Different Requirements of CBFB and RUNX2 in Skeletal Development among Calvaria, Limbs, Vertebrae and Ribs

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Abstract: RUNX proteins, such as RUNX2, regulate the proliferation and differentiation of chondrocytes and osteoblasts. Haploinsufficiency of RUNX2 causes cleidocranial dysplasia, but a detailed analysis of Runx2−/− mice has not been reported. Furthermore, CBFB is required for the stability and DNA binding of RUNX family proteins. CBFB has two isoforms, and CBFB2 plays a major role in skeletal development. The calvaria, femurs, vertebrae and ribs in Cbfb2−/− mice were analyzed after birth, and compared with those in Runx2+/− mice. Calvarial development was impaired in Runx2+/− mice but mildly delayed in Cbfb2−/− mice. In femurs, the cortical bone but not trabecular bone was reduced in Cbfb2−/− mice, whereas both the trabecular and cortical bone were reduced in Runx2+/− mice. The trabecular bone in vertebrae increased in Cbfb2−/− mice but not in Runx2+/− mice. Rib development was impaired in Cbfb2−/− mice but not in Runx2+/− mice. These differences were likely caused by differences in the indispensability of CBFB and RUNX2, the balance of bone formation and resorption, or the number and maturation stage of osteoblasts. Thus, different amounts of CBFB and RUNX2 were required among the bone tissues for proper bone development and maintenance.

Keywords: RUNX1; RUNX2; RUNX3; CBFB; cleidocranial dysplasia; calvaria; limb bone; vertebra; rib; osteoblast

1. Introduction

RUNX2 belongs to RUNX family transcription factors composed of RUNX1, RUNX2 and RUNX3, and is required for the commitment of multipotent mesenchymal cells to an osteoblast lineage of cells, the proliferation of osteoblast progenitor cells and the expression of major bone matrix protein genes, including Col1a1, Col1a2, Spp1, Ibsp and Bglap and Bglap2, a transcription factor Sp7 and a protease Htral [1,2]. Additionally, RUNX1 and RUNX3 induce bone formation [3,4]. RUNX2 and RUNX1 induce chondrocyte proliferation and maturation, and RUNX3 is partly involved in chondrocyte maturation [5,6]. Furthermore, RUNX2 enhances bone resorption by inducing Tnfsf11 expression in osteoblasts [7,8]. Notably, Haploinsufficiency of RUNX2 causes cleidocranial dysplasia, which is characterized by open fontanelles and sutures, hypoplastic clavicles, supernumerary teeth and short stature [9–11].

CBFB is a cotranscription factor, which is ubiquitously expressed. CBFB heterodimerizes with RUNX family transcription factors, and enhances their DNA binding capacity [12,13]. Cbfb-deficient (Cbfb−/−) mice die between embryonic days 11.5–13.5 due to the absence of definitive hematopoiesis in the liver, similarly to Runx1−/− mice, indicating
that RUNX1 and CBFB are essential for definitive hematopoiesis [14–16]. The knock-in of green fluorescent protein (GFP) into the coding region of Cbfb maintained sufficient function in hematopoietic cells to bypass the early embryonic lethality [17]. Furthermore, the introduction of Cbfb into Cbfb−/− mice using Cbfb transgenic mice under the control of Tek1 or Gata1 promoter, which directs Cbfb to hematopoietic progenitor cells, rescued definitive hematopoiesis in Cbfb−/− mice [18,19]. These mice survived until birth, but showed severely impaired intramembranous and endochondral ossification. Although the phenotypes were milder than those in Runx2−/− mice, the osteoblast differentiation and chondrocyte maturation were severely impaired, indicating that CBFB is also required for RUNX2-dependent bone development [17–19]. Moreover, the functions of CBFB in osteoblast differentiation and chondrocyte maturation were confirmed by generating Cbfb conditional knockout mice using Sp7-Cre, Col2a1-Cre or Prrx1-Cre transgenic mice and Dermo1-Cre knock-in mice [20–25]. CBFB regulates the functions of RUNX family proteins not only by enhancing their DNA binding capacity, but also by stabilizing the proteins [23,24].

CBFB has two functional isoforms, CBFB1 and CBFB2, which are formed by alternative splicing using donor splicing sites located inside exon 5 and at the 3′ terminus of exon 5, respectively and an acceptor splice site located at the 5′ terminus of exon 6 [12,26]. Cbfb1 expression is about three times higher than Cbfb1 expression in many tissues, including the calvaria, limbs, liver, thymus and brain [26]. Cbfb1-specific deletion by mutating the donor splicing signal sequence showed normal skeletal development, whereas Cbfb2-specific deletion by mutating the donor splicing signal sequence showed impaired endochondral and intramembranous ossification due to the delay in chondrocyte maturation and osteoblast differentiation during embryogenesis. Cbfb2 mRNA was upregulated in Cbfb1−/− mice, and the level of Cbfb was similar to that in wild-type mice, whereas Cbfb1 mRNA was reduced in Cbfb2−/− mice and the level of Cbfb was one-fourth of that in wild-type mice. However, CBFB1 was more potent than CBFB2 in chondrocyte and osteoblast differentiation and in DNA binding with RUNX2 in vitro [26]. The protein levels of RUNX1, RUNX2 and RUNX3 in Cbfb1−/− mice were similar to those in wild-type mice, whereas those in Cbfb2−/− mice were reduced compared with those in wild-type mice, although the levels of the reduction were different among RUNX family proteins and tissues [26].

Although open fontanelles and sutures and hypoplastic clavicles are observed in Runx2−/− mice [10], the development of the other bones have not been evaluated. Furthermore, Cbfb deletion in the skeletal progenitors is lethal at the newborn stage [24], and the requirement of CBFB in skeletal development after birth remains to be investigated. The expression levels of Cbfb mRNA in Cbfb2−/− mice were about one-fifth of that in wild-type mice, and half of the Cbfb2−/− mice survived beyond 4 weeks of age. Here, we show the differential requirement of CBFB and RUNX2 in the bone development of calvaria, limbs, vertebrae and ribs after birth by comparing Cbfb2−/− and Runx2−/− mice.

2. Results

2.1. Protein Levels of RUNX1, RUNX2, RUNX3 and CBFB in Cbfb2−/− and Runx2−/− Mice

The protein levels of RUNX1, RUNX2, RUNX3 and CBFB were examined using calvaria, trabecular bone from tibiae and femurs, ribs and vertebrae at 4 weeks of age (Figure 1). The reduction of RUNX1 in the Cbfb2−/− mice was mild, except in the ribs. In the ribs, all RUNX family and CBFB proteins were more severely reduced in the Cbfb2−/− mice with severe rib deformity than those with mild rib deformity, compared with those in wild-type mice (Figure 1F,G). The reduction of RUNX2 protein in the calvaria and trabecular bone of Cbfb2−/− mice were 30 and 46%, respectively (Figure 1A,B,D,E), while that in the ribs was more severe, 75% (Figure 1F,G). In contrast, the level of RUNX2 protein in the vertebrae of Cbfb2−/− mice was comparable with that of wild-type mice (Figure 1H,I). Among the RUNX family, the protein level of RUNX3 was most affected by the absence of CBFB2 and the reduction of RUNX3 protein in Cbfb2−/− mice was 58–81% in the tissues (Figure 1). The reduction of CBFB protein in the Cbfb2−/− mice was constant among
the tissues, 76–85% (Figure 1). In calvaria, the protein level of RUNX2 in the Runx2+/− mice was half of that in wild-type mice, and those of RUNX1, RUNX3 and CBFB were comparable with those in wild-type mice (Figure 1A,C).

