Ectopic expression of Smurf2 and acceleration of age-related intervertebral disc degeneration in a mouse model

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OBJECTIVE Lumbar intervertebral disc degeneration, an age-related process, is a major cause of low-back pain. Although low-back pain is a very common clinical problem in the aging population, no effective treatment is available, largely owing to lack of understanding of the molecular mechanisms underlying disc degeneration. The goal of this study was to characterize how ectopic expression of Smurf2 driven by the collagen Type II alpha 1 (Col2a1) promoter alters disc cell phenotype and associated cellular events, matrix synthesis, and gene expression during disc degeneration in mice.

METHODS To characterize how ectopic expression of Smurf2 in Col2a1-promoter working cells affects the disc degeneration process, the authors performed histological and immunohistochemical analysis of lumbar spine specimens harvested from wild-type (WT) and Col2a1-Smurf2 transgenic mice at various ages (n ≥ 6 in each age group). To elucidate the molecular mechanism underlying Smurf2-mediated disc degeneration, the authors isolated cells from WT and Col2a1-Smurf2 transgenic lumbar intervertebral discs and performed Western blot and real-time RT-PCR (reverse transcription polymerase chain reaction) to examine the protein and mRNA levels of interesting targets.

RESULTS The authors demonstrated that approximately 30% of WT mice at 10–12 months of age had started to show disc degeneration and that the disc degeneration process was accelerated by 3–6 months in Col2a1-Smurf2 transgenic mice. Chondrocyte-like cell proliferation, maturation, and fibrotic tissue formation in the inner annulus were often accompanied by fibroblast-to-chondrocyte differentiation in the outer annulus in transgenic discs. The chondrocyte-like cells in transgenic discs expressed higher levels of connective tissue growth factor (CTGF) than were expressed in WT counterparts.

CONCLUSIONS The findings that ectopic expression of Smurf2 driven by the Col2a1 promoter accelerated disc degeneration in Col2a1-Smurf2 transgenic mice, and that higher levels of CTGF protein and mRNA were present in Col2a1-Smurf2 transgenic discs, indicate that Smurf2 accelerates disc degeneration via upregulation of CTGF.

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KEY WORDS Smurf2; Col2a1 promoter; intervertebral disc degeneration; connective tissue growth factor; CTGF

Cartilage is a specialized type of connective tissue. Depending on its location in vivo, cartilage functions as growth plate cartilage, fibrocartilage, or articular cartilage. Growth plate cartilage is transient and is responsible for long-bone lengthening during development via the process of endochondral ossification, which consists of mesenchymal cell–chondrocyte differentiation (chondrogenesis), chondrocyte proliferation and matrix synthesis, chondrocyte maturation into hypertrophic chondrocytes, and replacement of hypertrophic tissue by bone. Articular cartilage covers the ends of long bone and functions in a stable state throughout postnatal life. However, during osteoarthritis, articular chondrocytes recapitulate the maturation process that normally occurs in the growth plate during endochondral ossification, leading to articular cartilage degradation. Fibrocartilage is uniquely situated within specific joint spaces to ensure smooth articulation and stress absorption. For example, menisci in knee joints and discs in intervertebral joints are the major fibrocartilage tissues found in the human body.
They possess complex geometrical shapes with highly organized matrix structures, which are necessary for proper tissue function.

The meniscus exhibits regional and zonal variations in its cellular composition and microstructure.\textsuperscript{15,18,35} The meniscus is wedge shaped and located at the periphery of the articular surface of the tibia.\textsuperscript{24} The outer two-thirds of the meniscus is fibrocartilage, where fibroblastic cells are fusiform in shape and mainly express Type I collagen and small amounts of Type II collagen and proteoglycans. The inner one-third of the meniscus consists of articular cartilage tissue in which the rounded chondrocytes balance the slow matrix turnover.\textsuperscript{36,39}

