マウス精巣におけるプリオン遺伝子mRNA発現パターンについて

誌名	The journal of reproduction and development
ISSN	09168818
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発行元	Japanese Society of Animal Reproduction
巻/号	50巻5号
掲載ページ	p. 565-570
発行年月	2004年10月

Journal of Reproduction and Development, Vol. 50, No. 5, 2004

—Original—

Expression of *Prnp* mRNA (Prion Protein Gene) in Mouse Spermatogenic Cells

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Abstract. The *Prnp* (prion protein) gene, which encodes a soluble protein anchored to the cell surface by glycosylphosphatidylinositol (GPI), might be involved in cell-to-cell interaction. The expression of *Prnp* is strongly observed not only in the brain, but also in non-neuronal tissues. In order to examine the *Prnp* expression sites in mouse testes, we carried out Northern blot and *in situ* hybridization analyses. By Northern blot analysis, two kinds of *Prnp* transcripts (major band of 2.2 kb, and minor band of 1.1 kb) were detected in testes. The 2.2-kb transcript was observed in testes throughout the postnatal development, whereas the 1.1-kb transcript was observed in testes from 2 to 70 weeks old. *In situ* hybridization analysis showed that the positive signals for *Prnp* mRNAs were predominantly observed in spermatogenic cells, but not in somatic cells such as Sertoli cells, Leydig cells and peritubular myoid cells. The signals were observed moderately in spermatogonia, and strongly in spermatocytes and round spermatids, but not in elongate spermatids and spermatozoa. These results suggest that *Prnp* may be involved in germ cell differentiation during mammalian spermatogenesis.
Key words: *Prnp*, *In situ* hybridization, Northern blot, Spermatogenesis

(J. Reprod. Dev. 50: 565–570, 2004)

Transmissible spongiform encephalopathy (TSEs) or prion diseases which show a transmissible neurodegenerative disease common to medical (new variant Creutzfeldt-Jakob disease etc.) and veterinary medical fields (scrapie of sheep, bovine spongiform encephalopathy, etc.) manifest as sporadic, infectious, or genetic disorders characterized by the accumulation of an abnormal isoform (PrP^{sc}) of cellular prion protein (PrP^{c}). PrP^{c} expression has been investigated in most of organs, and differences in amount of expression in each organ have been reported in the hamster [1] and sheep [6]. Concerning the expression pattern and the functional analysis of PrP^{c}, much information is available for the central nervous system. Especially, detailed analysis has been done on the hamster brain [23], and on hamster brain

Accepted for publication: June 9, 2004
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developmental stage [13, 14]. Although many studies on digestive and immune systems have also been carried out, there is little data on reproductive organs such as the testis.

PrP^c is classified as a GPI-anchored glycoprotein [26]. Since the expression of PrP^c is frequently seen on the cell membrane surface, it has been speculated that PrP^c may participate in cell signaling or adhesion [17, 24]. PrP^c also participates in regulation of copper/zinc superoxide dismutase (Cu/Zn SOD: SODI) as a copper binding protein, suggesting that PrP^c may be related to copper metabolism and anti-oxidative responses [3, 4]. Since the oxidative stress gives severe damage to testes and the *SodI* mRNA expression is present in seminiferous tubules [7], PrP^c may play an important role in spermatogenesis. Moreover, it has been found by using the $Prnp^{-/-}$mouse that the prion-like protein gene (Doppel gene; *Dpl*) exists in a downstream region of the prion gene and is specifically expressed in wild-type mouse testes [16]. It is antipated that the relationship between the two genes in the testes will be clarified in the future.

In order to examine the role of PrP^c during spermatogenesis, we examined the *Prnp* expression in mouse testes by using Northern blot and *in situ* hybridization analyses. The present study has revealed that *Prnp* transcripts are abundantly expressed in spermatogenic cells, but not in somatic cells, suggesting the possible role of PrP^c in germ cell differentiation during mammalian spermatogenesis.

Materials and Methods

Experimental animals

C57BL/6J male mice were used in the present study. They were maintained in our closed colony under a standardized laboratory condition. At 1, 2, 4, 8 and 70 weeks of age, male mice were sacrificed under pentobarbital anesthesia, and the testes were isolated and used for the following RNA analysis. We used the 8-week-old mouse as a representative of a mature mouse, and the 70-week-old as a representative of senescent one. For *in situ* hybridization, 8-week-old male mice were perfused with Bouin's fixative, and the testes were excised and immersed in the same fixative for 12–24 h. Subsequently, they were processed for embedding in paraffin.

