The high mobility group transcription factor Sox8 is a negative regulator of osteoblast differentiation

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Bone remodeling is an important physiologic process that is required to maintain a constant bone mass. This is achieved through a balanced activity of bone-resorbing osteoclasts and bone-forming osteoblasts. In this study, we identify the high mobility group transcription factor Sox8 as a physiologic regulator of bone formation. *Sox8*-deficient mice display a low bone mass phenotype that is caused by a precocious osteoblast differentiation. Accordingly, primary osteoblasts derived from these mice show an accelerated mineralization ex vivo and a premature expression of osteoblast differentiation markers. To confirm the function of Sox8 as a negative regulator of osteoblast differentiation we generated transgenic mice that express *Sox8* under the control of an osteoblast-specific *Col1a1* promoter fragment. These mice display a severely impaired bone formation that can be explained by a strongly reduced expression of *runt-related transcription factor 2*, a gene encoding a transcription factor required for osteoblast differentiation. Together, these data demonstrate a novel function of Sox8, whose tightly controlled expression is critical for bone formation.

Introduction

Bone is a highly specialized tissue whose primary functions are to provide mechanical support and physical protection of soft tissues and to act as a storage system for mineral homeostasis. There are two distinct types of bone that can be classified based on their different development (Olsen et al., 2000). Bones of the skull as well as the clavicles are formed by intramembranous ossification, where mesenchymal cells directly differentiate into bone-forming osteoblasts. In contrast, the other skeletal elements form by endochondral ossification, where a cartilage intermediate is subsequently replaced by bone through the activity of osteoclasts and osteoblasts (Kronenberg, 2003). Besides the growth plate the most critical region for skeletal development and growth is the periosteal bone collar, where the bone matrix is deposited by primordial osteoblasts and specialized hypertrophic chondrocytes (Bianco et al., 1998).

Once the vertebrate skeleton has reached its final size and shape, bone is constantly remodeled, thereby providing the possibility to adapt to changes in daily activities and to contribute to mineral homeostasis. This remodeling process is coordinated on several levels to achieve a balance between bone formation by osteoblasts and bone resorption by osteoclasts, thereby maintaining a sufficient bone mass (Amling et al., 2000). The disturbance of this balance can lead to bone loss and osteoporosis, one of the most prevalent degenerative diseases. Therefore, the identification of mechanisms regulating the differentiation and activity of bone-forming osteoblasts is an important step toward the identification of novel therapeutic options to regain bone mass in osteoporotic patients.

Osteoblasts differentiate from mesenchymal precursor cells in a process requiring the coordinated activity of transcription factors and other signaling proteins (Harada and Rodan, 2003). Among these molecules, the *runt*-related transcription factor 2 (Runx2) is generally considered to be a master regulator of osteoblast differentiation and bone formation based on overwhelming genetic evidence (Karsenty, 1999; Komori, 2002). First, *Runx2*-deficient mice fail to form bone, which is explained by a complete absence of osteoblasts (Komori et al., 1997; Otto et al., 1997). Second, mice lacking one allele of *Runx2* display a defect in intramembranous ossification

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Abbreviations used in this paper: Bsp, bone sialoprotein; Lrp5, low density lipoprotein receptor-related protein 5; Osc, osteocalcin; Osx, osterix; Phex, phosphate-regulating gene with homologies to endopeptidases located on the X-chromosome; Runx2, runt-related transcription factor 2; Tnsalp; tissue-nonspecific alkaline phosphatase.

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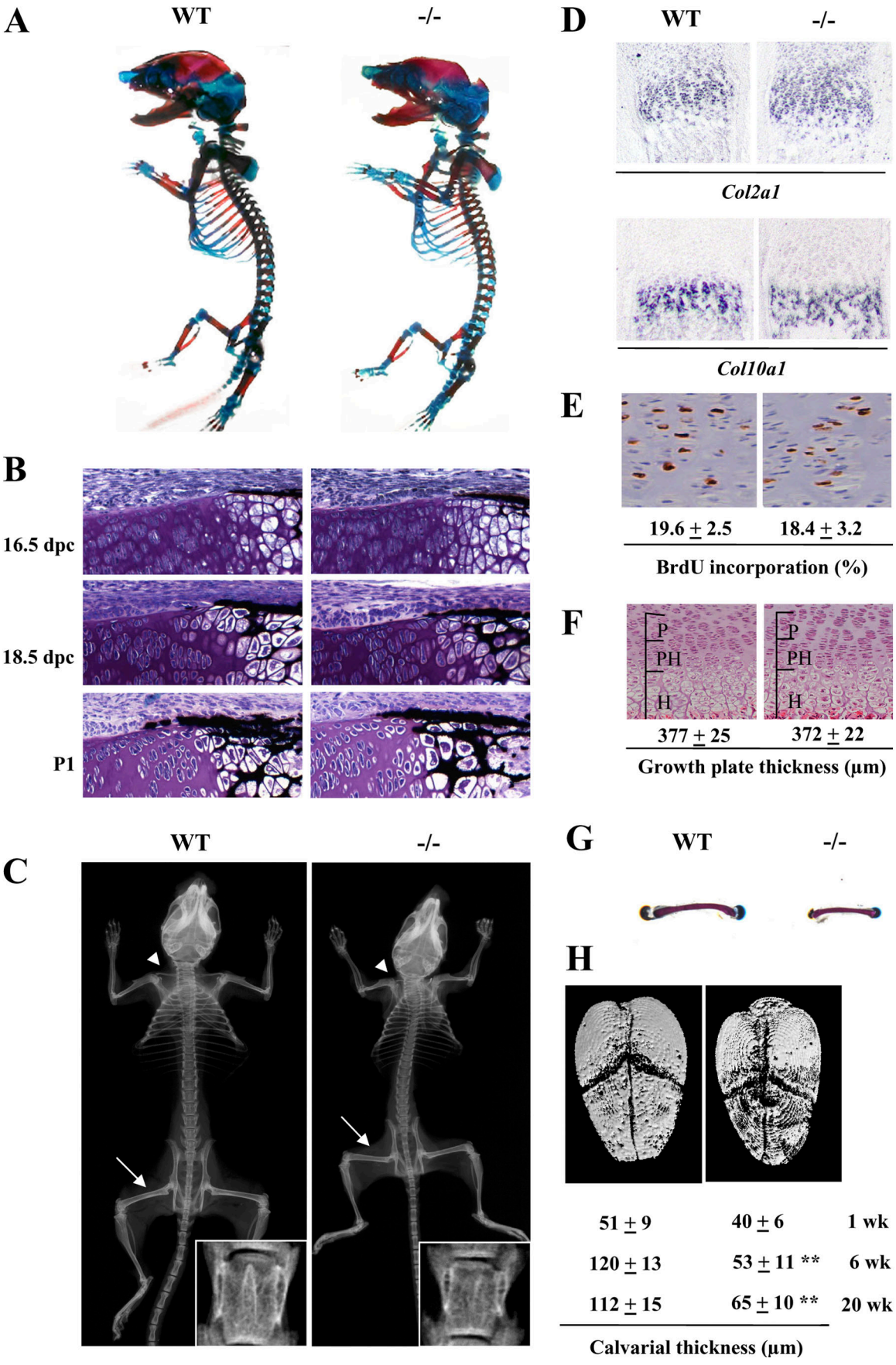
A
WT
-/-
B
16.5 dpc
18.5 dpc
P1
C
WT
-/-
D
WT
-/-
Col2a1
Col10a1
E
19.6 ± 2.5
18.4 ± 3.2
BrdU incorporation (%)
F
P
PH
H
377 ± 25
372 ± 22
Growth plate thickness (μm)
G
WT
-/-
H
51 ± 9
40 ± 6
1 wk
120 ± 13
53 ± 11 **
6 wk
112 ± 15
65 ± 10 **
20 wk
Calvarial thickness (μm)

that is reminiscent of cleidocranial dysplasia, an autosomal dominant disorder caused by mutations of the human *Runx2* gene (Lee et al., 1997; Mundlos et al., 1997, Otto et al., 1997). Third, transgenic mice expressing a dominant-negative variant of *Runx2* in differentiated osteoblasts display an osteopenic phenotype, thereby demonstrating that Runx2 is also required for the functional activity of osteoblasts (Ducy et al., 1999). This action of Runx2 is, at least in part, mediated through osterix (Osx), another transcription factor required for osteoblast differentiation, acting downstream of Runx2 (Nakashima et al., 2002).

Although Runx2 has an additional function in the regulation of chondrocyte hypertrophy (Takeda et al., 2001), the most important transcription factor in terms of chondrogenesis is Sox9, because it is required for determination, proliferation, and differentiation of mesenchymal progenitors into chondrocytes (De Crombrugghe et al., 2001). Accordingly, endochondral bone formation is inhibited at an early phase and does not take place in the absence of Sox9 (Akiyama et al., 2002). Similarly, human patients with heterozygous *SOX9* mutations suffer from campomelic dysplasia, a severe skeletal malformation syndrome (Foster et al., 1994; Wagner et al., 1994). Sox9 belongs to a family of high mobility group transcription factors with close resemblance to the male sex-determining factor Sry in their DNA-binding domain and many different functions during mammalian development (Wegner, 1999; Bowles et al., 2000). Among these Sox proteins, Sox9 exhibits closest similarity to Sox8 and Sox10, and together these three proteins form the group E of the Sox family. Sox10 has been studied extensively and is required in derivatives of the neural crest and glial cells of the central nervous system at various phases of development (Herbarth et al., 1998; Southard-Smith et al., 1998; Britsch et al., 2001; Stolt et al., 2002; J. Kim et al., 2003). In contrast, much less is known about the physiologic role of Sox8.

