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Mechanical stress and inflammation have opposite effects on Wnt signaling in human chondrocytes

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Abstract

Dysregulation of Wingless and Int-1 (Wnt) signaling has been strongly associated with development and progression of osteoarthritis (OA). Here, we set out to investigate the independent effects of either mechanical stress (MS) or inflammation on Wnt signaling in human neocartilage pellets, and to relate this Wnt signaling to OA pathophysiology. OA synovium-conditioned media (OAS-CM) was collected after incubating synovium from human end-stage OA joints for 24 h in medium. Cytokine levels in the OAS-CM were determined with a multiplex immunoassay (Luminex). Human neocartilage pellets were exposed to 20% MS, 2% OAS-CM or 1 ng/mL Interleukin-1β (IL-1β). Effects on expression levels of Wnt signaling members were determined by reverse transcription-quantitative polymerase chain reaction. Additionally, the expression of these members in articular cartilage from human OA joints was analyzed in association with joint space narrowing (JSN) and osteophyte scores. Protein levels of IL-1β, IL-6, IL-8, IL-10, tumor necrosis factor α, and granulocyte-macrophage colony-stimulating factor positively correlated with each other. MS increased noncanonical *WNT5A* and *FOS* expression. In contrast, these genes were downregulated upon stimulation with OAS-CM or IL-1β. Furthermore, Wnt inhibitors *DKK1* and *FRZB* decreased in response to OAS-CM or IL-1β exposure. Finally, expression of *WNT5A* in OA articular cartilage was associated with increased JSN scores, but not osteophyte scores. Our results demonstrate that MS and inflammatory stimuli have opposite effects on canonical and noncanonical Wnt signaling in human neocartilage. Considering the extent to which MS and inflammation contribute to OA in individual patients, we hypothesize that targeting specific Wnt pathways offers a more effective, individualized approach.

KEYWORDS
human neocartilage, inflammation, mechanical stress, osteoarthritis, Wnt signaling

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1 | INTRODUCTION

Although considered a whole joint disease, the most prominent hallmark of osteoarthritis (OA) is the progressive degradation of the articular cartilage.[1] Articular cartilage is a highly specialized tissue, responsible for smooth movement of the joints and distribution of mechanical pressure.[2] The extracellular matrix (ECM) of the cartilage contains primarily collagens and proteoglycans. The negative charge of the proteoglycans attracts water into the cartilage that acts as a buffer zone for the underlying subchondral bone. With cartilage loading, water is being pushed out of the cartilage, causing a strain on the tissue. While physiological mechanical strains promote beneficial effects on the cartilage, hyperphysiological mechanical strains or mechanical stress (MS) can cause damage and dysregulate critical underlying pathways leading to a catabolic and proinflammatory response.[3] This is one of the causes of inflammation of the synovium, which occurs in >70% of patients suffering from all grades of knee OA.[4] Together, MS and inflammation create microtraumas in the articular cartilage.[5] However, the degree to which MS and synovial inflammation contribute to cartilage degeneration in OA can be expected to highly differ between patients, making these important parameters to study separately to open new avenues for more personalized care and treatment.

The Wingless and Int-1 (Wnt) signaling pathway is an evolutionary highly conserved pathway that plays a critical role in both embryonic development and tissue homeostasis throughout life. Wnt proteins activate different Wnt pathways, depending on the Wnt ligand, frizzled receptor, and coreceptors. Either the β-catenin-dependent canonical Wnt pathway or the noncanonical Wnt pathway is activated, where the latter can be further divided into the planar cell polarity (PCP) and the Wnt/Ca^{2+} pathway.[6–8] Wnt signaling is usually tightly regulated and dysregulation of Wnt signaling has been linked to severe skeletal disorders.[9] Furthermore, it is evident that aberrant Wnt signaling in the joint can play a key role in development and progression of OA.[8,10–15] Overactive Wnt signaling, due to decreased levels of Wnt inhibitors, such as *FRZB* and *DKK1* and increased levels of Wnt ligands, such as *WNT5A* and *WNT5B*, can lead to chondrocyte hypertrophy, increased catabolic activity and decreases anabolic activity, resulting in cartilage degeneration.[6,13] Whereas it is clear that Wnt signaling is dysregulated in OA, determining the extent to which MS and inflammation individually cause aberrant Wnt signaling may contribute to a better understanding of the effectiveness of Wnt-based treatments in patients prone to develop OA, or decrease progression of patients with early to moderate OA.

Here, we set out to investigate how MS and inflammation individually affect Wnt signaling. Thereto, we employed human primary articular OA chondrocytes into a three-dimensional (3D) neocartilage pellet model with high-quality physiological cartilage. We exposed neocartilage to MS, which induced cartilage damage as was previously demonstrated,[16] or exposed neocartilage to OA synovium-conditioned media (OAS-CM) or Interleukin-1β (IL-1β). For the OAS-CM, we characterized the inflammatory status based on the concentration of different OA-relevant cytokines. Subsequently, we determined the effects of these OA-related stimuli on the expression of ligands, inhibitors, transcription factors, and receptors of canonical and noncanonical Wnt signaling that are dysregulated in OA.[17] Finally, we investigated the relation between messenger RNA (mRNA) levels of these Wnt members in OA articular cartilage and radiographic scores.

