ORIGINAL ARTICLE

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Core Binding Factor Beta (*Cbfβ*) Controls the Balance of Chondrocyte Proliferation and Differentiation by Upregulating Indian hedgehog (Ihh) Expression and Inhibiting Parathyroid Hormone-Related Protein Receptor (PPR) Expression in Postnatal Cartilage and Bone Formation

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ABSTRACT

Core binding factor beta (Cbfβ) is essential for embryonic bone morphogenesis. Yet the mechanisms by which *Cbfβ* regulates chondrocyte proliferation and differentiation as well as postnatal cartilage and bone formation remain unclear. Hence, using *paired-related homeobox transcription factor 1*-Cre (*Prx1*-Cre) mice, mesenchymal stem cell–specific *Cbfβ*-deficient ($Cbf\beta^{f/f}$*Prx1*-Cre) mice were generated to study the role of Cbfβ in postnatal cartilage and bone development. These mutant mice survived to adulthood but exhibited severe sternum and limb malformations. Sternum ossification was largely delayed in the $Cbf\beta^{f/f}$*Prx1*-Cre mice and the xiphoid process was noncalcified and enlarged. In newborn and 7-day-old $Cbf\beta^{f/f}$*Prx1*-Cre mice, the resting zone was dramatically elongated, the proliferation zone and hypertrophic zone of the growth plates were drastically shortened and disorganized, and trabecular bone formation was reduced. Moreover, in 1-month-old $Cbf\beta^{f/f}$*Prx1*-Cre mice, the growth plates were severely deformed and trabecular bone was almost absent. In addition, *Cbfβ* deficiency impaired intramembranous bone formation both in vivo and in vitro. Interestingly, although the expression of Indian hedgehog (Ihh) was largely reduced, the expression of parathyroid hormone-related protein (PTHrP) receptor (PPR) was dramatically increased in the $Cbf\beta^{f/f}$*Prx1*-Cre growth plate, indicating that that *Cbfβ* deficiency disrupted the Ihh-PTHrP negative regulatory loop. Chromatin immunoprecipitation (ChIP) analysis and promoter luciferase assay demonstrated that the Runx/Cbfβ complex binds putative Runx-binding sites of the *Ihh* promoter regions, and also the Runx/Cbfβ complex directly upregulates *Ihh* expression at the transcriptional level. Consistently, the expressions of Ihh target genes, including *CyclinD1*, *Ptc*, and *Pthlh*, were downregulated in *Cbfβ*-deficient chondrocytes. Taken together, our study reveals not only that Cbfβ is essential for chondrocyte proliferation and differentiation for the growth and maintenance of the skeleton in postnatal mice, but also that it functions in upregulating Ihh expression to promoter chondrocyte proliferation and osteoblast differentiation, and inhibiting PPR expression to enhance chondrocyte differentiation.

KEY WORDS: GENETIC ANIMAL MODELS; SIGNALING PATHWAYS; DEVELOPMENT; OSTEOBLASTS; GROWTH PLATE; INDIAN HEDGEHOG

Introduction

Core binding factors (CBFs) are heterodimeric transcription factors composed of two subunits: the core binding factor alpha (CBFα) and core binding factor beta (CBFβ).[1] CBFα subunits are encoded by three genes, namely *Runt-related transcription factor 1* (*Runx1)* (*Cbfα2*), *Runx2* (*Cbfα1*), and *Runx3* (*Cbfα3*),[2] each of which plays important roles in skeletal

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Additional Supporting Information may be found in the online version of this article.

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development. Runx2 is a key transcription factor associated with osteoblast differentiation and chondrocyte hypertrophy.[3] Runx3 is indispensable for endochondral ossification if the *Runx2* gene dosage is reduced,[4] and Runx1 cooperates with Runx2 to regulate sternal morphogenesis.[5,6]

The non-DNA-binding subunit, Cbfβ, cooperates with Cbfα to form DNA-protein complexes and protects the Cbfα subunits from degradation.[7] *Cbfβ*$^{-/-}$ embryos died from an absence of fetal liver hematopoiesis at mid-gestation.[8,9] This barrier had impeded further research in understanding the role of Cbfβ in skeletal development until the generation of the three mice models (*Cbfβ*$^{GFP/GFP}$ knock-in mice, and *Tek*-GFP/*Cbfβ* and *Gata1-Cbfβ* transgenic mice) in 2002.[10–12] *Cbfβ*$^{GFP/GFP}$ knock-in mice died soon after birth. *Cbfβ*$^{-/-}$ embryos that were rescued by *Tek*-GFP/*Cbfβ* (*Cbfβ*$^{-/-}$Tg[*Tek*-GFP/*Cbfβ*]) and *Gata1-Cbfβ* (*Cbfβ*$^{-/-}$Tg[*Gata1-Cbfβ*]) transgene died around birth. These mouse models enabled the study of Cbfβ's role in embryonic skeletal development.[10–12] The role of *Cbfβ* in postnatal bone formation was unexplored until the generation of *Cbfβ/Runx2* double transgenic mice, which exhibited severe osteopenia.[13] However, the physiological defects caused by *Cbfβ* deficiency in postnatal mice have not yet been clarified. To further explore the role of Cbfβ in skeletal development, we generated mesenchymal stem cell (MSC)-specific *Cbfβ* conditional knockout mice by crossing *Cbfβ*$^{f/f}$ mice[14] with (*paired-related homeobox transcription factor 1*) *Prx1*-Cre mice.[15] Cre expression driven by the *Prx1*-promoter was first detected in the forelimb mesenchyme at embryonic day (E) 9.5 and then in all MSCs at E10.5.[15] *Cbfβ*$^{f/f}$*Prx1*-Cre mice survived into adulthood, *Cbfβ*$^{f/f}$*Prx1*-Cre mice displayed short limbs, short statures, inhibited osteoblastogenesis, inhibited chondrocyte differentiation, and impaired trabeculae formation. In addition, Cyclin D1, Indian hedgehog (Ihh), and parathyroid hormone-related protein receptor (PPR) expression were dysregulated in the growth plates of the *Cbfβ*$^{f/f}$*Prx1*-Cre mice.

Materials and Methods

Generation of Cbfβ conditional knockout mice

Cbfβ$^{f/f}$ mice (B6.129P2-*Cbfβ*^{tm1Itan}/J) mice[14] and *Prx1*-Cre (B6.Cg-Tg[*Prx1*-Cre]1Cjt/J)[15] (Jackson Laboratory, Bar Harbor, MI, USA) were crossed to generate *Cbfβ*$^{f/+}$*Prx1*-cre mice, and their progeny were intercrossed to obtain *Cbfβ*$^{f/f}$*Prx1*-cre mice. Mice were housed in the animal room of University of Alabama at Birmingham (UAB) (Birmingham, AL, USA). All research procedures using mice were approved by the UAB Animal Care and Use Committee and conformed to NIH guidelines.

Statistical analysis and data quantification analysis

All data were presented as the mean ± SD. Statistical significance was assessed using Student's *t* test performed with the SPSS 16.0 software (SPSS Incorporation, Chicago, IL, USA). Values of $p < 0.05$ were considered significant, and labeled $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in the graphs. The results are representative of at least six individual experiments ($n = 6$).

