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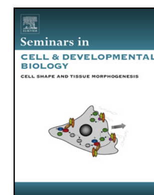

Review

Analysis of genetics and DNA methylation in osteoarthritis: What have we learnt about the disease?

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ABSTRACT

Osteoarthritis (OA) is a chronic musculoskeletal disease characterised by the destruction of articular cartilage, synovial inflammation and bone remodelling. Disease aetiology is complex and highly heritable, with genetic variation estimated to contribute to 50% of OA occurrence. Epigenetic alterations, including DNA methylation changes, have also been implicated in OA pathophysiology. This review examines what genetic and DNA methylation studies have taught us about the genes and pathways involved in OA pathology. The influence of DNA methylation on the molecular mechanisms underlying OA genetic risk and the consequence of this interaction on disease susceptibility and penetrance are also discussed.

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1. Introduction

Osteoarthritis (OA), one of the leading causes of global disability [1], is a chronic musculoskeletal disease that affects the structure and function of synovial joints, including the hips, knees, spine, feet and hands. OA is characterised by cartilage degeneration but within the OA affected joint, pathological changes occur in multiple tissues. They include subchondral bone remodelling, synovial inflammation and ligament fibrosis, and together result in loss of joint function [2]. The symptoms of OA include joint stiffness and chronic pain, leading to decreased joint movement and limitations in physical activity. In addition to mobility problems, OA-related pain is associated with poor sleep, fatigue and negative effects on mood [3], causing a considerable reduction in the quality of life.

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Furthermore, individuals with OA have an excess of all-cause mortality compared to the general population; this is strongest for individuals with co-morbidities such as diabetes, cancer, cardiovascular disease and walking disability [4]. OA has a significant socioeconomic burden, due to direct health care costs and to indirect costs associated with loss of productivity. The overall cost of OA is estimated to be 1–2.5% of the gross national product [3].

OA is a multifactorial disease. Its main risk factors are age, genetics, gender, obesity, and prior injury or abnormal alignment [5]. The knee and hip joints are the most commonly affected joints, with knee OA estimated to affect 250 million people worldwide [5]. OA is more common in females than males and is very rare before 40 years of age, but rises steeply after this. The global prevalence of radiographic knee OA peaks at 50 years of age, and hip OA prevalence continues to rise with increasing age [1]. Due to increasing obesity levels and an aging population, the incidence of OA is predicted to double within the next 15–20 years [6]. In the absence of disease-modifying drugs for OA, the only treatment options currently available are analgesia, physical therapy and joint replacement.

The aetiology of OA is poorly understood, especially the early molecular processes that mark the onset of OA prior to the presence of radiographically detected joint damage. However, molecular studies and clinical observations have indicated that OA is a heterogeneous disease, with different pathophysiological pathways leading to the same end phenotype. In order to identify new therapeutic targets, improve existing joint tissue regeneration approaches and discover biomarkers of disease, we need to increase our understanding of the biological processes underlying OA development and progression. Genetic, transcriptomic, epigenetic and proteomic analyses have all played crucial roles in identifying pathways that are dysregulated in OA. This review will examine what we have learnt from genetic and epigenetic analysis of OA about the molecular pathways and processes underlying disease susceptibility and pathophysiology. The molecular interplay between genetics and epigenetics in OA disease risk will also be considered and potential directions for future research discussed.

2. OA genetic susceptibility loci

2.1. The genetics of OA

Epidemiological and family-based genetic studies have demonstrated that genetics plays a significant contribution to OA susceptibility. Heritability was estimated to range from 39 to 79% depending on gender, disease severity and the affected joint site [7,8]. These studies also demonstrated that OA is not a single-gene disorder, but is a complex polygenic trait composed of multiple risk loci, each of which confers a small effect on disease susceptibility [9]. Although genetic heterogeneity and small-size effects have made the identification of OA risk loci challenging, the potential utility of risk loci as a diagnostic tool and means to discover new disease pathways explain why genetics is an active area of OA research.

2.2. Identification of OA susceptibility loci

The identification of OA susceptibility loci has progressed over the last 10 years from linkage scans to case-control association studies. These studies utilise the principle that an allele that increases OA risk will be more common in a group of affected individuals than in a control group of individuals without OA. Initial case-control studies focused on genotyping for DNA variants located within the promoter and coding regions of genes with known roles in cartilage biology or OA pathology such as *GDF5* and *ACAN*. To date, these candidate gene studies have looked at single nucleotide polymorphisms (SNPs) in over 200 genes for evidence of an association with OA in different ethnic populations. Of all of these SNPs, 199 were subjected to replication in a recent meta-analysis study in the European population, and only two (*COL11A1* and *VEGF*) were found to be significantly associated with OA [10]. One more locus identified by the candidate gene approach that was reproducibly and robustly associated with OA in both East Asian and European populations is the rs143383 SNP located within the 5′UTR of the growth differentiation factor 5 (*GDF5*) gene. This SNP was first identified as a hip susceptibility locus in Japanese individuals, and was subsequently found to be significantly associated with knee OA at the genome-wide level in Europeans through meta-analysis [11,12]. The OA risk allele of rs143383 leads to reduced *GDF5* expression in joint tissues [13], and mice carrying only one functional copy of *Gdf5* have increased susceptibility to OA [14].

