CARTILAGE
2023, Vol. 14(1) 67–75

DOI: 10.1177/19476035221144747
journals.sagepub.com/home/CAR
SAGE

CCN4/WISP1 Promotes Migration of Human Primary Osteoarthritic Chondrocytes

Ritchie G.M. Timmermans[1,2], Arjen B. Blom[1], Niek G.C. Bloks[2], Rob. G.H.H. Nelissen[3], Enrike H.M.J. van der Linden[3], Peter M. van der Kraan[1], Ingrid Meulenbelt[2], Yolande F.M. Ramos[2*], and Martijn H.J. van den Bosch[1*]

Abstract

Objectives. Previously, we have shown the involvement of cellular communication network factor 4/Wnt-activated protein Wnt-1-induced signaling protein 1 (CCN4/WISP1) in osteoarthritic (OA) cartilage and its detrimental effects on cartilage. Here, we investigated characteristics of CCN4 in chondrocyte biology by exploring correlations of CCN4 with genes expressed in human OA cartilage with functional follow-up. *Design.* Spearman correlation analysis was performed for genes correlating with *CCN4* using our previously established RNA sequencing dataset of human preserved OA cartilage of the RAAK study, followed by a pathway enrichment analysis for genes with $\rho \geq |0.6|$ Chondrocyte migration in the absence or presence of CCN4 was determined in a scratch assay, measuring scratch size using a live cell imager for up to 36 h. Changes in expression levels of 12 genes, correlating with *CCN4* and involved in migratory processes, were determined with reverse transcription-quantitative polymerase chain reaction (RT-qPCR). *Results.* Correlation of *CCN4* with $\rho \geq |0.6|$ was found for 58 genes in preserved human OA cartilage. Pathway analysis revealed "neural crest cell migration" as most significant enriched pathway, containing among others *CORO1C*, *SEMA3C*, and *SMO*. Addition of CCN4 to primary chondrocytes significantly enhance chondrocyte migration as demonstrated by reduced scratch size over the course of 36 h, but at the timepoints measured no effect was observed on mRNA expression of the 12 genes. *Conclusion.* CCN4 increases cell migration of human primary OA chondrocytes. Since WISP1 expression is known to be increased in OA cartilage, this may serve to direct chondrocytes toward cartilage defects and orchestrate repair.

Keywords

osteoarthritis, CCN4/WISP1, cartilage, chondrocyte, migration

Introduction

A prominent hallmark of osteoarthritis (OA) is progressive degradation of articular cartilage.[1] The articular cartilage is a highly specialized connective tissue required for smooth movement of the joints, with chondrocytes being the sole cell type present.[2] Cartilage has limited regenerative capacity after incurred damage. In the usual process of wound healing, neighboring cells proliferate and migrate toward the wound to initiate repair.[3] In cartilage, however, damaged sites are not healed effectively, which is likely due to the lack of neighboring chondrocytes. Due to the vast density and pressure of the extracellular matrix (ECM), and the presence of a unique pericellular matrix (PCM) surrounding

[1]Experimental Rheumatology, Radboud university medical center, Nijmegen, The Netherlands
[2]Section Molecular Epidemiology, Department of Biomedical Data Sciences, Leiden University Medical Centre, Leiden, The Netherlands
[3]Department of Orthopaedics, Leiden University Medical Centre, Leiden, The Netherlands
*Shared last author.

Supplementary material for this article is available on the Cartilage website at http://cart.sagepub.com/supplemental.

Corresponding Author:
Martijn H.J. van den Bosch, Experimental Rheumatology, Radboud university medical center, Geert Grooteplein Zuid 28, Nijmegen 6525 GA, The Netherlands.
Email: martijn.vandenbosch@radboudumc.nl

the chondrocyte, chondrocytes are very limited in their ability to migrate.[4] That chondrocytes are able to migrate after damage occurs has been demonstrated in *in vitro* and *ex vivo* studies.[5-7] In OA cartilage, chondrocyte clusters are observed near lesioned sites, but are thought to be mainly a result of proliferation, rather than migration.[4] Therefore, promoting chondrocyte migration in damaged articular cartilage toward cartilage lesions could be beneficial to cartilage regeneration and remodeling.

Wnt signaling is an important pathway that can regulate cell migration.[8,9] This pathway is involved in almost every aspect of embryonic development and controls tissue homeostasis later in life.[10] Dysregulation of Wnt signaling has been associated with severe skeletal disorders. Over the years, it has become clear that dysregulated Wnt signaling also plays a prominent role in OA development and progression.[11-14] In several experimental mouse models of OA, we observed strong upregulation of several Wnt ligands in the synovial tissue.[15] Likewise, we further demonstrated that expression of the canonical cellular communication network factor 4/Wnt-activated protein Wnt-1-induced signaling protein 1 (CCN4/WISP1) was upregulated in both the synovium and the articular cartilage of mice that developed OA.[15] In macroscopically lesioned areas compared to preserved areas of articular cartilage from human OA joints, CCN4 was upregulated, positively correlating with the Mankin score.[14] Moreover, we demonstrated that in humans, *CCN4* expression was epigenetically regulated via DNA methylation, and that high CCN4 levels were detrimental for cartilage integrity.[14] Furthermore, CCN4 has been recognized as an oncogene, controlling cell survival, migration, and proliferation in tumorigenic cells.[16]

In the present study, we aimed to further characterize functions of CCN4, relevant to OA. Thereto we explored RNA sequencing data on preserved and lesioned OA articular cartilage[17] to identify genes correlating with *CCN4* expression in preserved OA cartilage, on which we performed pathway analysis. This revealed enrichment for genes involved in cell migration, which was then tested in a scratch assay with human primary articular chondrocytes.

