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Sox9-expressing Precursors Are the Cellular Origin of the Cruciate Ligament of the Knee Joint and the Limb Tendons

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Summary

Sox9 expression defines cell progenitors in a variety of tissues during mouse embryogenesis. To establish a genetic tool for cell-lineage tracing and gene function analysis, we generated mice in which the *CreERT2* gene was targeted to the endogenous mouse *Sox9* locus. In *Sox9^{CreERT2/+};R26R* embryos, tamoxifen activated Cre recombinase exclusively in *Sox9*-expressing tissues. To determine the suitability of this mouse line for developmental stage-specific gene recombination, we investigated the cellular origins of the cruciate ligaments of the knee joint and the limb tendons, in which precursor cells have not been defined. The cells in these tissues were labeled after tamoxifen treatment before or at the stage of chondrogenic mesenchymal condensation, indicating that ligament and tendon cells originated from *Sox9*-expressing cells and that cell fate determination occurred at mesenchymal condensation. This mouse line is a valuable tool for the temporal genetic tracing of the progeny of, and inducible gene modification in *Sox9*-expressing cells. *genesis* 48:635–644, 2010.

Keywords

Sox9; CreERT2; cell fate; cruciate ligament; limb tendon

INTRODUCTION

The Cre/loxP site-specific DNA recombination system is a powerful tool in the field of mouse genetics. The conditional ablation or induction of specific genes allows the control of gene activity in a tissue- and time-dependent manner, thus permitting analysis of gene function and cell-lineage tracing *in vivo* (Zou *et al.*, 1994). To achieve a more precise control of Cre activity both spatially and temporally, ligand-dependent chimeric Cre recombinases have been developed (Feil *et al.*, 1996). A fusion polypeptide of Cre recombinase and a mutant ligand-binding domain of the estrogen receptor, *CreERT2*, are inserted into the mouse genome, and the synthetic estrogen receptor ligand, tamoxifen, activates Cre recombinase, resulting in the excision of loxP-containing alleles at specific times during embryogenesis and after birth (Indra *et al.*, 1999).

Sox9 is a member of the SOX family of transcription factors that contains a high-mobility group DNA binding domain and was identified as a causative gene of Campomelic

dysplasia, a rare skeletal dysplasia associated with XY sex reversal. Campomelic dysplasia is an autosomal-dominant condition caused by haploinsufficiency of the gene via heterozygous mutation in and around *SOX9* gene. The distinct clinical features of this disease include a disproportionately short stature, bowing of the limbs, low ears, a depressed nasal bridge, talipes equinovarus, elongated philtrum, and micrognathia (Foster *et al.*, 1994). Our recent cell-lineage tracing analysis using *Sox9-Cre* knock-in mice indicate that cells in a variety of tissues are derived from *Sox9*-expressing precursors (Akiyama *et al.*, 2005). Furthermore, the conditional gene ablation of *Sox9* using the Cre/loxP-mediated recombination system reveals that *Sox9* plays crucial roles in cell-lineage determination at an initial stage of organogenesis, as well as in cell proliferation and differentiation and in the maintenance of cellular phenotypes in a stage-specific manner (Akiyama *et al.*, 2002; Chabossier *et al.*, 2004; Mori-Akiyama *et al.*, 2003; Seymour *et al.*, 2007; Stolt *et al.*, 2003). Thus, the control of DNA recombination at different stages during organogenesis using tamoxifen-inducible *CreERT2* under the control of the endogenous *Sox9* promoter should open new avenues for the study of gene function and cell-lineage tracing.

In this study, we used homologous recombination to introduce a *CreERT2* construct, encoding a tamoxifen-inducible Cre, into the 3' untranslated region (UTR) of the endogenous mouse *Sox9* gene and created a *Sox9^{CreERT2}* mouse line. We report here that tamoxifen administration to offspring of crosses between *Sox9^{CreERT2}* and *Rosa26 reporter (R26R)* mice (Soriano, 1999) efficiently induced expression of the Cre reporter in *Sox9*-expressing tissues.

In our previous report, *Sox9*-expressing mesenchymal cells contribute to the formation of the ligaments, tendons and synovium in *Sox9^{Cre/+};R26R* embryos (Akiyama *et al.*, 2005), but the cellular origins of these tissues have not been well defined. Using this mouse line, we found that cells in the cruciate ligament of the knee joint and limb tendons, the Achilles tendon and the patellar tendon, originated from *Sox9*-expressing precursors.

RESULTS

Generation of a *Sox9-CreERT2* Mouse Line

We generated a knock-in allele in mouse ES cells in which a *CreERT2* was integrated into the 3' UTR of the endogenous mouse *Sox9* gene (Fig. 1). The construct consisted of a 5' internal ribosome entry sequence (IRES) followed immediately by the *CreERT2* open reading frame with a SV40 polyadenylation sequence. Consequently, the *Sox9* mRNA transcribed from the targeted allele incorporated the nucleotide sequence of *IRES-CreERT2*, thus forming a bicistronic mRNA. Heterozygous *Sox9^{CreERT2/+}* mice were viable and fertile, developed normally, and were not smaller than their wild-type littermates. Homozygotes *Sox9^{CreERT2/+}* mice were also viable and fertile, indicating that the knock-in gene did not disrupt *Sox9* expression, which is essential for normal growth (Bi *et al.*, 2001). *Sox9^{CreERT2/+}* mice were maintained by crossing with wild-type C57BL/6 mice and were backcrossed eight times. All heterozygous *Sox9^{CreERT2/+}* mice in this study were analyzed on a C57BL/6 genetic background.

