Chapter 13
Cell–Cell Interactions, Cell Polarity, and the Blood–Testis Barrier

Elizabeth I. Tang, Dolores D. Mruk, Will M. Lee, and C. Yan Cheng

Abstract The study of polarity and its role in cell–cell interactions, such as spermatid polarity and adhesion in the seminiferous epithelium, and blood–testis barrier (BTB) in the testis during the epithelial cycle of spermatogenesis in recent years has yielded some unexpected and interesting observations. Similar to other polarized tissues, Sertoli and germ cells in the seminiferous epithelium express many of the component proteins of the Par-, Scribble- and Crumb-based polarity protein complexes. These polarity proteins are working in concert with non-receptor protein kinases, adhesion proteins, and cytoskeletons to confer spermatid and Sertoli cell polarity, and these proteins are also involved in germ cell transport in the epithelium during the epithelial cycle. In this review, we summarize the latest findings in the field. Based on the available data in the literature, it is increasingly clear that polarity proteins are crucial in (1) conferring spermatid and Sertoli cell polarity, (2) regulating spermatid adhesion and transport, and (3) regulating BTB dynamics in the testis during the epithelial cycle. We also highlight specific areas of research that deserve attention in future years. This information should be helpful to investigators in other blood–tissue and epithelial barriers in the field.

Keywords Blood–testis barrier • Cell polarity • Cell–cell interactions • Ectoplasmic specialization • F-actin • Germ cell • Microtubule • Sertoli cell • Spermatogenesis • Testis

E.I. Tang • D.D. Mruk • C.Y. Cheng (✉)
The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, 1230 York Avenue, New York, NY 10065, USA
e-mail: Y-Cheng@popcbr.rockefeller.edu; ccheng@rockefeller.edu

W.M. Lee
School of Biological Sciences, University of Hong Kong, Hong Kong, China

13.1   Introduction

13.1.1   Concept of Cell–Cell Interactions, Cell Polarity, and the BTB in the Testis

The two major functions of the mammalian testis are to produce testosterone, a hormone synthesized by the interstitial Leydig cells to maintain sexual characteristics of the male, and spermatozoa in the seminiferous tubules for fertilization to safeguard the continuation of a species. One man produces >300 million spermatozoa, as compared to ~10 and 50 million in a male mouse and rat, respectively, via spermatogenesis each day following puberty until death (Auharek et al. 2011; Johnson et al. 1980; Mauss and Hackstedt 1972). This thus illustrates there are tightly regulated and robust cellular events that take place in the testis to maintain this tempo of sperm output. Spermatogenesis is a complex cellular process, and it can be divided into three discrete events: (1) mitosis, for self-renewal of spermatogonial stem cells (SSC) and spermatogonia; (2) meiosis, to ensure that the amount of genetic material carried in each spermatid is half that of spermatocytes; and (3) spermiogenesis, for the development of spermatids into functional spermatozoa, such that during fertilization, the fusion of a single sperm and an ovum reconstitute the genetic material similar to other somatic cells. These events all take place in the seminiferous epithelium of the seminiferous tubule—the functional unit in the testis that produces sperm—via the epithelial cycle of spermatogenesis (Fig. 13.1). These events are supported by testosterone produced by Leydig cells in the interstitium and also estrogen produced by Sertoli and germ cells (Sharpe 1994; O'Donnell et al. 2001; Carreau and Hess 2010; Carreau et al. 2010; Verhoeven et al. 2010; Wang et al. 2009; McLachlan et al. 2002; Mruk and Cheng 2004b). Recent studies have shown that peritubular myoid cells in the tunica propria also play an important role in supporting spermatogenesis (Welsh et al. 2009; Qian et al. 2013). It is conceivable that there are extensive cell–cell interactions in the testis, in particular at the Sertoli–Sertoli and Sertoli–germ cell interface (Fig. 13.2) during the epithelial cycle of spermatogenesis (Fig. 13.1).

As noted in Fig. 13.2 illustrating the cross section of a typical seminiferous tubule, such as in the rat testis, during the 14 stages of epithelial cycle from I through XIV (Fig. 13.1), elongating/elongated spermatids are highly polarized cells in which their heads are all pointing toward the basement membrane (BM) with their tails toward the tubule lumen (Fig. 13.2), such that the maximal number of developing spermatids can be packed in the tubule to allow the simultaneous development of millions of germ cells. It is noted that spermatid polarity is affected during toxicant- or drug-induced disruption in spermatogenesis such as following treatments with cadmium or adjudin [1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, a potential male contraceptive under development in our laboratory (Cheng et al. 2005, 2011a)] in which exposure of rats to these toxicants induces misorientation of elongating/elongated spermatids in the epithelium (Cheng et al. 2011b; Wong et al. 2008b). This loss of spermatid polarity appears to be
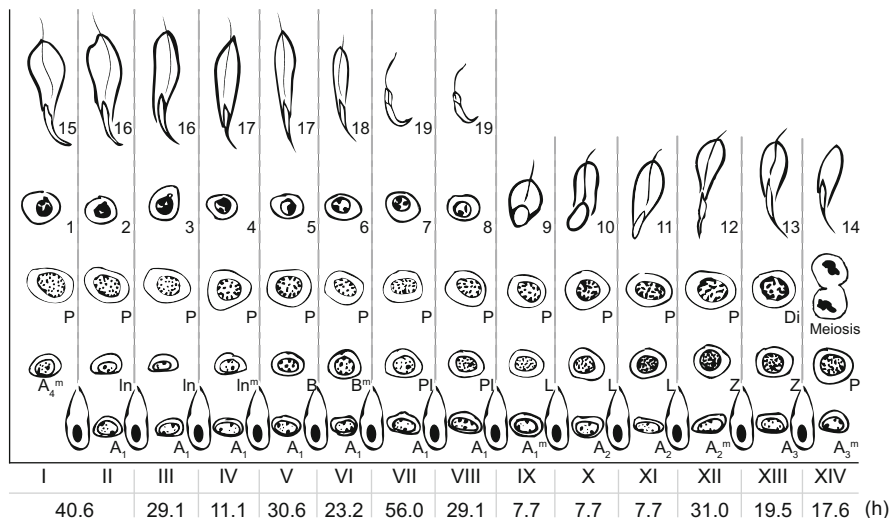
Fig. 13.1 Stages of the seminiferous epithelial cycle of spermatogenesis. The epithelial cycle of spermatogenesis in the rat is divided into 14 (I–XIV) stages and lasts 12.8 days, and the duration, in hour (h), of each cycle from I through XIV is shown. It is crucial to note that each stage does not contain one type of germ cell; rather each stage is unique and contains germ cells at different stages in their maturation and development. For instance, in the seminiferous epithelium of a stage VIII tubule, only type A1 spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, and step 8 and 19 spermatids are found. However, a type A1 spermatogonium (at the *bottom* of the figure on the *left panel*) will go through the epithelial cycle ~4.5 times until it finally matures into step 19 spermatid, taking a total of ~58 days to complete. Spermatogonia, type A (A) containing A_1, A_2, A_3 and A_4, type A_1 undergo mitosis (A_1^m), type A2 undergo mitosis (A_2^m), type A3 undergo mitosis (A_3^m), type A4 undergo mitosis (A_4^m), intermediate (ln), type In undergo mitosis (In^m), and type B (B), type B undergo mitosis (B^m). Spermatocytes, preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P), and diplotene (Di). Spermatids, round spermatids (steps 1–8), and elongate spermatids (steps 9–19). This figure was prepared based on the earlier reports and reviews (Dym and Clermont 1970; de Kretser and Kerr 1988; Mruk et al. 2008). Sertoli cell nucleus near the base of the seminiferous epithelium in each stage of the cycle is also shown

