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Expression of Genomic Functional Estrogen Receptor 1 in Mouse Sertoli Cells

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Abstract

There is no consensus whether Sertoli cells express estrogen receptor 1 (*Esr1*). Reverse transcription-polymerase chain reaction, Western blot, and immunofluorescence demonstrated that mouse Sertoli cell lines, TM4, MSC-1, and 15P-1, and purified primary mouse Sertoli cells (PSCs) contained *Esr1* messenger RNA and proteins. Incubation of Sertoli cells with 17β-estradiol (E2) or ESR1 agonist stimulated the expression of an estrogen responsive gene *Greb1*, which was prevented by ESR inhibitor or ESR1 antagonist. Overexpression of *Esr1* in MSC-1 enhanced E2-induced *Greb1* expression, while knockdown of *Esr1* by small interfering RNA in TM4 attenuated the response. Furthermore, E2-induced *Greb1* expression was abolished in the PSCs isolated from *Amh-Cre/Esr1*-floxed mice in which *Esr1* in Sertoli cells were selectively deleted. Chromatin immunoprecipitation assays indicated that E2-induced *Greb1* expression in Sertoli cells was mediated by binding of ESR1 to estrogen responsive elements. In summary, ligand-dependent nuclear ESR1 was present in mouse Sertoli cells and mediates a classical genomic action of estrogens.

Keywords

Esr1, estrogen, gene regulation, Sertoli cells, testis, mouse

Introduction

Several lines of evidence from cell-based and animal model studies and human genetic data have generally concluded that estrogen, once considered as a female hormone, also plays important roles in the male reproductive system.[1-9] Either estrogen insufficiency or estrogen overexposure causes impaired spermatogenesis and infertility,[10-15] suggesting that maintenance of a delicate balance between androgens and estrogens is critical for normal testicular function.[15,16] The testis is 1 of the 2 major organs which produces estrogen in males. Here, the testosterone synthesized by Leydig cells is converted to estrogens by aromatase in almost all cell types in the testis.[3] In addition to feedback regulation on the hypothalamus and pituitary gland that indirectly influences testicular functions,[17,18] there is convincing evidence showing direct estrogen action within the testes that includes the modulation of testicular androgenesis and spermatogenesis.[2,4,8,10,11,15,19-21]

Estrogen is well established to elicit a myriad of biological processes in target tissues primarily through its binding to estrogen receptors (ESRs) ESR1 and ESR2 (also known as ERα and ERβ, respectively). Estrogen receptors belong to the steroid/thyroid hormone superfamily of nuclear transcription factors encoded by 2 different genes. There are 2 different but interrelated ESR-dependent estrogen signaling pathways commonly described as genomic and nongenomic mechanisms. The classic estrogen signaling pathway is through nuclear ESR-mediated genomic activity. The binding of estrogen to ESRs causes the dimerization of ESRs either as homodimers (eg, ESR1/ESR1 and ESR2/ESR2) or as heterodimers (eg, ESR1/ESR2 and ESR/other transcription factor) and interacts with the consensus DNA sequences called estrogen response elements (EREs) present in the promoter region of the target gene to alter the transcription activity. The nongenomic mechanism refers to membrane ESR-initiated rapid estrogen responses. This pathway is mediated mainly by nonclassical, membrane-associated ESRs through cross talk with other membrane receptors or direct activation of various kinase cascades to exert the biological effects.[22-25]

Testicular ESR1-mediated local estrogen actions have been demonstrated to be essential for male reproduction.[2,26] Although both ESRs are present in the testes, deletion of *Esr1* in mice causes male infertility, whereas an adverse testicular phenotype is not observed in mice with a targeted disruption

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of *Esr2*, suggesting that ESR2 has a very limited function in the testes.[7,20,27] However, the mechanism by which ESR1-mediated estrogen activity affects male fertility is not completely understood. The fundamental question regarding which testicular cell types express functional ESR1 remains uncertain. Leydig cells are generally considered to express ESR1 in several species examined, such as fish, rodents, domestic animals, primates, and humans.[7] Studies in *Esr1* knockout mice reveal that the androgenesis in Leydig cells is enhanced in the absence of ESR1.[28,29] This protein is also detected in the seminiferous tubules. Transplantation experiments in mice demonstrate that germ cells lacking *Esr1* develop normally in the wild-type seminiferous tubules, and the mature sperm can fertilize wild-type oocytes to generate offspring.[19,30] Hence, ESR1 has been postulated to play a role in testicular somatic cells that provide an environment for gametes to develop and mature.[2] This notion is concurred by a recent study in mice that estrogen-dependent ESR1 action is required for germ cell survival and most likely involves the support of Sertoli cell functions.[31] Sertoli cells are the somatic epithelial cells that line the seminiferous tubules of the testes in continuous contact with spermatogenic cells. It is known that these cells play critical roles in nursing and support of spermatogenic cell differentiation and maturation in response to a variety of hormone actions. However, there is no consensus regarding whether these cells express *Esr1*. In several earlier reports, no ESR1 protein was detected in Sertoli cells by immunohistochemistry in bank vole, bird, rat, mouse, dog, cat, goat, boar, monkey, and human (reviewed by Carreau and Hess[7]), whereas numerous more recent studies indicate that ESR1 is present in the Sertoli cells of multiple species including the hystricognath rodent, rat, cat, boar, pig, and human.[31-40] The expression of ESRs in Sertoli cells from rat testes has recently been studied in more detail.[31,35] The transcripts and proteins of both *Esr1* and *Esr2* are detected in premature and adult rat Sertoli cells. Furthermore, estrogen treatment of primary rat Sertoli cells reveals a membrane ESR-mediated rapid signal, involving the activation of the mitogen-activated protein kinases.[34,35]

Mice are one of the most common laboratory animals used in the studies of reproductive biology, but it is still debatable whether mouse Sertoli cells express *Esr1*. The results of the present study demonstrate the presence of both ESR1 and ESR2 in mouse Sertoli cell lines as well as primary Sertoli cells (PSCs). The ESR1 in mouse Sertoli cells mediates the classic genomic mechanism of estrogen action in the transactivation of its target gene *Greb1* (gene regulated by estrogen in breast cancer protein 1) expression.

Materials and Methods

Animals

All animals were housed on 12-hour light–dark cycles with food and water provided ad libitum. All mice were maintained as required under the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The use of animals in this study has been approved by the Animal Care and Use Committee of the University of Louisville. All the mice were killed under ketamine anesthesia and all efforts were made to minimize their discomfort.

Primary Cell Culture and Cell Lines

Primary Sertoli cells were isolated from 30-day-old wild-type, *Amh-Cre*$^-$/*Esr1*$^{fl/fl}$ and *Amh-Cre*$^+$/*Esr1*$^{fl/fl}$ mice using a procedure described previously[41] with a minor modification. Briefly, the testes were decapsulated and incubated with a collagenase type II solution (0.5 mg/mL; Sigma, St Louis, Missouri) to separate interstitial cells and seminiferous tubules. The dispersed seminiferous tubules were cut into small pieces and digested with a solution containing 1 mg/mL trypsin (Sigma) and 10 μg/mL DNase I (Sigma) at 32°C for 30 minutes. The reaction was stopped by adding trypsin inhibitor (Sigma) and Hanks balanced salt solution (HBSS; Invitrogen, Carlsbad, California). The supernatant that contained germ cells was discarded. The pellet was incubated with a collagenase type II solution at 32°C for 15 minutes and settled down by unit gravity sedimentation. The cell pellet, containing Sertoli cells, was rinsed with HBSS 3 times and plated with a 1:1 mixture of Dulbecco modified Eagle medium (DMEM) and F12 Ham medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) overnight and the residual germ cells were hypotonically removed.

