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Characteristic Markers of the WNT Signaling Pathways Are Differentially Expressed in Osteoarthritic Cartilage

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Abstract

Objective: It is well known that expression of markers for WNT signaling is dysregulated in osteoarthritic (OA) bone. However, it is still not fully known if the expression of these markers also is affected in OA cartilage. The aim of this study was therefore to examine this issue. *Methods:* Human cartilage biopsies from OA and control donors were subjected to genome-wide oligonucleotide microarrays. Genes involved in WNT signaling were selected using the BioRetis database, KEGG pathway analysis was searched using DAVID software tools, and cluster analysis was performed using Genesis software. Results from the microarray analysis were verified using quantitative real-time PCR and immunohistochemistry. In order to study the impact of cytokines for the dysregulated WNT signaling, OA and control chondrocytes were stimulated with interleukin-1 and analyzed with real-time PCR for their expression of WNT-related genes. *Results:* Several WNT markers displayed a significantly altered expression in OA compared to normal cartilage. Interestingly, inhibitors of the canonical and planar cell polarity WNT signaling pathways displayed significantly increased expression in OA cartilage, while the Ca^{2+}/WNT signaling pathway was activated. Both real-time PCR and immunohistochemistry verified the microarray results. Real-time PCR analysis demonstrated that interleukin-1 upregulated expression of important WNT markers. *Conclusions:* WNT signaling is significantly affected in OA cartilage. The result suggests that both the canonical and planar cell polarity WNT signaling pathways were partly inhibited while the Ca^{2+}/WNT pathway was activated in OA cartilage.

Keywords

articular cartilage < tissue, biopsy < research methods, osteoarthritis < diagnosis, signaling molecules < cytokines and growth factors

Introduction

Osteoarthritis (OA) is the most common form of arthritis and has a prevalence of about 10% in people over 55 years of age.[1] The hallmark of OA involves joint pain and reduced mobility that is caused by erosion of the cartilage extracellular matrix and alterations in the underlying bone. Although the extracellular matrix is the functional element in articular cartilage and its degradation is central in the pathogenic process in OA, there is now abundant evidence that chondrocytes play a critical role in cartilage degeneration. For instance, OA chondrocytes secrete a variety of enzymes and cytokines, resulting in cleavage of type II collagen.[2] OA chondrocytes, compared to normal chondrocytes, are further in another differentiation state, detected by their increased expression of collagen types I, III, and X.[3] Therefore, increasing the knowledge about the cellular alterations in OA chondrocytes is essential for generating new therapies for patients suffering from OA.

WNT signaling has been shown to regulate crucial aspects of cell fate determination, cell migration, cell polarity, and

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organogenesis during embryonic development and is therefore of importance to study in OA cartilage. WNT regulates at least 3 distinct signaling pathways; the canonical β-catenin pathway regulates cell fate determination, while the 2 noncanonical pathways (planar cell polarity and Ca^{2+}/WNT pathway) control cell movement and tissue polarity.[4] Signaling through these 3 different WNT pathways is dependent upon WNTs, which are secreted glycoproteins. Today, 19 different WNT proteins have been discovered that bind to several different types of receptors, but the most widely recognized WNT receptors are the frizzled proteins (FZD). The FZD receptors are 7-transmembrane–spanning proteins and constitute a family of at least 10 different G protein–coupled receptors. Downstream of this ligand-receptor interaction, the 3 WNT signaling pathways diverge. With regard to the canonical WNT signaling, FZD coreceptors such as LRP5/6 are also required for translocation of the signal. Several secreted WNT antagonists, such as DKK, SFRP, and WIF, exert a key level of regulation of canonical WNT signaling. The activation of FZD and LRP5/6 triggers phosphorylation of DVL, which disrupts the β-catenin degradation complex, consisting of AXIN, APC, and GSK-3β. This complex normally degrades β-catenin through phosphorylation; however, when WNTs bind FZD, β-catenin levels are stabilized and translocated into the nucleus, resulting in transcription of downstream canonical WNT markers. The noncanonical pathways are less studied. They function independently of β-catenin.[5] Still, the FZD receptor is of importance for signaling but also coreceptors such as ROR2 and RYK, but not LRP5/6.[6]

A few studies have been performed investigating the expression of WNT markers during cartilage development, demonstrating an altered expression depending on the differentiation state of the chondrocytes.[7-12] Compared to the knowledge obtained concerning the importance of WNT signaling during cartilage development, relatively little is known regarding the role of WNT signaling in the maintenance and destruction of cartilage. Interleukin-1 is known to increase cartilage matrix degradation, and chondrocytes stimulated with this cytokine increase their expression of WNT5A while decreasing their expression of WNT11.[13] Further evidence for the importance of WNT signaling in OA cartilage is the increased levels of β-catenin in chondrocytes within areas of degenerative cartilage.[14,15] Moreover, microarray analysis of the underlying bone suggests that the WNT network is altered in OA bone and may play a role(s) in OA pathogenesis.[16]

Differential regulation of WNT markers during cartilage development, recent findings concerning the importance of WNT signaling for OA, as well as the shortage of knowledge regarding the expression pattern of WNT markers in OA cartilage prompted us to comprehensively investigate the expression of WNT markers in human OA cartilage. We have earlier reported a broad gene expression comparison of human OA and normal donor cartilage and here use this unique database to focus on investigating the expression of WNT markers.[17] This knowledge will expand our current understanding of OA pathogenesis and provide relevant insight into the phenotypical alterations and mechanisms involved in OA pathogenesis.

Materials and Methods

Collection of Biopsies

Cartilage biopsies from 5 OA patients (74 ± 8 years old) undergoing total knee replacement and 8 control donors (57 ± 16 years old) with macroscopically healthy cartilage and with a Mankin score ≤1 were collected from organ donors (24-48 hours, *post mortem*) (normal donors [NDs]) as described earlier.[17] These biopsies were further used for immunohistological detection of WNT markers (see below). The rest of the biopsies were used for RNA isolation and subsequent microarray analysis. ND and OA cartilage was also collected for subsequent cell cultures. The donation of cartilage was approved by the ethical committees at the Medical Faculties at Gothenburg University and Charité-Universitätsmedizin Berlin.

RNA Isolation

Cartilage biopsies were processed for RNA isolation.[17] Briefly, after mechanical disruption, isolation of total RNA was performed using the Qiagen RNeasy Mini Kit (Hilden, Germany), and the integrity and purity were analyzed using the Agilent Bioanalyzer and NanoDrop spectrophotometer (Santa Clara, CA). All samples were of similar RNA quality and integrity with limited signs of degradation typical for primary tissues.

Microarray Analysis

Gene expression was performed using Affymetrix oligonucleotide microarray HG-U133plus2.0 (Santa Clara, CA) according to the manufacturer's recommendations, as previously described.[17] Genes were regarded as differentially expressed when fulfilling specific change call criteria. In this study, the limit was set to at least ≥75% possible significant change calls. Functional classification of genes involved in WNT signaling was conducted with annotations from the Gene Ontology Annotation Database.[18] Downstream *t* test statistics applying the Welch test on $\log_2$-transformed signal values were performed to obtain a significance level. Expression differences were given as fold changes (FCs); only significantly altered genes that displayed a mean fold change of FC ≥2 or ≤–2 were selected for further analysis. Hierarchical cluster analysis was performed as described previously.[17]

Pathway Analysis

In order to identify significant differently expressed pathways in OA and ND cartilage, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database[19] was searched using DAVID software tools.[20,21] All genes on the array fulfilling the specific criteria used in this study (see above) were included in the gene list.

