Src Kinases as Therapeutic Targets

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Src is the prototypal member of Src Family tyrosine Kinases (SFKs), a large non-receptor kinase class that controls multiple signaling pathways in animal cells. SFKs activation is necessary for the mitogenic signal from many growth factors, but also for the acquisition of migratory and invasive phenotype. Indeed, oncogenic activation of SFKs has been demonstrated to play an important role in solid cancers; promoting tumor growth and formation of distant metastases. Several drugs targeting SFKs have been developed and tested in preclinical models and many of them have successfully reached clinical use in hematologic cancers. Although in solid tumors SFKs inhibitors have consistently confirmed their ability in blocking cancer cell progression in several experimental models; their utilization in clinical trials has unveiled unexpected complications against an effective utilization in patients.

Keywords: Src ; src family tyrosine kinases ; cancer metastasization ; cancer migration ; cancer invasion ; epithelial-to-mesenchymal transition ; clinical trial ; targeted therapy ; dasatinib

1. Introduction

Non-receptor tyrosine kinases represent a large cytosolic enzyme family, the most representative of which in mammals is the Src Family tyrosine Kinases (SFKs), including Src, the first ever described tyrosine kinase proto-oncogene. To date tyrosine kinases (TKs) represent also the most representative class of targeted proteins in anticancer therapy [1]. Ten additional kinases with homology to Src have been identified: Blk (B-lymphoid tyrosine kinase), Fgr (gardner-rasheed feline sarcoma), Fyn (proto-oncogene tyrosine-protein kinase Fyn), Frk (Fyn-related kinase), Hck (hematopoietic cell kinase), Lck (lymphocyte specific kinase), Lyn (tyrosine-protein kinase Lyn), Yes (yamaguchi sarcoma), Yrk (Yes-related kinase), and Srms (Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites) [2][3][4].

Although several studies have demonstrated the presence of some functional redundancy between co-expressed SFKs, there is also plenty of evidence for non-overlapping functions. However, of the 11 SFK members, Src, Fyn, and Yes have been most frequently implicated in tumorigenesis and metastasis formation [5]. Indeed, Src, Fyn, and Yes, but also Frk, are widely expressed in a variety of tissues, whereas for the other members the protein expression is more tissue-restricted with a prevalence in cells of hematopoietic origin. Nonetheless, in contrast to the widely characterized agonistic role of SFKs in cancer, noteworthy specific examples of antagonistic role have been reported. Frk was described as tumor suppressor in different cancers, at least partly by protecting the tumor suppressor Phosphatase and TENsin homolog (PTEN) from degradation [6]. However, other reports of a potential pro-oncogenic function of Frk also exist, such as in studies evaluating the therapeutic potential of SFKs in liver and pancreatic cancer cell lines [7][8]. At status quo, the cellular roles of SFKs, specifically in the context of cell proliferation and invasion, should be evaluated on a tissue-specific basis and identification and characterization their cellular substrates will be helpful in deciphering the context-specific function of SFKs.

Yrk has been described only in adult chicken and it was detected in hematopoietic cells, cerebellum, spleen, lung, and skin [9][10]. In solid tumors, an increased expression of many members of the family was generally observed, and also for those SFKs with a prevalent expression in normal hematological cells, a de novo presence was frequently reported in non-hematological cancer tissues (Table 1) [11][12].

Elevated protein levels were shown for Src, Frk, Lyn, Blk, Hck, and Srms, in different tumors, with Yes that demonstrated the highest number of positive cases in a variety of tumors.

SFKs interact directly with several tyrosine kinase receptors, G-protein-coupled receptors, steroid receptors, signal transducers, and activators of transcription, leading to a diverse array of biological functions from cell survival to metastases [13]. The importance of SFKs in metastatic spreading is a consolidated evidence and it has been associated with different mechanisms including the promotion of tumor cell motility, epithelial-to-mesenchymal transition (EMT) as well as adaptation of resident cells in the secondary microenvironment [14][15].

Specifically, Src overexpression or overactivation was associated with the aberrant formation of invadopodia, critical morphological structures employed by cancer cells to intravasate into the bloodstream and extravasate into secondary sites during the metastatic process [16][17].

