Age- and cell-related gene expression of aromatase and estrogen receptors in the rat testis

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Abstract

Spermatogenesis is a complex and coordinated process leading to the formation of spermatozoa. This event, which is under the control of numerous endocrine and paracrine factors, seems to also be controlled by estrogens which exert their effects via nuclear estrogen receptors (ESRs) ESR1 and ESR2. Estrogens are synthesized by aromatase which is biologically expressed in the rat testis. The objective of our study was to clarify the gene expression patterns of aromatase and ESRs according to age and in the two compartments of the adult rat testis. In the adult, transcripts of aromatase vary according to the germ cell type and to the stages of seminiferous epithelium, a maximum being observed at stage I. The ESR1 gene is highly expressed in the adult testis and in stages from VIIIc–d to XIV. Moreover, both ESR mRNA levels are higher in purified round spermatids than in pachytene spermatocytes, suggesting a putative role of estrogens in the haploid steps of spermatogenesis. The variability of the results in the expression of both ESRs led us to explore the putative presence of variants in the rat testis. Concerning ESR1, we have shown the presence of the full-length form and of one isoform with exon 4 deleted. For ESR2, besides the wild type, three isoforms were observed: one with exon 3 deleted, another with an insertion of 54 nucleotides, and the last one with both modifications. Therefore, the stage-regulated expression of aromatase and ESR1 genes in the rat testis suggests a likely role of estrogens in spermatogenesis.

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Introduction

The seminiferous epithelium supports the spermatogenesis which is a complex process leading to the formation of spermatozoa from spermatogonia. Tubules are composed by specific associations of germ cells and Sertoli cells, which progress synchronously through the stages of seminiferous epithelium. In rat, 14 stages (I–XIV) have been described (Leblond & Clermont 1952, Parvinen 1982, Wing & Christensen 1982). During their progression, germ cells undergo structural and functional changes under the control of several endocrine, paracrine, and autocrine factors (Gnessi *et al.* 1997, Franca *et al.* 1998, McLachlan *et al.* 2002). Among them, estrogens synthesized by the microsomal enzymatic complex aromatase are recognized as potential regulators of spermatogenesis in several species (O'Donnell *et al.* 2001, Carreau & Hess 2010).

Gene modification in mice and men has brought us valuable information: a decrease in quantity and quality of spermatids in aromatase-deficient male mice (aromatase knockout; ArKO) was observed, resulting

in the infertility of 1-year-old animals (Robertson *et al.* 1999). Conversely, hypertrophy and hyperplasia of Leydig cells associated with infertility were observed in mice overexpressing aromatase (Li & Rahman 2008). Moreover, some men with the congenital aromatase deficiency syndrome present impaired quality and quantity of spermatids associated with a reduction in the motility and in the number of spermatozoa (Jones *et al.* 2006).

In the rat testis, several studies concerning aromatase expression at transcript (Janulis *et al.* 1996, Levallet & Carreau 1997, Levallet *et al.* 1998a,b, Bourguiba *et al.* 2003a,b, Silandre *et al.* 2007) and protein levels (Levallet *et al.* 1998a, Carpino *et al.* 2001) as well as its biological activity (Levallet *et al.* 1998a, Bourguiba *et al.* 2003a,b) have been published. Aromatase is expressed both in the interstitial tissue and in the seminiferous tubules. Sertoli cells are the main source of estrogens in immature rats, whereas in adults, estrogens are synthesized by Leydig and germ cells (Carreau *et al.* 2009).

Genomic effects of estrogens are mediated by nuclear estrogen receptors (ESRs) ESR1 and ESR2 (Heldring *et al.* 2007), which are both expressed in testicular cells of

