Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors

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Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved August 10, 2005 (received for review June 7, 2005)

The transcription factor Sox9 is expressed in all chondroprogenitors and has an essential role in chondrogenesis. *Sox9* is also expressed in other tissues, including central nervous system, neural crest, intestine, pancreas, testis, and endocardial cushions, and plays a crucial role in cell proliferation and differentiation in several of these tissues. To determine the cell fate of *Sox9*-expressing cells during mouse embryogenesis, we generated mice in which a Cre recombinase gene preceded by an internal ribosome entry site was inserted into the 3′ untranslated region of the *Sox9* gene (*Sox9-Cre* knock-in). In the developing skeleton, *Sox9* was expressed before *Runx2*, an early osteoblast marker gene. Cell fate mapping by using *Sox9-Cre;ROSA26* reporter (*R26R*) mice revealed that *Sox9*-expressing limb bud mesenchymal cells gave rise to both chondrocytes and osteoblasts. Furthermore, a mutant in which the *Osterix* gene was inactivated in *Sox9*-expressing cells exhibited a lack of endochondral and intramembranous ossification and a lack of mature osteoblasts comparable with *Osterix*-null mutants. In addition, *Sox9*-expressing limb bud mesenchymal cells also contributed to tendon and synovium formation. By using *Sox9-Cre;R26R* mice, we also were able to systematically follow *Sox9*-expressing cells from embryonic day 8.0 to 17.0. Our results showed that *Sox9*-expressing cells contributed to the formation of all cell types of the spinal cord, epithelium of the intestine, pancreas, and mesenchyme of the testis. Thus, our results strongly suggest that all osteo-chondroprogenitor cells, as well as progenitors in a variety of tissues, are derived from Sox9-expressing precursors during mouse embryogenesis.

chondrogenesis | Osterix | chondrocytes | osteoblasts

Limb skeleton is formed as a cartilage model that undergoes endochondral bone formation. At the initiation of limb development, undifferentiated mesenchymal cells in the lateral plate mesoderm receive proliferation signals from the apical ectodermal ridge. These cells start to aggregate and form mesenchymal condensations, which are the primordia of the limb skeleton, then differentiate into chondrocytes and generate a cartilage skeleton. Cells surrounding the nascent cartilage form the perichondrium and periosteum, specialized structures consisting of thin layers of mesenchymal cells. The cells surrounding the zone of hypertrophic chondrocytes begin to differentiate into osteoblasts and, together with blood vessels and osteoclasts, invade the mineralized cartilage matrix and replace cartilage by bone.

Specific transcription factors regulate the differentiation pathways of chondrocytes and osteoblasts. Sox9, a transcription factor with a high-mobility group DNA-binding domain, activates chondrocyte-specific marker genes, such as *Col2a1*, *Col11a2*, and *Aggrecan* (1–3). Sox9 is expressed in all chondroprogenitors and chondrocytes except hypertrophic chondrocytes (4, 5). Campomelic dysplasia, a human disease that is caused by heterozygous mutations in the *Sox9* gene, is characterized by a general hypoplasia of endochondral bones (6, 7). We have recently shown that inactivation of *Sox9* in limb buds by using the Cre recombinase/*loxP* recombination system before chondrogenic mesenchymal condensations form results in the complete absence of mesenchymal condensations and subsequent cartilage and bone formation (8). Moreover, inactivation of *Sox9* during or after mesenchymal condensations results in a very severe chondrodysplasia, which is characterized by an almost complete absence of cartilage in the endochondral skeleton (8). Hence, Sox9 is required at sequential steps in chondrogenesis before and after mesenchymal condensations. These results also suggest that Sox9 may be involved in the determination of both chondrogenic and osteogenic cell lineages in limb development.

Runx2 is a member of the Runt-domain family of transcription factors, which form heterodimers with a single ubiquitous polypeptide called Cbfb (9). In addition to the Runx DNA-binding domain, Runx2 contains an active transactivation domain, rich in glutamine and alanine residues, and activates the *Osteocalcin* (*Oc*) and *Col1a1* genes (10, 11). Cleidocranial dysplasia, a genetic disease in humans that is characterized by hypoplastic clavicles, large open spaces between the frontal and parietal bones of the skull, and other skeletal dysplasias, is caused by heterozygous mutations in the *Runx2* gene (12). *Runx2*-null mice are characterized by a block in osteoblast differentiation and the absence of both endochondral and intramembranous bone formation, indicating that Runx2 has an essential role in the differentiation of mesenchymal progenitors into osteoblasts (10, 13, 14). Thus, Runx2 is an initial marker of the osteogenic cell lineage.

