

Loss of Sox9 function results in defective chondrocyte differentiation of mouse embryonic stem cells *in vitro*

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ABSTRACT The transcription factor Sox9 plays an important role during chondrogenesis. After early conditional inactivation of Sox9 in mesenchymal limb bud cells of mice, mesenchymal condensations as well as cartilage and bone are completely absent in the developing limbs. We analyzed chondrogenic differentiation of Sox9^{-/-} mouse embryonic stem cells *in vitro*, using two clones with different targeted mutations. We found that the development of mature and hypertrophic chondrocytes is completely inhibited in the absence of Sox9 confirming that Sox9 is required for the formation of cartilage. In contrast, Sox9^{+/-} mouse embryonic stem cells showed continuous but reduced differentiation into mature chondrocytes. Interestingly, the formation of early chondrogenic condensations expressing characteristic marker genes such as scleraxis, Sox5 and Sox6 was not inhibited in the absence of Sox9 *in vitro*. Thus, we propose that the earliest step of chondrogenesis could be regulated by a non cell-autonomous function of Sox9.

KEY WORDS: chondrogenesis, *in vitro* differentiation, mesenchymal condensations, Sox9

Introduction

Sox9 is a member of the SOX (Sry-related high mobility group box) family of transcription factors that share a common 79 amino acid DNA binding motif, known as the high-mobility group (HMG) domain, with the mammalian testis-determining factor SRY (Wegner, 1999; Bowles *et al.*, 2000). Evidence exists that Sox9 plays an important role during cartilage development (de Crombrughe *et al.*, 2001). During mouse embryogenesis, Sox9 is expressed in all regions of cartilage formation including the sclerotomal parts of the somites that give rise to the axial skeleton, the cartilaginous elements of the limb buds which are formed by the lateral plate mesoderm, and in neural crest-derived mesenchymal cells of the craniofacial region (Zhao *et al.*, 1997; Ng *et al.*, 1997). In these structures, mesenchymal cells which differentiate into chondroprogenitor cells and form condensations have been shown to express Sox9 (Wright *et al.*, 1995) as well as the cell adhesion molecule N-cadherin (Oberlander and Tuan, 1994), the basic-helix-loop-helix transcription factor scleraxis (Cserjesi *et al.*, 1995) and the transcription factors Sox5 and Sox6 (Lefebvre

et al., 1998). These cells continue to express Sox9 during further differentiation, which is characterized by expression and deposition of cartilage-specific matrix components such as collagen type II and aggrecan. Sox9 is completely downregulated when chondrocytes acquire a hypertrophic shape and start to express Col10a1 (Zhao *et al.*, 1997). Sox9 binds to target sites in promoters or enhancers of cartilage-specific genes such as Col2a1 (Lefebvre *et al.*, 1997; Zhou *et al.*, 1998), Col11a2 (Bridgewater *et al.*, 1998), and aggrecan (Sekiya *et al.*, 2000), and has been shown to activate the expression of these genes *in vitro*. Furthermore, Col2a1 has been identified as a direct target gene of Sox9 *in vivo* (Bell *et al.*, 1997; Zhou *et al.*, 1998).

Heterozygous mutations in the human SOX9 gene cause the skeletal malformation syndrome campomelic dysplasia (CD) and associated XY sex reversal (Foster *et al.*, 1994; Wagner *et al.*, 1994), which establishes SOX9 as an essential factor for

Abbreviations used in this paper: EB, embryoid body; ES, embryonic stem (cell); HMG, high mobility group; HR, homologous recombination; PNA, peanut agglutinin; SOX, Sry-related high mobility group box.

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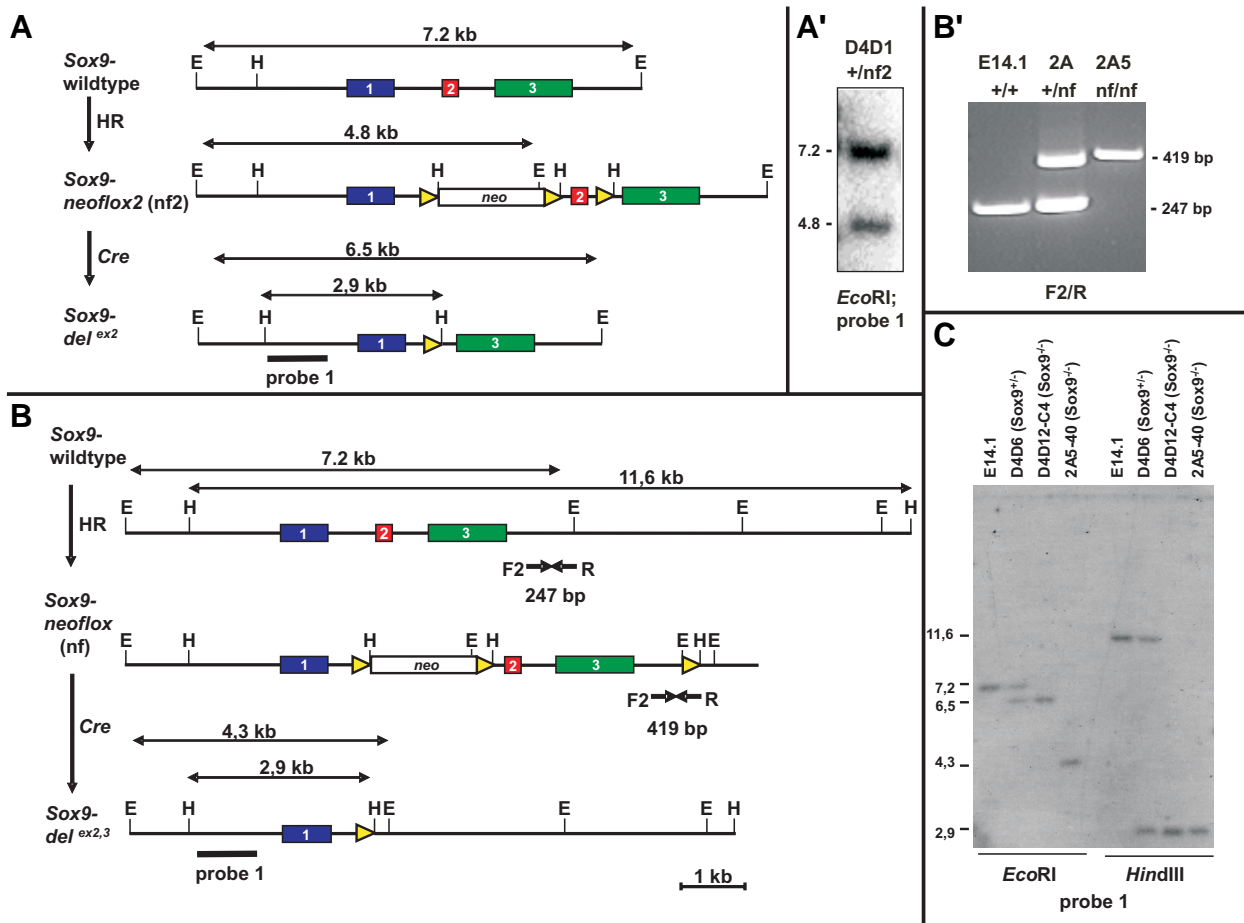


