

DEVELOPMENT AND DISEASE

Regulation of the chondrocyte phenotype by β -catenin

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SUMMARY

β -Catenin regulates important biological processes, including embryonic development and tumorigenesis. We have investigated the role of β -catenin in the regulation of the chondrocyte phenotype. Expression of β -catenin was high in prechondrogenic mesenchymal cells, but significantly decreased in differentiated chondrocytes both *in vivo* and *in vitro*. Accumulation of β -catenin by the inhibition of glycogen synthase kinase-3 β with LiCl inhibited chondrogenesis by stabilizing cell-cell adhesion. Conversely, the low level of β -catenin in differentiated articular chondrocytes was increased by post-translational stabilization during phenotypic loss caused by a serial monolayer culture or exposure to retinoic acid or interleukin-1 β . Ectopic expression of β -catenin or

inhibition of β -catenin degradation with LiCl or proteasome inhibitor caused de-differentiation of chondrocytes. Transcriptional activation of β -catenin by its nuclear translocation was sufficient to cause phenotypic loss of differentiated chondrocytes. Expression pattern of Jun, a known target gene of β -catenin, is essentially the same as that of β -catenin both *in vivo* and *in vitro* suggesting that Jun and possibly activator protein 1 is involved in the β -catenin regulation of the chondrocyte phenotype.

Key words: Cartilage, Chondrocytes, Differentiation, De-differentiation, β -Catenin, Chick

INTRODUCTION

Chondrocytes in cartilage are differentiated from mesenchymal cells during embryonic development, which can be mimicked by micromass culture of mesenchymal cells *in vitro* (Sandell and Adler, 1999; DeLise et al., 2000). Both *in vivo* and *in vitro* chondrocyte differentiation require precartilaginous condensation and its progression to cartilage nodule. The differentiated chondrocyte phenotype in normal mature cartilage is characterized by the synthesis and maintenance of cartilage-specific extracellular matrix (ECM) molecules, including type II collagen and sulfated proteoglycan. The differentiated phenotypes of chondrocytes are unstable, with rapid loss of their markers by exposure to interleukin (IL)-1 β (Goldring et al., 1994; Demoor-Fossard et al., 1998) and retinoic acid (RA) (Cash et al., 1997; Weston et al., 2000) or during *in vitro* culture (Lefebvre et al., 1990; Yoon et al., 2002). The de-differentiated cells redifferentiate to chondrocytes when cultured three-dimensionally (Bonaventure et al., 1994; Yoon et al., 2002). Homeostasis in the synthesis of cartilage ECM is also destroyed during osteoarthritis and rheumatoid arthritis, which is characterized by degradation and insufficient synthesis of cartilage matrix by chondrocytes and increased

numbers of apoptotic chondrocytes (Poole, 1999; Sandell and Aigner, 2001). Pro-inflammatory cytokines such as IL1 β play a predominant role in structural and biochemical changes of chondrocytes during cartilage destruction (Martel-Pelletier et al., 1999; Choy and Panayi, 2001).

Although the maintenance of the differentiated chondrocyte phenotype is important for cartilage homeostasis, the detailed mechanisms of the maintenance and loss of differentiated chondrocyte phenotypes remain largely unknown. Cell-to-cell interaction mediated by N-cadherin is an important regulator of both precartilaginous condensation and its progression to cartilage nodule during chondrocyte differentiation (Sandell and Adler, 1999; DeLise et al., 2000). Based on the importance of the strictly regulated expression of N-cadherin, it is believed that expression of N-cadherin-related cytoskeletal components such as α - and β -catenin play a role in chondrocyte differentiation. In addition to the stabilization of cell-cell adhesion by interacting with cadherin, β -catenin is also engaged in the regulation of gene expression by acting as a transcriptional co-activator in the regulation of several biological functions (Ben-Ze'ev and Geiger, 1998; Willert and Nusse, 1998). In the presence of Wnt signal, β -catenin escapes from ubiquitin-dependent proteolytic degradation via the 26S

proteasome, and the accumulated β -catenin translocates into the nucleus in association with members of the T cell-factor (TCF)/lymphoid-enhancer-factor (LEF) family of transcription factors to stimulate transcription of target genes.

The role of β -catenin in the regulation of chondrogenesis or phenotypic loss of chondrocytes during cartilage destruction remains largely unknown. To address this issue, we investigated the function of β -catenin in the regulation of phenotypic changes of chondrocytes (i.e., differentiation, de-differentiation, and redifferentiation). In addition to *in vivo* examination, we employed micromass culture of embryonic mesenchymal cells as a model system to study chondrogenesis, a serial monolayer culture or treatment of articular chondrocytes with RA or IL1 β to study de-differentiation, and three-dimensional culture of de-differentiated cells in alginate gel beads to study redifferentiation. We focused our effort on RA and IL1 β , which are known to modulate chondrocyte phenotypes. RA is a well-characterized soluble mediator that inhibits chondrogenesis and induces de-differentiation of chondrocytes (Cash et al., 1997; Hering, 1999; Weston et al., 2000). IL1 β plays a major role in joint cartilage destruction in arthritis and induces de-differentiation of chondrocytes by inhibiting expression of cartilage-specific type II collagen and proteoglycan (Goldring et al., 1994; Demoor-Fossard et al., 1998).

MATERIALS AND METHODS

Cell culture

Mesenchymal cells were derived from the wing buds of Hamburger-Hamilton stage 23/24 chicken embryos and maintained as micromass culture to induce chondrogenesis as described previously (Chang et al., 1998; Oh et al., 2000). Briefly, the cells at a density of 2.0×10^7 cells/ml in Ham's F-12 medium were spotted as 15 μ l drops into culture dishes, and cultured up to 5 days in the presence of various pharmacological agents, as described in each experiment. Rabbit articular chondrocytes were released from cartilage slices and cultured in Dulbecco's modified Eagle's medium (Kim et al., 2002a; Yoon et al., 2002). The confluent primary culture, designated as passage (P) 0, was treated with RA or IL1 β or subcultured up to P4 to induce de-differentiation. De-differentiated fibroblastic cells were cultured in alginate gel beads, as described previously, to induce redifferentiation (Yoon et al., 2002). Briefly, cells suspended by trypsin treatment were rinsed with washing solution (0.15 M NaCl, 20 mM HEPES, pH 7.4) and suspended in 1.25% sodium alginate (Sigma) prepared in 20 mM HEPES, 0.15 M NaCl, pH 7.4, at a cell density of 2×10^6 cells/ml. The cell suspension was slowly dropped into a gelation solution (102 mM CaCl₂, 5 mM HEPES, pH 7.4). After instantaneous gelation, the beads were allowed to further polymerize in the gelation solution for 10 minutes under gentle stirring and were then washed three times in five volumes of washing solution. Cells in alginate gel beads were cultured in complete medium for various periods and refed every other day. For recovery of cells, alginate gel beads were solubilized with two volumes of 50 mM EDTA and 10 mM HEPES, pH 7.4. After 10 minutes of incubation at 37°C, the cell suspension was centrifuged at 400 *g* for 5 minutes, and the cell pellet was extracted with lysis buffer as described below. Differentiation status was determined by Alcian Blue staining or the expression of type II collagen by western or northern blot analysis.

