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Chondrocyte-specific ablation of Osterix leads to impaired endochondral ossification

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Abstract

Osterix (Osx) is an essential transcription factor required for osteoblast differentiation during both intramembranous and endochondral ossification. Endochondral ossification, a process in which bone formation initiates from a cartilage intermediate, is crucial for skeletal development and growth. Osx is expressed in differentiating chondrocytes as well as osteoblasts during mouse development, but its role in chondrocytes has not been well studied. Here, the *in vivo* function of Osx in chondrocytes was examined in a chondrocyte-specific Osx conditional knockout model using Col2a1-Cre. Chondrocyte-specific Osx deficiency resulted in a weak and bent skeleton which was evident in newborn by radiographic analysis and skeletal preparation. To further understand the skeletal deformity of the chondrocyte-specific Osx conditional knockout, histological analysis was performed on developing long bones during embryogenesis. Hypertrophic chondrocytes were expanded, the formation of bone trabeculae and marrow cavities was remarkably delayed, and subsequent skeletal growth was reduced. The expression of several chondrocyte differentiation markers was reduced, indicating the impairment of chondrocyte differentiation and endochondral ossification in the chondrocyte-specific Osx conditional knockout. Taken together, Osx regulates chondrocyte differentiation and bone growth in growth plate chondrocytes, suggesting an autonomous function of Osx in chondrocytes during endochondral ossification.

Keywords

Osterix; Col2a1-Cre; Chondrocytes; Endochondral ossification

1. Introduction

Bone formation through endochondral ossification is essential for the longitudinal growth of long bones as well as for bone development and repair. Endochondral ossification is a process in which bone formation begins from a cartilage template and then replaced by the bone [1,2]. In this process, both chondrocytes and osteoblasts are involved in bone growth and development. Specifically, longitudinal bone growth is mediated by the continuous cell division and action of chondrocytes [3]. Chondrogenesis, the process that precedes endochondral ossification, initiates through the condensation of mesenchymal cells. These cells differentiate into proliferating chondrocytes and then transition into prehypertrophic chondrocytes before maturing into hypertrophic chondrocytes [3–5]. Upon the invasion of blood vessels, the terminally differentiated hypertrophic cells undergo apoptosis, and invading osteoclasts and osteoblasts form bone.

The transcription factor Osterix (Osx), which acts downstream of Runx2, has an essential role in osteoblast differentiation during both intramembranous and endochondral ossification [6,7]. Many studies have aimed to define the function of Osx in osteoblast differentiation and bone formation. Null mutations of Osx result in a complete absence of bone formation, whereas osteoblast-specific deletion of Osx using Col1a1-Cre led to osteopenia due to reduced osteoblast differentiation in adult mice [6,8]. Likewise, conditional ablation of Osx in osteoblasts via tamoxifen-induced Cre activity revealed the importance of Osx in postnatal bone formation and maintenance [9,10]. During mouse skeletal development, Osx expression is observed in perichondrium, prehypertrophic and hypertrophic chondrocytes as well as osteoblasts [6,10]. An in vitro study that investigated the effects of Osx gene silencing in ATDC5 chondroprogenitor cells suggested that Osx is involved in chondrogenic gene activation and may positively regulate the chondrocyte differentiation [11]. Despite this interesting report, it has been still unclear whether Osx plays a role in chondrocytes during endochondral ossification. Here, we evaluated a chondrocyte-specific Osx knockout to determine whether this gene is important for chondrocyte differentiation during endochondral ossification. Conditional ablation of Osx in chondrocytes was induced by Col2a1-Cre, which is a line that expresses an active Cre in all differentiating chondrocytes under the control of the type II collagen promoter (Col2a1) [12]. Osx deficiency in chondrocytes (Osx^{flox/-};Col2a1-Cre) resulted in impaired endochondral bone formation with delayed chondrocyte differentiation, reduced formation of bone trabeculae and marrow cavities, and reduced skeletal growth. The delay in chondrocyte differentiation was confirmed by the reduced expression of chondrocyte differentiation markers in Osx^{flox/-};Col2a1-Cre. These results demonstrate that Osx may be involved in regulating chondrocyte differentiation as well as osteoblast differentiation during endochondral ossification. Our data provide a novel insight into the role of Osx in chondrogenesis and bone growth.

