A Wnt/β-catenin Negative Feedback Loop Inhibits IL-1-induced MMP Expression in Human Articular Chondrocytes

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Abstract

Objective. Recent animal studies suggest that activation of Wnt/β-catenin signaling in articular chondrocytes might be a driving factor in the pathogenesis of osteoarthritis (OA) by stimulating amongst others the expression of matrix metalloproteinases (MMPs). This study aimed to investigate the role of Wnt/β-catenin signaling in IL-1β-induced MMP expression in human chondrocytes.

Methods. Primary cultures of human, mouse and bovine articular chondrocytes as well as human mesenchymal stem cells (hMSCs) and mouse embryonic fibroblasts (MEFs) were used. Multiple strategies for activation and inhibition of signaling pathways were used. Reporter assays and co-immunoprecipitations were used to study the interaction between β-catenin and NF-κB.

Results. In contrast to animal chondrocytes, in human chondrocytes Wnt/β-catenin is a potent inhibitor of MMP1, -3 and -13 expression and generic MMP activity both in basal conditions and after IL-1β stimulation. This effect is independent of TCF/LEF transcription factors but is due to an inhibitory protein-protein interaction between β-catenin and NF-κB. Furthermore we show that IL-1β indirectly activates β-catenin signaling by inducing canonical Wnt7B expression and by inhibiting the expression of canonical Wnt antagonists.

Conclusion. Our data reveal an unexpected anti-catabolic role of Wnt/β-catenin signaling in human chondrocytes by counteracting NF-κB-mediated MMP expression induced by IL-1β in a negative feedback loop.
Introduction

Osteoarthritis (OA) is the most common form of arthritis and a leading cause in mobility associated disability. OA affects the whole joint and is characterized by progressive degeneration of articular cartilage, mild signs of inflammation and typical bone changes (1, 2). Although all tissues in the joint are affected by the disease, it is believed that the articular chondrocyte is a major cellular mediator of OA pathogenesis through actively promoting cartilage matrix degradation by expressing matrix metalloproteinases (MMPs) and aggrecanases in response to adverse environmental signals by for example pro-inflammatory cytokines. Particularly, increased production of MMP-1, MMP-3 and MMP-13 by chondrocytes has been associated with cartilage degradation in OA (3-5). Pro-inflammatory cytokines like interleukin-1 (IL-1) are potent inducers of cartilage degradation by activating pro-catabolic NF-κB signaling in chondrocytes, which results in amongst others the expression of MMPs in cartilage (6-9).

A role for Wnt/β-catenin in OA is predominantly based on observations in animal models: i) in postnatal mouse models, conditional activation of β-catenin signaling in cartilage results in increased articular cartilage degeneration by stimulating endochondral ossification and other phenotypes resembling OA (10); ii) activation of Wnt/β-catenin signaling in rabbit and mouse chondrocytes stimulates the expression of cartilage matrix degrading MMPs (11, 12); iii) in a spontaneous guinea pig OA model, development of OA is associated with increased β-catenin expression in cartilage (11); and iv) pro-catabolic factors like IL-1 implicated in OA development induce expression of various Wnt proteins resulting in the activation of β-catenin (12, 13). These findings were subsequently corroborated by observation of increased nuclear β-catenin staining in
human OA cartilage compared to control (10). In addition, increased expression of the Wnt target gene (WISP-1) was found in both mouse OA models and in human OA cartilage (14). Likewise, differential expression of various Wnt-related genes has been documented in human joint disorders (15-17). For example, canonical Wnt1 and non-canonical Wnt5A have been implicated in rheumatoid arthritis (RA) (15, 16) while the canonical Wnt7B is upregulated in OA cartilage and RA synovium (17). Interestingly, inhibition of β-catenin signaling in articular chondrocytes also causes OA-like cartilage degradation in a Col2a1-ICAT transgenic mouse model (18). Taken together, multiple lines of evidence have led to the hypothesis that low levels of Wnt/β-catenin signaling are required for maintenance of normal cartilage function and that deregulation of this pathway may contribute to the development and progression of OA. Consequently, the Wnt/β-catenin signaling pathway has been identified as a potential therapeutic target for intervention in OA.

Up to date, functional data on the role of Wnt/β-catenin signaling in human chondrocytes are however still scarce. In this study, we therefore systematically evaluated the role of canonical Wnt signaling in human chondrocytes in comparison with animal chondrocytes. Our study reveals an unexpected and remarkable species difference in regulation of MMP expression by Wnt/β-catenin signaling. In contrast to its pro-catabolic role in animal models, in human chondrocytes Wnt/β-catenin signaling potently inhibits MMP expression and can effectively counteract pro-catabolic NF-κB signaling activated by IL-1β in a negative feedback loop.
Materials and methods

Human cartilage samples. Cartilage was obtained from 8 patients (62 ± 10 years) with osteoarthritis (OA) and one patient (67 years) with rheumatoid arthritis (RA) undergoing total knee replacement surgery. Knee cartilage was harvested from regions with no macroscopically evident degeneration.