related to the subsequent loss of spermatid adhesion in the epithelium, causing their eventual departure from the testis prematurely (Wong and Cheng 2009; Wong et al. 2008a), thereby leading to infertility (Siu et al. 2009; Mok et al. 2011).

Besides spermatids, Sertoli cells in the epithelium are also highly polarized cells in which their nuclei are located restrictively near the basement membrane (Fig. 13.1). The ultrastructures that constitute the blood–testis barrier (BTB), such as the actin-based tight junction (TJ), basal ectoplasmic specialization (basal ES), and gap junction, as well as the intermediate-based desmosome, are also restricted near the basement membrane which also physically divides the seminiferous epithelium into the basal and the adluminal (apical) compartments (Fig. 13.2). On the other hand, the testis-specific actin-rich anchoring device only found at the Sertoli–spermatid (steps 8–19) interface is restricted to the adluminal compartment (Fig. 13.2). Collectively, these findings illustrate that the Sertoli cell is a highly polarized cell type in the epithelium. Furthermore, Sertoli cells are cyclic in nature during the epithelial cycle. For instance, the protein secretory and phagocytic
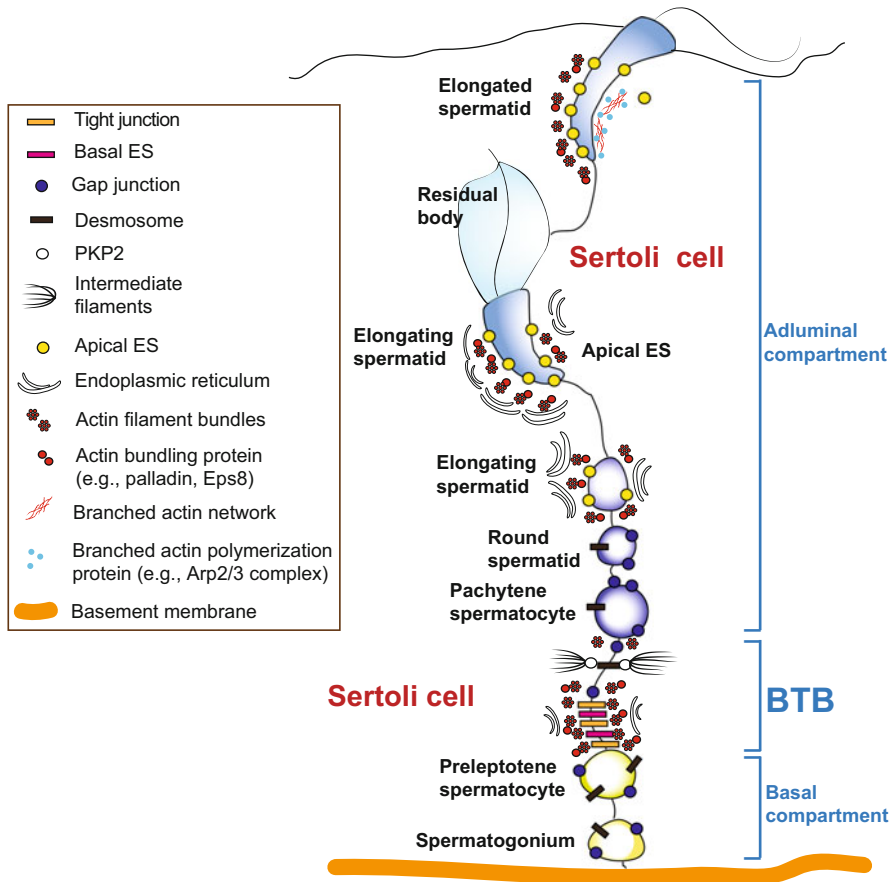
Fig. 13.2 A schematic drawing of the cross section of a seminiferous tubule, illustrating different cell junctions at the Sertoli–Sertoli and Sertoli–germ cell interface in the rat testis. The BTB, which is constituted by coexisting actin-based TJ (tight junction), basal ES (ectoplasmic specialization) and gap junction, as well as intermediate filament-based desmosome, physically divides the seminiferous epithelium into the basal and the adluminal compartment. The basal compartment is in close contact with the basement membrane, a modified form of extracellular matrix in the mammalian testis. Apical ES is restricted to the Sertoli–spermatid interface of step 8–19 spermatids, whereas gap junction and desmosome are at the Sertoli–spermatid interface of step 1–7 spermatids. Preleptotene spermatocytes transformed from type B spermatogonia at stage VII of the cycle will be transported across the BTB at late VII–VIII of the cycle (see Fig. 13.1). Thus, the actin-based cytoskeleton undergoes extensive restructuring, and this event is tightly coordinated with the tubulin-based cytoskeleton which serves as the track for the transport of germ cells across the BTB. This mechanism is also used to transport developing spermatids across the adluminal compartment during spermiogenesis

activities of Sertoli cells are stage-dependent. It is also noted that at each stage of the epithelial cycle, Sertoli cells are catered to specific cellular events that are unique to that specific stage. For example, in the rat testis, meiosis takes place at

stage XIV of the cycle (Fig. 13.1), whereas the release of sperm at spermiation and the transit of preleptotene spermatocytes at the BTB take place at stage VIII of the cycle (Figs. 13.1 and 13.3). Thus, at stage VIII, Sertoli cells are working in concert with elongated spermatid to prepare for apical ES degeneration at the adluminal compartment, and Sertoli cells per se are also undergoing restructuring in which a "new" BTB behind and the "old" BTB above the preleptotene spermatocytes (note: preleptotene spermatocytes transformed from type B spermatocytes are the only germ cell type that are transported across the BTB to enter the adluminal compartment for further development) in transit are being assembled and disassembled, respectively. Furthermore, these spermatocytes are connected in "clones" via intercellular bridges (Weber and Russell 1987; Fawcett 1961), making their transport across the BTB a highly coordinated and regulated cellular event. These findings thus illustrate Sertoli cells are highly cyclic in function during the epithelial cycle in which the Sertoli cell performs different functions according to the cycle of the epithelium (Fig. 13.1). As such, in order for spermatogenesis to proceed flawlessly during the epithelial cycle, Sertoli cells must be highly polarized, and perhaps compartmentalized, ultrastructurally and functionally, so that different functions can be performed at discrete domains of a Sertoli cell throughout spermatogenesis. This is particularly important since it is known that a single Sertoli cell is in close contact with ~30–50 germ cells at different stages of their development (Wong and Russell 1983; Weber et al. 1983) to support these germ cells functionally, structurally, and nutritionally, as each type of germ cell has distinct functional needs that are different from each other.

