Experimental chondrocyte hypertrophy is promoted by the activation of discoidin domain receptor 2

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Abstract. The aim of the present study was to assess the association between chondrocytes and the extracellular matrix (ECM), and determine whether this contributes to osteoarthritis (OA). Chondrocyte hypertrophy was measured in articular cartilage samples from early-stage OA patients. In addition, rat chondrocytes were cultured and divided into four groups (A to D): Group A was an untreated control group, group B was incubated with chicken collagen II, group C was transfected with the discoidin domain of discoidin domain receptor-2 (DDR2) and group D was transfected with full-length DDR2. The expression levels of DDR2 and hypertrophic markers in each group were then measured by quantitative polymerase chain reaction (qPCR) and western blot analyses. Chondrocyte hypertrophy was identified in samples of early-stage OA patients. In rat chondrocyte cultures, the relative mRNA and protein expression levels of hypertrophic markers were determined as: Group D > B > C > A. In conclusion, transfection with DDR2 induced the expression of hypertrophic markers, as assessed by qPCR and western blot analyses. DDR2 therefore promoted chondrocyte hypertrophy and terminal differentiation.

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Abbreviations: CII, collagen II; OA, osteoarthritis; ECM, extracellular matrix; DDR2, discoidin domain receptor 2; MMPS, matrix metalloproteinases; PTHrP, parathyroid hormone-related protein; MAPK, mitogen-activated protein kinase; ALP, alkaline phosphatase; FD, over length discoidin domain receptor 2; DS, discoidin domain motif; RIPA, radioimmunoprecipitation assay; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs

Introduction

Osteoarthritis (OA) is a progressive degenerative disease characterized by the destruction of articular cartilage, accompanied by subchondral bone sclerosis and synovial inflammation. Chondrocytes and extracellular matrix (ECM) are the major components of articular cartilage. Cartilage ECM contains large quantities of collagen II (CII) fibrils for tensile strength and glycosaminoglycans for osmotic swelling properties that confer compressive strength (1). CII, composed of a triple helix of three identical chains, forms fibrils stabilized by intermolecular crosslinks (2). The fibrils provide tensile strength and constrain the swelling of aggrecan, which provides cartilage tissue with compressive stiffness (3,4).

Excessive strain results in morphological, molecular and mechanical changes in chondrocytes and the ECM, which damages the structure and function of cartilage (5,6). The degradation of matrix molecules impairs the articular cartilage and may induce subsequent damage to the collagen network during attempted matrix repair (7). Collagen degradation and loss may be significant, since damage to the collagen network is generally considered to be irreparable (8).

The early stages of OA are accompanied by the activation of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and matrix metalloproteinases (MMPs) that cause the degradation of proteoglycan and collagen (9). Subsequently, MMPs (predominantly MMP13) are released, which results in significant degradation of the cartilage matrix and chondrocyte apoptosis (10). MMP-13 expression is considered to be involved in the excessive ECM cleavage during the development of OA (11-13).

Chondrocyte hypertrophy is increasingly recognized as a critical factor during the pathogenesis of OA. In normal physiology, chondrocytes transform into osteoblasts and undergo apoptosis and matrix mineralization, which results in the formation of bone tissue (14). However, during the early stages of OA, hypertrophy may occur and a prominent spatial reorganization of human superficial chondrocytes has been reported (15).

Fragments of matrix molecules are involved in cellular feedback mechanisms in cartilage explants and chondrocyte culture systems. Certain fragments induce the expression of active proteinases, resulting in increased matrix degradation (16,17), whereas others increase matrix synthesis (18,19) and induce

cellular proliferation (20). Different matrix fragments derived from fibronectin or collagen may therefore signal and amplify catabolic processes in chondrocytes to remove tissue components for repair or initiate reparative signals (21-24). Of note, several studies have suggested that CII fragments may influence chondrocyte differentiation during endochondral ossification (14,17).