Candidate gene studies have been largely superseded by hypothesis-free genome-wide association scans (GWAS), which involve genotyping a large number of cases and controls for set of SNPs selected to cover the majority of common genomic variants. Given the large number of SNPs analysed by GWAS and statistical tests performed, a SNP is said to be significantly associated with a disease or trait at the genome-wide level only if the p value for the association is $<5 \times 10^{-8}$. A p value of $<5 \times 10^{-5}$ is considered suggestive of an association. Several highly powered OA GWAS have been performed where by $\geq 500\,000$ SNPs were genotyped in sample sizes exceeding 20 000 individuals after replication. Together, candidate gene and GWAS studies identified 14 OA susceptibility loci that reached genome-wide significance after replication in at least one independent cohort and 11 loci suggestive of an association (Table 1; [10,11,15–27]).

As it can be seen in Table 1, the identification of OA loci has confirmed that genetic risk shows joint-specific effects, with loci often contributing to disease risk at a particular skeletal site. Furthermore, some loci are associated with OA in both males and females, whereas others are sex-specific, suggesting gender differences in molecular mechanisms underlying OA susceptibility. While the majority of European OA risk loci have not been studied in South East Asians, European replication analysis of the *DVWA, HLA-DQB1* and *BTNL2* SNPs indicate that these loci are specific to Japanese and Chinese populations [28,29]. Further analysis is required to determine whether there are genuine ethnic differences in genetic aetiology, or, as reported for the *HLA-DQB1* and *BTNL2* SNPs, the lack of replication between populations is solely the result of the SNPs marking different haplotypes [29]. Several OA susceptibility regions contain or flank genes involved in chondrogenesis and/or endochondral ossification, implicating skeletal development in OA pathogenesis [30]. A number of OA loci contain genes that have no known role in the synovial joint, and even for those loci containing skeletogenesis genes, detailed functional analyses are required to determine the molecular mechanisms through which these loci increase susceptibility to OA.

2.3. Functional analysis of susceptibility loci

There is a considerable disparity between the number of human disease/phenotype loci discovered by GWAS and functional characterisation, with the biological mechanism linking genotype to phenotype reported for only a tiny proportion of these associations. The reasons for this are two-fold. Firstly, recombination occurs at hotspots across the region, forming linkage disequilibrium (LD) blocks containing several SNPs that are inherited together as a haplotype. Due to this, the GWAS-identified SNP marks the LD block where genetic susceptibility resides, but is unable to pinpoint which of the SNPs within the region is the causal variant. The LD blocks marked by the OA SNPs range in size from 1 bp

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Table 1
Replicated genome-wide significant and genome-wide suggestive OA genetic susceptibility loci. All SNPs listed were replicated in an independent cohort from the same ethnic group, with a threshold replication p value < 0.005, and a combined P value less than the discovery P value. The combined discovery plus replication P value is shown. The LD block refers to the genomic region spanning all SNPs with an $r^2 \geq 0.8$ of the OA SNP. The number of SNPs and genes within each LD block are also given. LD was calculated by LDlink [43] using The 1000 Genomes Project phase 3 data [98] from individuals of European (EUR; n = 503) or East Asian (EAS; n = 504) descent. When known, the effect of the risk allele on gene expression or protein function is stated. TJR; total joint replacement. THR; total hip replacement. *These SNPs were subject to meQTL analysis in [38].

