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Src family kinases (SFKs) and cell polarity in the testis

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Abstract

Non-receptor Src family kinases (SFKs), mostly notably c-Src and c-Yes, are recently shown to be expressed by Sertoli and/or germ cells in adult rat testes. Studies have shown that SFKs are involved in modulating the cell cytoskeletal function, and involved in endocytic vesicle-mediated protein endocytosis, transcytosis and/or recycling as well as intracellular protein degradation events. Furthermore, a knockdown to SFKs, in particular c-Yes, has shown to induce defects in spermatid polarity. These findings, coupled with emerging evidence in the field, thus prompt us to critically evaluate these findings to put forth a developing concept regarding the role of SFKs and cell polarity, which will become a basis to design experiments for future investigations.

Keywords

Src family kinases; c-Src; c-Yes; testis; spermatogenesis; spermatids; Sertoli cells; cell polarity

Introduction

In a typical cross-section of an adult mammalian testis, cell polarity across the seminiferous epithelium is readily notable (Figure 1) [1]. For instance, Sertoli cell nuclei are located mostly near the basement membrane of the tunica propria. Furthermore, peritubular myoid cells are restrictively localized to the myoid cell layer behind the acellular zone of the tunica propria namely the basement membrane (also known as basal lamina) and the type I

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collagen layer (Figure 1). On the other hand, elongating/elongated spermatids are mostly found in the adluminal compartment until these cells are line-up near the tubule lumen, with their heads all point to the basement membrane and their tails to the tubule lumen (Figure 1). This morphological setting of cell polarity is being used to support spermatogenesis. It is envisioned that the robust cellular output of spermatozoa in the tens of millions daily in the seminiferous tubules from an adult male (including rodents and humans) is analogous to a car manufacturing plant. This requires highly orchestrated alignment of developing germ cells (i.e., vehicles) that move through the assembly line (conferred by actin and/or microtubule (MT)-based tracks) with well aligned machineries (e.g., endocytic vesicles aided by GTPases) to support the assembly of different components (e.g., various integral membrane proteins, adaptor proteins) onto the developing germ cells, and the removal of some necessary wastes (e.g., residual bodies). Nonetheless, the concept of cell polarity in particular the involving molecules and the signaling pathways that confer cell polarity in the testis have not been carefully investigated. For instance, it was first reported in 2008 that the Par (partitioning defective)-based polarity complex and its binding partners, namely Par3, Par6 and Cdc42, were expressed by Sertoli and/or germ cells in the testis, and these proteins are involved in apical-basal polarity of developing spermatids and cell adhesion function [2]. Thereafter, the other two known polarity protein complex modules that work in concert with the Par-based polarity complex to support cell polarity [3, 4], namely the Scribble- [5] and the Crumbs-based [6] complexes, were also identified in the testis with their functions partially characterized [7, 8]. Furthermore, many of the planar cell polarity (PCP) proteins necessary to modulate PCP including Van Gogh-like 2 (Vangl 2) were later shown to be expressed in the testis, also with defined functions and possibly interacting with other polarity proteins (e.g., Scribble) to modulate spermatogenesis [9]. Since the subject regarding the role of polarity proteins and PCP proteins in testis functions has recently been reviewed [8, 10, 11], in order to avoid redundancy, we focus herein on a rapidly developing field on the involvement of Src family kinases (SFK) in regulating cell polarity in the testis. However, there are emerging evidence that SFKs are also involved in PCP in the testis, most notably spermatids, so that some brief discussions on PCP are provided wherever appropriate, such as noted in Table 1. Since this is a rapidly developing field, but studies from other epithelia are of importance to reproductive biologists, we seek to summarize these findings and to provide a thoughtful basis for future investigation.

