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Smad3 Controls β -1,3-Glucuronosyltransferase 1 Expression in Rat Nucleus Pulposus Cells: Implications of Dysregulated Expression in Disc Disease

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Abstract

Objective—To study the regulation of expression of β -1,3-glucuronosyltransferase 1 (GlcAT-1), an important regulator of glycosaminoglycan (GAG) synthesis, by Smad3 in nucleus pulposus (NP) cells.

Methods—GlcAT-1 expression was examined in rat NP and anulus fibrosus (AF) cells treated with transforming growth factor β (TGF β). The effects of Smad signaling and Smad suppression on GlcAT-1 were examined in rat NP cells. GlcAT-1 expression was analyzed in the discs of Smad3-null mice and in degenerated human NP tissue.

Results—TGF β increased the expression of GlcAT-1 in rat NP but not rat AF cells. Suppression of GlcAT-1 promoter activity was evident with dominant-negative ALK-5 (DN-ALK-5). Cotransfection with Smad3 strongly induced promoter activity independent of TGF β . Bioinformatics analysis indicated the presence of several Smad binding sites in the promoter; deletion analysis showed that the region between –274 and –123 bp was required for Smad3 response. DN-Smad3, Smad 3 small interfering RNA, and Smad7 strongly suppressed basal as well as TGF β -induced promoter activity. Induction of promoter activity by Smad3 was significantly blocked by DN-Smad3; Smad7 had a very small effect. Lentiviral transduction of NP cells with short hairpin RNA Smad3 resulted in a decrease in GlcAT-1 expression and accumulation of GAG. Compared to wild-type mice, significantly lower expression of GlcAT-1 was seen in the discs of Smad3-null mice. Analysis of degenerated human NP tissue specimens

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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.

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showed no positive correlation between GlcAT-1 and TGF β expression. Moreover, isolated cells from degenerated human tissue showed a lack of induction of GlcAT-1 expression following TGF β treatment, suggesting an altered response.

Conclusion—Our findings demonstrate that in healthy NP cells, the TGF β –Smad3 axis serves as a regulator of GlcAT-1 expression. However, an altered responsiveness to TGF β during disc degeneration may compromise GAG synthesis.

The intervertebral disc comprises an outer ligament, the annulus fibrosus (AF), which encloses a gel-like tissue, the nucleus pulposus (NP). While sparse, cells in the NP secrete a complex extracellular matrix that contains fibrillar collagens and the proteoglycan aggrecan. Glycosaminoglycan (GAG) components of the aggrecan molecule provide a robust hydrodynamic system that accommodates applied biomechanical forces (1,2). Surprisingly, while the importance of aggrecan secretion and function has been discussed by many investigators, the mechanisms of control of GAG synthesis are poorly understood.

In the NP, the principal GAG is chondroitin sulfate. Bound to the aggrecan core protein and associated with hyaluronic acid, chondroitin sulfate chains form a giant polydispersed supramolecular structure. The high osmotic pressure of the aggregate serves to accommodate applied biomechanical forces (3). Recent work from our laboratory suggests that galactose β -1,3-glucuronosyltransferase 1 (GlcAT-1), the enzyme that catalyzes the transfer of glucuronic acid to the core protein Gal-Gal-Xyl-*O*-Ser trisaccharide, is required for the synthesis of chondroitin sulfate and heparan sulfate in NP cells (4–9).

Several transcription factors, including hypoxia-inducible factor, activator protein 1 (AP-1), Sp-1, and tonicity-responsive enhancer binding protein (TonEBP), control GlcAT-1 expression in NP cells (8–11). Other powerful regulators of GAG and aggrecan synthesis include bone morphogenetic protein 2 (BMP-2) and transforming growth factor β (TGF β) (10). Previous studies have shown increased TGF β 1 synthetic activity in degenerated human NP (12), in degenerative discs from old animals, and in an animal model of disc injury (13,14). Moreover, several investigators noted that degenerated, painful human discs contained elevated levels of both TGF β and its receptors (15–17). These findings lent considerable strength to the hypothesis that elevated TGF β expression and secretion is linked to disc injury and reparative response. Interestingly, despite elevated levels of TGF β , sulfated GAG and aggrecan content is markedly lower in diseased tissue than in healthy tissue, suggesting decreased biosynthesis (18,19).