13.2 The Seminiferous Epithelial Cycle of Spermatogenesis and Ectoplasmic Specialization

13.2.1 The Epithelial Cycle of Spermatogenesis

The seminiferous epithelial cycle refers to the specific pattern of cellular association between Sertoli and germ cells and the cellular events pertinent to spermatogenesis that take place within the epithelium (de Kretser and Kerr 1988; Hess and de Franca 2008; Mruk et al. 2008). The cycle of events is divided into 14 stages (I–XIV) in the rat (Fig. 13.1) versus 12 (I–XII) and 6 (I–VI) stages in the mouse and human testis, respectively (Hess and de Franca 2008; Amann 2008; Mruk et al. 2008; Parvinen and Vanha-Perttula 1972). Each stage has its own defining characteristics; for instance, in the rat testis, preleptotene spermatocytes are found only in stages VII–VIII of the cycle, spermiation is restricted to stage VIII (lasts ~29.1 h), and meiosis is limited to stage XIV (lasts ~17.6 h) (Fig. 13.2). The duration of a complete epithelial cycle (stages I–XIV) is about 12.8 days in the rat testis. However, a type A1 spermatogonium takes ~58 days to develop into a step 19 (mature) spermatid. Thus, a type A1 spermatogonium goes through the
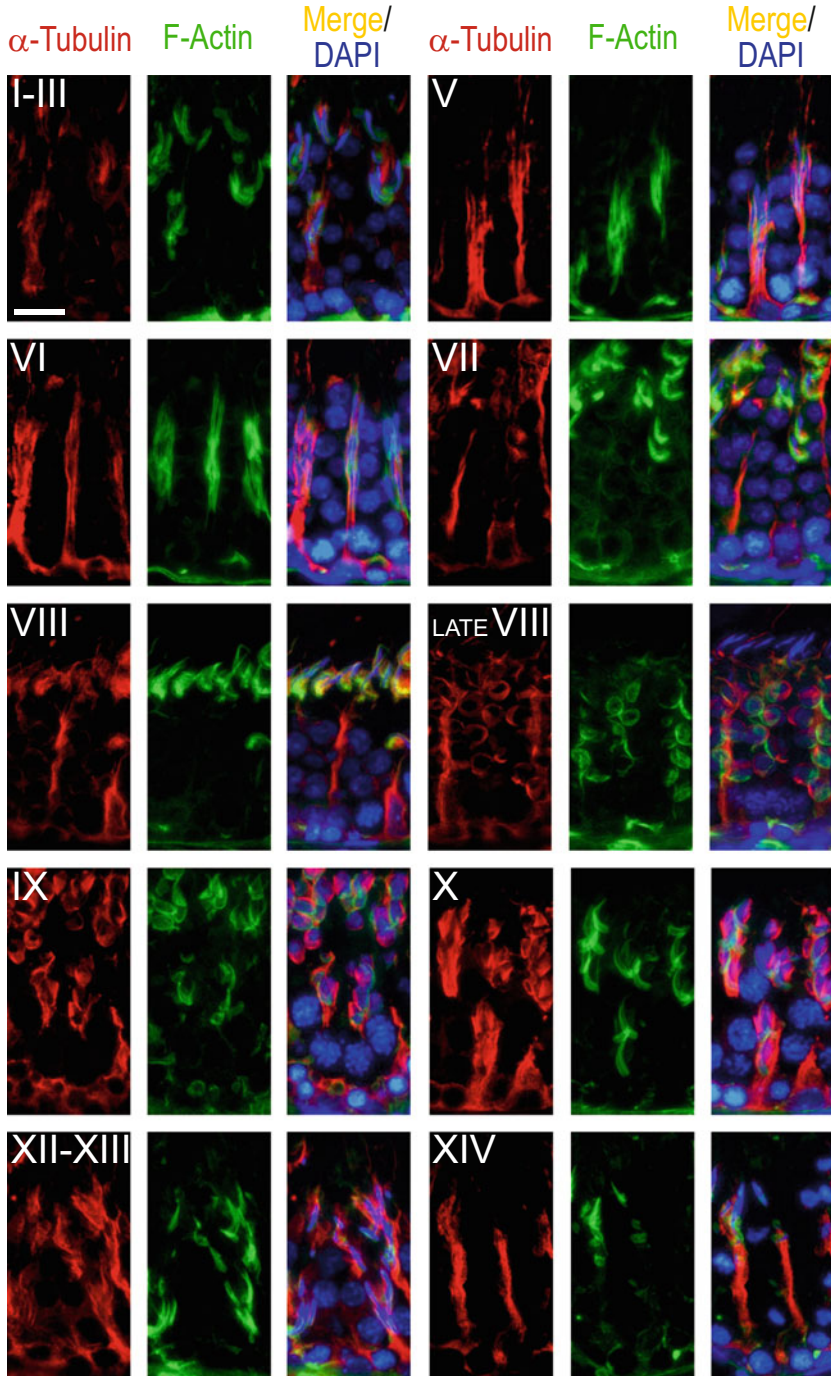
Fig. 13.3 Stage specific expression of actin and α-tubulin. Dual-labeled immunofluorescence analysis was performed in which α-tubulin (*red*) was found to co-localize with and F-actin (*green*) throughout stages I–XIV of the seminiferous epithelial cycle in the rat. Stage I–XIV of the epithelial cycle can also be found in Fig. 13.1. Scale bar, 30 μm

epithelial cycle a total of 4.5 times (Fig. 13.1). In short, Sertoli-germ cell associations differ with each stage, as each stage contains specific types of germ cells progressing through the epithelial cycle (Fig. 13.1). If a discrete section of a seminiferous tubule at stage VIII is visualized under a transillumination microscope, one will witness the progression to stages IX through XIV and then I through VIII again in 12.8 days (Fig. 13.1) (Parvinen 1982; Mruk et al. 2008; Lie et al. 2011).