Cell culture and Cartilage explant culture. Primary human and bovine articular chondrocytes were isolated from cartilage of knee joints as described before (8). Human fetal chondrocytes were obtained as previously described (19). P0 or P1 chondrocytes were used in all experiments. Human chondrocytes, bovine chondrocytes, HEK293T cells and mouse embryonic fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Human bone marrow-derived mesenchymal stem cells (hMSCs) were isolated from aspirates as described previously (20) and cultured in αMEM with 10% FBS and penicillin-streptomycin. For cartilage explants culture, human cartilage was sliced into small cubes, and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. Mouse cartilage was isolated from femoral heads of 2-month-old C57B mice and maintained in DMEM with 10% FBS and 1% penicillin-streptomycin. Normal human articular chondrocytes as healthy control were derived from a donor (45 years) without joint disease (Lonza).

Recombinant proteins and Reagents. Recombinant human Wnt3A, IL-1β, DKK-1 and recombinant mouse Wnt3A (R&D Systems) were used. The GSK3 inhibitor BIO was obtained from Sigma-Aldrich.
**RNA isolation and Quantitative RT-PCR.** Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized from total RNA with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) was performed with the MyiQ real-time PCR detection system (Bio-Rad) using the standard curve based method (21). GAPDH was used as internal control. Primer sequences are listed in Supplemental Table 1.

**Immunoprecipitation and Western blot.** Immunoprecipitation (IP) was performed using the Dynabeads Co-Immunoprecipitation kit (Invitrogen). Nuclear and cytoplasmic proteins were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific). Total cell proteins were collected in RIPA buffer (Cell Signaling) supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Antibodies used for Western blot and Co-IP were anti-β-catenin (BD Biosciences), pro-MMP1 (R&D Systems), pro-MMP13 (R&D Systems), TATA-binding protein (TBP) (Millipore), GAPDH (Sigma-Aldrich), NF-κB p50(C-19) (Santa Cruz), NF-κB p65(A) (Santa Cruz), FLAG (Origene), TCF4 (Millipore), LEF1 (Millipore).

**MMP activity assay.** Generic MMP activity in human chondrocytes and culture media was measured using the SensoLyte 520 generic MMP activity kit (AnaSpec). MMP activity was normalized for protein concentrations of total cell lysates measured using the Pierce BCA Protein Assay Kit (Thermo Scientific).

**Plasmid constructs and Viral transduction.** Human Wnt3 and Wnt7B cDNAs (Origene) were cloned in to a lentiviral vector pBOB (Addgene Plasmid 12335) (22). shRNA sequences against human TCF4 and LEF1 were cloned into pLKO.1-TRC cloning vector.
(Addgene plasmid 10878) (23). pLKO.1 vectors containing a scrambled shRNA (Addgene plasmid 18640) (24) and an shRNA sequence against human β-catenin (Addgene plasmid 18803) (25) were used. Lentiviral vectors and packaging vectors were transfected into HEK293T cells to produce lentiviruses. Lentiviruses were harvested and used to infect chondrocytes in the presence of 6 µg/ml polybrene (Sigma-Aldrich).

**siRNA transfection.** Mouse embryonic fibroblasts (MEFs) were transfected with ON-TARGETplus SMARTpool siRNA (Thermo Scientific) using the X-tremeGENE siRNA transfection reagent (Roche).

**Reporter assay.** Human chondrocytes were infected with Cignal™ lentiviruses containing the NF-κB responsive reporter or a TCF/LEF reporter (SA Biosciences) together with lentiviruses constitutively expressing Renilla luciferase (SA Biosciences) in the presence of 6 µg/ml polybrene (Sigma-Aldrich). Luciferase activity was measured using Dual-Glo luciferase assay kit (Promega). Activity of firefly luciferase was normalized for Renilla luciferase activity.

**Statistical analysis.** Data were expressed as the mean ± SD and analyzed by two-tailed student’s t-tests or one-way ANOVA. P-values < 0.05 were considered statistically significant. *, P < 0.05; **, P < 0.01.
Results

Canonical Wnt signaling inhibits MMP mRNA expression in human chondrocytes.

To investigate the effect of Wnt/β-catenin signaling on MMP expression, human articular chondrocytes isolated from knee joints of OA patients undergoing total knee replacement surgery were treated with recombinant human (rh) Wnt3A or the GSK3 inhibitor BIO. Both treatments activated canonical Wnt signaling in human chondrocytes as evidenced by an increase in total β-catenin protein levels, nuclear localization of β-catenin and activation of a canonical Wnt signaling responsive TCF/LEF reporter (Supplemental Fig. 1A-C). The specificity of Wnt3A for activation of the canonical pathway was demonstrated by co-incubation with the extracellular antagonist for canonical Wnt signaling DKK-1, which blocked Wnt3A-induced TCF/LEF reporter activity (Supplemental Fig. 1C). Furthermore, treatment with Wnt3A stimulated chondrocyte proliferation, an established effect of canonical Wnt signaling in many cell types (Supplemental Fig. 1D) (26, 27).

