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·*Original Article*·

# Spatial and temporal expression of *c-mos* in mouse testis during postnatal development

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**Abstract**

**Aim:** To immunolocalize the c-mos gene product and to investigate its spatial and temporal expression in mouse testis during postnatal development. **Methods:** Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization techniques were used to examine *c-mos* mRNA and indirect immunofluorescence was used to localize c-Mos protein in mouse testis on postnatal days 14, 21, 25, 28, 30, 35, 49 and 70. **Results:** *c-mos* mRNA remained low on postnatal days 14–21, increased abruptly from day 25 and peaked on day 30. Its levels decreased a little on day 35 and became almost stable thereafter until day 70. *c-mos* mRNA was localized in the nucleus and cytoplasm of the spermatocytes and round spermatids. The nuclear staining was much stronger than the cytoplasmic staining. Using a polyclonal anti-c-Mos antibody, Western blotting detected a single band at 43 kDa in testis lysate. c-Mos protein was exclusively localized to the elongating spermatids and was first detected on postnatal day 30. The number of c-Mos-positive spermatids increased progressively till day 49 and stabilized thereafter. **Conclusion:** The c-mos gene displays a spatial and temporal expression pattern in the mouse testis during postnatal development at both the mRNA and protein level. This suggests that *c-mos* might play important roles in spermatogenesis. *(Asian J Androl 2008 Mar; 10: 277–285)*

**Keywords:** proto-oncogene; *c-mos*; spermatogenesis; postnatal development

## 1 Introduction

Proto-oncogenes, such as *c-abl* [1] and *int-1* [2], are expressed in a developmentally regulated pattern in germ cells. The expression of specific genes in unique spatial and/or temporal patterns is believed to be important in regulating development and differentiation. The cellular and stage specificity of expression of these genes suggests that their gene products might function during germ cell development.

The proto-oncogene *c-mos* encodes a widely expressed protein serine/threonine kinase. Transcripts of *c-mos* are highly expressed in the mouse reproductive tract (testis, ovary and epididymis) and in near-term embryos and in rat testis and embryos [3]. *c-mos* mRNA

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has also been detected in undifferentiated teratocarcinoma cells [4]. Interestingly, *c-mos* transcripts decrease in teratocarcinoma cell lines stimulated to differentiate into embryo-like tissues. These observations suggest a role for *c-mos* in mammalian reproduction and in very early stages of development.

*c-mos* product accumulates during postnatal development in the skeletal muscle and exhibits protein kinase activity [5], suggesting that *c-mos* might play a role in skeletal muscle development.

Research of *c-mos* function has recently been focused on the process of oocyte maturation. As a protein kinase, it plays an important role in upregulating mitosis promoting factor (MPF) activity at various stages of final oocyte maturation. The c-mos-MPF system is associated with important features of the last stages of oocyte maturation and cell cycle control [6]. In contrast, its function in male reproduction is poorly understood. A 60-kDa Mos protein has been identified from the testis of the anuran amphibian, *Rana esculenta*, and it was proven to exert a new role associated with the regulation of spermatogonial proliferation [7]. The purpose of the present study is to localize the c-mos gene and to investigate its temporal and spatial expression pattern in mouse testis during postnatal development. The results are then discussed with regard to the possible functions of the c-mos gene during spermatogenesis.

## 2 Materials and methods

### *2.1 Animals*

Intact male BALB/c mice were obtained from the Animal Center of the Chinese Academy of Sciences (Shanghai, China), and were divided into eight groups according to their postnatal age (14, 21, 25, 28, 30, 35, 49 and 70 days after birth). The testes were removed immediately after the animals were killed by cervical dislocation. The tissues were either homogenized for Western blot assay, stored in RNAlater (Ambion, Austin, TX, USA) for reverse transcription-polymerase chain reaction (RT-PCR), or fixed in 4% paraformaldehyde or in Bouin's solution for *in situ* hybridization or indirect immunofluorescence, respectively.

### *2.2 Materials*

An RNA easy extract kit was purchased from Qiagen (Hilden, Germany). rTaq DNA polymerse, gel extraction kit, T vector and T4 DNA ligase were from Takara (Dalian, China). The first strand cDNA synthesis kit, the restriction endonucleases and the expression vector pET28a(+) were from Promega (Madison, WI, USA). Isopropylthio-β-D-galctopyrano-side (IPTG), *Escherichia coli* host DH16B and competence *E. coli* BL21 were from Tiangen (Beijing, China). Freuds adjuvant and FITC-labeled goat anti-rabbit IgG were from Sigma (St. Louis, MO, USA). The IgG purification kit was from Millipore (Billerica, MA, USA). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG was from DingGuo (Shanghai, China). Protein molecular weight markers were from Fermentas (Burlington, Ontario, CA, USA). All other chemicals were of analytical grade.

