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

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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 monocyctic 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)/runt-related gene (Runx)2, which...
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 Cbfa1 locus show a phenotype similar to that of cleidocranial dysplasia in humans, in whom mutations of Cbfa1 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 Cbfa1 by using this knockout model. An alternative model, which expressed the dominant negative form of Cbfa1 (DN-Cbfa1), 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 Cbfa1 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 Cbfa1 plays important roles in matrix formation and mineralization.

In the process of osteoblast differentiation, Cbfa1 seems to function in the commitment of the osteoblast lineage from multipotential mesenchymal cells because Cbfa1-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 Cbfa1 operates in the process of bone formation. To understand fully the functions of Cbfa1 in the processes of osteoblast differentiation, matrix production, and mineralization, we generated transgenic mice that overexpress Cbfa1 specifically in osteoblasts under the control of type I collagen promoter. Unexpectedly, Cbfa1 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 Cbfa1 inhibits the late stage of osteoblast maturation, restricting Cbfa1’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 Cbfa1 transgenic mice was generated using the 2.3-kb proximal promoter of pro-α I collagen gene and type II Cbfa1 cDNA, which starts from exon 1 (Enomoto et al., 2000), to express Cbfa1 specifically in osteoblasts (Fig. 1 A) (Rossert et al., 1995). We also generated β-galac-

Figure 1. Generation of transgenic mice. (A) Diagrams of the DNA constructs used to generate pro-α1 (I) collagen promoter Cbfa1 transgenic mice. DNA fragments covering the entire coding region of the mouse type II Cbfa1 isoform and 2.3 kb pro-α1 (I) collagen gene promoter region were used. *SV40 splice donor/acceptor signals; **SV40 polyadenylation signal. (B and C) α-Galactosidase staining in pro-α1 (I) collagen promoter α-galactosidase transgenic mice at E15.5. (C) Longitudinal section of forelimb. Staining was observed specifically in osteoblasts around the diaphysis and in immature osteoblastic cells around the metaphysis. Sections were counterstained with eosin. (D) Northern blot hybridized with Cbfa1 probe. DNA was extracted from tissues of 4-wk-old Cbfa1 transgenic mice, and 20 μg of total RNA was loaded per lane. St, stomach; Te, testis; Lu, lung; Sp, spleen; Li, liver; Th, thymus; Ki, kidney; He, heart; Br, brain; Mu, muscle; Bo, bone; Ca, cartilage; Sk, skin. (E) Northern blot analysis comparing the transgene and endogenous Cbfa1 levels of expression in long bones of newborn Cbfa1 transgenic mice. 20 μg of total RNA was loaded. Bar, 100 μm.

tosidase transgenic mice using the same pro-α1 (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 Cbfa1 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
line from one of them, the other F0 mice were affected too severely to be bred and were analyzed directly. Although these F0 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 tibia 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

Figure 2. Radiological analysis. (A–C) X-ray analysis of 6-wk-old Cbfa1 transgenic mice from the established line. Whole skeletons of transgenic mice are proportionally shorter and more radiolucent (A). The transgenic mouse suffered from fractures in the tibia, fibula, and calcanei, and fracture healing is observed in these regions (A and B). The radioluency is caused mainly by thinner cortices because metaphyseal trabeculation is similarly observed in femurs (C). (D) Radiograph of 9-wk-old F0 transgenic mice. Whole skeletons are also more radiolucent, and fracture healing is observed in tibiae, fibulae, and calcanei in both lower limbs. WT, wild-type mouse; Tg, transgenic mouse.

Figure 3. Histological appearance of transgenic bone. Longitudinal sections through the proximal tibia of wild-type (A and C) and transgenic (B and D) mice at 3 (A and B) and 6 wk of age (C and D). Cortical bone in transgenic mice is thin and porous at both 3 and 6 wk of age. Transgenic mice show reduced trabeculation at 3 wk of age but not at 6 wk of age. Undecalcified sections were stained with toluidine blue. Bar, 1 mm.
pQCT analysis. Diaphyses of femurs from wild-type and transgenic female mice at 3 mo of age were analyzed by pQCT. (A and B) pQCT images from wild-type (A) and transgenic (B) mice. Note the increased width of the marrow cavity and the concomitant reduction in cortical thickness in transgenic mice. Mineral densities are shown as different colors according to the standard mineral density gradients. (C–F) Cortical thickness (C), endosteal circumference (D), periosteal circumference (E), and bone mineral density (F) were measured for wild-type (white bars) and transgenic (black bars) mice. Error bars show means ± SEM (n = 4). *P < 0.05 and **P < 0.01 between wild-type and transgenic mice as determined by one-way ANOVA. Bars, 1 mm.

