17β-Estradiol regulates cyclin A1 and cyclin B1 gene expression in adult rat seminiferous tubules

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Abstract

Spermatogenesis, which is the fundamental mechanism allowing male gamete production, is controlled by several factors, and among them, estrogens are likely concerned. In order to enlighten the potential role of estrogen in rat spermatogenesis, seminiferous tubules (ST) from two groups of seminiferous epithelium stages (II–VIII and IX–I) were treated with either 17β-estradiol (E2) agonists or antagonists for estrogen receptors (ESRs). In this study, we show that cyclin A1 and cyclin B1 gene expression is controlled by E2 at a concentration of 10^{-9} M only in stages IX–I. This effect is mimicked by a treatment with the G-protein coupled estrogen receptor (GPER) agonist G1 and is abolished by treatment with the ESR antagonist ICI 182 780. Moreover, using letrozole, a drug that blocks estrogen synthesis, we demonstrate that these genes are under the control of E2 within rat ST. Thus, germ cell differentiation may be regulated by E2 which acts through ESRs and GPER, expressed in adult rat ST.

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Introduction

Spermatogenesis is the mechanism by which spermatogonium, a diploid cell, is transformed in spermatozoon, a highly differentiated haploid cell. This phenomenon takes place in the seminiferous tubules (ST) in which Sertoli cells support germ cells. In adult rat ST, Sertoli and germ cells are organized in 14 stages of the seminiferous epithelium (I–XIV; Leblond & Clermont 1952, Parvinen 1982). Each stage corresponds to specific associations between either four or five different germ cells types. During their maturation, germ cells undergo structural and physiological changes that are under the control of numerous endocrine, paracrine, and/or autocrine factors including gonadotropins, steroids, cytokines, and growth factors (Gnnessi et al. 1997, Franca et al. 1998, McLachlan et al. 2002).

Among these factors, there is growing evidence that estrogens, synthesized by the cytochrome P450 aromatase, could be concerned with the regulation of spermatogenesis (for review, Carreau & Hess (2010)). Indeed, in men with a congenital cytochrome P450 aromatase deficiency, a decrease in the quality and motility of spermatozoa has been reported and two cases of sterility have been described (for review, Rochira et al. (2005)). Moreover, in 1-year-old mice in which the cytochrome P450 aromatase deficiency is genetically induced (ArKO), failures in spermatogenesis are reported with a decrease in the number and quality of spermatids, leading to infertility (Robertson et al. 1999). Furthermore, studies have shown that estrogens are involved in several aspects of rodent spermatogenesis: proliferation of gonocytes and spermatogonia, control of apoptosis, and in some steps of spermiogenesis (for reviews, O’Donnell et al. (2001) and Carreau & Hess (2010)). Cytochrome P450 aromatase has been shown to be expressed in the adult rat testis at the transcript and protein levels and to be biologically active in adult Leydig and germ cells (for review, Carreau et al. (2011)). Moreover, cytochrome P450 aromatase mRNA levels are regulated according to age, cell type, and stages of the seminiferous epithelium (Bois et al. 2010).

The effects of estrogen are mediated by classical estrogen receptors (ESRs), ESR1 and ESR2. After the ligand binding, ESRs act as transcription factors, recruit cofactors (Hall & McDonnell 2005), and directly regulate the expression of target genes containing estrogen responsive elements (ERE) or indirectly regulate by interacting with transcription factors such as ESRs.
as activating protein-1 (AP-1) complex (c-Fos/c-Jun), Sp-1, or nuclear factor κB (NFκB). These interactions between ESRs and transcription factors modify the binding affinity of cofactors (Hall & McDonnell 2005) and thus regulate the expression of genes without ERE (O’Lone et al. 2004). Despite the discrepancies, ESRs are found expressed in rat testis at the transcript (Bois et al. 2010) and protein levels (for review, Carreau & Hess (2010)).

