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Chondrocyte-Specific Inhibition of β-Catenin Signaling Leads to Dysplasia of the Caudal Vertebrae in Mice

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Study Design. To inhibit β-catenin specifically signaling in chondrocytes *Col2-ICAT* transgenic mice were generated. Anomalies in caudal vertebrae were detected during embryonic and postnatal stages of *Col2-ICAT* transgenic mice.

Objective. To determine the role of canonical β-catenin signaling in caudal vertebral development.

Summary of Background Data. β-catenin signaling plays a critical role in skeletal development. *Col2-ICAT* transgenic mice were generated to selectively block β-catenin signaling by overexpression of the *ICAT* gene in chondrocytes.

Methods. Tails of E16.5 transgenic embryos and adult *Col2-ICAT* transgenic mice and their wild-type littermates were collected and analyzed. Skeletal preparation, 3-dimensional micro-computed tomographic and histological analyses were performed to evaluate changes in the structure of caudal vertebrae. Bromodeoxyuridine labeling was performed to evaluate changes in chondrocyte proliferation in caudal vertebrae.

Results. Skeletal preparation and 3-dimensional micro-computed tomographic analyses revealed bone deformation and angulated deformities in tail tissue in *Col2-ICAT* transgenic mice. Histological studies revealed abnormal bone development and dysplastic caudal vertebrae in *Col2-ICAT* transgenic mice. Inhibition of β-catenin signaling in cartilage resulted in vertebral dysplasia leading to aberrant resegmenting process. Thus, 2 poorly developed sclerotomes failed to fuse to form a complete vertebrae. BrdU labeling revealed a decreased chondrocyte proliferation in both cartilageous templates of transgenic embryos and the growth plate of adult *Col2-ICAT* transgenic mice.

Conclusion. Wnt/β-catenin signaling plays an important role in vertebral development. Inhibition of β-catenin signaling in chondrocytes results in caudal vertebra deformity in mice, which may occur as early as in the stage of sclerotome formation.

Key words: β-catenin, ICAT, chondrocyte, caudal vertebral dysplasia.
Level of Evidence: N/A
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The metameric vertebral columns develop from the somites, the repeated segmental units in the paraxial mesoderm. Sometogenesis in the mouse embryo initiates with the generation of presumptive somatic mesoderm at the primitive streak and the tail-bud mesenchyme. Somites, which are blocks of mesodermal cells, are formed in a coordinated craniocaudal sequence from the paraxial mesoderm.[1–3] Mesenchymal cells in the caudal somites then differentiate into chondrocytes with subsequent formation of a cartilaginous template. Endochondral ossification of such a template eventually leads to the formation of caudal vertebrae.[4–6]

In the mouse embryo, mesodermal formation begins at embryonic day 6.5 (E6.5).[7]

The sequential formation of somites along the anterior-posterior axis is under control of multiple signaling gradients involving the Wnt (wingless-related MMTV integration site), fibroblast growth factor, and retinoic acid pathways.[8] The Wnt family of signaling molecules have been detected in the primitive streak and tail-bud mesenchyme. Among the

Wnt family members detected, *Wnt3a* is expressed in cells fated to give rise to embryonic mesoderm, from E6.5 on. A null mutation of *Wnt3a* leads to lack of caudal somites from the level of umbilicus at E12.5, have a disrupted notochord, and fail to form a tail bud.[9]

The canonical Wnt signaling pathway is mediated by β-catenin, which accumulates in the cytoplasm in the presence of Wnts, and translocates into the nucleus. In the nucleus, β-catenin interacts with a T cell-specific factor or a lymphoid enhancer factor (LEF) to activate the downstream target genes.[10] Inhibition of β-catenin and T cell-specific factor (ICAT) is a small peptide that binds the armadillo repeats of β-catenin and interferes with the formation of the β-catenin and T cell-specific factor/LEF complex, thereby disrupting this pathway.[11–13] ICAT expression starts at a very low level in the precartilaginous mesenchymal sclerotome, which coincides with the temporal and spatial expression pattern of *Col2a1* mRNA during mouse embryonic development (at about E12).[14–16]

