Wnt-7a Causes Loss of Differentiated Phenotype and Inhibits Apoptosis of Articular Chondrocytes via Different Mechanisms*

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Although regulation of chondrogenesis and cartilage development by Wnt signaling is well established, the function of Wnt in the maintenance and destruction of cartilage remains largely unknown. Here we investigated the involvement and regulatory mechanisms of Wnt signaling in cartilage destruction. We found that interleukin-1β, the primary pro-inflammatory cytokine involved in cartilage destruction, induces expression of Wnt-5a and -7a in primary culture articular chondrocytes. The level of β-catenin was also increased in chondrocytes of arthritic cartilage, suggesting the association of Wnt/β-catenin signaling with arthritic cartilage destruction. In addition, our results show that Wnt-7a induces dedifferentiation and inhibits NO-induced apoptosis of primary culture articular chondrocytes. Wnt-7a induces dedifferentiation of articular chondrocytes by stimulating transcriptional activity of β-catenin, whereas NO-induced apoptosis is inhibited via the activation of cell survival signaling, such as phosphatidylinositol 3-kinase and Akt, which block apoptotic signaling cascade. Our results collectively suggest that Wnt-7a is associated with cartilage destruction by regulating the maintenance of differentiation status and the apoptosis of articular chondrocytes via different mechanisms.

Cartilage development is initiated by the differentiation of mesenchymal cells into chondrocytes. Chondrogenesis is induced by precartilage condensation, which requires increased cell-cell adhesion mediated by N-cadherin (1). Precartilage condensation develops into cartilage nodules in which differentiated chondrocytes are located. Chondrocytes express cartilage-specific matrix molecules, such as type II collagen, with a loss of cell-cell contacts (1). Both cell-cell adhesion (2) and loss of cell-cell contacts (3) during precartilage condensation and cartilage nodule formation appear necessary for chondrogenesis. Chondrogenesis is additionally regulated by a variety of soluble factors including the Wnt family of proteins (1). Several members of Wnt family are expressed and differentially regulate cartilage development (1, 4–7). For instance, Wnt-7a is known to inhibit chondrogenesis both in vivo and in vitro (3, 8, 9). It is suggested that stabilization of cell-cell adhesion by the sustained expression of N-cadherin and β-catenin induced by Wnt-7a is responsible for the inhibition of chondrogenesis of mesenchymal cells (3, 9). However, many of cellular effects regulated by Wnt signals are exerted by the modulation of β-catenin, which acts as a transcriptional co-activator (10, 11). Wnt signals inhibit glycogen synthase kinase-3β, which inhibits proteolysis and facilitates cytosolic accumulation of β-catenin. Accumulated β-catenin translocates to 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.

Differentiated chondrocytes in articular cartilage maintain homeostasis by synthesizing cartilage-specific matrix molecules. However, this homeostasis is destroyed during pathogenesis of cartilage disease, such as arthritis. Cartilage destruction during arthritis involves the loss of differentiated phenotype (dedifferentiation) and apoptotic death of chondrocytes, which is caused by the production of pro-inflammatory cytokines such as interleukin (IL)-1β (12, 13). It is believed that production of NO by pro-inflammatory cytokines plays an important role in apoptosis and dedifferentiation of articular chondrocytes (14, 15). Previous reports from our group show that apoptosis and dedifferentiation induced by NO are regulated by a complicated protein kinase signaling cascade, including down-regulation of phosphatidylinositol (PI) 3-kinase and Akt, activation of extracellular signal-regulated protein kinase (ERK) and p38 kinase, and inhibition of protein kinase C (PKC) α and ε (16–21).

In addition to the regulation of chondrogenesis and limb development, Wnt proteins may be involved in maintenance and destruction of cartilage. This possibility is indirectly supported by the observation that several Wnt proteins (Wnt-1, -2, -5a, -10b, -11, and -13) and frizzled receptors (frizzled-2, -3, -5, -6, and -7) are expressed in synovial tissue of arthritic cartilage (22). In addition, a secreted frizzled-related protein (FrzB-2) that acts as an antagonist for the frizzled receptor is strongly