Results

Spatial and temporal expression of Cbfβ in wild-type mouse skeleton

Expression of Cbfβ in skeletons at postnatal 1-day-old (P1) mice was detected by immunohistochemistry (IHC) using paraffin sections. The results showed that Cbfβ is highly expressed in proliferative chondrocytes, hypertrophic chondrocytes, and osteoblasts in long bones (Supporting Fig. S1*A*), vertebrae (Supporting Fig. S1*B*), and ribs (Supporting Fig. S1*C*).

MSC-specific *Cbfβ*-deficient mice exhibited dwarfism with skeletal malformation and delayed ossification

MSC-specific *Cbfβ*-deficient mice were generated by crossing *Cbfβ*$^{f/f}$ mice with *Prx1*-cre mice, and genotypes were confirmed by PCR analysis (Fig. 1*L*). Efficient ablation of Cbfβ expression in chondrocytes and osteoblasts in ribs, vertebrae, and long bones in *Cbfβ*$^{f/f}$*Prx1*-Cre mice was confirmed by IHC (Supporting Fig. S2*A–C*). Although a small portion (~10%) of homozygotes died of asphyxiation problems soon after birth, most survived into adulthood. Male homozygotes were fertile. Female homozygotes were able to conceive, although dystocia would occur, probably due to an abnormal pelvis (Fig. 2*A*, upper panel and lower panel). Postnatal 7-day-old (P7) *Cbfβ*$^{f/f}$*Prx1*-Cre mice exhibited dwarfism (Fig. 1*A*, *B*) with shortened limbs (Fig. 1*A–D*) compared to wild-type (WT) cohorts. Bone morphology of P7 mice was analyzed by Alizarin red S/Alcian blue staining (Fig. 1*B–I*). Although limbs were shortened, the epiphyseal cartilage (light grey color) was elongated (Fig. 1*C*, *D*) in the *Cbfβ*$^{f/f}$*Prx1*-Cre mice. In addition, mutant mice had widened sutures and enlarged fontanelles, with delayed ossification of the parietal and frontal bones (Fig. 1*F*). Ossification of the sternum (Fig. 1*H*) and hyoid bone (Fig. 1*I*) were also delayed in mutant mice compared to WT mice. The xiphoid process was non-calcified and abnormally enlarged (Fig. 1*H*). Spines and ribs did not show notable morphological changes in P7 mutant mice (Fig. 1*E*, *G*), but they did show delayed ossification in P1 mutant mice (Supporting Fig. S2*D*, *E*). Thus, defects may be compensated for as the mice aged. Finally, the short-limb deformity observed in the mutant mice (Fig. 1*A–D*) persists with age, which was confirmed again by Alizarin red S/Alcian blue (Fig. 1*J*) and μCT analysis (Fig. 1*K*) of 1-month-old (P30) mice. Notably, X-ray analysis showed that 6-week-old *Cbfβ*$^{f/f}$*Prx1*-Cre mice displayed several characteristics of cleidocranial dysplasia (eg, short stature, short limb, and absent clavicles) (Fig. 2*A*). Taken together, these results indicate that loss of Cbfβ results in dwarfism, limb and sternum malformation, and delayed skeletal ossification during postnatal skeletal development.

Loss of *Cbfβ* impaired skeletal development in newborn mice

Next, to examine the role of Cbfβ in the differentiation of chondrocytes, osteoblasts, and osteoclasts in newborn mice, hematoxylin and eosin (H&E) staining, Safranin O staining, Goldner's trichrome staining, and TRAP staining were performed on paraffin sections of femurs (Fig. 2*B–F*). Compared with WT mice, newborn *Cbfβ*$^{f/f}$*Prx1*-Cre mice had elongated growth plates and shortened diaphysis (Fig. 2*B*, *C*). Although the resting zone was elongated, the proliferation zone was shortened and the proliferative columns, which were presented in the WT growth plates, were disrupted in newborn mutant mice (Fig. 2*C*). The hypertrophic zone was also slightly deformed in newborn mutant mice (Fig. 2*C*). Furthermore, the number and thickness of trabecular bones and the number of osteoblasts were significantly reduced in newborn *Cbfβ*$^{f/f}$*Prx1*-Cre mice (Fig. 2*D*, *E*), the osteoclast number did not change (Fig. 2*D–F*). These results indicate that Cbfβ is important for chondrocyte proliferation and