Wnt/β-catenin signaling also plays a role in the differentiation process of mesenchymal progenitors toward chondrocyte lineage. β-catenin is highly expressed in prechondrogenic mesenchymal cells but significantly decreased in differentiated chondrocytes.[17] The canonical Wnt signaling represses chondrogenesis, and inactivation of β-catenin in mesenchymal progenitor cells induces chondrocyte differentiation under conditions allowing only osteoblasts to form *in vitro*.[18,19] However, the findings are not always consistent. For example, Yano *et al*[20] have shown that constitutively active LEF-1 in undifferentiated mesenchymal cells promoted chondrogenic differentiation, whereas downregulation of LEF-1 or silencing β-catenin suppressed the chondrocyte gene expression. The expression level of the Wnt mRNA was low in the resting and hypertrophic zones of the growth plate. In contrast, LEF-1 expression is much higher in proliferative and prehypertrophic zones.[21] Inhibition of Wnt/β-catenin signaling or inactivation of β-catenin leads to reduced chondrocyte proliferation and increased chondrocyte apoptosis.[22–24] Despite these findings, the role of the Wnt/β-catenin signaling pathway in resegmentation process remains largely undefined.

A previous study has demonstrated that cartilage-specific inhibition of β-catenin in the *Col2-ICAT* transgenic mice results in severe osteoarthritis-like phenotype.[25] In this study, we aimed to investigate the role of β-catenin signaling in the development of caudal vertebrae.

MATERIALS AND METHODS

Col2-ICAT Transgenic Mice and Genotyping

The use of animals was approved by the Shanghai Laboratory Animal Use Committee. The *Col2-ICAT* transgenic mouse (C57BL/6J) was generated and reported before.[24,25] The Flag-tagged *ICAT* (*Flag-ICAT*) complementary DNA includes the 5′ *Nde* I site *Col2a1* promoter, β-globin intron cassette, SV40 poly (A), and *Col2a1* enhancer. The generation of 2 separate lines of *Col2-ICAT* transgenic mice and their wild-type (WT) littermates were genotyped by polymerase chain reaction.[23,24]

Skeletal Preparation

Skeletal preparation was performed to compare possible differences between E16.5 *Col2-ICAT* transgenic and WT embryos (n = 6). The phenotype of 6-month-old *Col2-ICAT* transgenic mice and their WT littermates (n = 6) were also analyzed. The skin, viscera, and adipose tissue were carefully removed after they were killed. The whole skeletons were fixed in 95% ethanol for 2 to 5 days followed by fixation in acetone for another 1 to 2 days, and stained with 0.015% Alcian Blue and 0.005% Alizarin Red for 1 to 3 days. Images of the mouse skeletons were captured with a camera (Sony H10, Tokyo, Japan).

Three-Dimensional Reconstruction Analyses

The caudal vertebrae from 6-month-old *Col2-ICAT* transgenic mice (n = 6) and their WT littermates (n = 6) were dissected, and fixed in 4% paraformaldehyde overnight followed by washing for 2 hours. Three-dimensional reconstruction analyses were performed with a Micro-CT 80 scan machine (SCANO Medical AG, Bassersdorf, Switzerland). The deformed regions were first located with scout views of the whole caudal vertebrae. The abnormal bones and part of the neighboring vertebrae underwent fine scanning for 160 slices with 20-μm slice increments. The x-ray source voltage was 70 kVp, the source current was 114 μA, and the integration time was 400 ms. A reconstruction of the bitmap data set was used to build the 3-dimensional images.

