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·Original Article·

Characterization of Spindlin1 isoform2 in mouse testis

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Abstract

Aim: To investigate the expression of Spindlin 1 (Spin 1) isoform2 and assess its function in mouse testis. **Methods:** First, reverse-transcription polymerase chain reaction (RT-PCR) was used to determine whether Spin1 isoform2 is present in mouse testis. Then the expression patterns of the isoform between newborn and adult mice testes were compared by immunoblot analysis. Finally, the diversity of its localization in mice testes at different ages (days 0, 7, 14, 21, 28 and 60) was observed by immunohistochemistry. The localization of the protein in mouse sperm was also investigated by immunofluorescence. **Results:** The RT-PCR results show that Spin1 isoform2 is present in mouse testis. As shown by immunoblot analysis, the isoform was more highly expressed in adult testes compared with newborn testes. Interestingly, Spin1 isoform2 did not show up in the cytoplasm of primary spermatocytes until day 14. Also, the protein exists at the tail of the mouse sperm. **Conclusion:** Spin1 isoform2 is a protein expressed highly in adult testis, which might be involved in spermatogenesis and could be necessary for normal sperm motility. *(Asian J Androl 2008 Sep; 10: 741–748)*

Keywords: Spindlin 1; spermatogenesis; sperm motility; isoform; mouse testis; primary spermatocyte; meiosis

1 Introduction

Spindlin1 (Spin1), first reported as a maternal transcript in mice, has been suggested to play an important role during the transition from oocyte maturation to embryo development [1, 2]. It has also been demonstrated that the protein localizes to spindle of oocyte undergoing maturation division. Since mouse Spin1 was first reported in 1997 by Oh *et al.* [1], a series of homologous genes have been discovered in chicken, gibel carp and humans [3–5]. There are two Spin-type genes in the chicken: *chSpin-Z* localizing on the long arm of the Z chromosome is transcribed in various tissues of adult chickens and *chSpin-W* representing the counterpart gene that is transcribed most prominently in ovarian granulosa and thecal cells. The function of chSpin is associated with chromosomes during mitosis [3]. Spin in gibel carp is a gene specifically expressed in oocyte and plays an important role by interacting with β-tubulin during oocyte maturation and egg fertilization [4]. Spin 1, a homologous gene in humans, contributes to tumorigenesis [5].

All the genes above belong to the Spin/Ssty protein family, which contains a conserved motif of approxi-

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mately 50 amino acids (Spin/Ssty repeat). Three modules of Spin/Ssty repeats are thought to be independent functional units and are considered to be necessary for the structural and functional integrity of all known Spin family proteins [6]. Ssty, as a member of Spin/Ssty family, is present on the long arm of the mouse Y chromosome (Yq). Partial Yq deletion can lead to reduction of Ssty expression and result in severe sperm defects and sterility. Therefore, Ssty is considered to be essential for normal sperm differentiation [7–11].

According to the NCBI Gene Database, there are two isoforms of Spin1 in mice. All previous research on Spin1 has focused on the isoform1 (Spin1 transcript variant 1; NP_035592). However, the other isoform (Spin1 transcript variant 2; NP_666155), obtained from sequencing work [12], has not been studied until now. The full length of its cDNA is 1 064 bp, encoding a 262-amino-acid protein. The C-terminus of Spin1 isoform2, containing three Spin/Ssty repeats, is identical to that of Spin1 isoform1. Oh *et al*. [1, 2] reported that Spin1 isoform1 is associated with oocyte maturation, which is only expressed in mouse oocyte and early embryos. Therefore, we are very interested in the function of Spin1 isoform2 in mouse testis.

Spermatogenesis is the main function of the testis. It is a well-characterized developmental process for the genesis of male germ cells [13]. This process is regulated by programmed gene expression [14, 15]. Studies on the genes that were specifically expressed at different stages of testis development could reveal their function, especially in spermatogenesis [16]. Therefore, in the present study, the expression patterns of Spin1 isoform2 in mice testes of different ages were investigated using western blot analysis and immunohistochemisty.

