Lasp1 misexpression influences chondrocyte differentiation in the vertebral column

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ABSTRACT  The mouse mutant wavy tail Tg(Col1a1-lacZ)304ng was created through transgene insertion and exhibits defects of the vertebral column. Homozygous mutant animals have compressed tail vertebrae and wedge-shaped intervertebral discs, resulting in a meandering tail. Delayed closure of lumbar neural arches and lack of processus spinosi have been observed; these defects become most prominent during the transition from cartilage to bone. The spina bifida was resistant to folic acid treatment, while retinoic acid administration caused severe skeletal defects in the mutant, but none in wild type control animals. The transgene integrated at chromosome 11 band D, in an area of high gene density. The insertion site was located between the transcription start sites of the Rpl23 and Lasp1 genes. LASP1 (an actin binding protein involved in cell migration and survival) was found to be produced in resting and hypertrophic chondrocytes in the vertebrae. In mutant vertebrae, temporal and spatial misexpression of Lasp1 was observed, indicating that alterations in Lasp1 transcription are most likely responsible for the observed phenotype. These data reveal a yet unappreciated role of Lasp1 in chondrocyte differentiation during cartilage to bone transition.

KEY WORDS: transgene insertion, cartilage bone transition, folic acid, retinoic acid, collagen

Introduction

Malformations of the vertebral column, like scoliosis, skyphosis, or spina bifida, are frequent congenital disorders in human. However, the precise genetic basis underlying these defects is known only in surprisingly few instances, and many disorders are multifactorial, involving either multiple genes or gene/environmental interactions (Helwig et al., 1995). The mouse provides a useful model for human congenital defects, in particular of the skeleton. Over 190 spontaneous mouse mutants exhibiting a skeletal phenotype are known (reviewed in Kibar et al., 2007; Searle et al., 1989), and the responsible genes have been identified in a number of them. Targeted mutation of specific genes facilitated the study of their function in distinct developmental processes, including bone and skeletal development. Yet, this approach does not find and identify genes previously not suspected to play a role in skeletogenesis.

Insertional mutagenesis results from the often random integration of foreign DNA sequence (e.g. a retrovirus or a transgene) within or near a gene. We previously reported (Kleiter et al., 2002) that the wavy tail Tg(Col1a1-lacZ)304ng mouse line was produced by pronuclear injection of a 15kb reporter gene construct in which the Col1a1 promoter was fused to a LacZ reporter gene. Beta galactosidase staining faithfully reflected endogenous Col1a1 transcription in transgenic animals (Ghaffari-Tabrizi N., in preparation). No other transgenic mouse line carrying this construct showed any skeletal abnormalities, indicating that transgenic expression itself did not account for the skeletal deformations observed, suggesting instead that the insertion had disrupted or influenced the expression of an endogenous gene. Isolation of the

Abbreviations used in this paper: Col, collagen; ECM, extracellular matrix; Lasp, Lim and SH3 protein; RA, retinoic acid.

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insertion site has been reported before (Kleiter et al., 2000), the transgene locus mapped on chromosome 11 close to the Hoxb cluster and Erbb2, where a 185 kb region including the insertion site was isolated and searched for transcribed regions. 6 genes were identified: Lasp1, Rpl23, Mllt6, Pip5k2b, Psmb3, Zfp144 (Kleiter et al., 2002). The insertion site was located between the transcription start sites of Lasp1 and Rpl23, with the Lasp1 transcription start site situated just 6,785 bp from the insertion site. Previous reports had demonstrated that neither Rpl23 expression nor the expression of the other genes Mllt6, Pip5k2b, Psmb3, Zfp144 was perturbed in transgenic animals (Kleiter et al., 2000).

