Title: Genome-wide mapping of estrogen and androgen receptor binding sites in germ cells and Sertoli cells of adult male rat

1. Introduction:

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. The process of spermatogenesis is under tight regulation of pituitary gonadotropins (LH and FSH), and testicular steroid hormones. Androgen and estrogen are two important steroid hormones involved in male reproduction. Androgen is crucial for maintenance of blood-testis-barrier (BTB), meiosis, Sertoli-spermatid adhesion, spermiogenesis, and sperm release (Smith & Walker, 2014). Estrogen also regulates key steps in spermatogenesis such as sperm release and germ cell survival (O'Donnell et al. 2001; Kumar et al. 2015; 2016). Therefore, the actions of the sex hormones, androgen and estrogen are crucial for maintenance of spermatogenesis.

Testicular steroid hormone production is controlled by gonadotropins, LH and FSH, which are released from the anterior pituitary (Walker & Cheng, 2005). 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 (McLachlan et al. 1995). Testosterone produced by Leydig cells diffuses into seminiferous tubules and acts via the androgen receptor present on the Sertoli cells, as germ cells lack androgen receptor. The effects of estrogen in the germ cells are brought about by estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), which exert their function in a ligand-dependent manner. Whereas, the effects of estrogen and androgen in the Sertoli cells are brought about by estrogen receptor beta (ERβ) and androgen receptor (AR).

Estrogen receptors (ERα and ERβ) and androgen receptor (AR) 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 conformation thereby regulating the expression of various genes (Wierman, 2007). The importance of these receptors in spermatogenesis has been highlighted by their knockout models.

To better understand the role of estrogen and androgen in spermatogenesis, one approach is to identify responsive genes in the cell types of the seminiferous epithelium. A few studies have been carried out to study the gene expression pattern of sex hormone responsive genes in testes (Zhou et al. 2011; Balasinor et al. 2010; Sadate-Ngatchou et al. 2004; Zhou et al. 2005). However, most of them have utilized hormone overexposure or depletion models, concomitantly leading to a disturbed hormonal background. This may lead to overexpression of the genes due to physiologically irrelevant levels of hormones 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 and Sertoli cells from adult rat testes to identify estrogen

and androgen responsive genes using genome-wide next-generation sequencing based approach.

2. Objectives:

i. Identification of genome-wide binding sites of ERα, ERβ and AR in germ cells and Sertoli cells separately using ChIP-seq
ii. Validation of ERα, ERβ and AR binding sites thus obtained by ChIP-qPCR
iii. To study expression of estrogen/androgen regulated genes obtained from ChIP-seq experiments using seminiferous tubule culture treated with estrogen/androgen or their agonists and antagonists

3. Material and Methods:

3.1 Animals 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%) and light:dark cycle of 12h:12h 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).

3.2 Enrichment of adult rat germ cells

To study the estrogen receptor binding sites in germ cells, cell population enriched in germ cells were obtained from adult rat testes. 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 marker as mentioned in Kumar et al. 2017. 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.

3.3 Enrichment of adult rat Sertoli cells

To study the binding sites of ERβ and AR in Sertoli cells, we first isolated an enriched population of Sertoli cells from adult rat testes. Briefly, following the protocol mentioned in section 3.2, for germ cell enrichment, the supernatant following DNase and trypsin treatment was passed through a 70 µm, followed by 40 µm nylon mesh filter. 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. The remaining cells were crossed-linked as mentioned in section 3.2 and stored at -80ºC for chromatin immunoprecipitation.

3.4 Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

ChIP for ERα and ERβ in germ cells, and ERβ and AR in Sertoli cells was performed following a protocol described in Raut et al. 2020. Briefly, the cross-linked cells (germ cells and Sertoli cells) were lysed by re-suspending in sodium dodecyl sulfate (SDS) buffer and incubated on ice for 15 min. Thereafter, chromatin was sheared in a water bath sonicator 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 mixture 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- ERα (1 µg) and anti-ERβ (1 µg) (Sigma-Aldrich, USA) for germ cells; and anti-ERβ (1 µg) and anti-AR (1 µg) for Sertoli cells (Santa Cruz Biotechnology, Inc. USA), or rabbit IgG (Mock reaction) overnight at 4 ºC. 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 NaHCO3) 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. The samples from six individual ChIP experiments (from six animals) were pooled for each sample. After performing ChIP-qPCR for the known EREs and AREs to evaluate the efficiency of the ChIP experiment, samples were subjected to NextGen Illumina Sequencing at Sandor Lifesciences, India.

