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DNA hypomethylation during MSC chondrogenesis occurs predominantly at enhancer regions

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Regulation of transcription occurs in a cell type specific manner orchestrated by epigenetic mechanisms including DNA methylation. Methylation changes may also play a key role in lineage specification during stem cell differentiation. To further our understanding of epigenetic regulation in chondrocytes we characterised the DNA methylation changes during chondrogenesis of mesenchymal stem cells (MSCs) by Infinium 450 K methylation array. Significant DNA hypomethylation was identified during chondrogenic differentiation including changes at many key cartilage gene loci. Integration with chondrogenesis gene expression data revealed an enrichment of significant CpGs in upregulated genes, while characterisation of significant CpG loci indicated their predominant localisation to enhancer regions. Comparison with methylation profiles of other tissues, including healthy and diseased adult cartilage, identified chondrocyte-specific regions of hypomethylation and the overlap with differentially methylated CpGs in osteoarthritis. Taken together we have associated DNA methylation levels with the chondrocyte phenotype. The consequences of which has potential to improve cartilage generation for tissue engineering purposes and also to provide context for observed methylation changes in cartilage diseases such as osteoarthritis.

Our skeleton acts as an essential framework for the overall structure of our body. Accurate generation of this frame is dependent upon carefully controlled chondrocyte differentiation and cartilage formation, processes that underpin the development of the long and short bones of the skeleton¹. Chondrocytes, the sole cartilage cell type, develop from condensations of mesenchymal progenitor cells in a process known as chondrogenesis². During chondrogenesis progenitors commit to a tightly coordinated chondrocyte differentiation program determined by temporal and spatial expression of multiple growth factors and transcription factors³.

Epigenetic mechanisms, such as DNA methylation and histone modifications, provide cell-type specific regulation of gene expression essential for differentiation and maintenance of cell phenotype⁴. DNA methylation is a reversible process catalysed by DNA methyltransferases (DNMTs) on the fifth carbon of cytosine residues at CpG dinucleotides to form 5-methylcytosine (5mC)⁵. DNA methylation at gene promoter or enhancer sequences is frequently associated with gene repression where it correlates with the presence of inhibitory histone modifications and prevents the binding of transcription factors⁶. Loss of DNA methylation occurs passively during cell replication or by conversion of 5mC back to cytosine via oxidised intermediates with the assistance of ten eleven translocation (TET) proteins⁷.

During development and cell differentiation DNA methylation is dynamic, correlating with changes in gene expression^{8,9}. The role, whereabouts and dynamics of DNA methylation during chondrogenesis remains poorly understood. In chondrocytes the regulation of genes such as MMP13, IL1, iNOS, chondromodulin, collagen 9 and GDF5 is influenced by DNA methylation at specific CpGs¹⁰⁻¹⁵. Similarly DNA methylation may regulate gene expression during chondrogenesis where COL10A1 induction correlates with its promoter demethylation, and interference with DNA methylation during chondrogenesis by treatment with 5-aza-C alters gene expression^{16,17}.

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While addition of methylation by DNMT3A has been found to regulate SOX9 expression in limb bud mesenchymal cells, and by DNMT3B to regulate cartilage metabolism and homeostasis^{18,19}.

At the genome-wide level histone modifications are regulated during chondrogenesis and found to correlate with gene expression²⁰. In contrast DNA methylation at gene promoters, assessed by reduced representation bisulfite sequencing (RBBS), did not correlate with gene expression²⁰. Utilising the Infinium 450 K methylation array the effect of ageing on cartilage DNA methylation and the similarity between MSC-derived cartilage methylation in comparison with cartilage engineered from articular chondrocytes have been studied^{21,22}.

Herein we focus directly on the differentially methylated CpGs during chondrogenesis in order to better define the chondrocyte methylome. Methylation changes are contrasted with gene expression to infer causality, while chromatin state information is integrated in order to further characterise regions of dynamic methylation. Further, a chondrocyte-specific methylation profile is established by comparison with cartilage and non-cartilage tissue methylation profiles, and the alterations in DNA methylation in osteoarthritis correlated. Interpretation of the epigenetic changes during chondrogenesis can provide context for those seen during deterioration of chondrocyte function in diseases such as osteoarthritis and also improve understanding of the processes governing differentiation of MSCs into chondrocytes for tissue regeneration purposes.

Results

Human MSCs were differentiated into chondrocytes by culture in chondrogenic differentiation medium for 14 days in a scaffold-free Transwell insert. A cartilage disc is formed with a homogenous extracellular matrix and concomitant rapid upregulation of chondrocyte gene expression indicative of differentiation, which is highly reproducible between different MSC donors^{23,24}. In order to identify CpG methylation changes during MSC chondrogenic differentiation a DNA methylation profile was generated with the Infinium HumanMethylation450 BeadChip. There was no change in median methylation level of all CpG sites between MSCs (Day0) and MSC-derived chondrocytes (Day14). However, differential methylation analysis identified 5950 differentially methylated CpG loci (DMLs) with a significant, greater than 10%, change in methylation level at Day14 compared with Day0 (Fig. 1A and Supplementary Table 1). The vast majority of these (5802 DMLs) become hypomethylated during chondrogenesis, and the extent of methylation change is greater for CpGs becoming hypomethylated compared to hypermethylated (Fig. 1B). A number of DMLs are found at key cartilage gene loci such as ACAN and SOX9 (Fig. 1C,D). 12 of the DMLs associated with genes exhibiting significant expression changes during chondrogenesis were selected for pyrosequencing analysis in an independent chondrogenic differentiation of MSCs (Supplementary Fig. 1)²³. Significant hypomethylation was confirmed for CpGs at all the associated genes, besides ACAN owing to the absence of hypomethylation in one MSC donor. Despite the variation in donor methylation the extent of methylation change per CpG during chondrogenesis are moderately correlated between the different MSC donors (Supplementary Fig. 2).

Grouping of DMLs in differentially methylated regions (DMRs) are considered an indication of regions with the potential to regulate gene transcription²⁵. We defined DMRs as regions containing >1 differentially methylated CpG with a maximum separation of 1000 bp. 1276 DMRs are found during MSC chondrogenesis (Supplementary Table 2), including a DMR found at the promoter of SNORC (C2orf82), the transcript of which we found previously as the most upregulated during MSC chondrogenesis, and a DMR in WWP2 a key cartilage development protein also upregulated during chondrogenesis (Fig. 1E,F)^{23,26}. GO term analysis of the genes with which these DMLs and DMRs are associated identifies terms consistent with the differentiation of cells into chondrocytes (Fig. 1G,H).

We previously identified expression changes in greater than 2000 genes during MSC chondrogenesis²³. Intersection of DNA methylation changes with gene expression changes identifies DMLs at 25% of upregulated genes and 15% of downregulated genes (Fig. 2A). There is a greater enrichment of DMLs in the more upregulated genes (Fig. 2B).

