Emerging role for SRC family kinases in junction dynamics during spermatogenesis

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Abstract

SRC family kinases (SFKs) are known regulators of multiple cellular events, including cell movement, differentiation, proliferation, survival and apoptosis. SFKs are expressed virtually by all mammalian cells. They are non-receptor protein kinases that phosphorylate a variety of cellular proteins on tyrosine, leading to the activation of protein targets in response to environmental stimuli. Among SFKs, SRC, YES and FYN are the ubiquitously expressed and best studied members. In fact, SRC, the prototypical SFK, was the first tyrosine kinase identified in mammalian cells. Studies have shown that SFKs are regulators of cell junctions, and function in endocytosis and membrane trafficking to regulate junction restructuring events. Herein, we briefly summarize the recent findings in the field regarding the role of SFKs in the testis in regulating spermatogenesis, particularly in Sertoli–Sertoli and Sertoli–germ cell adhesion. While it is almost 50 years since the identification of the oncogene *v-Src* encoded by Rous sarcoma transforming virus, the understanding of SFK involvement during spermatogenesis in the testis remains far behind that in other epithelia and tissues. The goal of this review is to bridge this gap.

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Introduction

During spermatogenesis, type A spermatogonia undergo either mitotic proliferation for self-renewal or differentiation into type B spermatogonia by 8 and 6 dpp in mouse and rat testes, respectively (Clermont & Perry 1957, Bellve *et al.* 1977). These germ cell types reside behind the blood–testis barrier (BTB) at the basal compartment of the seminiferous epithelium (Fig. 1). Type B spermatogonia subsequently give rise to preleptotene and then leptotene spermatocytes, which are the only germ cell types to be transported across the BTB with the aid from Sertoli cells in rodents (de Kretser & Kerr 1988, Smith & Braun 2012). This reliance on Sertoli cells for their transport across the BTB is necessary since the germ cells do not possess either filopodia or lamellipodia found in other motile cells such as macrophages, fibroblasts or neutrophils (Cheng & Mruk 2002). The developing germ cells enter the adluminal compartment to further differentiate and transform into zygotene, pachytene and diplotene spermatocytes to enter meiosis I/II (Clermont 1972, Parvinen 1982, Hess & de Franca 2008, Hermo *et al.* 2010a). Thereafter, haploid cells (step 1, round spermatids) derived from meiosis undergo spermiogenesis, which consists of extensive morphological changes, to form elongating and elongated spermatids via 16 and 19 steps in mouse

and rat testes, respectively (Hess & de Franca 2008, Xiao *et al.* 2014a). Eventually, sperm line up near the tubule lumen to prepare for their release at spermiation during stage VII–VIII of the epithelial cycle. After spermiation, testicular sperm enter the epididymis so that they can undergo post-testicular maturation to become fully functional sperm, capable of undergoing capacitation and acrosome reaction to fertilize the egg in the female reproductive tract.

It is envisioned that spermatid transport by Sertoli cells across the seminiferous epithelium in the adluminal compartment is one of the crucial cellular events to support spermatogenesis, in particular, spermiogenesis because without the timely transport, spermiation fails, and infertility occurs. But the biology underlying spermatid transport remains unclear (Vogl *et al.* 2008, 2013, Hermo *et al.* 2010b, O'Donnell *et al.* 2011, Xiao *et al.* 2014a, Cheng & Mruk 2015, Tang *et al.* 2016). Recent studies have shown that this cellular event relies on the support of F-actin- and microtubule (MT)-based cytoskeletons, likely involving specific regulatory proteins modulating biogenesis, organization, polymerization and depolymerization of cytoskeletal networks (O'Donnell & O'Bryan 2014, Wen *et al.* 2016). An actin-based testis-specific cell–cell anchoring junction ectoplasmic specialization (ES) is present at the interface of elongating/elongated spermatids