Sox9-specific Cre Induction by Tamoxifen Treatment

To test the inducible properties of the Cre recombinase, male *Sox9^{CreERT2/+}* mice were crossed with female *R26R* mice. *R26R* mice carry the *LacZ* gene inserted into the ubiquitously expressed *Rosa26* locus, which is preceded by a transcriptional stop cassette flanked by loxP sites (Soriano, 1999). Pregnant females were injected once with tamoxifen intraperitoneally at embryonic day (E) 13.5, and were sacrificed 48 h after injection. The presence of Cre activity was monitored by detection of β -galactosidase expression using X-

gal staining. Strong X-gal staining was detected in the cartilages of the appendicular and axial skeleton, as well as in the whisker follicles and ears of *Sox9^{CreERT2/+};R26R* embryos at E15.5. (Fig. 2a–d). The calvariae that were formed via intramembranous ossification were X-gal negative (Fig. 2b). No X-gal staining was observed in control *R26R* embryos harvested from pregnant females that were injected with tamoxifen and control *Sox9^{CreERT2/+};R26R* embryos harvested from pregnant females that were injected with the vehicle alone, i.e., corn oil (Fig. 2a). *Sox9*-expressing tissues, including brain, heart, lung, kidney, testis, intestine, and pancreas of E16.5 *Sox9^{CreERT2/+};R26R* embryos harvested from pregnant females that were injected with tamoxifen at E12.5 were X-gal positive, while weak endogenous β -galactosidase activity was detected in the kidney and intestine of control *R26R* embryos and control *Sox9^{CreERT2/+};R26R* embryos (Fig. 2e). This weak endogenous β -galactosidase activity was nonspecific X-gal staining.

Tamoxifen-mediated Cre Induction in Osteo-chondroprogenitor Cells of Limb Buds

Our recent cell-lineage analysis using *Sox9-Cre* knock-in mice suggested that *Sox9*-expressing limb bud mesenchymal cells give rise to both chondrocytes and osteoblasts, indicating that *Sox9* defines osteo-chondroprogenitors (Fig. 3a) (Akiyama *et al.*, 2005). Strong X-gal staining was detected in all chondrocytes of the growth plate including hypertrophic chondrocytes of E16.5 *Sox9^{CreERT2/+};R26R* embryos harvested from pregnant females that were injected with tamoxifen at E10.5 before chondrogenic mesenchymal condensation and at E12.5 during chondrogenic mesenchymal condensation (Ng *et al.*, 1997) (Fig. 3b, c). However, the positive X-gal staining detected in periosteum, in which preosteoblasts were located, was detected exclusively in embryos from females injected with tamoxifen at E10.5, and not in embryos from females injected at E12.5 (Fig. 3b, c, arrow). This suggests that segregation of osteoblast and chondrocyte lineages occurred in mesenchymal condensation.

Stage-specific Cell-lineage Tracing of the Cruciate Ligaments of the Knee Joint Using *Sox9^{CreERT2/+};R26R* Mice

We reported previously that *Sox9*-expressing mesenchymal cells contribute to the formation of the ligaments, tendons and synovium in *Sox9^{Cre/+};R26R* embryos (Akiyama *et al.*, 2005). In *Sox9^{Cre/+};R26R* embryos harvested at E17.0, strong X-gal staining was observed in the cruciate ligaments of the knee joint, the Achilles tendon and the patellar tendon (Figs. 4d, 5d, and 6d). In addition, a recent report using *Col2a1-Cre/R26R* mice showed that the cruciate ligaments of the knee joint are derived from *Col2a1*-expressing cells (Hyde *et al.*, 2008). First, we analyzed the expression of *Sox9* in the cruciate ligaments via X-gal staining using *Sox9-LacZ* mice that were generated previously by our group (Bi *et al.*, 1999). At E13.5, *Sox9* was expressed in cartilage primordia in the presumptive knee joint of *Sox9^{LacZ/+}* embryos. At this stage, the interzone started to form, and *Sox9* was expressed in some cells of the interzone (Fig. 4a). At E14.5, *Sox9* expression was restricted to chondrocytes, and no expression of *Sox9* was observed in the developing joint (Fig. 4b). At E15.5, when the cruciate ligaments form (Hyde *et al.*, 2008), *Sox9* expression was absent in the cells of the cruciate ligaments (Fig. 4c).

Next, to determine the origins of the cruciate ligament cells, we examined *Sox9^{CreERT2/+};R26R* newborn mice using X-gal staining after single injection of tamoxifen at various time points between E11.5 and E15.5. X-gal positive cells were detected in the cruciate ligaments of *Sox9^{CreERT2/+};R26R* newborn mice that were injected at E11.5 before chondrogenic mesenchymal condensation (Fig. 4e). The cruciate ligament cells of *Sox9^{CreERT2/+};R26R* newborn mice that were injected with tamoxifen at E12.5 and E13.5 were also positive for X-gal staining (Fig. 4f, g). Tamoxifen injection at E14.5 led to marked decrease in the number of X-gal positive cells which were detected only in and around the

bony insertions of the cruciate ligaments (Fig. 4h). No labeled cells were detected in newborn mice that were injected with tamoxifen at E15.5 (Fig. 4i). Thus, these results suggest that the cells in the cruciate ligaments of the knee joint originated from *Sox9*-expressing condensed mesenchymal cells and that these cells are committed to ligament cells before the joint cavity is formed.

