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REVIEW ARTICLE

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The role of c-Src kinase in the regulation of osteoclast function

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Abstract The targeted disruption of c-Src impairs osteoclast bone resorbing activity, causing osteopetrosis. Although it has been reported that restoring only the c-Src adaptor function at least partly rescues the skeletal phenotypes, the importance of c-Src kinase activity remains controversial. We here highlight the contributions of the Src adaptor and kinase activities in cytoskeletal organization and osteoclast function using adenovirus vectors containing various mutants of Src or Pyk2. In addition, we describe the importance of c-Src in mitochondria, where it phosphorylates cytochrome *c* oxidase (Cox). Src-induced Cox activity is also required for bone resorbing activity of osteoclasts that require high levels of ATP. Thus, c-Src kinase activity not only on the plasma membrane but also within mitochondria is essential for the regulation of osteoclastic bone resorption.

Key words c-Src · Kinase · Mitochondria · Osteoclast · Pyk2

Introduction

Non-receptor type tyrosine kinase c-Src is a member of a family of nine protein–tyrosine kinases that associate with the cytoplasmic surface of the cellular membrane.[1] Activated mutants of Src are oncogenic, and study of these mutants has implicated c-Src in the control of cell growth and proliferation. However, c-Src is highly expressed in terminally differentiated cells such as platelets and neurons, indicating a physiological role for the protein unrelated to growth control.[2] Soriano et al.[3] reported that targeted disruption of the c-*src* gene in mice induced osteopetrosis, a disorder characterized by decreased bone resorption, without showing any obvious morphological or functional abnormalities in other tissues or cells. They also showed that the osteopetrotic phenotype of c-*src*-disrupted mice is cell-autonomous and occurs in mature osteoclasts.

Osteoclasts are multinucleated, terminally differentiated cells which degrade mineralized matrix during normal and pathological bone turnover.[4] Osteoclastic bone resorption involves the proliferation and homing of the hemopoietic osteoclast progenitors to bone, their differentiation and fusion to form multinucleated cells, and the migration of osteoclasts to and between the resorption sites. Osteoclasts attach to the bone surface and form a tight sealing zone (or "clear zone") enclosing the resorption lacunae, which was frequently compared to a large lysosome. Following the insertion of secretory vesicles, a highly convoluted membrane called the "ruffled border" is formed facing the bone surface.[5] Integrins have been suggested to play a role in osteoclast activity by mediating osteoclast adhesion and regulating the cytoskeletal organization required for both cell migration and formation of sealing zone. v-*src*-transformed cells contain high concentrations of Src as well as other tyrosine phosphorylated proteins in podosome,[6,7] specialized adhesion sites that are structurally and functionally distinct from focal adhesions.[6,8–10] Although many of the same proteins are present in podosomes and focal adhesions, focal adhesions are relatively stable, whereas podosomes are dynamic attachment structures, undergoing assembly and disassembly within minutes.[11–13] Activation of Src may therefore be associated with a shift from stable focal adhesions with actin stress fibers to more dynamic podosome assemblies, possibly regulating cell motility. Indeed, several papers have suggested a role for Src kinase activity in cell spreading and migration.[14–18]

It remains uncertain, however, whether catalytically active Src is required for normal osteoclast function.

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Schwartzberg et al.[19] previously reported that osteoclast-specific expression of kinase-dead Src mutants rescued the $Src^{-/-}$ osteopetrotic phenotype, suggesting that c-Src may act as an adaptor molecule and that c-Src kinase activity may not be critical for bone resorption.[19] In addition, no adverse effects of kinase-dead Src expression in $Src^{+/+}$ and $Src^{+/-}$ animals was observed.[19,20] On the other hand, we have recently reported that down- or up-regulation of c-Src activity modulates osteoclastic bone resorption not only in vitro but also in vivo,[18] leaving the question of the contribution of c-Src kinase activity unsettled.

Here, we show that not only adaptor function but also the kinase activity of c-Src are important in osteoclastic bone resorption, taking advantage of the ability of adenoviral vectors to transduce foreign genes into osteoclasts.[21] In addition, we also found that c-Src in mitochondria regulates osteoclast function via cytochrome c oxidase activity.[22] In this review, we would like to introduce the role of c-Src kinase activity in both plasma membrane and mitochondria.

