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## TGF- $\beta$ Signaling Plays an Essential Role in the Growth and Maintenance of Intervertebral Disc Tissue

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### Summary

TGF- $\beta$  signaling plays a critical role in cartilage and spine tissue development at embryonic stage but its role in postnatal intervertebral disc (IVD) tissue growth and maintenance remain poorly understood. In the present studies, we have deleted the *Tgfb2* gene in inner annulus fibrosus cells of the disc tissue and surrounding growth plate chondrocytes using *Col2a1-CreER<sup>T2</sup>* transgenic mice. We found that TGF- $\beta$  signaling is required for normal growth plate cartilage and endplate cartilage growth at postnatal stage. The expression of *Mmp13* gene is significantly up-regulated in primary disc cells of *Tgfb2* conditional knockout mice. Deletion of the *Mmp13* gene under *Tgfb2* null background completely reverses the abnormal disc phenotype found in *Tgfb2* knockout mice.

### Keywords

TGF- $\beta$ ; Col2a1-CreER<sup>T2</sup>; Intervertebral disc; Conditional knockout; MMP-13

### Introduction

Low back pain, which is often related to intervertebral disc degeneration, affects approximately 80% of the aging population and causes a significant socio-economic burden [1]. In an autopsy study, 97% of individuals age 50 years or older showed disc degeneration [2]. The intervertebral disc (IVD) is a specialized tissue that permits rotation as well as flexion and extension of the human spine. It consists of an annulus fibrosus that encloses the nucleus pulposus, a highly viscous gel-like tissue that produces aggrecan and other proteoglycans, providing the disc with its critical water-retaining characteristics. The superior and inferior boundaries of the IVD are formed by the growth plate and cartilage

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### Competing interest statement

The authors have no conflict of interest to disclose.

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endplate [3]. Genetic mouse models are important tools to target specific genes in specific cell populations such as IVD and surrounding cells at different developmental and postnatal stages. Since the cells of annulus fibrosus, nucleus pulposus, growth plate cartilage, and endplate tissues express type II collagen [4], we have generated *Col2a1-CreER<sup>T2</sup>* transgenic mice [5, 6]. We bred these transgenic mice with Rosa26 reporter mice and analyzed the cell population(s) targeted in *Col2a1-CreER<sup>T2</sup>* transgenic mice. In addition, we investigated the Cre-recombination efficiency of the targeted disc cells. Taking advantage of the inducible manner of Cre expression in *Col2a1-CreER<sup>T2</sup>* transgenic mice, we also performed a time-course experiment to investigate the fate of targeted disc cells.

Similar to long bone, the development of IVD tissue starts from mesenchymal cell condensation at around E10.5 in mice. At approximately E12.5, the condensed mesenchyme forms the IVD, and the loose mesenchyme undergoes chondrogenesis to form the vertebral body. Notochord cells derived from the mesoderm are then removed from the vertebral region and expand into the IVD region to form the nucleus pulposus. The region around the nucleus pulposus forms the annulus fibrosus. The inner annulus fibrosus contains hyaline cartilage and the outer annulus fibrosus is more fibrous. The nucleus pulposus is formed in the middle of the IVD region at E15.5–E17.5 stage in mice [7]. It has been demonstrated that defects in the development of IVD tissue was detected in *Tgfb2* cKO embryos targeted by *Col2a1-Cre* transgenic mice (hereafter referred to as *Tgfb2<sup>Col2</sup>*). The development of annulus fibrosus and the expansion of nucleus pulposus tissue to the IVD region were delayed in E15.5 to E17.5 *Tgfb2<sup>Col2</sup>* embryos [7]. In addition, it has also been reported that spinal column of E18.5 *Tgfb2<sup>Col2</sup>* embryos show the neural arch defect from a dorsal-lateral aspect in addition to the craniofacial defects [8]. The *TGF $\beta$ -2* knockout phenotype has no overlap with the phenotypes observed in the *TGF $\beta$ -1* and *TGF $\beta$ -3* null mice [9–12].

Although TGF- $\beta$  signaling plays a critical role in axial skeletal development [7, 8, 13], its roles in postnatal growth and maintenance of disc tissue remain poorly understood. In the present studies, we have bred *Col2a1-CreER<sup>T2</sup>* transgenic mice with *Tgfb2<sup>fx/fx</sup>* mice [13] and generated inducible *Tgfb2* cKO mice (hereafter referred to as *Tgfb2<sup>Col2ER</sup>*). We specifically deleted the *Tgfb2* gene in inner annulus fibrosus cells and growth plate chondrocytes at the postnatal stage. Defects in disc tissue growth and maintenance were observed in *Tgfb2<sup>Col2ER</sup>* mice. Our studies demonstrate that *Col2a1-CreER<sup>T2</sup>* transgenic mice are an important tool to target cells of IVD and surrounding tissues and TGF- $\beta$  signaling plays an essential role in the development of IVD and surrounding tissues.