Stage-specific Cell-lineage Tracing of the Limb Tendons of *Sox9^{CreERT2/+};R26R* Mice

Tendons are interconnective tissues between muscles and bones. Although specific molecules, including Scler-axin and Tenomodulin, play important roles in tendon development, the cellular origins of the tendons have not been defined. First, we performed cell-lineage tracing of the Achilles tendon, which is the largest tendon in the zeugopod/autopod. *Sox9*-expressing cells were detected exclusively in cartilage primordia in the autopod of the hind limbs of *Sox9^{LacZ/+}* embryos at E13.5 and E14.5, at which stages the Achilles tendon was not yet established (Fig. 5a, b). *Sox9* expression was negative in the calcaneus with the exception of chondrocytes at E15.5, which is the stage of formation of the Achilles tendon (Fig. 5c). The Achilles tendon cells of *Sox9^{CreERT2/+}; R26R* newborn mice that were injected tamoxifen at E11.5, E12.5, and E13.5 were X-gal positive (Fig. 5e–g), whereas only a few cells were labeled after tamoxifen injection at E14.5 and X-gal positive cells were only detected at the bony insertions of the Achilles tendon (Fig. 5h). No labeled cells were detected in newborn mice that were injected with tamoxifen at E15.5 (Fig. 5i).

Similar results were observed in the other tendon. The cartilage primordium of tibia was observed in *Sox9^{LacZ/+}* embryos at E13.5 (Fig. 6a). At E14.5 the patellar tendon in the stylopod/zeugopod were established, but no X-gal positive cells were observed in this tendon and at a later stage. (Fig. 6b, c). The patellar tendon cells of *Sox9^{CreERT2/+}; R26R* newborn mice injected tamoxifen at E12.5 were X-gal positive (Fig. 6e). No labeled cells were detected in newborn mice that were injected with tamoxifen at E13.5 and later stages (Fig. 6f–h). Interestingly, X-gal positive cells were extensively found in the Achilles tendon and in patellar tendon when tamoxifen was injected before and just at mesenchymal condensations (Figs. 5g and 6e), but the number of X-gal positive cells rapidly decreased when tamoxifen was injected after mesenchymal condensations. These results indicate that the cells of the tendon originated from *Sox9*-expressing chondrogenic mesenchymal cells in the cartilage primordia. These cells are committed to the tendon cells at, and just after, mesenchymal condensation. Once the tendon forms and attaches to the cartilage, *Sox9*-expressing cells in the cartilage primordia no longer contribute to it.

DISCUSSION

Recent mouse genetic approaches using the conditional alleles of the *Sox9* gene revealed essential roles for *Sox9* in the development of a variety of tissues during mouse embryogenesis, including cartilage, spinal cord, testis, and pancreas (Akiyama *et al.*, 2002; Chabossier *et al.*, 2004; Mori-Akiyama *et al.*, 2003; Seymour *et al.*, 2007; Stolt *et al.*, 2003). In these tissues, *Sox9* defines progenitor cells and determines cell-lineages at the initial step of organogenesis. During the skeletal development, genetic ablation of the *Sox9* gene results in complete absence of endochondral skeletal elements (Akiyama *et al.*, 2002). In the developing spinal cord, *Sox9* expression is detected throughout the ventricular neuroepithelial layer, in which neural progenitor cells are located (Stolt *et al.*, 2003). During the formation of testis, *Sox9* is detected in mesenchymal progenitors (Akiyama *et al.*, 2005). In vitro culture of the urogenital ridges of *Sox9*-null gonads results in gonads that lack testicular cords that initiate ovarian development (Kobayashi *et al.*, 2005). During pancreas development, *Sox9* inactivation induces severe pancreatic hypoplasia, which results from the

depletion of the progenitor pool (Seymour *et al.*, 2007). On the basis of these mouse genetic studies, we previously generated mice carrying the Cre recombinase gene inserted into the 3'UTR of the *Sox9* gene. This mouse line has been useful for the tracing of *Sox9*-expressing precursors and for specific gene ablation in progenitor cells at the early stage of tissue development (Akiyama *et al.*, 2005).

The expression and functions of *Sox9* are also restricted at later stages of tissue development: *Sox9* is expressed exclusively in chondrocytes in the skeletons, Sertoli cells in the testis, epithelial cells in the intestine, and ductal cells in the pancreas. Thus, we took advantage of the developmental stage-specific expression of *Sox9*, to generate mice carrying an inducible form of the Cre recombinase gene, *CreERT2*, under the control of the endogenous *Sox9* promoter. The properties of tamoxifen-inducible Cre recombinase expression were assessed by crossing *Sox9^{CreERT2/+}* mice with the Cre-dependent *R26R* strain (Soriano, 1999). As expected, intra-peritoneal injection of tamoxifen induced genomic recombination exclusively in *Sox9*-expressing tissues, which included cartilage, brain, lung, heart, intestine, pancreas, kidney and testis. To determine whether the *Sox9^{CreERT2/+}* mouse line is useful for cell-lineage tracing at different developmental stages, we confirmed the properties of X-gal positive cells in the limb buds of *Sox9^{CreERT2/+};R26R* embryos by tamoxifen treatment at different time points. Positive X-gal staining was observed in all chondrocytes of the growth plate and periosteum, in which the preosteoblasts are located, of E16.5 *Sox9^{CreERT2/+};R26R* embryos harvested from pregnant females that were injected with tamoxifen at E10.5, whereas positive X-gal staining was also observed in all chondrocytes and in the cruciate ligaments, but not in periosteum, of E16.5 *Sox9^{CreERT2/+};R26R* embryos harvested from pregnant females that were injected with tamoxifen at E12.5. Tamoxifen treatment at different developmental stages of limb buds resulted in the identification of cell-lineage segregation in *Sox9^{CreERT2/+};R26R*. Thus, tamoxifen-inducible Cre recombinase expression in *Sox9^{CreERT2/+};R26R* mice is useful for developmental-stage-specific cell-lineage tracing in various tissues.

