Sox5 and Sox6 are required for notochord extracellular matrix sheath formation, notochord cell survival and development of the nucleus pulposus of intervertebral discs

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SUMMARY
The notochord has major roles in vertebral column formation: indirectly by inducing sclerotome cell differentiation; and directly by forming the nucleus pulposus of intervertebral discs. Sox5 and Sox6 encode Sry-related HMG box transcription factors that act redundantly to promote chondroblast differentiation in all cartilages of the mouse embryo. We show that Sox5 and Sox6 are expressed in the notochord cell lineage and required for notochord late development.

In Sox5−/−/Sox6−/− embryos, the notochord formed a typical rod-like structure. It fulfilled its inductive functions, as indicated by expression of sonic hedgehog and sclerotome specification. However, the notochord failed to become surrounded with an extracellular matrix sheath. This phenotype was associated with a downregulation of extracellular matrix genes, including the genes for collagen 2, aggrecan and perlecan in both notochord cells and surrounding chondrocytic cells of presumptive inner annuli and vertebral bodies. The mutant notochord then underwent an aberrant, fatal dismantling after sclerotome cell migration. Its cells became removed first from intervertebral spaces and then from vertebral bodies, and it progressively underwent apoptosis. Meanwhile, the development of inner annuli and vertebral bodies was dramatically impaired. Consequently, the vertebral column of Sox5−/−/Sox6−/− fetuses consisted of a very deficient cartilage and was devoid of nuclei pulposi. In Sox5−/−/Sox6+/− and more severely in Sox5+/−/Sox6+/− embryos, the notochord sheath was thinner, but cells survived. By birth, nuclei pulposi were rudimentary, and its cells poorly swelled and still expressing sonic hedgehog.

Hence, Sox5 and Sox6 are required for notochord extracellular matrix sheath formation, notochord cell survival and formation of nuclei pulposi. Through these roles and essential roles in cartilage formation, they are central transcriptional regulators of vertebral column development.

Key words: Sox5, Sox6, Notochord, Nucleus pulposus, Intervertebral disc, Cartilage, Vertebral column, Mouse

INTRODUCTION
The development of the vertebral column relies on the proper specification and differentiation of several cell lineages. In recent years, important transcription factors, growth factors and signaling molecules have been identified that control such cells as sclerotome cells, chondrocytes and osteoblasts (Christ et al., 2000; de Crombrugghe et al., 2001; Karsenty and Wagner, 2002). However, little is known about notochord cells (Fleming et al., 2001).

The notochord is a midline structure of mesodermal origin that forms during gastrulation in chordate embryos (Hogan et al., 1994). By mouse embryonic day 9.5-11.5 (E9.5-E11.5), it consists of a solid rod underlying the neural tube. The cells are small, condensed and enveloped altogether within a sheath of extracellular matrix. This sheath contains many collagens, proteoglycans and glycoproteins, typically found in cartilage, basement membranes, or mesenchymal and fibrous tissues (Gotz et al., 1995; Hayes et al., 2001).

The notochord has crucial roles in inducing ectoderm, endoderm, and mesoderm derivatives in the early embryo (Cunliffe and Ingham, 1999; Cleaver and Krieg, 2001). It may also have an important mechanical role, and thereby constitute a primitive axial skeleton (Adams et al., 1990). In vertebrate embryos, the notochord has major roles later on during vertebral column formation (Christ et al., 2000; Pourquié et al., 1993). Intervertebral discs are joint-like structures that connect vertebrae. They feature a highly hydrated nucleus pulposus core, surrounded with a cartilaginous inner annulus and a fibrous outer annulus. At E10.5-E11.5, signals from the notochord induce sclerotome cell migration, condensation, and differentiation around the notochord and neural tube. The so-formed perinotochordal tube has a metameric pattern with the alternation of more condensed and less condensed zones.
Between E12.5 and E15.5, the more condensed zones develop into outer and inner annuli and the less condensed zones into vertebræ. The notochord thus has indirect roles in the formation of vertebræ and annuli. Simultaneously, it undergoes profound changes. Notochord cells located in vertebral bodies are removed and probably relocated into intervertebral regions (Aszódi et al., 1998; Rufai et al., 1999). In these latter regions, they proliferate and undergo hypertrophy to form the nuclei pulposi. The notochord thus also directly participates in vertebral column formation.

Several molecules are known to control the early notochord (Cunliffe and Ingham, 1999). For example, the T-box transcription factor brachury (T) determines notochord cell differentiation and survival (Herrmann and Kispert, 1994), and the secreted factors sonic hedgehog (Shh) and noggin mediate inductive actions (Chiang et al., 1996; McMahon et al., 1998; Teillet et al., 1998). In vitro studies have suggested that the notochord sheath maintains the structural scaffold and internal pressure of the notochord (Carlson and Kenney, 1980; Adams et al., 1990), but its exact roles in vivo have not been determined. The cellular origin and molecular control of this sheath remain unknown. Moreover, the mechanisms that underlie the transformation of the notochord into nuclei pulposi are also largely unknown. Several mouse mutants have an abnormal development of the notochord and vertebral column. These include mice that lack collagen 2 (Li et al., 1995; Aszódi et al., 1998), the paired box transcription factors Pax1 and Pax9 (Wallin et al., 1994; Peters et al., 1999), and the homeobox transcription factor Bapx1 (Triblioni and Luftkin, 1999; Lettice et al., 1999; Akazawa et al., 2000). In these mice, vertebral body cartilages fail to develop properly. The notochord forms normally, but fails to be removed from vertebral bodies and to develop into nuclei pulposi. Bapx1, Pax1 and Pax9 are expressed in sclerotome cells, but not in notochord cells. It was therefore proposed that the transformation of the notochord into nuclei pulposi requires mechanical pressure from the vertebral body cartilage matrix. As of today, no regulatory factor expressed in notochord cells and controlling cell fate and differentiation beyond early embryonic stages has been identified.