The purity of Sertoli cell preparations was verified by performing (1) reverse transcription-polymerase chain reaction (RT-PCR) analysis of the putative marker genes, (2) microscopic examination of their morphology following fixation with 10% formalin and stained with hematoxylin and eosin, and (3) immunostaining of a Sertoli cell-specific marker GATA-2 using an avidin–biotin immunoperoxidase method (Figure 1I-L). The transcripts of putative Sertoli cell marker genes (eg, *Fshr* [follicle-stimulating hormone receptor] and *Pem* [placenta and embryos oncofetal gene]) were readily detectable by RT-PCR, whereas Leydig cell marker genes (eg, *Lhcgr* [luteinizing hormone receptor] and *Cyp17* [17α-hydroxylase]), myoid cell marker genes (eg, *Alp* [alkaline phosphatase] and *Fn1* [fibronectin 1]), and germ cell maker genes (eg, *Prm2* [protamine2] and *Stra8* [stimulated by retinoic acid gene 8]) were not detected using the same RT-PCR conditions. Over 92% of isolated cells were positively immunostained for GATA-2. The mouse Sertoli cell lines, TM4 and 15P-1, were purchased from ATCC (Manassas, Virginia), and the MSC-1 cell line was kindly provided by Dr Griswold Washington State University (Pullman, Washington). MA10 cells (a mouse Leydig cell line) were a gift from Dr Ascoli (The University of Iowa, Iowa City, Iowa). All the cells were maintained in a mixture of DMEM and F12 medium supplemented with 10% FBS (Sigma) and antibiotic–antimycotic solution (Invitrogen). On the day of hormonal treatments, the medium was changed to phenol red- and serum-free DMEM and cells were then incubated with 17β-estradiol (E2, 10^{-10} mol/L; Sigma), ESR inhibitor ICI 182,780 (10^{-8} mol/L), ESR1 agonist PPT (4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol) (10^{-7} mol/L), ESR1 antagonist MPP (1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride)

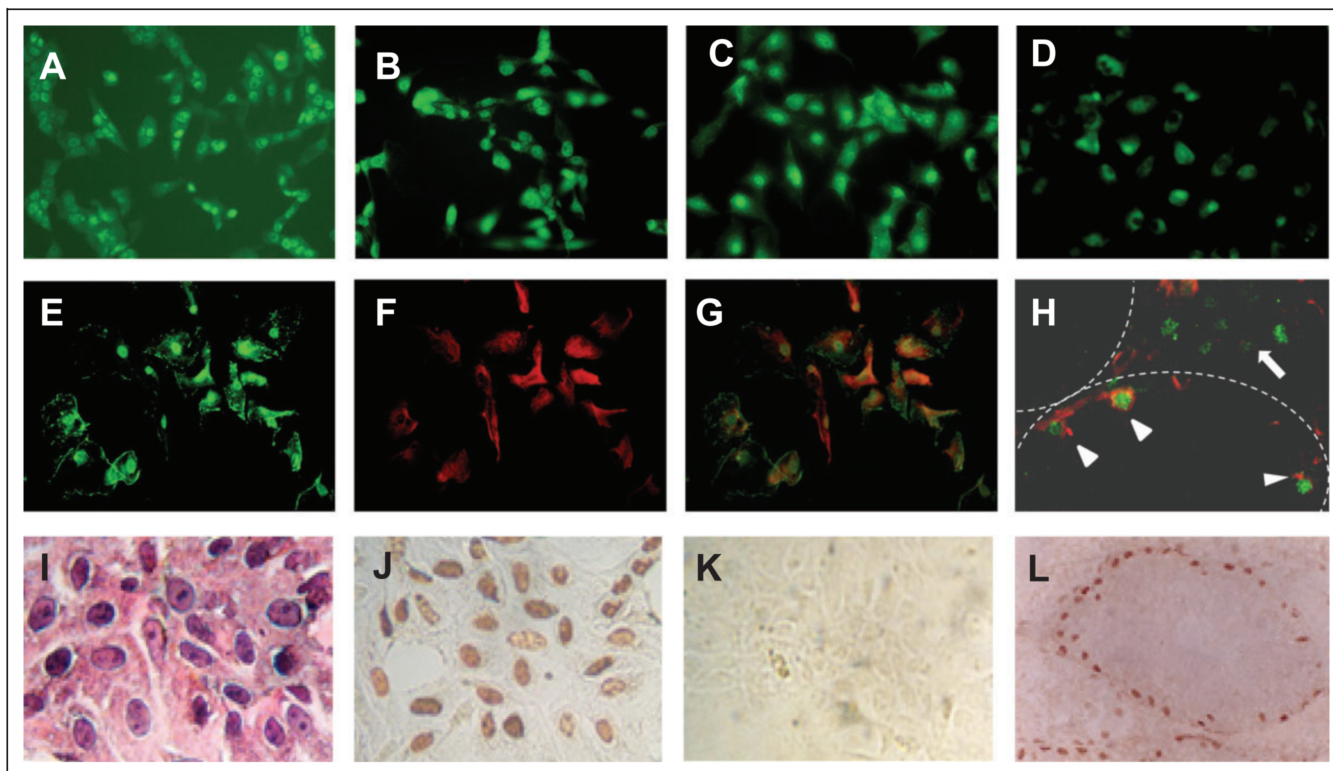

Figure 1. Immunofluorescence showing membrane, cytoplasmic, and nuclear staining of ESR1 in MA10 (A), TM4 (B), 15P-1 (C), MSC-1 (D), and PSCs (E) cells. To differentiate ESR1-positive Sertoli cells from germ cells in isolated PSCs (G) and the testicular section (H), double labeling of ESR1 (E, green) and a Sertoli cell marker vimentin (F, red) was performed. Arrow heads in panel (H) indicate Sertoli cells in a somniferous tubule, and an arrow indicates Leydig cells in the interstitial space that is stained positively for ESR1. Panel (I) is isolated PSCs stained with hematoxylin and eosin. Panel (J) shows that the nuclei of isolated PSCs are immunostained for a Sertoli cell-specific marker GATA-2. Panel (K) is a negative control, in which GATA-2 antibody was omitted and panel (L) is a positive control, in which only the nuclei of Sertoli cells in the seminiferous tubules were immunostained for GATA-2. The immunostaining procedure was performed by an avidin–biotin immunoperoxidase method as described previously.[42] Mag (A) to (H) and (L) = ×350 and (I) to (K) = ×500. ESR indicates estrogen receptor; mag, magnification; PSC, primary mouse Sertoli cell. (The color version of this figure is available in the online version at http://rs.sagepub.com/.)

