On the relation between genetic variation and osteoarthritis

Hollander, W. den

5

TRANSCRIPTIONAL ASSOCIATIONS OF OSTEOARTHRITIS MEDIATED LOSS OF EPIGENETIC CONTROL IN ARTICULAR CARTILAGE

Wouter den Hollander[1], Yolande F.M. Ramos[1], Nils Bomer[1,2], Stefan Elzinga[1], Ruud van der Breggen[1], Nico Lakenberg[1], Wesley J. de Dijcker[1], Eka D. Suchiman[1], Bouke J. Duijnisveld[4], Jeanine J. Houwing-Duistermaat[5], Eline P. Slagboom[1,2,3], Steffan D. Bos[1,3], Rob G.H.H. Nelissen[4], Ingrid Meulenbelt[1,3].

[1]Dept. of Molecular Epidemiology, LUMC, Leiden, The Netherlands. [2]IDEAL The Netherlands. [3]Genomics Initiative, sponsored by the NCHA, Leiden, The Netherlands. [4]Dept. of Orthopaedics, LUMC, Leiden, The Netherlands.[5]Dept. of Biostatistics and Bioinformatics, LUMC, Leiden, The Netherlands.

https://doi.org/10.1002/art.39162

ABSTRACT

Objective To identify osteoarthritis (OA) progression modulating pathways in articular cartilage and their respective regulatory epigenetic and genetic determinants in end stage disease.

Methods Transcriptional activity of CpGs (t-CpGs) was assessed using gene expression and DNA methylation data of respectively 33 and 31 pairs of preserved and lesioned articular cartilage. Disease responsive t-CpGs were identified by means of differential methylation between preserved and lesioned cartilage. Transcriptionally relevant genetic determinants were addressed by means of proximal SNPs near the OA responsive t-CpGs. Statistical analyses were corrected for age, sex, joint and technical covariates. A random effect was included to correct for possible correlations between paired samples.

Results Of the 9838 transcribed genes in articular cartilage, 2324 correlated with the methylation status of 3748 t-CpGs, both negative (N=1741) and positive (N=2007) correlations were observed. Hypomethylation and hypermethylation (FDR<0.05, $|\Delta\beta|>0.05$) were observed for 62 and 25 t-CpGs, respectively, covering 70 unique genes. Enrichment for developmental and ECM maintenance pathways indicated possible reactivation of endochondral ossification. Finally, we observed 31 and 26 genes of which, respectively, methylation and expression was additionally affected by genetic variation.

Conclusion We identified tissue specific genes involved in OA disease progression, reflected by genetic and pathological epigenetic regulation of transcription, primarily at genes involved in development. Therefore, transcriptionally active SNPs near these genes may serve as putative susceptibility alleles. Our results comprise an important step in understanding the reported widespread epigenetic changes occurring in OA affected articular cartilage and subsequent development of future treatments targeting disease driving pathways.

INTRODUCTION

Osteoarthritis (OA) is the most prevalent arthritic disease among the elderly (1) and is currently recognized as a disease of the whole joint. (2) A well described hallmark of OA is articular cartilage degradation. (3) The single cell type present in articular cartilage is the articular chondrocyte, which is a highly specialized, maturational arrested, non-proliferating cell. To ensure articular cartilage integrity throughout life it needs to adapt its behaviour in response to external signals, such as mechanical stress, ageing or micro-traumas. (4) To facilitate these adaptations, the chondrocyte requires phenotypic plasticity with proper dynamic control of gene expression to shift between active metabolic and maturational arrested states. In this respect, chondrocytes in OA affected cartilage were shown to have lost their maturational arrested state, regain growth-plate morphology and start to proliferate, while degrading and calcifying the articular cartilage matrix. (5;6)

A likely candidate for maintaining the chondrocyte phenotype is through epigenetic control of gene expression, such as DNA methylation; a biochemical process which is utilized by cells to adapt to environmental challenges such as age or disease by dynamic control of gene expression. (7;8) In this respect, methylome wide studies of articular cartilage in OA have revealed numerous differentially methylated loci between healthy and diseased tissue, while only a small minority of these loci were subsequently studied in terms of gene expression differences. (9-12) Therefore, up to now it remains unclear to what extent the large number of differentially methylated CpGs in OA confer relevant gene expression changes in articular cartilage. Moreover, a growing body of literature describes how aberrant gene expression is influenced, in addition to DNA methylation, by genetic risk alleles in complex genetic diseases, a mechanism outlines previously in OA. (13-17) These reports imply the need for combining multiple levels of genome wide data to gain a more robust understanding of the transcriptional processes that occur with complex genetic diseases, such as OA.

