IMMUNOHISTOCHEMICAL EXPRESSION OF MEMBRANE PROTEIN PALMITOYLATED 6 (MPP6) IN MICE TESTIS

Gewaily M.S[1],MansourA.A.[1], FayedM.H.[1],WakayamaT.[2]and Iseki S.[2]

[1]Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt

[2]Department of Histology and Embryology, Graduate School of Medical Science, Kanazawa University, Japan

ABSTRACT

Spermatogenesis is a process by which sperms are produced through successive mitosis of spermatogonia, meiosis of spermatocytes and maturation of spermatids.MPP6 mRNA was abundant in testis, brain, and kidney with lower levels detectable in other tissues.in the present study,the cellular localization of MPP6 in the mouse testis was analyzedbyenzyme histochemistry and immunofluorescence staining using polyclonal anti-rat antibody and paraffin tissue sections of mice testes.The histochemicalstaining revealed that the MPP6 waslocalized in the cytoplasm of elongated spermatid near to the lumen of seminiferous tubules while not expressed in other spermatogenic cells or in Sertoli cells.The expression of MPP6 in the elongated spermatids refers to the role of it at the level of spermiogenesis and suggest important role during maturation of elongated spermatids.

Keywords: MPP6, MAGUK, Mouse testis, IHC

INTRODUCTION

Male fertility is required for large numbers of normal spermatozoa produced by a complex process known as spermatogenesis. Spermatogenesis is the transformation of spermatogonial cells into

spermatozoa over an extended period of time within seminiferous epithelium of the seminiferous tubule ***(Hess and Franca, 2005 and Cheng et al. 2010)*** and occurs during the 14 stages of the seminiferous epithelial cycle of spermatogenesis in rats, 12 stages in mice, and 6 stages in human ***(Cheng et al. 2010).*** This process can be subdivided into three major steps: (i) the proliferation of spermatogonia by mitosis; (ii) meiosis reducing the chromosome number from diploid to haploid. These cells, now called primary spermatocytes, divide to form secondary spermatocytes, and then divide again to form round spermatids; (iii) the successful transformation of the round spermatid into the complex structure of the spermatozoon, this phase alsocalled spermiogenesis ***(de Kretser et al.,1998)***. During this process, germ cells multiply first by repeated mitotic divisions and then by meiosis, which involves the duplication of chromosomes, genetic recombination, and then reduction of chromosomes through two cell divisions to produce spherical haploid spermatids that differentiate into highly compacted spermatozoa for release into the lumen of seminiferous tubules ***(Hess and Franca, 2008).*** Each of these steps represents a key element in the spermatogenic process. Defects which occur in any of them can result in the failure of the entire process and lead to the production of defective spermatozoa and reduction or absence of sperm production. Therefore, it is essential to understand the regulatory mechanism of this process to be able to deal with any defect and improve the efficiency of it ***(de Kretser et al., 1998)***.

The MAGUKs (membrane-associated guanylatekinase homologues) constitute a family of peripheral membrane proteins that function in tumor suppression and receptor clustering by forming multiprotein complexes containing distinct sets of transmembrane, cytoskeletal, and cytoplasmic signaling proteins ***(Tseng et al.,2001).*** This family functions

as scaffolding proteins at the sites of membrane specializations, linking transmembrane proteins to the membrane cytoskeleton and to signal transduction pathways ***(Anderson, 1996, Craven and Bredt, 1998*** and ***Dimitratos et al.,1999)***.

MPP6 is also known as VAM1 (Veli Associated MAGUK1) and considered as one of MAGUK molecules.VAM-1 mRNA was abundant in human testis, brain, and kidney with lower levels detectable in other tissues. The primary structure of VAM-1, predicted from cDNA sequencing, consists of 540 amino acids including a single PDZ domain near the N-terminus, a central SH3 domain, and a C-terminal GUK (guanylate kinase-like) domain ***(Tseng et al.,2001)***. So, the aim of this study is to analyze the expression and cellular localization of MPP6 or VAM1 in the testes of adult mice by immunohistochemistry.

