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Expression of Zonular Occludens-1 in Mouse Testis

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생쥐 정소내 Zonular Occludens-1 발현

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ABSTRACT: Spatiotemporal expression of two isoforms of zonular occludens-1 (ZO-1), tight junctional protein, was examined in mouse testis. By RT-PCR, transcripts encoding two isoforms of ZO-1; ZO-1 α+ and ZO-1 α- were detected in testis. Two different forms of ZO-1 antigens with Mr. 225 and 200 kDa were detected in western blot of extract of neonatal to adult testis, coinciding with the result of RT-PCR. The relative amount of ZO-1 α- versus ZO-1 α+ increased as the mice matured. In immunostaining using the pan antibody which detected both isoforms, ZO-1 was localized in the intercellular spaces in the Sertoli cell - Sertoli cell contacts in periphery of seminiferous tubule as well as Sertoli cell - germ cells contacts within the seminiferous tubule. The expression of ZO-1 was ubiquitous in both junctional area and cytoplasm of seminiferous tubule components. However, more intense signals were found in Sertoli cell junctional areas according to sexual maturation. The changes in the relative amount of both isoforms and spatial distribution of ZO-1 at the periphery of seminiferous tubule might be important for functional appearance of blood testis barrier and spermatogenesis.

Key words: Zonular occludens-1, Testis, Mouse.

요 약: 생쥐 정소에서 밀착결합단백질의 일종인 zonular occludens-1 (ZO-1)의 발현을 조사하였다. RT-PCR결과 ZO-1의 2가지 isoform인 ZO-1 α+, ZO-1 α-의 발현을 확인하였다. 생쥐 신생 및 성체의 정소에서 분자량 225 및 200 kDa의 2종의 ZO-1의 단백질항원의 발현을 확인되어 RT-PCR의 결과와 일치하였다. ZO-1 α+에 대한 ZO-1 α-의 상대적 발현량은 성숙에 따라 증가하였다. 2종의 ZO-1항원을 동시에 인식하는 항체를 사용한 면역염색을 통해 세정관 외곽의 Sertoli세포 사이의 접촉부위 및 Sertoli세포와 생식세포 접촉부위에서 ZO-1의 존재를 확인하였다. ZO-1은 세정관내 세포들 사이의 결합부위 및 세포질에서 공통적으로 발현되지만 성숙에 따라 Sertoli세포의 결합부위에서 강한 신호가 검출되었다. 2종의 ZO-1 항원의 상대적 발현량의 변화 및 세정관 외곽의 분포의 강화는 기능적 혈액정소장벽의 출현 및 정자형성의 진행과 관련된 것으로 사료된다.

INTRODUCTION

Tight junction (TJ) creates a regulated paracellular barrier to the movement of water, solutes, and immune cells between both epithelial and endothelial cells. Basally located tight junctions between Sertoli cells in the postpubertal testis are the largest and most complex junctional complexes known and TJ is an important structural element in the formation of blood testis barrier (BTB). TJ is a multimolecular membrane specialization comprising multiple integral membrane proteins with four putative transmembrane domains, occludin and claudins (Furuse et al., 1993; 1998), and several associated peripheral proteins. The latter forms a cytoplasmic plaque which interact with actin filaments (Anderson & Van Itallie, 1995; Fanning et al., 1998).

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Zonula occludens-1 (ZO-1), TJ peripheral membrane protein of Mr. 205 ~ 225 kDa (Stevenson et al., 1986; Itoh et al., 1993) encoded by *TJP1* in human (chromosome 15q13) and *Tjp1* in mouse (chromosome 7) (Mohandas et al., 1995) establishes a link between the transmembrane protein occludin and the actin cytoskeleton (Fanning et al., 1998). ZO-1 has alternatively spliced isoforms, the longer isoform having an extra 80 amino acid domain known as the α-domain (Willott et al., 1993, see Fig. 1). Although the function of the α-domain is unclear, ZO-1 α+ isoform is found in conventional epithelial TJs while ZO-1 α- is present in endothelial junctions and highly specialized epithelial TJs characteristic of Sertoli cells and renal podocytes. This pattern of ZO-1 isoform expression may influence the plasticity of TJs (Balda & Anderson, 1993). These proteins are seen at the cell membrane interface, where they engage in the formation of TJ and thus construct diffusion barrier between blood and adluminal compartment of seminiferous tubule. In rodent, ultrastructure of TJ was found before puberty (Nagano& Suzuki, 1976) but functional BTB crucial for normal progression of spermatogenesis appears around puberty (Russell & Peterson, 1985; Russell et al., 1989). Pathologic condition in BTB has known to be related to diverse fertility status in men (Landon & Pryor, 1981; Cavicchia et al., 1996). Although recent works have focused on the cloning and tissue distribution of the ZO-1, little is known about their expression in testis and the mechanism regulating the TJ assembly and sealing capacity of BTB. In this study, spatiotemporal expression of ZO-1 was examined in mouse testis.

