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c-Src and c-Yes are Two Unlikely Partners of Spermatogenesis and their Roles in Blood-testis Barrier Dynamics

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Abstract

Src family kinases (SFKs), in particular c-Src and c-Yes, are nonreceptor protein tyrosine kinases that mediate integrin signaling at focal adhesion complex at the cell-extracellular matrix interface to regulate cell adhesion, cell cycle progression, cell survival, proliferation and differentiation, most notably in cancer cells during tumorigenesis and metastasis. Interestingly, recent studies have shown that these two proto-oncogenes are integrated components of the stem cell niche and the cell-cell actin-based anchoring junction known as ectoplasmic specialization (ES) at the: (1) Sertoli cell-spermatid interface known as apical ES and (2) Sertoli-Sertoli cell interface known as basal ES which together with tight junctions (TJ), gap junctions and desmosomes constitute the blood-testis barrier (BTB). At the stem cell niche, these SFKs regulate spermatogonial stem cell (SSC) renewal to maintain the proper population of SSC/spermatogonia for spermatogenesis. At the apical ES and the BTB, c-Src and c-Yes confer cell adhesion either by maintaining the proper phosphorylation status of integral membrane proteins at the site which in turn regulates protein-protein interactions between integral membrane proteins and their adaptors, or by facilitating androgen action on spermatogenesis via a nongenomic pathway which also modulates cell adhesion in the seminiferous epithelium. Herein, we critically evaluate recent findings in the field regarding the roles of these two unlikely partners of spermatogenesis. We also propose a hypothetical model on the mechanistic functions of c-Src and c-Yes in spermatogenesis so that functional experiments can be designed in future studies.

Introduction

In the mammalian testis such as in rodents, spermatogenesis takes place in the seminiferous epithelium of the seminiferous tubule via an intricate process in which a diploid spermatogonium is theoretically capable of producing 4096 haploid spermatids via cycles of mitosis and meiosis¹⁻⁴ even though ~75% of germ cells undergo apoptosis to avoid overwhelming the fixed number of Sertoli cells per testis.⁵ Spermatogenesis refers to the development of spermatozoa from spermatogonial stem cells (SSC) and spermatogonia, which can be divided into several discrete cellular events, which include: (1) SSC/spermatogonial self-renewal via mitosis, (2) mitotic proliferation and differentiation of spermatogonia, and differentiation of Type B spermatogonia into preleptotene spermatocytes, (3) cell cycle progression in spermatocytes, (4) meiosis including meiosis I

and II that form secondary spermatocytes and spermatids, respectively, (5) spermiogenesis and (6) spermiation. These events are supported exclusively by the Sertoli cell since Sertoli and germ cells are the *only* cellular components that constitute the seminiferous epithelium in the mammalian testis⁶⁻⁹ (Fig. 1). The seminiferous epithelium, however, is anatomically segregated into the basal and the adluminal (apical) compartments by specialized junctions between adjacent Sertoli cells near the basement membrane that create the blood-testis barrier (BTB), so that meiosis I and II and postmeiotic spermatid development can take place in a specialized microenvironment (i.e., the adluminal compartment), segregated from the host's systemic circulation (Fig. 1). Moreover, preleptotene spermatocytes transformed from Type B spermatogonia must traverse the BTB while differentiating into leptotene spermatocytes at Stage VIII of the epithelial cycle to enter the apical compartment to prepare for meiosis I and II which occur at Stage XIV of the cycle in the rat testis. Thereafter, round spermatids (step 1 spermatids) are transformed into elongated spermatids (step 19) via spermiogenesis so that spermatozoa can be released into the tubule lumen at spermiation,¹⁰ and all of these cellular events also involve the movement of developing germ cells across the seminiferous epithelium^{8,11} (Fig. 1). It is therefore conceivable that tremendous restructuring events are taking place in the seminiferous epithelium throughout spermatogenesis, especially at the BTB during the transit of preleptotene spermatocytes into the adluminal compartment, the movement of spermatids across the epithelium and at the luminal edge when spermatozoa are released from the Sertoli cell epithelium at spermiation.¹⁰⁻¹³ Interestingly, the events of spermiation and BTB restructuring that take place simultaneously at Stage VIII of the cycle but at opposite ends of the seminiferous epithelium (Fig. 1), were recently shown to be tightly regulated during spermatogenesis.^{11,14}

The BTB is one of the tightest blood-tissue barriers found in the mammalian body to protect developing spermatocytes to undergo meiosis I and II, as well as spermatid development during spermiogenesis from deleterious immune factors, toxicants and/or unwanted substances/hormones.^{11,13,15,16} Unlike other blood-tissue barriers which are created by endothelial tight junctions (TJs) of the capillaries (e.g., the blood-brain barrier, the blood-ocular/retina barrier), the BTB is constituted by multiple coexisting junction types, that is, TJ, basal ES [basal ectoplasmic specialization, a testis-specific atypical adherens junction (AJ) type], gap junction (GJ) and desmosome and is in proximity of the basement membrane (a modified form of extracellular matrix in the testis).^{11,17,18} Also, unlike other blood-tissue barriers (e.g., the blood-brain barrier) which create a tightly “sealed” ultrastructure, the BTB undergoes extensive restructuring at Stage VIII of the seminiferous epithelial cycle to allow the transit of preleptotene spermatocytes to enter the adluminal compartment for further development. Yet, the BTB integrity cannot be compromised, even transiently, during the passage of preleptotene spermatocytes to avoid the host's immune system from mounting an immune response to specific antigens that arise transiently during meiosis I and II and spermiogenesis. Recent studies have shown that this is possibly mediated via the intricate but coordinated actions of cytokines and testosterone, in which cytokines disrupt the “old” BTB above preleptotene spermatocytes in transit while androgens promote the assembly of “new” TJ-fibrils below migrating spermatocytes, likely by regulating the kinetics of endocytosis of integral membrane proteins (e.g., occludin and N-cadherin).^{11,14}

