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Targeted Disruption of *Cbfa1* Results in a Complete Lack of Bone Formation owing to Maturational Arrest of Osteoblasts

T. Komori,* H. Yagi,* S. Nomura,† A. Yamaguchi,‡ K. Sasaki,* K. Deguchi,* Y. Shimizu,* R. T. Bronson,§ Y.-H. Gao,‡ M. Inada,* M. Sato,‖ R. Okamoto,* Y. Kitamura,† S. Yoshiki,‡ and T. Kishimoto*
*Department of Medicine III
†Department of Pathology
Osaka University Medical School
2–2 Yamada-oka Suita
Osaka 565, Japan
‡Department of Oral Pathology
Showa University School of Density
Hatanodai 1–5–8, Shinagawa-ku
Tokyo 142, Japan
§Department of Pathology
Tufts University Schools of Medicine
and Veterinary Medicine
Boston, Massachusetts 02111
‖First Department of Anatomy
Osaka City University Medical School
1–4–54 Asahimachi, Abeno-ku
Osaka, 545, Japan

Summary

A transcription factor, Cbfa1, which belongs to the *runt*-domain gene family, is expressed restrictively in fetal development. To elucidate the function of Cbfa1, we generated mice with a mutated *Cbfa1* locus. Mice with a homozygous mutation in *Cbfa1* died just after birth without breathing. Examination of their skeletal systems showed a complete lack of ossification. Although immature osteoblasts, which expressed alkaline phophatase weakly but not *Osteopontin* and *Osteocalcin*, and a few immature osteoclasts appeared at the perichondrial region, neither vascular nor mesenchymal cell invasion was observed in the cartilage. Therefore, our data suggest that both intramembranous and endochondral ossification were completely blocked, owing to the maturational arrest of osteoblasts in the mutant mice, and demonstrate that Cbfa1 plays an essential role in osteogenesis.

Introduction

Cbfa1 (core-binding factor), also called Pebp2αA (polyoma enhancer-binding protein), is a transcription factor that belongs to the *runt*-domain gene family. Three *runt*-domain genes (*Cbfa1/Pebp2αA*, *Cbfa2/Pebp2αB*, and *Cbfa3/Pebp2αC*) have been identified (Bae et al., 1993, 1995; Ogawa et al., 1993a). They have a DNA binding domain, *runt*, which is homologous with the Drosophila pair-rule gene *runt* (Kania et al., 1990), and they form heterodimers with cotranscription factor Cbfb/Pebp2β and acquire enhanced DNA-binding capacity in vitro (Ogawa et al., 1993b; Wang et al., 1993). Cbf specifically recognizes a consensus sequence, PuACCPuCA, which was originally identified in the polyoma virus enhancer (Kamachi et al., 1990) and murine leukemia virus enhancers (Wang and Speck, 1992). The consensus sequence has also been found in T cell-specific genes (*TCRα*, *TCRβ*, *TCRδ*, *TCRγ*, and *CD3ε*) (Hallberg et al., 1992; Prosser et al., 1992; Redondo et al., 1992; Hsiang et al., 1993; Giese et al., 1995), enzymes (myeloperoxidase, neutrophil elastase, granzyme B serine protease) (Nuchprayoon et al., 1994; Wargnier et al., 1995), and cytokines and their receptors (GM-CSF, IL3, CSF-1) (Cameron et al., 1994; Zhang et al., 1994; Frank et al., 1995; Takahashi et al., 1995). In recent studies, Cbf-related factors were shown to interact with the promoter region of the *Osteocalcin* gene (Geoffroy et al., 1995; Merriman et al., 1995; Banerjee et al., 1996).

CBFA2 and CBFB are frequently involved in chromosomal translocations in acute leukemia (Miyoshi et al., 1991; Liu et al., 1993). *Cbfa2* is widely expressed in mouse embryo and adult tissues (Simeone et al., 1995; Satake et al., 1995), and *Cbfb* expression is ubiquitous (Ogawa et al., 1993b; Wang et al., 1993; Satake et al., 1995). The heterodimerization of Cbfa2 and Cbfb is required for their DNA-binding capacity in vitro (Ogawa et al., 1993b; Wang et al., 1993). The targeted disruption of *Cbfa2* resulted in the embryonic death at midgestation, owing to hemorrhage in the central nervous system, and blocked fetal liver hematopoiesis. The phenotype of *Cbfb*-deficient mice (Sasaki et al., 1996; Wang et al., 1996a) was similar to that of *Cbfa2*-deficient mice (Okuda et al., 1996; Wang et al., 1996b). Therefore, it was confirmed that Cbfb is essential for the function of Cbfa2 in vivo. As Cbfb is also necessary for the DNA binding of Cbfa1 in vitro (Ogawa et al., 1993a), it was suggested that *Cbfa2* is the earliest gene necessary for embryogenesis among the *runt*-domain gene family (Sasaki et al., 1996; Wang et al., 1996a).

In contrast, the expression of *Cbfa1* seemed to be restricted. It was detected in T cell lines and NIH3T3 cells but not in B cell lines (Ogawa et al., 1993a). It was also detected in thymus and testis but not in the tissues including brain, lung, heart, spleen, liver, and kidney (Satake et al., 1995). Consistent with these observations, there is a Cbf site in the regulatory regions of many T cell-specific genes, including the T cell receptor α, β, γ, and δ genes, and Cbfa1 binds to T cell receptor β enhancer and stimulates the enhancer activity in vitro (Ogawa et al., 1993a). These observations led to the speculation that Cbfa1 is likely to be involved in T lymphocyte-specific transcriptional regulation (Satake et al., 1995). However, the function of Cbfa1 in vivo remains to be clarified.

The skeletal tissue is composed of various types of mesenchymal cells, among which are osteoblasts, chondrocytes, myoblasts, and bone-marrow stromal cells, including adipocytes. These cell lineages are believed to originate from common mesenchymal progenitors (Aubin et al., 1993). These progenitors acquire specific phenotypes depending on the maturational stage of the particular cell type during the differentiation process. In the case of skeletal muscles, the muscle-specific transcription factors of the MyoD family, which belong to the basic Helix-Loop-Helix family, are necessary for determining the pathway of differentiation into the muscle lineage and are required for the differentiation from a determined myoblast to a fully differentiated myotube (Weintraub, 1993). In addition, peroxisome proliferator-activated receptor γ2 (PPARγ2) plays an important role in determining the pathway of differentiation into the adipocyte lineage (Tontonoz et al., 1994). However, the specific transcription factors that are necessary for differentiation into the osteoblast lineage and the maturation of osteoblasts have not been clarified. Identification of these factors is necessary in order to understand the molecular mechanism involved in osteoblast differentiation and skeletogenesis.