Recently, the existence of estrogen pathways starting at the plasma membrane has been found. As a novel ESR, G-protein coupled estrogen receptor (GPER) has been shown to be implicated in estrogen responses (for review, Maggiolini & Picard (2010)) in the mouse spermatogonial cell line GCI (Sirianni et al. 2008), rat Sertoli cells (Lucas et al. 2010), pachytene spermatocytes (Chimento et al. 2010) and in round spermatids (Chimento et al. 2011). 17β-Estradiol (E2) binding to GPER activates the mitogen-activated protein kinase (MAPK) pathway and can result in the activation of transcription factors such as c-Fos or c-Jun and thus regulate the expression of target genes involved in cell proliferation or apoptosis (Lucas et al. 2008, 2010, Chimento et al. 2010, 2011).

Cyclins A1 and B1, by their association with cyclin-dependent kinases (CDKs), are involved in the control of cell cycle. Cyclin A1, with either CDK2 or CDK1, is implicated in G1/S and G2/M transitions, respectively (Pagano et al. 1992), is essential for the spermatocyte entry in the first meiotic division (Liu et al. 1998), and is expressed exclusively in germ cells (for review, Wolgemuth et al. 2004). Cyclin B1 with CDK1 forms the mitosis promoting factor (MPF) complex and is involved in the control of G2/M transition. Both cyclin B1 and CDK1 are regulated according to the maturation degree of germ cells, with the highest level observed in late spermatocytes, just before the first division of meiosis, but they are still present in round spermatids (Gromoll et al. 1997, Godet et al. 2000), suggesting a function in spermatid maturation in addition to their well-known role in the progression of the cell cycle.

Previous studies have demonstrated that the activation of GPER and ESR1 inhibited the expression of cyclin A1 and cyclin B1 and enhanced the expression of Bax in purified pachytene spermatocytes (Chimento et al. 2010). In addition, the cyclin B1 and Bax gene expressions are down- and up-regulated, respectively, by E2 through an activation of MAPK pathway by ESR1 and GPER in purified round spermatids of adult rats (Chimento et al. 2011). Thus, E2 acts as a regulator of the proliferation/apoptosis balance in rat ST, especially in germ cells.

Therefore, the purpose of our work was to study the potential effect of E2 on cyclin A1 and cyclin B1 gene expression in adult rat ST. We have chosen to use a culture of ST from 90-day-old rats to maintain fundamental interactions between Sertoli and germ cells within tubules. Two groups of stages of the seminiferous epithelium have been defined, II–VIII and IX–I, and treated with either E2 agonists or antagonists for ESRs.

Materials and methods

Animals

Sprague–Dawley rats (Centre Universitaire de Ressources Biologiques, Caen, France) were housed under standard conditions (14 h light:10 h darkness cycle and controlled room temperature (21 ± 1 °C)) with food and water ad libitum. All animal procedures were carried out in accordance with the French Government Regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l’Agriculture).

Tissue collection

Ninety-day-old Sprague–Dawley rats were killed by decapitation. Then, testes were decapsulated and placed in PBS.

Culture of ST from 90 day-old rat testis

The interstitial tissue was mechanically removed under microscopic control. Two groups of stages were identified with a transillumination microscope and separated: the group II–VIII corresponding to the darkest zones of the ST (containing stages II–VIII) and the group IX–I which appears clear under the microscope (corresponding to stages IX–I). ST were cut in 5-mm-length pieces and 5 cm of each group was cultured in 500 μl of Ham’s F12/DMEM medium without Phenol Red (pH 7.4) supplemented with 2% of Replacement Serum 3 (Sigma–Aldrich), antibiotics (penicillin 50 000 IU/l, streptomycin 50 mg/l, kanamycin 50 mg/l, fungizon 0·25 mg/l; Dutscher, Brumath, France), and 10 μg/ml of insulin (Sigma–Aldrich; Boujrad et al. 1995) in a humidified atmosphere with 5% CO2 and 95% air at 32°C. After a pre-treatment for 1 h with or without 10−7 or 10−5 M of ICI 182 780 (Sigma–Aldrich), tubules were incubated for 24 h with 10−9 or 10−6 M of E2 (Sigma–Aldrich; Chimento et al. 2010), with 10 ng/ml of follicle stimulating hormone (hFSH; Organon, Levallois-Perret, France), or with 10−6 M of letrozole (Sigma–Aldrich) which allows complete inhibition of aromatase activity (Mor et al. 2001), or with 10−6 M of G1 (Merck).
Squash preparations

