Blood-testis barrier and spermatogenesis: lessons from genetically-modified mice

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The blood-testis barrier (BTB) is found between adjacent Sertoli cells in the testis where it creates a unique microenvironment for the development and maturation of meiotic and postmeiotic germ cells in seminiferous tubes. It is a compound proteinous structure, composed of several types of cell junctions including tight junctions (TJs), adherence junctions and gap junctions (GJs). Some of the junctional proteins function as structural proteins of BTB and some have regulatory roles. The deletion or functional silencing of genes encoding these proteins may disrupt the BTB, which may cause immunological or other damages to meiotic and postmeiotic cells and ultimately lead to spermatogenic arrest and infertility. In this review, we will summarize the findings on the BTB structure and function from genetically-modified mouse models and discuss the future perspectives.

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INTRODUCTION

Blood-testis barrier (BTB) is found between adjacent Sertoli cells within the seminiferous tubules.1–9 The BTB divides the seminiferous tubules into the basal and apical (adluminal) compartments. Meiosis, spermiogenesis and spermiation take place in the apical compartment; whereas, spermatogonial cell division and differentiation to preleptotene spermatocytes occur in the basal compartment.10,11 Thus, the BTB creates a unique microenvironment for meiotic and postmeiotic cells by forming an immunological barrier that separates meiotic and postmeiotic germ cells from blood circulation (reviewed in12).

The BTB consists of several types of cellular junctions including tight junctions (TJs), gap junctions (GJs) and adherence junctions, and many junctional proteins are involved in the establishment of BTB (reviewed in12–14). Defects in these proteins can cause BTB disfunction which may elicit immune responses against meiotic and postmeiotic cells, ultimately leading to spermatogenetic failure and male infertility. Furthermore, functions of BTB may also be compromised due to the defects of genes that regulate the formation and function of cell junctions. In this article, we will review recent findings in BTB functional genes obtained from genetically-modified mice.

TECHNOLOGIES USED FOR BTB FUNCTION STUDY

In vitro method

Since Sertoli cells cultured at high density in vitro show the ability to form junctions that mimic BTB to some extent,15,16 an in vitro system based on the culture of primary Sertoli cells has been established and used as a model for BTB study.17–21 Because it is relatively easy, quick and cheap, many studies have utilized this method to investigate the structure and function of the cell junctions.17–24 However, since the main function of BTB is to provide microenvironment for meiotic and postmeiotic cell development, this in vitro system is not suitable for the study of major aspects of BTB function because coculture of germ cells with Sertoli cells cannot achieve meiosis.25 Moreover, the BTB structure and/or function may also be affected by germ cells. Therefore, this primary Sertoli cell culture system is insufficient for in-depth study of the structure, function and regulation of BTB in the testis.

In vivo method

Genetically-modified mice have been widely used to understand the functional roles of specific gene in development. There are two basic technical approaches used to produce genetically-modified mice, namely, transgenic and knockout (KO) mice.26–28 The transgenic mouse approach involves pronuclear injection into a zygote, where the gene of interest will randomly integrate into the mouse genome.29 The second approach, pioneered by Oliver Smithies and Mario Capecchi, involves modification of embryonic stem cells with a DNA construct containing DNA sequences homologous to the target gene.28 Embryonic stem cells with deletion of the target gene are selected and then injected into the mouse blastocysts. This manipulation causes absence of the gene (null) from all the cells of mouse. This approach, usually called conventional KO technology, is appropriate for investigating the physiological function of tissue or cell type-specific genes.30 A refined version of the KO technology, conditional KO (cKO), which is based on tissue and cell type-specific deletion of a gene of interest, shows significant advantages over conventional KO, especially for those genes whose conventional KO causes embryonic lethality.31 The most
widely used approach at present for cKO is the Cre-LoxP system, which involves a ‘floxed’ mouse line bearing alleles of the gene to be deleted with recombinase-specific sites (i.e. two LoxP repeats flanking critical exons) and a transgenic mouse line expressing the Cre-recombinase driven by a promoter with a desired temporal and/or spatial expression pattern.\cite{32,35} The gene of interest flanked by two LoxP sites will be deleted or disrupted when Cre-recombinase is expressed in specified tissues or cell types at a specific development time point.\cite{35,36-38} Most resulting cKO mice have no evident developmental abnormalities in tissues except the one of interest and thus can be used for studies of gene function in a specified tissue or cell type at specific time point.