13.2.2   Ectoplasmic Specialization

When the cross section of a seminiferous tubule, such as in the rat testis, is examined under electron microscope, a testis-specific actin-rich anchoring junction called ectoplasmic specialization (ES) is readily noted which is known to confer cell polarity, cell adhesion, and cellular structural support (Russell and Peterson 1985; Vogl et al. 2008; Cheng and Mruk 2002; Mruk and Cheng 2004a). In the testis, ES is either found in the basal compartment at the Sertoli cell–cell interface at the BTB called basal ES or in the adluminal (apical) compartment at the Sertoli–spermatid interface called apical ES (Fig. 13.2). ES is typified by the presence of actin filament bundles that lie perpendicular to the apposing plasma membranes of either Sertoli–Sertoli (basal ES) or Sertoli–spermatid (apical ES), which are sandwiched in between cisternae of the endoplasmic reticulum and the apposing plasma membranes (Fig. 13.2). Ultrastructurally, apical and basal ES are indistinguishable except that since actin filament bundles are only found in Sertoli cells and not in germ cells, a single array of actin filament bundles is found at the apical ES, whereas a double array of actin microfilaments is found at the basal ES. Once apical ES appears at the interface of Sertoli cells and step 8 spermatids in stage VIII tubules, it becomes the only anchoring device from steps 8 to 19 spermatids, replacing desmosome and gap junction at the Sertoli–spermatid (1–7) interface, and persists until its degeneration at spermiation. Unlike apical ES, basal ES at the Sertoli cell–cell interface never exists alone; rather it coexists with either TJ or gap junction, which together with desmosome constitutes the BTB, one of the tightest blood–tissue barriers in the mammalian body (Franca et al. 2012; Cheng and Mruk 2012; Pelletier 2011; Wong and Cheng 2005) (Fig. 13.2). In fact, when the force that is required to disrupt adhesion induced by apical ES versus desmosome is compared and quantified, ES is at least twice as strong as desmosome to confer steps 8–19 spermatid adhesion to the Sertoli cell (Wolski et al. 2005). This observation is unusual since desmosome, the predominant anchoring junction in skin, is considered to be the strongest adhesive junction in the mammalian body (Green and Simpson 2007; Thomason et al. 2010; Jamora and Fuchs 2002). This unusual strength of the ES apparently is contributed by the extensive network of actin filament bundles (Russell et al. 1988) (Fig. 13.2). It is of interest to note that while the appearance of apical ES at the Sertoli–spermatid (step 8) interface at stage VIII of the cycle replaces desmosome and gap junction between Sertoli cells and

step 1–7 spermatids, apical ES is a hybrid atypical adherens junction (AJ) (Wong et al. 2008a; Yan et al. 2007) since it is constituted by proteins that are found in AJ (e.g., nectins, afadins, N-cadherin, β-catenin), TJ (e.g., JAM-C, CAR), gap junction (e.g., connexin 43), and focal adhesion complex (or focal contact) (e.g., α6β1-integrin, laminin-α3,β3,γ3, FAK, c-Src, c-Yes) (Cheng and Mruk 2010). Regardless of the ultrastructural similarity between the apical and basal ES, proteins that are found at the basal ES/BTB are quite different from those at the apical ES (Cheng and Mruk 2010).

13.2.3   The Uniqueness of the Seminiferous Epithelium Versus Other Epithelia

As noted in Fig. 13.1, the seminiferous epithelium is unique compared to other epithelia due to the seminiferous epithelial cycle of spermatogenesis. This is a unique cellular process in the mammalian body due to the cyclic events of the epithelial cycle such that millions of sperm can be produced from the tubules via spermatogenesis. Furthermore, as germ cells change shape, orientation, and size during maturation, they undergo extensive "adhesion" and "de-adhesion," which involves major restructuring of junctions at the cell–cell interface. Additionally, germ cells are transported progressively from the basal to the adluminal compartment across the epithelium throughout the epithelial cycle so that step 19 spermatids can be lined up at the adluminal edge of the apical compartment to prepare for their release once they are transformed into spermatozoa at late stage VIII of the cycle (Figs. 13.1 and 13.2). Thus, it is envisioned that actin filament bundles at the ES must undergo cyclic reorganization from their "bundled" to "de-bundled" configuration and vice versa to facilitate the transport of preleptotene spermatocytes and spermatids across the BTB and the epithelium. It is of interest to note that germ cells per se are not motile cells, since they lack cellular structures, such as lamellipodia and filopodia found in fibroblasts, macrophages, and keratinocytes, and they rely solely on the Sertoli cell for their transport across the BTB and the epithelium. Thus, the transport of spermatids across the epithelium during spermiogenesis requires the presence of tubulin-based cytoskeleton/microtubules which serve as the track for cargo (e.g., spermatids) to be transported across the epithelium. In short, the actin- and tubulin-based cytoskeletons are working in concert via yet-to-be defined mechanism(s) to facilitate germ cell transport across the epithelium.

13.3 Polarity Proteins on Cell Polarity and Cell Adhesion During Spermatogenesis

13.3.1 Why Is Polarity Important During Spermatogenesis?

Spermatids are highly polarized cells in which the heads of spermatids point toward the basement membrane, while the tails point toward the tubule lumen. Spermatid polarity is crucial to maximize the production of sperm during spermatogenesis since the arrangement of polarized spermatids in the epithelium as noted in Fig. 13.1 allows the maximal number of spermatids that can be packed and developed simultaneously in the epithelium in the tubule. Similarly, polarized Sertoli cells can coordinate cellular events across the epithelium more efficiently so that signals can be sent across the Sertoli cell orderly and Sertoli cell can also communicate with its neighboring cells effectively during the epithelial cycle. In the testis, cell polarity is conferred by the Par (partitioning defective)-based protein complex (e.g., Par6, Par3, Cdc42, aPKC, Pals1, and PatJ), the Scribble-based complex (e.g., Scribble, Dlg, Lgl), and the Crumbs-based complex (e.g., CRB-3, Pals1, Patj) (Wong and Cheng 2009; Wong et al. 2008a). Each of these polarity protein complexes recruits its own binding partners, thus conferring cellular asymmetry; this is because a multiprotein complex can be effectively created for each protein complex and also because there is mutually exclusive distribution between Par-/CRB- and Scribble-based protein complexes across an epithelial cell, such as the Sertoli cell (Iden and Collard 2008; Assemat et al. 2008; Head et al. 2013; Goldstein and Macara 2007). During spermiogenesis, most of the cytosol is eliminated from the developing spermatids and transported to the residual body to be scavenged and cleaned up by the Sertoli cell (Fig. 13.2). Thus, there is scant cytosol remaining in the more mature spermatids, such as step 8–19 spermatids, particularly in the head region where apical ES is present both to anchor spermatids to the Sertoli cell and to confer spermatid polarity (*note*: acrosome that is found at the spermatid head represents a giant proteasome containing acrosin, a serine protease with trypsin-like specificity, and is to be used by the sperm to penetrate the zona pellucida at fertilization (Honda et al. 2002)). The mechanism(s) through which polarity proteins expressed by germ cells (Wong et al. 2008b; Su et al. 2012b) involved in conferring or regulating spermatid polarity is still not known.

