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REVIEW ARTICLE

An intracellular trafficking pathway in the seminiferous epithelium regulating spermatogenesis: a biochemical and molecular perspective

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Abstract

During spermatogenesis in adult rat testes, fully developed spermatids (i.e. spermatozoa) at the luminal edge of the seminiferous epithelium undergo "spermiation" at stage VIII of the seminiferous epithelial cycle. This is manifested by the disruption of the apical ectoplasmic specialization (apical ES) so that spermatozoa can enter the tubule lumen and to complete their maturation in the epididymis. At the same time, the blood–testis barrier (BTB) located near the basement membrane undergoes extensive restructuring to allow transit of preleptotene spermatocytes so that post-meiotic germ cells complete their development behind the BTB. While spermiation and BTB restructuring take place concurrently at opposite ends of the Sertoli cell epithelium, the biochemical mechanism(s) by which they are coordinated were not known until recently. Studies have shown that fragments of laminin chains are generated from the laminin/integrin protein complex at the apical ES via the action of MMP-2 (matrix metalloprotease-2) at spermiation. These peptides serve as the local autocrine factors to destabilize the BTB. These laminin peptides also exert their effects on hemidesmosome which, in turn, further potentiates BTB restructuring. Thus, a novel apical ES-BTB-hemidesmosome regulatory loop is operating in the seminiferous epithelium to coordinate these two crucial cellular events of spermatogenesis. This functional loop is further assisted by the Par3/Par6-based polarity protein complex in coordination with cytokines and testosterone at the BTB. Herein, we provide a critical review based on the latest findings in the field regarding the regulation of these cellular events. These recent findings also open up a new window for investigators studying blood–tissue barriers.

Keywords: *Testis; seminiferous epithelial cycle; spermatogenesis; Sertoli cells; germ cells; blood–testis barrier; anchoring junction; ectoplasmic specialization; hemidesmosome*

Abbreviations: AJ, adherens junction; BTB, blood–testis barrier; CAR, coxsackie and adenovirus receptor; $CdCl_2$, cadmium chloride; Co-IP, co-immunoprecipitation; DS, desmosome-like junction, a testis-specific cell–cell intermediate filament-based anchoring junction type sharing the properties of desmosome and GJ; ECM, extracellular matrix; EEA-1, early endosome antigen-1; EM, electron microscopy; ERK, extracellular signal-regulated kinase; ES, ectoplasmic specialization, a testis-specific atypical AJ type restricted to Sertoli–Sertoli cell interface at the BTB known as basal ES, and Sertoli–spermatid interface called apical ES; FAC, focal adhesion complex, also known as focal contact, which is a cell-cell actin-based cell-matrix anchoring junction type; FAK, focal adhesion kinase; FITC, fluorescein 5′-isothiocyanate; GJ, gap junction; GSK, glycogen synthase kinase, GSK3β is a Ser/Thr protein kinase identical to Tau protein kinase 1 crucial to intracellular signaling function; IFNγ, interferon γ; ILK, integrin-linked kinase; JAM, junctional adhesion molecules, a class of TJ-integral membrane proteins consisting of JAM-1, 2, and 3 also called JAM-A, B, and C; JNK, c-Jun N-terminal protein kinase also known as SAPK; MAPK, mitogen activated protein kinase; MEK1, MAP/ERK kinase 1; MEKKs, MAP/ERK kinase kinases, upstream signaling molecules of p38 MAP kinase; MESNA, 2-mercaptoethanesulfonic acid; α2-MG, $α_2$-macroglobulin, a non-specific protease inhibitor; MMP, matrix metalloproteases, 25 MMPs (i.e. MMP1 through MMP25) are found in ECM of multiple epithelia, and eight MMPs are currently found in the testis, including MMP-1, 2, 9, 14, 18, 23, 24 & 28 with MMP2 and MMP9 in Sertoli cells and MMP7 in spermatocytes; MT1-MMP, membrane type 1-matrix metalloprotease; p130Cas, a 130 kDa protein encoded by *Crkas* gene also

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called Crk-associated protein, a substrate of src family of non-receptor protein tyrosine kinases, upon its phosphorylation, it acts as an adaptor for proteins with SH2 domains; Par, partitioning defective family of polarity proteins, such as Par3 and Par6; PTK, protein tyrosine kinase; PTKi, protein tyrosine kinase inhibitor; SH domains, Src homology domains that are involved in the interaction with phosphorylated Tyr resides on other proteins (SH2 domains) or with Pro-rich regions of other proteins (SH3 domains); Src gene, sarcoma-inducing gene of Rous sarcoma virus encoding the PTK Src; TBC, tubulobulbar complex; TER, transepithelial electrical resistance; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteases, TIMP 1–4 are all found in testes; TJ, tight junction; TNF-α, tumor necrosis factor-α; ZO-1, zonula occludens-1

Introduction

In the mammalian testis, spermatogenesis takes place in the seminiferous epithelium of the seminiferous tubule, which is the functional unit that produces spermatozoa (Figures 1A, 1B). In rats, spermatozoa (1n, haploid) are formed from more primitive germ cells known as spermatogonia (2n, diploid) (Figure 1C) in ~58 days via 14 stages of development known as the seminiferous epithelial cycle of spermatogenesis (LeBlond and Clermont, 1952; de Kretser and Kerr, 1988). Spermatogenesis is divided into four phases: mitosis, meiosis, spermiogenesis and spermiation, and these events are concomitant with the movement of developing germ cells from the basal to the adluminal (or apical) compartment (Figure 1C). These two compartments are created by the blood–testis barrier (BTB) via specialized junctions between Sertoli cells so that post-meiotic germ cell development takes place behind the immunological barrier conferred by the BTB in a specialized microenvironment (Figure 1C). This thus avoids the production of anti-sperm antibodies against sperm-specific antigens that arise during spermiogenesis in the host body.

Type A spermatogonia are composed of A single (A_s) spermatogonia (i.e. self-renewing germline stem cells that occupy a niche near the basement membrane), A paired (A_{pr}) and A aligned (A_{al}), and all are undifferentiated germ cells. Type A spermatogonia reproduce via mitosis, but some, however, transform to differentiated type A spermatogonia (A_1, A_2, A_3, A_4). Type A spermatogognia differentiate into intermediate spermatogonia (In) and then type B spermatogonia (B) (Figure 1C) (de Rooij and Russell, 2000; Hess and de Franca, 2008). At stage VII of the epithelial cycle, type B spermatogonia differentiate into preleptotene, followed by leptotene spermatocytes, which are the primary spermatocytes that cross the BTB while differentiating into zygotene spermatocytes at stages VIII–IX (Russell, 1977c; Parvinen, 1982), illustrating that transit of primary spermatocytes across the BTB associates with cell cycle progression (e.g. duplication of genetic materials and condensation of chromosomes) in preparation for metaphase I. Once these cells enter the adluminal compartment (Figure 1C), pachytene spermatocytes differentiate into diplotene spermatocytes (tetraploid, 4n). These cells undergo diakinesis and enter the first division of meiosis (Div I) to become secondary spermatocytes, followed immediately by the second division of meiosis (Div II) to form spermatids (haploid, 1n) at stage XIV the epithelial cycle in rats. Thereafter, spermatids undergo extensive morphological changes in a process known as spermiogenesis (Figure 1C). This is typified by: (i) formation of the acrosome, (ii) condensation of chromatin, (iii) packaging of unwanted cytosolic or cellular substances into the residual body, and (iv) elongation of the tail. These processes are accompanied by the migration of developing spermatids from the basal compartment towards the edge of the tubule lumen so that fully developed spermatids (i.e. spermatozoa) can be emptied into the lumen at spermiation. These events are associated with extensive interactions at the Sertoli-germ cell and Sertoli–Sertoli cell interface, in particular at the BTB, to allow transit of primary spermatocytes and progression of the germ cell cycle (Figure 1). Interestingly, restructuring of the BTB, spermiation and cell cycle progression in primary spermatocytes crossing the BTB take place *simultaneously* at stage VIII of the epithelial cycle, yet these events occur at opposite ends of the seminiferous epithelium (Figure 1) (Clermont, 1972; Parvinen, 1982). Herein, we provide a biochemical model to explain how these events are coordinated and regulated based on recent findings in the field.

The blood–testis barrier (BTB)

