Published in final edited form as:

J Bone Miner Res. 2013 March ; 28(3): 660–671. doi:10.1002/jbmr.1778.

Overexpression of H1 Calponin in Osteoblast Lineage Cells Leads to a Decrease in Bone Mass by Disrupting Osteoblast Function and Promoting Osteoclast Formation

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Abstract

H1 calponin (CNN1) is known as a smooth muscle-specific, actin-binding protein which regulates smooth muscle contractive activity. Although previous studies have shown that CNN1 has effect on bone, the mechanism is not well defined. To investigate the role of CNN1 in maintaining bone homeostasis, we generated transgenic mice overexpressing *Cnn1* under the control of the osteoblast-specific 3.6-kb *Col1a1* promoter. *Col1a1-Cnn1* transgenic mice showed delayed bone formation at embryonic stage and decreased bone mass at adult stage. Morphology analyses showed reduced trabecular number, thickness and defects in bone formation. The proliferation and migration of osteoblasts were decreased in *Col1a1-Cnn1* mice due to alterations in cytoskeleton. The early osteoblast differentiation of *Col1a1-Cnn1* mice was increased, but the late stage differentiation and mineralization of osteoblasts derived from *Col1a1-Cnn1* mice were significantly decreased. In addition to impaired bone formation, the decreased bone mass was also associated with enhanced osteoclastogenesis. Tartrate-resistant acid phosphatase (TRAP) staining revealed increased osteoclast numbers in tibias of 2-month-old *Col1a1-Cnn1* mice, and increased numbers of osteoclasts co-cultured with *Col1a1-Cnn1* osteoblasts. The ratio of *RANKL* to *OPG* was significantly increased in *Col1a1-Cnn1* osteoblasts. These findings reveal a novel function of CNN1 in maintaining bone homeostasis by coupling bone formation to bone resorption.

Keywords

H1 CALPONIN; BONE REMODELING; CYTOSKELETON; OSTEObLAST; OSTEocLAST

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Additional Supporting Information may be found in the online version of this article.

Disclosures

All authors state that they have no conflicts of interest.

Authors' roles: Study design: NS and LC. Study conduct: MC, SC, CL, YX, YZ, LZ, QH. Data collection: NS. Data analysis and interpretation: NS and LC. Drafting manuscript: NS. Revising manuscript content: XD, DC, and LC.

Introduction

Calponin is an actin filament-associated protein of 34 to 37kDa (292–330 amino acids) that was first found in chicken gizzard smooth muscle.⁽¹⁾ There are three isoforms of calponin according to their distinct theoretical isoelectric points (pI): h1 calponin is basic (pI = 9.4), h2 calponin is near neutral (pI = 7.5), whereas the acidic calponin has a lower pI of 5.2.⁽²⁾

H1 calponin (CNN1) is known to be a marker of smooth muscle differentiation, which can bind to actin, tropomyosin, and calmodulin, and is involved in the regulation of smooth muscle contractive activity and cell proliferation.⁽³⁾ In addition to its expression in smooth muscle cells (SMCs), it is also expressed in human osteosarcoma cell lines and osteosarcoma tissues.^(4,5) These observations suggest that CNN1 may also play a role in bone-related cells.

There are two types of bone formation process, intramembranous ossification and endochondral ossification. Both types of ossification process involve in condensation of mesenchyme, differentiation of mesenchymal cells into osteoblasts, and secretion and mineralization of extracellular matrix.^(6,7) Previous studies have demonstrated that *Cnn1* was expressed in osteoblastic cell line MC3T3-E1 cells and bone tissues.⁽⁸⁾ *Cnn1* knockout (KO) mice showed increased ossification⁽⁸⁾; however, the mechanism is not clear. During endochondral ossification, bone formation is secondary to chondrogenesis.⁽⁹⁾ The chondrocyte maturation process is accelerated in conventional *Cnn1* KO mice,⁽⁸⁾ which may affect the following bone formation. It is not known if the bone phenotype observed in *Cnn1* conventional KO mice is an osteoblast cell autonomous effect or is also related to altered chondrogenesis. In addition, in postnatal and adult skeleton, bone mass is maintained by bone remodeling through the coupling of osteoblastic function to the osteoclastic activity. The direct effects of CNN1 on osteoblasts and osteoclasts remain to be defined.

To investigate the role of CNN1 in bone formation and bone remodeling and their underlying mechanisms, we generated transgenic mice in which the *Cnn1* gene is overexpressed in osteoprogenitors and osteoblasts. We analyzed the bone phenotype of these mice and found that overexpression of *Cnn1* in osteoblasts led to a significant decrease in bone mass at the adult stage by inhibiting osteoblast migration, proliferation, and mineralization, and also by promoting osteoclastogenesis. Our studies provide novel insights into the functional mechanism of CNN1 in regulation of bone remodeling.

Subjects and Methods

Generation of *Cnn1* transgenic mice

To study the role of *Cnn1* in osteogenesis, we generated transgenic mice with osteoblast-specific expression of *Cnn1*. Briefly, the transgenic construct contains a rat 3.6-kb collagen type I alpha 1 (*Col1a1*) promoter, 1.6-kb rat intron,⁽¹⁰⁾ *Cnn1* cDNA, and *SV40* polyadenylation signal fragments. The linearized transgenic fragments were digested with *SacII-Sal I*. Then the fragments purified by DNA purification kit (Qiagen, Hilden, Germany) were microinjected into *FVB/NCrIVR* mouse fertilized eggs and surgically implanted to pseudopregnant *FVB/NCrIVR* dams. The founders were screened by PCR using mouse tail genomic DNA. Transgenic expression in tissues was assessed by RT-PCR using two sets of transgene-specific primer *Cnn1* (F: 5'-ACACGGCGTCACCTCTATG-3', R: 5'-TGAGTGTGTGTCGAGTGTTC-A-3'). All experiments with mice were performed according to protocols approved by the Institutional Animal Care and Use Committee at Daping Hospital.