Discoidin domain receptor-2 (DDR2) is a member of the tyrosine kinase receptor superfamily. DDR receptors may regulate cell proliferation, adhesion, migration and tumor metastasis (23). DDR2 transmits information to the nucleus and ECM, which is important in the physiological and pathological differentiation of cells. In addition, collagen integration in DDR2 results in receptor activation, which impacts chondrocytes and the ECM. The increase in DDR2 expression levels in chondrocytes is a key event in the pathogenesis of OA (24). *In vitro* studies have suggested that CII fragments may exacerbate OA by stimulating the induction and activation of MMPs, which further degrades the collagen matrix and initiates a positive feedback loop (14,17,25).

During OA, early cartilage damage causes the depletion of proteoglycans, resulting in CII exposure, which then interacts with chondrocytes by binding to DDR2 (26). CII activation via DDR2 may also induce overexpression of hypertrophic markers, such as MMP13. Thus, CII and DDR2 may together contribute to chondrocyte hypertrophy (14). In the present study, the expression levels of DDR2 and the corresponding chondrocyte hypertrophic markers were assessed using gene recombination technology.

Materials and methods

Ethical approval. The Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and the Animal Ethics Committee of Chongqing Medical University approved this study.

Samples from OA patients

Hematoxylin and eosin (H&E) staining of samples from OA patients. Samples were obtained from patients attending the First Affiliated Hospital of Chongqing Medical University, China. The sample inclusion criteria were that the patients fit the clinical diagnostic criteria of OA and that hypertrophy was visible by light microscopy. The samples with fewer pathological changes and a more integrated morphology of cartilage were selected. Slides were fixed in formalin (Dingguo Biotechnology Inc., Shanghai, China) for 24 h and then soaked in 15% EDTA (Dingguo Biotechnology Inc.) for one month for decalcification. The sections were then stained with hematoxylin for 5 min, incubated with hydrochloric acid ethanol [Chongqing Chuandong Chemical (Group) Co., Ltd., Chongqing, China] stained with eosin and observed by light microscopy (Olympus, Tokyo, Japan).

Immunohistochemical (IHC) staining for chondrocyte hypertrophic markers. Sections were heated at 60˚C for 20 min, then deparaffinized in dimethylbenzene. The sections were then dehydrated in graded ethanol solutions and endogenous peroxidase activity was inhibited with 3% H_2O_2. Antigen retrieval was conducted in 15% EDTA at 37˚C for 10 min and the sections

were blocked using goat serum (Beijing ComWin, Inc., Pekin, China). For staining, the samples were incubated overnight at 4˚C with the appropriate primary antibodies: Anti-collagen X (Bioss, Inc., Pekin, China; dilution, 1:200), anti-alkaline phosphatase (ALP; A3687; Sigma-Alrich, St. Louis, MO, USA; dilution, 1:200), anti-MMP13 (Bioss, Inc.) or anti-DDR2 (Bioss, Inc., dilution, 1:100); control sections were incubated with non-immune rabbit serum. All sections were fixed using neutral balata (Solarbio Inc., Pekin, China) and analyzed by optical microscopy (Olympus).

Quantitative polymerase chain reaction (qPCR) analysis of CII and DDR2. Fresh samples were defined as early- or end-stage OA as determined by the Mankin scoring system (28). The samples with a Mankin score <4 were considered to be early-stage OA and >8 was defined as end-stage OA. Total RNA was isolated using an RNA isolation kit (Stratagene, La Jolla, CA, USA). Primers were designed using the Primer-Blast tool from the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov/pubmed/; Bethesda, MA, USA). The primer sequences were as follows: CII forward 5'-GATTCGCCTC-GGGGCTC-3' and reverse 5'-GGGCCCAGGGGTTCCA-3'; DDR2, forward 5'-TCTGCACCCGTTGATATGCC-3' and reverse 5'-TGTAGTGAACTTGCCCAGCA-3'; GAPDH forward 5'-CCGTATTCAGCATTCTATGCTCT-3' and reverse 5'-CAGGCCCCTCCTGTTGTTAT-3'. qPCR reactions were performed at 95˚C for 2 min, followed by 95˚C for 30 sec, 52˚C for 30 sec and 72˚C for 30 sec, with a final extension at 72˚C for 3 min. The relative expression levels were calculated using the ΔΔCt method (29). GAPDH served as the internal control.