Concept of the BTB

The BTB is also known as the seminiferous epithelium barrier and the Sertoli cell barrier. The concept of the BTB was first described at the beginning of the last century. When dyes were administered to rodents via the jugular vein, all organs were stained except for seminiferous tubules in the testis and the brain (for reviews, see Miller *et al.*, 2008; Setchell, 2008). Subsequent detailed morphological studies have shown that the BTB is different from other blood–tissue barriers, such as the blood–brain barrier (BBB). For instance, the BBB is constituted by tight junction (TJ) of capillary endothelial cells and contributed in part by pericytes (Bernacki *et al.*, 2008), whereas the microvessels in the interstitium of the testes contribute relatively little barrier function to the BTB. However, myoid cells in the tunica propria (Figure 1) of rodents are known to contribute to barrier function since lanthanum, an electron-opaque marker,
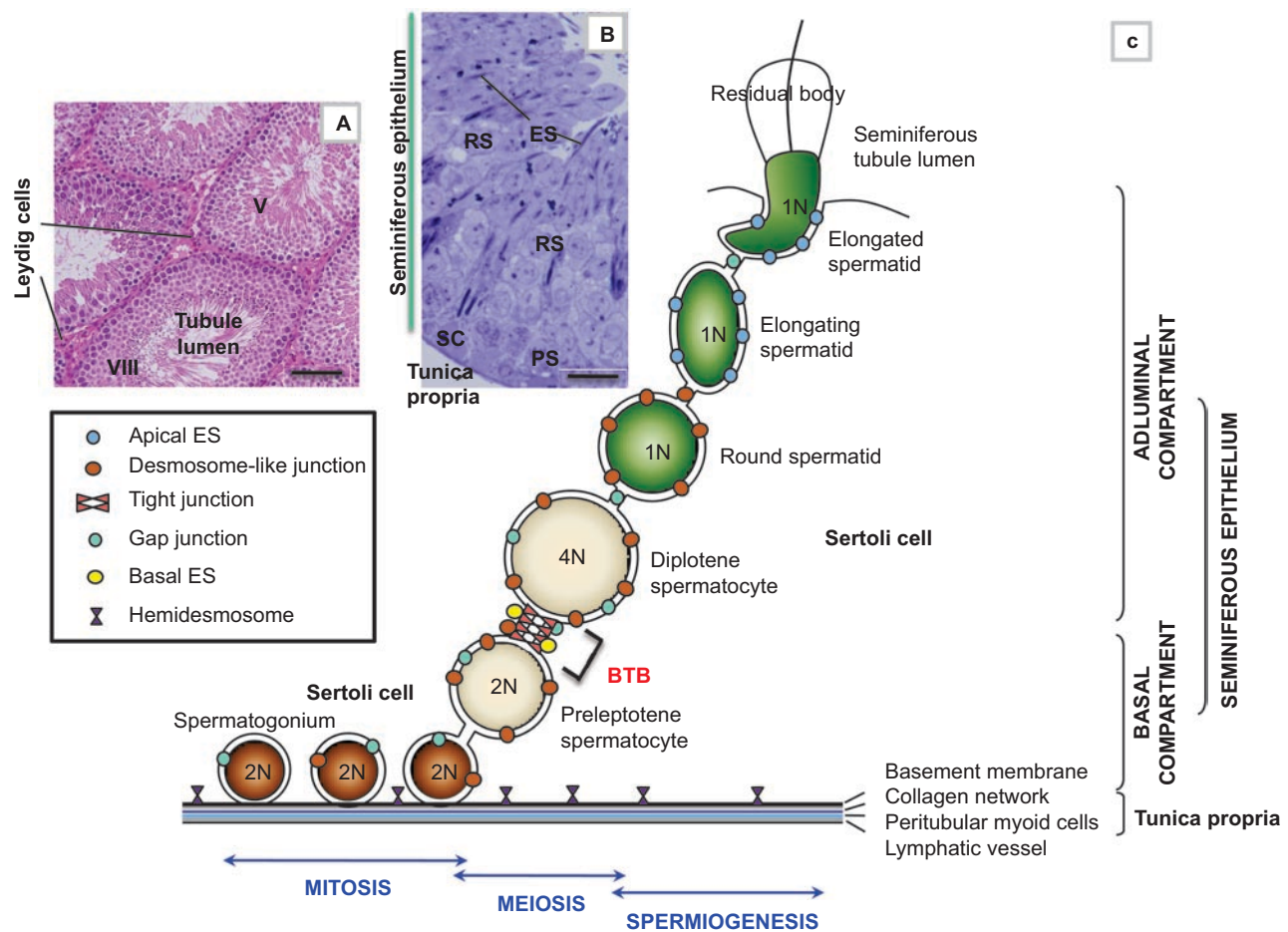
Figure 1. The general morphological features of mammalian testes, and a schematic drawing of the seminiferous epithelium illustrating the relative location of different junction types, and the adluminal (apical) and basal compartments of the seminiferous epithelium created by the blood–testis barrier (BTB) in adult rat testes. (A) This micrograph is a cross-section of an adult rat testis, showing several seminiferous tubules (e.g. a stage V and a stage VIII seminiferous tubule), which are the functional units that produce spermatozoa. The sperm producing function of the seminiferous tubule during spermatogenesis is also supported by testosterone produced by Leydig cells which are found in the interstitium. Bar = 80 μm. (B) Magnified view of a stage V tubule, illustrating that different germ cell types (e.g. PS, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatid) are tightly associated with Sertoli cells (SC, Sertoli cell nucleus) found in the seminiferous epithelium. Bar = 8 μm. (C) Schematic drawing illustrating that the seminiferous epithelium is composed of Sertoli cells and germ cells at different stages of their development, lying on the tunica propria which is composed of two acellular zones: basement membrane (a modified form of extracellular matrix) and type I collagen network; and two cellular zones: peritubular myoid cells and lymphatic vessel. The BTB, located near the basement membrane, is constituted by the coexisting tight junction, gap junction, desmosome-like junction, and basal ectoplasmic specialization (basal ES), between adjacent Sertoli cells. The BTB also physically divides the seminiferous epithelium into the basal and adluminal (apical) compartments and confers cell polarity in the seminiferous epithelium. From step 8 through step 19 spermatids, the apical ectoplasmic specialization (apical ES) is the only anchoring device that maintains cell adhesion and orientation of a developing spermatid onto the Sertoli cell in the seminiferous epithelium. Preleptotene spermatocytes differentiated from type B spermatogonia are the germ cells in transit at the BTB at stage VIII of the seminiferous epithelial cycle while differentiating into leptotene and zygotene spermatocytes. Once these primary spermatocytes enter the adluminal compartment, they differentiate into pachytene spermatocytes and diplotene spermatocytes to begin meiosis I, to be followed immediately by meiosis II to form haploid round spermatids at stage XIV of the epithelial cycle in rats. Round spermatids undergo spermiogenesis via steps 1 through step 19 spermatids in rats, which is typified by the condensation of the genetic materials. The genetic substances are tightly packed into the spermatid head concomitant with the formation of the acrosome above the head region *and* the elongation of the spermatid tail. During these morphological changes, developing spermatids are also in transit near the BTB towards the luminal edge of the seminiferous epithelium progressively, then migrate towards the basement membrane (such as at stage IV-V of the epithelial cycle) (see Figure 1B) and moving towards the luminal edge again (such as stage VI-VII of the epithelial cycle) until the fully developed spermatids (i.e. spermatozoa) are emptied into the seminiferous tubule lumen at spermiation at stage VIII of the epithelial cycle which is associated with disruption of the adhesion protein complexes at the elongated spermatid–Sertoli cell interface, and the shedding of the residual bodies. The Sertoli cell also serves as a scavenger by engulfing the residual bodies via phagocytosis. As a result of this germ cell maturation process and the continuous movement of developing germ cells, in particular elongating spermatids, across the seminiferous epithelium, there is extensive restructuring, namely disassembly and reassembly, of junctions at the Sertoli–Sertoli and Sertoli–germ cell interface during spermatogenesis. Also noted are the three phases of spermatogenesis: mitosis, meiosis, and spermiogenesis (see text for details). 1N, haploid; 2N, diploid; 4N, tetraploid.

failed to penetrate ~85% of seminiferous tubules beyond the myoid cell layer (Dym and Fawcett, 1970). Interestingly, the myoid cell layer is less efficient in blocking dyes and markers from passing through the BTB in primates (Dym, 1973). The reason for this is not known. In short, the BTB in mammals is constituted significantly by specialized junctions between Sertoli cells (Figures 1 and 2).

Functions of the BTB

The BTB is a physiologically important structure in the testis, and it confers several functions which are essential for spermatogenesis (Pelletier, 2001; Cheng and Mruk, 2002; Mruk and Cheng, 2008a; Setchell, 2008). First, it functions as a "fence" by selectively restricting the
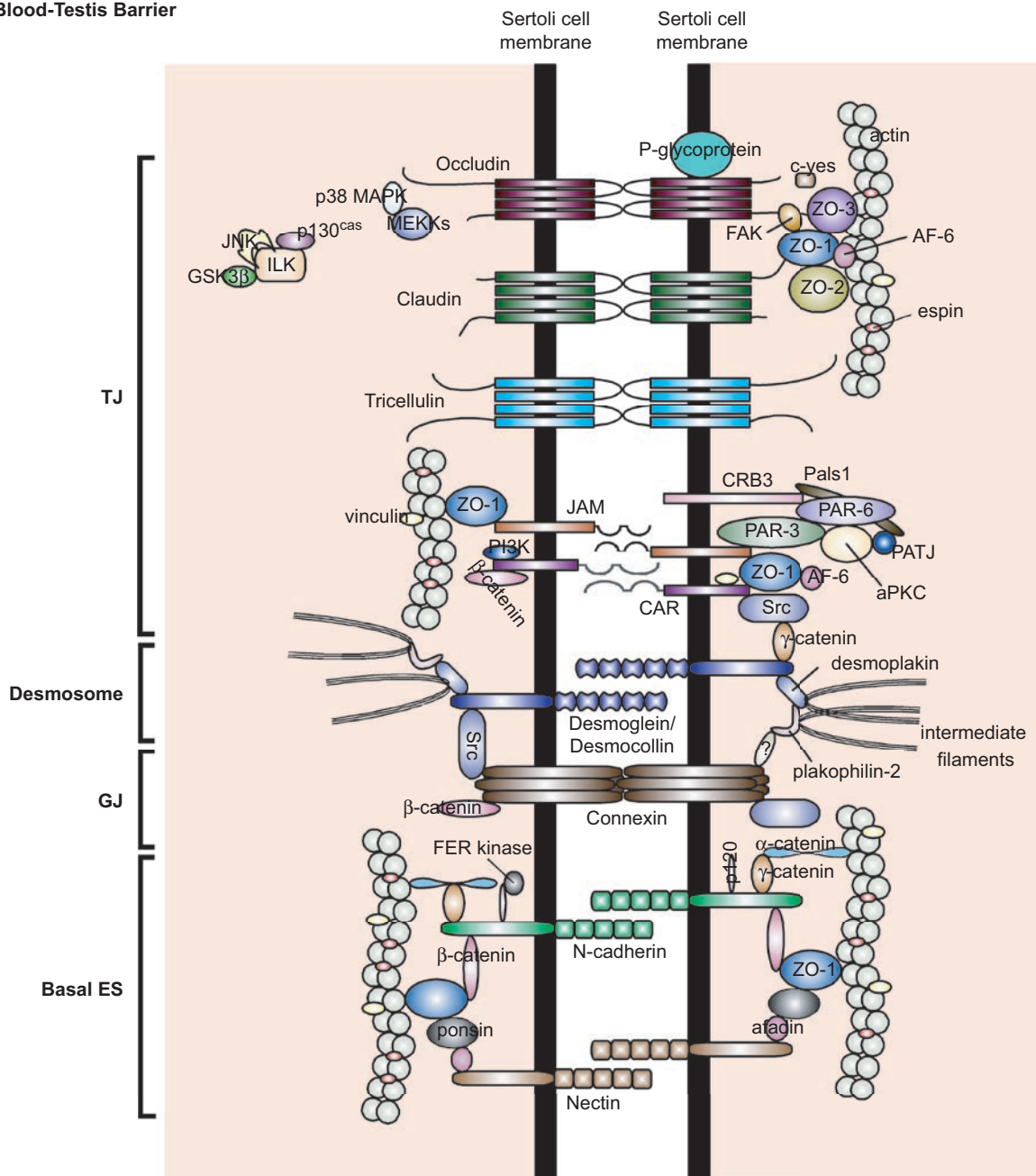
The Blood-Testis Barrier

