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Osteoarthritis and Cartilage

WWP2 confers risk to osteoarthritis by affecting cartilage matrix deposition via hypoxia associated genes

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Objective: To explore the co-expression network of the osteoarthritis (OA) risk gene *WWP2* in articular cartilage and study cartilage characteristics when mimicking the effect of OA risk allele rs1052429-A on *WWP2* expression in a human 3D *in vitro* model of cartilage.
Method: Co-expression behavior of *WWP2* with genes expressed in lesioned OA articular cartilage ($N = 35$ samples) was explored. By applying lentiviral particle mediated *WWP2* upregulation in 3D *in vitro* pellet cultures of human primary chondrocytes ($N = 8$ donors) the effects of upregulation on cartilage matrix deposition was evaluated. Finally, we transfected primary chondrocytes with miR-140 mimics to evaluate whether miR-140 and WWP2 are involved in similar pathways.
Results: Upon performing Spearman correlations in lesioned OA cartilage, 98 highly correlating genes ($|\rho| > 0.7$) were identified. Among these genes, we identified *GJA1*, *GDF10*, *STC2*, *WDR1*, and *WNK4*. Subsequent upregulation of WWP2 on 3D chondrocyte pellet cultures resulted in a decreased expression of *COL2A1* and *ACAN* and an increase in *EPAS1* expression. Additionally, we observed a decreased expression of *GDF10*, *STC2*, and *GJA1*. Proteomics analysis identified 42 proteins being differentially expressed with WWP2 upregulation, which were enriched for ubiquitin conjugating enzyme activity. Finally, upregulation of miR-140 in 2D chondrocytes resulted in significant upregulation of *WWP2* and *WDR1*.
Conclusions: Mimicking the effect of OA risk allele rs1052429-A on *WWP2* expression initiates detrimental processes in the cartilage shown by a response in hypoxia associated genes *EPAS1, GDF10,* and *GJA1* and a decrease in anabolic markers, *COL2A1* and *ACAN*.

Introduction

Globally, osteoarthritis (OA) is a highly prevalent and disabling joint disease which confers high social and economic burden to society. Risk factors for OA include sex, abnormal joint loading, obesity, metabolic diseases, and genetic factors[1]. To discover genes and underlying disease pathways, large genome wide association meta-analyses have been performed and multiple robust single nucleotide polymorphisms (SNPs) were identified significantly conferring risk to initiation and progression of OA[2–4]. Similar to other complex traits, these risk alleles have subsequently been found to affect expression of positional genes *in cis* in disease relevant tissues, also known as allelic imbalance (AI)[5,6]. Founded by

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this mechanism, we previously used RNA sequencing data of OA articular cartilage to report on genome-wide AI expression of SNPs in cartilage specific genes, as such, providing an AI expression database to *in silico* check functional aspects of identified and/or future OA risk SNPs[7]. One of the top findings was SNP rs1052429 located in the 3′UTR of the *WWP2* gene showing highly significant AI, with risk allele rs1052429-A marking higher expression of *WWP2* relative to rs1052429-G. Among the OA risk SNPs identified in a large genome-wide meta-analysis of Icelandic and UK knee OA patients was rs34195470, located in *WWP2* gene and a proxy of our AI SNP rs1052429 ($r^2 = 0.6$)[2]. Recently, rs34195470 was confirmed being OA risk SNP in the largest genome-wide meta-analysis so far, including individuals from 9 populations[4]. Based on these data, we could make a firm hypothesis that *WWP2,* with risk alleles rs34195470-G and rs1052429-A, confers robust risk to human OA which is marked by increased expression of *WWP2*. We also previously identified transcription of *WWP2* in cartilage being epigenetically regulated[8], as well as being responsive in the OA pathophysiological process[9]. Moreover, *WWP2* was previously shown to be a marker for hypertrophic chondrocytes in OA knee joints[10].

WWP2 is a member of the Nedd4 superfamily, a small group within the E3 ubiquitin ligase enzymes and is involved in post-translational modifications. The WWP2 protein contains four double tryptophan (WW) domains, which allow specific protein–protein interactions and it is expressed in multiple organs throughout the body[11]. More specifically to cartilage, Nakamura *et al.*[12] showed that WWP2 interacts with SOX9 to form a complex that facilitates nuclear translocation of SOX9, as such enabling SOX9 transcriptional activity. Despite the association between the risk allele and higher expression levels of *WWP2* in human cartilage, the effect of *WWP2* knockout (KO) in mice with age-related and surgically induced models of OA showed that lack of *WWP2* expression resulted in increased expression of catabolic cartilage markers *RUNX2* and *ADAMTS5*[13]. In a different context, *WWP2* was found to be a host gene for microRNA-140 (miR-140), a miRNA highly expressed in cartilage and shown to be differentially expressed between preserved and lesioned OA cartilage[9]. As such, it was suggested that expression of miR-140 and the C-terminal transcript of *WWP2* (*WWP2-C*, also called *WWP2* isoform 2) are co-regulated[14,15].

In the current study, we set out to explore how increased levels of *WWP2*, conferring risk to OA, affect cartilage matrix. To get gain insight in the *WWP2* pathway, we started with exploring a *WWP2* co-expression network in our previous whole-transcriptome OA cartilage dataset[9]. Moreover, to study the effect of the genetic risk allele (increased levels of *WWP2*), we functionally assessed the effect of lentiviral-mediated upregulation of *WWP2* in a 3D *in vitro* model using primary human chondrocytes. Apart from conventional anabolic and catabolic cartilage markers, genes identified in the *WWP2* co-expression network were used as a read-out to evaluate the effect of *WWP2* upregulation. Since WWP2 is involved in post-translational modifications, we explored the effect of *WWP2* upregulation on protein level by performing proteomic analysis. Finally, we explored the effects of upregulation of miR-140 in primary chondrocytes by transfection with miR-140 mimics.