The LASP1 protein (for LIM and SH3 protein 1) consists of a LIM, an SH3 domain and 2 actin binding modules. The protein was first identified in a screen for genes amplified in breast cancer (Tomasetto et al., 1995). Northern blot analysis showed that Lasp1 mRNA was transcribed in various tissues (e.g. colon, intestine, stomach, kidney, brain (Schreiber et al., 1998a)), with its expression being restricted to specific cell types in the organs investigated (e.g. gastric parietal cells within the stomach (Chew et al., 1998). The actin binding domain mediates interaction between LASP1 and actin at cell membrane extensions (Chew et al., 1998; Nakagawa et al., 2006; Schreiber et al., 1998b) and is necessary for cell migration and survival in response to growth factors and extracellular matrix (ECM) proteins (Lin et al., 2004).

So far, expression of Lasp1 has not been reported in skeletal tissues. Here we report that Lasp1 transcripts were detected in resting and hypertrophic chondrocytes during skeletal development in the wild type. Wavy tail Tg(Col1a1-lacZ)304ng mutant mice had aberrant Lasp1 expression in proliferating chondrocytes and the periosteum. Our results suggest that misexpression of Lasp1 is the most likely cause for the mutant defects. These data reveal a yet unappreciated role of Lasp1 in chondrocyte differentiation during cartilage to bone transition.

**Results**

Phenotypic characterization of the insertional mutant Tg(Col1a1-lacZ)304ng (wavy tail)

Only one of several transgenic lines created with the same DNA construct (see Methods) exhibited developmental abnormalities, and only when bred to homozygosity. (Heterozygous carriers remained asymptomatic). Even homozygous animals appeared entirely normal at birth and accordingly, no deviations from the normal phenotype were detectable in embryos or fetuses. The first signs of a skeletal defect became apparent at the end of the first postnatal week. Homozygous mutant animals had multiple bends of the tail but not kinks. This deformation was most pronounced at ~15 days after birth (Fig. 1). Transgenic mice were fertile and viable. The phenotype was observed in 100% of all mutant animals and was maintained upon breeding the mutation into a different mouse strain (C57Bl/6).

Longitudinal histological sections of 10 day (P10) mutant tails showed vertebral bodies of normal thickness but reduced length (Fig. 1 A,B). At P10 growth plates appeared compressed and disrupted, mainly due to thinner zones of proliferating and hypertrophic cartilage; the wavy tail phenotype combines a reduction in DNA synthesis of mutant chondrocytes. Quantification of BrdU positive cells in serial sections of the same level of P10 tail vertebrae revealed a significantly reduced number of proliferating cells in mutant (H) compared to wild type (G) tail vertebrae and intervertebral discs. Alizarin red alcian blue staining of the 3rd lumbar vertebra of P10 wild type (I) and mutant animals (J); mutant neural arches failed to close in time and the processus spinosi were missing in the lumbar vertebral column at this stage. Abbreviations: hz, hypertrophic zone; pz, proliferating zone; rz, resting zone.

Fig. 1. Wavy tail Tg(Col1a1-lacT)304ng mutants have compressed tail vertebrae and wedge-shaped intervertebral discs. Longitudinal sections through wild type (A) and Tg(Col1a1-lacZ)304ng (B) tail of P10 animals showing scoliosis of the tail and decreased size of tail vertebrae. P10 tail vertebrae of wild type (C) and mutant (D) P10 animals stained with Alcian blue. Wavy tail mutants have reduced size and alterations in the shape of the tail vertebrae (Fig. 1 C-F). As determined by BrdU incorporation, the number of proliferating cells in serial sections of the same level of P10 tail vertebrae revealed a significantly reduced number of proliferating cells in mutant (H) compared to wild type (G) tail vertebrae and intervertebral discs. Alizarin red alcian blue staining of the 3rd lumbar vertebra of P10 wild type (I) and mutant animals (J); mutant neural arches failed to close in time and the processus spinosi were missing in the lumbar vertebral column at this stage. Abbreviations: hz, hypertrophic zone; pz, proliferating zone; rz, resting zone.
cells was significantly reduced in mutant growth plates (Fig. 1 G,H). Proliferating cells were determined by counting BrdU positive cells in longitudinal serial epiphyseal sections of three different wild type and mutant P10 tail vertebrae n(wt) = 30; mean value 44.8 ± 6.3 SD; n(wavy tail) = 27; mean value 32.7 ± 11.5 SD (df = 55 two sample t-test p = 0.00006).

