

Cross-talk between Src kinases and Rho small GTPases regulates biomineralization and simplify imaging of the mineralization process

ABSTRACT

The process of physiological mineralization that occurs during bone ossification is a tightly regulated cascade of molecular events leading to formation of bony skeleton. Its deregulation associated with aging leads to pathological mineralization not only in osseous but also in soft tissues. Recent discoveries in the field of bone biology indicate the participation of the Src family of tyrosine kinases as well as the Rho family of small GTPases in mineral formation. Cross-talk between these two signaling pathways is activated during an early step of the mineralization process, and leads to reorganization of the cytoskeleton of mineralization-competent cells and to matrix vesicles release. The understanding of mechanisms of the cross-talk between Src kinases and Rho small GTPases that regulate the mineralization process is crucial for the development of novel simply imaging techniques and therapeutic strategies in relation to pathological mineralization.

INTRODUCTION

In its simplified form the signal transduction mechanism in the cell is a direct interaction between specific proteins in a strictly determined order. A number of key proteins may participate in several signal transduction pathways. Different types of kinases and adaptor molecules without any enzymatic activity are the main elements of signal transduction chains in cells. Actually, the major molecular principles underlying the signal transduction mechanism are represented by specific protein associations and protein phosphorylation (or dephosphorylation). Phosphorylation of proteins leads to immediate changes in their conformation and properties. The balance between phosphorylation and dephosphorylation normally determines intracellular signal transduction.

Members of the Src family of tyrosine kinases and of the Rho family of small GTPases act as molecular switches and play pivotal roles in signal transduction. An autoinhibitory mechanism has been proposed for both families of kinases. It involves an intramolecular interaction of the kinase domain with the C-terminal SH2 and SH3 domains in case of Src at phosphorylation state [1] as well as with PH domain in case of ROCK in the absence of Rho binding [2,3]. Indeed, the activity of kinases from both families influences cell polarity, cell adhesion, cytokinesis, neurite retraction and transcription factor activity [4]. Moreover, contractile processes in smooth muscle and nonmuscle cells have been linked to the Src [5] as well as Rho/ROCKs pathways [6,7]. Cytoskeleton reorganization is essential for various cellular events, including changes in cell morphology, cell motility, adhesion and cytokinesis. Various signaling pathways, together with the mineralization process, link the external stimuli to the machinery controlling actin polymerization and organization.

Mineralization-competent cells, such as chondrocytes during endochondral ossification or osteoblasts during intramembranous ossification, release matrix vesicles (MVs), originating from cellular microvilli [8-11]. MVs are engaged at the early steps of mineralization providing an optimal environment for the nucleation process of HA formation from Ca^{2+} and P_i [12,13]. It is now established that MVs bud from the parent plasma membrane of mineralization-competent cell in an acto-myosin contractility-dependent manner. In consequence, the MV membrane is enriched in many proteins, particularly tissue non-specific alkaline phosphatase (TNAP), nucleotide pyrophosphatase phosphodiesterase 1 (NPP1), inorganic phosphate (P_i) transporter, Ca^{2+} and phospholipid binding proteins belonging to the annexin (AnxA) family (AnxA1, AnxA2, AnxA5, AnxA6 and AnxA11), matrix metalloproteinases (MMPs) family (MMP-9, MMP-13), collagens (types II and X) and inorganic phosphate (P_i)-phospholipid complexes [14-20]. Lipid composition of MVs membrane differs from that of plasma membrane they bud from - MVs are enriched in cholesterol, glycolipids and phospholipids whereas the latter has high affinity for calcium ions, stabilizes the deposited

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Abbreviations: ALP - alkaline phosphatase; AnxA - vertebrate annexin; BSP - bone sialoprotein; CHK - CSK homologous kinase; CRD - cysteine-rich domain; CSK - c-Src tyrosine kinase; CysD - cysteine-rich domain; HA - hydroxyapatite; LIMK - LIM kinase; LPA - lysophosphatidic acid; MAPK - mitogen-activated protein kinase; MARCK - myristoylated alanine-rich C kinase, MLCK - myosin light chain kinase; MMP - matrix metalloproteinase; MVs - matrix vesicles; NPP1 - nucleotide pyrophosphatase phosphodiesterase 1; OCN - osteocalcin; OPN - osteopontin; PKC - protein kinase C; P_i - inorganic phosphate; PHD - pleckstrin homology domain; PI3-K - phosphoinositide 3-kinase; PP1 - 4-amino-5-(methylphenyl)-7-(t-butyl)pyrazolo (3,4-d) pyrimidine; PP2 - 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d] pyrimidine; PP_i - inorganic pyrophosphate; PLA, PLC, PLD - phospholipases A, C, D; PTH - parathyroid hormone; RBD - a Rho-binding domain; ROCK - Rho-associated coiled-coil kinase; Src - sarcoma proto-oncogene tyrosine-protein kinase; Src-11 - Src Inhibitor-1, 4-(4'-phenoxyanilino)-6,7-dimethoxyquinazoline, 6,7-dimethoxy-N-(4-phenoxyphenyl)-4-quinazolinamine; RANKL - receptor activator of nuclear factor- κ B ligand; RSV - Rous sarcoma virus; TNAP - tissue non-specific alkaline phosphatase; Y-27632 - ROCK inhibitor, (R)-(+)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide dihydrochloride

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mineral and protects phospholipids from degradation by phospholipases (PL-ases) [8].

Physiological mineralization occurs in extracellular matrix (ECM) of skeletal tissues and is strictly regulated for the proper formation, development and function (rearrangement and repair) of the skeleton. The currently held mechanism regulating MVs release postulates that ANK and NPP1 function to suppress mineralization by increasing the extracellular concentration of the calcification inhibitor, pyrophosphate (PP_i), while TNAP functions to promote mineralization by decreasing the concentration of PP_i and increasing the concentration of the mineralization promoter, P_i [21]. The presence of vesicular trafficking molecules (e.g. Rab proteins) and of the on-site protein synthesis machinery suggests that cell polarization and apical targeting are required for the incorporation of specific lipids and proteins at the site of MVs formation. Pathological mineralization such as vascular or cancer calcification recapitulates physiological mineralization. Smooth muscle cells or osteosarcoma cells involved in such calcification behave as mineral competent cells - they are able to release MVs, have high TNAP activity and produce collagen [9,22]. MVs release from microvilli may be driven by the action of actin-severing proteins (gelsolin, cofilin 1) and contractile motor proteins (myosins) [9]. However, the questions concerning regulation and mechanisms of MVs release, incorporation of proteins into MVs membrane and influence of ECM factors on mineral formation still remain without answer. During osteogenesis the actomyosin cortex [8,14] and focal contacts, where integrins bind the actin cytoskeleton, through vinculin, to the matrix collagen [23] are subject to many changes. The intracellular signaling pathways that transduce and integrate all signals mentioned above are still largely unknown. In this review we describe the cross-talk between signal transduction pathways dependent on Src kinases and Rho small GTPases which may regulate the mineralization process under physiological as well as pathological conditions.

STRUCTURE AND FUNCTION OF SRC KINASES

Src is a proto-oncogenic tyrosine kinase originally discovered by J. Michael Bishop and Harold E. Varmus, for which they won the 1989 Nobel Prize in Physiology or Medicine. It belongs to a family of non-receptor-associated protein tyrosine kinases called Src family kinases. Src kinases control a variety of cellular processes ranging from proliferation, differentiation, motility, adhesion, and transcription to the control of cell survival and angiogenesis [5,24].

HOMOLOGUES OF SRC

Src was initially discovered as the oncogenic protein of the retrovirus Rous sarcoma virus (v-Src). A highly conserved cellular homolog (c-Src) was subsequently discovered. Much of the original research on both homologs paved the way for our current understanding of how oncogenes cause cell transformation [24]. Although many oncogenes of the nonreceptor tyrosine kinases group have been identified after the discovery of *v-src*, the studies are still focused on c-Src as a prototype oncoprotein [5]. Mutations in *src* gene cause functional activation of the protein product. *v-src*

codes virus protein, which lacks the C-terminal inhibitory phosphorylation site (Tyr⁵²⁷) and is therefore constitutively active as opposed to *c-src* coding normal cellular protein, which is only activated under certain circumstances when required (e.g. growth factor signaling). *v-src* is therefore an instructive example of an oncogene whereas *c-src* is a proto-oncogene.

There are 8 Src kinases in vertebrates: Src, Fyn, Yes, Lck, Lyn, Hck, Fgr and Blk [25]. These proteins are closely related to each other and share the same regulatory mechanism. They interact with the intracellular domains of growth factor/cytokine receptors, G protein-coupled receptors, integrins and other non-receptor tyrosine kinases such as Jak and ZAP-70 [24]. Fyn and Yes are ubiquitously expressed, while Lck, Lyn, Hck, Fgr and Blk have limited expression, although high levels of c-Src are found in platelets, neural tissue and osteoclasts [24,25].

