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Sex hormones regulate lipid metabolism in adult Sertoli cells: A genome-wide study of estrogen and androgen receptor binding sites

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ABSTRACT

Optimal functioning of Sertoli cells is crucial for spermatogenesis which is under tight regulation of sex hormones, estrogen and androgen. Adult rat Sertoli cells expresses estrogen receptor beta (ERβ) and androgen receptor (AR), both of which regulate gene transcription by binding to the DNA. The present study is aimed to acquire a genome-wide map of estrogen- and androgen-regulated genes in adult Sertoli cells. ChIP-Seq was performed for ERβ and AR in Sertoli cells under physiological conditions. 30,859 peaks in ERβ and 9,594 peaks in AR were identified with a fold enrichment >2 fold. Pathway analysis for the genes revealed metabolic pathways to be significantly enriched. Since Sertoli cells have supportive functions and provide energy substrates to germ cells during spermatogenesis, significantly enriched metabolic pathways were explored further. Peaks of the genes involved in lipid metabolism, like fatty acid, glyceride, leucine, and sphingosine metabolism were validated. Motif analysis confirmed the presence of estrogen- and androgen-response elements (EREs and AREs). Moreover, transcript levels of enzymes involved in the lipid metabolic pathways were significantly altered in cultured Sertoli cells treated with estrogen and androgen receptor agonists, demonstrating functional significance of these binding sites. This study elucidates a mechanism by which sex hormones regulate lipid metabolism in Sertoli cells by transcriptionally controlling the expression of these genes, thereby shedding light on the roles of these hormones in male fertility.

1. Introduction

Sertoli cells, initially identified by Enrico Sertoli in 1865, play a crucial role in the process of spermatogenesis. They are somatic cells that act as structural elements of the seminiferous epithelium and provide nutrients and energy to developing germ cells, and hence are also called 'nurse cells' or 'supporting cells' [1]. Adequate functioning of the Sertoli cell is required for appropriate spermatogenesis which is required for male fertility. The process of spermatogenesis is under tight regulation of steroid hormones and gonadotropins. It is imperative to note that, Sertoli cells act as the key hormonal target for the action of steroid hormones estrogen and androgen in the seminiferous epithelium [2].

Testicular steroid hormone production is controlled by gonadotropins, LH and FSH, which are released from the pituitary [3]. LH binds to its receptor present on Leydig cells and promotes the production of testosterone, whereas FSH acts on Sertoli cells to produce various factors necessary for spermatogenesis, including inhibin and estrogen [4]. Since

Abbreviations: ERβ, estrogen receptor beta; AR, androgen receptor; ChIP-Seq, chromatin immunoprecipitation sequencing; ERE, estrogen response elements; ARE, androgen response elements; TSS, transcription start site; UTR, untranslated region; E2, 17β-estradiol; DHT, dihydrotestosterone; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; DMEM, Dulbecco's Modified Eagle Medium; KEGG, Kyoto Encyclopedia of Genes and Genomes; Rn18s, 18S ribosomal RNA; Stra8, stimulated by retinoic acid 8; Sycp3, synaptonemal complex protein 3; Acrv1, acrosomal vesicle protein 1; Rhox5, Rhox homeobox family member 5; Hsd3b, hydroxy-delta-5-steroid dehydrogenase 3 beta- and steroid delta-isomerase 5; Acta2, actin alpha 2 smooth muscle; Arpc1b, actin related protein 2/3 complex subunit 1B; Stx5a, syntaxin 5a; Acsl6, Acyl-CoA synthetase long-chain family 6; Gpat2, Glycerol-3-phosphate acyltransferase 2; Acads, Acyl-CoA dehydrogenase short chain; Dgkz, Diacylglycerol kinase zeta; Dgka, Diacylglycerol kinase alpha; Acox3, Acyl-CoA oxidase 3; Sphk1, Sphingosine kinase 1; Oxct1, 3-oxoacid CoA transferase 1; Acaca, Acetyl-CoA carboxylase alpha; Acsl1, Acyl-CoA synthetase long-chain family 1; Mgll, Monoglyceride lipase; Acadm, Acyl-CoA dehydrogenase medium chain; Ivd, Isovaleryl-CoA dehydrogenase; Auh, AU RNA binding methylglutaconyl-CoA hydratase.

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testosterone is produced locally in the testes, the intratesticular testosterone (iTT) levels are much higher compared to serum. It has been previously demonstrated that these high iTT levels are crucial for the maintenance of normal spermatogenesis and very low levels of iTT can result in infertility or abnormal spermatogenesis [5]. Testosterone produced by Leydig cells diffuses into seminiferous tubules and acts via the androgen receptor present on the Sertoli cells. Several studies suggest that the action of testosterone on spermatogenesis is by paracrine regulation through Sertoli cells [6–8]. Testosterone is crucial for maintenance of blood-testis-barrier (BTB), meiosis, Sertoli-spermatid adhesion, spermiogenesis, and sperm release [9]. Estrogen also regulates key steps in spermatogenesis such as sperm release and germ cell survival [10–12]. Estrogen is produced from testosterone by an enzyme, aromatase, whose inhibition has an adverse effect on germ cells, specifically, round and elongated spermatids [13,14]. This suggests that actions of the sex hormones, androgen and estrogen, are crucial for maintenance of spermatogenesis.

The effects of androgen and estrogen on Sertoli cells are brought about by androgen receptor (AR) and estrogen receptor beta (ERβ), respectively, which belongs to class 1 family of nuclear receptors. Upon ligand binding, the receptor dimerize and translocate into the nucleus where they interact with the target DNA, either directly through hormone response elements (HREs) which are palindromic DNA sequences, or indirectly via co-transcription factors. This binding brings about changes in the chromatin thereby regulating the expression of various genes [15]. The importance of AR and ERβ in spermatogenesis has been highlighted by their ablation and knockout models. AR knockout (ARKO) male mice were infertile and demonstrated severe defects in male sex differentiation having female-like appearance, small testes size with cryptorchidism, reduced number of Leydig and Sertoli cells, and complete androgen insensitivity [16–18]. Sertoli cell specific AR knockout studies demonstrated normal development of reproductive organs; however, the mice were infertile with a significant reduction in the number of spermatocytes and spermatids [18,19]. The phenotype observed in the knockout model suggests that AR on Sertoli cell is indispensible for spermatogenesis, especially for meiosis. ERβ null mutant male mice generated by Antal et al. were also found to be infertile [20]. In addition, similar effects were seen in the aromatase knockout male mice [21].

Very few studies have investigated estrogen and androgen responsive genes in the testes by using ablation or over-exposure animal models [22–25]. To better understand the role of these steroid hormones in spermatogenesis, it is important to identify hormone responsive genes under unperturbed conditions. We have previously reported ERα and ERβ responsive genes in the adult rat male germ cells under normal physiology [26]. In the present study, we identified estrogen- and androgen-responsive genes in adult Sertoli cells under unperturbed conditions using a genome-wide next generation sequencing-based approach and have uncovered the involvement of these steroid receptors in lipid metabolism in adult Sertoli cells.

2. Materials and methods

2.1. Animal acquisition

Male Holtzman rats (75–90 days old) were obtained from the Institute's animal house facility. The animals were maintained under controlled temperature (22 ± 1 °C), humidity (55 ± 5%) conditions in a fixed 12-h light, 12-h dark cycle with free access to food and water. The use of animals for the study was approved by the Institute's Animal Ethics Committee (Ethics Approval No. 05/17). The animal experimental work for this study was carried out at ICMR-National Institute for Research in Reproductive Health, Mumbai, India.

2.2. Adult Sertoli cells enrichment

To identify the estrogen and androgen receptor binding sites in adult Sertoli cells, Sertoli cell population was obtained from adult rat testes with modifications in the protocol previously described by Kumar et al. [27]. Briefly, adult male rats were sacrificed by cervical dislocation. 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 Dulbecco's Modified Eagle Medium- F-12 (DMEM-F12) and treated with trypsin (1 mg/mL) followed by DNase (0.5 mg/mL) (HiMedia Laboratories, India) and incubated at 37 °C on the shaker (80 rpm) for 8 min and 5 min, respectively. The tubules were allowed to settle down and the supernatant was passed through 70 μm nylon mesh. The filtrate thus obtained was passed through a 40 μm nylon mesh. The filters were then back washed using Hanks Balanced Salt Solution (HBSS) and the resulting mixture was suspended in water and centrifuged for 8 min at 800 rpm to pellet the Sertoli cells. A fraction of the Sertoli cells suspension was set aside to check for purity using gene expression markers (listed in Supplementary Table S1) as mentioned previously in Kumar et al. [27]. The remaining cells were crossed-linked using 1% (v/v) formaldehyde for 10 min. at room temperature, followed by addition of 125 mM glycine to block further crosslinking. The cross-linked cells were washed twice with chilled phosphate buffered saline containing protease inhibitors (cOmplete™, Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Germany) and stored at -80°C for chromatin immunoprecipitation.

2.3. Chromatin immunoprecipitation (ChIP)

ChIP for ERβ and AR was performed on the Sertoli cells following a protocol previously described by Raut et al. [26]. Briefly, the crosslinked Sertoli cells were lysed by re-suspending in sodium dodecyl sulfate (SDS) buffer (1% SDS in Tris-EDTA buffer) and incubated on ice for 15 min. 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. This was followed by centrifuging for 10 min at 13,000 rpm, and 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 obtained was diluted 10-fold using ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl, 167 mM NaCl) and pre-cleared using Protein A agarose beads (Merck Millipore, USA) for 60 min with slow rotation. Pre-cleared chromatin was then subjected to immune-precipitation using anti-ERβ (1 μg) (Sigma-Aldrich, USA), anti-AR (1 μg) (Santa Cruz Biotechnology, Inc. USA), or rabbit IgG (Mock reaction) overnight at 4 °C. 10% of the pre-immuno-precipitated lysate was set aside as 'input' for normalization. The immuno-precipitated DNA was then washed sequentially with low salt (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, 150 mM NaCl), high salt (0.01% SDS, 1% Triton X-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 at 4 °C on rotation. 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 obtained was then reversed cross-linked with 5 M NaCl at 65 °C overnight. DNA was purified using ExpinTM CleanUp SV (GeneAll Biotechnology Co. Ltd., Korea), eluted in nuclease-free water and stored at −20 °C.

