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Potential directions for drug development for osteoarthritis

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Background: Osteoarthritis (OA) is a frustrating disease for both patient and physician because neither cause nor cure is known and there are currently no disease-modifying drugs. *Objective*: To review current therapeutic approaches as well as new findings regarding OA pathoetiology that could form the basis of future direction for the development of drugs to prevent or slow down disease progression. *Methods*: After reviewing disease progression in human OA, as demonstrated by histological analyses, the reasons for cartilage erosion are explored and possible therapeutic approaches are highlighted. *Results/conclusions*: OA may be an epigenetic disease. This new concept can explain many aspects of the disease and provide reasons why therapeutic approaches until now have met with little success.

Keywords: aggrecanases, articular chondrocyte, DNA methylation, drug development, epigenetics, matrix metalloproteases, osteoarthritis, pathoetiology

Expert Opin. Drug Discov. (2008) 3(5):1-12

1. Introduction

Osteoarthritis (OA) is a degenerative condition that is caused by erosion of articular cartilage at diarthrodial joints [1-3]. As a result cartilage can no longer fulfill its role as shock absorber. It is one of the most common disabling conditions of elderly people in the Western world. As people reach retirement age, 60% of men and 70% of women will suffer from OA to some extent [4]. Although the disease does not cause death, it severely affects the quality of life by making movement difficult and painful and significantly limiting everyday activities. As life expectancy increases, the number of people seeking relief from the pain and lack of mobility due to OA will increase every year. Joint replacements, so far the only 'cure' for arthritis, have met with enormous success and have transformed the lives of millions of people. But they constitute drastic surgical intervention and, on average, last only 15 years, when revisions may become necessary. Therefore, a non-surgical treatment that either prevents OA or significantly slows progression would be of enormous benefit. Current treatment regimes (paracetemol, NSAIDs, Cox-2 inhibitors, viscosupplementation with hyaluronans [5,6]) can do no more than ameliorate the symptoms, such as pain and inflammation, but do not prevent the underlying tissue catabolism, so the disease still progresses. The holy grail of future drug development for OA would be disease-modifying drugs [7,8], analogous to the disease-modifying antirheumatic drugs in the treatment of rheumatoid arthritis [9,10].

2. Pathoetiology of OA

Any search for disease-modifying drugs for OA must start with a thorough understanding of the changes that characterize the disease process in humans. Animal models of OA have been useful but do not replicate several features

of the human disease, such as the decade-long progression or the formation of clusters of abnormal chondrocytes (see Section 2.5).

2.1 Changes in the cartilage matrix in OA

As already mentioned, the key feature of OA is the loss of shock-absorbing capacity of articular cartilage, a result of the destruction of the extracellular matrix (ECM). Other joint tissues, that is, the synovial capsule, the ligaments, and the subchondral bone, are also affected, but it is the erosion of cartilage that causes the loss of mobility and pain. To understand why cartilage erosion has such a devastating effect means understanding the function of the components of the ECM. Cartilage is a highly hydrated gel in which a small number (< 3%) of chondrocytes are embedded. Approximately 70 – 80% of the wet weight of cartilage is water [11], which is bound to the highly negatively charged aggrecan molecules that in turn swell. The amount it swells depends on the collagen network, which is comprised of type II collagen along with collagen types IX and XI. The latter provide tensile strength and compressive stiffness, whereas aggrecan and water provide elasticity. Other minor collagens, types III, VI, XII and XIV, also play a role in the cartilage matrix, as do other collagen-binding small proteoglycans, such as decorin, fibromodulin, lumican and perlecan, along with cartilage oligomeric matrix protein (COMP). Mutations in their genes give rise to defective cartilage and to early-onset OA [12]. This illustrates the functional importance of even minor components of the cartilage matrix. However, most OA patients have no demonstrable defects in structural proteins. Therefore, the cause of their arthritis does not seem to involve alterations in ECM molecules.

2.2 Histological grading of OA severity

The availability of human cartilage samples following joint replacement operations (with appropriate ethical permission) as well as new histological techniques and antibodies have enabled researchers not only to map the various stages of cartilage erosion quite precisely but also to relate the cellular changes associated with OA to the stage of cartilage erosion. The femoral head is particularly useful for such studies, as patients with osteoporosis (OP) frequently fracture (#) the neck of the femur (#NOF). The fracture is typically treated by hemi-arthroplasty so that the femoral head is available for research. It has long been known that there is an inverse relationship between OP and OA [13]. Indeed, femoral heads from OP patients typically have healthy cartilage, which therefore constitutes an easily accessible control with which to compare OA cartilage.

