

## Original Article

# Chronic stress reduces spermatogenic cell proliferation in rat testis

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**Abstract:** Male reproductive dysfunction induced by mental stress and environmental factors has increased greatly in recent years. Previous studies of the male rat reproductive system under stress conditions evaluated changes in physiology and pathophysiology. However, no genome-wide study has been applied to such models. Here we studied the histopathologic changes in testes of rats under different durations of stress and used RNA sequencing (RNA-seq) to investigate the testicular transcriptome and detect differentially expressed genes. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) and immunohistochemistry were used to verify these. Chronic stress resulted in significant histopathologic changes in seminiferous tubules and RNA-seq showed that growing numbers of genes were dysregulated with increasing stress exposure. Gene Ontology (GO) analysis showed that many biological processes of cell proliferation-associated terms were highly significantly enriched among down-regulated genes, from chronically stressed groups. Proliferating cell nuclear antigen (PCNA) was used as a key marker of cell proliferation. RT-qPCR and immunohistochemistry indicated that PCNA mRNA and protein expression levels were greatly decreased with prolonged stress, thereby contributing to the attenuation of spermatogenic cell proliferation in the rat testis. This could provide a new scientific basis for the study of male reproductive dysfunction caused by stress.

**Keywords:** Stress, cell proliferation, testis, spermatogenic cell, RNA sequencing, PCNA

## Introduction

In modern societies, the rapid pace of life has greatly increased individual mental pressures. The mental disorders caused by such factors and their influence on reproductive function have attracted the attention of scholars. Not only female patients with reproductive dysfunction show significant increases in stress, but also male patients with reproductive dysfunction face severe problems.

Stress is a comprehensive response of the body to cope with danger through the joint participation and regulation of the nervous, endocrine, and other systems [1]. Through proper regulation of the metabolism of each system,

the body reinforces its homeostatic ability, thus effectively reducing the impact of risk factors on the body [2]. Moderate stress increases the body's ability to resist external risk factors, while excessive or long-term stress damages the body and can disrupt normal psychologic and physiologic functions [3]. Male fertility depends on the production of large numbers of spermatozoa by spermatogenesis in the testicular seminiferous epithelium, where germ cells are produced by sequential phases of mitosis and meiosis [4]. A previous study indicated that enforced swimming (3 min for 15 days) in male rats decreased the numbers of mature spermatids, while fertility was not disrupted [5], probably because the duration of stress was shorter than the spermatogenic cycle [6]. Other studies

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have indicated that stress induced changes in the levels of corticosterone [7] and testosterone [8, 9], suggesting that germ cells might be damaged under stress by increased corticosterone and decreased testosterone levels. However, the detailed mechanism of action and the effects of stress on the proliferation of spermatogenic cells are not clear, and no genome-wide study has been applied in rat testis under stress condition. Given that spermatogenesis is dependent on the proliferation and division of various spermatogenic cells, here we focused on identifying whether stress would affect the proliferation of testicular germ cells. Using an RNA sequencing (RNA-seq) approach, quantitative reverse transcription polymerase chain reaction (RT-qPCR) and immunohistochemistry, we investigated the changes in testicular spermatogenic cell proliferation and pathologic changes in rats with different durations of restraint and ice-water swimming, in attempts to provide a theoretical basis for the study of male reproductive dysfunction caused by stress.

### Materials and methods

#### *Animals*

Male Sprague Dawley (SD) rats (Beijing Vital River Laboratory Animal Technology Co. Ltd.) initially weighing  $200 \pm 20$  g were used in this study. The rats (four per cage) were housed at a constant temperature of 22°C and a relative humidity of 50-60% (v/v) under a 12/12-h light/dark cycle. The rats were given free access to food and water for at least 1 week prior to experimentation. All operations were approved by the Institutional Review Board for Animal Experiments at Fudan University. There were four experimental groups: control, 3-days, 14-days and 21-days of restraint stress added to ice water swimming (RS+IS; n = 8 rats per group).

#### *Models of restraint stress and ice-water swimming*

According to a previous study [10], models of restraint stress and ice-water swimming were established. Briefly, the rats were placed in a restrainer that allowed them to stretch their legs but not move otherwise, with no access to food and water for 8 h (from 09:00 to 17:00) each day. Then the restraint stressed rats were placed in ice cold water to swim for 5 min each

day. The process lasted for 3, 14, and 21 days in the different RS+IS groups. The control rats were left in their cages for the same time without food or water. All rats were given food and water *ad libitum* in the rest time.

#### *Tissue preparation*

Sixty minutes after restraint stress and ice-water swimming, the rats were anesthetized deeply. Tissue used for staining were harvested and fixed immediately in 10% formalin. Sections were subsequently dehydrated in a graded ethanol series and embedded in paraffin. They were then prepared for hematoxylin and eosin (H&E) and immunohistochemical staining and examined under a light microscope (Olympus IX73; Olympus, Tokyo, Japan). Tissues used for RNA-seq and RT-qPCR were removed rapidly, snap-frozen in liquid nitrogen then stored at -80°C.

#### *RNA extraction and cDNA synthesis*

Total RNA was isolated from tissue samples using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's protocol. RNA concentrations and purity were determined using a NANODROP 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 100-200 ng of total RNA was reverse transcribed using the Quantitect reverse transcription kit (Qiagen, Hilden, Germany). Samples were assayed three times, and mean values were recorded.

#### *RNA-seq library preparation*

For each group, three testis samples were taken, and 12 cDNA libraries were constructed. Aliquots of 3 µg RNA per sample were used as initial material. Ribosomal RNA was removed using Epicentre Ribo-Zero™ Gold kits (Rat) (Epicentre, an Illumina company, Madison, WI, USA). The libraries were constructed according to the recommendations of NEBNext® Ultra™ Directional RNA Library Prep kits of Illumina. Briefly, RNA fragmentation and short RNA strands were carried by using NEBNext First Strand Synthesis Reaction Buffer (5 ×). Using random hexamer primers and M-MuLV reverse transcriptase, first-strand cDNA was synthesized. Second-strand cDNA was synthesized subsequently with DNA polymerase I and RNase H. Purified second-strand cDNA terminals were repaired and then we added poly (A)

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and an adapter. Fragments of approximately 300 base pairs (bp) were selected using UNG enzyme. Clustering was performed in a cBot cluster generation system by the TruSeq PE Cluster Kit v4-cBot-HS (Illumina). After cluster generation, the library was sequenced using the Illumina HiSeq-4000 platform and 150 bp paired-end reads were generated.

