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Research Article

Genome-wide identification of estrogen receptor binding sites reveals novel estrogen-responsive pathways in adult male germ cells

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Spermatogenesis occurs in the seminiferous epithelium that shows the presence of estrogen receptors alpha (ERα) and beta (ERβ), both of which regulate gene transcription by binding to the DNA. Estrogen responsive phases of spermatogenesis are well documented; however, the genes regulated remain inexplicit. To study the regulation of genes by estrogen in male germ cells, we performed chromatin immunoprecipitation (ChIP) sequencing for ERα and ERβ under normal physiological conditions. A total of 27 221 DNA binding regions were enriched with ERα and 20 926 binding sites with ERβ. Majority of the peaks were present in the intronic regions and located 20 kb upstream or downstream from the transcription start site (TSS). Pathway analysis of the genes enriched by ChIP-Seq showed involvement in several biological pathways. Genes involved in pathways whose role in spermatogenesis is unexplored were validated; these included prolactin, GnRH, and oxytocin signaling. All the selected genes showed the presence of estrogen response elements (EREs) in their binding region and were also found to be significantly enriched by ChIP-qPCR. Functional validation using seminiferous tubule culture after treatment with estrogen receptor subtype-specific agonist and antagonist confirmed the regulation of these genes by estrogen through its receptors. The genes involved in these pathways were also found to be regulated by the respective receptor subtypes at the testicular level in our *in vivo* estrogen receptor agonist rat models. Our study provides a genome-wide map of ERα and ERβ binding sites and identifies the genes regulated by them in the male germ cells under normal physiological conditions.

Introduction

Estrogen is a steroid hormone involved in many reproductive functions in females as well as males. Traditionally considered to be a 'female hormone', its pivotal role in male reproduction has been uncovered in the past few decades. The increased focus on estrogen in males is mainly attributed to detrimental effects on male fertility observed in various estrogen receptor knockout and ablation models. Estrogen is known to be important in a number of male physiological processes, such as regulation of the hypothalamic-pituitary-testicular axis, testicular descent and spermatogenesis, efferent ducts and epididymal functions, sexual behavior, regulation of growth hormone insulin-like growth factor-1 axis, bone growth and maintenance of skeletal health, body composition and glucose metabolism, and vasomotor stability [1,2]. Male fertility is attributed to normal spermatogenesis, i.e. a process by which immature spermatogonia undergoes proliferation and differentiation to give rise to mature spermatozoa [3]. Estrogen is intimately involved in the process of spermatogenesis, specifically regulating spermiation and germ cell survival [4–6]. The effects of estrogen are brought about by estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which exert their function in a ligand-dependent manner [7,8]. ERα is expressed in pachytene spermatocytes and round spermatids in the rat testes [9]. ERβ is localized in spermatogonia, spermatocytes, and spermatids [10–12]. The

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functions of these receptors have been highlighted by their knockout as well as receptor agonist models. ERα knockout male mice demonstrated altered spermatogenesis and infertility [13,14]. Mice lacking ERβ due to disruption of exon 3 in the ERβ gene were found to be fertile [15,16]. However, in these studies although full-length transcript was absent, transcript variants of the gene were found to be present, suggesting there might be some residual activity of ERβ. Antal et al. [17] generated complete ERβ-null mutant and the males were observed to be sterile suggesting a role of ERβ in male fertility, however, the reason for infertility in these null mutant is not clear.

ERα and ERβ belong to Class 1 of nuclear hormone receptor superfamily and are composed of six domains; the N-terminal A/B domain; DNA-binding domain (DBD) termed C domain; the D domain possessing signals for nuclear localization; the ligand-binding domain (LBD) called the E domain; the C-terminal F domain [18]. The DBD contains two zinc finger structures that are responsible for recognition of specific DNA sequence known as estrogen response elements (EREs) which are present within the regulatory region of genes. Classically, upon binding of estrogen to its receptor, conformational changes in the LBD are induced; the receptor then translocates into the nucleus and interacts with the DNA region containing EREs. This conformational change also recruits co-activating or co-repressing factors leading to modulation of transcriptional activity [18].

To better understand the role of estrogen in spermatogenesis, one approach is to identify estrogen responsive genes in the cell types of the seminiferous epithelium. A few studies have been carried out to study the gene expression pattern of estrogen responsive genes in testes. However, most of them have utilized different estrogen overexposure or depletion models, concomitantly leading to a disturbed hormonal background [19,20]. This may lead to overexpression of the genes due to physiologically irrelevant levels of estrogen and may not replicate the phenomenon that happens *in vivo*. In addition, there are no studies done to illustrate the cell-type specific regulation of estrogen in the testis. Here, we used a physiological, unperturbed system of germ cells from adult rat testes to identify estrogen responsive genes using a genome-wide next-generation sequencing-based approach.

Materials and methods

Animals

Male Holtzman rats (75 days–90 days old) were obtained from the Institute's animal house facility. The animals were maintained under controlled temperature (22 ± 1°C) and humidity (55 ± 5%) conditions in a fixed 12-h light, 12-h dark cycle with free access to food and water. All the animal experiments were performed at ICMR-National Institute for Research in Reproductive Health (ICMR-NIRRH) after approval by the Institute's Animal Ethics Committee.

Reagents and chemicals

All the reagents and chemicals used are from HiMedia unless mentioned otherwise.

Germ cell enrichment

To study the estrogen receptor binding sites in germ cells, cell population enriched in germ cells were obtained from adult rat testes as previously described [21]. The animals were sacrificed and testes were dissected out, detunicated and the tubules were teased apart. Interstitial cells were lysed by hypotonic treatment by suspending the tubules in distilled water twice for 1 min and decanting the supernatant. The tubules were then resuspended in DMEM F12 and treated with trypsin (1 mg/ml) followed by DNase (0.5 mg/ml) twice and incubated at 37°C on the shaker (80 rpm) for 8 and 5 min, respectively. The tubules were allowed to settle down and the supernatant was passed through 70 μm nylon mesh. The filtrate was passed through a 40 μm nylon mesh to separate Sertoli cells. The filtrate thus obtained was centrifuged at 800 rpm for 8 min at 4°C. The supernatant was discarded and the resulting pellet of germ cells was resuspended in Hank's balanced salt solution (HBSS). A fraction of the germ cell suspension was set aside to check for purity using gene expression markers (listed in Supplementary Table S9) as mentioned in Kumar et al. [22]. The remaining cells were crossed-linked using 1% (v/v) formaldehyde for 10 min at room temperature, after which 125 mM glycine was added to block further crosslinking. The crosslinked cells were washed twice with chilled phosphate buffered saline containing protease inhibitors (Roche Diagnostics) and stored at −80°C for chromatin immunoprecipitation.

