Histol Histopathol (2009) 24: 25-30

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

ZNT7 and Zn^{2+} are present in different cell populations in the mouse testis

Zhi-Hong Chi[1], Wan-Yu Feng[1], Hui-Ling Gao[1], Wei Zheng[1], Liping Huang[2] and Zhan-You Wang[1]

[1]Department of Histology and Embryology, China Medical University, Shenyang, PR China and [2]Department of Nutrition and Rowe Program in Genetics, USDA/ARS/Western Human Nutrition Research Center, University of California at Davis, Davis, California, USA

Summary. The aim of the present study was to investigate the relation of the spatial distribution between ZNT7 and chelatable zinc ions in the mouse testis. Immunohistochemical results demonstrated a wide distribution of ZNT7 in both seminiferous tubules and interstitial tissues of the testis. The spermatocytes and spermatids in the seminiferous tubules showed strong ZNT7 immunoreactivity whereas zinc autometallographic (AMG) staining was absent. Spermatozoa showed a high level of free zinc, but were void of ZNT7 immunoreactivity. No ZNT7 immunoreactivity and AMG grains were found in spermatogonia. Both ZNT7 and chelatable zinc were detected in Sertoli and Leydig cells. Furthermore, double immunofluorescence study demonstrated that the ZNT7 staining overlapped with that of TGN38 (a trans-Golgi marker), suggesting that ZNT7 was localized in the Golgi apparatus in the ZNT7-positive cells. In conclusion, ZNT7 and chelatable zinc were distributed in different cell populations except for Sertoli and Leydig cells in the mouse testis. ZNT7 may be involved in zinc transportation into the Golgi apparatus for protein packaging in the mouse testis.

Key words: Zinc, Zinc transporter, Autometallography, Testis, Mouse

Introduction

Roles of zinc in male reproduction have been studied for more than five decades. Studies indicate that zinc is intimately involved with many aspects of sperm morphology, physiology, and biochemistry (Hidiroglou and Knipfel, 1984). Particularly, high zinc concentrations are found in most mammalian seminal plasma, spermatozoa, testis, epididymis, and prostate (Mawson and Fischer, 1951; Wetterdal, 1958; Byar et al., 1969; Sorensen et al., 1998). Interestingly, semen is rich in zinc. Low zinc in semen is associated with poor sperm count and motility (Hidiroglou and Knipfel, 1984). During spermiogenesis, DNA chromatin in the sperm nucleus is tightly packed and this condensed structure, which is important for successful fertilization, is stabilized by zinc (Delgado et al., 1984). High zinc concentrations in semen helps to prevent premature capacitation and acrosome reactions (Andrews et al., 1994). Once zinc concentrations become diluted within the female tract, the capacitation and acrosome reactions occur for fertilization. Zinc may also regulate the synthesis and secretion of testosterone by sensitizing Leydig cells to the stimulation of gonadotropins (McClain et al., 1984; Fabris, 1994). Furthermore, zinc affects testis development as it has been shown that dietary zinc deficiency results in necroses of precursors of germ cells, hypogonadism, inhibition of spermatogenesis, and abnormal morphology of spermatozoa (Hidiroglou and Knipfel, 1984; Merker and Gunther, 1997).

Zinc transporter (ZNT, Slc30) family members are involved in transportation of zinc from the cytosol into the extracellular space or pumping zinc into different intracellular organelles (Colvin et al., 2003; Kambe et al., 2004). To date, at least 8 Znt genes, named Znt1 to Znt8, have been cloned and characterized (Seve et al., 2004). ZNT members are predicted to have 6 transmembrane domains and a large histidine-rich intracellular loop between domains 4 and 5 which is proposed to be the zinc binding site (Palmiter and Huang, 2004). Among the ZNTs, ZNT7 was identified by searching the expressed sequence tag (EST) databases with the amino acid sequence of ZNT1 (Kirschke and Huang, 2003). Immunocytochmistry and electron microscopy have demonstrated that ZNT7 is localized in the Golgi apparatus of the cell (Chi et al.,

Offprint requests to: Zhan-You Wang MD, PhD, Department of Histology and Embryology, China Medical University, Shenyang 110001, P.R. China. e-mail: wangzy@mail.cmu.edu.cn

2006). Exposure of Chinese hamster ovary (CHO) cells overexpressing ZNT7 to high zinc caused an accumulation of zinc in the Golgi apparatus, suggesting that ZNT7 facilitated zinc transport from the cytosol into the Golgi complex (Kirschke and Huang, 2003).

