Wnt/ β -catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: its possible role in joint degeneration

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A fine balance between anabolic and catabolic mechanisms maintains extracellular matrix homeostasis in articular cartilage, and shifts toward degradation are associated with joint conditions such as osteoarthritis. To test the possible involvement, relevance and significance of the Wnt/ β -catenin-signaling pathway in those catabolic shifts, rabbit articular chondrocyte cultures were subjected to experimental activation of β -catenin signaling by Wnt3A treatment or forced expression of constitutive-active β -catenin (CA- β -catenin). Both interventions provoked strong gelatinase activity and stimulated gene expression of matrix metalloprotease-3 and -13 and a disintegrin-like and metalloprotease with thrombospondin motif (ADAMTS)-4 and -5 proteases. Furthermore, Wnt3A treatment additively enhanced the effects of interleukin-1 β , a well-known catabolic culprit of proteoglycan matrix loss. To determine whether Wnt/ β -catenin signaling is associated with age-associated osteoarthritic changes in articular cartilage *in vivo*, we analyzed the presence and intracellular distribution of β -catenin in a spontaneous guinea pig osteoarthritis model. Healthy articular chondrocytes in young guinea pig knees contained barely detectable levels of β -catenin. In contrast, the protein was highly abundant in osteoarthritic-like chondrocytes present in older guinea pig joints, and was localized not only in the cytoplasm but also the nucleus, a clear reflection of activated Wnt signaling. These and other data suggest that Wnt/ β -catenin signaling is a powerful stimulator of chondrocyte matrix catabolic action and may be part of mechanisms leading to excessive remodeling and degradation of cartilage matrix in age-associated joint pathologies.

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Wnt proteins play central roles in a variety of developmental processes and events, which include organogenesis, cell differentiation, morphogenesis and tissue remodeling.^{1–3} Activation of Wnt signaling is initiated by binding of Wnt proteins to Frizzled receptors and co-receptors, such as low-density lipoprotein receptor-5 or -6.^{4–7} Interactions of Wnt with the receptors are regulated and modulated by endogenous antagonists in the extracellular milieu, which include secreted Frizzled-related proteins, Wnt-inhibitory factors, Cereberus and Dickkopf.⁸ In the canonical Wnt/ β -catenin-signaling pathway, binding of Wnt to cell-surface Frizzled receptors leads to inhibition of β -catenin phosphorylation and proteasome-mediated degradation; stabilized β -catenin translocates to the nucleus, where it interacts with resident lymphoid enhancer factor/T-cell (LEF/TCF) transcription factors to activate Wnt target genes.^{9,10}

Articular cartilage is an essential component for the continuous functional performance of synovial joints throughout life. Its ability to provide mechanical resilience is due to its abundant and unique extracellular matrix and the structural and functional characteristics of matrix macromolecular components. Aggrecan, the most prominent proteoglycan in cartilage, is a very large molecule rich in chondroitin and keratan sulfate chains, which binds large quantities of water and ions and provides mechanical elasticity. Collagens, and in particular collagen II, are organized in a highly structured fibrillar network that holds spatial and physical arrangement of tissue and provides tensile strength. These two major matrix components are aided in their physiologic functions by an array of additional matrix components, which include collagen VI, IX and XI; leucine-rich proteoglycans; hyaluronan and link protein.

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Such complex matrix composition and structure are maintained by a fine balance of anabolic and catabolic mechanisms operating in the chondrocytes, which preserve matrix organization and functional integrity through life. Numerous studies conducted over the past years have provided ample evidence that these homeostatic mechanisms are altered in joint pathologies, including osteoarthritis (OA), in which a shift toward catabolism leads to progressive erosion of structural integrity and eventual loss of functional performance. This shift involves the activation of degradation pathways that rely on expression, activation and action by matrix metalloproteases (MMPs) and other matrix proteases.^{11,12} It is also well established that inflammatory cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , represent major culprits in eliciting such homeostatic-to-catabolic shifts.^{12–14}