REGULATION OF SRC ACTIVITY

The protein product of *src* is a cytoplasmic 60 kDa protein with tyrosine-specific protein kinase activity that associates with the cytoplasmic face of the plasma membrane. It is activated as a result of disruption of regulatory processes that normally suppress its activity [26]. The protein consists of three domains, an N-terminal SH3 domain, a central SH2 domain and a C-terminal tyrosine kinase domain (Fig. 1). The main difference between v-Src and c-Src is found in the structure of their C-terminal regions. The last "tail" 19 amino acids of c-Src contain Tyr-527, which plays a regulatory role controlling kinase activity whereas in v-Src these 19 amino acids are replaced by 12 amino acids present in all known RSV strains [5]. The SH2 and SH3 domains cooperate in the auto-inhibition of the kinase domain. c-Src is phosphorylated on an inhibitory tyrosine near the C-terminus of the protein. This produces a binding site for the SH2 domain which, when bound, facilitates binding of the SH3 domain to a low affinity polyproline site within the linker between the SH2 domain and the kinase domain. Binding of the SH3 domain results in misalignment of residues within the kinase active site and results in enzyme inactivation. This allows for multiple mechanisms for c-Src activation: dephosphorylation of the C-terminal tyrosine by a protein tyrosine phosphatase, binding of the SH2 domain by a competitive phosphotyrosine residue, as seen in the case of c-Src binding to focal adhesion kinase, or competitive binding of a polyproline binding site to the SH3 domain, as seen in the case of the HIV NEF protein. Phosphorylation of Tyr⁵²⁷ by an enzyme

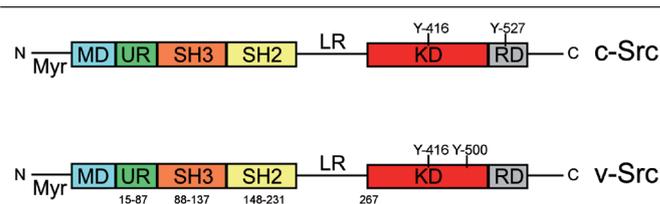


Figure 1. Domain structure of c-Src and v-Src kinases. Myr; amino-terminal myristoylation sequence, MD: membrane association domain, UR: unique region, SH2 and SH3: Src-homology-2 and Src-homology-3 protein-interaction domains, LR: linker region, KD: kinase domain that contains autophosphorylation site Tyr⁴¹⁶, RD: carboxy-terminal regulatory domain that contains Tyr⁵²⁷. v-Src proteins differ from c-Src in several ways, but one important difference is the substitution of the negative regulatory carboxy-terminal region.

that is closely structurally related to c-Src, termed c-Src tyrosine kinase (CSK), negatively regulates the kinase activity of Src by inducing a 'closed' conformation – that is, by promoting the association between the Src kinase domain and its SH2 domain [1]. For c-Src, autophosphorylation of Tyr⁴¹⁸ and dephosphorylation of Tyr⁵³⁰ is required to switch the kinase from the inactive "closed" form to the active "open" form. c-Src can be inactivated by two kinases, c-Src kinase (CSK) and CSK homologous kinase (CHK), both of which phosphorylate Tyr⁵³⁰ of c-Src. The activity of the Src kinase family can also be regulated by phosphatases (e.g. SHP1), binding to adaptor proteins (e.g. Cbp) and proteasomal degradation. Protein phosphatase 2A inhibits Src in a concentration-time-dependent manner [27].

PP1 and PP2 are potent and selective inhibitors of Src-family tyrosine kinases. Src Inhibitor-1 (Src-I1, 4-(4'-phenoxyanilino)-6,7-dimethoxyquinazoline, 6,7-dimethoxy-N-(4-phenoxyphenyl)-4-quinazolinamine is a potent, competitive, dual site (both the ATP- and peptide-binding) Src kinase inhibitor (IC₅₀=44 nM and 88 nM for Src and Lck, respectively). It has been shown that PP1 or PP2 may be used in parallel with Src-I1 for Src kinase inhibition [28-30].

FUNCTIONS AND DISEASES DEPENDENT ON SRC

In 1979, J. Michael Bishop and Harold E. Varmus discovered that normal chickens have *c-src* gene that is structurally closely-related to *v-src* [31]. This discovery changed the current thinking about cancer from a model wherein cancer is caused by a foreign substance (a viral gene) to one where a gene that is normally present in the cell can cause cancer. Src kinases also transmit integrin-dependent signals to cell movement and proliferation. Hallmarks of *v-src* induced transformation are rounding of the cell and formation of actin rich podosomes on the basal cell surface. These structures are correlated with increased invasiveness, a process thought to be essential for metastasis. *c-src* proto-oncogene may play a role in the regulation of embryonic development and cell growth. Mutations in this gene could be involved in the malignant progression of colon cancer. Some data showed a requirement for c-Src in colorectal carcinogenesis. Elevation of c-Src or down-regulation of SHP-2 and/or PTP-PEST may promote cancer metastases and invasion by regulating villin-induced cell migration and cell invasion [32]. A function for Src tyrosine kinases in normal cell growth was first demonstrated with the binding of family member p56lck to the cytoplasmic tail of the CD4 and CD8 co-receptors on T-cells [33]. The importance of the association between the cell surface receptors and Src kinases of T cells is discussed in relation to receptor-dependent cell activation and HIV infectivity.

Src kinases are key upstream mediators of both the PI3-K and MAPK signaling pathways and play important roles in a variety of other cellular processes such as proliferation, differentiation, motility and adhesion [5]. They are also involved in control of cell survival and angiogenesis [24]. The expression of Src and phospho-Src (p-Src) is closely related to tumor migration, invasion and metastasis. Src and p-Src can be used as an auxiliary indicator to determine a malignant phenotype of bone tumors, and the combined detec-

tion of Src and p-Src may indicate poor prognosis for osteosarcoma patients [34].

STRUCTURE AND FUNCTION OF RHO FAMILY OF SMALL GTPases

The mammalian Rho-family of small GTPases is composed of 20 intracellular signaling molecules, the best documented of which are: Ras homolog gene family member A (RhoA), Ras-related C3 botulinum toxin substrate (giving three Rac isoforms – Rac1, Rac2, and Rac3) and cell division control protein 42 (Cdc42). ROCKs (aka Rho-associated coiled-coil kinases) are kinases activated by RhoA GTPases. They belong to the AGC group of protein kinase A, G, and C families (PKA/PKG/PKC) of serine-threonine kinases, which are key modulators of processes implicated in Rho-mediated actin reorganization, such as formation of stress fibers and focal adhesions, smooth muscle contraction and tumor cell invasion [35-39]. ROCKs are expressed not only in mammals (human, rat, mouse, cattle) but also other vertebrates (chicken, zebrafish, *Xenopus*) and invertebrates (*C. elegans*, mosquito, *Drosophila*).

The Rho family of small GTPases plays a central role in regulating actin organization through downstream effectors, the activity of which is controlled by interactions with the active, GTP-bound forms of the Rho family members [4,40]. The GTPases switch between an active GTP-bound state to an inactive GDP-bound state. Guanine-nucleotide exchange factor (GEF) promotes the release of GDP in exchange of GTP, while GTPase activating protein (GAP) increases the intrinsic hydrolytic GTPase activity, which leads to GDP generation from GTP, whereby the GTPase becomes inactivated. Rho-kinases are involved in many aspects of cell motility, centrosome positioning and cell-size regulation, giving rise to an increase in myosin light chain (MLC) phosphorylation and thereby inducing actomyosin-based contractility [2,7,41]. ROCKs phosphorylate and activate a number of proteins involved in actin filament assembly e.g. actin-binding kinase (LIMK), myosin light chain kinase (MLCK) or ezrin-radixin-moesin (ERM) proteins, which in turn promotes downstream phosphorylation and inactivation of the actin depolymerizing factor cofilin [42-44]. Several other Rho effector proteins have been described including signal transduction molecules such as myosin phosphatase target subunit 1 (MYPT1), insulin receptor substrate 1 (IRS1), enzymes of the PI3-K/Akt pathway, citron, rhotekin, rhophilin, protein kinase N and Rho-associated kinases [6,35,37,45,46].

HOMOLOGUES OF ROCKs

There are two highly homologous isoforms of ROCKs: ROCK1 (ROCKI, aka ROK β) and ROCK2 (ROCKII, aka ROK α), which have 65% of amino acids in common and 92% identity within their kinase domains [47,48]. In human these proteins may have different molecular mass: ROCK1 of 160 kDa, whereas ROCK-2 of 150-160 kDa. ROCK1 is a major downstream effector of the small GTPase RhoA, whereas ROCK2 shares structural similarity with myotonic dystrophy kinase [35,37]. The tissue distribution of the two enzymes also differs: ROCK1 has a ubiquitous tissue distribution, whereas ROCK2 is preferentially expressed in brain and in muscle. In

mouse, ROCK1 is mainly expressed in the lung, liver, spleen, kidney and testis, whereas ROCK2 is distributed mostly in the brain, heart, lung, muscle and placenta [7,47,48].

REGULATION OF ROCKs ACTIVITY

ROCKs are the downstream effector molecules of the Rho GTPase which increases ROCKs activity when bound to them. ROCKs are activated by disruption of intramolecular autoinhibition. Generally ROCK proteins consist of an N-terminal kinase domain (KD), a coiled-coil region (CR) containing a Rho-binding domain (RBD) and a C-terminal pleckstrin homology domain (PHD) containing a cysteine-rich domain (CysD) (Fig. 2). In case of the Rho family kinases an autoinhibitory mechanism which involves an intramolecular interaction between the kinase domain and the PH domain in the absence of Rho binding has been proposed [2,3]. Thus, the kinase activity is "switched off" when ROCKs are intramolecularly folded and "switched on" when Rho-GTP binds and disrupts the autoinhibitory interaction within ROCKs [7]. Rho is not the only activator of ROCKs, they can be regulated also by lipids, in particular arachidonic acid, and protein oligomerization which induces N-terminal transphosphorylation [7].