## Materials and Methods

### Characterization of *Col2a1-CreER<sup>T2</sup>* transgenic mice

*Col2a1-CreER<sup>T2</sup>* transgenic mice were previously generated in our laboratory [5, 6] and these mice were crossed with Rosa26 reporter mice in which the expression of *Escherichia coli*  $\beta$ -galactosidase can be induced by Cre-mediated recombination [14]. Offspring were genotyped by polymerase chain reaction (PCR) using specific primers for detecting Cre insertion and R26R alleles. The sequences of PCR primers for genotyping *Col2a1-CreER<sup>T2</sup>* mice are: 5'-CCTGGAAAATGCTTCTGTCGGTTTGCC-3' (forward primer) and 5'-GAGTTGATAGCTGGCTGGTGGCAGAG-3' (reverse primer) and the size of the PCR product is 600-bp. The sequences of PCR primers for genotyping Rosa26 reporter mice are: R1295: 5'-GCGAAGAGTTTGTCTCAACC-3'; R523: 5'-GGAGCGGAGAAATGGATATG-3' and R26F2: 5'-AAAGTCGCTCTGAGTTGTTAT-3'. The 600-bp PCR product was detected in wild-type mice and the 325-bp PCR product was detected in homozygous Rosa26 mice. Both 600- and 325-bp PCR products were detected in heterozygous Rosa26 reporter mice.

Tamoxifen was administered to 2-week-old *Col2a1-CreER<sup>T2</sup>;R26R* mice (1 mg/10g body weight, x5 days, i.p. injection). Mice were sacrificed 1, 2, 3, and 4 months after tamoxifen injection. The disc tissues were harvested and fixed in 0.2% glutaraldehyde, decalcified and processed for frozen sectioning followed by X-Gal staining. Nuclear Fast Red staining was performed as a counter stain. The Cre recombination efficiency was determined by counting the lacZ positive cells divided by the total number of cells in growth plate, cartilage endplate, inner and outer annulus fibrosus and nucleus pulposus regions (n=4).

### Generation of *Tgfb2* conditional knockout mice

*Col2a1-CreER<sup>T2</sup>* transgenic mice were bred with *Tgfb2<sup>flx/flx</sup>* mice (obtained from Jackson lab) [15]. Tamoxifen was administered to P1 pups (1 mg/10g body weight, single dose, i.p. injection). Mice were sacrificed when they were 2 weeks old. Tamoxifen was also administered to P14 *Tgfb2<sup>Col2ER</sup>* mice (1 mg/10g body weight, x 5 days, i.p. injection). Mice were sacrificed at 3 months old.

### Histological analysis

The disc tissues were dissected from *Col2a1-CreER<sup>T2</sup>;R26R* mice, *Tgfb2<sup>Col2ER</sup>* mice, *Tgfb2<sup>Col2ER</sup>;Mmp13<sup>Col2ER</sup>* mice, and their corresponding Cre-negative control mice. Samples were fixed in 10% formalin, decalcified in 14% EDTA, and embedded in paraffin. Serial sections were taken at 3 levels with 15  $\mu$ m apart within the midsagittal region of the intervertebral bodies. Sections were cut at 3  $\mu$ m thickness. The sections were stained with Alcian blue/H&E (AB/HE) and Safranin O/Fast green (SO/FG).

### Disc cells culture and real-time PCR analysis

14-day-old mice were genotyped and then sacrificed. Primary mouse disc cells were isolated from the intervertebral disc tissues of Cre-negative and *Tgfb2*-flox mice through digestion with 1 mg/ml Collagenase A (Roche). The cells were then counted and plated in DMEM containing 10% FBS. 12 hours after the seeding, the cells were infected with Ad-Cre (Vector Biolabs) or an Ad-GFP (Vector Biolabs) (control), at 100 MOI for 24 hours. The cells were allowed to recover for 48 hours before harvest. The cells were then subjected to RNA isolation.

Total RNA was prepared using PureLink<sup>TM</sup> RNA Mini Kit (Invitrogen) according to the manufacturer's protocol. 1  $\mu$ g of total RNA was used to synthesize cDNA using iScript<sup>TM</sup> cDNA Synthesis Kit (Bio-Rad). Real-time PCR amplifications were performed using gene specific primers and SYBR green real-time PCR kit. The primers and sequences used for the real-time PCR analysis are shown in Table 1. The thermal cycling conditions were as follows: an initial denaturation at 95°C for 3 min, followed by 40 cycles of 20 seconds of denaturing at 95°C, 20 seconds of annealing at 58°C, and 20 seconds of extension at 72°C. The expression levels of the target genes were normalized to that of  $\beta$ -actin in cDNA samples.

