SCIENCE CHINA
Life Sciences

• COVER ARTICLE •

January 2013 Vol.56 No.1: 1–12
doi: 10.1007/s11427-012-4411-y

Transcriptome profiling of the developing postnatal mouse testis using next-generation sequencing

GONG Wei[1,2], PAN LinLin[1], LIN Qiang[1], ZHOU YuanYuan[1,2], XIN ChengQi[1], YU XiaoMin[1], CUI Peng[1], HU SongNian[1] & YU Jun[1*]

[1]*CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100029, China;*
[2]*Graduate University of Chinese Academy of Sciences, Beijing 100049, China*

Received October 18, 2012; accepted November 9, 2012; published online December 20, 2012

Mammalian testis development is a complex and highly sophisticated process. To study the dynamic change of normal testis development at the transcriptional level, we investigated mouse testes at three postnatal ages: 6 days postnatal, 4 weeks old, and 10 weeks old, representing infant (PN1), juvenile (PN2), and adult (PN3) stages, respectively. Using ultra high-throughput RNA sequencing (RNA-seq) technology, we obtained 211 million reads with a length of 35 bp. We identified 18837 genes that were expressed in mouse testes, and found that genes expressed at the highest level were involved in spermatogenesis. The gene expression pattern in PN1 was distinct from that in PN2 and PN3, which indicates that spermatogenesis has commenced in PN2. We analyzed a large number of genes related to spermatogenesis and somatic development of the testis, which play important roles at different developmental stages. We also found that the MAPK, Hedgehog, and Wnt signaling pathways were significantly involved at different developmental stages. These findings further our understanding of the molecular mechanisms that regulate testis development. Our study also demonstrates significant advantages of RNA-seq technology for studying transcriptome during development.

next-generation sequencing, transcriptome, mouse testis, development

Citation: Gong W, Pan L L, Lin Q, et al. Transcriptome profiling of the developing postnatal mouse testis using next-generation sequencing. Sci China Life Sci, 2013, 56: 1–12, doi: 10.1007/s11427-012-4411-y

The mammalian testis is the male reproductive organ, which produces spermatozoa and androgens. Spermatozoa are formed by germ cells through three phases: (i) proliferation and differentiation of spermatogonia, (ii) meiosis of spermatocytes, and (iii) spermiogenesis. The self-renewing spermatogonial stem cells differentiate into type B spermatogonia through mitotic divisions. Type B spermatogonia continue dividing into spermatocytes. After DNA duplication, the tetraploid primary spermatocytes undergo two rounds of meiosis to successively produce secondary spermatocytes and spermatids. Haploid spermatids are transformed into sperm cells through at least four distinct morphological changes: nuclear condensation, acrosome formation, flagellum formation, and cytoplasmic reduction. Spermatogenesis involves not only spermatogenic cells, but also multiple somatic cells in the testicular tissues, such as Sertoli cells and Leydig cells. Sertoli cells are located within the seminiferous tubules, which support, protect, and nourish germ cells. Leydig cells are the major cell type in the testicular interstitial tissues and they secrete androgens (mostly testosterone), which promote gonad development and maintain spermatogenesis and male secondary sex characteristics. Testis development is a complex and highly sophisticated process. Profiling the transcriptome of the

*Corresponding author (email: junyu@big.ac.cn)

developing postnatal mouse testis can provide important clues for understanding the regulatory mechanisms of testis development.

Large-scale techniques to analyze the transcriptome, such as expressed sequence tags (EST) and cDNA microarrays, have emerged over the past decade, and have been applied to gene expression studies of the mouse testis [1–12]. The advent of serial analysis of gene expression (SAGE) has made these technologies more efficient and O'Shaughnessy et al. have used SAGE to generate gene expression profiles from the somatic cells of fetal and adult mouse testes [13]. They identified 12976 unique transcripts and found that most of the Sertoli or Leydig cell-specific genes are up-regulated in adult mice. Wu et al. [14] sequenced the transcriptomes of mouse type A spermatogonia, pachytene spermatocytes, and round spermatids and, again using SAGE, estimated that the germ-cell transcriptome was comprised of more than 30000 transcripts. Yao et al. [15] identified 19323 unique tags from both young adult and aged mouse testes. The levels of transcripts associated with spermatogenesis, such as protamine 2, transition protein 1, and poly-A binding protein, were overwhelmingly high, whereas housekeeping genes and marker genes for Sertoli and Leydig cells were generally expressed at a low level. Meanwhile, other researchers have combined different techniques, such as differential display competitive PCR, DNA array, and subtracted cDNA libraries to study gene expression in mouse testes during development [16,17].

Comparative analyses and reinvestigations among the acquired datasets have also been done. For instance, Divina et al. [18] constructed SAGE libraries of adult mouse testes. They produced 24529 unique tags, and determined predominant expression of 829 genes in germ cells and 944 genes in somatic cells when compared with the publicly available SAGE libraries. Chan et al. analyzed the data generated by Wu et al., and identified 27504 species of transcript in type A spermatogonia, pachytene spermatocytes, and round spermatids [19]. Later, Chan's group [20] constructed GermSAGE, a web-based database for studying germ cell transcriptomes of male mice. Efforts have also been made to define cell-specific gene expression. For instance, Ike et al. [21] examined changes in testicular gene expression before and after haploid germ cell differentiation using microarray analysis and found that 1315 genes are differentially expressed. Xiao et al. [22] performed gene expression profiling of mouse testes at different differentiation stages using DNA microarrays, and characterized 2058 spermatogenesis-related genes. Waldman Ben-Asher et al. [23] used microarrays to compare five distinct meiotic stages during postnatal development of mouse testes, and detected 790 differentially expressed genes. They also suggested that gene expression related to meiosis might be regulated at the chromosomal level.

The above accomplishments focused on different types of spermatogenic or somatic cell, or were performed on mutant mice, or focused on one developmental stage, but all pave the way for understanding the gene expression profiles of mouse testes. Here, we report our study on the mouse testis transcriptome using ultra high-throughput RNA sequencing (RNA-seq) technology, where we obtained a global view of gene expression profiles during normal development of the mouse testis. RNA-seq has been developed for mapping and quantifying transcriptomes [24]. It has several advantages over other transcriptome technologies, such as higher resolution and sensitivity, a large dynamic range of gene expression levels, and an ability to identify novel transcribed regions and splicing isoforms of known genes [25]. In the present study, we investigated mouse testes of three ages: 6 days postnatal, 4 weeks old, and 10 weeks old, representing infant (PN1), juvenile (PN2), and adult (PN3) stages, respectively. Using the Life Technologies SOLiD system, we obtained 211 million reads with a length of 35 bp. We identified 1722 significantly up-regulated genes and 2712 significantly down-regulated genes in PN2 compared to PN1, and 190 significantly up-regulated genes and 185 significantly down-regulated genes in PN3 compared to PN2. We analyzed these dynamic changes in the transcriptome during postnatal development. This study sheds new light on gene expression and the regulation of the mouse testis development.

1 Materials and methods

1.1 Sample collection

Male Balb/c mice aged 6 days (n=35), 4 weeks (n=20), and 10 weeks (n=4) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). Mice were killed by cervical dislocation, and then testes of different developmental stages were dissected and pooled. All samples were immediately frozen in liquid nitrogen. The animal experiments were approved by the Animal Care and Welfare Committee at the Beijing Institute of Genomics, Chinese Academy of Sciences.

1.2 RNA extraction

Total RNA was extracted from mouse testes using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and was stored at −80°C. Messenger RNA was isolated following the protocol of the Qiagen Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany) and underwent two rounds of oligo(dT) selection.

1.3 RNA-seq library construction

A mouse testis RNA-seq fragment library for each of the three developmental stages investigated was constructed based on the procedure of the SOLiD Whole Transcriptome Analysis Kit (Applied Biosystems, Foster City, CA, USA).

Briefly, 1 μg mRNA (no more than 8 μL) was used for enzymatic fragmentation. For each mRNA sample, a reaction mixture was assembled on ice. The mixture was incubated at 37°C for 10 min and then placed on ice. An Ambion flashPAGE fractionator was sequentially loaded with 250 μL lower running buffer, a precast gel cartridge, and 300 μL upper running buffer. Then 10 μL flashPAGE gel loading buffer A40 was added to the fragmented RNA. The mixture was heated at 95°C for 2 min and loaded on the Ambion flashPAGE fractionator. The RNA fraction of 50–150 nt in length was selected by electrophoresis and then purified by the flashPAGE Reaction Clean-up Kit (Applied Biosystems).

