factor-α in a model of herniated disc resorption. J Clin. Invest., **105**: 143-150.

Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K,Tamura F, Sakai D (2008) Transplantation of mesenchymal stem cells in a canine disc degeneration model. J Orthop Res **26**: 589-600.

Ikeda T, Nakamura T, Kikuchi T, Umeda S, Senda H, Takagi K (1996) Pathomechanism of spontaneous regression of the herniated lumbar disc: Histologic and immunohistochemical study. J Spinal Disord **9**: 136-140.

Jones P, Gardner L, Menaga J, Williams G, Roberts S (2008) Intervertebral disc cells as competent phagocytes *in vitro*: implications for cell death in disc degeneration. Arthritis Res Ther **10**: R86.

Kang JD, Georgescu HI, McIntyle-Larkin L, Stefanovic-Racic M, Donaldson WF 3rd, Evans CH (1996) Herniated lumbar intervertebral discs spontaneously produce matrix metalloproteinases, nitric oxide, interleukin-6, prostaglandin E2. Spine **21**: 271-277.

Katsuno R, Hasegawa T, Iwashina T, Sakai D, Mikawa Y, Mochida J (2008) Age-related effects of cocultured rat *nucleus pulposus* cells and macrophages on nitric oxide production and cytokine imbalance. Spine **33**: 845-849.

Le Maitre CL, Hoyland JA, Freemont AJ (2007) Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1**b** and TNF**a** expression profile. Arthritis Res Ther **9**: R77.

McCarron RF, Wimpee MW, Hudkins PG, Laros GS (1987) The inflammatory effect of *nucleus pulposus*. A possible element in the pathogenesis of low back pain. Spine **12**: 760-764.

Nachemson A (1969) Intradiscal measurements of pH in patients with lumbar rhizopathies. Acta Orthop Scand **40**: 23-42.

Park JB, Chang H, Kim KW (2001) Expression of Fas ligand and apoptosis of disc cells in herniated lumbar disc tissue. Spine **26**: 618-621.

Park JB, Chang H, Kim YS (2002) The pattern of interleukin-12 and T-helper types 1 and 2 cytokine expression in herniated lumbar disc tissue. Spine **27**: 2125-2128.

Satoh K, Konno S, Nishiyama K, Olmarker K, Kikuchi S (1999) Presence and distribution of antigen-antibody complexes in the herniated *nucleus pulposus*. Spine **24**: 1980-1984.

Schuppe HC, Meinhardt A (2005) Immune privilege and inflammation of the testis. Chem Immunol Allergy **88**: 1-14.

Spitzer G, Verma DS, Fisher R, Zander A, Vellekoop L, Litam J (1980) The myeloid progenitor cell – its value in predicting hematopoietic recovery after autologous bone marrow transplantation. Blood **55**: 317-323.

Virri J, Gronblad M, Seitsalo S, Habetemarian A, Kappa E, Karahaju E (2001) Comparison of the prevalence of inflammatory cells in subtypes of disc herniations and associations with straight leg raising. Spine **26**: 2311-2315.

Wang XM, Terasaki PI, Rankin GW Jr., Chia D, Zhong HP, Hardy S (1993) A new microcellular cytotoxicity test based on calcein AM release. Hum Immunol **37**: 264-270.

Weiler C, Nerlich BE, Boos N (2005) Expression and distribution of tumor necrosis factor alpha in human lumbar intervertebral discs: A study in surgical specimen and autopsy controls. Spine **30**: 44-53.

Wildner G, Diedrichs-Möhring M (2004) Autoimmune uveitis and antigenic mimicry of environmental antigens. Autoimmun Rev **3**: 383-387.

Yoshino H, Ueda T, Kawahata M, Kobayashi K, Ebihara Y, Manabe A, Tanaka R, Ito M, Asano S, Nakahata T, Tsuji K (2000) Natural killer cell depletion by anti-asialo GM1 antiserum treatment enhances human hematopoietic stem cell engraftment in NOD/Shi-scid mice. Bone Marrow Transplantation **26**: 1211-1216.

Journal of Cellular Physiology

The Relationship Between the Wnt/β-Catenin and TGF-β/BMP Signals in the Intervertebral Disc Cell

AKIHIKO HIYAMA,[1,2]* DAISUKE SAKAI,[1,2] MASAHIRO TANAKA,[1] FUMIYUKI ARAI,[1] DAISUKE NAKAJIMA,[3] KOICHIRO ABE,[3] AND JOJI MOCHIDA[1,2]

[1]*Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Isehara, Kanagawa, Japan*

[2]*Research Center for Regenerative Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan*

[3]*Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan*

Degeneration of the lumbar intervertebral disc (IVD) is a cause of low back pain. In osteoarthritis patients, an increase in β-catenin accumulation has been reported. However, the molecular mechanisms involved in IVD remain unclear. In the present study, we examined the relationship of Wnt/β-catenin and transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) signals in the IVDs. We found that treatment of nucleus pulposus (NP) cells with the Wnt/β-catenin activator lithium chloride (LiCl) results in the increased expression of β-catenin mRNA and protein, and cell proliferation is decreased due to the activation of the Wnt/β-catenin signals through the suppression of c-myc and cyclin-D1. In addition, T-cell-specific transcription factor (TCF) promoter activity was found to increase the following stimulation with LiCl alone, and was further increased when BMP2 was added, in comparison to the control group. We further observed the effects of treatment with PD98059, a specific inhibitor of the mitogen-activated protein kinase pathway, on TCF promoter activity in NP cells. These effects were largely attenuated by PD98059. Moreover, when transfected IVDs were co-transfected with R-Smad expression plasmids, there was a significant decrease in TCF reporter activity. We thereafter evaluated the effects of increased Wnt/β-catenin activity on the transcriptional activity of the Smad binding element (SBE). As a result, LiCl suppressed the activity of SBE reporter activity. The present study demonstrates for the first time that there are opposing effects between the Wnt/β-catenin and TGF-β/BMP signals in IVDs, which is consistent with the Wnt/β-catenin signals contributing to the pathogenesis of IVD degeneration.
J. Cell. Physiol. 226: 1139–1148, 2011. © 2010 Wiley-Liss, Inc.

It has been some time since the concept of discogenic low back pain was reported. Intervertebral disc (IVD) degeneration is a common cause of pain in these patients, and although the efficacy of surgical treatment is confirmed, new post-operative functional disorders have also been reported. In recent years, a regenerative medicine approach has attracted much attention (Thompson et al., 1991; Sakai et al., 2003; Masuda et al., 2004; Hiyama et al., 2008a, 2008b). However, in order to achieve consistent therapeutic effects, it is necessary to elucidate the molecular mechanism underlying the expression of IVD degeneration. The IVD acts as a shock absorber, has a jelly-like consistency in its center, or nucleus pulposus (NP) due to altered proteoglycan (PG) metabolism, and distributes pressure away from the spine and maintains the trunk. IVD cells exhibited degenerative change with advancing age. The most significant biochemical change to occur in disc degeneration is the loss of PG. Therefore, in elucidating IVD degeneration, the metabolism of IVD cells, or net change in PG content, is important. We previously reported the outcome of an analysis of the Smad proteins, in which the transforming growth factor-β (TGF-β) family was found to dominantly induce PG synthesis in IVD cells (Hiyama et al., 2008a, 2008b). Although it has been established that Smads function as a molecular switch to activate the transcription of various target genes, it is not yet clear how they become involved in IVD degeneration via dysregulated signaling. In addition, during IVD embryogenesis, the cells of the notochord play a critical role in initiating tissue formation, and may be directly responsible for development of the NP. In some species, including humans, these notochordal cells are eventually lost, usually through apoptosis or terminal differentiation, and are replaced by chondrocyte-like cells (Erwin and Inman, 2006). The free cytoplasmic β-catenin pool thereafter moves to the nucleus and activates the transcription of the T-cell-specific transcription factor (TCF)/lymphoid enhancer factor (LEF) family, which mediates the transcription of several genes encoding such proteins as c-myc and cyclin D1. The β-catenin molecule forms a complex with TCF/LEF to form a functional transcription factor (Gordon and Nusse, 2006). These interactions suggest that β-catenin may therefore play an essential role in the control of cellular proliferation, differentiation, cell polarity, adhesion, apoptosis, and tumorigenesis by regulating both cell–cell interactions and gene transcription (Nelson and Nusse, 2004; Clevers, 2006). Interestingly, (TGF-β/bone morphogenetic protein (BMP)) signals inhibits glycogen synthase kinase-3β (GSK3β) and

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: Japan Orthopaedics and Traumatology Foundation 0120.
Contract grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan.
Contract grant sponsor: Tokai University School of Medicine Research.

*Correspondence to: Akihiko Hiyama, Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa, 259-1193, Japan. E-mail: a.hiyama@tokai-u.jp

Received 7 April 2010; Accepted 8 September 2010

Published online in Wiley Online Library (wileyonlinelibrary.com), 13 October 2010.
DOI: 10.1002/jcp.22438

activates the Wnt/β-catenin signals in various cell types (Cheon et al., 2004). The analyses of Wnt signals using mouse models have been indispensable in elucidating the formation of various organs, and many of these studies have demonstrated the importance of the β-catenin pathway (Wells et al., 2007; Harada et al., 1999). However, there have been no reports on the role of Wnt/β-catenin signals in IVD cells or on the impact of Wnt/β-catenin signals on the metabolism of IVD cells, and as a result the degenerative process remains poorly characterized.

Therefore, it is expected that the clarification of the mechanisms controlling Wnt/β-catenin signals (inhibition and activation) as well as the mechanism of Wnt/β-catenin transcriptional activation will clarify disc differentiation and the subsequent degenerative IVD process. We herein focused on Wnt/β-catenin signals, and studied the expression of these signals and the mechanisms controlling TGF-β/BMP signals, which are important for PG synthesis in IVD cells.

Materials and Methods

Plasmids and reagents

To determine the β-catenin-TCF/LEF transcriptional activity, NP cells and annlus fibrosus (AF) cells were transiently transfected with the Tcf/Lef reporter gene Topflash (optimal Tcf-binding site) or Fopflash (mutated Tcf-binding site) (Upstate Biotechnology Inc., Lake Placid, NY). The aggrecan reporter plasmid (Agg-luc) was provided by Dr. Michael C. Naski (The University of Texas Health Science Center at San Antonio). The aggrecan promoter carries 1.2 kb of the proximal mouse promoter (Reinhold et al., 2006). The Smad binding element (SBE)4-luc, SBE2-luc, and MBE6-luc plasmids, which have four copies of the Smad binding site upstream of the luciferase gene, two copies of the Smad binding site, and two copies of the mutant Smad binding site, respectively, were purchased from Addgene (Cambridge, MA) (SBE4-luc:16495, SBE2-luc:16500, and MBE6-luc:16497).The c-myc reporter plasmid (16601) was also purchased from Addgene. The R-Smad expression plasmids (Smad1, Smad2, Smad3, Smad5, and Smad8) and the empty backbone plasmid were provided by Dr. Miyazono at the University of Tokyo at Tokyo in Japan. As an internal transfection control, we used the empty vector pGL4.74 (Promega, Madison, WI) containing the *Renilla reniformis* luciferase genes. Lithium chloride (LiCl) has been shown to promote Wnt/β-catenin signals through the β-catenin pathway. In addition, Dickkopf-1 (DKK1) (R&D Systems, Minneapolis, MN) is a secreted protein that acts as a soluble inhibitor of the Wnt signaling pathway. In some experiments, to inhibit MEK activation, the MEK inhibitor PD98059 (Calbiochem, San Diego, CA) dissolved in dimethyl sulfoxide (DMSO) was added to the cell cultures.