2.4. ChIP-Sequencing

After performing ChIP for ERβ and AR, qPCR was performed for the

known binding sites of ERβ and AR (Arpc1b ERE and Stx5a ARE) [28] to check the efficiency of ChIP experiment. The samples from six individual ChIP experiments (from six animals) were pooled, and sequenced using next generation sequencing at Sandor Lifesciences, India. Briefly, the immuno-precipitated DNA was quantified by Qubit2.0. Agilent Bioanalyser was used for size distribution for quality control of library preparation. 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. DNA fragments with ligated adapter molecules on both ends were selectively enriched in the final PCR reaction. Products were purified using AMPure XP system (Beckman Coulter, USA). The final libraries were quantified using Kapa qPCR Kit for adapter ligated molecules and were sequenced on 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).

2.5. Pathway and motif analysis

KEGG and DAVID Databases were used for the analysis of pathways enriched of the genes obtained by ERβ and AR ChIP-Seq. Gene Ontology for molecular functions and biological processes were performed using the PANTHER database. To obtain estrogen and androgen response elements (EREs and AREs), TRANSFAC database was used, which is a motif discovery algorithm and uses the established consensus sequence for EREs (AGGTCAnnnTGACCT) and AREs (AGAACAnnnTGTTCT) [29].

2.6. ChIP-qPCR

Data obtained after ChIP-Seq was validated by ChIP-qPCR. ChIP for ERβ and AR was performed inadult Sertoli cells followed by qPCR. Briefly, amplification reactions of 20 μL (in duplicate) were set up containing 1.6 μL of DNA (Input/ChIP/IgG), 10 pM of respective primers and Takyon SYBR Green Mastermix (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-Aldrich and the sequences are mentioned in Supplementary Table S2. Fold enrichment of ChIP over mock was calculated by normalizing with the input sample as described previously [27]. Data analysis was performed for ChIP-qPCR results obtained from four different animals.

2.7. Sertoli cell in vitro culture and treatment with ERβ and AR agonists

Adult Sertoli cells were first isolated as described above. The Sertoli cells pellet obtained was resuspended in DMEM-F12 without phenol red and with glutamine and HEPES (pH 7.4) (Gibco, USA), supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), selenite (5 ng/mL) (Sigma-Aldrich, USA), penicillin (50,000 U/L), streptomycin (50 mg/mL), and kanamycin (10 mg/mL) (Gibco, USA). For comparative analysis, Sertoli cells from three animals were first pooled and then seeded into three petri plates, treated with 17-β estradiol (E2) (10^{-9} M), and dihydrotestosterone (DHT) (10^{-8} M). Only DMSO (dimethyl sulfoxide) was used as the control. The concentrations of the drugs were decided based on the previous studies in the lab [11]. The cells were incubated at 32°C for 24 h with 5% CO2. After 24 h, Sertoli cells were treated with TrypLE™ Express Enzyme (1X) (Gibco, USA) for 8 min at 37°C, followed by the addition of charcoal stripped FBS (15%) in DMEM-F12 to stop the reaction. The cells were then collected in eppendorf tubes and centrifuged at 4500 rpm for 4 min 30 s. The cell pellet obtained was resuspended in TRI Pure reagent (Roche Diagnostics, Germany) and used for RNA extraction.

2.8. RNA extraction and qPCR

Total RNA was extracted from Sertoli 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. 2 μg of the RNA extracted was then reversed transcribed using High Capacity Reverse Transcription system (Applied Biosystems, USA) 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. Rn18S (18S ribosomal RNA) was used as the reference gene. Pfaffl method was used for normalization with the reference gene to calculate relative expression. Primers for the reference (Rn18S) and all target genes were synthesized by Sigma-Aldrich, India. The primer sequences for all the genes are mentioned in Supplementary Table S3. Melt curve analysis was performed for checking specificity, and all products obtained yielded the predicted melting temperature (Supplementary Figure S1). All procedures and analysis for Real Time PCR followed MIQE guidelines as mentioned in Supplementary Table S4.

2.9. Statistical analysis

The statistical analysis was performed using Graph Pad Prism (version 6, Graph Pad Inc., USA). For comparison between groups, unpaired Student's *t*-test was used. The level of significance was set at $p < 0.05$.

3. Results

3.1. Genome-wide mapping of ERβ and AR binding sites in Sertoli cells

The purity of the isolated adult Sertoli cells was confirmed using cell-type specific markers. The adult Sertoli cells showed the presence of transcripts of Sertoli cell marker (*Rhox5, Vim, Fshr, Wt1*), and absence of germ cell (*Stra8, Sycp3, Acrv*), Leydig cell (*Hsd3b*), and peritubular myoid cell markers (*Acta2*) (Supplementary Figure S2). ChIP was performed in adult Sertoli cells using anti-ERβ and anti-AR antibodies. The efficiency of ChIP antibodies was evaluated by performing ChIP-qPCR for known regions consisting of validated EREs and AREs (as a positive control, Supplementary Figure S3). After evaluation of the efficiency of the ChIP grade antibodies, the ChIP-ed DNA along with Input was subjected to Illumina next generation sequencing. A total of 30,859 peaks (12,047 genes) in ERβ ChIP and 9,594 peaks (5,990 genes) in AR ChIP were identified with p value <0.01 and fold enrichment >2 fold (Table 1). All the peaks identified for ERβ and AR ChIP are listed in Supplementary Table S5 and S6, respectively. Regional distribution of the peaks obtained showed that a majority of the peaks were in the intronic regions, followed by splice site region, and within 5 kb upstream or downstream of the enriched gene in both ERβ and AR peaks (Fig. 1A and Fig. 1C). Approximately 60–65 % of the peaks lay within 20 kb proximity from the Transcription Start Site (TSS) suggesting

Table 1
Characteristics of data obtained from ChIP-Seq of ERβ and AR in adult rat Sertoli cells.

Parameters	ERβ ChIP	AR ChIP
Total No. of Reads Sequenced	24796658	30190070
Total No. of Reads Uniquely Mapped	19996725	28275523
Average Read Length	76	76
Total No. of Peaks	30860	9594
Genes associated with Peaks	12047	5990
Exclusive Genes	7814	1757
Common Genes	4233	

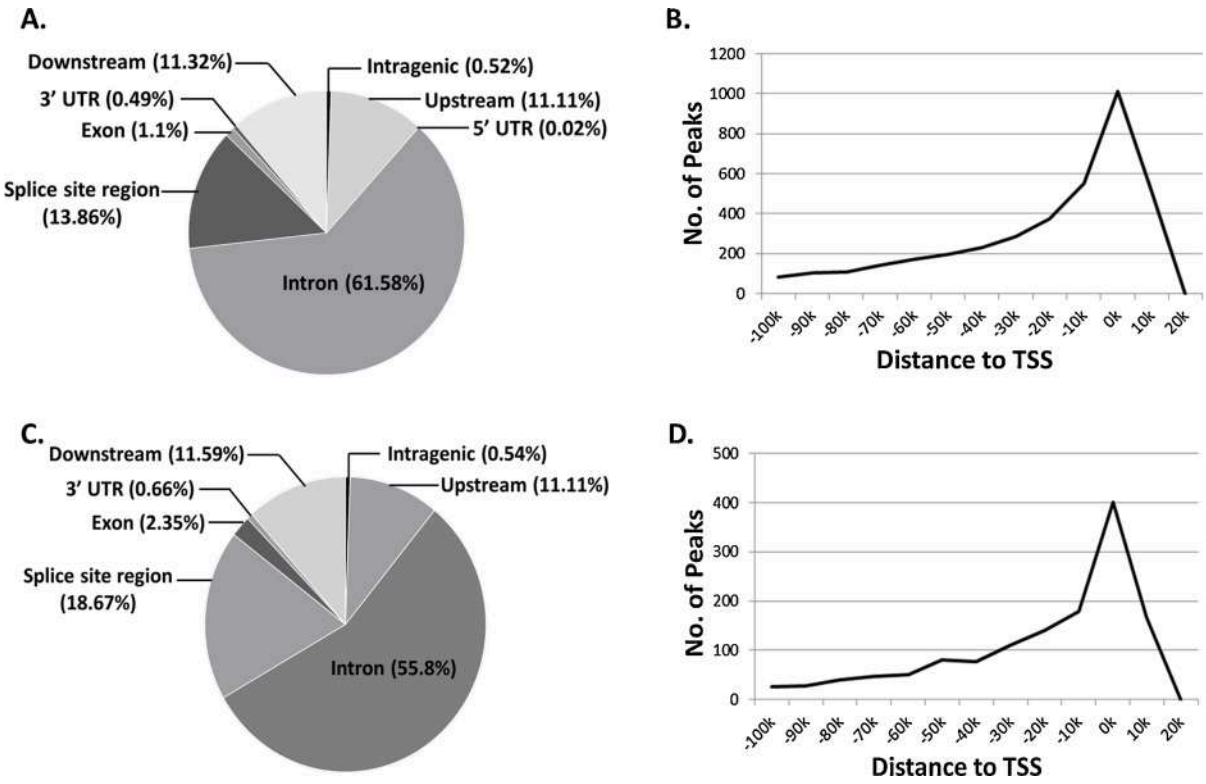

Fig. 1. Distribution of ERβ and AR binding sites in rat genome. Regional distribution (left panel) and Distance to Transcription Start Site (TSS) (right panel) of binding sites obtained by ERβ (A and B) and AR (C and D) ChIP-Seq in adult Sertoli cells. The pie diagram of regional distribution (A and C) shows binding sites obtained in intron, exon, splice site region, upstream, downstream, intragenic, and 3′ and 5′ UTR. The graph of distance to TSS (B and D) illustrates the distance of between the peak and TSS of the gene.

functional relevance of these binding sites (Fig. 1B and Fig. 1D).