Sox5 and Sox6 encode two highly identical transcription factors, respectively, L-Sox5 and Sox6 (Lefebvre, 2002). These factors feature an Sry-related HMG box domain, which mediates DNA binding, and a coiled-coil domain, which mediates homo- and heterodimerization (Lefebvre et al., 1998). Sox5 also encode a short protein (Sox5) that lacks the N-terminal half of L-Sox5, including the dimerization domain. The transcripts for L-Sox5 and Sox6 are co-expressed in all cartilages, and expressed in a few other tissues. The transcript for Sox5 is expressed exclusively in testis. In vitro experiments have suggested that L-Sox5 and Sox6 cooperate with Sox9, a distant relative and master chondrogenic factor (Bi et al., 1999; Akiyama et al., 2002), in the direct activation of the collagen 2 gene (Col2a1) (Lefebvre et al., 1998). The roles of Sox5 and Sox6 in humans are unknown.

We recently inactivated Sox5 and Sox6 in the mouse and revealed that the two genes have essential, redundant roles in chondrogenesis (Smits et al., 2001). Whereas Sox5+/– mice and Sox6+/– mice are born with minor cartilage defects, Sox5+/–/Sox6+/– fetuses develop a generalized and dramatic chondrodysplasia. Sox5+/–/Sox6+/– prechondrocytes develop normal prechondricle condensations, but their differentiation into chondroblasts is delayed and impaired. These cells express cartilage extracellular matrix genes at low or undetectable levels and poorly proliferate. They are unable to persist as a pool of proliferating chondroblasts in epiphyses and to establish cartilage growth plates in metaphyses. Instead, they precociously undergo an aberrant maturation into hypertrophic chondrocytes. By E16.5, when the fetuses die, cartilages are rudimentary and matrix deficient, and are starting to be invaded by bone-forming cells.

We show here that Sox5 and Sox6 are expressed in notochord cells and surrounding sclerome-derived cells, and that Sox5+/–/Sox6+/– embryos have severe defects in notochord development. Sox5 and Sox6 are dispensable for early inductive functions of the notochord, but indispensable for notochord sheath formation, cell survival and development into nuclei pulposi. Hence, Sox5 and Sox6 are required to direct the fate and differentiation of notochord cells and chondrocytes, two cell types with major roles in vertebral column formation.

**MATERIALS AND METHODS**

**Mouse mutants and embryo analysis**

The generation of Sox5/Sox6 mutant mice has been described (Smits et al., 2001). Mice were analyzed on the 129/SvEv × C57BL/6 genetic background, and skeletal phenotypes were fully penetrant. Sox5+/– and Sox6+/– mice were indistinguishable from wild-type littermates in all assays. Therefore, we refer to wild-type and single heterozygous mice as controls. Genotyping, skeletal preparations and histological analyses were performed as described (Smits et al., 2001). Unless otherwise indicated, embryo sections were stained with Alcian Blue and nuclear Fast Red. All experiments were repeated with two or more pairs of control and mutant littermates, and, unless otherwise indicated, figures show data from one representative experiment.

**RNA in situ hybridization**

RNA in situ hybridization was performed using 35S-labeled antisense probes (Smits et al., 2001). Pictures were taken using a red filter for RNA signals and under blue fluorescence for nuclei stained with the Hoechst 33258 dye. T and Car3 cDNA probes were generated by RT-PCR of ~500 bp of the 3′ untranslated sequences. The Fmod cDNA probe corresponded to 100 bp of the open reading frame and 200 bp of the 3′ untranslated sequence. The Col3a1 (Metsäranta et al., 1991), Laml1 (Laurie et al., 1989), Pax1 (Deutsch et al., 1988), Shh (Echelard et al., 1993) and other cDNA probes (Smits et al., 2001) were as described.

**Cell proliferation assay**

Pregnant mice were injected with 5-bromo-2′-deoxyuridine (BrdU) (Zymed Laboratories) (10 μl/g of mouse) and sacrificed 2 hours later. Embryos were fixed in 4% paraformaldehyde, dehydrated through a graded ethanol series and embedded in paraffin wax. BrdU-labeled DNA was detected in 7 μm sections using a BrdU immunostaining kit (Zymed laboratories). Sections were counterstained with Hematoxylin.

