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TGF β Regulates Galectin-3 Expression through Canonical Smad3 Signaling Pathway in Nucleus Pulposus Cells: Implications in Intervertebral Disc Degeneration

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Abstract

Galectin-3 is highly expressed in notochordal nucleus pulposus (NP) and thought to play important physiological roles; however, regulation of its expression remains largely unexplored. The aim of the study was to investigate if TGF β regulates Galectin-3 expression in NP cells. TGF β treatment resulted in decreased galectin-3 expression. Bioinformatic analysis using JASPAR and MatInspector databases cross-referenced with published ChIP-Seq data showed nine locations of highly probable Smad3 binding in the *LGALS3* proximal promoter. In NP cells, TGF β treatment resulted in decreased activity of reporters harboring several 5' deletions of the proximal Galectin-3 promoter. While transfection of NP cells with constitutively active (CA)-ALK5 resulted in decreased promoter activity, DN-ALK5 blocked the suppressive effect of TGF β on the promoter. The suppressive effect of Smad3 on the Galectin-3 promoter was confirmed using gain- and loss-of-function studies. Transfection with DN-Smad3 or Smad7 blocked TGF β mediated suppression of promoter activity. We also measured Galectin-3 promoter activity in Smad3 null and wild type cells. Noteworthy, promoter activity was suppressed by TGF β only in wild type cells. Likewise, stable silencing of Smad3 in NP cells using sh-Smad3 significantly blocked TGF β -dependent decrease in Galectin-3 expression. Treatment of human NP cells isolated from tissues with different grades of degeneration showed that Galectin-3 expression was responsive to TGF β -mediated suppression. Importantly, Galectin-3 synergized effects of TNF- α on inflammatory gene expression by NP cells. Together these studies suggest that TGF β , through Smad3 controls Galectin-3 expression in NP cells and may have implications in the intervertebral disc degeneration.

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Keywords

Intervertebral disc; nucleus pulposus; Galectin-3; TGF β ; Smad3; disc degeneration

INTRODUCTION

The intervertebral disc (IVD) is a unique tissue in that it permits flexion and extension, as well as rotation of the spine. It comprises an outer fibrocartilagenous annulus fibrosus (AF), which encloses a gel-like tissue, the nucleus pulposus (NP). Cells of the NP secrete a complex extracellular matrix that primarily consists of proteoglycan, aggrecan, and fibrillar collagens. Assembly of these macromolecules provides a robust hydrodynamic system that accommodates applied biomechanical forces to the spine [1–3]. During degenerative disc disease, the loss of proteoglycans decreases the water-binding capacity of the system, resulting in a failure of the tissue to resist compressive forces. While AF and endplate cartilage is sclerotomal in origin, cells of the NP are derived from the notochord [4], an embryonic tissue with limited blood supply. Galectin-3, also known as eBP (immunoglobulin E binding protein), Mac-2, CBP-30, and L-29, has been used as a molecular marker of the notochord. Gotz et al. [5] have suggested that this protein is a phenotypic indicator of cells of the NP. However, our previous studies have shown ubiquitous expression of Galectin-3 in all the compartments of the disc, casting doubt on this notion [6]. Galectin-3, a major non-integrin laminin-binding protein, serves as a receptor of glycation endproducts (AGEs) and its functions also include cell adhesion and cell cycle regulation [7]. Several matrix molecules including fibronectin, hensin, elastin, collagen IV, have been identified to interact with Galectin-3 [8–11]. Extracellular Galectin-3 is required for cell adhesion in organogenesis and tumorigenesis [12] and allergic inflammation [13]. Galectin-3 and its binding proteins have also been shown to play a role in many other inflammatory diseases, including rheumatoid arthritis [14], osteoarthritis [15], juvenile idiopathic arthritis [16], and antigen-induced arthritis [17].

Thus, due to important biological function of this lectin, there is a considerable interest in the regulation of Galectin-3 expression. In the context of skeletal tissues, expression is mediated by Runx-2 [18, 19]. Our previous work has shown that Galectin-3 expression in NP is partially controlled by HIF-1, a major determinant of NP niche function [20]. However, mechanisms that control the expression of Galectin-3 in intervertebral disc under pathological conditions have received little attention. We and others have shown that TGF β levels increase significantly during degenerative disc disease [21–23]. Therefore, the objective of the investigation was to examine if Galectin-3 expression by NP cells is regulated by TGF β and if expression is altered during the disease process. Results of the study show for the first time that TGF β suppresses Galectin-3 expression through canonical Smad3 signaling and that Galectin-3 synergizes catabolic actions of TNF- α on NP cells implying its possible role in pathogenesis of disc disease.

