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/enviromental 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 transgene 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

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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 size and alterations in the shape of the tail vertebrae (Fig. 1 C-F). As determined by BrdU incorporation, the number of proliferating
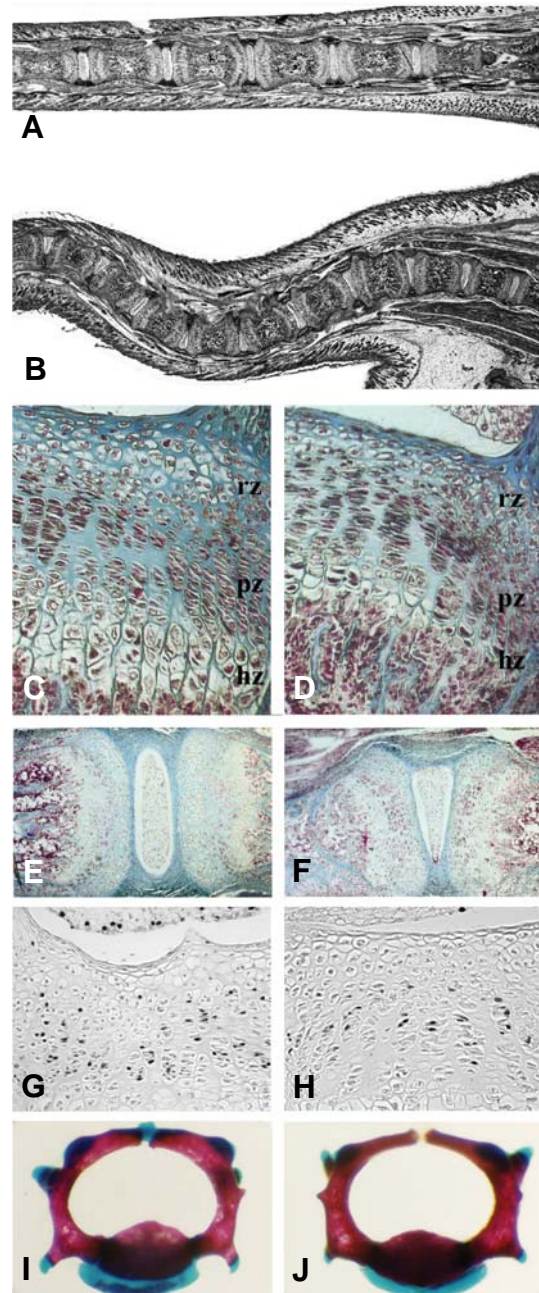
Fig. 1. Wavy tail Tg(Col1a1-lacT)304ng mutants have compressed tail vertebrae and wedge-shaped intervertebral discs. *Longitudinal sections through wild type* **(A)** *and Tg(Col1a1-lacZ)304ng* **(B)** *tail of P10 animals showing scoliosis of the tail and decreased size of tail vertebrae. P10 tail vertebrae of wild type* **(C)** *and mutant* **(D)** *P10 animals stained with Azan. Mutant intervertebral discs* **(F)** *are wedge-shaped and the proliferation zone of mutant vertebrae is compressed compared to wild type control samples* **(E)**. *BrdU incorporation demonstrates a significant 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.*

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 lumbal 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

