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PERSPECTIVES

The Role of the Proteasome in Bone Formation and Osteoclastogenesis

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Abstract

The findings that proteasome inhibitors are bone-anabolic in normal and pathological bones corroborate mounting evidence on the critical role of the proteasome in regulating activity of key osteoblastogenic transcription factors (*e.g.*, RUNX2, ATF4) and signaling pathways (*e.g.*, Hedgehog, BMP, Wnt/β-catenin). E3 ubiquitin ligases such as Smurf1, β-TrCP1, WWP1, and related adapter proteins (*e.g.*, Schnurri 3) regulate protein activity of RUNX2 or ATF4 by promoting their proteasomal-dependent degradation. Therefore, knocking down each of these ligases results in increased bone mass, and increasing Smurf1 expression (by factors such as TNFα or sustained PTH administration) may lessen bone formation. The clinically approved proteasome inhibitor bortezomib is bone-anabolic in patients with multiple myeloma, and various other proteasome inhibitors consistently increase bone mass in experimental animals. Proteasome inhibitors impact various signaling pathways in osteogenic cells; BMP signaling is induced by enhancing Hedgehog signaling-induced BMP-2 expression and by preventing degradation of relevant Smad receptors. Bortezomib stabilizes the Wnt signaling mediator β-catenin by reducing expression of the Wnt inhibitor DKK1 and also independently of Wnt signaling. The canonical and noncanonical NF-κB pathways are tightly regulated by the proteasome, and inhibition of this pathway promotes osteoblastogenesis and suppresses osteoclast differentiation. *In vivo* proteasome inhibition simultaneously increases osteoblastogenesis by stabilizing RUNX2 and reduces osteoclast numbers directly and indirectly by lowering the RANKL/OPG ratio. Increased bone mass resulting from proteasome inhibition is, therefore, mediated by multiple mechanisms of action that involve different cellular components, various signaling pathways within each cell type, and, possibly, systemic endocrine factors. *IBMS BoneKEy*. 2010 April;7(4):147-155.

Keywords: Proteasome; bone formation; bortezomib; osteoblasts; osteoclasts; RUNX2

Introduction

Clinical and experimental research on proteasome inhibitors has revealed that post-transcriptional proteolytic processing by the ubiquitin-proteasome pathway is critical for regulating the differentiation and/or survival of bone cells and subsequent bone remodeling. Proteasome activity is conferred by six catalytic active sites and is responsible for degrading ubiquitinated peptides in the cell. Formation of ubiquitin-protein conjugates requires three constitutively and broadly expressed enzymes that participate in a cascade of ubiquitin-transfer reactions: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Specificity of ubiquitination is determined by E3 enzymes, and proteins polyubiquitinated by these enzymes are targeted to undergo degradation, primarily by the 26S proteasome. The remaining three catalytic sites form an immunoproteasome that is relatively restricted to use in hematopoietic cells (1;2).

The role of the proteasome in bone formation appears to occur mainly through regulation of the master transcription factor in osteoblasts, runt-related transcription factor 2 (RUNX2)/Cbfa1, and additional components associated with bone morphogenetic proteins (BMPs) and Wnt signaling (Fig. 1). These proteasomal effects on osteoblastogenesis and on the

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production of osteoclastogenic factors in osteogenic cells indirectly impact osteoclastogenesis, but the proteasome is also directly involved in osteoclastogenesis because it controls important signaling pathways, such as the NF-κB pathway, in osteoclast precursor cells. It is, therefore, not surprising that proteasome inhibitors exert significant effects on bone remodeling in various bone-associated diseases.