* The abbreviations used are: Tcf, T cell factor; Lef, lymphoid enhancer factor; ERK, extracellular signal-regulated protein kinase; I-β, inhibitory α; PI, phosphatidylinositol; RT, reverse transcription; SNP, sodium nitroprusside; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; OA, osteoarthritic; IL, interleukin.
expressed in osteoarthritic cartilage and may regulate chondrocyte apoptosis (23). A previous report from our group also indicated that chondrocytes express low level of β-catenin, and accumulation of β-catenin is sufficient to cause dedifferentiation of chondrocytes (24), suggesting the possibility that Wnt signaling is involved in cartilage destruction. A number of earlier studies also demonstrate that the Wnt signal prevents apoptosis in various cell types (25, 26). However, there is currently no evidence to confirm direct roles for Wnt proteins in cartilage destruction and empty vessel, respectively, regulation of apoptosis and dedifferentiation of articular chondrocytes. In this study, we investigated the roles and regulatory mechanisms of Wnt-7a in the maintenance of differentiated phenotype of primary culture articular chondrocytes and NO-induced apoptosis of articular chondrocytes.

EXPERIMENTAL PROCEDURES

**Tissue Specimens**—Human osteoarthritic (OA) cartilage was obtained from 13 female patients (aged between 51 and 72 years) undergoing arthroplasty for the osteoarthritic knee joints. Transfer of material was approved by the appropriate Human Subjects Committees. All of the cases satisfied the American College of Rheumatology classification criteria for osteoarthritis of the knee (27), and cases of secondary osteoarthritis and inflammatory diseases like rheumatoid arthritis were excluded. Cartilage tissues were sampled down to the subchondral bone from tibial plateau of each specimen within 60 min of operation and treated for immunohistochemistry as described below.

**Primary Culture of Articular Chondrocytes**—Articular chondrocytes from cartilage slices of 2-week-old New Zealand White rats were isolated with 0.2% collagenase type II, as reported previously (28). The cells were treated with Wnt-7a conditioned medium in the absence or presence of various pharmacological reagents, as specified in each experiment. Redifferentiation of dedifferentiated chondrocytes by a serial subculture was induced by three-dimensional culture in alginate gel beads, as described earlier (29). De- and re-differentiation of chondrocytes were determined by examining the suppression of type II collagen and the onset of type I collagen expression with Western blot analysis using mouse monoclonal antibody against type II collagen (Chemicon, Temecula, CA) or reverse transcription (RT)-PCR. Hypertrophic maturation of chondrocytes was caused by maintaining primary chondrocytes as micromass culture as described by Stanton et al. (33) and subsequent determination of type X collagen expression (29).

**Preparation of Conditioned Medium for Wnt-7a**—Control or Wnt-7a conditioned medium was prepared as described previously by Lyu et al. (30). Briefly, stable mouse fibroblast L929 cell lines expressing mouse Wnt-7a or empty vector were generated by transfection of L929 cells with Wnt-7a cDNA and empty vector, respectively. Wnt-7a was confirmed by RT-PCR. After growing to 90% confluency, control and Wnt-expressing L929 cells were washed and maintained in serum-free Dulbecco’s modified Eagle’s medium for 36 h. The conditioned media were clarified by centrifugation at 10,000 g for 5 min followed by filtration (0.2-mm pore size) and concentrated 20 times by ultrafiltration in Amicon-stirred cells (Millipore) using a YM membrane with a 10-kDa molecular mass cut-off.

**Immunocytochemistry and Immunofluorescence Microscopy**—Cartilage tissue and alginate gel beads were fixed in 4% paraformaldehyde overnight at 4 °C, dehydrated with graded ethanol, embedded in paraffin, and sectioned at 4-μm thickness. The sections were stained by standard procedures using mouse monoclonal antibodies against type II collagen (Chemicon) or β-catenin (BD Transduction Laboratories, Lexington, KY) and visualized by developing with a kit purchased from DAKO Co. (Carpinteria, CA) (24). Sulfated glycosaminoglycan was visualized by developing with a kit purchased from Bio-Rad Laboratories ( Hercules, KY) and visualized by developing with a kit purchased from Bio-Rad Laboratories (Hercules, KY) and visualized by developing with a kit purchased from Bio-Rad Laboratories (Hercules, KY). Sections were dehydrated with graded ethanol, immersed in xylene, and mounted with coverslips.

**RT-PCR**—Primary culture chondrocytes were treated with 5 ng/ml of IL-1β or Wnt conditioned medium for the indicated time periods. Total RNA was isolated using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). cDNA was synthesized with oligo dT primer and reverse transcriptase (Invitrogen), as previously described (18). Sequence of primers and PCR conditions were summarized in Table I. In case of Wnt genes, the primers were designed based on the sequence of human homologue and sequencing of PCR products for Wnt-5a and -7a showed 92 and 94% homology to human Wnt-5a and -7a, respectively (data not shown).