Histological Evaluation

Tail samples from 6-month-old mice of both genotypes (WT and *Col2-ICAT* transgenic) were subjected to histological analysis with different staining methods to reveal the potential pathological changes. The caudal vertebrae of E16.5 and 6-month-old WT and *Col2-ICAT* transgenic mice were fixed in 4% paraformaldehyde, decalcified, dehydrated, and embedded in paraffin. Serial midsagittal sections (6-μm thick) of the caudal vertebrae were cut and stained with hematoxylin/eosin, the widely used staining method in histological diagnosis, and safranin O/fast green, a common staining method for cartilage and bone, respectively. A morphometric study was performed using a light microscope (Olympus B×50; Tokyo, Japan) with camera (Olympus DP71; Tokyo, Japan) and image analysis system (CMIAS-99B; Beijing, China).

BrdU Labeling and Staining

For adult mice, bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) was intraperitoneally injected into 6-month-old WT and *Col2-ICAT* transgenic mice 1 day and 4 hours before they were killed (10 mg/mL, 100 mg/kg body weight). For E16.5 embryos, *Col2-ICAT* transgenic mice were bred with WT mice, and the pregnant mice were intraperitoneally injected with BrdU 2 hours before they were killed. The caudal vertebrae of adult mice and embryos were collected and fixed in 4% paraformaldehyde, decalcified, dehydrated, and embedded in paraffin. Serial midsagittal sections (6-μm thick) of the caudal vertebrae were cut and stained using a BrdU

immunohistochemistry kit (Chemicon, Billerica, MA), for cell proliferation.

Statistical Analysis

Data were expressed as means ± standard error of the mean. An unpaired Student t test was performed using SPSS version 16.0 software (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

RESULTS

The mice were able to ambulate and move their tails, suggesting that the neural tube development had not been affected. We observed that all *Col2-ICAT* transgenic mice developed at least one angulated deformity in their distal tails (Figure 1A). Radiographical images showed an aberrant vertebral development, leading to reduced lengths of the caudal vertebrae (Figure 1B). Skeletal preparation and 3-dimensional (3D) reconstruction analyses showed the paraxial locations of the sesamoid-like bones in most transgenic mice. The deformed bones were either solitary or attached to neighboring vertebra, and in some cases, single or multiple bones with different sizes were dislocated on the opposite side (Figure 1C, D). All *Col2-ICAT* transgenic mice showed decreased numbers of the caudal vertebrae compared with their WT littermates (Figure 1E, unpaired Student t test, *$P < 0.05$, n = 6).

The abnormal bones displayed some histological characteristics of vertebrae, including cartilage endplate, endochondral ossification, and intervertebral disc tissue (nucleus pulposus and annulus fibrosus) between the abnormal bone and the consecutive vertebrae (Figure 2A, B). In some cases, sparse cartilaginous tissues were detected opposite to the abnormal vertebra, and ossification in such tissue was rare. BrdU labeling revealed a significant reduction in the number of positive cells in the growth plate chondrocytes of *Col2-ICAT* transgenic mice compared with that in the WT littermates (Figure 2C).

The E16.5 *Col2-ICAT* transgenic embryos developed macroscopic deformities in their tails (Figure 3A). Alcian blue staining of the caudal vertebrae showed that at the embryonic E16.5 stage, the cartilaginous templates have already formed although endochondral ossification has not yet been initiated (Figure 3B). The proliferation of the chondrocytes was reduced in the caudal vertebrae of *Col2-ICAT* transgenic embryos compared with that in WT littermates, as evidenced by significant lower numbers of BrdU positive cells (Figure 3C, D, unpaired Student t test, *$P < 0.05$, n = 6).

A normal caudal vertebra is developed from 2 sclerotomes located on the 2 sides of the notochord, and these 2 sclerotomes become gradually fused in the middle. Histological examination demonstrated that the defective resegmentation of the caudal vertebrae in the *Col2-ICAT* transgenic embryos resulted in an axis excursion to 1 side leading to angle formation. The poorly developed semicolumn failed to fuse with the other half to form a complete vertebra, thereby prevent the proper translocation of the notochord to the nucleus pulposus (Figure 4A, B).