Recently, we identified a second transcription factor required for osteoblast differentiation, called Osterix (Osx) (15), that is specifically expressed in osteoblasts of all endochondral and membranous bones. Osx contains a DNA-binding domain consisting of three C2H2-type zinc fingers at its C terminus that share a high degree of sequence identity with similar motifs in Spl, Sp3, and Sp4. In addition, Osx also contains a proline- and serine-rich transactivation domain and activates the *Oc* and *Col1a1* genes. In *Osx*-null mutant mice, no endochondral or intramembranous bone formation occurs (15). The mesenchymal cells in *Osx*-null mutant mice do not deposit bone matrix, and cells in the periosteum and the condensed mesenchyme of membranous skeletal elements cannot differentiate into osteoblasts, although these cells express normal levels of *Runx2*. Arrest in osteoblast differentiation in *Osx*-null mutant mice occurs at a later step than that in *Runx2*-null mice. Indeed,

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: E*n*, embryonic day *n*; IRES, internal ribosome entry site.

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expression of *Osx* requires Runx2, indicating that Osx acts downstream of Runx2.

On the basis of our previous mouse genetic studies, we hypothesized that Sox9 is expressed in osteo-chondroprogenitors during endochondral bone formation before chondrogenic and osteogenic cell lineages are distinct (8). To test this hypothesis, we generated mice carrying the Cre recombinase gene inserted into the 3′-UTR of the *Sox9* gene and crossed these mice with the Rosa26 reporter (R26R) strain to follow the cell fate of *Sox9*-expressing cells during limb development. We also crossed this Cre line with mice carrying a floxed *Osx* allele to inactivate the *Osx* gene in *Sox9*-expressing cells. Our results indicated that Sox9 defines common progenitors of chondrogenic and osteogenic cell lineages.

Materials and Methods

Generation of *Sox9-Cre* Knock-In Mice and Floxed *Osx* Mice. A Sox9 clone was isolated from a mouse 129/Sv genomic DNA library. The targeting vector spanned a 7.7-kb fragment of the *Sox9* gene and an *internal ribosome entry site (IRES)-Cre-pA/FRT-flanked PGK-neo-bpA* cassette was inserted into a HpaI site within the 3′-UTR in exon3. An *MC1-tk-pA* herpes simplex virus thymidine kinase expression cassette was added onto the 3′ arm of homology to enrich for homologous recombinants by negative selection with 1-(2′-deoxy-2-fluoro′-β-D-arabinofuranosyl)-5-iodouracil (FIAU). The targeting vector was introduced into 129SvEv AB1 mouse ES cells and G418/FIAU-resistant ES cell clones were initially screened by Southern blot analysis of BamHI-digested genomic DNA by using a 3′ probe external to the region of vector homology. Mouse chimeras were generated by injection of mutant ES cell clones into C57BL/6 host blastocysts, and the chimeras obtained were bred with C57BL/6 mice to generate *Sox9-Cre* knock-in heterozygous mice.

An Osx clone was also isolated from a mouse129/Sv genomic DNA library. To construct the targeting vector for the floxed *Osx* allele, an 8.0-kb fragment was subcloned. Exon 2 was flanked by two *loxP* sites; the first was in intron 1 and the second in the 3′-flanking region. An *FRT-flanked PGK-neo-bpA* cassette and an *IRES- EGFP-pA* cassette, which contained a splicing acceptor signal at the 5′ end, were inserted into the BamHI site 3′ to the second *loxP* site. The target vector was introduced into 129SvEv AB1 ES cells, and G418-resistant ES cell clones were initially screened by Southern blot analysis of EcoRV-digested genomic DNA with a 3′ probe external to the region of homology. Homologous recombination was verified by using a 5′ probe external to the region of homology. 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 generate floxed *Osx* heterozygous mice. The *FRT-flanked PGK-neo-bpA* cassette was removed by Flp-mediated recombination with *ACTFlpe* transgenic mice (16).

In a first cross, *Sox9-Cre* knock-in mice were mated with mice heterozygous for the floxed *Osx* allele. The offspring inheriting both the Cre recombinase gene and the floxed allele were then mated with $Osx^{lacZ/wt}$ heterozygous mice (15) to obtain embryos with the $Osx^{flox/lacZ}$ and the *Sox9-Cre* knock-in allele. Routine mouse genotyping was performed by PCR. The following primer pairs were used: floxed *Osx* allele (5′-CTTGGGAACACTGAAGCTGT-3′ and 5′-CTGTCTTCACCTCAATTCTATT-3′); *Osx-lacZ* allele (5′-CGGATAAACGGAACTGGAAA-3′ and 5′-TAATCACGACGCGCTGTATC-3′); and *Sox9-Cre* knock-in allele (5′-TCCAATTTACTGACCGTACACCAA-3′ and 5′-CCTGATCCTGGCAATTTCGGCTA-3′).