Recent studies by this and other groups have revealed that the Wnt/ β -catenin-signaling pathway can also elicit matrix catabolism.^{15–19} We found that activation of this pathway occurs in the hypertrophic zone of the growth plate in developing and growing long bones in which the extracellular matrix is subjected to physiologic remodeling and degradation to permit replacement of hypertrophic cartilage with endochondral bone. MMPs, such as MMP-9 and -13, play essential roles in such matrix-restructuring and removal process.²⁰ Ablation of MMP-9 and -13 genes, singly or in combination, causes accumulation of hypertrophic cartilage and its matrix and a severe delay in endochondral bone deposition.^{21,22} We found also in cultures of chick embryo growth plate chondrocytes, that experimental activation of Wnt/ β -catenin signaling provokes rapid gene expression of MMPs and other proteases, and degradation of the proteoglycan matrix, indicating that this pathway acts directly on the chondrocytes' regulatory machinery to elicit matrix catabolism.¹⁵ The present study was conducted to extend our previous work to mammalian articular chondrocytes, seeking to determine whether acute activation of the Wnt/ β -catenin pathway would be able to derange normal matrix homeostasis characteristic of these cells and shift it toward catabolism. To obtain evidence that the pathway has relevance and significance in joint degeneration *in vivo*, we asked whether activation of Wnt/ β -catenin signaling accompanies age-dependent articular cartilage degeneration in a spontaneous OA guinea pig model. The results obtained provide strong support for both predictions.

MATERIALS AND METHODS

Chondrocyte Culture and Viral Infection

Chondrocytes were isolated from articular cartilage of the distal ends of femur and humerus of 6-week-old New Zealand rabbits by digestion with 0.25% trypsin and 2 mM EDTA for 15 min, followed by digestion with 0.15% collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 4–5 h. Primary cells were maintained in MEM containing 10% fetal bovine serum (FBS). Two days after

plating, the medium was changed to 10% FBS-MEM containing 30% of conditioned medium collected from wild-type (WT) L-cell cultures or L-Wnt-3A-producing cell cultures described below, or treated with the recombinant Wnt3A protein (rWnt3A) of mouse (Chemicon, Temecula, CA, USA) or IL-1 β (R&D Systems Inc., Minneapolis, MN, USA). When indicated, chondrocyte cultures were infected with adenovirus encoding a constitutive active form of β -catenin (CA- β -catenin) that lacks the amino-terminal phosphorylation site or β -galactosidase 1 day after plating. For detection of sulfated proteoglycans associated with cell layer, chondrocyte cultures were fixed with 10% formalin for 10 min, incubated with 70% ethanol for 5 min and stained with 1% Alcian blue in 5% acetic acid (pH 1) for 2 h at room temperature.²³ Intensity of the staining was analyzed by Image J software. Experiments were repeated a minimum of 2–3 times and comparable results were obtained.

Preparation of Wnt3A-Containing Conditioned Medium

The L-Wnt3A cell line stably expressing Wnt-3A and the control WT L-cell line, were purchased from the American Type Culture Collection (Manassas, VA, USA). Medium conditioned by L-Wnt3A cells or WT L-cells over a 72-h period (heretofore termed Wnt3A-Containing Conditioned Medium (Wnt3A-CM) or WT-CM, respectively) was collected, processed and used according to the manufacturer's protocol.

Transfection and Reporter Assays

Freshly isolated chondrocytes were cultured until sub-confluent and then transfected with one of the following plasmids using TransIT-LT1 transfection reagent (Mirus Bio Corp., Madison, WI, USA): an Ax2-Luc promoter reporter vector, which contains Axin2 promoter, exon 1 and intron 1;²⁴ or a dominant-negative form of β -catenin (EnR β cat) expression vector encoding β -catenin and a repressor element of engrailed.²⁴ Luciferase activity was measured 2 days after transfection using Bright-Glo luciferase assay kit (Promega Corp. Madison, WI, USA).

RNA Isolation and RT-PCR

Total RNA was isolated by the guanidine isothiocyanate method as described previously.²⁵ Real-time PCR was performed using SYBR green (Sigma), following the manufacturer's instruction. The average of threshold cycle value (C_t value) was calculated from the triplicate reactions. Averaged C_t values were then normalized to the averaged C_t value of a control gene, GAPDH. Adjusted average C_t values were used to calculate relative expression levels to the control group.