Fig. 1. *Sox9* gene targeting strategies and inactivation by Cre-mediated recombination in ES cells. The *Sox9*-neoflox2 (**A**) and *Sox9*-neoflox (**B**) alleles, carrying loxP-flanked MC1-neo-pA cassettes (*neo*, open boxes) inserted into intron 1 and a third loxP site inserted into intron 2 or into the 3' untranslated region, respectively, were generated by homologous recombination (HR) in ES cells. The loxP-flanked *neo* cassettes and either, *Sox9* exon 2 (**A**) or exon 2 and 3 (**B**) were removed by transient expression of Cre recombinase in vitro resulting in the *Sox9*-delex2 and *Sox9*-delex2,3 alleles, respectively. Colored boxes, *Sox9* exons 1-3; triangles, loxP sites; E, EcoRI; H, HindIII. Expected restriction fragment sizes detected by the hybridisation probe 1 (**A,B**), or the lengths of PCR fragments amplified with primers F2 and R (**B**) are indicated. (**A'**) Southern blot analysis of the heterozygous *Sox9*-neoflox2 clone D4D1 shows the wildtype 7.2 kb EcoRI and the floxed 4.8 kb EcoRI band. (**B'**) PCR analysis of wildtype and *Sox9*-neoflox ES cell clones demonstrates replacement of the wildtype allele with the mutant allele in a homozygous *Sox9*-neoflox clone. (**C**) Southern Blot analysis of wildtype (E14.1), heterozygous D4D6 and homozygous *Sox9*-delex2 (D4D12-C4) and *Sox9*-delex2,3 (2A5-40) ES cell clones. Homologous recombination and Cre-mediated deletion was confirmed using probe 1 showing a 6.5 kb EcoRI and a 2.9 kb HindIII fragment in homozygous *Sox9*-delex2 clone D4D12-C4 and a 4.3 kb EcoRI and a 2.9 kb HindIII fragment in homozygous *Sox9*-delex2,3 clone 2A5-40.

skeletogenesis and for testis determination. Defects are due to haploinsufficiency, indicating that a reduced dosage of SOX9 causes the developmental abnormalities. Characteristic features in CD patients are defective skeletal structures such as bowing and angulation of the tibiae and femora, hypoplastic scapulae and pelvic bones, undermineralized vertebrae and craniofacial malformations. Possibly due to defective tracheobronchial cartilages and narrow upper airways, most patients die in the neonatal period from respiratory distress (Houston *et al.*, 1983; Mansour *et al.*, 1995). Heterozygous *Sox9*-mutant mice generated by gene targeting phenocopy many of the features seen in CD patients, and also show typical abnormalities in cartilage primordia and premature skeletal mineralization (Bi *et al.*, 2001; Kist *et al.*, 2002).

In mouse chimeras, *Sox9*^{-/-} cells were excluded from cartilage primordia and mature cartilage tissue throughout embryonic

development (Bi *et al.*, 1999). Moreover, *Sox9*^{-/-} teratomas did not form any cartilage tissues (Bi *et al.*, 1999) and both, in chimeras and teratomas, *Sox9*^{-/-} cells failed to produce type II collagen. Furthermore, cartilage and bone was completely absent in the limbs of mice after conditional inactivation of the *Sox9* gene in early mesenchymal limb bud cells, and severe chondrodysplasia was described when *Sox9* was conditionally inactivated after mesenchymal condensations had formed (Akiyama *et al.*, 2002).

As an alternative to homozygous *Sox9* knockout mice, we analyzed differentiation of *Sox9*^{-/-} embryonic stem (ES) cells *in vitro* to elucidate which steps of chondrocyte differentiation are affected by loss of Sox9 function. Using the model system of ES cell differentiation *via* embryoid bodies (EBs), we have recently demonstrated that the process of chondrocyte differentiation is closely recapitulated *in vitro*, finally resulting in hypertrophic and calcifying cells (Kramer *et al.*, 2000; Hegert *et al.*, 2002; Kramer

et al., 2005). Here, we demonstrate that murine *Sox9*^{-/-} ES cells differentiate *in vitro* into pre-cartilage condensations which express early chondrocytic marker molecules such as the transcription factors *Sox5*, *Sox6* and *scleraxis* and also bind peanut-agglutinin. However, *Sox9*^{-/-} cells fail to develop further and are not able to form cartilage nodules and hypertrophic chondrocytes *in vitro*.

Results

Generation of Sox9^{+/-} and Sox9^{-/-} ES cells

By homologous recombination with a *Sox9* targeting vector, several correctly targeted ES cell clones were identified carrying the modified allele *Sox9-neoflox2* (Kist *et al.*, unpublished). One of the *Sox9-neoflox2* clones, D4 was used to generate *Sox9*^{+/-} ES cells by transient *in vitro* expression of Cre recombinase. Upon complete recombination the *loxP*-flanked *neo* cassettes are removed together with exon 2, resulting in the deleted allele *Sox9-del^{ex2}* (Fig. 1A). Several partially or completely recombined ES cell clones were identified by Southern blot analysis (data not shown). Three particular clones, D4D1, D4D6 and D4D12, were generated from the *Sox9-neoflox2* cells and characterised in detail (Fig. 1A,C). Clone D4D1 is identical to the parental clone D4 in which no recombination by Cre recombinase had occurred. The 4.8 kb *EcoRI* fragment in clone D4D1 indicates the presence of the *neo* cassette in the targeted allele (Fig. 1A'), while in clone D4D6, the 6.5 kb *EcoRI* and the 2,9kb *HindIII* fragment indicate the deleted allele (Fig. 1C). Surprisingly, in clone D4D12, the *Sox9* wildtype allele on the non-targeted chromosome had been replaced by the deleted *Sox9* allele, resulting in a *Sox9*^{-/-} genotype, lacking exon 2. This clone was subcloned because a very weak wildtype band was detectable after prolonged exposure possibly due to contaminating wildtype cells (data not shown) and the resulting subclones D4D12-C4 and

D4D12-D6 were verified by Southern Blotting showing only the *EcoRI* 6,5 kb and the 2,9 kb *HindIII* fragment, respectively (Fig.1C). These subclones were used for the differentiation experiments.

In addition, an ES cell clone with a different modified allele, *Sox9-neoflox* (Kist *et al.*, 2002), was used to generate a *Sox9*^{-/-} clone lacking both, exon 2 and 3 (Fig. 1B). From ES cell clone 2A, carrying the *Sox9-neoflox* allele (Kist *et al.*, 2002) in a heterozygous configuration, clone 2A5 was selected after cultivation with high concentrations of G418. PCR analysis showed that this clone carried the *Sox9-neoflox* allele in a homozygous configuration (Fig. 1B'). Replacement of the wildtype allele on the non-targeted chromosome by the mutant allele, is known to occur spontaneously and has been used before to generate homozygous ES cell clones (Mortensen *et al.*, 1992; Lefebvre *et al.*, 2001). After Cre expression, the subclone 2A5-40 was