Methods

Correlation Analysis with CCN4 *in Preserved OA Articular Cartilage*

Genome-wide correlations ($\rho \geq 0.6$) between mRNA levels of *CCN4* and other genes were calculated in preserved human OA cartilage in our previously established RNA sequencing dataset on human OA cartilage derived from knee and hip joints of patients that are enrolled in the research osteoarthritis and articular cartilage (RAAK) study.[17] The online tool DAVID was used to perform pathway enrichment on the genes correlating with *CCN4* with genes expressed in human OA cartilage (RAAK) as a background, selecting the gene ontology (GO) terms Biological Processes (GOTERM_BP_DIRECT), Cellular Component (GOTERM_CC_DIRECT), and Molecular Function (GOTERM_MF_DIRECT).[17,18]

Cell migration Assay

To investigate the effects of CCN4 on chondrocyte migration, primary chondrocytes were isolated from preserved regions of articular cartilage from human OA knee joints, and digested O/N at 37 °C in 1.5 mg/ml collagenase B (Roche, Basel, Switzerland) dissolved in DMEM GlutaMax™ (Gibco, Waltham, MA), supplemented with 100 mg/l sodium pyruvate (Invitrogen, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin (pen/strep, Lonza, Basel, Switzerland). Cells were expanded for two passages in medium supplemented with 10% FCS (Sigma, St. Louis, MO), sodium pyruvate, and pen/strep, as described above. After two passages, cells were seeded in 24-well plates ($N = 6$ donors) with a density of 8.75×10^4 cells/well in medium with 0.1% FCS, sodium pyruvate and pen/strep. The next day, a thin scratch was made using a sterile 200 µl pipette tip. Three groups were compared: (1) Control medium, (2) medium with 0.1 µg/ml rhCCN4 (Peprotech, London, UK), and (3) medium with 0.5 µg/ml rhCCN4. The migration of cells was recorded by time-lapse imaging for 36 h using a Zeiss Axiovert 200M microscope (Boston Microscopes, Wilmington, MA) with a stage incubator (Okolab, Ottaviano, Italy). The distance between the edges of the scratch was measured at four timepoints using Fiji-ImageJ v1.52. Chondrocyte numbers were measured at endpoint by fixing the cells in 4% paraformaldehyde and staining them with crystal violet. Crystal violet stained cells were dissolved in 10 v/v % acetic acid and absorbance was read at 590 nm using a CLARIOstar Plus plate reader (BMG Labtech, Ortenberg, Germany).

Reverse transcription quantitative real-time PCR (RT-qPCR)

Chondrocytes ($N = 9$ donors) were collected in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) 48 h after addition of CCN4, and RNA was extracted according to manufacturer's protocol. DNA contamination was removed using DNase I (Thermo Fisher Scientific, Waltham, MA) and RNA was reverse transcribed into cDNA using 1.9 µL ultra-pure water, 2.4 µL 10 × DNAse buffer, 2.0 µL 0.1 M DTT, 0.8 µL 25 mM dNTP, 0.4 µg oligo dT primer, and 1 µL 200 U/µL M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA), and 0.5 µL 40 U/µL RNAsin (Promega, Madison, WI). cDNA was amplified using *Power* SYBR™ Green PCR Master Mix (Applied Biosystems™, Foster City, CA) and mRNA expression was measured using

Table 1. Primer Sequences.

Gene	Forward sequence	Reverse sequence
GAPDH	5'-TGCCATGTAGACCCCTTGAAG-3'	5'-ATGGTACATGACAAGGTGCGG-3'
SDHA	5'-TGGAGCTGCAGAACCTGATG-3'	5'-TGTAGTCTTCCCTGGCATGC-3'
CCND1	5'-ATCAAGTGTGACCCGGACTG-3'	5'-CTTGGGGTCCATGTTCTGCT-3'
CLMN	5'-TCATCGGGCAGATTAGCGAC-3'	5'-AGAGGTGGGTTGCACTTTTCT-3'
COL6A3	5'-ATGAACAAGCAGGACGTCGT-3'	5'-GCAGAGCTGACCAGGATGTT-3'
CORO1C	5'-GGACTGCACGGTCATGGTAT-3'	5'-CCACGATGCCGACTCTCTTT-3'
EML1	5'-AGAGGAAACCGGAATCGCAC-3'	5'-ACATAGCCTTCTTCTGCACTG-3'
MXRA5	5'-ATGTTGCAGAAGGTCGCAGA-3'	5'-TTTTCCCACGGACTTTGGCT-3'
PLXNA2	5'-GGAAAAGCAACTGCCTCCCT-3'	5'-ATGCTGCCCTTGTGGATGTC-3'
PTPRK	5'-CGATGAGCAGTGTGGAGAAGG-3'	5'-GTTCTTCTGTTGCTGCTGCTTTTG-3'
RAI14	5'-GAGGGCAAGACCGCTTTCCA-3'	5'-GTCCGGTAGTATCTTGGGCTG-3'
SEMA3C	5'-CAAAGATCCCACACACGGCT-3'	5'-ACTTGGTCCTCTGATCTCCTCC-3'
SMO	5'-CTGCTCATCTGGAGGCGTAC-3'	5'-GCTTAGAGAAGGCCTTGGCA-3'
TNFAIP6	5'-TCCATATGGCTTGAACGAGCA-3'	5'-GCCTTAGCTTCTGCGTAGGT-3'

the Quantstudio™ 1 Real-Time PCR System (Applied Biosystems™, Foster City, CA). -ΔCt expression levels were calculated using two housekeeping genes *GAPDH* and *SDHA*, with the following formula: $\Delta Ct = Ct_{\text{gene of interest}} — Ct_{\text{average housekeeping genes}}$. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method with $\Delta\Delta Ct = \Delta Ct_{\text{treated}} — \Delta Ct_{\text{Control.}}$ Primer sequences are listed in **Table 1**.

