

Differential Expression of Genes Involved in Early Events of Spermatogenesis in Mice

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Abstract

Spermatogenesis is a highly complex process involved in the transmission of genetic information from one generation to next generation. The process takes place in the seminiferous tubules of the testis and is regulated by genes that control cell division, cell-cell interaction and morphogenetic changes in both the somatic and germ cell lineages in a highly orchestrated pattern. The first wave of spermatogenesis in mouse is typically characterized by the differential expression of genes involved in spermatogenesis. The purpose of this study was to analyze the differential expression of genes involved in Spermatogonial Stem Cells (SSCs) self-renewal, proliferation and differentiation using microarray approach in the testes of 35 vs. 5 Days Post Partum (dpp) mice. Our results demonstrate that genes involved in SSC self-renewal and proliferation were significantly down-regulated while genes involved in SSC differentiation were significantly up-regulated in the testes of 35 vs. 5 dpp mice. There was up-regulation in the expression of genes involved in cell cycle regulation. Pro-apoptotic genes were found to be up-regulated on the contrary anti-apoptotic genes were down-regulated in the testes of 35 vs. 5 dpp mice. Thus, our study helps in understanding the differential expression profile of genes involved in early stages of spermatogenesis in mice.

Keywords: Spermatogenesis; Spermatogonial stem cells (SSCs); Microarray; days post partum (dpp)

Introduction

Spermatogenesis is a complex and well-orchestrated process, in which Spermatogonial Stem Cells (SSCs) divide and differentiate to produce unlimited numbers of mature spermatozoa. This process is continuous throughout the adult life in most mammals. The seminiferous epithelium consists of only one somatic cell type, sertoli cell [1] and many different germ cell types [2]. Sertoli cells play a nurturing role in coordinating and supporting important events of spermatogenesis which occurs in mitotic, meiotic and post meiotic phases. Spermatogenesis is initiated by the division of stem cells (primitive type A spermatogonia) to form type B spermatogonia. These differentiated spermatogonial cells enter a meiotic prophase as pre-leptotene spermatocytes. The spermatocytes continue to mature as they go through the leptotene, zygotene and pachytene phases of meiosis. At the end of meiotic prophase, they undergo the final meiotic division to form haploid spermatids. These spermatids undergo spermiogenesis to form mature spermatozoa [3]. This complex process is orchestrated through expression of thousands of genes encoding proteins which are developmentally regulated during spermatogenesis and play essential roles during specific phases of germ cell development. Both transcriptional and translational control mechanisms are responsible for temporal and stage-specific expression pattern of genes [4,5].

Each cell contains the same genome, but not all the genes are used in each cell. Some genes are expressed when needed and many genes are used to specify features unique to each type of cells. In order to understand how genes are controlled and expressed at specific time and which genes function uniquely in special cells, an important step is defining gene expression profiles i.e. comparing patterns of gene expression in different tissues and at different developmental stages, in normal versus treated or diseased states. This can be accomplished by RT-PCR, RNase protection assays or Northern blot analysis, but these methods focus on only a few genes at a time. A more promising approach for analyzing multiple genes simultaneously is the hybridization of entire cDNA populations to

nucleic acid arrays such as microarray, a method adopted for high-throughput analysis of gene expression [6-9].

In a similar vein, microarray technology can be applied to gain a comprehensive view of expression pattern of genes involved in early stages of spermatogenesis with the purpose of studying the mechanisms and regulation of spermatogenesis at the genetic level. In rodents, first wave of spermatogenesis sets the basis for spermatogenesis process in adult animals. In mouse, at 0 days post partum (dpp), gonocytes are in the quiescent stage, around 3 dpp gonocytes are converted into SSCs which then migrate to the basement membrane of seminiferous tubules, at 5 dpp proliferation of SSCs starts, at 10 and 20 dpp spermatocytes and spermatids appear, and at 35 dpp first appearance of spermatozoa thereby marking the end of the first wave of spermatogenesis [10,11]. In our earlier studies, we deduced the expression patterns of Oct-4 as well as Plzf and functional significance of Oct-4 during the early stages of spermatogenesis in mice [12,13]. Both Oct-4 and Plzf are known to be expressed in undifferentiated SSCs and considered to be important for SSC self-renewal. The results of study showed Oct-4 and Plzf expression to be highest in the testes of 10 dpp mice both at the mRNA and protein level [12]. Apart from Oct-4 and Plzf, the SSC self-renewal and proliferation process involves many other genes which are crucial to maintain SSCs in the undifferentiated state. Therefore, the main aim of the present study is to elucidate the expression pattern of all those

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genes that are involved in the process of SSC self-renewal during the early events of spermatogenesis in mice using microarray approach. The study would also help in deducing the expression pattern of genes that are involved in SSC differentiation process and those which are expressed only in the differentiated germ cells.

Materials and methods

Experimental animals

Adult CD1 male and female mice were bred and maintained in institute's mice colony to get the 5 and 35 dpp mice for the study. All animals were housed at 25°C with 12 hour light: 12 hour dark photoperiod and were given water and a standard diet ad libitum. All animal experimental protocols were approved by the Institutional Animal care and Ethics committee (IAEC), National Institute for Research in Reproductive Health, Mumbai (IAEC# 08/09) in accordance with the guidelines of committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) established by Govt. of India on animal care.

