Zona Occludens-2 Is Critical for Blood–Testis Barrier Integrity and Male Fertility

Jianliang Xu, Farhana Anuar, Safiah Mohamed Ali, Mei Yong Ng,
Dominic C.Y. Phua, and Walter Hunziker

Epithelial Cell Biology Laboratory, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore 138673

Submitted December 24, 2008; Revised July 21, 2009; Accepted August 11, 2009
Monitoring Editor: Keith E. Mostov

Tight junction integral membrane proteins such as claudins and occludin are tethered to the actin cytoskeleton by adaptor proteins, notably the closely related zonula occludens (ZO) proteins ZO-1, ZO-2, and ZO-3. All three ZO proteins have recently been inactivated in mice. Although ZO-3 knockout mice lack an obvious phenotype, animals deficient in ZO-1 or ZO-2 show early embryonic lethality. Here, we rescue the embryonic lethality of ZO-2 knockout mice by injecting ZO-2(–/–) embryonic stem (ES) cells into wild-type blastocysts to generate viable ZO-2 chimera. ZO-2(–/–) ES cells contribute extensively to different tissues of the chimera, consistent with an extraembryonic requirement for ZO-2 rather than a critical role in epiblast development. Adult chimera present a set of phenotypes in different organs. In particular, male ZO-2 chimera show reduced fertility and pathological changes in the testis. Lanthanum tracer experiments show a compromised blood–testis barrier. Expression levels of ZO-1, ZO-3, claudin-11, and occludin are not apparently affected. ZO-1 and occludin still localize to the blood–testis barrier region, but claudin-11 is less well restricted and the localization of connexin-43 is perturbed. The critical role of ZO-2 for male fertility and blood–testis barrier integrity thus provides a first example for a nonredundant role of an individual ZO protein in adult mice.

INTRODUCTION

Tight junctions (TJs) between adjacent Sertoli cells are responsible for establishing the blood–testis barrier (BTB), which divides the seminiferous epithelium into an adluminal and a basal compartment (reviewed in Lui *et al.*, 2003; Wong and Cheng, 2005; Yan *et al.*, 2007). In spermatogenesis, germ cells migrate during their differentiation from the serosal into the adluminal compartment, a process that depends on their functional interaction with Sertoli cells. Spermatogonia and preleptotene spermatocytes are found in the serosal compartment, where they have access to the blood circulation. Germ cells at later developmental stages are present in the adluminal compartment. The chemical composition of this compartment, established by the vectorial secretory and endocytic activity of the Sertoli cells (Griswold, 1988), is critical for germ cell viability. Although TJs establish an effective barrier between the basal and adluminal compartment, dynamic regulation is required to allow for the transmigration of germ cells (reviewed in Yan *et al.*, 2008). In the adluminal side, spermatids remain attached to the Sertoli cells via ectoplasmic specializations, a specialized actin-based adherens junction type that is also important for spermatid movement.

TJs do not form an absolute barrier but rather selectively facilitate or restrict the diffusion of solutes, in particular ions. This selective permeability is critical in establishing distinct electrolyte compositions within different compartments in a tissue or organ (reviewed in Tsukita *et al.*, 2001; Anderson *et al.*, 2004; Van Itallie and Anderson, 2004, 2006; Kohler and Zahraoui, 2005). In addition to regulating paracellular permeability, TJs participate in the maintenance of apical–basal cell polarity and the regulation of vesicular membrane transport (reviewed in Kohler and Zahraoui, 2005), as well as in cell differentiation and proliferation (reviewed in Matter and Balda, 2003, 2007).

At the molecular level, TJ integral membrane proteins, in particular claudins (Cldns), occludin (Ocln), and junctional adhesion molecules (JAMs), engage through their luminal domains in homo- and/or heterotypic interactions with corresponding proteins on adjoining cells (reviewed in Balda and Matter, 2000). Claudins, a family of ~24 proteins in mammals, are key components of the paracellular pore and the Cldn repertoire is thought to determine ion selectivity and overall permeability properties. On the cytoplasmic side, these TJ transmembrane proteins are tethered to the actin cytoskeleton via adaptor or scaffolding molecules, in particular the three ZO proteins (ZOPs), ZO-1, ZO-2, and ZO-3. These three closely related and widely expressed proteins belong to the membrane-associated guanylate kinase-like protein superfamily (reviewed in Gonzalez-Mariscal *et al.*, 2000). The nuclear localization of some ZOPs under particular conditions (Islas *et al.*, 2002; Traweger *et al.*, 2003; Jaramillo *et al.*, 2004; Kausalya *et al.*, 2004) and a growing number of regulatory proteins that associate with these adaptors (reviewed in Gonzalez-Mariscal *et al.*, 2003) suggest that they also regulate intracellular signaling pathways, possibly in response to cell–cell contact cues.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-12-1236>) on September 9, 2009.

Address correspondence to: Walter Hunziker (hunziker@imcb.a-star.edu.sg).

Abbreviations used: Cx43, connexin-43; ES, embryonic stem; KO, knockout; PCR, polymerase chain reaction; TJ, tight junction; WT, wild type; ZO, zonula occludens; ZOP, zonula occludens protein.

The relevance of individual ZOPs for the assembly and the function of TJs have been explored in tissue culture cells using small interfering RNA-mediated silencing or inactivation by homologous recombination (Medina *et al.*, 2000; Umeda *et al.*, 2004, 2006; Adachi *et al.*, 2006; McNeil *et al.*, 2006; Hernandez *et al.*, 2007; Traweger *et al.*, 2008). Although the absence of an individual ZOP often results in delayed TJ assembly, their importance for cell polarity or paracellular permeability remains inconclusive. This probably reflects cell type differences, varying efficiencies of silencing, or the use of different experimental conditions and assays to monitor TJ functionality. Recently, each of the three ZOPs has been inactivated in mice, unraveling requirements for ZO-1 and ZO-2 during early stages of embryonic development (Katsuno *et al.*, 2008; Xu *et al.*, 2008). In contrast, mice lacking ZO-3 show no apparent phenotype (Adachi *et al.*, 2006; Xu *et al.*, 2008).