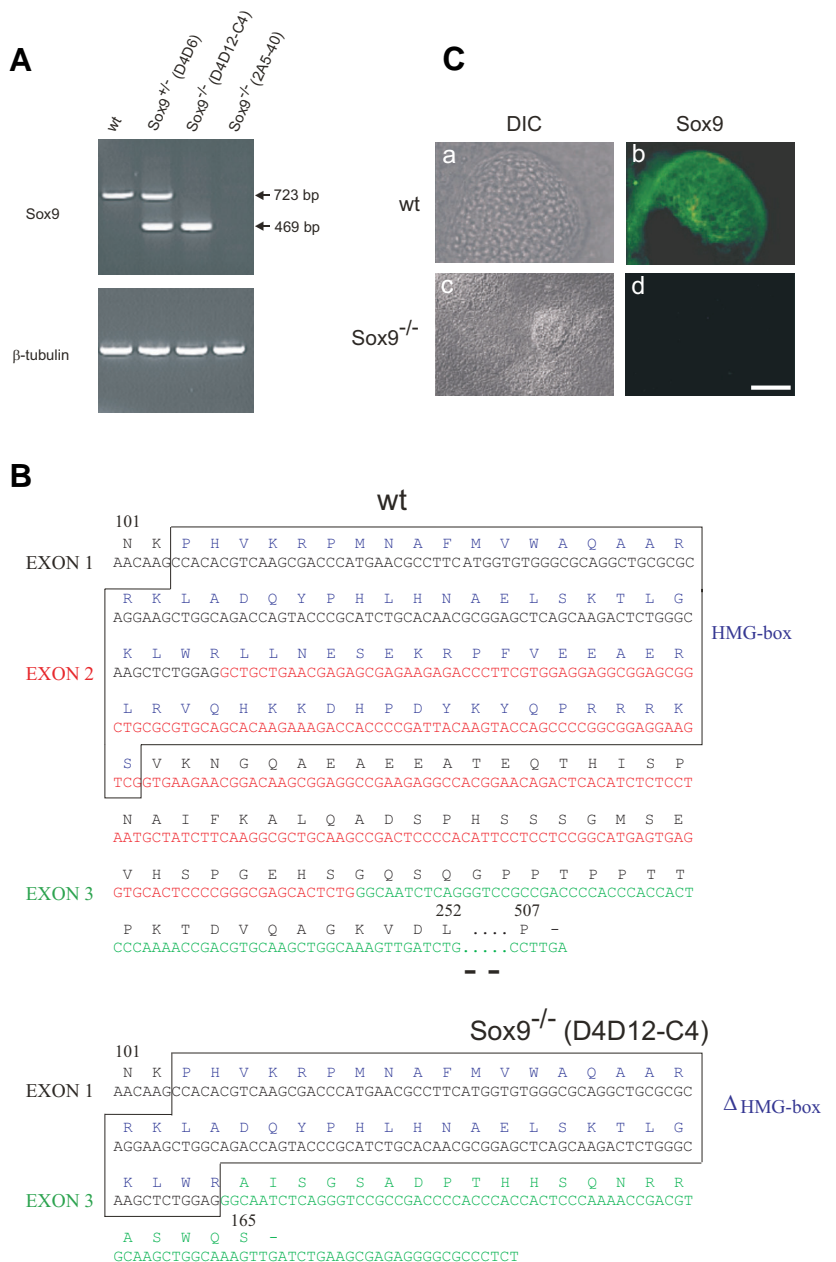


Fig. 2. Loss of functional Sox9 expression in Sox9^{-/-} embryoid bodies. (A) RT-PCR analysis of RNA isolated from wildtype (wt), heterozygous (Sox9^{+/-}) and homozygous (Sox9^{-/-}) embryoid bodies generated from ES cell clones D4D6, D4D12-C4 or 2A5-40. Oligonucleotide primers specifically binding in exon 1 and 3 of the Sox9 gene could not amplify any wildtype fragment (723 bp) in both homozygous mutant ES cell clones. Instead, a 469 bp fragment was detected for the Sox9-del^{ex2} clone D4D12-C4, while no other fragment was detected for the Sox9-del^{ex2,3} clone 2A5-40. (B) DNA sequencing of the 469 bp RT-PCR product revealed splicing of exon 1 to exon 3 resulting in a frameshift mutation, caused by the lack of the 254 bp-long exon 2. This nucleotide sequence predicts an aberrant peptide which would code only for the first half of the HMG box (blue; D HMG-box; 42 amino acids) followed by an altered amino acid sequence and a stop codon at position 166. (C) During *in vitro* differentiation, Sox9 protein was detected by immunostaining in wildtype EB outgrowths (a,b) but not in Sox9^{-/-} (D4D12-C4 and 2A5-40) EB outgrowths (c,d). Representative areas are shown (wildtype and clone D4D12-C4). DIC = differential interference contrast. Scale bar, 100 μm.

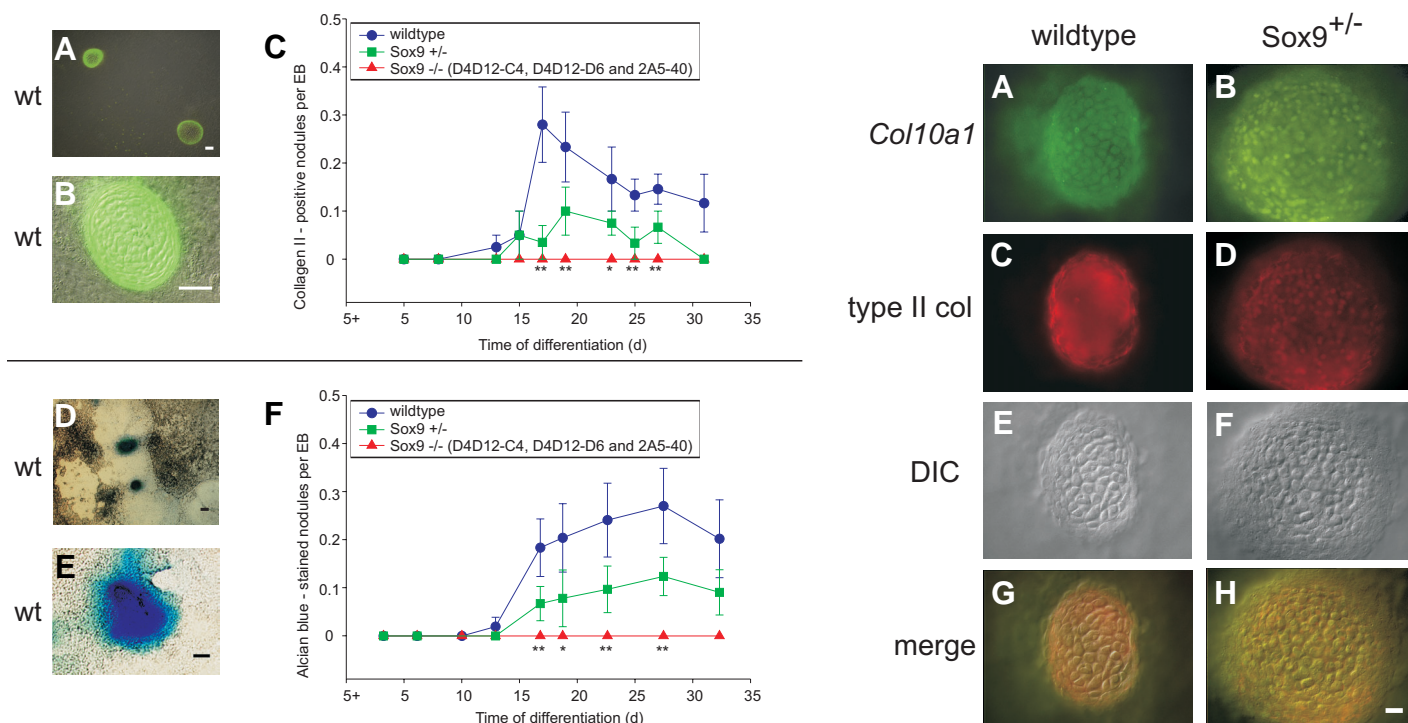


Fig. 3 (Left). *Sox9*^{-/-} ES cells are not able to differentiate into mature chondrocytes forming cartilage nodules. The number of cartilage nodules (A,B,D,E) was determined in embryoid body (EB) outgrowths of wildtype (wt), *Sox9*^{+/-} (D4D6) and *Sox9*^{-/-} (D4D12-C4, D4D12-D6 and 2A5-40) ES cells after immunostaining for type II collagen (A-C) and after Alcian blue staining (D-F). Whereas in wildtype EBs, the first nodules were found 13 days after plating (5+13 d), and two days later in *Sox9*^{+/-} EBs (5+15 d), no such structures could be detected in *Sox9*^{-/-} EBs (C,F). In *Sox9*^{+/-} EBs, the number of nodules appeared to be reduced but the differences were not statistically significant. Mean values \pm SEM from at least three independent experiments are shown. Because we found less than 1 nodule per EB in the wildtype EBs we analyzed approximately 200 EBs per day. Significant differences between wildtype and *Sox9*^{-/-} EBs: *: $p \leq 0.05$; **: $p \leq 0.01$. Scale bars, 100 μ m.

