Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures

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Targeted disruption of core binding factor α1 (Cbfa1) showed that Cbfa1 is an essential transcription factor in osteoblast differentiation and bone formation. Furthermore, both in vitro and in vivo studies showed that Cbfa1 plays important roles in matrix production and mineralization. However, it remains to be clarified how Cbfa1 controls osteoblast differentiation, bone formation, and bone remodelling. To understand fully the physiological functions of Cbfa1, we generated transgenic mice that overexpressed Cbfa1 in osteoblasts using type I collagen promoter. Unexpectedly, Cbfa1 transgenic mice showed osteopenia with multiple fractures. Cortical bone, which was thin, porous, and enriched with osteopontin, was invaded by osteoclasts, despite the absence of acceleration of osteoclastogenesis. Although the number of neonatal osteoblasts was increased, their function was impaired in matrix production and mineralization. Furthermore, terminally differentiated osteoblasts, which strongly express osteocalcin, and osteocytes were diminished greatly, whereas less mature osteoblasts expressing osteopontin accumulated in adult bone. These data indicate that immature organization of cortical bone, which was caused by the maturational blockage of osteoblasts, led to osteopenia and fragility in transgenic mice, demonstrating that Cbfa1 inhibits osteoblast differentiation at a late stage.

Introduction

Bone is a mineralized tissue that is composed of an organic matrix. Type I collagen constitutes ~95% of the organic matrix, and the remaining 5% is composed of proteoglycans and noncollagenous proteins such as osteopontin and osteocalcin. Bone formation and maintenance are carried out by the coupled activities of osteoblasts and osteoclasts. Osteoblasts are bone-forming cells that synthesize and mineralize extracellular matrix, whereas osteoclasts are bone-resorbing cells that remove mineralized matrix. Osteoblasts arise from multipotential mesenchymal cells and further differentiate into bone-lining cells and osteocytes, the latter of which are the most abundant cells in bone and work as mechanosensors (Nijweide et al., 1996). Osteoclasts are derived from hematopoietic precursor cells formed by the fusion of mononuclear cells at the bone sites to be resorbed. Dysfunction of and imbalance between them can lead to bone metabolic disease states such as osteoporosis, which is marked by progressive bone loss and increased risk of fracture, or more rarely, osteopetrosis. Many factors influence the activities of these cells, and BMPs, TGFβs, FGFs, and IGFs, which are known to be local regulators of bone formation, have positive effects on osteoblast differentiation. Recent studies have suggested that many of them might target an important transcription factor, core binding factor α1 (Cbfa1)*/runx-related gene (Runx)2, which

*Abbreviations used in this paper: ALP, alkaline phosphatase; Cbfa1, core binding factor α1; HPRT, hypoxanthine guanine phosphoribosyl transferase; MMP, matrix metalloproteinase; OPG, osteoprotegerin; pQCT, quantitative computed tomography; RANKL, receptor activator of NF-κB ligand; RT, reverse transcriptase; Runx, runt-related gene; Tg, transgenic; TRAP, tartrate-resistant acid phosphatase; WT, wild-type.

Key words: Cbfa1; osteoblast; osteocyte; transgenic mice; osteopenia
directs a pathway in bone formation (for review see Yamaguchi et al., 2000).

Cbfa1 plays a pivotal role in osteogenesis (Komori and Kishimoto, 1998). Mice heterozygously mutated in the Cbfal locus show a phenotype similar to that of cleidocranial dysplasia in humans, in whom mutations of Cbfal have been found (Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). A homozygous mutation of this gene in mice induced a complete lack of bone formation with arrest of osteoblast differentiation (Komori et al., 1997; Otto et al., 1997). However, the complete lack of osteoblasts and neonatal lethality makes it difficult to examine the postnatal function of Cbfal by using this knockout model. An alternative model, which expressed the dominant negative form of Cbfal (DN-Cbfal), developed an osteopenic phenotype in mice and was used to indicate the indispensability of the gene for postnatal bone formation by regulating the functions of mature osteoblasts (Ducy et al., 1999). Furthermore, a large number of recent in vitro studies also implied that Cbfal is a positive regulator that can upregulate the expression of bone matrix genes, including type I collagen, osteopontin, bone sialoprotein, osteocalcin, and fibronectin. (Banerjee et al., 1997; Ducy et al., 1997; Sato et al., 1998; Harada et al., 1999; Xiao et al., 1999; Kern et al., 2001; Lee et al., 2000; Prince et al., 2001). All of these studies have indicated that Cbfal plays important roles in matrix formation and mineralization.

