

## Temperature dependent expression of *cdc2* and *cyclin B1* in spermatogenic cells during spermatogenesis

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### ABSTRACT

p34<sup>cdc2</sup> and Cyclin B1 are key components of cell cycle controlling machine and are believed to play a fundamental role in gametogenesis. It is also well known that, in scrotal mammals, spermatogenesis depends greatly on the maintenance of comparatively low temperature in the scrotum. To investigate whether the expression of *cdc2* and *cyclin B1* in spermatogenic cells during spermatogenesis is actually a temperature dependent event, in situ hybridization, Western blotting and immunohistochemistry analysis were used to study the expression of *cdc2* and *cyclin B1* in normal and cryptorchid testis. Results showed that the abdominal temperature had no significant influence on the transcription of *cdc2* and *cyclin B1* in the spermatogonia and pachytene/diplotene primary spermatocytes, but it blocked the translation of them. Due to the deficiency of p34<sup>cdc2</sup> and Cyclin B1, the spermatogonia and pachytene/diplotene primary spermatocytes were unable to form MPF, hence, they couldn't undergo karyokinesis. The development of primary spermatocytes was arrested at the G2 to M phase transition. We also found that testosterone could regulate the Cyclin B1 expression in spermatogenic cells. Muscular injection of testosterone could recover spermatogenesis in the unilateral scrotal testis which was influenced by the contralateral cryptorchid testis, but it could not salvage the spermatogenesis block in the cryptorchid testis.

**Key words:** *Temperature, spermatogenesis, cdc2, cyclin B1.*

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## INTRODUCTION

Temperature influences the development of germ cells and the reproductive cycle of living beings. Gametogenesis in many kinds of insects, lower vertebrate animals and hibernating animals as well as scrotal mammals depends on a suitable low- temperature condition to generate germ cells and acquires maturation competence thereafter.

Among mammals, generation of male germ cells depends mostly on the comparatively low temperature condition in scrotum. The temperature rise of testis induced by cryptorchidism, extreme heat in summer, body fever, tight clothing, sauna or other occupational high temperature conditions will cause the block or abnormality of spermatogenesis[1]. So far, the studies on the temperature influence on heat shock protein, heat shock factor, cold-inducible RNA binding protein, insulin-like growth factor-I receptors, ubiquitin,  $\alpha$ -,  $\beta$ - and  $\gamma$ -DNA polymerase and topoisomerase-I as well as some other enzymes have been made. However, no conclusion is reached that these changes have direct connection with the block of spermatogenesis[2]. Spermatogenesis is an accurately controlled and timed process. It includes mitotic divisions of spermatogonia, meiotic divisions of spermatocytes, the differentiation and maturation of spermatids and so on. It is well known that all cell divisions are inspired by activated maturation-promoting factors (MPF) which consists of catalytic subunit p34<sup>cdc2</sup> and regulatory subunit Cyclin B1. The activation of MPF can be achieved through sequential phosphorylation and dephosphorylation of tyrosine and threonine residues within the ATP-binding-site of p34<sup>cdc2</sup> subunit[3]. It has been demonstrated that high levels of expression of cdc2 and cyclin B1 in rat and mouse testis play an important role in spermatogenesis and sperm maturation[4-7]. So far, there has been no report on whether the spermatogenesis block induced by high temperature is related to the abnormal expression of cdc2 and cyclin B1. In this study, we studied the influence of temperature on the expression of cdc2 and cyclin B1 in rabbit spermatogenic cells and discussed the possible mechanism of spermatogenesis block by abdominal high temperature.

## MATERIALS AND METHODS

### *Animals*

Adult male New Zealand white rabbits weighted 5 kg were obtained from animal facilities of Shanghai Children's Hospital. After the rabbits were anesthetized by injecting pentobarbital sodium (24 mg/kg) via ear vein, one testis of each "test rabbit" was brought into the abdomen, sutured to the inner surface of its wall and designated as contralateral cryptorchid testis, while the other testis remained in the scrotum to act as an euthermic control, designated as unilateral scrotal testis. The "control rabbits" were performed a pseudo-operation and their testes were designated as control testes. 20 d (the duration of spermiogenesis in rabbit) later, half of the "test rabbits" were given testosterone (5 mg/day) by intra-femoral muscle injection for 38 d. On the 58th day (the average duration of spermatogenesis in a rabbit is about 2 months [8]) after the surgical operation, the testes were dissected for analysis.

### *Preparation of slides*

The testes slices were fixed at 4°C overnight in 4 % paraformaldehyde and embedded in paraffin. The paraffin embedded slices were cut into 7  $\mu$ m sections which were placed onto Silane or Gelatin coated slides for in situ hybridization or immunohistochemistry analysis respectively.

### *In situ hybridization analysis*

The pLXSN- cdc2 and pLXSN- cyclin B1 constructs were kindly provided by Dr. A. Koff[9] and Dr. J. Pines[10] respectively. The cdc2 and cyclin B1 cDNA were obtained from above plasmids through digestion with BamH I and EcoR I endonuclease restrictedly and then reconstructed into pSPT18 vector. Amplification, purification and identification of pSPT18- cdc2 and pSPT18- cyclin B1 plasmids were performed as described in reference[11]. The linearized pSPT18- cdc2 and pSPT18- cyclin B1 were obtained by digestion with EcoR I or Hind III and then transcript in vitro with T7 or SP6 RNA polymerase (DIG RNA labeling kit, Bohringer Mannheim) into sense or antisense DIG-cRNA probes, respectively[12].

