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Expression and localization of the spermatogenesis-related gene, *Znf230*, in mouse testis and spermatozoa during postnatal development

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***Znf230*, the mouse homologue of the human spermatogenesis-related gene, *ZNF230*, has been cloned by rapid amplification of cDNA ends (RACE). This gene is expressed predominantly in testis, but its expression in different testicular cells and spermatogenic stages has not been previously analyzed in detail. In the present study, the cellular localization of the Znf230 protein in mouse testis and epididymal spermatozoa was determined by RT-PCR, immunoblotting, immunohistochemistry and immunofluorescence. It is primarily expressed in the nuclei of spermatogonia and subsequently in the acrosome system and the entire tail of developing spermatids and spermatozoa. The results indicate that Znf230 may play an important role in mouse spermatogenesis, including spermatogenic cell proliferation and sperm maturation, as well as motility and fertilization. [BMB reports 2008; 41(9): 664-669]**

INTRODUCTION

Spermatogenesis is characterized by successive periods of regulated cell proliferation, meiosis, and haploid differentiation, in which many gene products are involved. Identification of these genes and studies on their spatial and chronological expression are essential for understanding the biology of spermatogenesis and male infertility (1-3).

The zinc finger gene family, one of the largest gene families in mammals, is widely reported to be involved in mammalian spermatogenesis and can be divided into several subfamilies (4-6). In accordance with their diverse structures, zinc finger proteins have been assigned various functions (7-11), and until now, more than 25 zinc finger genes have been reported to play a role in spermatogenesis. Some of these genes, for instance *ZNF76*, *Sperizin*, *ZFY*, *ZFX*, *mZNF8*, *Zfp35* and *Znf313*, are supposed to participate in the transcriptional regulation of spermatogenesis and are expressed ubiquitously during meiosis and early spermatid development (12-17).

Znf230, which is also called *Rnf141*, is a new gene that was cloned in our laboratory by rapid amplification of cDNA ends several years ago as the mouse homologue of the human gene, *ZNF230* (18, 19). It belongs to the ring finger subfamily (C3HC4) of the zinc finger gene family. It is expressed predominantly in testis. In COS cells transfected with the recombinant vector, pEGFP-Znf230, the Znf230 protein localizes mainly in the nucleus. The recombinant protein exhibits DNA binding activity and its ring finger domain may function as an activator module in transcription. We therefore hypothesized that Znf230 may function as a testis-specific transcription factor during mouse spermatogenesis (18, 20). However, its expression in different testis cell types and in spermatozoa as well as its role during spermatogenesis have not been studied in detail. In the present study, both the expression pattern and the subcellular localization of the spermatogenesis-related gene, *Znf230*, were investigated by RT-PCR, immunoblotting, immunohistochemical and immunofluorescence analyses of testicular tissues and epididymal spermatozoa.

RESULTS

Production and characterization of a polyclonal antibody to Znf230

A recombinant plasmid containing Znf230 cDNA fused to GST was identified by restrictive endonuclease digestion. The plasmid was digested with *BamH*I and *Not*I in combination, and the digest was separated by 1.5% agarose gel electrophoresis. The expected 750-bp band representing Znf230 cDNA was observed and was further confirmed by DNA sequencing. A Znf230 polyclonal antibody was generated by immunizing New Zealand White rabbits with the GST- Znf230 protein purified as described in Materials and Methods. The specificity of the purified antiserum was confirmed by western blotting. On immunoblots, the purified antibody specifically recognized the GST-Znf230 protein and the dominant band was of the expected 52-kDa molecular size. No bands were evident when the same sample was subjected to immunoblotting with the

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pre-immune rabbit serum (Fig. 1A).