We next explored the effect of Wnt/β-catenin signaling on the expression of MMPs and chondrocyte markers. To our surprise activation of canonical Wnt signaling either by Wnt3A or BIO significantly decreased the mRNA expression of MMP1, MMP3 and MMP13 in human chondrocytes (Fig. 1). In agreement with a recent study, the expression of chondrocyte markers Col2A1 and Sox9 was decreased (Supplemental Fig. 1E) (27). We further explored the effect of Wnt3A on the catabolic gene expression. The decrease in MMP mRNA expression by Wnt3A was dose-dependent and gradual, first measurable 6 hours after the start of treatment with maximum inhibition after 72 hours (Supplemental Fig. 1F&G). The inhibition could be reversed by DKK-1, which also efficiently blocked...
Wnt3A-induced expression of the established Wnt target gene Axin2 (Supplemental Fig. 1H&I). Notably, blocking of endogenous canonical Wnt signaling by DKK-1 slightly, but significantly increased basal transcription levels of MMP1 and -13 mRNA (Supplemental Fig. 1H). Taken together, these data suggest that Wnt3A represses MMP mRNA expression in human chondrocytes via a signaling cascade downstream of the Frizzled/LRP receptor complex and probably through β-catenin.

In sharp contrast, stimulation of mouse cartilage explants with recombinant mouse (rm) Wnt3A or BIO induced MMP3 and MMP13 mRNA expression (Fig. 1), which is in accordance with previously reported data (12). Similarly stimulation of bovine chondrocytes with BIO induced MMP3 and MMP13 expression (Fig. 1). The species difference could not be explained by chondrocyte dedifferentiation, an established effect of culturing chondrocytes in monolayer, since direct treatment of human cartilage explants with Wnt3A also reduced MMP mRNA expression (Fig. 1). To exclude that the observed species difference in the regulation of MMP mRNA expression by canonical Wnt signaling in chondrocytes was due to an OA-induced change in cell response, the experiments were repeated using human chondrocytes from a donor without degenerative cartilage disease and from a donor with RA. Also in cells from these donors, Wnt3A inhibited MMP1, -3 and -13 expression (Fig. 1). Similar results were obtained using human fetal chondrocytes excluding an age-related effect (Fig. 1). Finally, activation of canonical Wnt signaling in multipotent human mesenchymal stem cells (hMSCs) which were able to differentiate into chondrocytes also reduced MMP mRNA expression (Fig. 1), suggesting that a decrease in MMP1, -3 and -13 mRNA expression upon activation of canonical Wnt signaling is a conserved response in various human cell types. In
conclusion, these data point to a remarkable species difference in regulation of MMP mRNA expression by Wnt/β-catenin signaling in human and animal chondrocytes.

**Wnt/β-catenin signaling inhibits IL-1β-induced MMP expression and activity in human chondrocytes.** IL-1β is a potent activator of MMP expression in human chondrocytes and has been implicated in cartilage degradation in OA (6-9). To test the effect of canonical Wnt signaling on IL-1β-induced MMP1 and -13 mRNA expressions, human chondrocytes were co-stimulated with IL-1β and Wnt3A or BIO. As expected, IL-1β potently induced expression of MMP1 and -13 at the mRNA and proMMP1 and -13 at the protein level (Fig. 2A). Co-stimulation with Wnt3A or BIO blocked, while co-incubation with DKK-1 further increased IL-1β-induced MMP expression (Fig. 2A). Both Wnt3A and BIO inhibited proMMP1 and -13 protein expression under basal conditions and after co-stimulation with IL-1β (Fig. 2B). The inhibitory effect of Wnt3A on MMP1 and -13 mRNA and protein expression was associated with a decrease in generic MMP activity secreted in the culture media and in cell extracts. This effect was observed in both basal conditions and after co-treatment with IL-1β (Fig. 2C). BIO did not decrease generic MMP activity in basal conditions but inhibited IL-1β-induced MMP activity. These data suggest that activation of Wnt/β-catenin signaling in human chondrocytes negatively regulates the expression and activity of a set of MMP family members in both basal conditions and after stimulation by IL-1β.

**Crosstalk of Wnt/β-catenin and IL-1β signaling pathways in human chondrocyte.** The potentiating effect of DKK-1 on MMP1- and -13 expression in human chondrocytes in both basal and IL-1β stimulated conditions suggested the presence of endogenous canonical Wnt family members in human chondrocytes repressing MMP expression.
Using a qPCR survey, we identified relatively abundant mRNA expression of the non-canonical Wnt5A and lower expression levels of the non-canonical Wnt4, -5B, -9A and the canonical Wnt7B (Supplemental Table 2). Wnt7B was the only canonical Wnt upregulated by IL-1β. The mRNA expression of Wnt7B peaked at 24hrs after IL-1β stimulation, which coincided with the peak in MMP1 and MMP13 mRNA expression and after which both MMP1 and -13 mRNA and Wnt7B mRNA started to decrease. Interestingly, IL-1β treatment simultaneously downregulated mRNA expression of several canonical Wnt signaling inhibitors including DKK-1, FRZB and WIF-1 (Fig. 3A). DKK1 and WIF1 mRNA expression started to decrease from 4 hours after stimulation and reached the lowest expression levels at 24 hours (Fig. 3A). The decrease in FRZB mRNA expression was more gradual (Fig. 3A). As a consequence of the opposite effects on the expression of Wnt7B and its antagonists, β-catenin protein accumulated in both nuclear and cytoplasmic compartments upon stimulation of human chondrocytes with IL-1β (Fig. 3B).