In situ hybridization was performed according to the descriptions of application manual [12]. Antisense DIG-actin cRNA probe (300 ng/ml) was used as positive control, antisense DIG-cdc2 or DIG- cyclin B1 cRNA probe (300 ng/ml) was used to detect cdc2 or cyclin B1 transcripts respectively, while sense DIG- cdc2 or DIG- cyclin B1 cRNA probe (300 ng/ml) was used as negative control. Non-probe and non anti-DIG antibody tests were also performed.

### *Western blotting analysis*

Total proteins were extracted by homogenizing pieces of testis from “control” or “test” animals in EB (Extraction buffer: 20 mM Hepes, pH 7.5, containing 80 mM  $\beta$ -glycerophosphate, 20 mM EGTA, 15 mM MgCl<sub>2</sub>, 200  $\mu$ M PMSF, 50 mM NaF, 1 mM DTT and 3  $\mu$ g/ml leupeptin), followed by ultracentrifugation (100,000  $\times$  g, 1 h, 4°C). Proteins were separated by SDS-PAGE in 12 % gel and transferred to nitrocellulose membranes. The membranes were rinsed in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1 % Tween-20) and blocked with block solution (5 % non-fat milk in TBST) for 30 min, followed by antibody (anti-PSTAIRES polypeptide polyclonal antibody, 5  $\mu$ g IgG/ml, or anti-Cyclin B1 monoclonal antibody, 0.4  $\mu$ g IgG/ml, Santa Cruz Biotechnology Inc.) incubation in block solution for 1 h. After washing (3  $\times$  5min) with TBST, the membranes were then incubated with a 1:1,000 HRP-conjugated goat anti-mouse IgG (Sigma) in block solution for 30 min. Then the membranes were again washed (3  $\times$  10 min) with TBST, the western blotting analysis was performed with ECL (Amersham).

### *Immunohistochemical analysis*

The dewaxed and rehydrated sections were incubated in 10  $\mu$ g/ml Saponin for 30 min, and then washed (3  $\times$  5 min) with TBS (10 mM Tris-HCl, pH 8.0 and 150 mM NaCl). After being blocked with block solution (1.5 % normal sheep serum in TBS) for 1 h, the specimens were incubated with antibody (anti-p34<sup>cdc2</sup> or anti-Cyclin B1 monoclonal antibody, 2  $\mu$ g IgG/ml, Santa Cruz Biotechnology) in block solution for 30 min. Having been washed (3  $\times$  5 min) with block solution, the sections were incubated with biotin-conjugated sheep anti-mouse IgG (Vector Laboratories) in block solution for 30 min, washed with TBS for 3  $\times$  5min, then incubated for 30 min with avidin biotin enzyme reagent, they were washed again with TBS for 3  $\times$  5 min, rinsed in 1 % Triton X-100/TBS and in distilled water for 30 seconds respectively. Each section was color reacted and mounted as per se in the reference[12].

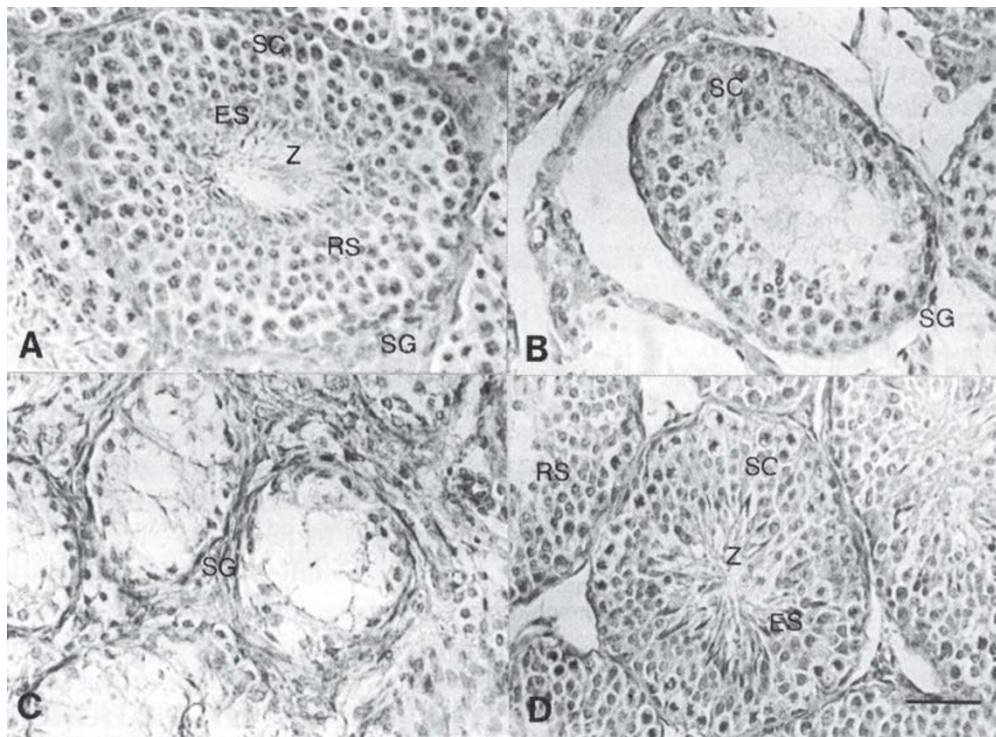
The negative controls were performed by using normal sheep serum in substitution for primary and secondary antibody respectively.