2 Materials and methods

2.1 Sample collection

All pregnant Institute of Cancer Research (ICR) mice used in these studies were obtained from the Lab Animal Center of Nanjing Medical University (Nanjing, China) and were maintained under a controlled environment of 20ºC–22ºC, 12:12 h LD cycle, at 50%–70% humidity, with food and water provided *ad libitum*. After delivery, the testes of the male offspring were collected at different postpartum times (days 0, 7, 14, 21, 28 and 60) and fixed in Bouin's solution for histological examination. Mature sperm were obtained from the epididymis by making small incisions throughout the epididymis cauda followed by extrusion and resuspension in phosphate buffered solution (PBS).

2.2 Reverse-transcription polymerase chain reaction (RT-PCR)

Multiple tissues from adult mice, including hearts, livers, spleens, lungs, kidneys, brains, stomachs, intestines, skeletal muscle, testes and ovaries were collected and homogenized. Total mRNA was extracted according to the Trizol RNA isolation protocol (Gibco BRL, Grand Island, NY, USA) and reverse-transcribed into cDNA with AMV reverse transcriptase (Promega, Madison, WI, USA). The cDNA was PCR amplified according to the manufacturer's instructions and conditions as follows: denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s; 35 cycles. Primers were 5'-CCCCATTCGGGAAG ACAC-3' and 5'-ACAGGGAAGGATTCACAGG-3' for Spin1 isoform2; and 5'-ATGGCCTCTGCGTCA AGTCC-3' and 5'-CTAGGATGTTTTCACCAAAT-3' for Spin1 isoform1. The primers for Spin1 isoform2 were designed at exon2 and exon4, respectively, and the PCR products span two introns, as shown in Figure 1D. β-actin was used as the positive control.

2.3 Expression of recombinant protein and preparation of antibody

The full length coding sequence of Spin1 was subcloned into pET28a expression vector (GE Healthcare, San Francisco, CA, USA) coding for six N-terminally located histidine residues.

For this purpose, PCR was performed with primers containing the following restriction sites: NdeI for the forward primer and XhoI for the reverse primer. PCR amplification was performed with polymerase mix (BD Bioscience, Piscataway, NJ, USA) using an initial denaturing step at 95ºC for 5 min, followed by 30 cycles of incubation at 95ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s, and a final extension step of 7 min at 72ºC. The construct was used for transformation of competent BL21 (DE3) cells. These cells were grown in LB medium (10 g of tryptone, 10 g of yeast extract and 5 g of NaCl) containing Kanamycin (50 μg/mL). When the absorbance at 600 nm reached 0.6 (approximately 1.7×10^8 cells/mL), isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mmol/L. After 6 h of induction at 30ºC, cells were collected and resuspended in 200 mL

of 20 mmol/L Tris-HCl, 500 mmol/L NaCl, 8 mol/L urea buffer. The cells were sonicated for 10 min on ice, then centrifuged at 10 000 × *g* at 4ºC for 30 min. The clear supernatant was filtered through a 0.22 μm membrane and then purified through a Ni^{2+} affinity column by AKTA Basic (Amersham Biosciences) under denaturing conditions according to the manufacturer's protocol using HiTrap Chelating HP 1 mL. The purity of the recombinant protein was confirmed by 12% SDS-PAGE. Purified protein was refolded by dialysis against a linear decrease gradient of 6, 4 and 2 mol/L urea buffer.

Polyclonal antibodies were raised by immunization of a male New Zealand White rabbit with the purified recombinant protein. The rabbit received approximately 100 μg of recombinant protein with complete Freund's adjuvant for the primary injection. Two additional boosts with 50 μg of protein in incomplete Freund's adjuvant were administered on the 14th and 21st days, respectively. The antibody titer of preimmune and immune sera was determined using ELISA. When the antibody titer of immune rabbit serum reached 10^5–10^6, rabbits were killed to collect the serum.

2.4 Protein extraction and immunoblot analysis

Mice testes at days 0 and 60 were collected and washed three times in chilled PBS, then treated with lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% [w/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 2% [w/v] dithiothreitol (DTT), 2% [v/v] immobilized pH gradient [IPG] buffer, pH 3–10) in the presence of 1% (v/v) protease inhibitors-cocktail kit (Pierce Biotechnology, Rockford, Illinois, USA). The mixture was homogenized (Ultra Turrax, IKA, Germany) at 11 000 rpm for 5 min on ice. After centrifugation at 40 000 × *g* at 4ºC for 1 h, the supernatant was collected and stored at –70ºC until use. The concentration of extracted protein was determined by Bio-Rad DC protein assay (10) kit (Bio-Rad Laboratories, Mississauga, ON, Canada) using bovine serum albumin (BSA) as standard protein.

