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TECHNOLOGY REPORT

# Generation of Mice Harboring a *Sox5* Conditional Null Allele

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**Summary: *Sox5* belongs to the Sry-related HMG box gene family, which encodes transcription factors controlling cell fate and differentiation in many lineages. *Sox5* produces a long L-Sox5 protein in neuronal, glial, neural crest, cartilage, and other cells, and a short Sox5 protein in spermatids. $Sox5^{-/-}$ mice have revealed essential roles for L-Sox5 in development but their neonatal death has prevented postnatal studies. We show here that we have generated mice harboring a conditional null allele for L-Sox5 ($Sox5^{fl+}$) by flanking the fifth coding exon with *loxP* sites. Cre recombinase-mediated conversion of $Sox5^{fl+}$ into $Sox5^{fl-}$ abolishes L-Sox5 expression. Expectedly, $Sox5^{fl+/fl+}$ mice are indistinguishable from wildtype mice, and $Sox5^{fl-/fl-}$ mice from $Sox5^{-/-}$ mice. Moreover, the chondrodysplasia of $Sox5^{fl+/fl+}Sox6^{fl+/fl+}$*Prx1Cre* mice demonstrates that the two redundant chondrogenic *Sox* genes can be efficiently inactivated in a cell type-specific manner. This *Sox5* conditional null allele will be valuable in further uncovering the in vivo roles of L-Sox5. genesis 46:294–299, 2008.** **© 2008 Wiley-Liss, Inc.**

**Key words:** Sox5; gene targeting; Cre recombinase; mouse

*Sox5* belongs to a family of 20 genes in mice and humans that encode transcription factors with a highly conserved Sry-related high-mobility-group (HMG) box DNA-binding domain (review in Lefebvre *et al*., 2007). Most *Sox* genes are expressed with a specific spatial and temporal pattern from development onto adulthood, and play critical roles in determining cell fate and differentiation. *Sox5* is expressed in several cell lineages. It is expressed in spermatid cells as a short transcript that encodes a 43 kDa Sox5 protein, whose function remains unknown (Connor *et al*., 1994). It is expressed in neuronal cells, glial cells, neural crest cells, notochord cells, chondrocytes, cranial ganglia cells, pancreatic epithelial cells, and possibly a few other cell types as a long transcript encoding a 75 kDa protein, known as L-Sox5 (for "long Sox5") (Hiraoka *et al*., 1998; Lefebvre *et al*., 1998; Lioubinski *et al*., 2003; Morales *et al*., 2007; Perez-Alcala *et al*., 2004; Smits and Lefebvre, 2003; Stolt *et al*., 2006). Highly identical to Sox6 and Sox13, L-Sox5 has two functional domains: the DNA-binding HMG box domain, located in the C-terminal half of the protein, and a homodimerization coiled-coil domain, located in the N-terminal half. Sox5 corresponds to the C-terminal half of L-Sox5 only. We previously generated mice harboring null alleles for L-Sox5 ($Sox5^{-/-}$) and/or Sox6, and used them to demonstrate that the two proteins have critical, redundant roles in chondrocytes and notochord cells (Smits and Lefebvre, 2003; Smits *et al*., 2001; Smits *et al*., 2004). $Sox5^{-/-}$ mice died at birth from respiratory distress due to a cleft secondary palate and narrow thoracic cage and $Sox5^{-/-}Sox6^{-/-}$ embryos died in utero with a very severe skeletal dysplasia. All cartilaginous elements remained rudimentary, chondrocytes failing to overtly differentiate, proliferate, and produce a bona fide cartilage extracellular matrix. The replacement of cartilage by bone, a process known as endochondral ossification, was significantly delayed and disturbed. These double null mutants and mice harboring a *Sox6* conditional null allele recently helped demonstrate important, redundant roles for the two genes in gliogenesis (Stolt *et al*., 2006). While it is likely that *Sox5* also has critical roles in chondrocytes and glial cells postnatally, and in other developmental, physiological and possibly pathological processes, studies on these roles are difficult or even impossible using the *Sox5* null allele, because of the early lethality of $Sox5^{-/-}$ mice. These studies would be greatly facilitated or made possible, however, using mice harboring *Sox5* conditional null alleles. We report here on the generation of such mice using DNA homologous recombination in embryonic stem (ES) cells and the Cre-*loxP* and FLPe-*Frt* strategies (Cheah and Behringer, 2001; Kilby *et al*., 1993; Nagy, 2000).