By using these genetic approaches, especially conventional and conditional gene KOs, about 400 genes involved in murine spermatogenesis have been inactivated,\cite{39-42} but only a few have been associated with the structure and function of BTB. In the following sections, we will summarize BTB-associated genes identified from genetically-modified mice.

**DEFECTS IN SPERMATOGENESIS IN KO MICE OF BTB-ASSOCIATED PROTEINS**

The BTB-associated genes are classified into two major groups based on their roles in BTB structure and function. The first group (Table 1) includes the known structural components of the BTB, and the other (Table 2) consists of those that regulate BTB formation, integrity and function. Since many of the mechanisms involved in this process are unknown, these may also include structural components.

**Function of BTB structural components**

Several cellular junctions function together to establish BTB with each type of cellular junction composed of multiple structural proteins. Deficiency in these proteins would cause significant damage to BTB and consequently spermatogenic failure.

**Claudin-11 (Cldn-11)**

Claudins, 20–27 kDa phosphoproteins, are the main constituents of the TJ in mammalian body.\cite{43-46} They are intercellular adhesion molecules with variable pore-like properties.\cite{47,48} To date, about 24 different claudins have been identified and many of them show a distinct organ-specific distribution.\cite{49,50,51} In mice and rat testes, Cldn-11 is specifically expressed in the Sertoli cells and responsible for the formation of the typically parallel tight junctional strands between Sertoli cells.\cite{52} In mouse testis, Cldn-11 expression peaks between postnatal day 6–16, coinciding with the BTB formation.\cite{92,93}

Cldn-11 KO mice were the first mouse model used for the study of BTB.\cite{39} In prepubertal and adult Cldn-11 KO mice, the lumens of the seminiferous tubules are narrow and often filled with Sertoli cells.\cite{53,54} Adult mouse testes lacking Cldn-11 in Sertoli cells are devoid of a mature BTB and show increased apoptotic germ cells.\cite{55} Cldn-11 KO Sertoli cells lose polarity and detach from the basement membrane of seminiferous tubules. They experience an epithelial to fibroblastic cell transformation and proliferate actively while still maintaining the expression of Sertoli cell specific differentiation markers. As expected, Cldn-11 KO mice are sterile.\cite{39}

**Ocludin (Ocln)**

Ocludin, a 65 kDa protein, was the first component of the TJ strand identified.\cite{55-58} It expresses in Sertoli cells, together with claudins, serving as a key component of TJ in BTB.\cite{56} In mouse, Ocln is detected by immunofluorescence in testis cords as early as embryonic day 13.5.\cite{55} By postnatal day 14, it is detected as focal wavy bands toward the base of seminiferous tubules that contain a number of germ cells.\cite{97} By postnatal day 23 and in adult mice, these bands are present in all tubules at all stages of seminiferous epithelial cycle.\cite{57,58} As in mice, Ocln is also detected at all stages of the seminiferous epithelial cycle in dogs and Korean wild rabbits Lepus coreanus.\cite{98,99} However, in rats, Ocln protein expression is stage-specific, expressing heavily in Sertoli cells in seminiferous tubes of all stages except stage VIII, where it is not detectable by immunostaining.\cite{98,99} Interestingly, Ocln is not expressed in seminiferous tubules of guinea pigs (Cavia porcellus) and humans.\cite{57,58}

Compared to Cldn-11 KO mice, the abnormalities of spermatogenesis in Ocln KO mice increase slowly and gradually with ageing.\cite{59-61} In testis of 6-week-old Ocln KO mice, the seminiferous

| Table 1: Defects of spermatogenesis in the BTB structural genetically-modified mice |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Gene**        | **Abbreviation** | **Type of junction** | **Techniques for gene modification** | **Fertility** | **Defects of BTB in genetically-modified mice** | **Defects of somatic cells in genetically-modified mice** | **Defects of germ cells in genetically-modified mice** | **References** |
| **Claudin 11**  | Cldn11 (Osp; Otm; Claudin11; Claudin-11) | TJ | KO | Infertility | Tight junctions diminished | Sertoli cells lose polarity, keep proliferation and show compromised differentiation | Spermatogenesis arrests at spermatids, increased germ cell apoptosis | 39,40 |
| **Ocludin**     | Ocln (Ocl; Al503564) | TJ | KO | Progressive infertility | ND | ND | Germ cell loss in testes of old mice | 41 |
| **Tight junction protein 2** | ZO-2 (Tip2) | TJ | KO | Infertility | Increased permeability | Sertoli cell vacuolation | Germ cell loss | 42 |
| **Tight junction protein 3** | ZO-3(Tip3) | TJ | KO | No obvious abnormality | ND | ND | ND | 43 |
| **Gap junction protein, alpha 1** | Cx43 (Gja1; Npm1; Cnx43; Gja-1; AU042049; AW546267; Cnx3alpha1; connexin43) | GJ | cKO (Amh-Cre) | Infertility | ND | Sertoli cell vacuolation | Spermatogenesis arrests at spermatagonia | 44,45 |
| **Catenin (cadherin associated protein), beta 1** | Ctnnb1 (Btc; Mesc; Ctnb) | AJ | cKO (AmhR2-Cre) | No obvious abnormality | Infertility | ND | ND | 46 |
| **References**  |                  |                  |                  |                  |                  |                  |                  | 47,48 |