The structure of c-Src

Src family tyrosine kinases have a common domain organization, with each segment designated as a Src-homology (SH) region. The N-terminal segments includes the SH4 domain, which is a myristoylation and membrane-localization signal, as well as a "unique" domain, which differs among family members. This region is followed in the peptide chain by the SH3 domain, the SH2 domain, the tyrosine kinase (SH1) domain, and a short C-terminal tail, which includes a critical tyrosine residue (Fig. 1A). SH2 and SH3 domains mediate protein–protein interactions in cellular signaling cascades, and are found in many proteins outside the Src family. The SH3 and SH2 domains and the C-terminal tail all have roles in regulating Src kinase activity. It is now clear that phosphorylation of Tyr-527 by a specific kinase, Csk (C-terminal Src family kinase),[23] inhibits Src catalytic activity by creating an intramolecular binding site for the Src SH2 domain. The interaction is believed to result in auto-inhibition by locking the molecule in an inactive state. Displacement of SH2 domains by C-terminal tail dephosphorylation or by competitive binding of optimal SH2/SH3 domain ligands, allows the kinase domain to open, exposing Tyr-416 to phosphorylation. In open state, phosphorylation of Tyr-416 in the activation loop of the kinase domain further upregulates the enzyme (Fig. 1B).[24,25]

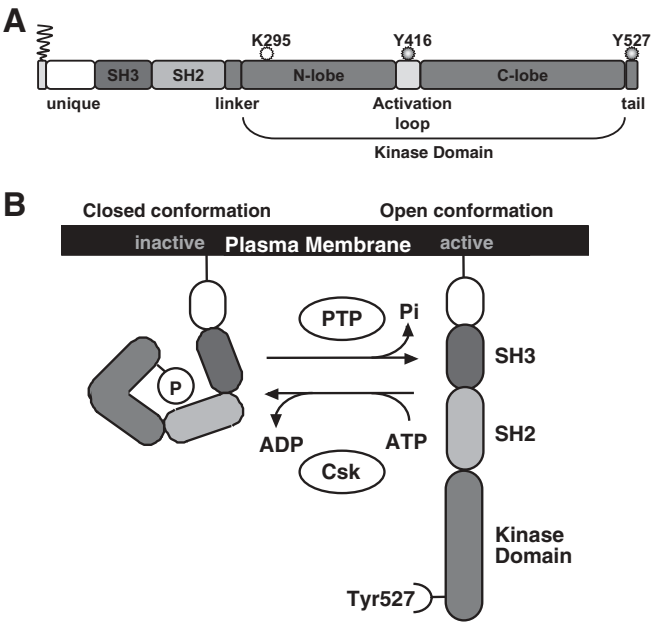

Fig. 1A,B. The structure of c-Src. **A** Schematic illustrations of the c-Src. N-terminal segment includes membrane-localization signal, myristoylation site (also called SH4 domain). The Src homology 3 domain, proline-rich binding site, SH2 domain, which binds to phosphotyrosine, and kinase domain follow in order. There are also a short, C-terminal tail, which includes a critical tyrosine residue. **B** The restrained conformation of c-Src is stabilized by intramolecular interactions among the kinase domain, the SH2/SH3 domains, and the phosphorylated C-terminal tail. Displacement of SH2 and/or SH3 domains, either by C-terminal tail dephosphorylation or by competitive binding of optimal SH2/SH3 domain ligands, allows the kinase domain to open, exposing Tyr-416 to phosphorylation

Pyk2-dependent recruitment of c-Src to the plasma membrane is necessary for bone resorption

Proline-rich tyrosine kinase 2 (Pyk2) has been identified as a major adhesion-dependent tyrosine kinase in osteoclasts, both in vivo and in vitro.[26–28] Pyk2 is a member of the focal adhesion kinase family, highly expressed in cell of the central nervous system and cells of hematopoietic lineage. Pyk2 and FAK share approximately 45% of the overall amino acid identity and have a high degree of sequence conservation surrounding binding site of SH2- and SH3-domain-containing proteins.[29] Ligation of $\alpha_v\beta_3$ integrin either by ligand binding or by antibody-mediated clustering increased Pyk2 tyrosine phosphorylation in osteoclasts. Moreover, in adherent osteoclasts, Pyk2 is tightly associated with c-Src, via its SH2 domain.[17,27] Upon osteoclast adhesion, Pyk2 translocates into the Triton X-100 insoluble cytoskeletal fraction and was concentrated at the cell periphery, co-localizing with F-actin.[27] Furthermore, Sanjay et al.[17] reported that Pyk2 is autophosphorylated upon integrin activation and that Pyk2 autophosphorylation at Y402 is required for the Pyk2/c-Src complex formation via the interaction with the Src SH2 domain. To examine the importance of the binding of Src to Pyk2, we constructed adenovirus vector carrying autophosphorylation site-mutated Pyk2 [$Pyk2^{Y402F}$]. More than 90% of wild-type osteoclast-like cells (OCLs) formed in vitro[30,31] display typical rounded appearances with a clear actin ring formation (Fig. 2A, upper panel). Adenovirus-mediated $Pyk2^{Y402F}$-overexpressed OCLs did not exhibit one large actin ring (Fig. 2A, lower panel) and strongly inhibited the bone-resorbing activity in proportion to $Pyk2^{Y402F}$ expression (Fig. 2B).[21] These data lead us to speculate that the interaction of

