Opposing Roles of WNT-5A and WNT-11 in Interleukin-1β Regulation of Type II Collagen Expression in Articular Chondrocytes

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Je-Hwang Ryu and Jang-Soo Chun
From the Department of Life Science, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea

Interleukin (IL)-1β is a major catabolic cytokine that plays a pivotal role in cartilage destruction. This study examined the possible involvement and regulatory mechanisms of Wnt signaling in IL-1β-induced inhibition of type II collagen expression in chondrocytes. Treatment of chondrocytes with IL-1β up-regulated Wnt-5a and down-regulated Wnt-11 expression. Conditioned medium from Wnt-5a-expressing cells inhibited type II collagen expression, whereas knockdown of Wnt-5a by siRNA blocked the inhibitory effects of IL-1β on type II collagen expression. In contrast to the inhibitory effects of Wnt-5a, Wnt-11 stimulated type II collagen expression. Wnt-5a and Wnt-11 did not cause accumulation of β-catenin or activation of the β-catenin-T-cell/lymphoid enhancer (Tcf/Lef) transcriptional complex. Instead, we found that Wnt-5a activated c-Jun N-terminal kinase and that an inhibitor of this kinase blocked Wnt-5a inhibition of type II collagen expression. In contrast, Wnt-11 activated protein kinase C and an inhibitor of this kinase blocked Wnt-11 stimulation of type II collagen expression. Collectively, these results indicate that Wnt-5a and Wnt-11 signaling through distinct non-canonical Wnt pathways have opposing effects on type II collagen expression by chondrocytes.

To maintain cartilage homeostasis, articular chondrocytes synthesize cartilage-specific extracellular matrix molecules, including type II collagen and sulfated proteoglycans (1). This homeostasis is lost in cartilage diseases such as rheumatoid arthritis and osteoarthritis, eventually leading to cartilage destruction. Cartilage destruction involves a loss of differentiated chondrocyte phenotypes (i.e. dedifferentiation), which is characterized by the cessation of type II collagen expression, and onset of fibroblastic type I and type III collagen expression (2). A variety of soluble factors are known to cause chondrocyte dedifferentiation. One of the best studied is interleukin (IL)-1β, which plays a key role in cartilage destruction (3). We have also shown that Wnt-7a, which is up-regulated by IL-1β, causes dedifferentiation of chondrocytes (4).

Wnt is a family of secreted glycoproteins that can be separated into two classes: the Wnt-1 class (e.g. Wnt-1, -3a, -7a, and -8), which activates the canonical Wnt/β-catenin pathway; and the Wnt-5a class (e.g. Wnt-4, -5a, and -11), which activates the noncanonical Wnt pathway (5). The canonical Wnt pathway involves stabilization of cytoplasmic β-catenin and its translocation into the nucleus, where it acts as a transcriptional coactivator. Noncanonical Wnt pathways are independent of β-catenin signaling and involve activation of protein kinase C (PKC), calmodulin-dependent kinase II, and c-Jun N-terminal kinase (JNK). Several Wnt isoforms are known to regulate chondrogenesis and cartilage development. For instance, forced expression of Wnt-1, -7a, or -14 inhibits chondrocyte differentiation within mesenchymal condensations (6–8). In addition, Wnt-5a and Wnt-5b are reported to promote chondrocyte differentiation and to inhibit chondrocyte maturation (9). In contrast, Wnt-4 and Wnt-8 have been shown to accelerate chondrocyte hypertrophy (10, 11).

In addition to the regulation of chondrogenesis and limb development, Wnt proteins appear to regulate the maintenance of differentiated chondrocyte phenotypes. This possibility is indirectly supported by the observation that several Wnt proteins and frizzled receptors (Fz) are expressed in the synovial tissue of arthritic cartilage (12). More direct evidence was provided by our previous observations that Wnt-3a and Wnt-7a inhibit type II collagen expression in articular chondrocytes via the canonical pathway (4, 13); however, the Wnt isoforms regulated by proinflammatory cytokines in chondrocytes and the role of the regulated Wnt isoforms in chondrocyte dedifferentiation have not been identified. Therefore, in the present study, we identified Wnt isoforms regulated by IL-1β in chondrocytes and examined the role of the identified Wnt isoforms in chondrocyte-specific type II collagen expression. We report here that Wnt-5a and Wnt-11 have opposite effects on type II collagen expression via distinct noncanonical Wnt pathways.