**TUNEL assay**

The Alkaline Phosphatase In Situ Cell Death Detection kit (Roche) was used according to the manufacturer’s instructions, with the following modifications. Before incubation with the terminal deoxynucleotidyl transferase (TdT), sections were blocked for 20 minutes with 0.3 mg/ml bovine serum albumin in phosphate buffered saline. The TdT enzyme was used at a 1:20 dilution and the converter alkaline phosphatase at a 1:2 dilution.
RESULTS

Sox5<sup>−/−</sup>/Sox6<sup>−/−</sup> embryos lack nuclei pulposi

Histological analysis of E15.5 Sox5<sup>−/−</sup>/Sox6<sup>−/−</sup> fetuses indicated that in addition to developing severe cartilage deficiencies, these fetuses were also failing to develop intervertebral discs (Fig. 1). In the thoracic region, which, together with the lumbar region, corresponds to the vertebral column segment that is the most developmentally advanced, vertebral bodies could be distinguished from intervertebral discs in control embryos (Fig. 1A). Vertebral bodies contained a core of hypertrophic chondrocytes, flanked with prehypertrophic cells and proliferating chondroblasts. Intervertebral discs contained a nucleus pulposus core surrounded with inner annulus cartilage and outer annulus mesenchyme. In Sox5<sup>−/−</sup>/Sox6<sup>−/−</sup> fetuses, intervertebral areas could be identified only by the presence of outer annulus. There were no nuclei pulposi, and inner annulus cartilage was indistinguishable from vertebral body cartilage. Both cartilages had a deficient extracellular matrix, and vertebral bodies featured no hypertrophic chondrocytes. Interestingly, small groups of condensed cells were present in vertebral body regions and at the junction with intervertebral regions, but never within intervertebral regions. Their aspect strongly suggested that these cells were remnants of the notochord (see Fig. 4). In the most developed (upper lumbar) region (Fig. 1B), these notochord cells were found only at the junction between vertebral and intervertebral spaces. They were often forming trains heading to the outer mesenchyme. Because a similar phenotype, with abnormal removal of the notochord and absence of nuclei pulposi, had not been described in mouse mutants, we embarked upon a detailed analysis.

Sox5 and Sox6 are co-expressed in the notochord, nucleus pulposus and cartilage

The expression pattern of Sox5 and Sox6 during vertebral column development was analyzed by RNA in situ hybridization of mouse embryo sections (Fig. 2). At E11.5, at the onset of vertebral column formation, Sox5 and Sox6 were both expressed in notochord cells and in sclerotome cells surrounding the notochord and neural tube. From E11.5 to E15.5, and until birth (data not shown), Sox5 and Sox6
remained expressed in notochord cells throughout differentiation into nucleus pulposus cells. They also remained expressed in sclerotome-derived prechondrocytes and chondroblasts in vertebral bodies and inner annuli, but not in outer annuli. At all stages, the signal for \textit{Sox5} RNA was slightly weaker in the notochord and nucleus pulposus than in surrounding tissues, whereas the signal for \textit{Sox6} RNA was similar in all these tissues.

**\textbf{Sox5 and Sox6 are required for notochord sheath formation}**

At E11.5, the notochord of both control and \textit{Sox5}\textsuperscript{−/−}/\textit{Sox6}\textsuperscript{−/−} embryos was a rod of condensed cells, with the mutant notochord generally containing fewer cells (Fig. 3A). Control embryos had started to deposit a notochord sheath, which stained with Alcian Blue, whereas sclerotome-derived cells had not yet accumulated any cartilage matrix. The notochord sheath was totally missing in mutant embryos. By E13.5, the control notochord sheath had become much thicker (Fig. 3B). It was evenly thick in vertebral and intervertebral areas, despite a strong difference in abundance of cartilage matrix between these two areas. In mutant littermates, vertebral bodies had started to deposit some cartilage matrix, but the notochord sheath was still undetectable. \textit{Sox5} and \textit{Sox6} thus control notochord sheath formation.

We tested the RNA levels for notochord sheath components
Sox5 and Sox6 control notochord development in E11.5 to E14.5 embryos (E13.5, Fig. 3C). As previously described (Smits et al., 2001), Sox5−/−/Sox6−/− chondroblasts expressed Col2a1 at a partially reduced level, and the genes for aggrecan (Agc1) and perlecan (Hspg2) at virtually undetectable levels. Expression of these genes was similarly reduced in Sox5−/−/Sox6−/− notochord cells. Control embryos expressed...
the gene for laminin γ1 (Lamc1) in notochord cells and intervertebral mesenchyme, but not in chondroblasts. Mutant notochord cells expressed Lamc1 at a similar or slightly reduced level. We also tested the expression level of genes for other basement membrane proteins, including collagen 4 (Col4a1) and nidogen, and for other mesenchymal and fibrous tissue proteins, including fibronectin, collagen 1 (Col1a1) and collagen 3 (Col3a1), which were detected by immunolocalization in the notochord sheath of mouse or rat embryos (Gotz et al., 1995; Hayes et al., 2001). However, these genes were not expressed in control notochord cells, and their expression in other cells was not affected by the Sox5+/Sox6+ mutation (data not shown).

We also tested whether the Sox5+/Sox6+ mutation affected expression of non-matrix genes in the notochord. We chose genes highly expressed in notochord cells, but not expressed in chondrocytes: Shh (Echelard et al., 1993), T (Wilkinson et al., 1990) and Car3 (carbonic anhydrase 3) (Lyons et al., 1991). Their RNA levels were normal in the mutant notochord (Fig. 3D).

Hence, Sox5 and Sox6 are not needed for the initial formation of the notochord, but are required for notochord sheath formation. They are required in notochord cells and chondrocytes to express genes for common components of the cartilage matrix and notochord sheath.

