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Transforming Growth Factor β Controls CCN3 Expression in Nucleus Pulposus Cells of the Intervertebral Disc

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Objective. **To investigate transforming growth factor β (TGFβ) regulation of CCN3 expression in cells of the nucleus pulposus.**

Methods. **Real-time reverse transcription–polymerase chain reaction and Western blot analyses were used to measure CCN3 expression in the nucleus pulposus. Transfections were used to measure the effect of Smad3, MAPKs, and activator protein 1 (AP-1) on TGFβ-mediated CCN3 promoter activity. Lentiviral knockdown of Smad3 was performed to assess the role of Smad3 in CCN3 expression.**

Results. **CCN3 was expressed in embryonic and adult intervertebral discs. TGFβ decreased the expression of CCN3 and suppressed its promoter activity in nucleus pulposus cells. DN-Smad3, Smad3 small interfering RNA, or DN-AP-1 had little effect on TGFβ suppression of CCN3 promoter activity. However, p38 and ERK inhibitors blocked suppression of CCN3 by TGFβ, suggesting involvement of these signaling pathways in the regulation of CCN3. Interestingly, overexpression of Smad3 in the absence of TGFβ increased CCN3 promoter activity. We validated the role of Smad3 in controlling CCN3 expression in Smad3-null mice and in nucleus pulposus cells transduced with lentiviral short hairpin Smad3. In terms of function, treatment with recombinant CCN3 showed a dose-dependent decrease in the proliferation of nucleus pulposus cells. Moreover, CCN3-treated cells showed a decrease in aggrecan, versican, CCN2, and type I collagen expression.**

Conclusion. **The opposing effect of TGFβ on CCN2 and CCN3 expression and the suppression of CCN2 by CCN3 in nucleus pulposus cells further the paradigm that these CCN proteins form an interacting triad, which is possibly important in maintaining extracellular matrix homeostasis and cell numbers.**

The intervertebral disc is a unique structure that permits rotation as well as flexion and extension of the human spine. It is comprised of a gel-like nucleus pulposus surrounded circumferentially by a ligamentous anulus fibrosus. Although cells comprise only ~1% of the tissue volume, they are critically important in maintaining a healthy disc (1). Nucleus pulposus cells secrete and organize a complex extracellular matrix that mainly contains the proteoglycan aggrecan and a small proportion of fibrillar collagens. This macromolecular assembly provides a robust hydrodynamic system that accommodates applied biomechanical forces to the spine (2–4). During degenerative disc disease, loss of disc cells, limited proteoglycan synthesis, and a shift toward synthesis of a fibrotic matrix decrease the water-binding capacity of the disc and result in a failure of the tissue to resist compressive loads (5).

Growth factors, in particular transforming growth factor β (TGFβ), are known to up-regulate aggrecan synthesis in the disc, and there is evidence that this molecule influences the activities of members of the CCN family of proteins (6,7). CCN proteins signal through heparan sulfate proteoglycans and integrins,

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interacting with, and regulating, the activity of many growth factors. They play important roles in such processes as cell differentiation, proliferation, extracellular matrix synthesis, and angiogenesis (8–12). The antiproliferative activities of CCN3 have been documented using embryonic fibroblasts, gliomal cells, vascular smooth muscle cells, and osteogenic mesenchymal cells (13–16); this activity is most likely mediated through association with, and enhancement of, the activities of Notch-1 and connexin 43 (15–17). In skeletal tissues, expression of a mutant, truncated CCN3 protein lacking the von Willebrand factor C domain results in enlarged axial and appendicular skeletal elements, increased bone mineralization, severe joint malformations, and disrupted growth plate organization (18). In epiphyseal chondrocytes, CCN3 is up-regulated early during differentiation, and treatment with the recombinant CCN3 protein causes enhanced expression of type X collagen, suggesting a requirement for CCN3 during the chondrocyte maturation process (19). Other than a single report that indicates CCN3 expression during early development of the notochord, the anlage of the nucleus pulposus (16), little is known about the regulation of CCN3 expression and function in the developing intervertebral disc.

The main goal of this investigation was to examine the expression and regulation of CCN3 in cells of the nucleus pulposus and to determine if CCN3 expression is regulated by TGFβ. Results of this study showed that TGFβ suppressed CCN3 gene expression in a Smad- and activator protein 1 (AP-1)–independent manner; importantly, Smad3 exerted an opposite effect on CCN3 expression. A second goal was to examine the effects of CCN3 on proliferation and on the expression of extracellular matrix components of nucleus pulposus tissue. The studies suggested that during the pathogenesis of degenerative disc disease, the interaction between TGFβ and members of the CCN family of proteins may provide a reparative function.

MATERIALS AND METHODS

Plasmids and reagents. The human CCN3 reporter plasmid (amino acids –1257 to +317) was from Kurt Engeland (Universitat Leipzig, Leipzig, Germany). Plasmid Smad2 was kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD), TAM67 by Nancy Colburn (National Institutes of Health [NIH], Bethesda, MD), DN-AP-1 and A-Fos by Charles Vinson (NIH, Bethesda, MD), AP-1 reporter plasmid by Silvio Gutkind (NIH, Bethesda, MD), psiLv-H1-EGFP, pLVTHM-ShSmad3, and pLVTHM by David Danielpour (Case Western Reserve University, Cleveland, OH), DN-p38 plasmids p38αAF, p38β2AF, p38γAF, and p38δAF by Jiahui Han (Scripps Research Institute, La Jolla, CA), and DN-ERK-1 (ERK-1K71R) and DN-ERK-2 (ERK-2K52R) by Melanie Cobb (University of Texas Southwestern Medical Center, Dallas, TX). Expression plasmids for Smad3 (ID no. 11742), DN-ALK-5 (ID no. 14834), CA-ALK-5 (ID no. 14833), lentiviral packaging psPAX-2 (ID no. 12260), and pMD2.G (ID no. 12259) were obtained from Addgene. As an internal transfection control, vector pGL4.10 (Promega) containing *Renilla reniformis* luciferase gene was used.

The amount of transfected plasmid, the pretransfection period after seeding, and the posttransfection period before harvesting were optimized for rat nucleus pulposus cells using pSV β-galactosidase plasmid (Promega) (20). Smad3-null mouse embryonic fibroblasts were provided by Dr. Rik Derynck (University of California, San Francisco, San Francisco, CA). Anti-CCN3 rabbit polyclonal K19M antibody was used for CCN3 detection (21). Recombinant human TGFβ3 and CCN3 were purchased from R&D Systems.

