Sox5 and Sox6 are needed to develop and maintain source, columnar, and hypertrophic chondrocytes in the cartilage growth plate

Patrick Smits, Peter Dy, Srijeeit Mitra, and Véronique Lefebvre

Department of Biomedical Engineering and Orthopaedic Research Center, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195

Sox5 and Sox6 encode Sry-related transcription factors that redundantly promote early chondroblast differentiation. Using mouse embryos with three or four null alleles of Sox5 and Sox6, we show that they are also essential and redundant in major steps of growth plate chondrocyte differentiation. Sox5 and Sox6 promote the development of a highly proliferating pool of chondroblasts between the epiphyses and metaphyses of future long bones. This pool is the likely cellular source of growth plates. Sox5 and Sox6 permit formation of growth plate columnar zones by keeping chondroblasts proliferating and by delaying chondrocyte prehypertrophy. They allow induction of chondrocyte hypertrophy and permit formation of prehypertrophic and hypertrophic zones by delaying chondrocyte terminal differentiation induced by ossification fronts. They act, at least in part, by down-regulating Ihh signaling, Fgf3, and Runx2 and by up-regulating Bmp6. In conclusion, Sox5 and Sox6 are needed for the establishment of multilayered growth plates, and thereby for proper and timely development of endochondral bones.

Introduction

Cartilage has essential roles in endochondral ossification, a multistep process whereby most bones develop in vertebrates. This process starts in the embryo with the formation of cartilage primordia that already prefigure definitive bones. These primordia then develop growth plates that enlarge skeletal elements and are progressively replaced by bone from fetal stages until adulthood.

Cartilage primordia arise upon condensation of prechondrogenic mesenchymal cells. These cells differentiate sequentially into prechondrocytes and chondroblasts. Prechondrocytes activate Col2a1 (collagen type 2), Agc1 (aggrecan), and other cartilage-specific extracellular matrix genes. Chondroblasts up-regulate these genes and activate others like Mat1 (matrilin-1). They produce large amounts of extracellular matrix and actively proliferate. Several types of signaling molecules and transcription factors control skeleton patterning and cell differentiation at these early stages (DeLise et al., 2000; Hall and Miyake, 2000; de Crombrugghe et al., 2001; Karsenty and Wagner, 2002). The latter include three Sry-related HMG box-containing proteins, L-Sox5, Sox6, and Sox9. L-Sox5 (a long product of Sox5) and Sox6 are highly identical to each other but related to Sox9 only in the HMG box DNA-binding domain (Lefebvre, 2002). Sox5, Sox6, and Sox9 are coexpressed in all cartilage primordia of the mouse embryo and may cooperate to directly activate Col2a1 (Ng et al., 1997; Zhao et al., 1997; Lefebvre et al., 1998). Sox9 is required for prechondrogenic cell condensation, prechondrocyte and chondroblast differentiation, and activation of Sox5, Sox6, and cartilage matrix genes (Bi et al., 1999; Akiyama et al., 2002). Sox5 and Sox6 have essential, redundant roles in early chondroblasts (Smits et al., 2001). Although Sox5+/− and Sox6+/− mice are born with minor cartilage defects, Sox5−/−/Sox6−/− embryos develop a severe, generalized chondrodysplasia due to considerable delay and impairment of chondroblast proliferation and expression of cartilage matrix genes. They die around embryonic day 16.5 (E16.5) with rudimentary and matrix-deficient cartilage primordia.

The fate of each chondroblast is determined by its location in cartilage primordia (Karsenty and Wagner, 2002; Kronenberg, 2003). Although epiphyseal chondroblasts are...
maintained at an early differentiation stage throughout gestation, diaphyseal and metaphyseal chondroblasts mature to form growth plates and induce the formation of primary ossification centers. Upon undergoing prehypertrophy, they stop proliferating and sequentially activate Ppr (receptor for parathyroid hormone and parathyroid hormone–related peptide), Ihh (Indian hedgehog), and Col10a1 (collagen type 10). They also induce the flanking perichondrium to form a bone collar. Next, they undergo hypertrophy, stop expressing Col2a1, Agc1, and other early markers, progressively down-regulate Ppr and Ihh, up-regulate expression of Col10a1, and induce cartilage matrix mineralization. As they terminally differentiate, they stop expressing Col10a1, activate Opn (osteopontin) and Mmp13 (matrix metalloproteinase-13), and undergo apoptosis. Bone collar chondro/osteoclasts, osteoblasts, and blood vessels then invade and replace the cartilage by bone. Various signaling molecules control these processes. They include Ihh, PTHrP (parathyroid hormone–related peptide), and PPR (Karaplis et al., 1994; Lanske et al., 1996; St-Jacques et al., 1999), FGFR3 (fibroblast growth factor receptor-3) and its ligand FGF18 (Colvin et al., 1996; Deng et al., 1996; Liu et al., 2002; Ohbayashi et al., 2002), bone morphogenetic proteins (BMPs; Zou et al., 1997), and Wnt proteins (Hartmann and Tabin, 2000; Yang et al., 2003). Few transcription factors have yet been demonstrated to control these processes. Cbfa1/Runx2 is expressed in late chondroblasts and in prehypertrophic and hypertrophic chondrocytes (Inada et al., 1999; Kim et al., 1999). It induces prehypertrophy as shown by a block of chondrocyte differentiation before prehypertrophy in several skeletal elements of Runx2–/– mouse embryos and by maturation of transgenic chondroblasts forced to express Runx2 (Takeda et al., 2001). However, because Runx2–/– chondrocytes properly mature in some skeletal elements, additional factors must contribute to induce chondrocyte maturation. Sox5, Sox6, and Sox9 are turned off upon chondrocyte prehypertrophy (Lefebvre et al., 1998). Sox9+/- embryos precociously mineralize cartilage primordia and develop enlarged hypertrophic zones (Bi et al., 2001). Thus, Sox9 might delay chondrocyte hypertrophy. When Sox5+/–/Sox6+/- fetuses die, their cartilage primordia contain few prehypertrophic chondrocytes but no hypertrophic cells. They are being invaded by thick bone collars but feature no cartilage growth plates and no ossification centers (Smits et al., 2001). Therefore, these fetuses do not allow for a full assessment of the roles of Sox5 and Sox6 in the growth plate.

