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Research Article

Maturation Promotion Factor Decreased With Germ Cell Removal in the Experimental Unilateral Cryptorchid of Mice

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Abstract

Maturation promotion factor (MPF) was found to be involved in both mitotic and meiotic cell cycle. The arm of the present study was to verify the changes of the subunits of MPF in cryptorchid spermatocytes, and to further explain the mechanism of spermatogenic losses in cryptorchidism patients. DNA end labeling (TUNEL) were used to observe morphological and apoptotic characteristics of spermatogenic cells; Immunohistochemical analysis was used to detect changes of Cdc2, Cyclin B1 and Cyclin B2 in response to heat-stress from cryptorchidism leading to spermatocyte losses; Real-time polymerase chain reaction (RT-PCR) and western blotting were used to test the expression of Cdc2, Cyclin B1 and Cyclin B2 in testis at mRNA and protein levels. The results show that staining specific to all subunits of MPF was positive in most spermatogenic cells and there has no significant difference between abdominal testis and control, and in abdominal testis, staining of Cdc2, Cyclin B1, and Cyclin B2 were also observed in most AC and MC. However, sthe overall expression of Cdc2, Cyclin B1 and Cyclin B2 in abdominal testis was much less than that of the scrotal testes at mRNA and protein levels. These result not only promote us to retrospect the previous report that subunits of MPF were down-regulated transcriptionally in cryptorchidism, but also to focus on the relationship between MPF and apoptosis and fusion of spermatogenetic cells.

ABBREVIATIONS

MPF: Maturation Promotion Factor; **AC:** Apoptotic spermatocytes; **MC:** Multinucleated Giant Cells

INTRODUCTION

Cryptorchidism is one of the most common congenital anomalies in boys. More than 80% of cryptorchidism are isolated problems while the rest are generally associated with other congenital malformations, chromosomal disorders, and syndromes [1]. However, it is important to realize that the terms cryptorchidism and undescended testis, even in isolated cases, include the spectrum of a disease complex with a great variety in pathogenesis, pathophysiology, epidemiology, treatment options, and prognosis especially in relation to fertility [2]. An explanation for infertility of cryptorchidism is massive germ cells death caused by an insufficient endogenous defense system against transpose ons. Hadziselimovic et al. (2011) reported that a group of boys with cryptorchidism are at high risk of infertility. They regularly have defective mini puberty and exhibit impaired expression of genes that are important for transpose on silencing, including DDX4, MAEL, MOV10L1, PIWIL2, PIWIL4, and TDRD9 [3]. This suggests that gene instability induced by impaired expression of transpose on-silencing genes may contribute to the development of azoospermia.

It is well known that both mitotic and meiotic cell cycle are supervised by the maturation phase promotion factor (MPF), a complex of Cdc2 kinase (Cdc2) and cyclin B (include cyclinB1 and cyclin B2) [4,5]. As a 34 kDa catalytic subunit, Cyclin B is a regulator of Cdc2. The activation of MPF promotes the transition of G2 to M phase in cell cycle progression[6], and various molecular mechanisms of MPF activation during spermatocyte maturation have been illuminated[7,8]. Cdc2 is activated by its own phosphorylation on Thr161 and dephosphorylation on Thr14/Tyr15 of Cdc2, and it is dependent on its association with Cyclin B to form MPF [9,10]. It has been demonstrated that high level expression of Cdc2, Cyclin B1, and Cyclin B2 in mouse testis play an important role in spermatogenesis and sperm maturation [11,12]. However, so far, there has no report on whether spermatogenesis block of cryptorchidism patients is correlate to the abnormal expression of Cdc2, Cyclin B1, and Cyclin B2. Therefore, the unilateral experimental cryptorchid model is used in this study to verify the changes of Cdc2, Cyclin B1 and Cyclin B2 proteins in cryptorchid spermatocytes, and to further explain the mechanism of spermatogenic losses in cryptorchidism patients.