SNP	p value	Association	Study	Nearest gene	LD size (bp)	Co-ordinates(hg19)	SNPs	Genes	Effect of risk allele
EUR Genome-wide significance ($p < 5 \times 10^{-8}$)									
rs3204689*	1.10×10^{-11}	hand	[15]	*ALDH1A2*	145,170	chr15:58208165-58353335	56	1	reduced *ALDH1A2* expression in OA cartilage [15]
rs143383*	6.20×10^{-11}	knee	[11]	*GDF5*	136,077	chr20:33890061-34026138	113	2	reduced *GDF5* expression in joint tissues [13]
rs6976*	7.24×10^{-11}	TJR	[16]	*GLT8D1*	291,582	chr3:52546820-52838402	362	11	reduced *GNL3* and *SPCS1* expression in OA cartilage [32]
rs4836732*	6.11×10^{-10}	THR female	[16]	*ASTN2*	34,913	chr9:119266695-119301607	2	1	N/A
rs9350591*	2.42×10^{-9}	hip	[16]	*FILIP1*	188,719	chr6:76199219-76387937	91	2	no eQTL or AEI identified in OA cartilage [37]
rs6094710*	7.90×10^{-9}	hip	[17]	*NCOA3*	193,415	chr20:46095649-46289063	13	2	reduced *NCOA3* expression in OA cartilage [33]
rs4730250	9.17×10^{-9}	knee	[18]	*COG5*	146,676	chr7:107061020-107207695	73	3	reduced *HBP1* in cartilage, reduced *DUS4L* in fat pad [34]
rs10492367*	1.48×10^{-8}	hip	[16]	*PTHLH*	14,790	chr12:28010407-28025196	5	0	N/A
rs835487*	1.64×10^{-8}	THR	[16]	*CHST11*	2726	chr12:105059930-105062656	7	1	N/A
rs11842874*	2.10×10^{-8}	knee & hip	[19]	*MCF2L*	12,723	chr13:113684333-113697055	11	1	increased *MCF2L* in TKR synovium [36]
EAS Genome-wide significance ($p < 5 \times 10^{-8}$)									
SNP	p value	Association	Study	Nearest gene	LD size (bp)	Co-ordinates(hg19)	SNPs	Genes	Effect of risk allele
rs143383	1.80×10^{-13}	hip	[12]	*GDF5*	136,077	chr20:33890061-34026138	108	2	reduced *GDF5* expression in joint tissues [13]
rs7639618	7.30×10^{-11}	knee	[20]	*DVWA*	105,061	chr3:15197987-15303047	80	3	reduced binding of DVWA to tubulin [20]
rs7775228	2.43×10^{-8}	knee	[21]	*HLA-DQB1*	1681	chr6:32657255-32658935	4	0	
rs10947262	6.73×10^{-8}	knee	[21]	*BTNL2*	8499	chr6:32365707-32374205	23	1	
EUR Genome-wide suggestive ($p < 5 \times 10^{-5}$)									
SNP	p value	Association	Study	Nearest gene	LD size (bp)	Co-ordinates(hg19)	SNPs	Genes	Effect of risk allele
rs12107036*	6.71×10^{-8}	TKR female	[16]	*TP63*	1	chr3:189600160	1	1	N/A
rs8044769*	6.85×10^{-8}	female	[16]	*FTO*	40,613	chr16:53798523-53839135	9	1	N/A
rs12982744*	7.80×10^{-8}	hip	[22]	*DOT1L*	31,988	chr19:2152018-2184005	25	1	N/A
rs10948172*	7.92×10^{-8}	male	[16]	*SUPT3H*	580,638	chr6:44696262-45276899	240	2	no eQTL identified in TKR cartilage [38]
rs3815148*	8.00×10^{-8}	knee &/or hand	[23]	*HBP1*	411,553	chr7:106786142-107197694	328	4	reduced *HBP1* expression in cartilage and synovium [34]
rs12901499	7.50×10^{-6}	knee & hip	[24]	*SMAD3*	32,681	chr15:67337826-67370506	29	1	no eQTL or AEI identified in OA cartilage [39]
rs4907986	1.29×10^{-5}	hip	[10]	*COL11A1*	52,163	chr1:103519589-103571751	26	1	N/A
rs833058	1.35×10^{-5}	hip male	[10]	*VEGF*	1	chr6:43731854	1	0	N/A
rs225014*	2.02×10^{-5}	hip female	[25]	*DIO2*	38,878	chr14:80634365-80673242	20	1	increased *DIO2* expression in OA cartilage [35]
EAS Genome-wide suggestive ($p < 5 \times 10^{-5}$)									
SNP	p value	Association	Study	Nearest gene	LD size (bp)	Co-ordinates(hg19)	SNPs	Genes	Effect of risk allele
D-repeat D13/D14	1.3×10^{-6}	knee	[26]	*ASPN*	N/A	chr9:92456205-92482506	1	1	increased suppression of TGF-beta activity [31]
rs10980705	2.6×10^{-5}	knee	[27]	*LPAR1*	993	chr9:113802193-113803185	2	0	N/A