Src family kinases (SFKs) and cell polarity in the testis

Introduction

Src family of non-receptor protein kinases (SFKs) are widely expressed by virtually all mammalian cells, consisting of at least nine members: Src, Yes, Fyn, Lck, Hck, Fgr, Lyn, Blk and Yrk [12, 13]. SFKs are known to play a prominent role in various cellular events, regulating numerous cell functions including cell migration, cell proliferation, cell differentiation, cell cytoskeletons, cell survival, apoptosis, calcium signaling, androgen signaling and spermatogenesis; and since SFKs are oncogenes so that they are also involved in pathogenesis of diseases, such as tumorigenesis, HIV-1 infection and Alzheimer's disease [12, 14–21]. In short SFKs are involved in virtually all aspect of cellular function in epithelia and endothelia. Interestingly, all SFK members share some common structural features

along their polypeptide sequences which have recently been reviewed [18] and summarized in Figure 2. However, the involvement of SFKs in cell polarity is an emerging concept. In the testis, while many of the SFK members including c-Src, c-Yes, Fyn, Lyn, Lck and Hck have been identified, the two best studied SFK members are c-Src and c-Yes [18, 22–25]. As such, much of the discussion herein is focused on these two SFK members.

SFKs and spermatid polarity in the testis – their role on Sertoli cell cytoskeletal function

The first indication that SFKs may be involved in cell polarity in the testis was the earlier study of c-Yes when this protein was first found to be localized restrictively to the basal region of the seminiferous epithelium, consistent with its localization at the basal ectoplasmic specialization (basal ES)/blood-testis barrier (BTB) (see Figure 1) [26]. Furthermore, c-Yes was also restrictively expressed and localized at the apical ES at the Sertoli cell-spermatid interface in stage V–VIII tubules [26]. This pattern of localization illustrates that the c-Yes protein is localized in a polarized fashion. Furthermore, the localization of c-Yes across the seminiferous epithelium is almost superimposable to F-actin [26], implicating c-Yes may be playing a role in modulating F-actin dynamics in the testis. This speculation is reached based on studies showing that all three polarity protein modules, including the Par-, the Scribble- and the Crumbs-based polarity complexes in the testis exert their regulatory effects via the actin-based cytoskeleton [2, 5, 6]. Furthermore, the use of a selective c-Yes inhibitor, SU6656, was found to induce Sertoli cell TJ-permeability barrier disruption due to defects in the organization of the actin microfilaments across the cell cytosol in which treatment of Sertoli cells with SU6656 led to extensive actin filament truncation [26]. Collectively, these findings support the notion that c-Yes, a non-receptor protein tyrosine kinase, may be involved in cell polarity in the testis through its effects on the organization of actin-based cytoskeleton. However, the proof that c-Yes is indeed involved in spermatid polarity was not known until a report published in subsequent years that a knockdown of c-Yes in the testis in vivo led to defects in spermatid polarity [27]. Histological analysis of testes following c-Yes knockdown by as much as 75% have shown that many step 19 spermatids failed to undergo c-Yes knockdown-induced spermiation, and considerably number of these spermatids displayed obvious defects in polarity [27] (Figure 3). For instance, in control testes, spermatids are aligned almost perpendicular to the basement membrane with their heads point to the basal region of the epithelium and their tails to the tubule lumen. However, after c-Yes knockdown by RNAi, many elongated spermatid heads deviated by ~90–180° from their intended orientation [27] (Figure 3). More important, the spatial expression of polarity protein Par6 in c-Yes silenced testes was grossly mis-localized [27]. For instance, Par6 that was localized at the tip of spermatid heads in normal testes moved away from the site, instead, Par6 was localized to the convex (dorsal) side of spermatid heads and no longer tightly associated with spermatids [27]. These findings thus provide the first direct proof that c-Yes is playing a role in conferring spermatid polarity. Since c-Yes is a non-receptor protein tyrosine kinase, it remains unknown if c-Yes plays a role in activating or inactivating the Par-, the Scribble- and/or the Crumbs-based protein complexes through changes in the phosphorylation status of any of these proteins. However, studies in *Drosophila* and other mammalian tissues and cells (e.g., epithelial stem cells) including humans have shown that the Crumbs/CRB-Hippo/Warts/LATS kinase signaling cascade can phosphorylate and *inhibit* YAP/TAZ (Yes-associated

protein, a family of transcriptional co-activators that serve as a sensor for cell polarity, involving in cell proliferation and suppressing apoptotic genes), whereas integrins and SKFs can phosphorylate and *activate* YAP (Yes-associated protein)/TAZ [28–30]. Thus, it is likely that there is an intimate relationship between c-Yes and the Crumbs-based polarity complex in which c-Yes may modulate the Crumbs-Hippo signaling pathway through phosphorylation. In this context, it is of interest to note that a knockdown of Crumbs homolog-3 (CRB3) in the testis by RNAi was found to cause defects in spermatid polarity [6], but the involvement of c-Yes or c-Src in this event has not been examined.