3.5 Pathway 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. Estrogen and androgen response elements (EREs and AREs) were identified in the promoter region of the enriched genes by TRANSFAC database using established consensus sequence for EREs (AGGTCAnnnTGACCT) and AREs (AGAACAnnnTGTTCT).

3.6 ChIP-qPCR

Data obtained after ChIP-Seq was validated by ChIP-qPCR. Primers were designed for the genes selected for validation spanning the EREs and AREs. 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 ºC followed by 40 cycles of 95 ºC for 20 s, primer annealing temperature for 10 s and extension at 72 ºC for 1 min and final extension at 72 ºC for 5 min. A no-template control was also included. Primers for all the selected target genes were synthesized by Sigma-Aldrich, India. Fold enrichment of ChIP over mock was calculated by normalizing with the input sample as described previously Kumar et al. 2017. Data analysis was performed for ChIP-qPCR results obtained from four different animals.

3.7 Seminiferous tubule culture

To check for the regulation of selected enriched genes by ERα and ERβ in germ cells, seminiferous tubule cultures were set up and treated with estrogen receptor specific agonist and antagonist. 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, U.S.A.), supplemented with ITS (5 µg/ml) (insulin, transferrin, selenite) (Sigma–Aldrich), penicillin (50 000 U/l), streptomycin (50 mg/l), and kanamycin (10 mg/ml) (Gibco, U.S.A.) in a 4-well plate (Himedia, India). The cultures were treated with ERα agonist PPT (4,40,400-(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% CO2 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 (17β-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 (Kumar et al. 2015). 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.

3.8 Adult Sertoli culture

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 ITS (5 µg/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. 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 and centrifuged at 4500 rpm for 4 min 30 s. The cell pellet obtained was resuspended in TRI Pure reagent (Roche Diagnostics, Germany) and processed for RNA extraction.

3.9 RNA extraction and qPCR

Total RNA was extracted from germ cells or 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. 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. Melt curve analysis was performed for checking specificity, and all products obtained yielded the predicted melting temperature.

4. Results:

4.1 Genome-wide binding sites of estrogen and androgen

4.1.1 Genome-wide identification of ERα and ERβ binding sites in 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, which constitute the other cell types in the testes. Since germ cells shows presence of ERα and ERβ, ChIP was performed using anti-ERα and anti-ERβ antibodies, along with anti-rabbit immunoglobulin G (anti-IgG) 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) (data not shown). Immuno-precipitated DNA from ERα, ERβ, and IgG (mock) reactions along with Input DNA was subjected to NextGen Illumina sequencing. A total of 27,221 binding sites were obtained in ERα ChIP and 20,926 binding sites in ERβ ChIP reactions (Table 1) with a p value of <0.01 and fold enrichment >2. 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. 60% of the peaks lie within 20 kb upstream or downstream from the Transcription Start Site (TSS) in both ERα and ERβ ChIP.

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

Similarly, adult Sertoli cells were enriched from rat testes and purity of the isolated adult Sertoli cells was confirmed using cell-type specific markers. Since, Sertoli cells show presence of ERβ and AR, ChIP was performed using anti-ERβ and anti-AR antibodies along with IgG as mock control. The efficiency of ChIP antibodies was evaluated by performing ChIP-qPCR for known regions consisting of validated EREs and AREs (as a positive control). 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 in ERβ ChIP and 9,594 peaks in AR ChIP were identified (Table 1) with p value <0.01 and fold enrichment >2 fold. 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. Approximately 60–65 % of the peaks lay within 20 kb proximity from the Transcription Start Site (TSS) suggesting functional relevance of these binding sites.

Table 1: Characteristics of the data obtained by ChIP-Seq of ERα and ERβ in germ cells; and ERβ and AR in Sertoli cells

	Germ Cells		Sertoli Cells	
	ERα ChIP	**ERβ ChIP**	**ERβ ChIP**	**AR ChIP**
Total No. of Reads	10916745	14486314	19996725	28275523
Average read length	75	75	76	76
Total No. of peaks	27221	20926	30860	9594
No. of genes	11096	8985	17690	6224
Common genes	5398		4233	
Exclusive genes	5698	3587	13457	1991

4.2 Gene functions and pathways enriched by genes identified

To understand the molecular functions of genes enriched after ChIP-Seq with ERα and ERβ in germ cells, and ERβ and AR in Sertoli cells, gene ontology analysis was performed using PANTHER and DAVID Database. To study the pathways enriched, pathway analysis of the enriched genes was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database.