Sox5–/–/Sox6–/– notochord cells are removed from intervertebral spaces and vertebral bodies

From E13.5 to E15.5, the control notochord developed into nuclei pulposi, and sclerotome cells into vertebral body and inner annulus chondroblasts. Because these transformations started earlier in the thoracolumbar region than in the cervical and caudal regions, all steps could be visualized simultaneously in E14.5 embryos (Fig. 4A). In the distal tail, the notochord was still a continuous rod, intervertebral regions highly condensed, and vertebral bodies starting to accumulate some cartilage matrix. In the proximal tail, the notochord was starting to bulge in intervertebral regions. In the thoracic region and in the E15.5 lumbar region, notochord cells were completely removed from the vertebral bodies, but an acellular notochord sheath was still visible. Nuclei pulposi, vertebral body and inner annulus cartilage were fully developing.

During the same time period, the notochord of Sox5–/–/Sox6–/– embryos underwent an aberrant dismantlement. First, notochord cells disappeared from presumptive intervertebral areas, but not from vertebral bodies (Fig. 4A, top two panels). The vertebral bodies were underdeveloped, but the intervertebral mesenchyme was normally condensed. Next, notochord cells formed trains that seemed to be moving out of the vertebral column between vertebral and intervertebral spaces (Fig. 4A, bottom two panels). The vertebral bodies featured some cartilage matrix, but the intervertebral mesenchyme was still condensed. The hybridization of Sox5+/Sox6+ embryo sections with a Car3 RNA probe convincingly illustrated the disruption of the notochord and diversion of its cells (Fig. 4B). As shown earlier (Fig. 1B), Sox5+/Sox6+ notochord cells were removed from vertebral bodies by E15.5, thus with a delay of approximately 2 days compared with control cells, and never developed nuclei pulposi.

We also analyzed transverse sections to ascertain that the notochord interruptions seen in sagittal sections were not due to waviness of the notochord (Fig. 4C,D). At E12.5, the notochord was still visible and expressing Shh in the cervical and caudal intervertebral spaces in both control and Sox5+/Sox6+ embryos. In the thoracolumbar intervertebral spaces, notochord cells were identifiable in control embryos, but not in Sox5+/Sox6+ embryos. Sox5+/Sox6+ notochord cells were therefore vanishing from intervertebral regions.

Hence, Sox5 and Sox6 are needed to maintain the notochord in intervertebral regions and to remove the notochord from vertebral bodies in a timely manner.

Sox5 and Sox6 are needed for notochord cell survival

We tested whether the reduced numbers of Sox5+/Sox6+ notochord cells in E11.5 embryos, and the removal of notochord cells from intervertebral spaces in E12.5-E14.5 embryos resulted from abnormal cell proliferation or from cell death. As we have shown previously that Sox5 and Sox6 are required to promote chondroblast proliferation, we first tested notochord cell proliferation (Fig. 5A). In both control and mutant embryos, notochord cells were proliferating actively at E11.5, and less actively at E13.5. At both stages, mutant notochord cells were proliferating at a similar or higher rate than control cells. Thus, the loss of mutant notochord cells was not due to a decreased rate in cell proliferation.

Cell death was assessed by the TUNEL assay (Fig. 5B). At E11.5, few apoptotic cells were detected in control notochords, but many dying cells were seen in mutant notochords. Both control and mutant intervertebral mesenchymes featured dying cells, whereas only mutant vertebral mesenchymes featured some. At E13.5, control notochord cells were not dying, whereas Sox5+/Sox6+ notochord cells were undergoing massive apoptosis in prevertebral and intervertebral regions. Cells were not dying in control cartilage, but a few cells were still dying in Sox5+/Sox6+ intervertebral mesenchyme.

Hence, apoptosis explains the progressive loss of all Sox5+/Sox6+ notochord cells, but not the selective loss in intervertebral spaces.

Notochord disintegration occurred in embryos lacking T (Herrmann and Kispert, 1994) and Im5a (integrin-α5) (Goh et al., 1997). However, we found that Im5a was expressed in intervertebral cells, but not in notochord and prevertebral cells. Moreover, its expression was not affected by the Sox5+/Sox6+ mutation (data not shown). As shown above, T was expressed at normal levels in Sox5+/Sox6+ notochord cells (Fig. 3D). Therefore, the cause of apoptosis of Sox5+/Sox6+ notochord cells was not an altered expression of T or Im5a.

Sox5 and Sox6 promote inner annulus chondroblast differentiation

The exclusion of Sox5+/Sox6+ notochord cells from intervertebral spaces could result either from a notochord defect or from an intervertebral defect. In favor of the latter, histological analysis suggested that the Sox5+/Sox6+ intervertebral mesenchyme was impaired in its development (Fig. 4A). To test this hypothesis further, we analyzed expression of cell-specific markers.

Pax1 and Pax9 are indispensable for cell fate specification and differentiation of sclerotome cells. As expected (Deutsch...
et al., 1988), Pax1 was highly expressed in presumptive intervertebral regions in E11.5 wild-type embryos (Fig. 6A). It was correctly expressed in Sox5+/−/Sox6+/− sclerotome cells, indicating that these cells were correctly specified. From E11.5 to E13.5, control cells underwent chondrogenesis, and Pax1 expression became confined to narrow zones of intervertebral mesenchyme, to perichondrium and to outer annuli. By E15.5, it remained expressed only in outer annuli, whereas, in mutant embryos, it was still highly expressed in lower lumbar intervertebral areas.