(10^{-7} mol/L), ESR2 agonist Diarylpropionitrile (DPN) (10^{-7} mol/L), ESR2 antagonist PHTPP (4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol) (10^{-7} mol/L; Tocris, Minneapolis, Minnesota), or vehicle dimethyl sulfoxide (Sigma) for 16 hours unless indicated elsewhere.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from the testes and the isolated testicular cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was adjusted to a concentration of approximately 1 μg/μL. Total RNA of 1 μg was reverse transcribed into complementary DNA (cDNA) using random primers (Invitrogen) and avian myeloblastosis virus (AMV) reverse transcriptase (Promega Corporation, Madison, Wisconsin).[43] The cDNA was amplified by PCR using the primer sets of the target gene and a housekeeping gene, ribosomal protein large subunit 19 (*Rpl19*), as an internal control for both cDNA quantity and quality. The PCR primers, as listed in Table 1, were designed according to the sequences obtained from GenBank using the Vector NTI 12.0 program (Invitrogen) and synthesized by Operon Technologies (Alameda, California). All primers were designed to amplify products that covered more than 1 exon. Each PCR cycle consisted of denaturation for 45 seconds at 94°C, annealing for 1 minute at 57°C, and extension for 1 minute at 72°C. Avian myeloblastosis virus was omitted in reactions used as procedure controls. The amplified products were separated by electrophoresis. For semiquantitative analysis, the intensity of specific bands was scanned using the TotalLab (Nonlinear USA Inc, Durham, North Carolina) image analysis software. The results were presented as the ratio of target gene to *Rpl19*.

Western Blotting

Sertoli cells were homogenized by sonication in an ice-cold lysis buffer containing protease inhibitors (Roche, Indianapolis, Indiana). The protein concentrations were measured using the Bradford method (Bio-Rad laboratories, Hercules, California). An equal quantity of proteins was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 3% nonfat milk and

Table 1. Primer Sequences for Semiquantitative RT-PCR, ChIP, and Genotyping.

Gene	Primer Sequence (5′-3′)	PCR Cycles
RT-PCR		
Greb1	F: TGCAGCATACAACACGTACCA R: GGGCTTTTGATGTGTTCATG	27 for TM4, 34 for MSC-1
Esr1	F: CCGCAGCTGTCTCCTTTCCT R: CGGTTCTTGTCAATGGTGCA	35 for all cell lines, 32 for TM4
Esr2	F: CGCTCAGGGACCGAGGAAAGTACGT R: GTCATGGCTGAGTATTCGTGACGG	39 for all cell lines
Rpl19	F: CTCAGGCTACAGAAGAGGCTT R: GGACAGAGTCTTGATGATCTC	Same as the coamplified target gene
ChIP		
ERE1	F: TTGGAAGATCCACCGCAAACT R: AGACAGGCTCGGGCATGTATC	40 for TM4
ERE2	F: TCACCCACAGTGCTGCGAGA R: GCCCTTGACCGAGGAGATGA	40 for TM4
Genotyping		
Amh-Cre	F: GCGGTCTGGCAGTAAAAACTATC R: GTGAAACAGCATTGCTGTCACTT	30 cycles
Floxed and Δ*Esr1*	F: TTGCCCGATAACAATAACAT R: GGCATTACCACTTCTCCTGGGAGTCT	30 cycles
Floxed Esr1	F: GTGTCAGAAAGAGACATT R: GGCATTACCACTTCTCCTGGGAGTCT	30 cycles

Abbreviations: ChIP, chromatin immunoprecipitation; ESR, estrogen receptor; ERE, estrogen response element; F, Forward primer; R, Reverse primer; RT-PCR, reverse transcription-polymerase chain reaction; *Rpl19*, ribosomal protein large subunit 19.

incubated overnight with rabbit anti-ESR1 (H184, 1:500), rabbit anti-ESR2 antibody (h-150, 1:500; Santa Cruz Biotech, California), and mouse anti-ESR1 (1:500; Abcam, Cambridge, Massachusetts), respectively, and detected by ECL (Amersham, Piscataway, New Jersey). Peroxidase-conjugated anti-rabbit or anti-mouse immunologublin G (IgG, 1:2000; Vector Laboratories, Burlingame, California) was used as the secondary antibody. Immunoblotting signals were detected using the Amersham ECL plus Western blotting detection system (GE Healthcare Biosciences, Pittsburgh, Pennsylvania). All the membranes were reprobed with anti-β actin or β-tubulin antibody (Sigma), which served as a loading control. The intensity of specific bands was scanned using the TotalLab (Nonlinear USA Inc) image analysis software. The results were presented as the ratio of target protein to β-actin or β-tubulin.

Immunofluorescence

The coverslips of cultured cells and frozen sections of the testes were fixed in freshly prepared 2% paraformaldehyde and permeabilized with 0.01% saponin. The coverslips and frozen sections were then incubated with goat antivimentin (1:150; Sigma) and rabbit anti-ESR1 (H184, 1:50) overnight at 4°C. Subsequently, ESR1 and ESR2 expression levels were detected with fluorescein isothiocyanate-labeled donkey antirabbit IgG and vimentin was detected by Texas red-labeled donkey antigoat IgG (1:100; Jackson, West Grove, Pennsylvania). The coverslips and frozen sections were covered with a 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Santa Cruz Biotech) to visualize the nuclei and then were viewed and photographed with an Olympus fluorescence microscope (B & B Microscopes, LTD, Pittsburgh, Pennsylvania). Replacement of the primary antibody with normal rabbit or mouse serum was used as a procedure control.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assays were performed using an Imprint Ultra ChIP kit (Sigma) according to the manufacturer's instructions. Briefly, TM4 cells were cultured with DMEM medium supplemented with 10% FBS in 500 cm^2 dishes. The chromatin was cross-linked by treating the cells with 1% formaldehyde for 10 minutes at room temperature. The cells were harvested and homogenized with a glass Dounce homogenizer (Fisher Scientific, Pittsburgh, Pennsylvania). The cross-linked chromatin was fragmented by sonication (3 × 10 minutes) with a sonic dismembrator model 150 (Fisher Scientific, Pittsburgh, Pennsylvania). The ESR1 (H-184) and ESR2 (H-150) antibodies (Santa Cruz Biotech) at a 1:50 dilution were used for immunoprecipitation, respectively. The DNA was then purified and used for PCR with the primer sets listed in Table 1 and the conditions as follows: 40 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, and extension for 1 minute at 72°C.

Overexpression of *Esr1* in MSC-1 Cells

MSC-1, the cell line expressing low amount of endogenous *Esr1*, was selected for overexpression of a human *ESR1* cDNA. To generate stable ESR1 expression clones, the cells were cultured to approximately 60% confluence in a 6-well plate and were transfected with 2 μg of either *pcDNA3-ESR1* or *pcDNA3* vector (a control) using Lipofectamine 2000 (Invitrogen). The

cells were then cultured in a medium containing DMEM, 10% FBS, and 600 μg/mL G418 (Cellgro Molecular Genetics, Manassas, Virginia) for 14 days. The G418-resistant clones were selected and subcultured for subsequent RT-PCR and Western blot analyses. The overexpression of ESR1 protein levels in these clones was determined by Western blot.