Similar events come to pass at the apical ES, an *atypical* AJ type similar to the basal ES at the BTB but restricted to the interface of Sertoli cells and developing spermatids (step 8-19 in the rat testis) at spermiation—involving the breakdown of apical ES and the release of mature spermatozoa from Sertoli cell cytoplasmic crypts to the tubule lumen, which occurs synchronously with the translocation of preleptotene spermatocytes across the BTB.^{10,11,14,19} Apical ES is the only anchoring device once it appears between Sertoli cells and step 8 spermatids which persists through step 19 and 16 in rats and mice, respectively^{7,19} to maintain cell adhesion and spermatid polarity (orientation)²⁰ and structurally it is a hybrid AJ type, possessing the properties, as well as the constituent proteins of AJ, focal contact, TJ and GJ found in other epithelia.²¹⁻²³ Although adhesion protein complexes such as the N-cadherin/catenin complex can be found at both the apical and basal ES, other protein complexes, such as $\alpha 6\beta 1$ -integrin/laminin333 and JAM-C/ZO-1, are restricted to the apical ES^{11,20} and biologically active laminin fragments generated at the apical ES were shown to regulate the BTB restructuring at Stage VIII of the epithelial cycle,²⁴ thereby coordinating these two cellular events, namely spermiation and BTB restructuring, that take place at the opposite ends of the seminiferous epithelium. Prior to spermiation, the apical ES undergoes extensive restructuring to prepare for its degeneration so that adhesion protein complexes can be recycled via endocytic vesicle-mediated protein trafficking involving endosomes²⁵⁻²⁷ and it is metamorphosed into an endocytic device formerly designated as apical tubulobulbar complex (TBC), manifested by tubular invaginations in the Sertoli cell cytoplasm which provides a complementary cuff for the docking of spermatid head.^{11,27-29} In short, the role of the apical TBC is to recycle the endocytosed junctional components, such as the $\alpha 6\beta 1$ -integrin/laminin333 complex, and the JAM-C/ZO-1 complex, from the “old” apical ES to be reused for the “new” apical ES that arises during spermiogenesis.¹¹

In this chapter, based on recent findings in the literature, we provide a critical evaluation on how two well studied members of SFKs (Src family kinases), namely c-Src and c-Yes, which are known to perform considerably overlapping but also discrete cellular functions in several epithelia and are mostly restricted to the cell-matrix interface at the focal contacts,^{30,31} work together to regulate spermatogenesis via their effects on: (1) spermatogonial stem cell/spermatogonial self-renewal and (2) cell adhesion which is mediated via their actions on cell adhesion protein complexes at the apical ES and/or BTB, as well as nongenomic androgen action. Initially, we provide a brief comparison of c-Src and c-Yes structurally and functionally and their divergent roles on cell adhesion during carcinogenesis since this information will be helpful in designing functional experiments for reproductive biologists.

General Structure of SFKs and a Comparison Between c-Src and c-Yes

Currently, SFKs, such as Src, Yes, Fyn, Lck, Hck, Fgr, Lyn, Blk and Yrk, constitute a well-defined subfamily of cytoplasmic nonreceptor protein tyrosine kinases known to regulate various cellular events, such as cell differentiation, proliferation, migration and adhesion.^{32,34} Genetic models of SFK-knockout mice were shown to display impaired immune function, developmental defects and deficiency in multiple epithelia (Table 1). Each member of the SFK family shares similar physicochemical characteristics, that is, they have

an apparent molecular weight of 53-62 kDa and a backbone structure composed of four src-homology (SH) domains for protein-protein interactions (Fig. 2A): specifically, an N-terminal SH4 domain, followed by SH3 and SH2 domains and finally a kinase (SH1) domain near the C-terminus.^{32,34,35} The myristoylation and palmitoylation sites near the N-terminus of SH4 domain in all SFKs are needed for membrane localization,^{36,37} so that SFK kinases can be recruited to specific cellular domains/sites in response to changes in the environment, pathogenesis of a disease (e.g., tumorigenesis) and/or during development. Interestingly, c-Src and Blk are nonpalmitoylated since there is a cluster of basic amino acid residues in c-Src/Blk which is able to interact with phospholipids for membrane anchoring. Also, reversible palmitoylation has been shown to affect the distinctive localization, trafficking and activation of SFKs. For example, mono-palmitoylated c-Yes was found to accumulate at the Golgi pool of caveolin which was then transported to the plasma membrane through the exocytic pathway, whereas c-Src rapidly cycled between the plasma membrane and late endosomes/lysosomes.^{38,39} In addition, palmitoylation probably increases the affinity of SFKs (e.g., c-Yes but not c-Src) for the lipid-enriched membrane microdomain at cell junctions, which plays a role in down-regulation of the SFK transforming activities and thus the functional differences between c-Src and c-Yes.⁴⁰⁻⁴² Summy et al, suggested that the unique subcellular localization of c-Yes modulated its effects on actin remodeling in Src-deficient fibroblasts.⁴³ It is noted that, although myristoylation exerts a positive effect on kinase activity, the resulting membrane association may negatively regulate intracellular stability of c-Src (and also Blk) because they may be more susceptible to ubiquitin-mediated degradation^{44,45} This seemingly contributes to the signaling specificity between c-Src and c-Yes, which will be further discussed below.

SH3 and SH2 domains are located behind the SH4 domain (Fig. 2A), which bind to proline-rich motifs and phosphotyrosine residues found in other proteins, respectively. Thus, these two domains are critical for mediating inter- and intramolecular protein-protein interactions. The SH1 domain at the C-terminus is the kinase/catalytic domain, harboring the autophosphorylation site, i.e., Tyr419 in c-Src⁴⁶ and Tyr424 in c-Yes⁴⁷ (Fig. 2A). Tyr530 in c-Src and Tyr535 in c-Yes at the C-terminal region, however, are inhibitory in nature since their phosphorylation lead to a blockade of the corresponding SFK activity. Endogenous negative regulators of SFKs, namely C-terminal Src kinase (Csk) and Csk-homologous kinase (Chk), keep SFKs in an “off” state in the absence of stimulation⁴⁸ and a conformational change as shown in Figure 2B would allow phosphorylation of the stimulatory tyrosine to activate SFKs.³³ It has been reported that the association of c-Src with the plasma membrane is relatively dynamic and is regulated by its intrinsic kinase activity and its SH2 domain⁴⁹ These observations thus indicate that SFKs are capable of interacting with a large number of partner proteins and substrates via their inherent SH domains, which pave the way for their ability to integrate and transmit diverse cellular signals.