### *RNA-seq expression analysis*

For data analysis, reads were quantified with alignment free software Kallisto, version 0.44.0 [11], and reference genome assembly Rnor\_6.0 downloaded from the Ensembl database ([ftp://ftp.ensembl.org/pub/release-93/fasta/rattus\\_norvegicus/](ftp://ftp.ensembl.org/pub/release-93/fasta/rattus_norvegicus/)). Transcripts per million (TPM) read values of gene levels were generated using Kallisto and analysis was performed using the Sleuth R Package [12]. The statistical significance of any difference in expression levels between normal and stress samples was assessed using the DESeq2 R Package [13], applying  $P < 0.05$  cutoff values to select differentially expressed genes (DEGs). Visualization of DEG expression data was done using pHeatmap, ggplot2, VennDiagram and Sleuth R Package.

### *Gene ontology (GO) analysis*

To understand the functions of significant DEGs, we applied the cluster profiler R package [14] to realize a GO gene enrichment analysis. Thresholds of  $P < 0.01$  and  $q$  Values  $< 0.05$  (adjusted for the false discovery rate, FDR) were used to select the significantly enriched GO terms, and were visualized with the enrichplot R package [15].

### *RT-qPCR*

Primers for proliferating cell nuclear antigen (PCNA; 5'-AGTTTCTGCGAGTGGGGAG-3' and 5'-GGAGACAGTGGAGTGGCTTT-3'),  $\beta$ -actin (5'-AGGGAAATCGTGCATGACAT-3' and 5'-CCTCGGGGCATCGGAA-3') were synthesized by Sangon Biotech Co. Ltd. (Shanghai, P. R. China). The results of RNA sequencing were validated by RT-qPCR. The cDNA sequences from RT reactions were used as templates for subsequent qPCRs, which was performed with the LightCycler 96 real-time PCR system (Roche Molecular Biochemicals, Basel, Switzerland). The 20  $\mu$ L reaction volume contained 1 mL forward primer, 1 mL reverse primer, 6.5 mL of

double distilled H<sub>2</sub>O, 1.5 mL of cDNA, and 10 mL of 2  $\times$  Hi SYBR Green qPCR Mix (HaiGene). Three samples were taken for each gene to perform the qPCRs, and the experiments were repeated three times for each. For quantification of gene expression levels the 2<sup>- $\Delta\Delta$ CT</sup> method was employed [16]. The internal control gene used for these analyses was that encoding  $\beta$ -actin.

### *Immunohistochemistry*

After deparaffinization and microwave antigen retrieval, sections were incubated for 30 min in 3% H<sub>2</sub>O<sub>2</sub> in cold methanol. The tissues were blocked for 1 h at room temperature with 5% (w/v) bovine serum albumin to avoid nonspecific staining and incubated with a monoclonal antibody specific for rabbit PCNA (Abcam, Cambridge, UK) (1:200) overnight at 4°C. Then, the tissues were incubated with biotinylated secondary antibody (Zhongshan Goldenbridge Biotech., P. R. China) for 30 min and subsequently with horseradish peroxidase (HRP)-conjugated biotin for 30 min. Finally, 3, 3'-diaminobenzidine (DAB) was used as a chromogen. The immunohistochemically stained sections were counterstained with hematoxylin.

### *Cell counting*

Four rats from each group were used for morphologic observations. The numbers of PCNA positive cells in each section were counted (per 400  $\times$  field of view) in five view fields of the spermatogenic tubules. Two independent observers counted and calculated the mean numbers of PCNA positive cells.

### *Statistical methods*

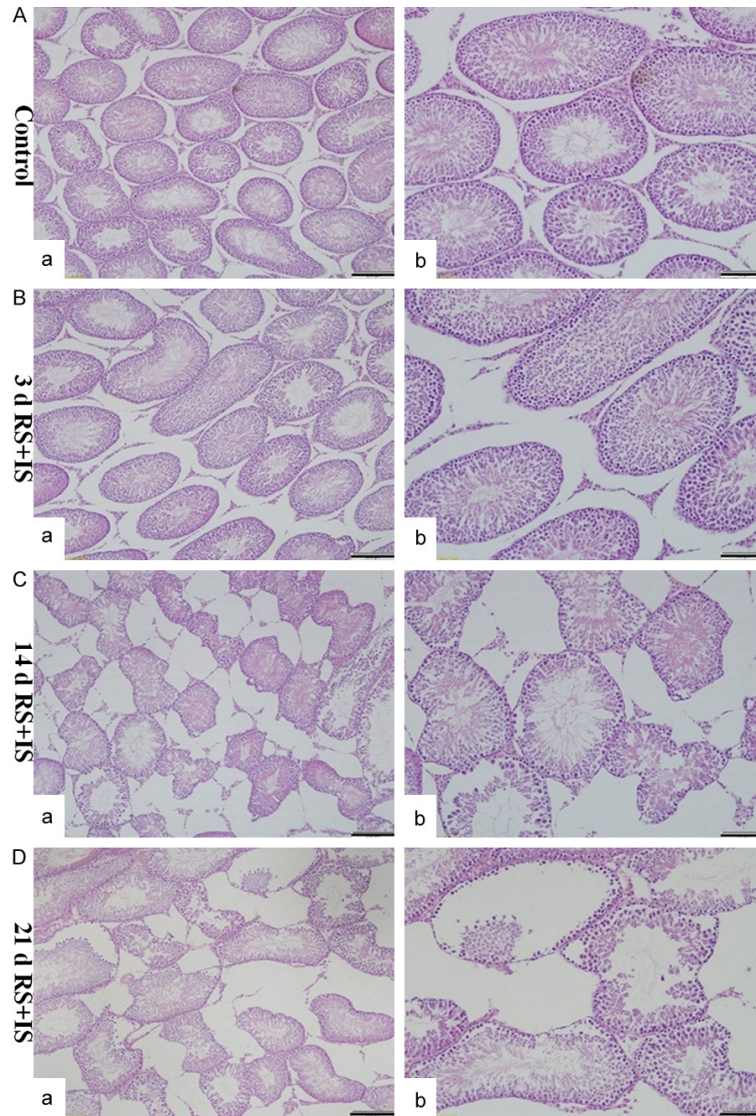
Immunohistochemically positive cell count data were compared by one-way analysis of variance (ANOVA). Student's  $t$ -tests were used to compare the mean results of RT-qPCR results. Data are presented as the mean  $\pm$  standard error of the mean (SEM) and significance was set at  $P < 0.05$  for all statistical tests.