MATERIALS AND METHODS

1. Reverse transcription and polymerase chain reaction

All solutions were prepared with water treated with 0.1% diethylpyrocarbonate (DEPC). Testis and liver were removed from adult (8 weeks after birth) mice (ICR strain), and blood was cleared from the organs by blotting to filter paper on ice. Trizol (GIBCO BRL, USA) was immediately added, the sample was homogenized with homogenizer. RNA was ethanol precipitated, solubilized in D.W. and quantitated according the manufacturer's manual. Reverse transcription (RT) was carried out in 50 μl of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 5 mM $MgCl_2$ containing 1 mM each of dATP, dGTP, dCTP, dTTP, 20 units of RNase inhibitor, and 50 pmol oligo(dT)20-M4 adaptor primer (Takara). The tubes (Gene Amp thin-walled tubes) were incubated at 37°C for 2 min, 5 units of AMV reverese transcriptase XL (Takara, Japan) were added, and the tubes were transferred to a PCR thermal cycler (Takara, model 480). Reverse transcription was conducted for 1 hr at 42°C. The samples were then heated for 5 min at 99°C and then placed on ice. At this point the samples were either used directly for PCR. Polymerase chain reaction (PCR) of cDNA equivalent to 10 ng of total RNA from testis was carried out in 100 μl of 1x PCR buffer (10 mM Tris-HCl, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM each of the 4dNTPs, 1 unit *Taq* polymerase (Takara), 25 pmol each of the appropriate 3' and 5' primers, and 5 μl of the reverse transcription reaction. The primers for ZO-1 were 5'-GCTGGTGTGGGTCTCTGA-3' and 5'-GGTCTCTGCTGGCTTGTT-3' (Fig. 2). Due to the alternative splicing of α motif in ZO-1 transcript, this primer set give rise to amplicons of ZO-1 α+ and ZO-1 α- diagnostic fragments of 773 bp and 513 bp, respectively. The basic PCR program used was incubation at 94°C for 1 min, followed by a cycle program

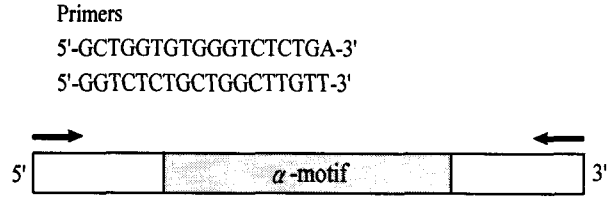

Fig. 2. Primer used for cDNA amplification of ZO-1. Arrows mark the position and direction of primers used for PCR.

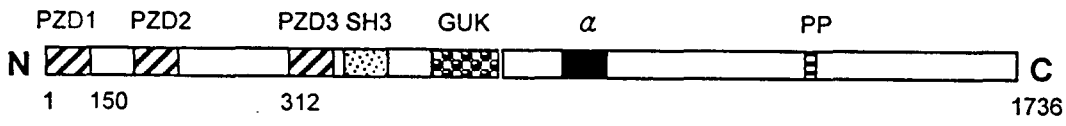

Fig. 1. Representation of peptide structure of mouse ZO-1. Functional domains including the α-domain were presented above the boxed area. Numbers below the box mean the amino acid number. PZD, PSD-95DlgZO-1 domain; SH3, Src homology 3 domain; GUK, guanylate kinase domain; PP, protein phosphatase domain.

of 95℃ for 30 sec, 55℃ for 40 sec, and 72℃ for 40 sec. The last cycle was concluded with a 10 min extension at 72℃. Following 35 cycles of amplifications, the PCR products (20 ㎕) were run on 2.5% agarose gels containing 0.5 ㎍/㎖ ethidium bromide and photographed under UV light.