## **RESULTS**

### Effects of temperature on spermatogenesis in rabbit testis

## Temperature influences expression of *cdc2* and cyclin B1 in spermatogenic cells

Spermatogonia, spermatocytes, round spermatids, elongated spermatids and spermatozoa can be found in the seminiferous epithelium of the control testis (Fig 1A), while the number of spermatids and elongated spermatids decreased in the seminiferous tubules of unilateral scrotal testis (Fig 1B). In the contralateral cryptorchid testis, there were no spermatogenic cells in all stages of seminiferous epithelium except spermatogonia and some pachytene/diplotene primary spermatocytes (Fig 1C). The interstitial tissue of the contralateral cryptorchid testis appeared to be atrophied. Intramuscular injection of testosterone could make the spermatogenesis recover in the unilateral scrotal testis (Fig 1D), but it could not improve the spermatogenesis block in the contralateral cryptorchid testis.



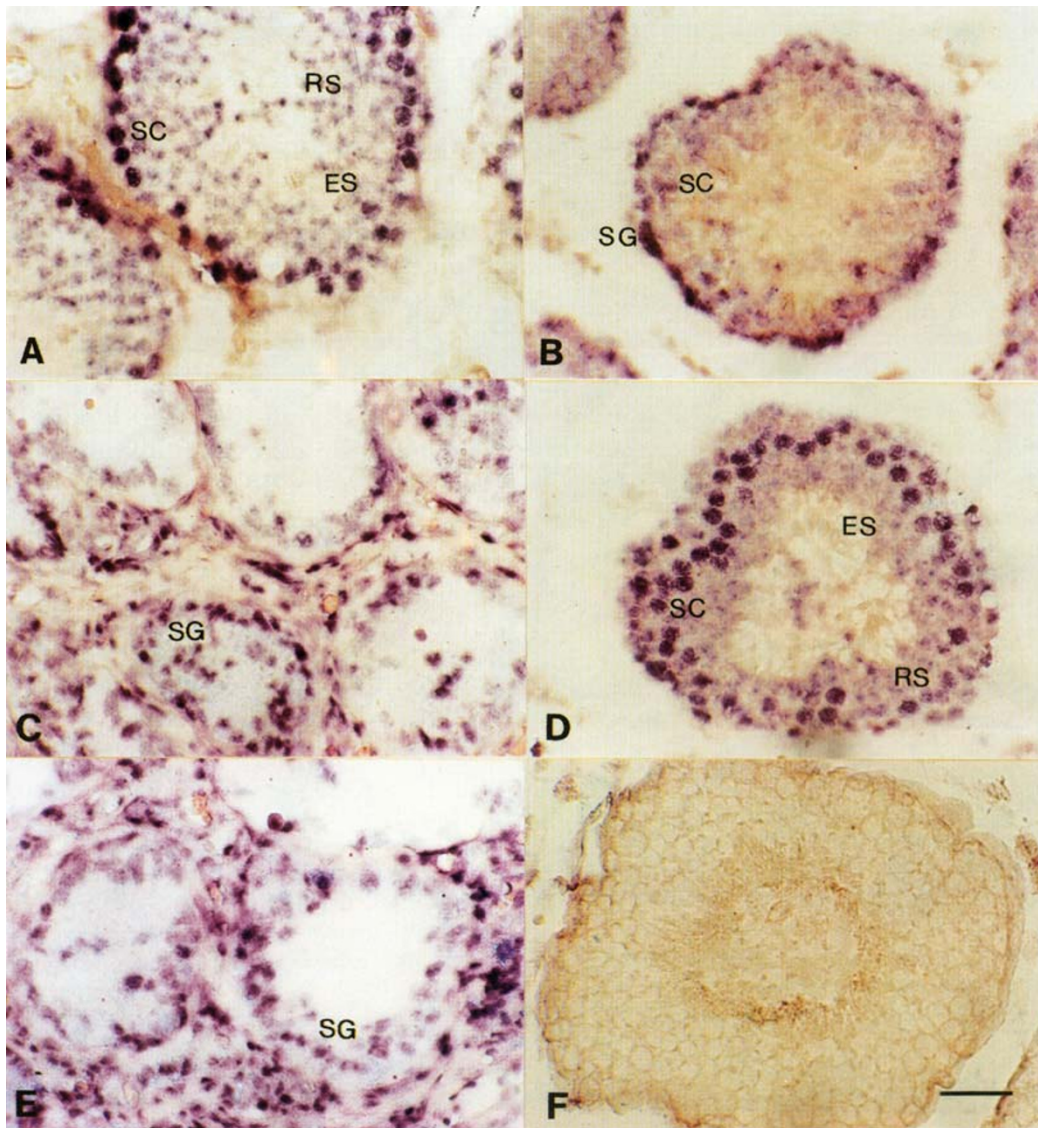
**Fig 1.** Effects of temperature on spermatogenesis in rabbit testis **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis **D:** Unilateral scrotal testis after treatment with testosterone **SG:** Spermatogonium, **SC:** Spermatocyte, **RS:** Round Spermatid, **ESm:** Elongated Spermatid, **Z:** Spermatozoon. Black bar: 50  $\mu$ m