The primer sequences used for real-time PCR amplification are as follows: 5'-ATGGACAGGACTGAACGGCTTGC T-3' and 5'-GGTCTGCATTGTTTGGCCAGTGTCA-3' for 4–312 of rabbit HPRT (AF020294), 5'-GAAGTTCCTTGGCT TGGAGGTGACA-3' and 5'-TGGCAGCATCAACAGCAT CTCTTG-3' for 258–454 of rabbit MMP-3 (M25664),

5'-GGGTCCCTGATGTGGGTGAATACAAC-3' and 5'-GGA GAAAAGCGTGAGCCAGAAGA-3' for 956–1238 of rabbit MMP-13 (AF059201), 5'-CGGAACGGTGGCAAGTATTGT GAG-3' and 5'-GGCAGGTGAGTTTGCATTGGTCTC-3' for 23–236 of rabbit disintegrin-like and metalloprotease with thrombospondin motif-4 (ADAMTS-4) (AF247707), 5'-AGG GCAAGTGTGTGGACAAGACCA-3' and 5'-GAAAAGATT TACCTTGGCTGGGCAG-3' for 520–756 of rabbit ADA MTS-5 (ADAMTS-11) (AF317415), 5'-CCTCACCCCGAGA ATCAAATGGAG-3' and 5'-GAACACAACACCTTTCACCA CGACCT-3' for 12–273 of rabbit aggrecan (L38484), and 5'-GGTGACTACTGGATAGACCCCAACCA-3' and 5'-TGA AGTGAAGCCGCCATTGATG-3' for 187–379 of rabbit type II collagen (D83228).

Measurement of Proteoglycan Release

Chondrocyte cultures were pre-labeled with 2 μ Ci/ml of 35 S-labeled-sulfate for 16 h in DMEM containing 50 μ g/ml of ascorbic acid and 10% FBS, washed three times with Hank's balanced solution and incubated with 10% FBS-MEM in the absence or presence of 300 ng/ml of mouse rWnt3A. Media were collected over time and 35 S-labeled proteoglycans released into the media were precipitated with cetylpyridinium chloride and the radioactivity was measured by scintillation counting.²⁶

Gelatin Zymography

Chondrocyte cultures were incubated with adenovirus encoding β -galactosidase as control or CA- β -catenin for 6 days. The cultures were then washed twice with serum-free MEM, incubated with serum-free MEM for 20 min and cultured on fresh serum-free MEM. After 24 h, the medium was harvested and spun at 3000 r.p.m. for 10 min, and the supernatant was kept at -70°C until use. Parallel samples were used to measure protein content so that each sample could be loaded on the basis of protein content. For gelatin zymography, samples were denatured by mixing with an equal volume of SDS sample buffer for 10 min at room temperature. Aliquots containing equal protein amounts were separated on gelatin-containing 8% sodium dodecylsulfate–polyacrylamide gels. After electrophoresis, proteins in the gels were renatured by incubation with 2.5% Triton X-100 buffer and then developing buffer overnight. Proteolytic activity and gelatin digestion were visualized by staining the gels with 0.1% Coomassie Blue R250 solution, followed by de-staining with 50% methanol and 10% acetic acid solution.

Guinea Pig Osteoarthritis Model

Hartley guinea pigs are a widely used model of spontaneous OA,²⁷ and were maintained in one of the author's (T Koike) laboratory following Institutional Animal Care and Use Guidelines. Fifty 4-month-old female Hartley albino guinea pigs were purchased from Bioindustry Division, Oriental Yeast Co. Ltd. (Tokyo, Japan) and housed individually in

38 $\times$ 34 $\times$ 18-cm wire cages. Food (Guinea Pig Chow GOC4; Bioindustry Division, Oriental Yeast Co. Ltd.) and water were available for *ad libitum* consumption from the beginning to months 4 ($n = 5$), 6 ($n = 10$), 8 ($n = 10$), 10 ($n = 10$) and 12 ($n = 15$). Animals were killed by exposure to carbon dioxide. Serial tissue sections were prepared from medial plateau of knee joints of 4- to 12-month-old Hartley guinea pigs, and used to determine the intracellular distribution of β -catenin by immunohistochemistry. Pathological changes were assessed by microscopic examination of sections stained with hematoxylin and eosin (H–E) or safranin-O. Grading was carried out by three independent researchers, using Mankin's criteria, with some modifications.^{27,28} Extent and severity of osteoarthritic histological lesions were set from grade 0 to 8 (0, normal; 1, mild surface irregularities, no cleft; 2, irregular surface, 1–3 superficial clefts; 3, >3 clefts and/or loss of cartilage to superficial zone; 4, 1–3 clefts extending into the middle zone; 5, >3 clefts and/or loss of cartilage extending into the middle zone; 6, 1–3 clefts extending into the deep zone; 7, >3 clefts extending into the deep zone and/or loss of cartilage to deep zone; and 8, clefts extending to zone of calcified cartilage). Proteoglycan loss was graded from 0 to 6 (0, uniform staining throughout articular cartilage; 1, loss of staining in superficial zone only and for less than half the length of the condyle or plateau; 2, loss of staining in superficial zone for half the length or greater of the condyle or plateau; 3, loss of staining in superficial and middle zones for less than half the length of the condyle or plateau; 4, loss of staining in superficial and middle zones for half the length or greater of the condyle or plateau; 5, loss of staining in all three zones for less than half the length of the condyle or plateau; and 6, loss of staining in all three zones for half the length or greater of the condyle or plateau). The two scores were combined and the total scores of the three independent investigators were averaged.