MATERIALS AND METHODS

1. Animal preparation

The present study was performed on the testes of wild type mice(Musmusculus) of129Sv/C57BL6 strain that were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animal experiments were performed according to Guidelines for the Care and Use of Laboratory Animals in Kanazawa University, Japanand grown under 12L: 12D laboratory conditions.

2. Tissue preparation

Mice have been anesthetized with sodium pentobarbital, killed by bleeding from the right atrium, and perfused transcardially in vivo with cold physiological saline. To make tissue specimens for

immunohistochemistry, the animals will be further perfused transaortally with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The testes will be dissected out, further immersed in the same fixative for 4 h, dehydrated in a graded alcohol series for 24 h, and embedded in paraffin. Then, they will be cut into 4μm-thick sections utilizing a routine procedure.

2.1. Haematoxylin and Eosin

The paraffin sections from mice testes were subject to routine staining by H&E to investigate the basic cellular structure of seminiferous tubules.

2.2. Enzyme IHC (Immunohistochemistry)

The tissue sections of mouse testes were treated with 0.3% H2O2 in methanol for 10 min to inhibit intrinsic peroxidase activity and with 5% low fat milk for 30 min to prevent nonspecific antibody binding. Subsequently, the sections were incubated overnight at room temperature with Rat anti-MPP6 antibody (prepared in the lab of histology and embryology, Kanazawa University, Japan) in a dilution of 1:400. After the sections were washed in PBS, the sites of immunoreaction were visualized by incubating the sections successively with biotinylated horse anti-rat IgG antibody **(Vector, Burlingame, CA)** for 1 h, horseradish peroxidase-conjugated streptavidin **(DAKO)** for 1h. The immune reaction was detected by immPACT™ DAB **(Vector, Burlingame, CA)**. Haematoxylin was used as a counter staining for the nuclei.

2.3. Immunofluorescence (IF):

The 4% paraformaldehyde- fixed cryostat sections of the mouse testis were first treated with 5% low fat milk (skimmed milk) to prevent non-specific antibody binding and then incubated overnight at 4C° with rat polyclonal antisera against MPP6 (1:400). After the sections were washed in PBS, the immunoreaction was visualized by incubating the sections with anti-rat IgG antibody conjugated with Alexa Fluor 594 **(Molecular Probes)** at 1:400 for single Immunoflourscent microscopy. The sections were counterstained in the nucleus with bisbenzimide H33258 (Hoechst 33258) (**Sigma-Aldrich**) while PNA (Pea nut Lectin) was used for acrosomal staining to help in stages differentiation. To investigate sertoli cells, we used mouse monoclonal anti-tyrosine tubulin (Sertoli cell marker) antibody followed by anti-mouse IgG antibody conjugated with Alexa Fluor 594 **(Molecular Probes)** at 1:400 for double immunoflourscent microscopy ***(Wakayama et al, 2007)***. The sections were then subjected to observation with an immunofluorescence microscope **(BX50/BXFLA; Olympus, Tokyo)**.

RESULTS

1. Expression of MPP6 in the wild typeadult mouse testis

To demonstrate the histological structure of seminiferous tubules, we stained the testes tissue sections by H&E. Sertoli cells as well as spermatogonia appear towards the periphery of seminiferous tubules and rest on the basement membrane.in the middle layer, there are different stages of spermatocytes followed by round spermatid while the elongated spermatids are found in the most inner layer towards the lumen of seminiferous tubules**(Fig.1A,)**. The mature sperms are found in the lumen of epididymis after releasing from the seminiferous tubules (**Fig.1C**).

On the level of immunohistochemistry, when the testis tissue sections of adult wild type mice were stained with rat polyclonal anti-MPP6 antibody, the positive reaction of Mpp6 was restricted to the cytoplasm of elongated spermatids towards the lumen of seminiferous tubules **(Fig.1B)**. On the other hand, there is no reaction of MPP6 with in the lumen of epididymis in which the elongated spermatids released after spermiation**(Fig.1D)**.