Expression of Znf230 during mouse testis development

Western blot and RT-PCR analyses were conducted to determine the expression pattern of Znf230 during mouse testis development. Analysis of mouse testes on postnatal (pn) days 1, 3, 6, 9, 12, 18, and 25 and of adult mouse testes by western blotting showed that a protein of about 26 kDa, the expected size of full-length Znf230, was detected from pn day 1 to adulthood. The protein expression remained relatively stable with only a temporary moderate decline on pn day 18 (Fig. 1B). Meanwhile, the expression of *Znf230* mRNA in testicular tissue was confirmed by RT-PCR with specific primers that amplified a sequence with an expected size of 234 bp. The level of gene expression remained constant from pn day 1 to adulthood (Fig. 1C). Thus, the results of the RT-PCR analysis were consistent with those obtained by western blotting, and they both showed that the gene remained relatively stable and was expressed throughout postnatal development of the mouse testis.

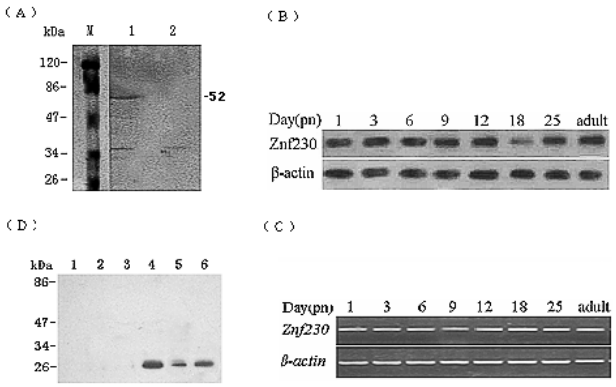

Fig. 1. (A) Western blot analysis of the specificity of the Znf230 antibody for GST- Znf230 (52-kDa) using purified antiserum or normal rabbit serum obtained before immunization. M: Protein marker; 1: purified antiserum; 2: rabbit serum obtained before immunization. (B) Western blot analysis at different developmental stages including pn day 1, 3, 6, 9, 12, 18, 25, and adulthood. The purified antibody was used to detect a protein of about 26-kDa, the expected size for full-length Znf230. β-actin was used as a control. (C) mRNA analysis of *Znf230* in mouse testis during postnatal development by RT-PCR amplification of samples from newborn mice at pn day 1, 3, 6, 9, 12, 18, 25, and from adult mice. The mRNA expression of *Znf230* (234 bp) was detected in all stages. Mouse β-*actin* was co-amplified as an internal control. (D) Adult mouse testis, sperm acrosome, and sperm tail lysates were loaded into Lanes 1 and 4, lanes 2 and 5, and lanes 3 and 6, respectively. The samples were separated by SDS-PAGE, transferred to PVDF membranes and probed with pre-immune rabbit serum (lanes 1, 2 and 3), or with purified antibody (lanes 4, 5 and 6).

The cellular distribution of Znf230 during mouse spermatogenesis

To identify when and where *Znf230* was expressed during the spermatogenic process, we studied its expression in tissue sections obtained from mice of different ages based on the germ cell stages observed in each section. Testicular tissue sections from pn day 6, 12, 18, and 25 mice as well as those from adult mice were analyzed with the purified antibody and the results are summarized below.

As shown in Fig. 2 and Fig. 3, the gene was expressed in spermatogonia, round and elongated spermatids, and spermatozoa, but not in spermatocytes. Also the gene product was not expressed in Sertoli cells or Leydig cells. On pn day 6, seminiferous tubules contained mainly Sertoli cells and spermatogonia, and the Znf230 protein was detected in the nuclei of spermatogonia (Fig. 2A and 3A). On pn day 12, the majority of the germ cells were spermatogonia and early primary spermatocytes, and Znf230 was also detected in the nuclei of spermatogonia (Fig. 2B and 3B). On pn day 18, round spermatids were first identified in some seminiferous tubules. Analysis at higher magnification showed that immunoreactivity was only