2. Materials and methods

2.1. Generation of chondrocyte-specific Osx knockout

To delete Osx specifically in chondrocytes, Osx^{flox/-};Col2a1-Cre were generated by crossing Osx floxed mice (Osx^{flox/flox}) [13], Osx heterozygous mice (Osx^{+/-}) [6], and

Col2a1-Cre transgenic mice [12]. $Osx^{+/-}$;Col2a1-Cre male mice were crossed with $Osx^{flox/flox}$ female mice. Embryos from pregnant $Osx^{flox/flox}$ females were analyzed. PCR genotyping of embryos was performed using genomic DNA from the yolk sac or tail. The primer sets and reactions for PCR genotyping have been described previously [8,9]. All procedures concerning animal experiments were conducted with the approval of Kyungpook National University.

2.2. Radiographic imaging and skeletal preparation

For radiographic analysis, newborn pups (P0) were imaged at 10–50 kV for 30 s using a cabinet X-ray system, XPERT 80™ (Kubtec, Fairfield, CT). For skeletal preparation, embryos were stained with alcian blue and alizarin red to visualize skeletons as described previously [8,9].

2.3. Histological analysis

Paraffin-embedded embryo blocks were sectioned at 5 μ m and stained with hematoxylin and eosin (H&E), alcian blue, and von Kossa. To detect osteoclasts, the sections were subjected to TRAP staining and counterstained with methyl green. To assay in vivo cell proliferation, BrdU was intraperitoneally injected into pregnant females at 100 μ g per gram of body weight 2 h before sacrifice. BrdU incorporation was detected using a BrdU staining kit (Zymed) following the manufacturer's procedure. Statistical differences were assessed by the *t*-test.

2.4. Immunohistochemistry

For immunostaining, deparaffinized sections were incubated in 3% H_2O_2 for 10 min at room temperature to quench endogenous peroxidase activity. After incubating in a serum blocking solution to minimize nonspecific staining, the sections were exposed to an *Osx* antibody (ab22552, Abcam) diluted 1:500 or a platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody (sc-1506, Santa Cruz) diluted 1:500 in a blocking solution overnight at 4 °C. The next day, the slides were incubated with a biotinylated secondary antibody (VectaStain ABC Kit, Vector Laboratories) at room temperature for 10 min and then treated with the ABC reagents (Vector Laboratories). To visualize the signal, the sections were incubated with DAB substrate (Invitrogen) and counterstained with 0.25% methyl green.

2.5. Quantitative real-time PCR

Total RNA was prepared from the rib cages and limbs of embryos. Total RNA was extracted using the TRI reagent (Sigma–Aldrich) and subjected to quantitative real-time PCR as described previously [8,14]. Primer sequences used were as follows: *Osx*, 5'-CGTCCTCTCTGCTTGAGGAA-3' and 5'-CTTGAGAAGGGAGCTGGGTA-3'; *Cre*, 5'-CGAACGCACTGATTTTCGAC-3' and 5'-GGCAACACCATTTTTTCTGAC-3'; *ColIII*, 5'-CACACTGGTAAGTGGGGCAAGACCG-3' and 5'-GGATTGTGTTGTTTCAGGGTTCGGG-3'; *ColIX*, 5'-CCTGGGTTAGATGGAAAA-3' and 5'-AATCTCATAAATGGGATGGG-3'; *Runx2*, 5'-AAATGCCTCCGCTGTTATGAA-3' and 5'-GCTCCGGCCCAAAATCT-3'; *Ihh*, 5'-GACTCATTGCCTCCCAAGAACTG-3' and 5'-CCAGGTAGTAGGGT CACATTGC-3';