We used the advantage of *Sox9^{CreERT2/+}* mice to perform cell-lineage tracing in the cruciate ligaments of the knee joint via stage-specific single injection of tamoxifen into pregnant females. During knee joint formation, the cartilage primordia are separated by the formation of an interzone at E13.5, which becomes a joint cavity (Hyde *et al.*, 2007). The cells in the interzone are flattened and cease to express *Col2a1* (Hyde *et al.*, 2008). In *Sox9^{LacZ/+}* embryos, *Sox9* expression was detected in the interzone cells of the knee joint up to E13.5 and, thereafter, *Sox9* expression was detected exclusively in chondrocytes. In contrast, in *Sox9^{CreERT2/+};R26R* newborn mice, the cells in the cruciate ligaments were labeled when tamoxifen was injected before E14.5. Thus, these results suggest that the cruciate ligament cells of the knee joint originate from *Sox9*-expressing chondrogenic cells of the cartilage primordia. This conclusion is in keeping with a recent study using *Col2a1-Cre/R26R* mice (Hyde *et al.*, 2008). Although many cells in the presumptive joint interzone of these mice do not express *Col2a1* at E13.5, all the cells in this zone were derived from resident anlagen cells that expressed *Col2a1* previously. These authors concluded that the cruciate ligaments originate from *Col2a1*-positive cells.

Tendon progenitor cells express *Scleraxis* (*Scx*) and are induced between E9.5 and E12.5 in the syndetome and limb bud mesenchyme. During limb bud development, all of the limb tendons are derived from lateral plate mesenchyme, and tendon progenitors are thought to be localized under the ectoderm, in locations that follow the proximal-to-distal outgrowth of the limb bud (Schweitzer *et al.*, 2001). By E12.5, the *Scx*-expressing cells align as loosely organized progenitors between differentiating muscles and corresponding cartilage condensations, and then condense and differentiate to give rise to overtly distinct tendons, which form by E13.5. However, this assertion is based on the continuity of *Scx*-expressing

cells, and not based on a direct lineage from *Scx*-expressing cells. (Brent *et al.*, 2003; Murchison *et al.*, 2007; Pryce *et al.*, 2009). In this study, we injected tamoxifen once into *Sox9^{CreERT2/+};R26R* pregnant females to determine the origins of the cells of the limb tendons. We observed previously that positive X-gal cells are present in tendons of *Sox9^{Cre/+};R26R* embryos (Akiyama *et al.*, 2005), which suggests that *Sox9*-expressing cells differentiate into tendon cells. Interestingly, although *Sox9* expression was not detected in the cells of the limb tendons in *Sox9^{LacZ/+}* embryos, tendon cells were labeled in *Sox9^{CreERT2/+};R26R* newborn mice if tamoxifen was injected before and at the stage of chondrogenic mesenchymal condensation. The tendon cells of the Achilles tendon were still labeled in *Sox9^{CreERT2/+};R26R* newborn mice injected tamoxifen at E14.5, which is the stage of the formation of these tendons. Thus, our results strongly suggest that at least part of the cells of the tendons originate from *Sox9*-expressing condensed mesenchymal cells of the cartilage primordia and possibly contribute to the formation of the tendon.

We concluded that the *Sox9-CreERT2* knock-in mouse line is a very valuable tool for the temporal genetic tracing of cellular progeny and for the inducible genetic activation or inactivation of *Sox9*-expressing cells.

METHODS

Generation of *Sox9-CreERT2* Knock-in Mice

A *Sox9* clone was isolated from a mouse 129/SvEv genomic DNA library (Bi *et al.*, 1999; Chen and Behringer, 1995). The targeting vector spanned a 7.7 kb fragment of the *Sox9* gene and a 5' IRES-CreERT2-pA/FRT-flanked PGK-neo-bpA cassette was inserted into HpaI site located within the 3'UTR of 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 via 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 that was 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 crossed with C57BL/6 mice to generate heterozygous *Sox9^{CreERT2/+}* mice. Mouse-tail DNA was used for PCR genotyping. The following primer pairs were used: Cre, 5'-TCCAATTTACTGACCGTACAC CAA-3' and 5'-CCTGATCCTGGCAATTTCCGGCTA-3' (545 bp fragment) (Ovchinnkov *et al.*, 2000); and R26R, 5'-AATGGCTTTTCGCTACCTGGAG-3' and 5'-TGGTGTTTT GCTTCCGTCAGC-3' (200 bp fragment) (Nakamura *et al.*, 2006).

Tamoxifen Treatment

Tamoxifen-inducible Cre activity was evaluated using the *R26R* mice. Tamoxifen (T5648, Sigma, St. Louis, MO) was dissolved in ethanol and diluted in corn oil (C8267, Sigma) at a concentration of 10 mg/ml, as described previously (Danielian *et al.*, 1998). Three milligrams of tamoxifen were injected into the peritoneal cavity of pregnant females at the indicated time points.

Histological Analysis

Whole-mount X-gal staining was performed as described previously (Hogan *et al.*, 1994). Decalcification of newborn mice was performed over the period of 1 week using 20% EDTA at 4°C. For the histological analyses, embryos and newborn mice were embedded in paraffin, sectioned at a thickness of 5 μ m, and counter-stained with nuclear fast red.