Quantitative Real-Time PCR Analysis

The following assay-on-demand mixes were used from Applied Biosystems (Foster City, CA) (order number in parentheses): *DKK1* (Hs00183740_m1), *FOSL1* (Hs00759776_s1), *FZD1* (Hs00268943_s1), *SFRP4* (Hs00180066_m1), *WNT5A* (Hs00180103_m1), which were all FAM dye labeled. 18s rRNA (4310893E) labeled with VIC/TAMRA was used as endogenous control. PCR was performed with the Applied Biosystems 7900HT real-time PCR System. cDNA from 6 NDs and 5 OA donors, corresponding to 1 ng total RNA isolated from the cartilage biopsies, and 2.5 ng total RNA from the monolayer cultured cells isolated from 3 NDs and 3 OA donors were analyzed in duplicates. Raw data were analyzed by using SDS v2.2.2 software (Applied Biosystems), and the $2^{-\Delta\Delta Ct}$ value was used to obtain the gene expression for each particular gene. Statistical analysis was performed using the Mann-Whitney *U* test (cartilage biopsies) and Wilcoxon paired signed-rank test (monolayer cultured cells). Values of $P < 0.05$ were considered to indicate statistically significant differences.

Immunohistochemistry

Immunohistochemistry was performed on 5 OA and 4 ND sections in duplicates from articular cartilage for AXIN2, DKK1, DKK3, and WNT5A. Slides for labeling with AXIN2 and DKK3 were digested with trypsin 0.05% EDTA, while labeling with DKK1 and WNT5A was preceded by protease treatment (1 mg/mL; Sigma-Aldrich, St. Louis, MO). Additional enzyme treatment with hyaluronidase (8000 U/mL; Sigma-Aldrich) was performed for DKK1. The sections were blocked with 3% BSA. Primary antibodies (rabbit polyclonal anti-AXIN2, rabbit polyclonal anti-DKK, rabbit polyclonal anti-DKK3, and goat polyclonal anti-WNT5A) were purchased from Abcam (Cambridge, MA), Sigma-Aldrich, and R&D Systems (Minneapolis, MN), respectively. An isotype control for each primary antibody was used as control: normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for AXIN2 and DKK1 as well as rabbit polyclonal IgG (Abcam) for DKK3 and normal goat IgG for WNT5A. The primary antibodies and the isotype controls were visualized using HRP-conjugated secondary antibodies (sheep anti-rabbit HRP [Millipore, Billerica, MA] and donkey anti-goat HRP). The secondary antibodies were visualized using the TSA-Direct Cy3 kit (Perkin Elmer, Boston, MA) according to the manufacturer's instructions. The nuclei were stained with 4',6-diamidino-2-phenylindol (DAPI) (Sigma-Aldrich). Digital pictures were taken with the NIS-Elements D Ver.3.00 (Nikon, Tokyo, Japan).

Analysis of Protein Interaction Networks

To investigate possible interactions among proteins coded by genes that were differentially regulated (defined by having a mean FC ≥ 3 or ≤ -3) between NDs and OA donors, the protein interaction analysis was performed as described previously.[17]

Chondrocyte Isolation

Chondrocytes were isolated from 3 ND and 3 OA cartilage biopsies as described previously.[22] The isolated cells were expanded in medium consisting of DMEM/F12 (Invitrogen, Paisley, UK) supplemented with L-ascorbic acid (0.025 mg/mL; Apotekets production unit, Umeå, Sweden), gentamicin sulfate (50 mg/L; Gibco, Paisley, UK), amphotericin B (250 µg/mL), and L-glutamine (2 mM; Gibco) and 10% human serum.

Cytokine Stimulation of Chondrocytes

Chondrocytes, isolated from 3 NDs and 3 OA donors, in passage 1 were stimulated with 10 ng/mL of human recombinant interleukin-1β (Sigma-Aldrich) for 24 hours or left unstimulated. Gene expression of WNT markers was then studied in duplicates using real-time PCR, as described above.

Results

Characterization of OA and ND Cartilage

For an extensive verification of the NDs and OA phenotypes, see Karlsson *et al*.[17] Briefly, all OA donors displayed an Ahlbäck score of 2 to 3. Mankin scoring of the cartilage biopsies demonstrated significant differences between ND and OA articular cartilage biopsies (0.8 ± 0.15 and 6.4 ± 1.3, respectively).

Pathway Analysis in OA versus ND Cartilage

In order to explore pathways that could be differentially expressed in OA versus ND cartilage, the KEGG pathway database was searched using the genes regarded as significantly differentially expressed as input. The analysis demonstrated 26 pathways that show a significantly different expression in OA compared to ND cartilage, whereas 10 of the most significantly expressed pathways are listed in

Table 1. Differentially Expressed Pathways in Osteoarthritic (OA) versus Normal Donor (ND) Cartilage

KEGG ID	Pathway Term	Count	*P* Value
hsa04512	Extracellular matrix receptor interaction	29	9.28E-10
hsa04510	Focal adhesion	43	4.12E-7
hsa05130	Pathogenic *Escherichia coli* infection	18	1.10E-5
hsa05200	Pathways in cancer	52	1.81E-4
hsa05222	Small cell lung cancer	18	1.81E-3
hsa04810	Regulation of actin cytoskeleton	34	3.11E-3
hsa04310	Wnt signaling pathway	26	3.53E-3
hsa04115	p53 signaling pathway	15	3.81E-3
hsa04722	Neurotrophin signaling pathway	22	5.53E-3
hsa05221	Acute myeloid leukemia	13	6.87E-3

Note: Pathway analysis using the KEGG pathway database with significantly different expression in OA compared to ND cartilage. Only 10 (of 26) of the most significant differentially expressed pathways are shown. Count indicates the total number of genes from the input list that belong to the corresponding term; only significantly expressed genes that displayed a mean fold change (FC) of ≥2 or ≤–2 were included in the gene list.

Table 1. The presence of the WNT signaling pathway demonstrates that the WNT network is dysregulated in OA compared to ND cartilage.

WNT Markers Are Differentially Regulated in OA Cartilage

Due to the results obtained from the KEGG pathway analysis, a cluster analysis was performed of all genes denoted to be involved in WNT signaling. The analysis revealed 2 distinct clusters, one with OA cartilage and one with ND cartilage (**Fig. 1**). These separate clusters thus demonstrate that the expression of WNT markers is significantly altered in OA cartilage.

Increased Expression of Genes Encoding Inhibitors of the WNT Signaling Pathway in OA Cartilage

Our results from the microarray analysis demonstrate that the expression of 32 genes associated with WNT signaling was significantly regulated between OA and ND cartilage (**Table 2**). Of interest to note is that several inhibitors of the canonical and planar cell polarity WNT signaling pathways displayed an increased expression in OA cartilage. The expression of important extracellular inhibitors of the canonical pathway, including *DKK1*, *DKK3*, *WIF1*, *SFRP1*, and *SFRP4*, was significantly upregulated in the OA biopsies, with a 3.9, 3.9, 3.1, 8.1, and 14.2 FC, respectively. Several homologs of the FZD receptor (*FZD1*, *FZD7*, *FZD8*, and *FZD10*) also displayed increased expression in OA cartilage. Expression of intracellular and intranuclear inhibitors was also upregulated in the OA biopsies, including genes involved in β-catenin phosphorylation and its destruction, such as *AXIN2*, *SOX17*, and *TAX1BP3*. Furthermore, the transcriptional repressor *TLE4* displayed a 2.3-fold higher expression in OA than in ND biopsies, whereas *TCF7L2* was downregulated 2.5-fold. On the other hand, genes transcribed when the canonical WNT signaling is activated, such as *FOSL1* and *PPARD*, exhibited an increased expression in OA cartilage. Our results further demonstrate that the planar cell polarity pathway was inhibited. Expression of several markers for this pathway, such as *PRICKLE2*, *DVL2*, and *DAAM2*, was significantly downregulated 2.1-, 3.4-, and 3.8-fold, respectively, in OA cartilage. However, genes denoted to the Ca^{2+}/WNT pathway displayed an increased expression. Among them, *WNT5A*, *CAMKII*, *NFAT5*, and *NFATC2* expression was significantly upregulated 16.0-, 3.2-, 4.3-, and 8.2-fold, respectively, in OA cartilage. These extended microarray results are summarized in **Figure 2**.