DISCUSSION

Wnt/β-catenin/Axin2 signaling molecules have been reported to be involved in the oscillatory process for segmentation.[26] However, their precise role in segmentation remains largely undefined. Abnormal development of the caudal vertebrae was observed in the mice with aberrant Wnt/β-catenin signaling. *Wnt3a* mutant mice display a complete absence of tail-bud development.[9,27] In contrast, the mice with the gain-of-function mutation in low-density lipoprotein receptor-related protein 6 (*Lrp6*), a coreceptor of Wnt signaling, have a neural tube defect, leading to the formation of the crooked tails.[28,29] Consistently, mice deficient of *Dkk1* (Dickkopf 1), a secreted Wnt antagonist, display vertebral phenotypes, including tail kinks and vertebral fusion.[30] Furthermore, inactivation of
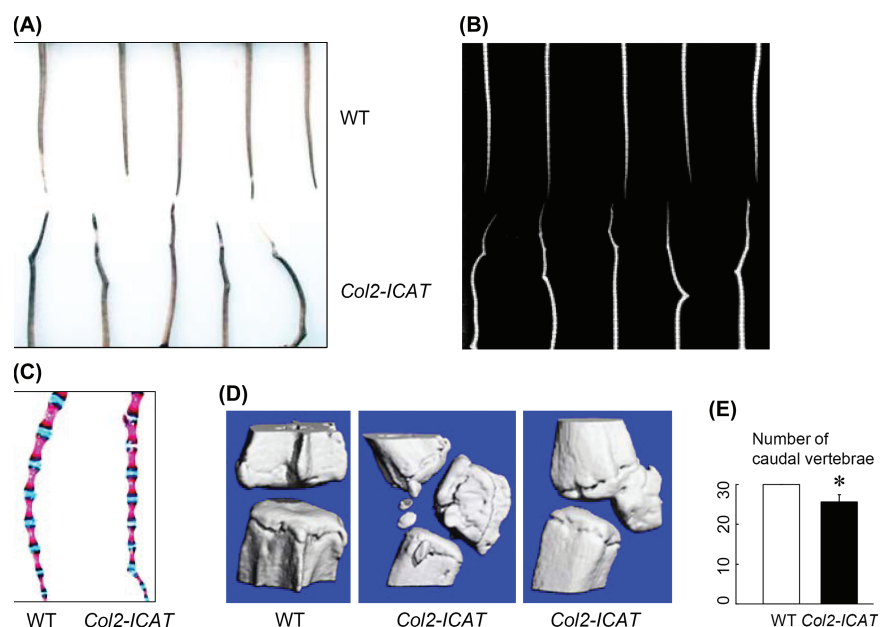