ST at \( t=0 \) and \( t=24 \) h of culture were submitted to squash as previously described by Kotaja et al. (2004). Briefly, ST of 2 mm length were taken in a PBS volume of 40 μl, layered on slices, and covered with a coverslide triggering the spreading of tubular cells. Then, samples were fixed with liquid nitrogen, the coverslide was removed, and the slices were immersed in cold ethanol 96% for 2 min and air dried.

RNA extraction

The TRI reagent solution (Sigma–Aldrich) was used to extract total RNA according to the manufacturer’s instructions. The concentration of RNA samples was determined by the absorbance at 260 nm. The purity and the integrity of the RNAs were checked by measuring the optical density at 260 and 280 nm, followed by an electrophoresis on 1% agarose gel stained with 0·01% ethidium bromide (v/v; Sigma–Aldrich).

Reverse transcription and real-time PCR assay

Two hundred and fifty nanograms of total RNA was reverse transcribed for 90 min at 37 °C with 100 IU Moloney Murine Leukemia Virus Reverse Transcriptase, 10 IU RNasin, 0·1 μM M dNTP (Promega) and 0·25 μM of each primer (Eurogentec, Angers, France) in a total volume of 20 μl. The negative control was obtained by adding water instead of diluted cDNA. Primers were designed to be placed on different exons and are presented in Table 1. PCR amplification, an increase of 0·5 \( ^\circ \)C every 10 s from 50 to 95 °C was allowed to obtain the melting curve. PCR efficiency, determined for each set of primers using a range dilution of reverse transcription (RT) products (1:5, 1:10, 1:20, 1:50, 1:100), comprised between 96 and 105%. Relative levels of specific mRNA were obtained with the formula \( 2^{-\Delta \Delta C_t} \), the reference being L19 transcripts (Tena-Sempere et al. 2002).

Sequence analyses

Each amplified cDNA was sequenced (Cogenics, Takeley, UK). Then, the sequences were subjected to Basic Local Alignment Search Tool (BLAST) homology search (www.ncbi.nlm.nih.gov/BLAST/; Altschul et al. 1990). Promoter regions of cyclin A1 and cyclin B1 gene were analyzed using TF Search and Softberry online software. The threshold was fixed at 0·85.

Terminal dUDP transferase nick end labeling

Squash preparations were rehydrated in PBS buffer and apoptosis was evaluated using the Terminal dUDP Transferase Nick End Labeling (TUNEL) Apoptosis Detection Kit (Upstate, Lake Placid, NY, USA) according to the manufacturer’s instructions. A positive control was performed using a DNase treatment. After the labeling of DNA breaks, samples were mounted with a specific medium containing DAPI (UltraCruz Mounting Medium, Santa Cruz, Heidelberg, Germany) and observed using a fluorescence microscope (Leica, Nanterre, France).

