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Increased DNA Methylation and Reduced Expression of Transcription Factors in Human Osteoarthritis Cartilage

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Objective. **To analyze the methylome of normal and osteoarthritic (OA) knee articular cartilage and to determine the role of DNA methylation in the regulation of gene expression in vitro.**

Methods. **DNA was isolated from human normal (n = 11) and OA (n = 12) knee articular cartilage and analyzed using the Infinium HumanMethylation450 BeadChip array. To integrate methylation and transcription, RNA sequencing was performed on normal and OA cartilage and validated by quantitative polymerase chain reaction. Functional validation was performed in the human TC28 cell line and primary chondrocytes that were treated with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC).**

Results. **DNA methylation profiling revealed 929 differentially methylated sites between normal and OA cartilage, comprising a total of 500 individual genes. Among these, 45 transcription factors that harbored differentially methylated sites were identified. Integrative analysis and subsequent validation showed a subset of 6 transcription factors that were significantly hypermethylated and down-regulated in OA cartilage (*ATOH8*, *MAFF*, *NCOR2*, *TBX4*, *ZBTB16*, and *ZHX2*). Upon 5-aza-dC treatment, TC28 cells showed a significant increase in gene expression for all 6 transcription factors. In primary chondrocytes, *ATOH8* and *TBX4* were increased after 5-aza-dC treatment.**

Conclusion. **Our findings reveal that normal and OA knee articular cartilage have significantly different methylomes. The identification of a subset of epigenetically regulated transcription factors with reduced expression in OA may represent an important mechanism to explain changes in the chondrocyte transcriptome and function during OA pathogenesis.**

Osteoarthritis (OA) is a chronic musculoskeletal disease characterized by degradation of articular cartilage and remodeling of other tissues in synovial joints, leading to joint malfunction, pain, and disability (1). It is estimated that 10–12% of adults worldwide have symptomatic OA and this number is predicted to increase 50% over the next 20 years (2). Despite the high prevalence and socioeconomic burden of the disease, OA pathophysiology is not entirely understood, and to date, no disease-modifying agents have been approved for OA treatment (3).

The pathogenesis of OA is multifactorial, with aging, obesity, and genetic susceptibility being the main risk factors (4). During OA development, articular chondrocytes (the only cell type in articular cartilage that is responsible for maintaining tissue homeostasis) undergo marked transcriptional and phenotypic changes that compromise their function and lead to cartilage degradation (5). However, genome-wide association studies have shown only a small genetic variance component for OA (6), and recent evidence has pointed to epigenetic regulation as a key driver of the transcriptional alterations observed in OA chondrocytes (7–9).

Epigenetic mechanisms, which are defined as heritable changes in gene expression that do not change the DNA sequence, include DNA methylation, post-translational histone modifications that alter chromatin structure, and complex noncoding RNA networks (10). DNA methylation consists of the addition of a methyl

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group to a cytosine in a CpG-containing nucleotide to form 5-methylcytosine. Genomic localization of the CpG sites is a determinant of the functional consequences of DNA methylation. Methylation in promoter and enhancer regions is known to correlate with gene silencing, whereas methylation in gene body regions usually correlates with increased gene expression (11). During OA development and in adulthood, DNA methylation is an essential mechanism to ensure cell-specific gene expression, and consequently, cells from different tissues often exhibit unique methylation landscapes. On the other hand, aberrant epigenetic alterations have been suggested to play a pivotal role in different pathologies, such as cancer and neurodegenerative diseases (12).

The role of DNA methylation in OA has begun to be elucidated. Several candidate gene studies have identified alterations in the methylation status of genes involved in OA pathogenesis, such as *MMP3*, *MMP9*, *MMP13*, *ADAMTS4* (13–15), *IL1B* (16), *NOS2* (17), *GDF5* (18), *SOD2* (19), and *SOX9* (20). These methylation changes have been proposed to contribute to the differential gene expression observed in OA cartilage. More recently, genome-wide approaches have been used to compare the methylomes of normal and OA chondrocytes (21–24). These studies clearly showed that there is a distinct methylation landscape in OA cartilage compared with healthy cartilage in the hip and knee (21,23). Moreover, they showed that hip and knee cartilage have different methylation profiles regardless of disease status (24).

The aim of this study was to comprehensively compare the methylome of normal and OA knee articular cartilage using an Infinium HumanMethylation450 BeadChip array. In order to identify functional changes in gene expression due to differences in methylation, we focused the second part of the study on transcription factors, which are proteins that bind to specific DNA sequences, thereby controlling the rate of transcription of several genes. Using integrative approaches, we identified a group of hypermethylated transcription factors with reduced expression in OA articular cartilage. We used in vitro approaches to experimentally validate the link between DNA methylation and gene expression.

MATERIALS AND METHODS

Human cartilage samples. Macroscopically normal human knee cartilage from donors without a history of previous joint pathology was provided by tissue banks. OA cartilage was obtained from patients undergoing knee replacement surgery. Cartilage samples were harvested from identical locations on the weight-bearing region of the medial femoral condyle. All human cartilage samples were macroscopically assessed and scored according to the method described by Outerbridge (25), and histologic analysis was performed on Safranin O–stained sections. Based on the macroscopic and histologic analyses, samples were classified as normal or OA. The tissue donors included in the present study are described in Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39643/abstract.