### *2.3 Extraction of RNA*

Total cellular RNA was extracted from normal BALB/c mouse testis using RNA easy extract kit according to the manufacturer's protocol. DNase was added to eliminate genomic DNA contamination. The purity of RNA was confirmed by formaldehyde denaturing agarose gel electrophoresis, and the concentration of RNA was determined with a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA).

### *2.4 Semi-quantitative RT-PCR*

One microgram total RNA was reverse-transcribed using the First Strand cDNA Synthesis system (Pro-mega, Madison, WI, USA) according to the instructions. Two microliters of the RT reactions were used for PCR amplification of the *c-mos* cDNA in the presence of 0.25 mmol/L dNTPs, 0.2 μmol/L forward (5'-GCGGA TCC CAA GTC ATC TAC GGT GCCA-3') and reverse (5'-GC AAG CTT GGT CCC TTT GGA GCA GTT-3') primers and 1 unit of Taq DNA polymerase in 1 × reaction buffer (Takara, Dalian, China). For housekeeping control, 2 μL of the same RT reaction was amplified using primer pairs (forward: 5'-TGT GAT GGT GGG AAT GGG TCA G-3'; reverse: 5'-TTT GAT GTC ACG CAC GAT TTC C-3') specific for mouse β-actin. The RT-PCR products were analyzed by electrophoresis in a 1.2% agarose gel.

### *2.5 Bacterial expression of a recombinant histidine-c-Mos protein and generation of polyclonal antiserum*

A 6 × histidine-c-Mos fusion protein ($His_6$-c-Mos) was synthesized using a bacterial expression system. Briefly, the *c-mos* PCR product amplified from the cDNA of a 70-day-old mouse was subcloned into the T Easy vector. The BamHI/HindIII fragment of this vector was

then subcloned into the pET28a(+) vector, resulting in the *c-mos* cDNA fragment linked to a 6 × His affinity tag coding sequence. A $His_6$-c-Mos fusion protein was expressed in *E. coli BL21* induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). The supernatant and the precipitate were separated by centrifugation after the bacterial pellet was ultrasonically disrupted. The molecular mass and output of c-Mos were checked by SDS-PAGE. The expressed c-Mos was purified by Ni-NTA affinity chromatography (Pierce, Rockford, IL, USA). After the eluted $His_6$-c-Mos protein was extensively dialyzed, rabbits were immunized with it for polyclonal antibody production.

Antibody titer was determined by ELISA and all antisera were purified with protein A (Millipore, Billerica, MA, USA).

*2.6 Western blot analysis*

Proteins from mouse testis were prepared by homogenization of the testis in lysis buffer, which components as described by Nakamura *et al.* [8]. Purified recombinant c-Mos protein and extracted testicular total protein were run on 12% SDS-PAGE in parallel followed by transfer to nitrocellulose membrane. After blocking, the membrane was incubated with the purified rabbit antiserum (1:800) at 4ºC overnight. Control blot was incubated with preimmune serum. After washing, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (H + L) (1:4 000). Then, protein bands were visualized using ECL western blotting detection reagent.

*2.7 In situ hybridization*

The digoxigenin-labeled *c-mos* probe was designed and synthesized by Boster (Wuhan, China). The procedure was carried out according the manufacturer's protocol. Briefly, freshly dissected tissues were fixed by immersion in phosphate-buffered 4% paraformaldehyde (pH 7.0) at room temperature for 24 h. Tissues were dehydrated through graded ethanol solutions and embedded in paraffin. Paraffin sections were cut at 4 μm, adhered to polylysine-coated slides and dried at 60ºC overnight. Paraffin was removed by xylene at 64ºC for 30 min, and the sections were rehydrated through graded ethanol to water. $H_2O_2$ treatment (3%, room temperature for 10 min) was used to eliminate endogenous peroxidase activity. The slides were rinsed three times with water, incubated at 37ºC in pepsin for 15 min, rinsed three more times with phosphate buffered saline (PBS) (0.02 mol/L phosphate; 0.5 mol/L NaCl) for 5 min each time, and finally rinsed once in water for 5 min. Prehybridization was performed at 40ºC for 4 h in moist chambers with the prehybridization buffer provided in the kit. No washing was needed and hybridization was done for 16 h at 42ºC. The slides were thoroughly washed sequentially in 2 × SSC (0.3 mol/L NaCl; 0.03 mol/L $C_6H_5O_7Na_3$) for 30 min at 37ºC with agitation, 0.5× SSC for 15 min and 0.2 × SSC for 15 min. Then, the slides were incubated with blocking buffer for 30 min at 37ºC. For detection of hybridization signals, the slides were incubated with biotinylated anti-digoxigenin at 37ºC for 90 min followed by three 5-min PBS washes. Sections were incubated sequentially with anti-biotin protein at 37ºC for 20 min, and peroxidase conjugated biotin for 20 min at 37ºC. Diaminobenzidine (DAB) was then added as substrate. After color was observed, the reaction was stopped by dipping slides in water. The sections were stained with hematoxylin if necessary.