Methods

Sample description

All material included in this study is obtained as part of the Research Arthritis and Articular Cartilage (RAAK) study. The RAAK-study is aimed at biobanking of joint materials of patients who underwent a total joint replacement surgery due to OA. Classification of macroscopically preserved and lesioned OA cartilage was done as described previously[16]. For all sample characteristics see Table S1. The RAAK-study is approved by the medical ethics committee of the Leiden University Medical Center (P08.239/P19.013).

RNA-sequencing

Lesioned OA cartilage was collected from hip and knee joints ($N = 35$ samples), snap frozen in liquid nitrogen, pulverized and homogenized in TRIzol (Invitrogen), and RNA was isolated using Qiagen RNeasy Mini Kit (Qiagen). Paired-end 2×100 bp RNA-sequencing (Illumina HiSeq2000 and Illumina HiSeq4000) was performed. Data from both Illumina platforms were integrated and analyzed with the same in-house pipeline. Additional details are described in Supplementary methods.

Creating a co-expression network

We explored co-expression behavior of WWP2 with progression of OA by correlating (Spearman correlation) *WWP2* expression levels in our RNA sequencing dataset with expression levels of all genes expressed in OA articular cartilage ($N = 20{,}048$ genes)[9]. To correct for multiple testing, the Benjamini-Hochberg method was used, as indicated by the false discovery rate (FDR), with a significance cutoff value of 0.05. To include the most informative genes a threshold of $|\rho| > 0.7$ and FDR < 0.05 were selected, corresponding to approximately the top 1% of the total significant correlations.

Lentiviral transduction

The full length WWP2 plasmid was digested and inserted into the XhoI/XbaI sites of the pLV-CMV-IRES-eGFP lentiviral backbone (kindly provided by Prof. Dr. Hoeben). The pLV-CMV-IRES-eGFP lentiviral backbone without the WWP2 insert was used as a control. Additional details are available in the Supplementary methods.

In vitro 3D pellet cultures

3D pellet cultures were formed by adding 2.5×10^5 cells in their expansion medium to a 15 ml Falcon tube and subsequently expose them to centrifugal forces (1,200 rpm, 4 min). Chondrogenesis was initiated in serum-free chondrogenic differentiation medium. Additional details are available in the Supplementary methods.

RT-qPCR

RNA was isolated from the samples using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Roche Applied Science). Subsequently, RT-qPCR was performed adjusting for housekeeping genes GAPDH and SDHA. Additional details are available in the Supplementary methods.

Quantitative proteomics using TMT labeling

Lysis, digestion, TMT labeling and mass spectrometry analysis was essentially performed as described previously[8]. All searches and subsequent data analysis, including Percolator and abundance ratio calculation, were performed using Proteome Discoverer 2.4 (Thermo Scientific). Additional details are available in the Supplementary methods.

Histochemistry

Sections of the 3D chondrocyte pellet culutes were stained for glycosaminoglycan (GAG) deposition using the Alcian Blue staining. The staining was quantified using Fiji. Additional details are available in Supplementary methods.

Transfection with miR-140 mimics

Primary chondrocytes were transfected with hsa-miR-3p mimic (Invitrogen) or a control mimic at 5 nM final concentration using Opti-MEM (Gibco) and Lipofectamine RNAiMax Transfection reagent (Invitrogen) according to manufacturer's protocol. Additional details are available in Supplementary methods.

An overview of the applied strategy can be seen in Fig. 1. The RNA sequencing data of the articular cartilage is deposited at ArrayExpress (E-MTAB-7313). Further data generated and used in this study is not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Results

Co-expression network of WWP2

To identify genes that are regulated by, or co-expressed with, *WWP2* in OA cartilage, we used RNA sequencing data of lesioned OA cartilage [$N = 35$ samples, Table S1(A)] to perform Spearman correlation between expression levels of *WWP2* and genes expressed in cartilage [$N = 20{,}048$ genes, Table S2, Fig. 1(A)]. We identified 98 genes highly correlating ($|\rho| > 0.7$) to *WWP2*. These 98 genes were significantly enriched for, amongst others, GO-terms Extracellular exosome (GO:0070062, 36 genes), characterized by expression of *GJA1* (encoding gap junction alpha 1), *SMO* (encoding smoothened frizzled class receptor), and *WDR1* (encoding WD repeat domain 1), and Myelin sheath (GO:0043209, 10 genes), characterized by expression of *WDR1*, *RALA* (encoding RAS like proto-oncogene A), and *CCT5* (encoding chaperonin containing TCP1 subunit 5) (Table S3). As shown in Fig. S1, genes highly correlating to *WWP2* ($N = 98$) formed a highly interconnected network, i.e., genes that are all highly correlating with each other. In this network we identified direct and indirect relations with *WWP2*, including *GJA1* ($\rho = -0.81$, 70 connections, i.e., highly correlating to 70 genes in the network), *WNK4* (encoding WNK lysine deficient protein kinase 4, $\rho = 0.81$, 37 connections), *ACAN* (encoding aggrecan, $\rho = 0.78$, 16 connections), and *STC2* (encoding stanniocalcin 2, $\rho = 0.77$, 17 connections).