RESULTS

Expression of Galectin-3 is regulated by TGF β in NP cells

Expression of Galectin-3 in mature rat tissues was studied using real-time PCR. As expected, a robust basal expression of Galectin-3 mRNA is seen in both healthy NP and AF tissues, with NP showing a trend of higher expression than the AF (Fig. 1A, B). To explore the premise that TGF β regulates Galectin-3 expression, NP cells were treated with TGF β and expression of Galectin-3 was analyzed. Figure 1C shows that treatment with TGF β results in decreased Galectin-3 mRNA levels as early as 4 h with suppression persisting at 24 h. In addition, we measured the level of Galectin-3 protein from treated rat NP cells by Western blot analysis. TGF β treatment significantly decreases Galectin-3 protein levels (Fig. 1D, E).

The Proximal Promoter of *LGALS3* Contains Conserved Smad3 Binding Sites

To gain insight into regulation of Galectin-3 transcription, proximal promoter regions of *LGALS3* were analyzed using Evolutionarily Conserved Regions (ECR) genome browser (<http://ecrbrowser.dcode.org/>) to determine inter species conservation, which was high from -6 to -2 kb of the human promoter when compared to mouse (Fig. 2A). Next, ten kb of the mouse *LGALS3* promoter region was scanned using both the JASPAR core database (<http://jaspar.genereg.net/>) and the Genomatix MatInspector database for matches to Smad3 binding matrices. The searches were filtered to show only relative scores of matrix similarity above .80. A total of 41 Smad3 binding sites were identified by the databases, with eight reported by both programs (Fig. 2B). Processed ChIP-seq data published on Array Express (<http://www.ebi.ac.uk/arrayexpress>) was analyzed to verify predicted sites. Analysis of ChIP-Seq data (E-GEOD-36673) shows that stimulation by TGF β in mouse neural stem cells induced Smad3 binding upstream of the transcription start site of *LGALS3* in four regions, each approximately 440 bp in length (Fig. 2B). The sites predicted by JASPAR and MatInspector were cross-referenced with these sequences, revealing 9 locations of highly probable Smad3 binding (Fig. 2C). Noteworthy, the sequence at -1943/-1494 bp identified by ChIP-Seq did not contain any binding sites predicted by the programs. In addition to predicted Smad3 sites in regions identified by ChIP-Seq, 5 more Smad3 binding sites were identified in ~1.5 kb of the *LGALS3* promoter region immediately proximal to the transcription start site. Multiz alignment was performed for predicted Smad3 binding sites using the Ensembl lastz interface. Sites one, five, six, seven, nine, and fourteen demonstrated high levels of sequence conservation between mouse, rat and human, suggesting their physiological importance in regulating gene expression (Fig. 2D).

TGF β decreases Galectin-3 promoter activity in NP cells

To investigate if the regulation of expression is at the transcriptional level, we first co-transfected NP cells with luciferase reporter constructs harboring either a 2 kb (-1970/+50) *LGALS3* proximal promoter or different length 5' deletions of this promoter and measured their relative activities (Fig. 3A). Results showed that the 2 kb fragment exhibits the highest basal activity among all of the promoter fragments tested (Fig. 3B). We then measured the activity of all reporters following TGF β treatment for 24 h. Following treatment with TGF β , reporter activity significantly decreases (Fig. 3C, D). Considering that the 2 kb fragment exhibited the highest basal activity, we chose this reporter for further studies. We confirmed

the specificity of the TGF β response in NP cells using a CA-ALK-5 construct and a dominant-negative TGF β RI/ALK-5 receptor construct. The galectin-3 reporter showed decreased activity when cells were co-transfected with CA-ALK5 in absence of TGF β (Fig. 3E). On the other hand, when cells were co-transfected with DN-ALK5, the TGF β -mediated suppression of Galectin-3 promoter activity was blocked (Fig 3F). The results of these experiments suggest that TGF β plays a role in controlling Galectin-3 expression through ALK5 in NP cells.