## Results

Administration of tamoxifen to *Col2a1-CreER<sup>T2</sup>;R26R* mice induced efficient Cre-recombination in IVD cells up to 4 months after tamoxifen induction (Fig. 1A). High Cre-recombination efficiency was observed in growth plate chondrocytes. It reached 80% in 1-month-old mice and remained high (70%) in 4-month-old mice (Table 2). These results suggest that the cell turnover rates are very low in growth plate chondrocytes of the disc surrounding tissue. Although *Col2a1-CreER<sup>T2</sup>* mice can target cartilage endplate cells, the efficiency is relatively low. While only 10% of endplate cartilage cells were X-Gal positive by 1 month after tamoxifen induction, the efficiency increased to 37% by 4 months after

tamoxifen induction (Fig. 1B and Table 1). *Col2a1-CreER<sup>T2</sup>* mice can also efficiently target inner annulus fibrosus cells, with 73 to 81% efficiency in mice of different ages (Fig. 1B and Table 1). In contrast, the targeting efficiency for outer annulus fibrosus cells was very poor. These results are expected since it is well documented that inner annulus fibrosus cells are enriched in type II collagen while outer annulus fibrosus cells have a high type I collagen content [16]. We did not observe X-Gal positive cells in the nucleus pulposus region (Fig. 1A). This is probably because of low type II collagen expression in nucleus pulposus cells. Immunohistochemistry (IHC) showed that type II collagen expression was undetectable in nucleus pulposus cells (data not shown). Another possible reason is that tamoxifen may not be able to reach the nucleus pulposus due to the lack of a blood supply to the nucleus pulposus region. However, this may not be the case because tamoxifen can induce Cre-recombination in nucleus pulposus cells in *aggrecan-CreER<sup>T2</sup>* transgenic mice [17]. In this study, we provide evidence that *Col2a1-CreER<sup>T2</sup>* transgenic mice can efficiently target growth plate chondrocytes and inner annulus fibrosus cells in the IVD, suggesting that these mice are an important tool to selectively target floxed genes specifically expressed in inner annulus fibrosus cells in the disc tissue and surrounding growth plate chondrocytes.

To determine the role of TGF- $\beta$  signaling in the growth and maintenance of disc tissue at postnatal stage, we generated *Tgfr2<sup>Col2ER</sup>* mice using the *Col2a1-CreER<sup>T2</sup>* transgenic mice to specifically inactivate TGF- $\beta$  signaling in inner annulus fibrosus cells in the IVD and surrounding growth plate chondrocytes. In P14 early postnatal mice, the cartilage endplate tissue was well developed in wild-type mice. In contrast, significant reduction in the length and area of cartilage endplate tissue was observed in *Tgfr2<sup>Col2ER</sup>* mice. A significant loss of matrix tissue and endplate cartilage cells was observed in *Tgfr2<sup>Col2ER</sup>* mice (Fig. 2A). In 3-month-old postnatal mice, significant loss of growth plate chondrocytes in disc tissue was found in *Tgfr2<sup>Col2ER</sup>* mice. In addition, growth plate chondrocytes lost their columnar structure compared to their Cre-negative control mice. Disorganized chondrocytes formed clusters in the growth plate region of *Tgfr2<sup>Col2ER</sup>* mice (Fig. 2B). These results demonstrated that TGF- $\beta$  signaling is required for normal growth of disc and surrounding tissues at the postnatal stage. To determine changes in gene expression of disc cells, we isolated primary disc cells from these mice. We found that mRNA expression of *Col10a1* (11-fold increase), *Mmp13* (14-fold increase), *Adams4* (4-fold increase), and *Adams5* (15-fold increase) was significantly up-regulated in *Tgfr2<sup>Col2ER</sup>* mice (Fig. 3A), indicating that disc cell differentiation process was accelerated and several genes (and probably their coding proteins) involved in matrix degradation were activated. To confirm the effect of TGF- $\beta$  on *Mmp13* expression *in vitro*, we treated RCS chondrogenic cells with 5ng/ml of TGF- $\beta$  up to 48 hours and found that TGF- $\beta$  significantly inhibited *Mmp13* expression at 24 and 48 time points (Fig. 3B). This finding is consistent with the *in vivo* observation. To explore the possible interaction of TGF- $\beta$  with PTHrP and *Ihh* signaling, we examined the expression of *Pthr1* and *Ptc1* in RCS cells in which *Tgfr2* siRNA was transfected. We found that silencing of *Tgfr2* expression significantly up-regulated *Ptc1* expression but had no effect on *Pthr1* expression (Fig. 3C), suggesting that TGF- $\beta$  may have inhibitory effect on *Ihh* signaling in chondrocytes.