Immunohistochemistry and immunofluorescence microscopy

Wing buds of chicken embryos and spots of micromass culture were fixed in 4% paraformaldehyde for 24 hours at 4°C, dehydrated with

graded ethanol, embedded in paraffin wax and sectioned at 4 μ m thickness. The sections were stained by standard procedures using Alcian Blue or antibodies against type II collagen (Chemicon, Temecula, CA) and β -catenin and Jun (BD Transduction Laboratories, Lexington, KY), and visualized by developing with a kit purchased from DAKO (Carpenteria, CA). Immunofluorescence microscopy was also used to determine expression and distribution of type II collagen and β -catenin (Yoon et al., 2002). Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 5% fetal calf serum in PBS for 30 minutes. The fixed cells were washed and incubated for 1 hour with antibody (10 μ g/ml) against β -catenin or type II collagen. The cells were washed, incubated with rhodamine- or fluorescein-conjugated secondary antibodies for 30 minutes, and observed under a fluorescence microscope.

Northern and western blot assay

Total RNA was isolated by a single-step guanidinium thiocyanate-phenol-chloroform method, using RNA STAT-60 (Tel-Test B, Friendswood, TX) according to the manufacturer's protocol. Total RNA (3 μ g) was fractionated on formaldehyde/agarose gel. Rabbit type II collagen transcript was detected with a 370-bp partial cDNA probe as previously described (Kim et al., 2002a; Yoon et al., 2002). The probe (542 bp) for β -catenin transcript was generated by RT-PCR using a sense primer corresponding to nucleotides -18 to +10 and an antisense primer corresponding to nucleotides +501 to +524 of β -catenin. For western blotting, whole cell lysates prepared as previously described (Kim et al., 2002a) were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Proteins were detected using antibodies purchased from the following sources: type II collagens from Chemicon, rabbit anti-chick N-cadherin polyclonal antibody from Sigma-Aldrich, rabbit anti-human α -catenin polyclonal antibody from Santa Cruz (Santa Cruz, CA), and mouse β -catenin or Jun monoclonal antibodies from BD Transduction Laboratories.

Transfection and reporter gene assays

Retroviral vector (5 μ g) containing cDNA for S37A β -catenin was transfected to articular chondrocytes using LipofectaminePLUS (Gibco-BRL, Gaithersburg, MD) or infected with viral supernatant for 90 minutes. The transfected cells, which were cultured in complete medium for 48 hours, were used for further analysis as indicated in each experiment. To investigate β -catenin-TCF/LEF signaling, cells were transiently transfected with 1 μ g of the TCF/LEF reporters, TOPFlash (optimal LEF-binding site) or FOPFlash (mutated LEF-binding site) (van de Wetering et al., 1997) (Upstate Biotechnology Inc., Lake Placid, NY), and 1 μ g of pCMV- β -galactosidase. After incubation with IL1 β or RA for 72 hours, luciferase activity was measured and normalized for transfection efficiency using β -galactosidase activity.

Immunoprecipitation

Chondrocytes were lysed in Nonidet P-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 8.0) containing inhibitors of proteases [10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 10 μ g/ml aprotinin and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatases (1 mM NaF and 1 mM Na₃VO₄). After preclearing with protein A sepharose for 1 hour, proteins (500 μ g) were incubated with antibodies against β -catenin or N-cadherin. The immune complex was then precipitated by the incubation with protein A sepharose for 1 hour at 4°C. After washing with lysis buffer, the immunocomplex was analyzed by SDS-polyacrylamide gel electrophoresis and western blotting (Kim et al., 2002a).

Preparation of Triton X-100 insoluble and nuclear fractions
Triton X-100 insoluble cytoskeletal fraction was prepared as

described by Stolz et al. (Stolz et al., 1992). For the preparation of nuclear proteins, chondrocytes were washed with PBS and homogenized in a buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol containing inhibitors of proteases and phosphatases). Following addition of 0.6% (v/v) Nonidet P-40, the cells were incubated for 15 minutes on ice and then centrifuged at 13,000 *g* for 30 seconds at 4°C. The pellet was suspended in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol) containing inhibitors of proteases and phosphatases and centrifuged at 13,000 *g* for 5 minutes at 4°C. The resulting supernatant (nuclear fraction) was stored at -70°C until further analysis.

RESULTS

β -Catenin functions as a negative regulator of chondrogenesis

Chondrogenesis from mesenchymal cells during wing bud development of chicken embryo was detected in day 6 embryos, as determined by the expression of type II collagen via immunohistochemistry or accumulation of sulfated proteoglycan via Alcian Blue staining (Fig. 1A). During limb bud development, β -catenin was highly expressed in prechondrogenic mesenchymal cells in day 5 embryos but significantly decreased in differentiated chondrocytes in day 6 and thereafter. Visualization by higher magnification clearly showed the opposite expression pattern of type II collagen and β -catenin (Fig. 1B), suggesting a possible negative role of β -catenin in chondrocyte differentiation and cartilage development.