Figure 1 A schematic diagram illustrating the relative localization of the BTB and different junction types at the Sertoli cell–cell and Sertoli–germ cell interface in adult rat testes to support spermatogenesis (Sertoli and germ cell arrangement at a specific stage is omitted). The blood–testis barrier (BTB) divides the seminiferous epithelium into the adluminal and basal compartments, which are composed of only Sertoli and germ cells at different stages of their development, lying above the tunica propria. The BTB is constituted by coexisting actin-based tight junction (TJ), basal ectoplasmic specialization (ES) and gap junction (GJ), as well as intermediate filament-based desmosome. During spermatogenesis, spermatogonia, primary spermatocytes (i.e., preleptotene/leptotene spermatocytes) residing in the basal compartment (annotated with a black asterisk), and more-developed primary spermatocytes (i.e., zygotene, pachytene and diplotene spermatocytes), secondary spermatocytes and step 1–7 spermatids residing in the adluminal compartment (annotated with a white asterisk), are supported by desmosome and gap junction at the Sertoli–germ cell interface. Apical ES first appears at the interface of step 8 spermatid–Sertoli cell in stage VIII tubules, replacing desmosome and gap junction as the only anchoring device until step 19 spermatids, which transform to become sperm to prepare for their release at spermiation in stage VII–VIII tubules when apical ES undergoes degeneration. The tunica propria is comprised of the basement membrane, the type I collagen layer, myoid cell layer, lymph and endothelia cells of the lymphatic vessel.

(step 8–19 vs 8–16 spermatids in the rat and mouse testis, respectively) and Sertoli cells in the adluminal compartment, which is designated as apical ES. Once apical ES appears in step 8 spermatids at stage VIII of the epithelial cycle (Russell 1977b, de Kretser & Kerr 1988, Xiao *et al.* 2014a), it is the only anchoring device at the site until spermiation. ES is also found between adjacent Sertoli cells at the BTB known as the basal ES (Russell & Peterson 1985, Vogl *et al.* 2000, 2008, Cheng & Mruk 2015). Different from apical ES, basal ES coexists with actin-based tight junction (TJ) and gap junction,

which localize together with the intermediate filament-based desmosome, and as a whole, they confer to the BTB integrity (Vogl *et al.* 2008, 2013, Pelletier 2011, Cheng & Mruk 2012) (Fig. 1). At stages VII–VIII of the epithelial cycle in both mouse and rat testes, elongated spermatids in adluminal compartment are preparing for their release into the tubule lumen at spermiation, necessitating apical ES degeneration at the Sertoli cell–spermatid interface. In the meantime, BTB also undergoes extensive remodeling to accommodate the transit of preleptotene/leptotene spermatocytes (residing in the basal compartment) across the immunological barrier (de Kretser & Kerr 1988, Hess & de Franca 2008). Conceivably, these two synchronized processes during spermatogenesis involve substantial cytoskeletal rearrangement and turnover of cell junctions, such as ES at the apical ES and BTB/basal ES sites, which is known to support spermatid transport (Mruk & Cheng 2004) and translocation of spermatocytes through the BTB (Russell 1977a). However, the molecules and/or the mechanism(s) underlying these concerted and coordinated cellular events, and how apical ES and BTB morph cyclically to facilitate germ cell transport are left largely unrevealed. Studies during the past decade support the notion that SRC family kinases (SFKs) are active participants in spermatogenesis (Maekawa *et al.* 2002, Goupil *et al.* 2011, Xiao *et al.* 2012, 2014b, Chojnacka & Mruk 2015, Mruk *et al.* 2017), and may play a role in BTB restructuring and disassembly of Sertoli cell–spermatid junctions. Herein, we focus primarily on the better studied SFKs, such as SRC and YES in spermatogenesis, and evaluate their involvement in cell junction dynamics, and how they influence cytoskeletal organization and endocytic vesicle-mediated protein trafficking events to support junction remodeling. We also provide a hypothetical model based on these studies that phosphorylation of cell adhesion protein by either SRC or YES serves as a hub to determine if the protein should be targeted to endosome-mediated protein degradation vs its recycling. This model could serve as a point of reference for future studies on BTB restructuring and SFK's role in regulating spermatid adhesion to Sertoli cells.