Although we have previously shown that GlcAT-1 and aggrecan expression in NP cells responds to TGF β and involves the interplay of several signaling proteins (10,20,21), the role of Smad signaling has not been elucidated. In this study, we show that Smad3 positively regulates GlcAT-1 expression, thus providing further insights into the complex regulation of GlcAT-1 expression by TGF β . Moreover, we demonstrate, for the first time, that in the disease state GlcAT-1 expression no longer correlates with TGF β levels and is unresponsive to TGF β treatment.

MATERIALS AND METHODS

Plasmids and reagents

Flag-Smad3N (amino acids [aa] 1–146; MH-1 domain), Flag-Smad3NL (aa 1–211; MH-1 domain and linker), Flag-Smad3C (aa 199–424; MH-2 domain), Flag-Smad3LC (aa 146–425; MH-2 domain and linker), and Flag-Smad3 Δ C (aa 1–381; lacking the C-terminal 43 amino acids and functioning as dominant-negative Smad3 [DN-Smad3]) vectors were provided by Dr. Rik Derynck (University of California at San Francisco). Plasmids were kindly provided by Dr. Bert Vogelstein (John Hopkins University, Baltimore, MD) (Smad2),

Dr. Adam Glick (The Pennsylvania State University, State College, PA) (Flag-Smad7), Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York, NY) (3TP-Lux reporter), and Dr. David Danielpour (Case Western Reserve University, Cleveland, OH) (pLVTHM–short hairpin Smad3 [shSmad3]). Expression plasmids for Smad3 (no. 11742), DN-ALK-5 (no. 14834), and CA-ALK-5 (no. 14833) and for lentiviral packaging psPAX2 (no. 12260) and pMD2.G (no. 12259) were obtained from Addgene. GlcAT-1 reporter constructs have been described previously (9). As an internal transfection control, pRL-TK vector (Promega) containing the *Renilla reniformis* luciferase gene was used. Transfection methodology has been optimized for rat NP cells (22). Recombinant human TGF β (rhTGF β) was from R&D Systems. Tissue samples from Smad3-null and wild-type mice were from Dr. Aviva Symes (Uniformed Services University, Bethesda, MD). Smad3-null and wild-type mouse embryonic fibroblasts (MEFs) were from Dr. Rik Derynck. PC12 cells, which do not show Smad3 activation in response to TGF β treatment due to lack of TGF β receptor type II (TGF β RII), were used as controls in some experiments (23).

Isolation and treatment of NP and AF cells

Rat and human NP cells and rat AF cells were isolated using a method previously described by our group (22). NP and AF cells and MEFs were maintained in Dulbecco's modified Eagle's medium and 10% fetal bovine serum (FBS) supplemented with antibiotics. In some experiments, cells were treated with rhTGF β (10 ng/ml).

Human tissue collection and grading

Lumbar disc tissue specimens were collected as surgical waste from individuals undergoing elective spinal surgery. Informed consent for sample collection was obtained from each patient in accordance with the Thomas Jefferson University Institutional Review Board guidelines. Assessment of disease state was performed using Pfirrmann grading (24). This grading scheme uses T2-weighted magnetic resonance imaging (MRI) and image analysis by 3 independent observers. Additional data on the donors and the nucleus tissue specimens used for cell isolation are available from the corresponding author upon request.

Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from NP cells using RNeasy Mini Columns (Qiagen). The purified, DNA-free RNA was converted to complementary DNA (cDNA) using SuperScript III reverse transcriptase (Invitrogen). Template cDNA and gene-specific primers were added (for rat GlcAT-1 [NCBI #NM_001128184], forward 5'-ATGCCAGTTTGATGCTACTGCAC-3' and reverse 5'-TGTTCTCCTGCTTCATCTTCGGT-3') to Fast SYBR Green Master Mix (Applied Biosystems), and messenger RNA (mRNA) expression was quantified using the StepOnePlus real-time PCR system (Applied Biosystems). β -actin and GAPDH were used to normalize the expression. All of the primers used were synthesized at Integrated DNA Technologies, Inc., Coralville, IA.

Immunofluorescence microscopy

Cells were plated in 96-well flat-bottomed plates (4×10^3 /well) and treated with TGF β for 30 minutes to 24 hours. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) for 10 minutes, blocked with PBS containing 5% FBS, and incubated with antibodies against GlcAT-1 (1:200; Novus Biologicals) or Smad3 (1:200; Cell Signaling Technology) at 4°C overnight. As a negative control, cells were incubated with isotype IgG under similar conditions. After washing, the cells were incubated with Alexa Fluor 488–conjugated anti-mouse secondary

antibody (Invitrogen), at a dilution of 1:200 for 45 minutes at room temperature. Cells were imaged using a confocal laser scanning microscope (Olympus FluoView).