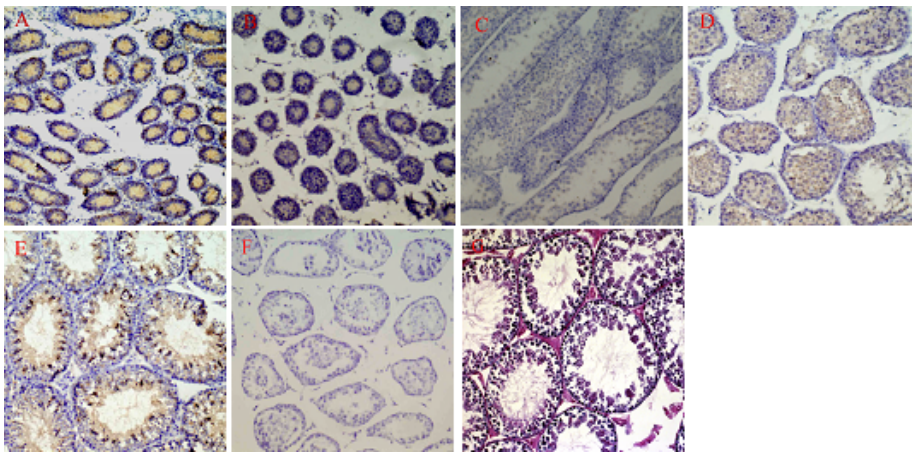

Fig. 2. Immunodetection of Znf230 in seminiferous tubules from 6-day-old (A), 12-day-old (B), 18-day-old (C), 25-day-old (D), and adult mice (E). Sections were incubated with the polyclonal antibody (A-E). No immunoreactivity was observed when the sections were incubated with pre-immune serum (F). PAS staining (G) indicated that the immunoreactive region consisted of the acrosome system. Original magnifications: (A-G) ×200.

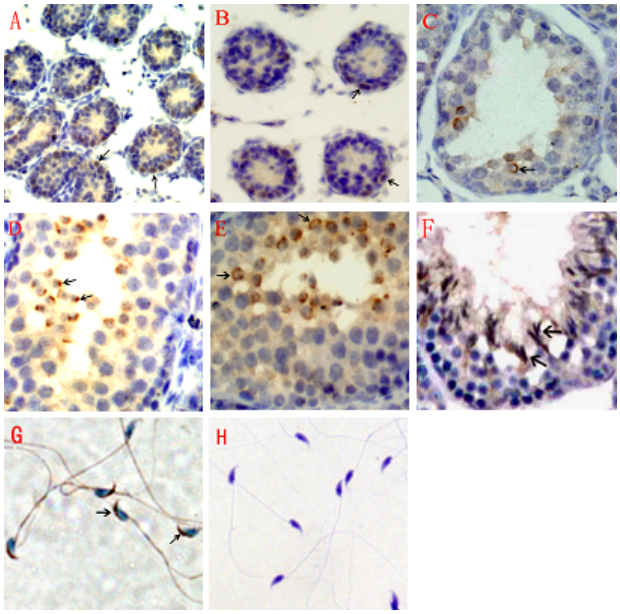

Fig. 3. Specific cellular localization of Znf230 in spermatogenic cells from 6-day-old (A), 12-day-old (B), 18-day-old (C), and adult mice (D-F). Znf230 was mainly expressed in the nuclei of spermatogonia (A-B). On pn day 18, it began to appear in the acrosome of round spermatids (C). The changing shape of the forming acrosomal system expressing Znf230, from the Golgi phase (D), through the cap phase (E) to the acrosome phase (F), was observed in spermatids. The protein was also apparent in the Golgi phase (D) and the cap phase (E) in 25-day-old mice. Znf230 was mainly associated with the acrosomal membrane of spermatids (C and E). Znf230 was expressed in the acrosome region and tail of epididymal spermatozoa (G). No immunoreactivity was observed when epididymal spermatozoa were incubated with pre-immune serum (H). Arrows indicate positive staining. Original magnifications: (A-H) ×400.

