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Role of non-receptor protein kinases in spermatid transport during spermatogenesis*

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Abstract

Non-receptor protein tyrosine kinases are cytoplasmic kinases that activate proteins by phosphorylating target protein tyrosine residues, in turn affecting multiple functions in eukaryotic cells. Herein, we focus on the role of non-receptor protein tyrosine kinases, most notably, FAK, c-Yes and c-Src, in the transport of spermatids across the seminiferous epithelium during spermatogenesis. Since spermatids, which are formed from spermatocytes via meiosis, are imotile haploid cells, they must be transported by Sertoli cells across the seminiferous epithelium during the epithelial cycle of spermatogenesis. Without the timely transport of spermatids across the epithelium, the release of sperms at spermiation fails to occur, leading to infertility. Thus, the molecular event pertinent to spermatid transport is crucial to spermatogenesis. Herein, we provide a critical discussion based on recent findings in the field. We also provide a hypothetical model on spermatid transport, and the role of non-receptor protein tyrosine kinases in this event. We also highlight areas of research that deserve attention by investigators in the field.

Keywords

Testis; spermatogenesis; seminiferous epithelial cycle; FAK; c-Src; c-Yes; blood-testis barrier; cell adhesion; spermiogenesis; spermiation

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1. Introduction

Production of spermatozoa is one of the two major functions of the mammalian testes besides sex steroids testosterone and estradiol-17 β [1-5]. Testosterone is produced exclusively by Leydig cells found in the interstitial space between seminiferous tubules [5-7], whereas estradiol-17 β is the product of Leydig cells, Sertoli cells and spermatozoa in adult mammals including rodents and humans [3, 4, 8] (Figure 1). Besides regulating secondary sexual characteristics of the male such as, accessory glands like the prostate and seminal vesicles, and regulating other organ and body functions (e.g., bone metabolism and blood pressure), steroids also contribute significantly to the production of spermatozoa via these effects on spermatogenesis that takes place exclusively in the seminiferous epithelium, which is composed of only Sertoli and germ cells [3, 7, 9-12]. Spermatogenesis is a complex, but tightly regulated series of cellular events which involve the formation of spermatozoa (haploid, 1n) from spermatogonial stem cells and spermatogonia (diploid, 2n) in the seminiferous epithelium [2, 13, 14] (Figure 1). This process is comprised of four discrete cellular events: (i) renewal of spermatogonial stem cells (SSC) and spermatogonia via mitosis and differentiation of type B spermatogonia to pachytene spermatocytes, (ii) meiosis, (iii) spermiogenesis, and (iv) spermiation, the eventual release of spermatozoa transformed from step 19 spermatids.

The seminiferous epithelium is physically divided by the Sertoli cell blood-testis barrier (BTB), one of the tightest blood-tissue barriers in the mammalian body [15], into the basal and the adluminal compartments (Figure 1). Except for self-renewal of SSC and spermatogonia, and the differentiation of type B spermatogonia to preleptotene spermatocytes, which take place in the basal compartment outside the BTB; meiosis, spermiogenesis and spermiation all take place in the adluminal compartment, which is a specialized microenvironment behind the BTB [16-20]. Unlike other blood-tissue barriers, such as the blood-brain barrier (BBB), the blood-retinal barrier (BRB), and the gut barrier [21-24], which are constituted almost exclusively by tight junctions (TJ), the BTB is constituted by coexisting TJ, basal ectoplasmic specialization (basal ES, a testis-specific F-actin-rich adherens junction (AJ) restricted to the BTB), gap junction (GJ) and desmosome [16, 25-31] (Figure 1). While the BTB is one of the tightest blood-tissue barriers, unlike other tissue barriers, it undergoes rapid remodeling during the epithelial cycle of spermatogenesis. For example, at stage VIII of the epithelial cycle, remodeling of the BTB is necessary to accommodate the transport of preleptotene spermatocytes which are connected in clones via intercellular bridges (also known as tunneling nanotubes, TNT) across the BTB while transforming to leptotene spermatocytes, so that spermatocytes undergo meiosis I and II in the adluminal compartment of the epithelium [20, 32-35]. Interestingly, the BTB function cannot be compromised, even transiently, to avoid the production of antibodies against antigens residing on germ cells, many of which are expressed transiently during spermatogenesis [36]. At present, detailed molecular mechanism(s) that governs BTB remodeling during the transit of preleptotene spermatocytes at the immunological barrier remain unknown. Emerging evidence has supported the notion that a “new” BTB is assembled behind transiting preleptotene spermatocytes at the BTB while the “old” BTB above these spermatocytes is disassembled [33, 34, 37] so that

spermatocytes connected in clones are transported across the BTB and the immunological barrier integrity is also maintained [38, 39]. Recent studies have illustrated the likely the involvement of non-receptor protein kinases, in particular FAK (focal adhesion kinase) and two members of the Src kinase family c-Src and c-Yes in the transport of preleptotene spermatocytes at the BTB [40-43].