2. Western blot analysis of ZO-1

Testis were removed from neonatal, immature (1 and 2 weeks after birth), pubertal (4 weeks after birth), and adult (8 weeks after birth) mice (ICR strain). Blood was cleared from organ by blotting to filter paper, and tunica albuginia was decapsulated on ice. TM4 Sertoli cell line and testis were homogenized in RIPA buffer containing 0.1 % sodium deoxycholate, 0.1 % NP-40, 20 mM NaF, 10 mM sodium orthovanadate, and protease inhibitor cocktail (CompleteTM, BMS). After centrifugation at 12,000 g for 10 min, supernatant was collected. After quantitation of protein content according to Bradford (1976), protein extracts were mixed with sample lysis buffer (Laemmli, 1970), boiled for 5 min at 100℃, and subjected to SDS-PAGE in 6 % polyacrylamide gel. After electrophoresis, proteins were electrotransferred to PVDF membrane. Immunodetection of ZO-1 was done according to Towbin et al (1979). Membrane was blocked with 5% BSA in Tris buffered saline (TBS). After brief washing twice in TBS, 1:1,000 diluent (0.5 ㎍/㎖ in 1% BSA in TBS) of rabbit anti rat ZO-1 antibody (polyclonal IgG, Zymed) which detect both isoforms of ZO-1 was applied to blot and incubated for 1 hr at RT. After washing (3X each for 10 min) with TBS, anti rabbit IgG antibody conjugated with alkaline phosphatase (Promega) (1:1,000 diluent in 1 % BSA in TBS) was applied and incubated for 1 hr at RT. After washing (3X each for 10 min) with TBS, blot was equilibriated with alkaline phosphatase buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) for 10 min. Alkaline phosphatase coloring reagent (Promega) was applied and incubated until the bands appeared. Relative amount of ZO-1 α+ versus ZO-1 α- was calculated after the image analysis using the Bioprofill program (Vilber Lourmat, version 96, France).

3. Immunocytochemical localization of ZO-1

Testis was fixed with 5% formaldehyde in PBS (pH 7.4) for 1hr and processed for paraffin section. After deparaffination with xylene, slide was incubated in PBS for 1hr at RT and then blocked in 5% BSA in PBS. Anti ZO-1 polyclonal antibody (Zymed) was diluted with 1:100 in 0.5 % BSA in PBS and applied to tissue section and incubated for 1hr in humidified chamber at RT. After washing with PBT (0.1 % Triton X-100 in PBS) each for 10 min, goat anti rabbit IgG antibody (peroxidase conjugate, Promega) was applied and incubated for 30 min. After washing with PBT for 3 times, coloring was done using diaminobezidine solution (DAKO) and counter-stained with hematoxylin, After permanent mounting, observation was done and photography was made on Kodak color reversal film (iso 400).

RESULTS

1. ZO-1 mRNA detection in testis

Two different sized amplicons of ZO-1 mRNA (773 and 513 bp) was detected after RT-PCR of the total RNA from testis and liver. Amplicon size exactly matched to the both isoforms of ZO-1 (Fig. 3). However, relative amount of ZO-1 α versus ZO-1 α+ transcript was higher in testis than liver.

2. Western blot analysis of ZO-1 in testis

Two different isoforms of ZO-1 of Mr. 225kDa and 200kDa were detected in neonatal to adult testis (Fig. 4A). The relative amount of two isoforms changed according to sexual maturation; the relative amount of fast migrating band (ZO-1 α-) vs. slow migrating antigen (ZO-1 α+) increased from 0.7 to 1.4 in neonatal and adult testis, respectively (Fig. 4B). In Sertoli cell line TM4, ZO-1 α+ isoform expression was higher than its counterpart.

3. Immunocytochemical localization of ZO-1 in testis

ZO-1 antibody gave rise the signals in the seminiferous

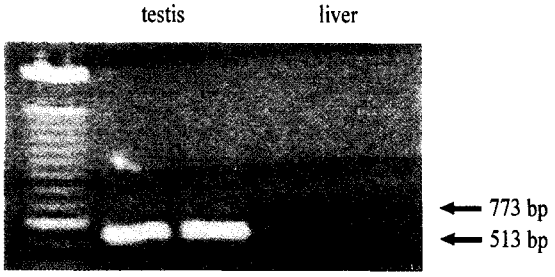

Fig. 3. RT-PCR of ZO-1 from testis and liver using primers designed to detect both ZO-1 α+ and ZO-1 α-.

