

## Review

# Polarity proteins and actin regulatory proteins are unlikely partners that regulate cell adhesion in the seminiferous epithelium during spermatogenesis

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**Summary.** In mammalian testis, spermatogenesis takes place in the seminiferous epithelium of the seminiferous tubule, which is composed of a series of cellular events. These include: (i) spermatogonial stem cell (SSC) renewal via mitosis and differentiation of SSC to spermatogonia, (ii) meiosis, (iii) spermiogenesis, and (iv) spermiation. Throughout these events, developing germ cells remain adhered to the Sertoli cell in the seminiferous epithelium amidst extensive cellular, biochemical, molecular and morphological changes to obtain structural support and nourishment. These events are coordinated via signal transduction at the cell-cell interface through cell junctions, illustrating the significance of cell junctions and adhesion in spermatogenesis. Additionally, developing germ cells migrate progressively across the seminiferous epithelium from the stem cell niche, which is located in the basal compartment near the basement membrane of the tunica propria adjacent to the interstitium. Recent studies have shown that some apparently unrelated proteins, such as polarity proteins and actin regulatory proteins, are in fact working in concert and synergistically to coordinate the continuous cyclic changes of adhesion at the Sertoli-Sertoli and Sertoli-germ cell interface in the seminiferous epithelium during the epithelial cycle of spermatogenesis, such that developing germ cells remain attached to the Sertoli cell in the epithelium while they alter in cell shape and migrate across the epithelium. In this review, we highlight the physiological significance of endocytic vesicle-mediated protein trafficking events under the influence of polarity and actin regulatory proteins in conferring cyclic events of cell adhesion and

de-adhesion. Furthermore, these recent findings have unraveled some unexpected molecules to be targeted for male contraceptive development, which are also targets of toxicant-induced male reproductive dysfunction.

**Key words:** Testis, Polarity proteins, PAR6, Cdc42, GTPase, Actin regulators, Eps8, Arp3, Anchoring junction, Ectoplasmic specialization, Seminiferous epithelial cycle

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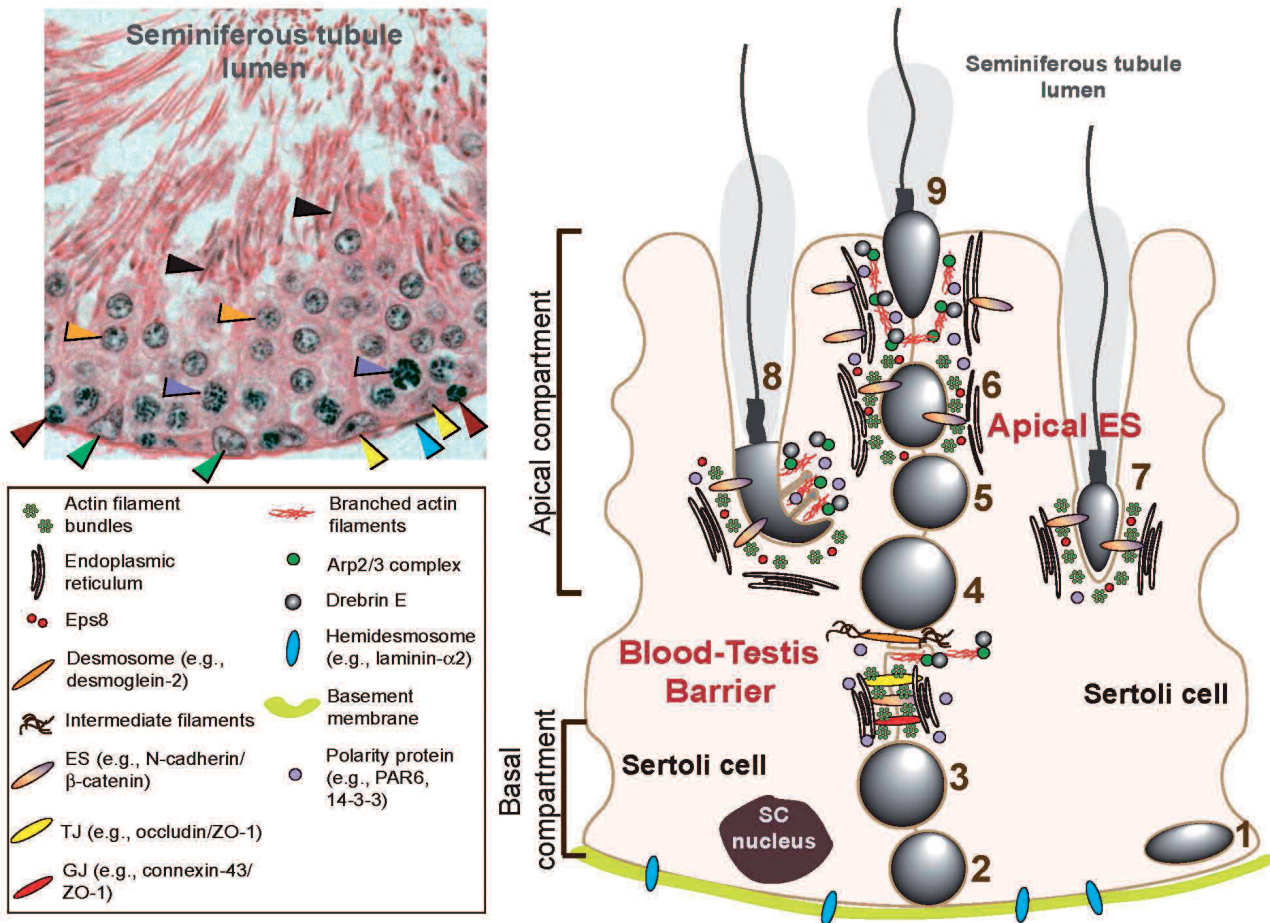
## Introduction