AJ: adhesion junctions; BTB: blood-testis barrier; GJ: gap junctions; ND: not determined; KO: knockout; TJ: tight junction. -ZO-2 KO embryonic stem (ES) cells were injected into wild type blastocysts to generate viable ZO-2 chimera.

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Table 2: Defects of spermatogenesis in the BTB regulatory genetically-modified mice

| Gene                      | Abbreviation (other names) | Protein localization in testes | Techniques used for function analysis | Fertility of genetically-modified mice | Defects of BTB in genetically-modified mice | Potential targets of BTB junction type | Defects of somatic cells in genetically-modified mice | Defects of germ cells in genetically-modified mice | Reference |
|---------------------------|-----------------------------|--------------------------------|---------------------------------------|---------------------------------------|--------------------------------------------|----------------------------------------|---------------------------------------------|-----------------------------------------------|-----------|
| Androgen receptor         | Ar (Tfm; AW320017)          | Leydig cells, peritubular myoid cells and Sertoli cells | cKO (Amh-Cre)                        | Infertility                           | Increased permeability                    | TJ                                      | Sertoli cell vacuolation                    | Spermatogenesis arrest at the diapente stage | 49-61     |
| Adenomatosis polypsis coli| Apc (CC1; Min; mAPC; AI047805; AU020952; AW124434) | Leydig cells, Sertoli cells and spermatids | cKO (Amh-Cre)                        | Infertility                           | Increased permeability                    | TJ, AJ                                  | Sertoli cell vacuolation and lacking apical extensions | Abnormal differentiation and desquamation    | 46        |
| AT rich interactive domain 4A (RBP1-like) | Arid4a (Rbbp1; MrrBbp1; A630009N03; A630067N03Rik) | Sertoli cells | KO                              | Progressive infertility               | Increased permeability in testes of old mice | TJ                                      | Sertoli cell vacuolation                     | Spermatogenesis arrest at spermatocytes or spermatids | 62        |
| AT rich interactive domain 4B (RBP1-like) | Arid4b (BCAA; BRCA4; Rbbp1; MrrBbp1; A630009N03; A630067N03Rik) | Sertoli cells | KO                              | Progressive infertility               | Increased permeability                    | TJ, GJ, AJ                              | Sertoli cell vacuolation                     | Spermatogenesis arrest at spermatids          | 68        |
| Basigin                   | Bsg (HT-7; CD147; EMMPRIN; A1115436; A1325119) | Leydig cells, Sertoli cells, spermatocytes and spermatids | KO                                | Infertility                           | Increased permeability                    | AJ                                      | Sertoli cell vacuolation                    | Spermatogenesis arrest at spermatids          | 63-65     |
| Ets variant gene 5        | Etv5 (ERM; 11100005E01Rik; B430401F14Rik) | Sertoli cells and subpopulation of gonocytes | KO                                | Infertility                           | Increased permeability                    | ND                                      | Sertoli cell vacuolation                     | SSCs loss during first wave of spermatogenesis | 66,67     |
| Fatty acid desaturase 2   | Fads2 (Fads2; 2900042M13Rik) | Leydig cells, peritubular myoid cells, Sertoli cells and germ cells (ubiquitously expressed) | KO                                | Infertility                           | Increased permeability                    | TJ, GJ, AJ                              | Sertoli cell vacuolation                     | Spermatogenesis arrest at spermatids          | 68        |
| GATA binding protein 4    | Gata4 (Gata-4) | Fetal pre-Sertoli cells, Sertoli cells, fetal Leydig cells, fibroblast-like interstitial cells and peritubular myoid cells Postnatal: Leydig cells and Sertoli cells | cKO (Amhr2-Cre) | Progressive infertility               | Increased permeability                    | GJ                                      | Sertoli cell vacuolation in older cKO testes | Spermatocyte and spermatid desquamation | 69-76     |
| Retinoblastoma 1           | Rb (Rb1; pRb; Rb-1) | Sertoli cells and germ cells with stage dependent | cKO (Amh-Cre)                        | Progressive infertility               | Increased permeability in testes of old mice | TJ                                      | Sertoli cell vacuolation in older cKO testes | Spermatogenesis arrest at spermatocytes and round spermatids | 77        |
| SRY (sex determining region Y)-box 8 | Sax8 | Sertoli cells | KO                              | Progressive infertility               | Increased permeability in testes of old mice | TJ                                      | Sertoli cell vacuolation in older cKO testes | Abnormal differentiation and desquamation | 78-80     |
| TYRO3 protein tyrosine kinase 3 | Tyro3 (Brt; Dtk; Rse; Sky; Tf; Etk-2; A1323366) | Sertoli cells | KO                              | Infertility                           | Increased permeability in testes of old mice | ND                                      | Increase permeability in testes of old mice | Degeneration of germ cells of different stages | 81,82     |
| AXL receptor tyrosine kinase | Axl (Ar; Ufo; Tyro7; A1323647) | Sertoli cells | KO                              | Infertility                           | Increased permeability in testes of old mice | ND                                      | Increase permeability in testes of old mice |                | 81,82     |
| c-mer proto-oncogene tyrosine kinase | Merk (Eyk; Mer; Nyk; nfly12) | Leydig cells and Sertoli cells | KO                              | Infertility                           | Increased permeability in testes of old mice | ND                                      | Increase permeability in testes of old mice |                | 81,82     |