RNA extraction from the testes of 5 and 35 dpp mice

In the current study, 5 animals (10 testes) from each age group (i.e. 5 and 35 dpp mice) were used to extract RNA. The total of 10 testes (from 5 animals) from 5 dpp mice was pooled in one tube and 10 testes (from 5 animals) from 35 dpp mice were pooled in another tube. RNA extraction was carried out from the testes of 5 and 35 dpp mice. This was referred to as Set I or first biological replicate. We made such three different sets or biological replicates on different occasions from same number of animals. Briefly, 5 and 35 dpp mice were asphyxiated with CO₂. Testes were retrieved from 5 and 35 dpp mice, homogenized at 4°C in 1ml of TRIzol reagent (Invitrogen, CA, USA) and total RNA was extracted as per the manufacturer's instructions. The 1 µl (1 unit/1 µg of RNA) of DNase I was added and incubated at 37°C for 20 min in order to remove the DNA contamination. The RNA obtained was quantified and checked for purity by spectrophotometer at A_{260/280} nm (Shimadzu, Japan). The ratio of ≥1.8 was considered to be acceptable.

Microarray processing

Total RNA extracted from the testes of 5 and 35 dpp mice was quantified using the NanoDrop ND-1000A spectrophotometer (Thermo Scientific, MA, USA). The quality of RNA was ascertained using the Agilent Bioanalyzer 2100 using the NanoChip protocol (Agilent Technologies, CA, USA). An acceptance criterion of RNA integrity value (RIN) of ≥7.0 was used. The cRNA preparation and array hybridization were performed using Illumina microarray technology (Illumina, CA, USA). A total of 500 ng of isolated total RNA was converted to biotinylated-cRNA following the Ambion Illumina TotalPrep RNA Amplification Kit procedure (Applied Biosystems/Ambion, TX, USA). In brief, RNA was converted into first strand cDNA for 2 hrs at 42°C, primed with an oligo(dT) primer bearing a T7 promoter, and catalyzed by ArrayScript reverse transcriptase. Second strand cDNA was synthesized by adding DNA polymerase I, and RNase H, and incubation was carried out for 2 hrs at 16°C. After cDNA purification by proprietary cDNA filter cartridge, eluted cDNA was used for *in vitro* transcription with T7 RNA polymerase (Ambion MEGAscript IVT technology). *In vitro* transcription was carried out at 37°C for 14 hrs, yielding with multiple copies of biotinylated antisense RNA molecules from each mRNA in the sample. Labeled cRNA was purified by cRNA filter cartridge. The quality of eluted biotin-cRNA was verified using the Bioanalyzer RNA Nano Chips according to the manufacturer's protocol. Measurement of cRNA yield was performed

using a NanoDrop 1000A Spectrophotometer. A total of 750 ng of labeled cRNA was then prepared for hybridization to Illumina Mouse WG6 v2.0 expression array beadchip by preparing a probe cocktail that includes GEX-HYB Hybridization Buffer. A total hybridization volume of 30 µl was prepared for each sample and 30 µl loaded into a single array on the Illumina Mouse WG6 v2.0 Expression Beadchip. Illumina Mouse WG6 v2.0 Expression Beadchip allows for six samples and targets 45281 probes corresponding to well-documented RefSeq 30854 transcripts. A total of 6 different labeled samples can be loaded into 6 individual arrays per beadchip. The chip was hybridized at 58°C for 16 hrs in an oven with a rocking platform. After hybridization, the chip is washed using protocols outlined in the Illumina manual. Upon completion of the washing, the chips are coupled with Cy3 and scanned in the Illumina BeadArray Reader confocal scanner (BeadStation 500GXDW; Illumina). The scanner operating software converts the signal on the array into a text file for analysis. All data is MIAME compliant. The Illumina microarray processing and data analysis was performed by Bionivid, Bengaluru, India.

Microarray data analysis

The txt file containing raw data obtained through BeadArray reader was subjected to GeneSpring Gx v12.0 software for further data processing, normalization, statistical and biological analysis to identify differentially expressed genes between 35 and 5 dpp testicular tissues. Raw data obtained in .txt (Sample Probe Profile) format was inter array normalized by shifting to 50th percentile and intra array normalized to median of all samples. Normalized ratios were transformed by Log₂ to obtain fold change. Volcano plot based method was used to find out genes that are differentially expressed by 1.5 fold between 35 and 5 dpp testes samples by applying Unpaired Student t-Test for p-value calculation and Benjamini-Hochberg based False Discovery Rate (FDR) correction. Genes that are 1.5 fold and above differentially expressed with a p-value of less than 0.05 were considered as true differentials (Significant Differentials). Hierarchical clustering of differentially regulated genes was done using Pearson uncentered distance matrix and average linkage rule to establish gene clusters that differentiate the two groups of samples. Significant Gene Ontology (GO) categories and pathways were identified and filtered based on p-value less than 0.05 and considered as differentially regulated.

