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Direct regulation of genes involved in sperm release by estrogen and androgen through their receptors and coregulators

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ABSTRACT

Steroid hormones, estrogen and androgen, control transcription in various reproductive and non-reproductive tissues. Both hormones are known to be important for control of sperm release from the seminiferous epithelium (spermiation), a process characterized by extensive remodeling of actin filaments and endocytosis. Earlier studies with an estrogen (E2)-induced rat model of spermiation failure revealed genes involved in actin remodeling (Arpc1b and Evl) and endocytosis (Picalm, Eea1, and Stx5a) to be differentially regulated. Further, among these genes, Arpc1b and Evl were found to be estrogen-responsive whereas Eea1 and Stx5a were androgen-responsive and Picalm was responsive to both hormones in seminiferous tubule cultures. Yet, the mechanism by which these genes are regulated by estrogen and androgen in the testis was unclear. Here, we report the presence of a functional estrogen response element (ERE) upstream of Arpc1b and Evl genes and androgen response element (ARE) upstream of Picalm, Eea1, and Stx5a genes. Chromatin immunoprecipitation in control versus E2-treated testes revealed significant changes in estrogen receptor beta (ERβ) recruitment along with coregulators to the EREs upstream of Arpc1b and Evl genes and androgen receptor (AR) at AREs upstream of Picalm, Eea1, and Stx5a genes. Enrichment patterns of these EREs/AREs with coregulators, activating and repressing histone modifications along with RNA polymerase II recruitment, correlated with the observed expression patterns of these genes upon E2 treatment. Taken together, our results reveal direct targets of estrogen and androgen in the testes and provide insights into transcriptional control of sperm release by the two steroid hormones.

1. Introduction

Spermatogenesis in mammals is a precisely coordinated and highly complex process that results in the formation of the mature spermatozoa from precursor undifferentiated spermatogonial stem cells. It broadly consists of phases of spermatogonial proliferation and differentiation, meiosis, spermiogenesis (round spermatid differentiation), and spermiation (sperm release) [1]. Spermatogenesis is regulated by various endocrine factors including androgens and estrogens. Androgens are known to be important for progression of germ cells through meiosis, spermiogenesis, and spermiation (sperm release) [2] whereas estrogens are important for spermiation and germ cell survival [3–5]. These sex steroid hormones also control metabolism in Sertoli cells, thus providing nutritional support for spermatogenesis [6]. Androgens mediate their biological action via androgen receptors (AR) whereas estrogens mediate their action via two cognate receptors, estrogen receptor (ER) α and β [7,8].

In the adult mammalian testis, AR is localized to the somatic cell types—Sertoli cells, peritubular myoid cells, and Leydig cells [9,10] whereas germ cells are devoid of AR. ERα was only recently reported within seminiferous tubules and is less abundant than ERβ. ERα expression has been found in pachytene spermatocytes and round spermatids [11]. ERβ localization has been shown in

Abbreviations: AR, androgen receptor; ARE, androgen response element; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; ER, estrogen receptor; ERE, estrogen response element; E2, 17β-estradiol; H3ac, histone H3 lysine 9 and 14 acetylation; H3K4me2, histone H3 lysine 4 dimethylation; H3K27me3, histone H3 lysine 27 trimethylation; H3K9me2, histone H3 lysine 9 dimethylation; HAT, histone acetyltransferase; HDAC, histone deacetylase; HRE, hormone response element; MNase, Micrococcal nuclease; NCoR1, nuclear receptor corepressor; RNA Pol II, RNA polymerase II; Src1, steroid receptor coactivator 1; TBC, tubulobulbar complex; TSS, transcription start site; T, testosterone.

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Sertoli cells [12], spermatogonia, spermatocytes, and spermatids [13,14].

Steroid hormones such as androgens and estrogens mediate their action on gene transcription via their cognate receptors. Steroid receptors are ligand-regulated transcription factors belonging to the nuclear receptor superfamily. The classical mechanism of steroid hormone action involves ligand binding, receptor dimerization, nuclear translocation, binding to hormone response elements (HREs), recruitment of coregulators, and chromatin remodelling leading to gene transcription control [2,15,16]. Several studies have attempted to identify genes regulated by androgens and estrogens in the testis, mostly using microarray approaches [17–22]. However, there is no clear information about direct target genes and their mechanism of regulation by these hormones.

While studying the role of estrogen during spermiation using adult rat model, genes involved in actin remodeling (*Arpc1b* and *Evl*) and endocytosis (*Picalm*,*Stx5a*, *Eea1*) were found to be differentially regulated in the testis [23]. All these gene products were found to be associated with tubulobulbar complexes (TBCs), actin-rich endocytic devices of mature spermatids during spermiation. Using seminiferous tubule cultures, it was further discerned that the actin remodeling genes were estrogen-responsive whereas the endocytosis-related genes were androgen-responsive [24]. In the present study, we demonstrate that these genes are direct targets of 17β-estradiol (E2) and testosterone (T), respectively, in the testis. We also show a mechanism by which E2 and T act in order to activate or repress genes involved in spermiation.