4.2.1 Pathways enrich by estrogen receptor in germ cells

Gene ontology of molecular functions showed that majority of the genes were involved in binding and catalytic activity. 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. Additionally, 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. Furthermore, 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. Genes from these pathways were selected for further functional validation.

4.2.2 Pathways enrich by estrogen and androgen receptor in Sertoli cells

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. 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 three pathways enriched in both ERβ and AR were metabolic pathways, pathways in cancer, and PI3K-Akt signaling pathway. Additionally, pathways crucial in spermatogenesis, namely, tight junction, cell adhesion molecules, gap junctions, and adherens junction were also enriched. Since Sertoli cells act as a metabolic source for developing germ cells, metabolic pathways were further explored for validation.

4.3 Motif analysis of the response elements in genes identified

Motif analysis was performed using TRANSFAC and TF Bind for all the genes selected for validation to identify genomic regions bound by ERα and ERβ in germ cells, ERβ and AR in Sertoli cells. In germ cells, genes involved in Prolactin, GnRH, and Oxytocin signaling pathway were selected. The peaks selected ERα ChIP and ERβ ChIP showed presence of estrogen response elements (EREs). For Sertoli cells, genes involved in lipid metabolism were selected and the peaks of ERβ ChIP showed the presence of EREs and the peaks from AR ChIP showed the presence of androgen response elements (AREs). The EREs and AREs were detected on the basis of similarity score to the consensus ERE and ARE. Table 2 & 3 summarizes the peaks of genes selected in germ cells and Sertoli cells, respectively. 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). All the selected genes showed 5–17 fold enrichment by ChIP-Seq.

Table 2: Motif analysis for selected genes after ChIP-Seq of ERα and ERβ in germ cells

	Receptor	Gene	Score	ERE Sequence	Fold Enrichment
Prolactin Pathway	ERα	***Shc4***	0.779	GTAGTTCACAACTATCTGT	10.4
		Prlr	0.766	ATAGGTCAATGATTCACTG	7.2
		Akt2	0.742	TGTGCTCACCCTGAGTTTC	7.2
		Rela	0.761	GAAGGTCAAATGGGAAAGT	7.2
		Stat3	0.757	TGACGTCAACCTGGTCTAT	5.4
		Prl	0.732	AATAGACAGCTTCACCTAA	5.4
		Gsk3b	0.759	CCAGCTCAAAACAAGATAT	5.4
	ERβ	***Pik3ca***	0.734	GGGGCTCACTGACACTCAC	7.6
		Jak2	0.759	TAGTGTATATTTGACCATT	5.7
		Socs7	0.754	GCATGTCACATAGAGAGAA	5.7
GnRH Pathway	ERα	***Gnrhr***	0.739	ACAGTTACACTTGGCCTTC	7.2
		Nras	0.763	GCAGCTCAGAATAGTGTGA	5.4
		Mapk14	0.737	TAGGCTCAACCTCAGTGTG	5.4
		Egfr	0.774	TTCCGTCACCGAAGCTTGG	5.4
	ERβ	***Grb2***	0.751	AAAATCAACTCTGACAAAC	7.6
Oxytocin Pathway	ERα	***Adcy5***	0.738	GAAGGCCAGAGCGGCTGGG	9
		Jun	0.797	TTTTGTCACCGAGCCCTTG	7.2
		Calml5	0.781	TCTGGTCAAAGACCCGGAA	7.2
		Adcy6	0.733	GTGGGACAAAGAACATTAA	7.2
	ERβ	***Prkcb***	0.735	TCCCTCACCACTGACCCTA	5.7

Table 3: Motif analysis for selected genes involved in lipid metabolism after ChIP-Seq ERβ and AR in Sertoli cells