2 | METHODS

2.1 | Conditioned media from OA synovium

OAS-CM was obtained as described previously.[18] In short, synovium from anonymous patients undergoing total knee joint replacement surgery as a result of end-stage OA ($n = 10$) was collected in the Radboud University Medical Center and cultured for 24 h in DMEM with 0.1% BSA (0.3 g tissue/mL). Debris was removed by centrifugation and aliquots of OAS-CM were stored at −20°C until further use.

2.2 | Immunoassays

Protein levels of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and MCP-1 in the OAS-CM were measured with a multiplex immunoassay on a Bio-Plex 200 system using a multiplex cytokine kit (Luminex, Bio-Rad Laboratories), according to the manufacturer's protocol.

2.3 | Cell culture

The effects of MS and a proinflammatory environment on Wnt signaling were investigated in our previously established human 3D in vitro neocartilage pellet model.[16,19] Human primary articular chondrocytes were isolated from macroscopically preserved areas of anonymized patients ($n = 12$) undergoing total knee joint replacement due to end-stage OA included in the Research in Articular Osteoarthritis Cartilage (RAAK) study[20] at Leiden University Medical Center (Leiden, The Netherlands), or at Radboud University Medical Center (Nijmegen, the Netherlands). There was no overlap between donors of synovium tissue and primary chondrocytes. Human primary articular chondrocytes were expanded in culture for two passages in DMEM (high glucose, Gibco), supplemented with 10% fetal bovine serum (FBS, Biowest), antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco), and 5 ng/mL bFGF (Peprotech). Subsequently, pellets (2.5×10^5 cells/pellet) were created by centrifugation. The following day, the medium was replaced with a serum-free chondrogenic medium (CDM), consisting of DMEM (high glucose, Gibco), supplemented with 50 µg/mL ascorbic acid (Sigma-Aldrich), 40 µg/mL L-proline (Sigma-Aldrich), 100 µg/mL sodium pyruvate (Sigma-Aldrich), 0.1 µM dexamethasone (Sigma-Aldrich), 1% insulin-transferrin-selenium +, antibiotics (100 U/mL

penicillin and 100 µg/mL streptomycin, Gibco), and 10 ng/mL human transforming growth factor-β1 (hTGF-β1) (Peprotech) and refreshed every 3–4 days, as previously described.[16,19]

2.4 | Exposure to MS, IL-1β, and OAS-CM

MS was applied using a Mach-1 mechanical testing system (Biomomentum) as previously described.[16] At two consecutive days, neocartilage pellets ($n = 12$ donors) were transferred to a sterile 0.9% phosphate buffered saline solution (B. Braun) and subjected twice to MS (10 min, 20% strain, frequency of 5 Hz, with a 10-min recovery interval between MS), and pellets were collected 12 h after MS. To largely reach a uniformity of strain for each donor, the diameters of the neocartilage pellets within each donor were measured and averaged, where the standard deviation of the average diameter was less than 5% of the average diameter. Before 24-h incubation with OAS-CM (2%) or IL-1β (1 ng/mL, Bio-Techne), pellets were cultured in the absence of dexamethasone (4 days prior) and TGF-β (1 day prior) to avoid the inhibition of an inflammatory response. To reflect biological variation across patients, three neocartilage pellets from each donor were exposed separately to 2% OAS-CM of three independent donors.

2.5 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Per donor, two replicate neocartilage pellets were collected in TRIzol (Invitrogen). RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, and was treated with RNase-Free DNase (Qiagen) to remove DNA contamination. RNA concentrations and quality were measured using the NanoDrop. RNA was reverse-transcribed into complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Subsequently, cDNA was amplified using FastStart SYBR Green Master (Roche) and mRNA expression was measured in triplicates in a MicroAmp Optical 384-Well Reaction Plate (ThermoFisher Scientific), using the QuantStudio Flex Real-Time PCR system (Applied Biosystems), with the following cycling conditions: 10 min 95°C; 10 s 95°C, 30 s 60°C, 20 s 72°C (45 cycles); 1 min 65°C and 15 s 95°C. Primer efficiency was tested using a cDNA dilution series, and primers with an efficiency between 90% and 110% were considered reliable. *GADPH* and *SDHA* were used as housekeeping genes. Fold changes (FCs) were calculated using the $2^{-\Delta\Delta Ct}$ method with $\Delta Ct = Ct_{gene\ of\ interest} - Ct_{average\ housekeeping\ genes}$ and $\Delta\Delta Ct = \Delta Ct_{condition} - \Delta Ct_{Control}$. Genes were selected based on their differential expression in OA articular cartilage and/or were representative of either canonical or non-canonical Wnt signaling.[17] Primer sequences are listed in Table 1.

2.6 | Association of mRNA levels in preserved OA cartilage with radiographic scores

Gene expression levels of Wnt signaling members from macroscopically unaffected or preserved OA cartilage (RAAK study; $N = 37$) were extracted from our previously established RNA sequencing data set (Table 2).[17,19,21] Radiographic scores (Kellgren–Lawrence, joint space narrowing [JSN], and osteophytes) were previously determined

TABLE 1 Primer sequences.