In the process of osteoblast differentiation, Cbfal seems to function in the commitment of the osteoblast lineage from multipotential mesenchymal cells because Cbfal-deficient calvarial cells had the potential to differentiate into both adipocytes and chondrocytes but completely lacked the ability to differentiate into the osteoblastic lineage (Kobayashi et al., 2000). However, after cells commit to the osteoblastic lineage it remains to be clarified how Cbfal operates in the process of bone formation. To understand fully the functions of Cbfal in the processes of osteoblast differentiation, matrix production, and mineralization, we generated transgenic mice that overexpress Cbfal specifically in osteoblasts under the control of type I collagen promoter. Unexpectedly, Cbfal transgenic mice showed severe osteopenia and suffered from bone fractures within a few weeks after birth. Osteopenia and fragility of bone were caused by the inhibition of osteoblast maturation, and immature osteoblasts accumulated in the bone of adult mice. These data indicate that Cbfal inhibits the late stage of osteoblast maturation, restricting Cbfal’s positive function to the early differentiation stage in the process of osteoblast development.

**Results**

Transgenic mice showed osteopenia with multiple fractures

The construct for Cbfal transgenic mice was generated using the 2.3-kb proximal promoter of pro-α(I) collagen gene and type II Cbfal cDNA, which starts from exon 1 (Enomoto et al., 2000), to express Cbfal specifically in osteoblasts (Fig. 1 A) (Rossert et al., 1995). We also generated β-galactosidase transgenic mice using the same pro-α(I) collagen promoter. The promoter directed the expression of the β-galactosidase gene to osteoblasts and immature osteoblastic cells. (Fig. 1, B and C). Bone-specific expression of the transgene was confirmed also by Northern blot analysis using Cbfal transgenic mice (Fig. 1 D). The transgene was expressed only in bone not in other tissues including muscle, brain, heart, kidney, thymus, liver, spleen, lung, testis, stomach, skin, and cartilage.

Although transgenic mice were born normally, they quickly started to display bone fractures (as early as 1 wk after birth), and within 3 wk most of them suffered from bone fracture. Bone fractures were found most frequently in tibiae, fibulae, calcanei, and femurs, whereas fractures in upper limbs and ribs were found also, indicating that the skeleton of transgenic mice was extremely fragile. Teeth of transgenic mice were also fragile and sometimes broken. The phenotype of teeth will be described elsewhere in detail. We generated seven F₀ transgenic mice with fractures. Although we succeeded in establishing a
Osteopenia in Cbfa1 transgenic mice | Liu et al. 159

line from one of them, the other F<sub>0</sub> mice were affected too severely to be bred and were analyzed directly. Although these F<sub>0</sub> mice suffered from severe fractures (Fig. 2 D), they showed phenotypes similar to the established line in radiological, histological, and Northern blot analyses, and the data from this line is described here in detail. The transgene expression of the line was about five times higher than endogenous Cbfa1 expression (Fig. 1 E). The body weight of transgenic (Tg) mice became progressively lower than that of wild-type (WT) littermates during development and reached nearly half of the normal average value at 4 wk of age (Tg, male 9.4 ± 0.4 g, n = 15; Tg female, 9.2 ± 0.5 g, n = 13; WT, male 18.1 ± 0.4 g, n = 61; WT, female 15.2 ± 0.4 g, n = 51, p < 0.01). The fragility of bones and teeth seemed to cause the growth retardation. The serum concentration of calcium was similar between wild-type and transgenic mice at 6 wk of age (WT, 9.5 ± 0.2 mg/dl, n = 6; Tg, 9.1 ± 0.2 mg/dl, n = 9, P > 0.1).

Radiological analysis showed that the whole skeleton of transgenic mice was proportionally shorter, and their bone was generally more radiolucent (Fig. 2, A and D). The fracture healing was observed in tibiae, fibulae, and calcanei, and cortices were thinner (Fig. 2, B and C). The histological appearance of tibiae clearly showed osteopenia in the transgenic mice (Fig. 3). The cortical bone of transgenic mice was porous and thinner at both 3 and 6 wk of age. The analysis by peripheral quantitative computed tomography (pQCT) also showed thinner cortical bone and enlarged marrow cavity in 3-mo-old transgenic mice (Fig. 4, A–E). The trabecular bone was decreased at 3 wk of age but not at 6 wk of age in transgenic mice (Fig. 3). This was also demonstrated by pQCT analyses in 3-mo-old mice, which showed similar amounts of mineral content in the
trabecular region (WT, 0.34 ± 0.02 mg/mm; Tg, 0.43 ± 0.04 mg/mm, n = 4, P > 0.05). Maintenance of trabecular bone and thinner porous cortical bone were observed also in histological analyses of 3- and 8-mo-old transgenic mice (unpublished data).