several species (Carreau & Hess 2010). However, data concerning their localization in adult rat testis are not consistent. Indeed, ESR1 has been immunodetected only in Leydig cells by Fisher *et al.* (1997) and by Saunders *et al.* (1998), but was also revealed in the seminiferous compartment by Pelletier *et al.* (2000). About ESR2, all studies are in agreement concerning its localization in seminiferous tubules but data are in conflict regarding its presence in germ cells. While Saunders *et al.* (1998) and van Pelt *et al.* (1999) found ESR2 in Sertoli cells and in different germ cell types (spermatogonia, pachytene spermatocytes (PS), and round spermatids (RS)), Pelletier *et al.* (2000) localized ESR2 was only found in Sertoli cells. ESR transcripts have been studied using *in situ* hybridization: ESR1 mRNAs have been found in Leydig cells (Mowa & Iwagana 2001) and spermatids (Pelletier *et al.* 2000); ESR2 mRNA has been revealed in Sertoli cells and spermatocytes (Shugrue *et al.* 1998) while van Pelt *et al.* (1999) retrieved them also in A spermatogonia and RS. It is of note that the presence of ESRs either at transcript or protein level has never been observed in peritubular cells (Fisher *et al.* 1997, Saunders *et al.* 1997, 1998, Shugrue *et al.* 1998, van Pelt *et al.* 1999, Pelletier *et al.* 2000, Mowa & Iwagana 2001).

The synchrony of germ cell development suggests the existence of precise and coordinated cyclic programs of gene expression (Kimmins *et al.* 2004, Johnston *et al.* 2008). Indeed, the expression of numerous genes implicated in biological pathways and processes in spermatogenic cells and Sertoli cells is regulated in a stage-dependent manner (Johnston *et al.* 2008). As several testicular cell types express aromatase and ESRs and since data published are not in accordance, we wish to carefully clarify the expression patterns of aromatase and ESRs in rat testis in relation to age but also in purified germ cells, Sertoli cells, Leydig cells, and in seminiferous tubules at defined stages of the cycle of adult rat. In addition, we enlighten the presence of variants of estrogen receptors ESR1 and ESR2 in the adult testis.

Materials and methods

Animals

Sprague–Dawley rats (Janvier, Le Genest Saint-Isle, France) were housed under standard conditions (12 h light:12 h darkness cycle and controlled room temperature) with food and water provided *ad libidum*. 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) and approved by the local ethical committee of the University of Caen Basse-Normandie.

Sprague–Dawley rats were housed at the Animal Center of Turku University (Turku, Finland) in a

controlled environment with access to food and water *ad libidum*. All experiments were preapproved by the Committee on the Ethics of Animal Experimentation of the University of Turku.

Tissue collection

Rats (10, 20, 30, 70, and 90 days old) were killed by decapitation either after CO₂ anesthesia or not, and tissues (testis, brain, and pituitary) were dissected, placed in PBS, or flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Collection of seminiferous tubules from adult rat testes

Heterogeneous seminiferous tubules were prepared from decapsulated testes of 70-day-old rats. Interstitial tissue was eliminated mechanically under microscopic control. Seminiferous tubules from decapsulated testes of 90-day-old rats were subjected to transillumination-assisted microdissection as previously described (Toppari & Parvinen 1985) to collect 5 cm of each stage or group of stages (I, II–III, IV–V, VI, VIIa–b, VIIc–d, VIII, IX–XI, XII, and XIII–XIV). Samples were flash-frozen and stored at -80°C until RNA extraction.

Purification of immature rat Sertoli cells

Sertoli cells were isolated from testes of 10-, 20- and 30-day-old rats by sequential enzymatic digestions according to the method described by Dorrington *et al.* (1975). Sertoli cells were seeded at a density of 100 000, 200 000, and 650 000 cells/cm² for 10-, 20-, and 30-day-old rats respectively, and cultured for 72 h in Ham's F12/DMEM (Gibco) supplemented with 2% ultrosor SF (v/v; Biosepra, Cergy-Pontoise, France) in a humidified atmosphere of 5% CO₂ and 95% air at 32 °C. On day 3, germ cells were removed by a hypotonic treatment with 20 mM Tris–HCl (pH 7.2; Sigma), and 5 days after plating, Sertoli cells were rinsed with PBS and stored at -80°C until RNA extraction.

Purification of adult rat Leydig, Sertoli, and germ cells

Testes of 70-day-old Sprague–Dawley rats were decapsulated and submitted to a first enzymatic digestion with collagenase–dispase 0.05%, DNase 0.001%, and soybean trypsin inhibitor (STI) 0.005% (w/v; Sigma) for 15 min at 37 °C. After centrifugation, Leydig cells contained in the supernatant were purified on a discontinuous Percoll gradient (Papadopoulos *et al.* 1985). After several washes in PBS buffer, Leydig cells were stored at -80°C until RNA extraction. The pellet was submitted to a second enzymatic digestion with