Canonical Smad signaling regulates Galectin-3 promoter activity in NP cells

To investigate whether suppression of Galectin-3 by TGF β is mediated via the Smad signaling pathway, we co-transfected NP cells with plasmids encoding full-length Smad3 and different length of Galectin-3 promoter fragments. Transfection with Smad3 significantly decreased the activity of 2 kb Galectin-3 promoter in a dose-dependent manner (Fig. 4A) and the same trend was seen with other reporters harboring different length of promoter fragments (Fig. 4B). Figure 4C shows that DN-Smad3 abolished the suppression of Galectin-3 promoter activity by TGF β . In contrast to Smad3, co-transfection with Smad2 elicited no significant response (Fig. 4D), while transfection with Smad4 showed a small but significant decrease in Galectin-3 promoter activity (Fig. 4E). Interestingly, when Smad4 was co-transfected with Smad3, no appreciable synergistic effect on activity suppression was seen (Fig. 4F). To further delineate the involvement of TGF β -induced Smad signaling in the suppression of Galectin-3, we co-transfected the Galectin-3 reporter with a plasmid encoding Smad7 in the presence of TGF β . Figure 4G shows that Smad7 abolished the TGF β suppression of Galectin-3 promoter activity even at a dose of 50 ng. To further investigate the role of Smad3 in the transcriptional regulation of Galectin-3, we measured Galectin-3 promoter activity in Smad3-WT (wild-type) and Smad3-null MEFs. Wild type cells (Fig. 5A), in contrast to null cells (Fig. 5B), evidenced TGF β suppression of the Galectin-3 promoter activity. When Smad3 was overexpressed in MEFs, derived from Smad3-null mice in the presence or absence of TGF β , a significant decrease in Galectin-3 promoter activity was seen (Fig. 5C, D).

Smad3 controls Galectin-3 expression in NP cells

We further validated the role of Smad3 in regulating Galectin-3 expression in rat NP cells. We stably suppressed Smad3 expression by transduction with a lentivirus expressing Smad3 shRNA. Figure 6A shows that there is a robust GFP expression by the virally transduced cells, indicating high level of transduction efficiency and transgene expression. As expected, cells transduced with shSmad3 showed a significant decrease in the Smad3 protein levels compared to cells transduced with a control shRNA (Fig. 6B, C). Importantly, silencing of Smad3 partially blocked the suppressive effect of TGF β on the expression of Galectin-3 mRNA (Fig. 6C) and protein (Fig. 6D, E).

NP cells isolated from degenerated human discs retain TGF β responsiveness of Galectin-3

Next, we evaluated the expression levels and TGF β responsiveness of Galectin-3 in NP cells isolated from NP tissues with different grades of degeneration. Level of Galectin-3 is suppressed to varying degree by TGF β treatment in tissues from Pfirrmann grades 1 to 5 (Fig. 7A). Next, we measured mRNA expression in NP cells isolated from tissues with

advanced stages of disease (G 3–5). Again Galectin-3 mRNA expression is suppressed by TGF β in NP cells from all the tissue samples, while expression of CCN2, a known TGF β target gene is induced (Fig. 7B). As expected, patient-to-patient variation in gene expression was seen.

Galectin-3 synergizes catabolic and inflammatory actions of TNF- α on NP cell

To delineate the functional consequences of TGF β mediated decrease in Galectin-3 levels, we treated NP cells with TNF- α in presence or absence of Galectin-3 for 24 h and measured expression of inflammatory and catabolic genes known to be TNF- α targets during disc disease. As expected, TNF- α treatment significantly increased expression of IL-1 β (Fig. 7C), IL-6 (Fig. 7D), CCL-2 (Fig. 7E) and CCL20 (Fig. 7G). On the other hand, expression of all these target genes remained unaffected by treatment of cells with Galectin-3 alone. Interestingly, when Galectin-3 was included with TNF- α , it synergized TNF- α action and resulted in further induction in expression of all the genes measured compared to TNF- α alone except for CCL20.