Figure 2. Protein complexes and their peripheral regulators and adaptors that constitute the BTB in the rat testis. As described in the text, the BTB in the testis is constituted by integral membrane proteins of the: (i) tight junction (TJ) – occludins, claudins, tricellulin, JAMs, and CAR; (ii) desmosome-like junction – desmogleins, desmocollins; (iii) gap junction (GJ) – connexins; and (iv) basal ES – N-cadherins, nectins. Each integral membrane protein is associated with its corresponding adaptors (e.g., ZOs, plakophilins, catenins, afadins), regulatory proteins (e.g., FAK, MAPKs, ILK, FER kinase), polarity proteins (e.g. Par, Pals1). All these protein complexes, in turn, attach to actin via actin-binding proteins (e.g. vinculin, afadin, espin) except for the desmosome-like junction which uses intermediate filaments (e.g. vimentin) for attachment.

passage of water, electrolytes, ions, food, amino acids, hormones and paracrine factors from both the interstitium and basal compartment into the apical compartment. As such, Sertoli cells can regulate the "type" and amount of water, ions, nutrients and hormones needed for haploid spermatid development. Second, the BTB provides an immunological barrier to sequester antigens that are expressed transiently during post-meiotic germ cell development from the host's immune system to prevent the production of anti-sperm antibodies. Third, the BTB also confers cell polarity in the testis. For instance, all Sertoli cell nuclei are located near the basement membrane, and the cytoplasmic processes of each Sertoli cell connects to ~30–50 germ cells at different stages of development (Weber *et al.*, 1983) some of which are connected via intercellular bridges and referred to as germ cell "clones" (Fawcett, 1961; Kato *et al.*, 2004; Greenbaum *et al.*, 2006). A recent study has illustrated that cell polarity proteins such as Par (partitioning-defective) proteins (e.g. Par3, Par6) are crucial proteins that function in the orientation of elongating spermatids, as well as in Sertoli–Sertoli cell adhesion at the BTB (Wong *et al.*, 2008b) (Figure 2). However, unlike most other blood–tissue barriers, the BTB undergoes extensive restructuring at stages VIII–IX of the seminiferous epithelial cycle to facilitate the transit of preleptotene/leptotene spermatocytes so that meiosis I and II can be completed in the apical compartment at stage XIV of the epithelial cycle (Hess and de Franca, 2008).

Structural features of the BTB

Integral membrane proteins that constitute the BTB

At present, the BTB is known to be constituted by co-existing TJ, basal ectoplasmic specialization (ES), gap junctions (GJ) and desmosome-like junctions (Figure 2). Recent studies have identified TJ (e.g. occludin/ZO-1, claudin/ZO-1, JAM-A/ZO-1 and JAM-B/ZO-1), basal ES (e.g. N-cadherin/β-catenin, nectin-2/afadin, CAR/β-catenin), GJ (e.g. connexin43), and desmosome-like (e.g. desmoglein-2/desmocollin-2, connexin43/plakophilin-2) protein complexes at the BTB in the rat testis (Vogl *et al.*, 2000; 2008; Cheng and Mruk, 2002; Pointis *et al.*, 2005; Wang and Cheng, 2007; Yan *et al.*, 2007; 2008b; Mruk and Cheng, 2008a; Setchell, 2008; Wong *et al.*, 2008a) (Figure 2). Many of these are integral membrane proteins composed of one or several transmembrane, extracellular and intracellular domains (Figure 2). However, only intracellular domains bind to adaptor proteins, thereby recruiting other regulatory proteins (e.g. FAK, c-Src), as well as anchoring the multiprotein complex to cytoskeletal proteins (Figure 2). Thus, the interacting domains between apposing cell adhesion protein complexes residing on two adjacent Sertoli cells that constitute the BTB can be efficiently

disassembled and reassembled via changes in adhesive function, such as changes in the phosphorylation status of integral membrane proteins and/or their adaptors (Gumbiner, 2000; Zhang *et al.*, 2005), affecting the association between the adhesion protein complex and the cytoskeleton.

Interactions of regulatory proteins with structural protein complexes

The regulation of the BTB, particularly its restructuring which facilitates the transit of preleptotene spermatocytes at stage VIII-IX, has remained largely unknown since the initial discovery of the BTB more than a century ago (Mruk and Cheng, 2008a; Setchell, 2008). This is largely due to the lack of information on the regulatory proteins that are integrated components of the BTB, which ultimately regulate the tight and adhesive function of the BTB. The first report on the likely presence of a regulatory protein at the BTB appeared in 2003 when studies by immunohistochemistry and immunofluorescence microscopy illustrated the localization of focal adhesion kinase (FAK) at the BTB in adult rat testes (Siu *et al.*, 2003b). This observation was difficult for investigators to appreciate at the time because FAK, a non-receptor tyrosine kinase known to regulate diverse cellular functions, is most notably a regulatory protein kinase at the cell–matrix focal adhesion complex which is a downstream modulator of integrins (Boutros *et al.*, 2008). Subsequent studies by co-immunoprecipitation showed that FAK structurally interacted with occludin and ZO-1 (Siu *et al.*, 2009b; 2009c) and that both proteins localized to the BTB in the adult rat testis, as well as to the Sertoli cell TJ barrier *in vitro* which mimics the BTB *in vivo* (Siu *et al.*, 2009a). Since the expression and localization of FAK at the BTB was stage-specific, being lowest at stage VIII and coinciding with BTB restructuring (Siu *et al.*, 2009a), it was postulated that FAK may be involved in BTB dynamics via its effects on the occludin/ZO-1 complex. Indeed, when FAK was silenced by FAK-specific siRNA duplexes versus non-targeting (control) siRNA duplexes in Sertoli cells cultured *in vitro* with an established TJ permeability barrier that mimicked the BTB *in vivo* (Siu *et al.*, 2005), the knockdown of FAK led to a transient disruption of the Sertoli cell TJ barrier (Siu *et al.*, 2009b). This finding was confirmed when the distribution of occludin in Sertoli cells was examined following FAK RNAi since there was considerable redistribution of occludin from the Sertoli–Sertoli cell interface to the cell cytosol, thereby perturbing Sertoli cell TJ barrier function (Siu *et al.*, 2009b). Additionally, there was a loss of association between occludin and ZO-1 following knockdown of FAK during the Sertoli cell TJ-barrier disruption as shown by co-immunoprecipitation (Siu *et al.*, 2009b). These findings are significant because they illustrate for the first time that TJ integral

membrane proteins at the BTB are structurally associated with FAK, a regulatory protein normally found at the focal adhesion complex. Furthermore, it was noted that while the steady-state level of occludin in the Sertoli cell epithelium following FAK knockdown did not decrease, a significant decline in occludin phosphorylation at Tyr and Ser, but not Thr, residues was detected, illustrating that changes in occludin redistribution that led to a disruption of the Sertoli cell TJ barrier were mediated by changes in the phosphorylation status of occludin (Siu *et al.*, 2009b). These findings are also consistent with earlier observations in MDCK cells which demonstrated that occludin incorporated into TJ fibrils is phosphorylated (Sakakibara *et al.*, 1997; Tsukamoto and Nigam, 1999), whereas the small pool of occludin at the basolateral region of epithelial cells that are not assembled into TJ fibrils are not or less phosphorylated (Cordenonsi *et al.*, 1997; Sakakibara *et al.*, 1997). Certainly, additional regulatory protein kinases and phosphatases at the BTB will be identified, and this area of research will continue to be open for future investigation.

Drug transporters

The best studied drug transporters in the testis are P-glycoprotein and multidrug resistance protein 1 (MRP1). Both are important regulatory proteins of the ATP-binding cassette (ABC) superfamily that regulate drug transport across the BTB and into/out of the apical compartment of the seminiferous epithelium (for a review, Setchell et al., 2008). Both P-glycoprotein and MRP1 are efflux pumps that are present in multiple epithelial and endothelial cells (Couture *et al.*, 2006; Miller *et al.*, 2008). P-glycoprotein is the product of multidrug resistance 1 gene: Mdr 1a and 1b in rodents, and Mdr1 in humans, whereas MRP1 is a distant member of the ABC superfamily related to P-glycoprotein (Schaller *et al.*, 1995; Fromm, 2004). P-glycoprotein is found in Sertoli cells, peritubular myoid cells, late spermatids (Melaine *et al.*, 2002; Bart *et al.*, 2004) and spermatogonia (Trezise *et al.*, 1992). MRP1 is also localized to Sertoli cells in mouse and human (Flens *et al.*, 1996; Bart *et al.*, 2004). ABCG2 (breast cancer resistance protein, BCRP, an efflux pump) is another ABC transporter also found at the BTB (Robey *et al.*, 2009). Recent studies have shown that P-glycoprotein is an integrated component of the BTB since it structurally interacts with TJ proteins occludin, claudin-11 and JAM-A, but not with the basal ES protein N-cadherin as demonstrated by co-immunoprecipitation experiments (Su and Mruk, 2009) (Figure 2). Equally important, in rats treated with adjudin (50 mg kg^{-1} b.w., by gavage), a drug that was shown to induce germ cell depletion from the epithelium (Cheng *et al.*, 2005), there was a transient induction in P-glycoprotein and a significant increase in its association with occludin, claudin 11 and JAM-A (Su and Mruk, 2009). These findings illustrate that Sertoli cells which constitute the BTB, possess the ability

to pump drugs, such as adjudin, out of the seminiferous epithelium in order to protect post-meiotic germ cell development. This is likely mediated by its interaction with occludin and JAM-A to "seal" the BTB. Besides putative drug transporters, recent studies have also identified multiple organic cation transporters (OCT) and organic cation/carnitine transporters (OCTN) expressed by Sertoli cells, regulating cation transport across the BTB (Maeda *et al.*, 2007). This is an important area that requires additional research since drug transporters in Sertoli cells that constitute the BTB determine which and how much of a therapeutic compound or contraceptive enters the adluminal compartment.