Isolation of nucleus pulposus cells and treatments of cells. Rat nucleus pulposus and anulus fibrosus cells were isolated using a previously reported method (20). Nucleus pulposus cells and mouse embryonic fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics. In some experiments, cells were treated with recombinant human TGFβ3 (10 ng/ml), recombinant CCN3 (50–500 ng/ml), or recombinant CCN2 (100 ng/ml), all from R&D Systems.

Immunohistologic studies. Freshly isolated rat and mouse spines or whole embryos were immediately fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and then embedded in paraffin. Transverse and coronal sections, 6–8 μm in thickness, were deparaffinized in xylene, rehydrated through graded ethanol, and stained with Alcian blue and with hematoxylin and eosin. For localizing CCN3, sections were incubated with the anti-CCN3 antibody (K19M) in 2% bovine serum albumin in PBS at a dilution of 1:100 at 4°C overnight. After thoroughly washing the sections, the bound primary antibody was incubated for 45 minutes at room temperature with Alexa Fluor 488–conjugated anti-rabbit secondary antibody (Invitrogen), at a dilution of 1:200. Sections were visualized using a fluorescence microscope (Olympus).

Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from nucleus pulposus cells using RNeasy Mini Columns (Qiagen). Before elution from the column, RNA was treated with RNase-free DNase I (Qiagen). The purified, DNA-free RNA was converted to complementary DNA (cDNA) using SuperScript III reverse transcriptase (Invitrogen). Template cDNA and gene-specific primers (for rat CCN3, 5′-TCATTGGAACCTGTACCTGCCACT-3′ [forward] and 5′-TCCCTGGGCACCTGTTACATTTCT-3′ [reverse]) were added to Fast SYBR Green Master Mix (Applied Biosystems) and messenger RNA (mRNA) expression was quantified using the StepOnePlus Real-Time PCR system (Applied Biosystems). GAPDH and β-actin were used to normalize the mRNA expression. Melting curves were analyzed to verify the specificity of the RT-PCR and the absence of primer–dimer formation. Each sample was analyzed in duplicate, and each analysis included a template-free control. All of the primers were synthesized by Integrated DNA Technologies.

Immunofluorescence microscopy. Cells were plated in 96-well flat-bottomed plates (4×10^3 cells/well) and were treated for 6–24 hours with TGFβ. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized for 10 minutes with 0.2% Triton X-100 in PBS, blocked by overnight incubation at 4°C with PBS containing 5% FBS, and incubated with antibodies against CCN3 (1:200 dilution). As a negative control, cells were reacted with isotype IgG under similar conditions. After washing, the cells were incubated with Alexa Fluor 488–conjugated anti-rabbit secondary antibody (Invitrogen) at a dilution of 1:200 for 45 minutes at room temperature. Cells were imaged using a laser scanning confocal microscope (Olympus FluoView).

Western blotting. Cells were placed on ice immediately following treatment and washed with ice-cold Hanks' balanced salt solution. All of the wash buffers and final resuspension buffer included a 1× protease inhibitor cocktail (Roche), NaF (5 m*M*), and Na_3VO_4 (200 μM). Total cell proteins were resolved on 8–12% sodium dodecyl sulfate–polyacrylamide gels and transferred by electroblotting to PVDF membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk in TBST (50 m*M* Tris, pH 7.6, 150 m*M* NaCl, 0.1% Tween 20) and incubated overnight at 4°C in 3% nonfat dry milk in TBST with the anti-CCN3 (1:600 dilution) or anti–β-tubulin (1:2,000 dilution) antibody (both from DSHB) or anti-GAPDH (1:2,000 dilution; Cell Signaling Technology). Immunolabeling was detected using enhanced chemiluminescence reagent (Amersham Biosciences).

Transfections and dual-luciferase assay. One day before transfection, cells were transferred to 24-well plates at a density of 4×10^4 cells/well. To measure the effect of TGFβ, cells were transfected with 500 ng of CCN3 reporter plasmids with 500 ng of the pRL-TK plasmid. In some wells, cells were treated with the inhibitors SB203580 (10 μM), SB202190 (10 μM), or PD98059 (10 μM) (all from Calbiochem). To investigate the effect of Smad3 or AP-1 on CCN3 promoter activity, cells were cotransfected with A-Fos (100–300 ng), TAM67 (100–300 ng), DN-Smad3 (100–300 ng), Smad3 (100–300 ng), or backbone vector with 400 ng of CCN3 reporter and 300 ng of pRL-TK plasmid in the presence or absence of TGFβ (10 ng/ml). To investigate the effects of p38 and ERK signaling, cells were transfected with 100–300 ng of dominant-negative p38α, β2, γ, or δ isoforms or 100–300 ng of DN-ERK-1 or ERK-2. For each transfection, plasmids were premixed with the transfection reagent (Lipofectamine 2000; Invitrogen). The next day, the cells were harvested and a dual-luciferase reporter assay system (Promega) was used for sequential measurements of firefly and *Renilla* luciferase activities. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20; Turner Designs). At least 3 independent transfections were performed, and all analyses were carried out in triplicate.

Lentiviral particle production and viral transduction. Two days before transfection, HEK 293T cells were seeded in 10-cm plates (1.3×10^6 cells/plate) in DMEM with 10% heat-inactivated FBS. Cells were transfected with 2.5 μg of psiLv-H1-EGFP, pLVTHM-LacZ, and shSmad3 plasmids, along with 1.875 μg of psPAX-2 and 0.625 μg of pMD2.G. After 16 hours, the transfection medium was removed and replaced with DMEM containing 5% heat-inactivated FBS and penicillin/streptomycin. Lentiviral particles were harvested at 48 and 60 hours posttransfection. One day before transduction, nucleus pulposus cells were plated in DMEM with 5% heat-inactivated FBS. Cells (in 10-cm plates) were transduced with 5 ml of conditioned medium containing viral particles along with 6 μg/ml of Polybrene. After 24 hours, the conditioned medium was removed and replaced with DMEM containing 5% heat-inactivated FBS with 5 μg/ml of doxycycline. Cells were harvested for protein extraction 5 days after viral transduction.