Fig. 4 (Right). Wildtype and *Sox9*^{+/-} embryoid bodies (EBs) form nodules consisting of hypertrophic chondrocytes. Wildtype (A,C,E,G) and *Sox9*^{+/-} (B,D,F,H) ES cells differentiated into Col10a1-(A,B) and type II collagen (C,D)-positive cells. These cells were embedded in abundant extracellular matrix and formed cartilaginous nodules (E,F). Merging of the pictures demonstrates that type II collagen is evenly distributed between the Col10a1-expressing cells derived from wildtype and *Sox9*^{+/-} ES cells (G,H). *Sox9*^{-/-} ES cells do not form cartilage nodules. EBs were analyzed at 5+28 d by in situ hybridization for Col10a1 mRNA combined with immunostaining for type II collagen. DIC, differential interference contrast. Scale bar, 100 μ m.

selected which only carried the *Sox9-de^{px2,3}* alleles as verified by Southern Blotting showing 4,3 kb *Eco*RI and 2,9 kb *Hind*III fragments, respectively (Fig. 1C).

To investigate the *Sox9* transcripts generated from the *Sox9-de^{px2}* allele, we performed RT-PCR with RNA isolated from ES cell clones D4D6 and D4D12-C4 and sequence-specific primers located in exon 1 and exon 3. We found that the fragment amplified from the transcripts of the deleted allele (469 bp) was 254 bp shorter than the wildtype fragment (723 bp) and no *Sox9* wildtype transcripts could be detected in clone D4D12-C4 (Fig. 2A). DNA sequencing of the mutant 469 bp fragment showed that exon 2 was completely deleted and exon 1 was spliced to exon 3 resulting in a frameshift mutation. If a protein was to be translated from the mutant transcript of the *Sox9-de^{px2}* allele, it would consist of 165 instead of 509 amino acids, carrying a truncated HMG domain followed by 21 out-of-frame amino acids encoded by exon 3 (Fig. 2B). RT-PCR with RNA isolated from ES cell clone 2A5-40 carrying the *Sox9-de^{px2,3}* allele did not result in any amplification product (Fig. 2A). By immunostaining we were not able to detect any Sox9 protein in the knock-out clones D4D12-C4 (Fig. 2C) and 2A5-40

(data not shown) during differentiation.

***Sox9*^{-/-} cells are unable to develop into mature chondrocytes in vitro**

ES cells differentiate *in vitro* into highly organized cartilage nodules (Kramer *et al.*, 2000). To test for the chondrogenic *in vitro* differentiation capacity in the absence of Sox9, wildtype, *Sox9*^{+/-} and *Sox9*^{-/-} ES cells were analyzed by counting type II collagen-positive cartilage nodules (Fig. 3 A,B) in EB outgrowths. We found that cells of the *Sox9*^{-/-} clones D4D12-C4 and D4D12-D6 and of the different *Sox9*^{-/-} clone 2A5-40 did not differentiate into these highly organized cartilage structures during EB cultivation up to 5+31 d (Fig. 3C). In contrast, in *Sox9*^{+/-} EB outgrowths, nodules appeared but the number was reduced in comparison to the wildtype control (Fig. 3C). The first nodules were detected in wildtype EBs at 5+13 d, their number increased up to 5+17 d and decreased thereafter. Similarly, in *Sox9*^{+/-} EB outgrowths, the first nodules formed at 5+15 d, the maximum number was detected at 5+19 d and decreased later. The mean values for the number of type II collagen-positive nodules in *Sox9*^{+/-} EB outgrowths never reached the wildtype levels, although the differ-

ences were not statistically significant. These results were confirmed by Alcian blue staining, which showed that deep blue stained nodules (Fig. 3D,E) were completely absent in *Sox9*^{-/-} EB outgrowths up to 5+33 d, and that their number was reduced in *Sox9*^{+/-} EBs (Fig. 3F). Alcian blue specifically stains acidic proteoglycans found in the extracellular matrix of cartilage tissue. The maximum number of Alcian blue-stained nodules was observed at later stages as compared to type II collagen-positive nodules, in both wildtype and *Sox9*^{+/-} EBs. This is probably due to the fact that the Alcian blue-stained proteoglycans and type II collagen are subsequently expressed during terminal chondrogenic differentiation in EBs. In summary, these data demonstrate that Sox9 is required for the formation of highly organized cartilage nodules *in vitro*.

To characterize the terminal stage of chondrocyte differentiation in more detail, the expression of *Col10a1* was analyzed, a marker for hypertrophic chondrocytes appearing at the latest differentiation steps during endochondral differentiation *in vivo*. *In situ* hybridization for *Col10a1* mRNA (Fig. 4 A,B) combined with immunostaining for type II collagen (Fig. 4 C,D) demonstrated that hypertrophic chondrocytes co-expressing *Col10a1* and type II collagen were present in wildtype and *Sox9*^{+/-} EBs but not in *Sox9*^{-/-} EBs (not shown). These results show that chondrogenic cells in *Sox9*^{+/-} ES cell-derived nodules become hypertrophic. Hypertrophic cells of wildtype and *Sox9*^{+/-} EBs did not show any obvious differences regarding their morphology or expression and distribution of type II collagen (Fig. 4 C-F). However, *in situ* hybridization showed that expression of *Col10a1* was always stronger in *Sox9*^{+/-} EBs and the number of *Col10a1*-expressing cells seemed to be increased compared to the wildtype (Fig. 4 A,B).

Sox9^{-/-} ES cells are able to form pre-cartilage condensations *in vitro*

To see whether already early steps of chondrogenic differentiation were affected after complete loss of Sox9 function *in vitro*, we tested the EBs for the presence of pre-cartilage condensations. To this end we analyzed wildtype and *Sox9*^{-/-} (clones D4D12-C4, and 2A5-40) EB outgrowths for expression of *Sox5* (Fig. 5 A-C) and *Sox6* (Fig. 5 J-L) and found that both markers are coexpressed with type II collagen in pre-cartilage condensations of wildtype and *Sox9*^{-/-} EB outgrowths (Fig. 5). Control slides hybridized with the respective sense probes and the secondary antibody alone were negative (data not shown). Condensations in both, wildtype and *Sox9*^{-/-} (D4D12-C4, and 2A5-40) EB outgrowths, displayed intensive binding of PNA (Fig. 5 S-U), demonstrating that pre-cartilage condensations are formed in the absence of Sox9. Identical results were obtained for the *Sox9*^{-/-} clone D4D12-D6 (data not shown).

To analyze condensation formation on a quantitative level, we analyzed wildtype, *Sox9*^{+/-} and *Sox9*^{-/-} (D4D12-C4) EBs for the formation of N-cadherin- and *scleraxis*-positive cell condensations. We found *scleraxis*- (Fig. 6 A-C) and N-cadherin- (Fig. 6 D-F) positive cell condensations in EB outgrowths of wildtype, *Sox9*^{+/-} and *Sox9*^{-/-} ES cells during cultivation. There were no obvious morphological differences between these pre-cartilage structures among the three genotypes and we did not find any significant quantitative differences in the number of *scleraxis*-positive condensations between wildtype, *Sox9*^{+/-} and *Sox9*^{-/-}

EBs (Fig. 6 J). The same results were obtained when we studied expression of *scleraxis* and N-CAM, which are also characteristically co-expressed by cells forming pre-cartilage condensations (data not shown).