Overview of SFKs and findings in testis

As non-receptor protein tyrosine kinases, SFKs are known to phosphorylate large number of substrates on tyrosine, leading to the activation of protein targets in response to environmental stimuli; and many of the substrates are phosphorylated by multiple SFKs (Takeda *et al.* 2010, Espada & Martin-Perez 2017), which suggests the complementarities of SFK functions. SFKs are widely involved in signaling events that regulate various epithelial functions, such as cell migration, cell proliferation and differentiation (e.g., neuronal differentiation), cell survival and apoptosis, calcium

signaling, androgen signaling and spermatogenesis. Since the overexpression and/or hyperactivation of SFKs is commonly observed in pathogenesis of diseases, such as tumorigenesis (e.g., prostate cancer progression), HIV-1 infection and dementia (Engen *et al.* 2008, Xiao *et al.* 2012, Anguita & Villalobo 2017, Castoria *et al.* 2017, Coiras *et al.* 2017, Espada & Martin-Perez 2017, Kim *et al.* 2017, Nygaard 2018), specific SFK inhibitors have been used and/or investigated to treat various diseases including cancers and neurodegenerative diseases. In brief, the physiological role of SFKs on many other cellular functions has been the subject of several recent reviews (Xiao *et al.* 2012, Castoria *et al.* 2017, Coiras *et al.* 2017, Espada & Martin-Perez 2017, Kim *et al.* 2017, Nygaard 2018).

There are at least eight SFK members, including SRC, YES, FYN, LYN, LCK, HCK, FGR and BLK, which are expressed in mammals. Among them, SRC, YES and FYN are the best studied and found in a variety of cell types, while the others are predominant in hematopoietic cells (Thomas & Brugge 1997, Espada & Martin-Perez 2017). Also, SRC, YES and FYN are the most ubiquitously expressed SFKs, able to compensate for the loss of one another since only triple knockouts show embryonic lethality (Stein *et al.* 1994). Emerging evidence has shown that SRC, YES and FYN are F-actin regulators in the testis, and may operate in cell adhesion at the Sertoli cell–cell and/or Sertoli cell–spermatid interface via their effect on actin-binding/regulatory proteins, such as Eps8 (epidermal growth factor receptor pathway substrate 8), an actin barbed end capping and bundling protein that confers actin filaments to a bundled configuration to maintain ES integrity in the seminiferous epithelium (Maekawa *et al.* 2002, O'Donnell *et al.* 2011, Cheng & Mruk 2012, Xiao *et al.* 2013, Chojnacka & Mruk 2015, Mruk *et al.* 2017). SRC and YES are expressed by both Sertoli and germ cells, and confirmed to be an integrated component of BTB and apical ES in the rat testis (Xiao *et al.* 2012, Chojnacka & Mruk 2015). FYN has also been found to localize to ES and can phosphorylate and promote plakoglobin (an ES/desmosome protein) interaction with A-catenin (Maekawa *et al.* 2002, Mruk *et al.* 2017). Revealed by immunohistochemistry, RT-PCR or immunoblot analyses, the other SFKs have also been shown to be present in testis and likely involved in spermatogenesis (Kierszenbaum 2006, Lalancette *et al.* 2006, Bordeleau & Leclerc 2008, Goupil *et al.* 2011, Xiao *et al.* 2012, Singh *et al.* 2017). Additionally, besides their full-length forms, some SFKs (e.g., FYN, HCK) are found in testis as truncated variants devoid of intact kinase domain, which probably function in spermiogenesis as adaptor proteins and/or competitive inhibitors independent from their kinase activity (Kierszenbaum 2006, Lalancette *et al.* 2006, Bordeleau & Leclerc 2008, Kierszenbaum *et al.* 2009). Still, the exact functional details of different SFKs as well as their truncated forms in spermatogenesis

are yet to be fully elucidated. It is of interest to note that studies have illustrated the role of SFKs in supporting drug transporter function, which is explicitly important regarding the BTB uptake of nutrients, drugs and even toxicants into the adluminal compartment of the seminiferous tubule (Klein & Cherrington 2014). For instance, phosphorylation by YES is crucial to support OCT (organic cation transporter) activity in murine kidneys, such as OCT2 (Sprowl *et al.* 2016). By using SRC kinase inhibitor PP2, SFK has been shown required for a lysophosphatidic acid 1 receptor-mediated signaling cascade that regulates P-glycoprotein transport activity at the blood–brain barrier (Banks *et al.* 2018). Whether there is a similar function of SFKs at the BTB remains to be examined.