Cortical bone was immature and invaded by osteoclasts
Osteocytes in bone increase during growth. At 6 wk of age, both trabecular and cortical bone contained many osteocytes in wild-type mice but had few osteocytes in transgenic mice (Fig. 3 and Fig. 5, A and B). The drastic decrease of osteocytes was observed until 1 yr of age (Fig. 5, C–F; unpublished data). The decrease of osteocytes in transgenic mice is not due to cell death, since empty lacunae, which represent the death of osteocytes, were absent in bone. Numerous tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts were observed on the surface of trabecular bone but not in cortical bone of wild-type mice, whereas they were observed not only on trabecular bone but...
but also on cortical bone, especially in the cavities in transgenic mice (Fig. 5, A and B). Polarization microscopy revealed that cortical bone of transgenic mice at 3 mo of age was composed mainly of immature bone, described as woven bone, in which collagen fibers ran in all directions, instead of mature lamellar bone, which has highly ordered parallel collagen fibers as seen in wild-type mice (Fig. 5, C and D). The composition of bone matrix was also different. Osteopontin protein was restricted to trabecular bone in wild-type mice, whereas it was distributed widely and extensively in the whole bone, including cortical bone, in transgenic mice (Fig. 5, E and F).

Osteoblast function was impaired in both matrix production and mineralization

The cause of osteopenia was examined in detail by histomorphometrical analyses at 3 and 6 wk of age. The trabecular bone volume (BV/TV) of transgenic mice was significantly decreased at 3 wk of age but not at 6 wk of age (Fig. 6 A). Although the number of osteoblasts in transgenic mice was more than twice that in wild-type mice at both 3 and 6 wk of age, matrix production by osteoblasts was impaired severely in transgenic mice as shown by the decreased thickness of newly deposited matrix (osteoid thickness; O.Th) (Fig. 6, B–D). Osteocytes were decreased dramatically in transgenic mice, with their number in cortical bone about one-tenth of that in wild-type mice (Fig. 6 E). The number of osteoclasts in the trabecular bone of transgenic mice was equivalent to that of wild-type mice, but osteoclasts in transgenic mice were less activated at 6 wk of age as shown by an ~50% decrease of osteoclast surface (Oc.S) and eroded surface (ES) (Fig. 6, F–H). Therefore, the maintenance of trabecular bone volume in 6-wk-old transgenic mice seems to be due to decreased osteolytic activity.

To perform kinetic analyses of bone formation and mineralization, calcein was injected twice at a 3-d interval in 3-wk-old mice and a 7-d interval in 6-wk-old mice. Calcein injections formed two consecutive labels in most parts of 3- and 6-wk-old wild-type bone (Fig. 7 A). In contrast, transgenic bone showed only a single band of labeling or dual lines with decreased distance between them, and these bands were interrupted frequently by engraved cavities (Fig. 7 B). Trabecular dynamic histomorphometry was performed on the longitudinal sections of 3- and 6-wk-old bone. In transgenic mice, the mean distances between the two labels were short at both 3 and 6 wk of age (Fig. 7 C), and the mineralizing surface was decreased at 6 wk of age (Fig. 7 D), resulting in the significantly decreased bone formation rate at 6 wk of age (Fig. 7 E). Cortical dynamic histomorphometry was performed on cross sections of 3-wk-old bone. In transgenic mice, the mean distances between the two labels in both periosteum and endosteum were short (Fig. 7 F), and the mineralizing surface in periosteum was decreased (Fig. 7 G), resulting in the significantly decreased bone formation rates in both periosteum and endosteum (Fig. 7 H). Further, pQCT analysis showed that bone mineral density was decreased in the cortical bone of transgenic mice (Fig. 4 F). These data indicate that osteoblast function was impaired in both matrix production and mineralization in transgenic mice.

