Journal of Steroid Biochemistry and Molecular Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

# Delineating the regulation of estrogen and androgen receptor expression by sex steroids during rat spermatogenesis

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ARTICLE INFO

*Keywords:*
Estrogen receptor
Androgen receptor
Spermatogenesis
Gene regulation

ABSTRACT

Estrogen receptors (ERα and β) and androgen receptor (AR) regulate various critical processes during spermatogenesis. Since spermatogenesis is very sensitive to hormonal stimuli and perturbations, it is important to understand the regulation of expression of these receptors by sex steroid hormones. Although many studies have reported deregulation of steroid receptors on endocrine disruption, there is no consensus on the regulation of their expression by steroid hormones during spermatogenesis, and a lack of clear understanding of the mechanism of regulation. Here, we evaluated the receptor expressions in a well-established exogenous estradiol administration model. We then investigated the mechanisms by which the individual receptors regulate their expression by binding to the respective hormone response elements upstream of these receptor genes. By further employing *in vitro* and *in vivo* models of ER and AR stimulation or antagonism, we delineated their regulation in a receptor subtype-specific manner. Our results indicate that ERα positively regulates expression of both the ERs; whereas, ERβ and AR negatively regulate expression of both ERβ and AR by direct binding to upstream regulatory regions. The perturbations in the levels of steroid receptors could be an important factor contributing to spermatogenic defects and male sub-fertility after estradiol and ER agonist treatment. Our study delineates the direct contribution of the individual steroid receptors in the regulation of their own expression.

## 1. Introduction

Spermatogenesis is under intricate hormonal regulation of the hypothalamo-pituitary-testis axis. Although pituitary hormones are master regulators of reproductive function, many vital functions are performed by locally secreted sex steroids such as androgens and estrogens, which are the final effector hormones. Estrogens signal mainly *via* their two cognate receptors estrogen receptor (ER) α and β, encoded by the ERα gene (Esr1) and the ERβ gene (Esr2), respectively, while androgens mediate their effects through the androgen receptor (AR) encoded by the Ar gene. Both ERs and AR belong to the nuclear hormone receptor family. They act as ligand-activated transcription modulators by either binding directly to estrogen or androgen responsive elements (EREs or AREs, specific short sequences of DNA), or by associating with other transcription factors [reviewed in 1–3].

Both, androgens and estrogens play crucial roles during spermatogenesis. It is well established that testosterone is absolutely essential for maintaining spermatogenesis *via* the AR present on Sertoli, Leydig and peritubular myoid cells. Since germ cells do not express AR, the androgen-dependent functions of germ cells are thought to be mediated through Sertoli cells [4–6]. Through cell type-specific AR knockout mice models, AR signaling has been shown to be involved in various stages of germ cell development, like meiotic progression, transition from round to elongated spermatids (*i.e.*, spermiogenesis), and sperm release (*i.e.*, spermiation) [reviewed in 7]. Androgens are specifically required for germ cell adhesion to Sertoli cells during spermiogenesis [8], maintenance of the blood-testis barrier [9], and endocytosis during sperm release [10,11]. In the testis, estrogen is produced by Leydig cells, Sertoli cells, and germ cells by the aromatization of testosterone [reviewed in 12]. While both the ERs are present in the germ cells like

*Abbreviations:* AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; ER, estrogen receptor; ERE, estrogen response element; E2, 17β-estradiol; T, testosterone; DES, diethylstilbestrol; BPA, bisphenol A; SERM, selective estrogen receptor modulator; MNase, Micrococcal nuclease; RIPA, radioimmunoprecipitation assay lysis buffer; HBSS, Hank's balanced salt solution; PPT, 4,4',4"-(4-Propyl-[$^1$H] pyrazole-13,5-triyl); DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; MPP, 1,3- bis (4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy)phenol)-1*H*-pyrazole; PHTPP, 4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo(1,5-a)pyrimidin-3-yl)phenol; CPA, cyproterone acetate; DMSO, dimethyl sulfoxide; Src1, Steroid Receptor Coactivator 1; SMRT, Silencing Mediator for Retinoid and Thyroid hormone receptors; RNA Pol II, RNA polymerase II

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https://doi.org/10.1016/j.jsbmb.2018.04.018
Received 22 August 2017; Received in revised form 18 April 2018; Accepted 26 April 2018

**Table 1**
Primer sequences of gene expression studies.

| Gene | Primer Sequences | Annealing temp. | Amplicon length | Efficiency |
|---|---|---|---|---|
| Estrogen receptor 1 (Esr1) NM_012689.1 | F AATTCTGACAATCGACGCCAG<br>R GTGCTTCAACATTCTCCCTCCTC | 56 °C | 345 bp | 1.97 |
| Estrogen receptor 2 (Esr2) NM_012754.1 | F CTTGCCCACTTGGAAACATC<br>R CCAAAGGTTGATTTTATGGCC | 60 °C | 152 bp | 2.15 |
| Androgen receptor (Ar) NM_012502.1 | F CCCATCGACTATTACTTCCCACC<br>R TTCTCCTTCTTCCTGTAGTTTGA | 59 °C | 291 bp | 1.82 |
| 18S rRNA (Rn18s) NR_046237.1 | F CCGCAGCTAGGAATAATGGA<br>R AGTCGGCATCGTTTATGGTC | 61 °C | 246 bp | 2.05 |

pachytene and round spermatids, only ERβ is expressed in spermatogonia and Sertoli cells [13,14]. In the adult testis, estrogen is essential for germ cell survival [15,16] and spermiation [17,10]. Estrogen signaling *via* its two receptors has distinct roles during spermatogenesis; ERα is important during spermiogenesis, while ERβ regulates spermatocyte apoptosis and spermiation [18,10,11].