The L-Sox5 protein is encoded by 15 exons (CE1 to CE15), spread over 374 kb of mouse genomic DNA (Fig. 1a). The coiled-coil dimerization domain is encoded by CE5 to CE7, and the HMG box DNA-binding domain, by CE13 and CE14. We previously generated a null allele for

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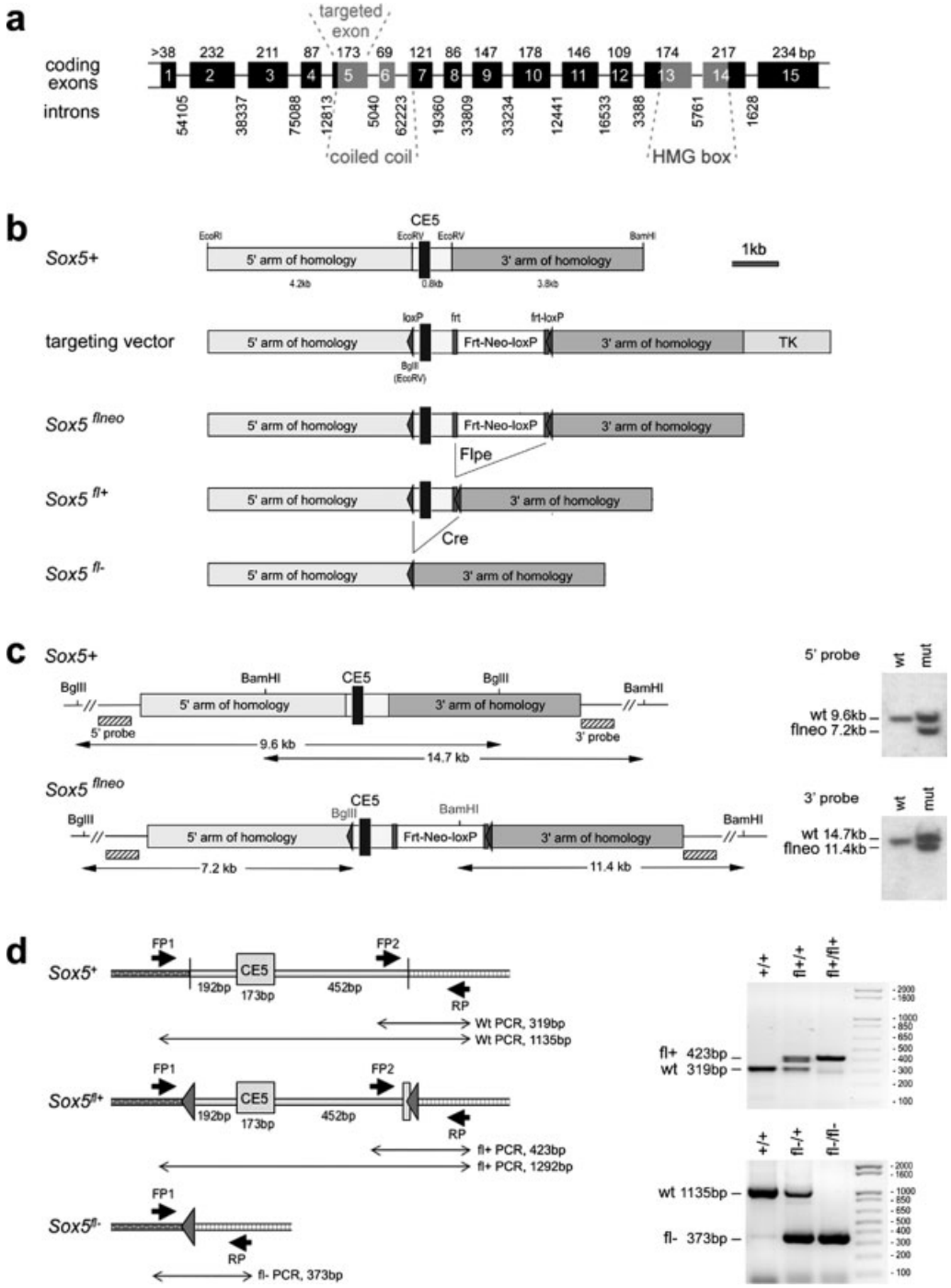