Divergent Roles of c-Src and c-Yes During Carcinogenesis

As a proto-oncogene, aberrant and elevated c-Src activity and its protein levels are often associated with the development and/or progression of miscellaneous human cancers as recently reviewed.^{33,50,51} For instance, the activation of c-Src has been detected in more

than 70% of human colorectal cancer patients but only ~50% for c-Yes.^{52,53} During cancer cell invasion and metastasis, c-Src was found to mediate AJ disruption and cytoskeletal turnover by targeting cell adhesion-related molecules such as integral membrane proteins (e.g., E-cadherin), actin-binding proteins (e.g., villin), as well as activators of Rho GTPases (e.g., Tiam1), to promote cell migration.⁵⁴⁻⁵⁶ Moreover, a c-Src/ β -actin complex in integrin-mediated cell adhesion at focal contact was found to modulate peripheral actin dynamics in colon cancer cells.⁵⁷ Equally important, a blockade of c-Src activity was shown to maintain AJ/TJ barrier function and to suppress tumor dissemination.⁵⁸⁻⁶⁰

Despite the fact that c-Src is expressed predominantly in tumors and its overexpression and/or hyperactivation is most frequently observed in human epithelial and non-epithelial cancers,^{61,62} distinctive profiles of c-Yes at the membrane lipid microdomains are found during carcinogenesis which cannot be substituted by c-Src. For instance, in human colorectal cancer HT29 cell line, c-Yes knockdown, but not c-Src, was found to induce cell apoptosis, and morphological changes, which also accompanied by E-cadherin/ β -catenin accumulation at the AJ and, as such, an adaptor function unique to c-Yes other than its intrinsic kinase activity in cell migration was proposed.⁶³ However, the role of c-Yes in germ cell apoptosis remains to be investigated. In human melanoma cells, c-Yes, but not c-Src, became constitutively active after being treated with GD3 ganglioside in lipid rafts, thereby triggering the p130Cas and paxillin signaling pathway to facilitate cell growth and invasion.⁶⁴ These results are in agreement with those discussed earlier that the interaction of c-Src with membrane-associated targets are dynamic events and that activation of c-Src can cause it to associate with the plasma membrane while at the same time making it less stable.^{39,44,49} Yet, the preferential localization of c-Yes at the membrane microdomains allows this SFK to function in specific cellular domains in response to changes in the environment during growth or pathogenesis. Additionally, the lysosomal localization of c-Src as earlier reported^{38,65} probably distinguishes it from c-Yes even more. Abnormal aggregation of lysosomes at the perinuclear region which occurs during tumor invasion and metastasis can be induced by c-Src through its SH2 domain via a kinase activity-independent manner.⁶⁶ All of these findings clearly demonstrate that the distinct cellular localizations of c-Src and c-Yes likely confer their unique characteristics as regulators of membrane trafficking and junction dynamics during carcinogenesis, some of which are also applicable to spermatogenesis.

SFKs in the Testis

Several members of the SFK family, such as Src, Yes, Fyn, Lck, Hck, Fgr, Lyn and Blk have been found in rodent testes, except Yrk which is absent in mammals.⁶⁷⁻⁶⁹ Interestingly, a different isoform of c-Src exists in the testis versus the one found in the nervous system and, in addition to the full-length kinases, some members of the SFK family are found in the testis as truncated variants (e.g., Fyn, Hck).^{46,70,71}

By using the technique immunohistochemistry and an antibody prepared against c-Src at its C-terminus which partially cross-reacted with c-Yes, Fyn and Fgr due to their sequence similarity, c-Src was shown to be highly expressed in Sertoli and germ cells⁷² and it localized both to the BTB and the apical ES in the adult rat testis (Fig. 3), consistent with an

earlier report.⁷³ The expression of c-Src in the testis was also shown to be stage-specific, with a moderate decrease at the BTB during Stages VII-VIII of the epithelial cycle, but an increase at the apical ES at spermiation, in particular at the site where spermiation just occurs, suggesting that these are the structures of the residual bodies undergoing phagocytosis by Sertoli cells (Fig. 3). Recent studies have also shown that c-Src structurally associates with p-FAK, β 1-integrin, laminin α 3-, β 3- and γ 3 at the apical ES, as well as with CAR, desmoglein-2, connexin 43 and MTMR2 at the BTB.⁷⁴⁻⁸² Furthermore, c-Yes was found to structurally interact with occludin, FAK, N-cadherin, β -catenin, β 1-integrin and actin, but not with CAR, JAM-A, paxillin and vimentin.⁸³ Although much work is needed to determine if other SFK members, besides c-Src and c-Yes, also interact with these proteins at or near the Sertoli-Sertoli and Sertoli-spermatid interface, the significance of SFKs, in particular c-Src and c-Yes, in cytoskeletal and cell junction dynamics is apparent.