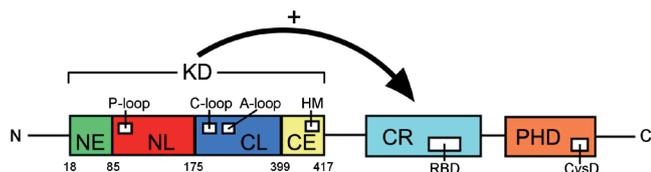


Figure 2. Domain structure of ROCK kinase. KD: kinase domain, CR: coiled-coil region, PHD: pleckstrin homology domain, NE: N-terminal extension, NL: β -rich N-terminal lobe, CL: helix-rich C-terminal lobe, CE: C-terminal extension. Functionally important motifs are indicated inside the domains: P-loop: phosphate binding loop (or Glycine-rich loop) with the GxGx ϕ G motif, C-loop: catalytic loop, A-loop: activation loop, HM: hydrophobic motif, RBD: Rho binding domain, CysD: putative Zn binding cysteine-rich domain.

Y-27632 (*R*)-(+)-*trans*-4-(1-aminoethyl)-N-(4-ayridyl)cyclohexane carboxamide dihydrochloride) is highly potent, cell-permeable, selective ROCK-1 inhibitor ($K_i=140$ nM). It also inhibits ROCK-2 with equal potency, but the inhibition is competitive with respect to ATP. It was shown that inhibition of ROCKs with Y-27632 significantly increased the lifespan of mice with spinal muscular atrophy (SMA). The same studies evaluated the therapeutic potential of a clinically approved ROCKs inhibitor fasudil [49]. SAR407899 is a novel, an ATP-competitive and selective Rho-kinase inhibitor equipotent against human and rat-derived ROCK-2 ($K_i=36$ nM and 41 nM, respectively) with promising antihypertensive activity [50]. Simvastatin beneficial action can be attributed to inhibition of ROCKs and activation of PI3-K/Akt what is crucial for its cardio protective and insulin resistance effects, involved in the pathophysiology of metabolic syndrome (MS) [46]. Other ROCKs inhibitors such as Wf-536, H1152, and RKI-1447 reduced tumor progression in melanoma, hepatocellular, lung and breast cancers [51-55].

FUNCTIONS AND DISEASES DEPENDENT ON ROCKs

ROCKs are acting in a wide range of different cellular phenomena. They are downstream effector proteins of the small GTPase Rho, which is one of the major regulators of

actin organization and thus a regulators of the cytoskeleton and cell migration. Rat ROCKs were discovered as the first effectors of Rho, which induce the formation of stress fibers and focal adhesions by phosphorylating MLC [36]. Different substrates can be phosphorylated by ROCKs, including LIMK, MLCK and MLC phosphatase and these substrates, once phosphorylated, regulate actin filament organization and contractility. ROCK1 inhibit the depolymerization of actin indirectly, whereas ROCK2 activate LIMK, which in turn phosphorylates ADF/cofilin, thereby inactivating its actin-depolymerization activity. This results in stabilization of actin filaments and an increase in their numbers as well as in reduction of the amount of actin monomers that are required to form new filaments necessary for migration [7,42]. ROCKs also regulate cell migration by promoting cell-substratum contacts. They increase the activity of the motor protein, myosin II, by two different mechanisms: firstly, by phosphorylation of the MLC leading to increased ATPase activity of myosin II; secondly, by inactivation of MLC phosphatase leading to increased levels of phosphorylated MLC. Thus in both cases, ROCK activation by Rho induces formation of actin stress fibers, actin filament bundles of opposing polarity (containing myosin II, tropomyosin, caldesmon, MLCK) and consequently, of focal contacts, which are immature integrin-based adhesion points with the extracellular substrate [7,56]. ROCKs are homologous to other metazoan kinases such as myotonic dystrophy kinase (DMPK), DMPK-related cell division control protein 42 (Cdc42)-binding kinases (MRCK) and citron kinase (CRIK) [7].

ROCKs play an important role in cell cycle control inhibiting the premature separation of the two centrioles in G1, and are proposed to be required for contraction of the cleavage furrow, which is necessary for the completion of cytokinesis [7,57-61]. ROCK regulates also MLC phosphorylation and actomyosin contractility which regulate membrane blebbing, a morphological change seen during cell apoptosis [7]. The pro-apoptotic protease, caspase 3, activates ROCK kinase activity by cleaving the C-terminal PH domain and as a result the autoinhibitory intramolecular fold of ROCK is abolished. ROCKs contribute to neurite retraction inducing growth cone collapse by activating actomyosin contractility. It is also possible that phosphorylation of the collapsin response mediator protein-2 (CRMP2) by ROCK inhibits CRPM2 function of promoting axon outgrowth [7].

ROCKs regulate cell-cell adhesion. Loss of ROCKs activity leads to loss of tight junction integrity in endothelial cells, whereas in epithelial cells inhibition of them decreases tight junction integrity. Active ROCKs in these cells seem to stimulate the disruption of E-cadherin-mediated cell-cell contacts by activating actomyosin contractility [7]. Other ROCKs targets are: NHE1 (a sodium hydrogen exchanger, involved in focal adhesions and actin organization); intermediate filament proteins: vimentin, GFAP (glial fibrillar acidic protein), NF-L (neurofilament L protein); F-actin binding proteins: adducin, EF-1 α (elongation factor 1 α), MARCKS (myristoylated alanine-rich C kinase substrate), caponin (unknown function), and ERM (involved in linkage of the actin cytoskeleton to the plasma membrane).

Recent observations suggest that Rho family GTPases signaling plays an important role in many diseases including diabetes, neurodegenerative diseases and cancers. ROCKs inhibitors are proposed as potential therapeutic treatment to prevent unwanted cell migration, with the aim of staving off metastatic cancer cell invasion [48]. RhoA-GTP stimulates the phospholipid phosphatase activity of PTEN (phosphatase and tensin homologue), a human tumor suppressor protein which mutations are responsible for PTEN hamartoma tumor syndromes. This stimulation depends on ROCKs activity and MLC phosphorylation [62]. Moreover, overexpression of PTEN may attenuate the adhesion, migration and invasion capabilities of osteosarcoma cells [63].

INVOLVEMENT OF SRC KINASES IN THE MINERALIZATION PROCESS

CHONDROCYTES

A non-receptor protein kinase Src plays a crucial role in fundamental cell functions such as proliferation, migration, and differentiation. While inhibition of Src is reported to contribute to chondrocyte homeostasis, its regulation at a subcellular level by chemical agents that attenuate ER stress and mechanical stimulation has not been fully understood. Cytosolic Src activity as well as membrane-bound Src activity in C28/I2 human chondrocytes was determined. In response to TNF α and IL1 β , both cytosolic and plasma membrane-bound Src proteins were activated, but activation in the cytosol occurred earlier than that in the plasma membrane [64]. Matrix metalloproteinase-13 (MMP-13) may contribute to the breakdown of articular cartilage during arthritis. Stimulation with thrombin led to increased secretion of MMP-13 in cultured human chondrocytes [22]. Thrombin acts through the PAR1/PAR3 receptors and activates PKC δ and c-Src, resulting in EGFR transactivation and activation of PI3K, Akt, and finally AP-1 on the MMP-13 promoter, thereby contributing to cartilage destruction during arthritis. It was also found that gene and protein expression levels of the focal adhesion complex and small Rho GTPases are upregulated with increasing passage number and are closely linked to chondrocyte dedifferentiation. Inhibition of focal adhesion kinase (FAK), but not of small Rho GTPases, induced the loss of fibroblastic traits and the recovery of collagen type II, aggrecan, and SOX9 expression levels in dedifferentiated chondrocytes. Based on these findings, a strategy to suppress chondrogenic dedifferentiation by inhibiting the FAK or Src pathways while maintaining the expansion capability of chondrocytes in a 2D environment was proposed [65]. Periodic mechanical stress activates the integrin β 1-dependent Src-dependent PLC γ 1-independent Rac1 mitogenic signal in rat chondrocytes through ERK1/2 [66]. This finding suggests that periodic mechanical stress promotes chondrocyte proliferation and matrix synthesis in part by activating the ERK1/2 mitogenic signal through the integrin β 1-Src-PLC γ 1/Rac1-ERK1/2 pathway, which links these important signaling molecules into mitogenic cascades. It has recently been shown that the Src-PLC γ 1-ERK1/2 signal transduction pathway is involved in cartilage tissue integration by affecting chondrocyte migration [67]. For example, chondrosarcoma is a primary malignant bone cancer, with a potent capacity to invade locally and

cause distant metastases, especially to the lungs. Berberine, an active component of the *Ranunculaceae* and *Papaveraceae* families of plant, did not induce cell apoptosis in human primary chondrocytes and chondrosarcoma cells but reduced the migration and invasion of chondrosarcoma cancer cells. Incubation of chondrosarcoma cells with berberine reduced mRNA transcription for, and cell surface expression of, the $\alpha_v\beta_3$ integrin, with additional inhibitory effects on PKC δ , c-Src, and NF- κ B activation. Thus, berberine may be a novel anti-metastasis agent for the treatment of metastatic chondrosarcoma [68].

OSTEOBLASTS

Src has been proven to be crucial in the process of differentiation and mineralization of mouse osteoblasts [69]. An elevated activity of Src family kinases was demonstrated in highly metastatic human osteosarcoma sublines [70]. It was also reported that c-Src kinase activity seems to be involved in the development of the osteosarcoma malignant phenotype [71]. Osteosarcoma is the most frequent primitive malignant tumor of the skeletal system, characterized by an extremely aggressive clinical course that still lacks an effective treatment. Treatment of human osteosarcoma cell lines with a new pyrazolo[3,4-*d*]pyrimidine derivative, namely SI-83, an Src inhibitor, impaired cell viability with kinetics different from that known for the Src inhibitor PP2 [72]. Moreover, SI-83, by inhibiting Src phosphorylation, decreased the osteosarcoma tumor mass *in vivo* in a mouse model. Finally, SI-83 showed selectivity for osteosarcoma, since it had a far lower effect in primary human osteoblasts. Taken together, these results suggest that the new pyrazolo[3,4-*d*]pyrimidine derivatives may provide therapeutic benefit by preventing the growth of bone sarcomas with potentially low side effects in the nonneoplastic cells [72].

