# Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice

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**The transcription factor Osterix (Osx) is required for osteoblast differentiation and bone formation during embryonic development, but it is not known whether Osx has an essential function in postnatal bone growth and in bone homeostasis. Conditional deletion of *Osx* at several time points postnatally revealed that Osx was essential for osteoblast differentiation and new bone formation in growing and adult bones. Additionally, inactivation of *Osx* in bones severely disrupted the maturation, morphology, and function of osteocytes. These findings identify Osx as having an essential role in the cell-specific genetic program of osteocytes. Interestingly, *Osx* inactivation also led to the massive accumulation of unresorbed calcified cartilage in a large area below the growth plate of endochondral bones. This specific area was also marked by an unanticipated almost complete lack of bone marrow cells and a marked decrease in the density and size of osteoclasts. This diminished density of osteoclasts could contribute to the lack of resorption of mineralized cartilage. In addition, we speculate that the abnormally accumulated, mainly naked cartilage represents an unfavorable substrate for osteoclasts. Our study identifies Osx as an essential multifunctional player in postnatal bone growth and homeostasis.**

osteoblast differentiation | skeletal homeostasis | transcription factor | osteocyte | cartilage resorption

The identification of master transcription factors essential for osteoblast differentiation and bone formation during embryonic development has greatly advanced our knowledge of bone biology. However, the transcriptional control of postnatal bone formation and homeostasis remain poorly understood. Normal skeletal growth and homeostasis depends on the coordinated activities of three types of bone cells: osteoblasts, osteoclasts, and osteocytes. Factors secreted by the mesenchyme-derived osteoblasts also control the differentiation and activity of the bone-resorbing osteoclasts derived from hematopoietic stem cells. Conversely, bone resorption by osteoclasts releases factors important for bone formation. Osteocytes, which make up over 90–95% of all bone cells in adult animals, are derived from mature osteoblasts and are embedded inside the bone matrix. It has been suggested that osteocytes are mediators of mechanical and hormonal stimulations to control the activity of both osteoblasts and osteoclasts (1). Osteocytes are regulators of mineralization and mineral homeostasis (2, 3), and by controlling *Sost* expression also act as modulators of Wnt signaling (4).

Three transcription factors [β-catenin, Runx2, and Osterix (Osx)] are required for osteoblast differentiation and bone formation during embryonic development (5–9). Osx, which acts downstream of Runx2, is a zinc-finger-containing transcription factor essential for embryonic osteoblast differentiation and bone formation (8). During development, *Osx* is specifically expressed in osteoblast lineage cells and, at lower levels, in prehypertrophic chondrocytes, but not in osteoclasts. Osx-null mutant mice, which die at birth, develop a complete cartilaginous skeleton, but no bone formation takes place in either the endochondral or membranous skeleton. All skeletal elements of these Osx-null mice are characterized by the presence of *Runx2*-expressing precursor cells, which are arrested in their differentiation and unable to express osteoblast markers. Recent studies have shown that genetic variants in the region of *Osx* are associated with bone mineral density (BMD) in both children and adults, suggesting that Osx may continue to play an important role in the postnatal skeleton (10, 11). However, despite the crucial role of Osx in osteoblast differentiation and bone formation during development, an essential role for Osx in postnatal bone growth and homeostasis has not yet been demonstrated.

To investigate the functions of Osx in skeletal growth and homeostasis, we conditionally ablated Osx postnatally through tamoxifen activation of the CreER recombinase (12). Our findings suggest that Osx acts as an essential and central factor of bone homeostasis after birth, because it is required not only for new bone formation, osteocyte maturation, and function, but also for cartilage resorption.

## Results

**Postnatal Osx Inactivation Leads to Severely Altered Bone Structures.** In the floxed *Osx* allele, a cassette containing *IRES-EGFP* preceded by a *LOXP* site was inserted 3′ to the poly-A site, whereas the other *LOXP* site was in the first intron of the *Osx* gene. In mice harboring this allele, *EGFP* expression occurs only in *Osx*-expressing cells when the *Lox*P sites recombine (13). Immunohistochemical (IHC) analyses showed that there were abundant EGFP-positive cells on the surfaces of trabeculae and cortex in the humerus of 1-mo-old $Osx^{\Delta EX2/+}$ heterozygous mice (Fig. 1*A*). Prehypertrophic and hypertrophic chondrocytes were also positive for EGFP in these mice. This extends previous findings (8) indicating that *Osx* continues to be expressed in osteoblasts and hypertrophic chondrocytes postnatally. In addition, EGFP-positive cells were seen embedded inside the cortex and trabeculae, indicating that *Osx* is expressed in osteocytes as well (Fig. 1*A*). Overall expression of *Osx* is highly specific for osteoblasts, osteocytes, and (pre)hypertrophic chondrocytes.

