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DNA methylation in osteoarthritic chondrocytes: a new molecular target

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Summary

Objective: To review the current knowledge of the mechanism of DNA methylation, its association with transcriptional silencing, possible mechanisms of hyper- and hypomethylation and how epigenetic changes may relate to the pathogenesis of osteoarthritis (OA).

Methods: Journal literature was searched using Pubmed. Since there are very few publications directly on epigenetic phenomena in OA, the search was extended to give an overview of epigenetic mechanisms as they relate to the molecular mechanisms of the disease.

Results: While the epigenetics of cancer cells have been intensively investigated, little attention has so far been paid as to whether epigenetic changes contribute to the pathology of non-neoplastic diseases such as OA. This review explains the mechanisms of DNA methylation, its role in transcriptional regulation, and possible demethylation mechanisms that may be applicable to OA. Preliminary evidence suggests that changes in DNA methylation, together with cytokines, growth factors and changes in matrix composition, are likely to be important in determining the complex gene expression patterns that are observed in osteoarthritic chondrocytes.

Conclusion: Early evidence points to a role of epigenetics in the pathogenesis of OA. Since epigenetic changes, although heritable at the cellular level, are potentially reversible, epigenetics could be a new molecular target for therapeutic intervention, especially early in the disease.

Key words: Osteoarthritis, Cartilage articular, Epigenetics, DNA methylation, Matrix metalloproteases, Inflammatory cytokines.

Abbreviations

#NOF	fracture-neck-of-femur
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
CpG	cytosine–phosphate–guanine
Dnmt	DNA methyltransferase
EBNA	EBV nuclear binding factor
EBV	Epstein–Barr virus
GR	glucocorticoid receptor
GRU	glucocorticoid responsive unit
IL-1β	interleukin-1 beta
MBD	methyl-CpG-binding domain
^{Me}C	methylated cytosine
MeCP	methyl-CpG-binding protein
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappaB
NO	nitric oxide
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
oriP	plasmid containing the latent replication origin
PCNA	proliferating cell nuclear antigen
SAM	*S*-adenosyl-methionine
Sp1	specific protein 1
Tat	tyrosine aminotransferase
TNF	tumor necrosis factor
κB	kappaB

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Introduction

Osteoarthritis (OA) represents a disease group with different underlying pathophysiological mechanisms, however, one common key event is the destruction of the extracellular matrix of articular cartilage by aggrecanases and collagenases. There are two possible sources for these enzymes: the synovium and the articular chondrocytes. It has been shown that degradative enzymes are present in the inflamed synovium. Inflammatory episodes could be important for the initiation of the disease or a low level of inflammation could contribute to progression of the disease. During OA, an increasing number of articular chondrocytes undergo a phenotypic modulation[1] either to cells with hypertrophic characteristics[2,3] or to cells that may be regarded as 'degradative' chondrocytes[4]. These latter cells can synthesize all the known cartilage-degrading proteases and thus probably play a decisive role in the progression of the disease. The phenotype of normal chondrocytes, as that of all adult somatic cells, is stabilized by the epigenetic status of the cell. This raises the question as to whether the destabilization of the chondrocytic phenotype in OA is at least in part the result of changes in this epigenetic status. While

the role played by epigenetics in cancer has been investigated extensively[5–7], few studies have addressed the question whether epigenetic changes contribute to the pathogenesis of other diseases such as OA. This review presents and discusses what is known about pathological changes in epigenetic status and whether this might be of relevance for OA.

Methods

We used Pubmed (www.ncbi.nlm.nih.gov/entrez) as the search engine. Initially, we searched for "DNA methylation + chondrocytes" and "DNA methylation + OA". This was followed by searches for review papers regarding the mechanism of DNA methylation, using the search combinations "epigenetics + review" and "DNA methylation + review". No specific date limits were applied, but more recently published papers were selected to a greater extent. Many references cited in the primary papers were also read. Some topics, which are of particular relevance for OA were followed in greater depth, e.g., "NF-κB + methylation" and "IL-1β + methylation". From all available papers a selection was made, judged according to relevance and importance.

Results

WHAT IS EPIGENETICS?

Epigenetics (literally "beyond genetics") is defined as heritable changes in DNA without changes in the sequence. While the genetic code is identical for every somatic cell in the body, epigenetic changes are generally confined to specific cells/tissues or even cells within a tissue. Epigenetic control mechanisms involve modifications of chromatin. Broadly speaking, there are two major epigenetic modifications: (1) DNA methylation and (2) histone modifications, such as acetylation, methylation and phosphorylation. Both are parts of the same epigenetic program and usually work hand-in-hand in gene-silencing[8,9]. On the whole, conformationally relaxed chromatin (euchromatin) indicates transcriptionally active regions and is associated with hypomethylated DNA and acetylated histones, whereas compact chromatin (heterochromatin) is transcriptionally silent, hypermethylated and bound to non-acetylated histones. Since histone acetylation and chromatin condensations are frequently a consequence of DNA methylation, the latter is probably the principal mechanism by which cells maintains a stable chromatin configuration that represses transcription. Methylation patterns are normally stable, and inherited upon mitosis in adult cells. Deviations are associated with diseases (recently reviewed in Refs. 10–13).