Although knockout mice have highlighted an importance for two ZOPs in mammalian development, the embryonic lethality (e.g., ZO-1 and ZO-2) and the lack of an apparent phenotype (e.g., ZO-3) have hampered an analysis of these proteins in the physiology of adult organs, such as the testis. To circumvent this problem, we explored whether the embryonic lethality of ZO-2(−/−) mice could be rescued by injecting ZO-2(−/−) embryonic stem (ES) cells into wild-type (WT) blastocysts to generate chimera. Indeed, viable ZO-2 chimera with an almost exclusive Agouti colored coat were readily obtained, suggesting that ZO-2 may be more critical for the function of extraembryonic tissues than for development of the embryo proper. Adult chimera mice presented with a set of reproducible phenotypes that varied in severity, likely reflecting different extents of chimerism. In particular, male ZO-2 chimera showed reduced fertility and tracer diffusion experiments established defects in the BTB. Although the presence of ZO-1 at the region of the BTB was not altered in the absence of ZO-2, Cldn11 was less well restricted and the localization of connexin-43 (Cx43) was perturbed. Thus, ZO-2 plays a nonredundant role in testis physiology and in particular in maintaining the integrity of the BTB.

MATERIALS AND METHODS

Generation of ZO-2 Chimera Mice

Two independently generated ZO-2(−/−) ES clones (clone 54 and 91) (Xu *et al.*, 2008) were injected into blastocysts derived from C57BL/6 mice to generate chimera. One pregnant mother was sacrificed at embryonic day (E)15.5 to collect chimera embryos. The other pregnant females were kept until they gave birth to chimera offspring. All animals were maintained in a single pathogen-free facility with the oversight of the institutional veterinarian.

Histological Analysis

Freshly dissected testes were fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The slides were viewed with a DM4000 B microscope (Leica, Wetzlar, Germany), and images were taken with a DFC300 FX camera (Leica).

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Freshly dissected testes were homogenized with a mortar, and RNA was extracted with an RNeasy Fibrous Tissue extraction kit (QIAGEN, Valencia, CA). Semiquantitative RT-PCR analysis was carried out with the One-Step RT-PCR kit (QIAGEN). The reaction was performed with 10 ng of mRNA in a 25- μ l volume. The primer sequences and the thermal cycle conditions are provided in Supplemental Data 1.

Immunostaining

Freshly dissected testes were embedded in OCT and then processed on a cryostat to obtain 5- μ m-thick sections. Paraffin sections were dewaxed and the antigen was retrieved by steaming the slides for 12 min in a 2100 Retriever

(Pick Cell Laboratories, Amsterdam, The Netherlands). Immunostaining was carried out with rabbit polyclonal antibodies to ZO-1, ZO-2, Occludin (Zymed Laboratories, South San Francisco, CA), Cldn11 (Santa Cruz Biotechnology, Santa Cruz, CA), Cx43 (Cell Signaling Technology, Danvers, MA), Sox9 (Abcam, Cambridge, United Kingdom) and/or mouse monoclonal antibodies against ZO-1 and ZO-2 (Zymed Laboratories), and suitably labeled secondary antibodies, essentially as described previously (Xu *et al.*, 2008). Nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI).

Lanthanum Permeability

Freshly dissected testes were immediately fixed in freshly made 5.5% glutaraldehyde containing 1% lanthanum nitrate for 3 h. After rinsing with phosphate-buffered saline (PBS), the samples were postfixed in 1% osmium tetroxide for 1 h, rinsed in phosphate buffer, dehydrated in ethanol, and embedded in resin. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and then viewed with a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan) essentially as detailed previously (Xu *et al.*, 2008).

Apoptosis Assay

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were done on cryosections of testes with the TMR Red In Situ Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol.

Western Blot Analysis

Freshly dissected testes were homogenized with a mortar and lysed for 15 min in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl₂, and 0.5% Triton X-100) on ice. Lysates were sonicated and cleared by centrifugation (13,000 $\times$ g for 15 min) at 4°C. The supernatant was fractionated by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis.

Testosterone Measurements

Blood (200 μ l) was obtained from the mouse tails and clotted at room temperature for 15 min. The clotted blood was then centrifuged, and the serum was collected. The concentration of testosterone in the serum was measured by chemiluminescence immunoassay as part of a routine diagnostic service provided by the National University Hospital of Singapore.

RESULTS

Rescue of The Early Embryonic Lethality of ZO-2(−/−) Mice by Injecting ZO-2(−/−) ES Cells into Wild-Type Blastocysts

Although ZO-2 is recruited to junctional sites in the mouse blastomere as early as the 16-cell stage (Sheth *et al.*, 2008), the absence of ZO-2 only affects embryo development after implantation ~E6.5–E7.5, before mesoderm differentiation (Xu *et al.*, 2008). To further explore the role of ZO-2 in embryonic development, we analyzed its expression at E6.5 and E7.5 by immunohistochemistry. As shown in Figure 1, A–C, ZO-2 is strongly expressed in E6.5 embryos, both in the extraembryonic and embryonic tissues, where it localizes to sites of cell–cell contact in the endoderm and ectoderm cell layers. In particular, ZO-2 is prominently expressed in the visceral endoderm. After the induction of mesoderm differentiation around E7.5, ZO-2 continues to be expressed in the ectoderm and endoderm, but is absent from the mesoderm (Figure 1, D–F).

Because embryoid bodies derived in vitro from ZO-2(−/−) ES cells express mesoderm markers (e.g., T antigen/brachyury) (Xu *et al.*, 2008), the developmental arrest of ZO-2(−/−) embryos in vivo is unlikely to reflect a direct role for ZO-2 in mesoderm development but rather a requirement for embryo viability before E7.5. To explore this possibility, ZO-2(−/−) ES cells were microinjected into C57BL/6 blastocysts to generate ZO-2 chimera embryos. At E15.5, one of the pregnant foster mothers was sacrificed, and the six embryos she carried were dissected out. The embryos did not reveal any gross anatomical anomalies. Genotyping using yolk sac tissue showed the presence of ZO-2(−/−) cells in all six embryos (data not shown). To confirm the

Figure 1. ZO-2 expression in the gastrulating mouse embryo. Sections of E6.5 (A–C) and E7.5 (D–F) embryos labeled with antibodies to ZO-2 (A and D; green) or DAPI (B and E; blue) and visualized by fluorescence microscopy. C and F show merged images.

presence of ZO-2(–/–) cells, the skin of the embryos was analyzed by immunohistochemistry. Although ZO-2 was abundantly expressed in the skin of E15.5 WT embryos (Figure 2A), it was only weakly expressed in the skin of chimera mice and expression was restricted to a few isolated cells (Figure 2B). Given the high contribution of ZO-2(–/–) ES cells to viable chimera mice (Figure 2C; see below), these observations indicate that ZO-2 may primarily be required for extraembryonic development. Alternatively, the expression of ZO-2 by a limited number of endoderm and/or ectoderm cells could be sufficient to rescue the developmental arrest at E6.5–E7.5.