A hybridization mixture was prepared on ice: 2 μL adaptor mix A, 3 μL hybridization solution, and 3 μL fragmented RNA sample. The mixture was incubated at 65°C for 10 min, then at 16°C for 5 min. Then the RNA ligation reagents were added to the sample. The ligation reaction mixture was incubated at 16°C for 16 h. The sample was added to a 20 μL reverse transcription (RT) master mix and incubated at 42°C for 30 min to synthesize cDNA. After the RT reaction, 10 μL of the mixture was transferred to a fresh tube and 1 μL RNase H was added. The new mixture was incubated at 37°C for 30 min. For each sample, 1 μL RNase H-treated cDNA was added to one PCR master mix and four replicate PCRs were run. The PCR products were purified using the Qiagen MinElute PCR Purification Kit (Qiagen) and were then run on a native 6% polyacrylamide gel. The cDNA fragments ranging from 140 to 200 bp were excised from the gel for SOLiD sequencing.

1.4 SOLiD-sequencing read mapping

The mouse reference sequence (Release mm9, July 2007) from UCSC, which contains 21896 genes, was employed for SOLiD-sequencing read mapping. An exon-exon junction database, which was composed of 34 nt flanking sequences from both donor and acceptor sequences, was also applied [26]. The read mapping procedure is described by Cui et al. [27]; however, some modifications were made as outlined below. For mapping the full length (35 bp) and 30 bp truncated reads (by removing the last 5 bp from 35 bp reads), and junction mapping, we allowed three mismatches, whereas for 25 bp truncated reads (by removing the last 10 bp from 35 bp reads), only two.

1.5 Gene expression analysis

Gene expression values were measured by read counts that were normalized against total uniquely-mapped reads and RPKM values (reads per kilobase of exon model per million mapped reads; [24]). Hierarchical clustering was performed using the Bioconductor function "heatmap" in R language [28]. Gene ontology (GO) was predicted by WEGO (Web Gene Ontology) in terms of cellular component, molecular function, and biological process [29,30]. Differentially expressed genes were identified by the DEGseq package in R language [31]. To gain an overview of gene networks, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were analyzed by GenMAPP 2.1 [32–36].

2 Results

2.1 Sequence read mapping

We acquired 210.9 million sequencing reads with a length of 35 bp. Reads were recursively mapped to both the mouse reference sequence from USCS and an exon-exon junction database [26]. The characteristics of the data are summarized in Table 1. About 26.4 to 34.7 million reads were uniquely mapped to each library, which represents 42%–50% of all generated reads. The majority of these unique reads (56%–59%) were mapped to exonic sequences of known or predicted genes from the public databases (Table 2). The data quality and reads per library were both comparable to other mammalian transcriptome datasets routinely generated in our laboratory.

2.2 Gene expression profile analysis

Using a threshold of >2 reads from a given library, we identified a total of 18837 genes: 18060 for PN1, 18128 for PN2, and 17700 for PN3 (Figure 1A). We also calculated the numbers of annotated genes with different expression levels. Genes with 100–999 mapped reads represented 38%–44% of the total annotated genes in the three libraries, whereas only one gene, *Tnp2* (Transition nuclear protein 2), with

Table 1 Sequencing data summary of mouse testes libraries

	PN1	PN2	PN3
Raw reads	81704088	76850818	52387161
Filtered rRNA reads	269001	87116	114516
(/raw reads)	(0.33%)	(0.11%)	(0.22%)
Uniquely-mapped reads	34687710	35329252	26441992
(/raw reads)	(42.46%)	(45.97%)	(50.47%)
Multiply-mapped reads	6451404	5947158	4397719
(/raw reads)	(7.90%)	(7.74%)	(8.39%)
Exon-exon junction reads	2058357	2297519	1823624
(/raw reads)	(2.52%)	(2.99%)	(3.48%)
Mapped reads	43197471	43573929	32663335
(/raw reads)	(52.87%)	(56.70%)	(62.35%)

Table 2 Distribution of reads uniquely-mapped onto genes

	PN1	PN2	PN3
Uniquely-mapped reads	34687710	35329252	26441992
Unique reads mapped to exons	20503000	20237707	14798772
(/uniquely-mapped reads)	(59.11%)	(57.28%)	(55.97%)
Unique reads mapped to introns	6882366	4467063	3157790
(/uniquely-mapped reads)	(19.84%)	(12.64%)	(11.94%)
Unique reads mapped to exon-intron junctions	1202934	1149293	819197
(/uniquely-mapped reads)	(3.47%)	(3.25%)	(3.10%)

read counts of more than 100000, was detected in both PN2 and PN3 (Figure S1A).

Similarly, genes with RPKM values ranging from 1 to 10 represented 39%–45% of the total annotated genes in the libraries, whereas only six genes in PN2 and eleven genes in PN3 with RPKM values of more than 1000 were detected (Figure S1B, Table S1). Of these, five genes were highly expressed in both libraries, including *Tnp2*, *Prm1* (Protamine 1), *Prm2*, *Smcp* (sperm mitochondria-associated cysteine-rich protein), and *Ropn1l* (Ropporin 1-like).

We also used the bioconductor function "heatmap" to analyze hierarchical clustering (Figure 1B), and the gene expression pattern in PN1 is distinct from that in PN2 and PN3. These results indicate that spermatogenesis has commenced in PN2.

To facilitate the biological interpretation of the transcriptional survey, we used GO assignments for the three libraries in terms of cellular component, molecular function, and biological process (Figure S2). For the cellular component category, genes involved in "cell" and "cell part" are dominant. With respect to molecular function, "binding" appears to be the most abundant GO category, followed by "catalytic activity". For biological processes, "cellular process" and "metabolic process" are all highly represented. In addition, the number of genes in each GO classification is only slightly different among the three libraries. This result suggests that most of the annotated genes are expressed throughout the postnatal developmental stages of the testis.

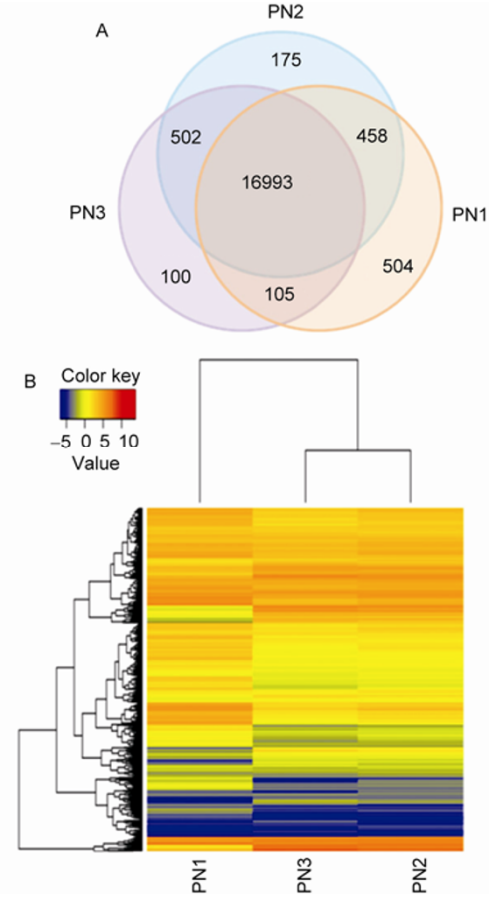

Figure 1 Annotated genes in the three libraries. A, Venn diagram of annotated gene numbers. B, A heat map of all annotated genes. Columns, libraries; rows, genes. Color key indicates gene expression value; blue, lowest; red, highest. PN1, testes from 6-day-old mice; PN2, testes from 4-week-old mice; PN3, testes from 10-week-old mice.

2.3 Significant gene expression changes

We employed the DEGseq package in R language for identifying and handling differentially expressed genes (DEGs) among the libraries. To avoid the influence of read number variation in each library, we used RPKM value to define DEGs in neighboring time points of the three samples. MARS (MA-plot-based method with random sampling model) was selected in the DEGseq package, and DEGs with fold changes >2 (P-value<0.01) were chosen to analyze the significant gene expression changes in the three libraries (Figure S3). We obtained 1722 up-regulated genes and 2712 down-regulated genes based on a comparison between PN1 and PN2, and 190 up-regulated genes and 185 down-regulated genes in a comparison between PN2 and PN3. This result was consistent with our hierarchical clustering analysis.