By E15.5, the genes for the mesenchymal markers collagen 3 (Fig. 6B), collagen 1 and fibronectin (data not shown) were no longer expressed in control and mutant vertebral body chondroblasts and in control inner annulus chondroblasts. In the mutant intervertebral regions, they were being inactivated in the thoracic and higher lumbar regions (data not shown), but...
were still expressed in the lower lumbar region (Fig. 6B). The differentiation process of mutant inner annulus cells was thus severely delayed, but not blocked.

The RNAs for general cartilage markers, such as aggrecan (Fig. 6B) and link protein (data not shown), were highly expressed in the cartilages of control embryos, but almost undetectable in mutant intervertebral and vertebral cells. The RNA for fibromodulin, which is expressed at high levels in inner annulus chondroblasts, was undetectable in mutant intervertebral cells (Fig. 6B). This virtual absence of expression of general and inner annulus-specific chondroblast markers revealed that the cells were not only delayed, but also impaired in their differentiation process. These defects may therefore have contributed to remove notochord cells from intervertebral regions.

**Sox5 and Sox6 are both needed for notochord development**

To assess the relative contribution of Sox5 and Sox6 to notochord development, we analyzed compound mutants. At E12.5, the notochord sheath was normal in Sox5+/+/Sox6+/+ embryos, and slightly thinner in Sox5+/−/Sox6+/− and Sox5+/+/Sox6−/− embryos (Fig. 7A). It was significantly thinner in Sox5+/−/Sox6−/− embryos, and very thin in Sox5+/+/Sox6−/− embryos. Thus, Sox5 and Sox6 were both needed for notochord sheath formation, and Sox6 was slightly more important than Sox5.

Notochord cells started to bulge in intervertebral spaces from E13.5 in Sox5+/−/Sox6−/− embryos, as well as in control littersmates, but mutant cells started to be removed from vertebral bodies between E14.5 and E16.5 with a short delay relative to control cells (Fig. 7B). This delay coincided with a delay in cartilage development. By E16.5, control nuclei pulposi were fully developing, and its cells were swollen, whereas nuclei pulposi were underdeveloped in Sox5+/−/Sox6−/− embryos, and its cells were hardly swollen (Fig. 7B,C).

At birth, the nuclei pulposi of control mice appeared as swollen, translucent structures in skeletal preparations (Fig. 7D). Those of Sox5+/−/Sox6+/−, Sox5−/−/Sox6+/− and Sox5+/−/Sox6−/− mice were less expanded in width, but swollen and translucent. The nuclei pulposi of Sox5+/−/Sox6−/− and Sox5+/−/Sox6−/− mice were small, often fragmented and opaque. The cells in Sox5+/−/Sox6−/− nuclei pulposi were still incompletely swollen (Fig. 7E) and still expressing Shh (Fig. 5). Normal proliferation, but massive apoptosis of Sox5−/−/Sox6−/− notochord cells. (A) BrdU labeling of proliferating cells in E11.5 and E13.5 control and Sox5−/−/Sox6−/− embryos. Three pairs of littermates were tested per age (1-6). For each embryo, notochord cells were counted in five to nine mid-sagittal sections in the thoracolumbar region, totaling 300-1000 cells. The percentage of BrdU-positive cells is presented as the mean±s.d. for all sections. Representative pictures are shown underneath. BrdU-labeled cells are brown. At both stages, mutant notochord cells proliferate at a rate that is similar to or higher than that of control cells. (B) TUNEL assay in the thoracolumbar region of control and Sox5−/−/Sox6−/− embryos. At E11.5, few cells are dying in the control notochord (red, arrowheads), and many more in the mutant notochord. Cells are also dying in intervertebral mesenchyme (IM) in both embryos (arrows). At E13.5, no cells are dying in the control notochord, but massive apoptosis is occurring in the mutant notochord. No cells are dying in control cartilages, but few cells are dying in mutant intervertebral mesenchyme (arrow). VB, vertebral bodies.
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Fig. 6. Impaired development of Sox5+/−/Sox6−/− intervertebral mesenchyme. (A) In situ hybridization of Pax1 RNA in mid-sagittal sections of control and Sox5+/−/Sox6−/− embryos in the lower lumbar vertebral column. At E11.5, Pax1 is expressed in pre-intervertebral cell condensations in both embryos (arrowheads). At E12.5 and E13.5, Pax1 is expressed in control embryos in presumptive annuli (arrowheads) and perichondrium (arrows). These expression domains are wider at E12.5 than at E13.5 in the control, but similar in the mutant. At E15.5, Pax1 expression is restricted to outer annuli in control embryos (asterisk). In Sox5+/−/Sox6−/− embryos, Pax1 is still expressed in intervertebral mesenchyme (arrowheads). (B) In situ hybridization of RNAs for extracellular matrix proteins in mid-sagittal sections of the cervical region of E15.5 control and Sox5+/−/Sox6−/− embryos. Wild-type inner annulus cells (arrowheads) are differentiated chondroblasts: they are no longer expressing Col3a1, and are expressing Agc1 and Fmod. Sox5+/−/Sox6−/− intervertebral cells (arrowheads) are still exhibiting an undifferentiated mesenchymal phenotype: they are expressing Col3a1 at high levels, and Agc1 and Fmod at low or undetectable levels.

