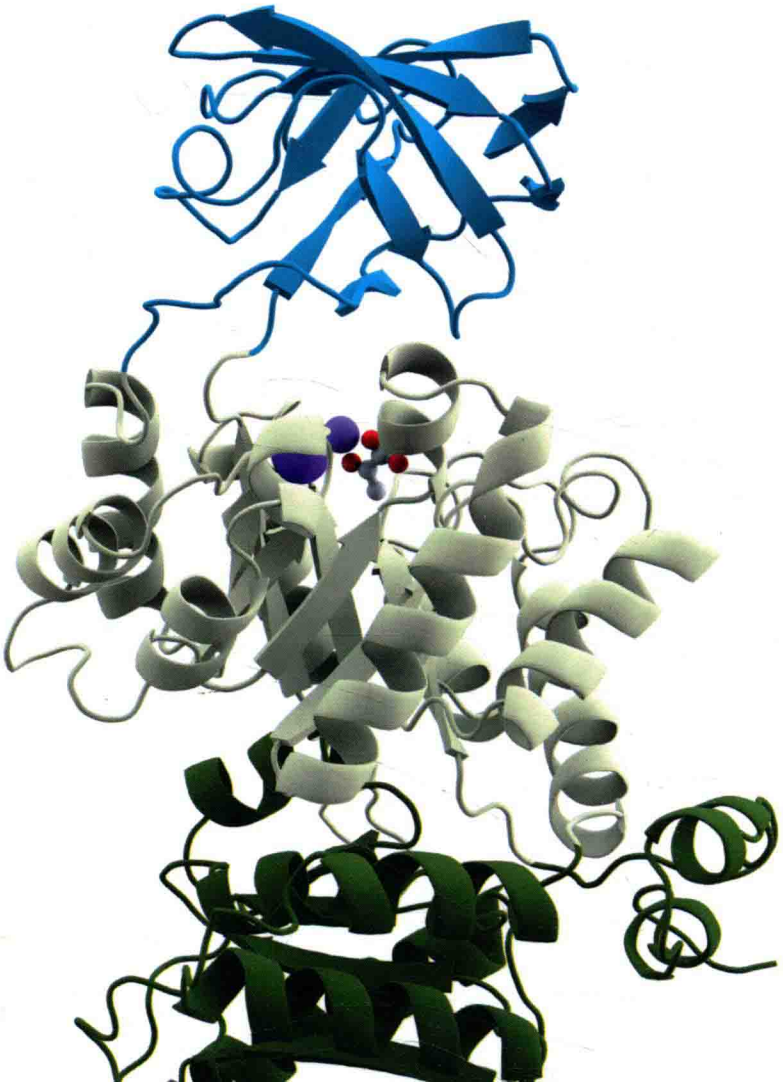
Protein Kinases
Comprehensive Researches

Michelle McGuire



Protein Kinases: Comprehensive Researches

Edited by **Michelle McGuire**

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Preface

This book of advanced researches aims to serve as a resource guide in the field of protein kinases. The aim of this book is to educate readers on protein kinases. As regulators of protein function, protein kinases are concerned with the control of cellular functions through complicated signaling pathways, enabling fine tuning of physiological functions. This book is an integrated effort, with contributions from experts in modern science from across the globe. Existing literature is reviewed in this book, and occasionally, new data on the function of protein kinases in different systems is also provided. The book discusses broad topics related to the role of c-Src tyrosine kinase in bone metabolism, protein kinases and pancreatic islet function, kinases in spinal plasticity etc.

The information contained in this book is the result of intensive hard work done by researchers in this field. All due efforts have been made to make this book serve as a complete guiding source for students and researchers. The topics in this book have been comprehensively explained to help readers understand the growing trends in the field.

I would like to thank the entire group of writers who made sincere efforts in this book and my family who supported me in my efforts of working on this book. I take this opportunity to thank all those who have been a guiding force throughout my life.

Editor

Contents

1

The Crucial Role of c-Src Tyrosine Kinase in Bone Metabolism

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1. Introduction

c-Src belongs to the SRC Family of non receptor tyrosine kinases (SFKs), which includes at least ten members (Lyn, Fyn, Lck, Hck, Fgr, Blk, Yrk, Gfr, Yes and c-Src) sharing high homology in their domain structure (Brown & Cooper, 1996).

Due to its proto-oncogene nature, c-Src is the SFK most frequently associated with malignancy (Yeatman, 2004). Over 100 years ago, Peyton Rous observed that injection of cell-free extracts from tumours grown in chickens caused the development of the same type of tumour in host animals. This observation prompted the hypothesis that a filterable agent was the cause of the tumour (Rous, 1911a, 1911b). In support of this notion, in 1955 Rubin showed that the Rous'filterable agent was a virus, called Rous Sarcoma Virus (RSV), which was found to play a direct role in inducing cell malignancy (Rubin, 1955). In the '60s and '70s, the tools of modern molecular biology provided the genetic definition of v-Src, a viral oncogene included within the RSV genome. v-Src was observed not to be required for virus replication but to be the causative agent of cancer (Martin, 1970; Duesberg & Vogt, 1970). Shortly thereafter, it was shown that v-Src had a counterpart in eukaryotic cells, named c-Src (Takeda and Hanafusa, 1983). c-Src is involved in many physiological functions of the cells. It carries a regulatory domain lacking in v-Src (Fig. 1), therefore, its activity is under tight molecular control. c-Src was the first of several proto-oncogenes discovered in the vertebrate genome and, in 1989, this discovery earned Bishop and Varmus the Nobel Prize in Physiology or Medicine, for the description of "the cellular origin of retroviral oncogenes".