Regulation of the BTB

Coordinated effects of cytokines and testosterone to maintain BTB integrity during transit of preleptotene/leptotene spermatocytes across the BTB

As discussed above, junction restructuring in different epithelia such as the seminiferous epithelium is regulated by changes in protein phosphorylation. However, unlike other blood–tissue barriers, the BTB cannot "open", even transiently at stages VIII–IX, to allow preleptotene/leptotene spermatocytes to cross the BTB, as this would expose post-meiotic germ cell antigens to the host's immune system. Based on earlier morphological studies (Siu *et al.*, 2005), it was postulated that during preleptotene spermatocyte transit, "new" TJ fibrils are first formed below the migrating spermatocyte followed by the dissolution of "old" TJ fibrils above the spermatocyte, thus permitting the migration of spermatocytes across the BTB without eliciting an immune response (Figure 3). Recent findings have provided the biochemical basis for such a mechanism to operate at the BTB. Cytokines such as TGF-β3 and TNFα are putative products of Sertoli and germ cells, and their receptors are also expressed by these cells even though mostly restricted to Sertoli cells (De *et al.*, 1993; Skinner, 1993; Le Maguersse-Battistoni *et al.*, 1995; Caussanel *et al.*, 1997). While these cytokines are known to regulate many other testicular functions (Lui *et al.*, 2003a; Loveland *et al.*, 2007), recent findings have shown that they also play a critical role in the disruption of BTB integrity, possibly at stage VIII of the epithelial cycle (Lui *et al.*, 2003b; 2003c; Siu *et al.*, 2003a; Li *et al.*, 2008). For instance, the integrity of the Sertoli cell TJ permeability barrier *in vitro* (Lui *et al.*, 2001; Siu *et al.*, 2003a) and *in vivo* (Li *et al.*, 2006; Xia *et al.*, 2006; 2009) could be reversibly perturbed following inclusion of recombinant TGF-β3 and/or TNFα to Sertoli cell cultures having an established TJ barrier *in vitro* or administered intratesticularly *in vivo*. These findings are also consistent with reports from other epithelia regarding the disruptive effects of cytokines on TJ barrier function
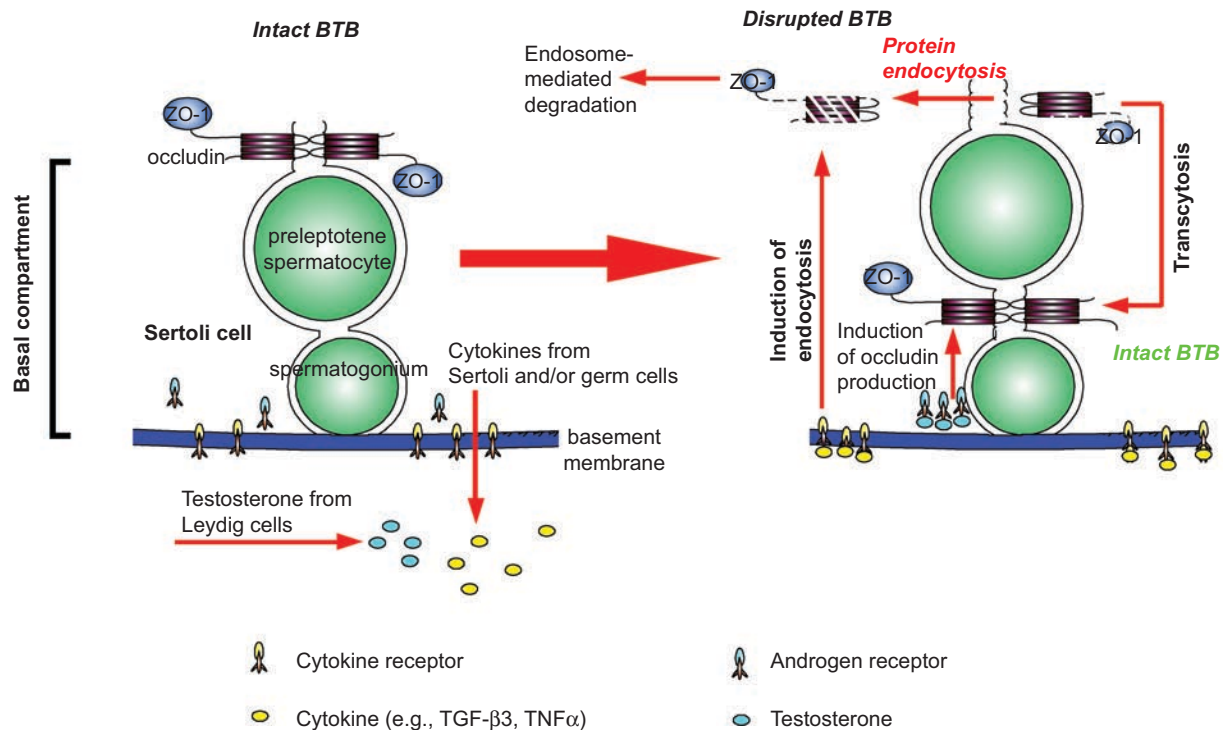
Figure 3. A hypothetical model illustrating the likely mechanism utilized by the testis to facilitate the transit of primary preleptotene spermatocytes at the BTB while maintaining the immunological barrier function. At the time of primary preleptotene spermatocytes in transit at the BTB, such as at stage VIII of the epithelial cycle, testosterone produced by Leydig cells in the interstitium enters the BTB microenvironment, which is known to promote BTB function, binds to androgen receptor to exert is effects or mediates its action via nongenomic pathway, such as by c-Src and ERK (Fix *et al.*, 2004; Cheng *et al.*, 2007) (see left panel). This promotes the production of BTB proteins (e.g. claudin-11, occludin) (Kaitu'u-Lino *et al.*, 2007; Yan *et al.*, 2008c) for the assembling of "new" TJ fibrils behind a migrating primary preleptotene spermatocyte (see right panel). Furthermore, testosterone also appears to promote transcytosis of internalized integral membrane proteins, perhaps from the apical region to the basal region of the migrating spermatocytes to facilitate "new" TJ fibril assembly (Yan *et al.*, 2008c). Cytokines (e.g. TGF-β3, TNFα) released from Sertoli and germ cells also bind to their corresponding receptors at the BTB to activate the downstream signaling function, which, in turn, induces protein endocytosis at the "old" BTB site above the migrating spermatocytes but targeted to degradation via the endosome-mediated pathway (Yan *et al.*, 2008c) (left panel). Thus, "new" TJ fibrils behind the migrating spermatocytes continue to maintain the immunological barrier while the "old" TJ fibrils above the spermatocytes in transit are disrupted to facilitate cell movement. This thus provides a novel mechanism to maintain the immunological barrier during the transit of primary preleptotene spermatocytes across the BTB.

(Walsh *et al.*, 2000). On the other hand, testosterone was shown to promote BTB integrity in mice *in vivo* (Meng *et al.*, 2005; Wang *et al.*, 2006), as well as enhance the Sertoli cell TJ barrier *in vitro* (Janecki *et al.*, 1992; Chung and Cheng, 2001). Using biotinylation in conjunction with endocytosis assays to assess the kinetics of protein internalization (Le *et al.*, 1999) and dual-labeled immunofluorescence analysis to assess changes in protein distribution in Sertoli cells having an assembled TJ permeability barrier, it was shown that TGF-β2/-β3, TNFα and testosterone enhanced clathrin-mediated endocytosis of integral membrane proteins at the BTB (e.g. occludin, N-cadherin) (Yan *et al.*, 2008c; Xia *et al.*, 2009). Interestingly, TGF-β2 promoted endosome-mediated *degradation* of endocytosed proteins from the BTB, but testosterone promoted endosome-mediated *recycling* of endocytosed proteins (Yan *et al.*, 2008c). These findings are physiologically significant because they illustrate for the first time that cytokines and

testosterone, whose effects on BTB integrity are opposite, may be working synergistically to accelerate protein endocytosis and degradation (i.e. cytokines) above a migrating preleptotene spermatocyte, while promoting protein endocytosis (i.e. testosterone) below this primary spermatocyte so as to assemble "new" TJ fibrils (Figure 3). This provides an efficient mechanism to regulate the movement of preleptotene spermatocytes across the BTB without compromising the immunological barrier. Nonetheless, additional work is needed to expand the regulatory role of testosterone on protein transcytosis at the BTB. For instance, which molecules mediate androgen-induced transcytosis? How is cross talk between cytokines and testosterone mediated in light of the fact that these biomolecules exert different effects on protein endocytosis, recycling, and/or transcytosis? Finally, what are the key proteins that take part in protein recycling, transcytosis, and endosome- or ubiquitin-mediated protein degradation?

