Biochemical and Biophysical Research Communications 457 (2015) 451–456

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Bcl6 promotes osteoblastogenesis through Stat1 inhibition

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ARTICLE INFO

Article history:
Received 26 December 2014
Available online 15 January 2015

Keywords:
Osteoblast
Differentiation
Bone
Bcl6
Stat1

ABSTRACT

Bone mass is tightly controlled by a balance between osteoclast and osteoblast activities. Although these cell types mature via different pathways, some factors reportedly regulate differentiation of both. Here, in a search for factors governing osteoblastogenesis but also expressed in osteoclasts to control both cell types by one molecule, we identified B cell lymphoma 6 (Bcl6) as one of those factors and show that it promotes osteoblast differentiation. Bcl6 was previously shown to negatively regulate osteoclastogenesis. We report that lack of Bcl6 results in significant inhibition of osteoblastogensis *in vivo* and *in vitro* and in defects in secondary ossification center formation *in vivo*. Signal transducer and activator of transcription 1 (Stat1) reportedly attenuates osteoblast differentiation by inhibiting nuclear translocation of runt-related transcription factor 2 (Runx2), which is essential for osteoblast differentiation. We found that lack of Bcl6 resulted in significant elevation of Stat1 mRNA and protein expression in osteoblasts and showed that Stat1 is a direct target of Bcl6 using a chromatin immune-precipitation assay. Mice lacking both Bcl6 and Stat1 (DKO) exhibited significant rescue of bone mass and osteoblastic parameters as well as partial rescue of secondary ossification center formation compared with Bcl6-deficient mice *in vivo*. Altered osteoblastogenesis in Bcl6-deficient cells was also restored in DKO *in vitro*. Thus, Bcl6 plays crucial roles in regulating both osteoblast activation and osteoclast inhibition.

1. Introduction

Bone homeostasis is regulated cooperatively by bone-forming osteoblasts and bone-resorbing osteoclasts, and such activity is termed coupling [1–3]. To date, several transcription factors essential for either osteoblast or osteoclast differentiation have been identified. Runt-related transcription factor 2 (Runx2) and Sp7 were both identified as essential for osteoblastogenesis, and both Runx2- or Sp7-deficient mice exhibit complete abrogation of bone formation [4–6]. Likewise, c-Fos and nuclear factor of activated T cells 1 (NFATc1) were both found to be essential for osteoclast differentiation, and lack of either c-Fos or NFATc1 resulted in failure of osteoclastogenesis [7,8]. Sp7 reportedly acts downstream of Runx2 in osteoblasts [6], while NFATc1 was shown to be a c-Fos target in osteoclasts [7,9]. B cell lymphoma 6 (Bcl6) is a transcriptional repressor so named because it was identified as a transcript in B cell lymphoma cells [10,11]. Previously, we demonstrated that Bcl6 inhibits osteoclast differentiation by attenuating transcription of osteoclastic genes, such as *NFATc1* [12]. Signal transducer and activator of transcription 1 (Stat1) has been shown to inhibit osteoblastogenesis by interacting with Runx2 and blocking its nuclear translocation [13]. However, how Stat1 expression is regulated in osteoblasts is not known.

To date, various molecules including Semaphorin 3A [14], Zfp521-Ebf1 [15], DC-STAMP [16], and Atp6v0d2 [17] have all been shown to regulate both osteoblasts and osteoclasts by expressed in each cell type or through regulation of osteoclast activity, and all

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http://dx.doi.org/10.1016/j.bbrc.2015.01.012
0006-291X/

are considered therapeutic targets in strategies to increase bone mass.

Here, we found that Bcl6 is expressed in osteoblasts and demonstrate that it regulates osteoblastogenesis through Stat1 inhibition. Bcl6-deficient mice exhibited significant reduction of bone mass and attenuated osteoblast differentiation with significant elevation of Stat1 expression in osteoblasts.