Src was found to be up regulated in anoikis (form of apoptosis) resistance of human osteosarcoma Saos-2 cells, and pharmacological inhibition of its activity resulted in the restoration of anoikis sensitivity. Moreover, these studies indicated that cell survival is mediated by the Src-dependent activation of the PI3-K/Akt pathway in a manner independent of FAK activity [73]. Other data showed that vitamins A and D stimulate synchronized molecular responses downstream of a ligand-bound cytoplasmic RXR/VDR heterodimers, including PI3K/Akt activation, and lead to suppression of osteoblast apoptosis and increased cell survival [74,75]. The Ca²⁺-dependent binding of AnxA2 to the plasma membrane or phosphatidylserine vesicles, but not to the sub membranous cytoskeleton, stimulates the pp60c-src-dependent tyrosine phosphorylation of AnxA2 [76]. Dynamic remodeling of the actin cytoskeleton is required for cell spreading, motility, and migration and can be regulated by tyrosine *ts-v*-Src kinase phosphorylation of AnxA2 at Tyr²³, which plays an essential role in regulation of the cofilin-dependent actin cytoskeletal dynamics in the context of cell scattering and branching morphogenesis [77].

The Src proto-oncogene negatively regulates osteoblast activity, and, as such, its inhibition is a potential means to prevent bone loss. ALP activity and nodule mineralization were increased in primary calvaria cells and in SV40-im-

mortalized osteoblasts from Src mice, whereas expression of the bone matrix protein osteopontin (OPN) remained unchanged [78]. Deletion/reduction of Src expression not only inhibits bone resorption, but also stimulates osteoblast differentiation and bone formation, suggesting that the osteogenic cells may contribute to the development of the osteopetrotic phenotype in Src-deficient mice [78]. Dasatinib is a new dual Src/Bcr-Abl tyrosine kinase inhibitor initially developed for the treatment of chronic myeloid leukemia. It has also yielded promising results in preclinical studies in various solid tumors. Dasatinib significantly increased the activity of ALP and the level of calcium deposition in mesenchymal stem cells cultured with DAG; it upregulated the expression of bone sialoprotein (BSP) and OPN genes independently of DAG; and it markedly downregulated the expression of receptor activator of nuclear factor- κ B ligand (RANKL) gene and protein (decrease in RANKL/OPG ratio), the key factor that stimulates osteoclast differentiation and activity [79]. These data suggest a dual role for dasatinib in (i) stimulating osteoblast differentiation, which leads to a direct increase in bone formation, and (ii) downregulating RANKL synthesis by osteoblasts, which leads to an indirect inhibition of osteoclastogenesis. Thus, dasatinib is a potentially interesting candidate drug for the treatment of osteolysis through its dual effect on bone metabolism. Moreover it is also a popular clinical drug for the treatment of prostate cancer bone metastasis. Bidirectional interactions between osteoblasts and prostate cancer cells are critical in the progression of prostate cancer in bone.

It was found that dasatinib inhibits proliferation of primary mouse osteoblasts isolated from mouse calvaria and of the immortalized MC3T3-E1 cell line [80]. Dasatinib also inhibited fibroblast growth factor-2-induced osteoblast proliferation, but strongly promoted osteoblast differentiation, as reflected by stimulation of ALP activity, osteocalcin secretion and osteoblast mineralization. To determine how dasatinib blocks proliferative signaling in osteoblasts, the expression of a panel of tyrosine kinases, including Src, Lyn, Fyn, Yes and Abl, was tested. Among the Src family kinases, only Src was activated at a high level. Abl was expressed at a low level in osteoblasts. Phosphorylation of Src-Y⁴¹⁹ or Abl-Y²⁴⁵ was inhibited by dasatinib treatment. Knockdown of either Src or Abl by lenti-shRNA in osteoblasts enhanced osteoblast differentiation, suggesting that the effect of dasatinib is due to inhibition of both Src and Abl [80]. Moreover, low dasatinib concentrations used by Garcia-Gomez *et al.* [81] showed convergent bone anabolic and reduced bone resorption effects, which makes it potentially useful for the treatment of bone diseases such as osteoporosis, osteolytic bone metastasis and myeloma bone disease. It was also reported that ugonin K, a flavonoid isolated from *Helminthostachys zeylanica* (L.) Hook, induced cell differentiation and mineralization of MC3T3-E1 mouse osteoblast-like cells by increasing ALP activity and expression of BSP and OCN. The subsequent bone nodule formation was concentration-dependently inhibited by estrogen receptor antagonist ICI 182,780, suggesting that an estrogen receptor-dependent pathway was involved [82]. Moreover, ugonin K raised the level of phosphorylated c-Src and such phosphorylation was significantly attenuated by ICI 182,780 treatment. Application of c-Src specific inhibitor PP2 con-

centration-dependently repressed ugonin K-induced osteogenesis. Excepting MC3T3-E1 cells, results obtained from ALP activity assay revealed that ugonin K also stimulated osteoblastic differentiation of human MG-63 osteosarcoma cells and rat primary osteoblasts isolated from femora [82]. These results demonstrated that ugonin K stimulated osteogenesis acts through an estrogen receptor-dependent activation of a "non-classical" signaling pathway mediated by phosphorylation of c-Src, and that a transactivation potential toward estrogen receptor- α through a "classical pathway" is not precluded.

OSTEOCYTES

Mechanical stimulation of the skeleton promotes bone gain and suppresses bone loss, ultimately resulting in improved bone strength and fracture resistance. Most research into mechanotransduction (MTD) mechanisms is typically focused on understanding the signaling pathways that stimulate new bone formation in response to load. Latest data investigated that the high bone mass phenotype of mice with global deletion of either Pyk2 or Src suggests a role for these tyrosine kinases in repression of bone formation. These results indicate on Pyk2 and Src function as molecular switches that inhibit MTD in mechanically stimulated osteocyte culture experiments [83]. Once activated by oscillatory fluid shear stress (OFSS), Pyk2 and Src translocate to and accumulate in the nucleus, where they associate with methyl-CpG-binding domain protein 2 (MBD2), a protein involved in DNA methylation and the interpretation of DNA methylation patterns. OFSS-induced Cox-2 and OPN expression was enhanced in Pyk2 KO osteoblasts, while inhibition of Src enhanced OCN expression in response to OFSS. Src kinase activity increased in the nucleus of osteocytes in response to OFSS and an interaction activated between Src (Y418) and Pyk2 (Y402) increased in response to OFSS. Thus, as a mechanism to prevent an over-reaction to physical stimulation, mechanical loading may induce the formation of a Src/Pyk2/MBD2 complex in the nucleus that functions to suppress anabolic gene expression [83].

Destructive repair is the pathological feature of osteonecrosis characterized with the elevated vascular permeability and persistent bone resorption, which is associated with higher VEGF expression, activated c-Src, and vascular leakage. Latest findings provide a new insight into VEGF and c-Src mode of reaction in triggering destructive repair of osteonecrosis. Cao *et al.* [84] showed that VEGF enhanced Src bioactivity through promoting dephosphorylation of Src at Tyr⁵²⁷ and phosphorylation of Src at Tyr⁴¹⁶. Src specific pp60(c-src) siRNA significantly reduced Src expression in endothelial cells as well as in osteoclasts. VEGF destroyed the junctional integrity of endothelial cells resulting in higher endothelial permeability. However, Src blockade significantly relieved VEGF induced actin stress and inhibited caveolae and VVOs formation, meanwhile further stabilized the complex β -catenin/VE-cadherin/Flk-1 through decreasing phosphorylation of VE-cadherin, ultimately decreasing VEGF-mediating higher vascular permeability. VEGF promoted osteoclasts formation and function without affecting the adhesion activity and cytoskeleton. Blockade of Src significantly impaired cytoskeleton resulting in a lower adhe-

sion activity through down-regulation of phosphorylation of Src, Pyk2 and Cbl, ultimately inhibited osteoclasts formation and function, and finally may have great potential as an effective therapy targeting destructive repair in osteonecrosis [84].

OSTEOCLASTS

Bone-remodeling imbalance induced by decreased osteoblastogenesis and increased bone resorption by osteoclasts is known to cause skeletal diseases such as osteoporosis. Activation of osteoclasts and their acidification-dependent resorption of bone is thought to maintain proper serum calcium levels. Osteoclast dysfunction alone does not generally affect calcium homeostasis. Indeed, mice deficient in Src, show signs of osteopetrosis, but without hypocalcemia or defects in bone mineralization. Mice deficient in cholecystokinin-B receptor (Cckbr) and gastrin receptor that affects acid secretion by parietal cells, have the expected defects in gastric acidification but also secondary hyperparathyroidism, osteoporosis and modest hypocalcemia [85]. Although neither Src- nor Cckbr-deficient mice have this latter phenotype, the combined deficiency of both genes results in osteopetrorickets. The diseases mentioned above have distinct phenotypes, depending on the site or sites of defective acidification. Vascular endothelial growth factor (VEGF)-stimulated angiogenesis is critical for endochondral ossification that occurs during bone development and bone repair. Under these circumstances, VEGF production appears to be driven by low oxygen tension. Hypoxia, desferrioxamine (hypoxia mimetic), and recombinant VEGF all increased AnxA2 mRNA and protein levels in osteoblastic cells. Moreover, VEGF stimulated AnxA2 expression via a pathway involving Src and MEK kinase [86]. These data demonstrate that AnxA2 expression in osteoblasts is under the control of VEGF, which may have implications for both angiogenesis and bone mineralization under low oxygen conditions.