MMP13 plays a critical role in cartilage function and the development of osteoarthritis [18, 19]. In order to determine if the phenotype observed in *Tgfr2<sup>Col2ER</sup>* mice is due to the activation of *Mmp13* expression, we have deleted the *Mmp13* gene under the *Tgfr2* deletion background by generating *Tgfr2/Mmp13* double KO (dKO) mice. Histologic analysis of spine sections showed that proteoglycan levels and cell numbers in growth plate cartilage area were significantly decreased in *Tgfr2<sup>Col2ER</sup>* mice. In contrast, increased proteoglycan levels and the preservation of the growth plate cartilage morphology were seen in *Tgfr2/Mmp13* dKO mice (Fig. 4). Deletion of the *Mmp13* gene alone had no significant disc phenotype (Fig. 4). These results suggest that *Mmp13* is a critical downstream target gene of

TGF- $\beta$  signaling in disc cells and inhibition of TGF- $\beta$  signaling may lead to growth plate and endplate tissue destruction through activation of *Mmp13* gene expression.

## Discussion

Two major cell types were identified in the nucleus pulposus region, notochord-like cells and chondrocyte-like cells. It has been reported that the immature disc nucleus pulposus consists of notochordal cells. With nucleus pulposus maturation, notochordal cells disappear and are replaced by chondrocyte-like mature nucleus pulposus cells in the human spine. It seems that the notochordal cells disappear at different stages in postnatal and adult animals in different species [20]. In humans, notochordal cells could be lost as early as 4 years of age [21]. In the rabbit, notochordal cells disappeared by 11 weeks of age [22]. In the mouse, notochordal cells are survived and greatly reduced at postnatal stage. The changes in disc phenotype coincide with early signs of disc degeneration [23]. Therefore, it is important to determine the origin of chondrocyte-like mature nucleus pulposus cells. It has been reported that postnatal transition from a notochordal to a cartilaginous nucleus pulposus is accomplished by the migration of chondrocyte-like cells from the hyaline cartilage endplate and inner annulus fibrosus region into the ectopic notochordal nucleus pulposus region at the postnatal stage [24–26]. A study using a rabbit model suggests that chondrocytes from the cartilage endplate can migrate toward the nuclear pulposus region as notochordal cells undergo apoptosis [21]. *In vivo* analysis of cell fate has been facilitated by the creation of Cre-dependent conditional reporter lines to track the fate of cell populations expressing Cre under the control of tissue-specific promoters [27]. Our findings from *Col2a1-CreER<sup>T2</sup>;R26R* mice indicate that no X-Gal positive cells migrated from the cartilage endplate and annulus fibrosus cells during the postnatal 4 month period, suggesting that chondrocyte-like cells in the nucleus pulposus region are not derived from the surrounding annulus fibrosus or cartilage endplate, at least at the early postnatal stage in mice.

Although TGF- $\beta$  signaling has been shown to play a critical role in spine and disc tissue development at embryonic stage [8, 13], its role in disc tissue growth and maintenance at postnatal stage have not been reported. Since cellular environments and gene expression profiles could be different at embryonic and postnatal stages, functions of TGF- $\beta$  signaling at these two developmental stages could be different. Our present study demonstrated that TGF- $\beta$  signaling is required for normal growth plate and endplate cartilage function at the postnatal stage. Loss of TGF- $\beta$  signaling in growth plate chondrocytes and inner annulus fibrosus cells led to the loss of matrix tissue and endplate cartilage cells and abnormal growth plate cartilage morphology. Since the expression of *Mmp13* gene was significantly up-regulated in disc cells of *Tgfb2<sup>Col2ER</sup>* mice, we also examined the role of *Mmp13* in disc tissue phenotype of *Tgfb2<sup>Col2ER</sup>* mice. We found that deletion of the *Mmp13* gene under *Tgfb2* null background completely reversed disc phenotype observed in *Tgfb2<sup>Col2ER</sup>* mice. These findings demonstrate that TGF- $\beta$ -*Mmp13* signaling plays an essential role in postnatal disc tissue growth and the maintenance.