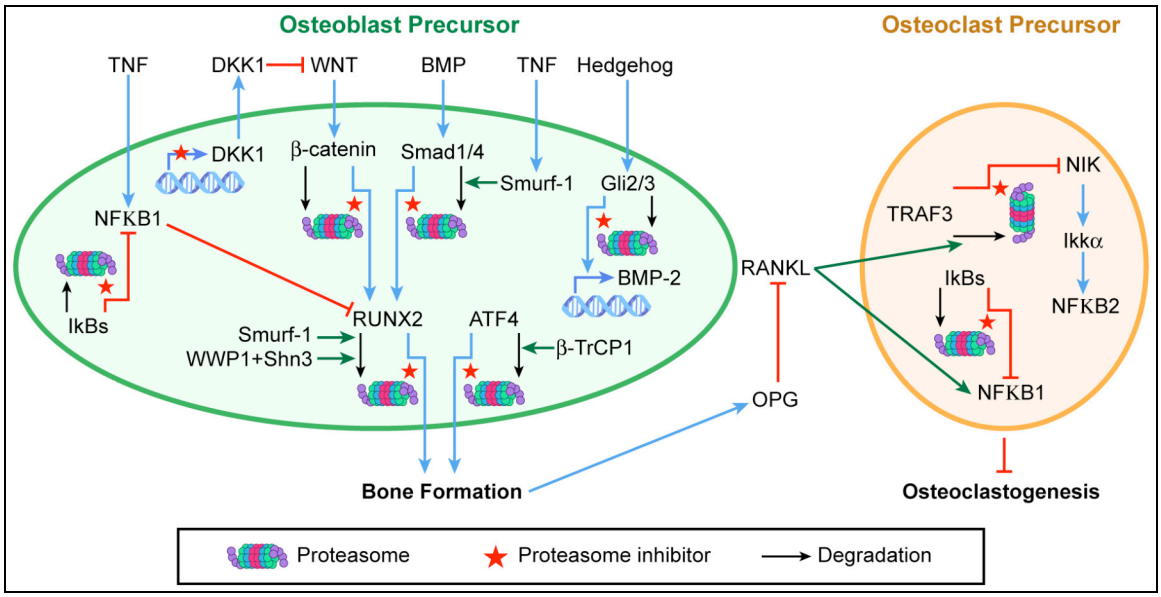

Fig. 1. Potential molecular targets in osteogenic cells and osteoclast precursors induced by proteasome inhibition. Osteoblastogenesis is stimulated by multiple mechanisms. BMP signaling is induced by enhancing Hedgehog signaling-induced BMP-2 expression and by preventing degradation of relevant Smad receptors. Stabilization of the Wnt mediator β-catenin is induced independently of Wnt and possibly also by reduced levels of the Wnt inhibitor DKK1. Preventing degradation of IκBs (inhibitors of NF-κB) inactivates the canonical NF-κB pathway (NFKB1), attenuating NF-κB inhibitory effects on osteoblastogenesis. The transcription factors RUNX2 and ATF4, which are induced by BMP and Wnt/β-catenin pathways, are further stabilized by preventing completion of proteasomal degradation by E3 ubiquitin ligases, such as Smurf-1, WWP1, and β-TrCP1. Osteoclast differentiation is suppressed directly through inhibition of the canonical (NFKB1) and noncanonical (NFKB2/p100) NF-κB pathway by preventing degradation of IκBs and TRAF3, respectively. Osteoclast numbers are also reduced indirectly due to increased numbers of mature osteoblasts that produce higher levels of OPG. Simultaneous induction of osteoblastogenesis and suppression of osteoclastogenesis result in increased bone mass (see text for details).

Proteasomal Regulation of RUNX2 and BMP Signaling

Steady-state and paracrine-regulated levels of RUNX2 are tightly controlled post-translationally in osteogenic cells by E3 ubiquitin ligases such as Smad ubiquitin regulatory factor 1 (Smurf1), Smurf2, and WW-domain-containing protein 1 (WWP1). The clinically approved proteasome inhibitor bortezomib (also known as Velcade) seems to enhance RUNX2 expression only in primitive mesenchymal stem cells (MSCs) (3), but it substantially increases RUNX2 activity in osteogenic cells *in vitro* at low nanomolar concentrations and clinically in myelomatous bone (4). Smurf1, the most studied of the E3 ubiquitin ligases, directly interacts with RUNX2 and induces its degradation in a ubiquitin- and proteasome-dependent manner (5;6). Transgenic mice overexpressing Smurf1 in osteoblasts have impaired bone formation during postnatal life (6). Conversely, Smurf1 knockout mice are born normal but exhibit age-dependent increases in bone mass (7).