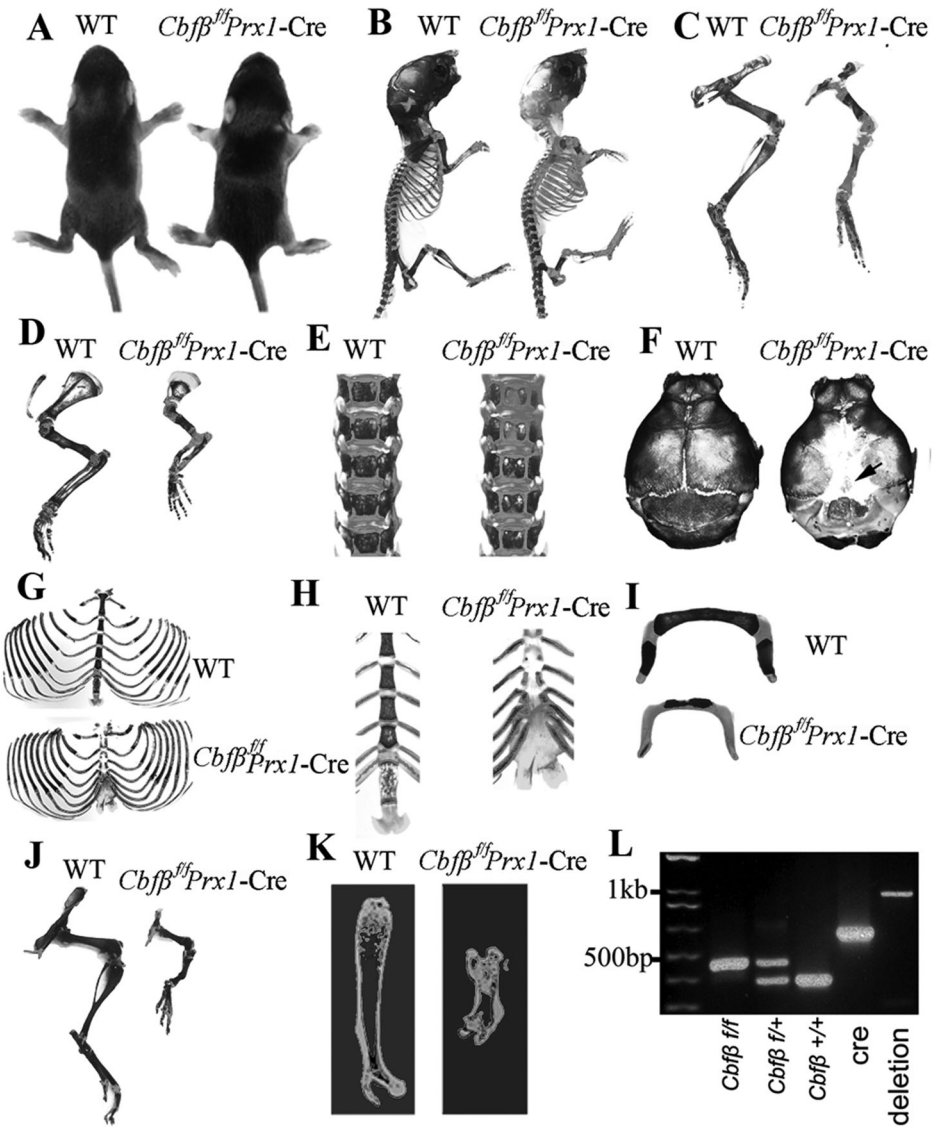

Fig. 1. *Cbfβ*$^{f/f}$*Prx1*-Cre mice had dwarfism with shortened limbs. (*A*) Gross morphology of postnatal 7-day-old (P7) *Cbfβ*$^{f/f}$*Prx1*-Cre and wild-type (WT) mice. (*B–I*) Skeletal analysis by Alizarin red S/Alcian blue staining of P7 *Cbfβ*$^{f/f}$*Prx1*-Cre and WT mice. Long bones were shorter (*C*, *D*), sutures and fontanelles were widened (black arrow) (*F*), and ossification of parietal bones (*F*), frontal bone (*F*), sternum (*H*), and hyoid bone (*I*) was delayed in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. Development of ribs (*G*) and spine (*E*) was not affected in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. (*J*) Skeletal analysis by Alizarin red S/Alcian blue staining of P30 *Cbfβ*$^{f/f}$*Prx1*-Cre and WT mouse limb. (*K*) μCT analysis of P30 *Cbfβ*$^{f/f}$*Prx1*-Cre and WT mouse femur. (*L*) PCR was used to determine *Cbfβ* alleles (*f/f*, *f*/+, +/+, or deletion) and the presence of Cre.

maturation in newborn mice and that it may play a lesser role in osteoclasts.

Loss of *Cbfβ* impaired growth plate development in P7 mice

Continuous postnatal skeletal development is required for normal development toward adulthood. H&E staining on paraffin sections of P7 mouse femur showed that growth plate and trabecular bone development were delayed in mutant mice (Fig. 3*A*, *B*). Comparable with the newborn mutant mice (Fig. 2*B*, *C*), P7 mutant mice also had shortened femurs, elongated growth plates, shortened diaphysis (Fig. 3*A*), elongated resting zones, and shortened and disorganized proliferation zones (Fig. 3*B*). Although the hypertrophic zone was slightly deformed in newborn mutant mice (Fig. 2*C*), it was notably shortened in the P7 *Cbfβ*$^{f/f}$*Prx1*-Cre mice (Fig. 3*B*). Moreover, proliferating cell nuclear antigen (PCNA) staining showed that the number of proliferating chondrocytes was decreased (Fig. 3*C*, *D*), and immunofluorescent (IF) staining showed that collagen type X (ColX) expression was reduced in mutant mice compared to WT (Fig. 3*E*). These results confirmed the retardation of chondrocyte proliferation and hypertrophy in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. Micromass culture of growth plate chondrocytes from newborn mice and Alcian blue staining confirmed that chondrocyte differentiation in vitro was also drastically delayed in the absence of *Cbfβ*

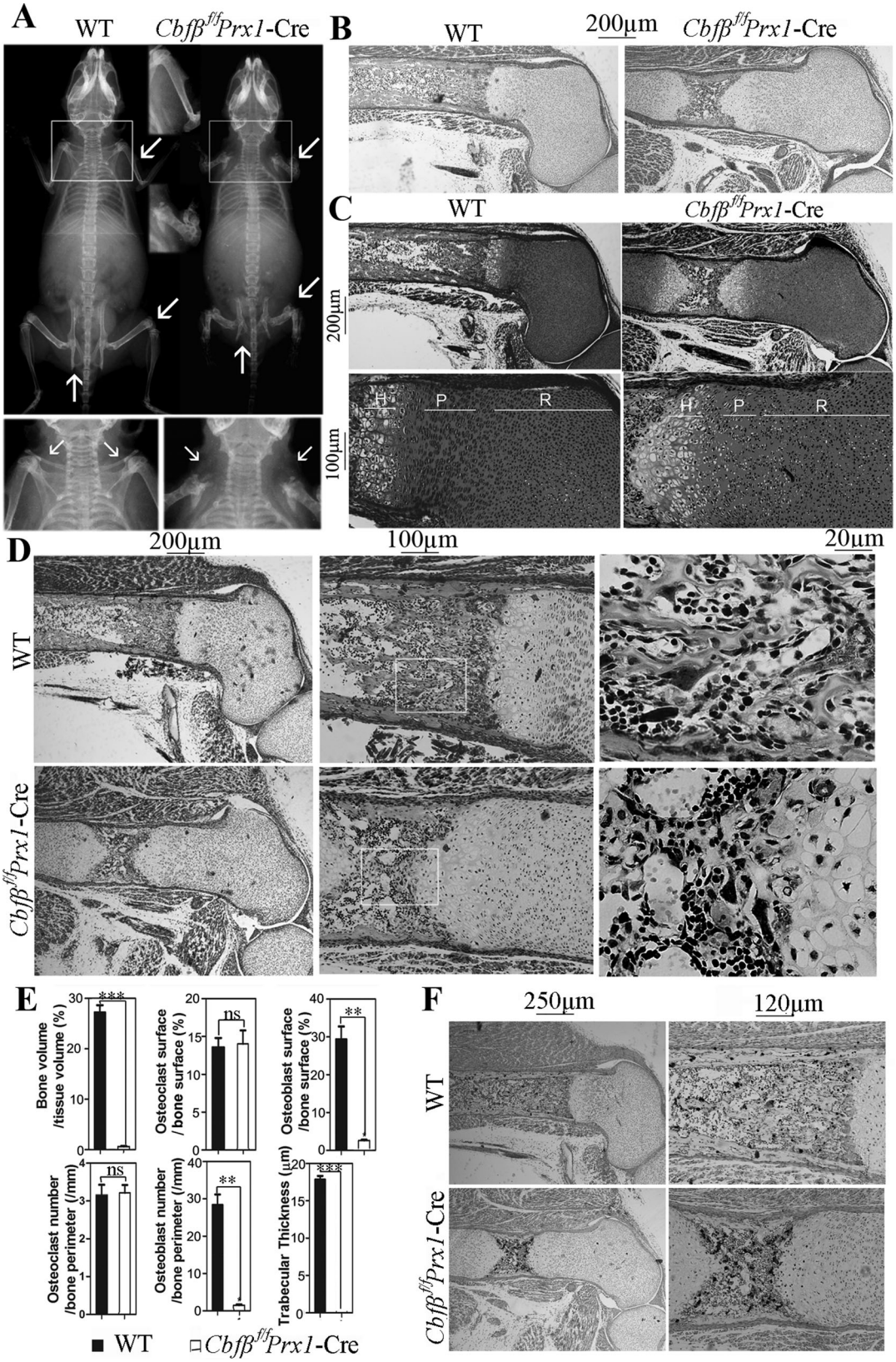

Fig. 2. *Cbfβ* deficiency resulted in cleidocranial dysplasia-like phenotype in adult mice and skeletal defects in newborn mice. (*A*) Whole-body X-ray of 6-week-old mice showed cleidocranial dysplasia-like phenotype of *Cbfβ*$^{f/f}$*Prx1*-Cre mice, including shortened stature, shortened long bones (upper lane, marked by arrowhead), and absent clavicles (lower lane marked by arrowhead and upper lane marked by arrowhead and square). (*B*) H&E staining of paraffin sections of femurs from newborn *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. (*C*) Safranin O staining of paraffin sections of femurs from newborn *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. (*D*) Goldner's trichrome staining of paraffin sections of femurs from newborn *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. (*E*) Quantification data of Goldner's trichrome staining were presented as mean ± SD, $n \geq 6$, ns = nonsignificant, $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT. (*F*) TRAP staining of paraffin sections of femurs from newborn *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. P = proliferation zone; R = resting zone; H = hypertrophic zone.