MTT assay. Cell proliferation was measured by MTT assay. After treatment for 24 or 72 hours, MTT diluted in serum-free DMEM was added to the culture medium to a final concentration of 0.5 mg/ml. At the end of the incubation period (2 hours at 37°C), the medium was removed, and the precipitated formazan crystals were solubilized in DMSO. Product formation was measured by reading the absorbance at 560 nm using a microplate reader (SpectraFluor Plus; Tecan).

Cell cycle analysis. Following treatment, single-cell suspensions were prepared from cell cultures and fixed for 1 hour in ice-cold 70% ethanol. Cells were washed and resuspended in PBS with 5% FBS. Cells were incubated with 50 μM propidium iodide for 30 minutes at 37°C. Cell cycle analysis was conducted using a Coulter Epics XL-MCL system with XL System II software.

Statistical analysis. All measurements were performed in triplicate. Results are presented as the mean ± SEM. Differences between groups were analyzed by Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Expression of CCN3 in the intervertebral disc. Sagittal sections of neonatal (Figure 1A) and skeletally mature (Figure 1B) rat intervertebral discs were stained with an antibody to CCN3 or were counterstained with hematoxylin and eosin and with Alcian blue (Figures 1C and D). (Negative controls were also prepared, and the results are available upon request from the author.) As indicated in Figures 1A and B, CCN3 was expressed by cells of the nucleus pulposus, anulus fibrosus, and cartilaginous end plate. In the embryonic nucleus pulposus, staining was localized to the cytosol as well as to the pericellular matrix. In contrast, at this early stage in the anulus fibrosus, staining was primarily cellular. In skeletally mature animals, both discal tissues showed cellular as well as pericellular staining. CCN3 expression in the embryonic discs was more prominent than that in discs from skeletally mature animals.

Expression of CCN3 in native tissues and cultured cells was studied using Western blot analysis. Both nucleus pulposus and anulus fibrosus tissues expressed a 54-kd band, representing the full-length secreted CCN3 (Figure 1E), while a prominent 48-kd band, representing

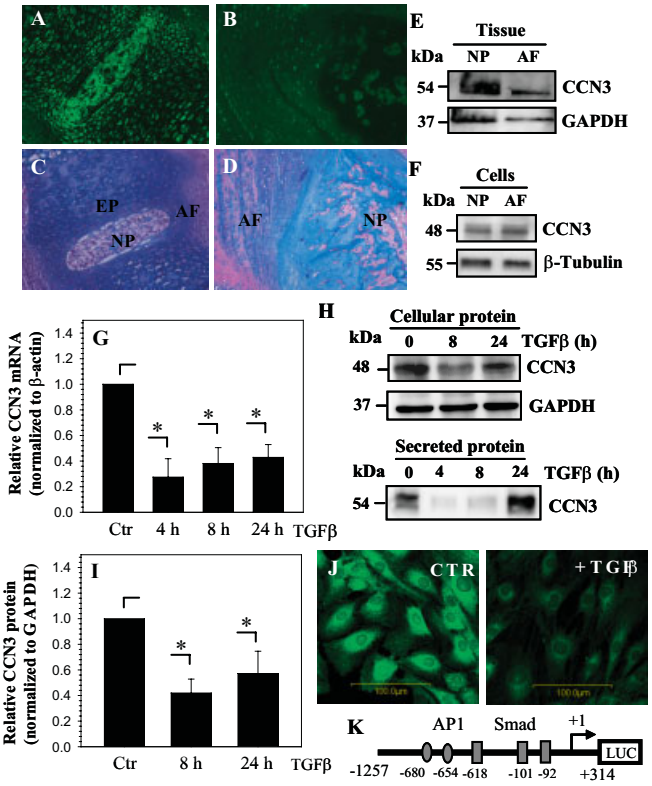

Figure 1. Transforming growth factor β (TGFβ) suppression of CCN3 expression in the intervertebral disc. **A–D,** Sagittal sections of embryonic (**A** and **C**) and skeletally mature (**B** and **D**) rat discs treated with anti-CCN3 antibody (**A** and **B**) or stained with hematoxylin and eosin and with Alcian blue (**C** and **D**). CCN3 is expressed in the nucleus pulposus (**NP**) and anulus fibrosus (**AF**) of both the neonatal and skeletally mature rat. **EP** = end plate cartilage. Original magnification × 20. **E** and **F,** Western blot analysis of CCN3 expression in rat nucleus pulposus and anulus fibrosus tissue (**E**) and cultured cells (**F**). A 54-kd CCN3 band is present in tissue extracts, while a 48-kd band is present in cultured cells. GAPDH and β-tubulin were used as controls. **G,** Real-time reverse transcription–polymerase chain reaction analysis of CCN3 mRNA expression by nucleus pulposus cells treated with TGFβ for the indicated times. TGFβ significantly decreased CCN3 mRNA expression. Values are the mean ± SEM of 3 independent experiments. ∗ = $P < 0.05$. **H,** Western blot analysis of CCN3 in nucleus pulposus cells and conditioned media treated with TGFβ for the indicated times. GAPDH was used as control. **I,** Densitometric analysis of CCN3 protein expression by nucleus pulposus cells treated with TGFβ for the indicated times. TGFβ significantly decreased CCN3 protein expression. Values are the mean ± SEM of 3 independent experiments. ∗ = $P < 0.05$. **J,** Immunofluorescence analysis of nucleus pulposus cells left untreated or treated with TGFβ. TGFβ-treated cells show decreased CCN3 expression. Original magnification × 20. **K,** Human CCN3 promoter construct (amino acids –1257 to +317) used to examine whether TGFβ regulates CCN3 promoter activity. A few of the major transcription factor binding sites are indicated. **Arrow** indicates the transcription start site at +1. AP-1 = activator protein 1; Luc = luciferase.

intracellular CCN3, was expressed in cultured nucleus pulposus and anulus fibrosus cells (Figure 1F). Western blot analysis of conditioned media from nucleus pulposus cells showed that full-length CCN3 was secreted into the media (Figure 1H).