Src with Pyk2, mediated by the SrcSH2 domain and the Pyk2 Tyr(P)-402, is required for the normal organization of the osteoclast actin cytoskeleton and for bone resorption. Although the recruitment of Src to autophosphorylated Pyk2 at adhesion sites is a key event in bone resorption, it does not address the question of whether Src kinase activity is required or if the adaptor functions of Src SH2 and SH3 domains are sufficient. To examine whether or not the upregulation of Src kinase activity is sufficient to rescue the decreased pit-forming activity of $Pyk2^{Y402F}$-expressing OCLs, we coinfected OCLs with $AxPyk2^{Y402F}$ and adenovirus carrying kinase-dead C-terminal Src family kinase ($AxCsk^{KD}$), which prevents the phosphorylation of the negative regulatory Tyr-527 and increases Src kinase activity in OCLs.[18] The inhibitory effect of $AxPyk2^{Y402F}$ on osteoclast function was, however, not rescued by co-infection with $AxCsk^{KD}$, leading us to conclude that high Src kinase activity alone is not sufficient to rescue the decreased bone resorbing activity in the $Pyk2^{Y402F}$-expressing OCLs and that the Pyk2-dependent recruitment of Src is necessary for bone resorption.

c-Src kinase activity is required for osteoclast function

To further confirm the importance of Src kinase activity in osteoclasts, we constructed an adenovirus carrying kinase-dead Src (Src^{K295M}). In contrast to the report by Schwartzberg et al.,[19] Src^{K295M}-expression completely disrupted the actin cytoskeleton in osteoclasts and bone-resorbing activity was strongly inhibited in proportion to the expression level of Src^{K295M} protein.[21] These results strongly suggest that it is the presence of the kinase activity of Src in the osteoclast adhesion structures and not just the Src protein that is necessary for actin ring formation and bone resorption. However, the autophosphorylation of Src promotes the adoption of the fully open active conformation,[24,25] and thus, Src^{K295M} might not be an effective adaptor.

To rule out the possibility that the dominant negative effects of Src^{K295M} could be due to a loss of adaptor function, we constructed $AxSrc^{K295M+Y527F}$, which prevents the phosphorylation of the negative regulatory Tyr-527 and promotes the availability of the SH2 and SH3 domains to bind other proteins. The morphology and bone-resorbing activity of $Src^{K295M+Y527F}$-expressing osteoclasts was similar to that of osteoclasts infected with $AxSrc^{K295M}$, further establishing that it is the absence of kinase activity, not the possible failure to bind to Pyk2 and/or other proteins in the adhesion complex, that causes the loss of bone resorbing activity.[21] To further confirm the importance of Src kinase activity, we investigated the functional changes in osteoclasts infected with $AxSrc^{Y416F+Y527F}$. The Y416F mutation reduces kinase activity about 50% by preventing the phosphorylation of a tyrosine on the activation loop of Src.[32,33] Despite its open conformation, $Src^{Y416F+Y527F}$ partially inhibited osteoclast function, consistent with its reduced kinase activity. $AxSrc^{Y416F+Y527F}$-infected osteoclasts exhibited a few small actin rings and still retained partial bone-resorbing activity (about 70% of control).[21] These results lead us to conclude that the localization of catalytically active Src in adhesion structures is required for actin ring formation and bone resorption, at least in vitro.

c-Src kinase activity is required for the efficient rescue of the $c\text{-}Src^{-/-}$ osteoclast phenotype

