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Generation of transgenic mice for conditional overexpression of Sox9

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Abstract

Sox9 belongs to the family of Sry-related high-mobility group box transcription factors controlling cell fate, cell proliferation and differentiation in various tissues, including cartilage, testis, the central nervous system, kidney, and gastrointestinal. Mice conditionally lacking *Sox9* have revealed essential roles for Sox9 in these tissues. To gain further insight into the role of Sox9 in different tissues and at different stages of development, we have generated a transgenic mouse line to express *Sox9* in a Cre recombinase-dependent manner. The construct contained the human cytomegalovirus enhancer and chicken β -actin promoter, and a floxed monomeric red fluorescence protein 1 (mRFP1) cassette to direct ubiquitous expression of *mRFP1*. Cre expression removed the *mRFP1* gene, allowing expression of *Sox9* and enhanced green fluorescent protein. Expectedly, overexpression of *Sox9* in chondrocytes using *Col2a1-Cre* mice suppressed chondrocyte hypertrophy, and delayed terminal differentiation and subsequent ossification. Misexpression of *Sox9* in hypertrophic chondrocytes using *Col10a1-Cre* knock-in mice also delayed terminal differentiation. This Sox9 conditional transgenic mouse line will be a valuable tool to uncover tissue-specific and developmental stage-specific functions of Sox9.

Keywords

Sox9; *Col10a1*; Transgenic mouse; Conditional expression

Introduction

Sox9 is a transcription factor with a highly conserved Sry-related high-mobility group DNA-binding domain and a transactivation domain. During mouse embryogenesis, Sox9 is expressed in a variety of tissues with a specific and temporal pattern, and controls cell lineage commitment and the subsequent cell proliferation and differentiation in these tissues. Recent mouse genetic approaches reveal that Sox9 plays essential roles in organogenesis in several organs. For example, Sox9 is required for testogenesis [1], chondrogenesis [2], and endocardial cushion formation in cardiogenesis [3].

In chondrogenesis, *Sox9* is expressed in all chondroprogenitors and chondrocytes, although its expression is diminished in hypertrophic chondrocytes. Our previous studies reveal that *Sox9* regulates cell proliferation and differentiation at various steps of chondrogenesis [2, 4]. Inactivation of *Sox9* in limb buds before chondrogenic mesenchymal condensation results in the complete absence of mesenchymal condensation and of subsequent cartilage and bone formation [2]. Moreover, inactivation of *Sox9* in and after mesenchymal condensation arrests differentiation of condensed mesenchymal cells at condensations and impairs the proper proliferation and maturation of differentiated chondrocytes. Heterozygous *Sox9* mutants are small and display delayed chondrogenic mesenchymal condensation and enlargement of the hypertrophic zone, in association with premature mineralization [5]. These data provide strong evidence that an adequate level of *Sox9* is required for both mesenchymal condensation and the physiological inhibition of hypertrophic conversion of proliferating chondrocytes. Furthermore, transgenic mice expressing *Sox9* ectopically in limb bud mesenchyme under the control of the *Prx1* promoter exhibit ectopic cartilage formation in association with the induction of ectopic expression of *Sox5* and *Sox6*, without any patterning defects in limb bud development [6]. The overexpression of *Sox9* under the control of the *Col2a1* promoter results in chondrodysplasia because of dramatic inhibition of chondrocyte proliferation [7]. These mouse genetic studies indicate that *Sox9* is a master regulator of chondrogenesis and of the tightly controlled phases of proliferation and differentiation.

To study the *in vivo* functions of *Sox9* using a tool that allows its controlled spatiotemporal expression, we developed conditional *Sox9* transgenic mice, *CAG-mRFP1^{flox}-Sox9-EGFP* (*CAG-Sox9*), to express *Sox9* in a Cre recombinase-dependent manner. Using these mice, we generated mutant mice in which *Sox9* was conditionally overexpressed or misexpressed in chondrocytes using *Col2a1-Cre* or *Col10a1-Cre* mice, resulting in impairment of endochondral bone formation.

