Loss of cyclin-dependent kinase 1 impairs bone formation, but does not affect the bone-anabolic effects of parathyroid hormone

Bone mass is maintained by a balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Although recent genetic studies have uncovered various mechanisms that regulate osteoblast differentiation, the molecular basis of osteoblast proliferation remains unclear. Here, using an osteoblast-specific loss-of-function mouse model, we demonstrate that cyclin-dependent kinase 1 (Cdk1) regulates osteoblast proliferation and differentiation. Quantitative RT-PCR analyses revealed that Cdk1 is highly expressed in bone and is down-regulated upon osteoblast differentiation. We also noted that Cdk1 is dispensable for the bone-anabolic effects of parathyroid hormone (PTH). Cdk1 deletion in osteoblasts led to osteoporosis in adult mice due to low bone formation, but did not affect osteoclast formation in vivo. Cdk1 overexpression in osteoblasts promoted proliferation, and conversely, Cdk1 knockdown inhibited osteoblast proliferation and promoted differentiation. Of note, we provide direct evidence that PTH’s bone-anabolic effects occur without enhancing osteoblast proliferation in vivo. Furthermore, we found that Cdk1 expression in osteoblasts is essential for bone fracture repair. These findings may help reduce the risk of nonunion after bone fracture and identify patients at higher risk for nonresponse to PTH treatment. Collectively, our results indicate that Cdk1 is essential for osteoblast proliferation and that it functions as a molecular switch that shifts osteoblast proliferation to maturation. We therefore conclude that Cdk1 plays an important role in bone formation.

Bone mass is maintained by a balance between bone formation by osteoblasts and bone resorption by osteoclasts. Disruption of this balance in bone turnover leads to bone-related diseases such as osteoporosis. As the global population ages, the proportion of patients with osteoporosis has increased dramatically, a trend that is associated with enormous health care costs.

Treatment strategies for osteoporosis include inhibiting bone resorption (2), promoting bone formation (3), or both (4). Although many effective antiresorptive drugs are available, including bisphosphonates, estrogen, selective estrogen receptor modulators, and anti-receptor activator of NF-κB ligand antibodies, the only Food and Drug Administration-approved bone-anabolic drugs are parathyroid hormone (PTH) and PTH-related protein (5).

Growing clinical evidence suggests that intermittent PTH administration increases the number of osteoblasts, resulting in stimulation of new bone formation (6). However, the mechanisms by which PTH increases osteoblast numbers are not completely understood. For example, although PTH exerts an anabolic effect by reducing osteoblast apoptosis in mice (7), PTH-stimulated bone formation is associated with an increase in osteoblast apoptosis in humans (8). Moreover, the effects of PTH on osteoblast proliferation remain controversial. One study has shown that PTH inhibits osteoblast proliferation and increases differentiation (9), whereas others have found that PTH stimulates osteoblast proliferation (10, 11). Furthermore, although recent genetic studies have uncovered various mechanisms regulating osteoblast differentiation, including transcription factors, growth hormones, and noncoding RNAs (1, 12–14), the mechanisms regulating osteoblast proliferation are largely unknown. To develop a strategy to promote bone formation, investigations of the regulatory mechanisms of osteoblast proliferation are overdue.

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Loss of cyclin-dependent kinase 1 impairs bone formation

Generally, cell proliferation is promoted by the activity of cell cycle regulators (15). The cell cycle is regulated by cyclins and cyclin-dependent kinases (Cdks) (16). Cdks are a group of kinases consisting of 20 members in mammals, which play critical roles in cell cycle control, transcription, and development (15). However, despite the theoretical importance of Cdks, the physiological roles of Cdks in bone metabolism have not been defined. Moreover, no Cdks have thus far been found to regulate bone mass through in vivo cell-specific loss-of-function experiments.

In this study, we generated osteoblast-specific Cdk1-knockout mice to investigate the role of cyclin-dependent kinase 1 (Cdk1) in regulating bone formation.

Results

Cdk1 plays an important role in osteoblast proliferation

To investigate the functions of Cdks in bone, we first examined the expression of Cdks in the femurs of 3-month-old mice using quantitative PCR (qPCR) analysis. Cdk1 was expressed at higher levels than other Cdks. *, p < 0.05 versus Cdk1 expression. B, Cdk1 expression in murine tissues. Note that Cdk1 is expressed well in bone tissue. C, changes in Cdk1 mRNA and protein expression during osteoblast differentiation, as determined via qPCR (upper figure) and Western blotting (lower figure), respectively. Cdk1 expression gradually decreased over the course of osteoblast differentiation. *, p < 0.05 versus day 0. D, the relative number of MC3T3-E1 cells treated with vehicle or RO-3306 was counted for 5 days. Note that RO-3306–treated MC3T3-E1 cells showed impaired proliferation compared with proliferation of vehicle-treated cells. *, p < 0.05 versus controls. E, relative number of MC3T3-E1 cells infected with pcDNA (control) or pcDNA Cdk1 (Cdk1 overexpression) was counted at days 1, 3, and 5. Cdk1 overexpression significantly increased MC3T3-E1 cell numbers. *, p < 0.05 versus controls. All data represent the mean ± standard deviation (S.D.). n.s., not significant. ALP, alkaline phosphatase.
confirm Cdk1 expression in vitro, we used MC3T3-E1 cells, a murine preosteoblast cell line (12). Interestingly, Cdk1 expression gradually decreased during osteoblast differentiation (Fig. 1C). To study the importance of Cdk1 kinase activity, we tested whether inhibiting Cdk1 would affect osteoblast proliferation. To this end, we treated MC3T3-E1 cells with RO-3306, a specific inhibitor of Cdk1 (19). As expected, compared with the proliferation of vehicle-treated cells, the proliferation of MC3T3-E1 cells was significantly impaired by RO-3306 treatment (Fig. 1D), which agrees with the results in other types of cells (17, 19, 20). Conversely, overexpressing Cdk1 significantly promoted MC3T3-E1 cell proliferation (Fig. 1E). Taken together, these results indicate that Cdk1 plays an important role in osteoblast proliferation in vitro.