EXPERIMENTAL PROCEDURES

Articular Chondrocytes and Cartilage Explant Cultures—Rabbit articular chondrocytes were isolated from the cartilage...
of 2-week-old or 8-month-old New Zealand White rabbits as described previously (14). Cartilage slices were dissociated enzymatically for 4 h in 0.2% collagenase type II (381 units/mg solid; Sigma) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen). Individual cells were suspended in DMEM supplemented with 10% (v/v) fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin and plated on culture dishes at a density of 22040 cells/cm². After 3 days in culture, the cells were treated with IL-1 or Wnt-containing conditioned medium and transfected with a reporter gene or siRNA in the presence of 10% (v/v) serum. Type II collagen expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. For cartilage explant cultures, rabbit joint cartilage was dissociated using Metafectene (Biontex Laboratories GmbH, Munich, Germany) and stable cell lines were established by selection with G418 (0.75 mg/ml). Expression of Wnt-5a and Wnt-11 in stable cell lines was confirmed by RT-PCR analysis. To collect conditioned medium, Wnt-5a-producing L cells or Wnt-11-producing NIH3T3 cells were maintained in serum-free DMEM for 36 h. Conditioned medium was clarified by centrifugation at 10,000 × g for 5 min and concentrated 20-fold by ultrafiltration in Amicon-stirred cells (Millipore, Billerica, MA) using a YM membrane with a 10-kDa molecular mass cut-off as described below. The epitope for the antibody is localized in the triple helix of type II collagen and the antibody recognizes α1(II) chain of collagen.

**Preparation of Conditioned Medium Containing Wnt-5a or Wnt-11—**Wnt-5a-producing L cells were obtained from the American Type Culture Collection. Rabbit Wnt-11 was cloned from chondrocytes (GenBank™ accession number DQ388601), and a mammalian Wnt-11 expression vector was constructed in pcDNA3.0 (Invitrogen). NIH3T3 cells were transfected with the Wnt-11 expression vector or empty vector using Metafectene (Biontex Laboratories GmbH, Munich, Germany), and stable cell lines were established by selection with G418 (0.75 mg/ml). Expression of Wnt-5a and Wnt-11 in stable cell lines was confirmed by RT-PCR analysis. To collect conditioned medium, Wnt-5a-producing L cells or Wnt-11-producing NIH3T3 cells were maintained in serum-free DMEM for 36 h. Conditioned medium was clarified by centrifugation at 10,000 × g for 5 min and concentrated 20-fold by ultrafiltration in Amicon-stirred cells (Millipore, Billerica, MA) using a YM membrane with a 10-kDa molecular mass cut-off as described below.

**Table 1**

| Gene       | Primer sequencea | Expected size | A_{T}b | Homologyc |
|------------|------------------|---------------|--------|-----------|
| Col2a1     | Sense 5'-gac ccc atg cag tac atg cag-3' | 370            | 62     |           |
|            | Antisense 5'-agt cgg cat tga tgg tct cc-3' | 299            | 62     |           |
| GAPDH      | Sense 5'-tca cca tct tcc agg cag ga-3' | 452            | 51     | 94        |
| Wnt-1      | Sense 5'-cgt cta ctt cga gaa atc gcc caa ctt c-3' | 459           | 60     | 94        |
| Wnt-2      | Sense 5'-tca cag aca tct ggc gag tac tgc ggg c-3' | 574           | 60     | 92        |
| Wnt-3a     | Sense 5'-ttc tgc agg aac tac tgt gag atc atg c-3' | 463           | 52     | 89        |
| Wnt-4      | Sense 5'-aga agc ctc atg aac ctc cgc aac aat g-3' | 476           | 56     | 91        |
| Wnt-5a     | Sense 5'-agc ctt gtt cct agt aag gaa act-3' | 452           | 51     | 94        |
| Wnt-5b     | Sense 5'-aca gac gcc aac tcc tgg cgg tca tta g-3' | 361           | 60     | 94        |
| Wnt-7a     | Sense 5'-agc ctc cta ctc tga tcc tcc gcc aag gtt c-3' | 463           | 52     | 89        |
| Wnt-10b    | Sense 5'-agt ttc ctc ggg att tct tgg att cct c-3' | 463           | 52     | 89        |
| Wnt-11     | Sense 5'-tgc aga ctc tgg tgg cgg att atg taa g-3' | 476           | 56     | 91        |
| Wnt-13     | Sense 5'-agc cag gaa gtt cat gct cag g-3' | 452           | 51     | 94        |
| Wnt-14     | Sense 5'-gcg ctc gtt gtt cct cct cc-3' | 452           | 51     | 94        |
| Fx2        | Sense 5'-cgg ctc ctt cgg gcg tc-3' | 463           | 52     | 90        |
| Fx3        | Sense 5'-cat acc acg cgc ttt tgc ggt-3' | 452           | 51     | 94        |
| Fx4        | Sense 5'-ttg tgt atg acc aag gcg gc-3' | 319           | 52     | 96        |
| Fx5        | Sense 5'-cac cgg caa tct cga cgg tct cga cag tct aggc-3' | 574           | 60     | 90        |
| Fx7        | Sense 5'-agt gtc tct cta aac cta tgg tag c-3' | 459           | 60     | 96        |
| Fx8        | Sense 5'-act ctc cta cgc aag gta ggt gca ggc-3' | 459           | 60     | 96        |