Figure 1. *Col2-ICAT* transgenic mice display an aberrant vertebral development resulting in angulated deformity in tails as evidenced by macroscopic observation (**A**) and radiographic analysis (**B**). The results of whole-mount Alizarin red/Alcian blue staining showed abnormal bone formation in the tails of the *Col2-ICAT* transgenic mice (**C**). The results were further confirmed by micro-CT analysis (**D**). The quantitative data showed that numbers of caudal vertebrae of the *Col2-ICAT* transgenic mice were significantly reduced compared with the WT littermates (**E**). $P < 0.05$, unpaired Student t test (n = 6). WT indicates wild-type; CT, computed tomographic.
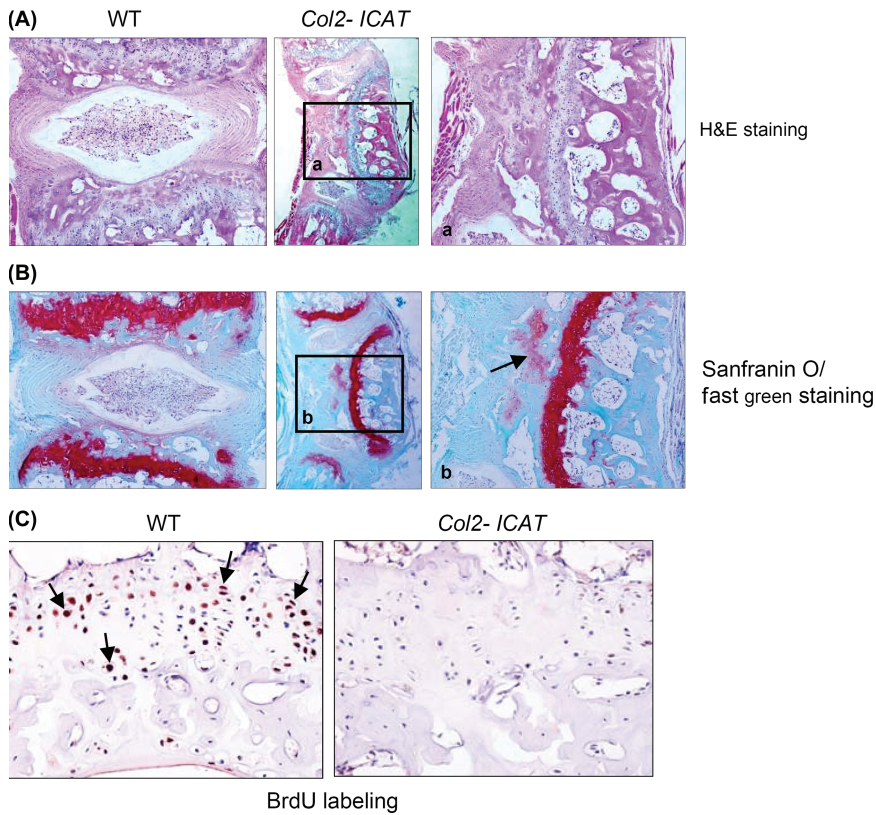
Figure 2. Tail-tissue deformity of adult *Col2-ICAT* transgenic mice. Histological analysis with H&E (**A**) and safranin O/fast green (**B**) staining demonstrated the dysplasia of caudal vertebrae in *Col2-ICAT* transgenic mice compared with their WT littermates. Disorganized bone due to vertebral dysplasia exhibits a typical cartilage endplate, endochondral ossification, and IVD-like structure between the abnormal bone and the adjacent vertebra. Sparse cartilaginous tissue was also found opposite to the abnormal vertebra with limited bone formation inside (black arrow). Right and left panels, ×100, middle panels, ×40. The results of BrdU labeling demonstrated that chondrocyte proliferation in growth plate of caudal vertebrae from the *Col2-ICAT* transgenic mice was significantly reduced compared with their WT littermates (×200, **C**). H&E indicates hematoxylin/eosin; IVD, intervertebral disc; BrdU, bromodeoxyuridine; WT, wild-type.

sFRP (Frizzled-related proteins) 1, 2, and 5, which are the secreted Wnt antagonists, induces the formation of fused somites in early mouse embryos.[31] These findings demonstrate the importance of the balanced Wnt signaling in the normal development of caudal vertebrae.