We also performed GO analysis for DEGs. The genes up-regulated in PN2 compared to PN1 were enriched (>10%) in cellular components related to "cell", "cell part", "organelle", and "organelle part" (Figure 2A). Molecular function analysis showed that "catalytic activity" and "binding" are dominant. Regarding biological processes, genes involved in "metabolic process", "cellular process", "multicellular organismal process", "developmental process", "localization", "pigmentation", and "biological regulation" are highly expressed (>10%). In addition to these GO categories mentioned above, genes down-regulated in PN2 compared to PN1 are also enriched (>10%) in "extracellular region", "extracellular region part", "macromolecular complex", "cellular component organization", "establishment of localization", and "response to stimulus".

Between PN2 and PN3, the GO terms of "cell", "cell part", "organelle", "organelle part", "catalytic activity", "binding", "metabolic process", "cellular process", "multicellular organismal process", "pigmentation", "localization", and "biological regulation" are highly represented and are associated with both up- and down-regulated genes, whereas "extracellular region", "extracellular region part", "macromolecular complex", and "establishment of localization" are also categories related to down-regulated genes (Figure 2B).

2.4 Differentially expressed biomarkers of testis-specific cells

To clarify the growth of different cell types, we analyzed gene expression changes of cell biomarkers during postnatal testis development. There are multiple spermatogonial stem

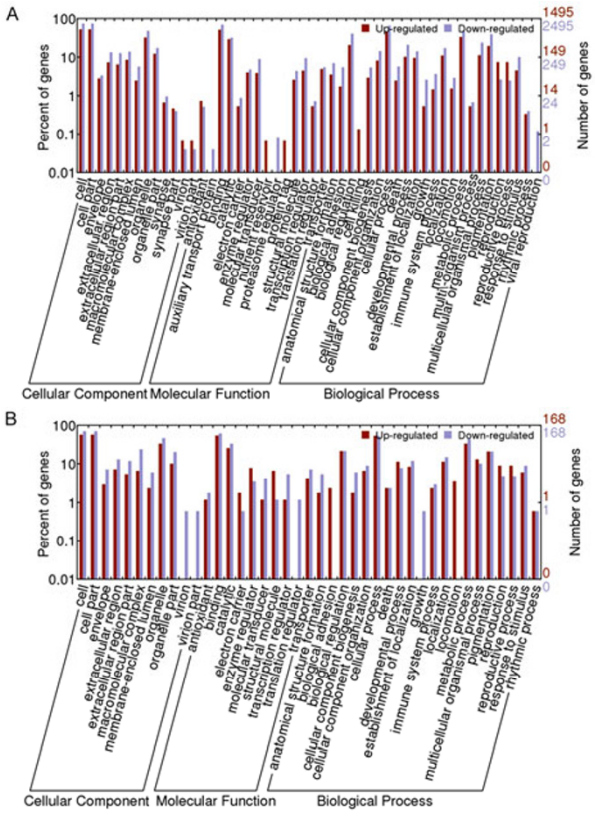

Figure 2 Histograms of gene ontology classification of DEGs between PN1 and PN2 (A) and between PN2 and PN3 (B).

cell biomarkers, including ITGA5 (integrin alpha 5), ITGA6 (integrin alpha-6), ITGB1 (integrin beta-1), CD9, THY1 (thymus cell antigen 1), CD24, EPCAM (epithelial cell adhesion molecule), GPR125 (G protein-coupled receptor 125), GFRA1 (GDNF family receptor alpha 1), KIT (kit oncogene), PTPRC (protein tyrosine phosphatase, receptor type, C), CD34, LY6A (lymphocyte antigen 6 complex, locus A), and POU5F1 (POU domain, class 5, transcription factor 1) [37–39]. Except for *Gpr125*, we found that the genes in this list were all present in the three libraries. Furthermore, except for *Pou5f1* and *Ly6a*, the biomarkers were expressed most highly in PN1 (Table 3).

We next evaluated several biomarkers of Sertoli cells, including AMH (anti-Müllerian hormone), SOX9 (Sry-box containing gene 9), SRY (sex determining region of Y chromosome), WT1 (Wilms' tumor 1 homolog), VIM (vimentin), KRT18 (keratin 18), FSHR (follicle stimulating hormone receptor), TRF (transferrin), SHBG (sex hormone binding globulin), and 4930486L24Rik (testin) [40–42]. Except for *Trf*, the other biomarkers were expressed more highly in PN1 than in PN2 and PN3. *Amh*, *Sox9*, *Wt1*, *Vim*, and *Shbg* were significantly down-regulated in PN2 (Table 3).

We also analyzed biomarkers specific to Leydig cells, including steroidogenic enzymes such as STAR (steroidogenic acute regulatory protein), CYP11A1 (cytochrome P450, family 11, subfamily a, polypeptide 1), HSD3B6 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6), CYP17A1 (cytochrome P450, family 17, subfamily a, polypeptide 1), and HSD17B1 (hydroxysteroid (17-beta) dehydrogenase 1), as well as LHCGR (luteinizing hormone/choriogonadotropin receptor), THBS2 (thrombospondin 2), and INSL3 [42–45]. We observed that genes, including *Insl3*, *Cyp17a1*, *Hsd3b6*, and *Cyp11a1*, were up-regulated in PN2, and down-regulated in PN3, whereas *Hsd17b3* was down-regulated significantly in PN2, then up-regulated significantly in PN3. The remaining biomarkers, including *Star*, *Thbs2*, and *Lhcgr*, were expressed most highly in PN1 (Table 3).

Table 3 Differentially expressed biomarkers in testis-specific cells[a)]

Accession number	RPKM			Gene
	PN1	PN2	PN3	
Spermatogonial stem cell				
NM_133911	0	0	0	*Gpr125*
NM_010738	1.15	2.77	0.88	*Ly6a*
NM_013633	2.01	1.89	5.03	*Pou5f1*
NM_010578	98.70	13.66	12.66	*Itgb1*
NM_133654	49.38	8.47	5.12	*Cd34*
NM_001111059	47.60	8.15	4.86	*Cd34*
NM_008397	37.46	3.63	2.19	*Itga6*
NM_008532	22.88	7.29	3.78	*Epcam*
NM_007657	17.34	2.75	1.21	*Cd9*
NM_009382	10.32	0.55	0.09	*Thy1*
NM_010279	7.90	1.02	0.70	*Gfra1*
NM_021099	11.10	5.03	5.08	*Kit*
NM_009846	6.79	0.87	1.08	*Cd24*
NM_010577	6.31	0.78	0.67	*Itga5*
NM_011210	0.31	0.24	0.21	*Ptprc*
Sertoli cell				
NM_133977	1.64	2.56	1.90	*Trf*
NM_011701	183.51	29.02	14.02	*Vim*
NM_011367	157.67	21.96	6.87	*Shbg*
NM_007445	124.89	5.76	2.45	*Amh*
NM_144783	113.71	23.51	18.07	*Wt1*
NM_011448	33.53	5.20	2.61	*Sox9*
NM_178098	12.18	4.02	1.43	*4930486L24Rik*
NM_013523	7.37	1.02	0.66	*Fshr*
NM_010664	5.93	0.31	0.27	*Krt18*
Leydig cell				
NM_013564	139.31	1656.41	719.59	*Insl3*
NM_007809	87.80	101.60	31.99	*Cyp17a1*
NM_013821	0.16	10.90	5.39	*Hsd3b6*
NM_019779	50.51	59.07	41.55	*Cyp11a1*
NM_008291	99.29	10.76	27.38	*Hsd17b3*
NM_011485	30.27	10.50	5.84	*Star*
NM_011581	7.66	0.17	0.16	*Thbs2*
NM_013582	5.16	2.51	0.99	*Lhcgr*

a) The underlined values indicate significant differential expression of a gene between two libraries.