To elucidate the function of Cbfa1, we generated mutated mice in which the *Cbfa1* gene locus was targeted. The mutated mice died just after birth and showed a complete lack of bone formation. Although the development of cartilage was nearly normal, ossification was completely blocked throughout the body. Here, we show that Cbfa1 is essential for the maturation of osteoblasts and both intramembranous and endochondral ossification.

Results

Immediate Postnatal Death of *Cbfa1*-Deficient Mice

To disrupt the *Cbfa1* gene, 1.2 kb of exon 1, which contains the first 41 amino acids of the *runt* domain, was replaced with PGK-*neo* (Figure 1A). The targeting vector was electroporated into the E14 line of embryonic stem (ES) cells and selected by G418 and gancyclovir. Targeted ES cells were injected into blastocysts of C57BL/6 mice. The chimeras were mated with C57BL/6 mice, and the *Cbfa1* mutation was transmitted through the germline. The heterozygous ($Cbfa1^{+/-}$) mice resembled normal mice in gross appearance. In addition, their body weight was not significantly different from that of wild-type mice at four weeks of age (+/−, 18.6 ± 3.4 g; +/+, 20.8 ± 2.6 g in male. +/−, 16.7 ± 2.9 g; +/+, 17.0 ± 2.7 g in female), and they were fertile.

After intercrossing of heterozygous mice, no homozygous ($Cbfa1^{-/-}$) mice were observed in our analysis of the genotype of 4-week-old litters (data not shown). However, a few newborns were found dead in every litter, and their genotype was always $Cbfa1^{-/-}$. At embryonic day 18.5 (d18.5), however, we found live homozygous embryos at the frequency predicted by Mendelian law (Figure 1B; data not shown). After intercrossing heterozygous mice, we observed their delivery and noticed that small newborns scarcely breathed and soon died; all of these mice were homozygous. It was ascertained that they were alive just after delivery because they reacted to stimulation for several minutes; however, they soon became cyanotic. The weight of homozygous embryos at d18.5 was approximately 80% of that of heterozygous and wild-type embryos (+/+, 1.16 ± 0.21 g; +/−, 1.17 ± 0.14 g; −/−, 0.93 ± 0.22 g), and they had short legs (Figure 2A). To confirm that the *Cbfa1* gene was disrupted, we carried out RT–PCR of the *runt* domain (Figure 1C). Homozygous embryos lacked amplified cDNA of the *runt* region. Since the *runt* domain

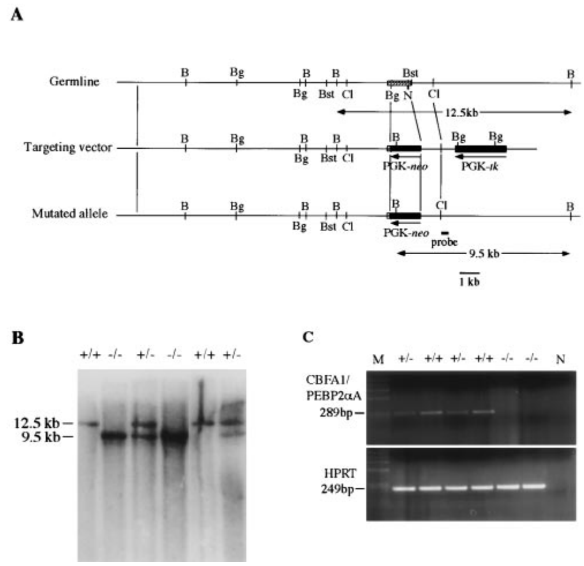

Figure 1. Generation of Mutant Mice

(A) Structure of the targeting vector and partial restriction map of genomic *Cbfa1* locus and mutated allele after homologous recombination. Exon 1 is depicted as a hatched box, and PGK-*neo* and PGK-*tk* as closed boxes. B, BamHI; Cl, ClaI; Bg, BglII; Bst, BstEII; N, NotI. ClaI, BglII, and BstEII sites between 3′ ClaI and BamHI sites have not been determined.

(B) Southern blot analysis of fetal DNA. Genomic DNA isolated from livers of embryos was digested with BamHI and hybridized with the ClaI-SalI* probe shown in (A) (asterisk denotes the site in the cloning vector). Bands are indicated corresponding to wild type (12.5 kb) and mutant (9.5 kb) genes.

(C) RT–PCR analysis of *runt* region of *Cbfa1*. Total RNA was isolated from livers of wild type (+/+), heterozygous (+/−), and mutant (−/−) embryos at d18.5. N, no cDNA in the reaction mixture of PCR.

is essential for DNA binding and heterodimerization with Cbfb, the function of Cbfa1 should have been abolished in homozygous embryos.

Absence of Ossification in $Cbfa1^{-/-}$ Mice

Since mutant ($Cbfa1^{-/-}$) embryos and newborns uniformly showed dwarfism and had short legs, embryos and newborns were examined by soft X-ray and double stained by Alizarin red and Alcian blue to evaluate the development of skeletal systems (Figures 2B and 2C). Soft X-ray examination of the wild-type embryos at d18.5 revealed that various skeletal components including the skull, mandibula, upper and lower extremities, ribs, and vertebrate were well calcified. In mutant embryos at d18.5, however, parts of the tibia, radius, and vertebrae were weakly calcified, and no calcification was found in the skull, mandibula, humerus, and femur. Furthermore, Alizarin red staining of mutant embryos at d15.5–d16.5 showed an absence of calcification throughout the body, unlike wild-type embryos, which exhibited well-calcified skeletons. Calcification stained by Alizarin red increased from d15.5 to birth in wild-type mice. In contrast, weak staining by Alizarin red was observed in the tibia, fibula, radius, and ulna, and pinpoint staining in the dorsal arch of vertebrae and the dorsal part of ribs of d17.5–18.5 mutant embryos and newborns. Conversely, the development of cartilage in mutant mice seemed to

