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WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes

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Wnt-1 inducible signaling pathway protein 2 (WISP-2/CCN5) is a recently identified adipokine that has been described as an important mediator of canonical Wnt activation in adipogenic precursor cells. In osteoarthritis (OA), the most common form of arthritis, chondrocytes exhibit aberrant and increased production of pro-inflammatory mediators and matrix degrading enzymes such as IL-1β and MMP-13. Although recent evidence suggests a role for Wnt signaling in OA physiopathology, little is known about the involvement of WISP-2 in cartilage degradation. In the present study, we determined the expression of WISP-2 in healthy and OA human chondrocytes. WISP-2 expression is modulated along chondrocyte differentiation and downregulated at the onset of hypertrophy by inflammatory mediators. We also investigated the effect of WISP-2 on cartilage catabolism and performed WISP-2 loss-of-function experiments using RNA interference technology in human T/C-28a2 immortalized chondrocytes. We demonstrated that recombinant human WISP-2 protein reduced IL-1β-mediated chondrocyte catabolism, that IL-1β and WNT/b-catenin signaling pathways are involved in rhWISP-2 protein and IL-1β effects in human chondrocytes, and that WISP-2 has a regulatory role in attenuating the catabolic effects of IL-1β in chondrocytes. Gene silencing of WISP-2 increased the induction of the catabolic markers MMP-13 and ADAMTS-5 and the inflammatory mediators IL-6 and IL-8 triggered by IL-1β in human primary OA chondrocytes in a Wnt/β-catenin dependent manner. In conclusion, here we have shown for the first time that WISP-2 may have relevant roles in modulating the turnover of extracellular matrix in the cartilage and that its downregulation may detrimentally alter the inflammatory environment in OA cartilage. We also proved the participation of Wnt/β-catenin signaling pathway in these processes. Thus, targeting WISP-2 might represent a potential therapeutical approach for degenerative and/or inflammatory diseases of musculoskeletal system, such as osteoarthritis.

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INTRODUCTION

Osteoarthritis (OA) is a multifactorial joint degenerative disease characterized by progressive destruction of articular cartilage, changes in subchondral bone, osteophyte formation, and synovial inflammation[1]. It is the most prevalent type of arthritis, but its etiology is still incomplete[2,3]. Recently, inflammation has been recognized as contributing to the symptoms and progression of OA[4]. Chondrocytes, as the only resident cells in articular cartilage, preserve the integrity of the cartilage itself. However, during OA, high mechanical stress, extracellular matrix (ECM) degradation products, pro-inflammatory cytokines, and adipokines activate chondrocytes to stimulate the production of inflammatory mediators, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α and degradative enzymes, such as matrix metalloproteinase (MMP)-13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5[2]. In addition, IL-1β and TNF-α also induce other pro-inflammatory cytokines, such as IL-6, and chemokines, like IL-8.

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These mediators act synergistically to promote and perpetuate chondrocyte catabolic responses[2].

Recent evidence from animal experiments and clinical studies highlights a role for Wnt signaling in OA pathology[5]. Progressive joint destruction in OA has been associated with overactivation of Wnt signaling[6]. The canonical Wnt pathway is initiated by the binding of Wnt ligands to frizzled receptors and co-receptors (LRP5/6), causing the inactivation of glycogen synthase kinase-3β (GSK-3β), which leads to intracellular β-catenin accumulation and nuclear translocation. Once in the nucleus, β-catenin interacts with the LEF/TCF family of transcription factors and activates Wnt target genes[7]. GSK-3β phosphorylation is a key step, and inhibition of this enzyme can lead to β-catenin stabilization and initiation of target gene expression independent of Wnt binding. Wnt ligands as Wnt-7b and Wnt target genes including Wnt-1 inducible signaling pathway protein 1 (WISP-1)[5] were found to be upregulated in OA cartilage, as well as nuclear β-catenin and the co-receptor LRP5[8]. Pro-catabolic factors such as IL-1β induced the expression of various Wnt proteins, resulting in the activation of β-catenin[9], and activation of Wnt/β-catenin signaling stimulated the expression of cartilage ECM-degrading MMPs[10].

Wnt-1 inducible signaling pathway protein 2 (WISP-2), also named CCN5, is a 27 kDa matricellular protein that belongs to the CCN family. WISP-2 is considered a novel adipokine, as it was recently identified in a proteomics analysis of the secretome of human adipose tissue[11]. It is a secreted protein, highly expressed in mesenchymal stem cells (MSCs) and preadipocytes[12]. Grünberg et al. described WISP-2 as an important mediator of canonical WNT activation in adipogenic precursor cells, keeping the adipocytes in an undifferentiated state. They suggest that this adipokine may be involved in the development of obesity-related metabolic complications[13]. Frequently, WISP-2 is used as an indicator of canonical Wnt activation[14]. The identity of the WISP-2 receptor is currently unknown, although a Frizzled receptor would seem a likely possibility since the Frizzled co-receptor LRP5/6 is phosphorylated by WISP-2 in 3T3-L1 adipocytes[13].

To date, little is known about the role of this adipokine in cartilage pathophysiology. It has been demonstrated that WISP-2 expression was downregulated in human OA chondrocytes following sear stress[15]. WISP-2 has been found to be closely related to the pathogenesis of inflammatory arthritis[16] and the modulation of bone turnover[17]. In a previous study, we identified WISP-2 in synovium, infrapatellar fat pad, and chondrocytes obtained from OA patients[18], being its expression in infrapatellar fat pad adipocytes higher in OA patients than in healthy subjects. Therefore, the aim of this study was to analyze WISP-2 expression in OA chondrocytes, its effect on cartilage catabolism using recombinant WISP-2 protein, and loss-of-function experiments and to elucidate the role of WNT/b-catenin pathway.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), human recombinant interleukin (IL)-1β, human recombinant tumor necrosis factor alpha (TNF-α), lipopolysaccharide (LPS), human recombinant IL-6, human recombinant leptin, and glycogen synthase kinase 3 (GSK-3) inhibitor 6-bromoindirubin-3′-oxime (BIO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant adiponectin and visfatin were obtained from BioVendor (Karasek, Brno, Czech Republic). Human recombinant WISP-2 protein (hrWISP-2) was purchased from PeproTech (Rocky Hill, NJ, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, L-glutamine, penicillin-streptomycin mixture, and trypsin-EDTA were purchased from Lonza Group (Basel, Switzerland). Pronase and collagenase P were obtained from Roche Applied Science (Penzberg, Germany).

Cell culture and treatments

The murine chondrogenic cell line ATDC-5 (purchased from RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM–Ham's F-12 medium supplemented with 5% FBS, 10 µg/mL human transferrin, 3×10^{-8} M sodium selenite, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Chondrogenic ATDC-5 cells were differentiated into mature chondrocytes as previously described[19]. Briefly, cells were seeded at a density of 6×10^4 cells per well in six-well plates with ATDC-5 standard medium supplemented with insulin (10 µg/mL). The differentiation medium was replaced every two days for 21 days. Differentiation was qualitatively characterized by increased formation of cell nodules. In other experiments (data not shown), differentiation was further analyzed by a sequential increase in the levels of type II collagen, aggrecan and type X collagen mRNA expression, as previously published[20]. The immortalized human juvenile costal chondrocyte cell line T/C-28a2 (a kind gift from Dr. M.B. Goldring, Hospital for Special Surgery, NYC, USA) was cultured in DMEM–Ham's F-12 medium supplemented with 10% FBS, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Human primary chondrocytes were isolated from healthy, or OA articular cartilage samples obtained from knee and hip joints of patients undergoing total joint replacement surgery. All the procedures were made under informed patient consent and approved by the local ethics committee, according to the declaration of Helsinki. Human primary chondrocytes were isolated and cultured as previously described[21]. They were cultured in DMEM/Ham's F-12 medium supplemented with 10% of FBS, 4 mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Cells were seeded in monolayer up to the high density and used in the first passage of culture to avoid dedifferentiation.