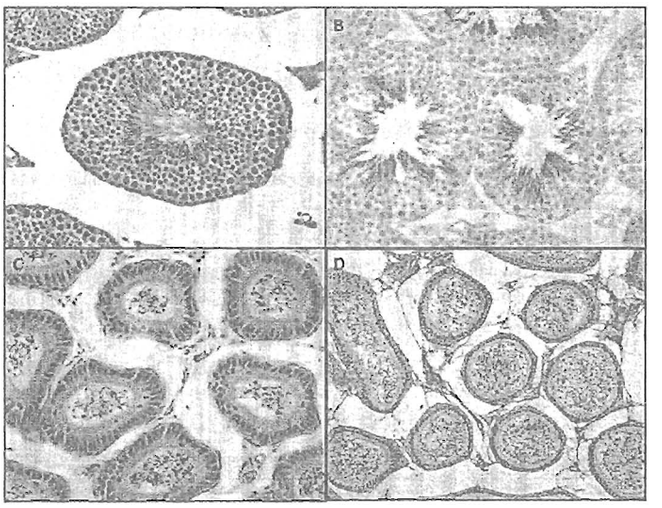

Fig. (1): A, C) Histological structures of adult mice testis by using H&E staining of paraffin sections of mice testes and epididymis showing cellular components of seminiferous tubules and epididymis.B and D; Immunohistochemical staining of mice testes and epididymis by using rat polyclonal anti-MPP6 antibody showing positive immunoreactivity in the cytoplasm of elongated spermatid near the lumen of seminiferous tubules while, there is no reaction in the epididymis. Bar=25μm (A&B) and 50 μm in (C&D).

2. Cellular localization of MPP6 during spermatogenesis

To examine the cellular localization of MPP6, we performed immunofluorescence histochemistry in paraffin-embedded mouse testicular sections using rat polyclonal anti-MPP6 antibody. The immunoreactivity was localized exclusively in the seminiferous tubules (**Fig.2-4**). The immunoreactivity in the interstitial tissue, presumably in Leydige cells, was non-specific, because replacement of the primary antibody with normal rat serum resulted in the same immunostaining(not shown). Some seminiferous epithelia were immunostained at the portion close to the lumen. The immunopositive cells close to the lumen represented elongating spermatids (**Fig.2A,C**).

In the mouse testis, spermiogenesis, i.e., the differentiation of spermatids, is divided into 16 steps based on the progression of acrosome formation and shape of nucleus in spermatids and, using these steps and the nuclear morphology of spermatogenic cells, spermatogenesis is classified into 12 stages represented by different segments of seminiferous tubules ***(Russell et al.1990)***.

At stage Iseminiferous tubule (**Fig.3A**), MPP6 immunoreactivity appeared weakly in the cytoplasm of elongated spermatid near the lumen of the tubules. Then immunoreactivity appeared stronger gradually in the cytoplasm of these cells in the following stages (**Fig.3B-F,)**. The positive immunoreaction reached the maximal intensity at stage VII to VIII (**Fig.3F**). The immunoreactivity was not detected at stageIX to X (**Fig.4A,B**) while appeared weak in the following two stages of XI and XII (**Fig.4C,D**). There is no any cellular localization of MPP6 in the Sertoli cells (**Fig.5**).