Gene	Forward sequence	Reverse sequence
GAPDH	5′-TGCCATGTAGACCCCTTGAAG-3′	5′-ATGGTACATGACAAGGTGCGG-3′
SDHA	5′-TGGAGCTGCAGAACCTGATG-3′	5′-TGTAGTCTTCCCTGGCATGC-3′
WNT5A	5′-TCGCCAAGGAGTTTGTGGAT-3′	5′-CCATCTTATACACAGCCCTGC-3′
WNT5B	5′-TCGCCAAGGAGTTTGTGGAT-3′	5′-CCATCTTATACACAGCCCTGC-3′
WNT9A	5′-GTTCCAGTTCCGCTTTGAGC-3′	5′-AAGGCAGTCTCCTTGAAGCC-3′
WNT16	5′-AACACCACGGGCAAAGAAAAC-3′	5′-ATCAACTTGGCGACAGCCT-3′
TCF7L1	5′-CGATCCAAAGACAGGAATCCCC-3′	5′-GATTTGTCCGACAGCTCCG-3′
TCF7L2	5′-CCGACGTAGACCCCAAAACA-3′	5′-ATTTGTCCTACGGTGCCAGG-3′
LEF1	5′-GGTAGCAATTGGCAGCCCTA-3′	5′-CAAACCAGTCTGCTGAACGC-3′
CCN4	5′-CCTGCTTGGGGTTCATAGGA-3′	5′-TGGGTCTCTCAAGGCTCTGT-3′
JUN	5′-GAGCTGGAGCGCCTGATAAT-3′	5′-CCCTCCTGCTCATCTGTCAC-3′
FOS	5′-GGAGAATCCGAAGGGAAAGGA-3′	5′-AGTTGGTCTGTCTCCGCTTG-3′
DKK1	5′-TGACAACTACCAGCCGTACC-3′	5′-CAGGCGAGACAGATTTGCAC-3′
FRZB	5′-ATTGACTTCCAGCACGAGCC-3′	5′-CGAGTGGCGGTACTTGATGAG-3′
FZD1	5′-AGTTCGTGCCAATCCTGACA-3′	5′-TGTTCAAAGGAGCCTGCGAA-3′
FZD5	5′-AGCACCATCGTTGTGTCTTGA-3′	5′-TCCACCAATCACCATCGCTT-3′

TABLE 2 Baseline characteristics of RAAK samples with OA phenotypes.

Characteristic	*n* = 37
Age-year	
Mean (SD)	69.8 (7.7)
Sex-no (%)	
Female	29 (78%)
Male	8 (22%)
Joint type-no (%)	
Hip	12 (32%)
Knee	25 (68%)
KL score (SD)	2.7 (0.6)
JSN score (SD)[a]	4.7 (1.2)
OP score (SD)[a]	2.8 (2.1)

Abbreviations: JSN, joint space narrowing; KL score, Kellgren–Lawrence score; OA, osteophytosis; OP, osteophytes; RAAK, Research in Articular Osteoarthritis Cartilage.

[a]The JSN score (0–6) or OP score (0–6) is defined as the sum of JSN grades (0–3) or OP grades (0–3), determined at the superior and inferior areas of the acetabular–femoral hip joint or at the medial and lateral areas of the tibiofemoral knee joint, respectively.

by two experienced readers prior and independent of the current study.[21] In short, osteophytes and JSN were graded using the OARSI atlas, with 0–3 scores for lateral and medial compartments of the tibiofemoral knee joints and for inferior and superior compartments of the acetabular–femoral hip joint by consensus opinion. Association of gene expression levels with radiographic scores was determined by applying a generalized estimation equation (GEE).

2.7 | Statistical analysis

Graph creation and statistics were carried out in GraphPad Prism v.9, unless stated otherwise. Correlations between the cytokines were carried out using a Spearman correlation test. Statistical significance of mean differences in $-\Delta Ct$ levels between MS, OAS-CM, and IL-1β with their respective controls was estimated using a GEE. Since perfect pairs were absent, by applying a GEE, we could adjust for dependencies among donors by adding a random effect for sample donors. We followed a linear GEE model, with $-\Delta Ct$ levels as dependent variable and condition as a factor. Associations of mRNA levels in preserved OA cartilage with JSN score and osteophytosis (OP) score were carried out with a linear GEE model, using age, sex, KL score, and joint type as covariates, and are depicted as odds ratios (OR).[16,22] GEE analyses were carried out using IBM SPSS Statistics 25. The cytokine concentrations and FCs are described using the mean ± difference between 95% CI and the mean. ORs are described using the mean and the 95% CI. Differences in expression levels of Wnt genes in preserved OA articular cartilage from knees and hips were calculated with a linear GEE model, using age and sex as covariates. *p* Values of the correlation analysis, RT-qPCR analysis, associations of Wnt signaling with radiographic scores, and differences in gene expression between knees and hips were corrected for multiple testing using the Benjamini–Hochberg method, with a significant cutoff value of 0.05, as is indicated by the false discovery rate (FDR).

3 | RESULTS

3.1 | Cytokine levels in undiluted conditioned media derived from OA synovium

To gain insight into the level of inflammatory factors secreted from OA synovium, we performed a Luminex assay of the OAS-CM to determine concentrations of 10 different OA-relevant cytokines. As shown in Figure 1, high levels of IL-6 ($1.31 \times 10^5 \pm 9.74 \times 10^4$ pg/mL) and IL-8 ($1.56 \times 10^5 \pm 1.39 \times 10^5$ pg/mL) were observed. Relative to these two cytokines, we detected low levels of IL-1β (190.7 ± 158.1 pg/mL), IL-10 (100.6 ± 62.5 pg/mL), TNF-α ($1.16 \times 10^3 \pm 1.01 \times 10^3$ pg/mL), IFN-γ (0.9 ± 0.6 pg/mL), GM-CSF (29.3 ± 41.3 pg/mL), and monocyte chemoattractant protein-1 (MCP-1) (623.1 ± 74.6 pg/mL). Furthermore, IL-4 and IL-17 were too low to detect (*data not shown*).