The reduced proliferation resulted in the formation of considerably shorter (reduced to 2/3) tail vertebrae compared to wild type controls, but vertebrae were still straight. (Fig. 1 E,F). The intervertebral discs were also shorter and, most conspicuously, were grossly deformed and wedge-shaped. In horizontal sections, only every third disc was found to be symmetric, whereas the others were strongly deformed with regular alteration of the orientation of the «wedge», producing the characteristic multiple smooth bends of the mutant tail without causing kinks. Quantification of BrdU positive cells in serial sections of the same level of P10 tail vertebrae demonstrated a significantly reduced number of proliferating cells in mutant intervertebral discs n(wt) = 15; mean value 15.6 ± 4.3 SD; n(wavy tail) = 12; mean value 10.6 ± 4.2 SD p=0.006, df=25 (Fig. 1 G,H). No defects in long bone and skull morphology were observed, thickness and length of these bones seemed unaffected in homozygous transgenic animals (data not shown).

Alcian blue and alizarin red skeletal staining demonstrated delayed closure of neural arches in the lumbar region. In the wild type, neural arches closed around P10, the most dorsal part and the processus spinosi still being cartilaginous; in contrast, the neural arches of mutant animals were still open and no processus spinosi were present at the same stage (Fig.1 I,J). Eventually, closure of the neural arches was completed in mutants at P14, the animals thus exhibiting only temporarily the symptoms of spina bifida occulta. In contrast to the full penetrance of tail scoliosis, delayed closure of the neural arches in the lumbar vertebrae was observed in roughly 50% of mutant animals. No alteration in the proliferation rate of chondrocytes in dorsal neural arches was observed by BrdU incorporation and staining of histological sections in P10 and P14 old animals (data not shown).

**Lasp1 expression is altered in wavy tail Tg(Col1a1-lacZ)304ng mutant mice**

The location of the transgene insertion site in close vicinity to *Lasp1* and the presence of conserved genomic sequence in this

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**Fig. 2. Transgene arrangement and chromosomal location site.**

Chromosomal location of the transgene insertion site (A). The insertion site was mapped between the *Col1a1* and the *Wnt3* locus, it always cosegregated with *Zfp144* on chromosome 11 band D (Kleiter et al., 2000). A screen for transcribed regions showed that six genes (*Zfp144-Psmb3-Pip5k2b-Mllt6-Rpl23-Lasp1*) are located in a 200kb area surrounding the insertion site (Kleiter et al., 2002). LacZ staining of transgenic tail vertebrae (B). P10 mutant tail vertebrae show beta-galactosidase staining in bone and periosteum in blue. The distribution is similar to that observed for endogenous *Col1a1* transcription. A schematic drawing illustrates location and arrangement of the transgene. The insertion is localized 6,785kb upstream of the *Lasp1* transcription start. A 3.5kb fragment of the *Col1a1* upstream regulatory region inserted next to *Lasp1*, this part of the upstream (~5.3 to –8.7) regulatory region was oriented in the same transcriptional direction as the *Lasp1* gene. The adjacent part of the first intron of *Col1a1* is located in the same transcriptional orientation. A complete copy of the transgene consisting of the *Col1a1* upstream region (~8.3 to 0 kb) exon1 and intron1 fused to nls Lacz was located in the opposite transcriptional direction. (C) VISTA Global Alignments of the mouse with rat and human *Lasp1* loci. VISTA plot showing sequence similarity in pairwise sequence alignments between the mouse, rat and human sequences, respectively: Peaks are shown relative to their position in the reference mouse sequence (horizontal axis) and percent identity (50–100%) is shown on the vertical axis. Color code is predominantly pink (non coding sequence), exons are illustrated in blue. Several conserved regions (mouse to rat) are located in the area of the transgene insertion, suggesting the presence of transcriptional control elements; the arrow indicates the transgene insertion site. The arrowhead denotes transcriptional orientation of the *Lasp1* gene.
area (Fig. 2) suggested \textit{Lasp1} as the most likely candidate gene affected in this insertional mutant. In addition, expression of the other genes close to the insertion site was not altered (Kleiter et al., 2002).