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 in cortical bone of transgenic mice (unpublished data).

Figure 5. Decreased osteocytes, osteoclast invasion, collagen structure, and osteopontin deposition in cortical bone of transgenic mice. (A and B) TRAP staining of cortical bone in tibiae of wild-type (A) and transgenic (B) mice at 6 wk of age. Many TRAP-positive osteoclasts are observed in the cavities of cortical bone in transgenic mice (B). Note that osteocytes are diminished greatly in transgenic mice. (C and D) Polarized microscopy of cortical bone in tibiae of wild-type (C) and transgenic (D) mice at 3 mo of age. Cortical bone in transgenic mice shows the woven pattern instead of the lamellar collagen deposition seen in wild-type mice. (E and F) Immunohistochemical analysis in cortical bone of wild-type (E) and transgenic (F) mice at 3 mo of age using antiosteopontin antibody. Osteopontin is deposited heavily in cortical bone of transgenic mice. Note that osteocytes are spaced regularly in wild-type bone, but a few osteocytic cells are spaced irregularly in transgenic bone. Bars, 100 μm.
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 trabecular 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 Cbfa1 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 Cbfa1 was similar to that of osteopontin but different from that of osteocalcin at any age, although some overlaps of Cbfa1 and osteocalcin expression were observed, especially at younger ages. It indicates that a major population of Cbfa1 highly positive cells consists of less mature osteoblasts. In transgenic mice, endogenous Cbfa1-positive cells were increased, and the level of the expression seemed to be upregulated.

Cbfa1 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 up-regulated during osteoblast differentiation (Stein et al., 1990). To determine 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 and transgenic mice. The expression of osteopontin was examined by RT-PCR, we also examined the expression of endogenous Cbfa1 and osteocalcin (OC) probes. The Cbfa1 probe detects the expression of osteopontin 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. Ct, cortical bone; Tb, trabecular bone. Bars: (A–H) 200 μm; (I–P) 100 μm.

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 repressors may play an important role in the transcriptional regulation of osteocalcin by repressing Cbfa1-dependent activation at an early stage of osteoblast differentiation.

Overexpression of DN-Cbfa1 at a late stage of osteoblast differentiation caused a decrease in the bone formation rate and decreased expression of the genes encoding main bone matrix proteins and resulted in osteopenia (Ducy et al., 1999). Surprisingly, our transgenic mice overexpressing Cbfa1 at both early and late stages of osteoblast differentiation also showed an osteopenic phenotype, although the mechanism for osteopenia was different. The two kinds of transgenic mice had a common feature because Cbfa1 function was suppressed in fully differentiated osteoblasts and the fully differentiated osteoblasts were diminished in our Cbfa1 transgenic mice. Therefore, both transgenic mice lacked at least Cbfa1-dependent function of fully differentiated osteoblasts, which finally caused osteopenia in both transgenic mice. It indicates that Cbfa1 inhibits osteoblast differentiation at a late stage, but some level of Cbfa1 is required for the expression of the genes encoding main bone matrix proteins. It also indicates that the transcriptional regulation of bone matrix genes by Cbfa1 is dependent on the maturational stage of osteoblasts as discussed about osteocalcin expression in the previous paragraph.

It was reported recently that RANKL is essential for osteoclastogenesis and that OPG inhibits osteoclastogenesis (Aubin and Bonnelye, 2000), and it was suggested that Cbfa1 is involved in the regulation of RANKL or OPG expression (Gao et al., 1998; Kitazawa et al., 1999; Thirunavukkarasu et al., 2000). However, both RANKL and OPG decreased gradually during development in transgenic mice (Fig. 9). It was suggested that osteoprogenitor cells have more potential to support osteoclast development than more differentiated cells (Manolagas, 2000). Therefore, the decrease of RANKL may reflect the relative decrease of osteoprogenitor cells because Cbfa1 seemed to accelerate the early stage of osteoblast differentiation (Fig. 8). In transgenic mice, cortical bone mass but not trabecular bone mass was reduced severely without acceleration of osteoclastogenesis. However, the enlarged bone marrow cavity and the presence of numerous osteoclasts in cortical bone showed that cortical bone loss was a result of osteolytic activity. This seemed to cause by the immature composition of cortical bone, which contains abundant osteopontin with the small cell attachment motif Arg-Gly-Asp recognized by integrins and promotes the attachment of osteoclasts to the extracellular matrix (Fig. 5; Young et al., 1993). The expression of bone sialoprotein, which also has the Arg-Gly-Asp motif, was increased in Northern blot analysis (Fig. 9).