In 1977, Brugge and Erikson immunoprecipitated a 60-kDa phosphoprotein from RSV-transformed fibroblasts. The protein was called $pp60^{v\text{-}src}$, but it is now usually referred as v-Src. Both v- and c-Src have tyrosine kinase activity (Collett et al, 1978; Oppermann et al, 1979). c-Src also autophosphorylates itself at tyrosine residues (Hunter & Sefton, 1980), and is the prototype of a large family of kinases that we now know are involved in the regulation of cell growth and differentiation.

v-Src lacks the C-terminal domain that in c-Src has a negative regulatory role on its tyrosine kinase activity. Consequently, v-Src shows constitutive activity and transforming ability (Jove & Hanafusa, 1987). In addition, v-Src contains point mutations throughout its coding

region that probably contribute to the high intrinsic activity and transforming potential of the protein.

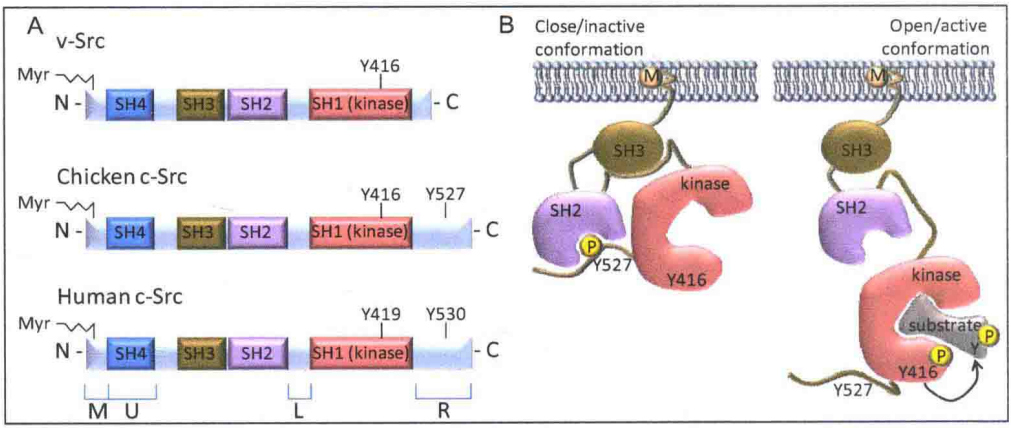

Fig. 1. Structure and activation of Src proteins
A) Comparison of protein structure of viral (v-)Src, chicken and human cellular (c-)Src, with indication of Src homology (SH) and membrane-binding (M), unique (U), linker (L) and regulatory (R) domains. (B) Representation of inactive (left) and active (right) conformation of chicken c-Src.

Aberrant activation of c-Src results in a wide variety of cellular phenotypic changes, including morphological transformation and acquisition of anchorage and growth-factor independence, that are implicated in the development, maintenance, progression, and metastatic spread of several human cancers, such as prostate, lung, breast, and colorectal carcinomas (Irby & Yeatman, 2000). Indeed, a number of human malignancies display increased c-Src expression and activation, confirming its involvement in oncogenesis (Alvarez et al, 2006).

c-Src is ubiquitous and physiologically expressed at high levels in a variety of cell types, including neurons and platelets (Brown & Cooper, 1996). Nonetheless, the very first mouse model of c-Src deficiency (Soriano, 1991) showed an unexpected prominent bone phenotype characterized by increased bone mass and lack of bone resorption, unveiling a previously unrecognized role of c-Src in bone cells (Boyce et al, 1992; Marzia et al, 2000).

In this review, we will describe the structure and function of c-Src and will highlight its crucial physiological and pathogenetic role in bone metabolism.

2. c-Src structure, activation and function

2.1 c-Src structure

c-Src shares with the other SFK members a conserved domain structure consisting of four consecutive Src Homology (SH) domains (Fig. 1A). The N-terminal segment includes the SH4 domain, as well as an "unique" domain of 50-70 residues that display the greatest divergence among the family members (Koegl et al., 1994 ; Resh, 1999). The SH3, SH2 and SH1 (catalytic) domains follow in order in the polypeptide chain. There is also a short, C-

terminal "tail" which includes a hallmark of Src kinases, that is an autoinhibitory phosphorylation site [Tyrosine (Tyr) 527 in chicken, Tyr 530 in human] (Cooper & King, 1986). This tail is not present in the v-Src isoform (Fig.1A).

The SH4 domain is a 15-amino acid sequence whose myristoylation allows binding of SFK members to the inner surface of the plasma membrane. The unique domain has been proposed to be important for mediating interactions with receptors or proteins that are specific for each family member. Serine and threonine phosphorylation sites have also been identified in the unique domains of c-Src and Lck (Winkler et al, 1993).