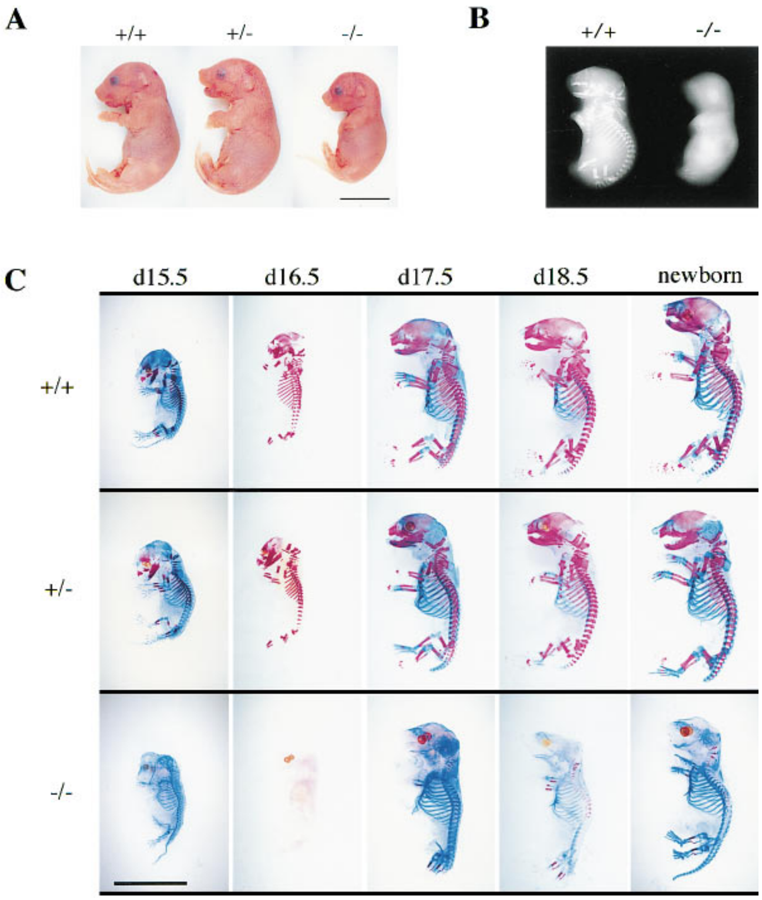

Figure 2. Examination of Skeletal System

(A) Gross appearance of wild type (+/+), heterozygous (+/−), and mutant (−/−) embryos at d18.5. Mutant embryo is small and has short legs. Bar = 1 cm.

(B) X-ray of d18.5 wild type and mutant embryo. Mutant embryo has a barely calcified skeleton.

(C) The skeleton from d15.5–d18.5 embryos and newborns of wild type, heterozygous, and homozygous genotype. Embryos and newborns were double stained with Alizarin red and Alcian blue except for d16.5 embryos, which were stained with only Alizarin red. Bar = 1 cm.

be normal upon staining with Alcian blue. Heterozygous (*Cbfa1*$^{+/-}$) embryos and newborns showed no significant abnormality in skeletal development except for clavicles and cranium (Figure 2C). They exhibited hypoplastic clavicles and nasal bones and retarded ossification of parietal, interparietal, and supraoccipital bones.

Histological sections of skeleton from d18.5 embryos were examined by staining with alkaline phosphatase (ALP), von Kossa's method, and tartrate-resistant acid phosphatase (TRAP) (Figure 3). In d18.5 wild-type embryos, tibiae consisted of three parts: two cartilaginous epiphysis at the proximal and distal ends and a bony diaphysis in the middle (Figure 3A). In the diaphysis, well-calcified bones were observed in the cortical and metaphysial regions (Figure 3C). These calcified bones were surrounded by numerous ALP-positive osteoblasts (Figure 3E). Many TRAP-positive osteoclasts were scattered on the surface of calcified bones and cartilage (Figure 3G). A large bone-marrow cavity was formed at the middle of the diaphysis (Figure 3A). In d18.5 mutant embryos, the middle part of tibia remained as calcified cartilage without formation of the bone-marrow cavity (Figures 3B and 3D). Neither vascular nor mesenchymal cell invasion was observed in the calcified cartilage. Although ALP-positive cells appeared in the perichondrial region of the calcified cartilage, no bone was formed (Figures 3D and 3F). A few TRAP-positive cells appeared adjacent to the calcified cartilage at the perichondrium (Figure 3H), but the size of the cells and the number of nuclei were reduced in comparison with those in wild-type embryos (Figures 3J and 3I). Femurs of mutant embryos were composed of noncalcified cartilage, and neither ALP-positive nor TRAP-positive cells appeared at perichondrium of the femurs (data not shown).

Well-calcified bones were formed between brain and subcutaneous connective tissue at the calvaria of d18.5 wild-type embryos (Figure 3K). The bone surface was covered with numerous ALP-positive osteoblasts (Figure 3M). Several TRAP-positive osteoclasts were also observed on the bone surface (data not shown). In d18.5 mutant embryos, only a thin layer of fibrous connective tissue was observed between the brain and subcutaneous connective tissue (Figure 3L). ALP-positive cells were detected in the fibrous connective tissues, but no calcified bone was observed (Figure 3N). No TRAP-positive cells were found in the calvarial region of mutant embryos (data not shown).