Increased Gene Expression of FOSL1, FZD8, NFATC2, SFRP1, SFRP4, and WNT5A in OA Cartilage

To verify the microarray data, a quantitative real-time PCR analysis was performed for 7 of the most highly differentially expressed WNT-related genes comparing OA and ND cartilage according to the microarray analysis. The real-time PCR verification demonstrated that the extracellular inhibitors *SFRP1*, *SFRP4*, as well as the ligand *WNT5A* were significantly upregulated in OA cartilage (**Table 3**). The intracellular target and effector genes *FOSL1* and *NFATC2* also displayed a significant upregulation in OA cartilage, whereas the receptor *FZD8* displayed a similar trend but not a significant upregulation ($P = 0.052$). On the other hand, no significant difference was detected with regard to the expression of the downstream target gene *PPARD* in OA and ND cartilage ($P = 0.082$) (**Table 3**).

Increased Protein Expression of AXIN2, DKK1, DKK3, and WNT5A in OA Cartilage

The spatial localization of AXIN2, DKK1, DKK3, and WNT5A was studied in OA and ND cartilage biopsies using immunohistochemistry in order to verify the microarray data. These markers were investigated due to their involvement in inhibiting the canonical WNT pathway (DKK1, DKK3, and AXIN2) as well as activating the Ca^{2+}

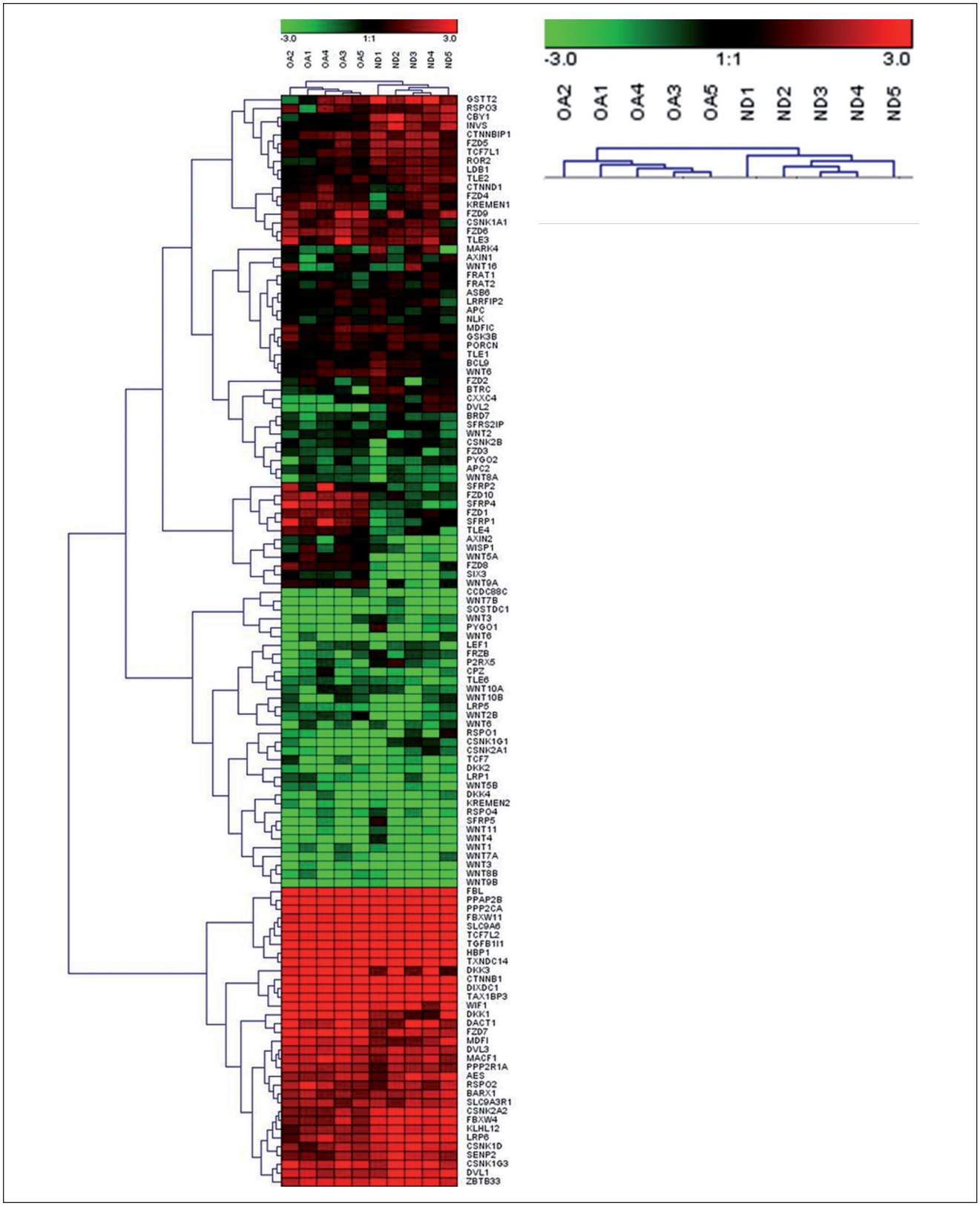

Figure 1. Hierarchical cluster analysis of genes involved in WNT signaling. Hierarchical cluster analysis of genes known to be associated with WNT signaling demonstrated that cartilage from the 5 donors with normal cartilage (ND1-ND5) formed one cluster and cartilage from osteoarthritic donors (OA1-OA5) formed another cluster, showing significantly altered WNT signaling in OA cartilage.

Table 2. WNT Related Genes Revealed in the Microarray Analysis

Gene Name	Gene Symbol	Probe ID	Public ID	FC OA versus ND	*P* Value
Extracellular					
Wingless-type MMTV integration site family, member 5	WNT5A	205990_s_at	NM_003392	16.0	6.92735E-15
Secreted frizzled-related protein 4	SFRP4	204051_s_at	AW089415	14.2	1.38979E-25
Secreted frizzled-related protein 1	SFRP1	202037_s_at	NM_003012	8.1	1.45767E-12
Dickkopf homolog 1	DKK1	204602_at	NM_012242	3.9	4.24038E-6
Dickkopf homolog 3	DKK3	202196_s_at	NM_013253	3.9	1.18684E-5
WNT inhibitory factor 1	WIF1	204712_at	NM_007191	3.1	2.26111E-5
Membrane bound					
Frizzled homolog 8	FZD8	227405_s_at	AW340311	13.4	8.92757E-26
Frizzled homolog 10	FZD10	219764_at	NM_007197	6.7	6.72813E-30
Vang-like 1	VANGL1	219330_at	NM_024062	4.8	5.55668E-17
Sulfatase 1	SULF1	212353_at	AI479175	4.3	4.29776E-11
Frizzled homolog 7	FZD7	203706_s_at	NM_003507	4.0	3.13684E-24
Frizzled homolog 1	FZD1	204452_s_at	AF072872	3.8	3.07887E-13
Intracellular					
Peroxisome proliferator–activated receptor δ	PPARD	242218_at	AI201116	12.8	9.55763E-8
FOS-like antigen 1	FOSL1	204420_at	BG251266	9.1	3.88083E-11
Transcript factor NFAT1 isoform C	NFATC2	226991_at	NM_001136021	8.2	4.32651E-13
Nuclear factor of activated T-cells 5, tonicity responsive	NFAT5	215092_s_at	NM_001113178	4.3	3.4648E-4
Casein kinase 1 ε	CSNK1E	226858_at	—	3.5	2.73695E-8
Calmodulin-dependent protein kinase II α	CAMK2A	213108_at	NM_015981	3.2	1.36801E-6
SRY-box 17	SOX17	219993_at	NM_022454	2.7	2.70202E-7
Transducin-like enhancer of split 4 E (sp1) homolog	TLE4	204872_at	NM_007005	2.3	2.82611E-8
Axin 2	AXIN2	222696_at	BF684446	2.3	7.11705E-14
Tax1-binding protein 3	TAX1BP3	209154_at	AF234997	2.1	5.9216E-12
DIX domain containing 1	DIXDC1	214724_at	AF070621	2.1	5.99097E-16
F-box and WD repeat domain containing 4	FBXW4	221519_at	AF281859	−2.0	7.8246E-19
Prickle homolog 2	PRICKLE2	225968_at	BG285881	−2.1	4.51586E-11
Protein phosphatase 2 regulatory subunit B, α isoform	PPP2R2A	202313_at	NM_002717	−2.1	1.24529E-10
HMG-box transcription factor 1	HBP1	209102_s_at	AF019214	−2.2	3.19294E-15
C-terminal–binding protein 2	CTBP2	201218_at	N23018	−2.4	5.83668E-12
Casein kinase 2, α prime polypeptide	CSNK2A2	203575_at	NM_001896	−2.4	4.59298E-13
Transcription factor 7–like 2	TCF7L2	216511_s_at	—	−2.5	1.03935E-12
Disheveled-associated activator of morphogenesis 2	DAAM2	212793_at	BF513244	−3.4	2.16355E-17
Disheveled, dsh homolog 2	DVL2	218756_at	NM_004422	−3.8	2.2239E-9