SFK and Spermatogonial Stem cell proliferation

The initial step of spermatogenesis involves differentiation of spermatogonial stem cells (SSC) [which are a subset of A_{single} spermatogonia (A_s)^{84,85}] to differentiated Type A spermatogonia (e.g., A_1 , A_2 , A_3 , A_4), to be followed by Type B spermatogonia and then spermatocytes.^{84,86} However, this also requires proliferation of SSC in the stem cell niche for self-renewal to maintain the proper population of SSC. The stem cell niche is located in the basal compartment of the epithelium, near the base of the Sertoli cell but above the basement membrane where several tubules meet and it borders the interstitial space.⁸⁷ Recent studies have shown that GDNF (glial cell line-derived neurotrophic factor)⁸⁸ released from the Sertoli cell into the stem cell niche binds onto its receptor GFR α -1, which, in turn, recruits Ret transmembrane receptor [note: GFR α -1 is expressed exclusively by A_s spermatogonia and possibly A_{paired} (A_{pr}) spermatogonia whereas Ret is expressed by all premeiotic germ cells⁸⁹⁻⁹²] to the site. Then, the activated GDNF/GFR α -1/Ret protein complex induces the c-Src/c-Yes complex at the stem cell niche, which in turn, activates PI3-K (phosphoinositide 3-kinase)/Akt (also known as PKB, protein kinase B, which is a nonreceptor Ser/Thr protein kinase) downstream to up-regulate *N-myc* (neuroblastoma derived myelocytomatosis viral related oncogene) expression (and other genes essential for self-renewal, such as *Bcl6b*, *Erm*, *Lhx1*) to elicit SSC proliferation for self-renewal.^{88,93} In short, GDNF-induced SSC self-renewal via mitotic division at the stem cell niche in rodent testes is mediated by c-Src/c-Yes, consistent with findings in the field regarding the role of SFK and stem cell proliferation.⁹⁴

c-Src on Cell Adhesion, Blood-Testis Barrier and Spermatogenesis

c-Src was first found to be expressed by both Sertoli cells and germ cells at various stages of development and it was shown to be most abundantly associated with elongated spermatids in the adult rat testis.⁹⁵ c-Src expression,⁹⁶ in particular its activated form, p-c-Src-Tyr419,⁷⁴ was later found to peak at the luminal edge of the seminiferous epithelium at the site of the apical ES just prior to spermiation, suggesting a role of c-Src in the degeneration of the apical ES at Stage VIII of the epithelial cycle. This notion was subsequently assessed and elaborated in studies using the adjuvin model, which is a potential male contraceptive that primarily targets the apical ES⁹⁷⁻¹⁰¹ to induce premature spermatid release from the

epithelium in rat testes without compromising the BTB. c-Src was found to structurally interact with the N-cadherin/ β -catenin complex and c-Src was found to be induced in both its protein level and intrinsic kinase activity during adjudin-induced AJ restructuring that led to germ cell loss.⁷² It is likely that c-Src alters the phosphorylation status of adhesion protein complexes at the apical ES, mediating a loss of protein-protein interactions at the apical ES as the result of an increase in protein endocytosis and possibly endosome-mediated phosphoprotein degradation. Indeed, c-Src is one of the SFK members that has been implicated to be involved in late endosomal-lysosomal intracellular trafficking³⁸ and there is evidence that loss of E-cadherin function in epithelial cells requires Src-dependent ubiquitination and lysosomal degradation, to cause a breakdown of cadherin-based cell adhesion.^{102,103} Moreover, upon activation, c-Src is translocated to late endosomes³⁸ from the perinuclear region to the plasma membrane, where it becomes less stable and targeted for degradation.^{44,45} This thus facilitates internalization of adhesion proteins. This concept on the involvement of c-Src in intracellular protein trafficking involving endocytic vesicles and phagosomes, and it is consistent with findings shown in Fig. 3 in which c-Src was intensely localized at the “degenerating” apical ES, associating with residual bodies shortly after spermiogenesis has taken place in which c-Src was localized with phagosome-like structures (see Fig. 3C, D vs B, E and F).

Findings in other epithelia in which c-Src regulates cell adhesion via its effects on endocytic vesicle-mediated protein trafficking events are also consistent with recent studies that reported the involvement of c-Src in protein trafficking and junction restructuring events at the BTB. c-Src was found to form a functional complex with GJ protein connexin 43, desmosomal protein plakophilin-2 and basal ES proteins N-cadherin and β -catenin and was proposed to regulate the phosphorylation status and binding of occludin to ZO-1, to perturb the Sertoli cell TJ-barrier.⁸² These findings are consistent with studies in other epithelia that activation of c-Src is believed to be involved in the dissociation of TJs. For example, two highly conserved tyrosine residues (Tyr398 and Tyr402 in humans) at the C-terminal region of occludin in epithelial cells could be phosphorylated exclusively by c-Src which would prevent the binding of ZO-1 and its homologs (e.g., ZO-2, ZO-3), but not F-actin, to occludin, thus negatively regulating TJ reassembly in Caco2 cells.¹⁰⁴ In keeping with this, it has been reported that decreased Ser/Thr phosphorylation and induced tyrosine phosphorylation of occludin occur at the same time during the disruption of epithelial TJ.¹⁰⁵ A study using the calcium switch model also revealed that during TJ biogenesis, upon G α 12-stimulated tyrosine phosphorylation of ZO-1/ZO-2 by c-Src, claudin-1 and occludin became disassociated from the ZO-1 complex, thereby destabilizing the TJ-barrier. These findings thus support the notion that c-Src activation can lead to a loss of TJ-barrier function.¹⁰⁶

In addition, *c-src*^{-/-} mice were found to be infertile (Table 1), however, it is not known at present if this is the result of a failure in SSC self-renewal since SFK signaling has been recognized as essential for self-renewal of mouse SSC^{88,93,107,108} (see above). A study from our laboratory also showed that when a selective SFK inhibitor PP1 was administered intraperitoneally to adult rats, there was a loss of spermatocytes and round spermatids.⁷² Moreover, c-Src is intimately involved in nongenomic androgen action in the testis and

androgen is known to be involved in spermatogenesis and to be essential for meiosis and germ cell development.^{109,110} A more recent study has shown that the nongenomic action of androgen that regulates Sertoli-germ cell adhesion, mostly notably spermatid adhesion in the seminiferous epithelium using a novel cell adhesion assay involving seminiferous tubules, is mediated by c-Src, which can also modify androgen-regulated genes transcriptionally.¹¹¹