Immunohistochemistry

After deparaffinization, sections were blocked with 10% normal goat serum and 0.1% BSA in PBS, and then incubated with rabbit antibody human β -catenin (Sigma) at 1:2000 dilution for 1 h at room temperature. After rinsing, sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) and the reaction was visualized by developing with a ABCComplexes kit (DAKO North America Inc., Carpinteria, CA, USA) and counterstained with 0.1% methyl green for 5 min. Degree of β -catenin staining was evaluated by three investigators and set according to the following three levels: negative, threshold or positive. Only sections judged positive by all investigators were counted as positive.

Statistical Analyses

The data were validated by Student's *t*-tests. The results of immunohistochemical analyses for β -catenin in guinea pig OA model were evaluated by chi-square tests.

RESULTS

Rapid Induction of Matrix Loss and Protease Expression by Wnt3A

First, we determined whether extracellular matrix metabolism is rapidly affected by acute experimental activation of Wnt/ β -catenin signaling. Articular chondrocytes were isolated from the knee and elbow joints of New Zealand rabbits, maintained in primary monolayer culture for a few days until sub-confluent and then treated with Wnt3A-CM, a Wnt protein that signals through β -catenin;²⁹ companion cultures received control vehicle or medium (WT-CM). Reporter²⁴ was indeed activated in Wnt3A-CM-treated cultures within 24 h, whereas it remained at background levels in control cultures and was inhibited by coexpression of plasmid-encoded EnR β cat, a dominant-negative form of β -catenin³⁰ (Figure 1a). Similar levels of reporter activity were elicited by treatment with rWnt3A (Figure 1a).

Microscopic examination showed that the chondrocytes in control WT-CM cultures exhibited a typical and normal polygonal morphology (Figure 1b, WT-CM), and were surrounded by an abundant proteoglycan-rich extracellular matrix strongly stainable with Alcian blue (Figure 1c). They also contained conspicuous amounts of RNAs encoding aggrecan and collagen IX, a further reflection of their highly differentiated and stable status (Figure 1e). In comparison, chondrocytes in parallel Wnt3A-CM cultures had acquired a dull morphology and barely stained with Alcian blue, indicating that they had undergone massive matrix loss and shedding (Figure 1c and d, Wnt3A-CM). Their steady-state levels of aggrecan and collagen IX RNAs were reduced as well, indicating that their overall differentiated phenotype had decreased (Figure 1e).

Having found that Wnt3A-CM treatment leads to decreased matrix gene expression and increased proteoglycan

Figure 1 Wnt3A inhibits cartilage matrix synthesis and stimulates gene expression of matrix-degradation enzymes, gelatin-degrading activity and proteoglycan release in rabbit articular chondrocytes. (a) Sub-confluent mouse chondrocyte cultures were transfected with the indicated DNA plasmids and exposed to 30% WT-CM, Wnt3A-CM or rWnt3A. Luciferase activity was measured after 24 h of additional incubation. Values represent averages and s.d. of two samples; * $P < 0.05$. (b) Phase-contrast images of chondrocytes receiving 30% of WT-CM or Wnt3A-CM from day 2 to 5. The bar represents 100 μ m. (c, d) The cultures were stained with Alcian blue on day 7. Photo pictures represent the entire culture wells (c), and the intensity was measured by Image J software (d). Values represent averages and s.d. of two samples; * $P < 0.05$. (e, f) RNA was prepared from cultures receiving 30% WT-CM as control (open columns) or Wnt3A-CM (solid columns) from day 2 to 4, and used to quantify aggrecan and collagen 2 (Col II) mRNA levels (e), or the indicated MMP and ADAMTS mRNAs (f) by real-time PCR. Values represent averages and s.d. of three samples; * $P < 0.05$ compared with control. (g) The cultures were pre-labeled with 35 S-labeled sulfate and then incubated with 300 ng/ml of rWnt3A. Macromolecular-associated radioactivity released into the medium was determined after 1–4 days. Values represent averages and s.d. of three samples; * $P < 0.05$ compared with control.

shedding, we asked whether it may also trigger increases in matrix protease gene expression. To this end, semi-confluent chondrocyte cultures were incubated in WT-CM or Wnt3A-CM for 1 to 2 days, and total cellular RNAs were subjected to real-time PCR. In Wnt3A-CM cultures, gene expression of MMP-3, and -13, and ADAMTS-4 and -5 was indeed four- to ninefold higher than that in companion control WT-CM cultures (Figure 1f). Treatment with rWnt3A also stimulated gene expression of these matrix degradation enzymes (data not shown).