To investigate the coherency of secreted cytokine levels between patients, we explored correlations between the different cytokines. Overall, this showed significant positive correlations between levels of IL-1β, IL-6, IL-8, IL-10, TNF-α, and GM-CSF (Figure 2). Notably, MCP-1 did not show any significant correlation with the other cytokines. Some of the strongest correlations were observed for IL-8 with IL-6 ($\rho = 0.92$, FDR = 1.22×10^{-2}) and GM-CSF ($\rho = 0.89$, FDR = 1.22×10^{-2}).

3.2 | Effects of MS and inflammation on canonical and noncanonical Wnt signaling

To determine the individual effects of MS and inflammation on Wnt signaling, we measured mRNA levels of genes involved in Wnt signaling by RT-qPCR. As shown in Figure 3, upon applying 20% MS, specifically mRNA expression of noncanonical Wnt ligand *WNT5A* (FC = 3.62 ± 3.10, FDR = 4.35×10^{-2}) and *WNT5B* (FC = 1.85 ± 0.66, FDR = 8.12×10^{-2}) was consistently upregulated, albeit not FDR significant for *WNT5B* (Figure 3A). Moreover, we observed a high and significant upregulation of transcription factor *FOS* (FC = 18.12 ± 19.47, FDR = 1.68×10^{-6}, Figure 3C). Notable in Figure 3D is the absence of response of Wnt ligand scavenger *FRZB* and canonical Wnt inhibitor *DKK1*, which are usually among the most consistent downregulated genes associated with OA pathophysiology.[11,15]

On the other hand, exposure to 2% OAS-CM downregulated expression of noncanonical Wnt ligands *WNT5A* (FC = 0.65 ± 0.19, FDR = 9.27×10^{-4}), *WNT5B* (FC = 0.49 ± 0.11, FDR = 1.89×10^{-3}), and *WNT16* (FC = 0.47 ± 0.42, FDR = 1.87×10^{-3}, Figure 3A), and

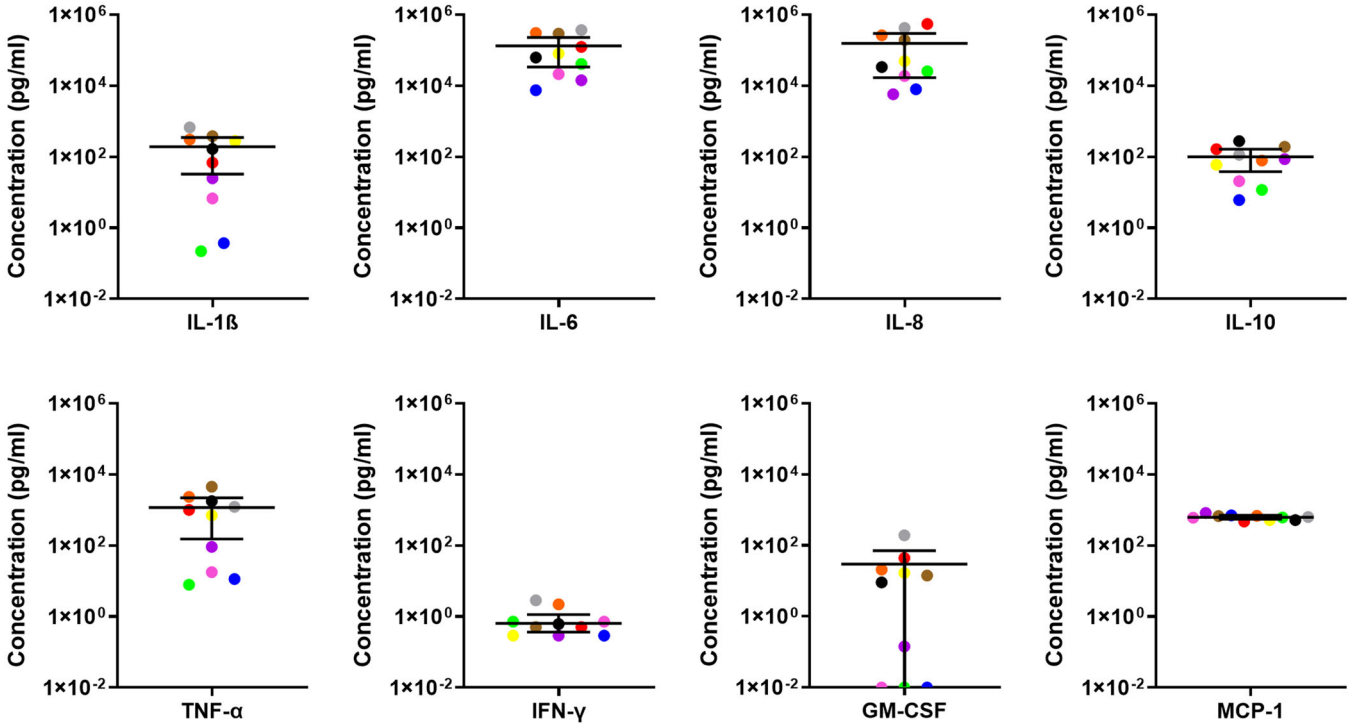

FIGURE 1 Cytokine levels in undiluted OAS-CM. Levels of IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, TNF-α, IFN-γ, GM-CSF and MCP-1, measured by Luminex in OAS-CM ($n = 10$). Graphs depict the mean with 95% confidence intervals, and each colored dot represents a single OAS-CM. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL-1β, Interleukin-1β; MCP-1, monocyte chemoattractant protein-1; OA, osteophytosis; OAS-CM, OA synovium-conditioned media; TNF-α, tumor necrosis factor α.