Histological Analysis. β-gal staining of embryos was performed as described in ref. 17. For the histological analysis, embryos were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 7 μm were stained with alcian blue, hematoxylin, and Treosin (Statlab, Lewisville, TX). Immunohistochemical staining of sections was performed by using peroxidase chromogens (Zymed, South San Francisco, CA)/TrueBlue substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) with rabbit polyclonal anti-Sox9 antibody (1:100) (1). RNA *in situ* hybridization analysis was carried out as described in ref. 8. Pictures of hybridization signals were superimposed with blue fluorescence images of cell nuclei stained with Hoechst 33258 dye.

Results

Generation of *Sox9-Cre* Knock-In Mice. To follow the fate of *Sox9*-expressing cells throughout embryonic development, we generated mice that express Cre recombinase in a *Sox9*-specific pattern. The perinatal lethality of heterozygous *Sox9* mutant mice precludes the generation of mice in which specific DNAs are inserted into the coding regions of the *Sox9* gene (18, 19). Thus, to overcome this problem, we inserted *IRES-Cre-pA* into the 3′-UTR of the *Sox9* gene in exon3 just 5′ to the polyadenylation signal (Fig. 5, which is published as supporting information on the PNAS web site). In contrast to *Sox9* heterozygous mutant mice, which died perinatally with cleft palate, respiratory distress, hypoplasia, and bending of many skeletal structures derived from cartilage precursors (18, 19), heterozygous *Sox9-Cre* knock-in mice were viable and fertile and showed no noticeable phenotypic changes.

***Sox9* Expression Precedes *Runx2* and *Osx* Expression During Limb Bud Development.** *Sox9* was expressed in the chondrogenic cell lineage during limb bud development. Indeed, in embryonic day (E)10.0 *Sox9* started to be expressed in limb bud mesenchyme, then it was expressed in the cells in mesenchymal condensations and in chondrocytes (Fig. 1*A*). In contrast, the expression of *Runx2* and *Osx*, early markers of the osteogenic cell lineage, was first detected in E10.5 and E13.5 in limb bud mesenchyme, respectively, and followed *Sox9* expression (Fig. 1*A*), indicating that *Sox9* expression occurs before the determination of an osteogenic cell lineage in limb buds.

Sox9-Expressing Limb Bud Mesenchymal Cells Give Rise to Chondrocytes and Osteoblasts. We hypothesized that *Sox9*-expressing limb bud mesenchymal cells give rise to both the chondrogenic and osteogenic cell lineages. To confirm this hypothesis, we crossed *Sox9-Cre* knock-in mice with R26R mice. Expression of Cre recombinase in R26R mice results in activation of the *lacZ* gene, and, therefore, robust β-gal staining provides an indelible marker of cells that have expressed *Sox9* at any time during development (20–22). In *Sox9-Cre*;*R26R* E10.5 embryos, β-gal staining was detected in limb bud mesenchyme, and, in E13.5 embryos, all cells in cartilage primordia and perichondrium were β-gal positive (Fig. 1*B*). Indeed, the β-gal positive region in the limb buds of *Sox9-Cre*;*R26R* embryos was markedly wider than in the limb buds of $Sox9^{lacZ/wt}$ embryos, which represents only a chondrogenic cell population (Fig. 1*C*). In E17.0 limb buds, all chondrocytes as well as perichondrial, periosteal, and osteoblast cells were β-gal positive (Fig. 1*B*), indicating that *Sox9*-expressing limb bud mesenchymal cells give rise to both chondrogenic and osteogenic cell lineages. In addition, β-gal positive cells were detected in tendons and synoviums in E17.0, indicating that *Sox9*-expressing limb bud mesenchymal cells also give rise to tendon cells and synovial cells in limb bud development.