Materials and methods

Generation of mutant mice

The *CAG-mRFP1^{flox}-Sox9-EGFP* construct was generated by insertion of the following four DNA fragments into pcDNA3.1(–) (Invitrogen, CA): (1) a *SalI*-*XhoI* fragment containing the human *CAG* promoter derived from TriEX-2 (Novagen); (2) a *NotI*-*SalI* fragment encompassing an expression cassette that contained the loxP site-flanked *mRFP1* gene and four tandem polyadenylation sites (4xpA) [8]; (3) a *SalI*-*NotI* fragment containing the 3xHA-epitope-tagged human *Sox9* cDNA sequence; and (4) a *Bam*HI-AflIII fragment containing an *internal ribosome entry site (IRES)*-*EGFP-pA* sequence. The 12.7-kb *PmeI*-*PmeI* *CAG-mRFP1^{flox}-Sox9-EGFP* fragment was microinjected into the pronuclei of fertilized C57BL/6 oocytes to generate transgenic mice. Routine genotyping was performed by PCR using the primers 5'-TCCCCGACTACTTGAAGCTG-3' and 5'-CTTGCCCATGTAGGTGGTCT-3', which amplify 317 bp of *mRFP1*.

A *Col10a1* clone was isolated from a mouse C57BL/6 genomic DNA library. The targeting vector spanned a 13.1-kb fragment of the *Col10a1* gene, and an *IRES-Cre-pA/FRT-flanked PGK-neo bpA* cassette was inserted into a *SmaI* site within the 3'-untranslated region (UTR) in exon 3. 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/C57BL/6 hybrid mouse ES cells [9], and G418/FIAU-resistant ES cell clones were initially screened by Southern blot analysis of *EcoRV*-digested genomic DNA by using 5' 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 *Col10a1-Cre* knock-in heterozygous mice. Routine geno-typing was performed by PCR using the primers 5'-TCCAATTTACTGACCGTACACCA-3' and 5'-CCTGATCCTGGCAATTCGGCTA-3', which amplify 540 bp of *Cre*.

Histological analysis

For histological analysis, embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 μ m were stained with hematoxylin and eosin, Safranin O staining or the von Kossa reaction, and nuclear fast red. Immunohistochemical staining was performed using a peroxidase-conjugated polymer (Zymed) and DAB with rabbit polyclonal anti-HA (1:500, Covance). RNA in situ hybridization analysis was performed as described previously [10].

Quantitative RT-PCR

Total RNA was extracted from the ribs of newborn mice using the TriPure isolation reagent (Roche Applied Science). One microgram of total RNA was prepared to synthesize cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). PCR amplification was performed using a LightCycler Instrument and the LightCycler FastStart DNA Master SYBR Green 1 Kit (Roche Applied Science), according to the manufacturer's instructions. The sequences of the primers were as follows: *Gapdh*, forward primer, 5'-TGTCCTGTCGTGGATCTGAC-3'; reverse primer, 5'-CCTGCTTACCCACCTTCTTG-3'; *Col2a1*, forward primer, 5'-CTGGTACCCCTGGAAATCCT-3'; reverse primer, 5'-CCTGCTTACCCACCTTCTTG-3'; *Aggrecan*, forward primer, 5'-CGGGACACCAATGAGACCTA-3'; reverse primer, 5'-GGGATGTGCGATAAAAGACC-3'; *Sox9*, forward primer, 5'-AACATGGAGGACGATTGGAG-3'; reverse primer, 5'-TCCCTCAAATGGTAATGAG-3'.

Statistical analyses

We used the Student's *t* test to calculate *P* values. Differences were considered significant at *P* < 0.05.