Validation of microarray data by Q-PCR analysis

The relative expression levels of genes which were differentially expressed in microarray were validated by Q-PCR using SYBR Green chemistry. For this purpose, we chose representative genes which were significantly up-regulated and down-regulated from selective GOs. We selected those GOs which were related to spermatogenesis, reproduction, reproductive process or germ cell development etc. Similarly, pathways which are involved in spermatogenesis were selected. From these different GOs and pathways, significantly up or down-regulated genes were selected as candidates for further validation by Q-PCR. For Q-PCR analysis the RNA samples used were the same as those used for microarray analysis. Briefly, DNase I treated RNA samples from the testes of 5 and 35 dpp mice were converted into cDNA. The relative expression levels of selected candidate genes were estimated by CFX96 real-time PCR system (Bio-Rad, CA, USA) with respect to *Gapdh* as housekeeping control. The primers used for Q-PCR analysis are listed in Table 1. For each primer pair, reaction efficiency was estimated by the amplification of serial dilution of mouse testicular cDNA pool over a 10-fold range.

Gene	Accession no	Primer Sequence	Product size (bp)
<i>Pou5f1</i>	NM_013633	Forward 5'-cgggctgggtggatcctcga-3' Reverse 5'-ttcacggcattggggcggtc-3'	277
<i>Zbtb 16 (Plzf)</i>	NM_001033324	Forward 5'-gcaggagaccagcaaggcga-3' Reverse 5'-gcagagacccaggaggagg-3'	196
<i>Gfra-1</i>	NM_010279	Forward 5'-ggctaggaggagagatgct-3' Reverse 5'-ctggatgaccagggactt-3'	231
<i>Bcl6b</i>	NM_007528	Forward 5'-gcagcagtgaagaaggacc-3' Reverse 5'-agccacagcctcacagtct-3'	206
<i>Etv5</i>	NM_023794	Forward 5'-gggagagacaaaaccacca-3' Reverse 5'-atgggtgtgcagttcttcc-3'	219
<i>Stra8</i>	NM_009292	Forward 5'-ttctcgtgttccacaagt- 3' Reverse 5'-tacctgccacttgaggctg- 3'	263
<i>Crem</i>	NM_001110854	Forward 5'-tgaactgatgaggagactgacc-3' Reverse 5'-atittcaagcacaccacacg- 3'	280
<i>Mea 1</i>	NM_010787	Forward 5'-gaggaatccaacgcccagaga-3' Reverse 5'-ctgtaggaccctgatgtggc-3'	139
<i>Sycp1</i>	NM_011516	Forward 5'-catgctcgaacaggttgcta-3' Reverse 5'-ttcgctggctcaattatc-3'	215
<i>Bak 1</i>	NM_007523	Forward 5'-ggtgacctcttttgctg-3' Reverse 5'-taccacgaattggccaaca-3'	157
<i>Bad</i>	NM_007522	Forward 5'-agcgtacgcacacctatcct-3' Reverse 5'-caatggctgtgcatggtt-3'	242
<i>Bcl2</i>	NM_177410	Forward 5'-gctcaacaggagatgta-3' Reverse 5'-ccagaatccaactcacacc-3'	185
<i>Mcl1</i>	NM_008562	Forward 5'-tgccagctgggagtgatt Reverse 5'-actgcgctccaagcttcc-3'	277
<i>Cdk10</i>	NM_194446	Forward 5'-gaaggttcttccctgccc-3' Reverse 5'-ccacaatggttgatggcg-3'	244
<i>Cul3</i>	NM_016716	Forward 5'-cctggaagaagactgcta-3' Reverse 5'-ccccttgctgtagatgtt-3'	181
<i>Plk1</i>	NM_011121	Forward 5'-tggagcaacttcggcatcat-3' Reverse 5'-gcctgcaacacctctttg- 3'	261
<i>Gapdh</i>	NM_008084	Forward 5'-aacttggcattgtggaagg-3' Reverse 5'-acacattggggtaggaaca-3'	223

Table 1: Sequence of primers used for validation of microarray data.

The amplification conditions were as follows: initial denaturation at 94°C for 2 min followed by 40 cycles comprising denaturation at 94°C for 30 sec, primer annealing at desired temperature for each primer pair for 30 sec, and extension at 72°C for 45 sec. The final extension was carried out for 7 min at 72°C. The fluorescence emitted at each cycle was collected for the entire period of 30 sec during the extension step of each cycle. The homogeneity of the PCR amplicons was verified by running the products on 1.2% (w/v) agarose gels in 1X TBE buffer and also by studying the melt curve. Mean C_t values generated in each experiment using the CFX Manager software (Bio-Rad) were used to obtain the standard curve, and the cDNA concentrations in the samples were computed and normalized to *Gapdh*. The relative expression levels of different genes in the testes of 35 vs. 5 dpp mice were calculated by $2^{-\Delta\Delta C_t}$ method [14].

Statistical analysis

The data obtained on fold change expression from Q-PCR was represented as mean \pm SD. Statistical analysis of the results was performed by one-way analysis of variance (ANOVA). All the statistical analysis was done using GraphPad Prism software version 5.0 (GraphPad software, CA, USA). The fold change in the expression was considered to be significant if the values obtained were less than 0.05 ($p < 0.05$).

Results

Overall gene expression profile

To characterize the genes that are associated with spermatogenesis (germ cell development, proliferation and differentiation) and the genes that are differentially regulated, we examined the gene expression profiles in the testes of 35 vs. 5 dpp mice. After normalization of raw data for all three biological replicates, 10437 genes were found to be differentially expressed between 35 and 5 dpp time points. Out of 10437, 3826 were up-regulated and 6611 were down-regulated with statistically significant changes in gene expression between 35 and 5 dpp testes. The differentially regulated genes were classified according to the molecular function of their cognate protein and their involvement in biological processes and cellular component distribution. Figure 1 and Figure 2 represent Volcano plot and Hierarchical cluster of differentially expressed genes respectively in the testes of 35 vs. 5 dpp mice.