Inactivation of Conditional *Osx* Alleles by *Sox9-Cre* Results in Lack of Bone Formation. Cell fate mapping by using *Sox9-Cre* knock-in mice indicated that *Sox9*-expressing limb bud mesenchymal cells give rise to osteoblasts. To confirm this result, we generated mutant mice in which both *Osx* alleles were inactivated in *Sox9*-expressing cells. *Osx* is a transcription factor that is specif-

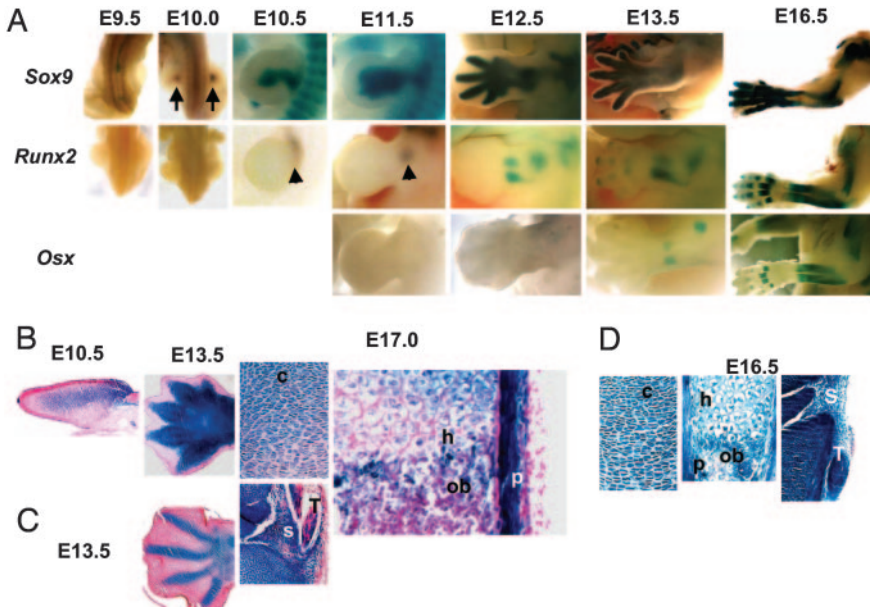

Fig. 1. Comparison of *Sox9*, *Runx2*, and *Osx* expression during embryonic development with *lacZ* expression in *Sox9-Cre*;*R26R* compound heterozygous embryos. (*A*) Expression of *Sox9*, *Runx2*, and *Osx* during limb bud development. β-gal activity in limb buds of *Sox9* heterozygous (*Sox9*$^{lacZ/wt}$) (19), *Runx2* heterozygous (*Runx2*$^{lacZ/wt}$) (14), and *Osx* heterozygous (*Osx*$^{lacZ/wt}$) (15) embryos from E9.5 to E16.5. The arrows indicate the expression of *Sox9* in E10.0. The arrowheads indicate the expression of *Runx2* in E10.5 and E11.5. (*B*) Sections of limb buds in *Sox9-Cre*;*R26R* compound heterozygous embryos from E10.5 to E17.0 stained with whole-mount β-gal staining. c, resting and proliferating chondrocytes; h, hypertrophic chondrocytes; p, periosteum; ob, osteoblasts; S, synovium; T, tendon. (*C*) Sections of limb buds in *Sox9* heterozygous (*Sox9*$^{lacZ/wt}$) embryos in E13.5 stained with whole-mount β-gal staining. (*D*) Sections of limb buds in *Prx1-Cre*;*R26R* compound heterozygous embryos in E16.5 with whole-mount β-gal staining. The abbreviations are the same as in *B*.

ically expressed in osteoblasts. We recently reported that Osx is a downstream gene of Runx2 in bone formation and that the *Osx*-null mutant mice lack bones completely (15). To generate the conditional *Osx*-null mice, we used mice carrying the floxed *Osx* allele, in which the DNA segment including exon2 was flanked by *loxP* sites (Fig. 2*A*). Both heterozygous and homozygous animals were viable and fertile and showed no noticeable phenotypic change. Using the mating scheme described in *Materials and Methods*, we produced mutants carrying the *Sox9-Cre* gene in which one *Osx* allele was replaced by the *lacZ* gene (15) and the other was replaced by a floxed *Osx* allele (Fig. 2*B*). The conditional *Osx*-null mutant mice resulting from expression of the *Sox9-Cre* gene, which were recovered with the expected Mendelian frequency, died in the immediate postnatal period because of the absence of bones. Staining of skeletal preparations of E17.5 conditional null mutant mice embryos with alcian blue and alizarin red indicated virtually no mineralization in any facial and skull bones generated by intramembranous bone formation, although very small parts of the maxilla, mandible, and parietal bones were calcified (Fig. 2*C*). Other skeletal elements formed by endochondral bone formation, including the ribs, limb skeletons, and vertebrae, were hypoplastic, bent, and often deformed, comparable with the phenotype in the *Osx*-null mice. Histological analysis showed that the humerus of E17.5 wild-type embryos displayed a typical cellular organization of bone trabeculae, bone collars, and a zone of hypertrophic chondrocytes adjacent to the chondro-osseous junction (Fig. 2*D*). In contrast, the humerus of E17.5 *Osx*-null and conditional *Osx*-null mutant mice had no bone collars and no bone trabeculae. The abnormalities in the humerus were present in all other endochondral skeletal elements (data not shown).