Western blotting

Anti-GlcAT-1 (1:500) and anti- β -tubulin antibody (1:2000; Developmental Studies Hybridoma Bank) were used for the detection of GlcAT-1 and β -tubulin protein levels, respectively, using methods described previously (8–10).

Transfections and dual luciferase assay

Cells were transferred to 24-well plates at a density of 4×10^4 cells/well one day before transfection. To measure the effect of TGF β , cells were transfected with 500 ng of GlcAT-1 reporter plasmids and 500 ng of pRL-TK plasmid. To investigate the effect of Smad signaling on GlcAT-1 promoter activity, cells were cotransfected with DN-Smad3 (100–300 ng), Smad3 (100–300 ng), Smad7 (50–300 ng), or backbone vector with 400 ng GlcAT-1 reporter and 300 ng pRL-TK plasmid in the presence or absence of TGF β (10 ng/ml). Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. A Dual-Luciferase Reporter Assay system (Promega) was used for sequential measurements of firefly and *Renilla* luciferase activities. At least 3 independent transfections were performed, and all analyses were carried out in triplicate.

Lentiviral particle production and viral transduction

Lentiviral particles were produced in HEK 293T cells using pLVTHM-GFP and pLVTHM-shSmad3 as previously described (13). NP cells were transduced with viral particles along with 6 μ g/ml Polybrene (13). Cells were harvested for protein extraction 5 days after viral transduction.

Immunohistologic studies

Whole mouse embryos were immediately fixed in 4% paraformaldehyde in PBS and then embedded in paraffin. Coronal sections measuring 6–8 μ in thickness were used to localize GlcAT-1 (anti-GlcAT-1; 1:200) using methods described previously (13). Some tissue sections were stained with both Alcian blue and hematoxylin and eosin for morphologic evaluation.

Statistical analysis

All measurements were performed in triplicate, and data are presented as the mean $\pm$ SEM. Differences between groups were analyzed by Student's *t*-test and one-way analysis of variance.

RESULTS

Effect of TGF β on GlcAT-1 expression in rat NP cells

Rat NP cells were treated with TGF β , and GlcAT-1 expression was analyzed. Figure 1A shows that treatment with TGF β resulted in increased GlcAT-1 mRNA levels in rat NP cells. The expression of GlcAT-1 in rat NP cells was also evaluated using immunofluorescence microscopy. TGF β treatment caused an increase in GlcAT-1 protein expression, although the staining was primarily cytosolic (Figure 1B). We also measured GlcAT-1 protein expression using Western blot analysis. Figure 1C indicates that expression of GlcAT-1 protein was increased. To investigate the regulation of expression, we used luciferase reporter constructs containing –1,170 to +61 bp (pGT-I-D), –274 to +61 bp (pGT-I-M), and –123 to +61 bp (pGT-I-P) fragments of the human GlcAT-1 promoter (see schematic drawing in Figure 1D) and measured their activities in response to TGF β .

treatment. There was an increase in the activity of the -1,170 to +61 bp GlcAT-1 promoter (pGT-I-D); the shorter promoter fragments (pGT-I-M and pGT-I-P) did not show any change in activity (Figure 1E).

To investigate whether GlcAT-1 regulation was unique to the NP, we measured GlcAT-1 mRNA expression and promoter activity in rat AF cells. There was no increase in GlcAT-1 expression and promoter activity in rat AF cells following TGF β treatment (Figure 1F). Since PC12 cells do not express TGF β type II receptors, they were used as a negative control. As expected, they evidenced no change in GlcAT-1 promoter activity, nor was there an increase in TGF β -responsive 3TP-Lux reporter activity (Figure 1G). We further confirmed the specificity of the TGF β response in rat NP cells using a dominant-negative TGF β RI/ALK-5 receptor construct. When cells were transfected with this construct in the presence (Figure 1H) or absence (Figure 1I) of TGF β , GlcAT-1 promoter activity was suppressed. The results of these experiments indicate that TGF β plays a role in the basal expression of GlcAT-1 in rat NP cells.

Control of GlcAT-1 promoter activity by Smad signaling

We monitored activation of the Smad-responsive reporter 3TP-Lux by TGF β in rat NP cells (Figure 2A). Following TGF β treatment, 3TP-Lux activity was strongly induced in rat NP cells. Next, to investigate whether induction of GlcAT-1 by TGF β is mediated via the Smad signaling pathway, we cotransfected rat NP cells with plasmids encoding full-length Smad2 and Smad3 (Figures 2B and C). Cotransfection with Smad2 elicited no response (Figure 2B). In contrast, transfection with Smad3 robustly increased GlcAT-1 promoter activity in a dose-dependent manner (Figure 2C).