### *Effects of temperature on the transcription of cyclin B1 in rabbit testis*

Significant cyclin B1 transcripts could be detected in the spermatogonia and pachytene/diplotene spermatocytes of the control testis (Fig 2A) but the signal slightly decreased in spermatocytes of unilateral scrotal testis (Fig 2B). In the contralateral cryptorchid testis, we still detected the signal in the survived spermatogonia and



pachytene/diplotene primary spermatocytes (Fig 2C). Treatment of testosterone could restore cyclin B1 transcription in spermatogenic cells of unilateral scrotal testis to normal level (Fig 2D), but the effect on the survived spermatogonia in contralateral cryptorchid testis was not so distinct (Fig 2E compare with 2C).

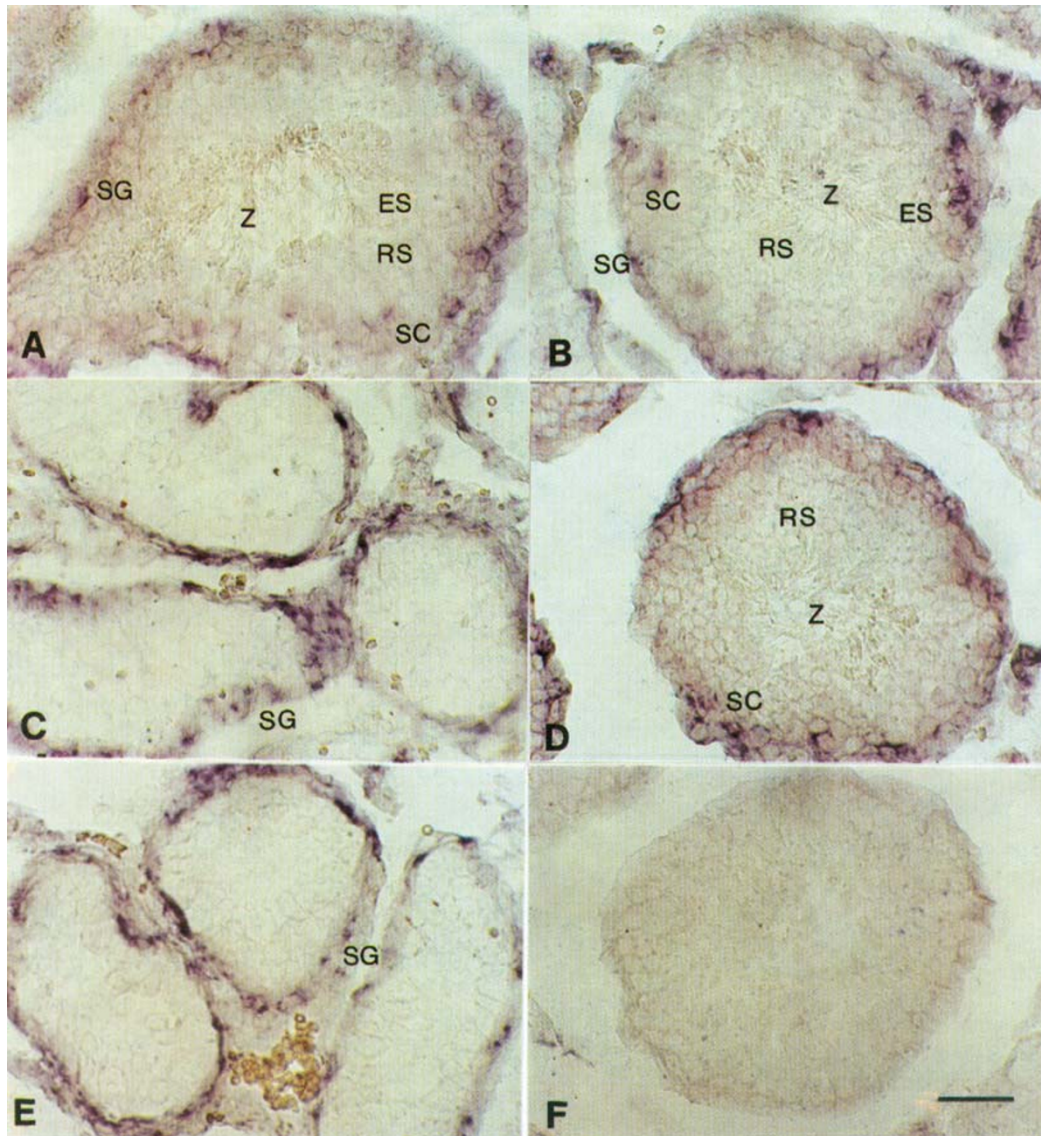


**Fig 2.** In situ hybridization analysis of the cyclin B1 transcripts in rabbit testis **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis **D:** Unilateral scrotal testis after treatment with testosterone **E:** Contralateral cryptorchid testis after treatment with testosterone **F:** Negative control **SG:** Spermatogonium, **SC:** Spermatocyte, **RS:** Round Spermatid, **ES:** Elongated Spermatid, Black bar: 50  $\mu$ m