On the other hand, step 19 spermatids at stage VII of the epithelial cycle that are anchored to the Sertoli cell via a testis-specific AJ known as apical ES (it is restricted to the apical/adluminal compartment at the Sertoli-step 8-19 spermatid interface) also undergo extensive remodeling to prepare for their release at late stage VIII of the cycle at spermiation. Restructuring of apical ES involves the initial formation of giant endocytic vesicles called apical tubulobulbar complex (apical TBC) [26, 44], which is analogous to cellular events of endocytic vesicle-mediated trafficking found in other epithelial cells. Apical TBC first appears at the concave (ventral) side of spermatid heads, being used to recycle proteins at the “old” apical ES to assemble a “new” apical ES that appears in stage VIII tubules, as well as to eliminate unwanted cellular debris from spermatids that arise during spermiogenesis [44, 45]. These restructuring events eventually cover the entire spermatid head at early stage VIII of the cycle, and to prepare for their release at spermiation, involving degeneration of the apical ES at late stage VIII [44-46]. However, the molecules and/or the mechanism(s) that trigger the initial transition from intact apical ES to a remodeling/restructuring apical ES at stage VII, and the progressive degeneration at early stage VIII to its eventual progression to cover the entire apical ES for its break down at late stage VIII remain unknown. Studies during the last decade also support the possible involvement of non-receptor protein kinases such as FAK, c-Yes and c-Src [41, 42, 47-50], and the generation of biologically active laminin fragments at the apical ES to serve as autocrine factors [51, 52] in this transition.

While much research is needed to better understand these two specific cellular events, basal ES/BTB and apical ES restructuring to facilitate germ cell transport, we provide a critical and timely discussion based on recent findings in the field. We envision that a better understanding of BTB and apical ES restructuring/remodeling during the epithelial cycle will provide some insightful information in developing novel male contraceptives by perturbing spermatogenesis. This information also offers potential insights of some unexplained male infertility, such as oligospermia and azoospermia. Since the subject on BTB restructuring during the epithelial cycle has recently been reviewed [37, 38, 53], we focus most of our discussion on the transport of spermatids across the seminiferous epithelium during spermatogenesis in this short review.

2. Cytoskeletons in Sertoli cells

Cytoskeletons are cellular scaffolds found in mammalian cells, including Sertoli and germ cells in the testis, that confer cell shape, intracellular transport and trafficking, cell polarity, cell locomotion, and cell division [20, 26, 54-59]. In the Sertoli cell, cytoskeletons based on actin microfilaments, intermediate filaments and microtubules are present. Interestingly, the relative expression of these cytoskeletons in the seminiferous epithelium are not identical but expressed stage-specifically as illustrated by the spatiotemporal expression of vimentin (a component of intermediate filament in Sertoli cells [60, 61]) and F-actin (Figure 1). In the

testis, the best studied cytoskeleton in the Sertoli cell is the actin-based cytoskeleton since bundles of actin microfilaments are abundant at the ES [26, 46, 62-64]. Actin microfilaments that lie perpendicular to the Sertoli cell plasma membrane are bundled into distinctive hexagonal blocks and are sandwiched in-between cisternae of endoplasmic reticulum and the apposing plasma membranes of either Sertoli cell or spermatid. This junction type is called apical ES in the adluminal compartment at the Sertoli cell-spermatid interface or basal ES in the basal compartment at the interface between two adjacent Sertoli cells. These actin filament bundles, readily detected by electron microscopy, were first identified at the BTB in the 1970s [65] and are not found in other mammalian epithelia (Figure 1). The term ES, which was not coined until the late 1970s, describes the testis-specific anchoring device at the Sertoli-spermatid and Sertoli cell-cell interface known as apical and basal ES, respectively [32, 46, 66], which, in essence, represents regional specialization of F-actin network at these sites. On this note, it is of interest to mention that germ cells *per se*, in particular post-meiotic spermatids, are immotile cells lacking the typical features found in motile cells (e.g., macrophages, fibroblasts, and metastatic cancer cells) such as lamellipodia and filopodia. Also, the distinctive features of the actin filament bundles found in the apical and basal ES are limited to the Sertoli cell and no visible ultrastructures are found in spermatids. Thus, most of the studies on cytoskeletons are focused on the Sertoli cell with one notable exception in which cytoskeletons are found in the mid-piece and the elongating tail of spermatids during spermiogenesis [67, 68]. More important, few reports are found in the literature that probe the role of cytoskeletons in spermatogenesis at the molecular level except morphological studies. Nonetheless, it is generally accepted that cytoskeletons in the Sertoli cell are important for germ cell transport, Sertoli and spermatid polarity, cell adhesion and the release of sperms at spermiation [62, 69, 70].