Statistical Analysis

Transcriptome-wide correlations with WISP1 in preserved OA articular cartilage were calculated using a Spearman correlation with a Benjamini-Hochberg test to correct for multiple testing, and were carried out using R. Statistical differences of the distance between the edges of the scratch over time were tested using a two-way analysis of variance (ANOVA) with a Dunnett's test to correct for multiple comparisons. Differences in scratch closure rate, chondrocyte numbers and mRNA expression levels were tested with a one-way ANOVA and are described using the mean ± difference between 95% confidence interval (CI) and the mean. Statistics and creation of the graphs were carried out in GraphPad Prism 9.0.0. *P*-values ≤ 0.05 were considered statistically significant.

Results

Correlation Analysis for CCN4/WISP1 in Preserved Human OA Cartilage

To characterize new functions of CCN4 in human cartilage, we explored our previously established RNA sequencing dataset of macroscopically preserved OA articular cartilage[17] to identify genes correlating with *CCN4*. We identified 58 genes with a $\rho \geq 0.6$ that significantly correlated with *CCN4* (**Table 2**). *Echinoderm microtubule-associated protein-like 1* (*EML1*, $\rho = 0.7521$, $P = 1.90 \times 10^{-8}$) showed the strongest and most significant correlation with *CCN4*. Other notable correlations were with *Collagen type VI alpha 3* (*COL6A3*, $\rho = 0.6574$, $P = 1.78 \times 10^{-5}$), a collagen exclusively found in the pericellular matrix of articular cartilage[19] and *Smoothened* (*SMO*, $\rho = -0.664$, $P = 1.24 \times 10^{-5}$), a part of Hedgehog signaling which is implicated in the pathogenesis of OA.[20]

Analysis for these 58 genes demonstrated enrichment for particular pathways. Most significant enrichment was the GO term "neural crest cell migration" (GO 0001755; $P = 7.03 \times 10^{-3}$), containing *Coronin-1C* (*CORO1C), Semaphorin-3C* (*SEMA3C*), and *SMO* (**Table 3**). In addition to these genes, we observed several other genes correlating to *CCN4*, such as the aforementioned *COL6A3* and *Plexin-A2* (*PLXNA2*), that are also involved in migratory processes.

CCN4/WISP1 Addition Increases Cell Migration in Primary Chondrocytes

Since we found cell migration as most significant enriched pathway, we determined whether CCN4 indeed affected migration of chondrocytes. To this end, we performed a scratch assay to measure chondrocyte migration at four different timepoints with and without addition of recombinant human CCN4. After 36 h, we observed that in the absence of CCN4, the distance between the edges of the scratch in untreated chondrocytes was reduced by 73.7% ± 4.4%, whereas in the 0.1 µg/ml CCN4 treated chondrocytes, it was reduced by 87 ± 2.4% ($P = 4.00 \times 10^{-3}$, **Figure 1A** and **1D**, Supplementary Table S1). Additionally, we observed that the scratch closure rate was highest in the first 12 h, and increased directly after exposure to CCN4 (**Figure 1B**, Control = 15.9 ± 2.5 µm/h vs 0.1 µg/ml rhCCN4 = 22.1 ± 4.2 µm/h, $P = 2.32 \times 10^{-2}$). To rule out that the increased

Table 2. Genes Correlating with *CCN4* ($\rho \geq |0.6|$) in Preserved OA Articular Cartilage.