## Notes

a Primers for Col2a1 and GAPDH are designed from known rabbit sequences. Primer sequences for tested Wnt and Fz are based on human sequences.

b A_{T}, annealing temperature.

c PCR products of rabbit Wnt and Fz were sequenced and compared with corresponding human sequences.

d Blank space, Wnt and Fz isoforms are not detected in rabbit chondrocytes by various PCR conditions.
previously (4). Currently, there are no appropriate methods to determine the exact activity of the Wnt proteins in the conditioned medium. However, the activity of Wnt proteins in conditioned medium was ascertained by measuring transcriptional activity of β-catenin using TOPFlash assay and by determining activation of noncanonical pathways. In addition, control conditioned medium was obtained from a stable cell line transfected with empty vector and used as a control for Wnt-conditioned medium.

**Reporter Gene Assay**—β-Catenin-Tcf/Lef transcriptional activity was determined by a reporter gene assay as described previously (15). Briefly, chondrocytes were transfected with 1 μg of reporter gene (TOPFLASH; optimal Lef-binding site) or control gene (FOPFLASH; mutated Lef-binding site) (Upstate Biotechnology, Lake Placid, NY) and 1 μg of pCMV-β-galactosidase using Metafectene. Following culture for 24 h, the cells were treated with Wnt-5a- or Wnt-11-conditioned medium for an additional 24 h. Luciferase activity was measured and normalized for transfection efficiency as measured by β-galactosidase activity.

**RT-PCR Analysis and Quantitative Real-time PCR (qRT-PCR)**—Total RNA was isolated using RNA STAT-60 (Tel-Test, Woodlands, TX) and reverse-transcribed with ImProm-II™ reverse transcriptase (Promega, Madison, WI). The generated cDNA was amplified by PCR with Taq polymerase (TakaRa Bio, Shiga, Japan). PCR primers and conditions are summarized in Table 1, and qRT-PCR was performed using a chromo 4 cycler (Bio-Rad) and SYBR Premix Ex Taq™ (TakaRa Bio). All qRT-PCR reactions were performed in duplicate, and the amplification signal from the target gene was normalized by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal in the same reaction.

**Knockdown of Wnt-5a by siRNA**—Rabbit Wnt-5a was cloned from chondrocytes (GenBank™ accession number DQ388600), and the following three siRNA sequences were selected using siRNA Wizard™ (InvivoGen, San Diego, CA): 5’-GAA GAA ACT GTG CCA CTT GTA-3’ (no. 1), 5’-GCG AAG ACC GGC ATC AAA GAA-3’ (no. 2), and 5’-GAG CGC GGC CAT CCT GAA-3’ (no. 3). Complementary oligonucleotides for siRNAs including restriction enzyme sites, 7-mer hairpin loops (TCAAGAG), 21-mer nucleotide sense, and 21-mer nucleotide antisense were designed according to the manufacturer’s specifications (InvivoGen). Annealing of sense and antisense oligonucleotides was performed in a solution containing 0.15 M NaCl by heating at 80 °C for 2 min and then cooling to 35 °C. Annealed siRNAs were ligated into Acc65I/HindIII-digested psiRNA-hH1GFP:zeo vector (InvivoGen). Cells were transfected with the siRNAs using Metafectene. Two of the tested siRNA (no. 2 and no. 3) caused effective knockdown of Wnt-5a.

**Immunohistochemistry**—Rabbit joint cartilage explants were fixed for 24 h at 4 °C in phosphate-buffered saline containing 4% paraformaldehyde, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and cut into 4-μm sections as described previously (15). Dewaxed paraffin sections were stained by standard procedures using an antibody against type II collagen (Chemicon International) and visualized with a
In Situ Hybridization—Digoxigenin-conjugated riboprobes for type II collagen, Wnt-5a, and Wnt-11 were synthesized using digoxigenin RNA labeling mix (Roche Diagnostics Corp., Indianapolis, IN). Briefly, the cDNA fragment of rabbit type II collagen was amplified by PCR using specific primers (sense, 5'-GGG TCT CCT GCC TCC TCC TGC TC-3'; antisense, 5'-CTC CAT CTC TGC CAC GGG GT-3'). The cDNA encoding rabbit Wnt-5a and Wnt-11 was inserted into the pcDNA3.0 vector. The full-length DNA plasmids were cut with PstI and SacI for Wnt-5a or with BamHI and SacI for Wnt-11. The products were cloned into the pSPT-18 vector (Roche Diagnostics Corp.), linearized with EcoRI or HindIII, and transcribed with SP6 or T7 RNA polymerase, respectively, to generate antisense and sense probes. For hybridization, dewaxed paraffin sections of cartilage explants were treated with 0.2 N HCl for 10 min and then permeabilized for 10 min at 37 °C with 20 μg/ml proteinase K. After acetylation for 10 min with 0.25% acetic anhydride, the sections were incubated with hybridization buffer (40% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 4× SSC, 10 μM dithiothreitol, 1 mg/ml yeast tRNA, and 1 mg/ml salmon sperm DNA) containing denatured sense or antisense digoxigenin-labeled riboprobes. The sections were treated for 30 min at 37 °C with 10 μg/ml RNase A and processed using an anti-digoxigenin detection assay kit (Roche Diagnostics Corp.). Hybridization signals were visualized with a solution of 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-iodo-phosphate (Roche Diagnostics Corp.).