To examine whether increases in gene expression of matrix-degrading enzymes reflect matrix loss, we tested proteoglycan release directly. The cultures were pre-labeled with ^{35}S -labeled sulfate and treated with rWnt 3A, and release of ^{35}S -labeled proteoglycan from the cell layer was monitored over time. Significant increases in proteoglycan release by Wnt3A was observed from day 1 of treatment and continued for up to 4 days (Figure 1g).

Matrix Loss and Protease Gene Expression Induced by CA- β -Catenin

To obtain more direct evidence that β -catenin signaling can rapidly trigger matrix loss and protease gene expression, semi-confluent chondrocyte cultures were infected with adenovirus particles encoding CA- β -catenin tagged with hemagglutinin (HA) epitope or control β -galactosidase-encoding viral particles (adeno- β -gal). CA- β -catenin-expressing cells were flatter and stained poorly with Alcian blue (Figure 2a–c, Ad-CA- β -cat) compared with respective control adeno- β -galactosidase-expressing cultures (Figure 2a–c, Ad- β -gal). Immunostaining for HA epitope revealed that the CA- β -catenin virus infection had led to proper expression of transgene products (Figure 2d). This was also confirmed by immunoblot analysis; expression of HA-tagged protein was detected in cultures infected with CA- β -catenin virus, but not in control cultures, whereas ERK proteins (used as internal control) were expressed in both cultures (Figure 2e).

Real-time PCR analysis revealed that CA- β -catenin cultures expressed lower levels of collagen II than control (Figure 2f). In addition, CA- β -catenin-expressing cultures displayed higher levels of RNAs encoding MMP-3 and -13 and ADAMTS-5 compared with controls (Figure 2g). It should be noted that since control adeno- β -galactosidase chondrocytes displayed a normal polygonal appearance and strong Alcian blue staining (Figure 2a), and closely resembled uninfected chondrocytes (see Figure 1b), adenovirus infection *per se* and expression of β -gal clearly had no obvious adverse effects on their phenotype.

To investigate whether MMP activity and levels had changed as well, the cultures were maintained for 5 days after virus infection and then switched to serum-free medium for 16 h. Aliquots of the resulting serum-free conditioned media were analyzed for the presence of protease activity by gelatin zymography. Both control and CA- β -catenin virus-infected culture media contained major protease bands. The most

prominent bands displayed electrophoretic positions corresponding to pro-MMP-2, and a fainter band likely represented MMP-13 (Figure 2h). In general, the intensities of the bands were stronger in CA- β -catenin culture medium than control samples.

β -Catenin Signaling and IL-1 β Signaling in Catabolic Action

As it is widely known, IL-1 β is a potent inducer of cartilage matrix degradation. To analyze the possible mechanisms of the catabolic action of Wnt/ β -catenin signaling, we investigated whether Wnt/ β -catenin signaling modulates the action of IL-1 β . We treated confluent chondrocyte cultures with different doses of IL-1 β and/or Wnt3A for 48 h, stained the proteoglycan matrix with Alcian blue and spectrophotometrically measured the intensity of staining. Proteoglycan levels were significantly decreased in a dose-dependent manner by treatment with IL-1 β or Wnt3A; a combination of IL-1 β plus Wnt3A caused a greater loss and did so in an IL-1 β dose-dependent manner (Figure 3).