Results

Generation of CAG-Sox9 transgenic mice

To gain further insight into the tissue-specific and/or developmental stage-specific roles of *Sox9* in vivo, we generated transgenic mice that overexpressed *Sox9* (tagged with a hemagglutinin epitope, HA) conditionally under the control of the CAG promoter in a Cre recombinase-dependent manner (Fig. 1a). *Sox9* expression under the control of the CAG promoter was blocked by an mRFP1 cassette that included four tandem polyadenylation sites (4xpA) to terminate transcription flanked by two loxP sites (flox). The expression of

Cre recombinase in transgenic tissues led to the excision of the floxed mRFP1 cassette, resulting in the expression of *Sox9* and *EGFP*. An IRES sequence was placed between *Sox9* and the *EGFP* gene for access of ribosomes to the bicistronic RNA for parallel translation of both *Sox9* and *EGFP*, rendering the EGFP protein a marker for the expression of HA-tagged Sox9 protein. Hence, this system allowed cell-specific overexpression or misexpression of *Sox9* that was restricted to Cre recombinase-expressing cells and their cellular progeny. We then isolated eight *CAG-Sox9* transgenic founders that expressed mRFP1 and were normal in phenotype. We selected one transgenic line that exhibited significant mRFP1 expression levels (data not shown). All tissues exhibited mRFP1 expression, indicating ubiquitous expression of the transgenic cassette. In general, hemizygous *CAG-Sox9* mice were viable and fertile, and manifested no gross behavioral or phenotypic abnormalities.

We next verified that the expression of the *Sox9* transgene in *CAG-Sox9* mice was induced in a Cre recombinase-dependent manner. To analyze the expression of the *Sox9* transgene, we crossed *CAG-Sox9* mice with *Sox9-Cre* mice, in which a Cre recombinase gene preceded by an IRES was inserted into the 3' UTR of the endogenous *Sox9* gene [4]. Mating of *CAG-Sox9* mice with *Sox9-Cre* mice, in which the expression of Cre recombinase was limited to endogenous *Sox9*-expressing cells and their progeny, led to a restricted pattern that was similar to the endogenous *Sox9* expression pattern. *Sox9* is expressed in a relatively wide range of tissues including cartilage [2], skin [11], pancreas, kidney, digestive tract [12], and brain [13]. In these tissues, fluorescent color conversion to EGFP from mRFP1 was observed in *CAG-Sox9;Sox9-Cre* mice (Fig. 1b). *CAG-Sox9;Sox9-Cre* mice showed strong EGFP fluorescence, whereas tissues of their *CAG-Sox9* littermates lacking the *Sox9-Cre* allele exhibited minimal EGFP fluorescence that can be considered as background levels. The presence of the *mRFP1* and Cre transgenes in *CAG-Sox9;Sox9-Cre* mice was confirmed by PCR amplification from DNA extracted from mouse tails (Fig. 1c). Western blot analysis showed that HA-tagged Sox9 protein was expressed in E16.5 forelimbs in *CAG-Sox9;Sox9-Cre* embryos, whereas this protein was not detectable in *CAG-Sox9* littermates, demonstrating that HA-tagged *Sox9* protein expression was efficiently induced by Cre recombinase (Fig. 1d). Quantitative RT-PCR using RNA extracted from neonatal rib cartilages exhibited a significant increase in the levels of *Sox9* and its downstream chondrocyte markers, such as *Col2a1* and *Aggrecan* (Fig. 1e). These data led us to conclude that the *CAG-Sox9* conditional transgenic system worked in a Cre recombinase-dependent manner.

Conditional *Sox9* overexpression in chondrocytes using *Col2a1-Cre* mice

To verify the functions of *Sox9* transgene in *CAG-Sox9* mice, we crossed *CAG-Sox9* mice with *Col2a1-Cre* transgenic mice, which express Cre recombinase in differentiated chondrocytes, to study the effect of *Sox9* overexpression in chondrocytes. We found that EGFP expression was detectable throughout the cartilaginous skeletal elements of *CAG-Sox9;Col2a1-Cre* embryos at E15.5 (Fig. 2a). In *CAG-Sox9;Col2a1-Cre* embryos, HA-tagged Sox9 was detected in proliferating chondrocytes (Fig. 2b). In addition, misexpression of *Sox9* was also detected in hypertrophic chondrocytes, which revealed persistent expression of *Sox9* throughout the growth plate (Fig. 2b). Histological analysis showed that E15.5 *CAG-Sox9;Col2a1-Cre* embryos exhibited delayed hypertrophic conversion of the proliferating chondrocytes (Fig. 2c) and that E17.5 *CAG-Sox9;Col2a1-Cre* embryos exhibited an expansion of hypertrophic zone and a delay in the formation of the primary ossification center in forelimb skeletal elements (Fig. 2d). The von Kossa staining showed that bone formation around the primary ossification center was delayed, and mineralized hypertrophic chondrocytes remained undegraded in the shaft in *CAG-Sox9;Col2a1-Cre* embryos. To characterize the phenotype further, we examined the expression of markers of specific stages of chondrocyte differentiation in the *CAG-Sox9;Col2a1-Cre* mice (Fig. 2d).