Lentiviral particle-mediated upregulation of WWP2

The effect of upregulation of *WWP2* was studied on cartilaginous matrix deposition in *in vitro* 3D chondrocyte pellet cultures, by creating a lentiviral particle mediated upregulation of *WWP2*. 3D pellet cultures were harvested after three or seven days of culturing and gene ($N = 16$ pellet cultures of $N = 8$ donors) and protein ($N = 16$ pellet cultures of $N = 4$ donors) expression levels were measured [Table S1(B)]. First, we confirmed whether *WWP2* upregulation was successful by measuring both gene and protein expression levels at day zero of the 3D chondrocyte pellet culture, and we observed a significant increase in *WWP2* gene expression levels [$P = 1.0 \times 10^{-5}$, Figs. S2(A)–(B)], which was confirmed on protein level [$P = 3.2 \times 10^{-6}$, Fig. S2(C)].

Effect of WWP2 upregulation on cartilage matrix deposition

Next, we evaluated effect of *WWP2* upregulation on expression levels of conventional cartilage genes during 3D pellet culture of

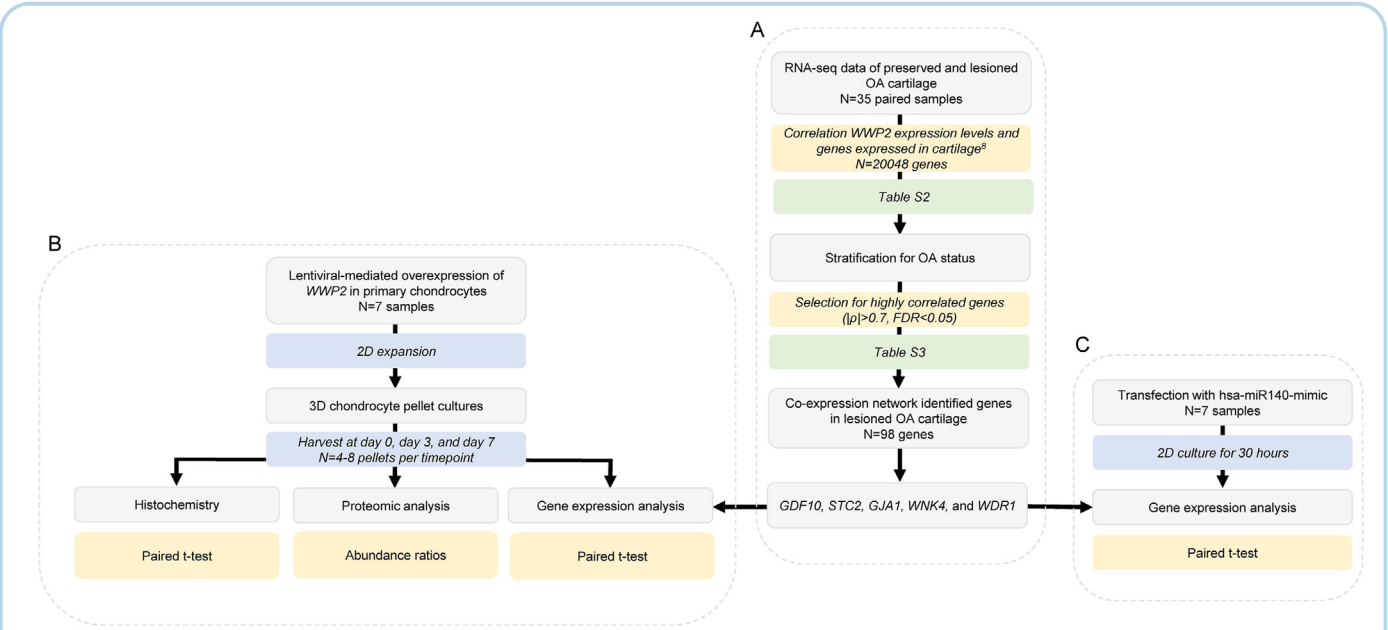

Fig. 1

Osteoarthritis and Cartilage

Schematic overview of applied strategy.