To inactivate Osx postnatally, *CAG-CreER;* $Osx^{floxed/-}$ mice were injected with tamoxifen starting at several different time points after birth. IHC analyses with an anti-EGFP antibody showed that there were abundant EGFP-positive cells on the surface of the trabeculae and cortex, as well as embedded inside the bone matrix (Fig. 1*B*). Hypertrophic chondrocytes were also weakly positive for EGFP. This findings confirms that the floxed *Osx* allele was efficiently removed in *Osx*-expressing bone cells upon tamoxifen injections.

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DEVELOPMENTAL BIOLOGY

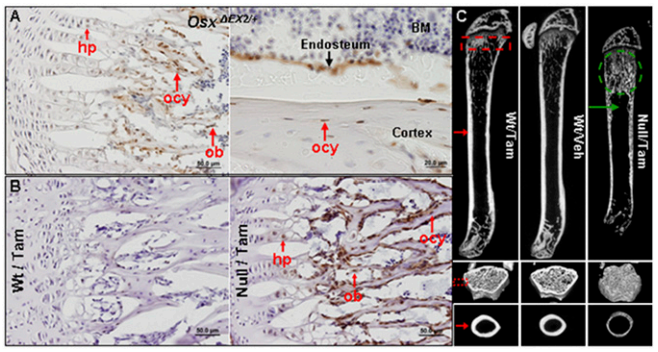


**Fig. 1.** *Osx* expression pattern and skeletal phenotypes of $Osx^{postnatal}$ mutant mice. (*A*) anti-eGFP IHC analysis on frozen humerus sections of 1-mo-old $Osx^{\Delta EX2/+}$ mouse. (*B*) anti-eGFP IHC analysis showed that Osx was effectively deleted in *Osx*-expressing cells in the humeri of 2-mo-old $Osx^{postnatal}$ mutant. hp, hypertrophic chondrocyte; ob, osteoblast; ocy, osteocyte. (*C*) μCT images of the femurs of 2-mo-old mice. Massive accumulation of mineralized tissue (green dotted circle) and complete absence of trabeculae (green arrow). For schedules of tamoxifen injections, see *Materials and Methods*. Wt/Tam, tamoxifen-treated wild-type mice; Wt/Veh, vehicle-treated wild-type mice; Null/Tam, tamoxifen-treated $Osx^{postnatal}$ mutant mice.

The $Osx^{postnatal}$ mutants grew at slower rates than the tamoxifen- or vehicle-treated wild-type controls (Fig. S1*A*). In these mice, radiography showed the presence of dense mineralized tissue under the growth plates of all long bones (Fig. S1*B*). Microcomputed tomography (μCT) images of the femurs of $Osx^{postnatal}$ mutants revealed a number of marked phenotypic changes. First, there was a zone of intensely mineralized tissue extending from right beneath the growth plate into the metaphysis. In contrast, there was a complete absence of trabeculae below this zone of hypermineralization, and a much thinner and porous cortical bone. There was also a complete absence of cortex around and beyond the primary spongiosa (Fig. 1*C*). These phenotypes were observed in the P24 $Osx^{postnatal}$ mutants 10 d after the first tamoxifen injection. The abnormal mineralized tissue progressively increased in size toward the diaphysis of femurs and also in lumbar vertebrae of $Osx^{postnatal}$ mutants from P24 to 3 mo. Also, when Osx was inactivated at 4 mo, and the mice were killed 3 mo later, the very thin cortical bone showed multiple microfractures (Fig. S2*A*). Evidently, postnatal Osx inactivation led to severely altered bone structures, suggesting that Osx continues to play a crucial role in the postnatal skeleton.

**Osx Is Required for Osteoblast Differentiation and Bone Formation During and After the Postnatal Growth Period.** To examine the role of Osx in osteoblast differentiation and bone formation during and after the postnatal growth period, the $CAG\text{-}CreER;Osx^{floxed/-}$ mice and wild-type controls were injected with tamoxifen or vehicle starting at weaning or at 4 mo, and then with calcein shortly before sacrifice at 1.5 mo or 7 mo, respectively. In the femurs and vertebrae of both tamoxifen- and vehicle-treated wild-type controls, bone formation was clearly indicated by the double calcein fluorescent lines lining the cortexes and trabeculae. In contrast, in the $Osx^{postnatal}$ mutants, there were almost no intact double or single lines on the surfaces of the trabeculae and cortex, except a few faint, short, single lines and dots (Fig. 2*A* and Fig. S2*B*). Histomorphometry of lumbar vertebrae of 1.5-mo-old mice (Table S1) provided further evidence that new bone formation in $Osx^{postnatal}$ mutants was dramatically reduced. The absence of new bone formation was also supported by the greatly decreased levels of *Col1a1* mRNA throughout long bones, as seen by in situ hybridization (Fig. 2*C*). Histological analyses further showed that there were very few morphologically mature osteoblasts and almost no osteoid on the endosteum in the $Osx^{postnatal}$ mutants (Fig. S2*C* and Fig. 2*B*). Along with drastically decreased *Osx* expression (~1% of the controls), qPCR measurements of osteoblast-specific marker RNAs, such as *Col1a1*, *Bsp*, and *Oc*, were all markedly reduced in the $Osx^{postnatal}$ mutants, despite elevated *Runx2* expression and sustained *Atf4* expression (Fig. 2*D*). Furthermore, primary osteoblast lineage cells isolated either from calvariae or from the bone marrow mesenchymal progenitor cells of the $Osx^{postnatal}$ mutants completely failed to form any mineralized nodules in vitro (Fig. 2*E*). No difference, however, was seen in the number of bone marrow-derived CFU fibroblasts (CFU-Fs) between tamoxifen-treated wild-type and $Osx^{postnatal}$ mutant mice (Fig. S2*D*), suggesting that progenitor cells were not affected. Together, these data provided solid evidence that Osx is required for osteoblast differentiation and bone formation far beyond birth and the initial growth period.