*2.8 Indirect immunofluorescence*

The testes were fixed in Bouin's solution for 10–18 h, embedded in paraffin, cut into 6 μm sections and mounted onto polylysine-coated slides. After deparaffinization and rehydration, sections were washed for 5 min in PBS and incubated in 10% goat serum for 60 min at room temperature. Purified anti-c-Mos antiserum (1:400) was applied overnight at 4ºC. After three 5-min PBS washes, the sections were incubated with FITC-conjugated goat anti-rabbit IgG (1:100) for 1 h in the dark at room temperature. Finally, the tissues were washed again and mounted in glycerol/PBS (75% glycerol in PBS). Control sections were incubated with either pre-immune serum or the purified anti-c-Mos antiserum that had been previously incubated with the $His_6$-c-Mos protein following the same procedure with the primary antibody. Digital photographs of fluorescent sections were taken using a laser scanning confocal microscope (LSM-510, Carl Zeiss, Jena, Germany). Thereafter, these sections were re-stained with hematoxylin/eosin (HE) for morphological observation.

## 3 Results

*3.1 Preparation of recombinant c-Mos protein*

Both the soluble and particulate fractions of the sonicated bacterial lysate were analyzed for the presence of

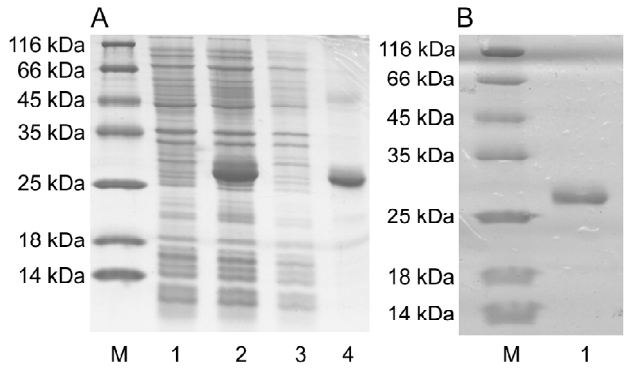


Figure 1. Expression of c-Mos recombinant protein and purification. (A): Proteins were separated on 12% acrylamide gel and stained with Coomassie blue. Lane M, molecular mass markers; Lane 1, cells carrying the vector with the insert before Isopropylthio-β-D-galctopyranoside (IPTG) induction; Lane 2, total cellular protein after IPTG induction; Lane 3, supernatant protein inducted by IPTG after centrifugation; Lane 4, insoluble protein in the inclusion inducted by IPTG after centrifugation. (B): c-Mos recombinant protein purified with Ni-NTA affinity chromatography. Lane M, molecular mass markers; Lane 1, affinity chromatography purified c-Mos recombinant protein.

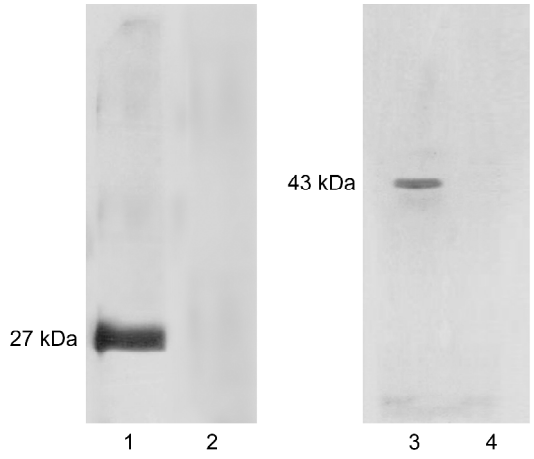


Figure 2. Western blot analysis. Lane 1, purified c-Mos recombinant protein reacted with anti-c-Mos antibodies (1:800); Lane 2, purified c-Mos recombinant protein did not react with pre-immune serum (1:800); Lane 3, protein sample obtained from the testes of male BALB/c mice reacted with anti-c-Mos antibodies (1:800); Lane 4, protein sample obtained from the testes of male BALB/c mice did not react with pre-immune serum (1:800).