Lentiviral overexpression of Wnt7B as well as Wnt3, another canonical Wnt not detected in the qPCR survey as control, inhibited MMP1 and -13 mRNA expression like stimulation with Wnt3A in human chondrocytes (Fig. 3C and Supplemental Fig. 2A). Wnt7B and Wnt3 stimulated the expression of the canonical Wnt target gene Axin2 (Supplemental Fig. 2B). Knockdown of Wnt7B in human chondrocytes increased MMP1 and -13 mRNA expressions in both basal condition and after stimulation with IL-1β (Fig. 3D and Supplemental Fig. 2C). Taken together, these data suggest that Wnt7B is a likely candidate for the endogenous canonical Wnt negatively regulating MMP expression in human chondrocytes. Furthermore, these data point to a role of canonical Wnt signaling
as a negative feedback loop controlling MMP expression downstream of IL-1β receptor activation.

**TCF4 and LEF1 are not involved in Wnt-mediated repression of MMP expression in human chondrocytes.** Since Wnt/β-catenin showed an inhibitory effect on MMP expression, we next tested if knockdown of β-catenin by lentiviral shRNA expression could restore MMP expression levels. The knockdown efficiently decreased β-catenin expression (Supplemental Fig. 2D) and strongly elevated MMP expression levels (Fig. 4A&B). The inhibitory effect of Wnt3A was almost completely eliminated by β-catenin knockdown (Fig. 4A&B).

Previous studies suggested that the transcription factors TCF4 and LEF1 acting together with β-catenin might act as transcriptional repressors of target gene expression (28-30). We therefore examined the roles of TCF4 and LEF1 in regulation of MMP expression. TCF4 and LEF1 were effectively depleted in human chondrocytes by lentiviral shRNA-mediated knockdown (Supplemental Fig. 2E-G). The knockdown of both genes showed minimal effects on MMP expression (Fig. 4C), suggesting that the repression of MMP expression in human chondrocytes is not mediated by the conventional complex of β-catenin and TCF/LEF opening the possibility that MMP repression is mediated by β-catenin independently of its DNA binding partners, for example by crosstalk with other pathways involved in MMP expression. In contrast, β-catenin, TCF4 and LEF1 were required for Wnt3A-induced MMP expression in mouse embryonic fibroblasts (MEFs) as knockdown of all three genes eliminated the pro-catabolic effect of Wnt3A on MMP13 expression (Fig. 4D and Supplemental Fig. 2H-K) suggesting the involvement of the β-catenin:TCF/LEF complex in regulation of MMP expression in mouse cells (31).
β-Catenin suppresses MMP expression through an inhibitory interaction with NF-κB. It has been shown that IL-1β-induced expression of MMP1 and -13 in human chondrocytes is dependent on NF-κB activation (7-9). In addition, an interaction between β-catenin and NF-κB has been recently described in various human cell types (32-34). We therefore tested whether an interaction between NF-κB and β-catenin might explain β-catenin-mediated inhibition of MMP expression in human chondrocytes. In agreement with previous studies, knockdown of RELA/NF-κB p65 abrogated the expression of basal and IL-1β-induced MMP1 and MMP13 mRNA expression, while knockdown of NF-κB p50, the active form of NFkB1, only diminished expression of MMP13 while MMP1 mRNA expression was increased (Fig. 5A and Supplemental Fig. 3A&B) (7-9). This increase in MMP1 mRNA was not reflected in an increase in MMP1 protein expression, which is most likely explained by a lag phase between an increase in mRNA expression and protein expression (Fig. 5A). MMP3 expression was regulated by both p50 and p65 (Supplemental Fig. 3C). We next examined the effect of Wnt3A on NF-κB activity, using lentiviral transduction of a NF-κB responsive promoter reporter construct in human chondrocytes. Treatment of Wnt3A significantly decreased basal and IL-1β-induced NF-κB reporter activity (Fig. 5B). This effect was dependent on β-catenin, since knockdown of β-catenin abolished a Wnt3A-induced decrease in NF-κB promoter reporter activity and enhanced IL-1β-induced reporter activation (Fig. 5B). Using a co-immunoprecipitation (Co-IP) assay, we found that β-catenin was able to form a protein complex with NF-κB p65 in human chondrocytes under basal condition (Fig. 5C). The interaction between β-catenin and NF-κB p65 was weakened upon activation of NF-κB by IL-1β and strengthened after Wnt3A induced stabilization of β-catenin. Our data
suggest that activation of NF-κB signaling by IL-1β in human chondrocytes may require dissociation of β-catenin from the NF-κB signaling complex. Co-immunoprecipitation of β-catenin with NF-κB p65 was also found in bovine chondrocytes and mouse embryonic fibroblasts (Supplemental Fig. 4A). Wnt3A was able to decrease basal and IL-1β-induced NF-κB activity in MEFs (Supplemental Fig. 4B), suggesting that the interaction between β-catenin and NF-κB alone may not explain the species differentiation in the regulation of MMP expression by Wnt/β-catenin signaling.