We employed micromass culture of chick embryonic mesenchymal cells to determine the role of β -catenin in chondrogenesis. Immunohistochemical staining of type II collagen and β -catenin (Fig. 2A) in cross-sections of micromass culture spot (upper panel) revealed that β -catenin is absent in cartilage nodules where type II collagen expressing chondrocytes are localized. The expressed β -catenin in chondrifying mesenchymal cells is localized in cell-cell contacts (lower panel). Expression of β -catenin and N-cadherin was high at the condensation period and decreased thereafter, a pattern opposite to that of type II collagen (Fig. 2B, upper panel). Treatment of chondrifying mesenchymal cells with 10 nM phorbol 12-myristate 13-acetate (PMA) to downregulate protein kinase C, 10 μ M SB203580 to inhibit p38 kinase, or 10 ng/ml epidermal growth factor, conditions that inhibit chondrogenesis (Chang et al., 1998; Oh et al., 2000; Yoon et al., 2000) (Fig. 2B, middle and lower panels), blocked the decrease of β -catenin and N-cadherin expression. By contrast, inhibition of extracellular signal-regulated kinase with 10 μ M PD98059, which enhances chondrogenesis (Oh et al., 2000), potentiated the decrease of β -catenin and N-cadherin expression (Fig. 2B, middle and lower panels). Treatment with 5 mM LiCl, which causes accumulation of β -catenin by the inhibition of glycogen synthase kinase (GSK)-3 β via its phosphorylation (Stambolic et al., 1996), resulted in inhibition of type II collagen expression (Fig. 2C, upper and middle panels) and proteoglycan synthesis (Fig. 2C, lower panel) and sustained expression of N-cadherin (Fig. 2C, middle panel), which clearly indicating a negative role of β -catenin in chondrocyte differentiation.

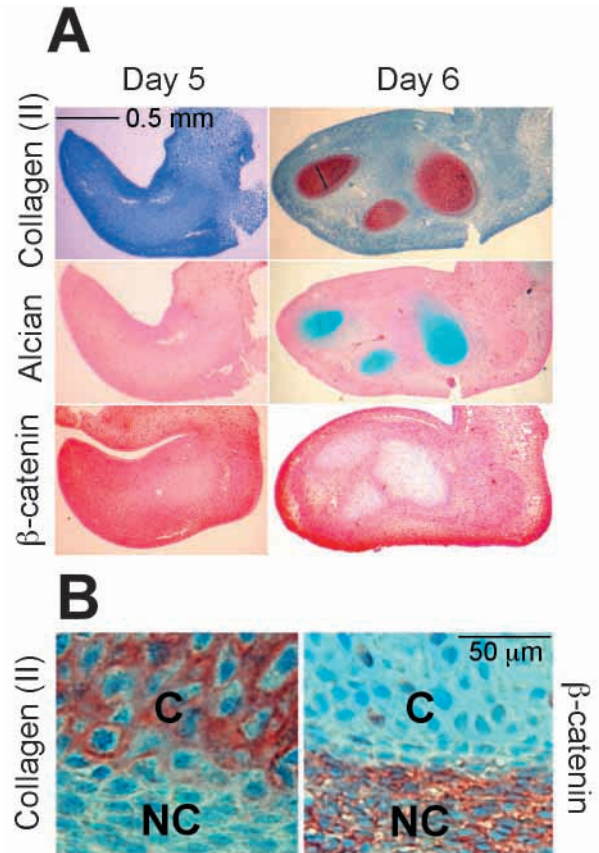


Fig. 1. Expression pattern of β -catenin in developing wing buds of chicken embryo. (A) Type II collagen and β -catenin in wing buds of day 5 and 6 chicken embryos were detected by immunohistochemistry. Accumulation of sulfated proteoglycan was detected by Alcian Blue staining. (B) Higher magnification in area between differentiating cartilage (C) and non-cartilage (NC) at day 6 embryo.

Accumulation and transcriptional activity of β -catenin causes phenotype loss of differentiated chondrocytes

We next investigated whether β -catenin is also associated with the maintenance of differentiated chondrocyte phenotypes using rabbit articular chondrocytes. The β -catenin level was low in differentiated chondrocytes and significantly increased as cells underwent de-differentiation by a serial monolayer culture (Fig. 3A, upper panel), 1 μ M RA treatment (Fig. 3A, middle panel), or 5 ng/ml IL1 β treatment for 72 hours (Fig. 3A, lower panel). All of the culture conditions caused reduction of type II collagen expression (Fig. 3A,B lower panel) and proteoglycan synthesis (Fig. 3B, upper panel). The elevated β -catenin protein level in de-differentiated cells was decreased when de-differentiated cells redifferentiate by three-dimensional culture in alginate gel (Fig. 3C). Therefore, the expression level of β -catenin is differentially regulated during de- and redifferentiation with an inverse relationship to the degree of chondrocyte differentiation status.

Northern blot analysis indicated that de-differentiation did not accompany any changes in β -catenin transcript levels (Fig. 4A, upper panel). Phosphorylation of GSK-3 β was significantly increased in de-differentiating cells, indicating the