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 14-h light, 10-h dark cycle. Animals were maintained on a soy-free diet consisting of in-house prepared rat pellets with free access to food and water. 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 μg/kg body weight daily for 10 days. The drug was suspended in saline and administered by subcutaneous route, as described earlier [3]. Control animals ($n = 6$) received only saline. After completion of treatment for 10 days, the animals were euthanized by cervical dislocation and the testes from both control and treated animals were dissected out and further processed for chromatin immunoprecipitation (ChIP).

2.3. ChIP

The testes from six control and six treated adult rats were processed to obtain a single cell suspension, which were further used for ChIP studies. Testes were dissected out, decapsulated, and seminiferous tubules were teased apart. The tissue was mechanically homogenized and passed through a 70 μm nylon mesh to obtain a single cell suspension. Cells (2×10^7) were immediately crosslinked using 1% (v/v) formaldehyde at room temperature for 10 min with intermittent shaking, followed by addition of 125 mM glycine to stop the reaction. Crosslinked cells were washed twice in ice-cold phosphate-buffered saline containing protease inhibitor cocktail tablets (Roche Diagnostics). Cells were lysed using sodium dodecyl sulfate (SDS) lysis buffer (1% SDS in Tris-EDTA buffer) for 15 min on ice. In order to perform ChIP for transcription factors (ERβ, AR, RNA Pol II), chromatin was sheared in a water bath sonicator (Equitron, India) using 53 kHz frequency with 30 s ON/OFF pulses for 16 cycles to obtain chromatin in the range of 200–500 bp. For histone ChIP, chromatin was subjected to Micrococcal nuclease (MNase—obtained from *Staphylococcus aureus*, USB Corporation, OH, USA) treatment (3 U for 1×10^6 starting cell count) for 3 min at 37 °C followed by immediate addition of 1 mM EDTA on ice to obtain mono nucleosomal DNA of ~150 bp in length. Sheared chromatin (30 μg) was diluted 10-fold with dilution buffer precleared with protein A Agarose/salmon sperm DNA pre-blocked beads (Millipore, Temecula, CA, USA) with slow rotation at 4 °C for 1 h. Precleared chromatin was incubated with appropriate antibody (Table 1) or rabbit IgG (mock sample) at the same concentration as antibody overnight with slow rotation at 4 °C. Pre-immunoprecipitated (10%) lysate was saved as 'input' for normalization later. The samples were then washed once with low salt, high salt, and LiCl and washed twice with Tris-EDTA buffer and eluted using elution buffer (SDS, $NaHCO_3$). Crosslinking was reversed by incubation for 4 h at 65 °C, followed by incubation with proteinase K (Roche Diagnostics, Germany) for 1 h at 45 °C. DNA was purified by phenol/chloroform extraction and ethanol precipitated using glycogen as a carrier. Purified DNA from immunoprecipitates was analyzed by qPCR.

2.4. ChIP-qPCR

Enrichment in ChIP was quantified by qPCR using SYBR Green on Light Cycler 96 real time PCR system (Roche Diagnostics). Details of the regions analyzed along with primer sequences (Sigma-Aldrich) are given in Table 2. Amplification reactions were set up of 20 μl containing 1.6 μl of DNA (Input/ChIP/mock), 10 pM of respective primers, and SYBR green mastermix 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-

Table 1
Antibodies used for ChIP experiments in this report.

Antibody target	Host species	Concentration used (one reaction contains ~30 μg chromatin)	Vendor (product code)
ERβ	Rabbit	5 μg per reaction	Abcam (ab3577); Cambridge, MA, USA
AR	Rabbit	2 μg per reaction	Abcam (74272)
Src1	Rabbit	8 μg per reaction	Affinity Bioreagents (PA1-840); Colorado, USA
NCoR1	Goat	5 μg per reaction	Santa Cruz Biotechnology (sc-1611); Santa Cruz, CA, USA
H3ac	Rabbit	4 μg per reaction	Upstate (06-599); Temecula, CA, USA
H3K4me2	Rabbit	4 μg per reaction	Upstate (07-030)
H3K27me3	Rabbit	4 μg per reaction	Abclonal (A2363); Massachusetts, USA
H3K9me2	Rabbit	4 μg per reaction	Upstate (07-212)
RNA Polymerase II	Mouse	5 μg per reaction	Millipore (05-623); Temecula, CA, USA