2.5 Differentially expressed genes in cellular signaling pathways

After analyzing the expression levels and GO classification of DEGs, we examined their effect on cellular signaling pathways. As we mentioned above, the gene expression profiles of PN2 and PN3 are more similar to each other than to that of PN1. We therefore focused our attention on DEGs between PN1 and PN2 in the MAPK, Hedgehog, and Wnt signaling pathways. First, we found that genes such as *Pdgfrb*, *Grb2*, *Mras*, *Map2k1* (*Mek1*), and *Mapk1* (*Erk2*) in the ERK (extracellular signal-regulated kinase) cascade, as well as *Atf4*, were significantly down-regulated in PN2, whereas the expression of *Mapk6* increases (Figure 3). In the JNK (c-Jun N-terminal kinase) and p38 MAPK pathways, genes such as *Rac1*, *Cdc42*, *Casp6*, *Map3k1*, *Mapk9*, *Mapk12*, *Mapk13*, *Mapk14*, *Jun*, and *Trp53* were also down-regulated. Second, when comparing PN1 and PN2 with respect to the Hedgehog signaling pathway, we found that *Dhh*, *Smo*, *Ptch1*, *Ptch2*, *Gas1*, and *Gli1* were significantly down-regulated in PN2; however, *Sufu* and *Stk36* were significantly up-regulated in PN2 (Figure 4). Third, when comparing the Wnt signaling pathway with respect to PN1 and PN2, we noticed that *Wnt5a*, *Wnt6*, *Fzd2*, *Dvl3*, *Apc*, *Ctnnb1*, *Prkci*, and *Prkcm* were significantly down-regulated in PN2, while *Dvl1*, *Prkcq*, and *Lef1* were significantly up-regulated in PN2 (Figure 5).

2.6 Differentially expressed transcription factors

We integrated the 1675 non-redundant mouse transcription factors in the TFdb (Riken Transcription Factor Database) with the 1727 mouse transcription factors collected by Ravasi et al. [46,47], and identified 125 significantly up-regulated and 420 significantly down-regulated transcription factors in PN2 compared to PN1, and 10 significantly up-regulated and 17 significantly down-regulated transcription factors in PN3 compared to PN2.

We analyzed a few selected transcription factors that play crucial roles in mouse testis development. We found

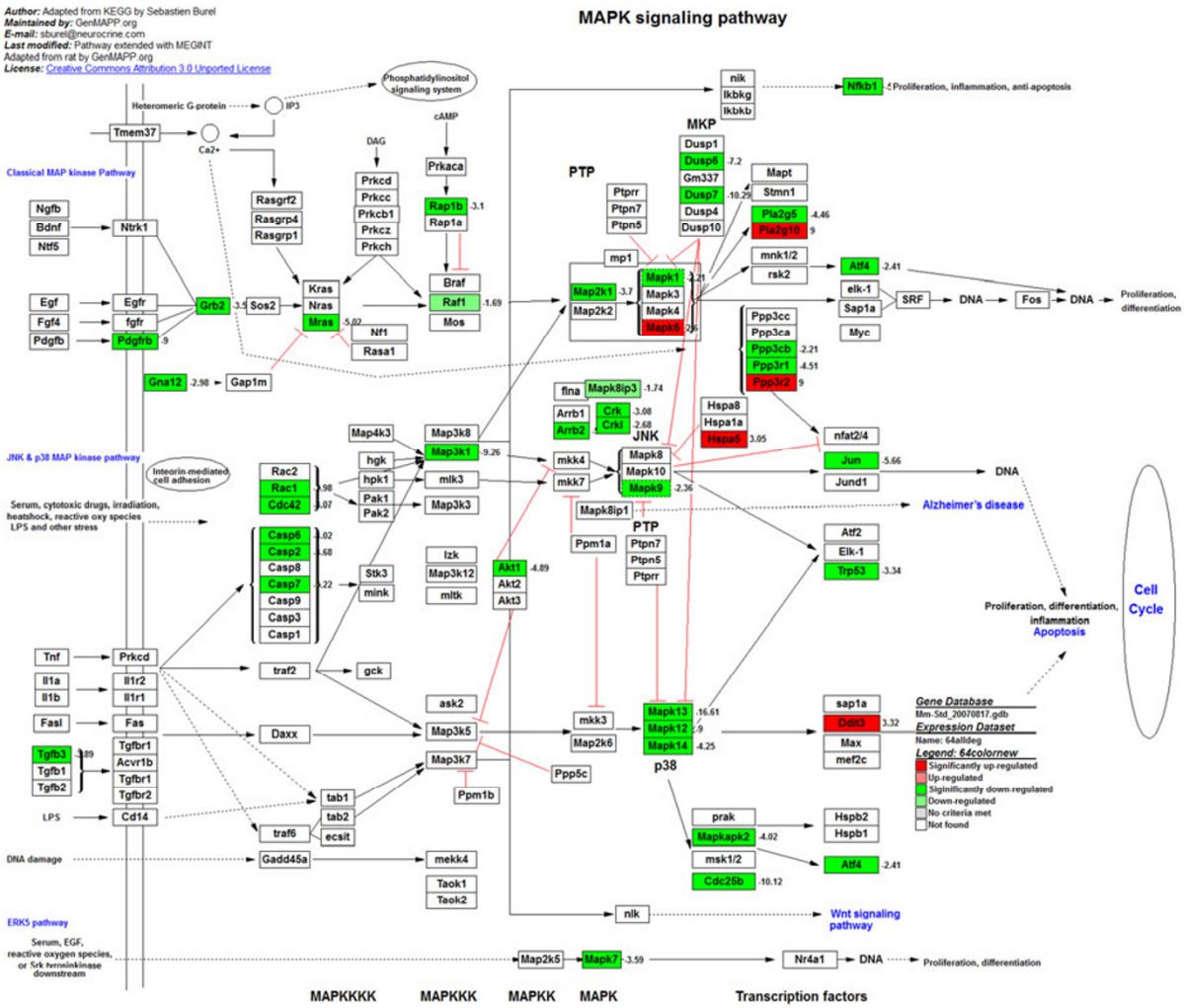

Figure 3 DEGs in the MAPK signaling pathway. DEGs are colored according to fold change. Red, up-regulated, fold change >2; pink, up-regulated, fold change ⩽2; green, down-regulated, fold change >2; aqua, down-regulated, fold change ⩽2.

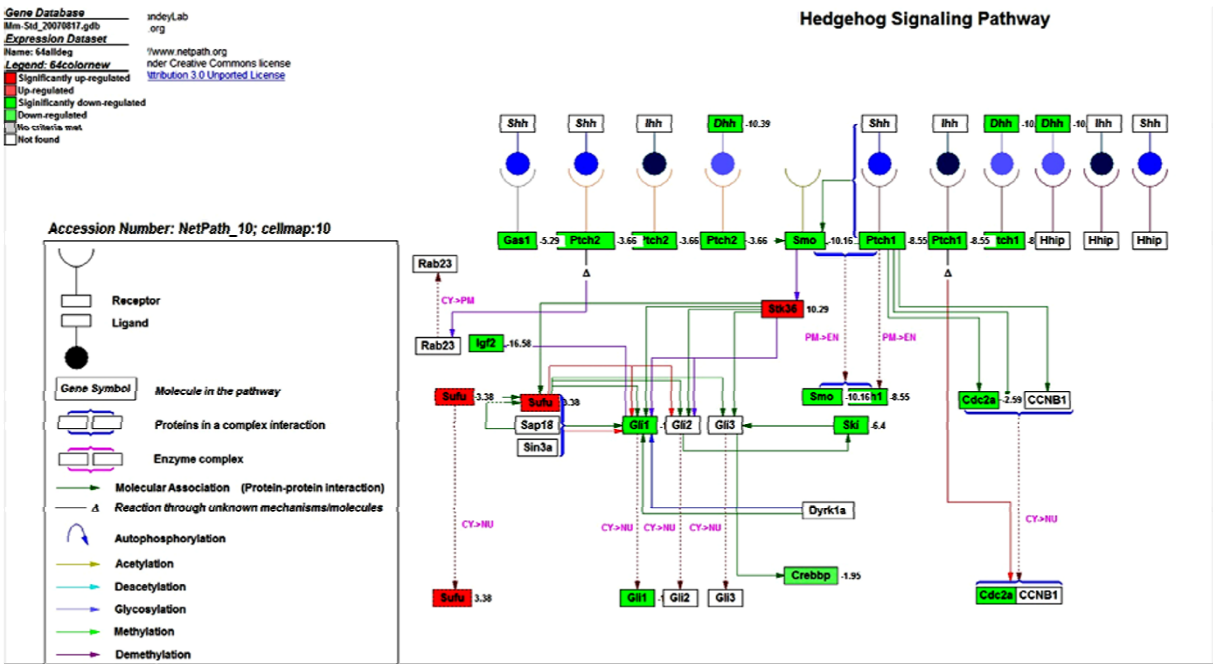

Figure 4 DEGs in the Hedgehog signaling pathway. DEGs are colored according to fold change. Red, up-regulated, fold change >2; pink, up-regulated, fold change ⩽2; green, down-regulated, fold change >2; aqua, down-regulated, fold change ⩽2.

