Focal adhesion kinase (FAK), as its name implied, is an important mediator of integrin-based signaling function in mammalian cells at the focal adhesion complex (FAC, also known as focal contact) at the cell-extracellular matrix interface. FAK is intimately related to cell movement, such as in macrophages, fibroblasts and also tumor cells. In the testis, however, FAK and two of its phosphorylated forms, p-FAK-Tyr\(^{407}\) and p-FAK-Tyr\(^{397}\), are not found at the FAC since there is no ultrastructure analogous or similar to FAC in the mammalian testis vs. other epithelia. Instead, FAK and its two phosphorylated forms are detected along the seminiferous epithelium in the rat testis at the cell-cell interface in a testis-specific adherens junction (AJ) known as the ectoplasmic specialization (ES). ES is an F-actin-rich ultrastructure in which bundles of actin filaments are sandwiched in-between plasma membrane and cisternae of endoplasmic reticulum not found in other mammalian epithelial/endothelial cells. The ES is restricted to the interface of Sertoli cells and spermatids (step 8–19) known as the apical ES, and to the Sertoli cell-cell interface known as the basal ES. Interestingly, the basal ES is also an integrated component of the blood-testis barrier (BTB), coexisting with tight junction (TJ) and gap junction (GJ), and it is conceivable that actin filament bundles at the ES undergo extensive organization, converting from their “bundled” to “de-bundled/branching” configuration to facilitate transport of germ cells across the epithelium and at the BTB during the epithelial cycle.

A recent report (Lie et al. PNAS 2012; 109:12562–12567) has demonstrated that the stage-specific and spatiotemporal expression of p-FAK-Tyr\(^{407}\) and p-FAK-Tyr\(^{397}\) are crucial to the regulation of these events via their stage-specific and spatiotemporal expression during the epithelial cycle mediated by their effects on the organization of the actin filament bundles at the ES, involving actin binding/regulatory proteins. In this Commentary, we will critically evaluate these findings in light of other recent reports in the field. While these ideas are based on studies in the BTB in the rat testis, this information should be applicable and helpful to investigators studying other tissue barriers.

Introduction

The seminiferous tubule is the functional unit in the mammalian testis, such as in human males, which produces millions of spermatozoa each day since puberty.\(^1\)–\(^3\) This is made possible since spermatogonial stem cells (SSC) found in the SSC niche,\(^4\),\(^5\) such as in rodents, are capable of self-renewal and give rise to type A spermatogonia. Each of the type A spermatogonia known as Asingle (or As), being considered as a SSC,\(^6\) undergoes a series of mitotic divisions while differentiating into more advanced germ cells, to be followed by meioysis to give rise to 4096 spermatids, which, theoretically can transform into spermatozoa,\(^5\),\(^6\) and each spermatozoan is capable of fertilizing an egg. Even though many of these germ cells enter apoptosis to avoid overwhelming the fixed number...
of Sertoli cells in the testis, such that a Sertoli:germ cell ratio of ~1:30–1:50\(^7,8\) can be maintained, there are virtually unlimited supplies of sperm cells in the mammalian testis throughout the entire adulthood due to the self-renewal capability of SSC to give rise to spermatogonia.

In the cross-section of a seminiferous tubule, SSC, spermatogonia (different type A spermatogonia, as well as intermediate and type B spermatogonia), and preleptotene spermatocytes differentiated from type B spermatogonia, are all located in the basal compartment, outside the blood-testis barrier (BTB), created by tight junction (TJ), basal ectoplasmic specialization [ES, a testis-specific adherens junction (A)\(1\)], gap junction (GJ) as well as desmosome between adjacent Sertoli cells near the basement membrane (Fig. 1). Preleptotene spermatocytes, however, are the only germ cells that can cross the BTB that take place at stage VIII of the epithelial cycle while transforming and differentiating into leptotene and zygotene spermatocytes, so that late pachytene spermatocytes can undergo diakinesis to prepare for meiosis I/II behind the BTB in the specialized microenvironment in the seminiferous epithelium known as the adluminal compartment (Fig. 1). In short, meiosis I/II and all the post-meiotic spermatid development are segregated from the systemic circulation due to the BTB, and spermatid development during spermiogenesis rely exclusively on the structural and nourishment supports of the Sertoli cell in the adluminal compartment (Fig. 1).