In a previous study we have described functional DNA methylation differences between knee and hip articular cartilage, independent of OA pathophysiology. (10) Although the entire epigenomic profile of knee and hip articular cartilage is primarily defined by differentially methylated regions (DMRs) between the two joints, literature suggests highly gene specific DNA methylation changes in association with OA onset and progression. (9;11;12;14-17) Therefore, in the current study we set out to identify gene specific DNA methylation differences, independent of the joint, between pre-served and lesioned cartilage in patients undergoing total joint replacement surgery due to primary end-stage OA. Moreover, we combined DNA methylation changes with a previously assessed gene expression dataset of overlapping samples (18) to assess OA related changes in the epigenetically regulated transcriptome. Finally, by integrating the results with genome wide single nucleotide poly-

morphism (SNP) data, we aim to identify OA relevant, tissue specific genetic variants that influence gene expression in articular cartilage. The applied consecutive stepwise approach will provide novel OA susceptibility genes, as well as the respective transcriptional determinants. To our knowledge this is the first study comprehensively combining genetic, epigenomic and transcriptomic data to gain a functional understanding of joint independent DNA methylation changes in relation to OA pathophysiology.

MATERIALS AND METHODS

THE RAAK COHORT.

Ethical approval was obtained from the medical ethics committee of the LUMC (P08.239) and informed consent was obtained from all participants. Participant details are listed in **Supplementary Table S1**. For sampling details see (10;14;18).

METHYLATION DATA.

Methylation data were obtained and processed as previously. (10) In short, DNA was isolated using the Promega Wizard Genomic DNA Purification kit according to the manufacturer's protocol. Next, the DNA was bisulphite treated using the ZymoResearch EZ DNA Methylation kit. DNA methylation was assessed using Illumina Infinium HumanMethylation450 BeadChips. Samples were randomly dispersed, while sample pairs were assured to be on the same chip. Using the *minfi* and *lumi* R-packages the methylation dataset was filtered for probes that contained SNPs or mapped ambiguously to the genome and colour channels were separately quantile normalized. Validation and replication using the EpiTYPER platform were done so as previously reported, (14) primer sequences are listed in **Supplementary Table S2**.

EXPRESSION DATA.

Normalized expression data from the RAAK study were processed and normalized as described previously (GSE57218). (18) RT-qPCR validation primers are listed in **Supplementary Table S2**

GENOTYPE DATA.

Using Illumina HumanOmniExpressExome chips genome wide genotyping data was constructed for 216 samples from the RAAK study. SNPs with <95% call rate, Hardy-Weinberg equilibrium $<10^{-4}$, minor allele frequency <0.01 or located on the sex chromosomes were removed prior to imputation together with Leiden Longevity Study data against the 1000 Genomes V3 March 2012 reference panel. (19) Next, SNPs that were homozygous in all the overlapping samples with the methylation

(N=23) and expression (N=24) datasets were removed prior to analyses, as were SNPs of which the imputation quality of 0.4 was not met. (20;21)

STATISTICAL ANALYSES.

All statistical procedures were carried out in R-3.0.2. Analyses were corrected for technical covariates as well as sex, joint and age. To correct for putative correlations between preserved and OA affected AC from the same joint, a random effect for patient ID was included using the *lme4* package. (22) Correction for multiple testing was performed using the bonferroni procedure per gene in the functional methylation analysis as well as the genetic analysis, all other multiple testing corrections were performed using the Benjamini-Holm method. Methylation measurements are reported as β-values. (10;23) CpGs were considered differentially methylated when the mean paired difference was at least 0.05β, as smaller differences would be hard to address statistically and/or interpret biologically. Pathway enrichment was performed using the online annotation tools DAVID and STRING-DB. A full analysis summary scheme is shown in **Figure 1.**

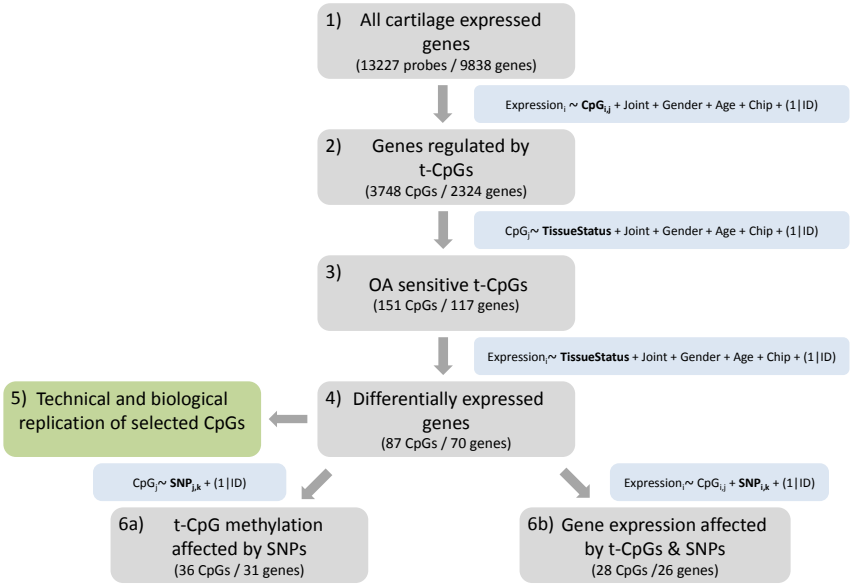

Figure 1. Overview of the applied analysis strategy.