collagenase–dispase 0.05%, DNase 0.001%, and STI 0.005% (w/v; Sigma) for 30 min at 37 °C and to a final enzymatic digestion with hyaluronidase 0.1%, STI 0.005%, and DNase 0.001% (w/v; Sigma) during 30 min at 37 °C. After centrifugation, the supernatant and the pellet, containing Sertoli cells and germ cells, were filtered through glass wool to remove spermatozoa. After several washes in PBS buffer, cells were seeded at a density of 650 000 cells/cm² in Ham's F12/DMEM (Gibco) as previously described by Silandre *et al.* (2007) and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 32 °C. After 24 h of culture, the medium containing germ cells was washed with PBS, and germ cells were purified (PS and RS) by unit gravity sedimentation through a BSA (Roche Diagnostics) gradient (0.2–2.75%; w/v) in a Sta-Put apparatus (Bellve *et al.* 1977). The fractions enriched with PS and RS were identified under microscope as reported elsewhere (Levallet *et al.* 1998a). To estimate the contamination of the germ cell fractions by Leydig cells, a 3 β -hydroxysteroid dehydrogenase histochemical staining (Klinefelter *et al.* 1987), a specific marker of Leydig cells, was realized, showing <1% contamination. For both germ cell preparations, the purity was higher than 95% (the major contamination was by other germ cells; Silandre *et al.* 2007). Cells were washed with PBS buffer and stored at –80 °C until RNA extraction. Sertoli cells were cultured for an additional period of 48 h in Ham's F12/DMEM (Gibco) supplemented with 2% ultrosor SF (v/v; Biosepra) in a humidified atmosphere of 5% CO₂ and 95% air at 32 °C, and remaining germ cells were removed by a hypotonic treatment. Five days after plating, Sertoli cells were washed with PBS buffer and stored at –80 °C until RNA extraction.

RNA extraction

The TRI Reagent solution (Sigma) was used to extract total RNA according to the manufacturer's instructions.

The concentration of samples was determined by the absorbance at 260 nm. The purity and the integrity of the RNAs were checked by measuring optical density at 260 and 280 nm followed by an electrophoresis on 1% agarose gel stained with 0.01% ethidium bromide (v/v; Sigma).

Reverse transcription and PCR assay

Two micrograms of total RNA were reverse transcribed for 90 min at 37 °C with 200 IU Moloney murine leukemia virus reverse transcriptase, 20 IU RNasin, 0.2 μ g random hexamers, and 500 μ M dNTP in a total volume of 40 μ l. Four microliters of cDNA were used for PCR which was performed using 1.5 IU Taq DNA polymerase (Promega) in a PCR buffer containing 200 μ M dNTP, 1.5 mM MgCl₂, and 25 pmol of each primer (Eurogentec, Angers, France) in a total volume of 50 μ l. The negative control was realized by adding water instead of cDNA. PCR primers are presented in Table 1. PCRs were performed as follows in the Stratagene Gradient Cycler (Agilent Technologies, Massy, France): an initial step at 95 °C for 5 min and then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and a final step of elongation at 72 °C for 10 min. Resulting PCR products were separated by electrophoresis on a 2% (w/v) agarose gel stained with 0.01% (v/v) ethidium bromide (Sigma).

Purification of PCR products

PCR fragments were eluted from the agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions.

Reverse transcription and real-time PCR assay

In total, 250 ng of total RNA were reverse transcribed for 90 min at 37 °C with 100 IU Moloney Murine

Table 1 Sequences of oligonucleotides used for PCR

Gene (GenBank accession number)	Primers name, position, and sequence
ER α (X61098)	5'ER α 5'- ₆₁₀ GATCATGGAGTCTGCCAAGG 3'ER α 5'- ₁₄₉₅ CAGAGACTTCAAGGTGCTGGA 5'ER α (nested) 5'- ₇₇₂ CATCGATAAGAACCGGAGGA 3'ER α (nested) 5'- ₁₂₄₅ ATCTCCAACCAGGCACACTC
ER β (U57439)	5'ER β 5'- ₆₅₇ GAGGAAGCTTAGTGGGAGCA 3'ER β 5'- ₁₄₂₆ GCCAGGAGCATGTCAAAGAT 5'ER β (nested) 5'- ₇₅₁ CTGGGTATCATTACGGCGTT 3'ER β (nested) 5'- ₁₄₀₄ CCAGAATCCCTTCTACGCAC
ER β 2 (AF042059.1)	5'ER β (nested) 5'- ₇₅₁ CTGGGTATCATTACGGCGTT 3'ER β 2 5'- ₉₇₉ GCCAGTGAGGGTCTTCTGAG