Chondrocyte culture and analysis

Chondrocyte culture. One specific pathogen-free grade Sprague Dawley rat (4-weeks old), weighing 200 g, obtained from the Laboratory Animal Center of Chongqing Medical University, was sacrificed by the cervical dislocation method following inhalation anesthesia with diethyl ether. Costicartilage from the ribs was harvested and cut into fragments of ~1 mm³. The samples were trypsinized for 10 min at 37˚C and digested with CII for 4 h, with cell suspensions collected during the incubation. Penicillin-streptomycin (North China Pharmaceutical Group Corporation, Shijiazhuang, China) solution was then added and the cells were incubated at 37˚C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco).

Induction of chondrocyte hypertrophy. Chondrocyte hypertrophy was induced using CII isolated from chicken (Sigma-Aldrich) after the cells had adhered and been passaged, and de-differentiation had not occurred over three passages. CII (C-9301; Sigma-Aldrich) was dissolved in 0.25% acetic acid at a concentration of 1 mg/ml and incubated with the cells for 48 h.

Analysis of mRNA and protein in chondrocytes

Plasmid construction. Total RNA was isolated from rat brain tissue homogenates using an RNA isolation kit (Stratagene). Primers for the discoidin domain of DDR2 (DS) and the full-length protein (FD) were designed as determined by the sequences in the NCBI database. The primer sequences were as follows: FD, forward 5'-GCTAG-

CATGATCCCGATTCCCAGAATGC-3' and reverse 5'-CTCGAGTCACTCGGCTCCTTGCTGAAGAAGC-3'; DS, forward 5'-GGTACCATGATCCCGATTCCCAGAATGC-3' and reverse, 5'-CTCGAGTCAGACTGTCATTTCATCATC-CAGCATCC-3'. qPCR reactions were conducted and the PCR products were separated on sepharose gels. The gels were melted and placed on a spin column (Takara Bio., Inc., Dalian, China) centrifuging at a rotation speed of 20,000 x g the DNA was then eluted. Target DNA was mixed with rTaq (Toyobo Co., Ltd., Osaka, Japan) and dATP, heated at 72°C for 40 min and ligated to a pGEM-T vector (Promega Corporation, Madison, WI, USA).

Transformation and DNA extraction. DH5α-competent cells (CWBIO, CW0808) were thawed at room temperature and placed on ice. The target DNA was added to the cells, which were mixed uniformly with a tripette and placed back on ice for 30 min. The cells were then incubated in a 42°C water bath for 60 sec and immediately placed on ice for 5 min. Subsequently, 1 ml media (without ampicillin) was added and the cells were incubated at 37°C for 1 h with agitation. The cells were then plated onto ampicillin-containing agar plates and incubated for 16-20 h. Single colonies were selected and DNA was isolated using phenol - ethanol extraction following standard methods.

Plasmid linking and transfection. The target DNA and pcDNA3.1(+) vector (Invitrogen Life Technologies, Carlsbad, CA, USA) were digested with the following restriction endonucleases (all from Invitrogen Life Technologies): *Nhe*I and *Xho*I for FD, and *Kpo*I and *Xho*I for DS. Inserts were then ligated to pcDNA3 using 2X Rapid Ligation with T4 DNA Ligase (Roche Diagnostics, Mannheim, Germany). The cells were divided into four groups: Group A, control of untreated chondrocytes; group B, chondrocytes incubated with CII, without transfection; group C, chondrocytes transfected with the discoidin domain; and group D, chondrocytes transfected with full-length DDR2. The cells were transfected using 4 µg plasmid, 10 µl Lipofectamine (Invitrogen Life Technologies) and 2 ml serum-free DMEM per well, and were incubated in 5% CO_2 for 6 h at room temperature. The media were then replaced with DMEM containing 10% fetal calf serum.