AJ: adhesion junctions; BTB: blood-testis barrier; GJ: gap junctions; KO: knockout; ND: not determined; SCC: spermatogonial stem cell; TJ: tight junction. The potential targets of BTB junction type classification is based on the BTB basic gene expression level change in the modified mice. Besides cKO, a transgenic mice is also included in Ar studies. "KO refers to Arid4a KO and Arid4b haploinsufficiency (Arid4a" Arid4b"). The KO mouse is a triple KO for Tyro3, Axl and Mertk.

Tubules and spermatogenesis are histo-cytologically indistinguishable from those in wide-type mice. Around 40–60 weeks of age, the seminiferous tubules of KO mice display atrophy. The atrophic tubules are devoid of germ cells, but retain Sertoli cells along the basement membrane. The exact mechanisms underlying this age-dependent effect in testis of Ocln KO mice remain unknown.
Zonula occludens (ZO) proteins

TJ integral membrane proteins such as claudins and Ocln are tethered to the actin cytoskeleton by adaptor proteins, notably the closely related ZO proteins ZO-1, ZO-2 and ZO-3. These three closely related and widely expressed ZO proteins belong to the membrane-associated guanylate kinase-like protein superfamily. ZO-1 protein is also known as TJ protein 1 (TJPI). It is detected at the inter-Sertoli cell junctions in testis of guinea pig and mouse. In normal human testis, ZO-1 and ZO-2 are observed at the adherent site of adjacent Sertoli cells. The nuclear localization of some ZO proteins is also reported in particular conditions. Recently, these three ZO proteins have been deleted in mice. Although ZO-3 KO mice lack an obvious phenotype, mice deficient in ZO-1 or ZO-2 shows early embryonic lethality. By microinjecting ZO-2 KO embryonic stem cells into wild-type mouse blastocysts, Xu et al. (2009) generated viable ZO-2 chimera. The adult chimera presented a set of phenotypes in different organs. Male ZO-2 chimera showed reduced fertility and pathological changes in the testis. Lanthanum tracer experiments showed a compromised BTB function in these mice. Based on the gene expression and localization analyses, the authors found that the expression level of ZO-1, ZO-3, Cldn-11 and Ocln is not apparently affected when compared to the controls. ZO-1 and Ocln still localize to the BTB region, but Cldn-11 and Connexin43 are mislocalized from BTB. These results indicate there is limited redundance between ZO-2 and other ZO proteins in adult mice.