found in round spermatids, while the other cells, including spermatogonia, did not appear to express Znf230 (Fig. 3C). The immunostaining in spermatids was restricted to a small area between the nucleus and cell membrane and took the shape of an acrosome. On pn day 25, the number of Znf230-positive cells had increased due to proliferation of the round spermatids, and most of these cells were Znf230-positive (Fig. 2D). In the testes of adult mice that had completed the first wave of spermatogenesis, all germ cell types (from spermatogonia to spermatozoa) could be seen along the seminiferous tubules. Under higher magnification of the sections, the round and elongated spermatids exhibited the highest level of immunoreactivity to the anti-Znf230 antibody (Fig. 3D-3F). In these cells, Znf230 protein expression was always restricted to the acrosomal system, depicting the changing shape of this structure. The immuno-positive structures showed two different morphologies: in early round spermatids, the Znf230 staining was limited to a granule close to the nucleus that matches the so-called Golgi phase of the acrosome (Fig. 3D); as development of round spermatids progressed, the positive structure took on a flattened cap shape covering approximately half of the nucleus (Fig. 3E). Znf230 expression was also observed in the head of elongated spermatids. Immunoreactivity in these cells was observed in an elongated area corresponding to the acrosome (Fig. 3F). In both round and elongated spermatids, the strongest immunoreactivity seemed to be localized to the acrosomal membrane (Fig. 3C and 3E). Znf230 localization in the acrosomal system was confirmed by PAS (periodic acid-Schiff) staining in comparison with section which was stained specifically to the acrosome structure (Fig. 2G). Finally, Znf230 was analyzed in spermatozoa obtained from mouse epididymis. Once again, the immunoreactivity was present in the acrosome of these cells. However, it is interesting to note that Znf230 was also detected in the tail of spermatozoa (Fig. 3G). Nonspecific immunostaining was not observed with the pre-immune rabbit serum (Fig. 2F and 3H).

Cellular and subcellular localization of Znf230 in epididymal spermatozoa

To confirm that epididymal spermatozoa contain the Znf230 protein and to verify its subcellular localization, we used indirect immunofluorescence using the prepared Znf230 antibody. The results were the same: positive immunostaining (green) was observed over the acrosomal region of the sperm head and on the entire length of the sperm tail including the middle piece, principal piece and end piece (Fig. 4F). Specificity of the immunolabeling was indicated by the lack of immunoreactivity in sperm cells where the primary antibody was pre-immune serum (Fig. 4C).

Immunoblot analysis

To confirm that Znf230 was indeed present in the testis and in the acrosome and tail of spermatozoa, we carried out immunoblot analysis. The results of this analysis indicated that the antibody detected a single band of 26-kDa in extracts of adult testes, sperm acrosomes and sperm tails, while no bands were observed in negative control blots (Fig. 1D). These results strongly indicated that Znf230 detected by the antibody was indeed present in the testis and in the sperm acrosome and tail.

DISSCUSSION

During mammalian spermatogenesis, spermatogenic stem cells proliferate to produce spermatogonia, which differentiate into primary spermatocytes. After a prolonged meiotic prophase, the latter undergo two consecutive meiotic divisions to generate haploid spermatids. Spermatids then undergo marked morphological changes from an initially round to a progressively elongated shape with condensed nuclei, and eventually form spermatozoa (21). In pn day 6 mice, the seminiferous epithelium is essentially organized into type-A spermatogonia cells and Sertoli cells. Type-B spermatogonia ap-

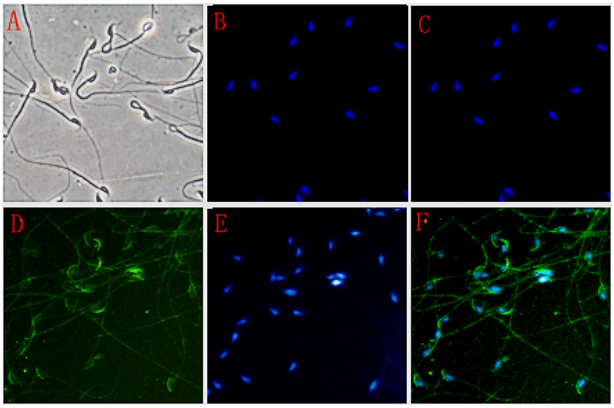

Fig. 4. The domain-specific localization of Znf230 by indirect immunofluorescence on mouse spermatozoa from the epididymis. The polyclonal antibody identified the domains of the acrosome as well as those of the tail. Znf230 localized to the acrosomal region of the sperm head and the entire length of the sperm tail including the middle piece, principal piece and end piece. (A-C) Epididymal sperm incubated with pre-immune serum as a negative control. (D-F) Sperm incubated with antiserum. (A) Phase contrast picture. (D) Indirect-immunofluorescence micrography picture (green). (B) and (E) Sperm nuclei stained with DAPI (blue). (C) and (F) Merge of A with B and D with E. Magnifications: (A-F) ×400.