Preparation of Wnt3A, Wnt5a-containing conditioned medium

The L-Wnt3A cell line stably expressing Wnt-3A, Wnt-5a and the control WT L-cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). Medium conditioned by L-Wnt3A, L-Wnt5A or WT L-cells over a 72 h period (hereafter termed Wnt3A-containing conditioned medium (Wnt3A-CM), Wnt5A-containing conditioned medium (Wnt5A-CM) or WT-CM, respectively) were collected, processed, and used according to the manufacturer's protocol.

Isolation of intervertebral disc cells

NP and AF cells were isolated from a total of 32 female Sprague-Dawley (SD) rats (6–9 months of age) using a previously reported method (Hiyama et al., 2008a, 2008b). The gel-like NP was separated from the AF by using a dissecting microscope. The isolated cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics in a humidified atmosphere containing 5% CO_2 at 37°C. When confluent, NP and AF cells were harvested and subcultured in 10-cm dishes. We used the low-passage (<3) cells cultured in a monolayer.

Immunofluorescence microscopy

NP cells were plated in flat-bottom 96-well plates (5,000 cells/well) and were treated with LiCl (20 mM) or BMP2 (100 ng/ml) for 24 h. After incubation, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in phosphate buffered saline (PBS) for 10 min, blocked with PBS containing 5% FBS, and incubated with antibodies against β-catenin (1:200; catalog no. 9562; Cell Signaling, Inc, Danvers, MA), GSK3β (1:200; catalog no. 9315; Cell Signaling) or Wnt3a (1:200; catalog no. 2721; Cell Signaling) at 4°C for 18 h. After washing, the cells were incubated with anti-rabbit AlexaFluor488 secondary antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:50 and 10 μm DAPI for 1 h at room temperature. The cells were imaged by laser-scanning confocal microscopy. For quantification purposes, a minimum of 100 cells, spanning five different microscopy fields, were scored for staining.

Immunohistological studies

Freshly isolated 3-week-old rat and embryonic mouse (E9.5 and E15.0) spinal tissue specimens were immediately fixed in 4% paraformaldehyde in PBS and were embedded in paraffin. Transverse and coronal sections were deparaffinized in xylenes, rehydrated through a graded ethanol series, and stained with hematoxylin and eosin. For localizing Wnt3a, sections were incubated with an anti-Wnt3a antibody (Cell Signaling) in 2% bovine serum albumin in PBS at a dilution of 1:200 at 4°C overnight. After thoroughly washing the sections, the bound primary antibody was incubated with a biotinylated universal secondary antibody, at a dilution of 1:20 (Vector Laboratories, Burlingame, CA) for 10 min at room temperature. Sections were incubated with a streptavidin/peroxidase complex for 5 min and washed with PBS, and the staining was developed using the 3′-3-diaminobenzidine substrate (Vector Stain Universal Quick Kit; Vector Laboratories).

MTT assay

The effects on cellular proliferation were measured using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the ability of live cells to utilize thiazolyl blue and convert it into the water-insoluble dark blue formazan stain. Exponentially growing NP cells were seeded into a 24-well plate at 1.5×10^4 cells/well. After LiCl stimulation (20 mM for 24–48 h), cells were treated with MTT (5 g/L, Sigma[Q6]) for 2 h at 37°C, DMSO (Me_2SO) was added into each well, and the reaction was incubated for 30 min. Subsequently, the cells were transferred to a 96-well plate. A 96-well microtitre plate reader (GE health care, Buckinghamshire, UK) was used to quantify the A_{590}. The number of control cells that is viable cells not exposed to any treatment, was defined as 100%.

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

IVD cells were cultured in 6-cm dishes (5×10^5 cells/dish) 1 day before treatment with LiCl (20 mM) for 24 h. Following treatment, cells were lysed, and the total RNA was isolated using the Trizol reagent (Invitrogen). As mentioned above, cDNA was synthesized by the reverse transcription of mRNA. The real-time PCR analyses were performed in duplicate using 96-well plates with the Fast SYBR green master mix (Life Technologies, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Two microliters of cDNA per sample were used as a template for real-time PCR; 1 μl forward primer and 1 μl reverse primer were added to 20 μl SYBR green master mix. The reactions were synthesized in a 20-μl reaction volume with the following conditions: Initial step at 50°C for 2 min, followed by 95°C for 10 min; 40 cycles of (denaturation, 95°C for 3 s, and hybridization/elongation, at 60°C for 30 s). All primers (β-catenin, GSK3β,

cyclin-D1, c-myc, and GAPDH) were designed based on the coding sequences from Genbank (AF121265.1, NM_032080.1, NM_080782.3, NM_012603.2, and NM_017008.3, respectively), and were synthesized by Takara Bio Inc. (Tokyo, Japan). The relative quantitation of real-time RT-PCR data was performed using the delta–delta C_t method (Livak and Schmittgen, 2001). Data are reported as the average value of the range of the calculated fold difference, which incorporates the standard deviation of the delta–delta C_t value in the fold difference calculation as delta–delta C_t + SD and delta–delta C_t − SD. The statistical analyses were calculated using the SPSS software program (SPSS 14.0, Chicago, IL) using a one-way ANOVA with Tukey's HSD post-hoc pairwise comparisons ($P < 0.05$).

Transfections and dual luciferase assay

NP cells were transferred to 24-well plates at a density of 6×10^4 cells/well 1 day before transfection. The next day, to investigate the effect of LiCl and BMP2 on Topflash activity, NP cells were treated with LiCl (20 mM) or BMP2 (100 ng/ml) with 900 ng of the Topflash reporter plasmid. To evaluate Tcf transcription activity through the Smad pathway, which is downstream from the TGF-β/BMP signals, we used an expression plasmid. NP and AF cells were co-transfected with 500 ng of a R-Smad (Smad1, Smad2, Smad3, Smad5, or Smad8) or the backbone vector with 400 ng of the Topflash reporter plasmid. We then evaluated the effects of an increased Wnt/β-catenin activity on the transcription activity of the SBE4 and SBE2 promoter, which lies upstream of the luciferase gene and is the specific promoter for the target gene of the Smad complex. At the same time, we also conducted an evaluation for cases involving the MBE6 reporter, which is a mutant vector. Briefly, NP and AF cells were transfected with 900 ng of the SBE4 reporter, the SBE2 reporter or the MBE6 reporter plasmid. The cultured cells were treated with or without LiCl (20 mM) for 24 h, and the luciferase reporter activity was measured. Thereafter, to evaluate the effects of ERK signaling on the transcription activity of SBE and TCF, NP cells were treated with or without 100 ng/ml BMP2 and 1, 10, or 100 mM PD98059 with 900 ng of the Topflash reporter plasmid or 900 ng of the SBE4 reporter plasmid. In addition, to evaluate the effect of Wnt3a on the transcriptional activity of SBE and aggrecan, NP and AF cells were co-transfected with 100–500 ng of Wnt3a, or the respective empty backbone vectors, with 400 ng of SBE4 reporter plasmid or the aggrecan reporter plasmid. The pGL4.74 plasmid containing the *R. reniformis* luciferase gene was used as an internal control for normalization of all transfection experiments. Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. Forty-eight hours after the initial transfection, the cells were harvested and a Dual-Luciferase reporter assay system (Promega) was used for the sequential measurements of the firefly and renilla luciferase activities. Transfection efficiency for rat nucleus and annulus cells was about 50–60%. The luciferase activities and calculation of the relative ratios were quantified using a Turner Designs Luminometer Model TD-20/20 (Promega) (TD-20/20; Turner Designs, CA).

Statistical analysis

All measurements were performed in triplicate and were repeated with two independent cultures. Data are presented as the mean ± SD. To test for significance, data were analyzed using an unpaired Student's *t*-test; the obtained *P*-values are indicated in the text and figures ($^*P < 0.05$). $P < 0.05$ was considered to be statistically significant.

Results

To investigate the role of the Wnt/β-catenin signals in the IVD, we first determined whether rat IVD cells express β-catenin mRNA. Figure 1A shows that treatment for 6–24 h with LiCl (20 mM), which mimics Wnt/β-catenin signals activation, results in increased β-catenin mRNA levels in both NP and AF cells. In order to assess the relationship with TGF-β/BMP signals caused by the activation of Wnt/β-catenin signals, we performed a reporter assay to confirm the TCF activity. The results revealed the Topflash reporter activity to increase by 7.16-fold following stimulation with LiCl (20 mM), and was further increased by 9.5-fold when BMP2 (100 ng/ml) was added, when compared to the control group (Fig. 1B). In addition, we determined the expression levels of the β-catenin protein in rat NP cells following treatment with LiCl (20 mM) or BMP2 (100 ng/ml). As shown in Figure 1C, an immunofluorescence analysis with an anti-β-catenin antibody demonstrated that LiCl and BMP2 treatment induces the expression of total β-catenin levels and promotes the nuclear translocation of β-catenin in NP cells. LiCl and BMP2 treatment for 24 h increased the average proportion of β-catenin-positive NP cells (LiCl) compared to untreated controls (Cr) (LiCl: 84.51 ± 4.45%, BMP2: 53.34 ± 3.50%, Cr: 39.04 ± 9.82%; $P < 0.05$). To further explore the effect of LiCl suppression on GSK3β gene and protein expression in NP cells, we performed a real-time PCR and immunofluorescence analysis.
Figure 1D shows that there is a marked suppression in GSK3β mRNA levels when NP cells were treated with LiCl (20 mM), and an immunofluorescence analysis showed the cytoplasmic staining to decrease by treatment with an anti-GSK3β antibody in LiCl-treated NP cells. From these data, we demonstrated that the inhibition of GSK3β by LiCl induced the nuclear translocation of β-catenin in NP cells.

Furthermore, when NP and AF cells were co-transfected with R-Smad expression plasmids, a significant decrease was observed in the Topflash reporter activity both in NP cells (Fig. 2A) and in AF cells (Fig. 2B). We thereafter evaluated the effects of increased Wnt/β-catenin activity on the transcriptional activity of the SBE4 reporter and the SBE2 reporter, in which the luciferase genes lie downstream of the SBE, which is the specific promoter for the target genes of the Smad complex. At the same time, we also conducted an evaluation for cases involving the MBE6 reporter, which is a mutant vector. As a result, treatment with LiCl inhibited SBE4 and SBE2 reporter activity, whereas the MBE6 reporter failed to suppress activity both in NP cells (Fig. 2C) and in AF cells (Fig. 2D). The ERK signaling cascade transmits signals from a variety of extracellular stimuli to multiple cellular processes, including cell proliferation, differentiation, and development. Recently, the existence of crosstalk between Wnt and ERK pathways was reported. Therefore, we further examined the effect of mitogen-activated protein kinase signaling on TCF reporter activity in NP cells. NP cells were transfected with the Topflash or SBE4 reporter plasmid and treated with BMP2 or LiCl and PD98059 for 24 h. PD98059 prevented the BMP2 or LiCl-induced increase in Topflash and SBE4 reporter activity (Fig. 3A–C). In addition, to investigate the further effect of Wnt/β-catenin signals, we analyzed ERK1 and ERK2 mRNA expression by real-time PCR. Figure 3D shows that treatment with LiCl (20 mM) for 24 h resulted in decreased ERK1 and ERK2 mRNA levels in NP cells. Conversely, ERK1 and ERK2 mRNA levels were not affected by LiCl treatment in AF cells (Fig. 3E).