SH3 and SH2 are protein-binding domains widely present in other molecules, such as lipid kinases, protein and lipid phosphatases, cytoskeletal proteins, adaptor molecules and transcription factors (Mayer & Baltimore, 1993). The SH3 domain consists of small, β-barrel modules and is important for intra- as well as inter-molecular interactions, regulating c-Src catalytic activity, localization and recruitment of substrates. Proline-rich sequences in target molecules mediate the interactions with SH3 (Ren et al, 1993). The other domain regulating c-Src interaction with proteins is SH2, which preferentially binds to polypeptide segments containing a phosphotyrosine (Mayer et al, 1991; Pawson, 1995).

The catalytic domain (SH1) is the most conserved domain in all tyrosine kinases. It contains an ATP-binding pocket and the tyrosine-specific protein kinase activity. As it will be described in the next paragraph, the first step of c-Src activation is the autophosphorylation of Tyr416 (in chicken, Tyr419 in human), while phosphorylation of Tyr527 by c-Src kinase (CSK) and CSK homologous kinase (CHK) results in its inhibition (Kmiecik & Shalloway, 1987; Cartwright et al, 1987; Piwnica-Worms et al, 1987; Okada & Nakagawa, 1989).

2.2 c-Src activation

c-Src is normally maintained in an inactive or "closed" conformation, where the SH2 domain is engaged with the phosphorylated Tyr527, the SH3 domain binds the SH2-kinase linker sequence and the Tyr416 is dephosphorylated. Dephosphorylation of Tyr527 disrupts its intramolecular interaction with the SH2 domain and this open conformational state allows autophosphorylation of Tyr416, resulting in c-Src activation (Fig. 1B) (Yamaguchi & Hendrickson, 1996).

Phosphorylation of Tyr527 can be removed by several protein phosphatases that function as activators of c-Src, such as protein tyrosine phosphatase-α (PTPα) (Zheng et al, 1992), PTP1, SH2-containing phosphatase 1 (SHP1) and SHP2 (Jung & Kim, 2002). The most direct evidence for a role of c-Src activation in cancer among these phosphatases is for PTP1B, which is present at high levels in breast cancer cell lines (Jung & Kim, 2002). In addition, the direct binding of focal-adhesion kinase (FAK) (Schaller et al, 1994) or its molecular partner CRK-associated substrate (CAS, also known as p130CAS) to the SH2 and the SH3 domains of c-Src also results in the open, active configuration of c-Src, since the intramolecular interactions that maintain the closed configuration are displaced (Thomas et al, 1998).

2.3 c-Src functions

c-Src plays a key role in regulating the assembly and disassembly of cell-cell (adherens junctions) and cell-matrix (focal adhesions) adhesion (Yeatman, 2004) (Fig.2). Adherens

junctions are maintained by homotypic interactions between E-cadherin molecules present on neighboring cells, and loss of E-cadherin is a key event in the epithelial-to-mesenchymal transition of cancer cells. It has been shown that increased c-Src signalling correlates with decreased E-cadherin expression and decreased cell-cell adhesion (Irby and Yeatman, 2002; Nam et al, 2002). Moreover, constitutively active c-Src can phosphorylate the cadherins, resulting in loss of the cadherin–catenin complex function, thereby promoting cell invasiveness (Irby & Yeatman, 2002; Behrens et al, 1989) (Fig.2).

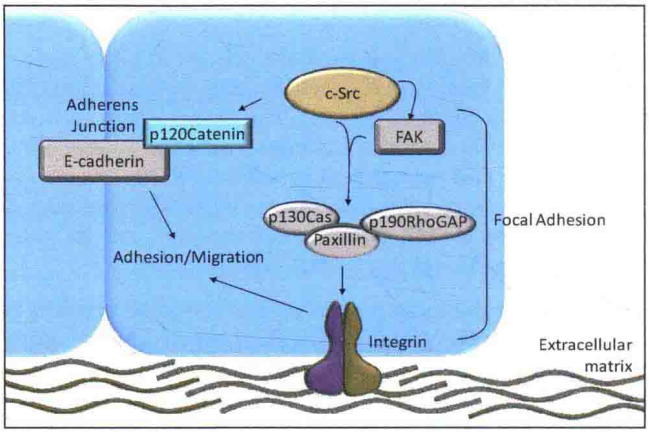

Fig. 2. c-Src involvement in cell adhesion signals.
Molecular interactions among c-Src and the components of both adherens junction and focal adhesion structure.