Table 2 Sequences of oligonucleotides used for real-time PCR and size of PCR products

Gene (GenBank accession number)	Primer name, position, and sequence	Size of PCR product (bp)
L19 (NM_031103)	5'L19 5' ₃₅₈ -GGAATCTAAGAAGATTGACCGTC 3'L19 5' ₄₇₇ -GCCTTGCTGCGCTTCAGTTT	120
Aromatase (M33986)	5'Aro 5' ₁₅₄₆ -CGTCATGTTGCTTCTCATCG 3'Aro 5' ₁₆₉₅ -TACCGCAGGCTCTCGTTAAT	150
ESR1 (X61098)	5'ESR1 5' ₅₄₅ -AATTCTGACAATCGACGCCAG 3'ESR1 5' ₈₈₉ -GTGCTTCAACATTCTCCCTCCTC	345
ESR2 (U57439)	5'ESR2 5' ₂₁₀₇ -CTTGCCCACTTGGAACATC 3'ESR2 5' ₂₂₅₈ -CCAAAGGTTGATTTTATGGCC	152

Leukemia Virus Reverse Transcriptase, 10 IU RNasin, 0.1 µg random hexamers, and 500 µM dNTP (Promega) in a total volume of 20 µl. Five microliters of diluted cDNA (1:20) were used for quantitative PCR with 10 µl of 2× iQ SYBR Green Supermix (Bio-Rad) and 0.25 µM of each primer (Eurogentec) in a total volume of 20 µl. The negative control was realized by adding water instead of diluted cDNA. Primers were designed to be placed on different exons except for ESR2 primers, and are presented in Table 2. PCR was performed as follows in the iCycler IQ real-time PCR detection system (Bio-Rad): an initial step at 95 °C for 2 min and 30 s and then 45 cycles of 95 °C for 10 s and 60 °C for 60 s. At the end of the amplification, an increase in 0.5 °C every 10 s from 50 to 95 °C allowed to obtain the melt curve. PCR efficiency, measured for each set of primers using a range dilution of reverse transcription (RT) products (1:5; 1:10; 1:20; 1:50; 1:100), was comprised between 96 and 105%. Relative levels of specific mRNA were obtained with the formula $2^{-(C_t \text{ gene} - C_t \text{ ref})}$, the reference being L19 transcripts (Tena-Sempere *et al.* 2002).

Sequence analyses

Each cDNA amplified 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).

Statistical analysis

Results are means ± S.E.M. of three to five experiments, each sample performed in duplicates. Statistical analysis was performed using the software 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 accepted at $P \leq 0.05$.

Results

Evolution of aromatase gene expression in rat testis

The aromatase mRNA expression in Sertoli cells was low at 10 days; it was four times higher in cells from 20-day-old rats, and at 30 days it fell to the same level as observed at 10 days. In adult, aromatase transcripts were undetectable in Sertoli cells in our experimental conditions (Fig. 1), whereas the seminiferous tubules contained similar levels as the Leydig cells (Fig. 2A). In whole gonad, the levels of aromatase mRNA increased between 10 and 30 days of age and remained at that level in the adult testis (data not shown), suggesting a germ cell expression of that enzyme. Indeed, the aromatase mRNA level was twofold higher in PS compared with RS (Fig. 2B). Within the seminiferous tubules, the aromatase expression varied according to the stages of the seminiferous epithelium: it began to increase in stage XIII and reached its maximum at stage I, whereafter it progressively decreased until stage IV and was stable until stage XII (Fig. 2C).

Evolution of ESR1 gene expression and identification of variants in the rat testis

In order to study the ESR1 expression in the rat testis, we used primers designed to amplify the sequence coding for DNA-binding domain (Table 2). Changes in the expression of ESR1 transcripts in the whole testis

Figure 1 Aromatase gene expression in rat Sertoli cells according to age. Measures were done using real-time RT-PCR. Results are expressed as means ± S.E.M. ($n=3$). The average value for S 10 d is fixed at 1, and different letters (a and b) denote significant differences ($P \leq 0.01$). S, Sertoli cells and d, days.