c-Yes on Cell Adhesion, Blood-Testis Barrier and Spermatogenesis

Functional redundancy between c-Yes and c-Src in many epithelia examined to date, coupled with the lack of specific agonists and/or antagonists against individual kinases have made it difficult to discern the physiological function of c-Yes *versus* c-Src during spermatogenesis. A recent study has reported the cellular localization of c-Yes in the seminiferous epithelium of adult rat testes.⁸³ It has shown that c-Yes is present at the BTB in almost all stages of the epithelial cycle, with the highest expression occurring at Stages VIII-IX, coinciding with BTB restructuring to accommodate the transit of preleptotene spermatocytes at the site (Fig. 4). This pattern of stage-specific expression for c-Yes is somewhat different from c-Src in that the expression of c-Src at the BTB around stages VIII-IX was found to be considerably reduced. Thus, it is increasingly clear that c-Yes and c-Src, while both are members of the SFK family, are probably playing different roles in the seminiferous epithelium during the epithelial cycle of spermatogenesis. Notably, c-Yes was found to interact with occludin- and N-cadherin-based adhesion protein complexes at the BTB.⁸³ The involvement of c-Yes in TJ formation/maintenance has been reported in other epithelial cells. In T84 human intestinal cell line and MDCK II epithelial cells, c-Yes was shown to form a functional complex with occludin; and when the calcium depletion-repletion model was used to study the latter cell type, there was a temporary surge of tyrosine phosphorylation of occludin correlating with TJ reassembly.^{112,113} Indeed, when BTB function was found to be enhanced during adjuvin-mediated apical ES disruption at the Sertoli-spermatid interface in adult rat testis, the protein levels of several TJ and basal ES proteins, as well as c-Yes, were found to be induced at the BTB.^{83,114}

Other studies have shown that cytokines, such as TGF- β 3 and TNF α , are crucial regulators of BTB dynamics.^{115,116} During TGF- β 3-induced loss of Sertoli cell-TJ barrier integrity, a significant decline in the c-Yes steady-state protein level was found to precede the reduction of other integral membrane proteins at the BTB, such as occludin and N-cadherin at the TJ and basal ES, respectively, which thus illustrates the importance of c-Yes in supporting TJ-barrier function.⁸³ More important, when c-Yes activity was blocked by a selective SFK inhibitor SU6656 preferentially to block c-Yes function, occludin and N-cadherin at the Sertoli-Sertoli cell interface were found to become mis-localized and to associate more with the endocytic vesicle protein clathrin,⁸³ further demonstrating the significance of c-Yes in conferring cell adhesion at the Sertoli cell BTB.

Besides at the BTB, c-Yes was also detected at the apical ES but subsided considerably to an almost nondetectable level by late Stage VIII just prior to spermiation, coinciding with the degeneration of the apical ES.⁸³ This pattern of expression of c-Yes is different from c-Src which, in contrast, most notably expressed at Stage VIII at both the apical ES and the BTB, but it remained intensely expressed at the “degenerating” apical ES site, associated mostly

with ultrastructures of residual bodies following spermiation (see Fig. 3 versus Fig. 4). It is noteworthy that these differential expression patterns of c-Yes and c-Src around the time of spermiation presumably indicates their participation in the degeneration of apical ES, as well as the subsequent endocytic recycling events when the “old” apical ES under dissolution is being used to assemble the “new” one for newly differentiated step 8 spermatids during spermiogenesis (Fig. 5). As noted above, following biosynthesis, c-Yes is transported to the plasma membrane from the Golgi pool of caveolin in COS-1 cells³⁹ to be used for endocytic vesicle-mediated protein trafficking events, such as protein recycling since caveolin is a marker for protein transcytosis in cells including those in the testis.^{117,118} In line with this possibility, c-Yes knockout mice revealed a deficiency in pIgA-pIgR transcytosis which would not be compensated by c-Src; and the formation of a pIgR-c-Yes-EGFR complex is necessary for the pIgA internalization and recruitment to the recycling endosome in polarized epithelial cells.^{119,120} The possibility that c-Yes is involved in protein recycling events during spermatogenesis, such as for recycling of proteins from the “old” apical ES and the “old” BTB site to the “new” apical ES and “new” BTB under “construction” via its role in endocytic vesicle-mediated protein trafficking, such as endocytosis, recycling, transcytosis and endosome-/ubiquitin-mediated protein degradation (Fig. 5), must be carefully evaluated in future studies.

In this context, it is of interest to note that Par6, a component of the Par3/Par6 polarity complexes known to regulate the junction restructuring at the apical ES,²⁰ reaching its lowest expression level at the apical ES in misaligned and depleting spermatids following treatment of rats with adjuvin, was found to have a tighter association with SFK member(s).¹²¹ All of these results suggest that c-Yes or c-Src activity at the apical ES is being used to induce undesired phosphorylation of apical ES constituents such as that of Par6, to evoke protein endocytosis [note: Par6, Par5 (also known as 14-3-3) and Cdc42 (a component of the Par6-based protein complex^{20,122}) were recently shown to be involved in regulating protein endocytosis at the Sertoli cell BTB,^{123,124} thereby destabilizing the apical ES and leading to elongating/elongated spermatid loss from the epithelium.

An Emerging Model by Which SFKs Regulate Spermatogenesis in Particular Blood-Testis Barrier Dynamics in the Seminiferous Epithelium

The model depicted in Figure 5 regarding the role of SFKs, most notably c-Src and c-Yes, in spermatogenesis, was prepared based on recently published studies in the rodent testis as described in the text above. First, c-Src and c-Yes play a critical signaling role to mediate GDNF-stimulated SSC proliferation for self-renewal of SSC via PI3K/PBK downstream (see also Fig. 1); without this, aspermatogenesis would result due to the lack of differentiated Type A and B spermatogonia. Second, c-Src and c-Yes are crucial for spermatid adhesion to the epithelium at the apical ES, possibly working in conjunction with FAK (focal adhesion kinase)⁷⁸ to maintain the proper phosphorylation status of proteins at the apical ES (e.g., integrins, laminins, nectins, JAM-C) via structural interactions between c-Src and/or c-Yes with these proteins^{75,77,83,121} in response to testosterone^{74,76,111,125} via the nongenomic pathway.^{110,111} This action of SFKs, in concert with FAK,¹²⁶ maintain cell adhesion. This is also applicable to the Sertoli-Sertoli cell interface at the BTB via the

