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 precartilage 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)-1β (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 IL-1β 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 precartilage 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 IL1β 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 IL1β, 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). IL1β 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 2.0×10^5 cells/ml
in Ham’s F-12 medium were spotted as 15 μl 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
modified Eagle’s medium (Kim et al., 2002a; Yoon et al., 2002). IL1β 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).

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 μm
thickness. The sections were stained by standard procedures using
Aclian 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 (Carpinteria, 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 μg/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 guanidine thiocyanate-
phenol-chloroform method, using RNA STAT-60 (Tel-Test B,
Friendswood, TX) according to the manufacturer’s protocol. Total
RNA (3 μg) 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-
polyacrylamid 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-cat ein 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 μg) containing cDNA for S37A β-catenin was transfection 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 μg of the TCF/LEF reporters,
TOPFlash (optimal LEF-binding site) or TOPFlash (mutated LEF-
binding site) (van de Wetering et al., 1997) (Upstate Biotechnology
Inc., Lake Placid, NY), and 1 μg of pCMV-β-galactosidase. After
incubation with IL1β 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
μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 1 mM
of 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatases
(1 mM NaF and 1 mM Na3 VO4). After preclearing with protein A
sepharose for 1 hour, proteins (500 μg) 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 immune complex
was analyzed by SDS-polyacrylamid 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 μM 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 mM 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)-3β 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.

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 μM RA treatment (Fig. 3A, middle panel), or 5 ng/ml IL1β 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-3β was significantly increased in de-differentiating cells, indicating the
inhibition of GSK-3\(\beta\) activity (Fig. 4A, lower panel). Because GSK-3\(\beta\) activity is primarily responsible for the degradation of \(\beta\)-catenin via ubiquitin-proteasome system, the inhibition of GSK-3\(\beta\) indicated that post-translational accumulation of \(\beta\)-catenin contributes to the increased levels of \(\beta\)-catenin in cells treated with RA or IL1\(\beta\). This was further supported by the observation that treatment of chondrocytes with LiCl, which inhibits GSK-3\(\beta\), resulted in increased phosphorylation of GSK-3\(\beta\), accumulation of \(\beta\)-catenin and reduction of type II collagen expression (Fig. 4B). In addition, block of \(\beta\)-catenin degradation by the inhibition of 26S proteasome by MG132 also resulted in increased levels of \(\beta\)-catenin and cessation of type II collagen expression (Fig. 4C).

To determine molecular mechanisms of \(\beta\)-catenin regulation of the chondrocyte phenotype, we examined a role of \(\beta\)-catenin functions as a cytoskeletal component by determining its participation in N-cadherin-mediated cell-to-cell adhesion and as a nuclear signaling molecule by activating the TCF/LEF transcription factor. During chondrocyte de-differentiation caused by 1 \(\mu\)M RA treatment for 72 hours, expression of cell adhesion machinery components such as N-cadherin and \(\alpha\)-catenin as well as \(\beta\)-catenin was significantly increased, whereas expression of these molecules did not change during IL1\(\beta\) (5 ng/ml for 72 hours)-mediated de-differentiation (Fig. 5A, upper panel). Treatment of cells with RA, but not IL1\(\beta\), also increased association of \(\beta\)-catenin with N-cadherin as determined by immunoprecipitation experiments (Fig. 5A, lower panel). Thus, RA-induced de-differentiation accompanied not only increased expression of \(\beta\)-catenin but also enhanced association with N-cadherin, while IL1\(\beta\)-induced de-differentiation did not accompany these changes. This was further demonstrated by examining localization of N-cadherin and \(\beta\)-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\(\beta\), increased localization of N-cadherin and \(\beta\)-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 \(\beta\)-catenin acts as a nuclear signaling molecule during de-differentiation through its function as a co-activator of TCF/LEF family of...
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
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 μM 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 μM 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 precartilage 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 precartilage 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;
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. 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 condensing 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.

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**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.
expression of N-cadherin, increased cell-to-cell adhesion, and RA effects on chondrocytes, RA treatment caused increased chondrocyte markers. Consistent with our current observation of chondrocytes, rather than cessation of the expression of N-cadherin appears to be involved in morphological changes that the function of 
\[\beta\]-catenin causes loss of 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 
\[\beta\]-catenin as a transcriptional co-activator. This was clearly demonstrated by the observation that de-differentiation of chondrocytes caused by IL1\[\beta\] accompanied transcriptional activation by 
\[\beta\]-catenin (Fig. 5) without any modulation of cell-to-cell adhesion (Fig. 5). In addition, forced expression of S37A \[\beta\]-catenin, which dramatically increased 
\[\beta\]-catenin-TCF/LEF activity without modulation of N-cadherin expression, caused de-differentiation of chondrocytes (Fig. 7), indicating that the function of \[\beta\]-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\[\beta\] (Fig. 5), RA-induced \[\beta\]-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 \[\beta\]-catenin to the membrane in epithelial and breast cancer cells (Vermeulen et al., 1995; Sanchez et al., 1996). Although increased cadherin expression can modulate \[\beta\]-catenin signaling by depleting the cytoplasmic pool of \[\beta\]-catenin, our results indicate that this is not the case in chondrocytes as RA increased \[\beta\]-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 \[\beta\]-catenin appears to contribute to arthritic disease. Indeed, we observed that levels of \[\beta\]-catenin were significantly increased in osteoarthritic cartilage that is obtained from individuals undergoing total knee arthroplasty with loss of type II collagen and proteoglycan. The increase in \[\beta\]-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 transcriptionally competent \[\beta\]-catenin stimulated expression of cyclooxygenase 2 in articular chondrocytes (Kim et al., 2002b). Therefore, our results suggest that accumulation of \[\beta\]-catenin may play a role in the inflammatory responses and destruction of cartilage during arthritic disease.

Although it is clear that \[\beta\]-catenin causes loss of chondrocyte phenotype by activating transcription of genes, the mechanisms of chondrocyte phenotype loss by \[\beta\]-catenin need to be further characterized. \[\beta\]-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 \[\beta\]-catenin-TCF/LEF indirectly regulates type II collagen expression by modulating an unknown \[\beta\]-catenin-LEF/TCF target gene that may inhibit type II collagen expression and accumulation of sulfated proteoglycan. Therefore, it is of interest to identify \[\beta\]-catenin target genes in chondrocytes to define \[\beta\]-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 Frl1 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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