The context of the DMLs was further examined by examination of genomic features and chromatin states. In comparison with the distribution of probes across gene features in the array there is an enrichment of DMLs in gene body and intergenic regions (Fig. 3A). These DMLs are also less likely to be found in or around CpG island areas, where CpG islands are >200 bp in length with an observed/expected CpG ratio >60%. The hypomethylation occurs at all genomic features and locations, with shore CpGs up to 2 kb from a CpG island, shelf CpGs 2–4 kb, and open sea CpGs elsewhere in the genome (Fig. 3B,C). A 15 chromatin state model of MSC-derived chondrocytes has previously been generated from the integration of multiple histone mark ChIP-seq data as part of the Roadmap Epigenomics project²⁰. CpG methylation levels during chondrogenesis were intersected with five functional categories of chromatin states from MSC-derived chondrocytes. Hypomethylation at DMLs was found at all chromatin states but was most extensive (>25% for the enhancer state (Fig. 3D). Taking all CpGs into account an empirical cumulative distribution frequency plot showed that the enhancer state exhibited the greatest hypomethylation during chondrogenesis (Fig. 3E). Assessment of the distribution of DMLs across chromatin state categories indicates that a large proportion are found at enhancer states (Fig. 3F).

We previously identified DNA methylation levels in normal human hip chondrocytes from neck of femur fracture patients (GSE63695)²⁷. The final methylation level of DMLs during MSC chondrogenesis were compared with CpG methylation levels in healthy cartilage (NOF) and a number of other tissues with methylation data available from TCGA (<https://cancergenome.nih.gov/>). The level of methylation at DMLs at Day14 is more similar to the level of methylation in human articular chondrocytes (NOF) than Day0 (Fig. 4A). In other tissues these DMLs have a higher level of methylation indicating a chondrocyte specific demethylation during chondrogenesis. For example DMLs at the CD109 and VGLL4 gene loci are methylated at levels similar to the other non-cartilage tissues at Day 0 but following chondrogenesis the methylation level is reduced to those found in articular chondrocytes (Fig. 4B,C). To further characterise the chondrocyte DNA methylome tissue-specific methylation markers were sought. 1464 CpGs were identified with an average methylation beta value < 0.5 in chondrocytes and

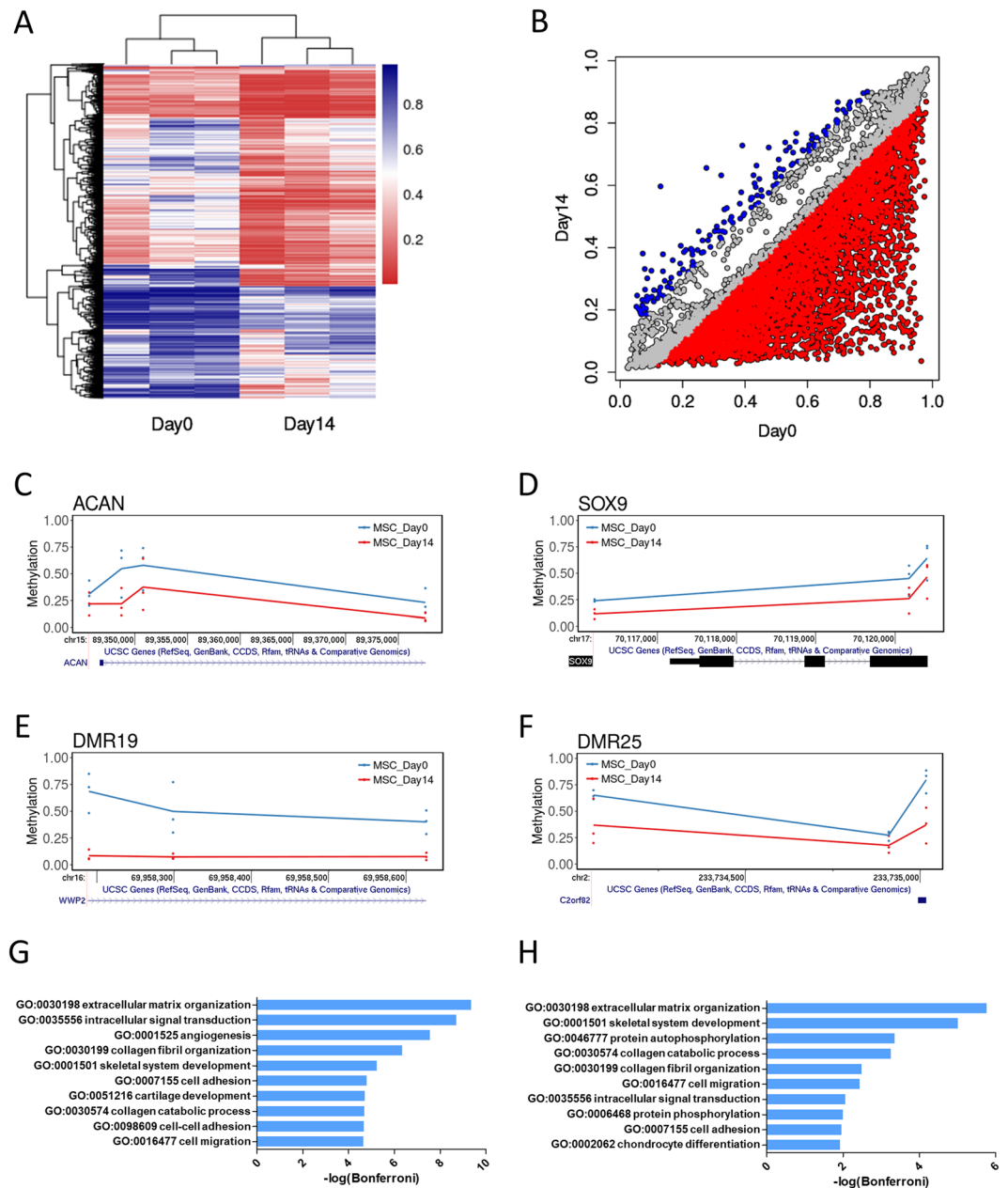


Figure 1. Differentially methylated CpGs during MSC chondrogenesis. **(A)** Heatmap of significant CpGs in MSC Day0 and Day14. **(B)** Correlation plot of significant CpGs Beta values at Day0 and Day14. Red indicates >10% hypomethylation, blue >10% hypermethylation at Day14. **(C–F)** Genomic vignette of significant CpGs at **(C)** ACAN and **(D)** SOX9 loci; or significant DMRs **(E)** DMR19 at WWP2 and **(F)** DMR25 at C2orf82 loci. Each donor is represented by a single dot and the average methylation for each timepoint represented by a line. Blue for MSC Day0, red for MSC Day14. **(G,H)** GO term analysis of **(G)** DML and **(H)** DMR genes.

>0.8 in >90% of tissues (Fig. 4D). These CpGs are more highly methylated in MSCs than cartilage, both before and after chondrogenesis. The COL11A2 and microRNA miR-140 gene loci include a number of CpGs exhibiting hypomethylation only in chondrocytes (Fig. 4E,F).