structural interactions of c-Src with connexin 43⁸² and desmoglein-2⁷⁹ and also c-Yes with occludin and N-cadherin,⁸³ so that c-Src and c-Yes can maintain the proper phosphorylation status of integral membrane proteins and their peripheral adaptors at the BTB. This, in turn, confers proper protein-protein interactions between integral membrane proteins and their adaptors at the BTB so that these proteins can be assembled to the TJ-fibrils at the BTB to maintain the immunological barrier integrity. For instance, it was shown that a loss of FAK, which is a component of the occludin-ZO-1 adhesion protein complex,¹²⁶ by RNAi using FAK-specific siRNA duplexes was found to induce dissociation between occludin and ZO-1, which was accompanied by unwanted phosphorylation of occludin.¹²⁷ Perhaps c-Src and c-Yes also regulate endocytic vesicle-mediated protein trafficking events (e.g., endocytosis, transcytosis, recycling) via the effects of c-Yes on actin filament dynamics.⁸³ It is obvious that the action of c-Src and c-Yes on different cellular events pertinent to spermatogenesis in particular their role in BTB dynamics as depicted in the model shown in Figure 5 will require additional investigation in the years to come, however, this model provides a framework for investigators in the field, including our laboratory, to design functional experiments. For instance, besides mediating the effects of GDNF to induce SSC proliferation, can c-Src and c-Yes confer adhesion of SSC to the Sertoli cell at the stem cell niche to maintain proper signaling between Sertoli cells and SSC to fine tune spermatogonial differentiation via gap junctions and/or desmosomes? If this is the case, what is the significance of cell adhesion conferred by SFK kinases to the maintenance of SSC population at the stem cell niche?

Conclusion and Future Perspectives

As briefly summarized above, it is increasingly clear that members of the SFK, most notably c-Src and c-Yes, are two unlikely partners that regulate spermatogenesis, most notably the BTB and the apical ES function. Both c-Src and c-Yes have been defined as crucial signaling molecules at the cell-extracellular matrix interface, similar to FAK, since they regulate focal adhesion (or focal contact) function, pertinent to cell migration during development (e.g., embryogenesis) and pathogenesis (e.g., tumorigenesis and metastasis), mediating integrin-based signaling functions;¹²⁸⁻¹³⁰ thus, no studies were found in the literature regarding the role of SFK members at the cell-cell interface in the seminiferous epithelium. In fact, their significance at the cell-cell AJ in the seminiferous epithelium was not known until the 2000s¹³¹ when c-Src was first found to be a component of the apical ES at the Sertoli-spermatid interface,^{72,96} most notably the integrin-based adhesion protein complex when examined by co-immunoprecipitation.⁷⁸ Since then, c-Src and its functional partner FAK, as well as c-Yes, were also found to be structurally associated with proteins at the BTB, such as occludin,⁸³ N-cadherin,⁸³ connexin 43,⁸² CAR⁸⁰ and desmoglein-2.⁷⁹ Thus, these two SFKs have become two unlikely partners in regulating cell adhesion in the seminiferous epithelium. As discussed herein, members of SFK such as c-Src and c-Yes, are also involved in GDNF-mediated SSC proliferation for self-renewal to maintain the proper SSC/spermatogonia population at the stem cell niche. It remains to be investigated if c-Src and/or c-Yes are involved in other aspects of spermatogenesis, most notably, cell cycle progression, meiosis and germ cell apoptosis. Since, cancer cells are known to utilize c-Src signaling to regulate cell cycle progression during tumorigenesis^{132,133} and other studies

have shown that androgen-induced Src activation regulates cell cycle progression, apoptosis, migration and differentiation in cancer cells, such as in prostate and breast cancer;¹³⁴⁻¹³⁶ thus, it is logical that future studies should include an investigation on the role of c-Src and c-Yes on cell cycle progression and meiosis in spermatocytes and cell apoptosis in spermatocytes and spermatids during spermatogenesis. Furthermore, much work is needed to delineate the role of c-Src and c-Yes in endocytic vesicle-mediated protein trafficking at the apical ES and the BTB, in particular how these nonreceptor protein tyrosine kinases work in concert with FAK to mediate protein endocytosis, recycling and transcytosis to maintain the homeostasis of apical ES and BTB function. For instance, endocytic vesicle-mediated protein trafficking events are known to involve SFK¹³⁷ and recent studies have demonstrated unequivocally the involvement of Src in protein endocytosis and/or recycling during carcinogenesis.¹³⁸⁻¹⁴¹ In short, we have provided a working hypothetical model to investigate the role of two unlikely partners, c-Src and c-Yes, on multiple cellular events pertinent to spermatogenesis, such as at the apical ES and the BTB.

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Figure 1.

A graphic representation of the cellular events that take place in the seminiferous epithelium during spermatogenesis. Shown on left panel are relative locations of different germ cell types and their intimate association with the two adjacent Sertoli cells that constitute the seminiferous epithelium, overlying the tunica propria of the seminiferous tubule in the rat testis. The blood-testis barrier (BTB) formed by *co-existing* tight junction (TJ), basal ectoplasmic specialization (basal ES), gap junction and desmosome between adjacent Sertoli cells divides the seminiferous epithelium into two compartments: the basal and the adluminal (apical) compartments.^{11,14,15} At the basal ES, spermatogonial stem cell (SSC) and early undifferentiated Type A spermatogonia undergo self-renewal via mitosis, to be followed by their differentiation into differentiated Type A and then Type B spermatogonia, which are the germ cells to be transformed to preleptotene spermatocytes. Preleptotene spermatocytes are the only germ cells that are in transit at the BTB to enter the adluminal compartment while differentiating into leptotene spermatocytes at Stage VIII of the epithelial cycle, to be followed by zygotene and pachytene spermatocytes to prepare for meiosis I and II (see right panel) which takes place at Stage XIV of the epithelial cycle. Apical ES is the *only* anchoring device found at the Sertoli cell-elongating spermatid interface (step 8-19 spermatids) to maintain spermatid adhesion, cell polarity (orientation) and cell-cell communication. Apical ES is typified by the presence of actin filament bundles sandwiched in between cisternae of endoplasmic reticulum and the apposing plasma members of the Sertoli cell and the spermatid, whereas desmosome and gap junction provide spermatogonia/spermatocytes/step 1-7 spermatids mechanical adhesion onto Sertoli cells and cell-cell communication, respectively. Immediately prior to spermiogenesis, apical ES undergoes extensive endocytic vesicle-mediated protein trafficking (e.g., endocytosis, transcytosis, recycling and endosome-/ubiquitin-mediated protein degradation) and