Targeted disruption of the *c-src* gene in mice has been demonstrated to cause osteopetrosis as a result of reduced bone resorption and therefore much interest has been focused on the role of this gene in bone cells [87]. The recognized defect in Src mice consists of an alteration in the bone resorbing function. Increased numbers of inactive osteoclasts lacking a ruffled border on the bone surface is a remarkable feature of this bone phenotype [88-90]. Impairment of osteoclastic bone resorption leads to decreased bone remodeling, which results in small size, failure in incisor eruption, thickened growth plate, poorly developed cortex, persistence of endochondral primary spongiosa with widening and extension of the trabecular bone in the distal metaphysis and diaphysis, and reduced bone marrow tissue that fills the very little remaining space of the bone cavity [87]. Osteoclast differentiation and bone resorption are also dependent on cells of the osteoblast lineage [91,92]. However, Lowe *et al.* [90] demonstrated that osteoblasts derived from *c-src* knockout mice successfully contributed to normal osteoclast differentiation and were morphologically indistinguishable from wild-type mice. This leads to the conclusion that the inherited defect concerns exclusively mature osteoclasts and is autonomous from the bone marrow microenvironment. However, a detailed molecular analysis of

osteoblast function has not been performed. Examination of the skeletal phenotype in older Src mice has indicated that bone mass continues to increase with age, suggesting a continued imbalance between bone resorption and formation [93]. Nevertheless, a high Src expression has been found in mouse osteoclasts, where it is implicated in the regulation of cell growth, migration, and survival [24]. Cytoskeletal changes in osteoclasts such as formation of actin ring is required for bone-resorbing activity. The tyrosine kinase Src is a key player in the massive cytoskeletal rearrangement taking place in osteoclasts, thereby in bone destruction. In order for Src to be activated, trafficking to the inner plasma membrane via myristoylation is of importance. Myristoleic acid inhibited RANKL-induced osteoclast formation *in vitro*, especially at later stages of differentiation [94]. Myristoleic acid attenuated tyrosine phosphorylation of c-Src and Pyk2, which associates with Src, by RANKL. When myristoleic acid was co-administered with soluble RANKL into mice, RANKL-induced bone loss was substantially prevented. Bone dissection clearly revealed that the number of multinucleated osteoclasts was significantly diminished by myristoleic acid. On the other hand, myristoleic acid treatment had little or no influence on early osteoclast differentiation markers, such as c-Fos and NFATc1, and proteins related to cytoskeletal rearrangement, including DC-STAMP, integrin α v and integrin β 3 *in vitro*. These data suggest that myristoleic acid is capable of blocking the formation of large multinucleated osteoclasts and bone resorption, likely through suppressing activation of Src and Pyk2 [94]. That is why Src inhibitors have also been studied for their potential activity in bone resorption for the therapy of osteoporosis in rodent models [28-30].

Latest data suggest that silibinin, the major active constituent of silymarin, the mixture of flavonolignans extracted from blessed milk thistle, is a powerful antioxidant and has anti-cancer effects against carcinoma cells [95]. This study demonstrated that silibinin had bone-forming and osteoprotective effects in *in vitro* cell systems. Silibinin accelerated cell proliferation and promoted matrix mineralization by enhancing bone nodule formation by calcium deposits. In addition, silibinin treatment induced activation of osteoblastogenic biomarkers such as ALP, collagen type 1, connective tissue growth factor, and bone morphogenetic protein-2 (BMP-2). Silibinin reversed the enhanced secretion of RANKL, essential for osteoclastogenesis, by differentiated murine osteoblastic MC3T3-E1 cells. It was also found that silibinin elevated activities of tartrate-resistant acid phosphatase, cathepsin K and MMP-9, through disturbing TRAF6-c-Src signaling pathways [95]. These results demonstrate that silibinin was a potential therapeutic agent promoting bone-forming osteoblastogenesis and blocking osteoclastic bone resorption.

Some data demonstrated that osteoprotegerin inhibited differentiation of osteoclasts. Osteoprotegerin induced podosome reassembly and detachment of peripheral adhesive structures. This was due to the ability of osteoprotegerin to modulate phosphorylation of Tyr⁴⁰² in Pyk2, Tyr⁴¹⁶ and Tyr⁵²⁷ in Src, and to regulate Pyk2/Src association and intracellular distribution in osteoclasts [96]. This evidence suggests that Src may function as an adaptor protein that

competes for Pyk2 and relocates it from the peripheral adhesive zone to the central region of osteoclasts in response to osteoprotegerin treatment.

INVOLVEMENT OF ROCK KINASES IN THE MINERALIZATION PROCESS

CHONDROCYTES

Coordinated proliferation and differentiation of growth plate cells is required for normal growth and development of the skeleton, but little is known about the ROCK-induced intracellular signal transduction pathways regulating these processes. RhoA and its effector kinases ROCK1/2 are responsible for increased chondrocytes proliferation and delayed hypertrophic chondrocytes differentiation, as shown by decreased induction of ALP activity, mineralization, and expression of the hypertrophic markers: collagen X, bone sialoprotein and MMP-13 [97]. In contrast, inhibition of ROCKs by Y-27632 diminishes chondrocyte proliferation, accelerates hypertrophic differentiation and rescues the effects of RhoA overexpression in chondrogenic ATDC5 cells [97]. RhoA overexpression increased the expression of ROCK-1 but inhibited the expression of collagen II, aggrecan, L-Sox5, and Sox6 in developmental dysplasia of the hip acetabulum chondrocytes compared with normal cells [98]. The effects of RhoA overexpression were rescued by the addition of Y-27632, and ROCKs inhibition resulted in an increase in all the above markers in control cells [99]. Disruption of chondrocyte proliferation and/or differentiation by gene mutations commonly results in chondrodysplasias that are characterized by skeletal deformities and reduced growth [100-102]. Mutations in genes encoding ECM molecules, growth factors, receptors, and transcription factors have been identified as causes of several chondrodysplasias. Excessive mechanical stress on the cartilage can cause degradation of the matrix leading to osteoarthritis [103]. Recent findings suggest that inhibition of Rho/ROCK pathway may serve to prevent cartilage degradation. Y-27632 counteract dedifferentiation of monolayer-cultured chondrocytes, and may be a useful reagent to maintain chondrocytic phenotypes *in vitro* for chondrocyte-based regeneration therapy [104].

OSTEOBLASTS

Cell adhesion to ECM results in clustering of integrins in focal adhesions, structures that contain multiple signaling and cytoskeletal proteins as well as are important sites of signal transduction and integration [105]. ROCKs play an important role in generating the contractile force by increasing MLC phosphorylation [2,106]. Soluble factors from serum such as lysophosphatidic acid (LPA) are thought to activate Rho since they induce actin stress fibers and focal adhesions in a Rho dependent manner [107]. Some evidence suggest that Rho is regulated by integrin-mediated cell adhesion to ECM, which in turn ECM governs osteogenic differentiation by modulating ERK activity. Furthermore, RhoA has been widely implicated in integrin-mediated signaling and mechanotransduction. Inhibition of RhoA and ROCKs in MC3T3-E1 pre-osteoblasts cultured on substrates of varying compliance reduced ERK activity, whereas constitutively

active RhoA enhanced it. Inhibition of RhoA, ROCKs, or the MAPK pathway delayed the onset of osteogenesis as shown by altered OCN and BSP gene expression, ALP activity, and mineralization [108]. These data have demonstrated that one possible mechanism by which ECM rigidity regulates osteogenic differentiation involves MAPK activation downstream of the RhoA-ROCK signaling pathway.

RhoA/ROCK signaling pathway are investigated as a novel and important regulator of mineralization process in bone tumors. An AMPK activator, 5-aminoimidazole-4-carboxamide- β -D-ribofuranoside (AICAR), and a ROCK inhibitor, fasudil hydrochloride, significantly stimulated endothelial nitric oxide synthase (eNOS), bone morphogenetic protein-2 (BMP-2), and osteocalcin mRNA expression as well as promote bone formation in osteoblastic MC3T3-E1 cells. Also, measurement of ROCKs activities revealed that both agents significantly suppressed phosphorylation of the myosin-binding subunit of MLC phosphatase [109]. These findings suggest that the AMPK activator and the ROCKs inhibitor are able to stimulate mineralization of osteoblasts through modulating the mevalonate pathway and that they could be candidates for drugs for the treatment of osteoporosis.

Mesenchymal-to-epithelial transition (MET) of osteosarcoma Saos-2 cells is induced by transcription factor Pax3 and is associated with altered expression and activity of numerous proteins involved in signal transduction pathways that regulate cytoskeleton remodeling. Actomyosin contractility induced *via* Rho/ROCK signaling is required for the formation of circumferential actin bundles, epithelial discoid cell shape and regulation of membrane protrusions in these cells [110]. Homozygous mutations of Pax3 in mice result in numerous developmental anomalies including spina bifida, loss of skeletal muscles, skeletal abnormalities and defects to numerous neural crest derived tissues [111,112]. Inhibition of RhoA/ROCK signaling reduced ERK activity, diminished runt-related transcription factor 2 activity, and altered alkaline phosphatase activity in osteoblasts [108]. During osteogenic differentiation matrix stiffness is regulated by integrin-mediated mechanotransduction and the ROCK-MAPK-ERK pathway [113]. It has recently been reported that Rac negatively regulates the TGF- β -stimulated VEGF synthesis via inhibition of MAPK in osteoblast-like MC3T3-E1 cells [114]. Additionally, Rac1, when activated by kindlin-2, is essential for adhesion, spreading and proliferation of osteoblasts [115].