Knockdown of *Esr1* in TM4 Cells

TM4, the cell line expressing high levels of endogenous *Esr1*, was selected for *Esr1* small interfering RNA (*siRNA*) transfection using a procedure as described previously.[43] The cells were used for transfection once they reached approximately 50% confluence. The *Esr1 siRNA*, purchased from Santa Cruz Biotech, consisted of a pool of 3 to 5 target-specific *siRNAs* against mouse *Esr1* (Gene ID: 13982). Typically, a mixture of 0.25 μg *Esr1 siRNA*, 100 μL DMEM, and 4 μL Lipofectamine 2000 (Invitrogen) was added to the cells in a 12-well plate and incubated overnight. The transfection medium was replaced with 0.5 mL DMEM containing 10% FBS for an additional 24 hours and these cells were used for subsequent experiments. A control *siRNA* (Santa Cruz Biotech) consisting of a scrambled RNA sequence was transfected in parallel with *Esr1 siRNA* as a specificity control for the *Esr1* knockdown. The RT-PCR and Western blotting were used to determine the effectiveness of *Esr1 siRNA* in suppressing *Esr1* messenger RNA (mRNA) and protein levels in TM4 cells.

Sertoli Cell-Specific *Esr1* Ablation in Mice

Floxed *Esr1* male mice (provided by Drs Andree Krust and Pierre Chambon, Institute for Genetics and Cellular and Molecular Biology, Strasbourg, France)[44] were mated with female *Amh-Cre* mice (purchased from Jackson Laboratories, Bar Harbor, Maine), where the Cre recombinase is specifically expressed in Sertoli cells under the control of the anti-Müllerian hormone promoter[45] to obtain biogenic heterozygous females (ie, $Amh\text{-}Cre^{+}/Esr1^{flox/wt}$). These heterozygous females were bred with male $Esr1^{flox/flox}$ to generate Sertoli cell-specific *Esr1* mutant mice ($Amh\text{-}Cre^{+}/Esr1^{flox/flox}$). Tail biopsies were obtained and used for genotyping as described previously.[43] The primer sets listed in Table 1 were used to determine the deletion of the floxed *Esr1* allele by PCR as described previously.[46] The efficiency of the excised floxed *Esr1* allele in the Sertoli cells was evaluated by performing semiquantitative RT-PCR with PSCs isolated from the testes of $Amh\text{-}Cre^{+}/Esr1^{flox/flox}$ and $Amh\text{-}Cre^{-}/Esr1^{flox/flox}$ mice.

Statistical Analysis

The data presented are the mean ± standard error of the mean. All results were analyzed by 1-way analysis of variance and the Tukey multiple comparison posttest using the version 3.06 Instat program (Graphpad Software, San Diego, California). A *P* value <.05 was considered as statistically significant.

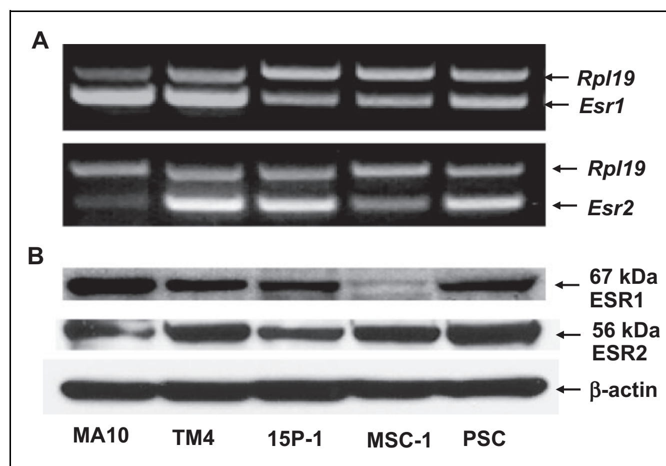

Figure 2. The RT-PCR (A) and Western blot (B) analyses demonstrate that similar to mouse Leydig cells (MA10), all Sertoli cell lines (TM4, 15P-1, and MAC-1) and primary Sertoli cells (PSC) express both *Esr1* and *Esr2*. MA10 serves as a positive control cell line. Ribosomal protein large subunit 19 (*Rpl19*) and β-actin serve as internal controls for RT-PCR and Western blotting, respectively. ESR indicates estrogen receptor; RT-PCR, reverse transcription-polymerase chain reaction.

Results

Mouse Sertoli cell Lines and PSCs Express *Esr1* and *Esr2*

Reverse transcription PCR detected the expression of *Esr1* and *Esr2* mRNA in 3 established mouse Sertoli cell lines, TM4, 15P-1, and MSC-1 as well as PSCs isolated from 30-day-old mice (Figure 2A). A mouse Leydig cell line MA-10 was used as a positive control. No PCR-amplified products were observed when reverse transcriptase was omitted (data not shown).

Comparable with MA-10 cells, Western blot analysis revealed the presence of 67-kDa ESR1 and 56-kDa ESR2 proteins in all 3 Sertoli lines and PSCs (Figure 2B). Both monoclonal and polyclonal antibodies to ESR1 demonstrated the same results (data not shown). These proteins were absent when the primary antibodies were replaced by normal mouse or rabbit serum (data not shown). In agreement with the RT-PCR results, relatively high levels of ESR1 expression were observed in the TM4, 15P-1 cell lines, and PSCs but not in the MSC-1 cell line.

Immunofluorescent staining of the cultured Sertoli cells showed that the ESR1 protein was primarily localized in the nuclei of all 3 Sertoli cells lines and PSCs (Figure 1). To a lesser extent, ESR1 immunofluorescence was also observed in the membrane and cytoplasm of these cells (Figure 1). In the isolated PSCs (G) and the testicular sections (H), we performed double-labeling immunofluorescent staining for ESR1 (E, green) and a somatic cell marker vimentin (F, red) to differentiate ESR1-positive Sertoli cells from germ cells. Estrogen receptor 1 was localized to the nuclei of vimentin-positive Sertoli cells in frozen sections of the adult testes and isolated PSC (Figure 1H). Both monoclonal and polyclonal ESR1 antibodies showed the same immunostaining pattern. No specific

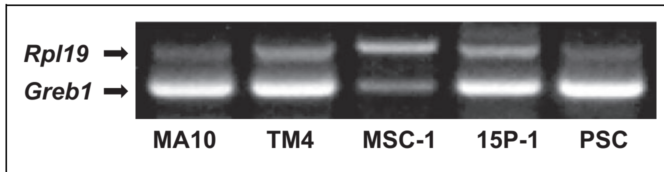

Figure 3. The RT-PCR analysis demonstrates that all Sertoli cell lines and primary Sertoli cells express *Greb1* (A). Ribosomal protein large subunit 19 (*Rpl19*) serves as an internal control for RT-PCR. RT-PCR indicates reverse transcription-polymerase chain reaction.

immunofluorescence was detected when ESR1 antibodies were omitted (data not shown).

The Expression of Greb1 in Mouse Sertoli Cell Lines and PSCs

Estrogen-dependent modulation of target gene transcription is well established as the classic genomic function of ESR1 and ESR2. Reverse transcription PCR showed that all 3 Sertoli cell lines, PSCs, and MA-10 expressed mRNA for a well-characterized estrogen responsive gene *Greb1* (Figure 3), which suggests that *Greb1* can be used as an endogenous reporter gene to investigate ESR1-mediated gene expression in mouse Sertoli cells.