Similar to menisci, intervertebral discs also vary in cell phenotype and matrix structure from one region to another.\textsuperscript{9,10,35,34} An intervertebral disc consists of an annulus fibrosus ring, a nucleus pulposus core, and 2 endplates. The outer annulus is made up of highly ordered collagen lamellae in which Type I collagen fibers are aligned with elongated fibroblastic cells.\textsuperscript{34} The inner annulus differs from the outer section, containing spherical cells, more widely spaced layers, and greater amounts of Type II collagen and proteoglycans.\textsuperscript{9,31} The central nucleus, a hydrogel-like tissue, is predominantly composed of proteoglycans and Type II collagen. The highly negatively charged glycosaminoglycan in the nucleus provides osmotic properties that enable the nucleus to maintain height and turgor against compressive loads.\textsuperscript{23,42} The nucleus cells that produce and maintain the nucleus matrix are larger clusters of notochordal cells and relatively smaller chondrocyte-like cells.\textsuperscript{23} The endplate, situated at the articular surface of the intervertebral disc and the adjacent vertebrae,\textsuperscript{20} is a layer of articular cartilage–like tissue containing chondrocyte-like cells embedded within a cartilage matrix. The annulus, the nucleus, and the endplates are interconnected to form the most important part of the motion segment of the spine.

Low-back pain, a very common clinical problem, is frequently caused by lumbar intervertebral disc degeneration.\textsuperscript{12,34,20} Disc degeneration begins as early as the 2nd decade of life and is an inevitable consequence of aging.\textsuperscript{4} Thus, it is difficult to distinguish the physiological process of disc aging from that of degeneration; however, when structural failure of a disc is observed in combination with accelerated or advanced signs of aging, the disc is considered to be a degenerative disc.\textsuperscript{2} The process of disc aging/degeneration has been clearly described at macroscopic and histological levels. For example, with increasing age, water content decreases and collagen content increases in the nucleus. Clefts and tears first occur in the nucleus and endplates and then extend to the annulus, and loss of the annulus-nucleus boundary and the superficial layer of the endplates, fibroblast-chondrocyte differentiation, chondrocyte cloning, and annulus migration into the nucleus are frequently detected in aging/degenerating discs. In the late stage of disc degeneration, unsuccessful repair of structural failure leads to the formation of scar or fibrotic tissue, a process that results in the progression of structural failure rather than repair.\textsuperscript{1,2,23,33} Little is known about the regulation of disc aging or the progression of structural failure. It is known that connective tissue growth factor (CTGF) and its expression inducer transforming growth factor–β (TGFβ) are the key factors for regeneration of connective tissue during cutaneous wound healing and fibrosis formation when high CTGF expression by fibroblasts persists.\textsuperscript{17,19} However, it is not clear whether CTGF expression is altered in degenerative disc cells or in the cells that are prone to become degenerative or fibrotic tissue during disc aging or degeneration.

We have previously shown that ectopic expression of Smurf2, an E3 ubiquitin ligase, under the control of collagen Type II alpha 1 (Col2a1) promoter induces osteoarthritis in adult knee joints of Col2a1-Smurf2 transgenic mice.\textsuperscript{46} We found that when articular cartilage underwent chronic degeneration in Col2a1-Smurf2 transgenic mice, similar alterations occurred simultaneously in meniscus. Thus, we reasoned that Col2a1-Smurf2 transgenic mice might exhibit a disc degeneration phenotype. The goal of this study was to characterize how ectopic expression of Smurf2 in Col2a1 promoter working cells in mice altered disc phenotype and associated mechanisms.

