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Ectopic expression of cyclooxygenase-2-induced dedifferentiation in articular chondrocytes

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Abbreviations: COX, cyclooxygenase

Abstract

Cyclooxygenase-2 (COX-2) is known to modulate bone metabolism, including bone formation and resorption. Because cartilage serves as a template for endochondral bone formation and because cartilage development is initiated by the differentiation of mesenchymal cells into chondrocytes (Ahrens *et al.*, 1977; Sandell and Adler, 1999; Solursh, 1989), it is of interest to know whether COX-2 expression affect chondrocyte differentiation. Therefore, we investigated the effects of COX-2 protein on differentiation in rabbit articular chondrocyte and chick limb bud mesenchymal cells. Overexpression of COX-2 protein was induced by the COX-2 cDNA transfection. Ectopic expression of COX-2 was sufficient to causes dedifferentiation in articular chondrocytes as determined by the expression of type II collagen via Alcian blue staining and Western blot. Also, COX-2 overexpression caused suppression of SOX-9 expression, a major transcription factor that regulates type II collagen expression, as indicated by the Western blot and RT-PCR. We further examined ectopic expression of COX-2 in chondrifying mesenchymal cells. As expected, COX-2 cDNA transfection blocked cartilage nodule formation as determined by Alcian blue staining. Our results collectively suggest that COX-2 overexpression causes dedifferentiation in articular chondrocytes and inhibits chondrogenic differentiation of mesenchymal cells.

Keywords: chondrocytes; cyclooxygenase 2; cell dedifferentiation; cell differentiation; collagen type II; mesenchymal cells

Introduction

Chondrocytes in cartilage are differentiated from mesenchymal cells during embryonic development. Differentiated chondrocytes, which are the only cell type found in normal mature cartilage, synthesize sufficient amounts of cartilage-specific extracellular matrix (ECM) to maintain matrix integrity. This homeostasis is destroyed in degenerative diseases, such as osteoarthritis and rheumatoid arthritis (Sandell and Aigner, 2001). Arthritis is characterized by structural and biochemical changes in chondrocytes and cartilage, including degradation of cartilage matrix, insufficient synthesis of ECM because of loss of chondrocyte phenotype. However, the differentiated phenotype is unstable both *in vivo* and *in vitro* and thus lost by a process designated "dedifferentiation" upon exposure of cells to IL-1β (Goldring *et al.*, 1994; Demoor-Fossard *et al.*, 1998), nitric oxide (Amin and Abramson, 1998), or retinoic acid (Cash *et al.*, 1997; Weston *et al.*, 2000) and during serial monolayer culture (Lefebvre *et al.*, 1990; Yoon *et al.*, 2002). However, little is known about dedifferentiation in articular chondrocytes.

COX is known to exist in two isoforms, COX-1 and COX-2. And two COXs are identified similar sequence (Smith *et al.*, 2000). COX-1 is considered a constitutive enzyme, being found in most mammalian cells (Dubois *et al.*, 1998). COX-2, on the other hand, is undetectable in most normal tissues (Wu, 1995). COX-2 is an inducible enzyme, becoming abundant in activated macrophages and other cells at sites of inflammation by various stimuli including cytokines. Expression of COX-2 increased PGE_2 (Namkoong *et al.*, 2005). PGE_2 induced various inflammation reactions (Smith *et al.*, 1996).

Mice lacking COX-2 but not COX-1 expression display reduced bone resorption in response to parathyroid hormone (PTH) or 1,25-hydroxyl vitamin D3 (Okada *et al.*, 2000). In addition to bone

resorption, COX-2 may also have a role in bone formation. Systemic or local injection of PGE_2 stimulates bone formation (Weinreb *et al*., 1997; Suponitzky and Weinreb, 1998). Increased lamellar bone formation in response to mechanical strain is mediated by COX-2 (Duncan and Turner, 1995; Forwood, 1996).

The crucial events in adult bone formation are the recruitment, proliferation, and differentiation of mesenchymal stem cells with endochondral and intramembranous bone formation at the injury site (Bruder *et al*., 1994). In endochondral ossification, mesenchymal cells first differentiate into chondrocytes, which subsequently undergo terminal differentiation and apoptosis, leading to calcification of the matrix.