Interestingly, using electron microscopy, extensively bundles of actin filaments are noted at the Sertoli cell-cell interface at the BTB, coexisting with either TJs or GJs, and this ultrastructure is known as the basal ES (Fig. 1). Similar network of actin filament bundles are also found at the Sertoli cell-spermatid interface (from step 8–19 spermatids in the rat testis) known as the apical ES, except that once the apical ES appears, it is the only anchorage device, replacing GJs and desmosomes. Unlike the basal ES, the apical ES never coexists with other junctions (Fig. 1). This unusual network of actin filament bundles confers strong adhesive function to the BTB, making it one of the tightest blood-tissue barriers in mammals,\(^6,9-11\) and it also confers strong adhesion to developing spermatids, making the apical ES one of the strongest anchoring junctions, almost twice as strong as the desmosome when the “force” required to tear the apical ES apart vs. the desmosome was quantified.\(^12\)

Interestingly, the apical and the basal ES undergo extensive restructuring during the epithelial cycle of spermatogenesis since spermatids are transported by the Sertoli cell across the seminiferous epithelium during the epithelial cycle, whereas preleptotene spermatocytes are also transported across the BTB at stage VIII of the epithelial cycle. Thus, it is envisioned that these actin filament bundles must be reorganized continuously throughout the epithelial cycle, converting their “bundled” to a “de-bundled” configuration, such that these strong adhesion junction can be “opened/disassembled” to facilitate the transport of spermatids and preleptotene spermatocytes across the epithelium and the BTB, respectively.

### Proteins that Regulate the Organization of Actin Filament Bundles at the ES

**Eps8 and the Arp2/3 complex.** Eps8 (epidermal growth factor receptor pathway substrate 8), an actin barbed end capping and bundling protein,\(^13,14\) was shown to confer actin filament bundles at the ES in the rat testis, maintaining the ES integrity during the epithelial cycle via its stage-specific spatiotemporal expression at the apical and basal ES.\(^15\) However, it is also noted that in stage VII tubules, virtually no Arp3 is detected at the BTB (see white arrowheads in Figure 2B). In stage VIII tubules, when spermatiation and BTB restructuring take place simultaneously, the expression of Eps8 at the apical and the basal ES is virtually non-detectable so that actin filament bundles can be converted
Figure 1. A schematic drawing of a hypothetical model depicting the likely role of two FAK phosphorylated forms, p-FAK-Tyr^{397} and p-FAK-Tyr^{407}, in conferring plasticity to the actin filament bundles at the ES via their stage-specific and spatiotemporal expression in the seminiferous epithelium during the epithelial cycle of spermatogenesis. On the left panel is a schematic drawing of the seminiferous epithelium of a stage VII tubule wherein intact filament bundles at the apical ES and the basal ES are maintained by an upregulation on the expression of p-FAK-Tyr^{397} and p-FAK-Tyr^{407}, respectively, via the intrinsic activity of Eps8 and other actin filament bundling proteins (e.g., palladin) to confer ES adhesion function at both sites. However, in stage VIII tubules (right panel), downregulation of p-FAK-Tyr^{397} but changes in the association of p-FAK-Tyr^{407} with Arp3 (or with other actin bundling proteins)—even though the expression of p-FAK-Tyr^{407} remains upregulated at this stage—favor re-organization of the actin filament bundles such as from their "bundled" to their "un-bundled/branched" configuration in the microdomain within the BTB (see text for details) to induce BTB restructuring at the basal ES to accommodate the transport of preleptotene spermatocytes across the BTB and spermiation at the apical ES. Many questions (annotated by "?"") remain unanswered as noted in this hypothetical model. For instance, it is not known if biologically active fragments released at the apical ES during spermiation or at the hemidesmosome can regulate the spatiotemporal expression of p-FAK-Tyr^{397} and p-FAK-Tyr^{407}, and/or the interaction between p-FAK-Tyr^{407} and actin regulatory proteins (e.g., Arp3, ) to recruit these proteins to the site to induce actin filament reorganization.

