces spermatogonia proliferation activating ERK and c-*fos* in other species lead us to hypothesize that E2 could be involved in the regulation of mitosis of mouse spermatogonia, activating rapid EGFR/ERK/c-*fos* signaling through GPR30. Further studies in primary cultures of spermatogonia and/or cocultures on a feeder layer of Sertoli cells are necessary to validate our data obtained in GC-1 cells. Based on our findings, new studies may lead to the elucidation of the physiological role of estrogen-activated rapid signaling pathways involved in the regulation of male fertility and/or testicular tumorigenesis.

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