To better characterize the abnormalities of endochondral bones in the conditional *Osx*-null mutant mice, we examined the expression of the *Oc* gene, a late osteoblast marker, by *in situ* hybridization (Fig. 2*E*). In E17.5 wild-type embryos, *Oc* was strongly expressed in bones. In contrast, Oc was not expressed in E17.5 *Osx*-null and conditional *Osx*-null embryos. Thus, these results indicate that osteoblast differentiation was arrested in the mutant embryos in which the *Osx* gene was inactivated in *Sox9*-expressing limb mesenchymal cells. Hence, *Sox9*-expressing mesenchymal cells are osteo-chondroprogenitors that differentiate into both chondrocytes and osteoblasts. The absence of alizarin red staining in membranous skeletal elements in Osx$^{flox/LacZ}$;*Sox9-Cre* embryos also indicated that Sox9-expressing cells are the progenitors of osteoblasts in membranous bones.

***Paired-Related Homeobox Gene-1 (Prx1)*-Expressing Limb Bud Mesenchymal Cells Give Rise to All of the Mesenchymal Cell Lineages in Limb Buds.** *Prx1* is a homeobox-containing gene that starts to be expressed in all limb bud mesenchymal cells at E9.0 in advance of *Sox9* (23, 24). Mice homozygous for a mutant *Prx1* allele exhibited the loss or malformation of facial, limb, and vertebral skeletal structures because of a defect in the formation and growth of chondrogenic and osteogenic precursors (25). We previously reported that the conditional *Sox9*-null mutants harboring a *Prx1-Cre* transgene showed a complete lack of both cartilage and bone in limbs (8). Therefore, these findings strongly suggest that *Prx1*-expressing limb bud mesenchymal cells give rise to *Sox9*-expressing osteo-chondroprogenitors. In contrast to the expression of the *Prx1* transgene, *Sox9* is only expressed in a subpopulation of limb mesenchymal cells. Indeed, in *Prx1-Cre*;*R26R* embryos (23, 24), all of the cells in skeletal elements, the fibroblasts in connective tissues, and the tendon cells and the synovial cells were β-gal positive (Fig. 1*D*). Hence, *Prx1*-expressing undifferentiated limb bud mesenchymal cells differentiate into all of the mesenchymal cell populations during limb bud development.

***Sox9* Defines Progenitors in Testis, Intestine, Spinal Cord, and Pancreas During Mouse Embryogenesis.** We also examined the fates of *Sox9*-expressing cells in other tissues during embryogenesis.