RESULTS

TRANSCRIPTIONALLY ACTIVE CPG DINUCLEOTIDES (T-CPGS) IN ARTICULAR CARTILAGE.

Recently, we have assessed the late stage transcriptomic profile of articular cartilage of patients who underwent total joint replacement surgery, due to primary OA (GSE57218, **Supplementary Table S1**). (18) For the 13277 probes (covering 9838 unique genes, **Figure 1, step 1**) that were expressed to detectable extent in articular cartilage, we set out to explore whether they associated with DNA methylation of proximal CpGs. To identify articular cartilage relevant CpGs in terms of transcriptional association, DNA methylation data of CpGs within 10kb of annotated genes was correlated to respective gene expression data of 13 sample pairs (4 knees and 9 hips) of preserved and lesioned articular cartilage. After multiple testing correction for the number of CpGs for each individual gene, we observed 3748 CpGs that significantly correlated with proximal gene expression, covering a total of 2324 unique genes (24%, **Supplementary Table S3, Figure 1, step 2**), hereafter indicated as transcriptionally active CpGs (t-CpGs). Notably, both negative (e.g. *SPINT2*, *CILP*, *BFSP1*, *TMEM140*, **Figure 2A-D**) as well as positive correlations (e.g. *COL1A2*, *THBS2*, *MSX1*, *RUNX3*, **Figure 2E-H**) were observed for the, respectively, 1741 and 2007 t-CpGs.

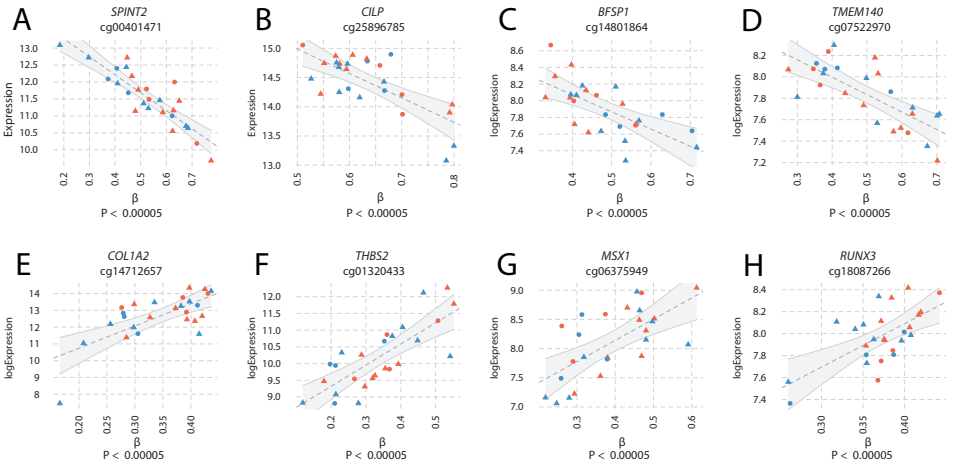

Figure 2. Examples of epigenetically regulated cartilage expressed genes by DNA methylation. Preserved and lesioned samples are respectively coloured blue and red, while knee and hip samples are respectively depicted as circles and triangles. In grey the 95% confidence intervals are plotted. (**A-D**) Examples of down regulated genes upon increased methylation. (**E-H**) Examples of genes of which expression is positively correlated to increased DNA methylation.

OA ASSOCIATED DNA METHYLATION CHANGES AT T-CPGS.

Next, we determined in a total of 31 sample pairs (17 knees and 14 hips) which of the detected 3748 t-CpGs were sensitive to the ongoing OA disease process, as reflected by differential methylation between paired preserved and lesioned cartilage. In total we observed 5282 differentially methylated CpGs (FDR<0.05, |Δβ|>0.05, **Figure 3A, Supplementary Table S4**), of which 2188 and 3094 were respectively hyper- and hypomethylated. Among these OA associated CpGs 151 overlapped with the observed t-CpGs, covering a total of 117 unique genes (**Figure 1, step 3**). Hypermethylation was observed in 59 OA responsive t-CpGs, while hypomethylation was seen in 92 OA responsive t-CpGs covering respectively 46 and 75 genes. Among those are genes known to be involved in OA pathophysiology (e.g. *FOXA2*, *RUNX1*, *COL6A3* and *CD44*, **Figure 3B-E**), as well as multiple genes not earlier reported (e.g. *UACA*, *DLX5*, *DYSF* and *IGFBP7*, **Figure 3F-I**). Next, to focus solely on genes of which expression is involved in OA progression, we selected t-CpG regulated genes of which expression was additionally significantly different between preserved and lesioned tissue. As of such, we continued with 25 and 62 respectively hyper- and hypomethylated t-CpGs, covering 70 unique genes (**Figure 1, step 4**). Subsequent gene enrichment analysis revealed significant enrichment among the 70 genes for pathways earlier reported to be implicated in OA pathophysiology, such as ECM maintenance and developmental processes (**Supplementary Table S5** and **Supplementary Figure S1**).