Despite the widespread expression of *Sox8* (Pfeifer et al., 2000; Schepers et al., 2000), *Sox8*-deficient mice appeared remarkably normal, exhibiting mainly a significant overall weight reduction (Sock et al., 2001). The lack of a more severe phenotype may be explained by the fact that in many tissues *Sox8* is coexpressed with *Sox9* and *Sox10*. Because Sox8 has similar biochemical properties as Sox9 and Sox10 in vitro, the Sox8 deficiency is possibly compensated by the remaining production of either of these two Sox proteins. Previously, we had however noticed, that ossification of tarsal bones in the hindfeet of *Sox8*-deficient mice was disturbed (Sock et al., 2001), arguing that Sox8 might have a previously uncharacterized role in skeletal biology. This finding prompted us to analyze the skeletal phenotype of *Sox8*-deficient mice.

Here, we show that *Sox8*-deficient mice display an osteopenic phenotype that is caused by an intrinsic osteoblast differentiation defect. Primary osteoblasts derived from these mice show an accelerated differentiation and mineralization compared with wild-type cells, where *Sox8* expression ceases upon differentiation. The fact that transgenic mice that express *Sox8* under the control of an osteoblast-specific *Col1a1* promoter fragment display severe defects in bone formation demonstrates that the down-regulation of *Sox8* is required for osteoblast differentiation and an essential step in bone remodeling.

Results

Osteopenia in *Sox8*-deficient mice

To determine whether Sox8 has a physiologic function in skeletal biology, we embarked on a full characterization of the skeletal phenotype of *Sox8*-deficient mice. We first studied their skeletal development and stained embryos with alcian blue and alizarin red without finding gross abnormalities compared with wild-type littermates (Fig. 1 A). To rule out subtle defects of endochondral ossification, we histologically analyzed the hindlimbs from wild-type and *Sox8*-deficient embryos at 16.5 and 18.5 dpc, as well as from newborn mice. Again, we did not observe significant differences between the two groups (Fig. 1 B).

In contrast, the radiographic analysis of whole skeletons at 6 wk old revealed that *Sox8*-deficient mice were reduced in size and displayed an increased radiolucency as well as a reduction of clavicle size (Fig. 1 C). To determine whether the overall size reduction of the *Sox8*-deficient mice is caused by defects in the growth plate, we next analyzed the differentiation of growth plate chondrocytes, where *Sox8* has been shown to be expressed (Sock et al., 2001). Using in situ hybridization, we did not observe a difference between wild-type and *Sox8*-deficient mice concerning the expression of *Col2a1*, a marker of nonhypertrophic chondrocytes, and *Col10a1*, a marker of hypertrophic chondrocytes (Fig. 1 D). Incorporation of BrdU into chondrocytes of the proliferative zone also failed to reveal a difference in the proliferation rate of growth plate chondrocytes between the two groups (Fig. 1 E). Histologically, the growth plate organization and thickness was the same in wild-

Figure 1. **Analysis of endochondral and intramembranous ossification in *Sox8*-deficient mice.** (A) Staining of wild-type (WT) and *Sox8*-deficient (−/−) skeletons with alcian blue and alizarin red at 17.5 dpc of embryonic development. No gross abnormalities were observed in the absence of Sox8. (B) Staining of undecalcified tibia sections with Toluidine blue and silver nitrate at 16.5 and 18.5 dpc, as well as in newborn wild-type and *Sox8*-deficient mice. No significant defects of endochondral ossification were observed in the absence of Sox8. (C) Radiographic analysis of skeletons from wild-type and *Sox8*-deficient mice at 6 wk old. The *Sox8*-deficient mice are smaller, show an increased radiolucency (arrows) and display hypoplastic clavicles (arrowheads) compared with wild-type littermates. A high power magnification of vertebral bodies is shown in the inset. (D) In situ hybridization analysis for the expression of *Col2a1* and *Col10a1* in the growth plates from wild-type and *Sox8*-deficient mice 2 d after birth showing normal chondrocyte differentiation in the absence of Sox8. (E) Analysis of chondrocyte proliferation by BrdU-incorporation assays at 1 wk old. No significant difference in the percentage of BrdU-positive cells was observed between the two groups. (F) Hematoxylin/eosin staining of tibial growth plates at 1 wk old. No difference in growth plate organization and thickness was observed between the groups. P, Proliferative zone, PH, prehypertrophic zone, H, hypertrophic zone. (G) Staining with alcian blue and alizarin red reveals a size reduction of *Sox8*-deficient clavicles at 6 wk old. (H) μCT imaging of the calvariae from 6-wk-old wild-type and *Sox8*-deficient mice showing hypomineralization in the absence of Sox8. The calvarial thickness is decreased in *Sox8*-deficient mice at 6 and 20 wk old. Values represent means ± SEM ($n = 6$). Asterisks indicate statistically significant differences as determined by *t* test between the two groups ($n = 6$). **, $P < 0.005$.

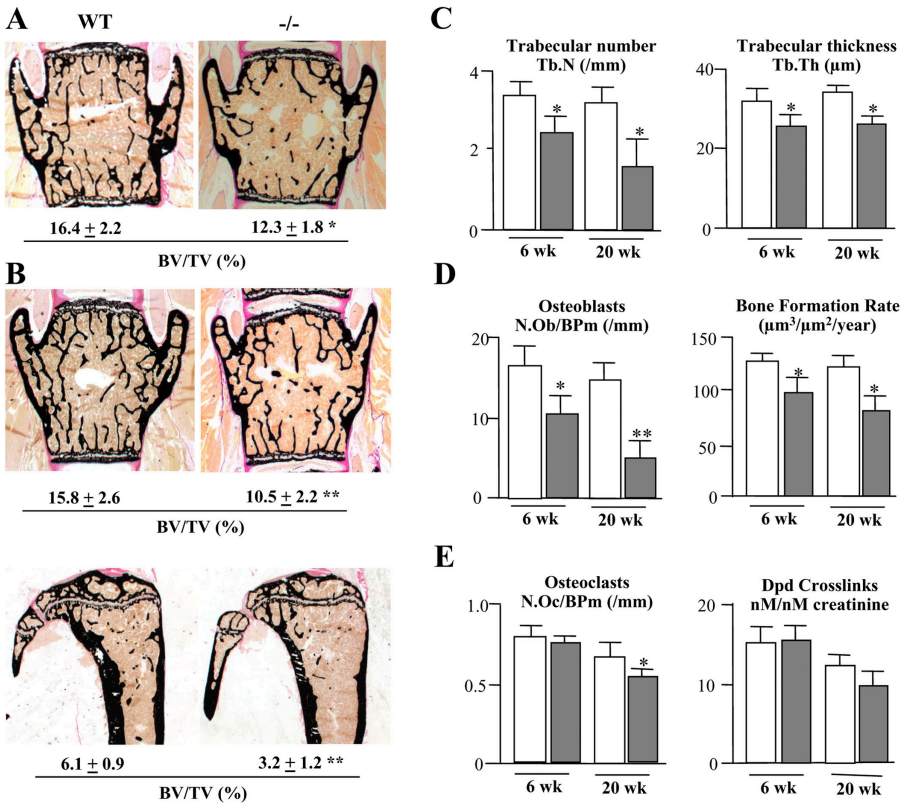

Figure 2. **Osteopenia in *Sox8*-deficient mice.** (A) Von Kossa staining of undecalcified vertebral sections and quantification of the trabecular bone volume (BV/TV, bone volume per tissue volume) reveals an osteopenic phenotype in *Sox8*-deficient mice at 6 wk old. (B) The same was observed in sections from vertebral bodies and tibiae at 20 wk old. (C) Histomorphometric quantification shows that trabecular number (Tb.N) and trabecular thickness (Tb.Th) are significantly decreased in *Sox8*-deficient mice (gray bars) compared with wild-type littermates (white bars) at both ages. (D) Histomorphometric quantification of trabecular bone formation parameters. The number of cuboidal osteoblasts (Ob.N/BPm, osteoblast number per bone perimeter) is strongly decreased in *Sox8*-deficient mice compared with wild-type littermates. This results in a significant reduction of the bone formation rate as determined by dynamic histomorphometry after dual calcein injection. (E) Quantification of bone resorption parameters. Although the numbers of osteoclasts (N.Oc/BPm, osteoclast number per bone perimeter) are decreased in *Sox8*-deficient mice at 20 wk old, there is no significant change in bone resorption as determined by measuring the urinary deoxypyridinoline (Dpd) cross-links. Values represent means ± SEM. Asterisks indicate statistically significant differences as determined by *t* test between the two groups ($n = 6$). *, $P < 0.05$; **, $P < 0.005$.

type and *Sox8*-deficient mice at 1 wk old (Fig. 1 F) and thereafter (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200408013/DC1). Together, these data demonstrate that the loss of Sox8 does not affect chondrocyte proliferation and differentiation in the growth plate.

Therefore, it was likely that the size reduction of the *Sox8*-deficient mice is caused by defects in postnatal bone formation. This possibility is underscored by the finding that bones formed by intramembranous ossification were also affected in the absence of Sox8. Besides the reduction of the clavicle size (Fig. 1 G) we observed a hypomineralization of the calvariae using µCT analysis (Fig. 1 H). The quantification of the calvarial thickness revealed that the absence of Sox8 leads to a 50% reduction at the age of 6 and 20 wk. This suggested that *Sox8*-deficient mice display an osteopenic phenotype that is caused by an impaired bone formation.