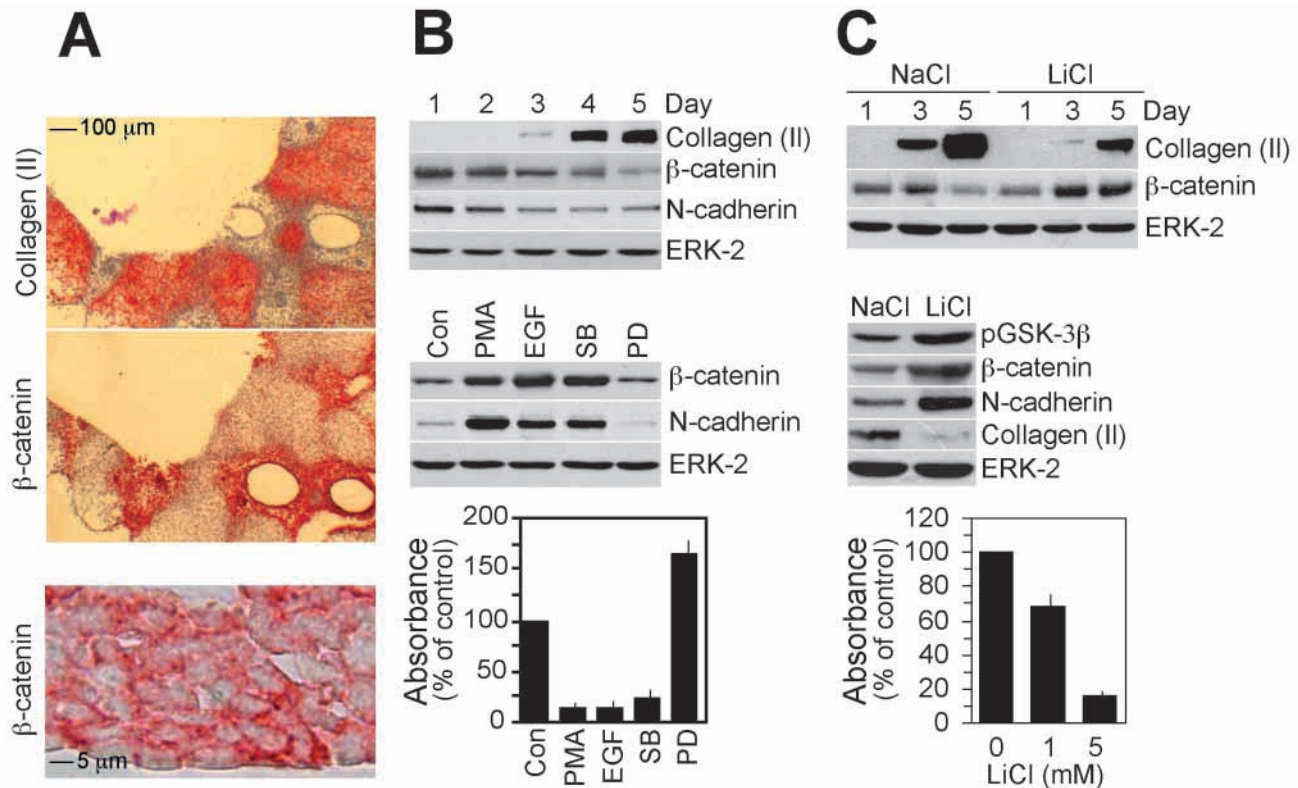


Fig. 2. Expression of β -catenin negatively regulates chondrogenesis in vitro. (A) Mesenchymal cells from chicken embryo were maintained as a micromass culture for 4 days, and distribution of type II collagen and β -catenin was detected by immunocytochemical staining of cross-sections of a micromass culture spot (top and middle). Subcellular localization of β -catenin was determined in day-2 culture (bottom). (B) Mesenchymal cells were maintained as micromass culture for the indicated period to induce chondrogenesis (top). Alternatively, the cells were cultured for 4 days in the presence of vehicle alone as a control, 10 nM phorbol ester (PMA), 10 ng/ml epidermal growth factor (EGF), 10 μ M SB203580 (SB) or 10 μ M PD98059 (PD) (middle and bottom). Type II collagen, β -catenin and N-cadherin were detected by Western blotting (middle). Synthesis of sulfated proteoglycan was determined by Alcian Blue staining and quantified (bottom). (C) Chondrifying mesenchymal cells during micromass culture were treated with 5 mM NaCl as a control or 5 mM LiCl for indicated periods (top), for 5 days (middle) or for 5 days with the indicated concentrations of LiCl (bottom). Expression of type II collagen, β -catenin, N-cadherin and phosphorylated GSK-3 β was determined by western blotting (top and middle panels). Accumulation of sulfated proteoglycan at day 4 culture was determined by Alcian Blue staining and quantified (bottom). ERK-2 was detected as loading control of blots.

inhibition of GSK-3 β activity (Fig. 4A, lower panel). Because GSK-3 β activity is primarily responsible for the degradation of β -catenin via ubiquitin-proteasome system, the inhibition of GSK-3 β indicated that post-translational accumulation of β -catenin contributes to the increased levels of β -catenin in cells treated with RA or IL1 β . This was further supported by the observation that treatment of chondrocytes with LiCl, which inhibits GSK-3 β , resulted in increased phosphorylation of GSK-3 β , accumulation of β -catenin and reduction of type II collagen expression (Fig. 4B). In addition, block of β -catenin degradation by the inhibition of 26S proteasome by MG132 also resulted in increased levels of β -catenin and cessation of type II collagen expression (Fig. 4C).

To determine molecular mechanisms of β -catenin regulation of the chondrocyte phenotype, we examined a role of β -catenin functions as a cytoskeletal component by determining its participation in N-cadherin-mediated cell-to-cell adhesion and as a nuclear signaling molecule in activating the TCF/LEF transcription factor. During chondrocyte de-differentiation caused by 1 μ M RA treatment for 72 hours, expression of cell adhesion machinery components such as N-cadherin and α -

catenin as well as β -catenin was significantly increased, whereas expression of these molecules did not change during IL1 β (5 ng/ml for 72 hours)-mediated de-differentiation (Fig. 5A, upper panel). Treatment of cells with RA, but not IL1 β , also increased association of β -catenin with N-cadherin as determined by immunoprecipitation experiments (Fig. 5A, lower panel). Thus, RA-induced de-differentiation accompanied not only increased expression of β -catenin but also enhanced association with N-cadherin, while IL1 β -induced de-differentiation did not accompany these changes. This was further demonstrated by examining localization of N-cadherin and β -catenin in Triton-X 100 insoluble cytoskeletal fractions. Western blot analysis (Fig. 5B) and indirect immunofluorescence microscopy (Fig. 5C) clearly indicated that RA, but not IL1 β , increased localization of N-cadherin and β -catenin in the cytoskeletal fraction. In addition, changes of cell morphology including stress fiber formation were seen only in cells treated with RA (Fig. 5D).

We next assessed the possibility that β -catenin acts as a nuclear signaling molecule during de-differentiation through its function as a co-activator of TCF/LEF family of

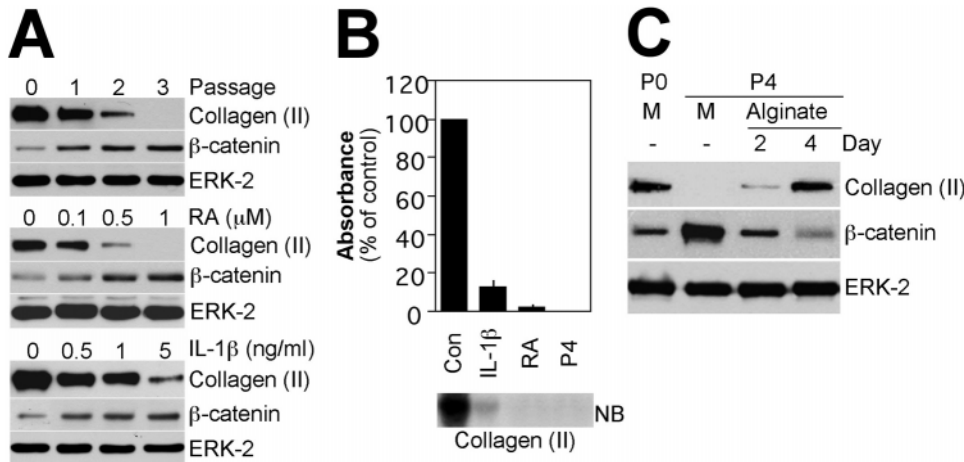


Fig. 3. Expression patterns of β-catenin during de- and redifferentiation of chondrocytes. (A) Articular chondrocytes were maintained as a serial monolayer up until the indicated passages (top) or P0 chondrocytes were treated with 1 μM retinoic acid (RA) (middle) or 5 ng/ml IL1β (bottom) for 72 hours. Expression patterns of type II collagen and β-catenin were determined by western blot analysis. (B) Chondrocytes were untreated or treated with IL1β or RA or P0 chondrocytes were passed to P4 (P4). Accumulation of sulfated proteoglycan was determined by Alcian Blue staining (top) and type II collagen transcript level was determined by

northern blot (NB) analysis (bottom). (C) Chondrocytes were cultured as monolayer (M) to P0 or to P4. P4 cells were detached and three-dimensionally cultured for 4 days in alginate gel (Alginate). Type II collagen and β-catenin expression was determined by western blot analysis. ERK-2 was detected as loading control of blots.