The seminiferous tubule is the functional unit of the testis that produces spermatozoa from spermatogonia via spermatogenesis in ~58, 35 and 64 days in rats, mice, and humans respectively (de Kretser and Kerr, 1988; Franca et al., 1998). More than 100 million sperms are produced from a man each day since puberty at ~12-14 years of age throughout his entire life span (Neaves et al., 1984; de Kretser and Kerr, 1988; Johnson et al., 2001). Spermatogenesis takes place in the seminiferous epithelium (Fig. 1) which is composed of only Sertoli and germ cells. It is regulated by the pituitary hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH), as well as testosterone and estrogen that are released by Leydig cells located in the interstitial space (Sharpe, 1994; O'Donnell et al., 2001; Carreau and Hess, 2010; Verhoeven et al., 2010). However, post-meiotic germ cell development during spermiogenesis and spermiation (the release of sperm) (O'Donnell et al., 2011) is sequestered behind the blood-testis barrier (BTB), an ultrastructure established by specialized junctions between Sertoli cells near the basement membrane (Wong and Cheng, 2005; Setchell, 2008; Vogl et al., 2008; Cheng et al., 2010; Cheng and Mruk, 2010). Thus, many of the cellular events associated with

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spermatogenesis take place in a specialized microenvironment behind the BTB known as the apical (adluminal) compartment (Fig. 1). As such, the barrier imposed by adjacent Sertoli cells in the seminiferous epithelium prevents the access of hormones, electrolytes, ions, paracrine factors, biomolecules, water, and all other substances to germ cells undergoing meiosis I and II, and all events of post-meiotic cell development, including the release of sperm at spermiation (Mruk et al., 2008; Cheng and Mruk, 2009, 2010; Franca et al., 2011; O'Donnell et al., 2011). Despite the fact that many of the cyclic events during spermatogenesis that occur in

germ cell “clones” (which are connected by cytoplasmic bridges) (Fawcett et al., 1959; Fawcett, 1961; Hamer et al., 2003) take place in a synchronized fashion, most of the necessary paracrine factors and hormones possibly needed to coordinate these events are “excluded” from the apical compartment because of the BTB. Thus, it is conceivable that germ cell development relies exclusively on Sertoli cells in the seminiferous epithelium, and these events must be coordinated via precisely regulated and coordinated “communications” at the cell-cell interface, such as via communicating gap junctions.



**Fig. 1.** Relative distribution of polarity proteins (e.g., PAR6, 14-3-3) and actin regulatory proteins (e.g., Eps8, Arp2/3 complex, drebrin E) at the apical ES and the BTB in the seminiferous epithelium of adult mammalian testes. The left panel is the cross-section of an adult rat testis showing the seminiferous epithelium of a stage VIII seminiferous tubule. The blood-testis barrier (BTB) has physically divided the epithelium into the basal and apical or adluminal compartments as shown in the right panel. Sertoli cell nuclei (green arrowheads) are restricted to the basal compartment near the basement membrane with the peritubular myoid cells (see blue arrowhead) lying outside the basement membrane. Both spermatogonia (yellow arrowheads) and preleptotene spermatocytes (red arrowheads) are found in the basal compartment. However, preleptotene spermatocytes are in transit at the BTB at stage VIII and differentiate into pachytene spermatocytes (purple arrowheads) so that they can undergo meiosis I and II at stage XIV of the epithelial cycle. Also seen are round spermatids (step 8 spermatids, orange arrowheads) and elongated spermatids (step 19 spermatids, black arrowheads). The apical ES in the apical compartment, and the basal ES at the BTB in the basal compartment are typified by the presence of actin filament bundles (maintained by the intricate actions of Eps8, Arp2/3 complex and drebrin E) sandwiched in between cisternae of endoplasmic reticulum and the apposing plasma membranes of either the Sertoli cell and the spermatid (step 8-19 spermatids) (for apical ES) or the two Sertoli cells (for basal ES at the BTB). **1**, spermatogonial stem cell (SSC) or spermatogonium (undifferentiated or differentiated type A); **2**, type B spermatogonium; **3**, preleptotene spermatocyte; **4**, zygotene, pachytene or diplotene spermatocyte; **5**, round spermatid; **6**, elongating spermatid (step 8); **7**, elongating spermatid; **8**, elongating spermatid; **9**, elongated spermatid. Apical ES, once it appears, is the only anchoring device between the Sertoli cell and the elongating/elongated spermatid to confer cell adhesion and polarity (or orientation) to the developing spermatids until spermiation.