PTHrP, 5'-GAACATCAGCTACTGCATGACAAG-3' and 5'-TCTGATTTCGGCTGTGTGGATC-3'; Sox9, 5'-ACGGCTCCAGCAAGAACAAG-3' and 5'-TTGTGCAGATGCGGGTACTG-3'; TRAP, 5'-TCCTCGGAGAAAATGCATCAT-3' and 5'-GCAGTTAAGCTCCTGGACCAA-3'; PECAM-1, 5'-TGCGATGGTGTATAACGTCA-3' and 5'-GCTTGG CAGCGAAACACTAA-3'; and β -actin, 5'-TCACCCACACTGTGCCCATCTACGA-3' and 5'-GGATGCCACAGGATTCCATACCCA-3'. Real-time PCR amplification was carried out using SYBR green master mix (Roche Applied Science) in a LightCycler 480 (Roche Applied Science) as follows: initial denaturation at 95 °C for 5 min; 45 cycles of amplification with denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s; 1 cycle of melting curves at 95 °C for 5 s, 65 °C for 1 min, and 97 °C continuous; and a final cooling step at 40 °C for 30 s. All samples were run in duplicate. The quantitative real-time PCR results were analyzed using the comparative cycle threshold (C_T) method as previously described.

2.6. Reverse transcription (RT)-PCR

Total RNA extracted from the cartilaginous rib cage was used for RT-PCR. Reverse transcription was performed with M-MLV reverse transcriptase (Promega) using 2 μ g of total RNA for 50 min at 42 °C, followed by 5 min at 95 °C. The newly synthesized cDNA was used to amplify Cre under the following conditions: initial denaturation at 94 °C for 2 min; 30 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Primer sequences for Cre were 5'-CGACCAAGTGACAGCAATGCTGTTTCA-3' and 5'-CACCAGCTTGCATGATCTCCGGT ATT-3'.

2.7. In situ hybridization

Deparaffinized sections were rehydrated with ethanol and post-fixed in 4% PFA. These sections were prehybridized with 50% formamide in 2 $\times$ saline sodium citrate buffer at 60 °C for 30 min. Digoxigenin (DIG)-labeled RNA probes, synthesized according to the manufacturer's instructions, were pre-warmed to 85 °C and hybridized to sections overnight at 60–75 °C. Color development was performed with a NBT/BCIP color development substrate solution (Promega Laboratories) at room temperature. Mouse type II collagen (ColII) and type X collagen (ColX) cDNAs were used as templates for the synthesis of DIG-labeled RNA probes. All steps for in situ hybridization were performed in RNase-free conditions.

3. Results

3.1. Chondrocyte-specific deletion of *Osx* results in skeletogenic defects

To verify the role of *Osx* in chondrogenesis, conditional floxed *Osx* (*Osx*^{lox/lox}) mice [13] and *Osx* heterozygous (*Osx*^{+/-}) mice [6] were crossed to Col2a1-Cre transgenic mice in which Cre is expressed in chondrocytes under the control of Col2a1 promoter [12]. These crosses generated chondrocyte-specific *Osx* knockout, *Osx*^{lox/-};Col2a1-Cre, which contain one conditional *Osx* allele and one *Osx* null allele as well as Col2a1-Cre transgene (Fig.

1A). In the same littermate, wildtype ($\text{Osx}^{\text{flox/+}}$), Osx heterozygote ($\text{Osx}^{\text{flox/-}}$), and chondrocyte-specific Osx heterozygote ($\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$) were also generated (Fig. 1A). $\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$ having the same phenotype as $\text{Osx}^{\text{flox/+}}$ and $\text{Osx}^{\text{flox/-}}$ littermate were used as a control of $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ in this study (data not shown). Even though $\text{Osx}^{\text{+/+}};\text{Col2a1-Cre}$ were not generated from the same littermate of this study, a previous study reported no specific phenotypic alteration of Col2a1-Cre [12]. $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$, which were generated at the expected Mendelian ratio, died within 30 min after birth. They had difficulty in breathing and protecting internal organs due to weak skeletons. Perinatal lethality of $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ was similar to that observed in Osx homozygous null mutants [6]. Finally, $\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$ and $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ were analyzed at embryonic stages.

To investigate the skeletal phenotype of the mutants, radiographic analysis and skeletal preparations were performed. At postnatal day 0 (P0), radiography revealed that skeletal elements were very weak, thin, and bent in $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ mutants compared to $\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$ controls (Fig. 1B). In addition, severe bending was observed in the ribs and long bones of $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ stained with alcian blue and alizarin red (Fig. 1C–F). The mineralized bones, stained with alizarin red, were remarkably shorter than the controls. At E15.5, mineralization of primary ossification centers was observed in the long bones of control embryos. However, mineralization was not apparent in $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ embryos (Fig. 1G and H), indicating that mutants lacking the Osx gene in chondrocytes fail to undergo skeletogenesis to the same degree as control littermates.