The extracts of mice testes at days 0 and 60 were subjected to 12% SDS-PAGE. 100 μg testicular protein extract was loaded in each lane and the resolved proteins were transferred to a nitrocellulose membrane. After being blocked with blocking solution (5% non-fat milk powder in Tris-buffered saline [TBS; pH 7.4]) for 2 h, the membrane was incubated with anti-spin rabbit serum (1:1 000) or a polyclonal antibody against β-tubulin (Abcam, Cambridge, MA, USA; 1:2 000) diluted in blocking solution at 4ºC overnight. After washing with TBS three times, the membrane was incubated with horseradish peroxidase-labeled goat antirabbit IgG (1:1 000; Beijing ZhongShan Biotechnology, China) for 1 h at 37ºC. After three washes, immunoreactivity was detected using an enhanced chemoluminescence reaction kit (Amersham Biosciences) and the images were captured by FluorChem 5500 (Alpha Innotech, San Leandro, CA, USA). Molecular weights of the detected proteins were deduced by comparison with recombinant molecular weight standards (New England BioLabs, Ipswich, MA, USA).

For quantification of the data, the images were analyzed using Adobe PhotoShop (San Jose, CA). Boxes of the same size were drawn around the appropriate band, and the average pixel intensity was measured. The relative amount of Spin1 isoform2 was calculated as the ratio of its average pixel intensity to that of the β-tubulin loading control. Three repeated experiments were performed independently.

2.5 Immunohistochemistry

Bouin's fixed testes were embedded in paraffin, sectioned at 5 μm, and mounted on silane-coated slides. For immunohistochemistry, sections were dewaxed and rehydrated through descending grades of alcohol to distilled water, followed by incubation in 2% hydrogen peroxide to quench the endogenous peroxidase activity and washed in PBS. Subsequently, they were blocked with goat serum (Beijing ZhongShan Biotechnology, China) for 2 h and incubated with primary antibody (anti-spin rabbit serum; 1:2 000) overnight at 4ºC. Following three washes in PBS, sections were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (Beijing ZhongShan Biotechnology, China) for 1 h at room temperature. Immunoreactive sites were visualized brown with diaminobezidine (DAB) and mounted for bright field microscopy (Axioskop 2 plus, Zeiss, Germany). As one negative control, sections were incubated with the preimmune rabbit serum in place of the primary antibody. In the other negative control, the sections were incubated with anti-spin rabbit serum, which was preabsorbed with the spin recombinant protein.

2.6 Immunofluorescence

Mouse sperm samples were fixed with 4% paraform-

aldehyde/PBS for 1 h, permeabilized with 0.2% Triton X-100 /PBS for 20 min at 37ºC, and then blocked with goat serum (Beijing ZhongShan Biotechnology, China) for 2 h at room temperature. Following incubation with a 1:1000 dilution of anti-spin serum overnight at 4ºC, sperm were incubated with the secondary anti-rabbit IgG labeled with fluorescein isothiocyanate (FITC, Beijing ZhongShan Biotechnology, China) at 1:100 dilution for 1 h at room temperature and observed under ZEISS Axioskop plus2 fluorescent microscopy at an excitation wave of 470 nm. Negative controls were performed by the replacement of the anti-spin rabbit serum with preimmune rabbit serum.

2.7 Statistical analysis

Data were expressed as mean ± SME. Student's *t*-test was used for statistical comparison. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Identification of Spin1 isoform2 in mouse testis and other tissues

To analyze the tissue-specific expression pattern of Spin1 isoform2, we performed Reserve-transcription polymerase chain reaction analysis with total RNA from different mouse tissues. The data showed that Spin1 variant2 is transcribed in adult mouse testis and that Spin1 transcripts, leading to isoform2, are also present in other tissues, including hearts, livers, spleens, lungs, kidneys, brains, stomachs, intestines, skeletal muscle and ovaries (Figure 1A). β-actin was performed as the positive con-

