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

Katy Schmidt,1 Thorsten Schinke,2,3 Michael Haberland,2,3 Matthias Priemel,2,3 Arndt F. Schilling,2,3 Cordula Mueldner,2,3 Johannes M. Rueger,2,3 Elisabeth Sock,1 Michael Wegner,1 and Michael Amling2,3

1Institute of Biochemistry, Friedrich-Alexander-University, Erlangen-Nürnberg, Erlangen 91054, Germany
2Department of Trauma, Hand and Reconstructive Surgery and 3Experimental Trauma Surgery and Skeletal Biology, Center for Biomechanics, Hamburg University School of Medicine, Hamburg 20246, Germany

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 (Krohnemberg, 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...
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 (Ox), 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 (Herbart 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 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 1 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.

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
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.

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 units 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
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 D-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 D-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).

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 Osc 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), Osc (osterix), Osx (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).
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
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 formation in transgenic mice at 2 wk old. Force to failure as determined by three-point bending assays was significantly decreased in transgenic mice (Fig. 7 E).
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, Lrp5, 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 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.

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.
found strong and specific staining of wild-type sections in the primary spongyosa, 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 osteopetrotic 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 Osm, are required for the differentiation of mesenchymal precursor cells into osteoblasts (Ducy et al., 2000; Komori, 2002; Nakashima et al., 2002). Because Osm 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; Geofroy 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 pronuclei of fertilized oocytes from C57Bl/6 mice according to standard procedures (Bonnetot 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 rib of each mouse were thereafter dehydrated in ascending alcohol concentrations and embedded in methyl methacrylate 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 Micro-Imaging 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 Axiovid 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 Col1A1 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 cryo sections using specific antibodies against type I collagen (Novocastra) 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 10% FBS, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate. Total RNA was extracted using 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 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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