MATERIALS AND METHODS

Animals

Two-month-old male C57BL/6J mice weighted 20 grams

were obtained from the animal center of Chinese Academy of Sciences in Shanghai. In a standard animal facility, the mice were fed under the controlled temperature25°C. Thirty mice were divided into 3 groups. Unilateral testicular control with right testis removal: the rest left testis marked as Uni-S. In the unilateral cryptorchid model, the right testis was fixed in the abdominal cavity by suturing its capsule to the abdominal wall, marked as Cry-C, and the left testis was kept free into the scrotum, marked as Cry-S. In another control group, sham-operation, which are similar to unilateral cryptorchid model but not fix the testis in the abdominal cavity, in right side testis was performed, marked as Con-S. The mice were sacrificed at 10 and 14 days after operation, then the testes were removed. A part of testes was stored at the temperature -80°C to obtain mRNA and protein extracts, and the other part was fixed in the Bouin's solution for immunohistochemical analysis. The experiment described above was repeatedly carried out for three times.

DNA end labeling of tissue sections (TUNEL methods)

After being fixed overnight in Bouin's solution and embedded in paraffin wax, the cryptorchid and scrotal testis samples were sectioned in 5-μm thickness. To identify the apoptotic cells in cryptorchid and scrotal testis samples, the ApopTag® Peroxidase in Situ Oligo Ligation (ISOL) Kit (Chemicon International, Inc., Cat #S7200.) was applied according to the protocols. The apoptotic spermatocytes (AC) were counted from 10 tubule cross-sections of each specimen under 400 amplification using Olympus BH-2 microscope. Meanwhile, hematoxylin and eosin (HE) staining was performed as usual to distinguish the cytoplasm of multinucleated giant cells (MC). MC was observed and counted as AC.

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from the testis, and the reverse transcription was performed using RNAiso Plus (Takara, Tokyo, Japan). RT-PCR was performed on an ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green dye according to the manufacturer's protocol. All of the primers are listed in (Table 1). β-Actin served as an internal reference gene. The relative amount of mRNA was calculated using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$ method).

Western blotting

Identical amounts of total protein were extracted from the testis and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. A gel transfer was performed, and the membranes were then blocked with blocking buffer for 2 hours at room temperature. The membranes were subjected to western blotting using rabbit anti-Cdc2 (at a dilution of 1:1000, Chemicon International Company), rabbit anti-Cyclin B1 (1:1000, Santa Cruz Biotechnology, Inc. Dallas, USA), and rabbit anti-Cyclin B2 (1:1000, Abcam, Cambridge, UK) overnight at 4°C with horseradish peroxidase-labeled secondary antibodies for 1 hour at 4°C. Finally, the signals were detected using an ECL Advance system (Amersham Biosciences, Piscataway, NJ, USA). The relative protein expression levels were determined by normalization to β-actin.

Immunohistochemistry

The serial 5 μm-thickness sections were mounted on poly-*L*-Lysine (Sigma)-coated glass slides. Tissue sections were incubated with 10% normal bovine serum in PBST (PBS, pH7.4, containing 0.05% Tween-20), pH 7.5 for 1 hour to block the nonspecific binding. Cdc2, Cyclin B1, and Cyclin B2 antigens in tissue sections were recognized respectively by rabbit anti- Cdc2 (at a dilution of 1:400, Chemicon International Company), rabbit anti-Cyclin B1 (1:500, Santa Cruz Biotechnology, Inc. Dallas, USA), and rabbit anti-Cyclin B2 (1:200, Abcam, Cambridge, UK) overnight at 4 °C. After washing for 3 times in PBST, the sections were incubated with biotin-conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology, Inc. Dallas, USA) for 1 hour at room temperature. After washing, strepavidin-HRP was added to bind to biotin. DAB was used as substrate for the peroxidase reaction. Sections were counterstained with hematoxylin. As controls, the primary antibodies were neutralized by the blocking peptides (Santa Cruz Biotechnology, Inc. Dallas, USA). The negative controls were performed by using normal sheep serum in substitution for primary and secondary antibody respectively.

Statistical analysis

The data were processed by SPSS13.0 (SPSS Inc., Chicago, IL, USA). All the data were expressed in mean ± SD, and results were analyzed for statistical significance using one-way analysis of variance (ANOVA) or *t*-test. The significance level was set at 0.05.