Another protein that is associated with post-translational activity of RUNX2 is the zinc finger adapter protein Schnurri 3 (Shn3), which promotes RUNX2 polyubiquitination and proteasome-dependent degradation by recruiting WWP1 to RUNX2 (8). Mice deficient in Shn3 unexpectedly displayed

adult-onset osteosclerosis, bone matrix deposition, and augmented activity of osteoblasts, which express elevated levels of RUNX2 target genes. Knockdown of WWP1 in osteogenic cells increased RUNX2 protein levels and matrix mineralization (8). These studies underscore the important role of some E3 ubiquitin ligases in regulating proteasomal degradation of RUNX2 in osteogenic cells, which has subsequent effects on the bone phenotype.

The ubiquitin-proteasome pathway is directly involved in BMP signaling. Smurf1 interacts with BMP-activated Smad1 and Smad4, triggering their ubiquitination and degradation (9). This E3 ubiquitin ligase also interacts directly with and degrades MEKK2, an upstream kinase in the JNK signaling cascade that promotes osteoblastogenesis by sensitizing osteoblasts to BMP signaling (7). BMP-2 protects RUNX2 from Smurf1-catalyzed proteolysis by stimulating RUNX2 acetylation, which occurs via a Smad-dependent mechanism (10). In contrast, the inflammatory cytokine tumor necrosis factor alpha (TNFα) increases Smurf1 and Smurf2 expression in osteoblasts, resulting in degradation of Smad1 and RUNX2 and subsequent impaired osteoblastogenesis (11;12).

Using a panel of inhibitors that bind to specific catalytic beta subunits of the 20S proteasome, Garret *et al*. demonstrated that, within 5 days of applying proteasome inhibitors, bone formation was stimulated in bone organ cultures and markedly increased *in vivo*, mediated by upregulation of BMP-2 expression (13). This group further demonstrated that Hedgehog signaling stimulates osteoblast differentiation by inducing BMP-2 expression and that the effect of proteasome inhibitors on osteoblastogenesis occurs by blocked proteolytic processing of the Hedgehog signaling pathway mediators Gli3 and, possibly, Gli2 (13;14). Thus, the ubiquitin-proteasome pathway tightly regulates the Hedgehog signaling pathway and is involved in maintaining levels of BMP ligands and transcriptional modulators associated with BMP signaling in osteoblasts.

An interesting connection between Smurf1 and RUNX2 activities in osteoblasts was demonstrated by Jilka and colleagues in their attempts to unravel the mechanism of action of parathyroid hormone (PTH) in bone (15;16). While intermittent administration of PTH promotes bone formation, sustained PTH treatment induces bone loss. Intermittent administration of PTH has been suggested to increase osteoblast number and promote bone formation by stimulating the development of osteoblasts and inhibiting osteoblast apoptosis (16). The prosurvival effects of PTH require RUNX2 expression, but RUNX2 levels are subsequently decreased by PTH-induced RUNX2 degradation mediated by Smurf1. During intermittent PTH treatment, RUNX2 returns to basal levels and permits another episode of RUNX2-induced osteoblast survival and differentiation, but sustained PTH treatment maintains a low expression of RUNX2 and negatively affects osteoblast numbers. Although it is unclear whether PTH directly stimulates Smurf1 expression, functional assays using proteasome inhibitors or dominant-negative mutants of Smurf1 resulted in abrogated Smurf1-mediated RUNX2 degradation due to PTH treatment (15).