Chromatin immunoprecipitation (ChIP)

After germ cell enrichment, chromatin immunoprecipitation (ChIP) was performed for ERα and ERβ following a protocol described previously by Kumar et al. [22]. Briefly, the crosslinked cells were pelleted and resuspended in sodium dodecyl sulfate (SDS) buffer (1% SDS in Tris-EDTA buffer) and incubated on ice for 15 min for lysis. Thereafter, chromatin was sheared in a water bath sonicator (Equitron, India) at 53 kHz frequency for 16 cycles with 30 s ON/OFF pulses to yield chromatin fragments of ∼500 bp. After centrifuging for 10 min at 13 000 rpm, the supernatant was collected. The remaining pellet was then subjected to MNase treatment (40 U) (Takara, Japan) for 3 min at 37°C followed by addition of EDTA (1 mM) to stop the reaction. The reaction was centrifuged and the supernatant was again collected and pooled with the previous one. The sheared chromatin was diluted 10-fold using ChIP dilution buffer (0.01% SDS, 1.1% TritonX 100, 1.2 mM EDTA, 16.7 mM Tris HCl, 167 mM NaCl) and pre-cleared using Protein A agarose beads (Millipore, U.S.A.) for 60 min with slow rotation (10 rpm). Pre-cleared chromatin was then subjected to immunoprecipitation using anti-ERα (1 μg) (Millipore, Cat No. CS200620), anti-ERβ (1 μg) (Sigma, Cat No. E1276) or rabbit IgG (mock reaction) overnight at 4°C. Ten percentage of the pre-immunoprecipitated lysate was set aside as 'input' for normalization later. The immuno-precipitated DNA was then washed sequentially with low salt (0.01% SDS, 1% TritonX 100, 2 mM EDTA, 20 mM Tris HCl, 150 mM NaCl), high salt (0.01% SDS, 1% TritonX 100, 2 mM EDTA, 20 mM Tris HCl, 500 mM NaCl), and LiCl buffer (0.25 M LiCl, 1% NP40, 1% NaDox, 1 mM EDTA, 10 mM NaCl) for 10 min each on rotor at 4°C. This was followed by two washes with TE buffer (10 mM Tris HCl, 1 mM EDTA) for 5 min each. The samples were then eluted using elution buffer (1% SDS, 0.1 M $NaHCO_3$) and the supernatant was collected. The chromatin was then reversed crosslinked with 5 M NaCl at 65°C overnight. DNA was purified using column extraction kit (GeneAll Expin^{TM} CleanUp SV), eluted in nuclease-free water and stored at −20°C.

ChIP sequencing

Enrichment in ChIP was first confirmed by qPCR for the known binding sites of ERα and ERβ to check the efficiency of ChIP. The samples from six individual ChIP experiments (from six animals) were pooled, and subjected to next-generation sequencing at Sandor Lifesciences, Hyderabad. Briefly, the immuno-precipitated DNA was quantified by Qubit2.0 and Agilent Bioanalyser was used for size distribution as a QC step for chip sequencing library. ChIP Sequencing libraries were generated using NEBNext® Ultra™ II DNA kit for Illumina. A total amount of 200–500 pg input material was used for ChIP sequencing. The fragmented DNA overhangs were converted into blunt ends via exonuclease/polymerase activities (end repair). After adenylation of 3′ ends of DNA fragments, adapter oligonucleotides were ligated. DNA fragments with ligated adapter molecules on both ends were selectively enriched in final PCR reaction. Products were purified using AMPure XP system (Beckman Coulter, Beverly, U.S.A.). The final libraries were quantified using Kapa qPCR Kit for adapter-ligated molecules and was sequenced on the PE75 Illumina platform. The sequence was aligned to the rat genome *Rattus norvegicus Rnor_6.0* using Bowtie 1.2 and peak calling was performed by MACS14 (Model-based Analysis of ChIP-Seq 14).

Pathway and motif analysis

PANTHER Database was used to study molecular functions of the genes obtained by ChIP-Seq. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database was used for pathway analysis of the enriched genes. To look for estrogen receptor binding sites, a de novo motif discovery algorithm, TF Bind was used to find the existing EREs. The algorithm uses the ERE motif, A(A/G)GNNANNNTGACC(C/T) which is similar to the established consensus canonical ERE.

ChIP-qPCR

Data obtained after ChIP-Seq was validated by ChIP-qPCR. ChIP-qPCR was performed in six animals for analysis. Briefly, amplification reactions of 20 μl (in duplicate) were set up containing 1.6 μl of DNA (Input/ChIP/mock), 10 pM of respective primers and Takyon mastermix (SYBR Green) (Eurogentec, Belgium) with the following program which consisted of initial denaturation of 2 min at 95°C followed by 40 cycles of 95°C for 20 s, primer annealing temperature for 10 s and extension at 72°C for 1 min and final extension at 72°C for 5 min. A no-template control was also included. Primers for all the selected target genes were synthesized by Sigma–

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Aldrich and the sequences are mentioned in Supplementary Table S4. Fold enrichment of ChIP over mock was calculated by normalizing with input sample as described previously [22].

Seminiferous tubule culture and hormonal treatment

To check for the regulation of selected genes by ERα and ERβ, seminiferous tubule cultures were set up and treated with hormones as described earlier [5]. Briefly, adult male rats were sacrificed by cervical dislocation. Testes were excised, decapsulated and placed in PBS. The tubules were teased apart and interstitial tissue was mechanically removed. Eight seminiferous tubule fragments (∼1 cm each) were cultured per well in 0.5 ml of DMEM-F12 without phenol red and with glutamine and HEPES (pH 7.4) (Gibco, Life Technologies, GrandIsland, U.S.A.), supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenite (5 ng/ml) (Sigma–Aldrich), penicillin (50 000 U/l), streptomycin (50 mg/l), fungizone (0.125 mg/l), and kanamycin (10 mg/ml) (Gibco, U.S.A.) in a 24-well plate (Himedia, India). The cultures were treated with ERα agonist PPT (4,4′,4″-(4-propyl-(1H)-pyrazole-1,3,5-triyl)trisphenol) (10^{-7} M) or ERβ agonist-DPN (2,3-bis (4hydroxyphenyl)-propionitrile) (10^{-7} M) (Tocris Bioscience, U.K.) and incubated for 24 h in a humidified atmosphere with 5% CO_2 and 95% air at 32°C. For antagonist studies, a pre-treatment for 1 h was given with ERα antagonist-MPP (1,3bis(4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy)phenol)1H-pyrazole) (10^{-6} M) or ERβ antagonist-PHTPP (4-(2-phenyl-5,7-bis(trifluoromethyl)pyrazolo(1,5-a)pyrimidin-3yl)phenol) (10^{-6} M) (Tocris Bioscience, U.K.) followed by addition of E2 (estradiol) (10^{-9} M) (Sigma, U.S.A.) for 24 h. The doses for all the hormones, agonist and antagonist treatments were selected based on the previous studies in the laboratory [5]. Only dimethylsulfoxide (DMSO) (Sigma, St Louis, MO, U.S.A.) was used as vehicle control. There were six animals in each treatment group ($N = 6$). After 24 h of treatment, tubule segments were collected in TRI Pure reagent (Roche Diagnostics, Germany) for RNA extraction.

In vivo estrogen receptor agonists treatments

To study gene regulation by ERα and ERβ in the testis, *in vivo* ER agonist administration models were employed [23]. PPT (>99%, Axon Medchem, Groningen, Netherlands) and DPN (>99%, Tocris Biosciences, Bristol, U.K.) were administered at the dose of 0.05 mg/kg/day to adult male rats for 60 d. The treatment dosage and regime were based on our earlier studies [6,23]. The drugs were dissolved completely in vehicle, DMSO : Saline (75 : 25), and administered subcutaneously as described earlier. The animals in the control group received the vehicle alone. There were at least six males in each of the treatment and control groups. After 60 d of treatment, control and treated males were sacrificed, both the testes were excised out, immediately snap frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

RNA extraction and RT-PCR

Total RNA was extracted from the seminiferous tubule segments or 100 mg of frozen testis tissue or isolated germ cell pellets using TRI Pure reagent according to the manufacturer's protocol. The concentration of RNA samples was determined by the absorbance at 260 nm (Nanovue; GE Healthcare, Sweden). The purity and the integrity of the RNA extracted were checked by measuring the ratio of optical density at 260 and 280 nm. An amount of 2 μg of the RNA extracted was then reversed transcribed using High Capacity Reverse Transcription system (Applied Biosystems, U.S.A.) according to the manufacturer's instruction. qPCR was performed in Roche LightCycler 96 (Roche, Switzerland) using Takyon SYBR green mastermix (Eurogentec, Belgium). The PCR program was same as described previously. Primers for the reference (*Rn18S*) and all target genes were synthesized by Sigma–Aldrich. The primer sequences for all the genes are mentioned in Supplementary Table S5. Melt curve analysis was performed for checking specificity, and all products obtained yielded the predicted melting temperature (Supplementary Figure S6). All procedures and analysis for Real Time PCR followed MIQE guidelines as mentioned in Supplementary Table S8.