ZO-2(–/–) ES Cells Contribute Extensively to Viable Chimera Mice

Chimera embryos derived from two independent ZO-2(–/–) ES cell clones, 54 and 91, were allowed to develop to term. Of 33 and 29 offspring derived from clones 54 and 91, respectively, the coat of 17 and 15 animals was almost ex-

clusively Agouti color (Figure 2C). Of these, three males and one female were mated with WT C57BL/6 females or males, respectively. Of the 30 offspring obtained, all were Agouti and, based on genotyping, heterozygous for ZO-2. Thus, viable chimera mice were obtained with a high contribution of ZO-2(–/–) ES cells, including transmission into the germline.

To further characterize the extent of chimerism, expression of ZO-2 and, as controls, ZO-1 and ZO-3, was analyzed in the liver, kidney, intestine, and testis of two male ZO-2 chimera. As shown in Figure 3A, all four tissues of WT animals expressed significant levels of ZOPs, including ZO-2 (Jesaitis and Goodenough, 1994). As expected, ZO-2 protein levels were strongly reduced in the livers, kidneys, and testes of the chimera and were undetectable in the intestines. Expression levels of ZO-1 and ZO-3 mRNA (Figure 3B) or protein (Figure 3A) were apparently not altered in the chimera, indicating that the absence of ZO-2 was not compensated by a significant up-regulation of ZO-1 and/or ZO-3 expression.

The low ZO-2 protein levels detected by Western blot in the testes of male chimera were confirmed by immunohistochemistry. ZO-2 was readily visualized in all seminiferous tubules of WT testes, in which it colocalized with ZO-1 (Figure 3, D–F). In testes from chimera males, however, only the occasional seminiferous tubule stained for ZO-2 (Figure 3C and G–I), with the expression being restricted to a few cells in these tubules (Figure 3C, arrows). Although the expression and localization of ZO-1 was not altered (Figure 3, G–I), the diameter of the seminiferous tubules was significantly smaller in the ZO-2 chimera (Supplemental Data 2).

Figure 2. Rescue of early embryonic lethality of ZO-2(–/–) embryos and generation of viable ZO-2 chimera. (A and B) Rescue of ZO-2(–/–) early embryonic lethality. ZO-2(–/–) ES cells were injected into WT blastocysts and transferred into pseudopregnant female mice. Embryos were harvested at E15.5 and processed for immunohistochemistry. Skin sections from control WT (A) or chimera embryos (B) were labeled with antibodies to ZO-2 (green) and DAPI (blue). Note the strong and homogenous expression of ZO-2 in WT embryonic skin and its absence from most cells in the chimera. (C) Generation of viable chimera. Chimera offspring obtained from two independent ZO-2(–/–) ES cell clones. Approximately 50% of the offspring presented with a coat that was almost completely Agouti colored, indicative of an extensive contribution of the ZO-2(–/–) ES cells to the animals.

Compromised Male Fertility and Histological Changes in Seminiferous Tubules of ZO-2 Chimera

Given that the majority of cells of the seminiferous tubules from ZO-2 chimera did not express ZO-2 (Figure 3C), we characterized the effect of the loss of ZO-2 expression on male fertility. Mating experiments in which one ZO-2 chimera male was allowed to pair with two C57BL/6 females over a 4-wk period were set up. Once pregnant, females were isolated and allowed to give birth. As shown in Figure 4A, only 50% (4/8) of the chimera males were able to impregnate females, leading to 54 offspring or 6.7 pups per male (Figure 4B). In contrast, 100% (5/5) of the ZO-2(+/-) heterozygous and 100% (6/6) of the WT males impregnated the available females, resulting in 15 and 14.3 offspring per male, respectively. Based on a small scale mating experiment of female ZO-2 chimera with WT males, no apparent

Figure 3. Expression of ZOPs in different tissues and localization in testis of ZO-2 chimera. (A) Western blot analysis. Liver, kidney, intestine, and testis of two chimera derived from two independent ZO-2(-/-) ES cell clones were processed for Western blot analysis using antibodies to ZO-1, ZO-2, and ZO-3. Madin Darby canine kidney cell lysates and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as controls. Note that ZO-2 expression is strongly reduced in liver, kidney, and testis and not detected in the intestine of the chimera. ZO-1 and ZO-3 protein levels are not significantly different in the tissues of the chimera compared with WT controls. The apparent molecular mass of the different proteins is indicated. (B) RT-PCR analysis. Testes of two chimera derived from two independent ZO-2(-/-) ES cell clones were processed for RT-PCR analysis using primers specific to ZO-1, ZO-2, and ZO-3, or actin as a control. (C-I) Immunohistochemical analysis of the testis. Sections of testes from WT controls (D-F) or ZO-2 chimera (C and G-I) were labeled with antibodies to ZO-1 (red) or ZO-2 (green) and DAPI (blue) to stain nuclei. ZO-2 is largely absent in the chimera testes and only found in the occasional seminiferous tubule, in which expression is restricted to a few isolated cells (C, arrows). F and I show merged images. Note the similar distribution of ZO-1 to the region of the BTB in seminiferous tubules of WT and ZO-2 chimera.

effect on the fertility of the female ZO-2 chimera was observed (data not shown).

To determine whether the reduced fertility of male ZO-2 chimera correlated with histological changes in the testis, we

Figure 4. Compromised fertility of male ZO-2 chimera. (A and B) Male fertility. Individual WT (+/+; n = 6), ZO-2 heterozygous (+/-; n = 5) or chimera (+/+;-/-; n = 8) male mice were each paired with two C57Bl6 females for 4 wk. Once pregnant, females were separated from the males. The ability of males to impregnate the females (A) and the number of offspring per male (B) were determined. Males were 6 mo old and females were 8–12 wk old. All offspring derived from the ZO-2 chimera were heterozygous, indicating germ line transmission of the inactivated ZO-2 gene, and fertility of female ZO-2 chimera was normal (data not shown). (C) Testis weight. Individual testes from WT controls or ZO-2 chimera males were dissected and their weight determined. (D and E) Histology. Hematoxylin-stained sections of testes from WT control (D) and ZO-2 chimera (E). Note the smaller diameter, the occasional vacuolarization, and the reduced number of spermatocytes observed for the seminiferous tubules of the chimera.