Figure 2 Aromatase gene expression according to compartment (A), to germ cell type (B), and to the stage of the seminiferous epithelium (C) of the adult rat testis. Measures were done using real-time RT-PCR. (A) Results are expressed as means $\pm$ S.E.M. ($n=3$), and the average value for L 70d is fixed at 1. (B) Results are expressed as means $\pm$ S.E.M. ($n=5$), the average value for PS is fixed at 1, and different letters (a and b) denote significant difference ($P \leq 0.05$). (C) Results are expressed as means $\pm$ S.E.M. ($n=4$), the average value for stage I is fixed at 1, and different letters (a, b, c, and d) denote significant differences ($P \leq 0.05$). L, Leydig cells; ST, seminiferous tubules; PS, pachytene spermatocyte; RS, round spermatid and d, days.

during development were observed: their levels did not vary between 10 and 30 days, whereas there was a sevenfold increase between 30 and 70 days (Fig. 3A). In Sertoli cells, the amount of mRNA increased eightfold

from 10 to 30 days, whereas in adult cells, the transcript levels fell to the same level as recorded at 10 and 20 days of age (Fig. 3B). In the adult, ESR1 mRNA level was sixfold higher in the seminiferous tubules than in the

Figure 3 ESR1 gene expression in the whole rat testis (A) and in rat Sertoli cells (B) according to the age. Measures were done using real-time RT-PCR. Results are expressed as means of three experiments $\pm$ S.E.M. (A) The average value for WT 10d is fixed at 1, and different letters (a and b) denote significant differences ($P \leq 0.001$). (B) The average value for S 10d is fixed at 1, and different letters (a, b, and c) denote significant differences ($P \leq 0.05$). WT, whole testis; S, Sertoli cells and d, days.

Leydig cells (Fig. 4A). In germ cells, ESR1 expression was fourfold higher in RS than in PS (Fig. 4B). In the seminiferous tubules, ESR1 expression was low in stages I–VIIa–b and then increased starting at stage VIIc–d until stage VIII (20-fold higher in stage VIII than in stage VIIa–b), and the level of expression was maintained until stage XIV (Fig. 4C).

ER α mRNA spliced variants have been reported in rat tissues (Friend *et al.* 1997), and thus, we designed primers flanking putative deleted regions to check the presence of these variants in the testis (Table 1). In the rat brain, the fragments of 886 and 613 bp correspond to the size expected for the full-length form and for the form deleted of exons 5 and 6, ER α 5,6 respectively (Friend *et al.* 1997). In the testis, two fragments of 886 and 550 bp were observed (Fig. 5). After sequencing using internal primers (Table 1), the sequence obtained from the 886-bp fragment presented 99% of homology with ESR1 sequence (X61098). The sequence obtained from the 550-bp fragment had a homology of 100% with ESR1 sequence with deleted exon 4 (X61098). The sequence of this variant was submitted to GenBank (GU111761).

Evolution of ESR2 gene expression and identification of variants in the rat testis

Although it seems to have an increase in the 70-day-old whole testis, there was no significant difference in ESR2 expression within the whole testis (Fig. 6A). In Sertoli cells, there was no significant change in ESR2 expression between 10 and 30 days; however, a

significant increase of 20-fold at 70 days was observed (Fig. 6B). In adult testis, ESR2 was expressed at the same level in the seminiferous tubules and in the Leydig cells (Fig. 7A), whereas the receptor was four times more expressed in RS than in PS (Fig. 7B). There were no significant differences according to the stages of the seminiferous epithelium (Fig. 7C).

Petersen *et al.* (1998) have described several ER β spliced variants in rat tissues. Using primers flanking the variable region of ESR2 mRNA previously described (Table 1), we amplified four cDNAs in the rat testis and pituitary (Fig. 8). The sizes of the PCR products in the pituitary and in the testis were 825, 771, 708, and 654 bp, which correspond to the sizes expected for ER β 2, ER β 1, ER β 2 δ 3, and ER β 1 δ 3 respectively. After sequencing using internal primers (5'-ER β (nested)/3'-ER β (nested) for ER β 1 and ER β 1 δ 3, and 5'-ER β (nested)/3'-ER β 2 for ER β 2 and ER β 2 δ 3 (Table 1)), the sequences presented at least 98% of homology with the ER β 2 (AF042059.1), ER β 1 (U57439), ER β 2 δ 3 (AF042061.1), and ER β 1 δ 3 sequences (AF0420690.1) respectively.