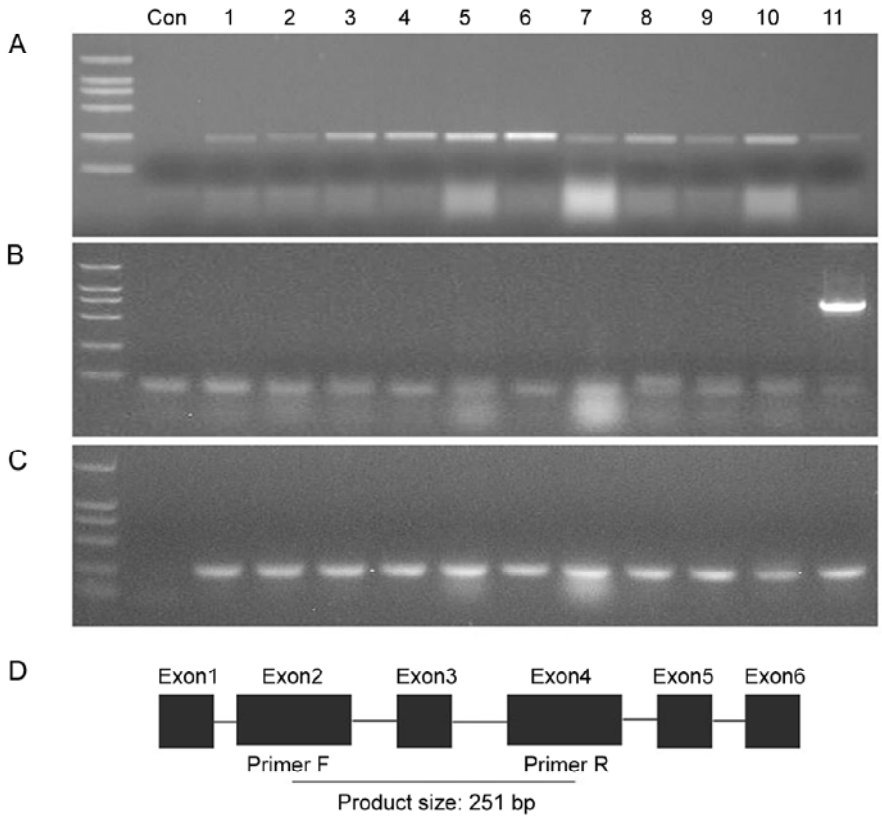

Figure 1. Reserve-transcription polymerase chain reaction (RT-PCR) in multiple tissues. Spindlin 1 (Spin1) isoform2 was expressed extensively in different tissues; while Spin1 isoform1 was proven to be present only in ovary tissue. β-actin was performed as a positive control. Spin1 isoform2 (A), Spin1 isoform1 (B), β-actin (C), primers for Spin1 isoform2 (D) were designed at exon2 and exon4, respectively, and the expected size of the product was 251 bp. Con: control; 1: heart; 2: liver; 3: spleen; 4: lung; 5: kidney; 6: brain; 7: stomach; 8: intestine; 9: skeletal muscle; 10: testis; 11: ovary.