Ensembl ID	Gene Name	ρ[a]	*P*-value[b]
ENSG00000066629	*EML1*	0.7667	7.87×10^{-8}
ENSG00000131389	*SLC6A6*	0.7521	1.90×10^{-7}
ENSG00000184905	*TCEAL2*	–0.6796	7.15×10^{-6}
ENSG00000152894	*PTPRK*	0.6793	7.24×10^{-6}
ENSG00000110880	*CORO1C*	0.6782	7.59×10^{-6}
ENSG00000134531	*EMP1*	0.6756	8.45×10^{-6}
ENSG00000149948	*HMGA2*	0.6752	8.62×10^{-6}
ENSG00000169439	*SDC2*	–0.6751	8.66×10^{-6}
ENSG00000213694	*S1PR3*	0.6744	8.90×10^{-6}
ENSG00000136052	*SLC41A2*	0.6737	9.18×10^{-6}
ENSG00000075223	*SEMA3C*	0.6723	9.74×10^{-6}
ENSG00000142149	*HUNK*	0.6716	1.00×10^{-5}
ENSG00000079156	*OSBPL6*	0.6699	1.07×10^{-5}
ENSG00000120278	*PLEKHG1*	0.6695	1.09×10^{-5}
ENSG00000165959	*CLMN*	0.6666	1.23×10^{-5}
ENSG00000128602	*SMO*	–0.6664	1.24×10^{-5}
ENSG00000267100	*ILF3-DT*	–0.6653	1.30×10^{-5}
ENSG00000070882	*OSBPL3*	0.6616	1.51×10^{-5}
ENSG00000038945	*MSR1*	0.6598	1.62×10^{-5}
ENSG00000133816	*MICAL2*	0.6580	1.74×10^{-5}
ENSG00000163359	*COL6A3*	0.6574	1.78×10^{-5}
ENSG00000185989	*RASA3*	0.6560	1.88×10^{-5}
ENSG00000145685	*LHFPL2*	0.6555	1.92×10^{-5}
ENSG00000175352	*NRIP3*	0.6526	2.15×10^{-5}
ENSG00000164237	*CMBL*	–0.6496	2.42×10^{-5}
ENSG00000253293	*HOXA10*	–0.6485	2.52×10^{-5}
ENSG00000130508	*PXDN*	0.6471	2.66×10^{-5}
ENSG00000106351	*AGFG2*	–0.6445	2.93×10^{-5}
ENSG00000211445	*GPX3*	–0.6412	3.32×10^{-5}
ENSG00000118257	*NRP2*	0.6409	3.36×10^{-5}
ENSG00000134369	*NAV1*	0.6384	3.69×10^{-5}
ENSG00000110092	*CCND1*	0.6373	3.84×10^{-5}
ENSG00000076356	*PLXNA2*	0.6361	4.01×10^{-5}
ENSG00000272168	*CASC15*	0.6352	4.15×10^{-5}
ENSG00000107249	*GLIS3*	0.6317	4.71×10^{-5}
ENSG00000244682	*FCGR2C*	0.6295	5.10×10^{-5}
ENSG00000171017	*LRRC8E*	0.6288	5.23×10^{-5}
ENSG00000260314	*MRC1*	0.6277	5.43×10^{-5}
ENSG00000237452	*BHMG1*	–0.6256	5.86×10^{-5}
ENSG00000167191	*GPRC5B*	–0.6216	6.74×10^{-5}
ENSG00000163517	*HDAC11*	–0.6216	6.74×10^{-5}
ENSG00000146411	*SLC2A12*	0.6212	6.83×10^{-5}
ENSG00000171488	*LRRC8C*	0.6207	6.94×10^{-5}
ENSG00000236609	*ZNF853*	–0.6207	6.94×10^{-5}
ENSG00000156273	*BACH1*	0.6171	7.87×10^{-5}
ENSG00000115468	*EFHD1*	–0.6165	8.02×10^{-5}
ENSG00000170275	*CRTAP*	–0.6162	8.10×10^{-5}
ENSG00000101825	*MXRA5*	0.6126	9.17×10^{-5}
ENSG00000123610	*TNFAIP6*	0.6098	1.01×10^{-4}
ENSG00000124731	*TREM1*	0.6097	1.01×10^{-4}
ENSG00000168913	*ENHO*	–0.6087	1.05×10^{-4}
ENSG00000039560	*RAI14*	0.6034	1.25×10^{-4}

(continued)

Table 2. (continued)

Ensembl ID	Gene Name	ρ[a]	*P*-value[b]
ENSG00000136010	*ALDH1L2*	0.6031	1.26×10^{-4}
ENSG00000109436	*TBC1D9*	0.6031	1.26×10^{-4}
ENSG00000176723	*ZNF843*	–0.6031	1.26×10^{-4}
ENSG00000123329	*ARHGAP9*	0.6027	1.27×10^{-4}
ENSG00000170190	*SLC16A5*	0.6020	1.31×10^{-4}
ENSG00000167642	*SPINT2*	–0.6008	1.35×10^{-4}

CCN4 = cellular communication network factor 4; OA = osteoarthritic.
[a]Transcriptome wide correlations were calculated using a Spearman correlation.
[b]P-values were corrected for multiple testing with the Benjamini-Hochberg method.

Table 3. Gene Enrichment Analysis for 58 Genes Correlating with $CCN_4/WISP_1$ in Preserved OA Articular Cartilage.

Category[a]	Term	Count	%[b]	Genes	*P*-value
GOTERM_BP_DIRECT	GO:0001755~neural crest cell migration	3	5.5	*SEMA3C, SMO, CORO1C*	7.03×10^{-3}
GOTERM_BP_DIRECT	GO:0045444~fat cell differentiation	3	5.5	*CCND1, LRRC8C, HMGA2*	1.78×10^{-2}
GOTERM_BP_DIRECT	GO:0031175~neuron projection development	3	5.5	*CLMN, EFHD1, PTPRK*	3.19×10^{-2}
GOTERM_BP_DIRECT	GO:0007165~signal transduction	8	14.5	*ARHGAP9, RASA3, TNFAIP6, MRC1, HUNK, HMGA2, PTPRK, CORO1C*	4.13×10^{-2}
GOTERM_CC_DIRECT	GO:0005887~integral component of plasma membrane	10	18.2	*MSR1, SLC6A6, MRC1, SLC2A12, PLXNA2, LRRC8C, S1PR3, PTPRK, LRRC8E, SLC16A5*	1.21×10^{-2}
GOTERM_CC_DIRECT	GO:0005886~plasma membrane	19	34.5	*MSR1, NRP2, OSBPL6, SLC41A2, OSBPL3, SDC2, SLC2A12, HDAC11, EMP1, SPINT2, TREM1, SLC6A6, GPRC5B, SMO, MRC1, LRRC8C, PLXNA2, S1PR3, LRRC8E*	1.76×10^{-2}
GOTERM_MF_DIRECT	GO:0005096~GTPase activator activity	4	7.3	*ARHGAP9, RASA3, TBC1D9, AGFG2*	4.48×10^{-2}
GOTERM_MF_DIRECT	GO:0008134~transcription factor binding	4	7.3	*CCND1, GPX3, HDAC11, HMGA2*	4.68×10^{-2}