As noted earlier, Schwartzberg et al.[19] previously reported that osteoclast-specific expression of the kinase-dead Src^{K295M} mutant partially restored normal trabecular bone volume and tooth eruption in the $Src^{-/-}$ mice. We then introduced c-Src mutants into $c\text{-}Src^{-/-}$ osteoclasts to determine the influence of endogenous c-Src levels in our previous experiments. Under these in vitro experimental conditions, $Src^{-/-}$ OCLs were completely unable to form resorption pits in our assay (Fig. 4). Interestingly, Src^{K295M} and $Src^{K295M+Y527F}$, which did not notably affect the cytoskeletal organization of OCLs, restored (Fig. 3A), albeit extremely minimally (less than 3%), some bone-resorbing activity in $Src^{-/-}$ osteoclasts (Fig. 3B).[21] This minimal effect could be due to the adaptor function of c-Src or to the very low residual kinase activity in these mutants. In contrast, the expression of $Src^{Y416F+Y527F}$, which retains about 50% kinase activity, induced the formation of actin rings and very significantly increased bone-resorbing activity to ~70% of control (Fig. 3).[21] These results clearly show that c-Src kinase activity is indeed required to rescue the disruptive effect of c-Src deficiency on the cytoskeletal organization and bone-resorbing activity of osteoclasts.

The data from our study clearly show that at the level of the individual osteoclast, the kinase activity of Src is indeed required for bone resorption. Most interestingly, although $Src^{-/-}$ OCLs were 100% devoid of resorbing activity, cells reconstituted with Src^{K295M} demonstrated a very minimal (about 3%) but significant ($P < 0.001$) level of pit-forming activity (Fig. 3B), which was still much less than the >70% restoration of pit formation by OCLs reconstituted with $Src^{Y416F+Y527F}$. This minimal restoration of activity by Src^{K295M} suggests a possible explanation of the apparent discrepancy between our results with individual OCLs and the findings of Schwartzberg in animals. It is known that there are severalfold more osteoclasts in $Src^{-/-}$ than in wild type animals, and the degree of osteopetrosis in these animals is moderate.[34] Even a small increase in the activity of the larger number of osteoclasts acting over a longer time than our in vitro bone resorbing assay could induce sufficient additional bone resorption to account for the partial rescue reported by Schwartzberg et al.[19]

c-Src is localized in mitochondria

To define additional candidate proteins implicated in c-Src signaling, we performed a two-hybrid screen using kinase-

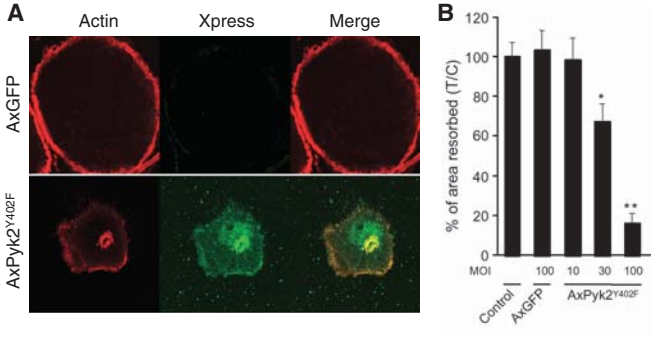

Fig. 2. A Double immunofluorescence staining of F-actin (*red*) and Xpress-tagged Pyk2^{Y402F} (*green*) in osteoclast-like cells (OCLs) infected with AxGFP or AxPyk2^{Y402F}. Adenovirus-mediated Pyk2^{Y402F} overexpression disrupted the one large actin ring formation. **B** Dentine-resorbing activity of OCLs expressing the Pyk2 mutants. OCLs 24 h after the infection at an indicated multiplicity of infection (*MOI*; a measure of titer, which reflects how many viruses infected a cell) were restored by digesting the collagen gel with 0.2% collagenase for 20 min at 37°C and an aliquot of the crude OCL preparation was transferred onto dentine slices (diameter 5 mm) as well as on 96-well culture plates and further cultured for 12 h. The resorbed area on dentine slices was measured using an image analysis system, and the number of TRAP-positive OCLs on culture plates was counted by light microscopy. The bone-resorbing activity of the cells was expressed as the resorbed area per osteoclast. The values are the mean ± SD ($n = 8$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with non-infected OCLs)