Whole-mount skeletal preparations

Mice were euthanized and skinned. Skeletons were prepared and stained as described.^(11,12) Briefly, after removing the skin, the whole body was fixed in 95% ethanol and stained with Alcian blue and Alizarin Red S (Sigma Aldrich, St. Louis, MO, USA). Soft tissues were removed with KOH treatment.

Radiologic imaging and dual beam X-ray absorptiometry

Femoral bones of 2- and 4-month-old mice were stored in 70% ethanol at 4°C. Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DXA; PIXImus Mouse 11 densitometer; GE Medical System, Madison, WI, USA). Femurs and tail vertebrae of 2-month-old mice were analyzed by high-resolution X-ray using a Faxitron MX20-Digita (Faxitron X-ray, Lincolnshire, IL, USA).

Micro-computed tomography

The femoral middle cortical bones and cancellous bones of the distal metaphysis were scanned by micro-computed tomography (μ CT) (μ CT-80; Scanco Medical AG, Bassersdorf, Switzerland) as reported.⁽¹³⁾ Two dimensional images were used to generate three-dimensional reconstructions and to calculate morphometric parameters defining cortical bone and trabecular bone mass and microarchitecture. These include cortical thickness (Ct.Th), bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), structure model index (SMI) and connective density (Conn.D).

Cortical bone mechanical properties

Freshly dissected femurs of 2-month-old mice were used for testing the biomechanical strength using a three-point bending test performed on an Instron (5565) tester (Instron Corp., Canton, MA, USA).⁽¹⁴⁾ Femurs were centered anterior side up on the rounded supports so that the actuator, moving at 6 mm/min, made contact with the midshaft of the bone. Bone mechanical properties of maximum load and Young's modulus were determined.

Bone histology

Mice were weighed and injected with calcein (30 mg/kg body) (Sigma Aldrich) at 10 and 3 days prior to sacrifice. The right tibias were fixed with 40% ethanol overnight and dehydrated in a graded ethanol series. The bones were embedded in a mixture of methyl methacrylate (MMA) and dibutyl phthalate for analysis of parameters of bone formation. Five- μ m-thick midfrontal sections of the distal tibias were used for Von Kossa and toluidine blue staining or Goldner's trichrome staining as previously described,^(13,15) and 10- μ m-thick sections were used for fluorescence observation. The left tibias were fixed in 4% paraformaldehyde (PFA) at 4°C overnight and decalcified before embedding in paraffin. Six- μ m-thick sections were stained for osteoclasts using tartrate-resistant acid phosphatase (TRAP) kit (Sigma Aldrich). OsteoMeasure (OsteoMetrics, Decatur, GA, USA) bone analysis software was used for histomorphometric analyses. The regions of interest for trabecular bone were measured in an area 1.5 mm in length, from 0.3 mm below the growth plate of the proximal tibias ($n = 4-5$). All histomorphometric parameters are reported in accordance with the ASBMR nomenclature.⁽¹⁶⁾

Preparation of neonatal calvarial cells

Primary mouse calvarial cells were prepared as described.⁽¹⁷⁾ Briefly, calvaria from mice less than 3 days old were dissected, isolated and subjected to sequential digestions in collagenase I (1 mg/mL) (Gibco, Langley, OK, USA) and 0.25% trypsin (Thermo Scientific

Hyclone, Logan, UT, USA) for 20, 40, and 90 minutes. Cells from the third digest were collected, counted and plated in a modified essential medium (α -MEM) with 10% fetal bovine serum (FBS) (Thermo Scientific Hyclone) containing 100 U/mL of penicillin and streptomycin (Thermo Scientific Hyclone).⁽¹⁸⁾ These cells were 95% osteoblast or osteoblast precursors.⁽¹⁹⁾ Passage 1 osteoblasts were used for all studies. For osteoblastic differentiation assay, primary cells were seeded at 8×10^4 cells/well in 24-multiwell plates or 4×10^5 cells/well in six-multiwell plates. After the cell confluence, the medium was supplemented with 50 μ g/mL ascorbic acid, 10 mM β -glycerophosphate and 10^{-8} M dexamethasone (Sigma Aldrich) and replaced every 3 days for 7, 14, and 21 days.

Cell proliferation assay

Osteoprogenitors were used to detect cell proliferation. Cell proliferation analysis was performed by cell counting. Cells were seeded at 2×10^4 cells/well in 24-multiwell plates on the first day, and the cell number was counted using counting chamber every day. For bromodeoxyuridine (BrdU) incorporation assay, cells were labeled with 4 μ g/mL BrdU (Sigma Aldrich) for 4 hours at 37°C and detected by anti-BrdU monoclonal antibody (Sigma Aldrich) as reported.⁽²⁰⁾

Cell-cycle analysis

P1 passage osteoblasts were seeded in 6-cm dishes at 2×10^5 cells/dish. After the cells had grown to subconfluence, cells were digested by 0.25% trypsin and washed twice by PBS, then cell suspension was fixed in 70% ethanol for at least 2 days at 4°C. For cell-cycle analysis, cells were permeabilized and treated with propidium iodide, which stains DNA quantitatively. Then, 1×10^4 cells were used for flow analysis (FACSCalibur; BD, San Jose, CA, USA).^(21,22) The proliferation index (PI), a measure to evaluate cell proliferation ability, where $PI = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100\%$,⁽²³⁾ and the percentage of G1 phase cells were evaluated.