RESULTS

The weight changes of unilateral cryptorchid testes

Comparing the weight changes with time between different groups, we found that weights of abdominal testes (Cry-C) decreased significantly ($P < 0.01$) after the operation (Table 1), but no significant changes ($P > 0.05$) were found for scrotal testes. By the day 14 after operation, weights of abdominal testes (Cry-C) were reduced to about one third those of controls; and at days 10 and 14 were also significantly smaller than scrotal testes as showed in (Table 1) (*t*-test).

Table 1: The testis weight (mg) changes in cryptorchid models.

Days after operation	Uni-S	Cry-S	Cry-C	Con-S	*t*-test between Cry-C and Cry-S*
10	93.46 ± 0.67	88.68 ± 8.38	47.63 ± 5.88	90.85 ± 2.47	$P = 0.000$
14	98.47 ± 8.76	86.93 ± 5.57	30.53 ± 6.82	91.63 ± 9.62	$P = 0.000$

Uni-S are testes of the unilateral testis removal group on 10 and 14 days after the operation; Cry-S are scrotal testes of the unilateral cryptorchidism group on 10 and 14 days after the operation; Cry-C are abdominal testes of the unilateral cryptorchidism group on 10 and 14 days after the operation; Con-S are testes of the sham-operation with no significant difference between the day points. The testis weights in mg were shown as mean ± SD.
*Note: By t-test of random experimental design, doubly truncated to obtain P value.

Induction of apoptosis and necrosis in cryptorchid models

TUNEL-labeled slides show that apoptotic spermatocytesin abdominal testes (Cry-C) group occurred at day 10 in a significantly greater quantity, which reduced with the time, compared with those in the scrotal testes(Con-S), and MC were found in the tubules only at days 10 and 14 after operation (Table 2, Figure 1).

The paralleled distributions of Cdc2, Cyclin B1 and Cyclin B2 in spermatocytes revealed by immunohisochemistry

To explore the potential mechanism that apoptotic spermatocytesin abdominal testes occurred more than control, we found that there was non-specific reaction in controls to immunohistochemical detection (IHC). Cdc2, Cyclin B1, and Cyclin B2 signal were positive expression in spermatogonia, Sertoli cells, and spermatocytes in sham-operated mouse control (Figure 2). In the scrotal testis (Cry-S10, Cry-S14), the distribution pattern of Cdc2, Cyclin B1, and Cyclin B2 signal was found similar to the control. Similarly, in the abdominal testes (Cry-C10, Cry-C14), the positive immunohistochemical staining of Cdc2, Cyclin B1, and Cyclin B2 protein was also found in spermatogonia, Sertoli cells, and spermatocytes (Figure 2). In addition, in most AC and MC, staining of Cdc2, Cyclin B1, and Cyclin B2 were also observed, MC constitutes a large part of the loss of spermatogeniccells resulted from cryptorchidism, especially at the later stage (Cry-C10 and Cry-C14) (Figure 2).

The expression of Cdc2, Cyclin B1 and Cyclin B2 at mRNA and protein levels in cryptorchid testes

To further explore the differences of Cdc2, Cyclin B1, and Cyclin B2 expression at mRNA and protein levels in cryptorchid testes, we examined mRNA and protein expression of Cdc2, Cyclin B1, and Cyclin B2. The results show that Cdc2, Cyclin B1, and Cyclin B2 expressed in Cry-S10 group and Cry-S14 group testis was lower than that of the Con-S10 group and Con-S14 group testis at mRNA and protein level (Fig 3), while Cdc2, Cyclin B1 and Cyclin B2 expressed in Cry-C10 and Cry-C14 testis was

Table 2: The occurrences of apoptotic in cryptorchid testes.

Group	Apoptotic spermatocytes/tubule section	Multinucleated giant cells/tubule section
Uni-S10	0.13 ± 0.06	0
Uni-S14	0.16 ± 0.05	0
Cry-S10	0.18 ± 0.12	0
Cry-S14	0.20 ± 0.09	0
Cry-C10	3.71 ± 1.44*	2.11 ± 1.77*
Cry-C14	3.30 ± 2.00*	2.06 ± 1.21*
Con-S	0.22 ± 0.13	0

Uni-S10 and Uni-S14 are scrotal testes of the unilateral testis removal group on 10 and 14 days after the operation, respectively; Cry-S10, Cry-S14 are scrotal testes of the unilateral cryptorchidism group on 10 and 14 days after the operation, respectively; Cry-C10, Cry-C14 are abdominal testes of the unilateral cryptorchidism group on 10 and 14 days after the operation, respectively; Con-S is the pooled data of the sham-operation for there is no significant difference between the day points. Numbers were shown as mean ± SD. Notes: *indicates P < 0.01 compared with Con-S by Dunnett t (2-sided) test after one-way ANOV Aanalysis.