In situ hybridization revealed that the expression domain of *Col10a1*, which is a definitive molecular marker of hypertrophic chondrocytes, was expanded in *CAG-Sox9;Col2a1-Cre* embryos, although the expression domain of *Col2a1* had no significant difference. The signal intensity of *Mmp13*, which is a marker of terminally differentiated hypertrophic chondrocytes, was significantly decreased in *CAG-Sox9;Col2a1-Cre* embryos compared with *CAG-Sox9* embryos. These results indicate that terminal differentiation of hypertrophic chondrocytes and endochondral bone formation were inhibited with increased *Sox9* expression in *CAG-Sox9;Col2a1-Cre* embryos.

Misexpression of *Sox9* in hypertrophic chondrocytes resulted in delayed terminal differentiation

In *CAG-Sox9;Col2a1-Cre* embryos, overexpression of *Sox9* in all differentiated chondrocytes resulted in delayed terminal differentiation of hypertrophic chondrocytes. In endochondral bone formation, expression of *Sox9* is shut down in hypertrophic chondrocytes. To understand the biological significance of this shutdown of *Sox9* expression in hypertrophic chondrocytes, we first generated *Col10a1-Cre* knock-in (*Col10a1-Cre*) mice, in which the Cre recombinase gene was inserted into the 3' UTR of the endogenous *Col10a1* gene (Fig. 3a, b). We first crossed *Col10a1-Cre* with the *Rosa26* reporter (R26R) strain [14], and in E16.5 *Col10a1-Cre;R26R* embryos, X-gal staining was specifically detected in hypertrophic zones (Fig. 3c). Histological analysis revealed that all hypertrophic chondrocytes were stained with X-gal during chondrogenesis (Fig. 3d). *Col10a1-Cre* mice displayed Cre expression and activity that were restricted to hypertrophic chondrocytes, resulting in specific expression of *Sox9* in hypertrophic chondrocytes in *CAG-Sox9;Col10a1-Cre* embryos. Indeed, expression of the HA-tagged *Sox9* protein was detected in hypertrophic chondrocytes using an anti-HA antibody in E16.5 *CAG-Sox9;Col10a1-Cre* embryos (Fig. 3e). In E17.5 *CAG-Sox9;Col10a1-Cre* embryos, histological analysis revealed that endochondral bone formation was impaired in *CAG-Sox9;Col10a1-Cre* embryos, compared with *CAG-Sox9* littermates (Fig. 3f). The von Kossa staining indicated that mineralization eventually occurred in *CAG-Sox9;Col10a1-Cre* embryos, although the mineralized domain was markedly decreased. These observations suggest that terminal differentiation of hypertrophic chondrocytes was delayed as a consequence of *Sox9* misexpression in hypertrophic chondrocytes.

To test this hypothesis, we analyzed the expression of genes that are markers of specific stages of chondrogenic differentiation in *CAG-Sox9;Col10a1-Cre* embryos. In situ hybridization revealed that the expression domains of *Col10a1* and *Mmp13* were expanded, and especially *Mmp13* expression was extended to the bone marrow cavity in *CAG-Sox9;Col10a1-Cre* embryos (Fig. 3f). These results indicate that misexpression of *Sox9* in hypertrophic chondrocytes inhibited the terminal differentiation of hypertrophic chondrocytes and delay of cartilage replacement by bone.