Osteoblast-specific Cdk1-knockout mice have low bone mass

Because Cdk1-null mice exhibit early embryonic lethality (17, 20), we generated conditional osteoblast-specific Cdk1-knockout mice to investigate the role of Cdk1 in bone metabolism. To achieve this, we crossed Cdk1lox/lox (hereafter, “control”) mice with transgenic mice expressing Cre recombinase under the control of the Osterix promoter (hereafter, “Osx–Cre mice”) to generate Osx–Cre/Cdk1lox/lox’ mice (hereafter, “Cdk1osb mice”) (21). These mutant mice were recovered in the expected Mendelian ratio, indicating that embryonic development can proceed without Cdk1 expression in osteoblasts. Although the deletion of Cdk1 was confirmed in the calvaria of 3-month-old mice by qPCR and Western blotting, no significant differences in the expression of other Cdks were detected (Fig. 2A and Fig. S1). Osx–Cre expression alone has been shown to negatively affect skeletal growth in young mice, but the effect of Cre is diminished by 12 weeks of age (22, 23). Thus, we decided to use 3-month-old mice for analysis. To confirm any potential effect of Osx–Cre on bone morphology in our studies, control mice were compared with Osx–Cre mice via microcomputer tomography (μCT) and histological analysis. Indeed, bone histomorphometric analysis of the vertebrae and μCT analysis of the distal femur in 3-month-old mice showed no significant differences in bone volume between control and Osx–Cre mice, in line with previous reports (Fig. S2). These results show that the Osx–Cre allele per se had a minimal effect on the bone histomorphometric and μCT analyses in our study. A bone histomorphometric analysis of the vertebrae of 3-month-old Cdk1osb mice demonstrated a significant decrease in bone mass compared with that of control mice as measured by BV/TV (Fig. 2B). This effect was likely caused by a significant decrease in the number of osteoblasts, which may result in a decrease in the bone formation rate, whereas the osteoclast surface was not affected (Fig. 2C). μCT analysis indicated that bone volume/total volume, bone mineral density, trabecular number, trabecular thickness, and cortical thickness were also decreased in the long bones of Cdk1osb mice compared with control mice (Fig. 2D). Accordingly, serum levels of procollagen type 1 N-terminal propeptide (P1NP), a biomarker correlated with bone formation, was decreased in Cdk1osb mice (Fig. 2E). However, serum C-terminal telopeptide of type 1 collagen (CTX-I), a biomarker for bone resorption, showed no difference between Cdk1osb mice and control mice (Fig. 2E). Collectively, these results indicate that Cdk1 is important for bone formation.

Osteoblast proliferation is decreased in osteoblast-specific Cdk1-knockout mice

We aimed to examine the molecular mechanism behind the decrease in osteoblast numbers observed in Cdk1osb mice. To test whether decreased bone formation was caused by altered osteoblast survival and/or proliferation, we performed TUNEL and BrdU assays and examined proliferating cell nuclear antigen (PCNA) and Ki67 expression. TUNEL assays performed in femoral sections from 3-month-old female mice failed to reveal any difference in apoptotic cells between Cdk1osb mice and control mice (Fig. 3A). However, BrdU assays performed in femoral sections from 3-month-old female mice suggested a 55% decrease in the number of proliferating osteoblasts in Cdk1osb mice compared with control mice (Fig. 3B). Moreover, there were fewer PCNA- and Ki67-positive osteoblasts in the Cdk1osb sections than in the control sections (Fig. 3C). To further test whether the decrease in bone formation was caused by decreased proliferative activity of osteoblastic progenitors and/or a decreased pool of osteoblast progenitors, we analyzed the expression of Osx and Runx2. Although Runx2 expression was similar in control and Cdk1osb mice, Osx expression levels were lower in Cdk1osb mice than in control mice (Fig. 3D). Thus, the decrease in bone formation is likely caused by decreased proliferative activity of osteoblastic progenitors rather than by a decreased progenitor pool. Collectively, these results indicate that Cdk1 plays an important role in osteoblast proliferation in vivo.

Cdk1 regulates osteoblast differentiation

Because Cdk1 regulates osteoblast proliferation, we wondered whether Cdk1 also affects osteoblast differentiation. To address this question in vitro, we isolated calvarial osteoblasts from WT mice, treated them with RO-3306 or vehicle, and then compared their behavior during differentiation. Cdk1-inhibited osteoblasts formed more mineralized nodules than vehicle-treated osteoblasts (Fig. 4A). To test whether osteogenic differentiation is affected by the Cdk1 status when progenitors are stimulated with osteogenic BMP2, we treated ST2 mesenchymal progenitor cells with RO-3306 followed by BMP2. As expected, inhibiting Cdk1 promoted osteoblastic differentiation of ST2 cells (Fig. 4B), as evidenced by alkaline phosphatase (Alp) and osteocalcin (Ocn) expression. Furthermore, knocking down Cdk1 with shRNA promoted osteoblast differentiation, as confirmed by the elevated expression of the osteoblast differentiation markers Col1a1 and Ocn (Fig. 4C). To address this question in vivo, we performed in situ hybridization analysis for osteoblast differentiation markers. Consistent with our observations in vitro, Cdk1 deletion resulted in accelerated osteoblast differentiation, which was confirmed by the elevated expression of the osteoblast differentiation markers Col1a1 and Ocn (Fig. 4D). In addition, qPCR analysis revealed increased expression of osteoblast differentiation marker genes (Alp, Col1a1, and Ocn) in the calvaria of 3-month-old Cdk1osb mice (Fig. 4E). These results indicate that the biological function of Cdk1 in osteoblasts correlates with their ability to differentiate. Over-
Loss of cyclin-dependent kinase 1 impairs bone formation