We next tested whether canonical Wnt signaling could downregulate other NF-κB target genes. We tested this hypothesis by evaluating the effect of Wnt3A treatment on expression of IL-6 and SERPINA1, two established target genes of the NF-κB pathway in human chondrocytes (Supplemental Fig. 3C). The mRNA expression of both genes was upregulated by IL-1β but downregulated by stimulation with Wnt3A (Fig. 5D). Wnt3A treatment was also able to decrease IL-1β-induced IL6 mRNA expression but failed to significantly counteract the effect of IL-1β on SERPINA1 mRNA expression (Fig. 5D). These results suggest that Wnt/β-catenin signaling negatively regulates a subset of NF-κB target genes most likely via an inhibitory protein-protein interaction with NF-κB.
Discussion

Cumulating evidence, mainly based on experimental animal models for OA, has suggested an important role for Wnt/β-catenin signaling in the pathogenesis of OA by driving, amongst others, hypertrophic differentiation of chondrocytes and the expression of matrix degrading MMPs in articular cartilage. Here we propose that this hypothesis requires revisiting in human osteoarthritis. In remarkable contrast to animal models, we provide evidence that in human chondrocytes Wnt/β-catenin signaling is a potent inhibitor of MMP1, -3 and -13 mRNA and protein expression and is part of a negative feedback loop counteracting pro-catabolic NF-κB signaling in human chondrocytes activated by pro-inflammatory cytokines. Thus in human articular cartilage β-catenin may have an anti-catabolic role by inhibiting MMP expression opposed to its established pro-catabolic role in animal cartilage. Similar observations were made in human chondrocytes from OA, RA and healthy adult donors as well as in human fetal chondrocytes and adult bone marrow-derived MSCs, indicating that the inhibitory response of Wnt/β-catenin on MMP expression is conserved across various human mesenchymal cell types, irrespective of age and disease status.

It has been shown that IL-1β induces Wnt7A expression and β-catenin accumulation in rabbit chondrocytes (13). We did not detect significant Wnt7A expression in human chondrocytes. However, we have found that IL-1β increased Wnt7B expression and decreased expression of several Wnt inhibitors in human chondrocytes. Together, their concerted action may be responsible for the indirect increase in β-catenin protein levels after IL-1β stimulation. Differential expression of Wnt proteins has previously been shown in joint diseases. In particular Wnt7B was found significantly upregulated in OA
cartilage (17). We showed that overexpression of Wnt7B repressed MMP expression while knockdown of Wnt7B enhanced MMP expression. This implies that upregulation of Wnt7B as observed in OA cartilage may be considered as an attempt of human chondrocytes to reduce the expression of MMPs to slow down matrix degradation. Moreover, we provide evidence that Wnt7B might be a driving factor in an anti-catabolic negative feedback loop induced by pro-catabolic IL-1 signaling, although we cannot exclude roles for other canonical Wnts. In addition, IL-1β-induced loss of Wnt inhibitor expression is another import mechanism for negative feedback on MMP expression as DKK-1 enhances both basal and IL-1β-induced MMP expression. These inhibitors may act independently of the change in Wnt7B, or, based on time course experiments showing that the IL-1β-induced increase in Wnt7B expression coincides with a decrease in the expression of Wnt antagonists, may potentiate the effect of increased Wnt7B expression.

It has been shown that the interaction of β-catenin with TCF/LEF transcription factors can either stimulate or repress transcription in a promoter and context dependent manner (28-30). In mouse chondrocytes β-catenin and TCF/LEF transcription factors are required for induction of MMP13 expression (31). Indeed knockdown of either TCF4 or LEF1 abrogates Wnt3A-induced MMP expression in mouse embryonic fibroblasts. The observation that knockdown of TCF4 or LEF1 did not relieve the β-catenin-mediated repression of MMP expression in human chondrocytes, clearly demonstrates that the canonical transcriptional function of β-catenin is not involved in inhibiting MMP expression in these cells. These data in mouse and human cells pinpoint the species specific regulation of MMP expression to a differential role of TCF/LEF transcription factors in MMP expression regulation. Why humans in evolution have lost the propensity
to regulate MMP3 and -13 expression by the β-catenin:TCF/LEF transcription complex remains elusive.

Previous studies have shown that β-catenin inhibits NF-κB-mediated signaling in human cancer cells by a protein-protein interaction between β-catenin and NF-κB (32-34). In human chondrocytes, it is well known that activation of NF-κB signaling by pro-inflammatory cytokines like IL-1β potently induces cartilage matrix degradation by stimulating the expression and activity of various MMPs. We therefore tested whether crosstalk between β-catenin and NF-κB could explain the inhibitory effects of canonical Wnt signaling on MMP expression in human chondrocytes. We extend the observations made in cancer cells to human chondrocytes and show that activated β-catenin signaling can inhibit a number of NF-κB target genes. We furthermore show that activation of NF-κB weakens the protein-protein interaction while stabilization of β-catenin strengthens the interaction between NF-κB and β-catenin. These data strongly suggest that the TCF/LEF-independent repressive effect of β-catenin on MMP expression is mediated by negative cross-regulation of the NF-κB signaling pathway. Remarkably, co-immunoprecipitation assays using mouse embryonic fibroblasts and bovine chondrocytes demonstrate the presence of a protein complex between NF-κB and β-catenin in these cells. In addition, we showed that Wnt3A was able to decrease NF-κB reporter activity in mouse cells. These data suggest that mouse β-catenin was able to inhibit NF-κB-mediated signaling and that this interaction cannot explain the species difference in regulation of MMP expression by canonical Wnt signaling. Our data suggest that in animal cells the canonical Wnt/β-catenin signaling pathway via TCF/LEF prevails over the inhibitory
effects on NF-κB signaling in regulation of MMP-3 and -13 expressions in remarkable contrast to human cells in which the non-canonical effect on NF-κB is dominant.