β -Catenin Nuclear Localization in Osteoarthritic Cartilage with Age

We next asked whether articular cartilage undergoing osteoarthritic changes with age *in vivo* displays signs of activation of β -catenin signaling. Thus, we resorted to Hartley guinea pigs of different ages, which represent a widely used animal model of age-dependent OA.²⁷ Serial sections of knee joints of 4-, 6-, 8-, 10- and 12-month-old guinea pigs were processed for analysis of the presence and intracellular distribution of β -catenin in articular chondrocytes. Accumulation and/or nuclear translocation of β -catenin are widely recognized and well-documented signs of activation of the Wnt/ β -catenin-signaling pathway.^{9,10} Tibial articular cartilage in young healthy 4-month-old animals was very well organized (Figure 4a), stained strongly and homogeneously with safranin-O, indicating a high proteoglycan content (Figure 4b), and displayed undetectable staining for β -catenin in either cytoplasm or nucleus (Figure 4c and d). On the other hand, articular cartilage in 6- to 8-month-old animals often displayed an irregular surface and fissures (Figure 4e, arrows), was thinner and stained poorly with safranin-O (Figure 4f), and did exhibit strong β -catenin staining (Figure 4g). Analysis at higher magnification revealed that β -catenin was present in both cytoplasm and nucleus (Figure 4h). Analysis of older 10- and 12-month-old specimens showed that cartilage degeneration and thinning had become severe (Figure 4i), safranin-O-positive matrix was markedly reduced (Figure 4j) and staining for β -catenin was now very prominent and characterized even the most superficial chondrocytes facing the synovial space (Figure 4k and l).

Comparisons of multiple guinea pigs from each age group and microscopic evaluation by independent observers showed that there was a statistically significant dependent

Figure 2 CA- β -catenin inhibits cartilage matrix gene synthesis and stimulates activity and gene expression of matrix degradation enzymes in rabbit articular chondrocytes. Primary rabbit articular chondrocytes were infected with adenovirus encoding control β -galactosidase or HA-tagged CA- β -catenin on day 1. (a–c) Phase-contrast images of live cultures on day 5 (a), and photo pictures of Alcian blue-stained culture wells on day 7 (b). The bar represents 100 μ m. The intensity was measured by Image J software (c). Values represent averages and s.d. of two samples; * $P < 0.05$ compared with control. (d, e) After adenovirus infection, infection was confirmed by immunostaining for HA epitope (d), and cultures were lysed in SDS sample buffer on day 3 for immunoblot analysis with antibodies to HA-tag or ERK (e). (f, g) Total RNA isolated from control β -galactosidase (open columns) or CA- β -catenin (solid columns) cultures was used to quantify collagen 2 (Col II) mRNAs by real-time PCR (f). Gene expression levels for matrix degradation enzymes in β -galactosidase (open columns) or CA- β -catenin (solid columns) virus-infected cultures were quantified by real-time PCR. Values represent averages and s.d. of three samples (g); * $P < 0.05$ compared with control. (h) Primary cultures infected with control β -galactosidase or CA- β -catenin virus were maintained until day 7 and then incubated with serum-free medium for 16 h. Aliquots of the latter medium were subjected to gelatin.

relation between articular cartilage defects with age and strong β -catenin staining of chondrocytes. Thus, whereas none of the 4-month-old specimens were positive, about 50% of the 6- to 8-month-old and over 72% of 10- to 12-month-old specimens were positive (Table 1). When specimens from

all ages were re-grouped according to their joint phenotype, the ratio of β -catenin-positive cells significantly increased with severity of osteoarthritic changes (Table 2). When changes became very severe, most of the specimens showed positive β -catenin staining (Table 2, score 9–11). Because of

Figure 3 Additive effects on matrix loss by Wnt3A and IL-1 β . Rabbit articular cultures were treated with 3, 10 or 30% of Wnt3A-CM or WT-CM (30%) in the presence of 0.1 or 0.5 μ g/ml of IL-1 β or vehicle for 2 days after confluence. The cultures were then stained with Alcian blue and the results were quantified by Image J. Values represent averages and s.d. of two samples; * P < 0.05, ** P < 0.01.

the low number of old animals used for the latter studies, statistical significance could not be established conclusively (Table 2). Figure 5 shows the average values of grading scores in β -catenin-negative and β -catenin-positive groups, and indicates that there were clear differences in osteoarthritic severity between the two groups.

DISCUSSION

The results of the study show that the fine balance between anabolic and catabolic mechanisms maintaining matrix homeostasis in cartilage cells is rapidly disrupted upon acute experimental activation of Wnt/ β -catenin signaling. Both Wnt3A treatment and adeno-driven overexpression of constitutive active β -catenin trigger a major and sudden loss of proteoglycans normally decorating the cellular periphery and inter-territorial matrix of rabbit articular chondrocytes. We have also shown that there is a close relationship between osteoarthritic changes occurring in Hartley guinea pig limb joints over age and presence of large amounts of cytoplasmic and nuclear β -catenin. The Wnt/ β -catenin-signaling pathway thus appears to be an effective, efficient and powerful stimulator of chondrocyte matrix catabolic action. The pathway may conceivably be part of mechanisms leading to excessive remodeling and degradation of cartilage matrix in age-associated joint conditions in mammals.