All SFKs share well-conserved structural domains across their polypeptide sequences as noted in Fig. 2. Each member contains the following functional domains: (i) an N-terminal SRC homology (SH) 4 domain which contains the lipid modification sites, allowing for fatty acid acylation of SFK and subsequent linking to the plasma membrane; (ii) a non-conserved region 'unique' to each SFK member but with no clear function assigned, which, together with the SH4 domain, forms an N-terminal intrinsically disordered region (IDR) maintained in a compact yet dynamic state; (iii) an SH3 domain that binds to specific proline-rich sequences of partner proteins; (iv) an SH2 domain that binds to specific tyrosine phosphorylation sites of partner proteins; (v) a

Figure 2 A schematic diagram illustrating the different functional and structural domains of members of the non-receptor SRC family kinases (SFKs). (A) This is the general layout of the different functional domains of SFKs. From the N-terminal, there is a penultimate glycine (Gly2, i.e. the second amino acid from the N-terminus) co-translationally myristoylated (M) which is used for SFK membrane targeting and to elicit signal transduction. A cysteine residue (Cys3, i.e. the third amino acid from the N-terminus) of SFK is post-translationally palmitoylated (P), to be used for subcellular trafficking of SFK such as to the plasma membrane. This is followed by 4 SRC homology (SH) domains: SH4, SH3 and SH2 domains and then the SH1/kinase domain having the intrinsic kinase activity. (B) This illustrates the general layout of different SFK members, and the different colored domains correspond to the functional domains shown in (A). Y, Tyr.

polyproline linker between SH2 and SH1 domains; (vi) an SH1 intrinsic tyrosine kinase catalytic domain and (vii) a negative regulatory tail region for auto-inhibition of kinase activity (Brown & Cooper 1996, Thomas & Brugge 1997, Arbesu *et al.* 2017). Under normal physiological conditions, SFK is inactive via auto-inhibition in which SH2 domain binds to the inhibitory phosphotyrosine (p-Tyr, Tyr530 in SRC vs Tyr535 in YES) at the C-terminal tail, whereas SH3 domain interacts with polyproline linker between SH2 and SH1 domains, which thus blocks the SH1/intrinsic kinase domain by stabilizing SFK into an inactive conformation. SFKs can be activated following ligand binding to the SH2 and/or SH3 domain or by dissociation of inhibitory p-Tyr and unlatching as catalyzed by various protein tyrosine phosphatases. This in turn leads to a conformational change, allowing phosphorylation of the stimulatory Tyr in the SH1/kinase domain (Tyr419 in SRC vs Tyr424 in YES) to confer intrinsic kinase activity in the SH1 domain (Thomas & Brugge 1997, Roskoski 2015, Espada & Martin-Perez 2017). Although the three folded domains (SH3, SH2 and SH1) are regulatory/catalytic domains responsible for the classical mechanism of SFK activity, recent studies have shown that the intrinsically unfolded domains (SH4 and Unique), as well as their interactions with SH3 domain, may represent a new modulator of SFK functions (Perez *et al.* 2013, Amata *et al.* 2014, Arbesu *et al.* 2017).