In addition, we determined whether the treatment of NP cells with LiCl (20 mM) resulted in a decreased cell proliferation. An MTT assay was performed to assess the rates of Wnt/β-catenin-induced NP cell proliferation.
Figure 4A shows that cell proliferation decreased due to the activation of the Wnt/β-catenin signals compared to untreated controls (24 h; 70.39 ± 3.19%, control; 100 ± 7.25%, 48 h; 92.27 ± 7.00%, control; 164.67 ± 3.5%). Furthermore, we determined the expression levels of c-myc and cyclin-D1

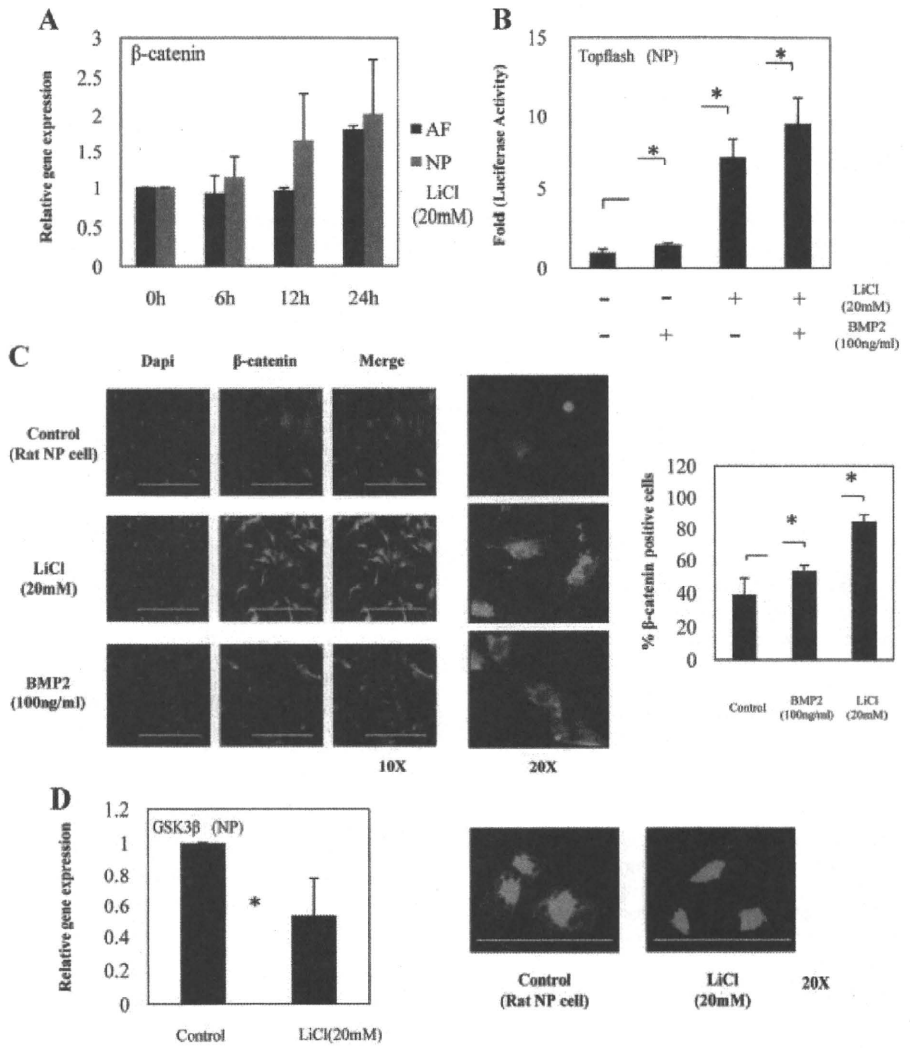


Fig. 1. LiCl and BMP2 activate β-catenin expression in IVD cells. A: Real-time RT-PCR of β-catenin mRNA levels in the presence or absence of 20 mM LiCl for 6–24 h, both in NP cells and AF cells. B: NP cells were co-transfected with the Topflash reporter plasmid and the pGL4.74 vector, and were treated with LiCl (20 mM) or BMP2 (100 ng/ml). The luciferase activity was measured 24 h after transfection. C: Confocal imaging of β-catenin nuclear translocation in NP cells after treatment with LiCl (20 mM) or BMP2 (100 ng/ml) for 24 h. Left: Cells were stained with DAPI to identify healthy nuclei. Middle: Cells stained with an antibody to β-catenin. Right: Cells stained with β-catenin and DAPI. Scale bar: 200 μm (original magnification 10× or 20×). D: Real-time reverse transcription-polymerase chain reaction of GSK3β mRNA levels in the presence or absence of LiCl (20 mM) for 24 h in NP cells. NP cells were stained for immunofluorescence with the anti-GSK3β and fluorescent-labeled secondary antibodies (right). Scale bar: 200 μm (original magnification 20×). The values represent the mean ± SD of three independent experiments (*P < 0.05).

mRNA, which regulate cell proliferation following treatment with LiCl (20 mM). Figure 4B shows that treatment with LiCl for 24 h results in decreased c-myc and cyclin-D1 mRNA levels in NP cells. This result was similar to the c-myc reporter activity observed following LiCl treatment (Fig. 4C). We next examined the morphological changes in NP cells after exposure to LiCl (20 mM) or DKK1 (100 ng/ml) (Fig. 4D). Figure 4D shows that treatment with LiCl decreased the number of NP cells, whereas treatment with DKK1 did not decrease the number of NP cells. In addition, there was no marked influence on the cellular morphology.

We further investigated the role of the Wnt ligand in the IVD, and first determined whether rat IVDs express Wnt3a. Wnt3a is a secreted ligand and activates members of the Frizzled family of receptors. Wnt3a is one of the closely related Wnt family members and has multiple roles in development and cell fate. Recently, some groups have reported that Wnt3a induces the expression of chondrocyte marker genes, and is a key regulator of the chondrocyte life cycle during embryonic development. Figure 5A shows the expression of Wnt3a in the neonatal rat (3 weeks of age) and embryonic mouse (day E9.5 and E15.0). The immunohistological analyses revealed that Wnt3a is expressed in notochordal cells and NP cells. Much of the staining was nuclear. However, some staining was observed in the cytosol of the NP cells. In addition, we determined the expression levels of Wnt3a protein in rat NP cells following treatment with LiCl (20 mM) or BMP2 (100 ng/ml). As shown in Figure 5B, the immunofluorescence analyses with an anti-Wnt3a antibody

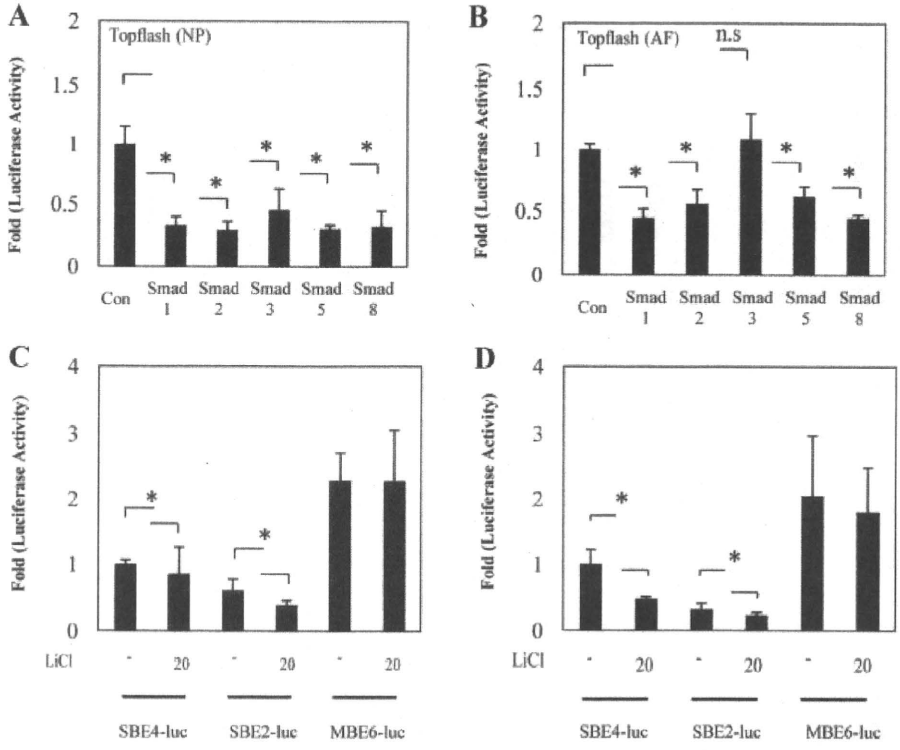


Fig. 2. The TGF-β/BMP and Wnt/β-catenin signals produce opposite effects. A, B: Subconfluent rat NP (A) and AF (B) cells were co-transfected with the Topflash reporter plasmid (400 ng) with either R-Smad expression plasmids (500 ng) or the respective empty backbone vectors (500 ng). The cells were cultured for 48 h after transfection, and reporter activity was measured. C, D: The SBE4 reporter (900 ng), the SBE2 reporter (900 ng), or the MBE6 reporter (900 ng) were transfected into rat NP (C) and AF (D) cells along with the pGL4.74 vector (100 ng). The cultured cells were treated with LiCl (20 mM) for 24 h and luciferase reporter activity was measured thereafter. The values represent the mean ± SD of three independent experiments (*$P < 0.05$).

demonstrated that LiCl or BMP2 treatment induced total Wnt3a protein expression and promoted the nuclear translocation of Wnt3a in NP cells. To further evaluate the activation of Wnt3a, we measured the activity of the Topflash reporter in NP and AF cells. These cells were transfected with plasmids encoding TCF and were treated with Wnt3A-CM, a Wnt protein that signals through β-catenin; companion cultures received a control vehicle, medium (WT-CM), or DKK1. Figure 5C shows that Topflash reporter activity was induced 50-fold in Wnt3A-CM-treated cultures within 24 h in NP cells. In contrast, Topflash reporter activity was activated by 10-fold in Wnt3A-CM-treated cultures within 24 h in AF cells. DKK1 addition suppressed Topflash reporter activity. Similar levels of reporter activity were elicited by treatment with the Wnt3a expression plasmid (data not shown).

In order to determine whether the Wnt3a protein directly interacts with BMP-2 and TGF-β signaling, the SBE4 reporter plasmid was co-transfected with the WT-Wnt3a expression plasmid into NP and AF cells. Figure 5D shows that SBE4 reporter activity is unresponsive to Wnt3a treatment in a dose-dependent manner in both NP and AF cells. To understand the Wnt/β-catenin signals in IVD cells, we also examined the Wnt5a protein, which stimulates non-canonical Wnt signaling. Both NP and AF cells were transiently transfected with either the Topflash reporter or the Fopflash reporter along with the pGL4.74 vectors. The cultured cells were treated with either Wnt3a- or Wnt5a-CM for 24 h, and luciferase reporter activity was measured. Figure 6A shows that Topflash reporter activity is activated by Wnt3a-CM treatment both in NP cells and in AF cells, whereas Topflash reporter activity is not affected by Wnt5a-CM treatment.

In aging IVDs, there is a well-recognized loss of cells, PG, and type II collagen. While the loss of PG is the primary chemical change in IVD degeneration present in lower back pain patients, little is known concerning the events that control this loss. Therefore, to further investigate the possible mechanisms of Wnt3a or Wnt5a protein in NP cells, we examined the effect of Wnt3a or Wnt5a protein treatment on aggrecan promoter activity in NP cells. We measured the basal activity of the aggrecan reporter in IVD cells. Figure 6B shows that NP cells displayed a significantly higher (1.8-fold) basal level of aggrecan reporter activity than AF cells. We then determined if Wnt3a or Wnt5a mediated its effects on aggrecan through the Wnt/β-catenin signals. Figure 6C shows that when NP cells were co-transfected with the aggrecan reporter plasmid and either WT-Wnt3a or WT-Wnt5a or the respective empty backbone vectors, the aggrecan reporter activity was not affected in a dose-dependent manner by either WT-Wnt3a or by the WT-Wnt5a expression plasmids.