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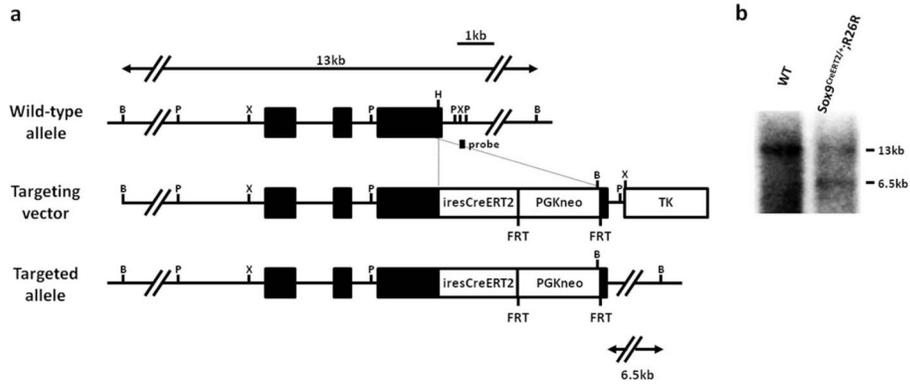


FIG. 1. Targeting strategy for the insertion of the tamoxifen-inducible *CreERT2* construct into the 3' UTR of the *Sox9* gene. (a) Structure of the genomic *Sox9* gene, targeting vector, and targeted allele. Exons are depicted as filled boxes, and intronic sequences are shown as solid lines. *IRES-CreERT2-pA* and the *FRT-PGK-neo-bpA* cassettes are depicted as open boxes. The *FRT-PGK-neo-bpA* cassette was not removed in the final targeted allele. DNA fragments identified in the Southern blot analysis are indicated by double arrows, with the restriction enzymes and the probe. *iresCreERT2*, *IRES-CreERT2-pA*; *PGKneo*, *PGK-neo-bpA*; B, BamHI; P, PstI; X, XbaI; H, HpaI. (b) Southern blot analysis of genomic DNA isolated from the tails of mice carrying the wild-type and targeted allele. The DNA is digested with BamHI and then hybridized with the 3' probe. Southern blotting reveals that the wild-type and targeted alleles are detected as 13 kb and 6.5 kb fragments, respectively.