Given the serious consequences of increased catabolic activity and matrix loss in joint pathologies, there has been continuous interest and research efforts directed toward identification of possible culprits and toward finding therapeutic means to block or reverse those deleterious cascades. As pointed out above, these efforts have led to the recognition that a number of inflammatory cytokines and

factors such as IL-1 β , IL- α , IL-17, IL-18, TNF- α and retinoids act to shift cartilage homeostasis toward degradation and can be significant contributors to joint pathologies.^{12–14} Indeed, anti-TNF- α therapies are currently used to treat patients affected by rheumatoid arthritis.³¹ By demonstrating that the Wnt/ β -catenin pathway can trigger matrix degradation as effectively and rapidly and can further potentiate IL-1 β action, our study now adds this pathway to the list of possible major contributors to joint pathology. We do not yet know how exactly the pathway can destabilize the epiphyseal chondrocyte phenotype in such concerted and encompassing manner, resulting in the coordinate stimulation of gene expression of multiple MMPs and ADAMTSs, and concurrent stimulation of gelatinase activity. The encompassing nature of this response indicates that it must operate in a central and vital manner within chondrocytes' regulatory circuits. In this regard, it is interesting to note that the MMPs and ADAMTSs boosted by experimental activation of Wnt/ β -catenin pathway are quite similar to those triggered by treatment with IL-1 β or TNF- α in chondrocytes.¹¹ Several studies have indicated that signaling pathways involving MAPK, NF- κ B and PI3/Akt are likely to mediate the catabolic responses of chondrocytes to those inflammatory cytokines, as also revealed by the fact that specific inhibitors to those pathways can counteract cytokine effects on matrix protease gene expression.^{32–38} It is plausible then that the Wnt/ β -catenin-signaling pathway may feed into, or cross-talk with, central pathways utilized by inflammatory cytokines and would ultimately elicit a similar catabolic response by chondrocytes. Our finding that Wnt/ β -catenin signaling and IL-1 β additively enhance matrix catabolic action greatly encourages this idea. In addition, further support comes from studies suggesting that (a) treatment of cultured chondrocytes with IL-1 β or retinoic acid does induce Wnt/ β -catenin signaling, as delineated by β -catenin nuclear relocalization, increased Wnt-7A expression and reporter assays;³⁶ and (b) Wnt-3A treatment induces articular chondrocyte de-differentiation through transcriptional activation of β -catenin and c-Jun AP-1 pathway.³⁸ Notably, it has been found in cancer cell lines that Wnt/ β -catenin signaling directly activates gene expression of MMPs such as MMP-7, presumably by formation of activated β -catenin-LEF/TCF complexes and direct interaction with response elements on those genes.³⁹ In sum, it seems likely that the Wnt/ β -catenin pathway may be part of integrated signal transduction mechanisms through which chondrocytes respond to deranging and catabolic cues, activate the expression of MMPs and ADAMTS genes and corresponding proteolytic activity, and undermine their phenotypic status and ultimately tissue function.

Chondrocyte cytoarchitecture has long been recognized as a key parameter influencing the phenotypic stability of chondrocytes, including articular chondrocytes.^{40,41} It is well recognized that a change from a round to a more flattened cell shape does not favor chondrocyte function and can have

serious repercussions.^{40,41} Alterations in cell shape and even cell hypertrophy are documented to occur in OA.⁴² Hence, it is of considerable interest that the matrix catabolic shifts triggered by activation of Wnt/ β -catenin signaling are accompanied by a shift in chondrocyte cytoarchitecture that changes from polygonal to rather flatten and dull. In this connection, it has been suggested recently that Wnt signaling may regulate pathologic cartilage degeneration by mechanisms including chondrocyte de-differentiation^{35,38} and blocking of NO-induced apoptosis via activation of cell-survival signaling, phosphatidylinositol-3-kinase and Akt.³² Hence, changes in cytoarchitecture could contribute to de-stabilization of the chondrocyte phenotype or even

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Table 1 Relationship between age-related osteoarthritic changes and positive β -catenin immunostaining in Hartley guinea pig knee joint articular cartilage

Age (average of scores)	β -Catenin staining			Total	
	Negative	Positive	Positive (%)		
4 months (0.6)	0	5	0	5	$\left. \begin{array}{l} P < 0.05 \\ P < 0.01 \end{array} \right\}$
6–8 months (4.65)*	11	9	55	20	
10–12 months (6.52)**	7	18	72	25	