Receptor	Gene	Score	Input Sequence	Fold Enrichment
ERβ	***Acsl6***	0.741	GGAGGTCAGAGGAGGCCAA	15.81
	Gpat2	0.750	TTGGGTCAGTGACAGGGAT	15.81
	Acads	0.745	CTGTGTCACGATCGCGGGA	10.54
	Dgkz	0.753	TGAGGTTTCTGTGCCCATC	10.54
	Dgka	0.739	CCAGGTCATTTCCAGTGCC	10.54
	Sphk1	0.768	CTGGGTCACTGGGATGCCT	10.54
	Acox3	0.798	ACAAGTCCACAGGACCTGG	10.54
AR	***Oxct1***	0.886	AGTGGTTCATGTACT	17.64
	Acaca	0.841	AGTTTTAGACTGTTTTAT	17.64
	Acsl1	0.872	ATGCACTGGCTGTTCAA	13.23
	Mgll	0.866	ATGCACACGGTGTGTTT	13.23
	Acadm	0.837	AGGCCAGCCTGACCT	8.82
	Ivd	0.744	AGAAGATGCCCAGTATC	8.82
	Auh	0.899	TGTGCTGTCTGTTCTCT	8.82

4.4 Validation of the genes identified by ChIP-qPCR

To validate the data obtained from ChIP-Seq in germ cells and Sertoli cells, ChIP-qPCR for the selected genes was performed. The primers for all the genes were designed spanning the EREs and AREs present in the peaks of the genes identified.

4.4.1 ChIP-qPCR validation for germ cells

A total of 20 genes were selected from prolactin, GnRH, and oxytocin signaling pathway from both ERα and ERβ ChIP (Table 2). Among these, ten genes were selected from prolactin signaling pathway, of which seven genes (Shc4, Prlrl, Akt2, Rela, Stat3, Prl, Gsk3b) had ERα binding sites and three genes (Pik3ca, Jak2, Socs7) had ERβ binding sites. Five genes were selected from GnRH signaling pathway, of which four genes (Gnrhr, Nras, Mapk14, Egfr) had ERα binding sites and one gene (Grb2) had ERβ binding site. Similarly, in oxytocin signaling pathway five genes were selected, of which four genes (Adcy5, Jun, Calml5, Adcy6) had ERα binding sites and one gene (Prkcb) had ERβ binding site. All of the selected genes showed significant enrichment in the ChIP for corresponding ERα or ERβ binding sites as compared with IgG (Mock) (Figure 1).