2. Materials and methods

2.1. Mice

C57BL/6 background wild-type mice were purchased from Sankyo Labo Service (Tokyo, Japan). *Bcl6*$^{-/-}$ and *Stat1*$^{-/-}$ mice were established as previously described [18,19], Bcl6 KO, Bcl6-Stat1 DKO, and control littermates were injected intraperitoneally with 16 mg/kg calcein (Dojindo Co.) at 6 and 1 days before sacrifice to evaluate bone formation rate, and were necropsied at 6 weeks of age. Hindlimbs were removed, fixed with 70% ethanol, and subjected to DEXA analysis to measure bone mineral density, for micro CT analysis and for bone-histomorphometric analysis, as previously described [12,20]. Animals were maintained under specific pathogen-free conditions in animal facilities certified by the Keio University School of Medicine animal care committee. All animal procedures were approved by the Keio University School of Medicine animal care committee.

2.2. Cell culture

To assess osteoblast formation *in vitro*, primary osteoblasts isolated from newborn mouse calvaria were cultured for 72 h in αMEM (Sigma–Aldrich Co., St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS, JRH Biosciences Lenexa, KS, USA) supplemented with or without BMP2 (300 ng/ml, PeproTech Ltd., Rocky Hill, NJ, USA) for three days. Osteoblastogenesis was evaluated by Alp staining and realtime or RT PCR analysis. For osteoclast culture, osteoclast progenitor cells were isolated from mouse BM cells and cultured in the presence of M-CSF (50 ng/ml) and recombinant soluble RANKL (25 ng/ml, PeproTech Ltd., Rocky Hill, NJ, USA) as described [12,20].

For realtime PCR, total RNA was isolated from cultured cells using an RNeasy mini kit (Qiagen), and cDNA was synthesized using oligo (dT) primers and reverse transcriptase (Wako Pure Chemicals Industries). Quantitative PCR was performed using SYBR Premix ExTaq II reagent and a DICE Thermal cycler (Takara Bio Inc., Shiga, Japan), and results were quantified using the ddCt method. *β-actin* (*Actb*) expression served as an internal control. Primers for *Bcl6*, *Alp*, *Col1a1*, *Sp7* and *Actb* were as follows.

Bcl6-forward: 5′-AGACGCACAGTGACAAACCATACA-3′
Bcl6-reverse: 5′-CTCCACAAATGTTACAGCGATAGG-3′
Alp-forward: 5′-CACCATTTTTAGTACTGGCCATCG-3′
Alp-reverse: 5′-GCTACATTGGTGTTGAGCTTTGG-3′
Col1a1-forward: 5′-CATGTTCAGCTTTGTGGACCTC-3′
Col1a1-reverse: 5′-CCTTAGGCCATTGTGTATGCAG-3′
Sp7-forward: 5′-GATGGCGTCCTCTCTGCTTG-3′
Sp7-reverse: 5′-AGGGCTAGAGCCGCCAAAT-3′
β-actin-forward: 5′-TGAGAGGGAAATCGTGCGTGAC-3′
β-actin-reverse: 5′-AAGAAGGAAGGCTGGAAAAGAG-3′

For western blotting, whole cell lysates were prepared using RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 10 mM Tris–HCl, pH 7.5) supplemented with a protease inhibitor cocktail (Sigma–Aldrich Co.). Equivalent amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane (EMD Millipore Corporation). Proteins were detected using anti-Stat1 (Cell Signaling Technology, Inc.) and anti-Actin (Sigma–Aldrich Co., St Louis, MO) antibodies. No bands were detected using the anti-Stat1 antibody in Stat1-deficient cell lysates (data not shown).

For immunohistochemical staining, cultured cells were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature. Subsequently, cells were incubated in 5% BSA-PBS and then stained with rabbit anti-Runx2 antibody (Santa Cruz) followed by alexa488-conjugated goat anti-rabbit Igs' antibody (Invitrogen Corp.). Nuclei were counterstained using DAPI (Chemical Dojin).

2.3. Chromatin immunoprecipitation (ChIP)

ChIP was performed on osteoblastic MC3T3E1 cells using the ChIP-IT Enzymatic Kit (ActivMotif Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. Immunoprecipitation was performed using a rabbit anti-Bcl6 antibody (N-3, Santa Cruz) as described [21]. DNA was purified using a Qiaquick PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA) and analyzed using the following primers: *Stat1* promoter: 5′-GAGTCAGTTCTGTGATGCCTTTG-3′ (sense) and 5′-TCTAAAGAGTGAGTTCCAGGACA-3′ (antisense).

2.4. Statistical analyses

Statistical analyses were performed using an unpaired two-tailed Student's *t*-test and ANOVA ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant, throughout the paper). All data are expressed as the mean ± SD.