Connexin-43 (Cx43)

Cx43, also known as GJ protein alpha 1 (Gja1), is the predominant testicular GJ protein located between adjacent Sertoli cells and between Sertoli cells and germ cells. It is colocalized with Ocln, ZO-1 and N-cadherin at the base of the epithelium, and also observed at the focal sites in the epithelium. To study the function of Cx43 in spermatogenesis, mice with Cx43 specifically deleted in Sertoli cells have been generated. Studies on these mice revealed that the expression of Cx43 in Sertoli cells is required for normal testicular development and initiation of spermatogenesis. Adult Sertoli cell-specific Cx43 KO mice are sterile with a dramatic reduction in size and weight of testes. Their spermatogenesis is arrested at spermatogonia in 95% of seminiferous tubules with the number of spermatogonia dramatically decreased and Sertoli cells increased. Sertoli cell-only syndrome and Sertoli cell clusters are also noted in these mice.

Cadherin associated protein beta 1 (β-catenin)

Cadherin associated protein beta 1 (Cttnb1, β-catenin) is a multifunctional molecule that functions in intercellular adhesion and signal transduction. It is colocalized with N-cadherin between adjacent Sertoli cells in the seminiferous tubules near the basal and the lower one-third of the adluminal compartments, and also at cell-cell contacts sites between Sertoli cells and spermatocytes in testes of Sprague–Dawley rats. N-cadherin is considered as a structural component of BTB, so the colocalization of β-catenin with N-cadherin at the inter-Sertoli cells contact point suggests that it is also an integral component of BTB. β-catenin is also an essential component of the WNT/β-catenin signaling pathway, which plays important roles in multiple developmental processes including testis development.

By crossing with mice expressing Cre recombinase driven by the anti-Mullerian hormone (AMH) type II receptor promoter (Amhr2) in Sertoli cells, Cttnb1 is specifically deleted in Sertoli cells. Histological examination of testes of adult (>12 weeks) Cttnb1 cKO mice does not show any abnormalities in testicular morphology. Constitutively activated β-catenin in Sertoli cells leads to continuous proliferation and compromised differentiation of Sertoli cells. Compared with the controls, Sertoli cells in the adult mutant mice still express AMH and glial cell-derived neurotrophic factor (GDNF) at high levels, which are normally expressed only in immature Sertoli cells. Defective differentiation of germ cells and increased apoptosis were observed in these mutant mice. As expected, the epididymus of the adult mutant mice are devoid of sperm. Besides, as a structural component of BTB, it also plays an essential role in the regulation of Sertoli cell proliferation and differentiation. Actually, it has been reported to regulate cell proliferation and differentiation through the WNT/β-catenin signaling pathway. As for its role in BTB, based on the observation that its deficiency does not cause detectable reproductive defects, we speculate that β-catenin may just serve as an adaptor for N-cadherin. To confirm or refute this speculation, more studies are required.

Function of BTB regulatory elements

Androgen receptor

Androgen receptor (Ar), a member of the steroid hormone receptor superfamily, mediates androgen action and plays an important role in male reproduction (reviewed in ). In testes, Ar can be detected in Sertoli cells, peritubular myoid cells and cells in the interstitial spaces including Leydig cells and perivascular smooth muscle cells. It has been reported that the Sertoli cell-specific Ar cKO mice are infertile, due to spermatogenic arrest predominately at the diplotene stage with almost no sperm observed in the epididymis. The defects in BTB structure of these cKO mice are associated with the reduced expression of BTB proteins like Cldn-11, ZO-1, Ocln and gelsolin and with a significantly enhanced expression of vimentin. It is noteworthy that the Ar cKO mice had a partial defect in androgen sensitivity when carry this floxed allele, and a marked reduction in AR protein levels in different tissues including the testis and show defects in spermatogenesis. The BTB in Ar cKO mice is disrupted, possibly due to the reduced expression of Cldn-3. These results from Ar mouse models indicate that the function of AR in Sertoli cells is essential for the maintenance of fully competent Sertoli cell function in BTB integrity as well as the sustenance of appropriate hormone levels to support the completion of spermatogenesis.

Adenomatous polyposis coli (Apc)

Mutations in Apc, a multifunction tumor suppressor protein, are associated with the development of various human cancers, including colon, liver, ovarian, endometrial and testicular cancers. In a mouse model that expresses a truncated form of Apc in Sertoli cells, despite having normal embryonic and early postnatal testicular development, premature germ cell loss and Sertoli cell only (SCO) seminiferous tubules were observed. The cKO of Apc does not affect the Sertoli cell quiescence, apoptosis or differentiation, as evidenced by the absence of proliferating cell nuclear antigens and DNA damages in Sertoli cells, as well as AMH expression. However, these Sertoli cells lose their apical extensions, which normally enclose germ cells at late stages of spermatogenesis. As for the BTB structure, ZO-1 and N-cadherin proteins are seen as diffused and away from the BTB site in Apc cKO testes. As a result, deficiency of the Apc in Sertoli cells disrupts the BTB and causes spermatogenic failure most probably by affecting localization of junctional proteins.