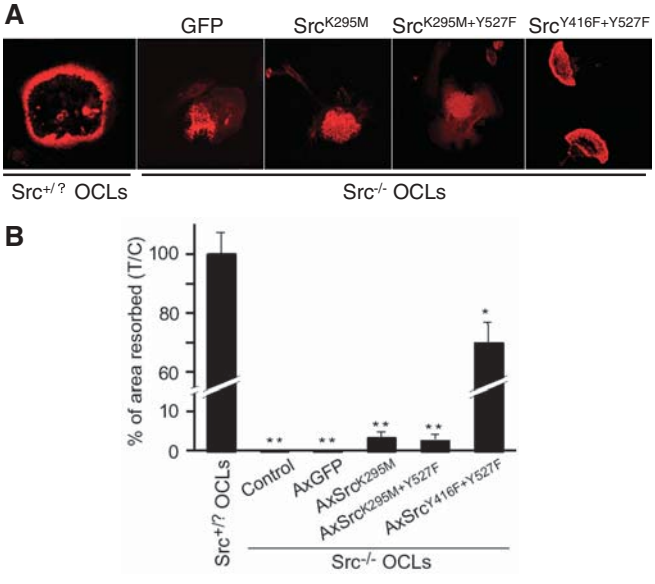

Fig. 3. A F-actin staining in Src$^{-/-}$ OCLs infected with AxGFP, AxSrc^{K295M}, AxSrc$^{K295M+Y527F}$, or AxSrc$^{Y416F+Y527F}$. Although Src^{K295M} and Src$^{K295M+Y527F}$ did not notably affect the cytoskeletal organization of Src$^{-/-}$ OCLs, the expression of Src$^{Y416F+Y527F}$ partly rescued the formation of actin rings. **B** Dentine-resorbing activity of Src$^{-/-}$ OCLs expressing the Src mutants. In contrast to Src^{K295M} and Src$^{K295M+Y527F}$, the expression of Src$^{Y416F+Y527F}$, which retains about 50% kinase activity, very significantly increased bone-resorbing activity. The values are the mean ± SD ($n > 8$; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with Src$^{+/?}$ OCLs)

dead c-Src as a bait. We screened a mouse brain library and obtained several clones that encode a mitochondrial protein. Mitochondria, the cellular energy plants, generate ATP through oxidative phosphorylation (OXPHOS). OXPHOS, defined as the oxidation of fuel molecules by

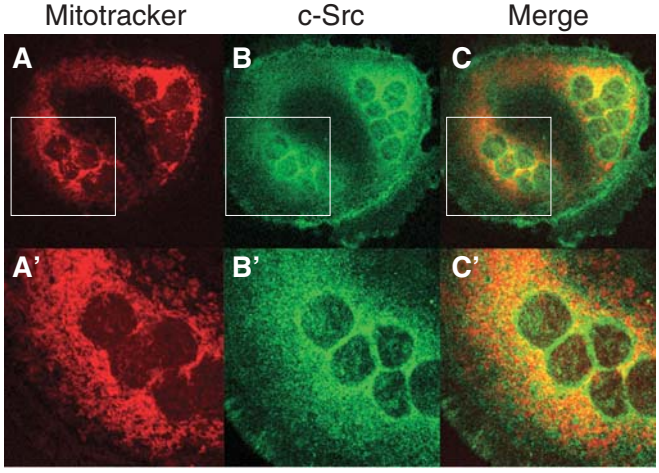

Fig. 4. Mitochondrial localization of c-Src. **A**–**C** Double immunofluorescence staining of Mitotracker and c-Src in osteoclasts. The localizations of Mitotracker (*red*) and c-Src (*green*) were assessed by confocal microscopy. **A′**–**C′** Close-up of the boxed region from **A**–**C**. c-Src staining was detected throughout the cytoplasm as well as at the cell periphery and many of these colocalized with mitochondria in osteoclasts

oxygen and the concomitant transduction of this energy into ATP, is the final process of the complicated biochemical network involved in cellular energy production. The OXPHOS molecular system, which is embedded in the lipid bilayer of the mitochondrial inner membrane, consists of electron acceptors, coenzyme Q and cytochrome c, and five multisubunit protein complexes (complexes I–V). The OXPHOS system comprises about 70 nuclear gene products and 13 mitochondrial gene products.[35,36]

To address whether c-Src might be present in mitochondria, we performed the dual staining of osteoclasts for c-Src and mitochondria using c-Src antibody and Mitotracker. As shown in Fig. 4, the merged image of c-Src (green) and mitochondria (red) revealed that c-Src protein appeared to colocalize with mitochondria. In close inspection of magnified images, c-Src clearly exhibited mitochondrial localization, as evidenced by colocalization with Mitotracker. The immunofluorescent results were also verified by immunoelectron microscopy. c-Src was found to be associated with the inner mitochondrial membrane in Src$^{+/?}$ osteoclasts. A large fraction of the gold particles were close to, or superimposed on, the inner mitochondrial membrane (Fig. 5). In contrast, no mitochondria showed labeling in the c-Src$^{-/-}$ osteoclasts (negative control).

