Regulation of osteoblastic differentiation by the proteasome inhibitor bortezomib

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ABSTRACT

In eukaryotic cells, degradation of most intracellular proteins is carried out by the ubiquitin-proteasome pathway. Recent investigations suggest that bone metabolism is also regulated by this pathway. The clinical efficacy of bortezomib, a 26S proteasome inhibitor used as an anticancer drug, has been linked to an increase in bone formation. In the present study, we show that proteasome inhibitors induce expression of osteoblastic differentiation-related genes such as osteocalcin and alkaline phosphatase in C2C12 cells. In contrast, myogenic differentiation is inhibited. Among the proteasome inhibitors tested, bortezomib induced the greatest increase in osteocalcin expression. Although these effects were similar to that of bone morphogenetic protein (BMP) 2, proteasome inhibitors did not induce transcriptional activity of Smad1/4-dependent reporter or BMP2 signaling target gene expression. Transient transfection of osteocalcin promoter-luciferase constructs with bortezomib resulted in an increase in luciferase activity. Mutation of OSE2, but not OSE1, sites of the osteocalcin promoter diminished the bortezomib-induced activity. Also, Runx2 binding activity and protein levels were induced by bortezomib treatment. These results suggest that the bortezomib induces osteoblastic differentiation by modifying the activity of Runx2, and that the function of the proteasome in controlling degradation of differentiation-related transcription factors plays an important role in osteoblast differentiation.

Introduction

The ubiquitin-proteasome pathway plays a pivotal role in the selective degradation of intracellular proteins which is important in the control of basic cellular processes including regulation of differentiation and proliferation (Schwartz & Ciechanover 2009). Given its essential role, ubiquitination is a highly regulated process that involves the sequential action of three enzymes termed ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). The specificity of ubiquitination is often controlled by E3, which facilitates the transfer of ubiquitin to appropriate targets. Poly-ubiquitination of proteins promotes their translocation to the 26S proteasome for degradation (Ciechanover 1998). Recent investigations suggest that the ubiquitin-proteasome pathway is the therapeutic target in several diseases (Dahlmann 2007).

Bone mass is dependent on the balance between bone formation by osteoblasts and bone resorption by osteoclasts. The differentiation and function of osteoblasts is regulated by a number of regulatory factors including bone morphogenetic proteins (BMPs) and Wnt, key signal transduction molecules (such as the Smads and β-catenin), and critical transcription factors (including Runx2 and ATF4). The Wnt signaling pathway has been reported to be involved in regulation of bone formation (Tamura *et al.* 2010). According to the model of canonical Wnt action, in cells lacking a Wnt signal, glycogen synthase kinase (GSK)-3β phosphorylates β-catenin, inducing rapid degradation of β-catenin via the ubiquitin-proteasome pathway (MacDonald *et al.* 2009). E3 ubiquitin ligases such as Smad-ubiquitination-regulatory factor 1 (Smurf1), beta-transduction repeat-containing protein 1 (β-TrCP1), WW-domain-containing protein 1 (WWP1), and related adapter proteins (e.g., Schnurri-3 (Shn3)) regulate the protein activity of Smads, Runx2 or ATF4 via ubiquitination, thus promoting their proteasome-dependent degradation (Yang *et al.*

2004; Jones *et al.* 2006; Xing *et al.* 2010). Experiments in mice revealed that knocking down Smurf1 results in increased bone mass, while overexpressing Smurf1 decreases bone formation (Zhao *et al.* 2004; Yamashita *et al.* 2005). Shn3 deficient mice also display osteosclerosis, increased bone matrix deposition, and augmented osteoblast activity (Jones *et al.* 2006). These observations suggest that proteasome activity is critical for the activation of osteogenic transcription factors and that bone metabolism is regulated by the ubiquitin-proteasome pathway.

Many structural classes of proteasome inhibitors are known (Borissenko & Groll 2007; de Bettignies & Coux 2010). Among them, certain proteasome inhibitors are bone-anabolic agents in both normal and pathological conditions. Garrett et al. demonstrated that systemic administration of epoxomicin and proteasome inhibitor-1 increased bone volume and bone formation rates in mice (Garrett *et al.* 2003). A strong correlation between the capacity of these compounds to inhibit proteasomal activity in osteoblasts and their bone-forming activity was also demonstrated. Bortezomib (Velcade™, PS-341) is a modified dipeptidyl boronic acid derived from leucine and phenylalanine, and a novel cytotoxic chemical entity that potently and specifically inhibits the proteolytic activity of the proteasome and thus the degradation of poly-ubiquitinated proteins destined for catalysis by the proteasome. Bortezomib is used for the treatment of multiple myeloma and mantle cell lymphoma (Shah & Orlowski 2009). Multiple myeloma is a plasma cell malignancy characterized by the formation of bone lesions. In these patients, bortezomib exhibits bone-anabolic activity, with increased serum levels of osteogenic markers such as osteocalcin, and increased numbers of osteoblasts in bone biopsies (Giuliani *et al.* 2007). Recent results from animal studies showed that bortezomib also increases bone mass in normal bone and in those with ovariectomy-induced bone loss (Oyajobi *et al.* 2007; Mukherjee *et al.* 2008; Pennisi *et al.* 2009). However, it is not known currently whether the proteasome

inhibitor may have a direct effect on determination of cell fate to differentiation. Also, the molecular basis of action of the proteasome inhibitor on the regulation of cell differentiation remains poorly understood.

In this study, we investigate the effects of proteasome inhibitors on differentiation of mesenchymal progenitor cells. Here we show that bortezomib induces osteoblast differentiation by altering the activities of Runx2. These results indicate that the function of the proteasome in controlling degradation of signaling molecules plays an important role in the determination of cell fate during differentiation.