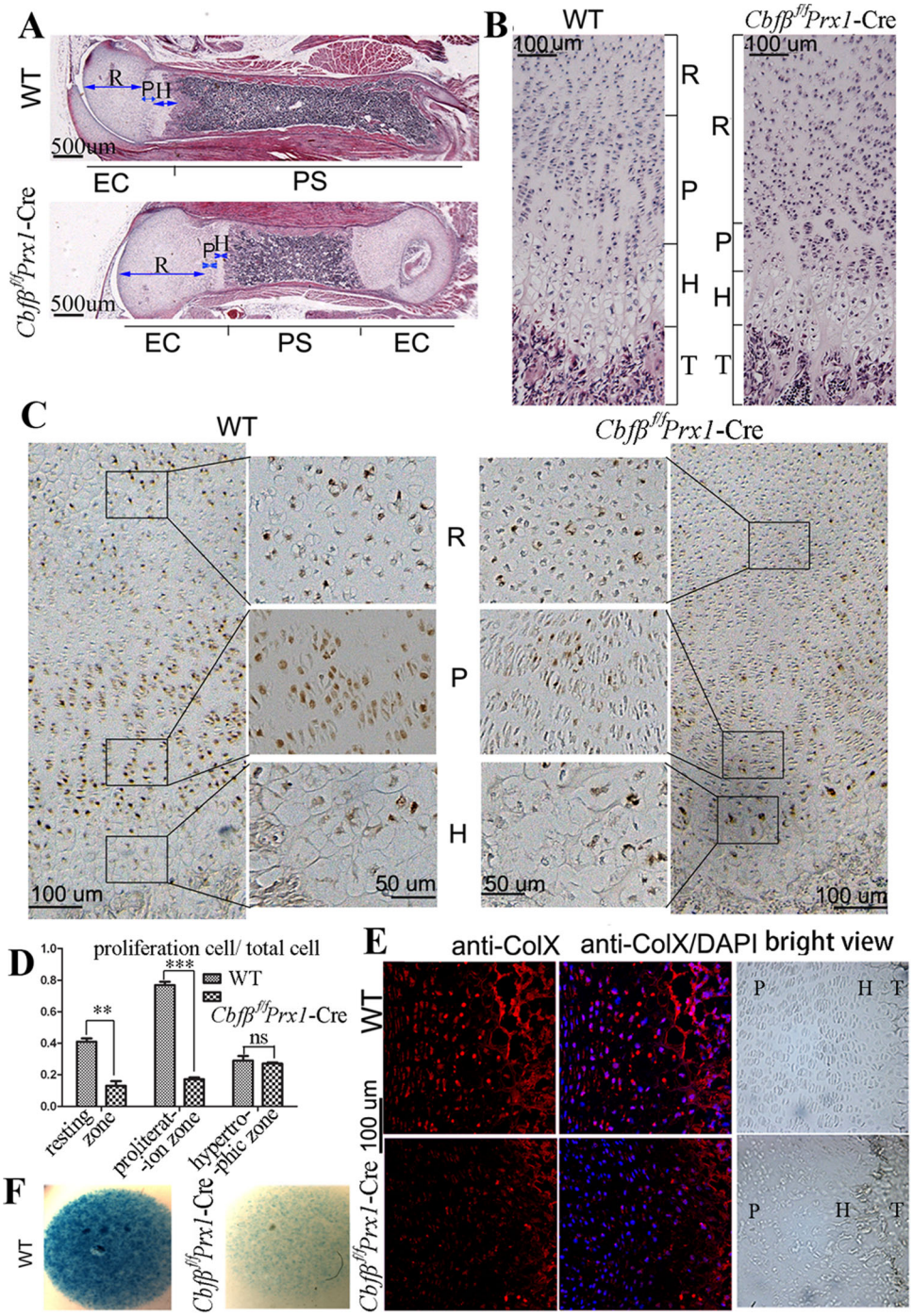

Fig. 3. *Cbfβ* deficiency retards the development of primary spongiosa and delays chondrocyte proliferation and maturation. (*A*, *B*) H&E staining of paraffin sections of femurs from P7 *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. Femur and primary spongiosa were shortened, while the epiphyseal growth plate was elongated in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. Columnar structure of proliferative chondrocyte zone was lost in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. (*C*) PCNA staining of paraffin sections of femurs from P7 *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. Second and third columns show magnified images of areas in black boxes. (*D*) The ratio of proliferating cells in total cells in the three chondrocyte zones of WT and *Cbfβ*$^{f/f}$*Prx1*-Cre mice from (*C*). Data were presented as mean ± SD, $n \geq 6$, ns = nonsignificant, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the same zone in WT mice. (*E*) IF staining using frozen sections of P7 mouse femurs showed that ColX expression was decreased in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. Bright field views were co-presented on the right panel. (*F*) Micromass culture of growth plate chondrocytes of newborn mice. PS = primary spongiosa; EC = epiphyseal cartilage; R = resting zone; P = proliferation zone; H = hypertrophic zone; T = trabecular bone.

(Fig. 3*F*). Taken together, these results demonstrate that Cbfβ plays important roles in growth plate formation in the early stages of postnatal development.

Loss of *Cbfβ* blocked Ihh-cyclin D1 signaling and the Ihh-PTHrP negative feedback loop