Chondrocytes were seeded in six-well plates (2.5×10^5 cells per well) and treated in serum-free conditions as indicated in each case, after overnight serum starvation. All the treatments concentrations were selected based on previous studies and in the absence of chondrocyte cell toxicity.

Immunocytochemical assays

Immunohistochemistry was automatically performed using an Autostainer-Link 48 immunostainer from Dako-Agilent (Santa Clara, CA, USA). Briefly, the slides were incubated at room temperature in: (1) heat-induced epitope retrieval solution at high pH (Dako-Agilent) for 20 min at 97 °C; (2) human polyclonal antibody to WISP-2 (ABK1-A4795) from Abyntek Biopharma (Derio, Biscay, Spain) at 1:100 for 30 min; (3) EnVision®+ Dual Link System-HRP (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse and goat anti-rabbit immunoglobulins) (Dako-Agilent, K4065) for 20 min; (4) DAB + substrate-chromogen solution (1 mL of substrate buffer solution containing hydrogen peroxide and 20 µL of 3,3′-diaminobenzidine tetrahydrochloride chromogen solution) for 10 min; and (5) EnVision FLEX hematoxylin for 15 min.

RNA isolation and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell culture with TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and NucleoSpin RNA/Protein Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions; and reverse-transcribed using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) at 42 °C for 30 min, followed by 2 min incubation at 95 °C. Then, SYBR-green-based quantitative real-time PCR (RT-qPCR) was performed in a Stratagene MX3005P thermal cycler using a standard protocol (10 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C) with RT^2 SYBR Green qPCR Mastermix and specific PCR primers (Qiagen, Hilden, Germany) (mouse GAPDH, 140 bp, PPM02946E, reference position 309, Gen-Bank accession no. NM_008084.2; human GAPDH, 175 bp, PPH00150E, reference position 1287, Gen-Bank accession no. NM_002046.3; mouse WISP-2, 135 bp, PPM02952F, reference position 1044, Gen-Bank accession no. NM_016873.2; human WISP-2, 123 bp, PPH00981B, reference position 1257, Gen-Bank accession no. NM_003881.2; mouse MMP-13, 88 bp, PPM03675A, reference position 1145, Gen-Bank accession no. NM_008607; human MMP-13, 61 bp, PPH00121B, reference position 1380, Gen-Bank accession no. NM_002427.3; human ADAMTS-5, 170 bp, PPH30803E, reference position 7506-7527, Gen-Bank accession no. NM_011782.2; human ADAMTS-4, 4410 bp, PPH14490A, reference position 2675, Gen-Bank accession no. NM_005099; human IL-6, 98 bp, PPH00560C, reference position 816, Gen-Bank accession no. NM_000600.3; human IL-8, 126 bp, PPH00568A, reference position 326, Gen-Bank accession no. NM_000584.3; human IL-1β, 126 bp, PPH00171C, reference position 574, Gen-Bank accession no. NM_000576.2). No-template controls were included to eliminate any non-specific amplification, and melting curves were generated to ensure a single gene-specific peak. Gene expression changes were determined by the comparative ΔΔCt method in MxPro qPCR Software version 4.10 (Stratagene, La Jolla, CA, USA), expressed

as relative fold change compared to control (C-), and normalized to GAPDH housekeeping gene.

Protein extraction and western blot analysis

After treatment, cells were rapidly washed with ice-cold phosphate-buffered saline and scraped in lysis buffer for protein extraction (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail from Thermo Fisher Scientific (Waltham, MA, USA)). Lysed cells were centrifuged at 14.000 × *g* for 20 min. SDS-PAGE and blotting procedure were performed as previously described[22]. Immunoblots were incubated with the pertinent antibody against (WISP-2 diluted 1:400 (Abyntek Biopharma, Derio, Biscay, Spain); MMP-13, Phospho-GSK-3α/β, GSK-3α/β, Phospho-NF-κβ p65, NF-κβ p65, Phospho-p44/p42 MAPK (Erk1/2), and Phospho-p38 MAPK diluted 1:1000 form Cell Signaling (Danvers, MA, USA); MAPK 1/2 (Erk1/2), p38/SAPK2, Phospho-JNK 1/2, and JNK/SAPK1 diluted 1:1000 from Upstate (Syracuse, NY, USA); ADAMTS-5 diluted 1:500 (Abcam, Cambridge, UK) The immune complexes were detected using anti-rabbit or anti-mouse horseradish-peroxidase-labeled secondary antibodies diluted 1:5000 (both from GE Healthcare, Chicago, IL, USA) and visualized with Immobilon Western Detection kit (Millipore, MA, USA) To confirm equal loading in each sample, the membranes were stripped in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and re-blotted with anti-GAPDH antibody diluted 1:2000 (Sigma Aldrich, MO, USA). In all figures showing images of gels, the bands for each picture were obtained from the same gel, although they may have been spliced for clarity. The images were captured with ChemiDoc MP Imaging System and analyzed with Image Lab 6.0.1 Software, both from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

WISP-2 gene knockdown

For siRNA transfection experiments, T/C-28a2 cells and human primary OA chondrocytes were seeded at 2×10^5 cells per well in six-well plates and incubated overnight with DMEM/Ham's F-12 with 10% FBS. Before transfection, the medium was changed to serum and antibiotics free medium. Transfections were performed using TriFECTa RNAi Kit following the manufacturer's instructions (Integrated DNA Technologies, Coralville, IA, USA). Gene silencing was made with 10 nM of three DsiRNAs that specifically target WISP-2 and 10 nM of nontargeting universal negative control RNA duplex that does not interact with any known sequence. Transfection with siRNA duplexes was performed using the cationic lipid siLentFect Lipid Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's recommendations. Incubation was continued for 48 h after siRNA transfection. WISP-2 gene knockdown was verified at mRNA and protein levels and supported with two control sequences provided by the manufacturer: a TYE 563-labeled transfection control and a positive control DsiRNA that targets the *HPRT1* gene and is prevalidated to give more than 90% knockdown of HPRT (presented as Supplementary Data). At 48 h after transfection, cells were treated with recombinant human IL-1β (0.5 ng/mL) for 24 h. A specific pharmacological inhibitor (BIO 1 μM) was added 1 h before stimulation.

Statistical analysis

Data are reported as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed with GraphPad Prism 9.3.1 software (GraphPad Software, La Jolla, CA, USA). When assuming a normal distribution, we used two-sided unpaired t test with Welch's correction or one-way ANOVA test corrected with Bonferroni's multiple comparison test. When normal distribution was not assumed, we used Mann–Whitney test. *P*-values less than 0.05 were considered significant.