Figure 1. Western blot analysis of RUNX family proteins and CBFB. Proteins were extracted from calvaria (A–C), the trabecular bone in femurs and tibiae (D,E), ribs (F,G) and vertebrae (H,I) at 4 weeks of age in Cbfb2+/+, Cbfb2−/−, Runx2+/−, and Runx2−/− mice. Representative data are shown in (A), (D), (F), and (H). β-ACTIN was used as an internal control. The intensities of bands were normalized against β-ACTIN, and the normalized values in Cbfb2+/+ or Runx2+/− mice were set as 1. The means ± SD of the relative levels of Cbfb2−/− mice (B,E,G,I) and Runx2−/− mice (C) are shown. The number of mice analyzed: B, n = 3–5; C, n = 3; E, n = 4–5; G, n = 4; I, n = 4.

2.2. Skeletal Development of Cbfb1−/−, Cbfb2−/− and Runx2−/− Newborns

Cbfb1−/− mice showed normal skeletal development in the newborn stage (Figure 2B,E,H,K,N,Q), and the trabecular bone volume, trabecular thickness, trabecular number, cortical area and cortical thickness were similar to those in wild-type mice at 10 weeks of age according to micro-CT analysis (Supplementary Figure S1). Mineralization of the interparietal bone, supraoccipital bone (Figure 2A,C), ribs (Figure 2G,I), sternum (Figure 2D,F,G,I), vertebrae (Figure 2J,L), scapulae (Figure 2M,O) and pelvic bones (Figure 2P,R) was mildly delayed, and clavicles were thinner (Figure 2D,F) in Cbfb2−/− newborns than those in wild-type newborns. In the scapulae in Cbfb2−/− newborns, the whole area, mineralized area and the ratios of mineralized area in the whole area were lower than those in wild-type newborns (Figure 2S). Mineralization in the calvaria was apparently reduced (Figure 3A,B), clavicles were severely hypoplastic (Figure 3C,D) and mineralization of sternum (Figure 3C,F), scapulae (Figure 3I,J) and pelvic bones (Figure 3K,L) was mildly delayed in Runx2+/− newborns than those in wild-type newborns. In the scapulae in Runx2+/− newborns, the whole area, mineralized area and the ratios of mineralized area in the whole area were lower than those in wild-type newborns (Figure 3M). There were no apparent differences in mineralization of ribs and vertebrae between wild-type and Runx2+/− newborns (Figure 3E–H). The same phenotypes were confirmed in all newborns analyzed.
Runx2+–/– newborns (Figure 3E–H). The same phenotypes were confirmed in all newborns analyzed. In the crossing of Cbfβ2+–/– mice, the frequency of Cbfβ2−/– mice in the offspring during the embryonic stage was 24% (Supplementary Table S1). However, it was slightly reduced to 21% between postnatal day 0 (P0) and P2 and reduced further to 11% at 4 weeks of age. Thus, about half of the Cbfβ2−/– mice were estimated to have died by 4 weeks of age.

**Figure 2.** Skeletal development in Cbfβ2+/+, Cbfβ1−/−, and Cbfβ2−/− newborn mice. Cbfβ1+/+ mice were similar to Cbfβ2+/+ mice, and are not shown here. (A–C) Lateral view of skulls. (D–F) Neck and chest. (G–I) Lateral view of ribs and vertebrae. (J–L) Enlarged pictures of vertebrae. (M–O) Forelimbs. (P–R) Hind limbs. The arrow and arrowhead in (C) show delayed mineralization of interparietal bone and supraoccipital bone, respectively. The arrow and arrowheads in (F) show thin clavicle and delayed mineralization of sternum, respectively. The arrows in (O) and (R) show delayed mineralization of scapula and pelvic bones, respectively. Scale bars: 0.2 cm. (S) Whole area and mineralized area of scapulae and the ratios. The number of mice analyzed: Cbfβ1+/+, n = 3; Cbfβ1−/−, n =2; Cbfβ2+/+, n = 5; Cbfβ2−/−, n = 4. *** p < 0.001.
Figure 3. Skeletal development in Runx2+/+ and Runx2+/− newborn mice. (A,B) Lateral view of skulls. (C,D) Neck and chest. (E,F) Lateral view of ribs and vertebrae. (G,H) Enlarged pictures of vertebrae. (I,J) Forelimbs. (K,L) Hind limbs. The arrow in (D) shows hypoplastic clavicle, and arrowheads in (D,F) show delayed mineralization of sternum. The arrows in (J,L) show delayed mineralization of scapula and pelvic bones, respectively. Scale bars: 0.2 cm. (M) Whole area and mineralized area of scapulae and the ratios. Six Runx2+/+ mice and nine Runx2+/− mice were analyzed. ***p < 0.001.

In the crossing of Cbfb2+/− mice, the frequency of Cbfb2−/− mice in the offspring during the embryonic stage was 24% (Supplementary Table S1). However, it was slightly reduced to 21% between postnatal day 0 (P0) and P2 and reduced further to 11% at 4 weeks of age. Thus, about half of the Cbfb2−/− mice were estimated to have died by 4 weeks of age.
2.3. Delayed Closure of Posterior Frontal Suture in Cbfb2−/− Mice

The processes of the closure of posterior frontal suture are unique and include the processes of endochondral ossification [27–29]. Since its closure was completely interrupted in Runx2+/− mice [27], the processes were examined in Cbfb2−/− mice at four different ages (Figures 4–7). In wild-type mice, the frontal suture was open in the image of micro-CT at P7 (Figure 4A,B). In the histological analysis of wild-type mice, Col1a1-positive osteoblasts accumulated on both sides of frontal bone, and the frontal suture cells condensed between them (Figure 4E,F,I,J,M,N,Q,R). In Cbfb2−/− mice at P7, the frontal suture was more widely open in the micro-CT image (Figure 4C,D) and the condensation of the suture cells was not apparent (Figure 4G,H,K,L,O,P,S,T). In wild-type mice at P10, the frontal suture was still not completely closed in the micro-CT image (Figure 5A,B) and some chondrocytes in the suture expressed Col2a1, but most of them were hypertrophic chondrocytes, which expressed Col10a1 (Figure 5E,F,I,J,M,N,Q,R,U,V). In Cbfb2−/− mice at P10, the frontal suture was still apparently open in the micro-CT image (Figure 5C,D), frontal suture cells condensed, most of them expressed Col2a1 and a few expressed Col10a1 (Figure 5G,H,K,L,O,P,S,T,W,X). Thus, there were more Col2a1-positive cells and less Col10a1-positive cells in Cbfb2−/− mice compared with wild-type mice, indicating that the process of endochondral ossification was delayed in Cbfb2−/− mice. In wild-type mice at P14, the frontal suture was nearly closed in the micro-CT image (Figure 6A,B) and the cartilaginous tissue in the suture was replaced with bone, which was surrounded by Col1a1-positive osteoblasts (Figure 6E,F,I,J,M,N,Q,R,U,V). In Cbfb2−/− mice at P14, the frontal suture was still open in the micro-CT image (Figure 6C,D), and the chondrocytes in the suture were hypertrophic and expressed Col10a1 (Figure 6G,H,K,L,O,P,S,T,W,X). At P28, the frontal suture was completely closed in both wild-type and Cbfb2−/− mice in the micro-CT images (Figure 7A–D) and the suture was also completely closed in the histological analysis in both groups (Figure 7E–H). Thus, the closure process of the posterior frontal suture was delayed, but not interrupted in Cbfb2−/− mice. Incisor development was similar between wild-type and Cbfb2−/− mice according to the micro-CT analyses (Figures 4A,C, 5A,C, 6A,C and 7A,C). The same findings were confirmed in all mice analyzed.