Next, we studied the expression profiles of those genes that are involved in spermatogonial development, SSC self-renewal, proliferation and differentiation processes. To achieve this, we selected GO categories such as “Spermatogenesis”, “Reproduction”, “germ cell development”, “reproductive process”, “gene expression”, “cell

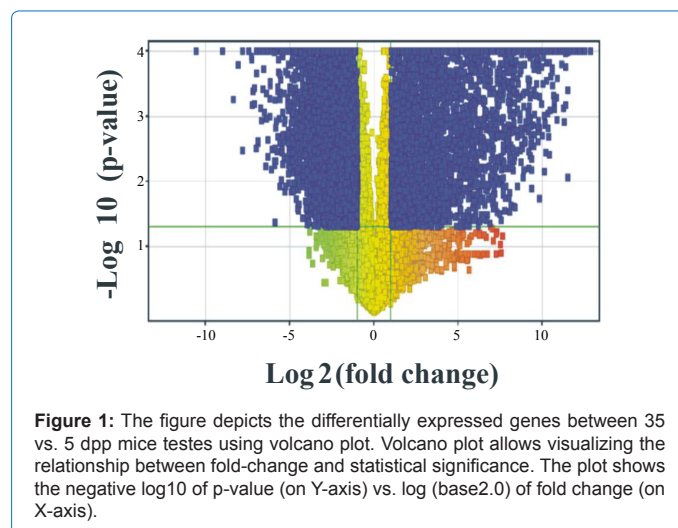


Figure 1: The figure depicts the differentially expressed genes between 35 vs. 5 dpp mice testes using volcano plot. Volcano plot allows visualizing the relationship between fold-change and statistical significance. The plot shows the negative log10 of p-value (on Y-axis) vs. log (base2.0) of fold change (on X-axis).

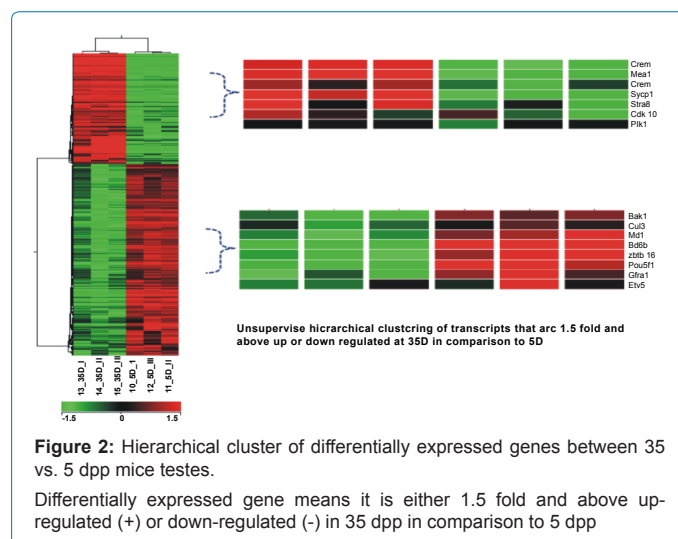


Figure 2: Hierarchical cluster of differentially expressed genes between 35 vs. 5 dpp mice testes. Differentially expressed gene means it is either 1.5 fold and above up-regulated (+) or down-regulated (-) in 35 dpp in comparison to 5 dpp

differentiation” and “Regulation of gene expression”. In each of these GOs significant numbers of genes were differentially regulated. We also selected the pathways which were differentially regulated between 5 and 35 dpp mice testes. Only those pathways were selected which were relevant to spermatogenesis and also known to be involved in apoptosis and cell cycle regulation. The pathways chosen were “trans membrane receptor protein tyrosine kinase signaling pathways”, “apoptosis” and “cell cycle”. The GOs which were differentially regulated and the number of genes differentially expressed amongst these GOs are listed in Table 2. The pathways and the number of genes amongst them which were differentially regulated are listed in Table 3.

Identification of down-regulated genes associated with spermatogenesis in the testes of 35 vs. 5 dpp mice

We evaluated the GOs mentioned in Table 2 to analyze the genes which were down-regulated and were associated with SSC self-renewal, proliferation or differentiation and regulation of gene expression in spermatogenesis. In GO category “Spermatogenesis”, out of the total 198 genes detected 116 were differentially regulated, in GO category “Regulation of gene expression” out of the 1782 genes detected 772 were differentially regulated. Some of the pathways also were significantly dysregulated such as “ESC pluripotency pathways” and “Trans membrane receptor protein tyrosine kinase signaling pathway”. These constituted the genes which are important in SSC self-renewal and thus control the undifferentiated state of testicular stem cells. “Trans membrane receptor protein tyrosine kinase signaling pathway” showed 82 differentially regulated genes out of 129 genes that were detected in microarray experiment. “ESC pluripotency pathway” showed 111 dysregulated genes out of 235 genes that were detected in microarray experiments. The microarray data showed that the genes such as *Pou5f1*, *Zbtb16*, *Etv5*, *Bcl6b*, *Gfra-1* etc. involved in SSC self-renewal or proliferation were significantly down-regulated in the testes of 35 vs. 5 dpp mice. These all were categorized into different GOs as mentioned in Table 2 and Table 3.