RESULTS

WISP-2 mRNA and protein basal expression along ATDC-5 differentiation

To evaluate whether WISP-2 mRNA and protein levels change along chondrocyte differentiation, we differentiated murine ATDC-5 cells into mature and hypertrophic chondrocytes. As shown in Fig. 1A, WISP-2 mRNA expression increased during the process of differentiation of ATDC-5 cells. This increase is significant after 7 and 14 days of differentiation in comparison to undifferentiated cells (day 0) (Fig. 1A, upper panel). However, WISP-2 mRNA expression dramatically declined at 21 days in hypertrophic

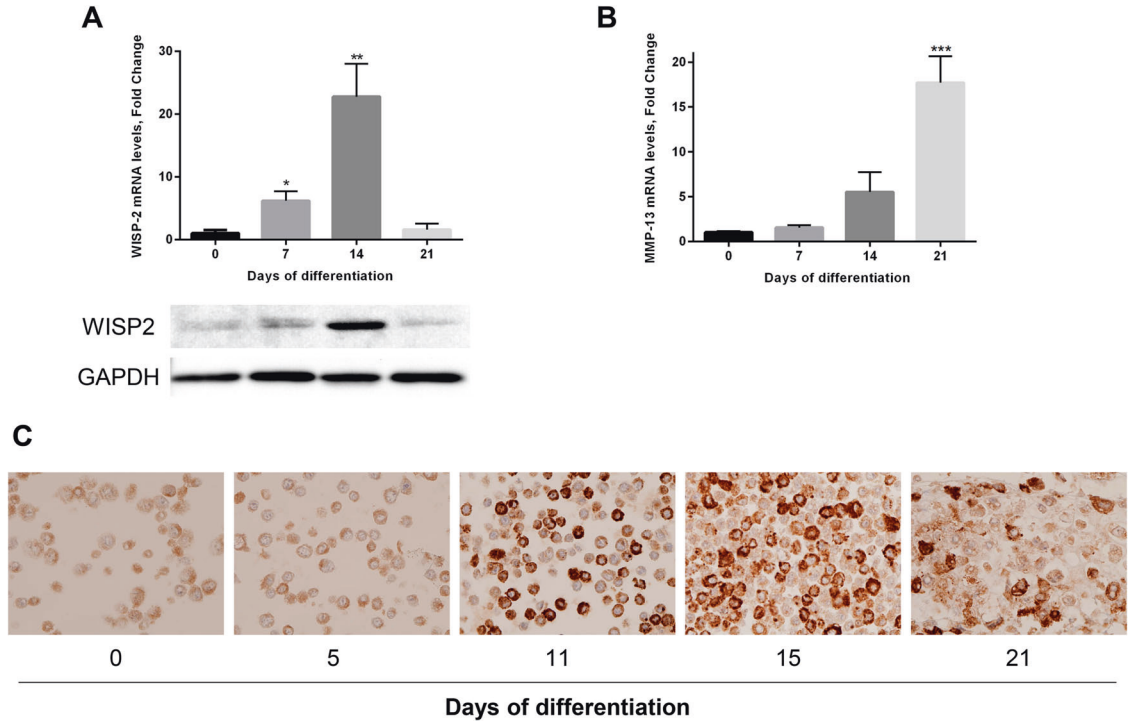


Fig. 1 WISP-2 mRNA and protein constitutive expression along ATDC-5 differentiation. A WISP-2 mRNA and protein expression along ATDC-5 differentiation after 7, 14, and 21 days. Cell lysates underwent western blot analysis using WISP-2 antibody. GAPDH was used as a loading control. Values are the mean ± SEM of at least four independent experiments (*$p < 0.05$, **$p < 0.01$ vs. control). **B** mRNA levels of MMP-13 along ATDC-5 differentiation. Values are the mean ± SEM of at least four independent experiments (***$p < 0.001$ vs. control). **C** Immunocytochemical images showing WISP-2 protein expression in ATDC-5 cells along cell differentiation at 0, 5, 11, 15, and 21 days.