Mitogen-activated protein kinases (MAPK)

In studies using cytokines to perturb Sertoli cell TJ barrier function both *in vitro* and *in vivo*, an activation of selected members of the MAPK family was shown to be associated with these disruptive effects. For instance, TGF-β3 was shown to activate p38 MAPK to impair the Sertoli cell TJ permeability barrier *in vitro* (Lui *et al.*, 2003b). This cytokine-mediated activation of MAPK appears to be specific since the use of MAPK inhibitors could delay and partially block the adverse effects of TGF-β3 on BTB integrity *in vivo* (Lui *et al.*, 2003c; Xia *et al.*, 2006) and *in vitro* (Lui *et al.*, 2003b). These findings reveal that MAPK activation may be associated with the transcriptional regulation of other genes that regulate BTB integrity such as proteases which may facilitate endosome-mediated degradation of integral membrane proteins at the BTB. This may also lead to the activation and/or production of critical proteins needed for endocytosis (Doherty and McMahon, 2009; Galletta and Cooper, 2009), endosome- or ubiquitin-mediated degradation and/or recycling of endocytosed proteins (Chen and Sun, 2009; Mittal and McMahon, 2009; Sorkin and Goh, 2009) and transcytosis (Tuma and Hubbard, 2003). Furthermore, JNK (c-Jun N-terminal protein kinase), a member of the MAPK family, was shown to regulate the production of α_2-macroglobulin, a non-specific protease inhibitor, to limit proteolysis during seminiferous epithelial restructuring in the cadmium-induced testicular injury model (Wong *et al.*, 2005). Collectively, these findings illustrate the involvement of MAPK in cytokine-mediated disruptive effects on the Sertoli cell BTB function.

In this context, it is of interest to note that at the time of preleptotene/leptotene spermatocyte transit across the BTB, these cells differentiate into zygotene spermatocytes by undergoing chromatin condensation to prepare for metaphase I. It is known that MAPK also plays an essential role in regulating germ cell cycle progression event. For instance, treatment of pachytene spermatocyte cultures with okadaic acid, a Ser/Thr phosphatase inhibitor, was shown to prematurely induce metaphase I by accelerating chromatin condensation. This was accompanied by an increase in intrinsic MAPK activity (Wiltshire *et al.*, 1995; Cobb *et al.*, 1999). This okadaic acid-induced chromosomal condensation could be abolished by preincubating pachytene spermatocytes with an MEK1/2 inhibitor, PD98005 (Di Agostino *et al.*, 2002) or U0126 (Petronczki *et al.*, 2003). These findings also illustrate the involvement of MAPK in germ cell cycle events such as preparation of the chromatin for diakinesis while these cells are migrating across the BTB. This prompts us to speculate whether MAPK regulation of junction restructuring between Sertoli cells at the BTB also plays a role in germ cell cycle progression at stage VIII of the epithelial cycle. It is worth noting that germ cells such as spermatocytes and spermatids develop as a clone, that is, they are connected by intercellular bridges to synchronize differentiation (Fawcett *et al.*, 1959; Dym and Fawcett, 1971; Ren and Russell, 1991). Other studies have shown that spermatogonia undergo apoptosis also in clones following exposure to irradiation (Huckins, 1978a; 1978b), illustrating that factor(s) which regulate programmed cell death can diffuse through the intercellular bridges to coordinate apoptosis among developing germ cells within a clone. Intercellular bridges that connect preleptotene spermatocytes during transit at the BTB were also detected in adult rat testes by electron microscopy (Mruk and Cheng, 2008b). While the biology of intercellular bridges is not known, recent studies have shown that TEX14 (Greenbaum *et al.*, 2006; Greenbaum *et al.*, 2009) and δ-tubulin (Kato *et al.*, 2004) are crucial components of intercellular bridges. Furthermore, Sertoli–Sertoli and Sertoli–germ cells are also connected by gap junctions (Pointis and Segretain, 2005) and desmosome-like junctions (also known as desmosome-gap junctions) (Ren and Russell, 1992), both of which are used for signaling and communication to coordinate different events pertinent to spermatogenesis, such as germ cell cycle progression, meiosis, BTB restructuring to facilitate spermatocytes in transit, and others. Thus, it is likely that the "signal(s)" that regulate germ cell cycle progression, such as DNA condensation in preparation of the subsequent diakinesis, can also pass between primary spermatocytes in clones via the intercellular bridges to coordinate this event; signals can also pass between Sertoli cells *and* between Sertoli cells and germ cells to coordinate BTB restructuring and germ cell cycle progression simultaneously, such that these events can be mediated by MAPK. This possibility should be vigorously evaluated in future studies.

Polarity proteins

Two polarity protein complexes are found in adult rat testes. These are (i) the CRB (Crumbs) complex: CRB3/Pals1/PATJ and (ii) the Par (partitioning-defective) complex: Par3/Par6/aPKC (Wong *et al.*, 2008a). Initially identified in *Caenorhabditis elegans* and *Drosophila melanogaster* as proteins which confer cell polarity during development, their homologs were subsequently found in mammalian cells, mostly restricted to TJs (Shin *et al.*, 2006; Suzuki and Ohno, 2006). It was shown that Par6 localized mostly to the BTB and apical ES in the seminiferous epithelium of adult rat testes (Wong *et al.*, 2008b). The apical ES is the only anchoring device present between Sertoli cells and elongating/elongated spermatids (i.e., steps 8–19 in the rat) (Wong *et al.*, 2008a), and the TJ is not found between Sertoli and germ cells. However, developing spermatids must be properly oriented within the seminiferous epithelium,

that is, their heads must point towards the basement membrane in order to maximize the number of elongated spermatids that are packed into the limited space of the seminiferous epithelium. Thus, the apical ES was proposed to function in spermatid polarity since its identification in the 1970s (Russell and Clermont, 1976; Russell, 1977b; Mruk and Cheng, 2004b; Vogl *et al.*, 2008; Wong *et al.*, 2008a). However, the proteins at the apical ES that function in spermatid polarity were not known until recently. Studies by immunohistochemistry, single-/dual-labeled immunofluorescence analysis and co-immunoprecipitation have all demonstrated unequivocally that several polarity proteins such as Par3 and Par6 are integral components of the apical *and* basal ES at the BTB (Wong *et al.*, 2008b). Equally important, Par6 expression at the apical ES diminished when the polarity of elongated spermatids was lost, followed by their premature depletion from the seminiferous epithelium during adjudin-induced germ cell loss from the testis (Wong *et al.*, 2008b). In addition, the Par6/Pals1 complex was found to structurally interact with JAM-C, an integral membrane protein at the apical ES, in the normal testis, but during adjudin-induced apical ES disruption, Par6 interacted considerably more with c-Src (Wong *et al.*, 2008b). These findings are significant because they illustrate that the Par6/Pals1 complex can shift its association with either JAM-C or Src, and this contributes to an intact or disrupted apical ES, respectively. This is also an efficient mechanism to regulate adhesion and de-adhesion during movement of developing spermatids across the adluminal compartment during spermiogenesis.

These findings prompted us to examine if the Par6 polarity protein complex also regulates cell adhesion at the Sertoli–Sertoli cell interface at the BTB during the transit of preleptotene/leptotene spermatocytes at stage VIII of the epithelial cycle. When Sertoli cells were cultured *in vitro* with an established TJ barrier that functionally and structurally mimicked the BTB *in vivo* (except that the apical ES was lacking since elongating/ elongated spermatids were absent from this *in vitro* system), the knockdown of Par3 or Par6 by RNAi was shown to cause redistribution of JAM-A from the cell surface (i.e., it became cytosolic-bound), thereby disrupting cell adhesion (Wong *et al.*, 2008b). Furthermore, the silencing of Par 6, but not Par3, also induced redistribution of N-cadherin from the Sertoli cell surface and into the cytosol (Wong *et al.*, 2008b). These findings suggest that Par proteins may regulate cell adhesion via their effects on protein endocytosis. Indeed, knockdown Par5 (also known as 14-3-3) by RNAi also led to the redistribution of N-cadherin, moving away from the cell surface and into cytosol (Wong *et al.*, 2009), analogous to results from Par6 knockdown experiments (Wong *et al.*, 2008b). This was accompanied by increased endocytosis of

biotinylated N-cadherin and JAM-A (Wong *et al.*, 2009). Taken collectively, Par polarity proteins at the BTB, besides conferring cell polarity, also regulate cell adhesion between Sertoli cells, and this is similar to their function at the apical ES.

A local regulatory mechanism at the BTB

In most blood–tissue barriers, except the BTB, barrier function is conferred by the TJ, which is functionally and structurally supported by the adherens junction (AJ) and desmosome junction. These three segregated entities are collectively referred to as the junctional complex. The gap junction lies behind the junctional complex (Alberts *et al.*, 2002). In contrast, the BTB is composed of co-existing TJ, basal ES, desmosome-like junction and gap junction (Cheng and Mruk, 2002; Mruk and Cheng, 2008a; Setchell, 2008; Vogl *et al.*, 2008). While these observations were reported almost four decades ago, the physiological significance of this anatomical layout of cell junctions in the seminiferous epithelium has remained unknown until recently. It has been speculated that gap and desmosome-like (also known as desmosome-gap junctions because they have ultrastructural features of both desmosome and gap junctions; see Ren and Russell, 1992; Russell, 1977a) junctions at the BTB may function in relaying signals between Sertoli cells for junction restructuring to facilitate transit of preleptotene spermatocytes at stage VIII of the epithelial cycle (Mruk and Cheng, 2004b), but the molecular and biochemical mechanism(s) remain unknown. Recent studies using Sertoli cells cultured *in vitro* with an established TJ-permeability barrier and a "loss-of-function" approach by RNAi, however, have shed new insights on the physiological significance of coexisting junctions at the BTB. For instance, silencing of desmosome-gap junction proteins using specific siRNA duplexes has demonstrated the significance of desmosome-like junctions in the regulation of BTB dynamics. While knockdown of desmoglein-2 (an integral membrane protein of desmosomes) alone by RNAi did not interfere with Sertoli–Sertoli cell adhesive function at the BTB, silencing of both desmoglein-2 and desmocollin-2 (another integral membrane protein of desmosomes) induced redistribution and internalization of CAR and ZO-1, but not occludin and N-cadherin (Lie *et al.*, 2009a). Moreover, silencing of both desmoglein-2 and desmocollin-2 affected TJ barrier function as manifested by a significant decline in transepithelial electrical resistance (TER) across the Sertoli cell epithelium (Lie *et al.*, 2009a). Studies using protein endocytosis assays demonstrated that loss of CAR from the cell surface was indeed mediated by an increase in its endocytosis (Lie *et al.*, 2009a). Likewise, silencing of connexin 43 (an integral membrane protein at the desmosome-gap junction) alone did not affect