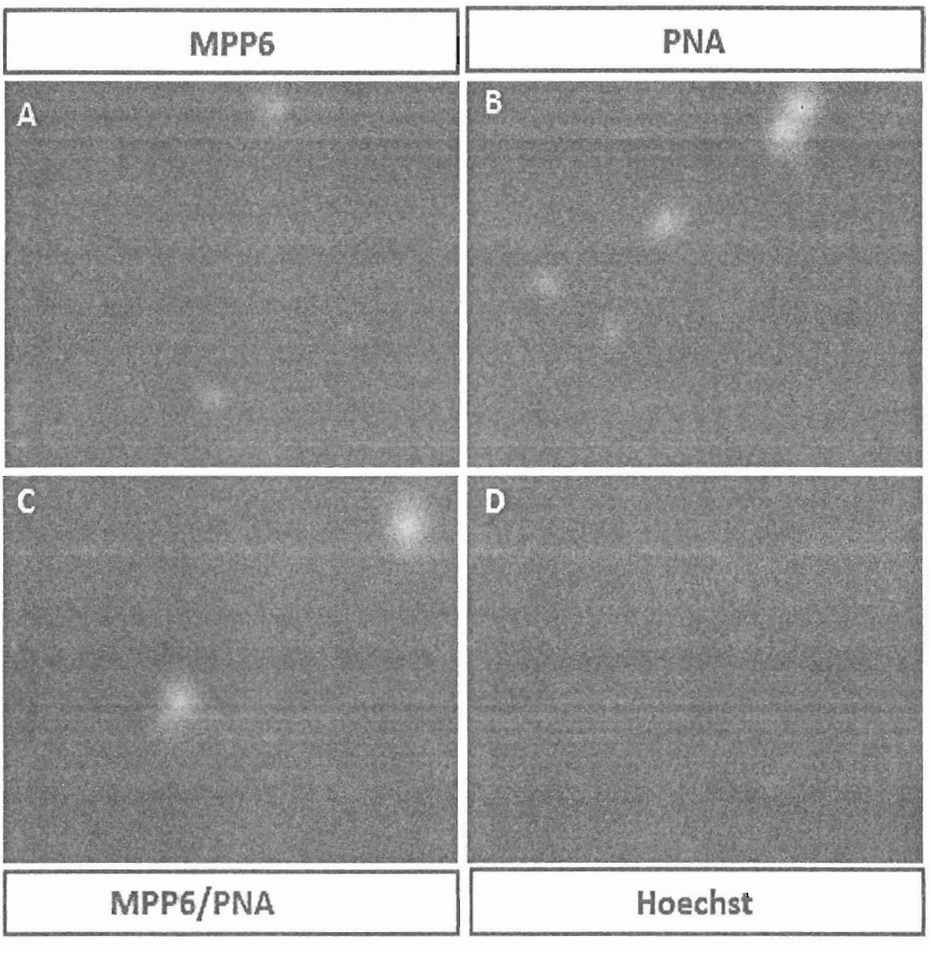

Fig. (2): Immunofluorescence microscopy showing localization of MPP6 immunoreactivity (red) in adult mouse testis. Paraffin sections of 10-week old testis were immunostained with rat polyclonal anti-MPP6 antibody. MPP6 is expressed in the elongated spermatid of the seminiferous tubules (A). B image is PNA lectin staining (green) for acrosomal formation. C refers to merge between MPP6 and PNA. D image is nuclear staining (blue). Bar=50 μm.