3. Results

3.1. Bcl6 is expressed in osteoblasts and required for osteoblastogenesis

Previously, we reported that Bcl6 negatively regulates osteoclastogenesis [12], however, it was not known whether it functions in osteoblasts. Thus, we analyzed *Bcl6* expression in primary osteoblasts using realtime PCR and found that it is expressed in osteoblasts at levels similar to those seen in osteoclasts (Fig. 1A). As previously reported [12], we found that osteoclastogenesis was activated in Bcl6-deficient mice. However, when we analyzed osteoblastic parameters in Bcl6-deficient mice, we found that osteoblast surface per bone surface (Ob.S/BS) was significantly low in Bcl6-deficient compared with control mice *in vivo* (Fig. 1B). We then isolated osteoblastic cells from Bcl6-deficient and wild-type neonatal calvaria, cultured them with or without bone morphogenetic protein 2 (BMP2) to promote osteoblast differentiation, and evaluated osteoblastogenesis by alkaline phosphatase (Alp) staining (Fig. 1C) and realtime PCR for osteoblastic factors such as *Alp*, *Col1a1* and *Sp7* (Fig. 1D). We found that osteoblast differentiation was inhibited in Bcl6-deficient compared with wild-type cells, as determined by Alp staining (Fig. 1C). Likewise, *Alp*, *Col1a1* and *Sp7* expression was significantly inhibited in Bcl6-deficient compared with wild-type cells (Fig. 1D). In contrast, osteoblastic MC3T3-E1 cells overexpressing Bcl6 exhibited significantly accelerated osteoblast differentiation (Fig. S1), suggesting that Bcl6 regulates osteoblastogenesis.

3.2. Stat1 is negatively regulated by Bcl6 in osteoblasts

Next, we analyzed molecular mechanisms underlying regulation of osteoblastogenesis by Bcl6. To do so, we analyzed activation of Smad 1,5,8, which are required to promote osteoblast

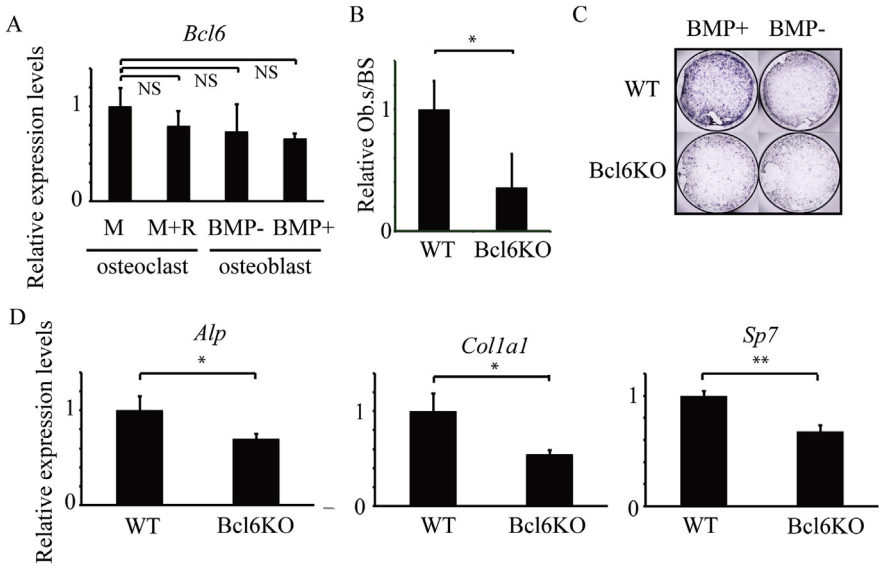

Fig. 1. Bcl6 KO mice exhibit inhibited osteoblastogenesis *in vivo* and *in vitro*. (A) *Bcl6* expression in osteoblasts or osteoclasts was determined by realtime PCR. M, M-CSF alone, M + R, M-CSF plus RANKL. Data represent mean *Bcl6* expression relative to *β-actin* ± SD (NS: not significant, $n = 3$). (B) Bone histomorphometrical analysis of osteoblast surface per bone surface (Ob.S/BS) of tibias from six weeks old wild-type (WT) and Bcl6 KO (KO) female mice. Data are mean relative values ± s.d. of Ob.S/BS. ($^*P < 0.05$, $n = 5$). (C, D) Primary osteoblasts isolated from wild-type (WT) and Bcl6 KO (KO) mice were cultured in the presence or absence of BMP2 (300 ng/ml) for three days, and osteoblastogenesis was evaluated by Alp staining (D) or realtime PCR (E). Data represent means ± s.d of *Alp*/*β-actin*, *Col1a1*/*β-actin* or *Sp7*/*β-actin* levels ($^*P < 0.05$, $^{**}P < 0.01$, $n = 3$). Representative data of two (B) and at least three (A, C and D) independent experiments are shown.