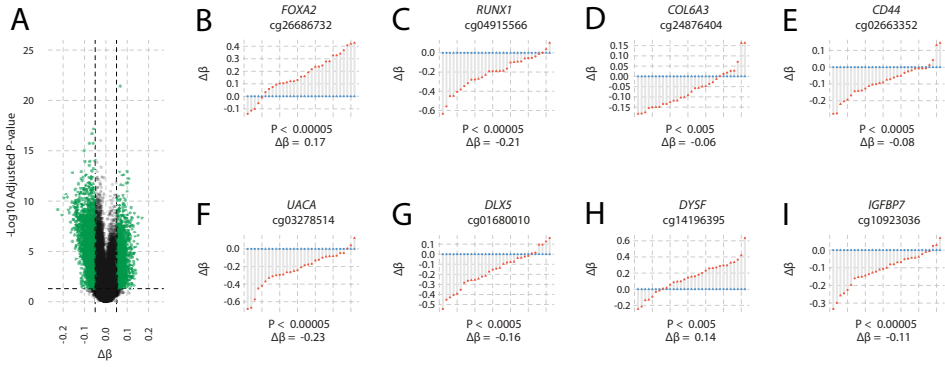

Figure 3. (**A**) Volcano plot showing the cut-offs taken to identify all differentially methylated CpGs between preserved and lesioned cartilage, significant (FDR < 0.05) differentially methylated (|Δβ| > 0.05) CpGs are depicted as green dots. (**B-E**) Significant differential methylation between preserved and lesioned cartilage in known OA associated genes. (**F-I**) Significant differential methylation between preserved and lesioned cartilage in genes not earlier implicated in OA. Preserved samples are set to 0 and depicted in blue, while its paired lesioned sample is depicted in red. Knee and hip joints are respectively shown as circles and triangles.

TECHNICAL VALIDATION AND BIOLOGICAL REPLICATION OF OA RESPONSIVE T-CPG DINUCLEOTIDES.

Using the EpiTYPER® platform, a commonly used technique for measuring DNA methylation, (14) we set out to technically validate 8 CpGs in 17 pairs of preserved and lesioned samples. We found a high degree of similarity between the two techniques, reflected by large Pearson correlation coefficients (mean r>0.85, **Supplementary Figure S2, Figure 1, step 5**). Next, we addressed the previously observed relation between the replicated CpGs and respective gene expression. Except for *IGFBP7*, we were able to validate the transcriptional involvement of all selected t-CpGs and/or disease associated dysregulation of the respective gene (**Supplementary Figure S3**). For biological validation, DNA methylation of the selected CpGs was measured in an additional 31 pairs of preserved and lesioned cartilage. All CpGs showed highly similar, significant DNA methylation changes as were seen in both the discovery and validation samples (**Supplementary Figure S3**).

THE INFLUENCE OF GENETIC FACTORS ON T-CPG METHYLATION AND EXPRESSION IN ARTICULAR CARTILAGE.

Finally, we investigated the stable regulatory genetic environment, as reflected by transcriptionally active SNPs in proximity of the 70 genes. Presence of such SNPs may causally affect cartilage homeostasis of epigenetically controlled genes and confer potential OA susceptibility. The genotypes of all SNPs (dbSNP build 138) 10kb up- and downstream of the 70 genes were assessed in 23 sample pairs of the methylation dataset. Using multivariate analysis with methylation as dependant variable, we identified 36 OA responsive t-CpGs that are significantly affected by at least one SNP (**Supplementary Table S6, Figure 1, step 6a**), covering 31 unique genes. In parallel, using multivariate analysis with expression as dependent variable, we explored whether epigenetic regulation of the 70 genes was additionally affected by the alleles of proximal SNPs. As of such, we observed 26 genes of which expression was modulated by the local genetic background in conjunction with 28 t-CpGs (**Supplementary Table S7, Figure 1, step 6b**). For the *ESR*, *NAV2* and *WLS*, we observed t-CpGs and SNPs that modulated gene expression jointly.

As example, three notable genes of which we have observed transcriptomic, epigenetic and genetic involvement in OA progression are *VIT* (**Figure 4A-D**), *ROR2* (**Figure 4E-H**) and *WLS* (**Figure 4I-M**). All genes were differentially expressed between preserved and lesioned cartilage (**Figure 4A**, **4E**, **4I**), which was modulated by differential DNA methylation (**Figure 4B**, **4F**, **4J**), also reflected by significant differential methylation at the respective t-CpGs (**Figure 4C**, **4G**, **4K**). Moreover, rs11884419 and rs13292198 influenced gene expression and t-CpG methylation of *ROR2* and *VIT*, respectively (**Figure 4D**, **3H**). Additionally, rs12028757 jointly affected t-CpG methylation and *WLS* gene expression (**Figure 4L-M**).