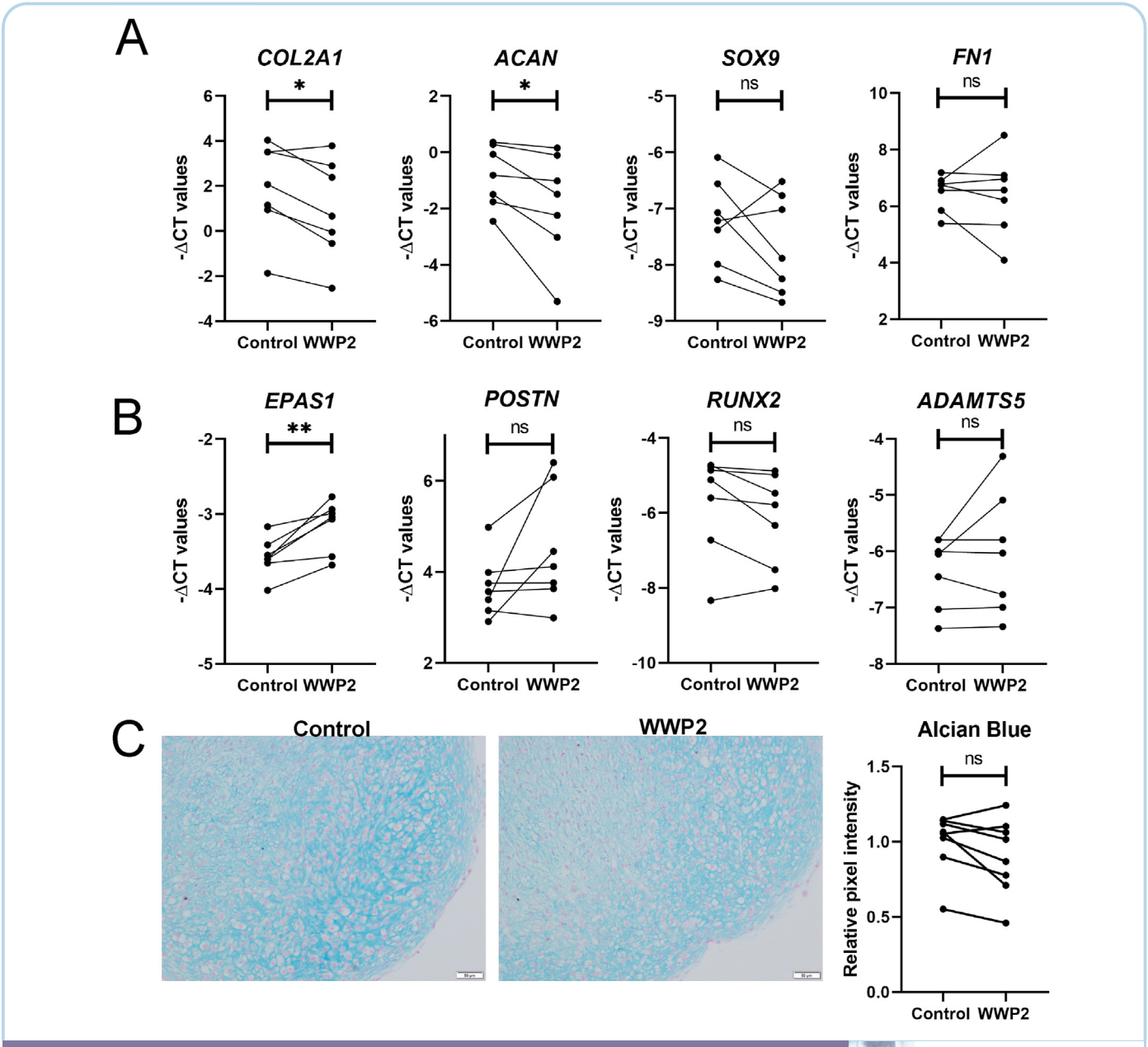

Fig. 2

Osteoarthritis and Cartilage

mRNA expression levels of cartilage matrix markers (A) and cartilage degeneration markers (B) for pellets with WWP2 upregulation and their controls at day 7 ($N = 11–12$ pellet cultures, $N = 7$ donors). (C) Alcian Blue staining visualizing GAGs deposition in WWP2 overexpressed pellets and their controls after 7 days of culturing ($N = 26$ pellet cultures, $N = 8$ donors). The scale bar indicates 50 um. Ns: not significant, $*P < 0.05$, $**P < 0.005$ upon performing a Paired sample *t*-test.

seven days [Fig. 1(B)]. As shown in Fig. 2(A), we found significant reduced gene expression of *ACAN* (FC = 0.80, $P = 0.04$) and *COL2A1* (encoding collagen type 2 alpha chain 1, FC = 0.77, $P = 0.01$), in *WWP2* upregulated pellets compared to their controls at day seven (Table S4). Moreover, we showed significant increased gene expression of degeneration markers *EPAS1* (encoding endothelial PAS domain protein 1, FC = 1.56, $P = 0.004$) [Fig. 2(B)]. Notably, *SOX9*, *ADAMTS5*› and *RUNX2*, which were previously linked to WWP2 function, were not consistently changed upon WWP2 upregulation. Moreover, we stained 3D pellet cultures for presence of glycosaminoglycans (GAGs) using Alcian Blue staining, and observed a trend towards decreased Alcian Blue intensity when comparing *WWP2* upregulated pellets with controls [Fig. 2(C)].

Effect of WWP2 upregulation on genes correlated to WWP2

To investigate functional relationships between *WWP2* and identified correlating and highly interconnected genes, we selected

GDF10 (encoding growth differentiation factor 10, $\rho = 0.72$, 18 connections), *STC2* ($\rho = 0.77$, 17 connections), *GJA1* ($\rho = -0.81$, 70 connections), *WDR1* (encoding WD repeat-containing protein 1, $\rho = -0.70$, 5 connections), and *WNK4* ($\rho = 0.81$, 37 connections) from the network (Fig. 1) to use as read-out of WWP2 upregulation in 3D chondrocyte pellet cultures [Fig. 1(B)]. As shown in Fig. 3, we observed significant decreased gene expression of *GDF10* (FC = 0.62, $P = 0.002$) and *STC2* (FC = 0.73, $P = 0.04$) with upregulation of *WWP2*. Albeit not significant, gene expression of *GJA1* (FC = 0.76, $P = 0.08$) was also consistently lower in *WWP2* upregulated pellets. Together, these data suggest that *GDF10*, *STC2*, and *GJA1* are downstream of *WWP2* either by direct or indirect activity. In contrast, *WNK4* and *WDR1* did not show consistent changes in expression with upregulation of *WWP2*, suggesting *WNK4* and *WDR1* are rather upstream in the pathway of *WWP2*.

