c-Abl-dependent Molecular Circuitry Involving Smad5 and Phosphatidylinositol-3-Kinase Regulates Bone Morphogenetic Protein-2-induced Osteogenesis*

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Background: BMP-2-induced signal transduction requires receptor-specific Smads for osteogenesis.
Results: c-Abl regulates BMP-2-induced canonical Smad and noncanonical PI 3-kinase in osteoblasts.
Conclusion: BMP-2-stimulated c-Abl tyrosine kinase potentiates osteoblast and osteoclast differentiation.
Significance: c-Abl is essential for bone remodeling.

Skeletal remodeling consists of timely formation and resorption of bone by osteoblasts and osteoclasts in a quantitative manner. Patients with chronic myeloid leukemia receiving inhibitors of c-Abl tyrosine kinase often show reduced bone remodeling due to impaired osteoblast and osteoclast function. BMP-2 plays a significant role in bone generation and resorption by contributing to the formation of mature osteoblasts and osteoclasts. The effects of c-Abl on BMP-2-induced bone remodeling and the underlying mechanisms are not well studied. Using a pharmacological inhibitor and expression of a dominant negative mutant of c-Abl, we show an essential role of this tyrosine kinase in the development of bone nodules containing mature osteoblasts and formation of multinucleated osteoclasts in response to BMP-2. Calvarial osteoblasts prepared from c-Abl null mice showed the absolute requirement of this tyrosine kinase in maturation of osteoblasts and osteoclasts. Activation of phosphatidylinositol kinase PI 3-kinase/Akt by BMP-2 leads to osteoblast differentiation. Remarkably, inhibition of c-Abl significantly suppressed BMP-2-stimulated PI 3-kinase activity and its downstream Akt phosphorylation. Interestingly, c-Abl regulated BMP-2-induced osteoclastogenic CSF-1 expression. More importantly, we identified the requirements of c-Abl in BMP-2 autoregulation and the expressions of alkaline phosphatase and osteonectin that are necessary for osteoblast differentiation. c-Abl contributed to BMP receptor-specific Smad-dependent transcription of CSF-1, osteonectin, and BMP-2. Finally, c-Abl associates with BMP receptor IA and regulates phosphorylation of Smad in response to BMP-2. We propose that activation of c-Abl is an important step, which induces two signaling pathways involving noncanonical PI 3-kinase and canonical Smads to integrate BMP-2-induced osteogenesis.

Balanced activities of two principal cell populations, osteoblasts and osteoclasts with other cells in the bone matrix, contribute to bone remodeling. Many protein factors, including bone morphogenetic protein-2 (BMP-2), stimulate mesenchymal cells to differentiate into osteoblast precursors and induce maturation of the osteoblasts via expression of osteoblastic gene markers (1, 2). Dimeric BMP-2 contains two receptor binding domains. Wrist epitope binds with high affinity to BMP receptor I and the low affinity kunkel epitope binds receptor II (3–6). Binding of BMP-2 to type I receptor is affected by type II receptor and induces the formation of receptor oligomer (7, 8). In this complex, the constitutively active receptor II transphosphorylates the type I receptor at the GS domain located upstream of the serine-threonine kinase domain, resulting in an increase in kinase activity of the type I receptor, which determines the specificity of the intracellular signaling by BMP-2 (9). Thus BMP receptor I acts as a downstream effector of type II receptor and phosphorylates Smads 1/5/8 (R-Smads) in their C-terminal SXS motif leading to their activation. R-Smads contain MH1 and MH2 domains joined by a linker region (10). MH1 binds to the MH2 domain in the absence of receptor activation and ensures inhibition of receptor function. Phosphorylation of R-Smads releases this interaction resulting in oligomerization with Smad4 and translocation to the nucleus. In the nucleus, the MH-1 domain binds to specific DNA sequences and recruits other nuclear proteins to stimulate or repress gene transcription (10, 11). In addition to the activation of Smads, BMP-2 activates non-Smad signaling pathways, including the MAPK family: p38, ERK1/2, and JNK (9, 12, 13).

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In fact, BMP-2-stimulated p38 occurs via activation of TAK1/TAB1 (14, 15). Both TAK1 and Smad5 null mice show similar phenotypes, indicating non-Smad signaling may contribute to BMP signal transduction (16). In fact, activation of p38 MAPK and JNK induces osteoblast differentiation (17).

Role of receptor and nonreceptor tyrosine kinases in osteoblast differentiation has been reported (2). Two nonreceptor tyrosine kinases Abl1 (c-Abl) and Abl-related gene (Arg) contribute to normal development and regulate cell cycle, cell survival, actin cytoskeleton, oxidative stress, and DNA damage response (18, 19). Although c-Abl is activated by TGFβ in rodent models of fibrosis, it antagonizes the epithelial mesenchymal transition and cytostatic and oncogenic actions of TGFβ (20–23). c-Abl-deficient mice exhibit low viability and defective hematopoiesis, cardiac hyperplasia, and decreased systolic blood pressure (24–27). c-Abl has an SH3-SH2-tyrosine kinase domain cassette. C-terminal to this segment, it contains a DNA binding domain. A long C-terminal stretch contains proline-rich motifs, which interact with SH3 domains of Crk, Grb2, and Nck. Also, binding sites for p53, Rb, ATM, and RNA polymerase II are also present in this segment (19). Nuclear localization and export signals have been identified in c-Abl, which confer upon its localization in the nucleus and cytoplasm (28–30). Tyrosine phosphorylation of this enzyme regulates its catalytic activity, subcellular localization of its substrates, and SH2 domain binding (31).