Statistical analysis

Results were expressed as means ± s.e.m. of three experiments, with each sample run in duplicate. Statistical analyses were performed using the software

**Table 1 Sequences of oligonucleotides used for real-time PCR and size of PCR products**

<table>
<thead>
<tr>
<th>Gene (GenBank accession number)</th>
<th>Primers name, position and sequence (5’–3’)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cycline A1 (NM_001011949.1)</strong></td>
<td>5’ CyclA1 5’1189-CAGTACTTTAGGCGGCAAGG 3’ CyclA1 5’1292-TCAAGAATGGTCTAGCTTCC</td>
<td>94</td>
</tr>
<tr>
<td><strong>Cycline B1 (NM_171991.2)</strong></td>
<td>5’ CyclB1 5’350-ACCAGAGGTGGAATCCCGGATG 3’ CyclB1 5’454-GAGCTTTGAGGAGGGATATTC</td>
<td>96</td>
</tr>
<tr>
<td><strong>L19 (NM_031103)</strong></td>
<td>5’ L19 5’355-GGAATCTAAGAAGATTGACGCTC 3’ L19 5’477-GCCCTTGTCGCTTCAGTTT</td>
<td>120</td>
</tr>
<tr>
<td><strong>Protamine 2 (NM_012873.1)</strong></td>
<td>5’ P2 5’167-GGCTTACAGGATCCACAAGA 3’ P2 5’309-ATAGTGGCACCCTGACATTC</td>
<td>143</td>
</tr>
<tr>
<td><strong>Transition protein 2 (NM_017057.2)</strong></td>
<td>5’ TP2 5’242-TGACACTCTGTATTACCCACCTTCC 3’ TP2 5’391-ATTCGTCCTGACATTC</td>
<td>150</td>
</tr>
<tr>
<td><strong>SCF (M59966.1)</strong></td>
<td>5’ SCF 5’516-TGGTGATGCGATGAAAG 3’ SCF 5’660-TCAGATGGCACCAGGAAAGTC</td>
<td>145</td>
</tr>
</tbody>
</table>
GraphPad Instat (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by a Tukey test (comparison of at least three groups of data) or unpaired Student’s t-test (comparison of two groups of data) was used to identify significant differences. Statistical significance was set at $P \leq 0.05$.

**Results**

**Selection and quality of the two groups of adult rat ST**

In order to control the proper selection of the two groups of ST stages (II–VIII and IX–I), we measured the relative levels of transition protein 2 ($TP2$), cyclin A1, and cyclin B1 transcripts, which were shown to be regulated through the seminiferous epithelium in the rat and more expressed in stages IX–I than in stages II–VIII (Johnston et al. 2008). The expressions of $TP2$, cyclin A1, and cyclin B1 genes were around five-, three-, and two-fold higher in group IX–I than in group II–VIII respectively (Fig. 1A–C). These results confirmed the good selection of the two groups of stages II–VIII and IX–I, and thus the enrichment of haploid cells and spermatocytes in groups II–VIII and IX–I respectively.

In order to control the quality of ST after 24 h of culture, we verified the relative levels of cyclin A1 and cyclin B1 mRNA in the two groups of stages II–VIII and IX–I before ($t = 0$) and after ($t = 24$ h) 24 h of culture. There was no difference in cyclin A1 and cyclin B1 mRNA relative levels between $t = 0$ and $t = 24$ h in the two groups of stages (Fig. 1B and C). As Yan et al. (1999) found a positive effect of FSH on stem cell factor ($SCF$) gene expression in ST, we treated ST with 10 ng/ml of hFSH. In our culture conditions, we have observed that this treatment for 24 h induced an increase of $SCF$ mRNA relative levels in the two groups of stages (Fig. 1D). Moreover, there was no change in apoptosis levels measured by TUNEL between $t = 0$ and $t = 24$ h (data not shown).