We then examined the tyrosine phosphorylation of mitochondrial proteins from c-Src-overexpressing HEK 293 cells to determine if c-Src functions in mitochondria. Western blots of proteins suspended on two-dimension non-denaturing/denaturing gels with anti-phosphotyrosine suggested that one of the tyrosine-phosphorylated mitochondrial proteins was the cytochrome *c* oxidase (Cox).[22] Cox, which contains 13 subunits, is the terminal oxidase of cell respiration. The three major subunits of Cox are encoded by mitochondrial DNA and form the functional core of the enzyme; this core is surrounded by 10 nuclear-coded small subunits. Cox reduces dioxygen to water with four

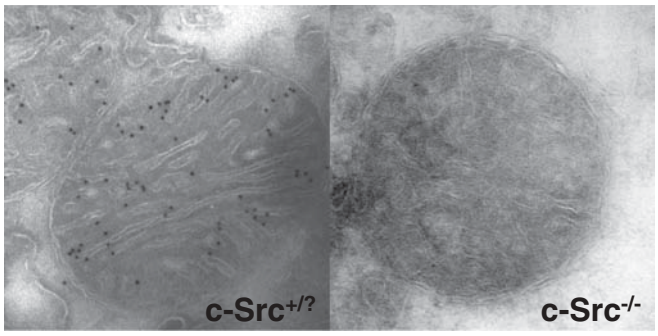

Fig. 5. Immunogold labeling of c-Src in isolated mitochondria from c-Src$^{+/?}$ and c-Src$^{-/-}$ osteoclasts. As predicted from confocal microscopy data, there is strong labeling of mitochondria. Many of gold particles are associated with the inner mitochondrial membrane in c-Src$^{+/?}$ osteoclasts, whereas no labeling was detected in the mitochondria of c-Src$^{-/-}$ osteoclasts

electrons from cytochrome *c* and four protons taken up from the mitochondrial matrix, without the formation of reactive oxygen species. The energy generated by the passage of electrons down the electron transport chain creates a proton gradient across the membrane that drives ATP synthase to make ATP from ADP.

To examine the functional consequences of the tyrosine phosphorylation of Cox by c-Src, we investigated whether or not c-Src kinase activity affected Cox activity. For this purpose, we used Src family tyrosine kinase-deficient mouse embryonic fibroblasts[37]. Cox activity in Src$^{-/-}$ Yes$^{-/-}$ Fyn$^{-/-}$ cells (SYF) was significantly decreased compared to Yes$^{-/-}$ Fyn$^{-/-}$ cells (Src++). Furthermore, c-Src re-introduction in SYF cells restored Cox activity to the higher level of Src++ cells.[22] These results lead us to speculate that c-Src can promote Cox activity in mouse embryonic fibroblasts. Further support for this conclusion was provided by an experiment showing that Cox activity in the enriched mitochondrial fraction was inhibited by the Src family inhibitor PP2. As in the SYF fibroblasts, Cox activity was reduced in c-Src$^{-/-}$ OCLs formed in vitro (Fig. 6A), providing further evidence that c-Src regulates Cox activity. To test whether modulating c-Src kinase activity in OCLs would affect Cox activity and bone resorption, we used adenovirus vectors containing kinase-dead Src (AxSrc^{K295M}), wild-type C-terminal Src family kinase (AxCsk^{WT}), or kinase-dead Csk (AxCsk^{KD}). Downregulation of c-Src kinase activity by Src^{K295M} or Csk^{WT} overexpression inhibited Cox activity in OCLs, while upregulation of Src kinase activity by AxCsk^{KD} infection induced higher Cox activity (Fig. 6B).[22] Thus, Cox activity is positively correlated with c-Src kinase activity.