A recent study demonstrated no difference in number or rate of proliferation of osteoblasts from c-Abl null mice. Rather, these cells underwent senescence (32). Another study in rats treated with the c-Abl inhibitor STI 571 (imatinib, Gleevec) showed significant effects on both osteoclasts and osteoblasts in the same bone leading to dysregulated bone remodeling (33). Similarly, c-Abl inhibition in children with chronic myeloid leukemia is associated with growth retardation (34). The mechanism by which c-Abl affects bone remodeling is not known. We have shown recently that BMP-2 stimulates osteogenesis by promoting differentiation/maturation of osteoblasts, which in turn contribute to osteoclastogenesis via increased expression of CSF-1 (35–37). We implicated requirement of tyrosine kinase(s) in BMP-2-induced signal transduction during osteoblast differentiation (37). In this report, we show an essential role of c-Abl tyrosine kinase in BMP-2-stimulated osteoblast differentiation and osteoclastogenesis. We demonstrate that c-Abl is required for the expression of osteoblastic and osteoclastic genes in response to BMP-2. Finally, we establish the existence of a cross-talk between BMP-specific Smad and c-Abl.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant BMP-2 was obtained from Wyeth Pharmaceuticals, Cambridge, MA. Tissue culture reagents were purchased from Invitrogen. c-Abl inhibitor STI 571 (imatinib, Gleevec) was obtained from Selleckchem (Houston, TX). FuGENE HD was obtained from Roche Diagnostics. Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phospho-c-Abl (Tyr-412), phospho-c-Abl (Tyr-245), c-Abl, phospho-Akt (Ser-473), Akt, phospho-Smad1/5, and GST-Crk were purchased from Cell Signaling (Boston). Smad1/5 antibody was obtained from Santa Cruz Biotechnology. Osterix antibody was purchased from Abcam (Cambridge, MA). β-Actin antibody, acid phosphatase kit, and Fast Garnet dye for tartrate-resistant alkaline phosphatase (TRAP) activity and Alizarin Red S were obtained from Sigma. TRIZol reagent for RNA isolation was purchased from Invitrogen. Luciferase reporter assay kit was purchased from Promega (Madison, WI). ELISA kit to detect CSF-1 was obtained from R & D Systems (Minneapolis, MN). Polyvinylidene membrane for Western blotting was purchased from PerkinElmer Life Sciences. Kinase-dead c-Abl KR (pSRaMSVc-Abl(K290)R-tKNeo) expression plasmid was provided by Dr. Charles Sawyer, UCLA (38). Constitutively active Smad5 containing S463D/S465D mutation was a kind gift from Dr. David Kimelman, University of Washington. BMP-2 promoter-driven luciferase (BMP-2-Luc), CSF-1 promoter luciferase (CSF-1-Luc), Osterix promoter-driven luciferase (Osx-Luc) reporter plasmids were described previously (35, 37, 39, 40).

**Cell Culture and Adenovirus Infection**—Mouse calvarial 2T3 osteoblast cells are responsive to BMP-2 and undergo bone matrix formation in vitro. These cells have been characterized extensively (36, 37, 41–45). These cells were grown in α-MEM in the presence of 10% fetal bovine serum. The cells were infected with adenovirus vector expressing dominant negative c-Abl or green fluorescence protein (control infection) essentially as described (35, 37, 40, 46). Mouse primary calvarial osteoblasts were prepared by sequential digestion with trypsin and collagenase as described (36, 47). The c-Abl inhibitor STI 571 was dissolved in sterile PBS. Genistein was dissolved in DMSO. The cells were pretreated with the indicated concentration of inhibitors. Control cells received the vehicle.

**Coculture Assay**—Cocultures of 2T3 and mouse spleen cells were done as described previously (35, 39). In brief, 2T3 cells were cultured for 24 h followed by plating 10⁶ mouse spleen cells in the presence of 10⁻⁸ M 1,25-dihydroxyvitamin D₃, 10⁻⁷ M dexamethasone, and 300 ng/ml recombinant BMP-2 or bovine serum albumin (vehicle control). Medium was replaced every 2 days. At 6 days adherent cells were fixed in 10% formalin for 5 min and treated with a 1:1 mixture of ethanol and acetone for 1 min. The cultures were then dried and stained for TRAP activity using the acid phosphatase kit and Fast Garnet dye (Sigma). TRAP-positive multinucleated cells with three or more nuclei were photomicrographed using a light microscope.