**FIG. 1.** Design and generation of *Sox5* conditional null mice. (**a**) Schematic of the coding region of the mouse *Sox5* gene. Coding exons are drawn to scale relative to each other. Intervening introns are not drawn to scale. The size of each exon and intron is indicated in bp. The targeted exon and the exons coding for the functional domains of L-Sox5 (HMG box and coiled coil dimerization) are indicated. (**b**) Schematic of the *Sox5* targeted segment, targeting vector, and new alleles. The targeting region consisted in a 4.2 kb *Eco*RI/*Eco*RV 5′ arm of homology, a 0.8 kb *Eco*RV/*Eco*RV core region containing CE5, and a 3.8kb *Eco*RV/*Bam*HI 3′ arm of homology. The targeting vector featured a *Bgl*II site and a *loxP* site that replaced the 5′ *Eco*RV site of the 0.8 kb core region, an *frt-neo*$^r$*-frt-loxP* cassette replacing the 3′ *Eco*RV site of the 0.8 kb core region, and a *TK* cassette inserted 3′ of the 3′ arm of homology. The *Sox5*$^{flneo}$ allele has the same features as the targeting vector, but lacks the *TK* cassette. Flpe-mediated excision of the *frt-neo*$^r$*-frt-loxP* cassette from the *Sox5*$^{flneo}$ allele resulted in the *Sox5*$^{fl+}$ allele, and Cre-mediated recombination of the *loxP* sites resulted in the *Sox5*$^{fl-}$ allele. The *loxP* and *Frt* sites are drawn at a larger scale than the other DNA sequences. (**c**) Southern analysis of ES cell clones. The schematics show the position of 5′ and 3′ external probes (blocks filled with diagonal lines), the restriction enzymes used to digest genomic DNA, and the fragments hybridizing with the probes (double arrows with the fragment sizes in bp). The pictures show the signals that were obtained on Southern blots with DNA from wild-type (wt) and *Sox5*$^{flneo/+}$ (mut) ES cell clones. (**d**) PCR genotyping of mice carrying *Sox5*$^{+}$, *Sox5*$^{fl+}$, and *Sox5*$^{fl-}$ alleles. The schematics represent the *Sox5* CE5 exon and flanking intron sequences. Vertical lines in the *Sox5*$^{+}$ allele represent the *Eco*RV sites, in which *loxP* and *frt*/*loxP* sites are inserted in the *Sox5*$^{fl+}$ allele. PCR primers are shown as thick, short arrows and PCR products as thin, long, double-headed arrows. The pictures show the PCR products obtained using DNA from mice carrying the *Sox5* alleles indicated on top of the lanes.