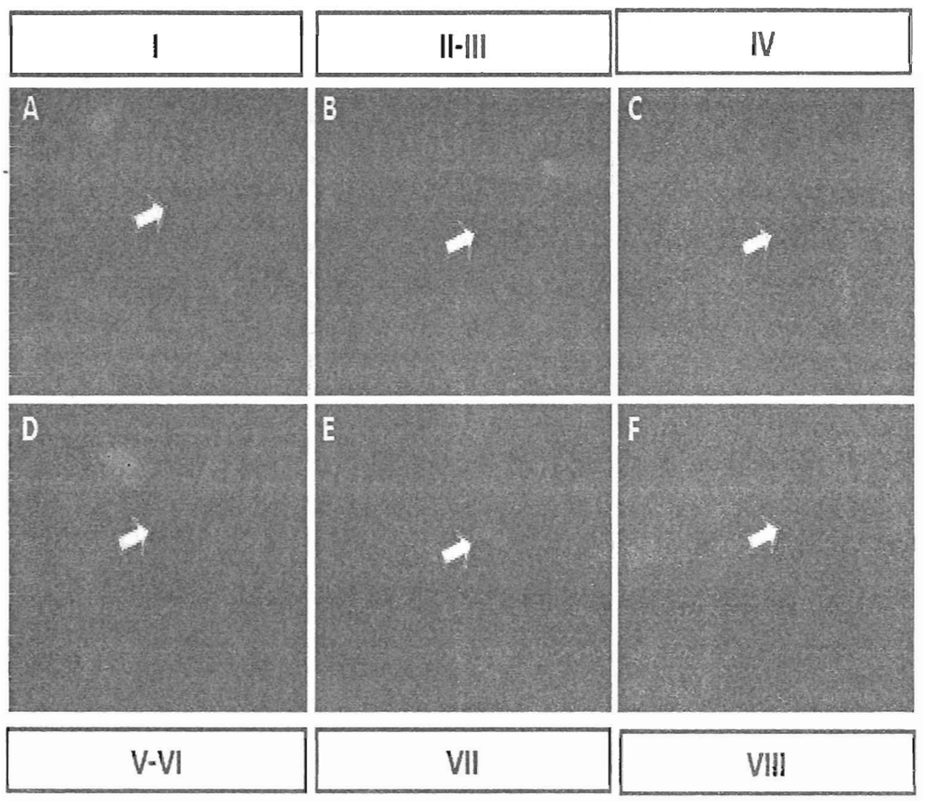

Fig. (3): Immunofluorescence microscopy showing cellular localization of MPP6 at stagesI-VIII (A-F respectively) during spermatogenesis. The expression of MPP6 (red)(white arrow) restricts in the elongated spermatid and appeared weak in stage I (A), then increased gradually in the following stages until reach maximal density in stages; VII-VIII. Bar =25μm.