Results

Regulation of gene expression by proteasome inhibitors in C2C12 cells

To evaluate a potential role for proteasome inhibitors in mesenchymal cell differentiation, we used the C2C12 cell line. C2C12 cells are a multipotent cell line and are a well-characterized model system which have been reported to differentiate into not only myotubes, but also osteoblasts, depending upon the specific culture conditions, when incubated in the presence of BMP (Katagiri *et al.* 1994). RT-PCR was used to analyze the transcript levels of osteoblastic or myogenic specific genes following treatment with proteasome inhibitors. Osteocalcin mRNA expression was induced by proteasome inhibitors. In contrast, myogenin and MCK mRNA expression was reduced by bortezomib, lactacystin, epoxomicin or MG-132 (Fig. 1A). Expression of *M-CSF* was not changed after treatment with any proteasome inhibitors (Fig. 1A). The expression levels were also determined by qRT-PCR assay. The level of osteocalcin mRNA was increased by over 50-fold in response to bortezomib compared to untreated cells (Fig. 1B). ALP mRNA was also upregulated by treatment with proteasome inhibitors. In contrast, the level of myogenin expression declined to 10% upon treatment with bortezomib or epoxomicin. To confirm the role of bortezomib in the regulation of mRNA expression, we analyzed the dose effects of culturing cells in the presence of bortezomib. The level of osteocalcin mRNA increased significantly after addition of 10 nM bortezomib and further increased with doses up to 100 nM (Fig. 1C). Also, addition of bortezomib caused a dose-dependent decrease in the expression of myogenin (Fig. 1C). These findings indicate that proteasome inhibitors can induce osteoblastic phenotype-specific gene expression, thereby suppressing their ability to express myogenic genes.

Bortezomib inhibits myotube formation in C2C12 cells

Next, we examined the effects of bortezomib on myotube formation. By day 4 in low mitogen medium, control C2C12 cells had become fused to form multinucleated myotubes (Fig. 2A). Similar to findings in the presence of BMP2 (Nakashima *et al.* 2005), no myotubes were detected in cells cultured in the presence of bortezomib (Fig. 2A), indicating that bortezomib inhibits myotube formation.

Regulation of miR expression by bortezomib in C2C12 cells

More than 500 miRNAs have been discovered in mammals, and some of them are expressed in a tissue-specific manner, which suggests that they have specific roles in the specification of tissues during differentiation. Among them, miR-206 is unique in that it is only expressed in skeletal muscle including C2C12 cells (Sato *et al.* 2009b). We recently identified osteoblastic differentiation-related miRNAs including miR-34b (manuscript in preparation). Therefore, we investigated the regulation of expression of these miRNAs by bortezomib, analyzed by a qRT-PCR assay. miR-206 expression was dose-dependently down-regulated by bortezomib in C2C12 cells (Fig. 2B), similar to our previous findings that BMP2 decreases miR-206 expression (Sato *et al.* 2009b). In contrast, miR-34b expression significantly increased after treatment with bortezomib dose-dependently (Fig. 2B). BMP2 and canonical Wnt signaling such as Wnt3a also induced the same changes in expression of these miRNAs (Fig. 2B). These results indicate that bortezomib regulates not only tissue specific mRNA but also miRNA expression which regulates both myogenic and osteogenic differentiation, and that the

effects of bortezomib may be involved in regulating the differentiation pathways.

Proteasome inhibitors do not enhance β-catenin-mediated and Smad1/4-mediated transcriptional activation

Since canonical Wnt signaling is known to be modulated by proteasomal proteolysis, we analyzed the effects of proteasome inhibitors on regulation of this signaling pathway. We transfected with Topflash, the reporter plasmid that carries six tandem repeats of the Lef1/Tcf binding site, and then treated with proteasome inhibitors. Although the promoter activity of Topflash was strongly enhanced by transfection of an expression plasmid carrying an activating form of β-catenin (β-cateninΔGSK), proteasome inhibitors did not increase β-catenin-mediated transcriptional activation (Fig. 3A). To further address the involvement of canonical Wnt by which the proteasome inhibitor induces osteoblastic differentiation, we used small molecular inhibitors IWR-1 to block activation of canonical Wnt signaling pathway (Chen *et al.* 2009). This inhibitor could not diminish the response of osteocalcin mRNA induction by bortezomib in C2C12 cells (Fig. 3B). In addition, canonical Wnt signaling-regulated gene transcripts such as *OPG* and *RANKL* could not be detected following treatment with either of these proteasome inhibitors in these cultures (data not shown). These results indicate that proteasome inhibitors may not be involved in regulating the canonical Wnt signaling pathway in these cultures.

BMP2 signaling results in the direct transcriptional activation of BMP-responsive promoters by transcription factors that include Smad1/4 (Wrana & Attisano 2000). Therefore, to explore the effect of proteasome inhibitors on Smad1/4-dependent transcriptional activation, we transfected IdWT4F-luc (a luciferase

reporter plasmid that contains four copies of a 29-bp BMP responsive fragment) (Katagiri *et al.* 2002) into C2C12 cells. Although BMP2 significantly enhanced transcriptional activity of IdWT4F-luc, none of the proteasome inhibitors could up-regulate these activities (Fig. 3C). BMP2 target genes such as *Id1*, *2* and *3* are known to be induced by BMP2 treatment (Miyazono & Miyazawa 2002). However, bortezomib could not enhance these mRNA levels (Fig. 3D). Moreover, small molecule inhibitor dorsomorphin (Yu *et al.* 2008a) to block specific BMP/Smad signaling pathway did not regulate the response of osteocalcin mRNA induction by bortezomib (data not shown). These results indicate that these proteasome inhibitors do not activate the Smad1/4-dependent BMP2 signaling pathway.

Bortezomib activates osteocalcin gene promoter activity

To investigate the mechanisms by which bortezomib activates *osteocalcin* transcription, we cloned an approximately 0.7-kilobase pair mouse genomic DNA fragment corresponding to the 5'-flanking promoter region of the mouse osteocalcin (OG2) gene. The *OG2* promoter region was ligated into a pGL4.11 to examine its responsiveness to proteasome inhibitor stimulation. Transient transfection of pOC661-luc into C2C12 cells with bortezomib, but not lactacystin, epoxomicin or MG-132, resulted in a significant increase in luciferase activity (Fig. 4A). This analysis implies the presence of transcriptional machinery that is sensitive to interference by bortezomib and that regulates transcriptional activity through interaction with the *OG2* gene promoter downstream from nucleotide position -661. This region contains two osteoblast-specific *cis*-acting elements (termed osteoblastic specific element: OSE1 and OSE2) (Ducy & Karsenty 1995). To determine whether these two sites contributed to the induction of

transcriptional activity by bortezomib, we introduced mutations into each by replacing two nucleotide sequences. The luciferase activity of pmOSE1-luc was enhanced by bortezomib in a similar fashion to that observed in pOC661-luc (Fig. 4B). In contrast, pmOSE2-luc did not induce luciferase activity by bortezomib (Fig. 4B), indicating that the OSE2 site contributes to the inducible transcriptional activity of the osteocalcin gene by bortezomib.