DISCUSSION

This is the first study to demonstrate that, in the intervertebral disc, the expression of Galectin-3 was regulated by TGF β in a Smad3-dependent manner. TGF β , a pleiotropic cytokine expressed by NP cells is shown to control expression of many important extracellular matrix proteins such as aggrecan and collagen 2 and enzymes that contribute to glycosaminoglycan (GAG) synthesis like *B3GAT3* [24, 25]. In addition, TGF β controls expression of CCN2, an important matricellular protein concerned with matrix homeostasis and anti-inflammatory activities in the disc [21, 26–28]. Importantly, we and others have shown previously that the levels of TGF β are elevated during disc degeneration [21, 29]. It was therefore of interest to understand if this cytokine has a role in modulating Galectin-3 expression, a molecule which is highly expressed in notochord and considered by many as a NP phenotypic marker, despite persistent expression throughout disc [6]. Extensive bioinformatic analysis of the *LGALS3* promoter for Smad3 binding sites using JASPAR and MatInspector databases, and of published ChIP-Seq data that showed regions of Smad3 binding as well as presence of multiple conserved sites suggested that the regulation of expression is likely at the transcript level. The observation that TGF β causes a decrease in the expression levels of Galectin-3 mRNA and protein by primary NP cells also supports the hypothesis that regulation was mediated at the transcript level. This notion was further supported by transient transfection assays that showed that TGF β decreased the activities of multiple Galectin-3 promoter fragments. Importantly, 5' deletion analysis indicated that the regulatory region is present between –136/+50 bp of the promoter. Noteworthy, JASPAR database predicted a Smad3 response element at –99/–83 with high conservation between mouse, rat and human, which is most likely active in the NP cells. The specificity of TGF β -mediated suppression of Galectin-3 expression was further validated by gain- and loss-of-function experiments. Results of transfections with a dominant-negative TGF β RI/ALK5 (DN-ALK5) and CA-ALK5 showing that inhibition of ALK5 signaling blocked TGF β -mediated Galectin-3 transcription, while ALK5 activation in absence of TGF β ligand

decreased Galectin-3 expression, suggests that TGF β controlled Galectin-3 expression through ALK5.

Additional gain- and loss-of-function experiments were performed to determine if Smad signaling downstream of ALK5 regulated Galectin-3 promoter activity. That overexpression of Smad3 and Smad4, but not Smad2, decreased Galectin-3 promoter activity, while Smad3 silencing inhibited the promoter activity clearly indicated that the Galectin-3 promoter activity was responsive to Smad3. Importantly, we found that DN-Smad3 blocked TGF β dependent inhibition of Galectin-3 promoter activity, supporting the importance of canonical Smad signaling in controlling Galectin-3 transcription. Further insights into the suppression mechanism were provided by results that clearly showed that Smad7 exerted effects similar to DN-Smad3 on TGF β dependent Galectin-3 reporter activity. Recent studies indicate that Smad7 down-regulates TGF β activity by blocking signaling at the receptor level either by interfering with TGF β binding to type I receptors and preventing the activation of Smad2/3, or by recruiting Smurf, an E3 ubiquitin ligase, to receptors subsequently targeting them for proteasome-mediated degradation [30, 31]. Recently, Zhang and colleagues reported 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 Galectin-3 promoter activity are therefore consistent with these earlier mechanistic findings, although it remains to be elucidated whether Smad7 functions via receptor-dependent or receptor-independent mechanisms in NP cells.

We then used MEFs from Smad3-null mice to determine if there was cell type specificity to the TGF β response. We observed that Smad3 null cells failed to suppress Galectin-3 promoter activity; when these cells were transfected with Smad3, Galectin-3 promoter activity was suppressed. These results provided further validation of the contribution of Smad3 signaling in Galectin-3 promoter regulation and suggested that the response was similar between NP cells and MEFs as has been observed in case of CCN2 regulation by TGF β [21]. Next, we used lentiviral-mediated delivery of shRNA to stably suppress Smad3 in NP cells; suppression of Smad3 significantly blocked the repressive effect of TGF β on Galectin-3 mRNA and protein expression. These studies highlighted the importance of this canonical pathway in controlling Galectin-3 expression. Together, the results of these functional studies indicate that by controlling the activity of Smad3, TGF β controls the expression of Galectin-3 in NP cells.