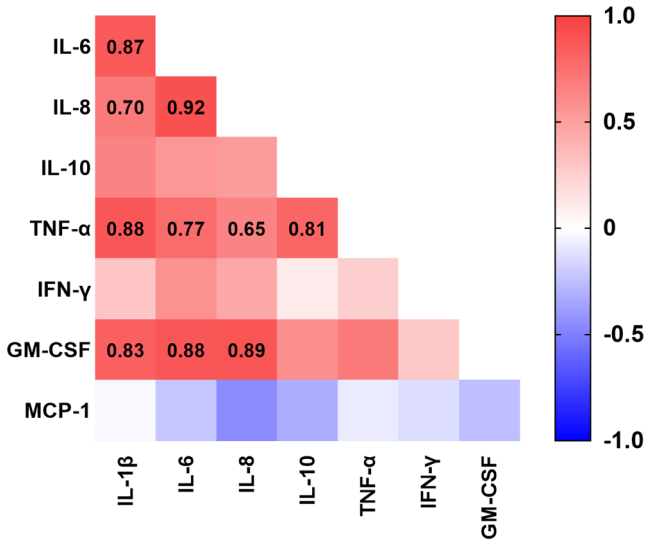

FIGURE 2 Correlation matrix between cytokine levels in OAS-CM ($n = 10$). Correlations were calculated using a Spearman correlation test. p Values were corrected for multiple testing using the Benjamini–Hochberg method. Only significant correlations (FDR ≤ 0.05) display the Spearman (ρ) correlation coefficient. FDR, false discovery rate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, Interleukin; MCP-1, monocyte chemoattractant protein-1; OAS-CM, OA synovium-conditioned media; TNF-α, tumor necrosis factor α.

noncanonical transcription factor *JUN* (FC = 0.64 ± 0.16, FDR = 9.27×10^{-4}, Figure 3C). Concurrently, exposure to 2% OAS-CM decreased expression of the canonical transcription factor *TCF7L1* (FC = 0.63 ± 0.18, FDR = 2.68×10^{-3}, Figure 3B), *DKK1* (FC = 0.66 ± 0.14, FDR = 3.44×10^{-2}), and *FRZB* (FC = 0.51 ± 0.13, FDR = 2.48×10^{-4}) (Figure 3D). Moreover, frizzled receptors *FZD1* (FC = 0.66 ± 0.16, FDR = 2.15×10^{-4}) and *FZD5* (FC = 0.71 ± 0.29, FDR = 9.98×10^{-5}, Figure 3E) were down-regulated upon exposure to 2% OAS-CM while the response to MS was less consistent. Furthermore, we compared the effects of OAS-CM on Wnt signaling to IL-1β, a cytokine commonly used to mimic OA-associated processes in vitro although our data indicate its levels are relatively low in OAS-CM. We observed overall similar effects of OAS-CM and IL-1β on the expression of Wnt ligands and inhibitors (Supporting Information Figure S1).

3.3 | Associations of Wnt mRNA levels in OA articular cartilage of joints with JSN and osteophytes

Given the fact that the responses of members of Wnt signaling to the OA-related triggers (20% MS and 2% OAS-CM) are highly specific and different, we next wanted to explore whether these different responses could mark endotypes of that pathophysiological process in preserved cartilage. Hereto, we related expression patterns of these Wnt members in preserved regions of OA articular cartilage to