DNA methylation occurs through the addition of a methyl group to cytosine (C) to form methylated cytosine (^{Me}C). This happens particularly to those cytosines that are 5′ to guanines (G), the so-called CpG sites, where 'p' represents the phosphate connecting the two nucleotides. This is mediated by enzymes known as *DN*A *m*ethyl*t*ransferases (Dnmts), which catalyze the transfer of a methyl group from *S*-adenosyl-methionine (SAM) to the cytosine base to form ^{Me}C (see Fig. 1). Methyl donors are folic acid and choline, which are dietary requirements, as are the co-factors vitamins B6 and B12 and zinc. Several methyltransferases have been identified: Dnmt1, Dnmt3a and Dnmt3b and the splice variants Dmnt1a and Dmnt1b (reviewed by Pradhan and Esteve[14]). Dnmt1 is responsible for maintaining established methylation patterns during cell division.

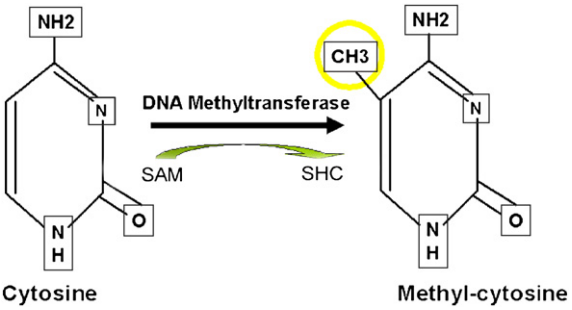

Fig. 1. The chemistry of DNA methylation. SAM is the methyl donor and becomes converted to *S*-adenosyl homocysteine through the action of DNA methyltransferases.

Expression is induced at the entrance into the S-phase[15], its abundance is reduced to low levels at the G0-phase and the enzyme is not detectable in growth-arrested cells. During mitosis, Dmnt1 is targeted to replication foci through its interaction with the DNA polymerase clamp proliferating cell nuclear antigen (PCNA)[16,17], detects hemi-methylated CpGs in the parent DNA strand and replicates the methylation pattern on the newly-formed strand. Dnmt3a and Dnmt3b are known to play key roles in the *de novo* methylation of primarily unmethylated DNA and are important in establishing the methylation pattern during development[14]. In early fetal development, tissue differentiation is to a large extent determined through mechanisms involving methylation changes of the genomic DNA, resulting in phenotype-specific activation of expressed genes together with silencing of non-expressed genes.

About 70% of CpG sites within the mammalian genome are methylated[6]. It is thought that methylation evolved initially as a protective mechanism in limiting expression of foreign DNA, such as transposons, intragenomic parasites and proviral DNA, which threatens the orderly expression of the genome. Around 40% of human DNA consists of such 'junk' DNA, thus silencing this potentially harmful DNA by methylation is of obvious importance for host defense. However, DNA methylation is also essential for normal cellular functions, in particular imprinting of specific genes, X chromosome inactivation in the female and, most importantly in the present context, cell-type specific gene expression through permanent silencing of all genes that are not expressed in a particular somatic cell.

THE ROLE OF EPIGENETICS IN TRANSCRIPTIONAL REGULATION

Gene expression is controlled by multiple mechanisms: (1) at a genetic level, the DNA sequence of the promoter and other regulatory regions is important, in particular which binding sites for regulatory proteins are present; (2) at the molecular level, the availability of nuclear regulatory factors, such as transcription factors, enhancers and suppressors will influence gene transcription; and (3) at an epigenetic level, it will be determined whether or not the gene is permanently silenced in the particular somatic cell so that neither (1) nor (2) can play a role. For example, the silencing of interferon regulatory factor 4 in leukemic cells was due to hypermethylation of CpG sites in the promoter[18]. The opposite situation occurs during muscle differentiation, where loss of methylation at one specific CCGG site in the promoter region of myogenin was correlated with transcriptional

activation of this gene[19]. The fact that methylation can override the activities of transcription factors is illustrated by the regulation of the Apaf-1 gene, which plays a central role in DNA damage-induced apoptosis[20]. Some leukemia cell lines express the factor, while others do not. The expression of factors regulating Apaf-1 expression, such as E2F-1, p53 and specific protein 1 (Sp1), did not differ between Apaf-1 positive and Apaf-1 negative cells, and the inactivation could be attributed to silencing by methylation[20]. Other examples of genes, whose tissue-specific expression has been shown to be controlled by DNA methylation, can be found in the review by Ehrlich[21].

How does methylation silence gene transcription? There are three possibilities (see Fig. 2): (1) access of transcription factors may be prevented directly by the presence of methyl-groups; (2) the latter attract methyl-binding proteins and histone deacetylases, which itself may prevent transcription factor binding and/or (3) cause remodelling to transcriptionally inactive heterochromatin (reviewed in Refs. 10,22–24).

