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Sox9 is required for notochord maintenance in mice

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Abstract

Sox9 encodes a HMG-box transcription factor that has been implicated in numerous developmental processes including chondrogenesis, formation of cardiac valves, and neural crest, testis and spinal cord development. Here we show that *Sox9* is expressed in the notochord and the sclerotome during mouse development suggesting that the gene may play additional roles in the development of the axial skeleton. We used ubiquitous mosaic inactivation of a conditional *Sox9* allele by Cre/*loxP*-mediated recombination in the mouse to screen for novel functions of *Sox9*, and revealed that its absence results in severe malformations of the vertebral column. Besides its established role in chondrogenesis, *Sox9* is required for maintaining the structural integrity of the notochord. Mutant embryos establish a normal notochord; however, starting from E9.5, the notochord disintegrates in a cranial to caudal manner. The late requirement in notochord development uncovered a function of notochord-derived signals in inducing segmentation of the ventral sclerotome and chondrogenesis. Thus, *Sox9* is required for axial skeletogenesis by regulating notochord survival and chondrogenesis.

Keywords: *Sox9*; Cytokeratin 19; Cre/*loxP*; Notochord; Vertebral column; Sclerotome; HMG-box

Introduction

The vertebral column is a metamerically organized axial structure that mediates the rigidity as well as the mobility of the vertebrate body. The development of this organ relies on the proper specification and continued interaction of two embryonic tissues: the somite-derived sclerotome and the notochord. Somites are segmentally repeated units in the paraxial mesoderm that form in a periodic fashion by epithelialization of mesenchymal cells at the anterior end of the unsegmented presomitic mesoderm (PSM). Later on, the ventral part of the somite deepithelializes and forms the mesenchymal sclerotome. The dorsal somite half remains epithelial, forming the dermomyotome, which will give rise to striated muscles and the dermis of the body. Cells in the ventral area of the sclerotome migrate medially and form the perinotochordal tube. This sclerotomal compartment is initially unsegmented but acquires a metameric organization of highly condensed areas representing the future intervertebral discs and intervening regions that will undergo cartilage differentiation to form the vertebral bodies (Christ and Wilting, 1992). The other vertebral regions, neural arches and ribs, can be traced back to lateral and dorsal sclerotomal regions (for reviews on somitogenesis and vertebral column development, see Christ et al., 2000; Pourquié, 2001; Saga and Takeda, 2001; Gossler and Tam, 2002).

Somites do not only differentiate along the dorso-ventral axis, they also become compartmentalized along the anterior–posterior (AP) axis into two halves (reviewed in Christ et al., 2004). This subdivision is established at the anterior end of the PSM under the control of intrinsic Notch-Delta signaling in conjunction with the transcription factor Mesp2 (Saga and Takeda, 2001). AP-somite polarity is maintained during later sclerotome differentiation under the combined control of Uncx4.1 and Tbx18 transcription factors (Leitges et al., 2000; Mansouri et al., 2000; Bussen et al., 2004). This maintenance is important for vertebral development since each somite half differentially contributes to the forming vertebra (Aoyama and Asamoto, 2000; Huang et al., 2000). In addition, the vertebral

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column does not simply copy the metameric organization of the paraxial mesoderm but becomes newly organized such that the posterior half of the anterior somite and the anterior half of the posterior somite will together form one vertebra. This process, that has been coined resegmentation or Neugliederung (Remak, 1850), is crucial for vertebral mobility since myotomes follow the original metamery of somites thus linking two adjacent vertebrae together.

The notochord is an embryonic midline structure of mesodermal origin that underlies the neural tube along its entire length. In higher vertebrates, the notochord is a transient structure serving both a structural role and acting as a local signaling center for adjacent tissues. Being a tissue resembling cartilage, the initial rod-like structure of the notochord confers rigidity to the early vertebrate embryo before vertebral elements take over this function. Then, notochord cells in the intervertebral regions proliferate and undergo hypertrophy to form the nuclei pulposi whereas notochord cells located in the vertebral bodies are ossified. Positioned centrally in the embryo, the notochord acts as a source of secreted factors that pattern adjacent tissues along the left–right and dorso-ventral axes. In this role, the notochord specifies ventral fates in the neural tube, controls aspects of left–right asymmetry, induces pancreatic fate and controls arterial versus venous identity of the major blood vessels (for review, see Stemple, 2005).