13.3.2 Role of Par-Based Polarity Proteins on Cell Adhesion and Polarity in the Testis

Studies in the testis have shown that a knockdown of either Par3 or Par6 specifically by RNAi without detectable off-target effects impedes Sertoli cell TJ barrier. In these studies, proteins at the Sertoli cell–cell interface, such as JAM-A and α-catenin, became mis-localized, as these proteins no longer tightly localized to

the Sertoli cell BTB, but relocated to the cell cytosol, thereby destabilizing the BTB function via a loss of Sertoli cell adhesion (Wong et al. 2008b). Furthermore, it appears that Par3 and Par6 regulate the localization of different BTB proteins differentially since the knockdown of Par3, but not Par6, induces mis-localization of ZO-1, whereas the knockdown of Par6, but not Par3, induces mis-localization of N-cadherin selectively (Wong et al. 2008b). These changes in protein localization appear to be the result of changes in the kinetics of endocytic vesicle-mediated protein trafficking, since the knockdown of Par5 (also known as 14-3-3) is found to accelerate the endocytosis of JAM-A and N-cadherin, thereby destabilizing the Sertoli cell BTB (Wong et al. 2009), illustrating the role of polarity proteins in cell adhesion function. Par6 was also found to be crucial to confer spermatid polarity in the rat testis. Adjudin induces spermatid loss; however, prior to spermatid loss, there is a loss of spermatid polarity as evidenced by the presence of misoriented spermatids in rats treated with the drug. Treatment of rats with adjudin was associated with considerable loss of Par6 surrounding the spermatid heads, which were pointed in all directions in the epithelium (Wong et al. 2008b). More important, this loss of spermatid polarity occurs before a disruption of spermatid adhesion onto the Sertoli cell in the epithelium is detected, seeming to suggest that spermatid polarity and adhesion are two intimately related events regulated by polarity proteins, such as Par3 and Par6, during spermatogenesis in the testis.

13.3.3   Role of Scribble-Based Polarity on Cell Polarity and Adhesion Is Mediated by Changes in the Actin-Based Cytoskeleton in the Testis

Scribble, Lgl (lethal giant larvae), and Dlg (discs large) are found to be expressed by both Sertoli and germ cells in the rat testis (Su et al. 2012b). Scribble is localized most notably at the Sertoli cell–cell interface when Sertoli cells establish a functional TJ-permeability barrier in vitro but it is also found in the cell cytosol (Su et al. 2012b). Scribble is also localized predominantly to the BTB in the seminiferous epithelium in vivo in virtually all stages of the epithelial cycle in the rat testis (Su et al. 2012b), illustrating polarity protein Scribble is involved in Sertoli cell polarity and adhesion, and it is involved in BTB dynamics during the epithelial cycle. While the knockdown of Scribble or Dlg1 alone fails to modulate the Sertoli cell TJ-barrier function, the simultaneous knockdown of Scribble and its two integral component proteins Dlg1 and Lgl2 by RNAi using specific siRNA duplexes with no detectable off-target effects is shown to promote the Sertoli cell TJ-permeability barrier, making it "tighter" (Su et al. 2012b), illustrating its role in inducing BTB restructuring during the epithelial cycle. This promoting effect of Scribble on the Sertoli cell BTB function is supported by studies using immuno-fluorescence microscopy since a considerable increase in occludin and β-catenin

localization to the Sertoli cell–cell interface is found in the Sertoli cell epithelium following the simultaneous knockdown of Scribble, Dlg1 and Lgl2 (Su et al. 2012b). More important, when Scribble/Dlg1/Lgl2 is knocked down in the testis in vivo, an increase in occludin localization to the BTB is detected in stage VIII tubules. This change is accompanied by a loss of spermatid polarity in the adluminal compartment in which spermatids no longer orientate properly with their heads pointing toward the basement membrane; instead spermatid alignment is disarrayed, concomitant with a downregulation of laminin-γ3 (Su et al. 2012b), an apical ES protein limited to the spermatid which forms a bona fide adhesion complex with α6β1-integrin in the Sertoli cell (Yan and Cheng 2006; Koch et al. 1999). Interestingly, this loss of spermatid polarity, downregulation of laminin-γ3 at the apical ES, and the increase in occludin at the BTB are associated with changes in the organization of F-actin at these sites; when F-actin is visualized by rhodamine phalloidin, more F-actin is found at the BTB whereas reduced levels of F-actin are detected at the apical ES (Su et al. 2012b). Collectively, these findings thus illustrate that the Scribble-based polarity protein complex supports spermatid polarity and adhesion, while it also promotes BTB dynamics possibly via restructuring, illustrating its antagonistic effects on the apical and basal ES in the testis during the epithelial cycle.

13.4 Actin- and Microtubule-Based Cytoskeletons and Their Role in Cell–Cell Interactions, Cell Polarity, and BTB Function

13.4.1 Cross Talk Between Actin and Tubulin Cytoskeletons

Across different cell types, both the actin filament and microtubule networks play critical roles in a variety of processes such as cell division, cell polarization, transport, and migration. Although actin- and tubulin-based cytoskeletons are usually portrayed as functionally independent networks, recent research has begun to unravel the cooperative interaction between them via some routes of communication or cross talk (Gavin 1997; Goode et al. 2000). The Sertoli cell is a highly polarized cell, as earlier described, and also is very dynamic via cyclic changes functionally, adapting to the evolving shape of germ cells as they progress through different stages of the epithelial cycle. This is made possible due to the dynamicity of both actin and microtubule (MT) cytoskeletons, both of which exemplify stage specificity within the seminiferous epithelial cycle. For example, at late stage VIII in the rat, both actin and tubulin (e.g., α-tubulin) levels diminish significantly at the apical ES at the onset of the spermiation (Fig. 13.3). Spermiation is a highly regulated temporal event; if not tightly regulated, it can result in premature or delayed release of spermatids (O'Donnell et al. 2011; Mruk and Cheng 2004b). As shown in Fig. 13.3, actin and α-tubulin co-localize in the
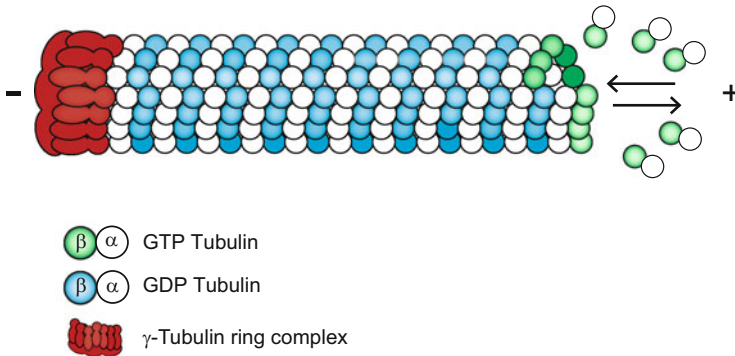
Fig. 13.4 Microtubule structure in mammalian cells including the Sertoli cell in the testis. Microtubules (MTs) are assembled from αβ-tubulin dimers. β-tubulin designates the MT plus (+) end, and α-tubulin the minus (−) end. Polymerization of a MT occurs through the interaction of the α-subunit of an incoming dimer with the β-subunit of a preexisting dimer on a MT protofilament. GTP is bound to both α and β-subunits; however, only the β-tubulin GTP exhibits GTPase activity. GTP hydrolysis occurs as the MT is assembled, leaving most of the MT comprised of GDP-tubulin and the growing plus end as the only region where GTP is still bound to β-tubulin, known as the GTP-cap. γ-tubulin forms a protein complex called the γ-tubulin ring complex and is responsible for nucleation and stabilization of MTs

majority of the 14 stages of the epithelial cycle in the rat testis (*note:* α- and β-tubulin are the structural components of tubulin-based cytoskeleton; see Fig. 13.4). Though this is a morphological observation, it lays the foundation for expanding this information on how actin and MT cytoskeletons work in concert to regulate spermatogenesis.