pear on pn day 8, and the first meiotic spermatocytes appear on pn day 10. From pn day 18 to pn day 25, round spermatids increase in number and progressively differentiate into condensing spermatids (22). With this chronology in mind, we have found that the *Znf230* gene is expressed in mouse testicular tissues throughout postnatal development and this was confirmed by immunoblotting and RT-PCR analysis. The expression level remains stable from pn day 1 to adulthood.

In the testis, it is possible to distinguish the series of distinct developmental stages of spermatogenic cells based on their morphology and position in the seminiferous tubule (23). In the present investigation, Znf230 is mainly expressed in the nuclei of spermatogonia within the immature testis (at pn day 6 and pn day 12), but the expression gradually decreases and is absent from the spermatogonia nuclei by pn day 18. However, the gene product starts to appear again in the acrosome of round and elongated spermatids and persists as the morphology changes from the Golgi phase, through the cap phase to the acrosome phase (Fig. 3). The Znf230 protein is also localized in the acrosome and throughout the tail of epididymal spermatozoa. No immunochemical signal is detected in spermatocytes, Sertoli cells or Leydig cells. The specific Znf230 expression in spermatogonia, spermatids and spermatozoa suggests that the gene may be involved in several functions during spermatogenesis, including cell proliferation, sperm maturation, fertilization and motility.

Since molecular mechanisms in spermatogenesis are largely unknown, it is worthwhile to investigate novel proteins that are expressed at specific developmental stages of male germ cells. It is interesting and curious that Znf230 is detected in the nuclei of spermatogonia in the beginning of spermatogenesis and then gradually appears in the acrosome and tail of haploid cells, while spermatogonia lose Znf230 expression. This shift in protein localization is very rare. It is interesting to note that expression of the germ cell nuclear factor (*GCNF*) gene is similar to that of Znf230. The former is initially detected in the nuclei of spermatogonia and primary spermatocytes. Subsequent expression in round spermatids and pachytene spermatocytes coincides with a loss of expression in spermatogonia and primary spermatocytes. In addition, GCNF is also localized in the acrosomal cap region of spermatozoa. The reason for the change in expression is that GCNF has two transcripts, a larger transcript that is expressed earlier and a smaller transcript that is expressed at later time points (24). Similarly, Znf230 was also reported to be expressed as two transcripts (1kb and 4.4kb) in mouse testes (18), and the same protein is translated from both transcripts. It is very likely that the two transcripts may be expressed in different stages, one in the nuclei of spermatogonia, and the other in the acrosome and tail of haploid cells. Further investigations will be required to prove this hypothesis and to determine which transcript is expressed earlier.

In summary, Znf230, as a new zinc finger protein, is presumed to be a novel nuclear protein of spermatogonia and an acrosomal and sperm tail protein of spermatids and spermatozoa. As an important spermatogenesis-related protein, the biochemical structure, function and role of Znf230 in different stages of spermatogenesis should be studied in greater detail in the future.

MATERIALS AND METHODS

Animals

BALB/c male mice were divided into eight groups of 4 animals each according to their postnatal age (postnatal day 1, 3, 6, 9, 12, 18, 25 and adults). The left testes of male mouse pups and adult mice were fixed in formalin for immunohistochemical analysis. The other testes were frozen at -70°C subsequent protein or RNA extraction and the epididymides were taken as a source of sperm. Two adult male New Zealand White rabbits were used for antibody production. All of the animal experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (USA).