In addition, Src is a potential target in tumor associated cells. In fact, Src kinase inhibition counteracts those mechanisms underlining some tumor-induced phenotypic switches, including stimulation of cancer associated fibroblasts (CAFs), angiogenesis, and immune infiltrate [18][19][20]. Although Src is ubiquitously expressed, the primary phenotype associated with mutant Src−/− mice is osteopetrosis, a condition caused by the failure to resorb bone. This phenotype results from the blockade of osteoclast maturation and in particular from the inhibition of actin dynamics and its organization, a phenomenon essential in resorbing bone [21]. These data have designated Src as an attractive therapeutic target for the prevention and treatment of bone metastases [22].

Table 1. Distribution of Src Family tyrosine Kinase (SFK) proteins in normal and solid tumor tissues according to Human Protein Atlas database available from http://www.proteinatlas.org.

		Solid Tumor Distribution	
Protein Kinase	**Tissue Distribution**	**Level of Expression**	**Tumor (*)**
Src	most	strong	cervical, head and neck, pancreatic, skin, urothelial
		moderate	colorectal, lung, stomach
		weak	carcinoid, cervical
Yes	most	moderate	most (>60% = breast, colorectal, head and neck, liver, ovarian, prostate, testis, thyroid, urothelial)
Frk	most	strong	thyroid
		weak to moderate	carcinoid, colorectal, endometrial, liver, melanoma, renal, urothelial
Lyn	most	moderate to strong	liver, stomach
		weak	carcinoid, head and neck, thyroid
Fyn	brain, endocrine tissues, female tissues, hematopoietic cells, liver	moderate	glioma
		weak	carcinoid, thyroid
Blk	hematopoietic cells, lung	moderate to strong	endometrial
Fgr	hematopoietic cells, lung	weak	Carcinoid, colorectal, renal, thyroid

Hck	hematopoietic cells, lung	strong	endometrial, lung, renal, stomach
		weak	carcinoid, glioma, liver, ovarian, pancreas, skin
Lck	hematopoietic cells	negative	none
Srms	gastrointestinal, male tissue (**)	strong	colorectal, ovarian, prostate
		moderate	most

* only positivity >20% cases are reported; ** based on mRNA expression.

2. SFKs Structure and Regulation

SFKs display large sequence and structural similarity. The most variable long sequence among SFKs is localized in the “unique domain” connecting the SH3 and SH4 domains, but to which has been assigned specific regulatory role only in few cases [23][24][25][26][27][28][29][30][31][32][33][34][35]. Src, the prototype kinase of SFKs, is a 60-kDa protein able to associate with the plasma, perinuclear, and endosomal membrane via a N-terminal anchoring region that includes the SH4 domain, rich in positively charged residues [36]. During intense cytoskeleton dynamic and cell migration, SFKs are targeted to focal adhesions (FAs), a phenomenon that is regulated mainly by the SH2 domain [37]. Recent studies have reported the presence of Src also in the nuclear compartment, and this localization seems to be relevant in the progression of some solid tumors [38][39]. While the Src enzymatic activity is localized in the SH1 domain, the SH2 and SH3 domain through intra-protein and protein–protein interaction can control the catalytic activity and the recognition of substrates. In fact, protein–protein interaction and Tyr phosphorylation status determine the existence of a dynamic equilibrium between closed-inactive and open-active form of SFKs. The open conformation allows the phosphorylation of Tyr419 (in human Src) in the activation cycle, which improves catalytic activity. In the canonical pathway, Src activation is initiated by dephosphorylation of pTyr530 (in human Src) followed by a conformational change that permits autophosphorylation at Tyr419 with full activation [40].