To determine such roles, we mostly analyzed here embryos with three null alleles (3NA) of Sox5 and Sox6 (Sox5+/–/Sox6−/– and Sox5−/–/Sox6−/–). These embryos live until birth and show severe growth plate chondrocyte defects. We show that Sox5 and Sox6 are needed to form and maintain a pool of highly proliferating chondroblasts between epiphyses and metaphyses, to form columnar chon-
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Sox5 and Sox6 control growth plate chondrocytes, delay chondrocyte prehypertrophy but promote hypertrophy, and to delay terminal differentiation of chondrocytes on contact with ossification fronts. Sox5 and Sox6 act in part by controlling the domain of action and expression of major regulatory factors.

**Results**

**Chondro dysplasia of 3NA embryos**

By E14.5, all cartilage primordia of 3NA embryos were hypoplastic and weakly stained with Alcian blue (Fig. 1 A). Some were also bent. In comparison, only the Meckel’s, costal, and a few other cartilages were hypoplastic in 2NA embryos (Fig. 1 A), and all 4NA cartilage primordia were rudimentary and hardly stained with Alcian blue (Smits et al., 2001). 1NA and wild-type embryos were indistinguishable from each other at this and later stages, and therefore were used as controls throughout this work. The onset of mineralization was delayed in all 3NA endochondral skeletal elements, such as in vertebral bodies (Fig. 1 B). The two types of 3NA mice died at birth from respiratory distress and featured virtually identical skeletal defects (Fig. 1, C–G). They had a foreshortened snout, micrognathia, short trunk, and short, bent limbs (Fig. 1 C). Forelimb (Fig. 1 D) and other long bones were shorter than normal, but not always thinner, and some were bent. Cervical vertebrae start to ossify later than lumbar and sacral vertebrae. In 3NA newborns, the former had not yet started to mineralize (Fig. 1 E), whereas the latter were ectopically mineralized (Fig. 1 F). The sternum was the most affected 3NA element (Fig. 1 G). It was short and thick, and intersternebrae were ectopically mineralized. Thus, 3NA embryos developed generalized cartilage primordium hypoplasia and skeleton growth and mineralization defects that strongly suggested roles for Sox5 and Sox6 in chondrocyte maturation.

**Precocious prehypertrophy of chondrocytes in 3 and 4NA cartilage primordia**

3NA chondroblasts started to differentiate with a significant delay compared with control cells but ultimately formed close-to-normal cartilage primordia. For instance, 3NA tibia chondroblasts were surrounded by less cartilage extracellular matrix and were expressing Agc1 at a lower level than control cells at E13.5 (Fig. 2 A). In contrast, 3NA humerus chondroblasts, which develop earlier, were expressing Agc1 and other markers at close-to-normal levels and had accumulated a fair amount of cartilage matrix (Fig. 2 B). The onset of chondrocyte prehypertrophy was delayed in all 3NA cartilage primordia, as seen for Ppr activation in E13.5 tibias. Interestingly, once the first core cells became prehypertrophic, their neighbors became prehypertrophic more rapidly in mutants than in controls, as seen for Ppr and Col10a1 activation in E13.5 humeri. The sternum was the most affected 3NA element (Fig. 2 C). Sternebral but not intersternebral chondroblasts were prehypertrophic in E16.5 control sternums, as seen by cell enlargement and Ppr expression. In contrast, both sternebral and intersternebral chondrocytes were already prehypertrophic in 3NA sternums. 4NA humeri were poorly developed by E14.5 (Fig. 2 D). The expression domain of Mat1, a marker of both chondroblasts and prehypertrophic chondrocytes, was wide in control humeri and overlapped only partially with that of Ppr. In contrast, few cells were expressing Mat1 in 4NA humeri and most of them were also expressing Ppr. Thus, these data
demonstrated that Sox5 and Sox6 are needed to prevent chondroblast premature prehypertrophy.