Several methyl-CpG binding domains (MBD) have been identified (reviewed in Refs. 25,26), some of which form part of methyl-CpG-binding protein (MeCP) complexes. Of particular importance with regard to transcriptional repression are MeCP1 and MeCP2[27]. Both contain transcriptional repression domains and are associated with histone deacetylase[28,29]. MeCP1 contains MBD2, while MeCP2 contains the transcriptional suppressor mSin3A[29]. These methyl-binding proteins are preferentially recruited to methylated DNA and their associated histone deacetylase activity leads, under normal circumstances, to hypoacetylated histones[30]. Methyl-binding proteins thus link these two epigenetic processes. Trichostatin A, a specific inhibitor of histone deacetylase, can reverse the process, which may lead to upregulation of non-methylated or sparely methylated promoters, but not hypermethylated genes, indicating that CpG island methylation is dominant for a silent state[30]. On the other hand, some studies suggest that histone modifications are a pre-requisite for DNA methylation, suggesting that histone modifications are the primary events in gene silencing[31,32,8].

Whatever the sequence of events in gene silencing, the methylation status in the promoter regions determines whether the gene is silenced or can be transcribed if the appropriate regulatory factors are present. Promoters vary considerably with regard to the number of available CpG sites. At one extreme are those that contain only a few CpG sites. These are the so-called 'sparse CpG' promoters (Fig. 3 left-hand side). For example, only seven CpG sites are found in the 2000 bp 5′ flanking region of matrix metalloproteinase (MMP)-3. Similarly, the degradative proteases MMP-9, -13 and the aggrecanase ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs) have sparse CpG promoters[33]. At the other extreme, some promoters have a high concentration of CpGs, the so-called "CpG island" promoters (Fig. 3 right-hand side). For example, in the collagen type II α1 gene there are 65 CpGs in the 850 bp region upstream of the start of exon1. All housekeeping and tumor suppressor genes have CpG islands in their promoters and these islands contain multiple binding sites for transcription factors[34], especially for Sp1[35,36]. Binding of Sp1 keeps the islands methylation-free, which makes intuitive sense, since these genes must be expressed in all cells. About half of the mammalian genes have CpG islands within or near their promotor regions [37]. The remaining CpG-elements are found more or less randomly distributed throughout the genome.

The efficiency of repression by DNA methylation may depend on (1) methylation of a single crucial CpG site[38]; (2) the total number of methylated CpGs; or (3) the presence of an enhancer element[39,40], see Fig. 3. It is of note that the two methyl-CpG-binding proteins MeCP1 and MeCP2 differ in their preference for CpG sites. MeCP1 requires multiple, closely spaced CpGs and thus binds preferentially to CpG island promoters, but MeCP2 binds preferentially to single CpG sites, where it covers approximately 12 bp of DNA[41,27]. If the promoter is a weak promoter with no enhancer element, then methylation will repress transcription in both sparse and island promoters [Fig. 3(B)]. However, if the promoter contains a strong enhancer element [Fig. 3(C)], transcription may not be repressed in a gene with a sparse CpG promoter, possibly because the enhancer element prevents binding of the methyl-binding proteins. However, transcription will continue to be repressed in a gene with an island promoter even in the presence of an enhancer element. It should be noted that not all CpGs

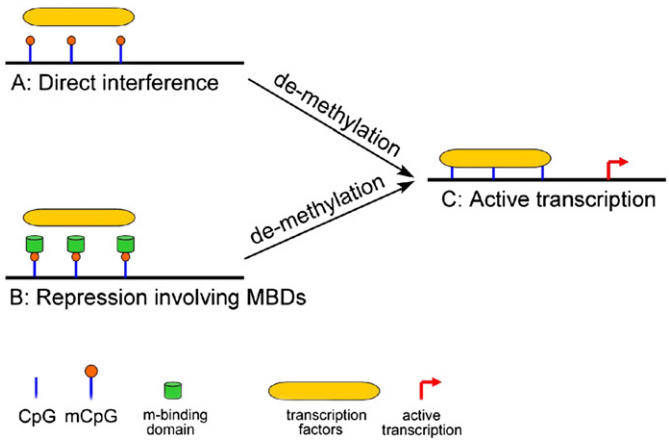

Fig. 2. Mechanisms of silencing. (A) Binding of transcription factors is prevented directly by the presence of methyl groups on CpG sites. (B) Methyl-binding domains have attached to the methylated cytosines and this prevents binding of transcription factors. A third mechanism is the tight packaging of methylated DNA, which is generally associated with methylated DNA and deacetylated histones (not shown). (C) Demethylation would be required for active transcription of the gene.