Both Sertoli cells and germ cells express actin, however the organization of actin in each of the two cell types differ. F (filamentous)- and G (globula)-actin constitute the actin-based cytoskeleton in Sertoli cells. Findings in the mouse testis show that germ cells express a different type of actin called T-actin 1 and T-actin 2, which share ~40% homology with actin found in Sertoli cells [71]. However, GC actin is not organized into well-defined ultrastructures characteristic of Sertoli cells. For intermediate filament-based cytoskeleton in the Sertoli cell, vimentin is the predominant structural component [60], whereas keratins are the component of intermediate filament-based cytoskeleton in germ cells that facilitate the shaping of the spermatid head [58, 68]. It is known that intermediate filament-based cytoskeleton that forms around the nucleus provides mechanical support and the scaffolds for protein recruitment and serve as a platform for cell signaling [72, 73]. The tubulin-based cytoskeleton is comprised of microtubules, which are tubular polymers formed by α and β tubulin heterodimers. Microtubules confer cell shape and most importantly, and serve as the “track” (analogous to a railroad track) for directional intracellular transport of “cargoes” (e.g., spermatids) from the minus (–) to the positive (+) end [55, 56, 74]. Microtubules in Sertoli cells are arranged parallel to the long axis of the cell, thus conferring polarity to the seminiferous epithelium [75]. Herein, we focus our discussion mostly on the actin-based cytoskeleton since few studies are found in literature that investigate the role of non-receptor protein kinases on the other two cytoskeletons. Nonetheless, this illustrates much research is

needed to examine the functional significance of intermediate filament- and tubulin-based cytoskeletons on spermatogenesis in the years to come.

2.1. Non-receptor protein kinases and actin-based cytoskeleton

As noted above, actin microfilaments are concentrated at the ES in the seminiferous epithelium. These bundles of actin microfilaments, together with the adhesion protein complexes (such as $\alpha 6 \beta 1$ -integrin-vinculin-paxillin, nectin-2/-3-afadin, and JAM-C-ZO-1 at the apical ES [50, 76-80], and N-cadherin- β -catenin and nectin-2-afadin at the basal ES [78, 81]) that utilize actin for attachment, thus confer adhesive strength to the ES which is localized either at the apical ES, or at the basal ES/BTB. Actin filaments at the ES are either organized into “bundled” or “un-bundled/branched” configuration, depending on the stage of the epithelial cycle mediated by the corresponding regulatory proteins that organize these microfilaments accordingly. Thus, ES can be rapidly remodeled via rapid conversion of the actin microfilaments from their “bundled” and “un-bundled/branched” configuration and *vice versa* to facilitate the transport of: (i) spermatids across the adluminal compartment (at the apical ES) and (ii) preleptotene spermatocytes across the BTB (at the basal ES). Studies have shown that this rapid conversion of actin microfilaments from their “bundled” and “un-bundled/branched” configuration is made possible via the spatiotemporal expression of two different types of actin regulatory proteins. First, the actin bundling proteins: Eps8 (epidermal growth factor receptor pathway substrate 8, an actin barbed end capping and bundling protein) [82] and palladin (an actin bundling protein) [83] are expressed at the ES to confer actin filament bundling during the epithelial cycle. Second, the branched actin polymerization inducing proteins: Arp3 (actin-related protein 3) which together with Arp2 form the Arp2/3 complex, when the Arp2/3 complex is activated by N-WASP (neuronal Wiskott-Aldrich Syndrome protein), the complex causes barbed end nucleation of an existing microfilament [84]; and filamin A, an actin cross-linker that effectively induces F-actin branching [85]; both of which are expressed at the ES stage-specifically in the rat testis (Figure 2). Studies have shown that these actin regulatory proteins physically interact with non-receptor protein tyrosine kinases, such as the interaction between FAK and the Arp2/3 complex [86], and between FAK and Eps8 [42]. Also, FAK is known to modify F-actin organization via its effects and/or interactions with the Arp2/3 complex in mammalian cells [86, 87]. In the testis, while FAK is not associated with Arp3 or Eps8, p-FAK-Tyr⁴⁰⁷ interacts with N-WASP, thus FAK is involved in actin polymerization at the Sertoli cell basal ES/BTB [40]. For instance, overexpression of FAK phosphomimetic mutant Y407E, a constitutively active p-FAK-Tyr⁴⁰⁷ mutant, in Sertoli cells with an established functional TJ-barrier that mimics the Sertoli cell BTB *in vivo*, was found to induce actin polymerization [40], illustrating FAK is playing an active role in modulating the organization of the F-actin bundles at the ES. On the other hand, c-Yes structurally interacts with FAK [41] and Eps8, but not Arp3 [42] in the rat testis. More importantly, a knockdown of c-Yes by RNAi was shown to induce actin polymerization at the Sertoli cell BTB [42], which is likely mediated by changes in the spatiotemporal expression of p-FAK-Tyr⁴⁰⁷ at the basal ES/BTB. This postulate was supported by observations in which a knockdown of c-Yes by RNAi was found to induce mis-localization of p-FAK-Tyr⁴⁰⁷ at the apical ES where p-FAK-Tyr⁴⁰⁷ was no longer restricted mostly to the concave (ventral) side of the tip of the spermatid head, instead, it was found on the convex (dorsal) side of the spermatid

