

The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6

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To examine whether the transcription factor Sox9 has an essential role during the sequential steps of chondrocyte differentiation, we have used the Cre/loxP recombination system to generate mouse embryos in which either Sox9 is missing from undifferentiated mesenchymal cells of limb buds or the Sox9 gene is inactivated after chondrogenic mesenchymal condensations. Inactivation of Sox9 in limb buds before mesenchymal condensations resulted in a complete absence of both cartilage and bone, but markers for the different axes of limb development showed a normal pattern of expression. Apoptotic domains within the developing limbs were expanded, suggesting that Sox9 suppresses apoptosis. Expression of Sox5 and Sox6, two other Sox genes involved in chondrogenesis, was no longer detected. Moreover, expression of Runx2, a transcription factor needed for osteoblast differentiation, was also abolished. Embryos, in which Sox9 was deleted after mesenchymal condensations, exhibited a severe generalized chondrodysplasia, similar to that in Sox5; Sox6 double-null mutant mice. Most cells were arrested as condensed mesenchymal cells and did not undergo overt differentiation into chondrocytes. Furthermore, chondrocyte proliferation was severely inhibited and joint formation was defective. Although Indian hedgehog, Patched1, parathyroid hormone-related peptide (Pthrp), and Pth/Pthrp receptor were expressed, their expression was down-regulated. Our experiments further suggested that Sox9 is also needed to prevent conversion of proliferating chondrocytes into hypertrophic chondrocytes. We conclude that Sox9 is required during sequential steps of the chondrocyte differentiation pathway.

[Keywords: Sox9; Sox5/Sox6; chondrocyte differentiation; osteochondroprogenitors; apoptosis]

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At the onset of endochondral bone formation, a number of patterning molecules target the mesenchyme by outlining the three-dimensional coordinates that determine the shape, number, and size of the primordia of skeletal elements. In parallel to these patterning effects, the multistep process of endochondral bone formation is initially marked by the acquisition in undifferentiated mesenchymal cells of chondrogenic potency. These committed mesenchymal cells first undergo condensation, by which cells become closely packed, followed by the

overt differentiation of cells within these condensations into chondrocytes. These differentiated chondrocytes then sustain a series of sequential changes that include unidirectional proliferation, conversion to hypertrophic chondrocytes, ability to calcify the extracellular matrix, cell death, and replacement by bone. A number of secreted polypeptides are known to cooperatively regulate the rates of proliferation of chondrocytes and their transition to hypertrophy (Karaplis et al. 1994; Lanske et al. 1996; Vortkamp et al. 1996; St-Jacques et al. 1999; Hartmann and Tabin 2000; Vortkamp 2001). However, until recently, much less was known about intracellular events, especially about the transcription factors that control the onset of chondrogenesis in undifferentiated mesenchymal cells as well as the progression of chondrocytic differentiation.

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Sox9 is a transcription factor with a high-mobility-group (HMG-box) DNA-binding domain exhibiting a high degree of homology with that of the mammalian testis-determining factor, SRY. During chondrogenesis, Sox9 is expressed in all chondroprogenitors and all differentiated chondrocytes, but not in hypertrophic chondrocytes (Ng et al. 1997; Zhao et al. 1997). The human skeletal dysmorphism syndrome, campomelic dysplasia, which is characterized by hypoplasia of most endochondral bones, is caused by heterozygous mutations in and around Sox9 (Foster et al. 1994; Wagner et al. 1994). Previous studies based on mouse embryo chimeras derived from Sox9^{-/-} embryonic stem (ES) cells showed that the mutant cells were excluded from chondrogenic mesenchymal condensations and could not express chondrocyte-specific markers, such as *Col2a1*, *Col11a2*, and *Aggrecan* (Bi et al. 1999). Other studies showed that Sox9 bound to and activated chondrocyte-specific enhancer elements in the *Col2a1*, *Col11a2*, *Aggrecan*, and *CD-RAP* genes in vitro, consistent with the notion that these genes were direct targets of Sox9 (Lefebvre et al. 1997; Bridgewater et al. 1998; Xie et al. 1999; Sekiya et al. 2000). Moreover, ectopic expression of Sox9 in transgenic mice activated the endogenous *Col2a1* gene in vivo (Bell et al. 1997).

Two other members of the Sox family, Sox5 and Sox6, also play an essential role in chondrocytic differentiation. Sox5 and Sox6 are coexpressed with Sox9 in all chondroprogenitors and all differentiated chondrocytes (Lefebvre et al. 1998). In vitro studies showed that Sox5 and Sox6 cooperate with Sox9 to activate the *Col2a1* enhancer and the *Col2a1* gene. Whereas Sox5-null and Sox6-null mutant mice have relatively mild skeletal phenotypes, Sox5; Sox6 double-null mutants develop a severe, generalized chondrodysplasia characterized by the virtual absence of cartilage (Smits et al. 2001). In these double mutants, chondrogenic cells are largely arrested at the stage of chondrogenic mesenchymal condensations, and hence, these experiments showed that Sox5 and Sox6 are needed for overt differentiation of chondrocytes.

Although our studies with mouse embryo chimeras derived from Sox9^{-/-} ES cells strongly suggested that Sox9 is needed for chondrogenic mesenchymal condensation (Bi et al. 1999), they did not address a number of critical questions regarding other potential roles of Sox9 during endochondral bone formation. First, given previous speculation about a common progenitor for both the chondrocyte and osteoblast lineages (Grigoriadis et al. 1988; Ahdjoudj et al. 2001) and the expression of *Runx2* in chondrogenic mesenchymal condensations (Otto et al. 1997), we asked whether inactivation of Sox9 before mesenchymal condensation affected establishment of the osteoblast lineage in endochondral skeletal elements. Second, whereas the normal levels of Sox9 expression in mesenchymal condensations of Sox5; Sox6 double-null mutants indicated that Sox5 and Sox6 were not required for Sox9 expression (Smits et al. 2001), it was possible that Sox9 might control expression of Sox5 and Sox6. Third, given that specific patterning factors

control the axes of limb development, we wanted to examine whether inactivation of Sox9 before mesenchymal condensation would affect expression of these limb-patterning genes. Finally, although in vitro results suggested the possibility that Sox9 was involved during chondrogenesis beyond mesenchymal condensation, there is no reported in vivo evidence that supports this hypothesis.

The perinatal lethality of Sox9 heterozygous mutant mice, which almost perfectly reproduces the skeletal abnormalities of human campomelic dysplasia patients, precludes, however, the generation of Sox9 homozygous null mutants (Bi et al. 2001) and, hence, studies of the functions of Sox9 during embryonic development using conventional genetic methods. To determine the roles of Sox9 during the sequential steps of chondrogenesis and chondrocytic differentiation, we therefore conditionally inactivated the Sox9 gene using the Cre recombinase/*loxP* (Cre/*loxP*) recombination system of bacteriophage P1 (Gu et al. 1994) both in undifferentiated mesenchymal cells and after chondrogenic mesenchymal condensation.

Results

Generation of floxed Sox9 mice and Sox9 conditional null mutant mice

The Cre/*loxP* system was developed in order to restrict inactivation of a target gene in a cell- or tissue-specific manner (Gu et al. 1994). To inactivate Sox9 in chondrogenic cell lineages, we used mice carrying Sox9^{fllox}, a Sox9 allele in which the DNA segment that includes exons 2 and 3 was flanked by *loxP* sites (Fig. 1A). Exons 2 and 3 code for part of the HMG-box DNA-binding domain and the transactivation domain of Sox9. Both heterozygous and homozygous animals were viable and fertile and showed no noticeable phenotypic change. Crossing of the Sox9^{fllox} allele with animals expressing the Cre recombinase ubiquitously (deleter-Cre; Schwenk et al. 1995) leads to severe bone defects and perinatal death, a phenotype identical to that found in Sox9 heterozygous mutant mice (Bi et al. 2001; Kist et al. 2002; data not shown), indicating that the recombined allele represents real loss of Sox9 function. Using the mating scheme described in the Materials and Methods section, we produced animals that were heterozygous for Sox9^{fllox} carrying either a *Prx1-Cre* or a *Col2a1-Cre* transgene. In embryos harboring the *Prx1-Cre* transgene, Cre is expressed in all mesenchymal cells of limb buds starting around embryonic day 9.5 (E9.5) (Martin and Olson 2000; Logan et al. 2002). In addition, it is also expressed in mesenchymal precursor cells of sternum (data not shown). The Sox9^{fllox} heterozygotes that harbor *Prx1-Cre* are viable and fertile and show the same phenotype as Sox9 heterozygous embryos in their limbs (Bi et al. 2001), that is, hypoplasia of scapula and pelvic bones and bending of radius, ulna, tibia, and fibula, as well as absence of deltoid protuberance of the humerus (H. Akiyama, unpubl.). Ninety-five percent of Sox9^{fllox} heterozygotes harboring the *Col2a1-Cre* transgene died around 10 d after birth, displaying dwarfism and severe kyphosis