Delayed chondrocyte hypertrophy and terminal differentiation in 3NA cartilage primordia

By E14.5, chondrocytes in the core regions of control humeri still exhibited a moderate size, typical of prehypertrophic cells, but they had already induced matrix mineralization (Fig. 3 A). They had inactivated Ppr, were still expressing Col10a1, and had activated Opn and Mmp13 (Fig. 3 B). Thus, these chondrocytes had rapidly progressed from prehypertrophy to terminal differentiation. Flanking chondrocytes were fully enlarged, had induced cartilage mineralization, and were expressing Col10a1 at the highest level. Thus, they were overtly hypertrophic. Both core and flanking cells were surrounded with a mineralized bone collar in which osteoclasts were expressing Mmp9. Mmp9-positive cells were also detected in the cartilage core, indicating the onset of endochondral ossification. In 3NA humeri, both core and flanking cells were still prehypertrophic. Indeed, they were moderately enlarged, were not mineralizing the cartilage matrix, were expressing Ppr strongly and Col10a1 weakly, and were not expressing Mmp13. The perichondrium had not developed into a mineralized bone collar but contained Mmp9-expressing osteoclasts. Interestingly, chondrocytes expressing Opn were found adjacent to the bone collar and were thus terminally differentiating. By E15.0, the core of control humeri was ossifying (Fig. 3, C and D). It contained Mmp9-expressing osteoclasts/chondroclasts and cells expressing Opn and Mmp13, which were either terminal chondrocytes or mature osteoblasts. The core of 3NA humeri was still cartilaginous, but its cells were terminally differentiating as seen by Ppr inactivation and Opn and Mmp13 activation. The cartilage matrix was mineralized only next to the bone collar and was starting to be invaded by bone. By E15.5, the 3NA humerus core was fully ossified (Fig. 4 A). Thus, 3NA chondrocytes were delayed and impaired in undergoing hypertrophy but terminally differentiating next to ossification fronts.

Defective columnar and hypertrophic zones in 3NA growth plates

To determine the roles of Sox5 and Sox6 in established growth plates, we compared the development of control and 3NA humeri from E13.5 until birth by measuring the elongation of their epiphyses, columnar and hypertrophic regions, and primary ossification centers (Fig. 4 A). The hypertrophic region was defined by enlarged cells, and thus contained late prehypertrophic, hypertrophic, and terminal chondrocytes. The columnar region contained metaphyseal chondroblasts and early prehypertrophic chondrocytes. Mutant and control humeri were similar at E13.5 (Fig. 4 B). From that time on, mutant epiphyses elongated at the same rate as controls. In contrast, the mutant columnar and hypertrophic zones became dramatically smaller than the controls, remaining only at 43% and 63% of the height of the respective control zones at birth. The mutant bone segments grew at 65–70% of the rate of controls. High magnification pictures of newborn humerus sections showed that the shortness of the mutant columnar region was not due to a
more compact cell organization or a failure of the cells to organize into columns (Fig. 4 C). The mutant region contained half as many cell layers as the control, corresponding to the height reduction. In contrast, the mutant hypertrophic zone contained 25% fewer cell layers but was 37% shorter than normal. Therefore, its reduced height was in part due to the smaller size of the cells. We measured that mutant hypertrophic cells were on average 83% as high as control cells in the four layers abutting the ossification front. Because endochondral bone forms by replacement of these layers and because 3NA bone was expanding at 65–70% of the normal rate, the 3NA bone grew slowly not only because hypertrophic chondrocytes were smaller but probably also because terminal chondrocytes died at a slower rate. Together with the absence of columnar and hypertrophic cells in 4NA elements (Smits et al., 2001), these data demonstrate that Sox5 and Sox6 ensure optimal growth of endochondral bones by specifically promoting the formation and maintenance of columnar and hypertrophic chondrocytes.

Reduced proliferation and precocious prehypertrophy of 3NA growth plate chondroblasts