DNA isolation and methylation profiling. DNA was extracted from snap-frozen cartilage samples using a DNeasy Plant Maxi kit (Qiagen). The resulting DNA solution was precipitated with 1.5 volumes of cold isopropanol for 30 minutes at −20°C followed by centrifugation at 4°C for 1 hour. Finally, the pellet was washed twice in 70% ethanol and resuspended in AE buffer (10 m*M* Tris HCl, 0.5 m*M* EDTA, pH 9.0) at a concentration of 75 ng/μl. Next, 300 ng of DNA was treated with bisulfite, using an EZ DNA Methylation Kit (Zymo Research), and bisulfite-converted DNA was used to hybridize Infinium HumanMethylation450 BeadChips (Illumina).

DNA methylation data analysis. Raw intensity data were processed using a Bioconductor minfi package (26) and normalized for technical variations using SWAN (27). Poorly performing probes ($P > 0.01$ in more than 1 sample) and probes that interrogated high frequency single-nucleotide polymorphisms or mapped on sex chromosomes were removed from the analysis. The final data set included 446,950 probes. The β value, which represents the percentage of methylation at each CpG site, was calculated from the normalized intensity values. The β values at all CpG sites were compared between OA and normal samples using an F test after including age, body mass index (BMI), and sex as covariates; *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Differentially methylated CpG sites were defined as having Benjamini-Hochberg–adjusted *P* values less than 0.05 and a difference in mean β values of >0.15 between normal and OA groups. To define differentially methylated genes, the methylation data were mapped to the genome using Illumina v1.2 annotation from the Bioconductor package IlluminaHumanMethylation450kanno.ilmn12.hg19, which is based on University of California, Santa Cruz (UCSC) hg19 (NCBI RefSeq database release 37).

Gene Ontology (GO) enrichment analyses were performed using a WEB-based GEne TeT AnaLysis Toolkit WebGestalt. Pathway analysis was performed for differentially methylated genes, using a Bioconductor SPIA package with topologies derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. In both cases, a hypergeometric test was used to test for enrichment after adjustment for multiple testing using the Benjamini-Hochberg method. An adjusted *P* value of less than 0.05 was considered significant.

Enrichment analysis for different genomic regions was performed by comparing the percentage of differentially methylated sites (differential methylation rate) with the total number of sites present in the array after filtering (assay rate). Statistical analysis was performed using Fisher's test with adjustment for multiple testing using the Benjamini-Hochberg method. A corrected *P* value of less than 0.05 was considered significant. An outline of the analyses used in the present study is shown in Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39643/abstract.

RNA isolation and sequencing. Genome-wide transcriptomic analysis was performed in a set of 8 normal and 10 OA knee articular cartilage samples. RNA was isolated from cartilage stored at −20°C in Allprotect Tissue Reagent (Qiagen).