recombinant c-Mos protein. The target protein with an apparent molecular weight of 27 kDa was found predominantly in the insoluble fraction (Figure 1A). After the insoluble inclusion was lysed using lysis buffer (100 mmol/L $NaH_2PO_4$, 10 mmol/L Tris-HCl, 8 mol/L Urea, pH 8.0) and purified by Ni-NTA affinity chromatography, only one single band was detected. When the purified protein was detected with His monoclone antibody (data not shown), the result demonstrated that the fusion protein was in-framed with the His-tag and its molecular weight was consistent with what we calculated in advance.

*3.2 Generation of polyclonal antibody against recombinant c-Mos protein*

The ELISA data indicated that the antibody titer was ideal, demonstrating that absorbance values at 405 nm using anti-c-Mos antibodies in three rabbit sera were higher than that of pre-immunized serum (control), with the titer reaching 1:102 400 (data not shown). Western blot analysis showed that purified c-Mos protein and extracted testicular protein of male BALB/c mice both reacted with anti-c-Mos antibodies. Only a single protein band was detected with the molecular weight of 43 kDa in the testes, which was consistent with the molecular weight of testicular c-Mos protein reported previously [9]. In the control blots, where pre-immune sera and natural rabbit IgG were used as primary antibody, the band was not shown (Figure 2). The reproducibility in the sera of three rabbits making the antibody is available. Those results guarantee the quality and specificity of the antibody against recombinant c-Mos, which made the indirect immunofluorescence results credible.

*3.3 c-mos mRNA expression in postnatal testes*

*3.3.1. Semi-quantitative RT-PCR*

Semi-quantitative RT-PCR was performed to determine whether or not *c-mos* mRNA displayed an age-dependent expression pattern. One pair of primers, which could amplify a 505-bp-long *c-mos* fragment, was chosen to perform RT-PCR. The results showed a remarkable age-dependent change of *c-mos* mRNA expression. *c-mos* mRNA was detected in the same low level on day 14 as day 21. The expression of *c-mos* mRNA increased abruptly on day 25, and it soon reached a peak on day 30. A slight drop was observed from day 30 to 35, *c-mos* mRNA remained stable at this level afterward with the development of the mouse until day 70 (Figure 3).

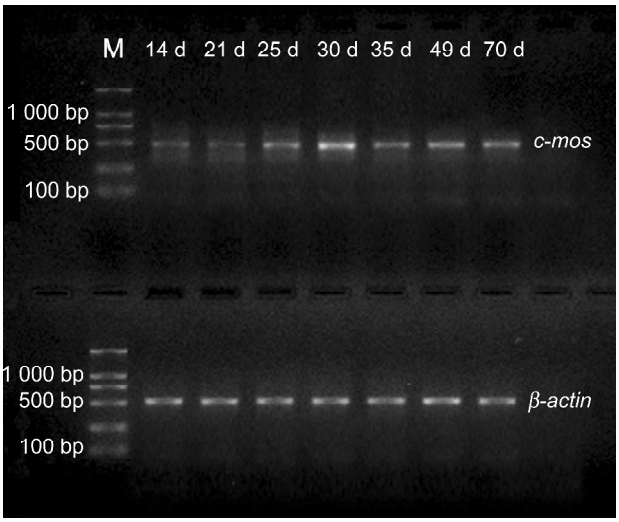


Figure 3. Semi-quantitative reverse transcription-polymerase chain reaction results of *c-mos* mRNA expression in postnatal testes. Upper lane, amplification of c-mos gene from the total RNA of testis; lower lane, amplification of β-actin gene as constitutive control from the total RNA of testis; M lane, DNA marker DL2000; 14 d, 21 d, 25 d, 30 d, 35 d, 49 d, 70 d lane, mice of 14, 21, 25, 30, 35, 49, 70 days after birth, respectively.