There are a number of MT regulatory proteins that not only associate with MTs but also with the actin cytoskeleton. For example, CLIP-170 (cytoplasmic linker protein of 170 kDa) is one MT regulatory protein that associates with myosin VI, which is an ATP-dependent actin motor protein, thus linking the actin and MT networks together. CLIP-170 is also known to be involved in the dynein and dynactin pathway (Akhmanova et al. 2005). In general, motor proteins are either plus- or minus-end-directed along MTs. Some formins, such as mDia1, are actin-nucleating proteins that also can stabilize MTs. Both CLIP-170 and mDia1 are present in testis and thus may be the subject of future studies on cross talk between the actin and tubulin networks in regulating key events of spermatogenesis. Table 13.1 summarizes results of findings that illustrate the likely function of microtubule regulatory proteins in microtubule dynamics and their role in male fertility via the use of genetic (such as gene knockout KO or N-ethyl-N-nitrosourea-induced mutation) or knockdown (KD) models. However, the molecular mechanism(s) by which these proteins regulate spermatogenesis remain largely unknown.

Table 13.1 KO/KD studies of microtubule regulatory proteins

Protein	Function(s)	KO/KD/ Mutation	Phenotype	Reference(s)
CLASP1 & 2	MT stability: promote MT rescue, suppress MT catastrophe	KD	Decreased axon and dendrite length in neurons; defects in mitotic spindle and delayed metaphase	(Drabek et al. 2012; Mimori-Kiyosue et al. 2006)
CLIP-170	Regulation of MT dynamics, cell polarity, MT-dependent organelle transport	KO	Male mice were viable, but subfertile with abnormal spermatid head shape	(Akhmanova et al. 2005)
EB1	Regulation of MT dynamics and interaction with cellular elements	KD	Disruption in PKC signaling pathways, cell migration, spindle pole movement	(Schober et al. 2012; Bruning-Richardson et al. 2011)
KATNAL1	MT severing	ENU-induced mutation	Premature release of immature sperm and male infertility	(Smith et al. 2012)
KATNB1	MT severing	ENU-induced mutation	Mice were sterile; decrease in sperm production; abnormal spermatid head shape	(O'Donnell et al. 2012)
MCAK	Regulation of MT dynamics and mitosis, depolymerizes MTs	KD	Defects in chromosome congression and segregation	(Stout et al. 2006; Ganem et al. 2005; Kline-Smith et al. 2004)
mDia1	Actin polymerization and MT stabilization	KD	Decreased MT stabilization	(Bartolini et al. 2012)
Tau	MT assembly and stabilization; predominant in neurons	KO	Mice develop normally, delay in neuronal maturation, some behavioral effects reported	(Dawson et al. 2001; Harada et al. 1994; Denk and Wade-Martins 2009)
Stathmin	Regulation of MT dynamics and mitosis	KO	Mice displayed normal phenotype, but developed axonopathy of CNS and PNS as they aged	(Schubart et al. 1996; Liedtke et al. 2002)
		KD	Delayed mitotic entry in HeLa cells	(Silva and Cassimeris 2013)

(continued)

Table 13.1 (continued)

Protein	Function(s)	KO/KD/ Mutation	Phenotype	Reference(s)
XMAP215	Regulation of MT dynamics, MT polymerase	KD	Reduced axon growth	(Lowery et al. 2013)

CLASP CLIP-170 associated protein, *CLIP-170* cytoskeletal linker protein 170, *EB1* end-binding protein 1, *KATNAL1* katanin p60 subunit A-like1, *KATNB1* p80 regulatory subunit of katanin, *MCAK* mitotic centromere-associated kinesin, *mDia1* mammalian protein diaphanous homolog 1, *XMAP215 Xenopus* microtubule-associated protein 215; KO, knock-out; KD, knockdown; ENU, N-ethyl-N-nitrosourea.

13.4.2 *Functional Role of Actin- and Tubulin-Based Cytoskeletons in the Testis*

The current understanding of spermatid transport via MTs and the involvement of actin-based cytoskeleon in the seminiferous epithelium remains elusive. Microtubules are abundant in Sertoli cells, and research thus far has implicated their role by serving as the tracks for translocation of spermatids throughout the epithelial cycle. As germ cells mature, they adopt an elongate shape; concomitant with elongation, spermatids are enveloped by the Sertoli cell, and these cells, unlike macrophages, neutrophils, and fibroblasts, are not motile cells. Instead, their transport across the epithelium during the epithelial cycle relies completely on Sertoli cells, and as such, it is logical to speculate that the "cargoes" (i.e., germ cells) require the presence of a "track" (i.e., polarized microtubules) for their transport (Redenbach and Vogl 1991). Indeed, microtubules, which are intrinsically polar, are arranged with their plus and minus ends directed basally and apically, respectively, within the Sertoli cell. Studies have revealed the presence of microtubule motor proteins in the testis, such as dynein and kinesin, which are MT minus and plus-end-directed motor proteins (Hall et al. 1992), supporting the idea that microtubules are in part responsible for the organized movement of spermatids across the seminiferous epithelium. Dynein is classified as a minus-end-directed motor protein, but when it forms a complex with dynactin, an adaptor, it can also be targeted to the plus end (Kardon and Vale 2009). During the epithelial cycle, germ cells are transported progressively up from the basal to the apical region of the seminiferous epithelium; however, during stage V in the rat, developing spermatids actually return to the basal region and are found deep inside the Sertoli cell crypts. This suggests the importance of bidirectional transport along the MTs during spermatogenesis. It has been proposed that the cytoplasmic side of the endoplasmic reticulum (ER) of the apical ES comes into contact with the microtubules, MTs thus confer ER the ability to move along the tracks while the actin filaments of the apical ES anchor the spermatid (Hall et al. 1992; Russell 1977).