transcription factors. Most of the expressed β-catenin is localized in cell-to-cell contacts in chondrocytes, and RA (1 μM, 72 hours) or IL1β (5 ng/ml, 72 hours) dramatically increased nuclear localization of β-catenin (Fig. 6A). Western blot analysis also showed significantly increased levels of β-catenin in the nuclear fraction (Fig. 6B, lower panel). Transcriptional activation by β-catenin was examined by TCF/LEF reporter gene assay using TOPFlash (optimal TCF/LEF-binding site) and FOPFlash (mutated TCF/LEF-binding site). These reporter gene assays indicated a transcriptionally active role for β-catenin (Fig. 6B, upper panel). Consistent with the increased β-catenin-TCF/LEF activity, expression of known β-catenin target genes such as Jun (Mann et al., 1999), but not connexin 43 (van der Heyden et al., 1998), was increased (Fig. 6B, lower panel). Therefore, accumulation of β-catenin in de-differentiating chondrocytes appears to alter the gene expression profile of the cell by activating the TCF/LEF family of transcription factors.

To access the function of β-catenin more directly in the

regulation of the chondrocyte phenotype, S37A β-catenin, a stable non-ubiquitinatable form of β-catenin (Easwaran et al., 1999), was ectopically expressed in chondrocytes. Transfection of β-catenin caused a dramatic increase of TCF/LEF activity (Fig. 7A, middle panel) and reduced accumulation of proteoglycan (Fig. 7A, right panel). Ectopic expression of β-catenin also caused a significant reduction of type II collagen expression and enhanced expression of the β-catenin target gene Jun (Fig. 7A, left panel). Double staining of type II collagen and β-catenin in differentiated chondrocytes transfected with S37A β-catenin indicated that cells highly expressing β-catenin are negative for type II collagen staining (Fig. 7B), indicating that β-catenin expression was sufficient to cause de-differentiation of chondrocytes.

Regulation of Jun expression by β-catenin

Because the above results indicate that accumulation of β-catenin during de-differentiation causes increased expression of Jun, we next examined the role of β-catenin on

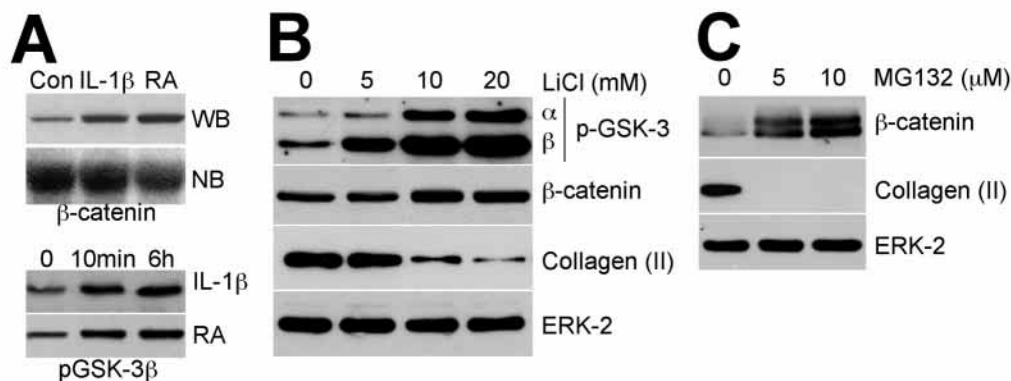


Fig. 4. Post-translational accumulation of β-catenin during de-differentiation of chondrocytes. (A) Chondrocytes were untreated or treated with 5 ng/ml IL1β or 1 μM RA for 72 hours, and β-catenin and phosphorylated GSK-3β was determined by western (WB) or northern (NB) blotting. (B) Chondrocytes were treated with the indicated concentrations of LiCl for 48 hours. β-Catenin, type II collagen and phosphorylated GSK-3β was determined by western blotting. (C) Chondrocytes were treated with the indicated concentrations of MG132 for 48 hours. β-Catenin and type II collagen was determined by western blotting. ERK-2 was detected as loading control of blots.