Assessing chondrocyte hypertrophy

qPCR assessing the expression levels of DDR2, MMP13, ALP and collagen X mRNA. The mRNA expression levels of DDR2, MMP13, ALP and collagen-X were measured in each group. Primers were designed using the Primer-Blast tool. The primers were as follows: DDR2 (rat), forward 5'-GCTGAAGCGAGGTACAGGAC-3' and reverse 5'-AAGGATCTGGGCCACAGGAA-3'; MMP13 (rat), forward 5'-TGGGAAGGAGAGACTCCAGG-3' and reverse 5'-AAGAAGAGGGTCTTCCCCGT-3'; Col10a1 (encoding type X collagen), forward 5'-TGCTAGTGTCCTTGAC-GCTG-3' and reverse 5'-GCCCATTGAGGCCCTTAGTT-3'; ALP, forward 5'-GCACCTGCCTTACCAACTCT-3' and reverse 5'-AACCAACACCAAAAGAGGAAAT-3'; and GAPDH, forward 5'-CCGTATTCAGCATTCTATGCTCT-3' and reverse 5'-CAGGCCCCTCCTGTTGTTAT-3'. Rat chondrocyte mRNA served as the template and the cDNA
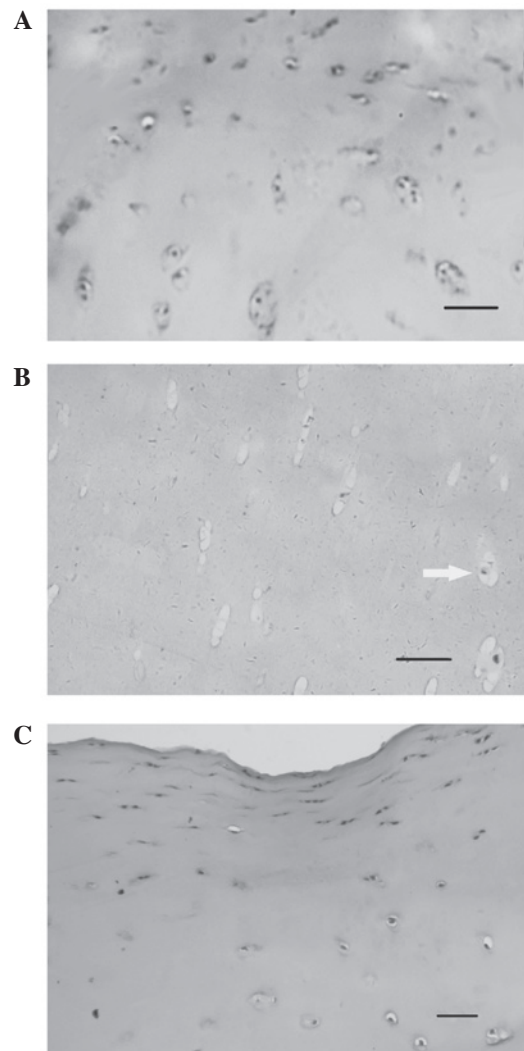

Figure 1. Hematoxylin & Eosin staining of cartilage samples from OA patients. (A) Early-stage OA section; (B) end-stage OA section; (C) early-stage OA, indicative of chondrocyte hypertrophy. Scale bar, 50 µm. OA, osteoarthritis.

was synthetized. qPCR was performed using the following cycling conditions: 95°C for 2 min, followed by 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final extension of 72°C for 3 min. The relative expression levels were assessed using the ΔΔCt method and GAPDH served as the internal control.