Like RUNX2, the transcription factor activating transcription factor 4 (ATF4) is also required for PTH treatment to increase bone anabolism (17). ATF4 plays a critical role in directly increasing expression of osteocalcin and increasing terminal differentiation of osteoblasts (18). ATF4 seems to be rapidly degraded by E3 ubiquitin ligase β-TrCP1 in most cell types, but, interestingly, it accumulates in osteoblasts, seemingly due to a lack of proteasomal degradation (19). Importantly, proteasome inhibitors increase ATF4 levels in various cell types (19). These findings indicate that extrinsic factors, such as PTH, mediate osteoblastogenesis by affecting expression and activity of factors associated with the ubiquitin-proteasome system and suggest that therapy that combines intermittent administration of PTH and a proteasome inhibitor, given simultaneously or in cycles, may induce synergistic effects on bone formation.

Proteasomal Regulation of β-catenin

β-catenin is a known target of the ubiquitin-proteasome pathway, and RUNX2 is a direct target of Wnt signaling, which, in osteogenic cells, is mediated through stabilization and prevention of β-catenin proteasomal degradation (20). Therefore, physiological and pharmacological compounds that affect proteasome activity or the status of β-catenin phosphorylation are likely to impact expression of target genes that are necessary for proper osteoblastogenesis. In canonical β-catenin-dependent Wnt signaling, an intracellular complex, which includes GSK-3b (glycogen synthase kinase 3b), axin, and the tumor suppressor gene product APC, phosphorylates β-catenin, which targets β-catenin for ubiquitin-mediated proteasomal degradation. However, activation of canonical Wnt signaling by Wnt ligands binding to Frizzled receptors, alone or complexed with low-density lipoprotein receptor-related proteins (LRPs) 5/6, prevents β-catenin degradation and leads to its accumulation and translocation to the nucleus, where it binds the TCF/LEF family of transcription repressors, turning them into transcriptional activators. Conversely, inhibitors of Wnt signaling, such as Dickkopf-1 (DKK1), secreted Frizzled-related proteins (sFRPs), and sclerostin, block Wnt ligands from interacting with Frizzled receptors and permit phosphorylation and subsequent degradation of β-catenin. The role of GSK-3b in regulating β-catenin activity is underscored by studies of its pharmacologic inhibition with lithium chloride, in which proteasomal degradation of β-catenin was prevented and bone mass was increased in mice with bone loss due to LRP5 mutation (21) and in a mouse model of myeloma (22).

Oyajobi *et al*. (23) showed that the proteasome inhibitor bortezomib inhibits DKK1 expression in osteogenic cells and that bortezomib-induced bone formation is blocked by DKK1. Interestingly, however, Qiang *et al*. (24) demonstrated that bortezomib stimulates osteoblast differentiation induced by β-catenin/TCF signaling but through a Wnt-independent pathway. Their *in vitro* study showed that bortezomib treatment resulted in accumulation of the active form of β-catenin and in subsequently increased TCF transcriptional activity; these resultant effects did not depend on changes in downstream targets of the Wnt pathway (*i.e.*, GSK-3b and disheveled proteins) or expression of Wnt ligands, antagonists, and cell-surface receptors (24). Although contradictory to earlier findings by Giuliani *et al*. (4) that bortezomib had no effect on β-catenin levels, these results emphasize the role of the proteasome in controlling degradation of activity of β-catenin in osteogenic cells, either through Wnt-dependent or -independent mechanisms.

Proteasomal Regulation of the NF-κB Pathway

Emerging findings indicate that the canonical and noncanonical/alternative NF-κB pathways are involved in bone remodeling and that signaling through NF-κB directly affects osteoclastogenesis (25) and osteoblastogenesis (26). The NF-κB family of transcription factors consists of NFKB1 (p50 and its precursor, p105), NFKB2 (p52 and its precursor, p100), RelA (p65), RelB, and c-Rel. In the canonical pathway, activated IKKβ phosphorylates the inhibitory subunits IκBα, IκBβ, or IκBε (IκBs), which leads to their proteasomal degradation; as a result, NF-κB homodimers and heterodimers, comprised mainly of RelA, RelC, and p50, accumulate in the nucleus. The RelA/p50 heterodimers are predominantly regulated by IκBα. Inflammatory stimuli, such as TNFα, activate the canonical NF-κB pathway through induction of phosphorylation and subsequent degradation of IκBs.