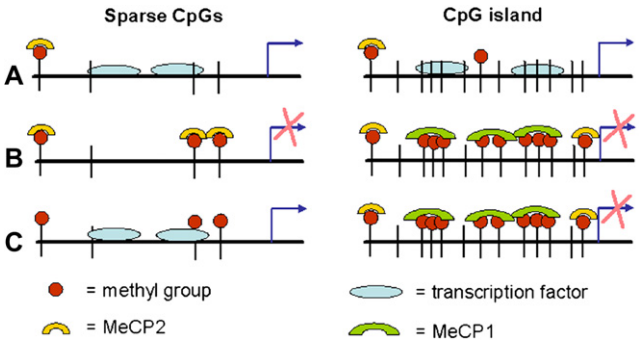

Fig. 3. The effects of methylation on transcription in two types of promoters. Non-methylated CpG sites are shown as vertical lines with red circles illustrating methylated CpGs. A sparse CpG promoter is illustrated on the left, an island promoter on the right. (A) If the majority of CpGs is not methylated in either promoter, transcription factors (blue ovals) can bind and initiate transcription. Note that an occasional methylated CpG may not be sufficient to prevent transcription. (B) Here most CpGs are methylated, which attract methyl-binding proteins. MeCP2 (yellow) is capable of binding to individual CpGs, while MeCP1 (green) requires several closely spaced CpGs. This prevents transcription factors binding and thus transcription in either promoter. (C) If the promoter contains a strong enhancer element, upstream or downstream from the promoter region (not shown in this diagram), then this may overcome the silencing effects of CpG methylation in sparse CpG promoters, but not in island promoters. The enhancer may act by preventing the binding of methyl-binding proteins (as shown in the diagram).

need to be unmethylated for transcription to take place, neither do all CpGs require methylation for repression of transcription. Binding of methyl-binding proteins will promote chromatin condensation into an inactive conformation so that gene expression can be affected at a distance from the methylated region[25].

Examination of methylation status in promoters with sparse CpGs has been neglected, largely because it was thought that methylation of many CpGs was needed to repress gene transcription. This may be true for promoters with enhancer elements, but, as Fig. 3(B) illustrates, silencing of genes by DNA methylation is feasible in genes with sparse CpG promoters. It is possible that the likelihood of pathological demethylation is greater in sparse island promoters.

DEMETHYLATION MECHANISMS

In essence, demethylation of the DNA can occur through passive or active mechanisms. Active mechanisms would involve a direct removal of the methylated moieties, while passive mechanism would involve inhibition of the maintenance methylation during cell division[42]. Both active and passive mechanisms are thought to occur during the epigenetic reprogramming that occurs in the zygote after fertilization[43]. The paternal genome is rapidly demethylated in the fertilized oocyte without cell division, hence must involve active demethylation[44], whereas methylation of the maternal genome is lost more gradually between the one-cell and eight-cell stage, suggesting passive demethylation[45,43].

Passive demethylation: This results if Dnmt1 is not able to catalyze the methylation of the daughter DNA strand. The may be due to the presence of DNA binding proteins or factors inhibiting the enzyme directly. It is theoretically feasible for specific transcription factors to bind to the DNA and prevent methylation of the newly-synthesized strand at a specific and limited site only, see Fig. 4(A). On the other hand, there maybe an overall inhibition of the enzyme, resulting in complete non-methylation of new strand, see Fig. 4(B).

This mechanism does not require any demethylases, but is comparatively inefficient, as it is dependent on several replication cycles: three rounds of replication demethylate only 87% of CpGs[46]. Such mechanism would thus not be suitable for rapid reprogramming of gene expression. The experimental demethylation agent 5-aza-cytidine or 5-aza-2′-deoxycytidine mimics passive demethylation by the second mechanism.

Active demethylation: Theoretically, there are three possible biochemical mechanisms: (1) a direct replacement of the methyl group with hydrogen; (2) excision of the methylated base while keeping the phosphodiester backbone intact; or (3) excision of the methylated cytosine[46], possibly together with adjacent nucleotides (see Fig. 5).

However, enzymes with relevant activities have been rather elusive. Although the methyl-binding domain MBD2 was credited with a demethylase activity that cleaved the methyl residue[47,48], this was not confirmed by other

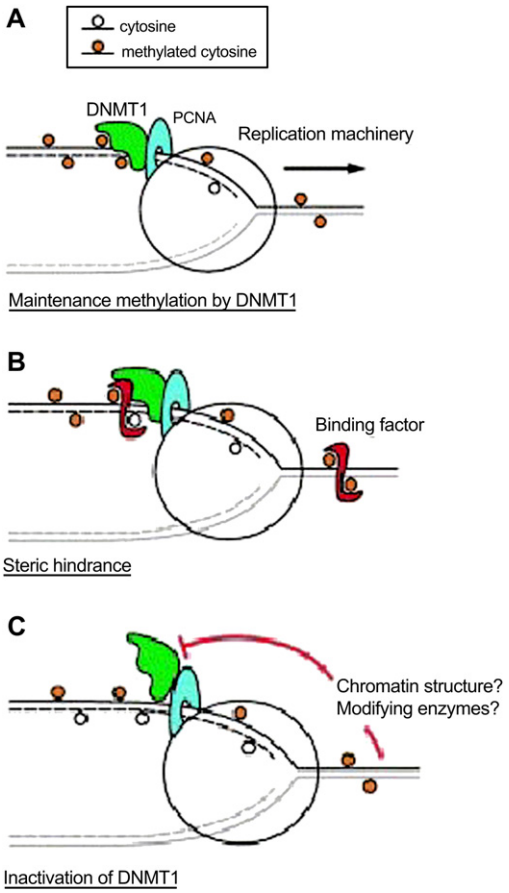

Fig. 4. (A) Following DNA replication, the methylation status is normally faithfully reproduced by the maintenance Dnmt1, which associates with the DNA clamp PCNA. (B) Specific DNA binding factors may prevent the methylation of specific CpG sites by temporarily inhibiting Dnmt1 through steric hindrance or (C) the enzyme itself may be completely inhibited so that no cytosines are methylated on the new DNA strand (reproduced with permission from Ref. 46).