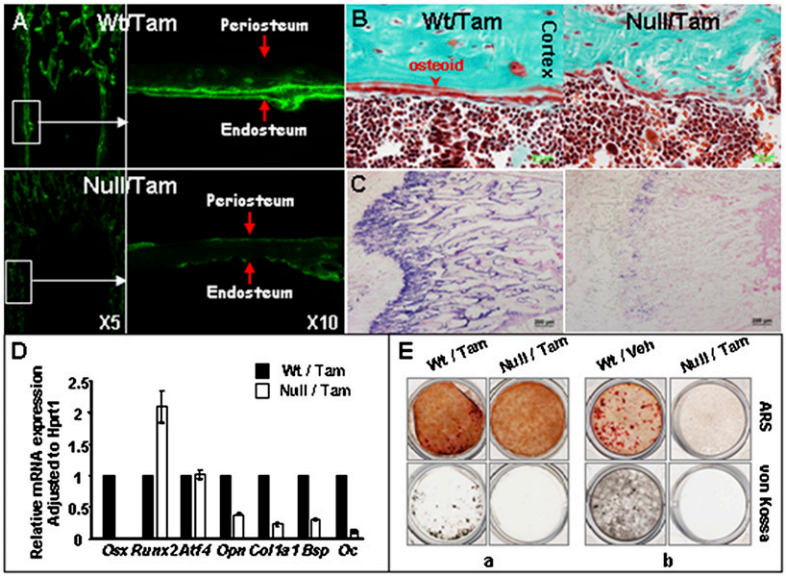


**Fig. 2.** Osx is required for osteoblast differentiation and bone formation during and after the postnatal growth period. (*A*) Calcein incorporation. The second of two calcein injections was performed 5 d after the first injection and 2 d before sacrifice. (*B*) Goldner staining. (*C*) *Col1a1* in situ hybridization on femur sections of 6-wk-old tamoxifen-treated control and $Osx^{postnatal}$ mutant. In *A* and *B*, plastic sections were prepared from 6-wk-old tamoxifen-treated control and $Osx^{postnatal}$ mutant. (*D*) Quantitative PCR analysis of osteoblast markers. RNA was isolated from the humeri of P24 tamoxifen-treated mice. (*E*) In vitro osteogenic assays. (*a*) Primary osteoblasts were isolated from the calvariae of P24 tamoxifen-treated $Osx^{postnatal}$ mutant and control mice. (*b*) BMSC was isolated from 2-mo-old tamoxifen-treated $Osx^{postnatal}$ mutant and vehicle-injected controls. The cells were cultured in osteogenic media for 14 d. ARS, alizarin red staining.

**Major Role of Osx in Both Osteocyte Maturation and Function.** We examined the morphology of the *Osx*-null osteocytes using acid-etched scanning electronic microscopy (SEM). As shown in Fig. 3*A*, the osteocytes in the $Osx^{postnatal}$ mutant were markedly deformed. There was a decreased number of osteocytes close to both periosteum and endosteum in the mutant. Moreover, these osteocytes were covered with very few dendrites. The number of dendrites in osteocytes found in the middle of the mutant cortex was also noticeably decreased, and the overall density of the dendrite network in the mutant cortex was much reduced. In addition, the expression levels of *Dmp1*, *Phex*, and *Sost*, which are highly expressed in normal osteocytes, were significantly reduced in the $Osx^{postnatal}$ mutants (Fig. 3*C*). *Fgf23* expression was elevated in bones of $Osx^{postnatal}$ mutants (Fig. S3*A*) but to a lesser extent than in *Dmp1*-null and *Hyp* mice (2, 3), Serum levels of both phosphorus and calcium were, however, unchanged (Fig. S3*A*). Figure 3*B* showed that in the $Osx^{postnatal}$ mutant there were very few mineral spherical particles in the process of being incorporated into the bone matrix, and the bone mineral density was lower than in the wild-type controls, indicating that the mineralization process in the $Osx^{postnatal}$ mutants was seriously compromised. Furthermore, transmission electron microscopy (TEM) images revealed that the collagen fibers surrounding osteocytes in the $Osx^{postnatal}$ mutant were disorganized, unlike in the wild-type controls (Fig. S3*B*).