2.4. Shortened Limb Bones, Rib Deformity and Abnormal Spinal Curvature in Cbfb2−/− Mice

At 4 weeks of age, the body weight was reduced in Cbfb2−/− and Runx2+/− mice compared with the respective control groups (Figure 8A,B). The lengths of the limb bones, including the ulna, femur and tibia, were significantly reduced in Cbfb2−/− mice, but the reduction was not significant in Runx2+/− mice compared with the respective control mice (Figure 8C–J). No apparent differences were observed in Cbfb2−/− and Runx2+/− mice compared with the respective control mice in histological analysis of femurs (Supplementary Figure S2). Rib deformities were observed in all Cbfb2−/− mice (8/8 mice), half of which were severe, and the area of the thoracic cage in Cbfb2−/− mice was reduced compared with that in wild-type mice (Figure 8K,L,O,P,S). In Cbfb2−/− mice with severe rib deformity, the expression of Col2a1 and Col10a1, which are expressed in immature and mature chondrocytes, respectively [30], but not Mmp13, which is highly expressed in terminal hypertrophic chondrocytes [30], in the ribs was extremely higher than that in wild-type mice and Cbfb2−/− mice with mild rib deformity, except for the part of permanent cartilage (Figure 8T). However, the expression of osteoblast marker genes, including Runx2, Sp7, Alpl, Spp1, Ibsp and Col1a1, was not significantly affected (Figure 8T), indicating that endochondral ossification was impaired in Cbfb2−/− mice with severe rib deformity due to the inhibited chondrocyte maturation. Half of the Cbfb2−/− mice showed abnormal spinal curvature (4/8 mice), and the lengths of the 1st lumbar vertebrae in Cbfb2−/− mice were shorter than those in wild-type mice (Figure 8O,P,U). On the other hand, the expression of these genes, except Runx2 in Runx2+/− mice, was not significantly different from that in wild-type mice (Supplementary Figure S3A). Furthermore, there was no rib deformity or abnormal spinal curvature in Runx2+/− mice (0/7 mice), although the lengths of the 1st lumbar vertebrae were shorter than those in wild-type mice (Figure 8M,N,Q,R,S,U).
Figure 4. Micro-CT and histological analyses of posterior frontal sutures in Chfb2+/+ and Chfb2−/− mice at P7. (A–D) Lateral (A,C) and dorsal (B,D) views of micro-CT images of skulls. The two lines in (B) and (D) show the anterior and posterior boundaries of the posterior frontal suture, and arrow heads indicate the location of coronal sections in the histological analyses. (E–H) H-E staining, (I–L) Safranin O staining, (M–T) In-situ hybridization using Col1a1 (M–P) and Col2a1 (Q–T) probes. The boxed regions in the left columns were magnified in the right columns. Scale bars: 0.5 cm (A–D) and 100 μm (E–T). Three Chfb2+/+ mice and two Chfb2−/− mice were analyzed.
Figure 5. Micro-CT and histological analyses of posterior frontal sutures in Cbfb2+/+ and Cbfb2−/− mice at P10. (A–D) Lateral (A,C) and dorsal (B,D) views of micro-CT images of skulls. The two lines in (B) and (D) show the anterior and posterior boundaries of the posterior frontal suture, and arrow heads indicate the location of coronal sections in the histological analyses. (E–H) H-E staining, (I–L) Safranin O staining. (M–X) In-situ hybridization using a Col1a1 (M–P), Col2a1 (Q–T) and Col10a1 (U–X) probes. The boxed regions in the left columns were magnified in the right columns. In-situ hybridization using the sense probes showed no significant signals. Scale bars: 0.5 cm (A–D) and 100 µm (E–X). Three Cbfb2+/+ mice and two Cbfb2−/− mice were analyzed.
Figure 6. Micro-CT and histological analyses of posterior frontal sutures in $Cbfb2^{+/+}$ and $Cbfb2^{-/-}$ mice at P14. (A–D) Lateral (A,C) and dorsal (B,D) views of micro-CT images of skulls. The two lines in (B,D) show the anterior and posterior boundaries of the posterior frontal suture, and arrow heads indicate the location of coronal sections in the histological analyses. (E–H) H-E staining. (I–L) Safranin O staining. (M–X) In-situ hybridization using a $Col1a1$ (M–P), $Col2a1$ (Q–T) and $Col10a1$ (U–X) probes. The boxed regions in the left columns were magnified in the right columns. Scale bars: 0.5 cm (A–D) and 100 μm (E–X). Three $Cbfb2^{+/+}$ mice and two $Cbfb2^{-/-}$ mice were analyzed.
Figure 7. Micro-CT and histological analyses of posterior frontal sutures in Chfb2+/+ and Chfb2−/− mice at P28. (A–D) Lateral (A,C) and dorsal (B,D) views of micro-CT images of skulls. The two lines in (B,D) show the anterior and posterior boundaries of the posterior frontal suture, and arrow heads indicate the location of coronal sections in the histological analyses. (E–H) H-E staining. (I–L) Safranin O staining. (M–T) In-situ hybridization using Col1a1 (M–P) and Col2a1 (Q–T) probes. The boxed regions in the left columns were magnified in the right columns. Scale bars: 0.5 cm (A–D) and 100 μm (E–T). Three Chfb2+/+ and Chfb2−/− mice were analyzed.
The development of limb bones, ribs and vertebrae in Cbf2<sup>−/−</sup> and Runx2<sup>+/−</sup> mice at 4 weeks of age. (A,B) Body weights of male mice. n = 21–29. (C–J) The lengths of the humerus (C,D), ulna (E,F), femur (G,H) and tibia (I,J) in female mice. The number of mice analyzed: Cbf2<sup>+/+</sup>, n = 6; Cbf2<sup>−/−</sup>, n = 4; Runx2<sup>+/+</sup>, n = 8; Runx2<sup>−/−</sup>, n = 7. (K–U) Three-dimensional images of frontal (K–N) and lateral views (O–R) of thoracic cage and vertebrae in micro-CT, the area of thoracic cage (S), real-time RT-PCR analysis using bony segments of rib RNA (T) and the length of the 1st vertebrae (U) in male mice. Cbf2<sup>−/−</sup> mice with mild or severe rib deformity were analyzed separately in (T). Scale bars: 1 mm (C,E,G,I) and 1 cm (K–R). Data are shown as the mean ± SD. * p < 0.05, **, ## p < 0.01 and ***, ### p < 0.001. The number of mice analyzed: Cbf2<sup>+/+</sup>, n = 4; Cbf2<sup>−/−</sup>, n = 8 (4 mild and 4 severe); Runx2<sup>+/−</sup>, n = 8; Runx2<sup>−/−</sup>, n = 7.
At 10 weeks of age, the body weight in Cbfb2−/− mice was similar to the control mice, but that in Runx2+/− mice was reduced compared with the control mice (Figure 9A,B). Suture and fontanelles were opened in Runx2+/− mice but not in Cbfb2−/− mice (Figure 9C–G), and the length of clavicles was mildly and severely reduced in Cbfb2−/− and Runx2+/− mice, respectively, at 14 weeks of age (Figure 9H–L). Although the rib deformity was observed in half of the Cbfb2−/− mice (7/14 mice), it was mild and the area of the thoracic cage was similar to that in wild-type mice (Figure 9H,J,M). There was no rib deformity in Runx2+/− mice, and the area of thoracic cage was similar to that in wild-type mice (Figure 9J,K,M). The abnormal spinal curvature was observed in about one-third of the Cbfb2−/− mice (5/14 mice). The frequencies of rib deformity and abnormal spinal curvature were two-thirds (22/33 mice) and one-third (12/33 mice), respectively, in Cbfb2−/− mice between 4 weeks and 10 months of age, whereas these deformities were not observed in Runx2+/− mice (0/26 mice) and wild-type mice (0/43 mice). Furthermore, severe rib deformities were not observed in the Cbfb2−/− mice after 10 weeks of age, suggesting that severe rib deformity is involved, at least in part, in being a cause of death in Cbfb2−/− mice after birth.