**Transfection and Luciferase Assay**—Plasmids (2 μg) bearing dominant negative Tcf-4, a deletion mutant lacking the N-terminal 30 amino acids (31), S37A β-catenin, or a reporter gene for β-catenin (24) were transfected into articular chondrocytes (day 2 cultures) using the LipofectAMINE reagent (Invitrogen), as described previously (24). Alternatively, primary chondrocytes from day 2 cultures were infected with control adenovirus or adenoenovirus encoding wild-type or dominant negative PKCα or PKCβ or wild-type Lef-1 as described in a previous report (21). Adenovirus expressing full-length Lef-1 and green fluorescent protein under different cytokonegator promoters was constructed using the adenoenovirus construction kit provided by Dr. B. Bogelstein (Johns Hopkins University). Infected or transfected cells were cultured for 36 h prior to treatment with other reagents. To determine β-catenin-Tcf/Lef transcriptional activity, the cells were transiently transfected with 1 μg of Tcf/Lef reporter gene, TOPFLASH (optimal Lef-binding site) or FOPFLASH (mutated Lef-binding site) (Upstate Biotechnology Inc., Lake Placid, NY) and 1 μg of pCMV-β-galactosidase. Luciferase activity was measured and normalized for transfection efficiency using β-galactosidase activity (24).

**Assay of Apoptosis and Caspase-3**—Data from DNA fragmentation and terminal deoxynucleotidyl transfer-mediated nick end labeling (TUNEL) assays confirm that NO induces apoptosis in primary articular chondrocytes (16). In this study, apoptotic cells were determined by TUNEL assay or quantified by counting surviving cells using a methylthiazol tetrazolium assay kit (Roche Applied Science), according to the manufacturer’s protocol. Activation of caspase-3 in articular chondrocytes was determined by measuring absorbance of the cleaved synthetic substrate of caspase-3, Ac-Asp-Glu-Val-Asp-chromophore p-nitroanilide, as described earlier (16).

**Immunoprecipitation and PI 3-Kinase Assay**—Chondrocytes were lysed in Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl) containing protease inhibitors (1 mM Pefabloc SC, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin A, 10 μM aprotinin, and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na3VO4) on ice for 30 min. Following centrifugation at 4 °C for 10 min at 12,000 × g, 500 μg of proteins from supernatant were incubated overnight with anti-phosphotyrosine antibody (BD Transduction Laboratories). PI 3-kinase activity was determined by using the immune complex, as described previously (20). Briefly, immune complexes were resuspended in 45 μl of kinase assay buffer (20 mM Tris-HCl, pH 7.6, 75 mM NaCl, 10 mM MgCl2, 1 mM EGTA), and the kinase reaction was initiated by adding 200 μg/ml phosphatidylinositol and [γ-32P]ATP (5 Ci/sample) at room temperature for 30 min. The reactions were terminated by adding 100 μl of 1 N HCl. The reaction products were extracted using 200 μl of CHCl3:MeOH (1:1) and resolved on a thin layer chromatography silica plate coated with potassium oxalate in solvent containing CHCl3:MeOH:H2O:NH4OH (60:47:11.5:2.2). The PI 3-kinase reaction product (phosphatidylinositol 3-phosphate) was identified by autoradiography.

**Western Blot Analysis**—Chondrocytes were lysed on ice for 30 min with buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatase inhibitors, as described above. The proteins were

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**Table 1: Oligonucleotide primers and PCR conditions**