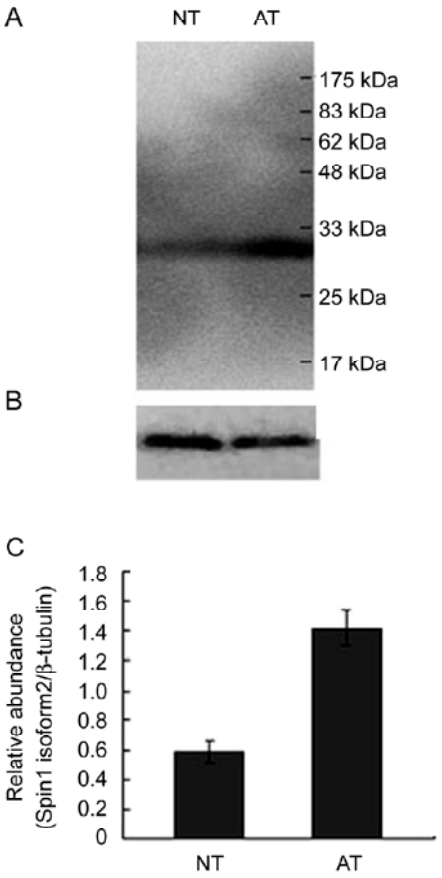

Figure 2. Immunoblot analysis of Spindlin 1 (Spin1) isoform2 in newborn (NT) and adult mice testes (AT). 100 μg protein extract was loaded in one lane. (A): Western blot was performed with anti-spin rabbit serum, showing a single band at expected size (approximately 29 kDa) in both NT and AT lanes. (B): β-tubulin was used as positive control. (C): Semi-quantiatation of Spin1 isoform2 compared with β-tubulin in newborn and adult mice testes. The gene was highly expressed in adult testes; the relative abundance of Spin1 isoform2 in adult testes is approximately three times greater than that in newborn testes. The experiment was repeated three times and the data was analyzed by Student's *t*-test ($P < 0.001$).

trol (Figure 1C). Moreover, Spin1 isoform1 was proven to be only present in ovary tissue (Figure 1B).

3.2 Expression level of Spin1 isoform2 in newborn and adult mice testes

The expression level of Spin1 isoform2 between newborn and adult testes was investigated using immunoblot analysis. Only a single band with an apparent molecular weight of approximately 29 kDa was detected in testicular protein extracts of newborn (day 0 post partum) and adult (day 60 post partum) mice (Figure 2A). β-tubulin was used as the positive control (Figure 2B). Semi-quantitation of Spin1 isoform2 demonstrated that the gene was highly expressed in adult testes compared with newborn testes ($P < 0.001$); the abundance of Spin1 isoform2 in adult testes is approximately three times that in newborn testes (Figure 2C).

3.3 Localization of Spin1 isoform2 in mice testes at different ages

No obvious signals were detected in the germ cells of mice testes at days 0 and 7. Only faint brown signals can be revealed in Leydig cells at days 7 (Figure 3A, B). Significant Spin1 isoform2 immunolabeling patterns showed up in the cytoplasm of primary spermatocytes after day 14, as well as Leydig cells (Figure 3C, D). From day 21, Spin1 isoform2 was expressed extensively in Leydig cells, sertoli cells and germ cells from the primary spermatocyte phase to the spermatozoa phase with the development of testis (Figure 3E–G). However, the signals in primary spermatocytes and Leydig cells at day 60 were conspicuously strong (Figure 3H).

As negative controls, both anti-spin serum preabsorbed with the spin recombinant protein and preimmune rabbit serum produced background levels of staining in mice testes (Figure 3I–L).

3.4 Localization of Spin1 isoform2 in mouse sperm

The localization of Spin1 isoform2 in mouse sperm was further examined by immunostaining using the anti-serum of the protein. Bright fluorescence staining was invariably observed in the centriole, principal piece and end piece of sperm (Figure 4A, B). Besides, a tenuous fluorescence signal is also visible in the mitochondrial sheath. Therefore, Spin1 isoform2 was conformed to localize to the tail of the mouse sperm.

In control experiments with preimmune serum, a little background staining was also detected (Figure 4C, D).

4 Discussion

In this study, we showed that Spin1 isoform2 is transcribed ubiquitously and that the transcript of Spin1 isoform2 is also present in mouse testis. At the same time, Spin1 isoform1 was proven to be specifically expressed. The amino-acid sequences of the two isoforms at the C-terminus are totally identical, and the