Statistical analysis

The statistical analysis was performed using Graph Pad Prism (version 6; Graph Pad Inc., San Diego, CA, U.S.A.). For comparison of two groups of data, unpaired Student's t-test was used. For comparison of three or more groups, one-way analysis of variance followed by Dunnett's test was used. The level of significance was set at $P < 0.05$.

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Results

Genome-wide identification of ERα and ERβ binding sites in rat germ cells

Germ cells were successfully enriched from adult rat testes. The enrichment was confirmed by the presence of germ cell markers (*Stra8, Sycp3,* and *Acrv1*) and the absence of Sertoli cell (*Rhox5*), Leydig cell (*Hsd3b*), and peritubular myoid cells (*Acta2*) markers (Supplementary Figure S1). ChIP was performed using anti-ERα and anti-ERβ antibodies in the enriched germ cell population. To confirm the specificity of the antibodies and efficiency of the ChIP experiment, ChIP-qPCR was performed to check for the region having known estrogen response element (ERE) in the *Picalm* gene promoter (Kumar et al. [22]) as a positive control, and for *β-actin* gene promoter with no ERE as a negative control (Supplementary Figure S2). Immunoprecipitated DNA from ERα, ERβ, and IgG (mock) reactions along with Input DNA was subjected to NextGen sequencing. A total of 27 221 binding sites were obtained in ERα ChIP and 20 926 binding sites in ERβ ChIP reactions with a *P* value of <0.01, after subtracting the IgG peak background (Table 1). All peaks obtained for ERα and ERβ ChIP are listed in Supplementary Tables S1, S2, respectively. There were 279 peaks commonly enriched with both ERα and ERβ ChIP indicating that there may be a hetero-dimerisation of the two receptors at these loci (Supplementary Table S3). The majority of the peaks were mapped to intronic regions, followed by 5 kb upstream and downstream regions of enriched genes in both ERα and ERβ ChIP. Sixty percentage of the peaks lie within 20 kb upstream or downstream from the TSS in both ERα and ERβ ChIP (Figure 1). The original data files of the ChIP-Seq study have been deposited in Gene Expression Omnibus (GEO) Database with an accession number GSE147079.

Enrichment of novel pathways in spermatogenesis

To understand the molecular functions of genes enriched after ChIP-Seq with ERα and ERβ, gene ontology analysis was done using Panther Database (PANTHER 14.1). Majority of the genes were involved in binding and catalytic activity (Figure 2A). Among the biological processes, more than 1000 genes (in each category) were involved in cellular, metabolic, biological regulation, and localization processes. The enriched genes were also involved in development, immune system, adhesion, reproduction, cell component organization, and proliferation (Figure 2B).

Pathway analysis of the enriched genes was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database. The top three pathways enriched for ERα and ERβ were metabolic pathways, olfactory transduction and pathways in cancer. Other important signaling pathways enriched were MAPK, Ras, Rap1, and cAMP signaling pathways. Several pathways which play a crucial role in spermatogenesis like the endocytosis, focal adhesion, and regulation of actin cytoskeleton were also enriched (Figure 2C). The top 15 pathways enriched in ERα and ERβ ChIP are listed in Supplementary Table S6, S7, respectively.

In addition, few novel pathways whose role in spermatogenesis or regulation by estrogen is not studied in the testes were also identified, namely prolactin, GnRH, and oxytocin signaling pathways (Table 2). Genes from these pathways were selected for further functional validation. The selected genes and their involvement in the respective pathways are shown in Supplementary Figures S3–S5.

Table 1 Characteristics of ChIP-seq data

	ERα	ERβ
Total No. of Reads	10916745	14486314
Average read length	75.30	75.35
Total No. of peaks	27221	20926
Overlapping peaks	279	
No. of genes	11096	8985
Common genes	5398	
Exclusive genes	5698	3587

Number of reads and peaks obtained by ChIP-Seq of ERα and ERβ in germ cells with a *P* value of <0.01.

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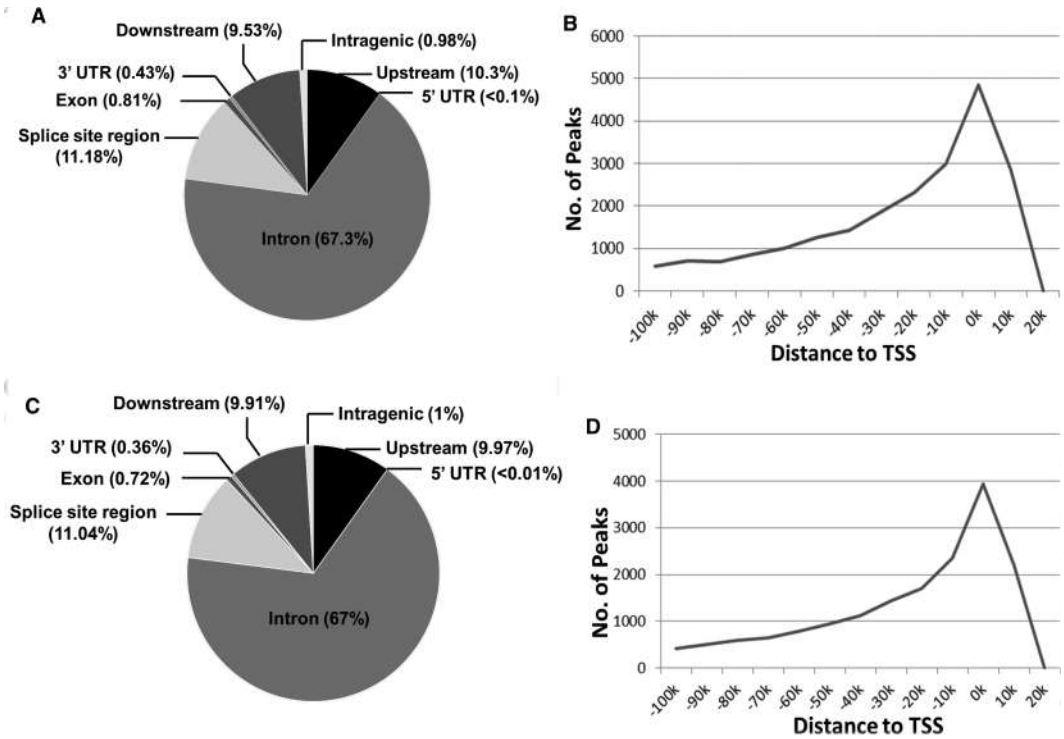

Figure 1. Distribution of ER binding sites across the rat genome.

Regional distribution (left panel) and distance from Transcription Start Site (TSS) (right panel) of the peaks obtained by ChIP of ERα (**A** and **B**) and ERβ (**C** and **D**). The pie diagram of the regional distribution (**A** and **C**) shows the distribution of peaks obtained in intronic, upstream, downstream, intragenic, exonic, splice site (SS), and 3′ and 5′ UTR regions.