Focal contacts formation and polymerization of actin at the cell periphery is regulated by Rac, whereas F-actin stress fibers creation and development of focal contacts into focal adhesions by modulating MLC phosphorylation are linked to activation of RhoA and the downstream ROCKs. ROCKs inhibitors stimulate migration of human osteoblastic cells by regulating actomyosin activity [116] and significantly increase osteoblast differentiation and biomineralization in a topography-dependent manner [117]. These pharmacological inhibitions could represent a new therapeutic way to speed bone formation around implanted metals and in regenerative medicine applications. Down-regulation of RhoA/ROCK signaling and a reduced ratio of RANKL/

OPG may be the possible molecular mechanisms causing bone cells phenotype alterations. It affects the downstream ERK-MAPK signaling causing reduced function and differentiation of bone cells, particularly osteoblasts [64]. RhoA-GTP stimulates, depending on ROCKs activity and MLC phosphorylation, the phospholipid phosphatase activity of PTEN [62]. The activated PTEN downregulates Akt activity by antagonizing the effect of the growth factor-stimulated activation of PI3K, which is essential for cell proliferation. In addition, RhoA activity seems to be related to FAK activity and upregulation of PTEN, both of which may attenuate the adhesion, migration and invasion capabilities of osteosarcoma cells [63].

Cell motility often depends on extracellular signals interacting with receptor tyrosine kinases (RTK), which trigger cytoskeleton changes. AnxA2 was found to be phosphorylated upon RTK activation. AnxA2 phosphorylation led to Rho/ROCK-dependent and actin-mediated changes in cell morphology associated with the control of cell adhesion [118]. This finding suggests that AnxA2 phosphorylation acted as a switch to transform a stationary cell to a more migratory type. The link between AnxA2 and ROCK is corroborated by the fact that: 1) AnxA2 and RhoA are co-localized in the F-actin network and AnxA2 may act upstream of the Rho/ROCK pathway by regulating F-actin remodeling [119]; 2) inhibition of the Rho/ROCK pathway restored suppression of cell motility by silencing of AnxA2 in renal cell carcinoma [120].

OSTEOCYTES

So far, there are any direct experimental evidences of ROCKs activities in osteocytes. As hypothesized, proteins which may be regulated by Rho-associated kinases in these cells are FAK and integrins [121]. Among FAK related proteins, there is proline-rich tyrosine kinase 2 (Pyk2), which induces cytoskeleton reorganization, cell detachment and apoptosis of osteocytes, and which may serve as an "off switch" to suppress the anabolic response of bone subjected to mechanical load [83].

OSTEOCLASTS

Different GTPases of the Rho family act during distinct steps of osteoclastogenesis: the fusion of mononuclear into multinuclear osteoclasts (OCs), podosome organization, cell migration and polarization, what is consistent with the emerging model that dissociates OCs differentiation and podosome dynamics from OCs adhesion [122,123]. Rac and Vav mediated lamellipodia formation and cytoskeletal reorganization [124-126], Cdc42 stimulated filopodia formation and actin fiber retraction [127], while Rho activated actin cable assembly and cell contraction, whereas the Rho inhibitor, C3 transferase, induced the loss of actin cables [127]. Normal cells had cell-to-substratum adhesion sites containing beta1 integrin, FAK, paxillin, vinculin and tyrosine phosphorylated proteins but these "focal complexes" were disassembled in cells with mutated Cdc42 or Rac [127]. Using RhoE-deficient cells, it was demonstrated that RhoE is indispensable for OCs migration and bone resorption by maintaining fast actin turnover in podosomes. Moreover,

RhoE activated cofilin, a podosome component, by inhibition of its phosphorylation by ROCKs [128]. Rac1 and Rac2 are essential for the formation of the podosome belt [129,130] and for bone resorption [138-142]. Deletion both of them in mature OCs caused osteopetrosis, an age-dependent change in osteoclast number, and a reduced number of osteoblasts *in vivo*, indicating that Rac proteins have critical role in skeletal metabolism [136]. P2X7R was involved in osteoclast differentiation, RANKL expression, MMPs and cathepsin secretion thus promoting bone resorption and osteolytic lesions [137]. Although several mechanisms which trigger the activation of Rho GTPases had been described, it is still not clear how their activation and inactivation are temporally and spatially restricted. Treatment with osteoprotegerin reduced bone resorption and caused a significant decrease in the expression of MMP-9, RhoV, ROCK1 and ROCK2 [138,139].

CROSS-TALK BETWEEN SRC KINASES AND RHO SMALL GTPASES REGULATES PHYSIOLOGICAL AND PATHOLOGICAL MINERALIZATION

The discovery of Src and ROCK family kinases and their protein substrates (Tab. 1) has been instrumental to the modern understanding of pathological mineralization as a disease where normal healthy cellular signaling has gone awry. They may be treated as vesicular transport regulators and their Ca- and nucleotide-dependent presence at the plasma membrane may be mediated by phosphorylation of annexins (AnxA2 and/or AnxA6) [77,118]. Proteins of the submembranous cytoskeletal system, which are substrates for these kinases, as F-actin is for Src, may be considered as vesicular transport platforms that control different steps of receptor transport and sorting to different cellular organelles [78,97]. Also, proteins and kinases of the cellular adhesion system such as FAK, Src, vinculin or paxillin may also be involved in the ROCK signaling pathway [116] and responsible for the association of plasma membrane integrins with the actin cytoskeleton in cell-cell as well as cell-extracellular matrix contact sites [23].

Parathyroid hormone (PTH) signaling during physiological mineralization is integrated to membrane Src kinase pathway through PTH-R and activates phospholipases (PLAses), which enzymatic activity generates LPA signaling through LPA-R to cytoplasmic ROCK1/2 kinase pathway (as depicted in Fig. 3). Dohn *et al.* [140] identified an essential role for catenin in Src-induced anchorage-independent cell growth. Src appeared to act depending on catenin at the level of RhoA suppressing the RhoA-ROCK-LIMK pathway. Oncogenic perturbation of the ROCK pathway *via* catenin-dependent mechanisms ultimately resulted in constitutive activation of the actin severing protein cofilin, whose activity was necessary but not sufficient to permit the transition to anchorage independence and tumorigenic potency. P2X7R triggered ROCK-RANKL-PI3K/AKT and VEGF pathways in osteoblasts promoting primary tumor development [137]. Regulation of membrane remodeling was also mediated in part by the regulation of Arf (ADP-ribosylation factor), GAPs (GTPase-activating proteins) acting as upstream regulators of Rho family proteins and providing a scaffold for Rho effectors and exchange factors.

Table 1. Principal substrates of Src and ROCK kinases involved in mineralization.

Kinase	Substrate	Function	Reference
Src	↑ PI3-K/ Akt	osteoblast anoikis resistance and survival VDR/RXR regulated suppression of osteoblast apoptosis and increased cell survival	[73-75]
	↑ AnxA2 phosphorylation ↑ cofilin	Ca ²⁺ -dependent binding to PM/PS vesicles actin cytoskeletal dynamics low oxygen-dependent VEGF-stimulated angiogenesis and ossification	[76,77,86]
	↑ ALP ↑ F-actin ↑ MMP-9	nodule demineralization and bone resorption RANKL synthesis, osteolysis and osteoclastogenesis	[78,79,95]
	↑ Pyk2/MBD2 ↑ RANKL ↓ BSP ↓ OCN ↓ OPN ↓ Cox-2	decreased osteoblast proliferation and osteoblastogenesis with bone nodule formation repression of bone formation by osteocytes myristoleic acid - blocking of the formation of large multinucleated osteoclasts and bone resorption	[80,82,83,94]
	↓ Cckbr	serum Ca ²⁺ - and acidification-dependent bone resorption during hyperparathyroidism, osteoporosis, osteopetrosis and hypocalcemia	[85]
	↑ VEGF ↑ Pyk2/Cbl ↓ RhoA ↓ ROCK ↓ LIMK ↑ catenin ↑ catherin ↑ cofilin	anchorage-independent cell growth and tumorigenicity	[84,147]
	ROCK	↓ ALP ↑ BSP ↑ OCN ↑ Collagen X ↑ MMP-13	increased osteoblast proliferation, delay of hypertrophic differentiation
↓ eNOS ↓ BMP-2		decreased osteoblast proliferation and mineralization	[115]
↑ Pax3 ↑ MLC phosphorylation		skeletal development, osteoblast cytoskeleton remodeling and membrane protrusions	[2,97,109,116-118]
↑ Rho A ↑ ERK ↑ Src ↑ LPA ↑ FAK ↑ vinculin ↑ paxillin		integrin-mediated cell adhesion to ECM integrin-mediated association to the actin cytoskeleton in cell-cell and cell-ECM associations creation of actin stress fibers, focal adhesions and mechanotransduction during osteogenesis	[23,105,113,122]
↑ AnxA2 phosphorylation		actin rearrangement and cell adhesion F-actin remodeling associated with the control of suppression of cell motility in carcinoma	[124-126]
↑ PLD phosphorylation		osteoblast differentiation degradation of MVs phospholipids and destabilization of minerals PTH-regulated hydrolysis of PC/PE and IL-6 production by osteoblasts during bone resorption	[8,95]
↑ MAPK ↑ FAK ↑ ERK1/2 ↑ OCN ↑ Collagen I		ECM regulated osteogenic differentiation integrin-mediated mechanotransduction during osteogenic differentiation	[114,119]

With multiple functional elements, the Arf GAPs could integrate signals and biochemical activities that resulted in coordinated changes in actin and membranes, which were necessary for a wide range of cellular functions [141]. Our earlier results showed that mineralization is decreased by phalloidin which stabilizes cytoskeleton and blocks actin filament polymerization, whereas cytochalasin D, an inhibitor of actin polymerization, was found to stimulate release of MVs [8]. Detachment of actin filaments may lead to reduction in membrane tension, indispensable for MV formation and release from microvilli which is driven by the action of

actin-severing proteins (gelsolin, cofilin) and contractile motor proteins (myosins) [9]. In addition to the already known proteins involved in MV-mediated mineralization, new proteins that may regulate PP_i and P_i homeostasis (inorganic pyrophosphatase 1), Ca²⁺ ion homeostasis (voltage-dependent Ca²⁺ channel and sorcin), intravesicular pH (vacuolar H⁺-ATPase and SLC4A7, a sodium bicarbonate cotransporter) or lipid composition of MVs membrane (sphingomyelin phosphodiesterase 3) were identified providing additional insights into MVs functions [9].