Discussion

The purpose of the present study was to clarify the mechanisms controlling the inhibition and activation of the Wnt/β-catenin signals and the mechanism of its transcriptional activation. Therefore, we conducted an analysis of the crosstalk between

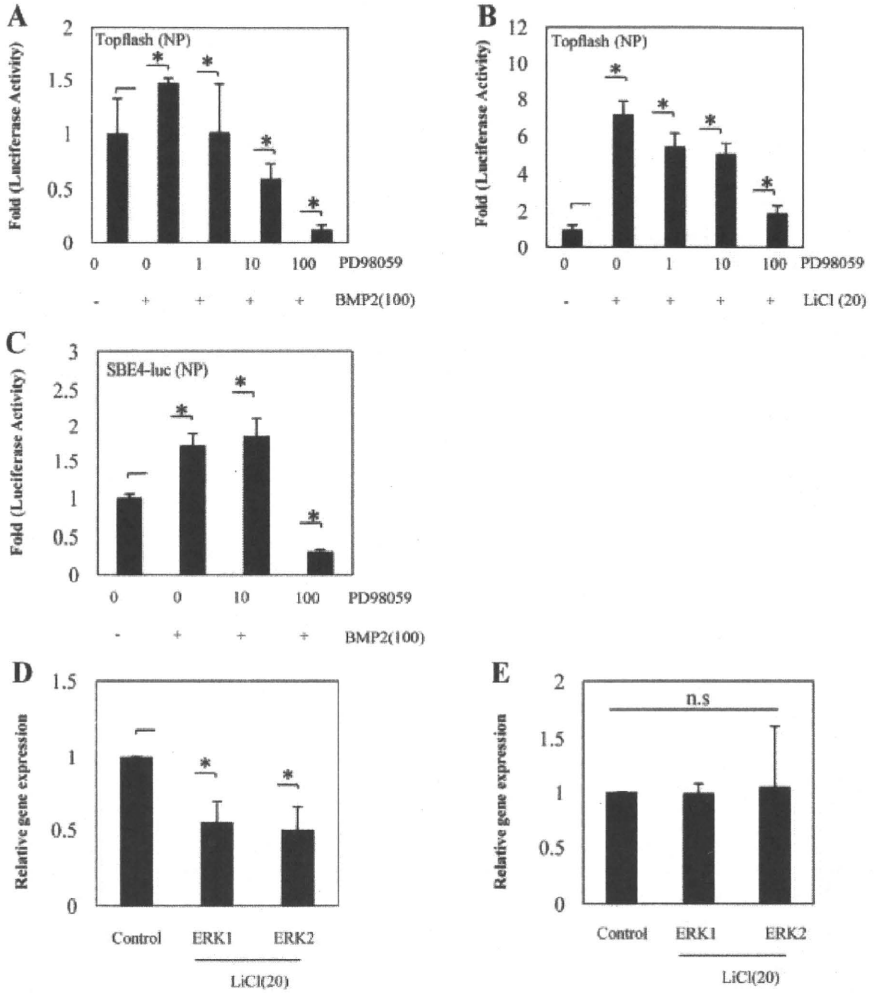


Fig. 3. Extracellular signal regulated-kinase (ERK) pathways regulate the Wnt/β-catenin signals. A, B: The Topflash reporter plasmid was transfected into rat NP cells along with the pGL4.74 vector, and was stimulated with BMP-2 (A) or LiCl (B) in the presence or absence of PD98059. Luciferase activity was measured 24 h after transfection. C: The SBE4 reporter plasmid was transfected into rat NP cells along with the PGL4.74 vector, and was stimulated with BMP-2 in the presence or absence of PD98059. The luciferase activity was measured 24 h after transfection. D, E: Total RNA from stimulated rat NP (D) and AF (E) cells was extracted and reverse transcribed into cDNA followed by real-time PCR, quantified, and normalized to untreated cells, which was arbitrarily set at 1.0. Values were normalized to the levels of GAPDH. The relative expression levels of ERK1, ERK2, and GAPDH were determined.

TGF-β/BMP signals, which was found to be important for PG synthesis in IVD cells.

However, there was a limitation with respect to the analyses and data that may affect the accuracy of these results. The limitation of the present study is the fact that we used LiCl to activate Wnt/β-catenin signaling. Selectivity is a key issue when GSK-3 inhibitors are used as pharmacological tools to demonstrate the involvement of GSK-3 in a cellular process. The selectivity of most of the available GSK-3 inhibitors is poorly known. At least 30 small molecule GSK-3 inhibitors have been developed (Alonso and Martinez, 2004; Meijer et al., 2004). Among those, LiCl has been widely used for research purposes. Clinically, lithium salts are prescribed for treatment of bipolar disorders, depression, and mania. GSK3β was firstly identified as a target of LiCl in 1996 (Klein and Melton, 1996). Several groups have used LiCl, an inhibitor of GSK3β (Berridge et al., 1989; Stambolic et al., 1996; Spencer et al., 2006), to examine these relationships. Inhibition of GSK3β leads to the accumulation of β-catenin and the activation of Wnt/β-catenin-dependent signals. It is important to note that GSK3β has other functions and that LiCl is therefore not specific for just activating the Wnt/β-catenin signals alone (Crabtree and Olson, 2002). However, we do not know the mechanism to regulate Wnt/β-catenin by LiCl in IVD. Therefore, we used LiCl as Wnt/β-catenin activator to get the first data. Moreover, we used WT-β-catenin instead of LiCl treatment, and also evaluated the effect of WT-β-catenin on reporter activity. These data were similar to results of LiCl treatment (data not shown). It means that activity of LiCl produced activation of Wnt/β-catenin signal.

First, in order to study whether NP cells express the TCF factors necessary for β-catenin-mediated transcriptional activation, LiCl was added to measure the levels of β-catenin nuclear translocation, and the transcriptional activation of Topflash. With GSK3β inhibition, the levels of β-catenin nuclear translocation and activation of Topflash substantially

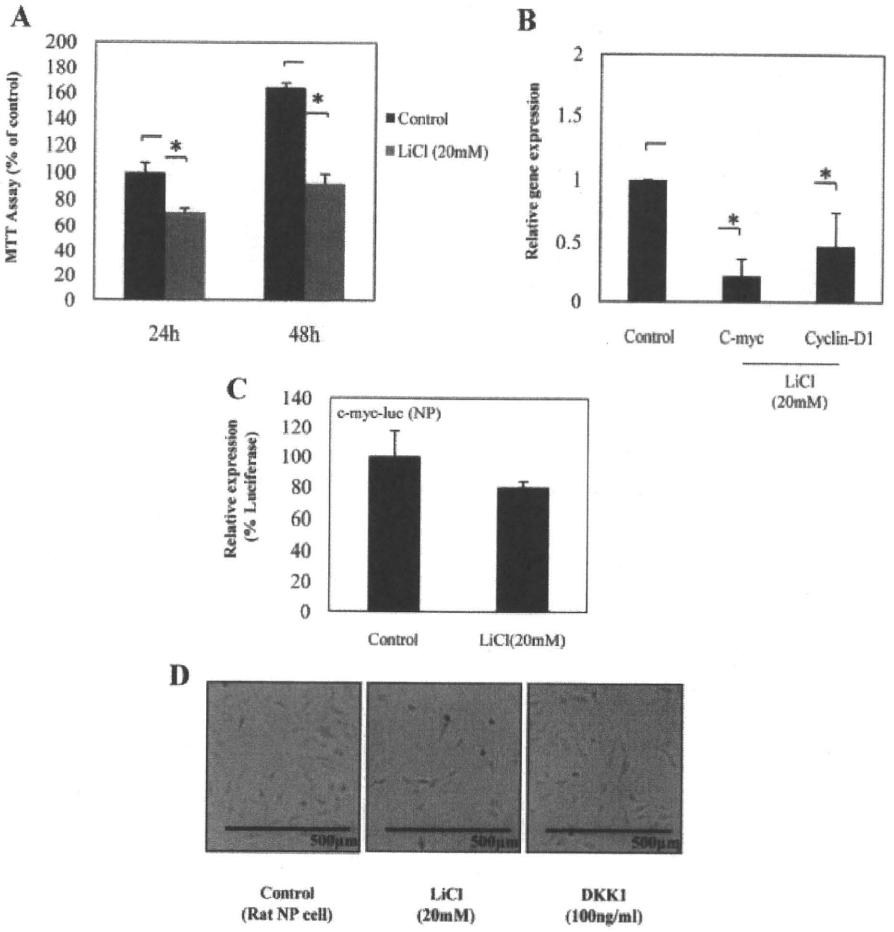


Fig. 4. The Wnt/β-catenin signals suppress IVD cell proliferation. A: The effect on cellular proliferation was also measured using the MTT assay as described in the Materials and Methods Section. *Statistically significant changes compared with the controls. B: We examined the effect of Wnt/β-catenin signaling on the expression levels of c-myc and cyclin-D1 in NP cells. The relative expression levels of c-myc, cyclin-D1, and GAPDH were determined by a real-time PCR analysis, quantified, and normalized to untreated cells, whose expression values were set to 1.0. GSK3β inhibition (activation of Wnt/β-catenin signals) caused suppression in c-myc and cyclin-D1 mRNA. C: The c-myc reporter (900 ng) was transfected into rat NP cells along with the pGL4.74 vector (100 ng). The cultured cells were treated with LiCl (20 mM) for 24 h and luciferase reporter activity was measured thereafter. D: Micrographs of NP cells harvested from 12-week-old SD rats were cultured in medium in the presence or absence of LiCl (20 mM) or DKK1 (100 ng/ml) for 24 h. Scale bar: 500 μm. The represented values indicate the mean ± SD of three independent experiments (*$P < 0.05$).

increased. In addition, when BMP2 was added with LiCl, Topflash activation increased further. This suggested that Wnt/β-catenin signals were important in IVD cells, and that TGF/BMP signal was involved in the activation of Wnt/β-catenin signals. The TGF-β superfamily binds to a type 2 receptor, a specific receptor existing on the membrane surface, and the bound type 2 receptor forms a complex with a type 1 receptor (Wrana et al., 1994; Zhang et al., 1996). Thereafter, when the type 1 receptor kinase is activated, it phosphorylates R-Smads, which are intracellular signal transduction molecules. The R-Smads include Smad2 and Smad3, which transmit TGF-β/activin signals, and Smad1, Smad5, and Smad8, which transmit BMP signals. When phosphorylated, R-Smads are able to bind to Smad4, called the Co-Smad, and the R-Smad/Co-Smad complexes formed in the cytoplasm can then enter the nucleus (Macías-Silva et al., 1996; Massagué, 1998). It is believed that once in the nucleus, the Smad complexes bind to SBEs in the promoters of target genes and, together with other transcription factors and transcription coupling factors, regulate the expression of these genes. Therefore, we focused on the Smad component of TGF/BMP signals and analyzed the R-Smad-mediated interaction of β-catenin. When R-Smad expression plasmids were transfected into IVD cells, Topflash activation markedly decreased. Furthermore, when IVD cells were treated with LiCl, the transcriptional activation of SBE was also inhibited. These results suggested that the Smad signal and the Wnt/β-catenin signals inhibit one another, and that they are involved in the maintenance of IVD homeostasis. However, it was confirmed that Topflash activation increases with the addition of BMP2, suggesting that Wnt/β-catenin signal activation is regulated by Smad-independent signals. Recently, the ERK inhibitor PD98059 was shown to prevent the inhibition of GSK3β and the nuclear accumulation of β-catenin induced by TGF-β/BMP signals (Grimes and Jope, 2001; Ding et al., 2005). Therefore, in the present study, in order to examine whether BMP-mediated activation of Wnt/β-catenin signals is induced by