Enhancement of Runx2 activity and protein levels by bortezomib in C2C12 cells

To further address whether the binding activity of nuclear proteins that interact with the OSE2 site is regulated by bortezomib, a binding assay was performed using an oligonucleotide containing the consensus Runx2 binding sequence as a probe. Binding activity of the probe in nuclear extracts prepared from C2C12 cells was very low, while bortezomib-treated nuclear extracts displayed binding activity at a similar level to that of nuclear extracts from osteoblastic SaOS-2 cells (Fig. 4C). The binding of proteins could be competed out with unlabeled oligonucleotide containing the probe. As expected, the binding was not abolished when mutated oligonucleotide was used as the competitor (Fig. 4C). These findings indicate that the Runx2 binding activity in nuclear extracts is regulated by bortezomib and is closely correlated to the promoter activity found using the reporter constructs in the site-directed mutation analyses (Fig. 4B).

To assess whether bortezomib induced-Runx2 also bound in vivo to its target site in the osteocalcin promoter, we performed a ChIP assay. Chromatin was prepared from C2C12 cells treated with bortezomib and immunoprecipitated with an antibody specific for Runx2. Then, PCR analyses were performed. Bortezomib induced interaction of Runx2 with chromatin fragment of the proximal mOG2 promoter that

contain OSE2 site (primers P1/P2). In contrast, Runx2 antibody failed to immunoprecipitate chromatin fragments of the proximal mOG2 promoter that contain OSE1 site (primers P3/P4) or mOG2 gene that contains no Runx2-binding site (primers P5/P6) (Fig.5A). This result demonstrates that bortezomib stimulation led to the recruitment of Runx2 at OSE2 site, indicating that OSE2 in the osteocalcin promoter might be involved by bortezomib. Furthermore, we determined if bortezomib could increase the levels of endogenous Runx2 protein in C2C12 cells. Western blot analysis shows that bortezomib significantly increased Runx2 protein levels in the studied cells (Fig. 5B). However, bortezomib did not increase the levels of Runx2 mRNA in these cells (data not shown).

Discussion

Proteasome inhibitors come in two different types: Synthetic and natural inhibitors, and each have been known to induce different biological effects, in particular in distinct cells (Borissenko & Groll 2007). The catalytic activities of the 26S proteasome are responsible for β1, β2, and β5-subunits of the 20S proteasome and their substrate specificities involve caspase-like, trypsin-like and chymotrypsin-like activity (Dick & Fleming 2010). Bortezomib is a reversible inhibitor that targets primarily chymotrypsin-like activity by the β5-subunit of the 20S proteasome and to a somewhat lesser extent also caspase-like activity by the β1-subunit (Oerlemans *et al.* 2008). MG-132 also inhibits chymotrypsin-like activity by the β5-subunit. Bortezomib has stronger affinity for the proteasome than the peptide aldehydes such as MG-132 (Borissenko & Groll 2007). The affinity for the different subunits is distinct for each of the four proteasome inhibitors and may contribute to their differing biological effects. Our observations also suggest the possibility that the induction of osteoblastic gene expression by bortezomib results from specific reversible binding to the β5-subunit. Further investigation is required to understand the mechanisms by which bortezomib specifically regulates osteoblast cell differentiation.

In this study, we have shown that bortezomib activates the osteocalcin gene promoter. Biochemical, molecular, and genetic studies have revealed that the OSE1-binding protein is ATF4 (Yang *et al.* 2004). In turn, complex of Runx2 and core binding factor β (cbfb) interacts with OSE2 (Ducy *et al.* 1997). Runx2 levels increase greatly during osteoblast differentiation, whereas homozygous gene deletion of Runx2 limits bone formation, and few differentiated osteoblasts are found in Runx2-deficient mice (Ducy *et al.* 1997). Ubiquitination of both Runx2 and ATF4 promote their proteasome-dependent degradation (Lassot *et al.* 2001; Zhao *et al.* 2003). From our

results, the OSE2 site, Runx2 binding activity in nuclear extracts and Runx2 protein levels are regulated by bortezomib. These observations implicate Runx2, but not ATF4, as a potential target for bortezomib treatment. Bortezomib did not alter Runx2 mRNA levels (data not shown). Bortezomib induced expression of the osteoblast phenotype by increasing Runx2 protein levels and activity without changing Runx2 mRNA levels and consequently stimulating the expression of osteocalcin and ALP, suggesting that the effects of bortezomib may be dependent on proteasome inhibition. Consistent with our studies, other investigators have also shown that bortezomib significantly increased Runx2 level in human osteoblast progenitors (Giuliani *et al.* 2007).

None of the proteasome inhibitors could regulate transcriptional activation of BMP-responsive promoters. Smurf1 has been found to interact with the Smad1 and Smad5, thereby triggering their ubiquitination and degradation (Zhu *et al.* 1999). In our experimental systems, treatment with proteasome inhibitors may not enhance ubiquitination of Smad1 or inhibit proteasomal-dependent degradation of Smad1, thus proteasome inhibitors could not up-regulate Id4WT-luc activity. Expression of Id1, 2 and 3 mRNA was not induced by treatment with proteasome inhibitors. These observations suggest that the effects of proteasome inhibitors are completely different to those of BMP2 in these cells. Our results indicated that proteasome inhibitors failed to stimulate Top-flash activity or to regulate *OPG* or *RANKL* expression (data not shown) (Spencer *et al.* 2006; Sato *et al.* 2009a), suggesting that proteasome inhibitors did not modulate canonical Wnt signaling. Accordingly, it has been reported that canonical Wnt has no effect on osteocalcin expression by C2C12 cells (Nakashima *et al.* 2005). Consistent with our studies, they have also shown that bortezomib did not alter nuclear or cytoplasmic active β-catenin levels in human osteoblast progenitors and osteoblasts (Giuliani *et al.* 2007). Taken together, our observations suggest that the regulation of

osteoblastic differentiation by proteasome inhibitors may not be dependent upon transcriptional activation of the β-catenin-mediated canonical Wnt signaling pathway.