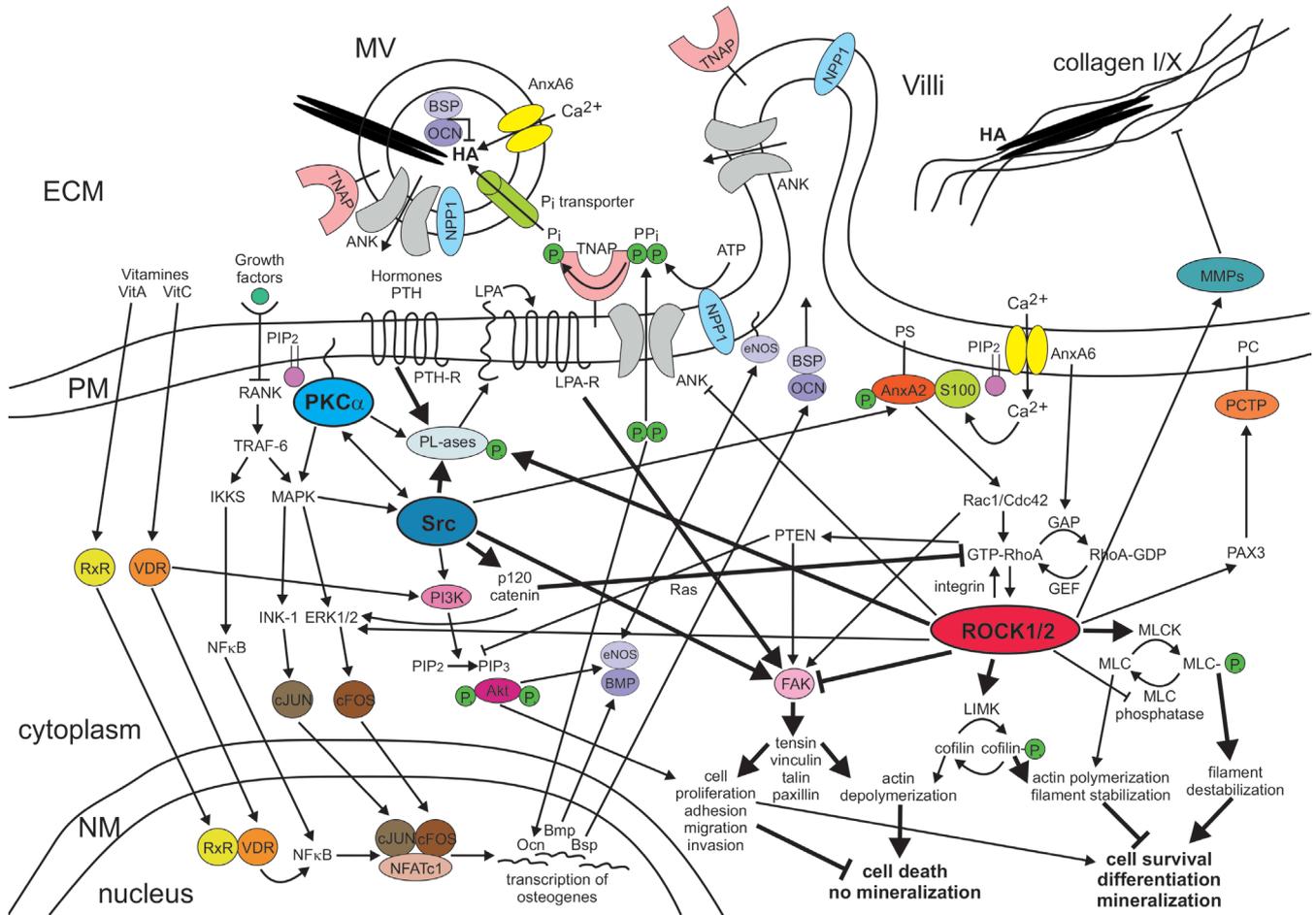


Figure 3. Scheme outlining functions of Src and ROCK kinases in the process of physiological mineralization. Parathyroid hormone (PTH) signaling is integrated to membrane Src kinase pathway through PTH-R and activates phospholipases (PLAses) the enzymatic activity of which generates lysophosphatidic acid (LPA) signaling through LPA-R to cytoplasmic ROCK1/2 kinase pathway. Src appears to act at the level of RhoA depending on p120 catenin for its activity and suppresses the RhoA-ROCK-LIMK pathway moving the force to the RhoA-ROCK-MLCK pathway. Growth factors and ROCK-RANK-PI3K/AKT pathways are induced in osteoblasts and promote bone tumor development. On the other hand, the RhoA-ROCK pathway stimulates the phospholipid phosphatase activity of PTEN, a human tumor suppressor protein and induces cellular focal adhesion. Src and ROCKs may also directly regulate FAK by an opposite action i.e., by regulating cell survival and mineralization. Detachment of actin filaments leads to reduction in membrane tension, indispensable for MV formation. MV release from microvilli is driven by actions of phosphorylated actin-severing proteins (e.g., cofilin) and contractile motor proteins (e.g. MLC) followed by cell differentiation and mineralization.

IMAGING OF THE MINERALIZATION PROCESS

Bone grafts are the most common transplants after the blood transfusions. This makes the bone tissue regeneration research of pressing scientific and social impact. Although contrast opacification of joint cavities (arthrography), tendon sheaths (tenography) and bursa (bursography) has been used less frequently in recent years, owing to increased reliance on Computed Tomography (CT) and Magnetic Resonance (MR) imaging, such diagnostic techniques still has important clinical applications. Because alternative techniques such as CT and MR imaging have influenced dramatically the manner in which Ultrasonography (US) as well as Radionuclide or power Doppler techniques are used, the benefits of US – which include lower cost, less time, less patient discomfort – ensure its unique place in clinical imaging. In its relative short existence, MR imaging has emerged as the most powerful but still most expensive imaging technique for the noninvasive diagnosis of a host of traumatic, degenerative, rheumatologic, infections and neoplastic conditions in the musculoskeletal system. But in case of mineralizing tumors the needle biopsy of bone or soft tissue is still irreplaceable diagnostic technique [142].

Bone is a complex hierarchical structure, where the interplay of organic and inorganic mineral phases at different length scale (from micron to atomic scale) affect its functionality and health. Thus, the understanding of bone tissue regeneration requires to image its spatial-temporal evolution (i) with high spatial resolution and (ii) at different length scale. Recently Italian scientists investigated the dynamics of collagen packing during *ex-vivo* mineralization of ceramic porous HA implant scaffolds using synchrotron high resolution X-ray phase contrast micro-tomography (XPC μ T) and synchrotron scanning micro X-ray diffraction (S μ XRD). While XPC μ T provided the direct 3D image of the collagen fibers network organization with micrometer spatial resolution, S μ XRD allowed to probe the structural statistical fluctuations of the collagen fibrils at nanoscale [143]. In particular they imaged the lateral spacing and orientation of collagen fibrils during the anisotropic growth of mineral nanocrystals. Beyond throwing light on the bone regeneration multiscale process, this approach can provide important information in the characterization of tissue in health, aging and degeneration conditions.