Scratch wound-healing migration assay

Cells were seeded in six-multiwell plates at 2×10^5 cells/well. After the cells had grown to confluence, the cell monolayer was wounded with 1-mL sterile plastic pipette tips, and washed twice with PBS, then incubated in culture medium again.^(24,25) The wound areas were photographed at 0 hour and 24 hours later using a microscope equipped with a digital camera, followed by analysis with image analysis software SPOT (Version 3.4.5, Silicon Graphics, Inc, Fremont, CA, USA), and the rate of cell migration was evaluated as the percentage of the invaded area relative to the initial wound area.

Analysis of alkaline phosphatase activity and staining

For detection of alkaline phosphatase (ALP) activity, cells were washed two times with PBS, then lysed with 10 mM Tris-HCl containing 2 mM $MgCl_2$ and 0.05% Triton X-100 (pH 8.2) at 4°C. Lysates were incubated in ALP detection buffer (Sigma Aldrich) for 30 minutes at 37°C. The reaction was stopped by 0.1 M NaOH and monitored at 405 nm. Total protein was measured using a Micro BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA) and read at 562 nm. The relative enzymatic activity of ALP was normalized to the total protein content of the sample (405 nm/562 nm). For ALP staining, cells were fixed with 4% PFA for 10 minutes and stained with ALP detection solution for 30 minutes at 37°C, and then rinsed with PBS to remove excess staining.⁽¹³⁾

Alizarin red staining of mineralized osteoblast cultures

Mineralized osteoblast cultures were fixed in 70% ethanol for 1 hour at 4°C. Then, cells were stained in 0.2% Alizarin red in 2% ethanol for 15 minutes. After being washed four

times with water, cells were dried at 37°C. Bound dye was dissolved with 0.5 N HCl, 5% SDS, and measured at 415 nm to quantify mineral content.⁽²⁶⁾ Total DNA was extracted and measured at 260 nm. The relative quantification of mineral content was normalized to the total DNA content of the sample (415 nm/260 nm).

Cytoskeleton staining of osteoblasts

Cells were seeded at 2×10^4 cells/well in 24-multiwell plates on the first day. After being cultured for 48 hours, cells were washed twice with PBS (37°C), and fixed in 4% PFA for 10 minutes. After being washed thrice with PBS, cells were blocked with 1% bovine serum albumin (BSA) for 30 minutes, and then incubated in 5 µg/mL Phalloidin-phalloidine (Enzo Alexis-Biomol, Farmingdale, NY, USA) for 60 minutes. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The pictures of cytoskeleton (F-actin) were taken using confocal microscope (Leica Microsystems ICS Sp2; Leica, Wetzlar, Germany). The fluorescence intensity of cytoskeleton was also measured.

Cocultures of osteoblastic cells and osteoclasts

For coculture experiments, osteoblasts (8×10^4 cells/well) were plated with marrow mononuclear cells (4×10^6 cells/well, purified by Percoll) in 1 ml of α -MEM containing 10% FBS, and 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ (Sigma Aldrich) in 12-multiwell plates.⁽²⁷⁾ On day 6, 50% of the medium was refreshed, and then the medium was replaced every 3 days. Osteoclast differentiation was evaluated by TRAP staining on day 14. All quantifications were performed on five fields per genotype, and specimen means were calculated.

Real-time PCR

mRNA of cells was extracted using TRIZOL reagent (Invitrogen, Grand Island, NY, USA), and cDNA were prepared using the PrimeScript RT-PCR kit (Takara, Shiga, Japan). Real-time PCR was performed in a Mx3000P PCR machine (Stratagene, Santa Clara, CA, USA) according to previous study.⁽¹³⁾ The sequences of the primers of *Cnn1*, *Cyclophilin A*, *Core binding factor a1 (Cbfa1)*, *Osteopontin (OP)*, *Osteocalcin (OC)*, *Collagen type I a1 (Col1a1)*, *TRAP*, matrix metalloproteinase 9 (*MMP9*), and *cathepsin K (Ctsk)* were used previously.⁽¹³⁾ Other sequences of primers are as follows: receptor activator of NF- κ B ligand (*RANKL*) (F: 5'-CTGATGAAAGGAGGGAGCACG-3', R: 5'-AGCAGGGAAGGGTTGG-ACAC-3'); osteoprotegerin (*OPG*) (5'-TCCCTTGCCCTGACCACT-CTT-3', R: 5'-CAGGTAACGCCCTTCCTCACA-3'); macrophage colony-stimulating factor (*M-CSF*) (5'-TTTTCCTGGGCATTGTGG-TCT-3', R: 5'-AGGAGGTTTCAGGGCTTCTTG-3'), *Matrix Gla protein (MGP)* (F: 5'-ACACAGAGGCAGACTCACAGGAC-3', R: 5'-GAGATTCGTAGCACAGGGTTG-3').