| Gene   | Sequence                        | Product T<sub>m</sub> |
|--------|---------------------------------|-----------------------|
| Type I collagen |                                    |                       |
| Sense   | 5'-ggtcttctgaggagaatgg-3'       | 441 62                |
| Antisense | 5'-ataagacagacgggcaagg-3'      |                       |
| Type II collagen |                                |                       |
| Sense   | 5'-gaacctcatgctacatcgg-3'       | 370 62                |
| Antisense | 5'-agctcctagattgtctc-3'        |                       |
| Type X collagen |                                |                       |
| Sense   | 5'-tggagagagaaacaggtctcgg-3'   | 408 50                |
| Antisense | 5'-ttttctcttctgcaactcaacca-3' |                       |
| Wnt-5a |                                    |                       |
| Sense   | 5'-agctgaagttctcatgaga-3'       | 452 52                |
| Antisense | 5'-catagacagacagaccaat-3'      |                       |
| Wnt-7a |                                    |                       |
| Sense   | 5'-agctgctagagaacagacgaggg-3'  | 452 52                |
| Antisense | 5'-gtgcaaggcttacagctacagc-3'  |                       |
| GAPDH |                                    |                       |
| Sense   | 5'-tcacacttcagggagcagga-3'      | 299 50                |
| Antisense | 5'-cactagcgaagggtggct-3'       |                       |
fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were employed to detect proteins: mouse anti-type II collagen monoclonal (Chemicon), mouse monoclonal antibodies against β-catenin, ERK-1/2 or PKCδ (BD Transduction Laboratories), rabbit polyclonal antibody against I-κB or PKCδ, mouse monoclonal against p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal antibody against Akt, phosphorylated Akt, and phosphorylated p38 kinase from Cell Signaling Technology (Beverly, MA).

RESULTS

Expression of Wnt and β-Catenin in Articular Chondrocytes and Cartilage—In addition to the regulation of chondrogenesis and limb development, several lines of evidence indirectly suggest that Wnt signaling is involved in cartilage destruction (22, 23). In addition, our previous observation indicated that chondrocytes express low levels of β-catenin, a downstream molecule of Wnt signaling, and accumulation of β-catenin causes dedifferentiation of chondrocytes (24). However, there is currently no evidence to confirm direct roles for Wnt proteins in cartilage destruction. In an attempt to examine whether Wnt signaling is associated with cartilage destruction such as dedifferentiation and apoptosis of chondrocytes, we first compared the β-catenin protein level between undamaged region of OA cartilage, which has a smooth surface, and the OA-affected damaged region (Fig. 1A). As expected, OA-affected cartilage showed significantly reduced staining for type II collagen and Alcian blue (Fig. 1B). Undamaged regions of the OA cartilage showed undetectable levels of the β-catenin, whereas the β-catenin level was significantly increased in the OA-affected damaged region. Positive staining of β-catenin was more evident in the deep zone than in the superficial zone of OA cartilage. Similar results were observed in other OA tissues examined (11 tissues from total 13 samples) (data not shown). We also tested expression of various Wnt molecules in pathogenic condition of primary culture chondrocytes using IL-1β, which is the primary inflammatory cytokine involved in cartilage destruction. Consistent with our previous observation (24), IL-1β increased β-catenin levels with a concomitant cessation of type II collagen expression (Fig. 1C). IL-1β also caused a significant increase of Wnt-5a and -7a transcript levels in primary culture chondrocytes, which is an opposite pattern of type II collagen expression (Fig. 1C). Other Wnt genes examined in this study (i.e. Wnt-1, -2, -3a, -4, -5b, and -10b) are barely detectable in cells treated with IL-1β up to 24 h, as determined by various PCR conditions (data not shown).

Wnt-7a Triggers Dedifferentiation of Primary Culture Articular Chondrocyte—Because the above results suggest a possibility that Wnt-5a and -7a cause a loss of differentiated phenotype of chondrocytes via β-catenin signaling, we investigated the effects of Wnt-7a on maintenance of the differentiated phenotype of primary culture articular chondrocytes. We used conditioned medium for Wnt-7a from L929 cells that secrete Wnt-7a protein. The data presented in Fig. 2A show that Wnt-7a suppressed type II collagen expression and synthesis of sulfated proteoglycan. The number of type II collagen-expressing chondrocytes was dramatically reduced upon Wnt-7a treatment (Fig. 2B). Wnt-7a also caused the onset of type I collagen expression (Fig. 2C). Expression of type X collagen, a marker for hypertrophic chondrocytes, was barely detectable in primary culture chondrocytes, and its expression pattern was not altered by Wnt-7a treatment (Fig. 2C). Therefore, suppression of type II collagen expression, induction of type I collagen expression, and no effects on type X collagen expression by Wnt-7a indicate that Wnt-7a causes dedifferentiation of primary culture articular chondrocytes. Wnt-7a also inhibited the redifferentiation of dedifferentiated chondrocytes. As depicted in Fig. 2D, three-dimensional culture of passage 4 cells, which do not express type II collagen, resulted in re-expression of type II collagen in alginate gel. Western blotting and immunohistochemical analyses revealed that the addition of Wnt-7a completely abolished re-expression of type II collagen. The above results indicate that Wnt-7a induces dedifferentiation of articular chondrocytes and also blocks redifferentiation of dedifferentiated chondrocytes.