Microtubules are polar cylindrical structures made from protofilaments of α-β-tubulin heterodimers. Protofilaments, which are assembled by the head-to-tail addition of αβ-tubulin subunits, arrange laterally to form a hollow tube (Fig. 13.3). MTs possess a property called dynamic instability, which describes both the

polymerization and depolymerization that occurs at the plus end of MTs (Wade 2007; Mitchison and Kirschner 1984). As listed in Table 13.1, there are a number of proteins that regulate microtubules. For example, MT plus-end tracking proteins (+TIPs) such as CLIP-170 and EB1 can stabilize MTs. It is conceivable that these proteins are helping to stabilize MTs as spermatids are transported directionally across the epithelium. Stabilizing agents ensure a "steady ride" as the spermatids migrate ultimately toward the apex of the epithelium. Severing proteins, another type of MT regulatory protein, like katanin, which has been studied in the testis (Smith et al. 2012; O'Donnell et al. 2012), may play an important role in regulating transport. Tubulin expression is stage specific, as immunohistological staining show (Fig. 13.3), for instance, in some stages of the epithelial cycle, the length of the MTs that appears as "stalks" in the seminiferous epithelium in late VIII-IX stages are shorter than in others such as at stages V-VII (Wenz and Hess 1998). This observation may be attributed to severing proteins which as the name suggests, sever or cut the MT, to promote generation of new MTs. Transport which occurs across the seminiferous epithelium is a continuous process; thus it is probable that generation of new MTs coincides with spermatid movement.

In epithelia, apicobasal polarity requires specific targeting of proteins to both the apical and basal regions of the cell type. In the testis, protein trafficking in the seminiferous epithelium is crucial for regulation of discrete cellular events of spermatogenesis, such as mitosis, meiosis, spermiogenesis, and spermiation. Most of the current research on protein trafficking and cell–cell communication in the testis has only begun to elucidate the intimate relationship between the actin cytoskeleton and regulation of these cellular events (Su et al. 2013). Although most studies focus on actin dynamics and protein trafficking, there is much yet to be uncovered. In addition, the microtubule cytoskeleton is also at play in regulating the events of spermatogenesis, but how it does so is still the subject of future research.

Cell junction protein recruitment and endocytosis are two types of processes, not only found in the seminiferous epithelium but across all epithelia. Polarity proteins such as Par3/Par6, Scribble, Lgl, 14-3-3, and Cdc42 are involved in regulating these processes in the testis (Wong et al. 2008b; Su et al. 2012b). For example, Par3/Par6, which are established polarity proteins, has been shown to confer spermatid adhesion at the apical ES (Wong et al. 2008a). Changes in cell adhesion regulated by Par proteins may likely coordinate protein endocytosis, which is in part regulated by the actin network (Wong et al. 2009). Microtubules also play a role in endocytosis, via transport of endosomes and lysosomes (Matteoni and Kreis 1987), but the mechanism in the testis has yet to be defined. As previously mentioned, CLIP-170 is one MT regulatory protein that is involved in MT dynamics. This protein was first discovered for its role in linking endocytic vesicles to the MTs (Pierre et al. 1992); in addition to its role as a + TIP, it is a likely player in endocytic trafficking in the testis.

13.5   Polarity Proteins and Cytoskeletons in the Apical ES–BTB–Basement Membrane (BM) Functional Axis

13.5.1   The Apical ES–BTB–BM Functional Axis

The concept of a local functional axis that coordinates and regulates cellular events taking place across the seminiferous epithelium during the epithelial cycle was first reported in 2008 (Yan et al. 2008). It was noted that overexpression of fragments of laminin chains at the apical ES or purified recombinant proteins perturbed the Sertoli cell TJ-permeability barrier function by downregulating expression of proteins at the BTB, such as occludin and N-cadherin, but also β1-integrin at the hemidesmosome (HD) at the Sertoli-BM interface (Yan et al. 2008). These findings thus illustrate that matrix metalloproteinase-2 (MMP-2) which is highly expressed at the apical ES at stage VIII of the epithelial cycle (Siu and Cheng 2004) is capable of inducing cleavage of laminin chains at the apical ES during its degeneration to prepare for spermiation to release biologically active fragments. These autocrine-like fragments in turn induce BTB restructuring to facilitate the transit of preleptotene spermatocytes across the BTB. These fragments also perturb HD function, which creates a positive regulatory loop to further potentiate BTB restructuring. This possibility was confirmed by silencing β1-integrin at the HD in Sertoli cells, which indeed was found to perturb the Sertoli cell TJ-permeability function (Yan et al. 2008). Taken collectively, these data illustrate the presence of a functional axis that links cellular events that occur at the apical ES in the adluminal compartment at spermiation with BTB restructuring near the BM in the seminiferous epithelium, and also HD in the BM, at stage VIII of the epithelial cycle. Subsequently studies have shown that MMP-9 is also capable of inducing cleavage of collagen chains, mostly collagen α3(IV), in the BM to release the NC1 (non-collagenous 1) domain peptide, which was shown to perturb the Sertoli cell TJ barrier function (Wong and Cheng 2013). While the purified NC1 recombinant protein was shown not to downregulate the expression of BTB-associated proteins such as CAR-ZO-1 and N-cadherin-β-catenin, it effectively induced mis-localization of these proteins at the Sertoli cell BTB, so that they no longer localized predominantly at the Sertoli cell–cell interface; instead, they were relocalized to the cell cytosol, thereby destabilizing the Sertoli cell TJ-barrier function (Wong and Cheng 2013). Studies using the phthalate-toxicant model have confirmed the presence of this local functional axis in which a disruption of the apical ES by phthalate induces BTB restructuring and can compromise its integrity (Yao et al. 2009, 2010). Collectively, these data demonstrate unequivocally that there is a functional autocrine-based regulatory axis that coordinates cellular events, such as spermiation and BTB restructuring, which take place simultaneously at the opposite ends of the epithelium at stage VIII of the epithelial cycle . In brief, apical ES degeneration as well as HD/BM restructuring contribute to BTB restructuring, which is further induced by re-organization of collagen network in the BM at the tunica propria by generating NC1 domain-containing peptide. A recent study has identified the biologically active domain of the laminin-γ3 chain that induces BTB restructuring, and synthetic peptide based on this

functional domain designated F5-peptide is known to perturb BTB integrity in vivo reversibly and it also induces germ cell loss from the epithelium, illustrating its potential to serve as an endogenously produced male contraceptive peptide (Su et al. 2012a). These findings using F5-peptide based on the biologically active fragment of laminin-γ3 chain illustrate that the apical ES-BTB-BM axis can be a target of male contraceptive development. In fact, studies have shown that this functional axis is a target of environmental toxicants, such as phthalates, BPA, cadmium, and PFOS (perfluorooctane sulfonate) (Wan et al. 2013b; Mazaud-Guittot 2011). A disruption of the critical regulatory components in this axis following exposure of men to these toxicants is likely the cause of reduced semen quality and sperm count as recently reported (Rolland et al. 2013; Toft et al. 2012).