### Methods

**Col2a1-Smurf2 Transgenic Mice**

We previously generated 3 lines of Col2a1-Smurf2 transgenic C57BL/6-SJL mice by cloning FLAG-tagged human Smurf2 cDNA downstream collagen Type II alpha 1 (Col2a1) promoter.\textsuperscript{45} We recently used materials and methods similar to those used in our previous study,\textsuperscript{45} including FLAG-tagged Smurf2 cDNA (Addgene) and Col2a1 promoter (Yoshihiko Yamada, NIH/NIDCR). In addition, the Smurf2 cDNA fragments were injected into fertilized C57BL/6 oocytes (Cyagen Biosciences). The cells with ectopic Smurf2 expression driven by the Col2a1 promoter in Col2a1-Smurf2 transgenic mice were identified by immunostaining with anti-FLAG M2 antibodies (Sigma). The positive cells for this staining were chondrocytes, osteoprecursors, and fibrocartilage cells, consistent with previous reports.\textsuperscript{37,41,45}

### Processing of Samples for Histological and Immunohistochemical Analysis

All procedures performed in mice were conducted in accordance with the policies and guidelines proposed in an animal protocol approved by Institutional Animal Care and Use Committee. Adult mouse knee joints and lumbar spine tissues were prepared for histology as described previously.\textsuperscript{33,45} Briefly, adult mouse knee joints and lumbar spine tissues were fixed, decalcified, and embedded in either paraffin or optimal cutting temperature compound (OCT). Five- and 10-μm-thick sections were cut for paraffin and OCT blocks, respectively, and 4–6 serial sagittal paraffin sections were harvested every 30 μm apart within the midsagittal region of knee joints and intervertebral bodies. Each group contained at least 6 mice; 3 sections from each sample were stained with Safranin-O–fast green (proteoglycan is red) and Alcian blue–H & E (cartilage is blue) as described previously.\textsuperscript{24,45} Phenotype was evaluated based on classification of age-related changes in lumbar intervertebral discs; OCT sections were stained with anti-CTGF (ab6992, abcam) as described previously.\textsuperscript{45}
Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) buffer was used to extract proteins from disc cells that were freshly isolated without culture. We used a modified method for isolation of disc cells based on previous reports. Briefly, lumbar discs were isolated from 6 mice in each group under a dissecting microscope and digested with 0.2% (w/v) Pronase (Roche) at 37°C for 1 hour and then with 0.02% (w/v) collagenase (Sigma) in DMEM/F-12. Cells were harvested by filtering the digested mixture through a 70-μm strainer. Proteins of 10–15 μg were separated by SDS-PAGE, blotted with anti-CTGF antibody (ab6992, abcam), and visualized through exposure of BioMax XAR film (Carestream).

Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted by using TRizol from cells similar to those used for Western blot. RNA was reverse transcribed into cDNA using SuperScript IV Reverse Transcriptase (ThermoFisher Scientific). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described by using SYBR Green. The primers for CTGF were 5′-CTG TCAAGTTTGAGCTTTCTGG-3′ and 5′-GGACTCAAA GATGTCATTTGTCGCC-3′; the primers for TGF-β1 were 5′-TGCTCGTCTTTGTACAACAGCA-3′ and 5′-GGTTTTCTCATAGATGGCGTGG-3′.

Results

Meniscus Aging and Degeneration in Wild-Type and Col2a1-Smurf2 Transgenic Mice

We previously showed that ectopically expressed Smurf2 driven by the Col2a1 promoter induces articular cartilage degeneration and osteophyte formation in adult knee joints in Col2a1-Smurf2 transgenic mice. Meanwhile, we also found that meniscus degeneration progresses at a pace similar to that of articular cartilage degeneration in these mice (Fig. 1). Specifically, in the wild-type
Smurf2 accelerates disc degeneration

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Transgenic Mice

CTGF Expression Is Increased in Intervertebral Discs in Col2a1-Smurf2 Transgenic Mice

Given the role of CTGF in chondrocyte maturation,22,26,28

Abnormal Chondrocyte Differentiation and Maturation Leads to Progression of Disc Degeneration in Col2a1-Smurf2 Transgenic Mice