Figure 5. Testicular apoptosis, Sertoli cell organization, and testosterone levels of ZO-2 chimera. (A and B) TUNEL staining. Apoptosis in testes from WT controls (A) or ZO-2 chimera (B) was visualized by fluorescence microscopy using TUNEL assay (red). Nuclei were stained with DAPI (blue). (C and D) Organization of Sertoli cells in seminiferous tubules. Sections of testes from WT controls (C) or ZO-2 chimera (D) were labeled with antibodies to the Sertoli cell maker Sox9 (green) and DAPI (blue) to stain nuclei. The asterisk indicates unspecific labeling in both WT and chimera. (E) Sertoli cell numbers. The number of Sertoli cells in 10 random seminiferous tubules in two different sections each were counted and the average number per tubule was determined. Note that despite the smaller diameter of chimeric seminiferous tubules, the organization and number of Sertoli cells in ZO-2 chimera is similar to that in WT tubules. (F) Testosterone levels. Blood testosterone levels were measured in three to four WT or ZO-2 chimera at 6 and 14 mo of age.

dissected the testes of 6- to 10-mo-old chimera. Testes from the chimera were significantly smaller and lighter (0.079 ± 0.005 vs. 0.129 ± 0.002 g) compared with those from WT males (Figure 4C). Histological analysis revealed pathological changes ranging from the loss of cells and vacuolarization in some tubules to the occasional degeneration of complete seminiferous tubules and the absence of spermatogenesis (Figure 4, D and E). Although little if any cell death was detected in WT testis by TUNEL analysis (Figure 5A), apoptosis was observed in some tubules in the testes of chimera (Figure 5B).

Interestingly, chimera seminiferous tubules of otherwise apparently normal histology had a significantly smaller diameter as compared with WT tubules (Figure 5, C and D, and Supplemental Data 2). Despite the smaller diameter, the organization of Sertoli cells (identified by their staining for Sox9; Kobayashi *et al.*, 2005) within the seminiferous tubule (Figure 5, C and D) as well as the number of Sertoli cells per tubule (Figure 5E) did not significantly differ from WT tubules. Furthermore, serum testosterone levels were comparable in WT and ZO-2 chimera (Figure 5F).

The Blood-Testis Barrier Is Compromised in ZO-2 Chimera Mice

To determine whether the absence of ZO-2 affects the formation of the BTB, we analyzed the expression and distribution of selected TJ markers in seminiferous tubules of apparently normal histology by immunohistochemistry. The expression of ZO-1, ZO-3 (Figure 3, A and B), and Cldn11 (Figure 6, A and B) mRNA or protein was not significantly affected in testes of ZO-2 chimera. Although more Ocln mRNA was detected in testes of chimera (Figure 6A), Ocln protein levels were comparable with those of controls (Figure 6B). Despite the noted smaller diameter of the chimera seminiferous tubules, the localization of ZO-1 (Figure 3, G-I), Ocln (Figure 6, F-H) and Cldn11 (Figure 7, G-I) within the seminiferous tubules from the ZO-2 chimera and WT testes was similar. However, although Cldn11 generally colocalized with ZO-1 (Figure 7, D-I) and, in WT controls with

ZO-2 (Figure 7, A-C), Cldn11 staining was found to spread into the adluminal region (Figure 7, G-I, arrows in I) in >60% of the chimera seminiferous tubules (Figure 7J).

Because TJ integrity in the testis is critical for maintaining the BTB, we carried out lanthanum tracer studies to assess whether this barrier was compromised in the ZO-2 chimera. In WT testes, lanthanum penetrated the open junctions of the myoid cell layer and the intercellular cleft around the spermatogonia but was unable to diffuse beyond the BTB toward the lumen of the tubule (Figure 8, A and B). Diffusion across the myoid cell layer was observed, on average, at 10 sites per WT tubule, and this was also the case in ZO-2 chimera tubules (data not shown). However, in ZO-2 chimera, lanthanum frequently diffused into the adluminal intercellular space between Sertoli cells and multiple spermatocytes (Figure 8, C and D), a hallmark of a compromised BTB. Analysis of ~25 histologically normal seminiferous tubules from two independent ZO-2 chimera revealed 25–50% leaky tubules, with a total of 10–19 sites in which lanthanum had permeated into the adluminal space (Figure 8E). In contrast, lanthanum was never observed in the adluminal space in the control seminiferous tubules monitored.

Perturbed Localization of Cx43 and Filamentous Actin within Seminiferous Tubules of ZO-2 Chimera

Because Cx43 (Singh *et al.*, 2005) and actin (Wittchen *et al.*, 1999; Mattagajasingh *et al.*, 2000) can interact with ZO-2, we analyzed whether the distribution of Cx43 or the organization of the actin cytoskeleton was affected in the testes of ZO-2 chimera.

Although Cx43 mRNA levels were increased in testes of ZO-2 chimera (Figure 9A), protein levels did not differ from those in controls (Figure 9B). However, in contrast to the accumulation of Cx43 in the region of the BTB, in which the protein partially colocalized with ZO-1 in control testes (Figure 9, C-E), Cx43 labeling was observed throughout the adluminal region of seminiferous tubules from ZO-2 chimera (Figure 9, F-H).

Figure 6. Expression of occludin and claudin-11 and localization of occludin in testis from ZO-2 chimera. (A) RT-PCR analysis. Testes of two chimera derived from two independent ZO-2(−/−) ES cell clones were processed for RT-PCR analysis using primers specific to OcIn, Cldn11, or, as a control, actin. (B) Western blot analysis. Testes of two chimera mice derived from two independent ZO-2(−/−) ES cell clones were processed for Western blot analysis using antibodies to OcIn and Cldn11. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. Note that OcIn and Cldn11 protein levels are not significantly different in the testes from the chimera compared with WT controls. The apparent molecular mass of the different proteins is indicated. (C–H) Immunohistochemical analysis. Sections of testes from WT controls (C–E) or ZO-2 chimera (F–H) were labeled with antibodies to ZO-1 (red) and OcIn (green). DAPI (blue) was used to stain nuclei. E and H show merged images. Note that OcIn still colocalizes with ZO-1 to the region of the BTB in ZO-2 chimera.

Also, the labeling of filamentous actin differed in the testes of chimera. In control seminiferous tubules, prominent actin staining was observed at the level of the BTB and along the lumen of the tubule, presumably at ectoplasmic specializa-

tions mediating the adhesion of between Sertoli cells and spermatids (Figure 10, A–C). In contrast, actin labeling was observed along the length of the adluminal region of tubules of ZO-2 chimera (Figure 10, D and E).