Wnt-7a Inhibits Type II Collagen Expression by Stimulating Transcriptional Activity of β-Catenin—We investigated the regulatory mechanism of Wnt signaling in dedifferentiation by focusing on the role of β-catenin in type II collagen expression. Wnt-7a increased the levels of β-catenin in primary culture chondrocytes (Fig. 3B). Wnt-7a also caused significant translocation of β-catenin to the nucleus (Fig. 3A) and increased β-catenin-Tcf/Lef complex transcriptional activity (Fig. 3C). Wnt-7a-induced transcriptional activity of β-catenin and inhibition of type II collagen expression were significantly blocked by ectopic expression of dominant negative Tcf-4 (Fig. 4, A and B). Moreover, expression of wild-type Lef-1 increased the transcriptional activity of β-catenin (Fig. 4C) and inhibited type II collagen expression in the absence of Wnt-7a (Fig. 4D). The role of β-catenin in the type II collagen expression was further characterized by transfecting a nonubiquitatable stable form of S37A β-catenin into chondrocytes. S37A β-catenin expression in the absence of Wnt proteins led to increased β-catenin transcriptional activity (Fig. 5A) and decreased the expression of type II collagen (Fig. 5B). Co-transfection of dominant negative Tcf-4 and S37A β-catenin blocked S37A β-catenin-induced in-
Fig. 2. Wnt-7a causes dedifferentiation and inhibits redifferentiation of articular chondrocytes. A, primary culture articular chondrocytes were treated with the indicated volumes (µl) of control or Wnt-7a conditioned medium for 48 h. Type II collagen expression (left panel) and sulfated proteoglycan synthesis (right panel) were determined by Western blotting and Alcian blue staining, respectively. B, type II collagen expressing cells were identified by immunostaining from cells treated with 100 µl of control or Wnt-7a conditioned medium for 48 h. C, chondrocytes were cultured in the presence of control (100 µl) or Wnt-7a (100 or 200 µl) conditioned medium for 48 h (upper panel), and expression levels of type I collagen (Col-I), type II collagen (Col-II), type X collagen (Col-X), and GAPDH were determined by RT-PCR. As positive or negative controls for type I and X collagens, chondrocytes were cultured at passage 0 and 4 (middle panel) or maintained as micromass culture for 15 days to induce hypertrophy (H, lower panel). Expression patterns of type I, type II, and type X collagen were determined by RT-PCR. D, chondrocytes were cultured as a monolayer (M) at passage 0 or 4. Passage 4 cells were three-dimensionally cultured for 4 days in alginate gel in the presence of 100 µl of control or Wnt-7a conditioned medium. Type II collagen was detected by Western blot analysis (upper panel) or immunohistochemistry from sections of alginate gel (lower panel). ERK was employed as loading controls. The data from a typical experiment are presented from at least five independent experiments.

Fig. 3. Stimulation of transcriptional activity of β-catenin by Wnt-7a. Chondrocytes were treated with 100 µl (A) or the indicated volume (µl) (B and C) of control or Wnt-7a conditioned medium for 48 h. Distribution of β-catenin was determined by immunofluorescence microscopy (A). Levels of β-catenin and ERK were determined by Western blotting (B). Transcriptional activity of β-catenin was determined using active (TOPFLASH) or inactive (FOPFLASH) Tcf/Lef reporter genes (C). The data show either results of a typical experiment (A and B) or the mean values and standard deviations (C) from at least five independent experiments. Con, control.

crease of transcriptional activity (Fig. 5A) and concomitantly blocked inhibition of type II collagen expression (Fig. 5B). Taken together, the above results indicate that Wnt-7a triggers inhibition of type II collagen expression in articular chondrocytes by stimulating the transcriptional activity of the β-catenin-Tcf/Lef complex.