Also, it remains to be determined if small RNAs that are abundantly found in germ cells are being used to regulate the spatiotemporal expression of these two activated FAK forms in the apical ES-BTB-basement membrane functional axis. Also, it remains to be determined if these small regulatory RNAs are being transported from germ cells to Sertoli cells via gap junctions at different stages of the epithelial cycle.
to a “de-bundled” state to facilitate these events (Fig. 2A). However, Arp3 is also not needed at the apical ES because of the release of the sperm at spermiation in stage VIII tubules. But the expression of Arp3 is high at the basal ES to facilitate BTB restructuring while the expression of Eps8 at the basal ES is virtually non-detectable (Fig. 2B vs. 2A). These findings illustrate an efficient physiological mechanism is in place in the testis to regulate the dynamic organization of actin filaments at the ES during the epithelial cycle from their “bundled” to their “debundled/branched” configuration via the intrinsic, but antagonistic, activities of two actin regulatory proteins. In short, Eps8 favors “bundled” and “debundled/branched” actin configuration, respectively, at the ES during the epithelial cycle of spermatogenesis.

Drebrin E and the Scribble/Lgl/Dlg complex. Recent studies have shown that the antagonistic actions of these two proteins are being assisted by other proteins at these sites, such as drebrin E, which was shown to have high affinity with Arp3, but not Eps8, and displaying similar spatiotemporal and stage-specific expression as of Arp3 in the seminiferous epithelium of rat testes. Furthermore, recent studies have shown that the F-actin network at the ES is also maintained and regulated by the Scribble/Lgl/Dlg polarity proteins. This conclusion is based on the observations that the simultaneous knockdown of Scribble and its two partner component proteins Lgl2 and Dlg1 in the testis in vivo was found to induce defects in spermatid polarity since adhesion proteins (e.g., laminin-γ3) were mis-localized, failing to confer proper spermatid polarity in stage VII-VIII tubules; and occludin was also failed to undergo proper re-distribution in stage VIII tubules by moving away from the BTB to facilitate restructuring of the barrier to accommodate the transit of preleptotene spermatocytes at the site, its “unwanted” presence in stage VIII tubules thereby strengthening the Sertoli cell TJ-permeability barrier. In short, the proper functioning of the ES in the epithelium during the epithelial cycle requires the stage-specific, spatiotemporal and also differential expression of Eps8 and Arp3, coupled with other supporting proteins (e.g., drebrin E), as well as other regulatory proteins [e.g., the Scribble/Dlg/Lgl complex, secreted Frizzled-related protein 1 (sFRP1)]. More important, the intrinsic activities of these proteins, at least the Arp2/3 complex, appear to be regulated by the differential expression of a crucial signaling molecule FAK at the ES.