**Effect of addition and depletion of E$_2$ in ST stages IX–I**

In order to study the effect of E$_2$ on cyclin A1 and cyclin B1 gene expression in ST, we chose to treat ST from stages IX–I and II–VIII with 10$^{-9}$ and 10$^{-6}$ M of E$_2$ for 12 and 24 h. There was no effect of E$_2$ on cyclin A1 and cyclin B1 after 12 h of treatment (data not shown) and similar data were observed in the group of stages II–VIII for cyclin A1 gene expression, as for cyclin B1 after 24 h of treatment with E$_2$ (Fig. 2C and D). In the group of stages IX–I, the relative level of cyclin A1 mRNA was enhanced by around 50% with the concentration of 10$^{-9}$ M of E$_2$ and there was no effect with the higher concentration of E$_2$ (Fig. 2A). Concerning cyclin B1 in the same group of stages, the relative level of mRNA was enhanced by around 50% with the concentration of 10$^{-9}$ M of E$_2$ and there was no effect with the higher concentration of E$_2$ (Fig. 2A). Concerning cyclin B1 in the same group of stages, the relative level of mRNA was enhanced by around 50% with the concentration of 10$^{-9}$ M of E$_2$ and there was no effect with the higher concentration of E$_2$ (Fig. 2A).

![Figure 1](https://www.endocrinology-journals.org) Characterization and quality of the two groups of stages II–VIII and IX–I. (A) Transition protein 2 ($TP2$), (B) cyclin A1, (C) cyclin B1, and (D) SCF relative gene expression in groups II–VIII and IX–I ST was measured by real-time PCR. Relative gene expression of $TP2$ was determined at $t = 24$ h and SCF was determined after incubation with or without hFSH (10 ng/ml) for 24 h. Results were expressed as means $\pm$ S.E.M. of samples of (A–C) one representative experiment or (D) three experiments. The average value for the group II–VIII was fixed at 1. Different letters mean significant differences ($P \leq 0.01$). *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$. [Downloaded from Bioscientifica.com at 11/28/2018 12:11:14PM via free access]
increased by 40% with a concentration of $10^{-9}$ M of E$_2$ and was reduced by 40% with $10^{-6}$ M of E$_2$ (Fig. 2B).

ST were also treated with $10^{-6}$ M of letrozole, which allows an inhibition of local aromatase activity, to evaluate if cyclin A1 and cyclin B1 gene expression was under the control of E$_2$ in basal conditions. After 24 h of treatment with letrozole, relative levels of cyclin A1 and cyclin B1 mRNA were reduced by 20 and 40%, respectively, in the group of stages IX–I (Fig. 3).

These results show that cyclin A1 and cyclin B1 gene expression was under the control of endogenous E$_2$ in stages IX–I of the rat seminiferous epithelium.

**Effect of a pre-treatment with ICI 182 780 on cyclin A1 and cyclin B1 gene expression regulated by E$_2$ in ST stages IX–I**

In order to determine the identity of receptors involved in the increase of cyclin A1 and cyclin B1 mRNA levels in response to E$_2$, we used the ESR antagonist ICI 182 780. Obviously, ESR antagonist inhibited the enhancement of cyclin A1 gene expression in response to $10^{-9}$ M of E$_2$ at both the concentrations used, $10^{-7}$ and $10^{-5}$ M (Fig. 4A). Concerning cyclin B1, the addition of $10^{-7}$ and $10^{-5}$ M of ICI 182 780 blocked the effects of $10^{-9}$ and $10^{-6}$ M of E$_2$ (Fig. 4B). So, the regulation of cyclin A1 and cyclin B1 gene expression by E$_2$ might be mediated by ESR1 and/or ESR2.

Moreover, cyclin A1 gene expression was increased by around 40% in the presence of $10^{-7}$ M of ICI 182 780 alone and cyclin B1 mRNA relative levels were enhanced by about 30% with both the concentrations of ICI 182 780 used alone (Fig. 4).