Together, these results indicate that *Sox9* inactivation does

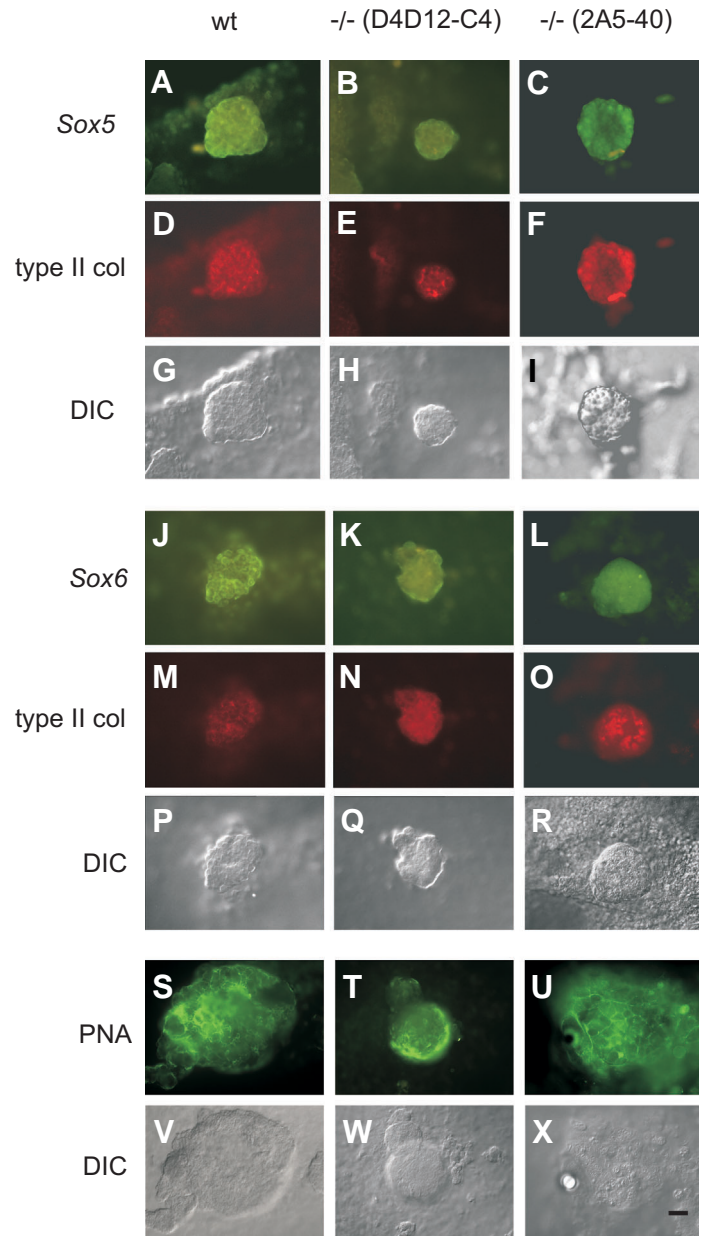


Fig. 5. Pre-cartilage condensations show expression of Sox5 and Sox6 and bind peanut agglutinin (PNA) in the absence of Sox9. *In situ* hybridization for Sox5 (A-C) and Sox6 mRNA (J-L) combined with immunostaining for type II collagen (D-F, M-O) and DIC microscopy (G-I, P-R) showed that mesenchymal cells in wildtype (wt; A,D,G,J,M,P) and *Sox9*^{-/-} EBs derived from the two different *Sox9*^{-/-} ES cell clones D4D12-C4 (B,E,H,K,N,O) and 2A5-40 (C,F,I,L,O,R) form condensations which express Sox5 and Sox6. Such condensations also bind fluorescein-labeled PNA in wildtype (S,V) and *Sox9*^{-/-} EBs derived from clones D4D12-C4 (T,W) and 2A5-40 (U,X). Representative areas from EB outgrowths are shown. Scale bar, 100 μ m.

not affect the formation of early pre-cartilage condensations during ES cell differentiation *in vitro*.

Col2a1 expression is downregulated but not completely abolished during *in vitro* ES cell differentiation in the absence of Sox9

Because Sox9 is an activator of the *Col2a1* gene both *in vivo* and *in vitro*, we performed conventional and quantitative RT-PCR of Sox9-deficient EBs. Using primer which amplify two splice variants of *Col2a1*, a juvenile and an adult form (Metsäranta *et al.*, 1991), we found that both were expressed in *Sox9*^{-/-} EBs of clones D4D12-C4 and 2A5-40 (Fig. 7A). This was confirmed by immunostaining of EB cultures for collagen type II. Both, in wildtype and *Sox9*^{-/-} EBs, type II collagen-fibers could be detected at late differentiation stages, scattered throughout the EB outgrowths (Fig. 7 C,D). These fibers were also found in *Sox9*^{+/-} EB outgrowths (not shown). To determine the level of *Col2a1* expression in wildtype and *Sox9*^{-/-} cells, EBs of clone 2A5-40 and D4D12-C4 were analyzed by real-time RT-PCR for expression of *Col2a1* using primers which do not discriminate between both splice variants. *Col2a1* was still expressed but clearly downregu-

lated in the absence of Sox9 (Fig. 7 B). Thus, Sox9 seems to be required for an upregulated expression of *Col2a1* but other factors may compensate for a basal expression at least *in vitro*.

Discussion

Sox9 is required for ES cell differentiation into cartilage nodules *in vitro*

Our results demonstrate that *in vitro* differentiation of *Sox9*^{-/-} ES cells into chondrocytes is disrupted at a stage characterised by the appearance of typical round-shaped chondrocytes organized in distinct nodules and expressing a high level of type II collagen. *Sox9*^{-/-} cells fail to form these cartilage nodules. In line with this, it has been demonstrated that Sox9-deficient cells could not differentiate into mature chondrocytes in mouse *Sox9*^{-/-} chimeras and teratomas (Bi *et al.*, 1999). Furthermore, conditional null mutant mice lacking Sox9 in mesenchymal cells of limb buds were not able to form cartilage and bone in limbs (Akiyama *et al.*, 2002). Since we found in previous studies that the formation of cartilage nodules in differentiating EBs can be induced after application of growth factors of the transforming growth factor β -family (Kramer *et al.*, 2000) we applied BMP-2, TGF- β_1 and TGF- β_3 to differentiating *Sox9*^{-/-} EBs. However, we did not detect any cartilage nodule in these EB outgrowths (data not shown) indicating that loss of Sox9 function cannot be rescued by these chondrogenic factors *in vitro*. In *Sox9*^{+/-} EB outgrowths, cartilage nodules were present, but the number of nodules appeared to be reduced compared to the wildtype, indicating that *Sox9* gene dosage is important for proper cartilage differentiation. This agrees with the observation that cartilage structures are defective and hypoplastic but not completely absent in CD patients and *Sox9*^{+/-} mice (Houston *et al.*, 1983; Bi *et al.*, 2001). Taken together, this demonstrates that Sox9 plays an essential role during chondrogenesis both *in vivo* and *in vitro*.