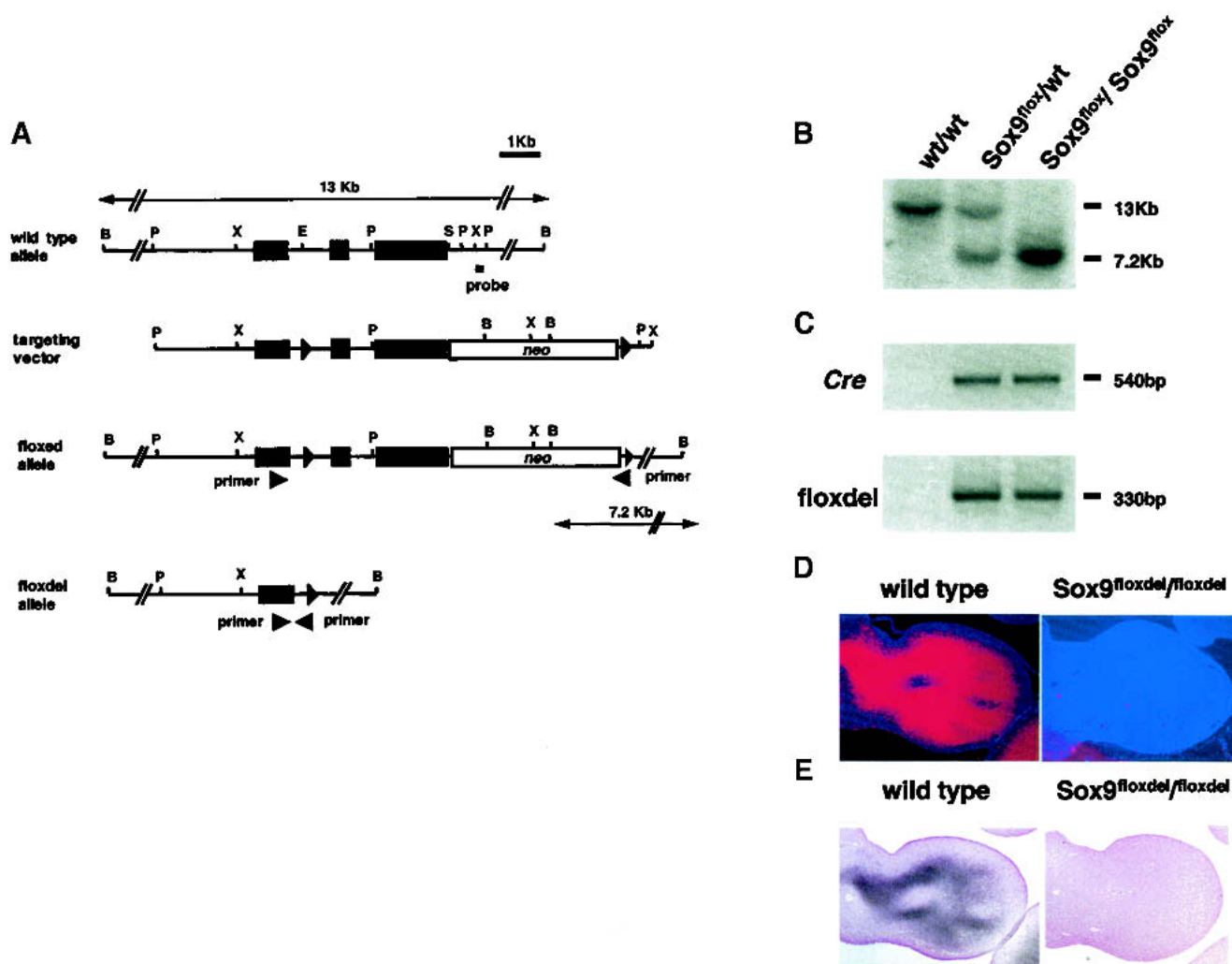


Figure 1. Targeting strategy for conditional inactivation of the gene for Sox9. (A) Structure of the genomic *Sox9* locus, targeting vector, and the homologous recombined allele. Exons are depicted as closed boxes, and intronic sequences are shown as solid lines. The *Neo* cassette is depicted as an open box. Forward and reverse primers used for PCR genotyping for floxed alleles are shown as arrowheads. DNA fragments revealed in Southern analysis are indicated as arrows with the restriction enzymes and the probe. B, *Bam*HI; P, *Pst*I; X, *Xba*I; E, *Eag*I; S, *Ssp*I. (B) Southern blot analysis of fetal genomic DNA. Genomic DNA isolated from the skin of wild-type, *Sox9*^{lox/wt}, *Cre*, and *Sox9*^{lox/flox}; *Cre* was digested with *Bam*HI and then hybridized with the 3' probe. The wild-type and floxed alleles were detected as 13-kb and 7.2-kb fragments, respectively. (C) PCR genotyping of *Cre* transgenes and floxed alleles. (D,E) Expression of *Sox9* mRNA (in situ hybridization, D) and *Sox9* protein (immunohistochemistry, E) in limb buds of E12.5 wild-type and *Sox9*^{lox/flox}; *Prx1-Cre* embryos. Both *Sox9* mRNA and *Sox9* protein were not detected in mutant limb buds.

with compression of cervical and thoracic vertebrae. Only 5% were viable beyond this point and fertile, probably due to mosaicism of *Sox9*^{loxdel}, the deleted allele of *Sox9*^{lox}, in chondrocytes.

In *Sox9*^{lox/flox}; *Prx1-Cre* and *Sox9*^{lox/flox}; *Col2a1-Cre* embryos, Cre-mediated conversion of *Sox9*^{lox} to *Sox9*^{loxdel} occurred and the floxed allele was detected only in embryos positive for either *Prx1-Cre* or *Col2a1-Cre* (Fig. 1B,C), indicating that Cre was capable of recombining the *loxP* sites within the *Sox9* gene in vivo.

Complete absence of cartilage and bone in limbs of *Sox9*^{lox/flox}; *Prx1-Cre* mutant embryos

The conditional *Sox9* null mutants resulting from expression of the *Prx1-Cre* transgene, which were recov-

ered with the expected Mendelian frequency, mostly died in the immediate postnatal period from respiratory distress due to the absence of a sternum. Newborns had very short limbs, but they looked otherwise normal (Fig. 2A). Staining of skeletal preparations from newborn mutant mice with alcian blue and alizarin red indicated a virtual absence of all skeletal components in forelimbs and hindlimbs (Fig. 2B). Occasionally, only rudiments of scapula, pubis, and ishium were detected. In situ hybridization and immunohistochemistry indicated a complete absence of *Sox9* RNA and protein in mutant limb buds, an indication that the inactivation of the *Sox9* genes was complete (Fig. 1D,E).

Histological analysis of wild-type limb buds showed condensed mesenchymal cells at 12.5 days postcoitum

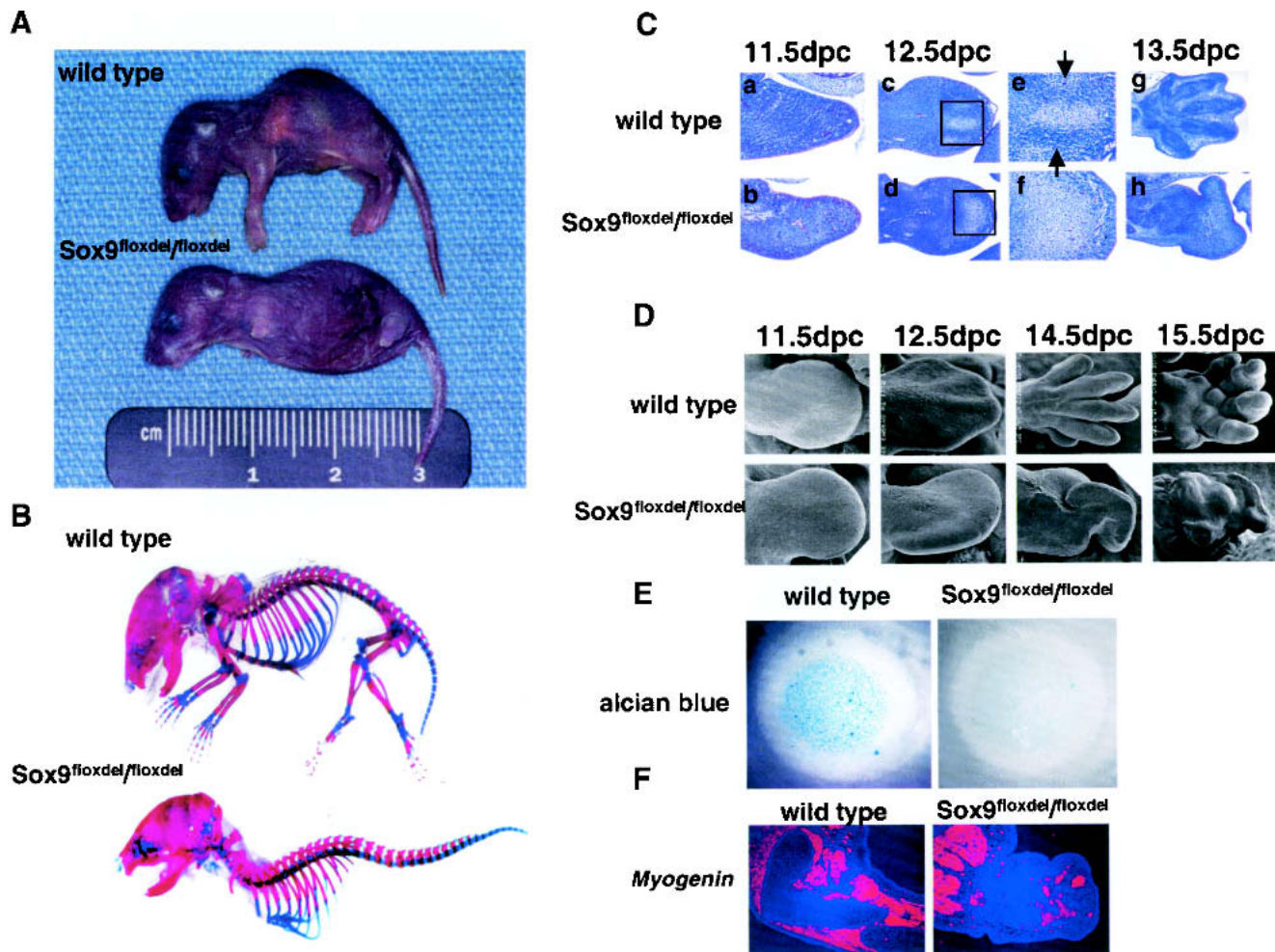


Figure 2. Analysis of skeletal phenotypes in *Sox9^{flox/flox}; Prx1-Cre* mice. (A) Gross appearance of newborn mice. Mutant newborns had very short limbs. (B) Skeletons of newborn mice stained by alcian blue followed by alizarin red. Cartilage and bone in the mutant limbs were completely absent. (C) Histological analysis of limb buds stained by hematoxylin and Treosin at 11.5 dpc (a,b), 12.5 dpc (c,d), and 13.5 dpc (g,h). Mutant limb buds had no discernible chondrogenic mesenchymal condensations at 12.5 dpc (c,d). Boxed regions at 12.5 dpc are shown at a high magnification (e,f). The arrows indicate chondrogenic mesenchymal condensations. (D) Morphological analysis of limb buds by scanning electron microscope. In the mutant limb buds, no distinct digit formation was observed, and the outgrowth of the mutant limb buds was arrested. (E) Micromass culture of dissociated mutant mesenchymal cells shows no alcian-blue positive cartilaginous nodules 7 d after plating. (F) Expression of *Myogenin* RNA in limb buds of E15.5 wild-type and mutant embryos. *Myogenin*-positive cells were present in the mutant limb buds.

(dpc), in which the digits differentiate into chondrocytes at 13.5 dpc (Fig. 2C). In contrast, mutant limb buds had no discernible chondrogenic mesenchymal condensations at 12.5 dpc, and, thereafter, no cartilages or bones were detectable. The lectin peanut agglutinin (PNA) bound to chondrogenic mesenchymal condensations of radius and ulna but not yet in the digit condensations in E12.5 wild-type embryos. In E12.5 mutant limb buds, there was very little localized binding of PNA (data not shown). Consistent with the absence of mesenchymal condensations in the mutant limb buds, no alcian blue-stainable cartilage nodules were observed in an in vitro high-density micromass culture using E12.5 mutant limb mesenchymal cells (Fig. 2E), indicating that *Sox9*^{-/-} cells lost their chondrogenic potency even if conditions of cell-cell interactions were provided in the micromass

culture. Histological examination of the mutant limb buds at 15.5 dpc revealed a small number of muscle fibrils and apparently normal skin development (data not shown). Although *myogenin*-positive cells were present in the mutant limb buds, an indication that myoblasts migrated into *Sox9*-deficient limb buds, the organization of muscle bundles were very abnormal (Fig. 2F).