head and localized almost to the base of the spermatid head [42] (Figure 3). Also, c-Yes knockdown at the Sertoli cell BTB also induces recruitment of more Eps8 to the Sertoli cell-cell interface [42]. Collectively, these findings illustrate FAK and c-Yes are intimately involved in the organization of F-actin bundles at the ES via their effects on actin barbed end nucleation proteins (e.g., N-WASP, Arp3) and actin bundling proteins (e.g., Eps8). In the sections below, we critically evaluate the highly restrictively spatiotemporal expression of p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁴⁰⁷, c-Yes and c-Src at the apical ES *versus* basal ES wherever appropriate during the epithelial cycle of spermatogenesis.

3. Spermatid transport during spermiogenesis is regulated by the spatiotemporal expression of p-FAK-Tyr³⁹⁷, p-FAK-Tyr⁴⁰⁷, and c-Yes at the apical ES

Non-receptor protein tyrosine kinases such as FAK, c-Yes and c-Src are cytoplasmic enzymes that activate proteins via phosphorylation of tyrosine residues in their target proteins, and play important roles in cell signaling [88]. Examples of non-receptor tyrosine kinases are FAK family (e.g., FAK), SRC family (e.g., c-Yes, c-Src) and JAK [Janus kinase, e.g., JAK1, JAK2, JAK3, tyrosine kinase 2 (TYK2)] family. Members of FAK and SRC family are expressed in rodent testes, and are involved in the regulation of spermatogenesis [50, 89-91]. Herein, we provide a critical review on the role of FAK, c-Src and c-Yes in regulating spermatid transport during spermatogenesis since more published work is found on these three non-receptor tyrosine kinases in the literature.

3.1. Focal adhesion kinase (FAK)

FAK is found in virtually all mammalian cells, and it is known to be involved in cell migration, adhesion, apoptosis, F-actin organization and others [90, 92]. Furthermore, FAK is the signal transducer that relates signals downstream of integrin-based receptors at focal adhesion complex (FAC or focal contact) in multiple epithelia following their activation by the corresponding ligands such as laminins, collagens and others [93, 94]. FAK, c-Src and c-Yes are mostly found at the cell-extracellular matrix (ECM) interface using actin for attachment known as FAC. In the testis, FAC is absent in the seminiferous epithelium, and FAK is an ES component at the Sertoli-spermatid and Sertoli cell-cell interface restrictively expressed at the apical and basal ES, respectively [50, 91, 95]. For instance, FAK structurally interacts with occludin at the basal ES [91] and with β 1-integrin [50, 96] at the apical ES. A knockdown of FAK in Sertoli cells cultured *in vitro* perturbs the TJ-permeability barrier, illustrating FAK is a BTB regulator [97]. Also, a knockdown of FAK was found to de-sensitize Sertoli cells in response to the cadmium-induced disruption of the TJ-barrier function, making the Sertoli cell BTB less sensitive to cadmium toxicity [97]. To date, six putative phosphorylation sites in FAK at tyrosine residues 397, 407, 576, 577, 861 and 925 are known, where p-FAK-Tyr³⁹⁷, -Tyr⁴⁰⁷ and -Tyr⁵⁷⁶ have been positively identified at the ES in the rat testis with each displaying differential expression during the epithelial cycle [94]. For instance, p-FAK-Tyr³⁹⁷ is highly expressed at the apical ES at stage VII to VIII until it is down-regulated at late stage VIII just prior to spermiation [40, 42, 50] (Figure 3). Furthermore, p-FAK-Tyr³⁹⁷ is almost exclusively localized at the convex