Preferential Expression of *Cbfa1* in Osteoblasts

To examine the expression of *Cbfa1*, sections from d18.5 wild-type embryos were hybridized with an RNA probe of *Cbfa1*, and skeletons were positively stained (Figures 4A–4D). In the skeleton, osteoblasts expressed *Cbfa1* more strongly than chondrocytes (Figure 4C), whereas osteoclasts did not show significant *Cbfa1* expression (Figures 4C and 4D; data not shown). Although thymocytes expressed *Cbfa1* (Figure 4E) as previously

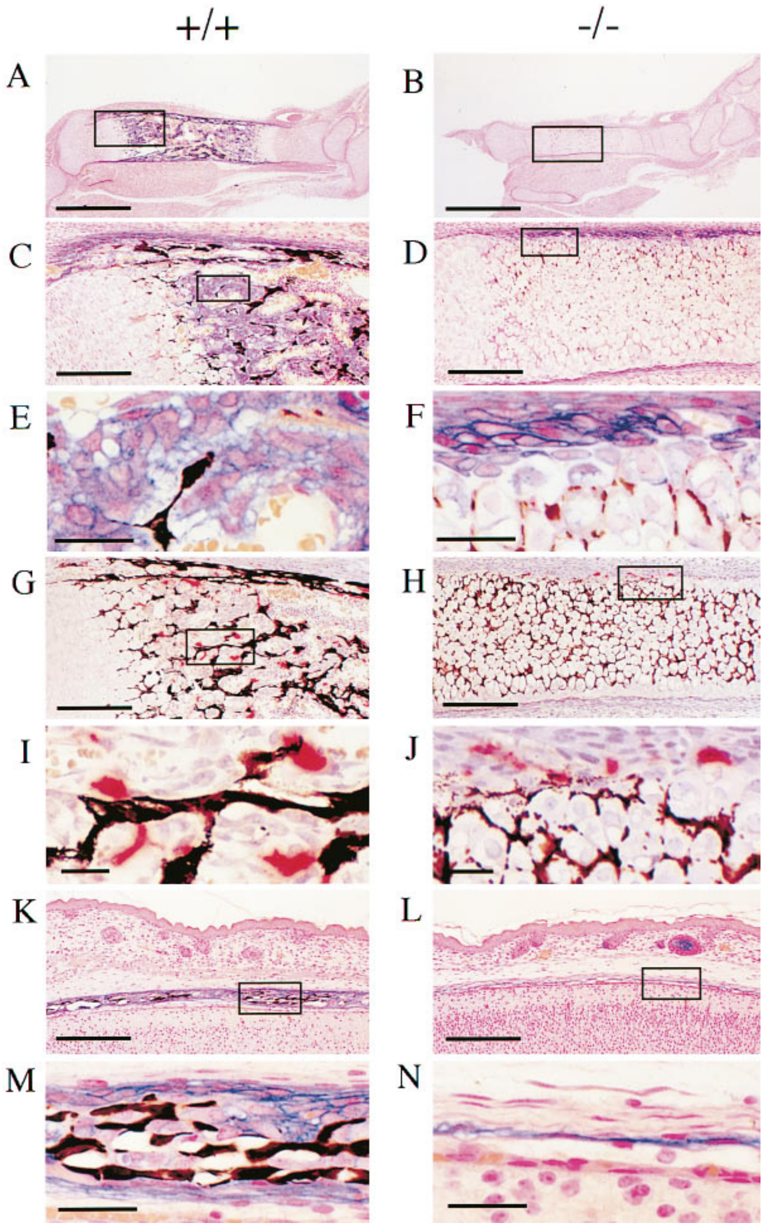

Figure 3. Histological Analysis of Skeleton of d18.5 Embryos

(A and B) Total view of tibia stained with ALP and von Kossa's method. Wild-type embryo (+/+) (A) shows the generation of bone marrow, which is lacking in mutant embryo (−/−) (B). Bar = 1 mm.

(C and D) Ossification and ALP-positive osteoblasts are seen in diaphysis of wild-type embryo (C). Mutant embryo shows calcified cartilage without subperiosteal ossification. Note that ALP-positive cells are observed at perichondrial region, but no invasion of ALP-positive cells into cartilage (D). Cells stained blue represent ALP-positive cells, and matrices stained black represent calcified matrices. Higher magnification of boxed region of (A) and (B). Bar = 0.2 mm.

(E and F) Cuboidal ALP-positive osteoblasts on the trabecular bone surface in the bone marrow (E) and flat ALP-positive osteoblasts at perichondrial region of mutant embryo (F). Note that mutant embryo shows no ossification at all. Higher magnification of boxed region of (C) and (D). Bar = 20 μm.

(G and H) Tibia stained by TRAP and von Kossa's method. Many TRAP-positive osteoclasts on calcified bone surface of wild-type embryo (G). Note a few TRAP-positive osteoclasts around the calcified cartilage, but no invasion of these cells into cartilage (H). Cells stained red represent TRAP-positive cells, and matrices stained black represent calcified matrices. Bar = 0.2 mm.

(I and J) Large mutinucleated osteoclasts in the bone marrow of wild-type embryo (I) and small mononuclear osteoclasts around cartilage of mutant embryo (J). Higher magnification of boxed region of (G) and (H). Bar = 20 μm.

(K and L) Calvaria from wild type (K) and mutant (L) embryos were stained by ALP and von Kossa's method. Cells stained blue represent ALP-positive cells, and matrices stained black represent calcified matrices. Bar = 0.2 mm.

(M and N) Ossification and cuboidal ALP-positive osteoblasts in wild-type embryo (M) and flat ALP-positive osteoblasts without ossification in mutant embryo (N). Note that calvaria of mutant embryo is composed of only a thin layer of osteoblasts. Bar = 20 μm.

described (Satake et al., 1995), there were no abnormal findings in the thymus of mutant embryos histologically (data not shown). Development of αβ T cells and γδ T cells was normal in the thymus by FACS analysis, although the number of thymocytes was significantly fewer in mutant embryos than in wild type and heterozygous embryos (data not shown). *Cbfa1* was also expressed in tendon and weakly in dermis (Figures 4F and 4G), but a histological study of the tendon showed no difference between wild type and mutant embryos (data not shown). In dermis, *Cbfa1* was expressed in fibroblasts. Although fibroblasts in the dermis of mutant embryos seemed to be slightly fewer in number than those in the dermis of wild-type embryos, the thickness of the dermis was similar (data not shown).

Although we did not detect significant *Cbfa1* expression in placenta, we found dilated blood vessels and deposits of mineral in the vascular walls of the placenta in mutant embryos (data not shown). mRNA of *Cbfa1* was detectable in livers by RT–PCR (Figure 1C), but we did not detect *Cbfa1* expression by in situ hybridization in tissues including brain, heart, lung, gut, liver, and muscle of d18.5 wild-type embryos; there were no abnormal findings histologically in these tissues of mutant embryos (data not shown).