We next performed a complete histomorphometric analysis of bone remodeling parameters in *Sox8*-deficient mice and wild-type littermates at 6 and 20 wk old. Von Kossa staining of undecalcified sections confirmed the osteopenia in *Sox8*-deficient mice at both ages (Fig. 2, A and B). The histomorphometric analysis revealed that the trabecular bone volume in vertebral bodies and tibiae of *Sox8*-deficient mice was decreased by >30% compared with wild-type littermates. Further analysis demonstrated that trabecular number and trabecular thickness were significantly decreased in *Sox8*-deficient mice at both ages (Fig. 2 C).

We next determined the numbers and activities of the two cell types involved in bone remodeling. The quantification of cuboidal osteoblasts demonstrated that their number was decreased by 70% in *Sox8*-deficient mice compared with wild-type littermates, thereby resulting in a significantly decreased bone formation rate (Fig. 2 D). The number of bone-resorbing osteoclasts was slightly decreased in *Sox8*-deficient mice at 20 wk old, but not at 6 wk old. In contrast, we did not observe a significant difference compared with wild-type littermates at both ages when we quantified the concentration of urinary collagen degradation products as a measure of bone resorption (Fig. 2 E). Together, these data demonstrate that the lack of Sox8 in mice causes an osteopenic phenotype that can be explained by an impaired osteoblast differentiation, thereby resulting in a decreased bone formation.

Accelerated osteoblast differentiation in the absence of Sox8

To determine whether the decreased bone formation of *Sox8*-deficient mice is caused by an intrinsic osteoblast differentiation defect, we next studied the behavior of wild-type and *Sox8*-deficient primary calvarial osteoblasts ex vivo. We first analyzed *Sox8* expression in these cells by Northern blotting and found a strong expression in nondifferentiated wild-type cultures. Importantly, this expression was completely abolished 5 and 10 d after the addition of ascorbic acid and β-glycerophosphate, two agents leading to terminal osteoblast differentiation and mineralization ex vivo (Fig. 3 A). We next determined the proliferation of wild-type and *Sox8*-deficient primary calvarial osteoblasts. Using a BrdU incorporation assay we found that the proliferation rate of *Sox8*-deficient cells was strongly reduced compared with wild-type cells, even before osteoblast differentiation was induced (Fig. 3 B). To analyze

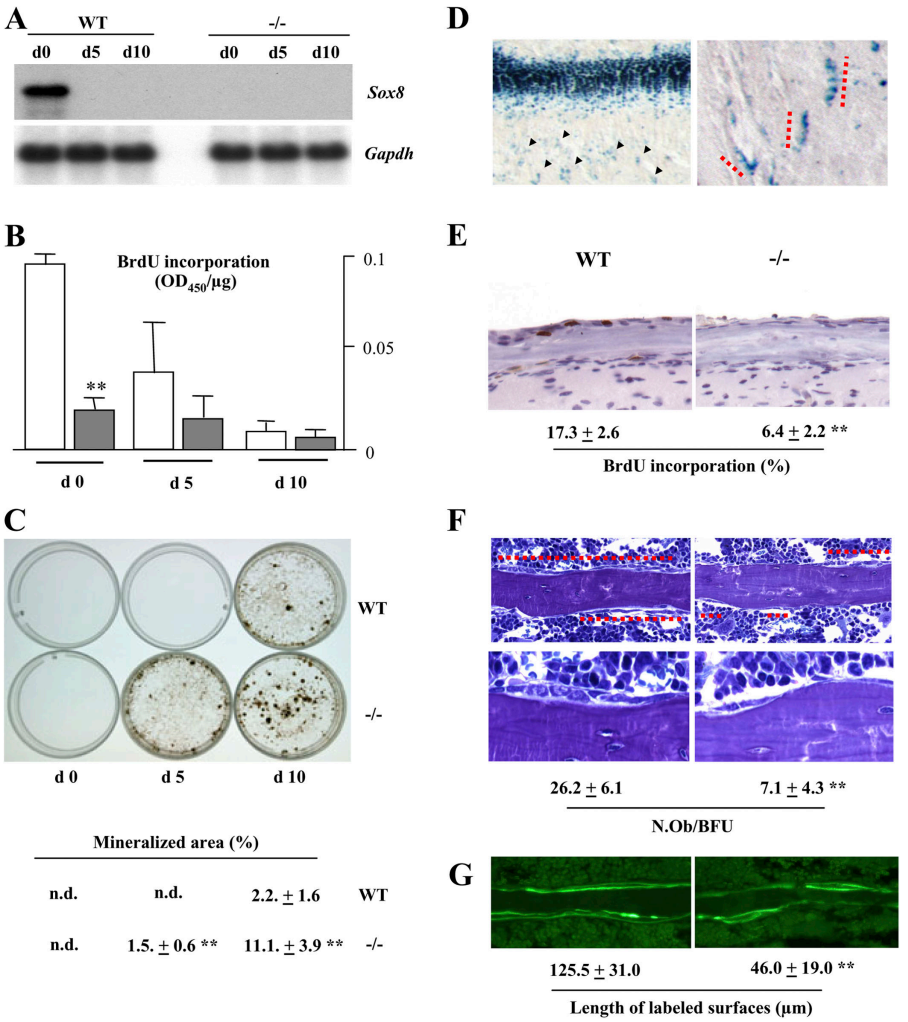

Figure 3. **Decreased proliferation and accelerated differentiation of *Sox8*-deficient osteoblasts.** (A) Analysis of *Sox8* expression in primary calvarial osteoblast cultures. Northern blot expression analysis shows that *Sox8* is expressed in nondifferentiated wild-type calvarial osteoblast cultures (d0), but not in the same cultures 5 and 10 d (d5 and d10) after the addition of ascorbic acid and β-glycerophosphate. (B) Analysis of osteoblast proliferation. A BrdU-incorporation assay with primary osteoblasts from wild-type (white bars) and *Sox8*-deficient (gray bars) mice reveals a strong reduction of osteoblast proliferation in the absence of Sox8, occurring before ascorbic acid and β-glycerophosphate were added to the culture medium (d0). (C) Analysis of ECM mineralization. Von Kossa staining reveals an accelerated mineralization of *Sox8*-deficient primary osteoblast cultures compared with wild-type cultures. n.d., not detectable. (D) X-Gal staining of tibia sections from *Sox8*-deficient mice at 1 wk old reveals *LacZ* expression in the growth plate, but also in cells of the primary spongiosa (arrowheads). Higher magnification reveals expression in groups of cells (indicated by the dotted red lines) at the bone surface which is characteristic of osteoblasts. (E) Analysis of osteoblast proliferation in vivo by BrdU-incorporation assays at 1 wk old. The percentage of BrdU-positive cells in calvarial sections is significantly decreased in *Sox8*-deficient mice compared with wild-type littermates. Note the flattened appearance of *Sox8*-deficient osteoblasts compared with wild-type controls, suggesting premature differentiation. (F) Toluidine blue staining of undecalcified vertebral bone sections shows that the teams of bone-forming osteoblasts (indicated by the dotted red lines) are shorter in *Sox8*-deficient mice. Quantification of the average numbers of osteoblasts within these bone-forming units (N.Ob/BFU) reveals a significant reduction in the absence of Sox8. (G) Fluorescent micrographs showing the calcein-labeled surfaces representing newly formed bone. The length of these surfaces is significantly decreased in *Sox8*-deficient mice. Values represent means ± SEM. Asterisks indicate statistically significant differences as determined by *t* test between the two groups ($n = 6$). **, $P < 0.005$.

osteoblast differentiation we performed Von Kossa staining of the mineralized matrix from wild-type and *Sox8*-deficient cells formed before (d0) as well as 5 and 10 d after the addition of ascorbic acid and β-glycerophosphate. Unexpectedly, we observed an accelerated mineralization of *Sox8*-deficient cells. In contrast to wild-type cultures, mineralized nodules were already detectable after 5 d of differentiation. After 10 d the mineralization was still much more pronounced (Fig. 3 C).

To correlate these results to the situation in vivo, we first analyzed *Sox8* expression in osteoblasts. Because the targeted deletion of the *Sox8* gene had introduced a *lacZ* reporter gene whose expression faithfully mimicked that of *Sox8*, we performed X-Gal staining of bone sections from *Sox8*-deficient mice. In addition to the expression in growth plate cartilage, we also detected staining in the primary spongiosa (Fig. 3 D). At higher magnification we observed *lacZ*-positive groups of cells at the bone surface suggesting expression in osteoblasts that typically act in teams when they form new bone (Parfitt, 1983). To analyze osteoblast proliferation in vivo we performed BrdU incorporation assays in the calvariae from wild-type and *Sox8*-deficient mice. Thereby we found that the percentage of proliferating cells was decreased by 65% in *Sox8*-deficient mice compared with wild-type littermates (Fig. 3 E). Having verified the decreased proliferation of osteoblast precursor cells in *Sox8*-deficient mice, we next analyzed their differentiation on trabecular bone surfaces. Toluidine blue staining revealed a striking morphologic difference between wild-type and *Sox8*-deficient mice at the sites of bone formation. Although in wild-type mice the bone-forming units at the trabecular bone surface are comprised of large teams of osteoblasts, we observed that the number of osteoblasts within these teams was decreased by 70% in *Sox8*-deficient mice (Fig. 3 F). This observation was corroborated by the finding that the length of labeled surfaces after dual calcein injection was significantly decreased in *Sox8*-deficient mice compared with wild-type littermates (Fig. 3 G). Together, these observations suggest that loss of Sox8 results in premature osteoblast differentiation at the expense of proliferation, thereby reducing the number of active osteoblasts within the bone-forming units.