(dorsal) side of the spermatid head from stage VII-VIII until late stage VIII [40, 42] (Figure 3), where two actin bundling proteins Eps8 [82] and palladin [83] are also found in stage VI-VII. However, both Eps8 and palladin are shifted to the concave (ventral) side of the spermatid heads in late stage VII and early VIII, to be co-localized with p-FAK-Tyr⁴⁰⁷ (Figures 2 and 3) and Eps8 and palladin are no longer expressed or considerably diminished at late VIII [48, 82, 83] (Figure 2). On the other hand, p-FAK-Tyr⁴⁰⁷ is localized predominantly at the concave (ventral) side of the spermatid head from stage VII-VIII until late stage VIII [40] (Figure 3) where the actin barbed end branching polymerization protein Arp3 is also predominantly expressed until it down-regulates to a virtually un-detectable level at late stage VIII [40] (Figure 2). Collectively, these data illustrate that the spatiotemporal expression of p-FAK-Tyr³⁹⁷/Eps8/palladin and p-FAK-Tyr⁴⁰⁷/Arp3 (and also p-FAK-Tyr⁴⁰⁷/Eps8/palladin) at the apical ES are critically important to spermatid transport during spermiogenesis (Figures 2, 3 and 4) via rapid organization of actin microfilaments from their “bundled” to “unbundled/branched” configuration and *vice versa*. In short, p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁴⁰⁷ serve as molecular “switches” that “turn on-or-off” the machinery (i.e., actin bundling or un-unbundling inducing proteins) that confers actin microfilaments to be assembled in their “bundled” or “unbundled/branched” configuration and *vice versa*. It is noted that spermatids are anchored onto the Sertoli cell in the seminiferous epithelium via their head (Figure 1). During the transport of spermatids across the seminiferous epithelium throughout the epithelial cycle, actin filament bundles surrounding the spermatid head at the convex and the concave side are to be reorganized differentially via a highly organized manner. If all the actin filament bundles at the apical ES are disrupted simultaneously, spermatids will become non-polarized and depleted from the epithelium prematurely, analogous to premature spermiation, as illustrated in rats treated with the environmental toxicant cadmium [98] or male contraceptive adjuvin [99-101].

Thus, actin filament bundles at the convex and the concave side of the spermatid head are unbundled and re-bundled differentially under the regulation of different regulators (i.e., p-FAKTyr³⁹⁷, p-FAK-Tyr⁴⁰⁷) and proteins (i.e., Eps8, palladin, Arp2/3 complex). Since p-FAK-Tyr⁴⁰⁷ is co-localized with Arp3 at stages VII to early VIII (*note*: the expression of both proteins are down-regulated at late stage VIII to facilitate spermiation) (Figure 2), and the Arp2/3 complex induces branched actin polymerization, effectively converting actin filaments to a branched and unbundled configuration whereas p-FAK-Tyr⁴⁰⁷ induces actin polymerization. Thus, p-FAK-Tyr⁴⁰⁷ serves as the “molecular switch” to turn the Arp2/3 complex “on-or-off” during spermatid transport to favor the appropriate configuration of the actin filament bundles at the concave (ventral) side of spermatid heads. Furthermore, in late stage VII to early stage VIII, actin bundling proteins are also found to be associated with p-FAK-Tyr⁴⁰⁷ (see Figure 2 vs. 3), which may also serve as the “molecular switch” to turn palladin and Eps8 activity “on-or-off” (Figure 3).

On the other hand, p-FAK-Tyr³⁹⁷ is co-localized with actin bundling proteins Eps8 and palladin at the convex side of spermatid heads (Figure 3), analogous to c-Yes (Figure 3) p-FAK-Tyr³⁹⁷ also acts as the “molecular switch” of the actin bundling proteins to effectively turn Eps8 and palladin “on-or-off” during spermatid transport to determine if the actin microfilaments at the site should assume a bundled configuration (Figure 4). While the