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 co-segregated 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.*
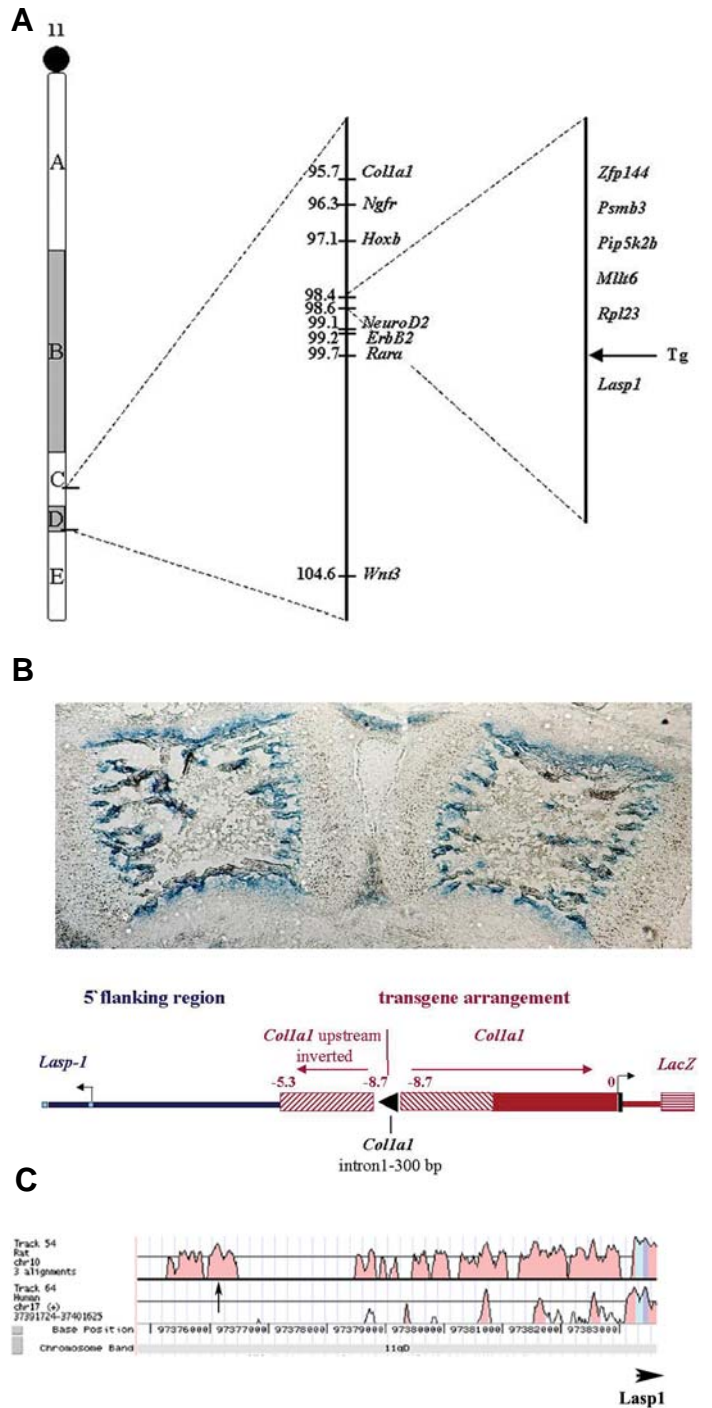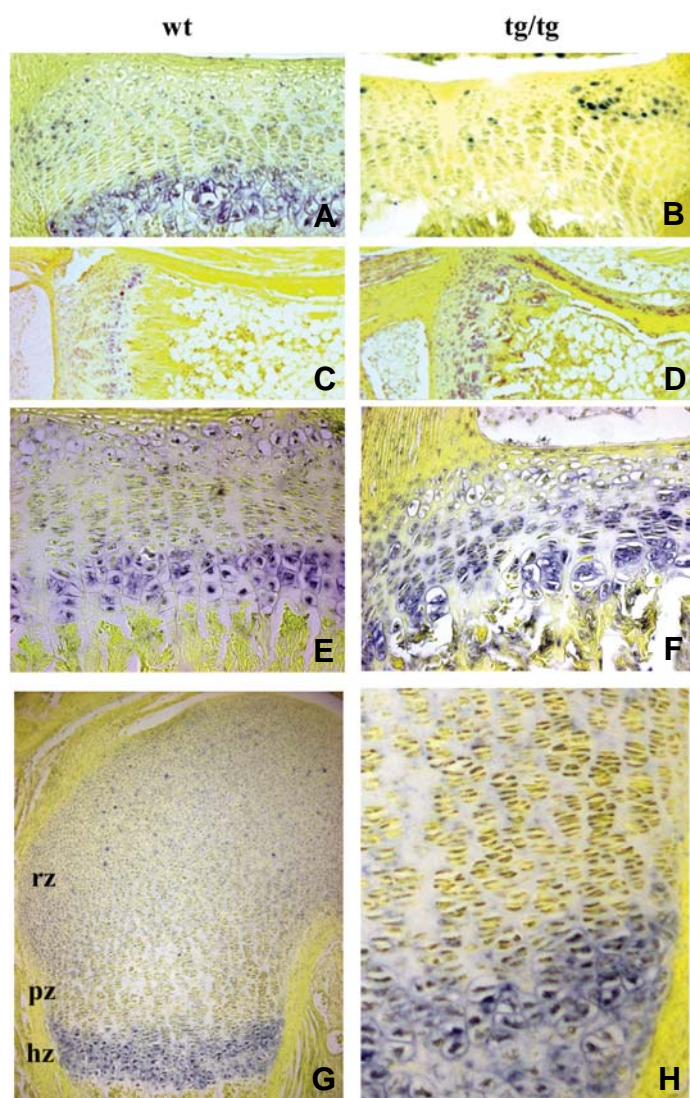
Fig. 3. *Lasp1* expression in developing bones. Lasp1 *expression in P7* **(A,B)** *and P14* **(C-F)** *tail vertebrae and E18.5 long bones* **(G,H)**. Lasp1 *is strongly transcribed in hypertrophic chondrocytes and to lesser extent in chondrocytes of the resting zone of wild type P7* **(A)** *and P14* **(E)** *growth plates, whereas the proliferative zone is devoid of* Lasp1 *expression. In P7 mutant growth plates* **(B)** Lasp1 *was detected in single cells of all zones, with the exception of the hypertrophic area. Ectopic expression of* Lasp1 *is detected in P14 mutant periosteum* **(D)**, *whereas no* Lasp1 *mRNA can be found in wild type* **(C)** *periosteum. A detailed view of P14 growth plates shows that* Lasp1 *is expressed in hypertrophic and resting chondrocytes of wild type* **(E)** *tissue. In contrast all mutant chondrocytes misexpressed* Lasp1, *including the compressed proliferative zone (proliferative zone: Lasp1+ cells n(wt)=9; mean value 16.3± 13.6 SD; n(wavy tail)=9; mean value 85.3± 16 SD (df = 15 two sample t-test p=4.13e-8).* **(F)**. *Abbreviations: hz, hypertrophic zone; pz, proliferating zone; rz, resting zone.*