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In the seminiferous epithelium, gap junctions (GJs, a cell-cell communication junction type) and desmosomes (an intermediate filament-based cell-cell anchoring junction type) are found at both Sertoli-Sertoli and Sertoli-germ cell interface including spermatocytes and spermatids (steps 1-8). Hemidesmosomes are intermediate filament based cell-matrix anchoring junction type at the Sertoli cell-basement membrane [note: the basement membrane is a modified extracellular matrix also known as basal lamina (Dym, 1994)] interface (Fig. 1) (Cheng and Mruk, 2002, 2010; Mruk and Cheng, 2004; Mruk et al., 2008). However, there is no “conventional” cell-cell actin-based adherens junction (AJ) in the seminiferous epithelium analogous to other epithelia. Instead, a specialized testis-specific actin-based cell-cell anchoring junction known as ectoplasmic specialization (ES) is found in the mammalian testis. ES is restricted to the interface of Sertoli cells and elongating spermatids (steps 8-19 in rats) known as apical ES and to the Sertoli-Sertoli cell interface at the BTB known as the basal ES (Fig. 1). Once apical ES appears, GJ and desmosome vanish entirely, and it becomes the only anchoring device that anchors developing spermatids (step 8-19 in rats) to the Sertoli cells in the epithelium during spermiogenesis. Interestingly, basal ES coexists with TJ and GJ, and these junctions together with desmosomes constitute the BTB in the mammalian testis (Fig. 1). Furthermore, ES is the most prominent anchoring junction in the seminiferous epithelium clearly visible under electron microscopy throughout spermatogenesis. Unexpectedly, recent studies have shown that this junction type is regulated by two groups of proteins, namely polarity and actin regulatory proteins. Recent findings also demonstrated that ES works in concert with GJ and desmosome to coordinate cellular events, in particular at the BTB, throughout spermatogenesis. While much research is needed to understand the signaling mechanism(s) underlying these events, these findings have unraveled some unexpected turns of events, revealing several molecular entities that are crucial for cell adhesion in the seminiferous epithelium, and functional studies can now be designed to probe the molecular and biochemical events pertinent to spermiogenesis and spermiation (Cheng and Mruk, 2011; O'Donnell et al., 2011).

### Ectoplasmic specialization

Ectoplasmic specialization (ES) is limited to the Sertoli-Sertoli cell interface at the BTB known as the basal ES; however, ES is also found at the Sertoli-spermatid (step 8-19 spermatids) interface known as the apical ES, which is the only anchoring device once it appears during spermiogenesis (Cheng and Mruk, 2009, 2010). It is known that in adult rats each testis contains  $\sim 30 \times 10^6$  Sertoli cells (Berndtson and Thompson, 1990) [ $\sim 4 \times 10^6$  Sertoli cells per testis in adult mice (Auharek and Franca, 2010)]. Sertoli cells cease to proliferate by  $\sim 15$ -17 days of age postpartum (Orth, 1982) and the

ratio of Sertoli:germ cell is  $\sim 1:50$  (Weber et al., 1983) [note: the ratio of spermatogonium:Sertoli cell was estimated to be 0.12:1 whereas spermatid:spermatogonium ratio is  $\sim 75:1$  in adult rats at 150-250 days of age postpartum (Berndtson and Thompson, 1990)], illustrating the significance of ES in the seminiferous epithelium simply from a spatial distribution stand-point. While ES is basically a testis-specific cell-cell actin-based AJ type, this ultrastructure is exclusively found in the testis, typified by the presence of actin filament bundles lying parallel to the plasma membrane and sandwiched in between cisternae of endoplasmic reticulum and the apposing plasma membranes of either Sertoli-Sertoli cell interface at the basal ES or Sertoli-spermatid interface at the apical ES (Fig. 1). While these ultrastructural features are found on both sides of the Sertoli cells in the basal ES at the BTB, they are restricted to the Sertoli cell side at the apical ES, and no such ultrastructures are visible in the elongating spermatids (steps 8-19). This may be due to the fact that relatively little cytosol is present in elongating spermatids from steps 8-19 whose cell bodies are occupied almost exclusively by the nucleus and the acrosome, and thus an extensive actin filament network cannot be sustained spatially. Nevertheless, spermatids were shown to express many of the structural proteins at the apical ES (e.g., N-cadherin, E-cadherin, nectins, laminins), peripheral adaptors (e.g.,  $\beta$ -catenin,  $\gamma$ -catenin, afadins) and non-receptor protein kinases (e.g., FAK, c-Src, c-Yes) (Mruk and Cheng, 2004; Zhang et al., 2005; Mruk et al., 2008) at or near the plasma membrane opposite to the actin filament bundles (Lie et al., 2010a,b, 2011; Wong et al., 2008a,b; Wong and Cheng, 2009). These findings thus suggest that adhesion protein complexes (e.g., N-cadherin/ $\beta$ -catenin, nectin/afadin, laminin/c-Src) residing in elongating spermatids can form putative inter-locking interactions with the corresponding domains of the adhesion complexes (e.g., N-cadherin/ $\beta$ -catenin, nectin/afadin, integrin/vinculin) residing in the Sertoli cell at the apical ES. Thus, developing spermatids are likely to contribute, at least in part, to the apical ES functionality without the distinctive network of actin filament bundles. This perhaps is physiologically necessary so that the metabolically inactive developing spermatids (relative to the Sertoli cells in the epithelium) do not have to express another set(s) of proteins to “de-polymerize” and “re-polymerize” the actin filament bundles similar to the Sertoli cells, but they can still “adhere” to the Sertoli cell via these putative cell adhesion protein complexes.