A

**Cdk1**

|       | Cdk1 ff | Cdk1 osb/− |
|-------|---------|------------|
| N.Ob/B.Pm (mm) | 28.07±0.97 | 12.24±6.98 * |
| BFR/BS (mcm³/mcm³/day) | 0.70±0.11 | 0.41±0.15 * |
| MAR (mcm/day) | 1.57±0.11 | 1.12±0.21 * |
| Oc.S/BS (%) | 11.58±3.37 | 12.31±3.40 n.s. |

B

**Male**

![Male Bone Images]

**Female**

![Female Bone Images]

C

**Calcein labeling**

**Toluidine blue staining**

**TRAP staining**

D

|       | Cdk1 ff | Cdk1 osb/− |
|-------|---------|------------|
| Tb.N (mm) | 3.33±0.47 | 2.60±0.34 * |
| Tb.Th (mcm) | 33.57±2.52 | 27.71±2.40 * |
| Tb.Sp (mcm) | 272.41±49.62 | 368.10±44.83 * |
| Cort.Th (mcm) | 176.87±9.47 | 153.26±14.94 * |
| BMD (mg/cm²) | 394.06±21.74 | 353.74±28.74 * |
| BV/TV (%) | 12.95±0.64 | 7.68±0.66 * |

E

**3D image**

![3D Images]

**P1NP**

![P1NP Graphs]

**CTX-1**

![CTX-1 Graphs]
all, Cdk1 is important for not only the proliferation but also the proper differentiation of osteoblasts.

**Cdk1 is dispensable for the bone-anabolic effect of PTH in vivo**

Intermittent PTH treatment enhances bone formation and increases osteoblast number by a variety of mechanisms, including stimulation of osteoblast proliferation (24). Because Cdk1 is important for osteoblast proliferation, we hypothesized that Cdk1 may be involved in the bone-anabolic response to intermittent PTH treatment in vivo. To test this hypothesis, Cdk1−/− mice and control mice were treated with either PTH or vehicle for 4 weeks (Fig. 5A). These mice were then analyzed by bone histomorphometry and μCT for bone parameters. As expected, PTH increased bone mass by 30% in control mice (Fig. 5B). Surprisingly, PTH also successfully increased BV/TV by 60% in Cdk1−/− mice (Fig. 5B) in the presence of low osteoblast proliferation. Bone histomorphometric analysis revealed that although the number of osteoblasts and the bone formation rate were substantially lower in Cdk1−/− mice compared to control mice, they were both increased to approximately the same levels by PTH (Fig. 5C). With regard to bone resorption, although there was a trend toward higher bone resorption induced by PTH administration in control and Cdk1−/− mice, there was no significant difference between control and Cdk1−/− mice (Fig. 5C). We then examined Ki67 expression to test whether the bone-anabolic effect of PTH in Cdk1−/− mice was caused by altered osteoblast proliferation. PTH treatment induced osteoblast proliferation, as shown by the markedly increased number of Ki67-positive cells in the control femoral sections; however, the effect of PTH on osteoblast proliferation was blunted in Cdk1−/− sections (Fig. 5D). Collectively, these results indicate that Cdk1 in osteoblasts may be dispensable for the bone-anabolic effect of intermittent PTH treatment, suggesting that the bone-anabolic effect of PTH is independent of osteoblast proliferative ability.

**Cdk1 is essential for bone fracture healing**

The bone fracture healing process consists of intramembranous and endochondral bone formation (25). To examine the role of Cdk1 during bone fracture repair, we performed femoral fractures on Cdk1−/− mice and control mice at 3 months of age. X-ray analysis revealed no significant differences between control and Cdk1−/− mice in callus formation at 6 weeks after surgery (Fig. 6A), indicating that the bone repair process was activated normally. μCT analysis demonstrated bony bridging across the fracture site at 6 weeks after surgery in control mice, suggesting bony union. However, no bony bridging was observed in Cdk1−/− mice, suggesting failed union (Fig. 6B). These results indicate that Cdk1 in osteoblasts is not essential for callus formation in bone fracture repair but is required for bony union. Finally, we tested whether the administration of PTH prevented nonunion after bone fracture in the Cdk1−/− mice. Indeed, the Cdk1−/− mice treated with PTH showed a trend toward a higher bony union rate (50%) than non-PTH–treated Cdk1−/− mice (Fig. 6C). In addition, the mean gap size was significantly lower in the PTH group than in the non-PTH group (41.5 versus 172.2 μm in the non-PTH–treated Cdk1−/− mice; p < 0.05) (Fig. 6D).

**Discussion**

In this study, we investigated the roles of Cdk1s in bone formation. First, we showed that Cdk1 is expressed in bone and that its protein levels are down-regulated upon osteoblast differentiation. Then, we demonstrated that deletion of Cdk1 in osteoblasts leads to osteoporosis due to a reduced number of osteoblasts and low bone formation. Finally, we found that the bone-anabolic effect of PTH is achieved without promoting osteoblast proliferation in vivo. Furthermore, we demonstrated that Cdk1 in osteoblasts plays an important role in bone fracture repair. To the best of our knowledge, this is the first study to demonstrate through in vivo osteoblast-specific loss-of-function experiments that Cdk1 has an important role in bone remodeling.

Ovariectomized mice (hereafter, “ovx mice”) have been widely used as a mouse model of osteoporosis (13). In fact, ovx mice display low bone mass due to increased osteoclastogenesis, which reflects the characteristics of postmenopausal osteoporosis. However, this mouse model does not reflect the phenotype of age-related osteoporosis because in age-related osteoporosis, bone formation is reduced due to low osteoblast numbers (26). We demonstrated here that Cdk1 regulates osteoblast proliferation and that Cdk1 deletion results in osteoporosis that resembles the phenotype of age-related osteoporosis. Thus, the use of this mouse model may be beneficial for the establishment of new treatment strategies for age-related osteoporosis. Furthermore, deleting Cdk1 in osteoblasts impairs osteoblast proliferation without altering the number of osteoclasts or bone resorption (Fig. 2C). Thus, increasing Cdk1 activity in osteoblasts to promote their proliferation may enhance bone formation without affecting osteoclast parameters.