area (Fig. 2) suggested *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 *Lasp1* during skeleton forma-tion. *Lasp1* transcripts were present in forming vertebrae as early as E14.5 and strong expression was detected in E17.5 hyper-trophic chondrocytes (data not shown). *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, *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 *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, *Lasp1* was transcribed in cells of all stages of chondrocyte differentiation (resting, prolifera-tive 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 *wavy tail* mutant is a temporary *spina bifida occulta* in the lumbar region. *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 verte-brae (data not shown). In addition, ectopic expression was found in the periosteum of the mutant.

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, *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 xyphoid 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 trails 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 adminis-tration in the *wavy tail* mutant. The 50% incidence of spina bifida was not changed in treated animals (data not shown) indicating that our mutant is folate-resistant.

Aberrant expression of collagen genes and Pax1 in wavy tail 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 extra cellular 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
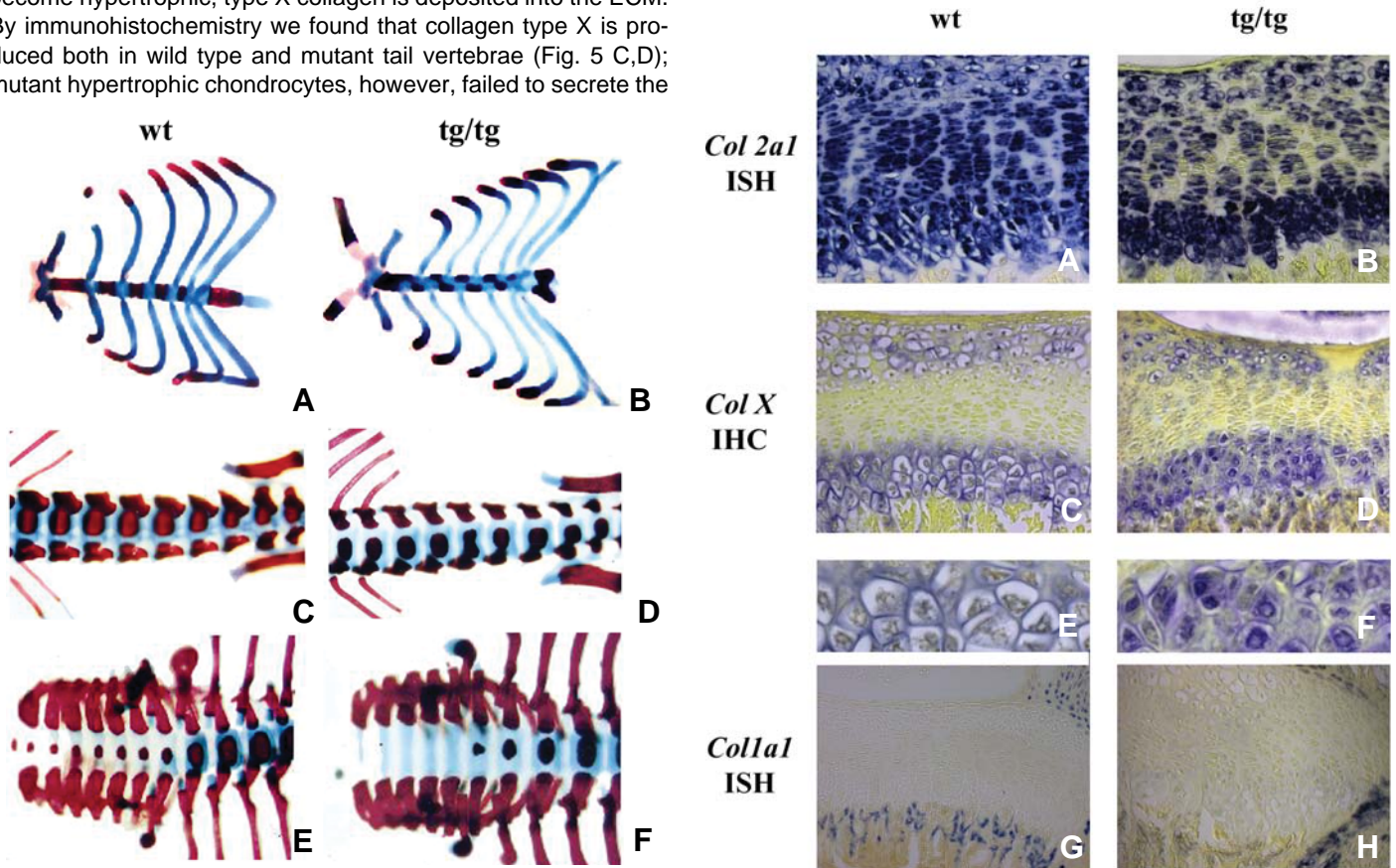
Fig. 4 (Left). Effect of retinoic acid (RA) on skeletal development of wild type and Tg(Col1a1-lacZ)304ng mutant neonates. *Analysis of skeletal elements of wild type and Tg(Col1a1-lacZ)304ng mutant neonates treated with 10 mg/kg RA at E9.5. Whole mount skeletons were prepared from wild type (A,C,E) 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 lumbal vertebral column: 6 lumbal 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 (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.*
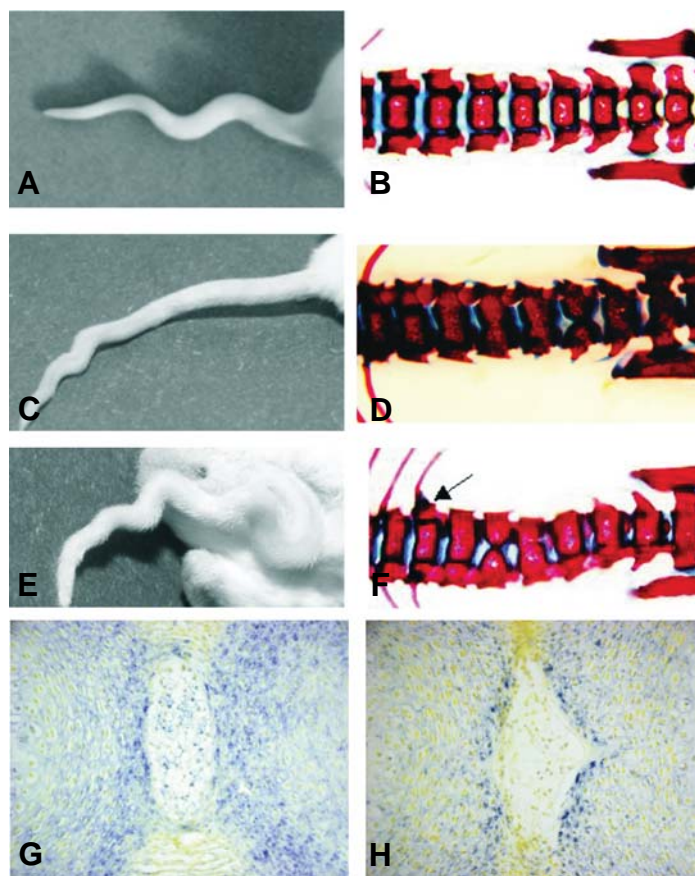
Fig. 6. Severe defects in undulated[ex]/wavy tail Tg(Col1a1-lacZ)304ng double mutants. *Skeletal structures of Tg(Col1a1-lacZ)304ng/Tg(Col1a1-lacZ)304ng, un[ex]/un[ex], and un[ex]/un[ex]/ Tg(Col1a1-lacZ)304ng/Tg(Col1a1-lacZ)304ng compound mutants. The tail vertebral column of* wavy tail *mutants* **(A)** *presents with scoliosis, the tail is bent but, in contrast to the un[ex]/un[ex] 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 lumbal vertebral column showing a structure similar to that of wild type vertebral bodies in the* wavy tail *mutant* **(B)***. un[ex]/un[ex] 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[ex]/wavy tail *Tg(Col1a1-lacZ)304ng* double mutant animals