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*Effects of temperature on the transcription of cdc2 in rabbit testis*

In the seminiferous epithelium of control and unilateral scrotal testis, *cdc2* transcripts in spermatogonia and pachytene/diplotene spermatocytes were fewer than those



**Fig 3.** *In situ* hybridization analysis of the *cdc2* transcripts in rabbit testis **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis **D:** Unilateral scrotal testis after treatment with testosterone **E:** Contralateral cryptorchid testis after treatment with testosterone **F:** Negative control **SG:** Spermatogonium, **SC:** Spermatocyte, **RS:** Round Spermatid, **ES:** Elongated Spermatid, **Z:** Spermatozoon. Black bar: 50 $\mu$ m



of cyclin B1 (Fig 3A, 3B), and mainly present in the cytoplasm. Weak signals of the *cdc2* were detected in the remains of spermatogenic cells in contralateral cryptorchid testis too (Fig 3C). Testosterone treatment could improve *cdc2* transcription in spermatocytes but not in spermatogonia of the unilateral scrotal testis (Fig 3D). However, the *cdc2* expression was not influenced by testosterone both in spermatogonia and in spermatocytes of contralateral cryptorchid testis (Fig 3E).

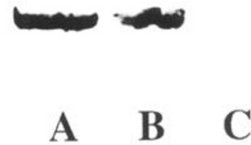
There was no positive signal detected with the *cdc2* and cyclin B1 sense probe, as well as non-probe and non-anti-DIG antibody (Fig 2F, 3F).

#### *Effects of temperature on the translation of p34<sup>cdc2</sup> and Cyclin B1 in rabbit testis*

Western blotting analysis showed that Cyclin B1 expressed in unilateral scrotal testis (Fig 4B) was significantly lower than that of the control testis (Fig 4A), while Cyclin B1 could not be detected in contralateral cryptorchid testis (Fig 4C). After the treatment of testosterone, Cyclin B1 expression increased in unilateral scrotal testis (Fig 5B) and reached normal level as compared with control testis (Fig 5A), but still could not be detected in contralateral cryptorchid testis (Fig 5C). In the unilateral scrotal testis (Fig 6B), p34<sup>cdc2</sup> expression was almost the same as that of the control testis (Fig 6A), but was not detected in the contralateral cryptorchid testis (Fig 6C). The injection of testosterone had little effects on p34<sup>cdc2</sup> expression in both the unilateral scrotal (Fig 6D) and contralateral cryptorchid testis (Fig 6E).



**Fig 4.** Western blotting analysis of the effects of temperature on expression of Cyclin B1 **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis Each lane was loaded with 240  $\mu$ g proteins



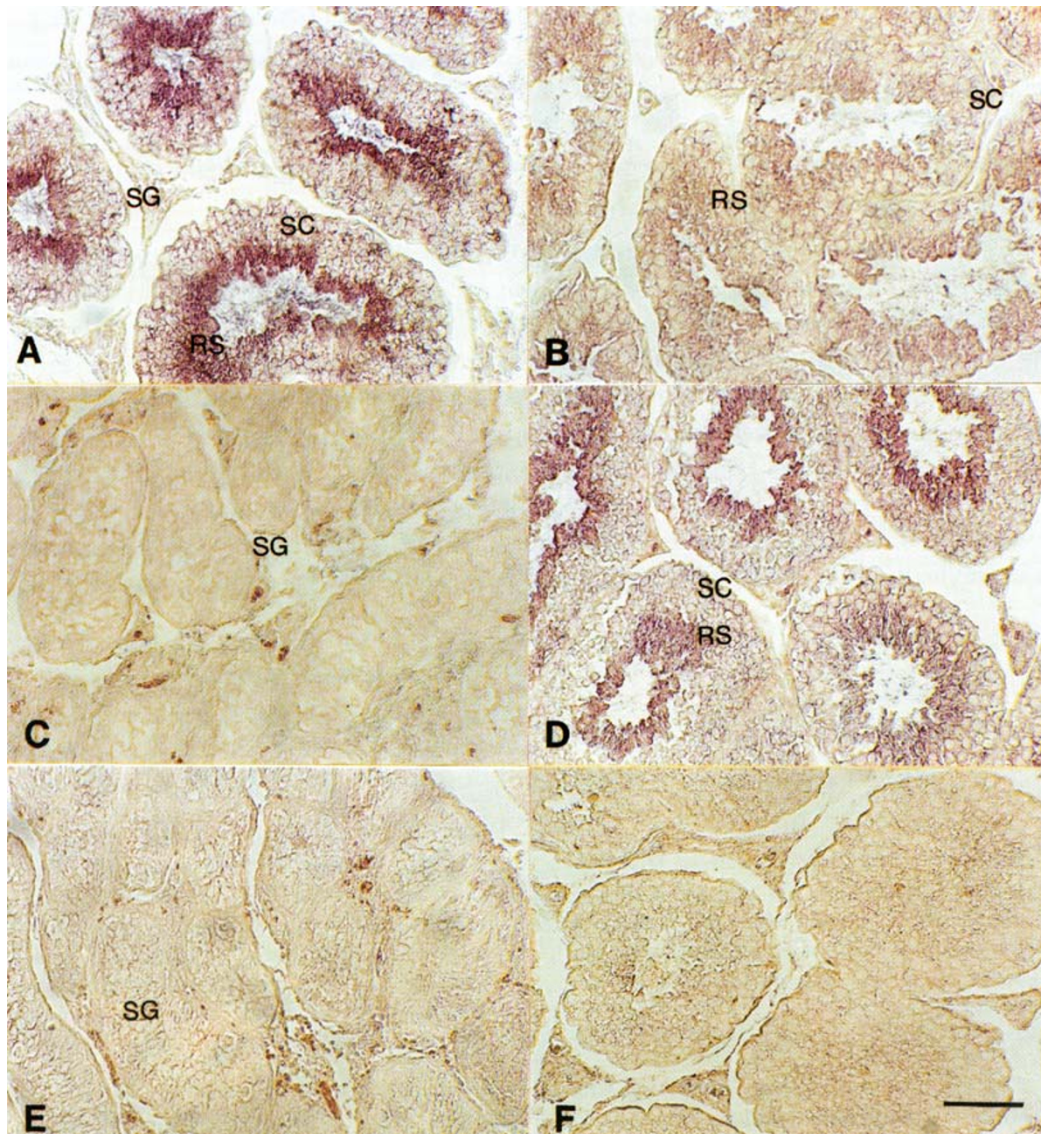
**Fig 5.** Western blotting analysis of expression of Cyclin B1 after treatment with testosterone **A:** Control testis **B:** Unilateral scrotal testis after treatment with testosterone **C:** Contralateral cryptorchid testis after treatment with testosterone Each lane was loaded with 120  $\mu$ g proteins