The first understanding of the cellular changes involved in OA was based on histological observations of post-mortem cartilage. Mankin *et al.* [14] developed a numerical scale, from 0 (normal cartilage) to 14 (severe degradation), that graded the loss of structural organization, cellular characteristics, loss of Safranin O staining and tidemark integrity. More recently, a new grading system has been proposed by the Osteoarthritis Research Society International (OARSI) [15], which divides the stages of degradation into six grades, with grades 1 – 4 representing cartilage changes and grades 5 – 6 including changes in subchondral bone. Both systems are based on the histological appearance of the cartilage at a particular site, rather than on a patient-based score for overall OA severity. The same patient may have OA cartilage with a high score in the weight-bearing region and almost normal cartilage in non-weight-bearing regions.

With today's techniques, we can go beyond documenting cartilage erosion. Immunocytochemistry allows defining the proteins produced by chondrocytes at specific stages in specific regions and, by incorporating fluorescent markers, metabolically active or apoptotic cells can be identified.

2.3 Matrix and cellular changes start at the superficial zone

Changes in the composition and subsequent loss of matrix always start at the surface – a fact difficult to reconcile with the hypothesis [16] that changes in subchondral bone precede cartilage changes. Proteoglycans are lost before cartilage collagen, because loss of Safranin O staining in the superficial zones (**Figure 1A – D**) occurs whereas the cartilage collagen remains, as seen by the Sirius red stain (**Figure 1E, F**). In healthy cartilage matrix, the presence of proteoglycans masks the Sirius red binding sites, hence only that region from which proteoglycans have been lost shows the bright Sirius red stain of fibrillar collagen. Comparing the cartilage from #NOF patients with that of OA patients shows interesting similarities, as well as crucial differences. As expected, hardly any proteoglycan loss has occurred in the cartilage from non-weight-bearing regions of most #NOF patients (**Figure 1A, E**), but in weight-bearing regions the whole cartilage is thinner and proteoglycans are absent from the upper third of the tissue (**Figure 1B, F**). In fact, the histological appearance of this cartilage is remarkably similar to that from OA patients in non-weight bearing regions (**Figure 1C, G**). The crucial difference is that this is the worst-scoring cartilage of a #NOF patient, whereas it may be the best-scoring cartilage of an OA patient, whose cartilage at the weight-bearing region will have been eroded right down to the subchondral bone. This suggests that the early stages of cartilage loss occur in both osteoporotic and osteoarthritic patients but do not reach clinical significance in OP patients, either because the process started at a later age or because the early changes have not progressed. One important but often not appreciated conclusion is that differences in cartilage erosion in OA and OP may be in degree rather than in kind.

2.4 Articular chondrocytes in OA undergo a change in phenotype

Normal articular chondrocytes have a long life, low metabolic activity, divide rarely and slowly turn over the