Changes in cartilage during OA may reflect a reinitiation of developmental pathways in the chondrocyte or a loss of chondrocyte phenotype due to disrupted epigenetic regulation^{28,29}. Previously we identified DMLs between the hip cartilage of OA patients and healthy controls²⁷. A comparison between CpGs altered in OA hip cartilage and MSC chondrogenesis indicated a small, but greater than expected, proportion (~10%, hypergeometric distribution $p < 0.001$) common to both (Fig. 5A and Supplementary Table 3). For these 481 common CpG there is a reduction in methylation during OA consistent with the changes during chondrogenesis (Fig. 5B). This is in contrast to the increase in mean methylation of all OA DMLs (Fig. 5C). In comparison with the methylation of other tissues many CpGs altered in OA are found at a lower level of methylation in chondrocytes (Fig. 5C).

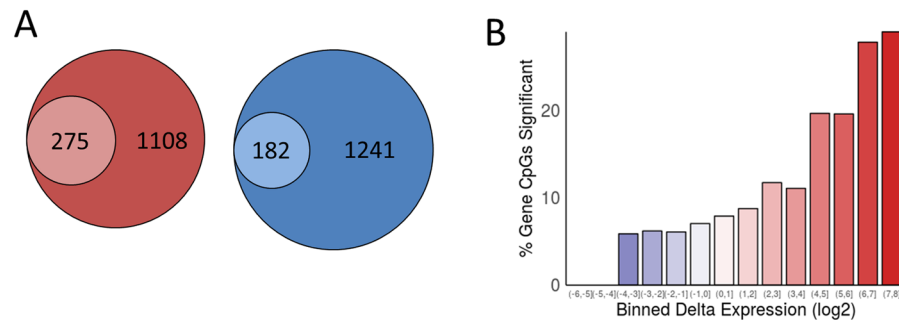


Figure 2. Comparison between methylation changes and gene expression changes. **(A)** Proportion of differentially expressed genes during MSC chondrogenesis with methylation changes. Outer circle represents total 2fold up- (red) or down- (blue) regulated gene number in chondrogenesis. Inner circle represents number of genes with at least 1 significant CpG. **(B)** Histogram showing enrichment of significant CpGs in comparison to direction and magnitude of binned log₂ gene expression changes.

Discussion

DNA methylation establishes and reinforces cell-specific gene expression patterns during development and cell differentiation⁹. Here we show that DNA methylation changes, predominantly in the form of hypomethylation, are also a characteristic of differentiation of MSCs into chondrocytes during cartilage formation. These DMLs are enriched at enhancer regions consistent with the key role of cell-type specific enhancers in regulating gene expression. During MSC chondrogenesis the methylation level at significant CpGs becomes closer to the level found in articular chondrocytes, however significant differences remain between MSC-derived chondrocytes and adult chondrocytes.

By RBBS only limited changes in promoter methylation were identified during MSC chondrogenesis in a 3D alginate model²⁰, but more recently by Infinium 450 K methylation array 1116 differentially methylated CpGs were identified across a timecourse of chondrogenic differentiation in a pellet model, albeit starting at Day14 (to Day49) in contrast to our comparison of undifferentiated vs. differentiated cells (Day0 vs. Day14)²¹. The vast majority of differentially methylated CpGs during chondrogenesis herein become hypomethylated indicative of a loss of repression at these locations. Although the impact of a defined percentage change in methylation will differ significantly on a CpG-specific basis there are clear inverse correlations between the extent of methylation and the level of gene expression^{30,31}. Epigenome editing has also confirmed the direct effect of DNA methylation at selected CpGs on gene expression^{30,32}. Transcription factor binding can cause local demethylation and thus the activation of the chondrocyte gene expression program might actively cause the loss of methylation³³. Generally global levels of DNA methylation increase during differentiation, though this can vary in a lineage-specific manner^{31,34,35}. At the gene level the shutdown of pluripotency genes occurs with increased methylation but the expression of lineage-specific genes is associated with hypomethylation^{31,34,35}.

Interpretation of the genomic landscape and chromatin states harbouring significant CpGs indicated their enrichment in non-CpG rich enhancer regions of the genome. Consistent with the open-sea localisation of significant CpGs enhancers are mostly CpG-poor with variable methylation while in contrast 75% of promoters are within CpG islands with low methylation³³. Hundreds of thousands of enhancers exist in the human genome, potentially contributing the major function of non-coding DNA regions³⁶. These cis-regulatory regions can drive transcription over long distance from their target gene, by for example forming chromatin loops, and offer both cell- and developmental-stage type specific regulation of gene expression³⁷. Cooperative action of transcription factors at enhancers and promoters facilitates chromatin access and activates gene expression³⁷. DNA methylation and DMRs at enhancers have been shown to correspond to enhancer activity of differentiation and cell-type specific genes⁸. Numerous enhancers play key roles in regulating chondrocyte specific gene expression and skeletal development³⁸. In particular both the regulation of SOX9 expression and the regulation by SOX9 are mediated by well-established enhancers^{39,40}. During chondrogenesis the regulation of enhancer-associated H3K4me1 or H3K27ac modifications at distal cis-regulatory elements, up to 50 kb from transcription start sites, correlates with gene expression suggesting that enhancer regions regulate many upregulated genes²⁰.

DMLs are enriched in genes associated with cartilage development GO terms, and grouping genes by the extent of their expression change during chondrogenesis indicated that the most upregulated genes contain more DMLs. However, many of the genes undergoing expression changes have no associated DNA methylation changes, and downregulated genes were also subject to hypomethylation. Consistent with this, hydroxymethylated cytosine (5hmC), a pathway intermediate generated during active demethylation, is found enriched at regulatory regions of genes during ATDC5 cell chondrogenic differentiation, although only a subset corresponded with changes in gene expression⁴¹. A number of factors may contribute to this disparity including the baseline methylation level and chromatin state, the particular transcription factors regulating expression, as well as the genomic location of CpGs on array. Interestingly the converse is also true whereby binding of transcription factors to promoters even in the absence of active transcription can cause the local loss of DNA methylation³³. To better establish correlation whole genome methylation in combination with chondrocyte ATAC-seq data and gene expression would highlight the possible overlap of methylation and transcription factor binding sites⁴².