Discussion

Our data have shown that the aromatase expression is not only regulated according to age and cell type but also in relation to the stages of the seminiferous epithelium, the maximum being observed between stages XIII and III.

As previously reported (Silandre *et al.* 2007), the aromatase expression in the whole testis was regulated according to age: it increased between 10 and 30 days, and was maximal from 30 days onwards. However, the maximal expression of aromatase in Sertoli cells was observed at 20 days, and then decreased until being undetectable at 70 days. This decrease of the aromatase expression in the adult Sertoli cells could be explained by a negative control of Sertoli cell aromatase expression by germ cells via paracrine regulatory factors (Boitani *et al.* 1981, Silandre *et al.* 2007, Bouraïma-Lelong *et al.* 2010). In adult animals, the aromatase gene expression ensured, in part, by germ cells (up to 50%) and by Leydig cells was demonstrated (Carreau & Hess 2010). This expression was dependent of germ cell type and mainly supported by PS (Silandre *et al.* 2007; our results). Stage-specific expression pattern should reflect transcript levels in germ cells, i.e. late spermatocytes and early RS, and it was reported that despite the protein being localized in PS and RS (Carpino *et al.* 2001), the aromatase activity was weak in PS and progressively increased until it reached a maximum in spermatozoa (Levallet *et al.* 1998a). This delay between transcript expression and protein activity could be explained by the fact that decoupling between

Figure 4 ESR1 gene expression according to the compartment (A), to the germ cell type (B), and to the stage of the seminiferous epithelium (C) of the adult rat testis. Measures were done using real-time RT-PCR. (A) Results are expressed as means of three experiments $\pm$ S.E.M., the average value for L 70 d is fixed at 1, and different letters (a and b) denote significant differences ($P \leq 0.001$). (B) Results are expressed as means of five experiments $\pm$ S.E.M., the average value for PS is fixed at 1, and different letters (a and b) denote significant difference ($P \leq 0.01$). (C) Results are expressed as means of four experiments $\pm$ S.E.M., the average value for stage I is fixed at 1, and different letters (a and b) denote significant differences ($P \leq 0.05$). Leydig cells; ST, seminiferous tubules; PS, pachytene spermatocyte; RS, round spermatid and d, days.

transcription and translation was a main characteristic of spermatogenic cells (Dadouné 1994, Eddy 2002), and by the fact that the aromatase (a glycoprotein) undergoes several posttranslational modifications like glycosylations (Sethumadhavan *et al.* 1991).

Concerning the nuclear receptor ESR1, we have demonstrated that the transcripts varied according 1) to age, 2) to the germ cell type, and 3) to the stage of the seminiferous epithelium, whereas the ESR2 expression seemed to be less concerned. The ESR1

Figure 5 ESR1 variants in the rat adult testis. Primers of both sides of the variable region of ER α mRNA were designed. Forty cycles of PCR were performed, and PCR products were separated on 1.5% agarose gel during 45 min. L, ladder; T, testis; B, brain; NC, negative control.

expression strongly increased between 30 and 70 days in the adult whole testis, although it drastically decreased in Sertoli cells during the same time, explained by the presence of ESR1 transcripts in Leydig cells and in germ cells. Mowa & Iwagana (2001) reported ESR1 messengers only in Leydig cells; however, our results are in accordance with those of Pelletier *et al.* (2000) who demonstrated the presence of ESR1 transcripts using *in situ* hybridization in developing spermatids. The increase of mRNAs in RS could be due to the use of a specific testis promoter. In fact, it was described that ESR1 transcripts can contain different 5'-UTR extremities arising from the utilization of various promoters as demonstrated in the human testis (Brand *et al.* 2002). This mechanism was described for the regulation of the expression of numerous genes (Eddy 2002) including the aromatase gene (Silandre *et al.* 2007). The ESR2 expression seemed to be more elevated in the adult testis, which could be explained by

Figure 6 ESR2 gene expression in the whole rat testis (A) and in rat Sertoli cells (B) according to age. Measures were done using real-time RT-PCR. Results are expressed as means $\pm$ S.E.M. ($n=3$). (A) The average value for WT 10d is fixed at 1. (B) The average value for S 10d is fixed at 1, and different letters (a and b) denote significant difference ($P \leq 0.001$). WT, whole testis; S, Sertoli cells and d, days.