The size of the columnar region is determined by the rate of cell proliferation within this zone and by the rate at which chondroblasts exit this zone to become hypertrophic. It may also depend on the rate at which epiphyseal chondroblasts enter this zone (Kobayashi et al., 2002). To measure the rate of chondroblast proliferation, we labeled proliferating cells in vivo with BrdU and counted the percentages of positive cells in small segments juxtaposed from the presumptive joint to the center of cartilage primordia or to the hypertrophic zone of growth plates (Fig. 5). We found that control chondroblasts proliferated at highly variable rates in different subregions of epiphyses and growth plates. They proliferated at the highest levels at the top of metaphyses and elongating humeri shafts primarily arise from cells proliferating maximally at the top of metaphyses, and that cells in the proximal half of E16.5 epiphyses arise from cells highly proliferating in periarticular regions. Ep, epiphysis; M, metaphysis; Ce, center; Col, columnar zone.
Reduced hypertrophic zone in 3NA growth plates. (A) Longitudinal sections through humeri of control and 3NA newborn mice. Consecutive sections were stained with Alcian blue or the von Kossa reagent, or hybridized with RNA probes, as indicated. Pictures focus on the lower part of growth plates and are aligned at the level of the ossification front. The von Kossa pictures show the last cell layers of the growth plates at high magnification. White arrows, expression domains of tested genes. Green arrows, cell layers between the gene expression domain and the ossification front. Blue arrows, layers of matrix-mineralizing chondrocytes. (B) Longitudinal sections through humeri of control and 4NA E15.5 fetuses. Consecutive sections were stained with Alcian blue or the von Kossa reagent, or hybridized with RNA probes, as indicated. Control pictures show the proximal growth plate of the humerus and mutant pictures show the entire humerus. The von Kossa pictures were taken at higher magnification.

Inability of chondrocytes to undergo overt hypertrophy in 3NA and 4NA long bone growth plates

Because chondrocytes no longer proliferate beyond early prehypertrophy, the number of cell layers in the hypertrophic region only reflects the time spent by the cells in this region. We determined if 3NA cells proceeded faster through late prehypertrophy, hypertrophy, or terminal differentiation by analyzing expression of stage-specific markers (Fig. 6 A). In newborn control humeri, Ppr is highly expressed in prehypertrophic chondrocytes and weakly expressed in hypertrophic and terminal cells. In 3NA humeri, its high expression domain was slightly reduced and its low expression domain strongly reduced. Col10a1 is highly expressed in prehypertrophic and hypertrophic chondrocytes and turned off in terminal chondrocytes. Its expression domain was strongly reduced in mutants. The terminal chondrocyte marker Mmp13 was similarly expressed in control and mutant growth plates. von Kossa staining revealed cartilage mineralization around several layers of lower hypertrophic and terminal chondrocytes in controls, but only around terminal chondrocytes in mutants. Thus, the shortening of the 3NA hypertrophic region was mostly due to reduction of the hypertrophic zone. In E15.5 4NA humeri (Fig. 6 B), Mmp13 was expressed in cells lining the bone collar and Ppr was expressed in cells adjacent to them. There was no Col10a1 expression and no cartilage matrix mineralization. Thus, 4NA cells were terminally differentiating immediately after reaching prehypertrophy. Thus, Sox5 and Sox6 promote chondrocyte hypertrophy and prevent terminal differentiation of prehypertrophic and hypertrophic cells.

Absence of growth plates in 3NA sternum and vertebrae

Control lumbar vertebrae featured short, bidirectional growth plates flanking the ossification centers of vertebral bodies and neural arches (Fig. 7 A). In contrast, growth plates were lacking in 3NA vertebrae and replaced with cartilage-mineralizing fully hypertrophic cells. Control sternums featured ossified sternebrae and intersternebral growth plates, whereas 3NA sternebrae and intersternebral were both mostly filled with fully enlarged, matrix-mineralizing chondrocytes (Fig. 7 B). These cells were hypertrophic or terminally differentiated, expressing Col10a1 and Mmp13, whereas the other chondrocytes were prehypertrophic, expressing Ppr and Ihh (Fig. 7 C). Thus, the abundance of hypertrophic chondrocytes in 3NA sternums contrasted with the reduction of hypertrophic zones in future long bones but indicated that 3NA chondrocytes were retaining the ability to undergo overt hypertrophy. Interestingly, 3NA sternum chondrocytes were maturing from the ventral to the dorsal side of the sternum, perpendicularly to the normal maturation gradient. The ventral side of the 3NA sternum was a nonmineralized perichondrium/periosteum, whereas the dorsal side was a continuous bone collar. Thus, terminal chondrocytes were developing against ossification fronts, as in long bones.
Changes in expression and activity of regulatory molecules in 3NA growth plates