Western Blot Analysis—Whole cell lysate were prepared as described previously (14). Proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The nitrocellulose sheet was blocked with 5% nonfat dry milk in Tris-buffered saline. The following antibodies were used to detect proteins: anti-type II collagen (Chemicon International), anti-β-catenin (BD Biosciences, San Jose, CA), anti-extracellular signal-regulated protein kinase (ERK)-1 (BD Biosciences), anti-pan-phospho-PKC (Cell Signaling Technology, Beverly, MA), and anti-phospho-JNK (New England Biolabs, Beverly, MA). The blots were developed using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection.

**RESULTS**

**IL-1β Up-regulates Wnt-5a and Down-regulates Wnt-11 in Chondrocytes**—We have previously shown that IL-1β up-regulates Wnt-7a, which causes inhibition of type II collagen expression in chondrocytes (4). In this study, we extended our study to the various isoforms of Wnt (Fig. 1A) and Fz (Fig. 1B) in primary cultures chondrocytes obtained from joint articular cartilage of 2-week-old rabbits or 8-month-old rabbits. We examined 11 Wnt isoforms and 9 Fz isoforms, and we detected 5 of each. As shown in Fig. 1, IL-1β up-regulated Wnt-5a and Wnt-7a and down-regulated Wnt-11. IL-1β also down-regulated the expression of Fz-1 and Fz-6. Because the functions of up-regulated Wnt-7a have already been reported (4), we selected Wnt-5a and Wnt-11 for functional characterization in chondrocytes. Consistent with the inhibitory effects on type II collagen expression in the primary chondrocytes cultures (Fig. 1), IL-1β suppressed type II collagen expression as determined by in situ hybridization and proteoglycan synthesis as demonstrated by Alcian blue staining in cartilage explants (Fig. 2). IL-1β also extensively removed type II collagen in explants as determined by immunohistochemistry. This might be due to not only by the inhibition of type II collagen expression but also induction and activation of matrix metalloproteinases (16).

**In Situ Hybridization analysis also indicated that IL-1β causes up-regulation of Wnt-5a and down-regulation of Wnt-11 in cartilage explants cultures (Fig. 2).** We further characterized the effects of IL-1β on expression of type II collagen, Wnt-5a and Wnt-11 in primary cultures of chondrocytes obtained from articular cartilage of 2-week-old rabbits. As shown in Fig. 3A, IL-1β down-regulated type II collagen expression as demonstrated by Western blotting and RT-PCR. IL-1β also up-regulated Wnt-5a and down-regulated Wnt-11 in a time-dependent manner. We next performed qRT-PCR analysis to compare the expression pattern of Wnt proteins and type II collagen (Fig. 3B). We found that Wnt-5a
expression was enhanced and Wnt-11 was reduced after 6 h of stimulation with IL-1β. Thus, qRT-PCR indicated that there is a strong correlation between type II collagen expression and the both down-regulation of Wnt-11 and the up-regulation of Wnt-5a.

Wnt-5a Inhibits Type II Collagen Expression in Chondrocytes—The above results suggested that Wnt-5a and Wnt-11 are involved in dedifferentiation of chondrocytes. Therefore, we first examined the role of Wnt-5a in type II collagen expression by using Wnt-5a conditioned medium prepared from L cells. As shown in Fig. 4, A and B, treatment of chondrocytes with Wnt-5a significantly reduced the expression of type II collagen in a dose- and time-dependent manner. Wnt-5a also enhanced the inhibitory effects of IL-1β on type II collagen expression (Fig. 4C). The role of Wnt-5a in the regulation of type II collagen expression was further characterized by knockdown of Wnt-5a using siRNAs. Among the three examined siRNAs, no. 2 and no. 3 effectively inhibited the up-regulation of Wnt-5a by IL-1β and resulted in a concomitant recovery of type II collagen expression (Fig. 5A). We therefore used siRNA no. 3 in subsequent experiments. Knockdown of Wnt-5a expression using the siRNA increased type II collagen expression in a dose-dependent manner (Fig. 5B). Treatment with an siRNA to block the up-regulation of Wnt-5a by IL-1β also abrogated the inhibitory effects of IL-1β on type II collagen expression (Fig. 5C). On the basis of these results, we hypothesized that Wnt-5a inhibits type II collagen expression, thereby mediating IL-1β-induced dedifferentiation of chondrocytes.