**Fig 6.** Western blotting analysis of the effects of temperature and testosterone on the expression of p34<sup>cdc2</sup> **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis **D:** Unilateral scrotal testis after treatment with testosterone **E:** Contralateral cryptorchid testis after treatment with testosterone Each lane was loaded with 120  $\mu$ g proteins



### Temperature influences expression of *cdc2* and cyclin B1 in spermatogenic cells

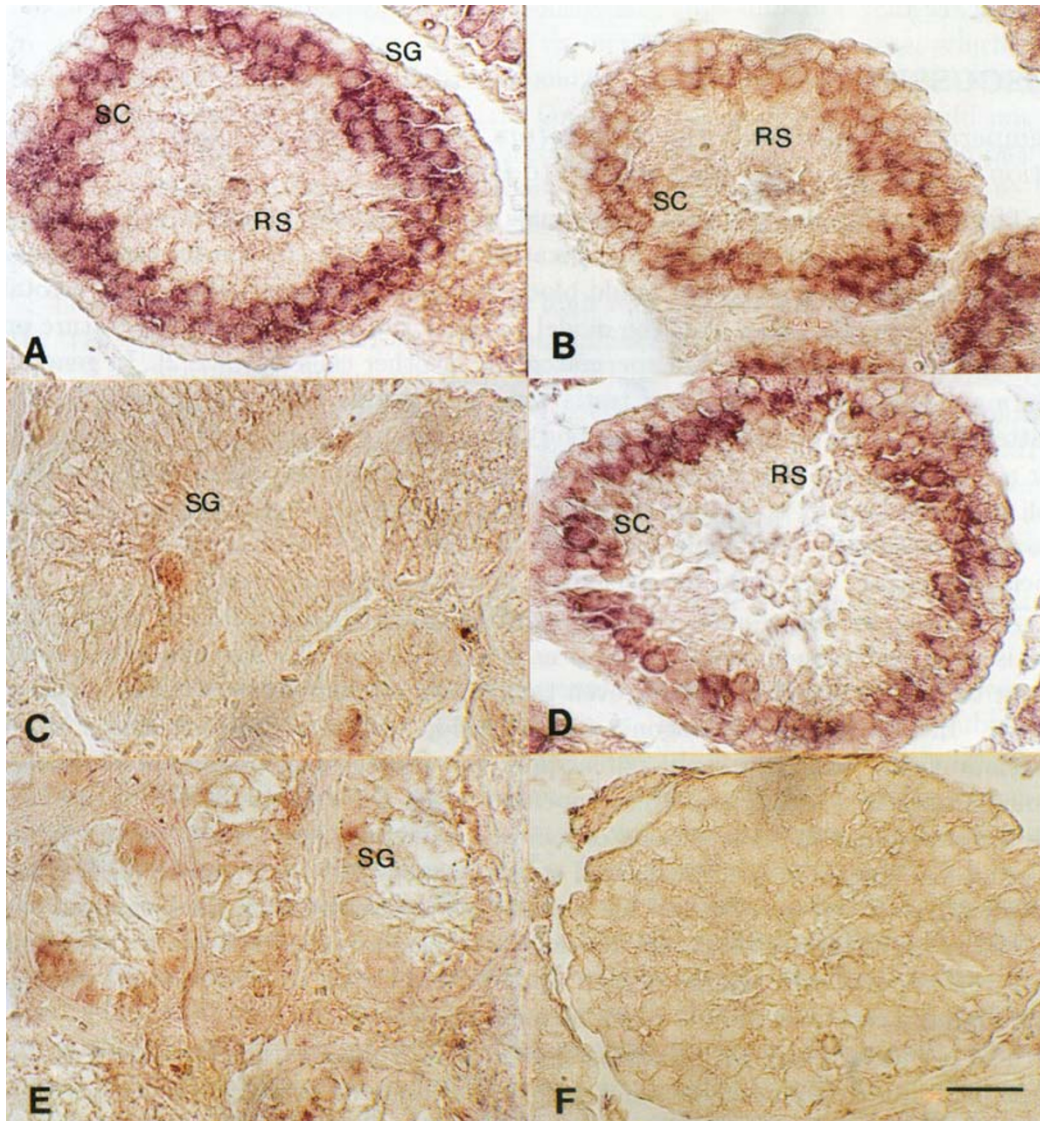
The immunohistochemical analysis results showed that Cyclin B1 existed in the spermatogenic cells of different developmental stages in the control testis and increased markedly in the round and elongated spermatids (Fig 7A). In unilateral scrotal testis, Cyclin B1 was significantly fewer than that of the control testis (Fig 7B). In contralateral-