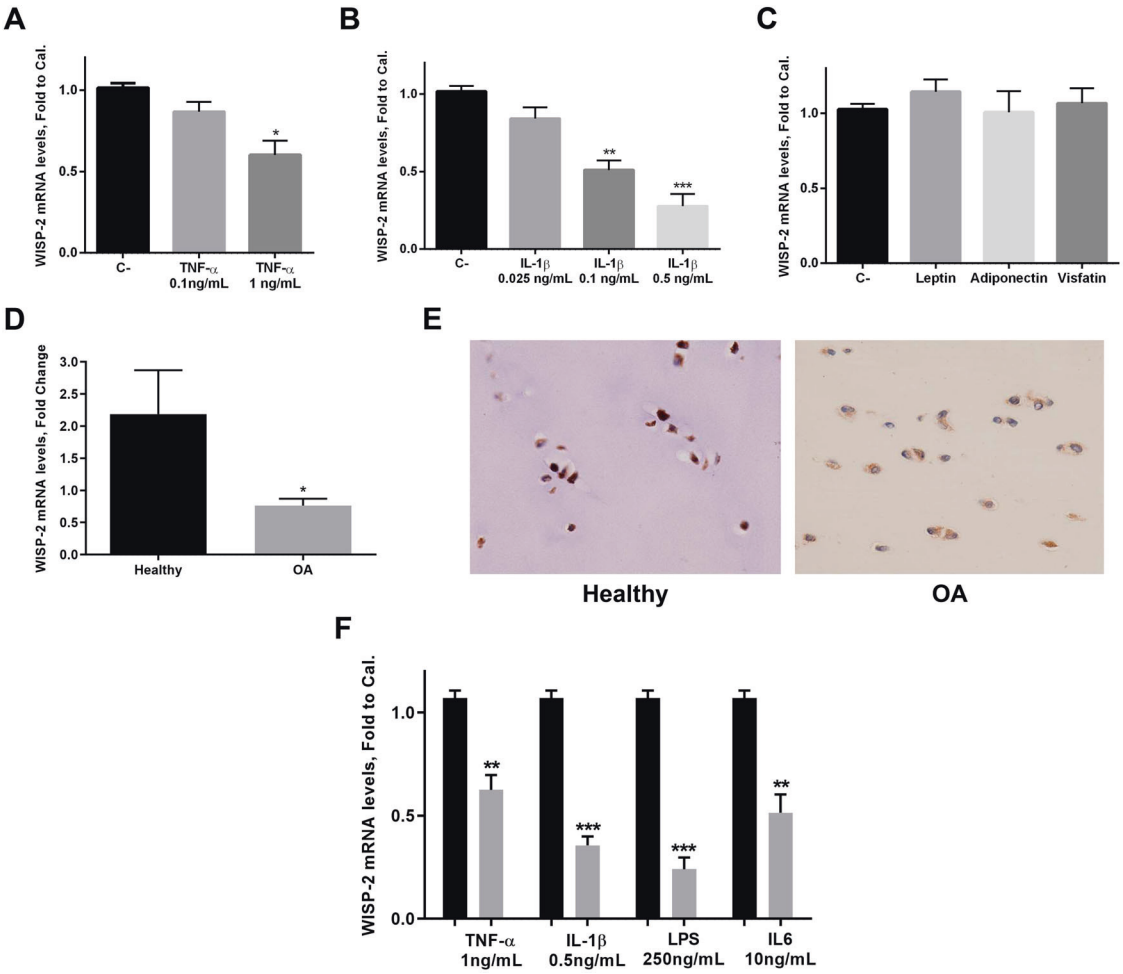


Fig. 2 WISP-2 mRNA and protein expression in human chondrocytes. A Human T/C-28a2 cells were treated with TNF-α 0.1 and 1 ng/mL, **B** IL-1β 0.025, 0.1 and 0.5 ng/mL, and **C** with leptin (800 nM), adiponectin (10 µg/mL) and visfatin (500 ng/mL) for 24 h. **D** WISP-2 mRNA basal expression in healthy and OA human primary chondrocytes. Values are the mean ± SEM of 4 independent human samples for the healthy cartilage, and 8 independent human samples for OA cartilage (*$p < 0.05$). **E** Immunocytochemical images showing WISP-2 protein expression in healthy and OA human cartilage. **F** Human primary OA chondrocytes were treated with TNF-α 1 ng/mL, IL-1β 0.1 ng/mL, LPS 250 ng/mL and IL-6 10 ng/mL for 24 h. WISP-2 mRNA expression was evaluated by RT-qPCR. Values are the mean ± SEM of at least three independent experiments (**$p < 0.01$, ***$p < 0.001$ vs. control).

chondrocytes. This effect was also evaluated in terms of protein expression as reported in Fig. 1A (lower panel). This decrease matches with a significant increment in MMP-13 expression at the same time point in ATDC-5 hypertrophic cells (Fig. 1B). The WISP2 protein expression along ATDC-5 differentiation was also assayed by immunocytochemistry (Fig. 1C)

Effect of pro-inflammatory cytokines and adipokines on WISP-2 mRNA expression in human chondrocytes

To further elucidate the pattern of WISP-2 expression under inflammatory conditions, we treated T/C-28a2 cells with pro-inflammatory cytokines TNF-α and IL-1β and with representative adipokines leptin, adiponectin, and visfatin. As shown in Fig. 2A, cells stimulated with TNF-α 1 ng/mL for 24 h showed a significant inhibition of WISP-2 mRNA expression. A more pronounced effect was observed when cells were stimulated with IL-1β 0.5 ng/mL (Fig. 2B) for 24 h. Nonetheless, neither leptin (800 nM) nor adiponectin (10 µg/mL) or visfatin (500 ng/mL) had significant effects on WISP-2 expression (Fig. 2C).

We also examined the constitutive expression of WISP-2 in human chondrocytes derived from healthy and OA joints. As shown in Fig. 2D, WISP-2 mRNA levels in OA chondrocytes are lower than those observed in chondrocytes obtained from healthy subjects. We further confirmed these results by immunocytochemistry in human healthy and OA cartilage. As shown in Fig. 2E, immunocytochemical techniques showed cytoplasmic positivity for WISP-2 in human chondrocytes. In osteoarthritis cartilage samples the intensity of immunostaining was weak, and the WISP-2 immunoreactivity rate (WISP-2-immunostaine-positive cells divided by total cells) was 59.92 ± 0.76% [Fig. 2 panel E (OA)] In healthy cartilage controls the intensity of immunostaining was stronger, with a WISP-2 immunoreactivity rate of 81.02 ± 0.98% [Fig. 2 panel E (Healthy)].

OA chondrocytes were also stimulated with cytokines TNF-α, IL-1β, IL-6, and an agonist of TLR4, the bacterial lipopolysaccharide (LPS). As shown in Fig. 2F, TNF-α as well as IL-1β, LPS, and IL-6 were able to decrease WISP-2 mRNA expression.

Effect of recombinant human WISP-2 protein and IL-1β stimulation in human chondrocyte catabolism

To elucidate if WISP-2 can interfere in IL-1β-mediated MMPs and aggrecanase production, T/C-28a2 chondrocytes were pre-treated with human WISP2 recombinant protein (500 ng/mL) one hour before being stimulated with IL-1β 0.5 ng/mL for 24 h. Compared to IL-1β-stimulated cells, chondrocytes pre-treated with rhWISP-2