**Bone Marrow Assay for Mature Osteoclast Formation**—Bone marrow cells were flushed from mice femur and tibia using α-MEM. 1 × 10⁶ nonadherent cells were cultured in α-MEM containing 10% serum in 24-well tissue culture plates in the presence of 10⁻⁸ M 1,25-dihydroxyvitamin D₃. Growth medium was replenished every 48 h for 7 days. TRAP staining was performed as described above. For TRAP activity assay, cells were fixed with 37% formaldehyde for 30 s, followed by washing with PBS. TRAP activity was measured at 405 nm using TRAP assay buffer, pH 4.7–5.0, containing sodium acetate, tartaric acid, glacial acetic acid, and phosphatase substrate (Sigma).

**Immunoblotting, Immunoprecipitation, and PI 3-Kinase Assay**—Cells were washed twice with PBS. The cells were lysed in RIPA buffer (20 mm Tris–HCl, pH 7.5, 5 mm EDTA, 150 mm NaCl, 1% Nonidet P-40, 1 mm Na₃VO₄, 1 mm PMSF, and 0.1% protease inhibitor mixture) for 30 min at 4 °C. The cell extracts
were centrifuged at 10,000 × g for 30 min at 4 °C. Protein was separated in the supernatant. Equal amounts of cell lysates were separated by SDS-PAGE. The separated proteins were transferred to PVDF membrane and immunoblotted using indicated antibodies. The protein bands were developed using HRP-conjugated secondary antibodies with ECL chemiluminescent reagent as described previously (35, 37, 39, 40, 45, 46, 48, 49). For PI 3-kinase activity, equal amounts of cell lysates were immunoprecipitated with anti-phosphotyrosine antibody. The immunoprecipitates were assayed for PI 3-kinase activity using phosphatidylinositol as a substrate in the presence of [γ-32P]ATP. The reaction products were separated by thin layer chromatography to detect PI 3-phosphate as described (37, 49).

c-Abl Immunocomplex Kinase Assay—The cell lysates were immunoprecipitated with c-Abl antibody as described (50). The immunoprecipitates were incubated with 1 μg of GST-Crk in the presence of 20 μCi of [γ-32P]ATP in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MnCl2). The labeled protein was then separated by SDS-PAGE, dried on a filter paper, and autoradiographed.

Alkaline Phosphatase Staining and Assay—Fixed cells in 10% formalin were stained using 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium as described previously (37). The stained structures were photomicrographed (×200). Lysates of cells were assayed for alkaline phosphatase activity using p-nitrophenyl phosphate as substrate as described (37, 46, 47).

Mineralized Bone Nodule Formation—Cells were grown to confluency in 24-well plates. Growth medium was supplemented with differentiation medium containing serum-free α-MEM, 100 mg/ml ascorbic acid, 5 mM β-glycerophosphate, and 7% FBS before addition of BMP-2. The cells were cultured with a fresh medium change every 2 days for 10 more days. The cells were washed with PBS and fixed in ice-cold ethanol (70%) for 1 h at 4 °C. The cells were then washed with water and stained for 5 min with 2% solution of Alizarin Red S, pH 4.0, for calcium detection. Unbound stains were removed by washing with water (45). Plates were dried and photographed. The stains were extracted in DMSO, and absorbance was determined at 590 nm for quantification. For von Kossa (for mineral) and Van Gieson (for collagen) staining, the cells were fixed with 10% formalin, ethanol-washed, and air-dried before staining with 5% silver nitrate followed by 5% sodium thiocyanate and counterstaining with 1% acid fuchsin in picric acid (Sigma) (36).

RNA Isolation and Real Time Quantitative RT-PCR—Total RNA was prepared from cells using TRIzol reagent as described previously (39, 45, 46). 2 μg of total RNA was used to make cDNA, which was subsequently used for amplification by quantitative PCR using the ABI Prism 7300 sequence detection system and analyzed by SDS 2.1 software and SYBR Green probe method (Applied Biosystems). The primer sequences and PCR conditions for BMP-2 and CSF-1 were described previously (39, 45). The PCR condition for osteon was 35 cycles (95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s). The primer sequences for Oss were as follows: forward, TGGAGAAGAAGGCCCCATTCCAC; reverse, ACTTCTTCTCCCGGCTTGT.

Transfection and Luciferase Assay—Cells were transfected with the indicated plasmids using FuGENE HD reagent, and luciferase activity was determined using luciferase assay kit as per the vendor’s instructions. The luciferase activity was quantified as described previously (35, 40, 45, 48).

Statistics—The significance of the data was determined by analysis of variance followed by Student-Newman-Keuls analysis as described (35, 39, 40, 45, 46, 48).