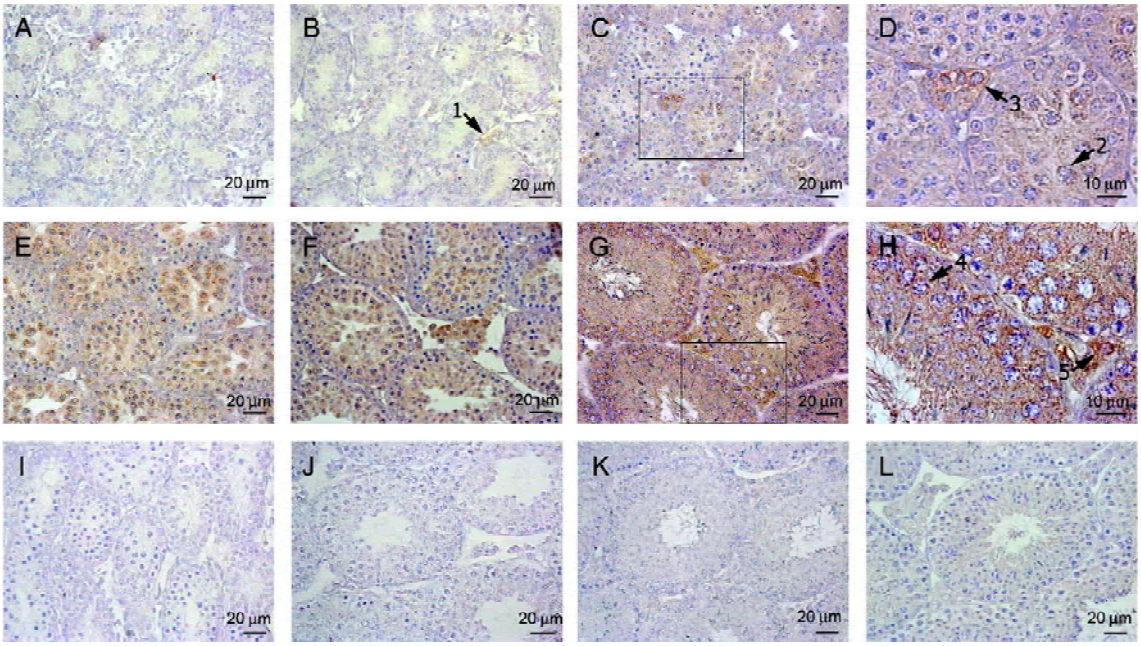

Figure 3. Expression of Spindlin 1 (Spin1) isoform2 by immunohistochemistry in mice testes at different ages. Anti-spin rabbit serum at day 0 (A), day 7 (B), day 14 (C). (D): Enlarged image of the region in (C). Anti-spin rabbit serum at day 21 (E), day 28 (F), day 60 (G). (H): Enlarged image in (G). Anti-spin rabbit serum preabsorbed with the spin recombinant protein at day 14 (I), day 28 (J), day 60 (K). (L): Preimmune rabbit serum at day 60. No obvious expression of Spin1 isoform2 was detected in mice testes at days 0 and 7 and only faint brown signals were revealed in Leydig cells at day 7 (arrow 1). At day 14, strong signals showed up in the cytoplasmic of primary spermatocytes (arrow 2), as well as in Leydig cells (arrow 3). From day 21, Spin1 isoform2 was expressed extensively in Leydig cells, sertoli cells and germ cells from the primary spermatocyte phase to the spermatozoa phase. At day 60, strong signals were observed in both primary spermatocytes (arrow 4) and Leydig cells (arrow 5).

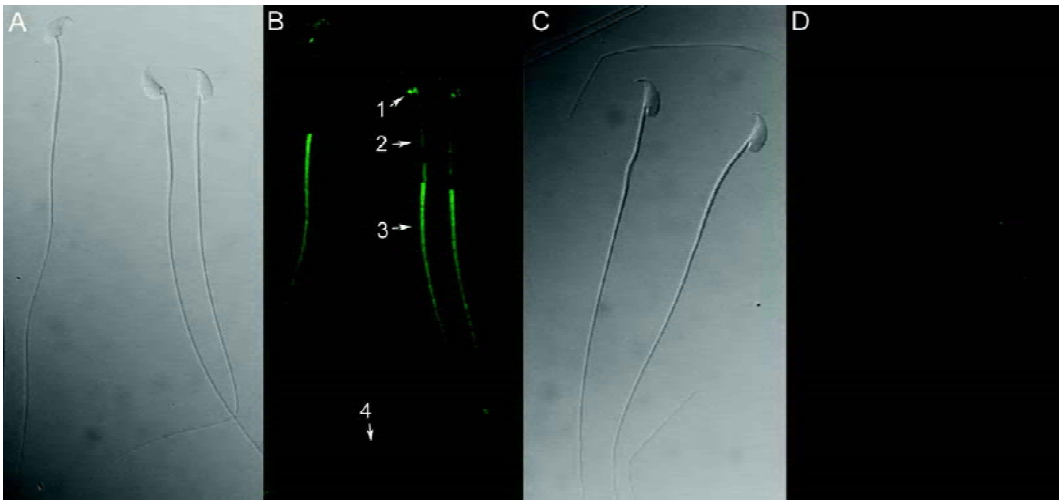

Figure 4. Localization of Spindlin 1 (Spin1) isoform2 in mouse sperm by immunofluorescence. Phase-contrast (A) and immunofluorescent photomicrograph (B) of sperm with anti-spin rabbit serum. Negative controls was performed with preimmune rabbit serum (C, D). Compared with negative control (little background signal), intense fluorescent staining was observed in the centriole, principal piece and end piece of sperm, just as the arrows show. Arrow 1: centriole; 2: mitochondrial sheath; 3: principla piece; 4: end piece. Tenuous fluorescence signal in the mitochondrial sheath was visible.