Based on these findings, we investigated the effects of COX-2 in the dedifferentiation of articular chondrocytes and chondrogenesis. Here, we suggest that COX-2 overexpression might be responsible for dedifferentiation of articular chondrocytes and chondrogenesis of mesenchymal cells.

Materials and Methods

Cell culture

As described previously (Oh *et al*., 2000; Yoon *et al*., 2000), mesenchymal cells, which were isolated from chicken embryo wing buds, were grown in micromass culture to induce chondrogenesis. Chondrifying mesenchymal cells were transfected with COX-2 cDNA. Articular chondrocytes were isolated from cartilage slices of 2-week-old New Zealand white rabbits by enzymatic digestion as described previously (Yoon *et al*., 2002). Cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 U/mg solid, Sigma) in DMEM (Gibco-BRL, Gaithersburg, MD). Individual cells were suspended in DMEM supplemented with 10% (v/v) fetal bovine-calf serum, 50 μg/ml streptomycin, and 50 units/ml penicillin, after which and they were then plated on culture dishes at a density of 5×10^4 cells/cm^2. The medium was changed every 2 days after seeding, and cells reached confluence in approximately 5 days. Differentiation status of articular chondrocytes was determined by examining the accumulation of sulfated glycosaminoglycan with Alcian Blue staining or expression of type II collagen was detected using antibodies purchased from Chemicon (Temecula, CA) by Western blot analysis as described previously (Yoon *et al*., 2002).

Immunoblot analysis

Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS, supplemented with protease inhibitors [10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride] and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The proteins were size-fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose sheet was then blocked with 3% non-fat dry milk in Tris-buffered saline. COX-2 was detected using antibody purchased from Cayman Chemical (Ann Arbor, MI), and Sox-9 and ERK-2 were detected using antibodies purchased from Santa Cruz Biotech. (Santa Cruz, CA). Blots were developed using a peroxidase-conjugated secondary antibody and visualized with an ECL system.

Immunohistochemistry and immunofluorescence microscopy

Wing buds of chicken embryos and spots of micromass culture were fixed in 4% paraformaldehyde in PBS for 40 min at room temperature. The cells were stained by standard procedures using Alcian blue. Rabbit joint cartilage explants or arthritic cartilage were in 4% paraformaldehyde in PBS for 24 h at 4°C, washed with PBS, dehydrated with graded ethanol, embedded in paraffin, and sectioned at 4 μm thickness. The sections were stained by standard procedures using Alcian blue or antibody against type II collagen and visualized by developing with a kit purchased from DAKO (Carpinteria, CA). Expression and distribution of type II collagen and COX-2 in rabbit articular chondrocytes were determined by indirect immunofluorescence microscopy, as described previously (Ryu *et al*., 2002). Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 5% FCS in PBS for 30 min. The fixed cells were washed and incubated for 1 h with antibody (10 μg/ml) against type II collagen or COX-2. The cells were washed, incubated with rhodamine-conjugated secondary antibodies for 30 min, and observed under a fluorescence microscope.

Transfection

To introduce cDNA for COX-2, mesenchymals cells and articular chondrocytes were transfected with plasmid containing COX-2 cDNA. Transfection of the expression vector was performed as described previously (Ryu *et al*., 2002). The COX-2 (1 μg) was introduced to cells using METAFECTENE (Biontex) using the procedure recommended by

the manufacturer. The transfected cells, which were cultured in complete medium for 48 h, were used for further assay as indicated in each experiment