Proteomics

To study the extent to which gene expression levels translate to protein levels, we performed proteomics analysis. Prior to differential expression analysis of pellet cultures with and without WWP2 upregulation, we explored protein expression levels of cartilage markers in our control pellet cultures at day three and day seven of 3D pellet culture. Upon comparing day three and day seven with day zero of control pellets, we observed increased protein expression of cartilage markers COL2A1 (FC = 2.68 and FC = 32.94, respectively), ACAN (FC = 5.28 and FC = 13.75, respectively), COMP (cartilage oligomeric matrix protein, FC = 5.31 and FC = 20.25, respectively), and FN1 (fibronectin, FC = 2.12 and FC = 3.51, respectively) (Table S5, Fig. S3). Moreover, mesenchymal markers CD44 (FC = 0.83 and FC = 0.46, respectively) and CD166 (FC = 0.79 and FC = 0.62, respectively) and IGFBP3 (insulin growth factor binding protein 3, FC = 0.10 and FC = 0.10, respectively) were downregulated on both days. Together, this indicates that cartilage-like matrix is produced by chondrocytes already at day three, but is increasing towards day seven. Notably, SOX9 was not detected in the proteomics analysis.

Next, we evaluated the effect of WWP2 upregulation on cartilage matrix deposition on protein level [Fig. 1(B)]. Since we observed increased protein expression of cartilage markers in control pellet cultures already on day three [Fig. S3(A)], we pooled day three and day seven for further analysis to increase power. Upon comparing pellet cultures with and without WWP2 upregulation, we found WWP2 still being significantly upregulated after three and seven days of culturing. Furthermore, we found 42 proteins significantly differentially expressed [Fig. 4(A), Table S6], of which GJA1 (FC = 0.54) was most significantly downregulated in WWP2 upregulated pellet cultures, confirming the downregulation observed on gene expression level (FC = 0.76). Oppositely, the observed changes in *ACAN and COL2A1* gene expression levels were not confirmed on protein level. Proteins encoded by *SOX9, EPAS1, RUNX2,* and *ADAMTS5* were either not identified or did not show unique peptides. Upon performing enrichment analysis on the 42 differentially expressed proteins, we found significant enrichment for ubiquitin conjugating enzyme activity (5 proteins, FDR = 0.002) and ubiquitin-protein transferase activity (6 proteins, FDR = 0.03), both terms characterized by expression of, amongst others, UBE2D4, UBE2L3, and UBE2D1. Furthermore, these 42 proteins showed significant protein–protein interactions [$P = 0.02$, Fig. 4(B)], also representing ubiquitin conjugating enzyme activity.

miR-140-3p and WWP2

Since it has been suggested that *WWP2* and miR-140 are co-expressed[14,15], we transfected primary chondrocytes with miR-140-3p mimics, to assess whether this miRNA regulates *WWP2* expression or similar genes as involved in the *WWP2* co-expression network [$N = 7$, Table S1(B), Fig. 1(C)]. To investigate whether miR-140-3p regulates *WWP2,* we first evaluated the effects of miR-140-3p mimic on expression levels of *WWP2* full length and *WWP2* splice variants isoform 2, isoform 4, and isoform 6 (Fig. S4). MiR-140 is suggested to be co-expressed with splice variant *WWP2* isoform 2, also called *WWP2-C,* as they share the promotor[15]. Isoform 4 is also known as *WWP2-N* and is a transcript that does not contain miR-140 (Fig. S4). As shown in Fig. 5(A), we observed significant increased expression levels of *WWP2* (FC = 1.22, $P = 0.02$) with upregulation of miR-140-3p. Moreover, we observed consistent increased expression of *WWP2* isoform 6 (FC = 1.29, $P = 0.06$), while significant decreased expression of isoform 4 (FC = 0.63, $P = 0.02$) (Table S7). Notably, we did not see effect on expression levels of *WWP2* isoform 2, also called *WWP2-C*. With respect to highly correlated genes, we observed increased expression of *WDR1* (FC = 1.79, $P = 1.00 \times 10^{-3}$), one of the genes that was not consistently changed with *WWP2* upregulation [Fig. 5(B)]. Albeit

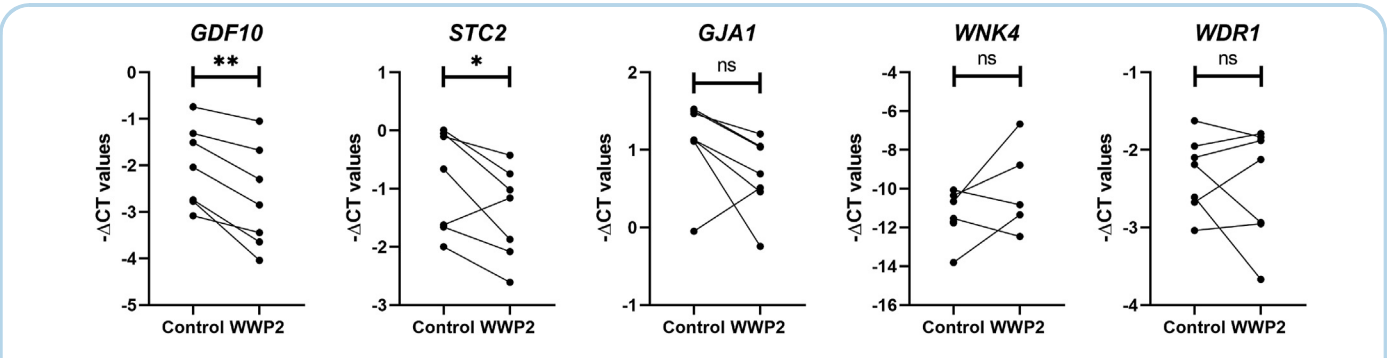

Fig. 3

Osteoarthritis and Cartilage

mRNA expression levels of genes correlating with WWP2 in WWP2 upregulated 3D chondrocyte pellet cultures compared to their controls after 7 days of culturing ($N = 6$–12 pellet cultures, $N = 7$ donors). Ns: not significant, $*P < 0.05$, $**P < 0.05$ upon performing a Paired sample *t*-test.