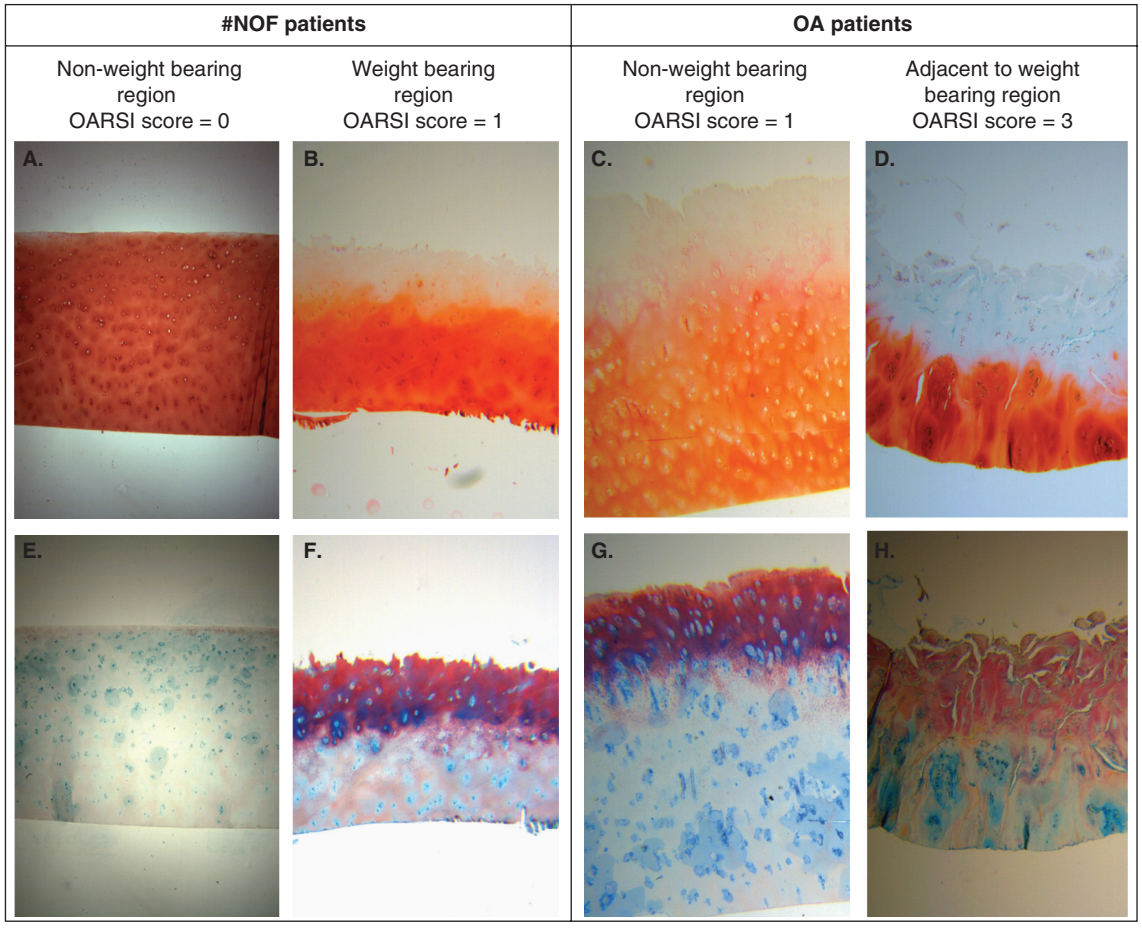

Figure 1. Cartilage erosion with increasing OA grade. A – D, Safranin O staining illustrates the loss of proteoglycans from the superficial zone. **E – H**, positive Sirius red stain of the cartilage collagen is only apparent in the region from which proteoglycans have been lost. In fracture of the neck of femur (#NOF) patients, healthy cartilage is present in non-weight-bearing regions (**A**, **E**), whereas loss of proteoglycans and some matrix fibrillations are apparent in weight-bearing regions (**B**, **F**). The latter is similar to early-grade OA cartilage (**C**, **D**). In high-grade OA cartilage, some cartilage has been eroded and the fibrillations and the regions of proteoglycan loss are more extensive (**D**, **H**). **A**, **E** from a 84-year-old female; **B**, **F** from a 85-year-old female; **C**, **G** from a 61-year-old male; **D**, **H** from a 73-year-old female.

cartilage matrix. The half-life of cartilage collagens has been estimated to exceed 100 years [17], whereas aggrecan components turn over in 3 – 24 years [18]. Normal adult articular chondrocytes synthesize cartilage matrix proteins, such as collagen types II, IX and XI, the components of aggrecan and COMP. Normal articular chondrocytes do not produce enzymes such as matrix metalloproteases (MMPs), ADAMTSs or inflammatory cytokines. Yet all these proteases and cytokines plus many more abnormal gene products are expressed by some, but not all, chondrocytes of OA patients. The location of cartilage cells that express abnormal genes, the so-called 'degradative' chondrocytes [19], follows the pattern of proteoglycan loss, starting at the surface of weight-bearing regions.

Chondrocytes in non-weight-bearing regions of the same patient, on the other hand, still resemble normal cartilage cells. This heterogeneity is usually overlooked in gene expression studies. There all chondrocytes from an OA patient are pooled, so as to obtain enough cells for RNA extraction. As a result, the abnormal gene expression of degradative chondrocytes will be confounded by still-normal chondrocytes.