transforms to an ultrastructure visible under electron microscopy and fluorescent microscopy at high resolution as the result of invagination of plasma membrane formerly designated apical tubulobulbar complexes (apical TBC), preparing for the “degeneration” of the apical ES to facilitate the release of sperms at spermiation.^{10,11,25,26} Underneath the seminiferous epithelium is the tunica propria, which is composed of four layers, beginning with the basement membrane, Type I collagen layer, the peritubular myoid cell layer and the lymphatic endothelium, sequentially.^{21,142} Between Sertoli cells and the basement membrane is an intermediate filament-based cell-matrix anchoring junction type called hemidesmosome. Recent studies as described in *text* and summarized herein have shown that c-Src and c-Yes are involved in multiple cellular events pertinent to spermatogenesis, such as SSC/spermatogonia self-renewal, BTB restructuring to accommodate the transit of preleptotene spermatocytes, apical ES dynamics to facilitate spermiogenesis and spermiation.

Figure 2.

A schematic drawing illustrating structural and functional domains of rat c-Src and c-Yes and their activation in epithelial cells including the seminiferous epithelium. A) Members of Src family kinases (SFKs), such as c-Src and c-Yes, are composed of four characteristic src-homology (SH) domains: SH4 domain at the N-terminus, followed by SH3 and SH2 domains and finally a kinase/SK1 domain which is joined to the SH2 domain by a short polyproline Type II helix called SH2-SK1 linker. Within the SH4 domain of c-Src/c-Yes there is a penultimate glycine (Gly2) residue cotranslationally myristoylated (M) to be used for membrane targeting and signal transduction. In addition to the myristoylation, an cysteine residue (Cys3) near the N-terminus of c-Yes is posttranslationally palmitoylated (P) to be used for its subcellular trafficking, such as membrane association due to an increase in its hydrophobicity following palmitoylation. Subsequent to the myristoylation/palmitoylation motif, there is a unique domain (50-70 residues in length, shown in red at the N-terminus) that confer structural uniqueness to each member of SFKs because of its sequence divergence across the family. But the function of this region is not well defined. B) Under normal physiological conditions, SFK is auto-inhibited through intramolecular interactions in which the SH2 domain binds to the inhibitory phosphotyrosine at the C-terminal tail, while the SH3 domain interacts with the SH2-SK1 polyproline linker so that

these two protein interacting motifs block the kinase domain and stabilize SFK in an inactive conformation (left). SFKs can be activated by binding of a ligand to the SH2/SH3 domain, or with dephosphorylation of the inhibitory phosphotyrosine by protein tyrosine phosphatases, thus leading to a conformational change which would allow the phosphorylation of the stimulatory tyrosine in the activation loop and to confer intrinsic kinase activity in kinase domain (SH1) (right).

Figure 3.

Distribution and cellular localization of c-Src in the seminiferous epithelium of adult rat testes. Paraffin sections of adult rat testes were used for immunohistochemistry using an antibody against c-Src (Santa Cruz, Cat. # sc-8056, Lot# K0507, 1:50 dilution) as shown in A-G, essentially as earlier described for c-Yes,⁸³ illustrating cellular localization and stage-specific expression of c-Src in the seminiferous epithelium. The specificity of this antibody was illustrated in an immunoblot shown in (H) using lysates of testes (T), seminiferous tubules (ST) from adult rats and Sertoli cells (SC, from 20-day-old rats following 4 days in cultures). As indicated by the manufacturer, this antibody specifically recognized c-Src, however, it also cross-reacted partially to c-Yes, Fyn and Fgr; however, based on the immunoblot shown in (H), this antibody cross-reacted strongly and mostly with c-Src. Immunoreactive c-Src was intense in the seminiferous epithelium throughout the epithelial cycle of spermatogenesis, localized in the basal compartment near the basement membrane consistent with its localization at the BTB and c-Src also associated with developing spermatids at the apical ES (A). At Stage VIII, the localization of c-Src at the BTB became considerably weakened, but it remained to be intensely localized near the luminal edge of the seminiferous tubule and c-Src was found to associate with the residual body derived from elongated spermatids/spermatozoa which have just departed the epithelium and the residual body appears being phagocytosed by Sertoli cells (D vs. B, C, E and F) [see also the magnified micrographs in the lower panels of B-G encircled with either a “yellow” or “blue”

box derived from the encircled “yellow” and “blue” boxed areas of the corresponding micrograph, illustrating representative areas of the adluminal (apical) compartment (e.g., apical ES, residual bodies) and basal compartment (e.g., BTB)]. Representative tubules of Stages (V, VIII, late VIII, X and XII) correspond B-F, respectively, *versus* control (Ctrl, E) which is a Stage X tubule and serves as a negative control in which mouse IgG was used in place of the anti-c-Src IgG to validate the specificity of the c-Src staining. Bar in A = 100mm; bar in B = 40 mm, which applies to C-G; bar in the micrograph behind b = 20mm which is an inset from B, this applies to all other insets. A color version of this figure is available online at www.landesbioscience.com/curie.

Figure 4.

Distribution and cellular localization of c-Yes in the seminiferous epithelium of adult rat testes. c-Yes localization in the seminiferous epithelium of adult rat testes was examined by immunohistochemistry as described.⁸³ c-Yes was found to be expressed stage-specifically at the BTB and apical ES. c-Yes was localized at the BTB in almost all stages of the epithelial cycle, with the highest expression at Stages VIII-IX (B, C vs. A, D), which is the opposite to c-Src whose localization at the BTB was the lowest at Stages VIII-IX (see Fig. 3). c-Yes was found both at the convex and the concave sides of the apical ES (A, B and D), except that it rapidly diminished at the apical ES shortly before spermiation. In a Stage IX tubule (C), the level of c-Yes in the apical compartment was virtually nondetectable except that it peaked at the BTB in Stages VIII-IX at the time of BTB restructuring to facilitate the transit of preleptotene spermatocytes. (E) Cross-sections of testes stained with normal mouse IgG instead of the anti-c-Yes antibody, illustrating the staining shown in A-D was specific for c-Yes. Bar in A = 55µm, which applies to B-E, bar in inset in A = 25µm, which applies to all insets in B-E.