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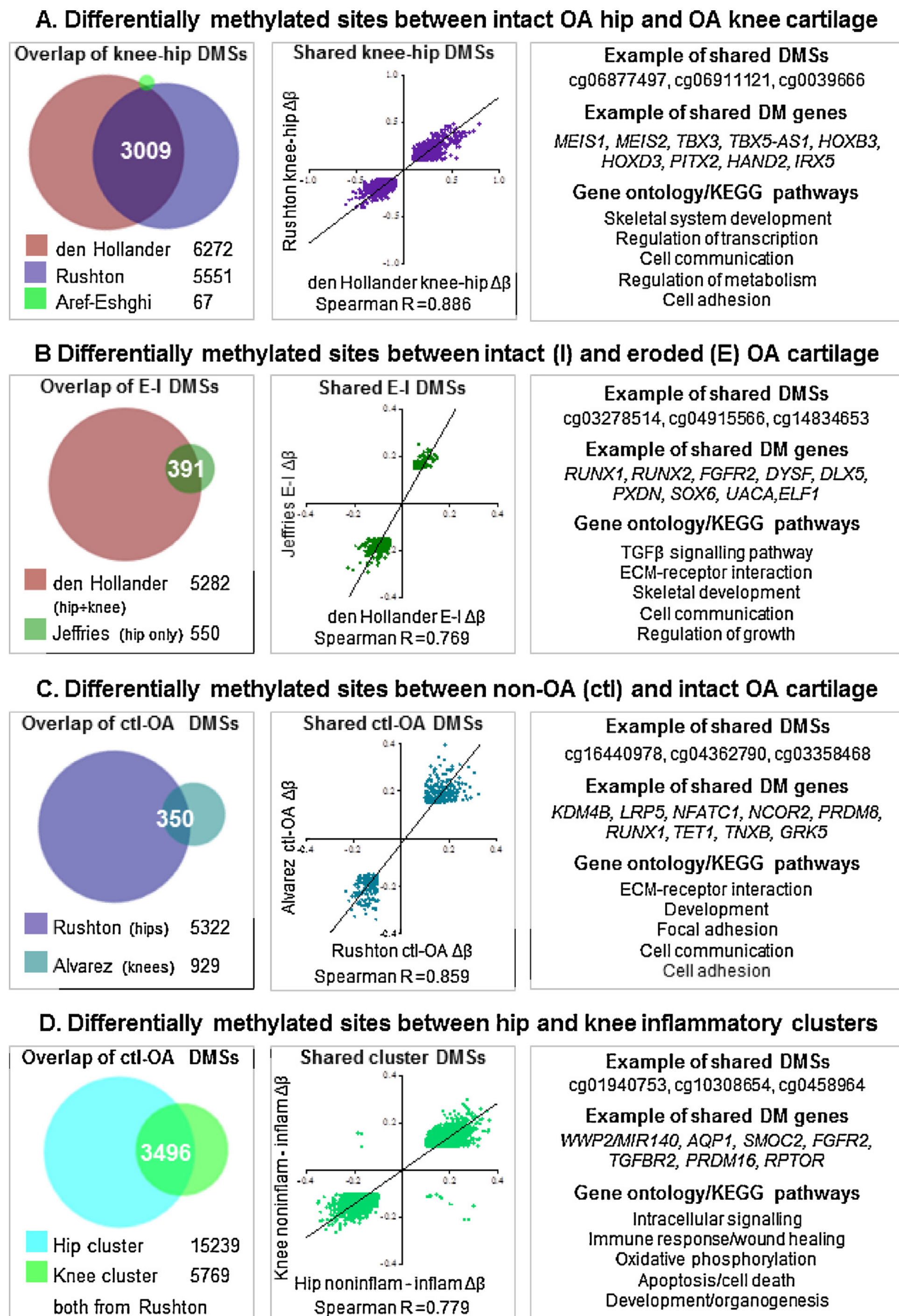

Fig. 1. Overlap between differentially methylated sites identified by cartilage methylome studies using the Illumina 450 K BeadChip. The differentially methylated site (DMS) lists were compared for studies where the complete lists of DMSs and their delta beta (Δβ) values were available online. (A) DMSs between intact OA hip cartilage and intact OA knee cartilage identified in the studies of Rushton [69], den Hollander [70] and Aref-Eshghi [73]. (B) DMSs between eroded and intact OA cartilage identified by Jeffries [71] and den Hollander [80]. (C) DMSs between control and intact OA hip and OA knee cartilage identified by Rushton [69] and Alvarez [75] respectively. (D) DMSs between non-inflammatory and inflammatory sub-clusters of OA hip and knee patients identified by Rushton [69]. Left; a Venn diagram of the overlapping probes between the studies, together with the number of DMSs identified by each study. Middle; a graph of the Δβ values for the shared DMSs, with the Spearman's rank correlation coefficient indicated. Right; the top three shared DMSs and examples of genes containing at least two shared DMSs. Gene ontology and KEGG pathway analysis was performed on the genes containing shared DMSs using GATHER [97] and the top terms are indicated.

to over 580 kb, and the number of putative causal SNPs may be in the hundreds (see Table 1), making it difficult to identify the SNP responsible for increased OA risk. Secondly, the bulk of SNPs identified by GWAS studies are located outside of open reading frames, with over 97% of the SNPs within the OA LD regions listed in Table 1 mapping to non-coding regions of the genome, including enhancers and insulators. These SNPs are thought to mediate disease susceptibility through subtle effects on target gene expression or transcript stability caused by allelic differences in transcription factor or microRNA binding.

Except for the *DVWA* and *ASPN* D-repeat variants, which have been shown to affect the function of the encoded proteins [20,31],

molecular characterisation of OA risk loci has focused on correlating genotype with expression of nearby genes through expression quantitative trait locus (eQTL) and allelic expression imbalance (AEI) analyses. In eQTL analysis, gene expression is stratified by genotype at the associated SNP in a large number of individuals, whereas AEI involves quantifying the mRNA levels of each allele in heterozygous individuals. eQTLs and/or AEI have been observed in cartilage for several OA SNPs including those within the *ALDH1A2*, *GDF5* and *NCOA3* genes (see Table 1; [13,15,32–35]). OA-associated eQTLs/AEI are also present in synovium and fat pad tissues from OA patients [13,34,36]. However for several OA loci, the OA SNP is not associated with expression of nearby genes in cartilage [37–39]. This may be because the SNPs are located within regulatory elements such as enhancers, many of which are known to regulate gene expression in a temporal and/or tissue specific manner. It has been hypothesised that a substantial amount of OA genetic susceptibility is operating during skeletal development, potentially through subtle effects on joint architecture that predispose to OA later in life [30,40,41], and this is supported by the association between the OA hip SNP rs4836732 and femoral head shape [42]. The functional effect of an OA SNP on gene expression may thus be limited to a particular time-point and/or cell type during skeletal development.