Interestingly, while the protein complexes that confer cell adhesion at the apical ES are better defined, the constituent protein complexes at the basal ES are less known (Table 1) because basal ES coexists with either TJ or gap junction (but not desmosome) (Mruk and Cheng, 2011b) (note: TJ, basal ES, gap junction, and desmosome constitute the BTB); thus, even if a protein is found to localize at the BTB, it is somewhat difficult to assign it to be a basal ES-, TJ- or gap junction-protein, since it is not technically possible at present to isolate

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basal ES without the associated TJ or gap junction for protein composition analysis. Thus, the information listed in Table 1 regarding the constituent proteins at the TJ and basal ES is largely based on earlier studies from other epithelia. If a selected protein was shown earlier to be associated with TJ (e.g., occludin) or AJ (e.g., N-cadherin) in other tissues, it would be classified accordingly at the BTB. For instance, N-cadherin would be assigned as a basal ES protein and occludin would be designated a TJ-protein.

It is likely that the unique ultrastructural features, such as the distinctive network of actin filament bundles at the ES, contribute to its unusual adhesive strength. The strength of ES was found to surpass that of desmosome, which is considered to be a strong adhesion junction in epidermis and in keratinocytes *in vitro* (Green et al., 2010; Green and Simpson, 2007; Thomason et al., 2010), as well as at the Sertoli cell-spermatid (pre-step 8 spermatids) interface in the testis (Wolski et al., 2005). For instance, the adhesive force conferred by apical ES was found to be almost twice as strong as desmosome when it was quantified by a micropipette pressure transducing system (Wolski et al., 2005), and the remarkable adhesive strength is largely the result of the extensive network of actin filament

bundles at the ES. Interestingly, apical ES is also exceedingly vulnerable to toxicants (e.g., cadmium) or chemicals/drugs (e.g., adjuvins) and it can be broken down by these toxicants and/or chemicals long before desmosome and gap junction are disrupted in the seminiferous epithelium (Chen et al., 2003; Yan et al., 2007; Cheng et al., 2011). Thus, earlier studies have shown that in rodents treated with toxicants, exfoliation of spermatids from the epithelium is the most typical phenotype in the testis (Boekelheide et al., 1989; Hew, et al., 1993; Wong et al., 2005; Moffit et al., 2007; Elkin et al., 2010; Wong et al., 2010a,b). The BTB is also highly sensitive to environmental toxicants (e.g., cadmium), more than other blood-tissue barriers (Setchell and Waites, 1970; Hew, et al., 1993; Wong et al., 2004). Such vulnerability of the ES to toxicants may be attributed to the hybrid nature of this junction type (Siu and Cheng, 2004; Yan et al., 2007; Wong et al., 2008). For instance, the primary molecular target of cadmium was shown to be E-cadherin because Cd<sup>2+</sup> competed for the same binding motif as Ca<sup>2+</sup> in E-cadherin (Prozialeck and Lamar, 1999; Prozialeck, 2000), but E-cadherin is usually shielded behind the TJ in virtually all other epithelia and/or endothelia, making the E-cadherin-based AJ less accessible to cadmium. In

**Table 1.** Adhesion protein complexes that confer cell-cell adhesion in the seminiferous epithelium of adult testes.

	Location	Adhesion protein complex
<b>Anchoring junctions</b>		
Apical ES	Sertoli cell-spermatid (step 8-19)	$\alpha$ 6 $\beta$ 1-integrin-laminin $\alpha$ 3 $\beta$ 3; N-cadherin- $\beta$ -catenin Nectin-2/3-afadin JAM-C-ZO-1 CAR-ZO-1
Desmosome	Sertoli cell-spermatocyte/spermatogonium Sertoli cell-spermatid (step 1-7)	Desmoglein-desmocollin Desmoglein-desmocollin
Hemidesmosome	Sertoli cell-basement membrane	$\beta$ 1-integrin/laminin $\alpha$ 2
<b>Communicating junctions</b>		
Gap junction	Sertoli cell-spermatid (step 1-19)	Connexin 43-plakophilin-2
<b>Blood-testis barrier</b>		
Tight junction	Sertoli-Sertoli cell	Occludin-ZO-1 N-cadherin- $\beta$ -catenin JAM-A-ZO-1 JAM-B-ZO-1 CAR-ZO-1
Basal ES	Sertoli-Sertoli cell	N-cadherin- $\beta$ -catenin Nectin-2-afadin JAM-A-ZO-1 CAR-ZO-1
Desmosome	Sertoli-Sertoli cell	Desmoglein-2-desmocollin-2
Gap junction	Sertoli-Sertoli cell	Connexin 43-plakophilin-2

This Table was prepared based on recent reviews (Mruk and Cheng, 2004; Mruk et al., 2008; Wong et al., 2008; Cheng and Mruk, 2009; 2010; Wong and Cheng, 2009; Cheng et al., 2010; Lie et al., 2011) based on studies in the rat testis. Apical ES, basal ES, tight junction and gap junction are using actin for their attachment; desmosome and hemidesmosome are using intermediate filament for their attachment. Focal contact (or focal adhesion complex), a cell-matrix anchoring junction type using actin for its attachment is absent in the testis. CAR, coxsackievirus and adenovirus receptor; JAM, junctional adhesion molecule; ZO-1, zonula occludens-1.