Figure 7. Localization of Cldn11 in testis from ZO-2 chimera. (A–I) Immunohistochemical analysis. Sections of testes from WT controls (A–F) or ZO-2 chimera (G–I) were labeled with antibodies to ZO-2 (A–C, red) or ZO-1 (D–F, red) and Cldn11 (green). DAPI (blue) was used to stain nuclei. Note that Cldn11 still extensively colocalizes with ZO-1 to the region of the BTB in ZO-2 chimera. C, F, and I show merged images. Areas where the Cldn11 labeling is delocalized and extends into the adluminal region are indicated (I, arrows). (J) Quantification. Fifty randomly chosen WT or chimera tubules were analyzed for the presence of delocalized Cldn11 labeling as shown in panel I. In >60% of the chimera tubules, the Cldn11 labeling extended into the adluminal domain.

J

Genotype	# of seminiferous tubules	
	punctate staining	delocalized staining
WT	50	0
	50	0
ZO-2 chimera	19	31
	14	36

Figure 8. Compromised blood–testis barrier function in testis from ZO-2 chimera. (A–D) Electron micrographs of lanthanum tracer studies. Freshly dissected testes were immediately fixed in the presence of lanthanum nitrate and then processed for EM analysis. At a few sites in the WT seminiferous tubule, the lanthanum tracer (black) permeates across the incomplete system of TJs of the myoid cell layer and into the intercellular space between Sertoli cells and spermatogonia (A and B). Diffusion is restricted at the levels of the BTB (B, arrow), and the tracer was never observed in the adluminal compartment (E). In contrast, in testes from ZO-2 chimera, diffusion of lanthanum tracer into the adluminal compartment and between Sertoli cells and multiple spermatocytes is observed (C and D). (E) Quantification of BTB leakage. The number of leaky seminiferous tubules and total leaky sites were determined in the testes of 8- and 13-mo-old WT and ZO-2 chimera mice. Permeability across the myoid cell layer in WT and chimera was indistinguishable (data not shown).

DISCUSSION

Inactivation of ZO-2 by homologous recombination in mice results in early embryonic lethality accompanied with arrest of cell proliferation and enhanced apoptosis in the embryo during gastrulation (Xu et al., 2008). In the present study, we show that injection of ZO-2(–/–) ES cells into WT blasto-

cysts rescues the embryonic lethality and allows the generation of viable chimera. Furthermore, we characterize a distinct testicular phenotype of male ZO-2 chimera mice, namely, a defect in the BTB and reduced fertility.

Before and during mesoderm differentiation, ZO-2 is highly expressed both in the endoderm and ectoderm of

Figure 9. Expression and localization of connexin-43 in testis from ZO-2 chimera. (A) RT-PCR analysis. Testes of two chimeras derived from two independent ZO-2(–/–) ES cell clones were processed for RT-PCR analysis using primers specific to Cx43 and actin as a control. (B) Testes of two chimera mice derived from two independent ZO-2(–/–) ES cell clones were processed for Western blot analysis using antibodies to Cx43. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. Note that Cx43 protein levels are not significantly different in the testes from the chimera compared with WT controls. The apparent molecular mass of the different proteins is indicated. (C–H) Immunohistochemical analysis. Sections of testes from WT controls (C–E) or ZO-2 chimera (F–H) were labeled with antibodies to ZO-1 (red) and Cx43 (green). DAPI (blue) was used to stain nuclei. E and H show merged images. Note that in the testes from ZO-2 chimera, Cx43 is delocalized and present along the adluminal domain (H, arrows), whereas it is absent from this region in WT samples (H, arrows).

Figure 10. Localization of filamentous actin in testis from ZO-2 chimera. (A–F) Immunohistochemical analysis. Sections of testes from WT controls (A–C) or ZO-2 chimera (D–F) were labeled with antibodies to ZO-1 (red) and actin (green). DAPI (blue) was used to stain nuclei. C and F show merged images. Note that in control tubules actin is present in the BTB area and enriched in the luminal region (asterisks) but absent from the adluminal region (C, arrows), whereas in the ZO-2 chimera is present along the adluminal domain (F, arrows).

mouse embryos. After gastrulation, ZO-2 expression is higher in the endoderm compared with the ectoderm but absent from mesoderm. Interestingly, injection of ZO-2(–/–) ES cells into WT blastocysts rescues the embryonic lethality of ZO-2(–/–) mice to give rise to viable chimera. The extensive Agouti colored coat, the prevalence of cells lacking ZO-2 in different tissues, and the germline transmission of the ZO-2(–/–) genotype confirm that a significant fraction of the tissues in chimera are derived from ZO-2(–/–) ES cells. During the generation of ES cell-blastocyst chimera, injected ES cells only contribute to the epiblast, whereas the mural trophoblast that gives rise to the yolk sac and placenta is derived from WT cells (Rossant and Spence, 1998). The rescue of the lethality of ZO-2(–/–) embryos thus suggests that ZO-2 is critical for extraembryonic development and that it may be dispensable for development of the epiblast. However, it cannot be ruled out that the presence of a few ZO-2(+/+) cells is sufficient to support epiblast development.

Mice with an almost exclusive Agouti-colored coat were obtained in relatively large numbers in the litters of foster mothers. These animals were viable and reached adulthood, despite strongly reduced ZO-2 protein levels in several vital organs, including liver, kidney, intestine, and testis. Expression levels of ZO-1 or ZO-3 protein were not increased as a possible compensatory mechanism in these tissues. Overall, chimera reproducibly presented with a common set of phenotypes. Although gross anatomy and histology of the intestine and liver seemed normal, abnormalities were observed in the kidney (data not shown) and testis. These phenotypes varied in severity between animals, probably reflecting different degrees of chimerism in the affected tissues and organs among individual chimera. To minimize variations in phenotype, chimera with an apparently complete Agouti-colored coat were selected for analysis.

Gross anatomy, histology, and physiology of the testes of male chimera were consistently affected. Size and weight of the organs were reduced and individual seminiferous tubules showed vacuolization and evidence for enhanced apoptosis. ZO-2 protein was often only detected in a few tubules of the chimera testes and, where present, it was only expressed by a few cells within these tubules. Nevertheless, only a fraction of the tubules showed degeneration and evidence of apoptosis. This could reflect a gradual deterioration of the integrity of the testis over time in the absence of ZO-2. Given the heterogeneity in the severity of the phenotype among individual animals, it is not possible to establish a clear correlation between phenotype progression and age. Despite a normal Sertoli cell organization, the diameter of

chimera seminiferous tubules was significantly smaller compared with WT tubules. The smaller tubule diameter, also observed in Cldn11 knockout (KO) mice (Gow *et al.*, 1999), may reflect the reduced spermatogenesis in the ZO-2 chimera. Because testosterone levels in ZO-2 chimera were comparable with those in WT males, lower spermatogenesis is a probable cause for the reduced fertility rate. Compared with the complete infertility of Cldn11 KO male animals (Gow *et al.*, 1999), the reduced fertility in the ZO-2 chimera could reflect the residual expression of ZO-2 in some seminiferous tubules or tubule regions and/or that fact that Cldn11 still localizes to a significant extent to the BTB region, as assessed by its colocalization with ZO-1. Hence, the phenotype in the ZO-2 chimera may be less severe compared with a complete lack to Cldn11 in Cldn11 KO mice (see below).