Fully differentiated osteoblasts were decreased and less mature osteoblasts were increased in transgenic mice

Because osteoblasts were increased but their function was impaired severely in transgenic mice, osteoblastic markers were investigated by in situ hybridization using probes of type I collagen, osteopontin, and osteocalcin. Mice at birth, 2 wk, 4 wk, 6 wk, 3 mo, and 8 mo of age were examined (Fig. 8; unpublished data). In wild-type mice at birth, most bone was covered by type I collagen-positive and osteopontin-positive
cells, but the number of osteocalcin-positive cells was very low (Fig. 8, A, C, and E). In transgenic mice at birth, osteopontin was expressed strongly in type I collagen–positive cells, but osteocalcin was barely detectable (Fig. 8, B, D, and F). The number of type I collagen–positive cells was increased by 60% in transgenic newborns compared with their wild-type counterparts (WT, 1528/mm²; Tg, 2531/mm², n = 5, p < 0.01). In wild-type mice, osteopontin-positive cells decreased gradually and osteocalcin-positive cells increased gradually during development, with osteocalcin-positive cells widely distributed at 3 mo of age (Fig. 8, K and M). However, in transgenic mice osteopontin-positive cells always covered most of the trabecular and cortical bone, but osteocalcin expression was much weaker than in their wild-type counterparts (Fig. 8, L, M, and N). Even in 8-mo-old transgenic mice, most bone was covered by osteopontin-positive cells, whereas only a small area of bone was covered by osteopontin-positive cells in the wild-type mice (unpublished data). Since osteopontin expression is detected earlier than osteocalcin expression in osteoblast differentiation (Yoon et al., 1987; Mark et al., 1988), these data suggest that osteoblasts were less mature in transgenic mice.

Endogenous Chfra1 expression was also examined using a probe containing the 3′ untranslated region, which does not hybridize with the transgene (Fig. 8, G, H, O, and P; unpublished data). In wild-type mice, the expression pattern of Chfra1 was similar to that of osteopontin but different from that of osteocalcin at any age, although some overlaps of Chfra1 and osteocalcin expression were observed, especially at younger ages. It indicates that a major population of Chfra1 highly positive cells consists of less mature osteoblasts. In transgenic mice, endogenous Chfra1-positive cells were increased, and the level of the expression seemed to be upregulated.

Chfra1 failed to induce expression of the genes related to bone matrix, mineralization, and osteoclastogenesis

Expression of the genes related to bone matrix proteins, including type I collagen, osteopontin, bone sialoprotein, osteocalcin, and matrix metalloproteinase (MMP)13, is con-
considered to be regulated by Cbfa1 (Yamaguchi et al., 2000). 

Alkaline phosphatase (ALP) is considered to be upregulated during osteoblast differentiation (Stein et al., 1990). To analyze the expression of these genes, Northern blot or reverse transcriptase (RT)-PCR analysis was performed using RNA from long bones of 1-mo-old wild-type mice. To analyze the expression of these genes, Northern blot or reverse transcriptase (RT)-PCR analysis was performed using RNA from long bones of 1-mo-old wild-type mice. By using RT-PCR, we also examined the expression of endogenous Cbfa1 (G, H, O, and P) probes. The Cbfa1 probe detects the expression of endogenous Cbfa1 but not the transgene. Note that the pattern of Cbfa1 expression is similar to that of osteopontin expression but not osteocalcin expression in wild-type mice (C, E, G, K, M, and O). Osteopontin-positive cells are increased markedly, but osteocalcin highly positive cells are decreased markedly in transgenic mice (K, L, M, and N). Ct, cortical bone; Tb, trabecular bone. Bars: (A–H) 200 μm; (I–P) 100 μm.

Figure 9. Expression of genes related to bone matrix, mineralization, and osteoclastogenesis. RNA was extracted from long bones without fractures at 4 wk of age for Northern blot analysis and at 4 and 11 wk of age for RT-PCR. 20 μg of total RNA was loaded and hybridized with probes of pro-α1(I) collagen (Col1(I)), osteocalcin (OC), MMP13, ALP, bone sialoprotein (BSP), and osteopontin (OP). Glycerinaldehyde-3-phosphate-dehydrogenase was used as an internal control. Representative data from four independent samples are shown. The expression of pro-α2(I) collagen (Col2(I)), RANKL, and OPG was examined by RT-PCR. HPRT was used as an internal control. Duplicate PCRs were performed in four independent samples. Representative data are shown. WT, wild-type mouse; Tg, transgenic mouse.