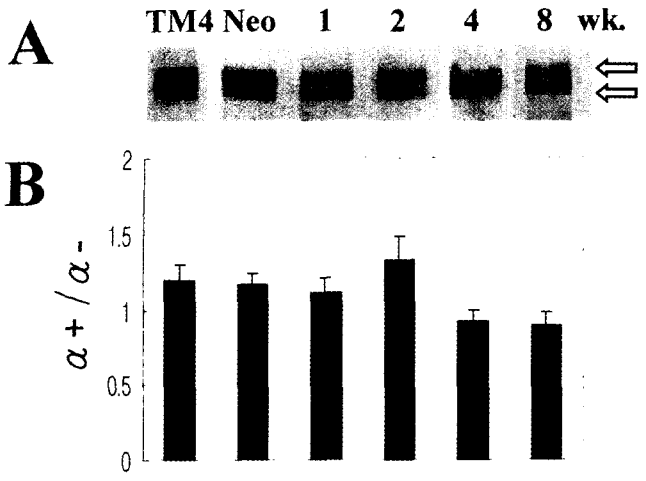

Fig. 4. Western blot of ZO-1 proteins from testis at different stage of development using an antibody detects both isoforms of ZO-1. (A) Western blot. Arrowheads indicate two isoforms of ZO-1; lane 1, TM4; lane 2, neonatal testis; lane 3, 1 week-old testis; lane 4, 2 weeks-old testis; lane 5, 4-weeks old testis; lane 6, 8 weeks-old testis. (B) Densitogram of relative amount of ZO-1 α+ and ZO-1 α- of A (Bar = SD, n = 4).

tubule of adult testes (Fig. 5A). When the control IgG was used, there was no specific signal in the seminiferous tubule (Fig. 5B). In next series, temporal change in ZO-1 expression was examined. In immature testis (Fig. 6A and B), the expression of ZO-1 was ubiquitous in the seminiferous tubule and interstitial cells. Both junctional area and cytoplasm of seminiferous tubule components showed ZO-1 expression. In pubertal and adult testis, more intense signals were found in Sertoli cells junctional areas at the periphery of seminiferous tubule (Fig. 6C and D).

DISCUSSION

At transcription level, both isoforms of ZO-1 transcripts were detected in RT-PCR (Fig. 3). Alternative RNA splicing generates isoforms of ZO-1 and it suggested unrecognized heterogeneity in junctional structure and function. In the RT-PCR of total RNA from adult testis ZO-1 α- form was dominant for ZO-1 α+. In liver, however, expression of two isoforms was similar. Similar result was obtained in protein expression. Both isoforms of ZO-1 were detected in mouse testis throughout the testis development examined (Fig. 4). Appearance of two isoforms of ZO-1 was also reported in other tissues including mouse embryos (Sheth et al., 1997). Because the antibody used in this experiment has ability to detect both ZO-1 isoforms, appearance of two immunoreactive bands on western blot reflects expression of two isoforms of ZO-1 protein. Previously, Balda and Anderson (1993) argued the correlation between expression of ZO-1 isoforms and junctional plasticity; ZO-1 α- is expressed in structurally dynamic junctions, whereas ZO-1 α+ in less dynamic junction. Therefore, it can be suggested that the dominant expression of ZO-1 α- in testis reflects more plastic nature of TJ in the seminiferous tubule. Interestingly, there was a increase in the ZO-1 α-/ZO-1 α+ ratio after puberty. It coincided with the formation of functional blood testis barrier (BTB) in testis at puberty in rodent (Russell et al., 1989) and human (Furuya et al., 1978). Although the difference in the ZO-1 α-/ZO-1 α+ ratio between immature and mature testis was minute, the apparent reduction in the relative amount of ZO-1 α+ suggested that expression pattern of ZO-1 isoforms is important for functionl differentiation of seminiferous epithelia. It also suggested that increase in ZO-1 α- can be used as a molecular marker for onset of terminal differentiation of seminiferous tubule and the formation of functional BTB. Differentiation of Sertoli cell starts after a period from the end of Sertoli cell proliferation during the fetal to postnatal development of testis (Gondos & Berndston, 1993). Hormones (McGuinness & Griswold, 1994) and testicular local regulators (Rosselli & Skinner, 1992) regulate the Sertoli cell differentiation and seminiferous remodeling. Although the direct evidence is lacking, temporal changes in ZO-1 isoform expression suggests that expression of ZO-1 in the seminiferous tubule is regulated by endocrine and paracrine factors. At puberty, remodeling of Sertoli cells according to the differentiation of germ cells is apparent in seminiferous tubule. Therefore, it is plausible that developmentally regulated expression of ZO-1 in seminiferous epithelia engaged in formation of functional BTB. It fulfills the requirement for cellular interaction in the seminiferous tubule at puberty and thereafter.