A well-characterized and critical role for TJs in the testis is the formation of the BTB. TJs between adjacent Sertoli cells establish a basal and adluminal compartment in the seminiferous epithelium. During normal spermatogenesis, germ cells progressively migrate through the extracellular space between Sertoli cells from the basal compartment across the BTB into the apical compartment to be released into the tubule lumen at spermiation. Despite the transient opening to allow germ cell transmigration, the BTB is considered among the tightest blood-tissue barriers in mammals (Wong *et al.*, 2004; Yan *et al.*, 2007). Besides restricting the diffusion of macromolecules and ions between the serosal and adluminal compartment, the BTB also plays a critical role in preventing the development of antibodies to germ cell specific antigens (Fijak and Meinhardt, 2006).

Lanthanum permeability studies revealed severe defects in the BTB of ZO-2 chimera. No differences were observed between control and ZO-2 chimera in the number of sites where the tracer diffused into the basal compartment of the seminiferous epithelium. This partial permeability barrier is associated with an incomplete system of tight junctions between cells of the myoid cell layer, located between the endothelium and the seminiferous epithelium (Dym and Fawcett, 1970). Whether ZO-2 is present in the myoid cell junctions is not known. In any case, the BTB at the level of tight junctions between Sertoli cells was severely compromised in ZO-2 chimera. Leakage of lanthanum into the adluminal compartment was never observed in controls, but occurred at ~20% of the sites where the tracer had diffused past the myoid cell layer in chimera tubules. Despite the smaller diameter of the seminiferous tubules in the chimera, ZO-1 and Occludin showed a similar discrete localization as in control tubules, indicating that the absence of ZO-2 did not

result in a complete reorganization or disassembly of the BTB. Consistent with this notion, not all ZO-2 deficient seminiferous tubules showed a compromised BTB barrier. This indicates that the absence of ZO-2 only leads to a mild or partial effect on BTB function, or that the loss of barrier function is a gradual process, or more prominent in seminiferous tubules associated with a particular stage of spermatogenesis.

Cldn11 is prominently expressed in testes and Cldn11 KO mice show a loss of TJ fibrils in Sertoli cells and present with male infertility (Gow *et al.*, 1999). Given that the colocalization of Cldn11 with ZO-1 at the BTB is only mildly perturbed in seminiferous tubules of ZO-2 chimera, it is conceivable that the BTB function is less severely affected in the chimera as compared with the Cldn11 KO mice. ZO-2 interacts with Cldns (Itoh *et al.*, 1999) and filamentous actin (Wittchen *et al.*, 1999). Thus, the absence of ZO-2 may compromise the tethering of Cldns to the actin cytoskeleton and this could result in a weakening of the BTB barrier to affect permeability of certain ions (e.g., lanthanum). In contrast, the lack of Cldn11 itself likely has a more profound effect on barrier integrity that may also affect the restriction of macromolecules. Indeed, disruption of filamentous actin weakens Cldn-Cldn-mediated adhesion (Vedula *et al.*, 2008), which conceivably could alter paracellular permeability properties of the TJ. Surprisingly, the distribution of filamentous actin in seminiferous tubules of ZO-2 chimera is perturbed. This could reflect a role for ZO-2 in organizing cortical actin, or the redistribution of actin that is not recruited to ectoplasmic specializations due to defects in spermatogenesis.

Chemical compounds that disrupt the BTB such as CdCl₂ and lindane induce the delocalization of ZOPs (Defamie *et al.*, 2003). Furthermore, dislocation of ZO-1 and ZO-2 from Sertoli cell TJs to the lateral membrane and the cytosol has been correlated with the disruption of the BTB in testicular carcinoma in situ (Fink *et al.*, 2006). These findings are thus consistent with a critical role for ZOPs in BTB function. Expression levels for ZO-1 or ZO-3 in chimera testes were not increased to compensate for the absence of ZO-2. Cldn11 KO mice show a loss of TJ fibrils in Sertoli cells (Gow *et al.*, 1999). Although we have not analyzed TJ fibrils in the testis of ZO-2 chimera, preliminary analysis of embryoid bodies lacking ZO-2 indicate that TJ fibrils still form in the absence of ZO-2 but that their organization and structure may be altered.

Disruption of the BTB is likely to affect the distinct composition of the fluids in the basal and adluminal compartments of the seminiferous tubule, required for germ cell maturation and viability. In addition to a direct role in structure and function of TJs between Sertoli cells, ZO-2 may also be involved in the adhesion between germ cells and Sertoli cells and the remodeling of junctions during germ cell migration and differentiation. Several TJ proteins, including ZO-1, JAMs, and Occludin, have been implicated in this process, which is critical for spermatogenesis (Wong *et al.*, 2004; Yan and Cheng, 2005). Because ZO-2 shuttles between the nucleus and cytoplasm (Jaramillo *et al.*, 2004; Kausalya *et al.*, 2004) and interacts with several transcription factors (Betanzos *et al.*, 2004; Huerta *et al.*, 2007), it may also regulate the expression of genes important for the physiology of the seminiferous epithelium and spermatogenesis.

In addition to their localization to TJs, ZOPs bind connexins (Kausalya *et al.*, 2001; Giepmans, 2004) and have been implicated in the regulation of connexin trafficking, as well as the assembly and stability of gap junctions and their localization and size (Segretain *et al.*, 2004; Hunter *et al.*, 2005; Laing *et al.*, 2005). ZO-2 associates with Cx43 (Singh *et*

al., 2005), the main gap junction protein in the testis (Pointis and Segretain, 2005; Lee *et al.*, 2007). Intriguingly, the predominant localization of Cx43 to the region of the BTB was perturbed in testes of ZO-2 chimera, where the protein was readily detected in the adluminal domain. Because Cx43 is essential for the initiation and maintenance of spermatogenesis (Brehm *et al.*, 2007; Sridharan *et al.*, 2007), its perturbed localization in ZO-2 chimera tubules may contribute to the reduced fertility of ZO-2 chimera males.