## Acknowledgments

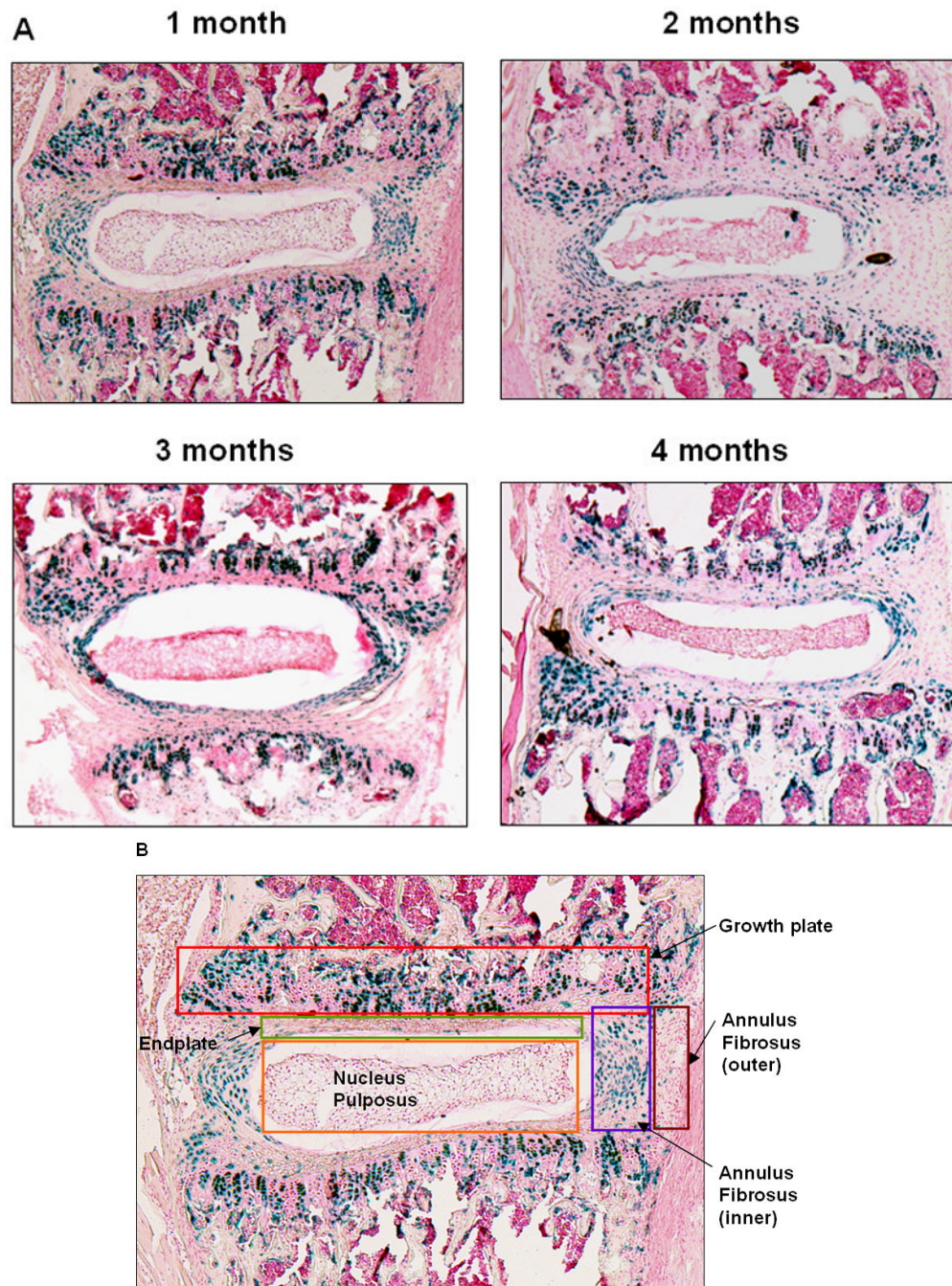
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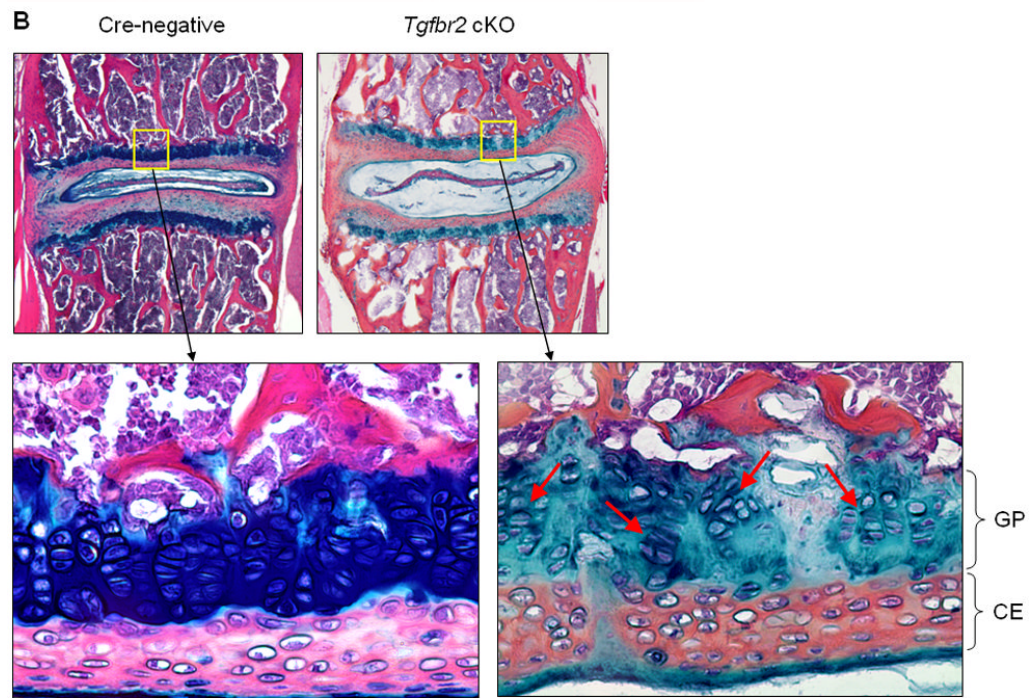
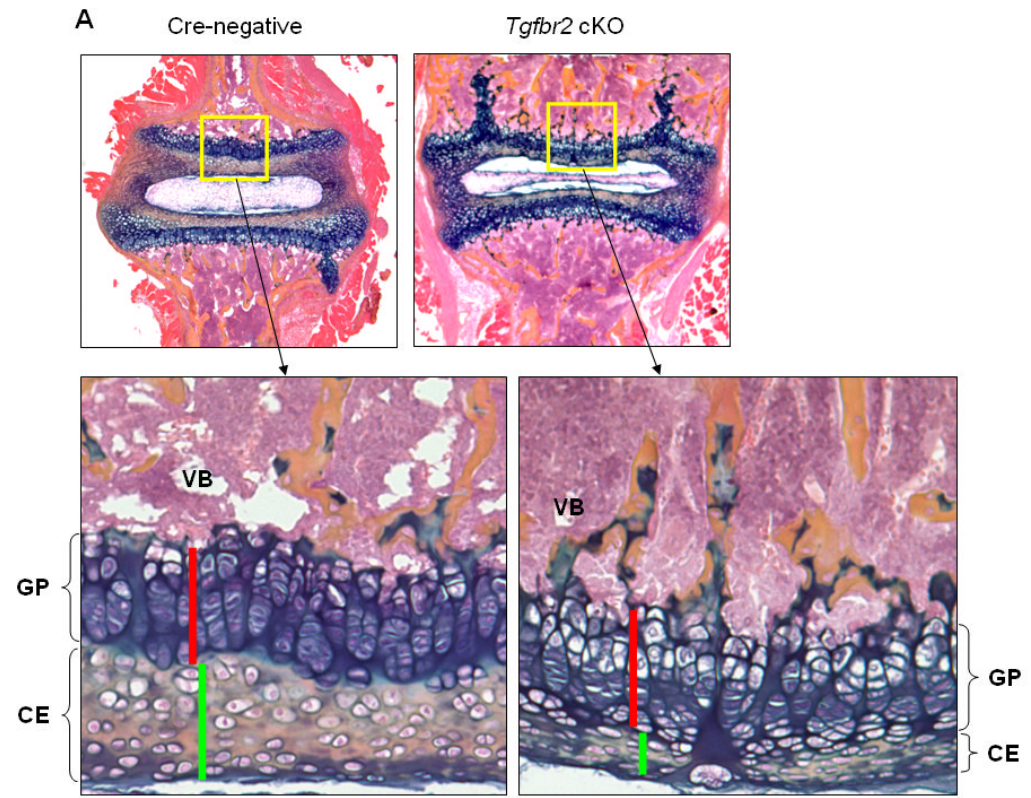
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**Fig. 1.** X-Gal staining of *Col2a1-CreER<sup>T2</sup>;R26R* mice. (A) Two-week-old *Col2a1-CreER<sup>T2</sup>;R26R* double transgenic mice were injected with tamoxifen (1 mg/10g body weight, x5 days, i.p. injection). Mice were sacrificed 1, 2, 3, and 4 months (a–d) after injections were completed and disc samples were fixed, decalcified and processed for frozen section preparation. X-Gal staining was performed and sections (L3/L4) were then counterstained with nuclear fast red. *Col2a1-CreER<sup>T2</sup>* targeted cells showed lacZ-positivity and stained blue. Recombination efficiency was evaluated by counting lacZ positive cells divided by the total cell number in the growth plate, cartilage endplate, inner and outer annulus fibrosus, and nucleus pulposus