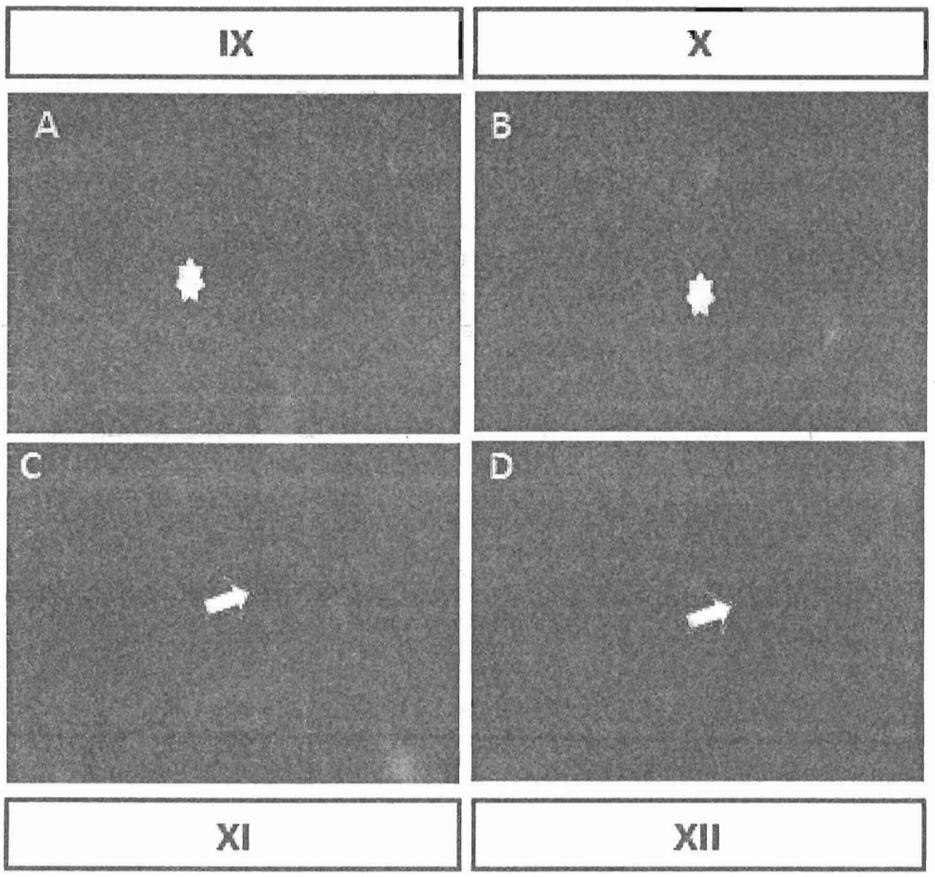

Fig. (4): Immunofluorescence microscopy showing cellular localization of MPP6 at stages IX-XII (A-D respectively) during spermatogenesis. The expression of MPP6 is absent in stages Ix and X(white star) while starts very weak in stages; XI-XII (white arrow). Bar =25μm.

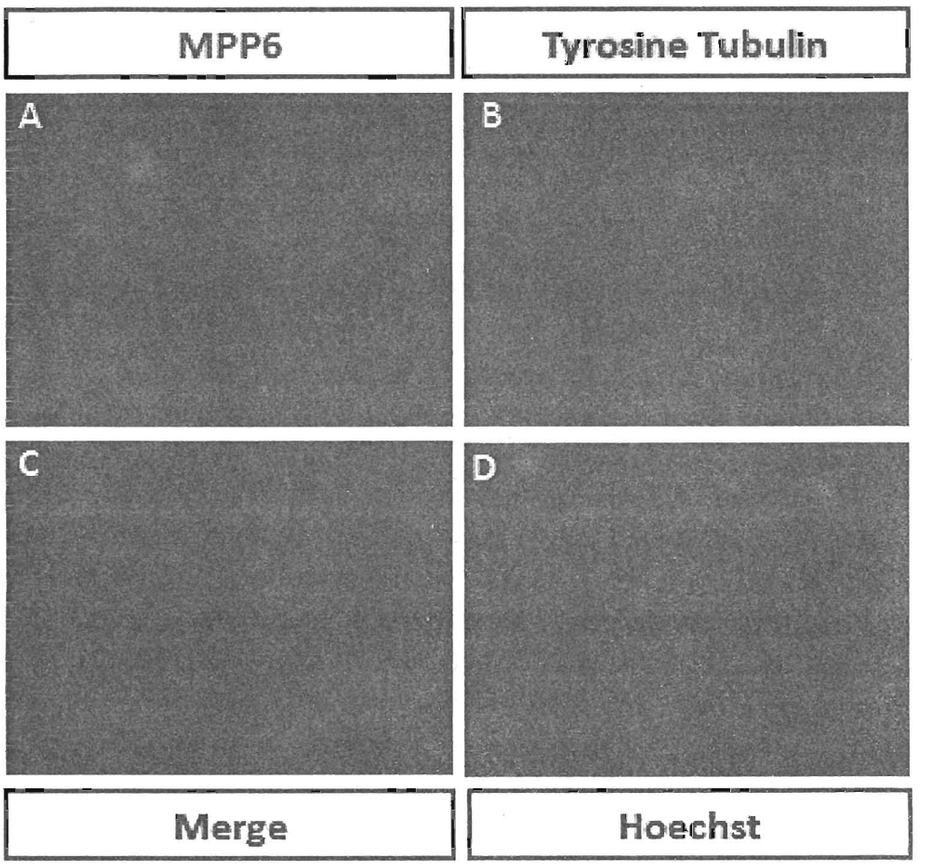

Fig. (5): double immunofluorescence of stage VII seminiferous tubule of adult wild type mouse with rat polyclonal anti-MPP6 antisera and mouse monoclonal anti-tyrosine tubulin (Sertoli cell marker) antibody. The immunoreactivity for MPP6 is demonstrated as green (A) and for tyrosine tubulin as red (B). There is no overlapping when the images are merged (C). Note that nucleus was stained with Hoechst (blue, D). Bar= 25 μm.