Serial sections from medial tibial plateau of 4-, 6-, 8-, 10- and 12-month-old Hartley guinea pig knee joints were stained with H-E and safranin-O and evaluated by a modified Mankin's score by three independent investigators. The average of scores was significantly different between the 4- and 6- to 8-month groups (* $P < 0.05$), and between 6- to 8-month and 10- to 12-month groups (** $P < 0.05$ to 6–8 months) as determined by a Student's *t*-test. Companion sections were stained with β -catenin antibodies, and degree of staining was evaluated by independent investigators and set to the following three levels: negative, threshold or positive. Only sections judged positive by all investigators were counted as positive. χ^2 -tests indicated lack of independence between age and β -catenin-positive results.

Table 2 Relationship between severity of joint changes and positive β -catenin staining

Grading score	β -Catenin staining		Total	
	Negative	Positive		
0–2	0	11	11	$P < 0.01$
3–5	6	6	12	
6–8	3	20	23	$P < 0.05$
9–11	0	4	4	

The results of β -catenin immunostaining in Table 1 were re-grouped according to osteoarthritic grading score (normal, grade 0–2; slight, grade 3–5; moderate, grade 6–8 and severe, grade 9–11). Values were analyzed by χ^2 -tests.

and stimulation of PI3/Akt pathway. Further studies have demonstrated that GSK3 β phosphorylation is required for activation of the Akt pathway.^{45,46} Thus, changes in both chondrocyte cytoarchitecture and cell-surface interactions with microenvironment, all resulting from Wnt/ β -catenin signaling-mediated loss of pericellular matrix, could further contribute to catabolic responses and phenotypic demise and destabilization of chondrocytes.

Translocation and accumulation of β -catenin into the nucleus are universally recognized as invariant signs of activation of Wnt/ β -catenin signaling, with consequent formation of β -catenin–LEF/CTF complexes interacting with response sequences and stimulating target gene expression. From this point of view then, presence of readily detectable and abundant nuclear β -catenin in 10-month-old degenerating guinea pig articular cartilage and its absence in younger and healthier 4-month-old tissue are very likely to reflect the activation of Wnt/ β -catenin signaling with age *in vivo*. Nuclear β -catenin could in turn contribute to genetically reprogram chondrocytes toward catabolism, with negative consequences on tissue structure, organization and

Figure 5 Comparison of osteoarthritic grading scores between β -catenin-positive and -negative groups. The results of β -catenin immunohistochemical staining of guinea pig knee specimens were subdivided into negative and positive groups. Averages and s.d. of modified Mankin's scores were calculated for each group, and the values were evaluated by a Student's *t*-test; * $P < 0.01$.

functionality. This possibility, although likely and conceivable, remains to be tested experimentally *in vivo*, although the results of our explant cultures do provide clear support for the same. In addition, several lines of observations from other studies strongly support its premise as well. Immunohistochemical analysis of human osteoarthritic cartilage has revealed the presence and accumulation of β -catenin in the chondrocytes;³² these data are in very good agreement with our data in guinea pigs and strongly point to the universality of these phenomena across mammals. Likewise, expression of several Wnt family members was found to

be upregulated in synovial cells and articular cartilage in patients with rheumatoid arthritis and OA.^{47,48} There are also interesting recent multi-scale genetic studies on single-nucleotide polymorphism in the secreted Frizzled-related protein 3 (sFRP3) gene that link joint disease to Wnt signaling. sFRP3 is a glycoprotein that antagonizes signaling of Wnt proteins by interference with Frizzled membrane receptor interactions.⁴⁹ Genetic analysis among members of 378 hip-affected sibling pair families has identified an Arg324Gly substitution at the carboxyl terminus of sFRP3 to be associated with hip OA in females.⁵⁰ *In vitro* testing of the mutant sFRP3 showed that it had a lower ability to antagonize Wnt signaling, suggesting that the increased incidence of hip OA is associated with increased Wnt signaling. A more recent study from the same laboratories has found that an identical mutation is associated with susceptibility to knee OA.⁵¹ Together, these and other studies provide compelling and intriguing support to the idea that activation or increased activity of the Wnt/ β -catenin-signaling pathway could play a causative role in joint diseases such as OA, where, as suggested above, it would act to shift matrix homeostasis toward catabolism and chondrocyte phenotype toward destabilization. These combinations of deleterious pathways would have serious repercussions for joint homeostasis and could contribute or even lead to joint dysfunction and disease.

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