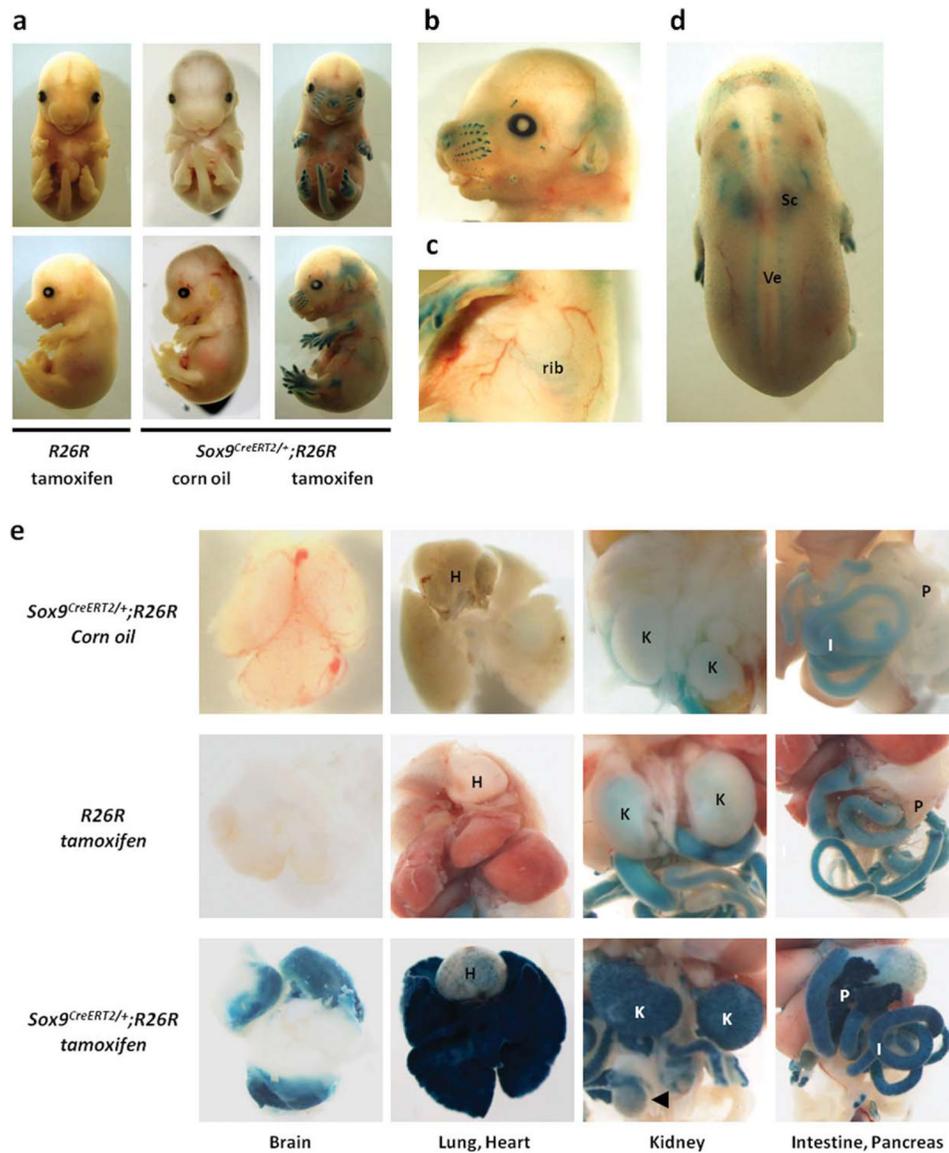


FIG. 2. *Sox9*-specific Cre induction by tamoxifen treatment. (a, b, c, d) Tamoxifen or vehicle control is injected into pregnant females at E13.5 and the females are sacrificed 48 h later. (a) X-gal positive staining in *Sox9^{CreERT2/+};R26R* embryos injected with tamoxifen is detected in appendicular and axial skeletons. No X-gal staining is observed in *R26R* embryos injected with tamoxifen and *Sox9^{CreERT2/+};R26R* embryos injected with corn oil. (b-d) Large magnification views of *Sox9^{CreERT2/+};R26R* embryos injected with tamoxifen. (b) X-gal positive staining is detected in whisker