Figure 9. Micro-CT analysis of calvaria, clavicles and thoracic cage in Cbfb2−/− and Runx2+/− mice. (A,B) Body weights of male mice at 10 weeks of age. n = 14–19. (C–M) Calvaria (C–F), clavicles and thoracic cage (H–K), unmineralized area in calvaria (G), the length of clavicles (L) and the area of thoracic cage (M) in male Cbfb2+/+, Cbfb2−/−, Runx2+/+ and Runx2+/− mice at 14 weeks of age. Scale bars: 1 cm. Data are shown as the mean ± SD. ** p < 0.001. The number of mice analyzed: Cbfb2+/+, n = 7; Cbfb2−/−, n = 4; Runx2+/+, n = 5; Runx2+/−, n = 6.
2.5. Micro-CT Analysis of Femurs and Vertebrae in Cbfb2−/− and Runx2+/− Mice at 4 Weeks and 10 Weeks of Age

From micro-CT analysis, the trabecular bone volume, trabecular thickness and trabecular number in Cbfb2−/− femurs were similar, but the trabecular bone mineral density was reduced compared with those in wild-type femurs at 4 weeks of age (Figure 10A,B). In contrast, all of the trabecular parameters were reduced in Runx2+/− femurs compared with those in wild-type femurs (Figure 10C,D). In the analysis of femoral cortical bone, the cortical area, cortical thickness and bone mineral density were reduced in both Cbfb2−/− and Runx2+/− mice compared with those in the respective control mice, but with more reduction in Cbfb2−/− mice (Figure 10E–H). The periosteal perimeter was increased in Cbfb2−/− mice but not in Runx2+/− mice, and the endosteal perimeter was increased in Cbfb2−/− and Runx2+/− mice, with a greater increase in Cbfb2−/− mice compared with those in the respective control mice (Figure 10E–H).

Bone volume, trabecular thickness and trabecular number in the 1st lumbar vertebrae were increased in Cbfb2−/− mice compared with those in wild-type mice, but their values in Runx2+/− mice were similar to those in wild-type mice at 4 weeks of age (Figure 10I–L). The trabecular bone mineral density was significantly reduced in the Runx2+/− mice, but not in the Cbfb2−/− mice (Figure 10J,L).

The trabecular bone volume, trabecular thickness, trabecular number and trabecular bone mineral density were comparable in Cbfb2−/− femurs but reduced in Runx2+/− femurs compared with those in the respective control mice at 10 weeks of age (Figure 11A–D). Cortical area, cortical thickness and cortical bone mineral density were reduced and the endosteal perimeter was increased in the Cbfb2−/− and Runx2+/− femurs compared with those in the respective control mice (Figure 11E–H). The periosteal perimeter was increased in the Cbfb2−/− femurs, but not in the Runx2+/− femurs (Figure 11E–H).

In the 6th lumbar vertebrae of Cbfb2+/+ and Cbfb2−/− mice and 1th lumbar vertebrae of Runx2+/+ and Runx2+/− mice, the bone volume of Cbfb2−/− mice and Runx2+/− mice were not significantly affected and the trabecular thickness was reduced in the Runx2+/− mice, but not in the Cbfb2−/− mice, and the trabecular number was increased, but the trabecular bone mineral density was reduced in the Cbfb2−/− and Runx2+/− mice compared with those in the respective control mice (Figure 11I–L).

2.6. Bone Histomorphometric Analysis of Femurs and Vertebrae and Serum Markers for Bone Formation and Resorption in Cbfb2−/− and Runx2+/− Mice at 10 Weeks of Age

In the bone histomorphometric analysis of femurs, the osteoblast parameters, including osteoid surface, osteoid thickness, osteoblast surface and osteoblast number, were reduced in the Runx2+/− mice, but not in the Cbfb2−/− mice, compared with those in the respective control mouse (Figure 12A–D). Osteoclast surface in the Cbfb2−/− mice and osteoclast number in the Runx2+/− mice were reduced, and the mineral apposition rate and bone formation rate were reduced in the Cbfb2−/− and Runx2+/− mice compared with those in the respective control mice (Figure 12B,D). The mineralizing surface was significantly reduced in the Runx2+/− mice, but not in the Cbfb2−/− mice (Figure 12B,D).