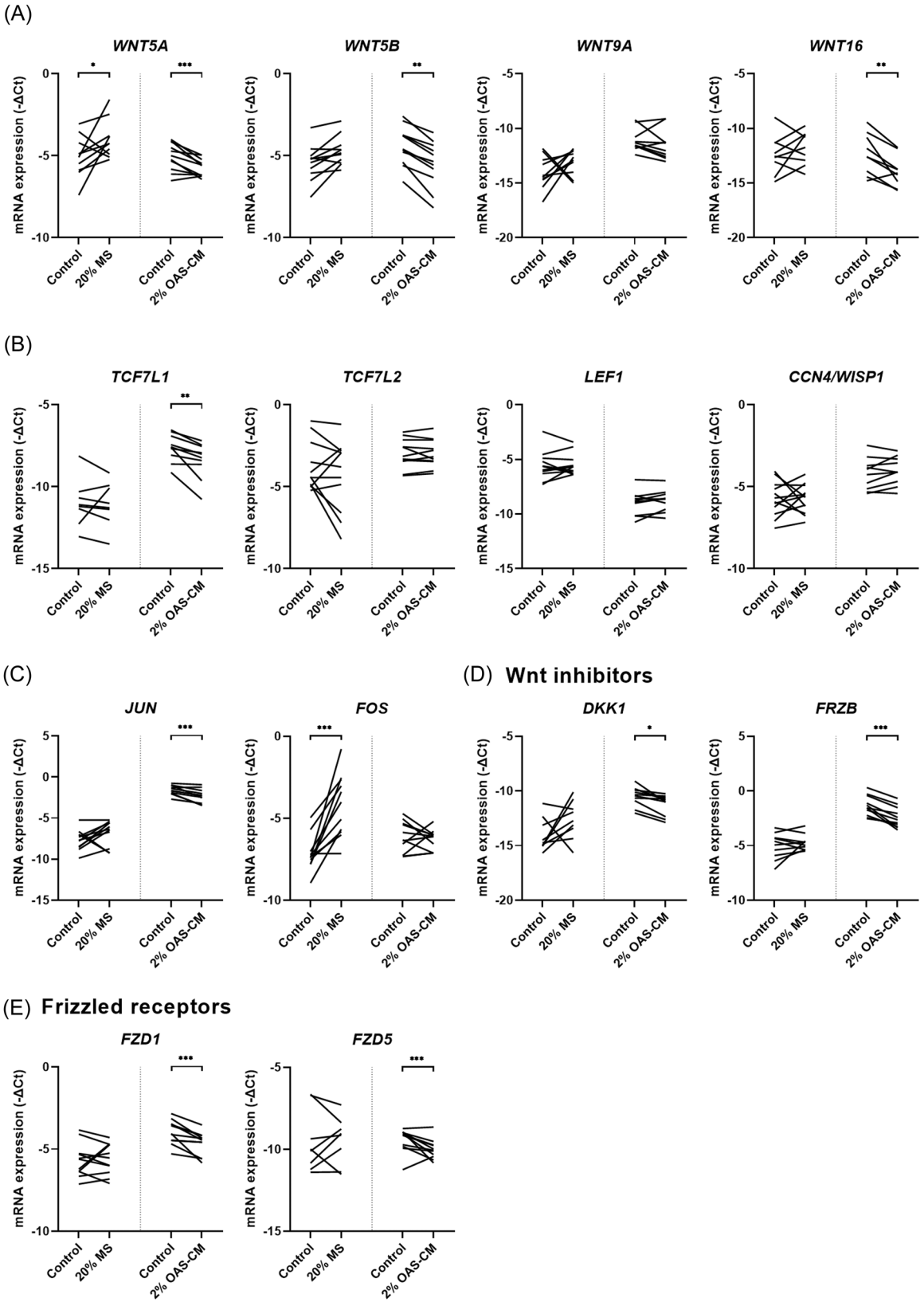

FIGURE 3 Mechanical stress and OAS-CM have opposite effects on Wnt signaling in human neocartilage. (A) Wnt ligands. (B) Canonical TFs and canonical target *CCN4/WISP1*. (C) Noncanonical TF *JUN* and its AP-1 associated TF *FOS*. (D) Canonical Wnt inhibitor *DKK1* and Wnt ligand scavenger *FRZB*. (E) Frizzled receptors 1 and 5. Lines represent differences in mRNA expression levels ($n = 12$) between control and 20% MS or control and OAS-CM within a single donor. *FDR ≤ 0.05, **FDR ≤ 0.01, and ***FDR ≤ 0.001. AP-1, activator protein 1; FDR, false discovery rate; mRNA, messenger RNA; MS, mechanical stress; OA, osteophytosis; OAS-CM, OA synovium-conditioned media; OAS-CM, OA synovial conditioned media; TF, transcription factor; Wnt, Wingless and Int-1.

TABLE 3 Associations of mRNA levels of Wnt signaling members with hallmarks of radiographic OA.

	JSN score[a]					OP score[a]				
		95% CI					95% CI			
Gene	OR[b]	Lower	Upper	*p* Value[b]	FDR[c]	OR[b]	Lower	Upper	*p* Value[b]	FDR[c]
WNT5A	1.65	1.27	2.15	0.00018	**0.0050**	1.05	0.88	1.25	0.6100	0.6832
WNT5B	1.18	0.97	1.44	0.1010	0.2724	1.07	0.96	1.19	0.2420	0.3640
WNT9A	0.87	0.71	1.05	0.1490	0.2980	1.15	1.02	1.29	0.0230	0.1073
WNT16	0.81	0.50	1.31	0.3820	0.5213	1.08	0.83	1.41	0.5570	0.6498
TCF7L1	1.04	0.91	1.20	0.5550	0.6498	1.00	0.92	1.08	0.9220	0.9220
TCF7L2	1.09	1.00	1.18	0.0450	0.1575	0.98	0.93	1.03	0.4680	0.5956
LEF1	1.63	1.07	2.47	0.0220	0.1073	1.14	0.91	1.42	0.2470	0.3640
CCN4	1.20	0.96	1.49	0.1070	0.2724	1.05	0.94	1.18	0.3910	0.5213
JUN	1.16	0.91	1.49	0.2240	0.3640	1.01	0.86	1.19	0.8670	0.8991
FOS	1.10	0.94	1.28	0.2430	0.3640	1.02	0.92	1.14	0.6930	0.7463
DKK1	0.74	0.55	0.98	0.0360	0.1440	0.89	0.75	1.04	0.1380	0.2972
FRZB	0.65	0.46	0.93	0.0170	0.1073	0.88	0.72	1.09	0.2310	0.3640
FZD1	1.16	0.98	1.38	0.0940	0.2724	1.12	1.02	1.23	0.0140	0.1073
FZD5	0.89	0.76	1.04	0.1380	0.2972	0.90	0.82	0.99	0.0220	0.1073

Abbreviations: CI, confidence interval; FDR, false discovery rate; JSN, joint space narrowing; KL score, Kellgren–Lawrence score; mRNA, messenger RNA; OA, osteophytosis; OP, osteophytes; OR, odds ratio; Wnt, Wingless and Int-1.