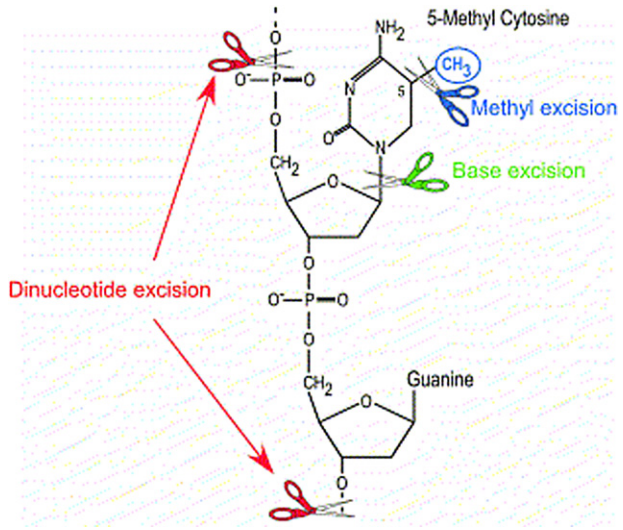

Fig. 5. Possible levels of action of demethylases: (1) methyl excision; (2) excision of the methylated base; and (3) excision of the nucleotide, maybe together with adjacent nucleotides, i.e., the whole CpG element (reproduced with permission from Ref. 46).

groups[28]. The third mechanism would result in DNA strand breaks and if the nucleotides on both strands were excised simultaneously, the total structure would be disrupted. So the second mechanism appears the most feasible. Indeed, there is evidence for a mechanism that resembles "base excision DNA repair" activity. In chick embryos, a ^{Me}C-DNA glycosylase has been identified, which acts preferentially on hemi-methylated DNA (which could have resulted from passive demethylation) and initiates demethylation by breaking the glycosidic bone of ^{Me}C[49]. Interestingly, the ^{Me}C-DNA glycosylase co-purifies with the guanine/thymidine (G/T) mismatch DNA glycosylase[50]. In humans, ^{Me}C-DNA glycosylase as well as the G/T mismatch glycosylase activity is associated with the MBD4 complex[51] as well as PCNA[52], binding selectively to hemi-methylated DNA, and initiating demethylation of the still methylated strand.

Probably the most efficient way to achieve demethylation of both strands is a passive–active mechanism, where inhibition of Dnmt1 is followed by activity of the ^{Me}C-DNA glycosylase and associated factors. This would achieve demethylation in one round of cell division and avoid double-stranded DNA breaks. The fact that ^{Me}C-DNA glycosylase catalyzes demethylation preferentially on hemi-methylated DNA strands supports this notion.

IS EPIGENETICS INVOLVED IN THE DEVELOPMENT AND PROGRESSION OF OA?

Alterations in epigenetic state are likely to have occurred in situations where there is a fundamental change in the gene expression repertoire (not just a single gene) *and* this changed expression is transmitted to daughter cells. In normal articular chondrocytes, most proteases are not expressed, hence these genes would be expected to be silenced by DNA methylation. However, osteoarthritic chondrocytes neo-express many genes involved in cartilage catabolism, for example matrix-degrading enzymes such as MMP-2[53], MMP-9[54–57], MMP-13[58–60] and ADAMTS-4 and -5[60,61]. These enzymes are expressed predominantly by chondrocytes in the superficial region that have started to proliferate and formed clones[62,4].

Expression might start in a few isolated cells near the surface; these cells might divide to produce doublets, quadruplets, etc. and, finally clusters of many cells. This is illustrated in Fig. 6. OA cartilage is graded in histological sections, using either the Mankin score (ranging from 0 to 14) or the more recently developed Osteoarthritis Research Society International (OARSI) grade (ranging from 0 to 6)[63]. When the Mankin score/OARSI grade is low, MMP-positive chondrocytes are present as single cells or as one cell of a cell doublet near the surface [Fig. 6(A)]. This figure shows that with increasing OA grade, cells divide to become doublets (B) and quadruplets (C) until, in high-grade OA, all cells are in the form of immunopositive clones [(D), shown for MMP-9, but also found for MMP-3, -13 and ADAMTS-4[4]]. One possible explanation for this is that whatever fundamental change had occurred in individual chondrocytes to cause the expression of aberrant genes, this change was transmitted to the daughter cells during cell division, i.e., the change was heritable at a cellular level, consistent with an epigenetic change. The challenge is obviously to investigate whether the *de novo* expression of aberrant gene was indeed accompanied by loss of DNA methylation in the relevant promoter region.