We next analyzed the expression of osteoblast differentiation markers in wild-type and *Sox8*-deficient primary osteoblasts by RT-PCR. In wild-type cultures expression of

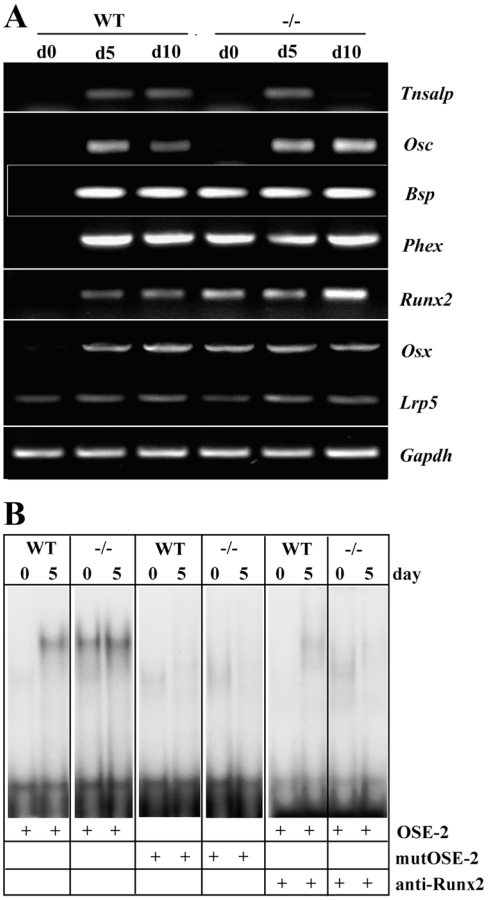

Figure 4. **Premature expression of osteoblast differentiation markers in *Sox8*-deficient osteoblasts.** (A) RT-PCR expression analysis of primary osteoblast cultures from wild-type (WT) and *Sox8*-deficient (−/−) mice at d0, d5, and d10 of differentiation. Note the premature expression of *Bsp, Phex, Runx2*, and *Osx* in *Sox8*-deficient cultures. *Tnsalp (tissue nonspecific alkaline phosphatase), Osc (osteocalcin), Bsp (bone sialoprotein), Phex (phosphate-regulating gene with homologies to endopeptidases located on the X-chromosome), Runx2 (runt-related transcription factor 2), Osx (osterix), Lrp5 (low density lipoprotein receptor-related protein 5), Gapdh (Glycerinaldehydephosphate dehydrogenase)*. (B) Analysis of Runx2 DNA-binding activity. An electrophoretic mobility shift assay using the Runx2-binding site (OSE-2; Ducy et al., 1997) reveals the presence of Runx2 in nuclear extracts from nondifferentiated *Sox8*-deficient osteoblasts, but not from wild-type osteoblasts (d0). Binding was not observed with a mutated binding site (mutOSE-2) or in the presence of an anti–Runx2-antibody. (C) Analysis of osteoblast differentiation markers in vivo. A Northern blot expression analysis using calvaria and femur RNA from wild-type and *Sox8*-deficient mice at 6 wk old reveals an elevated expression of *Osc, Bsp, Phex*, and *Runx2* in the absence of Sox8.

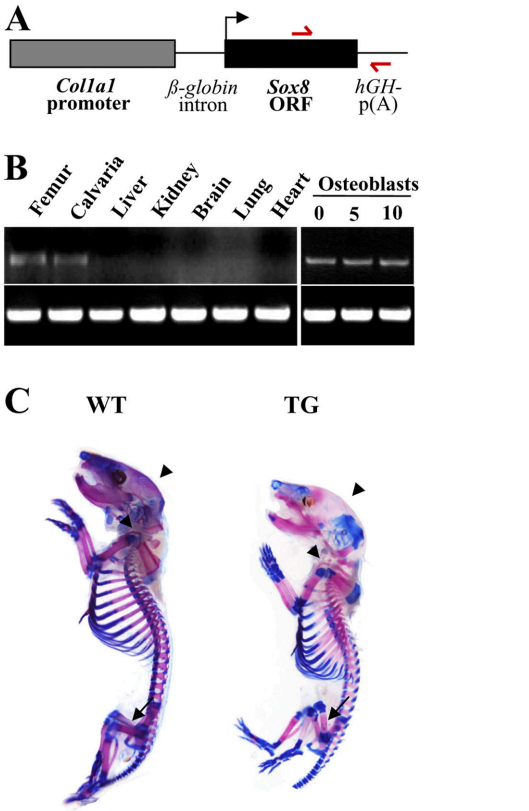

Figure 5. **Generation and skeletal analysis of *Col1a1-Sox8* transgenic mice.** (A) Schematic presentation of the DNA construct used for microinjection. Transgenic mice contain the Sox8-encoding ORF under the control of an osteoblast-specific *Col1a1* promoter fragment. The location of the primers used for the transgene-specific expression analysis is indicated in red. (B) Analysis of transgene expression. RT-PCR expression analysis using transgene-specific primers reveals expression in bone and calvaria, as well as in primary osteoblasts at all stages of differentiation. (C) Staining of skeletons from newborn wild-type (WT) and *Col1a1-Sox8* transgenic (TG) mice with alcian blue and alizarin red. Skeletal elements of transgenic mice are reduced in size and staining intensity (arrows). Additionally, their clavicles and calvariae are hypomineralized (arrowheads).

tissue-nonspecific alkaline phosphatase (Tnsalp), osteocalcin (Osc), bone sialoprotein (Bsp), phosphate-regulating gene with homologies to endopeptidases located on the X-chromosome (Phex), Runx2 and *Osx* was observed only 5 and 10 d after the addition of ascorbic acid and β-glycerophosphate (Fig. 4 A). In *Sox8*-deficient cells however, we observed a different expression pattern for all of these genes except *Osc*. Whereas *Tnsalp* was prematurely down-regulated in the absence of Sox8, *Bsp*, and *Phex*, two genes associated with ECM mineralization, were expressed in *Sox8*-deficient cells even without the addition of ascorbic acid and β-glycerophosphate (Fig. 4 A). A premature expression in *Sox8*-deficient cells was also observed for *Runx2* and *Osx*, two genes encoding transcription factors required for osteoblast differentiation. In contrast, we did not observe changes in the expression pattern of *low density lipoprotein receptor-related protein 5 (Lrp5)*, a gene that was recently identified to play a major role in bone formation in a Runx2-independent manner (Kato et al., 2002).

Premature *Runx2* expression in *Sox8*-deficient osteoblasts was also confirmed on the protein level using an electrophoretic mobility shift assay. The complex of Runx2 and OSE-2, an oligonucleotide containing the Runx2 binding site, was only observed after 5 d of differentiation in nuclear extracts from wild-type cells, whereas it was already present before the addition of ascorbic acid and β-glycerophosphate (d0) in nuclear extracts from *Sox8*-deficient cultures (Fig. 4 B). This complex was specific for Runx2, because it was not observed when a mutated OSE2 oligonucleotide was used, and because it disappeared when a Runx2-specific antibody was added to the binding reaction (Fig. 4 B).

To further confirm the premature differentiation of *Sox8*-deficient osteoblasts in vivo, we performed a Northern blot expression analysis using RNA from calvaria and femur of wild-type and *Sox8*-deficient mice at 6 wk old (Fig. 4 C). Thereby, we found an increased expression of *Bsp*, *Phex*, and *Runx2*, which is consistent with the RT-PCR expression analysis described above. Together, these data suggested that Sox8 is a negative regulator of osteoblast differentiation, potentially acting through Runx2.

Impaired bone formation in *Col1a1-Sox8* transgenic mice

The fact that *Sox8* expression is down-regulated upon differentiation of wild-type osteoblasts raised the hypothesis that this down-regulation is a prerequisite for osteoblast differentiation. To interfere with *Sox8* down-regulation in osteoblasts we generated transgenic mice that express *Sox8* under the control of an osteoblast-specific *Col1a1* promoter fragment that is active in osteoblast precursor cells and maintains its activity in differentiated osteoblasts (Rossert et al., 1995, 1996). The transgene consisted of the complete Sox8 ORF placed under the control of the 2.3-kb osteoblast-specific *Col1a1* promoter fragment (Fig. 5 A). Four transgenic founders with similar phenotype and transgene expression were obtained. Three of them were used to generate transgenic mouse lines (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200408013/DC1). The fourth had to be killed at 10 d old because of spontaneous fracture. Expression analysis of the transgene using RT-PCR confirmed its bone-specific expression, as well as the expected lack of down-regulation during osteoblast differentiation (Fig. 5 B).

For the phenotypic analysis of the *Col1a1-Sox8* transgenic mice we first stained skeletons of newborn mice with alcian blue and alizarin red. Compared with wild-type littermates the *Col1a1-Sox8* transgenic mice displayed a generalized weaker staining with alizarin red as well as a size reduction of most skeletal elements (Fig. 5 C). Intramembranous ossification was also affected, because we observed a severe hypomineralization of the calvariae and clavicles of the transgenic mice.