RESULTS

BMP-2-stimulated c-Abl Tyrosine Kinase Activity Is Required for Osteoclastogenesis—To systematically investigate a role for tyrosine kinase in BMP-2-induced signal transduction, we incubated 2T3 preosteoblasts with BMP-2. As expected, BMP-2 increased tyrosine phosphorylation of a panel of proteins (Fig. 1A). The broad spectrum tyrosine kinase inhibitor genistein inhibited BMP-2-stimulated tyrosine phosphorylation (Fig. 1A). One of the BMP-2-stimulated tyrosine-phosphorylated proteins migrated at 120 kDa (Fig. 1A, indicated by arrow). We hypothesized that this protein may represent c-Abl tyrosine kinase. One of the best substrates of c-Abl is the SH3 domain containing adaptor protein Crk, which binds to the PXXP motif of c-Abl (51). To assess directly the c-Abl tyrosine kinase activity, we used recombinant Crk protein as substrate in vitro. Immunocomplex kinase assay of c-Abl immunoprecipitates from BMP-2-stimulated cells showed increase in tyrosine kinase activity (Fig. 1B). STI 571, a c-Abl inhibitor, abolished BMP-2-stimulated phosphorylation of Crk (Fig. 1B). Auto-phosphorylation (Tyr-245) of c-Abl in the linker region between SH2 and kinase domains and in the activation loop of the kinase domain (Tyr-412) is associated with its increased kinase activity (52, 53). Therefore, we tested phosphorylation of these two sites in c-Abl. BMP-2 increased phosphorylation of c-Abl at Tyr-412 and Tyr-245 (Fig. 1C), which was significantly inhibited by STI 571 (Fig. 1C).

We have recently shown that BMP-2 stimulates osteoblast-aided osteoclastogenesis from mouse spleenocytes (39). We investigated the role of c-Abl in this process. As expected, BMP-2 enhanced the formation of multinucleated osteoclasts in this coculture assay (Fig. 1D). STI 571 significantly attenuated BMP-2-induced osteoclastogenesis (Fig. 1D, compare panel d with b). To confirm these results, we employed an adenovirus vector expressing dominant negative c-Abl. Expression of this kinase-dead c-Abl markedly decreased BMP-2-stimulated osteoclast formation (Fig. 1E). To further evaluate the contribution of c-Abl in osteoclastogenesis, we used calvarial osteoblasts as the feeder layer from c-Abl+/− mice in coculture assay. Calvarial osteoblasts from wild type mice showed weak basal multinucleated osteoclast formation as compared with that from c-Abl null mouse (Fig. 1F, compare panel c with a). BMP-2 significantly increased the formation of mature multinucleated osteoclasts in wild type mice. However, c-Abl−/− osteoblasts showed significant impairment in supporting osteoclast formation in response to BMP-2 (Fig. 1F, compare panel d with b). These results suggest that BMP-2-stimulated c-Abl tyrosine kinase regulates osteoblast-aided osteoclast formation/maturatation. To address whether c-Abl regulates osteoclastogenesis in normal bone marrow-derived cells, we examined the effect of STI 571 on osteoclast differentiation using mouse bone marrow culture in the presence of BMP-2. As shown in Fig. 1G, BMP-2 significantly increased
osteoclast formation. STI 571 inhibited BMP-2-induced osteoclastogenesis from normal bone marrow-derived cells (Fig. 1G, compare panel d with b). Similarly, STI 571 significantly inhibited BMP-2-stimulated TRAP enzyme activity in mouse bone marrow-derived cultures (Fig. 1H). To confirm this observation, we used adenovirus vector expressing dominant negative c-Abl to infect normal mouse bone marrow culture. Similar to STI 571, dominant negative c-Abl attenuated osteoclast differentiation and TRAP enzyme activity from normal bone marrow-derived cells (Fig. 1I, compare panel d with b, and f). Furthermore, bone marrow cells prepared from c-Abl null mice showed reduced osteoclast differentiation and TRAP activity (Fig. 1, K and L). These data indicate a significant role of c-Abl in osteoclast differentiation.
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**FIGURE 1. BMP-2-induced c-Abl tyrosine kinase activity is required for osteoclast formation.** A, genistein blocks BMP-2-induced tyrosine phosphorylation. 2T3 preosteoblasts were treated with 10 μM genistein prior to incubation with 100 ng/ml BMP-2 for 5 min. The cell lysates were immunoblotted with anti-phosphotyrosine antibody. Arrow indicates the position of a 120-kDa protein. B, 2T3 cells were incubated with 25 μM STI 571 followed by treatment with 100 ng/ml BMP-2 for the indicated periods of time. The cell lysates were immunoprecipitated with c-Abl antibody. The immunoprecipitates were used in an immunocomplex kinase assay using GST-Crk fusion protein as a substrate in the presence of [γ-32P]ATP as described under “Experimental Procedures” (50). Bottom panel shows immunoblotting of the cell lysates with c-Abl antibody. Con, control. C, 2T3 cells were treated with 25 μM STI 571 and incubated with BMP-2 for 10 min. The cell lysates were immunoblotted with antibodies against phospho-c-Abl (Y1412), phospho-c-Abl (Y245), and c-Abl antibodies. D, 2T3 cells were left untreated or treated with 25 μM STI 571 and cultured with mouse spleen cells in the absence and presence of 100 ng/ml BMP-2. At 7 days, the cells were fixed and stained for TRAP-positive multinucleated osteoclasts as described under “Experimental Procedures” (39). The bottom part of E shows quantification of multinucleated (MNC) TRAP-positive cells. **p < 0.001 versus BMP-2-stimulated. F, 2T3 cells were treated with Ad ΔN c-Abl or Ad GFP. The infected cells were incubated with mouse spleen cells in the presence of 100 ng/ml BMP-2. At 7 days, the cells were fixed and stained for TRAP-positive multinucleated osteoclasts as described under “Experimental Procedures.” The cells were treated with STI 571 followed by incubation with BMP-2. TRAP-positive multinucleated cells were detected as described above. F, TRAP activity was determined as described under “Experimental Procedures.” Mean ± S.E. of triplicate wells is shown. **p < 0.001 versus BMP-2-stimulated. **p < 0.001 versus BMP-2-stimulated. / and J, cultured mouse bone marrow cells were infected with Ad ΔN c-Abl or Ad GFP as described under “Experimental Procedures.” The cells were treated with STI 571 followed by incubation with BMP-2. TRAP-positive multinucleated cells were detected as described above. F, TRAP activity was determined as described under “Experimental Procedures.” Mean ± S.E. of triplicate wells is shown. **p < 0.001 versus BMP-2-stimulated. K, bone marrow-derived cells from wild type (WT) and c-Abl null mice were cultured for 7 days. TRAP-positive multinucleated cells were detected as described above. L, TRAP activity was determined as described under “Experimental Procedures.” Mean ± S.E. of triplicate wells is shown. **p < 0.001 versus wild type.
expression of CSF-1 mRNA (Fig. 4D). Similarly, expression of kinase-dead c-Abl blocked CSF-1 mRNA expression in response to BMP-2 (Fig. 4E). We have recently shown that BMP-2 increases CSF-1 expression by a transcriptional mechanism using a reporter plasmid in which the CSF-1 promoter drives the luciferase gene (39). BMP-2 increased reporter activity in 2T3 cells (Fig. 4, F and G). Inhibition of c-Abl either by STI 571 or by expression of dominant negative c-Abl significantly suppressed BMP-2-stimulated reporter activity (Fig. 4, F and G). These results suggest that c-Abl regulates osteoclastogenic CSF-1 expression by regulating its transcription.