Since we and others have already shown the expression levels of TGF β are elevated during disc degeneration [21, 23, 33], we next investigated whether changes in Galectin-3 expression may correlate with degenerative disc disease. Not surprisingly, the level of Galectin-3 protein was significantly suppressed by TGF β treatment in NP cells derived from graded human tissues (G1 to G5). Again, in contrast to CCN2 that showed increased expression, mRNA levels of Galectin-3 were repressed. These results are also in agreement with our previous studies that showed Smad3 nuclear localization by TGF β in human NP cells [35], and elevated tissue levels of CCN2 in degenerated specimens [21] confirming that TGF β activates canonical Smad signaling pathway in human NP cells. Noteworthy, it has been shown in other cell types that one of the functions of Galectin-3 is to regulate cell survival by suppressing signaling through TNF family of proteins, [14, 34–36]. Moreover,

studies by Lapis K et al. have shown that Galectin-3 can regulate the interaction between cells and elastin, an important component of the extracellular matrix of the intervertebral disc [37] and by forming complexes with integrins, externalized Galectin-3 also plays a role in cell adhesion and spreading, thereby initiating transmembrane signaling [38, 39]. On the other hand, Galectin-3 is also known to induce pro-inflammatory effects in RA by modulating the pattern of cytokine and chemokine production, indicating that the function is context dependent [40]. Our results supported this pro-inflammatory function of Galectin-3 as it was seen to potentiate deleterious effects of TNF- α on NP cells and resulted in synergistic increases in expression of inflammatory cytokines IL-1 β and IL-6 [41], chemokine, CCL2 and matrix degrading enzyme MMP-3, molecules that are implicated in disc degeneration [42]. Noteworthy, this synergism between Galectin-3 and TNF- α was target specific since such effect was not observed for CCL20. Importantly, since atypical interactions between matrix components and integrins and other co-receptors are known to promote catabolic responses [27, 43, 44] it is not unreasonable to assume that TGF β -dependent decrease in overall Galectin-3 levels under inflammatory conditions may provide some protection to the cells and alter overall secretory phenotype of cells.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids

Flag-Smad3 C (aa 1–381, lacks the C-terminal 43 amino acids and functions as DN-Smad3) vector and wild type and null Smad3 MEFs were provided by Dr. Rik Derynck, University of California at San Francisco. Smad7 (catalog #11733) and Smad3 (#11742) plasmids were obtained from Addgene. Galectin-3 promoter and its 5' deletion constructs have been described earlier [20]. The vector pRL-TK (Promega) containing Renilla luciferase gene was used as an internal transfection control. The amount of transfected plasmid, the pre-transfection period after seeding, and the post-transfection period before harvesting, have been optimized for NP cells using pSV β -galactosidase plasmid (Promega) [43]. rhTGF β 3 was from R&D systems. rTNF- α and Galectin-3 was from Peprotech.

Isolation of NP cells and treatments

Rat and human NP cells were isolated using a method reported earlier by Risbud et al. [45]. NP tissue from lumbar discs of 3–4 rats was pooled for each cell isolation. NP cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) and 10% FBS supplemented with antibiotics. To investigate effect of TGF β , cells were treated with 10 ng/ml TGF β for 4–24 h in serum-free media. To study effect of Galectin-3 on NP cells under conditions that mimic disc degeneration, cells were treated with TNF- α (10 ng/ml) with or without Galectin-3 (5 μ g/ml) for 24 h and gene expression measured.

Human tissue collection and grading

Both lumbar and cervical disc tissues were collected as surgical waste from individuals undergoing elective spinal surgical procedures. In accordance with Thomas Jefferson University's Institutional Review Board guidelines, informed consent for sample collection was obtained from each patient. Assessment of the disease state was performed using Pfirrmann grading [46].

Real-time RT-PCR analysis

Following treatments, total RNA was extracted from NP cells (5×10^5 cells/plate) using RNAeasy micro columns (Qiagen). Before elution from the column, RNA was treated with RNase free DNase I. 2 μ g of total DNA free-RNA was used to synthesize cDNA using SuperScript III cDNA synthesis kit (Invitrogen). Reactions were set up in triplicate in 96-well plates using 1 μ l cDNA with Fast SYBR Green PCR Master Mix (Applied Biosystems) to which gene-specific forward and reverse PCR primers were added. Each set of samples included a template-free control. PCR reactions were performed in a StepOnePlus real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. Expression of the gene of interest was first normalized by housekeeping gene Hprt1 and then data presented as relative to the corresponding control group. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

Protein extraction and Western blotting

NP cells (1×10^6 cells /plate) were placed on ice immediately following treatment and washed with ice-cold Hanks' Balanced Salt Solution. All wash buffers and final re-suspension buffer included 1X protease inhibitor cocktail (Roche), NaF (5 mM) and Na_3VO_4 (200 μ M). Proteins were resolved on 8–12 % SDS-polyacrylamide gels and transferred by electroblotting to PVDF membranes (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20) and incubated overnight at 4 °C in 3% non-fat dry milk in TBST with anti-Galectin-3 or anti-Smad3 (1:1000; Cell Signaling) or β -tubulin (1:2500, DHSB) or anti-GAPDH (1:2000, cell Signaling) antibodies. Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