CCN4/WISP1 = cellular communication network factor 4/Wnt-activated protein Wnt-1-induced signaling protein 1; OA = osteoarthritic; GO = gene ontology; GOTERM_BP_DIRECT = gene ontology terms Biological Processes; GOTERM_CC_DIRECT = gene ontology terms Cellular Component; GOTERM_MF_DIRECT = gene ontology terms Molecular Function.
[a]Gene enrichment was carried out using the online functional annotation tool DAVID, with gene ontology (GO) terms Biological Processes (GOTERM_BP_DIRECT), Cellular Component (GOTERM_CC_DIRECT), and Molecular Function (GOTERM_MF_DIRECT).
[b]Percentage involved genes/total genes.

scratch closure was the result of enhanced chondrocyte proliferation as a result of the CCN4 exposure, we conducted a crystal violet staining. As shown in **Figure 1C**, we did not observe any effect of CCN4 on crystal violet staining (Control = 0.228 ± 0.037 A.U. vs 0.1 µg/ml CCN4 = 0.216 ± 0.085 A.U., $P = 5.93 \times 10^{-1}$). Of note, while the 0.5 µg/ml CCN4 treated chondrocytes did show a similar trend on migration compared to the 0.1 µg/ml group, the difference with controls was not significant due to larger variation.

Finally, we investigated whether the genes involved in migration, matrix composition, and cytoskeletal reorganization that we identified to correlate with CCN_4 in preserved OA cartilage, could be downstream of CCN4 signaling in chondrocytes. We therefore determined mRNA levels of the three genes in the most significantly enriched pathway (e.g., *CORO1C*, *SEMA3C*, and *SMO)* and of nine other genes (e.g., *CCND1*, *CLMN*, *COL6A3*, *EML1*, *MXRA5*, *PLXNA2*, *PTPRK*, *RAI14*, and *TNFAIP6*) involved in aforementioned processes. However, no significant changes in expression of these 12 genes were detected after exposure to CCN4 for 48 h (**Figure 2**).

Discussion

In this study, we further characterized functions of CCN4/WISP1 on chondrocyte biology, related to OA pathophysiology. Correlation analysis on *CCN4* in OA cartilage with subsequent pathway enrichment analysis revealed "neural crest cell migration" as most significant enriched pathway. In line with this, we showed that addition of CCN4 to