TGFβ-induced decrease in CCN3 gene expression in nucleus pulposus cells. To explore the premise that TGFβ regulates CCN3 expression, nucleus pulposus cells were treated with TGFβ, and expression of CCN3 was analyzed by real-time RT-PCR. Figure 1G shows that treatment with TGFβ for 4, 8, and 24 hours significantly decreased CCN3 mRNA levels in nucleus pulposus cells. Western blot analysis indicated a similar decline in CCN3 protein expression with TGFβ treatment (Figure 1H). TGFβ treatment also decreased the levels of secreted CCN3 (Figure 1H), although at 24 hours, secreted levels had returned to baseline levels. Densitometric analysis of Western blots from 3 independent experiments was performed and showed significant suppression of CCN3 protein levels by TGFβ (Figure 1I). Likewise, immunofluorescence microscopy of TGFβ-treated nucleus pulposus cells showed that treatment caused a strong decrease in CCN3 expression (Figure 1J).

To investigate whether TGFβ regulates CCN3 promoter activity, we transfected nucleus pulposus cells with a human CCN3 promoter construct encompassing amino acid positions –1257 to +317 (Figure 1K) and measured promoter activity following treatment of cells with TGFβ. CCN3 promoter activity was significantly decreased with TGFβ treatment (Figure 2A). To confirm the involvement of TGFβ signaling, cells were cotransfected with increasing doses of DN-ALK-5, the primary TGFβ type I receptor in nucleus pulposus cells. Figure 2B shows that coexpression of the DN-ALK-5 blocks suppression of CCN3 promoter activity by TGFβ. CCN3 promoter activity also decreased when cells were cotransfected with constitutively activated CA-ALK-5 in the absence of TGFβ (Figure 2C), suggesting a role for TGFβ in the regulation of CCN3 expression in nucleus pulposus cells.

Independence of TGFβ-induced CCN3 suppression on Smad and AP-1 signaling. To elucidate the mechanism of CCN3 suppression by TGFβ, we first investigated the involvement of the Smad signaling pathway. Nucleus pulposus cells were cotransfected with DN-Smad3 (Figure 2D) or small interfering RNA (siRNA) against Smad3 (data available upon request from the author), and then treated with TGFβ. Figure 2D shows that blocking Smad3 function had no effect on

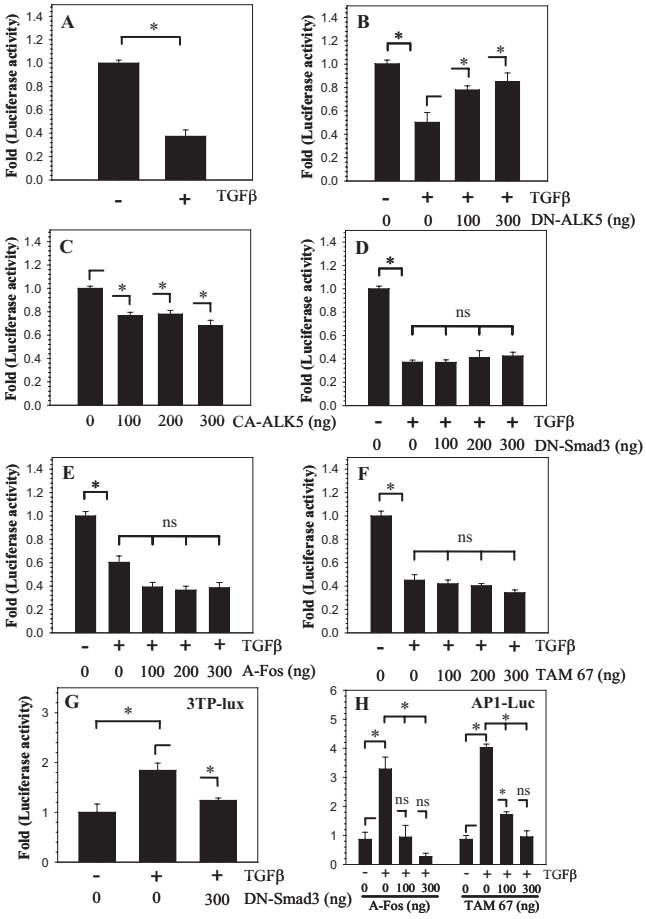

Figure 2. Transforming growth factor β (TGFβ)–induced decrease in CCN3 promoter activity in rat nucleus pulposus cells independent of Smad and activator protein 1 (AP-1) signaling. **A,** Decreased CCN3 promoter activity in cells transfected with a CCN3 promoter construct and left untreated or treated with TGFβ (10 ng/ml). **B,** Blockade of TGFβ-mediated suppression of CCN3 promoter activity in cells transfected with increasing doses of DN-ALK-5 in the presence of TGFβ. **C,** Decreased CCN3 promoter activity in cells transfected with increasing doses of CA-ALK-5 in the absence of TGFβ. **D–F,** No significant changes in CCN3 promoter repression by TGFβ in cells transfected with increasing doses of DN-Smad3 (**D**), A-Fos (**E**), or TAM 67 (**F**) in the presence of TGFβ. Control experiments were performed to validate dominant-negative plasmids. **G,** Increased 3TP-Lux reporter activity with TGFβ treatment and decreased activity with addition of DN-Smad3. **H,** Increased AP-1 reporter activity with TGFβ treatment and decreased activity with TAM67 and A-Fos treatment at the indicated concentrations. Values are the mean ± SEM of 3 independent experiments. $* = P < 0.05$. NS = not significant.

TGFβ suppression of CCN3 promoter activity. Next, we examined another important mediator of TGFβ action, the AP-1 pathway, which we previously showed to be induced by TGFβ in the nucleus pulposus (22). Cells were cotransfected with dominant-negative forms of the Fos (A-Fos) and Jun (TAM67) subunits of AP-1, which inhibit the transcriptional activity of endogenous AP-1. Figures 2E and F indicate that coexpression of either of the DN-AP-1 subunits had no effect on TGFβ-dependent actions on the CCN3 promoter activity. Control experiments were performed using Smad-responsive 3TP-Lux and AP-1–responsive reporters to validate the inhibitory function of DN-Smad3 (Figure 2G), TAM67, and A-Fos plasmids (Figure 2H).