Furthermore, we examined *Cbfa1* expression in developing limbs from d10.5–14.5 wild-type embryos. Until d12.5, we observed only faint signal from developing limbs, with no significant localization. Significant *Cbfa1* expression was first detected in the region surrounding cartilaginous condensation and in the tendon from d13.5 embryos. *Cbfa1* expression was more evident in the perichondrial region and tendon of limbs from d14.5 embryos (data not shown).

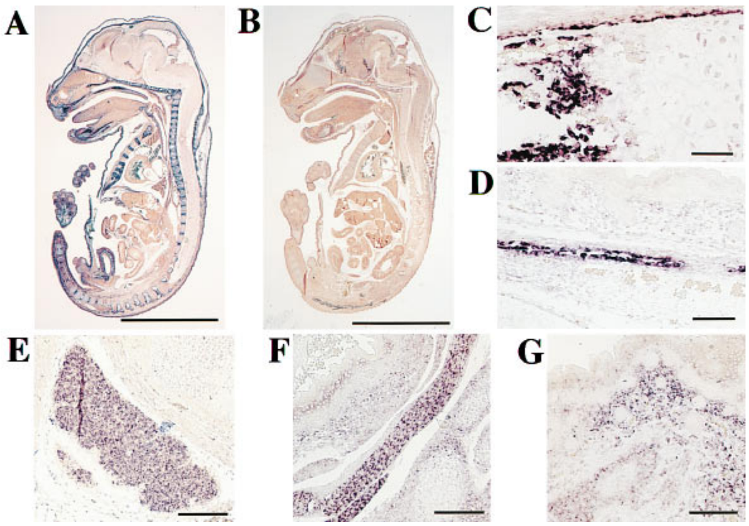

Figure 4. Spatial Expression of *Cbfa1*

(A and B) In situ hybridization of d18.5 wild-type embryos with *Cbfa1* antisense (A) and sense (B) probe. The pretreatment by proteinase K for in situ hybridization was done for 25 min for the analysis of skeleton and 10 min for the analysis of thymus. The pretreatment by proteinase K for 25 min reduced the signal from thymus.

(C and D) In situ hybridization of radius (C) and calvaria (D) of d18.5 wild-type embryos with *Cbfa1* antisense probe.

(E–G) In situ hybridization of thymus (E), tendon (F), and whiskers follicle (G) of d18.5 wild-type embryo with *Cbfa1* antisense probe.

Bar = 0.5 cm in (A) and (B); 0.1 mm in (C), (D), (F), and (G); and 0.2 mm in (E).

Expression of Noncollagenous Bone-Matrix Proteins

Sections from control (+/−) and mutant (−/−) embryos at d18.5 were hybridized by RNA probes of noncollagenous bone-matrix proteins including *Osteonectin*, *Osteopontin*, *Osteocalcin*, and Matrix Gla Protein (*MGP*) to determine the maturational stage of osteoblasts and chondrocytes (Figure 5). The osteoblasts in control embryos were hybridized by *Osteonectin*, *Osteopontin*, and *Osteocalcin* (Figures 5C, 5E, and 5G). The osteoblasts in mutant mice, which were flat shaped and observed only in the perichondrial region, were hybridized by *Osteonectin* (Figure 5D), but were only weakly hybridized by *Osteopontin* and not at all by *Osteocalcin* (Figures 5F and 5H). Chondrocytes in both control and mutant embryos were stained by *Osteonectin* and *MGP* (Figures 5C, 5D, 5I, and 5J). However, hypertrophied chondrocytes in mutant embryos expressed *Osteopontin* at barely detectable levels, while expression was more intense in control embryos (Figures 5F and 5E).

The expression of *ALP*, *Osteopontin*, and *Osteocalcin* was examined by Northern blot analysis using RNA from the skeleton (Figure 6A). Whereas wild-type embryos expressed all three, mutant embryos expressed *ALP* weakly, *Osteopontin* barely, and *Osteocalcin* not at all, findings that were consistent with the data from in situ hybridization (Figure 5).

Differentiation of Osteoblasts In Vitro

The effects of recombinant human BMP-2 (rhBMP-2) on ALP activity and Osteocalcin production in calvaria-derived cells were compared between the mix-culture of wild type and heterozygous embryos and the culture of mutant embryos. Exposure to rhBMP-2 for three days increased ALP activity in both cultures, but the activity in culture of mutant embryos was about one-third of that in the mix-culture of wild type and heterozygous embryos (data not shown). Prolonged culture for another three days in the presence of rhBMP-2 increased the ALP activity in culture of mutant embryos to more than half that in mix-culture of wild type and heterozygous embryos (Figure 6B). Although culture in the presence of rhBMP-2 for three days did not increase the production of Osteocalcin in either group (data not shown), prolonged culture for another three days in the presence of rhBMP-2 induced Osteocalcin in both groups; however, the Osteocalcin produced by mutant embryos was about one-third of that produced by wild type and heterozygous embryos (Figure 6C).

Discussion

The *Cbfa1*$^{-/-}$ newborns died without breathing. The injection of glucose had no effect, and the expression of surfactant proteins was normal (data not shown). Furthermore, we detected no abnormalities in the central nervous system of mutant mice. The absence of ossification of the ribs is considered to be a major cause of the lack of breathing and, moreover, of their death. Ribs lacking in ossification would not be strong enough to provide the negative pressure needed for lung expansion.

Since *Cbfa1*$^{-/-}$ embryos exhibited the complete lack of bone formation, we examined the expression of *Osteonectin*, *ALP*, *Osteopontin*, and *Osteocalcin* for the determination of maturational stage of osteoblasts. Osteonectin is a secreted phosphorylated calcium-binding glycoprotein that binds to type I collagen, and it is detected from early osteoprogenitor cells to osteocytes (Ibaraki et al., 1992; Hirakawa et al., 1994). Osteopontin is a sialic acid-rich phosphorylated glycoprotein. Osteocalcin, a calcium-binding protein also known as the bone Gla protein (BGP), is a vitamin K-dependent protein that can bind hydroxyapatite. *ALP* expression and *Osteopontin* expression appear in differentiating osteoblastic cells before the expression of *Osteocalcin*, and all three are downregulated in the late mineralization phase (Bronkers et al., 1987; Weinreb et al., 1990; Ibaraki et al., 1992; Stein and Lian, 1993; Malaval et al., 1994). Osteoblasts show increased ALP expression as they mature before mineralization phase (Rodan et al., 1988). Osteoblasts of mutant mice expressed *Osteonectin*, a low level of *ALP*, and barely detectable amounts of *Osteopontin* and *Osteocalcin* (Figures 3, 5, and 6A). These data indicate that the maturational arrest occurs at an early stage of osteoblast differentiation in *Cbfa1*$^{-/-}$ embryos.