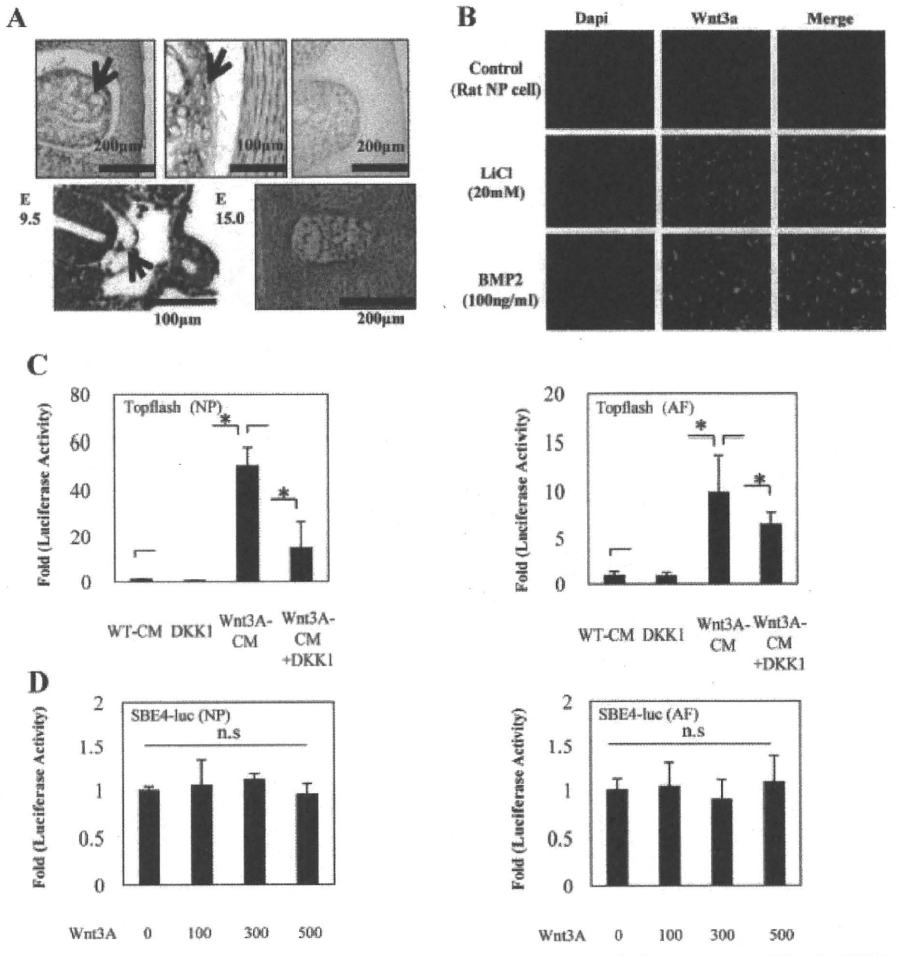


Fig. 5. Wnt3a is expressed in the IVD and activates Wnt/β-catenin signals. A: Sagittal sections of the IVD of neonatal rat (upper) and embryonic mouse (E9.5 and E15.0) (lower). Sections were treated with an anti-Wnt3a antibody and were counterstained with hematoxylin. Note that NP cells express Wnt3a protein (arrows in A). The original magnification is 10× or 20×. B: Detection of Wnt3a expression in NP cells by immunofluorescence microscopy after culturing cells with LiCl (20 mM) or BMP2 (100 ng/ml) for 24 h. Left: Cells were stained with DAPI to identify healthy nuclei. Middle: Cells stained with an antibody to Wnt3a. Right: Cells stained with Wnt3a and DAPI. Scale bar: 200 μm. C: The Topflash reporter plasmid was transfected into rat NP and AF cells along with the pGL4.74 vector, and were stimulated with DKK1 (100 ng/ml) or Wnt3A-CM for 24 h. D: Wnt3a was co-transfected with the SBE4 reporter into NP and AF cells.

ERK, PD98059 was used to inhibit ERK, therefore resulting in the concentration-dependent inhibition of the activation of Topflash and also suggesting the involvement of ERK in the activation of Wnt/β-catenin signals.

Furthermore, LiCl treatment was used to activate the Wnt/β-catenin signals, and the expression of both ERK1 and ERK2 genes was found to be markedly inhibited, thereby indicating the inhibitory involvement of the Wnt/β-catenin signals in TGF/BMP signaling. These data show that there was a time-lag between ERK1/2 or GSK3β phosphorylation and the nuclear translocation of β-catenin in response to BMP2 and LiCl. This may reflect a slow nuclear translocation of β-catenin following ERK1/2 activation/GSK3β inhibition.

Furthermore, in regard to the decrease in the number of cells, which is another important factor of IVD degeneration, it was found that the activation of Wnt/β-catenin signals led to cytostatic activity. With aging, notochord-like cells in the NP decreased in number and amyloid-like tissue replaced NP. We hypothesized that, with the activation of the Wnt/β-catenin signals, β-catenin target genes c-myc and cyclin-D1 may be involved in the initiation of cytostatic activity. It was previously suggested that Wnt signaling can promote both the c-myc and cyclin-D1 genes by the upregulation of β-catenin (He et al., 1998; Shtutman et al., 1999). Therefore, we analyzed the expression of these genes by a real-time PCR analysis and found that the expression was significantly inhibited, thereby indicating that these transcription factors regulate the growth of IVD cells. The results of the studies described herein suggest that this mechanism may be cell-specific, and LiCl may induce another pathway to suppress c-myc and cyclin-D1 gene expression.

Thereafter, stimulation by Wnt3a, the primary Wnt member of the Wnt/β-catenin signaling family, resulted in increased Topflash promoter activity and cytostatic effects, as observed with LiCl treatment, whereas a similar experiment using the Wnt antagonist Dickkopf (DKK)-1 did not induce any changes in activation. This finding suggested that the cytostatic activity in IVD cells by Wnt3a is mediated by Wnt/β-catenin signals. In

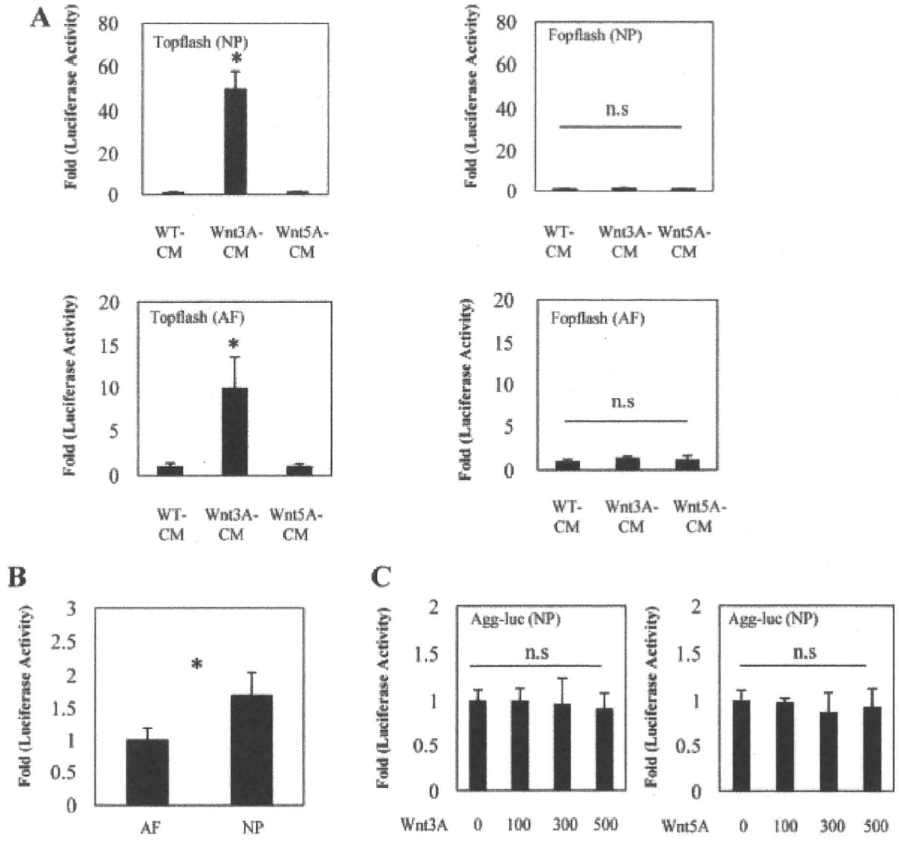


Fig. 6. Wnt3a and Wnt5a do not regulate aggrecan promoter activity. A: In order to define a functional mechanism between the Wnt3a and Wnt5a in NP cells (upper) and AF (lower) cells, cells were transfected with the Topflash (900 ng) or the Fopflash (900 ng) reporter plasmid and were treated with Wnt3A-CM or Wnt5A-CM. B: The aggrecan reporter plasmid was transfected into rat NP and AF cells along with the pGL4.74 vector, and the basal activities of the reporter were measured by a dual luciferase assay. C: NP cells were co-transfected with the aggrecan reporter plasmid with either Wnt3a, Wnt5a or empty vectors. The cells were cultured for 48 h after transfection, and the reporter activity was measured thereafter.

addition, the Topflash reporter activity for Wnt3a in NP cells was higher than that in AF cells. Their discovery suggests for the first time NP cells may have a high affinity for the Wnt3a protein than AF cells.

However, Wnt3a was not involved in PG synthesis associated with degeneration or regeneration of the IVD or in TGF/BMP signals crosstalk. Furthermore, the activation of Topflash was not observed after Wnt5a treatment, which activates a β-catenin-independent pathway, and Wnt5a was not found to be involved in the growth of cells (data not shown). Furthermore, Wnt5a was not involved in PG synthesis, as observed with Wnt3a.

In summary, based on the present findings, we concluded that the Smad signal and the Wnt/β-catenin signals inhibit each other, and that they are involved in the maintenance of IVD homeostasis. The present findings suggested that activation of Wnt/β-catenin signals by BMP2 may be regulated by Smad-independent signals. Furthermore, we have shown, for the first time, β-catenin target both genes c-myc and cyclin-D1 probably due to the stability of the NP cells in terms of cell proliferation. However, Wnt3a and Wnt5a were not involved in the PG synthesis associated with either the degeneration or regeneration of the NP cells using reporter assays. These results showed that different signal pathways might thus be involved in the regulation of the cell proliferation and PG synthesis in NP cells. However, we did not suggest whether the decrease of the cell number participated with low back pain from this study. We will think that it is necessary to investigate the relationship with low back pain and IVD degeneration at the molecular level in the future study. In addition, future studies will address the role of c-myc and cyclin-D1 and determine whether the regulation of cell growth by Wnt/β-catenin signals is specific to IVD cells.

Acknowledgments

We thank Dr. Kohei Miyazono and Dr. Michael C. Naski for kindly providing necessary reagents. We also thank Dr. Tadayuki Sato for helpful advice and excellent technical assistance. This work was supported by the Japan Orthopaedics and Traumatology Foundation (Grant 0120), a Grant-in-Aid for Scientific Research, and a Grant of The Science Frontier Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a grant from Tokai University School of Medicine Research.