In the bone histomorphometric analysis of vertebrae, osteoblast parameters, including osteoid surface, osteoid thickness, osteoblast surface and osteoblast number and eroded surface, in the Cbfb2−/− mice were similar to the control mice, but the eroded surface in Runx2+/− mice was reduced compared with the respective control mice (Figure 12E,H). Osteoclast parameters, including osteoclast surface, osteoclast number and eroded surface, in the Cbfb2−/− mice were similar compared with the control mice but reduced in Runx2+/− mice compared with those in the control mice (Figure 12E,H).
Figure 10. Micro-CT analyses of femurs and lumbar vertebrae in male mice at 4 weeks of age. (A–D) Three-dimensional trabecular bone architecture of distal femoral metaphysis (A,C) and quantification of the trabecular bone volume (bone volume/tissue volume, BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular bone mineral density (Tb.BMD) in Cbfb2+/+ and Cbfb2−/− mice (B) and Runx2+/+ and Runx2−/− mice (D). (E–H) Micro-CT images of cortical bone at mid-diaphysis in femurs (E,G) and quantification of the cortical area (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), periosteal perimeter (Ps.Pm), endosteal perimeter (Es.Pm) and cortical bone mineral density (Ct.BMD) in Cbfb2+/+ and Cbfb2−/− mice (F) and Runx2+/+ and Runx2−/− mice (H). The number of mice analyzed: Cbfb2+/+, n = 22; Cbfb2−/−, n = 14; Runx2+/+, n = 11; Runx2−/−, n = 8. (I–L) Three-dimensional trabecular bone architecture of 1st lumbar vertebrae (I,K) and trabecular bone parameters in Cbfb2+/+ and Cbfb2−/− mice (J) and Runx2+/+ and Runx2−/− mice (L). Scale bars: 1 mm. Data are shown as the mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001. The number of mice analyzed: Cbfb2+/+, n = 9; Cbfb2−/−, n = 7; Runx2+/+, n = 11; Runx2−/−, n = 8.
Cbfb2−/− mice (B) and Runx2+/+ and Runx2+/− mice (D). (E–H) Micro-CT images of the cortical bone at mid-diaphysis in femurs (E,G) and quantification of the cortical area (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), periosteal perimeter (Ps.Pm), endosteal perimeter (Es.Pm) and cortical bone mineral density (Ct.BMD) in Cbfb2+/+ and Cbfb2−/− mice (B) and Runx2+/+ and Runx2+/− mice (D). (E–H) The number of mice analyzed: Cbfb2+/+, n = 9; Cbfb2−/−, n = 19; Runx2+/+, n = 14; Runx2+/−, n = 17; Runx2−/−, n = 15. (I–L) Three-dimensional trabecular bone architecture of 6th lumbar vertebrae (I,K) and trabecular bone parameters in Cbfb2+/+ and Cbfb2−/− mice (J) and 1st lumbar vertebrae of those parameters in Runx2+/+ and Runx2+/− mice (L). Scale bars: 1 mm (A,C,E,G) and 0.5 mm (I,K). Data are shown as the mean ± SD. * p < 0.05, ** p < 0.01 and *** p < 0.001. The number of mice analyzed: Cbfb2+/+, n = 9; Cbfb2−/−, n = 14; Runx2+/+, n = 14; Runx2+/−, n = 13.
Figure 12. Bone histomorphometric analyses of trabecular bone in femurs and vertebrae in male mice at 10 weeks of age. (A–D) Bone histomorphometric analysis of femurs in Cbfb2+/+ and Cbfb2−/− mice (A, B) and Runx2+/+ and Runx2+/− mice (C, D). The osteoid surface (OS/BS), osteoid thickness (O.Th), osteoblast surface (Ob.S/BS), number of osteoblasts (N.Ob/BS), osteoclast surface (Oc.S/BS), number of osteoclasts (N.Oc/BS), eroded surface (ES/BS), mineral apposition rate (MAR), mineralizing surface (MS/BS) and bone formation rate (BFR/BS) are shown. BS, bone surface. The number of mice analyzed: Cbfb2+/+, n = 18; Cbfb2−/−, n = 14; Runx2+/+, n = 10; Runx2+/−, n = 14. (E–H) Bone histomorphometric analyses of lumbar vertebrae in Cbfb2+/+ and Cbfb2−/− mice (E, F) and Runx2+/+ and Runx2+/− mice (G, H). Scale bars: 0.2 mm. Data are shown as the mean ± SD. * p < 0.05, ** p < 0.01 and *** p < 0.001. The number of mice analyzed: Cbfb2+/+, n = 19; Cbfb2−/−, n = 14; Runx2+/+, n = 10; Runx2+/−, n = 11.
In the dynamic bone histomorphometric analysis of femoral cortical bone, mineral apposition rate, mineralizing surface, and bone formation rate in the periosteum and endosteum in Cbfb2\(^{-/-}\) mice were reduced compared with those in the control mice, whereas the mineralizing surface and bone formation rate, but not the mineral apposition rate in the periosteum, in Runx2\(^{+/+}\) mice were reduced compared with the control, and these parameters in the endosteum were unchanged compared with those in the control mice (Figure 13A–P).

**Figure 13.** Dynamic bone histomorphometric analysis of cortical bone at the mid-diaphyses of femurs and serum markers for bone formation and resorption in male mice at 10 weeks of age. (A–L) Cross-sections of Cbfb2\(^{+/+}\) (A,E,I), Cbfb2\(^{-/-}\) (B,F,J), Runx2\(^{+/+}\) (C,G,K) and Runx2\(^{+/+}\) (D,H,L) mice, into which calcine was injected twice. The boxed regions in (A–D) are magnified in (E,I,F,J,G,K,H,L), respectively. Scale bars: 200 \textmu m (A–D) and 100 \textmu m (E–L). (M–P) Mineral apposition rate (MAR), mineralizing surface (MS/BS) and bone formation rate (BFR/BS) in the periosteum (MAR, MS/BS, BFR/BS) and endosteum (MAR, MS/BS, BFR/BS) of Cbfb2\(^{+/+}\) and Cbfb2\(^{-/-}\) mice (M,N) and Runx2\(^{+/+}\) and Runx2\(^{-/-}\) mice (N,P). Cbfb2\(^{+/+}\), n = 8; Cbfb2\(^{-/-}\), n = 8; Runx2\(^{+/+}\), n = 22; Runx2\(^{-/-}\), n = 21. (Q) Serum markers for bone formation (P1NP) and resorption (TRAP5b). Data are shown as the mean ± SD. \(* p < 0.05\), \(** p < 0.01\) and \(*** p < 0.001\). The number of mice analyzed: Cbfb2\(^{+/+}\), n = 14; Cbfb2\(^{-/-}\), n = 16; Runx2\(^{+/+}\), n = 20; Runx2\(^{-/-}\), n = 18.
The serum markers for bone formation (total procollagen type 1 N-terminal propeptide: P1NP) and bone resorption (tartrate-resistant acid phosphatase 5b: TRAP5b) were reduced in the Cbfb2−/− and Runx2+/− mice compared with those in the respective control mice at 10 weeks of age (Figure 13Q).

Since the mineral apposition rate, mineralizing surface and bone formation rate in the endosteum were reduced in the Cbfb2−/− mice, but not in the Runx2+/− mice, compared with those in the respective control mice, we also examined TRAP-positive cells in the endosteum. The number of TRAP-positive cells was reduced in the Runx2+/− mice, but not in the Cbfb2−/− mice, compared with those in the respective control mice (Figure 14). Thus, the dynamic bone histomorphometric parameters in the endosteum of Runx2+/− mice were affected by the reduced bone resorption.

Figure 14. TRAP-positive cells in the endosteum of femurs at 4 weeks of age. (A–N) TRAP staining of femoral sections in Cbfb2+/+ (A–D), Cbfb2−/− (E–H), Runx2+/+ (I–L) and Runx2+/− (M–P) mice. The boxed regions in (A,E,I,M) are magnified in (B–D), (F–H), (J–L) and (N–P), respectively, and the boxed regions in (B–D), (F–H), (J–L) and (N–P) are magnified in (B’–D’), (F’–H’), (J’–L’) and (N’–P’), respectively. Black arrows indicate the TRAP-positive cells. Red lines show the area for counting in the endosteum. Scale bars: 500 μm (A,E,I,M) and 100 μm (B–D,F–H,J–L,N–P,B’–D’,F’–H’,J’–L’,N’–P’). (Q,R) The number of TRAP-positive cells in Cbfb2+/+ and Cbfb2−/− mice (Q) and Runx2+/+ and Runx2+/− mice (R). Data are shown as the mean ± SD. * p < 0.05. The number of mice analyzed: Cbfb2+/+ and Cbfb2−/−, n = 6; Runx2+/+ and Runx2+/−, n = 7.
2.7. Expressions of the Genes Related to Osteoblast and Osteoclast Differentiation, RUNX Family Genes and Cbfb

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using RNA from the tibiae and vertebrae at 4 weeks of age (Figure 15A,B). In Cbfb2−/− tibiae, the expressions of Sp7, Alpl, Col1a1, Tnfsf11, Cbfb1, Cbfb2 and Cbfb1+2 and the ratio of Tnfsf11/Tnfrsf11b were reduced, the expression of Runx1 was increased and the expression of Spp1, Bglap&Bglap2, Tnfrsf11b, Runx2 and Runx3 was unchanged compared with those in wild-type mice (Figure 15A). In Runx2+−/− tibiae, the expression of Spp1 and Runx2 and the ratio of Tnfsf11/Tnfrsf11b were reduced, the expression of Cbfb2 and Cbfb1+2 was increased and the other gene expressions were unchanged compared with those in wild-type mice (Figure 15B).