Sertoli cell TJ barrier function nor the localization of TJ and/or basal ES proteins (Li *et al.*, 2009). However, simultaneous knockdown of Cx43 and plakophilin-2 (an adaptor at the desmosome-like junction) in Sertoli cells *in vitro* by specific siRNA duplexes perturbed the TJ barrier, concomitant with a redistribution of occludin and ZO-1. Both proteins were internalized, resulting in a disruption of BTB integrity (Li *et al.*, 2009). These findings not only reveal the co-existence of cell junctions at the BTB; they also demonstrate that specific junction types, such as desmosome-like and gap junction at the BTB, function as signaling platforms so that restructuring events can be tightly coordinated. This would avoid an unnecessary "opening" of the BTB during primary spermatocyte transit across the BTB (Yan *et al.*, 2008a), and this would maintain the immunological barrier. As such, the unique morphological layout of co-existing junctions in the seminiferous epithelium has an important physiological function, that is, to maintain BTB integrity during spermatogenesis.

The apical ES–BTB–basement membrane local regulatory axis

Apical ES and laminin chains

Ectoplasmic specialization (ES)

The ES, a testis-specific cell–cell actin-based anchoring junction, was first described in the seminiferous epithelium of rodent testes in the 1970s (Russell and Clermont, 1976; Russell, 1977b; Russell and Peterson, 1985; Vogl *et al.*, 1991; 2000). It is restricted either to the Sertoli cell-elongating/elongated spermatid (i.e., step 8–19 spermatids) interface known as the apical ES appears, or to the Sertoli–Sertoli cell interface at the BTB known as the basal ES (Toyama *et al.*, 2003; Mruk and Cheng,
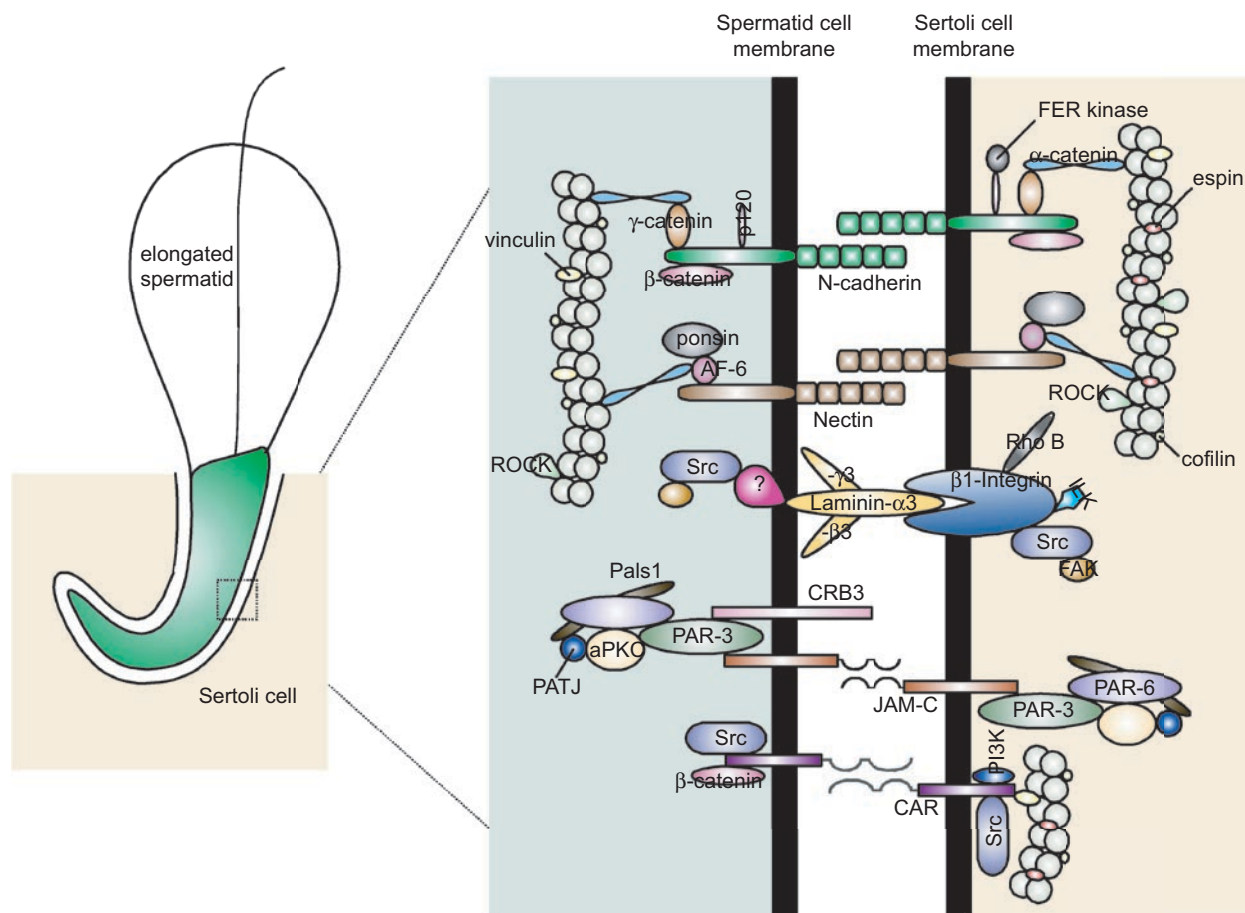

Figure 4. Protein complexes and their peripheral regulators and adaptors that constitute the apical ES in the rat testis. The presently known integral membrane proteins at the apical ES in adult rat testes are: cadherins, nectins, integrins, laminins/receptor protein(s) (note: laminins, such as α3, β3 and γ3, are cell surface proteins without any transmembrane domain, residing on elongating/elongated spermatids that anchor to spermatids at the apical ES via a yet-to-be identified laminin receptor protein), CRB3, JAM-C, and CAR. Each of these protein binds to its corresponding adaptors (e.g. catenins, afadins), polarity proteins (e.g. Par, Pals1), and protein kinases (e.g. c-Src, FAK), using actin for its attachment. Apical ES is an atypical adherens junction (AJ) type (Wong *et al.*, 2008a) since it is composed of proteins usually restricted to cell–matrix focal adhesion complex (or focal contact) (e.g. c-Src, FAK), and TJ (e.g. JAM-C, CAR).

2004a; 2004b; 2008a; Vogl *et al.*, 2008). Once the apical ES appears, whose function is to anchor and properly orientate spermatids to Sertoli cells in the seminiferous epithelium (Figures 1, 4), it is the only anchoring device present at this site (Russell and Clermont, 1976; Russell and Peterson, 1985; Vogl *et al.*, 1991; 1993). An *in vitro* study which quantified the physical force that can disrupt the apical ES versus the desmosome-like junction demonstrated that the apical ES has the strongest adhesive force (Wolski *et al.*, 2005). Ultrastructurally, the apical ES is typified by the presence of actin-filament bundles sandwiched between cisternae of endoplasmic reticulum and the Sertoli cell plasma membrane, and it is restricted only to Sertoli cells without any identifiable ultrastructures present on spermatids. However, several studies have shown that elongating spermatids express proteins that are putative components of the apical ES such as laminins (Koch *et al.*, 1999; Yan and Cheng, 2006), cadherins (Wu *et al.*, 1993; Lee *et al.*, 2003), Par3, Par6 (Wong *et al.*, 2008b), 14-3-3 (Par5) (Wong *et al.*, 2009) and others (Siu and Cheng, 2004b; Yan *et al.*, 2007) (Figure 4). Unlike the apical ES, the basal ES *co-exists* with TJ, gap junctions (GJ) and desmosome-like junctions (Vogl *et al.*, 2000; Mruk and Cheng, 2008a; Vogl 2008) to constitute the BTB (Figures 1C, 2).

Apical ES

The apical ES is known to date to be constituted by the cadherin/catenin (Johnson and Boekelheide, 2002; Lee *et al.*, 2003; Lee *et al.*, 2004), nectin/afadin (Ozaki-Kuroda *et al.*, 2002), and integrin/laminin (Palombi *et al.*, 1992; Salanova *et al.*, 1995; Salanova *et al.*, 1998; Koch *et al.*, 1999; Mulholland *et al.*, 2001) protein complexes (Figure 4). Of these, the cadherin–protein complex is detected at the apical ES at some stages of the epithelial cycle, such as stage V–VII (Johnson and Boekelheide, 2002; Lee *et al.*, 2004). One of the most studied protein complexes at the apical ES is the $\alpha6\beta1$-integrin/laminin-333 (Figure 4) (Salanova *et al.*, 1995; 1998; Mulholland *et al.*, 2001; Siu and Cheng, 2004c; Yan and Cheng, 2006). The apical ES differs from other epithelial AJ in many aspects. For instance, apical ES is composed of many proteins that are usually restricted to the anchoring junction at the cell–extracellular matrix (ECM) interface known as the focal contact or focal adhesion complex (e.g. integrins, laminins, FAK, Src, vinculin) which facilitates cell movement (Vogl *et al.*, 2000; Siu and Cheng, 2004b; Yan *et al.*, 2007). Furthermore, proteins restricted to gap junctions (e.g. connexin-43) and TJ (e.g. CAR, JAM-C) (Siu and Cheng, 2004b; Wang and Cheng, 2007; Yan *et al.*, 2007) are known to be integrated components of the apical ES. Thus, the apical ES has been referred to as an *atypical* AJ type (Wong *et al.*, 2008a).