As mentioned above, the N-terminal acylation determines the plasma membrane targeting of SFKs. SFKs usually localize in cell cytosol close to plasma membrane through myristoylation and/or palmitoylation (Thomas & Brugge 1997, Kasahara *et al.* 2004, Sato *et al.* 2009, Chu *et al.* 2014) (Fig. 2). Interestingly, the palmitoylation state in the SH4 domain of SFKs affects their subsequent cellular localization, thereby affecting the site(s) where a specific SFK exerts its regulatory function. For instance, YES is a mono-palmitoylated SFK, and it is transported through a Rab11-dependent pathway preferably from the Golgi pool of caveolin to the plasma membrane (Sato *et al.* 2009), implicating its possible role in supporting endocytosis, recycling and/or transcytosis of integral membrane proteins, such as those localized at the apical and the basal ES in testis to support spermatogenesis. SRC, on the other hand, is a non-palmitoylated SFK, rapidly transported between the plasma membrane and late endosomes or lysosomes (Kasahara *et al.* 2007, Sato *et al.* 2009), illustrating the possibility that it may be involved in endosome-mediated protein degradation. While future studies are needed to confirm these possibilities, the observations are important since they demonstrate that, although highly homologous, YES and SRC can work divergently, and they likely function in concert to modulate intracellular protein trafficking events to support protein turnover and homeostasis (Xiao *et al.* 2012). Accordingly, studies have shown that by late stage VIII of the epithelial cycle

in the rat testis, expression of YES increases at the BTB but decreases to an undetectable level at the apical ES around spermiogenesis, whereas that of SRC goes the opposite (Lee & Cheng 2005, Xiao *et al.* 2011, 2012, Chojnacka & Mruk 2015), suggesting that these two SFKs can indeed play differential roles in junction dynamics/turnover at the BTB and apical ES sites, thus supporting germ cell transport and/or spermiogenesis during the epithelial cycle of spermatogenesis (which we will discuss further below). Along this line, although how other SFKs (such as FYN which is dually palmitoylated) contribute to restructuring of the seminiferous epithelium has not been clearly defined, we suppose it might work differently with function(s) that cannot be substituted by dominantly expressed SRC. Consistent with this, when using cross-sections of adult rodent testes examined by immunohistochemistry, results have shown that there were differences in the localization patterns of SRC, YES and FYN in the seminiferous epithelium (especially from stage VII to late stage VIII tubules). For instance, unlike SRC or YES (Xiao *et al.* 2011, 2012, Chojnacka & Mruk 2015), FYN expression in the seminiferous epithelium had its own distinct pattern that is maintained at a relatively stable level both at the apical ES and BTB/basal ES sites throughout spermatogenesis (Maekawa *et al.* 2002) (Fig. 3). Also, while they were all detected in the basal ES/BTB, YES was more prominent in these stages at the site, whereas at the apical ES, SRC and FYN were more prominent when compared with YES (Maekawa *et al.* 2002, Xiao *et al.* 2011, 2012, Chojnacka & Mruk 2015). Furthermore, FYN was more extended that it appeared as track-like (or stalk-like) structures that expanded across the seminiferous epithelium (Maekawa *et al.* 2002) (Fig. 3). Collectively, these observations suggest that these SFKs may not be playing redundant functions in testis to modulate spermatogenesis. Instead, they may have different roles in supporting spermatogenesis.

Differential roles of SFKs on endocytic protein trafficking in the testis

During spermatogenesis, millions of germ cells at different developmental stages are formed along the entire length of the seminiferous tubules which are ~26-m in length, with a diameter at ~280 µm, in rat testes at 1 year of age (Gaytan *et al.* 1986), illustrating considerable cellular activity in the tubules, including turnover of cell junctions. For instance, step 8 spermatids first appear at stage VIII of the epithelial cycle when apical ES must be actively assembled to support spermiogenesis at the Sertoli cell-spermatid interface (Vogl *et al.* 1993, 2008, Cheng & Mruk 2010). Although the testis synthesizes different categories of proteins on a daily basis, due to the unusual cellular outputs to support spermatogenesis (e.g. the testis pair in rats and humans produce an upwards of ~50–70 to

Figure 3 A study by immunohistochemistry to illustrate the localization of FYN in the seminiferous epithelium using cross-sections of adult mouse testes. Immunohistochemistry was performed essentially as earlier described (Xiao *et al.* 2011), using a specific antibody against FYN (Santa Cruz, Cat #:sc-16, dilution 1:100). FYN (brown precipitate) is present in all stages of the seminiferous epithelial cycle. Roman numerals indicate the stages of the seminiferous epithelial cycle. FYN is localized at the BTB and the apical ES, appearing as stalk-like structures extending across the seminiferous epithelium. These results agree with previously published reports (Maekawa *et al.* 2002). Scale bar, 30 μ m, which apply to other corresponding micrographs.