Interestingly, Cdk1 expression is high in undifferentiated osteoblasts but low in mature osteoblasts. Inhibition of Cdk1 expression and/or activity promoted osteoblast differentiation and inhibited proliferation. These results suggest that Cdk1 functions to maintain osteoblasts in a proliferating state and to delay terminal differentiation, analogous to the function of Cdk1 in maintaining proliferation and self-renewal in mouse embryonic stem cells (27). Thus, down-regulation of Cdk1 expression in osteoblasts would be important for terminal osteoblast differentiation. However, the specific molecular

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**Figure 2. Osteoblast-specific Cdk1-knockout mice have low bone mass.** A, Cdk1 expression in Cdk1−/− mice and Cdk1+/+ mice. Cdk1 expression was lower in the calvaria of Cdk1−/− mice than in control mice at 3 months of age. However, Cdk2, 4, and 6 expression levels were similar between the two groups. *, p < 0.05 versus Cdk1+/+ mice. B and C, bone histomorphometric analysis of 3-month-old Cdk1−/− and Cdk1+/+ mice. Cdk1−/− mice showed decreased bone mass, as measured by BV/TV (B). The number of osteoblasts, the bone formation rate, and the mineral apposition rate were decreased in Cdk1−/− mice, whereas the osteoblast surface was not affected (C). Representative images of calcein labeling, toluidine blue staining, and TRAP staining. Black arrowheads show osteoblasts, and black arrows show osteoclasts. Scale bars, 100 μm. *, p < 0.05 versus Cdk1+/+ mice. D, μCT analysis of 3-month-old Cdk1−/− mice. All trabecular bone parameters described in the upper figure were low in Cdk1−/− mice. Lower figures depict 3D images of femoral trabecular bone from both groups. Representative 3D image of femoral trabecular bone from Cdk1−/− mouse was same as in Fig. S2. *, p < 0.05 versus Cdk1+/+ mice. E, serum P1NP and CTX-1 levels measured in 3-month-old mice. P1NP is decreased in Cdk1−/− mice, *, p < 0.05 versus Cdk1+/+ mice. All data represent the mean ± S.D. n.s., not significant.
Loss of cyclin-dependent kinase 1 impairs bone formation

A

Cdk1 ff
Cdk1osb-/-

TUNEL

n.s

B

Cdk1 ff
Cdk1osb-/-

BrdU

*

C

PCNA

Ki67

D

Osterix

Runx2

Osterix

n.s
The mechanism responsible for down-regulating Cdk1 during osteoblast differentiation remains to be elucidated. Because Cdk1 is an E2F target gene (17), we hypothesize that the expression level and/or functions of E2F or Cdk1 itself should be regulated by transcription factors involved in osteoblast differentiation, such as Runx2 and Osterix. Indeed, pRb, which is an upstream regulator of E2F, has been shown to directly interact with Runx2 (28). Moreover, many putative binding sites for pRb were observed in the osteoblast differentiation process.

Figure 3. Osteoblast proliferation is decreased in osteoblast-specific Cdk1-knockout mice. A, TUNEL assays were performed in 3-month-old Cdk1f/f and Cdk1osb/H11002/H11002 mice. No difference in apoptotic cells was detected between the two groups. B, in vivo BrdU assays suggested that the number of proliferating osteoblasts was decreased by 2-fold in Cdk1osb/H11002/H11002 mice compared with control mice. *, p < 0.05 versus Cdk1f/f mice. C, stable knockdown of Cdk1 with shRNA accelerated osteoblast differentiation. *, p < 0.05 versus controls. D, in situ hybridization in control and Cdk1osb/H11002/H11002 mice at P1. Note that Cdk1 deletion led to accelerated osteoblast differentiation. Black arrowheads represent osteoblast regions in which Col1α1 and Ocn had higher expression than in Cdk1f/f mice. E, differentiation markers (ALP, Ocn, and Col1α1) were up-regulated in Cdk1osb/H11002/H11002 mouse calvaria at 3 months of age. *, p < 0.05 versus control mice. All data represent the mean ± S.D. ALP, alkaline phosphatase; Ocn, osteocalcin.

Figure 4. Loss of Cdk1 expression promotes osteoblast differentiation in vitro and in vivo. A, Alizarin Red staining in calvarial cells isolated from WT mice treated with vehicle or RO-3306 for 14 days. Cdk1-inhibited osteoblasts formed more mineralized nodules than vehicle-treated osteoblasts. B, inhibition of Cdk1 in ST2 mesenchymal progenitor cells promoted osteoblast differentiation. *, p < 0.05 versus controls. C, stable knockdown of Cdk1 with shRNA accelerated osteoblast differentiation. D, in vivo BrdU assays suggested that the number of proliferating osteoblasts was decreased by 2-fold in Cdk1osb/H11002/H11002 mice compared with control mice. *, p < 0.05 versus Cdk1f/f mice. E, differentiation markers (ALP, Ocn, and Col1α1) were up-regulated in Cdk1osb/H11002/H11002 mouse calvaria at 3 months of age. *, p < 0.05 versus control mice. All data represent the mean ± S.D.
Figure 5. Cdk1 in osteoblasts is dispensable for bone-anabolic effect of PTH in vivo. A, schematic of the experimental protocol. B, bone histomorphometric analysis in Cdk1-/- mice and Cdk1ob/-/H11002 mice. PTH successfully increased BV/TV in both Cdk1ob/-/H11002 and control mice. *, p < 0.05 versus Cdk1-/- mice. n.s., not significant. C, number of osteoblasts and the bone formation rate were increased by PTH in both Cdk1ob/-/H11002 and control mice. D, fluorescence immunohistochemistry analysis of Ki67 (red) in the trabecular region of mouse femurs treated with PTH for 2 weeks. Nuclei are stained with DAPI (blue). Scale bars, 50 μm. *, p < 0.05 versus Cdk1-/- mice. n.s., not significant. All data represent the mean ± S.D.
these factors are present in the sequences upstream of the Cdk1 locus, and Runx2 has been shown to directly interact with Cdk1 (29).