2.4. Future directions for OA genetic analysis

The number of loci identified by GWAS studies is dependent on the number of samples used, and in order to identify additional OA susceptibility loci, more powerful GWAS studies with larger sample sizes are needed. Increased power may also be achieved by using a consistent definition of OA and by selecting a more severe disease phenotype, for example restricting analysis to patients that have undergone joint replacement. Previous GWAS studies have demonstrated the importance of sub-stratifying OA patients by joint site, gender and ascertainment criteria. Analysis of other endophenotypes such as joint space width, joint shape, synovitis and pain, should also be considered. In addition to identifying new OA susceptibility loci, it is crucial to functionally characterise existing loci to enhance our understanding of the OA disease process and discover new diagnostic and therapeutic targets. The identification the causal OA SNP within each locus, their target genes and mechanisms through which the SNPs predispose to OA remains the greatest challenge in the OA genetics field. In the future, such functional analyses will be aided by databases (e.g. LDlink [43]) that integrate genotyping data with publically available chromatin, transcription factor binding, and DNaseI hypersensitivity data.

3. DNA methylation analysis of OA tissues

3.1. Epigenetics and OA

The term epigenetics refers to heritable changes in gene expression and/or phenotype that occur without changes in the primary DNA sequence [44]. Epigenetic patterns are both plastic, permitting dynamic changes in gene expression during development and cell differentiation, and stable, allowing cellular identity to be maintained during mitotic cell divisions. As such, the epigenome of each cell or tissue is unique, and can undergo temporal and spatial changes in response to internal and external environmental factors that include diet, exercise and smoking.

Epigenetic mechanisms regulate gene expression either by affecting gene transcription or by acting post-transcriptionally. They include DNA methylation, histone modifications and non-coding RNAs (ncRNAs). In mammalian cells, DNA methylation predominantly occurs at CpG dinucleotides and involves the addition of a methyl group to a cytosine base. DNA methylation regulates transcription through effects on transcription factor and chromatin remodelling complex recruitment. Methylation within promoter regions is associated with gene repression, whereas methylation within gene bodies correlates positively with gene expression and has been implicated in splicing and transcription from alternative promoters [45,46]. There are over 150 known histone modifications. They include acetylation, phosphorylation, ubiquitination and methylation of assorted histone residues and have variable effects on transcription [47]. Gene promoters, enhancers, transcribed regions, and silenced regions are each associated with a specific combination of histone marks. ncRNAs regulate gene expression by affecting transcription, splicing or translation and include microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) such as *XIST* [48]. miRNAs interact through complementary base pairing in the 3′ untranslated region of the target mRNAs and lead to translational suppression or mRNA degradation [49].

Within the last 10 years, epigenetics has developed as a new and important area of OA research, with studies mainly focusing on miRNAs and DNA methylation. The importance of miRNAs in skeletal development and chondrogenesis was first demonstrated in mice with a cartilage-specific knock-out - of the miRNA processing enzyme DICER [50], and since then, many miRNAs that are involved in cartilage development and OA have been reported. These studies have been the topic of several reviews [51–53], and as such, this section will focus on DNA methylation.

3.2. Targeted DNA methylation analysis in OA cartilage

The majority of DNA methylation studies in OA have concentrated on cartilage, not only because of its crucial role in the disease, but also because it is composed of a single cell type, the chondrocyte; each cell type within a multicellular tissue such as synovium has its own epigenome, making it difficult to differentiate between genuine methylation differences and changes in cell proportions within the diseased tissue. Although the global levels of the DNA methyltransferase enzymes DNMT3A and DNMT1A are unchanged in OA cartilage [54], a potential role for DNA methylation in human OA disease pathogenesis was first suggested in 2005 [55]. This pioneering study found that increased expression of the genes encoding the cartilage-degrading enzymes MMP3, MMP9, MMP13 and ADAMTS4 correlated with demethylation of specific CpG sites within the promoter of these genes in OA cartilage. Since then, many studies have examined promoter methylation and mRNA levels for genes implicated in OA in cartilage, primary chondrocytes and transformed chondrogenic cell lines. Demethylation of specific promoter or enhancer sites in the *LEP*, *GDF*5, *NOS2*, *PHLPP1*, *SOST* and *IL8* genes have been associated with increased expression of these genes in OA cartilage or isolated chondrocytes [56–61], and increased methylation of *SOX9*, *SOD2* and *COL9A1* promoters with downregulation of these genes in OA cartilage [62–64].

None of these studies has conclusively proven that DNA methylation changes in chondrocytes contribute to OA pathogenesis because longitudinal studies of the same cartilage samples would be required to examine the direction of causality between altered methylation, gene expression and disease. Nevertheless, *in vitro* methylation reporter studies and treatment of cultured chondrocytes with demethylating agents have indicated that DNA methylation can have a direct functional impact upon the differentially methylated region. Furthermore, DNA methylation of specific CpG sites within *MMP13*, *GDF5*, *SOX9* and *COL9A1* promoters, and *NOS2* enhancer has been shown to alter transcription factor binding and is thereby thought to affect gene transcription in OA cartilage [57,58,62,64–66].