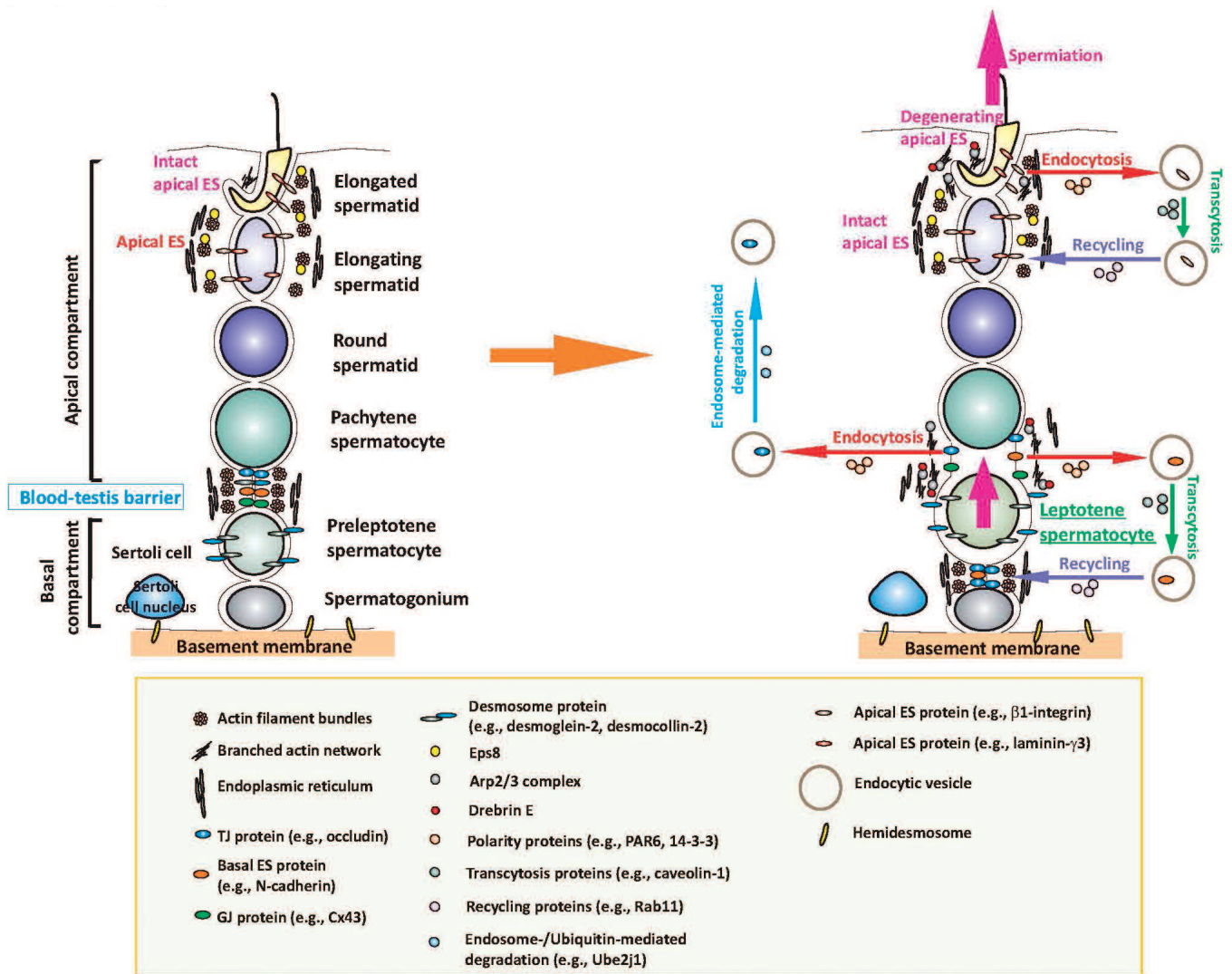
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contrast, E-cadherin found in the basal ES “coexists” with other TJ and gap junction proteins at the BTB, making it immediately accessible to cadmium. This thus explains the unusual vulnerability of the BTB to toxicants versus other blood-tissue barriers.

### Polarity proteins

Cell polarity in epithelia, such as apico-basal polarity, and cell asymmetry, is conferred by three different protein modules or complexes: (i) the

partitioning defective protein (PAR) complex [e.g., PAR3/PAR6/aPKC (atypical protein kinase C)], (ii) the Crumbs complex [e.g., Crumbs-3/PALS1 (protein associated with LIN7-1)/PATJ (PALS1-associated tight junction protein)], and (iii) the Scribble complex [e.g., Scribble/LGL1/2 (lethal giant larvae 1/2)/DLG1 (discs large 1)] (Assemat et al., 2008; Iden and Collard, 2008; Wong and Cheng, 2009; Cheng and Mruk, 2010). The PAR- and Crumbs-based modules are usually located at the TJ in the apical region of a cell epithelium, whereas the Scribble-based module is restricted to the basolateral