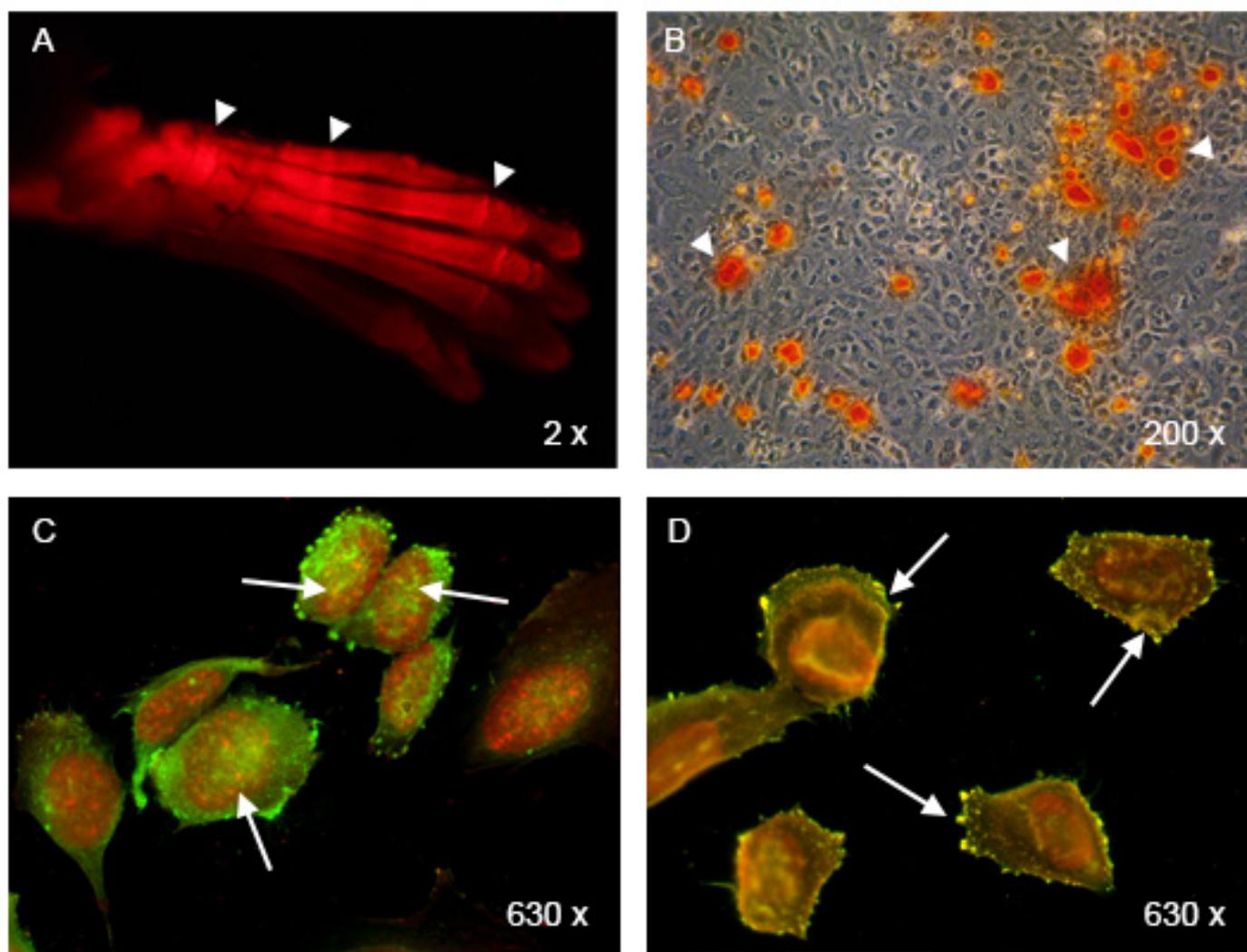


Figure 4. Mineralization of rat leg bone (A) and human Saos-2 osteosarcoma cells (B, C, D). A – Organ was cleared by PARTY method and stained with AR-S. Macrophotography was performed under 2 x magnification using a digital SLR camera (Nikon D7100), a lens with a focal length of 50 mm (AF-S NIKKOR 50 mm f/1.8 G) with extension tubes and excitation with 532 nm laser from home made macroscope. Image processing was carried out in Fiji software. B, C, D – The cell cultures were stimulated for mineralization by 7 days with AA and β -GP. Then probes were stained with AR-S and visualized under 200 x magnification of light microscope (Zeiss Axiovert 40C) with phase contrast and RGB filters (B) or fixed with 3% paraformaldehyde, stained with AnxA6-FITC (C, D, green color) together with Src-TRITC (C, red color) or ROCK-TRITC (D, red color) antibodies and visualized under 630 x magnification of fluorescent microscope (Zeiss Axio Observer.Z1) with DIC contrast and appropriate fluorescent filters. Image processing was carried out in AxioVision software. Red collar visible on low magnification images (A, B) indicates on calcium-phosphate minerals deposition (arrowheads). Yellow collar visible on high magnification merged images (C, D) indicates on proteins (AnxA6-kinases) colocalization (arrows).

Many of the mechanisms driving biomineralization remain unclear because the traditional biochemical assays used to investigate them are destructive techniques incompatible with viable cells. To determine the temporal changes in mineralization-related biomolecules at mineralization spots, time-lapse Raman imaging of mouse osteoblasts at a subcellular resolution throughout the mineralization process was performed due to Japan and China collaboration [144]. Raman imaging enabled to analyze the dynamics of the related biomolecules at mineralization spots throughout the entire process of mineralization. The HA and cytochrome c Raman bands were used as markers for osteoblastic mineralization and apoptosis, respectively, while β -carotene acted as a biomarker that indicated the initiation of mineral formation. They compared Raman images with Alizarin Red S (AR-S) staining assay, which is a conventional method to evaluate the mineral quality and quanti-

ty. Raman imaging of the KUSA-A1 culture visualized the mineralized spots more accurately than AR-S staining assay [145]. Raman imaging of HA serves as a powerful tool to identify the mineralized spots in an *in vitro* culture of osteogenic lineage cells. Mineral nodules formation in cultured human osteosarcoma cells after 7 days of stimulation for mineralization may be imaged not only by AR-S staining but also by von Kossa silver nitrate or lactate staining [146]. Both of mentioned above methods detect calcium and phosphate crystal deposits but only van Kossa reaction increases sensitivity and reduces background of received images.

Matrix vesicles are the initial sites of HA mineral formation. To evaluate the integrity of MVs, they were isolated from mineralizing Saos-2 cell cultures and examined under 50000 x magnification using transmission electron microscopy (Jeol JEM-1200EX TEM) [8,9]. MVs were recognized as spherical vesicle structures with a diameter ranging from 100

to 400 nm, delimited by a characteristic trilaminar membrane. In some cases, it was possible to observe needle-like electron-dense mineral deposits inside the MVs, showing their ability to mineralize. Moreover, after 6 h of incubation at 37°C in a mineralization buffer containing 2 mM Ca²⁺ and 3.42 mM P_i, MVs were able to form minerals. The formed minerals were collected, washed and analyzed by Fourier transform infrared (FTIR) spectroscopy [8,9]. It was proved that infrared spectrum of the mineral deposit formed by MVs of Saos-2 cells was identical to the spectrum of the HA standard.

The mineralization process may be also imaged using different macro- and microscopy techniques (Fig. 4). Rat leg was cleared by Perfusion-Assisted Rodent whole-body (PARTY) method (Matryba *et al.*, unpublished). Then cleared organ was stained with 0.003% Alizarin Red-S (AR-S). Macrophotography was performed under 2 x magnification using a digital SLR camera (Nikon D7100), a lens with a focal length of 50 mm (AF-S NIKKOR 50 mm f/1.8 G) with extension tubes and excitation with 532 nm laser from home made microscope. Image processing was carried out in Fiji software (Fig. 4A). Cultures of Saos-2 osteosarcoma cells were stimulated for mineralization by 7 days with 50 µg/ml ascorbic acid (AA) and 7.5 mM β-glycerophosphate (β-GP) [8,9]. Then probes were stained with 0.5% AR-S and visualized under 200 x magnification of light microscope (Zeiss Axiovert 40C) with phase contrast and RGB filters (Figure 4B) or fixed with 3% paraformaldehyde, stained with AnxA6-FITC (Fig. 4C, D) together with Src-TRITC (Fig. 4C) or ROCK-TRITC (Fig. 4D) antibodies and visualized under 630 x magnification of fluorescent microscope (Zeiss Axio Observer.Z1) with DIC contrast and appropriate fluorescent filters. Image processing was carried out in AxioVision software (Fig. 4B, C, D). Red collar visible on low magnification images (Fig. 4A, B) indicates on calcium-phosphate minerals deposition and increased mineralization process in bone epiphyses or in ECM surrounding osteoblast-like cells, respectively (arrowheads). Yellow collar visible on high magnification merged images (Fig. 4C, D) indicates on vesicular colocalization of AnxA6 with Src kinases in perinuclear region full of intracellularly formed MVs, whereas with ROCK kinases in plasma membrane region enriched with releasing MVs (arrows). We expect macro- and microscopy imaging techniques to help us to further elucidate mineralization mechanisms regulated by Src and ROCK kinases that have previously been unobservable.

CONCLUDING REMARKS

Biom mineralization is a complex process resulting in the deposition of mineral crystals in the extracellular matrix of various tissues. Because ectopic calcification taking place in many soft tissues such as articular cartilage, cardiovascular tissues, kidney, ligaments, and tendons is a process that shares many similarities with physiological formation of bones, teeth and cartilage [147] therefore, the understanding of mechanisms of the cross-talk between Src kinases and Rho small GTPases that regulate the mineralization process is crucial for the development of novel simply imaging techniques and therapeutic strategies in relation to pathological mineralization.

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Oddziaływanie pomiędzy kinazami Src a małymi GTPazami Rho reguluje biomineralizację i ułatwia obrazowanie procesu mineralizacji

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Słowa kluczowe: mineralizacja, kinaza Src, ROCK, przekazywanie sygnału, pęcherzyki macierzy pozakomórkowej, obrazowanie

STRESZCZENIE

Mineralizacja jest wieloetapowym procesem, który podlega ścisłej regulacji i prowadzi do utworzenia prawidłowego szkieletu kostnego. Rozregulowanie tego procesu, związane ze starzeniem organizmu, prowadzi do patologicznej mineralizacji, która może pojawić się nie tylko w układzie szkieletowym, ale także w tkankach miękkich. Wyniki najnowszych badań w dziedzinie biologii kości wskazują na udział rodziny kinaz tyrozynowych Src i rodziny małych GTPaz Rho w tworzeniu minerału. Oddziaływanie pomiędzy tymi dwoma szlakami przekazywania sygnału jest aktywowane we wczesnych etapach mineralizacji i prowadzi do przemodelowania szkieletu komórek kompetentnych w procesie mineralizacji oraz uwolnienia pęcherzyków macierzy pozakomórkowej. Zrozumienie mechanizmów oddziaływania pomiędzy kinazami Src a małymi GTPazami Rho, które reguluje proces mineralizacji, jest istotne dla rozwoju nowych łatwych technik obrazowania i podejść terapeutycznych wobec patologicznej mineralizacji.