At the cell periphery, activated c-Src forms complexes with FAK, which in turn interacts with a multitude of substrates, including CAS, paxillin, and p190RhoGAP, that play critical roles in promoting actin remodelling and cell migration (Fig. 2) (Guarino, 2010; Playford & Schaller, 2004). In cancer, deregulated focal adhesion signalling has been implicated in increased invasion and metastasis, and decreased patient survival (McLean et al, 2005). c-Src can also be activated downstream of tyrosine kinase growth factor receptors, such as epidermal growth factor (EGF) (Tice et al, 1999), platelet-derived growth factor (PDGF) (De Mali et al, 1999; Bowman et al, 2001), insulin-like growth factor (IGF)-1 (Arbet-Engels et al, 1999), fibroblast growth factor (FGF) (Landgren et al, 1995), colony-stimulating factor (CSF)-1 (Courtneidge et al, 1993) and hepatocyte growth factor (HGF) receptors (Mao et al, 1997) (Fig. 3). Ligand binding to receptor tyrosine kinases leads to receptor dimerization, kinase activation, and autophosphorylation of tyrosine residues. These phosphorylated tyrosines then serve as docking sites for the SH2 domains of several signalling molecules, including c-Src (van der Geer et al, 1994). For instance, the EGF receptor can bind to c-Src and phosphorylate tyrosine sites on its C-terminal loop. Conversely, c-Src can directly bind to the EGF receptor and phosphorylate the Y845 residue, resulting in increased Ras/ERK/MAPK activity and enhanced cell mitogenesis and transformation (Biscardi et al, 2000).

c-Src has also been implicated in signalling activated by integrins and G-protein coupled receptors (GPCRs). Indeed, clustering of integrins can lead to downstream signalling

pathways inducing activation of c-Src, FAK, Abl, and Syk (Miyamoto et al, 1995, Schlaepfer & Hunter, 1998). There is an increasing body of evidence for synergy between receptor tyrosine kinases and integrins, demonstrated by an increase in MAPK activation in response to various growth factors if integrins are preclustered (Miyamoto et al, 1996). The crosstalk between these pathways could be mediated by a common signalling molecule, including c-Src. A FAK-independent signalling pathway from integrins has also been described, in which caveolins act as adaptors, linking integrins and c-Src family kinases. Indeed, Wei et al. (1999) showed that caveolin is important for the association between β1 integrin and c-Src, and disruption of this interaction affected focal adhesions. On the other hand, c-Src can suppress the integrins attached to the extracellular matrix via phosphorylation of integrin subunits (Sakai et al, 2001; Datta et al, 2002). c-Src can also interrupt Rho-A function, which has an important role in actin filament assembly and stabilization of focal adhesions (Arthur & Burridge, 2001). c-Src activates FAK, Ras and phosphatidylinositol phosphate kinase, which indirectly affect integrin–actin cytoskeleton assembly (Brunton et al, 2004).

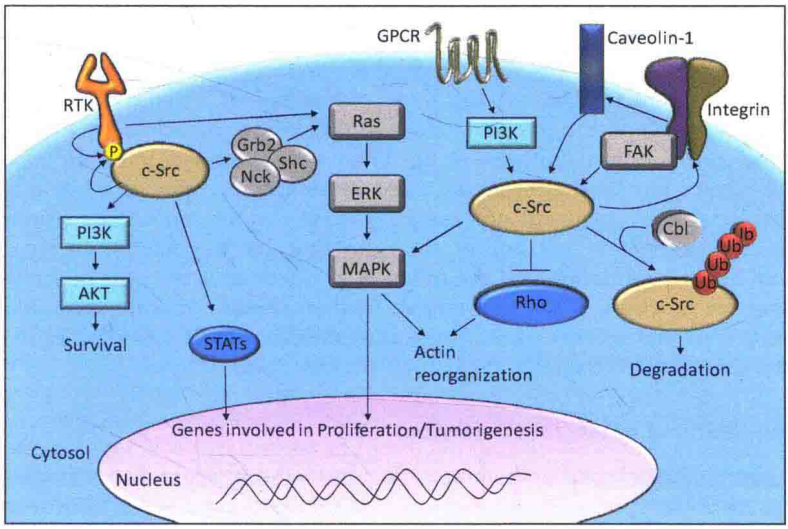

Fig. 3. c-Src-activating signals.
Extracellular signals involving Receptor Tyrosine Kinases (RTK), or G-Protein Coupled Receptors (GPCR) or integrins are shown to activate c-Src and its downstream pathways.

The localization of c-Src at the membrane–cytoskeletal interface in focal adhesions, lamellipodia and filopodia seems to be regulated by the small G-proteins RhoA, Rac1 and CdC42 (Timpson et al, 2001). The Cbl ubiquitin ligase has been shown to be important in suppressing v-Src transformation through ubiquitin-dependent protein degradation (Kim et al, 2004). Recent evidence indicates that the ubiquitin–proteasome pathway is deregulated in cancer cells, which might allow c-Src activation (Kamei et al, 2000).

Finally, there is also evidence that c-Src is activated through nitric-oxide signalling (Akhand et al, 1999) in mechanisms mainly implicated in cellular adhesion and motility (Gianni et al, 2010; Giannoni et al, 2005).

3. c-Src in the bone metabolism

Understanding the physiological role of SFKs has been aided by the advent of gene targeting and embryonic stem cell technology in the mouse. Targeted disruptions of all known mammalian SFK genes have been obtained in mice, with their phenotypes ranging from no overt defects to very distinct abnormalities in specific cell types and tissues. As stated above, the first SFK member to be disrupted was c-Src (Soriano et al, 1991). Although c-Src is ubiquitously expressed, mice lacking the *src* gene presented with overt alterations only in the bone tissue, suggesting a crucial role of this tyrosine kinase in the bone microenvironment that cannot be replaced by other SFK members, as probably occurs in the rest of the body.