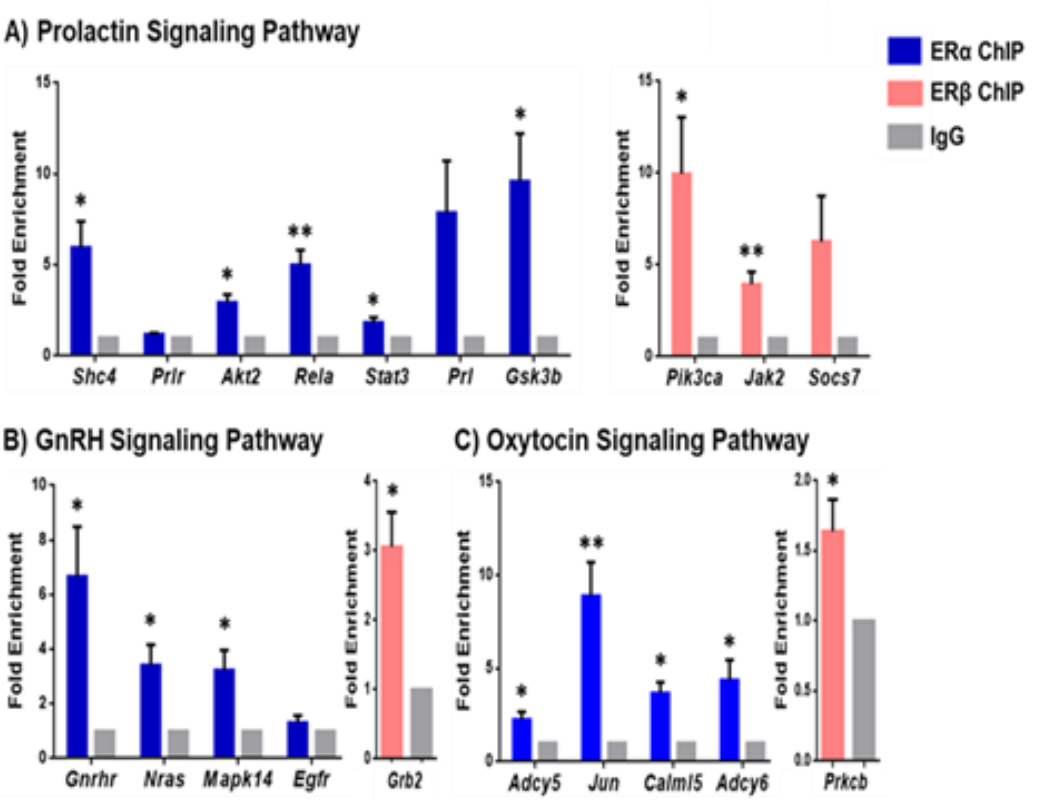

Figure 1: ChIP-qPCR validation done for genes involved in A) Prolactin, B) GnRH and C) Oxytocin Signaling pathway. The figure illustrates fold enrichment of ERα (blue bars) and ERβ (pink bars) ChIP over IgG Mock (grey bars). Values are represented as Mean ± SEM, N= 6, P value; * <0.05; ** < 0.01.

4.4.2 ChIP-qPCR validation for Sertoli cells

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). 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 (Figure 2).

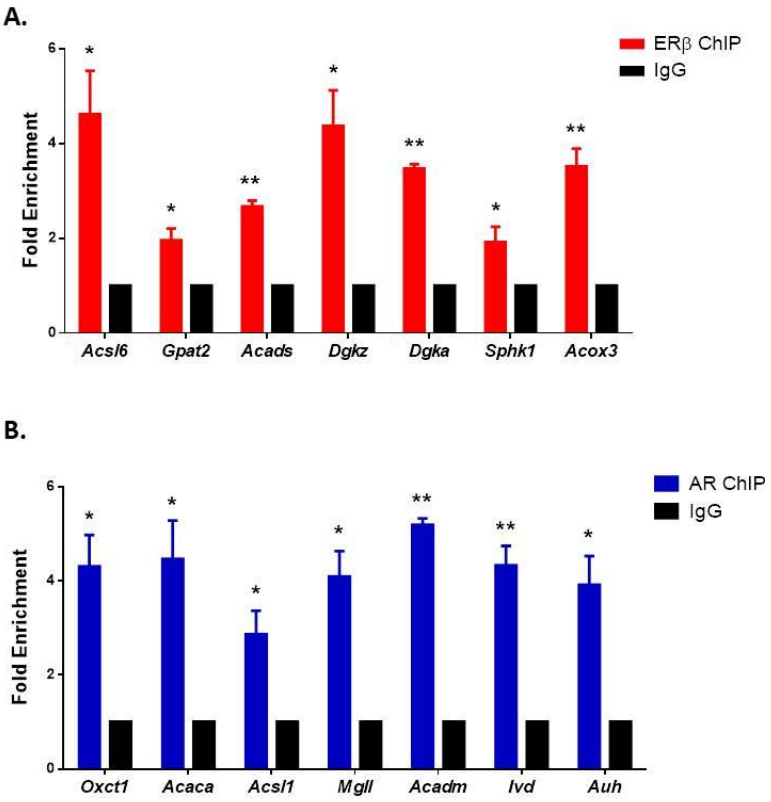

Figure 2: ChIP-qPCR validation done for genes identified by ChIP-Seq of A) ERβ and B) AR. The figure illustrates fold enrichment of ERβ (red bars) and AR (Blue bars) ChIP over IgG Mock (black bars). Values are represented as Mean ± SEM, N= 6, P value; * <0.05; ** < 0.01.

4.5 Integration of binding sites and transcription of the genes by estrogen and androgen

To ensure if the binding of estrogen and androgen receptor to these binding sites brings about any changes in the transcriptional level of these genes, the levels of mRNA were studied upon activation of the receptors using receptor specific agonists and antagonists.

4.5.1 Transcriptional regulation of genes by ERα and ERβ in germ cells

To study how estrogen regulates the expression of the selected genes, seminiferous tubules which mainly comprises of germ cells and Sertoli cells from adult rats were cultured in presence of estrogen receptor subtype specific agonists or antagonists. The alteration in the expression of the selected genes was studied after 24 h of treatment. All the genes in the prolactin signaling pathway show an up-regulation with ERα (PPT) or ERβ (DPN) agonist treatment and the effect was reversed by the corresponding antagonist treatment *in vitro* (Figure 3A). Similar results were observed for transcript levels of genes selected from GnRH and Oxytocin signaling pathway (Figure 3B & 3C).