Cox activity is correlated with osteoclastic bone resorption

To determine if reducing Cox activity affects the bone-resorbing activity of OCLs, we took advantage of the fact that nuclear-coded Cox subunit IV (CoxIV) is absolutely

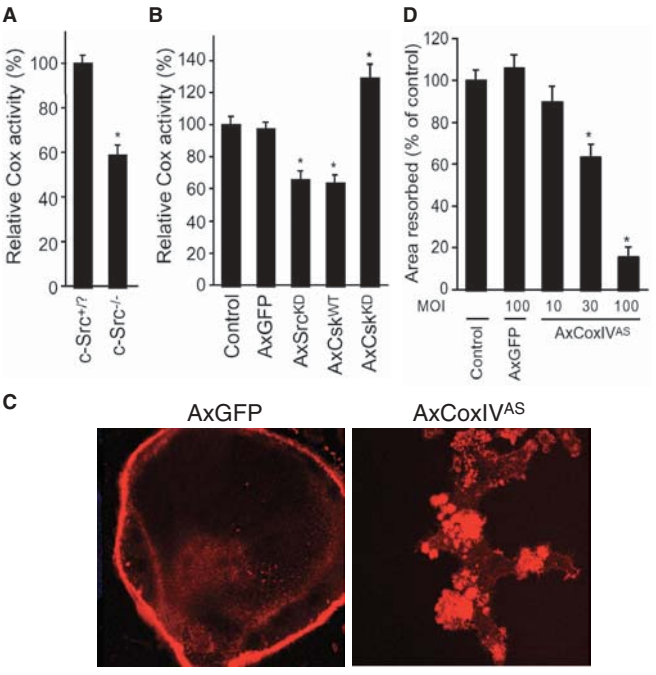

Fig. 6. A Cox activity in c-Src and c-Src$^{-/-}$ OCLs. Cox activity was reduced in c-Src$^{-/-}$ OCLs formed in vitro. The values are mean ± SD (n = 8; *P < 0.01 compared with Src$^{+/?}$ OCLs). **B** Cox activity in OCLs infected with AxGFP, AxSrc^{KD}, AxCsk^{WT}, or AxCsk^{KD} at an MOI of 100. The values are mean ± SD (n = 8; *P < 0.01 compared with uninfected OCLs). **C** Effects of CoxIV antisense (CoxIV^{AS}) on osteoclast morphology. Cocultures infected with AxGFP or AxCoxIV^{AS} at an MOI of 100 were plated on serum-coated glass coverslips for 12 h and then costained for F-actin using rhodamine phalloidin. **D** Dentine-resorbing activity of OCLs expressing CoxIV antisense. An aliquot of the OCL preparation was transferred to dentine and cultured for an additional 12 h. Bone-resorbing activity was progressively and severely inhibited as the MOI of AxCoxIV^{AS} increased. The values are mean ± SD (n = 8; *P < 0.01 compared with uninfected OCLs)

required for Cox activity.[38] So we constructed an adenovirus vector carrying CoxIV antisense (AxCoxIV^{AS}). The expression of CoxIV in OCLs infected with AxCoxIV^{AS} decreased in a dose-dependent manner. The decreasing levels of CoxIV correlated with decreased Cox activity in a biochemical assay; OCLs infected with AxCoxIV^{AS} showed 45% of basal Cox activity.[22] In addition, there was no significant change in c-Src kinase activity among OCLs infected with AxCoxIV^{AS}.

An actin ring was observed in 85% of the AxGFP-infected OCLs, whereas only 15% of OCLs infected with AxCoxIV^{AS} formed these rings (Fig. 6C). Consistent with the absence of the actin ring, bone-resorbing activity was progressively and severely inhibited by the infection with AxCoxIV^{AS} (Fig. 6D).[22] Interestingly, AxCoxIV^{AS} infection does not affect the survival of OCLs. These results strongly suggest that basal Cox activity is required for maintaining osteoclast morphology and for normal bone resorption. Consistent with these results, treating the cells with the classical Cox inhibitor KCN also decreased bone resorption. Diminished ATP levels by the failure of normal Cox could contribute to the observed loss of bone resorption Indeed, the complex I inhibitor rotenone and the complex

III inhibitor myxothiazol also prevented the bone-resorbing activity of OCLs in a dose-dependent manner, strongly suggesting that the rate of ATP generation by mitochondrial oxidative phosphorylation is critical for osteoclastic bone resorption. Furthermore, the inhibitory effect of $AxCoxIV^{AS}$ on bone resorption was not reversed by Csk^{KD} overexpression, even though c-Src kinase activity was increased in OCLs co-infected with $AxCoxIV^{AS}$ and $AxCsk^{KD}$ as much as it was in OCLs infected with $AxCsk^{KD}$ alone.[22] Taken together, these results indicate that Cox is a signaling effector downstream of c-Src that is necessary for bone resorption. Osteoclasts have a large number of mitochondria and express a high level of c-Src,[5,39,40] and a high level of ATP is required to support acid secretion by the osteoclast v-ATPase, as well as other functions that are required for bone resorption.[5] Thus, decreased Cox activity and the resulting reduction in ATP levels could explain, at least in part, the reduced bone-resorbing activity of $c\text{-}Src^{-/-}$ osteoclasts.