**Fig. 2.** Restructuring of adhesion at the apical ES and the BTB in the seminiferous epithelium during the epithelial cycle to facilitate the transit of developing germ cell across the epithelium is regulated by the intricate actions of polarity proteins (e.g., PAR6, 14-3-3, Cdc42) and actin regulatory proteins (e.g., Eps8, Arp2/3 complex, drebrin E) as depicted in this hypothetical model. The left panel illustrates the seminiferous epithelium of a stage VII tubule in which the integrity of the apical ES and basal ES is maintained by the rigid actin filament bundles. However, at stage VIII of the epithelial cycle when preleptotene spermatocytes are in transit in “clones” at the BTB and elongated spermatids begin the process of sperm release (i.e., spermiation), endocytosis of integral membrane proteins occurs at the basal ES (at the BTB) and apical ES, which is mediated by the intricate actions of the polarity proteins, Arp2/3 protein complex and drebrin E (and a concomitant loss and/or reduced Eps8 activity) to increase the actin network plasticity to facilitate the endocytic vesicle-mediated protein trafficking events. While some of the endocytosed proteins are degraded via the endosome- or ubiquitin-mediated pathway, many of the internalized proteins undergo transcytosis and recycling, so that these proteins can be “re-used” to establish the “newly” formed apical ES or basal ES and TJ/gap junction/desmosome components at the BTB. Thus, the extensive junction restructuring events that occur during the epithelial cycle do not lead to a disruption of the seminiferous epithelium.

domain. Since each of these protein modules recruits its own scaffolding proteins, adaptors, nonreceptor protein kinases and phosphatases, mutual exclusion of the Scribble module and the apically located Crumbs- and PAR-based modules, as well as the junctional complexes (composed of TJ-AJ plaques-desmosomes), thus confers apico-basal polarity in cell epithelium, including the seminiferous epithelium (Fig. 2). As such, TJ and basal ES at the BTB confer Sertoli cell polarity in the seminiferous epithelium, which is discernible even under light microscopy in which Sertoli cell nuclei are found localized near the basement membrane (Fig. 1). Under electron microscopy, it is noted that TJ and basal ES are restricted to the BTB near the basement membrane, whereas developing elongating spermatids are restricted to the apical compartment of the seminiferous epithelium (Fig. 1), displaying cellular asymmetry, and this polarity phenotype is the result of the polarity proteins at the BTB.

In this context, it is of interest to note that there is no TJ at the Sertoli-spermatid (step 1-19 in rats) interface in the seminiferous epithelium to confer spermatid polarity. Yet the heads of all developing spermatids are arranged in such a way with their heads all pointing toward the basement membrane, illustrating spermatid polarity (Fig. 1). Thus, apical ES, a testis-specific AJ, was speculated to be responsible for conferring spermatid polarity, since this is the only anchoring device at the Sertoli-spermatid interface (step 8-19 spermatids) when spermatid polarity becomes apparent (Mruk and Cheng, 2004; Wong and Cheng, 2009). Indeed, recent studies have demonstrated the presence of PAR6 at the apical ES and its involvement in conferring spermatid polarity (Wong, et al., 2008). For instance, it was found that in rats treated with adjuvins (Wong, et al., 2008) or cadmium (Cheng et al., 2011), the “prematurely” departing elongating spermatids found near the luminal edge of the seminiferous epithelium became “mis-oriented”, with their heads no longer pointing toward the basement membrane. More importantly, in these mis-oriented elongating spermatids, the intensely localized PAR6 usually found in the apical ES in normal rat testis reduced considerably, almost to a non-detectable level (Wong, et al., 2008). These findings thus provide the first proof that the PAR6-based polarity proteins are crucial to confer spermatid polarity at the apical ES.

### **Polarity proteins mediate ES-based adhesion function**

The first evidence that polarity proteins confer spermatid and Sertoli cell adhesion at the apical and basal ES, respectively, comes from studies using the adjuvins model *in vivo*, and the Sertoli cell *in vitro* culture system that mimics the Sertoli BTB *in vivo* (Cheng et al., 2005; Mruk et al., 2008; Wong and Cheng, 2009; Mok et al., 2011). It was found that within ~6-9-hr following treatment of adult rats with a single dose of adjuvins (50 mg/kg b.w., by gavage), apical ES was disrupted, which was manifested by a loss of spermatid polarity, with the

spermatid heads no longer pointing toward the basement membrane and a considerable loss of PAR6 staining at the apical ES. These changes were immediately followed by “premature” release of elongating spermatids from the seminiferous epithelium (Wong et al., 2008a,b) mimicking spermiation. These findings thus support the notion that polarity proteins confer spermatid adhesion in the seminiferous epithelium. This concept is further strengthened by earlier observations using Sertoli cells cultured *in vitro* with an established TJ-permeability barrier that functionally mimics the BTB *in vivo* [note: ultrastructures of TJ and basal ES are found in the Sertoli cell epithelium *in vitro* (Lui et al., 2001, 2003; Lee and Cheng, 2003; Siu et al., 2005)], in which the knockdown of either PAR6 or PAR3 by RNAi affected the distribution of JAM-A and  $\alpha$ -catenin (and also N-cadherin after PAR6 knockdown by RNAi) at the Sertoli-Sertoli cell interface. These proteins moved from the cell surface to cytosol, thereby destabilizing the Sertoli cell TJ-barrier function and altering cell adhesion properties (Wong, et al., 2008a,b). These initial findings were further strengthened by subsequent studies in which 14-3-3 (also known as PAR5) was also shown to be an integrated component of the basal ES and TJ at the BTB, and its knockdown by RNAi also induced mis-localization of N-cadherin and ZO-1 from the Sertoli-Sertoli cell interface to the cytosol, destabilizing the Sertoli TJ-barrier (Wong et al., 2009). But more importantly, 14-3-3 was found to regulate cell adhesion via its effects on the endocytic vesicle-mediated protein trafficking events, since a knockdown of 14-3-3 by RNAi induced a significant increase in protein endocytosis of both JAM-A and N-cadherin (Wong et al., 2009). Collectively, these findings thus illustrate that the loss of these adhesion proteins at the cell surface by RNAi destabilizes cell adhesion at the Sertoli-Sertoli cell interface.