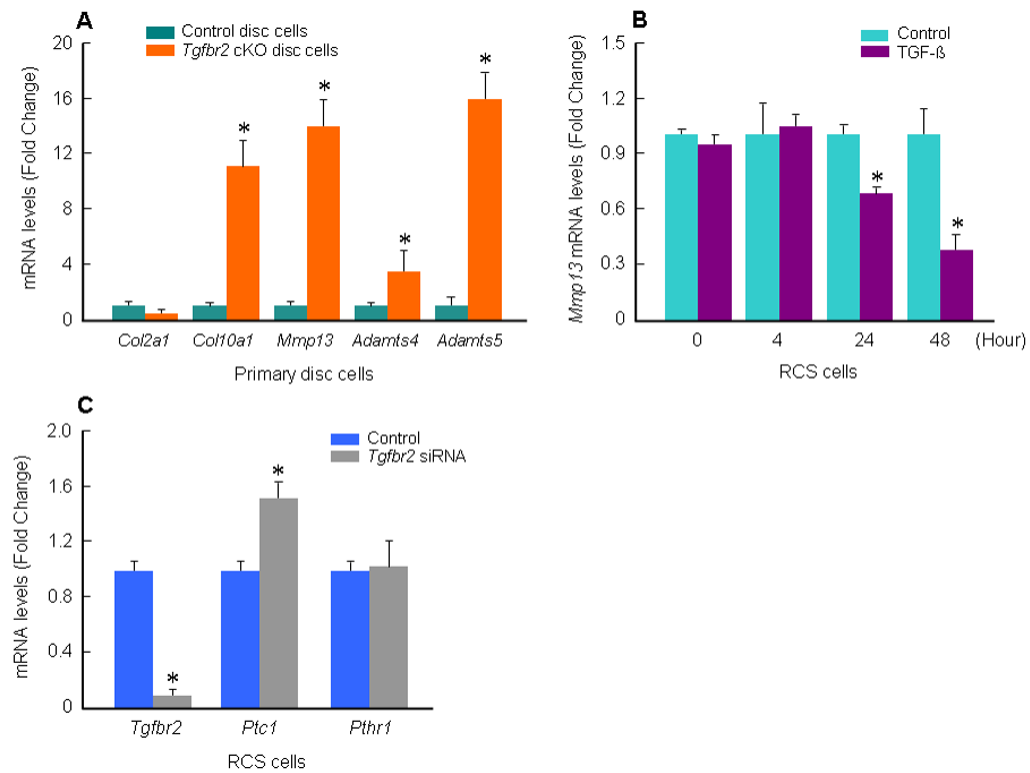


regions. Three non-consecutive histological sections from four different transgenic mice (n=4) were analyzed. High Cre-recombination efficiency in growth plate and inner annulus fibrosus cells was observed in *Col2a1-CreER<sup>T2</sup>* transgenic mice 1, 2, 3, and 4 months after tamoxifen induction. Relatively low Cre-recombination efficiency was found in cartilage endplate and outer annulus fibrosus cells. No lacZ-positive cells were found in the nucleus pulposus region. (B) Evaluation of Cre recombination efficiency. Tamoxifen was administered to 2-week-old *Col2a1-CreER<sup>T2</sup>;R26R* mice and mice were sacrificed 1 month after tamoxifen induction. X-Gal staining was performed and sections (L3/L4) were then counterstained with nuclear fast red. Total and X-Gal-positive cell numbers were counted in the growth plate, cartilage endplate, inner and outer annulus fibrosus, and nucleus pulposus regions.

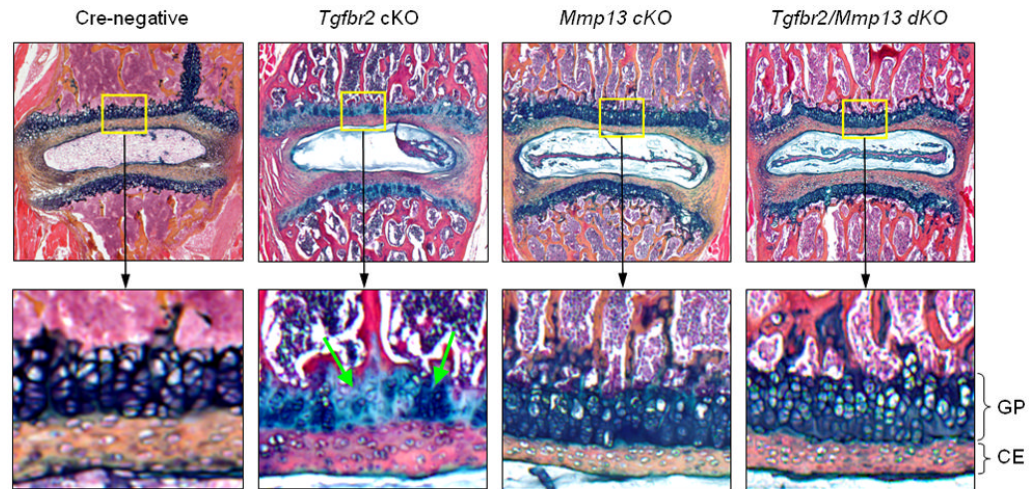


**Fig. 2.**

Inactivation of TGF- $\beta$  signaling leads to defects in the development and the growth of intervertebral disc tissue. (A) P14 *Tgfb $\beta$ 2<sup>Col2ER</sup>* mice were analyzed by histology. Histologic sections of disc samples (L3/L4) of P14 mice were stained with AB/H&E. Four Cre-negative (n=4) and three *Tgfb $\beta$ 2<sup>Col2ER</sup>* mice (n=3) were analyzed. Significant reduction in the length and area of cartilage endplate tissue (green bar) was observed in *Tgfb $\beta$ 2<sup>Col2ER</sup>* mice. GP: growth plate; CE: cartilage endplate; and VB: vertebral body. (B) AB/H&E staining were performed using histologic sections (L3/L4) of Cre-negative (n=5) and *Tgfb $\beta$ 2<sup>Col2ER</sup>* mice (n=6) mice. Growth plate chondrocytes lost their columnar structure and disorganized chondrocytes formed clusters (red arrows) in the growth plate region of *Tgfb $\beta$ 2<sup>Col2ER</sup>* mice.