The specific functions of ERs and AR underline their crucial roles during different phases of spermatogenesis. Their deregulation could affect numerous downstream targets, thereby, disrupting spermatogenesis and male fertility. Various studies have shown that estrogen-like chemicals in the environment can cause changes in ERα, ERβ, and AR gene expression in the testis, thus affecting spermatogenesis. Dietary exposure of diethylstilbestrol (DES), a synthetic estrogen, caused a 50% reduction in testicular AR expression in rats [19]. On the other hand, estradiol administered to neonatal rats increased AR and ERβ mRNA levels in testis [20]. Neonatal exposure of bisphenol A (BPA), a selective estrogen receptor modulator (SERM), in male mice disrupted spermatogenesis and increased the levels of ERα and ERβ in developing testis [21]. Also, administration of Aroclor 1254 (a polychlorinated bisphenol) to male mice led to disrupted spermatogenesis and increase in ERα and β expression [22]. Most of these studies are done in neonates and there is a dearth of studies in adults, where spermatogenesis is already ongoing. A study done by Kaushik et al. showed that adult male rats exposed to supraphysiological dose of estradiol, had increased ERα and AR transcript levels in the testis [23].

*In vivo* models of endocrine disruption, which involve administration of estradiol or SERMs like BPA, alter physiological levels of estradiol and androgens, and result in a combination of effects. Therefore, in these models it is not possible to identify whether the changes in ERs and AR levels are due to estradiol or androgen. Thus, there is no consensus on the regulation of expression of these steroid receptors by steroid hormones during spermatogenesis and a lack of clear understanding of the mechanism of regulation. In the present study, we evaluated the receptor expressions in a well-established exogenous E2 administration model [17], followed by investigation of the mechanism by which the individual receptors regulate expression by binding to EREs and AREs upstream of the ER and AR genes. We also delineate ER and AR expression in a receptor subtype-specific manner using *in vitro* and *in vivo* models.

## 2. Materials and Methods

### 2.1. Animals

Adult (90 days old) male Holtzman rats weighing ~300 g were obtained from the Institute's animal house facility. These animals were maintained at 22 ± 2 °C and 50–55% humidity in a fixed 12 h light, 12 h dark cycle. They were supplied with a diet of soy-free, in-house prepared rat pellets and water *ad libitum*. The Institutional Animal Ethics Committee approved the use of animals for the study.

### 2.2. Exogenous estradiol treatment and tissue collection

17β-Estradiol (Sigma-Aldrich, St Louis, MO, USA) was administered to six adult male rats at a dose of 100 mg/kg body weight daily for 10 days. The drug was suspended in saline and administered subcutaneously, as described earlier [17]. Control animals (n = 6) received only saline. After 10 days of treatment, the animals were sacrificed by cervical dislocation. One testis from both control and treated animals was dissected out and processed for chromatin immunoprecipitation (ChIP). The other testis was snap frozen for RNA and protein extraction.

### 2.3. RNA extraction and qRT-PCR

Total RNA was extracted using TRI pure (Roche Diagnostics; Mannheim, Germany) according to the manufacturer's instructions. The concentration of RNA samples was determined by the absorbance at 260 nm (Nanovue; GE Healthcare, Uppsala, Sweden). RNA extracted (2 µg) was then reverse transcribed using High Capacity Reverse Transcription system from Applied Biosystems (Foster City, CA, USA) according to the manufacturer's protocol. qRT-PCR was performed in a Light Cycler 96 real time PCR system using FastStart Essential DNA green master (Roche Diagnostics). Primers for all target genes as well as reference gene (18S rRNA) were synthesized by Sigma-Aldrich. Forward and reverse primer sequences and their amplicon sizes are listed in Table 1. Amplification reactions were set up of 20 µl containing 1.6 µl of cDNA, 10 pM of respective primers and SYBR green master with the following program, which consisted of initial denaturation of 10 min at 95 °C followed by 40 cycles of 95 °C for 10 s, primer annealing temperature for 10 s and extension at 72 °C for 10 s. Melt curve analysis was performed for checking specificity and all products obtained yielded the predicted melting temperature. Amplification reactions were prepared in duplicate and a no-template control was included. Primer PCR efficiencies (listed in Supplementary Table 2) were determined using a standard curve. Pfaffl method was used to calculate the relative gene expression [24]. Real-time PCR procedures and analyses follow the MIQE guidelines [25] (Supplementary Table 1).

### 2.4. Western blotting

Total testicular protein from control and treated animals was extracted in radioimmunoprecipitation assay lysis buffer (RIPA) (150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 1% deoxycholic acid, 10% sodium dodecyl sulfate) containing protease inhibitors. Forty micrograms of protein was resolved on 12% SDS–PAGE and transferred onto nitrocellulose membrane (GE; Amersham, Buckinghamshire, UK). The blots were washed with PBS, blocked with 5% nonfat dry milk and incubated with appropriate primary antibodies (Table 2) in 1% nonfat dry milk at 4 °C overnight. The membranes were washed with 0.01 M PBS containing 0.1% Tween 20 and incubated with appropriate secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich), developed with ECLPlus (GE) or Femto reagent (Pierce Biotechnology; Rockford, IL, USA). The bands were visualized and quantified by densitometry.