Wnt-7a Inhibits NO-induced Apoptosis in Articular Chondrocytes Independently of β-Catenin Signaling—We next examined the role of Wnt-7a in NO-induced apoptosis in primary culture articular chondrocytes. Treating cells with the NO donor sodium nitroprusside (SNP) resulted in apoptosis, and the addition of Wnt-7a significantly inhibited this NO-induced apoptosis (Fig. 6A). Furthermore, Wnt-7a blocked NO-induced apoptotic signaling cascade such as p38 kinase activation, inhibition of PKCα and ζ expression, IκB degradation, and p53 accumulation (Fig. 6B). The significance of PKCα and ζ in Wnt regulation of apoptosis was examined by overexpressing the PKC isoforms. Inhibition of apoptosis by Wnt-7a was completely abolished by overexpression of dominant negative PKCα or ζ, whereas overexpression of wild-type PKCα or ζ abolished NO-induced apoptosis (Fig. 6C). These results suggest that the inhibitory effects of Wnt-7a on apoptosis are exerted through regulation of apoptotic signaling molecules such as PKCα or ζ.

β-Catenin was investigated for a possible role in the inhibition of apoptosis by Wnt-7a. NO production caused a decrease in levels of β-catenin protein. These effects of NO were abolished by the addition of Wnt-7a (Fig. 7A). These NO-induced decreases were also blocked by direct inhibition of caspase-3 activity (Fig. 7B, upper panel). Indirect inhibition of caspase-3 activity via blocking p38 kinase activation with SB203580 or overexpression of PKCα or ζ (16–18) also blocked proteolysis of β-catenin (Fig. 7B, lower panel). The above results indicate that inhibition of proteolytic degradation of β-catenin by Wnt-7a is the result of caspase-3 activity inhibition, which in turn is due to blocking apoptotic signaling from molecules such as p38 kinase, PKCα and ζ, NF-κB, and p53. To further elucidate the role of β-catenin in apoptosis inhibition by Wnt-7a, S37A β-catenin was overexpressed in chondrocytes prior to NO production. Ectopic expression of S37A β-catenin dramatically increased β-catenin activity. This activity was decreased by NO but still remained significantly high (Fig. 8A). However, over-
expression of S37A β-catenin did not affect NO-induced apoptosis (Fig. 8B) nor apoptotic events such as activation of p38 kinase, inhibition of PKCa and ζ, degradation of I-xB, and accumulation of p53 (Fig. 8C). The noninvolvement of β-catenin in Wnt regulation of apoptosis was further investigated by inhibiting NO-induced degradation of β-catenin with the proteasome inhibitor MG132. Treatment with MG132 caused accumulation of β-catenin (Fig. 9A) and stimulated transcriptional activity (Fig. 9B). However, NO-induced apoptosis was not blocked under these conditions (Fig. 9B). Furthermore, stimulation and inhibition of β-catenin-Tcf/Lef complex transcriptional activity by overexpression of Lef-1 and dominant negative Tcf-4, respectively, did not affect NO-induced apoptosis (Fig. 9C). Taken together, these results strongly indicate that β-catenin is not involved in inhibition of NO-induced apoptosis by Wnt-7a.

Wnt-7a Inhibits NO-induced Apoptosis by Activating Cell Survival Signaling—We investigated whether Wnt proteins inhibited apoptosis by stimulating cell survival signals such as PI 3-kinase and Akt activity, based on our recent finding that inhibition of the PI 3-kinase/Akt pathway is necessary for NO-induced apoptosis (20). An in vitro kinase assay confirmed that NO inhibited PI 3-kinase activity (Fig. 10A, upper panel) and downstream Akt activity (as determined by examining phosphorylation status) (Fig. 10A, lower panel). Treatment with Wnt-7a in the absence of NO transiently increased Akt phosphorylation (Fig. 10C, upper panel) and blocked NO-induced down-regulation of Akt (Fig. 10C, lower panel) and PI 3-kinase activity (Fig. 10B). The possibility that Wnt-7a inhibits NO-induced apoptosis by modulating the PI 3-kinase/Akt signaling pathway was examined by blocking PI 3-kinase activity with the specific inhibitor LY294002. The blocking of NO-induced apoptosis by Wnt-7a was abolished by LY294002 (Fig. 11A). LY294002 additionally abolished Wnt-7a-induced modulation of apoptotic signals, such as the inhibition of p38 kinase activation, blocking of PKCa and ζ inhibition, suppression of I-xB degradation, and p53 accumulation (Fig. 11B). Also, LY294002 enhanced NO-induced apoptosis (Fig. 11C). The abolishment of Wnt-induced inhibition of apoptosis by PI 3-kinase inhibitor LY294002 did not cause re-expression of type II collagen (Fig. 11A). Furthermore, NO-induced inhibition of type II collagen expression was not altered during potentiation of NO-induced apoptosis by the inhibition of PI 3-kinase (Fig. 11C), suggesting that the effect of PI 3-kinase to
modulate apoptosis is not related to the expression of type II collagen. Taken together, these results clearly demonstrate that Wnt-7a inhibits NO-induced apoptosis in articular chondrocytes by stimulating cell survival signals such as PI 3-kinase and Akt, independent of β-catenin function.