### *3.3.2. In situ hybridization*

After *c-mos* mRNA was confirmed to display an age-dependent expression pattern by semi-quantitative RT-PCR, *in situ* hybridization was used to determine: (i) what kinds of cells in the seminiferous tubules transcribe *c-mos* mRNA; (ii) the relationship between the cells that transcribe *c-mos* mRNA and express c-Mos protein; and (iii) whether *c-mos* mRNA age-dependent expression tendency was consistent with the semi-quantitative RT-PCR result. A specialized *c-mos* mRNA probe was designed to perform *in situ* hybridization. The results showed that c-mos-positive stain localized in the nuclear and cytoplasm of the spermatocytes and round spermatids. The cytoplasmic stain was not as clear as the nuclear stain. On day 14, *c-mos* positive cells were detected in the early spermatocytes. The number of positive cells was very small, because the spermatocytes just began to appear at this time. To day 21, *c-mos* positive cells, which were still localized in spermatocytes, increased. With the proliferation of spermatocytes and appearance of round spermatids, *c-mos* positive cells increased dramatically and began to stain round spermatids on day 25. Then, both the number of positive cells

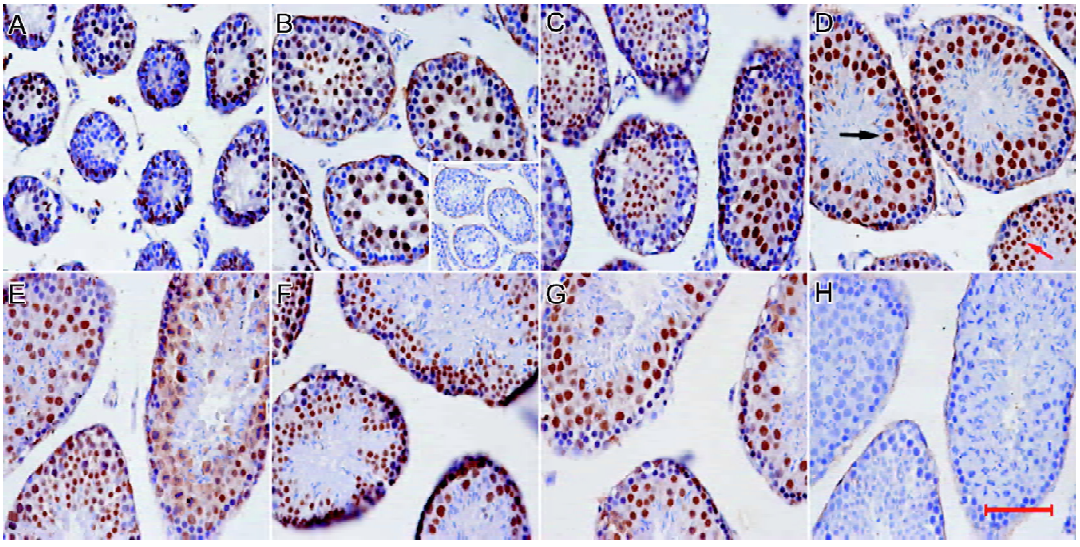


Figure 4. *In situ* hybridization pictures of *c-mos* mRNA expression in mouse postnatal testes. Paraffin sections of testes were hybridized with *c-mos* specific probe. (A)–(G): *c-mos* mRNA expressed in postnatal testes of 14, 21, 25, 30, 35, 49, 70 days, respectively; the positive signals were specifically detected from the nucleus and cytoplasm of the spermatocytes (black arrow in D) and round spermatids (red arrow in D). The insert of B and (H), negative control sections of postnatal day 21 and 35, respectively, were done without *c-mos* probe. Bar = 50 μm.