There has been longstanding debate whether animal models are suited for studying the pathogenesis of OA and it is believed that none of the current models can recapitulate all facets of human disease. Our observation of differential regulation of MMP expression by Wnt/β-catenin signaling may explain part of this discrepancy. Although evolutionarily conserved, our data indicate that the Wnt/β-catenin pathway is able to activate distinct subsets of target genes in a species-dependent manner which is at least partly explained by differential usage of canonical and non-canonical β-catenin signaling. We propose that differential usage of crosstalk of signaling networks may be responsible for species differences in cellular responses to Wnt and other extracellular signaling pathways (26, 35). Our findings suggest that animal models may not be suited for studying the role of Wnt/β-catenin signaling in human OA and questions whether strategies aimed at inhibiting β-catenin in chondrocytes will be successful in the management of OA.

Recently, it has been shown that Wnt3A can modulate the human articular chondrocyte phenotype by activating both β-catenin-dependent and -independent pathways (27). In line with our study, Nalesso et al. showed that chondrocyte proliferation and inhibition of MMP13 expression is β-catenin-dependent. In contrast, effects of Wnt3A on the expression of chondrocyte markers are β-catenin-independent. These data are supported by the observation that the non-canonical Wnt5A potently inhibits Col2A1 expression and induces MMP expression (36, 37). Taken together, these and our data suggest prominent roles for both canonical and non-canonical Wnt signaling...
in articular chondrocytes though the canonical pathway might not be a key event in the pathogenesis of human OA.

In summary, our study unravels an unexpected and novel role of Wnt/β-catenin signaling in human articular chondrocytes with respect of the expression of MMPs (Fig. 6). During OA development, β-catenin signaling is upregulated in human cartilage. This is at least partly due to increased Wnt7B expression and/or downregulation of Wnt inhibitors and can be further augmented by the activity of pro-inflammatory cytokines like IL-1β. Accumulated β-catenin in turn represses NF-κB activity and consequently expression of MMPs in human chondrocytes. Thus Wnt/β-catenin signaling is part of a negative feedback loop counteracting pro-catabolic NF-κB in osteoarthritis.
References


Figure Legends

**Figure 1.** Effect of Wnt/β-catenin activation on MMP mRNA expression. Human articular chondrocytes from 4 OA donors (each tested in triplicate) were stimulated with 200 ng/ml rhWnt3A or 1 µM BIO, or left untreated (control, CTL) for 48 hours. Mouse cartilage explants (n = triplicate cultures) were treated with 200 ng/ml rmWnt3A or 1 µM BIO for 5 days and bovine chondrocytes (n = triplicate cultures) were treated with 1 µM BIO for 48 hours. Human cartilage explants (n = triplicate cultures from one donor) were treated for 5 days and normal human articular chondrocytes, human MSCs, human RA chondrocytes and human fetal chondrocytes (n = triplicate cultures from one donor) were treated for 48 hours with 200 ng/ml rhWnt3A. mRNA expression was measured by qPCR. Data are expressed as mean fold change compared to CTL. *p<0.05, **p<0.01.

**Figure 2.** Wnt/β-catenin signaling reduces IL-1β-induced MMP expression and activity. **A,** Human chondrocytes were treated for 48 hours with 10 ng/ml IL-1β or IL-1β in combination with 200 ng/ml rhWnt3A, 1 µM BIO or 300 ng/ml DKK-1. MMP1 and -13 mRNA expression was measured by qPCR. *p<0.05, **p<0.01, n = 3 donors each tested in triplicate. **B,** Human chondrocytes were treated for 48 hours with 200 ng/ml rhWnt3A, 1 µM BIO and 10 ng/ml IL-1β and proteins were isolated from cell extracts. pro-MMP1 and pro-MMP13 were detected by immunoblot. **C,** Culture media (left) and cell lysates (right) of human chondrocytes were collected for generic MMP activity measurements after treatment with 200 ng/ml rhWnt3A, 1 µM BIO and 10 ng/ml IL-1β or combinations thereof for 72 hours. *p<0.05, **p<0.01, n = 3 donors each tested in triplicate.