Protein expression in the growth plates was detected by IF staining using P7 mouse femur sections (Fig. 4*A–D*, bright field views are co-presented in Supporting Fig. S3*A–D*) and Western blot using proteins from newborn mouse femoral cartilage (Fig. 4*E*, *F*). Expression of SRY-related high-mobility group-Box gene 9 (Sox9), a key transcription factor in the chondrocyte lineage, was similar in WT and *Cbfβ*$^{f/f}$*Prx1*-Cre mice (Figs. 4*A*; Supporting Fig. S3*A*). However, expression of Ihh was dramatically reduced in *Cbfβ*$^{f/f}$*Prx1*-Cre mice (Fig. 4*B*, *E*, *F*; Supporting Fig. S3*B*). Cyclin D1, a cell-cycle-regulating protein downstream of Ihh,[16] was also decreased in the proliferation zone of *Cbfβ*$^{f/f}$*Prx1*-Cre femurs (Fig. 4*C*, *E*, *F*; Supporting Fig. S3*C*), which may inhibit chondrocyte proliferation. Consistently, expression of other Ihh-targeted genes besides Cyclin D1 (eg, *Pthlh* and *Patched* [*PTC*]) were also reduced in the growth plates of *Cbfβ*$^{f/f}$*Prx1*-Cre mice. qRT-PCR using mRNA from newborn mouse femur showed that *Pthlh* expression was reduced by 30% in *Cbfβ*$^{f/f}$*Prx1*-Cre mice (Supporting Fig. S5*B*). IHC staining using P7 mouse femur sections showed that PTC expression was detected in the prehypertrophic zone in the growth plates in WT mice, but that it was greatly reduced in mutant mice (Fig. 4*G*). Ihh induces PTHrP expression in periarticular cells around prehypertrophic chondrocytes, which in turn suppresses chondrocyte differentiation through a feedback regulatory process, the "Ihh-PTHrP negative feedback loop."[17] Interestingly, although Ihh and *Pthlh* expression were reduced, PPR expression was increased in the *Cbfβ*$^{f/f}$*Prx1*-Cre growth plate. Upregulation of PPR in *Cbfβ*$^{f/f}$*Prx1*-Cre mice may increase the sensitivity of chondrocytes to PTHrP and retard chondrocyte hypertrophy even in the presence of a permissive dose of PTHrP resulting from decreased Ihh expression. In conclusion, *Cbfβ* deficiency may affect chondrocyte proliferation by inhibiting Ihh-cyclin D1 signaling and interfere with normal chondrocyte hypertrophy by disturbing the Ihh-PTHrP negative feedback loop.

Runx/Cbfβ complex regulated Ihh expression by binding to the *Ihh* promoter directly

In order to determine if the Runx/Cbfβ complex binds the promoter region of *Ihh*, chromatin immunoprecipitation (ChIP) assay was performed using anti-Cbfβ antibody and the primers shown in Fig. 4*H*. The ChIP input value using each primer pair represents the binding efficiency of its adjacent region. There were 16 predicted Runx-binding sites localizing in the *Ihh* promoter region (–3919/+27) (Fig. 4*H*). Primer pairs 4 and 5 resulted in the highest values (Fig. 4*I*), indicating that Runx2/Cbfβ complex may bind sites 9 through 16 most efficiently. A 1.4-kb *Ihh* promoter region (–1287/+162), which contains Runx-binding sites 9 through 16, was cloned into the pGL3-basic vector. Luciferase activity driven by the *Ihh* promoter (–1287/+162) was low in the *Cbfβ*$^{f/f}$*Prx1*-Cre chondrocytes and robustly increased (10-fold) after the expression of Cbfβ (Fig. 4*J*). Ectopic expression of Runx2 further promoted the luciferase signal. In conclusion, we believe that the Runx/Cbfβ heterodimer directly binds to the Runx-binding sites of the *Ihh* promoter regions and upregulates *Ihh* expression at the transcriptional level.

Trabecular bone formation is impaired in MSC-specific *Cbfβ*-deficient mice

Delayed ossification was observed in P1 (Supporting Fig. S2*D*, *E*), P7 (Fig. 1), and 6-week-old (Fig. 2*A*) *Cbfβ*$^{f/f}$*Prx1*-Cre mice. This was further confirmed by the H&E staining of P30 mice paraffin sections, which revealed a dramatic loss of trabecular bone in mutant mice (Fig. 5*A*, *B*). Notably, the growth plates were also severely deformed, protruding deep into the diaphysis (Fig. 5*B*, arrow). To investigate the potential role of Cbfβ in osteoblasts in vivo, IF staining using mouse femur sections was performed (Fig. 5*C–F*; Supporting Fig. S4). Expression of Runx2 (a master transcription factor in the osteoblast lineage) was similar in P7 WT and *Cbfβ*$^{f/f}$*Prx1*-Cre growth plates and trabecular bone, and increased in mutant perichondrium (Fig. 5*E*). Expression of Runx3, detected mainly in hypertrophic and prehypertrophic chondrocytes, was also similar between E18.5 and E16.5 WT and mutant femur (Supporting Fig. S4). However, Osx (Osterix) (Fig. 5*C*) and Opn (Osteopontin) (Fig. 5*D*), which are osteoblast-related genes, showed reduced expression in P7 *Cbfβ*$^{f/f}$*Prx1*-Cre trabecular bone compared with WT, indicating that osteoblastogenesis may be influenced. In addition, vascular endothelial growth factor (VEGF) expression was downregulated in the growth plates of newborn mutant mice (Fig. 5*F*). Decreased VEGF expression results in reduced angiogenesis, which may affect the population of MSC for normal trabecular bone formation. Taken together, these data demonstrate that Cbfβ not only regulates chondrocyte proliferation and maturation, but also influences trabecular bone formation for normal skeleton morphogenesis.

Loss of Cbfβ impaired osteoblastogenesis of calvarial cells in vitro

To further confirm the role of Cbfβ in bone ossification, we investigated the role of Cbfβ in osteoblastogenesis in vitro using osteoblast derived from calvarial cell primary culture (Fig. 6). Deletion of Cbfβ expression in *Cbfβ*$^{f/f}$*Prx1*-Cre osteoblasts was confirmed by quantitative RT-PCR (qRT-PCR) (Fig. 6*C*) and Western blot (Fig. 6*G*). *Cbfβ*$^{f/f}$*Prx1*-Cre osteoblasts showed reduced alkaline phosphatase (ALP) activity (Fig. 6*A*) and mineralization (Fig. 6*B*) compared to WT cells, indicating that osteoblastogenesis was impeded in mutant cells. qRT-PCR revealed that the osteoclastogenesis related cytokines *receptor activator of nuclear factor-kB ligand* (*RANKL*) was similarly expressed in *Cbfβ*$^{f/f}$*Prx1*-Cre and WT osteoblasts (Fig. 6*F*). Expression of the osteoblast-related gene *Ocn* (*Osteocalcin*) was decreased in *Cbfβ*$^{f/f}$*Prx1*-Cre cells on day 7 of osteoblastogenesis (Fig. 6*D*), and the expression of *Ocn*, *Opn*, *Alp*, and *integrin binding sialoprotein* (*Ibsp*) was also decreased in *Cbfβ*$^{f/f}$*Prx1*-Cre cells on day 14 of osteoblastogenesis (Fig. 6*E*, *F*). However, *Runx2* expression was increased (Fig. 6*D*, *E*), especially isoform 1 of *Runx2* (*Runx2-I*) (Fig. 6*H*). The expression of *Osx*, another master transcription factor of osteoblast differentiation, was also similar in WT and *Cbfβ*-deficient cells (Fig. 6*D*, *E*). Considering that *Osx* expression was decreased in *Cbfβ*$^{f/f}$*Prx1*-Cre trabecular bone (Fig. 5*C*), the mechanism by which Runx2 regulates Osx expression may be different in calvaria and long bones. Western blot revealed that protein levels of Runx2 and Runx3 were not affected, but that expression of Ocn and Runx1 was reduced in *Cbfβ*$^{f/f}$*Prx1*-Cre osteoblasts on day 14 and 21 of osteoblastogenesis. When *Runx1* expression was selectively rescued in the endothelial and hematopoietic systems of *Runx1*$^{-/-}$ embryos (*Runx1*$^{Re/Re}$ mice), these mice survived until birth and displayed