differentiation following BMP2 stimulation, using western blotting of osteoblast lysates [22,23]. We found that Smad 1,5,8 were equivalently phosphorylated in wild-type and Bcl6-deficient osteoblasts following BMP2 treatment (Fig. S2), suggesting that BMP2 signal was transduced to Smads in Bcl6-deficient cells.

Since Bcl6 is a transcriptional repressor, we searched for candidate targets in osteoblasts by screening for molecules that 1) exhibited Bcl6 consensus binding sites in their promoter regions and 2) regulated osteoblast differentiation. Based on these criteria, we identified *Stat1* as a candidate Bcl6 target (Fig. 2A). A Bcl6 consensus binding site located upstream of the *Stat1* gene was conserved between humans and mice (Fig. 2A), and Stat1 reportedly negatively regulates osteoblastogenesis [13]. We then undertook a chromatin immune-precipitation assay in osteoblastic MC3T3E1 cells and found that Bcl6 was recruited to the *Stat1* promoter in osteoblasts (Fig. 2B). Stat1 expression in Bcl6-deficient osteoblasts was significantly higher than that seen in wild-type osteoblasts at both the mRNA and protein levels (Fig. 2C, D). These results suggest that *Stat1* is directly repressed by Bcl6 in osteoblasts.

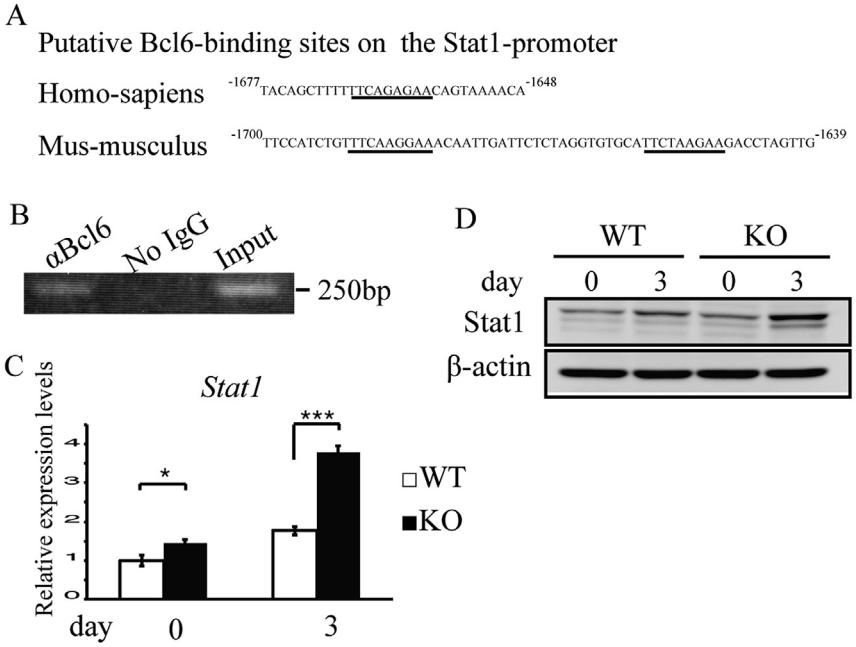

Fig. 2. Stat1 is a direct target of Bcl6 in osteoblasts. (A) Promoter sequence of human and mouse *Stat1* genes. Underlining indicates Bcl6 consensus binding sites. (B) Recruitment of Bcl6 to the *Stat1* promoter was analyzed in MC3T3E1 cells by a ChIP assay. (C, D) Stat1 expression was determined by realtime PCR (C) and western blotting (D). Data represent means ± s.d of *Stat1*/*β-actin* ($^*P < 0.05$, $^{***}P < 0.001$, $n = 3$). Representative data of two (B) and at least three (C, D) independent experiments are shown.