functional domains are also conserved. Therefore, we presumed that Spin1 isoform2 might take a similar role in testes to Spin1 isoform1 in ovaries.

By comparing the expression of Spin1 isoform2 between newborn and adult mice testes, we demonstrated that the protein is expressed at a higher level in adult testes than in newborn testes. The relative abundance of Spin1 isoform2 in adult testes is approximately three times that in newborn testes. There are only sertoli cells and undifferentiated spermatogonia cells in the seminiferous tubules of newborn testes, whereas the seminiferous tubules of adult testes contain not only sertoli cells and spermatogenous cells, but also various spermatogenic cells. In other words, there are many developmental germ cells in adult testes but not in newborn testes. Thus, the results of western blot analysis provide an important clue to its function. Spin1 isoform2 might be associated with testis development and spermatogenesis.

To further explore the function of the protein, we investigated the expression patterns of Spin1 isoform2 in testes at different ages (days 0, 7, 14, 21, 28 and 60) by immunohistochemistry. No obvious signals of Spin1 isoform2 were detected in mice testes at day 0, which was not match the Western blot analysis result. It is possible that the expression level of Spin1 isoform2 in newborn mice testes is too low to be visualized by immunohistochemistry. At day 7, faint brown signals can be revealed in Leydig cells. At day 14, significant Spin1 isoform2 immunolabeling patterns showed up in the cytoplasm of primary spermatocytes, as well as Leydig cells. From days 21 to 60, Spin1 isoform2 was expressed extensively in Leydig cells, sertoli cells and germ cells from the primary spermatocyte phase to the spermatozoa phase with the development of testis. However, strong signals can be viewed only in primary spermatocytes and Leydig cells at day 60. Spermatogenesis takes place in three major phases: proliferation and differentiation of spermatogonia, meiosis and spermiogenesis. We consider the aforesaid six time points to represent the major stages of germ cell development during the first wave of spermatogenesis: day 0, newborn testis with stem cell property; day 7, spermatogonia mitosis; day 14, spermatocyte meiosis; day 21, round spermatid production; day 28, elongated spermatid formation, also named spermiogenesis; day 60, normal postpubertal spermatogenesis [17]. Spin1 isoform2 showed up in the primary spermatocyte after day 14. While the day 14 was considered to be the time point of progressing the first wave of spermatocyte meiosis. In addition, the protein was also expressed highly in the cytoplasm of primary spermatocyte in adult testes. Accordingly, we presumed that Spin1 isoform2 might be associated with spermatocyte meiosis. It has been reported that Spin1 isoform1 is involved in the progression of the meiotic and first mitotic cell cycles and might be necessary for oocyte maturation and the initiation of development [1, 2]. Another member of the Spin/Ssty protein family, Ssty (Y-linked spermiogenesis specific transcript), has also been demonstrated to be required for normal spermatogenesis. Reduction of Ssty expression can result in severe sperm defects and sterility [7–11]. Therefore, it is possible that Spin1 isoform2 has a similar role and could be essential for spermatogenesis.

Interestingly, Spin1 isoform2 was also localized at the tail of mouse sperm. Microtubule is the major constituent of the sperm tail and is absolutely necessary for sperm movement. Spin1 isoform1 was reported to be associated with the spindle of ooycte [1, 2], and CagSpin in gible carp was confirmed to interact directly or indirectly with â-tubulin [4]. Therefore, we hypothesize that Spin1 isoform2 takes part in the organization of microtubule and might be absolutely necessary for normal sperm motility.

In conclusion, we have characterized Spin1 isoform2, which encode the 29 kDa protein in mouse testis for the first time. The results from the present study suggest that the protein is involved in spermatogenesis and could be essential for normal sperm motility.

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