**DISCUSSION**

Wnt proteins play major roles in chondrogenesis, limb development, and skeletal pattern determination. Wnt-3a is expressed in the apical ectodermal ridge and regulates outgrowth of the limb bud, whereas Wnt-7a is expressed in the dorsal ectoderm and regulates the specification of the dorso-ventral axis in the developing limb (4, 6, 32). Moreover, Wnt-7a inhibits chondrogenesis of mesenchymal cells (3, 8, 9). Although regulation of chondrogenesis and cartilage development by Wnt signaling is well established, the function of these proteins in the maintenance and destruction of cartilage remains largely unknown. We found in this study that IL-1β, the primary pro-inflammatory cytokine involved in cartilage destruction (12, 13), induced expression of Wnt-5a and -7a. Although it is not clear whether Wnt-5a and -7a expression by IL-1β is causative of IL-1β-induced dedifferentiation, strong co-relationships were observed between the kinetics of Wnt expression, accumulation of β-catenin, and cessation of type II collagen expression (Fig. 1C). Based on the result that Wnt-7a induces dedifferentiation of primary culture chondrocytes (i.e. suppression of type II collagen expression and induction of type I collagen expression), our study strongly suggests that IL-1β-induced expression of Wnt proteins contributes to the loss of a differentiated phenotype of chondrocytes. We also found the increased levels of β-catenin, a downstream molecule of Wnt signaling, in chondrocytes of arthritic cartilage. Although it remains to be determined whether the increase in β-catenin level in arthritic chondrocytes is due to the expression of Wnt-5a and -7a, in vitro observation in that Wnt-7a causes accumulation of β-catenin strongly suggests a role of Wnt proteins in β-catenin accumulation in arthritic chondrocyte.

We demonstrate that Wnt-7a causes the inhibition of type II collagen expression and the onset of type I collagen expression, which are typical markers of chondrocyte dedifferentiation. Recent literature (33) suggests that stimulation of β-catenin signaling accelerates the expression of type X collagen and maturation of chondrocyte, which is often associated with a concomitant decrease in type II collagen expression. This suggests a possibility that Wnt signaling accelerates chondrocyte maturation rather than causing dedifferentiation. However, Wnt-7a did not stimulate type X collagen expression in our experiments (Fig. 2C), indicating that Wnt signaling suppresses type II collagen expression via dedifferentiation rather than via maturation of chondrocytes. We additionally demonstrate that Wnt-7a causes suppression of type II collagen expression by stimulating the transcriptional activity of β-catenin-Tcf/Lef complex and inhibits NO-induced apoptosis by activating cell survival signaling independently of β-catenin. With the demonstration that Wnt-7a inhibits chondrogenesis by stabilizing cell-cell adhesion via sustained expression of N-cadherin and β-catenin (3, 9), our current results indicate that Wnt-7a regulates differentiation, dedifferentiation, and apoptosis of articular chondrocytes via different mechanisms. During chondrocyte dedifferentiation, Wnt-7a induced an increase in transcriptional activity of β-catenin-Tcf/Lef, which was sufficient to cause suppression of type II collagen expression. This finding was clearly confirmed by the observations that stimulation of transcriptional activity by Lef-1 virus
infection in the absence of Wnt caused suppression of type II collagen expression, whereas suppression of transcriptional activity by dominant negative Tcf-4 abolished Wnt-induced dedifferentiation. Furthermore, suppression of type II collagen expression caused by ectopic expression of S37A β-catenin was completely blocked by dominant negative Tcf-4. Thus, our results clearly indicate that the mechanisms underlying Wnt-induced dedifferentiation are distinct from those of differentiation and depend on the transcriptional activity of β-catenin. This conclusion is in agreement with the report by Bergwitz et al. (34) that