Previous studies have demonstrated an abundant expression of a ZNT family member, ZNT1 protein, in the testis (Elgazar et al., 2005), but little is known about the distribution and functional significance of other ZNTs in the mouse testis. It is well known that proacrosomal vesicles derived from the Golgi apparatus and the shape of Golgi apparatus is different in different stages of spermatocyte during the acrosomal biogenesis, (Kierszenbaum et al., 2007). Therefore, the present study aims to investigate the relation of the spatial distribution between ZNT7 and chelatable zinc ions in the mouse testis.

Materials and methods

Mice

Eight male CD-1 mice (8-10 weeks old, 30-35 g, provided by the experimental animal center of China Medical University) were used in the study. They were housed under a 12 h light/dark cycle with water and food available *ad libitum*. All procedures were carried out in accordance with the ethical standards of China Medical University.

Immunohistochemistry

Four mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and were transcardially perfused with isotonic saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH7.4). Testes were removed and fixed by immersion them into the fixatives for 3 h at 4°C. Fixed testes were then immersed in a 30% sucrose solution made in PB overnight at 4°C. Subsequently, the samples were cut in 10-µm sections on a cryostat (JUNG CM 1800, Leica, Germany) and mounted on glass slides. The sections were rinsed twice in Tris-buffered saline (TBS, pH 7.4), and treated with 3% hydrogen peroxide (H_2O_2) in PB for 10 min to reduce endogenous peroxidase activity. After rinsing with TBS, all tissue sections were preincubated for 1 h with 5% bovine serum albumin (BSA) and 3% goat serum in TBS to reduce nonspecific staining. Sections were then incubated with ZNT7 antiserum (an affinity-purified rabbit antibody specific for ZNT7) (Kirschke and Huang, 2003) diluted 1:100 in TBS containing 3% goat serum, 1% BSA, and 0.3% Triton-X 100 overnight at 4°C. After extensive washing in TBS, sections were incubated with a 1:200 diluted biotinylated anti-rabbit IgG for 1 h at room temperature (RT). The ABC Kit was then used to visualize the reaction sites. The ABC solution was diluted 1:100 in TBS. A brown color appeared in the section sites after incubation of the sections in 0.025% 3,3'-diaminobenzidine with 0.0033% H_2O_2 for 10 min at RT. Some sections were counterstained with hematoxylin to detect cell types of the mouse testis. All sections were then dehydrated in graded alcohols and covered by coverslips with neutral balsam for light microscopy. To assess nonspecific staining, a few sections were incubated with normal sera instead of the primary antibody. This procedure always resulted in a complete lack of immunoreactivity.

For confocal immunofluorescence microscopy, cryostat sections were preincubated with normal donkey serum (1:20) for 1 h and then incubated with the anti-ZNT7 antiserum at 1:100, and a monoclonal anti-TGN38 antibody (Sigma) at 1:100 overnight at RT. Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit (FITC-DAR) IgG and Texas Red-conjugated donkey anti-mouse (Texas Red-DAM) IgG (Jackson ImmunoResearch) were used as secondary antibodies for the primary anti-ZNT7 and anti-TGN38 antibodies, respectively. After rinsing with PBS, sections were incubated with DAR-FITC (1:50) and Texas Red-DAM (1:50) for 2 h at RT. Sections were then mounted and examined in a confocal laser scanning microscope (CLSM, SP2, Leica, Germany).

Zinc selenide autometallography (AMG)

Four mice were injected i.p. with sodium selenide dissolved in 0.1M PB (25 mg/kg). One and half hours later, animals were killed by perfusing transcardially with 2.5% glutaraldehyde solution in 0.1M PB. Tissue isolation and fixation were performed as described above. After fixing, samples were cut in 10 µm sections on a cryostat and collected on glassslides. Sections were then incubated in the AMG developer in a water bath for 1 h at 26°C as described previously (Danscher, 1982). Subsequently, sections were immersed in a 5% thiosulphate solution, rinsed with running tap water and counterstained with hematoxylin. Sections were then dehydrated in graded alcohols, covered with coverslips, and analyzed with a light microscope.