To identify the domains of Smad3 that mediate GlcAT-1 promoter activity, we used a number of Smad3 domain deletion constructs (Figure 2D). Figure 2E shows that when cells were cotransfected with these expression plasmids, only the full-length Smad3 construct caused significant induction of GlcAT-1 promoter activity. Sequence analysis revealed 11 putative Smad binding sites, at -9 to -6, -162 to -159, -291 to -288, -377 to -374, 3478 to 3475, 3616 to 3613, 3581 to -578, -636 to -633, -843 to -840, -1,055 to -1,052, and -1,070 to -1,067, both in *cis* and *trans* configuration in the promoter (Figure 2F). Due to the high number of Smad elements in the promoter and the complexity of generating and analyzing the contributions of each of the motifs, we chose not to perform site-directed mutagenesis. Instead, we examined the Smad3-responsive region of the GlcAT-1 promoter by measuring the activity of promoter deletion constructs in the presence of Smad3. Both the -1,170 to +61 bp (pGT-I-D) and -274 to +61 bp (pGT-I-M) fragments showed strong induction of activity by Smad3, while the shorter -123 to +61 bp (pGT-I-P) fragment was unresponsive.

Decreased GlcAT-1 promoter activity upon Smad3 suppression

To examine the involvement of Smad signaling in GlcAT-1 regulation, we cotransfected the GlcAT-1 reporter with a plasmid encoding DN-Smad3 in the presence or absence of TGF β . DN-Smad 3 suppressed the basal activity of the GlcAT-1 promoter (Figure 3A). Moreover, DN-Smad3 abolished the induction of GlcAT-1 promoter activity by TGF β . An inhibitory effect on GlcAT-1 promoter activity was seen with 100 ng DN-Smad3, and this was further enhanced when the concentration was increased to 300 ng (Figure 3B). In addition, when Smad3 expression was silenced using gene-targeting small interfering RNA (siRNA), TGF β -mediated induction of GlcAT-1 promoter activity was suppressed (Figure 3C). The specificity of siSmad3 was confirmed by examining its effect on the 3TP-Lux reporter in the presence of TGF β . As expected, siSmad3 significantly suppressed TGF β -induced 3TP-Lux activity (Figure 3D).

To further investigate the specificity and mechanism of action, we cotransfected Smad3 with increasing concentrations of a DN-Smad3 expression construct and measured GlcAT-1 promoter activity. Figure 4A shows that DN-Smad3 significantly suppressed Smad3-dependent induction, although complete inhibition was not achieved. Next, we cotransfected the GlcAT-1 reporter with a plasmid encoding Smad7, with or without TGF β . Figure 4B shows that Smad7 completely blocked TGF β induction of GlcAT-1 promoter activity, even at a dose of 100 ng. An inhibitory effect of Smad7 on basal GlcAT-1 promoter activity was also observed (Figure 4C). However, when Smad7 was cotransfected with Smad3, the induction of promoter activity was unaffected. Of note, a small but significant inhibitory effect was seen when the Smad7 plasmid concentration was increased to 200 ng (Figure 4D).

Control of GlcAT-1 expression in rat NP cells by Smad3

We further validated the role of Smad3 in regulating GlcAT-1 expression in rat NP cells. We suppressed Smad3 expression by transduction with a lentivirus expressing Smad3 shRNA. Figure 5A shows green fluorescent protein (GFP) expression in virally transduced cells, indicating a robust level of transgene expression. Moreover, comparison of an image of GFP expression (Figure 5A) to an image of transmitted light (Figure 5B) showed that a high percentage of cells were transduced with the lentivirus. Figures 5C and D show that there was a significant decrease in Smad3 protein expression in rat NP cells and that shRNA-mediated suppression was specific to Smad3. Thus, no compensatory increase in Smad2 expression was seen in Smad3-suppressed cells. Importantly, GlcAT-1 expression was reduced in the shSmad3-transduced cells compared to the cells transduced with control shRNA (Figures 5C and D). Moreover, shSmad3-transduced cells showed an ~30% reduction in GAG levels (Figure 5E). Intervertebral disc sections from wild-type mice (Figure 5F) and Smad3-null mice (Figure 5G) were immunostained with a GlcAT-1 antibody. The Smad3-null mouse NP showed less GlcAT-1 staining than the wild-type mouse NP. Similarly, when Smad3 was overexpressed in fibroblasts derived from Smad3-null mice, a significant increase in GlcAT-1 promoter activity was seen (Figure 5J).