Bortezomib is the first proteasome inhibitor in clinical use as an anti-myeloma agent. Multiple myeloma is characterized by the presence of bone lytic lesions due to increased osteoclastic activity and reduced osteoblast function. Osteolysis in multiple myeloma is related to the suppression of canonical Wnt signaling caused by DKK1. In addition, myeloma cells secrete interleukin-7 (IL-7), which leads to a reduction of osteoblast formation and differentiation (Giuliani *et al.* 2005). Treatment using bortezomib induces apoptosis of myeloma cells and suppresses DKK-1 and IL-7 secretion. However, recent clinical studies showed that bortezomib promoted osteoblastic activity in patients, irrespective of their response to treatment (Heider *et al.* 2006). In addition, bortezomib promotes bone formation in non-myelomatous bones by stimulating osteoblastogenesis (Pennisi *et al.* 2009). Our present study, showing that bortezomib acts directly on Runx2 activity, may support previous findings by other investigators demonstrating that bortezomib increases bone mass by simultaneously stimulating bone formation.

From our observations, bortezomib induced osteoblastic differentiation and inhibited myogenic differentiation in C2C12 cells. In contrast, no proteasome inhibitors were able to induce expression of chondrocyte-specific genes (e.g., Sox9) or adipocyte-specific genes (e.g., lipoprotein lipase) (data not shown). Interestingly, Mukherjee et al. (2008) showed that bortezomib induced osteoblastic differentiation and suppressed adipocyte differentiation in mesenchymal stem cells (Mukherjee *et al.* 2008). It is possible that the cells used in different studies could have varying potential to differentiate into other cell types. Taken together, these findings suggest that tight regulation by bortezomib is critical for osteoblastic differentiation.

Bortezomib has been shown to specifically regulate both miR-206 and miR-34b expression. These results indicate the possibility that not only differentiation-related mRNA but also microRNA expression is regulated by ubiquitination of transcription factors or signaling molecules and their proteasome-dependent degradation. Our observations provide the possibility that Runx2 regulates miR-206 and miR-34b expression in these cells.

In conclusion, we have shown that the proteasome inhibitor bortezomib induces osteoblastic differentiation, and that bortezomib inhibits the ubiquitin proteasome pathway leading to degradation of the osteogenic transcription factor Runx2. As such, these investigations may provide important new information pertaining to the molecular basis of the regulation of osteoblastic differentiation in bone tissue.

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Experimental Procedures

Cell cultures

Cells of the mouse cell line C2C12 were cultured in α-MEM containing 100 μg/mL kanamycin (Meiji, Tokyo, Japan) and supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Inc., Lenexa, KS) at 37^{o}C in 100-mm cell culture dishes (Corning, Corning, NY) in a humidified atmosphere of 5% CO_2 in air.

Compounds and Reagents

The proteasome inhibitors, bortezomib and epoxomicin, were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Lactacystin and MG132 were obtained from Cal-Biochem-Novabiochem (La Jolla, CA). Recombinant human BMP2 was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan). IWR-1 was purchased from Sigma-Aldrich (St Louis, MO).

Myotube formation

C2C12 cells were plated at 1×10^5 cells/cm^2. After 24 h, cells were cultured in α-MEM containing 3% FBS (low mitogen medium) and the cells were cultured with bortezomib (2 nM) or vehicle for 4 days. Cell morphology was viewed by phase contrast microscopy. Magnification: ×100

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells at the indicated time points using Isogen (Nippongene, Toyama, Japan) and RT-PCR was performed as previously described (Nakashima *et al.* 2005). The primer sequences for each gene were as follows: myogenin, 5'-TATGAGCGGACTGAGCTCAGCT-3' (forward) and 5'-CAGATGTGCACACTTGTCCAGG-3' (reverse); muscle creatine kinase (MCK), 5'-CAATAAGCTTCGCGATAAGGAG-3' (forward), 5'-AGGAAGCTTTTGTTGTCGTTG -3' (reverse); osteocalcin, 5'-CTGAGTCTGACAAAGCCTTC-3' (forward), 5'-GCTGTGACATCCATACTTGC-3' (reverse); macrophage colony-stimulating factor (M-CSF), 5'-TTGCCAAGGAGGTGTCAGAA-3' (forward), 5'-TATTGGAGAGTTCCTGGAGC-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TCCACCACCCTGTTGCTGTA-3' (forward), 5'-ACCACAGTCCATGCCATCAC-3' (reverse). To account for any difference in the amount of RNA, *GAPDH* was chosen as our endogenous control and amplified using the primers described above. The amplification products were electrophoresed on 2% agarose gels.

Quantification of gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) was performed using assay-on-demand TaqMan probes (Applied Biosystems, Foster city, CA) and the StepOne® real time PCR system. The relative level of gene expression was quantified using the comparative C_T method with *GAPDH* as the endogenous control.

Quantitation of miRNA expression

Total RNA was extracted from the cells and cDNA was synthesized with specific miRNA primers from the TaqMan MicroRNA Assay (Applied Biosystems). The resulting cDNA was amplified by PCR using the TaqMan MicroRNA assay system with the StepOne® real time PCR system. The relative level of miRNA expression was quantified using the comparative C_T method with sno234 RNA as the endogenous control.

Expression plasmids

The mutated β-catenin expression plasmid, β-cateninΔGSK, in which amino acid residues of the GSK phosphorylation site of β-catenin cDNA are deleted, was used (Obama & Ozawa 1997). The plasmid DNA was transfected into cells using Lipofectamine2000 (Invitrogen, Carlsbad, CA) as described previously (Sato *et al.* 2009a).

Reporter constructs and assay for luciferase activity

Luciferase reporter plasmids for the murine osteocalcin promoter were generated as follows. The 661-bp *osteocalcin* promoter fragment (-661 to +37) was isolated from mouse genomic DNA by PCR and subcloned into the pGL4.11 vector (Promega) to generate the luciferase reporter plasmid (pOC661-luc). The constructs with single site mutations (pmOSE1-luc and pmOSE2-luc) were generated by replacing the OSE1 and OSE2 sites in pOC661-luc, respectively (Ducy & Karsenty 1995). Site-directed mutagenesis was performed by the overlap extension technique using PCR. The following mismatched oligonucleotides were used: OSE1 site, 5'-TCGTGCATGCAGCAGAGAGC-3'; OSE2 site, 5'-ATCACCAACCGAAGCATCCT-3' (mutated nucleotides are underlined). The nucleotide sequences of each mutated

promoter region were verified by sequencing. Topflash is a Tcf reporter plasmid (Upstate Biotechnology, Lake Placid, NY). IdWT4F-luc has been described previously (Katagiri *et al.* 2002). For the luciferase assay, cells were plated in 24-well culture plates at a density of 1×10^4 cells/well and cultured for 24 hours. The cells were then treated with different proteasome inhibitors or other compounds for an additional 24-48 hours. The cell lysates were extracted, and luciferase activities were measured as described previously (Nakashima *et al.* 2005).