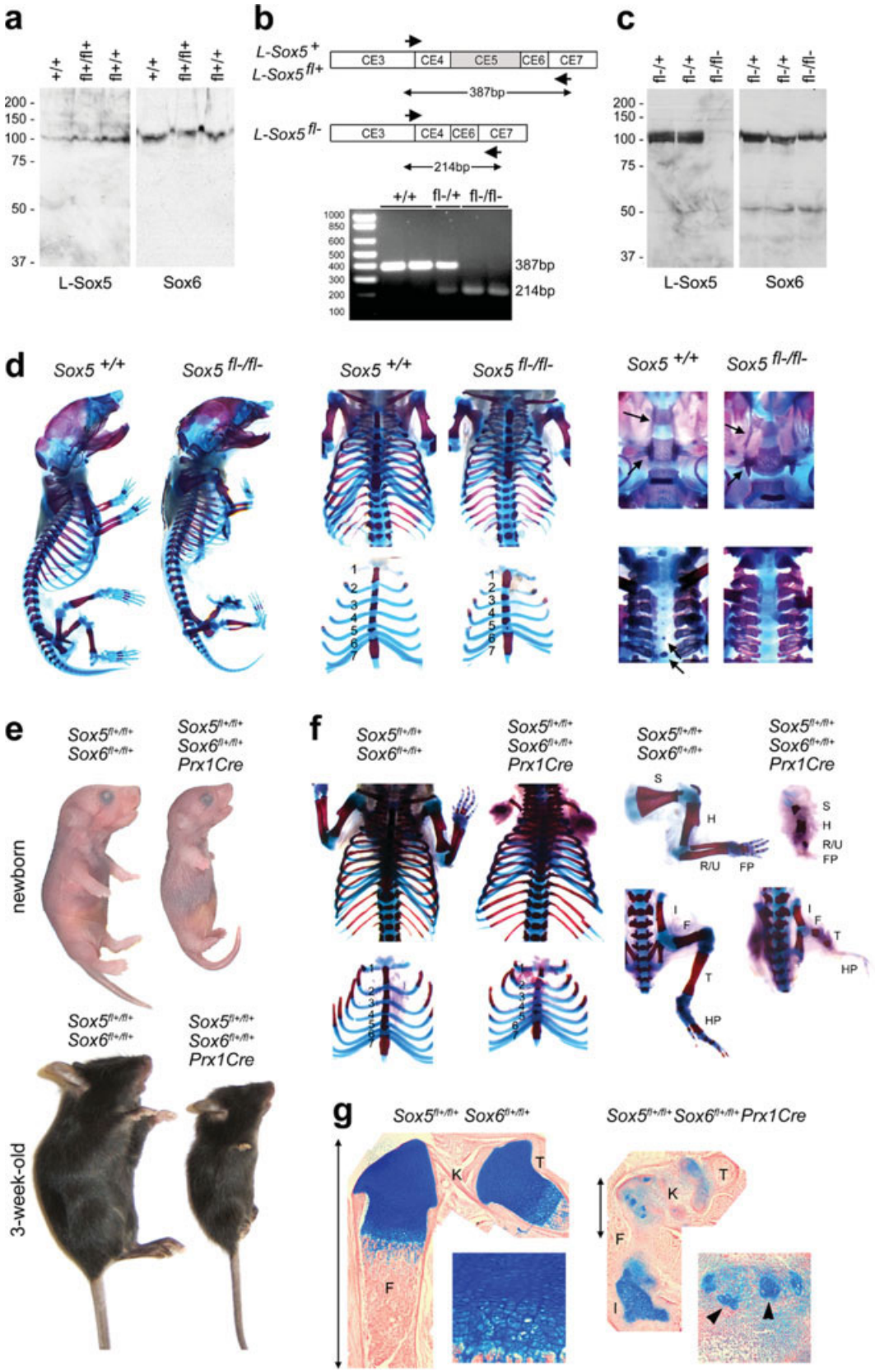
L-Sox5 (*Sox5*$^{-}$) by knocking in a *lacZ/neo*$^{r}$ cassette into CE5 (Smits *et al*., 2001). Here we generated a conditional null allele for L-Sox5 by flanking CE5 with *loxP* sites (Fig. 1b). We inserted the two *loxP* sites in the same orientation, such that Cre-mediated recombination would result in excising CE5. Splicing of CE4 into CE6 was predicted based on the gene sequence to create a frameshift rapidly followed by a stop codon, and thus to result in production of a short peptide lacking the two main functional domains of L-Sox5. It must be noted that this strategy was not designed to generate a conditional null allele for the short Sox5 protein, since the transcript for this protein only features the coding exons CE8 to CE15.