![Figure 15](image_url)  
Figure 15. Real-time RT-PCR analyses of the expression of the genes related to osteoblast differentiation and osteoclastogenesis, RUNX family genes and Cbfb isoforms using RNA from tibiae and vertebrae at 4 weeks of age. Real-time RT-PCR analyses were performed using RNA from the tibiae (A, B) and vertebrae (C, D) in Cbfb2+/+ and Cbfb2−/− mice (A, C) and Runx2+/+ and Runx2−/− mice (B, D). The primer pair for Bglap&Bglap2 amplifies both Bglap and Bglap2, and that of Cbfb1+2 amplified both Cbfb1 and Cbfb2. The ratios of Tnfsf11 and Tnfrsf11b are also shown. Data are shown as the mean ± SD. *p < 0.05, **p < 0.01 and ***p < 0.001. The values of Cbfb2+/+ or Runx2+/+ mice were defined as 1 and relative levels are shown. The number of mice analyzed: Cbfb2+/+, n = 16; Cbfb2−/−, n = 13; Runx2+/+, n = 19; Runx2−/−, n = 15 in the tibiae and Cbfb2+/+, n = 9; Cbfb2−/−, n = 6; Runx2+/+, n = 7; Runx2−/−, n = 7 in the vertebrae.
In Cbfβ2−/− vertebrae, the expression of Alpl, Cbfβ1, Cbfβ2, Cbfβ1+2 and the ratio of Tnfsf11/Tnfrsf11b were reduced, the expression of Runx1 was increased and the expression of Sp7, Spp1, Col1a1, Bglap&Bglap2, Tnfsf11, Tnfrsf11b, Runx2 and Runx3 was unchanged compared with those in wild-type vertebrae (Figure 15C). In Runx2−/− vertebrae, the expression of Runx2 and the ratio of Tnfsf11/Tnfrsf11b were reduced, but the expression of the other genes was unchanged compared with those in wild-type vertebrae (Figure 15D).

2.8. The Proliferation and Apoptosis of Osteoblast-Like and Osteoprogenitor-Like Cells in Femurs and Vertebrae

To investigate why the osteoblast parameters were reduced in Runx2+/− mice but not in Cbfβ2−/− mice in bone histomorphometric analysis of femurs (Figure 12A–D), the proliferation of osteoblast-like and osteoprogenitor-like cells was examined in the femurs. The frequencies of BrdU-positive osteoblast-like and osteoprogenitor-like cells in the trabecular and cortical bone of femurs in Cbfβ2−/− mice were comparable to those in wild-type mice, whereas those in the Runx2+/− mice were reduced compared with those in wild-type mice at P7 (Figure 16). To investigate why the osteoblast parameters were increased in Cbfβ2−/− mice but reduced in Runx2+/− mice in bone histomorphometric analysis of vertebrae (Figure 12E–H), the proliferation and apoptosis in osteoblast-like and osteoprogenitor-like cells were examined in the vertebrae. The frequencies of BrdU-positive osteoblast-like and osteoprogenitor-like cells were increased in Cbfβ2−/− mice but reduced in Runx2+/− mice compared with the respective control mice at 4 weeks of age (Figure 17). The frequencies of TUNEL-positive osteoblast-like and osteoprogenitor-like cells in the Cbfβ2−/− and Runx2+/− mice were comparable to those in the respective control mice (Supplementary Figure S4).

![Figure 16](image-url)

Figure 16. Proliferation of osteoblast-like and osteoprogenitor-like cells in the trabecular and cortical bone of femurs. (A–H) BrdU staining of trabecular bone (A–D) and cortical bone (E–H) in femurs from...
Cbfb2+/+ (A,E), Cbfb2−/− (B,F), Runx2+/+ (C,G) and Runx2−/− (D,H) mice at P7. The boxed regions in (A–H) are magnified in (A’–H’), respectively. Arrows in (A’–H’) show BrdU-positive osteoblast-like or osteoprogenitor-like cells. Scale bars: 100 µm (A–H) and 20 µm (A’–H’). (I,J) Frequencies of BrdU-positive osteoblast-like and osteoprogenitor-like cells in trabecular and cortical bone in Cbfb2+/+ and Cbfb2−/− mice (I) and Runx2+/+ and Runx2−/− mice (J). Data are shown as the mean ± SD. * p < 0.05, ** p < 0.01. The number of mice analyzed: Cbfb2+/+, n = 7; Cbfb2−/−, n = 5; Runx2+/+, n = 5; Runx2−/−, n = 9.

**Figure 17.** Proliferation of osteoblast-like and osteoprogenitor-like cells in vertebrae. (A–H) H-E staining (A,B,E,F) and BrdU staining (C,D,G,H) of the sections of 1st lumbar vertebrae in Cbfb2+/+ (A,C), Cbfb2−/− (B,D), Runx2+/+ (E,G) and Runx2−/− (F,H) mice at 4 weeks of age. The boxed regions in (A–H) are magnified in (A’–H’), respectively. Arrows in (C’,D’,G’,H’) show osteoblast-like or osteoprogenitor-like cells. Scale bars: 100 µm (A–H) and 20 µm (A’–H’). (I) The frequencies of BrdU-positive osteoblast-like and osteoprogenitor-like cells, which were counted in (C’,D’,G’,H’). Data are shown as the mean ± SD. * p < 0.05, ** p < 0.01. The number of mice analyzed: Cbfb2+/+, n = 3; Cbfb2−/−, n = 3; Runx2+/+, n = 3; Runx2−/−, n = 4. Two regions were counted in each mouse.

3. Discussion

The Cbfb2−/− mice expressed Cbfb about one-fifth of that in wild-type mice. Half of Cbfb2−/− mice died by 4 weeks of age, but half of them with mild rib deformity survived and enabled us to examine the functions of CBFB in bone development in young and
adult mice. The Runx2+/− mice were examined in detail and compared with Cbfb2−/− mice to evaluate the required amount of CBFB and RUNX2 in the development of calvaria, trabecular and cortical bone in femurs, vertebrae and ribs. The Cbfb2−/− mice showed differences in the phenotypes and their severities compared with Runx2+/− mice, and the bone volumes in each line were regulated differently among the bone tissues, indicating the different requirements of CBFB and RUNX2 for bone development and maintenance among the skeletal tissues. Although CBFB stabilizes RUNX2 protein and enhances the capacity of transcriptional activation [31], the dependency of RUNX2 on CBFB was also different among the skeletal tissues.