Wnt-11 Enhances Type II Collagen Expression in Chondrocytes—We next examined the role of Wnt-11, which is down-regulated by IL-1β, in type II collagen expression by using Wnt-11-containing conditioned medium prepared from NIH3T3 cells. In contrast to the inhibitory effects of Wnt-5a, type II collagen expression was significantly enhanced by exogenous Wnt-11 (Fig. 6A). This stimulation of type II collagen expression by Wnt-11 was dose-dependent (Fig. 6B). Wnt-11 treatment, however, did not affect the inhibition of type II collagen expression by IL-1β (Fig. 6C). The effects of Wnts on type II collagen mRNA expression were observed within 6 h of treatment with Wnt-5a (Fig. 6A) or Wnt-11 (Fig. 6A). However, because of long half-life of type II collagen mRNA (i.e. 15 h) (17), the effects of Wnts on type II collagen transcription are likely to be much earlier than this.

Wnt-5a and Wnt-11 Regulate Type II Collagen Expression by Distinct Noncanonical Wnt Pathways—We next investigated the mechanisms by which Wnt-5a and Wnt-11 regulate type II collagen expression. We first focused on the role of the canonical Wnt pathway by measuring the accumulation of β-catenin and the transcriptional activity of the β-catenin-Tcf/Lef complex. As shown in Fig. 7, treatment of chondrocytes with Wnt-5a or Wnt-11 conditioned medium did not cause the accumulation of β-catenin as determined by Western blotting or the activation of the β-catenin-Tcf/Lef transcrip-
noncanonical pathways in chondrocyte dedifferentiation. We demonstrated in this study that IL-1β, a major proinflammatory cytokine involved in cartilage destruction, up-regulates the expression of Wnt-5a and down-regulates the expression of Wnt-11. We also demonstrated that Wnt-5a inhibits type II collagen expression by activating the JNK pathway, whereas Wnt-11 enhances type II collagen expression by activating the PKC signaling pathway (see Fig. 9 for a summary of these pathways). Thus, our results suggest that Wnt-5a is associated with cartilage destruction, whereas Wnt-11 plays a role in the maintenance of cartilage homeostasis.

Several Wnt isoforms are expressed in the developing cartilage and bone, and they appear to have distinct roles. Among the various Wnt isoforms, Wnt-5a is known to regulate chondrogenesis, limb development, and longitudinal skeletal outgrowth (20–22). In addition, forced expression of Wnt-5a delays chondrocyte maturation by inhibiting type X collagen expression, which is a marker for hypertrophic chondrocytes (7). In the case of Wnt-11, its expression is restricted to the prehypertrophic chondrocytes of the cartilage elements, but it does not affect chondrogenic differentiation or hypertrophy of chondrocytes in chick micromass culture (9, 23); however, Wnt-11 stimulates chondrogenic differentiation of RCJ3.1 cells and synthesis of cartilage matrix (24). Although the functions of Wnt-5a and Wnt-11 in chondrogenesis and cartilage development have been studied extensively, the roles of these Wnt isoforms in the maintenance of differentiated chondrocyte phenotypes have not been elucidated. Thus, our results provide the first evidence that Wnt-5a and Wnt-11 directly participate in the regulation of differentiated chondrocyte phenotypes and cartilage homeostasis by regulating type II collagen expression.

IL-1β caused up-regulation of Wnt-5a in a biphasic pattern with the first peak at 6 h and the second peak at 36 h. We did not elucidate the mechanisms of biphasic regulation of Wnt-5a expression in this study. However, it is possible that at least two different signaling pathways modulated by IL-1β regulate Wnt-5a expression in chondrocytes. For instance, activation of an early signaling pathway may cause the first peak of Wnt-5a expression, whereas activation of a subsequent signaling pathway may cause the second peak. Nevertheless, our observations that Wnt-5a inhibits type II collagen expression and that knockdown of Wnt-5a by siRNA blocks the inhibitory effects of IL-1β on type II collagen expression clearly support the inhibitory role of Wnt-5a in type II collagen expression in articular chondrocytes. Because IL-1β causes up-regulation of Wnt-5a, it is very likely that up-regulation of Wnt-5a is necessary for IL-1β-induced inhibition of type II collagen expression. The inhibitory effects of Wnt-5a on type II collagen expression in chondrocytes are different from results recently obtained by our group in which it is shown that transforming growth factor-β3 stimulates Wnt-5a expression and ectopic expression of Wnt-5a stimulates chondrogenesis of chick wing bud mesenchymal cells (25). One possible reason for this discrepancy is the differences in intracellular signaling pathways activated by Wnt-5a. For instance, the stimulatory effects of Wnt-5a on chondrogenesis of mesenchymal cells are mediated by the activation of PKCα and p38 mitogen-activated protein kinase.