**Effect of G1, a GPER agonist, on cyclin A1 and cyclin B1 gene expression induced by E$_2$ in ST stages IX–I**

As ICI 182 780 induced an increase of cyclin A1 and cyclin B1 gene expression (Fig. 4), we aimed to determine whether these genes could be regulated through the GPER pathway. Indeed, it was shown that ICI 182 780 could act as an agonist of GPER (Thomas et al. 2005). So, we treated ST from stages IX–I for 24 h with the specific GPER agonist G1. Relative levels of cyclin A1 and cyclin B1 mRNA were enhanced by about 50% in the presence of G1 (Fig. 5). Moreover, this stimulation of cyclin A1 and cyclin B1 gene expression by G1 was identical to that observed with $10^{-9}$ M of E$_2$ (Fig. 5). These data suggest the involvement of GPER in the regulation of cyclin A1 and cyclin B1 gene expression by E$_2$ in rat ST from stages IX–I.

**Discussion**

Our study shows that cyclin A1 and cyclin B1 gene expression in rat ST is likely under estrogen control. In seminiferous epithelium, these two cyclins are expressed mainly in germ cells (Johnston et al. (2008), for review, Wolgemuth et al. (2004)), but we have chosen the culture of ST which allows cellular associations to be maintained between Sertoli and germ cells and between Sertoli cells themselves (for review, Mruk & Cheng (2004)). Moreover, germ cells
Our results clearly show that \( \text{E}_2 \) at the concentration of \( 10^{-9} \text{ M} \) had a positive effect on cyclin A1 and cyclin B1 gene expression. This effect was observed only in stages IX–I in which the proportion of spermatocytes is higher compared to the group II–VIII. As cyclin A1 and cyclin B1 are involved in the control of progression of the cell cycle, our results suggest that estrogens could be concerned in the progression of the germ cell maturation. These results are in agreement with those observed in 1-year-old mice after aromatase gene knockout (\( \text{ArKO} \)) which triggered a decrease in the number of spermatocytes, and round and elongated spermatids (Robertson et al. 1999, 2002). In vitro studies with human ST treated with low concentrations of \( \text{E}_2 \) \( (10^{-9} \text{ and } 10^{-10} \text{ M}) \) have shown a diminution of germ cell apoptosis determined by quantification of low molecular weight DNA and in situ end labeling, whereas higher concentrations of \( \text{E}_2 \) did not significantly inhibit germ cell death (Pentikäinen et al. 2000). Therefore, estrogens are probably involved in the control of the balance apoptosis/proliferation during spermatogenesis. Our data concerning the effect of \( \text{E}_2 \) on cyclin A1 and cyclin B1 gene expression apparently seem to disagree with our previous data obtained in purified adult rat pachytene spermatocytes treated for 6 h with 100 nM of \( \text{E}_2 \) or in purified round spermatids treated for 6 h with 1 \( \mu \text{M} \) of \( \text{E}_2 \). In fact, previously we had observed a decrease of cyclin A1 and cyclin B1 and an increase of \( \text{Bax} \) mRNA (Chimento et al. 2010, 2011). Nevertheless, in the present study, we have obtained at the concentration of \( 10^{-6} \text{ M} \) of \( \text{E}_2 \), a decrease in cyclin B mRNA as previously observed in round spermatids (Chimento et al. 2011). An explanation can be found in the effects produced by the agonists on ERK1/2 activation. Treatment of pachytene spermatocytes or purified round spermatids with \( \text{E}_2 \) \( (0.1 \text{ and } 1 \mu \text{M}) \) respectively or \( \text{G1} \) \( (1 \mu \text{M}) \) for 30 min increased ERK1/2 phosphorylation (Chimento et al. 2010, 2011). This effect on ERK1/2 phosphorylation is maintained for up to 6 h, but is lost at 24 h of treatment in GC2 pachytene spermatocyte cell line (Pezzi, personal communication). Moreover, using \( \text{E}_2 \) \( (1 \text{nM}) \) for 30 min did not increase ERK1/2 phosphorylation in pachytene spermatocytes or purified round spermatids (Chimento et al. 2010, 2011). These data support the hypothesis that low doses of \( \text{E}_2 \) as well as a long period of treatment with \( \text{E}_2 \) do not activate ERK1/2 and allow cyclins’ regulation. In fact, here we report an up-regulation of cyclin B1 using 1 \( \text{nM} \) of \( \text{E}_2 \) for 24 h and a down-regulation using 1 \( \mu \text{M} \). A similar mechanism occurs with the use of 1 \( \mu \text{M} \) of \( \text{G1} \) in GC2 pachytene spermatocyte cell line, wherein especially treatment for up to 6 h activates ERK1/2, and cyclin A1 and cyclin B1 are down-regulated (Pezzi, personal communication). Since here we reported an increase in cyclin A1 and cyclin B1 expression after 24 h treatment with 1 \( \mu \text{M} \) of