In all SFKs an important activation control is exerted by phosphorylation status in C-terminal Tyr residues (Tyr530 in human Src). The phosphorylation of Tyr530 determines conformational changes leading to intramolecular interaction with the SH2 domain and inactivating kinase activity [24]. The close association between spatial conformation and kinase activation, obligates researchers to carefully consider the interacting ability of SFKs with other proteins in critical cell compartments. The SH3 domain, consisting of 50–60 amino acids, binds proline rich sequences and it can interact with the linker domain (PPII-linker) on the back of the catalytic domain, thus promoting a "closed" conformation that prevents interaction with substrates. Several ligands are known to compete with the SH3/PPII-linker interaction: the progesterone receptor, p130Cas, AFAP-110, and Nef. The SH2 domain, consisting of about 100 amino acid residues, binds phosphorylated Tyr residues in C-terminal domain in the inactive conformation or, in the active form, on other proteins [25]. In the open form, the inhibitory tyrosine residues are accessible to phosphatase and dephosphorylation coincides with the destruction of all intramolecular interactions and the phosphorylation of the activating site that induces a complete kinase activation. Phosphorylation of pTyr530 works in conjunction with dephosphorylation of pTyr419, mediated by proline-enriched tyrosine phosphatase (PEP), in allowing the complete inactivation of SFKs in vivo [26]. However, the binding with external ligands still allows SFKs to open its spatial conformation and to remain active even in the presence of terminal C-tail phosphorylation. This indicates that phosphorylation of inhibitory tyrosine and the resulting intramolecular interaction are not sufficient to maintain SFK activity in a complete inhibited status in vivo [27]. In agreement with a context-dependent activation model, experimental evidence suggests that the activated phosphatase CD45 can also function as a negative regulator of SFK by dephosphorylating pTyr419 [28].

During tumor progression, Src activity becomes abnormally high and since activating mutations or amplification are very rare in human Src, an altered extrinsic control of phosphorylation by kinase or phosphatase and of interacting partners may represent an important mechanism for activation of Src. The phosphorylation of Tyr530 is determined by the action of different tyrosine kinases including Csk (C-terminal Src kinase) and Chk (Csk-homologous kinase) [29]. Csk kinase acts as the main negative regulator of Src and under basal conditions in vivo, 90–95% of Src is phosphorylated in Tyr530. The importance of phosphorylation of Tyr530 in the mechanism of inhibition of Src kinase activity is suggested by experimental

evidence in which knockout mutant shows a constitutive basal activity associated with oncogenic transformation [30]. Several tyrosine phosphatases (PTPs) are involved in terminal C-tail dephosphorylation mechanism. Their role in SFK regulation is complex showing tumor specificity and specific roles for the different members of the kinase family.

PTP1B phosphatase, which is upregulated in many human solid tumors as colon, lung, and breast cancer, is responsible for the dephosphorylation of Tyr530 and subsequent activation of Src and therefore, for increased tumorigenicity [31][32]. CD45, abundantly expressed in all nucleated cells of hematopoietic origin, is the most important phosphatase activating SFKs by dephosphorylation of the terminal C-tail [33]. In contrast to Lck and Fyn, the Src C-terminal phosphotyrosine does not appear subject to dephosphorylation by CD45 [34]. SHP-1 and SHP-2 are two tyrosine phosphatases localized in cytosol. SHP-1 is primarily present in cells of hematopoietic origin, SHP-2 is ubiquitous and experimental evidence has established that SHP-1 is involved in dephosphorylating and activation of Src [35]. However, SHP-1 and CD45 are also able to dephosphorylate Tyr-394 in the catalytic domain of Lck and to inactivate Lck, but not Src, suggesting that these phosphatases are not involved only in activation of SFKs but also in the modulation of their specificity [41][42]. SHP-2 can regulate SFKs activity in several ways: by an enzymatic mechanism involving terminal C-tail dephosphorylation, and a non-enzymatic mechanism linked to the control of Csk recruitment to the plasma membrane. In this case, SHP-2 catalyzes the dephosphorylation of the PAG protein (phosphoprotein associated with glycosphingolipid-enriched microdomains), which in turn are unable to recruit Csk. Thus, the activity of SHP-2 can result in the failure to activate the inhibitory control of Csk in membrane-associated complexes containing SFKs [43].

3. SFKs in Cancer Signal Transduction, Migration, and Invasion

Since the original identification of a transmissible agent responsible for the development of solid tumors in chickens, now known to be a retrovirus encoding the v-SRC, significant progress has been made in defining the functions of its human homolog, Src [44]. One of the aspects associated with oncogenic potential that emerged early on from the studies, was the ability of SFKs to stimulate cell motility. The relationship between Src activation and cancer invasion, the most advanced phase of solid tumor progression, appears to be significant in a wide number of preclinical human cancer models, thus prompting the optimistic use of SFK inhibitors in clinical trials [45]. The SH3 domain of SFKs permits the association with actin filaments that guide the translocation of SFKs towards the cell periphery where they can interact with other molecular partners allowing two major transduction events: (i) signaling from growth factor receptors, which mainly affects cell growth, (ii) signaling from adhesion receptors including integrins and E-cadherin, which mainly regulate the functions of the cytoskeleton [46]. To date, most evidence suggests that Src has a predominant role in the maintenance of the invasive phenotype, rather than of the cell cycle progression. FA junctions, that are key structure in regulating cell motility, are formed by the docking activity of SFKs, through the accessibility of SH2 domain.