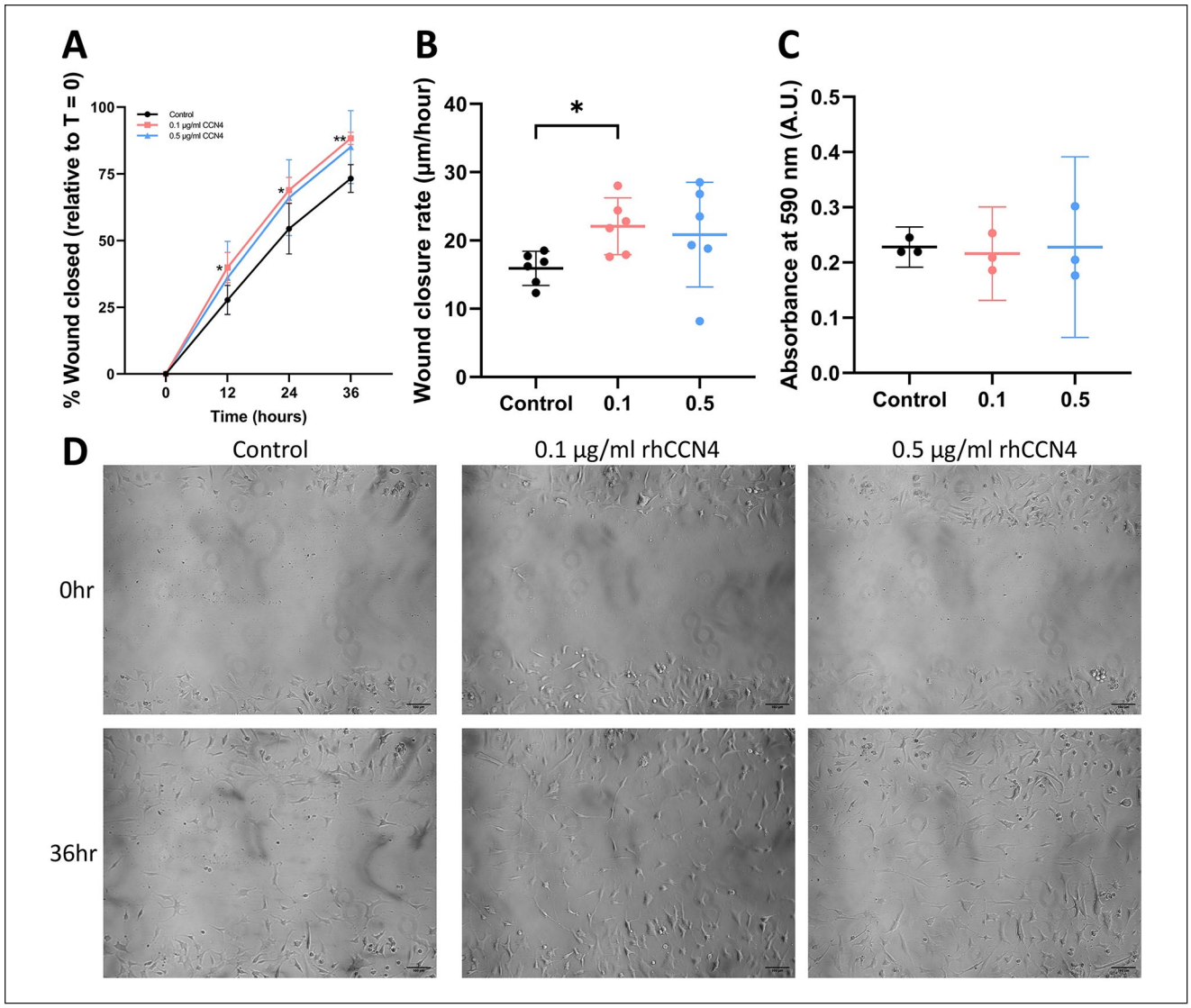

Figure 1. CCN4 addition increases chondrocyte migration. Scratch assay in human primary chondrocytes without and with addition of 0.1 µg/ml or 0.5 µg/ml recombinant human CCN4. (**A**) The distance between the edges of the scratch at four timepoints in percentage relative to T = 0 (N = 6 donors). (**B**) Scratch closure rate in the scratch assay in the first 12 h. (**C**) Cell proliferation determined by measuring absorbance at 595 nm of crystal violet stained chondrocytes dissolved in acetic acid (N = 3 donors). (**D**) Representative images of the scratch assay at T = 0 and T = 36. Percentages of the wound closed are presented in a continuous line graph, depicting the mean and 95% confidence intervals. Wound closure rates and crystal violet absorbances are presented in a scatter dot plot, depicting the mean and 95% confidence intervals, and each dot representing a single donor. *$P \leq 0.05$, ** $P \leq 0.01$ for 0.1 µg/ml CCN4. Scale bar = 100 µm A.U. = Absorbance units. CCN4 = cellular communication network factor 4.

human primary chondrocytes promoted migration in a scratch assay.

Given the limited healing capacity of cartilage, it was notable to observe that *CCN4* correlated with genes that are involved in migration. Promoting cell migration could assist in recruitment of chondrocytes to defect sites to initiate cartilage repair.[5] The effects of CCN4 on cell migration have been demonstrated in other cell types, especially tumorigenic cells, where it promotes migration, but also proliferation and cell survival.[16] In chondrocytes, CCN4 may induce proliferation.[21] Here, we repressed proliferation in our wound healing assay by seeding chondrocytes in low serum levels, and demonstrated that CCN4 promoted chondrocyte migration. By promoting both proliferation and migration in chondrocytes, CCN4 could aid in stimulating cartilage repair. Previously, we observed that CCN4 is highly expressed in chondrocyte clusters near cartilage lesions.[14] Since proteoglycan content and expression of matrix components were decreased in the presence of high CCN4 levels, we suggested that increased CCN4 was detrimental to