3.2. Gene function and pathways enriched of the genes identified

The binding sites identified by ChIP-Seq were linked to a total of 12,047 genes in ERβ and 5,990 genes in AR in the Sertoli cells, of these 4,233 genes were common to both (Table 1). To understand the functions of genes identified, gene ontology analysis was carried out using PANTHER Database (PANTHER 14.1). Molecular function analysis showed that the majority of the genes (>2000 in ERβ and >1000 in AR) were involved in binding and catalytic activity. A significant number of other genes in both ERβ and AR were involved in functions such as molecular transducer activity, transporter activity, molecular function regulator activity, transcription regulator activity, and structural molecule activity (Fig. 2A). Among biological processes, cellular and metabolic processes, as well as biological regulation and localization were the top processes enriched. Developmental, biological adhesion, reproduction, cellular component organization, and cell proliferation processes also showed enrichment. The top 10 biological processes enriched are listed in Fig. 2B.

Pathway analysis of the genes was carried out using the KEGG Pathway and DAVID Database. The top three pathways enriched in both ERβ and AR were metabolic pathways, pathways in cancer, and PI3K-Akt signaling pathway. Olfactory transduction and cAMP signaling were found to be exclusive to ERβ (Fig. 3A), whereas proteoglycans in cancer and regulation of actin cytoskeleton were exclusive to AR (Fig. 3B). Other prominently enriched pathways included MAPK signaling, focal adhesion, endocytosis, Ras signaling, Rap1 signaling (Fig. 3). Additionally, pathways involved in tight junction, cell adhesion molecules, gap junctions, and adherens junction were also enriched (data not shown).

3.3. Lipid metabolism genes are regulated by estrogen and androgen in Sertoli cells

Among metabolic pathways associated with both ERβ and AR in Sertoli cells, a significant number of genes were involved in lipid metabolism.. These included fatty acid synthesis, fatty acid degradation, lipid homeostasis, glycerolipid metabolism, glycerophospholipid metabolism, sphingolipid metabolism, biosynthesis of unsaturated fats, fat digestion and absorption, and regulation of lipolysis. All pathways associated with lipid metabolism and the number of genes obtained by ERβ and AR ChIP in Sertoli cells are listed in Supplementary Table S7. Few genes were found to be exclusive to ERβ and AR, whereas some genes were found to be overlapping between the two. Out of the 456 (ERβ) and 245 (AR) genes, 7 genes (*Acsl6, Gpat2, Acads, Dgkz, Dgka, Acox3, Sphk1*) having ERβ binding site and 7 genes (*Oxct1, Acaca, Acsl1, Mgll, Acadm, Ivd, Auh*) having AR binding sites were selected and further validated by ChIP-qPCR as well as by Sertoli cells *in vitro* culture upon agonist treatment. Tables 2 and 3 summarize the selected genes, pathways involved, their function, receptor binding site, and localization in the different cell types in testes. Almost all the genes selected have been reported to be localized in the testes (Table 2).

3.4. Presence of EREs and AREs in the peaks identified

Motif analysis was performed using TRANSFAC for all the genes selected for validation to identify genomic regions bound by AR and ERβ. The peaks selected from ERβ ChIP showed the presence of EREs and the peaks from AR ChIP showed the presence of AREs. The EREs and AREs were detected on the basis of similarity score to the consensus ERE and ARE. Table 3 summarizes the peaks of genes selected. The matrix score corresponds to the similarity to consensus sequence and all genes showed similarity in the range of 0.7 to 0.9 (i.e. 70%–90% similarity).

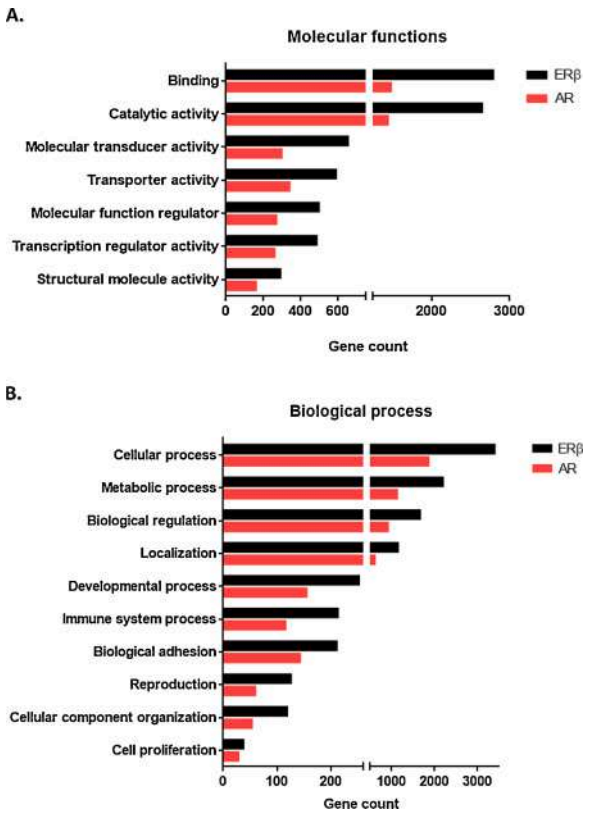

Fig. 2. Gene Ontology of the genes identified by ERβ and AR ChIP-Seq. Molecular functions (A) and Biological processes (B) of the genes obtained by ERβ (black bars) and AR (red bars) ChIP-Seq analysed by Panther Database.

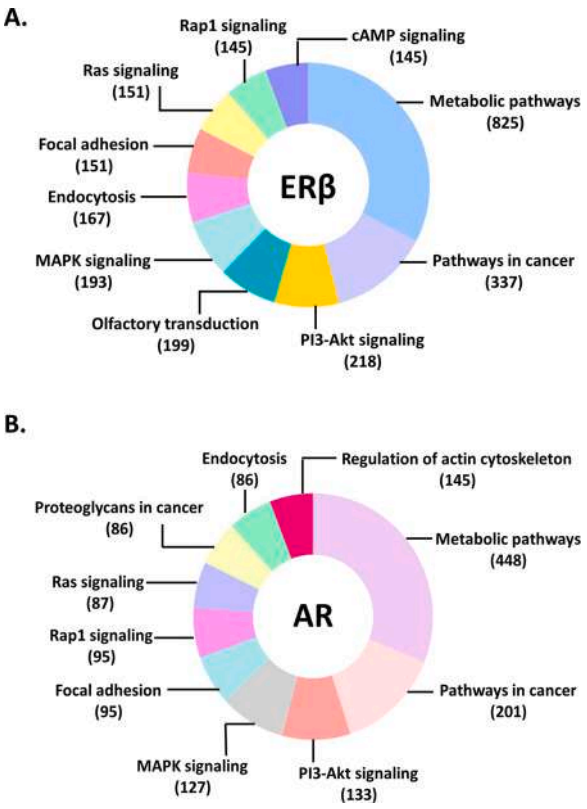

Fig. 3. Pathway analysis of the genes identified by ERβ and AR ChIP-Seq. Top 10 pathways of genes whose binding sites were obtained by ERβ (A) and AR (B) ChIP-Seq analysed by KEGG Pathway Database.

All the selected genes showed 8–17 fold enrichment by ChIP-Seq.

3.5. Validation of binding sites of ERβ and AR by ChIP-qPCR

Upon pathway and motif analysis, a significant number of genes were found to be involved in lipid metabolism. Since Sertoli cells are important for metabolic activity, and the regulation of lipid metabolism by sex hormones in adult Sertoli cells is unexplored, it was selected for further validation. ChIP-qPCR for a total of 14 genes that were involved in lipid metabolism was performed. Among these, seven genes (*Acsl6, Gpat2, Acads, Dgkz, Dgka, Acox3, Sphk1*) had ERβ binding sites, and seven genes (*Oxct1, Acaca, Acsl1, Mgll, Acadm, Ivd, Auh*) had AR binding sites. The genes selected had estrogen and androgen response elements (EREs and AREs), respectively (Table 3). Primers for ChIP-qPCR were designed spanning these response elements obtained in the binding regions of peaks of the genes. All the selected genes showed a significant enrichment after ChIP-qPCR for the corresponding ERβ and AR binding sites as compared to the IgG, thereby, confirming the binding of ERβ and AR to these genes (Fig. 4).

3.6. Integration of binding sites and transcriptional regulation of genes in lipid metabolism

In order to investigate the functional relevance of these binding sites obtained by ChIP-Seq of ERβ and AR, Sertoli cells from adult male rats were cultured *in vitro* and treated with ERβ and AR receptor agonists, 17-β estradiol (E2) (10^{-9} M) and dihydrotestosterone (DHT) (10^{-8} M), respectively. The alteration in the expression of the selected genes was studied after 24 h of treatment. All the seven genes (*Acsl6, Gpat2, Acads, Dgkz, Dgka, Acox3, Sphk1*) regulated by ERβ showed a significant up-regulation upon treatment with E2 (Fig. 5A). On the other hand, for AR, five genes (*Oxct1, Acsl1, Mgll, Acadm, Auh*) showed significant up-regulation, whereas two genes (*Acaca, Ivd*) showed significant down-regulation upon treatment with DHT (Fig. 5B). In addition, the genes regulated by ERβ were cross validated for their expression upon DHT treatment, and the genes regulated by AR were cross validated upon E2 treatment. The results obtained showed no significant difference in the expression of these genes in the adult Sertoli cells (Supplementary Figure S4). This suggests that the genes selected are regulated exclusively by estrogen and androgen in adult Sertoli cells.