Transfections and dual luciferase assay

NP 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 Galectin-3 reporter plasmids and 500 ng pRL-TK plasmid. To investigate the effect of Smad2 or Smad3 or Smad4 on Galectin-3 promoter activity, cells were co-transfected with Smad2 (50–200 ng), Smad3 (50–200 ng), Smad4 (50–200 ng), DN-Smad3 (50–200 ng), or backbone vector with 500 ng Galectin-3 reporter and 300 ng pRL-TK plasmid in presence or absence of TGF β (10 ng/ml). Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. 48 h after transfection, 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, CA). At least three independent transfections were performed, and all analyses were carried out in triplicate.

Lentiviral particle production and viral transduction

HEK 293T cells were seeded in 10 cm plates (1.3×10^6 cells/plate) in DMEM with 10% heat-inactivated FBS two days before transfection. Cells were transfected with 2.5 μ g of sh-Ctr or gene specific shRNA plasmids along with 1.875 μ g psPAX2, a packaging vector, and

0.625 µg pMD2.G, an envelope vector. After 16 h, transfection media was removed and replaced with DMEM with 5% heat-inactivated FBS and penicillin-streptomycin. Lentiviral particles were harvested at 48 h and 60 h post-transfection. Rat NP cells were plated in DMEM with 5% heat-inactivated FBS one day before transduction. Cells in 10 cm plates were transduced with 5 ml of media containing viral particles along with 6 µg/ml polybrene. After 24 h, media was removed and replaced with DMEM with 5% heat-inactivated FBS. Cells were harvested for protein extraction 5 days after viral transduction.

Statistical analysis

All experiments were repeated independently at 3 times, data is presented as mean + S.E. Differences between groups were analyzed by the Student's t-test; *p< 0.05.

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Fig. 1.

TGF β suppression of Galectin-3 expression in NP. A, B) Real-time RT-PCR analysis shows robust expression of Galectin-3 mRNA (A) and protein (B) in adult rat NP and annulus fibrosus (AF) tissues. NP tissue shows a trend of higher expression compared to AF. C) Real-time RT-PCR analysis of Galectin-3 expression by rat NP cells treated with TGF β . There was a decrease in Galectin-3 mRNA expression by TGF β treatment in a time-dependent fashion. D) Compared to controls, expression of Galectin-3 protein was significantly suppressed by 8 h or 24 h TGF β treatment in cells. E) Densitometric analysis of multiple blots from experiment described in C above. Values shown are mean + SE, of 3 independent experiments; * $p < 0.05$.

Fig. 2.

A) Analysis using the Evolutionary Conserved Regions Browser (<http://ecrbrowser.dcode.org/>) demonstrates sequence conservation between multiple vertebrate species 6 kb before and 2 kb into the *LGALS3* gene. ECR threshold is set at 77%. *LGALS3* promoter; red, 5' UTR; yellow, exons; blue, introns; salmon, transposons and simple repeats; green. B, JASPAR and MatInspector predicted binding sites 10 kb upstream of the mouse *LGALS3* transcription start site. ChIP-seq verified binding regions are labelled in grey and described by their start location. C, Binding sites predicted by JASPAR or MatInspector

within the ChIP-seq identified regions of the promoter (solid line) and the promoter region immediately proximal to the transcription start site (dashed). Filled boxes were predicted by only MatInspector, half-filled boxes were predicted by both MatInspector and JASPAR, and empty boxes were predicted by only JASPAR. D, Multiz alignment from Ensembl lastz database. Non-conserved bases are underlined, gaps in alignment (–), inability to match (.), red is the core matrix binding location, * sequences are from the negative strand.

Fig. 3.