Next, we determined whether or not the 3NA growth plate defects could be explained by changes in expression or activity of known regulatory molecules. Expression of Sox9, which may prevent chondrocyte hypertrophy, was unchanged in 3NA growth plates (unpublished data). Expression of Runx2, which can induce chondrocyte prehypertrophy, was strongly up-regulated in 3NA late chondroblasts, prehypertrophic chondrocytes, and periosteum cells in E13.5 and E14.5 humeri (Fig. 8 A). Thus, Sox5 and Sox6 may prevent prehypertrophy at least in part by down-regulating Runx2. Expression of Pthrp, which prevents chondroblast maturation, was similar in control and 3NA periarticular chondroblasts (Fig. 8 B). Ihh, which has major roles in the growth plate, including stimulation of chondroblast proliferation (St-Jacques et al., 1999), was expressed at similar levels in 3NA and control chondrocytes (Fig. 8 C). However, more 3NA cells were expressing Ihh than control cells between E13.5 and E15.0 due to precocious prehypertrophy and delayed hypertrophy, and fewer 3NA cells were expressing Ihh than control cells later on due to reduction of the hypertrophic region. Interestingly, Ptc1, which encodes an Ihh receptor and is an Ihh transcriptional target, was up-regulated in 3NA chondroblasts, peristeme, and primary spongiosa between E13.5 and birth (Fig. 8 D). Moreover, although it was widely expressed in early cartilage primordia but restricted to columnar chondroblasts in established growth plates in control embryos, it remained widely expressed in 3NA fetal cartilages. Thus, these Ptc1 data revealed an increase in Ihh signaling that was unexpected given the proliferation defect of 3NA chondroblasts. Hence, Sox5 and Sox6 unlikely control chondroblast proliferation by modulating Ihh expression or signaling. Fgfl8, which is expressed in the perichondrium and signals through FGFR3 in chondrocytes (Liu et al., 2002; Ohbayashi et al., 2002), was expressed at a normal level in 3NA skeletal elements (unpublished data). Fgfr3, which is expressed in columnar chondroblasts and inhibits cell proliferation (Deng et al., 1996; Colvin et al., 1996), was found expressed in a positive gradient from the top to the bottom of the columnar zone in control growth plates. Thus, this gradient was opposite to the cell proliferation gradient. Interestingly, Fgfr3 expression followed the same gradient in 3NA growth plates but was up-regulated earlier (Fig. 8 E). Therefore, Sox5 and Sox6 are likely to control chondroblast proliferation at least in part by delaying Fgfr3 up-regulation. Bmp2, Bmp4-7, Bmpr1a, and Bmpr1b are expressed in discrete domains in the growth plate, perichondrium, and periosteum and interact with the Ihh and FGF signaling pathways. Of these genes, only expression of Bmp6 was altered in 3NA elements (Fig. 8 F and not depicted). Its expression in developing joints was unchanged, but its expression in prehypertrophic chondrocytes was down-regulated. Expression of Wnt5a and Wnt5b, which control chondroblast proliferation and differentiation, was not altered in 3NA growth plates (unpublished data). As a result, Sox5 and Sox6 likely control growth plate chondrocytes at least in part through the key regulatory molecules RUNX2, IHH, FGFR3, and BMP6.

Discussion

This work has identified multiple, essential roles for Sox5 and Sox6 in cartilage growth plates and has allowed for a better understanding of the development and molecular control of these highly specialized structures. We have demonstrated that fetal chondroblasts proliferate at the highest level between epiphyses and metaphyses of future long bones. There-
Therefore, we propose that these cells must be the main cellular source of growth plates. Sox5 and Sox6 are needed for the optimal proliferation of these cells. They are also needed to develop and maintain growth plate columnar zones by allowing chondroblasts arising from the source to decrease their proliferation rate at a slow pace and delay prehypertrophy. Although no longer expressed beyond chondrocyte prehypertrophy, Sox5 and Sox6 are required for chondrocyte hypertrophy and to prevent precocious terminal differentiation of prehypertrophic and hypertrophic chondrocytes. The observation that Sox5/Sox6 mutant chondrocytes terminally differentiate only on contact with ossification fronts suggests an important role for ossification fronts in this last differentiation step. Finally, Sox5 and Sox6 were found to act in the growth plate at least in part through major regulatory factors. Sox5/Sox6 3NA mice and Sox9 mutant mice. This work would not have been possible using only 4NA mice because these mice die at ~E16.5 with no true growth plates (Smits et al., 2001). 3NA embryos constituted a good complementary model because their generalized chondrodysplasia is mainly due to growth plate defects. Their chondrocytes are defective at many differentiation steps but do not arrest at any specific step and do complete their differentiation program before mouse death. Therefore, they have allowed us to study the roles of Sox5 and Sox6 at each of these steps. The two types of 3NA mice, Sox5−/−/Sox6−/− and Sox5−/−/Sox6−/− mice, exhibit virtually identical growth plate phenotypes, indicating that Sox5 and Sox6 are redundant in all steps of chondrocyte maturation. They are more affected than 2NA mice but less affected than 4NA mice, demonstrating that the expression dosage of Sox5 and Sox6 is critical for proper chondrogenesis. Sox5/Sox6 3NA mice resemble Sox9−/− mice (Bi et al., 2001) by the developmental delay and matrix deficiency of cartilage primordia as well as by ectopic mineralization and bending of endochondral elements. However, major differences can be noticed. The onset of cartilage mineralization is precocious in Sox9−/− fetuses but delayed in Sox5/Sox6 3NA fetuses; endochondral elements are short but not thin in Sox5/Sox6 3NA newborns and thin but not always short in Sox9−/− newborns; columnar zones are short in Sox5/Sox6 3NA fetuses but normal in Sox9−/− fetuses; and hypertrophic zones are short in Sox5/Sox6 3NA fetuses but long in Sox9−/− fetuses. These differences are unlikely due to the degree of severity of the phenotypes because Sox5/Sox6 2NA, 3NA, and 4NA mice have qualitatively
identical defects. Thus, Sox5, Sox6, and Sox9 must have distinct functions during chondrocyte maturation.