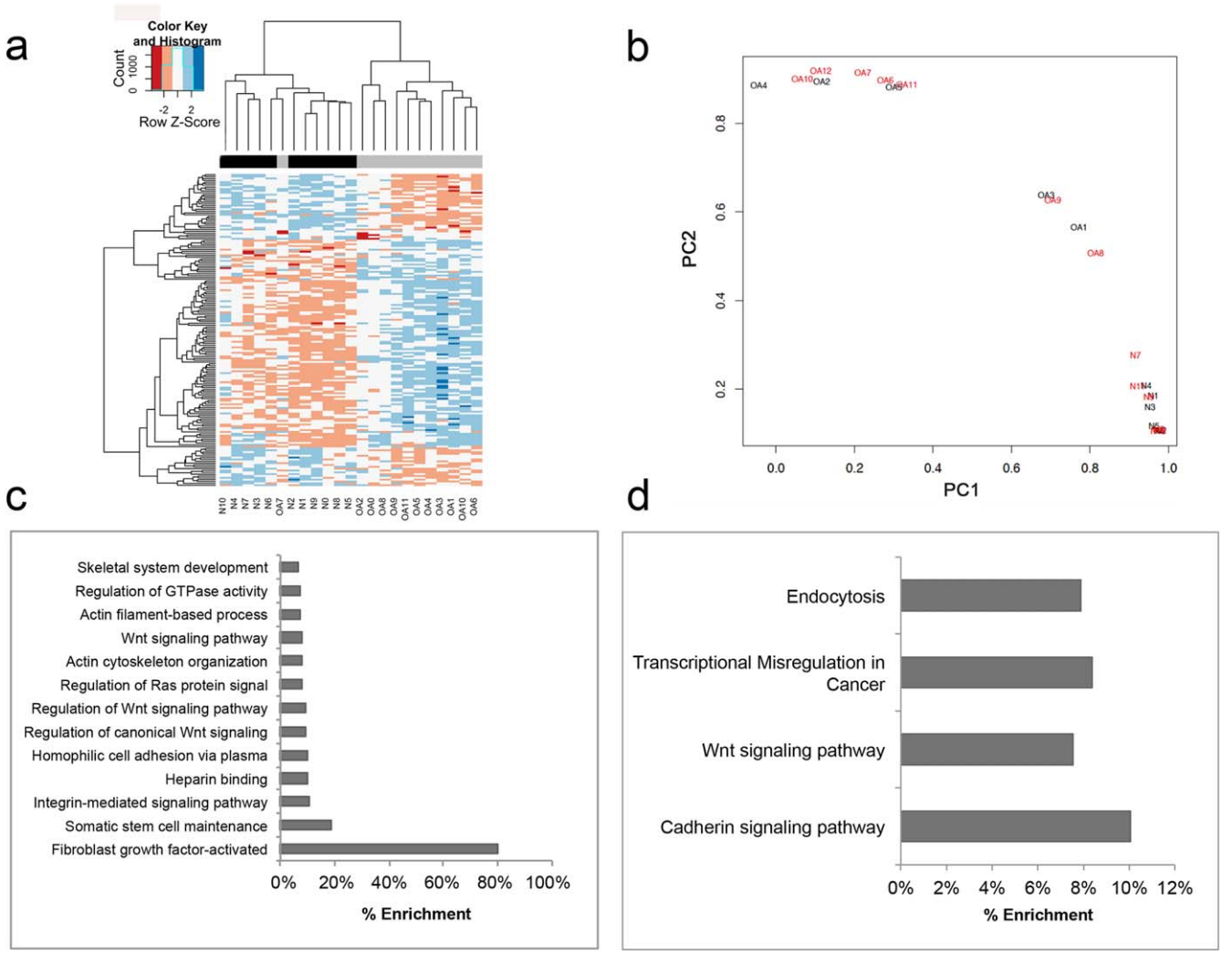

Figure 1. Genome-wide methylation analysis of human normal and osteoarthritic (OA) knee articular cartilage. **a,** Unsupervised hierarchical clustering of DNA methylation values in 11 normal samples and 12 OA samples. **b,** Principal components (PC) analysis of DNA methylation in the 929 differentially methylated CpG sites in normal (black) and OA (red) samples. **c,** Gene ontology term analysis of genes containing demethylated sites. **d,** Pathway analysis of the 929 sites that were differentially methylated in normal and OA samples.

Briefly, cartilage was pulverized using a 6770 Freezer/Mill Cryogenic Grinder (SPEX SamplePrep), and homogenized in QIAzol Lysis Reagent (Qiagen) at a concentration of 25 mg tissue sample per 700 μl QIAzol. Next, RNA was isolated using an RNeasy Mini Kit with on-column DNase digestion according to the protocol described by the manufacturer, followed by decontamination of proteoglycans using RNAmate (BioChain Institute). RNA purity was assessed using NanoDrop (ND-1000; Thermo Scientific), and sample quality was determined with an Agilent 2100 Bioanalyzer using an Agilent RNA 6000 Nano LabChip (no. 5067-1511). Normal and OA cartilage samples had mean $\pm$ SD RNA integrity numbers of 3.9 ± 1.1 and 5.6 ± 1.7, respectively. Sequencing messenger RNA (mRNA) libraries were prepared using Encore Complete RNA-Seq DR Multiplex Systems 1-8 and 9-16 (NuGEN). Libraries were amplified by polymerase chain reaction (PCR), gel purified, and an Illumina HiSeq 2000 system was used to generate a total of 8–30 million 100-bp reads.

RNA sequencing reads for each library were mapped to the UCSC human reference genome hg19 downloaded from the Illumina iGenome web site (online at http://support.illumina.com/sequencing/sequencing_software/igenome.ilmn) using TopHat2 (v2.0.9) and Bowtie2 (v2.1.0) with the default settings and options. The aligned reads were assembled into transcripts, and their abundances were estimated using Cufflinks version 2.1.1. Transcript abundances were measured in fragments per kilobase of exon per million fragments mapped, which describe the relative abundances of transcripts in an experiment after normalization to the transcript size and the total number of aligned reads in the sample. Genes with a Benjamini-Hochberg–adjusted P value of less than 0.05 were considered significantly differentially expressed.

Quantitative PCR (qPCR). Validation of the mRNA expression of selected transcription factors as detected by RNA sequencing was performed in an independent set of 13 normal

and 11 OA knee articular cartilage samples. Individual gene expression was assessed by qPCR using a LightCycler 480 instrument (Roche Diagnostics) and TaqMan gene expression assay probes (Applied Biosciences) for *ATOH8*, *FOXO3*, *KLF15*, *MAFF*, *NCOR2*, *NFATC2*, *RARA*, *TBX4*, *ZBTB16*, *ZHX2*, and *GAPDH*, as previously described (28). Statistical differences in qPCR experiments were determined by Student's *t*-test. *P* values less than 0.05 were considered significant.

Cell culture. Primary human articular chondrocytes were isolated from the joints of healthy subjects as previously described (28). After isolation, chondrocytes were plated at a density of 10^4 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM; Fisher Scientific) containing 10% calf serum and incubated at 37°C in 5% CO_2. First-passage chondrocytes were used in all experiments. The immortalized human chondrocyte cell line TC28 (29) was cultured in DMEM containing 10% calf serum, and only cells that had been maintained for <20 passages were used in all experiments.

The 5-aza-2′-deoxycytidine (5-aza-dC) experiments were performed using different methodologies depending on the cell type used, due to difference in the rate of proliferation. For primary chondrocytes, freshly plated cells were incubated with or without 10 μ*M* 5-aza-dC for at least 3 population doublings (~4–5 weeks in culture). TC28 cells were treated with 0, 1, 5, or 10 μ*M* 5-aza-dC for 48 hours. At the end of the treatment, RNA was collected using a Direct-Zol RNA MiniPrep Kit (Zymo Research), and gene expression was assessed by qPCR as described above.

RESULTS

General description of normal and OA cartilage methylomes. Genome-wide DNA methylation profiling was performed in 11 macroscopically normal knee cartilage samples obtained from healthy donors and 12 samples obtained from patients with OA. Unsupervised hierarchical clustering revealed that healthy and OA samples segregated into 2 different clusters (Figure 1a). The first cluster consisted of samples from 10 healthy controls and 1 OA patient, and the second cluster consisted of samples from 1 healthy control and 11 patients with OA.