**Figure 3.** Crosstalk of Wnt/β-catenin and IL-1β pathways. **A,** Time course evaluation of mRNA expression after stimulation of human chondrocytes treated with 10 ng/ml IL-1β.
mRNA expression of Wnt7B, MMP1 and -13 and the Wnt antagonists DKK1, FRZB and WIF1 was evaluated at indicated time points by qPCR. Data represent the mean fold change relative to non-treated cells which was set to 1. Not statistically significant (4hr: FRZB; 12hr: FRZB), p<0.05 (4hr: MMP1, MMP13, DKK1, WIF1; 72hr: WNT7B, MMP13), p<0.01 (other time points), n = 3 donors each tested in triplicate. B, Human chondrocytes were stimulated with 20 ng/ml IL-1β for 48 hours and nuclear and cytoplasmic β-catenin protein expression was detected by immunoblot. C, Human Wnt3 and Wnt7B were overexpressed using lentiviral transduction in human chondrocytes and MMP mRNA expression was measured by qPCR. Data are expressed using lentiviral GFP-transduced cells as control (Lenti-GFP). D, Wnt7B was knocked down by lentivirus-mediated expression of shRNA in human chondrocytes using scrambled shRNA (shScr) as control. MMP1 and -13 mRNA expression was measured 24 hours after stimulation with 10 ng/ml IL-1β and compared to control (CTL). n = triplicate cultures from one donor (C&D).

**Figure 4.** Effects of β-catenin, TCF4 and LEF1 knockdown. A-C, Human chondrocytes were transduced with lentiviruses expressing scrambled shRNA (shScr) and shRNA (sh) against human β-catenin (shCat) (A&B), TCF4 and LEF1 (C) and treated with 50 ng/ml rhWnt3A for 24 hours. MMP mRNA expression was measured by qPCR and expressed as fold change relative to control (CTL) (A&C) and proteins were detected by immunoblot (B). *p<0.05, **p<0.01, N.S. = Not Significant, n = 3 donors each tested in triplicate. D, MEFs were transfected with negative control siRNA (siNeg) and siRNA (si) against β-catenin (siCat), TCF4 and LEF1, and then treated with or without 200 ng/ml
rmWnt3A for 48 hours. MMP13 mRNA expression was measured by qPCR. n = triplicate cultures.

**Figure 5.** β-Catenin suppresses MMP expression through an inhibitory interaction with NF-κB. **A,** MMP mRNA was assessed by qPCR and protein expression was detected by immunoblot in human chondrocytes transduced with lentiviruses expressing scrambled shRNA (shScr) and shRNA against NFKB1 and RELA after treatment with 10 ng/ml IL-1β for 24 hours. qPCR data are expressed as fold change using untreated shScr-transduced cells as control. n = triplicate cultures from one donor. **B,** Human chondrocytes were transduced with lentiviruses expressing an NF-κB reporter and Renilla luciferase only or together with lentiviruses expressing scrambled shRNA and shRNA against β-catenin. Chondrocytes were treated with 100 ng/ml rhWnt3A, 5 ng/ml IL-1β or both for 24 hours and luciferase reporter activity was measured. Data are expressed as fold change compared to control (CTL). **p<0.01, n = 3 donors each tested in triplicate.** **C,** Protein lysates of human chondrocytes stimulated with 100 ng/ml rhWnt3A, 5 ng/ml IL-1β or both for 48 hours were isolated and subjected to immunoprecipitation using NF-κB p65 antibody as bait. Co-immunoprecipitated proteins were detected by immunoblot. **D,** Human chondrocytes were treated with 200 ng/ml rhWnt3A, 10 ng/ml IL-1β or both for 48 hours. IL6 and SERPINA1 mRNA expression was measured by qPCR. *p<0.05, **p<0.01, N.S. = Not Significant, n = 4 donors each tested in triplicate.

**Figure 6.** A Wnt/β-catenin negative feedback loop inhibits IL-1β-induced MMP expression in human chondrocytes. **A,** In animal cartilage, IL-1β indirectly activates canonical Wnt signaling by upregulation of Wnt ligands. Subsequently, the transcription complex of β-catenin with TCF/LEF induces the expression of MMP3 and -13 leading to
cartilage destruction. B, In human articular cartilage, IL-1β upregulates MMP1, -3 and -13 expression predominantly through activation of the NF-κB pathway resulting in cartilage degradation. Simultaneously IL-1β indirectly activates β-catenin through upregulation of Wnt7B expression and downregulation of canonical Wnt inhibitors like DKK-1, FRZB and WIF-1. Stabilized β-catenin interacts with and inhibits NF-κB which leads to the suppression of MMP expression in a negative feedback loop. It remains unclear whether the β-catenin pathway is directly involved in human cartilage degeneration.
Figure 1. Effect of Wnt/β-catenin activation on MMP mRNA expression. Human articular chondrocytes from 4 OA donors (each tested in triplicate) were stimulated with 200 ng/ml rhWnt3A or 1 µM BIO, or left untreated (control, CTL) for 48 hours. Mouse cartilage explants (n = triplicate cultures) were treated with 200 ng/ml rmWnt3A or 1 µM BIO for 5 days and bovine chondrocytes (n = triplicate cultures) were treated with 1 µM BIO for 48 hours. Human cartilage explants (n = triplicate cultures from one donor) were treated for 5 days and normal human articular chondocytes, human MSCs, human RA chondrocytes and human fetal chondrocytes (n = triplicate cultures from one donor) were treated for 48 hours with 200 ng/ml rhWnt3A. mRNA expression was measured by qPCR. Data are expressed as mean fold change compared to CTL. *p<0.05, **p<0.01.