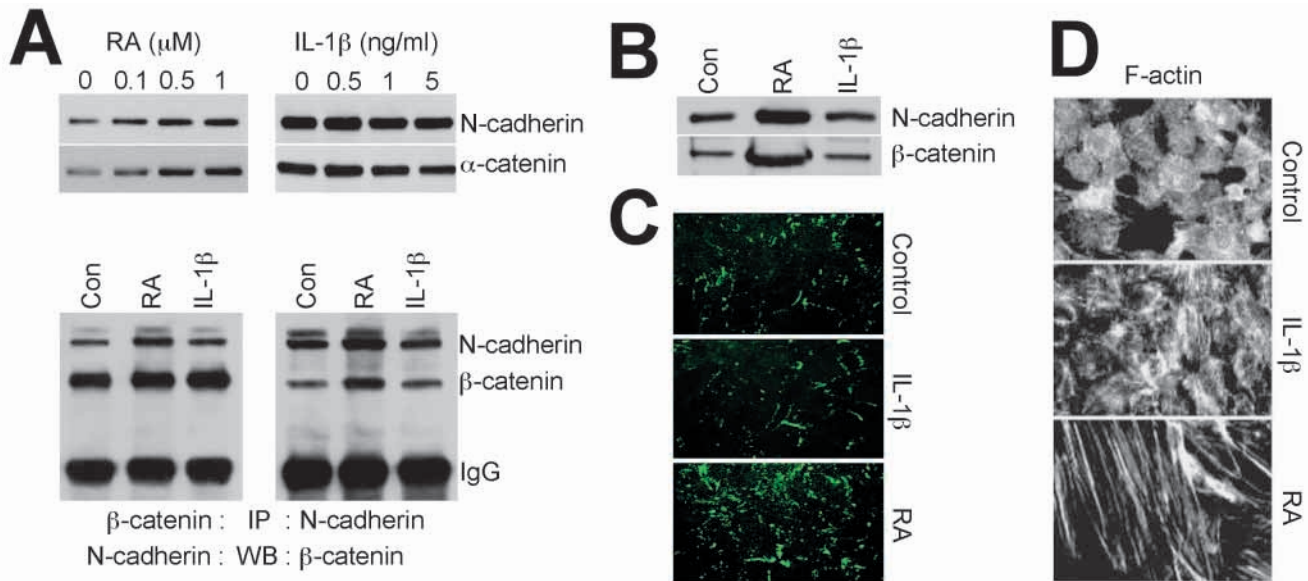


Fig. 5. β -Catenin function as a cytoskeletal component is not necessary for phenotype modulation of chondrocytes. (A) Rabbit articular chondrocytes were treated with the indicated concentrations of RA or IL1 β for 72 hours. Expression of N-cadherin and α -catenin was determined by western blotting (top). Chondrocytes were untreated (Con) or treated with 1 μ M RA or 5 ng/ml IL1 β for 72 hours. N-cadherin was detected by western blotting from the samples precipitated with β -catenin immunocomplex. Alternatively, N-cadherin was immunoprecipitated and β -catenin was detected by western blot analysis (bottom). (B,C) Chondrocytes were treated with vehicle alone as a control, RA or IL1 β . β -Catenin in Triton X-100 insoluble fraction was detected by western blotting (B) or indirect immunofluorescence microscopy (C). (D) Chondrocytes were untreated or treated with RA or IL1 β . The cells were fixed and immunostained for F-actin with pallodid-rhodamine and analyzed by immunofluorescence microscopy.

Jun expression during chondrogenic differentiation of mesenchymal cells. Distribution pattern of Jun is essentially same as that of β -catenin: it was highly expressed in prechondrogenic mesenchymal cells in day 5 embryos but absent in differentiated chondrocytes in day 6 and thereafter (Fig. 8A). Immunohistochemical staining of type II collagen and Jun in cross-sections of micromass culture spot showed that Jun staining in cartilage nodules, where type II collagen expressing chondrocytes are localized, is dramatically reduced (Fig. 8B). Thus, the expression and distribution pattern of β -catenin (Fig. 1) and Jun (Fig. 8) is essentially same during chondrogenesis both in vivo and in vitro. Expression level of Jun, as determined by western blot analysis, was high at the condensation period and decreased thereafter, a pattern similar to β -catenin and opposite to type II collagen (Fig. 8C). Treatment of chondrifying mesenchymal cells with 10 nM phorbol 12-myristate 13-acetate (PMA) or 1 μ M Go6976 to downregulate and inhibit protein kinase C, respectively, blocked the decrease of Jun and β -catenin levels with the inhibition of chondrogenesis. By contrast, inhibition of extracellular signal-regulated kinase with 10 μ M PD98059 potentiated the decrease of Jun and β -catenin levels with the enhancement of chondrogenesis (Fig. 8D). Therefore, the expression of Jun appears to be regulated by β -catenin during differentiation, as well as de-differentiation of chondrocytes.

DISCUSSION

This study demonstrates for the first time that β -catenin functions as a negative regulator of differentiated chondrocyte

phenotype. A decrease of β -catenin expression is required for chondrogenic differentiation of mesenchymal cells and the low level of β -catenin is necessary for the maintenance of differentiated chondrocyte phenotypes. Our results also indicate that the inhibitory role of β -catenin in chondrocyte differentiation is exerted by its ability to stabilize cell-to-cell adhesion, whereas loss of differentiated chondrocyte phenotypes is due to its ability to regulate gene expression profiles by acting as a transcriptional co-activator.

Cell-to-cell adhesion is an essential regulatory step in chondrogenesis by coordinating precartilaginous condensation and cartilage nodule formation (Oberlender and Tuan, 1994; Sandell and Adler, 1999; Woodward and Tuan, 1999; DeLise et al., 2000). N-cadherin is highly expressed and localized to the prechondroblastic cells during mesenchymal cell condensation, and perturbation of N-cadherin function inhibits cellular condensation and chondrogenesis (Oberlender and Tuan, 1994). The expression of N-cadherin is downregulated in the later stage of chondrogenesis, which appears to be required for the progression of precartilaginous condensation to cartilage nodules (Oberlender and Tuan, 1994; Chang et al., 1998; Oh et al., 2000; Yoon et al., 2000). The involvement of β -catenin in the regulation of chondrogenesis has been suggested from the observation that ectopic expression of members of Wnt genes such as Wnt1, Wnt7a and Wnt14 (Rudnicki and Brown, 1997; Hartmann and Tabin, 2001; Tufan and Tuan, 2001; Tufan et al., 2002a) or frizzled receptor for Wnt (Tufan et al., 2002b) inhibits chondrogenesis. In addition, it has been suggested that stabilization of N-cadherin-mediated cell adhesion is responsible for the Wnt inhibition of chondrogenesis (Tufan and Tuan, 2001; Tufan et al., 2002a;

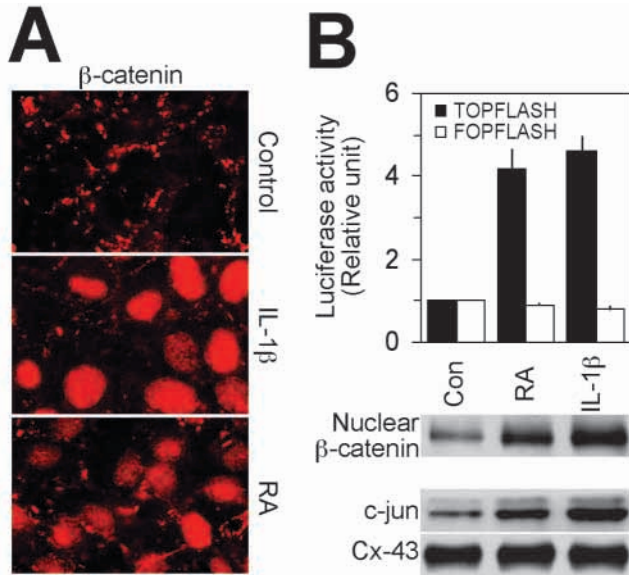


Fig. 6. β-Catenin function as a nuclear signaling molecule is sufficient to cause phenotype loss of chondrocytes. (A) Chondrocytes were treated with vehicle alone as a control, 1 μM RA or 5 ng/ml IL1β for 72 hours. The distribution of β-catenin was determined by immunofluorescence microscopy. (B) Chondrocytes were transfected with active (TOPFlash) or inactive (FOPFlash) TCF/LEF reporter gene for β-catenin and treated with vehicle alone as a control (Con), RA, or IL1β, and TCF/LEF reporter activity was monitored (upper panel). β-Catenin protein in nuclear preparation or Jun and connexin 43 (Cx43) in whole cell lysates were detected by western blotting from chondrocytes treated with RA or IL1β.