Discussion

Cbfa1 transgenic mice showed severe osteopenia and suffered from multiple fractures. Although trabecular bone was conserved, cortical bone was reduced severely. Cortical bone, which is composed of immature bone with a few osteocytes, was invaded by osteoclasts. Enlarged bone marrow cavity also implied the osteolysis of cortical bone by osteoclasts. However, osteoclastogenesis was not accelerated in transgenic mice. Osteoblast number was increased from an early developmental stage, but osteoblast function was impaired in both matrix production and mineralization. Osteoblast maturation was inhibited at a late stage, and less mature osteoblasts accumulated to form the impaired bone in adult mice. Therefore, failure in the terminal differentiation of osteoblasts resulted in osteopenia and fragility in transgenic mice, demonstrating that Cbfa1 inhibits osteoblast differentiation at a late stage.

Previous in vitro and in vivo data suggested that Cbfa1 plays an important role in maturation of osteoblasts. However, our data showed that the late stage of osteoblast maturation was inhibited in transgenic mice as indicated by the accumulation of osteopontin-positive cells and the decrease of highly osteocalcin-positive cells, osteocytes, and ALP and osteocalcin expression. The accumulation of less mature osteoblasts in transgenic mice seemed to be caused not only by the maturational blockage of osteoblasts but also by acceleration of osteoblast differentiation at an early stage of cell development because osteoblasts were increased in number at neonatal stage (Fig. 8). Furthermore, the proliferation and apoptosis of less mature osteoblasts in transgenic mice have to be considered, although their analyses in vitro were unsuccessful because of the loss of the transgene expression in
primary culture of calvaria-derived cells (unpublished data) as previously described (Krebsbach et al., 1993).

ALP activity is detected at an early stage of osteoblast differentiation and continues to increase during osteoblast maturation until the mineralization phase (Stein et al., 1990; Weinreb et al., 1990). In vitro experiments demonstrated that Cbfa1 transfection induced ALP activity in multipotential mesenchymal cells, C3H10T1/2 and C2C12 (Harada et al., 1999; Lee et al., 2000), indicating an important role for Cbfa1 in the induction of ALP activity. Although the difference in ALP expression level between transgenic and wild-type mice was not apparent at birth, it became evident during development (Fig. 9; unpublished data). This suggests that overexpression of Cbfa1 blocks osteoblast maturation at a certain stage in vivo.

Since osteocalcin expression is restricted to mature osteoblasts and odontoblasts, it is a convenient marker for fully differentiated osteoblasts (Mark et al., 1988; Stein et al., 1990). Cbfa1 induced osteocalcin expression in various cells in vitro including MC3T3-E1, C3H10T1/2, and skin fibroblasts (Ducy et al., 1997; Harada et al., 1999). Furthermore, Cbfa1 or related proteins bound osteocalcin promoter and strongly induced osteocalcin promoter activity in various cell lines, including C3H10T1/2 and nonosteoblastic cells, HeLa and F9, and Cbfa1 binding sites, were essential for osteocalcin expression (Geoffroy et al., 1995; Banerjee et al., 1996; Frendo et al., 1998; Javed et al., 1999; Xiao et al., 1999). These findings suggested that Cbfa1 is the most important factor for osteoblast-specific osteocalcin expression in vitro. However, a major population of Cbfa1 highly positive cells consisted of less mature osteoblasts in wild-type mice, and overexpression of Cbfa1 failed to upregulate osteocalcin expression in vivo (Figs. 8 and 9). These data indicate that other factors, which are induced at a late stage of osteoblast differentiation, are required for the regulation of osteocalcin expression or that some factors suppress osteocalcin expression at an immature stage of osteoblast differentiation in vivo. It has been shown that Groucho/TLE proteins repress Runx-dependent activation of tissue-specific gene transcription (Levanon et al., 1998; Javed et al., 2000), and TLE downregulates Cbfa1-mediated activation of osteocalcin expression (Javed et al., 2000). Further, Runx1 is known to interact with the corepressor mSin3A (Lutterbach et al., 2000). Thus, these repres...
tion of bone mass. Since Cbfa1 is an essential factor for osteoblast differentiation, many factors and substances that have an effect on bone mass will influence Cbfa1 expression or activation. Thus, our findings are expected to be of great benefit to future trials to increase bone mass.