Identification of up-regulated genes associated with spermatogenesis in the testes of 35 vs. 5 dpp mice

To identify and analyze the genes which were up-regulated in 35 vs. 5 dpp testes, we studied the GOs mentioned in Table 2. There were about 563 differentially regulated genes out of 1200 genes that

were detected in microarray experiment from “Cell Differentiation” ontology. Similarly, there were about 247 genes differentially regulated out of 479 and 476 genes detected in microarray experiment from each of “Reproduction” and “Reproductive process” ontologies respectively. The significantly up-regulated genes such as *Stra8*, *Mea1*, *Crem*, *Sycp1* etc. were involved and expressed in the differentiated germ cells and during the late stages of spermatogenesis in mice.

Differential regulation of genes involved in cell cycle and apoptosis

We further analyzed the expression of genes involved in cell cycle regulation and apoptosis pathway as mentioned in Table 3 during spermatogenesis. It was observed that the genes involved in cell proliferation such as *Cdk10*, *Cul3*, *Cdkl2* etc. were up-regulated. Some of the spermatocyte and spermatid cell cycle stage specific genes such as *Cul3* (S phase-Spermatocyte), *Siah11a* (M phase-Spermatocytes), *Plk1* (G2-M phase-spermatids) were also up-regulated. Analyzing the expression of genes involved in pro or anti-apoptosis revealed that pro-apoptotic genes like *Bak1* and *Bad* were up-regulated while anti-apoptotic genes such as *Bcl-2*, *Bcl-Xl* and *Mcl1* were down-regulated in the testes of 35 vs. 5 dpp mice.

Validation of microarray data by Q-PCR analysis

Although differences in gene expression during spermatogenesis were observed with the microarray, it was essential to verify these results using other methods. We monitored the mRNA levels of selected genes noted to be differentially expressed in the Illumina arrays by Q-PCR.

In order to validate the microarray data, we chose 4 representative down-regulated genes and 4 up-regulated genes from GOs which were significantly dysregulated as mentioned in Table 2. We studied the testicular expression of *Pou5f1*, *Zbtb16*, *Etv5* and *Bcl6b* genes which were down-regulated in 35 vs. 5 dpp mice according to microarray data. The respective fold change down-regulation of *Pou5f1*, *Zbtb16*, *Etv5* and *Bcl6b* genes was found to be 6.04, 3.59, 2.41 and 7.67 as per microarray data. Q-PCR analysis using primers for above mentioned genes showed 8.05, 5.12, 3.32 and 6.44 fold down-regulation of *Pou5f1*, *Zbtb16*, *Etv5* and *Bcl6b* genes respectively (Figure 3). Further, we studied the expression of *Gfra-1* which showed 2.71 fold down-regulation by microarray data and was categorized into “Trans membrane receptor protein tyrosine kinase signaling pathway” ontology (Table 3). Q-PCR

GO accession (Reference)	GO term	p-value	Differentially expressed	Total genes under GO term
GO: 0007283	Spermatogenesis	0.00000	116	198
GO: 0000003, GO: 0019952, GO: 0050876	Reproduction	0.00000	247	479
GO: 0007281	Germ cell development	0.05143	37	75
GO: 0022414	Reproductive process	0.00000	247	476
GO: 0010467	Gene expression	0.00030	834	1975
GO: 0030154	Cell differentiation	0.00000	563	1200
GO: 0010468	Regulation of gene expression	0.00020	772	1782

Table 2: Differentially expressed GOs and numbers of genes amongst GOs. Differentially expressed gene means it is either 1.5 fold and above up-regulated (+) or down-regulated (-) in 35 dpp in comparison to 5 dpp.

GO Accession (Reference)	Pathway	p-value	Differentially expressed	Total Genes in pathway
GO: 0007169	Trans membrane receptor protein tyrosine kinase signaling pathway	0.0001	82	129
WP1254	Apoptosis	0.0001	47	82
WP190	Cell cycle	0.002	45	88

Table 3: List of pathways differentially regulated and genes amongst these pathways. Differentially expressed gene means it is either 1.5 fold and above up-regulated (+) or down-regulated (-) in 35 dpp in comparison to 5 dpp.

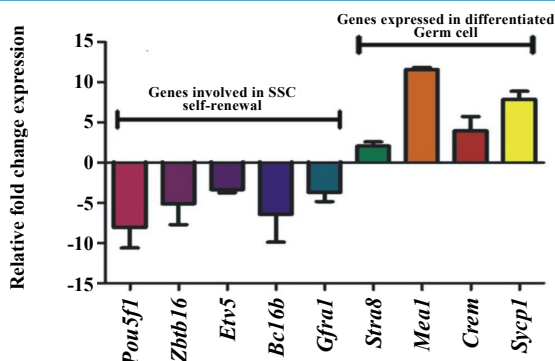


Figure 3: Validation by Q-PCR of differentially expressed genes involved in spermatogenesis in the testes of 35 vs. 5 dpp mice. The genes involved in SSC self-renewal or proliferation showed significant down-regulation (-) in the testes of 35 vs. 5 dpp mice. On the contrary, the genes involved in SSC differentiation and the ones which are expressed in differentiated germ cells showed significant up-regulation (+) in the testes of 35 vs. 5 dpp mice.