Our study showed that the bone-anabolic effect of PTH can be achieved despite impaired osteoblast proliferative ability. PTH has been shown to enhance the commitment of the progenitor proportion to an osteogenic fate in vitro (30). Collectively, these results suggest that the anabolic effect of intermittent PTH in vivo may result from an enhancement of the progenitor population to an osteogenic fate rather than promotion of osteoblast proliferation. Although PTH 1 receptor agonists are currently the only bone-anabolic agents indicated for patients with osteoporosis at a high risk of bone fracture, some patients do not respond to PTH treatment. To predict the effec-
tiveness of PTH in the early treatment period, several risk factors associated with nonresponse to PTH have been proposed. One study suggested that lower baseline bone turnover marker levels were associated with nonresponse (31), and another study suggested that lower early increases in bone turnover markers after starting PTH treatment were associated with nonresponses (32). Based on our research, we suggest adopting the latter strategy to predict response to PTH, because PTH can act effectively even in a lower bone formation state.

In addition to osteoblast proliferation and differentiation, Cdk1 influences bone fracture healing (Fig. 6B). Clinically, older age, malnutrition, alcoholism, and smoking are considered risk factors for nonunion after surgery (33). However, the molecular mechanisms by which these factors affect the development of nonunion are not understood. A recent clinical study showed that lower bone formation and/or a higher tendency for nondevelopment of nonunion are not understood. A recent clinical study suggested that lower early increases in bone turnover markers after starting PTH treatment were associated with nonresponse (34).

We suggest that the loss of Cdk1 in osteoblasts leads to low osteoblast numbers due to decreased proliferative activity of osteoblastic progenitors, resulting in low bone formation. Thus, we provide evidence that the low bone formation state caused by Cdk1 deficiency resulted in nonunion after bone fracture, confirming the hypothesis that low bone formation is a significant risk factor for nonunion. Because individual bone formation states can be clinically assessed by bone formation markers (32, 34), the use of a bone-anabolic agent to enhance bone formation in patients with a low bone formation state may lead to a reduced risk of nonunion after bone fracture. Although Cdk1 deficiency resulted in nonunion after bone fracture, no significant differences in callus formation were observed between groups. Thus, Cdk1 in osteoblasts may play a role in mineralization of cartilage into lamellar bone via endochondral ossification rather than in cartilage formation at the fracture site. In addition, the partial recovery of bone fracture healing by PTH administration in Cdk1osb−/− mice suggests that not only Cdk1 expression but also sufficient osteoblast numbers may be important for bone fracture healing.

Concerning the regulation of bone resorption by Cdk1, osteoclast-specific Cdk1-knockout mice constructed using the cathepsin-K promoter showed no significant differences in bone mass or osteoclast parameters, although in this study, we showed that bone formation was repressed in osteoblast-specific Cdk1-knockout mice. Thus, Cdk1 does not seem to be essential for osteoclasts but regulates bone metabolism through directly stimulating osteoblasts.

In conclusion, we have demonstrated that Cdk1 is required for proper osteoblast proliferation and differentiation and is important for bone formation. Because Cdk1 inhibitors are currently being tested for the treatment of various diseases, Cdk1 may be a potential therapeutic target for the treatment of diseases involving abnormal osteoblast proliferation, such as osteosarcoma. Moreover, increasing Cdk1 activity in osteoblasts to promote their proliferation may enhance bone formation without affecting osteoclast parameters. In this context, it will be important to develop new strategies for cell type–specific activation or inhibition of Cdk1.

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3 M. Saito, A. Takahashi, and H. Inose, unpublished data.

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**Experimental procedures**

**Animals**

Cdk1floxed mice (17) and the Osx−Cre mouse line (21) have been described previously. We crossed Osx−Cre mice with Cdk1floxed mice to obtain Cdk1osb−/−. All mice were maintained under standard conditions with food and water available ad libitum under a 12-h light/dark cycle. All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

**Histomorphometric analysis**

Bone histology and histomorphometry were performed at the L3 and L4 vertebrae in 3-month-old female mice, as described previously (35). In brief, vertebrae were dissected out and fixed in 4% formaldehyde for 18 h at 4 °C. Undecalcified bones were embedded in methyl methacrylate, and 5–7-μm sections were prepared on a rotating microtome. To determine the ratio of mineralized BV/TV, sections were stained with von Kossa reagent (3% silver nitrate) and counterstained with van Gieson solution (Wako). Bone formation rate (BFR) was analyzed by the calcine double-labeling method. Accordingly, mice were injected twice with 20 mg/kg calcine (Sigma) at 7 and 2 days, respectively, before sacrifice. BFR was calculated as the product of the mineral apposition rate (MAR) and the mineralizing surface/bone surface (MS/BS, \(BFR = MAR \times (MS/BS)\)). MAR is the distance between the midpoints of the two labels divided by the time between the midpoints of the interval. MS/BS represents the percentage of bone surface exhibiting mineralizing activity. For the analysis of osteoblast parameters (osteoblast number per tissue area), bone sections were stained with 1% toluidine blue. For the analysis of osteoclast parameters (osteoclast surface per bone surface), bone sections were incubated for 30 min in TRAP staining solution at 37 °C and then counterstained with hematoxylin. Osteoclasts were defined as multinucleated dark red cells along the bone surface. Histological sections were viewed under a microscope (Olympus) using a ×20 objective lens. Histomorphometric analysis was performed using the Osteomasure System (OsteoMetrics). For each group, 4–6 mice were analyzed.