Moreover, we found that Osx can activate the 2-kb *Sost* promoter (Fig. S3*C*) and specifically bind to a DNA fragment located within the promoter (Fig. 3*D*). Mutations in this binding site that prevented Osx binding inhibited activation of this promoter by Osx. A chromatin immunoprecipitation (ChIP) assay showed that Osx was able to interact with the same DNA fragment in the chromatin of intact cells (Fig. 3*D*), indicating that *Sost* is a direct target of Osx. Collectively, our findings suggest that Osx is needed for the maturation and function of osteocytes postnatally.

We noted that the number of BrdU-positive cells in the primary spongiosa of the $Osx^{postnatal}$ mutants was significantly higher than in the wild-type controls (Fig. S3*Da*). In contrast, the number of BrdU-positive cells in proliferating chondrocytes was unchanged in $Osx^{postnatal}$ mutants, implying that the function of these cells was unaffected. The increase in BrdU-positive cells was paralleled by an increase in Runx2-positive cells, which are likely to be preosteoblasts, in the primary spongiosa of the mutant (Fig. S3*Db*). It has been shown that SOST, which is an antagonist of Wnt signaling, was able to inhibit osteoblast proliferation in cell cultures (14). Given that *Osx* was highly expressed in osteocytes, and that loss of Osx led to decreased *Sost* expression, we speculated that the increased number of Runx2-positve preosteoblasts might be linked to the lower levels of *Sost* expression in osteocytes.

**Osx Inactivation Leads to Massive Accumulation of Calcified Cartilage.** Although postnatal *Osx* inactivation in osteoblast lineage cells caused the arrest of osteoblast differentiation and bone formation, we observed a large excess of mineralized tissue in the long bones of the $Osx^{postnatal}$ mutants. Both Safranin O and IHC analyses with antibodies against cartilage-specific matrix proteins (type X collagen and Aggrecan) revealed that the large accumulation of mineralized tissue in both the lumbar vertebrae (Fig. S4*A*) and the femurs (Fig. 4 *A* and *B* and Fig. S4*B*) of the $Osx^{postnatal}$ mutants was mainly calcified cartilage matrix.

By in situ hybridization we found no ectopic *Col2a1* and no *Col10a1* expression in the zone of excess cartilage of the

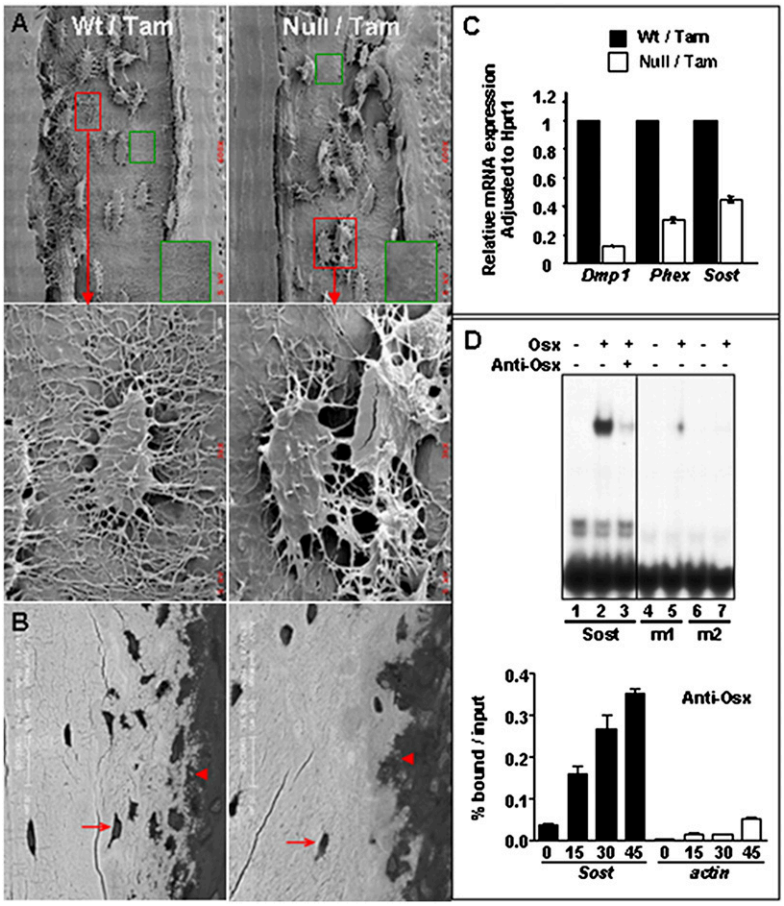


**Fig. 3.** Osx is required for osteocyte maturation and functions. (*A*) SEM images of the cortexes of humeri of 6-wk-old mice. (*B*) Back-scattered SEM images. Arrow, osteocytes; arrowhead, mineral spherical vaterites. (*C*) Quantitative PCR analysis of osteocyte markers. RNA was extracted from the humeri of tamoxifen-treated P24 mice. (*D*) *Sost* is a direct target of Osx. (*Upper*) EMSA using wild-type, m1, and m2 *Sost* oligos. Recombinant Osx was made in baculovirus (23). For oligo sequences, see Fig. S3*C*. (*Lower*) ChIP assay. Chromatin samples were prepared from BMP-2 treated MC3T3-E1 cells. Cells were harvested at 0, 15, 30, and 48 h after BMP-2 addition. The data are presented as percent of input after subtracting control IgG values.