Note: Genes whose expression is differentially regulated between osteoarthritic (OA) and normal donor (ND) cartilage and that are associated with WNT signaling. Genes are divided into genes encoding extracellular, transmembrane, and intracellular proteins. Expression differences are given as fold change (FC); only genes with an FC ≥2 or ≤−2 are shown. Genes with a positive FC are upregulated in OA cartilage when compared with ND cartilage.

pathway (WNT5A). Expression of AXIN2 and DKK1 in ND cartilage was localized to the uppermost cell layers, whereas their expression was extended to the cells located in the deeper layers in OA cartilage (**Fig. 3A-D**). Abundant expression of DKK3 and WNT5A was detected in OA cartilage, while a lower expression, somewhat more restricted to the superficial zone, was detected in ND cartilage (**Fig. 3E-H**).

WNT Signal Transduction Pathway Differentially Expressed in OA Cartilage

The protein-protein interaction network analysis of genes, classified both as signal transducers and differentially expressed in OA cartilage (performed with raw data originated from the microarray analysis), revealed 7 hub genes associated with the WNT signaling pathway

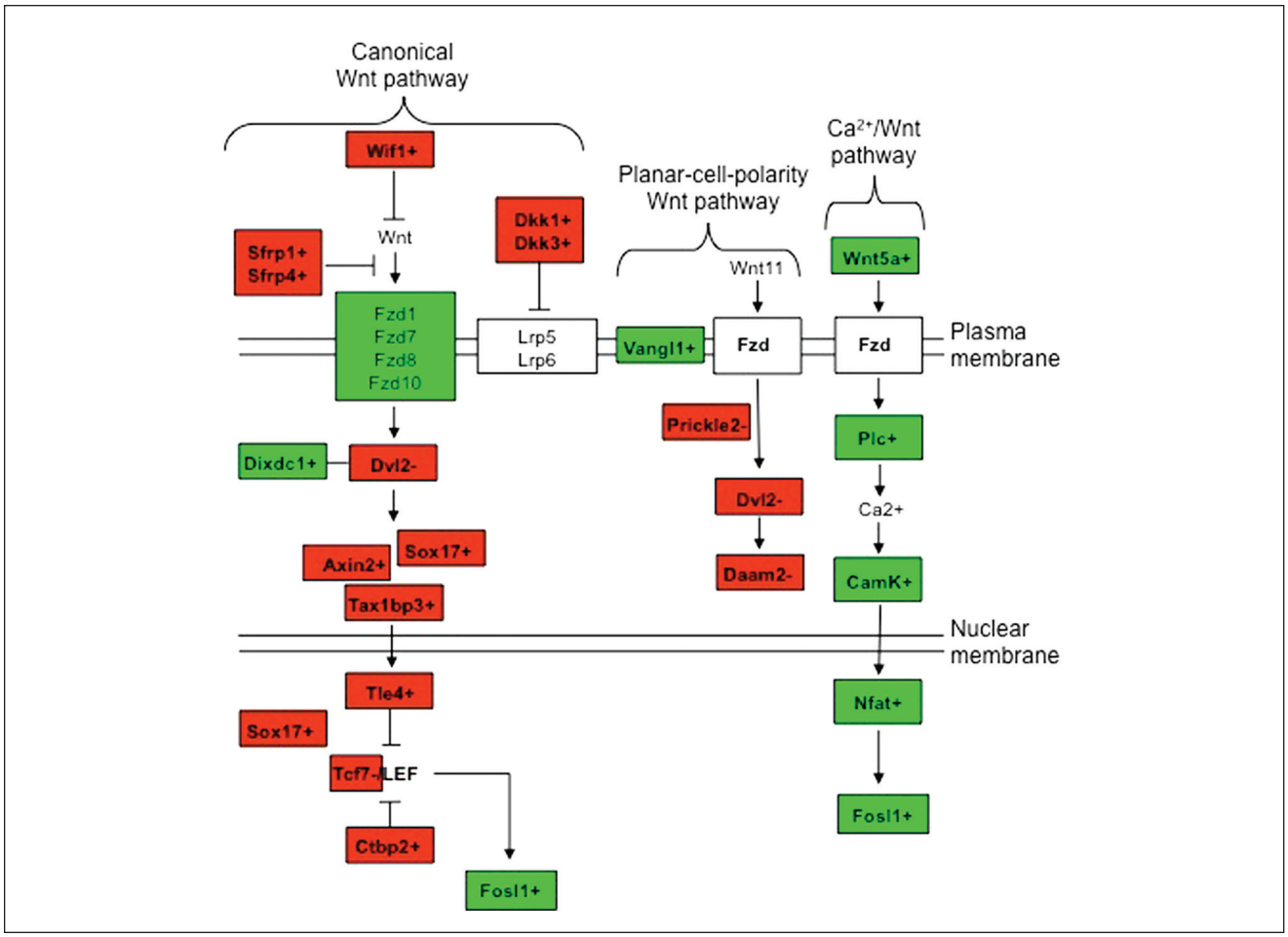

Figure 2. Schematic summary of the results obtained from the microarray analysis. Genes in red boxes negatively regulate the WNT signaling pathway, whereas the green boxes indicate a positive regulation of the network. A "±" sign after the gene name refers to a positive (higher gene expression in OA cartilage) or negative (lower gene expression in OA cartilage) fold change (FC) in the microarray analysis.

Table 3. Quantitative PCR Analysis of WNT-Related Genes

	ND	95% CI	OA	95% CI	*P* Value
SFRP4	1.0	0.87	33.64	16.98	0.004
FOSL1	1.0	0.72	14.26	8.29	0.004
WNT5A	1.0	0.37	10.66	5.55	0.004
SFRP1	1.0	0.72	8.35	7.67	0.030
NFATC2	1.0	0.32	7.90	2.54	0.004
FZD8	1.0	0.74	2.76	0.83	0.052
PPARD	1.0	0.36	0.58	0.13	0.082

Note: Quantitative real-time PCR verification of genes, which displayed the highest fold change (FC) in this extended microarray analysis. Error presenting the uncertainty with a 95% confidence interval (CI). Cartilage biopsies from 6 normal donors (NDs) and 5 osteoarthritis (OA) donors were studied; each sample was analyzed in duplicates.

including the receptors *FZD1*, *FZD7*, *FZD8*, and *FZD10*; the extracellular inhibitors *SFRP1* and *SFRPP4*; and the ligand *WNT5A* (**Fig. 4**). The presence of these hub genes demonstrates that the WNT signaling pathway is dysregulated in OA cartilage (for more general information, see **Table 4**).