**Cell culture**

Cells were purchased from the Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were maintained in α-minimal Eagle’s medium (Sigma) containing 2 mM L-glutamine, 100 units/ml penicillin, 10 μg/ml streptomycin, and 10% fetal bovine serum (FBS; Sigma) in 5% CO2. ST2 cells were cultured in RPMI 1640 medium (Sigma). Cells were treated with osteogenic medium (containing 10% FBS and 30 ng/ml recombinant human BMP2 (funakoshi)) with or without 10 μM RO-3306 (Sigma) to inhibit Cdk1 activity. For cell counting, we counted the total number of cells in the visual field of four different regions. The results are representative of more than four independent experiments.

**Transfection and infection**

Cdk1 cDNA fragments were amplified by PCR. The PCR fragments were cloned into the pcDNA3.2/V5-DEST vector.
Loss of cyclin-dependent kinase 1 impairs bone formation

(Invitrogen). For Cdk1 overexpression studies, MC3T3-E1 cells were seeded and transfected using the Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions. To establish stable cell lines, we constructed retroviruses expressing shRNA against Cdk1 using the RNAi-Ready pSIREN Vector (Clontech) and Platinum Retroviral Expression System (Cell Biolabs, Inc), as directed by the manufacturers. The shRNA sequence was 5’-AATTCTCTGAGAAATGGCC-AGAGCCTTTGGAATATCTCTTGAATATCCAAACGC- TCTGGCAGC-3’ (for Cdk1). Stable clones expressing shRNA against Cdk1 or the luciferase gene were selected using 3 μg/ml puromycin.

Quantitative real-time PCR analysis

To acquire RNA from mouse bones, we flushed mouse bone marrow out of the femur with PBS and extracted bone RNA with TRIzol reagent (Invitrogen); RNA from other tissues and cultured cells was also extracted using TRIzol reagent. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. We performed quantitative analysis of gene expression using the Mx3000p qPCR system (Agilent Technologies). Gapdh expression was used as an internal control. Primer sequences are available upon request.

Western blot analysis

For immunological detection, 20 μg of cell lysate was separated via SDS-PAGE (7.5–10% Tris gel). After the proteins were blotted onto a PVDF membrane, the membrane was incubated with the PVDF blocking reagent Can Get Signal (TOYOBO). Proteins were probed with primary antibodies against Cdk1 (MLB) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Horseradish peroxidase-conjugated goat anti-rabbit antibody was then added, and secondary antibodies were detected through autoradiography using enhanced chemiluminescence (ECL Plus, GE Healthcare).

Microcomputed tomography analysis

We obtained three-dimensional images of distal femurs via μCT (Comscan). We examined more than six mice for each group for bone morphometric analysis.

Biochemistry

Blood samples were collected through cardiac puncture, kept at room temperature for 30 min, and centrifuged at 12,000 × g for 15 min at 4 °C. Serum P1NP and C-terminal telopeptide of type 1 collagen (CTX-I) levels were measured by an ELISA kit for mouse P1NP (Cloud-Clone) and CTX-I (Ids) according to the manufacturer’s instructions. We performed quantitative analysis of gene expression using the Mx3000p qPCR system (Agilent Technologies). Gapdh expression was used as an internal control. Primer sequences are available upon request.

Immunohistochemistry and immunofluorescence

Frozen samples were embedded in 4% carboxymethylcellulose (CMC) sodium (Leica Microsystems A/S) and fixed in 4% paraformaldehyde prior to immunostaining. Then, 7-μm frozen tissue sections were immunostained overnight with anti-Osterix (Abcam, 1:600), anti-Runx2 (R&D Systems, 1:300), anti-PCNA (Abcam, 1:1000), and anti-Ki67 (Abcam, 1:1000) primary antibody at 4 °C, except anti-Runx2 that was used for 2 h at room temperature. We then applied goat anti-rabbit IgG H&L or goat anti-rat IgG H&L secondary antibody (Abcam, 1:200). After 4,6-diamidino-2-phenylindole (DAPI) staining, slides were mounted in Fluorescence Mounting Medium (DAKO) and stored at 4 °C in the dark. Detection by microscopy was performed on a Nikon Eclipse 80i microscope, and composite images were created using ImageJ (National Institutes of Health, Bethesda).

BrdU labeling

Mice were injected intraperitoneally with BrdU (100 μg/g body weight) 24 and 2 h before being sacrificed. Limbs were dissected and embedded in 4% CMC sodium. BrdU was detected using a BrdU immunohistochemistry kit (Abcam) according to the manufacturer’s protocol. After incubation with streptavidin–horseradish peroxidase conjugate, we used a tyramide signal amplification system (PerkinElmer Life Sciences) to detect fluorescent signals. Ki67-positive cells in the osteoids of femur were counted and normalized the counts to the total cell numbers in the same area. Ki67-positive hematopoietic cells in the bone marrow were not counted.

TUNEL assay

Osteoblast apoptosis in Cdk1osb/f and Cdk1osb−/− mice at the age of 12 weeks was examined via TUNEL assays. TUNEL assays were performed with the ApopTag system (Millipore) according to the manufacturer’s instructions. After applying anti-digoxigenin conjugate, we used a tyramide signal amplification system (PerkinElmer Life Sciences) to detect fluorescent signals.

In situ hybridization

In situ hybridization was performed using a DIG-labeled riboprobe according to the standard protocol, as described previously (36). Hybridizations were performed at 55 °C. Riboprobes for Ocn and type I collagen were provided by Dr. Shu Takeda (Tokyo Medical and Dental University, Tokyo, Japan). For detection, signals were developed using anti-DIG antibody conjugated with alkaline phosphatase. After antibody treatment, the sections were incubated with BM Purple (Roche Applied Science).

PTH treatment protocol

The study design is shown schematically in Fig. 5A. Control female mice and Cdk1osb−/− female mice at 12 weeks of age were treated with either vehicle or 80 μg/kg/day hPTH(1–34) (Bachem) for 4 weeks. Either vehicle or PTH was administered subcutaneously to the respective groups (5 days a week). All mice were sacrificed after 4 weeks of treatment.