To better understand the process of disc degeneration from nucleus breakdown at 10 months of age to expansion of it beyond the endplate into vertebra at 12 months of age in Col2a1-Smurf2 transgenic mice, we histologically examined serial disc sections from 11-month-old mice. Chondrocytes in the endplates underwent proliferation and formed column-like structures that Migration into the nucleus (Fig. 3B). Clefts and tears occurred in the front area of the migrating tissue, and scar/fibrotic tissue started to develop below the clefts and tears (Fig. 3B). Similarly, chondrocyte-like cells in the inner annulus also underwent proliferation, forming cell clusters (Fig. 3C and D), which transformed into large hypertrophic chondrocyte-like cells and migrated into the nucleus. Wide tears or tissue breakdown occurred within the “hypertrophic-like area” containing many hypertrophic cells, and fibrotic tissue was formed adjacent (Fig. 3C and D). In parallel with the cartilaginous formation in the inner annulus and endplates, cells in the outer annulus, which are normally fibroblast-like and predominantly express Type II collagen, differentiated into chondrocyte-like cells with expression of cartilage matrix proteins such as proteoglycans and Type II collagen (Figs. 2H, 3C, and 4F). In 8-month-old Col2a1-Smurf2 transgenic mice, when disc degeneration was at an early stage (i.e., loss of the annulus nucleus boundary [Fig. 4B] and minor breakdown of the superficial layer of the endplate [Fig. 4D]), we observed that the outer annulus cells became big and rounded, but without strong expression of Type II collagen (Fig. 4, compare B with A). These data, along with data from the 12-month-old Col2a1-Smurf2 transgenic mice, indicate that the degree of fibroblast differentiation into chondrocytes is parallel with the degenerative grade of discs.

CTGF Expression Is Increased in Intervertebral Discs in Col2a1-Smurf2 Transgenic Mice

Given the role of CTGF in chondrocyte maturation,22,26,28

Intervertebral Disc Aging and Degeneration in WT and Col2a1-Smurf2 Transgenic Mice

Given that ectopic expression of Smurf2 in Col2a1-promoter working cells induced meniscus degeneration in Col2a1-Smurf2 transgenic mice, the similarities between the meniscus and intervertebral disc prompted us to examine the phenotypic changes of intervertebral discs in these transgenic mice. We harvested lumbar spines from mice at various ages and performed histological and immunohistochemical analysis of the specimens. In 2-month-old WT mice, the matrix in the outer annulus was densely organized into lamellae, and the cells were fusiform in shape (Fig. 2A).9 The matrix in the inner annulus was loosely packed, and the cells were spherical.20 The matrix in the nucleus was a gel-like tissue, with loosely packed Type II collagen fibrils. The endplate was a layer of articular cartilage-like tissue, where the superficial zone contained Type II collagen, proteoglycans, and small flat cells (Fig. 2A; also see Fig. 4C). Compared with the discs of 2-month-old WT mice, discs of the age-matched Col2a1-Smurf2 transgenic mice exhibited decreased proteoglycan content in the inner annulus, nucleus, and superficial zone of the endplate and increased endplate thickness (Fig. 2B). In 6-month-old WT mice, the intervertebral disc structure and matrix components were similar to those in the 2-month-old WT mice (Fig. 2C). In the age-matched Col2a1-Smurf2 transgenic mice, however, tears in the annulus and matrix breakdown in the nucleus and endplate were often detected (Fig. 2D). Proteoglycan content in the inner annulus and nucleus was dramatically increased compared with that in the WT counterparts (Fig. 2; compare D with C), which was usually considered to be an attempt to initiate repair,2 similar to the alterations in articular cartilage from these transgenic mice.8 In the WT mice that were 10 months of age or older, more than one-third exhibited some disc degeneration (aging). For example, an increase in proteoglycan content in the inner annulus and nucleus was detected in 10-month-old mice (n = 3 of 8) (Fig. 2E), and annulus tears and nucleus matrix breakdown occurred in 12-month-old mice (n = 3 of 6) (Fig. 2G). These phenotypic changes in aging discs of the 10- to 12-month-old WT mice were similar to those in 6-month-old Col2a1-Smurf2 transgenic mice (Fig. 2, compare E and G with D). In contrast to the focal and minor disc degeneration (disc aging) seen in the 10- to 12-month-old WT mice, 10-month-old Col2a1-Smurf2 transgenic mice exhibited entire nucleus degredation (Fig. 2F). By 12 months, the nucleus degradation had expanded beyond the endplate into the calcified region of the vertebra (Fig. 2H). These data indicate that intervertebral disc degeneration is an age-related process,6 and ectopic expression of Smurf2 in Col2a1-promoter working cells greatly accelerates the degenerative process in Col2a1-Smurf2 transgenic mice.
wound healing, and fibrotic disease.\textsuperscript{19,17} Formation of scar tissue and fibrosis in the vicinity of structural defects observed in lumbar discs of \textit{Col2a1-Smurf2} transgenic mice could be due to an increase in CTGF expression levels. To test this hypothesis, we examined CTGF expression levels in lumbar discs by using immunohistochemical, Western blot, and real-time RT-PCR analyses. Immunostaining of specimens from 6-month-old WT mice with anti-CTGF antibody revealed that fibroblast-like cells in the outer annulus expressed a moderate level of CTGF (Fig. 5A), and this staining was barely detectable in the inner annulus and boundary cells. In the corresponding regions from the age-matched \textit{Col2a1-Smurf2} transgenic mice, however, much higher CTGF expression levels were detected in the chondrocyte-like cells differentiated from fibroblast-like cells in the outer annulus and in the enlarged inner annulus cells, which are normally small and spherical (Fig. 5B). By 10 months, these changes were more pronounced.