114x156mm (300 x 300 DPI)
Figure 2. Wnt/β-catenin signaling reduces IL-1β-induced MMP expression and activity. A, Human chondrocytes were treated for 48 hours with 10 ng/ml IL-1β or IL-1β in combination with 200 ng/ml rhWnt3A, 1 µM BIO or 300 ng/ml DKK-1. MMP1 and -13 mRNA expression was measured by qPCR. *p<0.05, **p<0.01, n = 3 donors each tested in triplicate. B, Human chondrocytes were treated for 48 hours with 200 ng/ml rhWnt3A, 1 µM BIO and 10 ng/ml IL-1β and proteins were isolated from cell extracts. pro-MMP1 and pro-MMP13 were detected by immunoblot. C, Culture media (left) and cell lysates (right) of human chondrocytes were collected for generic MMP activity measurements after treatment with 200 ng/ml rhWnt3A, 1 µM BIO and 10 ng/ml IL-1β or combinations thereof for 72 hours. *p<0.05, **p<0.01, n = 3 donors each tested in triplicate.

126x190mm (300 x 300 DPI)
Figure 3. Crosstalk of Wnt/β-catenin and IL-1β pathways. A, Time course evaluation of mRNA expression after stimulation of human chondrocytes treated with 10 ng/ml IL-1β. mRNA expression of Wnt7B, MMP1 and -13 and the Wnt antagonists DKK1, FRZB and WIF1 was evaluated at indicated time points by qPCR. Data represent the mean fold change relative to non-treated cells which was set to 1. Not statistically significant (4hr: FRZB; 12hr: FRZB), p<0.05 (4hr: MMP1, MMP13, DKK1, WIF1; 72hr: WNT7B, MMP13), p<0.01 (other time points), n = 3 donors each tested in triplicate. B, Human chondrocytes were stimulated with 20 ng/ml IL-1β for 48 hours and nuclear and cytoplasmic β-catenin protein expression was detected by immunoblot. C, Human Wnt3 and Wnt7B were overexpressed using lentiviral transduction in human chondrocytes and MMP mRNA expression was measured by qPCR. Data are expressed using lentiviral GFP-transduced cells as control (Lenti-GFP). D, Wnt7B was knocked down by lentivirus-mediated expression of shRNA in human chondrocytes using scrambled shRNA (shScr) as control. MMP1 and -13 mRNA expression was measured 24 hours after stimulation with 10 ng/ml IL-1β and compared to control (CTL). n = triplicate cultures from one donor (C&D).

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Figure 4. Effects of β-catenin, TCF4 and LEF1 knockdown. A-C, Human chondrocytes were transduced with lentiviruses expressing scrambled shRNA (shScr) and shRNA (sh) against human β-catenin (shCat) (A&B), TCF4 and LEF1 (C) and treated with 50 ng/ml rhWnt3A for 24 hours. MMP mRNA expression was measured by qPCR and expressed as fold change relative to control (CTL) (A&C) and proteins were detected by immunoblot (B). **p<0.01, n = 3 donors each tested in triplicate. D, MEFs were transfected with negative control siRNA (siNeg) and siRNA (si) against β-catenin (siCat), TCF4 and LEF1, and then treated with or without 200 ng/ml rmWnt3A for 48 hours. MMP13 mRNA expression was measured by qPCR. n = triplicate cultures.

94x50mm (300 x 300 DPI)
Figure 5. β-catenin suppresses MMP expression through an inhibitory interaction with NF-κB. A, MMP mRNA was assessed by qPCR and protein expression was detected by immunoblot in human chondrocytes transduced with lentiviruses expressing scrambled shRNA (shScr) and shRNA against NFKB1 and RELA after treatment with 10 ng/ml IL-1β for 24 hours. qPCR data are expressed as fold change using untreated shScr-transduced cells as control. n = triplicate cultures from one donor. B, Human chondrocytes were transduced with lentiviruses expressing an NF-κB reporter and Renilla luciferase only or together with lentiviruses expressing scrambled shRNA and shRNA against β-catenin. Chondrocytes were treated with 100 ng/ml rhWnt3A, 5 ng/ml IL-1β or both for 24 hours and luciferase reporter activity was measured. Data are expressed as fold change compared to control (CTL). **p<0.01, n = 3 donors each tested in triplicate. C, Protein lysates of human chondrocytes stimulated with 100 ng/ml rhWnt3A, 5 ng/ml IL-1β or both for 48 hours were isolated and subjected to immunoprecipitation using NF-κB p65 antibody as bait. Co-immunoprecipitated proteins were detected by immunoblot. D, Human chondrocytes were treated with 200 ng/ml rhWnt3A, 10 ng/ml IL-1β or both for 48 hours. IL6 and SERPINA1 mRNA expression was measured by qPCR. *p<0.05, **p<0.01, N.S. = Not Significant, n = 4 donors each tested in triplicate.
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