Scanning electron microscope analysis of mutant limb buds at 11.5 dpc showed a normal paddle shape with typical apical ectodermal ridges. In E12.5 mutant limb buds, no distinct digit formation was observed, and the outgrowth of the mutant limb buds was arrested by 13.5 dpc. The malformation of the limb buds grew progressively more severe with time, although prominent pads were seen on the palmar surface (Fig. 2D).

To further characterize the *Sox9* mutant limb buds, we

examined the expression of *Runx2*, which is required for osteoblast differentiation (Otto et al. 1997). In E12.5 wild-type forelimb buds, *Runx2* transcripts were detected in scapula, humerus, radius, and ulna, but not in metacarpals and digits (Fig. 3A). In contrast, little or no expression of *Runx2* was detected in the mutant limb buds, strongly suggesting that Sox9 is needed for *Runx2* expression at this stage. In wild-type E15.5 limbs, *Runx2* was expressed at high levels in osteoblasts of bone collars and bone trabeculae and at lower levels in prehypertrophic and hypertrophic chondrocytes, whereas *Bone sialoprotein (Bsp)*, an early marker of osteoblast differentiation (Chen et al. 1992), was expressed in osteoblasts of bone collars and bone trabeculae. In mutant E15.5 limbs, no expression of *Runx2* and *Bsp* was detectable in limb mesenchymal tissues (Fig. 3B). We conclude that no osteoblasts were present in Sox9 mutant limbs.

Complete inhibition of Sox5 and Sox6 in limb buds of Sox9^{flox/flox}; Prx1-Cre mutant embryos

To characterize the complete defect in chondrogenic mesenchymal condensations in mutant mice, we analyzed the expression of genes known to be expressed in

condensed mesenchymal cells. *Sox5* and *Sox6* are coexpressed with *Sox9* during chondrogenesis starting before mesenchymal condensation, and their gene products are required for overt chondrocyte differentiation (Lefebvre et al. 1998; Smits et al. 2001). Expression of *Sox5* and *Sox6*, as well as of *Col2a1*, an early marker of chondrogenic cells, was essentially abolished in the mutant limb buds (Fig. 3C). This indicated that Sox9 is needed for the expression of *Sox5* and *Sox6*.

No evidence for patterning defects in limb buds of Sox9^{flox/flox}; Prx1-Cre mutant embryos

To determine whether patterning defects occurred in Sox9-deficient limb buds, we examined the expression of a series of marker genes characteristic of each of the vertebrate limb axes (Cohn and Tickle 1996; Capdevila and Belmonte 2001). Expression of *fibroblast growth factor 8 (Fgf8)*, a marker of the apical ectodermal ridge, was identical in wild-type and mutant E12.5 limb buds (Fig. 4A). Likewise, expression of *Sonic hedgehog (Shh)*, a posterior marker, was very similar in wild-type and mutant limb buds. Expression of the dorsal marker *Lmx1b* and that of the ventral marker *En1* were also comparable in wild-type and mutant limb buds. In addition, expression

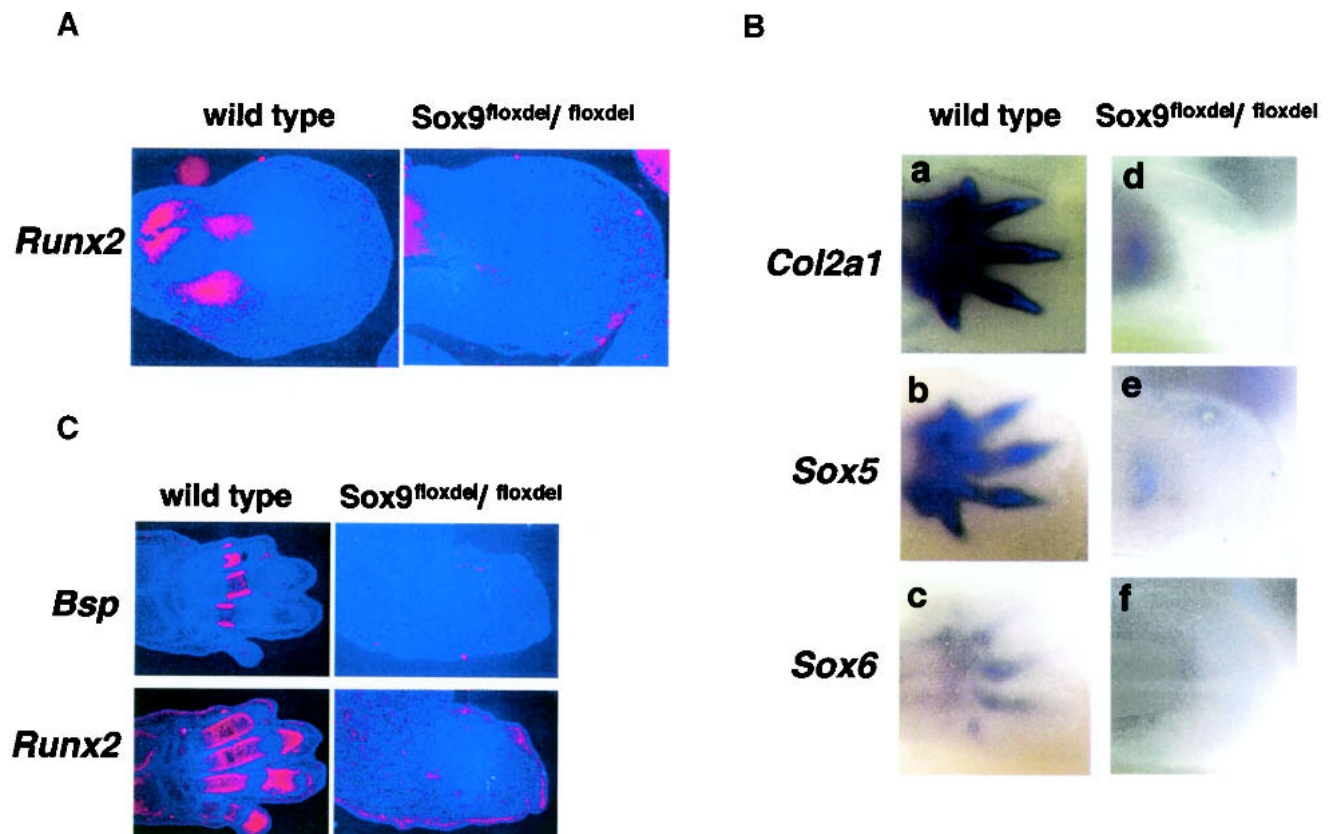


Figure 3. Expression of early markers of osteoblast differentiation and chondrogenic mesenchymal condensations in limb buds of Sox9^{flox/flox}; Prx1-Cre mice. (A) The limb buds of E12.5 wild-type and mutant embryos were hybridized with the *Runx2* probe. *Runx2* RNA was not detected in the mutant limb buds. (B) The limb buds of E15.5 wild-type and mutant embryos were hybridized with *Bsp* or *Runx2* probes. *Bsp* and *Runx2* transcripts were not detectable in mutant limb mesenchymal tissues. (C) Whole-mount RNA in situ hybridization showed lack of expression of *Col2a1* (a,d), *Sox5* (b,e), and *Sox6* (c,f) in E12.5 mutant limb buds.

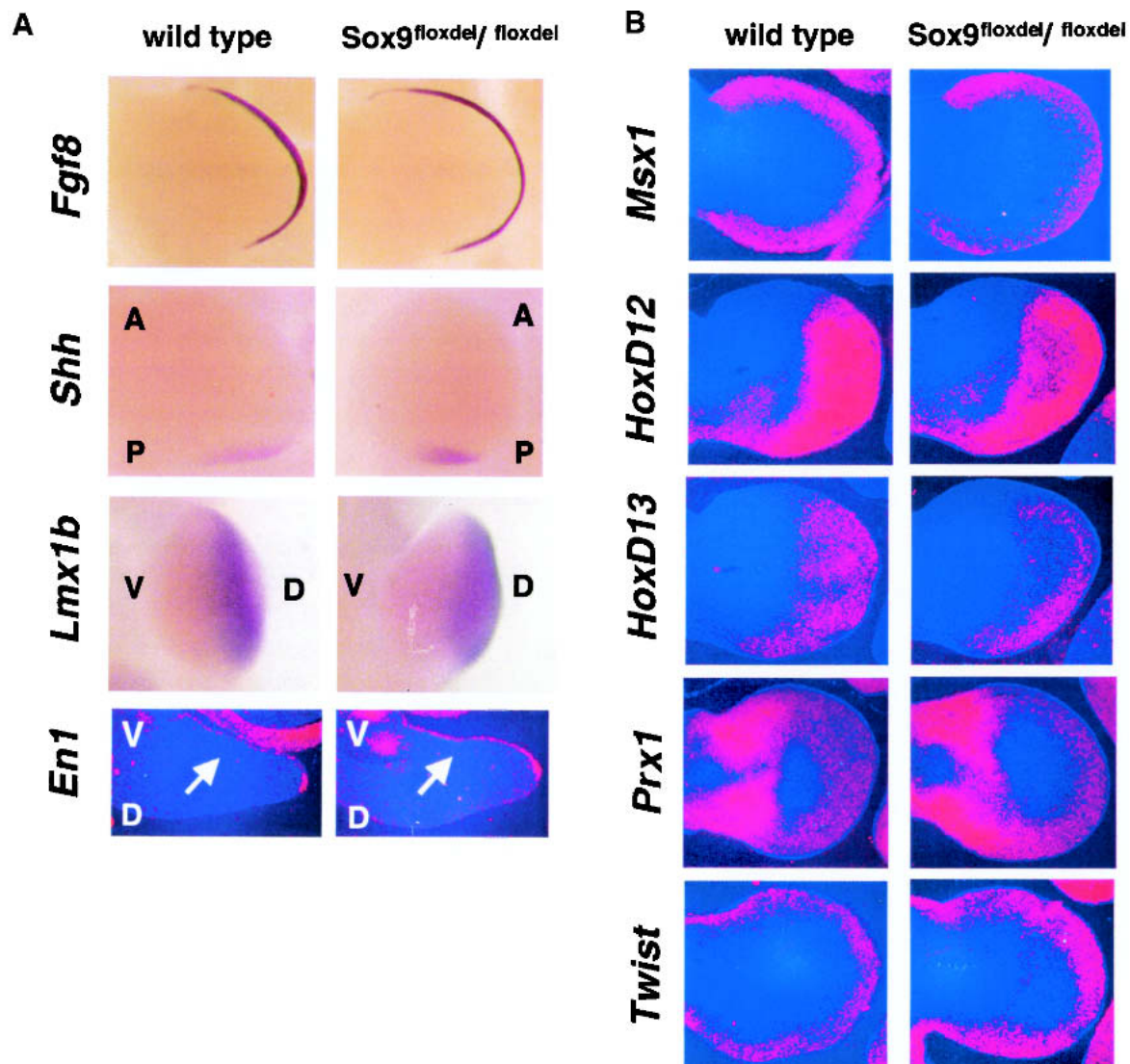


Figure 4. Expression of markers of limb patterning in *Sox9^{flox/flox}; Prx1-Cre* mice. The normal expression in limb buds of E11.5 (A) and E12.5 (B) mutant embryos of *Fgf8*, *Shh*, *Lmx1b*, *En1*, *Msx1*, *HoxD12*, *HoxD13*, *Prx1*, and *Twist* revealed no patterning defects in the mutant limb buds. A, anterior; P, posterior; V, ventral; D, dorsal. The arrows indicate the expression domain of *En1*.

of *Msx1*, *HoxD12*, *HoxD13*, *Prx1*, and *Twist* were essentially identical in wild-type and mutant limb buds (Fig. 4B). Thus, the absence of *Sox9* in E12.5 mutant limb buds did not cause misexpression of a series of limb patterning genes, further supporting the conclusion that *Sox9* is a true differentiation, not a patterning factor.