We examined the expression of \textit{Lasp1} during skeleton formation. \textit{Lasp1} transcripts were present in forming vertebrae as early as E14.5 and strong expression was detected in E17.5 hypertrophic chondrocytes (data not shown). \textit{Lasp1} was also expressed in resting and hypertrophic chondrocytes in E18.5 femur (Fig. 3 G,H).

In P7 and P14 wild type tail vertebrae, \textit{Lasp1} mRNA was detected in hypertrophic and resting chondrocytes of the growth plate, while no expression was found in proliferative cells (Fig. 3 A,B,E). Interestingly, we found an altered expression pattern in mutant vertebral growth plate tissue: at P7 no \textit{Lasp1} transcripts were detected in hypertrophic chondrocytes, and only sporadic expression was observed in individual cells throughout all zones of the growth plate (Fig. 3B). Then at P14, \textit{Lasp1} was transcribed in cells of all stages of chondrocyte differentiation (resting, proliferative and hypertrophic) in mutant tail vertebrae (Fig. 3 D,F). In addition, ectopic expression was observed in the periosteum of tail vertebrae (Fig. 3D).

A characteristic feature of the \textit{wavy tail} mutant is a temporary \textit{spina bifida occulta} in the lumbar region. \textit{Lasp1} expression was therefore examined in lumbar vertebrae at P8. Transcripts were found in resting and hypertrophic chondrocytes in a pattern that was not obviously different between wild type and mutant vertebrae (data not shown). In addition, ectopic expression was found in the periosteum of the mutant.

\textbf{Mutant animals are sensitive to low doses of retinoic acid and are folate resistant}

Retinoic acid (RA) is known to strongly affect the development of the vertebral column (Kessel and Gruss, 1991; Lohnes et al., 1993). Moreover, administration of low doses of retinoic acid can reduce the incidence of neural tube defects (Chen et al., 1994; Seller and Perkins, 1982). Remarkably, \textit{wavy tail} mutant animals exhibited increased sensitivity to RA. Low doses (10 mg/kg body weight) applied at day 9 of gestation caused perinatal death in all (17) animals born, but not in control animals which appeared asymptomatic (11). In contrast to the wild type situation, ossification centers of the cervical vertebrae were absent in 50% of the transgenic mice treated (Fig. 4F). In addition, the 6th lumbal vertebra was missing in 8 mutant animals (Fig. 4D). Furthermore malformations in the sternum were detected, as all RA treated neonates exhibited a widened and split xiphoid process, and the formation of a «crankshaft sternum» (asymmetrically attached ribs) was observed occasionally (Fig. 4 A,B). Unfortunately, due to the early death of the animals (defects in breathing caused by formation of a cleft palate), effects of RA on neural arch development in the lumbar region could not be further studied in RA-treated mice.

Clinical trials in humans have shown that maternal folic acid supplements prior to and during early pregnancy can prevent both the occurrence and the recurrence of neural tube defects (NTD). Nevertheless the mechanism by which folic acid prevents NTD is still poorly understood. We investigated the effect of folic acid administration in the \textit{wavy tail} mutant. The 50% incidence of \textit{spina bifida} was not changed in treated animals (data not shown) indicating that our mutant is folate-resistant.