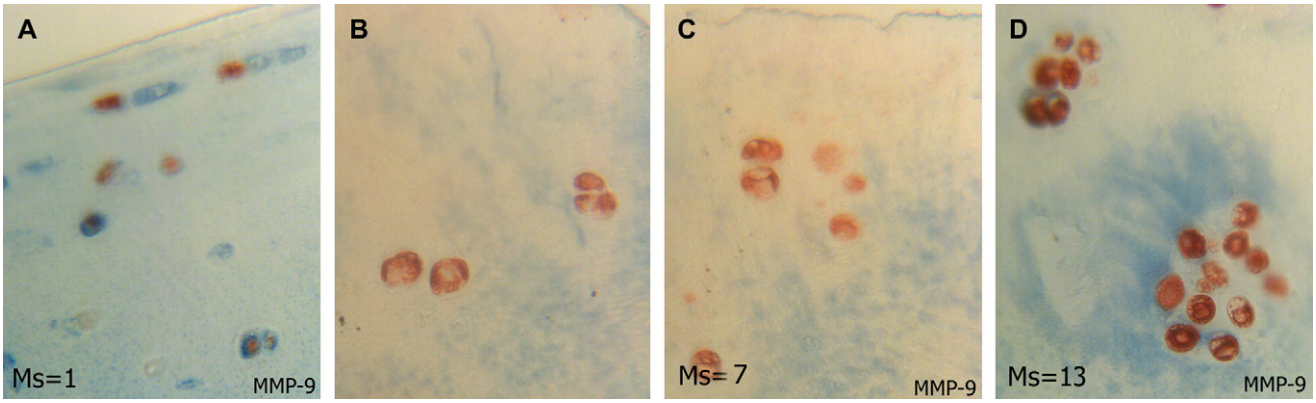

Fig. 6. Increase in the number of MMP-9 positive chondrocytes with increasing Mankin score. (A) At MS = 1, only a few single cells are immunopositive. (B) The positive cells are present in doublets at MS = 4. (C) Quadruplets are seen at MS = 7, while (D) in severe OA (MS > 10), all cells of the typical clones are immunopositive for MMP-9 (reprinted with permission from Ref. 4).

ARE CHANGES IN DNA METHYLATION INVOLVED INTO THE DEVELOPMENT AND PROGRESSION OF OA?

Aging is the most prominent risk factor for OA, but the explanations for this strong association remain largely unclear with some concepts for cellular changes emerging more recently (for review see Aigner *et al.*[64]). We would like to propose that changes in DNA methylation are not only part of these age-related changes within the cells but may also follow exposure to inflammatory cytokines, such as interleukin-1 (IL-1) β and tumor necrosis factor (TNF) α. These cytokines down-regulate the expression of chondrocytic genes, but induce expression of several matrix-degrading proteases when added to monolayer cultures of human or bovine articular chondrocytes (for reviews, see Refs. 65–68). Experimental data suggest that IL-1β is also produced *in vivo* by those OA chondrocytes that are positive for proteases[69]. Could cytokine induced changes in gene expression pattern include changes in DNA methylation status? This has not yet been investigated in OA, but, as outlined below, there is indeed some preliminary evidence for a link between cytokine activity and both hypermethylation of CpG islands as well as loss of methylation.

An example of IL-1β-induced methylation-dependent gene silencing in a CpG island promoter is the fragile X mental retardation one gene that is located in the active X chromosome[70]. In fragile X syndrome, this gene is abnormally hypermethylated, which leads to repression and characterizes (together with expansion of CGG repeats) the syndrome. IL-1β repressed the gene by hypermethylation of the CpG island, an effect that was mediated *via* nitric oxide (NO), since NO donors also caused gene silencing, whereas inducible NO synthase inhibitors prevented the IL-1β mediated repression and hypermethylation. Both NO and IL-1β seemed to act by increasing the activity, but not gene expression, of a DNA methyltransferase, presumably one of the *de novo* methyltransferases Dnmt3a or Dnmt3b.

Interestingly, those chondrocytic genes that might be repressed by IL-1β and/or TNFα during OA, for example, collagen II and aggrecan, also contain CpG islands in their promoters and NO is also induced in OA[71,72]. However, there is no evidence to date that repression of chondrocytic genes involves hypermethylation of the CpG islands. Poschl *et al.*[73] investigated whether the *loss* of aggrecan expression in OA was linked to *increased* methylation of the promoter and could not demonstrate hypermethylation as a possible cause for silencing of this chondrocytic gene. However, only a relatively short region of the promoter was examined and the possibility that changes in DNA methylation at non-examined CpG sites have taken place cannot, as yet, be excluded.