3.3. Stat1 loss rescues reduced bone mass seen in Bcl6-deficient mice

We next asked whether elevated Stat1 expression in Bcl6-deficient osteoblasts might underlie reduced bone mass and altered osteoblastogenesis by generating Bcl6 and Stat1 double mutant mice (DKO). DEXA analysis demonstrated that reduced bone mass seen in Bcl6 single knockout (Bcl6 KO) mice was significantly rescued in DKO mice (Fig. 3A). Altered trabecular bone formation in Bcl6-deficient mice, as assessed by toluidine blue staining and micro CT analysis, was restored in DKO mice (Fig. 3B, C). Bcl6-deficient mice exhibited impaired secondary ossification center formation, an effect partially rescued in DKO mice (Fig. 3B).

Bcl6-deficient mice show altered bone parameters such as significantly decreased bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) as well as elevated trabecular separation (Tb.Sp). Bone morphogenetic

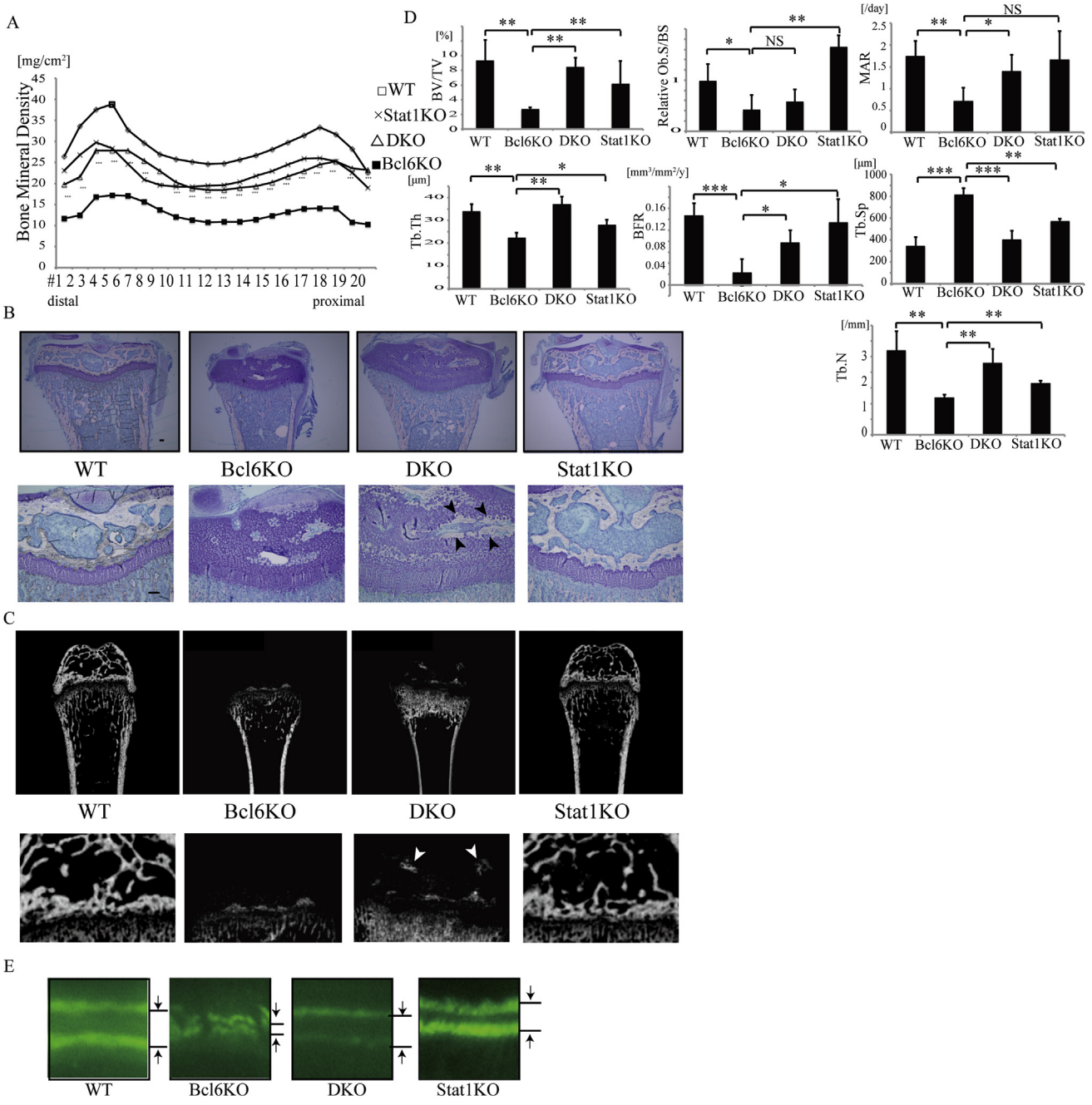

Fig. 3. Elevated Stat1 expression inhibits bone formation in Bcl6 KO mice *in vivo*. (*A*) Bone mineral density (BMD) of an equal longitudinal division of femurs from six weeks old wild-type (WT, open boxes), Bcl6 KO (Bcl6 KO, closed boxes), Stat1 KO (Stat1 KO, crosses) and Bcl6-Stat1 DKO female mice (DKO, open triangles). Data represent mean ± s.d of BMD (n = 3–5). (B, C) Toluidine blue staining (B) and micro CT analysis (C) of WT, Bcl6 KO, Stat1 KO and DKO bones. Lower panels in B and C show higher magnification images of upper panels. Arrowheads indicate ossification (B and C). Bar = 100 µm. (D) Bone morphogenetic analysis of six weeks old WT, Bcl6 KO, Stat1 KO and DKO female animals. Data represent means ± s.d of bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), osteoblast surface per bone surface (Ob.S/BS), mineral apposition rate (MAR) and bone formation rate (BFR) in WT, Bcl6 KO, Stat1 KO and DKO. Data represent means ± s.d (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS: not significant, $n = 3–5$). (E) Representative images of calcein labeling in WT, Bcl6 KO, Stat1 KO and DKO tibia bones. Calcein was injected into peritoneal cavity of WT, Bcl6 KO, Stat1 KO or DKO mice, one and five days before sacrifice.