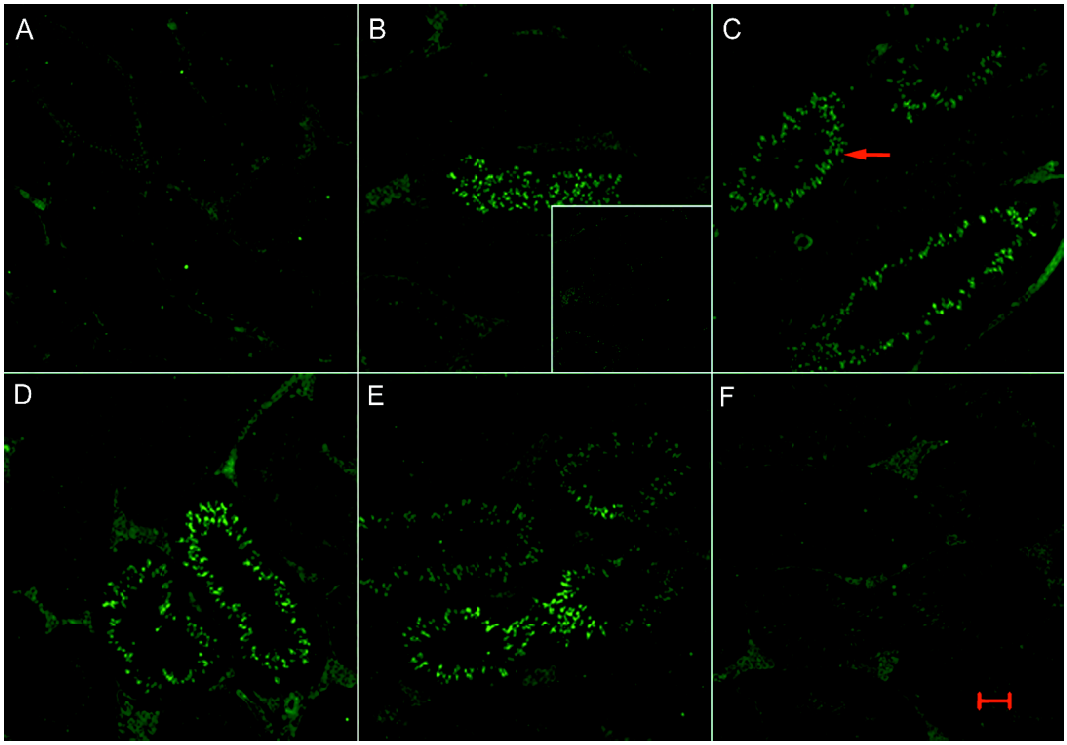


Figure 5. Indirect immunofluorescence staining pictures of c-Mos protein expression in postnatal testes. (A): Testicular section of postnatal day 28 stained with anti-c-Mos antiserum, c-Mos was not expressed at this stage; (B)–(E): Testicular section of postnatal day 30, 35, 49, 70, respectively, stained with anti-c-Mos antiserum. The positive stain was specifically detected from elongating spermatids (indicated by red arrow in C). The insert of B and (F), negative control sections of testis of postnatal day 30 and 70, respectively. Bar = 20 μm.

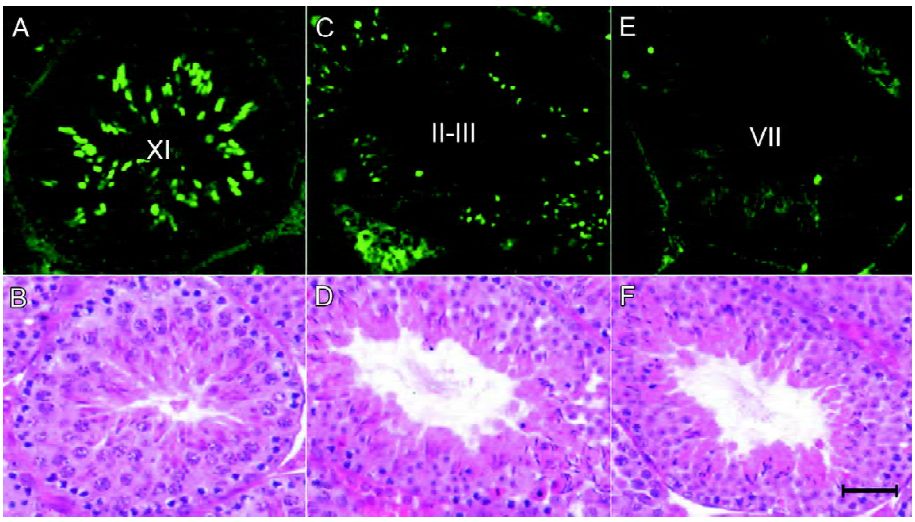


Figure 6. Indirect immunofluorescence staining of c-Mos protein in elongating spermatids of different stages. The c-Mos-positive cells in sections (A), (C) and (E) are elongating spermatids of stage XI, II–III, VII, respectively. (B), (D) and (F): The same sections to A, C and E, restained by HE after immunofluorescence staining. Bar = 50 μm.

and the intensity of positive staining reached a plateau on day 30 when almost all seminiferous tubules appeared c-mos-positive cells (Figure 4D). From day 35 to day 70, the stain intensity of *c-mos* positive cells decreased slightly in contrast with day 30, and maintained stable. Because other cells, such as Sertoli cells, spermatogonia, elongating spermatids and spermatozoa were all stainless, we confirmed that the positive stain in spermatocytes and round spermatids was specific to *c-mos* mRNA and was not caused by hybridizing with genomic DNA.