Western blot analysis of hypertrophic markers. The groups were divided as above, and the expression levels of β-actin, DDR2, MMP13, type X collagen and ALP were measured in each group. The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free milk in Tris-buffered saline for 1 h at room temperature and then probed with the appropriate antibodies, including anti-β-actin, anti-DDR2, anti-MMP13, anti-type X collagen and anti-ALP. Horseradish peroxidase-conjugated secondary antibodies (Bioss, Inc., dilution, 1:20,000 were then added and the membranes were incubated for 1 h. Bands were then visualized using a gel imaging analysis system (GelDoc XR; Bio-Rad, Hercules, CA, USA).
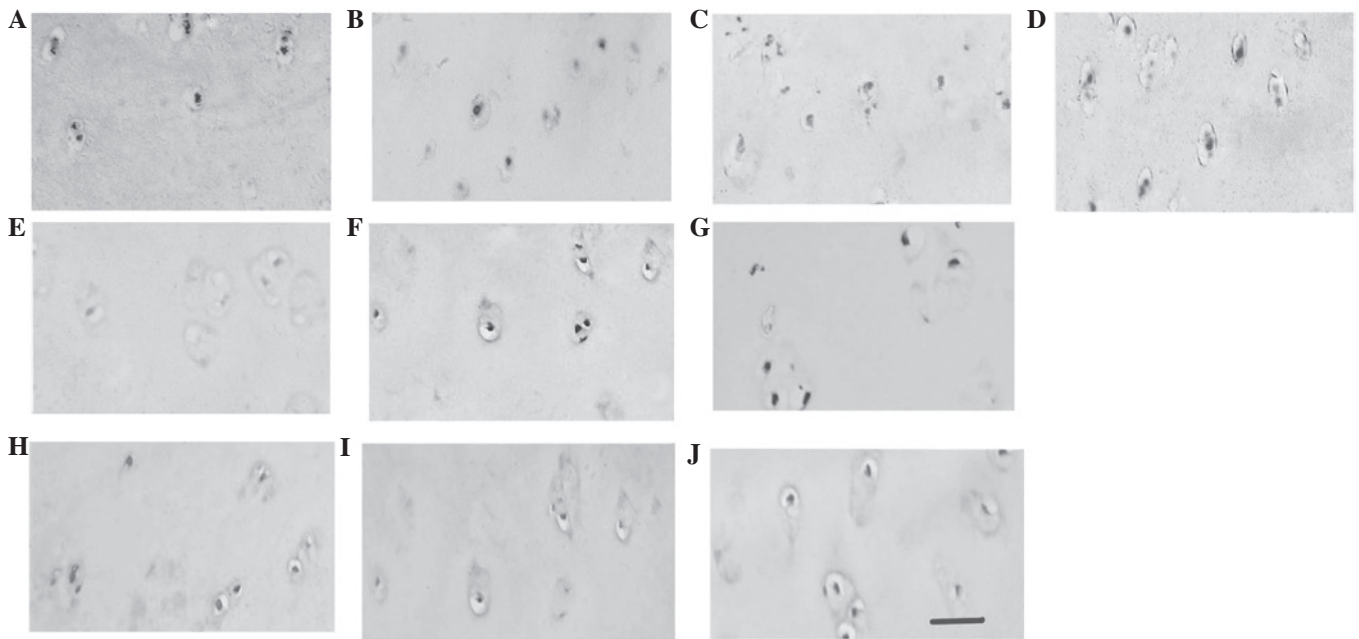
Figure 2. (A-E) Sections of early-stage osteoarthritis (Mankin score <4) samples, stained with antibodies against (A) type II collagen, (B) discoidin domain receptor 2, (C) matrix metalloproteinase 13, (D) alkaline phosphatase and (E) type X collagen, respectively. (F-J) Sections incubated with goat serum serving as control groups. Scale bar, 50 μm.

Results

H&E staining of cartilage samples from OA patients. Samples were isolated from OA patients and stained with H&E. As shown in Fig. 1A, the nuclei of several chondrocytes were stained blue, the cells were well-distributed and no apoptosis was observed. At end-stage OA (Fig. 1B), few chondrocytes were detected and clear vacuolar degeneration was visible. In addition, low chondrocyte nuclear staining was observed, consistent with the pathological changes associated with apoptosis (29). The sample shown in Fig. 1C (described from top to bottom) exhibited chondrocyte proliferation and hypertrophic chondrocyte differentiation. As shown in Fig. 1, proliferation and hypertrophy were observed in early-stage OA in chondrocytes, while apoptosis was detected only in late-stage OA.