Statistical analysis

Data were evaluated statistically in SPSS Windows, version 10.0. Results are shown as mean $\pm$ SD. Statistics were assessed using Student's *t* test, assuming double-sided independent variance, and *p* values were considered significant at **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Results

Generation of *Col1a1-Cnn1* transgenic mice

To achieve specific expression of *Cnn1* in osteoblasts, we prepared a construct containing the murine full-length *Cnn1* cDNA fused to the *SV40* polyadenylation signal cDNA and under control of the rat 3.6-kb *Col1a1* gene promoter⁽²⁸⁾ (Fig. 1A). The overexpression of *Cnn1* in bone tissue of *Col1a1-Cnn1* transgenic (Tg) mice was detected, but there was no

significant increase in *Cnn1* expression in other tissues of the transgenic mice such as cartilage, muscle, heart, kidney, and skin, and there was no *Cnn1* expression in spleen, lung, liver, et al. (Fig. 1B). These findings suggest that the exogenous *Cnn1* transgene is specifically expressed in bone tissue. The Tg mice were viable and bred normally. There was no obvious difference between their body sizes or length of femurs and tails compared to their wild-type (WT) littermate controls (data not shown).

Overexpression of *Cnn1* in osteoblasts results in delayed bone formation and decreased bone mass

To determine the effect of CNN1 on bone formation during bone development, skeletons from embryonic day 16.5 (E16.5) WT and Tg mice were stained with Alcian blue and Alizarin red. The results showed that bone formation was delayed in embryos of Tg mice (Fig. 1C), which was supported by delayed ossification in lumbar, hindlimb, and skull from Tg mice compared with that of WT mice (Fig. 1C, arrows). However, there was no obvious difference between the length of femurs of WT and Tg mice at E16.5. Furthermore, Tg mice showed normal cartilage (Supporting Fig. S2).

Radiological analysis and bone mineral density (BMD) measurements were performed to assess the difference of bone mass between adult WT and Tg mice. Radiological analysis of 2-month-old mice also showed decreased trabecula in femurs and tail vertebrae (Fig. 1D, arrows). The BMD of the whole femurs of Tg mice was reduced by 14.6% and 13.2% at 2 and 4 months, respectively, compared with that of WT mice (Fig. 1E). These observations indicate that overexpression of *Cnn1* in osteoblasts caused delayed ossification during bone development and decreased bone mass in adult mice.

***Col1a1-Cnn1* transgenic mice show impaired microarchitecture and biomechanical strength**

We used μ CT to determine the structural parameters of cortical and trabecular bone of femurs harvested from 2-month-old mice of both genotypes (Fig. 2A, B). Three-dimensional images showed that the thickness of cortical bone and the trabecular number of Tg mice were both decreased compared with those of WT mice, which was consistent with what we found in X-ray analysis. Quantification of structural parameters revealed that Ct.Th, BV/TV, Tb.Th, and Tb.N were decreased by 11.4%, 63.4%, 25.3% ($p < 0.05$), and 35.4% ($p < 0.01$), respectively (Fig. 2C–F). Tb.Sp in Tg mice was significantly increased by 53.4% ($p < 0.01$) (Fig. 2G). The decrease of trabecular connectivity was confirmed by a significant decrease in Conn.D (Fig. 2H) and increase in SMI (Fig. 2I) ⁽²⁹⁾ in Tg femurs compared with WT mice.

The biomechanical strength of freshly dissected femurs from 2-month-old WT and Tg mice was evaluated at room temperature by three-point bending test. The decrease in cortical width and compromised architecture of Tg femurs led to a reduction in the maximum load to failure (WT 18.92 ± 2.54 N versus Tg 15.25 ± 0.9 N, $p < 0.05$) and decreased Young's modulus (WT 3913.66 ± 587.3 MPa versus Tg 3589.51 ± 326.5 MPa, $p < 0.05$).

Collectively, using quantitative, noninvasive imaging and biomechanical techniques, we found that, compared with WT mice, the adult Tg mice with osteoblast-specific expression of *Cnn1* had reduced bone mass, compromised architecture, and reduced biomechanical strength.

Changes in bone formation in *Cnn1* Tg mice

To determine whether the reduced bone mass in *Cnn1* Tg mice was due to decreased bone formation, we performed static and dynamic bone histomorphometric analysis in sections of

undecalcified tibias at 2 months. Von Kossa staining showed that the trabeculae of Tg mice was less than those of WT mice (Fig. 2J, top panels) and BV/TV in Tg mice was decreased by 43% (Fig. 2K). Goldner's trichrome staining revealed that osteoid laid down on the trabecular bone in Tg mice was increased by 42% (Fig. 2J, middle panels; *L*). Among bone formation parameters, the osteoblast number lined trabecular bone (N.Ob/B.Pm) and percentage of bone surface occupied by osteoblasts (Ob.S/BS) was significantly decreased by 33% and 41%, respectively, in Tg mice compared with WT controls (Fig. 2M, N). Double labeling of calcein was used to analyze the dynamics of osteoblast activity and bone formation at the metaphysis of tibias. The distance between two labeling lines was reduced in Tg mice (Fig. 2J, bottom panels). Histomorphometric measurements showed that mineralized surface to bone surface (MS/BS), bone formation rate/bone surface (BFR/BS), and mineral apposition rate (MAR) (MS/BS, BFR/BS, and MAR are indices of osteoblast activity)⁽³⁰⁾ were also markedly reduced in Tg mice (Fig. 2O–Q). These data showed defected bone formation and bone mineralization in of Tg mice.