Transforming growth factor β (TGF β)–induced decrease in Galectin-3 promoter activity in rat nucleus pulposus cells. A) Galectin-3 promoter constructs of different lengths. Arrow indicates transcription start site. B) Transfection of cells with each of the four promoter constructs, ranging in size from –1970 bp to –136 bp. The –1970/+50 bp fragment exhibited the highest activity. C, D) Luciferase activity in rat nucleus pulposus cells transfected with the –1970 bp, –1075 bp (C); –280 bp, or –136 bp (D) Galectin-3 promoter fragment and left untreated or treated with TGF β for 24 hours. Decreased Galectin-3 promoter activity was observed with all promoter fragments. E, F), Decreased Galectin-3 promoter activity in cells transfected with increasing doses of CA-ALK-5 with (E) or without (F) the presence of TGF β G) Blockade of TGF β -mediated suppression of Galectin-3 promoter activity in cells transfected with increasing doses of DN-ALK-5 in the presence of TGF β . Values shown are mean + SE, of 3 independent experiments; * p <0.05.

Fig. 4.

Smad signaling controls Galectin-3 promoter activity in rat NP cells. A) Effect of Smad3 overexpression on -1970 kb Galectin-3 promoter activity. Note the dose-dependent increase in Galectin-3 promoter activity with increasing concentrations of Smad3. B) Effect of Smad3 over expression on different length Galectin-3 promoter fragments. C) NP cells were transfected with increasing doses of DN-Smad3, DN-Smad3 abolished suppression in 2 kb Galectin-3 promoter activity by TGFβ treatment. D) Effect of Smad2 overexpression on 2 kb Galectin-3 promoter activity. Smad2 overexpression did not influence Galectin-3 promoter

activity. E) Rat NP cells were co-transfected with Smad4 and 2 kb Galectin-3 promoter activity measured; decreased Galectin-3 promoter activity in cells transfected with Smad4 was seen. F) Rat NP cells were co-transfected with Smad3 and/or Smad4 and promoter activity measured. When Smad3 and Smad4 are added together, Galectin-3 promoter activity was further decreased. G) NP cells were co-transfected with increasing dose of Smad7 and 2 kb Galectin-3 promoter and luciferase activity measured following TGF β treatment. TGF β mediated induction in promoter activity was completely blocked by Smad7. Values shown are mean + SE, of 3 independent experiments; *p<0.05.

Fig. 5.

Regulation of 2 kb Galectin-3 promoter activity by Smad3. A, B) Galectin-3 promoter activity in Smad3-WT (wild-type, A) and Smad3-null (B) mouse embryonic fibroblasts measured following TGFβ treatment, only wild-type cells showed a decrease in Galectin-3 reporter activity. C, D) Dose-dependent decrease in Galectin-3 promoter activity in Smad3-null mouse embryonic fibroblasts transfected with increasing doses of Smad3 with (D) or without (C) TGFβ treatment. Values shown are mean + SE from three independent experiments, * $p < 0.05$.

Fig. 6.

Smad3 controls Galectin-3 expression in rat NP cells. A, 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 shSmad3 lentivirus (LV-ShSmad3). D) Densitometric analysis of multiple blots from experiment described in C above. E). Western blot analysis of human NP cells infected with control lentivirus (LV-shC) and lentivirus expressing shSmad3. The level of Galectin-3 is significantly increased following TGFβ

treatment in cells transduced with LV-ShSmad3 compared to control (LV-shC). F)
Densitometric analysis of multiple blots from experiment described in E above. Quantitative
data shown as mean + SE of 3 independent experiments. * $p < 0.05$.

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Fig. 7.

Expression of Galectin-3 in degenerated human NP tissue specimens and TGFβ responsiveness of Galectin-3 in NP cells isolated from degenerated tissue. A) Western blot analysis of Galectin-3 in human NP cells isolated from corresponding human NP samples. G1–G5 represent grades of disc degeneration of increasing severity. Expression of Galectin-3 was significantly suppressed by TGFβ treatment in each group. B) qRT-PCR analysis of Galectin-3 and CCN2 expression following TGFβ treatment of NP cells isolated from the disc tissues of six patients. Note that the expression of Galectin-3 was suppressed

in TGF β -treated cells. In contrast, CCN2 expression was increased in all samples. The expression levels of Galectin-3 and CCN2 in untreated controls were set at 1 (broken line). C–F) NP cells were treated with TNF- α (10 ng/ml) with or without Galectin-3 (5 μ g/ml) for 24 h and gene expression analysis was performed. TNF- α treatment resulted in induction in expression of IL-1 β (C), IL-6 (D), CCL2 (E), and CCL20 (F). While Galectin-3 had no effect on expression of any of the targets, it synergized TNF- α effect on expression of all targets except CCL20. Quantitative data shown as mean + SE of 3 independent experiments, * $p < 0.1$.