Tufan et al., 2002b). Our results indicated that most of the expressed β-catenin in chondrifying mesenchymal cells during condensation period or inter-nodular area was distributed at cell-cell contacts without any obvious nuclear localization (Fig. 2A). Furthermore, accumulation of β-catenin by the inhibition of GSK-3β blocked down regulation of N-cadherin

(Fig. 2C) and inhibition of chondrogenesis accompanied elevated expression of N-cadherin and β-catenin (Fig. 2B). Therefore, we postulate that the failure to downregulate β-catenin and N-cadherin blocks chondrogenesis by stabilizing cell-to-cell adhesion, rather than altering gene expression profiles by acting as a transcriptional co-activator.

Because the transforming group of Wnt family, such as Wnt1 and Wnt7a, but not nontransforming Wnts exerts their effects by accumulating cytosolic β-catenin (Shimizu et al., 1997), our current observation that accumulation of β-catenin inhibits chondrogenesis is in agreement with the inhibition of chondrogenesis by the transforming Wnts (Rudnicki and Brown, 1997; Stott et al., 1999; Tufan and Tuan, 2001). Although no direct evidence for the role of β-catenin in chondrogenesis is yet available, Hartmann and Tabin (Hartmann and Tabin, 2000) reported that misexpression of β-catenin in developing chicken wing buds accelerates chondrocyte maturation. Similar to our results, they observed lower levels of β-catenin mRNA in chondrocytes of the articular region of day 7.5 chick embryo limb buds, whereas significantly high levels of β-catenin were observed in the cells of the perichondrium and hypertrophic chondrocytes. Misexpression of β-catenin showed shortening of the cartilage elements with the slightly increased expression of markers for hypertrophic chondrocytes, including type X collagen leading to their conclusion that β-catenin promotes progression of differentiated chondrocytes to a hypertrophic state. Therefore, it may be possible that the inhibition of type II collagen expression by the accumulation of β-catenin during differentiation and de-differentiation of chondrocytes is due to maturation of differentiated chondrocytes into hypertrophic chondrocytes. However, we could not detect any increase of alkaline phosphatase activity in LiCl-treated micromass culture of mesenchymal cells and in articular chondrocytes treated with RA or IL1β or transfected with S37A β-catenin (data not shown), indicating that the loss of type II collagen expression is due to inhibition of chondrogenesis of mesenchymal cells and de-differentiation of articular chondrocytes. Our results

are in good agreement with the observations by others, which indicate expression of hypertrophic chondrocyte markers (type X collagen and alkaline phosphatase) during micromass culture of mesenchymal cells needs much longer culture period (1-3 weeks) (Mello and Tuan, 1999; Boskey et al., 2002), and that treatment with RA (Cash et al., 1997; Hering, 1999; Weston et al., 2000), IL1β (Goldring et al., 1994; Demoor-Fossard et al., 1998) or a serial subculture (Lefebvre et al., 1990; Yoon et al., 2002) causes de-differentiation of articular chondrocytes.

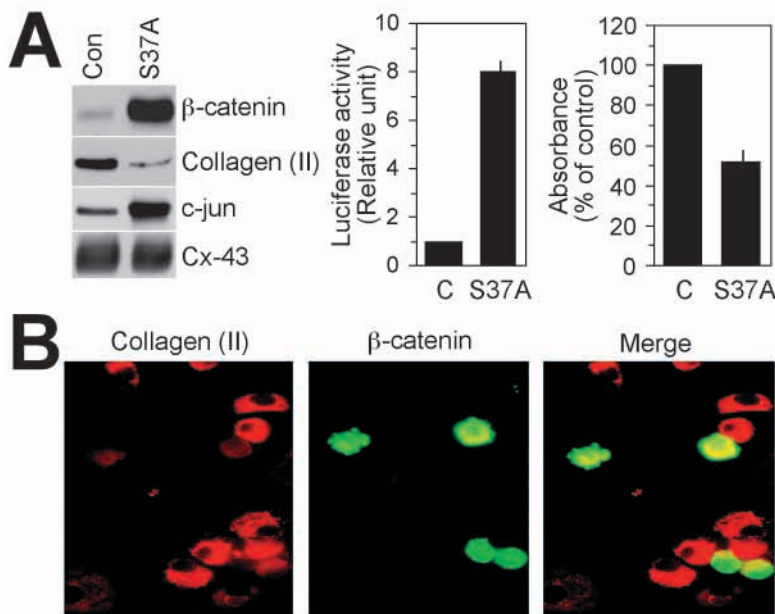


Fig. 7. Ectopic expression of S37A β-catenin causes phenotype loss of chondrocytes. (A) Chondrocytes were transfected with empty vector (Con) or S37A β-catenin. After 72 hours incubation, expression of type II collagen, β-catenin, Jun and connexin 43 (Cx43) was determined (left). TCF/LEF activity was determined by TOPFlash assay and accumulation of sulfated proteoglycan was determined by Alcian Blue staining and quantified in control cells (C) or cells transfected with S37A β-catenin (S37A) (right). (B) Type II collagen and β-catenin were double stained in chondrocytes transfected with S37A β-catenin and analyzed by immunofluorescence microscopy.