In summary, we provide evidence that ZO-2 may be more critical for the function of extraembryonic tissues than development of the epiblast. The loss of ZO-2 expression in the testis is associated with a reduced fertility of male ZO-2 chimera. A compromised BTB and a perturbed localization of Cx43 may contribute to the decreased fertility. This is to our knowledge the first instance where a particular ZOP is shown to be critical for the physiology of an adult organ in mammals.

ACKNOWLEDGMENTS

We thank M. Arun Kumar and Ng Chee Peng for help with blastocyst injections and transmission electron microscopy. W.H. is an adjunct faculty member at the Department of Physiology of the National University of Singapore. This work was supported by the Agency for Science, Technology and Research (A*STAR), Singapore.

REFERENCES

- Adachi, M., Inoko, A., Hata, M., Furuse, K., Umeda, K., Itoh, M., and Tsukita, S. (2006). Normal establishment of epithelial tight junctions in mice and cultured cells lacking expression of ZO-3, a tight-junction MAGUK protein. *Mol. Cell Biol.* 26, 9003–9015.
- Anderson, J. M., Van Itallie, C. M., and Fanning, A. S. (2004). Setting up a selective barrier at the apical junction complex. *Curr. Opin. Cell Biol.* 16, 140–145.
- Balda, M. S., and Matter, K. (2000). Transmembrane proteins of tight junctions. *Semin. Cell Dev. Biol.* 11, 281–289.
- Betanzos, A., Huerta, M., Lopez-Bayghen, E., Azuara, E., Amerena, J., and Gonzalez-Mariscal, L. (2004). The tight junction protein ZO-2 associates with Jun, Fos and C/EBP transcription factors in epithelial cells. *Exp. Cell Res.* 292, 51–66.
- Brehm, R., *et al.* (2007). A sertoli cell-specific knockout of connexin43 prevents initiation of spermatogenesis. *Am. J. Pathol.* 171, 19–31.
- Defamie, N., Berthaut, I., Mograbi, B., Chevallier, D., Dadoune, J. P., Fenichel, P., Segretain, D., and Pointis, G. (2003). Impaired gap junction connexin43 in Sertoli cells of patients with secretory azoospermia: a marker of undifferentiated Sertoli cells. *Lab. Invest.* 83, 449.
- Dym, M., and Fawcett, D. W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol. Reprod.* 3, 308–326.
- Fijak, M., and Meinhardt, A. (2006). The testis in immune privilege. *Immunol. Rev.* 213, 66–81.
- Fink, C., Weigel, R., Hembes, T., Lauke-Wettwer, H., Kliesch, S., Bergmann, M., and Brehm, R. H. (2006). Altered expression of ZO-1 and ZO-2 in Sertoli cells and loss of blood-testis barrier integrity in testicular carcinoma in situ. *Neoplasia* 8, 1019–1027.
- Giepmans, B. N. (2004). Gap junctions and connexin-interacting proteins. *Cardiovasc. Res.* 62, 233–245.
- Gonzalez-Mariscal, L., Betanzos, A., and Avila-Flores, A. (2000). MAGUK proteins: structure and role in the tight junction. *Semin. Cell Dev. Biol.* 11, 315–324.
- Gonzalez-Mariscal, L., Betanzos, A., Nava, P., and Jaramillo, B. E. (2003). Tight junction proteins. *Prog. Biophys. Mol. Biol.* 81, 1–44.
- Gow, A., Southwood, C. M., Li, J. S., Pariali, M., Riordan, G. P., Brodie, S. E., Danias, J., Bronstein, J. M., Kachar, B., and Lazzarini, R. A. (1999). CNS myelin and Sertoli cell tight junction strands are absent in *Osp/occludin-11* null mice. *Cell* 99, 649–659.
- Griswold, M. D. (1988). Protein secretions of Sertoli cells. *Int. Rev. Cytol.* 110, 133–156.