Construction of recombinant pGEX-Znf230

Total RNA was extracted from adult testes with the RNeasy Mini Kit and QIAshredder columns (Qiagen) and cDNA was obtained with random primers and Superscript II Reverse Transcriptase (Gibco BRL). The entire *Znf230* coding region was generated by RT-PCR using the following *BamH*I or *Not*I restriction site-containing primers: forward primer, 5′-CGGGA TCCCCATGGGACAGCAAATTTCGGAT-3′, and reverse primer,

5′-ATAAGAATGCGGCCGCACAGAAAGTCTTCCCCAA GTCA-3′. The PCR product was then cloned into the pGEM-T easy vector (Promega) for sequencing. The fragment released from the recombinant plasmid by cutting with *BamH*I and *Not*I, was subcloned into the prokaryotic expression vector pGEX-5X-3 (Phamacia). The resulting recombinant plasmid was termed pGEX-Znf230.

Preparation of polyclonal antibody to Znf230

The GST-Znf230 protein was expressed in *E. coli* BL21(DE3) and purified with glutathione-Sepharose 4B (Pierce) according to the manufacturer's instructions. The New Zealand White rabbits were immunized with the fusion protein to prepare the antiserum. Polyclonal antibody was purified to remove anti-GST present in the serum by incubating the antiserum with GST protein immobilized on glutathione-Sepharose 4B beads. The specificity of the antiserum was tested by western blotting with purified GST-Znf230 protein.

Immunoblotting

Whole testes of newborn mice at postnatal days 1, 3, 6, 9, 12, 18, and 25, and of adult mice were homogenized in ice-cold RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Sperm were collected and the sperm acrosome and tail fractions were prepared as described previously (25). Total proteins (20 μg) from each sample were electrophoresed on a SDS-polyacrylamide gel and subsequently electroblotted onto PVDF membranes (Millipore). After incubation in blocking buffer (5% nonfat milk-0.1% Tween 20-PBS) at 4°C overnight, the membrane was incubated with 1:7,000-diluted antiserum or β-actin antibody at room temperature for 2 h, and then incubated with 1:5,000-diluted goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase for 1 h at room temperature. Immunoreactive bands were visualized using ECL detection reagents (Amersham).

RT-PCR analysis

Total RNAs (2 μg) isolated from each sample were reverse transcribed. The obtained cDNAs (1 μl) were then used as templates for subsequent PCR amplification. The primers used to amplify a 234-bp fragment of *Znf230* were 5'-AGCACGTCAC CTTGGTTCGAGAAAG -3' (forward) and 5'-AATTCATGATCC GCGATGCTTCCAC-3' (reverse). A 517-bp fragment corresponding to β-actin was co-amplified as an internal control. The PCR profile was as follows: pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 30 s, with an extra final extension at 72°C for 5 min. The amplification products were then resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Control reactions were performed in the absence of the template cDNA.

Immunohistochemical methods

Sections of mouse testes were treated with 3% H_2O_2 in methanol for 20 min to inhibit intrinsic peroxidase activity and with 10% normal goat serum for 30 min to prevent nonspecific antibody binding. Immunohistochemical analysis of Znf230 was performed using the immunoperoxidase procedure of the Vectastain Elite ABC kit (Vector Laboratories), according to the manufacturer's instructions, with rabbit anti-Znf230 polyclonal antibody (dilution 1:300) and diaminobenzidine tetrahydrochloride (DAB) substrate. Negative control sections were prepared by replacing the primary antibody with non-immune serum from rabbit. Some sections were stained with PAS (periodic acid-Schiff staining) to identify the acrosome system (26). Epididymal spermatozoa immunoreactivity was investigated using the method described above.

Indirect immunofluorescence staining

To study Znf230 expression in spermatozoa, fresh mouse cauda epididymis was incised with a scalpel, spermatozoa were allowed to disperse into phosphate-buffered saline at 37°C for 30 min. Samples were dried on a glass slide and fixed with iced methanol. Slides were then covered with 10% goat serum for 30 min to block nonspecific antibody binding. They were then incubated at room temperature in a humidified chamber for 2 h with the purified antibody diluted 1:300. Slides were washed and goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma) diluted 1:100 was added for 1 h followed by staining of spermatozoa nuclei with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). Control samples were incubated with pre-immune serum. Slides were mounted and examined using a Nikon Microphot-FX microscope equipped with epifluorescence.

Acknowledgments

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