*Osx*$^{postnatal}$ mutant (Fig. 4*C* and Fig. S4*C*). This finding ruled out the possibility that the abnormal accumulation of cartilage tissue was produced by *Osx*-null preosteoblasts.

*Mmp13* is expressed by hypertrophic chondrocytes and osteoblasts, in which *Osx* is also expressed. We reasoned that deletion of *Osx* in hypertrophic chondrocytes and osteoblasts may cause reduced *Mmp13* expression and consequently hinder cartilage ECM remodeling. Indeed, we found that *Mmp13* expression in the long bones and calvariae and of the *Osx*$^{postnatal}$ mutants was significantly decreased (Fig. S4*D*). However, IHC analysis with an antibody that specifically recognizes the MMP-cleaved Aggrecan neopeptide revealed that Aggrecan was cleaved in the *Osx*$^{postnatal}$ mutants despite decreased *Mmp13* expression, presumably by other MMPs, such as MMP9, whose expression was unaffected in the *Osx*$^{postnatal}$ mutants (Fig. S4*D*).

Taken together, our findings suggest that inactivation of Osx results in the abnormal accumulation of calcified cartilage matrix, which is not caused by ectopic production of cartilage or by defective cleavage of Aggrecan. Thus, accumulation of mineralized cartilage tissue is very likely due to defective resorption.

In theory, tamoxifen administration will activate *CAG-CreER* in all cell types, including hematopoietic stem cell-derived osteoclasts. RNA analysis of in vitro differentiated osteoclasts indicated that these cells did not express *Osx*, suggesting that deletion of *Osx* in the cells of this lineage will not affect their osteoclastogenic potential in a cell-autonomous manner. Indeed, the monocytes isolated from the bone marrow cells of *Osx*$^{postnatal}$ mutants were able to form tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts as efficiently as the monocytes from the wild-type controls (Fig. S5*A*).

The findings in Fig. 4*A* and Fig. S4*A* show that while calcified cartilage was accumulating in the *Osx*$^{postnatal}$ mutants, the preexisting bone trabeculae were diminishing, as indicated by the absence of trabeculae right below the zone of calcified cartilages in the femurs and the markedly reduced trabeculae volume in the center of the lumbar vertebrae. In addition, the cortical bones of *Osx*$^{postnatal}$ mutants were much thinner and porous. These results suggested that the resorption defect was more severe in the mineralized cartilage than in the bone matrix. Histomorphometry analysis revealed that the density of osteoclasts in the ectopically accumulated mineralized cartilage was reduced at least three times in the femurs of *Osx*$^{postnatal}$ mutants compared with the same area below the growth plate in control mice (Fig. 5*B*). Moreover, the average size of osteoclasts on the surface of this ectopic mineralized cartilage was reduced by about half compared with that of osteoclasts in control bones (Fig. 5*C* and Fig. S5*B*).

In the cortex, however, the distribution rather than the total number of osteoclasts was changed. There were many more osteoclasts inside the thinner and porous cortical bone, but much fewer in the periosteum of the *Osx*$^{postnatal}$ mutants than in control bones The total number of TRAP-positive osteoclasts in the cortical bone region was, however, similar in mutants and control mice (Fig. S5*C* and Table S2), although the size of the osteoclasts and the intensity of TRAP staining were reduced in the mutants.

We also noted that the ratio of *Opg*/*Rankl* expression in long bones was higher in the *Osx*$^{postnatal}$ mutants than in the wild-type controls (Fig. S5*D*). This could account for the overall decrease in the size of osteoclasts, and the reduced TRAP (*Acp5*) expression in the femur of *Osx*$^{postnatal}$ mutants (Fig. S5*E*).