Increased Expression of DKK1, FOSL1, SFRP4, and WNT5A in Interleukin-1β–Stimulated Chondrocytes

Chondrocytes from both OA and ND cartilage were stimulated with interleukin-1β in order to study if the increased production of this cytokine in OA could explain the increased expression of WNT-related genes. Real-time PCR demonstrated that interleukin-1β stimulation induced significantly increased expression of *FOSL1* and *WNT5A* in chondrocytes derived from both ND and OA cartilage. The same trend was observed in *SFRP4* expression (except for OAChon3) (**Fig. 5A-C**). No consistent upregulated expression of *DKK1* was detected after interleukin-1β

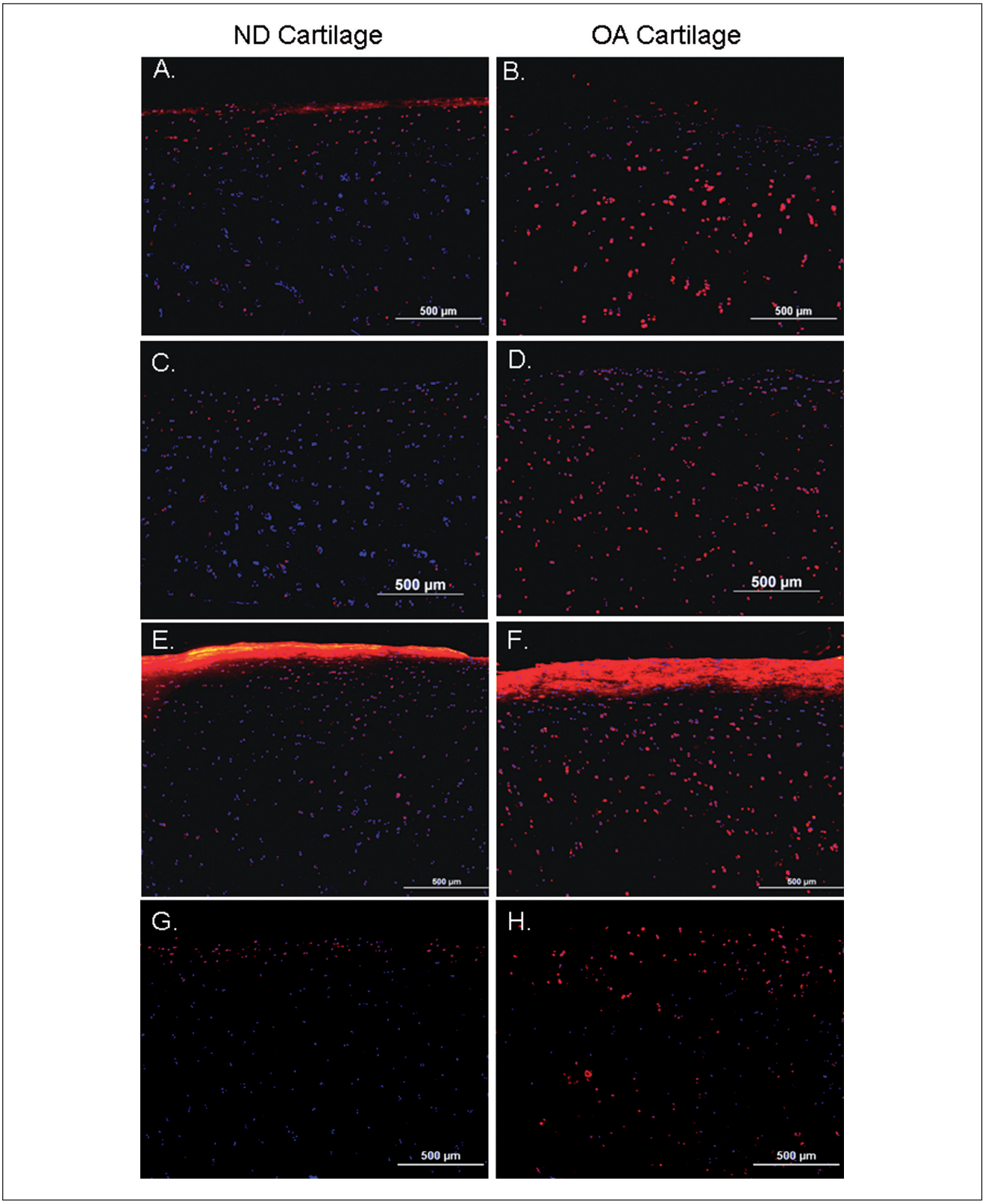

Figure 3. Verification of the microarray results using immunohistochemistry. Stainings with antibodies against AXIN2 (**A**, **B**), DKK1 (**C**, **D**), DKK3 (**E**, **F**), and WNT5A (**G**, **H**) in normal donor (ND) (**A**, **C**, **E**, **G**) and OA (**B**, **D**, **F**, **H**) cartilage. Nuclei were stained blue with 4',6-diamidino-2-phenylindol.