Discussion

In this study, we generated a novel conditional transgenic mouse line expressing a *Sox9* transgene in mouse organogenesis. Expression was driven by the ubiquitously expressed *CAG* promoter in a Cre recombinase-dependent manner. The use of a *CAG* promoter to drive the expression of the *Sox9* transgene offers a significant advantage over an alternative design using a more restrictive promoter, as the activation of the *Sox9* transgene will be determined by the expression pattern of the Cre transgene. Thus, *CAG-Sox9* mice have the potential to be used in a broader range of studies by crossing with tissue-specific Cre mouse lines. Especially our conditional *Sox9* expression system driven by the *CAG* promoter is well suited for persistent or ectopic expression of the *Sox9* transgene throughout tissue

development. In this study, we showed the expression of the *Sox9* transgene in chondrocytes using *Col2a1-Cre* and in hypertrophic chondrocytes using *Col10a1-Cre* mice. In endochondral bone formation, we used *Col2a1-Cre* mice to demonstrate that the *Sox9* transgene was active in all chondrocytes, including hypertrophic chondrocytes. Moreover, this system also has the significant advantage that EGFP fluorescence can be switched from mRFP1, thus allowing convenient monitoring of *Sox9* transgene expression in *CAG-Sox9* mice.

To confirm that overexpression of *Sox9* transgene in *CAG-Sox9* mice crossed with Cre driver mice functions in vivo, histological analyses were performed. A previous study showed that heterozygous *Sox9* mouse mutants exhibit an expansion of the zone of hypertrophic chondrocytes in association with premature mineralization, which suggests that *Sox9* inhibits the transition of proliferating chondrocytes to hypertrophy [5]. Our results indicate that persistent expression of *Sox9* throughout the growth plate impairs hypertrophic conversion in *CAG-Sox9;Col2a1-Cre* embryos. This is consistent with the finding that *Col2a1-Sox9* knock-in mice display a phenotype of dwarfism with a marked delay in the transition of proliferating chondrocytes to hypertrophy [7]. Thus, we provided strong evidence that the shutdown of *Sox9* expression just before hypertrophic conversion is required for the proper timing of the hypertrophic conversion of proliferating chondrocytes.

Terminal differentiation of hypertrophic chondrocytes is impaired by the overexpression of *Sox9* in *CAG-Sox9;Col2a1-Cre* and *CAG-Sox9;Col10a1-Cre* mice. The phenotype of *CAG-Sox9;Col10a1-Cre* embryos resembled that of *Sox9*-overexpression mice using a *Col10a1* BAC clone [15]. Recent studies showed that *Sox9* inhibits *Runx2* functions through direct binding to *Runx2* [16]. Furthermore, *Sox9* negatively regulates *Runx2* by enhancing *Bapx1* expression, which leads to the inhibition of terminal chondrocyte differentiation [17]. Therefore, these studies support the existence of a mechanism in which misexpression of *Sox9* in hypertrophic chondrocytes of *CAG-Sox9;Col2a1-Cre* and *CAG-Sox9;Col10a1-Cre* mice results in inhibition of terminal differentiation through suppression of *Runx2*.

Conclusion

Transgenic mice for *Sox9* conditional overexpression that we have generated will constitute a very valuable tool to further uncover the multiple tissue-specific functions of *Sox9* in vivo. It will facilitate studies on the roles of *Sox9* in developmental, physiological, and pathological processes from embryogenesis into adulthood.

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Abbreviations

IRES	Internal ribosome entry site
EGFP	Enhanced green fluorescent protein
mRFP1	Monomeric red fluorescence protein 1
CAG promoter	Human cytomegalovirus enhancer and chicken β -actin promoter

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Fig. 1.