RT-PCR

Primary cultured chondrocytes were transfected with COX-2 cDNA. Total RNA was isolated from the cell and reverse transcribed with Maxime RT-PCR PreMix kit (INTRON Biotechnology). The following primers (based on the sequences of the rabbit type II collagen and on the sequences of the human SOX-9 genes) and conditions were used in PCR: Type II collagen (370 bp product, annealing temperature 45°C, 30 Cycle), sense 5'-GACCCC-ATGCAGTACATGCG-3'; antisense 5'-AGCCGC-CATTGATGGTCTCC-3', SOX-9 (386 bp product, annealing temperature 45°C, 21 Cycle), sense 5'-GCGCGTGCACAAGAAGGACCACCCGGATTA-CAAGTA-3'; antisense 5'-CGAAGGTCTCGATGT-TGGAGATGACGTCGCTGCTCAGCTC-3'. GAP-DH was amplified for control and normalization purposes using the following primers and conditions;(299 bp product, annealing temperature 45°C, 30 Cycle), sense 5'-TCACCATCTTCCAGG-AGCGA-3'; antisense 5'-CACAATGCCGAAGTG-GTCGT-3'. Sequencing of the PCR products for rabbit SOX-9 showed that these gene fragment was 93% homologous respectively to the corresponding human genes.

Knockdown of COX-2 by siRNA

Rabbit COX-2 was cloned from chondrocytes (GeneBankTM accession number NM017237), and the following siRNA sequences were selected using siRNA WizardTM (Invitrogen, San Diego, CA): 5'-UGAUAGAGUGUCUUCAAUUCAGAGC-3' (no.1), 5'-CCUCUGAAUUCAAGACACUCUAUCA-3' (no. 2), 5'-AUGUCAUCUAGUCUGGAGUGGGA-GG-3' (no. 3). Cells were transfected with the siRNA (100 pM) using Metafectene. Three of the tested siRNAs caused effective knockdown of COX-2.

Data analyses and statistics

The results are expressed as the means ± S.E. values calculated from the specified number of determinations. A Student's *t*-test was used to compare individual treatments with their respective control values. A probability of $P < 0.05$ was taken as denoting a significant difference.