Sox9 is necessary for survival of mesenchymal cells in limb buds of Sox9^{flox/flox}; Prx1-Cre mutant embryos

The severe malformation and the arrest of outgrowth of mutant limb buds could have been due to an increase in apoptosis within the population of mesenchymal cells. To test this hypothesis, we performed TUNEL assays in E12.5 and E13.5 limb buds. In the limb buds of wild-type embryos, no apoptotic cells were seen at 12.5 dpc (data not shown), but starting at 13.5 dpc, apoptosis was ob-

served in the interdigital mesenchymal cells (Fig. 5a). In the mutant limb buds, no apoptosis was observed at 12.5 dpc, but extensive apoptosis was detected in mesenchymal cells at 13.5 dpc, in the area in which wild-type embryo chondrogenic mesenchymal condensations differentiate to form cartilage (Fig. 5d). Furthermore, immunohistochemistry showed that the expression of Bax was dramatically up-regulated in mutant limb buds and indicated large accumulation of cleaved caspase 3 in this domain of the mutant limb buds (Fig. 5b,c,e,f). These observations suggested that in the absence of chondrogenesis *Sox9^{-/-}* mesenchymal cells do not survive in the distal part of limb buds. Accumulating evidence shows that bone morphogenetic protein (BMP) signals induce, and BMP antagonists inhibit cell apoptosis in the interdigital spaces (Ganan et al. 1996; Zou and Niswander 1996; Merino et al. 1999a). In E13.5 wild-type limb buds,

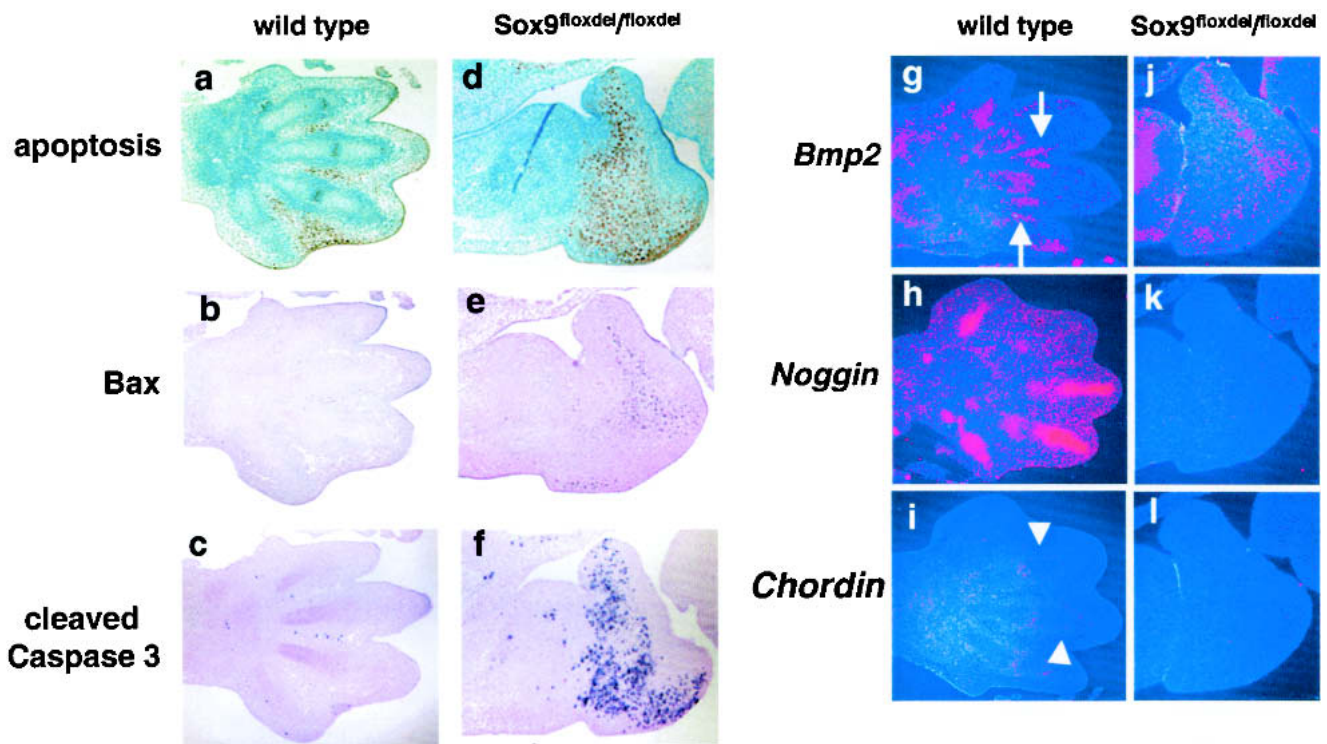


Figure 5. Analysis of apoptosis in *Sox9^{flox/flox}; Prx1-Cre* mice. TUNEL (a,d), immunohistochemistry of Bax (b,e) and cleaved caspase 3 (c,f), and in situ hybridization of *Bmp2* (g,i), *Noggin* (h,k), and *Chordin* (i,l) in limb buds of E13.5 wild-type and mutant embryos, respectively. The arrows indicate the expression of *Bmp2* in the vicinity of interdigital spaces. The arrowheads indicate the expression of *Chordin*.

Bmp2 was expressed in condensed cells, perichondrium, and mesenchymal cells in the vicinity of interdigital spaces (Fig. 5g). *Noggin* and *Chordin*, BMP antagonists, were expressed in chondrocytes and perichondrium of the digits, respectively (Fig. 5h,i). In contrast, in the mutant limb buds, *Bmp2* was expressed in a broad band of mesenchymal cells that paralleled the broad band of apoptotic cells (Fig. 5j), but expression of *Noggin* and *Chordin* was abolished (Fig. 5k,l). These results indicate that *Sox9* controls, either directly or indirectly, expression of *Noggin* and *Chordin*, and provide evidence for the hypothesis that *Sox9*^{-/-} mesenchymal cells are not protected by *Noggin* and *Chordin* from apoptotic signals generated by BMPs.

Severe chondrodysplasia in *Sox9^{flox/flox}; Col2a1-Cre* mutant embryos

Inactivation of *Sox9* by *Prx1-Cre*-mediated recombination, which took place in undifferentiated limb bud mesenchymal cells before chondrogenic mesenchymal condensation occurred, revealed *Sox9*'s essential roles in formation of chondrogenic mesenchymal condensations and in cell survival. However, the question remains whether *Sox9* is necessary for overt chondrocytic differentiation after formation of chondrogenic mesenchymal condensations. To address this question, we inactivated the *Sox9* gene in condensed mesenchymal cells and differentiated chondrocytes using *Col2a1-*

Cre transgenic mice. Only a few heterozygous *Sox9^{flox/wt}; Col2a1-Cre* mice survived beyond 10 d after birth and were able to mate. Very few newborn homozygous *Sox9^{flox/flox}; Col2a1-Cre* mice were found, indicating that embryos died before birth and were resorbed. However, until 16.5 dpc, *Sox9^{flox/flox}; Col2a1-Cre* mutant embryos were recovered at the expected Mendelian ratio. E12.5 mutants were undistinguishable from wild-type embryos (data not shown). However, from 14.5 dpc on, the mutants were easily distinguished, as they featured a round head with a short snout, short trunk, tail, and limbs, and a prominent abdomen, a phenotype that was very similar to that of the *Sox5; Sox6* double-null mutant embryos (Fig. 6A) (Smits et al. 2001). *Sox9^{flox/flox}; Col2a1-Cre* mutant embryos were characterized by a very severe and generalized chondrodysplasia (Fig. 6B). Alcian blue and alizarin red staining of skeletal preparations of E17.5 mutant embryos indicated that all of the skeletal elements formed by endochondral bone formation, including ribs, limbs, and vertebrae, were extremely small. Alcian blue staining was severely reduced, indicating that cartilage formation was markedly defective. In contrast, skeletal elements formed by intramembranous ossification lacked significant abnormality (Fig. 6B).

To assess the onset and efficiency of *Cre*-mediated gene deletion during limb bud development in *Sox9^{flox/flox}; Col2a1-Cre* embryos, we examined *Sox9* expression in the limb buds by immunohistochemistry using anti-