AT rich interactive domain 4A and AT rich interactive domain 4B

AT rich interactive domain 4A (Arid4a) and AT rich interactive domain 4B (Arid4b) are members of the ARID (AT-rich interaction...
domain) gene family. ARID4A and ARID4B proteins, also known as RB-binding protein 1 (RBBP1, RBP1) and RBBP1-like protein 1 (RBBP1L1), are the members of chromatin-remodeling complex and function as transcriptional repressors upon recruitment by RB. 135-138 In situ hybridization analysis reveal that Arid4a and Arid4b are expressed mainly in Sertoli cells of testes.62 Mice with complete deficiency of Arid4a and haploinsufficiency of Arid4b showed progressive loss of male fertility, accompanied by impaired BTB, hypogonadism and seminal vesicle agenesis/hypoplasia.62 These mice show spermatogenic arrest at meiotic spermatocytes or postmeiotic spermatids.62 These observations recapitulate the defects found in the Sertoli cell-specific Ar KO mice and the Sertoli cell-specific Rb KO mice.62 Gene expression evaluation revealed that ARID4A and ARID4B contribute to the optimal expression of Cldn-3 by functioning as positive coregulators in the context of the AR and RB complex.62 Furthermore, increased permeability of the BTB in the testes of Arid4a KO and Arid4b haploinsufficiency mice are observed based on a biotin tracer injection experiment. Together, Arid4a and Arid4b are critical for physiological function of Sertoli cells.

**Basigin (Bsg)**

Bsg is a transmembrane glycoprotein enriched with N-glycans.139,140 It is highly expressed in gonads and plays a crucial role in both male and female reproduction.141,142 In the mouse testis, Bsg is expressed in Sertoli cells, Leydig cells, spermatocytes and spermatids.64,65 Bsg KO males are sterile.65 The Bsg KO testes are devoid of elongated spermatids and mature spermatozoa but have numerous round spermatids.65 Significantly increased apoptotic germ cells and compromised integrity of the BTB are observed in Bsg KO testes. Immunolocalization analysis of BTB component proteins indicates that no obvious difference in the localization of Cxadr, Cx43 or Cldn-11 are seen between wild type and Bsg KO testes, however, the expression of N-cadherin was greatly reduced at the basal compartment of the seminiferous tubules (the site of the BTB) in Bsg KO mice.65 These results imply that Bsg deficiency can compromise BTB integrity.

**Ets-variant gene 5**

The Ets-variant gene 5 (Etv5), also known as Ets-related molecule or ERM, is a member of the PEA3 subfamily of the ETS family of transcription factors. It is mainly expressed in adult Sertoli cells.66,67 Mice with a targeted deletion of Etv5 can undergo first wave of spermatogenesis, but lose their spermatogonial stem cells during this time, and subsequently show SCO phenotype.66 The disappearance of spermatogonial stem cells in the mutants is attributed to the failure of spermatogonial stem cell proliferation without affecting their differentiation by lack of Etv5. The integrity of BTB in Etv5 KO mice is disturbed which was shown by biotin tracer experiment.67 Whether the BTB is regulated by Etv5 directly or indirectly is still unknown, which deserves the further analysis.

**Fatty acid desaturase 2**

Fatty acid desaturase 2 (Fads2) is responsible for the initial step in the enzymatic cascade of ω3- and ω6-polyunsaturated fatty acid synthesis from essential fatty acids.142-144 Fads2 KO mice are sterile, their testis weight is reduced to two-thirds of that of age-matched wild littermates.64 The lumen of the seminiferous tubules and epididymis of the adult mutants lacks spermatozoa.64 The epididymal ductuli fill with detritus and immature spermatids. Immunohistochemical studies revealed that Ocln, Cldn-11, JAM-A, ZO-1, Cx43 and β-catenin are dislocated throughout the basolateral and apical compartments of the Fads2 KO Sertoli cell membrane.65 Furthermore, transmission electron microscopic analysis highlighted that the well-structured TJ structures between Sertoli cells are missing in Fads2 KO testes. Finally, compromised selective permeation of BTB in KO testes has been revealed by the lanthanum nitrate and fluorescence dyes perfusion experiments.68