## **Results**

### *H&E staining showed pathological changes in the testis*

In the control group, the seminiferous tubules were regular in shape and orderly in arrange-

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**Figure 1.** H&E staining of the testis. The seminiferous tubules were regular in shape and orderly in arrangement in the control group (Aa, Ab) and RS+IS group at 3 days (Ba, Bb). The tubule basement membrane was separated from spermatogenic cells after 14 days stress exposure (Ca, Cb). After 21 days of stress exposure, pathologic changes of the seminiferous tubules were more obvious (Da, Db). Bars = 200  $\mu$ m in (Aa-Da); Bars = 100  $\mu$ m in (Ab-Db).

ment. The cell layers were clear, and the spermatogonia were closely connected to the basement membrane (**Figure 1Aa, 1Ab**). In the RS+IS groups, there were no significant pathologic changes in the testis at 3 days (**Figure 1Ba, 1Bb**). However, some irregular spermatogenic tubules and disordered cell arrangements were observed at 14 days. The tubule basement membrane was separated from spermatogenic cells (**Figure 1Ca, 1Cb**). After 21 days of stress exposure, pathologic changes of

the seminiferous tubules were more evident. The spermatogenic cell layer was thinner, and the basement membrane of seminiferous tubules had separated from the overlying cells (**Figure 1Da, 1Db**).

### Overview of RNA-seq data analysis

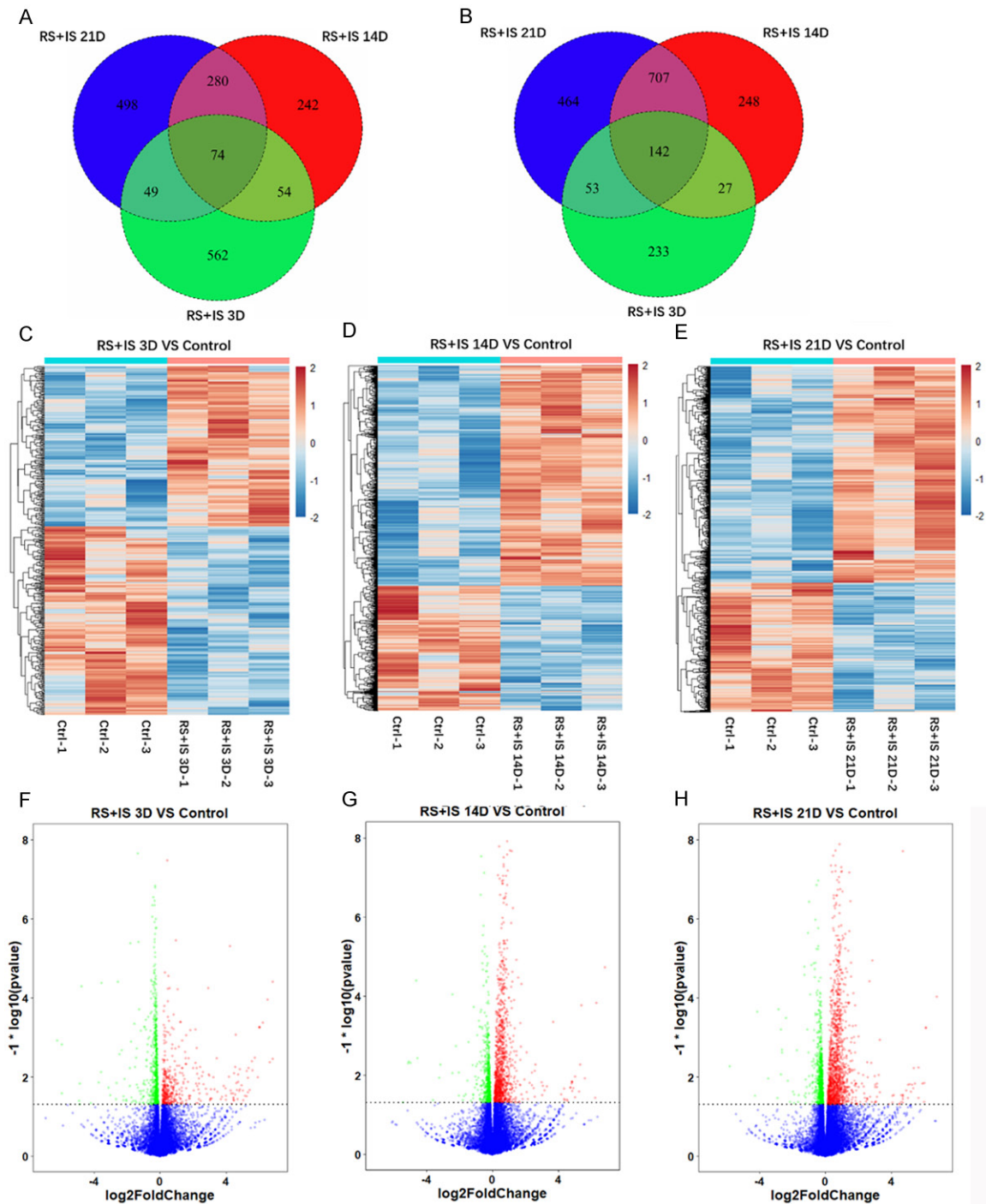
Using the Illumina platform, a total of 1,112,510,142 raw reads (265,530,024 raw reads from the control, 282,103,906 raw reads from the 3-day RS+IS group, 277,377,940 raw reads from the 14-day RS+IS group, and 287,498,272 raw reads from the 21-day RS+IS group) were identified. After trimming and discarding the raw reads with adapters (reads containing more than five adapter-polluted bases were regarded as adaptor-polluted reads and were filtered out), low-quality reads (those with the number of bases whose phred quality values were no more than 19 accounting for more than 15% were regarded as low-quality reads and filtered out) and reads with the number of N bases accounting for more than 5%, we obtained 1,080,388,272 clean reads for subsequent analysis.