Results

ZNT7 is highly expressed in the mouse testis

The distribution of ZNT7 immunoreactivity in the mouse testis was analyzed under a light microscope. No immunoreactivities were detected in control picture (Fig. 1a). In general, a distinct immunoreactivity of ZNT7 was observed throughout the sections. Most cells in the seminiferous tubules and interstitial tissue were ZNT7 positive (Fig. 1b). The immunoreactivity of ZNT7 was located in the cytoplasm of cells. In the seminiferous tubules, ZNT7 immunoreactivities were most intensive in the spermatocytes at all stages of meiosis and in the spermatids (Fig. 1b). However, the ZNT7 immunostaining patterns in the spermatocytes were distinctive in the different stages of the seminiferous tubules. In the stages VIII and IX, the staining pattern of

ZNT7 appeared semilunar whereas it was in the punctate form in the stages II and III (Fig. 1c,d). In non-counterstaining sections, ZNT7 immunoreactivities were absent in the zone where the spermatozoa and the spermatogonia located. The identification of stages in the seminiferous tubules was done according to the standards described previously (Parvinen and Ruokonen, 1982). In counterstaining sections, the spermatogonia were void of ZNT7 immunoreactivity (Fig. 1e). Most of the Sertoli cells in the seminiferous tubules showed weak staining of ZNT7 (Fig. 1f). The Leydig cells in the interstitial tissue, also exhibited ZNT7 immunoreactivity (Fig. 1g).

Dual immunofluorescence of ZNT7 and TGN38

To further test the subcellular distribution of ZNT7 in the mouse testis, double labeling of ZNT7 and TGN38 (a trans-Golgi marker) was performed. Under CLSM, the spermatocytes and spermatids were ZNT7-positive (Fig. 2a1). Almost all of cells in the seminiferous tubules were TGN38-positive (Fig. 2a2). After merging the ZNT7- and TGN38-labeled images from the two channels, ZNT7 and TGN38 immunostaining overlapped in the spermatocytes and spermatids (Fig. 2a3). The spermatogonia and spermatozoa were void of ZNT7 immunoreactivity (Fig. 2a3).

Zinc autometallographic reaction products in the mouse testis

One and half hours after an i.p. injection of a sodium selenite, AMG grains were detected in the seminiferous tubules under a light microscope. The general pattern of AMG staining in the mouse testis is shown in Fig. 3a. A disperse granular staining pattern of AMG grains was predominantly shown in the spermatozoa (Fig. 3b). Chelatable zinc was also detected in the Sertoli cells, but the level was much lower than that in the spermatozoa (Fig. 3c). There was no detectable free zinc in the