analysis demonstrated that altered bone volume and bone-forming parameters, such as BV/TV, Tb.Th, Tb.N, Tb.Sp, MAR and BFR, seen in Bcl6 KO mice were significantly rescued in DKO mice (Fig. 3D). Inhibited osteoblastic parameters in Bcl6-deficient compared with wild-type mice, as shown by reduced osteoblast surface per bone surface (Ob.S/BS), mineral apposition rate (MAR) and bone formation rate (BFR), were all increased in DKO mice (Fig. 3D, E). We previously showed that osteoclasts are activated in Bcl6-deficient mice [12], and it is possible that increased bone mass seen in DKO mice was due to decreased osteoclast activity. However, Stat1 deficiency also reportedly elevates osteoclast formation [13].

3.4. *Stat1 deficiency rescues altered osteoblastogenesis in Bcl6-deficient osteoblasts*

To determine if elevated bone mass and osteoblastic activity seen in DKO compared to Bcl6-deficient mice were due to increased osteoblastogenesis, we undertook *in vitro* culture of osteoblasts from wild-type (WT), Bcl6-deficient (Bcl6 KO), Stat1-deficient (Stat1 KO) or DKO mice and stimulated cells with BMP2 (Fig. 4). Osteoblastogenesis was evaluated by Alp staining and *Alp*, *Col1a1* and *Sp7* gene expression. We found that osteoblastogenesis, which was significantly inhibited in Bcl6-deficient compared with wild-type osteoblasts, was significantly restored in DKO osteoblasts (Fig. 4A, B). Stat1 reportedly attenuates osteoblastogenesis by inhibiting nuclear translocation of Runx2, which is required for osteoblast differentiation [13,24]. Indeed, immunostaining showed that nuclear translocation of Runx2 by BMP2 seen in wild-type osteoblasts was significantly inhibited in Bcl6-deficient osteoblasts but rescued in DKO mice (Fig. 4C, D).