The main phenotype associated with c-Src deletion is osteopetrosis, a bone remodelling disease in which excess of bone accumulates as a result of defective osteoclast bone resorption (Soriano et al, 1991). This mutation manifests itself by the failure of incisors to erupt, and the mutants have a much reduced survival rate after weaning. However, animals maintained on a soft food diet have been found to survive for at least a year and, on rare occasions, can breed, although some alterations in reproduction have been documented (Roby et al, 2005). In contrast with a general concept of c-Src involvement in cell proliferation, a detailed analysis of the bone phenotype of c-Src knock-out (KO) mice revealed the crucial role of the tyrosine kinase in regulating osteoclast activity, rather than formation and proliferation (Soriano et al, 1991). As discussed in more detail below, substantial evidence has been already provided in identifying c-Src as a key player in the correct cytoskeletal rearrangement necessary for bone resorption. Further studies pointed out the role of c-Src in bone metabolism, thus showing that the deletion of Src expression also enhances the differentiation and the function of osteoblasts, the cells of the bone tissue having osteogenic function, with a consequent further increase of bone mass (Marzia et al, 2000). Therefore, in this section we will introduce the bone cells and discuss in detail the multiple roles that c-Src exerts in the bone microenvironment.

3.1 c-Src regulation of osteoclast behaviour

Osteoclasts are multinucleated cells, originating from the myeloid tissue from which the mononuclear osteoclast progenitors arise and fuse into polykaria when their maturation is completed (Baron & Horne, 2005). They are terminally differentiated cells that resorb the mineralized matrix during physiological and pathological bone turnover by a peculiar extracellular mechanism involving specific domains of the plasma membrane. Indeed, during the bone resorbing process, the osteoclast is markedly polarized, in order to create three morphologically distinct areas of the plasma membrane: the basolateral membrane, which is not in contact with the bone; the tight sealing zone, which is closely apposed to the bone surface; and the ruffled border, a highly convoluted membrane that faces the resorbing surface (Baron & Horne, 2005).

The characterization of the phenotype of the c-Src KO mouse revealed that the most critical role of c-Src is related to osteoclast function rather than differentiation, since the number of osteoclasts in bones of c-Src KO mice is more than twice that in normal mice (Boyce et al, 1992). While osteoclasts also express other c-Src family kinases (Lowell et al, 1996), the deletion of any one of the genes encoding Fyn, Yes, Hck, and Fgr fails to produce

osteopetrosis (Stein et al, 1994). Moreover, re-expression of c-Src in c-Src KO osteoclasts restores *in vitro* the bone-resorbing activity (Miyazaki et al, 2004), implying that c-Src performs some specific functions in osteoclasts that cannot be compensated by these other SFKs. A possible exception is Hck, since its expression is upregulated in c-Src KO osteoclasts and c-Src KO/Hck KO double-mutant mice are significantly more osteopetrotic than the c-Src KO animals (Lowell et al, 1996). At the cellular level, c-Src KO osteoclasts present with a critical feature, that is the absence of the ruffled border (Boyce et al, 1992), suggesting a c-Src contribution to the regulation of exocytic and/or endocytic vesicle trafficking, as well as to the attachment and motility mediated by the adhesion structures.

Osteoclastic bone resorption involves a series of regulatory phases: migration of osteoclasts to the resorption site, their attachment to the calcified tissue and development of the ruffled border and the clear zone, followed by the secretion of acids and lysosomal enzymes into the space beneath the ruffled border (reviewed in Peruzzi & Teti, 2011). The formation of the sealing zone is essential for the osteoclastic bone resorption, since it forms a diffusion barrier and permits the directional secretion of lysosomal enzymes into the space beneath the ruffled border. In the ruffled border membrane, the vacuolar-type proton ATPase mediates the transport of protons into the resorption lacunae. Lysosomal enzymes of osteoclasts, such as cathepsin K, and metalloproteinase-9 are also secreted through this membrane and degrade the organic matrix of bone. To organize these highly polarized cellular structures, osteoclasts must adhere to the bone surface as the initial and essential phase for their activity (Coxon & Taylor, 2008), which involves the interaction of integrins with the extracellular matrix proteins within the bone. Among several integrins, osteoclasts express very high levels of αVβ3 integrin, and lower levels of the collagen/laminin receptor α2β1 and the vitronectin/fibronectin receptor αVβ1 (Nakamura et al, 2007; Horton, 1997; Horton & Rodan, 1996).

Like all members of the αV integrin family, the αVβ3 receptor recognizes the RGD (Arg-Gly-Asp) adhesion motif present in several matrix proteins such as vitronectin, bone sialoprotein II and osteopontin (Rupp & Little, 2001; Wilder, 2002; Horton, 1997). This interaction induces an integrin conformational change leading to the so-called outside-in signalling, which in turn triggers a number of intracellular events, including changes in cytosolic calcium, protein tyrosine phosphorylation and cytoskeletal remodelling (Duong & Rodan, 2000; Teitelbaum, 2007; Faccio et al, 2003). The engagement of the matrix by the αVβ3 integrin in osteoclasts and osteoclast precursors activates the non-receptor tyrosine kinase Pyk2, a member of the FAK family, by a mechanism that involves an increase in cytosolic Ca^{2+} and the binding of Pyk2 to the cytoplasmic domain of the β subunit (Fig.4) (Faccio et al, 2003; Duong & Rodan, 2000).