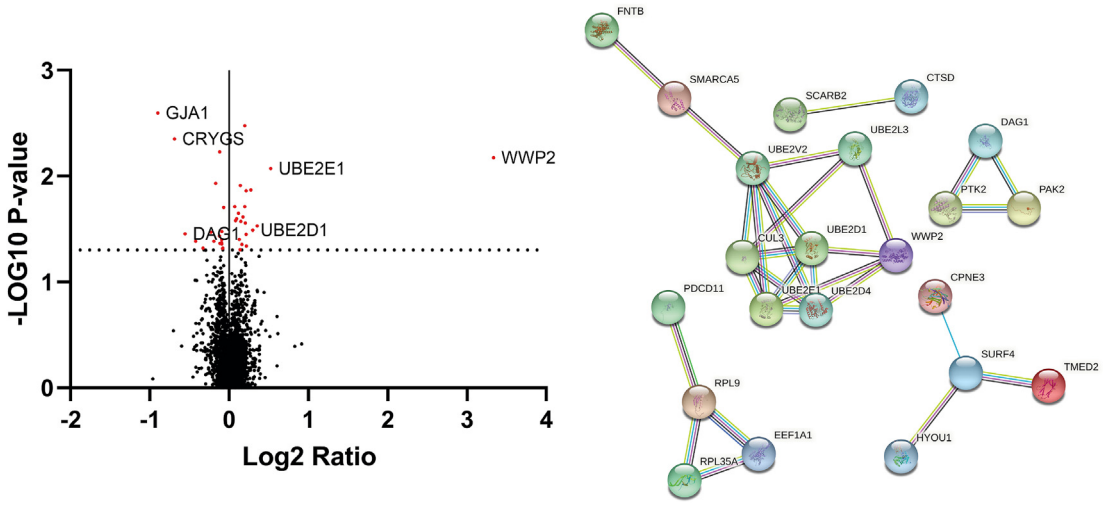

Fig. 4

Osteoarthritis and Cartilage

(A) Volcano plot of proteins differentially expressed between WWP2 transduced 3D pellet cultures and their controls at day three and day seven together ($N = 16$ pellets, $N = 4$ donors). The red dots indicate the significantly differentially expressed proteins. (B) Protein–protein interaction network in STRING.

not significant, we observed an increased expression of *STC2* (FC = 1.55, $P = 0.08$), which is also contradictory to the effects of *WWP2* upregulation. Moreover, we did not observe consistent effects on *GJA1* expression levels.

Discussion

By combining a genome-wide screen for cartilage specific AI[7] and large scale GWAS[2,4], we hypothesized that upregulated expression of *WWP2* confers robust risk to OA. Here, we set out to functionally investigate the role of *WWP2* in cartilage by exploring the *WWP2* co-expression network in a previously assessed RNA sequencing dataset[9]. Moreover, lentiviral-mediated upregulation of *WWP2* was shown to have detrimental effects on cartilage matrix deposition, as shown by downregulation of *COL2A1* and *ACAN* and upregulation of *EPAS1*. Apart from conventional anabolic and catabolic cartilage markers, genes identified in the *WWP2* co-expression network were used as read-out, showing *GDF10*, *STC2*, and *GJA1* being responsive to *WWP2* upregulation. Furthermore, to explore effects of miR-140-3p, that was suggested to be co-regulated with *WWP2*, we transfected primary chondrocytes with miR-140-3p mimics.

Based on AI expression of the OA risk SNP rs1052429, we hypothesized that *WWP2* confers risk to OA onset by upregulated expression, whereas *WWP2* exhibited FDR significantly lower expression in lesioned compared to preserved cartilage (FC = 0.78, FDR = 5.3×10^{-3}), together suggesting that lower expression levels of *WWP2* in lesioned OA cartilage are rather an attempt of chondrocytes to reverse the OA state than a cause to the OA process[17,18]. Concomitantly, co-expression network analyses showed 98 highly and significantly ($|\rho| > 0.7$, FDR < 0.05) correlating genes to *WWP2*, including *GJA1* ($\rho = -0.81$), *WNK4* ($\rho = 0.81$), *ACAN* ($\rho = 0.78$), and *STC2* ($\rho = 0.77$) (Table S2). Previously, it was shown that WWP2 interacts with SOX9 and that it regulates SOX9 transcriptional activity[12]. Although *SOX9* is highly expressed in cartilage, *SOX9* was not among the high and significant correlations ($\rho = 0.5$). On the other hand, *SOX9* was previously shown to regulate the expression of, amongst others *ACAN*[19,20], which was here shown to be highly correlated to *WWP2* ($\rho = 0.78$).