IHC staining for chondrocyte hypertrophic markers. Samples from early-stage OA patients were randomly divided into experimental (A, B, C, D and E) and control groups (F, G, H, I and J). The control samples were incubated with goat serum. Sample A was incubated with antibodies against CII. As shown in Fig. 2, CII expression in the ECM was detected in the early-stage OA tissue, but not in the control tissue, corresponding to ECM proliferation during chondrocyte hypertrophy. Compared with sample G, patient B exhibited positive staining for DDR2 in the areas surrounding the chondrocytes, demonstrating that DDR2 expression is associated with chondrocyte hypertrophy and terminal differentiation. Samples C, D and E (early OA) and H, I and J (control), were incubated with antibodies against MMP13, ALP and collagen X, respectively, but positive staining was only observed in the early-stage OA samples, indicating the expression of early cell hypertrophic markers in OA.

qPCR analysis of CII and DDR2 expression levels. qPCR was used to detect the mRNA expression levels of CII and DDR2

using template RNA isolated from the articular cartilage of early- and late-stage OA patients. The relative expression levels were calculated using the $\Delta\Delta$Ct method and GAPDH served as the internal control. Significantly increased CII and DDR2 expression levels were detected in early-stage OA compared with those in late-stage OA. The significance of the data was analyzed using Student's t-test.

qPCR analysis of DDR2, MMP13, ALP and collagen X mRNA expression levels. The DDR2, MMP13, ALP and collagen X mRNA expression levels in groups A-D were assessed as described above, using rat chondrocyte mRNA as the template. The relative expression levels were calculated using the $\Delta\Delta$Ct method and GAPDH served as the internal control. As shown in Fig. 3, the DDR2 mRNA expression levels varied significantly among the groups, with the highest expression levels detected in group D, followed by groups B, C and A, respectively. Notably, the expression levels of the hypertrophic markers, MMP13, ALP and collagen X, were similar to those of DDR2. Student's t-test was used to confirm that the differences were statistically significant.

Western blot analysis of protein expression levels of hypertrophic markers. The expression levels of DDR2, MMP13, collagen X and ALP were assessed using the appropriate specific antibodies, and compared with the expression levels of β-actin as the internal reference. As shown in Fig. 4, the DDR2 protein expression levels were greatest in group D, followed by groups B, C and A, respectively, mirroring the mRNA expression pattern. The expression patterns of MMP13, collagen X and ALP proteins were comparable with those of DDR2. In group A, low expression levels of DDR2, MMP13, collagen X and ALP were detected. As expected, β-actin expression levels were comparable among the groups.
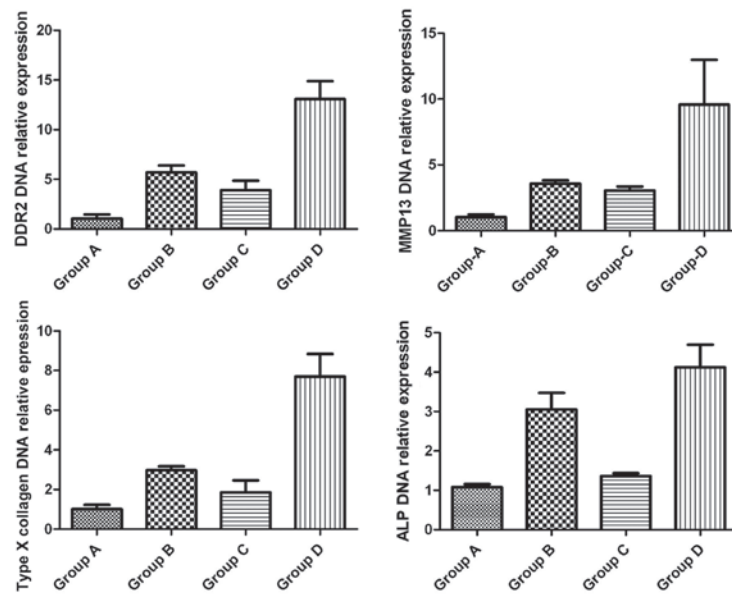
Figure 3. Quantitative polymerase chain reaction analysis of DDR2, MMP13, ALP and collagen X mRNA expression levels. Vertical coordinates indicate the target mRNA and A-D indicate the different treatment methods. (A) Untreated chondrocytes; (B) chondrocytes incubated with chicken type II collagen; (C) chondrocytes incubated with type II collagen and transfected with the discoidin domain of DDR2; and (D) chondrocytes incubated with type II collagen and transfected with full length DDR2. DDR2, discoidin domain receptor 2; MMP, matrix metalloproteinase; ALP, alkaline phosphatase.