Fig. 9. Wnt-7a inhibits NO-induced apoptosis independently of β-catenin signaling. A and B, chondrocytes were left untreated (control) or treated for 18 h with 5 μM of MG132 with or without 1.5 mM SNP, followed by determination of β-catenin and ERK expression levels (A) and cell viability and transcriptional activity of β-catenin (B). C, chondrocytes were transfected with dominant negative Tcf-4 (left panel) or infected with Lef-1 adenovirus (right panel). Following incubation for 24 h, the cells were treated with 1.5 mM SNP in the absence or presence of Wnt-7a conditioned medium for additional 18 h. Cell viability and transcriptional activity (TOPFLASH) were determined. The data are presented as the mean values with standard deviations or as the results of a typical experiment from at least five independent experiments.

Fig. 10. Wnt-7a blocks NO-induced down-regulation of PI 3-kinase/Akt pathway. A, chondrocytes were treated for the indicated periods with 1.5 mM SNP. PI 3-kinase activity was determined by an in vitro kinase assay (upper panel). The levels of total and phosphorylated Akt (pAkt) were determined by Western blotting (lower panel). B, chondrocytes were left untreated (−) or treated (+) for 18 h with 1.5 mM SNP in the presence of indicated volume (μl) of Wnt-7a conditioned medium, and PI 3-kinase activity was determined by in vitro kinase assay. C, chondrocytes were treated with 100 μl of Wnt-7a conditioned medium for the indicated time period (upper panel). Alternatively, chondrocytes were left untreated (−) or treated (+) for 18 h with 1.5 mM SNP in the presence of indicated volume (μl) of Wnt-7a conditioned medium (lower panel). Levels of total and phosphorylated Akt (pAkt) were determined by Western blotting. ERK-1 and -2 were detected as loading controls. The data are presented as the results of a typical experiment from at least five independent experiments.

Fig. 11. Wnt-7a blocks NO-induced apoptosis via PI 3-kinase/Akt pathway. Chondrocytes were left untreated (−) or treated (+) for 18 h with 1.5 mM SNP in the absence or presence of 100 μl of Wnt-7a conditioned medium and the indicated concentrations (μM) of LY204002 (LY). Apoptotic cells were quantified by TUNEL assay. The levels of the indicated proteins were determined by Western blotting. ERK was employed as loading controls. The data are presented as the mean values with standard deviations or as the results of a typical experiment from at least five independent experiments.
several Wnt proteins (including Wnt-1, -3a, -4, -7a, and -7b) suppress type II collagen reporter gene expression in NIH3T3 cells. Currently, it is not known how Wnt-7a and Lef-1 cause suppression of type II collagen expression. Lef-1 is a nuclear effector of the canonical Wnt signaling pathway, which binds the consensus DNA sequence (C/T)CCTGGAA and alters binding of other transcription factors to neighboring sites (35, 36). Lef-1 transcriptional activity is regulated by interactions with transcriptional co-activators and co-repressors. As a co-activator of Lef-1, β-catenin displaces co-repressors from Lef-1 and recruits other co-activators to induce gene transcription. The β-catenin/Lef-1 pathway also directly represses gene transcription in a manner depending upon Wnt, although the molecular mechanisms have not been defined yet (37, 38). Because human and mouse Col2a1 promoters contain sequences of TTTCCTGAA and CCTCTGGA, respectively, which are similar to the Lef-1-binding consensus sequence ((C/T)CCTGGAA), it is of interest to determine whether this sequence is involved in the suppression of type II collagen expression by Wnt-7a and Lef-1.

We additionally showed that Wnt-7a inhibits NO-induced apoptosis by activating survival signals (including PI 3-kinase and Akt activity) independently of β-catenin. Although it is proposed that Wnt may act as a pro-apoptotic signal (39), most reports suggest that Wnt proteins function as anti-apoptotic signals. For instance, Wnt-1 inhibits apoptosis in various cell types by activating the β-catenin-Tcf/Lef complex (25, 26, 40, 41). However, our result showing that ectopic expression of β37A β-catenin did not affect NO-induced apoptosis suggests inhibition of apoptosis by Wnt-7a is not mediated by β-catenin signaling. It is unlikely this result is due to low transfection efficiency given that accumulation of β-catenin following inhibition of proteolysis and ectopic expression of Wnt-3a and Tcf/Lef activity independently of β-catenin is of interest to determine whether this sequence is involved in the suppression of type II collagen expression by Wnt-7a and Lef-1.

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