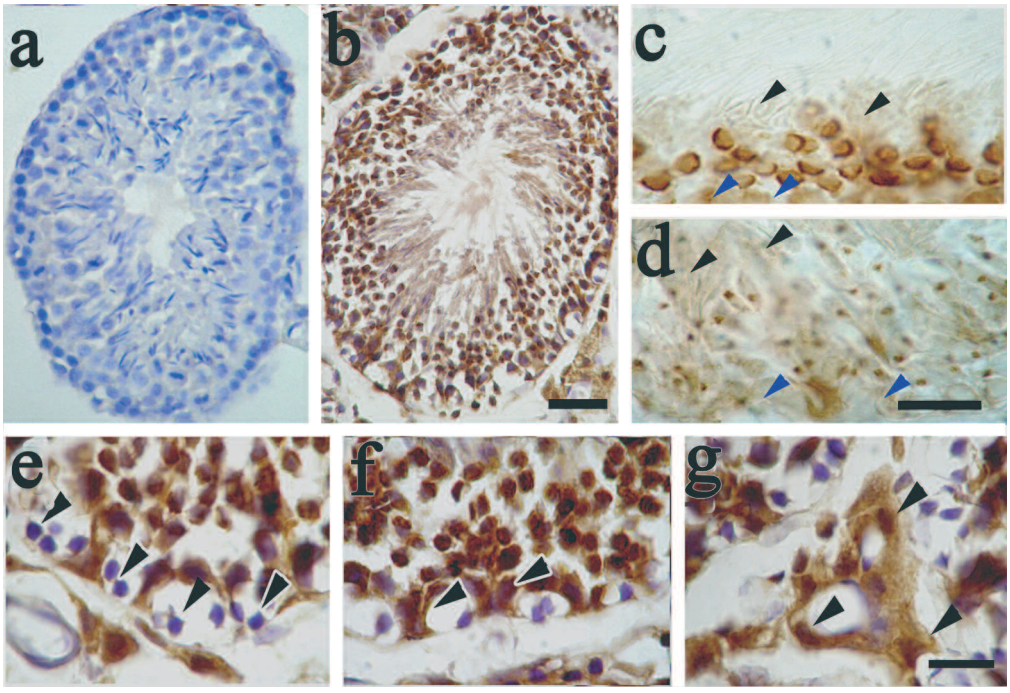

Fig. 1. Light microscopy photomicrographs of the testicular sections stained with an anti-ZNT7 antibody. No immunoreactivities were detected in control picture **(a).** General distribution of ZNT7 immunoreactivity in the seminiferous tubules is shown in **b.** The ZNT7 immunostaining patterns are different in respective stages of the seminiferous tubules. In stages VIII and IX, the pattern of ZNT7 immunoreactivity is semilunar **(c).** In stages II and III, ZNT7 immunoreactivity is present in the punctuate form **(d)**. In the zone where the spermatozoa (black arrows) and the spermatogonia (blue arrows) located, no ZNT7 immunoreactivities were detected **(c, d)**. ZNT7 immunoreactivity is absent in spermatogonia (arrowheads in **e)**. ZNT7 immunoreactivity is present in the Sertoli cells (arrowheads in **f**) and the Leydig cells (arrowheads in **g**). Scale bars: a, b, 50 µm; c-g, 10 µm.

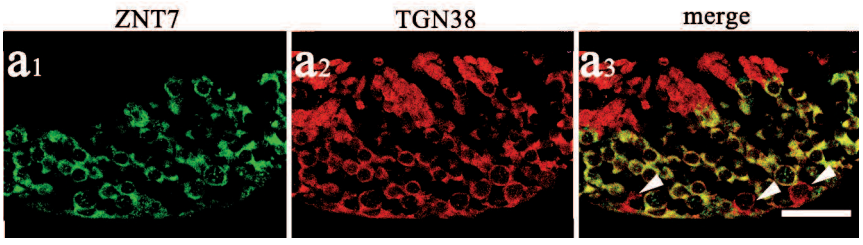

Fig. 2. Confocal fluorescence microcopy photomicrographs of ZNT7 and TGN38 in the seminiferous tubules. ZNT7 immunoreactivity is predominantly found in the spermatocytes and spermatids **(a1).** Almost all cells in the seminiferous tubules are TGN38-positive **(a2)**. Colocalization of ZNT7 and TGN38 is predominantly found in spermatocytes and spermatids (**a1-a3**). The arrowheads indicate that spermatogonia are devoid of ZNT7 immunoreactivity. Scale bar: 20 µm.