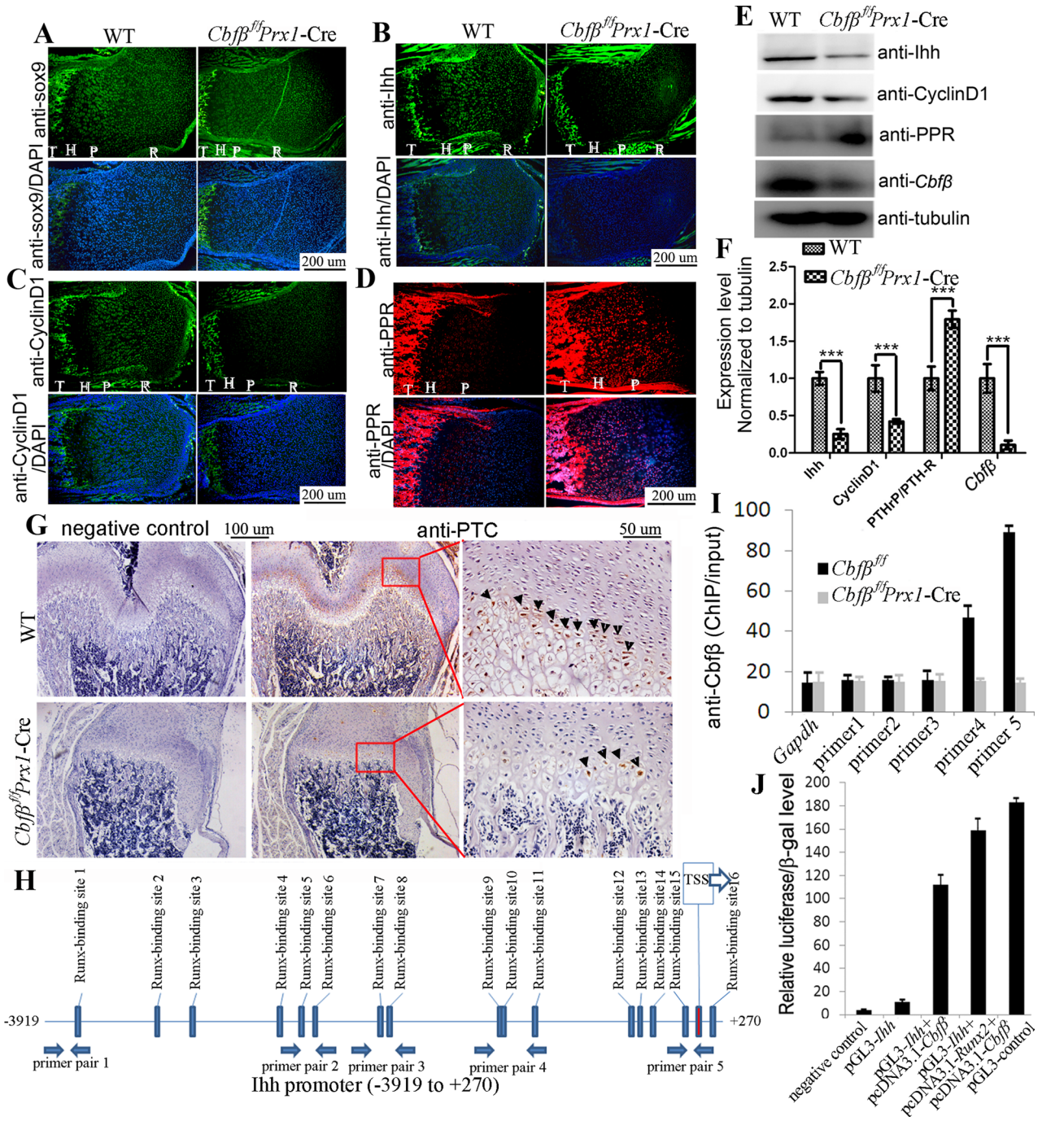

Fig. 4. Expression of Sox9, Ihh, CyclinD1, PTHrP-R, and Cbfβ in chondrocytes of *Cbfβ*$^{f/f}$*Prx1*-Cre mice and WT mice. Similar Sox9 expression (*A*), decreased Ihh expression (*B*), decreased Cyclin D1 expression (*C*), and increased PPR expression (*D*) was observed in femur growth plates of P7 *Cbfβ*$^{f/f}$*Prx1*-Cre mice compared to that of WT mice, as detected by IF staining using frozen sections. (*E*) The expression of Ihh, CyclinD1, PPR, and Cbfβ was confirmed by Western blot using protein lysates of femoral cartilage from *Cbfβ*$^{f/f}$*Prx1*-Cre and WT newborn mice. (*F*) Protein levels in *E* were quantified and normalized to tubulin. (*G*) The expression of Patched (PTC) in the prehypertrophic zone of the growth plates in P7 *Cbfβ*$^{f/f}$*Prx1*-Cre mice was reduced compared with that in WT mice, as detected by IHC staining using paraffin sections. Third column shows the magnified images of areas in red boxes. Arrowheads indicate positive stains. (*H*) Schematic display of the *Ihh* promoter region (–3919/+270). TSS (transcriptional start site), predicted Runx-binding sites and ChIP primer positions were indicated in the figure. (*I*) ChIP was performed using WT chondrocyte lysates, anti-Cbfβ antibody, and primers as indicated in *H*. (*J*) The *Ihh* promoter region (–1287/+162) was inserted into the pGL3-basic vector. Primary *Cbfβ*$^{f/f}$*Prx1*-Cre chondrocytes were transfected with pGL3-control, pGL3-*Ihh* + pcDNA3.1a-*Cbfβ*, or pGL3-*Ihh* + pcDNA3.1a-*Cbfβ* + pcNDA3.1a-*Runx2*. The β-GAL-expression plasmid was also cotransfected. Luciferase activity was detected 48 hours posttransfection, and normalized to β-GAL activity. Data were presented as mean ± SD, $n \geq 6$, ns = nonsignificant, $^{***}p < 0.001$. R = resting zone; P = proliferation zone; H = hypertrophic zone; T = trabecular bone.