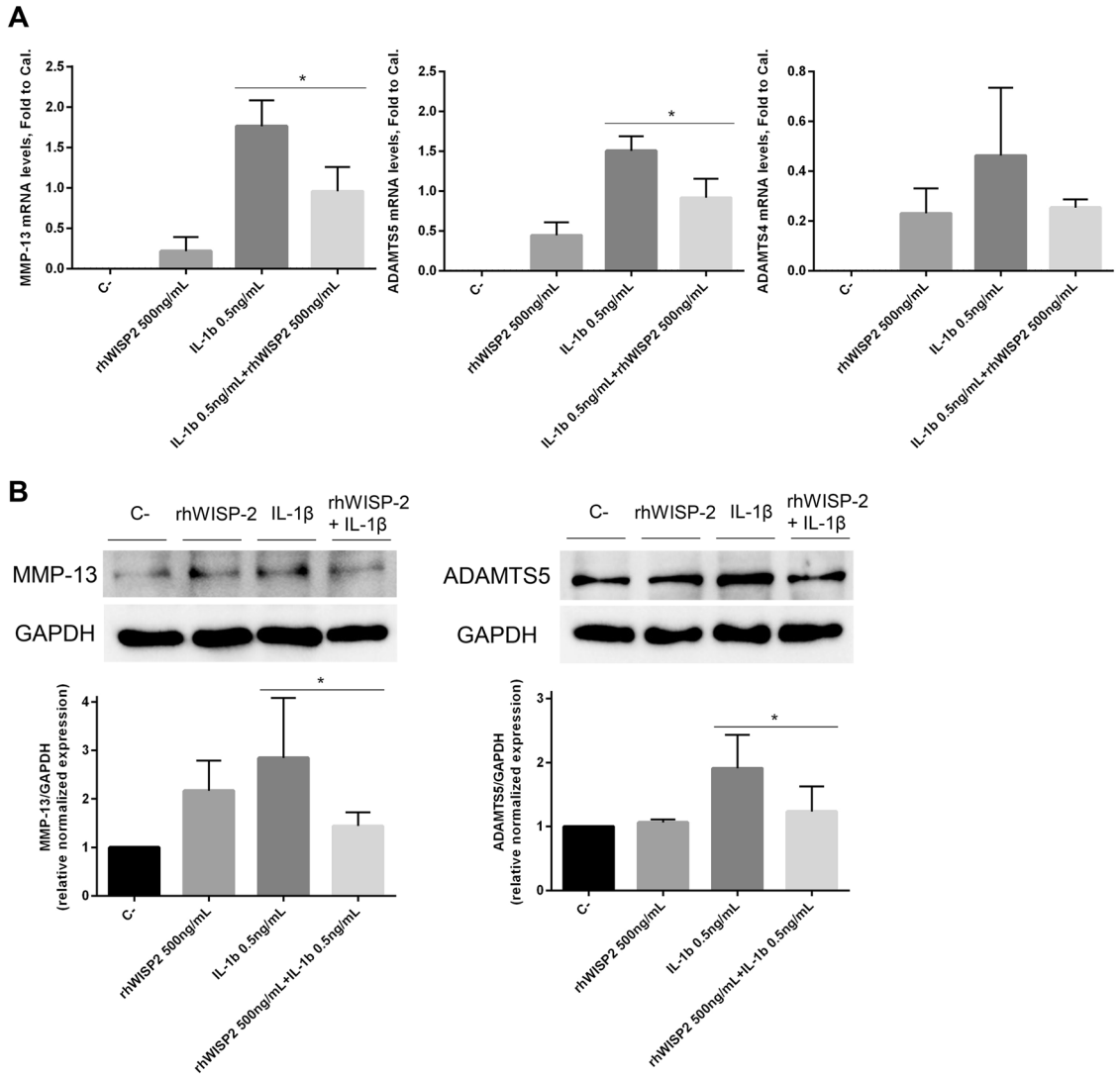


Fig. 3 Recombinant human WISP-2 protein reduces IL-1β-mediated chondrocyte catabolism. Human T/C-28a2 cells were pre-treated with rhWISP-2 (500 ng/mL) for one hour before being challenged with IL-1β 0.5 ng/mL for 24 h. Catabolism markers were evaluated in pre-treated and not pre-treated human chondrocytes. **A** Relative mRNA levels of MMP-13, ADAMTS-5, and ADAMTS-4 in human T/C-28a2 chondrocytes were determined by RT-qPCR. **B** Determination of MMP-13 and ADAMTS-5 protein expression by western blot in human T/C-28a2 chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown (lower panels). Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

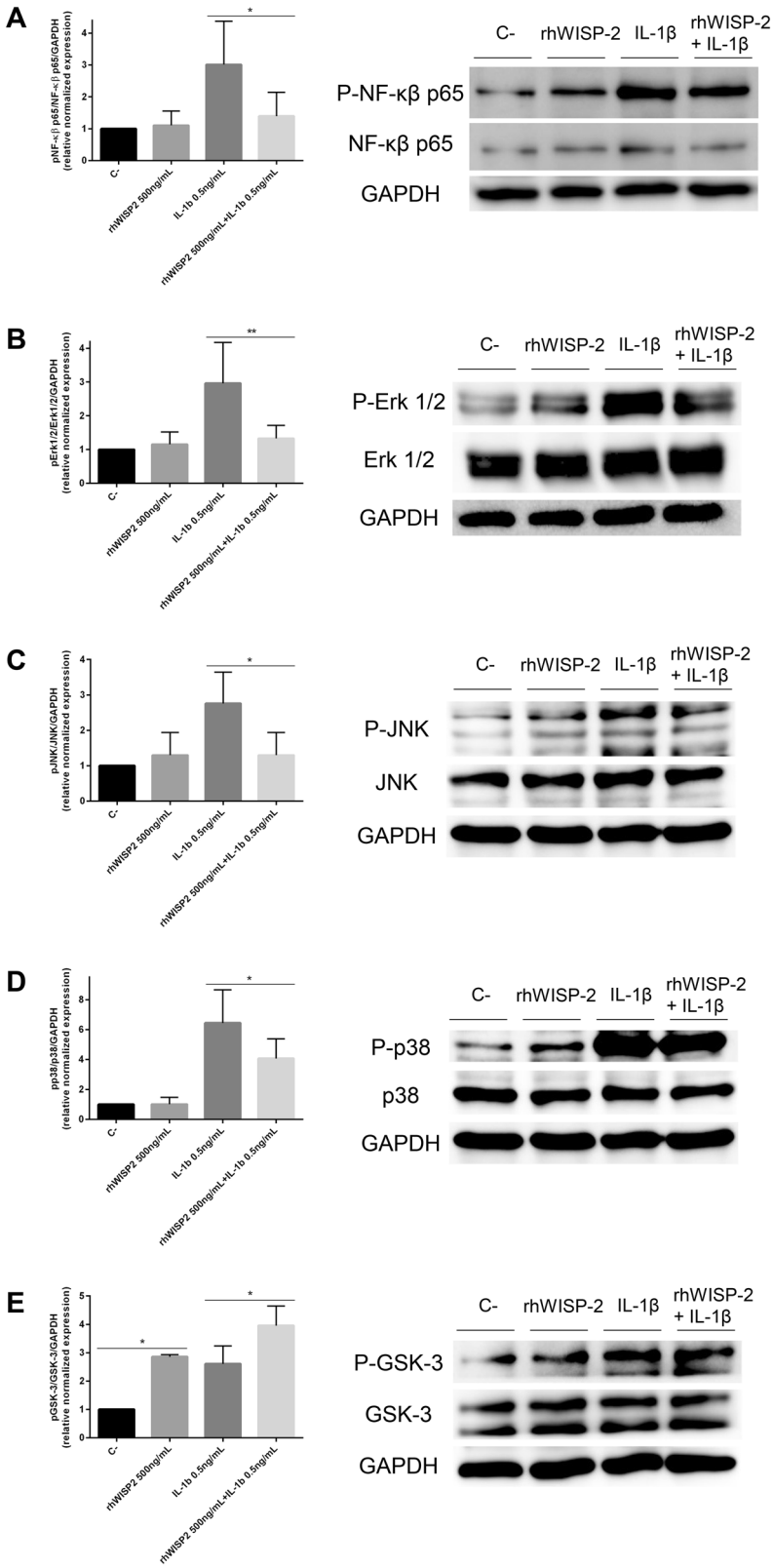
protein showed a significant reduction in MMP-13 and ADAMTS5 mRNA expression (Fig. 3A) These results were also confirmed at protein level (Fig. 3B) For completeness, ADAMTS4 was also examined since it is upregulated by inflammatory cytokines in chondrocytes. Interestingly, we observed that its mRNA expression is fairly lower than ADAMTS5. However, the pattern of response to rhWISP2 treatment is similar in both aggrecanases (Fig. 3A, right panel).

Downstream IL-1β and WNT/b-catenin signaling pathways involved in recombinant human WISP-2 protein and IL-1β stimulation action in human chondrocytes

In order to understand the molecular mechanisms involving WISP-2 we analyzed the downstream IL-1β signaling upon WISP-2 treatment. Human chondrocytes were pre-treated with human WISP2 recombinant protein (500 ng/mL) 1 h before being stimulated with IL-1β 0.5 ng/mL for 20 min. The obtained results clearly showed that rhWISP-2 significantly reduces IL-1β-induced NF-kβ p65 phosphorylation, Erk 1/2 phosphorylation, JNK phosphorylation and p38 phosphorylation (Fig. 4A–D) After confirming the WISP-2 inhibitory effect in the IL-1β signaling in human chondrocytes, we addressed Wnt/β-catenin signaling to elucidate if WISP-2 can act as a WNT agonist. For this purpose, we measured GSK-3 phosphorylation under the same experimental conditions and observed that rhWISP2 significantly activates Wnt signaling. Our results also showed that rhWISP2, in combination with IL-1β, has a cooperative effect, and that the Wnt pathway activation is significantly increased compared to single treatments (Fig. 4E).

WISP-2 siRNA knockdown stimulates chondrocyte catabolism and downregulates WNT/b-catenin signaling

Based on the data above showing WISP-2 downregulation under pro-inflammatory stimulation, we investigated the effect of WISP-2 gene silencing on cartilage ECM breakdown mediators. As shown in Fig. 5A, WISP-2 gene knockdown led to increased MMP-13 and ADAMTS-5 expression in comparison to T/C-28a2 cells transfected with a non-targeting control siRNA. Apart from cartilage-degrading enzymes, the catabolic effects of chondrocytes are mediated through several cytokines and chemokines. WISP-2 silencing increased IL-6 and IL-1β mRNA levels. The chemokine IL-



8 was also significantly upregulated upon WISP-2 gene knockdown. In addition, as shown in Fig. 5B, MMP-13 mRNA expression is increased also in OA human primary chondrocytes after WISP-2 gene silencing. This effect was also evaluated in terms of protein expression as reported in Fig. 5C.