Generation of *CAG-Sox9* transgenic mice. **a** The structure of the *CAG-Sox9* transgene was shown. Two genes coding for fluorescent proteins, mRFP1 and EGFP, were separated by four tandem polyadenylation sites. Gene expression was controlled by the ubiquitous CAG promoter. The mRFP1 cassette was flanked by two loxP sites. Before Cre-mediated recombination, only mRFP1 was expressed. After Cre-mediated recombination, the mRFP1 cassette was excised and the 3xHA-tagged *Sox9* and EGFP genes were expressed, instead of mRFP1. **b** Cre-mediated recombination was detected by color conversion from mRFP1 to EGFP in the cross of *CAG-Sox9* mice with *Sox9-Cre* mice. Widespread EGFP expression was detected in endogenous *Sox9*-expressing tissues, such as skin (neonate), cartilage in forelimbs (E 18.5), intestine, pancreas, brain, and kidney (4-week-old old animals). **c** Genomic PCR analysis of mRFP1 and Cre recombinase transgenes. **d** Western blot analysis was performed using an anti-HA antibody. Exogenous 3xHA-tagged Sox9 was detected in the forelimbs of E16.5 *CAG-Sox9; Sox9-Cre* embryos. **e** Quantitative RT-PCR analysis of *Sox9*, *Col2a1*, and *Aggrecan* in the rib cartilages of newborn *CAG-Sox9* and *CAG-Sox9; Sox9-Cre* mice (values shown as the average and standard deviation from five costal cartilages each; * $P < 0.05$)

Fig. 2.

Impairment of endochondral bone formation in *CAG-Sox9;Col2a1-Cre* mice. **a** High EGFP expression in the humeral growth plate of *CAG-Sox9;Col2a1-Cre* embryos. **b** The expression of Sox9 driven by the transgene in proliferating and hypertrophic chondrocytes in E15.5 *CAG-Sox9;Col2a1-Cre* embryos was detected using an anti-HA antibody. **c** Delayed hypertrophic conversion of the proliferating chondrocytes in E15.5 *CAG-Sox9;Col2a1-Cre* embryos. Histological analysis of the metacarpal bones stained with hematoxylin and eosin. **d** Expression of chondrocyte markers in *CAG-Sox9;Col2a1-Cre* embryos. Histological analysis of the forelimbs of *CAG-Sox9;Col2a1-Cre* embryos stained with Safranin O, von Kossa at E17.5. Sections of forelimbs of E17.5 *CAG-Sox9* and *CAG-Sox9;Col2a1-Cre* embryos were hybridized with *Col2a1*, *Col10a1*, and *Mmp13* probes

Fig. 3. Generation of *Col10a1-Cre* knock-in mice and delayed terminal differentiation in *CAG-Sox9;Col10a1-Cre* mice. **a** Targeting strategy for the insertion of Cre recombinase cDNA in the 3'-UTR of the *Col10a1* gene. Structure of the genomic *Col10a1* locus, targeting vector, and targeted allele were shown. Exons are depicted as *filled boxes*, and intronic sequences were shown as *solid lines*. The *IRES-Cre-pA* and the *FRT-PGK-neo-bpA* cassettes were depicted as *open boxes*. DNA fragments revealed in Southern blot analysis were indicated by *double arrows* with the restriction enzymes and the probe. RV; EcoRV. **b** Southern blot analysis of genomic DNA. Genomic DNA isolated from the tails of wild-type and *Col10a1-Cre* knock-in mice was digested with EcoRV and then hybridized with the 5' probe. The wild-type and mutant alleles were detected as 19- and 12.5-kb fragments, respectively. **c** Whole-mount X-gal staining of E16.5 *Col10a1-Cre;R26R* embryos. Positive X-gal staining was detected in all hypertrophic zones in skeletons. **d** Histological analysis of forelimbs of *Col10a1-Cre;R26R* embryos stained with X-gal at E14.5. Positive X-gal staining was detected in all hypertrophic chondrocytes. **e** The expression of *Sox9* driven by the transgene in hypertrophic chondrocytes of E16.5 *CAG-Sox9;Col10a1-Cre* embryos was performed using an anti-HA antibody. **f** Expression of chondrocyte markers in *CAG-Sox9;Col10a1-Cre* embryos. Histological analysis of forelimbs of *CAG-Sox9;Col10a1-Cre* embryos stained with Safranin O and von Kossa staining at E17.5. Sections of the forelimbs of E17.5 *CAG-Sox9* and *CAG-Sox9;Col10a1-Cre* embryos were hybridized with *Col2a1*, *Col10a*, and *Mmp13* probes