Role of MAPK signaling in TGFβ suppression of CCN3. We next investigated the involvement of the MAPK signaling pathway in TGFβ-mediated suppression of CCN3 promoter activity. Nucleus pulposus cells were treated with a p38 inhibitor (SB203580 or SB202190) or an ERK inhibitor (PD98059) or their combination in the presence of TGFβ. As shown in Figure 3A, treatment with the individual p38 and ERK inhibitors partially counteracted the suppressive effects of TGFβ on CCN3 promoter activity. The combination of p38 and ERK inhibitors rescued CCN3 promoter activity almost to basal levels (Figure 3A). As a control, cells were treated with p38 and ERK inhibitors without TGFβ, which did not cause a significant change in CCN3 promoter activity (data available upon request from the author). Real-time quantitative PCR analysis of cells treated with the combination of MAPK inhibitors in the presence of TGFβ confirmed this regulation at the mRNA level (Figure 3B). We then cotransfected nucleus pulposus cells with plasmids encoding DN-p38 isoforms α, β, γ, or δ to examine which isoform is important for mediating TGFβ suppression of CCN3. Figures 3C and D show that, individually, neither of the dominant-negative isoforms of p38 blocked the suppressive effects of TGFβ on CCN3 promoter activity (results not shown for DN-p38α and DN-p38γ). Similarly, cotransfections with DN-ERK-1 and DN-ERK-2 also showed little effect on CCN3 promoter activity (Figures 3E and F).

Positive regulation of CCN3 expression by Smad3. To further determine if Smad3 exerts an effect independent of TGFβ signaling on CCN3 promoter activity, we overexpressed Smad3 in nucleus pulposus cells in the absence of exogenous TGFβ. Surprisingly, cotransfection with Smad3 resulted in an almost 7-fold increase in CCN3 promoter activity (Figure 4A). Figure 4B shows that overexpression of Smad3 in the presence of TGFβ rescued the suppression of CCN3 promoter activity by the growth factor. We suppressed Smad3 expression in nucleus pulposus cells by transduction with lentivirus expressing Smad3 short hairpin RNA

(shRNA). Figure 4C shows that there was a significant decrease in Smad3 expression in nucleus pulposus cells and that shRNA-mediated suppression was specific to Smad3. CCN3 expression was reduced in the shSmad3-transduced cells compared to nontransduced cells or cells transduced with control shRNA (Figure 4C). Figure 4D shows robust expression of green fluorescent protein in virally transduced cells, indicating a high level of transgene expression. Moreover, comparison of the

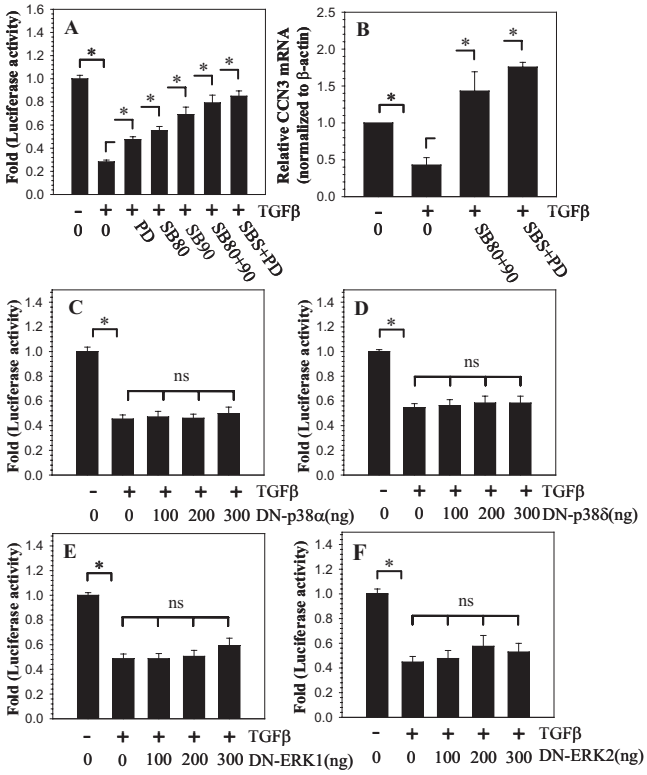

Figure 3. Partial dependence of transforming growth factor β (TGFβ)–mediated CCN3 suppression on p38 and ERK signaling in rat nucleus pulposus cells. **A,** CCN3 promoter activity in cells transfected with a CCN3 promoter construct and treated with TGFβ in the presence of inhibitors of p38 (SB203580 [SB80] or SB202190 [SB90]) and ERK (PD98059 [PD]) signaling, both individually and in combination (used at 10 μM each). Suppression of CCN3 promoter activity is partially blocked by individual inhibitors and almost fully blocked by their combination. **B,** Real-time reverse transcription–polymerase chain reaction analysis of cells treated with TGFβ and similar combinations of MAPK inhibitors (SB80+90 and SBS+PD), showing restoration of CCN3 expression. **C–F,** CCN3 promoter activity in cells transfected with increasing doses of dominant-negative isoforms of p38α (**C**) or p38δ (**D**), or dominant-negative ERK-1 (**E**) or ERK-2 (**F**) in the presence of TGFβ. Suppression of individual MAPK isoform function did not alter CCN3 promoter activity. Values are the mean ± SEM of 3 independent experiments. $* = P < 0.05$. NS = not significant.