### DEG analysis

The gene expression level was estimated from TPM values. Compared with the control

group, there were 1,194 DEGs in the 3-day RS+IS group (**Table S1**), including 455 upregulated genes and 739 downregulated genes ( $P < 0.05$ ), 1,774 DEGs in the 14-day RS+IS group (**Table S2**), including 1,124 upregulated genes and 650 downregulated genes ( $P < 0.05$ ), and 2,267 DEGs in the 21-day RS+IS group (**Table S3**), including 1,366 upregulated genes and 901 downregulated genes ( $P < 0.05$ ). Meanwhile, some same expression patterns were observed in three phenotypes (**Figure 2A, 2B**).

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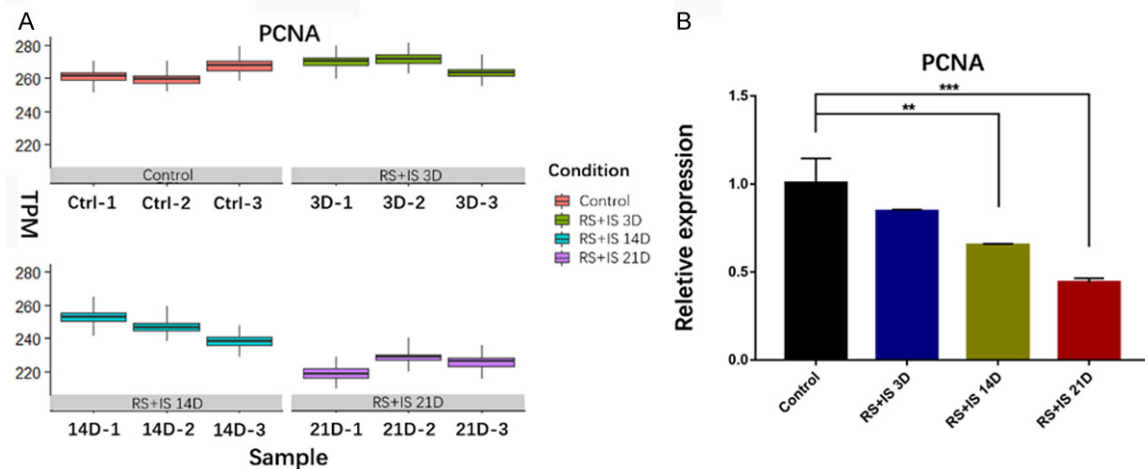


**Figure 2.** Different expression profile of RNA-Seq data between normal and stress group. “Control” represents the control group, “RS+IS” represents the stress group. A. Venn diagram of up regulated genes in different stress groups. B. Venn diagram of up regulated genes in different stress groups. C-E. The hierarchical cluster heat map of DEGs. “blue” shows low intensity indicates weak expression, “red” shows high intensity indicates strong expression. F-H. A volcano plot of DEGs. The horizontal dot line means a  $p$ -value of 0.05, and the red point in the plot shows the up regulated genes with statistical significance, the green point in the plot shows the down regulated genes with statistical significance.

Between the 14-day and 21-days RS+IS groups we identified 280 co-downregulated genes and

707 co-upregulated genes. Cluster analysis of DEGs produced heat maps (**Figure 2C-E**).

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**Figure 3.** TPM value of PCNA from RNA-seq data and validation of transcript expression by qPCR. A. TPM showing the expression levels of PCNA gene in different stress groups. With increasing stress exposure, expression of PCNA gene was decreased. B. PCNA expression are presented as the mean  $\pm$  SE ( $n = 3$ ).  $\beta$ -actin gene was used as a housekeeping internal control. Transcript expression was quantified relative to the expression level of  $\beta$ -actin using the comparative cycle threshold ( $\Delta$ CT) method. (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

Volcano plots of DEGs are also shown (Figure 2F-H). These data indicated that there were significant DEG patterns between the chronic stress and control groups. Based on our data analysis and the physiological characteristics of testicular spermatogenic cells, PCNA, a classical cell proliferation marker, was found to be significantly downregulated in the chronic stress groups (Figure 3A).

### RT-qPCR

PCNA mRNA expression levels measured by RT-qPCR showed significant changes after stress exposure (Figure 3B). Stress exposure for 14 and 21 days produced significant decreases in PCNA mRNA expression levels ( $P < 0.05$ ).