\textbf{Aberrant expression of collagen genes and \textit{Pax1} in \textit{wavy tail} \textit{Tg(Col1a1-lacZ)304ng} mutant animals}

To further characterize the defective development of mutant vertebral growth plate, we analyzed the expression of skeletal
marker genes. Collagens are components of the extracellular matrix (ECM) of cartilage and bone. Type II collagen is the major and the characteristic collagen of cartilage and is expressed in all chondrocytes. In situ hybridization (ISH) for Col2a1 performed on P14 tail vertebrae showed that in the wild type, all chondrocytes of the growth plate expressed Col2a1 (Fig. 5A). In the resting zone of mutant animals, sporadic cells failed to express Col2a1, while in the proliferative zone, whole chondrons were devoid of Col2a1 transcripts (30% of proliferating cells lacked Col2a1 transcripts, Fig. 5B). In most cases all cells of an individual chondron failed to express Col2a1, suggesting that these cells had arisen from a single non-expressing cell in the resting zone.

Collagen type X is a marker for terminally differentiating chondrocytes of the prehypertrophic and hypertrophic zones in the growth plate (Kong et al., 1993). Prehypertrophic chondrocytes produce collagen X, but do not secrete the protein. Once the cells become hypertrophic, type X collagen is deposited into the ECM. By immunohistochemistry we found that collagen type X is produced both in wild type and mutant tail vertebrae (Fig. 5 C,D); mutant hypertrophic chondrocytes, however, failed to secrete the protein (Fig. 5F), suggesting a defect in chondrocyte terminal differentiation.

Collagen type I is the major constituent of bone ECM while it is absent in (most) cartilage. It is therefore a marker of beginning osteogenesis. P14 wildtype and mutant vertebrae were analyzed for transcripts of Col1a1, which is expressed in osteoblasts, the perichondrium and the periosteum. In some mutant vertebrae expression of Col1a1 was specifically reduced in osteoblasts (Fig. 5H), whereas its transcription appeared to be unaffected in the perichondrium and the periosteum. Expression of the non-collagen chondrocyte markers Ihh, PTHrP and Bmp6 showed no differences between wild type and mutant tail vertebrae (data not shown).

The murine paired box-containing gene Pax1 is required for normal development of the vertebral column, the sternum, and the scapula (Balling et al., 1992). Expression of Pax1 was examined

![Fig. 4](image-url)  
**Fig. 4 (Left). Effect of retinoic acid (RA) on skeletal development of wild type and Tg(Col1a1-lacZ)304ng mutant neonates.** Analysis of skeletal elements of wild type and Tg(Col1a1-lacZ)304ng mutant neonates treated with 10 mg/kg RA at E9.5. Whole mount skeletons were prepared from wild type (A,C,E) and mutant (B,D,F) neonates. Ventral view of the sternum shows symmetric fusion of the sternbrae ossification centers in wild type (A) sternum. In contrast, mutant neonates (B) exhibit shifted fusion of the ossification centers resulting in a crankshaft sternum. Dorsal view of the lumbar vertebral column: 6 lumbar vertebrae in wild type (C) but only 5 vertebrae in mutant (D) animals. Dorsal view of the cervical vertebrae ossification centers in the wild type (E), these centers are missing in mutant neonates (F).

![Fig. 5](image-url)  
**Fig. 5 (Right). Chondrocyte marker gene expression in P 14 wild type and Tg(Col1a1-lacZ)304ng mutant tail vertebrae.** Analysis of chondrocyte marker gene expression using in situ hybridization and immunohistochemistry on P14 wild type and Tg(Col1a1-lacZ)304ng mutant tail vertebral sections. Wild type chondrocytes (A) express Col2a1 throughout the growth plate. In mutant growth plates (B) Col2a1 expression is lost in single cells and chondrons respectively. COLX protein is expressed and secreted in wild type hypertrophic chondrocytes (C,E). Mutant hypertrophic chondrocytes produce COLX (D), but fail to secrete it (F). Col1a1 transcripts are detected in wild type osteoblasts, periosteum, and perichondrium (G), mutant samples exhibited a loss of expression selectively in osteoblasts (H), whereas transcription in the perichondrium seems to be unaffected.
Severe defects in undulated+/wavy tail Tg(Col1a1-lacZ)304ng double mutants. Skeletal structures of Tg(Col1a1-lacZ)304ng/Tg(Col1a1-lacZ)304ng, un+/-un+/-, and un+/-un+/- Tg(Col1a1-lacZ)304ng compound mutants. The tail vertebral column of wavy tail mutants (A) presents with scoliosis, the tail is bent but, in contrast to the un+/-un+/- mutant (C), has no kinks. Compound mutant tails (E) develop kinks, bends and a strong upbending of the vertebral column immediately after the sacral region. Ventral view of the lumbar vertebral column showing a structure similar to that of wild type vertebral bodies in the wavy tail mutant (B). un+/-un+/- mutants (D) have a slightly compressed vertebral column. Double mutant animals (F) show stronger compression of the vertebrae, split vertebral bodies and a thickened ribjoint. Analysis of Pax1 expression using in situ hybridization. E18 wild type tail vertebrae (G) expressed Pax1 in the condensed cartilage of the vertebrae, the intervertebral discs, and the tissue surrounding the intervertebral discs. In mutant vertebrae (H) expression was weaker and completely missing in the intervertebral discs.