We constructed a targeting vector to generate a *Sox5*$^{fl neo}$ allele using 129SvEv genomic DNA sequences encompassing CE5 (Fig. 1b). We introduced a neomycin resistance cassette (*neo*$^{r}$) upstream of the 3′ *loxP* site for positive selection of ES cells (Meyers *et al*., 1998). We flanked it with *frt* sites to delete it through FLPe-mediated recombination. We introduced a thymidine kinase cassette (*TK*) 3′ of the construct for negative selection of ES cells with gancyclovir. Following electroporation of ES cells with the targeting vector and selection with neomycin and gancyclovir, we identified two clones heterozygous for the *Sox5*$^{fl neo}$ allele in Southern blot analysis (Fig. 1c). We used both clones to generate mouse male chimeras. These mice transmitted the new allele to their progeny. One mouse line was maintained and is described here. We induced conversion of *Sox5*$^{fl neo}$ into *Sox5*$^{fl+}$ by breeding chimeras with females carrying a *hACTB-FLPe* transgene (Rodriguez *et al*., 2000). This transgene expressed FLPe under the control of human β-actin sequences, and thus resulted in excision of the *frt*-flanked *neo*$^{r}$ cassette from *Sox5*$^{fl neo}$ almost ubiquitously in progeny that inherited from both *Sox5*$^{fl neo}$ and the transgene. Such progeny were then crossed with wildtype mice, and *Sox5*$^{fl+/+}$ progeny that did not carry the *hACTB-FLPe* transgene were used to derive a *Sox5*$^{fl+/fl+}$ mouse line. *Sox5*$^{fl neo}$ was converted into *Sox5*$^{fl-}$ by breeding chimeras with females carrying a *PrmCre* transgene, expressed exclusively in the male germ line (O'Gorman *et al*., 1997). *Sox5*$^{fl neo/+}$*PrmCre* male progeny were bred with wildtype females, which gave birth to *Sox5*$^{fl-/+}$ progeny. Those mice that did not carry the *PrmCre* transgene were intercrossed for analysis (see later). All mice were genotyped by PCR using strategies designed to readily identify the *Sox5*$^{+}$, *Sox5*$^{fl+}$, and *Sox5*$^{fl-}$ alleles (Fig. 1d).

*Sox5*$^{fl+}$ differs from *Sox5*$^{+}$ by the presence of *loxP* sites flanking CE5 and by an *frt* site inserted directly upstream of the 3′ *loxP* site. *Sox5*$^{fl+}$ was thus expected to express wildtype RNA and to do so at a normal level. Accordingly, *Sox5*$^{fl+/fl+}$ mice behaved normally and were externally indistinguishable from *Sox5*$^{+/+}$ and *Sox5*$^{fl+/+}$ littermates at all ages (data not shown). To verify that their level of L-Sox5 protein was normal, we prepared primary chondrocytes from newborn mouse rib cages and observed in western blot that L-Sox5 was running at the same level and was similarly abundant in cells from *Sox5*$^{+/+}$, *Sox5*$^{fl+/+}$, and *Sox5*$^{fl+/fl+}$ mouse littermates (Fig. 2a). All cultures also had the same level of Sox6 protein, proving that they contained similar proportions of differentiated chondrocytes. We thus concluded that *Sox5*$^{fl+}$ is a fully functional allele for L-Sox5.

*Sox5*$^{fl-}$ differs from *Sox5*$^{+}$ by replacement of CE5 and immediately flanking intron sequences with a *loxP* site. Its RNA was thus predicted to lack CE5 and to encode no functional L-Sox5 protein. Accordingly, RT-PCR performed with total RNA from *Sox5*$^{+/+}$, *Sox5*$^{+/fl-}$, and *Sox5*$^{fl-/fl-}$ embryo littermates and primers annealing in CE3 and CE7 yielded products of expected size for *Sox5*$^{+}$ and *Sox5*$^{fl-}$ RNA, i.e., including and excluding