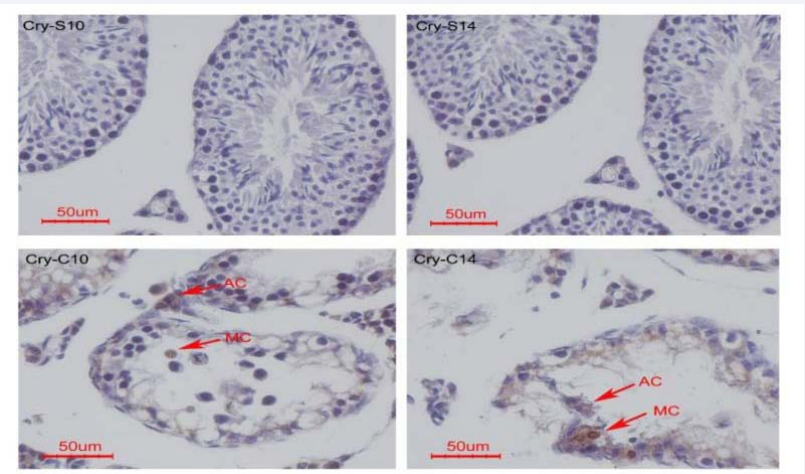

Figure 1 The multinuclear giant cells and apoptotic spermatocytes were presented individually by TUNEL Method in cryptorchid testes. Tunel apoptotic detection on sections of cryptorchid testes (Cry-C10) and contralateral scrotal testis (Cry-S10) on day 10 after the operation; and scrotal testis (Cry-S14) and abdominal testis (Cry-C14) on day 14 after the operation. The apoptosis positive spermatocytes and the apoptosis negative multinuclear giant cells (MC) were indicated by red arrowhead. Scale bar: 50μm.