by in situ hybridization. At E10.5 whole-mount in situ hybridization revealed no differences between wildtype and mutant embryos (data not shown). However, at E18.5, when Pax1 expression was found in vertebral chondrocytes and in intervertebral discs of wild type samples (Fig. 6G), no Pax1 transcripts were detected in intervertebral discs of wavy tail mutant animals (Fig. 6H), suggesting either a direct interaction with the Pax1 gene, or absence/delayed maturation of intervertebral discs.

Severe defects in undulated+/wavy tail Tg(Col1a1-lacZ)304ng double mutant animals

Previous studies have shown that three natural Pax1 (undulated) mouse mutants displayed phenotypes of different severity in axial skeletal elements (Balling et al., 1992). Wavy tail/Tg(Col1a1-lacZ)304ng mutants showed similar defects. To explore a possible genetic interaction, double mutant mice with undulated-extensive(un+/-) were generated. un+/- homozygous animals have skeletal defects in the lumbal region and the tail ((Dietrich and Gruss, 1995; Wallin et al., 1994); Fig. 6 C,D). At birth, double mutant animals showed a similar phenotype to un+/-ur+/. However, during the first week of postnatal development, i.e. when the phenotype of our mutant first becomes apparent, tails of double mutant mice sharply bent upwards immediately after the last sacral vertebra, forming a hair pin turn-like structure (Fig. 6E). Deformation of the tail was stronger than in each single mutant (Fig. 6 E,F). In addition, compressed lumbar vertebrae with split vertebral bodies (ventral fusion failed to occur), as well as thickened rib joints were seen (Fig. 6 compare wavy tail (B), ur+/-ur+/- (D) to double mutant (F)). Tg/Tg; ur+/-+ and Tg/+; ur+/-+ animals showed no differences to either single homozygous mutant.

Discussion

The most remarkable feature of wavy tail insertion mutant animals is that the mutant phenotype becomes apparent only after birth. We were unable to detect abnormalities in embryos and fetuses, and even at birth, homozygous animals were completely asymptomatic. Curvature of the tail started to develop only during the second postnatal week, coinciding with the juvenile growth phase (the phenotype becoming most pronounced at P14). These features suggested that the dysmorphogenesis was due to neuromuscular disorders. In humans the most common forms of scoliosis are associated with spinal muscular atrophy which is a neuromuscular spinal deformity with a long c-shaped curve. Single curves are seen in approximately 90% of patients. Thoracolumbar curves are most common, occurring in 80%, whereas thoracic curves are seen in approximately 20% of patients (Sucato, 2007). However, the wavy tail mouse mutant did not develop any of these characteristics, as only the tail was deformed and only during postnatal development. Other mouse models developed defects in the lumbal region or tail already around midgestation, like in the wavy tail/mutant these malformations were restricted to the vertebral column but not muscular development (flaky tail (Rothenagel et al., 1994), TgN(Imunsd)379Rpw(Schrick et al., 1995), Sickle tail (Semba et al., 2006), Jun (Behrens et al., 2003), Delta-like 1 (Teppner et al., 2007), or Tgtr2 (Baffi et al., 2006)).