To gain further insights into the signaling pathways at play, we tested the hypothesis that WISP-2 might be an activator of canonical Wnt signaling[13]. To investigate Wnt/β-catenin signaling activation we addressed GSK-3 phosphorylation in WISP-2-silenced T/C-28a2 chondrocytes. As shown in Fig. 5D, upon

Fig. 4 Downstream IL-1β and WNT/b-catenin signaling pathways involvement in recombinant human WISP-2 protein mechanism of action. Human T/C-28a2 cells were pre-treated with rhWISP-2 (500 ng/mL) for 1 h before being challenged with IL-1β 0.5 ng/mL for 20 min. IL-1β cascade and Wnt pathway activation were evaluated in pre-treated and not pre-treated human chondrocytes. **A** Determination of phosphorylated NF-kβ p65 and total NF-kβ p65 expression by western blot in human T/C-28a2 chondrocytes. **B** Determination of phosphorylated Erk 1/2 and total Erk 1/2 protein expression by western blot in human T/C-28a2 chondrocytes. **C** Determination of phosphorylated JNK and total JNK protein expression by western blot in human T/C-28a2 chondrocytes. **D** Determination of phosphorylated p38 and total p38 protein expression by western blot in human T/C-28a2 chondrocytes. **E** Determination of phosphorylated GSK-3 and total GSK-3 protein expression by western blot in human T/C-28a2 chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

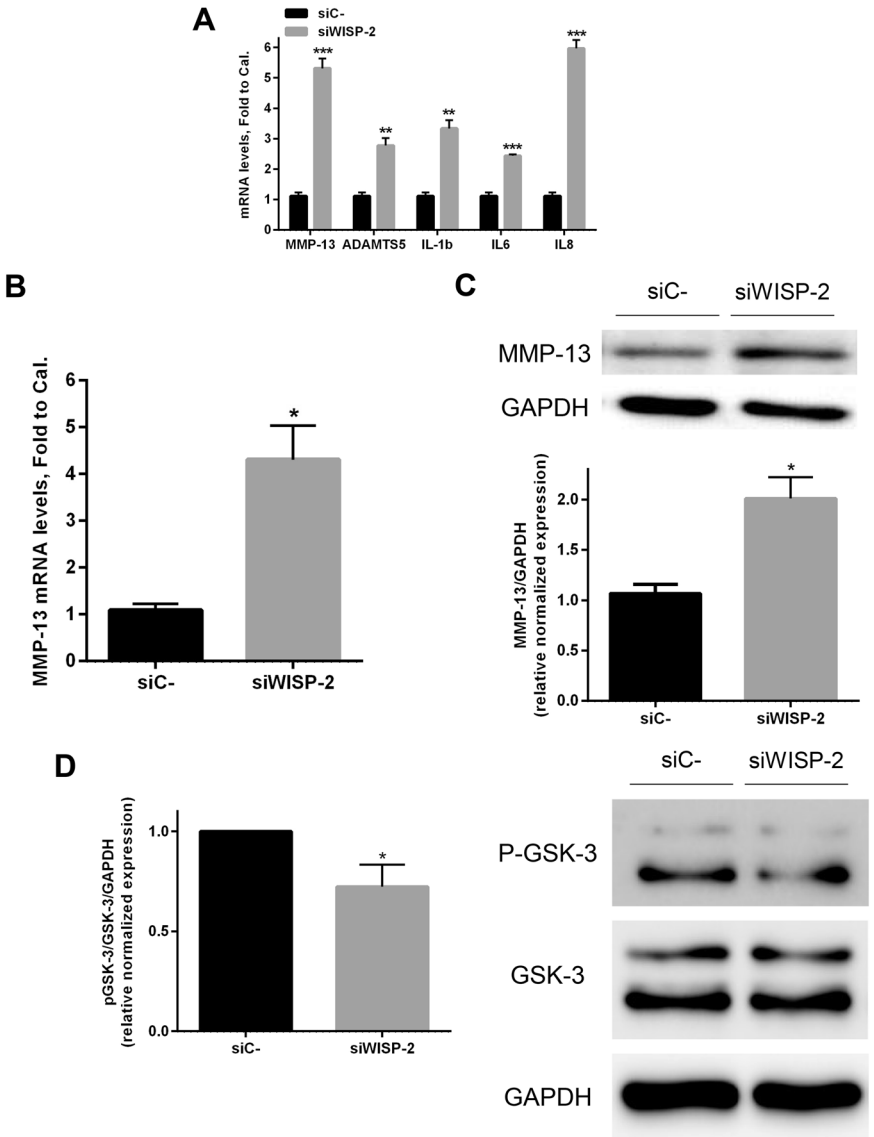


Fig. 5 Effect of WISP-2 gene knockdown on chondrocytes catabolic markers and in WNT/b-catenin pathway. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) for 48 h. **A** Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, IL-8, and IL-1β were determined in human T/C-28a2 chondrocytes by RT-qPCR. **B** Determination of human MMP-13 mRNA expression by RT-qPCR in human primary OA chondrocytes. **C** Determination of human MMP-13 protein expression by western blot in human OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. **D** Determination of phosphorylated GSK-3 protein expression by western blot in human OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

WISP-2 silencing there is a significant decrease of phosphorylation of GSK-3, suggesting that the process of accumulation and translocation of β-catenin to the nucleus, as well as the expression of downstream target genes are compromised.

Effect of WISP-2 siRNA knockdown on IL-1β-stimulated chondrocytes

Since IL-1β has been implicated in cartilage degradation[23], we determined the effect of WISP-2 silencing on IL-1β-stimulated T/C-