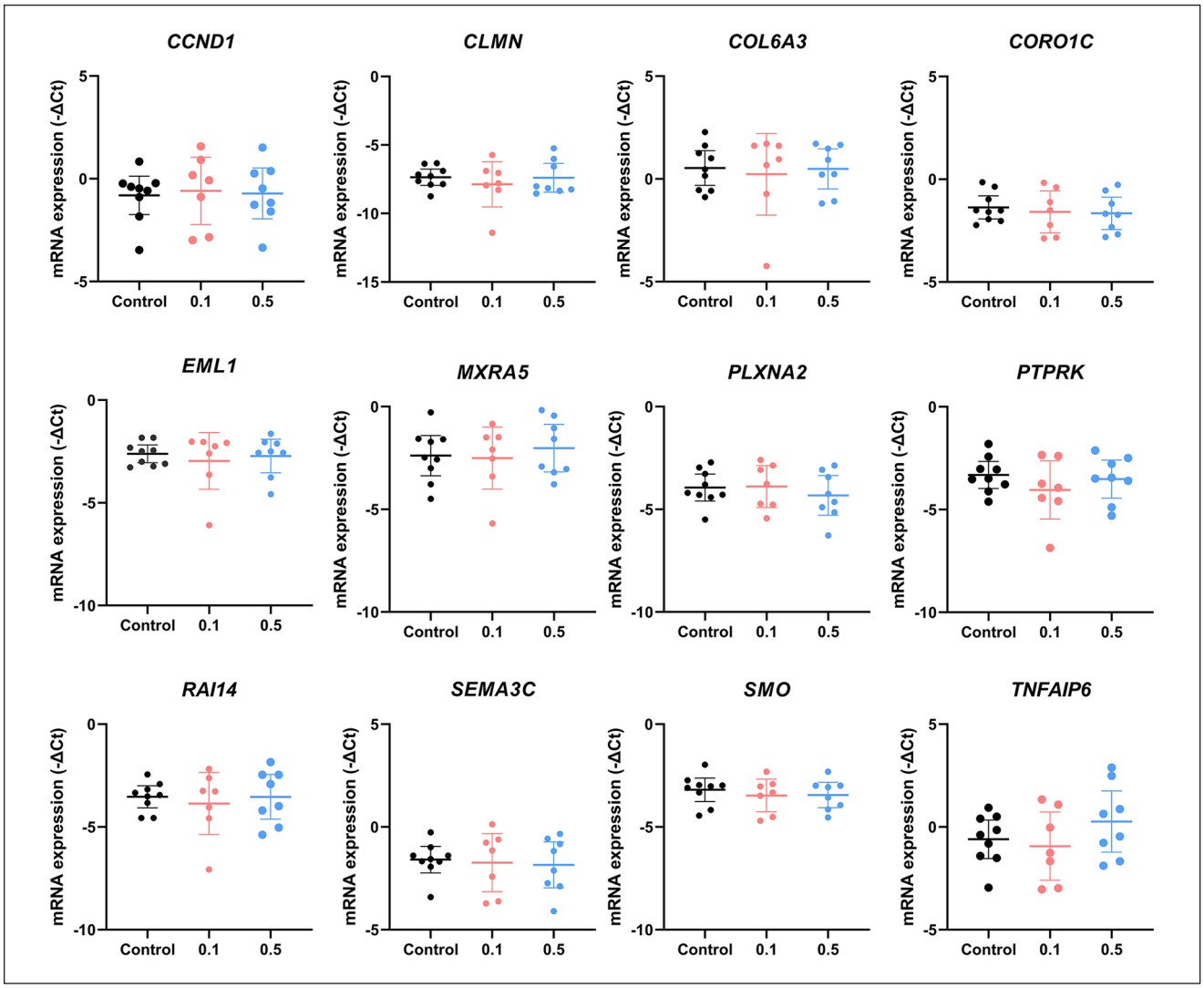

Figure 2. Gene expression in primary chondrocytes after exposure to CCN4. Reverse transcription quantitative real-time PCR (RT-qPCR) analysis of 12 genes correlating with *CCN4*, involved in migration, matrix composition and cytoskeletal reorganization (*CCND1*, *CLMN*, *COL6A3*, *CORO1C*, *EML1*, *MXRA5*, *PLXNA2*, *PTPRK*, *RAI14*, *SEMA3C*, *SMO*, and *TNFAIP6* after 48 h treatment with 0.1 µg/ml and 0.5 µg/ml CCN4 and their controls ($N = 9$ donors). Percentages of the wound closed are presented in a continuous line graph, depicting the mean and 95% confidence intervals. -ΔCt expression levels are presented in a scatter dot plot, depicting the mean and 95% confidence intervals, and each dot representing a single donor. CCN4 = cellular communication network factor 4.

cartilage ECM.[14] In light of the current findings, it is tempting to speculate, however, that this reduction in matrix deposition allows for the orchestration of chondrocytes toward damaged areas for its subsequent repair.

Pathway enrichment analysis in preserved OA cartilage revealed neural crest cell migration as most significant pathway containing these three genes, *CORO1C*, *SEMA3C*, and *SMO*. *SEMA3C* is part of a wide family of semaphorins, that are secreted factors that guide cell migration, while *SMO* is a part of Hedgehog signaling.[22,23] CCN4/Canonical Wnt signaling has been shown to interact with both semaphorins and Hedgehog signaling.[12,24] We investigated whether CCN4 affected transcription of these three genes, and of nine other genes correlating with *CCN4*, which are all involved in controlling cell shape and migration. However, we did not find any direct effects of CCN4 on expression of these genes at the 48-hour endpoint. Possibly, these genes are not directly downstream of CCN4, or expression changes occur within 48 h. In different types of cancer cells, by activating the αvβ3 integrin receptor, CCN4 can promote migration through the nuclear factor κB (NF-κB), activator protein 1 (AP-1) and intercellular adhesion molecule 1 (ICAM1), and epidermal growth factor receptor (EGFR)/extracellular signal-regulated kinase (ERK) pathways.[25] It would be interesting to investigate whether this also occurs in chondrocytes. Notably, chondrocytes treated

with higher concentration of CCN4 did not show significant increased migration, but had overall same direction of effects. Since the variability in this group was also larger, likely this is due to the relatively small number of samples.

In conclusion, we here demonstrated that CCN4 induces migration of human primary OA chondrocytes. This could aid in attracting chondrocytes to cartilage defects and repopulating cartilage scaffolds to stimulate cartilage regeneration.

Author Contributions

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

Acknowledgments and Funding

We thank all study participants of the RAAK study. The Leiden University Medical Center have and are supporting the RAAK. We thank all the members of our groups. We also thank Robert van der Wal, Peter van Schie, Shaho Hasan, Maartje Meijer, Daisy Latijnhouwers, Anika Rabelink-Hoogenstraaten, and Geert Spierenburg for their contribution to the collection of the joint tissue. Data are generated within the scope of the Medical Delta programs Regenerative Medicine 4D: Generating complex tissues with stem cells and printing technology and Improving Mobility with Technology. The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Dutch Arthritis Society (DAF-17-1-401).

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The RAAK study has been approved by the medical ethical committee of the Leiden University Medical Center (P08.239/P19.013). Written informed consent was obtained from all patients, and patients had the right to withdraw at any time. In the Radboud university medical center in Nijmegen, patients were informed about the potential anonymized use of the material for research and had the right to decline the use of their material for research (2018-4319). According to the Dutch law, informed consent and approval of an ethics committee was therefore not necessary.

Data Availability

Data is available upon request.

ORCID iDs

Ritchie G.M. Timmermans https://orcid.org/0000-0002-0787-0856
Yolande F.M. Ramos https://orcid.org/0000-0003-1459-413X