Analysis of the genomic sequence surrounding the insertion site showed that no open reading frame was disrupted, however the transgene inserted in a conserved region close to the Lasp1 transcription start site (Fig. 2A). This suggests Lasp1 as a likely candidate gene affected in the mutant. Interestingly, we found that Lasp1 is transcribed in the wild type growth plate, specifically in resting and hypertrophic chondrocytes. In contrast, Lasp1 expression is altered in mutant tail vertebrae (it is ectopically expressed in all zones of the growth plate and misexpressed in the periosteum) which further indicates that the transgene insertion influences Lasp1 transcription.

The transgene inserted approx. 6.7kb upstream of the Lasp1 transcription start. Vista plot analysis demonstrated that this area
is conserved between rat and mouse (Fig. 2C), suggesting that the insertion disrupted an upstream regulatory element of the Lasp1 gene, which could result in misexpression/lack of expression in chondrocytes. On the other side, the transgene itself contains strong Col1a1enhancer sequences, which could lead to ectopic expression of Lasp1 in the Col1a1 expression domain. The periosteum expresses high levels of Col1a1, thus the ectopic expression of Lasp1 in this tissue may be caused by the close vicinity of the Col1a1 enhancer to the Lasp1 transcription start.

Experiments in NIH3T3 cells indicated that Lasp1 is required for cell migration and survival (Lin et al., 2004), the mis- or overexpression of Lasp1 could lead to changes in chondrocyte cell migration, survival, and differentiation especially during periods of extensive growth (e.g. in the postnatal growth phase). Both increase and depletion of Lasp1 expression in COS-7, HEK293 and MCF-7 cells inhibited basal and growth factor-stimulated cell migration (Lin et al., 2004) and knock-down of Lasp1 in metastatic breast cancer lines resulted in inhibition of proliferation and migration due to a reduction of zyxin at focal contacts (Grunewald et al., 2006). LASP1 is also associated with actin (Butt et al., 2003; Schreiber et al., 1998b), therefore lack of expression or misexpression could lead to structural changes of the chondrocyte cytoskeleton, resulting in a disorganized appearance of growth plates. This becomes even more apparent when examining Col2a1 expression, which is partially lost in mutant chondrocytes. In contrast, Col1X was still expressed in hypertrophic chondrocytes, but secretion (of the protein) was inhibited, which further argues for defects in chondrocyte differentiation in the mutant. Interestingly, Pax1 expression was unaltered early during development (before manifestation of the wavy tail phenotype), but was lost in wavy tail mutant intervertebral discs, suggesting an absence of, or delayed maturation of these chondrocytes. Pax1 is involved in transducing proliferative signals from the notochord to sclerotome cells during skeleton formation (Furumoto et al., 1999). Thus the lack of Pax1 expression could be responsible for the reduced proliferation rate found in mutant chondrocytes.

Neural tube defects that can be prevented by folic acid provide excellent model systems to investigate its protective mechanism. Administration of folic acid results in a significant improvement of the excellent model systems to investigate its protective mechanism. The importance of folate-resistant, as the phenotype was linked to deregulation of Lasp1 gene transcription, which had not been associated with chondrocyte differentiation and function before. The known association of Lasp1 with the cytoskeleton suggests that mutant animals experience defects in cytoskeletal organization, which are most pronounced during the postnatal growth phase of vertebral bones. Lasp1 over-expression seems to have a negative effect on cartilage cell proliferation, on Collagen type X, Col2a1, Col1a1 and Pax1, but not on the expression of the non-collagen chondrocyte markers Ihh, PTHrP and Bmp6. In order to investigate these effects and to validate the important role of Lasp1 during cartilage to bone transition, analysis of deletion mutants or transgenic over-expression models will be required.