**DISCUSSION**

We therefore examined the role of the noncanonical Wnt pathway in the effects of Wnt-5a and Wnt-11 by focusing on the activation of PKC and JNK. We found that Wnt-5a transiently activated JNK, as demonstrated by its phosphorylation, but that it did not affect the phosphorylation of PKC. Pretreatment of cells with the JNK inhibitor SP600125 (18) but not the PKC inhibitor GF109203X (19) blocked the reduction of type II collagen expression (Fig. 8A). In contrast to the effects of Wnt-5a, Wnt-11 transiently activated PKC, as demonstrated by its phosphorylation, but it did activate JNK. Pretreatment with the PKC inhibitor GF109203X but not the JNK inhibitor GF109203X blocked the increase in type II collagen expression by Wnt-11 (Fig. 8B). These results indicate that Wnt-5a and Wnt-11 regulate type II collagen expression by distinct noncanonical Wnt pathways, namely by activating JNK and PKC, respectively.
whereas the inhibitory effects of Wnt-5a on type II collagen expression in chondrocytes are mediated by the activation of JNK without any effects on PKC activation. Indeed, Wnt-11 activated PKC\(|/H9251|\) in chondrocytes with a concomitant enhanced expression of type II collagen. In contrast to Wnt-5a, Wnt-11 is down-regulated by IL-1\(|/H9252|\) treatment, and exogenous Wnt-11 enhanced type II collagen expression. The stimulatory effects of Wnt-11 on cartilage matrix synthesis are in agreement with a report by Bergwitz et al. (24) that Wnt-11 increases type II collagen reporter gene activity in NIH3T3 cells. Interestingly, exogenous Wnt-11 did not affect the inhibition of type II collagen expression by IL-1\(|/H9252|\). Although we did not elucidate the molecular mechanisms, it is possible that exogenous Wnt-11 is

![Figure 7](image1.png)

**FIGURE 7.** The canonical Wnt pathway is not involved in the regulation of type II collagen expression by Wnt-5a and Wnt-11. Chondrocytes were treated with the indicated amount of control, Wnt-5a (A), or Wnt-11 (B) conditioned medium for 36 h. The transcriptional activity of \(\beta\)-catenin was determined using active (TOPFLASH) or inactive (FOPFLASH) \(\beta\)-catenin-Tcf/Lef reporter genes (upper panels). The levels of type II collagen and \(\beta\)-catenin expression were determined by Western blotting (lower panels). Cells were treated for 36 h with LiCl (10 mM) as a positive control for \(\beta\)-catenin signaling. The results are representative of five independent experiments.

![Figure 8](image2.png)

**FIGURE 8.** Wnt-5a and Wnt-11 regulate type II collagen expression via distinct noncanonical Wnt pathways. Chondrocytes were treated with 75 \(\mu\)l of Wnt-5a (A) or Wnt-11 (B) conditioned medium for the indicated amounts of time. Expression of type II collagen and phosphorylation of JNK and PKC were determined by Western blotting. Cells were treated for 30 min with IL-1\(|/H9252|\) as a positive control for JNK phosphorylation. Prior to treatment with Wnt-5a- or Wnt-11-containing conditioned medium, chondrocytes were treated for 30 min with the indicated concentrations of SP600125 or GF109203X to inhibit JNK or PKC, respectively. Expression of type II collagen and ERK was detected by Western blotting. The results are representative of at least five independent experiments.

![Figure 9](image3.png)

**FIGURE 9.** Schematic diagram summarizing the role of Wnt proteins in IL-1\(|/H9252|\)-induced dedifferentiation of articular chondrocytes. IL-1\(|/H9252|\) up-regulates Wnt-7a and Wnt-5a and down-regulates Wnt-11 in articular chondrocytes. Wnt-7a suppresses type II collagen expression via the \(\beta\)-catenin-Tcf/Lef pathway. Wnt-5a inhibits type II collagen expression by activating the JNK pathway, whereas Wnt-11 enhances type II collagen expression by activating the PKC pathway.
unable to override the inhibitory effects of other factors on type II collagen expression such as Wnt-5a and nitric oxide, which are produced by IL-1β. However, because Wnt-11 enhances type II collagen expression, down-regulation of Wnt-11 by IL-1β appears to provide a condition for the inhibition of type II collagen expression by IL-1β.

In addition to Wnt-5a and Wnt-11, we have previously shown that Wnt-7a suppresses type II collagen expression via the canonical Wnt pathway as described in Fig. 9 (4). In this study, we demonstrated that the canonical Wnt pathway does not mediate the inhibitory and stimulatory effects of Wnt-5a and Wnt-11, respectively, on type II collagen expression. This is consistent with the general classification of Wnt-5a and Wnt-11 as noncanonical Wnt family members (5). We further demonstrated that the JNK pathway mediates the action of Wnt-5a, whereas a PKC pathway mediates Wnt-11 action on type II collagen expression. Although Wnt-5a has been shown to activate both PKC (26) and JNK (27) pathways, our results indicate that the JNK pathway mediates Wnt-5a action in chondrocytes. Indeed, we previously showed that activation of JNK mediates IL-1β-induced suppression of type II collagen expression (28), supporting a negative role of the JNK pathway in cartilage matrix synthesis. Thus, the current observations of JNK activation by Wnt-5a and inhibition of Wnt-5a action by a JNK inhibitor are consistent with our previous results.