**Fig 7.** Immunohistochemical analysis of the effects of temperature and testosterone on the expression of Cyclin B1 **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis **D:** Unilateral scrotal testis after treatment with testosterone **E:** Contralateral cryptorchid testis after treatment with testosterone **F:** Negative control **SG:** Spermatogonium, **SC:** Spermatocyte, **RS:** Round Spermatid, **Black bar:** 100  $\mu\text{m}$



eral cryptorchid testis, Cyclin B1 could not be detected (Fig 7C). Though testosterone treatment could induce Cyclin B1 increase in the unilateral scrotal testis (Fig 7D), it was still unable to improve Cyclin B1 expression in contralateral cryptorchid testis (Fig 7E). p34<sup>cdc2</sup> was expressed mainly in the pachytene/ diplotene primary spermatocyte of control testis (Fig 8A). The expression of p34<sup>cdc2</sup> in the unilateral scrotal testis was



**Fig 8.** Immunohistochemical analysis of the effects of temperature and testosterone on the expression of p34cdc2 **A:** Control testis **B:** Unilateral scrotal testis **C:** Contralateral cryptorchid testis **D:** Unilateral scrotal testis after treatment with testosterone **E:** Contralateral cryptorchid testis after treatment with testosterone **F:** Negative control **SG:** Spermatogonium, **SC:** Spermatocyte, **RS:** Round Spermatid, **Black bar:** 50  $\mu$ m

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slightly weaker than that of the control testis (Fig 8B) while very few of p34<sup>*cdc2*</sup> could only be detected in some spermatogonia of the contralateral cryptorchid testis (Fig 8C). Testosterone injection could ameliorate p34<sup>*cdc2*</sup> expression in unilateral scrotal (Fig 8D) but no in contralateral cryptorchid testis (Fig 8E). The results above were accordant with those of Western blotting. We did not observe any significant immunostaining in the negative control groups (Fig 7F, 8F).

## DISCUSSION

*Temperature higher than that in scrotum leading to the blocking of the transition of male germ cell from mitosis to meiosis*

The spermatogenesis of scrotal mammals depends on the comparatively low temperature condition in scrotum. Temperature higher than the scrotal temperature (e. g. abdominal temperature) would block the spermatogenesis. Hence, the scrotal mammals were often chosen as the model to study the influences of temperature on the structures and functions of spermatogenic or other cells in testis[2]. In general, spermatogenic and other cells of testis were sensitive to heat, and if testis is submitted even to the so called “normal body temperature”(such as 37 °C in humans) for a long time, not only spermatogenesis will be blocked, but also Leydig and Sertoli cells will be hurt to a great extent[2], [13]. Cytological studies showed that heat would differentially hurt male germ cells in different developmental stages during spermatogenesis, especially the pachytene primary spermatocytes[2], [13].

According to our results, most of spermatogonia in contralateral cryptorchid testis were not harmed fatally by heat as yet, indicating that spermatogonia could resist to heat to a certain extent, even though the mechanism involved is not clear so far. In this case spermatogonia could develop to pachytene/diplotene primary spermatocytes, but they could not acquire the ability to complete the transition from mitosis to meiosis, and then appeared to go through apoptosis. Therefore, we could not find the descendants of meiosis: secondary spermatocytes and round spermatids, elongated spermatids and spermatozoon. It is well known, MPF is the major driver of cell division and all factors that interfering with the formation and activation of MPF will block cell division. We therefore analyzed the transcription and translation of each subunit of MPF (p34<sup>*cdc2*</sup> and Cyclin B1) to reveal the possible underlying mechanism of heat block of spermatogenesis.

The results of in situ hybridization showed that cyclin B1 transcripts were mainly expressed in the spermatogonia and pachytene/diplotene primary spermatocytes of control testis. With the advance of cell division, the hybridization signal decreased gradually from spermatocytes to round spermatids. When the spermatids become elongated, cyclin B1 transcripts were disappeared. *cdc2* was only transcribed in the spermatogonia and pachytene/diplotene primary spermatocytes before meiosis. In the contralateral cryptorchid testis, the cyclin B1 and *cdc2* transcripts still could be detected in

spermatogonia and pachytene/diplotene primary spermatocytes, which means that high temperature did not considerably block the transcription of cyclin B1 and *cdc2* in these cells.