3.2. Impaired endochondral bone formation caused by Osx deficiency in chondrocytes

To determine whether Osx regulates chondrogenesis *in vivo*, the histological architecture of cartilage and bone tissue was analyzed in the humerus of $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ during embryonic development (Fig. 2A and B). In control $\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$ embryos at E16.5, chondrocytic cells in the growth plate were well-organized, and the primary ossification center had formed within the diaphysis of the humerus. However, in $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$, hypertrophic chondrocytes were still abundant and expanded in the middle of the humerus, and the bone trabeculae and marrow cavity had not yet formed. At E17.5, even though the humerus had grown, the length of this bone in $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ mutants was still shorter than that of $\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$ controls. In $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$, the length of the growth plate, including prehypertrophic and hypertrophic chondrocytes, was increased relative to the total length, and calcium deposition into hypertrophic zones and bones was reduced. Although bone trabeculae and the bone marrow cavity were gradually forming in the proximal region of the humerus, delayed bone formation was still observed in the distal region of the humerus in $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$. At E18.5, defective chondrocyte differentiation and bone formation were observed in $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$.

Chondrocytic cell proliferation was examined in both $\text{Osx}^{\text{flox/+}};\text{Col2a1-Cre}$ controls and $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ mutants by BrdU incorporation. At E17.5, the number of BrdU-positive cells was decreased in the proliferative zone of $\text{Osx}^{\text{flox/-}};\text{Col2a1-Cre}$ embryos relative to the controls (Fig. 2C and D). However, no significant difference was statistically

examined in $Osx^{flox/-};Col2a1-Cre$ compared with $Osx^{flox/+};Col2a1-Cre$ at E17.5 as well as E18.5.

3.3. Defects in chondrocyte differentiation in $Osx^{flox/-};Col2a1-Cre$

To prove the deficiency of Osx in $Osx^{flox/-};Col2a1-Cre$, Osx expression was examined in bone tissues by immunostaining. As shown in previous reports [6,10], Osx expression was strong in osteoblasts and prehypertrophic chondrocytes, and weak in hypertrophic chondrocytes of $Osx^{flox/+};Col2a1-Cre$ controls at E16.5 and E17.5 (Fig. 3A). Expression of Osx was also observed in the perichondrium and periosteum as well as osteoblasts (data not shown). However, Osx expression was not detected only in the chondrocytes of $Osx^{flox/-};Col2a1-Cre$, whereas its expression was still observed in the perichondrium (Fig. 3A, data not shown), indicating that the $Col2a1-Cre$ had specifically deleted Osx in chondrocytes, but not in other cells. To confirm the reduced Osx expression in chondrocytes, quantitative real-time PCR analysis was performed in the cartilaginous rib cage of $Osx^{flox/-};Col2a1-Cre$ (Fig. 3B). Compared with that in $Osx^{flox/+}$ and $Osx^{flox/+};Col2a1-Cre$ controls, Osx expression was significantly reduced in $Osx^{flox/-};Col2a1-Cre$ mutants. The expression level of Cre mRNA was identical between $Osx^{flox/+};Col2a1-Cre$ and $Osx^{flox/-};Col2a1-Cre$ by quantitative real-time PCR as well as RT-PCR (Fig. 3B).