Generation of probes

The 495-bp DNA fragments, corresponding to the coding region of a *Prnp* cDNA (1–165 amino acids) were isolated from testis cDNAs by polymerase chain reaction, using the specific primers 5'-ATGGCGAACCTTGGCTACTG-3' (forward) and 5'-ACTGGCCTGTAGTACACTTG-3'(reverse); then they were subcloned into pCR^{II} plasmid (Invitrogen Corp., Carlsbad, CA USA). The clone was linearised with the appropriate restriction enzyme, and antisense RNA probes were generated by in vitro transcription using α-^{32}P UTP or DIG-labeled UTP with Sp6 or T7 RNA polymerase (Roche Diagnostics Corp., Mannheim, Germany).

Northern blot analysis

Total RNAs were extracted from testes and brains of 5 adult mice using a RNA extraction kit (Isogen; NPG, Toyama, Japan). Each 10 μg of total RNA sample was fractioned on 1% agarose formalin gel, and blotted onto a nylon membrane (Hybond N; Amersham Biosciences Corp., Piscastaway, NJ USA). Blots were hybridized with the appropriate ^{32}P-labeled RNA probe at 67 C as described previously [8]. Filters were finally washed with 0.1 × SSC-0.1% SDS at 75 C for 1 h and autoradiographed.

In situ hybridization

In situ hybridization was performed as described by Nam *et al.* [18] and Kanai *et al.* [9]. In short, deparaffinized sections were pretreated with 0.3% Triton X-100 in 10 mM phosphate buffer saline and 20 μg/ml proteinase K in Tris-HCl buffer (pH 7.5) containing $CaCl_2$, and then hybridized with DIG-labeled RNA probes in a solution containing 50% formamide, 10% dextran sulfate, 5 × SSC, 1 × Denharts' solution, 1% SDS, 100 μg/ml heparin, 10 mM DTT, and 1 mg/ml denatured tRNA and ssDNA at 55 C for 12–16 h. After treatment with RNase (20 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) at 37 C for 1 h, the specimens were finally washed twice with 0.1 × SSC at 65 C for 1 h. The signals were detected by an immunohistological method using alkaline phosphatase conjugated anti-DIG antibody and nitro blue tetrazolium as the chromogen (Roche Diagnostics).

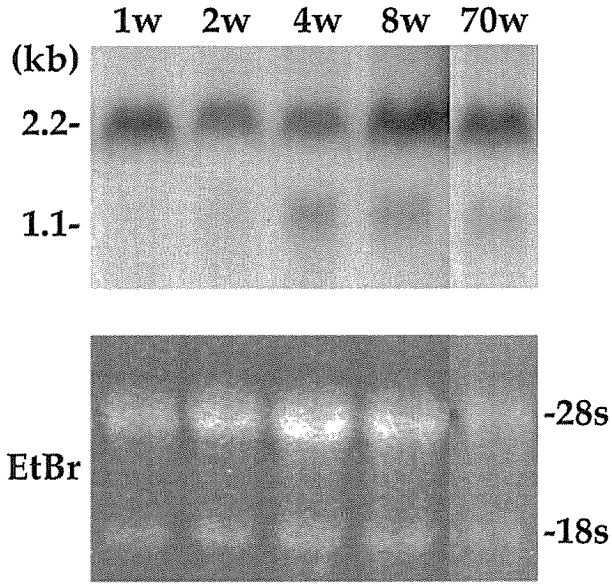

Fig. 1. Northern blot analysis shows *Prnp* expression pattern in the testis during postnatal development of mice. Two transcripts (approximately 2.2 kb and 1.1 kb) are expressed in the testis. The 2.2-kb major band is observed throughout the postnatal development and aging. In contrast, the 1.1 kb band is observed in the testes isolated from 2- to 70-week-old mice. Lower panels show the 28 S and 18 S rRNA bands stained with ethidium bromide (EtBr).

Results

In order to examine the *Prnp* expression in mouse testes, we first carried out Northern blot analysis using testes isolated from mice of various ages mice. We detected two kinds of the *Prnp* transcripts (major band of 2.2 kb, while minor band of 1.1 kb) in tests' samples (Fig.1). The 2.2 kb band was observed throughout the postnatal development, and its expression level was almost equal at all periods. In contrast, the 1.1 kb band was observed in testes from 2 weeks old.