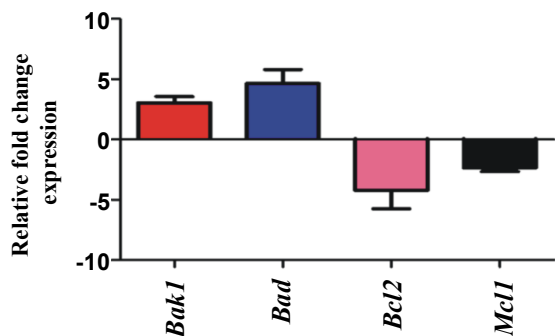


Figure 4: Validation by Q-PCR of differentially expressed pro and anti-apoptotic genes in the testes of 35 vs. 5 dpp mice. Pro-apoptotic genes (*Bak1* and *Bad*) were significantly up-regulated (+) and anti-apoptotic genes (*Bcl2* and *Mcl1*) were significantly down-regulated (-) in the testes of 35 vs. 5 dpp mice.

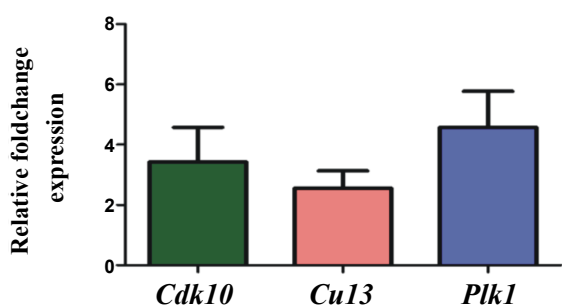


Figure 5: Validation by Q-PCR of differentially expressed genes involved in cell cycle regulation and proliferation in the testes of 35 vs. 5 dpp mice. The genes involved in cell cycle regulation and proliferation were significantly up-regulated (+) in the testes of 35 vs. 5 dpp mice.

data showed that there was 3.68 fold down-regulation of *Gfra-1* in the testes of 35 vs. 5 dpp mice (Figure 3).

Similarly, expression of those genes which were significantly up-regulated according to microarray data in the testes of 35 vs. 5 dpp mice was validated. We decided to study *Stra8*, *Crem*, *Mea1* and *Sycp1* genes. The respective fold up-regulation for *Stra8*, *Crem*, *Mea1* and *Sycp1*

genes was found to be 2.42, 3.69, 5.46 and 8.11 by microarray data. Q-PCR data showed 2.05, 11.59, 3.97 and 7.9 fold up-regulations for *Stra8*, *Crem*, *Mea1* and *Sycp1* genes respectively in the testes of 35 vs. 5 dpp mice (Figure 3).

To validate the genes that are differentially regulated and involved in the apoptosis, we chose *Bak1* and *Bad* genes (pro-apoptotic genes) which were up-regulated and *Bcl2*, *Mcl1* genes (anti-apoptotic genes) which were down-regulated. Microarray data showed up-regulation of *Bak1* and *Bad* by 2.65 and 2.9 folds respectively while down-regulation of *Bcl2* and *Mcl1* by 5.06 and 2.17 folds respectively. The results of validation by Q-PCR showed that there was 3.03 and 4.65 fold up-regulation of *Bak1* and *Bad* genes respectively in the testes of 35 vs. 5 dpp mice while for *Bcl2* and *Mcl1* the fold change down-regulation was found to be 4.22 and 2.35 respectively (Figure 4).

To validate the genes involved in cell cycle regulation and proliferation, we chose *Cdk10*, *Cul3* and *Plk1* genes which were up-regulated according to microarray data by 2.58, 2.53 and 2.14 folds respectively. Q-PCR data showed 3.42, 2.55 and 4.57 folds increased expression of *Cdk10*, *Cul3* and *Plk1* respectively in the testes of 35 vs. 5 dpp mice (Figure 5). The list of down-regulated and up-regulated genes from different GOs/ pathways and their expression/ function is illustrated in (Tables 4-7).

Discussion

In our earlier study, we demonstrated the expression pattern of Oct-4 and *Plzf* in the early stages of spermatogenesis in mice [12]. These two genes showed highest expression in the testes of 10 day old mice and are known to be important in SSC self-renewal and maintaining the undifferentiated state of testicular stem cells. Thus, study was conducted to understand the expression pattern of other genes involved in SSC self-renewal, proliferation and differentiation process. As mentioned earlier, it is not possible to study such a large number of genes using RT-PCR analyses. Therefore, we employed microarray approach using Illumina technology to study 5 and 35 dpp time points in testicular development of mice. The reason for choosing 5 and 35 dpp time points was, at 5 dpp the testes would be highly enriched with undifferentiated spermatogonial population on the contrary in 35 dpp testes there would be a wide range of differentiated germ cells population along with undifferentiated spermatogonial population. The present study helped us in understanding the stark differences in fold change expression of genes involved in SSC self-renewal versus differentiation.