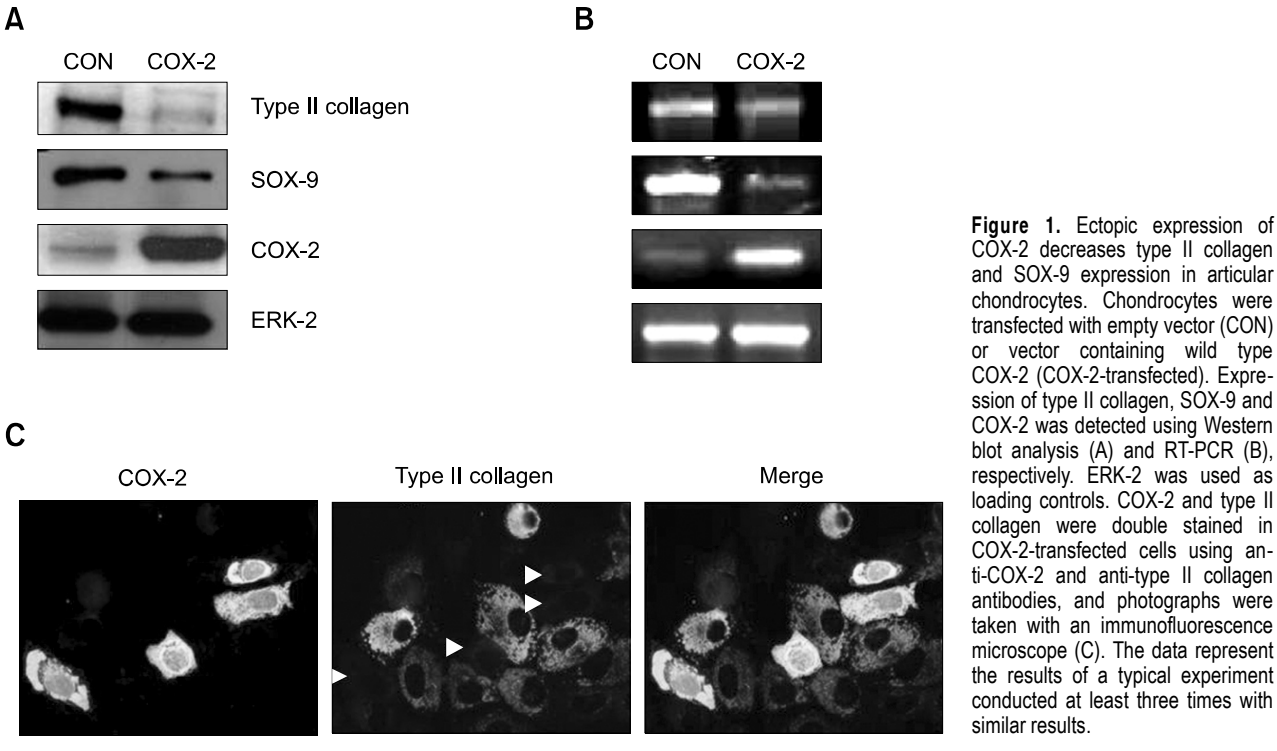

Figure 1. Ectopic expression of COX-2 decreases type II collagen and SOX-9 expression in articular chondrocytes. Chondrocytes were transfected with empty vector (CON) or vector containing wild type COX-2 (COX-2-transfected). Expression of type II collagen, SOX-9 and COX-2 was detected using Western blot analysis (A) and RT-PCR (B), respectively. ERK-2 was used as loading controls. COX-2 and type II collagen were double stained in COX-2-transfected cells using anti-COX-2 and anti-type II collagen antibodies, and photographs were taken with an immunofluorescence microscope (C). The data represent the results of a typical experiment conducted at least three times with similar results.

Results

Ectopic expression of COX-2 induces dedifferentiation of rabbit articular chondrocytes

To examine the effects of the expression of COX-2 as an central enzyme in the inflammatory cascade on articular cartilage chondrocyte differentiation, the protein was overexpressed by the COX-2 cDNA transfection. Ectopic expression of COX-2 inhibited type II collagen, a marker for differentiation of articular chondrocytes (Yoon *et al*., 2002) as determined by immunoblotting and RT-PCR, respectively (Figure 1A, B). Consistent with the inhibition of type II collagen expression, levels of SOX-9, a potent activator of the chondrocyte-specific enhancer of the pro alpha 1 (II) collagen gene (Lefebvre *et al*., 1997; DeLise *et al*., 2000) was decreased by the COX-2 overexpression (Figure 1A, B). To directly determine whether COX-2 expression is involved in the regulation of chondrocyte dedifferentiation, chondrocytes were transfected with COX-2 cDNA. Immunofluorescence double staining of COX-2 and type II collagen in chondrocytes transfected with COX-2 cDNA indicated that cells highly expressing COX-2 are negative for type II collagen staining (indicated by white arrow head), whereas cell do not express COX-2 are positive for type II collagen staining (Figure 1C).

To confirm the effects of COX-2 expression on chondrocyte differentiation, we used cartilage explant cultures which were transfected with COX-2 cDNA or treated with 5 ng/ml IL-1β for 24 h. Our previous data demonstrated that IL-1β induced COX-2 expression and also caused dedifferentiation of articular chondrocytes (Kim *et al*., 2003). Ectopic expression of COX-2 or IL-1β caused a dramatic loss of sulfated proteoglycan and type II collagen as determined Alcian blue staining and immunohistochemical staining, respectively (Figure 2). The role of COX-2 in the regulation of type II collagen expression was further characterized by knock-down of COX-2 using siRNAs. Three examined siRNAs (no. 1, no. 2, no. 3) effectively inhibited the COX-2 overexpression by COX-2 transfection or IL-1β and resulted in a concomitant recovery of type II collagen, respectively (Figure 3A and C). Similarly, knock-down of COX-2 using the three siRNAs blocked COX-2-transfected or IL-1β-stimulated dedifferentiation as indicated sulfated proteoglycan accumulation (Figure 3B and D).Taken together, these results indicate that ectopic expression of COX-2 appears to be sufficient to induce dedifferentiation of articular chondrocytes.