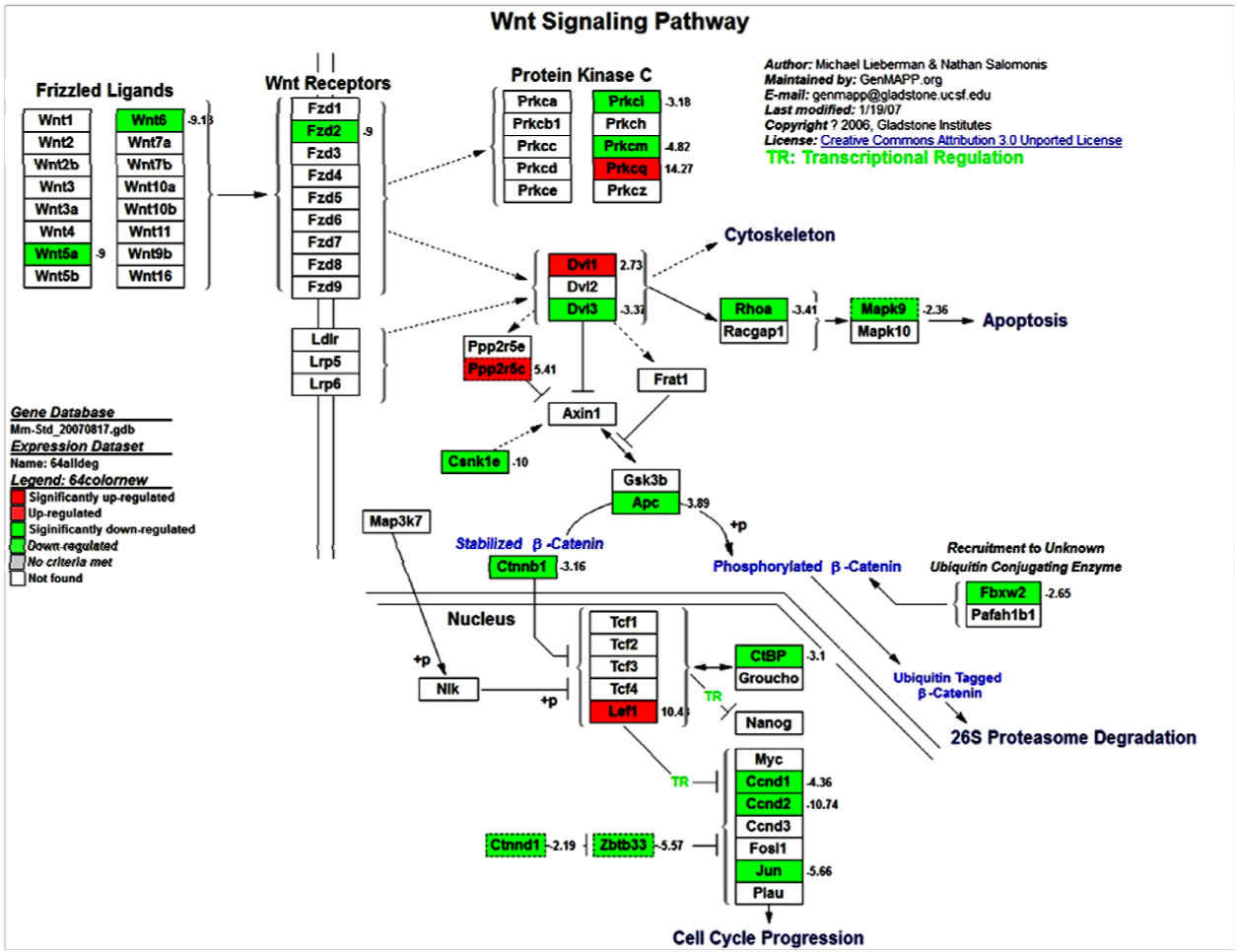

Figure 5 DEGs in the Wnt signaling pathway. DEGs are colored according to fold change. Red, up-regulated, fold change >2; pink, up-regulated, fold change ⩽2; green, down-regulated, fold change >2; aqua, down-regulated, fold change ⩽2.

that genes related to the self-renewal of spermatogonia, such as *Bcl6b*, *Gfra1*, *Gdnf*, and *Gja1*, and genes related to the differentiation of spermatogonia, including *Kitl*, *Sohlh1*, *Sox3*, and *Stra8*, were down-regulated during postnatal testis development [48] (Figure S4).

Sry (sex-determining region Y) together with *Nr5a1* (nuclear receptor subfamily 5, group A, member 1) and *Wt1* regulate activation of *Sox9* expression [49,50]. The expression of *Nr5a1*, *Wt1*, and *Sox9* were all decreased during mouse testis development and their expression levels were much higher in PN1 than in PN2 and PN3 (Figure S5). We also checked the downstream genes of *Sox9*, including *Amh*, *Cbln4*, and *Ptgds*. As mentioned above, *Amh* was expressed similarly to *Sox9*. *Cbln4* was expressed in PN1 and its transcription drops sharply in PN2 and PN3. In contrast, *Ptgds* (prostaglandin D2 synthase) was expressed at much higher levels in PN3. *Sox8*, which usually cooperates with *Sox9* to activate genes such as *Amh* and *Ptgds*, was transcribed less and less as development of the testis proceeded.

Crem was significantly up-regulated in PN2, but only slightly up-regulated in PN3. Correspondingly, the expression levels of *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, *Tesk1* (testis-specific kinase 1) and *Ldhc* (lactate dehydrogenase C) increased greatly as testis development proceeded (Figure S6). *Crem* has a coactivator in the testis called *Fhl5* (four and a half LIM domains 5), which is also a testis-specific transcription factor [51]. *Fhl5* was continuously up-regulated during the mouse testis development.

3 Discussion

In this study, we constructed three SOLiD fragment RNA-seq libraries of the developing testes from 6 dpn, 4-week, and 10-week-old mice. These time points represent mouse testis development, corresponding to the infant, juvenile, and adult stages, respectively. We assumed that transcriptome profiles of these time points would be distinct from each other, providing new data for understanding the molecular mechanisms of postnatal mouse testis development.

3.1 Gene expression abundance

By using the next-generation platform, we found that most of the highly expressed genes with RPKM values of more than 1000 are differentiation-related and involved in spermatogenesis, such as *Tnp2*, *Prm1*, *Prm2*, *Smcp*, and *Ropn1l*; all these genes are up-regulated in both PN2 and PN3 (Table S1). The transition nuclear proteins (TNP) constitute 90% of the chromatin basic proteins, replacing the histones temporarily, and are then switched by protamines (PRM) during spermiogenesis [52]. *Smcp* and *Ropn1l* affect sperm motility [53,54], while genes such as *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, and *Smcp* are transcribed in round spermatids, but when spermatids undergo nuclear condensation and elongation, their mRNAs are stored in a translationally repressed state in early elongating spermatids [55–58]. Therefore, these genes can be detected as early as PN2, before elongating spermatids appear. The testicular hormone *Insl3* (insulin-like 3), which is highly expressed during the juvenile period, plays a crucial role in mouse gubernacular development and testis descent [59]. Among the other five genes with abundant expression levels in PN3, *Gstm5* (glutathione S-transferase, mu 5) [60], *Dbil5* (diazepam binding inhibitor-like 5) [61], and *Fabp9* (fatty acid binding protein 9) [62] are required for spermatogenesis, *Dkkl1* (dickkopf-like 1) regulates postpubertal spermatocyte apoptosis and testosterone production [63], and *Gsg1* (germ cell-specific gene 1) is related to the morphological changes of round spermatids in spermiogenesis [64].

3.2 Differential expression of biomarkers in testis-specific cells

The parenchyma of the mouse testis consists of seminiferous tubules and interstitial tissue. Seminiferous tubules are formed from limiting membranes and seminiferous epithelium, where Sertoli cells and germ cells locate. A variety of germ cells exist in a mature testis, including spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and sperm cells. Interstitial tissue among seminiferous tubules contains many types of cells, such as fibroblasts, macrophages, and Leydig cells. Notably, spermatogenic cells, Sertoli cells, and Leydig cells are testis specific.