To further assess the impairment of endochondral bone formation in $Osx^{flox/-};Col2a1-Cre$ mutants, the expression of chondrocyte differentiation markers was examined in bone tissues by quantitative real-time PCR (Fig. 3C). The expression of $ColII$ and $ColX$, which mark proliferative chondrocytes and terminally differentiated prehypertrophic and hypertrophic chondrocytes, respectively, was reduced in $Osx^{flox/-};Col2a1-Cre$ embryos, indicating the impaired differentiation of Osx -deficient chondrocytes. The pattern of $ColIII$ expression was not altered, but in situ hybridization revealed that its density was decreased in the proliferating chondrocyte zone of $Osx^{flox/-};Col2a1-Cre$, due to the reduced numbers of total and proliferating cells (Fig. 3D). While mutants showed an increased number of hypertrophic chondrocytes, the level of $ColX$ expression as assessed by in situ hybridization was remarkably reduced in the expanded hypertrophic zones, suggesting a delay in the onset of hypertrophic chondrocyte differentiation and maturation (Fig. 3D). The expression of Indian hedgehog (Ihh), a key molecule in endochondral ossification [15], was decreased in $Osx^{flox/-};Col2a1-Cre$ (Fig. 3C). Consistently, PTHrP, a downstream target of the Hh signaling pathway, also showed reduced expression in mutant embryos (Fig. 3C). Expression levels of the transcription factors $Sox9$ and $Runx2$, which are essential for chondrogenesis [16], were also low in $Osx^{flox/-};Col2a1-Cre$ (Fig. 3C).

3.4. Alteration of osteoclast distribution in the humerus during embryonic development

During endochondral ossification, vascular invasion is required for osteoclasts to migrate, differentiate, and subsequently remove mineralized cartilage matrix and replace it with bone [17,18]. The presence of osteoclasts visualized by TRAP staining therefore indicates previous vascular invasion (Fig. 4A). Multinucleated TRAP-positive osteoclasts were only observed in the perichondrium of $Osx^{flox/-};Col2a1-Cre$ at E16.5. At E18.5, they were restricted to the proximal region where the bone trabeculae and marrow cavity had formed.

The number of TRAP-positive osteoclasts was statistically reduced in $Osx^{flox/-};Col2a1-Cre$ compared to $Osx^{flox/+};Col2a1-Cre$ (Fig. 4B). TRAP mRNA levels were also decreased in $Osx^{flox/-};Col2a1-Cre$ (Fig. 4C). PECAM-1, a specific marker of vascularization was observed to verify the reduced osteoclast invasion in $Osx^{flox/-};Col2a1-Cre$ (Fig. 4D). PECAM-1 was widely expressed in all periosteum of $Osx^{flox/+};Col2a1-Cre$ controls at E16.5 and E18.5. In $Osx^{flox/-};Col2a1-Cre$, however, PECAM-1 was detected only in perichondrium at E16.5, demonstrating a delay of vascular invasion. At E18.5, even though PECAM mRNA levels did not change (Fig. 4C), PECAM was restricted to the proximal region which fewer osteoclasts was observed in, leaving non-resorbed hypertrophic cartilage in the diaphysis of $Osx^{flox/-};Col2a1-Cre$ with little space for bone marrow cavity. These results suggest that vascular invasion, which is necessary for and precedes marrow cavity formation, was delayed in $Osx^{flox/-};Col2a1-Cre$ mutants.

4. Discussion

Osx is a bone transcription factor which is essential for osteoblast differentiation during bone formation [7]. *Osx* is specifically expressed in chondrocytes, osteoblasts, and osteocytes. To study the role of *Osx* in chondrocytes, we generated $Osx^{flox/-};Col2a1-Cre$ mutants. $Osx^{flox/+};Col2a1-Cre$ having the same phenotype as wildtype ($Osx^{flox/+}$) littermate was used as a control of $Osx^{flox/-};Col2a1-Cre$ mutants. The normal phenotype of *Osx* heterozygotes has already been reported in several in vivo studies using *Osx* mouse models [6]. In $Osx^{flox/-};Col2a1-Cre$, Cre recombinase was expressed under the control of the *Col2a1* promoter in epiphyseal chondrocytes, but not in osteoblasts or perichondrial fibroblasts [12]. *Osx* was expressed in the perichondrium and periosteum as well as chondrocytes and osteoblasts in the normal bone. However, *Osx* expression was still restricted to the perichondrium in $Osx^{flox/-};Col2a1-Cre$, indicating that the *Osx* gene was specifically excised in chondrocytes during endochondral ossification.