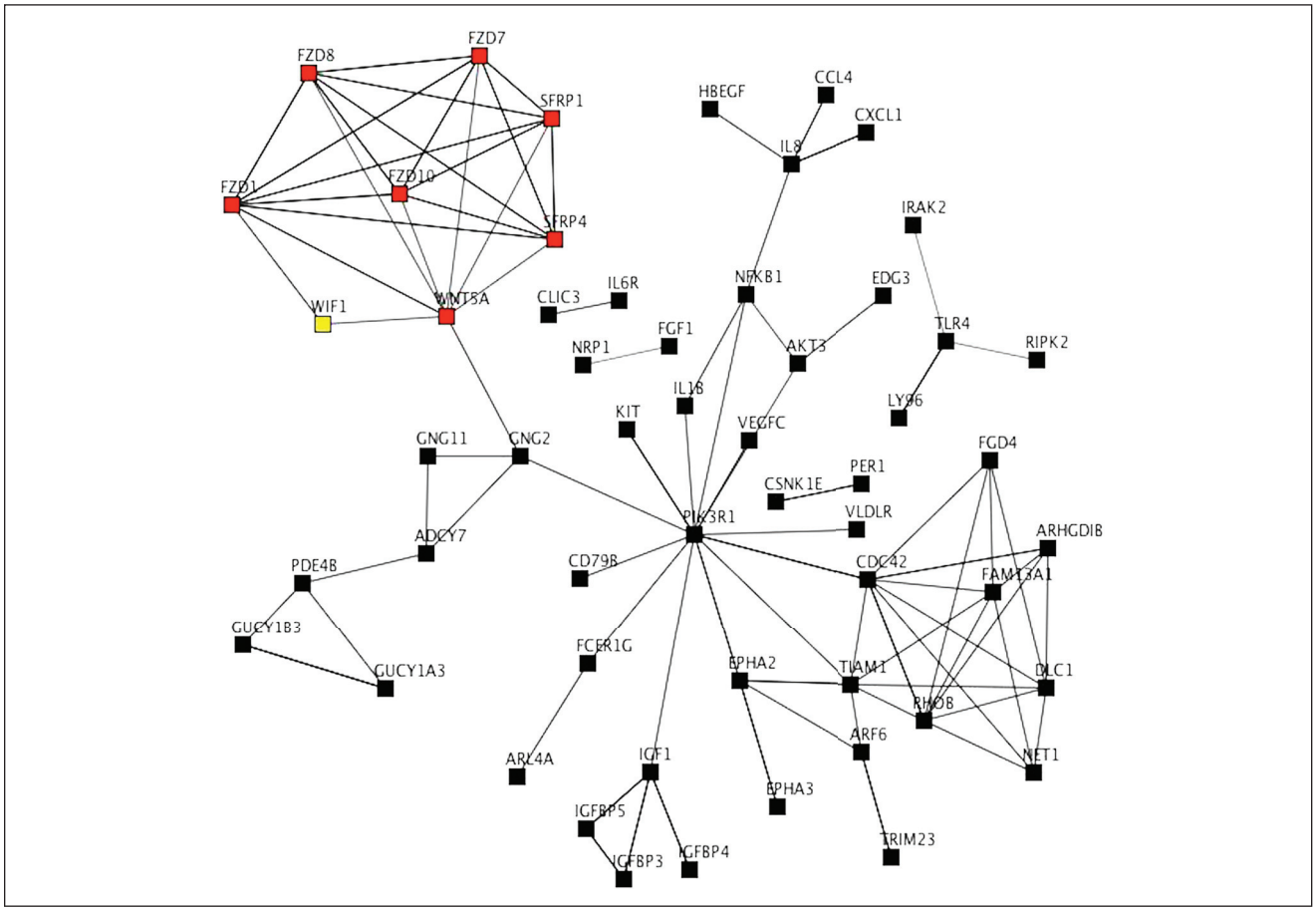

Figure 4. Protein-protein interaction network of genes differentially regulated comparing OA and ND cartilage and classified as signal transducers, generated with the STRING search tool. Hub genes linked to WNT signaling, identified as genes with interaction with at least 5 other regulated genes, are marked in red. Other WNT-associated genes are marked by yellow boxes.

stimulation, while a somewhat downregulated expression of *FZD1* was detected (**Fig. 5D** and **5E**). No significant differences in the expression of Wnt markers were detected between ND and OA chondrocytes cultured in monolayer (data not shown).

Discussion

This study is the first that comprehensively analyzed the expression of WNT markers in human OA cartilage using genome-wide microarrays. We have earlier reported the results from a broad gene expression comparison of ND and OA cartilage and here focus on the expression of WNT markers.[17] One important issue in this type of study is the source of biological material. In this study, we chose to analyze cartilage obtained *post mortem* from donors having no history of OA as well as patients diagnosed with OA. One drawback with this approach is that there is a difference in age between the 2 groups of donors compared, and it is further possible that these 2 groups of donors have different levels of physical activity. Both these factors can, of course, affect the gene expression of the chondrocytes.[23,24] Another alternative approach is to use macroscopically healthy and diseased cartilage from the same OA patient. This setup overcomes the issues described above, but on the other hand, possible genetic differences will not be detected, and what macroscopically appears to be healthy cartilage might still differ from "true" healthy cartilage. OA is further more common on the medial part of the knee so taking OA and ND cartilage from the same joint will thus likely result in a comparison of cartilage from different parts of the knee, which also is known to affect gene expression.[25,26] We have characterized our biopsies both macroscopically, microscopically, as well as using whole-genome microarray analysis and demonstrated typical traits of ND and OA cartilage as seen in the literature[17,27] and therefore consider the differences detected in WNT signaling in our study highly likely to be due to OA.

Table 4. Genes Revealed in the Protein-Protein Interaction Analysis

Gene Name	Gene Symbol	Probe ID	Public ID	FC OA versus ND	*P* Value
Interleukin 8	IL8	211506_s_at	AF043337	136.4	6.68882E-20
Chemokine ligand 4	CCL4	204103_at	NM_002984	29.1	1.38807E-17
Adenylate cyclase 7	ADCY7	203741_s_at	NM_001114	28.6	1.09951E-16
Fc fragment of IgE, high affinity I, receptor for γ polypeptide	FCER1G	1554899_s_at	—	20.4	5.63102E-12
Insulin-like growth factor 1	IGF1	209541_at	AI972496	17.6	4.53068E-8
Phosphodiesterase 4B, cAMP specific	PDE4B	203708_at	NM_002600	16.8	2.47481E-16
Wingless-type MMTV integration site family, member 5	WNT5A	205990_s_at	NM_003392	16.0	6.92735E-15
Secreted frizzled-related protein 4	SFRP4	204051_s_at	AW089415	14.2	1.38979E-25
Frizzled homolog 8	FZD8	227405_s_at	AW340311	13.4	8.92757E-26
Insulin-like growth factor binding protein 4	IGFBP4	201508_at	NM_001552	11.0	7.25381E-12
Interleukin-1β	IL1B	205067_at	NM_000576	10.4	4.28733E-16
Guanylate cyclase 1, soluble, α 3	GUCY1A3	221942_s_at	AI719730	9.6	1.45456E-12
Chloride intracellular channel 3	CLIC3	219529_at	NM_004669	8.4	3.06828E-9
Secreted frizzled-related protein 1	SFRP1	202037_s_at	NM_003012	8.1	1.45767E-12
Chemokine (C-X-C motif) ligand 1	CXCL1	204470_at	NM_001511	7.9	2.24479E-15
ADP ribosylation factor–like 4A	ARL4A	205020_s_at	NM_005738	7.3	3.32194E-9
Neuropilin 1	NRP1	210510_s_at	AF145712	6.9	2.562E-14
Frizzled homolog 10	FZD10	219764_at	NM_007197	6.7	6.72813E-30
Endothelial differentiation, sphingolipid G protein–coupled receptor 3	EDG3	228176_at	—	6.4	1.98944E-10
Fibroblast growth factor 1	FGF1	205117_at	X59065	6.3	6.13851E-12
Vascular endothelial growth factor C	VEGFC	209946_at	U58111	5.8	1.9044E-15
Insulin-like growth factor binding protein 5	IGFBP5	211959_at	AW007532	5.5	4.0204E-28
Interleukin-1 receptor–associated kinase 2	IRAK2	231779_at	AI246590	5.5	5.16393E-10
Guanine nucleotide binding protein γ 2	GNG2	224964_s_at	AK026424	5.2	4.62131E-13
Heparin-binding EGF-like growth factor	HBEGF	38037_at	M60278	4.9	9.22139E-11
Guanine nucleotide binding protein γ 11	GNG11	204115_at	NM_004126	4.8	1.66689E-15
EPH receptor A3	EPHA3	206070_s_at	AF213459	4.7	1.71399E-8
Lymphocyte antigen 96	LY96	206584_at	NM_015364	4.3	8.90125E-14
Insulin-like growth factor binding protein 3	IGFBP3	212143_s_at	BF340228	4.2	5.24352E-10
Frizzled homolog 7	FZD7	203706_s_at	NM_003507	4.0	3.13684E-24
Guanylate cyclase 1, soluble, β 3	GUCY1B3	211555_s_at	AF020340	3.9	8.58847E-8
Deleted in liver cancer 1	DLC1	210762_s_at	AF026219	3.9	2.07307E-11
Frizzled homolog 1	FZD1	204452_s_at	AF072872	3.8	3.07887E-13
Toll-like receptor 4	TLR4	221060_s_at	NM_003266	3.7	1.04917E-8
Cell division cycle 42	CDC42	210232_at	M35543	3.7	1.28184E-13
Casein kinase 1 ε	CSNK1E	226858_at	—	3.5	2.73695E-8
T-cell lymphoma invasion and metastasis 1	TIAM1	213135_at	U90902	3.5	2.20078E-11
Nuclear factor of κ light polypeptide gene enhancer in B-cells 1	NFκB1	209239_at	M55643	3.4	7.31975E-11
EPH receptor A2	EPHA2	203499_at	NM_004431	3.4	1.24766E-8
Receptor-interacting serine-threonine kinase 2	RIPK2	209545_s_at	AF064824	3.4	6.04088E-12
Rho GDP dissociation inhibitor β	ARHGDIB	1555812_a_at	—	3.1	1.29293E-12
WNT inhibitory factor 1	WIF1	204712_at	NM_007191	3.1	2.26111E-5
ADP ribosylation factor 6	ARF6	203312_x_at	NM_001663	2.3	1.20473E-10
Period homolog 1	PER1	36829_at	AF022991	–2.6	4.15062E-16
V-akt murine thymoma viral oncogene homolog 3	AKT3	212607_at	N32526	–3.0	3.91207E-15
Very low density lipoprotein receptor	VLDLR	209822_s_at	L22431	–3.2	3.87155E-14
CD79b molecule, immunoglobulin-associated β	CD79B	205297_s_at	NM_000626	–3.3	1.07823E-10
Phosphoinositide 3-kinase, regulatory subunit 1	PIK3R1	212249_at	AI934473	–4.2	9.10041E-13
Tripartite motif containing 23	TRIM23	210994_x_at	AF230398	–4.5	3.92373E-21
Neuroepithelial cell transforming 1	NET1	201829_at	AW263232	–4.8	1.24765E-44
FYVE, RhoGEF and PH domain containing 4	FGD4	230559_x_at	AI277617	–6.7	4.07098E-11
Interleukin 6 receptor	IL6R	226333_at	—	–6.8	1.22076E-29
Family with sequence similarity 13, member A	FAM13A1	202973_x_at	—	–7.0	1.13137E-17
V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	KIT	205051_s_at	NM_000222	–7.5	4.08763E-9
Ras homolog gene family, member B	RHOB	1553965_x_at	—	–8.4	7.02128E-17