**FIG. 2.** Analysis of *Sox5* conditional null mice. (**a**) Western blot demonstrating that primary chondrocytes from *Sox5*$^{+/+}$, *Sox5*$^{fl+/+}$, and *Sox5*$^{fl+/fl+}$ mouse littermates contained similar amounts of L-Sox5 and Sox6 protein. The two proteins migrate with an apparent Mr close to 100 k, as expected. (**b**) RT-PCR demonstrating splicing of CE4 into CE6 in the *Sox5*$^{fl-}$ RNA. The schematics show the CE3 to CE7 region in the *Sox5*$^{+}$ (identical in *Sox5*$^{fl+}$) and *Sox5*$^{fl-}$ RNAs, and the position of PCR primers (arrowheads) and PCR products (double arrows). The picture shows results of RT-PCR performed using *Sox5*$^{+/+}$, *Sox5*$^{fl-/+}$, and *Sox5*$^{fl-/fl-}$ whole-embryo RNA. (**c**) Western blot demonstrating that primary chondrocytes from *Sox5*$^{+/+}$ and *Sox5*$^{fl-/+}$ contained similar amounts of L-Sox5 protein whereas primary chondrocytes from *Sox5*$^{fl-/fl-}$ littermates had no L-Sox5 protein. All cells contained the same amount of Sox6 protein. (**d**) Skeletal preparations of *Sox5*$^{+/+}$ and *Sox5*$^{fl-/fl-}$ newborn littermates. The two pictures on the left show that the mutant mouse had an overall fairly normal skeleton. The middle pictures show that the mutant had shorter ribs (top) and sternum (bottom). Note that the seventh pair of ribs was so short that it did not attach to the sternum. The top right pictures show that the mutant had a cleft secondary palate, as demonstrated by failure of the palatal shelves (upper arrows) and pterigoid processes (lower arrows) to move toward the midline. The bottom right pictures show that the cervical vertebral bodies (arrows) of the control mouse had started to mineralize whereas those of the mutant had not. (**e**) Pictures of *Sox5*$^{fl+/fl+}$*Sox6*$^{fl+/fl+}$ and *Sox5*$^{fl+/fl+}$*Sox6*$^{fl+/fl+}$*Prx1Cre* littermates at birth and at three weeks of age. The mutants were overall smaller than the controls, and had extremely short limbs. The newborns are shown at a different magnification from the older pups. (**f**) Skeletal preparations of *Sox5*$^{fl+/fl+}$*Sox6*$^{fl+/fl+}$ and *Sox5*$^{fl+/fl+}$*Sox6*$^{fl+/fl+}$*Prx1Cre* newborn littermates. The left pictures show that the ribs and vertebrae of the mutant were normal, but that the sternum was very short, resulting in a bell-shaped thoracic cage. The upper right pictures show the upper limbs and the lower right pictures show the lower limbs. The more distal skeletal elements were more affected than the more proximal elements in size and in ability to stain with Alizarin red and Alcian blue. S, scapula; H, humerus; R/U, radius and ulna; FP, front paw; I, ilium; F, femur; T, tibia; HP, hind paw. (g) Histology analysis of *Sox5*$^{fl+/fl+}$*Sox6*$^{fl+/fl+}$ and *Sox5*$^{fl+/fl+}$*Sox6*$^{fl+/fl+}$*Prx1Cre* newborn littermates. Longitudinal sections through the hind limbs were stained with Alcian blue and nuclear fast red. The left-corner pictures show the femur (F), with the distal half indicated with a double arrow, the knee joint (K), and the proximal part of the tibia (T). Part of the iliac cartilage (I) is shown for the mutant limb. The right-corner pictures show a portion of the femur growth plate at high-magnification. Note in the mutant pictures that chondrocytes in the iliac cartilage and a few clusters (shown with arrowheads) of chondrocytes in the femur are surrounded with a fairly normal amount of cartilage matrix, whereas other chondrocytes have little cartilage matrix and are difficult to distinguish from other cell types.


a
+/+
fl+/fl+
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200 -
150 -
100 -
75 -
50 -
37 -
L-Sox5
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CE3
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CE5
CE6
CE7
387bp
L-Sox5 fl-
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**FIG. 2.**