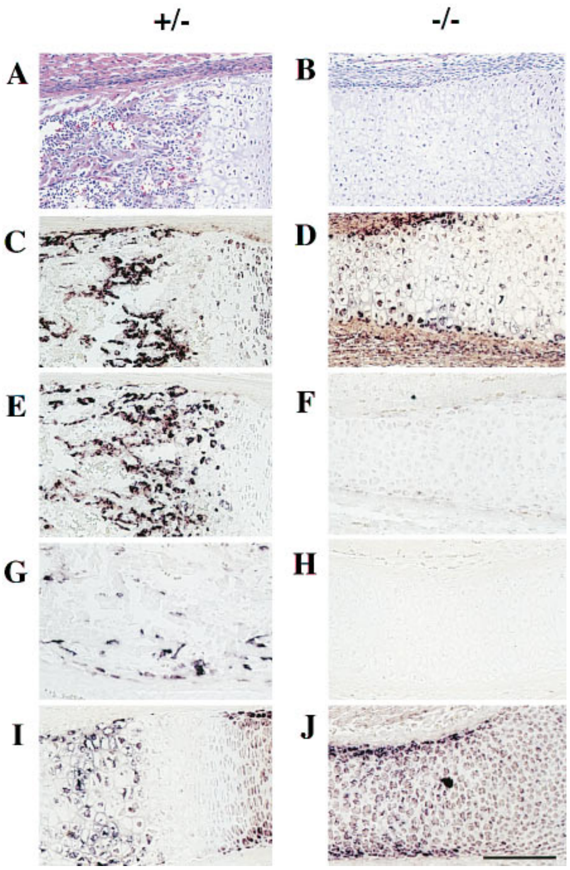

Figure 5. Distribution of *Osteonectin*, *Osteopontin*, *Osteocalcin*, and *MGP* mRNA

(A, C, E, G, and I) Radius from d18.5 control (+/−) embryo. (B, D, F, H, and J) Radius from d18.5 mutant (−/−) embryo. (A and B) Staining with hematoxylin and eosin. (C and D) In situ hybridization by *Osteonectin* antisense probe. (E and F) *Osteopontin* antisense probe. (G and H) *Osteocalcin* antisense probe. (I and J) *MGP* antisense probe. Bar = 0.15 mm.

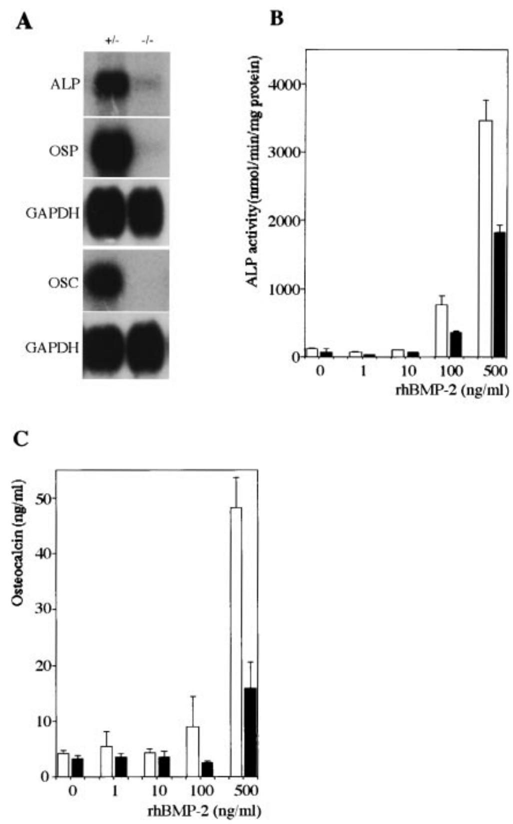

Figure 6. Expression of Maturational Markers for Osteoblasts

(A) Northern blot hybridized with *ALP*, *Osteopontin* (OSP), and *Osteocalcin* (OSC) probes. RNA was extracted from skeleton of d18.5 control (+/−) and mutant (−/−) embryos, and 15 μg of total RNA was loaded per lane. The filter hybridized with *ALP* probe was rehybridized with *Osteopontin* probe. Hybridization with the *GAPDH* probe was used as internal control for the loading of even amount of RNA for analysis.

(B and C) Effect of rhBMP-2 on ALP activity (B) and production of Osteocalcin (C) in calvaria-derived cells isolated from wild type, heterozygous, and mutant embryos at d18.5. The cells obtained from wild type and heterozygous mice were combined and are shown by open bars, and the cells obtained from mutant mice are shown by closed bars. ALP activity and concentration of Osteocalcin were examined after culture for six days with various concentrations of rhBMP-2.

Cbfa1 expression was restricted throughout fetal development (Figure 4; data not shown). We first detected significant expression of *Cbfa1* in the region surrounding cartilaginous condensation and in the tendon of d13.5 embryos. Thereafter, *Cbfa1* expression was evident in the perichondrial region and tendon. However, significant *Cbfa1* expression was not seen in the undifferentiated mesenchymal cells of d12.5 embryos. Furthermore, *Cbfa1*$^{-/-}$ embryos expressed *ALP* and *Osteonectin*, which are early markers of osteoblasts, in the perichondrial region. Therefore, Cbfa1 seems to play an essential role in the differentiation of immature osteoblasts that have been directed toward the osteoblastic lineage.

Osteocalcin, the promoter region of which has three Cbf sites and binds to Cbf-related protein (Geoffroy et al., 1995; Merriman et al., 1995; Banerjee et al., 1996), may be one of the target genes of Cbfa1. Although calvaria-derived cells isolated from *Cbfa1*$^{-/-}$ embryos showed less production of Osteocalcin than that from control in response to rhBMP-2, the increase of Osteocalcin synthesis was observed in the cells from mutant embryos at high dose rhBMP-2. This suggests that transcription factors other than Cbfa1 play important roles in BMP-2-induced Osteocalcin synthesis, at least in in vitro culture. Indeed, the down-regulation of *Osteocalcin* cannot explain the complete lack of osteogenesis of *Cbfa1*$^{-/-}$ mice, because knockout mice for the *Osteocalcin* gene showed increased bone formation (Ducy et al., 1996).