### Immunohistochemistry of PCNA

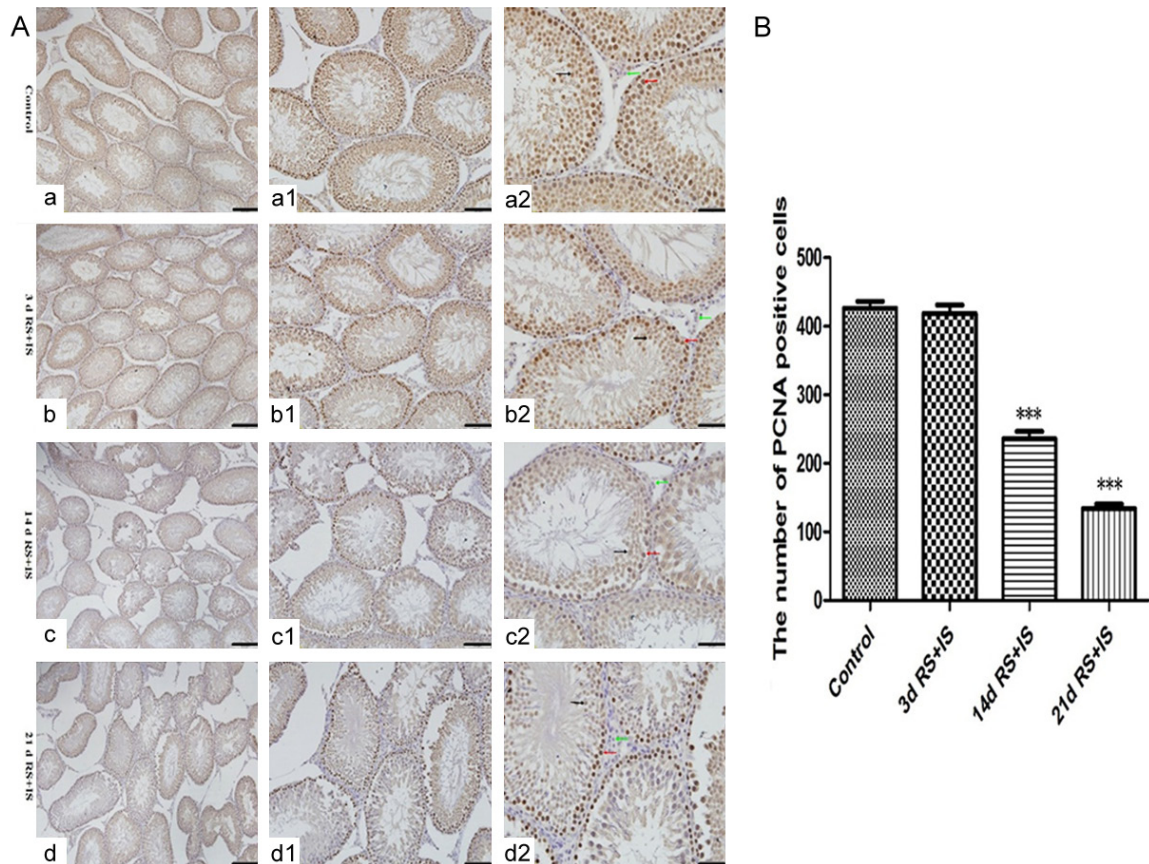
PCNA positive cells are those showing proliferative ability in the cell cycle. PCNA, which is expressed in the nucleus, was strongly positively stained in spermatogonia and positively stained in spermatocytes, but not in Leydig cells (Figure 4A). ANOVA for the numbers of PCNA positive cells in the testes revealed that there were significant differences among the groups ( $F_{[3,20]} = 206.561$ ;  $P < 0.001$ ). Compared with the control group ( $426.33 \pm 10.23$ ), the mean numbers of PCNA positive cells was significantly lower after 14 days ( $236.17 \pm 10.22$ ,

$P < 0.05$ ) and 21 days ( $134.00 \pm 6.56$ ,  $P < 0.05$ ) of stress exposure, although there was no significant difference at 3 days of stress exposure ( $418.83 \pm 12.08$ ,  $P > 0.05$ ; Figure 4B).

### Gene ontology (GO) analysis

GO analyses were performed on significant DEGs. These showed that among all the downregulated genes, nine GO terms ("biological processes") were significantly enriched ( $q < 0.05$ ) in the 14-day RS+IS group. 186 GO terms ("biological processes") were significantly enriched ( $q < 0.05$ ) in the 21-day RS+IS group (Tables S4, S5). Some of these terms, such as GO 1903046 ("meiotic cell cycle processes"), GO 0051321 (meiotic cell cycle), GO 0000280 (nuclear division), and GO 0071897 ("DNA biosynthetic processes"), indicate dysfunction in spermatogenic meiotic proliferation. In addition, we performed GO analyses in 280 co-downregulated genes between the 14- and 21-day stress groups and 25 GO terms were significantly enriched ( $q < 0.05$ ) (Table S6). After running gene set enrichment analysis and hypergeometric test, we found that many meiotic and cell cycle associated terms tended to cluster together. These, along with the GO results, reinforced our findings (Figure 5A). We have visualized these terms arranged by q-value and have illustrated the downregulated genes belonging to these terms in Figure 5B-D.