CE5, respectively (Fig. 2b). We also compared the level of L-Sox5 protein in primary chondrocytes prepared from $Sox5^{+/+}$, $Sox5^{+/fl-}$, and $Sox5^{fl-/fl-}$ littermates. While $Sox5^{+/+}$ and $Sox5^{+/fl-}$ extracts contained a high level of both L-Sox5 and Sox6 protein, $Sox5^{fl-/fl-}$ extracts only contained Sox6 (Fig. 2c). $Sox5^{fl-/fl-}$ mice had the same phenotype as $Sox5^{-/-}$ mice: they died at birth, and skeletal preparations revealed a narrow thoracic cage, irregularly mineralized sternum, cleft secondary palate, and delayed mineralization of cervical vertebral bodies (Fig. 2d). These data thus proved that $Sox5^{fl-}$ is a bona fide null allele for L-Sox5.

We next tested Cre-mediated recombination of $Sox5^{fl+}$ in somatic cells in vivo using the *Prx1Cre* transgene, which expresses Cre recombinase at a high level in early limb bud mesenchymal cells and at a low level in several other regions (Logan *et al.*, 2001). Like $Sox5^{fl+/fl+}$ and $Sox6^{fl+/fl+}$ mice, $Sox5^{fl+/fl+}Sox6^{fl+/fl+}$ mice appeared normal in every respect (Dumitriu *et al.*, 2006, and data not shown). $Sox5^{fl+/fl+}$*Prx1Cre* and $Sox6^{fl+/fl+}$*Prx1Cre* mice looked normal at birth, with their limbs, like those of $Sox5^{-/-}$ and $Sox6^{-/-}$ newborns, hardly, if at all, smaller than those of control littermates (data not shown). Unlike $Sox5^{-/-}$ mice and $Sox6^{-/-}$ mice, which die at birth or before weaning, $Sox5^{fl+/fl+}$*Prx1Cre* and $Sox6^{fl+/fl+}$*Prx1Cre* mice were viable and fertile, and unlike $Sox5^{-/-}Sox6^{-/-}$ fetuses, which die around embryonic day 16.5, $Sox5^{fl+/fl+}$ $Sox6^{fl+/fl+}$ *Prx1Cre* mice were born alive. These latter mutants had an overall small size and strikingly short limbs (Fig. 2e). Most reached weaning age, but were then sacrificed because of severe limb handicap. Skeletal preparations revealed that they were born with a short sternum, but with an otherwise virtually normal craniofacial and axial skeleton (Fig. 2f and data not shown). Their appendicular skeleton, however, was very abnormal. Cartilage could be seen upon Alcian blue staining in the most proximal elements, i.e., the ilium and scapula, but not in the distal elements. The humerus, radius, ulna, femur, and tibia nevertheless showed a short core of mineralized bone, staining with alizarin red, but the paws also failed to stain with this dye. These skeletal preparations strongly suggested that these mutants had the same skeletal defects as $Sox5^{-/-}Sox6^{-/-}$ fetuses in the elements derived from cells expressing the *Prx1Cre* transgene. Histology analysis supported this conclusion (Fig. 2g and data not shown). While the ilium and scapula cartilage looked fairly normal, the other skeletal elements of the limbs were severely reduced in size and underdeveloped. Most of their chondrocytes had the typical appearance of $Sox5^{-/-}Sox6^{-/-}$ chondrocytes: they were surrounded with little if any cartilage extracellular matrix staining with Alcian blue, and were unable to develop cartilage growth plates. Consequently, endochondral bones and synovial joints were missing or very abnormal.

These results demonstrate that the *Sox5* conditional null allele that we have generated will constitute a very valuable tool to specifically block expression of L-Sox5 in specific cell types in vivo. It will allow or greatly facilitate studies on the roles of L-Sox5 in multiple developmental, physiological, and even pathological processes in the mouse from embryogenesis onto adulthood.