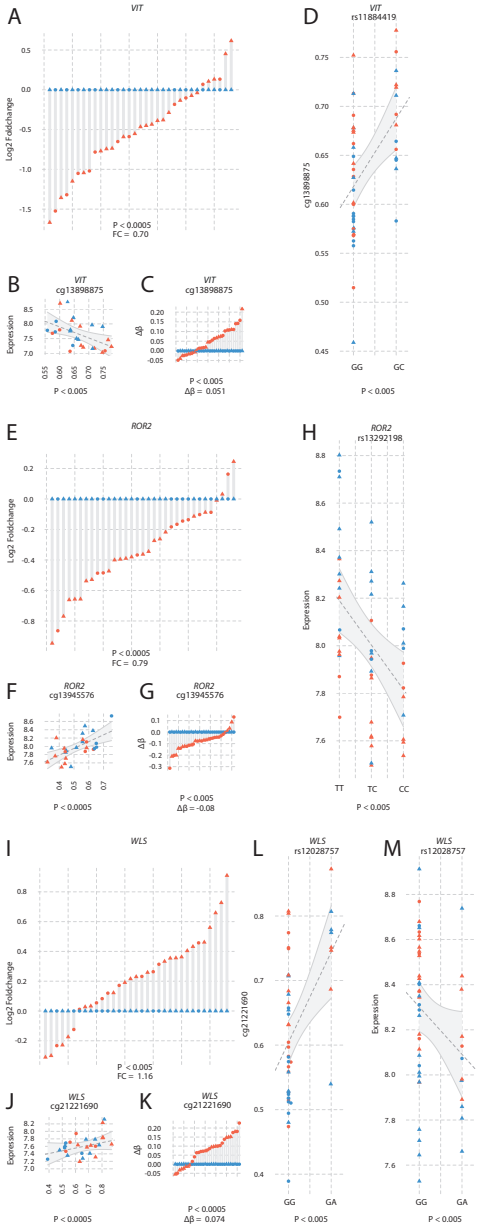

Figure 4. Examples of cumulative evidence for putative causal involvement of *VIT*, *ROR2* and *WLS* in OA pathophysiology. Preserved and lesioned samples are respectively coloured blue and red, while knee and hip samples are respectively depicted as circles and triangles. (**A, E, I**) Significant differential methylation was observed between preserved and lesioned cartilage for all three genes. (**B, F, J**) A significant direct relation between expression and respective CpG methylation was observed. (**C, G, K**) As expected, a significant difference in methylation was observed between preserved and lesioned tissue as well. (**D, H, L, M**) In conjunction with DNA methylation, expression was regulated by proximal SNPs.

DISCUSSION

The here presented study encompasses the first comprehensive multi-level integration of genome wide data to gain a more accurate understanding of OA associated changes in the epigenetically regulated transcriptome of articular chondrocytes. By stepwise integrating transcriptomic and epigenetic data in relation to cartilage OA severity, we identified 70 unique genes with OA responsive t-CpGs likely affecting expression in articular cartilage. Subsequent pathway analyses showed significant enrichment for genes that act within skeletal development. Moreover, we have shown that for 31 and 26 OA cartilage relevant genes, respectively, methylation and expression is additionally affected by genetic variation proximal to these genes.

Although the observed enrichment of OA responsive t-CpGs among genes within developmental pathways either marks disease advancement or an adaptation of the preserved cartilage to the adjacent lesioned tissue, our data shows that changes in epigenetically regulated control of developmental genes and OA progression are markedly linked. This could indicate either specific, dynamic regulation of expression of the genes in these pathways by the challenged articular chondrocytes in an attempt to cope with end-stage OA, or alternatively, chondrocytes at end stage disease have lost their ability to epigenetically control expression of essential genes involved in skeletal development and consequently recuperate growth plate morphology and start cartilage debilitating expression, a well described hallmark of OA. The latter hypothesis is supported by the here observed difference of epigenetic control of skeletal development associated genes with OA, such as *VIT, ROR2* and *WLS*. Markedly, in this respect, are also the results of comprehensive genome wide searches for genetic variants conferring risk for OA that have resulted in robust genome wide significant signals at genes implicated in these developmental pathways (24).