In immunostaining, there was a ubiquitous expression of ZO-1 in seminiferous tubules and interstitial cells (Fig. 5). Moreover, cytoplasm as well as junctional area in the seminiferous tubule showed positive signals (Fig. 6). In pubertal and adult testis, intense signals were found in Sertoli cells junctional areas at the periphery of seminiferous tubule (Fig. 6C

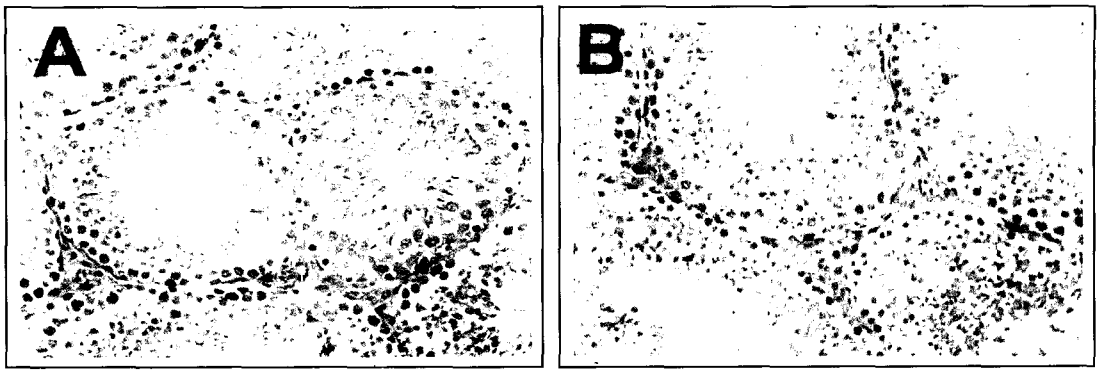

Fig. 5. Localization of ZO-1 protein from testis at different stage of development using an antibody detects both isoforms of ZO-1 (200×). A, ZO-1 antibody; B, control IgG. Signals are found in Sertoli cells, germ cells and interstitial cells of seminiferous tubules. Signals are not confined to Sertoli cell junctional area at the periphery of seminiferous tubule.

and D). It suggested that differentiation of Sertoli cells accompany the recruitment of ZO-1 to Sertoli cell junction at the periphery of seminiferous tubule.

The localization of ZO-1 at the junctional areas between Sertoli cell - germ cells as well as the inter-Sertoli cells junctions suggested that ZO-1 not only engaged in BTB between Sertoli cells but also Sertoli germ cells interaction. Recently, it was reported that second PZD domain of ZO-1 interacts with