Literature Cited

Alonso M, Martinez A. 2004. GSK-3 inhibitors: Discoveries and developments. Curr Med Chem 11:755–763.

Berridge MJ, Downes CP, Hanley MR. 1989. Neural and developmental actions of lithium: A unifying hypothesis. Cell 59:411–419.
Cheon SS, Nadesan P, Poon R, Alman BA. 2004. Growth factors regulate beta-catenin-mediated TCF-dependent transcriptional activation in fibroblasts during the proliferative phase of wound healing. Exp Cell Res 293:267–274.
Clevers H. 2006. Wnt/beta-catenin signaling in development and disease. Cell 127:469–480.
Crabtree GR, Olson EN. 2002. NFAT signaling: Choreographing the social lives of cells. Cell 109:S67–S79.
Ding Q, Xia W, Liu JC, Yang JY, Lee DF, Xia J, Bartholomeusz G, Li Y, Pan Y, Li Z, Bargou RC, Qin J, Lai CC, Tsai FJ, Tsai CH, Hung MC. 2005. Erk associates with and primes GSK-3beta for its inactivation resulting in upregulation of beta-catenin. Mol Cell 19:159–170.
Erwin WM, Inman RD. 2006. Notochord cells regulate intervertebral disc chondrocyte proteoglycan production and cell proliferation. Spine 31:1094–1099.
Gordon MD, Nusse R. 2006. Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem 281:22429–22433.
Grimes CA, Jope RS. 2001. The multifaceted roles of glycogen synthase kinase 3 beta in cellular signaling. Prog Neurobiol 65:391–426.
Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, Taketo MM. 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J 18:5931–5942.
He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. 1998. Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512.
Hiyama A, Mochida J, Omi H, Serigano K, Sakai D. 2008. Cross talk between Smad transcription factors and TNF-alpha in intervertebral disc degeneration. Biochem Biophys Res Commun 369:679–685.
Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K, Tamura F, Sakai D. 2008. Transplantation of mesenchymal stem cells in a canine disc degeneration model. J Orthop Res 26:589–600.
Klein PS, Melton DA. 1996. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci USA 93:8455–8459.
Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25:402–408.
Macías-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, Wrana JL. 1996. MADR2 is a substrate of the TGFbeta receptor and phosphorylation is required for nuclear accumulation and signaling. Cell 87:1215–1224.
Massagué J. 1998. TGF-beta signal transduction. Annu Rev Biochem 67:753–791.
Masuda K, Oegema TR, Jr., An HS. 2004. Growth factors and treatment of intervertebral disc degeneration. Spine 29:2757–2769.
Meijer L, Flajolet M, Greengard P. 2004. Pharmacological inhibitors of glycogen synthase kinase 3. Trends Pharmacol Sci 25:471–480.
Nelson WJ, Nusse R. 2004. Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303:1483–1487.
Reinhold MI, Kapadia RM, Liao Z, Naski MC. 2006. The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis. J Biol Chem 281:1381–1388.
Sakai D, Mochida J, Yamamoto Y, Nomura T, Okuma M, Nishimura K, Nakai T, Ando K, Hotta T. 2003. Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc: A potential therapeutic model for disc degeneration. Biomaterials 24:3531–3541.
Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Zéev A. 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 96:5522–5527.
Spencer GJ, Utting JC, Etheridge SL, Arnett TR, Genever PG. 2006. Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis in vitro. J Cell Sci 119:1283–1296.
Stambolic V, Ruel L, Woodgett JR. 1996. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr Biol 6:1664–1668.
Thompson JP, Oegema TR, Jr., Bradford DS. 1991. Stimulation of mature canine intervertebral disc by growth factors. Spine 16:253–260.
Wells JM, Esni F, Boivin GP, Aronow BJ, Stuart W, Combs C, Sklenka A, Leach SD, Lowy AM. 2007. Wnt/beta-catenin signaling is required for development of the exocrine pancreas. BMC Dev Biol 12:4.
Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. 1994. Mechanism of activation of the TGF-beta receptor. Nature 370:341–347.
Zhang Y, Feng X, We R, Derynck R. 1996. Receptor associated Mad homologues synergize as effectors of the TGFb response. Nature 383:168–172.

自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究

Clinical trial of disc repair with activated nucleus pulposus cells by mesenchymal stem cells

Keywords
椎間板変性
再生
活性化髄核細胞
骨髄間葉系幹細胞
共培養

持田 譲治
東海大学医学部外科学整形外科学

Summary

Degeneration of intervertebral discs of lumbar spine is a major cause of low back complaints and it is an irreversible occurrence without any available treatment. The authors have already demonstrated an important role for the nucleus pulposus in preserving overall disc structure. Therefore, we have designed an innovative method with activation of nucleus pulposus cells by mesenchymal stem cells using co-culture method. Similarly to the animal studies, cell proliferation of human nucleus pulposus cells by autologous mesenchymal stem cells having direct cellular interaction through the membrane pores was much greater than other culture systems such as incubated alone or co-culture regularly. No apparent tumorigenic findings were noted. Furthermore, no abnormality of the chromosome of the activated nucleus pulposus cells was determined. Based on *in vitro* and *in vivo* studies with various animal models and *in vitro* study in the human material, the clinical application was sent to the associated council of Ministry of health, Labour and Welfare and our project was approved in 2008. The authors started the clinical application from February 2009 and have already finished the transplantation of the activated nucleus pulposus cells into the degenerated disc in nine cases. This is a report in detail.

Mochida, Joji
Department of Orthopaedic Surgery, Surgical Science, Tokai University School of medicine
E-mail : jomo@is.icc.u-tokai.ac.jp

はじめに

わが国の腰痛患者数は800万人前後とする報告がある。腰痛の原因は多彩であり，脊椎に起因しない場合もあるが，一方脊椎疾患による腰痛の６，７割では椎間板変性がinitial triggerとなっている。米国の保険会社の医療費支払い額のデーターでは腰痛疾患が第２位であり,大きな問題となっている。良性疾患ではあるが絶対数の多い腰痛疾患の中核となる椎間板変性進行を予防する，あるいはその進行を抑制する取り組みは医療経済上も極めて重要である。

本稿では，「厚生労働省ヒト幹細胞を用いる臨床研究に関する指針」に従って承認された『自家骨髄間葉系幹細胞により活性化された椎間板髄核細胞を用いた椎間板再生研究』に至る経過と，すでに実施された９症例の概要を紹介する。

椎間板変性と腰痛

椎間板変性が進行すると外層線維輪部から感覚神経の終末が血管とともに椎間板内に侵入する。疼痛発現機序については，その神経終末部での炎症による化学的刺激や物理的な負荷が関与するとされる。

一方，椎間板変性が進行すると，後方関節部に物理的な負荷が加わり，関節軟骨の破綻が生じ関節症性疼痛が生じる。椎骨のすべり，骨棘や椎間板の後方への膨隆によって後方の神経根が圧迫され下肢痛だけでなく腰痛も出現する。さらに椎間不安定性や椎間高減少により，後方の筋肉，腱，靱帯などの軟部組織に炎症が生じ，軟部組織起因性腰痛が生じる。

したがって，椎間板変性の進行を遅らせる，またはすでに変性が始まった椎間板組織を再生の方向に向かわせることは，椎間板が原因となる一時的あるいは二次的な腰痛を予防しその克服につながると考えられる[1]。

椎間板再生，変性抑制の方法

椎間板の再生，変性抑制を目的として国内外で継続されている研究は以下の5つに分けられる。①椎間板細胞基質の活性亢進のためのサイトカイン，成長因子の注入，②遺伝子導入，③細胞移植，④組織工学的手法による椎間板組織の作製，⑤同種椎骨椎間板椎骨ユニット移植など，が基礎研究から橋渡し研究に進み，一部では臨床研究として開始されている。東海大学整形外科学領域では③の細胞移植療法を選択し，過去16年間にわたる研究を継続している[1]。椎間板変性は細胞数に依存することが判明しており，この細胞移植療法は中等度までの変性に対応できる抑制力，再生力をもつことから，臨床上も現実的な手法である。一方①では変性の自然経過例に過大適応されてしまう危惧があり，②は組織再生の個体差が大きく，安全性評価が未解決であり，④では体外での作製が困難であり，⑤は良性疾患である椎間板変性の治療における同種移植に関しての反対意見が大勢を占めている。

東海大学における椎間板変性抑制，再生研究

東海大学では細胞移植療法をその手法として選択し，移植細胞として髄核細胞そのものと骨髄間葉系幹細胞を選択した[2]。2つの実験系を動物実験で立案，実施した結果，髄核細胞の移植を選択した。

骨髄間葉系幹細胞の移植による椎間板組織，特に髄核細胞の導出は良好ではあるが，この幹細胞の髄核腔内での様態が人に応用する上ではさらに検索が必要と考えられた。一方，髄核細胞移植は元々ある細胞の復元であり，生物学的に極めて自然な手技と考えられる。高い細胞学的安全性があり，自家細胞を用いれば組織拒絶反応がなく，中等度までの椎間板変性に対応可能であることから，臨床応用を考える際に最も現実的な細胞であると判断した。しかし，髄核細胞そのものは細胞数が少なく，細胞活性も通常は低い。そこで，骨髄間葉系幹細胞のもつfeeding cellとしての力を利用し，また体外での培養期間を可及的に短くすることを意図し，細胞間接着を伴う共培養で髄核細胞を活性化することを考案した。

骨髄間葉系幹細胞による髄核細胞の活性化実験(動物，ヒト)

細胞間接着を伴う骨髄間葉系幹細胞との共培養による髄核細胞の活性化法を図1に示す。この方法(Group C)で

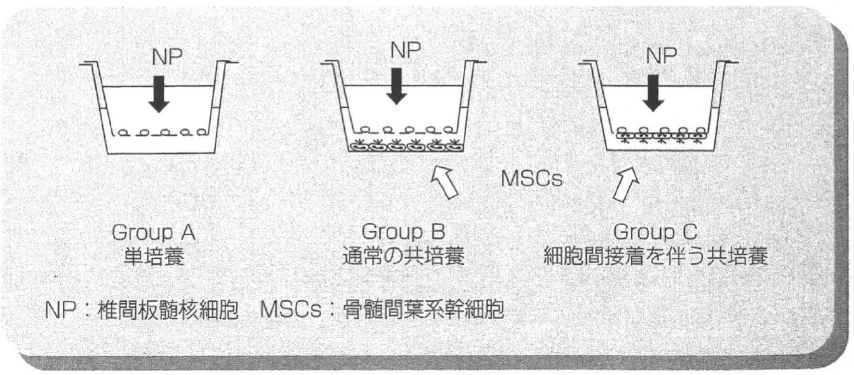


図1

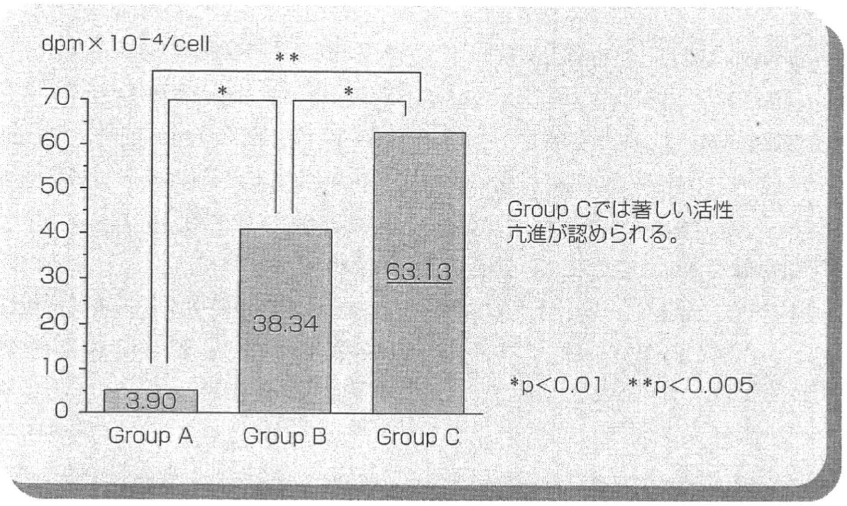


図2 プロテオグリカン(白色家兎)