Ectopic expression of COX-2 inhibits chondrogenesis of mesenchymal cells

Micromass culture of dissociated chick limb bud mesenchymal cells is frequently used as a model system to study chondrogenesis (Ahrens *et al*., 1977; Sandell and Adler, 1999). Chondrogenic differentiation of mesenchymal cells is accompanied by morphological changes such as precartilage condensation and cartilage nodule formation. We further examined whether ectopic expression of COX-2 regulated chondrogenesis of mesenchymal cells. Precartilage condensation was verified by staining the cells with peanut agglutinin which

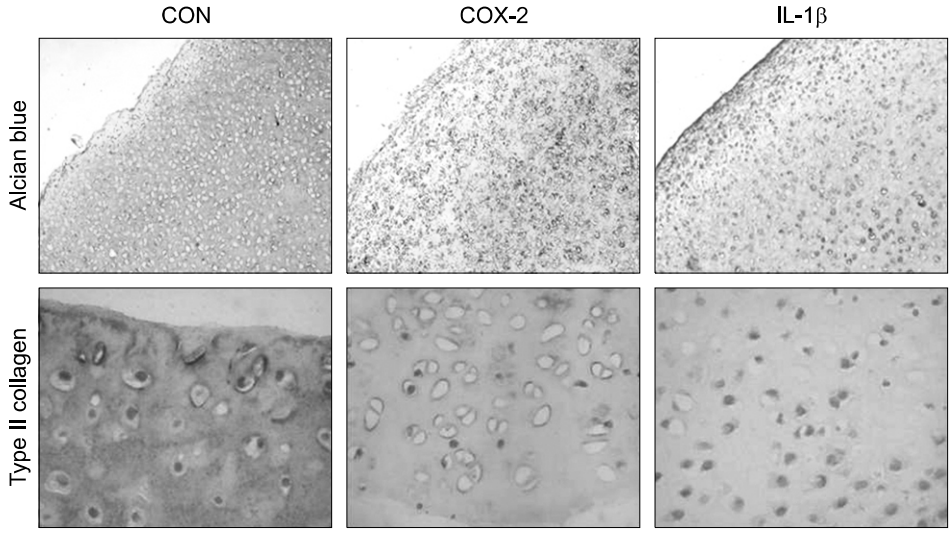

Figure 2. Ectopic expression of COX-2 causes dedifferentiation of articular chondrocytes. Cartilage explants were transfected with empty vector (untreated) or transfected with wild type COX-2 (COX-2-transfected) or treated with 5 ng/ml IL-1β (IL-1β) for 24 h. Type II collagen and proteoglycan were detected by immunohistochemical staining (× 400) and Alcian blue staining (× 200), respectively. The data represent results of typical experiment conducted at least four times.