Strikingly, in the area of excess mineralized cartilage in the femurs of *Osx*$^{postnatal}$ mutants, there was an almost complete absence of bone marrow cells in contrast to the presence of bone marrow cells in the same area under the growth plate of control femurs (Fig. 5*A* and Fig. S5*F*). There was also an increase in blood vessels stained by anti-collagen type IV in the area of mineralized cartilage in *Osx*$^{postnatal}$ mutants (Fig. S5*F*). However,

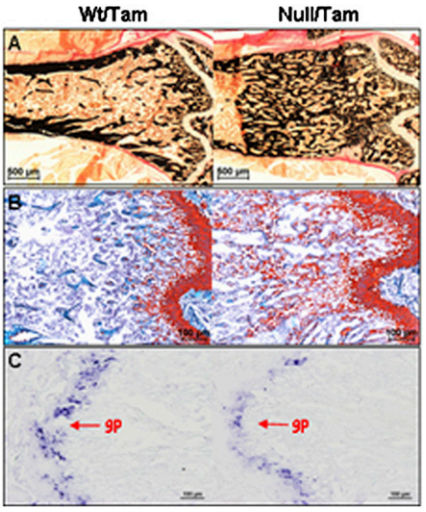


**Fig. 4.** Inactivation of Osx led to massive accumulation of calcified cartilage below the growth plate. (*A*) von Kossa. (*B*) Safranin O (SafO) staining. Plastic sections of femurs of tamoxifen-treated 2-mo-old mice were used for staining. (*C*) In situ hybridization of *Col2a1* RNA. Decalcified frozen sections were prepared from the humeri of tamoxifen-treated 6-wk-old mice. gp, growth plate.

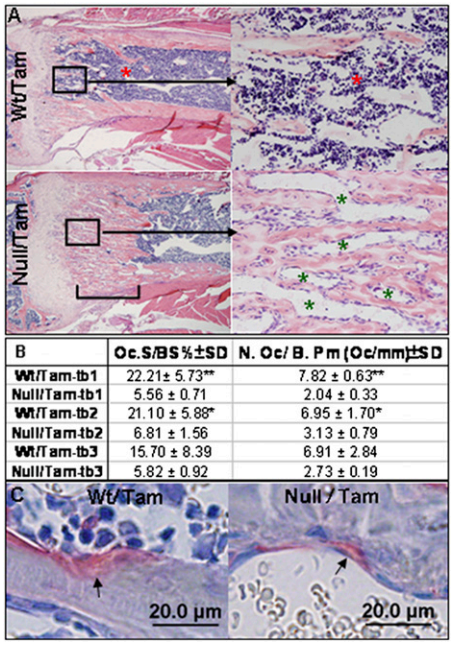


**Fig. 5.** Absence of bone marrow cells and reduced density of osteoclasts in the region of ectopic mineralized cartilage. (*A*) H&E staining showed that in the zone of mineralized cartilage (marked by the bracket) in the femur of 6-wk-old *Osx*$^{postnatal}$ mutant, there are almost no bone marrow cells (red asterisks). The spaces between the mineralized cartilages are filled by capillaries (green asterisks). Bone marrow cells are found below the zone of mineralized cartilage in the mutant. (*B*) Histomorphometry analysis of osteoclasts (Oc) in metaphysis (excluding cortex) of femurs of tamoxifen-treated 2-mo-old control and *Osx*$^{postnatal}$ mutants. BS represents mineralized tissue surface. Tb-1: the area measured from the growth plate to 500 μm distally; tb-2: the area measured from the end of tb-1 to 500 μm distally; tb-3: the area measured from the end of tb-2 to 700 μm distally. $n$ = 3/genotype group. $^{*}P < 0.05$, $^{**}P < 0.001$. (*C*) TRAP staining showed that the size of osteoclasts found in the *Osx*$^{postnatal}$ mutant was much smaller than the ones in the tamoxifen-treated control mice. Arrows indicate osteoclasts.

in the marrow cavity of the remainder diaphysis below the zone of excess cartilage, bone marrow cells were present in apparently normal amounts. Thus in the endochondral bones of $Osx^{postnatal}$ mutant mice, bone marrow cells were largely absent in the area where accumulation of unresorbed cartilage was found.

## Discussion

Our findings indicate that Osx has multiple essential functions in postnatal bone growth and homeostasis. First, several lines of evidence show that inactivation of *Osx* during and after the major postnatal growth period causes an arrest of osteoblast differentiation and of new bone formation. Our study provides clear evidence that a specific transcription factor essential for embryonic skeletal development is equally essential for osteoblast differentiation and new bone formation postnatally.

To study the Osx postnatal functions, we inactivated *Osx* after birth using *CAG-CreER*, a ubiquitously expressed rather than osteoblast-specific Cre recombinase. Inactivation of *Osx* by a ubiquitously expressed recombinase allowed us to explore the potential function of Osx in cells of the osteoblast lineage before expression of osteoblast-specific marker genes A recent study showed that mice, in which the conditional allele of *Osx* was inactivated by using a *Cre* transgene driven by a osteoblast-specific *Col1a1* promoter (15), developed a much milder phenotype compared with the $Osx^{postnatal}$ mutant mice described here. Unlike our $Osx^{postnatal}$ mutant mice, in which osteoblast differentiation and bone formation was completely arrested, these mice exhibited only a moderately decreased osteoblast activity. One major difference between these two conditional mice models is that in the *Col1a1-Cre* model, *Osx* was ablated only in differentiated *Col1a1*-expressing osteoblasts, whereas in the *CAG-CreER* model, *Osx* was deleted in all cell types, including cells involved in all stages of osteoblast differentiation. Another difference is that in the $Osx^{flox/-}$*;Col1a1-Cre* mice, inactivation of *Osx* began around E14.5 when the osteoblast-specific *Col1a1* promoter became active, in contrast to the $Osx^{postnatal}$ mutants described here. A subsequent study that used a *Col1a1-CreERT2* transgene to delete *Osx* postnatally produced only a modest decrease in bone formation and bone mineral density (16). No osteocyte anomalies and no abnormal cartilage accumulation were described in either of these two studies; furthermore, the number and function of osteoclasts was also unchanged. The phenotype of the $Osx^{flox/-}$*;Col1a1-Cre* mice indicated that Osx was needed for the optimal function of *Col1a1*-expressing osteoblasts, whereas the present mouse model demonstrated that Osx was not only essential for osteoblast differentiation and bone formation in postnatal mice, but also for cartilage resorption and osteocyte maturation and function.