Identification and characterization of differentially methylated sites in knee articular cartilage from controls and OA patients. We identified a total of 929 differentially methylated sites between normal and OA cartilage that comprised a total of 500 individual genes (for the complete list of differentially methylated sites, see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39643/abstract). Among these, 356 sites (38.3%) were hypomethylated, and 573 sites (61.7%) were hypermethylated in OA cartilage. These differences were observed after performing statistical analysis with correction for sex and BMI. However, when the analysis was corrected for age as a covariate, no differentially methylated sites were observed.

Principal components analysis using the 929 differentially methylated sites revealed complete segregation of normal and OA samples (Figure 1b). The gene-associated CpGs with the highest differences in methylation between both groups are shown in Table 1. In addition to the differences in the magnitude of single CpG methylation, we observed several genes that harbored multiple differentially methylated sites that were hypermethylated, such as *LRP5*, *NCOR2*, *NFATC1*, *PRDM8*, and *TBX4*, or hypomethylated in OA cartilage, such as *RUNX1*. As shown in Figure 1c, GO term analysis of genes containing differentially methylated sites showed significant overrepresentation of genes involved in different signaling pathways, such as integrins ($q < 0.01$), Wnt ($q < 0.001$), and fibroblast growth factor ($q < 0.01$) (for the complete list, see Supplementary Table 3, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39643/abstract).

We next carried out a pathway analysis using the KEGG database for all genes containing differentially methylated sites. The differentially enriched canonical pathways identified were endocytosis ($q < 0.05$), transcriptional misregulation in cancer ($q < 0.05$), Wnt signaling pathway ($q < 0.01$), and cadherin signaling pathway ($q < 0.01$) (Figure 1d).

Differentially methylated site enrichment analysis for CpG genomic features. Recent studies suggest that the effects of DNA methylation on gene expression are highly dependent on the CpG genomic location (11). Therefore, we performed an enrichment analysis of all of the differentially methylated sites identified in this study for their position in a gene or relative to a CpG island. Compared with all sites included in the 450K methylation array, differentially methylated sites were significantly enriched in intergenic and low-density CpG regions (Table 2). In the intragenic regions, differentially methylated sites were enriched in the gene body and depleted in promoters. In addition, 52% of the differentially methylated sites were in regions predicted to be functional enhancers as opposed to only 21% of all the CpGs included in the methylation array, indicating that differentially methylated sites are significantly ($P < 0.001$) enriched in enhancers.

Integrative analysis of DNA methylation and gene expression of transcription factors in normal and OA articular cartilage. To increase our functional understanding of the methylation changes observed in OA cartilage, we focused on transcription factors, because they act as master regulators of gene expression in eukaryotic cells. As shown in Supplementary Table 4 (available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39643/abstract), we identified 45 transcription factors that harbored at least 1 differentially methylated

Table 1. Most differentially hypomethylated and hypermethylated CpG sites in knee articular cartilage from normal donors and OA patients*

Illumina probe ID	Associated gene	$\Delta\beta$, normal vs. OA	P	P_{corr}
Hypomethylated in OA				
cg21465150	*TRPV3*	−0.45	1.96×10^{-4}	0.041057162
cg07371504	*TRPV3*	−0.43	2.18×10^{-4}	0.043347392
cg20092122	*BST2*	−0.40	1.26×10^{-6}	0.004419322
cg06880930	*CPNE2*	−0.34	3.06×10^{-6}	0.006008504
cg13030790	*RUNX1*	−0.33	2.35×10^{-5}	0.015638525
cg15591803	*DENND2D*	−0.31	1.32×10^{-7}	0.00200339
cg01675238	*JMJD7*	−0.31	4.34×10^{-6}	0.00688781
cg22714290	*LOC148696*	−0.30	5.86×10^{-6}	0.007902923
cg09966895	*ODZ4*	−0.30	6.13×10^{-7}	0.003304806
cg12273284	*CAMK1D*	−0.29	2.73×10^{-5}	0.016527922
cg03744842	*CCM2*	−0.29	3.31×10^{-6}	0.006090967
cg11854227	*ILDR1*	−0.28	6.16×10^{-7}	0.003250758
cg20054157	*SFTA1P*	−0.27	1.05×10^{-6}	0.004161572
cg00694560	*ILDR1*	−0.27	3.87×10^{-10}	6.27085×10^{-5}
cg21108085	*CD82*	−0.26	1.39×10^{-8}	0.000615655
cg25758828	*PAX8*	−0.26	5.12×10^{-5}	0.021732678
cg16707506	*CCM2*	−0.26	1.04×10^{-5}	0.01049113
cg07168232	*LAMB3*	−0.26	8.71×10^{-6}	0.009589979
cg19919590	*LAPTM5*	−0.25	9.11×10^{-6}	0.009808833
cg03357727	*ULK4*	−0.25	4.42×10^{-6}	0.006881195
Hypermethylated in OA				
cg03358468	*KDM4B*	0.44	3.22×10^{-7}	0.002562628
cg18536148	*TBX4*	0.39	2.60×10^{-6}	0.005864998
cg15517343	*NFATC1*	0.34	2.79×10^{-7}	0.00260294
cg14196395	*DYSF*	0.33	1.94×10^{-5}	0.014468514
cg01789499	*PRDM8*	0.33	9.45×10^{-6}	0.009995321
cg10890644	*TUBAL3*	0.32	1.10×10^{-4}	0.031613525
cg26819718	*C10orf11*	0.32	7.99×10^{-5}	0.027232593
cg24274579	*TBX4*	0.30	1.36×10^{-6}	0.004576905
cg03311556	*PRDM8*	0.30	3.07×10^{-6}	0.005983571
cg22079102	*KDM4B*	0.30	2.90×10^{-6}	0.005832481
cg13738327	*LRP5*	0.30	6.44×10^{-5}	0.024342721
cg14419393	*ASPSCR1*	0.30	9.20×10^{-5}	0.028974493
cg06365535	*TBX4*	0.30	1.19×10^{-7}	0.001863844
cg06440348	*PRDM8*	0.29	1.61×10^{-6}	0.004952817
cg01851968	*VASN*	0.29	9.97×10^{-5}	0.03017708
cg22902505	*PRDM8*	0.29	1.15×10^{-6}	0.00431253
cg07727358	*FGFRL1*	0.29	1.86×10^{-5}	0.01429792
cg14619259	*SPRY4*	0.29	4.73×10^{-5}	0.021123812
cg16536399	*NFATC1*	0.28	2.99×10^{-5}	0.017365227
cg06458239	*ZNF549*	0.28	5.73×10^{-8}	0.001210523