[a]The JSN score or OP score is defined as the sum of JSN grades (0–3) or OP grades (0–3), determined at the superior and inferior areas of the acetabular–femoral hip joint or at the medial and lateral areas of the tibiofemoral knee joint, respectively.

[b]Odds ratios describe the association between mRNA levels and JSN grades or OP grades. Odds ratios and *p* values were generated by generalized estimating equation, with mRNA expression level in preserved OA cartilage as dependent variable, and age, sex, KL score, and joint type as covariates.

[c]*p* Values were corrected for multiple testing using the Benjamini–Hochberg method, as indicated by the false discovery rate (FDR). FDR ≤ 0.05 are shown in bold.

hallmarks of radiographic OA (i.e., JSN and osteophytes scores; Table 3). We observed that *WNT5A* mRNA levels were significantly positively associated with JSN scores (OR = 1.65, 95% CI = 1.27, 2.15), independent of age, sex, KL score, and joint type. Albeit not FDR significant, decreased mRNA levels of *FRZB* (OR = 0.65, 95% CI = 0.46, 0.93) and *DKK1* (OR = 0.74, 95% CI = 0.55, 0.98) were also associated with higher JSN scores.

4 | DISCUSSION

In this study, we investigated the effects of two processes relevant for OA etiology, namely, MS and inflammation, on Wnt signaling in human primary chondrocyte neocartilage pellets. We observed that MS activated transcription of noncanonical Wnt ligands in neocartilage pellets, while exposure to proinflammatory stimuli resulted in inhibition. Furthermore, the inflammatory stimuli decreased the expression of (canonical) Wnt inhibitors, which could result in the activation of Wnt signaling. Finally, we associated *WNT5A* mRNA levels in preserved articular cartilage from OA joints with higher JSN scores.

As a multifactorial and complex disease, OA shows high heterogeneity in disease pathophysiology between patients. Many but not all patients develop synovial inflammation in the joint, and physically active patients can be very different from inactive patients, considering the response of cartilage to mechanical events.[23] Moreover, while it is clear that overactive Wnt signaling extensively contributes to OA (progression), we now observed that MS and inflammation have different effects on canonical and noncanonical Wnt signaling. This is interesting in light of developing Wnt inhibitors specific for canonical and noncanonical Wnt signaling, which may be more effective in some patients as compared with others, due to the heterogeneous nature of OA. The latter is probably also related to the different effects that MS and synovial inflammation and the combination have on articular cartilage.

Here, we applied 2% OAS-CM which previously has been shown to reduce sulfated glycosaminoglycan (sGAG) production by 50% in pellet cultures of bone marrow-derived mesenchymal stem cells that were differentiated into chondrocytes.[24] Furthermore, the addition of tofacitinib and oxozeaenol, in the presence of OAS-CM, has previously been demonstrated to rescue the sGAG content, confirming that factors secreted by the OA synovium are responsible for the observed effects.[18] The high levels of IL-6 and IL-8 in the OAS-CM and the presence of other cytokines indicate a strong proinflammatory potential of the OAS-CM. Although the importance and relevance of IL-1β in OA is under discussion,[25] we observed

similar effects of exposure to OAS-CM and IL-1β alone on most members of Wnt signaling. Therefore, IL-1β seems a good model cytokine to study the effects of synovial inflammation on Wnt signaling in case OAS-CM is not available.

MS resulted in upregulation of *WNT5A* and *WNT5B*, with the latter not being FDR significant. These two ligands signal through the noncanonical pathway. While WNT5A and WNT5B are highly similar in structure, they have different functions in chondrocytes.[26] WNT5A induces cytokine production and senescence, whereas WNT5B stimulates collagen type I secretion and is responsible for inhibiting the production of ECM components and chondrocyte differentiation.[27,28] In all donors, we observed an increase in expression of the transcription factor *FOS* and in 10 out of 12 donors for *JUN*. Although mostly *JUN* is associated with the noncanonical PCP/JNK pathway, FOS and JUN dimerize, forming the activator protein 1 (AP-1) transcription factor. AP-1, and can via inhibition of microRNA-30a (miR-30a) stimulate expression of the aggrecanase ADAMTS5, a matrix-degrading enzyme that plays an important role in cartilage degradation and OA.[29] We previously showed the MS-induced *ADAMTS5* expression in the neocartilage.[16] The applied MS in this model may upregulate *ADAMTS5* via AP-1 and miR-30a, however, this remains to be established. In contrast, we observed opposite effects on noncanonical Wnt signaling after exposure to proinflammatory stimuli compared with MS. Both OAS-CM and IL-1β exposure led to strong downregulation of the canonical Wnt inhibitor *DKK1* and Wnt ligand scavenger *FRZB*, which has previously been demonstrated for IL-1β.[11] Multiple genome-wide association studies have identified a nonsynonymous single-nucleotide polymorphism residing in the *FRZB* gene that reduces the ability of FRZB to antagonize Wnt signaling, and linked this to increased risks of OA.[15,30] Moreover, high levels of both FRZB and DKK1 have been linked to reduced progression of OA.[31] In line with this, we observed that both *DKK1* and *FRZB* mRNA levels, although not FDR significant, were negatively associated with JSN scores. Reduced DKK1 and FRZB levels can lead to excessive canonical Wnt signaling with chondrocyte hypertrophy and increased protease activity as a consequence.[6] We did not observe any effects on *DKK1* and *FRZB* in the mechanically stressed neocartilage. This is different compared with mechanically injured cartilage explants, which previously were shown to have decreased *DKK1* and *FRZB* expression.[14] Chondrocytes may respond differently to certain types of MS and should be taken into consideration when selecting preclinical models for drug testing.[3]