*3.4 c-Mos protein expression in postnatal testes*

The testis immunofluorescence results revealed that c-Mos protein was expressed in a stage-specific and age-dependent manner during spermatogenesis, which was highly restricted and was found only in the elongating spermatids. In mouse testis, no Mos-positive cells could be detected from day 14 to 25 (data not shown). On day 28, c-Mos was still not identified in whole testis. On day 30, c-Mos was first detected in the elongating spermatids, whereas only a few seminiferous tubules were c-Mos positive at this time. c-Mos-positive cells increased dramatically from day 35 to 49 because of the proliferation of the elongating spermatids, and more seminiferous tubules were c-Mos positive. From day 49 to 70, the number of c-Mos-positive cells was almost stable (Figure 5).

A detailed analysis of the seminiferous epithelium in various stages of spermatogenesis showed that c-Mos protein was first detected in the early elongating spermatids of Stage X, reached maximal levels in the elongating spermatids of Stage XII, and then decreased obviously in the elongating spermatids of Stages II–III. c-Mos protein had almost completely disappeared by late stage elongating spermatids (Stage VII). No c-Mos protein was detected in the Sertoli cells, spermatogonia, spermatocytes and round spermatids (Figure 6). The specificity of the c-Mos antiserum for the c-Mos protein was demonstrated using two methods. The positive staining pattern of c-Mos protein was specifically blocked when the antibody was pre-incubated with the $His_6$-c-Mos protein(data not shown). The testis sections incubated with the pre-immune serum were c-Mos-negtive.

## 4 Discussion

*c-mos* proto-oncogene encodes Mos proteins whose molecular weight vary in different tissues because of post-transcription modification. Ovary c-Mos protein is 39 kDa, whereas testis c-Mos protein is 43 kDa [9]. Although the significance of the post-transcription modification of the *c-mos* mRNA has yet to be determined, it is perhaps related to their specialized functions in different tissues.

The transcripts found in testis RNA are estimated to be approximately 1.7 kb by Northern analysis [10]. Analysis of purified populations of spermatogenic cell types detected *c-mos* RNA in the earliest haploid postmeiotic germ cell, the round spermatid [11]. Another investigation showed that 1.7 kb *c-mos* RNA was detected in pachytene spermatocytes and in early spermatids. Goldman *et al.* [11] reported that the *c-mos* transcript was detected in total cellular RNA of 35 days old but not 20- or 6-day-old mouse testes. A Northern blot study showed that *c-mos* transcript was detected beginning at day 25, with the more sensitive S1 nuclease assay revealing that very low levels of *c-mos* mRNA were detectable in testis as early as 1 day after birth [3]. In addition, there is controversy in the literature concerning the time of c-Mos protein expression, premeiotic or postmeiotic [12]. Therefore, expression of *c-mos* gene in the mouse testis remains to be clarified.

Here, for the first time, we localized *c-mos* mRNA in spermatocytes and round spermatids with *in situ* hybridization, which is in agreement with the result of spermatogenic cell separation and blot hybridization reported by Mutter *et al.* [13]. For separating spermatogenic cells, different methods applied resulted in variation of cell purity and inevitable damage of cells, which might be the reason of controversial expression of *c-mos* in previous studies. *c-mos* mRNA was localized in both cytoplasm and nucleus, even the stain in the nucleus was stronger. However, mRNA was thought to normally localize in the cytoplasm. c-mos gene is a small gene, just 1 176 bp long, which is transcribed as a whole exon in the nucleus but no intron needs to be spliced in the process of post-transcription modification in the cytoplasm; that is, the precursor of *c-mos* mRNA in the nucleus is quite like its mature formation in the cytoplasm. The probe could detect both forms of mRNA; this is probably the reason why the nucleus and the cytoplasm were both stained. Sufficient precursor mRNA was transcribed to prepare for the translation of c-Mos protein, whereas perhaps only a small part of precursor mRNA was transported to the cytoplasm to become mature mRNA; the rest was stored as a reservation in the nucleus, thus the

stain of nucleus was stronger than that of cytoplasm.