* OA = osteoarthritis; P_{corr} = corrected P.

site. Among these differentially methylated transcription factors, 31 were hypermethylated, and 14 were hypomethylated. Whereas some of the differentially methylated transcription factors, such as *RUNX2*, *NFATC1*, and *NCOR2*, had previously been associated with OA pathology (30–32), the majority of the differentially methylated transcription factors have not been studied in the context of cartilage biology or OA development.

Next, we compared the methylation data and the gene expression data to determine whether these differentially methylated transcription factors were also differentially transcribed. As shown in Table 3, only 10 differentially methylated transcription factors overlapped between both data sets. Of these, 7 were hypermethylated in OA cartilage whereas 3 were hypomethylated. Based on the CpG methylation status of these 10 differentially methylated transcription factors, the hierarchical clustering analysis was able to separate healthy and OA donors (Figure 2a). Interestingly, all of the differentially methylated transcription factors that were also differentially expressed were transcriptionally repressed in OA cartilage.

To investigate the putative functionality of the observed link between methylation and transcription, we selected those differentially methylated transcription factors with a negative association between methylation and transcription for further study, because they are more

Table 2. Enrichment analysis of DM sites for genomic features*

Genomic region	No. of DM sites	DM rate, %	No. of assay sites after filtering	Assay rate after filtering, %	P_{corr}	Status
Enhancer	487	52	93,053	21	2.20×10^{-16}	Enriched
No enhancer	442	48	353,903	79	2.20×10^{-16}	Depleted
Island	367	40	287,941	64	2.20×10^{-16}	Depleted
No island	562	60	159,015	36	2.20×10^{-16}	Enriched
Genic	653	70	4,336,801	75	4.50×10^{-4}	Depleted
Intergenic	276	30	110,155	25	4.50×10^{-4}	Enriched
Position within a gene						
TSS1500	55	6	62,471	14	6.27×10^{-15}	Depleted
TSS200	28	3	48,681	11	2.20×10^{-16}	Depleted
5′-UTR	91	10	38,707	9	NS	–
First exon	18	2	21,436	5	7.03×10^{-6}	Depleted
Body	418	45	149,763	34	4.62×10^{-13}	Enriched
3′-UTR	43	5	15,743	4	NS	–
Position within a CpG island						
N_shore	103	11	57,319	13	NS	–
N_shelf	48	5	22,449	5	NS	–
Island	94	10	143,319	32	2.20×10^{-16}	Depleted
S_shore	81	9	44,754	10	NS	–
S_shelf	41	4	20,100	4	NS	–

* DM = demethylation; P_{corr} = corrected P; TSS = transcription start site; 5′-UTR = 5′-untranslated region; NS = not significant; N_shore = north CpG island shore; N_shelf = north CpG island shelf; S_shore = south CpG island shore; S_shelf = south CpG island shelf.

likely to be epigenetically regulated in terms of gene transcription (11). These included *ATOH8*, *MAFF*, *NCOR2*, *NFATC1*, *TBX4*, *ZBTB16*, and *ZHX2*.

We first confirmed differential mRNA expression using qPCR analysis in an independent cohort of patients. As shown in Figure 2b, a significant reduction in mRNA levels was observed for *ATOH8*, *MAFF*, *NCOR2*, *TBX4*, *ZBTB16*, and *ZHX2* in OA samples when compared with control samples. Because no differences were observed for *NFATC1* expression levels, this gene was excluded from subsequent experiments.