4. Discussion

Osteoclasts and osteoblasts cooperatively regulate bone mass [2,3]. Osteoclast differentiation is promoted by stimulation with the cytokines M-CSF and RANKL, while osteoblastogenesis is activated by BMP2 or Wnt [25,26]. Various other factors are activated downstream of these regulators during cell differentiation [27,28]. Osteoclasts and osteoblasts are derived from hematopoietic and mesenchymal stem cells, respectively, and their differentiation is regulated by cell type specific regulators such as c-Fos and NFTAc1 in osteoclasts or Runx2 and Sp7 in osteoblasts [4,6,29]. However, here, we found that Bcl6, which inhibits osteoclast formation [12], promotes osteoblastogenesis. Thus, Bcl6 plays crucial roles in the differentiation of both cell types. We previously showed that Bcl6 inhibits expression of osteoclastic genes such as *NFATc1*, *Cathepsin K* and *DC-STAMP* in osteoclast progenitors [12], while the present study demonstrates that Bcl6 alters Stat1 expression in osteoblasts (Fig. 4E). Bcl6 expression was not affected by BMP2 treatment and was stable during osteoblastogenesis. Bcl6 loss in osteoblasts significantly elevated Stat1 expression, attenuating Runx2 nuclear translocation stimulated by BMP2. Thus, Bcl6 plays no role in basal osteoblastogenesis in the absence of BMP2 but rather inhibits osteoblast differentiation by attenuating Runx2 nuclear activity (Fig. 4E). We previously reported that decreased bone mass seen in Bcl6-deficient mice is due to accelerated osteoclastogenesis [12]. However, our results suggest that reduced bone mass in Bcl6-deficient mice could be due to both inhibited osteoblastic activity and accelerated osteoclast formation. To date, bone diseases such as osteoporosis have been treated with either anti-bone resorptive or bone-forming agents, and reagents regulating both cell types