Extraction of nuclear proteins and assay for Runx2 binding activity

Runx2 binding activity was quantified using the TransAM™ AML-3/Runx2 family transcription factor assay kit (Active Motif, Carlsbad, CA). In brief, nuclear lysates of C2C12 cells after treatment with 100 nM bortezomib or vehicle were incubated in ELISA wells containing the Runx2 consensus binding site oligonucleotide (5′-AACCACA-3′). The activated Runx2 contained in nuclear extract specifically binds to this oligonucleotide. Binding was measured by incubation with a primary antibody to AML-3/Runx2 followed by a secondary horseradish peroxidase-conjugated antibody that recognized the primary antibody. Following a final step of multiple washes, a colorimetric reaction was performed and quantified by spectrophotometry at 450 nm. The positive control nuclear extract and negative lysis buffer controls were also included. Results are expressed as a fold increase in the treated samples compared with the control samples and were calculated according to nuclear content in treated cells/nuclear content in control cells.

Chromatin immunoprecipitation (ChIP) Assay

A ChIP assay was performed as described previously (Sato *et al.* 2009a) using ChIP-IT® Express Chromatin Immunoprecipitation Kits (Active Motif). Chromatin

solutions were incubated overnight at 4 °C with rotation, and with the following antibody solutions: anti-Runx2 (Santa Cruz Biotechnology) or control IgG (Sigma-Aldrich). Fractions of the purified ChIP DNA or inputs were used for PCR analysis. The reaction was performed with Taq DNA polymerase (Qiagen) for 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C. PCR primer pairs were generated to detect DNA segments located near the OSE2 at -137/-131 (primers P1 and P2) or OSE1 at -55/-48 (primers P3 and P4) in mouse osteocalcin gene 2 (mOG2) proximal promoter (see Fig. 5A). PCRs using primers P5 and P6 of mOG2 gene region (+177/+311) served as negative controls. All PCR primer sequences used in this study were described in Yu et al. (Yu *et al.* 2008b). The PCR products were separated on 3% agarose gels and visualized with ultraviolet light. All ChIP assays were repeated at least two times.

Western blot analysis

Whole cell extracts were separated, transferred to a PVDF membrane, and probed with anti-Runx2 (Santa Cruz) or anti-β-actin antibodies (Santa Cruz) using the ECL prime detection system (GE lifesciences).

Statistical analysis

All experiments were repeated three to four times and representative results are shown. The data are reported as the mean ± standard deviation, and were analyzed by Student's t-test, where values of $p < 0.05$ were considered significant.

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FIGURE LEGENDS

Figure 1

Regulation of gene expression by proteasome inhibitors in C2C12 cells

C2C12 cells were plated at 1×10^5 cells/cm^2. After 24 h, cells were cultured without or with the indicated doses (A and C) or 100 nM (B) of proteasome inhibitors for a further 6 h in 10% FBS. Total RNA was extracted from the cells and then mRNA expression of osteocalcin, myogenin, muscle creatine kinase (MCK) and macrophage colony stimulating factor (M-CSF) was determined by RT-PCR (A). The level of osteocalcin, myogenin and alkaline phosphatase (ALP) transcripts was evaluated by quantitative RT-PCR (qRT-PCR) (B, C). Data are shown as means ± S.D. Each assay represents a separate experiment performed in triplicate. Asterisks indicate significant differences ($p < 0.05$, t test for paired data).

Figure 2

Regulation of myotube formation and microRNA expression by bortezomib in C2C12 cells

(A) C2C12 cells were cultured in low-mitogen medium without or with bortezomib for 4 days. Cell morphology was viewed by phase contrast microscopy. Magnification: ×100. *Scale bar*, 30 μm (B) C2C12 cells were cultured with indicated doses of bortezomib, BMP2, or vehicle for a further 6 h (bortezomib) or 48 h (BMP2). Wnt3a-C2C12 cells were cultured as previously described (Nakashima *et al.* 2005). Total RNA was extracted from the cells, and then the expression of miR-206 and miR-34b was

estimated. The expression levels were normalized against those of sno234 RNA. Data are shown as means ± S.D. Each assay represents a separate experiment performed in triplicate. Asterisks indicate significant differences ($p < 0.05$, t test for paired data).

Figure 3

Proteasome inhibitors do not enhance either β-catenin-mediated or Smad1/4-mediated transcriptional activation

(A and C) C2C12 cells were transiently transfected in 24-well plates with 0.1 μg of a reporter plasmid (Top-flash) (A) or a IdWT4F-luc (C). After the cells were cultured without or with lactacystin (5 nM), epoxomicin (5 nM), MG-132 (5 nM), and bortezomib (2 nM) for a further 48 h, luciferase activity was determined. Transfection of β-cateninΔGSK (A) or addition of BMP2 (300 ng/mL) were used as positive controls. The activity is represented as the relative induction compared to Top-flash (A) or IdWT4F-luc (C) with vehicle. (B) C2C12 cells cultured without or with indicated doses of canonical Wnt inhibitor IWR-1 and bortezomib (100 nM) for a further 6 h. Total RNA was extracted from the cells and then the level of osteocalcin mRNA expression was estimated by qRT-PCR. (D) C2C12 cells cultured without or with the indicated doses of proteasome inhibitors or BMP2 for a further 24 h. Total RNA was extracted from the cells and then the level of Id1, Id2 and Id3 mRNA expression was estimated by qRT-PCR. Data are shown as means ± S.D. Each assay represents a separate experiment performed in triplicate. Asterisks indicate significant differences ($p < 0.05$, t test for paired data).