Because this phenotype did not lead to postnatal lethality, we next analyzed wild-type and transgenic mice at 2 wk old. Using contact radiography we observed an increased radiolucency in transgenic mice demonstrating a low bone mass phenotype (Fig. 6 A). Additionally, we found a size reduction of all skeletal elements compared with wild-type littermates, including the clavicles. The severe hypomineralization of calvariae that

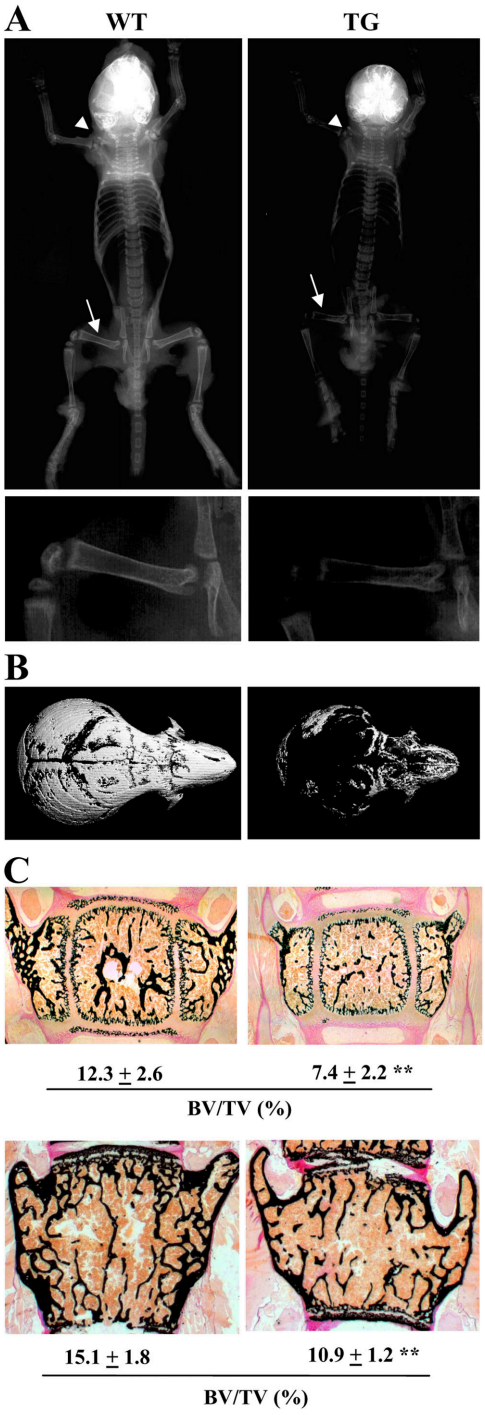

Figure 6. **Bone phenotype of *Col1a1-Sox8* transgenic mice.** (A) Radiographic analysis of skeletons from wild-type and *Col1a1-Sox8* transgenic mice at 2 wk old. Transgenic mice are smaller, display increased radiolucency compared with wild-type littermates (arrows) and have hypoplastic clavicles (arrowheads). (B) μCT imaging reveals that the *Col1a1-Sox8* transgenic mice have severe defects in calvarial bone formation and fontanelle closure. (C) Histological analysis of vertebral bodies from wild-type and transgenic mice at 2 wk (top) and 10 wk (bottom) old. Von Kossa staining of undecalcified sections reveals a low bone mass phenotype in *Col1a1-Sox8* transgenic mice. Histomorphometric quantification shows that the trabecular bone volume (BV/TV) is significantly reduced in transgenic mice at both ages. Values represent means ± SEM. Asterisks indicate statistically significant differences between the two groups ($n = 5$). **, $P < 0.005$.

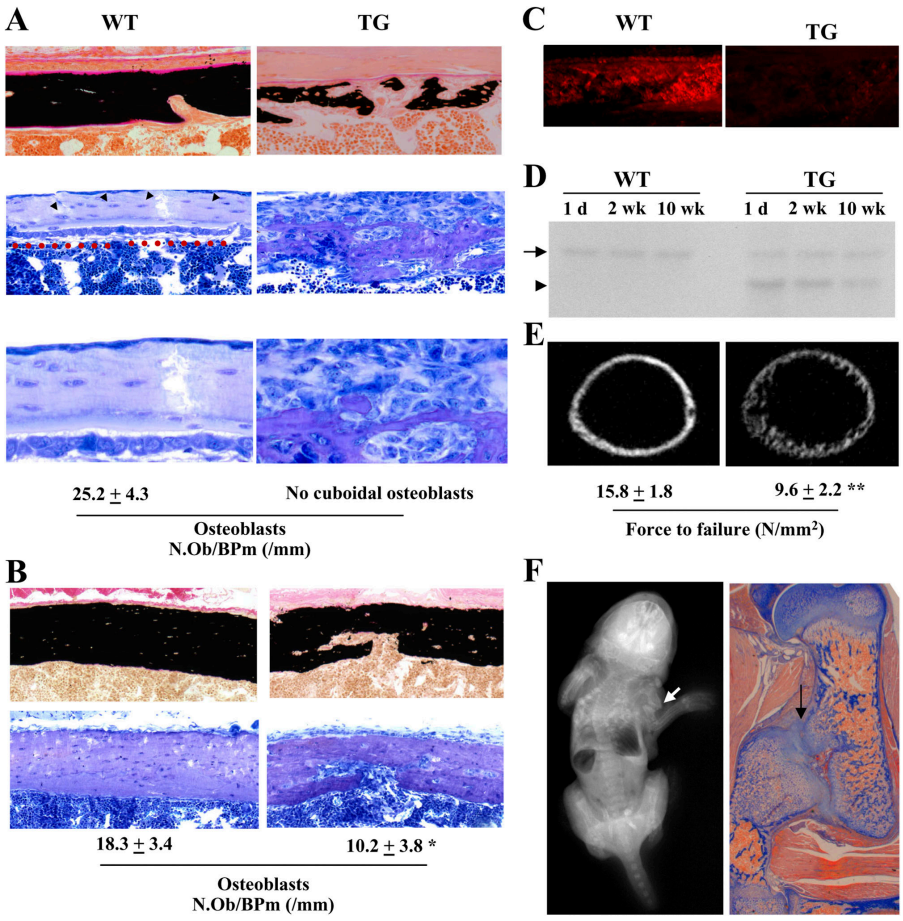

Figure 7. **Increased cortical porosity in *Col1a1-Sox8* transgenic mice.** (A) Von Kossa staining of undecalcified tibia sections from wild-type and *Col1a1-Sox8* transgenic mice at 2 wk old shows that transgenic mice display extensive cortical porosity. Toluidine blue staining reveals a severely impaired bone formation in *Col1a1-Sox8* transgenic mice. Note that the typical morphology of osteoblasts (located above the dotted red line) and osteocytes (arrowheads) is not observed in transgenic mice. Quantification of osteoblast number was not possible in transgenic mice, because they did not contain the characteristic cuboidal osteoblasts, in contrast to wild-type littermates. (B) Von Kossa and Toluidine blue staining of undecalcified tibia sections from wild-type and *Col1a1-Sox8* transgenic mice at 10 wk old reveals a partial recovery from the severe phenotype, although the number of osteoblasts is still significantly reduced in the transgenic mice. (C) Immunohistochemistry using an antibody against type I collagen shows that the amount of this protein is largely reduced in cortical bone of transgenic mice at 2 wk old. (D) Northern blot expression analysis using RNA from calvariae of wild-type and *Col1a1-Sox8* transgenic mice at different ages reveals that the expression of the transgene (arrowhead) is strongly down-regulated over time compared with the endogenous *Sox8* expression (arrow). (E) Cross-sectional μCT scans from femora of wild-type (WT) and *Col1a1-Sox8* transgenic (TG) mice confirming the increased cortical porosity in transgenic mice at 2 wk old. Force to failure as determined by three-point bending assays is significantly reduced in transgenic femora indicating decreased biomechanical stability. (F) A spontaneous fracture of the humerus (white arrow) was observed by contact radiography in a *Col1a1-Sox8* transgenic founder (left). Mallory staining of an undecalcified section (right) shows that callus formation occurred at the fracture site (black arrow). Values represent means ± SEM. Asterisks indicate a statistically significant difference between the two groups ($n = 5$). *, $P < 0.05$; **, $P < 0.005$.

was still prominent in the transgenic mice at 2 wk old was further visualized by μCT imaging (Fig. 6 B). Histomorphometric analysis of vertebral sections demonstrated that the trabecular bone volume in transgenic mice was reduced by 40% at 2 wk old and by 30% at 10 wk old compared with wild-type littermates (Fig. 6 C).

The phenotype of the *Col1a1-Sox8* transgenic mice was especially pronounced in cortical bone. In fact, Von Kossa staining of undecalcified tibia sections revealed an incomplete cortical bone formation in *Col1a1-Sox8* transgenic mice at 2 wk old (Fig. 7 A). Toluidine blue staining showed that in wild-type mice cortical bone was correctly organized with several osteocytes of flattened appearance and large teams of osteoblasts forming new bone. In contrast, the cortical bone matrix of transgenic mice was disorganized, and osteoblast differentiation was obviously impaired. Therefore, histomorphometric quantification of osteoblast indices was not possible in *Col1a1-Sox8* transgenic mice at that age, because bone-forming cells with the characteristic cuboidal appearance were not detectable (Fig. 7 A). At 10 wk old this phenotype was still present, albeit less severe. Osteoblasts were now quantifiable, but their number was significantly reduced by 45% (Fig. 7 B).

One possible reason for the partial recovery of the *Col1a1-Sox8* transgenic mice came from the observation that the production of type I collagen was strongly decreased in transgenic mice (Fig. 7 C). Because the transgenic expression of *Sox8* is driven by the promoter of this gene, we speculated that *Sox8* expression in the transgenic mice may be down-regulated over time, thereby preventing a more severe phenotype. This was confirmed by Northern blot expression analysis using calvarial RNA from wild-type and transgenic mice at birth, 2 and 10 wk old. Whereas the expression level of the transgene (that could be separated from the endogenous mRNA based on the reduced size) was initially approximately five times higher than the endogenous expression level, we observed a strong decrease of transgene expression at 10 wk old (Fig. 7 D).