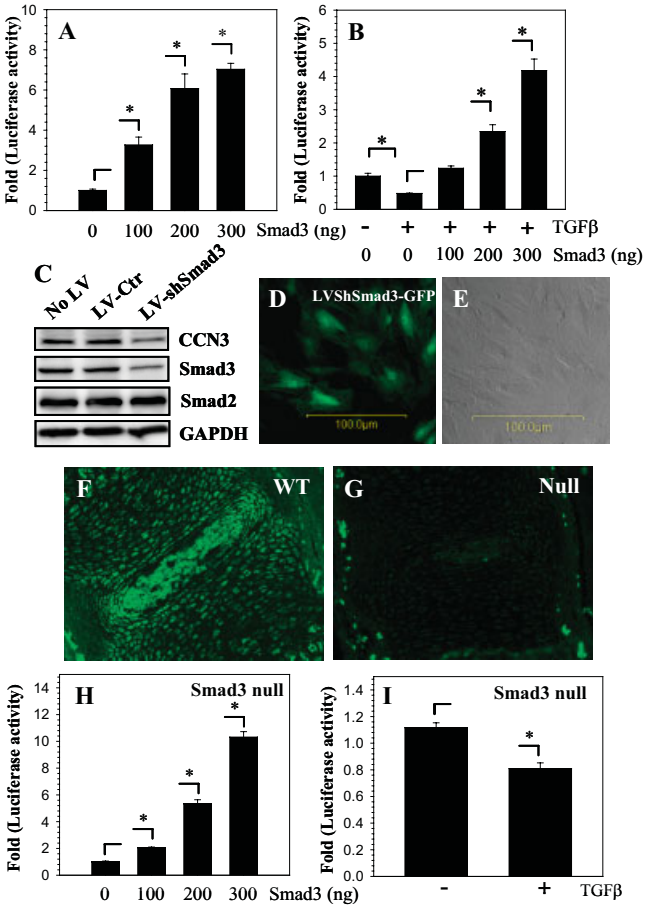

Figure 4. Increases in CCN3 promoter activity induced by Smad3. **A,** Dose-dependent increase in CCN3 promoter activity of rat nucleus pulposus cells transfected with Smad3. **B,** Counteractivation of transforming growth factor β (TGFβ)–induced suppression of CCN3 promoter activity in rat nucleus pulposus cells by Smad3 overexpression. **C,** Western blot analysis of nucleus pulposus cells transduced with short hairpin Smad3 lentivirus (LV-shSmad3), as compared with no lentivirus (No-LV) and control lentivirus (LV-Ctr). Smad3 suppression resulted in a concomitant decrease in CCN3 expression. Smad3 silencing had no effect on Smad2 expression. **D** and **E,** Photomicrographs of rat nucleus pulposus cells transduced with shSmad3 lentivirus. The green fluorescent protein (GFP) reporter expression in cells transduced with shSmad3 lentivirus (**D**), as compared with the transmitted-light image of the cells (**E**), shows efficient viral transduction. Original magnification × 20. **F** and **G,** Photomicrographs of sagittal sections of intervertebral disc from a wild-type (WT) mouse (**F**) and a Smad3-null mouse (**G**) treated with anti-CCN3 antibody, showing higher expression of CCN3 in the section from the wild-type mouse. Original magnification × 20. **H,** Dose-dependent increase in CCN3 promoter activity in Smad3-null mouse embryonic fibroblasts transfected with increasing doses of Smad3. **I,** Decreased CCN3 promoter activity in Smad3-null mouse embryonic fibroblasts treated with TGFβ. Values in **A**, **B**, **H**, and **I** are the mean ± SEM of 3 independent experiments. $* = P < 0.05$.

green fluorescence protein image to the transmitted-light image showed that most of the cells were transduced with the lentivirus (Figures 4D and E).

To confirm these results, sections of intervertebral disc from wild-type mice (Figure 4F) and Smad3-null mice (Figure 4G) were stained with a CCN3 antibody. The Smad3-null mouse disc showed less CCN3 expression than the wild-type disc (Figure 4G). Similarly, when Smad3 was overexpressed in embryonic fibroblasts derived from Smad3-null mice, a significant increase in CCN3 promoter activity was seen (Figure 4H). Last, treatment of Smad3-null mouse embryonic fibroblasts with TGFβ decreased CCN3 expression (Figure 4I).

CCN3-modulated proliferation of nucleus pulposus cells. To delineate the effect of CCN3 on nucleus pulposus cell proliferation, we treated cells with an increasing dose of recombinant CCN3 (10–500 ng/ml) for 24 or 72 hours. Figures 5A and B show phase-contrast microscopy images of cells treated with 100 ng/ml of CCN3 for 24 hours (Figure 5A) compared with control (Figure 5B). There was no appreciable differ-

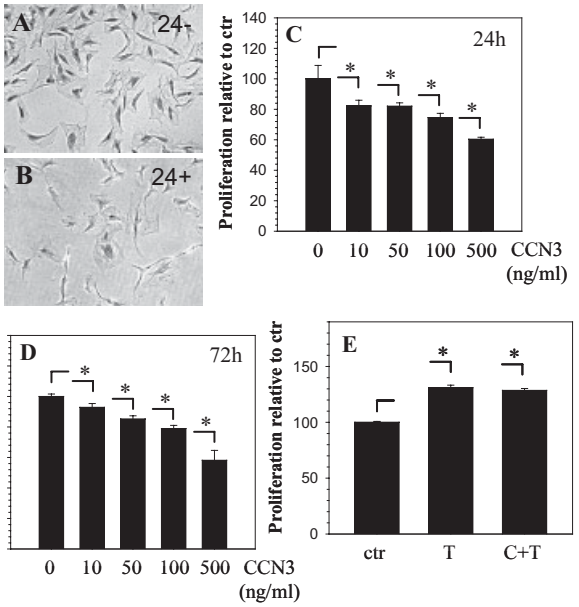

Figure 5. Antiproliferative effect of CCN3 on nucleus pulposus cells. **A** and **B,** Phase-contrast images of untreated control cells (24–) (**A**) and cells treated for 24 hours with 100 ng/ml of recombinant CCN3 (24+) (**B**). Crystal violet stained; original magnification $\times$ 10. **C** and **D,** Effects of increasing doses of recombinant CCN3 on cell proliferation for 24 hours (**C**) and 72 hours (**D**), as determined by MTT assay. Cell proliferation decreased with increasing doses of CCN3 at both time points. **E,** Effects of transforming growth factor β (TGFβ) (T) or CCN3 in combination with TGFβ (C+T) on the proliferation of nucleus pulposus cells after 72 hours of treatment. TGFβ treatment increased cell proliferation, while treatment with CCN3 plus TGFβ produced no significant change. Values in **C–E** are the mean $\pm$ SEM of 3 independent experiments. $* = P < 0.05$.