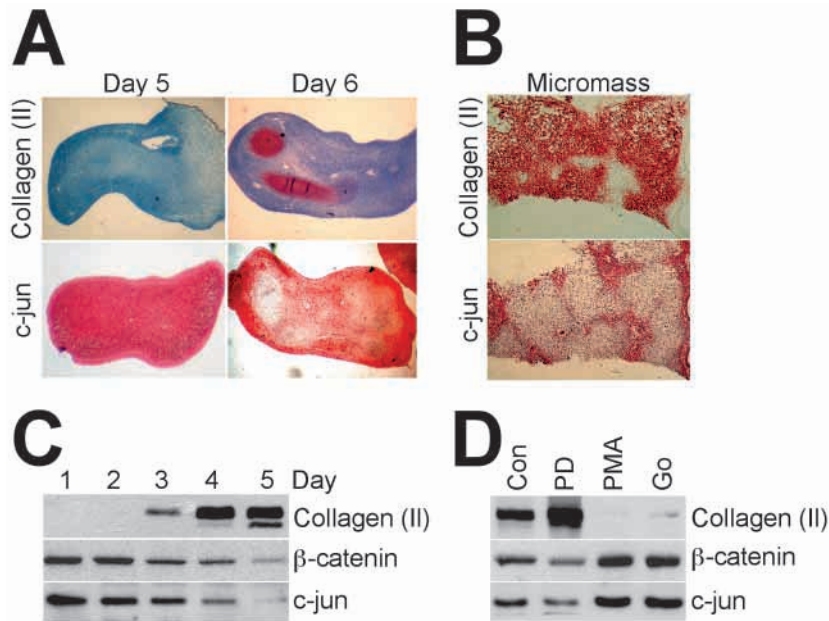


Fig. 8. Expression pattern of Jun during chondrogenesis in vivo and in vitro. (A) Type II collagen and Jun in wing buds of day 5 and 6 chicken embryos were detected by immunohistochemistry. (B) Mesenchymal cells from chicken embryo were maintained as a micromass culture for 4 days, and distribution of type II collagen and Jun was detected by immunocytochemical staining of cross-sections of a micromass culture spot. (C) Mesenchymal cells were maintained as micromass culture for the indicated period to induce chondrogenesis. (D) Alternatively, the cells were cultured for 4 days in the presence of vehicle alone as a control (Con), 10 nM phorbol ester (PMA), 1 μ M Go6976 (Go) or 10 μ M PD98059 (PD). Type II collagen, β -catenin and Jun were detected by western blotting.

On the bases of the experiments by Hartmann and Tabin (Hartmann and Tabin, 2000) that suggest β -catenin misexpression promotes chondrocyte maturation and on our in vitro experiment that indicates inhibition of chondrogenesis by the accumulation of β -catenin in chondrifying mesenchymal cells, it is possible that β -catenin inhibits initial chondrogenic differentiation of mesenchymal cells and also promotes maturation of the differentiated chondrocytes that is caused by escaping from β -catenin inhibition of chondrogenesis. Our current observation of the de-differentiation of articular chondrocytes, rather than maturation by the accumulation of β -catenin in articular chondrocytes, is different from the suggestions made by Hartmann and Tabin (Hartmann and Tabin, 2000). Therefore, further investigation is necessary to reconcile these observations. It is of interest to determine the effects of β -catenin accumulation on chondrocyte phenotype in three-dimensionally cultured chondrocytes that mimics in vivo condition of chondrocytes.

In contrast to chondrocyte differentiation, phenotype loss or de-differentiation of chondrocytes is caused by the action of β -catenin as a transcriptional co-activator. This was clearly demonstrated by the observation that de-differentiation of chondrocytes caused by IL1 β accompanied transcriptional activation by β -catenin (Fig. 6) without any modulation of cell-to-cell adhesion (Fig. 5). In addition, forced expression of S37A β -catenin, which dramatically increased β -catenin-TCF/LEF activity without modulation of N-cadherin expression, caused de-differentiation of chondrocytes (Fig. 7), indicating that the function of β -catenin as a nuclear signaling molecule is sufficient to cause phenotypic loss of chondrocytes. Because changes in cell morphology and actin cytoskeleton such as stress fiber formation were observed in cells treated with RA but not IL1 β (Fig. 5), RA-induced β -catenin association with N-cadherin appears to be involved in morphological changes of chondrocytes, rather than cessation of the expression of chondrocyte markers. Consistent with our current observation of RA effects on chondrocytes, RA treatment caused increased expression of N-cadherin, increased cell-to-cell adhesion, and

the recruitment of cytoplasmic β -catenin to the membrane in epithelial and breast cancer cells (Vermeulen et al., 1995; Sanchez et al., 1996). Although increased cadherin expression can modulate β -catenin signaling by depleting the cytoplasmic pool of β -catenin, our results indicate that this is not the case in chondrocytes as RA increased β -catenin protein in both the cytoskeletal and nuclear fractions.

Because loss of differentiated phenotype of chondrocytes is associated with cartilage destruction during arthritis (Sandell and Aigner, 2001), accumulation of β -catenin appears to contribute to arthritic disease. Indeed, we observed that levels of β -catenin were significantly increased in osteoarthritis-affected cartilage that is obtained from individuals undergoing total knee arthroplasty with loss of type II collagen and proteoglycan. The increase in β -catenin protein levels was also evident in experimental rheumatoid arthritic cartilage caused by type II collagen injection in DBA/1 mice (data not shown). In addition, we recently showed that ectopic expression of cyclooxygenase 2 in articular chondrocytes (Kim et al., 2002b). Therefore, our results suggest that accumulation of β -catenin may play a role in the inflammatory responses and destruction of cartilage during arthritic disease.

Although it is clear that β -catenin causes loss of chondrocyte phenotype by activating transcription of genes, the mechanisms of chondrocyte phenotype loss by β -catenin need to be further characterized. β -Catenin may cause cessation of type II collagen expression and proteoglycan synthesis either directly or indirectly. The known type II collagen promoter/enhancer sequence in human, mouse and rat does not contain the canonical TCF/LEF-binding motif CCTTTGA/TA/TC (van de Wetering et al., 1997). Thus, we postulate that β -catenin-TCF/LEF indirectly regulates type II collagen expression by modulating an unknown β -catenin-LEF/TCF target gene that may inhibit type II collagen expression and accumulation of sulfated proteoglycan. Therefore, it is of interest to identify β -catenin target genes in chondrocytes to define β -catenin regulation of chondrocyte phenotype. In this study we

identified that Jun is a target gene of β -catenin in articular chondrocytes. Expression pattern of Jun is essentially same as that of β -catenin both in vivo and in vitro, and ectopic expression of β -catenin caused induction of Jun expression, indicating that Jun expression is regulated by β -catenin during phenotype modulation of chondrocytes. Indeed, it has been shown that Jun and Fra1 are direct target genes of β -catenin in colorectal carcinoma cells (Mann et al., 1999). Recent study by Tufan et al. (Tufan et al., 2002a) also indicated that AP-1 transcription factor is a target of Wnt-7a signal during chondrogenesis. Therefore, it is likely that AP-1 transcription factor is associated with the β -catenin regulation of chondrocyte differentiation and de-differentiation.

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