The comparable serum levels of Ca and PO₄ in WT and Tg mice (data not shown) suggest that defects in bone formation found in Tg mice may not be due to systemic alteration in mineral homeostasis.

Overexpression of *Cnn1* suppresses osteoblast proliferation

To further explore the mechanism for the altered osteogenesis in Tg mice, we cultured osteoblasts from neonatal mouse calvaria, and evaluated their proliferation activity. We first confirmed that the mRNA expression of *Cnn1* was increased in Tg osteoblasts both on undifferentiated and differentiated stage at day 7 (Fig. 3A). Furthermore, *Cnn1* expression was significantly decreased after induced for differentiation both in WT and Tg osteoblasts compared with undifferentiated osteoblasts (Fig. 3A), which is consistent with a previous study.⁽⁸⁾

We found that the osteoblast number of Tg mice was decreased compared with that of WT osteoblasts at basal condition (Fig. 3B). BrdU labeling assay showed that DNA replication of Tg osteoblasts was also decreased (Fig. 3C, D), which was further demonstrated by cell-cycle analysis. This result showed that Tg mice had decreased proliferation index and increased percentage of osteoblasts at G1 stage, which indicated that Tg osteoblasts were arrested at G1 stage (Fig. 3E, F). These results suggest that overexpression of *Cnn1* may suppress cell proliferation through the inhibited DNA synthesis.

CNN1 inhibits osteoblast migration

Cell migration is important for bone marrow stromal cells and osteoblasts during bone formation,^(31–33) and calponin can affect cell migration.^(34,35) We performed a scratch wound assay using mouse primary calvarial osteoblasts to examine the effect of CNN1 on osteoblast migration. Twenty-four hours after scratch, the wounded area was almost completely invaded by WT osteoblasts. In contrast, there was still a large empty area without cell invasion in the Tg osteoblast group (Fig. 4A). Quantification of cell migration showed that the percentage area of invaded by osteoblasts from the Tg group was significantly decreased compared with that of WT cells (Fig. 4B). These results indicate that overexpression of *Cnn1* inhibits osteoblast migration.

CNN1 affects cytoskeleton rearrangement

CNN1 might combine with F-actin and affect cytoskeleton function.⁽³⁵⁾ Phalloidin-phalloidine staining showed that actin filaments were arranged regularly in cytoplasm of WT osteoblasts (Fig. 5A). However, in Tg osteoblasts, actin filaments in cytoplasm were arranged disorderly, and most of them were aggregated under the cell membrane (Fig. 5A).

Furthermore, the actin filaments in cytoplasm of Tg osteoblasts were thinner than that in WT osteoblasts (Fig. 5A), although there was no significant difference between the mean fluorescence intensity of both types of whole osteoblasts (Fig. 5B).

Overexpression of *Cnn1* affects osteoblast differentiation and mineralization

To determine changes in osteoblast differentiation, we compared the formation of ALP-positive colonies, ALP activity, and numbers of mineralized nodules (Alizarin red staining) in cultures of cells derived from WT and Tg mice. The results showed that the numbers of crystal violet-staining cells and ALP activity of Tg osteoblasts were similar to those of WT osteoblasts on day 7 and 14 cultures (Fig. 6A, B). Then we examined the expression of genes related to osteoblast differentiation. The expression levels of *Cbfa1*, *OC*, and *Col1a1* of Tg osteoblasts were significantly increased, but the expression of *OP* was decreased compared with that in WT osteoblasts on day 7 culture (Fig. 6C). However, on day 14, the expressions of *OP*, *OC*, and *Col1a1* were decreased in Tg osteoblasts relative to WT osteoblasts (Fig. 6D). These data suggest that CNN1 promotes early osteoblast differentiation.

On day 14 and 21 of cell cultures, Tg osteoblasts had fewer mineralized nodule formation and decreased relative absorbance of Alizarin red (Fig. 6E, F). The expression of *MGP*, a gene inhibiting mineralization, was increased, which is consistent with the decreased Alizarin red staining (Fig. 6D). These results suggest that overexpression of *Cnn1* promotes early osteoblast differentiation, but inhibits late stage osteoblast differentiation and mineralization.

Because *Cnn1* is also highly expressed in osteoprogenitors,⁽⁸⁾ we performed colony-forming unit fibroblastic (CFU-F) assay to analyze the effect of *Cnn1* on the number of mesenchymal progenitors. The result showed that the CFU number of Tg bone marrow stromal cells (BMSCs) was significantly decreased compared with WT BMSC after being cultured for 7 days, which indicated that Tg mice had fewer mesenchymal progenitors (Supporting Fig. S3).

Overexpression of *Cnn1* in osteoblasts promotes osteoclast formation

Bone remodeling is a process involving tight coupling between bone formation and bone resorption. Since the Tg mice showed a decrease in bone mass, we examined if the observed bone phenotype is related to the changes in osteoclast formation. We found that the number of TRAP-positive osteoclasts in trabecular bone of Tg mice was increased compared with those in WT mice (Fig. 7A, B).