>300 million sperm daily (Johnson *et al.* 1980, 1983), and given the fixed number of Sertoli cells following puberty in humans (at ~12 years of age) and rodents (at ~17–19 days of age) to support germ cell development (Sharpe *et al.* 2003), it is physiologically impossible for *de novo* synthesis of all the required apical ES proteins (e.g., N-cadherin, B-catenin, nectin 2, nectin 3, afadin, laminins, A6 integrin, B1 integrin, JAM3 and others) vs basal ES proteins (e.g., N-cadherin, B-catenin, nectin 2, afadin, occludin, ZO-1, JAM1, JAM2, claudins and others) (Cheng & Mruk 2002). Instead, some of the proteins at the ES must be rapidly recycled to support protein turnover at the cell junctions during spermatogenesis. Corresponding to such mechanism, at late stage VII of the epithelial cycle, apical and basal ES undergo extensive remodeling to transform into a transient ultrastructure designated apical and basal TBC (tubulobulbar complex) respectively (Young *et al.* 2012b, Du *et al.* 2013, Vogl *et al.* 2013, 2014, Lyon *et al.* 2015), and proteins necessary to support endocytic vesicle-mediated protein trafficking, including clathrin, N-WASP, cortactin (Young *et al.* 2009a,b, 2012a), zyxin and vinculin (Young & Vogl 2012), Arp3 (Lie *et al.* 2010),

Eps8 (Lie *et al.* 2009), and a newly identified Ca^{2+} signaling machinery (Lyon *et al.* 2017), are recruited to the site. This structure has been carefully examined in recent years (Du *et al.* 2013, Lyon *et al.* 2015, 2017), and believed to serve as an endocytic device to support protein endocytosis and recycling (Vogl *et al.* 2013, 2014), so that internalized ES proteins can be used to assemble new ES during junction formation/restructuring in stage VIII of the epithelial cycle.

As previously discussed, SFK localization and function require endocytic trafficking. This, in turn, modulates the endocytic events. SFK has been shown to regulate the trafficking/internalization of receptor tyrosine kinases (RTKs) such as FGFR (fibroblast growth factor receptor) and EGFR (epidermal growth factor receptor), as well as integrin-mediated adhesion complexes. SFK targets and phosphorylates components of the endocytic machinery such as dynamin, Eps8, clathrin and clathrin-associated adaptor complex AP2 (assembly polypeptide 2) (Sandilands & Frame 2008, Sato *et al.* 2009, Reinecke & Caplan 2014, Malaga-Trillo & Ochs 2016). Endocytic internalization of junctional protein complexes can also solicit SFK activity. For instance, during gap junction internalization, Cx43 (connexin 43, a gap junction integral membrane protein) was found to be phosphorylated and associated more with activated SRC. Interaction with and phosphorylation by SFK are critical for endocytosis at tight and adherens junctions between epithelial/endothelial cells as well (Gillieron *et al.* 2008, Carette *et al.* 2010, Morton *et al.* 2013, Reinecke & Caplan 2014, Chichger *et al.* 2015, Van Itallie & Anderson 2018). Nevertheless, the role of SFK on endocytic protein trafficking in the testis was not investigated until recently. Using an *in vitro* model of primary cultures of Sertoli cells, which is known to mimic much of the Sertoli cell BTB *in vivo* (Byers *et al.* 1986, Janecki *et al.* 1991, 1992, Grima *et al.* 1992, Nicholls *et al.* 2009), it was noted that large endocytic vesicles at Sertoli cell–cell interface implicating the presence of the basal TBC ultrastructures as reported earlier (Russell 1977a, 1979, Du *et al.* 2013, Lyon *et al.* 2015, 2017) were detected in these cultures under electron microscopy (Xiao *et al.* 2014b). This finding further supports the notion that this *in vitro* system faithfully reproduces information of the *in vivo* Sertoli cell BTB. When this *in vitro* system was used and coupled with biochemical assays to monitor the events of protein endocytosis and recycling, intracellular protein degradation and phagocytosis, it was shown that SRC and YES had differential effects on endocytic vesicle-mediated protein trafficking events (Xiao *et al.* 2014b). In brief, when knocking down of SRC or YES by RNAi using the corresponding specific siRNA duplexes vs non-targeting negative control siRNA duplexes, SRC knockdown was delayed, whereas YES knockdown enhanced protein endocytosis considerably at the Sertoli cell BTB (Xiao *et al.* 2014b). Thus, under physiological conditions, SRC and YES promote protein