Omoeteyama and Takagi first reported a role for *Osx* in chondrocytes [11]. They investigated the in vitro effects of *Osx* gene silencing in the chondrogenic cell line ATDC5. In the absence of *Osx*, chondrogenic genes were down-regulated, suggesting that *Osx* is involved in chondrogenic gene activation and chondrocyte differentiation. However, it is still insufficient to explain the function of *Osx* during chondrocyte differentiation. In our study, in vivo patterns of endochondral bone formation were impaired, and the expression of chondrogenic marker genes was clearly reduced in chondrocyte-specific *Osx* knockouts. Although the hypertrophic zone was expanded in $Osx^{flox/-};Col2a1-Cre$, a reduction in *ColX* expression was observed by in situ hybridization and quantitative real-time PCR. These results indicate that loss of *Osx* in chondrocytes causes impaired chondrocyte differentiation and maturation, followed by the reduced matrix production. Therefore, these in vivo results clearly demonstrate that *Osx* is a positive regulator of chondrocyte differentiation.

Chondrocyte proliferation and differentiation are regulated by a coordinated balance of positive and negative signals from various transcription factors [19]. One master paracrine regulator of endochondral ossification is *Ihh* [4,5]. *Ihh* produced by prehypertrophic chondrocytes promotes chondrocyte proliferation. In particular, in growth plate, *Ihh* controls chondrocyte differentiation via a negative-feedback mechanism involving the reciprocal

action of PTHrP. Recently, several transcription factors important for osteoblast differentiation have been shown to activate *Ihh* transcription in chondrocytes. *Runx2* and *ATF4* directly bind to the *Ihh* promoter and activate its transcription [20,21]. Likewise, *Msx2*, a regulator of skeletal development, promotes chondrocyte maturation by activating *Ihh* transcription [22]. The in vitro study of *Osx* gene silencing in chondrocytes did not discuss whether *Ihh* expression was affected by the loss of *Osx* [11]. In the current study, *Ihh* and PTHrP were significantly down-regulated in *Osx*^{fllox/-};Col2a1-Cre. This result suggests that *Osx*, like other bone transcription factors, may control *Ihh* transcription. However, *Osx* did not activate transcription driven by *Ihh* promoter in ATDC5 chondrocytic cells (unpublished data), indicating that *Osx* did not regulate a transcriptional activity of *Ihh* gene and may control another or novel downstream target genes, not *Ihh*, during chondrocyte differentiation. Further studies will be required to determine downstream target genes of *Osx* in *Osx*-mediated chondrocyte differentiation.

Osteoblast-specific inactivation of *Osx* did not affect osteoclast differentiation or cause functional defects in bone resorption [8,9]. TRAP-positive multinucleated osteoclasts were likewise observed in chondrocyte-specific *Osx* mutants. However, these cells were restricted to the perichondrium at E16.5 and the proximal region near the bone trabeculae and marrow cavity at E18.5. During endochondral ossification, vascular invasion is required for osteoclast migration and marrow cavity formation [17,18]. The altered distribution of osteoclasts could therefore be explained by a delay of vascular invasion which was demonstrated by the reduced PECAM-1 signals in *Osx*^{fllox/-};Col2a1-Cre. We therefore attribute the disrupted bone formation of chondrocyte-specific *Osx* knockouts to reduced hypertrophic chondrocyte maturation and vascular invasion, not to impaired osteoclast differentiation. Taken together, this study demonstrates that *Osx* regulates chondrocyte differentiation during endochondral ossification and thus has critical functions in chondrocytes as well as osteoblasts.

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Fig. 1.

Skeletal phenotypes of *Osx*^{flox/-}; *Col2a1-Cre* mutants. (A) Breeding scheme to generate chondrocyte-specific *Osx* knockout, *Osx*^{flox/-}; *Col2a1-Cre*. *Col2a1-Cre* was used to excise the *Osx* gene in chondrocytes. (B) X-ray images of newborn embryos. (C) Gross appearance and skeletal preparation at P0. (D) Higher magnification of the rib cage, forelimb, and hindlimb.

Osx^{flox/-}; *Col2a1-Cre* showed endochondral bone defects compared to *Osx*^{flox/+}; *Col2a1-Cre* controls at P0. Higher magnifications of the humerus (E and G) and femur (F and H) in *Osx*^{flox/+}; *Col2a1-Cre* (upper) and *Osx*^{flox/-}; *Col2a1-Cre* (lower) at P0 (E and F) and E15.5 (G and H). In *Osx*^{flox/-}; *Col2a1-Cre*, no mineralization was not observed in the long bones at E15.5, and severe bendings were observed in the ribs and long bones at P0.