Differentiation of osteoblasts is controlled by several transcription factors, including Osx (57). We and others have shown previously that Osx is a BMP-2-inducible gene (40, 57).
To gain more insight for the role of c-Abl in osteoblast differentiation, we tested the effect of STI 571 on the abundance of Osx protein in 2T3 preosteoblasts. Inhibition of c-Abl attenuated BMP-2-stimulated Osx protein levels (Fig. 5A). Expression of dominant negative c-Abl also inhibited Osx protein expression by BMP-2 (Fig. 5B). Both STI 571 as well as kinase-dead c-Abl blocked BMP-2-induced expression of Osx mRNA, indicating a possible transcriptional regulation of this gene (Fig. 5, C and D). Therefore, we determined transcription of Osx using a reporter construct in which luciferase gene is driven by the Osx promoter (40). As expected, BMP-2 increased transcription of Osx. Both STI 571 and dominant negative c-Abl significantly abrogated BMP-2-stimulated Osx transcription (Fig. 5, E and F).

Autoregulation of BMP-2 is a prominent process during BMP-2-induced osteoblast differentiation (36). We have previously shown that BMP-2 increased expression of its own mRNA and protein (44, 45). Both STI 571 and dominant negative c-Abl blocked the expression of BMP-2 protein and mRNA (Fig. 6, A–D). Furthermore, using a BMP-2 promoter-driven reporter plasmid, we showed that inhibition of c-Abl by STI 571 or expression of kinase-dead c-Abl significantly abrogated BMP-2-stimulated transcription of Osx (Fig. 6, E and F). These results together demonstrate a significant role of c-Abl in the expression of osteoclastogenic and osteoblastic genes during osteoblast differentiation.

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c-Abl Regulates BMP Receptor-specific Smad-dependent Transcription of Target Genes—BMP-2 uses its receptor-specific Smads to induce its biological activities. Smad5 plays a significant role in induction of osteoblastic genes that are necessary for osteoclastogenesis and osteoblast differentiation (39, 40). We showed that BMP-2 increased osteoclastogenic CSF-1 expression by Smad5-dependent transcriptional mechanism (39). Using CSF-1 promoter-driven reporter plasmid, we tested the role of c-Abl in Smad5-mediated transcription of CSF-1. Both STI 571 and expression of dominant negative c-Abl significantly inhibited BMP-2-stimulated transcription of CSF-1 (Fig. 7, A and B). We have reported earlier that Smad5 also regulates expression of Osx and BMP-2 (35, 37). Therefore, using reporter plasmids, we tested the effect of c-Abl inhibition on transcription of osteoblastogenic Osx and BMP-2. Both STI 571 and dominant negative c-Abl significantly inhibited Smad5-stimulated transcription of Osx (Fig. 7, C and D). Similarly, inhibition of c-Abl markedly blocked BMP-2 transcription induced by Smad5 (Fig. 7, E and F).