Next, we evaluated the expression pattern of the genes which are expressed in differentiated germ cells such as type B spermatogonia, spermatocytes and spermatids. These differentiated germ cell types would be present in the testes of 35 dpp mice. The earlier studies revealed the differential expression of genes involved in spermatogenesis, wherein the authors determined the expression profiles of 1176 known mouse genes in 6 different types of mouse spermatogenic cells viz. primitive type A spermatogonia, type B spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, round spermatids and elongating spermatids [15]. This study also demonstrated the utility of cDNA microarray in analyzing the gene expression changes at different stages of spermatogenic cells in spermatogenesis. In the present study, we found differential expression of 10437 transcripts which correlated with the findings of Shima et al. [16] where they found differential expression of 11260 transcripts at 35 dpp. Our microarray data showed that genes involved in SSC self-renewal and proliferation such as *Pou5f1*, *Zbtb16*, *Gfra-1*, *Etv5*, *Bcl6b* etc. were significantly down-regulated in 35 vs. 5 dpp testicular tissues. These results did not correlate with the study

Gene	GO category	Fold change (-)	Gene expression/ Function
<i>Pou5f1</i> (POU domain, class 5, transcription factor 1)	Nucleus; gene expression; regulation of gene expression; cell differentiation	6.04	SSC Self-renewal [21]
<i>Zbtb16</i> (<i>Plzf</i>) (zinc finger and BTB domain containing 16)	reproduction; nucleus; germ cell development; spermatogenesis; regulation of gene expression; reproductive process; cell differentiation	3.59	SSC Self-renewal [22]
<i>Etv5</i> (Ets variant gene 5)	nucleus ; regulation of gene expression	2.41	SSC Self-renewal [23]
<i>Bcl6b</i> (B cell CLL/lymphoma 6, member B)	nucleus ; regulation of gene expression	7.67	SSC Self-renewal [24]
<i>Nanos 2</i>	reproduction; spermatogenesis ; regulation of gene expression; reproductive process ; cell differentiation	7.6	Maintaining undifferentiated state of SSCs [25]
<i>Nanos 3</i>	reproduction ; spermatogenesis ; regulation of gene expression ; reproductive process ; cell differentiation	6.47	Germ cell development [26]
<i>Sohlh1</i> (Spermatogenesis-and oogenesis-specific basic helix-loop-helix-containing protein 1)	reproduction ; nucleus ; spermatogenesis ; gene expression ; regulation of gene expression ; reproductive process ; cell differentiation	2.26	Essential for spermatogonial differentiation and expressed in type A spermatogonia [27]
<i>Cyp26b1</i> (Cytochrome P450 26B1)	reproduction ; spermatogenesis ; reproductive process ; cell differentiation	4.29	Expressed in sertoli cells, Meiosis inhibitory factor [28]
<i>Sox2</i> (SRY-box containing gene 2)	reproduction ; nucleus ; gene expression ; regulation of gene expression ; reproductive process ; cell differentiation	2.10	Pluripotency marker [24]

Table 4: Genes from different GOs: fold change down-regulation (-) of genes from microarray experiment and their reported expression or function in the context of spermatogenesis.

Gene	GO category	Fold change (+)	Expression/ Function
<i>Stra8</i> (Stimulated by Retinoic Acid gene 8)	Reproduction ; germ cell development; spermatogenesis ; fertilization ; reproductive process ; cell differentiation	2.42	Role in premeiotic phase of germ cells, required for transition into meiosis [29]
<i>Mea 1</i> (Male enhanced antigen 1)	Reproduction; spermatogenesis; reproductive process; cell differentiation	5.46	Functions in late stages of spermiogenesis, expression in pachytene spermatocytes, cytoplasm of elongated spermatids [30]
<i>Crem</i> (cAMP responsive element modulator)	Reproduction ; nucleus ; spermatogenesis ; gene expression ; regulation of gene expression ; reproductive process	3.69	Regulation of genes required for spermatid maturation [31]
<i>Zfp42</i> (Zinc finger protein 42 or Reduced expression protein 1)	Nucleus ; gene expression ; regulation of gene expression	8.31	Expression in spermatocytes [32]
<i>Zfp35</i> (Zinc finger protein 35)	Reproduction ; nucleus ; spermatogenesis ; gene expression ; regulation of gene expression ; reproductive process ; cell differentiation	4.44	Expression in pachytene spermatocytes [33]
<i>Tnp1</i> (Transition protein 1)	reproduction ; nucleus ; spermatogenesis ; reproductive process ; cell differentiation	64.65	Expressed during spermiogenesis [34]
<i>Mtl5</i> (metallothionein-like 5)/ Tesmin	reproduction ; nucleus ; spermatogenesis ; reproductive process ; cell differentiation	105.95	Expressed in spermatocytes [35]
<i>Prm1</i> (Protamine 1)	reproduction ; nucleus ; germ cell development ; spermatogenesis ; reproductive process ; cell differentiation	2884	Expressed in spermatids [36]
<i>Hils1</i> (histone H1-like protein in spermatids 1)	reproduction ; nucleus ; spermatogenesis ; gene expression ; regulation of gene expression ; reproductive process ; cell differentiation	2451	Spermatid-specific linker histone. H1-like protein implicated in chromatin remodeling [37]

Table 5: Genes from different GOs: fold change up-regulation (+) of genes from microarray experiment and their reported expression or function in the context of spermatogenesis.

carried out by Kokkinaki et al. [17] wherein they showed that when gene expression pattern for *Gfra-1*, *Zbtb16* and *Pou5f1* was analyzed in the testes of 6, 21, 60 dpp and 8 months old mice, there was no change in *Gfra-1* and *Zbtb16* expression across this age group while that of *Pou5f1* was higher in 6 day old mice compared to the other three age groups. In our study, the expression of *Stra8*, *Mea1*, *Crem*, *Sycp1* etc. which are expressed or required during early or late stages of spermatocytes and spermatid formation were found to be significantly up-regulated in the testes of 35 vs. 5 dpp mice.