Altered TGF β responsiveness of GlcAT-1 in human disc disease

Next, we evaluated the expression of GlcAT-1 and TGF β 1 in degenerated human NP tissue specimens graded by MRI analysis (Figure 6A). Real-time RT-PCR analysis showed increased expression of TGF β 1 mRNA in degenerated human NP tissue compared with normal control tissue. However, there was no increase in GlcAT-1 expression in tissue specimens at later stages of degeneration (grades 4 and 5). Not surprisingly, there was considerable variation between patients within the same grade. We then measured the TGF β responsiveness of GlcAT-1 expression in human NP cells isolated from tissue samples with grade 3–5 degeneration (details are available from the corresponding author upon request). Surprisingly, GlcAT-1 mRNA expression was slightly suppressed in cells treated with TGF β , although expression of CCN2, a known TGF β target gene, was induced (Figure 6B). Finally, to investigate whether TGF β treatment results in the activation and nuclear translocation of Smad3 in these human cells, we localized Smad3 in treated cells. A pronounced increase in Smad3 nuclear staining was seen in TGF β -treated NP cells compared to untreated control cells (Figure 6C).

DISCUSSION

This is the first study to demonstrate that the expression of GlcAT-1, a key enzyme required for chondroitin sulfate biosynthesis, is regulated by TGF β in a Smad3-dependent manner in NP cells. Treatment of NP cells with TGF β resulted in the induction of GlcAT-1 expression and promoter activity. Moreover, this response was unique to NP cells. Our study revealed

that regulation was mediated through Smad3 in addition to the previously described noncanonical signaling pathway (10) (see schematic diagram in Figure 6D). TGF β is expressed by NP cells, and the levels of TGF β are elevated during disc degeneration. Accordingly, an increase in TGF β levels, resulting from inflammatory or degenerative stimuli, would be expected to preserve GlcAT-1 expression and GAG synthesis. However, the disconnect between TGF β and GlcAT-1 levels in later stages of degeneration and the lack of GlcAT-1 induction following TGF β treatment in cultured cells suggests that the disease process may alter cellular responsiveness to this growth factor. This may explain the inability of cells to mount a reparative response to enhance GAG synthesis despite elevated TGF β levels during disc degeneration (12–17).

A major function of the cells of the NP is to maintain the water-binding properties of the matrix by synthesizing aggrecan and GAGs (20,25). GlcAT-1 plays a key role in this process since this enzyme catalyzes a critical step in the synthesis of chondroitin sulfate: the transfer of glucuronic acid to the core protein Gal-Gal-Xyl-*O*-Ser trisaccharide (4,6). The expression of GlcAT-1 is regulated by TGF β and BMP-2 in NP cells through a noncanonical signaling network comprising MAPK and transcription factors that include TonEBP, AP-1, and Sp-1 (10). In the present study, it was evident that TGF β regulated GlcAT-1 expression, since treatment with this growth factor resulted in robust expression of GlcAT-1 at both the mRNA and protein levels. This finding, taken together with the observed increase in GlcAT-1 promoter activity after TGF β treatment, indicated that regulation occurred at the transcript level. The specificity of TGF β -mediated induction of GlcAT-1 expression was further validated using transfections with a dominant-negative TGF β RI/ALK-5 (DN-ALK-5). Results of these studies confirmed that TGF β induced GlcAT-1 expression through ALK-5 activation. Furthermore, we concluded that ALK-5 signaling controlled basal expression of GlcAT-1.

To determine the promoter region responsive to TGF β , we performed deletion analysis. Consistent with previous findings, it was evident that the region between –1,170 and –274 bp was required for growth factor response (10). Since the NP is unique both embryologically and functionally, it was important to ascertain if regulatory control of GlcAT-1 was specific for this cell type (26,27). For this purpose, AF and PC12 cells were used as controls; PC12 cells lack TGF β RII, and hence do not respond to TGF β (23). As expected, neither AF nor PC12 cells induced GlcAT-1 expression, confirming that there are profound differences in the responses of the 2 disc cell types to growth factor treatment (10). This finding is consistent with the results of a recent study indicating differing responses of AF and NP cells to BMP-2 treatment; BMP-2 had no effect on GAG synthesis by AF cells, while NP cells were responsive (28). Based on all of these observations, it is not unreasonable to assume that GlcAT-1 regulation is both context- and cell type-specific.