We next analyzed whether the disturbed cortical bone formation in the *Col1a1-Sox8* transgenic mice leads to a decreased biomechanical stability. Cross-sectional μCT scans of the femora confirmed the cortical porosity in transgenic mice at 2 wk old. When the stability of these femora was analyzed in three-point–bending assays, the force required to cause bone failure was significantly decreased in transgenic mice (Fig. 7 E). This reduced biomechanical stability is also relevant in vivo, because one founder animal displayed a spontaneous fracture of the humerus. This was observed by contact radiography and histologically confirmed by Mallory staining to verify callus

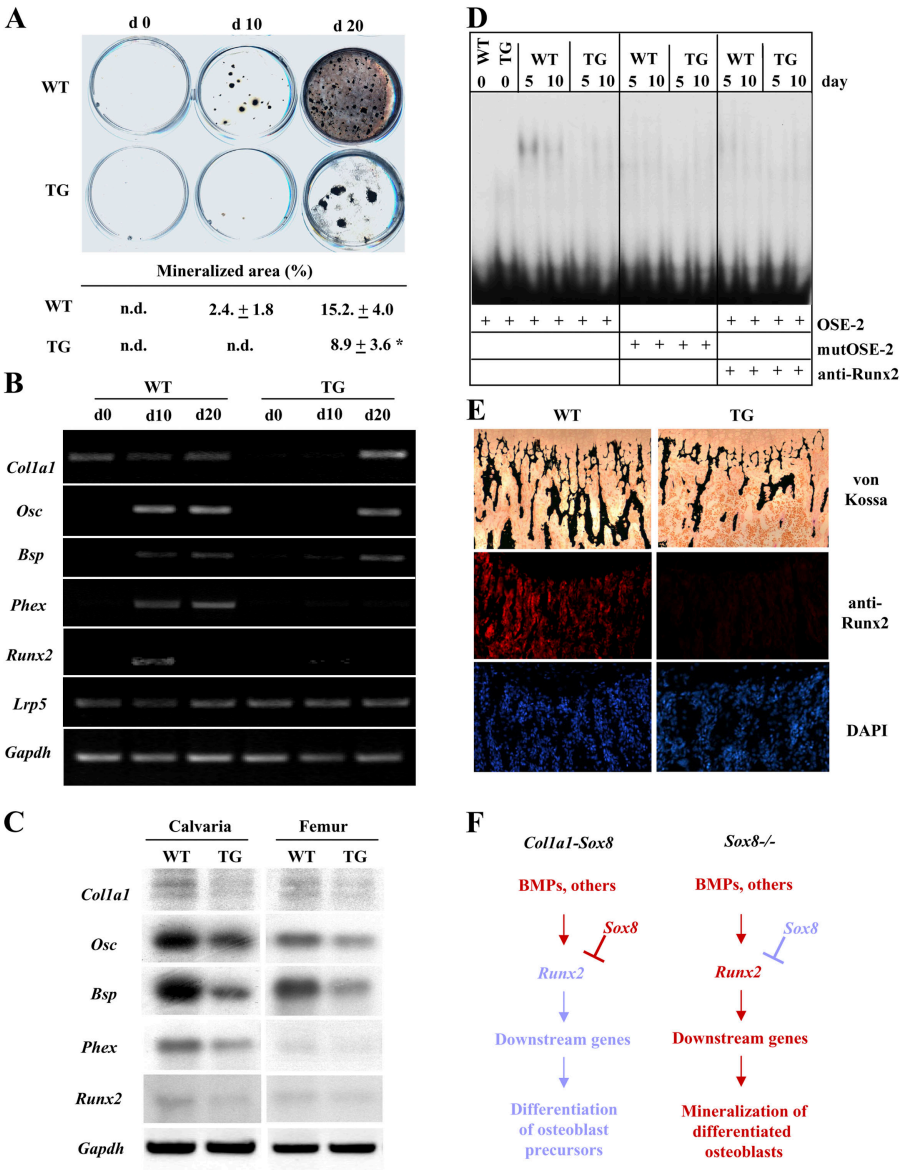

Figure 8. **Decreased *Runx2* expression in *Col1a1-Sox8* transgenic mice.** (A) Von Kossa staining of primary osteoblast cultures from wild-type and *Col1a1-Sox8* transgenic mice after 0, 10, and 20 d of incubation with ascorbic acid and β-glycerophosphate. Mineralization of the transgenic cultures is delayed and reduced compared with wild-type cultures. (B) RT-PCR expression analysis of primary osteoblast cultures from wild-type and *Col1a1-Sox8* transgenic mice at d0, d10, and d20 of differentiation. Note the decreased expression of *Col1a1*, *Osc*, *Bsp*, *Phex*, and *Runx2* in transgenic cultures. (C) A Northern blot expression analysis using calvaria and femur RNA from wild-type and *Col1a1-Sox8* transgenic mice at 2 wk old reveals a reduced expression of *Col1a1*, *Osc*, *Bsp*, *Phex*, and *Runx2* in transgenic bones. (D) An electrophoretic mobility shift assay using the Runx2-binding site (OSE-2) reveals the presence of Runx2 in nuclear extracts from differentiating wild-type cells (d5 and d10), but not in cells derived from transgenic mice. This binding was not observed with a mutated binding site (mutOSE-2) or in the presence of an anti–Runx2-antibody. (E) Analysis of tibia sections from wild-type and *Col1a1-Sox8* transgenic mice at 2 wk old. Von Kossa staining shows the osteoporotic phenotype in the primary spongiosa of transgenic mice (top). Immunohistochemistry using an antibody against Runx2 reveals that the expression of Runx2 is down-regulated in *Col1a1-Sox8* transgenic mice (middle). The counter-staining with DAPI is shown below. (F) Schematic presentation explaining the phenotypes of both mouse models and illustrating the deduced role of Sox8 as a negative regulator of osteoblast differentiation. In osteoblast precursor cells the presence of Sox8 limits the amount of *Runx2* expression keeping the cells in a proliferative state. In differentiated osteoblasts *Sox8* is not expressed, and Runx2 can activate the osteogenic cascade leading to bone formation.

formation (Fig. 7 F). Together, these data demonstrate that *Col1a1-Sox8* transgenic mice display a severe osteoporosis that is caused by an impaired bone formation.

Reduced expression of *Runx2* in osteoblasts from *Col1a1-Sox8* transgenic mice

To confirm that the phenotype of the *Col1a1-Sox8* transgenic mice is caused by an intrinsic osteoblast differentiation defect we analyzed the behavior of primary calvarial osteoblasts from transgenic mice and wild-type littermates. In contrast to wild-type cultures, no mineralized bone nodules were observed after 10 d of differentiation in transgenic cultures. After 20 d only few areas were mineralized in transgenic cultures, whereas wild-type cultures displayed many mineralized nodules (Fig. 8 A). We next performed an RT-PCR expression analysis for osteoblast differentiation markers. In contrast to the *Sox8*-deficient cultures, we observed a delayed and reduced expression of *Osc*, *Bsp*, *Phex*, *Runx2*, and *Col1a1* compared with wild-type cultures, whereas *Lrp5* expression was not significantly changed (Fig. 8 B). Again, these results were confirmed in vivo by Northern blot expression analysis using RNA from calvaria and femur of *Col1a1-Sox8* transgenic mice and wild-type littermates at 2 wk old (Fig. 8 C).

We further performed an electrophoretic mobility shift assay to analyze the presence of Runx2 in wild-type and transgenic osteoblast cultures on the protein level. In nuclear extracts from wild-type cells the complex of Runx2 and the OSE-2 oligonucleotide was present after 5 and 10 d of differentiation (Fig. 8 D). Again, the specificity of this complex was confirmed by its absence using a mutated OSE2 oligonucleotide or a Runx2-specific antibody. Most importantly however, no complex formation was detectable when nuclear extracts from transgenic cells were used (Fig. 8 D). To analyze whether this finding is also relevant in vivo, we performed immunohistochemistry using a Runx2-specific antibody on tibia sections from wild-type and *Col1a1-Sox8* transgenic mice. Whereas we

found strong and specific staining of wild-type sections in the primary spongiosa, such a staining was barely detectable in *Col1a1-Sox8* transgenic mice (Fig. 8 E). Together, these results suggest that Sox8 limits *Runx2* expression in osteoblast precursor cells, and that the down-regulation of *Sox8* is required to allow their differentiation (Fig. 8 F).

Discussion

Several transcription factors have been demonstrated to play crucial roles in cell-specific differentiation processes. These include Sox9 and Sox10, two related members of the Sox-family, that serve important functions in chondrogenesis and gliogenesis (Britsch et al., 2001; De Crombrugghe et al., 2001; Akiyama et al., 2002; Stolt et al., 2002). In this study we describe a novel function for Sox8, the third member of group E of the Sox family, as a negative regulator of osteoblast differentiation. This is underscored by several findings. First, *Sox8*-deficient mice display an osteopenic phenotype that is caused by a precocious osteoblast differentiation. Second, *Sox8*-deficient primary osteoblasts differentiate earlier than wild-type cells, where *Sox8* expression ceases upon differentiation. Third, *Col1a1-Sox8* transgenic mice that retain *Sox8* expression in differentiated osteoblasts display an osteoporotic phenotype caused by a severely impaired bone formation. Together, these data demonstrate that Sox8 is a physiological regulator of bone formation whose expression needs to be tightly controlled during the osteoblast differentiation process. Obviously, this is the first function of Sox8 that cannot be compensated by other members of the Sox family (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200408013/DC1). In contrast, we did not find any defects of growth plate chondrogenesis in the absence of Sox8, suggesting that the size reduction of the *Sox8*-deficient mice is a result of impaired bone formation. This is consistent with a contribution of bone formation to skeletal growth, as it has been shown for example in a mouse model of osteoblast ablation (Corral et al., 1998).