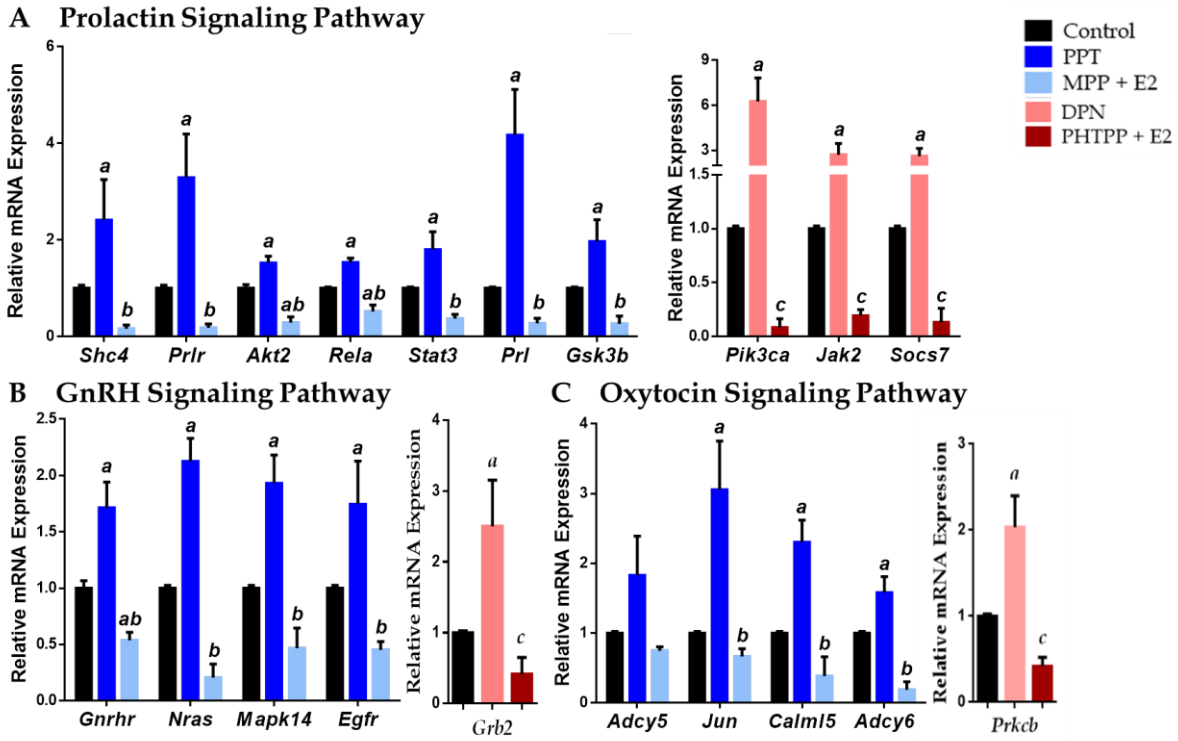

Figure 3: Relative expression of genes involved in A) Prolactin, B) GnRH and C) Oxytocin signaling pathway after treatment with PPT (ERα agonist), MPP + E2 (ERα antagonist + estradiol), DPN (ERβ agonist) and PHTPP + E2 (ERβ antagonist + estradiol) as compared to the control (DMSO). Values are represented as Mean ± SEM, N= 6, a: significant with respect to the control (DMSO); b, c: significant with respect to the ERα and ERβ agonist, respectively.

4.5.2 Transcriptional regulation by ERβ and AR in Sertoli cells

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) and dihydrotestosterone (DHT), 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 (Figure 4A). 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 (Figure 4B). 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 (data not shown). This suggests that the genes selected are regulated exclusively by estrogen and androgen in adult Sertoli cells.