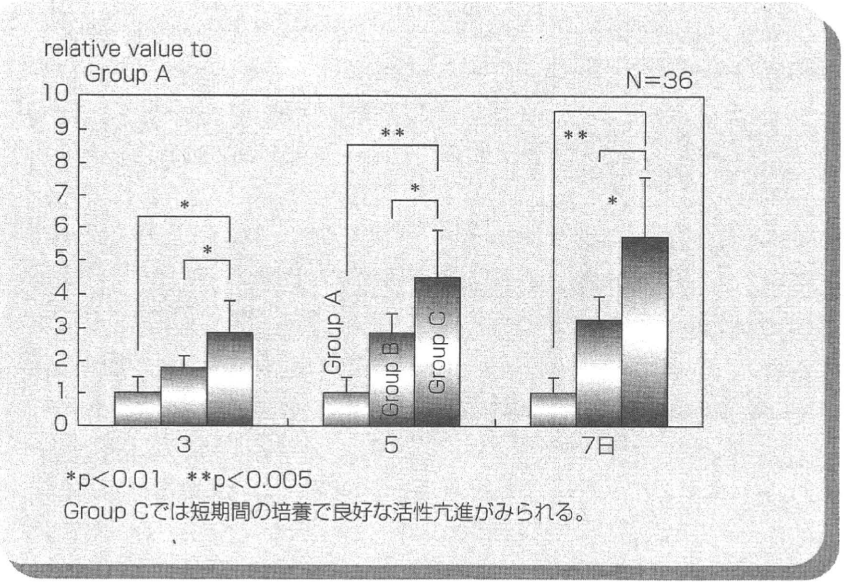


図3 プロテオグリカン(ヒト)

2週間で活性化された日本白色家兎の1髄核細胞あたりのDNA活性は，髄核細胞単独による培養(単培養：Group A)の27倍に，同じく1髄核細胞あたりの細胞基質プロテオグリカンの活性は16倍に亢進した(**図2**)[3]。

ヒト骨髄間葉系幹細胞との細胞間接着を伴う共培養で活性化されたヒト椎間板髄核細胞では，5日間という短期間の細胞間接着を伴う共培養で，単培養した髄核細胞の約5倍のDNA活性と，同じく5倍の細胞基質のプロテオグリカン活性の亢進が認められた(**図3**)[4]。活性化されたヒト椎間板髄核細胞には染色体異常や腫瘍化などは一切認められず，細胞活性化手法としての安全性が示された。

臨床研究の進捗

「厚生労働省ヒト幹細胞を用いる臨床研究に関する指針」に基づき，2007年4月に「厚生労働省ヒト幹細胞臨床研究に関する審査委員会」に『ヒト骨髄間葉系幹細胞との細胞間接着を伴う共培養で活性化されたヒト椎間板髄核細胞を用いた臨床研究』を申請した。審査委員会からの数回にわたる質疑，回答を経て，2008年1月にその実施が承認された。

良性疾患である椎間板再生に幹細胞を使用することの是非，適応される病態と年齢，活性化髄核細胞標準書の評価，バリデーションマスタープランの評価，GMP衛生管理基準書の評価などがその内容であり，質疑は細部に及んでいた。同審査委員会より同指針制定後第5例目の案件として承認されたが，10症例に対する第Ⅰ相試験を主眼とした臨床研究である。承認後細部の準備を重ね，2009年2月から臨床研究を開始した。

臨床研究の概略

図4に本臨床研究の対象となる患者の適応項目を示す。20歳代の腰椎椎間板ヘルニア，腰椎分離症，腰椎椎間

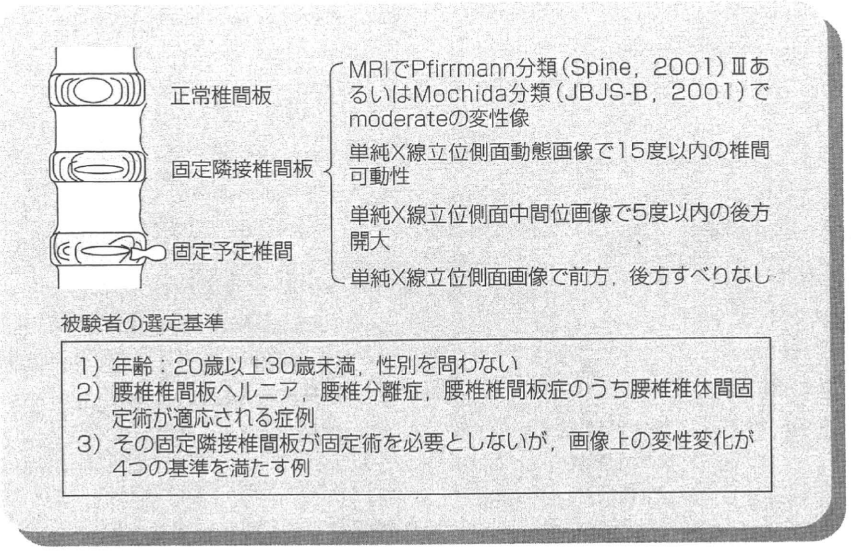


図4

板症などで当該椎間板切除＋同椎間固定を行う症例で，隣接椎間板に中等度の変性がみられる症例が対象である。

椎間固定時に摘出した椎間板組織より髄核細胞を分散し，同じく手術中に腸骨から採取した骨髄液より骨髄間葉系幹細胞を比重分離法で集積する。各々4日間単培養後，細胞間接着を伴う共培養を3日間行い，活性化された髄核細胞を7日間で隣接椎間板内に移植する。活性化髄核細胞移植後1週，2週，1，3，6ヵ月，1，2，3年時に，移植椎間板の変性状態を画像上，定性的，半定量的に評価する。また同時期に血液生化学的検査，全身状態の検診を行う。

臨床研究の結果概略

2010年8月までに9症例(男性8例，女性1例)で移植術が実施され，その経過観察期間は最長1年7ヵ月，最短1ヵ月である。

Cell processing centerで実施された髄核細胞の7日間の活性化の間，エンドトキシン，マイコプラズマ(PCR法)，マイコプラズマ(培養法)，細菌培養(好気性，嫌気性)，15種類のウイルス検査が，それぞれ事前に決められた基準に準じて，受け入れ時，中間管理時，最終製品時(髄核細胞活性化終了時)などに実施されたが，9症例ともにそのいずれの時期においても感染を示す所見は一切認められなかった。また細胞間接着を伴う共培養終了時の髄核細胞の生存率は91～98％であり，細胞数の増加率は4.2～6.3倍であった。細胞数の増加だけではなく，髄核細胞の中のどの構成細胞が増加しているかについての検索を行い，データーをまとめている[5]。

その経過観察期間のすべてにおいて自他覚所見上，有害事象は一切みられなかった。また血液生化学的検査では，第1，第2例目で移植後の短期間でGPT値が一過性に正常，異常値の境界値を示したが，活性化髄核細胞移植と直接関係する事象ではなく，短期間で正常値に復帰した。

画像上の経過観察では，当該移植椎間高の移植前高に対する1/3以上の狭小化，5度以上の後方開大，不安定性出現などは全くみられず，MRI上もPfirrmann Ⅲの状態を維持している。MRI上のT2値やADC(apparent diffusion coefficient)値もオプションとして検索している。

考　察

体外で他細胞により活性化された椎間板細胞を移植する本臨床研究は，国内外で初めての実施である。椎間板変性は良性の疾患であるが，その進行は脊柱のもつ体重の支持，関節，神経のコンテナーという役割を破綻させ，腰痛や下肢障害を引き起こす。高齢化社会の到来によりその頻度が増加し，また病態の増悪化も著しい。椎間板変性の加齢変化以上の進行は，脊椎の種々疾患のinitial triggerとなる。したがって椎間板の機能を温存する手法の開発が望まれ，活性化髄核細胞移植術の臨床応用はその先端に位置している。

今回の臨床研究は，体外で骨髄間葉系幹細胞で活性化された髄核細胞を体内に移植する過程における安全性を主眼とした研究である。最長3年までの

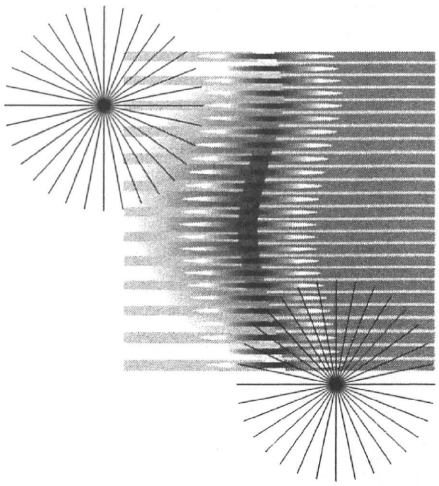
経過観察が義務付けられているが，移植が行われた9症例には有害事象は一切みられず，血液生化学的データーも正常である。また，本稿では他誌への投稿と重複するため，現在までの移植椎間板の状態に関して詳細なデーターを示していない。しかし画像上，移植が有効であると判定できる基準を満たしたまま,経過観察が続けられている。10例の移植を終了し，全例で2年以上の経過観察が終了した時点で新たな報告をしたいと考えている。

共同研究者
【整形外科学】酒井大輔，山本至宏，渡辺拓也，檜山明彦，芹ケ野健司，田村　太，田中真弘，新井文征，岩品　徹，大見博子，大熊正彦，渡辺雅彦
【再生医療科学】加藤俊一，浅原孝之，中村雅登
【Cell processing center】中村嘉彦
【臨床薬理学】小林広幸
【血液腫瘍内科学】安藤　潔
【再生医学センター】中井知子

●文　献

1）Mochida J : New strategy for disc repair : novel preclinical trials. J Orthop Sci **10** : 112-118, 2005
2）Sakai D, Mochida J, Yamamoto Y, et al : Transplantation of mesenchymal stem cells embedded in Atelocollagen gel to the intervertebral disc : a potential therapeutic model for disc degeneration. Biomaterials **24** : 3531-3541, 2003
3）Yamamoto Y, Mochida J, Sakai D, et al : Upregulation of the viability of nucleus pulposus cells by bone marrow-derived stromal cells : significance of direct cell-to-cell contact in coculture system. Spine **29** : 1508-1514, 2004
4）Watanabe T, Sakai D, Yamamoto Y, et al : Human nucleus pulposus cells significantly enhanced biological properties in a coculture system with direct cell-to-cell contact with autologous mesenchymal stem cells. J Orthop Res **28** : 623-630, 2010
5）酒井大輔，持田譲治：細胞レベルからの椎間板再生―細胞移植療法のその先に―．日本腰痛学会雑誌 **15** : 95-98, 2009

椎間板再生の展望



持田譲治
東海大学医学部外科学系整形外科学教授

二足動物であるヒトは，古代より腰痛に悩まされてきた記録がみられ，また現在では高齢化社会の到来により，腰痛や関連する下肢症状を訴える患者数も増加しています。わが国の腰痛患者数のデータは流動的ですが，800万人前後とする報告が多いようです。腰痛の原因は多彩であり，脊椎に起因しない内臓疾患による場合もありますが，脊椎疾患に起因する腰痛の60～70％が椎間板変性によるものです。