Mouse femoral fracture model

Anesthetized mice were placed on their backs, and the right leg was maximally flexed at the knee. An anterior longitudinal midline incision was made centered over the knee joint using a No. 15 scalpel blade. A subsequent incision was made just medial to the patella and extensor mechanism. The extensor mechanism was elevated and displaced in a lateral fashion using forceps. After the extensor mechanism was subluxated laterally, the distal end of the femur was exposed. Using a blade, the
Loss of cyclin-dependent kinase 1 impairs bone formation

femur was broken transversely at approximately the distal third of the femoral bone. Then, we inserted a 25-gauge needle from the troclear groove of the femur into the medullary canal in a retrograde manner. Through the fracture site, we made sure that the needle was completely inserted into the distal and proximal parts of the bone. The depth of insertion could be manually felt due to the resistance upon meeting the cortical bone of the greater trochanter. The needle was cut flush with the distal end of the femur with wire cutters. The extensor mechanism was pulled back to its anatomic location using forceps. The incision was then closed with 5–0 nonabsorbable sutures.

The callus index was defined as the ratio of the maximum callus diameter to the bone diameter at the same level as the callus (37). We measured the callus index at regular intervals using radiography during the healing period to observe callus growth and remodeling. For PTH treatment after bone fracture, 3-month-old Cdk1osb+/− female mice were fractured and then treated with 80 μg/kg/day hPTH(1–34) for 6 weeks.

Statistics
All data are presented as the means ± S.D. (n ≥ 3). We performed statistical analysis using Student’s t test, and p < 0.05 was considered statistically significant.

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References
1. Karsenty, G., Kronenberg, H. M., and Settembre, C. (2009) Genetic control of bone formation. Annu. Rev. Cell Dev. Biol. 25, 629–648 CrossRefMedline
2. Liberman, U. A., Weiss, S. R., Bröll, J., Minne, H. W., Quan, H., Bell, N. H., Rodriguez-Portales, J., Downs, R. W., Jr., Dequeker, J., and Favus, M. (1995) Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. The Alendronate Phase III Osteoporosis Treatment Study Group. N. Engl. J. Med. 333, 1437–1443 CrossRefMedline
3. Morley, P., Whitfield, J. F., and Willicock, G. E. (2001) Parathyroid hormone: an anabolic treatment for osteoporosis. Curr. Pharm. Des. 7, 671–687 CrossRefMedline
4. McClung, M. R., Grauer, A., Boonen, S., Bolognese, M. A., Brown, J. P., Diez-Perez, A., Langdahl, B. L., Reginster, J. Y., Zanchetta, J. R., Wasserman, S. M., Katz, L., Maddox, J., Yang, Y. C., Libanati, C., and Bone, H. G. (2014) Romosozumab in postmenopausal women with low bone mineral density. N. Engl. J. Med. 370, 412–420 CrossRefMedline
5. Augustin, M., and Horwitz, M. J. (2013) Parathyroid hormone and parathyroid hormone-related protein analogs as therapies for osteoporosis. Curr. Osteoporos Rep. 11, 400–406 CrossRef
6. Jilka, R. L., O’Brien, C. A., Ali, A. A., Roberson, P. K., Weinstein, R. S., and Manolagas, S. C. (2009) Intermittent PTH stimulation periosteal bone formation by actions on post-mitotic preosteoblasts. Bone 44, 275–286 CrossRefMedline
7. Jilka, R. L., Weinstein, R. S., Bellido, T., Roberson, P., Parfitt, A. M., and Manolagas, S. C. (1999) Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. J. Clin. Invest. 104, 439–446 CrossRefMedline
8. Lindsay, R. Z., Zhou, H., Cosman, F., Nieves, J., Dempster, D. W., and Hodsman, A. B. (2007) Effects of a 1-month treatment with PTH(1–34) on bone formation on cancellous, endocortical, and periosteal surfaces of the human ilium. J. Bone Miner. Res. 22, 495–502 CrossRefMedline
9. Qin, L., Li, X., Ko, J.-K., and Partridge, N. C. (2005) Parathyroid hormone uses multiple mechanisms to arrest the cell cycle progression of osteoblastic cells from G1 to S phase. J. Biol. Chem. 280, 3104–3111 CrossRefMedline
10. Shirakawa, J., Harada, H., Noda, M., and Ezura, Y. (2016) PTH-induced osteoblast proliferation requires upregulation of the ubiquitin-specific peptidase 2 (Usp2) expression. Calcif. Tissue Int. 98, 306–315 CrossRefMedline
11. Onishi, T., Zhang, W., Cao, X., and Hruska, K. (1997) The mitogenic effect of parathyroid hormone is associated with E2F-dependent activation of cyclin-dependent kinase 1 (cdk2) in osteoblast precursors. J. Bone Miner. Res. 12, 1596–1605 CrossRefMedline
12. Inose, H., Ochi, H., Kimura, A., Fujita, K., Xu, R., Sato, S., Iwakawa, M., Sunamura, S., Takeuchi, Y., Fukushima, S., Saito, K., Nakamura, T., Itoh, H., Ito, H., Arai, Y., Shinomiyama, K., and Takeda, S. (2009) A microRNA regulatory mechanism of osteoblast differentiation. Proc. Natl. Acad. Sci. U.S.A. 106, 20794–20799 CrossRefMedline
13. Takeda, S., Eleferiou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K. L., Armstrong, D., Ducy, P., and Karsenty, G. (2002) Leptin regulates bone formation via the sympathetic nervous system. Cell 111, 305–317 CrossRefMedline
14. Liu, J. B., Stein, G. S. van Wijnen, A. J., Stein, J. L., Hassan, M. Q., Gaur, T., and Zhang, Y. (2012) MicroRNA control of bone formation and homeostasis. Nat. Rev. Endocrinol. 8, 212–227 CrossRefMedline
15. Suryadina, R., Sadowski, M., and Sarcevic, B. (2010) Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates. Biosci. Rep. 30, 243–255 CrossRefMedline
16. Lim, S., and Kaldis, P. (2013) Cdks, cyclins, and CKIs: roles beyond cell cycle regulation. Development 140, 3079–3093 CrossRefMedline
17. Diril, M. K., Ratnacaram, C. K., Padmakumar, V. C., Du, T., Wasser, M., Coppola, V., Tessarollo, L., and Kaldis, P. (2012) Cyclin-dependent kinase 1 (Cdk1) is essential for cell division and suppression of DNA re-replication but not for liver regeneration. Proc. Natl. Acad. Sci. U.S.A. 109, 3826–3831 CrossRefMedline
18. Satyanarayana, A., Berthet, C., Lopez-Molina, J., Coppola, V., Tessarollo, L., and Kaldis, P. (2008) Genetic substitution of Cdk1 by Cdk2 leads to embryonic lethality and loss of mitotic function of Cdk2. Development 135, 3389–3400 CrossRefMedline
19. Vassilev, L. T. (2006) Cell cycle synchronization at the G2/M phase border by reversible inhibition of CDK1. Cell Cycle 5, 2555–2556 CrossRefMedline
20. Saito, M., Mulati, M., Talib, S. Z., Kaldis, P., Takeda, S., Okawa, A., and Inose, H. (2016) The indispensable role of cyclin-dependent kinase 1 in skeletal development. Cell 6, 20622 CrossRefMedline
21. Rodda, S. J., and McMahon, A. P. (2006) Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. Development 133, 3231–3244 CrossRefMedline
22. Davey, R. A., Clarke, M. V., Sastra, S., Skinner, J. P., Chiang, C., Anderson, P. H., and Zajac, J. D. (2012) Decreased body weight in young Osterix-Cre transgenic mice results in delayed cortical bone expansion and accrual. Transgenic Res. 21, 400–406 CrossRefMedline
23. Karuppaiyah, K., Yu, K., Lim, I., Chen, J., Smith, C., Long, F., and Ornitz, D. M. (2016) FGF signaling in the osteoprogenitor lineage non-autonomously regulates postnatal chondrocyte proliferation and skeletal growth. Development 143, 1811–1822 CrossRefMedline
24. Pettway, G. J., Megancik, J. A., Koh, A. J., Keller, E. T., Goldstein, S. A., and McCauley, L. K. (2008) Parathyroid hormone mediates bone growth through the regulation of osteoblast proliferation and differentiation. Bone 42, 806–818 CrossRefMedline
25. Marsell, R., and Einhorn, T. A. (2011) The biology of fracture healing. Injury 42, 551–555 CrossRefMedline
26. Almeida, M. (2012) Aging mechanisms in bone. Bonekey Rep. 1, 102 CrossRefMedline
27. Zhang, W. W., Zhang, X. J., Liu, H. X., Chen, J., Ren, Y. H., Huang, D. G., Zou, X. H., and Xiao, W. (2011) Cdk1 is required for the self-renewal of mouse embryonic stem cells. *J. Cell. Biochem.* **112**, 942–948 CrossRef Medline