![Figure 3](image-url)

**Figure 3** Effect of letrozole on (A) cyclin A1 and (B) cyclin B1 gene expression in seminiferous tubules (ST) from groups II–VIII and IX–I. Treatment with letrozole \( (10^{-6} \text{ M}) \) was done for 24 h and measurements were obtained by real-time PCR. Results were expressed as means of three experiments \( \pm \text{S.E.M.} \). The average values for controls in the group of stages II–VIII were fixed at 1. Different letters mean significant differences \( (P \leq 0.01) \).

need Sertoli cells which support and produce the factors essential for their normal development. Finally, it has been shown in co-cultures of Sertoli cells and germ cells that the effect of FSH on aromatase expression was lower when Sertoli cells were cultured with germ cells (Bouraima-Lelong et al. 2010), suggesting a sophisticated dialog between these two types of cells.

This model of ST culture was developed by Parvinen (1982) and allowed experiments up to 72 h. Our culture conditions are the same as those used previously in our laboratory by Boujrad et al. (1995) and we did not observe any enhancement of apoptosis level after 24 h of culture. However, we decided to conduct our study with two groups of stages because the expression of cyclin A1 and cyclin B1 genes is regulated through the level of maturation of germ cells and the stage of seminiferous epithelium (Godet et al. 2000, Johnston et al. 2008). Thus, these data allowed us to define two groups of stages (II–VIII and IX–I) in which the cyclin A1 and cyclin B1 gene expression was different.
G1, this effect could depend on the absence of ERK1/2 activation. Moreover, in purified pachytene spermatoocytes and round spermatids, when ERK1/2 is activated, apoptosis is triggered as a consequence of Bax up-regulation, in ST, long-term E2 and G1 treatments are not associated with apoptosis as demonstrated by the lack of changes in Bax gene expression (data not shown). These observations suggest a central role for ERK1/2 in the mechanism controlling meiosis progression, an event regulated by estrogens.

In our study, the two E2 concentrations used (10^{-9} and 10^{-6} M) differentially affected the expression of cyclin A1 and cyclin B1 genes. So, the effect of E2 could be related to the dose, the time of incubation, the cell environment (isolated germ cells or whole ST), and the type of ESRs concerned. These effects are probably focused directly by germ cells because these cells express the ESRs (ESR1, ESR2) and GPER (Tirado et al. 2004, Chimento et al. 2010, 2011).

As adult germ cells produce estrogens (for review, Carreau & Hess (2010)), we treated ST with letrozole, an aromatase inhibitor, to determine the effects on cyclin A1 and cyclin B1 gene expression of long-term E2 deprivation. This treatment caused a decrease in the gene expression of both the cyclins in stages IX–I, confirming the presence of endogenous androgens and therefore an involvement of E2 in the control of meiosis progression of germ cells.

To demonstrate the involvement of ESRs in this mechanism, we used an ESR selective inhibitor, ICI 182 780, alone and in combination with E2 at the doses effective in controlling cyclin A1 and cyclin B1. We observed that the presence of ICI for 24 h reversed the effects produced by E2 on cyclin A1 and cyclin B1 gene expression, confirming the involvement of ESRs in this process.