Figure 4

Regulation of osteocalcin promoter activity and Runx2 binding activity by proteasome inhibitors in C2C12 cells

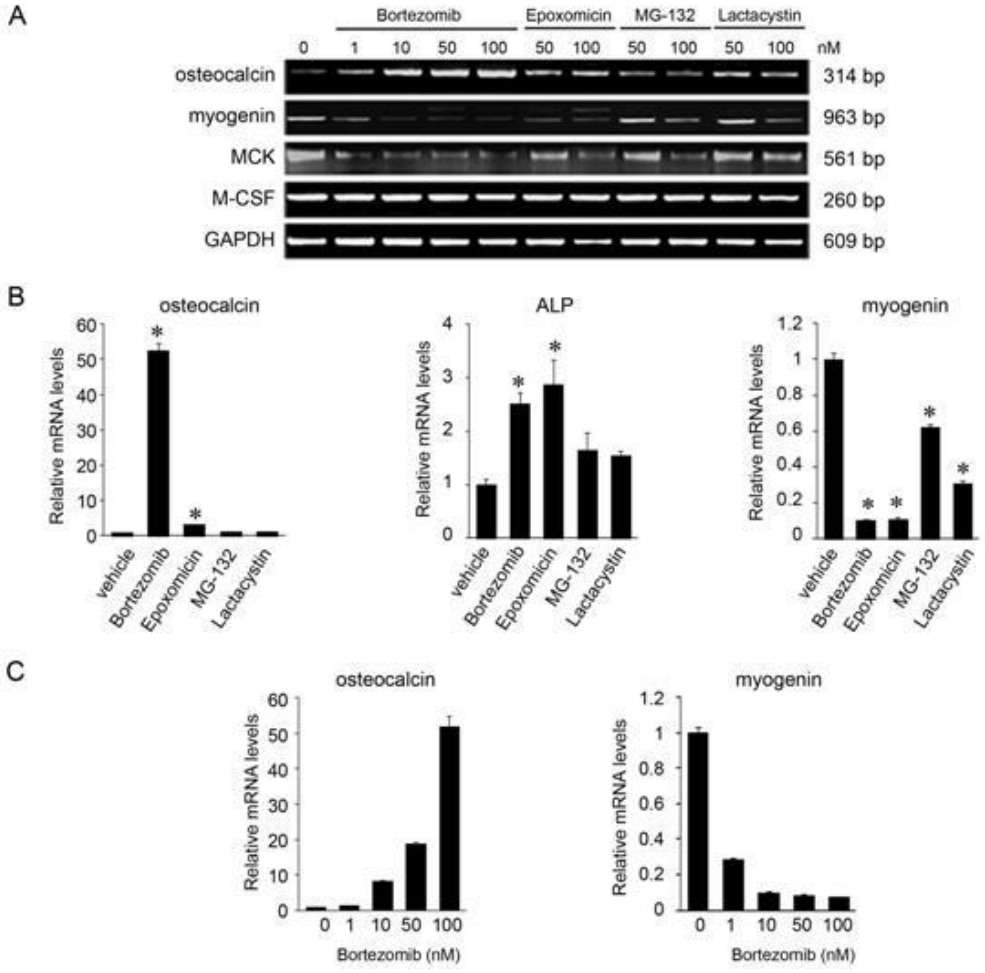
(A) C2C12 cells were transiently transfected in 24-well plates with 0.1 μg of a pOC-661luc. After the cells were cultured with lactacystin (5 nM), epoxomicin (5 nM), MG-132 (5 nM), bortezomib (2 nM) or vehicle for a further 48 h, luciferase activity was determined. The activity is represented as the relative induction compared to pOC-661luc with vehicle. (B) C2C12 cells were transiently transfected with 0.1 μg of a reporter plasmid (pOC-661luc, pmOSE1-luc or pmOSE2-luc). After the cells were cultured with or without 2 nM bortezomib for a further 24 h, luciferase activity was determined. The activity is represented as the relative induction compared to pOC661-luc with vehicle. (C) C2C12 cells were cultured with 100 nM bortezomib or vehicle for a further 3 h. Nuclear lysates were prepared and Runx2 binding activity was quantified. The competitors - wild type (WT) and mutated (MT) Runx2 consensus binding sites were used at a 500-fold molar excess. Nuclear lysates from osteoblastic SaOS-2 cells were used as the positive control. Results are expressed as a fold increase in the bortezomib-treated lysates compared with the vehicle control lysates. Data are shown as means ± S.D. Each assay represents a separate experiment performed in triplicate. Asterisks indicate significant differences ($p < 0.05$, t test for paired data).

Figure 5

Regulation of interaction of Runx2 at OSE2 site and Runx2 protein levels by bortezomib in C2C12 cells

C2C12 cells were cultured without or with 100 nM of bortezomib for a further 6 h in 10% FBS. (A) ChIP assay. Protein/DNA complexes from cells were precipitated without antibody (input), or with a Runx2 antibody or IgG. PCR amplification was performed using primers for OSE1 (P3/P4) or OSE2 (P1/P2). The positions used for PCR analyses of ChIP DNAs are shown (upper). PCR using input DNA was used as the positive control. PCR using primers P5/P6 of mOG2 gene region (+177/+311) was used as the negative control. (B) Effect of bortezomib on Runx2 protein levels. The levels of Runx2 protein in the cells were determined by Western blot analysis using a Runx2 antibody (top blot). Protein from MC3T3-E1 cells was used as the positive control. Blots were re-probed for β-actin as control (bottom blot).