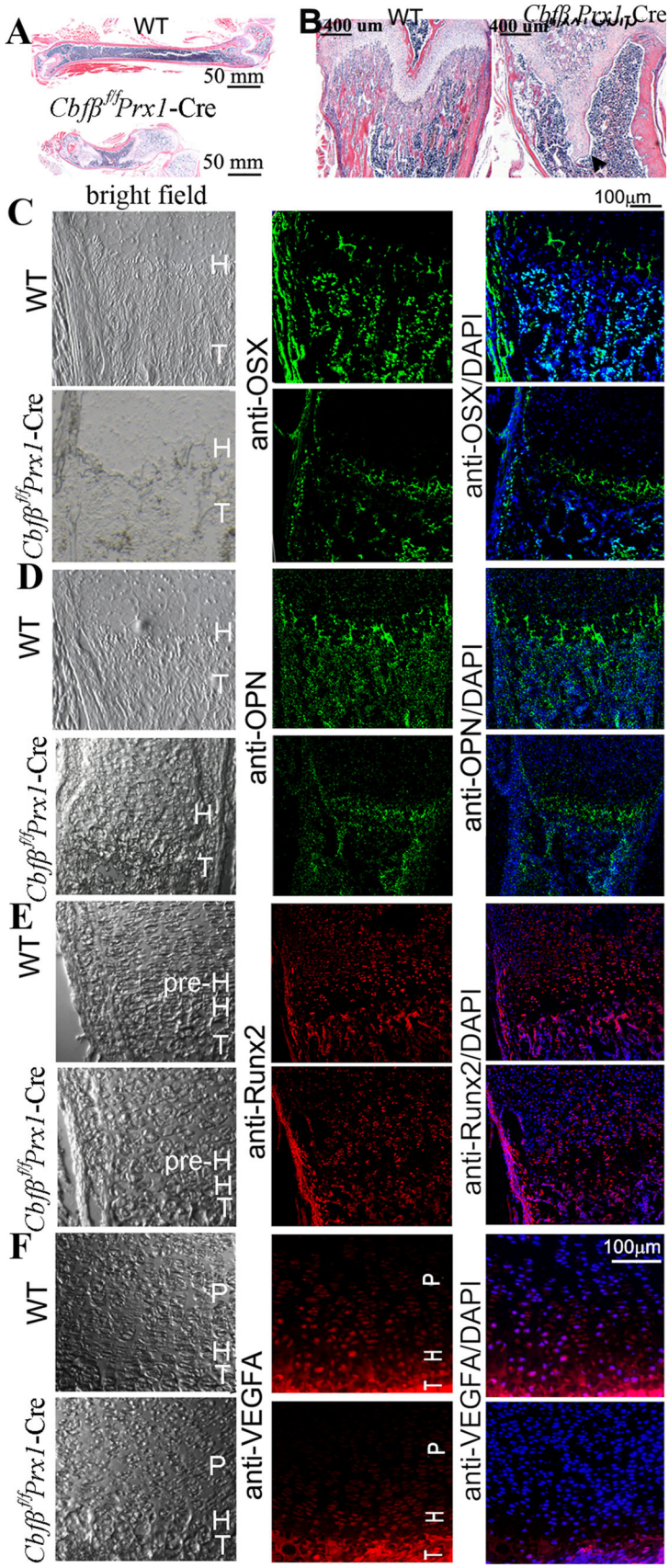

Fig. 5. Mice lacking *Cbfβ* had delayed ossification. (*A*, *B*) H&E staining of the femurs of P30 (postnatal 1-month-old) WT and *Cbfβ*$^{f/f}$*Prx1*-Cre mice. The femur was shortened and trabecular bone was lost in *Cbfβ*$^{f/f}$*Prx1*-Cre mice. The epiphyseal growth plate protruded deep into the diaphysis abnormally (marked by arrowhead). (*C–F*) IF staining using frozen sections of femurs of P7 (*C–E*) and newborn (*F*) WT and *Cbfβ*$^{f/f}$*Prx1*-Cre mice. In the *Cbfβ*$^{f/f}$*Prx1*-Cre mice, expression of OSX (*C*), OPN (*D*), and VEGFA (*F*) was decreased, but expression of Runx2 (*E*) was not changed. Bright field views of *C–F* are co-presented on the left panel. T = trabecular bone; H = hypertrophic zone; P = proliferation zone; pre-H = prehypertrophic zone.