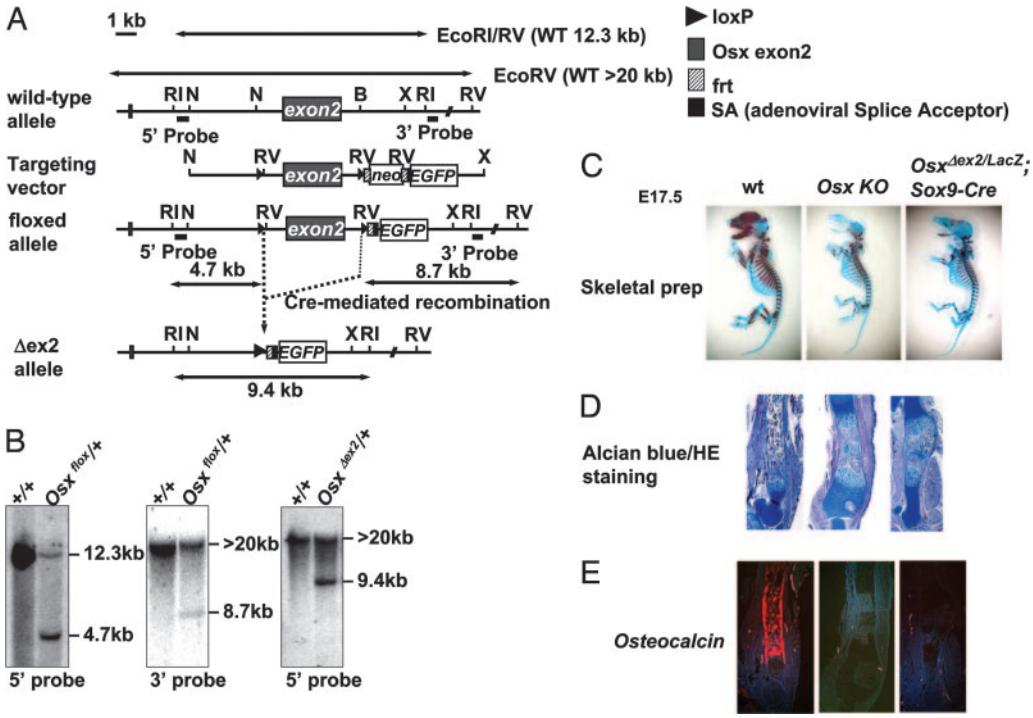

Fig. 2. Targeting strategy for conditional inactivation of the *Osx* gene. (*A*) Structure of the genomic *Osx* locus, targeting vector, and the homologous recombined allele. Exons are depicted as filled boxes, and intronic sequences are shown as solid lines. The *FRT-flanked PGK-neo bpA* and the *IRES-EGFP-pA* cassettes are depicted as open boxes. DNA fragments revealed in Southern analysis are indicated as arrows with the restriction enzymes and the probes. RI, EcoRI; N, NheI; B, BamHI; X, XbaI; RV, EcoRV. (*B*) Southern blot analysis of genomic DNA. (*C–E*) Analysis of skeletal phenotypes in *Osx* $^{flox/lacZ}$*;Sox9-Cre* knock-in mice. Skeletons in E17.5 embryos stained with alcian blue followed by alizarin red showed no mineralization of bones in *Osx*-null and the conditional *Osx*-null mutants (*C*). Histological analysis of humerus stained by alcian blue, hematoxylin, and Treosin revealed no bone trabeculae or mineralization in *Osx*-null and the conditional *Osx*-null mutants in E17.5 (*D*). Expression of *Oc* mRNA was not detected in humerus of E17.5 *Osx*-null and the conditional *Osx*-null mutants (*E*).

Whole-mount β-gal staining in *Sox9-Cre* knock-in;*R26R* embryos was first detected in E8.0 in notochord, node, and endodermal linings (Fig. 3*A*). From E10.5, β-gal staining was very strong in nonneural ectoderm throughout embryonic development, hampering the observation of β-gal staining of internal organs. For this reason, we examined β-gal staining in sections made from paraffin-embedded whole-mount β-gal stained E17.0 embryos in which the skin had been removed. In the male gonad, Sox9 was expressed in Sertoli cells (Fig. 3*B*). In contrast, all of the mesenchymal cells including Sertoli cells and Leydig cells in *Sox9-Cre*;*R26R* embryos were β-gal positive, but the germ cells were β-gal negative (Fig. 3*B*). In the intestine, because *Sox9* was expressed in stem cells and progenitor cells in the bottom of crypts in villi, all of the epithelial cells were β-gal positive; furthermore, the neurogenic cells derived from the neural crest in which *Sox9* was expressed (4, 5, 26) were also β-gal positive (Fig. 3*B*). In the spinal cord, *Sox9* expression was detected throughout the ventricular neuroepithelial layer in which neural progenitor cells are located and also in the cells migrating outward along the dorsoventral axis. Indeed, all of the neural cells, neurons and glia, in the spinal cord of *Sox9-Cre*;*R26R* embryos were β-gal positive. In the pancreas, *Sox9* was expressed