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Although these studies have highlighted the potential role of abnormal methylation in OA pathogenesis, there are limitations of these targeted analyses. Firstly, not every CpG site within the regulatory region of a gene can be analysed and so it is difficult to irrefutably conclude that gene expression differences are not due to changes in DNA methylation. Secondly, these studies concentrate on genes already linked to OA and thus do not identify new pathways that may be important in the disease process. Because of this, several groups have started to perform genome-wide DNA methylome studies, as discussed below.

3.3. Genome-wide methylome studies

The majority of the OA methylome studies published up until February 2016 have utilised the Illumina HumanMethylation BeadChip 27K and 450K arrays [67–75]; these measure methylation of over 27,000CpG sites located in almost 14,500 genes and ~485,000 sites covering 99% of RefSeq genes, respectively. Other methylome studies have assessed methylation through reduced representation bisulphite sequencing (RRBS, [76]; [77]), the Agilent human promoter microarray 244K arrays [78], or immunoprecipitation with an antibody against 5′hydroxymethylcytosine followed by sequencing (5hmC Me-DIP-seq; [79]). The experimental designs of cartilage methylome studies have fallen into four main categories; A) comparison of non-OA cartilage to non-damaged cartilage from the OA joint [68,69,73,75]; B) comparison of non-damaged intact OA hip and knee cartilage [69,70,73]; C) comparison of intact and eroded cartilage from the same joint [71,72,78,80]; and D) comparison of cultured chondrocytes from patients with and without OA [79]. In addition to the cartilage/chondrocyte methylome, methylation has also been studied in the femoral head trabecular bone of osteoporotic and OA patients [67] and in the subchondral bone underlying intact and eroded cartilage in the same joints [74]. These methylome studies are summarised in Table 2. Although they have only examined a small proportion of the estimated 28 million CpG sites within the human genome, these studies have been very informative in pinpointing genomic regions undergoing OA-associated methylation changes and genes and pathways subject to these changes.

The majority of the differentially methylated CpG sites (DMSs) or regions (DMRs) identified in these studies are depleted in promoter regions, and enriched in gene bodies, intergenic regions and enhancers. As enhancers located within one gene may actually regulate expression of a completely different gene, the target genes of DMS/DMR identified in methylome studies are not automatically known. This could explain why only ~10% of the DMRs between OA hip and knee cartilage are associated with differential expression of the nearest gene [70]. Furthermore, gene body methylation has been associated with mRNA splicing and transcription from alternative promoters [45,46]. This makes identifying the effect of the methylation change on gene expression challenging, and this should be taken into account when performing gene ontology and pathway analysis of genes containing DMSs/DMRs within non-promoter regions.

In spite of technical and biological differences in study designs, several key conclusions can be drawn from the genome-wide methylation studies published so far. Firstly, the cartilage methylome is different between hip and knee cartilage irrespective of disease status or degree of joint damage [69,70,73], and even patients with disease of the same joint can have distinct epigenomes [68,69,81]. These observations have important diagnostic, therapeutic and tissue engineering implications. Secondly, genes involved in immunological functions are enriched for DMSs in OA versus non-OA cartilage [68,69], and eroded versus intact cartilage from the same joint [71]. Furthermore, a subgroup of OA hip and OA knee patients are characterised by altered methylation of inflammatory genes [68,69], and for the hip inflammatory cluster, this is associated with increased transcription of *TNF*, *IL1A*, *IL6*, *MMP13*, *ADAMTS5* and zinc transporter genes [81]. Together, these studies suggest that DNA methylation plays a significant role in regulating the inflammation in cartilage, and emphasises the contribution of inflammation to OA pathology. The TGFβ signalling pathway also appears to be important in OA, with components of this pathway and its downstream target genes being enriched for DMSs associated with OA status [69], disease progression [72], cartilage erosion and methylation changes to the underlying subchondral bone [71,74], and altered hydroxymethylation levels in OA chondrocytes [79]. In addition, these methylome studies have provided further evidence for a role of skeletal development and limb morphogenesis in OA susceptibility. Genes encoding homeobox-containing transcription factors (including the *HOX* genes that play crucial roles in limb development [82]), are differentially methylated between hip and knee cartilage [69,70], in late knee OA [72], and in femoral head trabecular bone from OA patients [67]. Finally, cartilage degradation also affects the methylome of underlying subchondral bone, with genes involved in ERK/MAPK, PI3K and NFAT signalling undergoing damage-associated methylation changes in cartilage and subchondral bone [74].