In the alternative pathway, NIK (NF-κB-inducing kinase) activates IKKα, which phosphorylates NFKB2. This results in proteasomal removal of an inhibitory C-terminal IκBδ domain from NIK, generating the p52 subunit, which leads to accumulation of p52/RelB heterodimers in the nucleus. TRAF3, TRAF2, and cIAP1/2 are critically involved in promoting proteasomal degradation of NIK. The alternative NF-κB pathway is activated in response to certain TNF family members,

such as CD40L and receptor activator of NF-κB ligand (RANKL), but certain ligands can activate both pathways (27).

Both the canonical and noncanonical/alternate NF-κB pathways are tightly regulated by the ubiquitin-proteasome system and are required for proper osteoclastogenesis. Mice deficient for both NFKB1 and NFKB2, but not those deficient for either individually, develop skeletal defects that are reflected by induction of osteopetrosis due to a defect in osteoclast differentiation (28). In osteoclast precursors, binding of RANKL to RANK activates TRAF6 (tumor necrosis factor receptor-associated factor 6), resulting in activation of IKK, which promotes phosphorylation and subsequent proteasomal degradation of IκB, an inhibitor of NF-κB (25). In Paget's disease, mutations in the adapter protein p62 (sequestosome-1) disrupt its proteasomal processing, which sensitizes osteoclast precursors to RANKL- and TNFα-induced NF-κB signaling in osteoclasts (29;30). Indeed, proteasome inhibitors suppress osteoclastogenesis in part by reduced NF-κB activity that results from preventing IκB degradation and disrupting the p62-TRAF6 cascade in osteoclast precursors (31;32).

There is also evidence that RANKL promotes osteoclastogenesis by activating the noncanonical pathway, because it prevents proteasomal degradation of NIK, seemingly by controlling activity of a complex composed of TRAF3, TRAF2, and cIAP1/2 (33). Interestingly, TNFα suppresses osteoclastogenesis in certain conditions by preventing TRAF3 degradation, which results in sustained accumulation of p100 in osteoclast precursors, presumably via decreased NIK levels (33). Further investigations are needed to clarify whether altering the noncanonical pathway is also involved in proteasome inhibitors' suppressive effects on osteoclastogenesis. Taken together, these findings suggest that proteasome inhibitors promote bone formation also indirectly by regulating the NF-κB pathway in osteoclast differentiation.

In contrast to the positive effects of the NF-κB pathway in osteoclastogenesis, canonical NF-κB signaling seems to exert negative effects on osteoblastogenesis. Time- and stage-specific inhibition of an endogenous IKK inhibitor in differentiated osteoblasts substantially increases osteoblast activity, but not osteoclast activity, and results in increased trabecular bone mass in young mice (26). It is assumed, therefore, that proteasomal regulation of NF-κB activity also plays a direct role in regulating the rate of bone formation.

In Vivo Consequences of Systemic Proteasome Inhibition on Bone

As mentioned above, the bone-anabolic effects of various proteasome inhibitors were demonstrated in rodents (13), but advances and clinical interest in understanding the effects of proteasome inhibition on bone formation are attributed to clinical observations of the effects of bortezomib treatment in patients with multiple myeloma. In this hematological disease, malignant plasma cells typically reside and accumulate in the hematopoietic bone, resulting in induction of osteolytic bone disease in >80% of patients with active myeloma. Unique to most bone malignancies, myelomatous osteolysis is localized to areas adjacent to tumor growth and is often characterized by increased activity of osteoclasts and suppressed osteoblastogenesis (34). The introduction of bortezomib into clinical treatment of myeloma has opened novel avenues for this and other malignancies because bortezomib has shown high anti-tumor efficacy even in patients with relapsed refractory disease. Zangari *et al*. (35-37), followed by others (38-40), demonstrated the bone-anabolic effects of bortezomib in patients with myeloma. Animal studies further showed that bortezomib increased bone mass in normal bones and in those with ovariectomy-induced bone loss (3;23;41).