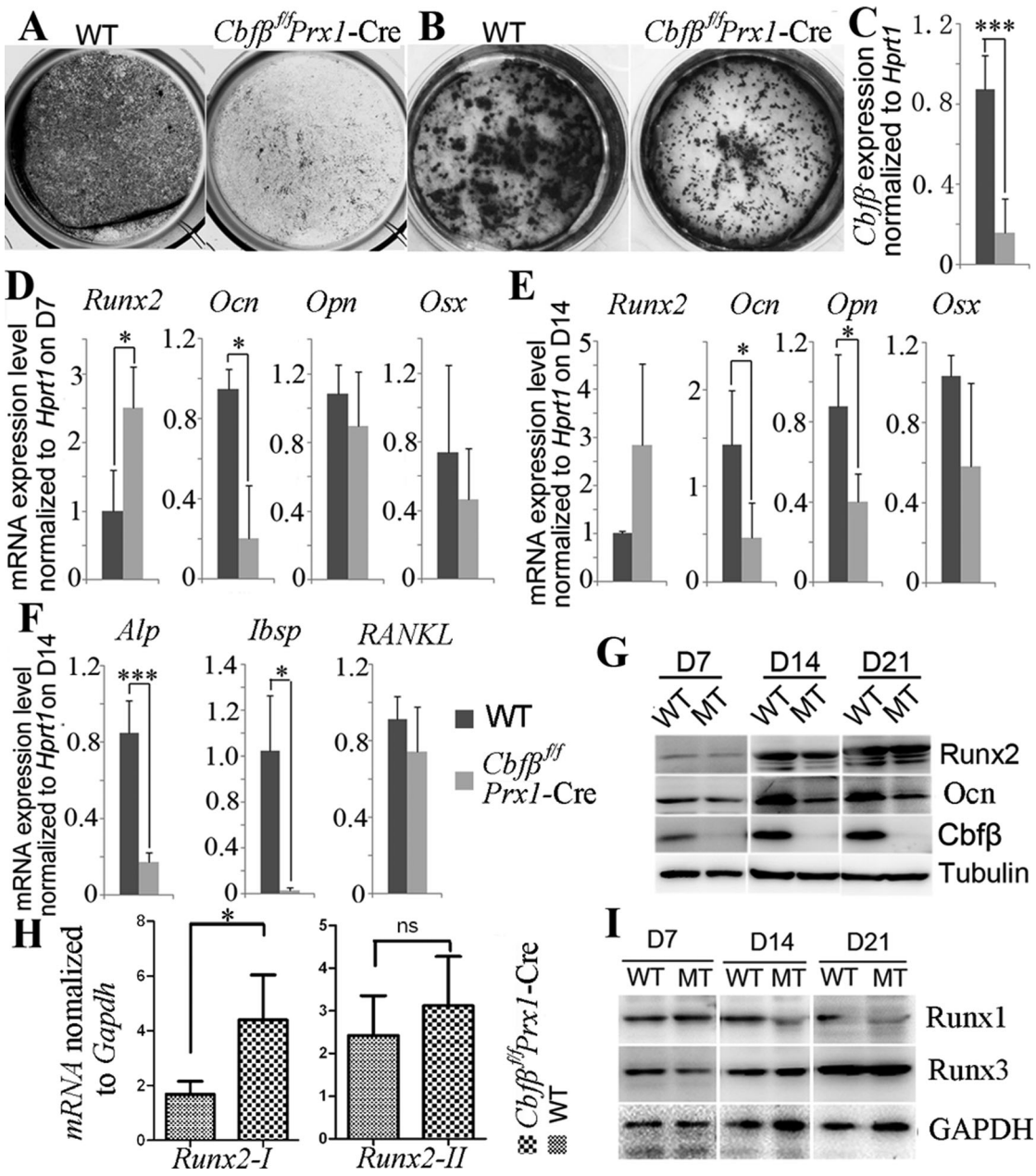

Fig. 6. Cbfβ is required for osteoblastogenesis of calvarial cells. Calvarial cells from *Cbfβ*$^{f/f}$*Prx1*-Cre and WT newborn mice were used for primary culture. Osteoblastogenesis was detected by (*A*) ALP staining on day 14 of osteoblastogenesis and mineralization was detected by (*B*) Von Kossa staining on day 21 of osteoblastogenesis. (*C*) *Cbfβ* expression levels in calvarial cells were detected by qRT-PCR and normalized to *Hprt1*. (*D*, *E*) Expression of *Runx2*, *Opn*, *Ocn*, and *Osx* in calvarial cells on day 7 (D7) (*D*) and day 14 (D14) (*E*) of osteoblastogenesis was detected by qRT-PCR and normalized to *Hprt1*. (*F*) Expression of *Alp*, *Ibsp*, and *RANKL* in calvarial cells on day 14 of osteoblastogenesis was detected qRT-PCR and normalized to *Hprt1*. (*G*) Western blot was applied to detect the protein levels of Runx2, Ocn, and Cbfβ in WT and mutant (MT) calvarial cells on days 7, 14, and 21 (D7, D14, and D21, respectively) of osteoblastogenesis. (*H*) Expression levels of *Runx2-I* and *Runx2-II* in calvaria were determined by qRT-PCR and normalized to *Gapdh*. (*I*) Western blot was applied to detect the protein levels of Runx1 and Runx3 in WT and mutant calvarial cells on days 7, 14, and 21 of osteoblastogenesis. Results were presented as mean ± SD, $n \geq 6$, ns = nonsignificant, $^{*}p < 0.05$, $^{***}p < 0.005$.

disrupted mineralization in some skull elements,[6] indicating that Runx1 plays a role in calvarial osteoblastogenesis. Thus, Runx1 hyposufficiency in *Cbfβ*$^{f/f}$*Prx1*-Cre cells may partially contribute to the impeded osteoblast differentiation. Taken together, these results indicate that Cbfβ deficiency affects osteoblast differentiation in vitro.

Discussion

The Ihh-PTHrP negative feedback loop maintains chondrocyte proliferation and counters chondrocyte hypertrophy.[17] We report that Cbfβ plays a dual function in this loop by regulating the expression of Ihh and PPR (Fig. 4*B*, *D*), thereby affecting the

balance of chondrocyte proliferation and maturation. The Runx/Cbfβ complex binds the promoter region of the *Ihh* gene so as to regulate its expression (Fig. 4*H–J*). Consistently, diminished Ihh expression was also observed in *Runx2*$^{-/-}$ and *Runx2*$^{-/-}$*Runx3*$^{-/-}$ mice.[4] It is therefore possible that Cbfβ also regulates the Ihh-PTHrP negative feedback loop by interacting with Runx2 and Runx3, thereby regulating chondrocyte proliferation and maturation. Thus, Cbfβ deficiency results in impaired growth plates development (Figs. 2 and 3) and severe skeletal malformation (Fig. 1).

Runx2 is a master regulator of the commitment and differentiation of pluripotent MSCs to osteoblasts.[3] As a subunit of the CBF complex, Cbfβ interacts with Runx2 to stabilize its interaction with DNA. pGL3-3X*OSE2* was constructed by inserting three *OSE2*,[18] a Runx2 binding sequence, into the pGL3-promoter. If only Runx2 and Cbfβ were co-expressed, luciferase driven by the promoter with the *OSE*2 sequence increased 1.7-fold (Supporting Fig. S5*A*), indicating that Runx activities were deregulated in the *Cbfβ*-deficient cells. Thus, *Cbfβ*$^{f/f}$*Prx1*-Cre mice at birth displayed a similar, but less severe, phenotype to *Runx2*$^{-/-}$ mice[3] (eg, reduced ossification and inhibited chondrocyte hypertrophy). Consistently, expression of Runx2 targeted genes, *ColX* (Fig. 3*E*) and *Ocn* (Fig. 6*D*, *E*), were also downregulated in *Cbfβ*-deficient mice. Although *Cbfβ* has been reported to protect the Cbfα subunits from degradation,[7] only Runx1 expression was reduced in *Cbfβ*$^{f/f}$*Prx1*-Cre calvarial cells compared to WT (Fig. 6*I*), and Runx2 and Runx3 were similarly expressed in WT and *Cbfβ*$^{f/f}$*Prx1*-Cre calvarial cells and long bones (Figs. 6*G*, *I*, 5*E*; Supporting Fig. S4). In fact, Runx2 expression was higher in *Cbfβ*$^{f/f}$*Prx1*-Cre perichondrium compared to WT (Fig. 5*E*). Expression of *Runx2-I*, a specific *Runx2* isoform expressed in the perichondrium and proliferating chondrocytes,[19] was also increased in *Cbfβ*$^{f/f}$*Prx1*-Cre calvaria (Fig. 6*H*). Thus, the protection mechanism of Runx proteins may vary depending on the cell type and the differentiation stage. In addition, the high expression of Runx2 in the mutant mice, where it would not normally have such high expression, may indicate the immaturity of the cells.

Although the Runx2$^{-/-}$ skeletal system showed a complete lack of ossification,[3] *Cbfβ*-deficient mice only have delayed ossification. The delayed mineralization observed in the ribs and spines in some mice at birth became less severe as the mice aged. This indicates that Runx2 remains partially active without Cbfβ. The in vitro promoter assay showed that the two isoforms of *Runx2* (ie, *Runx2-I* and *Runx2-II*) retained some transcriptional activity in the absence of Cbfβ.[13] qRT-PCR showed that *Runx2-I* expression was increased in *Cbfβ*-deficient calvaria (Fig. 6*H*). The partially rescued phenotype of *Cbfβ*-deficient mice may be a combined action of the remaining activity of Runx2-I and Runx2-II.

Cbfβ may form a complex with Runx2 or Runx3 to regulate Ihh expression, and thereby regulate chondrocyte proliferation and maturation. Recent studies have shown that the Cbfβ/Runx1 complex plays an important role in chondrogenesis and chondrocyte proliferation.[20] Soung and colleagues[21] reported that Runx1 is required for endochondral ossification during skeletal development. Moreover, Runx1 and Cbfβ both have much higher expression levels in MSCs and chondrocytes than Runx2 and Runx3 do.[21] In addition, Runx2 is usually considered as a positive regulator in chondrocyte maturation rather than in chondrocyte proliferation.[22,23] All these studies indicate that the Runx1/Cbfβ complex may exert a more important role than Runx2/Cbfβ and Runx3/Cbfβ in chondrocyte proliferation. To our knowledge, our study is the first report of Cbfβ regulating chondrocyte proliferation. Additional studies are needed to characterize the mechanism underlying the roles of Runx/Cbfβ complexes in regulating the chondrocyte proliferation in vivo.

In summary, we investigated the role of Cbfβ in postnatal cartilage and bone development. We found that Cbfβ is a key factor for chondrocyte proliferation, chondrocyte differentiation, and the maintenance of growth plates and trabecular bone in postnatal mice. Cbfβ upregulated Ihh and downregulated PPR in postnatal growth plates and thereby controlled the proliferation of chondrocytes to prehypertrophic chondrocytes. Our results also indicate that Cbfβ may interact with Runx1 to regulate chondrocyte proliferation.

Disclosures

All authors state that they have no conflicts of interest.

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