DISCUSSION

In the present study, we examined expression and cellular localization of MPP6 in the mouse testesby enzyme histochemistry as well as immunofluorescence and this is , to our knowledge, is the first work on it all over the world.Our results revealed that MPP6 protein is expressed in adult mice testis. This result is agreed with that revealed by ***Tseng et al. (2001)*** usingwestern blot analysis.

The immunoreactivity for MPP6 in wild-type testes demonstrates a characteristic pattern in the seminiferous tubules during different stages of spermatogenesis. It is restricted to the cytoplasm of elongated spermatid. At stage XI of spermatogenesis, MPP6 immunoreactivity first appeared weakly in the cytoplasm of step 9 spermatid (stage XI) near the lumen of the tubules. Then immunoreactivity appeared stronger gradually in the cytoplasm of these cells in the following stages. The immunoreactivity reached the maximum intensity at stage VII to VIII at which spermiation occur. The absence of Mpp6 localization in the spermatogenic cells may be due to lack of its role during the early stages of spermatogenesis and spermatogenic cells proliferation. On the other hand, the gradual increase of MPP6 expression inside the elongated spermatid during late stages leads us to suppose its role during sperm cells maturation and increase of this role towards spermiation. Furthermore, the histochemistry of MPP6 on the epididymis revealed no MPP6 expression that refers to stop of its role after spermiation.

Regarding to dependence of MPP6 on the formation of mammalian seminiferous tubules, Dlg was reported to function in gamete development in Drosophila testes ***(Papagiannouli and Mechler 2009)***. Furthermore, the defect of MAGUKs, such as Dlg1 ***(Bolino et al. 2004, Boles A, et al. 2009, Cotter Let al. 2010)*** and MPP5 ***(Özçelik et al.,2010)*** affectedthe myelination of nerve due to the disruption of the phosphatidylinositol metabolism with myotubularin-related 2 (MTMR-2)

and phosphatase and tensin homolog (PTEN). It has been demonstrated that MAGUK family members, such as Dlg1 and MPP7, interact with each other in epithelial cells ***(Bohl et al., 2007).*** Either P4.1G or P4.1B deficient mice represented to affect the myelination of Schwann cells but these mice did not affect spermatogenesis ***(Terada et al., 2012)***.

We suggest that there is a relation between MPP6 and CADM1 (cell adhesion molecule1) which considered a one of various transmembrane proteins that have been reported to occur in the seminiferous epithelium of mouse testis, where it is localized in the spermatids from steps 7 through 16 ***(Wakayama et al. 2003)***.

Our results cleared that there is no evidence of MPP6 expression in Sertoli cells, Although Sertoli cells are known to express adhesion molecules such as N-cadherin, nectin-2, PVR, junctional adhesion molecule-B, coxackie and adenovirus receptor, and integrin α6β1 ***(Johnson and Boekelheide 2002; Mueller et al. 2003; Siu and Cheng 2004; Mirza et al. 2006; Wakayama et al. 2007)***. During stage IX, however there is no immunoreactivity of MPP6 in the spermatogenic cells, there is strong immunopositive reaction inside the Sertoli cells that could be interpreted as the engulfed residual bodies inside the Sertoli cells. Thisfinding could be explained as; there is a role of MPP6 in the shedding of residual bodies from elongated spermatids and their phagocytosis of by Sertoli cells.

In conclusion, MPP6 was localized in the cell cytoplasm of elongating spermatids suggesting essential role in spermiogenesis. Moreover, it is interesting to assume the involvement of MPP6 in shedding off of residual bodies from elongated spermatids before spermiation. The similar localization of MPP6 and CADM1 in the elongated spermatids suggests interaction between both molecules to achieve an essential role in spermatogenesis and male fertility that need further investigation.

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