follicles, whereas X-gal staining is absent in calvariae. (c) X-gal positive staining is detected in ribs, (d) scapula (Sc), and vertebrae (Ve). (e) Pregnant females are injected once intraperitoneally with tamoxifen or corn oil at E12.5 and are sacrificed at E16.5. X-gal positive staining is detected in brain, lung, heart, kidney, intestine, pancreas, and testis of *Sox9^{CreERT2/+};R26R* embryos injected with tamoxifen. H, K, I, and P indicate heart, kidney, intestine, and pancreas, respectively. The arrow head indicates testis.

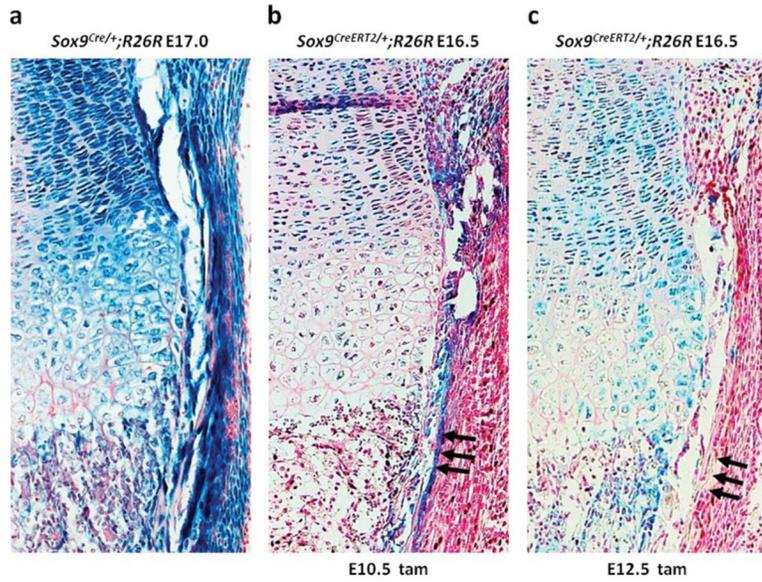
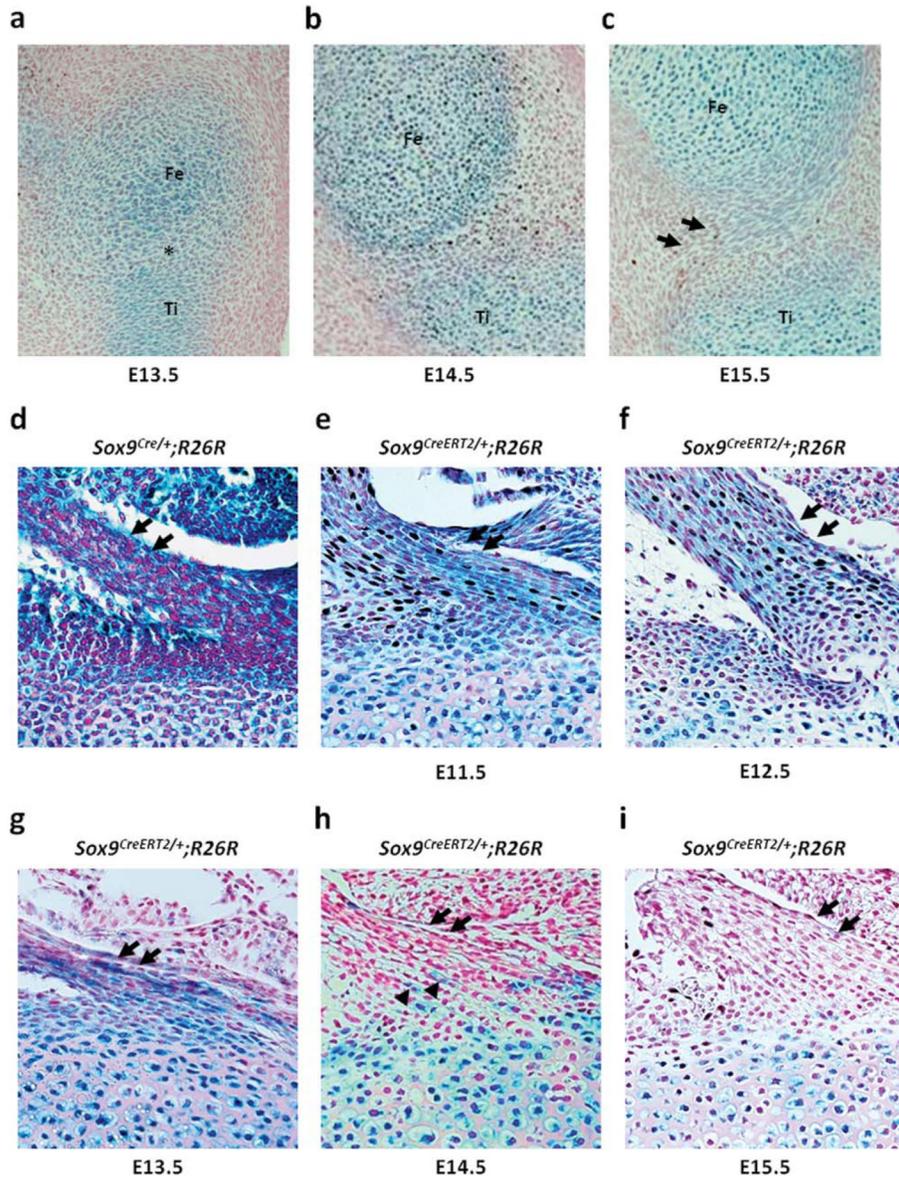
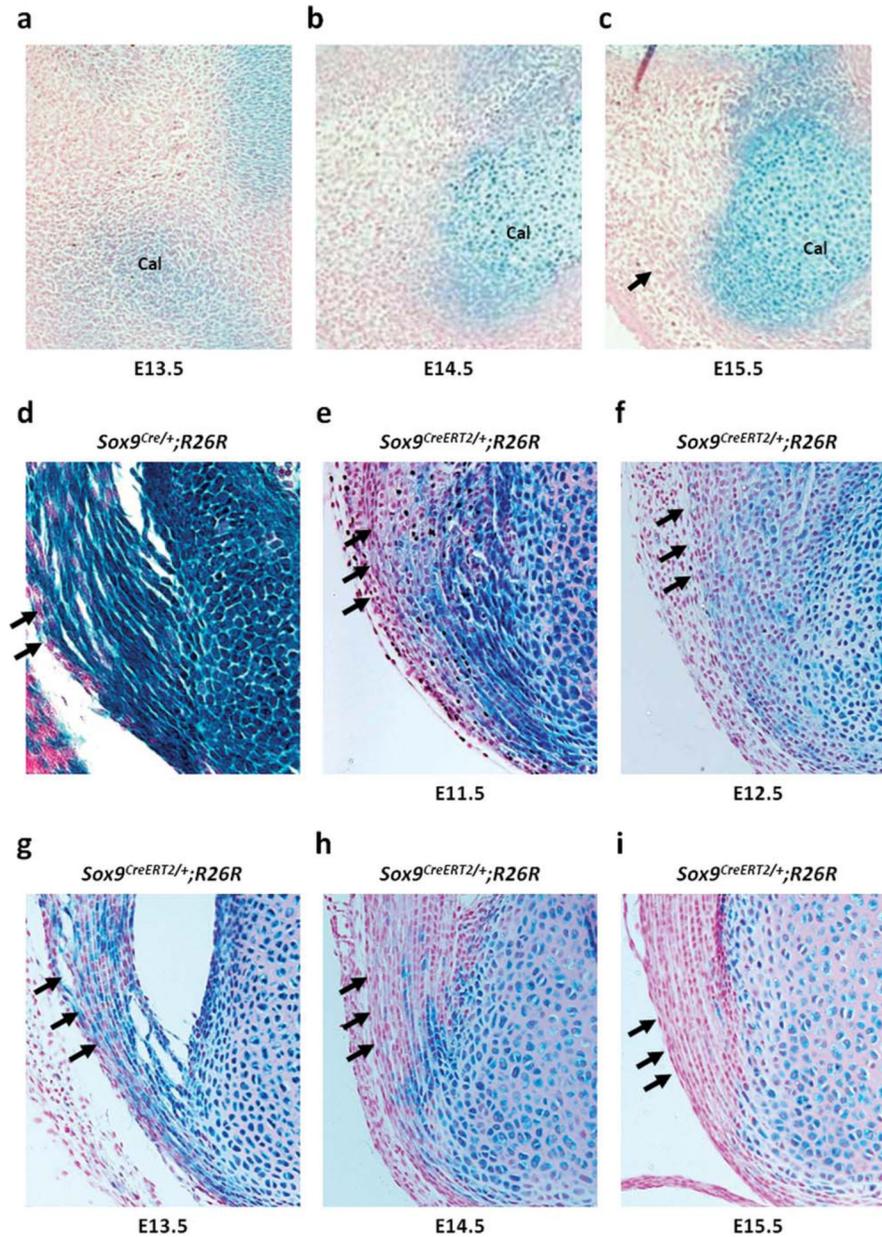