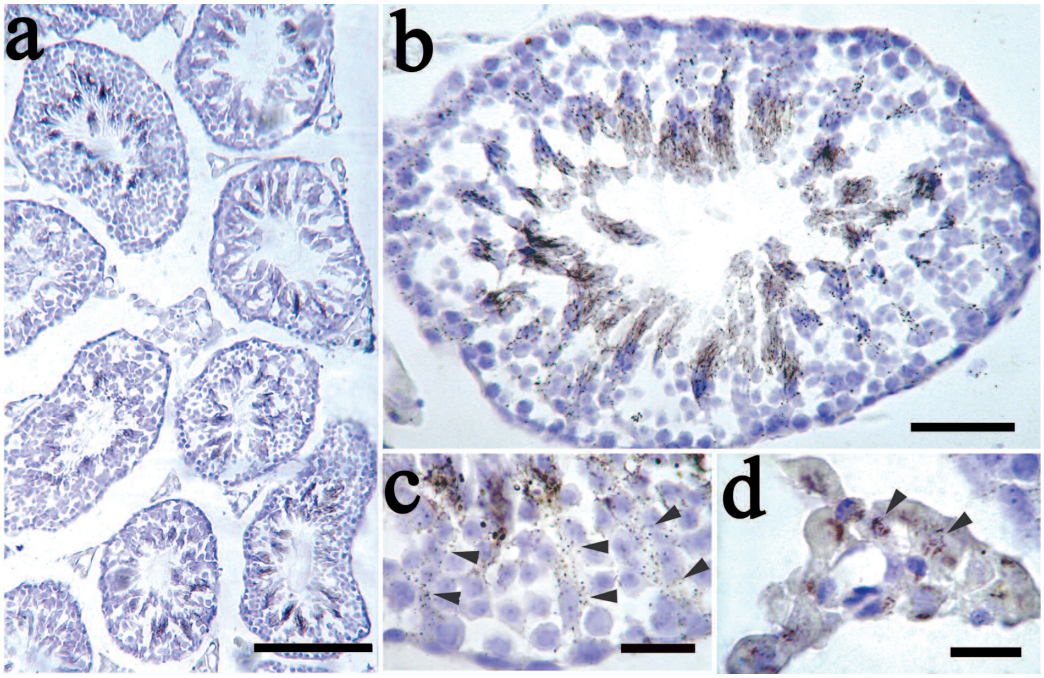

Fig. 3. Light microscopy photomicrographs of the testicular sections stained by autometallography (AMG). The general distribution of zinc-AMG grains in the seminiferous tubules of the testis is shown in **a**. AMG grains are predominately located in the spermatozoa in the seminiferous tubules **(b)**. Zinc-AMG grains are also found in the Sertoli cells (arrowheads in c) and in the Leydig cells (arrowheads in d). Scale bars: a, 200 μm; b, 50 μm; c, 10 μm; d, 5 μm.

spermatogonia, spermatocytes and spermatids. In the interstitial regions, Leydig cells were AMG-positive (Fig. 3d). However, the AMG grains in the Leydig cells were less than that in the spermatozoa (Fig. 3b,d).

Discussion

In the present study, for the first time, the regional distribution of ZNT7 in the mouse testis was investigated by immunohistochemistry. Our immunohistochemical results demonstrated that the ZNT7 immunoreactivity was predominantly present in the spermatocytes, spermatids sustentacular cells, and Leydig cells of the testis. Double immunofluorescence study indicated that ZNT7 is localized in the Golgi apparatus of the cell. In addition, chelatable zinc was present in the spermatozoa and to a less extent in Sertoli and Leydig cells.

ZNT7 is believed to facilitate the translocation of the cytoplasmic zinc into the Golgi apparatus, where zinc is presented to the newly synthesized metalloproteins and/or metalloenzymes (Huang et al., 2002; Kirschke and Huang, 2003; Suzuki et al., 2005; Chi et al., 2006; Ishihara et al., 2006). Interestingly, the staining patterns of ZNT7 in the spermatocytes were different in respective stages (Fig. 1c). The changes in the ZNT7 staining patterns in the spermatocytes were in good agreement with an earlier electron microscopic study in which it demonstrated that the Golgi apparatus in the spermatocytes had morphologic changes during the epithelial cycle of seminiferous tubules (Brokelmann, 1963; Gardner, 1966; Susi et al., 1971; Haraguchi et al., 2004).

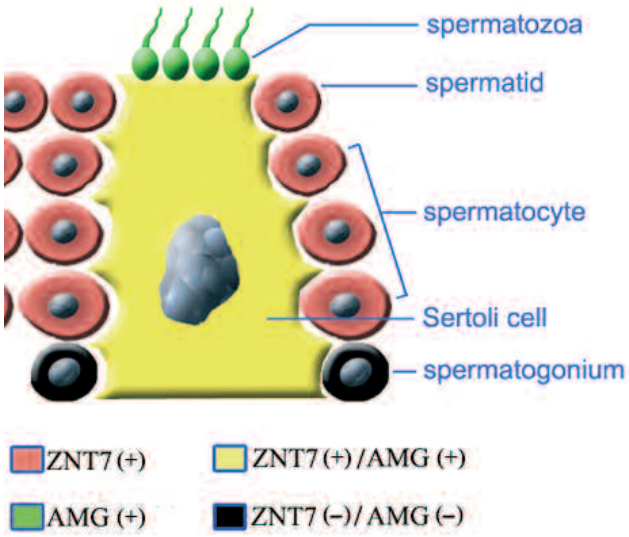

Fig. 4. Schematic drawings of the spatial distribution of ZNT7 immunoreactivity and chelatable zinc ions in the seminiferous tubules according to the results of immunohistochemistry and AMG in the present study.