4. Discussion

With an alarming increase in the deleterious effects of the environmental endocrine disruptors which are mainly estrogenic and/or anti-androgenic, on different aspects of male fertility, it has become imperative to study the mechanisms by which these sex hormones regulate spermatogenesis through regulation of target genes. Our previous studies demonstrated that exogenous administration of E2 disrupted spermatogenesis by altering hormone levels leading to spermiation failure and germ cell apoptosis [23,30,31]. To delineate the effects caused by sex steroid disruption, we used estrogen and androgen receptor stimulation and antagonism *in vitro* and *in vivo* and distinguished genes regulated by ER and AR [11,12,27,32]. Furthermore, our goal was to expand our identification of estrogen and androgen targets across the whole genome in different cell types of the seminiferous epithelium under physiological conditions. We performed ChIP-Seq for ERα and ERβ in enriched germ cells and identified a list of estrogen regulated genes, and novel pathways regulated by estrogen in germ cells [26]. The present study focuses on identification of estrogen and androgen receptor regulated genes in adult Sertoli cells under physiological conditions.

ChIP-Seq for ERβ and AR in adult Sertoli cells identified a total of 30,859 and 9,594 *in vivo* binding sites, respectively. The majority of the

Table 2
Functions of genes obtained by ChIP-Seq of ERβ and AR involved in lipid metabolism and their localization in the testes.

Gene Name	Gene Symbol	Pathway	Gene Function	Receptor binding site obtained	Localization in testis	References
Acyl-CoA dehydrogenase, short chain	***Acads***	Fatty acid metabolism and degradation	Catalysis the initial step of the mitochondrial fatty acid beta-oxidation pathway	ERβ	Testes + Sertoli Cell + Germ Cells – Leydig Cells +	[71]
Acyl-CoA oxidase 3, pristanoyl	***Acox3***	Fatty acid metabolism and degradation, biosynthesis of unsaturated fatty acids	Involved in the desaturation of 2-methyl branched fatty acids	ERβ	Sertoli cell + Germ Cell NA Leydig cell +	[72]
Acyl-CoA synthetase long-chain family member 6	***Acsl6***	Fatty acid metabolism, biosynthesis, and degradation	Involved in formation of acyl-CoA from fatty acids, ATP, and CoA	ERβ	Sertoli cell – Germ cells + Leydig cell +	[73]
Diacylglycerol kinase, alpha	***Dgka***	Glycerolipid and glycerophospholipid metabolism	Plays an important role in the re-synthesis of phosphatidylinositol and phosphorylating diacylglycerol to phosphatidic acid	ERβ	Testes +	[74]
Diacylglycerol kinase zeta	***Dgkz***	Glycerolipid and glycerophosphollipid metabolism	Regulates intracellular levels of diacylglycerol levels and involved in signal transduction	ERβ	Sertoli cell NA Germ cells + Leydig cells NA	[75]
Glycerol-3-phosphate acyltransferase 2, mitochondrial	***Gpat2***	Glycerolipid and glycerophosphollipid metabolism	Catalysis the first and rate-limiting step in the de novo glycerolipid synthesis	ERβ	Sertoli cell -Germ cells + Leydig cells -	[76]
Sphingosine kinase 1	***Sphk1***	Sphingolipid metabolism	Catalysis the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P), a lipid mediator with both intra- and extracellular functions	ERβ	Sertoli cell -Germ cells + Leydig cells -	[77]
AU RNA binding methylglutaconyl-CoA hydratase	***Auh***	Lipid metabolism, Leucine metabolism	Catalysis the hydration of 3-methylglutaconyl-CoA to 3-hydroxy-3-methyl-glutaryl-CoA	AR	Testis +	[78]
Acetyl-CoA carboxylase alpha	***Acaca***	Fatty Acid metabolism and biosynthesis	Catalysis the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis	AR	Testis + Sertoli cells + Germ cells + Leydig cells +	[79,80, 81]
Acyl-CoA dehydrogenase, mediumchain	***Acadm***	Fatty acid metabolism and degradation	Catalysis the initial step of the mitochondrial fatty acid beta-oxidation pathway	AR	Testes + Sertoli cells + Germ cells NA Leydig cells NA	[71,82]
Acyl-CoA synthetase long-chain family 1	***Acsl1***	Fatty acid metabolism, biosynthesis, and degradation	Converts free long-chain fatty acids into fatty acyl-CoA esters, and play a key role in lipid biosynthesis and fatty acid degradation	AR	NA	NA
Isovaleryl-CoA dehydrogenase	***Ivd***	Leucine and Fatty acid metabolism	It is a mitochondrial matrix enzyme that catalyzes the third step in leucine catabolism	AR	Testis + Seminiferous Tubules + Leydig cells +	[83]
Monoglyceride lipase	***Mgll***	Glycerolipid metabolism, regulation of lipolysis	Catalysis the conversion of monoacylglycerides to free fatty acids and glycerol	AR	Sertoli cells + Germ cells + Leydig Cells +	[84]
3-oxoacid CoA transferase 1	***Oxct1***	Fatty acid metabolism, Leucine metabolism	Catalysis the hydration of 3-methylglutaconyl-CoA to 3-hydroxy-3-methyl-glutaryl-CoA	AR	Sertoli cell + Germ cells + Leydig cells +	[85]

Table 3
Motif analysis for selected genes involved in lipid metabolism after ChIP-Seq of ERβ and AR.

Gene	TF Motif	No. of motifs	Matrix score	Input sequence	Fold enrichment
Acsl6	**V$ER**	1	0.741	GGAGGTCAGAGGAGGCCAA	15.81
Gpat2	**V$ER**	2	0.750	TTGGGTCAGTGACAGGGAT	15.81
Acads	**V$ER**	4	0.745	CTGTGTCACGATCGCGGGA	10.54
Dgkz	**V$ER**	2	0.753	TGAGGTTTCTGTGCCCATC	10.54
Dgka	**V$ER**	2	0.739	CCAGGTCATTTCCAGTGCC	10.54
Sphk1	**V$ER**	2	0.768	CTGGGTCACTGGGATGCCT	10.54
Acox3	**V$ER**	2	0.798	ACAAGTCCACAGGACCTGG	10.54
Oxct1	**V$AR**	1	0.886	AGTGGTTCATGTACT	17.64
Acaca	**V$AR**	2	0.841	AGTTTTAGACTGTTTTAT	17.64
Acls1	**V$AR**	2	0.872	ATGCACTGGCTGTTCAA	13.23
Mgll	**V$AR**	1	0.866	ATGCACACGGTGTGTTT	13.23
Acadm	**V$AR**	3	0.837	AGGCCAGCCTGACCT	8.82
Ivd	**V$AR**	1	0.744	AGAAGATGCCCAGTATC	8.82
Auh	**V$AR**	1	0.899	TGTGCTGTCTGTTCTCT	8.82

binding sites (60-65%) identified were located within 20Kb proximity from the TSS suggesting that ERβ and AR might be playing a crucial role in the transcription of these genes.

Pathway analysis of the enriched genes showed the top three pathways in both ERβ and AR target genes were metabolic pathways, pathways in cancer, and PI3K-Akt signaling pathway. During

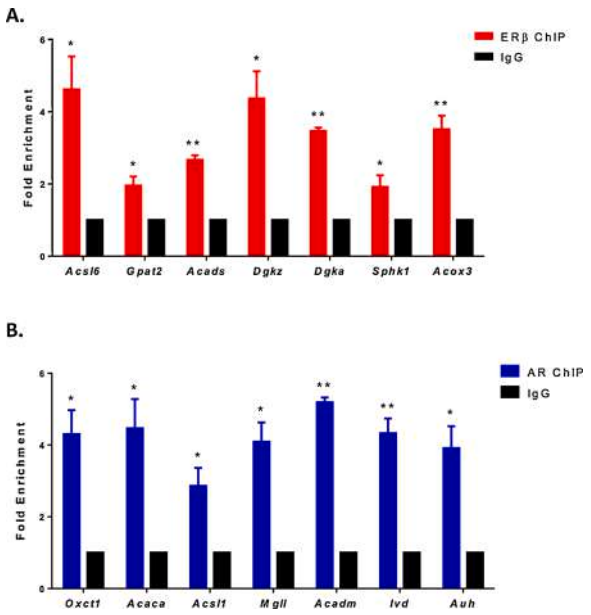

Fig. 4. ChIP-qPCR validation for genes involved in lipid metabolism. Fold enrichment obtained by ChIP-qPCR by ERβ (red bars) and AR (blue bars) ChIP as compared to IgG (black bars). Values are represented as mean ± SEM; N = 6; * P < 0.05; ** P < 0.01.