It has been proposed that Sox9 plays an inhibitory role for the switch from prehypertrophic to hypertrophic chondrocytes, because *Sox9* is completely switched off in hypertrophic chondrocytes *in vivo* (Zhao *et al.*, 1997; Bi *et al.*, 2001). Furthermore, in prehypertrophic chondrocytes of mice lacking the receptor for the parathyroid hormone-related peptide, Sox9 phosphorylation is abolished and these mice show accelerated differentiation of hypertrophic chondrocytes (Lanske *et al.*, 1996; Huang *et al.*, 2001). Our data suggest that *Col10a1* expression and the number

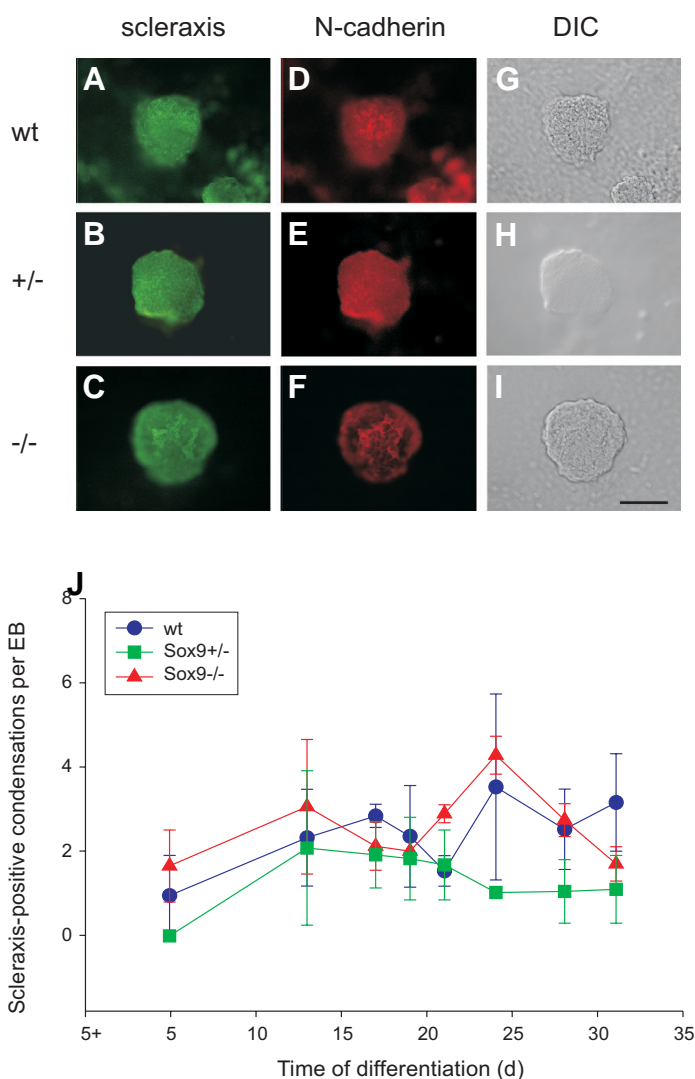


Fig. 6. Loss of Sox9 function does not affect the formation of pre-cartilage condensations *in vitro*.

In situ hybridization using an anti-sense probe detecting scleraxis mRNA (A,B,C) combined with immunostaining for N-Cadherin (D,E,F) and DIC microscopy (G,H,I) showed that mesenchymal cells in wildtype (wt; A,D,G), *Sox9*^{+/-} D4D6 (B,E,H) and *Sox9*^{-/-} D4D12-C4 (C,F,I) EBs form condensations (G,H,I) which express scleraxis (A,B,C) and N-Cadherin (D,E,F). Representative areas from EB outgrowths are shown. DIC, differential interference contrast. Scale bar, 100 μ m. Scleraxis-positive pre-cartilage condensations were found in EB outgrowths of the three ES cell clones during *in vitro* differentiation from 5 days (5+5 d) up to 31 days (5+31 d) after EB plating (J). Data are shown for wildtype, the heterozygous clone D4D6 and the homozygous clone D4D12-C4. The number of scleraxis-positive condensations did not differ significantly between EBs of the three genotypes. Mean values from at least three independent experiments are shown. Approximately 50 EBs were analyzed per time point.

of *Col10a1*-expressing cells increase in *Sox9*^{+/-} nodules. These results indicate that a reduced level of Sox9 may promote the formation of hypertrophic chondrocytes. In line with this, *Sox9*^{+/-} mice showed an enlarged zone of chondrocyte hypertrophy in the growth plates of the long bones (Bi *et al.*, 2001).

Formation of pre-cartilage condensations is not affected in Sox9^{-/-} EBs

We found that during ES cell differentiation *in vitro*, Sox9-deficient cells form early pre-cartilage condensations which express *Sox5*, *Sox6*, *scleraxis* and *N-cadherin*, markers characteristic for such pre-cartilage condensations (Oberlender and Tuan, 1994; Lefebvre *et al.*, 1998; Brown *et al.*, 1999). Furthermore, the condensations strongly bind the lectin PNA (DeLise *et al.*, 2000). These results were not only obtained with both subclones from the Sox9^{-/-} clone D4D12, lacking exon 2, but also with the independent Sox9^{-/-} clone 2A5-40, lacking exons 2 plus 3.

In mouse chimeras, Sox9^{-/-} cells were located adjacent to condensing wildtype mesenchymal cells in 11.5 and 12.5 d p.c. embryos and did not take part in the formation of mesenchymal condensations (Bi *et al.*, 1999). Moreover, conditional null mutant mice, in which Sox9 was inactivated in mesenchymal cells of limb buds before these cells condense, were no longer able to form mesenchymal condensations (Akiyama *et al.*, 2002). These results suggested that condensation formation is a cell-autonomous process under the control of Sox9. On the other hand, zebrafish with homozygous null mutations in the *sox9a* gene, an ortholog of the mammalian Sox9 gene, are able to form mesenchymal condensations in the first two pharyngeal arches (Yan *et al.*, 2002). We have shown in the present study, that a homogeneous population of Sox9-deficient cells is able to form pre-cartilage condensations and express early molecular markers *in vitro*. Thus, our data indicate that the function of Sox9 during condensation formation is rather not cell-autonomous.

In vivo, development is not only temporally controlled as in EBs, but is also spatially controlled by a combination of distinct signaling molecules present at defined concentrations. Because morphogenetic development is not possible within EBs, such spatially controlled signals might be lacking, resulting in condensation of mesenchymal cells in Sox9^{-/-} EBs. In line with this, a variability of morphogenetic signals around pre-cartilage condensations in EB outgrowths might influence their cellular fate. In fact, we found that in wildtype EBs only some of the condensations develop into cartilage nodules, and the mean number of scleraxis-positive condensations does not decrease significantly during culture. Thus, the *in vitro* system offers the possibility to analyze the potency of cell-autonomous differentiation effects. The formation of specifically shaped mesenchymal condensations *in vivo* may be regulated by antagonistic factors produced by ectodermal or non-condensing mesenchymal cells located close to the place of the condensations (Zanetti and Solursh, 1986). For example, in limb buds, the size and shape of mesenchymal condensations is controlled by inhibitory factors produced by ectodermal cells such as FGF2 and FGF8 (Mohtah *et al.*, 2002). Sox9 could be an antagonist of such condensation-inhibiting factors, which are expressed *in vivo* by the overlying ectoderm. The absence of Sox9 would then result in the complete loss of condensations *in vivo*. In contrast, in the EB *in vitro* differentiation system, the release of such condensation-inhibiting factors from

adjacent tissue can not occur and therefore condensations can form even in the absence of Sox9. Similarly, it has been found that *scleraxis* null mutant embryos fail to form mesoderm, whereas in EBs of *scleraxis*^{-/-} ES cells, mesodermal markers were expressed at a similar level as in wildtype EBs (Brown *et al.*, 1999) indicating that the role of *scleraxis* during mesoderm formation is not cell-autonomous but depends on the environment. Another example are mice lacking a transcription factor, the serum response factor (Srf). These *Srf*^{-/-} mice stop developing at the onset of gastrulation and do not form mesoderm (Arsenian *et al.*, 1998). However, *Srf*^{-/-} ES cells differentiated *in vitro* into mesodermal cell types although this process was impaired *in vivo* (Weinhold *et al.*, 2000) suggesting that the function of Srf to promote mesoderm formation is non-cell-autonomous. Our data indicate that a non cell-autonomous function may also apply to Sox9 regarding the formation of pre-cartilage condensations.