Figure 4 Effect of a pre-treatment with ICI 182 780 on (A) cyclin A1 and (B) cyclin B1 gene expression regulated by E2 in the stages IX–I of the seminiferous epithelium. ICI 182 780 (10^{-7} and 10^{-5} M) was added 1 h before a 24-h treatment with E2 (10^{-9} and 10^{-6} M). Measurements were taken using real-time PCR. Results were expressed as means of three experiments ± s.e.m. The average values for controls without ICI 182 780 were fixed at 1. Different letters mean significant differences (P < 0.05). *P < 0.05, **P < 0.01, and ***P < 0.001. E2, 17β-estradiol.

Figure 5 Effect of a 24-h treatment with G1 (10^{-6} M) and E2 (10^{-9} M) on (A) cyclin A1 and (B) cyclin B1 gene expression in the stages IX–I of the seminiferous epithelium. Measurements were done using real-time PCR. Results were expressed as means of three experiments ± s.e.m. Different letters mean significant differences (P < 0.001).
The effect of estrogens on the expression of target genes can be related to several mechanisms: either binding to their receptor on ERE or by interaction with other transcription factors which bind themselves at alternative DNA sequences (O’Lone et al. 2004). As cyclin A1 and cyclin B1 have no putative ERE in their promoting sequences (data not shown), we can suggest that other factors are involved in the regulation of cyclin A1 and cyclin B1 gene expression by E2 in rat ST, such as AP-1 or Sp-1 (for review, Björnström & Sjöberg (2005)). Moreover, it has been shown that an activation of the cyclin D1 promoter by E2 is observed in the presence of ESR1, whereas E2 linked to ESR2 induced an inhibition (Liu et al. 2002).

In addition to the classical ESRs acting as transcription factors, ESRs have been shown to be associated with the plasma membrane: GPER and ESRs (for review, Watson et al. (2007)). In that context, we have chosen to test if GPER could be implicated in cyclin A1 and cyclin B1 gene expression regulated by E2. As GPER is able to trigger responses consisting in the activation of protein kinases responsible for the phosphorylation of transcription factors c-Fos and c-Jun, the rapid effects induced by E2 can lead to the regulation of target genes containing CRE or AP-1 sites (for review, Björnström & Sjöberg (2005)). The differential expression of c-FOS and C-JUN according to the stages of the seminiferous epithelium could be responsible for the difference in cyclin A1 and cyclin B1 gene expression in the two groups of stages II–VIII and IX–I in basal conditions and in the presence of E2 (Schultz et al. 1995). So, we treated ST with the specific agonist of GPER, G1. The concentration of 10⁻⁶ M of G1 induced an increase in cyclin A1 and cyclin B1 gene expression to levels identical to those observed with 10⁻⁹ M E₂. Moreover, the treatment with ICI 182 780 alone induced an enhancement of relative levels of cyclin A1 and cyclin B1 mRNA. This can be explained by the fact that this ESR antagonist can act as a GPER agonist (Thomas et al. 2005). So, the regulation of cyclin A1 and cyclin B1 gene expression in ST seems to be mainly mediated by GPER. GPER, through the activation of ERK 1/2, regulated the cyclin A1 and cyclin B1 gene expression in purified pachytyne spermatocytes and round spermatids respectively (Chimento et al. 2010, 2011).

So, in this work, we have demonstrated that the expression of cyclin A1 and cyclin B1 genes, regulators of cell cycle progression, is under the control of E2 in ST from stages IX to I. Taking into account the cellular localization of cyclin A1 and cyclin B1 transcripts and/or proteins, we can suggest that this hormone could regulate the differentiation of meiotic germ cells. This effect implicates ESRs (ESR1 and/or ESR2) and GPER which are expressed in the ST, suggesting a complex and/or redundant regulation mechanism of genes expression by E2 in adult rat ST.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
C B, C D, and H B-L carried out the seminiferous tubules cultures, all the molecular studies were carried out by C B, C D, and P D, and S C supervised this work.

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