DISCUSSION

Previous studies on the notochord mostly focused on its formation, function and control in early organogenesis. This study has mainly focused on the notochord at later stages, when it directly contributes to vertebral column formation. We have shown that Sox5 and Sox6 are not necessary in the early notochord for the formation of a typical rod-like structure, the expression of the important regulatory T and Shh genes, and for fulfilling inductive functions. However, they are necessary at later stages for notochord sheath formation, cell survival and development of the nuclei pulposi.

Sox5 and Sox6 are required for notochord sheath formation

Many types of matrix molecules are present in the notochord sheath, but little is known about their function and gene regulation. The short tail (Ts) mouse lacks the notochord sheath (Center et al., 1988), but its genetic alteration is unknown.

The first genetic alterations causing abrogation of notochord sheath formation were identified in the laminin β1 and laminin γ1 genes in the zebrafish (Parsons et al., 2002). Lamc1-null mouse embryos die too early (E5.5) to study the role of laminin γ1 in notochord sheath formation in this species (Smyth et al., 1999), but crucial functions of laminin 1 have likely been conserved through evolution. We found that notochord cells express Lamc1 at the time of sheath formation, but Lamc1 expression is not or hardly affected by the Sox5+/−/Sox6−/− mutation, ruling out that Sox5 and Sox6 mediate notochord sheath formation by controlling Lamc1 expression.

Although the notochord sheath may contain mesenchymal, fibrous tissue and basement membranes components, the genes for such components were not expressed in wild-type notochord cells at the time of sheath formation, and those that were expressed in surrounding tissues were not affected by the Sox5+/−/Sox6−/− mutation. Hence, Sox5 and Sox6 must mediate notochord sheath formation by controlling other genes.

The notochord sheath also contains cartilage matrix components. The expression of genes for these components is partially or severely downregulated in Sox5+/−/Sox6−/−.
chondroblasts (Smits et al., 2001) and we found here that their expression is similarly affected in Sox5–/–/Sox6–/– notochord cells. Col2a1 expression is partially downregulated. However, as Col2a1–/– mouse embryos have a thick notochord sheath (Aszödi et al., 1998), the notochord phenotype of Sox5–/–/Sox6–/– embryos must be caused by the altered expression of more genes. The wild-type notochord sheath intensely stains with Alcian Blue, indicating that it contains sulfated glucosaminoglycans. Accordingly, expression of the genes for the sulfated proteoglycans aggrecan and perlecan is high in notochord and surrounding chondrocytic cells. It is virtually abrogated in Sox5–/–/Sox6–/– embryos. Mouse embryos that lack either gene develop a chondrodysplasia similar to that of Sox5–/–/Sox6–/– embryos, but it has not been described whether they have a similar notochord phenotype (Rittenhouse et al., 1978; Watanabe and Yamada, 1999; Arikawa-Hirasawa et al., 1999; Costell et al., 1999). Nevertheless, it is reasonable to propose that these proteoglycans have crucial roles both in cartilage and the notochord sheath, and that Sox5 and Sox6 mediate notochord...
sheath formation by controlling these genes and possibly other genes in notochord and surrounding cells.

The cellular origin of the notochord sheath is unknown. The phenotypic comparison of Sox5+/-Sox6+/- embryos and laminin 1-deficient zebrafish embryos strongly suggests that Sox5 and Sox6 mediate notochord cell survival essentially through their roles in notochord sheath formation. The primary defect of both mutants is the lack of notochord sheath, and it is directly followed by notochord cell apoptosis upon sclerotome cell condensation. The sheath may act on notochord cells in one or several ways. It may provide vital cell-matrix interactions, store essential growth factors or establish a barrier against death signals or mechanical pressure from surrounding tissues.

Sox5 and Sox6 are required for proper relocation of notochord cells

Sox5+/-Sox6-/- notochord cells are aberrantly removed from intervertebral areas, while their rates of apoptosis and proliferation are not different between vertebral and intervertebral regions, and mutant sclerotome cells are normally condensed and specified. We therefore propose that Sox5+/-Sox6-/- notochord cells may be removed from intervertebral regions because of their specific lack of notochord sheath. Sox5+/-Sox6-/- embryos remove notochord cells from vertebral bodies later than wild-type embryos. Previous studies have suggested that the wild-type notochord is removed upon swelling pressure from the vertebral body cartilage matrix. The