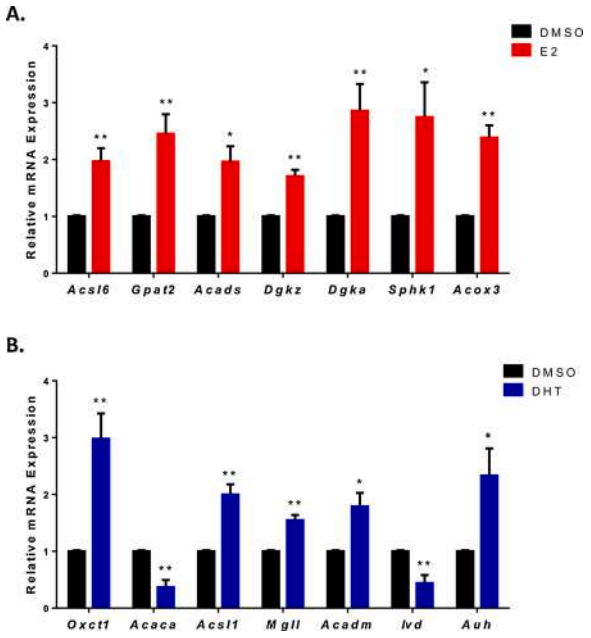

Fig. 5. Regulation of genes involved in lipid metabolism. Expression of genes in primary culture of adult Sertoli cells regulated by estrogen (A) and androgen (B) after 17β-estradiol (E2) (10^{-9} M) and dihydrotestosterone (DHT) (10^{-8} M) treatment, respectively. Only DMSO is used as the control. Values are represented as mean ± SEM; N = 6; * P < 0.05; ** P < 0.01.

spermatogenesis, proliferation and differentiation of germ cells is reliant on and occurs in close proximity to Sertoli cells. Sertoli cells provide metabolic support to the developing germ cells delivering essential nutrients and energy. Various metabolic pathways including glucose, lipid, and protein metabolism take place in Sertoli cells [33]. It is well known that the sex hormones, estrogen and androgen, regulate metabolism in different tissues. Estrogens are known to regulate a plethora of energy metabolic pathways, including glucose transport, glycolysis, tricarboxylic acid cycle, mitochondrial respiratory chain, adenosine nucleotide translocator, and fatty acid β-oxidation and synthesis [34,35]. Similarly, androgen is also known to play a significant role in different metabolic pathways like glycolysis, amino acid synthesis, lipid synthesis, and nucleotide synthesis [36,37]. In our study, 825 genes for ERβ and 448 genes for AR were identified which were involved in metabolic pathways in adult Sertoli cells. Since estrogen and androgen modulate a plethora of genes involved in metabolism; it is not surprising to find enrichment of pathways in cancer as one of the top 10 pathways. Testicular cancer, specifically germ cell tumor is the most common type of cancer observed in the testes. Although, Sertoli cell cancer is rare, 30% of all Sertoli cell tumors are known to have an endocrine origin [38]. It has been previously reported that estrogen and androgen are involved in the pathophysiology of testicular cancers. Estrogen is shown to promote the growth of testicular cancer [39]; androgen, on the other hand, suppresses testicular cancer growth [40]. The third pathway to be enriched significantly was PI3K-Akt signaling. The PI3K-Akt pathway is activated in response to different extracellular signals and is involved various biological processes such as metabolism, proliferation, cell survival, and cell growth. Estrogen receptor is known to activate PI3K-Akt signaling in myocardial hypothalamus and uterus [41,42]. Testosterone, also activates PI3K-Akt pathway through AR and activates proliferation in myoblast cells [43]. It is interesting to note that in the present study, the genes involved in this pathway have estrogen and androgen receptor binding sites, as identified by our ChIP-Seq data, and therefore, these hormones, may also regulate the genes involved in this pathway in adult Sertoli cells.

Glucose metabolism in Sertoli cells is widely studied and glucose is crucial for various functions such as formation of the blood-testis barrier, spermatocyte survival, as well as protein synthesis [44–46]. Earlier studies have reported estrogen and androgen to be modulators of glucose metabolism in Sertoli cells [47–49]. However, glucose is not the only source of energy, since Sertoli cells maintain their viability and produce ATP even in the absence of glucose [50]. During the process of spermatogenesis, majority of the germ cells undergo apoptosis. Germ cell apoptosis has been estimated to result in the loss of up to 75% of the potential numbers of mature sperm cells in the adult testis. These apoptotic bodies are phagocytosed by Sertoli cells. This process enables recycling of lipids in the Sertoli cells, which can be further metabolized and used as source of energy for production of ATP. Additionally, inactivation of genes involved in lipid metabolism also leads to impairment in the process of spermatogenesis [51]. Sertoli cells are also shown to produce significantly higher ATP and it has been previously demonstrated that they can utilize lipids to produce energy via the β-oxidation pathway [46]. All these evidences suggest that lipid metabolism in Sertoli cells is important for normal fertility. In our study, we found various pathways in lipid metabolism including fatty acid synthesis and degradation, glycerolipid metabolism, glycerophospholipid metabolism, sphingolipid metabolism, biosynthesis of unsaturated fats, fat digestion and absorption, and regulation of lipolysis to be enriched (Supplementary Table S7). It is well established that estrogen and androgen have a role in lipid metabolism and show altered expression of genes associated with lipid metabolism in adipose tissue, liver, and prostate cancer [52,53]. In our previous study, we performed ChIP-Seq for ERα and ERβ in rat germ cells and also found metabolic pathways to be significantly enriched [26]. We carried out a comparative analysis of these ERβ target genes in germ cells and Sertoli cells; and found 375 genes (having ERβ binding sites) to be exclusively present in Sertoli cells (data not shown). Upon, pathway analysis of these genes, we identified lipid metabolism to be significantly enriched, indicative of lipid metabolism being a target of sex steroids exclusively in Sertoli cells. From these, 14 genes were selected for further validation, 7 genes having ERβ and AR binding sites each (Table 2).

Fatty acyl CoA synthetase is a group of enzymes that play an important role in intermediary metabolism converting fatty acids into fatty acyl-CoA, which acts as the primary substrate for β-oxidation. It is then catalyzed by a group of enzymes called acyl CoA dehydrogenases

and oxidases. The end product of this oxidation leads to production of acetyl CoA which enters TCA cycle, ultimately leading to generation of ATPs (Fig. 6). In our study, we selected five enzymes to be involved in fatty acid metabolism; *Acsl1, Acsl6, Acads, Acox3*, and *Acadm,* of which *Acsl6, Acads*, and *Acox3* displayed ERβ binding sites; whereas *Acsl1* and *Acadm* displayed AR binding sites. Motif analysis revealed presence of cognate response elements, which were significantly enriched by ERβ and AR as compared to IgG, as demonstrated by ChIP-qPCR in adult Sertoli cells. In addition, we studied the functional relevance of these binding sites by performing estrogen and androgen receptor agonist treatment on the primary culture of adult Sertoli cells. We observed a significant alteration in the relative mRNA level of these enzymes suggesting that binding of the receptor causes transcriptional activation of these genes. Increase in estrogen levels is known to cause an increase in fatty acid utilization, suggesting that estrogen plays a role in free fatty acid (FFA) clearance [54,55]. Besides, evidence also supports that an increase in oxidation of fatty acids is observed upon androgen supplementation [56]. Our study suggests that these effects could be through direct transcriptional regulation of enzymes responsible for fatty acid catabolism. Acetyl CoA carboxylase (*Acaca*) is involved in catalyzing malonyl CoA production, which is an initial step in fatty acid synthesis (Fig. 6), and was found to be enriched with AR peaks. Interestingly, we found that androgen treatment significantly decreased the expression of *Acaca.* This corroborates with the previous finding where treatment with synthetic androgen decreased the expression of *Acaca* in prostate cancer cells [57]. Since up-regulation of the genes involved in fatty acid oxidation and down-regulation of genes in fatty acid synthesis upon steroid receptor agonist treatment was observed, it suggests that estrogen and androgen stimulate fatty acid oxidation and inhibit fatty acid synthesis.

Another important pathway of lipid metabolism is that of glycerolipids and glycerophospholipids which play a central role in triglyceride metabolism and energy balance. Glycerol-3-phosphate acyltransferases (GPATs) are key enzymes that catalyze the first and rate-limiting step in *de novo* glycerolipid synthesis. Another group of enzymes known as diacylglycerol kinases (DGKs) regulate the intracellular levels of diacylglycerol and play a crucial role in DAG signaling and lipid metabolism. Glycerolipid metabolism also has a tight interconnection with fatty acid metabolism as monoglyceride lipase (Mgll) catalyzes the hydrolysis of monoglycerides into glycerol and fatty acids (Fig. 6). In our study we identified a number of genes to be involved in glycerolipid metabolism, of which 4 genes, *Gpat2, Dgka, Dgkz*, and *Mgll* were selected for functional validation. *Gpat2, Dgka, Dgkz* are regulated by estrogen, whereas, *Mgll* is regulated by androgen. The results obtained showed that upon E2 or DHT treatment in adult Sertoli cells, the expression of all the four genes was significantly up-regulated (Fig. 5). This suggests that both estrogen and androgen promote the metabolism of glycerolipids and glycerophospholipid. Although, very few studies have shown the direct effect of sex hormones on glyceride metabolism, there are a few evidences suggesting a link between the same [58,59]. Our study gives a direct link for regulation of the enzymes involved in glyceride metabolism in adult Sertoli cells which could be further explored for its physiological functions.

The relationship between leucine and lipid metabolism is well established. Leucine supplementation inhibits lipogenesis, and promotes lipolysis, thereby affecting lipid metabolism [60,61]. We studied expression of three genes, *Ivd, Auh*, and *Oxct1*, involved in leucine metabolism, all of which showed AR enrichment. Upon treatment with DHT, the mRNA levels of *Auh* and *Ocxt1* showed up-regulation, whereas, the expression of *Ivd* showed down-regulation (Fig. 5). It has been previously reported that androgen receptor regulates the uptake of leucine in prostate cancer cells by regulating the expression of its transporters [62]. In the present study, we report that androgen also controls the transcription of the genes involved in leucine metabolism. This might play a crucial role in Sertoli cells metabolism as it has been shown earlier that amino acid also acts as a source of energy in Sertoli cells.