Figure 1


A
Bortezomib
Epoxomicin
MG-132
Lactacystin
0 1 10 50 100 50 100 50 100 50 100 nM
osteocalcin
myogenin
MCK
M-CSF
GAPDH
314 bp
963 bp
561 bp
260 bp
609 bp
B
osteocalcin
ALP
myogenin
Relative mRNA levels
vehicle
Bortezomib
Epoxomicin
MG-132
Lactacystin
C
osteocalcin
myogenin
Relative mRNA levels
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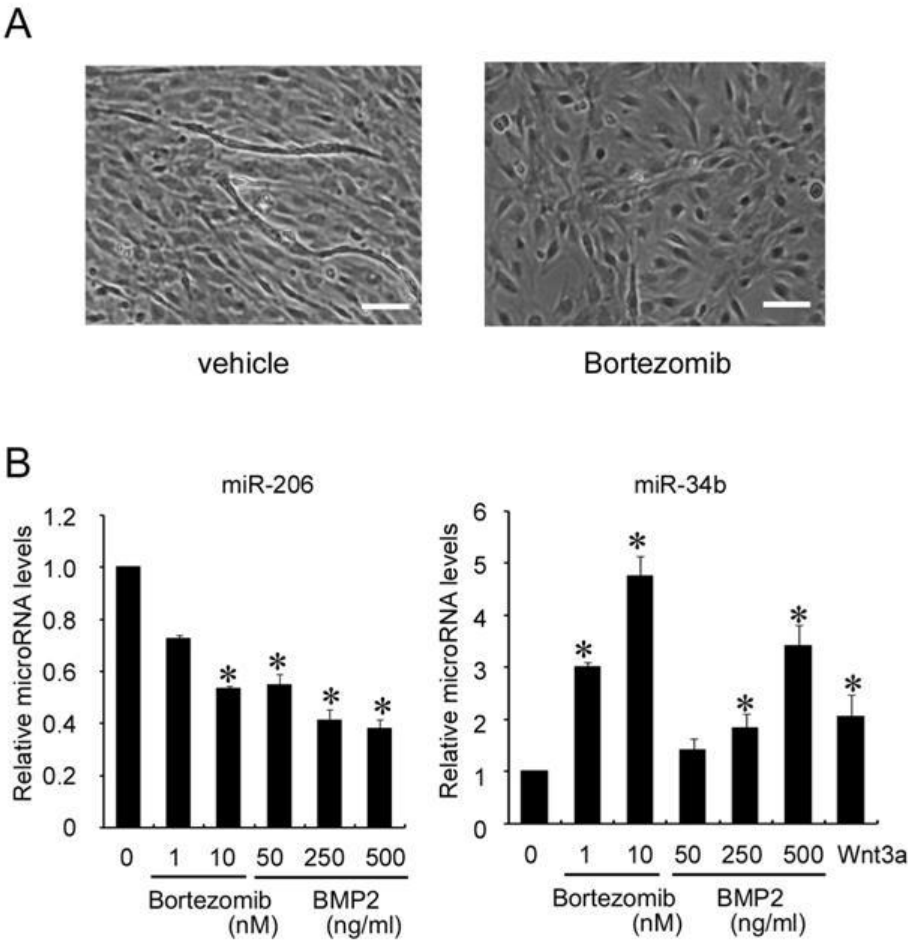
Bortezomib (nM)

Figure 2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vehicle
Bortezomib
B
miR-206
Relative microRNA levels
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1.0
0.8
0.6
0.4
0.2
0
0 1 10 50 250 500
Bortezomib (nM)
BMP2 (ng/ml)
miR-34b
Relative microRNA levels
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5
4
3
2
1
0
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Bortezomib (nM)
BMP2 (ng/ml)

Figure 3

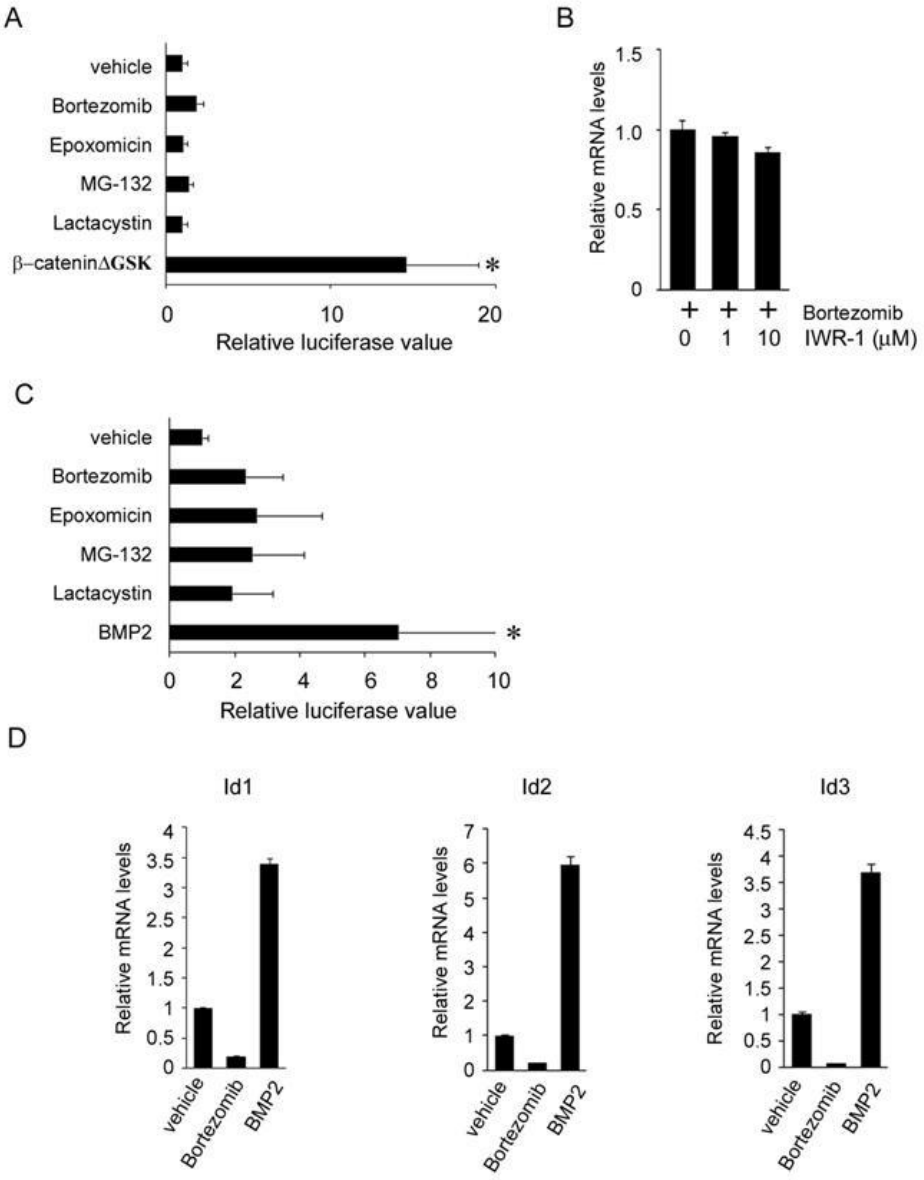

A
vehicle
Bortezomib
Epoxomicin
MG-132
Lactacystin
β–cateninΔGSK
*
0
10
20
Relative luciferase value
B
Relative mRNA levels
1.5
1.0
0.5
0
+ + +
0 1 10
Bortezomib
IWR-1 (μM)
C
vehicle
Bortezomib
Epoxomicin
MG-132
Lactacystin
BMP2
*
0 2 4 6 8 10
Relative luciferase value
D
Id1
Id2
Id3
Relative mRNA levels
vehicle
Bortezomib
BMP2