The disparity between MSC-derived cartilage and endogenous cartilage are well established owing to differences in lineage and developmental environment of the cells⁴³. At the epigenetic level the DNA methylation

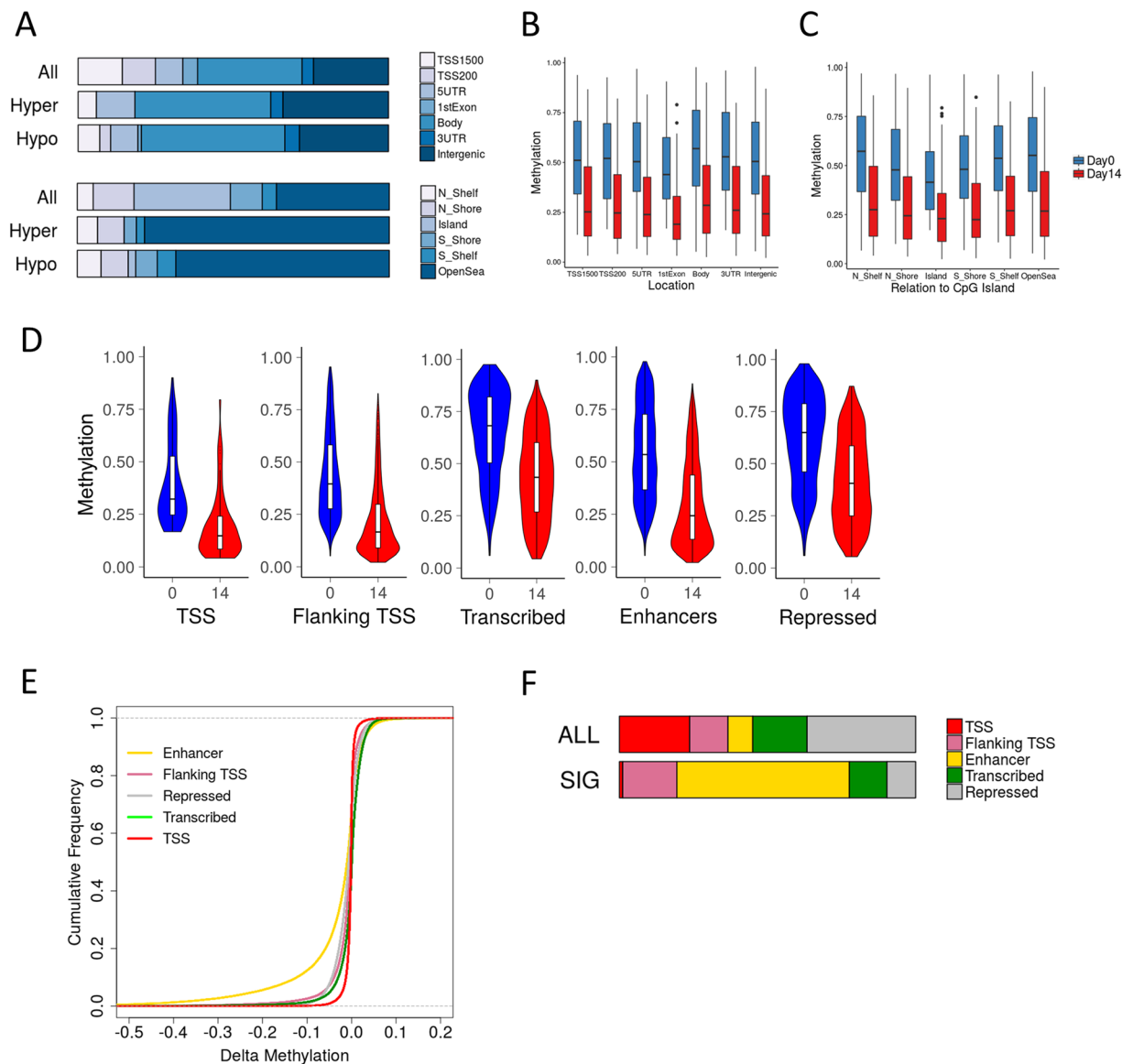


Figure 3. Genomic features and chromatin state of DMLs. **(A)** Proportion of significant hypo- and hyper-methylated CpGs overlapping gene and CpG island features. The distribution of all CpG probes on the array are presented for comparison. **(B,C)** Average methylation of significant hypo- and hyper-methylated CpGs overlapping **(B)** gene and **(C)** CpG island features in MSC Day0 and Day14. **(D–F)** Roadmap Epigenomics project MSC-derived chondrocyte-specific chromatin states were collapsed into five functional categories: TSS, Flanking TSS, Transcribed, Enhancers and Repressed. **(D)** Average methylation of DMLs overlapping the five chromatin state categories in MSC Day0 and Day14. **(E)** Cumulative frequency plot of methylation change at all CpGs during MSC chondrogenesis at each chromatin state category. **(F)** Proportion of DMLs overlapping chromatin state categories. The distribution of all CpG probes on the array are presented for comparison.

profiles are also quite distinct²¹, however at many DMLs in chondrogenesis we identify a reduction in methylation level to the level found in adult articular chondrocytes, validating the utility of the MSC chondrogenesis model for chondrocyte differentiation and supporting the association of these loci with the chondrocyte phenotype. Notably these CpGs are found highly methylated in other tissues supporting the chondrogenesis-specific nature of the methylation changes. In particular the region containing WWP2 and miR-140 undergoes extensive demethylation during chondrogenesis and is found highly methylated in all other tissues further cementing the cartilage-specific function of these genes^{26,44}. Genes with unexplored but possible functions in cartilage were also found hypomethylated in cartilage including CD109, a TGF- β co-receptor, and VGLL4 a regulator of Wnt signalling^{45,46}. Previously methylation differences were also identified between MSC-derived cartilage from young and old donors however it is unclear whether these differences are due to baseline differences in the MSCs or as a result of differences in the differentiation capacity of the donors²².

Providing context for the changes in OA we found a small overlap between methylation changes in MSC chondrogenesis and those CpGs altered in OA, with these DMLs again enriched in enhancers²⁷. Our data indicates that