WNT16 is a ligand that activates mostly noncanonical Wnt signaling and can only weakly activate canonical Wnt signaling in high doses. In certain situations, WNT16 can counteract excessive canonical Wnt signaling. Additionally, *Wnt16*$^{-/-}$ mice produce lower levels of lubricin and exhibit increased chondrocyte apoptosis, resulting in more severe OA, which suggests a protective effect of Wnt16 on cartilage homeostasis.[32] Moreover, *WNT16* is strongly upregulated in end-stage OA articular cartilage.[17] However, we observed reduced expression of *WNT16* after exposure to OAS-CM and IL-β, while no effect of MS was observed. Late *WNT16* upregulation may be an attempt of the chondrocyte to protect cartilage from excessive damage, while in early phases it may remain unchanged.[33,34]

The members of the TCF/LEF family are transcription factors regulating the transcription of target genes from the β-catenin-dependent/canonical Wnt pathway. After exposure to IL-1β, we observed that *TCF7L1* expression was decreased while *LEF1* was increased. LEF1 is thought to act as a strong transcriptional activator and TCF7L1 is considered a transcriptional repressor.[35] We speculate that exposure to IL-1β may cause more active transcription of canonical Wnt target genes.

Previously, we have demonstrated the involvement of CCN4/WISP1 in OA. CCN4/WISP1 is upregulated in both cartilage and synovium in mice with experimental OA, and aggravates cartilage degeneration in multiple OA mice models.[10,12] Furthermore, CCN4/WISP1 expression is increased in lesioned areas from human OA cartilage compared with macroscopically preserved areas, and mostly present near cartilage lesions. Surprisingly, we did not find any effects of MS nor the proinflammatory stimuli on *CCN4/WISP1* in the neocartilage. Possibly, to detect CCN4/WISP1 changes a different timing is required. Alternatively, in in vivo OA models, the CCN4/WISP1 response is very likely a combination of MS and inflammation, which we here aimed to separate.

Commonly, CDM contains dexamethasone to promote chondrogenesis. Dexamethasone, however, has strong anti-inflammatory properties, and has been shown to inhibit Wnt signaling.[36] Therefore, to prevent that the dexamethasone in our study would counteract the inflammatory effects of the OAS-CM and IL-1β, neocartilage pellets were cultured without dexamethasone 4 days before the exposure. This difference in composition of the CDM with OAS-CM as compared with the mechanically stressed neocartilage may have resulted in differences in basal expression levels of several Wnt genes in the control groups, such as *WNT9A* and *LEF1* (Figure 3) and should be taken into account in future studies.

Associating Wnt signaling in preserved OA cartilage to JSN scores and OP scores revealed, only higher *WNT5A* mRNA levels to be associated with higher JSN scores. We further observed that in knees, expression of 10 out of 14 genes was significantly lower (e.g., *WNT5A* and *WNT5B*) or higher (e.g., *FRZB*) compared with hips (Supporting Information Figure S2). In the neocartilage, MS increased *WNT5A* expression but not exposure to OAS-CM. Given these observations, it could be promising to further investigate the potential of WNT5A as a marker for MS. This would require more in-depth research measuring the long-term effects of OA-related pathologies on WNT5A in cartilage (e.g., ex vivo explants). Increased WNT5A levels have been shown to repress chondrogenic differentiation and synthesis of cartilage matrix components.[37] It is tempting to speculate that the increased levels of *WNT5A* we observed as the result of MS will affect the propensity of preserved cartilage to enter an OA disease state.

Taken together, MS activated the transcription of noncanonical Wnt signaling, while the inflammatory stimuli can promote canonical Wnt signaling by downregulating *DKK1* and *FRZB*. In addition, we found that WNT5A mRNA levels in preserved OA articular cartilage

are associated with a specific radiographic phenotype, namely, JSN. On the basis of these results, we think that during OA, certain patients may experience more MS-based events while others experience more inflammatory-based events. Therefore, the efficacy of inhibitors that specifically target canonical or noncanonical Wnt signaling may depend on the underlying etiology of the OA subtype and we advocate that targeting specific Wnt pathways could offer a more effective, individualized approach.

AUTHOR CONTRIBUTIONS

Study concept and design: Ritchie G. M. Timmermans, Arjen B. Blom, Ingrid Meulenbelt, Martijn H. J. van den Bosch, and Yolande F. M. Ramos. *Acquisition of material and data*: Ritchie G. M. Timmermans, Rob G. H. H. Nelissen, and Demiën Broekhuis. *Data analysis*: Ritchie G. M. Timmermans, Arjen B. Blom, Ingrid Meulenbelt, Martijn H. J. van den Bosch, and Yolande F. M. Ramos. *Preparation of the manuscript*: Ritchie G. M. Timmermans, Arjen B. Blom, Peter M. van der Kraan, Ingrid Meulenbelt, Martijn H. J. van den Bosch, and Yolande F. M. Ramos. *Critical reviewing and approval of the manuscript*: All authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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