**FIG. 2.** Lumbar disc degeneration in \textit{Col2a1-Smurf2} transgenic mice. Safranin-O staining of lumbar intervertebral discs from WT (A, C, E, and G) and \textit{Col2a1-Smurf2} transgenic mice (B, D, F, and H) at various ages as indicated. \textbf{A and B}: Proteoglycan content was decreased in transgenic discs in 2-month-old mice. In WT mice (A) (n = 7), the matrix in the outer annulus was densely organized lamellae with spindle-like cells and that in the inner annulus was loosely packed (A, arrowhead) with spherical cells (A, \textit{black arrow}). The superficial zone of the endplate was a thin layer of proteoglycan-rich tissue (A, \textit{red arrow}). The nucleus was a gel-like tissue predominantly containing proteoglycans (stained to be red) with loosely packed Type II collagen fibrils (A, \textit{asterisk}). In the transgenic mice (B) (n = 7), the thickness of endplate was increased (B, \textit{black arrows}); the proteoglycan content in the inner annulus, nucleus, and endplates was decreased. \textbf{C and D}: Compensatory proteoglycan synthesis in transgenic mice at 6 months of age. Compared with WT disc (C), the proteoglycan content in transgenic disc (D) was increased in the inner annulus, nucleus, and endplates (n = 7). Arrows in D indicate annulus tears; \textit{arrowhead} and \textit{asterisk} indicate matrix breakdown in the endplate and nucleus, respectively. \textbf{E and F}: Mild disc aging in WT and nucleus degradation in transgenic mice at 10 months of age. Three of 8 WT mice exhibited increased proteoglycan content in the inner annulus and nucleus (E). Transgenic mice exhibited severe nucleus degeneration (F) (n = 6). \textbf{G and H}: Disc aging in WT and severe disc degeneration in transgenic mice at 12 months of age. In WT mice (G), 3 of 6 mice exhibited annulus tears (G, \textit{arrow}) and nucleus matrix breakdown (G, \textit{arrowhead}). In transgenic mice (H, n = 6), nucleus degradation expanded beyond the endplate to the growth plate of vertebra (H, \textit{arrows}). \textit{Asterisk} in (H) indicates inner annulus undergoing degeneration. \textit{Blue arrow} in (H) indicates fibroblast-chondrocyte differentiation in the outer annulus. Bar = 100 \textmu m. Figure is available in color online only.
FIG. 3. Abnormal chondrocyte proliferation and maturation leads to disc degeneration progression in Col2a1-Smurf2 transgenic mice. A–C: Sections were stained with Alcian blue–H & E (AHE). In 11-month-old WT mice (A), AHE staining detected outer annulus tears (A, arrow) and nucleus matrix degradation (A, asterisk) in intact discs. In 11-month-old transgenic mice (B and C) (B’ and C’ are higher-magnification images of boxed areas in B and C), endplate chondrocytes underwent proliferation and formed column-like structures and migrated into the nucleus (B, asterisk; B’, yellow arrow); clefts and tears were formed in the front area of the cartilaginous tissue (B’, yellow arrowheads); and fibrotic tissue started to form below the clefts and tears (B’, red arrow). Annulus chondrocyte-like cells formed cell clusters (C’, yellow arrow), within which the cells retained their hypertrophic characteristics (C’, arrowhead). Wide tears occurred within the hypertrophic-like tissue (C’, asterisk), and fibrotic tissue was formed adjacently (C’, thick arrow). Thin arrow and thick arrows in C indicate fibroblast-chondrocyte differentiation in the outer annulus and bony tissue in the outmost annulus, respectively. D: Consecutive section of C but stained with Safranin-O fast green. Thick arrow in D represents maturing cartilage in the connective area between the vertebra epiphysis and outer annulus. Thin arrow, arrowheads, asterisk, and thick arrow in D’ represent structures similar to those in C’. Bars = 100 μm. Figure is available in color online only.
in the inner annulus, i.e., more rounded chondrocyte-like cells with stronger CTGF staining in this area (Fig. 5D).