Other studies have shown that the TGF- $\beta$ 3- or TNF $\alpha$ -induced disruption of the Sertoli cell TJ-barrier function is mediated by an increase in clathrin-mediated protein endocytosis at the BTB (Yan et al., 2008; Xia et al., 2009), to be followed by an increase in endosome- or ubiquitin-mediated protein degradation (Su et al., 2010). A recent report has shown that the cytokine-induced endocytic vesicle-mediated protein trafficking events that destabilize BTB are also regulated by polarity protein Cdc42 (Wong, et al., 2010), which is a component of the PAR-based polarity module (Iden and Collard, 2008; Wong and Cheng, 2009). Overexpression of a dominant negative mutant of Cdc42 abolished the TGF- $\beta$ 3-induced enhancement of protein endocytosis in the Sertoli cell epithelium (Wong et al., 2010a,b).

In short, these findings demonstrate unequivocally that polarity proteins are crucial to confer spermatid and Sertoli cell adhesion in the seminiferous epithelium at the apical and basal ES, respectively. This is likely mediated via their maintenance of the proper localization of adhesion proteins at the cell-cell interface by regulating the endocytic vesicle-mediated protein trafficking events. This, in turn, determines the levels of

adhesion proteins at the cell surface to form interlocking adhesion at the Sertoli-spermatid or Sertoli-Sertoli cell interface. However, as discussed earlier, ES is structurally associated with an extensive network of actin filament bundles that contribute to the unusual “adhesive strength” of the apical ES, as shown in the study using a micropipette pressure transducing system (Wolski et al., 2005). Does this network of actin filament bundles at the apical and basal ES interfere with the endocytic vesicle-mediated protein trafficking events? If it does, what mechanism is in place in the testis to “rapidly” disassemble and reassemble this actin network during spermiogenesis? These questions will be addressed in the following sections.

### Actin regulating proteins

Actin-based networks in a eukaryotic cell, such as the actin filament bundles at the ES in the Sertoli cell, are composed of filamentous actin (F-actin) (Lie et al., 2010a,b). F-actin is a polymer assembled by globular actin subunits (G-actin), with the fast-growing end known as the barbed end, and the slow-growing end called the pointed end (Pellegrin and Mellor, 2007; Bugyi and Carlier, 2010; Lie et al., 2010a,b). In the testis, the Sertoli cell is equipped with a number of actin regulatory proteins that confer actin bundling [e.g., espin (Bartles et al., 1996),  $\alpha$ -actinin (Yazama et al., 1991), Eps8 (epidermal growth factor receptor pathway substrate 8) (Lie et al., 2009)], capping [e.g., Eps8 (Lie et al., 2009)], nucleation [or actin branching, e.g., Arp2/3 (actin-related protein 2/3) complex (Lie et al., 2010a,b)] and severing [e.g., cofilin (Guttman et al., 2004), gelsolin (Guttman et al., 2002)]. A recent study has also demonstrated an actin binding protein (also known as microfilament-associated protein) drebrin E (developmentally regulated brain protein E) in the rat testis which structurally interacts with Arp3 but not Esp8, apparently contributes to the restricted temporal and spatial expression and function of Arp3 at the ES in the testis (Li et al., 2011). The combined effects of these proteins thus induce polymerization and depolymerization of the actin filament bundles (Lie, et al., 2010), conferring the plasticity of the ES at the Sertoli-spermatid (apical ES) and Sertoli-Sertoli (basal ES) cell interface. This is also necessary for the formation and trafficking of the endocytic vesicles, such as their internalization from the plasma membrane and the subsequent cleavage from the entry site [such as by dynamins (Lie et al., 2006, 2008; Vaid et al., 2007)], as well as endosome-mediated intracellular trafficking via transcytosis or recycling (Welling and Weisz, 2010; Kelly and Owen, 2011; Mruk and Cheng, 2011a). It is conceivable that the presence of these extensive actin filament bundles at the ES would make it difficult for endocytic vesicle formation and its subsequent internalization. In fact, it is known that towards the end of spermiogenesis, such as in step 17-18 spermatids, actin nucleation occurs at stage VII of the epithelial cycle (Lie et al., 2010a,b), beginning from the concave