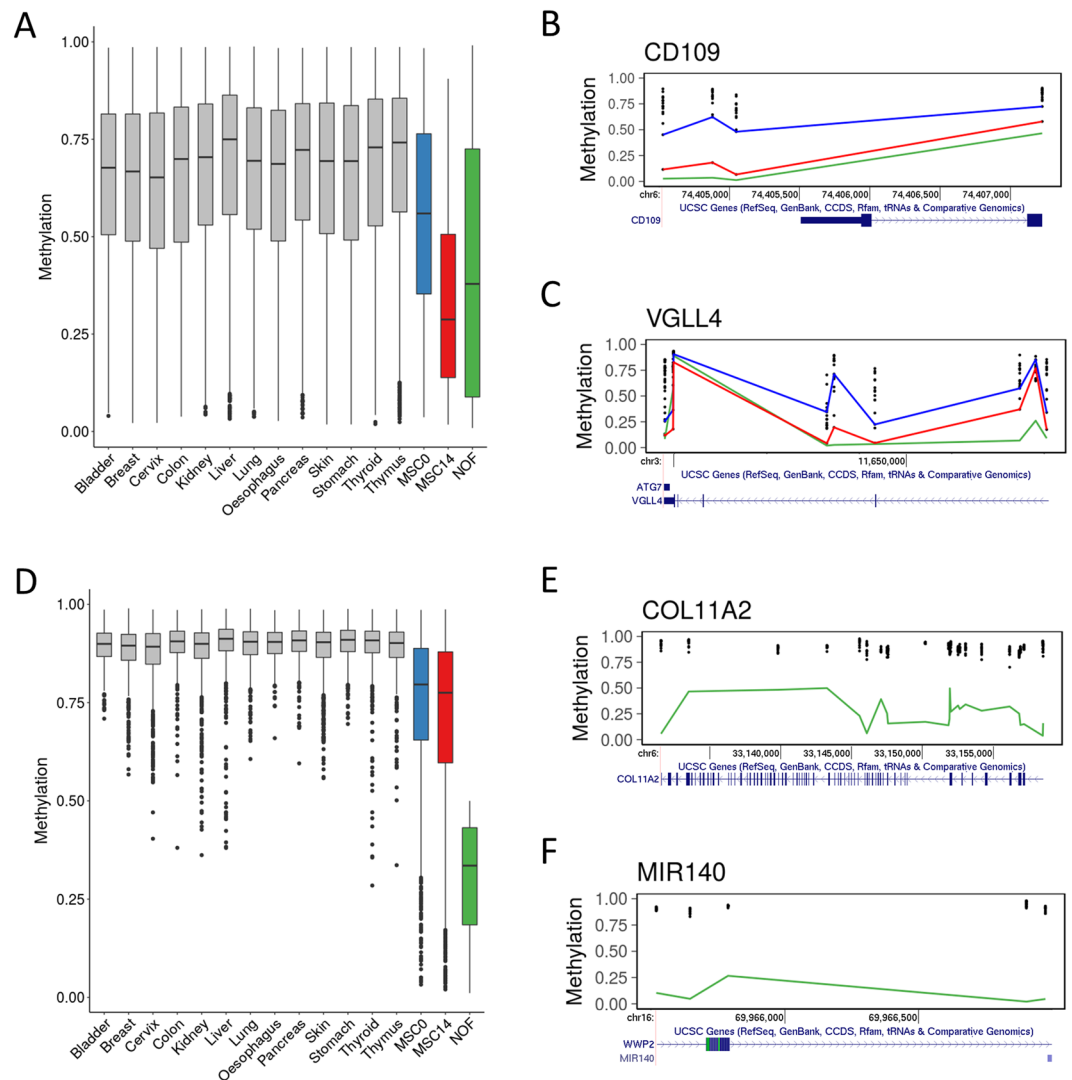


Figure 4. Methylation in chondrogenesis compared with human articular chondrocytes and other tissues. Mean methylation levels at Day0 and Day14, in human articular chondrocytes from neck of femur fracture patients (NOF), and in normal samples from a number of TCGA tissues were quantile normalised. **(A)** Average methylation of significant CpGs during MSC chondrogenesis in comparison with NOF and other tissues. **(B,C)** Genomic vignette of significant CpGs at **(B)** CD109 and **(C)** VGLL4 loci. **(D)** Methylation level of ‘cartilage-specific’ hypomethylated CpGs in NOF compared with other tissues. **(E,F)** Genomic vignette of ‘cartilage-specific’ hypomethylated CpGs at **(E)** COL11A2 and **(F)** MIR140 loci. **(B,C,E,F)** Each tissue is represented by a single dot. Lines indicate the methylation in MSC Day0 (blue), MSC Day14 (red), and NOF (green).

where there was previously no evidence of methylation changes in gene promoters during OA, such as ACAN and p21WAF1/CIP1, that dynamic methylation at enhancers regulating such genes should be considered^{47,48}. A number of studies have also identified DNA methylation changes in OA or damaged cartilage but it remains to be determined whether these correspond to enhancer regions^{49–55}. The subset of chondrogenesis DMLs also significantly altered in OA become hypomethylated consistent with the cells reactivating developmental pathways^{28,29}. However the majority of CpGs in OA become hypermethylated and more similar to the level in other cell types indicative of a loss of chondrocyte identity. Synthesis of all OA DNA methylation studies by meta-analysis could provide a comprehensive catalogue of DMLs altered in disease for future comparison.

Despite the modest number of MSC donors used in this study our array analysis and pyrosequencing validation identifies consistent hypomethylation in the donors, although the extent of demethylation can vary significantly. Both male and female donors were used in this study, but sex chromosome CpGs are filtered from the analysis and we have not observed any sex specific differences in chondrocyte methylation previously²⁷. We endeavoured to use MSC at low passage number and young donor age both for optimal chondrogenic differentiation and to limit the impact of epigenetic drift on the MSCs⁵⁶. Many tissues and cell types experience an increase in methylation with age at a subset of CpGs^{30,57}. Accordingly, the impact of age disparity between young MSC donors and aged NOF adult cartilage donors should be considered.

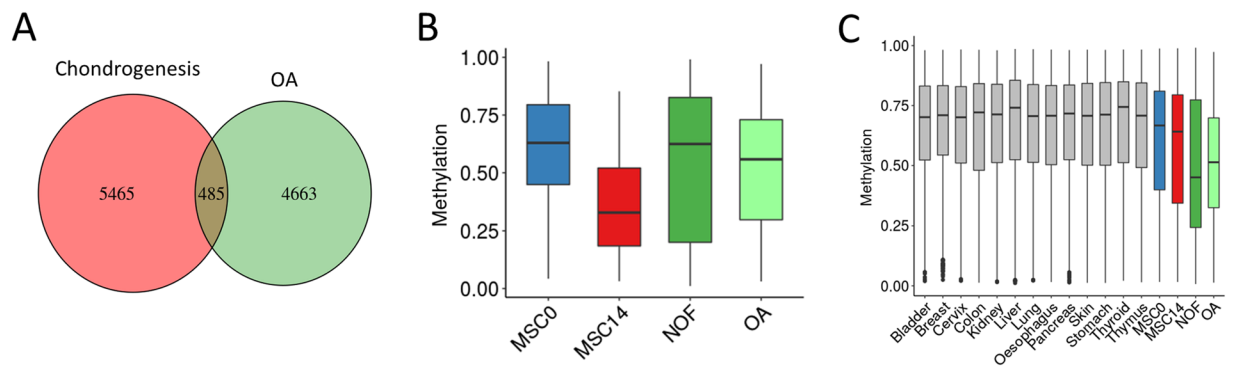


Figure 5. Overlap between DMLs in chondrogenesis and OA. **(A)** Overlap between differentially methylated CpGs in MSC chondrogenesis and in hip OA. **(B)** Average methylation of CpGs common to chondrogenesis and OA. **(C)** Average methylation of all significantly differentially methylated CpGs in OA compared with other tissues.

In conclusion, considerable demethylation changes to the epigenetic landscape occur during MSC chondrogenesis especially at sites marked by enhancer modifications. Comparison with other tissues, including healthy and OA cartilage, associates CpGs to the chondrocyte phenotype and provides context for changes in disease.

Experimental Procedures

Human bone marrow stem cell culture. Human bone marrow mesenchymal stem cells (MSC) (from four donors, 18–25 years of age) were isolated from human bone marrow mononuclear cells (Lonza Biosciences, Berkshire, UK) by adherence over 24 hours to tissue culture plastic and were expanded in monolayer culture in Mesenchymal Stem Cell Growth Medium (Lonza) supplemented with 5 ng/ml fibroblast growth factor-2 (R&D Systems, Abingdon, UK). Cultures were maintained in a humid atmosphere of 5% CO₂/95% air at 37°C. Once cells reached confluence, they were passaged (P1) using Trypsin/EDTA at a split ratio of 1:3. Experiments were performed using cells between P2–P7. The phenotypes of all MSC donors were tested previously²³.