Whilst genome-wide analyses have shown that the chondrocyte methylome is heterogeneous and impacted by factors including joint of origin and disease status, it is challenging to identify the molecular mechanisms through which these methylome differences may alter the chondrocyte phenotype. Each methylome study has identified hundreds or thousands of DMSs/DMRs, making it difficult to decide which of these sites to follow up with functional studies. One way to select DMSs/DMRs for detailed analysis may be to identify those that have been reported in multiple studies with similar experimental design. However, comparing DMSs/DMRs between studies using different techniques to measure methylation is currently arduous as it requires overlaying methylation data for individual CpG sites measured using array technology with sequencing data generated by RRBS or MeDIP-seq, not all of which are publicly available. Even for studies that used the 450K array, the DMSs/DMRs identified are not directly comparable because of differences in probe exclusion criteria, P value correction and threshold, and minimum differential methylation level used to determine significance. Despite these issues, there is a significant overlap between the DMSs identified using the 450K array among datasets using similar study designs (Fig. 1). For example, 34.1% of DMSs identified between OA hip and knee cartilage were reported in two independent studies [69,70], with all sites showing methylation differences in the same direction and to similar extents (Fig. 1A). Likewise, ~71% of the DMSs between intact and eroded OA hip cartilage were differentially methylated between intact and eroded cartilage when hip and knee samples are combined (Fig. 1B; [71,80]). Although hip and knee cartilage are epigenetically distinct, over a third of the DMSs between non-OA and OA knee cartilage are also differentially methylated in the same direction between non-OA and OA hip cartilage (Fig. 1C; [69,75]). Given that the cartilage samples used in these studies are from different geographical locations, it suggests that these shared pathways are largely independent of environmental effects. DMSs that undergo similar OA-associated methylation changes irrespective of the affected joint site, OA subtype or lifestyle factors therefore represent good candidates for further targeted studies.

3.4. Future directions for DNA methylation analysis in OA

Over the next few years, there will be increasing numbers of genome-wide methylation studies on cartilage and other joint tissues. More detailed functional characterisation of genes and regions implicated in OA through genetic, transcriptomic and

Table 2
Summary of genome-wide DNA methylation and 5′hydroxymethylation (5hmC) studies of cartilage, chondrocytes and bone. RRBS; reduced representation bisulphite sequencing. NOF; neck-of-femur fracture. THR; total hip replacement. TKR; total knee replacement. Intact; macroscopically normal cartilage distal from the OA lesion. Eroded; macroscopically damaged and degraded cartilage.

study	Assay	Samples	Country
Delgardo-Calle [67]	Illumina 27 K array	femoral head trabecular bone from 27 NOF and 26 OA THR female patients	Spain
Fernández-Tajes [68]	Illumina 27 K array	intact knee cartilage for 23 TKR patients and 18 post-mortem controls	Spain
Rushton [69]	Illumina 450 K array	intact cartilage from 23 THR, and 73 TKR OA patients, and 21 NOF controls	UK
den Hollander [70]	Illumina 450 K array	intact and eroded OA cartilage pairs from 14 TKR and 17 THR patients	Netherlands
Jeffries [71]	Illumina 450 K array	intact and eroded cartilage pairs from 24 OA THR patients	USA
Moazedi-Fuerst [78]	Agilent promoter array	intact and eroded cartilage pairs from 5 TKR female patients	Austria
Taylor [79]	5hmC meDIP seq	cultured chondrocytes from 4 TKR OA patients and 4 ACL reconstruction controls	USA
Zhang [72]	Illumina 450 K array	outer lateral, inner lateral and inner medial tibial plateau from 12 TKR OA patients	Japan
Aref-Eshghi [73]	Illumina 450 K array	intact cartilage from 5 THR and 6 TKR female OA patients and 7 female NOFs	Canada
Bonin [77]	RRBS	paired intact and eroded knee cartilage from 10 TKR OA patients	USA
Jeffries [74]	Illumina 450 K array	subchondral bone underlying intact and eroded cartilage from 12 THR patients	USA
Alvarez [75]	Illumina 450 K array	intact knee cartilage for 12 TKR patients and 11 controls	USA

methylome analysis will also be performed. Together, these studies have the potential to identify new etiological pathways and OA phenotypes, diagnostic and prognostic biomarkers, and therapeutic targets. The majority of the existing methylation studies have not been able to distinguish between 5′methylcytosine and its oxidised form, 5′hydroxymethylcytosine (5hmC). 5hmC is both an intermediate in DNA demethylation and a stable epigenetic mark associated with gene expression [83]. Global levels of this mark are increased in OA knee chondrocytes, and further studies of the role of 5hmC in OA joint tissues are expected. In view of the increasing number of methylome studies and the amount of DMSs/DMRs identified, it would be useful to establish a database of results readily accessible by researchers. Given that the majority of methylome studies have used the Illumina BeadChip technology, a consensus should be reached on probe exclusion criteria, gene annotation files, the multiple test correct method, and P value and differential methylation thresholds used to identify DMSs. This would allow direct comparison between studies, with data from one study potentially being used for replication analysis in a separate cohort of samples. These methylome analyses are expensive and generally have used relatively small sample sizes with no replication, potentially leading to a high degree of false positives. However, by combining samples from all published 450 K studies, a meta-analysis could be performed, producing a list of reliable DMS/DMR that can be studied in more detail.