2.5 Abnormal chondrocytes increase with disease progression

Degradative chondrocytes increase in number and location as the disease progresses (**Figure 2**). Moreover, when a chondrocyte expresses one abnormal gene, it also seems to express other abnormal genes. This suggests that an overall permanent switch in the phenotype of the chondrocytes has occurred rather than short-term upregulation of one or more factors. Another feature is that the degradative chondrocytes have high metabolic activity, as visualized by Celltracker green fluorescence (**Figure 2D**, **F**). In contrast to healthy

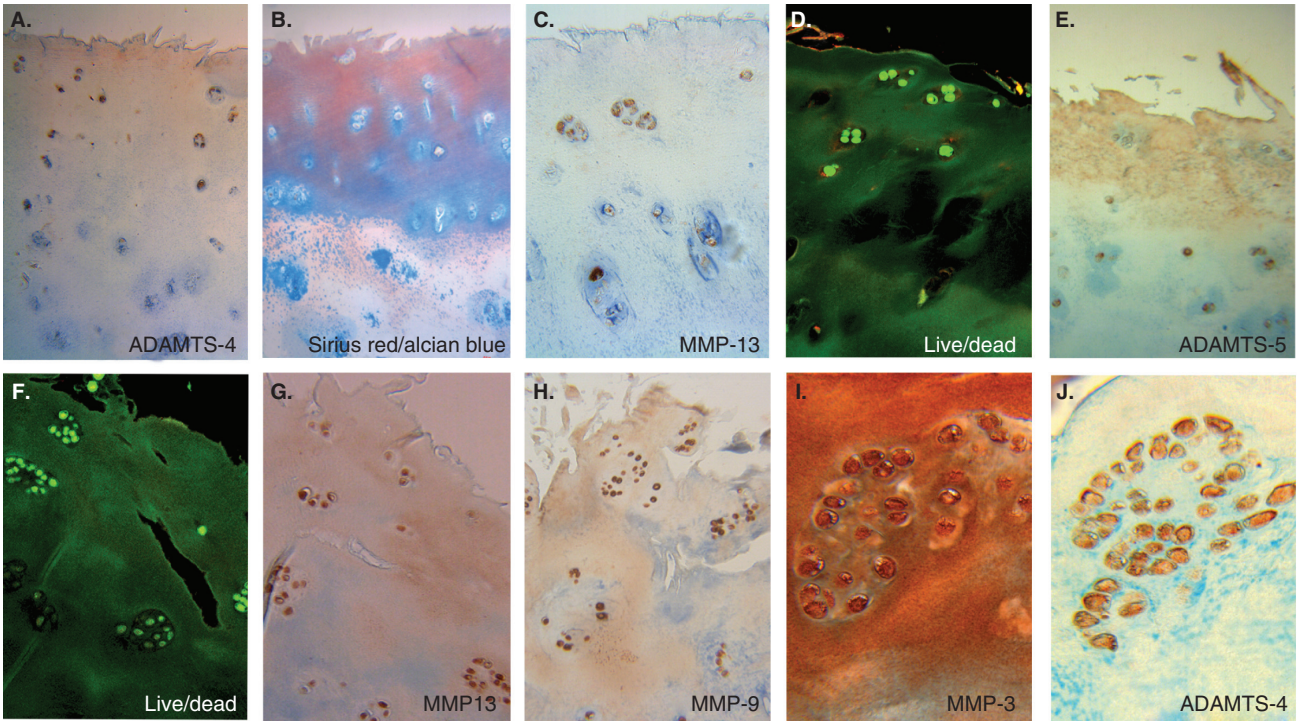

Figure 2. Location of protease-expressing 'degradative' chondrocytes in low-grade and high-grade osteoarthritis. A – E, low grade OA (OARSI ~ 1 – 2); **F – J**, high grade OA (OARSI ~ 3); staining as indicated. Degradative chondrocytes are first apparent in the superficial zone as single cells or doublets (**A**) and their localization corresponds to the region of proteoglycan loss (**B**). The cells continue to divide (**C**) and daughter cells are highly metabolically active (**D**, **F**). In high-grade OA, the typical clones are apparent, in which all cells are immunopositive for all the enzymes examined. Enzyme activity is also present in the matrix (**A**, **E**, **G**, **H**, **I**), which is specially apparent after hyaluronidase treatment (**E**, **I**). **C**, **J** had not been pretreated with hyaluronidase**.**

articular chondrocytes, degradative chondrocytes undergo cell division, as evidenced by the frequent presence of doublets and quadruplets in low-grade OA (**Figure 2C, D**) and the typical clusters of 16 – 32 cells in high-grade OA (**Figure 2F – J**). The abnormal expression of proteases and other genes is inherited by all daughter cells so that all cells of a cluster are of the degradative abnormal phenotype.