Chondrogenic differentiation. MSC were resuspended in chondrogenic culture medium consisting of high glucose DMEM containing 100 µg/ml sodium pyruvate (Lonza), 10 ng/ml TGF-β3 (PeproTech, London, UK), 100 nM dexamethasone, 1x ITS-1 premix, 40 µg/ml proline, and 25 µg/ml ascorbate-2-phosphate (all from Sigma-Aldrich, Poole, UK). 5 × 10⁵ MSC in 100 µl medium were pipetted onto 6.5 mm diameter, 0.4-µm pore size polycarbonate Transwell filters (Merck Millipore, Watford, UK), centrifuged in a 24-well plate (200 g, 5 minutes), then 0.5 ml of chondrogenic culture medium added to the lower well as described²⁴. Media were replaced every 2 or 3 days up to 14 days. Donor 1 chondrogenesis was performed in triplicate yielding an R² > 0.997 between the replicates. Consequently, Donor 2 and Donor 4 were measured in singlicate. Donor 3 provided an additional baseline Day0 sample. Further sample information is provided in Supplementary Table 4.

DNA isolation, bisulphite treatment and methylation array. Genomic DNA was extracted from cells prior to the induction of chondrogenesis (Day0) and from Day14 cartilagenous discs by disruption in Invitrogen PureLink Genomic Digestion Buffer (Life Technologies, Paisley, UK) using a small disposable plastic pestle and an aliquot of Molecular Grinding Resin (G-Biosciences, St. Louis, MO) followed by proteinase K digestion for 1 hour at 37°C then nucleic acid purification with Invitrogen PureLink Genomic DNA Kit according to manufacturer's instructions (Life Technologies). 1 µg of genomic DNA was bisulphite converted using the EpiTect Bisulfite Kit (Qiagen, Manchester, UK). DNA methylation profiling of the samples was carried out by Cambridge Genomic Services (Cambridge, UK), using the Illumina Infinium HumanMethylation450 Beadchip array (Illumina Inc., San Diego, USA).

Analysis of infinium 450K methylation data. Methylation analysis was performed using the R/Bioconductor package Minfi. Raw IDAT files were read and preprocessed and probes were filtered for high detection p-value ($P > 0.05$), location on sex chromosomes and potential SNP contamination. Array normalization was carried out using the preprocessFunnorm function to generate M values for statistical testing and Beta values between 0 and 1, with 0 being unmethylated and 1 fully methylated, indicative of the percentage CpG methylation per probe. Differentially methylated probes were identified by fitting a paired linear model followed by statistical analysis using an empirical Bayes method (R/Bioconductor package Limma) then filtered by significance threshold ($P < 0.05$, F-test, after correction for multiple testing using the Benjamini–Hochberg method). Annotation of probes was performed with R/Bioconductor package Illumina HumanMethylation450kdb, with mapping to the hg19 genome build. Enriched gene ontology (GO) pathways were identified by interrogation of the annotated genes with significant CpGs with DAVID⁵⁸. Differentially methylated regions (DMRs) were identified by R/Bioconductor package DMRcate. DMRs are defined as regions containing >1 differentially methylated CpG with a maximum separation of 1000 bp.

Integration with gene expression. Gene expression was profiled by Illumina whole-genome expression array for Day0 and Day14 MSC chondrogenesis triplicate biological samples and expression analysis was

performed in the R/Bioconductor Limma package²³. For genes with multiple probes gene expression values were averaged then intersected with DNA methylation Beta values associated with gene loci. Correlation of significant CpGs with gene expression was performed on binned log₂ chondrogenesis gene expression changes to identify the percentage CpGs at gene loci with methylation changes.

Chromatin states. To assign MSC-derived chondrocyte chromatin states to probes, we downloaded the NIH Roadmap Epigenomics Mapping Consortium E049 15-state chromHMM model and mapped probes based on their locations in the genome⁵⁹ (<http://www.roadmapepigenomics.org/>). Roadmap chromatin states were collapsed into five functional states: Transcription Start Site (TssA, TssBiv), Flanking Transcription Start Site (TssAFlnk, BivFlnk), Transcribed (Tx, TxFlnk, TxWk), Enhancer (Enh, EnhG, EnhBiv), and Repressed (ZNF/Rpts, Het, ReprPC, ReprPCWk, Quies). All plots were generated using the ggplot2 package in R.

Comparison with articular chondrocyte and TCGA normal tissue 450 K methylation data. Publicly available 450 K DNA methylation profiles were downloaded for human articular cartilage neck of femur fracture control (NOF) and osteoarthritic diseased (OA) (GEO GSE63695) and from The Cancer Genome Atlas (TCGA) for non-cartilage tissue healthy control samples (<https://cancergenome.nih.gov/>)²⁷. All tissue (cartilage, TCGA and Day0/14 MSC chondrogenesis) Beta values underwent quantile normalisation. Chondrocyte-specific hypomethylated CpGs were defined as CpG sites with an average methylation beta value < 0.5 in NOF cartilage and > 0.8 in > 90% of TCGA non-cartilage tissues.

Pyrosequencing. DNA was isolated from Day0 MSCs and Day14 cartilagenous discs as above then 500 ng bisulphite converted with the EZ DNA Methylation Kit (Zymo Research, California, USA) following the manufacturer's instructions. Pyrosequencing PCR was performed using the PyroMark PCR Kit (Qiagen) in a 20 µl reaction volume in duplicate with 1 µl bisulphite-converted DNA following the manufacturer instructions. Pyrosequencing assays were designed using PyroMark assay design software 2.0 (Qiagen), and the sequencing was performed using a PyroMark Q24 Advanced platform (Qiagen) with the recommended kit, following the instructions of the manufacturer and as previously detailed⁶⁰. Sequences of forward and biotinylated-reverse PCR primers and sequencing primers are listed in Supplementary Table 5.

Data availability

MSC chondrogenesis 450 K methylation data is deposited at GEO (GSE129266).