model depicted in Figure 4 is a hypothetical concept, it is supported by a recent *in vivo* study in which a phosphomimetic mutant FAK Y397E, which rendered p-FAK-Tyr³⁹⁷ constitutively active in transfected Sertoli cells, was overexpressed in the testis *in vivo* [48]. It was noted that the presence of constitutively active p-FAK-Tyr³⁹⁷ at the apical ES in the testis via overexpression of FAK-Y397E well beyond late stage VIII when its expression should have been down-regulated and turned off in normal testes, defects in spermiation were detected in the epithelium in which many step 19 spermatids failed to undergo spermiation, and instead were trapped inside the epithelium some of which were found near the basal compartment [48] due to a failure in spermatid transport. The persistent presence of p-FAK-Tyr³⁹⁷ at the apical ES was found to impede the spatiotemporal expression of Eps8 and palladin as well as Arp3 [48], which in turn perturbed the “de-bundling” of actin filaments at the apical ES, which is necessary to allow apical ES degeneration to facilitate spermiation. Instead, F-actin remained at the apical ES at stage VIII in testes overexpressed with p-FAK-Tyr³⁹⁷ when it should have been disorganized at late stage VIII of normal testes [48]. These changes thus impeded the localization of cell adhesion proteins nectin-2 and nectin-3 [48]. Instead of being re-localized, such as via endocytosis, transcytosis and recycling to assemble “new” apical ES for the step 8 spermatids just transformed from step 7 spermatids in stage VIII of the epithelial cycle, both nectin-2 and -3 remained at the apical ES to induce spermatid adhesion, making spermatids embedded in the epithelium even in late stage VIII and IX tubules, long after spermiation had occurred [48]. In summary, both p-FAK-Tyr³⁹⁷ and – Tyr⁴⁰⁷ serve as “molecular switches” that work closely with actin bundling proteins Eps8 and palladin, as well as actin un-bundling/branching protein Arp3 to elicit rapid re-organization of F-actin at the apical to facilitate spermatid transport during spermiogenesis.

3.2. Src family kinase (SFK)

SFK is a non-receptor protein tyrosine kinase family known to be involved in integrin-based signaling at FAC to regulate cell adhesion, cell movement, proliferation, survival, differentiation, endocytic vesicle-mediated trafficking and tumorigenesis [102-104]. Interestingly, FAK and c-Yes or c-Src are binding partners of each other at the apical ES of the testis and are components of the β 1-integrin-based adhesion protein complex [41, 50, 79, 96], and they are also the emerging target of chemotherapy [102, 105]. Among the 9 members of SFK, namely Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn and Frk, that are known to date, c-Src and c-Yes are known to be expressed in both Sertoli and germ cells, and localized to the basal ES/BTB and apical ES in adult rat [42, 43, 106-108] testes, displaying spatiotemporal expression in the testis [41]. c-Src and/or c-Yes structurally interacts with FAK, β 1-integrin, laminin-333 at the apical ES [41, 42, 79, 109]. While both c-Yes and c-Src are members of the SFK family, studies have shown that they play different roles in regulating cellular functions [43]. Besides studies by immunohistochemistry to identify c-Src in the seminiferous epithelium of rat testes, illustrating its likely involvement in spermiation [107, 108], few studies are found in the literature exploring the functional role of c-Src in spermatid transport and spermiation. We thus focus our discussion on c-Yes because more functional data are available. As noted in Figure 3, c-Yes is expressed almost exclusively at the convex side of spermatid heads in stages VI-VIII until it is considerably down-regulated to an almost un-detectable level at late stage VIII [41]. As such, its site of

spatiotemporal expression at the apical ES virtually overlaps with p-FAK-Tyr³⁹⁷ (Figure 3) and also Eps8 and palladin at stage VI to early V [82, 83]. These observations are important since they illustrate that c-Yes may work in concert with p-FAK-Tyr³⁹⁷ to confer F-actin its bundled configuration surrounding the spermatid head at the apical ES at these stages, and to facilitate spermiation by late stage VIII, its expression is considerably reduced to a level virtually un-detectable [41] (Figure 3). In fact, this notion is supported by findings in which a knockdown of c-Yes in Sertoli cells was found to promote the rate of actin polymerization [42], illustrating c-Yes at the convex side of spermatid heads indeed is being used to play a role in conferring bundling of actin microfilaments to maintain the apical ES integrity. More important, besides regulating actin polymerization kinetics, a knockdown of c-Yes in the testis *in vivo* was found to induce mis-localization of p-FAK-Tyr⁴⁰⁷ at the apical ES, in which it was no longer restricted to the concave side of spermatid heads, instead p-FAK-Tyr⁴⁰⁷ was detected on the convex side of the spermatid heads, in addition, it was diffusing away from the concave side of spermatid heads [42] (Figure 3), illustrating the F-actin network at the ES was perturbed. Also, nectin-3 failed to become down-regulated to an almost un-detectable level at late stage VIII, instead, nectin-3 was detected at the apical ES, perturbing spermatid transport and spermiation [42]. Collectively, these findings illustrate c-Yes is working in concert with p-FAK-Tyr³⁹⁷ and – Tyr⁴⁰⁷ to confer actin filament bundles at the ES during the epithelial cycle, regulating spermatid transport as shown in Figure 4.