endocytosis and protein retention at the Sertoli cell–cell interface, respectively. Interestingly, though both SRC and YES knockdown delayed the disappearance of biotinylated/endocytosed proteins (e.g., JAM1, CAR, both are Sertoli cell BTB-associated proteins) from the cell cytosol, YES knockdown decelerated the kinetics of protein recycling back to the Sertoli cell surface (Xiao *et al.* 2014b), whereas SRC knockdown considerably impeded Sertoli cell phagocytic activity. Taken collectively, these data support the notion that under physiological conditions, YES may be actively involved in protein recycling and SRC in protein degradation and phagocytosis of Sertoli cells. In agreement with this, when TBC is most prominent between late spermatid and Sertoli cell in stage VII tubules, YES has been found at the concave side of spermatid heads in the rat testis which corresponds to the site of apical TBC (Vogl *et al.* 2014). SRC was strongly associated with residual bodies in stage VIII tubules when its localization was assessed by immunohistochemistry using a specific anti-SRC antibody (Xiao *et al.* 2014b). These findings thus support the concept that under physiological conditions, SRC promotes protein endocytosis at the ES, but directs the endocytosed proteins to the intracellular endosome-mediated degradation pathway. On the other hand, YES also promotes protein endocytosis at the ES, but directs endocytosed proteins to the pathway of protein recycling (perhaps also transcytosis) (Fig. 4). Whether internalization and intracellular sorting of junctional proteins are dependent or independent of SRC/YES-mediated tyrosine phosphorylation and whether SRC/YES could function as docking protein in these endocytic trafficking events in the testis have not yet been investigated. But studies from our lab and others have shown that tyrosine phosphorylation of component proteins is essential to the regulation of apical ES and BTB dynamics, and SRC kinase activity is required for germ cell departure from the epithelium (apical

ES disruption) and adhesion of germ cells to Sertoli cells (apical ES assembly) (Zhang *et al.* 2005, Shupe *et al.* 2011), implicating that SFK-mediated tyrosine phosphorylation may have a role to play in endocytosis and membrane trafficking during junction restructuring at spermatogenesis.

SRC and YES on F-actin organization in the seminiferous epithelium

As noted above, both SRC and YES are involved in endocytic vesicle-mediated protein trafficking events, in which SRC promotes endocytosed proteins to enter the endosome-dependent degradation pathway, whereas YES promotes endocytosed proteins to be recycled, illustrating these two SFKs have contrasting roles on the fate of the endocytosed proteins in Sertoli cells during spermatogenesis. While this observation is physiologically important, the underlying biochemical and/or molecular mechanism(s) remains unclear. However, other studies have shown that endocytic vesicle-mediated protein trafficking is a cytoskeleton-dependent effect, intimately supported by actin- and/or microtubule-based cytoskeletons (Echarri & Del Pozo 2015, Kim & Gadila 2016, West & Harris 2016), involving Rab GTPases (Progida & Bakke 2016, Gibieza & Prekeris 2017, Rout & Field 2017). Studies have shown that the use of a selective YES inhibitor, SU6656, was found to perturb F-actin organization (Xiao *et al.* 2011). For instance, treatment of Sertoli cells with SU6656 at 20 nM induced the retraction of actin microfilaments from cell peripheries, with F-actin wrapping around cell nuclei instead of stretching across the entire cell cytosol as in control cells (Xiao *et al.* 2011). Furthermore, actin microfilaments were extensively truncated following inhibition of YES activity by SU6656 (Xiao *et al.* 2011), thereby perturbing the ES function. This observation is also consistent with findings that when YES was silenced