The important characteristics of disease progression of human OA may be summarized as follows:

1. Chondrocytes change phenotype to 'degradative' chondrocytes, which express matrix-degrading enzymes as well as many other gene products that are not normally expressed by articular chondrocytes.
2. These 'degradative' chondrocytes are first found in the superficial zone, then co-localize with the region of aggrecan loss.
3. Degradative but not normal chondrocytes proliferate to form clones. Up to 64 cells have been observed in one clone. Because all cells within a clone produce the degradative enzymes, it seems reasonable to infer the abnormal gene expression pattern is transmitted to daughter cells.
4. With increasing severity of OA, degradative chondrocytes increase in number, first, as a result of cell division of the abnormal chondrocytes, and second, because cartilage cells in the intermediate and deep zones change phenotype to degradative chondrocytes.

3. Aggrecanases and MMPs are involved in cartilage erosion

Even before it was shown that some OA chondrocytes expressed matrix-degrading enzymes, biochemical studies had identified and characterized the enzymes involved in cartilage erosion. The first major group of enzymes to be identified were the MMPs, a family of over 23 secreted and cell surface-bound zinc-dependent endopeptidases that degrade numerous substrates at neutral pH [20-22]. Of particular importance for degrading articular cartilage are collagenase-3 (MMP-13), stromelysin (MMP-3) and the gelatinases MMP-2 and MMP-9, to name just a few. These enzymes are released *in vivo* as inactive pro-enzymes and require activation by fibrinolysin or active MMP-3. MMP production is generally accompanied by MMP inhibitors (TIMPs), but in OA the balance between them is altered so that there is an MMP excess over its inhibitors, resulting in matrix destruction [22].

3.1 Protease inhibitors as targets for drug development

An obvious therapeutic approach is to increase natural enzyme inhibitors, restoring the normal balance between MMPs and inhibitors, or to develop new inhibitors with the aim of preventing cartilage destruction. Several synthetic oral inhibitors of MMPs have been developed. They are effective *in vitro* and in animal models of OA [23,24]. However, trials in humans have been disappointing, either because of adverse side effects, such as musculoskeletal pain and tendonitis, or because the drugs lacked efficacy. Inasmuch as MMPs also play a role in many other physiological processes [25,26], the incidence of adverse effects resulting from general use is not surprising.

The lack of efficacy has been attributed to the fact that the drugs administered orally might not diffuse to the joint in adequate amounts and/or not penetrate into the cartilage. Alternatively, the synthetic inhibitors may not inhibit all relevant MMPs and non-inhibited enzymes could conceivably compensate for the absence of the inhibited MMPs [21]. Moreover, the first stage in cartilage erosion is loss of aggrecan, not collagen, with aggrecanase activity preceding that of MMPs. As long as aggrecan is present in the cartilage matrix, collagen is protected from degradation even when activated MMPs are present [27]. Aggrecanase-1 and -2 (ADAMTS-4 and -5) are the main enzymes involved in aggrecan degradation [28]. ADAMTS-5 appears to be the most important aggrecanase in mice, as targeted deletion of ADAMTS-5 but not of ADAMTS-4 prevents the development of OA following meniscal destabilization [29-31]. However, in humans, both aggrecanases play a role [32]. Theoretically, if ADAMTS-4 and -5 rather than the MMPs were targeted, initial cartilage loss would be prevented.

An alternative explanation for the lack of efficacy is that degradative chondrocytes themselves are a major source of the degradative enzymes as the disease progresses. In order to neutralize the degradative enzymes from the chondrocytes, the inhibitors would have to diffuse into the cartilage matrix. The amount of diffusing inhibitor may be insufficient in the light of the continually produced enzymes by an increasing number of chondrocytes. A far more productive strategy for therapeutic treatment would appear to be to prevent abnormal protease expression by chondrocytes. But this requires understanding what regulates expression of the degradative proteases.

4. Inflammatory cytokines as targets for drug development

Inflammatory cytokines such as IL-1β, TNF-α and oncostatin M (OSM) [33,34] are known to induce expression of the degradative enzymes [35-39]. In addition, other inflammatory mediators such as nitric oxide [40-42], reactive oxygen species [43-45] and prostaglandin E2 [46,47] also contribute to cartilage destruction. It is IL-1β and/or TNF-α, however, which, perhaps in synergy with OSM, seem to play a central role in OA pathology.