**GATA binding protein 4**

Transcription factor GATA binding protein 4 (Gata4) has been implicated in the development and function of the mammalian testis.145 During fetal testicular development, Gata4 is expressed in pre-Sertoli cells, Sertoli cells, Leydig cells, fibroblast-like interstitial cells and peritubularmyoid cells.74,75 After birth, Gata4 is found mainly in the Sertoli cells and adult Leydig cells.69-72 Mice, whose Gata4 conditionally is deleted in Sertoli cells, develop age-dependent testicular atrophy and are infertile.73 Histological analysis demonstrated that the older cKO testes displayed Sertoli cell vacuolation, germ cell depletion, multinucleated giant cells and syncytia of degenerating spermatids.73 Biotinylated tracer injection experiments indicate that the BTB appeared intact in young cKO mice (2.5 months), but it had a compromised integrity in the 6-month-old cKO mice.73 Furthermore, biotinylated germ cells, including multinuclear giant cells were evident in seminiferous tubules of 6-month-old cKO mice.73 Thus, the older Gata4 cKO mice develop increased permeability of the BTB with the advancing of age.

**Retinoblastoma 1 (RB)**

RB protein, encoded by Rb gene, is a negative regulator of the cell cycle and the first tumor suppressor found.146,147 The Sertoli cell-specific Rb KO mice displayed progressive infertility in males.77 Initially, loss of Rb in Sertoli cells has no gross effect on Sertoli cell function and the mice are fertile at 6 week of age.77 However, by the age of 10–14 weeks, the cKO mice demonstrated severe Sertoli cell dysfunction and infertility.77 The most striking defects in mature Sertoli cell function are increased permeability of the BTB by biotin tracer experiment.77 Detailed analysis found that TJ components, Cldn-3 and Occludin, are downregulated in Rb cKO Sertoli cells.77 The progressive loss of integrity of BTB in the Rb cKO testes suggested that Rb was initially dispensable for the formation of the BTB but might be indispensable for its remodeling as maturing germ cells crossed from the basal to adluminal compartment and this function might be directly related to the regulation of TJ genes.

**Sex determining region Y-box 8**

Sex determining region Y-box 8 (Sox8) is a member of the Sox family of developmental transcription factor genes and is closely related to Sox9, a key gene in testis determination pathway in mammals.148-151 In testis, it is expressed in the developing mouse testis around the time of sex determination and continues beyond 16 days post coitum in Sertoli cells.79 Sox8 KO mice exhibit a progressive male infertility phenotype.80 These KO males sporadically produced litters of reduced size at young ages and showed an age-dependent deregulation of spermatogenesis, characterized by sloughing of spermatocytes and round spermatids, spermatiation failure and a progressive disorganization of the spermatogenic cycle, which resulted in the inappropriate placement and juxtaposition of germ cell types within the epithelium.80 Cldn-3 was significantly decreased in the Sox8 KO testes.80 Furthermore, the use of biotin tracers showed increased BTB permeability in the Sox8 KO adult testes.80 Thus, Sox8 is essential in Sertoli cells for germ cell differentiation, partly by controlling the microenvironment of the seminiferous epithelium.
constitute the TAM family of receptor tyrosine kinases, characterized by a conserved sequence within the kinase domain and adhesion molecule-like extracellular domains.\textsuperscript{152,153} This small family of receptor tyrosine kinases regulates an intriguing mix of processes, including cell proliferation, survival, cell adhesion and migration and regulation of inflammatory cytokine release.\textsuperscript{152–154} Tyro3, Axl and Mer (TAM) receptor tyrosine kinases triple KO (TAM KO) male mice are infertile due to impaired spermatogenesis.\textsuperscript{81,82} These triple KO testes showed a progressive loss of germ cells from elongated spermatids to spermatagonia.\textsuperscript{82} Young adult TAM KO mice exhibited oligo‑astheno‑teratozoospermia and various morphological malformations of the sperm.\textsuperscript{82} With the progress of mice age, germ cells were eventually depleted from the seminiferous tubules. Furthermore, biotin can be detected in the seminiferous tubules of 20- to 30-week-old testes indicating that BTB was initially built in TAM KO mice, but subsequently compromised as the mice aged.\textsuperscript{82} Moreover, major inflammatory cytokines, including tumor necrosis factor-\(\alpha\), interleukin-6 and monocyte chemotactic protein 1 were upregulated in the testis of TAM KO mice, and predominantly located in Sertoli cells.\textsuperscript{82} It is therefore suggested that the TAM receptors are important in the maintenance of the immune homeostasis in the testis through the BTB.