Mechanistically, bortezomib promoted bone formation *in vivo* by preventing degradation of RUNX2 in osteogenic cells (4) and upregulating expression of the RUNX2 downstream target, Osterix, in osteoblasts from patients with myeloma (42). Bortezomib has also been shown to induce

differentiation of uncommitted MSCs into osteoblasts (3;24) and to reduce expression of the Wnt inhibitor DKK1 in bone (23), as well as DKK1 circulating levels (38). Bortezomib also reduced circulating levels of RANKL in patients with myeloma (38). Using the SCID-rab model for primary myeloma with myeloma cells purified from various patients, Pennisi *et al.* (41) further demonstrated that bortezomib effectively increases the number of osteoblasts and reduces the number of osteoclasts in healthy bone and that similar effects in myelomatous bone were not a consequence of reduced tumor burden but, rather, of direct, simultaneous stimulation of osteoblastogenesis and inhibition of osteoclastogenesis. Intriguingly, Zangari *et al*. (37) also found that the bone anabolism in myeloma patients responsive to bortezomib was preceded by a significant pulsatile increase in serum PTH levels without concomitant significant changes in calcium, magnesium, or phosphorus levels, which suggests involvement of a systemic *in vivo* mechanism through control of endogenous PTH variation. Overall, the remarkable effects of proteasome inhibition on bone formation and bone mass are clearly mediated by multiple mechanisms of action that involve different cellular components, various signaling pathways within each cell type, and possibly systemic endocrine factors.

Conclusion

Consequences of proteasome inhibition affect activities of many proteins within the cell; thus, proteasome inhibitors are likely to influence additional molecular pathways that regulate bone formation and osteoclastogenesis, such as the AP-1 pathway and the state of oxidative stress. Often contradictory, results of the *in vitro* studies do not conclusively elucidate mechanisms of action of proteasome inhibitors in MSCs, osteoblasts, osteoclasts, and committed osteoclast precursor cells. The roles of the proteasome in the survival and activity of osteocytes, which are the most abundant bone cells and which dictate bone strength, remain an open question. Further studies are needed to determine the effects of proteasome inhibitors on production of important osteocyte-produced factors that mediate bone remodeling, such as Wnt inhibitors (*e.g.*, sclerostin, DKK1) and osteoclastogenic factors (*e.g.*, RANKL). It is still unclear whether proteasome inhibitors affect the fate of bone-lining cells.

A valuable point to discern from the clinical effects of bortezomib on bone anabolism is that the majority of treated patients are elderly individuals, some of whom suffer from systemic osteoporosis. Despite the general notion that proteasome activity decreases with age in various cell types (2), inhibition of the ubiquitin-proteasome pathway appears to positively affect aging bone. In light of systemic toxicities of various proteasome inhibitors, it is encouraging that subtoxic concentrations stimulate MSCs to commit and differentiate to osteoblasts. As deduced from the synthesis of studies reviewed here, targeting specific factors in the ubiquitin-proteasome pathway (*e.g.*, specific ligases or adaptors) may induce similar bone anabolism and spare some side effects induced by treatment with proteasome inhibitors. Finally, the clinical observations showing bortezomib's transient effects on circulating levels of osteoblast markers, at least in patients with myeloma (35), strongly emphasize the requirement for studying the long-term consequences of proteasome inhibition on bone remodeling, particularly with regard to the fate of MSCs and bone strength, and for further unravelling the roles of the proteasome in bone biology.

Conflict of Interest: None reported.

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