The development of calvaria was impaired in the Runx2+/− mice, in which the level of RUNX2 protein was half of that in wild-type calvaria and the level of CBFB protein was like that in wild-type calvaria, while it was mildly delayed in the Cbfb2−/− mice, in which the level of RUNX2 protein was 70%, and that of CBFB protein was one-fifth of those in wild-type calvaria (Figures 1, 4–7 and 9). Furthermore, the RUNX2 protein of the calvaria in the mice, in which the neo gene was inserted in the intron of RUNX2, was reduced to two-thirds of that in wild-type mice, and the closure of the posterior frontal suture was later than Cbfb2−/− mice (Figures 4–7) [32]. Thus, the suture closure is likely to be dependent on the level of RUNX2 protein and RUNX2 protein is relatively stable in the low amount of CBFB in calvaria, probably due to the presence of the transcription factors and/or other cofactors, which stabilize RUNX2 protein and enhance the DNA binding capacity by interacting with RUNX2.

CBFB, its isoform CBFB2 and RUNX2 are required for chondrocyte maturation, which is an essential step for endochondral ossification, while RUNX1 induces chondrocyte proliferation and maturation and RUNX3 is involved in chondrocyte maturation [5,6,21,22,24–26,33,34]. The shortening of limb long bones in Cbfb2−/− mice were more severe than those in Runx2+/− mice, the area of the thoracic cage in Cbfb2−/− mice but not in Runx2+/− mice at 4 weeks of age were significantly smaller than those in wild-type mice, and rib deformity was observed in Cbfb2−/− mice but not in Runx2+/− mice (Figure 8). Furthermore, the expression of chondrocyte marker genes, including Col2a1 and Col10a1, in the mineralized parts of ribs in Cbfb2−/− mice but not in Runx2+/− mice was markedly higher than that in wild-type mice, and the expression levels were dependent on the severity of rib deformity (Figure 8T), indicating that the inside of the mineralized bone collar of ribs in Cbfb2−/− mice with severe rib deformity was still largely cartilaginous. These findings suggest that the process of endochondral ossification was delayed more severely in Cbfb2−/− mice than in Runx2+/− mice. Furthermore, the protein levels of RUNX1, RUNX2 and RUNX3 in ribs of Cbfb2−/− mice were 11%, 25% and 19% of those in wild-type mice, respectively, although RUNX1 reduction may have been partly intensified by a lower amount of hematopoietic cells that highly express RUNX1, as endochondral ossification was retarded in the ribs but not in the other skeletons of Cbfb2−/− mice [26] (Figures 1F,G and 8T, Supplementary Figure S2). Thus, the rib deformity in Cbfb2−/− mice is likely to be caused by the impaired endochondral ossification due to the severe reduction in all RUNX family proteins. Moreover, the expression levels of CBFB and RUNX2 were lowest in rib among calvaria, tibia, vertebra and ribs (Supplementary Figure S3B). It may also contribute to the impaired rib development in Cbfb2−/− mice. In contrast, vertebrae were shortened in both Cbfb2−/− and Runx2+/− mice (Figure 8U). RUNX2 plays a major role in chondrocyte proliferation and maturation, and CBFB also induces chondrocyte proliferation and maturation by stabilizing RUNX proteins and enhancing their DNA binding [31]. Therefore, RUNX2 and CBFB cooperatively accelerate the anterior-posterior axis development by regulating chondrocyte proliferation and maturation.

The trabecular bone in femurs was reduced in Runx2+/− mice but not in Cbfb2−/− mice in micro-CT analysis (Figures 10A–D and 11A–D), osteoblast parameters were reduced in Runx2+/− mice but not in Cbfb2−/− mice in bone histomorphometric analysis, and the bone formation rate was reduced in both Cbfb2−/− and Runx2+/− mice, but more severely in Runx2+/− mice (Figure 12A–D). Furthermore, the serum marker for bone formation, P1NP, was reduced more apparently in Runx2+/− mice than in Cbfb2−/− mice. (Figure 13Q).
Therefore, the reduction in trabecular bone in Runx2+/− mice but not in Cbfb2−/− mice is likely to be explained by the level of the reduction in bone formation, as bone resorption was similarly reduced in Cbfb2−/− and Runx2+/− mice (Figures 12B,D and 13Q). However, the mechanism of the reduction in bone formation was different between Cbfb2−/− and Runx2+/− mice. Osteoblast marker gene expression was reduced in Cbfb2−/− mice but not in Runx2+/− mice in real-time RT-PCR analysis (Figure 15A,B), while the number of osteoblasts and BrdU-positive cells in osteoblast-like cells were reduced in the trabecular bone of femurs in Runx2+/− but not in Cbfb2−/− mice (Figures 12B,D and 16). These findings suggest that osteoblast maturation was impaired, but the number of osteoblasts was maintained in Cbfb2−/− mice, while the number of osteoblasts was reduced but osteoblast maturation was unaffected in Runx2+/− mice. This is consistent with the previous report, which showed that the commitment of mesenchymal cells to osteoblasts and the proliferation of osteoblast progenitors are impaired in Runx2+/− mice, but osteoblast marker gene expression in mineralized calvaria are normal in Runx2+/− mice [27]. As the levels of RUNX2 protein were about half (54%) and RUNX1 and RUNX3 proteins were also reduced to 61% and 25%, respectively, in the trabecular bone of Cbfb2−/− mice (Figure 1D,E), other cofactors or transcription factors may have compensated for the deficiency of Cbfb and have strengthened the activity of RUNX family proteins, especially RUNX2, in the trabecular bone.

In contrast to the trabecular bone in femurs, cortical bone in femurs was reduced in both Cbfb2−/− and Runx2+/− mice in micro-CT analysis (Figures 10E–H and 11E–H). Although bone formation rate in the periosteum was reduced in both Cbfb2−/− and Runx2+/− mice, that of the endosteme was reduced in Cbfb2−/− mice but not in Runx2+/− mice in bone histomorphometric analysis (Figure 13M–P). Although osteoclast parameters were reduced in the trabecular bone in both Cbfb2−/− and Runx2+/− mice (Figure 12B,D), the number of TRAP-positive cells in the endosteme was reduced in Runx2+/− mice but not in Cbfb2−/− mice compared with that in the respective control mice (Figure 14). Furthermore, the endosteal perimeter was more markedly enlarged in Cbfb2−/− mice than Runx2+/− mice (Figures 10E–H and 11E–H). Thus, the bone resorption in the endosteme was more in Cbfb2−/− than in Runx2+/− mice, and it likely affected the bone formation rate in the endosteme. Furthermore, as the eroded surface was reduced in vertebrae in Runx2+/− mice but not in Cbfb2−/− mice (Figure 12F,H), the levels of the reduction of bone resorption were different not only between the trabecular and cortical bone, but also among the bone tissues in Cbfb2−/− and Runx2+/− mice, although the serum TRAP5b was similarly reduced in Cbfb2−/− and Runx2+/− mice (Figure 13Q).