Figure 4 A hypothetical model illustrating the differential roles of SRC and YES in endocytic vesicle-mediated protein trafficking events at the Sertoli–Sertoli cell (SC) and/or Sertoli–germ cell (GC) interface. The figure progresses through representative junction restructuring events from disassembly of junctions on the left to resassembly of junctions on the right. As described in text, SRC is involved in targeting endocytosed proteins for their eventual endosome-mediated degradation, whereas YES is involved in targeting endocytosed proteins for recycling and transcytosis.

by RNAi, actin microfilaments were disorganized and extensively truncated (Xiao *et al.* 2014b). This pattern of F-actin disruption is somewhat similar to the knockdown of SRC in Sertoli cells (Xiao *et al.* 2014b). Collectively, these findings illustrate that both SRC and YES are important regulators of F-actin under physiological conditions by promoting F-actin organization in Sertoli cells to support ES integrity and function. Changes in the organization of F-actin by SRC vs YES, presumably via their action on actin-regulating proteins and/or adhesion protein complexes, facilitate the transport of endocytic vesicles through the degradation vs recycling pathway, respectively. This possibility, while requiring additional future studies in the testis, is in fact supported by studies in other cells and tissues. For instance, mutations or deletions of SFKs in macrophages led to a considerable reduction in cytoskeletal dynamics, impeding cell polarization, migration and phagocytic activity (Baruzzi *et al.* 2008). On the other hand, SRC (but not FYN) also promotes proper spindle orientation to support prometaphase (Nakayama *et al.* 2012). In summary, additional studies are needed to better understand how SFKs modulate protein trafficking events through their regulatory role on actin-based cytoskeletons. Furthermore, SRC, FYN and LCK were shown to bind to the microtubule cytoskeleton components such as Tau and A-Tubulin (Klein *et al.* 2002, Lee 2005), but little is known in the testis. Thus, besides F-actin, future studies should also include analysis of the MT- and vimentin-based cytoskeletons to examine whether SFK modulates MT- and vimentin-based cytoskeletal organization.

Concluding remarks and future perspectives

There is emerging evidence to support the concept that SFKs regulate spermatogenesis through the differential effects of different SFK members on endocytic vesicle-mediated trafficking and F-actin organization in Sertoli cells. However, two questions remain unaddressed. First, what is the overall upstream regulator(s) of SRC and YES on Sertoli cell protein trafficking function? Does this involve specific types of germ cells or other regulators, such as testosterone, cytokines and others? Second, besides SFK members, are there other non-receptor protein kinases also taking part in these events, such as FAK (focal adhesion kinase)? This is important and studies have shown that FAK and its phosphorylated forms are known regulators of Sertoli cell BTB dynamics (Lie *et al.* 2012). On the other hand, due to the presence of several SH domains (Fig. 2) along the polypeptide sequence of SFKs, each member of SFK, such as SRC and YES, can interact with other proteins in Sertoli cells and can even recruit additional partner proteins to widen the scope of its function in the testis. For instance, it is known that SRC is an integrated component of the Cx43 and ZO-1 complex in Sertoli cells in the testis (Gilleron *et al.* 2008, Li *et al.* 2009). SFKs are also

known regulators to modulate spermatogonial stem cell proliferation and function (Braydich-Stolle *et al.* 2007), and FYN kinase is involved in GDNF signaling to modulate spermatogonial stem cell function (Braydich-Stolle *et al.* 2010). Furthermore, SRC is the downstream protein kinase of non-classical testosterone signaling pathway, shown to be involved in the release of sperm at spermiation (Shupe *et al.* 2011). It is expected that with recent advances in cell and molecular biology techniques, many of these questions will be answered within the next decade.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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