To determine if Smad signaling is involved in the regulation of GlcAT-1 promoter activity, gain- and loss-of-function experiments were performed. Smad3, but not Smad2, overexpression enhanced GlcAT-1 promoter activity, while Smad3 silencing studies revealed inhibition of activity. Thus, GlcAT-1 promoter activity was directly regulated by Smad3. It was clear that the full-length Smad3 was required for this regulation, since lack of either the MH-1 or MH-2 domain abolished Smad3 function. That differences exist was not surprising, since the MH-2 domain exhibits transcription activation domain activity, while the MH-1 domain is involved in DNA recognition and Smad4 binding (29).

Support for conducting Smad3 gain- and loss-of-function studies was provided by the observation that there are several Smad binding elements in the GlcAT-1 promoter. Interestingly, promoter deletion analysis suggested that, in contrast to TGF β , Smad3 enhanced the activity of the –274 to +61 bp promoter construct, indicating that in addition to

the Smad3 response element at -162 to -159 bp, Smad response elements between -1,170 and -274 bp were required for TGF β -dependent induction of activity. Moreover, it is evident that the putative Smad response motif (-9 to -6 bp), which is juxtaposed to the first Sp-1 (-5 to +4 bp) and the transcription start site, does not participate in Smad3- or TGF β -dependent activation of the promoter. A recent study by Hiyama et al demonstrated that members of the TGF β superfamily of growth factors recruited AP-1 and TonEBP to the GlcAT-1 promoter (10). Therefore, it is a reasonable assumption that simultaneous recruitment of Smad3 and these noncanonical factors to the promoter is required for TGF β -dependent activation; this hypothesis is currently under investigation.

Further support for the notion that Smad3 is important for controlling GlcAT-1 promoter activity was provided by the results of the experiments performed using Smad7, DN-Smad3, and siSmad3. We found that in the transfected cells, TGF β -dependent induction of GlcAT-1 promoter activity was suppressed. Insights into the mechanism of suppression were provided by studies indicating that Smad7 down-regulated TGF β activity by blocking signaling at the receptor level. It either interferes with binding to type I receptors, thus preventing the activation of Smad2/3 (30), or recruits Smurf, an E3 ubiquitin ligase, to receptors, targeting them for proteasome-mediated degradation (30,31). More recently, Zhang et al demonstrated that Smad7 binds to the Smad-responsive elements by its MH-2 domain, thereby disrupting the formation of the functional Smad-DNA complex (32). The results of our experiments that measured the effect of Smad7 on GlcAT-1 promoter activity in the presence or absence of Smad3 clearly suggest that in NP cells the latter mechanism was not the primary mode of action; Smad7 functions via a receptor-dependent pathway.

Another important test of the hypothesis that GlcAT-1 expression is influenced by Smad3 was the analysis of cells from Smad3-null mice. We noted that GlcAT-1 expression in the discs of Smad3-null mice was significantly lower than in wild-type mice. We confirmed this finding using lentiviral-mediated delivery of shRNA to suppress Smad3. This study confirmed that Smad3 is required for the maintenance of basal GlcAT-1 expression in NP cells. Moreover, using cells from the Smad3-null mice, we noted that overexpression of Smad3 caused a dose-dependent increase in GlcAT-1 promoter activity. The results of these investigations lend direct support to the notion that Smad3 is a functional modulator of GlcAT-1 promoter activity in NP cells in vivo.

We next investigated whether changes in GlcAT-1 expression may be linked to the development of degenerative disc disease. While the number of control disc tissue samples was very limited (due to practical difficulties in acquiring and grading MRIs of normal human discs), measurement of mRNA expression suggested that as the tissue became degenerated, there was an increase in the expression of TGF β but no concomitant increase in GlcAT-1 expression. These in vivo results, along with the response of cultured cells isolated from degenerated NP tissue specimens to TGF β treatment, suggested a lack of correlation between the expression of GlcAT-1 and TGF β levels during disease.