Both the capacity of c-Src to bind the αVβ3 integrin and the subsequent activation of the kinase are mediated by Pyk2, which mobilizes c-Src to the integrin. αVβ3 integrin occupancy induces phosphorylation of Pyk2, which then binds the SH2 domain of c-Src. The proposed association between phosphorylated Pyk2 and c-Src would prevent c-Src-Y527 inactivating phosphorylation, thus relieving auto-inhibition of kinase function.

The signalling downstream of c-Src activation involves tyrosine phosphorylation of a distinct set of proteins, including Pyk2 itself, Cbl, PI3K, paxillin, cortactin, vinculin, talin, tensin, and p130Cas, which are present in the osteoclast adhesion structures, called podosomes (Thomas & Brugge, 1997; Linder & Aepfelbacher, 2003; Buccione et al, 2004).

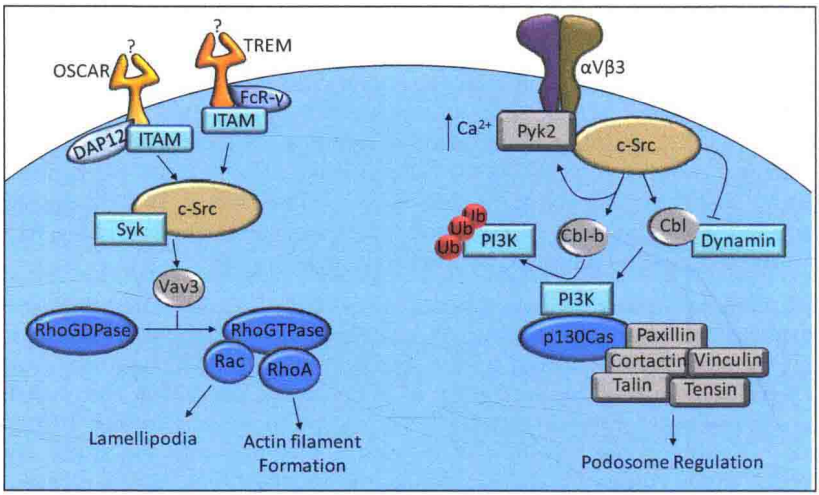

Fig. 4. c-Src involvement in osteoclast function.
Among several pathways regulating osteoclast activity, the cartoon shows c-Src activation and downstream effect depending on receptor signals.

Podosomes, which serve as attachment structures in osteoclasts and other highly motile cells, are more transient and dynamic than focal adhesion plaques (Destaing et al, 2003). As originally described (Marchisio et al, 1984; Marchisio et al, 1987), podosomes are small punctate structures with an F-actin-rich core surrounded by a ring of integrins and certain focal adhesion-associated proteins (e.g., paxillin, talin and vinculin). Cortactin, gelsolin, the actin-regulatory proteins Neuronal Wiskott–Aldrich Syndrome Protein (N-WASP), and Arp2/3 have also been identified in the podosome core (Linder & Aepfelbacher, 2003; Buccione et al, 2004). In osteoclasts, Src, Cbl, Pyk2, and various actin-associated proteins, including dynamin and filamin, are also associated with podosomes, whose rapid turnover (within minutes) is probably essential for the high mobility of the cells in which they occur (Fig. 4) (Destaing et al, 2003).

Among the substrates of c-Src activity involved in the mechanisms of bone resorption, Cbl plays a key role in promoting turnover or disassembly of podosomes (Sanjay et al, 2001). Indeed, c-Src, in association with Pyk2, recruits Cbl through its SH3 domain and promotes its activation by phosphorylation. Once activated, Cbl recruits PI3K and dynamin to the adhesion complex.

Since Cbl is an ubiquitin E3 ligase, it has been described to drive the negative feedback that has the potential to promote proteasomal degradation of the integrin-associated Pyk2/c-Src/Cbl complex (Fig. 4) (Yokouchi et al, 2001). Thus, Cbl is crucial in the integrin-mediated "inside-out" signalling, playing a key role in podosome detachment and subsequent disassembly. In this way, the c-Src/Pyk2/Cbl complex forms the basis for the cyclic attachment–detachment of single adhesion sites at the leading edge of lamellipodia in motile cells, and thereby participates in the assembly–disassembly of individual podosomes, ensuring cell adhesion while still allowing cell motility (Sanjay et al, 2001).