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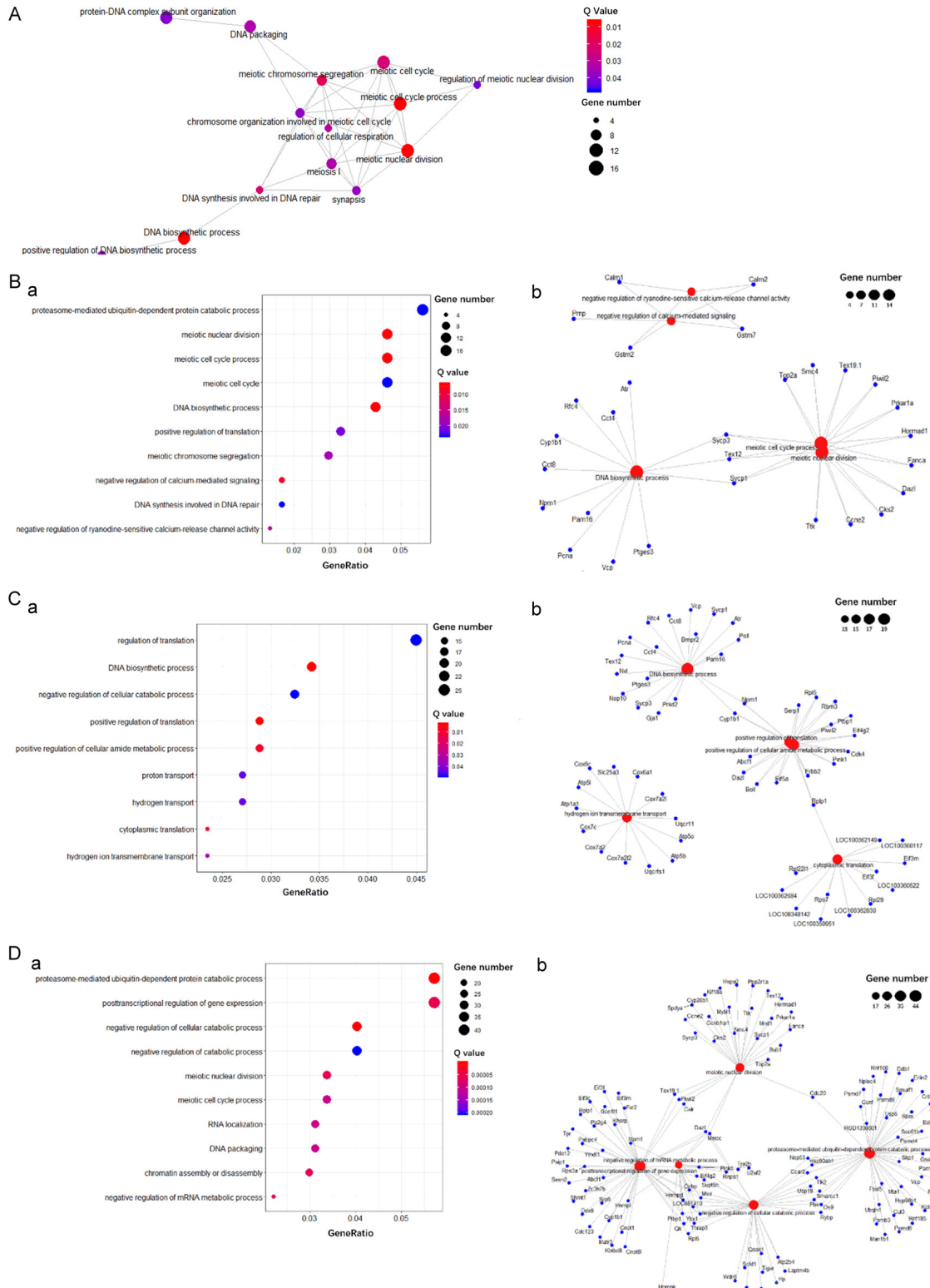
**Figure 4.** Immunohistochemistry expression of PCNA. (A) PCNA immunohistochemistry in the testis following different durations of stress exposure. Red arrow: strongly positive expression in spermatogonia, Black arrow: positive expression in spermatocyte, Green arrow: negative expression in Leydig cell. Bars = 200  $\mu$ m in (a-d); Bars = 100  $\mu$ m in (a1-d1); Bars = 50  $\mu$ m in (a2-d2). (B) Quantitative analysis. The data are shown as mean  $\pm$  SEM, \*\*\*P < 0.001 vs. control group (n = 6). With increasing stress exposure, the number of PCNA positive cells was decreased.

### Discussion

Stress is an inevitable life experience. Repeated and long-term stresses have negative effects on body function and metabolism [17], resulting in disturbances to body homeostasis in different organs and systems, especially the central nervous system [18]. In recent years, the number of male patients with reproductive dysfunction has increased significantly. Although current studies have indicated that stress is one of the causes of this [19, 20], the detailed mechanisms of action and pathological changes caused by stress still lack systematic and detailed studies. The testis is a highly proliferative tissue, where spermatozoa are produced from spermatogonia through mitosis and meiosis. This process requires suitable internal and external environments. Any perturbation could cause abnormal sperm production, resulting in infertility [21]. A previous study indicated that

the numbers of mature spermatids decreased in male rats exposed to forced swimming stress (3 min for 15 days) [5]. However, the cause of the decrease in mature sperm numbers remains unclear. Here, we successfully established rat models with restraint and forced ice-water swimming to investigate the histopathological changes in the testis under different durations of stress exposure. Chronic stress caused disruption to the spermatogenic cell layer of the testis and loss of spermatogenic cells. Seminiferous tubules were disordered, and the basement membrane was separated from the cells. Among these histopathological changes in testis under stress, thinning of the seminiferous epithelium is noteworthy. Because the proliferation and meiosis of spermatogenic cells are keys to normal spermatogenesis, if the proliferative capacity of spermatogenic cells decreases, pathologic changes involving such thinning may occur.

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**Figure 5.** Visualization of Gene ontology (GO). Significant enrichment ( $q$  value  $< 0.05$ , Top of 10) of Gene ontology terms and genes which belong to the terms. A. GO Enrichment map of Co-down-regulated genes between RS+IS at 14 days 21 days, which acquired from gene set enrichment analysis and hypergeometric test. B. Significantly enriched GO term of co-down-regulated genes between 14 days and 21 days of RS+IS groups. C. Significantly enriched GO term of down regulated genes between control group and 14 days group. D. Significantly enriched GO term of downregulated genes between control group and 21 days group.



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It is well known that male fertility depends on the production of a large number of spermatozoa during spermatogenesis [4]. This process occurs in testicular spermatogenic cells, where spermatozoa with structure and function are produced by successive mitotic and meiotic cell divisions. Therefore, the proliferation and division of spermatogenic cells are the most important factors in spermatogenesis. Previous studies have indicated that acute and chronic stress exposure results in male reproductive dysfunction [9, 22, 23]. In addition to an increase in adrenaline [24], chronic stress inhibits testosterone secretion and inhibits libido and spermatogenesis [25, 26]. Although stress is not the only cause of infertility, increasing evidence has demonstrated that chronic stress is an additional risk factor for infertility [22, 27]. As documented in the literature, stress leads to abnormal sperm morphology [28] and affects sperm activity [29] and output [27, 30], resulting in male reproductive dysfunction [31]. Moreover, stress elevates the levels of glucocorticoids [7], which inhibit testosterone synthesis in Leydig cells by binding to glucocorticoid receptors [8] and inhibits the proliferation of spermatogenic cells. However, the cause of this is unclear.