The expression of *Osteonectin*, *MGP*, and *Osteopontin* were also examined as markers of the differentiation of chondrocytes. Osteonectin is also expressed in chondrocytes (Nomura et al., 1988; Copray et al., 1989; Chen et al., 1991). MGP is a Gla-containing vitamin K-dependent protein and has been detected in chondrocytes in embryonic and adult bones (Hale et al., 1988; Ikeda et al., 1992). *Osteopontin* has been detected not only in osteoblasts but also in hypertrophied chondrocytes (Mark et al., 1988; Nomura et al., 1988; Franzen et al., 1989; Chen et al., 1991). Chondrocytes of both mutant and wild-type embryos expressed *Osteonectin* and *MGP* (Figure 5). However, in contrast to our findings in wild-type embryos, we did not detect significant *Osteopontin* expression in the hypertrophied chondrocytes of mutant embryos (Figure 5F). Furthermore, the calcification normally seen in the region of finally differentiated hypertrophied chondrocytes was observed in restricted parts of the cartilage of mutant embryos (Figures 2C and 3; data not shown). These data suggest that the final differentiation of chondrocytes is also impaired in mutant embryos.

In the present study, a few osteoclasts were observed around calcified cartilage in mutant mice, but they were small and mononuclear, indicating a lack of maturation (Figures 3H and 3J). Since we did not detect *Cbfa1* expression in osteoclasts (Figures 4C and 4D; data not shown), it is unlikely that the lack of osteoclast maturation is caused by osteoclasts themselves. The differentiation of osteoclasts is regulated by multiple factors produced by other cells. Among these factors is M-CSF, which is produced mainly by osteoblasts and bone-marrow stromal cells (Kodama et al., 1991a; Tanaka et al., 1993). The levels of *M-CSF* mRNA expression, as assessed by RT–PCR using mRNA both from skeleton and calvaria-derived cells, were almost the same in wild type and mutant embryos (data not shown). This suggests that M-CSF is not an essential factor in the lack of osteoclast differentiation. The contact of osteoclast progenitors with osteogenic cells, including bone-marrow stromal cells, is requisite for osteoclast differentiation (Takahashi et al., 1988; Kodama et al., 1991b). It is possible that the maturational arrest of osteoblasts is a major cause of the absence of osteoclast differentiation, but further studies are necessary to demonstrate this.

During the development of long bones in mammals, subperiosteal bone is formed around calcified cartilage before the formation of bone marrow. Osteogenic cells and blood capillaries then invade from the periosteal region into the calcified cartilage to form endochondral bone and the bone-marrow cavity. In the case of *Cbfa1*$^{-/-}$ embryos, however, no subperiosteal bone was formed around the calcified cartilage of the tibia and radius. The insufficient bone formation in this region in *Cbfa1*$^{-/-}$ embryos might have caused the lack of subsequent endochondral ossification and bone-marrow formation. Alternatively, it is possible that insufficient differentiation of chondrocytes affects the differentiation of osteoblasts and osteoclasts. The down-regulation of *Osteopontin* in hypertrophied chondrocytes of *Cbfa1*$^{-/-}$ embryos may be one of the causes of the inability of osteoblasts and osteoclasts to invade the cartilage, because *Osteopontin* has the putative Cbf binding sites in the 5′ flanking region (Miyazaki et al., 1990), and it is thought to promote the attachment of osteoblasts and osteoclasts to the extracellular matrix (Somerman et al., 1987; Reinholt et al., 1990; Miyauchi et al., 1991). In contrast to endochondral ossification, intramembranous ossification directly forms bones without cartilage formation. In the calvaria of *Cbfa1*$^{-/-}$ embryos, complete blockage of intramembranous ossification accompanied the maturational arrest of osteoblasts. Taken together, these findings indicate that the maturational block of osteoblasts is one of the primary causes of complete lack of osteogenesis.

Heterozygous (*Cbfa1*$^{+/-}$) embryos exhibited hypoplastic clavicles and nasal bones, and retarded ossification of parietal, interparietal, and supraoccipital bones. Heterozygous adult mice also had prominent hypoplasia of clavicles, although its degree was varied among the mice from a tiny ossification center at the acromial end to thin clavicle with normal length (unpublished data). These skeletal changes are similar to that of cleidocranial dysplasia, which is a dominantly inherited disease (Jarvis and Keats, 1974). A microdeletion in chromosome 6p21 has been shown in one family with cleidocranial dysplasia (Mundlos et al., 1995). Furthermore, a radiation-induced mutant mouse that carries similarities with cleidocranial dysplasia has been reported to have the deletion in chromosome 17 distal to *MHC* in an area that shows homology to human 6p (Mundlos et al., 1996). Since human *CBFA1* was mapped to chromosome 6p21 (Levanon et al., 1994), it is suggested that *CBFA1* is the gene that causes cleidocranial dysplasia.

Finally, our data demonstrate that the Cbfa1 is essential for osteogenesis, especially for the development of osteoblasts. However, the mechanisms by which the maturation of osteoblasts and osteoclasts is arrested need to be further clarified. The requirements for vascular invasion into cartilage also has to be investigated. And most importantly, the target genes regulated by Cbfa1 have to be clarified. Cbfa1 may regulate several genes, including *Osteopontin*, *Osteocalcin*, and some other known and unknown genes. Further examination of the mutant mice will elucidate these issues.