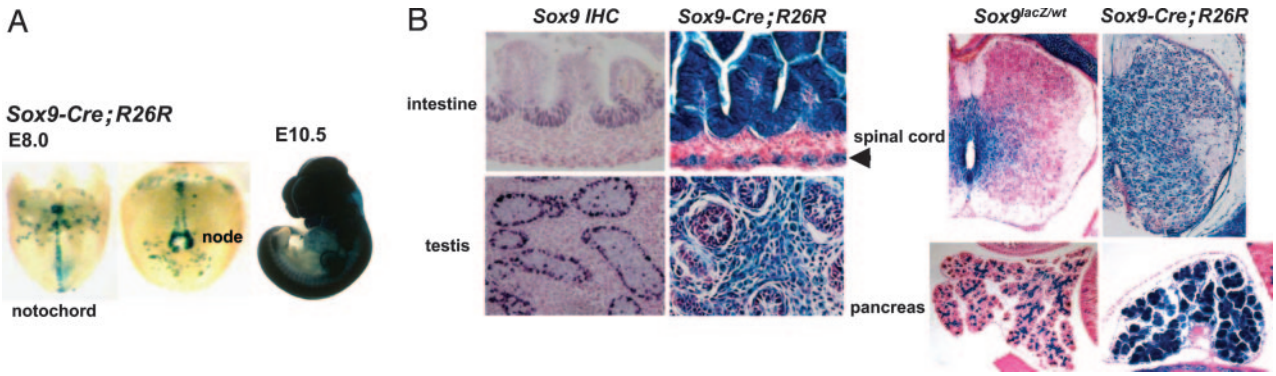

Fig. 3. Comparison of the pattern of cells expressing *Sox9* in E17.0 intestine, testis, spinal cord, and pancreas with the pattern of cells that are derived from *Sox9*-expressing cells in these embryos. (*A*) Contribution of *Sox9*-expressing cells during mouse embryogenesis. β-gal activity in *Sox9-Cre*;*R26R* compound heterozygous embryos in E8.0 and E10.5. (*B*) Comparison between the expression of *Sox9*, assessed by immunohistochemistry (intestine and testis) of wild-type embryos or by β-gal staining (spinal cord and pancreas) of *Sox9* heterozygous (*Sox9*$^{lacZ/wt}$) embryos, and the distribution of cells derived from *Sox9*-expressing cells in E17.0 *Sox9-Cre;R26R* embryos. The arrowhead indicates enteric neurons.

in ductal epithelial cells. In contrast, in Sox9-Cre;R26R embryos, not only the ductal epithelial cells but also all of the endocrine and exocrine cells were β-gal positive (Fig. 3*B*). These observations strongly support the hypothesis that Sox9 defines progenitors in these different tissues during mouse embryogenesis.

Discussion

We generated a mouse strain that expresses Cre recombinase in *Sox9*-expressing cells during embryogenesis. An *IRES-Cre-pA* cassette was inserted into the 3′-UTR of the *Sox9* gene, resulting in Cre recombinase expression directly under *Sox9* cis-regulatory control via production of a bicistronic *Sox9-Cre recombinase* mRNA. This approach avoided the perinatal lethality of mice that would have resulted from the insertion of *Cre* in the coding portion of Sox9 and inactivation of the *Sox9* gene. Expression of Cre recombinase was assessed by crossing *Sox9-Cre* knock-in mice to the Cre-dependent R26R strain. The advantage of using this genetic system to examine gene expression patterns is that the R26R strain provides a permanent lineage record through the constitutive β-gal expression in cells that express Cre recombinase even transiently in the precursor cells of different cell lineages (20–22).

During mouse limb development, *Sox9* expression was first detected in a subpopulation of limb mesenchyme in E10.0. In contrast, *Runx2* expression was detected in the lateral plate mesoderm from E10.5, and *Osx* expression was seen in limb mesenchyme from E13.5. These observations strongly suggest that the osteogenic lineage appeared after *Sox9*-expressing cells in limb bud development. Indeed, this hypothesis is supported by β-gal expression in *Sox9-Cre* knock-in;*R26R* compound heterozygous embryos. In these embryos, β-gal expression was detected in mesenchymal cells before mesenchymal condensation, whereas after mesenchymal condensations, β-gal expression was found in all of the skeletal components, including not only the chondrocytes but the perichondrium, the periosteum, and osteoblasts. Thus, these observations indicate that *Sox9*-expressing mesenchymal cells, present before mesenchymal condensations occur, are osteo-chondroprogenitors. Indeed, the inactivation of the *Osx* gene, which presumably takes place in *Sox9*-expressing mesenchymal cells before mesenchymal condensations, results in a complete block of osteoblast differentiation. Hence, Sox9 defines osteo-chondroprogenitors in the mesenchyme of developing limb buds.

We speculate that in endochondral skeletal elements, the osteogenic lineage cells segregate from *Sox9*-expressing osteochondroprogenitors at the stage of mesenchymal condensations. This idea is supported first by histological analysis, which showed that Sox9 was expressed in limb mesenchymal cells before mesenchymal condensations (Fig. 1*A*) (4, 5). In contrast, *Runx2* expression was detected at the stage of mesenchymal condensations (Fig. 1*A*) (27). Second, inactivation of the *Sox9* gene in limb buds before mesenchymal condensations prevented mesenchymal condensations and subsequent cartilage and bone formation, although the *Sox9*-null cells are present in their appropriate locations (8). In contrast, *Runx2*-null mutants form mesenchymal condensations normally (13, 14). Thus, these observations strongly suggest that the establishment of an osteogenic cell lineage occurs during mesenchymal condensations. We speculate that in endochondral skeletal elements, the process of mesenchymal condensation plays a major role in the establishment of the osteoblast lineage.