c-Yes and c-Src have differential function on Sertoli cell endocytic vesicle-mediated protein trafficking

For apico-basal polarity, such as those noted in mammalian cells and tissues in various organs including developing spermatids and in Sertoli cells in the seminiferous epithelium, accumulating evidence in the field based on studies in other epithelia has shown that it is supported by endocytic vesicle-mediated protein trafficking [31–34]. For instance, due to the presence of different sets of proteins in the apical *vs.* the basal region of a mammalian cell or tissue, this creates the apico-basal polarity. Indeed, the anchorage of spermatid heads into the Sertoli cell epithelium requires the support of adhesion protein complexes at the Sertoli cell-spermatid interface, so that the acrosomal region of spermatid heads can be aligned by pointing towards the basement membrane (Figures 1, 3). Additionally, intracellular trafficking that transports proteins to the spermatid head is considerably different from those being transported to the tail region [35–37], thereby supporting apico-basal polarity during spermiogenesis. Furthermore, the transport of residual bodies and phagosomes into the base of the seminiferous epithelium for their lysosomal degradation by Sertoli cells at stage VIII–IX of the epithelial cycle [38] also requires the tight coordination of lysosomal enzymes to be synthesized and be transported to the site for degradation of unwanted proteins and/or organelles *vs.* their storage for subsequent usage during fertilization [39, 40].

Earlier reports that examined the role of c-Src and c-Yes, two members of the non-receptor SFK, in endocytic vesicle-mediated intracellular trafficking of proteins have shown that these two protein kinases are playing differential role in this cellular events without much redundancy [18, 41, 42]. For instance, c-Yes is monopalmitoylated and once it is synthesized in mammalian cells, the newly produced c-Yes protein is transported from the Golgi pool of caveolin to be near the plasma membrane. Once c-Yes arrives its destination, it is being used to mediate endosome trafficking, possibly involving in protein transcytosis and/or recycling. On the other hand, c-Src, unlike c-Yes, is nonpalmitoylated and it is shuffled between plasma membrane and late endosomes/lysosomes in the mammalian cell. This thus suggests that c-Src may support endosome-mediated degradation intracellularly [41–43]. To further support this possibility, c-Yes knockout mice, although fertile, were shown to display defects in protein transcytosis/recycling [42, 44]. However, c-Src knockout mice were infertile [45], and VEGF (vascular endothelial growth factor) that was earlier shown to promote blood-brain barrier leakage [46] no longer capable of compromising the blood-brain barrier in these mice [45] due to defects in intracellular endosome-mediated protein degradation. In a recent report based on the use of different biochemical assays to track the fate of cell surface

labeled proteins using biotin, such as sulfo-NHS-SS-biotin, by monitoring protein endocytosis and recycling vs. protein degradation and phagocytosis. c-Yes and c-Src were found to play differential role in endocytic vesicle-mediated protein trafficking [47]. For instance, it was shown that c-Yes promoted endocytosed and biotinylated BTB integral membrane proteins to the pathways of transcytosis and/or recycling [47]. However, c-Src promoted endosome-mediated protein degradation such as facilitating the removal of residual bodies via the formation of phagosomes. These observations are physiologically significant since they illustrate that in the testis, these two SFK members are likely working in concert to determine the fate of endocytosed proteins at the Sertoli cell BTB such that they can either be recycled to form “new” TJ ultrastructures to support the assembly of “new” BTB behind the preleptotene under transport at the immunological barrier in stage VIII tubules by c-Yes (Figure 4), or promoted by c-Src to undergo intracellular degradation such that those from the “old” BTB site above the preleptotene spermatocytes at the immunological barrier can support the eventual breakdown of the “old” BTB ultrastructures to facilitate preleptotene spermatocyte transport (Figure 4). The combined efforts of c-Yes and c-Src thus provide a novel mechanism to support the timely breakdown and reassembly of the BTB ultrastructures surrounding the preleptotene spermatocytes under transport at the BTB. While the underlying molecular mechanism(s) by which c-Src and c-Yes modulate the BTB ultrastructures during preleptotene spermatocyte transport is not known, it is likely that c-Src or c-Yes mediates its effects via changes in the phosphorylation status of BTB components. These include the integral membrane proteins (e.g., occludin, CAR, JAMs, claudins) and/or their adaptors (e.g., ZO-1, ZO-2) at the Sertoli cell BTB, and actin regulatory proteins, such as actin bundling proteins palladin and Eps8 (and also for barbed end capping) and branched actin nucleation protein Arp3, actin nucleation protein formin 1. These changes in turn modulate protein endocytosis, transcytosis, recycling and/or degradation.