c-Abl Association with BMPRIA Induces Its Tyrosine Phosphorylation—Our results above demonstrate a role of c-Abl in BMP-2-induced osteogenic signal transduction. To characterize the mechanism, we examined association of c-Abl with BMPRIA. Coimmunoprecipitation experiments using c-Abl immunoprecipitates showed increased association of BMPRIA with c-Abl in response to BMP-2 (Fig. 8A). Reciprocal coimmunoprecipitation confirmed these results (Fig. 8B). Next, we determined the role of c-Abl tyrosine kinase activity in this association. Inhibition of c-Abl blocked BMP-2-induced association of BMPRIA with c-Abl, suggesting tyrosine kinase activity of c-Abl is necessary for this association (Fig. 8C). To examine whether the association of c-Abl induces tyrosine phosphorylation of BMPRIA, anti-phosphotyrosine immunoblotting was carried out with BMPRIA immunoprecipitates. As shown in Fig. 8D, BMP-2 enhanced tyrosine phosphorylation of BMPRIA. STI 571 inhibited BMP-2-stimulated BMPRIA tyrosine phosphorylation (Fig. 8D). Similarly, expression of dominant negative c-Abl blocked tyrosine phosphorylation of BMPRIA in
response to BMP-2 (Fig. 8E). These results indicate a significant role of c-Abl in tyrosine phosphorylation of BMPRIA.

c-Abl Controls BMP Receptor-specific Smad Phosphorylation—Phosphorylation of receptor-specific Smad in the C-terminal sites is required for BMP-2-induced signal transduction (9, 10). We first examined the effect of STI 571 on this phosphorylation. As expected, BMP-2 increased C-terminal phosphorylation of Smad1/5 in 2T3 preosteoblasts (Fig. 9A). Surprisingly, inhibition of c-Abl abrogated BMP-2-stimulated phosphorylation of Smad1/5 (Fig. 9A). Similarly, expression of dominant negative c-Abl blocked phosphorylation of Smad1/5 in response to BMP-2 (Fig. 9B). Next, we determined association of Smad1/5 with c-Abl. Immunoblotting of c-Abl immunoprecipitates with Smad antibody showed increased association of Smad1/5 with c-Abl in the presence of BMP-2 (Fig. 9C). STI 571 inhibited this association (Fig. 9C). Interestingly, BMP-2 increased phosphorylation of Smad1/5 associated with c-Abl (Fig. 9C). STI 571 blocked phosphorylation of Smad1/5 complexed with c-Abl (Fig. 9C). To confirm the role of c-Abl in Smad1/5 phosphorylation, we used calvarial osteoblasts from c-Abl null mice. Treatment of cells prepared from wild type mice with BMP-2 increased phosphorylation of Smad1/5 (Fig. 9D). In contrast to these results, BMP-2 was unable to stimulate phosphorylation of Smad1/5 in calvarial osteoblasts derived from c-Abl−/− mice (Fig. 9D). These results conclusively demonstrate a direct role of c-Abl in BMP-2 signal transduction involving receptor-specific Smads.

Smad5 Downstream of c-Abl Regulates BMP-2-induced Osteoclastic and Osteoblastic Marker Expression—Our results above have shown a role of c-Abl in expression of osteoclastogenic (CSF-1) and osteoblastogenic proteins (Osx and BMP-2) (Figs. 4–6). Furthermore, we show that c-Abl regulates Smad1/5 phosphorylation (Fig. 9). Therefore, using reporter transfection assays, we tested the mechanism of c-Abl-regulated expression of these proteins involving Smad5. As expected, expression of dominant negative c-Abl significantly reduced the transcription of CSF-1, Osx, and BMP-2 (Fig. 10, A–C). Consequently, expression of constitutively active Smad5 significantly prevented the dominant negative c-Abl-mediated inhibition of transcription of these genes (Fig. 10, A–C). Consequently, expression of constitutively active Smad5 reversed the dominant negative c-Abl-induced suppression of BMP-2-stimulated CSF-1, Osx, and BMP-2 mRNA expression (Fig. 10, D and F). These results demonstrate a direct role of Smad5 downstream of c-Abl in induction of osteoclastic and osteoblastic gene expression.