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References

- Berendsen, A. D. & Olsen, B. R. Bone development. *Bone* **80**, 14–18, <https://doi.org/10.1016/j.bone.2015.04.035> (2015).
- Onyekwelu, I., Goldring, M. B. & Hidaka, C. Chondrogenesis, joint formation, and articular cartilage regeneration. *J. Cell Biochem.* **107**, 383–392, <https://doi.org/10.1002/jcb.22149> (2009).
- Akiyama, H. Control of chondrogenesis by the transcription factor Sox9. *Mod. Rheumatol. / Jpn. Rheumatism Assoc.* **18**, 213–219, <https://doi.org/10.1007/s10165-008-0048-x> (2008).
- Bernstein, B. E., Meissner, A. & Lander, E. S. The mammalian epigenome. *Cell* **128**, 669–681, <https://doi.org/10.1016/j.cell.2007.01.033> (2007).
- Luo, C., Hajkova, P. & Ecker, J. R. Dynamic DNA methylation: In the right place at the right time. *Sci.* **361**, 1336–1340, <https://doi.org/10.1126/science.aat6806> (2018).
- Jones, P. A. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* **13**, 484–492, <https://doi.org/10.1038/nrg3230> (2012).
- Wu, H. & Zhang, Y. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell* **156**, 45–68, <https://doi.org/10.1016/j.cell.2013.12.019> (2014).
- Wiench, M. *et al.* DNA methylation status predicts cell type-specific enhancer activity. *EMBO J.* **30**, 3028–3039, <https://doi.org/10.1038/emboj.2011.210> (2011).
- Smith, Z. D. & Meissner, A. DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14**, 204–220, <https://doi.org/10.1038/nrg3354> (2013).
- Bui, C. *et al.* cAMP response element-binding (CREB) recruitment following a specific CpG demethylation leads to the elevated expression of the matrix metalloproteinase 13 in human articular chondrocytes and osteoarthritis. *FASEB J.* **26**, 3000–3011, <https://doi.org/10.1096/fj.12-206367> (2012).
- de Andres, M. C. *et al.* Loss of methylation in CpG sites in the NF-κB enhancer elements of inducible nitric oxide synthase is responsible for gene induction in human articular chondrocytes. *Arthritis Rheum.* **65**, 732–742, <https://doi.org/10.1002/art.37806> (2013).
- Hashimoto, K. *et al.* Regulated transcription of human matrix metalloproteinase 13 (MMP13) and interleukin-1β (IL1β) genes in chondrocytes depends on methylation of specific proximal promoter CpG sites. *J. Biol. Chem.* **288**, 10061–10072, <https://doi.org/10.1074/jbc.M112.421156> (2013).
- Reynard, L. N., Bui, C., Syddall, C. M. & 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.* **133**, 1059–1073, <https://doi.org/10.1007/s00439-014-1447-z> (2014).
- Aoyama, T. *et al.* Methylation in the core-promoter region of the chondromodulin-I gene determines the cell-specific expression by regulating the binding of transcriptional activator Sp3. *J. Biol. Chem.* **279**, 28789–28797, <https://doi.org/10.1074/jbc.M401273200> (2004).
- Imagawa, K. *et al.* Association of reduced type IX collagen gene expression in human osteoarthritic chondrocytes with epigenetic silencing by DNA hypermethylation. *Arthritis Rheumatol.* **66**, 3040–3051, <https://doi.org/10.1002/art.38774> (2014).
- El-Serafi, A. T., Oreffo, R. O. & Roach, H. I. Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: Differential effects of 5-aza-deoxycytidine and trichostatin A. *Differ.* **81**, 35–41, <https://doi.org/10.1016/j.diff.2010.09.183> (2011).
- Zimmermann, P. *et al.* Correlation of COL10A1 induction during chondrogenesis of mesenchymal stem cells with demethylation of two CpG sites in the COL10A1 promoter. *Arthritis Rheum.* **58**, 2743–2753, <https://doi.org/10.1002/art.23736> (2008).