Fig. 4 shows a general distribution of chelatable zinc and ZNT7 in the mouse seminiferous tubule. Spermatozoa exhibit AMG reaction but not ZNT7 immunoreactivity. It is known that spermatozoa contain high level of zinc (Mawson and Fischer, 1951;

Wetterdal, 1958; Byar et al., 1969; Sorensen et al., 1998). Apparently, ZNT7 is not the protein responsible for the zinc accumulation in spermatozoa. One proposed mechanism for the free zinc accumulation is lack of the expression of metallothioneins I and II (Elgazar et al., 2005). Sertoli cells provide the developing germ cells with appropriate mitogens, differentiation factors, energy, as well as protecting them from harmful agents and from host's own immune attack (Tsuruta et al., 1993; Petersen and Soder, 2006). Elgazar et al. demonstrated that ZNT1 was expressed in Sertoli cells (Elgazar et al., 2005). In the present study, free zinc and ZNT7 coexist in Sertoli cell, suggesting that Sertoli cell may be the zinc provider for the developing germ cells and ZNT7 may have a role in this process. In the testis of adult mammalian organisms, spermatogonia maintain their numbers by self-renewal and give rise to differentiating germ cells (Ehmcke et al., 2006). Zinc-regulating proteins, such as metallothioneins I and II, are expressed in spermatogonia (Elgazar et al., 2005). However, in the present study, we demonstrated that neither ZNT7 nor free zinc was detectable in spermatogonia, supporting the notion that spermatogonia are quiescent in zinc-containing protein synthesis.

Leydig cells of the testis are the main source for androgen synthesis and secretion, and play important roles in male reproductive system (Akhmerova, 2006). Both ZNT7 and free zinc were detected in Leydig cells, suggesting that ZNT7 is involved in zinc homeostasis in these cells.

In conclusion, the present study demonstrated a wide distribution of ZNT7 in the seminiferous tubules and interstitial tissues of the mouse testis. ZNT7 was localized in the Golgi apparatus of ZNT7-positive cells. ZNT7 and chelatable zinc distributed in different cell populations in the mouse testis except for Sertoli and Leydig cells.

Acknowledgements. Supported by Specialized Research Fund for the Doctoral Program of Higher Education (SRFDP-20060159001), Program for New Century Excellent Talents in University (NCET-04-0288), and the United States Department of Agriculture (CRIS-5603-515-30-014-00D).

References

Akhmerova L.G. (2006). Leydig cell development. Usp. Fiziol. Nauk. 37, 28-36.

Andrews J.C., Nolan J.P., Hammerstedt R.H. and Bavister B.D. (1994). Role of zinc during hamster sperm capacitation. Biol. Reprod. 51, 1238-1247.

Brokelmann J. (1963). Fine structure of germ cells and Sertoli cells during the cycle of the seminiferous epithelium in the rat. Z. Zellforsch. Mikrosk. Anat. 59, 820-850.

Byar D.P., Anderson J.E. and Mostofi F.K. (1969). The distribution of 65zinc in the prostate and other organs in control, castrated, and hypophysectomized rats. Invest. Urol. 7, 57-65.

Chi Z.H., Wang X., Wang Z.Y., Gao H.L., Dahlstrom A. and Huang L. (2006). Zinc transporter 7 is located in the cis-Golgi apparatus of mouse choroid epithelial cells. Neuroreport 17, 1807-1811.

Colvin R.A., Fontaine C.P., Laskowski M. and Thomas D. (2003). $Zn2^+$ transporters and $Zn2^+$ homeostasis in neurons. Eur. J. Pharmacol. 479, 171-185.

Danscher G. (1982). Exogenous selenium in the brain. A histochemical technique for light and electron microscopical localization of catalytic selenium bonds. Histochemistry 76, 281-293.

Delgado N.M., Magdaleno V.M., Merchant H., Rosado A. and Reyes R. (1984). Heparin-induced release of DNA template restrictions in human sperm zinc-depleted nuclei. Arch. Androl. 12, 211-216.

Ehmcke J., Hubner K., Scholer H.R. and Schlatt S. (2006). Spermatogonia: origin, physiology and prospects for conservation and manipulation of the male germ line. Reprod. Fertil Dev. 18, 7-12.