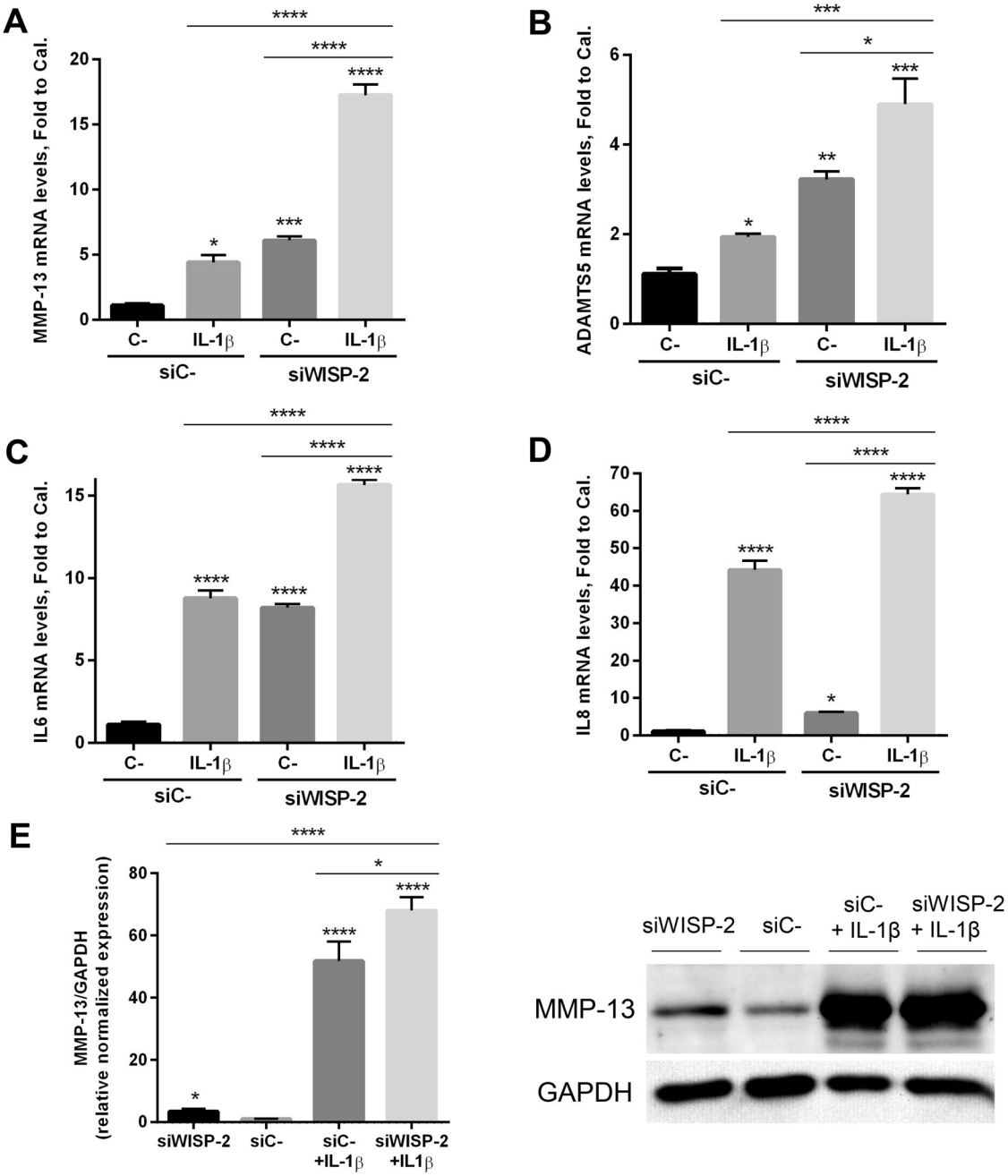


Fig. 6 Effect of WISP-2 siRNA knockdown on IL-1β-stimulated chondrocytes. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) in presence or not of IL-1β (0.5 ng/mL) for 24 h. **A**–**D** Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, and IL-8 in human T/C-28a2 chondrocytes were determined by RT-qPCR. **E** Determination of human MMP-13 protein expression by western blot in human primary OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

28a2 chondrocytes. As expected, IL-1β induced mRNA expression of MMP-13, ADAMTS-5, IL-6, and IL-8, while WISP-2 knockdown further increased IL-1β-induced expression of those genes (Fig. 6A–D). These results were confirmed also in human primary OA chondrocytes in terms of MMP-13 protein expression (Fig. 6E).

Effect of canonical Wnt/β-catenin signaling on WISP-2 siRNA knockdown mediated chondrocyte catabolism

To investigate the involvement of Wnt/β-catenin signaling, we transfected T/C-28a2 chondrocytes with siWISP-2 and then treated them with IL-1β in presence or not of selective GSK-3 inhibitor BIO. As shown in Fig. 7A–D, activation of canonical Wnt signaling by BIO significantly decreased the expression of MMP-13, as well as ADAMTS-5, IL-6, and IL-8. Moreover, BIO treatment blocked IL-1β-induced expression of those catabolic markers' mRNA. To note, blocking GSK-3 with BIO almost completely suppressed the IL-1β-mediated upregulation of MMP-13, ADAMTS-5, IL-6, and IL-8 in siWISP-2-transfected cells. This effect was confirmed in human primary OA chondrocytes at MMP-13 protein level (Fig. 7E).

DISCUSSION

The recently discovered adipokine WISP-2 was former identified as a Wnt-inducible protein[24]. In the last decade, evidence has

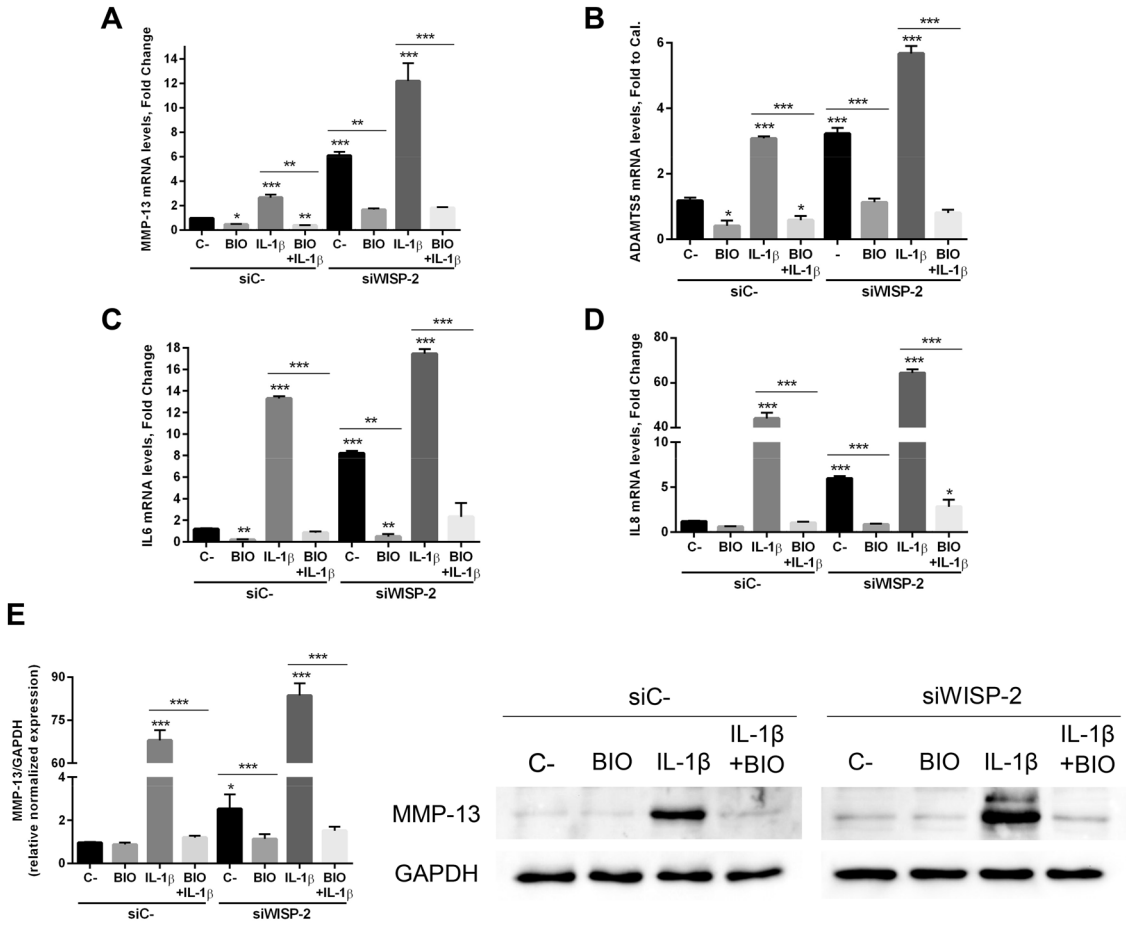


Fig. 7 Effect of canonical Wnt/β-catenin signaling on WISP-2 siRNA knockdown mediated chondrocyte catabolism. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) in presence or not of IL-1β (0.5 ng/mL) for 24 h in combination with 1 μM BIO. **A**–**D** Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, and IL-8 in human T/C-28a2 chondrocytes. **E** Determination of human MMP-13 protein expression by western blot in human primary OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

suggested a key role for Wnt signaling in the pathophysiology of OA. Canonical Wnt/β-catenin pathway leads to excessive catabolism and cartilage degradation and the hypertrophic differentiation of chondrocytes. However, Wnt signaling is necessary for maintenance of the articular cartilage, but an excessive activity is deleterious.