Source chondroblasts
The presence of a major chondroblast proliferation peak between epiphyses and metaphyses in fetal long bones leads us to revisit the main cellular source of the growth plate. Most studies with fetal growth plates have assumed that epithyseal chondroblasts (often referred to as reserve, resting, or periarticular chondrocytes) give rise to columnar chondroblasts. However, no definitive experimental evidence has validated this model. In contrast, our data strongly suggest that epithyseal chondroblasts elongate through cell proliferation mainly in the periarticular region and epithyseal side of the major proliferation peak, and that columnar zones are generated and renewed mainly by cells originating in the metaphyseal side of this peak. Two pieces of data support our model. First, sternum growth plates develop and are maintained in the absence of epithyseal cells. They present in pairs oriented back-to-back and sharing a pool of intersternebral chondroblasts. This pool is the likely equivalent of the major proliferation peak in long bones. Second, long bone growth plates become postnatally restricted to the region of the major proliferation peak and metaphysis. They are physically separated from articular cartilage by the secondary ossification centers that develop in epithyseal cells. Therefore, their cellular source cannot be in articular cartilage. Consistent with our model, a study in rabbits has suggested that postnatal growth plates find their cell source just above the columnar zone, in a zone (called resting zone) that likely corresponds to the fetal chondroblast proliferation peak (Abad et al., 2002).

Epiphyseal chondroblasts
When 4NA fetuses die, their epiphyses are still largely precartilaginous. In contrast, epithyseal chondroblasts differentiate with only a slight delay in 3NA embryos, and 3NA epithyseal cells are essentially normal at birth. These data indicate that epithyseal chondroblasts require Sox5 and Sox6 for development, but only at a low dosage. PTHrP/PPR signaling prevents ectopic maturation of epithyseal chondroblasts (Lanske et al., 1996; Chung et al., 1998; Kobayashi et al., 2002). Ectopic maturation of epithyseal chondroblasts does not occur in 3NA cartilages, which is consistent with the normal expression of Ppr and Pthrp detected in these cartilages. Therefore, PTHrP/PPR signaling does not require high levels of Sox5 and Sox6.

Columnar chondroblasts
3NA chondroblasts fail to proliferate at maximum levels at the top of the columnar zone and decrease their proliferation rate much faster than control cells. Thus, columnar chondroblasts require a high dosage of Sox5 and Sox6 to function optimally, in contrast to epithyseal chondroblasts. Nevertheless, Sox5 and Sox6 appear expressed at similar levels from the joint area to the prehypertrophic zone (Lefebvre et al., 1998; unpublished data), which means that their expression levels do not directly dictate the cell proliferation changes. Ihh signaling stimulates chondroblast proliferation (St-Jacques et al., 1999). However, because Ihh levels are likely inversely proportional to the gradient of cell proliferation in the columnar zone, Ihh signaling probably attenuates rather than generates this gradient. Interestingly, Ihh expression is not substantially altered in 3NA cartilages, and Ihh signaling actually functions better in these cartilages than in control cartilages. This result was revealed by up-regulation and expansion of the expression domain of Ptc1. It has been shown that proteoglycans or glycosaminoglycans facilitate the movement of Ihh through tissues (Gritli-Linde et al., 2001). Therefore, it is possible that the 3NA cartilage matrix deficiency allows for a better diffusion of Ihh, and hence, for increased signaling. The other possibility, that 3NA chondroblasts are more responsive to Ihh signaling, is not supported by the fact that Ptc1 is also up-regulated in 3NA perichondrium and bone cells, which do not express Sox5 and Sox6.

Minina et al. (2001) have shown that BMP signaling is required in addition to Ihh signaling to maintain chondroblast proliferation in limb explants, but a definitive role for BMP signaling in columnar chondroblasts has not been demonstrated in vivo. Nevertheless, inhibition of early chondroblast proliferation in Bmpr1b−/− mice (Baur et al., 2000; Yi et al., 2000) and continued expression of Bmpr1b and several BMP genes in the growth plate and perichondrium strongly suggest that BMP signaling must contribute to columnar chondroblast proliferation. Expression of BMP and BMP receptor genes is not altered in 3NA cartilages, except for Bmp6, which is expressed in prehypertrophic chondrocytes. Although the exact role of Bmp6 in growth plates has not been fully determined (Solloway et al., 1998), Sox5 and Sox6 appear to act upstream of BMP signaling, possibly in controlling chondroblast proliferation.