Data on DNA demethylation in relation to arthritis are also sparse. Kim *et al.*[74] found that inflammatory arthritis was associated with overall DNA hypomethylation in peripheral blood mononuclear cells, but did not investigate specific genes or chondrocytes. The first specific gene examined was MMP-9, whose expression is increased in OA[54]. The 670 bp promoter sequence[75] contains just six CpG sites. Using the methylation-sensitive restriction enzyme method, Roach *et al.* were able to demonstrate a difference in methylation status of AciI digestible sites: three CpG sites in the proximal promoter and two CpG sites in the distal promoter of MMP-9 were methylated in 4/5 control patients, but that at least one of these sites was unmethylated in 8/9 OA patients[33]. Further studies investigated the methylation status of the promoters of MMP-3, -13 and ADAM-TS4 in addition to MMP-9. Notably, all these promoters contain relatively few CpGs, which, as already mentioned, might favor pathological demethylation. The methylation status of these CpGs was examined in DNA extracted from the superficial zone of OA cartilage (which contained the protease-expressing chondrocytes) and, for controls, the deep zone from fracture-neck-of-femur (#NOF) patients (Fig. 7: presence of methylation is indicated by presence of bands and *vice versa*). The expectation had been that all CpG sites would be methylated in control samples and unmethylated in OA samples. This was not the case. Specific CpG

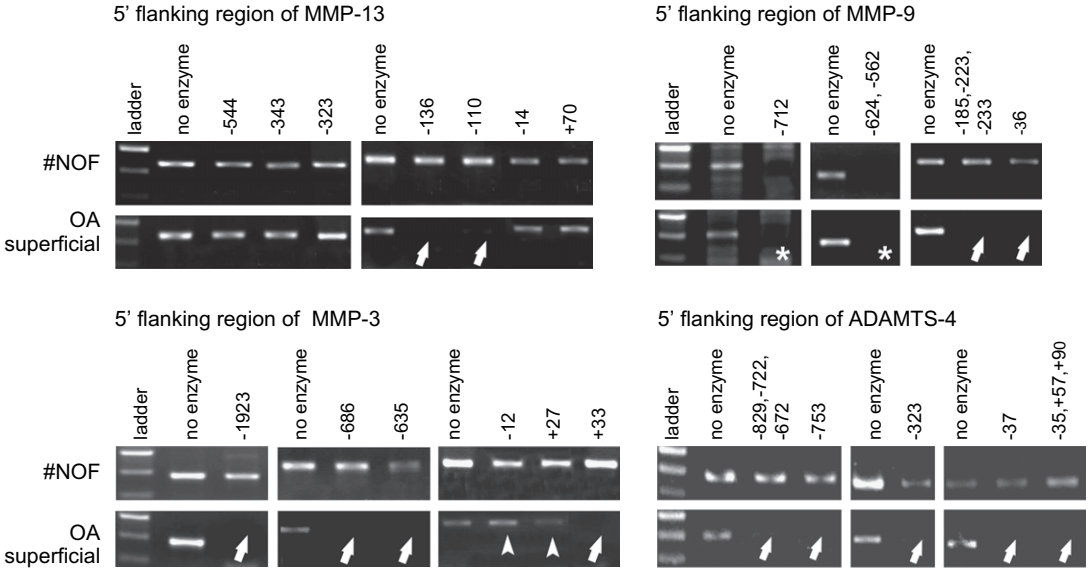

Fig. 7. Representative results of PCR amplifications of the promoter regions with or without digestion by methylation-sensitive restriction enzymes. A typical pattern of reactions is shown for DNA extracted from the deep zone of a patient with femoral neck fracture (#NOF) and from the surface zone around the weight-bearing area of an OA patient. The ladder shows 200 bp intervals and the location of CpG sites is indicated by the numbers (reprinted with permission from Ref. 4).

sites differed in their susceptibility to demethylation, ranging from sites with ubiquitous methylation in all patients (Fig. 7: arrow heads) to sites with no methylation in both control and OA samples (Fig. 7: *). However, there were CpG sites, where loss of methylation had occurred in most OA patients, whereas methylation was present in most #NOF patients (Fig. 7: arrows). It is feasible, though not yet proven, that these specific CpG sites are crucial for the epigenetic regulation of gene transcription, possibly along with the overall levels of methylated CpGs, since the overall percentage of non-methylated CpGs increased from 20% in controls to 48% in the degradative chondrocytes of OA patients. However, there was considerable variation between patients and, although these studies have shown an association between methylation status and gene expression, it has not yet been proven that loss of methylation is the cause of transcriptional activation for these degradative enzymes.

POSSIBLE DEMETHYLATION MECHANISMS IN OA

If demethylation of specific CpG sites was responsible for permitting aberrant gene transcription, by what mechanisms could this occur? One possibility is a random loss of methylation simply due to aging[6,34,76] or cellular senescence[77,78] *via* passive demethylation. However, the overall methylation content in OA cartilage does not change (unpublished data) and there does not appear to be random loss of methylation in all genes. So could demethylation mechanisms be targeted to specific genes? So far no studies have been carried out to determine this in chondrocytes or OA. There are, however, several studies is other systems, whose results are of importance inasmuch as they illustrate possible mechanisms that might also be applicable to OA.