The stimulatory effect of Wnt-11 on type II collagen expression is mediated by PKC phosphorylation, as demonstrated by the observations that Wnt-11 stimulates the phosphorylation of PKC and that inhibition of PKC blocks Wnt-11 action. In this study, as a measure of PKC activation, we assessed the level of PKC phosphorylation using a pan-phospho-PKC antibody (29, 30). Among the PKC isoforms known to be expressed in chondrocytes (α, ε, λ, and ζ) (15), the molecular weight of the band detected with the phospho-PKC antibody matched that of PKCα (data not shown). We have previously shown that the increased expression and activity of PKCα is necessary for chondrogenesis of mesenchymal cells (31) and that down-regulation and inhibition of PKCα in differentiated chondrocytes results in dedifferentiation (15). This agrees with our current observations that Wnt-11 activated PKCα and enhanced type II collagen expression. The activation of PKC by Wnt-11 also is consistent with the findings of Koyanagi et al. (30).

In addition to the regulation of Wnt isoforms, we also found that Fz-1 and Fz-6, which function as receptors for Wnt isoforms, are down-regulated by IL-1β in chondrocytes. We did not determine the significance of Fz down-regulation in this study. It has been shown that Fz receptors activate both canonical and noncanonical Wnt pathways depending on the types of Fz isoforms (32, 33). In addition, some Wnt proteins interact with multiple Fz isoforms which make it difficult to determine specificity of the Wnt-Fz interactions. For instance, Fz-1 interacts with Wnt-3a and Wnt-5a to activate canonical Wnt pathway (34), whereas it also acts as an antagonist of the canonical Wnt pathway (35). In addition, Fz-6 is known to repress Wnt-3a-induced canonical pathway (36). Because IL-1β up-regulates Wnt-7a, which activates canonical pathway, and Wnt-5a, which activates noncanonical pathway, it is likely that down-regulation of Fz-1 and Fz-6 by IL-1β might affect both canonical and noncanonical Wnt pathways in articular chondrocytes.

Because IL-1β modulates a variety of signaling pathways, it is difficult to assume the degree of contribution in the regulation of type II collagen expression by IL-1β-modulated Wnt expression as depicted in Fig. 9. Various signaling pathways including Wnt pathways may regulate type II collagen expression either independently or additively. For instance, although our observation that knockdown of Wnt-5a abrogates the inhibitory effects of IL-1β on type II collagen expression indicates a critical role of Wnt-5a in IL-1β action, it does not rule out the possibility that other signaling pathways are involved in mediating IL-1β action. Indeed, we recently found that IL-1β-induced activation of cyclin-dependent kinase 6 is required for the inhibitory role of IL-1β in type II collagen expression and also found that cyclin-dependent kinase 6 activation is independent to the modulation of Wnt signaling. Therefore, it is likely that up-regulated Wnt-5a participates as a critical component of IL-1β action, whereas down-regulated Wnt-11 acts as an additive secondary component based on the observation that exogenous Wnt-11 enhances type II collagen expression but does not affect the inhibitory role of IL-1β in type II collagen expression.

In summary, we have demonstrated here that Wnt-5a and Wnt-11 have opposing effects on chondrocyte-specific type II collagen expression via distinct noncanonical Wnt pathways involving JNK and PKC, respectively. Thus, our results provide strong evidence that Wnt signaling is involved not only in cartilage development but also in the maintenance of cartilage homeostasis.