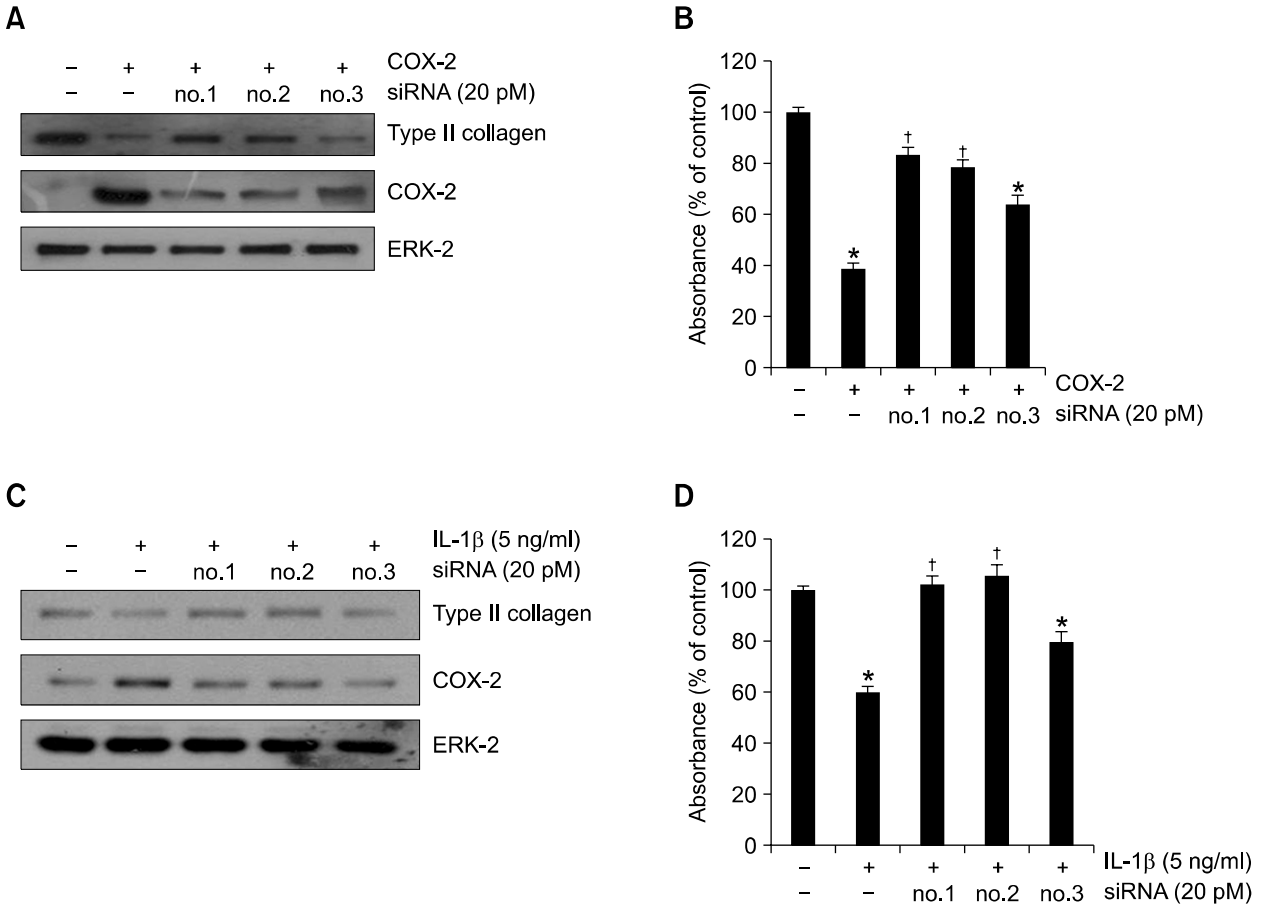

Figure 3. Knockdown of COX-2 rescues COX-2-induced dedifferentiation. Chondrocytes were transfected with empty vector (-) or three different constructs of rabbit COX-2 siRNA (no. 1, no. 2, and no. 3). Following a 4 h incubation, the cells were untransfected (-) or transfected with wild type COX-2 (COX-2-transfected). Transfected cells were cultured in complete medium for 24 h. Expression levels of type II collagen and COX-2 were determined by Western blot analysis (A) while accumulation of sulfated glycosaminoglycan was quantified by Alcian blue staining (B). After 4 h of siRNA transfection, the cells were untreated (-) or treated (+) with 5 ng/ml IL-1β for an additional 24 h. Expression levels of type II collagen and COX-2 were determined by Western blot analysis (C). ERK-2 was analyzed as loading controls. Accumulation of sulfated glycosaminoglycan was quantified by Alcian blue staining (D).The data represent the results of a typical experiment conducted at least three times with similar results.

specifically marks precartilage condensation. As expected, COX-2 cDNA transfection did not affect precartilage condensation as determined by peanut agglutinin staining (data not shown, Aulthouse and solursh, 1987), but it blocked cartilage nodule formation as determined by Alcian blue staining (Figure 4A) and accumulation of sulfated proteoglycans (Figure 4B). These results indicate that COX-2 overexpression inhibits progression from precartilage condensation to cartilage nodule, indicating inhibition of the chondrogenesis of mesenchymal cells.

Discussion

IL-1β is a major catabolic pro-inflammatory cytokine involved in cartilage destruction-associated processes, such as loss of the differentiated chondrocyte phenotype (dedifferentiation) and inflammation (Sandell and Aigner, 2001; Ghosh and Smith, 2002). We previously demonstrated that IL-1β induced COX-2 expression and dedifferentiation in primary culture chondrocytes (Kim, 2003).

We have also shown that ectopic expression of caveolin-1 contributes to the expression and activity of COX-2 (Kim *et al*., 2006) and that these caveolin-1-transfected cells causes dedifferentiation of articular chondrocytes (Yu *et al*., 2004).