Activation of ESR1 Induces Greb1 Expression in Mouse Sertoli Cells

To determine whether *Esr1* expressed in mouse Sertoli cells is functional, we first challenged mouse TM4 Sertoli cells with an ESR ligand and selective synthetic agonists or antagonists and then measured the *Greb1* mRNA levels. The expression of *Greb1* mRNA was markedly increased in TM4 cells when the cells were incubated with 17β-estradiol (E2) or a synthetic selective ESR1 agonist PPT (Figure 4). However, a synthetic selective ESR2 agonist, DPN, had no effect on *Greb1* mRNA levels.

When TM4 cells were treated with E2 and PPT (a selective ESR1 agonist), *Greb1* expression was increased in a dose- and time-dependent manner (Figure 5). However, when the cells coincubated with either ICI 182,780 (a nonselective ESR inhibitor) or MPP- (a selective ESR1 antagonist), E2-, and PPT-induced *Greb1* expression was significantly attenuated. Meanwhile, a selective ERS2 antagonist PHTPP did not affect *Greb1* mRNA levels (Figure 6). Together, these results suggest that regulation of *Greb1* expression in mouse Sertoli TM4 cells is mediated by ESR1.

ESR1-Dependent Modulation of Greb1 Expression in Mouse Sertoli Cells

To characterize ESR1-dependent transactivation of *Greb1* expression in mouse Sertoli cells, we used genomic approaches and performed both gain-of-function and loss-of-function experiments. Overexpression of human ESR1 in MSC-1, a

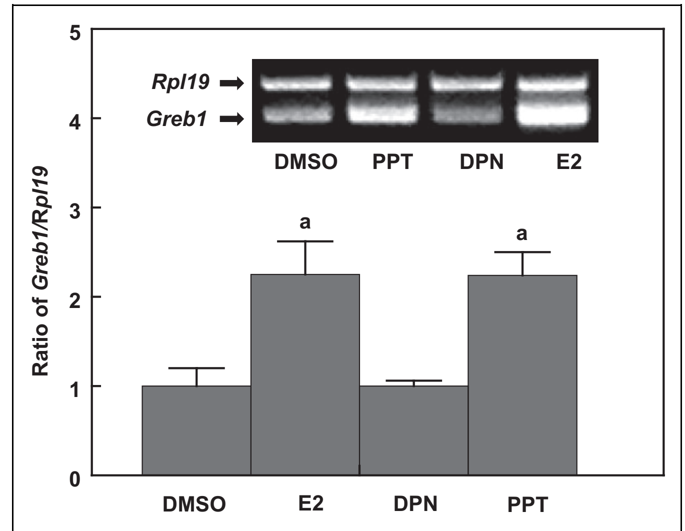

Figure 4. The RT-PCR analysis shows that 17β-estradiol (E2) and PPT (selective ESR1 agonist) but not DPN (selective ESR2 agonist) induce *Greb1* expression in TM4 cells. Ribosomal protein large subunit 19 (*Rpl19*) serves as an internal control for RT-PCR. Data are presented as mean ± standard error of the mean (SEM; n = 3). $^{a}P < .01$ compared to the vehicle dimethyl sulfoxide (DMSO). ESR indicates estrogen receptor; RT-PCR, reverse transcription-polymerase chain reaction.

mouse Sertoli cell line with a relatively low endogenous *Esr1* expression (Figure 7A), dramatically increased the responses to E2- or PPT-induced *Greb1* expression. This increase was curtailed by coincubation with either ICI 182,780 or MPP ESR inhibitors (Figure 7B and C). When *Esr1* was knocked down by siRNA in a mouse Sertoli cell line with a relatively high expression of endogenous *Esr1* TM4 cells, E2-induced *Greb1* expression was significantly attenuated (Figure 8). Moreover, ESR1-dependent modulation of *Greb1* expression was further examined in primary cultured mouse Sertoli cells. The Sertoli cells were isolated from the *Amh-Cre*$^{+}$/*Esr1*$^{fl/fl}$ bigeneric mice, in which the exon 3 of the mouse *Esr1* in Sertoli cells was selectively deleted by *Amh-Cre* (Figure 9A). The RT-PCR revealed that the mRNA levels of *Esr1* in purified Sertoli cells from these mice were markedly reduced (Figure 9B). These purified *Esr1*-deficient Sertoli cells exhibited a significant decrease in E2-induced *Greb1* expression (Figure 9C).

Estrogen Receptor 1 Binds to EREs in the Promoter Region of the Greb1 Gene

We finally determined whether the ESR1 or ESR2 physically interact with the promoter of *Greb1* gene in TM4 cells. Two previously identified canonical EREs (ERE1 and ERE2) were present in the *Greb1* gene at −3289 and −7666 bp upstream of its transcription start site (Figure 10A).[47,48] The ChIP analysis showed that only ESR1 but not ESR2 bound to both the ERE1 and the ERE2 (Figure 10B). The physical interaction between ESR1 and EREs in the *Greb1* promoter suggests that