4. Concluding remarks and future perspectives

Herein, we have critically evaluated recent findings supporting the role of non-receptor protein tyrosine kinases, most notably FAK, c-Yes and c-Src, in spermatid transport during spermiogenesis via their effects on the actin filament bundles at the apical ES. It is likely that these non-receptor protein tyrosine kinases serve as molecular switches to induce reorganization of actin microfilaments from their “bundled” to “un-bundled/branched” configuration (see Figure 4) via their effects on proteins that confer actin bundling and un-bundling. It is obvious that additional players will add onto the list of proteins that regulate spermatid transport during spermatogenesis, however the model depicted in Figure 4 will be helpful in the years to come. At present, there are questions that deserve immediate attention from investigators in the field. For instance, what molecule(s) and/or signaling pathway(s) are involved in coordinating both the events of spermiation and BTB restructuring which take place simultaneously at stage VIII but across the seminiferous epithelium? It is likely that biologically active fragments of laminin chains that are formed during apical ES degeneration at late stage VIII are involved in coordinating these events [51, 52], however, the biology of collagen fragments (e.g., non-collagenous domain 1, NC1) generated at the basement membrane that modulates BTB dynamics [110, 111] remains to be better elucidated. Also, does cytokine(s) (e.g., TGF- β 3, TNF α) play any roles in these events since studies have shown that cytokines released by Sertoli and/or germ cells into the microenvironment of the ES regulate cell adhesion [112-114]?

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Highlights

Spermatid transport during the epithelial cycle is crucial to spermatogenesis

Ectoplasmic specialization (ES) is the cellular structure that confers spermatid transport

Spatiotemporal expression of protein kinases and actin binding proteins during spermatogenesis regulates re-organization of microfilaments at the ES

A model depicting the role of protein kinases and actin binding proteins in spermatid transport

Figure 1. A schematic drawing illustrating the relative location of the F-actin-rich ectoplasmic specialization (ES) at the Sertoli-spermatid (apical ES) and the Sertoli cell-cell (basal ES) interfaces, and the spatiotemporal expression of vimentin and F-actin in the seminiferous epithelium that support the ES

(A) A schematic drawing that illustrates the cross-section of a seminiferous tubule at stage VII of the epithelial cycle, showing intact apical ES and basal ES and other junctions in the seminiferous epithelium. (B, C) The relative localization of vimentin (red fluorescence) and F-actin (green fluorescence) at the apical and/or basal ES at stage V – IX of the epithelial cycle are shown. Vimentin-based intermediate filaments are restricted to the basal

compartment or at the BTB where desmosomes (an intermediate filament-based cell-cell anchoring junction) are present, most notably surrounding the Sertoli cell nucleus, but virtually undetectable at the apical ES (**B**). On the other hand, F-actin is localized to both the basal and apical ES except at stage VIII, when F-actin is considerably diminished (**C**) to facilitate the release of sperms at spermiation in which actin microfilaments no longer assume a “bundled” configuration via degeneration of the apical ES. Scale bar, 50 μm , which applies to other micrographs in the same panel.

Figure 2. Spatiotemporal expression of Arp3, Eps8 and palladin at the apical ES at stages VII and VIII of the epithelial cycle in adult rat testes

Localization of barbed end nucleation inducing protein Arp3 (red fluorescence) that effectively “de-bundled” actin microfilaments at the apical ES to form a branched network in the seminiferous epithelium, which also co-localized with F-actin in stage VII tubules. Arp3 is annotated by a “yellow” arrow at the concave (ventral) side of the spermatid head. On the other hand, Eps8, an actin barbed end capping and bundling protein, and palladin, an actin bundling protein, both are being used to confer the bundling of actin microfilaments at the apical ES, were found to localize both at the concave (“yellow” arrow) and convex (dorsal) side (“white” arrow) of the spermatid head, and co-localized, at least in part, with F-actin. In short, conversion of F-actin to an “un-bundled” configuration at the concave side facilitates endocytic vesicle-mediated protein trafficking, such as endocytosis, transcytosis and recycling. But the spatiotemporal expression of Eps8 and palladin surrounding the spermatid head also allow rapid conversion of F-actin between “bundled” and “un-bundled” configuration to facilitate the transport of spermatids across the seminiferous epithelium. At stage VIII, when spermatids are prepared for spermiation, the expression of these proteins are considerably down-regulated. It is of interest to note that since Arp3 is predominantly expressed at the concave side of spermatid heads, this leads one to wonder how can actin microfilaments reorganize at the convex side of spermatid heads? We offer two explanations. First, this can be achieved by inactivation of palladin and/or Eps8 at the site. Second, it may be mediated by spatiotemporal expression of filamin A (an actin cross-linker protein that induces microfilaments to a branched/mesh-like network [85, 115]). Scale bar, 10 μ m, which applies to other micrographs in the same panel.