Laminin-333

Laminins are heterotrimers comprised of an α, β and γ chain to form a functional protein, which serves as the ligand of integrins, and they are usually restricted to the ECM where they serve as ligands for integrins to confer cell adhesion at the focal contact (Alberts *et al.*, 2002; Cheng and Mruk, 2002). To date, five α, four β, and three γ laminin chains have been reported in mammalian tissues, and different combinations of these laminin chains give rise to at least 17 functional laminin proteins (Aumailley *et al.*, 2005; Hallmann *et al.*, 2005). Laminin $\gamma3$ was the first non-basement membrane laminin to be detected at the apical ES in mouse testes (Koch *et al.*, 1999), forming a functional adhesive complex with $\beta1$-integrin at the apical ES (Siu and Cheng, 2004c). Subsequent studies have demonstrated that laminin $\gamma3$ forms a functional complex with $\alpha3$ and $\beta3$ chains, and it was designated laminin-333 (Yan and Cheng, 2006). Since laminin-333 lacks a transmembrane domain, it is restricted to the cell surface of elongating/elongated spermatids, whereas $\alpha6\beta1$-integrin is limited to Sertoli cells at the apical ES, and these two proteins form a bona fide complex that confers spermatid adhesion (Yan and Cheng, 2006). In focal contacts, the laminin–integrin complex can be remodeled by laminin proteolysis via metalloproteases (MMPs) (Giannelli *et al.*, 1997; Udayakumar *et al.*, 2003; Carragher and Frame, 2004). Interestingly, MMP-2 and MT1-MMP [membrane type 1- MMP is a membrane-anchored metalloprotease which forms a functional protein complex with tissue inhibitor of metalloproteases-2 (TIMP-2) to serve as a receptor for pro-MMP-2 and for its activation to MMP-2; see Werb, 1997; McCawley and Matrisian, 2001; Sternlicht and Werb, 2001], which are known to cleave laminins and release biologically-active fragments to affect cell migration and TJ barrier function (Giannelli *et al.*, 1997; Udayakumar *et al.*, 2003; Gurney *et al.*, 2006), were found to co-localize with laminin $\gamma3$, $\beta1$ integrin and TIMP-2 at the apical ES (Longin *et al.*, 2001; Siu and Cheng, 2004c). Equally important, studies by immunohistochemistry and/or dual-labeled immunofluorescence analysis have illustrated that the expression of MMP-2, MT1-MMP and TIMP-2 was stage-specific, being highest at early stage VIII prior to spermiation, and co-localizing with $\beta1$-integrin at the apical ES (Siu and Cheng, 2004c). These findings illustrate that the MMP-2/MT1-MMP/TIMP-2 complex may be used to cleave the laminin-333/$\alpha6\beta1$-integrin adhesion complex at the apical ES at spermiation, generating biologically-active fragments of laminin chains to regulate BTB restructuring and to facilitate preleptotene spermatocyte transit across the BTB. This is consistent with recent findings regarding the action of laminin fragments derived from laminin-5 on blood-brain barrier function and cell migration (Giannelli *et al.*, 1997;

Udayakumar *et al.*, 2003; Gurney *et al.*, 2006). These findings also imply that biologically active laminin fragments generated at the apical ES during spermiation coordinate the events of spermiation and BTB restructuring at stage VIII of the epithelial cycle.

Basement membrane and hemidesmosome

In adult rat testes, the basement membrane is a modified form of ECM (Dym, 1994), which together with the underlying collagen (type I) layer constitutes the noncellular zone of the tunica propria, and supports the seminiferous epithelium composed of Sertoli and germ cells (Figure 1). The basement membrane in mammalian testes is composed mostly of type IV collagen, laminin, entactin and heparan sulfate proteoglycans (Dym, 1994; Dobashi *et al.*, 2003; Siu and Cheng, 2004a; Harvey *et al.*, 2006). The hemidesmosome, an intermediate filament-based anchoring junction type restricted to the cell–matrix interface, provides adhesion between epithelial cells and the ECM via interactions between integrins (which function as receptors on the cell surface) and laminins or collagens (which function as ligands in the ECM). The presence of hemidesmosomes on the basal surface of Sertoli cells was first reported by Connell (1977), and hemidesmosomes are observed at the Sertoli cell-basement membrane interface by electron microscopy (Mruk and Cheng, 2004b; Siu and Cheng, 2004b). However, neither its composition nor function in the testis pertinent to spermatogenesis is presently known. Besides its presence at the apical ES, $\beta 1$-integrin was also shown to be a component of the hemidesmosome, and we have recently co-localized $\beta 1$-integrin with vimentin (an intermediate filament protein) and laminin $\alpha 2$ (a hemidesmosome protein) at the hemidesmosome in adult rat testes (Yan *et al.*, 2008d). However, the role of the hemidesmosome and basement membrane in regulating spermatogenesis and BTB dynamics remains largely unknown, except that earlier studies have shown that antibodies against the basement membrane or its components (e.g. laminins) caused extensive damage to the seminiferous epithelium which mimicked orchitis (Lustig *et al.*, 1978; 2000 Denduchis *et al.*, 1985).

An apical ES–BTB–hemidesmosome regulatory axis to coordinate the events of spermiation and BTB restructuring at stage VIII of the seminiferous epithelial cycle

Recent studies using purified recombinant proteins prepared against different domains of the laminin $\gamma 3$ or $\beta 3$ chains were shown to transiently perturb the Sertoli cell TJ permeability barrier by reducing the steady-state level of occludin at the Sertoli cell BTB (Yan *et al.*, 2008d). These observations are consistent with findings that fragments of laminin chains released at focal contacts can serve as biologically-active peptides to regulate cell adhesion and TJ barrier function in different epithelia and endothelia (for a review, see Yan *et al.*, 2008b). Furthermore, overexpression of laminin $\gamma 3$ domain IV in Sertoli cells following the establishment of a TJ barrier was shown to perturb barrier integrity associated with a significant decline of occludin and $\beta 1$-integrin, and an activation of ERK1/2 (Yan *et al.*, 2008d). Collectively these findings illustrate that biologically-active laminin fragments can *directly* regulate BTB dynamics (Figure 5), and this is possibly mediated via the ERK MAPK pathway, consistent with earlier findings which demonstrated the role of ERK in regulating BTB dynamics (Xia *et al.*, 2006). Since these Sertoli cell cultures were devoid of any elongating spermatids and no apical ES was present in this *in vitro* system, the declining $\beta 1$-integrin steady-state level appears to be the result of hemidesmosome disruption since both $\beta 1$-integrin and laminin $\alpha 2$ were found to co-localize to the hemidesmosome in rat testes (Yan *et al.*, 2008d). These findings suggest that laminin fragments can also regulate BTB dynamics *indirectly* via their action on hemidesmosome (Figure 5). To verify this possibility, $\beta 1$-integrin was silenced by RNAi in apical ES-absent Sertoli cell cultures having an established TJ permeability barrier. The knockdown of $\beta 1$-integrin led to a redistribution of occludin from the Sertoli cell surface and into the cell cytosol, interacting more with endosome-mediated endocytic vesicles as manifested by an enhanced association with EEA-1 (early endosome antigen-1, an endosome marker) (Yan *et al.*, 2008d). Collectively, these findings demonstrate the presence of a local autocrine-based regulatory axis that coordinates the events of spermiation and BTB restructuring at stage VIII of the epithelial cycle and designated the "apical ES–BTB–hemidesmosome" functional axis (Figure 5).

Collagens and biologically active fragments from collagen chains: a local BTB-basement membrane regulatory axis