Bellvé et al. [65] observed that only type A spermatogonia and Sertoli cells exist in the seminiferous tubules in 6-day-old mice. We suggest that the high proportion of spermatogonial stem cells in 6-day-old mice leads to high expression of biomarkers, such as *Itga6*, *Itgb1*, *Cd34*, *Epcam*, *Cd9*, *Thy1*, and *Gfra1*. When spermatogenesis starts and other germ cells become abundant, the percentage of spermatogonial stem cells decreases. Tagelenbosch and de Rooij [66] found that there are approximately 35000 spermatogonial stem cells in one testis of an adult male mouse, constituting about 0.03% of all germ cells. Thus, these biomarkers become down-regulated in PN2 and PN3.

The number of Sertoli cells accounts for 80% of all cells in the seminiferous epithelium in 6-day-old mice, then declines to only 3% in adult mice [65]. Hence, we assume that the down-regulated expression of most Sertoli cell biomarkers during puberty and thereafter might be related to the proliferation of spermatogenic cells. AMH causes the Müllerian duct to degenerate in XY embryos and promotes testicle differentiation. It has been reported that *Amh* and *Krt18* are expressed in immature testes, but are absent in adult testes [13,67,68]. Unexpectedly, we detected both of them in adult male mice. Follicle stimulating hormone (FSH) plays multiple roles in testicle development, such as increasing the number of Sertoli cells and regulating spermatogenesis. Our data show that FSH receptor is expressed

at a higher level in immature Sertoli cells, which is consistent with the findings of Dahia and Rao [69].

As we have discussed above, *Insl3* is required for testicle descent during puberty, which may account for its very high level of expression in PN2. *Thbs2* is a biomarker of fetal Leydig cells and is absent in adult Leydig cells [45]. However, we found that *Thbs2* is also present in PN2 and PN3. The expression pattern of *Lhcgr* is similar to that of *Fshr*; both of these hormone receptors are expressed most highly in PN1, but expression is not significantly different across the developmental stages. Among the steroidogenic enzymes, *Cyp17a1*, *Hsd3b6*, and *Cyp11a1* are up-regulated in PN2, and down-regulated in PN3, whereas *Hsd17b3* is down-regulated significantly in PN2, then up-regulated significantly in PN3. The gene expression changes of these enzymes may be controlled by a feedback regulation of testosterone and other male hormones.

Most biomarkers are down-regulated in PN2 and PN3. We suppose that the proportions of different cell types in the testis change as spermatogenesis occurs, and the proliferation of germ cells leads to a reduction in the proportion of somatic cells and spermatogonial stem cells, and to down-regulation of genes in these cells. We also noticed that genes like FSH and LH receptors have no significant differential expression across the developmental stages. We assume that testosterone levels increase in PN2 and inhibit the secretion of LH in the pituitary, which suppresses the transcription of *Lhcgr*. The expression pattern of *Fshr* may be due to the cycle of the seminiferous epithelium, which changes its expression levels periodically [70].

3.3 Differential expression of genes in cellular signaling pathways

The MAPK (Mitogen-activated protein kinase) pathway is involved in many male reproductive processes, including spermatogenesis, sperm maturation and activation, adhesion of Sertoli cells and spermatogenic cells, and dynamics of the blood-testis barrier [71]. Transcripts of rat *Pdgfrb* are detected at embryonic day 18 (E18), reach high levels on 5 dpn, and then decline to lower levels [72]. *Pdgfr* can activate gonocytes to migrate from the central region of the seminiferous tubule to the basal lamina of the seminiferous epithelium and initiate their differentiation to spermatogonial stem cells [73], which suggests that gene transcription of the MAPK pathway in the spermatogenic cells should be more active in PN1. Moreover, Sertoli cells in PN2 are nearly mature, which makes the MAPK pathway activated by FSH repressive. Therefore, the activity of the MAPK pathway is in decline.

The Hedgehog (Hh) signaling pathway is involved in cell differentiation, cell survival, tissue development, and stem cell proliferation in adult tissues. There are three Hh genes in mammals: Sonic hedgehog (*Shh*), Desert hedgehog (*Dhh*), and Indian hedgehog (*Ihh*) [74]. Patched (PTCH) and Smoothened (SMO) are the main receptors of Hh proteins. A classical Hh signaling pathway consists of Hh, PTCH-SMO complex, and downstream transcription factor, GLI. There are three genes in the *Gli* family, *Gli1*, *Gli2*, and *Gli3*. *Gli1* and *Gli2* activate downstream target genes, however, *Gli3* represses transcription.

Dhh-null male mice are infertile, owing to a complete absence of mature sperm [75]. *Dhh*, *Shh*, and *Ihh* are expressed at E11.5, and *Dhh* expression peaks at 6 dpn, while *Shh* and *Ihh* are expressed at relatively low levels at all stages of testicular development [74]. Our results are consistent with these findings, indicating that *Dhh* plays a more important role than *Shh* and *Ihh* in the Hh signaling pathway of mouse testicular development (Figure S7).

The three *Glis* are observed in spermatogonia and Sertoli cells in the first wave of spermatogenesis, and can be detected in spermatogonia and spermatocytes in adult mice, while *Ptch2* and *Smo* are also apparent in spermatogonia and spermatocytes; in contrast, *Sufu* is predominant in round spermatids [74]. Our results show that the expression levels of *Gli1*, *Ptch1*, *Ptch2*, and *Smo* decrease in PN2, whereas *Sufu* is transcriptionally activated. This demonstrates that the Hh signaling pathway in the mouse testes might be mainly involved in the early steps of spermatogenesis, and has less effect on round spermatids.

The Wnt signaling pathway in mouse development is complicated. It regulates the self-renewal and differentiation of spermatogonial stem cells; however, the balance of self-renewal and differentiation of spermatogonial stem cells is influenced not only by intracellular signals, but also by the microenvironment, termed the spermatogonial stem cell niche, which is formed by surrounding Sertoli cells [76]. By regulating the differentiation and maturation of Sertoli cells, the Wnt signaling pathway has an ability to support spermatogenesis [77]. We found that the transcriptional activity of the Wnt signaling pathway decreases in PN2. This phenomenon might be caused by the maturation of the testis; that is, Sertoli cells are maturing and the proliferation and differentiation of spermatogonial stem cells are becoming balanced.

Dvl1 expression first appears in pachytene spermatocytes, then increases in round and elongating spermatids, but becomes undetectable in mature sperm cells; however, its homologues, *Dvl2* and *Dvl3*, show relatively consistent expression [78]. *Dvl1* is detected in PN1, is significantly up-regulated in PN2, and is then down-regulated in PN3; while the expression levels of *Dvl2* and *Dvl3* decline slowly during testis development (Figure S8). We speculate that the transcription of *Dvl1* is active in PN2 and plays a vital role in spermiogenesis. Its expression is down-regulated in PN3, but is still higher than in PN1, which indicates that it continues to regulate spermatogenesis.

3.4 Differential expression of transcription factors

Transcription factors are important players in numerous

biological processes, including cell proliferation, tissue differentiation, apoptosis, and development, and regulate the expression of downstream genes by activating or repressing them.

By comparing the differential expression of several transcription factors and their related genes, we observed that the expression of genes associated with the self-renewal and differentiation of spermatogonia are down-regulated during postnatal testis development. *Sry* and its related genes are also similarly down-regulated. *Sry* is the sex-determining gene on the mammalian Y chromosome and can activate a series of genes that trigger the development of the male genital gland [49]. The expression of *Sry* is detectable in mice from 10.5 to 12.5 days post coitum until 28 dpn [79]. We found *Sry* is expressed in all three developmental stages, although its expression levels are very low (Figure S5). Genes related to *Sry*, such as *Nr5a1*, *Wt1*, *Sox9*, *Sox8*, *Amh*, and *Cbln4*, are more active in PN1, which indicates that their roles may be more important in the early developmental stage of the testis than in later stages.

CREM (cAMP response element modulator) is a key transcription factor in early round spermatids and activates many genes of spermiogenesis, including *Tnp1*, *Tnp2*, *Prm1*, *Prm2*, *Tesk1*, and *Ldhc* [80]. Mice lacking the *Crem* gene are sterile because of the cessation of sperm development [81]. *Crem* and its related genes are up-regulated in PN2 and PN3 compared to PN1, which indicates their importance in the later stage of testis development.