Motif analysis for presence of estrogen responsive elements

Motif analysis by TF Bind showed estrogen response elements (EREs) to be present in peaks obtained by ChIP for all the genes selected for validation. The motif similarity score to the consensus ERE for the different genes was in the range of 0.7 to 0.8 (i.e. 70% to 80% similarity). The similarity scores along with the input sequence corresponding to consensus ERE of all the selected genes are represented in the Table 3.

ChIP-qPCR validation

The ChIP-Seq data obtained was validated by ChIP-qPCR. A total of 20 genes were selected from prolactin, GnRH and oxytocin signaling pathway from both ERα and ERβ ChIP (Table 3). Among these, nine genes (*Shc4, Rela, Akt2, Nras, Mapk14, Adcy5, Adcy6, Jun, Calml5*) had only ERα binding sites, eight genes (*Prlr, Prl, Jak2, Stat3, Gsk3b, Pik3ca, Gnrhr, Egfr*) had both ERα and ERβ binding sites, and three genes (*Socs7, Grb2, Prkcb*) had only ERβ binding sites. The primers were designed covering the binding regions obtained by sequencing and spanning the ERE present in the respective genes. All of the selected genes showed significant enrichment in the ChIP for corresponding ERα or ERβ binding sites as compared with IgG (mock) (Figure 3).

Transcriptional regulation of genes by estrogen receptors

To study how estrogen regulates the expression of the selected genes, seminiferous tubules from adult rats were cultured in presence of estrogen receptor subtype specific agonists or antagonists. The expression pattern of these genes was further evaluated in our *in vivo* models of ER agonist treatment. All the genes in the prolactin signaling pathway show an up-regulation with ERα and/or ERβ agonist treatment and the effect was reversed by the corresponding antagonist treatment *in vitro*. Similar results were obtained *in vivo*, confirming the regulation of these genes by the ERs (Figure 4).

In the GnRH signaling pathway, the genes with only ERα (*Nars* and *Mapk14*) and ERβ (*Grb2*) binding sites were up-regulated by the respective agonist treatments both *in vivo* and *in vitro*; the trend was reversed by the

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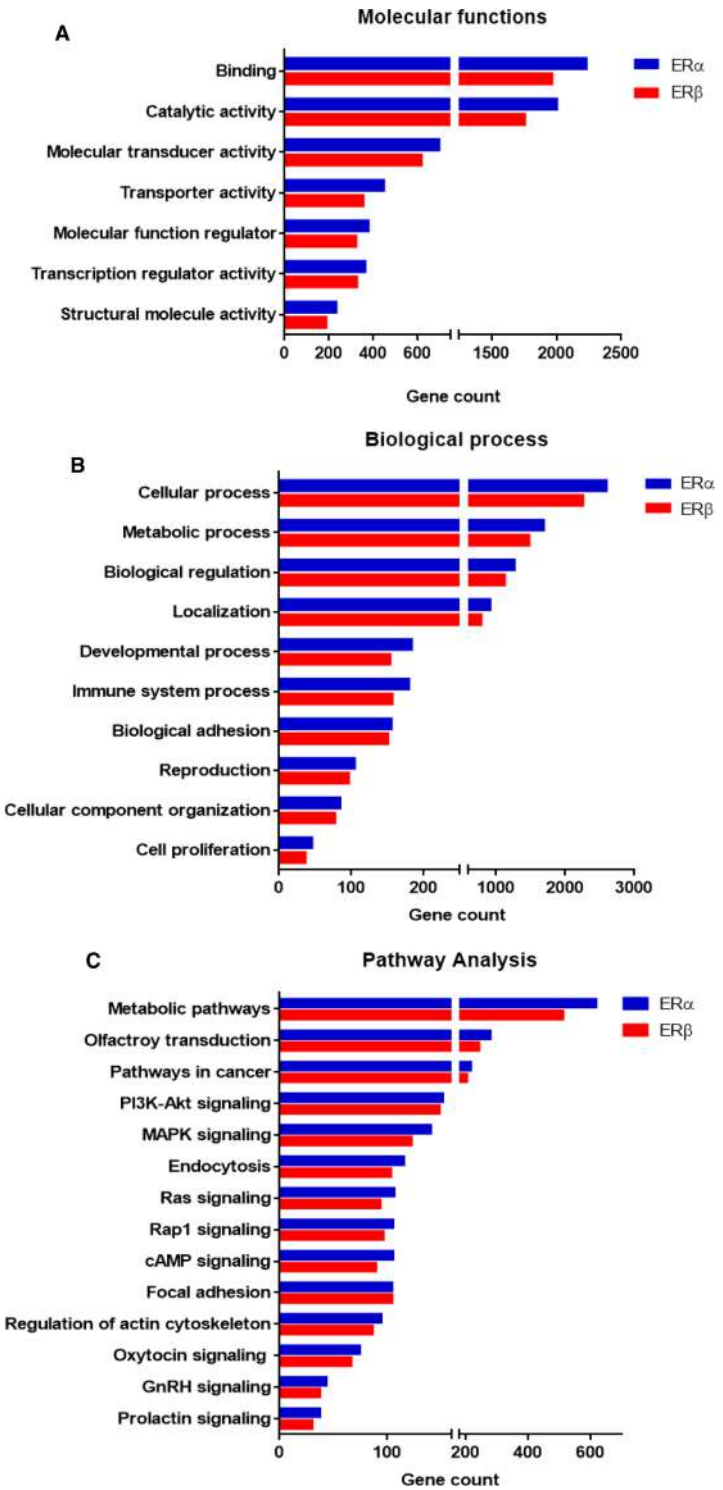

Figure 2. Gene ontology and pathway analysis.

Molecular functions (**A**) and biological processes involved (**B**) of the enriched genes obtained by ChIP-Seq of ERα (blue bars) and ERβ (red bars) analyzed by Panther database. Significant pathways enriched by the KEGG database which are involved in spermatogenesis (**C**).

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Table 2 Genes involved in validated signaling pathways

Signaling pathway	Total number of genes enriched	Genes enriched in ERα and ERβ ChIP	Genes exclusively enriched in ERα ChIP	Genes exclusively enriched in ERβ ChIP
Prolactin	41	26	11	4
GnRH	50	30	13	7
Oxytocin	83	53	21	13

Number of genes exclusive as well as common to ERα and ERβ obtained by ChIP-Seq in germ cells involved in prolactin, GnRH, and oxytocin signaling.

antagonist treatment *in vitro* (Figure 5A,B). For *Gnrhr*, both ERα and ERβ binding sites were observed. However, expression was found to be regulated only by ERβ, as there was a significant down-regulation after ERβ agonist treatment both *in vivo* and *in vitro*, which was reversed by the antagonist treatment *in vitro*. In case of *Egfr*, both the ERs regulate the gene expression. However, a down-regulation was observed *in vitro*, whereas an up-regulation was noted *in vivo* by the agonist treatments (Figure 5A,B).

In the oxytocin signaling pathway, the expression of *Adcy5* was down-regulated, whereas expression of *Adcy6* and Jun was up-regulated after *in vitro* and *in vivo* ERα agonist treatments. Expression of *Calml5* was not found to be affected by any of the treatments. The *Prkcb* gene expression was up-regulated by ERβ agonist treatment both *in vitro* and *in vivo* (Figure 5C,D).