Because cartilage, formed by differentiated chondrocytes, serves as a template for endochondral bone formation and because cartilage development is initiated by the differentiation of mesenchymal cells into chondrocytes (Ahrens *et al*.,

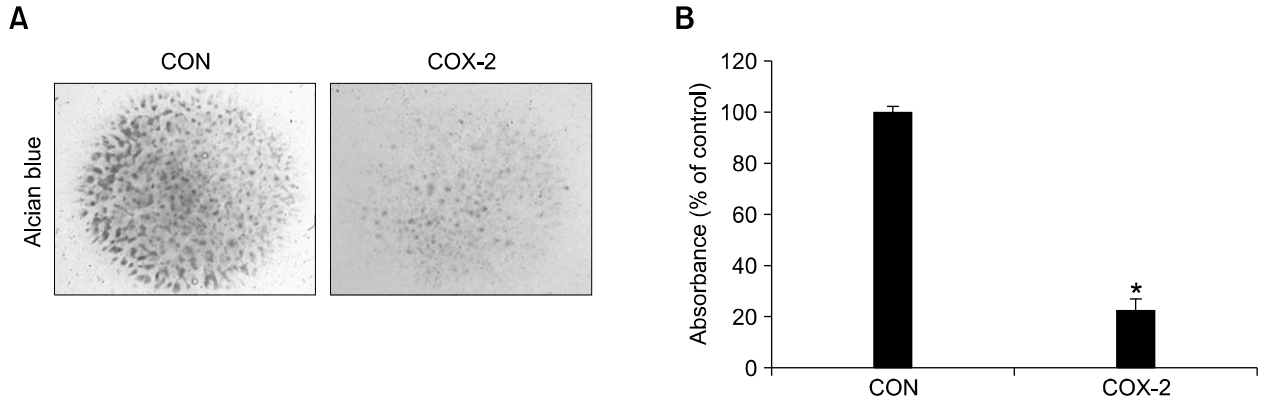

Figure 4. Ectopic expression of COX-2 inhibits progression of precartilage condensation to cartilage nodules. Mesenchymal cells were transfected with either empty vector (CON) or cDNA for COX-2 wild type (COX-2-transfected). The cells were cultured for 4 days. Accumulation of sulfate glycosaminoglycans was determined by Alcian blue staining (A) and quantified by measuring absorbance at 600 nm (B). The data represent the results of a typical experiment or mean values and SD (n = 4).

1977; Sandell and Adler, 1999; Solursh, 1989), we therefore examined in this study whether ectopic expression of COX-2 mediates differentiation of chondrocytes. As expected, COX-2 overexpression caused chondrocytes dedifferentiation and inhibited chondrogenesis.

The metabolites of COXs activity have long been suspected to have a role in skeletal reparative processes. The administration of PGE_2 has increased the rate of fracture healing in several animal models (Keller, 1996; Norrdin and Shith, 1998), indicating that the metabolites of COXs may be necessary for efficient bone healing. The effect of PGE_2 on chondrocytes depends on the culture system, microenvironment, and physiological conditions (Schwartz *et al*., 1998; Amin *et al*., 2000). PGE_2 exerts anabolic effects such as synthesis of proteoglycan and type II collagen and catabolic effects such as enhancing matrix degradation (Goldring *et al*., 1996; Abramson 1999). For example, PGE_2 has been shown to promote chondrocyte differentiation in addition to its role in inflammation by increasing type II collagen expression (Goldring *et al*., 1996, 1999; Schwartz *et al*., 1998). However, the expression and activation of COX-2 and resultant PGE_2 production are also believed to contribute to cartilage destruction by altering matrix degradation via matrix metalloproteinase (Abramson, 1999). In our culture system, we observed that the addition of exogenous PGE_2 did not affect chondrocyte dedifferentiation (data not shown). Therefore, our results suggest that ectopic expression of COX-2 causes dedifferentiation of rabbit articular chondrocytes and chondrogenic differentiation of mesenchymal cells via PGE_2-independent pathways.

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