its presence in germ cells (especially in RS) and in adult Sertoli cells. Conversely, ESR1 was highly expressed in Sertoli cells from young animals suggesting a switch of ESR expression in these cells. In addition, both ESR1 and ESR2 were more expressed in adult testes, suggesting a putative effect of estrogens in adult spermatogenesis. Indeed, the disruption of the ESR1 gene in mice induced infertility due to a lack of fluid resorption in efferent ducts and in the proximal part of the epididymis (Eddy *et al.* 1996) leading to an increase in spontaneous acrosome reactions and defects of flagellar (Joseph *et al.* 2010). Antal *et al.* (2008) demonstrated that ESR2 gene knock-out (ESR2KO) mice were infertile, despite the absence of histological abnormalities in the testis and normally mobile spermatozoa. Conversely, in the study from Krege *et al.* (1998), ESR2KO mice were fertile, and showed no apparent morphological or functional abnormalities in the testis possibly due to the presence of ESR2 forms lacking exon 3 which were still expressed in this model. It is of note that these studies were not realized in rats but in mice and phenotypes observed cannot be exactly transferred from one species to another. Moreover, expression of both ESRs is highly variable between species (Carreau & Hess 2010). In addition to Sertoli cells, Leydig cells and germ cells of adult animal expressed the ESR2, and as ESR1, its transcripts level was higher in RS than in PS. However, ESR2 expression was lower than that of ESR1 in adult seminiferous tubules (16-fold more ESR1 transcripts compared with ESR2). The ESR2 expression seemed to increase with the maturation degree of germ cells. In fact, van Pelt *et al.* (1999) found more transcripts in PS and RS compared with type A spermatogonia, which was supported by our data showing higher ESR2 mRNA levels in RS compared with PS. The fact that no significant variations of ESR2 expression were observed according to the stages of the seminiferous epithelium could be explained by its weak but widespread expression in somatic and germ cells (Pelletier *et al.* 2000, Mowa & Iwagana 2001; our results). Unlike previous data (Shugrue *et al.* 1998, van Pelt *et al.* 1999, Mowa & Iwagana 2001), the amount of detected ESR2 messengers in Leydig cells was identical to that in seminiferous tubules.

The presence of variants of both ESR1 and ESR2 (Fig. 9; Friend *et al.* 1997, Shupnik 2002) could explain the variability of the results in the quantification of their messengers. Three in-frame deletions of ESR1 have been reported (Fig. 9A): deletion of exon 4 (ER $\alpha\delta 4$), deletion of exons 3 and 4 (ER $\alpha\delta 3,4$), and deletion of exons 5 and 6 (ER $\alpha\delta 5,6$; Skipper *et al.* 1993, Friend *et al.* 1997, Shupnik 2002). Although Friend *et al.* (1997) reported ER $\alpha\delta 4$ and ER $\alpha\delta 5,6$ in the testis with the full-length form, we only found the form deleted of exon 4. This deletion occurring in DNA and in

Figure 7 ESR2 gene expression according to compartment (A), to germ cell type (B), and to the stage of the seminiferous epithelium (C) of the adult rat testis. Measures were done using real-time RT-PCR. (A) Results are expressed as means $\pm$ S.E.M. ($n=3$), and the average value for L 70 d is fixed at 1. (B) Results are expressed as means $\pm$ S.E.M. ($n=5$), the average value for PS is fixed at 1, and different letters (a and b) denote significant difference ($P \leq 0.05$). (C) Results are expressed as means $\pm$ S.E.M. ($n=4$), and the average value for stage I is fixed at 1. L, Leydig cells; ST, seminiferous tubules; PS, pachytene spermatocyte; RS, round spermatid; d, days.

hormone-binding domains suggested a modification of receptor activity. However, although smaller ER α forms were observed in other rat tissues (Geffroy-Roisne *et al.* 1992, Friend *et al.* 1997), the authors did not conclude that these proteins correspond to splice variants. Using

a specific antibody directed against the C-terminal region of the ESR1 protein, only the full-length form was detected by Chimento *et al.* (2010) in the whole adult rat testis and in purified germ cells as reported in immature rat Sertoli cells (Lucas *et al.* 2008).

Figure 8 ESR2 variants in the rat adult testis. Primers of both sides of the variable region of ER β mRNA were designed. Forty cycles of PCR were performed, and PCR products were separated on 1.5% agarose gel during 45 min. LM, ladder; T, testis; P, pituitary; NC, negative control.