Elgazar V., Razanov V., Stoltenberg M., Hershfinkel M., Huleihel M., Nitzan Y.B., Lunenfeld E., Sekler I. and Silverman W.F. (2005). Zinc-regulating proteins, ZnT-1, and metallothionein I/II are present in different cell populations in the mouse testis. J. Histochem. Cytochem. 53, 905-912.

Fabris N. (1994). Neuroendocrine-immune aging: an integrative view on the role of zinc. Ann. NY Acad. Sci. 719, 353-368.

Gardner P.J. (1966). Fine structure of the seminiferous tubule of the Swiss mouse. The spermatid. Anat. Rec. 155, 235-249.

Haraguchi C.M., Mabuchi T., Hirata S., Shoda T., Hoshi K. and Yokota S. (2004). Ubiquitin signals in the developing acrosome during spermatogenesis of rat testis: an immunoelectron microscopic study. J. Histochem. Cytochem. 52, 1393-1403.

Hidiroglou M. and Knipfel J.E. (1984). Zinc in mammalian sperm: a review. J. Dairy Sci. 67, 1147-1156.

Huang L., Kirschke C.P. and Gitschier J. (2002). Functional characterization of a novel mammalian zinc transporter, ZnT6. J. Biol. Chem. 277, 26389-26395.

Ishihara K., Yamazaki T., Ishida Y., Suzuki T., Oda K., Nagao M., Yamaguchi-Iwai Y. and Kambe T. (2006). Zinc transport complexes contribute to the homeostatic maintenance of secretory pathway function in vertebrate cells. J. Biol. Chem. 281, 17743-17750.

Kambe T., Yamaguchi-Iwai Y., Sasaki R. and Nagao M. (2004). Overview of mammalian zinc transporters. Cell. Mol. Life Sci. 61, 49-68.

Kierszenbaum A.L., Rivkin E. and Tres L.L. (2007). Molecular biology of sperm head shaping. Soc. Reprod. Fertil Suppl. 65, 33-43.

Kirschke C.P. and Huang L. (2003). ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. J. Biol. Chem. 278, 4096-4102.

Mawson C.A. and Fischer M.I. (1951). Zinc content of the genital organs of the rat. Nature 167, 859.

McClain C.J., Gavaler J.S. and Van Thiel D.H. (1984). Hypogonadism in the zinc-deficient rat: localization of the functional abnormalities. J. Lab. Clin. Med .104, 1007-1015.

Merker H.J. and Gunther T. (1997). Testis damage induced by zinc deficiency in rats. J. Trace Elem. Med .Biol. 11, 19-22.

Palmiter R.D. and Huang L. (2004). Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch. 447, 744-751.

Parvinen M. and Ruokonen A. (1982). Endogenous steroids in rat seminiferous tubules. Comparison of the stages of the epithelial cycle isolated by transillumination-assisted microdissection. J. Androl. 3, 211-220.

Petersen C. and Soder O. (2006). The sertoli cell--a hormonal target and 'super' nurse for germ cells that determines testicular size. Horm. Res. 66, 153-161.

Seve M., Chimienti F., Devergnas S. and Favier A. (2004). In silico identification and expression of SLC30 family genes: an expressed sequence tag data mining strategy for the characterization of zinc transporters' tissue expression. BMC Genomics 5, 32.

Sorensen M.B., Stoltenberg M., Henriksen K., Ernst E., Danscher G. and Parvinen M. (1998). Histochemical tracing of zinc ions in the rat testis. Mol. Hum. Reprod. 4, 423-428.

Susi F.R., Leblond C.P. and Clermont Y. (1971). Changes in the golgi apparatus during spermiogenesis in the rat. Am. J. Anat. 130, 251-267.

Suzuki T., Ishihara K., Migaki H., Nagao M., Yamaguchi-Iwai Y. and Kambe T. (2005). Two different zinc transport complexes of cation diffusion facilitator proteins localized in the secretory pathway operate to activate alkaline phosphatases in vertebrate cells. J. Biol. Chem. 280, 30956-30962.

Tsuruta J.K., O'Brien D.A. and Griswold M.D. (1993). Sertoli cell and germ cell cystatin C: stage-dependent expression of two distinct messenger ribonucleic acid transcripts in rat testes. Biol. Reprod. 49, 1045-1054.

Wetterdal B. (1958). Experimental studies on radioactive zinc in the male reproductive organs of the rat. Acta Radiol. Suppl. 156, 1-83.

Accepted June 27, 2008