Sphingolipids are abundantly expressed in the testes [63]. Sphingosine kinase 1 (*Sphk1*) is an enzyme that catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P), a lipid mediator that regulates varied functions like cell motility and proliferation,

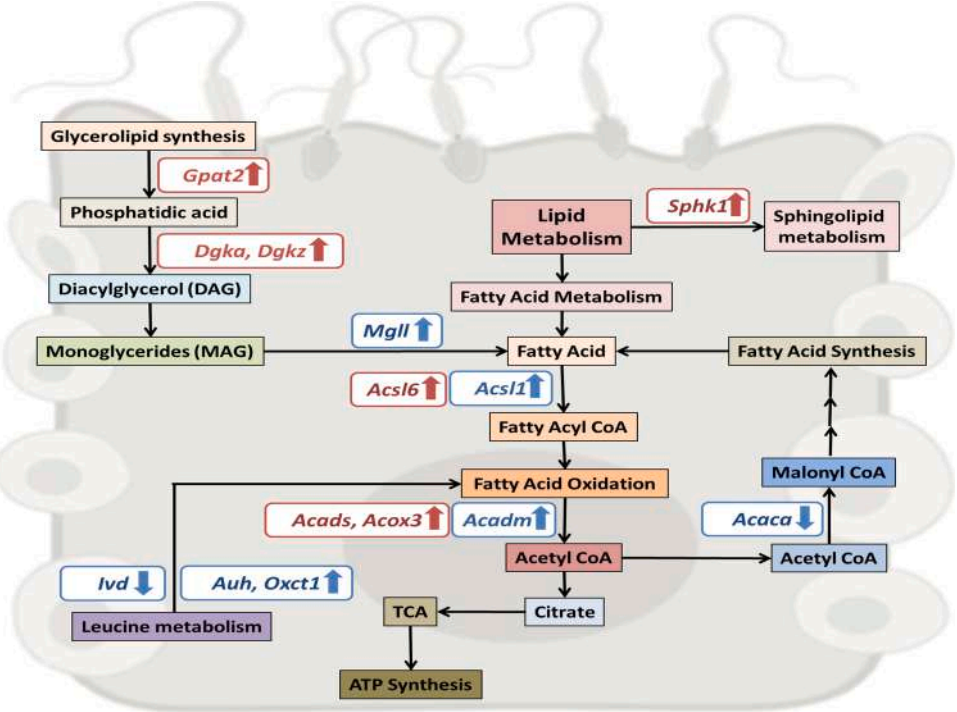

Fig. 6. Genes involved in lipid metabolism regulated by ERβ and AR. An overview of different lipid metabolic pathways whose genes were identified by ERβ and AR ChIP-Seq, and validated by *in vitro*Sertoli cell culture. The red boxes depict genes regulated by ERβ and blue boxes depict genes regulated by AR. Up arrow: increase in relative expression; down arrow: decrease in relative expression.

cytoskeletal organization, cell growth, and survival [64]. S1P acts as a survival factor in male germ cells and has been reported to have protective effects in early stages of spermatogenesis [65]. In addition, estrogen also activates signaling pathways via transcriptional activation of sphingosine kinase, as demonstrated in MCF-7 breast cancer cells [66]. We found that estrogen up-regulates *Sphk1* expression in Sertoli cells, suggesting that estrogen could alter S1P levels and thereby play a crucial role influence varied testicular functions.

Increase in the exposure to environmental endocrine disruptors has led to a significant decrease in the sperm concentrations and male fertility over the past few decades. There is evidence of transgenerational epigenome and transcriptome alterations in Sertoli cells upon perinatal exposure to environmental toxicants such as vinclozolin and dichlorodiphenyltrichloroethane (DDT) [67,68]. A study carried out showed that exposure to vinclozolin altered the transcriptome in the F3 generation in which 418 genes were differentially expressed in the F3 Sertoli cells, and the majority of these genes were involved in metabolism, transcription, and signaling [68]. Upon comparative analysis with our ChIP-Seq data, we observed that out of 418 genes, 161 genes were found to be enriched by ERβ and 81 genes by AR, highlighting transgenerational penetrance of environmental toxicants on hormone-mediated transcription of metabolism genes. Another similar study carried out upon exposure to DDT and vinclozolin obtained 573 mRNAs to be differentially expressed in the F3 generation, majority of which were involved in metabolism. Moreover, exposure to environmental toxicants also led to a remarkable increase in the frequency of testicular diseases [67]. Out of the 573 genes, 296 genes were observed to be enriched by ERβ and 177 genes by AR in our study. Further studies have shown that exposure to endocrine disruptors like BPA and genistein also alters Sertoli cells processes such as growth and proliferation [69,70]. Taken together, these evidences further strengthens the fact that abnormal Sertoli cells metabolism due to estrogenic and anti-androgen compounds can be a probable cause for male infertility.

5. Conclusion

This is the first study to investigate the estrogen and androgen binding sites in Sertoli cells under unperturbed conditions, using the ChIP-Seq approach. It provides a genome-wide map of ERβ and AR binding sites in adult rat Sertoli cells. It also highlights the pathways regulated exclusively as well as commonly, by estrogen and androgen. It further demonstrates that estrogen and androgen play an important role in lipid metabolism by transcriptionally regulating the expression of the genes. Knowledge of ERβ and AR binding sites in Sertoli cells will further shed light on the roles of these hormones in male fertility.

Author statement

Sanketa Raut: Conceptualization, Methodology, Formal analysis, Writing - Original Draft; **AnitaV.Kumar:** Conceptualization, Methodology, Writing - Review & Editing; **Sharvari Deshpande:** Methodology, Data curation, Writing - Review & Editing; **Kushaan Khambata:** Validation, Writing - Review & Editing; **N.H.Balasinor:** Conceptualization, Funding acquisition, Supervision, Writing - Review & Editing.

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Data availability

The data supporting this study are available in the article and the supplementary information and can be made available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2021.105898.

References

[1] C. Petersen, O. Söder, The Sertoli cell–a hormonal target and 'super' nurse for germ cells that determines testicular size, Horm. Res. Paediatr. 66 (4) (2006) 153–161.
[2] S.F. Sneddon, N. Walther, P.T. Saunders, Expression of androgen and estrogen receptors in sertoli cells: studies using the mouse SK11 cell line, Endocrinology 146 (12) (2005) 5304–5312.
[3] W.H. Walker, J. Cheng, FSH and testosterone signaling in Sertoli cells, Reproduction 130 (1) (2005) 15–28.
[4] R.I. McLachlan, N.G. Wreford, D.M. Robertson, D.M. De Kretser, Hormonal control of spermatogenesis, Trends Endocrinol. Metab. 6 (3) (1995) 95–101.
[5] J.Y. Chung, S. Brown, H. Chen, J. Liu, V. Papadopoulos, B. Zirkin, Effects of pharmacologically induced Leydig cell testosterone production on intratesticular testosterone and spermatogenesis, Biol. Reprod. 102 (2) (2020) 489–498.
[6] J. Wang, J. Li, W. Xu, Q. Xia, Y. Gu, W. Song, X. Zhang, Y. Yang, W. Wang, H. Li, K. Zou, Androgen promotes differentiation of PLZF+ spermatogonia pool via indirect regulatory pattern, Cell Commun. Signal. 17 (1) (2019) 57.
[7] R.S. Wang, S. Yeh, C.R. Tzeng, C. Chang, Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice, Endocr. Rev. 30 (2) (2009) 119–132.
[8] M.Y. Tsai, S.D. Yeh, R.S. Wang, S. Yeh, C. Zhang, H.Y. Lin, C.R. Tzeng, C. Chang, Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells, Proc. Natl. Acad. Sci. 103 (50) (2006) 18975–18980.
[9] L.B. Smith, W.H. Walker, The regulation of spermatogenesis by androgens, Semin. Cell Dev. Biol. 1 (2014) 2–13.
[10] L. O'Donnell, K.M. Robertson, M.E. Jones, E.R. Simpson, Estrogen and spermatogenesis, Endocr. Rev. 22 (3) (2001) 289–318.
[11] A. Kumar, K. Dumasia, R. Gaonkar, S. Sonawane, L. Kadam, N.H. Balasinor, Estrogen and androgen regulate actin-remodeling and endocytosis-related genes during rat spermiation, Mol. Cell. Endocrinol. 404 (2015) 91–101.
[12] K. Dumasia, A. Kumar, S. Deshpande, S. Sonawane, N.H. Balasinor, Differential roles of estrogen receptors, ESR1 and ESR2, in adult rat spermatogenesis, Mol. Cell. Endocrinol. 428 (2016) 89–100.
[13] I. Tsutsumi, J. Toppari, J.D. Campeau, Reduction of fertility in the male rat by systemic treatment with follicle regulatory protein, Fertil. Steril. 47 (4) (1987) 689–695.
[14] G. Shetty, H. Krishnamurthy, H.N. Krishnamurthy, A.S. Bhatnagar, N.R. Moudgal, Effect of long-term treatment with aromatase inhibitor on testicular function of adult male bonnet monkeys (M. radiata), Steroids 63 (7–8) (1998) 414–420.
[15] M.E. Wierman, Sex steroid effects at target tissues: mechanisms of action, Adv. Physiol. Educ. 31 (1) (2007) 26–33.
[16] S. Yeh, M.Y. Tsai, Q. Xu, X.M. Mu, H. Lardy, K.E. Huang, H. Lin, S.D. Yeh, S. Altuwaijri, X. Zhou, L. Xing, Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues, Proc. Natl. Acad. Sci. 99 (21) (2002) 13498–13503.
[17] A.J. Notini, R.A. Davey, J.F. McManus, K.L. Bate, J.D. Zajac, Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model, J. Mol. Endocrinol. 35 (3) (2005) 547–555.
[18] K. De Gendt, J.V. Swinnen, P.T. Saunders, L. Schoonjans, M. Dewerchin, A. Devos, K. Tan, N. Atanassova, F. Claessens, C. Lécureuil, W. Heyns, A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis, Proc. Natl. Acad. Sci. 101 (5) (2004) 1327–1332.
[19] C. Chang, Y.T. Chen, S.D. Yeh, Q. Xu, R.S. Wang, F. Guillou, H. Lardy, S. Yeh, Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells, Proc. Natl. Acad. Sci. 101 (18) (2004) 6876–6881.
[20] M.C. Antal, A. Krust, P. Chambon, M. Mark, Sterility and absence of histopathological defects in nonreproductive organs of a mouse ERβ-null mutant, Proc. Natl. Acad. Sci. 105 (7) (2008) 2433–2438.
[21] K.M. Robertson, L. O'Donnell, M.E. Jones, S.J. Meachem, W.C. Boon, C.R. Fisher, K.H. Graves, R.I. McLachlan, E.R. Simpson, Impairment of spermatogenesis in mice