FIG. 3. Identification of the segregation of osteoblastic and chondrogenic lineages in osteochondroprogenitor cells using tamoxifen-inducible Cre recombinase. Sagittal section of the tibia harvested at E17.0 from *Sox9^{Cre/+};R26R* embryos and at E16.5 from *Sox9^{CreERT2/+};R26R* embryos injected with tamoxifen at E10.5 and E12.5. (a) Positive X-gal staining is detected in all chondrocytes of the growth plate and in the periosteum of *Sox9^{Cre/+};R26R* embryos. (b) Positive X-gal staining is detected in all chondrocytes of the growth plate and in the periosteum of *Sox9^{CreERT2/+};R26R* embryos injected with tamoxifen at E10.5. (c) Positive X-gal staining is detected in all chondrocytes of the growth plate, but not in the periosteum, of *Sox9^{CreERT2/+};R26R* embryos injected with tamoxifen at E12.5. Arrows indicate the periosteum.

**FIG. 4.**

Cell-lineage tracing in the cruciate ligaments of the knee joint. Sagittal sections of the knee joint of *Sox9^{LacZ/+}* embryos and *Sox9^{CreERT2/+};R26R* newborn mice from the pregnant females that are injected once with tamoxifen at different time points between E11.5 and E15.5. (a) *Sox9* is detected in cartilage primordia of the femur, tibia, and fibula in the knee joint of E13.5 *Sox9^{LacZ/+}* embryos. The cells of the interzone between the cartilage primordia exhibit *Sox9* expression. (b) At E14.5, *Sox9* expression is restricted to chondrocytes. (c) At E15.5, the cruciate ligaments form and the ligament cells do not express *Sox9*. (d) The cruciate ligament cells are positive in the knee joint of E17.0 *Sox9^{Cre/+};R26R* embryos. (e–g) The cruciate ligament cells are positive for X-gal after tamoxifen injection at E11.5, E12.5, and E13.5. (h) The number of labeled cells is markedly decreased in newborn mice that are injected with tamoxifen at E14.5. (i) No labeled cells are detected when tamoxifen is injected at E15.5. Fe, Ti, and Fi indicate the cartilage primordia of the femur, tibia, and

fibula, respectively. Arrows indicate the cruciate ligaments. Arrow heads indicate X-gal positive cells.

**FIG. 5.**

Cell-lineage tracing of the Achilles tendon. Sagittal section of the zeugopod/autopod of the hindlimb of *Sox9^{LacZ/+}* embryos and *Sox9^{CreERT2/+};R26R* new-born mice from pregnant females that are injected once with tamoxifen at different time points from E11.5 to E15.5. (a) *Sox9* expression is detected in cartilage primordia of calcaneus of *Sox9^{LacZ/+}* embryos at E13.5. (b) At E14.5, *Sox9* expression is restricted to chondrogenic cells of cartilage primordia. (c) At E15.5, the Achilles tendon form and the cells of the tendon do not express *Sox9*. (d) The cells of the Achilles tendon of E17.0 *Sox9^{Cre/+};R26R* embryos are positive. (e–g) The cells of the Achilles tendon of *Sox9^{CreERT2/+};R26R* newborn mice that are injected with tamoxifen at E11.5, E12.5 and E13.5 are stained with X-gal. (h) The number of labeled cells of the Achilles tendon is markedly decreased in newborn mice injected with tamoxifen at E14.5. (i) No labeled cells of the Achilles tendon are detected in mice injected

with tamoxifen at E15.5. Cal indicates cartilage primordia of the calcaneus. Arrows indicate the Achilles tendon.

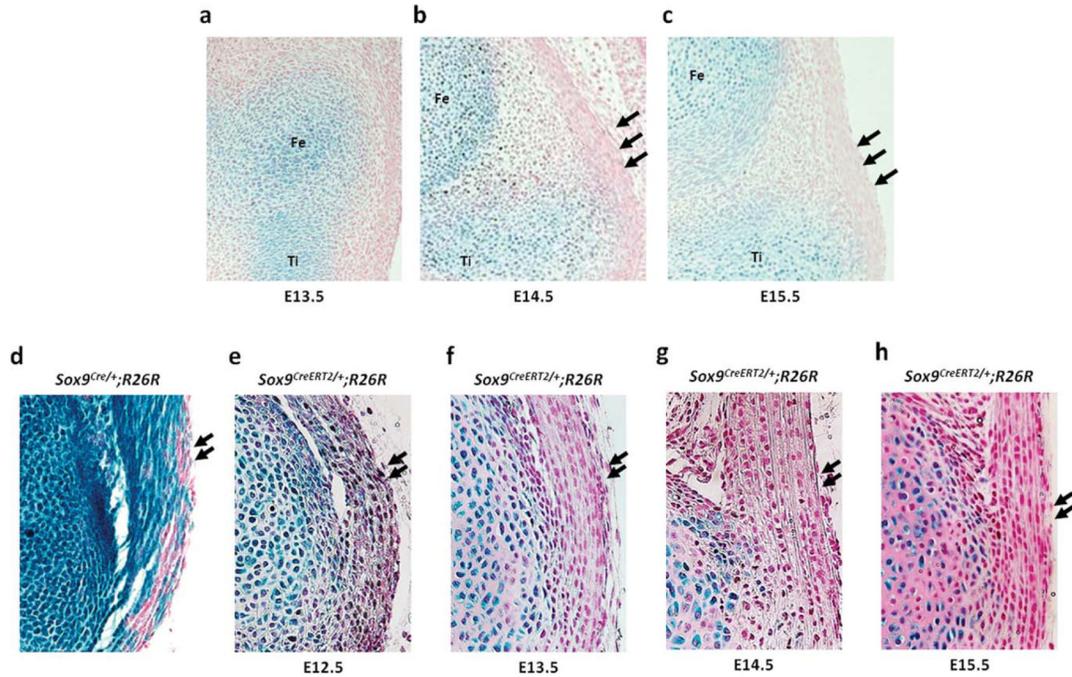


FIG. 6. Cell-lineage tracing of the patellar tendon. Sagittal section of the knee joint of *Sox9^{LacZ/+}* embryos and *Sox9^{CreERT2/+};R26R* new-born mice from pregnant females that are injected once with tamoxifen at different time points from E12.5 to E15.5. (a) *Sox9* expression is detected in cartilage primordia of tibia of *Sox9^{LacZ/+}* embryos at E13.5. (b, c) At E14.5 and E15.5, no X-gal positive cells in the patellar tendon. (d) The cells of the patellar tendon of E17.0 *Sox9^{Cre/+};R26R* embryos are positive. (e) Bony insertions of the patellar tendon to tibia of *Sox9^{CreERT2/+};R26R* newborn mice that are injected with tamoxifen at E12.5 is stained with X-gal. (f-h) No labeled cells of the patellar tendon are detected in mice injected with tamoxifen at E13.5, E14.5, and E15.5. Fe and Ti indicate the cartilage primordia of the femur and tibia, respectively. Arrows indicate the patellar tendon.