DISCUSSION

The concerted interaction between osteoblasts and osteoclasts maintains bone remodeling, which plays an important role in the maintenance of bone homeostasis. In this study, we have provided evidence that c-Abl mediates BMP-2 signal for bone remodeling by regulating receptor-specific Smad phosphorylation. Our results show that c-Abl negatively regulates BMP-2-induced Smad1/5 phosphorylation in 2T3 preosteoblasts, and this inhibition is reversed by inhibition of c-Abl. Furthermore, we have shown that c-Abl controls BMP-2-induced CSF-1 expression in calvarial osteoblasts. These findings suggest that c-Abl plays a critical role in BMP-2-dependent osteoclastic and osteoblastic gene expression.
role in common bone diseases, including inflammatory osteolysis, skeletal metastasis, and osteoporosis (58–60). Many factors, including BMP-2, are produced by osteoblasts, which maintain mature osteoblast formation and stimulate osteoclastogenesis. Our results represent the first demonstration of activation of the nonreceptor tyrosine kinase c-Abl by BMP-2, which contributes to osteoblast-aided mature osteoclast formation and osteoblast differentiation. We demonstrate that c-Abl contributes to BMP-2-induced PI 3-kinase/Akt signal transduction pathways, which control osteoblast differentiation. Furthermore, our results show requirement of c-Abl for BMP-2 autoregulation and expression of CSF-1 and Oxs by osteoblasts. Finally, we provide evidence that c-Abl regulates the canonical BMP-induced Smad phosphorylation, which contributes to osteogenic and osteoclastic gene expression. Thus, current data show two mechanisms, which emanate from BMP-2-stimulated c-Abl to converge on bone-specific gene expression and bone remodeling (Fig. 11).

c-Abl Mediates BMP-2 Signal for Bone Remodeling

Although we previously showed a role of tyrosine kinase in BMP-2-induced signal transduction and osteoblast differentiation, the involvement of specific tyrosine kinase was not determined (37). We identified that BMP-2 stimulated c-Abl tyrosine kinase in osteoblasts (Fig. 1, A–C). Osteoblast-produced factors are necessary for mature osteoclast formation (61). Thus, coculture of osteoblasts with osteoclast precursors has the capacity to form mature osteoclasts. However, BMP-2, which is a potent osteogenic growth and differentiation factor, significantly enhances the osteoclastogenic activity of the osteoblasts (39). Although BMP receptor-specific Smad may be required for osteoclastogenesis, our results using STI 571 and dominant negative c-Abl show that this nonreceptor tyrosine kinase regulates BMP-2-stimulated osteoblast-aided mature osteoclast formation (Fig. 1, D and E).

A previous report described that 50% of c-Abl null mice were osteoporotic (62). This study showed no difference in osteoclast differentiation of bone marrow-derived cells when compared with wild type cells, indicating c-Abl did not contribute to osteoclastogenesis. However, we show that calvarial osteoblasts from c-Abl null mice are defective in supporting osteoclastogenesis in a coculture assay (Fig. 1F). Furthermore, we provide evidence that c-Abl regulates osteoclast differentiation of normal mouse bone marrow-derived cells in response to BMP-2 (Fig. 1, G–J).

Highly specific and ordered expression of genes regulates osteoblast differentiation. Thus, BMP-2-induced osteoclastogenesis is initiated by expression of alkaline phosphatase at an early stage (63). We showed previously that tyrosine kinase-dependent signal transduction regulates BMP-2-stimulated alkaline phosphatase expression during osteoblast differentiation (37). A study using c-Abl null mice showed significant activity of alkaline phosphatase in osteoblasts (62). However, in this study using STI 571 and expression of dominant negative c-Abl, we show complete loss of BMP-2-stimulated alkaline phosphatase activity in 2T3 preosteoblasts (Fig. 2, A–D). In conjunction with these results, in calvarial osteoblasts prepared from c-Abl null mouse, we found complete prevention of alkaline phosphatase activity in response to BMP-2 (Fig. 2F). Furthermore, our results demonstrate that inhibition of c-Abl by its inhibitor or by dominant negative c-Abl expression significantly impaired osteoblast differentiation by BMP-2 (Fig. 2, F–I). This observation was also supported by our results showing marked impairment of bone nodule formation of cells isolated from c-Abl null calvaria (Fig. 2F). Thus, our data conclusively demonstrate a requirement of c-Abl tyrosine kinase in BMP-2-induced osteoblast differentiation.

We showed that BMP-2-stimulated class IA PI 3-kinase signaling was necessary for osteoblast differentiation and osteoblast-aided mature osteoclast formation (35, 37). This group of PI 3-kinases consists of two proteins, an 85-kDa regulatory and a 110-kDa catalytic subunit (64). The regulatory subunit contains SH3 and SH2 domains; the latter is involved in association with the activated receptor and nonreceptor tyrosine kinases (64, 65). The activated mutant of c-Abl, the viral oncogene product, was previously shown to activate PI 3-kinase in fibroblasts; however, overexpression of c-Abl was not sufficient to stimulate the lipid kinase activity (66). c-Abl contains a myris-
toyl group at its N terminus, which interacts with its kinase domain to render an autoinhibitory state (67). Moreover, the c-Abl SH2 domain binds phosphatidylinositol 4,5-bisphosphate, which inhibits its tyrosine kinase activity (68, 69). Activated PI 3-kinase also uses phosphatidylinositol 4,5-bisphosphate as substrate to produce phosphatidylinositol 3,4,5-trisphosphate (70, 71). Thus, activation of PI 3-kinase depletes cellular phosphatidylinositol 4,5-bisphosphate levels and as a consequence may release its inhibitory action resulting in the sustained activation of c-Abl. Activated c-Abl contains multiple phosphotyrosines, which recruit several SH2/SH3 domain-containing proteins, including PI 3-kinase in hematopoietic cells; however, direct association between p85 SH2 domain and c-Abl phosphotyrosines was not detected (31, 66, 72, 73). Rather, a role for intermediate proteins that directly bind to c-Abl has been proposed to recruit PI 3-kinase (74). In conjunction with these results, current data presented here show that BMP-2 stimulates PI 3-kinase activity in anti-phosphotyrosine immune complexes in a c-Abl tyrosine kinase-dependent manner, which results in downstream Akt phosphorylation (Fig. 3).