Materials and methods

Generation of transgenic mice

A DNA fragment covering the entire coding region of the mouse type II Cbfa1 isoform (Harada et al., 1999) was cloned into the mammalian expression vector pNASSB (CLONTECH Laboratories, Inc.) by replacing the β-galactosidase gene at NotI sites, giving rise to an intermediate vector for inserting a promoter. A DNA fragment containing the 2.3-kb osteoblast-specific promoter region for the mouse pro-α1 (I) collagen (Rossert et al., 1995), which was provided by B. de Crombrugghe (The University of Texas, Houston, Texas), was inserted into Xhol site of the intermediate vector to generate the final expression construct. The fragments from the final construct, including the 2.3-kb pro-α1 (I) promoter and Cbfa1, were injected into the pronuclei of fertilized eggs from C57BL/6 × C3H F1 (B6C3H F1) mice. Transgene integration and expression were identified by Southern and Northern blot analyses, respectively, using the whole length cDNA of type II Cbfa1 as a probe. A transgenic line was maintained on a Southern and Northern blot analyses, respectively, using the whole length cDNA of type II Cbfa1 as a probe. A transgenic line was maintained on a Southern and Northern blot analyses, respectively, using the whole length

Detection of β-galactosidase activity

To confirm the activity of the promoter used in this study, we also cloned the DNA fragment covering the 2.3-kb pro-α1 (I) promoter region into the EcoRI site of pNASSB to direct the expression of the β-galactosidase gene. The β-galactosidase transgenic embryos were analyzed at different days postcoitum. Detection of β-galactosidase activity was performed as described (Ueta et al., 2001). Stained embryos were embedded in paraffin and used to generate 7-μm sections, which were counterstained with eosin.

X-ray and pQCT analyses

Transgenic mice and their wild-type littermates were anesthetized and subjected to x-ray exposure in a micro-FX1000 (Fuji Film, Inc.). Long bones were dissected from killed mice and exposed to x-rays. In pQCT analysis, femurs were fixed with 10% buffered formalin for 24 h and measured using an XCT Research SA (Stratec Medizintechnik). Voxel size was 0.08 × 0.08 × 0.46 mm. The contour of the total bone was determined automatically by the pQCT software algorithm. The cortical and trabecular parameters were obtained at the diaphysis and 2 mm from distal epiphysis, respectively. The threshold values of 690 mg/cm² for the cortical region and 395 mg/cm² for the trabecular region were used in this experiment.

Histological analyses

For histological analyses, mice were killed at birth, 2 wk, 3 wk, 4 wk, 6 wk, 3 mo, 8 mo, and 1 yr of age. For the assessment of dynamic histomorphometric indices, mice were injected twice with calcine at a dose of 0.16 mg/10 g body weight and analyzed at 3 or 6 wk of age. The 3-wk group received dual injections at 6 and 3 d before sacrifice, and the 6-wk group received them at 8 and 1 d before sacrifice. Long bones were fixed with ethanol at room temperature for 20 min and then incubated with rabbit anti–mouse osteopontin antibody (IBL Co., Ltd.) overnight at 4°C. Localization of the first antibody was visualized by incubation with biotinylated Fabi, fragments of swine anti-rabbit IgG antibody (Dako) at room temperature for 40 min and then with the ABC reagents (Vector Laboratories). Finally, sections were stained by DAB substrate and counterstained with methyl green.

Northern blot and RT-PCR

Total RNA was extracted from long bones without fracture from newborn and 4- and 11-wk-old transgenic and wild-type mice by lithium chloride. 20 Mg of total RNA was denatured with formamide, subjected to electrophoresis on 1.0% agarose gels, and transferred onto nylon membranes. Membranes were hybridized with [32P]-labeled cDNA probes of pro-α1 (I) collagen, osteocalcin, MMP13, ALP, osteopontin, bone sialoprotein, and glyceraldehyde-3-phosphate-dehydrogenase as described previously (Inada et al., 1999). For RT-PCR, cdNA was amplified by Amp Taq DNA polymerase (Perkin Elmer) using the following primers: Pro-α2 (I) collagen, 5′-TGTCCTCTCCAGGGTTCACA-3′ and 5′-ACACGGAATTCTGTGGTACG-3′; RANKL, 5′-GTACACTGTCCCTTGGTAC-3′ and 5′-TGAAAATCCAAAGTACGTCG-3′; OPG, 5′-CACGTCTCCTGCTGATGGAAGA-3′ and 5′-AAACCCCATGACACATTCT-3′; hypoxanthine guanine phosphoribosyl transferase (HPR7), 5′-GCTGTTGAACAGCACCTCT-3′ and 5′-CACAGACTAGAACACTGC-3′. 18 (pro-α2 (I) collagen), 27 (RANKL), 28 (OPG), and 23 cycles (HPR7) of amplification were done with a Gene Amp PCR system 2400 (Perkin Elmer) (30 s at 94°C, 30 s at 55°C or 60°C, and 30 s at 72°C).

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