Discussion

The present study demonstrated that DDR2 was involved in OA pathogenesis, and was specifically linked to hypertrophy and terminal differentiation during OA development. Although several studies have assessed the role of DDR2 in tumor metastasis, to the best of our knowledge, this is one of the first studies investigating the effects of DDR2 during chondrocyte hypertrophy.

Chondrocytes in healthy articular cartilage are characterized by low expression levels of CII (30), and infrequent hypertrophy (31) and apoptosis (32,33). However, excessive collagen cleavage by collagenases occurs in the early stages of OA (34,35). In the present study, DDR2 and CII upregulation was observed in early-stage OA, but the expression levels were significantly reduced as OA progressed to late stage disease ($P<0.05$). The upregulation of DDR2 during the early stages of OA may be a response to articular cartilage damage and chondrocyte apoptosis. In addition, increased chondrocyte CII expression levels may compensate for articular cartilage lesions in early-stage OA; however, the expression levels decline when the number of functional chondrocytes is diminished.

qPCR revealed that the mRNA expression levels of DDR2 and hypertrophic markers were significantly different among the groups, suggesting that the transfections successfully generated an OA model. The pattern of gene expression levels of the hypertrophic markers between groups was: Group D > B > C > A, comparable with that of the DDR2 expression levels. This suggested that DDR2 induced the expression of the hypertrophic markers.

Chondrocytes transfected with DS expressed DDR2 at similar levels to those in the model group. However, expression of the discoidin domain on the cell surface, which may bind to CII ligands but not be activated, resulted in competitive inhibition with normal DDR2, and thus lower levels of active
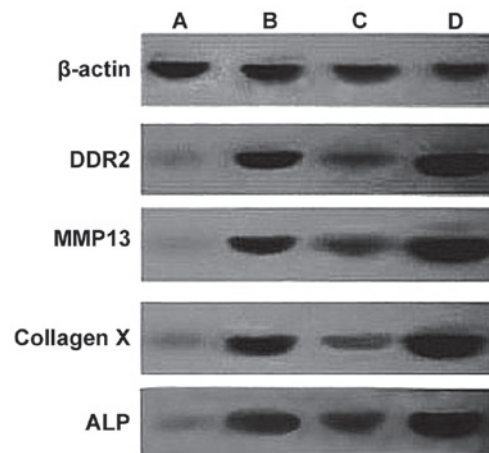

Figure 4. Western blot analysis of protein expression levels of hypertrophic markers. Lanes A-D indicate different treatments. (A) Untreated chondrocytes; (B) chondrocytes incubated with chicken type II collagen; (C) chondrocytes incubated with type II collagen and transfected with the discoidin domain; and (D) chondrocytes incubated with type II collagen and transfected with full length DDR2. DDR2, discoidin domain receptor 2; MMP matrix metalloproteinase; ALP, alkaline phosphatase.

DDR2 were expressed compared with those of the control. The expression levels of the hypertrophic markers were also reduced compared with those of the control. This suggested that the expression levels of DDR2 and hypertrophic markers are positively correlated, that CII exerts a positive feedback effect on DDR2, and that activation results in chondrocyte hypertrophy and terminal differentiation, mimicking OA.

MMP-13 knockout mice were identified to have significantly reduced cartilage structural damage during surgically induced OA, which was not associated with any reduction in aggrecanolysis, or changes in chondrocyte hypertrophy and apoptosis (36). In the present study, the activation of DDR2

led to the upregulation of MMP13, which was accompanied by increased expression levels of chondrocyte hypertrophic markers, including alkaline phosphatase and collagen X. This suggested that MMP13 is upregulated during hypertrophy and terminal differentiation, but is not directly responsible for articular cartilage hypertrophy.

The present study demonstrated that the cell surface receptor DDR2 is important in extracellular and intracellular communication. In conclusion, DDR2 regulates the cellular phenotype during the early stages of OA, in addition to the metabolism of chondrocytes and ECM. However, the signaling pathways that mediate these effects remain to be elucidated.

Acknowledgements

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