**FAK and Its Two Phosphorylation Forms:**

FAK and Its Two Phosphorylation Forms: p-FAK-Tyr407 and p-FAK-Tyr397

Earlier studies have demonstrated that there is a local functional axis designated the apical ES-BTB-basement membrane that coordinates cellular events that take place across the seminiferous epithelium during the epithelial cycle, such as the events of spermiation and BTB restructuring which occur simultaneously at the opposite ends of the seminiferous epithelium at stage VIII of the cycle. This functional axis and its physiological significance have
recently been confirmed by others using the phthalate-induced Sertoli cell injury model\textsuperscript{27-29} and reviewed.\textsuperscript{30-32} Studies have shown that the subtle changes detected at these sites during these cellular events, such as the release of sperm at spermiation and BTB restructuring that accommodate the transit of preleptotene spermatocytes across the BTB, are largely mediated by changes in the organization of the actin filament bundles, which in turn induce changes in cell adhesion functions at the apical and the basal ES.\textsuperscript{30,33} While the findings summarized above suggest that re-organization of the actin filament bundles at the apical and the basal ES during the epithelial cycle is regulated by Eps8 and the Arp2/3 complex plus several partner proteins (e.g., drebrin E, the Scribble/Dlg/Lgl, sFRP1), the underlying signaling molecules remain unknown. A recent report from our laboratory has shown that the two phosphorylated forms of FAK, p-FAK-Tyr\textsuperscript{397} and -Tyr\textsuperscript{407}, are the likely regulatory molecules in this axis that mediate their effects on the F-actin organization at the ES.\textsuperscript{34} It was shown that p-FAK-Tyr\textsuperscript{407} and -Tyr\textsuperscript{407} displayed stage-specific and spatiotemporal expression at the ES in which p-FAK-Tyr\textsuperscript{407} highly expressed and localized to the apical ES (restricted mostly to the concave side of spermatid heads where endocytic vesicle-mediated protein trafficking take place) and the basal ES at the BTB in stage VII-VIII tubules, likely being used to facilitate ES restructuring.\textsuperscript{22} This concept is supported by the finding that p-FAK-Tyr\textsuperscript{407} structurally interacts with Arp3 and N-WASP, but not Eps8, and such interaction increases by at least 3-fold following overexpression of a phosphomimetic mutant of FAK-Y407E.\textsuperscript{22} However, p-FAK-Tyr\textsuperscript{397} is also highly expressed at the apical ES but restricted almost exclusively to the convex side of spermatid heads and co-localized with β1-integrin (a putative apical ES protein), conferring spermatid adhesion in stage VII-VIII tubules until spermiation takes place at late stage VIII, but not detectable at the basal ES.\textsuperscript{22} In short, p-FAK-Tyr\textsuperscript{407} appears to be important to confer and promote the apical ES adhesion function, but it appears to have an antagonistic effect to the basal ES function. This possibility is also supported by the finding that overexpression of a non-phosphorylatable mutant FAK-Y397F promotes the Sertoli cell tight junction (TJ)-permeability barrier function, making the TJ barrier tighter.\textsuperscript{22} In short, these findings illustrate that p-FAK-Tyr\textsuperscript{407} promotes Sertoli cell BTB integrity at the basal ES and endocytic vesicle-mediated protein trafficking at the apical ES, whereas p-FAK-Tyr\textsuperscript{397} promotes apical ES adhesion and promotes basal ES disruption that perturbs the BTB integrity. However, it is striking to observe that the promoting effects of p-FAK-Tyr\textsuperscript{407} on the Sertoli cell BTB function is mediated via its association with Arp3 and an increase in actin polymerization rate,\textsuperscript{22} suggesting that while Arp3 promotes branched actin polymerization which favors the conversion of actin filaments from their “bundled” to their “debundled” and “branched” configuration, but if this intrinsic activity is confined to a specific cellular microdomain within the Sertoli cell BTB microenvironment, such as in the Sertoli cell cytosol instead of at the cell-cell interface, a functional and intact TJ-permeability barrier can still be maintained. Indeed, it was shown that when FAK Y407E was overexpressed in Sertoli cells and there was a -3-fold increase in association between FAK Y407E and the N-WASP/Arp3 complex, actin filament bundles in the Sertoli cell cytosol were found to be re-organized and re-distributed in which actin filament bundles were localized mostly at the Sertoli cell-cell interface instead of spreading across the Sertoli cell cytosol as seen in the control cells.\textsuperscript{22} Thus, it is likely that such an increase in FAK Y407E expression activates the Arp2/3-N-WASP complex to induce re-organization of the F-actin at the Sertoli cell BTB in which these bundles of actin filaments are re-organized and re-distributed to the Sertoli cell-cell interface that promotes the integrity of the Sertoli cell TJ-permeability barrier instead of eveny distributed across the cell cytosol. If this concept is true, it is likely that an actin cross-linking and bundling protein in the testsis, such as palladin,\textsuperscript{34} is involved in this event since Eps8 was shown not to be structurally associated/interacted with p-FAK-Tyr\textsuperscript{407}.\textsuperscript{22} In short, we speculate that palladin may be working in coordination with the Arp2/3-N-WASP complex in which actin filament bundles are “de-bundled” across the cell cytosol via the action of the Arp2/3-N-WASP complex, whereas new actin filament bundles are actively formed at the Sertoli cell-cell interface via the action of palladin. This speculation is supported by a recent report illustrating that palladin exhibits highly stage-specific and spatiotemporal expression at the ES in the rat testis, and its knockdown by RNAi was also found to perturb both the apical ES and the basal ES function, such as the loss of spermatid polarity and Sertoli cell TJ-barrier function, respectively.\textsuperscript{35} Nonetheless, effects of overexpression these FAK mutants on the distribution of palladin and/or association with a specific FAK mutant in Sertoli cells remain to be established. Furthermore, filamin A, a cross-linker of actin filaments in mammalian cells including Sertoli cells,\textsuperscript{36} was shown to be crucial to recruiting adhesion protein complexes (e.g., JAM-A/ZO-1, N-cadherin/β-catenin) to the BTB for its assembly during postnatal development in the rat testis,\textsuperscript{37} may also be involved in the BTB homeostasis mediated by p-FAK-Tyr\textsuperscript{407} and -Tyr\textsuperscript{397}. Nonetheless, these findings illustrate that much work is needed to better understand the intriguing functional and physiological interactions between FAK and its phosphorylated forms (e.g., p-FAK-Tyr\textsuperscript{407}, p-FAK-Tyr\textsuperscript{397}), actin regulatory proteins (e.g., Eps8, the Arp2/3 complex, palladin, filamin A) and polarity proteins (e.g., the Scribble/Lgl/Dlg complex, Par6, Cdc42). It is obvious that some other yet-to-be identified proteins may also take part in these events.