When addition of IL-1β and/or TNF-α to monolayer cultures of chondrocyte cell lines was found to induce expression of degradative enzymes, an explosion of studies utilized these experimental models to describe signal transduction and other regulatory pathways. Yet how relevant are these cell cultures to human OA? IL-1β is an important catabolic cytokine in rheumatoid arthritis [48] and the four IL-1β signaling cascades are functional in both normal and OA chondrocytes [49]. However, IL-1β is not significantly upregulated in OA chondrocytes. Moreover, OA is not a classical inflammatory arthropathy. Nevertheless, synovial inflammation is common in both early and late stages [50] and a relationship seems to exist between the levels of IL-1β and TNF-α in OA synovial fluids and the levels of catabolic enzymes and other inflammatory mediators such as prostaglandins and nitric oxide [38,46,51]. OA chondrocytes, especially in the superficial zone and in clonal clusters, are positive for IL-1β immunostaining [49,52,53]. IL-1β colocalizes with TNF-α, MMP-1, -3, -8, and -13, and with type II collagen cleavage epitopes in regions of matrix depletion found in OA cartilage [53,54]. Adenoviral transfer of IL-1β with OSM induces significant joint damage in a murine model [55]. It may be possible that the locally produced IL-1β is of importance in influencing chondrocyte responses in an autocrine–paracrine manner, rather than overall levels of the cytokine. This may become apparent only in selected chondrocytes. Its expression would therefore not be detectable when RNA is extracted from the entire OA cartilage.

4.1 Signal transduction mechanisms as possible drug targets?

If inflammatory cytokines are indeed important in the pathoetiology of human OA, what should be the targets of drug action? There are two possible approaches: the first depends on interfering with the signal transduction mechanisms of IL-1β/TNF-α with the aim of preventing *trans*-activation of the target genes; the second depends on preventing the expression of the abnormal cytokines and/or their target genes by preventing promoter activation. Il-1β, TNF-α and OSM have different membrane-based receptors (**Figure 3**), but their signal transduction cascades converge (**Figure 3**). Ultimately, this leads to binding of various transcription factors to the promoter regions of target genes and to gene *trans*-activations [2]. Details are beyond the scope of this review, but in principle it involves the MAPK family, which comprises extracellular signal-regulated protein kinases (ERKs), p38 kinase in its various isoforms and the C-Jun-N-terminal kinase (JNK) [25]. The activities