Fgf3 and Fgf18 negatively regulate chondroblast proliferation, acting at least in part by down-regulating Ihh expression and signaling (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1998; Liu et al., 2002; Minina et al., 2002; Ohbayashi et al., 2002). Fgf18 is expressed in the perichondrium, and its expression is unaffected in 3NA elements. Fgf3 is expressed in the columnar zone, and we found that its expression follows a positive gradient from the top to the bottom of this zone. This finding suggests that FGFR3 signaling generates the negative gradient of columnar chondroblast proliferation. Interestingly, 3NA chondroblasts up-regulate Fgfr3 earlier than control cells in the columnar zone, strongly suggesting that Sox5 and Sox6 control columnar chondroblast proliferation at least in part by delaying Fgfr3 up-regulation.

Chondrocyte prehypertrophy
In the cartilage primordia of future long bones, 3NA and 4NA chondroblasts activate the prehypertrophic marker Ppr later than control cells when considering the gestation time. However, when considering the time at which the cells become chondroblastic, 3NA chondroblasts activate Ppr faster than control cells. In the sternum primordium, activation of Ppr occurs so early and is so widespread that it is truly ectopic. Thus, Sox5 and Sox6 are needed to delay chondrocyte prehypertrophy. In seeking for the underlying mechanism, we found that Sox5 and Sox6 are needed to delay and down-regulate expression of the prehypertrophy inducer Runx2. However, their mode of action is likely indirect because Runx2 is also up-regulated in the perichondrium where Sox5
and Sox6 are not expressed. Significant changes in expression of Pthrp and Ppr, which delay prehypertrophy, are not detected in 3NA growth plates, indicating that Sox5 and Sox6 unlikely act upstream of PPR signaling. FGF signaling accelerates the onset and the pace of prehypertrophic chondrocyte differentiation in limb explants in vitro and BMP signaling antagonizes the effect of FGF signaling (Minina et al., 2002). Because Sox5 and Sox6 might control chondroblast proliferation at least in part by delaying Fgfr3 up-regulation and by down-regulating Runx2. Finally, Sox5 and Sox6 are needed to allow induction of chondrocyte hypertrophy and to delay terminal differentiation of prehypertrophic and hypertrophic chondrocytes induced by ossification fronts.

**Chondrocyte hypertrophy and terminal differentiation**

Chondrocytes become hypertrophic with a significant delay in 3NA cartilage primordia, and then form short hypertrophic zones because they terminally differentiate prematurely. In 4NA embryos, chondrocytes skip hypertrophy as seen namely by failure to express Col10a1, the most specific marker of hypertrophic chondrocytes, and directly differentiate from early prehypertrophy to the terminal stage. This critical role of Sox5 and Sox6 in allowing chondrocytes to undergo hypertrophy and in delaying terminal differentiation appears opposed to that of Sox9, which may prevent chondrocyte hypertrophy and accelerate terminal differentiation (Bi et al., 2001). Sox9 is expressed at a normal level in 3NA and 4NA chondroblasts and is correctly turned off in 3NA hypertrophic cells (Smits et al., 2001; unpublished data). Therefore, it is not involved in the phenotype of Sox5/Sox6 mutants. Because Sox5, Sox6 and Sox9 are no longer expressed in hypertrophic chondrocytes, their actions on these cells must be indirect. Runx2 is needed for chondrocyte prehypertrophy and may still be needed for hypertrophy because it was proposed to directly activate Col10a1 (Zheng et al., 2003). Therefore, its up-regulation in 3NA cells is at odds with the impairment of hypertrophy, unless Runx2 promotes prehypertrophy but delays hypertrophy, or cooperates with another yet unknown factor to specify chondrocyte hypertrophy. The signaling pathways that promote chondrocyte hypertrophy are still unknown, but the possibility that chondroblasts depend on Sox5 and Sox6 to express signaling molecules mediating hypertrophy of neighboring chondrocytes is not supported by observations made in Sox9/Col2-Cre mutants (Akiyama et al., 2002). In these mutants, cells in the core of cartilage primordia appear to undergo normal differentiation up to and including hypertrophy, whereas all other cartilage cells remain prechondrocytic and fail to activate Sox5 and Sox6. This suggests that chondrocyte hypertrophy is controlled cell autonomously. A mechanism whereby Sox5 and Sox6 might be required cell autonomously but indirectly for chondrocyte hypertrophy might be that chondroblasts need to surround themselves with a proper cartilage matrix, a function promoted by Sox5 and Sox6, to be able to undergo hypertrophy.

Despite hypertrophy impairment, 3NA and 4NA chondrocytes undergo terminal differentiation, a step that is not yet fully understood at the regulatory level. Several works have shown a block of chondrocyte differentiation at the mineralizing stage, for instance upon inactivation of Mmp9 (Vu et al., 1998), Vegf (Gerber et al., 1999), Runx2 (Komori et al., 1997; Otto et al., 1997), or Osx (Nakashima et al., 2002), but these works have not demonstrated whether chondrocytes are blocked at the hypertrophic or terminal stage. We have shown that 3NA and 4NA chondrocytes terminally differentiate only on contact with ossification fronts, strongly suggesting that chondrocyte terminal differentiation is induced at least in part by ossification fronts.