Loss of Sox9 affects the level of but does not completely abolish Col2a1 expression in vitro

Expression studies *in vivo* suggested that *Col2a1* is a target for Sox9, because *Sox9* and *Col2a1* are coexpressed in cartilage primordia throughout the developing skeleton and in other developing cartilage structures during embryogenesis (Zhao *et al.*, 1997; Ng *et al.*, 1997). It has also been shown that Sox9 binds to

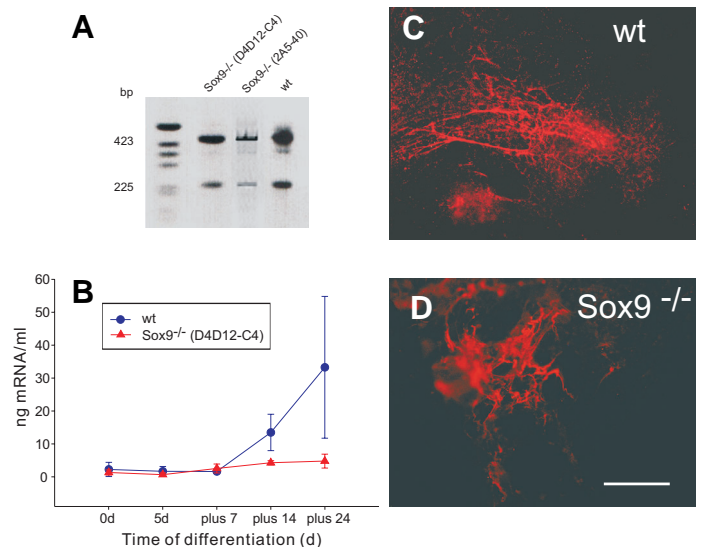


Fig. 7. Expression of *Col2a1* is clearly downregulated, but not completely abolished, during *in vitro* differentiation of Sox9-deficient ES cells. Both the adult (225 bp) and the juvenile (432 bp) splice variants of *Col2a1* were expressed in the absence of Sox9 as analyzed by conventional RT-PCR in 5+22 d EBs derived from wildtype (wt) and Sox9-deficient clones D4D12-C4 and 2A5-40 (A). However, the level of gene expression of *Col2a1* as analyzed by real time RT-PCR during differentiation of wildtype (wt) and Sox9^{-/-} ES cells (clones D4D12-C4 and 2A5-40) was clearly reduced in Sox9^{-/-} EBs compared to wildtype EBs (B). Data are shown for clone D4D12-C4. RNA isolated from undifferentiated ES cells (0d) and EBs at 5d, 5+7 d, 5+14 d and 5+24 was analyzed. Immunostainings showed that collagen II fibres were scattered throughout EBs derived from wildtype (C) and Sox9^{-/-} ES cells (D) at 5+28 d. Collagen type II fibre formation is independent of cartilage nodule formation which is missing in the absence of Sox9. Scale bar, 100 μ m.

specific sequence elements of the *Col2a1* enhancer and directs chondrocyte-specific *Col2a1* expression, both in transient transfection experiments and in transgenic mice (Lefebvre *et al.*, 1996; Bell *et al.*, 1997; Lefebvre *et al.*, 1997; Zhou *et al.*, 1998). In mouse chimeras, *Sox9*^{-/-} cells did not express *Col2a1*, and in *Sox9*^{-/-} teratomas, type II collagen was not detectable in any cell type (Bi *et al.*, 1999). In contrast, coexpression studies of *Sox9* and *Col2a1* in developing wild type mouse embryos revealed that *Col2a1* was expressed in several nonskeletal tissues which are negative for *Sox9* (Ng *et al.*, 1997), indicating that differentiating cells are able to express *Col2a1* in the absence of Sox9. Furthermore, type II collagen expression in cultured human articular chondrocytes does not correlate with the level of Sox9 expression (Aigner *et al.*, 2003). We found that Sox9-deficiency did not result in complete abolishment but obvious downregulation of *Col2a1* expression in differentiating EBs, as shown by real-time RT-PCR and by detection of type II collagen fibres in *Sox9*^{-/-} EB outgrowths. One possible explanation for this unexpected result could be that we generated a partially functional Sox9 protein by our gene targeting strategy. This can be ruled out, as we did not detect any Sox9 protein in cultured *Sox9*^{-/-} EBs. Furthermore, it has been shown recently that no functional Sox9 protein could be detected after conditional inactivation of *Sox9* in the lung using the same targeting strategy resulting in *Sox9-de^{ex2,3}* alleles (Perl *et al.*, 2005). Moreover, even if a truncated protein was still produced in the knock-out lines this mutant Sox9 protein would lack a functional DNA binding domain and the C-terminal transactivation domain (Südbeck *et al.*, 1996) and would thus not be able to function as a transcription factor.

Another explanation would be compensation of *Sox9* function *in vitro* by a protein with an overlapping function. The transcription factors *Sox5* and *Sox6* would be candidates. Both were expressed in Sox9-deficient condensations *in vitro* and might up-regulate *Col2a1* expression at least to moderate expression levels. Such a compensatory mechanism may depend on the prior formation of mesenchymal condensations. This agrees with the observation that in conditional null mutant mice, expression of *Col2a1* as well as *Sox5* and *Sox6* was inhibited when *Sox9* was inactivated in mesenchymal cells before condensations had been formed (Akiyama *et al.*, 2002).

In conclusion, this *in vitro* study unravels a mechanistic insight into the function of Sox9 during chondrogenic differentiation. We found that in contrast to the terminal step of differentiation characterized by the formation of cartilage nodules, the early step of pre-chondrogenic differentiation, the formation of pre-cartilage condensations, remained almost unaffected after loss of Sox9 function *in vitro*. In contrast, a block of this early differentiation step has previously been demonstrated *in vivo*. This indicates that the function of Sox9 during this process is not cell-autonomous. Thus, the *in vitro* differentiation of embryonic stem cells is a useful approach to bring new important insights into complex developmental processes.

Materials and Methods

Generation of Sox9^{+/-} and Sox9^{-/-} ES cells

Gene targeting of *Sox9* was achieved by electroporation of E14.1 ES cells (Kuhn *et al.*, 1991) with a targeting vector containing a *loxP*-flanked MC1-*neo*-pA cassette inserted into intron 1, a third *loxP* site inserted into intron 2, and a PGK-*tk*-pA negative selection cassette (Kist *et al.*,

unpublished data) generating the *Sox9-neoflox2* allele. The targeting vector is thus largely identical to the targeting vector described previously which resulted in the *Sox9-neoflox* allele (Kist *et al.*, 2002), except that the third *loxP* site is placed within intron 2 instead of downstream of exon 3. Cultivation, electroporation and drug selection of E14.1 ES cells was according to standard procedures (Matise *et al.*, 2000). Correctly targeted ES cell clones were identified and confirmed by Southern blot analysis using specific hybridisation probes (Kist *et al.*, unpublished). In order to generate *Sox9*^{+/-} ES cell clones by transient *in vitro* expression of Cre recombinase, correctly targeted *Sox9* ES cell clones were electroporated with *pCre-Pac* expression vector and selected with puromycin as described (Taniguchi *et al.*, 1998). Fortuitously, a *Sox9*^{-/-} ES cell clone, termed D4D12, was obtained in which the wildtype allele on the non-targeted chromosome had been replaced by the mutant allele lacking exon 2. The underlying mechanisms of such chromosome-specific loss of heterozygosity has been discussed elsewhere (Lefebvre *et al.*, 2001).

A different *Sox9*^{-/-} ES cell line was generated from a clone, termed 2A, carrying the *Sox9-neoflox* allele in a heterozygous configuration (Kist *et al.*, 2002). After drug selection with high concentrations of G418 of 10 and 12 mg/ml for 38 days, surviving clones were picked, genomic DNA was isolated and screened by PCR for homozygosity of the *Sox9-neoflox* allele. A clone, termed 2A5, was obtained which carried two *Sox9-neoflox* alleles. This clone was transfected with the *pCre-Pac* expression vector and cells were selected with puromycin as described above.