lacking a functional aromatase (Cyp 19) gene, Proc. Natl. Acad. Sci. 96 (14) (1999) 7986–7991.
[22] W. Zhou, O.U. Bolden-Tiller, S.H. Shao, C.C. Weng, G. Shetty, M. AbuElhija, P. Pakarinen, I. Huhtaniemi, A.A. Momin, J. Wang, D.N. Stivers, Estrogen-regulated genes in rat testes and their relationship to recovery of spermatogenesis after irradiation, Biol. Reprod. 85 (4) (2011) 823–833.
[23] N.H. Balasinor, R. D'Souza, P. Nanaware, S. Idicula-Thomas, N. Kedia-Mokashi, Z. He, M. Dym, Effect of high intratesticular estrogen on global gene expression and testicular cell number in rats, Reprod. Biol. Endocrinol. 8 (1) (2010) 72.
[24] P.I. Sadate-Ngatchou, D.J. Pouchnik, M.D. Griswold, Identification of testosterone-regulated genes in testes of hypogonadal mice using oligonucleotide microarray, Mol. Endocrinol. 18 (2) (2004) 422–433.
[25] Q. Zhou, J.E. Shima, R. Nie, P.J. Friel, M.D. Griswold, Androgen-regulated transcripts in the neonatal mouse testis as determined through microarray analysis, Biol. Reprod. 72 (4) (2005) 1010–1019.
[26] S. Raut, A.V. Kumar, K. Khambata, S. Deshpande, N.H. Balasinor, Genome-wide identification of estrogen receptor binding sites reveals novel estrogen-responsive pathways in adult male germ cells, Biochem. J. 477 (12) (2020) 2115–2131.
[27] A. Kumar, K. Dumasia, S. Deshpande, N.H. Balasinor, Direct regulation of genes involved in sperm release by estrogen and androgen through their receptors and co-regulators, J. Steroid Biochem. Mol. Biol. 171 (2017) 66–74.
[28] A. Kumar, K. Dumasia, S. Deshpande, R. Gaonkar, N.H. Balasinor, Actin related protein complex subunit 1b controls sperm release, barrier integrity and cell division during adult rat spermatogenesis, Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1863 (8) (2016) 1996–2005.
[29] K. Ikeda, K. Horie-Inoue, S. Inoue, Identification of estrogen-responsive genes based on the DNA binding properties of estrogen receptors using high-throughput sequencing technology, Acta Pharmacol. Sin. 36 (1) (2015) 24–31.
[30] R. D'Souza, M.K. Gill-Sharma, S. Pathak, N. Kedia, R. Kumar, N. Balasinor, Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats, Mol. Cell. Endocrinol. 241 (2005) 41–48.
[31] R. D'Souza, S. Pathak, R. Upadhyay, R. Gaonkar, S. D'Souza, S. Sonawane, M. Gill-Sharma, N.H. Balasinor, Disruption of tubulobulbar complex by high intratesticular estrogens leading to failed spermiation, Endocrinology 150 (4) (2009) 1861–1869.
[32] K. Dumasia, A. Kumar, L. Kadam, N.H. Balasinor, Effect of estrogen receptor-subtype-specific ligands on fertility in adult male rats, J. Endocrinol. 225 (3) (2015) 169–180.
[33] P.F. Oliveira, M.G. Alves, Sertoli Cell Metabolism and Spermatogenesis, Springer Briefs in Cell Biology, 2015.
[34] J.Q. Chen, T.R. Brown, J. Russo, Regulation of energy metabolism pathways by estrogens and estrogenic chemicals and potential implications in obesity associated with increased exposure to endocrine disruptors, Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1793 (7) (2009) 1128–1143.
[35] T. Wada, H. Tsuneki, T. Sasaoka, New Insights into metabolic regulation via bifurcated function of estrogen receptor α, Diabetes 62 (12) (2013) 3996–3998.
[36] S.J. Barfeld, H.M. Itkonen, A. Urbanucci, I.G. Mills, Androgen-regulated metabolism and biosynthesis in prostate cancer, Endocr. Relat. Cancer 21 (4) (2014) 57–66.
[37] V. Birzniece, Hepatic actions of androgens in the regulation of metabolism. Current Opinion in Endocrinology, Diabetes and Obesity 25 (3) (2018) 201–208.
[38] R. Littleton, R. Farah, J. Cerny, J. Ohorodnik, Sertoli cell testicular tumors, Urology 17 (6) (1981) 557–559.
[39] A. Bouskine, M. Nebout, B. Mograbi, F. Brucker-Davis, C. Roger, P. Fenichel, Estrogens promote human testicular germ cell cancer through a membrane-mediated activation of extracellular regulated kinase and protein kinase A, Endocrinology 149 (2) (2008) 565–573.
[40] H. Nakagawa, T. Ueda, S. Ito, T. Shiraishi, H. Taniguchi, N. Kayukawa, H. Nakanishi, S. Ushijima, M. Kanazawa, T. Nakamura, Y. Naya, Androgen suppresses testicular cancer cell growth in vitro and in vivo, Oncotarget 7 (23) (2016) 35224–35232.
[41] G.P. Cardona-Gomez, P. Mendez, L.M. Garcia-Segura, Synergistic interaction of estradiol and insulin-like growth factor-I in the activation of PI3K/Akt signaling in the adult rat hypothalamus, Mol. Brain Res. 107 (1) (2002) 80–88.
[42] A.A. Kazi, K.H. Molitoris, R.D. Koos, Estrogen rapidly activates the PI3K/AKT pathway and hypoxia-inducible factor 1 and induces vascular endothelial growth factor A expression in luminal epithelial cells of the rat uterus, Biol. Reprod. 81 (2) (2009) 378–387.
[43] D. Li, Q. Wang, K. Shi, Y. Lu, D. Yu, X. Shi, W. Du, M. Yu, Testosterone promotes the proliferation of chicken embryonic myoblasts via androgen receptor mediated PI3K/Akt signaling pathway, Int. J. Mol. Sci. 21 (3) (2020) 1152.
[44] R. Robinson, I.B. Fritz, Metabolism of glucose by Sertoli cells in culture, Biol. Reprod. 24 (5) (1981) 1032–1041.
[45] N.H. Jutte, R. Jansen, J.A. Grootegoed, F.F. Rommerts, O.P. Clausen, H.J. Van der Molen, Regulation of survival of rat pachytene spermatocytes by lactate supply from Sertoli cells, Reproduction 65 (2) (1982) 431–438.
[46] M.G. Alves, A.D. Martins, J.E. Cavaco, S. Socorro, P.F. Oliveira, Diabetes, insulin-mediated glucose metabolism and Sertoli/blood-testis barrier function, Tissue Barriers 1 (2) (2013), e23992.
[47] A.D. Martins, M.G. Alves, V.L. Simões, T.R. Dias, L. Rato, P.I. Moreira, S. Socorro, J. E. Cavaco, P.F. Oliveira, Control of Sertoli cell metabolism by sex steroid hormones is mediated through modulation in glycolysis-related transporters and enzymes, Cell Tissue Res. 354 (3) (2013) 861–868.
[48] L. Rato, M.G. Alves, S. Socorro, R.A. Carvalho, J.E. Cavaco, P.F. Oliveira, Metabolic modulation induced by oestradiol and DHT in immature rat Sertoli cells cultured in vitro, Biosci. Rep. 32 (1) (2012) 61–69.
[49] P.F. Oliveira, M.G. Alves, L. Rato, J. Silva, R. Sa, A. Barros, M. Sousa, R. A. Carvalho, J.E. Cavaco, S. Socorro, Influence of 5α-dihydrotestosterone and 17β-estradiol on human Sertoli cells metabolism, Int. J. Androl. 34 (2011) 612–620.
[50] M.F. Riera, M.N. Galardo, E.H. Pellizzari, S.B. Meroni, S.B. Cigorraga, Molecular mechanisms involved in Sertoli cell adaptation to glucose deprivation, Am. J. Physiol. Endocrinol. Metab. 297 (4) (2009) 907–914.
[51] S. Chung, S.P. Wang, L. Pan, G. Mitchell, J. Trasler, L. Hermo, Infertility and testicular defects in hormone-sensitive lipase-deficient mice, Endocrinology 142 (10) (2001) 4272–4281.
[52] L.M. Butler, M.M. Centenera, J.V. Swinnen, Androgen control of lipid metabolism in prostate cancer: novel insights and future applications, Endocr. Relat. Cancer 23 (5) (2016) 219–227.
[53] H. Szafran, W. Smielak-Korombel, The role of estrogens in hormonal regulation of lipid metabolism in women, Prz. Lek. 55 (5) (1998) 266–270.
[54] M.D. Jensen, M.L. Martin, P.E. Cryer, L.R. Roust, Effects of estrogen on free fatty acid metabolism in humans, Am. J. Physiol. Endocrinol. Metab. 266 (6) (1994) 914–920.
[55] P. Herrero, P.F. Soto, C.S. Dence, Z. Kisrieva-Ware, D.A. Delano, L.R. Peterson, R. J. Gropler, Impact of hormone replacement on myocardial fatty acid metabolism: potential role of estrogen, J. Nucl. Cardiol. 12 (5) (2005) 574–581.
[56] H.Y. Lin, Wang R.S. Yu IC, Y.T. Chen, N.C. Liu, S. Altuwaijri, C.L. Hsu, W.L. Ma, J. Jokinen, J.D. Sparks, S. Yeh, Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor, Hepatology 47 (6) (2008) 1924–1935.
[57] K.B. Singh, S.H. Kim, E.R. Hahm, S.K. Pore, B.L. Jacobs, S.V. Singh, Prostate cancer chemoprevention by sulforaphane in a preclinical mouse model is associated with inhibition of fatty acid metabolism, Carcinogenesis 39 (6) (2018) 826–837.
[58] S.F. Eisen, H.A. Brown, Selective estrogen receptor (ER) modulators differentially regulate phospholipase D catalytic activity in ER-negative breast cancer cells, Mol. Pharmacol. 62 (4) (2002) 911–920.
[59] P.M. Gowri, S. Sengupta, S. Bertera, B.S. Katzenellenbogen, Lipin1 regulation by estrogen in uterus and liver: implications for diabetes and fertility, Endocrinology 148 (8) (2007) 3685–3693.
[60] J. Jiao, S.F. Han, W. Zhang, J.Y. Xu, X. Tong, X.B. Yin, L.X. Yuan, L.Q. Qin, Chronic leucine supplementation improves lipid metabolism in C57BL/6J mice fed with a high-fat/cholesterol diet, Food Nutr. Res. 60 (1) (2016) 31304.
[61] Y. Cheng, Q. Meng, C. Wang, H. Li, Z. Huang, S. Chen, F. Xiao, F. Guo, Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue, Diabetes. 59 (1) (2010) 17–25.
[62] Q. Wang, C.G. Bailey, C. Ng, J. Tiffen, A. Thoeng, V. Minhas, M.L. Lehman, S. C. Hendy, G. Buchanan, C.C. Nelson, J.E. Rasko, Androgen receptor and nutrient signaling pathways coordinate the demand for increased amino acid transport during prostate cancer progression, Cancer Res. 71 (24) (2011) 7525–7536.
[63] R. Sandhoff, Very long chain sphingolipids: tissue expression, function and synthesis, FEBS Lett. 584 (9) (2010) 1907–1913.
[64] A. Cannavo, D. Liccardo, K. Komici, G. Corbi, C. de Lucia, G.D. Femminella, A. Elia, L. Bencivenga, N. Ferrara, W.J. Koch, N. Paolocci, Sphingosine kinases and sphingosine 1-phosphate receptors: signaling and actions in the cardiovascular system, Front. Pharmacol. 8 (2017) 556.
[65] M. Otala, L. Suomalainen, M.O. Pentikäinen, P. Kovanen, M. Tenhunen, K. Erkkilä, J. Toppari, L. Dunkel, Protection from radiation-induced male germ cell loss by sphingosine-1-phosphate, Biol. Reprod. 70 (3) (2004) 759–767.
[66] O.A. Sukocheva, L. Wang, N. Albanese, S.M. Pitson, M.A. Vadas, P. Xia, Sphingosine kinase transmits estrogen signaling in human breast cancer cells, Mol. Endocrinol. 17 (10) (2003) 2002–2012.
[67] I. Sadler-Riggleman, R. Klukovich, E. Nilsson, D. Beck, Y. Xie, W. Yan, M. K. Skinner, Epigenetic transgenerational inheritance of testis pathology and Sertoli cell epimutations: generational origins of male infertility, Environ. Epigenet. 5 (3) (2019) dvz013.
[68] C. Guerrero-Bosagna, M. Savenkova, M.M. Haque, E. Nilsson, M.K. Skinner, Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: molecular etiology of male infertility, PLoS One 8 (3) (2013), e59922.
[69] J. Kumi-Diaka, R. Rodriguez, G. Goudaze, Influence of genistein (4′, 5, 7-trihydroxyisoflavone) on the growth and proliferation of testicular cell lines, Biol. Cell 90 (4) (1998) 349–354.
[70] L.C. Ge, Z.J. Chen, H. Liu, K.S. Zhang, Q. Su, X.Y. Ma, H.B. Huang, Z.D. Zhao, Y. Y. Wang, J.P. Giesy, J. Du, Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: a comparative proteomic analysis, Biochimica et Biophysica Acta (BBA)-General Subjects 1840 (9) (2014) 2663–2673.
[71] M. Fukasawa, K. Atsuzawa, K. Mizutani, A. Nakazawa, N. Usuda, Immunohistochemical localization of mitochondrial fatty acid β-oxidation enzymes in rat testis, J. Histochem. Cytochem. 58 (2) (2010) 195–206.
[72] A. Nenicu, G.H. Lüers, W. Kovacs, M. Bergmann, E. Baumgart-Vogt, Peroxisomes in human and mouse testis: differential expression of peroxisomal proteins in germ cells and distinct somatic cell types of the testis, Biol. Reprod. 77 (6) (2007) 1060–1072.
[73] B.J. Hale, R.F. Fernandez, S.Q. Kim, V.D. Diaz, S.N. Jackson, L. Liu, J.T. Brenna, B. P. Hermann, C.B. Geyer, J.M. Ellis, Acyl-CoA synthetase 6 enriches seminiferous tubules with the ω-3 fatty acid docosahexaenoic acid and is required for male fertility in the mouse, J. Biol. Chem. 294 (39) (2019) 14394–14405.
[74] M. Hodgkin, S. Gardner, M. Wakelam, The identification of several stearoyl-arachidonyl selective diacylglycerol kinases in the particulate fraction of porcine testes, Biochem. Soc. Trans. 21 (4) (1993) 490S.
[75] H. Hasegawa, T. Nakano, Y. Hozumi, M. Takagi, T. Ogino, M. Okada, K. Iseki, H. Kondo, M. Watanabe, A.M. Martelli, K. Goto, Diacylglycerol kinase ζ is