The results of immunohistochemistry displayed that p34<sup>*cdc2*</sup> was expressed mainly in both spermatogonia and pachytene/diplotene primary spermatocytes. Cyclin B1 increased in the pachytene/diplotene primary spermatocytes before meiosis and reached its peak in the spermatids. Obviously, large amounts of Cyclin B1 existing in spermatids has no effect on adjusting the activity of p34<sup>*cdc2*</sup> kinase, which may be just a signal of the end of cell division and/or a necessary factor for the initiation of the metamorphosis of spermatids[4]. On the contrary, Cyclin B1 could not be detected in all cell types of the contralateral cryptorchid testis, even though p34<sup>*cdc2*</sup> could be detected in some spermatogonia, a fact which could be referred to the inhibitory effect of high temperature on periodic biosynthesis of Cyclin B1 and p34<sup>*cdc2*</sup>[14].

These results showed that abdominal high temperature did not affect seriously the transcription of cyclin B1 and *cdc2*, but inhibit the translation of Cyclin B1 and p34<sup>*cdc2*</sup> in the spermatogonia and pachytene/diplotene primary spermatocytes. Due to the deficiency of Cyclin B1 and p34<sup>*cdc2*</sup>, spermatogonia and pachytene/diplotene primary spermatocytes in contralateral cryptorchid testis were unable to form MPF, and therefore, they could not accomplish their nuclear division, and became arrested at the transition stage from mitosis to meiosis (G2/M phase).

#### *Testosterone is capable of adjusting the Cyclin B1 expression at translation level*

Our results showed that the spermatogenesis in unilateral scrotal testis was influenced by the contralateral cryptorchid testis. In some seminiferous tubules of unilateral scrotal testis, the number of round spermatids and metamorphosing spermatids decreased significantly and, in some of them even could not be observed. In the meantime, the expression of Cyclin B1 was also decreased. To examine the possible mechanism of these changes, half of “test rabbits” were treated with testosterone. Although testosterone injection could not improve blocked spermatogenesis in contralateral cryptorchid testis, it could recover spermatogenesis and increase the Cyclin B1 expression to almost the normal level in unilateral scrotal testis. These results indicated that testosterone could regulate Cyclin B1 expression in spermatogenic cells of unilateral scrotal testis. It has been reported that the metamorphosis of round spermatids depended on testosterone during spermatogenesis, but the mechanism was not mentioned [15]. As cryptorchidism could cause the decrease of testosterone level in many kind of animals[16-18], we believe that the decrease of Cyclin B1 expression due to the shortage of testosterone supply may be the main reason for the decrease and deficiency of round spermatids and other metamorphosing spermatids in unilateral scrotal testis. Therefore, we conclude that the spermatogenesis in scrotal mammals need to proceed not only in a comparatively low temperature condition, but also in a condition with



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adequate supply of testosterone to maintain the normal spermatids, especially their metamorphosis.

#### *High temperature inducing the deletion of MPF and the apoptosis of spermatogenic cells*

Abdominal temperature will increase the risk of apoptosis in spermatogenic cells, but its mechanism is not clear[18-20]. Though it is reported that p53 plays an important role in heat induced spermatogenic cell apoptosis[21], [22], but apoptosis also appears in the mouse cryptorchid testis which lacks p53[22]. So it is possible that the expression of p53 may not be the direct reason for heat induced spermatogenic cell apoptosis and spermatogenetic block[22], [23]. Eddy reported that if Cyclin B1 could not combine with p34<sup>*cdc2*</sup> to form heterodimer, the spermatogenesis would be blocked at the G2/M transition of the first meiosis, then all pachytene primary spermatocytes would go through apoptosis, leading finally to spermatids depletion[24]. Taken together, the above data lead us to infer that MPF is one of the most important factors that not only can promote cell division, but also prevent excessive apoptosis in spermatogenic cells during spermatogenesis.

#### *The possible activity of Y-box binding protein in spermatogenesis*

It is well known that low temperature can induce and impel the synthesis of some proteins. Among them, cold-shock protein is the most conspicuous. Y-box (inverted CCAAT box) binding proteins are special members of cold-shock protein family, which can bind DNA as well as mRNA to alter both transcription and translation of cells[25]. Like RNA chaperon, the conserved sequence of Y-box binding proteins destabilizes the RNA secondary structure and regulates cell translation in low temperature[26]. As Y-box binding proteins exist widely in the spermatocytes of scrotal mammals[27], we believe that they may have important effects on the inverted CCAAT box of *cdc2* gene promoter, and then control the transcription of p34<sup>*cdc2*</sup>. Meanwhile, they can also combine with cyclin B1 mRNA and play significant roles in cell cycle[28]. Recent report showed that a cold-shock protein, Rbm3, was decrease in Sertoli cells when mouse testis was exposed to heat stress by experimental cryptorchidism[29]. Thus, we would like to speculate that the further studies on the cold-shock proteins of spermatogenic cells at the scrotal low temperature may give insight to uncover the functions of cold- shock proteins in relation to spermatogenesis, including the control mechanism of gene transcription and translation in heat induced spermatogenetic block.

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