Figure 3. Spatiotemporal expression of c-Yes, p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁴⁰⁷ at the apical ES at stage VI-VIII of the epithelial cycle in adult rat testes

(A) At the apical ES, c-Yes (green fluorescence) is localized to the convex side of the spermatid head towards the tip of the head (“white” arrow) but also towards the back of the head (“red” arrow) in stage VI. In early VII, c-Yes is also detected at the concave side of spermatid heads (“yellow” arrow) which persists until late stage VII and no longer detected in stage VIII of the cycle. Expression of c-Yes at the convex side of spermatid heads is also considerably diminished in early VIII and almost non-detectable by late VIII. (B) p-FAK-Tyr⁴⁰⁷ (red fluorescence), an ES regulator, is expressed mostly at the concave side of spermatid heads, but the silencing of c-Yes by RNAi induces mis-localization of p-FAK-Tyr⁴⁰⁷, in which it diffuses away from the site. (C) Differential localization of p-FAK-Tyr³⁹⁷ and p-FAK-Tyr⁴⁰⁷ at the apical ES. p-FAK-Tyr³⁹⁷ is mostly expressed at the convex side of spermatid heads (“white” arrow) and persists through late stage VIII (but

considerably diminished), whereas, p-FAK-Tyr407 is restricted mostly to the concave side of spermatid heads (“yellow” arrow) and diminishes considerably by late VIII. When these findings are compared to Figure 2, it is likely that Arp3, Eps8 and palladin are regulated by these non-receptor protein tyrosine kinases. For instance, p-FAK-Tyr⁴⁰⁷, which is expressed predominantly at the concave side of spermatid heads, likely regulates Arp3 and Eps8 (both are found at the concave side of spermatids heads, see Fig. 2). Whereas p-FAK-Tyr³⁹⁷ and c-Yes, which are predominantly expressed at the convex side of spermatid heads, regulate Eps8 and palladin (both are also found at the convex side of spermatid heads, see Fig. 2) by activating the corresponding actin regulatory protein(s) to effectively confer actin microfilaments either in a “bundled” to “unbundled/branched” configuration so that actin filaments at the ES can be effectively transformed and re-organized during the epithelial cycle to facilitate spermatid transport. Scale bar, 12 μ m, which applies to other micrographs in the same panel.

Figure 4. A schematic model illustrating the role of non-receptor protein tyrosine kinases such as c-Yes and FAK on spermatid transport utilizing apical ES during the epithelial cycle

The left panel illustrates the status of the apical ES from late to early stage VIII of the epithelial cycle in which p-FAK-Tyr⁴⁰⁷ recruits (likely mediated by drebrin [116]) and activates the barbed end actin nucleation protein Arp3 [40]. This effectively converts actin filaments from “bundled” to “unbundled/branched” configuration, thereby destabilizing the apical ES adhesion proteins beginning at the concave side of the spermatid head. This also facilitates endocytic vesicle-mediated trafficking events, such as endocytosis, transcytosis and recycling so that “old” apical ES proteins can be re-used to assemble “new” apical ES when step 8 spermatids appear in stage VIII of the cycle [38, 44]. On the convex side of the spermatid head, the apical ES function is maintained by p-FAK-Tyr³⁹⁷ which recruits Eps8 and palladin to confer actin microfilaments in their bundled configuration [48]. In late stage VIII when the expression of Eps8 and palladin are considerably diminished, actin filaments can no longer be maintained to confer apical ES function. Furthermore, matrix

metalloprotease-2 (MMP-2) induces proteolytic cleavage of laminin chains to generate biologically active fragments, such as laminin- γ 3 domain IV, which can induce BTB restructuring [51, 52, 117]. As such, the events of spermiation and BTB restructuring that take place simultaneously at stage VIII of the epithelial cycle are coordinated.