To confirm the immunohistochemical result, we analyzed CTGF protein and mRNA levels in freshly isolated disc cells from transgenic and WT mice (Fig. 5E). Western blot showed that the protein levels of CTGF (36–38 kD) in disc cells from 6- and 10-month-old Col2a1-Smurf2 transgenic mice were significantly higher than those from the corresponding WT controls (Fig. 5E). Consistently, the mRNA levels of CTGF in the similar transgenic disc cells were 3-fold higher than those in the corresponding WT controls (Fig. 5G). Furthermore, the levels of CTGF protein and mRNA in 10-month-old WT mice were slightly higher than those in 6-month-old WT mice, and a similar
pattern was detected in 10- and 6-month-old transgenic mice (Fig. 5E and G), suggestive of a trend toward increased CTGF expression levels with increasing age and degenerative grade, consistent with previous results in human and mouse discs.\(^5,33,38\)

Given the ability of TGF\(\beta\) to induce CTGF expression in many types of cells,\(^19,21,38\) we examined whether TGF\(\beta\) mRNA levels were increased in the Col2a1-Smurf2 transgenic disc cells versus WT control cells. Indeed, a small but significant increase in the TGF\(\beta 1\) mRNA level was detected in transgenic disc cells from 6- but not 8-month-old mice versus WT control cells (Fig. 5F).

**Discussion**

During the process of osteoarthritis development in Col2a1-Smurf2 transgenic mice,\(^44,45\) the cartilage-like tissue in the inner meniscus demonstrated similar alternations
in young and adult mice, including matrix degradation and calcification, and the outer meniscus root, which is fibrotic tissue near the synovial membrane and capsule, exhibited chondrogenesis and ossification in old mice (Fig. 1F). It appeared that the meniscus degeneration in the transgenic mice was a primary, not a secondary, consequence of articular cartilage degeneration, because the degeneration in the articular cartilage and meniscus occurred and progressed almost simultaneously.