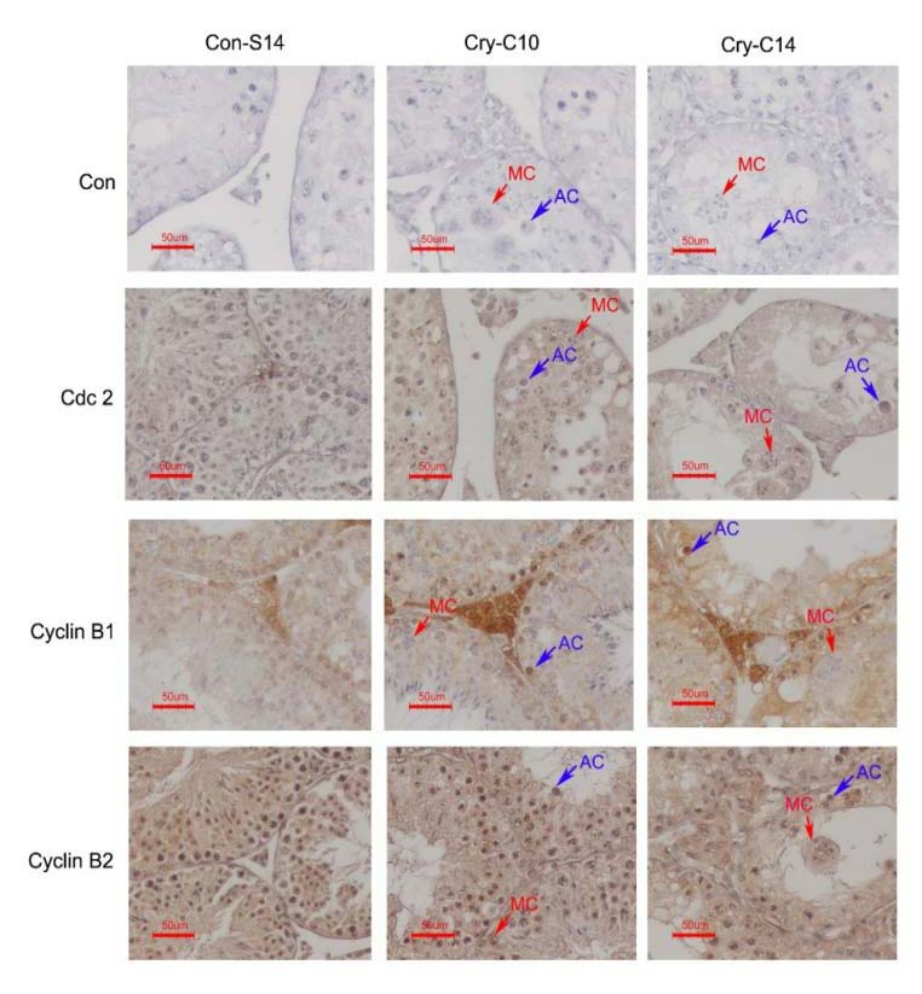

Figure 2 Immunohistochemical detection for Cdc2, Cyclin B1 and Cyclin B2 proteins in the experimental cryptorchidism. Negative controls are arranged in row 1, in which the primary antiserum was neutralized as outlined in the Method, here marked as Con; Cdc2, Cyclin B1 and Cyclin B2 immunohistochemical detections are arranged in row 2-4 in turn. Column Con-S14 is from scrotal testes of sham-operated group on 14 days after operation; Column Cry-C10 and Cry-C14 are from cryptorchid testes on 10 and 14 days after injury. In Column Cry-C10 and Cry-C14, AC: apoptotic spermatocytes; MC: multinuclear giant cells.

much less than that of the Con-S10 and Cry-S14 testis at mRNA and protein level (Figure 3). These findings reveal a possible positive relationship of Cdc2, Cyclin B1, and Cyclin B2 expression to apoptosis of spermatocytes.

DISCUSSION

It has been proved that high levels of expression of Cdc2, Cyclin B1, and Cyclin B2 in rat testis play an important role in spermatogenesis and sperm maturation [11,12]. Spermatogenesis block induced by high temperature is related to the reduced expression of Cdc2 and cyclin B1 [13]. However, in this study, we found that staining all subunits of MPF in most spermatogenic cells was positive and there has no significant difference between abdominal testis with others group testis. However, subunits of MPF (Cdc2, Cyclin B1 and Cyclin B2) expressed in abdominal testis was much less than that of the scrotal testes at mRNA and protein level. In addition, we found that weights of abdominal testes decreased significantly with the time after the operational injury, and the spermatogenetic cell removal in the cryptorchid testis was caused by the initial occurrence of AC and formation of MC subsequently.

Knudson et al. (1995) reported that the apoptosis cells and multinucleated giant cells in the cryptorchid testis are mainly from spermatocytes[14].Although apoptotic spermatocytes can lead to meiosis blockade, expression of the subunits of MPF (Cdc2, Cyclin B1, and Cyclin B2) did not observably decreased. However, the results of western blotting show that Cdc2, Cyclin B1, and Cyclin B2 was significantly reduced in cryptorchid testis. This difference may be caused by the unbalance of spermatogenetic cell apoptosis in which massive apoptosis of spermatocyte lead to the ratio of spermatogonia and Sertoli cells increased. Meanwhile, the mRNA levels of Cdc2, Cyclin B1, and Cyclin B2 reduced relative to spermatocytes in cryptorchidism also support this view. Thus, much apoptosis of spermatocyte is closely related to the decrease of subunits of MPF. According to our results, we need more evidence to demonstrate whether the reduction of the subunits of MPF is a cause or a result for the apoptosis of spermatocyte. However, the results of IHC show

