SMAD, ERK1/2 AND P3 KINASE PATHWAYS REGULATE TRANSFORMING GROWTH FACTOR-β-INDUCED AGGREGAN GENE EXPRESSION IN NUCLEUS PULPOSUS CELLS

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**Introduction**
In intervertebral disc, transforming growth factor-β (TGF-β) plays critical roles by inducing gene expression of cartilage-specific molecule. Conditional deletion of the TGF-β type II receptor in mice had been reported to lead to hypoplastic or missing intervertebral discs. Recently, we showed that cartilage intermediate layer protein (CILP) was associated with lumbar disc diseases. We also showed that CILP protein inhibited aggrecan mRNA expression and Smad2 phosphorylation induced by TGF-β1.

Aggrecan is the most important structural protein that constitutes NP tissues. It has been reported that aggrecan expression was induced by signaling pathways including Smads, ERK1/2, and p38 mitogen-activated protein kinase pathway in ATDC5 cells. However, in intervertebral disc cells, these mechanisms were less well understood. We, therefore, investigated expression mechanisms of the aggrecan gene induced by TGF-β in NP cells.

**Methods**
Quantitative analysis of TGF-β expression in human intervertebral disc tissues.
To investigate which TGF-β played a crucial roles, we examined expression levels of the TGF-β1, -β2 and -β3 in the human intervertebral discs by real-time PCR.

NP cell preparation and analysis of TGF-β-mediated signaling pathways.
Lumbar intervertebral discs from four consecutive levels (L2-3, L3-4, L4-5 and L5-S1) were dissected from the spines of adolescent Japanese white rabbits (1-1.5 kg, 6-9 weeks old) after sacrificing by injection of an excess amount of sodium pentobarbital. Tissue was harvested from the lumbar intervertebral discs from four consecutive levels (L2-3, L3-4, L4-5, and L5-S1) and treated with cycloheximide. The cells were also treated with SB-431542 (TGF-β type I receptor kinase inhibitor), LY294002 (phosphatidylinositol-3 kinase inhibitor), or U0126 (MEK1/2 inhibitor) at the indicated concentration and the changes in aggrecan gene expression were analyzed by real-time PCR.

Transfection and siRNA for R-Smads.
NP cells were suspended in sodium alginate (1.2% solution in 155 mM NaCl) and maintained for up to 14 days with Chondrocyte Differentiation Media. Cells were then recovered and seeded in a 12-well plate at a density of 1 x 10^5 cells/well. siRNA oligonucleotides for R-Smads were designed by Takara web site (http://www.takara-bio.co.jp) and transfected into the NP cell cultures. Suppression levels of the rabbit R-smads mRNA expression were confirmed by real-time PCR. The level of phosphorylated Smad2 was analyzed by Western blotting as previously described.

**Results**
Among TGF-β1, β2 and β3, TGF-β1 was the most prominently expressed molecule in human intervertebral discs. Addition of TGF-β1 to NP cells rapidly induced aggrecan gene expression and the induction was completely inhibited by TGF-β type I receptor kinase inhibitor (SB-431542). Next, we used cycloheximide to examine whether protein synthesis was required for aggrecan induction. Pretreatment with cycloheximide 30 min before TGF-β1 addition completely blocked transcription of aggrecan, indicating that de novo protein synthesis is essential for this response. To determine the nature of this signaling response, we examined whether R-Smads were involved in aggrecan expression. We detected that Smad2 was rapidly phosphorylated within 15 min of treatment with TGF-β1 and the phosphorylation persisted for 2 h.

We next examined the functional significance of this Smad pathway activated by TGF-β1 in the regulation of aggrecan expression using Smads siRNA. Inhibitory effects of the aggrecan expression using R-Smads siRNA were evaluated by real-time PCR (Fig.1). Aggrecan expression was significantly suppressed by siRNA of Smad2 or Smad3.

Combination of siRNA for Smad2 and 4 or Smad3 and 4 showed additional suppression or the aggrecan expression. We also investigated involvement of other signaling pathways in aggrecan expression using kinase inhibitors. Treatment of the NP cells with LY294002 and U0126 significantly suppressed aggrecan expression, and the inhibition of the aggrecan expression with LY294002 was stronger than that with U0126 (Fig.2). Finally, we investigated whether signaling cross-talk between Smad, P3 kinase and MEK1/2 pathways regulates TGF-β1-induced aggrecan gene expression by Western blotting analysis. We found that the inhibitors of MEK1/2 or P3 kinase did not affect Smad2 phosphorylation and, therefore, these pathways might act independently in aggrecan gene expression.

**Discussion**
In this study, we have demonstrated that R-Smads, MEK1/2 and P3 kinase pathways are required for the aggrecan expression following treatment with TGF-β1 in NP cells. TGF-β-induced R-Smad activation was most marked signaling that contributed to the aggrecan gene expression. Other pathways, independent with Smads, are also involved in the regulation of aggrecan expression. Possible interaction of R-Smads proteins with molecule downstream of MEK1/2 and P3 kinase pathways should be further determined.

**Reference**