In situ hybridization analysis revealed that the signals for *Prnp* mRNAs were predominantly observed in germ line lineage (Fig. 2A). No signals were detected in the sections treated with a sense probe (Fig. 2B). The signals were observed in spermatogonia, spermatocytes and round spermatids, and were especially strong in early pachytene spermatocytes and round spermatids (Fig. 2D-G). No signals were detected in elongate spermatids and spermatozoa. Such strong expression in spermatocytes and round spermatids may be partially due to the additional expression of the 1.1 kb transcript obtained by Northern blot analysis (Fig. 1). In contrast to high expression in spermatogenic cells, *Prnp*-positive signals could not be detected in interstitial cells such as Leydig cells (Fig. 2C), Sertoli cells and myoid cells.

Discussion

In Northern blot analysis, a 1.1 kb band was observed in testes. This band was observed from 2-week-old and older mice, and was also accepted at 70-week-old. Previous studies showed that two *Prnp* mRNAs were found in peripheral tissues of rodents as a result of alternative polyadenylation [5, 11, 21]. Although there was no detailed analysis about *Prnp* transcripts in testes, the 1.1 kb band we detected in mouse testes may be a mature transcript produced by the same mechanism. The probe which includes PrP ORF (open reading frame) hybridized with the prominent 2.2 kb *Prnp* mRNA expressed in wild-type adult mice testes, and also in *Prnd*$^{-/-}$ (prion-like protein gene, doppel) mice [2]. *Prnd* is located 16 kb downstream from *Prnp* and generates major transcripts of 1.7 kb and 2.7 kb as well as some unsual chimeric transcripts generated by intergenic splicing with *Prnp* [16]. To detect the chimeric transcripts in Northern blot analysis, we should use the probe consisting of *Prnp* exon1 and 2 cDNA, not *Prnp* exon3 including PrP ORF [10]. Because we used a probe which include PrP ORF in this study, the possibility that the 1.1 kb band is *Prnd* or chimeric ones may be low.

In situ hybridization analysis showed that a strong signal was detected from spermatocytes to round spermatids in adult mouse testes. Little signal was observed in Sertoli cells, Leydig cells, and myoid cells. Although previous studies showed that reactivities of *Prnp* mRNA were detected in rat myoid cells [27] or negative in mouse testes [12], our finding was different from these studies. The strongest expression was observed in pachytene spermatocytes followed by round spermatids. This result shows the possibility that the *Prnp* transcripts generated before meiosis may be translated in stage X or XI.

Reactive oxygen species are highly toxic agents that have an important role in male infertility. Copper/zinc superoxide dismutase (SOD1) is one