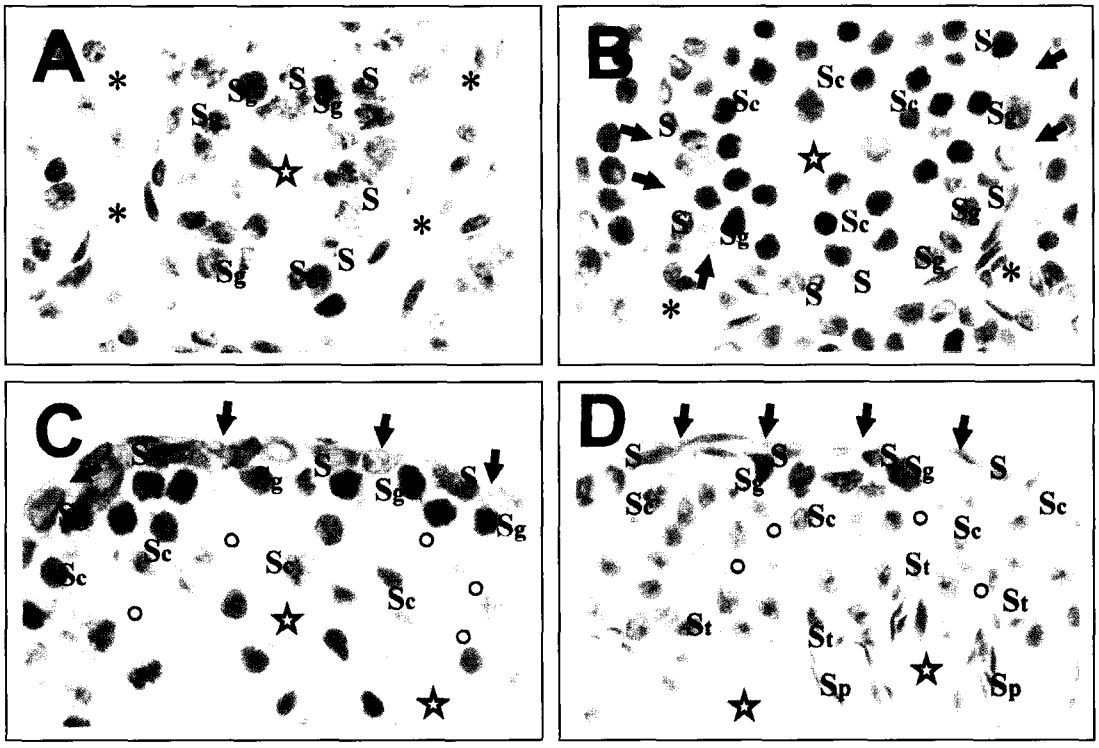

Fig. 6. Temporal expression of ZO-1 in the mouse seminiferous tubules at different stage of development (1,000×). Antibody detects both isoforms of ZO-1 was used. A, 1 week-old testis; B, 2 weeks-old testis; C, 3 weeks-old testis; D, 4 weeks-old testis. Arrows indicate signal between the Sertoli cells in the periphery of seminiferous tubule. Stars indicate signal in the Sertoli cell cytoplasm. Asterisks indicate signals in the interstitial cells. Open circles are signal between the Sertoli cell and germ cell. Sg, spermatogonia; Sc, spermatocytes; S, Sertoli cells; St, spermatids; Sp, spermatozoa.

connexin 43 in a yeast two-hybrid protein interaction screen (Giepmans & Moolenaar, 1998). It implicats that ZO-1 is a possible component of communicating junction and that Sertoli cells directly communicates with germ cells through gap junction. Therefore, it is tempting to speculate that expression of ZO-1 in the Sertoli cell - germ cell contact might be important for communication between them. Previously, it was reported that in the immature mouse testis, ZO-1 is present in the Sertoli cell plasma membrane in the absence of recognizable TJs. In the presence of TJs, however, ZO-1 is found only at the sites of junctional specializations associated with TJs and with elongating spermatids (Byers et al., 1991). More recently, Pelletier et al. (1997) reported that ZO-1 $\alpha+$ was predominant in isolated seminiferous tubule from young guinea pig and that ZO-1 $\alpha+$ localized in the junctions joining Sertoli cells to particular class of germ cells but the ZO-1 $\alpha-$ generally distributed in TJs between Sertoli cells and all classes of germ cells. The formation and maintenance of inter-Sertoli cell tight junction do not appear to be directly dependent on the presence of germ cells, but alterations in the TJ architecture and the ectoplasmic specializations was found in germ cell-free testis (Ribeiro & David-Ferreira, 1996), suggesting possible role of maturing germ cells in the functional organization of the BTB in normal testis. Taken together, it can be suggested that ZO-1 expression in the seminiferous tubule is harmonious to germ cell differentiation and that intercellular communication between germ cells and Sertoli cell is implicated in the regulation of ZO-1 expression.

In conclusion, the changes in the relative amount of both isoforms in testis and spatial distribution of ZO-1 at the periphery of seminiferous tubule might be important for functional appearance of blood testis barrier and spermatogenesis. Further studies about the defining factors that regulate assembly of tight junctions or alter their barrier properties may be helpful for comprehensive understanding of formation of BTB in seminiferous tubule.

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