In the integrin-mediated outside-in signalling, also the non-receptor tyrosine kinase Syk plays a pivotal role in osteoclast activity, since it associates with the β3 integrin subunit domain in a region close to the c-Src binding site and is activated by c-Src itself, a key event in organizing the cytoskeleton (Zou W et al., 2007). Recently, it has been shown that c-Src and Syk are also involved in the signal downstream the immunoreceptor tyrosine-based activation motif (ITAM)-bearing co-receptors, DAP12 and FcRγ (Fig.4). DAP12 and FcRγ are associated with the immunoreceptors OSCAR/PIR-A and TREM2/SIRPβ1, respectively, recently identified on the osteoclast surface (Mócsai et al, 2004). The c-Src-mediated phosphorylation of Syk kinase leads to activation of a number of cytoskeleton-regulating proteins, including the Vav family of guanine nucleotide exchange factor (GEFs). These proteins convert Rho GTPases from their inactive GDP to their active GTP conformation. Among these proteins, Vav3 is expressed in osteoclasts (Faccio et al, 2005), where it is triggered upon matrix-induced Syk activation and regulates RhoGTPase-dependent effect on actin cytoskeleton (Zou et al, 2007). In this context, the small GTPases Rac and Rho exert distinctive effects on osteoclasts. Indeed, Rac stimulation in osteoclast precursors prompts the appearance of lamellipodia, thus forming the migratory front of the cell, while RhoA stimulates actin filament formation which, in osteoclasts, allow organization of the sealing zone (Fig.4) (Fukuda et al, 2005).

The integrin-activated c-Src signalling also functions in other processes necessary for normal osteoclast function, among which adenosine triphosphate (ATP)-dependent events, especially those involved in cell motility, proton secretion, and the maintenance of electrochemical homeostasis (Baron, 1993). Indeed, c-Src promotes the maintenance of energy stores in osteoclasts by phosphorylating cytochrome c oxidase within the mitochondria (Miyazaki et al, 2003), which are very abundant in osteoclasts, consistent with the energy requirements of their activity (Miyazaki et al, 2006).

3.2 c-Src regulation of osteoblast differentiation

Osteoblasts are mononucleated cells of mesenchymal origin that synthesize and mineralize the bone matrix during bone accrual and remodelling events. Bone formation involves osteoblast maturation that requires a spectrum of signalling proteins including morphogens, hormones, growth factors, cytokines, matrix proteins, transcription factors, and their co-regulatory proteins. They act coordinately to support the temporal expression (i.e., sequential activation, suppression, and modulation) of other genes that represent the phenotypic and functional properties of osteoblasts during the differentiation process from osteoblast precursors (Jiang et al, 2002). Pre-osteoblasts are also responsible of the production of cytokines regulating osteoclastogenesis, that is receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG) and CSF-1, thereby coupling osteoblast and osteoclast function. Given the osteoblast-mediated regulation of osteoclast differentiation and bone resorption (Rodan & Martin, 1981; Suda et al, 1997) and the bone phenotype resulted by c-Src distruption (Soriano et al, 1991), several studies aimed at investigating the involvement of osteoblasts in c-Src KO phenotype have been performed. In 1993, Lowe et al. demonstrated that osteoblasts derived from these mice successfully contributed to normal osteoclast differentiation and showed unremarkable morphological features relative to wild-type (WT) mice, suggesting that the inherited defect is independent of the bone marrow microenvironment (Lowe et al, 1993). The first evidence of an osteoblast involvement in c-Src KO mouse bone phenotype derived from Marzia and coworkers, who

performed a detailed molecular analysis of the c-Src null osteoblasts (Marzia et al, 2000). This study clearly demonstrated that a decreased c-Src activity is responsible of enhanced osteoblast differentiation and *in vivo* bone formation, thereby highlighting the role of c-Src in maintaining osteoblasts in a poorly differentiated status.

Bone formation requires transcriptional mechanisms for sequential induction and repression of genes that support progressive osteoblast phenotype development. The Runx transcription factors and their co-regulators control cell differentiation and lineage commitment (Westendorf & Hiebert, 1999) by influencing the functional architecture of target gene promoters (Stein et al, 2000). Runx proteins are directed to subnuclear domains through the C-terminal nuclear matrix-targeting signal (NMTS) and interact with the DNA through the N-terminal runt homology domain (Zaidi et al, 2001). The Runx2 family member is essential for osteoblast maturation *in vivo* and its alteration is associated with the cleidocranial dysplasia (Komori et al, 1997). Runx2 is a target of several extracellular signals that regulate skeletal formation and homeostasis. The C-terminus of Runx2, which includes the NMTS, interacts with proteins involved in the transforming growth factor β/bone morphogenetic proteins (TGFβ/BMPs) (i.e., Smads), the transducin-like enhancer (TLE)/groucho and the c-Src/Yes tyrosine kinase (e.g., the Yes-associated protein, YAP) signalling pathways (Hanai et al, 1999; Yagi et al, 1999). Indeed, in response to c-Src/Yes signalling, YAP is phosphorylated and recruited by Runx2 to subnuclear sites of Runx2 target genes, resulting in their repression (Fig.5). Thus, c-Src controls osteoblast differentiation by regulation of Runx2–YAP interaction.

Another mechanism by which c-Src regulates osteoblast differentiation involves estrogens, which are known to control a variety of tissues, including the bone (Hall et al, 2001). Indeed, estrogen deficiency leads to accelerated bone loss which is the primary cause of postmenopausal osteoporosis (Manolagas et al, 2002). The estrogen receptors (ERs) belong to the nuclear receptor superfamily, acting as ligand-inducible transcription factors (Hall et al, 2001).