28. Thomas, D. M., Carty, S. A., Piscopo, D. M., Lee, J. S., Wang, W. F., Forrester, W. C., and Hinds, P. W. (2001) The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol. Cell* **8**, 303–316 CrossRef Medline

29. Rajgopal, A., Young, D. W., Mujeeb, K. A., Stein, J. L., Lian, J. B., van Wijnen, A. J., and Stein, G. S. (2007) Mitotic control of RUNX2 phosphorylation by both CDK1/cyclin B kinase and PP1/PP2A phosphatase in osteoblastic cells. *J. Cell. Biochem.* **100**, 1509–1517 CrossRef Medline

30. Wang, Y. H., Liu, Y., and Rowe, D. W. (2007) Effects of transient PTH on early proliferation, apoptosis, and subsequent differentiation of osteoblast in primary osteoblast cultures. *Am. J. Physiol. Endocrinol. Metab.* **292**, E594–E603 CrossRef Medline

31. Elraiyah, T., Ahmed, A. H., Wang, Z., Farr, J. N., Murad, M. H., and Drake, M. T. (2016) Predictors of teriparatide treatment failure in patients with low bone mass. *Bone Rep.* **4**, 17–22 CrossRef Medline

32. Krege, J. H., Lane, N. E., Harris, J. M., and Miller, P. D. (2014) PINP as a biological response marker during teriparatide treatment for osteoporosis. *Osteoporosis Int.* **25**, 2159–2171 CrossRef

33. Zura, R., Braid-Forbes, M. J., Jeray, K., Mehta, S., Einhorn, T. A., Watson, J. T., Della Rocca, G. J., Forbes, K., and Steen, R. G. (2017) Bone fracture nonunion rate decreases with increasing age: a prospective inception cohort study. *Bone* **95**, 26–32 CrossRef Medline

34. Inose, H., Yamada, T., Mulati, M., Hira, T., Ushio, S., Yoshii, T., Kato, T., Kawabata, S., and Okawa, A. (2018) Bone turnover markers as a new predicting factor for nonunion after spinal fusion surgery. *Spine* **43**, E29–E34 CrossRef Medline

35. Dempster, D. W., Compston, J. E., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., Ott, S. M., Recker, R. R., and Parfitt, A. M. (2013) Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR histomorphometry nomenclature committee. *J. Bone Miner. Res.* **28**, 2–17 CrossRef Medline

36. Obernosterer, G., Martinez, J., and Alenius, M. (2007) Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat. Protoc.* **2**, 1508–1514 CrossRef Medline

37. Eastaugh-Waring, S. J., Joslin, C. C., Hardy, J. R., and Cunningham, J. L. (2009) Quantification of fracture healing from radiographs using the maximum callus index. *Clin. Orthop. Relat. Res.* **467**, 1986–1991 CrossRef Medline