To analyze the influence of osteoblasts from *Cnn1* Tg mice on osteoclast formation, osteoclast formation assay in vitro was performed using marrow mononuclear cells cultured and cocultured with osteoblasts. After 10 days of culture, the results of TRAP staining showed that the numbers of osteoclasts was increased in cultures of bone marrow cells derived from Tg mice compared with those from WT mice (Fig. 7C, D). Real-time PCR analysis showed that the mRNA levels of *TRAP*, *MMP-9*, and *Ctsk* were upregulated in osteoclasts from Tg mice (Fig. 7E–G). Furthermore, the 3.6-kb *Col1a1* is also expressed in osteoclast lineage cells⁽²⁸⁾; however, there was no significant difference of osteoclastogenesis between WT and Tg mice (Supporting Fig. S4). These results indicate that overexpression of *Cnn1* in osteoblasts promotes osteoclast formation.

RANKL, *OPG*, and *M-CSF* are produced by osteoblast lineage cells and play an important role in osteoclast formation and differentiation. We further demonstrated that mRNA expression of *RANKL* and *M-CSF* was significantly upregulated in primary calvarial osteoblasts from newborn Tg mice (Fig. 7H, K). There was no difference in *OPG* expression

between osteoblasts isolated from Tg and WT mice (Fig. 7I), so the ratio of *RANKL* to *OPG* was also significantly increased (Fig. 7J). These data suggest that overexpression of *Cnn1* in osteoblasts stimulates osteoclast formation through upregulation of *RANKL* and *M-CSF* expression.

Discussion

In the present study, we investigated the role of CNN1 in bone development and remodeling by generating transgenic mice with *Cnn1* overexpressed under the control of the osteoblast *Col1a1* 3.6-kb promoter. The *Col1a1* 3.6-kb promoter can drive gene expression in osteoblast lineage cells,⁽²⁸⁾ so *Cnn1* was mainly expressed in preosteoblasts and mature osteoblasts in Tg mice. Our data demonstrate that CNN1 regulates bone homeostasis by affecting both osteogenesis and osteoclastogenesis.

The inhibited osteoblast proliferation and migration may partially be related to the impaired cytoskeleton resulting from overexpression of CNN1.

Osteoblast proliferation and migration are important steps for bone formation during bone remodeling and bone healing.^(24,31) CNN1 has high binding affinity to actin and influences cytoskeletal morphology and migration.^(25,36–38) Actin cytoskeleton also participates in anchorage-dependent cell division and proliferation.⁽³⁹⁾

Previous studies have shown that CNN1 is a negative regulator of cell proliferation. Overexpression of *Cnn1* in leiomyosarcoma and fibrosarcoma cell lines and in fibroblasts led to suppressed cell proliferation.^(3,34,40) Cell proliferation was increased in mesangial cells in *Cnn1* knockout mice.⁽⁴¹⁾ In this study, we found that the proliferation and DNA synthesis of osteoblasts from Tg mice was inhibited compared with cells from WT mice, more Tg cells were arrested at G1 stage, and the percentage of S-phase cells was decreased in Tg osteoblasts (data not shown), which all indicated a slowing of the cell cycle in Tg osteoblasts.

The cell cycle is coupled to the actin cytoskeleton at the transition to S-phase.^(3,42–44) We found that the actin filaments in cytoplasm of Tg osteoblasts were disorderedly arranged and most of them were aggregated under the cell membrane, which may be responsible for the reduced cell proliferation. Further-more, apoptosis of Tg osteoblasts was increased compared with WT osteoblasts, which may be another reason for the decreased number of osteoblasts in Tg mice (Supporting Fig. S1).

In addition to cell proliferation, the actin cytoskeleton is also essential for cell migration and contraction.^(39,45–47) In this study, we propose that impaired actin cytoskeleton in osteoblasts of Tg mice may lead to a decrease in cell migration. The inhibitory effects of CNN1 on cell migration were also found in other cells.^(34,48)

CNN1 inhibits late osteoblast differentiation and mineralization

Osteoblasts are cells responsible for bone formation. Previous studies have shown that cytoskeleton-related molecules participated in osteoblast differentiation.^(49,50) As a regulator of cytoskeleton, knockout of *Cnn1* in mice led to increased bone formation.⁽⁸⁾ However, the mechanism is unclear. In our study, *Cnn1* was specifically overexpressed in osteoblast population in Tg mice; we thus can determine the direct effect of CNN1 on osteoblast development.

In *Cnn1* Tg mice, bone formation was impaired at both embryonic and adult stages compared with WT mice. The numbers and thickness of trabecular bone and cortical bone

were decreased in Tg mice. Increased osteoid thickness and decreased distance between first and second calcein labeling line on bone surfaces in trabecular bone of Tg mice indicate impaired osteoblast bone formation.

In vitro neonatal calvarial cell culture study confirmed our hypothesis that overexpression of *CNN1* affected osteoblast differentiation. A previous study showed that expression of *CNN1* was decreased gradually during osteoblast differentiation, indicating that *CNN1* may be a negative regulator of osteoblast differentiation.⁽⁸⁾ In our study we found that although osteoblast ALP activity in Tg mice was similar to that in WT mice, the expressions of osteoblast differentiation marker genes, such as *Cbfa1*, *OC*, and *Col1a1*, were increased at early stage differentiation of Tg osteoblasts. However, in late stage of differentiation, the expressions of osteoblast marker genes including *OC*, *OP*, and *Col1a1* were all decreased. These results suggest that overexpression of *Cnn1* could promote early osteoblast differentiation, but inhibit late stage of differentiation. Cell culture study showed that overexpression of *Cnn1* also inhibited mineralization in osteoblast cell culture, and the expression of *MGP*, a potent inhibitor for mineralization,^(51,52) was increased in *Cnn1* Tg mice. This result suggest that increased expression of *MGP* may be related to the impaired mineralization.