Materials & Methods

Mice

Transgenic animals have been produced by pronuclear injection of a linearized 15 kb DNA fragment containing Col1a1 and nls-LacZ sequences. The reporter gene construct injected contained the transcription start site of the murine Col1a1gene with 8.7kb of upstream sequences, the entire exon1 (2 ATG codons mutated to TTG), intron1 (including 47bp of the LTR of the Moloney murine leukemia virus in the Mov31 insertion site (Harbers et al., 1984), and 13bp of exon2. These sequences were fused to the bacterial lacZ reporter gene (including the small intron and poly[A] signal of SV40 T) to yield a construct of 15.0kb (Fig. 2B). As reported before (Kleiter et al., 2002), a few (3-5) copies of the construct have inserted as concatamers with minor rearrangement within the insert (Fig. 2B). X-gal staining revealed that expression of this reporter gene faithfully reflected transcription of the endogenous Col1a1gene (Fig. 2B, unpublished results, Ghaifari-Tabrizi N.). The transgenic mouse line was originally produced in NMRI mice, and was bred to homozygosity in both FVB and C57Bl/6 mouse strains. Heterozygosity was determined by Southern blot analysis using a lacZreporter gene probe or by lacZstaining of P7 tail tips (Kleiter et al., 2000). As dizygous carriers of the insert were viable and fertile, the line was bred in homozygous condition. Dated pregnancies were obtained by detection of a vaginal plug (= E0.5). The mouse strain undulatedcontaining the Pax1 mutation untrans was a gift from Rudi Balling (GBF Braunschweig).

Skeletal analysis

Animals were sacrificed, skinned, and eviscerated. Alcian blue and Alizarin red whole mount skeletal staining was performed as previously described (Kessel and Gruss, 1991).

In situ hybridization and immunohistochemistry

Tissues were fixed overnight in 4% freshly prepared paraformaldehyde in PBS, skeletal components were decalcified in 2.5% paraformaldehyde/12.5% EDTA in PBS for 1 week. Samples were subsequently embedded in paraffin. In situ hybridization was performed on 8μm section according to (Henrique et al., 1995). Col1a1, Col2a1, and Pax1 in situ probes were generated as previously described (Aigner et al., 1995; Jaenisch et al., 1983; Peters et al., 1995). Plasmids containing Lasp1 (clone ID IMGAp998F101146) cDNA clones were obtained from the resource center of the human genome project (www.rzpd.de).

Immunohistochemistry was performed on 8μm sections. Sections were dewaxed and rehydrated. Proteinase K digestion was used for antigen retrieval prior to overnight incubation with the primary antibody solution (rabbit anti-ColX, 1:1000, BR Olsen, Harvard Medical School). Secondary antibody was goat anti-rabbit alkaline phosphatase-conjugated (Biorad), and color development was performed using BM Purple AP substrate (Roche).

LacZ staining

Beta galactosidase detection on cryo-sections was described previously (Schwarzler et al., 1997).

Administration of retinoic acid

All-trans retinoic acid was dissolved in corn oil and injected intraperi-
tone into the pregnant females (E9.5) at 10 μg/g body weight. Newborn animals were sacrificed and stained with Alcian blue and Alizarin red (Kessel and Gruss, 1991).

**Folic acid treatment**

Prenatal folic acid treatment began the day of vaginal plug discovery. Folic acid (3 mg/kg body weight; Sigma) was administered daily by intraperitoneal injection. PBS was used as the vehicle for these injections. A total of 56 growth zone and 26 intervertebral disc sections were analyzed for BrdU positive cells in three individual wild type and transgenic animals. Statistical analysis was performed using a two sample t-test.

**BrdU incorporation**

Animals were injected intraperitoneally with bromo-deoxyuridine (BrdU) solution (50mg/kg body weight in 0.1 M Tris-HCl buffer (Sigma)) and sacrificed two hours later. Proliferating cells were stained with peroxidase-coupled anti-BrdU antibody (Chemicon) and hematoxylin eosin. A small piece of tissue was placed in an Eppendorf tube and fixed in formalin for 24 hours. Tissue was then immersed in 30% sucrose solution for 24 hours, cryostate sectioned, and stained with Alcian blue and Alizarin red (Kessel and Gruss, 1991).

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