In the current study, we present genetic and epigenetic loci that are functionally relevant for OA responsive t-CpGs and cartilage expressed genes and should ideally now be followed up as candidate genes in large genome wide association (GWA) datasets to investigate whether these variants indeed confer a relatively large number of small effects that are responsible for the missing heritability observed in OA. We have observed a relatively small number of differentially expressed genes that are regulated by t-CpGs and/or SNPs. Although this could be due to statistical power and/or small effect sizes, it unquestionably highlights the importance of combining epigenome data, or gene specific epigenetic data for that matter, with other types of molecular data, to gain a robust understanding of and to biologically interpret the observed differences. Furthermore of note, we did neither observe established genetic OA susceptibility nor OA related epigenetic loci, which implies that our transcriptional, tissue relevant approach offers additional, compelling knowledge about

genes involved in mature articular cartilage homeostasis and late OA when compared to traditional GWA approaches. Even more so, the epigenetic and transcriptional effects of OA susceptibility genes such as *GDF5* (15) and *DIO2* (14) are relatively subtle, whereas we have aimed to select genes with larger effects in late OA. Also, we have possibly missed out on long distance or trans acting t-CpGs or SNPs, as a result of our applied 10kb cut-off. While we do not disregard the possible impact of long distance, transcriptional relevant loci, the measure of effect will likely be inversely correlated with the genomic distance. In order to study these effects accurately, larger sample sizes than our own would be required. Considering the earlier observed gene specific transcriptionally relevant and OA associated differentially methylated CpGs, we were unfortunately unable to address these as the applied methylation array lacks the density and subsequently does not measure these.

The *VIT, ROR2* and *WLS* genes are notable examples for which we here present functional epigenetic, genetic and transcriptional (**Figure 3**) differences depending on the late pathophysiological state of articular cartilage (25;26). *VIT* is a relatively under studied gene in both cartilage biology and osteoarthritis. Nonetheless, proteomic analysis of mouse hip cartilage revealed involvement in cartilage development (27). More specifically, vitrin, the protein product of the *VIT* gene, contains a Von Willebrand factor A domain, and is subsequently involved in ECM integrin signalling (28). Expression of *ROR2* drives chondrocyte expansion (29) and is known to be involved in regulating the TNFRSF11B/TNFSF11 protein (commonly referred to as OPG/RANK) ratio in articular chondrocytes, (30) a well described disrupted pathway in OA. (31) Down regulation of *ROR2* inhibits the chondrocytes regenerative capacities, while disruption of the OPG/RANKL ratio has been shown to induce calcification and bone formation. (29;31;32) Another major player in joint development and cartilage biology is the Wnt pathway (33-36), in which *ROR2* (37;38) and *WLS* (39;40) as well as a number of OA susceptibility genes are situated (34;41;42). While the role of Wnt signalling is evident in cartilage development and OA, *WLS* is specifically involved in the endochondral ossification process (39).

While OA related differences in methylation in articular cartilage have also been reported by others (9;11;12) , our results imply that changes in epigenetic control only lead to expression differences at a limited number of genes. More specifically, at genes involved in either maintaining the chondrocyte phenotype or adversely pursuing the endochondral ossification lineage. Moreover, the detected local SNPs that affected either methylation or gene expression in articular cartilage of epigenetically controlled genes may inherently affect proper cartilage homeostasis and potentially affect OA susceptibility. In this regard, we observed SNPs that influenced DNA methylation at t-CpGs, while no direct relation between the respective genotypes and gene expression was observed. Likely, a large number of factors obscure the direct regulatory mechanism between the local genetic background, t-CpG methylation and gene expression. Of note, these mechanisms likely arbitrate differential expression

among the genes in which we did observe differential t-CpG methylation but no difference in expression. SNPs and t-CpGs that appear to solely affect t-CpG methylation or expression respectively, are still of relevance, however, the transcriptional effects of these variants should be addressed in larger consortia. Further mechanistic studies, such as longitudinal measurements in animal experiments or actively perturbing the relevant genes in cell systems, are required to accurately address the hypothesis.

In conclusion, we have here shown that OA related epigenetic differences need to be integrated with other sources of molecular data, such as genomic and transcriptomic, to enhance our understanding of the pathophysiological processes of OA. Furthermore, by integration of multiple layers of genome wide data we have identified genes, such as *VIT*, *ROR2* and *WLS*, which are likely modulating OA pathophysiology and possibly reflect the loss of the chondrocyte's maturational arrested state. Although targeting DNA methylation seems unlikely to stand at the basis for developing treatments, it serves to deepen our understanding of the complex transcriptomic changes in OA affected articular cartilage.

ACKNOWLEDGMENTS

We thank all participants of the RAAK study. The RAAK studies were supported by the Leiden University Medical Centre, the Dutch Arthritis Association (DAA 101-402 and Reumafonds LRR) and the Centre of Medical System Biology and Netherlands Consortium for Healthy Aging both in the framework of the Netherlands Genomics Initiative (NGI). Furthermore, we acknowledge support by TreatOA and IDEAL, which are funded by the European Union's Seventh Framework Program (FP7/2007-2011) under respective grant agreement nos. 200800 and 259679. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Medical ethics approval LUMC P08.239