4 Conclusion

In this study, we identified the expression of 18837 genes in the mouse testis, and found the most highly expressed genes are involved in spermatogenesis. The gene expression pattern in PN1 is distinct from that in PN2 and PN3, which indicates that spermatogenesis has commenced in PN2. We also detected several genes in PN3, such as *Sry*, *Krt18*, and *Amh*, that were previously reported to be expressed only in immature testes. However, the read numbers of *Sry*, *Krt18*, and *Amh* in PN3 are low (only 9, 10, and 108, respectively) and have not been verified by the methods previously used by other authors. This suggests that RNA-seq has a higher sensitivity than other transcriptome technologies. We analyzed a large number of genes related to spermatogenesis and somatic development of the mouse testis, which play important roles at different developmental stages. For instance, genes expressed in spermatogonia, Sertoli cells, and Leydig cells are down-regulated, but genes associated with spermiogenesis are up-regulated as development preceeds. Down-regulated genes might be negatively affected by the proliferation and growth of spermatogenic cells, and also by the cycle of the seminiferous epithelium and the feedback regulation of hormones. We also found that the MAPK, Hedgehog, and Wnt signaling pathways are significantly involved at different developmental stages. These findings should help us to further understand how gene expression is regulated during testis development. Our study also shows that RNA-seq has great power for investigating the transcriptome during mouse testis development.

We would like to acknowledge Drs. Zhang Bing, Zhao WenMing, Song ShuHui, and Wu JiaYan for their advice on experiments and data analysis. We thank Dr. Luo YingFeng for his technical assistance, and Du ZhengLin and Yu CaiXia for their assistance with bioinformatics. This work was supported by the National Basic Research Program of China (2011CB944100, 2011CB944101), the National Natural Science Foundation of China (90919024), the Special Foundation Work Program of Ministry of Science and Technology (2009FY120100), and the National High Technology Research and Development Program of Ministry of Science and Technology of China (2012AA020409).

1 McCarrey J R, O'Brien D A, Skinner M K. Construction and preliminary characterization of a series of mouse and rat testis cDNA libraries. J Androl, 1999, 20: 635–639
2 Su A I, Cooke M P, Ching K A, et al. Large-scale analysis of the human and mouse transcriptomes. Proc Natl Acad Sci USA, 2002, 99: 4465–4470
3 Tanaka K, Tamura H, Tanaka H, et al. Spermatogonia-dependent expression of testicular genes in mice. Dev Biol, 2002, 246: 466–479
4 Rockett J C, Christopher Luft J, Brian Garges J, et al. Development of a 950-gene DNA array for examining gene expression patterns in mouse testis. Genome Biol, 2001, 2: RESEARCH0014
5 Sha J, Zhou Z, Li J, et al. Identification of testis development and spermatogenesis-related genes in human and mouse testes using cDNA arrays. Mol Hum Reprod, 2002, 8: 511–517
6 Pang A L, Taylor H C, Johnson W, et al. Identification of differentially expressed genes in mouse spermatogenesis. J Androl, 2003, 24: 899–911
7 Pang A L, Johnson W, Ravindranath N, et al. Expression profiling of purified male germ cells: stage-specific expression patterns related to meiosis and postmeiotic development. Physiol Genomics, 2006, 24: 75–85
8 Yu Z, Guo R, Ge Y, et al. Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. Biol Reprod, 2003, 69: 37–47
9 Guo R, Yu Z, Guan J, et al. Stage-specific and tissue-specific expression characteristics of differentially expressed genes during mouse spermatogenesis. Mol Reprod Dev, 2004, 67: 264–272
10 Clemente E J, Furlong R A, Loveland K L, et al. Gene expression study in the juvenile mouse testis: identification of stage-specific molecular pathways during spermatogenesis. Mamm Genome, 2006, 17: 956–975
11 Schultz N, Hamra F K, Garbers D L. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci USA, 2003, 100: 12201–12206
12 Shima J E, McLean D J, McCarrey J R, et al. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod, 2004, 71: 319–330
13 O'Shaughnessy P J, Fleming L, Baker P J, et al. Identification of developmentally regulated genes in the somatic cells of the mouse testis using serial analysis of gene expression. Biol Reprod, 2003, 69: 797–808
14 Wu S M, Baxendale V, Chen Y, et al. Analysis of mouse germ-cell transcriptome at different stages of spermatogenesis by SAGE: Biological significance. Genomics, 2004, 84: 971–981
15 Yao J, Chiba T, Sakai J, et al. Mouse testis transcriptome revealed using serial analysis of gene expression. Mamm Genome, 2004, 15: 433–451
16 Almstrup K. Analysis of cell-type-specific gene expression during