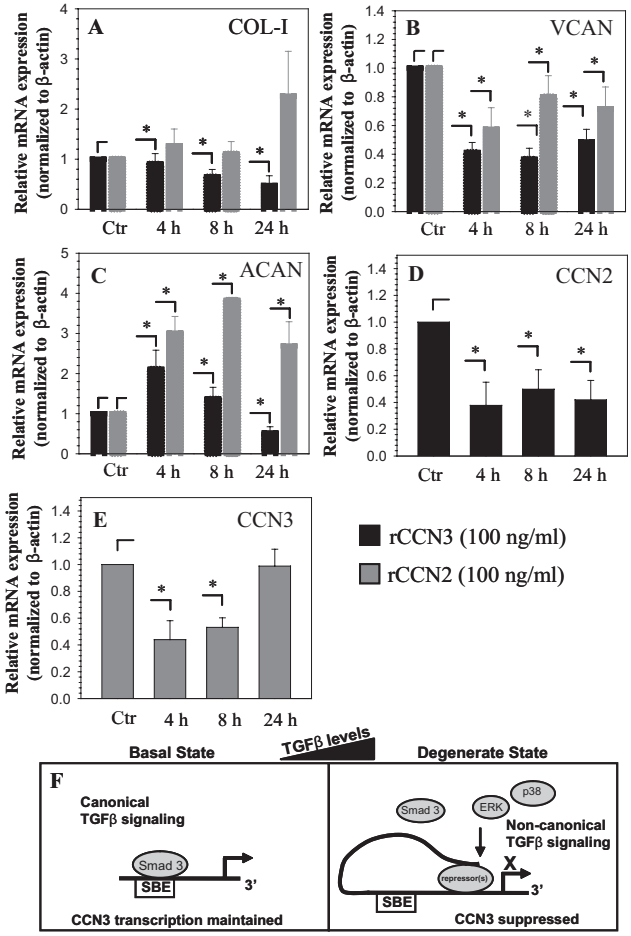

Figure 6. CCN3-induced decrease in the expression of extracellular matrix genes by nucleus pulposus cells. **A–D,** Real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis of cells treated with 100 ng/ml of either recombinant CCN3 (rCCN3) or rCCN2 for the indicated times. There are changes in the expression of type I collagen (COL-I) (**A**), versican (VCAN) (**B**), aggrecan (ACAN) (**C**), and CCN2 (**D**) as compared with controls. **E,** Real-time RT-PCR analysis of cells treated with CCN2 for the indicated times. CCN3 mRNA levels decreased at 4 and 8 hours, returning to baseline at 24 hours. Values in **A–E** are the mean $\pm$ SEM of 3 independent experiments. $* = P < 0.05$. **F,** Proposed model for the regulation of CCN3 expression by Smad3 and transforming growth factor β (TGFβ). In the basal state, when TGFβ levels are low, binding of Smad3 to Smad-binding element (SBE) maintains the baseline CCN3 expression. When TGFβ levels are elevated, phosphorylation of the TGFβ receptor type I/activin receptor–like kinase 5 (ALK-5) activates the noncanonical MAPK signaling pathways and phosphorylates the canonical effector Smad3. Enhanced noncanonical ERK and p38 signaling and possible recruitment of an as-yet-unknown repressor to the promoter may limit interaction of the Smads with the promoter, resulting in overall suppression of CCN3 expression.

ence in cellular morphology between the treated and untreated groups, although cell density appeared lower in the treated group. MTT assay showed that cell proliferation decreased with increasing doses of CCN3 at 24 and 72 hours (Figures 5C and D). To examine whether the effect on proliferation was due to modulation of the cell cycle, we treated cells with 100 ng/ml of CCN3, and cell cycle analysis was performed using fluorescence-activated cell sorting (FACS). The results from 4 independent experiments showed that CCN3 had no significant effect on the cell-cycle status of nucleus pulposus cells, with a mean ± SEM of 81.34 ± 4.7% of control cells in G_0/G_1 phase, 5.37 ± 2% in S phase, and 12.12 ± 5.91% in G_2/M phase, and with 76.9 ± 8.2% of CCN3-treated cells in G_0/G_1 phase, 8.02 ± 3.5% in S phase, and 14.83 ± 5.2% in G_2/M phase. To examine the effect of CCN3 on TGFβ-dependent cell proliferation, we treated the cells with TGFβ or with TGFβ together with CCN3 for 72 hours. Figure 5E shows that TGFβ treatment resulted in a small increase in cell proliferation, but when combined with CCN3, there was no significant difference in cell proliferation.

CCN3-modulated expression of critical extracellular matrix genes in the nucleus pulposus. Finally, we examined the effect of CCN3 treatment on the expression of critical extracellular matrix genes in the nucleus pulposus cells. As shown in Figures 6A–C, treatment with CCN3 decreased the expression of versican, aggrecan, and type I collagen after 24 hours. In addition, we treated cells with recombinant CCN2 and measured the expression of the same matrix genes (Figures 6A–C). In contrast to CCN3, CCN2 strongly induced aggrecan mRNA expression at all time points examined. To explore the possibility that these CCN proteins could regulate each other, as has been speculated in relation to other cell types (23,24), we treated the cells with either CCN2 or CCN3 and measured the expression of the corresponding CCN. CCN3 treatment decreased CCN2 expression by nucleus pulposus cells (Figure 6D), while CCN2 treatment decreased CCN3 expression transiently at 4 and 8 hours, but showed no effect at 24 hours (Figure 6E).

DISCUSSION

The experiments in this investigation demonstrated for the first time that CCN3 was expressed in nucleus pulposus cells of the intervertebral disc and that its basal expression was regulated by TGFβ. Growth factor suppression of CCN3 transcription was partially mediated through p38 and ERK signaling pathways, independent of Smad and AP-1 signaling. When treated with CCN3, nucleus pulposus cells elicited a dose-dependent decrease in cell number and lowered expression of versican, aggrecan, type I collagen, and CCN2. These studies also revealed that TGFβ signaling induces CCN3 promoter activity through Smad3 while also enhancing CCN3 repression via MAPK signaling. We propose that the repressive activity of MAPK signaling and as-yet-unknown transcription factors exceeds the stimulatory response generated by the Smads. To our knowledge, this is the first study in which Smad signaling and TGFβ signaling were decoupled, thereby promoting opposing effects on target gene transcription. We suggest that during disc degeneration, an increase in TGFβ activity and the corresponding changes in CCN3 and CCN2 expression, as we reported previously, could trigger a reparative response that may influence the remodeling response of the extracellular matrix and the proliferative status of the cells (22).