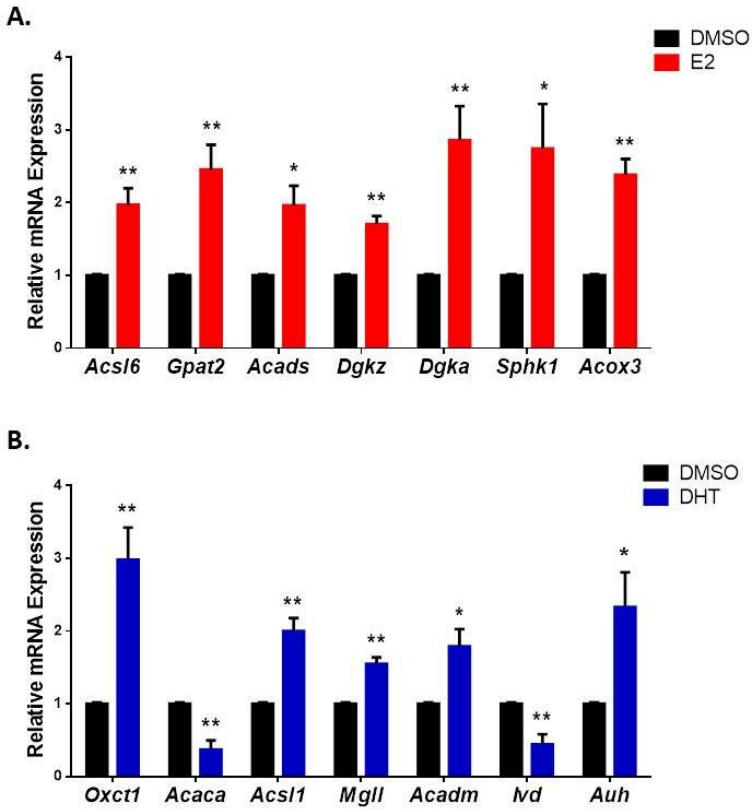

Figure 4: Relative expression of genes involved in lipid metabolism in adult Sertoli cell culture regulated by A) estrogen and B) androgen after estradiol (E2) and dihydrotestosterone (DHT) treatment, respectively. Values are represented as mean ± SEM; N = 6; P value; *<0.05; **<0.01.

5. 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 (ANOVA) followed by Dunnett's test was used. The level of significance was set at $p < 0.05$.

6. 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. Previous studies from our lab

have demonstrated that exogenous administration of E2 disrupted spermatogenesis by altering hormone levels leading to spermiation failure and germ cell apoptosis (Balasinor et al. 2010; D'Souza et al. 2005; 2009). 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 (Dumasia et al. 2015; 2016; Kumar et al. 2015; 2017). 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 unperturbed 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. We also performed ChIP-Seq for ERβ and AR in enriched Sertoli cells and identified number metabolic pathways to be regulated by estrogen and androgen.

The majority of the binding sites (60-65%) identified were located within 20Kb proximity from the Transcription Start Site (TSS) suggesting that ERβ and AR might be playing a crucial role in the transcription of these genes. Pathway analysis of the genes enriched by ERα and ERβ in germ cells and ERβ and AR in Sertoli cells showed involvement of these genes in a number of different pathways. The top pathway to be significantly enriched in both the cell types was metabolic pathway. During 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 (Oliveira et al. 2015). 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 (Chen et al. 2009; Wada et al. 2013). Similarly, androgen is also known to play a significant role in different metabolic pathways like glycolysis, amino acid synthesis, lipid synthesis, and nucleotide synthesis (Barfeld et al. 2014; Birzniece et al. 2018). Thus, it is not surprising that 617 genes in ERα and 511 genes in ERβ were involved in metabolic pathways in germ cells. Furthermore, 825 genes for ERβ and 448 metabolic genes for AR were identified adult Sertoli cells.