Both *in situ* hybridization and RT-PCR demonstrated that *c-mos* mRNA could be detected at low levels on day 14 in mouse testis. At this stage, only spermatogonia and spermatocytes appeared in the seminiferous tubules [14]. On day 21 to 25, the quantity of *c-mos* mRNA increased gradually with the appearance of round spermatids, which was confirmed by in situ hybridization results. c-Mos protein was first detected by indirect immunofluorescence staining on day 30 when elongating spermatids began to emerge. These results suggest that a delay exists between c-mos gene transcription and translation. Similar delays between transcription and translation are found for certain testis-specific proteins. *Cres* mRNA is mainly transcribed in round spermatids, whereas the protein is synthesized in elongating spermatids [15]. *CKLFSF2* mRNA is localized in the pachytene primary spermatocytes, not wholly consistent with the protein that is localized in meiotic and post-meiotic germ cells [16]. The *c-mos* mRNA increased dramatically from day 25 to 30, whereas the predominant increase of c-Mos protein occurred from day 35 to 49. These results reconfirmed the transcription-translation delay. *c-mos* mRNA reached a peak on day 30 when c-Mos protein began to be synthesized, whereas at the time of c-Mos protein increasing dramatically from day 35 to 49, the *c-mos* mRNA decreased a little compared with the level of day 30 and became almost stable. This is probably because sufficient *c-mos* mRNA is transcribed for preparation of the protein translation before the protein begins to be synthesized, but when the quantity of c-Mos protein is sufficient to function, the transcription of *c-mos* mRNA may decrease to a level that can maintain the protein function. Cellular mRNAs containing a complex 5' untranslated regions (UTR), whose translational efficiency can be very specifically regulated by their 5' UTR, provide post-transcriptional regulation. The 5' UTR of *c-mos* may regulate its protein expression in a spatial-temporal manner [17]. Translational control of *c-mos* mRNA by cytoplasmic polyadenylation is necessary for normal oocytes maturation, which requires three cis-elements in the 3' UTR: the polyadenylation hexanucleotide AAUAAA and two U-rich cytoplasmic polyadenylation elements located 4 and 51 nucleotides upstream of the hexanucleotide [18]. However, it is not clear whether it is also the case in the spermatogenic cells. The mechanisms for regulation of *c-mos* mRNA and c-Mos protein expression need to be elucidated further.

In rat testis, it has been reported that western immunoblot analysis revealed the presence of a 43-kD c-Mos protein in pachytene spermatocytes, but not in postmeiotic cell [12], which was not consistent with our result that c-Mos protein was detected in mouse postmeiotic haploid cells. The discrepancy is possibly owing to species differences. The functions of c-Mos in the spermatogenesis might be diverse due to various species. c-Mos protein was identified from spermatogonia of anuran amphibian and was proven to be involved in the regulation of spermatogonial proliferation [7].

In spite of a failure to find a spermatogenic phenotype in *c-mos* knockout mice [19], we still cannot conclude that there is no effect of *c-mos* ablation in spermatogenesis. One possibility for the lack of a detectable phenotype is that the phenotype of the knockout effect was not apparent or not detectable in the assays used. Another possibility is that a redundant protein replaces the Mos funtion and masks the effect of *c-mos* ablation. Because Mos is a MEK kinase, other activators of MEK might compensate for its absence. If *c-mos* is knocked-down in the adult mice with siRNA interference technology, the spermatogenic phenotype might be observed before the compensation is established.

Indirect immunofluorescence also revealed that although the number of Mos-positive cells varied a lot at different ages, the stain intensity was mainly dependent on developmental stages of the germ cells themselves rather than the age of the mouse. Both *in situ* hybridization and immunofluorescence experiments showed that within the testis the *c-mos* mRNA and c-Mos protein were expressed in a stage-specific manner. c-Mos protein appeared in early elongating spermatids of Stage X, whereas completely disappeared by late elongating spermatids of Stage VII, which shows that c-Mos protein functions at the stage of elongating spermatids. At this stage, a process of metamorphosis from a conventional cell to a unique cell capable of motility occurred. The age-dependent and stage-specific expression pattern of the *c-mos* gene strongly suggested its involvement in spermatogenesis. In mice, it is more prone to, as indicated by our results, function in the process of spermiogenesis rather than in the meiosis, because c-Mos protein expression corresponds approximately with the onset of spermatid elongation.

In conclusion, the *c-mos* proto-oncogene shows a specific spatial and temporal expression pattern in mouse

testis on both the mRNA and protein level, which might reveal its functions during spermatogenesis. More efforts should be made to elucidate the molecular mechanism of c-Mos protein function.

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