Interestingly, Smad3 localization studies and induced expression of CCN2 confirmed the activation of the canonical signaling pathway by TGF β in degenerated human cells (13). One possible explanation of this lack of induction in degenerated cells could be the failure to recruit TGF β -dependent noncanonical factors to the GlcAT-1 promoter (10). Notably, in addition to TGF β , GlcAT-1 expression has been shown to respond to a variety of stimuli (8,9), including interleukin-1 β (IL-1 β), which suppresses GlcAT-1 expression (7). It is therefore possible that in the degenerated disc the elevated secretion of IL-1 β by NP cells, and possibly other inflammatory factors, may override the inductive effects of TGF β on GlcAT-1 expression, resulting in suppressed levels. From a functional perspective, we conclude that the expression of GlcAT-1 is dependent on TGF β in the healthy intervertebral

disc. By controlling GAG as well as aggrecan synthesis, both the canonical and noncanonical TGF β signaling pathways promote NP cell function. On the other hand, in the degenerated disc, lack of GlcAT-1 responsiveness to TGF β may limit the ability of cells to increase GAG synthesis to compensate for the accelerated degradation of the aggrecan-rich matrix (Figure 6D). Importantly, decoupling of the relationship between GlcAT-1 and TGF β may further compromise cell function and exacerbate tissue degeneration. We propose that restoration of GlcAT-1 expression and activity in the NP may offer an approach to the treatment of disc degeneration.

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Figure 1.

Effect of transforming growth factor β (TGF β) on β -1,3-glucuronosyltransferase (GlcAT-1) expression in rat nucleus pulposus (NP) cells. **A**, Real-time reverse transcriptase–polymerase chain reaction analysis of GlcAT-1, indicating increased mRNA expression following TGF β treatment. **B**, Immunofluorescence detection of GlcAT-1 in rat NP cells. Cells treated with TGF β showed increased staining of GlcAT-1 compared to control (Ctr) cells. Original magnification $\times 20$. **C**, Western blot analysis, showing increased GlcAT-1 levels in TGF β -treated cells. **D**, Diagram showing a map of 5' deletion constructs of the human GlcAT-1 promoter. The transcription start site is marked as +1. **E**, Induction of GT-I-D, but not GT-I-M or GT-I-P, reporter activity after TGF β treatment. **F**, Lack of change in GlcAT-1 expression and promoter activity in rat anulus fibrosus (AF) cells after TGF β treatment. **G**, Lack of a TGF β -mediated increase in GlcAT-1 promoter (left panel) and 3TP-Lux (right panel) activity in PC12 cells, which lack TGF β receptor type II. **H** and **I**, Suppression of GlcAT-1 promoter activity by dominant-negative ALK5 (DN-ALK-5) in the presence (**H**) and in the absence (**I**) of TGF β . NP cells were transfected with GlcAT-1 reporter along with DN-ALK-5 construct or empty vector pcDNA3. Values in **A** and **E–I** are the mean $\pm$ SEM of 3 independent experiments. * = $P < 0.05$. NS = not significant. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

Figure 2.

Smad signaling controls GlcAT-1 promoter activity. **A**, TGF β reactivity of rat NP cells, assessed by measuring 3TP-Lux reporter activation. Only NP cells showed induction of 3TP-Lux activity following TGF β treatment. **B**, Effect of overexpression of Smad2 on GlcAT-1 promoter activity. A small inductive effect was observed with Smad2 at a dose of 300 ng. **C**, Robust dose-dependent increase in GlcAT-1 reporter activity with overexpression of Smad3 plasmid. **D**, Schematic diagram of expression constructs used to determine the importance of different Smad3 domains in mediating GlcAT-1 activity. **E**, Levels of reporter activity in cells transfected with different Smad3 constructs. Only full-length (FL) Smad3 caused a significant increase in reporter activity. N, NL, C, and LC = flag-Smads N, NL, C, and LC, respectively. **F**, Diagram of the human GlcAT-1 promoter constructs, showing important binding sites. AP-1 = activator protein 1; TonE = tonicity-responsive enhancer. **G**, Induction of GT-I-D (-1,170 to +61 bp) and GT-I-M (-274 to +61 bp) reporter activity by Smad3 coexpression. The shortest fragment, GT-I-P (-123 to +61 bp), was unresponsive to Smad3. Values in **A–C**, **E**, and **G** are the mean $\pm$ SEM of 3 independent experiments performed in triplicate. * = $P < 0.05$. See Figure 1 for other definitions.

Figure 3.

Smad3 suppression results in decreased GlcAT-1 promoter activity. **A** and **B**, GlcAT-1 promoter activity in rat NP cells transfected with the GlcAT-1 reporter along with a DN-Smad3 construct, in the absence (**A**) or presence (**B**) of TGF β . Note that DN-Smad3 blocked the inductive effect of TGF β on GlcAT-1 promoter activity and blocked basal promoter activity. **C** and **D**, GlcAT-1 promoter activity in rat NP cells transfected with GlcAT-1 (**C**) or 3TP-Lux (**D**) reporters along with Smad3 small interfering RNA (siSmad3) or control siRNA and treated with TGF β . Induction of both GlcAT-1 and 3TP-Lux reporters by TGF β was blocked by Smad3 siRNA. Values are the mean $\pm$ SEM of 3 independent experiments performed in triplicate. * = $P < 0.05$. See Figure 1 for other definitions.