mouse spermatogenesis. Biol Reprod, 2004, 70: 1751–1761
17 Ellis P J, Furlong R A, Wilson A, et al. Modulation of the mouse testis transcriptome during postnatal development and in selected models of male infertility. Mol Hum Reprod, 2004, 10: 271–281
18 Divina P, Vlcek C, Strnad P, et al. Global transcriptome analysis of the C57BL/6J mouse testis by SAGE: evidence for nonrandom gene order. BMC Genomics, 2005, 6: 29
19 Chan W Y, Lee T L, Wu S M, et al. Transcriptome analyses of male germ cells with serial analysis of gene expression (SAGE). Mol Cell Endocrinol, 2006, 250: 8–19
20 Lee T L, Cheung H H, Claus J, et al. GermSAGE: a comprehensive SAGE database for transcript discovery on male germ cell development. Nucleic Acids Res, 2009, 37: D891–897
21 Ike A, Tokuhiro K, Hirose M, et al. Comprehensive analysis of gene expression in testes producing haploid germ cells using DNA microarray analysis. Int J Androl, 2007, 30: 462–475
22 Xiao P, Tang A, Yu Z, et al. Gene expression profile of 2058 spermatogenesis-related genes in mice. Biol Pharm Bull, 2008, 31: 201–206
23 Waldman Ben-Asher H, Shahar I, Yitzchak A, et al. Expression and chromosomal organization of mouse meiotic genes. Mol Reprod Dev, 2010, 77: 241–248
24 Mortazavi A, Williams B A, McCue K, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods, 2008, 5: 621–628
25 Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet, 2009, 10: 57–63
26 Cui P, Lin Q, Ding F, et al. A comparison between ribo-minus RNA-sequencing and polyA-selected RNA-sequencing. Genomics, 2010, 96: 259–265
27 Cui P, Lin Q, Xin C, et al. Hydroxyurea-induced global transcriptional suppression in mouse ES cells. Carcinogenesis, 2010, 31: 1661–1668
28 Gentleman R C, Carey V J, Bates D M, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol, 2004, 5: R80
29 Ashburner M, Ball C A, Blake J A, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet, 2000, 25: 25–29
30 Ye J, Fang L, Zheng H, et al. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res, 2006, 34: W293–297
31 Wang L, Feng Z, Wang X, et al. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics, 2010, 26: 136–138
32 Ogata H, Goto S, Sato K, et al. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res, 1999, 27: 29–34
33 Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res, 2000, 28: 27–30
34 Kanehisa M, Goto S, Hattori M, et al. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res, 2006, 34: D354–357
35 Kanehisa M, Goto S, Furumichi M, et al. KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic Acids Res, 2010, 38: D355–360
36 Dahlquist K D, Salomonis N, Vranizan K, et al. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet, 2002, 31: 19–20
37 Yeh J R, Nagano M C. Spermatogonial stem cell biomarkers: improved outcomes of spermatogonial transplantation in male fertility restoration? Expert Rev Mol Diagn, 2009, 9: 109–114
38 Kubota H, Avarbock M R, Brinster R L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci USA, 2003, 100: 6487–6492
39 Phillips B T, Gassei K, Orwig K E. Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci, 2010, 365: 1663–1678
40 Suter L, Koch E, Bechter R, et al. Three-parameter flow cytometric analysis of rat spermatogenesis. Cytometry, 1997, 27: 161–168
41 Franke F E, Pauls K, Rey R, et al. Differentiation markers of Sertoli cells and germ cells in fetal and early postnatal human testis. Anat Embryol (Berl), 2004, 209: 169–177
42 O'Shaughnessy P J, Hu L, Baker P J. Effect of germ cell depletion on levels of specific mRNA transcripts in mouse Sertoli cells and Leydig cells. Reproduction, 2008, 135: 839–850
43 Haider S G. Cell biology of Leydig cells in the testis. Int Rev Cytol, 2004, 233: 181–241
44 O'Shaughnessy P J, Willerton L, Baker P J. Changes in Leydig cell gene expression during development in the mouse. Biol Reprod, 2002, 66: 966–975
45 Zhang F P, Pakarainen T, Zhu F, et al. Molecular characterization of postnatal development of testicular steroidogenesis in luteinizing hormone receptor knockout mice. Endocrinology, 2004, 145: 1453–1463
46 Kanamori M, Konno H, Osato N, et al. A genome-wide and nonredundant mouse transcription factor database. Biochem Biophys Res Commun, 2004, 322: 787–793
47 Ravasi T, Suzuki H, Cannistraci C V, et al. An atlas of combinatorial transcriptional regulation in mouse and man. Cell, 2010, 140: 744–752
48 Zhou Q, Griswold M D. Regulation of spermatogonia. StemBook. Cambridge (MA): Harvard Stem Cell Institute, 2008
49 Sekido R. SRY: A transcriptional activator of mammalian testis determination. Int J Biochem Cell Biol, 2010, 42: 417–420
50 Barrionuevo F, Scherer G. SOX E genes: SOX9 and SOX8 in mammalian testis development. Int J Biochem Cell Biol, 2010, 42: 433–436
51 Tanaka H, Baba T. Gene expression in spermiogenesis. Cell Mol Life Sci, 2005, 62: 344–354
52 Meistrich M L, Mohapatra B, Shirley C R, et al. Roles of transition nuclear proteins in spermiogenesis. Chromosoma, 2003, 111: 483–488
53 Nayernia K, Adham I M, Burkhardt-Gottges E, et al. Asthenozoospermia in mice with targeted deletion of the sperm mitochondrion-associated cysteine-rich protein (Smcp) gene. Mol Cell Biol, 2002, 22: 3046–3052
54 Anway M D, Ravindranath N, Dym M, et al. Identification of a murine testis complementary DNA encoding a homolog to human A-kinase anchoring protein-associated sperm protein. Biol Reprod, 2002, 66: 1755–1761
55 Kierszenbaum A L, Tres L L. Structural and transcriptional features of the mouse spermatid genome. J Cell Biol, 1975, 65: 258–270
56 Heidaran M A, Kistler W S. Transcriptional and translational control of the message for transition protein 1, a major chromosomal protein of mammalian spermatids. J Biol Chem, 1987, 262: 13309–13315
57 Kleene K C. Poly(A) shortening accompanies the activation of translation of five mRNAs during spermiogenesis in the mouse. Development, 1989, 106: 367–373
58 Kleene K C, Bagarova J. Comparative genomics reveals gene-specific and shared regulatory sequences in the spermatid-expressed mammalian Odf1, Prm1, Prm2, Tnp1, and Tnp2 genes. Genomics, 2008, 92: 101–106
59 Sozubir S, Barber T, Wang Y, et al. Loss of Insl3: a potential predisposing factor for testicular torsion. J Urol, 2010, 183: 2373–2379
60 Nakamura N, Mori C, Eddy E M. Molecular complex of three testis-specific isozymes associated with the mouse sperm fibrous sheath: hexokinase 1, phosphofructokinase M, and glutathione S-transferase mu class 5. Biol Reprod, 2010, 82: 504–515
61 Valentin M, Balvers M, Pusch W, et al. Structure and expression of the mouse gene encoding the endozepine-like peptide from haploid male germ cells. Eur J Biochem, 2000, 267: 5438–5449
62 Selvaraj V, Asano A, Page J L, et al. Mice lacking FABP9/PERF15 develop sperm head abnormalities but are fertile. Dev Biol, 2010, 348: 177–189
63 Dakhova O, O'Day D, Kinet N, et al. Dickkopf-like1 regulates postpubertal spermatocyte apoptosis and testosterone production. Endocrinology, 2009, 150: 404–412
64 Choi H S, Lee S H, Kim H, et al. Germ cell-specific gene 1 targets

testis-specific poly(A) polymerase to the endoplasmic reticulum through protein-protein interactions. FEBS Lett, 2008, 582: 1203–1209
65 Bellve A R, Millette C F, Bhatnagar Y M, et al. Dissociation of the mouse testis and characterization of isolated spermatogenic cells. J Histochem Cytochem, 1977, 25: 480–494
66 Tegelenbosch R A, de Rooij D G. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res, 1993, 290: 193–200
67 Gassei K, Ehmcke J, Wood M A, et al. Immature rat seminiferous tubules reconstructed *in vitro* express markers of Sertoli cell maturation after xenografting into nude mouse hosts. Mol Hum Reprod, 2010, 16: 97–110
68 Steger K, Rey R, Louis F, et al. Reversion of the differentiated phenotype and maturation block in Sertoli cells in pathological human testis. Hum Reprod, 1999, 14: 136–143
69 Dahia C L, Rao A J. Regulation of FSH receptor, PKIbeta, IL-6 and calcium mobilization: Possible mediators of differential action of FSH. Mol Cell Endocrinol, 2006, 247: 73–81
70 Heckert L, Griswold M D. Expression of the FSH receptor in the testis. Recent Prog Horm Res, 1993, 48: 61–77
71 Li M W, Mruk D D, Cheng C Y. Mitogen-activated protein kinases in male reproductive function. Trends Mol Med, 2009, 15: 159–168
72 Gnessi L, Emidi A, Jannini E A, et al. Testicular development involves the spatiotemporal control of PDGFs and PDGF receptors gene expression and action. J Cell Biol, 1995, 131: 1105–1121
73 Basciani S, Mariani S, Spera G, et al. Role of platelet-derived growth factors in the testis. Endocr Rev, 2010, 31: 916–939
74 Szczepny A, Hime G R, Loveland K L. Expression of hedgehog signalling components in adult mouse testis. Dev Dyn, 2006, 235: 3063–3070
75 Bitgood M J, Shen L, McMahon A P. Sertoli cell signaling by Desert hedgehog regulates the male germline. Curr Biol, 1996, 6: 298–304
76 Golestaneh N, Beauchamp E, Fallen S, *et al.* Wnt signaling promotes proliferation and stemness regulation of spermatogonial stem/progenitor cells. Reproduction, 2009, 138: 151–162
77 Tanwar P S, Kaneko-Tarui T, Zhang L, et al. Constitutive WNT/beta-catenin signaling in murine Sertoli cells disrupts their differentiation and ability to support spermatogenesis. Biol Reprod, 2010, 82: 422–432
78 Ma P, Wang H, Guo R, et al. Stage-dependent Dishevelled-1 expression during mouse spermatogenesis suggests a role in regulating spermatid morphological changes. Mol Reprod Dev, 2006, 73: 774–783
79 Hacker A, Capel B, Goodfellow P, et al. Expression of Sry, the mouse sex determining gene. Development, 1995, 121: 1603–1614
80 De Cesare D, Fimia G M, Sassone-Corsi P. CREM, a master-switch of the transcriptional cascade in male germ cells. J Endocrinol Invest, 2000, 23: 592–596
81 Blendy J A, Kaestner K H, Weinbauer G F, et al. Severe impairment of spermatogenesis in mice lacking the CREM gene. Nature, 1996, 380: 162–165

Supporting Information

Figure S1 Distribution of annotated genes with different expression levels in the three libraries measured by read counts (A) and RPKM values (B).

Figure S2 Histogram of gene ontology classification of annotated genes in the three libraries.

Figure S3 Summary reports generated by DEGseq. Left graphs illustrate gene expression abundance with RPKM values in neighboring time points of the three developmental stages. Right graphs show DEGs (red points) identified by MARS in neighboring time points of the three developmental stages.

Figure S4 Expression changes of transcription factors in spermatogonia.

Figure S5 Expression changes of *Sry* and its related genes.

Figure S6 Expression changes of *Crem* and its related genes.

Figure S7 The differential expression of *Dhh*, *Shh*, and *Ihh* in postnatal development of the mouse testis.

Figure S8 The differential expression of *Dvl1*, *Dvl2*, and *Dvl3* in postnatal development of the mouse testis.

Table S1 Genes with RPKM values >1000

The supporting information is available online at csb.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.