The drastic decrease of osteocytes that is a unique phenotype of the transgenic mice was caused by the inhibition of osteoblast maturation. Osteocytes are spaced regularly throughout the bone and communicate with each other and with osteoblasts and bone marrow stromal cells using their processes. Although the exact function of osteocytes remains unknown, they are considered to work as mechanosensors (Nijweide et al., 1996). Thus, the fragility of transgenic bone might be caused by the near absence of osteocytes, causing an inability to detect mechanical stress and microfractures.

Overexpression of Cbfa1 in osteoblasts increased osteoblast number but inhibited their terminal maturation, resulting in accumulation of less mature osteoblasts and osteopenia. Therefore, in an attempt to increase bone mass by Cbfa1 intermittent induction of Cbfa1 in osteogenic cells might permit a periodic increase of immature osteoblasts and their maturation. This concept might be related to the anabolic action on bone mass induced by the intermittent administration of parathyroid hormone (Dempster et al., 1993; Ishizuya et al., 1997; Jilka et al., 1999) because parathyroid hormone induces a protein kinase A–dependent transactivation of Cbfa1 (Selvamurugan et al., 2000).

We demonstrated using Cbfa1 transgenic mice that Cbfa1 negatively regulates osteoblast differentiation at a late stage of osteoblast development. However, Cbfa1 seems to regulate osteoblast differentiation positively at an early stage. These opposite functions of Cbfa1, depending on the maturational stage of osteoblasts, may play an important role in the regula-
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 NolI 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 (Rossier et al., 1995), was provided by B. de Crombrugghe (The University of Texas, Houston, Texas), was inserted into XhoI 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 mice. Transgene integration and expression were identified by Southern and Northern blot analyses, respectively, using the whole length anti–mouse osteopontin antibody (IBL Co., Ltd.) overnight at 4°C. The Southern and Northern blot analyses, using the whole length anti–mouse osteopontin antibody, were done. The fragments from the final construct were identified by Southern and Northern analyses, using the whole length anti–mouse osteopontin antibody, overnight at 4°C. The DNA fragments were injected into the pronuclei of fertilized eggs from C57BL/6 × C3H F1 mice. Transgene integration and expression were identified by Southern and Northern blot analyses, respectively, using the whole length anti–mouse osteopontin antibody (IBL Co., Ltd.) overnight at 4°C.

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 developmental stages. 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 (Fujifilm, 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 and the undecalified bones were embedded in glycolmethacrylate. 3-μm longitudinal sections from the proximal parts of tibiae and 20-μm cross sections from mid-diaphyses of femurs were stained with toluidine blue and analyzed using a semiautomated system (Osteoplan II; ZEISS). Nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt et al., 1987). Some of the sections were stained with TRAP. Bones from the other mice were treated with fixing reagents, sectioned, treated with hematoxylin and eosin and observed under polarized light (Bucay et al., 1998). To examine osteoblast markers, sections of tibiae at different ages were stained with antibodies to osteocalcin, osteopontin, and Cbfa1 as described previously (Inada et al., 1999).

Immunohistochemistry
Paraffin sections were blocked with 1% BSA containing 10% swine or rabbit serum 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 Fab, fragments of swine anti-rabbit IgG antibody (Dako) at room temperature for 40 min and then washed with the ABC reagents (Vector Laboratories). Finally, sections were stained with 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 formaldehyde, 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 (PerkinElmer) using the following primers: Pro-α2 (I) collagen, 5’-TGTGCTTCAGGGTTTCCA-3’ and 5’-ACACGGAATTTCTGGTGCAG-3’; RANKL, 5’-GTACCTGTCCTCTGTTGAC-3’ and 5’-TGGAACTCAGGATCTG-3’; OPG, 5’-CAGCCTTGCTCCGTATGAGA-3’ and 5’-AAACACCCGATGGACATTCT-3’; hypoxanthine guanine phosphoribosyl transferase (HPRT), 5’-GCTGTGAAGAGCACTCT-3’ and 5’-CACAGGCTAGACACCTG-3’. 18 (pro-α2 (I) collagen), 27 (RANKL), 28 (OPG), and 23 cycles (HPRT) of amplification were done with a Gene Amp PCR system 2400 (Perkin Elmer) (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C).

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References