FAs are protein complexes that play a critical role in dynamically controlling the coupling of actin filaments and the extracellular matrix. During migration, integrins, heterodimeric transmembrane receptors bind directly to the extracellular matrix (ECM) on the outside of the cell and link ECM to the actin cytoskeleton through FA. In turn, FAs contain signaling proteins that regulate cytoskeletal dynamics, including changes in actinomyosin contractility. The formation of FAs is dependent on the phosphorylation status and the presence of docking sites in participating proteins. SFKs can regulate the phosphorylation status of many substrates in the FA and participate directly in the formation of the complex through the docking site SH2. Src associates with FA in FAK-dependent manner. In addition, the association of Src with FAs plays a key role in transducing Src-mediated signals, in the promotion of FA formation/maturation and in enhancing cell migration. Src substrates in FA include FAK, p130Cas, tensins, paxillin, and crk[47][48]. Src and FAK in their activated states, form a functional bipartite kinase complex that is fundamental in the signal transduction and in formation of lamellipodia. In fact, phosphorylation of p130Cas by the FAK/Src complex provides a binding site for the SH2 domain of CrkII, which in turn serves as a docking site for Rac1 [49]. Involvement in FA activity has been described also for other members of SFKs including Fyn [50] and Yes, and through the use of deleted-mutant SFY (src-/-fyn-/-yes-/-) it was demonstrated a strong dependence of cancer cell migration and metastatic ability on these kinases [51]. Invadosomes, which include invadopodia, are F-actin based structures used by cancer cells to interact with and to degrade ECM. These structures were initially discovered in v-Src transformed chicken fibroblasts. Src may target different actin-binding proteins during distinct stages of invadosome formation and maturation to regulate actin dynamics and invadosome function. Various signaling pathways have been shown to be involved in the formation of invadosomes, resulting in focally releasing of metalloproteases (MMPs) that degrade ECM. During invadosome precursor formation, signaling proteins such as transmembrane growth factor receptors and/or SFKs organize with structural and adaptor proteins including Tks5, Nck1, and cortactin to recruit the Arp2/3 complex and mediate actin polymerization [52]. Cortactin is a multi-functional Src substrate that is involved in both the assembly and maturation of invadosomes and is important for invasive cell migration [53][54]. Src phosphorylates cortactin at three sites, Y421, Y466, and Y482, and cortactin phosphorylation is important for the formation of invadopodia [55]. In addition to its effects on the actin-severing activity of cofilin, phosphorylated cortactin

facilitates actin polymerization through its interactions with N-WASP and Arp2/3 [56][57]. MAbp1, in contrast to cortactin, impairs invadopodia formation and invasive cell migration and it is phosphorylated by Src at two sites, Y337 and Y347 [58]. The spatiotemporal phosphorylation of cortactin and mAbp1 can regulate invadosome dynamics, with cortactin present at invadopodia during early assembly and stabilization, while mAbp1 is present at mature podosome dots [58][59]. Src is necessary also during invadosome disassembly. Src phosphorylates paxillin, which through Erk signaling, activates the protease Calpain-2, which in turn cleaves multiple invadosome proteins including paxillin and cortactin.

Migration and invasion are acquired abilities in epithelial tumor cells and require the disassembly of adherent and tight-junctions and the inhibition of anoikis. These latter transformations are early steps in the EMT suggesting a role for SFKs in this phenomenon. Importantly, Src plays a control role in the expression of E-cadherin. Src inhibition induces in breast cancer cells a reverted epithelial phenotype associated with an increase of E-cadherin and a decrease of vimentin and it blocks cancer cell migration [60]. Oncogenic expression of Src in pancreatic tumor cells was also associated with a reduced expression of E-cadherin in favor of vimentin [61]. Similar results were obtained also in other tumors including prostate and renal cancer [62]. Given the involvement of SFKs activity in the EMT, cancer migration and invasion, Src family represents an attractive target for advanced stages of cancer progression.

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