米国の保険会社の医療費支払いの第１位は心疾患ですが，腰痛と関係する疾患は第２位であり，医療経済上も大きな問題となっています。腰痛の克服は国家戦略上も重要な位置づけとなってきており，その中核となる椎間板変性進行を予防する，あるいはその進行を抑制する取り組みも，近年とくに注目されてきています。

椎間板とは

椎間板は椎骨と椎骨に挟まれた軟骨様組織であり，中心部の髄核組織を線維輪組織が取り囲み，椎骨との境には終板があります。温泉まんじゅうのような構造の組織ですが，正常な組織では血行がなく，椎骨側からの拡散機構によって栄養を得ています。とくに椎間板の中心部にある髄核では血行はまったくなく，同部の酸素分圧も低くなっています。加齢変化や変性が進んでくると，椎間板周囲から血管が入り込んできます。関節軟骨とともに一度変性がはじまると，その組織の傷みの程度は戻らないとされています。

椎間板変性と腰痛

椎間板変性が進行すると外層線維輪部から血管が入り込み，同時に感覚神経の終末も血管とともに椎間板内に侵入します。この感覚神経終末が椎間板性疼痛発現につながるとされますが，その疼痛発現機序については，神経終末部での炎症による化学的刺激や物理的な負荷が関与するといわれています。

一方，椎間板変性が進行すると，椎間板高の減少，正常な椎間可動性の破綻（多くの場合は可動性の増大）や，前方，後方への椎骨のすべり出現によって，後方関節部に物理的な負荷が加わり，関節軟骨の破綻が生じます。その結果，関節症性の疼痛が生じます。椎間高の減少や椎間板の後方への膨隆，椎骨のすべり，骨棘によって後方の神経根が圧迫され，下肢痛や腰痛が出現します。さらに，このような椎間の不安定性や椎間高の減少によって，後方の筋肉，靭帯，腱などの軟部組織に炎症や傷害がおこり，軟部組織に起因する腰痛が生じます。

したがって，椎間板変性の進行は椎間板そのものが原因となる腰痛をひきおこすだけではなく，二次的に関節性あるいは軟部組織性の腰痛が生じることとなります。椎間板変性の進行を時間的に遅らせる，あるいは変性がはじまった椎間板の組織，細胞を再生の方向に向かわせることは，椎間板が原因となる一時的あるいは二次的な腰痛を予防し，克服するために重要と考えられます。

椎間板変性抑制，再生の方法

椎間板の変性抑制，再生のために，国内外でいろいろな研究が継続されています。

①椎間板細胞の基質の活性を高めるためのサイトカインや成長因子の注入療法
②遺伝子療法
③細胞移植療法
④組織工学的手法による椎間板組織の作成
⑤同種椎骨椎間板椎骨ユニットの移植

など，さまざまな取り組みが基礎研究，橋渡し研究，臨床研究として行なわれています。

筆者はこのなかから，③細胞移植療法を選択し，1994年以来研究を継続してきました。①では変性の比較的軽い椎間板が対象となり，変性の自然経過例に過大適応されてしまう可能性があり，②では安全性の課題が未解決であり，また組織再生の可否についても予測がしにくいという問題があります。④では体外での作成が困難であり，⑤は同種移植が本邦で受け入れられる下地は乏しい状況です。一方，椎間板の変性は細胞数に依存することが判明しており，また中等度までの変性に対応できる抑制力，再生力をもつことから，③の細胞移植療法には大きな期待が寄せられています。

東海大学における椎間板変性抑制，再生の試み

前節の検討から，東海大学では細胞移植療法をその手法として選択しました。1994年以来研究を継続していますが，移植細胞として髄核細胞そのものと骨髄間葉系幹細胞を選択しました。前者はもともと椎間板内にある細胞を移植する方法であり，後者は骨髄間葉系幹細胞により椎間板内で髄核あるいは椎間板組織の導出を意図したものです。二つの実験系を動物実験で立案，実施した結果，髄核細胞の移植を選択しました。

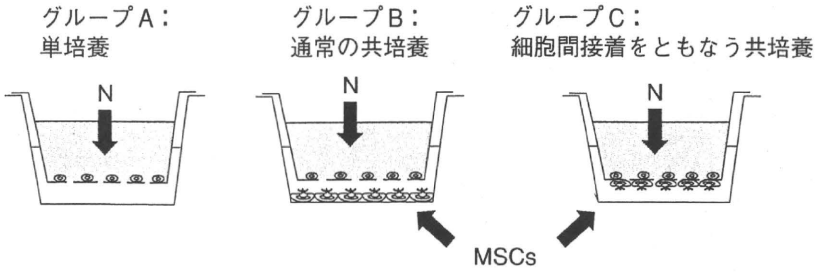
髄核細胞を選択した理由は，生物学的に自然な，もともと在る細胞の復元であり，高い細胞学的安全性があって，自家細胞を用いれば組織拒絶反応がなく，中等度までの椎間板変性に対応可能であることから，臨床応用を考える際にもっとも現実的な細胞であると判断したためです。しかし，髄核細胞自体は細胞数が少なく，細胞活性も高い状態ではありません。そこで，骨髄間葉系幹細胞のもつ feeding cell としての力を利用し，細胞間接着をともなう共培養で髄核細胞を活性化することを考案し，基礎実験を継続しました。

骨髄間葉系幹細胞による髄核細胞の活性化実験

図1に，細胞間接着をともなう骨髄間葉系幹細胞との共培養による髄核細胞の活性化法を示します。図2では，この方法によって活性化された日本白色家兎の髄核細胞1細胞あたりの DNA 活性と，同じく1細胞あたりの細胞基質のプロテオグリカンの活性を，髄核細胞単独による培養（単培養）ならびに通常の共培養と比較検討しています。

細胞間接着をともなう共培養によって髄核細胞の DNA 活性，プロテオグリカンの活性はともに著明に亢進しています。ヒト骨髄間

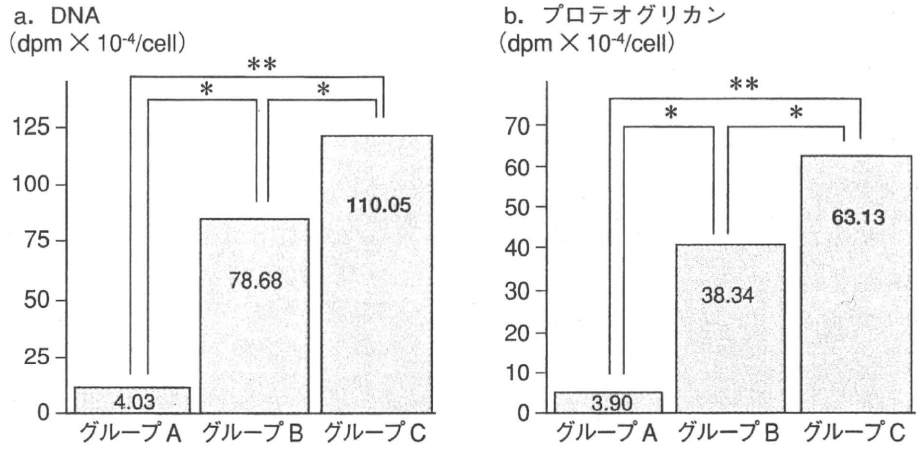
図1　骨髄間葉系細胞との共培養による髄核細胞の活性化法



N；椎間板髄核細胞，MSCs；骨髄間葉系幹細胞

中央と右の共培養法では，骨髄間葉系幹細胞と髄核細胞がお互いに影響を及ぼす

図2　培養グループ間における髄核細胞のDNA，プロテオグリカンの活性化比較



＊p＜0.01，＊＊p＜0.001

グループA：単培養，B：通常の共培養，C：細胞間接着をともなう共培養

グループCではDNAとプロテオグリカンのいちじるしい活性亢進が認められる

葉系幹細胞との細胞間接着をともなう共培養で活性化されたヒト椎間板髄核細胞も，わずか5日間という短期間の共培養により，単培養した髄核細胞の約5倍のDNA活性，ならびに同じく5倍の細胞基質のプロテオグリカンの活性の亢進が認められました。また，活性化されたヒト椎間板髄核細胞には染色体異常や腫瘍化などは一切認められず，細胞活性化手法としての安全性が示されました。

臨床研究の進捗

厚生労働省の「ヒト幹細胞を用いる臨床研究に関する指針」にもとづき，2007年4月に「厚生労働省ヒト幹細胞臨床研究に関する審査委員会」に対して，ヒト骨髄間葉系幹細胞との細胞間接着をともなう共培養で活性化されたヒト椎間板髄核細胞を用いた臨床研究を申請し，08年1月にその実施が承認されました。種々準備を重ね，09年2月から臨床研究を開始しました。10症例に対する第1相試験を主眼とした臨床研究であり，すでに7症例を終了し，経過観察中です。その詳細については厚生労働省への報告，ならびに別途専門誌へ投稿予定であり，本稿ではその詳細は記載できませんが，概略について紹介します。

●研究の概略と結果

20代の腰椎椎間板ヘルニア，腰椎分離症，腰椎椎間板症などで当該椎間板を切除して同

椎間を固定する必要がある患者において，その隣接椎間板に中等度の変性がみられる症例が対象となります。椎間固定時に摘出した椎間板から髄核細胞を分散し，同じく手術中に採取した骨髄液から骨髄間葉系幹細胞を導出します。おのおの4日間の単培養の後，細胞間接着をともなう共培養を3日間行ない，活性化された髄核細胞を7日間で隣接椎間板内に移植します。活性化髄核細胞移植後3年間，移植椎間板の変性状態を画像上で，定性的ならびに半定量的手法で評価します。また細胞移植後に血液生化学的検査，全身状態の検診を定期的に行ないます。

すでに実施された7症例では，その経過観察期間のすべてにおいて有害事象，血液生化学的データーの異常，画像上の異常はいっさい生じていません。安全な細胞移植療法であることが示されていますが，さらに症例を加え，長期的な経過観察の予定です。

●考　察

脊椎には，①体重の支持，②関節，③神経のコンテナーの役割があります。椎間板変性が進行すると，この三つの機能が損なわれることが多いですが，その治療の際には関節の機構を犠牲にした治療，すなわち椎間の固定術によって体重の支持機構や神経のコンテナー機構を復元する治療法が長年にわたり継続されてきました。

しかし，椎間固定術後の隣接椎間板の変性進行は多数例でみられ，新たな腰痛や下肢痛の原因となります。また椎間固定にいたらない変性段階の椎間板においても，その椎間板が腰痛や不安定性の原因となっていることも多くあります。したがって，椎間板変性を時間的に遅らせる，あるいは変性した椎間板組織を再生の方向に戻すという治療は，脊椎全体の機能の維持を考える際に，非常に重要かつ有効な方法と考えられます。

〈文　献〉

1）Watanabe T et al：Human nucleus pulposus cells significantly enhanced biological properties in a coculture system with direct cell-to-cell contact with autologous mesenchymal stem cells. J Orthop Res 28：623-630, 2010

2）酒井大輔，持田譲治：細胞レベルからの椎間板再生――細胞移植療法のその先に．日本腰痛学会雑誌 15：95-98，2009

3）Mochida J：New strategy for disc repair: Novel preclinical trials. J Orthop Sci 10：112-118, 2005

4）Yamamoto Y et al：Upregulation of the viability of nucleus pulposus cells by bone marrow-derived stromal cells: significance of direct cell-to-cell contact in coculture system. Spine 29：1508-1514, 2004

［もちだ・じょうじ／整形外科］