REFERENCES

1 Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. The Lancet 2011; 377 (9783):2115-26.
2 Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: A disease of the joint as an organ. Arthritis & Rheumatism 2012; 64 (6):1697-707.
3 Chen YA, Lemire M, Choufani S, Butcher DT, Grafodatskaya D, Zanke BW et al. Discovery of cross-reactive probes and polymorphic CpGs in the Illumina Infinium HumanMethylation450 microarray. Epigenetics 2013; 8 (2):203-9.
4 Dvir-Ginzberg M, Reich E. Chopping off the chondrocyte proteome. Biomarkers 2014;1-7.
5 Sun MM, Beier F. Chondrocyte hypertrophy in skeletal development, growth, and disease. Birth Defects Res C Embryo Today 2014; 102 (1):74-82.
6 Hosaka Y, Saito T, Sugita S, Hikata T, Kobayashi H, Fukai A et al. Notch signaling in chondrocytes modulates endochondral ossification and osteoarthritis development. Proc Natl Acad Sci U S A 2013; 110 (5):1875-80.
7 Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT et al. Dynamic changes in the human methylome during differentiation. Genome Res 2010; 20 (3):320-31.
8 Slieker RC, Bos SD, Goeman JJ, Bovee JV, Talens RP, van der Breggen R et al. Identification and systematic annotation of tissue-specific differentially methylated regions using the Illumina 450k array. Epigenetics Chromatin 2013; 6 (1):26.
9 Rushton MD, Reynard LN, Barter MJ, Refaie R, Rankin KS, Young DA et al. Characterization of the cartilage DNA methylome in knee and hip osteoarthritis. Arthritis Rheumatol 2014.
10 den Hollander W, Ramos YF, Bos SD, Bomer N, van der Breggen R, Lakenberg N et al. Knee and hip articular cartilage have distinct epigenomic landscapes: implications for future cartilage regeneration approaches. Ann Rheum Dis 2014.
11 Jeffries MA, Donica M, Baker L, Stevenson M, Annan AC, Humphrey MB et al. Genome-wide DNA methylation study identifies significant epigenomic changes in osteoarthritic cartilage. Arthritis Rheumatol 2014.
12 Fernandez-Tajes J, Soto-Hermida A, Vazquez-Mosquera ME, Cortes-Pereira E, Mosquera A, Fernandez-Moreno M et al. Genome-wide DNA methylation analysis of articular chondrocytes reveals a cluster of osteoarthritic patients. Ann Rheum Dis 2014; 73 (4):668-77.
13 Bell JT, Pai AA, Pickrell JK, Gaffney DJ, Pique-Regi R, Degner JF et al. DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol 2011; 12 (1):R10.
14 Bomer N, den Hollander W, Ramos YFM, Bos SD, van der Breggen R, Lakenberg N et al. Underlying molecular mechanisms of DIO2 susceptibility in symptomatic osteoarthritis. Annals of the Rheumatic Diseases 2014.
15 Reynard LN, Bui C, Syddall CM, Loughlin J. CpG methylation regulates allelic expression of GDF5 by modulating binding of SP1 and SP3 repressor proteins to the osteoarthritis susceptibility SNP rs143383. Hum Genet 2014; 133 (8):1059-73.
16 Hashimoto K, Oreffo RO, Gibson MB, Goldring MB, Roach HI. DNA demethylation at specific CpG sites in the IL1B promoter in response to inflammatory cytokines in human articular chondrocytes. Arthritis Rheum 2009; 60 (11):3303-13.
17 Hashimoto K, Otero M, Imagawa K, de Andres MC, Coico JM, Roach HI et al. Regulated transcription of human matrix metalloproteinase 13 (MMP13) and interleukin-1beta (IL1B) genes in chondrocytes depends on methylation of specific proximal promoter CpG sites. J Biol Chem 2013; 288 (14):10061-72.
18 Ramos YF, den Hollander W, Bovee JV, Bomer N, van der Breggen R, Lakenberg N et al. Genes Involved in the Osteoarthritis Process Identified through Genome Wide Expression Analysis in Articular Cartilage; the RAAK Study. PLoS ONE 2014; 9 (7):e103056.
19 Schoenmaker M, de Craen AJ, de Meijer PH, Beekman M, Blauw GJ, Slagboom PE et al. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. Eur J Hum Genet 2006; 14 (1):79-84.
20 Ramos YF, Metrustry S, Arden N, Bay-Jensen AC, Beekman M, de Craen AJ et al. Meta-analysis identifies loci affecting levels of the potential osteoarthritis biomarkers sCOMP and uCTX-II with genome wide significance. J Med Genet 2014; 51 (9):596-604.
21 Wood AR, Esko T, Yang J, Vedantam S, Pers TH, Gustafsson S et al. Defining the role of common variation in the genomic and biological architecture of adult human height. Nat Genet 2014; 46 (11):1173-86.