The volume of the trabecular bone in femurs and vertebrae was differentially regulated in both Cbfb2−/− and Runx2+/− mice. The trabecular bone volume in femurs was normal but increased significantly at 4 weeks of age and marginally at 10 weeks of age in vertebrae in Cbfb2−/− mice in micro-CT analysis (Figures 10B and 11A). Furthermore, osteoblast parameters were increased in the vertebrum but normal in the femurs in Cbfb2−/− mice in bone morphometric analysis (Figure 12B,F). Alpl expression was reduced and BrdU-positive cells were increased in the vertebrum in Cbfb2−/− mice (Figures 15C and 17I), suggesting an increase in immature osteoblasts. As osteoclast parameters were not reduced, the increase in immature osteoblasts is likely to be a cause of the decrease of trabecular bone in vertebrae in Cbfb2−/− mice. As the protein levels of RUNX1, RUNX2 and RUNX3 in Cbfb2−/− mice were about 81%, 100% and 42% of those in wild-type mice, respectively, RUNX2 protein seemed to be stabilized by other proteins than CBFB and RUNX2 is likely to have enhanced the commitment to osteoblasts and/or enhanced the proliferation of osteoblast progenitors by interacting with the unknown proteins. In Runx2+/− mice, the volume of trabecular bone in femurs was reduced but normal in vertebrae in micro-CT analysis (Figures 10D and 11D). The parameters for osteoblasts, osteoclasts and bone formation in bone morphometric analysis, the ratio of Tnfsf11/Tnfrsf11b expression and BrdU-positive cells were reduced in the trabecular bone in both femurs and vertebrae in Runx2+/− mice (Figures 12D,H, 15B,D, 16 and 17I). Therefore, the difference in the trabecular bone volume
between femurs and vertebrae was likely due to the levels of reduction in bone formation and resorption.

4. Conclusions

In the Cbfb2\(^{-/-}\) and Runx2\(^{+/−}\) mice, the bone volumes were regulated differently among bone tissues and between the trabecular and cortical bone. The differences were likely due to the balance of bone formation and resorption, which was different among bone tissues. Calvaria and clavicles were the exceptions. More than half the dosage of RUNX2 was required for the development. The protein levels of the RUNX family and CBFB, which determine the proliferation and differentiation of chondrocytes and osteoblasts, were different among bone tissues in the Cbfb2\(^{-/-}\) mice. The differences in the protein levels seemed to be caused by the amount of cofactors or transcription factors in the bone tissues, which can compensate for the deficiency of CBFB, and the amount of these factors is likely to determine the level of dependency of RUNX proteins on CBFB in the bone tissues. These cofactors or transcription factors need to be identified. The current study also indicated the importance of examining multiple skeletal tissues to evaluate the functions of target genes in bone development and maintenance.

5. Materials and Methods

5.1. Mice

Cbfb1\(^{-/-}\), Cbfb2\(^{-/-}\) and Runx2\(^{+/−}\) mice were generated as previously described \[35,36\]. The backgrounds of Cbfb1\(^{-/-}\) and Cbfb2\(^{-/-}\) mice were a mixed 129Ola/C57BL6 background, and that of Runx2\(^{+/−}\) mice was originally a mixed 129Ola/C57BL6 background and then backcrossed with C57BL/6N at least 12 times before this study. Prior to the investigation, all the experimental protocols were reviewed and approved by the Animal Care and Use Committee of Nagasaki University Graduate School of Biomedical Sciences (No. 1403111129-21). Animals were housed three per cage in a pathogen-free environment on a 12-h light cycle at 22 ± 2 °C, with standard chow (CLEA Japan, Tokyo, Japan) and free access to tap water.

5.2. Skeletal and Micro-CT Analyses

Whole skeletons were stained with alcian blue and alizarin red, as described previously \[36\]. Micro-CT analysis was performed using a micro-CT system (R\(_m\)CT; Rigaku Corporation, Tokyo, Japan). Data from the scanned slices were used for three-dimensional analysis to calculate femoral morphometric parameters. Trabecular bone parameters were measured on a distal femoral metaphysis. Cranio-caudal scans of approximately 2.4 mm (0.5 mm far from the growth plate), for 200 slices in 12-\(\mu\)m increments, were taken. The cortical bone parameters were measured in the mid-diaphysis of the femurs. The trabecular bone parameters in the vertebrae were measured in the 1st or 6th lumbar vertebrae. The threshold of the mineral density was 500 mg/cm\(^3\) in adult mice and 400 mg/cm\(^3\) in mice at 4 weeks of age.

5.3. Histological Analyses

Mice were fixed in 4% paraformaldehyde/0.1 M phosphate buffer and embedded in paraffin. Sections of 4 \(\mu\)m in thickness were stained with hematoxylin and eosin (H–E) or tartrate-resistant acid phosphatase (TRAP). For safranin O staining, the sections were stained with hematoxylin, fast green and safranin O. In-situ hybridization was conducted using mouse Col2a1, Col10a1 and Col1a1 antisense and sense probes, as described previously \[37\]. The sections were counterstained with methyl green. To analyze BrdU incorporation, we intraperitoneally injected BrdU into mice at 100 \(\mu\)g/g body weight 1 h before sacrifice and detected BrdU incorporation using a BrdU staining kit (Invitrogen, Carlsbad, CA, USA). The sections were counterstained with hematoxylin. TUNEL staining was performed using the ApopTag Peroxidase In-Situ Apoptosis Detection kit (Sigma Aldrich, St. Louis, MO, USA). The sections were counterstained with methyl green.
5.4. Bone Histomorphometric Analysis

Mice were intraperitoneally injected with calcein 7 and 2 days before sacrifice at a dose of 20 mg/kg body weight, and analyzed at 10 weeks of age. Mice were euthanized and the femurs, and lumbar vertebrae (L3–L5) were harvested and fixed in 70% ethanol for 3 days. Fixed bones were dehydrated with graded ethanol and infiltrated and embedded in the mixture of methyl methacrylate and 2-hydroxyethyl methacrylate (Fujifilm Wako pure chemical, Osaka, Japan). The bone histomorphometric analysis was performed in distal femurs and lumbar vertebrae using undecalcified 4-µm-thick sections as previously described [30]. The bone histomorphometric analysis of cortical bone was performed using 20-µm cross-sections from mid-diaphyses of femurs. The structural, dynamic and cellular parameters were calculated and expressed according to the standard nomenclature [38].

5.5. Serum Testing

The serum levels of total P1NP and TRAP5b were measured using the Rat/Mouse P1NP ELISA kit (Immunodiagnostic Systems, Boldon, UK) and Mouse TRAP assay (Immunodiagnostic Systems), respectively.

5.6. Real-Time RT-PCR and Western Blot Analyses

Total RNA was extracted using ISOGEN (Wako, Osaka, Japan). Real-time RT-PCR was performed using a THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and Light Cycler 480 real-time PCR system (Roche Diagnostics, Tokyo, Japan). Primer sequences are shown in Supplementary Table S2. The primer set for Bglap&Bglap2 amplifies both genes and that for Cbfb1+2 amplifies both isoforms. The expression of Tnfsf11 and Tnfsf11b was examined using TaqMan probes, Mm00441906_g1 and Mm1205928_m1 (Thermo Fisher Scientific, Tokyo, Japan). The values were normalized with those of Actb and Actb using Taqman probes, Mm02619580_g1 (Thermo Fisher Scientific), respectively. Western blot was performed using mouse monoclonal anti-CBFB [26], rabbit polyclonal anti-RUNX1 [24,26], anti-RUNX2 (Cell Signaling, Danvers, MA, USA), anti-RUNX3 (Cell Signaling) and anti-β-ACTIN (Santa Cruz Biotechnology, Dallas, TX, USA) antibodies.

5.7. Statistical Analysis

Values are shown as the mean ± SD. Statistical analyses were performed using the Student’s t-test. A p-value less than 0.05 was considered significant. p-values are indicated in the graphs by * as * p < 0.05, ** p < 0.01 and *** p < 0.001, and those of more than three groups were performed by ANOVA and the Tukey-Kramer post-hoc test. A p value < 0.05 was considered significant.

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