4. The molecular interplay between genetics and DNA methylation in OA disease susceptibility

4.1. Background

Studies in non-joint tissues have revealed that there is a symbiotic relationship between genetics and epigenetics, with genetic variation having genome-wide effects on DNA methylation, and in some cases, DNA methylation acting as a mediator between genotype and phenotype. Approximately 28% of common SNPs can create or delete CpG sites, and these CpG-SNPs include three common SNPs within the *GDF5* gene [84,85]. Genetic variation can also influence methylation of CpG sites, such that there are allelic differences in DNA methylation, with the associated DNA variant referred to as methylation quantitative trait locus (meQTL). meQTLs are common in the human genome and are enriched for non-coding disease SNPs, with 31% of non-MHC autoimmune disease SNPs associated with meQTLs [86]. A small number of meQTLs are accompanied by eQTLs, including 10 blood meQTLs that putatively mediate rheumatoid arthritis genetic susceptibility [87]. meQTLs can be tissue-specific, potentially explaining how the functional effect of genetic variation on gene expression can be limited to a single cell or tissue type.

4.2. DNA methylation as a mediator of OA genetic risk

Three cartilage studies have demonstrated the importance of integrating DNA methylation with genetic and gene expression data in order to investigate the molecular mechanisms mediating OA genetic risk. The first study examined the effect of local genetic variation on methylation levels of CpG sites that correlated with gene expression levels in cartilage [80]. 36CpG sites within 31 genes were identified, and the expression of 26 of these genes was affected by genotype in conjunction with DNA methylation. SNPs that influence DNA methylation may explain some of the missing heritability of common diseases, and as such, the authors suggested that these SNPs may be candidates for further OA genetic studies in larger datasets.

A second study performed a meQTL analysis in OA and non-OA cartilage for CpG sites surrounding 16 European OA genetic loci (see Table 1; [38]). Four meQTLs encompassing nine CpG sites were identified, with the OA risk allele of the *GLT8D1*, *SUPT3H*, *ALDH1A*2 and *GDF5* SNPs associated with reduced methylation of all nine CpG sites. These meQTLs were observed when all cartilage samples were combined and when only OA samples were analysed, indicating that the genetic effect of the OA SNPs on methylation is independent of disease status. Several of the cartilage meQTLs had previously been reported in non-joint tissues, and the *SUPT3H* and *ALDH1A2* meQTLs were also detected in the synovium and intrapatellar fat pad of OA knee patients, highlighting the importance of examining additional joint tissues when functionally analysing OA risk alleles.

Although not associated with a cartilage meQTL, targeted methylation analysis of the *DIO2* gene indicates that DNA methylation mediates the genetic risk of the OA SNP rs225014 at this locus [88]. DIO2 is upregulated in OA cartilage [35] and mice lacking this protein have reduced cartilage damage and synovitis in a mouse model of OA [89]. There is increased transcription of the risk allele relative to the wild type allele in cartilage, which is thought to mediate the OA susceptibility [35]. Risk allele carriers have increased methylation of a CpG site located approximately 2 kb upstream of the *DIO2* transcription start site in eroded cartilage relative to preserved cartilage from the same joint. Methylation of this site positively correlates with cartilage *DIO2* expression in carriers of the risk allele but not in individuals homozygous for the wild type allele, with demethylation of this site leading to reduced *DIO2* expression in OA hip chondrocytes [88].

4.3. DNA methylation as a modulator of the OA genetic risk

The *GDF5* rs143383 SNP is associated with OA and other musculoskeletal phenotypes, including congenital hip dysplasia and meniscal injury [90,91]. There is decreased expression of the OA-

risk T allele relative to the C allele in synovial joint tissues, and this is mediated by the allelic differences in the binding of transcriptional repressors including SP1 and SP3 [13,92]. Although there is no correlation between rs143383 genotype and 5′UTR methylation, DNA methylation affects the allelic imbalance of rs143383 and thus modulates the genetic effect of this SNP on *GDF5* gene expression. Demethylation of the 5′UTR increased expression of the C allele relative to the T allele, whilst methylation preferentially repressed the C allele, reversing the direction of the allelic imbalance [57,93]. This effect is mediated by a CpG site located 4 bp upstream of the rs143383 SNP, which alters SP1 and SP3 binding in an allelic and methylation-dependent manner. This CpG site is significantly demethylated in OA knee cartilage relative to OA hip cartilage, potentially explaining why this SNP is associated specifically with knee OA in Europeans. Furthermore, variability in methylation may underlie the inter- and intra-individual differences in the level of rs143383 allelic imbalance, potentially explaining the incomplete penetrance of diseases associated with this SNP.

The studies discussed above clearly underline that genetic and epigenetic mechanisms of gene regulation are not independent of each other, and that DNA methylation can mediate or modulate the functional effect of genetic loci on disease susceptibility. Methylation can be influenced by lifestyle and age, such that individuals with the same genotype have different phenotypes. Within an individual, the functional effect of a disease SNP may be limited to a specific developmental time point due to age-related DNA methylation changes as observed for the *NDUFB6* rs629566 SNP in human skeletal muscle [94]. DNA methylation analysis can help fine-map susceptibility variants and identify their target loci [95], and can also be used to predict chromatin interactions in primary tissues [96]. Therefore, it will be crucial for future genetic studies to integrate epigenetic data in order to elucidate the molecular events underlying OA disease susceptibility, penetrance and severity.

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