Note: All genes, both WNT and non-WNT related, revealed in the protein-protein interaction analysis. Expression differences are given as fold change (FC). Genes with a positive FC are upregulated in osteoarthritic (OA) cartilage when compared with normal donor (ND) cartilage. Performed with raw data originated from the microarray analysis.

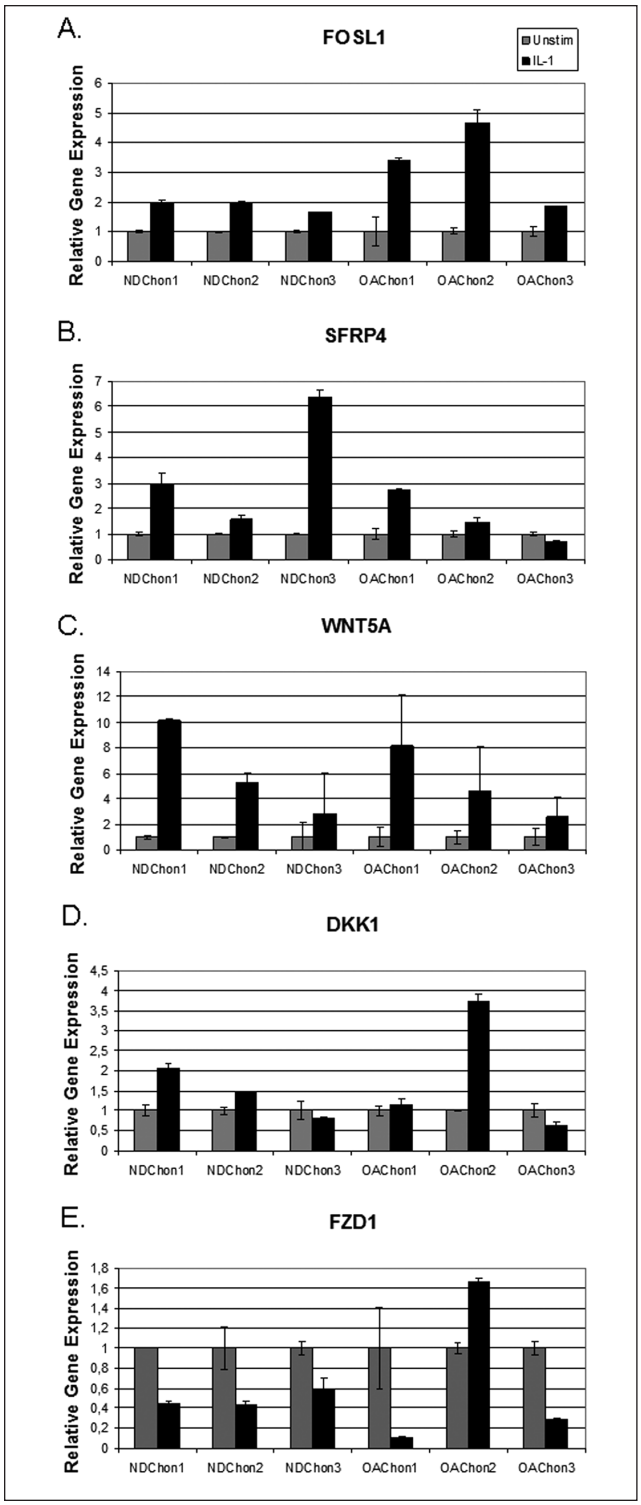

Figure 5. Real-time PCR analysis after IL-1β stimulation. Gene expression analysis of *FOSL1*, *SFRP4*, *WNT5A*, *DKK1*, and *FZD1* after interleukin-1β stimulation of chondrocytes from normal cartilage (indicated as NDChon1-3 in the chart) and OA cartilage (indicated as OAChon4-6 in the chart). Error bars present the uncertainty with a 95% confidence interval. Cartilage biopsies from 3 normal donors (NDs) and 3 OA donors were studied; each sample was analyzed in duplicates.

The KEGG pathway analysis, demonstrating differently expressed WNT signaling in OA compared to ND cartilage, encouraged us to further investigate the WNT pathway. This approach resulted in the discovery of an array of WNT-related genes, not previously associated with OA, displaying significantly altered expression in OA compared to ND human articular cartilage. Besides the results from the pathway analysis, the main finding of this study is the discovery of a panel of inhibitors of the canonical and planar cell polarity pathways displaying significantly increased expression in OA cartilage. Moreover, the protein-protein interaction network analysis, demonstrating that several hub genes within the WNT signaling pathway were dysregulated in OA compared to ND cartilage, gave further indications of a different signaling profile in OA-affected cartilage. Our results further demonstrate that the expression of several of these genes significantly increased after cytokine treatment, which at least partly might explain their increased expression in OA cartilage. In fact, the expression of IL-β was significantly increased (10.4 FC) in OA compared to healthy cartilage (**Table 4**). Moreover, previous studies have demonstrated upregulation of several WNT-related genes associated with IL-1β stimulation.[13,28,29]

In this study, both *DKK1* and *DKK3* were found to be highly expressed in OA cartilage. DKK1 interacts with LRP5 and LRP6 and the transmembrane receptors KRM1 and KRM2 and prevents activation of the canonical WNT network.[30] In accordance with our results, increased serum levels of DKK1 have been detected in patients with OA,[28,31] and it is likely that DKK1 detected in serum originates from the OA-affected cartilage. However, the impact of this increased expression is still unknown, but it has been associated both with reduced risk for cartilage loss[31] and chondrocyte apoptosis.[28] Additionally, in line with our results, Meng *et al.* observed an upregulation of DKK3 in experimentally induced OA cartilage.[32]