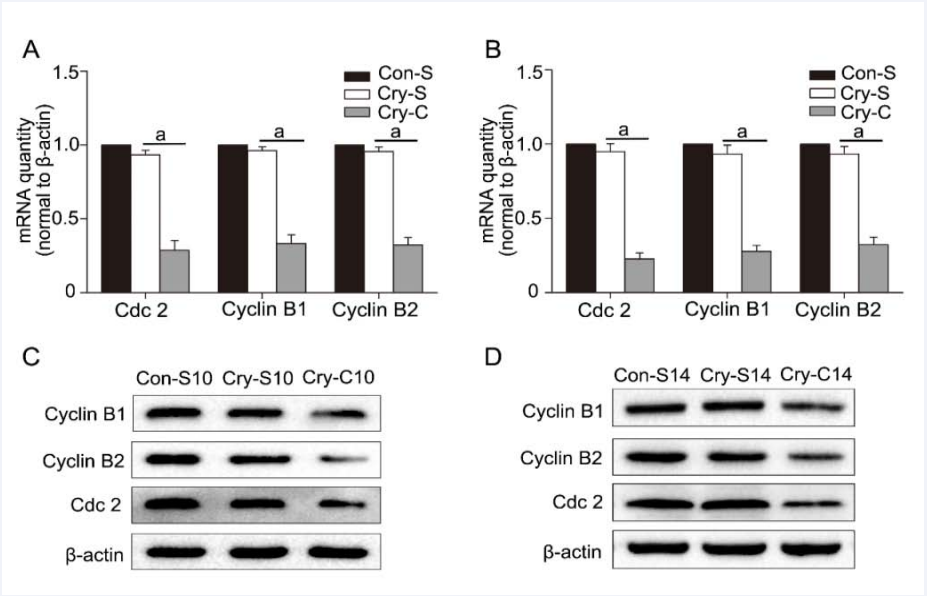

Figure 3 RT-PCR and Western blotting determine Cdc2, Cyclin B1 and Cyclin B2 expression at mRNA and protein level. The expression levels of Cdc2, Cyclin B1 and Cyclin B2 were detected in control testis, scrotal testis and abdominal testis on 10 (Con-S10, Cry-S10 and Cry-C10) and 14 (Con-S14, Cry-S14 and Cry-C14) days after operation. A: Expression of Cdc2, Cyclin B1 and Cyclin B2 in control testis, scrotal testis and abdominal testis on 10 days after operation at mRNA level. B: Expression of Cdc2, Cyclin B1 and Cyclin B2 in control testis, scrotal testis and abdominal testis on 14 days after operation at mRNA level. C: Expression of Cdc2, Cyclin B1 and Cyclin B2 in control testis, scrotal testis and abdominal testis on 10 days after operation at protein level. D: Expression of Cdc2, Cyclin B1 and Cyclin B2 in control testis, scrotal testis and abdominal testis on 14 days after operation at protein level. Notes: "a" indicates $P < 0.01$ compared with Cry-S.

that the subunits of MPF did not significantly reduce in apoptotic spermatocytes and multinucleated giant cells. Therefore, we can speculate that subunits of MPF decrement in cryptorchidism were caused by the reduction of spermatogenetic cells induced by spermatogenesis block. In addition, Skakkebaek et al (2005) pointed out that spermatocyte apoptosis, multinucleated giant cell formation, and spermatogenetic cell (shed form the wall of seminiferous tubule) were the main causes for reproductive barriers induced by cryptorchidism [15]. Expression of all subunits of MPF decrement in overall level was involved in those processes, but further research is needed to see whether the decrement of MPF results in spermatogenesis block. Overall, we demonstrate that the expression of all subunits of MPF in apoptotic spermatocyte and multinucleated giant cell fused by spermatocyte did not significantly reduce. We confirm that the relationship between obstacles of spermatocyte meiosis and the expression of MPF is not significant. However, spermatogenesis block may result in the decrease of gene expression at protein and mRNA level in cryptorchidism. Thus, this study promote us not only to retrospect the previous conclusion that subunits of MPF transcription was restrained in cryptorchidism, but also to focus on the relationship between the regulative activity of MPF and the apoptosis and fusion of spermatogenetic cells.

CONCLUSION

In conclusion, we demonstrated that subunits of MPF, which include Cdc2, Cyclin B1, and Cyclin B2, did not decrease in apoptotic spermatocytes and multinucleated giant cells fused by spermatocytes. However, the overall expression of Cdc2, Cyclin B1, and Cyclin B2 in abdominal testes was lower than that of the control testis at mRNA and protein level, which maybe reflects the loss of spermatocytes with high expression of MPF subunits. These results showed that the relationship between MPF and spermatogenetic cells apoptosis and fusion is complex, and need to further study.

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