In germ cells, since estrogen is involved in varied functions, it can be speculated 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 pathway 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 (Freeman et al. 2000). 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 (Freeman et al. 2000). Previous studies in our lab have shown that treatment with ERα (PPT) and ERβ (DPN) agonist leads to an increase in serum prolactin levels (Dumasia et al. 2015). Estrogen is known to regulate prolactin as well as its receptor in the brain (Pi et al. 2003), but this regulation in the testes is unexplored. To understand the regulation of prolactin and its downstream targets by estrogen receptors in testes, we analysed 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 (Coughlan et al. 2013). JAK/STAT pathway is mainly involved in proliferation as well as survival, therefore, play a pivotal role in germ cells. Hyperprolactinemia is associated with male infertility (Colao et al. 2004; De Rosa et al. 2006), 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 (Ciaramella et al. 2015). 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 (Eski et al. 2019). In our study, a total of 50 genes with ERα and ERβ binding sites involved in GnRH signaling were observed. Functional validation showed alteration in crucial genes like Gnrhr, Egfr, Mapk, Nras and Grb2 after estrogen receptor specific agonist treatment. Estrogen has been shown to regulate Gnrhr expression in other tissues cells such as ovarian cells and hippocampal neurons (Nathwani et al. 2000; Sarvari et al. 2016). We also found regulation of Gnrhr transcripts by ERβ in the testis in this study. This might be indicative of mechanism of regulation of GnRH signaling by estrogen in the testis as well. Oxytocin (OT), a peptide hormone, is known to be locally produced in the testes (Nicholson et al. 1984). The actions of OT are all mediated by its receptor, OTR, which is a transmembrane receptor belonging to the G-protein-coupled receptor superfamily. Apart from its role in steroidogenesis, oxytocin is thought to aid spermiation by regulating seminiferous tubule contractility. Frayne et al. demonstrated an early appearance of spermatozoa and residual bodies in oxytocin-treated rats as compared to the control and delay in the same in rats treated with oxytocin antagonist (Frayne et al. 1996). We identified a total of 83 genes involved in oxytocin signaling to be enriched by ERα and ERβ ChIP suggesting that estrogen could be involved in modulating the genes in downstream signaling of oxytocin receptor. This is the first study to demonstrate the role of estrogens in regulating prolactin, GnRH, and oxytocin pathway in the germ cells of the testes.

Sertoli cells provide the nutrients and energy to the germ 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 (Jutte et al. 1982; Alves et al. 2013). However, glucose is not the only source of energy, since Sertoli cells maintain their viability and produce ATP even in the absence of glucose (Riera et al. 2009). Additionally, inactivation of genes involved in lipid metabolism also leads to impairment in the process of spermatogenesis (Chung et al. 2001). 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 (Alves et al. 2013). 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. 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 (Butler et al. 2016; Szafran et al. 1998). We selected a total of 14 genes genes involved in lipid metabolism, like fatty acid, glyceride, leucine, and sphingosine metabolism for further validation, 7 genes having ERβ and AR binding sites each.

Fatty acyl CoA synthetase is a group of enzymes that play an important role in intermediary metabolism converting fatty acids into fatty acyl-CoA ultimately leading to generation of ATPs. 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 had ERβ binding sites; whereas Acsl1 and Acadm had AR binding sites. We studied the functional relevance of the binding sites obtained, 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 (Jensen et al. 1994; Herrero et al. 2005). Besides, evidence also supports that an increase in oxidation of fatty acids is observed upon androgen supplementation (Lin et al. 2008). 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, 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 (Singh et al. 2018).

Another important pathway of lipid metabolism is that of glycerolipids and glycerophospholipids which play a central role in triglyceride metabolism and energy balance. 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 (Figure 4). 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 the link between the same (Eisen et al. 2002; Gowri et al. 2007). 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. Furthermore, the relationship between leucine and lipid metabolism is well established. 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 (Figure 4). It has been previously reported that androgen receptor regulates the uptake of leucine in prostate cancer cells by regulating the expression of its transporters (Wang et al. 2011). 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), which acts as a survival factor in male germ cells and has been reported to have protective effects in early stages of spermatogenesis (Otala et al. 2004). 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) (Sadler-Riggleman et al. 2019; Guerrero-Bosagna et al. 2013). A 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 (Sadler-Riggleman et al. 2019). Out of the 573 genes, 296 genes were observed to be enriched by ERβ and 177 genes by AR in our study, highlighting transgenerational penetrance of environmental toxicants on hormone-mediated transcription of metabolism genes. 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.

7. Impact of the research in the advancement of knowledge or benefit to mankind:

There is an alarming increase in the deleterious effects caused by endocrine disruptors, which carry out their actions mainly through the steroid receptors. Estrogen and androgen responsive phases of spermatogenesis are well documented; however, the genes regulated by these sex hormones under unperturbed conditions remain inexplicit. This is the first study carried out that maps the androgen and estrogen binding sites in a cell-specific manner in germ cells and Sertoli cells. Identification of these genes gave a novel insight into different

pathways regulated by these steroid hormones in the testes. One such pathway is the prolactin signaling pathway. Hyperprolactinemia is associated with male infertility; however, the mechanism behind it is not yet well understood. Based on the leads obtained from this project, we are further studying the molecular mechanisms of this signaling. In addition, it also highlight how Sertoli cell metabolism is crucial for normal spermatogenesis.

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