Figure 5.

The involvement of c-Src and c-Yes in different cellular events during the seminiferous epithelial cycle of spermatogenesis. Based on recent findings in the field as summarized and discussed in the main text, c-Src and/or c-Yes are involved in at least three separate cellular events in the seminiferous epithelium during the seminiferous epithelial cycle of spermatogenesis as shown in (A), (B) and (C), namely (i) spermatid adhesion and the phagocytic events pertinent to the elimination of residual body at spermiogenesis (and possibly the endocytic vesicle-mediated protein trafficking of the “old” apical ES at spermiogenesis) (A), (ii) restructuring of the BTB to accommodate the transit of preleptotene spermatocytes across the BTB, with the “new” BTB being assembled before the “old” BTB is disrupted, so that pachytene spermatocytes can prepare for meiosis in the adluminal (apical) compartment (B) and (iii) self-renewal of spermatogonial stem cells (SSC) as well as the differentiation of SSC to spermatogonia (Type A) and the adhesion of SSC to the Sertoli cell at the stem cell niche via desmosomes and gap junctions (C). Since c-Yes was recently shown to be involved in the endocytic vesicle-mediated (e.g., clathrin) protein trafficking in the testis, in particular at the Sertoli cell BTB,⁸³ we postulate that c-Yes and c-Src may be involved in the endosome-mediated protein trafficking at the apical ES at spermiogenesis (e.g., phagocytosis of residual body derived from step 19 spermatids while transforming to spermatozoa and degeneration of the apical ES to facilitate spermiogenesis) and at the BTB during the transit of preleptotene spermatocytes, both events take place at Stage VIII of the epithelial cycle when c-Yes and c-Src was highest at the BTB and the apical ES, respectively (see also Figs. 3 and 4).

Table 1
Phenotypes in SFK-knockout mice*

Knockout(s)	Viability	Fertility	Phenotype(s)	Reference(s)
Triple knockouts				
<i>src^{-/-}yes^{-/-}fyn^{-/-}</i>	Embryonic lethality (E9.5 day)		Defects in cell-ECM signaling	143
<i>hck^{-/-}fgr^{-/-}lyn^{-/-}</i>	Increase in mortality vs. wild type		1 Hampered leukocyte recruitment 2 Impaired neutrophil phagocytosis 3 Altered macrophage activation	144,145
double knockouts				
<i>src^{-/-}yes^{-/-}</i>	Perinatal lethality		Reduced in body weight	146
<i>src^{-/-}fyn^{-/-}</i>	Perinatal lethality		1 Reduced in body weight 2 Impaired keratinocyte cell adhesion	146,147
<i>src^{-/-}hck^{-/-}</i>	Perinatal lethality		Osteopetrosis	148
<i>yes^{-/-}fyn^{-/-}</i>	Viable		Renal disease	146
<i>fyn^{-/-}lyn^{-/-}</i>	Increase in mortality vs. wild type		Severe lupus-like kidney disease	149
<i>hck^{-/-}fgr^{-/-}</i>	Viable	Fertile	1 Defects in adhesion-dependent neutrophil functions 2 Impaired integrin signaling, altered cytoskeletal structure and reduced motility in macrophages	150-152
Single knockout				
<i>src^{-/-}</i>	Postnatal lethality	Infertile	1 Osteopetrosis 2 Defects in tyrosine phosphorylation of cytoskeletal proteins in osteoclasts 3 An increase in Hck levels in osteoclasts 4 VEGF fails to compromise the BBB 5 Suppression of VEGF-induced FAK phosphorylation and formation of a FAK/ α v β 5 complex 6 Reduction in tumor-induced VP and in spontaneous tumor metastasis 7 Resistance to tumor cell extravasation during metastasis 8 Defects in development of mammary gland, uterine and ovarian	60,67,148,153-159
<i>yes^{-/-}</i>	Viable	Fertile	1 Defects in pIgA-pIgR transcytosis 2 VE-cadherin- β -catenin complex that maintained endothelial barrier function in lung did not dissociate after VEGF treatment 3 Resistance to tumor cell extravasation during metastasis	60,120,146
<i>fyn^{-/-}</i>	Viable	Fertile	1 Reduction in testis weight and presence of degenerated germ cells (3-4 weeks old) 2 Impaired TCR signaling 3 Neural dysfunctions	160-164

Knockout(s)	Viability	Fertility	Phenotype(s)	Reference(s)
<i>lck</i> ^{-/-}	Viable	Fertile	4 Defects in actin cytoskeleton	165-167
			5 Increases c-Yes in oocytes	
			1 Defects in T-cell development and TCR signaling	
			2 Impaired B-cell function and BCR signaling	
			3 Retinal dysplasia	
<i>hck</i> ^{-/-}	Viable	Fertile	Impaired phagocytosis in macrophages	151
<i>fgr</i> ^{-/-}	Viable	Fertile	Defects in eosinophil recruitment to the lung during allergic airway inflammation	151,168
<i>lyn</i> ^{-/-}	Viable	Fertile	1 Autoimmune disease and myeloproliferation	169-171
			2 Abnormal prostate gland morphogenesis	
<i>blk</i> ^{-/-}	Viable		Defects in T-cell development	172

* This table is not intended to be exhaustive. Instead, but it illustrates the importance of SFKs in immune function and development, especially in cell migration and cell adhesion. ECM: extracellular matrix; VEGF: vascular endothelial growth factor; BBB: blood-brain barrier; VP: vascular permeability; TCR: T-cell receptor; BCR: B-cell antigen receptor.