Figure 4

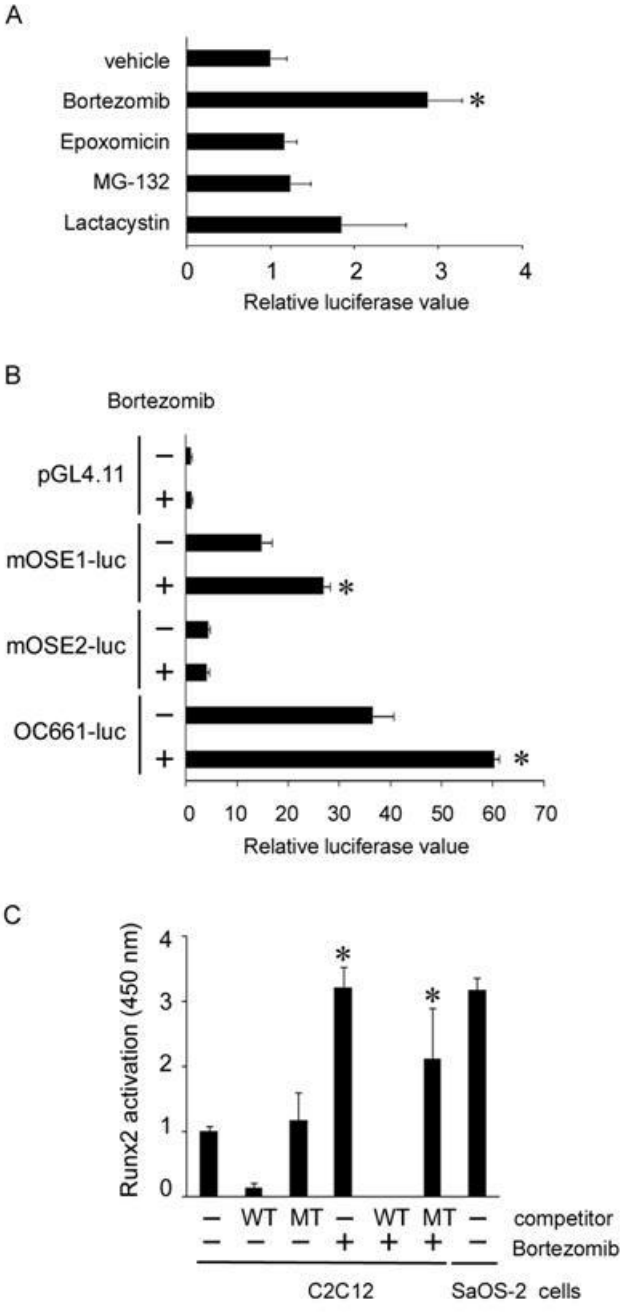
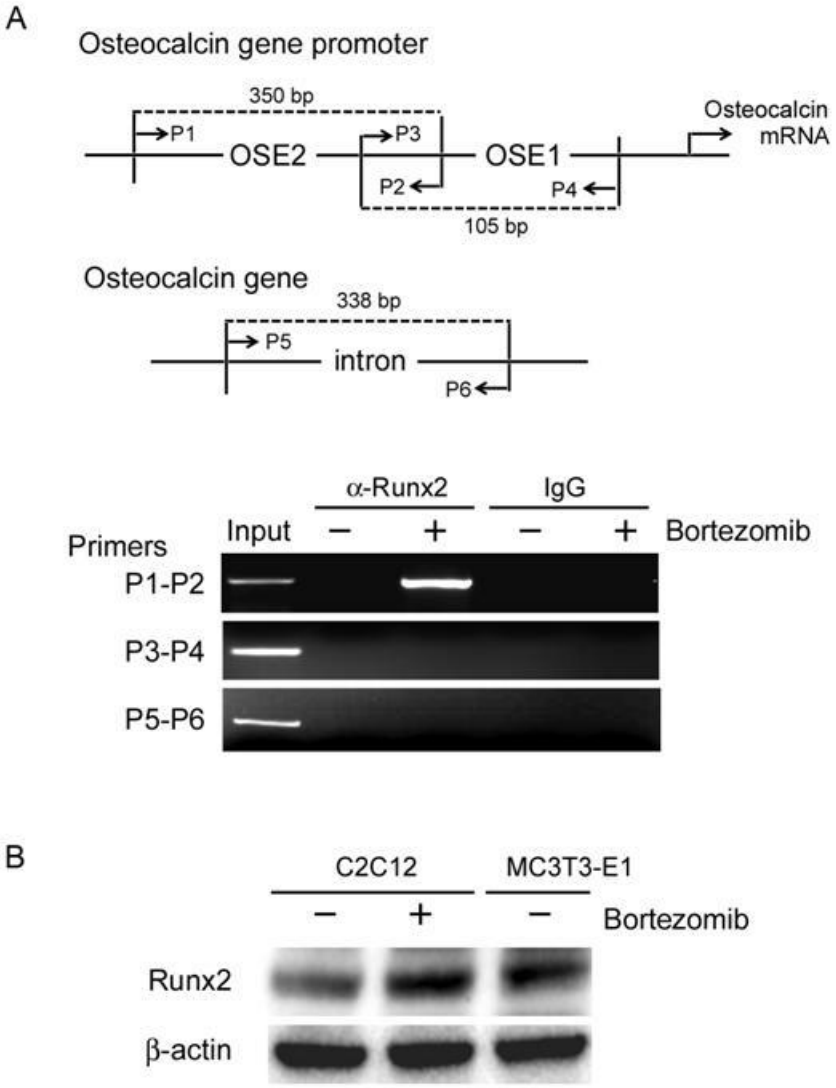

A
vehicle
Bortezomib
Epoxomicin
MG-132
Lactacystin
*
0 1 2 3 4
Relative luciferase value
B
Bortezomib
pGL4.11
mOSE1-luc
mOSE2-luc
OC661-luc
− +
*
*
0 10 20 30 40 50 60 70
Relative luciferase value
C
Runx2 activation (450 nm)
0 1 2 3 4
*
*
− WT MT − WT MT − competitor
− − − + + + − Bortezomib
C2C12
SaOS-2 cells

Figure 5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P1
OSE2
P3
P2
OSE1
P4
105 bp
Osteocalcin
mRNA
Osteocalcin gene
338 bp
P5
intron
P6
α-Runx2
IgG
Primers
Input
−
+
−
+
Bortezomib
P1-P2
P3-P4
P5-P6
B
C2C12
MC3T3-E1
−
+
−
Bortezomib
Runx2
β-actin