Our microarray data further demonstrated increased expression of other extracellular inhibitors of canonical WNT signaling, including *WIF1*, *SFRP1*, and *SFRP4* (results for *SFRP1* and *SFRP4* were also verified by real-time PCR). Verification by immunohistological stainings revealed expression of DKK1 and DKK3 in the entire sections of OA cartilage, but only in the surface zone in ND cartilage. This could be due to the phenotypic and metabolic changes that OA chondrocytes display among the zones in articular cartilage compared to ND chondrocytes. WIF1 and SFRP are antagonists to the WNT signaling network, resulting in an inhibition of both the canonical and planar cell polarity pathways.[33] Moreover, the protein interaction analysis conducted in this study revealed interactions between *FZD8*, *SFRP1*, and *SFRP4*, which give further support to the suggestion of inhibited WNT pathways. Our study is the first demonstrating WIF1 in OA cartilage, while both SFRP1 and SFRP4 have earlier been detected in OA

synovium[34] but have not previously been associated with OA chondrocytes. SFRP1, SFRP3, and SFRP5 have recently been demonstrated to be expressed in proliferating chondrocytes and at the prehypertrophic stage during mouse limb formation.[7] The significantly increased expression of *SFRP1* and *SFRP4* detected in OA cartilage might thus result in increased proliferation and hypertrophy characteristic of OA cartilage. Our results further demonstrate that the increased expression of *DKK1*, *FOSL1*, *SFRP4*, and *WNT5A* in OA cartilage might partly be due to the increased release of cytokines characteristic of OA chondrocytes. In accordance with our data, Weng *et al*. have correlated both tumor necrosis factor-α and interleukin-1β production to DKK1 expression in OA cartilage.[28]

Other signs of an inhibited canonical WNT signaling pathway in OA cartilage were suggested in this study by a high expression of several genes involved in β-catenin phosphorylation and its destruction, such as *AXIN2*, *SOX17*, and *TAX1BP3*. AXIN2 expression has been reported to increase in response to mechanical injury to human articular cartilage[35] and is known to assemble proteins involved in β-catenin phosphorylation and degradation. AXIN2 has further been suggested to participate in a negative feedback loop limiting the duration/intensity of a WNT-initiated signal.[36,37] Immunohistological staining performed in our study exhibited expression of AXIN2 in the uppermost layers of ND cartilage; meanwhile, OA cartilage displayed AXIN2 expression in the entire section, which could implicate that AXIN2 promotes restriction of WNT network signals in OA cartilage. SOX17 interacts directly with both β-catenin and TCF/LEF to trigger degradation of the β-catenin complex, thus inhibiting WNT signaling.[38] TAX1BP3, also referred to as TIP1, has been suggested to repress transcriptional activity of β-catenin, possibly by affecting the stability or subcellular localization of β-catenin.[39] Taken together, these findings could suggest that the activity/stability of β-catenin is affected in OA cartilage, thus potentially resulting in an inhibited canonical WNT signaling pathway. On the contrary, other studies have reported increased protein expression of β-catenin in human OA cartilage,[15] implicating an activated canonical WNT signaling. Additionally, the study also demonstrated significantly higher expression of the noncanonical ligand *WNT5A*, which is in line with our results (see below) and is further suggested by Zhu *et al.* as an upregulation of Ca^{2+}/WNT signaling pathway via β-catenin. Although the expression of β-catenin is not ascertained within this study, and we cannot rule out the possibility that downstream signaling is affected by other compensation mechanisms than those revealed in this microarray analysis, this thus suggests that the canonical WNT signaling pathway is partly inhibited in OA cartilage.

Finally, our analysis further revealed an increased expression in OA cartilage of several intranuclear inhibitors of canonical WNT signaling, such as *TLE4*, but also decreased expression of the transcription factor *TCF7L2* (also referred to as *TCF4*). This might result in an inhibited transcription of downstream WNT-related genes. On the other hand, the microarray data demonstrated increased expression in OA cartilage of *FOSL1* and *PPARD*. However, verification of the microarray expression by PCR only revealed a significantly increased expression of *FOSL1* in OA cartilage. *FOSL1* is involved in WNT signaling pathway (according to the KEGG database) and is suggested as a β-catenin/WNT signaling target gene, transcribed when WNT signaling is activated,[40,41] thus implying increased canonical WNT signaling. This is not in accordance with our results obtained for upstream genes within the canonical WNT pathway, suggesting decreased signaling through this pathway, but could thus indicate a partly inhibited canonical WNT signaling pathway. However, this gene is also part of another WNT signaling cascade, the Ca^{2+}/WNT signaling pathway,[42] which, suggested by our study, was activated in OA cartilage and thus might explain the increased expression of *FOSL1* (see discussion below). FOSL1, also referred to as FRA1, is further also part of the AP1 complex, which has been implicated in the regulation of matrix metalloproteinase 1 (MMP1) expression in OA cartilage.[43,44] Moreover, ectopic expression of FRA1 induces increased bone mass,[45] which is interesting because increased subchondral plate thickness is a characteristic of OA pathogenesis. However, because AP-1 genes have further been implicated in the pathogenesis of cancer in various tissues and are commonly activated in response to inflammatory stimuli,[46,47] the high expression in OA cartilage thus not unanimous demonstrates WNT activation.

With regard to markers of the planar cell polarity pathway, our study demonstrated that transcription of the gene encoding the transmembrane protein *VANGL1* was increased in OA cartilage. Furthermore, *DAAM2*, *PRICKLE2*, and *DVL2* encoding cytoplasmatic proteins, interacting with FZD and VANGL1, displayed decreased expression in OA cartilage. It has earlier been demonstrated that the proteins encoded by these genes interact with FZD and VANGL1, forming a multiprotein complex that transmit signaling to the nucleus. The decreased expression of these genes demonstrated in this study suggests not only the canonical WNT signaling pathway as partly inhibited in OA cartilage but also the planar cell polarity pathway.[48] However, our study further suggests that the Ca^{2+}/WNT signaling pathway was activated in OA cartilage. This was demonstrated by increased expression of the *WNT5A* ligand in OA cartilage, causing intracellular calcium flux leading to activation of Ca^{2+}-dependent effector molecules such as *CAMKII*, *NFAT5*, and *NFATC2*, which was also demonstrated to be upregulated in OA cartilage. Interestingly, CAMKII has been implicated as a key factor in impact-induced chondrocyte death and presumably acts downstream of an increased

concentration of cytoplasmic calcium.[49] Moreover, our study demonstrated that real-time PCR verification of the expression of *NFATC2* and *WNT5A* was in accordance with the results obtained from the microarray analysis. *NFATC2* is known to regulate chondrogenesis in adult mice, whereas an overexpression represses the mature phenotype and extinguishes the cartilage phenotype.[50] Extracellular matrix–degrading proteases have been suggested as downstream targets of *NFATC2* activation.[51] This further demonstrates the impact that WNT-related genes exert on the pathogenesis of OA and implicates *NFATC2* in the involvement of the OA chondrocyte phenotypic change and articular cartilage destruction process.

WNT5A microarray expression was further verified by immunohistological staining, demonstrating WNT5A expression in both the superficial zone and the deeper layers of OA cartilage. ND cartilage, on the other hand, displayed a somewhat more restricted WNT5A expression, located in the superficial zone. WNT5A has earlier been shown to exert an inhibitory effect on type II collagen expression in chondrocytes and is indicated to increase in gene expression related to IL-1β stimulation.[13,29] This is in accordance with our result that demonstrated a significantly higher *WNT5A* expression after cytokine stimulation. Increased *WNT5A* expression associated with IL-1β stimulation is further implicated in the regulation of MMPs responsible for promoting cartilage destruction.[29] This is interesting to note because we have earlier discussed (see above) the involvement of the *FOSL1*, a suggested downstream target gene of Ca^{2+}/WNT signaling pathway, in the regulation of MMP1.

This and the suggested activation of the Ca^{2+}/WNT signaling pathway and its effector molecules in OA cartilage could thus, to some extent, explain the increased apoptosis detected in OA chondrocytes as well as the altered matrix composition seen in OA cartilage. Our data, which is an extension of a previously published article,[17] provide a basis for future studies on the function of numerous newly identified genes in OA pathogenesis and on their suitability as drug targets in OA.

Conclusion

Our results demonstrate that WNT signaling is significantly altered in OA cartilage. The result suggests that the canonical and planar cell polarity pathways are partly inhibited while the Ca^{2+}/WNT signaling pathway is activated.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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