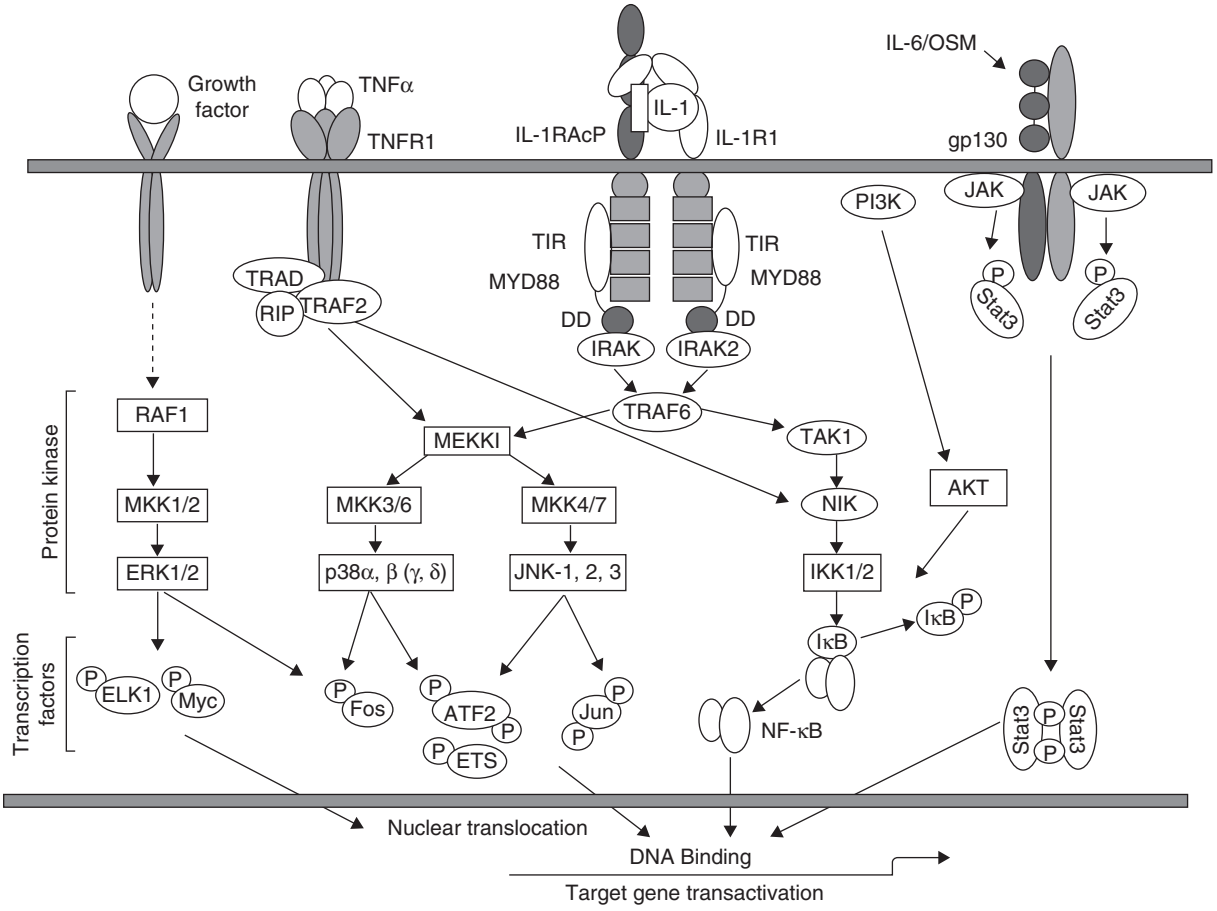

Figure 3. Signal transduction pathways in chondrocytes.

AKT: Protein kinase B; ATF-2: Activating transcription factor-1; DD: Death domain; ELF: E74-like factor; ERK: Extracellular signal-related kinase; ETS: E Twenty Six; Fos: v-Fos homolog osteosarcoma oncogene; gp 130: Glycoprotein 130; IκB: Inhibitor of kappa B; IL: Interleukin; IL-1RI: Type I IL-1 receptor; IL-1RAcP: IL-1 receptor accessory protein; IRAK: IL-1 receptor associated kinase; JAK: Janus activating kinase; JNK: c-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; MEKK1: Mitogen-activated ERK kinase kinase; MKK: MAPK kinase; Myc: Myogenic transcription factor; MYD88: Myeloid differentiation primary response gene 88; NF-κB: Nuclear factor kappa B; OSM: Oncostatin M; P: Phosphate; PI3K: Phosphoinositol 3-kinase; RAF: Receptor associated factor; RIP: Receptor-interacting protein; Stat3: Signal transducer and activator of transcription; TIR: Toll/IL-1 receptor domain; TNF-α: Tumor necrosis factor alpha; TNF-R1: Tumor necrosis factor receptor 1; TRADD: TNF receptor-associated death domain receptor; TRAF2: TNF receptor associated factor 2.

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of these kinases converge on several transcription factors, NF-κB in particular. The latter upregulates expression of the various MMPs and ADAMTSs, as well as of the inflammatory cytokines [56-58], but suppresses the synthesis of ECM proteins. Thus, NF-κB mediates the two major changes in gene expression in OA chondrocytes: suppression of normal chondrocyte gene products and induction of abnormal genes.

Available inhibitors for each of the JUN, ERK and p38 kinase isoforms are mostly used for research. They probably will not constitute effective treatment for OA, inasmuch as MAPK signaling pathways regulate many other physiological processes, including cell growth, differentiation, and apoptotic cell death [59]. Therefore, any one of these inhibitors, systemically applied, will almost certainly have adverse side effects.