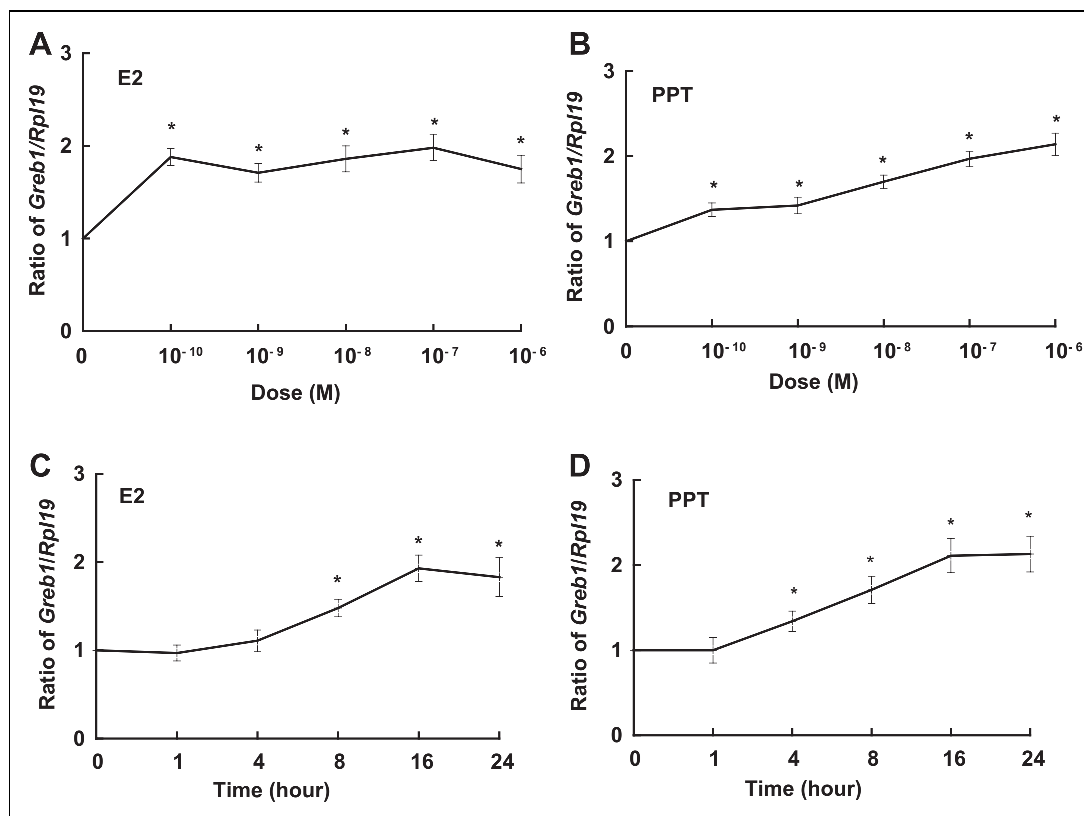

Figure 5. Dose-dependent (A and B) and time-dependent (C and D) induction of *Greb1* expression by 17β-estradiol (E2, A and C) and selective estrogen receptor 1 (ESR1) agonist PPT (B and D) in TM4 cells. Ribosomal protein large subunit 19 (*Rpl19*) serves as an internal control for reverse transcription-polymerase chain reaction (RT-PCR). Data are presented as mean ± standard error of the mean (SEM; n = 3). *P < .05 compared to dimethyl sulfoxide (DMSO) or time 0.

ESR1 contributes to the upregulation of *Greb1* expression in TM4 cells through a genomic action.

Discussion

Increasing lines of evidence now demonstrated that the testis is a site of estrogen production and is also a target of estrogen action.[2-4,15,16,26] Further evidence from the studies on *Esr* knockout mice suggests that ESR1 but not ESR2 expressed in the gonadal somatic cells, but not germ cells, plays a critical role in spermatogenesis.[20,26] However, whether Sertoli cells express *Esr1* is still controversial because although several studies showed its presence in these cells, other studies did not report any such expression.[7,34] In the present study, we utilized multiple approaches to determine whether *Esr1* is expressed in mouse Sertoli cells. The data demonstrate that mouse Sertoli cell lines and PSC express *Esr1*, and the protein is localized in nuclei, membrane, and cytoplasm. Our findings are consistent with several recent reports that Sertoli cells of the hystricognath rodent, rat, cat, boar, pig, and human express *Esr1*.[31-40] Although it appears difficult to reconcile, the possibilities of these conflicting results in the literature with regard to the presence of ESR1 in Sertoli cells may be due to the differences in the origin, condition, and age of the samples examined and the techniques and reagents used. In rat and human testes, the controversial results are most likely attributed to the various tissue preservations and antibodies used as well as to the origin of the tissues, which have been extensively discussed in recent studies.[35,38] The expression of *Esr1* in Sertoli cells is affected by various conditions including developmental stage, hormonal milieu, and even cell density in culture. For instance, the expression of *Esr1* in rat Sertoli cells is downregulated by estrogen[49] whereas ESR1 in pig Sertoli cells generally decreases with age.[32] Additionally, the expression levels of ESRs in mouse and rat Sertoli cells highly depend on cell density in vitro.[50] If the cell or tissue preservation methods are not optimal or the techniques and reagents used for detection are not sensitive enough, it is perceivable that low expression levels of *Esr1* mRNA or its protein in Sertoli cells may be difficult to detect.

The MSC-1 cell line, derived from a Sertoli cell tumor in the transgenic C57BL/6 X SJL-mixed hybrid adult mice, carries a transgene containing DNA encoding both small and large T antigen of the SV40 virus fused to the promoter for human Müllerian inhibiting substance. Several putative Sertoli cell marker genes are known to be expressed in this cell line, such as transferrin, clusterin, and inhibin βB.[51,52] The 15P-1 cell line, established from testicular cells of transgenic adult mice, expresses the large T protein of polyoma virus (PyLT) in the seminiferous epithelium. This cell line is derived from a Sertoli cell origin by its expression of Wilm tumor and kit ligand.[53]

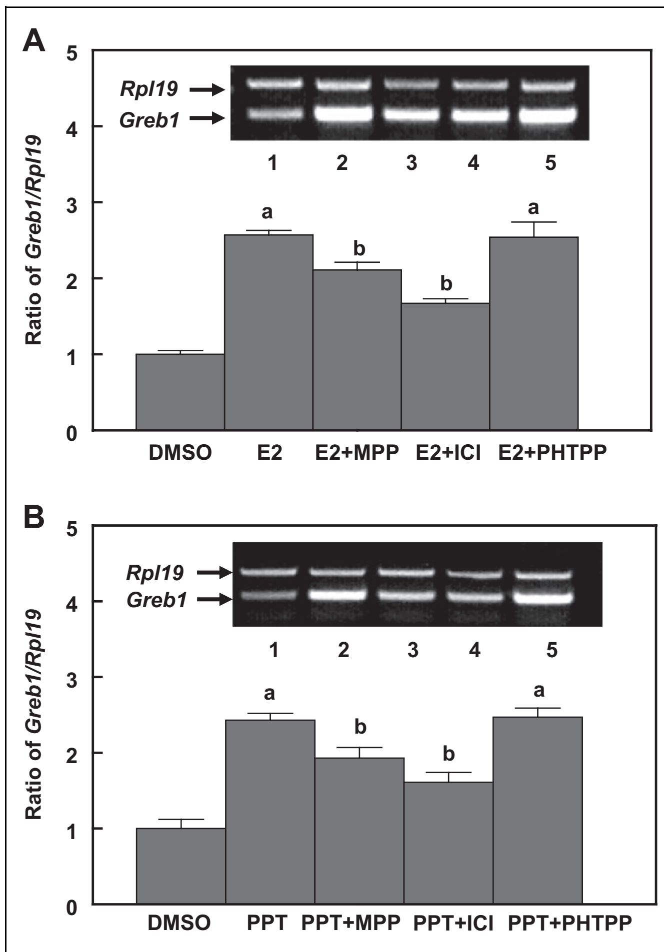

Figure 6. Induction of *Greb1* expression by 17β-estradiol (A) and the selective ESR1 agonist PPT in TM4 cells is attenuated by coincubation with either the selective ESR1 antagonist MPP or an ESR inhibitor ICI 182,780 but not selective ESR2 antagonist PHTPP. Ribosomal protein large subunit 19 (*Rpl19*) serves as an internal control for reverse transcription-polymerase chain reaction (RT-PCR). Data are presented as mean ± standard error of the mean (SEM; n = 3). $^{a}P < .01$ compared to dimethyl sulfoxide (DMSO) and $^{b}P < .05$ compared to PPT. ESR indicates estrogen receptor.