References

1. Goldring SR, Goldring MB. Clinical aspects, pathology and pathophysiology of osteoarthritis. J Musculoskelet Neuronal Interact. 2006;6(4):376-8.
2. Carballo CB, Nakagawa Y, Sekiya I, Rodeo SA. Basic science of articular cartilage. Clin Sports Med. 2017;36(3): 413-25. doi:10.1016/j.csm.2017.02.001.
3. Broughton GI, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg. 2006;117(Suppl 7): 12S-34S. doi:10.1097/01.prs.0000225430.42531.c2.
4. Morales TI. Chondrocyte moves: clever strategies. Osteoarthritis Cartilage. 2007;15(8):861-71. doi:10.1016/j.joca.2007.02.022.
5. Seol D, Yu Y, Choe H, Jang K, Brouillette MJ, Zheng H, *et al.* Effect of short-term enzymatic treatment on cell migration and cartilage regeneration: in vitro organ culture of bovine articular cartilage. Tissue Eng Part A. 2014;20(13-14):1807-14. doi:10.1089/ten.TEA.2013.0444.
6. Shiromoto Y, Niki Y, Kikuchi T, Yoshihara Y, Oguma T, Nemoto K, *et al.* Increased migratory activity and cartilage regeneration by superficial-zone chondrocytes in enzymatically treated cartilage explants. BMC Musculoskelet Disord. 2022;23(1):256. doi:10.1186/s12891-022-05210-2.
7. Lyman JR, Chappell JD, Morales TI, Kelley SS, Lee GM. Response of chondrocytes to local mechanical injury in an ex vivo model. Cartilage. 2012;3(1):58-69. doi:10.1177/1947603511421155.
8. Sedgwick AE, D'Souza-Schorey C. Wnt signaling in cell motility and invasion: drawing parallels between development and cancer. Cancers. 2016;8(9):80. doi:10.3390/cancers8090080.
9. Ono M, Masaki A, Maeda A, Kilts TM, Hara ES, Komori T, *et al.* CCN4/WISP1 controls cutaneous wound healing by modulating proliferation, migration and ECM expression in dermal fibroblasts via α5β1 and TNFα. Matrix Biol. 2018;68-69:533-46. doi:10.1016/j.matbio.2018.01.004.
10. Fuerer C, Nusse R, Ten Berge D. Wnt signalling in development and disease. Max Delbrück Center for Molecular Medicine meeting on Wnt signaling in development and disease. EMBO Rep. 2008;9(2):134-8. doi:10.1038/sj.embor.7401159.
11. van den Bosch MH, Gleissl TA, Blom AB, van den Berg WB, van Lent PL, van der Kraan PM. Wnts talking with the TGF-β superfamily: WISPers about modulation of osteoarthritis. Rheumatology. 2016;55(9):1536-47. doi:10.1093/rheumatology/kev402.
12. Sun C, Ma Q, Yin J, Zhang H, Liu X. WISP-1 induced by mechanical stress contributes to fibrosis and hypertrophy of the ligamentum flavum through Hedgehog-Gli1 signaling. Exp Mol Med. 2021;53(6):1068-79. doi:10.1038/s12276-021-00636-5.
13. van den Bosch MH, Blom AB, Schelbergen RF, Vogl T, Roth JP, Slöetjes AW, *et al.* Induction of canonical Wnt signaling

by the alarmins S100A8/A9 in murine knee joints: implications for osteoarthritis. Arthritis Rheumatol. 2016;68(1):152-63. doi:10.1002/art.39420.
14. van den Bosch MHJ, Ramos YFM, den Hollander W, Bomer N, Nelissen RGHH, Bovée JVMG, *et al.* Increased WISP1 expression in human osteoarthritic articular cartilage is epigenetically regulated and decreases cartilage matrix production. Rheumatology. 2019;58(6):1065-74. doi:10.1093/rheumatology/key426.
15. Blom AB, Brockbank SM, van Lent PL, van Beuningen HM, Geurts J, Takahashi N, *et al.* Involvement of the Wnt signaling pathway in experimental and human osteoarthritis: prominent role of Wnt-induced signaling protein 1. Arthritis Rheum. 2009;60(2):501-12. doi:10.1002/art.24247.
16. Liu Y, Song Y, Ye M, Hu X, Wang ZP, Zhu X. The emerging role of WISP proteins in tumorigenesis and cancer therapy. J Transl Med. 2019;17(1):28. doi:10.1186/s12967-019-1769-7.
17. Coutinho de Almeida R, Ramos YFM, Mahfouz A, den Hollander W, Lakenberg N, Houtman E, *et al.* RNA sequencing data integration reveals an miRNA interactome of osteoarthritis cartilage. Ann Rheum Dis. 2019;78(2):270-7. doi:10.1136/annrheumdis-2018-213882.
18. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1): 44-57. doi:10.1038/nprot.2008.211.
19. Guilak F, Nims RJ, Dicks A, Wu CL, Meulenbelt I. Osteoarthritis as a disease of the cartilage pericellular matrix. Matrix Biol. 2018;71-72:40-50.
20. Smith AE, Sigurbjörnsdóttir ES, Steingrímsson E, Sigurbjörnsdóttir S. Hedgehog signalling in bone and osteoarthritis: the role of smoothened and cholesterol. Febs J. Published online March 19, 2022. doi:10.1111/febs.16440.
21. Zhang M, Meng QC, Yang XF, Mu WD. TGF-β1/WISP1/Integrin-α interaction mediates human chondrocytes dedifferentiation. Eur Rev Med Pharmacol Sci. 2020;24(17):8675-84. doi:10.26355/eurrev_202009_22804.
22. Alto LT, Terman JR. Semaphorins and their signaling mechanisms. Methods Mol Biol. 2017;1493:1-25.
23. Carballo GB, Honorato JR, de Lopes GPF, Spohr TCLSE. A highlight on Sonic hedgehog pathway. Cell Commun Signal. 2018;16(1):11. doi:10.1186/s12964-018-0220-7.
24. Hayashi M, Nakashima T, Taniguchi M, Kodama T, Kumanogoh A, Takayanagi H. Osteoprotection by semaphorin 3A. Nature. 2012;485(7396):69-74. doi:10.1038/nature11000.
25. Feng M, Jia S. Dual effect of WISP-1 in diverse pathological processes. Chin J Cancer Res. 2016;28(6):553-60. doi:10.21147/j.issn.1000-9604.2016.06.01.