side of the elongating spermatid heads (Lie et al., 2010a,b). This assembly of actin networks is associated with the formation of a “giant” endocytic vesicle (Young et al., 2009a,b) previously designated tubulobulbar complex (TBC) (Russell, 1979b, a), which is presently known to be a transitional ES ultrastructure undergoing degeneration to prepare for spermiation at the apical ES and the restructuring of the BTB at stage VIII of the epithelial cycle (Cheng and Mruk, 2010). Eventually, this event spreads to the entire apical ES to prepare for spermiation (i.e., the release of sperm) (Cheng and Mruk, 2010; O'Donnell et al., 2011) at the end of spermiogenesis. This event is a critical step to maintain the homeostasis of spermatogenesis, since endocytosed apical or basal ES proteins can be transcytosed and recycled to assemble “new” apical and basal ES. Thus, de novo synthesis would not be required for each component protein necessary for spermatid adhesion during spermiogenesis, considering that each Sertoli cell has to “nurture” ~30-50 developing germ cells, making this an almost impossible task from a physiological standpoint (see Fig. 2). This concept is also supported by recent studies, which have shown that Eps8 (an actin barbed-end capping and bundling protein), Arp3 (an actin nucleation protein), and drebrin E (an Arp3 binding protein) display highly restricted temporal and spatial expression in the seminiferous epithelium at the apical and basal ES during the seminiferous epithelial cycle of spermatogenesis (Lie et al., 2008, 2010a,b, 2011). In short, Eps8 is being used to maintain the integrity of the actin filament bundles at the ES, whereas Arp3 is used to induce actin branching (with its action precisely regulated by an actin-binding protein drebrin E), causing the breakdown of the network of actin filament bundles at the ES to facilitate protein endocytosis, which is regulated by PAR6, 14-3-3, and Cdc42 at the site. It is through the concerted efforts of these proteins, as depicted in Fig. 2, that the integrity of the BTB can be maintained and proteins at the degenerating apical ES (in preparation for sperm release during spermiation) can be recycled to conserve protein utilization during spermiogenesis.

### Coordinated effects of polarity proteins and actin regulatory proteins to modulate ES-based adhesion function

#### Apical ES

As depicted in Fig. 2, endocytic vesicle-mediated protein trafficking events begin at the concave side of the apical ES at later stages of spermiogenesis, such as in step 17-18 spermatids (e.g. stage VII of the epithelial cycle). This is made possible by a surge in actin branching at the site mediated by a temporal and spatial reduction in Eps8, but an increase in Arp3 and drebrin E expression, thereby diminishing the rigid actin filament bundles to increase the “plasticity” at the apical ES microenvironment to facilitate the formation of endocytic vesicles. Subsequent protein endocytosis is

mediated by the combined action of PAR6, 14-3-3 and Cdc42. Internalized components (e.g., integrins, nectins, N-cadherin) from these degenerating apical ES are transcytosed and recycled to assemble “new” apical ES in newly formed elongating spermatids (step 8) via spermiogenesis. Thus, spermiogenesis is not an entirely “proteolytic” event; instead, many physiologically important components are being “re-used” to maintain the homeostasis of the seminiferous epithelium during spermatogenesis.

### Basal ES at the BTB

Since basal ES coexists with TJ and gap junction at the BTB, the events taking place at the basal ES thus affect integral membrane proteins at the TJ (e.g., occludins, JAM-A, JAM-B, claudins) and gap junction (e.g., connexin 43, connexin 33) as well. This also implies that the restructuring events that occur at the basal ES affect the integrity of the BTB. As shown in Fig. 2, besides de novo synthesis of proteins necessary to assemble “new” TJ-fibrils behind the preleptotene spermatocytes in transit at the BTB, restricted temporal and spatial expression of Arp3 and drebrin E, and a concomitant reduction in Eps8, at the site induce the loss of actin filament bundles to facilitate endocytic vesicle formation via an increase in cellular “plasticity”. This is further facilitated by a transient loss of PAR6 or 14-3-3 at the “old” BTB site, causing re-distribution of N-cadherin, occludin and JAM-A, destabilizing the “old” BTB. Internalized integral membrane proteins are transcytosed and recycled to the baso-lateral region of the transiting preleptotene spermatocytes to assemble “new” TJ-fibrils, creating a “new” BTB prior to the degeneration of the “old” BTB. This thus maintains the integrity of the BTB even during the transit of preleptotene spermatocytes that are interconnected by intercellular bridges as “clones”, and the immunological barrier will not be compromised during the cyclic events of spermatogenesis.

### Concluding remarks and future perspectives

While the concept depicted in Fig. 2 is a hypothetical model involving the combined efforts of polarity proteins and actin regulatory proteins to regulate cell adhesion and junction restructuring in the seminiferous epithelium during spermatogenesis, it is based on recently published findings in the field. However, much work is needed to provide some of the missing information. For instance, what triggers the temporal and spatial expression of Eps8, Arp3, and drebrin E (and other actin regulatory proteins) at the apical and basal ES during the epithelial cycle? Does this involve cytokines and/or steroids (e.g., testosterone, estradiol-17 $\beta$ ) or their concerted expression in the microenvironment? Also, what triggers the transient loss of polarity proteins at the ES to facilitate protein endocytosis? Is there a common regulator that triggers both events? What is the mechanism that coordinates the

transient loss of Eps8 and PAR-based polarity proteins but a surge in Arp3 to prepare for protein endocytosis at the site? It is expected that many of these questions will be answered in the years to come using genetic models or cell type specific knockdowns and/or overexpression of target genes.

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