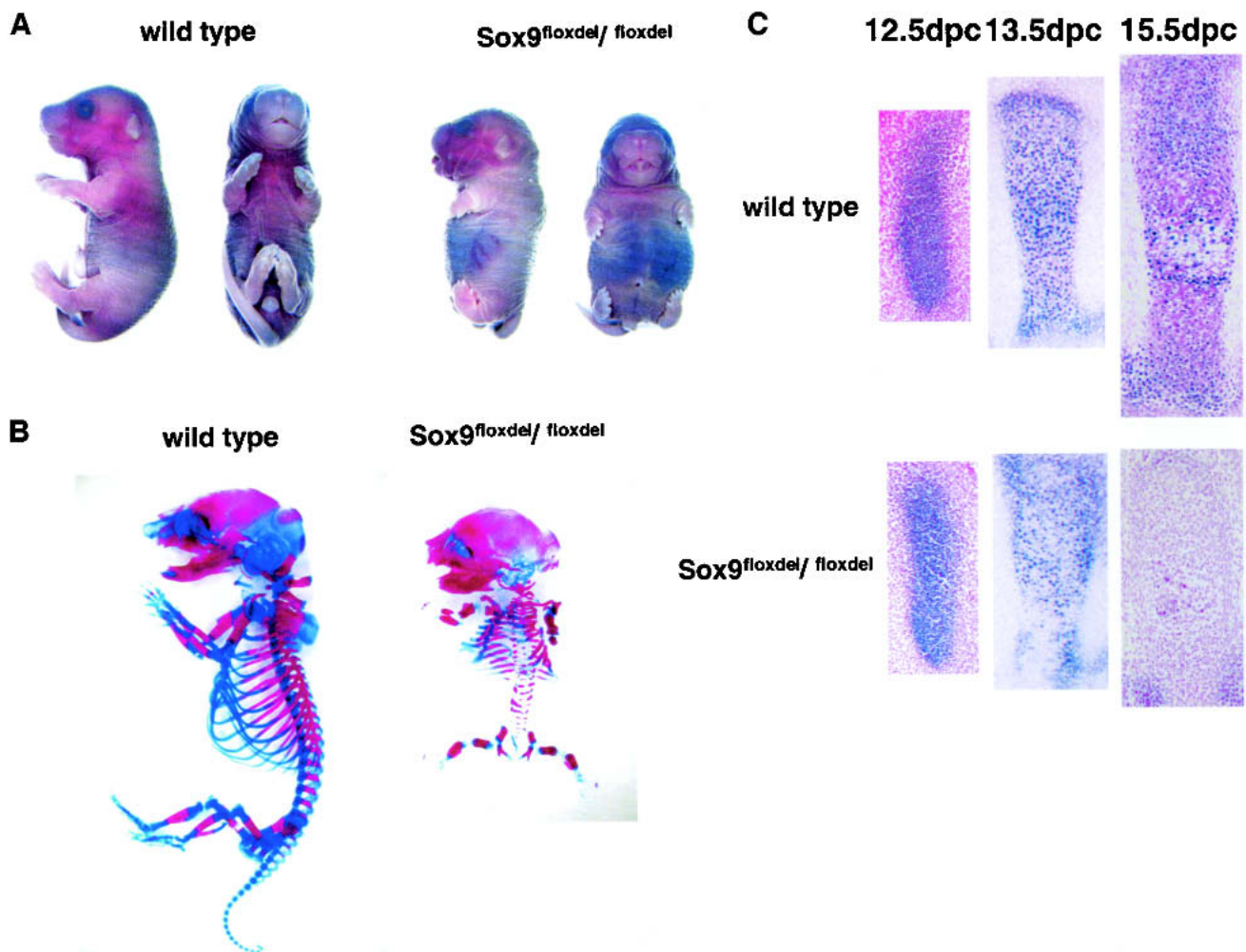


Figure 6. Severe, generalized chondrodysplasia in *Sox9^{lox/flox}; Col2a1-Cre* mice. (A) Gross appearance of E18.5 embryos. (B) Skeletons of E17.5 mice stained by alcian blue followed by alizarin red. Mutant embryos were characterized by a very severe and generalized chondrodysplasia. (C) Expression of Sox9 protein in metacarpals of E12.5, E13.5, and E15.5 wild-type and mutant embryos, respectively. The inactivation of *Sox9* occurred over a short window of time starting in condensed mesenchymal cells.

Sox9 antibody. In wild-type embryos, *Sox9* is expressed in condensed mesenchymal cells and then in the different chondrocytic cellular layers of the cartilage growth plate, except in hypertrophic chondrocytes, in which its expression is shut off. As shown in Figure 6C, in E12.5 mutant metacarpals, *Sox9* was expressed in condensed mesenchymal cells. From 13.5 dpc, the number of cells expressing *Sox9* was significantly less than in wild-type metacarpals, and at 15.5 dpc, only a few cells expressed lower levels of *Sox9* in the mutant, a result that was consistent with results of RNA in situ hybridization (data not shown). Thus, the inactivation of *Sox9* occurred over a short window of time starting in condensed mesenchymal cells.

Histological analysis of Sox9^{lox/flox}; Col2a1-Cre mutant embryos

Figure 7 shows the progression of chondrocyte differentiation and bone formation for two representative

skeletal elements, metacarpals and radius. In E12.5, *Sox9^{lox/flox}; Col2a1-Cre* metacarpals, chondrogenic mesenchymal condensations occurred normally (Fig. 7A, a,e). At 13.5 and 14.5 dpc, when deletion of the *Sox9* genes was proceeding, *Sox9^{-/-}* mutant cells remained mainly as condensed cells (Fig. 7A, b,c,f,g). Until 14.5 dpc, most chondrogenic cells accumulated little alcian blue-stainable extracellular matrix. At 15.5 dpc, hypertrophic chondrocytes appeared in the mid portion of metacarpals, but no orderly columns of proliferating chondrocytes were observed (Fig. 7A, d,h). The size of the metacarpals was also much reduced compared with wild-type metacarpals. In E13.5 and E14.5 mutant radius, cells with the aspect of hypertrophic chondrocytes surrounded by alcian blue-stainable extracellular matrix were also detected, although many cells remained in a condensed mesenchyme, indicating that some *Sox9*-expressing cells had entered the chondrocytic differentiation pathway. Again, in contrast to wild-type skeletal elements, the orderly parallel columnar structures of

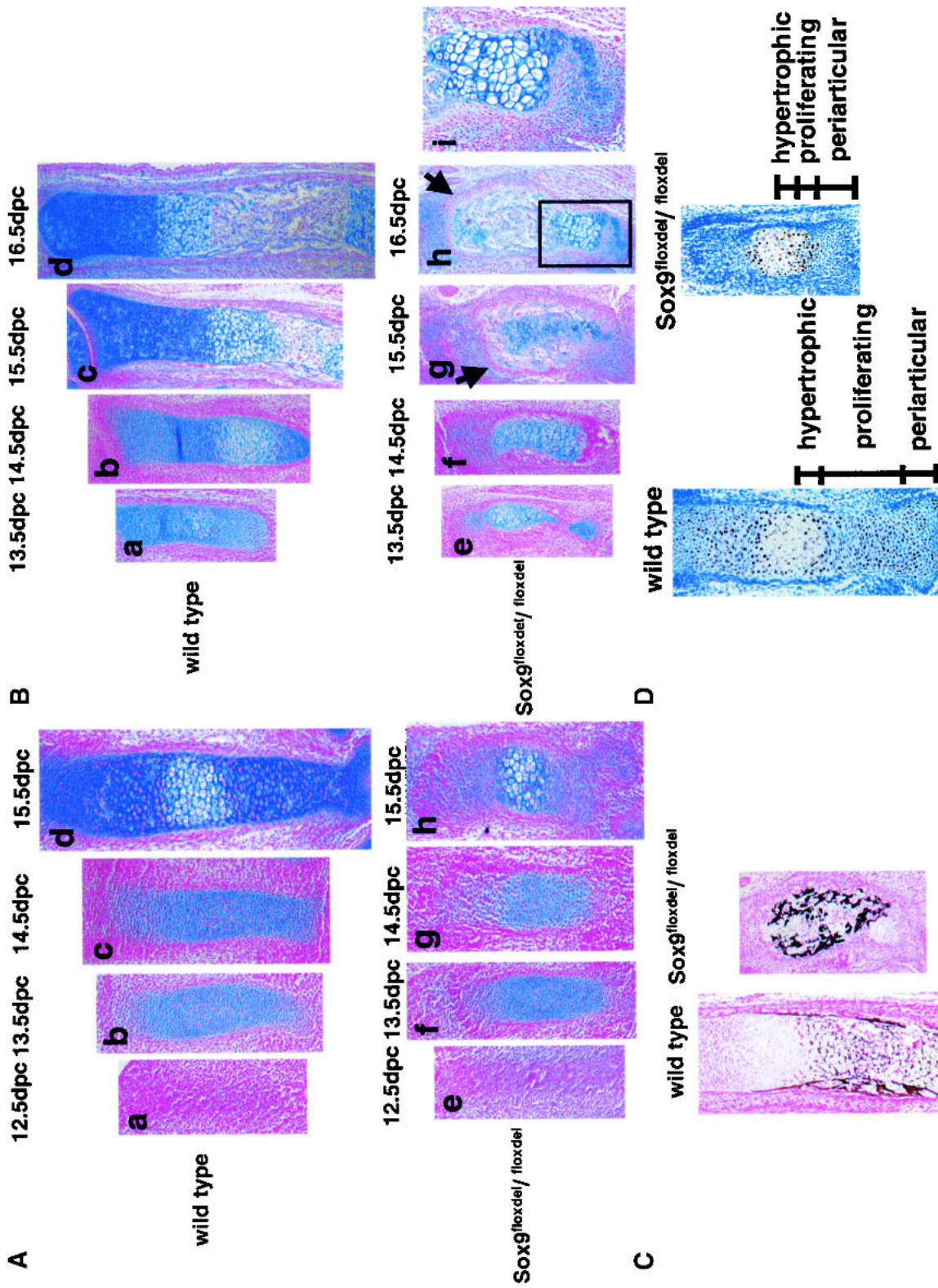


Figure 7. Histological analysis in *Sox9^{flox/flox}; Col2a1-Cre* mice. (A,B) Alcian blue and nuclear fast-red staining of metacarpals (A) and radius (B) during chondrocytic differentiation showed abnormal endochondral bone formation. The arrows indicate a thin layer of periosteum-like cells surrounding bone trabeculae and hypertrophic chondrocytes (B, g,h). A boxed region in B, h is shown at a high magnification as B, i. (C) Staining by von Kossa's method visualized mineral deposition in radius of E15.5 wild-type and mutant mice. (D) PCNA staining showed only a few PCNA-positive cells (brown nuclei) in metacarpals of E15.5 mutant embryos.

proliferating chondrocytes were not observed (Fig. 7B). Occasionally, small clusters of cells resembling proliferating chondrocytes were detected (Fig. 7B, g,h,i). In addition, bone formation took place, although it produced very deformed skeletal elements, a consequence of the deformity of cartilage primordia. Formation of bone trabeculae indicated that osteoblast differentiation occurred. In addition, mineralization took place in the malformed skeletal elements (Fig. 7C). We also noted that in the mutants, a thin layer of periosteum-like cells formed around the zone containing bone trabeculae and hypertrophic chondrocytes, separating this zone from the area of mesenchymal condensations (Fig. 7B, g,h).

Proliferative cell nuclear antigen (PCNA), a good marker for proliferating chondrocytes, was abundantly positive at 16.5 dpc in periarticular and columnar chondrocytes of wild-type growth plates. In contrast, in mutant growth plates, periarticular cells were PCNA negative, and only a few cells adjacent to hypertrophic chondrocytes were PCNA positive, indicating that the size of proliferating zones was dramatically reduced (Fig. 7D).

Formation of almost all joints was markedly delayed in the mutants; this was very striking in the carpal area,

in which most carpal skeletal elements were still fused together at 15.5 dpc (Fig. 8A). In the mutant limbs, the expression of *Noggin*, which is required for the formation of articular joints (Brunet et al. 1998), was dramatically reduced, and that of *Gdf5* and *Wnt14*, markers of joint formation (Storm and Kingsley 1996; Hartmann and Tabin 2001), was also down-regulated, suggesting that *Sox9* is involved, directly or indirectly, in joint formation.