Concluding remarks

c-Src, which is highly conserved throughout evolution and widely expressed, associates with the cytoplasmic surface of cellular membranes. It is generally thought that c-Src's regulation of cell adhesion, movement, and proliferation involves its activity as a plasma membrane-associated molecular switch that links a variety of extracellular cues to specific intracellular signaling pathways. Our study demonstrates that both the formation of a Pyk2/Src complex, presumably in the podosomes, and the kinase activity of Src are required for bone resorption by osteoclasts. Elucidation of the role of Src in osteoclast function requires the identification of the signaling elements whose activities might to be modulated by the recruitment of active Src kinase to Pyk2. Src-catalyzed phosphorylation of Cbl on Tyr-731 is reported to create a binding site for the regulatory p85 subunit of phosphatidylinositol 3-kinase (PI3K),[41,42] and induce its activation and recruitment to the cell membrane. PI3K in turn is involved in a reorganization of the cytoskeleton that results in cell spreading and migration of several cell types including osteoclasts. Expressing the Cbl^{Y731F} mutant in osteoclasts markedly reduced their bone resorbing activity, suggesting that phosphorylation of Cbl-Y731 and the subsequent recruitment and activation of PI3K may be critical signaling events downstream of c-Src in osteoclasts. These results provide further insight into how the attachment of osteoclasts to the bone surface regulates bone resorption and how Src participates in this regulation.

c-Src is required for maintaining the basal Cox activity in mouse embryonic fibroblasts and osteoclasts. Furthermore, basal Cox activity is important for bone-resorbing activity of mature osteoclasts as well as c-Src. On the other hand, the most prominent defects of the SYF cells were reduction of cell proliferation and motility, and it should be noted that these are also ATP-dependent events. We therefore conclude that c-Src in mitochondria regulates Cox activity and that the c-Src/Cox signaling pathway is critical for the bone-resorbing activity of osteoclasts. The downregulation of Cox activity in the absence of c-Src may be involved in the osteopetrotic phenotype of $c\text{-}Src^{-/-}$ mice.

Interestingly, c-Src has also been reported to be present on late endosomes in fibroblasts,[43] synaptic vesicles in PC12 cells,[44] secretory vesicles in chromaffin cells,[45] vesicular structures in osteoclasts,[39,46] and the Golgi apparatus in CHO cells,[47] suggesting that c-Src is involved in multiple intracellular processes as well. In addition, Lyn, another Src family kinase, was found in rat brain mitochondria.[48] More recently, Itoh et al.[49] reported that Dok-4 recruits c-Src in mitochondria as an anchoring molecule and regulates NF-κB activation in endothelial cells. The subcellular fractionation also confirmed the localization of c-Src in all fractions, suggesting that c-Src associates with not only mitochondria but also various intracellular membranes. The identification and characterization of signaling cascades of c-Src on various intracellular membranes present an interesting new avenue for further elucidating Src's role in regulating cell function.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by invasive synovial hyperplasia. Proliferation of the synovial cells leads to pannus tissue that invades the bare area between cartilage and bone, finally resulting in progressive bone and joint destruction in the affected joints. The ultimate goal of the treatment of RA is to prevent bone and joint destruction and preserve the daily activity of the patients. Recent studies have shown that osteoclasts are involved in the pathogenesis of bone and joint destruction and can be a potent therapeutic target of this disease, and that therapies that inhibit osteoclast formation or function can at least ameliorate the progression of these bone changes. We have previously demonstrated that local injection of Csk virus, which negatively regulates Src family tyrosine kinases, into rat ankle joints with adjuvant arthritis not only ameliorated inflammation but also suppressed bone destruction. There will be no cure for RA until its etiology is elucidated, but suppression of osteoclast activity by regulating various intracellular signaling pathways including c-Src might lead to a novel therapeutic strategy for preventing the joint breakdown associated with RA.

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