Concluding remarks and future perspectives

As briefly discussed herein, the involvement of SFKs in cell polarity, in particular spermatid polarity during spermiogenesis, is an emerging concept in the field. These findings should be carefully evaluated and expanded in future studies. However, there are many unanswered open questions. For instance, would the loss of SFKs, such as either c-Yes or c-Src, in Sertoli cells in the testis by using RNAi for its knockdown vs. its specific knockout (KO) lead to changes in the expression of polarity proteins (e.g., the Par-, the Scribble- or the Crumbs-based polarity complexes or any of their component proteins)? Besides the involvement of actin-binding and regulatory proteins, what role does MT- and/or MT-binding and regulatory proteins in SFK-mediated spermatid polarity? It is expected that many of these questions will be answered in the years to come.

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Figure 1. A schematic drawing that illustrates the general morphological features of Sertoli and germ cells in the epithelium of the seminiferous tubule

The blood-testis barrier (BTB) physically divides the seminiferous epithelium into the adluminal (apical) compartment and the basal compartment, which in turn lays on the basement membrane of the tunica propria. Undifferentiated and differentiated spermatogonia are found in the basal compartment. Type B spermatogonia differentiate into preleptotene spermatocytes, which are the germ cells that are being transported across BTB while transforming to leptotene spermatocytes to enter the adluminal compartment to form zygotene and pachytene spermatocytes to prepare for meiosis I/II. The most notable

anchoring junction in the testis is the actin-rich adherens junction (AJ) type called ectoplasmic specialization (ES), which is typified by the presence of an array of actin filament bundles found in the Sertoli cell near the plasma membrane and these bundles are sandwiched between the cisternae of endoplasmic reticulum and the apposing Sertoli cell-cell or Sertoli-spermatid plasma membranes. ES is either found at the Sertoli cell-cell interface called basal ES. The basal ES together with the tight junction (TJ) and gap junction all utilize F-actin for attachment. These actin-based junctions together with the intermediate filament-based desmosome constitute the BTB. At the Sertoli cell-spermatid (step 8–19 spermatids in the rat testis) interface, the ES is designated apical ES. The ES is an important structure to support Sertoli cell adhesion at the BTB and also spermatid adhesion. However, the ES is also an important cellular structure to support and Sertoli cell and spermatid polarity, and also spermatid PCP (planar cell polarity) during spermatogenesis.

Figure 2. A schematic drawing that illustrates the various function domains that are conserved among members of the SFK, and the conversion between the active and inactive state of SFKs Members of SFK (e.g., c-Src, c-Yes) consist of 4 SH (src-homology) domains: SH4 at the N-terminal region, followed by SH3 and SH2, and then a SH1 domain containing the catalytic kinase domain which is linked to the SH2 domain via a short polyproline type II helix called SH2-SH1 linker. In the SH4 domain of c-Src and c-Yes, there is a penultimate glycine (Gly2) residue co-translationally myristoylated (M) to be used for membrane targeting. Also, a cysteine residue (Cys3) near the N-terminus of c-Yes is post-translationally palmitoylated (P) to assist subcellular trafficking of a SFK protein (e.g., facilitating membrane association of c-Yes following palmitoylation). **(B)** Under normal physiological conditions, SFK is auto-inhibited through intramolecular interactions wherein the SH2 domain binds to the inhibitory phosphotyrosine at the C-terminal tail, while the SH3 domain interacts with the SH2-SH1 polyproline linker so that these two protein interacting motifs block the kinase domain and stabilize SFK in an inactive conformation (right). SFKs are activated through binding of a ligand to the SH2 and SH3 domain, or dephosphorylation of