deficient development of the notochord in Sox5/Sox6 compound mutants. (A) Histological analysis of notochord sheath formation in E12.5 embryos with all combinations of Sox5 and Sox6 wild-type and mutant alleles. The notochord sheath (arrow) becomes thinner as the number of mutant alleles increases. The mutation of Sox6 is more detrimental than that of Sox5. (B) Histological analysis of nucleus pulposus formation in control and Sox5+/-Sox6+/+ littersmates. Mid-sagittal sections were photographed in the thoracic region. At E13.5, the notochord is starting to bulge in intervertebral spaces in both embryos (arrows). By E14.5, notochord cells have been completely removed from vertebral bodies (VB) in the control embryo, but incompletely removed in the mutant (arrow). The formation of cartilage matrix is delayed in E13.5 and E14.5 mutant embryos. By E16.5, the notochord has completed its transformation into nuclei pulposi in both embryos. The notochord sheath (arrow) is still visible in the mutant vertebral bodies. The mutant nuclei pulposi are smaller, eccentric and often fragmented. IA, inner annulus; IM, intervertebral mesenchyme; NP, nucleus pulposus. (C) High-magnification pictures of nuclei pulposi in E16.5 embryos. Control cells are highly swollen, as indicated by a large volume of cytoplasm (white) per cell nucleus (red). Sox5+/Sox6+/- cells are incompletely swollen and Sox5+/-Sox6-/- cells hardly swollen. (D) Skeletal preparations of the vertebral column of newborn mice (P0) with various combinations of Sox5 and Sox6 null alleles. Non-mineralized cartilages and nuclei pulposi are stained with Alcian Blue, and mineralized cartilages and bones with Alizarin Red. Pictures show the L1 lumbar vertebra and flanking intervertebral discs. The nuclei pulposi (arrows) of Sox5+/-Sox6+/- and Sox5+/-Sox6+/- mice are less expanded in width than those of the control (Sox5+/Sox6+/+) mouse. The nuclei pulposi of Sox5+/-Sox6-/- and Sox5+/Sox6-/- mice are severely reduced in both width and height, and opaque instead of translucent. (E) Histological analysis of nuclei pulposi in control (Sox5+/Sox6+/+) and Sox5+/Sox6-/- newborn littersmates. Mutant cells are less swollen than control cells. (F) In situ hybridization of Shh RNA (top) and histological analysis (bottom) of sagittal sections of control (Sox5+/Sox6+/+) and Sox5+/Sox6-/- newborn littersmates. Pictures show a lumbar intervertebral disc and segments of flanking vertebrae. Nucleus pulposus cells (arrows) are still expressing Shh in the mutant, but not in the control. Hypertrophic chondrocytes (arrowheads) are expressing Shh in both mice. (G) In situ hybridization of Shh RNA in cross-sections of wild-type embryos. Pictures show a presumptive intervertebral disc region and the ventral part of the neural tube. Shh is expressed in E13.5 and E14.5 notochord cells (arrows), but no longer in E15.5 nucleus pulposus cells (arrow). At all three stages, Shh is expressed in the neural tube floor plate (arrowheads). (H) Picture of 1-week-old (P7) control and Sox6+/- littersmates. The Sox6+/- mouse is developmentally delayed and exhibits a kinked tail (arrow). (I) Histological analysis of longitudinal sections through the tail of 4-day-old (P4) control and Sox6-/- mice. Control nuclei pulposi (arrows) occupy the core region of intervertebral spaces. Mutant nuclei pulposi are small and located eccentrically, where the tail forms kinks.

Sox5 and Sox6 control notochord development

Sox5+/-Sox6-/- newborn littermates. By E16.5, the notochord has completed its transformation into nuclei pulposi (arrows) of L1 lumbar vertebra and flanking intervertebral discs. The nuclei pulposi are stained with Alcian Blue, and mineralized cartilages and bones with Alizarin Red. Pictures show the L1 lumbar vertebra and flanking intervertebral discs. The nuclei pulposi (arrows) of Sox5+/-Sox6+/- and Sox5+/-Sox6+/- mice are less expanded in width than those of the control (Sox5+/Sox6+/+) mouse. The nuclei pulposi of Sox5+/-Sox6-/- and Sox5+/Sox6-/- mice are severely reduced in both width and height, and opaque instead of translucent. (E) Histological analysis of nuclei pulposi in control (Sox5+/Sox6+/+) and Sox5+/Sox6-/- newborn littersmates. Mutant cells are less swollen than control cells. (F) In situ hybridization of Shh RNA (top) and histological analysis (bottom) of sagittal sections of control (Sox5+/Sox6+/+) and Sox5+/Sox6-/- newborn littersmates. Pictures show a lumbar intervertebral disc and segments of flanking vertebrae. Nucleus pulposus cells (arrows) are still expressing Shh in the mutant, but not in the control. Hypertrophic chondrocytes (arrowheads) are expressing Shh in both mice. (G) In situ hybridization of Shh RNA in cross-sections of wild-type embryos. Pictures show a presumptive intervertebral disc region and the ventral part of the neural tube. Shh is expressed in E13.5 and E14.5 notochord cells (arrows), but no longer in E15.5 nucleus pulposus cells (arrow). At all three stages, Shh is expressed in the neural tube floor plate (arrowheads). (H) Picture of 1-week-old (P7) control and Sox6+/- littersmates. The Sox6+/- mouse is developmentally delayed and exhibits a kinked tail (arrow). (I) Histological analysis of longitudinal sections through the tail of 4-day-old (P4) control and Sox6-/- mice. Control nuclei pulposi (arrows) occupy the core region of intervertebral spaces. Mutant nuclei pulposi are small and located eccentrically, where the tail forms kinks.

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Sox5+/-Sox6-/- embryos remove notochord cells from vertebral bodies later than wild-type embryos. Previous studies have suggested that the wild-type notochord is removed upon swelling pressure from the vertebral body cartilage matrix. The...
notochord persists in the cartilage-deficient Col2a1−/− vertebral bodies (Aszódi et al., 1998), and in the undifferentiated Bapx-1−/− vertebral bodies (Lettice et al., 1999; Tribioli and Lufkin, 1999; Akazawa et al., 2000). The delay in removing Sox5+/−/Sox6+/− notochord cells from vertebral bodies must result from the delay in cartilage development. It may also be facilitated by the lack of notochord sheath and apoptosis of notochord cells.