Recent studies have investigated the role of CCN family proteins in the development of OA. WISP-1/CCN4 and WISP-2/CCN5 were found expressed to a greater extent in OA and RA knee cartilage and all CCN genes were expressed in OA and RA synovial samples[25]. Moreover, our group has identified WISP-2 in cartilage, synovium, and infrapatellar fat pad, finding a significative increment in WISP-2 expression in OA infrapatellar fat pad in comparison to healthy controls[18]. WISP-1 regulates chondrocyte MMP and aggrecanase expression and is capable of inducing articular cartilage damage in models of OA[5]. By contrast, WISP-3/CCN6-induced repression of ADAMTS-5 expression and upregulation of MMP-10 in human chondrocytes[26]. Regarding the participation of WISP-2 in OA, there are currently no papers in the literature evaluating its action in chondrocytes. Thus, we have analyzed the effect of WISP-2 on cartilage catabolism using the recombinant protein and loss-of-function experiments in order to enlighten the role of this protein in chondrocyte pathophysiology. To the best of our knowledge, this is the first paper showing experimental evidence that WISP-2 is modulated by mediators of inflammation and its involvement in cartilage degradation.

We first analyzed the regulation of WISP-2 expression along chondrocyte differentiation observing an increment of WISP-2 expression in the first stages of chondrogenesis, followed by a dramatic decrease at the onset of hypertrophic stage. Our data in ATDC-5 cells, a well-established cell line to study chondrogenesis[27], are in agreement with those obtained by Schutze et al.[28]. Although these authors used bone marrow-derived MSC, that in their undifferentiated stage do not express none of the chondrogenic markers, the ATDC-5 chondrogenic cell line expressed them also in the chondrogenic stage. Our experimental set provides novel data since we analyzed WISP-2 expression up to the onset of hypertrophy, showing that both mRNA and protein expression of WISP-2 strongly decreased. Noteworthy, the marked reduction in WISP-2 expression at hypertrophic phase was coincident with a significant increase in MMP-13 expression, the main marker of this last stage[29], suggesting a relationship between these two factors.

Plenty of studies have described the involvement of cytokines in cartilage destruction. IL-1β is a well-known pro-inflammatory cytokine that is a potent inductor of MMP-13 expression[30]. Thus, we have studied the effect of pro-inflammatory cytokines and adipokines involved in rheumatic diseases, such as TNF-α, IL-1β, or leptin, on WISP-2 expression in chondrocytes. WISP-2 is regulated by transcription factors which are induced by inflammation, activation of the Wnt pathway, and hypoxia, processes increased in obesity[12]. Furthermore, IL-1β induces expression of Wnt

proteins, resulting in activation of Wnt pathway[9]. On the other hand, the endogenous activation of Wnt/β-catenin signaling, as observed in certain tumor cell lines, is characterized by a very low expression of WISP-2[13]. In contrast to other members of the CCN family, whose levels are increased under inflammatory conditions (i.e., WISP-3[31]), we have observed that either TNF-α, IL-1β, LPS, or IL-6 were able to reduce the expression of WISP-2 in human chondrocytes. Taking together, these results suggest that a "canonical" pro-inflammatory environment characterized by cytokines or TLR4 activation negatively modulates the expression of WISP-2. By contrast, the classic pro-inflammatory adipokines, leptin, and adiponectin did not alter the expression of WISP-2 in T/C-28a2 chondrocytes. We expected that these adipokines, which are well known contributors to create a pro-inflammatory environment in obesity and OA, might be able to regulate WISP-2. Remarkably, they did not.

One aspect that arises from our current investigations is that WISP-2 can minimize the catabolic effects of IL-1β in cartilage. As a matter of fact, recombinant WISP-2 was able to partially decrease the main metalloproteases and aggrecanases induced by IL-1β. In our experiments, recombinant WISP-2 was able to attenuate the IL-1β/NF-κβ as well as the Erk 1/2, JNK, and p38 signaling pathways. Therefore, these data, together with the observation that WISP-2 is able to increase the phosphorylation of GSK-3, suggested that WISP-2 may act as a WNT agonist with potential anabolic functions that are partially able to counteract the catabolic effects of IL-1β as one of the main mediators of inflammatory response in cartilage.

We confirmed these observations by silencing *WISP-2*. Indeed, the silencing of *WISP-2* increased the expression of MMP-13, but also was able to upregulate the expression of other aggrecanases and relevant pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8. Finally, the activation of canonical WNT pathway by BIO (a classic GSK-3β inhibitor) confirms the previously showed results and are in agreement with previous published literature. In concrete terms, WISP-2 may contribute to counteract the detrimental effects of inflammation. To this regard, CCN5/WISP-2 has been reported to have also protective effects counteracting the fibrosis in heart failure by inhibiting the TGF-β pathway[32]. In rheumatoid arthritis, Tanaka et al. reported that WISP-2 is prevalently expressed in arthritic synovial tissues[16] and specifically in fibroblast of the fibrotic area. In conclusion, there are potentially significant physiologic and pathophysiologic aspects glanced from this investigation.

In conclusion, we have shown for the first time that WISP-2 may have relevant roles in modulating the expression of enzymes involved in the turnover of extracellular matrix in the cartilage and that its downregulation may negatively alter the inflammatory environment in OA cartilage. We also proved the participation of Wnt/β-catenin signaling pathway in these processes. Thus, targeting WISP-2 may represent a possible therapeutical approach to OA. Further research is needed to define the contribution of WISP-2 in the complex metabolic network of degenerative/inflammatory diseases of musculoskeletal system such as osteoarthritis.

DATA AVAILABILITY

All the data are available in the manuscript. Data sets are available to readers promptly upon request.

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AUTHOR CONTRIBUTIONS

C.R.-F., M.G.-R., V.A., V.F., A.C.-B., J.P., F.L., J.C.-A., M.A.G.-G., A.M., D.A.E., Y.F., L.G.-C., and M.G.-C., participated in acquisition of data and samples, drafting the manuscript, analysis and interpretation of data, and statistical analysis. A.M. participated in scientific discussions, provided feedback, and critically revised the manuscript. O.G. and M.S. participated in conception and design of the study, in analysis and interpretation of data, critical revision of the manuscript, and scientific supervision of the experiments.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

This study was conducted with the approval of the Santiago University Clinical Hospital Ethics Committee (CAEIG 2014/310).

ADDITIONAL INFORMATION

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