Table 3 Motif analysis for selected genes after ChIP-Seq of ERα and ERβ

Pathway	Gene name	Gene Symbol	Receptor	Score	ERE Sequence	Fold Enrichment
Prolactin	***SHC adaptor protein 4***	***Shc4***	ERα	0.779	GTAGTTCACAACTATCTGT	10.4
	RELA proto-oncogene, NF-kB subunit	***Rela***	ERα	0.761	GAAGGTCAAATGGGAAAGT	7.2
	AKT serine/threonine kinase 2	***Akt2***	ERα	0.742	TGTGCTCACCCTGAGTTTC	7.2
	Prolactin	***Prl***	ERα	0.732	AATAGACAGCTTCACCTAA	5.4
			ERβ	0.759	TTGGCTTATATTAACTTTC	5.7
	Prolactin receptor	***Prlr***	ERα	0.766	ATAGGTCAATGATTCACTG	7.2
			ERβ	0.814	GACGGGCATTCTGACATAA	7.6
	Janus kinase 2	***Jak2***	ERα	0.790	TTAAATTTCGTTGACATGA	10.8
			ERβ	0.759	TAGTGTATATTTGACCATT	5.7
	Signal transducer and activator of transcription 3	***Stat3***	ERα	0.757	TGACGTCAACCTGGTCTAT	5.4
			ERβ	0.730	CCAGCTGGCCTGGACCTCA	7.6
	Phosphatidylinositol -4,5- bisphosphate 3-kinase, catalytic subunit alpha	***Pik3ca***	ERα	0.753	CAGGCTCAGCCTACATTGA	7.2
			ERβ	0.734	GGGGCTCACTGACACTCAC	7.6
	Glycogen synthase kinase 3 beta	***Gsk3b***	ERα	0.759	CCAGCTCAAAACAAGATAT	5.4
			ERβ	0.741	GCAAGATAACTTGAGAAAA	7.6
	Suppressor of cytokine signaling 7	***Socs7***	ERβ	0.754	GCATGTCACATAGAGAGAA	5.7
GnRH	***NRAS proto-oncogene, GTPase***	***Nras***	ERα	0.763	GCAGCTCAGAATAGTGTGA	5.4
	Mitogen activated protein kinase 14	***Mapk14***	ERα	0.737	TAGGCTCAACCTCAGTGTG	5.4
	Gonadotropin releasing hormone receptor	***Gnrhr***	ERα	0.739	ACAGTTACACTTGGCCTTC	7.2
			ERβ	0.764	AGACATTGATGTGAGCTAC	5.7
	Epidermal growth factor receptor	***Egfr***	ERα	0.774	TTCCGTCACCGAAGCTTGG	5.4
			ERβ	0.771	CTGTGTCAGACTGAACCTG	9.6
	Growth factor receptor bound protein 2	***Grb2***	ERβ	0.751	AAAATCAACTCTGACAAAC	7.6
Oxytocin	***Adenylate cyclase 5***	***Adcy5***	ERα	0.738	GAAGGCCAGAGCGGCTGGG	9
	Adenylate cyclase 6	***Adcy6***	ERα	0.733	GTGGGACAAAGAACATTAA	7.2
	Jun proto-oncogene, AP-1 transcription factor subunit	***Jun***	ERα	0.797	TTTTGTCACCGAGCCCTTG	7.2
	Calmodulin-like 5	***Calml5***	ERα	0.781	TCTGGTCAAAGACCCGGAA	7.2
	Protein kinase C, beta	***Prkcb***	ERβ	0.735	TCCCTCACCACTGACCCTA	5.7

Genes from prolactin, GnRH, and oxytocin signaling pathways validated, along with their motif similarity score, input sequence corresponding to the consensus estrogen response element (ERE), and fold enrichment obtained by ChIP-Seq of ERα and ERβ.

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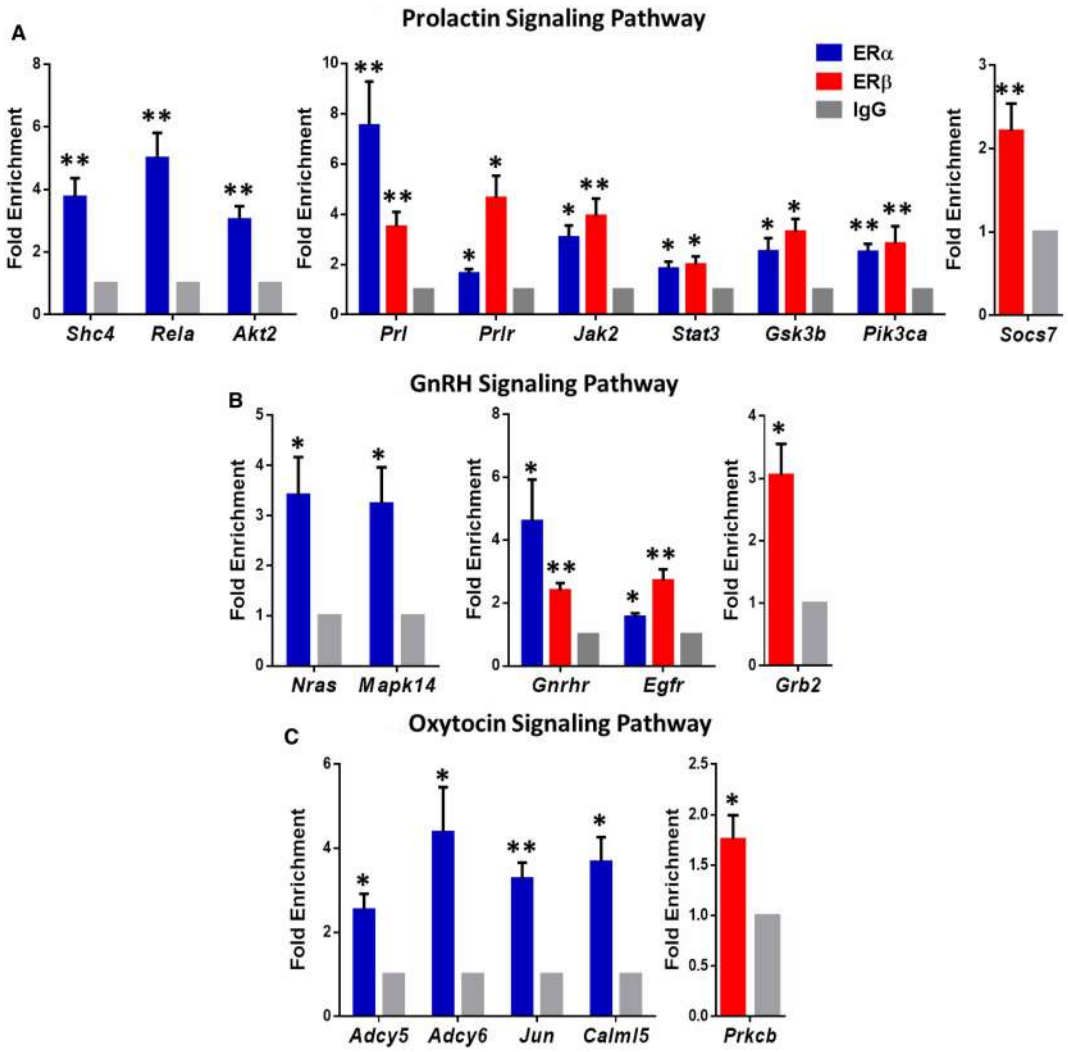

Figure 3. ChIP-qPCR validation for genes involved in (A) prolactin, (B) GnRH, and (C) oxytocin signaling pathway.
Fold enrichment of ERα (blue) and ERβ (red) ChIP over IgG (grey). Values are represented as mean ± SEM; $N = 6$; * denotes $P < 0.05$; ** denotes $P < 0.01$.