13.5.2   Role of Focal Adhesion Kinase (FAK), Polarity Proteins, and Cytoskeletons at the Apical ES–BTB–BM Functional Axis

While the apical ES–BTB–BM functional is crucial to coordinate cellular events that take place in the seminiferous epithelium during the epithelial cycle, the molecules that are involved in the regulation and the underlying molecular mechanism(s) remain unknown. Recent studies have shown that non-receptor protein tyrosine kinases, such as FAK (Lie et al. 2012) and c-Yes (Xiao et al. 2013); polarity proteins, such as Par6 (Xiao et al. 2013); and actin-based cytoskeleton (Su et al. 2012a) are critical players in this functional axis. For instance, studies using different mutants of FAK have shown that p-FAK-Tyr^{407} and p-FAK-Tyr^{397} are crucial to the integrity of the BTB and apical ES, respectively (Lie et al. 2012). In fact, these two phosphorylated forms of FAK are shown to display antagonistic effects on the BTB integrity in which p-FAK-Tyr^{407} promotes whereas p-FAK-Tyr^{397} disrupts the Sertoli cell TJ-permeability barrier function (Lie et al. 2012). However, p-FAK-Tyr^{397} plays a dominant role in maintaining spermatid adhesion via its effects on the adhesive function of the apical ES (Wan et al. 2013a). Thus, p-FAK-Tyr^{407} and p-FAK-Tyr^{397} likely serve as the molecular "switches" by turning "on" and "off" cell adhesion function at the Sertoli cell BTB and also Sertoli–spermatid interface along the apical ES–BTB–BM axis. It has been reported that overexpression of p-FAK-Tyr^{407} that promotes BTB integrity can block the F5-peptide-induced Sertoli cell TJ-permeability barrier disruption (Su et al. 2012a). Also, the F5-peptide-mediated BTB disruption and spermatid loss in vivo is accompanied by a mis-localization of p-FAK-Tyr^{407} in which this activated form of FAK is no longer restricted tightly to the BTB and the apical ES (Su et al. 2012a). This, in turn, perturbs the organization of F-actin at the apical ES and the BTB (Su et al. 2012a), such that actin filament bundles fail to be properly reorganized at both sites in response to the epithelial cycle of spermatogenesis, likely the result of a disruption in actin polymerization. Thus, a failure in F-actin

organization can no longer support adhesive function at the BTB and the apical ES, leading to unwanted BTB and apical ES restructuring or degeneration. This postulate is supported by studies in vitro since the promoting effects of p-FAK-Tyr[407] on BTB integrity is mediated, at least in part, via the Arp2/3-N-WASP protein complex that alters the kinetics of branched actin polymerization (Lie et al. 2012; Cheng et al. 2013). In this context, it is of interest to note that since the F5-peptide administered to the testis can be rapidly metabolized and cleared, its disruptive effects on spermatogenesis are reversible and germ cells gradually re-populate the epithelium (Su et al. 2012a).

13.5.3   The Role of c-Yes, p-FAK-Tyr[407], Par6, and F-actin on BTB and Apical ES Function at the Apical ES–BTB Axis

Studies have shown that FAK is the putative substrate of Src family kinases (SFK) such as c-Src and c-Yes in most epithelia including the seminiferous epithelium (Zhao and Guan 2010; Boutros et al. 2008; Xiao et al. 2012). Also, multiple proteins at the BTB and apical ES are binding partners of SFK and/or FAK (Xiao et al. 2012; Li et al. 2013; Cheng and Mruk 2012). In fact, the dual FAK/Src complex is one of the primary targets of chemotherapy (Bolos et al. 2010) and inflammatory and autoimmune diseases (Lowell 2011). Thus, it is not surprising that c-Yes is recently shown to be a crucial player in the apical ES–BTB axis (Xiao et al. 2013). For instance, a knockdown of c-Yes by RNAi was shown to perturb the Sertoli cell TJ-barrier function both in vitro and in vivo, mediated via a disorganization of F-actin at the BTB, in which actin microfilaments no longer tightly restricted to the BTB near the BM (Xiao et al. 2013). These findings are consistent with an earlier report by using SU6656, a selective inhibitor of c-Yes, to probe the role of c-Yes in modulating F-actin organization in Sertoli cells (Xiao et al. 2011). Interestingly, the knockdown of c-Yes in the testis that affects the BTB integrity also perturbs apical ES function, disrupting spermatid polarity and adhesion, which is mediated by a mis-localization of p-FAK-Tyr[407] and also polarity protein Par6 (Xiao et al. 2013), illustrating there is a feedback loop between the apical ES and the BTB. These changes, namely, mis-localization of p-FAK-Tyr[407] and Par6 at the apical ES, thus impede actin microfilaments at the apical ES, leading to mis-localization of adhesion protein nectin-3, causing defects in spermatid transport and spermiation, so that elongated spermatids are entrapped in the seminiferous epithelium even at the site close to the BM in stage IX tubules (Xiao et al. 2013). These data thus illustrate the intimate functional relationship between FAK/SKF (e.g., p-FAK-Tyr[407], c-Yes), polarity proteins (e.g., Par6), and cytoskeletons (e.g., actin microfilaments). Any changes on the cross talk between these proteins would impede cell adhesion function at the apical ES and/or the BTB, illustrating their pivotal role in the apical ES–BTB–BM functional axis.

13.6   Concluding Remarks and Future Perspectives

Findings discussed herein thus illustrate the intimate relationship between polarity proteins, cell–cell interactions at the Sertoli–Sertoli, and Sertoli–spermatid interface and cytoskeleton in the seminiferous epithelium during the epithelial cycle. It is also noted that non-receptor protein tyrosine kinases, in particular FAK and SFK (e.g., c-Yes and c-Src), are intimately involved in these events. At the time of this writing, no concrete data were found in the literature providing credible information regarding the mechanism(s) by which polarity proteins regulate cytoskeleton and vice versa in the testis. Except for in studies using different animal models, such as the adjudin model, it was shown that the loss of spermatid polarity due to a downregulation of Par6 was likely mediated by adjudin-induced truncation and defragmentation of actin filament bundles at the apical ES (Wong et al. 2008b). This thus destabilized the actin-based adhesion protein complexes at the apical ES, such as integrin–laminin, nectin–afadin, leading to premature loss of spermatids from the epithelium, analogous to "spermiation." Also, we have yet to integrate the concept regarding the role of tubulin-based cytoskeleton into the biology of spermatid transport using the apical ES and the biology of preleptotene spermatocyte transport at the BTB using the basal ES, and how actin- and tubulin-based cytoskeletons are working in concert to regulate germ cell transport. For instance, several actin regulatory proteins have been identified and studied in the testis; virtually no tubulin regulatory proteins have been investigated in the testis except for several microtubule motor proteins, such as dynein and kinesin. This is an area of research that deserves some attention in future years.

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