Severe down-regulation of Sox5 and Sox6 expression in Sox9^{flox/flox}; Col2a1-Cre mutant embryos

During chondrogenesis, *Sox5* and *Sox6* play essential roles during overt chondrocytic differentiation after mesenchymal condensation (Smits et al. 2001). Inactivation of the *Sox9* gene in limb buds before chondrogenic mesenchymal condensation abolished *Sox5* and *Sox6* expression (Fig. 3C). Here, we examined whether *Sox5* and *Sox6* expression was also down-regulated when the *Sox9* alleles were inactivated after mesenchymal condensations had been established. In E12.5 wild-type embryos, cells in sclerotomes overtly differentiated into chondro-

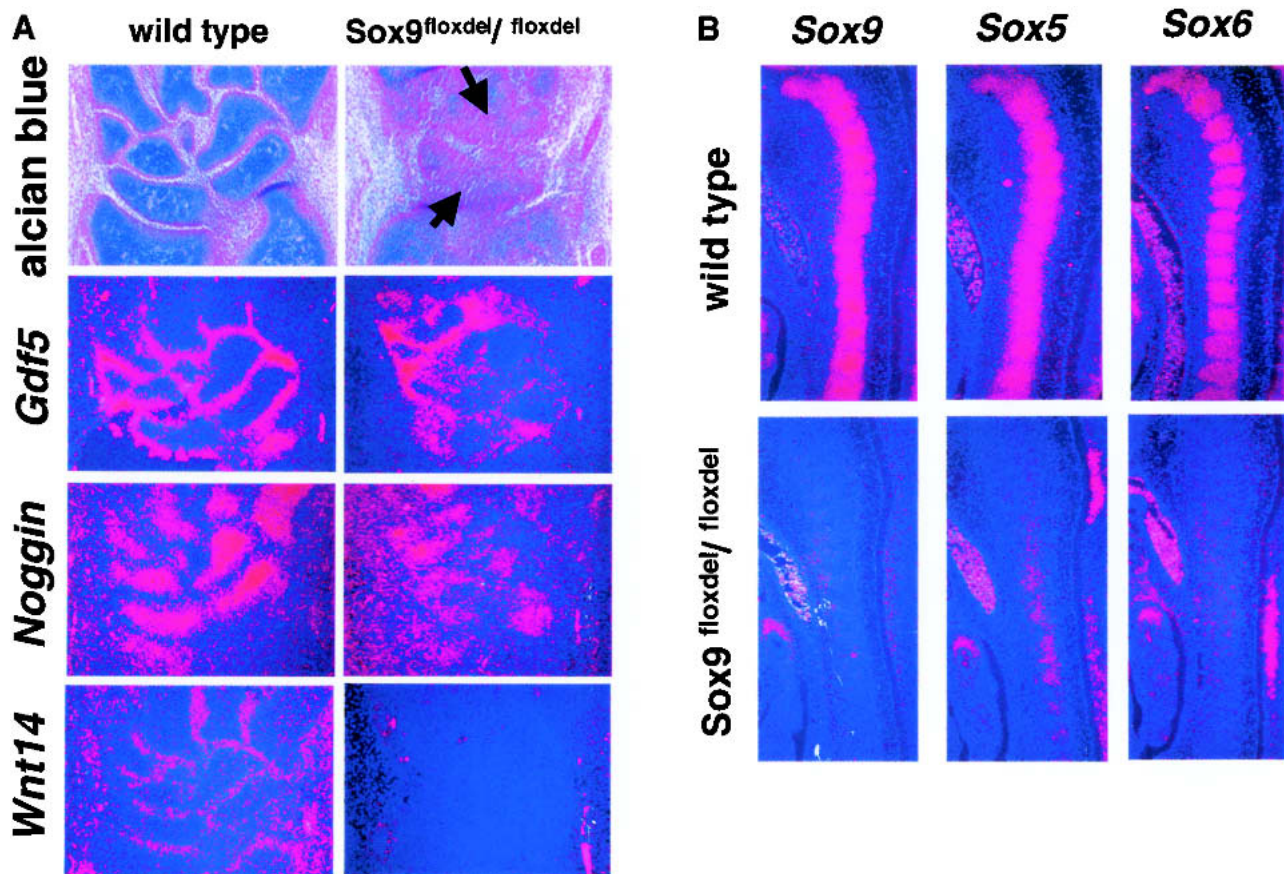


Figure 8. In situ hybridization analyses of markers of joint formation and of downstream genes of *Sox9* in chondrocytes. (A) Alcian blue and nuclear fast red staining and RNA in situ hybridization of *Gdf5*, *Noggin*, and *Wnt14* in carpal cartilage primordia of E15.5 wild-type and mutant mice. The arrows indicate fusion of carpal bones. (B) *Sox5* and *Sox6* are downstream genes of *Sox9* in chondrocytes. RNA in situ hybridization showed absence of expression of *Sox5* and *Sox6* in chondrocytes of E12.5 *Sox9^{flox/flox}; Col2a1-Cre* mice.

cytes and expressed high levels of *Sox9*, *Sox5*, and *Sox6* (Fig. 8B). In contrast, cells in sclerotomes of mutant mice showed a dramatic decrease in transcript levels, demonstrating that *Sox5* and *Sox6* are genetically downstream of *Sox9* in chondrocytes.

In *Sox5*; *Sox6* double-null mutant embryos, genes for cartilage extracellular matrix components, including *Col2a1*, *Aggrecan*, and *Cartilage oligomeric protein (Comp)*, were markedly down-regulated. In metacarpals of *Sox9^{flox/flox}*; *Col2a1-Cre* mutants, these genes were also severely down-regulated (Fig. 9A). Some cells adjacent to and in the zone of hypertrophic chondrocytes expressed these marker genes in mutant embryos, probably as a consequence of (1) the delayed inactivation of the *Sox9* alleles in some chondrogenic cells, and (2) the relatively long half-life of the mRNAs for these genes. In contrast, in E15.5 mutants, the levels of *Col10a1* expression were comparable with those in wild-type embryos, consistent with the presence of cells that have the aspect of hypertrophic chondrocytes in the mutant embryos.

Reduced signaling by the Indian hedgehog (Ihh)-Parathyroid hormone-related peptide (Pthrp) loop in Sox9^{flox/flox}; Col2a1-Cre mutant embryos

Ihh and *Pthrp* control the rate of chondrocyte proliferation and the transition of these cells to hypertrophy (Vortkamp et al. 1996); moreover, *Ihh* coordinates these processes with the onset of osteogenesis in bone collars (St-Jacques et al. 1999). Figure 9B shows that in E15.5 mutant metacarpal skeletal elements, *Pthrp receptor (PPR)* and *Ihh*, which are expressed in prehypertrophic chondrocytes (Vortkamp et al. 1996), were down-regulated. *Patched 1 (Ptc1)*, a transcriptional target of *Ihh* signaling expressed in proliferating chondrocytes and osteoblasts of bone collars (Vortkamp et al. 1996), was expressed only in a small number of cells in the mutant growth plates. Expression of *Pthrp*, another target of *Ihh* signaling, which is expressed in periarticular cells (Vortkamp et al. 1996), was also clearly down-regulated. Thus, transcripts for *Ihh*, *PPR*, *Ptc1*, and *Pthrp* were present at

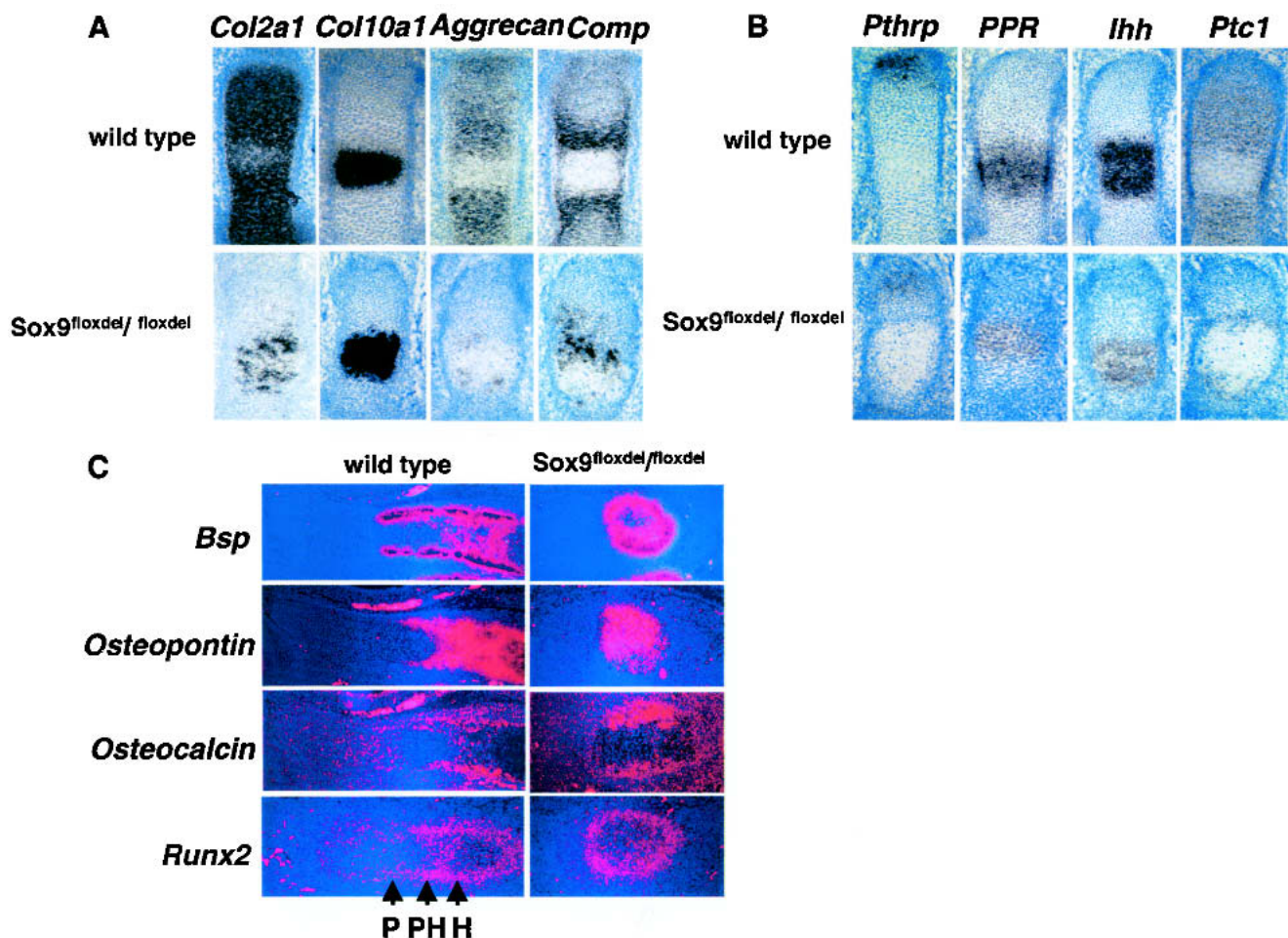


Figure 9. Expression of markers of chondrocytes, *Ihh*/*Pthrp* regulatory signaling molecules, and markers of osteoblast differentiation in *Sox9^{flox/flox}*; *Col2a1-Cre* mice. (A) Expression of *Col2a1*, *Aggrecan*, and *Comp*, but not *Col10a1* was dramatically down-regulated in E15.5 mutant metacarpals. (B) RNA in situ hybridization showed down-regulation of *Pthrp*, *PPR*, *Ihh*, and *Ptc1* in E15.5 mutant metacarpals. (C) Expression in radius of E15.5 mutant mice of *Bsp*, *Osteopontin*, *Osteocalcin*, and *Runx2* revealed normal osteoblast differentiation. P, proliferating; PH, prehypertrophic; H, hypertrophic.

lower levels in the mutants than in the wild-type embryos. Lower levels of *Ihh* expression probably contributed to the severe decrease in cell proliferation, whereas the reduced levels of *Pthrp* may have contributed to an accelerated transition to hypertrophy.