Here, we successfully established rat models with a combination of restraint and forced ice-water swimming and for the first time used RNA-seq approaches to investigate the changes of testicular transcriptome in rats under stress exposure. We found significant changes in the testicular transcriptome. Briefly, we identified 1,194 dysregulated genes in the 3-day RS+IS group, 1,774 dysregulated genes in the 14-day RS+IS group and 2,267 dysregulated genes in the 21-day RS+IS group. Based on our histopathology results, we focused on the changes in chronically stressed groups. Through GO analysis, we discovered many downregulated genes involved in the biological processes of cell proliferation and meiosis, such as DNA biosynthesis, meiotic nuclear division and meiotic cell cycle processes. Interestingly, we did not find enrichment patterns associated with cell proliferation or meiosis in the upregulated genes, although there were numbers of genes upregulated. Based on this bioinformatic evidence, we chose the gene encoding PCNA, one of the downregulated genes, to verify the proliferation of spermatogenic cells. PCNA, as the

auxiliary protein of DNA polymerase  $\delta$ , increases during the late part of the G1 phase of the mitotic cell cycle, peaks during the S phase and then decreases during the G2 and M phases. These changes are consistent with the rate of DNA synthesis [32-35], DNA repair, sister chromatid exchange and cohesion. They reflect cell cycle control [36] and play important roles in meiosis [37].

The TPM values of PCNA RNA levels from RNA-seq data indicated that PCNA in the testis decreased significantly in the rats stressed for 14 and 21 days. To verify the accuracy of the sequencing results, we used RT-qPCR and immunohistochemistry to detect PCNA mRNA expression and protein levels, and the results were consistent with those of the RNA-seq analyses. Because of the physiologic characteristics of testicular spermatogenic cells, we consider that the effect of stress on male reproductive function might arise from changes in their proliferative capacity. This decreased along with the duration of stress, which led to histopathologic changes and decreased numbers of spermatogenic cells. These results suggest that the pathological changes caused by stress are closely related to the attenuation of spermatogenic cell proliferation. This is the first study to provide a systematic view of mRNA changes in the testis under stress conditions and further link these to impaired spermatogenic cell proliferation. Our findings serve as a theoretical basis for studying male reproductive dysfunction caused by stress and for future clinical treatment. Moreover, the RNA-seq results indicated that there were significant changes in the testicular transcriptome in stressed rats, which suggests that the underlying mechanisms are complex rather than simple. Further studies are needed to clarify these.

### Conclusions

Here we have provided a systematic view of mRNA changes in the rat testis under stress conditions and showed dysfunction in spermatogenic cell proliferation. These findings suggested that chronic stress induced changes in gene expression in spermatogenic cells, which attenuated their proliferation. These findings could serve as a theoretical basis for male reproductive dysfunction caused by stress and offer options for future clinical treatment.

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## Disclosure of conflict of interest

None.

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**Table S4.** Enriched GO terms of downregulated genes in 14-day RS+IS group

ID	Description	Gene Ratio	Bg Ratio	p value	p.adjust	q value	gene ID	Count
GO:0045727	Positive regulation of translation	16/555	120/17363	1.38E-06	0.002873	0.002705	Abcf1/Cdk4/Cyp1b1/Dazl/Eif4g2/Eif5a/Erbp2/Rplp1/Boll/Npm1/Pink1/Piwil2/Ptbp1/Rbm3/Rpl5/Serp1	16
GO:0071897	DNA biosynthetic process	19/555	165/17363	1.42E-06	0.002873	0.002705	Atr/Bmpr2/Cct4/Cct8/Cyp1b1/Gja1/Nop10/Npm1/Nvl/Pam16/Pcna/Poll/Prkd2/Ptges3/Rfc4/Sycp1/Sycp3/Tex12/Vcp	19
GO:0002181	Cytoplasmic translation	13/555	92/17363	6.99E-06	0.008054	0.007584	Eif3f/Eif3m/Rplp1/LOC100360522/LOC100359951/LOC100362149/LOC100362684/Rps7/LOC100362830/LOC108348142/LOC100360117/Rpl2211/Rpl29	13
GO:0034250	Positive regulation of cellular amide metabolic process	16/555	137/17363	7.96E-06	0.008054	0.007584	Abcf1/Cdk4/Cyp1b1/Dazl/Eif4g2/Eif5a/Erbp2/Rplp1/Boll/Npm1/Pink1/Piwil2/Ptbp1/Rbm3/Rpl5/Serp1	16
GO:1902600	Hydrogen ion transmembrane transport	13/555	108/17363	4.05E-05	0.032782	0.030868	Atp1a1/Atp5b/Atp5l/Atp5o/Cox6a1/Cox6c/Cox7a2l/Cox7a2/Cox7a2l2/Cox7c/Slc25a3/Uqcr11/Uqcrfs1	13
GO:0015992	Proton transport	15/555	146/17363	6.91E-05	0.043248	0.040723	Atp1a1/Atp5b/Atp5l/Atp5o/Cox6a1/Cox6c/Cox7a2l/Cox7a2/Cox7a2l2/Cox7c/Slc25a3/Tmsb4x/Ucp2/Uqcr11/Uqcrfs1	15
GO:0006818	Hydrogen transport	15/555	147/17363	7.48E-05	0.043248	0.040723	Atp1a1/Atp5b/Atp5l/Atp5o/Cox6a1/Cox6c/Cox7a2l/Cox7a2/Cox7a2l2/Cox7c/Slc25a3/Tmsb4x/Ucp2/Uqcr11/Uqcrfs1	15
GO:0006417	Regulation of translation	25/555	339/17363	9.78E-05	0.048382	0.045558	Abcf1/Cdk4/Cyp1b1/Dazl/Ddx6/Eif3b/Eif3f/Eif3m/Eif4g2/Eif5a/Erbp2/Kbtbd8/Rplp1/Boll/Npm1/Pink1/Piwil2/Ptbp1/Rbm3/Rgs2/Rida/Rpl5/Rps23/Serp1/Zc3h7b	25
GO:0031330	Negative regulation of cellular catabolic process	18/555	205/17363	0.000108	0.048382	0.045558	Dazl/Eif4g2/Hp/Rps7/LOC100362830/Boll/Nop53/Os9/Pbk/Pink1/Poldip2/Ptbp1/Qsox1/Rpl5/Scfd1/Taf9/Timp1/Tlk2	18