The expression and localization of CCN3 in the intervertebral disc was age dependent. In the embryonic disc, there was robust expression in both anulus fibrosus and nucleus pulposus cells. In contrast, in the adult disc, there was a lower level of CCN3 protein expression in both tissue types. Immunofluorescence analysis of the mature disc indicated that staining was both cellular and pericellular, suggesting that the protein was being secreted and possibly trapped in the pericellular matrix. This notion was substantiated by Western blot analysis of adult discal tissues, where a prominent 54-kd band corresponding to the full-length secreted CCN3 protein was seen (25). From this perspective, CCN3, like CCN2, must be considered to be a matrix constituent of the disc. That intracellular levels of CCN3 are regulated by TGFβ was evident from studies of cultured nucleus pulposus cells. Treatment with TGFβ suppressed the expression of CCN3 mRNA as well as protein. We confirmed that TGFβ suppressed CCN3 activity by using a dominant-negative TGFβ receptor type I/activin receptor–like kinase 5 (ALK-5) construct. When cells were transfected with this construct in the presence of TGFβ, there was a dose-dependent restoration of CCN3 promoter activity. Likewise, in the absence of the growth factor, transfection with a constitutively active ALK-5 construct decreased CCN3 promoter activity. Results of these experiments suggest that TGFβ is concerned with the basal regulation of CCN3 in nucleus pulposus cells of the intervertebral disc.

We examined the mechanism by which TGFβ suppresses CCN3 expression by examining both Smad and non-Smad transduction pathways. With respect to MAPK and AP-1, Lafont et al (26) have previously shown that in adrenocortical cells, CCN3 suppression

was dependent on AP-1 signaling. To ascertain whether a similar regulation was functional in the nucleus pulposus, we transfected cells with dominant-negative Fos and Jun subunits of AP-1. Surprisingly, neither of these proteins influenced growth factor–dependent CCN3 promoter activity. In a parallel study, we found that treatment with p38 and ERK inhibitors partially blocked the TGFβ-mediated suppression of CCN3 promoter activity as well as the mRNA expression.

Thus, from a regulatory perspective, it would appear that in discal tissues, regulation of CCN3 expression by TGFβ is unique and more complex than was originally thought. Indeed, trying to delineate which MAPK isoforms are most likely to be required for suppression is not obvious. Transfection with individual dominant-negative isoforms of p38 (p38α, p38β, p38γ, and p38δ) or ERK (ERK-1 and ERK-2) showed no effect on CCN3 promoter activity. One interpretation of these results is that more than one isoform is required for CCN3 promoter suppression by TGFβ or, possibly, that there is some isoform redundancy. The importance of these and other noncanonical signaling pathways in mediating TGFβ suppression of CCN3 is under active investigation.

To examine the involvement of the Smad signaling pathway, the more conventional transducer of TGFβ signaling, we cotransfected cells with DN-Smad3 and Smad3 siRNA in the presence of TGFβ. The observation that neither strategy rescued CCN3 promoter activity suggested that Smad3 is not involved in the suppression of CCN3. This result was not surprising, since earlier studies indicated that in both mesangial and adrenocortical cells, TGFβ suppression of CCN3 is independent of Smad3 (23,26). Interestingly, in the course of the current investigation, we observed that overexpression of Smad3 caused a dose-dependent activation of CCN3 promoter activity. This result was further confirmed using Smad3-null mouse embryonic fibroblasts. When cells were transfected with increasing doses of Smad3, there was a dose-dependent increase in CCN3 promoter activity. In terms of disc cells, we observed a decrease in CCN3 protein levels in both the nucleus pulposus and anulus fibrosus of Smad3-null mice as compared to their wild-type littermates. We confirmed this Smad3-dependent regulation in nucleus pulposus cells by suppressing Smad3 levels using lentiviral transduction.

Thus, it is evident that Smad3 is more concerned with maintenance of basal CCN3 expression in nucleus pulposus cells than its control when high levels of TGFβ are present. Since the net effect of TGFβ treatment is suppression of CCN3 expression, one obvious interpretation of this result is that the stimulatory effect of Smad3 must be offset by inhibitory activities of a TGFβ-induced repressor molecule(s). It is possible that this could be achieved by limiting the binding of Smad3 to its response element by activation of the noncanonical pathway. Based on these observations, we propose a model of CCN3 regulation by TGFβ in nucleus pulposus cells that involves both canonical (Smad) and noncanonical (MAPK) signaling pathways (Figure 6F). Accordingly, TGFβ signaling through ALK-5 induces both activators and repressors of CCN3: the activating arm of this pathway is mediated by Smad3, while the repressive arm is through MAPK signaling. Activation of the latter arm and the generation of as-yet-unknown repressors could possibly override the stimulation generated by the Smads by limiting their access to the promoter.

In terms of a physiologic role of CCN3, we observed that it influenced matrix gene expression as well as the proliferative response of nucleus pulposus cells. When treated with CCN3, there was a dose-dependent decrease in cell number, similar to what has been observed in other cell types (13–17). FACS analysis indicated that the change in proliferation was not caused by an arrest of cell cycle progression in the G_2/M phase, as has been reported for glioma cells (27). As suggested by Fu et al and Sakamoto et al (15–17), CCN3 may exert its antiproliferative activity by its association with notch and Notch ligands and the connexin 43 signaling pathways (15–17). Consistent with previous reports, we also found that TGFβ caused an increase in nucleus pulposus cell proliferation (28,29). This effect was not blocked with addition of CCN3, indicating that TGFβ overrides the antiproliferative function of CCN3.

With regard to its effects on matrix genes, CCN3 caused alterations in the expression of aggrecan, versican, and type I collagen, matrix genes that are known to be expressed by cells of the nucleus pulposus. It also caused a similar decrease in the expression of CCN2, a CCN family member that we have shown to be upregulated by TGFβ in the nucleus pulposus and to promote aggrecan synthesis (22,30). The opposite effects of the two CCN proteins on aggrecan expression are noteworthy: CCN3 decreases, while CCN2 promotes, aggrecan expression. The opposing effects of TGFβ on CCN2 and CCN3 expression and the suppression of CCN2 by CCN3 lend considerable strength to the notion that these molecules may form a regulatory triad (23,24). Since, during disc degeneration, there is a trend toward increased TGFβ expression, the decrease in CCN3 expression and a concomitant increase in CCN2 may

promote a reparative response, enhancing matrix synthesis and promoting changes in cell number—events that are common in all tissues undergoing fibrotic degeneration. Whether the effects of TGFβ on CCN2 and CCN3 expression vary according to degenerative stage is not known. However, it would not be unreasonable to assume that each of the components of the regulatory circuit provides novel pharmacologic targets for the treatment of degenerative disc disease.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Risbud had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tran, Shapiro, Risbud.

Acquisition of data. Tran, Smith, Symes, Rittié, Perbal, Shapiro, Risbud.

Analysis and interpretation of data. Tran, Shapiro, Risbud.

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