Figure 4.

Smad3 regulation of GlcAT-1 promoter activity. **A**, GlcAT-1 promoter activity in rat NP cells transfected with GlcAT-1 reporter construct along with plasmids encoding wild-type Smad3 construct and DN-Smad3. Expression of Smad3 resulted in an increase in promoter activity, whereas simultaneous addition of DN-Smad3 resulted in partial blocking of the inductive effect of Smad3. **B** and **C**, GlcAT-1 promoter activity in rat NP cells transfected with the Smad7 construct along with the GlcAT-1 reporter in the presence (**B**) or absence (**C**) of TGF β . Smad7 expression suppressed both the inductive effect of TGF β on the promoter and its basal activity. **D**, GlcAT-1 promoter activity in rat NP cells cotransfected with Smad7 and Smad3. The induction of promoter activity was unaffected. A small but significant inhibitory effect was seen when the Smad7 plasmid concentration was increased to 200 ng. Values are the mean $\pm$ SEM of 3 independent experiments performed in triplicate. * = $P < 0.05$. See Figure 1 for definitions.

Figure 5.

Smad3 controls GlcAT-1 expression in the rat NP. **A** and **B**, Green fluorescent protein (GFP) expression (**A**) and transmitted light (**B**) in rat NP cells transduced with control lentivirus. GFP expression shows efficient viral transduction. **C**, Western blot analysis of rat NP cells transduced with control lentivirus (LV-C) or short hairpin Smad3 lentivirus (LV-Sh). Smad3 suppression resulted in a concomitant decrease in GlcAT-1 expression. Smad3 silencing had no effect on Smad2 expression. **D**, Densitometric analysis of 3 blots. β -tubulin expression was used as a loading control. Smad3 silencing significantly decreased GlcAT-1 expression. **E**, Significant decrease in sulfated glycosaminoglycan (sGAG) accumulation in Smad3-silenced cells compared to cells transduced with control virus. DMMB = dimethylmethylene blue. **F–I**, Sagittal sections of discs from wild-type (WT) mice (**F** and **H**) and Smad3-null mice (**G** and **I**) treated with an antibody against GlcAT-1. The wild-type mice showed higher expression of GlcAT-1 than the Smad3-null mice. Some sections (**H** and **I**) were stained with hematoxylin and eosin and Alcian blue for visualization of morphologic features. Original magnification $\times 20$. **J**, Dose-dependent increase in GlcAT-1 promoter activity in Smad3-null mouse fibroblasts transfected with increasing doses of Smad3. Values in **D**, **E**, and **J** are the mean $\pm$ SEM from 3 independent experiments. * = $P < 0.05$. See Figure 1 for other definitions.

Figure 6.

Expression of GlcAT-1 in degenerated human NP tissue specimens and altered TGFβ responsiveness of GlcAT-1 in NP cells isolated from degenerated tissue. G1–G5 represent grades of disc degeneration of increasing severity. **A**, Increase in TGFβ1 mRNA levels in degenerated tissue specimens. GlcAT-1 levels did not show a pronounced increase. The horizontal line represents expression levels of GlcAT-1 and TGFβ1 in healthy NP tissue. **B**, Quantitative reverse transcriptase–polymerase chain reaction analysis of GlcAT-1 and CCN2 expression following TGFβ treatment of NP cells isolated from the disc tissue of 7 patients. Note that the expression of GlcAT-1 was suppressed in TGFβ-treated cells. In contrast, CCN2 expression was increased in all but 1 (grade-5) sample. The expression levels of GlcAT-1 and CCN2 in untreated controls were set at 1 (broken line). **C**, Smad 3 localization in NP cells isolated from 1 grade-3, 1 grade-4, and 1 grade-5 tissue sample and treated with TGFβ for 30 minutes. Regardless of the grade, cells from all tissue samples evidenced Smad3 nuclear localization after treatment. **D**, Proposed model of the regulation of GlcAT-1 expression by TGFβ and Smad3. During disease, possible dysregulation of noncanonical signaling or the presence of inhibitory cytokines, such as interleukin-1β (IL-1β), may cause GlcAT-1 transcription to become unresponsive to TGFβ (broken line). AP-1 = activator protein 1; TonEBP = tonicity-responsive enhancer binding protein (see Figure 1 for other definitions). Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).