Changes in gene expression following DNA demethylation in cultured articular chondrocytes. One of the limitations of previous studies is that the consequences of differential methylation on transcriptional regulation were not validated in an experimental setting. Therefore,

Table 3. Differentially expressed transcription factors harboring differentially methylated CpG in knee articular cartilage from normal donors and OA patients*

Methylation status in OA, gene name	CpG genomic features	Gene expression status in OA	Average FPKM		Log_2†
			Normal	OA	
Hypomethylated					
BCL6	5′-UTR	Down-regulated	268.837	113.73410	−1.24107
RORA	Body	Down-regulated	22.9409	10.1887	−1.17095
ZNF395	Body	Down-regulated	57.0915	16.3765	−1.80165
Hypermethylated					
ATOH8	Body, enhancer	Down-regulated	51.8614	6.58004	−2.97849
MAFF	Body	Down-regulated	26.6943	4.13804	−2.68951
NCOR2	5′-UTR, body, enhancer	Down-regulated	57.9958	24.1427	−1.26436
NFATC1	Body	Down-regulated	23.4487	9.02882	−1.3769
TBX4	TS200, TS500, 5′-UTR, body, 3′-UTR, enhancer	Down-regulated	49.741	22.6369	−1.13576
ZBTB16	Body	Down-regulated	32.8472	13.4769	−1.28528
ZHX2	5′-UTR, enhancer	Down-regulated	36.2553	11.9678	−1.59903

* OA = osteoarthritis; FPKM = fragments per kilobase of exon per million fragments mapped; 5′-UTR = 5′-untranslated region.
† Fold expression.

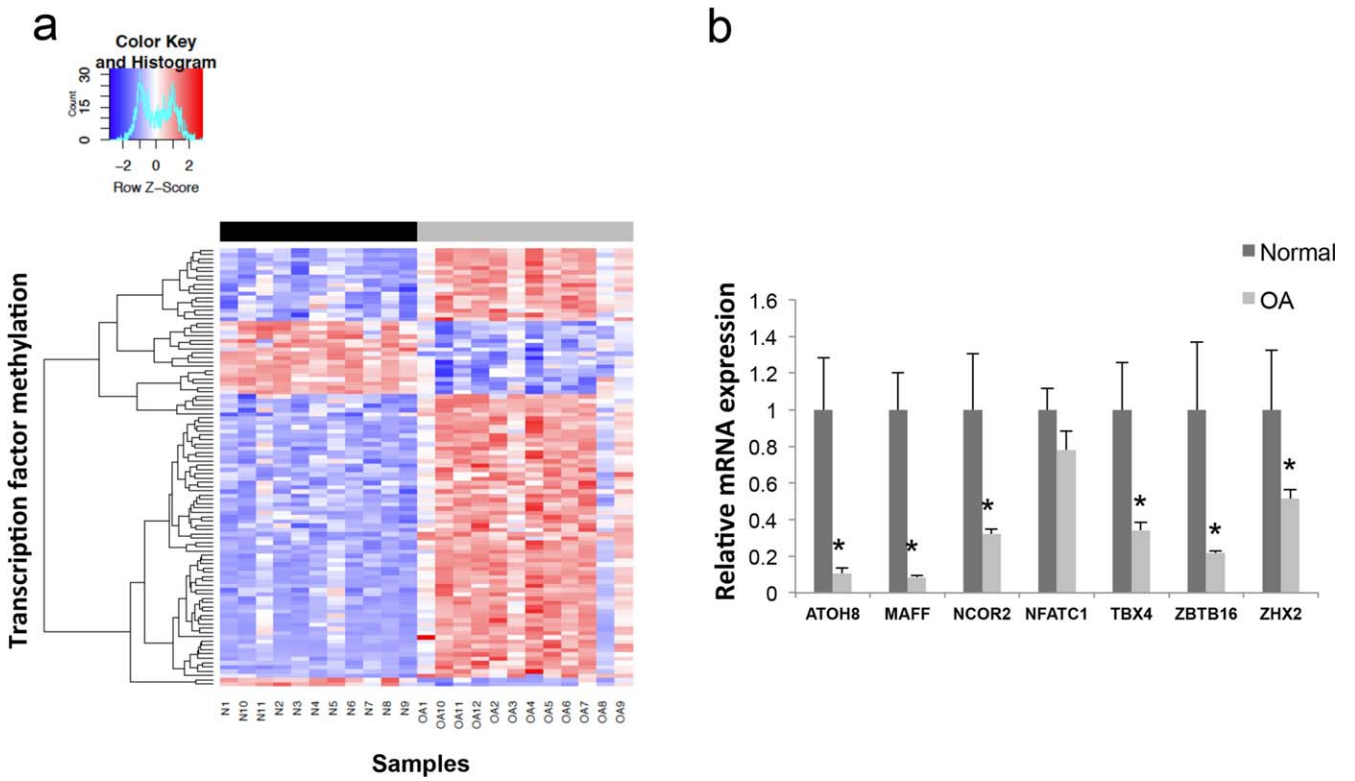

Figure 2. a, Heatmap of 102 differentially methylated CpG sites located in transcription factor genes that were differentially methylated in knee articular cartilage samples from normal subjects and patients with osteoarthritis (OA). Rows represent CpG sites and columns represent samples. Dendogram (top) shows clustering of the normal samples (solid bar) and the OA samples (shaded bar). **b,** Relative mRNA expression of selected transcription factors in knee articular cartilage samples from normal subjects (n = 13) and OA patients (n = 11), as assessed by real-time polymerase chain reaction. Values are the mean ± SEM. * = $P < 0.05$ versus normal. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/doi/10.1002/art.39643/abstract.

we tested the effects of the DNA methylation inhibitor 5-aza-dC on the expression of *ATOH8*, *MAFF*, *NCOR2*, *TBX4*, *ZBTB16*, and *ZHX2* in cultured human articular chondrocytes. We first treated TC28 cells with increasing concentrations of 5-aza-dC for 48 hours. There was a significant dose-dependent increase in mRNA levels for all of the genes tested (Figure 3a) except *ZBTB16*, which was not expressed under any condition (results not shown). *TBX4* showed the most striking differences in expression, being undetectable under basal conditions and strongly up-regulated by 5-aza-dC treatment.