During bone formation, osteoblasts first deposit an unmineralized bone matrix called osteoid, which mainly consists of type I collagen. The osteoid subsequently becomes mineralized by incorporation of small mineralized particles. This mineralization process is believed to be principally regulated by osteocytes (1). In the present mouse model, the abnormal *Osx*-null osteocytes could be derived from either existing osteocytes or *Osx*-null osteoblasts, which already had begun to express *Col1a1* (15) at the time of tamoxifen injections. The fact that some of the abnormal osteocytes in $Osx^{postnatal}$ mutants were surrounded by unorganized collagen fibers (Fig. S3*B*) suggested that some abnormal osteocytes might be derived from *Osx*-null mature osteoblasts. This finding suggested that Osx plays a key role in the maturation of osteocytes. Other lines of evidence support the notion that Osx is also required for osteocyte maintenance and functions. The defective mineralization process in the $Osx^{postnatal}$ mutants was likely due to the combined decreased expression of *Dmp1* and *Phex*, not to hypophosphatemia, because the blood phosphate levels were normal. Furthermore, the finding that Osx interacted with a specific site in the sclerostin promoter both in EMSA experiments and in intact cells, and activated this promoter in transfection assays, suggested that Osx is also a player in mature osteocytes. Preliminary results showed that inactivation of *Osx* by *Dmp1-Cre*, which is highly expressed in osteocytes postnatally, led to morphological osteocyte abnormalities similar to those in $Osx^{postnatal}$ mutants. Overall, our findings identify Osx as a critical transcription factor required for the maturation and function of osteocytes.

The endochondral bones of $Osx^{postnatal}$ mutants were also characterized by a massive accumulation of calcified cartilage, which during the growth period extended progressively more deeply toward the diaphysis. This process was driven by the continuous activity of growth plate-proliferating chondrocytes. The unique characteristic of these mice was that the growth plate chondrocytes continued their activity, but no new bone was being formed. The zone of excess cartilage did not contain *Col2a1*- or *Col10a1*-expressing cells, or active osteoblasts, as indicated by the virtual absence of *Col1a1*-expressing cells. This zone did contain cells in which the *Osx* promoter was active, as illustrated by EGFP positivity and also an abundance of Runx2-positive cells. Our findings clearly indicate that cartilage resorption is severely defective in $Osx^{postnatal}$ mutants. It is unlikely that this lack of resorption would be due to a selective increase in OPG production by hypertrophic chondrocytes, because OPG immunohistochemistry did not show a significant increase in staining in the hypertrophic zone of $Osx^{postnatal}$ mutant mice (Fig. S5*G*). Therefore, we suggest that the process of cartilage resorption is tightly coupled to Osx-dependent bone formation.

In osteopetrotic bones, cartilage and bone tissues accumulate and completely fill the marrow cavity (17). This suggests that osteoclasts are the cells that resorb both cartilage and bone matrices. In $Osx^{postnatal}$ mutant endochondral bones the overall number of osteoclasts and their size, as well as *TRAP* expression, was reduced. The reduction in osteoclast numbers was especially marked in the zone of excessive accumulated cartilage. Despite the overall reduction in *TRAP* expression and activity, our findings suggested that osteoclasts were functional in bone resorption. Indeed, the intrinsic ability of precusor cells from $Osx^{postnatal}$ mutants to differentiate into osteoclasts in culture was very similar to that of control cells. In addition, the presence of numerous osteoclasts inside the thin and porous cortical bones, and the gradual disappearance of preexisting bone trabeculae once *Osx* was ablated, strongly supported this view.