The role of two proteins CSF-1 and RANKL in osteoclastogenesis is well established. Our results demonstrate a role of c-Abl in expression of both these factors (Fig. 4A and data not shown). We have shown recently that the expression of osteoclastogenic CSF-1 and osteoblastic Osx by the osteoblasts depend upon PI 3-kinase/Akt signaling (35, 40). Our current results demonstrate that c-Abl regulates CSF-1 and Osx expression (Figs. 4 and 5). Also, BMP-2 autoregulation is mediated by c-Abl (Fig. 6). These data suggest that c-Abl regulation of PI 3-kinase/Akt signaling may be necessary for the expression of these genes.

Along with the noncanonical PI 3-kinase/Akt signaling, BMP-2 also uses canonical receptor-specific Smad for osteoblast differentiation and osteoblast-aided osteoclast matura-

![FIGURE 6. c-Abl regulates BMP-2-induced BMP-2 expression.](image-url)
However, Smad1 null murine embryonic fibroblasts retain BMP-2 responsiveness indicating that other Smads such as Smad5 control BMP-2 action (76). In fact, we have reported that Smad5 regulates expression of BMP-2, CSF-1, and Osx during osteoblast differentiation (37, 39, 40). In this study, we show that c-Abl contributes to Smad5-mediated expression of these genes (Fig. 7). Furthermore, we detect association between c-Abl and BMPRIA in the presence of BMP-2 and that the receptor undergoes tyrosine phosphorylation in a c-Abl-dependent manner (Fig. 8). Because we showed previously that PI 3-kinase contributes to Smad5-dependent transcription, current data demonstrate the presence of a cross-talk between BMPRIA and c-Abl, which acts upstream of PI 3-kinase to regulate Smad5 transactivation of osteogenic and osteoclastogenic genes. We have previously identified Smad-binding elements in the promoters of CSF-1 and Osx and demonstrated their interaction with BMP-2-stimulated Smad5, resulting in transcription of these genes during osteoblast differentiation (39, 40).
Phosphorylation of BMP receptor-specific Smad is necessary for binding to Smad4 and translocation to the nucleus to recruit transcriptional coactivators leading to direct binding to the Smad-binding element present in the promoter region of the target genes (11). We have shown previously BMP-2 stimulated increase in phosphorylation of Smads in 2T3 preosteoblasts. In this study, we show that BMP-2-induced activation of c-Abl tyrosine kinase is necessary for BMP receptor-specific Smad phosphorylation in preosteoblasts (Fig. 9, A and B). Because c-Abl does not have any serine/threonine kinase activity, it is somewhat unlikely that this tyrosine kinase would phosphorylate Smads in their C-terminal site. For Smad phosphorylation, binding to the type I receptor is essential. TGFβ receptor-specific R-Smads are anchored to the cell membrane by binding to the cytoplasmic protein SARA (77). However, this protein does not bind BMP receptor-specific Smads. One possibility may be that BMP-2-stimulated active c-Abl may bind BMP receptor-specific Smads to facilitate the phosphorylation of Smads by the BMP receptor I. In this context, we found that c-Abl coimmunoprecipitated with BMP receptor-specific Smads in response...
to BMP-2 in 2T3 cells (Fig. 9C). Interestingly, inhibition of c-Abl by STI 571 suppressed this complex formation (Fig. 9C). A corollary of this observation is that Smad should be phosphorylated in this immunocomplex. In fact, we found phosphorylated Smads in the BMP-2-induced c-Abl immunoprecipitates (Fig. 9C). Interestingly, the presence of these phospho-Smads was sensitive to STI 571 (Fig. 9C), indicating c-Abl regulation of this process. Furthermore, our results demonstrate the function of BMP-specific Smad downstream of c-Abl in BMP-2-induced osteoclastic and osteoblastic gene expression (Fig. 10). Because c-Abl forms a complex with Smad in a BMP-2 inducible manner (this study) and it can be present in the nucleus (30), our results point to an interesting possibility of the presence of this tyrosine kinase in the Smad-binding element of target genes. Further studies will be necessary to examine this mode of action of c-Abl in BMP-2-induced gene expression for osteoblast differentiation.

A study of patients with chronic myeloid leukemia treated with STI 571 showed hypophosphatemia and low serum calcium levels (78). In the treated group, expression of osteoblastic and osteoclastic markers was also inhibited, leading to attenuation of bone remodeling. BMP-2 plays an important role in bone remodeling. BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II. EMBO J. 19, 3314–3324

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