Furthermore, we also found that Tg mice have less number of mesenchymal progenitors of Tg mice, which may be another reason for less trabecular bone in Tg mice (Supporting Fig. S3).

CNN1 indirectly enhances osteoclast formation by promoting *RANKL* and *M-CSF* expression in osteoblasts

Osteoclasts are originated from the monocyte/macrophage hematopoietic lineages and are responsible for bone resorption.^(53,54) Two major mechanisms are involved in the regulation of osteoclast differentiation and its bone resorption activity.⁽⁵⁵⁾ One is the indirect regulation of osteoclast formation and function through expression of *M-CSF* and *RANKL/OPG* in mesenchymal cells and osteoblast lineage cells. The other mechanism is the direct regulation of osteoclast precursors and mature osteoclasts.^(56,57)

Bone mass is decreased in adult *Cnn1* Tg mice. In addition to the changes in osteoblast activity, we further demonstrated that osteoclast formation was increased in *Cnn1* Tg mice. Although the 3.6-kb *Col1a1* is also expressed in osteoclast lineage cells,⁽²⁸⁾ *Cnn1* overexpressed in osteoclast lineage did not have directly effect on osteoclastogenesis. The osteoclast phenotype observed in this study must be related to the indirect effect of osteoblasts on osteoclastogenesis. Using coculture experiments, we demonstrate that forced expression of *Cnn1* in osteoblasts promoted osteoclast formation and differentiation by upregulation of *RANKL* and *M-CSF*.

Previous studies had shown that overexpression of *Cbfa1* led to increased osteoclast differentiation and bone resorption, possibly through increased *RANKL* expression.^(58–60) Another study further suggested that *Cbfa1* could upregulate the expression of *RANKL* via a direct binding to promoter of the *RANKL*.⁽⁶¹⁾ In our study, we also found increased expression of both *Cbfa1* and *RANKL* in *Cnn1* overexpressed osteoblasts, so we propose that *Cnn1* may upregulate the expression of *RANKL* through the upregulation of *Cbfa1* expression. The mechanism still needs to be explored.

In summary, we analyzed the bone phenotype of embryonic and adult *Cnn1* Tg mice by overexpressing *Cnn1* in osteoblast lineage cells. We demonstrate that overexpression of *Cnn1* in osteoblasts leads to a decrease in bone mass by affecting osteoblast activities and promoting osteoclast formation. *CNN1* affects actin cytoskeletal morphology and inhibits

osteoblast proliferation, migration, differentiation, and mineralization. CNN1 also indirectly promotes osteoclast formation through upregulation of *RANKL* and *M-CSF* expression in osteoblasts. Our study reveals a novel function of CNN1 in maintaining bone homeostasis by coupling bone formation to bone resorption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported by grants from the National Natural Science Foundation of China (81030036, 81270012, 30901527), the Special Funds for Major State Basic Research Program of China (973 program) (2011CB964701, 2012CB518106), and the Committee of Science and Technology of Chongqing (CSTC, 2011jjA1468, SKLZZ201017). We thank Huabing Qi and Tujun Weng for helpful discussion.

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Fig. 1. Delayed bone formation and decreased bone mass in transgenic mice with *Cnn1* overexpressed in osteoblasts (Tg mice). (A) Construct of transgenic vector contains a rat 3.6-kb *Col1a1* promoter, 1.6-kb rat intron, and mouse *Cnn1* cDNA. (B) *Cnn1* overexpressed specifically in bone tissue. (C) Whole-mount staining of embryo at E16.5 using Alizarin red/Alcian blue. Tg mice had less staining areas in lumbar, hindlimb, and skull (arrows). (D) Faxitron X-ray analysis of femurs and tail vertebrae of mice at 2 months also revealed decreased bone density in Tg mice. (E) Bone mineral density of Tg mice was significantly reduced compared with that of WT mice at 2 and 4 months ($n = 6-7$). (Student's t test, $*p < 0.05$).

Fig. 2.

Quantitative μ CT of femurs and histological analysis of tibias showed impaired microarchitecture in Tg mice. (A, B) Three dimensional images of femoral cortical and trabecular bone from 2-month-old mice ($n = 5-6$). Tg mice showed decreased cortical thickness and trabecular number. (C-J) Quantification of structural parameters of femoral cortical and trabecular bone. Tg mice showed decreased Ct.Th, BV/TV, Tb.Th, Tb.N, and Conn.D. Tb.Sp and SMI in Tg mice was increased relatively to WT mice ($n = 5-6$). (J) Histological analysis of undecalcified tibias from 2-month-old mice. Top panels, Von Kossa and toluidine blue staining. Tg mice showed shorter and sparse trabecular bone (magnification, $\times 40$). Middle panels, Goldner's trichrome staining. Tg mice showed increased osteoid laid down on the trabecular bone (arrows, magnification, $\times 400$). Bottom panels, double-calcein labeling of undecalcified tibias. Distance specified by white arrows between labeled trabecular bone surfaces was decreased in Tg mice (magnification, $\times 400$). (K-Q) Quantitative histomorphometric measurements were performed on the spongiosa at the proximal tibias of 2-month-old mice ($n = 4-5$). In K, BV/TV, bone volume/tissue volume. In L, O.Th, osteoid thickness. In M, N.Ob/B.Pm, osteoblast number/bone perimeter. In N, Ob.S/BS, osteoblast surface to bone surface. In O, MS/BS, mineralized surface to bone surface. In P, MAR, mineral apposition rate. In Q, BFR/BS, bone formation rate/bone surface. These data showed decreased osteoblast number and bone formation, and impaired mineralization. The graphs show mean value $\pm$ SD. (Student's t test, * $p < 0.05$, ** $p < 0.01$).