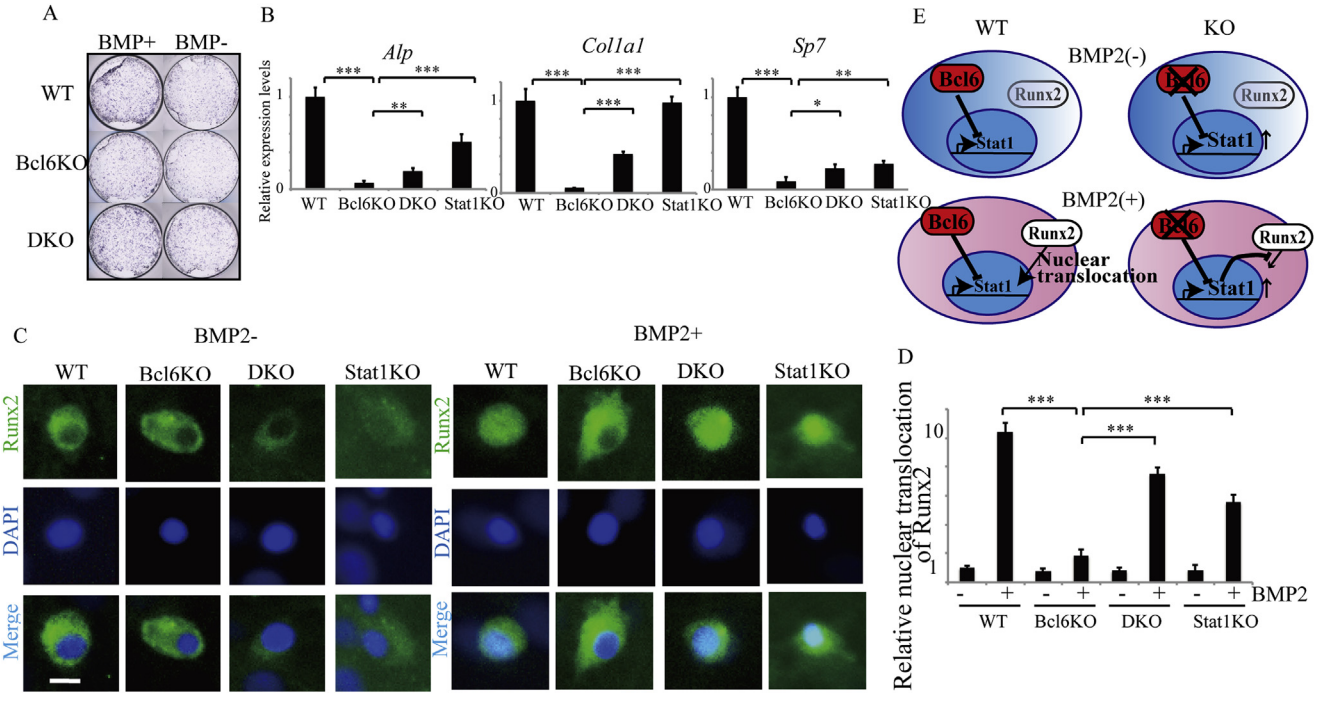

Fig. 4. Stat1 deficiency rescues inhibited osteoblastogenesis in Bcl6-deficient cells. (A, B) Primary osteoblasts were isolated from WT, Bcl6 KO, Stat1 KO and DKO mice and cultured in the presence or absence of BMP2 (300 ng/ml) for three days. Osteoblastogenesis was then evaluated by Alp staining (A) and realtime PCR for *Alp*, *Col1a1* and *Sp7* (B). Data represent means ± s.d of *Alp*/*β-actin*, *Col1a1*/*β-actin* or *Sp7*/*β-actin* levels ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$). (C, D) Primary osteoblasts isolated from WT, Bcl6 KO, Stat1 KO and DKO mice were cultured with or without BMP2 (300 ng/ml) for three days, stained with anti-Runx2 antibody and DAPI and observed under a fluorescence microscope (C), and cells showing nuclear Runx2 were counted (D). Data represent relative means ± s.d. of cells exhibiting nuclear Runx2 ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 3$). Bar = 10 μm. Representative data of two independent experiments are shown. (E) Bcl6 is required to promote osteoblastogenesis by inhibiting expression of *Stat1*, an attenuator of Runx2, a transcription factor essential for osteoblast differentiation. Bcl6 expression is stable during osteoblast differentiation and functions in osteoblastogenesis by inhibiting Stat1 expression at a time when Runx2 nuclear translocation is stimulated by BMP2.

concomitantly to increase bone mass have been sought. Interestingly, recent studies report several molecules that regulate both osteoclasts and osteoblasts [14,15,17], and here we demonstrate that Bcl6 could serve as a target to increase bone mass by both inhibiting osteoclasts and activating osteoblasts. Since deregulated Bcl6 expression reportedly promotes multiple myeloma cell growth [30], Stat1 inhibition could be a better option to activate osteoblasts in bones. Currently, a Stat1 inhibitor is being assessed to treat hematologic malignancies [31].

Runx2 is required to promote osteoblast differentiation, and Runx2-deficient mice show complete lack of bone formation leading to peri-neonatal lethality [4]. We demonstrated here that Bcl6 directly regulates expression of Stat1, an attenuator of Runx2, in osteoblasts, and that lack of Bcl6 significantly elevates Stat1 levels; however, Bcl6-deficient mice did not exhibit peri-neonatal lethality, although bone formation was severely altered. These results suggest that the Bcl6-Stat1 axis is not the sole system regulating Runx2 function. In addition, some phenotypes observed in Bcl6-deficient mice were fully restored by Stat1 deletion, while others were not or were only partially rescued, suggesting that Bcl6 and Stat1 may also have independent activities. Furthermore, Stat1 deletion in a Bcl6 KO background results in more moderate rescue of Bcl6 KO-associated phenotypes *in vitro* than it does in *in vivo*, suggesting that the Bcl6-Stat1 axis likely plays both direct and indirect roles in regulating osteoblastogenesis. Further studies are needed to determine how osteoblast differentiation is regulated by Bcl6 and Stat1.

Since osteoblast activity is coupled with osteoclastic activity, severe inhibition of osteoclasts by reagents such as bisphosphonate also alters osteoblast activity. Although, the number of patients diagnosed was small, some investigators report that inhibition of bone turnover beyond physiological levels promotes development of osteonecrosis of the jaws and severely suppressed bone turnover (SSBT) [32,33]. Osteoclastogenesis is reportedly activated in either Bcl6- or Stat1-deficient mice, as observed by our group or others, respectively [12,13]. However, we found that DKO mice exhibited higher bone mass compared with Bcl6-deficient mice (Fig. 3). The net increase in bone mass seen in DKO mice suggests that Stat1 inhibition is dominant over activation of osteoclasts and that Stat1 could be a therapeutic target to increase bone mass without inhibiting bone turnover, even under conditions of osteoclast activation.

Conflict of interest

The authors have no conflicting financial interests.

Acknowledgments

T. Miyamoto was supported by a Grant-in-aid for Scientific Research, Japan.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.012.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.012.

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