Indeed, ER expression is regulated by a c-Src/PKC-dependent mechanism involving osteoblast differentiation, with an increased responsiveness to estrogens in mature osteoblasts (Fig.5) (Longo et al., 2004). Estrogens are also responsible for an anti-apoptotic effect on osteoblasts, which is due to a rapid and sequential phosphorylation of the c-Src, Shc and ERK1/2 kinases. The c-Src/Shc/ERK signalling cascade rapidly phosphorylates the transcription factors Elk1, CREB and C/EBPβ with a mechanism that is retained when the receptor is localized outside the nucleus (Kousteni et al, 2003).

Beside the estrogen-mediated effect, other extracellular stimuli, such as mitogens and changes in the mechanical stress, are responsible of c-Src activation and of the downstream cascade involving the MAPK signalling. In this circumstance, the transcriptional target is the AP-1 complex, a heterodimer composed of members of the c-Fos, c-Jun, and activating transcription factor (ATF) families (Hess et al, 2004), which is an important regulator of bone development and homeostasis (Wagner & Eferl, 2005).

Of special interest in the regulation of c-Src activation and activity is the role of caveolae. They are small bulb-shaped invaginations located close to the cell surface representing specialized domains of the plasma membrane (Severs, 1988). Caveolin, a 21–24-kDa integral membrane protein, is a major structural and regulatory component of caveolae membranes (Rothberg et al., 1992). Several data suggest that caveolin may act as a scaffolding protein

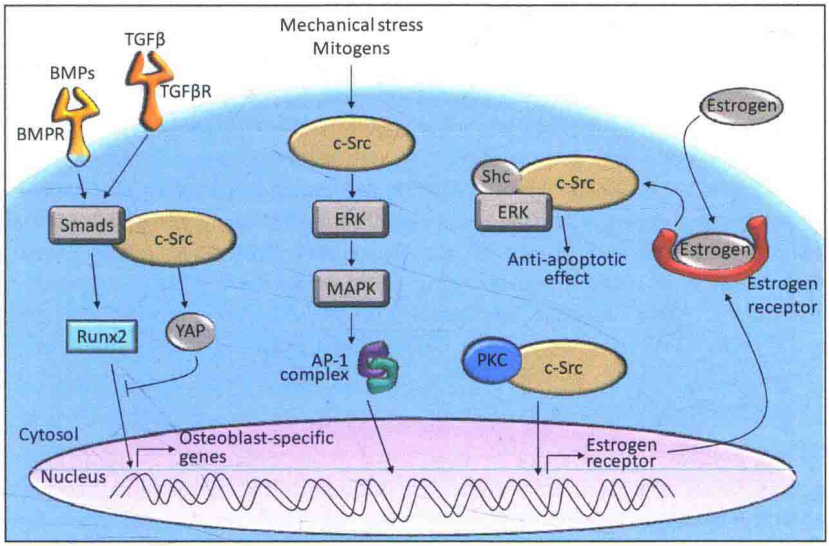

Fig. 5. c-Src involvement in osteoblast differentiation.
TGFβ/BMP signals, mechanical stress, mitogens and estrogen modulate c-Src activity and osteoblast differentiation.

within caveolae membranes, since both the N-terminal and C-terminal domains of caveolin face the cytoplasm (Dupree et al., 1993). Caveolin interacts with cytoplasmic signalling molecules including trimeric G proteins, Src family tyrosine kinases, and Ras-related GTPases. Thus, caveolin may serve as an oligomeric docking site for organizing and concentrating inactive signalling molecules within the caveolae membranes (Sargiacomo et al, 1995). Modification and/or inactivation of caveolin may be a common feature of the transformed phenotype. Caveolin can be phosphorylated by c-Src at Tyr14, an event that induces caveolar internalization by reorganizing the actin cytoskeleton (mediated by dynamin and PKC) (Mayor & Pagano, 2007).

On the other hand, caveolin has a role in regulating c-Src activation, since its interaction with c-Src, as well as with the other components of the SFKs, leads to the inhibition of auto-phosphorylation of these kinases, thus holding these molecules in the inactive conformation (Li et al, 1996).

Recently, we demonstrated that in osteoblasts c-Src regulates interleukin (IL)-6 and insulin-like growth factor binding protein (IGFBP)-5 expression (Peruzzi et al, 2012). More in details, c-Src controls IL-6 expression acting on STAT3, which is a downstream component of the IL-6 pathway and a transcription factor for IL-6 itself. At the same time, IL-6 stimulates the expression of IGFBP5 which, in turn, acts in an autocrine manner on osteoblasts inducing the c-Src activating-phosphorylation and inhibiting further osteoblast differentiation. On the other hand, in mature osteoblasts c-Src is barely expressed and therefore this loop is inactive, although IGFBP5 is still expressed under the control of Runx2. In this context, IGFBP5 has been observed to enhance osteoclast formation and bone resorption, thus unveiling its new role in the coupling between osteoblast and osteoclast activities (Peruzzi et al, 2012).