Primary human articular chondrocytes isolated from healthy donors were treated with 5-aza-dC for 4–5 weeks to ensure an appropriate reduction in methylation, because these cells divide very slowly, and 5-aza-dC requires active cell division in order to effectively demethylate the DNA. As opposed to TC28 cells, primary articular chondrocytes expressed detectable levels of all transcription factors assessed in this experiment. Following 5-aza-dC treatment, *ATOH8* and *TBX4* showed significantly increased expression levels (Figure 3b).

DISCUSSION

The current study is the first to compare the DNA methylation profile of knee articular cartilage from 11 normal and 12 OA donors, using the most comprehensive methylation array available, the Illumina Infinium HumanMethylation450 array. We identified 929 differentially methylated sites that are associated with 500 unique genes (see Supplementary Table 2, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39643/abstract), revealing that healthy and OA knee articular cartilage exhibit substantially different methylomes. Furthermore, comparative analysis of methylation and gene expression allowed us to identify several hypermethylated transcription factors with reduced expression in OA cartilage.

The current study greatly expands the findings reported by Fernandez-Tajes et al (21), who observed 91 differentially methylated sites when comparing knee cartilage samples from normal and OA patients using the Illumina Infinium HumanMethylation27 array, thus

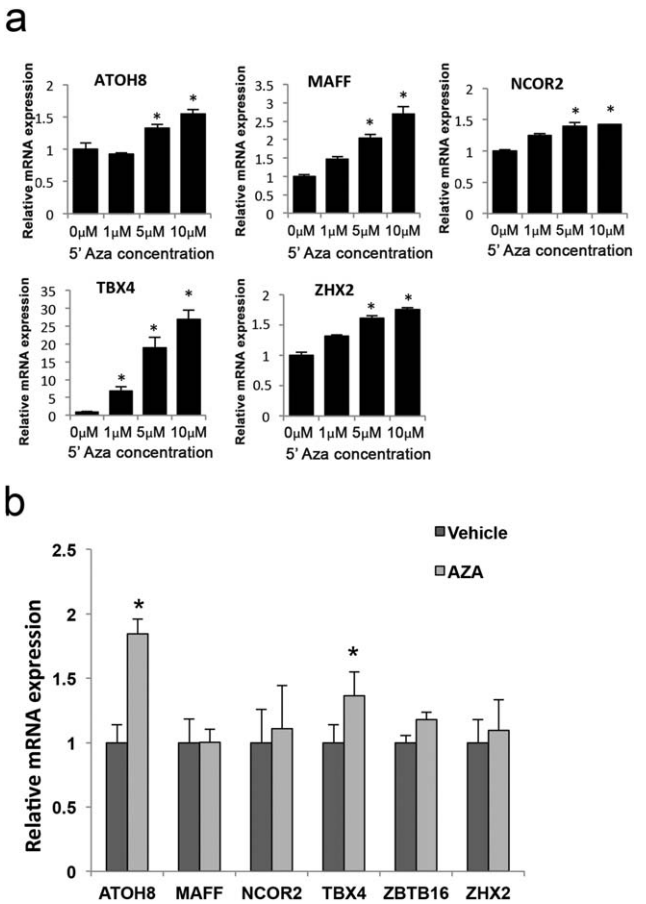

Figure 3. a, Relative mRNA expression of selected transcription factors in cultured immortalized human chondrocytes (TC28 cells) treated with different doses of the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC; 5′aza) for 48 hours. $* = P < 0.05$ versus 0 μ*M* 5-aza-dC. **b,** Relative mRNA expression of selected transcription factors in cultured primary human chondrocytes from normal donors (n = 5) treated with vehicle or 5-aza-dC (10 μ*M*) for 4 weeks. $* = P < 0.05$ versus vehicle. Values are the mean ± SEM.

offering broader insight into the epigenetic changes that occur during knee OA pathogenesis. We confirmed the methylation status of the most hypomethylated genes reported in that study, such as *RUNX1* and *KRT80*, as well as some of the hypermethylated genes, including *IGF2AS*, *SOCS1*, and *TBX4*. However, there is a substantial difference in the number of differentially methylated sites found in these 2 studies. This could be explained by differences in the array size and design, because the HumanMethylation27 array includes 27,000 CpG sites predominantly located in gene promoters, whereas the HumanMethylation450 array contains 480,000 probes within a broad range of genomic features, such as enhancers, promoters, untranslated regions, and gene bodies. In support of this notion, our enrichment analysis for specific genomic regions showed that differentially methylated sites are significantly depleted in promoters, particularly in those containing CpG islands (Table 2). This observation is in concordance with the finding that epigenetic modifications are more likely to occur in CpG-poor genomic regions such as enhancers (33,34).

Enhancer methylation is an active field of research, and it has been shown to negatively correlate with transcription factor binding, enhancer activity, and gene transcription (34–36). In this regard, we observed significant accumulation of differentially methylated sites in enhancer regions, both intergenic and intragenic; similar findings in hip articular cartilage have been reported (22,23), pointing to the possibility that specific genomic regions are more prone to undergo epigenetic changes during OA development.