18. Kumar, D. & Lassar, A. B. Fibroblast growth factor maintains chondrogenic potential of limb bud mesenchymal cells by modulating DNMT3A recruitment. *Cell Rep.* **8**, 1419–1431, <https://doi.org/10.1016/j.celrep.2014.07.038> (2014).
19. Shen, J. *et al.* DNA methyltransferase 3b regulates articular cartilage homeostasis by altering metabolism. *JCI Insight* **2**, <https://doi.org/10.1172/jci.insight.93612> (2017).
20. Herlofson, S. R. *et al.* Genome-wide map of quantified epigenetic changes during *in vitro* chondrogenic differentiation of primary human mesenchymal stem cells. *BMC Genomics* **14**, 105, <https://doi.org/10.1186/1471-2164-14-105> (2013).
21. Bomer, N. *et al.* Neo-cartilage engineered from primary chondrocytes is epigenetically similar to autologous cartilage, in contrast to using mesenchymal stem cells. *Osteoarthr. Cartil.* **24**, 1423–1430, <https://doi.org/10.1016/j.joca.2016.03.009> (2016).
22. Peffers, M. J. *et al.* Decoding the Regulatory Landscape of Ageing in Musculoskeletal Engineered Tissues Using Genome-Wide DNA Methylation and RNASeq. *PLoS One* **11**, e0160517, <https://doi.org/10.1371/journal.pone.0160517> (2016).
23. Barter, M. J. *et al.* Genome-Wide MicroRNA and Gene Analysis of Mesenchymal Stem Cell Chondrogenesis Identifies an Essential Role and Multiple Targets for miR-140-5p. *Stem Cell* **33**, 3266–3280, <https://doi.org/10.1002/stem.2093> (2015).
24. Murdoch, A. D. *et al.* Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: generation of scaffold-free cartilage. *Stem Cell* **25**, 2786–2796, <https://doi.org/10.1634/stemcells.2007-0374> (2007).
25. Rakyan, V. K., Down, T. A., Balding, D. J. & Beck, S. Epigenome-wide association studies for common human diseases. *Nat. Rev. Genet.* **12**, 529–541, <https://doi.org/10.1038/nrg3000> (2011).
26. Chantry, A. WWP2 ubiquitin ligase and its isoforms: new biological insight and promising disease targets. *Cell Cycle* **10**, 2437–2439, <https://doi.org/10.4161/cc.10.15.16874> (2011).
27. Rushton, M. D. *et al.* Characterization of the cartilage DNA methylome in knee and hip osteoarthritis. *Arthritis Rheumatol.* **66**, 2450–2460, <https://doi.org/10.1002/art.38713> (2014).
28. Loeser, R. F., Goldring, S. R., Scanzello, C. R. & Goldring, M. B. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum.* **64**, 1697–1707, <https://doi.org/10.1002/art.34453> (2012).
29. Reynard, L. N. Analysis of genetics and DNA methylation in osteoarthritis: What have we learnt about the disease? *Semin. Cell Dev. Biol.* **62**, 57–66, <https://doi.org/10.1016/j.semcdb.2016.04.017> (2017).
30. Field, A. E. *et al.* DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Mol. Cell* **71**, 882–895, <https://doi.org/10.1016/j.molcel.2018.08.008> (2018).
31. Laurent, L. *et al.* Dynamic changes in the human methylome during differentiation. *Genome Res.* **20**, 320–331, <https://doi.org/10.1101/gr.101907.109> (2010).
32. Maeder, M. L. *et al.* Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nat. Biotechnol.* **31**, 1137–1142, <https://doi.org/10.1038/nbt.2726> (2013).
33. Edwards, J. R., Yarychivska, O., Boulard, M. & Bestor, T. H. DNA methylation and DNA methyltransferases. *Epigenetics Chromatin* **10**, 23, <https://doi.org/10.1186/s13072-017-0130-8> (2017).
34. Berdasco, M. & Esteller, M. DNA methylation in stem cell renewal and multipotency. *Stem Cell Res. Ther.* **2**, 42, <https://doi.org/10.1186/scrt83> (2011).
35. Suelves, M., Carrio, E., Nunez-Alvarez, Y. & Peinado, M. A. DNA methylation dynamics in cellular commitment and differentiation. *Brief. Funct. Genomics* **15**, 443–453, <https://doi.org/10.1093/bfpg/ew017> (2016).
36. Consortium, E. P. An integrated encyclopedia of DNA elements in the human genome. *Nat.* **489**, 57–74, <https://doi.org/10.1038/nature11247> (2012).
37. Long, H. K., Prescott, S. L. & Wysocka, J. Ever-Changing Landscapes: Transcriptional Enhancers in Development and Evolution. *Cell* **167**, 1170–1187, <https://doi.org/10.1016/j.cell.2016.09.018> (2016).
38. Liu, C. F., Samsa, W. E., Zhou, G. & Lefebvre, V. Transcriptional control of chondrocyte specification and differentiation. *Semin. Cell Dev. Biol.* **62**, 34–49, <https://doi.org/10.1016/j.semcdb.2016.10.004> (2017).
39. Ohba, S., He, X., Hojo, H. & McMahon, A. P. Distinct Transcriptional Programs Underlie Sox9 Regulation of the Mammalian Chondrocyte. *Cell Rep.* **12**, 229–243, <https://doi.org/10.1016/j.celrep.2015.06.013> (2015).
40. Yao, B. *et al.* The SOX9 upstream region prone to chromosomal aberrations causing campomelic dysplasia contains multiple cartilage enhancers. *Nucleic Acids Res.* **43**, 5394–5408, <https://doi.org/10.1093/nar/gkv426> (2015).
41. Taylor, S. E. *et al.* Stable 5-Hydroxymethylcytosine (5hmC) Acquisition Marks Gene Activation During Chondrogenic Differentiation. *J. Bone Min. Res.* **31**, 524–534, <https://doi.org/10.1002/jbmr.2711> (2016).
42. Liu, Y. *et al.* Chromatin accessibility landscape of articular knee cartilage reveals aberrant enhancer regulation in osteoarthritis. *Sci. Rep.* **8**, 15499, <https://doi.org/10.1038/s41598-018-33779-z> (2018).
43. Vinatier, C. & Guicheux, J. Cartilage tissue engineering: From biomaterials and stem cells to osteoarthritis treatments. *Ann. Phys. Rehabil. Med.* **59**, 139–144, <https://doi.org/10.1016/j.rehab.2016.03.002> (2016).
44. Inui, M. *et al.* Dissecting the roles of miR-140 and its host gene. *Nat. Cell Biol.* **20**, 516–518, <https://doi.org/10.1038/s41556-018-0077-4> (2018).
45. Bizet, A. A. *et al.* The TGF-beta co-receptor, CD109, promotes internalization and degradation of TGF-beta receptors. *Biochim. Biophys. Acta* **1813**, 742–753, <https://doi.org/10.1016/j.bbamcr.2011.01.028> (2011).
46. Jiao, S. *et al.* VGLL4 targets a TCF4-TEAD4 complex to coregulate Wnt and Hippo signalling in colorectal cancer. *Nat. Commun.* **8**, 14058, <https://doi.org/10.1038/ncomms14058> (2017).
47. Poschl, E. *et al.* DNA methylation is not likely to be responsible for aggrecan down regulation in aged or osteoarthritic cartilage. *Ann. Rheum. Dis.* **64**, 477–480, <https://doi.org/10.1136/ard.2004.022509> (2005).
48. Sesselmann, S. *et al.* DNA methylation is not responsible for p21WAF1/CIP1 down-regulation in osteoarthritic chondrocytes. *Osteoarthr. Cartil.* **17**, 507–512, <https://doi.org/10.1016/j.joca.2008.09.006> (2009).
49. Alvarez-Garcia, O. *et al.* Increased DNA Methylation and Reduced Expression of Transcription Factors in Human Osteoarthritis Cartilage. *Arthritis Rheumatol.* **68**, 1876–1886, <https://doi.org/10.1002/art.39643> (2016).
50. Bonin, C. A. *et al.* Identification of differentially methylated regions in new genes associated with knee osteoarthritis. *Gene* **576**, 312–318, <https://doi.org/10.1016/j.gene.2015.10.037> (2016).
51. den Hollander, W. *et al.* Knee and hip articular cartilage have distinct epigenomic landscapes: implications for future cartilage regeneration approaches. *Ann. Rheum. Dis.* **73**, 2208–2212, <https://doi.org/10.1136/annrheumdis-2014-205980> (2014).
52. Fernandez-Tajes, J. *et al.* Genome-wide DNA methylation analysis of articular chondrocytes reveals a cluster of osteoarthritic patients. *Ann. Rheum. Dis.* **73**, 668–677, <https://doi.org/10.1136/annrheumdis-2012-202783> (2014).
53. Jeffries, M. A. *et al.* Genome-wide DNA methylation study identifies significant epigenomic changes in osteoarthritic cartilage. *Arthritis Rheumatol.* **66**, 2804–2815, <https://doi.org/10.1002/art.38762> (2014).
54. Steinberg, J. *et al.* Integrative epigenomics, transcriptomics and proteomics of patient chondrocytes reveal genes and pathways involved in osteoarthritis. *Sci. Rep.* **7**, 8935, <https://doi.org/10.1038/s41598-017-09335-6> (2017).
55. Zhang, Y. *et al.* Genome-wide DNA methylation profile implicates potential cartilage regeneration at the late stage of knee osteoarthritis. *Osteoarthr. Cartil.* **24**, 835–843, <https://doi.org/10.1016/j.joca.2015.12.013> (2016).
56. Bork, S. *et al.* DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. *Aging Cell* **9**, 54–63, <https://doi.org/10.1111/j.1474-9726.2009.00535.x> (2010).
57. Day, K. *et al.* Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape. *Genome Biol.* **14**, R102, <https://doi.org/10.1186/gb-2013-14-9-r102> (2013).

58. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57, <https://doi.org/10.1038/nprot.2008.211> (2009).
59. Roadmap Epigenomics, C. *et al.* Integrative analysis of 111 reference human epigenomes. *Nat.* **518**, 317–330, <https://doi.org/10.1038/nature14248> (2015).
60. Gee, F., Clubbs, C. F., Raine, E. V., Reynard, L. N. & Loughlin, J. Allelic expression analysis of the osteoarthritis susceptibility locus that maps to chromosome 3p21 reveals cis-acting eQTLs at GNL3 and SPCS1. *BMC Med. Genet.* **15**, 53, <https://doi.org/10.1186/1471-2350-15-53> (2014).

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Author contributions

M.J.B. designed the study, performed sample preparations, carried out analysis and wrote the manuscript. C.B. performed sample preparations and carried out analysis. R.G. performed sample preparations. K.C., A.J.S. and H.R.E. provided intellectual input and analysis. J.F. performed the pyrosequencing analysis. L.N.R. provided intellectual input. D.A.Y. obtained funding, designed the study, wrote the manuscript and has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Competing interests

The authors declare no competing interests.

Additional information

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