Fig. 2.

Histological examination of the humerus during endochondral bone formation. (A) Histological alterations in the humerus were examined by H&E, alcian blue, and von Kossa staining in serial sections from *Osx^{fllox/+};Col2a1-Cre* (*Osx con*) and *Osx^{fllox/-};Col2a1-Cre* (*Osx cKO*) embryos at E16.5, E17.5, and E18.5. Scale bar = 200 μm. (B) Quantitative analysis of the length of different chondrocyte zones. The percentages indicated for resting, proliferative, and pre- and hypertrophic zones were quantified over the total length of the growth plates. (C) Cell proliferation as measured by BrdU incorporation in the humerus at E17.5 and E18.5. Scale bar = 50 μm. (D) Quantification of the number of BrdU-positive cells per the same area of the proliferative zone of *Osx^{fllox/+};Col2a1-Cre* (black bar) and *Osx^{fllox/-};Col2a1-Cre* (gray bar) embryos at E17.5 and E18.5. There was no statistical significance for cell proliferation at E17.5 and E18.5.

Fig. 3.

Downregulation of Osx attenuates chondrogenic differentiation. (A) Immunohistochemical analysis of Osx expression in Osx^{flox/-};Col2a1-Cre mutants. Osx was expressed in the prehypertrophic and hypertrophic chondrocytes in Osx^{flox/+};Col2a1-Cre at E16.5 and E17.5. However, Osx expression was not detected in chondrocytes of Osx^{flox/-};Col2a1-Cre. Scale bar = 100 μ m. (B) Osx and Cre mRNA expression in chondrocytes by quantitative real-time PCR analysis. The expression of Osx was significantly decreased in Osx^{flox/-};Col2a1-Cre mutants. However, The expression level of Cre mRNA was identical between Osx^{flox/+};Col2a1-Cre and Osx^{flox/-};Col2a1-Cre. Comparable level of Cre expression was confirmed by RT-PCR. * $p < 0.05$. (C) Expression of chondrocyte differentiation markers by quantitative real-time PCR in the rib cage at E17.5. Relative expression levels of markers were reduced in Osx^{flox/-};Col2a1-Cre mutants (gray bar) compared to Osx^{flox/+};Col2a1-Cre controls (black bar). * $p < 0.05$. (D) Reduced ColII and ColX expression in the humerus at E17.5 as revealed by in situ hybridization. Scale bar = 200 μ m.

Fig. 4.

Reduced vascular invasion in $Osx^{flox/-};Col2a1-Cre$ mutants. (A) Altered distribution of osteoclasts in $Osx^{flox/-};Col2a1-Cre$. In $Osx^{flox/-};Col2a1-Cre$ mutants, TRAP-positive cells (red) were restricted to the perichondrium at E16.5 and detected in the bone matrix region at E18.5. Although multinucleated osteoclasts were clearly present, the distribution of osteoclasts was not identical in $Osx^{flox/-};Col2a1-Cre$ compared to $Osx^{flox/+};Col2a1-Cre$. Scale bar = 200 μ m. The lower panel is a higher magnification of the red box in the upper panel. Scale bar = 50 μ m. (B) Quantification of TRAP-positive osteoclasts. The number of TRAP-positive osteoclasts was statistically reduced in $Osx^{flox/-};Col2a1-Cre$ compared to $Osx^{flox/+};Col2a1-Cre$. * $p < 0.05$. (C) TRAP and PECAM mRNA expression in limbs by quantitative real-time PCR analysis. Real-time PCR analysis revealed that the expression of TRAP was significantly reduced in $Osx^{flox/-};Col2a1-Cre$, but PECAM mRNA was not altered. (D) Immunohistochemical analysis of PECAM-1 expression in $Osx^{flox/-};Col2a1-Cre$ mutants. Blood vessels stained by PECAM-1, a specific marker of vascularization by endothelial cells, were restricted only in perichondrium at E16.5 and to the proximal region with TRAP-positive osteoclasts at E18.5. Scale bar = 200 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)