22 Douglas Bates, Martin Maechler, Ben Bolker. lme4: Linear mixed-effects models using S4 classes. 2012.
23 Identification of new susceptibility loci for osteoarthritis (arcOGEN): a genome-wide association study. The Lancet 2001; 380 (9844):815-23.
24 Bos SDl, Slagboom PE, Meulenbelt I. New insights into osteoarthritis: early developmental features of an ageing-related disease. Current Opinion in Rheumatology 2008; 20 (5).
25 Pullig O, Weseloh G, Swoboda B. Expression of type VI collagen in normal and osteoarthritic human cartilage. Osteoarthritis Cartilage 1999; 7 (2):191-202.
26 Soder S, Hambach L, Lissner R, Kirchner T, Aigner T. Ultrastructural localization of type VI collagen in normal adult and osteoarthritic human articular cartilage. Osteoarthritis Cartilage 2002; 10 (6):464-70.
27 Wilson R, Norris EL, Brachvogel B, Angelucci C, Zivkovic S, Gordon L et al. Changes in the chondrocyte and extracellular matrix proteome during post-natal mouse cartilage development. Mol Cell Proteomics 2012; 11 (1):M111.
28 Manabe R, Tsutsui K, Yamada T, Kimura M, Nakano I, Shimono C et al. Transcriptome-based systematic identification of extracellular matrix proteins. Proc Natl Acad Sci U S A 2008; 105 (35):12849-54.
29 DeChiara TM, Kimble RB, Poueymirou WT, Rojas J, Masiakowski P, Valenzuela DM et al. Ror2, encoding a receptor-like tyrosine kinase, is required for cartilage and growth plate development. Nat Genet 2000; 24 (3):271-4.
30 Ermakov S, Trofimov S, Malkin I, Livshits G. A significant association exists between receptor tyrosine kinase-like orphan receptor 2 gene variants and the OPG/RANKL ratio in human plasma. Osteoporos Int 2012; 23 (7):1899-907.
31 Ramos YFM, Bos SD, van der Breggen R, Kloppenburg M, Ye K, Lameijer EW et al. A gain of function mutation in TNFRSF11B encoding osteoprotegerin causes osteoarthritis with chondrocalcinosis. Annals of the Rheumatic Diseases 2014.
32 Liu Y, Bhat RA, Seestaller-Wehr LM, Fukayama S, Mangine A, Moran RA et al. The orphan receptor tyrosine kinase Ror2 promotes osteoblast differentiation and enhances ex vivo bone formation. Mol Endocrinol 2007; 21 (2):376-87.
33 Kruger C, Kappen C. Expression of cartilage developmental genes in Hoxc8- and Hoxd4-transgenic mice. PLoS ONE 2010; 5 (2):e8978.
34 Casta+¦o Betancourt MC, Cailotto F, Kerkhof HJ, Cornelis FMF, Doherty SA, Hart DJ et al. Genome-wide association and functional studies identify the DOT1L gene to be involved in cartilage thickness and hip osteoarthritis. Proceedings of the National Academy of Sciences 2012; 109 (21):8218-23.
35 Leijten JC, Emons J, Sticht C, van GS, Decker E, Uitterlinden A et al. Gremlin 1, frizzled-related protein, and Dkk-1 are key regulators of human articular cartilage homeostasis. Arthritis Rheum 2012; 64 (10):3302-12.
36 Lories RJ, Corr M, Lane NE. To Wnt or not to Wnt: the bone and joint health dilemma. Nat Rev Rheumatol 2013; 9 (6):328-39.
37 Ford CE, Qian Ma SS, Quadir A, Ward RL. The dual role of the novel Wnt receptor tyrosine kinase, ROR2, in human carcinogenesis. Int J Cancer 2013; 133 (4):779-87.
38 Fukuyo S, Yamaoka K, Sonomoto K, Oshita K, Okada Y, Saito K et al. IL-6-accelerated calcification by induction of ROR2 in human adipose tissue-derived mesenchymal stem cells is STAT3 dependent. Rheumatology (Oxford) 2014; 53 (7):1282-90.
39 Lu C, Wan Y, Cao J, Zhu X, Yu J, Zhou R et al. Wnt-mediated reciprocal regulation between cartilage and bone development during endochondral ossification. Bone 2013; 53 (2):566-74.
40 Wang LT, Wang SJ, Hsu SH. Functional characterization of mammalian Wntless homolog in mammalian system. Kaohsiung J Med Sci 2012; 28 (7):355-61.
41 Evangelou E, Chapman K, Meulenbelt I, Karassa FB, Loughlin J, Carr A et al. Large-scale analysis of association between GDF5 and FRZB variants and osteoarthritis of the hip, knee, and hand. Arthritis & Rheumatism 2009; 60 (6):1710-21.
42 Loughlin J, Dowling B, Chapman K, Marcelline L, Mustafa Z, Southam L et al. Functional variants within the secreted frizzled-related protein 3 gene are associated with hip osteoarthritis in females. Proceedings of the National Academy of Sciences of the United States of America 2004; 101 (26):9757-62.

Supplemental figures and tables can be found at doi: 10.1002/art.39162.