Subsequently, we studied the differential expression of genes involved in cell cycle control and proliferation. The microarray data showed that the genes involved in cell cycle progression and which are expressed in cell cycle phase specific manner were up-regulated. There was up-regulation of genes such as *Cul3* expressed in S-phase of spermatocytes, *Siah1a*, *Senp1* and *Ccnb2* expressed in M-phase of spermatocytes, *Plk1* and *Rpa1* expressed in G2-M transition phase of

spermatids. These genes were also reported in a study published by Roy-Choudhury et al. [18] wherein they carried out microarray based analysis of cell cycle gene expression during spermatogenesis in the mouse.

Earlier study by Jahnukainen et al. [19] revealed that increased apoptosis occurring during the first wave of spermatogenesis is stage-specific and affects mid pachytene spermatocytes in the rat testis. The physiological apoptosis that occurs in immature testis is necessary for the maturation of this tissue. Thus, inhibition of the early apoptotic wave associated with the first round of spermatogenesis is followed by the accumulation of spermatogonia and infertility later in life. In immature testis of rats, two groups of populations have been shown to be prone to apoptosis. Firstly, type A spermatogonia undergo apoptosis by day 8 and then at 18 and 26 day pachytene spermatocytes undergo apoptosis [19]. In our study, we found increased expression of pro-apoptotic genes such as *Bak1* and *Bad* while down-regulation of anti-

Gene	GO category	Fold change (+)	Expression/function
<i>Cdk10</i> (Cell division protein kinase 10)	Cell cycle	2.58	Cell cycle progression [38]
<i>Cu13</i> (Cullin 3)	Cell cycle	2.53	Expressed in S-phase of spermatocytes [18]
<i>Plk1</i> (Serine-threonine protein kinase 1)	Cell cycle	2.14	Early trigger for G2-M transition [18]
<i>Senp1</i> (Sentrin-specific protease 1)	Cell cycle	2.17	M-Phase of spermatocytes [18]

Table 6: The genes involved in cell cycle regulation and proliferation which were significantly up-regulated (+) as per microarray data.

Gene	GO category	Fold change	Function
<i>Bak1</i> (Bcl2 homologous antagonist/killer)	Apoptosis pathway	2.65 (+)	Pro-apoptotic gene [39]
<i>Bad</i> (Bcl2 associated death promoter)	Apoptosis pathway	2.9 (+)	Pro-apoptotic gene [39]
<i>Bcl-2</i> (B-cell lymphoma 2)	Apoptosis pathway	5.06 (-)	Anti-apoptotic gene [40]
<i>Mcl1</i> (Induced myeloid leukemia cell differentiation protein)	Apoptosis pathway	2.17 (-)	Anti-apoptotic gene [41]
<i>Bcl-Xl</i> (B-cell lymphoma-extra large)	Apoptosis pathway	4.33 (-)	Anti-apoptotic gene [42]

Table 7: Differentially regulated pro and anti-apoptotic genes as per microarray data.

apoptotic genes such as *Bcl2*, *Bcl-Xl* and *Mcl1* in 35 vs. 5 dpp testes. The study by Jahnukainen et al. [19] also showed the increased expression of *Bax* and *Bad* at 18 and 26 day old rat testis in their study while the peak expression of *Bcl2* was seen only in 8 day old rat testis. Thus, our results demonstrated that mouse testes display similar pattern of pro or anti-apoptotic gene expression as that of rat testes during the early stages of spermatogenesis.

Gene expression in the testis can be regulated by a variety of factors, including hormones, cell-cell interactions and environmental cues, each of which can occur simultaneously or at distinct periods during the development of the animal leading to sexual maturity. Identification of the genes involved in the production of sperm has shown that both unique and ubiquitous genes play key roles in this process [20]. However, identifying and characterizing the genes necessary for spermatogenesis and their expression patterns at the genomic level has proven difficult due to the complex nature of testicular tissue which consists of numerous cell types, each contributing to the total testicular transcriptome. Therefore, the analysis of changes in gene expression based on major stages of development, especially with regard to germ cell development between 35 vs. 5 dpp testes would provide a powerful tool to determine the cellular processes involved in the formation of spermatozoa. Although the general patterns of expression are useful in looking at the testis as an entire tissue, the use of this time course in combination with more specific approach such as studying testis function during the first wave of spermatogenesis and other available datasets may prove to be an important method of completely describing the testis at the molecular level.

Conclusion

The process of spermatogenesis is typically characterized by the expression of plethora of genes at different stages of spermatogenesis in mammals. The first wave of spermatogenesis is crucial in order to set the process of spermatogenesis in adults. Our current study revealed the differential gene expression profile during the early stages of spermatogenesis in the testes of 35 vs. 5 dpp mice. From the results it can be concluded that the first wave of spermatogenesis is regulated by coordinated expression of number of genes involved in SSC self-renewal, proliferation, differentiation and cell cycle regulation. During the first wave of spermatogenesis, the spermatogenic cells undergo apoptosis which is essential to set the process of spermatogenesis in the adulthood. Thus, the present study sheds light on the expression pattern of different genes involved in the process of spermatogenesis in mice.

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