In this study, we provide new evidence of the role of β-catenin signaling in the pathogenesis of vertebral dysplasia. Our results reveal a reduced proliferation of the chondrocytes isolated from the caudal vertebrae in *Col2-ICAT* transgenic mice. Specific inhibition of β-catenin signaling in type II collagen (Col2) expressing chondrocytes affects early vertebral development when sclerotomes form. We have described previously that the mesenchymal sclerotomes express a low level of Col2.[23] Further, cartilage-specific inhibition of β-catenin in the *Col2-ICAT* transgenic mice results in severe osteoarthritis-like phenotype in some, but not all of *Col2-ICAT* transgenic mice.[24] Interestingly, all *Col2-ICAT* transgenic mice have deformities in the caudal vertebra including angulated and/or shortened tails during embryonic development. Osteoarthritis pathogenesis differs
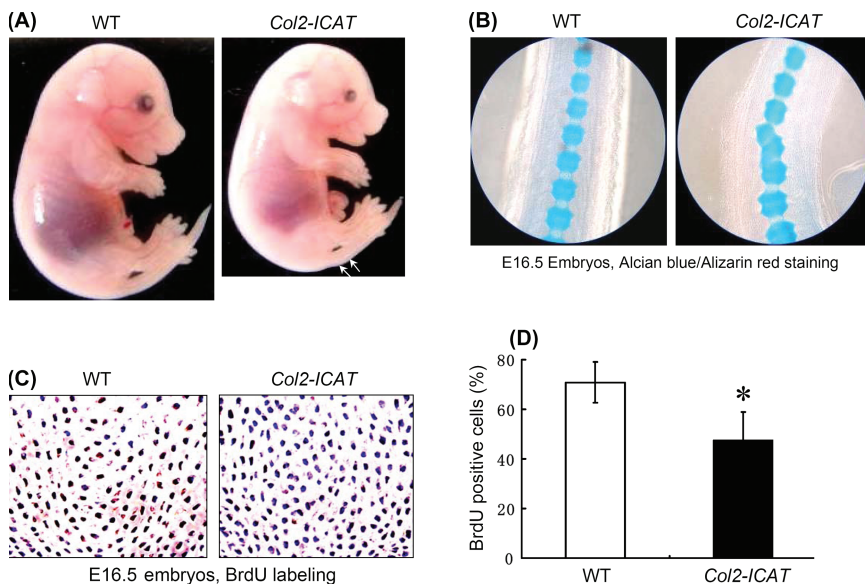

Figure 3. Defects in the development of caudal vertebrae at early embryonic stage. Macroscopic deformities (the white arrows) in tails were observed in E16.5 *Col2-ICAT* transgenic embryos (**A**). The results of Alizarin red/Alcian blue staining showed angulated tail and ectopic caudal vertebrae of E16.5 *Col2-ICAT* transgenic embryos (**B**) (×100). Cell proliferation was reduced in cartilaginous template of caudal vertebrae of E16.5 *Col2-ICAT* transgenic embryos compared with WT embryos (×400, **C**). The BrdU positive cells were significantly decreased in *Col2-ICAT* transgenic embryos compared with wild-type controls (**D**). *$P < 0.05$, unpaired Student t test (n = 6). BrdU indicates bromodeoxyuridine; WT, wild-type.
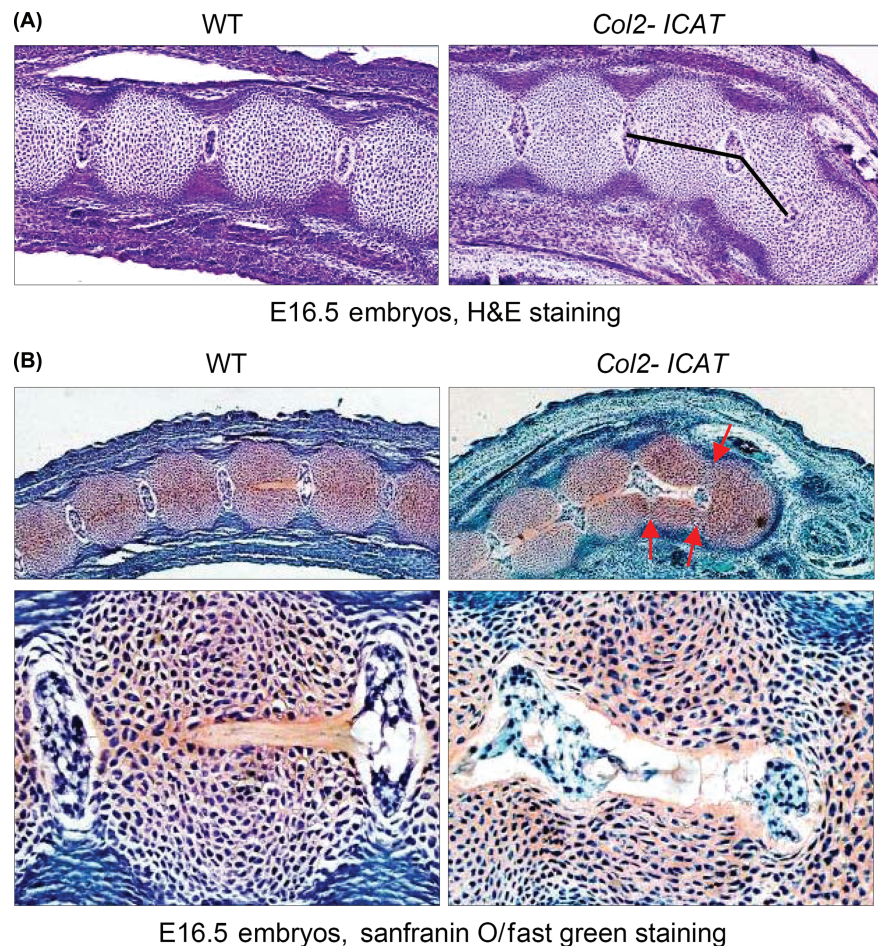
Figure 4. Ectopic resegmentation and formation of caudal vertebrae were observed in *Col2-ICAT* transgenic embryos. Histological analyses showed that ectopic resegmentation (arrows), axis excursion (**A**) as well as poorly formed dysplastic caudal vertebrae were observed in *Col2-ICAT* transgenic embryos (**B**). **A**, ×100; **B**, upper panel, ×100; lower panel, ×400. H&E indicates hematoxylin/eosin; WT, wild-type.