3NA cells undergo terminal differentiation even if they do not reach full hypertrophy, and 4NA cells do so immediately after reaching prehypertrophy, indicating that hypertrophy is a dispensable step in the chondrocyte pathway. Thus, Sox5 and Sox6 allow chondrocytes to add hypertrophy in their pathway, a step determinant for the proper elongation of en-
Docked chondrocyte elements. Here, they may also function by allowing cells to accumulate extracellular matrix and thereby protect themselves against bone invasion. In agreement with this model, 3NA sternum chondrocytes may be able to become hypertrophic because they undergo prehypertrophy so massively that ossification fronts cannot invade them all at once.

Conclusions
This work has demonstrated essential, redundant roles for Sox5 and Sox6 in chondrocyte differentiation and proliferation (Fig. 9) beyond their critical role in promoting differentiation of prechondrocytes into early chondroblasts (Smits et al., 2001). Sox5 and Sox6 ensure the development of early chondroblasts into epiphyseal, source, and columnar chondroblasts and delay chondrocyte prehypertrophy. They stimulate the proliferation of source chondroblasts, keep columnar chondroblasts proliferating, and prevent precocious prehypertrophy at least in part by delaying up-regulation of Fgfr3 and by down-regulating Runx2. They are also needed to allow development of hypertrophic chondrocytes and to prevent precocious terminal differentiation of prehypertrophic and hypertrophic chondrocytes. Because they are no longer expressed in chondrocytes beyond prehypertrophy, their actions in hypertrophic and terminal chondrocytes must be indirect, possibly extracellular matrix mediated. Thus, Sox5 and Sox6 are required for the establishment of cartilage growth plates and thereby for the proper and timely development of endochondral bones.

Materials and methods
Generation of Sox5/Sox6 mutant mice
The generation and genotyping of Sox5/Sox6 mutant mice was previously described (Smits et al., 2001). Mice were 129/SvEv × C57Bl/6 hybrids, and skeletal phenotypes were fully penetrant. 3 and 4NA mice were generated by crossing Sox5/Sox6 double heterozygous males with single or double heterozygous females. All experiments were repeated with two or more pairs of control and mutant littersmates.

Skeletostaining, histology analysis, and RNAsituation hybridization
Whole-mount staining of embryos, skeletal preparations, and histology were performed as described previously (Smits et al., 2001). Samples from control and mutant littersmates were embedded in the same paraffin blocks to minimize experimental variation. Sections stained with Alcian blue or with the von Kossa reagent were counterstained with nuclear fast red. The Bmp6 probe was an 893-bp EcoRI–SacI fragment of the mouse cDNA (a gift from Y. Furuta, University of Texas, Houston, TX), the Mmp13 probe was the first 699 nucleotides of the mouse cDNA, the Opn probe was a 1.3-kb EcoRI fragment of the rat cDNA (a gift from K. Nakashima and B. de Crombrugghe, University of Texas, Houston, TX), and the Ptc1 probe was the full-length mouse cDNA (a gift from R.L. Johnson, University of Texas, Houston, TX). The Apc1 and Col10a1 probes (gifts from E. Vuorio, University of Turku, Turku, Finland), the Fgfr3 probe (a gift from C.X. Deng, National Institutes of Health, Bethesda, MD), the il7r probe (a gift from A.P. McMahon, Harvard University, Cambridge, MA), the Matl probe (a gift from A. Aszödi, Max-Planck Institute, Martinsried, Germany), the Mmp9 probe (a gift from Z. Werb, University of California, San Francisco, San Francisco, CA), the Ppar probe (a gift from H.M. Kronenberg, Massachusetts General Hospital, Boston, MA), and the Runx2 probe (a gift from G. Karsenty, Baylor College of Medicine, Houston, TX) were as described previously (Smits et al., 2001).

Cell proliferation assay
Pregnant mice were injected with BrdU reagent (Zymed Laboratories; 10 μg of mouse) and killed 2 h later. BrdU-labeled nuclei were detected by immunostaining (Zymed Laboratories), and sections were counterstained with hematoxylin, as described previously (Smits and Lefebvre, 2003). Cell proliferation rates were determined by counting the percentages of BrdU-positive cells in consecutive segments distributed from the periarticular to the hypertrophic zone of growth plates. Reproducible data were obtained with three sets of control and mutant littersmates.

Image acquisition and manipulation
Samples were visualized on a microscope (model BX50; Olympus) equipped with Uplanapo 10×0.40 and Uplanapo 20×0.70; =0.17 lenses (Olympus). Images were captured with a digital camera (model DMC2; Polaroid) using accompanying software. In situ hybridization images were taken under dark field using a red filter for RNA signals and under blue fluorescence for nuclei stained with Hoechst 33258 dye. Figures were composed using Adobe Photoshop 7.0.

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