An important finding initially reported by Fernandez-Tajes et al (21) and confirmed in more recent studies (22,23,37) is a cluster of OA patients with a specific methylation signature enriched for inflammatory genes. Although corroboration of these findings in our study would have been of interest, a limitation of our study is that we did not have an adequate number of samples to perform this kind of analysis. It should be also pointed out that DNA methylation can be influenced by age, sex, and BMI (38). The differences in DNA methylation observed in the present study were not attributable to sex or BMI as covariates. However, when the data were corrected for age, we did not observe any significant differences in CpG methylation. This could be attributable to the age difference between donors who provided control samples and those who provided OA samples as well as the relatively low number of samples used in the study. Therefore, a limitation of our study is that the differential DNA methylation observed can be driven by both OA and aging, and caution should be taken when interpreting these results in the context of OA. Further studies including larger numbers of samples are needed to identify age-related versus OA-associated changes in articular cartilage methylome.

In recent studies, hip and knee articular cartilage showed inherent differences in DNA methylation, suggesting a joint-specific epigenetic landscape (23,24). In fact, den Hollander and colleagues (24) reported differential clustering of hip and knee samples independently of disease status, prompting the notion that knee and hip OA have similar pathology but are driven by different epigenetic mechanisms. However, we found some commonalities when comparing our results with those observed in the above-mentioned studies. For example, a vast portion of the most differentially methylated genes (see Supplementary Table 5, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.

wiley.com/doi/10.1002/art.39643/abstract) and, in particular, the differentially methylated transcription factors identified in our study (see Supplementary Table 6) were also differentially methylated when comparing hip OA and disease-free samples (23). We therefore cannot rule out the possibility that, despite having substantial differences in the methylation landscape, knee and hip articular chondrocytes share key common epigenetically controlled genes that may play a pivotal role in tissue homeostasis and OA pathogenesis.

Genome-wide expression studies have shown that aberrant gene transcription in articular chondrocytes is a hallmark of OA (39,40). It has been repeatedly reported that several catabolic proteins, such as extracellular matrix proteases or proinflammatory factors, have increased expression in OA cartilage, whereas key anabolic genes and components of the autophagic machinery are repressed (for review, see refs. 41 and 42). These transcriptional changes are thought to ultimately compromise the ability of chondrocytes to sustain cartilage homeostasis. However, the molecular mechanisms driving these transcriptional alterations remain elusive.

Recent studies identified specific transcription factors that coordinate broad cellular processes during cartilage development and OA pathogenesis. For instance, hypoxia-inducible factor 2α has been proposed to be a key driver of the catabolic response observed during OA development by inducing the expression of several catabolic genes (43). On the other hand, *SOX9* is a pivotal factor in chondrogenesis that induces the expression of different chondrocyte differentiation markers, and that is strongly repressed during OA development (44,45). Nevertheless, identification of key transcription factors and the mechanisms controlling their expression is paramount for the development of new strategies to treat OA.

In the current study, using an integrative approach, we identified several transcription factors that are differentially methylated and repressed in OA cartilage (Table 3). Moreover, we experimentally validated that modulation of DNA methylation is able to regulate transcription of these factors in cultured human articular chondrocytes. Several of the transcription factors identified have not been studied in the context of OA pathophysiology, thus presenting new opportunities for future studies aimed at characterizing their function in articular cartilage and OA development. *TBX4* is a member of a phylogenetically conserved family of transcription factors that are involved in the regulation of developmental processes. *TBX4* regulates limb development and is required for muscle and tendon morphogenesis (46). In addition, mutations in *TBX4* cause small patella syndrome in humans (47). *ZBTB16*, also known as *ZNF145*, enhanced the expression of *SOX9*, suggesting that *ZNF145* acts as a factor upstream of *SOX9*, which is the master regulator of chondrogenesis (48).

Methylation is a dynamic process that has been linked to gene silencing. Despite active research in the field, it remains unclear whether methylation leads to gene silencing or whether gene silencing precedes DNA methylation (11). In human chondrocytes, there is mounting evidence that specific genes are transcriptionally controlled by epigenetic mechanisms, including *SOX9* (20), *GDF5* (49), *MMP13* (15), and *IL1B* (16). The mechanisms controlling site-targeted methylation and demethylation in cartilage are not known. Generally, the addition of methyl groups to cytosines is catalyzed by the DNA methyltransferase (DNMT) family of proteins, which is comprised of 3 members: DNMT-1, DNMT-3a, and DNMT-3b. Whereas decreased DNMT-1 expression and activity have been observed during aging (50), no significant changes in any DNMT were found in articular cartilage from normal and OA donors (16,51). On the other hand, the TET family mediates the addition of hydroxyl groups to a methylcytosine to form 5-hydroxymethylcytosine, eventually leading to active demethylation through different mechanisms (52). Reduced TET1 expression and altered 5-hydroxymethylcytosine levels have been reported in OA articular cartilage, in a process that is partially mediated by proinflammatory cytokines (53,54). In this regard, it is important to point out that the methodology used in the current study and other recent studies (21–24,37) does not allow us to discern between cytosine methylation and hydroxymethylation. Future studies should be aimed at unraveling the precise molecular mechanisms underlying the profound changes in methylation and hydroxymethylation that are observed in OA articular cartilage.

In summary, our results show that normal and OA knee articular cartilage can be distinguished by their DNA methylation profiles. We identified a number of differentially methylated CpG sites and provided the first description of differential methylation among transcription factors in normal and OA cartilage. Furthermore, we identified several hypermethylated transcription factors with reduced expression in OA cartilage and experimentally validated the epigenetic control of their gene expression in human chondrocytes. These findings suggest that methylation-related changes in several important transcription factors represent an important mechanism that may explain changes in chondrocyte transcriptome and function in OA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lotz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Alvarez-Garcia, Sasho, Lotz.
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