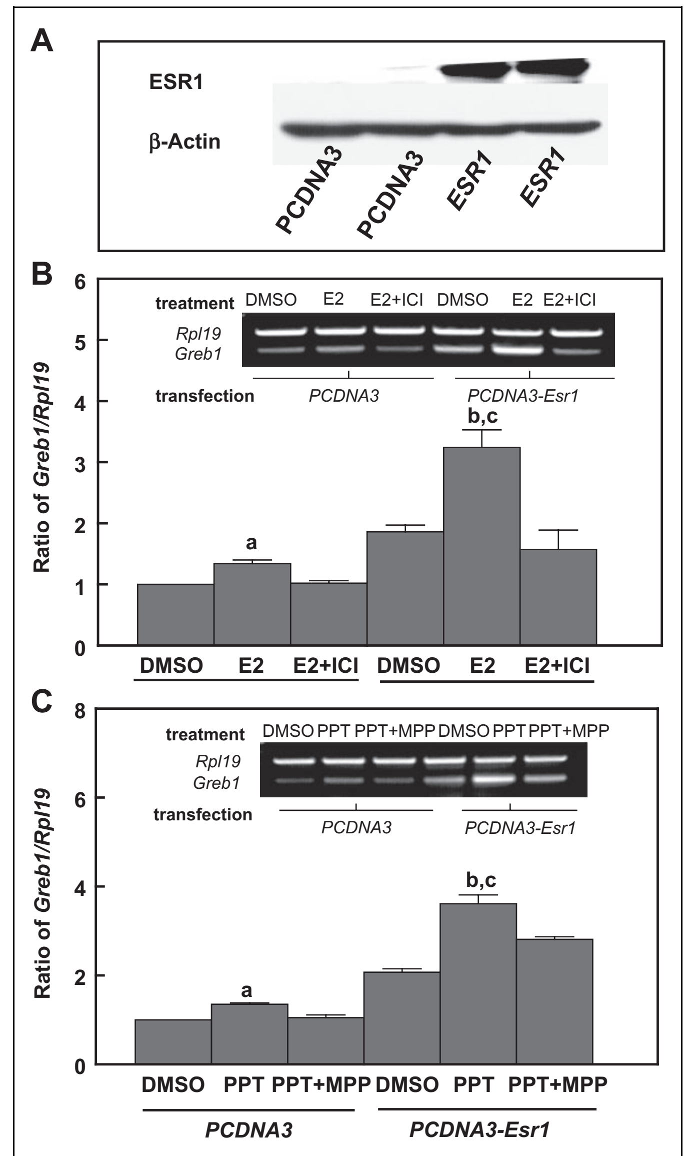

Figure 7. Western blot demonstrating overexpression of ESR1 protein levels in MSC-1 cells (A). Overexpression of *Esr1* in MSC-1 cells enhances 17β-estradiol (B) and the ESR1 agonist PPT-induced *Greb1* expression, which can be counteracted by coincubation with an ESR inhibitor ICI 182,780 (B) or the selective ESR1 antagonist MPP (C), respectively. The empty vector pcDNA3 is transfected in parallel with *Esr1* expression vector as a negative control for overexpression of ESR1. Ribosomal protein large subunit 19 (*Rpl19*) and β-actin serve as internal controls for reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively. Data are presented as mean ± standard error of the mean (SEM; n = 3). $^{a}P < .05$ and $^{b}P < .01$ compared to dimethyl sulfoxide (DMSO), $^{c}P < .01$ compared to E2 (B) and PPT (C) in PCDNA3. ESR indicates estrogen receptor.

The TM4 cell line is the first and the most well-characterized mouse Sertoli cell line which were generated by Mather from primary cultures of Sertoli cells isolated from 11- to 13-day-old mice.[54] This cell line possesses the most Sertoli cell characteristics, such as transferrin secretion, androgen receptor expression, response to FSH stimulation, and exhibition of phagocytotic activity.[54-57] Our results demonstrate the presence of *Esr1* mRNA and protein in all 3 cell lines. Although these immortalized Sertoli cell lines provide a pure population of cells for the study, they do not phenocopy all the properties of native Sertoli cells. For example, MSC-1 cells lack some of the immune privilege functions,[58] and TM4 cells do not secrete androgen-binding protein associated with primary Sertoli cells.[59] Therefore, we also include primary mouse Sertoli cells and testicular in situ immunolocalization of ESR1 in this study. These cell lines, freshly isolated primary Sertoli cells, and immunostained testicular tissues all demonstrate similar results, suggesting the presence of ESR1 in mouse Sertoli cells is unlikely to be an artifact.

To provide further evidence for the presence of ESR1 in mouse Sertoli cells, we examined its interaction with the promoter of *Greb1*, a known estrogen responsive gene. *Greb1* was

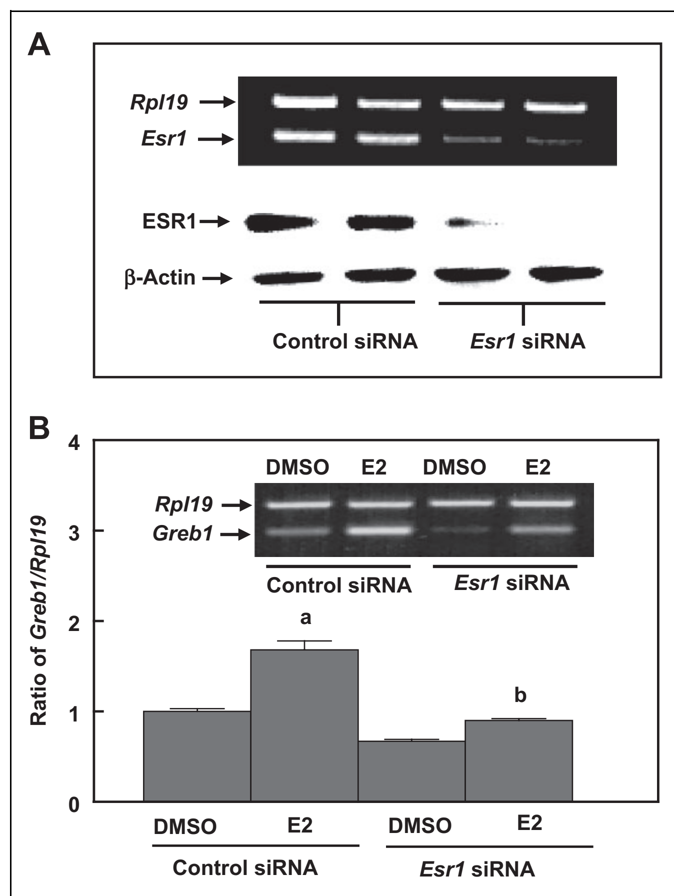

Figure 8. The RT-PCR and Western blot analyses show that *Esr1 siRNA* transfection markedly reduces *Esr1* messenger RNA (mRNA) and protein levels in TM4 cells (A). Knockdown of *Esr1* in TM4 cells abolishes 17β-estradiol (E2)-induced *Greb1* expression (B). A scrambled RNA sequence (control siRNA) is transfected in parallel with the *Esr1* siRNA as a specificity control for *Esr1* knockdown. Ribosomal protein large subunit 19 (*Rpl19*) and β-actin serve as internal controls for RT-PCR and Western blotting, respectively. Data are presented as mean $\pm$ standard error of the mean (SEM; n = 3). $^{a}P < .01$ compared to dimethyl sulfoxide (DMSO) and $^{b}P < .01$ compared to E2 in control *siRNA*. ESR indicates estrogen receptor; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA.