from that of vertebral dysplasia in that it develops in adults, and is influenced by multiple factors such as activity level, body weight, and sex.

Our results suggest that the reduced chondrocyte proliferation in the endplates of the *Col2-ICAT* transgenic mice is the critical defect responsible for vertebral dysplasia. Because the tail deformities in the *Col2-ICAT* transgenic mice were noticed immediately after birth, we postulated that such deformities may result from defective vertebral development. Therefore, we examined changes in caudal vertebrae in transgenic embryos.

In addition to chondrocyte proliferation, mesenchymal differentiation toward chondrocyte lineage may also be affected by inhibited β-catenin signaling. Thus, the poorly developed sclerotomes cannot fuse with the opposite parts to form a complete vertebra. Instead, they gradually develop into sparse cartilage tissues with very limited or no endochondral ossification. The opposite parts of the sclerotome may continue to develop after fusion failure, eventually leading to the formation of an abnormal vertebral column in a paraxial location. The results suggest that abnormal bone formation in the *Col2-ICAT* transgenic mice is deformed caudal vertebrae, but not ectopic bone.

The abnormal changes are observed only in the tail region, and no developmental defects are detected in other regions of the spinal column such as cervical, thoracic, and lumbar columns. ICAT expression starts at about E12 at a very low level

in the precartilaginous mesenchymal sclerotome, consistent with the temporal and spatial expression pattern of *Col2a1* mRNA during mouse embryonic development. Because ICAT inhibition of β-catenin signaling is leaky and starts at a later stage than Wnt3a, which is detected as early as E6.5, the tail deformity described in this study is milder than that of *Wnt3a* mutation mice. The neural tube development was not affected in our *Col2-ICAT* transgenic mice, likely because the *ICAT* gene express at a later stage (E12), after completion of neural tube closure (E10, about 26–28 somites).[32]

CONCLUSION

Tissue-specific inhibition of β-catenin signaling in cartilage causes the deformities of caudal vertebrae in mice, suggesting that β-catenin signaling plays a critical role in the caudal vertebral development.

➢ Key Points

- ❑ Chondrocyte-specific inhibition of β-catenin signaling results in caudal vertebra dysplasia in mice.
- ❑ Choncrocyte proliferation is decreased in both cartilageous templates of transgenic embryos

and the growth plate of adult *Col2-ICAT* transgenic mice.

❑ The poorly developed sclerotomes in *Col2-ICAT* transgenic mice failed to resegment and form regular caudal vertebrae.

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