**Fig. 3.** Gene expression analysis. (A) Total RNA was extracted from primary disc cells derived from P14 *Tgfb2*<sup>Col2ER</sup> mice and Cre-negative littermates. The mRNA expression of *Col2a1*, *Col10a1*, *Mmp13*, *Adamts4* and *Adamts5* was analyzed by real-time PCR. The expression of *Col10a1*, *Mmp13*, *Adamts4*, and *Adamts5* was significantly up-regulated in *Tgfb2*<sup>Col2ER</sup> mice. (B) Chondrogenic RCS cells were treated with 5ng/ml of TGF- $\beta$  for 4, 24 and 48 hours. The expression levels of *Mmp13* mRNA (normalized to the levels of  $\beta$ -actin mRNA) were analyzed by real time PCR. TGF- $\beta$  significantly inhibited *Mmp13* mRNA expression at 24 and 48 hour time points. (C) RCS cells were transfected with 20nM of *Tgfb2* siRNA or control siRNA for 48 hours and the total RNAs were extracted from cells and real time PCR was performed to assess changes in expression levels of *Tgfb2*, *Ptc1* and *Pthr1* mRNA. Knocking-down of the *Tgfb2* expression significantly enhanced *Ptc1* expression but had no effect on *Pthr1* expression. Unpaired Student *t*-test,  $p < 0.01$ ,  $n = 3$ .



TM induction: P14; Histology: 3-month-old, N=7

**Fig. 4.** Deletion of the *Mmp13* gene reverses the disc phenotype of 3-month-old *Tgfb2<sup>Col2ER</sup>* mice. Two-week-old *Tgfb2<sup>Col2ER</sup>* mice were injected with tamoxifen (1 mg/10g body weight, x5 days, i.p. injection) at 2-week-old. Mice were sacrificed at 3 months of age. The disc samples were fixed, decalcified and paraffin-embedded. The histological sections were stained with Alcian blue/H&E method.

Table 1

Primers and sequences for real-time PCR analysis

	Upper Primers	Lower Primers
<i>Tgfb<math>\beta</math>2</i>	5'-AGATGGCTCGTGAACTACCAA-3'	5'-AGAAATCCTGCTGCCTCTGGTCTTT-3'
<i>Col2a1</i>	5'-TGGTCCCTCTGGGCATCTCAGGC-3'	5'-GGTGAAACCTGCTGTTGCCCTC A-3'
<i>Col10a1</i>	5'-ACCCCAAGGACCTAAAGGAA-3	5'-CCCCAGGATACCCCTGTTTTT-3'
<i>Mmp13</i>	5'-TTTGAGAAACACGGGGAAGA-3'	5'-ACTTTTGTGGCCAAATCCAGG-3'
<i>Adamts4</i>	5'-GGCAGATGACAAAGATGGCAGCAT-3'	5'-AGACGAGTCAACCAACAAAGTTGACA-3'
<i>Adamts5</i>	5'-CCAAATGCCTTCAGCCACGATCA-3'	5'-AATGTCAAAGTTGCACCTGTGGGTG-3'
<i>Ptc1</i>	5'-TTCTGCTGCCCTGTCTCTTA-3'	5'-GCAAAACCCGGACGACACTT-3'
<i>Phr1</i>	5'-ACTCCTTCCAGGGATTTTTTGT-3'	5'-GAAAGTCCAAATGCCAGTGTTCCA-3'
$\beta$ -Actin	5'-TCAGGTACTGGTTCGGTCTG-3'	5'-ACCAGAGGCATACAGGGACAG-3'

Table 2

*Col2a1-CreERT2*-mediated Cre-recombination efficiency in disc cells

	1	2	3	4 (month)
Growth plate	79.9	74.7	73.5	70.6
Cartilage endplate	10.7	20.4	27.5	37.3
Inner annulus fibrosus	76.8	81.1	79.1	73.3
Outer annulus fibrosus	11.4	4.7	8.7	4.8
Nucleus Pulposus	0	0	0	0

Induction: 2-week-old; Sac: 1-, 2-, 3-, and 4-month-old after tamoxifen induction (n=4)