No inhibition of osteoblast differentiation in Sox9^{flox/flox}; Col2a1-Cre mutant embryos

Figure 9C shows that the levels of expression of *Runx2* in E15.5 mutant radius were comparable with those in wild-type embryos, as was the case for *Bsp* and *Osteopontin*. Moreover, *Osteocalcin* expression, which is just initiated at this stage in wild-type embryos, was also present in the mutants. We conclude that although the skeletal elements were extremely deformed, the process of osteoblast differentiation occurred normally in the mutants.

Discussion

Sox9 is essential for cartilage and bone formation in limb buds

After the onset of limb bud development, *Sox9* expression is induced in a subpopulation of undifferentiated pluripotent mesenchymal cells. These *Sox9*-expressing cells then undergo a process of cell aggregation, followed by overt differentiation into chondrocytes. Inactivation of *Sox9* in early limb buds, before condensations appeared, produced very short deformed limbs and resulted in a complete lack of chondrogenic mesenchymal condensations, subsequent cartilage formation, and bone development. The absence of *Sox9* in limb buds did not, however, produce patterning defects. The patterns of expression of markers that characterize the limb bud axes were essentially identical to those of wild-type embryos. In addition, the patterns of expression of *Msx1*, *Twist*, *Hoxd12*, *Hoxd13*, and *Prx1* in E12.5 mutant limb buds were all similar to those in wild-type embryos. Moreover, characteristic footpads that are normally present on the ventral side of limbs were observed in E15.5 mutants.

Inactivation of *Sox9* in limb buds both before and after mesenchymal condensations abolished expression of *Sox5* and *Sox6*, indicating that *Sox9* was required for expression of these two related *Sox* genes. In addition, inactivation of *Sox9* before chondrogenic mesenchymal condensations produced no transcripts for *Runx2*, which is ordinarily expressed at late stages of condensations and is required for osteoblast differentiation (Otto et al. 1997). This indicates that *Sox9* is needed for *Runx2* expression in early limb buds. However, if *Sox9* was inactivated after mesenchymal condensations, *Runx2* expression and osteoblast differentiation took place. Thus, in the absence of *Sox9* in early limb buds, there are no mesenchymal condensations, no chondrocyte differentiation, no establishing of the osteoblast lineage, and no osteoblast differentiation. These results are consistent with the hypothesis that in endochondral skeletal elements, *Sox9* is needed to establish osteochondrogenic

tor cells that will produce both chondrocytes and osteoblasts. We speculate that in endochondral skeletal elements, chondrocyte and osteoblast progenitors begin to segregate from each other at the stage of mesenchymal condensation. Once the mesenchymal condensations were established, inactivation of *Sox9* did not prevent osteoblast differentiation. We have no evidence to indicate that either *Sox5* and *Sox6* or *Runx2* are direct targets of *Sox9*.

Sox9 regulates cell survival of limb bud mesenchyme

In wild-type E13.5 embryos, mesenchymal cells in interdigital spaces undergo apoptosis. Inactivation of *Sox9* in *Sox9^{flox/flox}; Prx1-Cre* limb mesenchyme resulted in markedly increased apoptosis, an increased expression of *Bax*, and an increase in cleaved caspase 3 production. This cell death presumably contributed to the severe deformity of the mutant limbs. Cells within chondrogenic mesenchymal condensations and differentiating cartilages are unaffected by the apoptotic signals, which cause formation of interdigital spaces in wild-type limb buds. Our hypothesis is that the broad uninterrupted domain of apoptosis in *Sox9* mutant limb buds is due to normal proapoptotic signals, which, in wild-type limb buds, generate the interdigital spaces. Previous studies strongly suggest that BMPs have a proapoptotic activity in interdigital mesenchymal cells. BMP-beads implanted in chick embryonic limbs accelerate interdigital apoptosis (Ganan et al. 1996), whereas expression of a dominant-negative type I BMP receptor or exogenous administration of recombinant Gremlin, a BMP antagonist, reduces interdigital apoptosis (Zou and Niswander 1996; Merino et al. 1999b). The broad band of *Bmp2* expression, which parallels the broad band of apoptosis in the mutant limb buds, strongly supports the view that BMPs have a proapoptotic activity. In addition, the absence of *Noggin* and *Chordin* expression also provides indirect evidence that in wild-type limb buds, BMP antagonists expressed in digit chondrocytes protect the cells in and around the cartilages from proapoptotic BMP signals. Apoptosis in *Sox9* null mutant limb buds is unlikely to be an inherent property of *Sox9^{-/-}* cells, which would result from an inability to differentiate. In limbs of chimeric embryos derived from *Sox9^{-/-}* ES cells, the mutant cells survive (Bi et al. 1999).

Absence of mesenchymal condensations in Sox9 null mutants

The molecular mechanisms underlying the major cellular event of chondrogenic mesenchymal condensation remain largely unknown. A number of patterning factors must act at this step, as chondrogenic mesenchymal condensations prefigure the shape of future skeletal elements. In addition, in vitro experiments have suggested that several other molecules, including cell-adhesion molecules, are involved in this process (Hall and Miyake 2000). We have found that expression levels of *N-cadherin* and *NCAM* in E12.5 mutant limb buds are comparable with those in wild-type limb buds (H. Akiyama,

unpubl.). These results indicate that *Sox9* is not needed for expression of *N-cadherin* and *NCAM*, which have been proposed previously to mediate cell adhesion of chondrogenic mesenchymal cells (Widelitz et al. 1993; Oberlander and Tuan 1994). Although *Sox9* was reported recently to specifically bind an *N-cadherin* promoter region and to increase its activity in vitro (Panda et al. 2001), other transcription factors must control the levels of *N-cadherin* in vivo. The absence of alcian blue-stainable cartilage nodules in an in vitro high-density micro-mass culture using E12.5 mutant limb mesenchymal cells (Fig. 2E) indicates that even if conditions of cell-cell interactions exist in the micromass culture, *Sox9*^{-/-} cells are unable to differentiate. We hypothesize that *Sox9* controls expression of the cell-cell interaction machinery by a mechanism that does not involve changes in expression of *N-cadherin* and *NCAM*. Perhaps *Sox9* controls expression of other genes needed for mesenchymal condensations.

Sox9 is needed for chondrocyte differentiation after mesenchymal condensation

During endochondral bone formation in wild-type embryos, condensed mesenchymal cells differentiate into chondrocytes, and then these cells proliferate unidirectionally, forming parallel columns characteristic of cartilage growth plates. In E12.5 homozygous *Sox9*^{fllox/fllox} embryos harboring the *Col2a1-Cre* transgene, chondrogenic mesenchymal condensations were normal and the levels of *Sox9* in the cells of these condensations were comparable with those in wild-type digits. However, in E13.5 mutants, the number of *Sox9*-positive cells was markedly reduced, and by 15.5 dpc, almost all cells had become negative for *Sox9*. Thus, the *Sox9*^{fllox} alleles were inactivated over a window of time beginning at or immediately after the stage of chondrogenic mesenchymal condensations. The very severe chondrodysplasia and dwarfism of the mutants, which were the result of this inactivation, were characterized by an almost complete absence of cartilage in the endochondral skeleton. This was in contrast with a completely normal membranous skeleton. In the mutant endochondral skeletal elements, the majority of cells remained in mesenchymal condensations, and no parallel columns of proliferating chondrocytes were present. The severe decrease in cell proliferation was further demonstrated by the marked reduction in PCNA staining. *Sox9* might either control genes needed for cell proliferation or, alternatively, the decrease in cell proliferation could be a consequence of the lack of cartilage extracellular matrix components. However, hypertrophic chondrocytes, which expressed high levels of *Col10a1*, were often present in the center of skeletal elements. These results are consistent with the following interpretation: if inactivation of *Sox9* occurred in mesenchymal condensations, cells remained at this stage of differentiation, failing to produce a characteristic cartilage extracellular matrix. Those cells in which the *Sox9* genes were not inactivated in the condensations, proceeded into the chondrocyte differentia-

tion program. However, as the *Sox9* genes were promptly deleted, cells rapidly became hypertrophic. On the basis of this interpretation, our results indicate that *Sox9* is required for overt chondrocyte differentiation and for subsequent chondrocyte proliferation. The severe down-regulation of chondrocyte marker genes including *Col2a1*, *Aggrecan*, and *Comp* are in agreement with this conclusion. We propose that *Sox9* also prevents conversion of proliferating chondrocytes into hypertrophic chondrocytes. This hypothesis is supported by several lines of evidence. In the wild-type growth plate, expression of *Sox9* is abolished in hypertrophic chondrocytes; in addition, the levels of *Sox9* are higher in the prehypertrophic zone than in the proliferative zone. In *Sox9* heterozygous mice, the zone of hypertrophic chondrocytes was wider than in wild-type embryos, consistent with the notion that the levels of *Sox9* needed to inhibit hypertrophic chondrocyte maturation are not attained in the prehypertrophic cells of *Sox9* heterozygous mutants (Bi et al. 2001). The presence of abundant hypertrophic chondrocytes in the *Sox9*^{fllox/fllox}; *Col2a1-Cre* mutants is consistent with our hypothesis that *Sox9* prevents transition into hypertrophic chondrocytes. Recent preliminary experiments that generated embryos in which *Sox9* was overexpressed in chondrocytes indicated that hypertrophic chondrocyte differentiation was delayed in these embryos (H. Akiyama, unpubl.).