the inhibitory phosphotyrosine by protein tyrosine phosphatases. This leads to a conformational change which allows phosphorylation of the stimulatory tyrosine in the activation loop and to confer intrinsic kinase activity in kinase domain (SH1) (left).

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Figure 3. Defects in spermatid polarity following c-Yes knockdown by RNAi

Testes from adult rats received a transfection mixture containing 100 siRNA duplexes specific to c-Yes on day 1 and day 3, i.e., 2 transfections for RNAi to knockdown c-Yes by >70% as described [27]. Control testes received the same amount of non-targeting negative control siRNA duplexes. On day 5, rats were euthanized and testes were removed to freeze in liquid nitrogen. Frozen sections were obtained and cell nuclei stained for DAPI (4',6-diamidino-2-phenylindole). As noted herein, a knockdown of c-Yes, but not in control testes received the negative non-targeting control siRNA duplexes, caused extensive defects in spermatid polarity in which the head of spermatids no longer pointed towards the basement membrane, but deviating by 90° to 180° from the intended orientation (annotated by white arrowheads). Scale bar, 80 μ m and 40 μ m in the left and right panel.

Figure 4. A model illustrating the role of c-Src and c-Yes to support germ cell transport and polarity

In this hypothetical model, the BTB integrity is maintained by SFKs such as c-Yes and c-Src such as at stage VII of the epithelial cycle (left panel). For instance, it was shown that c-Src associates with CAR [48], whereas c-Yes associates with occludin and N-cadherin [26]. However, at stage VIII of the epithelial cycle, these two SFKs promote protein endocytosis in which c-Yes targets the endocytosed proteins (e.g., occludin, N-cadherin, CAR) to be recycled to the base of the preleptotene spermatocyte to support the reassembly of a "new" BTB before the "old" BTB is disassembled. At the same time, c-Src promotes the endocytosed proteins to undergo endosome-mediated protein degradation as recently reported [47] (middle panel). As such, the BTB integrity is maintained as noted in the right panel. As noted in Figure 3, SFKs (e.g., c-Yes) also promotes germ cell polarity which may also facilitate the directional transport of preleptotene spermatocytes across the BTB as noted herein.

Table 1

Recent findings of members of SFK that modulate cell polarity and/or PCP function