CONCLUSIONS

Based on the literature reviewed above, we conclude that:

1. BTB gene KO mice, once their BTB integrity is compromised, always show some common abnormalities, e.g. germ cell apoptosis, development arrest, aggregated Sertoli cells in apical compartment, SCO phenotype and infertility. This indicates that the BTB integrity is essential for normal spermatogenesis and male fertility
2. Deletion of genes encoding proteins involved in different types of junctions often causes different phenotypes in seminiferous tubules, suggesting that different junction types in the BTB may play distinct role in maintaining the integrity of BTB in structure and function
3. Deletion of different genes of the same cell junction composed of the BTB, e.g. Cldn, Ocnn and ZO-2, causes slightly different abnormalities in testicular tubules and fertility of animals, which indicates that these proteins function in non-redundant manner
4. Although the interactions between germ cells and Sertoli cells are believed to play a role in BTB function and integrity, a direct convincing evidence to support this hypothesis, where BTB is compromised after specific deletion of a gene in germ cells, is still lacking.

FUTURE PERSPECTIVES

BTB and male infertility

Unexplained male infertility accounts for 30%–40% of men with abnormal semen parameters.\textsuperscript{155} The causes of spermatogenic defects in infertile patients are multifactorial. Endocrine disruption of testicular development during neonatal period, due to environmental pollution, genetic and epigenetic factors, is the most frequent explanation invoked for unexplained male infertility.\textsuperscript{156–158} These factors have been associated with testicular dysgenesis, male infertility and recently testicular malignancy.\textsuperscript{155} It is predicted that these multifactors are associated with the BTB and could participate in the etiopathology of human male infertility by dysregulating BTB. For example, cKO of Cx43 in mouse Sertoli cells results in a very similar spermatogenic failure seen in infertile men.\textsuperscript{44,45,159,160} Azospermic patients with severe spermatogenic failure have been reported to show altered expression of Cx43 mRNA.\textsuperscript{161} Furthermore, significantly positive correlation is reported between the histological score and intensity of the testicular Cx43 expression in oligozoospermic men.\textsuperscript{162} Similar staining pattern of Cx43 are found in testes of healthy men and patients with hypospermatogenesis or spermatogenic arrest at meiotic and postmeiotic stages, while no staining is observed in the seminiferous tubules of patients with spermatogenic arrest at spermatagonia or SCO syndrome.\textsuperscript{163} It is thus suggested that, to understand the etiopathology of human infertility, the expression and localization of BTB proteins should be studied in men with spermatogenic defects and compared to those observed in BTB gene KO mice.

BTB and spermatogenic microenvironment or biomarkers

In testis, blood vessels, lymphatic vessels and nerves are only present in the interstitium between seminiferous tubules, but not inside the seminiferous tubules. The entry of nutrients (e.g. sugars, amino acids) and regulatory molecules (e.g. hormones, electrolytes), but not toxicants (e.g. environmental toxicants, drugs, chemicals) into the apical compartment where meiotic and postmeiotic germ cells reside is tightly regulated by BTB.\textsuperscript{12} The selectivity of BTB, thus, provides a unique microenvironment for the development of meiotic and postmeiotic germ cells in the apical compartment.\textsuperscript{12} The BTB may also function to prevent some molecules from emission from the apical compartment. It is, therefore, reasonable to think that if the integrity of BTB is compromised, some molecules that are only present within the seminiferous tubules normally may diffuse into the blood. These molecules can be used as circulation blood biomarkers of the integrity of BTB or the damage to the microenvironment of spermatogenesis.

BTB and cell specific conditional KO strategy

Normal BTB formation and function require numerous genes, many of which are ubiquitously expressed and function in other organs as well. Conventional KO of these genes may cause embryonic or perinatal lethality in homozygotes, e.g. ZO-1 or ZO-2 KO mice show early embryonic lethality.\textsuperscript{156,160} Even if the KO of a ubiquitously expressed gene is not lethal, it may cause alterations in the physiology of many organs, which could complicate the studies especially for reproduction, the process also regulated tightly by hypothalamic and pituitary. Therefore, the cKO approach shows obvious advantages over conventional KO. However, till now, only a few genes have been investigated by specific deletion in Sertoli cells for their role in BTB and spermatogenesis. Therefore, to delineate the function of BTB in spermatogenesis, much work is needed by using the conditional gene KO approach.

AUTHOR CONTRIBUTIONS

QHS, XHJ and IB conceived the ideas for preparing this review article, wrote the first draft and prepared the final version of the manuscript. WZ, SY, ZW and HJC modified the manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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