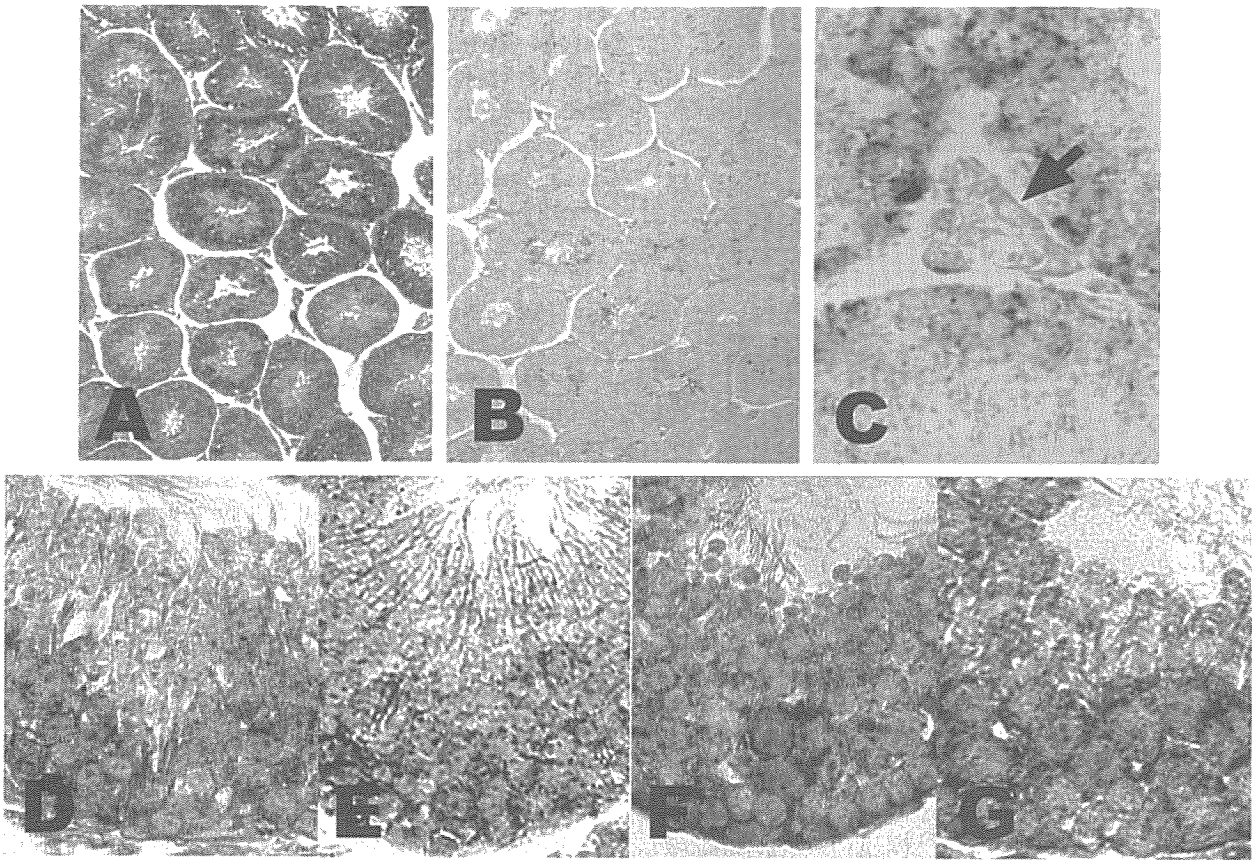

Fig. 2. *In situ* hybridization analysis shows *Prnp* expressions in spermatogenesis. Testis sections were hybridized with either DIG-labeled antisense (A) or sense (B) riboprobe for *Prnp*. *Prnp* mRNA is strongly expressed in spermatocytes, and weakly in round spermatids during each spermatogenesis stage. No signals are detected in elongate spermatids and spermatozoa. *Prnp*-positive signals cannot be detected in Leydig cells (C; arrowhead). A) Low amplification (antisense), B) low amplification (sense), C) Leydig cells, D) stage IV, E) stage VI, F) stage VII, G) stage X. A-B) × 40, C-G) × 250.

of the reactive oxygen scavengers. Its mRNA is determined in testes as two transcripts, and is localized in seminiferous tubules of rats [7]. Brown *et al.* [3, 4] and Wong *et al.* [28] reported that *Prnp*$^{-/-}$ mice have depleted levels of copper, and have shown a reduction in the activity of SODI, indicating that PrP may play a role in copper homeostasis, and that PrP itself can act as a superoxide dismutase in the brain. When the testicular copper concentration is higher than normal, degenerative changes, such as vacuolation and pyknosis, can be found in pachytene spermatocytes and early spermatids [29]. A copper metabolism system also exists in the testes. Therefore, PrP is one of the factors maintaining the system with SODI or other copper binding proteins within seminiferous tubules.

It has been reported that the *Prnp*$^{-/-}$ mutant mice are fertile both in male and female mice [22]. Moreover, our unpublished histological observation also showed no appreciable defect in spermatogenesis in *Prnp*$^{-/-}$ mice, suggesting that *Prnp* is not essential for spermatogenesis *in vivo*. While Prnd is strongly expressed in adult testes [16], these findings suggest that no defect in *Prnp*$^{-/-}$ mice is due to the complementation of *Prnd* in testicular function. In fact, Doppel is expressed in both Sertoli cells and spermatozoa in human testes [20], and *Prnd* deficiency resulted in male sterility [2]. A recent study showed that there was no evidence that Doppel compensates for the loss of PrP function in *Prnp*$^{-/-}$ /*Prnd*$^{-/-}$ mice (Prion/Doppel double knockout mice), but that it has an important anti-oxidant function necessary for sperm integrity

and male fertility [19].

PrP^c is classified as a GPI-anchored glycoprotein, which exists on the cell membrane surface *via* GPI [26]. It has been shown that the structure of PrP^c in mature spermatozoa differs from immature ones [25]. PrP^c was not separated from epididymal and ejaculated spermatozoa processed by PIPLC (phosphatidylinositol phospholipase C). Moreover, PrP^c in ejaculated spermatozoa had a truncated C terminus. A sperm antigen is classified broadly into three sorts, an acrosome membrane antigen, a sperm membrane (SM) antigen, and a sperm coating (SC) antigen. We surmise that PrP^c of spermatozoa is in the form of a SM or SC antigen.

In conclusion, the present study revealed that *Prnp* transcripts are apparently expressed in spermatogenic cells, not in testicular somatic cells, which indicates that *Prnp* may have a possible role in germ cell differentiation during mammalian spermatogenesis.

Acknowledgments

The authors wish to thank Mr. Iwao Tsugiyama, and Dr. Takuo Mizukami for their technical assistance. This work was supported in part by financial grants from the Ministry of Education, Culture, Sports, Science and Technology.

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