associated with chromatin, but dissociates from condensed chromatin during mitotic phase in NIH3T3 cells, J. Cell. Biochem. 105 (3) (2008) 756–765.
[76] E.R. Cattaneo, M. Pellon-Maison, M.E. Rabassa, E. Lacunza, R.A. Coleman, M. R. Gonzalez-Baro, Glycerol-3-phosphate acyltransferase-2 is expressed in spermatic germ cells and incorporates arachidonic acid into triacylglycerols, PLoS One 7 (8) (2012), e42986.
[77] K. Matsumoto, Y. Banno, T. Murate, Y. Akao, Y. Nozawa, Localization of sphingosine kinase-1 in mouse sperm acrosomes, J. Histochem. Cytochem. 53 (2) (2005) 243–247.
[78] L.E. Brennan, J. Nakagawa, D. Egger, K. Bienz, C. Moroni, Characterisation and mitochondrial localisation of AUH, an AU-specific RNA-binding enoyl-CoA hydratase, Gene. 228 (1-2) (1999) 85–91.
[79] M. Regueira, M.F. Riera, M.N. Galardo, E.H. Pellizzari, S.B. Cigorraga, S.B. Meroni, Activation of PPAR α and PPAR β/δ regulates Sertoli cell metabolism, Mol. Cell. Endocrinol. 382 (1) (2014) 271–281.
[80] M.T. Travers, M. Cambot, H.T. Kennedy, G.M. Lenoir, M.C. Barber, V. Joulin, Asymmetric expression of transcripts derived from the shared promoter between the divergently oriented ACACA and TADA2L genes, Genomics 85 (1) (2005) 71–84.
[81] M. Bajpai, G. Gupta, S.K. Jain, B.S. Setty, Lipid metabolising enzymes in isolated rat testicular germ cells and changes associated with meiosis, Andrologia 30 (6) (1998) 311–315.
[82] B.E. Hainline, D.J. Kahlenbeck, J. Grant, A.W. Strauss, Tissue specific and developmental expression of rat long-and medium-chain acyl-CoA dehydrogenases, Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression 1216 (3) (1993) 460–468.
[83] M. Uhlén, L. Fagerberg, B.M. Hallström, C. Lindskog, P. Oksvold, A. Mardinoglu, Å Sivertsson, C. Kampf, E. Sjöstedt, A. Asplund, I. Olsson, Tissue-based map of the human proteome, Science 347 (6220) (2015).
[84] J.E. Nielsen, A.D. Rolland, E. Rajpert-De Meyts, C. Janfelt, A. Jørgensen, S. B. Winge, D.M. Kristensen, A. Juul, F. Chalmel, B. Jégou, N.E. Skakkebaek, Characterisation and localisation of the endocannabinoid system components in the adult human testis, Sci. Rep. 9 (1) (2019) 1–4.
[85] H. Tanaka, N. Iguchi, Y. Miyagawa, M. Koga, J. Kohroki, Y. Nishimune, Differential expression of succinyl CoA transferase (SCOT) genes in somatic and germline cells of the mouse testis, Int. J. Androl. 26 (1) (2003) 52–56.