Fig. 3.

Inhibited osteoblast proliferation in *Cnn1* Tg mice. (A) *Cnn1* expression was significantly increased both in undifferentiated and differentiated Tg neonatal calvarial osteoblasts compared with WT osteoblasts. After being induced for differentiation, *Cnn1* expression was significantly decreased both in WT and Tg osteoblasts compared with undifferentiated osteoblasts. (B) Cell proliferation was evaluated by cell counting method. Along with increased cultured days, cell number of Tg osteoblasts was gradually decreased compared with that of WT osteoblasts at basal condition. (C, D) BrdU incorporation assay. Immunohistochemistry staining of BrdU showed decreased percent of BrdU-positive cells in Tg osteoblasts (arrows) (magnification, $\times 200$). (E, F) Cell cycle analysis. Tg mice showed decreased proliferation index and increased percentage of osteoblasts at G1 stage. Graphs show mean value $\pm$ SD. (Student's *t* test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In A: a and b, compared with WT osteoblasts on day 0; c, compared with Tg osteoblast on day 0; d, compared with WT osteoblasts on day 7.

Fig. 4. Suppressed migration of osteoblasts in Tg mice. (A) Scratch wounds were made in monolayer cultures of calvarial osteoblasts after plating for 24 hours (magnification, $\times 100$). Healing of the wound by cell migration was monitored 24 hours later. The micrographs showed delayed closure of the wound in the Tg osteoblast cultures compared with the WT control. (B) Quantification of areas of wound healing also showed decreased invaded areas in Tg osteoblasts. Graphs show mean value $\pm$ SD. (Student's *t* test, ** $p < 0.01$).

Fig. 5.

Changed F-actin cytoskeleton in Tg osteoblasts. (A) Representative images of F-actin cytoskeleton in osteoblasts staining with Phalloidin-phalloidine. Actin filaments in cytoplasm of WT osteoblasts were arranged regularly and regularly. However, in Tg osteoblasts, actin filaments were thinner and arranged disorderly, and most were aggregated under the cell membrane. (B) Measurement of mean fluorescence intensity. There was no difference between the mean fluorescence intensity of WT and Tg osteoblasts. Graphs show mean value $\pm$ SD.

Fig. 6.

Effect of overexpressed *Cnn1* on osteoblast differentiation. (A) ALP staining of differentiated osteoblasts. There was no difference between crystal violet-staining cells of both WT and Tg osteoblasts on days 7 and 14 cultured in osteoblastic differentiation medium. (B) Relative ALP activity (normalized to the total protein content of the sample) of Tg osteoblasts was also similar to that of WT osteoblasts. (C) Relative expressions of osteogenic markers detected by qRT-PCR on day 7. Tg mice showed increased mRNA expression of *Cbfa1*, *OC*, and *Col1a1*. (D) Relative expressions of osteogenic markers on day 14. The mRNA level of *OC*, *OP*, *Col1a1* was decreased in Tg osteoblasts, but *MGP* expression level was increased. (E) Alizarin red staining of mineralized osteoblasts on days 14 and 21 after osteogenic differentiation. Tg osteoblasts showed decreased number of mineralized nodules compared with WT mice. (F) Relative quantification of mineral content (normalized to the total DNA content of the sample). Cultured Tg osteoblasts showed decreased mineral content. Graphs show mean value $\pm$ SD. (Student's *t* test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Fig. 7.

Increased osteoclastogenesis in Tg mice. (A) TRAP staining of tibias from 2-month-old mice (magnification, $\times 100$). TRAP-positive osteoclasts lining trabecular bone surfaces in Tg mice were significantly increased compared with WT mice. (B) Quantitative histomorphometric measurements of osteoclast showed an increased number of osteoclasts in Tg mice ($n = 4-5$). N.Oc/B.Pm, osteoclast number/bone perimeter. (C) Representative images of osteoclast formation in vitro using an osteoblast and marrow mononuclear cell coculture system. Marrow mononuclear cells cocultured with Tg osteoblasts had increased formation of large, multinuclear TRAP-positive cells compared with marrow mononuclear cells cocultured with WT osteoblasts on day 14 (magnification, top panels $\times 100$, bottom panels $\times 400$). (D) Quantification of osteoclast formation in vitro also showed significantly increased number of TRAP-positive cells in Tg mice. (E-G) qRT-PCR was used to detect relative expressions of *TRAP*, *Ctsk*, and *MMP-9* of osteoclasts cultured for 14 days. Tg mice showed increased mRNA expression of *TRAP*, *Ctsk*, and *MMP-9*. (H-K) qRT-PCR analysis of *RANKL*, *OPG*, and *M-CSF* from neonatal calvarial cells showed increased expression of *RANKL* and *M-CSF* in Tg mice. Graphs show mean value $\pm$ SD. (Student's *t* test, ** $p < 0.01$, *** $p < 0.001$).