## METHODS

### Gene Targeting and Generation of Mutant Mice

The $Sox5^{flneo}$ targeting vector was constructed in the pBluescript $KS^{+/-}$ plasmid (Stratagene) using *Sox5* DNA clones obtained from a 129SvEv mouse genomic library. The *frt-neo$^r$-frt-loxP* neomycin resistance cassette (Meyers *et al.*, 1998) and *MC1tkpA* thymidine kinase cassette (Cheah and Behringer, 2001) were previously described. The 5′ *loxP* site and an adjacent *Bgl*II site were synthesized as an oligonucleotide. ES cell clones and mouse chimeras were generated by the Case Western Reserve University Transgenic Core. The targeting vector was electroporated into R1 ES cells, and recombinant clones were amplified in selection medium containing neomycin and gancyclovir. All mice were maintained on the 129SvE × C57BL/6J hybrid genetic background.

### Genotyping

Genomic DNA was prepared from ES cell clones and mice, and analyzed in Southern blot according to standard protocols. A 1.6 kb *Bam*HI/*Eco*RI genomic fragment located directly upstream of the 5′ arm of homology was used as 5′ external probe, and a 1.3 kb *Bgl*II genomic fragment located directly downstream of the 3′ arm of homology was used as 3′ external probe. Mice carrying $Sox5^{+}$ and $Sox5^{fl+}$ alleles were genotyped in a single PCR reaction using a forward "FP2" primer, 5′-GGC ATG GGC CCA AGA TGT TTA AGA-3′, and a reverse "RP" primer, 5′-CGA TGC ACA TGA TCA CAG TTG GGT-3′. Mice carrying the $Sox5^{fl-}$ allele were genotyped by PCR using a forward "FP1" primer, 5′-AAC TCA TGT ACT TGG GCT TCC CGA-3′, and the reverse RP primer. PCR was performed under identical conditions for all alleles, using TAQ polymerase in standard buffer containing 2 mM $MgCl_2$, with a denaturation step at 94°C for 2.5 min, 35 cycles at 94°C for 15 s, 65°C for 75 s, 72°C for 90 s, and an extension step for 7min at 72°C. Mice carrying *Sox6, Flpe,* and *Cre* alleles were genotyped as previously described. PCR products were visualized by ethidium bromide staining following DNA electrophoresis.

### Protein Analysis

Primary chondrocytes were isolated from the rib cages of newborn mice and cultured as described (Lefebvre *et al.*, 1994). Whole-cell extracts were made by lysing cells in SDS-PAGE loading buffer. Protein concentration was determined using the Dc protein assay kit (Bio-Rad). Western blot analyses were carried out according to a standard procedure and using an equal amount of protein for each sample. The blots were hybridized with custom-made rabbit polyclonal antibodies specifically recognizing L-Sox5 or Sox6 (Invitrogen). These antibod-

ies were generated using keyhole limpet hemocyanin linked to an L-Sox5 peptide corresponding to residues 1–15 (MLTDPDLPQEFERMSSK) or to a Sox6 peptide corresponding to residues 812–827 (SDYSSENEAPEPVSAN). Specific antibodies were purified from rabbit serum by peptide affinity chromatography. They were used at a 1:1,000 dilution (0.5 μg/ml). Signals were detected using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

### RNA Analysis

Total RNA was extracted from mouse embryos at day 12.5 of gestation using the TriZol reagent (Invitrogen). RT-PCR was achieved by synthesizing cDNA with random DNA hexamers using the ThermoScript RT-PCR System (Invitrogen) and performing PCR with TAQ polymerase using the forward primer 5′-AAT GAG CCA GAA GAC ACT CCC AGT-3′, and the reverse primer 5′-AGG AGG GAA CAC GGG AAT CAT CAA-3′. PCR consisted of one cycle at 94°C for 2.5 min, 36 cycles at 94°C for 15 s, 59°C for 30 s, 72°C for 1 min, and one cycle at 72°C for 10 min.

### Skeletal Preparations and Histology Analysis

Whole-mount skeletal preparations were made by staining cartilage with Alcian blue and mineralized cartilage and bone with Alizarin red, followed by partially digestion and clearing of soft tissues with KOH in glycerol (Hogan *et al*., 1994). Histology analysis was performed on paraffin-embedded sections, as previously described (Smits *et al*., 2001).

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