- Hernandez, S., Chavez Munguia, B., and Gonzalez-Mariscal, L. (2007). ZO-2 silencing in epithelial cells perturbs the gate and fence function of tight junctions and leads to an atypical monolayer architecture. *Exp. Cell Res.* 313, 1533–1547.
- Huerta, M., Muñoz, R., Tapia, R., Soto-Reyes, E., Ramírez, L., Recillas-Targa, F., González-Mariscal, L., and López-Bayghen, E. (2007). Cyclin D1 is transcriptionally down-regulated by ZO-2 via an E box and the transcription factor c-Myc. *Mol. Biol. Cell* 18, 4826–4836.
- Hunter, A. W., Barker, R. J., Zhu, C., and Gourdie, R. G. (2005). ZO-1 alters connexin43 gap junction size and organization by influencing channel accretion. *Mol. Biol. Cell* 16, 5686–5698.
- Islas, S., Vega, J., Ponce, L., and Gonzalez-Mariscal, L. (2002). Nuclear localization of the tight junction protein ZO-2 in epithelial cells. *Exp. Cell Res.* 274, 138–148.
- Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M., and Tsukita, S. (1999). Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J. Cell Biol.* 147, 1351–1363.
- Jaramillo, B. E., Ponce, A., Moreno, J., Betanzos, A., Huerta, M., Lopez-Bayghen, E., and Gonzalez-Mariscal, L. (2004). Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells. *Exp. Cell Res.* 297, 247–258.
- Jesaitis, L. A., and Goodenough, D. A. (1994). Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* discs-large tumor suppressor protein. *J. Cell Biol.* 124, 949–961.
- Katsuno, T., et al. (2008). Deficiency of zonula occludens-1 causes embryonic lethal phenotype associated with defected yolk sac angiogenesis and apoptosis of embryonic cells. *Mol. Biol. Cell* 19, 2465–2475.
- Kausalya, P. J., Phua, D. C., and Hunziker, W. (2004). Association of ARVCF with zonula occludens (ZO)-1 and ZO-2, binding to PDZ-domain proteins and cell-cell adhesion regulate plasma membrane and nuclear localization of ARVCF. *Mol. Biol. Cell* 15, 5503–5515.
- Kausalya, P. J., Reichert, M., and Hunziker, W. (2001). Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells. *FEBS Lett.* 505, 92–96.
- Kobayashi, A., Chang, H., Chaboissier, M. C., Schedl, A., and Behringer, R. R. (2005). Sox9 in testis determination. *Ann. N.Y. Acad. Sci.* 1061, 9–17.
- Kohler, K., and Zahraoui, A. (2005). Tight junction: a co-ordinator of cell signalling and membrane trafficking. *Biol. Cell* 97, 659–665.
- Laing, J. G., Chou, B. C., and Steinberg, T. H. (2005). ZO-1 alters the plasma membrane localization and function of Cx43 in osteoblastic cells. *J. Cell Sci.* 118, 2167–2176.
- Lee, N. P., Yeung, W. S., and Luk, J. M. (2007). Junction interaction in the seminiferous epithelium: regulatory roles of connexin-based gap junction. *Front. Biosci.* 12, 1552–1562.
- Lui, W. Y., Mruk, D., Lee, W. M., and Cheng, C. Y. (2003). Sertoli cell tight junction dynamics: their regulation during spermatogenesis. *Biol. Reprod.* 68, 1087–1097.
- Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S., and Benz, E. J., Jr. (2000). Characterization of the interaction between protein 4.1R and ZO-2. A possible link between the tight junction and the actin cytoskeleton. *J. Biol. Chem.* 275, 30573–30585.
- Matter, K., and Balda, M. S. (2003). Signalling to and from tight junctions. *Nat. Rev. Mol. Cell Biol.* 4, 225–236.
- Matter, K., and Balda, M. S. (2007). Epithelial tight junctions, gene expression and nucleo-junctional interplay. *J. Cell Sci.* 120, 1505–1511.
- McNeil, E., Capaldo, C. T., and Macara, I. G. (2006). Zonula occludens-1 function in the assembly of tight junctions in Madin-Darby canine kidney epithelial cells. *Mol. Biol. Cell* 17, 1922–1932.
- Medina, R., Rahner, C., Mitic, L. L., Anderson, J. M., and Van Itallie, C. M. (2000). Occludin localization at the tight junction requires the second extracellular loop. *J. Membr. Biol.* 178, 235–247.
- Pointis, G., and Segretain, D. (2005). Role of connexin-based gap junction channels in testis. *Trends Endocrinol. Metab.* 16, 300–306.
- Rossant, J., and Spence, A. (1998). Chimeras and mosaics in mouse mutant analysis. *Trends Genet.* 14, 358–363.
- Segretain, D., Fiorini, C., Decrouy, X., Defamie, N., Prat, J. R., and Pointis, G. (2004). A proposed role for ZO-1 in targeting connexin 43 gap junctions to the endocytic pathway. *Biochimie* 86, 241–244.
- Sheth, B., Nowak, R. L., Anderson, R., Kwong, W. Y., Papenbrock, T., and Fleming, T. P. (2008). Tight junction protein ZO-2 expression and relative function of ZO-1 and ZO-2 during mouse blastocyst formation. *Exp. Cell Res.* 314, 3356–3368.
- Singh, D., Solan, J. L., Taffet, S. M., Javier, R., and Lampe, P. D. (2005). Connexin 43 interacts with zona occludens-1 and -2 proteins in a cell cycle stage-specific manner. *J. Biol. Chem.* 280, 30416–30421.
- Sridharan, S., Brehm, R., Bergmann, M., and Cooke, P. S. (2007). Role of connexin 43 in Sertoli cells of testis. *Ann. N.Y. Acad. Sci.* 1120, 131–143.
- Traweger, A., Fuchs, R., Krizbai, I. A., Weiger, T. M., Bauer, H. C., and Bauer, H. (2003). The tight junction protein ZO-2 localizes to the nucleus and interacts with the heterogeneous nuclear ribonucleoprotein scaffold attachment factor-B. *J. Biol. Chem.* 278, 2692–2700.
- Traweger, A., Lehner, C., Farkas, A., Krizbai, I. A., Tempfer, H., Klement, E., Guenther, B., Bauer, H. C., and Bauer, H. (2008). Nuclear zonula occludens-2 alters gene expression and junctional stability in epithelial and endothelial cells. *Differentiation* 76, 99–106.
- Tsukita, S., Furuse, M., and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2, 285–293.
- Umeda, K., Ikenouchi, J., Katahira-Tayama, S., Furuse, K., Sasaki, H., Nakayama, M., Matsui, T., Tsukita, S., Furuse, M., and Tsukita, S. (2006). ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* 126, 741–754.
- Umeda, K., Matsui, T., Nakayama, M., Furuse, K., Sasaki, H., Furuse, M., and Tsukita, S. (2004). Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. *J. Biol. Chem.* 279, 44785–44794.
- Van Itallie, C. M., and Anderson, J. M. (2004). The molecular physiology of tight junction pores. *Physiology* 19, 331–338.
- Van Itallie, C. M., and Anderson, J. M. (2006). Claudins and epithelial paracellular transport. *Annu. Rev. Physiol.* 68, 403–429.
- Vedula, S.R.K., Lim, T. S., Kausalya, P. J., Lane, E. B., Rajagopal, G., Hunziker, W., and Lim, C. T. (2008). Quantifying forces mediated by integral tight junction proteins in cell-cell adhesion. *Exp. Mech.* 49, 3–9.
- Wittchen, E. S., Haskins, J., and Stevenson, B. R. (1999). Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J. Biol. Chem.* 274, 35179–35185.
- Wong, C. H., and Cheng, C. Y. (2005). The blood-testis barrier: its biology, regulation, and physiological role in spermatogenesis. *Curr. Top. Dev. Biol.* 71, 263–296.
- Wong, C. H., Mruk, D. D., Lui, W. Y., and Cheng, C. Y. (2004). Regulation of blood-testis barrier dynamics: an in vivo study. *J. Cell Sci.* 117, 783–798.
- Xu, J., Kausalya, P. J., Phua, D. C., Ali, S. M., Hossain, Z., and Hunziker, W. (2008). Early embryonic lethality of mice lacking ZO-2, but not ZO-3, reveals critical and nonredundant roles for individual zonula occludens proteins in mammalian development. *Mol. Cell Biol.* 28, 1669–1678.
- Yan, H. H., and Cheng, C. Y. (2005). Blood-testis barrier dynamics are regulated by an engagement/disengagement mechanism between tight and adherens junctions via peripheral adaptors. *Proc. Natl. Acad. Sci. USA* 102, 11722–11727.
- Yan, H. H., Mruk, D. D., and Cheng, C. Y. (2008). Junction restructuring and spermatogenesis: the biology, regulation, and implication in male contraceptive development. *Curr. Top. Dev. Biol.* 80, 57–92.
- Yan, H. H., Mruk, D. D., Lee, W. M., and Cheng, C. Y. (2007). Ectoplasmic specialization: a friend or a foe of spermatogenesis? *Bioessays* 29, 36–48.