Discussion

Several endocrine disrupting compounds found in the environment are estrogenic in nature and exposure to these xenoestrogens cause detrimental effect on male reproductive functions. The epidemiologic data have also shown an increase in human male reproductive function disorders and decline in sperm counts over the past 50 years, with the suggestion of a relation with the increase in the amounts of endocrine disruptors in the environment [24–28]. It has thus become increasingly important to understand the basic physiology and mechanisms by which estrogen regulates spermatogenesis. To address this, previous studies in our laboratory have demonstrated the specific roles of the two ERs in spermatogenesis and fertility using *in vitro* and *in vivo* models [5,6,22,23]. The present study focuses on identification of ERα and ERβ regulated genes in the germ cells to elucidate the significance of ERα and ERβ in adult rat testis under physiological conditions. Using ChIP-Seq approach, a total of 27 221 and 20 926 *in vivo* binding sites for ERα and ERβ, respectively, were identified in germ cells. Of these, only 279 sites were commonly enriched with ERα and ERβ ChIP, indicating that the ERs mainly bind as homo-dimers on the DNA as there are comparably few binding sites for the heterodimers. However, nearly half of the genes enriched have both ERα and ERβ binding sites, indicating a

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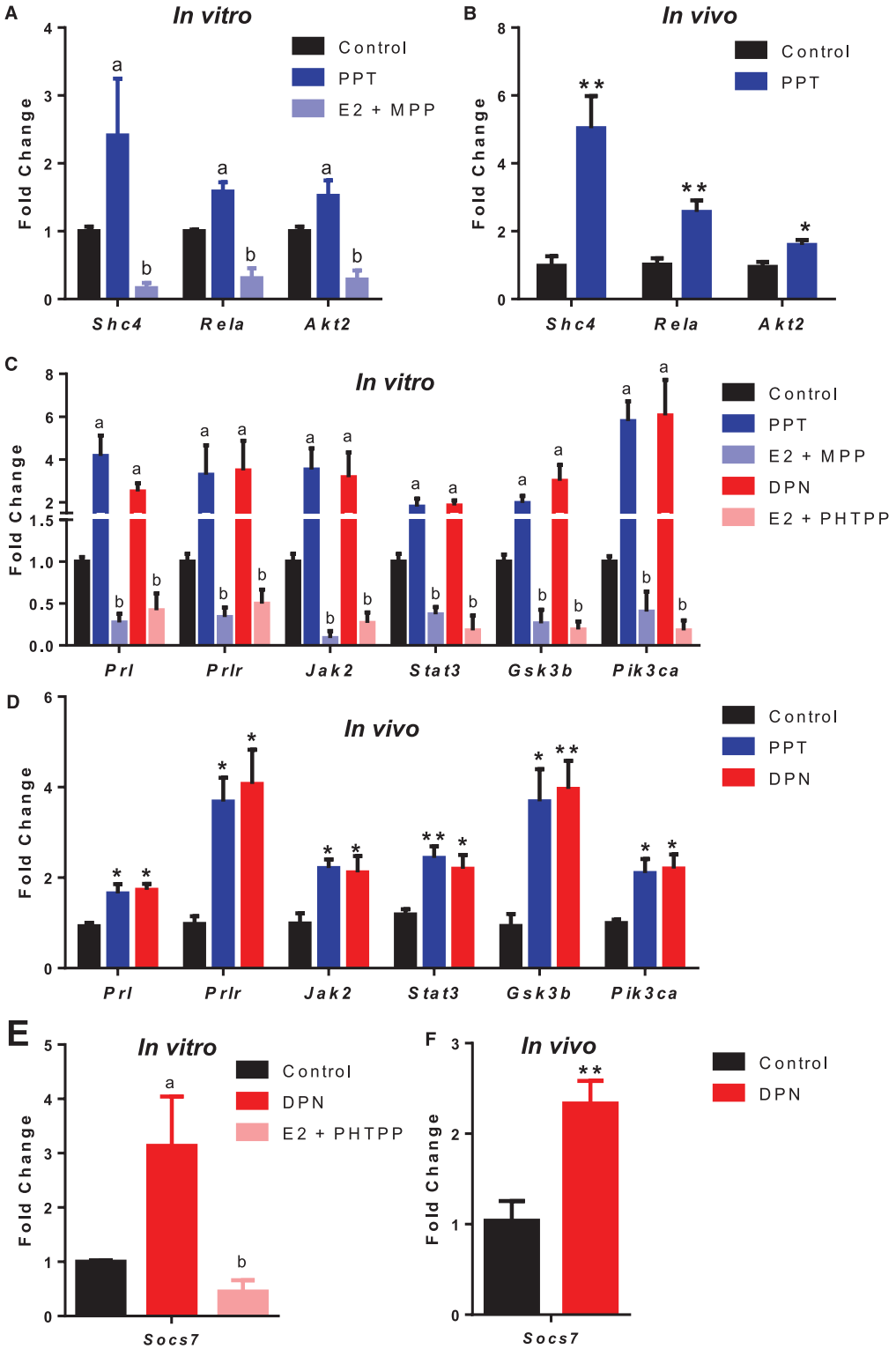

Figure 4. Regulation of genes involved in prolactin signaling pathway.

Expression of genes involved in prolactin signaling pathway in tubule culture (*in vitro*-**A**,**C**,**E**) and in the testis (*in vivo*-**B**,**D**,**F**) after ERα (PPT) and ERβ (DPN) agonist treatments (*in vitro* and *in vivo*) and their respective antagonists (*in vitro*). All values are represented as mean ± SEM; $n = 6$. For *in vitro* results: 'a' denotes significant with respect to the control (DMSO); 'b' denotes significant with respect to the corresponding agonist. For *in vivo* results: * $P < 0.05$, ** $P < 0.01$ with respect to control.