Member of SFK	Detection method	Cell/tissue type	PCP-related SFK activities	Ref
c-Src	Src 2–17 antibody, SFK inhibitors SU6656 and Bosutinib	Mouse auditory sensory epithelium	1. Relevant to Ptk7-mediated epithelial PCP regulation in vivo 2. Altered Src signaling may affect the cortical cytoskeleton	[49]
	Kras-Src biosensor, SFK inhibitor PP1	Bovine aortic endothelial cells	1. It plays an important role in regulating the shear stress-induced cell polarity 2. It regulates the polarized distribution of FAK 3. High shear stress-induced Src polarity localization is mainly mediated by actin cytoskeleton	[50]
	Src RNAi (siRNA), SFK inhibitors PP2 and SU6656	Osteoclasts	Phosphorylates FGD6 (guanine exchange factor) to form complexes containing IQGAP1, ARHGAP10, Talin-1/2, or Filamin A to regulate cell polarity and membrane recycling	[51]
	thermosensitive v-src mutant, mAb to avian Src (EC10), Src rabbit pAb	MDCK cells	1. Impacts on cortactin distribution and controls cortical actin dynamics 2. Promotes microtubule-dependent fusion of macropinosomes to the apical recycling endosome	[52]
	SFK inhibitor SU6656, SYF fibroblasts deficient of Src, Yes, and Fyn, rescue assay by re-expressing Src	RAT2 fibroblasts	Integrin/FAK/Src/p190RhoGAP pathway regulates nuclear reorientation required for cell polarization	[53]
	Src-2 antibody, Src pY416 antibody (2101)	FAK (R454) Knock-in mouse embryo fibroblasts (MEFs)	Reduces c-Src Y416 phosphorylation in R454 MEFs that exhibited enhanced focal adhesion formation, decreased migration, and defects in cell polarity	[54]
	Src RNAi (shRNA) Src antibody 2102P SFK inhibitor PP2	Acinar lung cancer cell	Regulates p190A RhoGAP phosphorylation, inhibiting RhoA-ROCK1 signalling and inducing polarity orientation	[55]
	Src RNAi (siRNA), Src antibody 36D10, SFK inhibitors PP1, PP2 and Dasatinib	Primary cultured spheroids of human colorectal adenocarcinoma	Critical for polarity switching of cancer tissue-originated spheroid	[56]
	Src RNAi (siRNA), SFK inhibitor PP2	Multiple cancer cell lines	CD133/integrin/Src/Akt/GSK3 β /B-catenin signaling induced by polarized cell migration is required for maintenance of cancer stem cell properties	[57]
Src42A, Src64B (mammalian Src orthologs)	Src mutants	<i>Drosophila</i> ectodermal cells	It requires for STAT92E (STAT transcription factor) apical localization and its interaction with Baz (a homolog of PAR3)	[58]
Src42A	Src mutants, genetic screen	<i>Drosophila</i> eye discs and larval wing imaginal disc	A key modulator of tumor invasion and tumor cell migration	[59]
c-Yes	c-Yes RNAi (siRNA)	Sertoli cells, testis	Its knockdown causes a disruption of the Sertoli cell tight junction-permeability barrier function, germ cell loss from the seminiferous epithelium, and also defects in spermatid polarity	[60]
	c-Yes mouse monoclonal (sc-8403)	Pancreatic epithelial cells	It co-localizes with occludin, Hippo pathway element YAP (c-yes associated protein) and TEAD (TEA-dependent) to regulate cell proliferation through the coordination of PCP with apical-basal polarity events	[61]
Fyn	SFK inhibitor Bosutinib, Fyn-null oocytes	Oocyte	Leads to defects in cortical actin organization and polarity, and the polarized association of Par-3 with the cortex overlying the meiotic spindle is completely disrupted	[62]

Member of SFK	Detection method	Cell/tissue type	PCP-related SFK activities	Ref
	Fyn antibody (sc-434), SFK inhibitor PP2	Hippocampal neurons	It is associated with raft-localized and is involved in neurite outgrowth and polarity formation	[63]
Lyn	Lyn RNAi (siRNA and shRNA), SFK inhibitor PP2, competition experiments using blocking peptide	Primary human neutrophils, neutrophil-like differentiated HL-60 cells	1. It is activated and recruited to leading edge of polarized neutrophils in response to chemo-attractants 2. Its depletion impairs β 2-integrin and Rap1 activation/translocation and recruitment of CrkL-C3G complex at the leading edge	[64]
	Lyn RNAi (siRNA) Bcr-Abl/Lyn inhibitor Bafetinib	Multiple cancer cells	It functions as a key modulator of SNAI family protein localization and stability through control of the Vav-Rac1-PAK1 (Vav-Rac1-p21-activated kinase) pathway, promoting EMT	[65]
	MDCK cells expressing tetracycline-inducible human Lyn	MDCK cells	1. Lyn mainly localizes to the plasma membrane in polarized MDCK cells and to endomembranes in non-polarized MDCK cells 2. Calcium-dependent cell-cell interactions recruit Lyn from endomembranes to the plasma membrane, and loss of cell-cell interactions relocates Lyn from the plasma membrane to endomembranes through Rab11-mediated recycling	[66]
SFKs	SFK inhibitor PP2	Vascular endothelium	This treatment leads to phosphorylation of a small pool of VE-cadherin on Y658 that binds to polarity protein LGN (Y658) which is the p120-catenin binding site assumed to be responsible for VE-cadherin internalization)	[67]