For subcloning, *Sox9*^{-/-} ES cell clones D4D12 and 2A5 were plated at low density and new subclones, termed D4D12-C4, D4D12-D6 and 2A5-40, were isolated and confirmed by Southern Blotting.

Cell culture and differentiation of EBs

Differentiation of chondrogenic cells *in vitro* was studied during differentiation of the *Sox9*^{+/-} ES cell clone D4D6 and the *Sox9*^{-/-} ES cell clones D4D12-C4 and D4D12-D6, lacking exon 2, and 2A5-40, lacking exon 2 and exon 3, in comparison to the wildtype ES cell line E14.1. ES cells were grown on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts in cultivation medium consisting of DME (INVITROGEN, Karlsruhe, FRG) supplemented with 15% FCS (INVITROGEN, Karlsruhe, FRG), non-essential amino acids (INVITROGEN, Karlsruhe, FRG, stock solution diluted 1:100), 2 mM L-glutamine (INVITROGEN, Karlsruhe, FRG) and 5x10⁻⁵ M β-mercaptoethanol (SERVA, Heidelberg, FRG), as described previously for line D3 (Kramer *et al.*, 2000). For differentiation, aliquots of 20 μl differentiation medium (containing 20% FCS instead of 15%) containing 800 cells were cultivated in «hanging drops» for 2 days and, after transfer on bacteriological petri dishes, in suspension for an additional 3 days (Kramer *et al.*, 2000). The 5 day («5 d») old EBs were plated separately onto gelatin (0.1%)-coated 24 well microwell plates for morphological analysis, or 15 EBs were plated onto a 6 cm tissue culture plate for Alcian blue staining and RT-PCR, or 10 EBs onto 2 well (21,3 x 20 mm) Lab-Tek chamber slides (NUNC, Wiesbaden, FRG) for immunostaining, *in situ* hybridization and test for PNA binding. Alcian blue stainings were performed as described previously (Kramer *et al.*, 2000). We performed at least three differentiation experiments per cell line and analyzed approximately 200 EBs per time point by Alcian blue staining. Data analysis was performed using the Sigma Plot 5.0 software (JANDEL, Corte Madeira, USA). The Student's t-test was used for statistical analysis.

Quantitative measurement of *Col2a1* gene expression by real-time-RT-PCR analysis

Samples of ten EBs of different developmental stages up to 26 days after plating (5+26 d) were collected, washed two times with PBS, and total RNA was isolated and reverse transcribed as described (Hegert *et al.*, 2002). Aliquots of 1 μl from the RT reactions were mixed with 10 pmol primer specific for *Col2a1* (sense: 5'-TTTCCTCCGCTACTGTCCACTG-3'; antisense: 5'-TGTATGTGAACCTGCTGTTGC=C-3'; product size: 161 bp) and real-time PCR was carried out with the iQ SYBR Green

Supermix (BIORAD, Munich, FRG) using an iCycler iQ thermal cycler (BIORAD, Munich, FRG) according to the manufacturer's instructions. The thermal cycling conditions were 95 °C for 2 minutes followed by 40 cycles of 95 °C for 40 seconds, 58 °C for 40 seconds and 72 °C for 40 seconds. To confirm the specificity of the amplified products, melting curves were performed at the end of the amplification by cooling samples to 58 °C for 1 minute and then increasing temperature to 95 °C at 0.05 °C/second with continuous fluorescence measurement. Each sample was tested in duplicate. For generation of standard curves, the PCR product was cloned into the vector pCR-TOPO (INVITROGEN, Karlsruhe, FRG). Plasmid DNA was isolated using QIAGEN-tip 100 anion-exchange columns (QIAGEN) and serially diluted in double-distilled water. Threshold cycles are adjusted to attain the highest possible correlation coefficient value for the standard curve provided by the manufacturer's software. According to their respective cycle numbers the concentrations of unknown samples were deduced from the standard curve.

Conventional RT-PCR analysis

RT-PCR reactions were carried out with sequence-specific primers as described previously (Kramer *et al.*, 2000; Hegert *et al.*, 2002). To study expression of *Col2a1* and *Sox9*, respectively, the following primers were used (oligonucleotide sequences are given in brackets in the order antisense-, sense-primer followed by the annealing temperature used for PCR, length of the amplified fragment and a reference): *Col2a1* 5' - AGGGGTACCAGGTTCTCCATC - 3', 5' - CTGCTCATCGCCGCGTCTTA - 3'; 60°C; 432 bp (splice variant A) and 225 bp (splice variant B); (Metsäranta *et al.*, 1991); *Sox9* 5' - TGGGTGGCAAGTATTGGTCAAACA - 3', 5' - TGAAGAAGGAGAGCGAGGAAGATAA - 3'; 57°C; 723 bp; (Lefebvre *et al.*, 1998). Electrophoretic separation of PCR products was carried out on 2% agarose gels.

Cloning of PCR fragments for sequence analysis

Reverse Transcription was performed as described above. cDNA was amplified by PCR using Vent-DNA-polymerase (NEW ENGLAND BIOLABS, Frankfurt, FRG). 723 bp and 469 bp long *Sox9* cDNA fragments amplified from RNA isolated from wildtype and *Sox9*^{-/-} (clone D4D12-C4) cells, respectively, were excised from a 2% agarose gel after electrophoretic separation, and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, FRG). These fragments were cloned blunt-ended into the plasmid vector pCR®-Blunt using the Zero Blunt™ PCR Cloning Kit according to the manufacturer's protocol (INVITROGEN, Karlsruhe, FRG). Clones carrying inserts of the expected length were selected after restriction enzyme digestion and their nucleotide sequence was verified by sequencing (MWG-Biotech, Ebersberg, FRG).

Fluorescence in situ hybridisation coupled with immunostaining

The combination of fluorescence *in situ* hybridization and immunostaining as well as cloning of the *scleraxis* and *Col10a1* cDNAs used to generate RNA probes by *in vitro* transcription have been described previously (Kramer *et al.*, 2000). The probes used to detect *Sox5* and *Sox6* have been described elsewhere (Lefebvre *et al.*, 1998). Ten EBs were plated per chamber slide and analyzed at different developmental stages. The monoclonal antibody II-I16B3 (Developmental Studies Hybridoma Bank, University of Iowa, USA) against type II collagen or the monoclonal anti-A-CAM antibody GC-4 (SIGMA, Taufkirchen, FRG) to detect N-Cadherin was applied in a 1:20 or 1:40 dilution, respectively. For immunostaining against Sox9 we used a polyclonal antibody from Santa Cruz (Heidelberg, FRG) diluted 1:200. Secondary antibodies were 1:100 diluted FITC-conjugated sheep F(ab) fragments against digoxigenin (BOEHRINGER, Mannheim, FRG) and Cy3- or FITC-conjugated goat anti-mouse or anti-rabbit secondary antibodies (DIANOVA, Hamburg, FRG) diluted 1:800 in PBS. Control

slides were hybridized with the respective sense probes and the secondary antibody alone.

PNA binding

To test for binding of Peanut agglutinin (PNA) to pre-cartilage condensations, EBs plated onto chamber slides were washed three times with PBS, fixed in 3.7% formaldehyde in PBS for 30 minutes at room temperature and washed again three times with PBS. FITC-labeled PNA (BIOMEDA, Foster City, USA) was applied at a concentration of 0.1 mg/ml and incubated for 45 minutes at room temperature. After washing four times in PBS, specimen were embedded in Vectashield mounting medium (VECTOR, Burlingame, USA).

Acquisition and processing of images

Slides were analyzed with the fluorescence microscope AXIOSKOP (ZEISS, Oberkochen, FRG) equipped with a 3 CCD color video camera (SONY, Cologne, Germany) using the acquisition software AXIOVISION (ZEISS, Oberkochen, FRG). Figures were assembled using the COREL DRAW software (COREL Corp., Ottawa, Canada).

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