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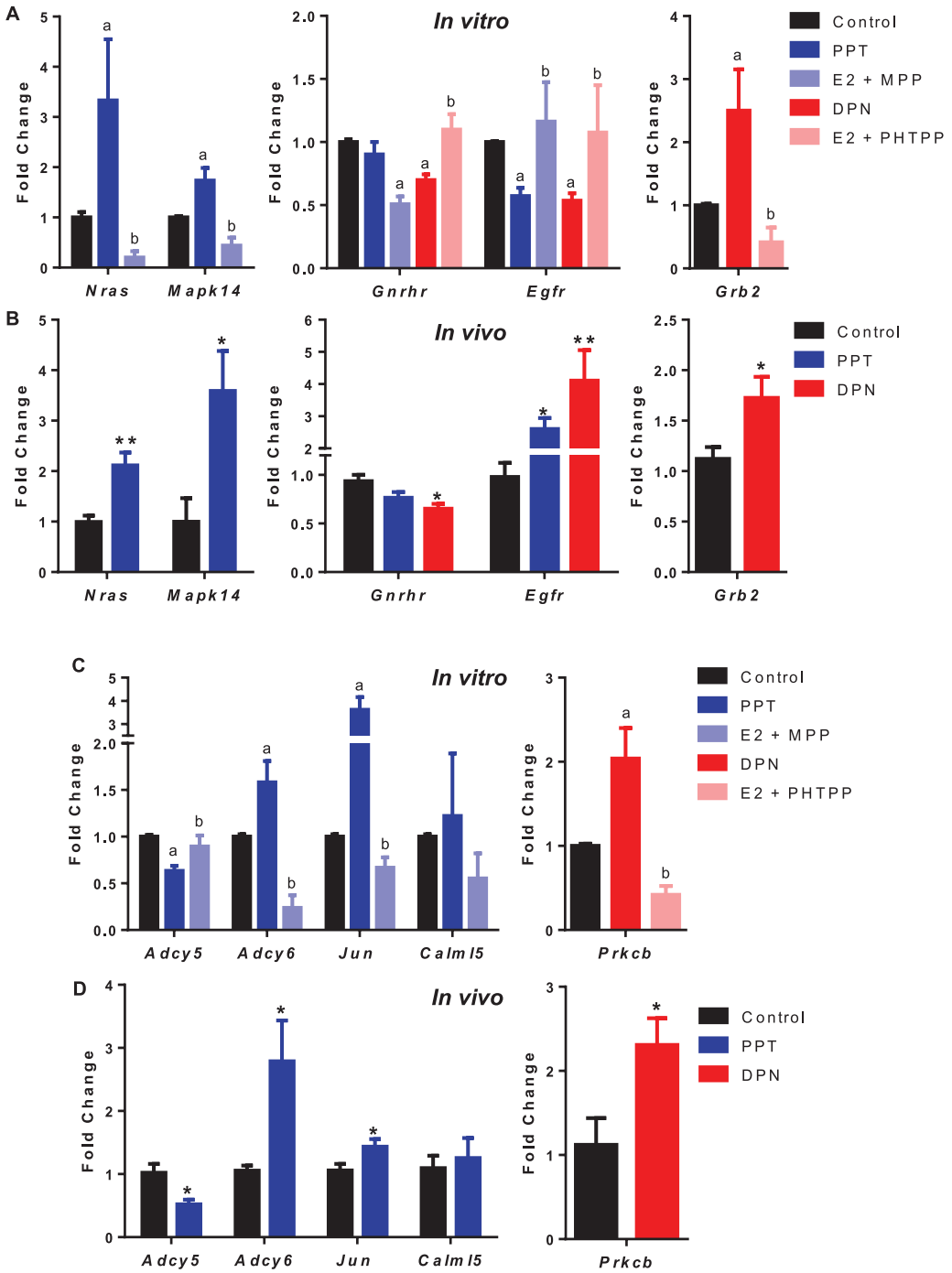

Figure 5. Regulation of genes in the GnRH and oxytocin signaling pathway.

Expression of genes involved in GnRH (**A** and **B**) and oxytocin (**C** and **D**) signaling pathway in tubule culture (*in vitro*-**A** and **C**) and in the testis (*in vivo*-**B** and **D**) after ERα (PPT) and ERβ (DPN) agonist treatments (*in vitro* and *in vivo*) and their respective antagonists (*in vitro*). All values are represented as mean ± SEM; $n = 6$. For *in vitro* results: 'a' denotes significant with respect to the control (DMSO); 'b' denotes significant with respect to the corresponding agonist. For *in vivo* results: * $P < 0.05$, ** $P < 0.01$ with respect to control.

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considerable overlap between the function of these receptors in the testis. Majority of the binding sites were present near TSS, which signifies that the ERs may play a role in transcriptional regulation of these genes.

Almost 60% of the binding sites obtained were present within 20 kb from the TSS, suggesting that the ERs may play a role in transcriptional regulation of these genes. However, it should be noted that binding of transcription factor to their binding sites may not always correlate or lead to gene expression changes. Binding of steroid receptors to their DNA response elements can also bring about conformational changes in chromatin structure of the neighboring domains, which may in turn create platforms for recruitment of other cofactors [29]. Therefore, the numbers of estrogen receptor binding sites in the genome are likely to be greater than the differentially regulated genes. Also, it is likely that the gene expression due to the binding of ERs to the genome can be tissue specific [30]. In addition, it has been previously demonstrated that apart from the canonical ERE DNA binding motifs, ERs also regulate gene transcription by binding to co-operating transcription factors such as Sp-1, AP-1, CEBP, Pitx1, Runx1 [31–34]. CEBPA (CCAAT/enhancer-binding protein alpha) binding motif was present in almost 27% of estrogen receptor binding sites suggesting a role of CEBPA as a cofactor to estrogen receptor. Fatima et al. [35] had shown that estrogen effects on transcription by ERα are associated with CEBPA. The interaction between ERα and Pitx1 (Paired like Homeo-domain 1) has been well studied and Pitx1 is shown to be a selective repressor for transcriptional activity of ERα target genes [34]. Similarly, Runx1 (Runt-related transcription factor 1) has also be demonstrated as a co-regulatory transcription factor of estrogen receptor [33,36]. In our study, we identified several DNA binding motifs to be enriched. This included CEBPA, Pitx1, Runx1, FoxL1, Smad3 to be present in 10% to 20% of the binding sites, suggesting a role of these cooperating factors in ER regulation of genes. In addition, we also obtained other binding motifs like Sox2, Oct2, Lhx3, Hoxd13. This suggests that an additional mechanism of transcriptional control by ERs may include mechanisms involving other tethering factors.

Zhou et al. [19] studied the effects of estrogen treatment in rat testes after suppression of GnRH and androgen signaling. They found a total of 1328 genes to be differentially altered, of which 522 genes showed ERα binding sites and 448 genes showed ERβ binding sites in our study (356 had both ER and ERβ binding sites). However, since GnRH and androgen signaling was suppressed, there was considerable of hormonal disturbance in the background and the effects observed cannot be directly attributed as estrogen induced. Another study by Yao et al. [37] identified estrogen receptor alpha binding sites in mouse efferent ductules and compared it to genes perturbed upon exogenous estradiol treatment in adult mice. Expression of 980 genes was perturbed upon E2 treatment. Comparative analysis of our ERα data set showed a total of 433 genes to be common between the two studies suggesting that estrogen regulation might be conserved across species in different tissues. The common genes found in our ChIP-Seq and these datasets are listed in Supplementary Table S10. Gene ontology and pathway analysis of the overlapping genes showed similar results as observed in our study (data not shown).

Pathway analysis of the genes enriched by ERα and ERβ showed involvement of these genes in a number of different pathways. The top three include metabolic pathways, olfactory transduction, and pathways in cancer. Testis is an important site for metabolic pathways since spermatogenesis is a high energy expedition process. Various energy metabolism pathways are involved in germ cell functioning including carbohydrate, lipid, and protein metabolism [38]. Estrogen receptor alpha and beta are known to regulate a plethora of energy metabolic pathways, including glucose transport, glycolysis, tricarboxylic acid cycle, mitochondrial respiratory chain, adenine nucleotide translocator, and fatty acid β-oxidation and synthesis [39,40]. Thus, it is not surprising that 617 genes in ERα and 511 genes in ERβ were involved in metabolic pathways. Interestingly, olfactory receptors (ORs) are also localized on germ cells [41,42] and are responsible for chemotaxis and sperm motility [43,44]. Estrogen regulates olfactory transduction in olfactory receptor neurons (ORNs) [45]. It can be speculated that estrogen regulates the expression and signaling of this pathway in germ cells, thereby influencing sperm motility. Sperm motility was also found to be reduced in our *in vivo* ERβ agonist treatment model [24]. Estrogen also induces proliferation of germ cells in rodents [46] and it has been shown to promote testicular germ cell cancer [47] which represents the most common solid tumors affecting the young men [48]. These pathways have been explored before for their function in testes as well as for their regulation by estrogen. Some of the anti-apoptotic (like *Bcl2* and *Aven*) and cell cycle regulating (like *Ccnb1*) genes enriched in this study were also found to be affected in our *in vivo* ER agonist models, where germ cell numbers were altered [6]. Estrogen also facilitates regulation and signaling of other fundamental pathways like MAPK, Ras, and cAMP signaling pathway. It increases cAMP levels and induces transcription of several genes as studied *in vitro* in breast and uterine cells [49]. It also activates downstream signaling of MAPK, Ras, and PI3K/Akt [50–52]. Previous

studies also show estrogen to be involved in regulation of actin cytoskeleton and endocytosis related genes in the testes [5,53]. Since estrogen is involved in such varied functions, this suggests that there must be an intricate mechanism by which ERs meticulously regulate the overall transcriptome in the male germ cells. In addition to these pathways, in our study, we identified a few pathways whose role in spermatogenesis is not clearly known, namely, prolactin, GnRH, and oxytocin signaling and studied them further.