During limb bud development in mouse embryos, undifferentiated limb bud mesenchymal cells that were derived from the lateral plate mesoderm differentiate and establish mesenchymal cell lineages, including chondrocytes, osteoblasts, and fibroblasts. Previous studies showed that *Prx1* is expressed in the early limb bud mesenchyme in mouse embryos (23) and that Cre recombinase activity is present in all mesenchymal cells, including osteo-chondroprogenitors, in E10.5 forelimb buds of *Prx1-Cre* transgenic mice (24). In E16.5 *Prx1-Cre;R26R* compound heterozygous embryos, β-gal activity was detected in all of the skeletal elements, including chondrocytes, perichondrium, periosteum, and osteoblasts, and all fibroblasts in connective tissues, tendon cells, and synovial cells. In these embryos, muscle and skin did not stain for β-gal, indicating that these cell lineages do not derive from Prx1-Cre expressing cells in limb buds (data not shown). In *Sox9-Cre*;*R26R* embryos not all tendon cells stained for β-gal. The difference between β-gal staining of tendons in *Prx1-Cre;R26R* and that of tendons in *Sox9-Cre;R26R* embryos may be due to the activation of the Prx1 promoter at the tendon insertion points of the skeletal muscles (23). We therefore concluded that *Prx1*-expressing undifferentiated limb bud mesenchyme gives rise to all mesenchymal cell populations, including *Sox9*-expressing osteo-chondroprogenitors, which differentiate into *Sox9*-expressing chondrocytes and *Runx2*-expressing osteoblasts during limb development (Fig. 4).

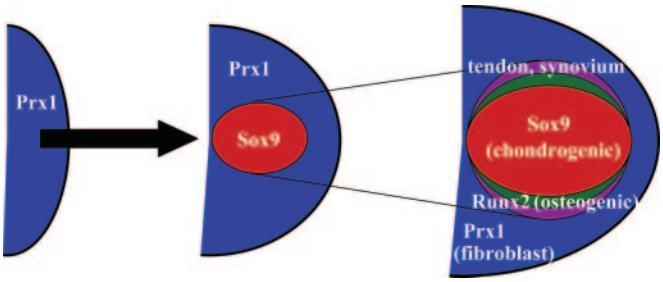

Fig. 4. Model of establishment of mesenchymal cell lineages during limb bud development. *Prx1*-expressing undifferentiated limb bud mesenchyme gives rise to *Sox9*-expressing osteo-chondroprogenitors, fibroblasts, tendon cells, and synovial cells. These *Sox9*-expressing osteo-chondroprogenitors form mesenchymal condensations, in which *Runx2*-expressing osteogenic cells are separated from *Sox9*-expressing chondrocytes.

The absence of membranous bones in $Osx^{flox/LacZ}$;*Sox9-Cre* embryos indicates that osteoblasts in membranous bones in the skull derive from *Sox9*-expressing precursor cells. In previous experiments, we used *Wnt1-Cre* to inactivate *Sox9* in delaminating cranial neural crest cells before frank emigration. In these embryos, endochondral bones did not form, but intramembranous bone formed normally (26). Together, these findings suggest that the segregation of two of the skeletal lineages arising from *Sox9*-expressing cranial neural crest cells, namely osteoblast progenitors of membranous bones and chondrocyte precursors of endochondral bones, occurs in the cranial neural crest before the expression of *Wnt-1 Cre* and, thus, before emigration of neural crest cells.

In *Sox9-Cre;R26R* compound heterozygous mice, β-gal expression was detected first in E8.0 embryos in node and notochord, and then in neural crest and its derivatives, including skin and dorsal root ganglia. Comparison of β-gal expression with *Sox9* expression revealed that *Sox9*-expressing cells gave rise to all mesenchymal cell lineages in the testis. *Sox9*-expressing cells also gave rise to all cell lineages in the spinal cord, pancreas, and epithelium of the intestines. These observations therefore indicate that *Sox9* is expressed in the progenitor cells of all of the cells that are present later in these tissues of mouse embryos. In addition, Sox9 may play crucial roles in cell proliferation and differentiation of these progenitors. Indeed, *Sox9*-null cells in the testis, spinal cord, and pancreas do not proliferate nor differentiate, as do wild-type *Sox9*-expressing cells in these tissues (H.A., unpublished data; refs. 28 and 29).

We thank Janie Finch for editorial assistance. This work was supported by the National Institutes of Health P01AR042919 (to B.d.C. and R.R.B.). DNA sequence analysis and veterinary services were supported by National Institutes of Health Grant CA16672.

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