Bone formation through osteoblasts is controlled on different levels (Harada and Rodan, 2003). Whereas endocrine and neuronal mechanisms are important to regulate the functional activity of osteoblasts, at least two transcription factors, Runx2 and Osx, are required for the differentiation of mesenchymal precursor cells into osteoblasts (Ducy et al., 2000; Komori, 2002; Nakashima et al., 2002). Because *Osx* is not expressed in the absence of Runx2, the latter one is generally considered to be a master regulator of osteoblast differentiation. Runx2 activates the expression of most genes associated with osteoblast differentiation and function, and its deficiency in mice results in a complete lack of bone formation (Ducy et al., 1997; Komori et al., 1997). Haploinsufficiency of Runx2 leads to cleidocranial dysplasia, a disorder characterized by defects in endochondral and intramembranous bone formation including short stature, open fontanelles, and hypoplastic clavicles (Lee et al., 1997; Mundlos et al., 1997; Otto et al., 1997). Given the important role of Runx2 in osteoblast differentiation, it is not surprising that its expression needs to be tightly regulated, which is underscored by the phenotype of transgenic mice that overexpress *Runx2* under the control of the *Col1a1* promoter. Unexpectedly, these mice displayed an osteopenia associated with spontaneous fractures (Liu et al., 2001; Geoffroy et al., 2002), thereby demonstrating that the expression level of *Runx2* needs to be limited to allow a coordinated osteoblast differentiation process.

The data presented in this manuscript provide evidence that Sox8 regulates osteoblast differentiation in a Runx2-dependent manner. In fact, the accelerated differentiation and mineralization of *Sox8*-deficient primary osteoblasts may be explained by the premature expression of *Runx2* in these cells. Likewise, the reduced proliferation of the *Sox8*-deficient cells can be explained by the same mechanism, because Runx2 has recently been demonstrated to trigger the exit of preosteoblasts from the cell cycle (Pratap et al., 2003). Furthermore, the severe phenotype of the *Col1a1-Sox8* transgenic mice is reminiscent of cleidocranial dysplasia, because we observed a size reduction of all skeletal elements including the clavicles as well as the characteristic failure of fontanelle closure. Again, this phenotype can be well explained by the strong down-regulation of Runx2 in these mice.

At least two other transcription factors, Twist and Stat1, have been demonstrated to attenuate Runx2-action in vivo (S. Kim et al., 2003; Bialek et al., 2004). Whereas Twist is required to prevent a premature Runx2-dependent osteogenesis during skeletal development, the function of Stat1 is more important in postnatal bone remodeling. In both cases the mechanism of action is posttranscriptional. Whereas the physical interaction of Twist and Runx2 decreases the DNA-binding activity of the latter one, the interaction with Stat-1 prevents the nuclear localization of Runx2 (S. Kim et al., 2003; Bialek et al., 2004). In this study, we have identified Sox8 as another molecule that exerts its inhibitory influence on osteoblast differentiation in a Runx2-dependent manner. As is the case for Stat1, this function is more important postnatally where Sox8 is required to assure a coordinated osteoblast differentiation process during bone remodeling. In contrast to Twist and Stat1 however, Sox8 acts on the transcriptional level, because the expression of *Runx2* is elevated in nondifferentiated primary osteoblasts lacking Sox8. Therefore, it is possible that Sox8 is a direct regulator of *Runx2* promoter activity, although such a mechanism needs to be established by future experiments. The same is the case for the identification of molecular mechanisms regulating *Sox8* expression during osteoblast differentiation. One possibility is that Sox8 is under the control of BMP signaling, because all group E Sox family members have been shown to be induced after implantation of a BMP-7 bead at the tip of the digits of chicken embryos (Chimal-Monroy et al., 2003).

Regardless of these open questions, the analyses of both mouse models described in this manuscript demonstrate that Sox8 is an important transcriptional regulator of osteoblast differentiation. Through its expression in osteoblast precursor cells Sox8 inhibits their terminal differentiation and keeps them in the proliferative stage. Upon differentiation, *Sox8* expression is down-regulated, which is required for an elevation of *Runx2* expression, consequently leading to the initiation of an osteogenic cascade ensuring bone matrix deposition and mineralization by the differentiated osteoblasts.

Materials and methods

Mice

Sox8-deficient mice and wild-type littermates were kept and genotyped as described previously (Sock et al., 2001). To rule out genetic background variations, we only analyzed mice that were backcrossed at least six times with C57Bl/6 mice. *Col1a1-Sox8* transgenic mice were obtained by injecting the transgene into the pronucleus of fertilized oocytes from C57Bl/6 mice according to standard procedures (Bonnerot and Nicolas, 1993). A plasmid containing the *Col1a1* promoter was provided by B. de Crombrugghe (M.D. Anderson Cancer Center, Houston, TX). The plasmid containing the β-globin-intron and growth hormone poly(A) was received from M. Blessing (Universität Leipzig, Leipzig, Germany). For details on the construct, genotyping and expression analysis, see Online supplemental material.

Skeletal analysis

For skeletal preparations newborn mice were dissected and fixed overnight in 95% ethanol. Staining with alcian blue and alizarin red was performed using standard protocols (McLeod, 1980). For radiographic and histologic analysis mice were killed, internal organs were removed and the whole skeletons were fixed in 3.7% PBS-buffered formaldehyde for 18 h at 4°C. The skeletons were analyzed by contact radiography using a Faxitron Xray cabinet (Faxitron Xray Corp.). For three-dimensional visualization the calvariae were scanned (40 kV/114 μA) in a μCT 40 (Scanco Medical) at a resolution of 12 μm. For the assessment of the cortical porosity, femora were scanned at the midshaft at a resolution of 10 μm. The raw data were manually segmented and analyzed with the μCT Evaluation Program V4.4A (Scanco Medical). For visualization, the segmented data were imported and displayed in μCT Ray V3.0 (Scanco Medical). After fixation the skeletons were incubated in 70% ethanol for 24 h. The lumbar vertebral bodies (L3–L5) and one tibia of each mouse were thereafter dehydrated in ascending alcohol concentrations and embedded in methylmetacrylate as described previously (Amling et al., 1999). For details on bone histology and histomorphometry, see Online supplemental material.

Image acquisition

Images were acquired using a Axioskop2 microscope (Carl Zeiss MicroImaging Inc.) with the Zeiss Plan Neofluar objective lenses: 1.25×/0.035; 2.5×/0.075; 10×/0.3; 20×/0.5; 40×/0.75. No immersion fluid was used. Pictures were taken at RT with a Zeiss Axiocam and Zeiss Axiovision Software V3.1. For white balancing and adjustment of brightness and contrast, Adobe Photoshop 7.0 was used.

In situ hybridization, BrdU labeling, X-Gal staining, and immunohistochemistry

In situ hybridization was performed according to standard procedures on 14-μm-thick sections using digoxigenin-labeled antisense riboprobes corresponding to the 3′-untranslated region of the *Col2a1*-cDNA and the third exon of the *ColXa1* gene. For BrdU labeling, 1-wk-old mice were injected with 100 μg BrdU per gram of body weight 2 h before dissection. Incorporated BrdU was detected by immunohistochemistry using a monoclonal anti-BrdU antibody obtained from DakoCytomation according to the manufacturer's instructions. Detection of β-galactosidase activity was performed following standard protocols (Hogan et al., 1994). In brief, tissue specimens were fixed in 1% PBS-buffered PFA for 2 d. For detection of β-galactosidase activity, sections were covered with X-Gal staining solution and incubated at 37°C until blue precipitates were detectable. Immunohistochemistry was performed on 14-μm cryotome sections using specific antibodies against type I collagen (Novacostra) and Runx2 (Santa Cruz Biotechnology, Inc.) as recommended by the manufacturer. Secondary antibodies conjugated to Cy3 immunofluorescent dye (Dianova) were used for detection.

Analysis of primary osteoblast differentiation

Primary osteoblasts were obtained by sequential collagenase digestion of calvariae from 3-d-old mice as described previously (Ducy et al., 2000). Osteoblast differentiation was induced at 80% confluency in α-MEM containing 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Total RNA was extracted using the TRIzol reagent (Invitrogen) immediately before differentiation (d0), as well as 5 and 10 or 20 d thereafter (d5, d10, d20). Northern blot analysis was performed according to standard protocols with the cDNAs encoding *Sox8* and *Gapdh* as probes. BrdU incorporation assays were performed using the Cell Proliferation ELISA Biotrak system obtained from Amersham Biosciences according to the manufacturer's instructions. Mineralization of the cultures was analyzed using Von Kossa staining as described previously (Ducy et al., 2000). The percentage of the mineralized area was quantified using computer aided image analysis (Image tool V2.0; University of Texas Health Science Center, San Antonio, TX). For details on the RT-PCR expression analysis and the electrophoretic mobility shift assays, see Online supplemental material.