Prolactin is a polypeptide hormone known to have multiple roles including osmoregulation, lactation, reproduction, growth and development, endocrinology and metabolism, brain and behavior, and immunomodulation [54]. It acts via prolactin receptor (Prlr) which is a membrane bound tyrosine kinase receptor and signals via the JAK/STAT, MAPK and PI3K pathway. Upon binding of prolactin to its receptor, the receptor dimerizes and brings about phosphorylation of the downstream messengers leading to activation of transcription of prolactin-regulated genes [54]. Previous studies in our laboratory have shown that treatment with ERα (PPT) and ERβ (DPN) agonist leads to an increase in serum prolactin levels [23]. However, the observed effects on spermatogenesis cannot be directly correlated to alterations in serum prolactin levels. Estrogen is known to regulate prolactin as well as its receptor in the brain [55,56] but this regulation in the testes is unexplored. To understand the regulation of prolactin and its downstream targets by estrogen receptors in testes, we analyzed the expression of genes identified by ChIP-Seq involved in prolactin signaling in germ cells. We identified a total of 41 genes, including *Prl* and *Prlr*, involved in prolactin signaling to be enriched by ERα and ERβ ChIP. All the ten selected genes in this pathway showed significant up-regulation after both the agonist treatments indicating that estrogen predominantly stimulates and activates the prolactin pathway via both ERα and ERβ in the germ cells. Interestingly, the expression of *Jak2* and *Stat3* were also found to be regulated by ERα and ERβ. Besides prolactin signaling, these proteins (JAK2 & STAT3) are an integral part of downstream signaling of several kinase cascades. The relationship between estrogen and JAK has been well studied in breast cancer and *Jak2*, is one of the direct estrogen target and it is known to up-regulate its expression [57,58]. JAK/STAT pathway is mainly involved in proliferation and survival, and therefore have a pivotal role in germ cells. Hyperprolactinemia is associated with male infertility [59,60], however, the exact molecular mechanisms of estrogen regulation of the prolactin signaling in testes needs to be further evaluated.

Gonadotropin releasing hormone (GnRH) is a peptide hormone secreted by hypothalamus and acts on its cognate receptor on the pituitary to release the gonadotropins, FSH, and LH. However, its transcript has been also found in the testes in rodents [61]. This locally produced hormone in the testes acts via its receptor GnRHR, which is present on various testicular cells, including interstitial cells and germ cells [62,63]. A recent study showed that GnRH agonist treatment resulted in significant reduction in serum testosterone levels and testicular size, and spermatogenic defects suggesting that GnRH has a role in spermatogenesis [64]. The role of estrogen in regulating GnRH and GnRHR expression in the hypothalamic neurons is well documented [65,66], however, the regulation of these genes in the testis is not known. We observed a total of 50 of genes with ERα and ERβ binding sites involved in GnRH signaling. This might be indicative of mechanism of regulation of GnRH signaling by estrogen in the testis as well. Functional validation showed alteration in crucial genes like *Gnrhr*, *Egfr*, *Mapk14*, *Nras*, and *Grb2* after estrogen receptor specific agonist treatment. Estrogen has been shown to regulate Gnrhr expression in cultured human granulosa-luteal cells in a time-dependent manner [67]. Specifically, ERβ regulates the expression of *Gnrhr* in hippocampal neurons in rats [68]. We also found regulation of *Gnrhr* transcripts by ERβ in the testis in this study. In addition, there is a cross-talk between EGFR and estrogen receptor [69]. Estrogen treatment suppresses *Egfr* expression in breast cancer cells *in vitro* [70], whereas, in human endometrium (*in vivo* conditions) estrogen treatment up-regulates *Egfr* expression [71]. This opposing regulation *in vitro* and *in vivo* corroborates with our results, suggesting an intricate mechanism of regulation of *Egfr* by the ERs.

Oxytocin (Oxt), a peptide hormone, is known to be locally produced in the testes [72]. The actions of OT are all mediated by its receptor, OXTR, which is a transmembrane receptor belonging to the G-protein-coupled receptor superfamily. The Gq/PLC/Ins3 is the main signaling pathway activated, along with MAPK and RhoA/Rho kinase pathways. In mammalian testes, OXTR is localized on Leydig cells and Sertoli cells [73,74]. In a transgenic mouse overexpressing oxytocin in the tubular compartment of the testis, there appeared to be a specific reduction in both testosterone and DHT within the testis [75]. Apart from its role in steroidogenesis, oxytocin is thought to aid spermiation by regulating seminiferous tubule contractility. Frayne et al. [76] demonstrated an early appearance of spermatozoa and residual bodies in oxytocin-treated rats as compared with the control and delay in the same in rats treated with oxytocin antagonist. We identified a total of 83 genes involved in oxytocin signaling to be enriched by ERα and ERβ ChIP. Interestingly, OXTR is not present

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on the germ cells which corroborates with our study since we did not observe any enrichment of oxytocin receptor in the germ cells. This suggests that estrogen might not be directly regulating the *Oxt* and *Oxtr* gene, but could be involved in modulating the genes in downstream signaling like *Adcy5*, *Adcy6*, *Jun*, and *Prkcb*.

Many of the genes picked for validation showed alteration in the transcript level upon estrogen receptor agonist treatment. However, there were a few genes which showed no effects. This suggests that the binding of ERs to the DNA does not always correspond to transcriptional regulation. It should also be noted that the changes observed at transcript levels of genes involved in various pathways may or may not correlate with the protein levels. To confirm the activation of the pathways by these genes further validation at protein level would be required.

Conclusion

In conclusion, our study gives a genome-wide map of ERα and ERβ binding sites in germ cells under physiological conditions. It provides information about genes and their pathways that are commonly as well as individually regulated by ERα and ERβ, thus highlighting their exclusive and overlapping roles in spermatogenesis. Knowledge of ER binding sites under unperturbed conditions in germ cells will help in understanding the role of estrogen during normal adult spermatogenesis.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

A.K. and N.B. conceptualized the study. A.K. and S.R. performed the ChIP and tubule culture experiments. K.K. performed the *in vivo* experiments. S.R., K.K. and S.D. analyzed the data. S.R. and K.K. prepared the manuscript. A.K., S.R., K.K., S.D. and N.B. approved the manuscript.

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Abbreviations

CEBPA, CCAAT/enhancer-binding protein alpha; ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; DMSO, dimethylsulfoxide; ERE, estrogen response element; GnRH, Gonadotropin releasing hormone; KEGG, Kyoto Encyclopedia of Genes and Genomes; LBD, ligand binding domain; ORNs, olfactory receptor neurons; ORs, olfactory receptors; OT, oxytocin; SDS, sodium dodecyl sulfate; TSS, transcription start site.

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