Regarding ER β , five different mRNAs were described by Price *et al.* (2000) (Fig. 9B): ER β 2 corresponds to ER β 1 containing an insertion of 54 nucleotides in the exon 6, which encodes a part of the ligand-binding domain (Maruyama *et al.* 1998, Petersen *et al.* 1998). Deletions of exon 3 (partial deletion of DNA-binding domain) lead to the formation of ER β 1 δ 3 and ER β 2 δ 3 (Petersen *et al.* 1998). Moreover, a deletion of exon 4

(partial deletion of the hinge region and of the hormone-binding domain) leading to the formation of ER β 1 δ 4 was described (Price *et al.* 2000). Studies on the activity of these receptors were carried out: proteins arising from messengers with the insertion of 54 nucleotides exhibited a ligand affinity weaker than those without. In addition, the deletion of the exon 3 induced a decrease in transcriptional activity (Petersen *et al.* 1998). Concerning ER β δ 4, it was localized in the cytoplasm, whereas other forms were found in the nucleus, and this protein was not able to bind estradiol (Price *et al.* 2000). Although the majority of ER α , ER β 1, and ER β 2 transcripts seemed to be in the testis, splice variants may contribute to the estradiol response, although internal exon deletion ESR1 mRNA forms do not exhibit steroid regulation (Friend *et al.* 1997). However, only one ESR2 protein was detected in the rat testis (Tirado *et al.* 2004, Lucas *et al.* 2008, Chimento *et al.* 2010). The transcript variants of rat ESR1 and ESR2 are issued from a single gene localized on chromosome 1 and chromosome 6 respectively (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In men, six variants were described in the testis (Aschim *et al.* 2004),

Figure 9 Schematic representation of rat ESR1 variants (Friend *et al.* 1997) (A) and ESR2 variants (Shupnik 2002) (B). Black arrows represent primer position, and black rectangle represents the insertion of 54 nucleotides; numbers 1–8 correspond to exons.

and their differential localization suggests that they could have specific functions in spermatogenesis.

Data concerning the evolution of the aromatase activity in germ cells have revealed that the biological aromatase activity was more elevated in RS than in PS (Levallet *et al.* 1998a, Bourguiba *et al.* 2003a,b), and we have shown that the expression of both ESRs evolved in the same manner. This may suggest that the expression of ESRs mRNAs could be regulated by estradiol, and a putative role of ESRs to mediate estrogen effects in spermatogenesis is becoming obvious. In that respect, previous studies have shown that estrogens can regulate the expression of ESR1 mRNA (full-length and variants) and particularly in pituitary cells (Friend *et al.* 1997, Schaussi *et al.* 2003, Bryant *et al.* 2006).

However, we cannot forget that estrogens can also exert rapid effects called ‘nongenomic’ activating signaling pathways. One of these receptors, G protein-coupled receptor 30 (GPR30) is a seven-transmembrane receptor. Estrogens and agonists were shown to induce MAPK activation and signaling via adenyl cyclase by this receptor (Prossnitz *et al.* 2007). Moreover, GPR30 was shown to be involved in proliferative effects of 17 β -estradiol in a mouse spermatogonial cell line GC-1 (Sirianni *et al.* 2008). In our laboratory, we have found that both GPR30 and ESR1 were involved in the control of apoptosis in PS by 17 β -estradiol (Chimento *et al.* 2010). Thus, it would be helpful to map GPR30 expression within the testis in order to investigate the part of rapid effects of estrogens in spermatogenesis.

While ESR2 seems less precisely regulated than ESR1, both ESRs are essential for fertility. Thus, it is necessary to deepen their mechanism(s) of action within testis. Moreover, membrane ESRs could be implicated in the nongenomic effects of estrogens (Chimento *et al.* 2010). Within the rat testis, there is precise regulation of the amount of aromatase and of ESR1 transcripts. Thus, a role of estrogens via nuclear ESR1 in the latest steps of spermatogenesis could be evoked which is in keeping with elevated aromatase activity (Levallet *et al.* 1998a).

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 Bois realized all molecular studies and participated with L Zanatta in the preparations of cells. M Nurmio, M Parvinen, and J Toppari prepared the different groups of seminiferous tubules stages. C Delalande and S Carreau supervised this work.

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