Upon studying the effect of upregulation of *WWP2*, we found *EPAS1* and *GDF10* being genes that had most consistent and significant changed levels of gene expression. *EPAS1*, encoding hypoxia-inducible factor 2 alpha, is known for its role in endochondral ossification and is a known cartilage degradation marker in OA[21,22]. *GDF10*, also known as bone morphogenic protein 3, is involved in osteogenesis as it inhibits osteoblast differentiation via *SMAD2* and *SMAD3*[18,23]. The latter also being previously identified as OA susceptibility gene[2]. Furthermore, lower expression level of *GDF10* was associated with OA severity in both bone and cartilage[24]. Interestingly, *GDF10* was shown to be a hypoxia inducible gene, like *EPAS1*, which is regulated by SOX9 and was identified as marker for differentiated chondrocytes as it inhibits adipogenesis and osteogenesis[25]. Both *EPAS1* and *GDF10* were not in the proteomics analysis, either because they were not measured (EPAS1) or they did not show unique peptides (GDF10). Additionally, we observed a decrease in *STC2* and *GJA1* expression. Downregulation of *GJA1* did not reach statistical significance on gene expression level, while on protein level GJA1 was the most significantly downregulated protein. STC2 is a glycoprotein and upregulation of *STC2* in mice has been shown to delay endochondral ossification[26,27]. Moreover, it was shown that *STC2* was higher expressed in healthy cartilage compared to osteophytic cartilage[28], suggesting its potential role in initiation and progression of OA in presence of higher *WWP2* expression. GJA1, also known as connexin 43, is a major protein of functional gap junctions which allows for cell–cell communication. More specific to cartilage, connexin 43 is essential in mechanotransduction[29]. Alterations in connexin 43 expression and localization affects this cell–cell communication, by which homeostasis to maintain cartilage tissue gets disturbed[30]. Notably, like EPAS1 and GDF10, the function of connexin 43 is regulated by oxygen

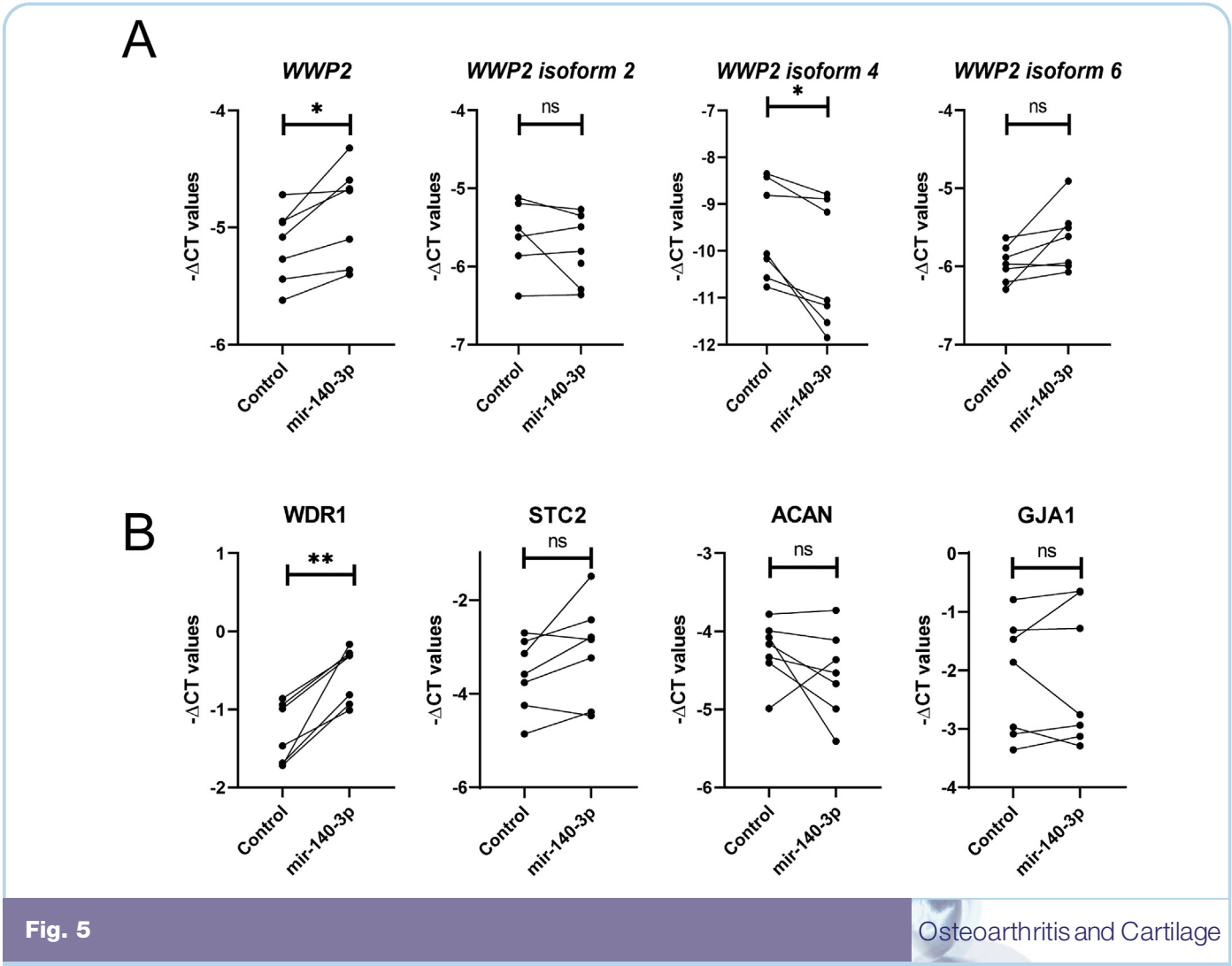

Fig. 5

Osteoarthritis and Cartilage

mRNA expression levels upon transfection with miR-140-3p. (A) expression levels of WWP2 and its isoforms. (B) Expression levels of genes correlated to WWP2 ($N = 8$ wells, $N = 4$ donors). Ns: not significant, $*P < 0.05$, $**P < 0.005$ upon performing a Paired sample *t*-test.

levels[31]. Upregulation of *EPAS1* and downregulation of *GDF10*, *STC2* and *GJA1* suggests that increased level of WWP2 has detrimental effects on cartilage matrix deposition, which acts via hypoxia associated chondrocyte dedifferentiation. This is in line with decreased gene expression levels of *COL2A1* and *ACAN*, two major cartilage markers.