It was stated earlier that methylation generally prevents binding of transcription factors and other DNA binding proteins. However, not all binding is inhibited by DNA methylation and binding of certain DNA binding proteins can actually initiate demethylation. Hsieh[79] used plasmids containing the latent replication origin (oriP) of the Epstein–Barr virus (EBV), transfected into human cells expressing the EBV nuclear binding factor EBNA-1. In this system an artificial CpG methylation pattern was generated in the plasmids *in vitro* by using the *Fnu*II, *Hha*I, *Hpa*II and *Sss*I methylases, which methylate the CpGs located near their respective recognition sequences or, in the case of *Sss*I, at all CpG sites. This pattern was maintained for months after transfection into human cells, confirming that the maintenance methyltransferase could maintain the artificially created CpG pattern during cell divisions. However, three *Hpa*II sites (CCGG) in the oriP region always became demethylated very quickly after transfection into the human cells expressing EBNA-1, but one *Hpa*II site did not. No EBNA-1 binding sites were present near this latter *Hpa*II, whereas the other three were close to several EBNA-1 binding motifs. Neither replication alone nor binding of EBNA-1 alone led to demethylation, but binding of EBNA-1 followed by replication led to demethylation of the CpGs at the three CCGG sites, but not at CpG sites adjacent to or in between the CCGG locations. Hemi-methylated DNA was detected after one round of replication, but demethylation of the second strand probably occurred without further replication, implicating a passive–active mechanism[79]. This study thus provided clear evidence that specific DNA binding proteins can protect specific CpGs from the activities of the maintenance Dnmt1, resulting in passive methylation of the first strand, possibly followed by active demethylation of the second strand.

The previous example depended on artificially methylated constructs. An example where DNA binding proteins achieved demethylation of specific CpGs in a tissue-specific gene in its natural chromatin context is the demethylation within an enhancer element of the rat liver-specific tyrosine aminotransferase (Tat) gene during development[80]. This gene is induced at birth by glucocorticoid in response to hypoglycemia, but is not inducible before birth. Induction is facilitated by two glucocorticoid responsive units (GRU) located at −2.5 bp and −5.5 bp. These GRUs contain numerous and overlapping sites for glucocorticoid receptor (GR) and transcription factors, such as C/EBP, HNF-3 and ets families. The −2.5 bp GRU also contains three closely spaced CpG sites, plus a fourth at −2341. Before birth, when the Tat gene is not inducible, these CpG sites are methylated, but non-methylated after birth. Thomassin *et al.*[80] treated rat hepatoma cells with dexamethasone. Short-term (<1 h) treatment caused reversible chromatin remodelling and HNF-3 binding, whereas prolonged treatment over 3 days resulted in progressive demethylation at all three sites as well as the fourth more distal CpG site, which was still within the 350 bp area of chromatin remodelling. These CpG sites were not re-methylated during subsequent 3 months culture without dex, as would be expected if a change in methylation status had occurred. If naïve methylated cells or demethylated cells were treated with glucocorticoid, transcription induction was faster and stronger in the demethylated cells. Moreover, binding of two further transcription factors was only possible in the demethylated cells. The authors propose that initial glucocorticoid and HNF-3 binding just prior to birth induce loss of methylation that primes the GRU to subsequent induction in response to hypoglycemia after birth.

These ground-breaking studies suggest that one mechanism for demethylation at defined CpG sites in specific genes involves specific DNA binding proteins/transcription factors that initially prevent Dnmt1 from methylating the cytosine on the newly-synthesized strand during one round of cell division, followed by active demethylation of the second strand. Could such a mechanism be involved in the demethylation of specific CpGs in the promoters of the aberrantly expressed degradative enzymes? At present, we do not yet know the answer, but the results from a study of B-cell maturation by Kirillov *et al.*[81] are of relevance. The immunoglobulin κ gene is demethylated at specific CpG sites during B-cell maturation, a process driven by several *cis*-acting regulatory elements, to which specific *trans*-acting factors bind in a stage and tissue-specific manner. Using mutation and complementation analysis, these authors showed that demethylation was dependent on a kappaB (κB) binding site and presence of the rel/NF-κB family of transcription factors. Cell lines with defective nuclear κB-binding proteins were unable to carry out demethylation, but this was restored by introducing relB.

Many of the degradative proteases that are induced in OA have NF-κB binding sites in their promoters. Moreover, NF-κB is activated by cytokines such as IL-1β[82–85] and TNFα. Hence NF-κB could be one of the DNA binding factors that initiates DNA demethylation, but other factors, such as members of the ets family, could also be involved. Clearly further research is required to determine the interrelations between the signal transduction of IL-1β and TNFα (and perhaps other inflammatory cytokines) and DNA methylation status of specific genes in chondrocytes during OA.

Conclusions

Methylation of genomic DNA clearly represents a very important mechanism in order to determine tissue and cell differentiation. DNA methylation is not so much a tool for the regulation of single genes, rather it is involved in the setup of a cellular phenotype, implicating as such fixed expression levels of many genes. DNA methylation is heritable and transmitted to daughter cells. This means that, under normal circumstances, a particular phenotype is stably propagated. However, when aberrant changes in methylation do occur, then these changes are also propagated to daughter cells, since a particular cell type has no way of "remembering" what its appropriate methylation status ought to be. Aberrant methylation patterns are involved in important pathologies such as tumorigenesis and complex non-Mendelian diseases, such as Alzheimer's, lupus erythematosus or psychiatric disorders. First experimental evidence suggests that changes in methylation patterns could also underlie the changed gene expression patterns observed in OA. To what extent this is the case and whether this can be influenced in terms of therapy should be a major interest for further research.

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