Previous studies have shown that three natural Pax1 (undu-

lated) 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[ex]) were generated. un[ex] 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[ex]/un[ex]*. 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), *un[ex]/un[ex]* (D) to double mutant (F)). *Tg/Tg; un[ex]/+* and Tg/+; *un[ex]/un[ex]* animals showed no differences to either single homozygous mutant.

Discussion

The most remarkable feature of *wavy tail* insertional 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 lumbar region or tail already around midgestation, like in the *wavy tail* mutant these malformation were restricted to the vertebral column but not muscular development (*flaky tail* (Rothnagel *et al.*, 1994), *TgN(Imunsd)379Rpw* (Schrick *et al.*, 1995), *Sickle tail* (Semba *et al.*, 2006), *Jun* (Behrens *et al.*, 2003), *Delta- like I (Teppner et al.,* 2007), or *Tgfbr2* (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 *Col1a1* enhancer 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, *ColX* 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 neural tube defects in some mutant mice strains like *Cart1* (Zhao *et al.*, 1993); *Cited2 (Barbera et al., 2002)* and *splotch* (Epstein *et al.*, 1991). On the other side, folate-resistant mutants offer the possibility to detect new pathways for NTD prevention, as has been demonstrated in the curly tail mutant, where inositol prevents NTD (Greene and Copp, 1997). The *wavy tail* mutant is folate-resistant, as the *spina bifida occulta* was not responsive to the administration of folic acid. In previous studies, folate-resistant neural tube defects were improved by alterations of RA signaling (Corcoran, 1998). In strong contrast, *wavy tail*/Tg(Col1a1-lacZ)304ng mutant embryos develop even more severe skeletal defects upon RA administration at E9.5. We could not identify RA response elements in the *Lasp1* regulatory sequences suggesting that RA signaling does not directly regulate *Lasp1* expression.

We have characterized a novel transgene insertional mutant with distinct skeletal defects in vertebral column formation. This 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 *Col1a1* gene 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 *Mov13* 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 concatemers with minor rearrangement within the insert (Fig. 2B). X-gal staining revealed that expression of this reporter gene faithfully reflected transcription of the endogenous *Col1a1* gene (Fig. 2B, unpublished results, Ghaffari-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 *lacZ* reporter gene probe or by *lacZ* staining 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 *undulated* containing the *Pax1* mutation un[ex] 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 IMAGp998F101146) cDNA clones were obtained from the resource center of the human genome project (www.rzpd.de).

Immunohistochemistry was performed on 8 μm 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-

toneally into 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. Animals were assessed morphologically for the presence or absence of NTDs.

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 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.

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