During the normal process of endochondral bone formation in the primary spongiosa, osteoblasts first use the cartilage as a scaffold to deposit a bone-specific matrix. During bone remodeling, αvβ3 integrin, which is expressed at high levels in osteoclasts, has a major role in the attachment of osteoclasts to this bone matrix and their subsequent function. αvβ3 integrin interacts with several bone matrix proteins, including bone sialoprotein (BSP), which is expressed at especially high levels in the primary spongiosa. Both loss- and gain-of-function mouse genetic experiments strongly support a role for Bsp in bone resorption (18, 19). Expression of *Bsp* and several other ECM components was markedly reduced in $Osx^{postnatal}$-null bones. The existence of an essentially naked cartilage scaffold not covered by bone was a unique abnormality in $Osx^{postnatal}$-null endochondral bones. We propose that the absence of bone formation on the surface of the cartilage scaffold and the reduced number and size of osteoclasts in the area of excess mineralized cartilage could account for the defective resorption of this cartilage.

The reduction in the overall number and size of osteoclasts and in *TRAP* expression in $Osx^{postnatal}$-null long bones could be attributed to an increase in the ratio of *Opg* to *Rankl* expression, which itself was a likely consequence of the observed decrease in *Sost* expression.

One other potential mechanism that might also have accounted for the failure of cartilage resorption is much less likely. We found that the expression of *Mmp13* in the femurs and calvariae of

the $Osx^{postnatal}$ mutant mice was significantly reduced (20, 21). Nevertheless, despite decreased *Mmp13* expression, the specific cleavage of Aggrecan by MMPs in the $Osx^{postnatal}$ mutant mice took place.

In $Osx^{postnatal}$-null mutants—specifically in the region of ectopic mineralized cartilage—we observed a clear correlation between the lack of new bone and the absence of bone marrow cells. These data suggested that in the primary spongiosa, Osx-dependent endochondral bone formation was required for the formation of bone marrow cells. Our findings are in agreement with a recent study that grafted endochondral bone progenitor cells under the kidney capsule (22). In this assay, these cells had an essential role in the generation of host-derived bone marrow and knockdown of *Osx* in these progenitor cells, which inhibited donor-derived osteogenesis and abolished formation of the hematopoietic stem cell niche and bone marrow cells in the host (22). Our findings represent the in vivo skeletal equivalent of the graft study. We speculate that the absence of mature osteoblasts and the lack of bone in the zone of ectopic mineralized cartilage causes the localized absence of bone marrow cells.

The $Osx^{postnatal}$-null mutant mice grew slower than wild-type controls. The moderate expansion of the hypertrophic zone of these mice could be due to reduced expression of *Mmp13*. Because the blood levels of phosphate were normal in $Osx^{postnatal}$-null mice, the slower growth and the expanded hypertrophic zone cannot be due to hypophosphatemia, as is the case in *Dmp1*-null mice. We propose that the decreased growth of $Osx^{postnatal}$-null limbs could be accounted for by the increase in size of the hypertrophic zone, lack of new bone formation, and the accumulation of unresorbed mineralized cartilage.

In summary, our findings suggest that postnatally, Osx has an essential role in osteoblast differentiation and bone formation. Osx is also needed for the maturation and full expression of the genetic program and hence the function of osteocytes after birth. Furthermore, our data strongly suggest that cartilage resorption is coupled to Osx-dependent endochondral bone formation. The lack of resorption of the accumulated unresorbed mineralized cartilage under the growth plate in the $Osx^{postnatal}$ mutants could be accounted for in part by the decrease in osteoclast function and density in this area and by the inefficiency of osteoclasts to resorb cartilage in absence of bone deposition on the surface of the cartilage scaffold. Our study thus identifies Osx as an indispensable multifunctional actor in postnatal skeletal growth and homeostasis.

## Materials and Methods

**Generation of $Osx^{postnatal}$ Mutant Mice.** The $Osx^{\Delta EX2/+}$ mice were generated by crossing $Osx^{floxed/floxed}$ and *Prm-Cre* mice. *CAG-CreER* transgenic mice (12) were purchased from Jackson Laboratory. The tamoxifen-treated *CAG-CreER;*$Osx^{floxed/-}$ mice are referred to throughout the text as $Osx^{postnatal}$ mutant mice; the vehicle or tamoxifen-treated *CAG-CreER;*$Osx^{floxed/+}$ mice are referred to as wild-type controls. Mice were injected intraperitoneally with tamoxifen 1.5–3.0 mg/10 g body weight (Sigma-Aldrich) or vehicle (corn oil containing 10% ethanol) at the desired postnatal days. $Osx^{postnatal}$ mutant mice and wild-type controls were injected three times with either tamoxifen or vehicle from P15 to P20 and killed at P24; four times (twice with 3 mg/10 g body weight and twice with 1.5 mg/10 g body weight) from P16 to P26 and killed at 6 wk; or four times (3 mg/10 g body weight) from P21 to P30 and killed at 2 mo. These mice are designated P24, 6 wk, and 2 mo.

**X-ray, μCT, and Histomorphometry Analyses.** See *SI Materials and Methods* for the following procedures: RNA isolation and quantitative PCR analyses; EMSA and transfection; in vitro osteoclastogenesis and in vitro osteoblastogenesis; immunohistochemical analysis and in situ hybridization; EM analysis of osteocytes; chromatin immunoprecipitation (ChIP) assay; and statistical analyses.

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