Wild-type notochord cells are believed to take refuge in intervertebral areas upon removal from vertebral bodies, but Sox5−/−/Sox6−/− notochord cells remain excluded from these areas and relocate between vertebral bodies and intervertebral areas. The repositioning of the cells, although ectopic, supports the relocation model proposed for wild-type notochord cells, and indicates that Sox5 and Sox6 are needed to maintain and relocate notochord cells in intervertebral areas.

**Sox5 and Sox6 promote nucleus pulposus cell differentiation**

Little is known about the genetic program of nucleus pulposus cells. They continue to express Sox5, Sox6 and T, but not Shh. They undergo hypertrophy, but do not activate the hypertrophic chondrocyte marker Col10a1 (data not shown). Their genetic program thus only partially overlaps with that of chondrocytes. Sox5+/−/Sox6−/− notochord cells die before developing nuclei pulposi, but the nuclei pulposi of Sox5−/−/Sox6−/− fetuses are severely impaired in their development. They remain rudimentary and their cells swell poorly and maintain Shh expression. Sox5 and Sox6 are the first genes for transcription factors shown to be expressed in these cells and needed for their differentiation.

Hypertrophic chondrocyte differentiation is also impaired in Sox5−/−/Sox6−/− embryos (Smits et al., 2001). Chondrocytes activate Cbfal and VEGF, but weakly express Col10a1, maintain Sox9 expression and poorly enlarge. These defects must be secondary consequences of the Sox5+/−/Sox6−/− mutation because Sox5 and Sox6 are no longer expressed in hypertrophic chondrocytes. Similarly, some of the defects of Sox5−/−/Sox6−/− nuclei pulposi may be secondary to defects in the earlier notochord, or in surrounding inner annuli.

**Sox5 and Sox6 promote inner annulus cell differentiation**

The vertebral column of Sox5−/−/Sox6−/− fetuses is mostly unsegmented by E15.5, even though the earlier vertebral column is properly patterned, with sclerotome cells condensing correctly and expressing Pax1. As previously described, the development of vertebral body cartilage is delayed and impaired (Smits et al., 2001) such that, by E15.5, it features no hypertrophic chondrocytes yet, and no growth plates. We have shown here that the differentiation process of inner annulus cartilage is similarly delayed and impaired, such that both cartilages were indistinguishable by E15.5. Moreover, mutant inner annulus cells do not activate Fmod, an abundant marker of both inner annulus and periarticular chondroblasts. We previously showed that periarticular chondroblasts in Sox5+/−/Sox6−/− limb cartilages also fail to activate Fmod (Smits et al., 2001). Therefore, the impaired differentiation of Sox5−/−/Sox6−/− inner annulus cells is a cell-autonomous defect, not a consequence of notochord defects.

**Sox5 and Sox6 have redundant roles in notochord development**

Sox5+/− and Sox6+/− embryos have limited notochord defects compared with Sox5+/−/Sox6−/− embryos, indicating that Sox5 and Sox6 have mostly redundant functions in notochord development, as in chondrogenesis (Smits et al., 2001). Nevertheless, the analysis of compound mutants has revealed that Sox6 is slightly more important than Sox5 for both notochord sheath formation and nucleus pulposus development. This functional difference between the two genes probably reflects the fact that Sox6 is expressed, relatively to Sox5, at a slightly higher level in notochord cells than in surrounding chondrocytes. This conclusion is consistent with the observation that L-Sox5 and Sox6 have indistinguishable DNA binding and transactivation activities in vitro (Lefebvre et al., 1998).

**Sox5 and Sox6 have critical roles in vertebral column formation**

This study and our previous study (Smits et al., 2001) have identified critical roles for Sox5 and Sox6 in the transcriptional control of notochord cells and chondrocytes (Fig. 8).

The differentiation of early notochord cells does not require Sox5 and Sox6, but requires T. Shh and noggin, secreted by notochord cells, contribute to induce sclerotome cell differentiation into prechondrocytes, Bapx1, Pax1, Pax9 and Sox9 act downstream of Shh at this step in the vertebral column.

Both pathways crucially rely on Sox5 and Sox6 at the next step of cell differentiation. Sox5 and Sox6 promote expression of extracellular matrix genes, and are thereby required for notochord sheath and cartilage matrix formation. Sox5 and Sox6 are required for proper proliferation of chondroblasts, but

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Fig. 8. Sox5 and Sox6 control essential steps in the notochord and chondrocyte differentiation pathways. Chondrocytes and notochord cells progress through four major differentiation steps during development. Major transcription factors known to control one or several of these steps are indicated. Biochemical (Shh and noggin) and physical (mechanical pressure) interactions occur between the two lineages at several steps. See the end of Discussion for details.
not notochord cells. By contrast, they are needed for the survival of notochord cells, but not chondroblasts. Jun also is needed for notochord cell survival, and Sox9 for chondroblast differentiation.

Later, mechanical pressure from cartilage likely induces notochord cell relocation from vertebral bodies into intervertebral areas. Sox5 and Sox6 promote notochord cell differentiation into nucleus pulposus cells. By contrast, Sox5 and Sox6 prevent chondroblast differentiation into hypertrophic chondrocytes. Runx2 may promote hypertrophic chondrocyte maturation in vertebral (Inada et al., 1999; Kim et al., 1999) and Sox9 may prevent chondrocyte hypertrophy (Bi et al., 2001).

In conclusion, Sox5 and Sox6 have essential functions in the development of cartilage, the notochord and nuclei pulposi. They are thereby critical transcriptional regulators of vertebral column scaffold formation.

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