Given the similarities between menisci and intervertebral discs, the degenerative phenotype in menisci in Col2a1-Smurf2 transgenic mice prompted us to examine the disc phenotype in transgenic mice. By comparing cell phenotype and matrix composition and architecture in Col2a1-Smurf2 transgenic mouse discs with those in WT controls at various ages (Figs. 2–4), we found that intervertebral discs underwent physiological aging in WT mice, and ectopic expression of Smurf2 driven by the Col2a1 promoter accelerated this aging process by 3–6 months, leading to disc degeneration. Although rodent models of disc degeneration have an increased propensity for disc repair due to high cell density and the presence of notochordal cells in the adult nucleus, Col2a1-Smurf2 transgenic mice exhibited a chronic disc degeneration phenotype. During the development of disc degeneration in this mouse model, we observed that many microscopic changes, such as fibroblast-to-chondrocyte differentiation, cell cloning, migration, and fibrosis, were similar to those occurring in humans. These similarities are probably attributable to the following: 1) The level of ectopic Smurf2 expression driven by the Col2a1 promoter in Col2a1-Smurf2 transgenic mouse chondrocytes was only 2.6-fold greater than that in WT controls, which might be just over a threshold level of Smurf2 expression in disc cells that can initiate certain molecular events associated with disc aging or degeneration progression. 2) Cells with ectopic Smurf2 expression driven by Col2a1 promoter were chondrocyte-like cells in the inner annulus for young adult Col2a1-Smurf2 transgenic mice and differentiated chondrocyte-like cells in the outer annulus for old transgenic mice. Therefore, most of the annulus cells in old Col2a1-Smurf2 transgenic mice were chondrocyte-like and expressed the transgene Smurf2, characteristics similar to those observed in cells in human degenerative discs, in which both inner annulus and nucleus cells were chondrocyte-like.

Given the role of CTGF in wound healing and fibrotic disease through its promotion of cell adhesion, migration, and proliferation along with matrix production and fibrosis formation, we speculated that the phenotypic changes such as chondrocyte-like cell clusters, migration of cartilaginous tissue into nucleus, and formation of fibrotic-like tissues in degenerative discs (Fig. 3) could be a result of increased CTGF levels in the intervertebral disc cells that were prone to becoming degenerative or fibrotic tissue. As expected, stronger staining for CTGF and higher CTGF protein and mRNA levels were detected in Col2a1-Smurf2 transgenic discs than in WT control discs (Fig. 5). Consistent with the CTGF expression pattern, TGFβ mRNA levels detected in the same transgenic discs were higher than those in WT control discs (Fig. 5F), although the increase was small or not significant. This finding is probably attributable to the following circumstances: TGFβ expression level does not always correlate with the levels of its biologically active polypeptides in the matrix environment due to posttranscriptional mechanisms, and CTGF can increase TGFβ activity by enhancing TGFβ binding to its receptors. Overall, when structural defects occur at an early stage of disc degeneration, disc cells attempt to initiate repair by activating chondrocyte maturation and matrix production, as well as CTGF gene expression. However, because the regenerated cartilaginous tissue cannot resist mechanical loading from daily activities, the result may be expansion of structural failure and persistence of CTGF expression, eventually leading to disc scarring and fibrosis.

Conclusions

Ectopic expression of Smurf2 under the control of Col2a1 promoter in mice induced disc degeneration by accelerating the age-related disc aging process. In addition, many phenotypic changes such as fibroblast-chondrocyte differentiation, chondrocyte cloning, and fibrotic tissue formation observed in Col2a1-Smurf2 transgenic degenerative discs were frequently detected in humans, and CTGF protein and mRNA levels were upregulated in these chondrocyte-like cells in Col2a1-Smurf2 transgenic discs. Thus, our findings indicate that Smurf2-mediated disc degeneration occurs via upregulation of CTGF, and this pathway may represent a novel mechanism for initiation or progression of disc degeneration in human beings.

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References

Smurf2 accelerates disc degeneration


**Disclosures**
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

**Author Contributions**
Conception and design: both authors. Acquisition of data: Wu. Analysis and interpretation of data: both authors. Drafting the article: Wu. Critically revising the article: both authors. Reviewed submitted version of manuscript: both authors. Approved the final version of the manuscript on behalf of both authors: Wu. Statistical analysis: Wu. Administrative/technical/material support: Wu. Study supervision: Wu.

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