Online supplemental material

Fig. S1 shows the histologic analysis of the growth plates from wild-type and *Sox8*-deficient mice at 1 and 6 wk old. Fig. S2 shows a comparison of transgene expression and phenotypes for the offspring derived from the three different founders carrying the *Col1a1-Sox8* transgene. Fig. S3 shows a Northern blot expression analysis for *Sox9* and *Sox10* in wild-type and *Sox8*-deficient osteoblasts and bones. Further comments on the data can be found in the legends. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200408013/DC1.

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References

Akiyama, H., M.C. Chaboissier, J.F. Martin, A. Schedl, and B. de Crombrugghe. 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* 16:2813–2828.

Amling, M., M. Priemel, T. Holzmann, K. Chapin, J.M. Rueger, R. Baron, and M.B. Demay. 1999. Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of a normal mineral ion homeostasis: formal histomorphometric and biochemical analyses. *Endocrinology.* 140:4982–4987.

Amling, M., S. Takeda, and G. Karsenty. 2000. A neuro (endo)crine regulation of bone remodeling. *Bioessays.* 22:970–975.

Bialek, P., B. Kern, X. Yang, M. Schrock, D. Sosic, N. Hong, H. Wu, K. Yu, D.M. Ornitz, E.N. Olson, et al. 2004. A twist code determines the onset of osteoblast differentiation. *Dev. Cell.* 6:423–435.

Bianco, P., F.D. Cancedda, M. Riminucci, and R. Cancedda. 1998. Bone formation via cartilage models: the "borderline" chondrocyte. *Matrix Biol.* 17:185–192.

Bonnerot, C., and J.F. Nicolas. 1993. Guide to techniques in mouse development. *Methods Enzymol.* 225:747–799.

Bowles, J., G. Schepers, and P. Koopman. 2000. Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev. Biol.* 227:239–255.

Britsch, S., D.E. Goerich, D. Riethmacher, R.I. Peirano, M. Rossner, K.A. Nave, C. Birchmeier, and M. Wegner. 2001. The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* 15:66–78.

Chimal-Monroy, J., J. Rodriguez-Leon, J.A. Montero, Y. Ganan, D. Macias, R. Merino, and J.M. Hurle. 2003. Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. *Dev. Biol.* 257:292–301.

Corral, D.A., M. Amling, M. Priemel, E. Loyer, S. Fuchs, P. Ducy, R. Baron, and G. Karsenty. 1998. Dissociation between bone resorption and bone formation in osteopenic transgenic mice. *Proc. Natl. Acad. Sci. USA.* 95:13835–13840.

De Crombrugghe, B., V. Lefebvre, and K. Nakashima. 2001. Regulatory mechanisms in the pathways of cartilage and bone formation. *Curr. Opin. Cell Biol.* 13:721–727.

Ducy, P., R. Zhang, V. Geoffroy, R.L. Ridall, and G. Karsenty. 1997. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell.* 89:747–754.

Ducy, P., M. Starbuck, M. Priemel, J. Shen, G. Pinero, V. Geoffroy, M. Amling, and G. Karsenty. 1999. A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* 13:1025–1036.

Ducy, P., M. Amling, S. Takeda, M. Priemel, A.F. Schilling, F.T. Beil, J. Shen, C. Vinson, J.M. Rueger, and G. Karsenty. 2000. Leptin inhibits bone for-

mation through a hypothalamic relay: a central control of bone mass. *Cell.* 100:197–207.

Foster, J.W., M.A. Dominguez-Steglich, S. Guioli, G. Kowk, P.A. Weller, M. Stevanovic, J. Weissenbach, S. Mansour, I.D. Young, P.N. Goodfellow, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature.* 372:525–530.

Geoffroy, V., M. Kneissel, B. Fournier, A. Boyde, and P. Matthias. 2002. High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. *Mol. Cell. Biol.* 22:6222–6233.

Harada, S., and G.A. Rodan. 2003. Control of osteoblast function and regulation of bone mass. *Nature.* 423:349–355.

Herbarth, B., V. Pingault, N. Bondurand, K. Kuhlbrodt, I. Hermans-Borgmeyer, A. Puliti, N. Lemort, M. Goossens, and M. Wegner. 1998. Mutation of the Sry-related Sox10 gene in *Dominant megacolon*, a mouse model for human Hirschsprung disease. *Proc. Natl. Acad. Sci. USA.* 95:5161–5165.

Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. Staining for beta-galactosidase (lacZ) activity. *In* Manipulating the Mouse Embryo. A Laboratory Manual, Second edition. Trans., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 373–375.

Karsenty, G. 1999. The genetic transformation of bone biology. *Genes Dev.* 13:3037–3051.

Kato, M., M.S. Patel, R. Levasseur, I. Lobov, B.H. Chang, D.A. Glass II, C. Hartmann, L. Li, T.H. Hwang, C.F. Brayton, et al. 2002. Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J. Cell Biol.* 157:303–314.

Kim, J., L. Lo, E. Dormand, and D.J. Anderson. 2003. SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron.* 38:17–31.

Kim, S., T. Koga, M. Isobe, B.E. Kern, T. Yokochi, Y.E. Chin, G. Karsenty, T. Taniguchi, and H. Takayanagi. 2003. Stat1 functions as a cytoplasmic attenuator of Runx2 in the transcriptional program of osteoblast differentiation. *Genes Dev.* 17:1979–1991.

Komori, T. 2002. Runx2, a multifunctional transcription factor in skeletal development. *J. Cell. Biochem.* 87:1–8.

Komori, T., H. Yagi, S. Nomura, A. Yamaguchi, K. Sasaki, K. Deguchi, Y. Shimizu, R.T. Bronson, Y.H. Gao, M. Inada, et al. 1997. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell.* 89:755–764.

Kronenberg, H.M. 2003. Developmental regulation of the growth plate. *Nature.* 423:332–336.

Lee, B., K. Thirunavukkarasu, L. Zhou, L. Pastore, A. Baldini, J. Hecht, V. Geoffroy, P. Ducy, and G. Karsenty. 1997. Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. *Nat. Genet.* 16:307–310.

Liu, W., S. Toyosawa, T. Furuichi, N. Kanatani, C. Yoshida, Y. Liu, M. Himeno, S. Narai, A. Yamaguchi, and T. Komori. 2001. Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J. Cell Biol.* 155:157–166.

McLeod, M.J. 1980. Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology.* 22:299–301.

Mundlos, S., F. Otto, C. Mundlos, J.B. Mulliken, A.S. Aylsworth, S. Albright, D. Lindhout, W.G. Cole, W. Henn, J.H. Knoll, et al. 1997. Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell.* 89:773–779.

Nakashima, K., X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, and B. de Crombrugghe. 2002. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell.* 108:17–29.

Olsen, B.R., A.M. Reginato, and W. Wang. 2000. Bone development. *Annu. Rev. Cell Dev. Biol.* 16:191–220.

Otto, F., A.P. Thornell, T. Crompton, A. Denzel, K.C. Gilmour, I.R. Rosewell, G.W. Stamp, R.S. Beddington, S. Mundlos, B.R. Olsen, et al. 1997. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell.* 89:765–771.

Parfitt, A.M. 1983. The physiologic and clinical significance of bone histomorphometric data. *In* Bone Histomorphometric Techniques and Interpretation. R.R. Recker, editor. CRC Press, Boca Raton, FL. 143–223.

Pfeifer, D., F. Poulat, E. Holinski-Feder, F. Kooy, and G. Scherer. 2000. The SOX8 gene is located within 700 kb of the tip of chromosome 16p and is deleted in a patient with ATR-16 syndrome. *Genomics.* 63:108–116.

Pratap, J., M. Galindo, S.K. Zaidi, D. Vradii, B.M. Bhat, J.A. Robinson, J.Y. Choi, T. Komori, J.L. Stein, J.B. Lian, et al. 2003. Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. *Cancer Res.* 63:5357–5362.

Rossert, J., H. Eberspaecher, and B. de Crombrugghe. 1995. Separate cis-acting DNA elements of the mouse pro-alpha 1(I) collagen promoter direct expression of reporter genes to different type I collagen–producing cells in transgenic mice. *J. Cell Biol.* 129:1421–1432.

Rossert, J.A., S.S. Chen, H. Eberspaecher, C.N. Smith, and B. de Crombrugghe. 1996. Identification of a minimal sequence of the mouse pro-alpha 1(I) collagen promoter that confers high-level osteoblast expression in transgenic mice and that binds a protein selectively present in osteoblasts. *Proc. Natl. Acad. Sci. USA.* 93:1027–1031.

Schepers, G.E., M. Bullejos, B.M. Hosking, and P. Koopman. 2000. Cloning and characterisation of the sry-related transcription factor gene sox8. *Nucleic Acids Res.* 28:1473–1480.

Sock, E., K. Schmidt, I. Hermanns-Borgmeyer, M.R. Bosl, and M. Wegner. 2001. Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. *Mol. Cell. Biol.* 21:6951–6959.

Southard-Smith, E.M., L. Kos, and W.J. Pavan. 1998. Sox10 mutation disrupts neural crest development in *Dom* Hirschsprung mouse model. *Nat. Genet.* 18:60–64.

Stolt, C.C., S. Rehberg, M. Ader, P. Lommes, D. Riethmacher, M. Schachner, U. Bartsch, and M. Wegner. 2002. Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes Dev.* 16:165–170.

Takeda, S., J.P. Bonnamy, M.J. Owen, P. Ducy, and G. Karsenty. 2001. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.* 15:467–481.

Wagner, T., J. Wirth, J. Meyer, B. Zabel, M. Held, J. Zimmer, J. Pasantes, F.D. Bricarelli, J. Keutel, E. Hustert, et al. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell.* 79:1111–1120.

Wegner, M. 1999. From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.* 27:1409–1420.