**Table S6.** Enriched GO terms of co-downregulated genes between 14- and 21-day RS+IS groups

ID	Description	Gene Ratio	Bg Ratio	p value	p.adjust	q value	gene ID	Count
GO:0140013	Meiotic nuclear division	14/303	177/17363	2.82E-06	0.006803	0.006251	Ccne2/Cks2/Dazl/Fanca/Hormad1/Piwil2/Prkar1a/Smc4/Sycp1/Sycp3/Tex12/Tex19.1/Top2a/Ttk	14
GO:1903046	Meiotic cell cycle process	14/303	188/17363	5.70E-06	0.006803	0.006251	Ccne2/Cks2/Dazl/Fanca/Hormad1/Piwil2/Prkar1a/Smc4/Sycp1/Sycp3/Tex12/Tex19.1/Top2a/Ttk	14
GO:0071897	DNA biosynthetic process	13/303	165/17363	6.68E-06	0.006803	0.006251	Atr/Cct4/Cct8/Cyp1b1/Npm1/Pam16/Pcna/Ptges3/Rfc4/Sycp1/Sycp3/Tex12/Vcp	13
GO:0050849	Negative regulation of calcium-mediated signaling	5/303	18/17363	1.11E-05	0.008503	0.007814	Calm1/Calm2/Gstm2/Gstm7/Prnp	5
GO:0060315	Negative regulation of ryanodine-sensitive calcium-release channel activity	4/303	11/17363	2.72E-05	0.016643	0.015294	Calm1/Calm2/Gstm2/Gstm7	4
GO:0045132	Meiotic chromosome segregation	9/303	93/17363	3.55E-05	0.018086	0.01662	Ccne2/Hormad1/Smc4/Sycp1/Sycp3/Tex12/Tex19.1/Top2a/Ttk	9

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GO:0045727	Positive regulation of translation	10/303	120/17363	4.82E-05	0.021057	0.01935	Abcf1/Cyp1b1/Dazl/Eif4g2/Rplp1/Npm1/Pink1/Piwil2/Ptbp1/Rpl5	10
GO:0051321	Meiotic cell cycle	14/303	235/17363	6.90E-05	0.023287	0.021399	Ccne2/Cks2/Dazl/Fanca/Hormad1/Piwil2/Prkar1a/Smc4/Sycp1/Sycp3/Tex12/Tex19.1/Top2a/Ttk	14
GO:0043161	Proteasome-mediated ubiquitin-dependent protein catabolic process	17/303	329/17363	7.07E-05	0.023287	0.021399	Ccnf/Crbn/Cul3/Rlim/Nop53/Nploc4/Os9/Pbk/Psmb3/Psmc2/Psmd12/RGD1308601/Sec61b/Smurf1/Tlk2/Ubqln1/Vcp	17
GO:0000731	DNA synthesis involved in DNA repair	5/303	26/17363	7.62E-05	0.023287	0.021399	Pcna/Sycp1/Sycp3/Tex12/Vcp	5
GO:0043457	Regulation of cellular respiration	5/303	28/17363	0.000111	0.030738	0.028247	Cisd1/Noa1/Nop53/Pink1/Vcp	5
GO:0034250	Positive regulation of cellular amide metabolic process	10/303	137/17363	0.000147	0.03318	0.030491	Abcf1/Cyp1b1/Dazl/Eif4g2/Rplp1/Npm1/Pink1/Piwil2/Ptbp1/Rpl5	10
GO:0006323	Dna packaging	11/303	164/17363	0.000147	0.03318	0.030491	H3f3b/Hells/Hist1h1c/Hist1h2ba/Hist1h2bo/Nap114/Npm1/Smc4/Sycp1/Sycp3/Top2a	11
GO:0007127	Meiosis i	9/303	112/17363	0.000152	0.03318	0.030491	Ccne2/Cks2/Hormad1/Piwil2/Sycp1/Sycp3/Tex12/Tex19.1/Top2a	9
GO:0051280	Negative regulation of release of sequestered calcium ion into cytosol	4/303	17/17363	0.000181	0.03683	0.033845	Calm1/Calm2/Gstm2/Gstm7	4
GO:0007129	Synapsis	6/303	49/17363	0.0002	0.036846	0.033859	Ccne2/Hormad1/Sycp1/Sycp3/Tex12/Tex19.1	6
GO:0002181	Cytoplasmic translation	8/303	92/17363	0.000205	0.036846	0.033859	Eif3f/Eif3m/LOC100360522/Rplp1/LOC100360117/LOC108348142/Rpl221l/Rpl29	8
GO:0051284	Positive regulation of sequestering of calcium ion	4/303	18/17363	0.000229	0.038708	0.035571	Calm1/Calm2/Gstm2/Gstm7	4
GO:0070192	Chromosome organization involved in meiotic cell cycle	7/303	72/17363	0.000257	0.038708	0.035571	Ccne2/Hormad1/Smc4/Sycp1/Sycp3/Tex12/Tex19.1	7
GO:2000573	Positive regulation of dna biosynthetic process	7/303	72/17363	0.000257	0.038708	0.035571	Atr/Cct4/Cct8/Cyp1b1/Npm1/Ptges3/Rfc4	7
GO:0031330	Negative regulation of cellular catabolic process	12/303	205/17363	0.000266	0.038708	0.035571	Dazl/Eif4g2/Hp/Nop53/Os9/Pbk/Pink1/Ptbp1/Qsox1/Rpl5/Scfd1/Tlk2	12
GO:0071824	Protein-dna complex subunit organization	12/303	207/17363	0.000291	0.040385	0.037111	Cand1/H3f3b/Hells/Hist1h1c/Hist1h2ba/Hist1h2bo/Nap114/Npm1/Psmc2/Smarca4/Sycp3/Taf7	12
GO:0040020	Regulation of meiotic nuclear division	5/303	35/17363	0.000331	0.042113	0.0387	Dazl/Hormad1/Piwil2/Prkar1a/Ttk	5
GO:1901020	Negative regulation of calcium ion transmembrane transporter activity	5/303	35/17363	0.000331	0.042113	0.0387	Calm1/Calm2/Gstm2/Gstm7/Ubqln1	5
GO:0033559	Unsaturated fatty acid metabolic process	8/303	101/17363	0.000389	0.047529	0.043676	Cyp1b1/Fads1/Fads2/Gstm2/Gstm7/MglI/Ptges3/Scd2	8