Concluding Remarks and Future Perspectives

As briefly reviewed herein, FAK, in particular its two phosphorylated forms, p-FAK-Tyr\textsuperscript{407} and -Tyr\textsuperscript{397}, are important regulators of ES dynamics in the seminiferous epithelium of the rat testis during the epithelial cycle via their effects on the network of actin filament bundles at the apical and the basal ES, which are mediated via their effects on actin regulatory proteins, such as the Arp2/3 complex.
They also suggest spermatid adhesion at the apical ES and also Sertoli cell adhesion at the BTB, thereby coordinating cellular events of spermatiation and BTB restructuring that take place simultaneously at opposite ends of the epithelium, such as in stage VIII tubules (Fig. 1), at the apical ES-BTB-basement membrane axis. However, there are many questions remain unanswered as illustrated in Figure 1. For instance, it is likely that other actin cross-linker and/or bundling proteins also take part in the events mediated by these FAK phosphorylated forms, such as filamin A and palladin, as Eps8 was shown not be a participating molecule.22 Furthermore, recent studies have shown that mammalian target of rapamycin complex 1 (mTORC1) and mTORC2,23-40 as well as intercellular adhesion molecule-1 (ICAM-1) and its soluble form (sICAM-1)41 are also having antagonistic effects on the Sertoli cell adhesion function in the testis. Furthermore, it remains to be determined the regulatory molecules that modulate the stage-specific and spatio-temporal expression of p-FAK-Tyr397 and -Tyr407 at the ES. For instance, are these changes mediated by the biologically active fragments of laminins released at the apical ES and/or at the hemidesmosome? Can it be possible that small regulatory RNA (e.g., miRNA) found in germ cells42-44 modulate the spatiotemporal expression of these FAK phosphorylated forms via the GJ communication channels?45,46 Many of these questions remain to be addressed in future studies. In short, it will be of interest to investigate the physiological and molecular relationship between these molecules in regulating BTB function at the apical ES-BTB-basement membrane axis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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