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. © 2013 American Society for Bone and Mineral Research.

KEY WORDS: H1 CALPONIN; BONE REMODELING; CYTOSKELETON; OSTEOBLAST; OSTEOCLAST

Introduction

Calponin is an actin filament-associated protein of 34 to 37 kDa (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 (pl): h1 calponin is basic (pl = 9.4), h2 calponin is near neutral (pl = 7.5), whereas the acidic calponin has a lower pl 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 knockouts (KO) mice showed increased ossification8; 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,10 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 dam. 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'-TGATGTGTCGCCAGTGTTCC-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 (μCT) (μCT-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-μm-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-μm-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-μm-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 α 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 × 104 cells/well in 24-multwell plates or 4 × 105 cells/well in six-multwell plates. After the cell confluence, the medium was supplemented with 50 μg/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 x 10⁴ 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 μg/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 x 10⁵ 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 x 10⁶ cells were used for flow analysis (FACScalibur; BD, San Jose, CA, USA). The proliferation index (PI) of osteoblast cells was calculated,

\[ PI = \frac{(S + G_2/M)}{(G_0/G_1 + S + G_2/M)} \times 100\% \]

and the percentage of G₁ phase cells was evaluated.

Scratch wound-healing migration assay

Cells were seeded in six-multiwell plates at 2 x 10⁵ cells/well. After the cells had grown to confluence, the cell monolayer was wounded with 1-mL sterile plastic pipette tips, and washed twice. After the cells had grown to confluence, the cell monolayer was wounded.

Analysis of alkaline phosphatase activity and staining

For detection of alkaline phosphatase (ALP) activity, cells were washed twice with PBS, then lysed with 10 mM Tris-HCl containing 2 mM MgCl₂ 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.

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Cytoskeleton staining of osteoblasts

Cells were seeded at 2 x 10⁴ 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-phalloidin (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 microscopy (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 x 10⁴ cells/well) were plated with marrow mononuclear cells (4 x 10⁵ cells/well, purified by Percoll) in 1 mL of α-MEM containing 10% FBS, and 10⁻⁸ M 1,25(OH)₂D₃ (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. The sequences of the primers of Cnn1, Cyclophilin A, Cbp/Cbfa1, Osteopontin (OP), Osteocalcin (OC), Collagen type 1 a1 (Coll1a1), TRAP, matrix metalloproteinase 9 (MMP9), and cathepsin K (Ctsk) were used previously. Other sequences of primers are as follows: receptor activator of NF-κB ligand (RANKL) (F: 5'- CAGGACTGAAAAAGGAGGAGCAGCAG-3'; R: 5'-ACAGAGGAAGGGTTGACGAC-3'), osteoprotegerin (OPG) (F: 5'-TCCCTTGCCCTGACACCTTT-3'; R: 5'-CAGGATACGCCGCTTCCGAC-3'), macrophage colony-stimulating factor (M-CSF) (F: 5'-TTTCTGGGCATTGTTGGTCT-3'; R: 5'-AGGAGGTTCAAGGCTTCTTGG-3'), Matrix GlA protein (MGP) (F: 5'-ACACAGAGGAGCAGACTACAGGAC-3'; R: 5'-GAGATTTCGATGACGCTGTT-3').

Statistical analysis

Data were evaluated statistically in SPSS Windows, version 10.0. Results are shown as mean ± 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 (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, etc. (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 (n = 6–7). (Student’s t test, *p < 0.05)
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 microarchitectural and biomechanical strength

We used μCT 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) 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
calcine 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) 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. 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, and calponin can affect cell migration. 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. 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 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 ± SD.

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, 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

![Fig. 7](https://example.com/fig7.png)

Fig. 7. Increased osteoclastogenesis in Tg mice. (A) TRAP staining of tibias from 2-month-old mice (magnification, ×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 ×100, bottom panels ×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 ± SD. (Student’s t test, **p < 0.01, ***p < 0.001).
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 Csk were upregulated in osteoclasts from Tg mice (Fig. 7E–G). Furthermore, the 3.6-kb Col1a1 is also expressed in osteoclast lineage cells; 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, 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. CNN1 has high binding affinity to actin and influences cytoskeletal morphology and migration. Actin cytoskeleton also participates in anchorage-dependent cell division and proliferation.

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. Cell proliferation was increased in mesangial cells in Cnn1 knockout mice. 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. 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. Furthermore, 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. 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.

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. As a regulator of cytoskeleton, knockout of Cnn1 in mice led to increased bone formation. 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 calcine 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. 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 Cola1, 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 Cola1 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, 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 maybe 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. Two major mechanisms are involved in the regulation of osteoclast differentiation and its bone resorption activity. 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.\textsuperscript{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,\textsuperscript{28} Cnn1 overexpressed in osteoclast lineage did not have direct 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 demonstrated 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.\textsuperscript{58–60} Another study further suggested that Cbfa1 could upregulate the expression of RANKL via a direct binding to promoter of the RANKL.\textsuperscript{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.

Disclosures

All authors state that they have no conflicts of interest.

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