In mammalian cells and tissues, four types of collagen, namely types I, II, III and IV, are found. Types I, II and III collagen are non-basement membrane collagens that confer tensile strength to tissues (Timpl and Brown, 1996; Siu and Cheng, 2004a). In the testis, type I collagen forms a collagen layer located immediately behind the basement membrane (Figure 1C) (Dym, 1994). It confers tensile strength to the seminiferous tubules. Type IV collagen and laminins are the major building blocks of the basement membrane in adult mammalian testes (Tung and Fritz, 1993; Dym, 1994; Enders *et al.*, 1995; Timpl and Brown, 1996; Alberts *et al.*, 2002; Siu and Cheng, 2004a). In the mammalian testis, collagens are products of Sertoli, germ and myoid cells (Skinner
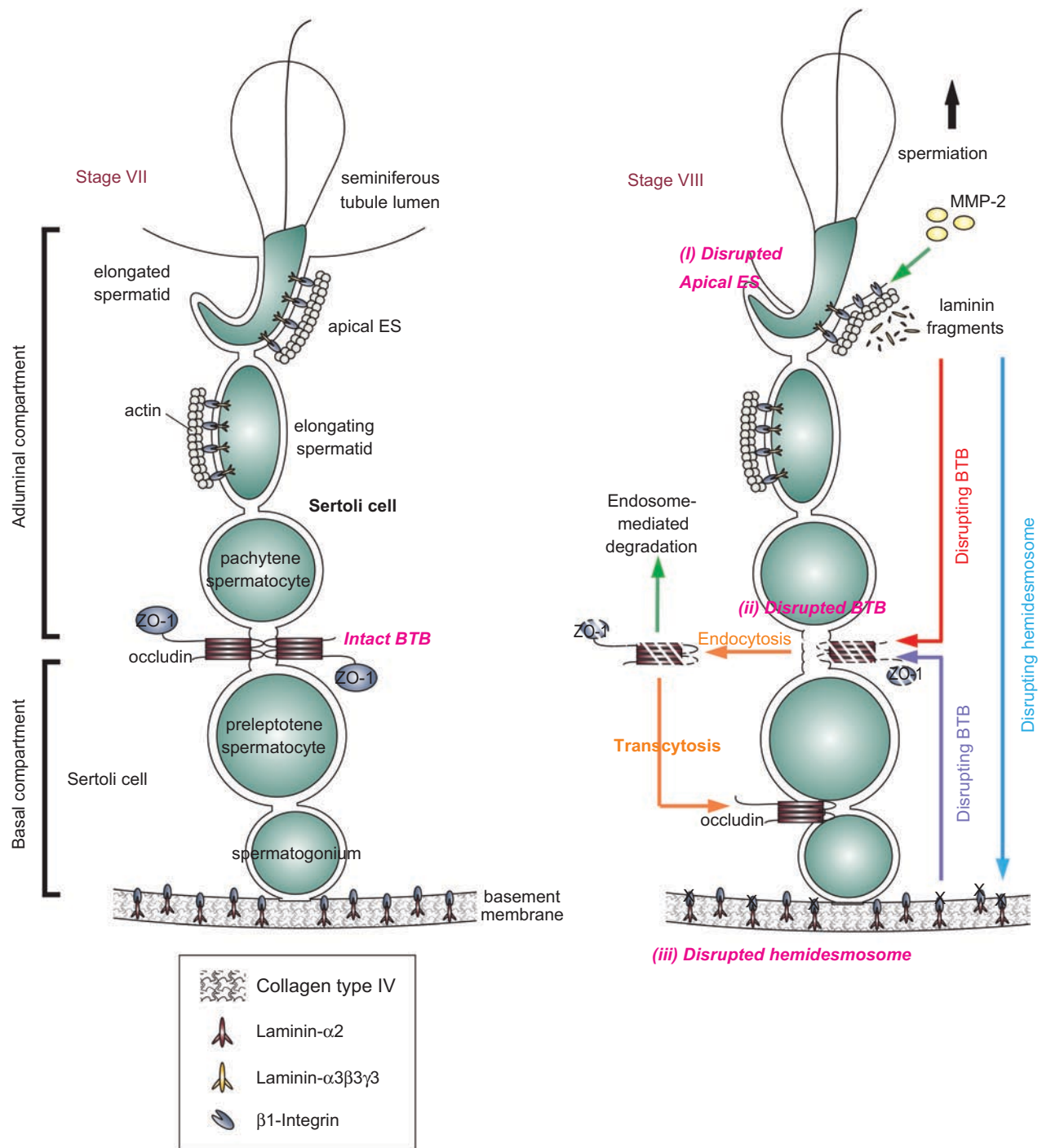
Figure 5. A schematic drawing illustrating the presence of a local regulatory loop known as the apical ES-BTB-hemidesmosome in the seminiferous epithelium to coordinate the events of spermiation and BTB restructuring that facilitate the transit of primary preleptotene spermatocytes at stage VIII of the seminiferous epithelial cycle. The left panel depicts the seminiferous epithelium of a normal rat testis at stage VII of the epithelial cycle. As described in the text, at the time of spermiation (see right panel), MMP-2 cleaves laminin-333 to release biologically active fragments at the time of apical ES disruption (step i), which can either destabilize BTB (step ii) *directly* by reducing the steady-state level of integral membrane proteins at the site via protein endocytosis and endosome-mediated degradation, or *indirectly* by perturbing the hemidesmosome function by reducing the steady-state level of β1-integrin. The disruption of the hemidesmosome (step iii), in turn, perturbs the BTB integrity via a mechanism that remains to be investigated. However, it likely involves FAK and/or cSrc. The endocytosed proteins may also be re-used to assemble "new" TJ fibrils behind the primary spermatocyte in transit via transcytosis. Using such a mechanism, the events of spermiation and BTB restructuring that take place at the opposite ends of the seminiferous epithelium can be coordinated at stage VIII of the epithelial cycle.

et al., 1985; Davis *et al.*, 1990; Richardson *et al.*, 1995; Siu *et al.*, 2003a). Collagens are also known to facilitate the retention of cytokines (e.g. TNFα and TGF-β3) in the ECM (O'Connor-McCourt and Wakefield, 1987; Huang *et al.*, 1988; Wong *et al.*, 2004), maintaining a pool of cytokines to affect epithelial cell function such as BTB dynamics in the testis and other epithelia (Walsh *et al.*, 2000; Al-Sadi *et al.*, 2009). Structurally, collagens (e.g. Type IV) possess a triple helical structure, consisting of three α chains. This triple helical molecule is also the building block of the collagen network known as the monomer (Siu and Cheng, 2004a). There are six collagen α chains, designated α1 to α6 (Timpl *et al.*, 1989; Hudson *et al.*, 1993). In rat and bovine testes, the basement membrane is composed mostly of collagen α3(IV) and α4(IV) chains instead of the classical α1(IV) and α2(V) chains in non-gonadal basement membranes (Enders *et al.*, 1995; Kahsai *et al.*, 1997; Frojdman *et al.*, 1998). Each collagen monomer is composed of a 7S noncollagenous domain composed of ~15 amino acid residues from the N-terminus, a middle collagenous domain having about 1400 residues of G-X-X repeats (G, glycine) and a carboxyl terminal noncollagenous (NC1) domain about ~230 residues from the C-terminus. It is of interest to note that peptides synthesized based on the NC1 domain of collagens were shown to be biologically active peptides, affecting junction dynamics in different epithelia (Tsilibary *et al.*, 1988; Davis *et al.*, 1990; Zhang *et al.*, 1994; Vogel *et al.*, 1997; Carragher *et al.*, 1999). For instance, the NC1 domain of the collagen α3(IV) chain was shown to inhibit angiogenesis, and suppress tumor growth (for reviews, see Siu and Cheng, 2004b; Hamano and Kalluri, 2005). Collagen fragments were also shown to disrupt cell adhesion via their effects on FAK, paxillin and talin at the focal adhesion complex (Carragher *et al.*, 1999), likely mediated by an activation of integrins. For instance, α3β1-integrin is one of the putative receptors for the NC1 domain (Borza *et al.*, 2006). Since MMP9, a matrix metalloprotease and product of Sertoli cells, has been localized to the basal compartment consistent with its localization at the BTB (Siu *et al.*, 2003a), and collagens are known to be putative substrates of MMPs (Siu and Cheng, 2004a; 2004b), biologically active collagen fragments can indeed be generated *in vivo* to modulate TJ barrier function. This possibility must be carefully evaluated in future studies.

TNFα, a cytokine found in the basement membrane, is a product of germ cells (e.g. round and elongating spermatids, and spermatocytes), macrophages, and Sertoli cells (De *et al.*, 1993; Skinner, 1993; Siu *et al.*, 2003a; Guazzone *et al.*, 2009). Two structurally related, but functionally distinct TNFα receptors, p55 and p75, are found in epithelial cells (Tartaglia and Goeddel, 1992), including Sertoli cells (De Cesaris *et al.*, 1999; Li *et al.*, 2006), that mediate the action of TNFα in the testis

(Guazzone *et al.*, 2009). A recent study has shown that TNFα was capable of perturbing Sertoli cell TJ permeability barrier function dose-dependently (Siu *et al.*, 2003a), illustrating that a putative component of the basement membrane can affect the BTB integrity. Additionally, TNFα stimulated the production and activation of MMP-9 by Sertoli cells. It also stimulated the production of collagen α3(IV) chain and TIMP-1 (tissue inhibitor of metalloproteases I) dose-dependently by Sertoli cells *in vitro* (Siu *et al.*, 2003a). Collectively, these findings suggest that activated MMP-9 induced by TNFα is used to cleave the existing collagen network in the basement membrane, perturbing the Sertoli cell-TJ barrier. This, in turn, creates a negative feedback that causes TNFα to induce collagen α3(IV) and TIMP-1 production in order to replenish the collagen network in the disrupted TJ barrier and to limit further unwanted proteolytic activity of MMP-9. This postulate is supported by a recent report illustrating that TNFα-mediated disruption of spermatogenesis as a result of Sertoli cell injury is mediated, at least in part, by MMP2 (Yao *et al.*, 2009). These findings also illustrate the presence of a local regulatory loop between the BTB and the basement membrane. Taking these findings together, we propose the presence of a local regulatory loop in the seminiferous epithelium known as the "apical ES–BTB–hemidesmosome/basement membrane" functional axis, which coordinates the events of spermiation and BTB restructuring at stage VIII of the seminiferous epithelial cycle of spermatogenesis. This axis also provides an effective mechanism to regulate the rapid restructuring of the BTB in response to changes in the microenvironment of the seminiferous epithelium during spermatogenesis.

Conclusion and future perspectives

There have been unprecedented advances in technology in the fields of cell biology, biochemistry, and molecular biology in the last two decades, many of which have been adopted by investigators in the field of male reproductive biology, including our laboratory, to expand understanding of testicular biology. These include: (i) the establishment of an *in vitro* culture system to study Sertoli cell TJ permeability barrier function that mimics the BTB *in vivo* (Byers *et al.*, 1986; Janecki and Steinberger, 1986; Janecki *et al.*, 1992); (ii) the use of specific siRNA duplexes to knockdown specific target genes in this system (Yan *et al.*, 2008d; Wong *et al.*, 2008b; 2009; Li *et al.*, 2009; Lie *et al.*, 2009a; Siu *et al.*, 2009b); (iii) the use of dual-labeled immunofluorescence analysis (Lie *et al.*, 2009b; Siu *et al.*, 2009c) and confocal microscopy (Xia *et al.*, 2009); and (iv) biochemical techniques to track the "fate" of integral membrane proteins via Sertoli cell surface protein biotinylation, endocytosis and recycling assays (Yan *et al.*,

2008c). Thus, many unresolved questions relating to the biology and regulation of spermatogenesis in the testis have begun to be tackled in recent years. Herein, we have attempted to provide a unified biochemical framework, based on recently published findings in the field on the regulation of BTB restructuring, which facilitates the transit of preleptotene spermatocytes, and how this event can be coordinated with spermiation (see Figure 5). It is understood that this model will continue to be updated and modified in the future by us and others. Nevertheless, this biochemical model does address several open questions in the field.

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