The development of the notochord is tightly interwoven with that of the vertebral column both structurally as well as functionally. The notochord is ensheathed by the ventral sclerotome-derived perinotochordal tube to become incorporated into the vertebral bodies and intervertebral discs, thus showing tight physical interaction. In addition, notochord-derived signals are pivotal for sclerotome induction and patterning. Notochord (and floor plate)-derived signals induce expression of *Pax1* and *Mfh1* in ventral somite halves that in turn are essential to maintain sclerotomal proliferation. Both in vitro experiments and genetic data suggest that notochord-derived Sonic hedgehog in conjunction with Noggin induces and maintains *Pax1*, and thus, sclerotomal fates (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan et al., 1995; Münsterberg et al., 1995; Borycki et al., 1998; Teillet et al., 1998). The notochord may play an additional role in sclerotome patterning by conferring a segmental organization to its ventral region. Ventral sclerotome is known to transiently lose its segmental character but to regain it after condensation around the notochord, suggesting that the notochord plays an underappreciated but important role in vertebral (re)segmentation (Grotmol et al., 2003; Fleming et al., 2004).

SOX9 encodes a transcription factor featuring a high-mobility group (HMG-box) DNA-binding domain similar to that of the testis-determining factor, *SRY*. Heterozygous mutations in and around *SOX9* cause campomelic dysplasia, a semilethal human skeletal disorder characterized by shortening and bowing of the long bones in affected patients (Foster et al., 1994; Wagner et al., 1994). Complete removal of *Sox9* function in mice has confirmed the role of the gene in chondrogenesis (Bi et al., 1999, 2001; Akiyama et al., 2002), and has revealed further requirements in testis development (Chaboissier et al., 2004; Barrionuevo et al., 2006), heart development (Akiyama et al., 2004), trunk neural crest (Cheung et al., 2005) and spinal cord development (Stolt et al., 2003). Expression of *Sox9* in the notochord and the sclerotome suggests an additional function for *Sox9* in notochord and vertebral column development, an assumption that is supported by weak vertebral column defects detected in *Sox9* heterozygous null mutants (Bi et al., 2001). Embryonic lethality of homozygous null mutants at E11.5 (Akiyama et al., 2004), however, has precluded the full analysis of the requirement for *Sox9* in vertebral column and notochord development, to date. To circumvent this early lethality, we have used a conditional approach of *Sox9* inactivation that allows the phenotypic characterization of highly mosaic *Sox9* mutant embryos until E15.5. Here, we show that *Sox9* is necessary for the development of the notochord and the perinotochordal sclerotome. Furthermore, our analysis uncovers multiple requirements for notochordal signaling in (ventral) sclerotome development.

Materials and methods

Generation of Ck19-Cre;Sox9$^{flox/flox}$ mice

Sox9$^{flox/+}$ mice, originally on a 129P2/OlaHsd×C57BL/6 mixed genetic background (Kist et al., 2002), were backcrossed onto the C57Bl/6 genomic background for three generations, before heterozygotes were mated inter se to generate mice homozygous for the *Sox9^{flox}* allele. *Sox9$^{flox/flox}$* mice were crossed with the *Ck19-Cre* transgenic mice that were also kept on a C57Bl/6 genetic background (Harada et al., 1999), and the resulting *Cre/+;Sox9$^{flox/+}$* offspring was backcrossed to *Sox9$^{flox/flox}$* mice to obtain *Cre/+;Sox9$^{flox/flox}$* mice. For timed pregnancies, plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Genotyping was carried out on genomic DNA derived from adult tails or embryonic yolk sacs using established PCR protocols for the *Ck19-Cre* allele (Lecureuil et al., 2002) and for the *Sox9* and *Sox9^{flox}* alleles (Kist et al., 2002).

Histological analysis

Skeletal preparations of embryos and of newborns were performed as previously described (Nagy et al., 2003; Bussen et al., 2004). Embryos for histological analyses were collected in PBS, fixed in Serra (ethanol: 37% formaldehyde:acetic acid, 6:3:1), embedded in paraffin and sectioned to 7 μm. Sections were stained with hematoxylin and eosin or with Alcian Blue and Nuclear Fast Red. Histochemistry for β-galactosidase was carried out as described (Echelard et al., 1994). Stained embryos were postfixed in 4% paraformaldehyde in PBS, paraffin-embedded and sectioned to 7 μm.

Immunohistochemical analysis

Paraffin sections were deparaffinized in xylene and rehydrated into PBS before antigens were recovered by boiling in sodium citrate buffer. Sections were blocked in 5% goat serum for 2 h before the primary antibody (an affinity purified anti-Sox9 rabbit antiserum directed against amino acids 182–229 of mouse Sox9 fused to amino acids 305–438, Stolt et al., 2003) was applied in a dilution of 1:200. After extensive washes in PBS/0.1% Tween-20, sections were incubated in secondary biotinylated antibody (Vector Laboratories) for 1 h at room temperature. After washing, sections were incubated with avidin–fluorescein (Vector Laboratories) for 30 min at room temperature, counterstained with DAPI and mounted with Vectashield mounting medium (Vector Laboratories).

Alternatively, endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol after the antigen retrieval step. Sections were incubated with Vectastain ABC reagent (Vector laboratories) diluted in blocking solution for 45 min after incubation with the secondary biotinylated antibody. Peroxidase-