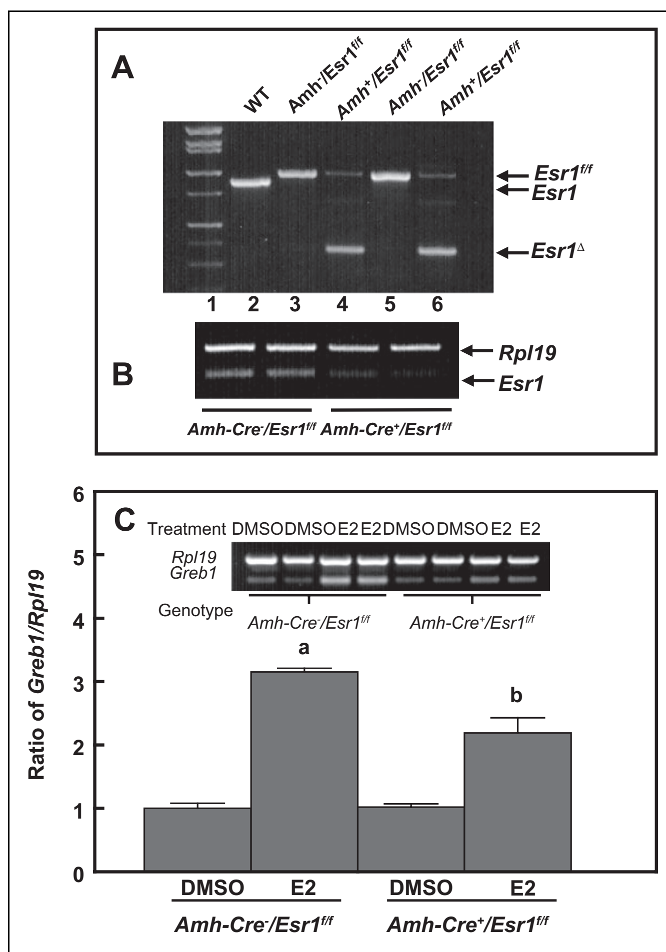

Figure 9. Panel (A) is a representative genotyping picture, which demonstrates that a deleted form of *Esr1* (*Esr1*$^{\Delta}$) is only detected in *Amh-Cre*$^{+}$/*Esr1*$^{flox/flox}$ mice (Lines 4 and 6) but not in *Amh-Cre*$^{-}$/*Esr1*-$^{flox/flox}$ mice (Lines 3 and 5). Panel (B) shows a dramatic decrease in *Esr1* messenger RNA (mRNA) levels in primary Sertoli cells isolated from the *Amh-Cre*$^{+}$/*Esr1*$^{flox/flox}$ testes. Panel (C) demonstrates that Sertoli cell-specific ablation of *Esr1* significantly curtails 17β-estradiol-induced *Greb1* expression. Ribosomal protein large subunit 19 (*Rpl19*) serves as an internal control for reverse transcription-polymerase chain reaction (RT-PCR). Data are presented as means $\pm$ standard error of the mean (SEM; n = 3). $^{a}P < .01$ and $^{b}P < .05$ compared to dimethyl sulfoxide (DMSO).

initially identified as an estrogen target gene in human breast tumor cells through a genome-wide screen.[47,60] There are 3 consensus EREs consisting of a 15-bp palindromic sequence presence in the 5′ flanking region of the *Greb1* gene, which are conserved in human and mouse.[47,48] Our results show that all 3 mouse Sertoli cell lines and mouse PSC express *Greb1* and its expression is estrogen inducible. More importantly, ChIP analysis indicated that ESR1 but not ESR2 binds to 2 proximal EREs in the mouse *Greb1* promoter region, which is in consonance with previous studies.[47,48,60] Therefore, *Greb1* was chosen as the endogenous reporter gene for functional analysis of ESR1 in mediating estrogen action to regulate target gene expression in mouse Sertoli cells. Pharmacological and genetic approaches were used in this study to investigate the functional significance of ESR1 in mouse Sertoli cells. The *Greb1* expression was induced in TM4 cells via ESR1 but not ESR2. The complementary genetic gain-of-function and loss-of-function experiments further confirmed the importance of ESR1 in the transactivation of *Greb1* expression in TM4 Sertoli cells. Furthermore, primary Sertoli cells isolated from a Sertoli cell-specific knockout mouse line also displayed a significant attenuation of estrogen-induced *Greb1* expression although *Esr1* mRNA levels in these Sertoli cells are noticeably reduced but not completely deleted. Taken together, these data obtained using multiple approaches in the present study clearly establish a classic genomic function of ESR1 in mouse Sertoli cells.

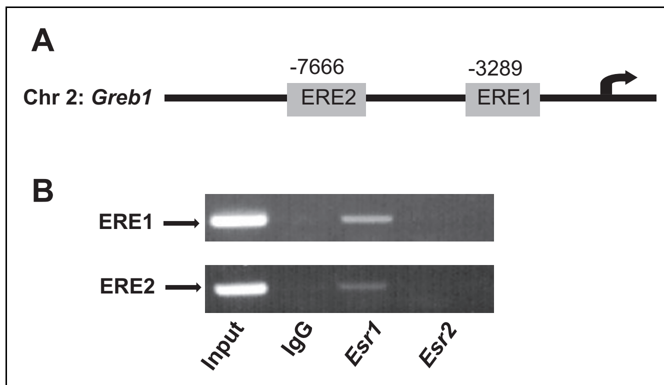

Figure 10. A schematic drawing illustrating the relative positions of 2 consensus estrogen responsive elements (ERE1 and ERE2) in the mouse *Greb1* upstream regulatory regions (A). Chromatin immunoprecipitation (ChIP) assays reveal that ESR1 but not ESR2 binding to both EREs in the *Greb1* promoter in mouse Sertoli cell line TM4 (B). Genomic DNA input and rabbit immunologublin G (IgG) serve as a positive and a negative control, respectively. ESR indicates estrogen receptor; ERE, estrogen response element.

Several mechanisms of ESR-mediated estrogen actions are well established.[22-25] A rapid estrogen-signaling cascade mediated by nonclassical membrane-associated ESRs in rat Sertoli cells has been recently characterized.[31,34,35] However, these studies do not differentiate whether the estrogen action is mediated by ERS1 or ESR2. Although our study focuses on the genomic action of ESR1 in mouse Sertoli cells, we do not exclude that a nonclassical membrane-associated ESR1 transduces rapid estrogen signals in mouse Sertoli cells. In fact, immunostaining of ESR1 in the membrane and cytoplasm in mouse Sertoli cell lines and PSCs was observed, suggesting the likelihood of existing membrane-associated ESR1 in these cells. Although a role of Sertoli cell ESR1 in the regulation of a target gene's expression has been demonstrated in this study, it remains to be established how ESR1-mediated estrogen signaling affects Sertoli cell functions that ultimately influence the spermatogenic processes.

In summary, the results of the present work indicate that Sertoli cell lines derived from either immature or adult mice, as well as primary Sertoli cells isolated from peripubertal mice, express both *Esr1* and *Esr2*. Importantly, ESR1 functions as a nuclear transcription activator to mediate the classic genomic action of estrogen to regulate *Greb1* expression in mouse Sertoli cells. This study provides essential information for our understanding of ESR1-mediated estrogen actions in the regulation of Sertoli cell functions, which may consequently influence germ cell development and maturation in the seminiferous tubules.

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Declaration of Conflicting Interests

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