REFERENCES
1. Delise, A. M., Fisher, L., and Tuan, R. S. (2000) Osteoarthr. Cartil. 8, 309–334
2. Sandell, L. J., and Aigner, T. (2001) Arthritis Res. 3, 107–113
3. Goldring, M. B., Fukudo, K., Birkhead, J. R., Dudek, E., and Sandell, L. J. (1994) J. Cell Biochem. 54, 85–99
4. Hwang, S.-G., Ryu, J.-H., Kim, I.-C., Jho, E. H., Jung, H.-C., Kim, K., Kim, S.-I., and Chun, I.-S. (2004) J. Biol. Chem. 279, 26597–26604
5. Church, V. L., and Francis-West, P. (2002) Int. J. Dev. Biol. 46, 927–936
6. Rudnicki, J. A., and Brown, A. M. (1997) Dev. Biol. 185, 104–118
7. Tufan, A. C., and Tuan, R. S. (2001) FASEB J. 15, 1436–1438
8. Hartmann, C., and Tabin, C. J. (2001) Cell 104, 341–351
9. Church, V., Nohno, T., Linker, C., Marcelle, C., and Francis-West, P. (2002) J. Cell Sci. 115, 4809–4818
10. Hartmann, C., and Tabin, C. J. (2000) Development 127, 3141–3159
11. Enomoto-Iwamoto, M., Kitagaki, J., Koyama, E., Tamamura, Y., Wu, C., Kanatani, N., Koike, T., Okada, H., Komori, T., Yoneda, T., Church, V., Francis-West, P. H., Kurisu, K., Nohno, T., Pacifici, M., and Iwamoto, M. (2002) Dev. Biol. 251, 142–156
12. Sen, M., Lauterbach, K., El-Gabalawy, H., Firestein, G. S., Corr, M., and Carson, D. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2791–2796
13. Hwang, S.-G., Yu, S.-S., Lee, S.-W., and Chun, J.-S. (2005) FEBS Lett. 579, 4837–4842
14. Yoon, Y.-M., Kim, S.-J., Oh, C.-D., Ju, I.-W., Song, W.-K., Yoo, Y.-J., Huh, T.-L., and Chun, J.-S. (2002) J. Biol. Chem. 279, 8412–8420
15. Ryu, J.-H., Kim, S.-J., Kim, S.-H., Oh, C.-D., Hwang, S.-G., Chun, C.-H., Oh, S.-H., Seong, J.-K., Huh, T.-L., and Chun, J.-S. (2002) Development 129, 5541–5550
16. Kozaci, L. D., Buttle, D. J., and Hollander, A. P. (1997) Arthritis Rheum. 40,
17. Askew, G. R., Wang, S., and Lukens, L. N. (1991) *J. Biol. Chem.* 266, 16834–16841.
18. Bennett, B. L., Sasaki, D. T., Murray, B. W., O’Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Manning, A. M., and Anderson, D. W. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 13681–13686.
19. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F. (1991) *J. Biol. Chem.* 266, 15771–15781.
20. Dealy, C. N., Roth, A., Ferrari, D., Brown, A. M., and Kosher, R. A. (1993) *Mech. Dev.* 43, 175–186.
21. Kawakami, Y., Wada, N., Nishimatsu, S. I., Ishikawa, T., Noji, S., and Nohno, T. (1999) *Dev. Growth Differ.* 41, 29–40.
22. Yang, Y., Topol, L., Lee, H., and Wu, J. (2003) *Development* 130, 1003–1015.
23. Loganathan, P. G., Nimmagadda, S., Huang, R., Scaal, M., and Christ, B. (2005) *Histochem. Biophys. Acta* 1338, 129–140.
24. Bergwitz, C., Wendlandt, T., Kispert, A., and Brabant, G. (2001) *Biochim. Biophys. Acta* 1338, 129–140.
25. Jin, E.-J., Park, J.-H., Lee, S.-Y., Chun, J.-S., Bang, O.-S., and Kang, S.-S. (2006) *Int. J. Biochem. Cell Biol.* 38, 183–195.
26. Weeraratna, A. T., Jiang, Y., Hostetter, G., Rosenblatt, K., Duray, P., Bittner, M., and Trent, J. M. (2002) *Cancer Cell* 1, 279–288.
27. Oishi, I., Suzuki, H., Onishi, N., Takada, R., Kani, S., Ohkawara, B., Koshida, I., Suzuki, K., Yamada, G., Schwabe, G. C., Mundlos, S., Shibuya, H., Takada, S., and Minami, Y. (2003) *Genes Cells* 8, 645–654.
28. Hwang, S.-G., Yu, S.-S., Poo, H., and Chun, J.-S. (2005) *J. Biol. Chem.* 280, 29780–29787.
29. Tsao, W.-C., Wu, H.-M., Chi, K.-H., Chang, Y.-H., and Lin, W.-W. (2005) *Exp. Cell Res.* 304, 234–243.
30. Koyanagi, M., Haendeler, J., Badorff, C., Brandes, R. P., Hoffmann, J., Pandur, P., Zielker, A. M., Kuhl, M., and Dimmeler, S. (2005) *J. Biol. Chem.* 280, 16838–16842.
31. Chang, S.-H., Oh, C.-D., Yang, M.-S., Kang, S.-S., Lee, Y.-S., Sonn, J.-K., and Chun, J.-S. (1998) *J. Biol. Chem.* 273, 19213–19219.
32. Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J., and Moon, R. T. (1996) *Curr. Biol.* 6, 1302–1306.
33. Sheldahl, L. C., Park, M., Malbon, C. C., and Moon, R. T. (1999) *Curr. Biol.* 9, 695–698.
34. Gazit, A., Yaniv, A., Bafico, A., Pramila, T., Igarashi, M., Kitajewski, J., and Aaronson, S. A. (1999) *Oncogene* 18, 5959–5966.
35. Roman-Roman, S., Shi, D. L., Stiot, V., Hay, E., Vayssiere, B., Garcia, T., Baron, R., and Rawadi, G. (2004) *J. Biol. Chem.* 279, 5725–5733.
36. Golan, T., Yaniv, A., Bafico, A., Liu, G., and Gazit, A. (2004) *J. Biol. Chem.* 279, 14879–14888.