The rate of chondrogenic differentiation in the cartilage growth plate as well as the timing and location of osteogenesis initiation in the perichondrium/periosteum are controlled by the *Ihh*/*Pthrp* regulatory loop (Vortkamp et al. 1996). *Ihh*, which is expressed mainly in prehypertrophic chondrocytes and in the immediately adjacent zone of hypertrophic chondrocytes, stimulates chondrocyte proliferation in the growth plate and osteogenesis in the periosteum (St-Jacques et al. 1999); in addition, it induces *Pthrp* expression in periarticular chondrocytes. *Pthrp* and its receptor *PPR*, which is expressed at high levels in prehypertrophic chondrocytes (Karaplis et al. 1994; Lanske et al. 1996), regulate the switch from a proliferative to a postproliferative state by delaying transition of chondrocytes into hypertrophy. In *Sox9*^{fllox/fllox}; *Col2a1-Cre* mice, the levels of expression of *Ihh* and its two downstream target genes *Pthrp* and *Ptc1*, as well as *PPR*, were down-regulated. Our results are consistent with the notion that in the mutant mice, the zone of prehypertrophic chondrocytes is markedly reduced. We believe that the resulting down-regulation of *Ihh* and *PPR* and of the *Ihh*/*Pthrp* signaling loop is an indirect consequence of the absence of *Sox9*. The expression of *Ptc1*, even in a narrow range, indicated that *Ihh* signaling was functioning in the mutant mice. In addition, although bone collar formation was delayed and disorganized, cartilage replacement by bone occurred normally. Perhaps the lower levels of *Ihh* in the *Sox9*^{fllox/fllox}; *Col2a1-Cre* are sufficient to induce osteogenesis but insufficient to stimulate normal chondrocyte proliferation.

Joint formation is initiated by the arrest of chondrogenic differentiation in the prospective joint interzone. *Wnt14* induces the expression of *Gdf5* and *Chordin* in

this distinct zone, and regulates the subsequent processes of joint formation (Hartmann and Tabin 2001). *Noggin* is expressed in differentiated chondrocytes. *Noggin*-deficient mice do not express *Gdf5* and fail to initiate joint formation (Brunet et al. 1998), indicating that *Noggin* is needed for joint formation after chondrogenic mesenchymal condensation. In *Sox9^{flox/flox}; Col2a1-Cre* mice, joint formation does not take place properly, and mesenchymal condensations remain partly fused to each other. Furthermore, expression of *Noggin* and *Gdf5* is down-regulated and that of *Wnt14* is abolished. One possible explanation is that the severe chondrodysplasia of *Sox9^{flox/flox}; Col2a1-Cre* embryos results in a low expression of *Noggin*, which, in turn, would inhibit expression of *Gdf5* and *Wnt14*. The virtual lack of *Wnt14* expression raises the possibility that *Sox9* may regulate the expression of other signaling molecules controlling *Wnt14* expression.

Sox9^{flox/flox}; Col2a1-Cre and *Sox5; Sox6* double-null mutants have similar phenotypes

The severe generalized chondrodysplasia of *Sox9^{flox/flox}; Col2a1-Cre* mice is very similar to the phenotype of *Sox5; Sox6* double-null mutant mice (Smits et al. 2001). Whereas chondrocytes in *Sox5; Sox6* double-null mutant mice expressed *Sox9* at levels comparable with those in wild-type chondrocytes, in *Sox9^{flox/flox}; Col2a1-Cre* mutants, the levels of *Sox5* and *Sox6* expression were virtually abolished. Hence, the chondrodysplasia phenotype of *Sox9* mutants can, in large part, be accounted for by the severe down-regulation of *Sox5* and *Sox6*. Previous *in vitro* results have shown that *Sox9*, *Sox5*, and *Sox6* cooperate with each other in activating the *Col2a1* gene (Lefebvre et al. 1998). Hence, we believe that in addition to being required for expression of *Sox5* and *Sox6*, *Sox9* has itself an important role in the activation of cartilage matrix genes. The low expression levels of cartilage matrix genes, and the resumption of proliferation after a long delay in *Sox5; Sox6* double-null mutant chondrocytes, are likely due to the presence of normal levels of *Sox9*.

Conclusion

Sox9 is required in several successive steps of the chondrocyte differentiation pathway during endochondral bone formation *in vivo* and for the proper progression of cells through the sequential steps of this process. Figure 10 presents a model that summarizes several of these different functions. We have presented evidence that *Sox9* is needed for the commitment of undifferentiated mesenchymal cells to a cell type that is both a chondroprogenitor and an osteoprogenitor. *Sox9* is further required for the establishment of chondrogenic mesenchymal condensations. At this stage, *Sox9* also controls antiapoptotic molecules such as *Noggin* and *Chordin* that inhibit signals responsible for formation of interdigital spaces. Later, *Sox9* is needed for overt differentiation of chondrocytes, for the proliferation of these cells, for the establishment of parallel columns of proliferating chon-

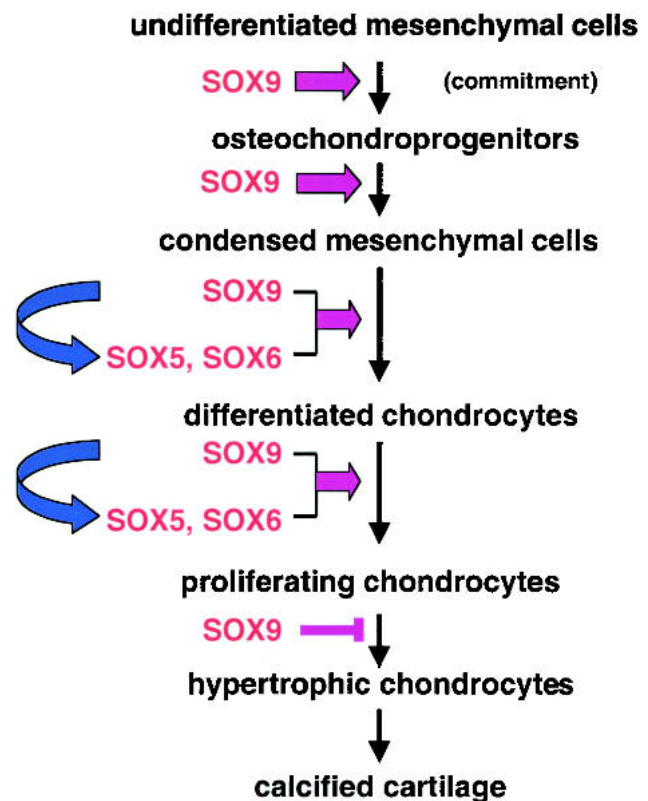


Figure 10. Functions of *Sox9* in the successive steps of the chondrocyte differentiation pathway during endochondral bone formation. *Sox9* commits undifferentiated mesenchymal cells in the lateral plate mesoderm to osteochondroprogenitors. *Sox9* is also needed for chondrogenic mesenchymal condensation, subsequent overt chondrocyte differentiation, and normal chondrocyte proliferation. Overt chondrocyte differentiation and normal chondrocyte proliferation are at least in part mediated by *Sox5* and *Sox6*, the expression of which requires *Sox9*. Finally, *Sox9* inhibits transition of proliferating chondrocytes to hypertrophy. It is also possible that *Sox5* and *Sox6* participate in inhibiting this last step.

drocytes in the growth plates of endochondral skeletal elements, and for proper joint formation. Finally, *Sox9* also inhibits the transition of chondrocyte into hypertrophic chondrocytes and, hence, controls subsequent endochondral ossification.

Materials and methods

Introduction of loxP sites into the *Sox9* gene and generation of *Sox9^{flox/wt}* and *Sox9^{flox/flox}; Cre* mice

A *Sox9* clone was isolated from a mouse 129/Sv genomic DNA library. To construct the targeting vector, a 5.5-kb *XbaI* fragment covering most of the *Sox9* gene was subcloned, a *loxP* sequence (5'-ATAACTTCGTATAGCATAACATTATACGAA GTTATGGCCCG-3') inserted into the *EagI* site in intron 1, and the fragment extended by adding 2.7 kb of upstream sequences (*PstI-XbaI* fragment). For the short vector arm, a 1.2-kb fragment starting from the *SspI* site after the *Sox9* polyadenylation signal was subcloned after a second *loxP* site, and the resulting construct fused with the 5' vector arm. Finally, an *Oct3/4neo* cassette was inserted upstream of the second *loxP* site. Because

the *Oct3/4* promoter is only expressed in undifferentiated ES cells, applying neomycin selection should result in an enrichment of undifferentiated ES cell clones. 129/SvEv ES clones harboring a *Sox9* floxed heterozygous mutation were screened by PCR and confirmed by Southern blot analysis with a *Sox9* probe located outside of the homology regions used for gene recombination. Mouse chimeras were generated by C57BL/6 host blastocyst injection of mutant ES cell clones, and chimeras obtained were bred with C57BL/6 mice to *Sox9*-floxed heterozygous mice. *Sox9*-floxed heterozygous mice were bred to homozygosity.

In a first cross, *Prx1-Cre* or *Col2a1-Cre* transgenic mice [Martin and Olson 2000; Ovchinnikov et al. 2000] were mated with mice heterozygous for the *Sox9*-floxed allele. The offspring inheriting either *Prx1-Cre* or *Col2a1-Cre* and a floxed allele were then mated with *Sox9*-floxed heterozygous mice to obtain embryos harboring either the *Prx1-Cre* or *Col2a1-Cre* transgene together with two *Sox9*-floxed alleles. Routine mouse genotyping was performed by PCR. The following primer pairs were used for *Cre* and floxed alleles: 5'-TCCAATTTACTGACCG TACACAA-3' and 5'-CCTGATCCTGGCAATTTCTGGCTA-3'; 5'-GTCAAGCGACCCATGAACGC-3' and 5'-TGTAAT GAGTCATACACAGTAC-3', respectively.

Histological analysis

Alcian blue and alizarin red staining of skeleton and scanning electron microscope analysis were performed as described previously [Hogan et al. 1994; Chen and Behringer 1995]. For the histological analysis, embryos were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 7 μ m were stained with hematoxylin and Treosin, with alcian blue and nuclear fast red, or with the von Kossa reaction and nuclear fast red. Immunohistochemical staining was performed by using peroxidase chromogens (Zymed)/TrueBlue substrate (KPL). The following antibodies were used: rabbit polyclonal anti-*Sox9* (1:150), rabbit polyclonal anti-Bax (Santa Cruz;1:200), and rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling Technology; 1:100). Cell proliferation analysis and TUNEL analysis were performed on paraffin-embedded sections using PCNA Staining Kit (Zymed) and ApopTag Plus peroxidase in situ apoptosis detection kit (Intergen) following the manufacturers' protocols. RNA in situ hybridization analysis was carried out as described previously [Conlon and Rossant 1992; Albrecht et al. 1997]. Pictures of hybridization signals were taken with a red filter and superimposed with blue fluorescence images of cell nuclei stained with Hoechst 33258 dye.

Micromass culture

For micromass culture, limb buds from E12.5 were isolated and were digested in 0.1% collagenase and 0.1% trypsin for 30 min at 37°C. Cell suspension was placed in DMEM, 10% FCS in 20- μ L drops at 2×10^7 cells/mL in 12-well plates. The cells were allowed to attach for 1 h, and then overlaid with 2 mL of DMEM, 10%FCS. After a 7-d culture, micromass cultures were stained with alcian blue for chondrogenic nodules as described previously [Akiyama et al. 1996].

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