To evaluate the effects of WWP2 upregulation on cartilage matrix deposition on protein level, we performed proteomics analysis. We did confirm upregulation of WWP2 on day zero, which was still present at day three and seven (Fig. 4, Fig. S2). Moreover, we found 42 significantly differentially expressed proteins upon comparing pellet cultures with and without WWP2 upregulation, of which GJA1 was most significantly differentially expressed and showing the highest fold change (FC = 0.49). The observed differences in gene expression levels of *COL2A1 and ACAN* with *WWP2* upregulation were not observed on protein level (Table S6), which might be due to the relatively low sample size ($N = 4$ donors) or due to suboptimal timepoint chosen to evaluate the effect of WWP2 on either gene or protein expression level. Since WWP2 is a E3 ubiquitin ligase and the differentially expressed proteins were significantly enriched for ubiquitin conjugating enzyme activity and ubiquitin-protein transferase activity, upregulation of WWP2 could also affect proteins cellular location, activity, and protein–protein interactions without changing expression levels itself, which is not captured by our read-outs. Moreover, it should be noted that the here observed fold differences were relatively low and additional validation and replication are necessary.

Since it has been suggested that miR-140 is co-expressed with *WWP2-C*[14], *WWP2* isoform 2, we generated upregulation of miR-140 in 2D primary chondrocytes to explore whether *WWP2* and miR-140 are involved in similar pathways. In the miR-140 upregulated cells, we observed significant increased expression levels of *WWP2,* indicating miR-140 indeed targets *WWP2*. Nonetheless, given the predicted *WWP2* target site of miR-140 (3′UTR of *WWP2* isoform 6[32]) and the absence of SNPs in this region in linkage disequilibrium with the OA risk SNP rs1052429, the genetic *WWP2* risk nor the AI is brought about via an aberrant miR-140 binding to *WWP2*. The fact that we did not observe consistent changes in

expression levels of *WWP2* isoform 2› suggests that *WWP2* isoform 2 and miR-140 indeed share the intron 10 (*WWP2* full length) promotor as hypothesized previously by Rice *et al.*[15]. Additional research is required to fully understand the role of miR-140 in the *WWP2* pathway and in OA pathophysiology in general.

Although lower expression of *WWP2* was observed in lesioned compared to preserved cartilage in our previous study[9], genetic evidence suggests that higher expression of *WWP2* predisposes to development of OA, indicating that downregulation in OA pathophysiology is merely a response to the pathophysiological process and a beneficial attempt of chondrocytes to reverse the OA state[7]. The latter shows that genes identified being differentially expressed between preserved and lesioned OA articular cartilage are a response to the OA pathophysiological process and not necessarily causal to the OA pathophysiological process. To identify genes causal to OA, genetic studies have to be performed. To our surprise, Styrkarsdottir *et al.*[2] reported on *WWP2* expression in adipose tissue as function of SNP rs4985453-G, a proxy of their identified OA risk allele rs34195470-G ($R^2 = 0.79$) and our AI SNP rs1052429-A ($R^2 = 0.77$), highlighting the OA risk allele being associated with lower expression levels of *WWP2*. Upon investigating the GTEx eQTL data of *WWP2* with the highlighted SNPs[33], we found only data showing consistently higher expression of *WWP2* as function of OA risk alleles of the respective SNPs across multiple tissues (Fig. S5), underscoring the aberrant effects observed here with *WWP2* upregulation. Although Mokuda *et al.*[13] showed that lack of *WWP2* expression in mice resulted in increased expression of *RUNX2* and *ADAMTS5*, we here did not observe consistent changes in expression of *RUNX2* or *ADAMTS5* upon upregulation of *WWP2* in our human chondrocyte pellet cultures and culturing for seven days. This difference could be due to translational limitations from mice to humans. Alternatively, we here create neocartilage, and the effect on *RUNX2* and *ADAMTS5* may be a temporal or time-dependent effect, which we do not observe at day seven of culturing.

In conclusion, our data provide support to our hypothesis that high levels of *WWP2* have detrimental effects on cartilage homeostasis. We identified *EPAS1, GJA1, GDF10,* and *STC2*, all genes involved in chondrocyte dedifferentiation, to be involved in the *WWP2* pathway. Moreover, we showed that miR-140 is likely involved in similar pathways as *WWP2* and miR-140 might play a role in regulating *WWP2* expression. Together these data contribute to a better understanding of how *WWP2* confers risk to OA and is a step towards translation from bench to bedside.

Data availability

The RNA sequencing data of the articular cartilage is deposited at ArrayExpress (E-MTAB-7313). Further data generated and used in this study is not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by M. Tuerlings, G.M.C. Janssen, P.A. van Veelen, Y.F.M. Ramos, and I. Meulenbelt. The first draft of the manuscript was written by M. Tuerlings, Y.F.M. Ramos, and I. Meulenbelt and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Supplementary data

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