Experimental Procedures

Construction of Targeting Vector

We screened the 129/Sv mouse genomic library in the Lambda Fix II phage vector (Stratagene) with cDNA of the *runt* domain of *Cbfa2*. After making a restriction map and carrying out sequencing, we obtained genomic fragments containing the *runt* domain of *Cbfa1*, *Cbfa2*, and *Cbfa3*, respectively. The fragment that contained the first exon of *Cbfa1* was used for disruption of the *Cbfa1* gene. To construct targeting vector, a 2.3 kb genomic ClaI–BglII fragment was cloned into a ClaI–BamHI site of pBluescript SK (Stratagene) that contained PGK-HSV-*tk* at its SspI site. An EcoRI–BglII fragment that contained PGK-*neo* was blunt-end ligated into a blunt-ended XbaI site. To obtain the final targeting vector, a 1.3 kb genomic BstEII–ClaI fragment was blunt-end ligated into a blunt-ended NotI site, and an 11.2 kb genomic SalI*–ClaI fragment was inserted into a SalI–ClaI site of pBluescript SK that contained a 2.3 kb genomic ClaI–BglII fragment, PGK-HSV-*tk*, and PGK-*neo* (asterisk denotes a site in cloning vector).

Generation of Mutant Mice

Culture, selection of ES cells, and screening of targeted clones were carried out as described (Komori et al., 1993). Selected clones were screened by Southern blot analysis of BamHI-digested genomic DNA probed with the ClaI–SalI fragment (SalI site is a site in the cloning vector). The mutated ES clone was injected into blastocysts of C57BL/6J and transferred into uteri of pseudopregnant ICR females. The resulting chimeric animals were backcrossed to C57BL/6J, and heterozygous mutants were identified by genomic Southern blotting tail-tip DNA. Brother–sister mating was then carried out to generate homozygous mutants.

RT–PCR

cDNA, which was made from total RNA of livers, was amplified by Amp Taq DNA polymerase (Perkin Elmer) using the following primers: *Cbfa1*, 5′-CCGCACGACAACCGCACCAT-3′ and 5′-CGCTCCG GCCCACAAATCTC-3′; *HPRT*, 5′-GCTGGTGAAAAGGACCTCT-3′

and 5′-CACAGGACTAGAACACCTGC-3′. Thirty cycles of amplification were done with a Gene Amp PCR system 2400 (Perkin Elmer) (30 s at 94°C, 30 s at 60°C or 50°C, and 30 s at 72°C).

X-Ray Examination and Skeletal Preparation

Radiograms of the wild type and mutant mice were taken by soft X-rays (type SRO-M50, SOFRON, Tokyo, Japan). Embryos from d15.5–d18.5 and newborn mice were eviscerated and fixed in 100% ethanol for 4 days and transferred to acetone. After 3 days, they were rinsed with water and stained for 10 days in staining solution consisting of 1 vol 0.1% Alizarin red S (Sigma, St. Louis, MO) in 95% ethanol, 1 vol 0.3% Alcian blue 8GX (Sigma) in 70% ethanol, 1 vol 100% acetic acid, and 17 vol ethanol. After rinsing with 96% ethanol, specimens were kept in 20% glycerol/1% KOH at 37°C for 16 hr and then at room temperature until the skeletons became clearly visible. For storage, specimens were transferred into 50%, 80%, and finally, 100% glycerol.

Histological Examination

Undecalcified 4 μm sections were prepared from d18.5 embryos and stained with hematoxylin and eosin, ALP, TRAP, or von Kossa's method. ALP activity was determined histochemically by incubation for 30 min with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N,N-dimethylformamide, 2 mM $MgCl_2$, 0.6 mg/ml fast blue BB salt (Sigma) in 0.1 M Tris–HCl (pH 8.5) at room temperature. TRAP activity was detected by incubation for 30 min with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N,N-dimethylformamide, 0.6 mg/ml fast red AL salt (Sigma) in 0.1 M acetate buffer solution (pH 5.0) at 37°C.

In Situ Hybridization

Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared using a DIG RNA labeling kit (Boehringer Mannheim GmbH biochemica, Manheim, Germany) according to the manufacturer's instructions. A 0.6 kb PstI–HindIII fragment of *Cbfa1* cDNA, a 1.0 kb fragment of mouse *Osteonectin* cDNA, a 1.2 kb fragment of mouse *Osteopontin* cDNA, a 0.47 kb fragment of mouse *Osteocalcin* cDNA (Hirota et al., 1994), and a 0.5 kb fragment of mouse *MGP* cDNA (Nomura et al., 1993) were used to generate antisense and sense probes. Hybridization was carried out as described (Nomura et al., 1993).

Isolation and Culture of Calvaria-Derived Cells

Calvaria from d18.5 embryos were cut into small pieces and cultured for 10–14 days in three-dimensional collagen gel (Cellmatrix, Nitta Gelatin, Co., Osaka Japan) with α-modified Minimum Essential Medium (α-MEM) containing 10% FBS. The cells outgrowing from the explants were retrieved by incubation for 30 min with 0.2% collagenase (Wako Pure Chemical Industries, Osaka, Japan) in PBS (−) at 37°C. The cells obtained from wild type and heterozygous mice were combined, and they were cultured with α-MEM containing 10% FBS. After reaching subconfluent state, the cells were removed from each culture flask and inoculated into multi-well plates at a density of 1×10^4 cells/cm^2. They were cultured with various concentrations of rhBMP-2, which was produced in Chinese hamster ovary cells, and purified as described previously (Wozney et al., 1988). rhBMP-2 was provided by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan.

Northern Blot

Total RNA extracted from skeleton of d18.5 embryos by lithium chloride was transferred into nylon membrane and hybridized with a ^{32}P-labeled 0.78 kb fragment of rat *ALP* cDNA (Noda et al., 1987), a 1.2 kb fragment of mouse *Osteopontin* cDNA, and a 0.47 kb fragment of mouse *Osteocalcin* cDNA (Hirota et al., 1994). Filters were rehybridized with a ^{32}P-labeled 0.85 kb fragment of mouse *GAPDH*.

Measurement of ALP Activity and Osteocalcin Production

The cultured cells were sonicated in 0.1 M Tris buffer (pH 7.2) containing 0.1% Triton X-100. ALP activity was determined using *p*-nitrophenylphosphate as a substrate in 0.05 M 2-amino-2-methylpropanol and 2 mM $MgCl_2$ (pH 10.5). The amount of *p*-nitrophenol released was estimated by measuring absorbance at 410 nm. Protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Amounts of Osteocalcin secreted into the culture medium during the last 3 day culture were measured by RIA using a mouse Osteocalcin assay kit (Biomedical Technologies Inc., Stoughton, MA).

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