5. Is induction of abnormal genes due to epigenetic 'unsilencing'?

An alternative strategy to targeting signal transduction pathways would be to target the epigenetic mechanisms that induce the cell type specific gene expression pattern of somatic cells. In each cell type, only those genes are expressed that are appropriate to its phenotype, with all other genes silenced. How is this selectivity achieved and maintained? Could a defect in selectivity underlie the inappropriate expression of abnormal genes in OA? All somatic cells contain the full gene apparatus of a person, but only a fraction of the genes is expressed in a given phenotype. Gene expression is regulated by two basic mechanisms, depending on whether the gene is part of the repertoire of the particular cell type or whether the gene is

permanently silenced. All those genes in the first category are regulated by specific transcription factors in combination with cofactors, for example, enhancers and repressors. All other (i.e., never-expressed) genes are permanently silenced by the epigenetic mechanisms outlined below. Many studies have examined the promoter regulation of specific genes of relevance in OA in order to understand both the downregulation of normal chondrocytic genes, such as *COL2A1*, and the activation of abnormal genes, such as the MMPs. If transcriptional activation of abnormal genes can be prevented, synthesis of the degradative proteases may also be prevented, as would cartilage erosion. MMP-13 is an example of a gene product that results from *de novo* activation in OA. Activation of the MMP-13 promoter depends on transcription factors Runx-2, NF-κB, members of the ETS family of transcription factors and AP-1 [60-62]. On the other hand, repression of *COL2A1* involves repression of Sp1 by Sp3 [63], EGR-1 [64] and ESE-1 [65].

Most studies of promoter regulation have used PCR-generated promoter constructs. Although very useful for analyzing transcription factors, the studies do not take account of the epigenetic mechanisms that, *in vivo*, can override transcriptional activation and thus cause genes to be silenced.

To illustrate the differences between epigenetic and transcriptional control of gene expression, a useful analogy is the process involved in entering a room through a door. If the door is locked, depressing the door handle will not open the door and entry is prevented. This is analogous to the permanent silencing of a gene that cannot be activated, even when the right transcription factors are present. However, unlocking the door is also not sufficient to permit entry. Rather the door handle must still be depressed after unlocking in order to open the door. This is analogous to binding of the required transcription factors to the promoter region. PCR-generated promoter constructs are analogous to a door that has been unlocked so that only the relevant transcription factors are required.

5.1 Epigenetic silencing of genes

Epigenetics is defined as heritable changes in gene function that cannot be explained by changes in DNA sequence. Epigenetic information is encoded by DNA methylation, histone modifications and changes in chromatin compaction. Conformationally relaxed chromatin (euchromatin) indicates transcriptionally active regions and is associated with a low level of DNA methylation, acetylation of histones-3 and -4, as well as methylation of histone-3 at lysine-4. Compact chromatin (heterochromatin) is transcriptionally silent, has a high degree of DNA methylation, and is bound to non-acetylated histones with methylation at histone-3, lysine-9 and -27 [66]. In the course of development, the extensive reprogramming of gene expression by epigenetic gene-control mechanisms takes place to obtain appropriate tissue-specific gene-expression patterns [67]. Epigenetics also lies at the heart of phenotypic variations in health and disease, so targeting the epigenome holds great promise for preventing and treating complex human diseases [68].

DNA methylation is the major heritable component of epigenetics. DNA is methylated when methyl groups are added to 5′-cytosines next to guanines (CpG sites) by DNA methyltransferases (Dnmts) [69,70]. Dnmt3a and Dnmt3b cause *de novo* methylation of primarily unmethylated DNA and play a major role in establishing the DNA methylation pattern during development. Dnmt1 is responsible for maintaining established methylation patterns during cell division. Methylated CpG sites attract methyl-binding proteins, which recruit a variety of co-repressors, such as histone deacetylases [71-73], histone methyl transferases and heterochromatin coating factors like HP1 [73,74]. The methyl-binding proteins thus act as a bridge between DNA methylation and histone modifications [75,76]. Histone methylation at histone 3-lysine 9 may also induce DNA methylation [74]. Gene silencing thus depends on both histone modification and DNA methylation, and is maintained in a manner specific for the cell type during mitosis, although the precise mechanisms are not well understood [77]. Deviations from the DNA methylation pattern can result in either too much DNA methylation, which can lead to abnormal silencing of essential genes, or too little DNA methylation and the likelihood of abnormal expression of previously silenced genes [78,79]. An important point is that the abnormal DNA methylation status is transmitted to daughter cells, because cells have no memory of their cell type specific epigenetic status. Moreover, unlike repair by DNA excision, no mechanisms are known that normalize altered DNA methylation patterns. This is why abnormal DNA methylation patterns can have such devastating effects. In the last 20 years, the study of epigenetics has expanded greatly and there is convincing evidence that the methylation status is altered in disease and that the alteration is maintained and passed onto daughter cells. Such changes have been reported for cancer [80], for autoimmune diseases [81] and for the inflammatory rheumatic diseases, rheumatoid arthritis and lupus erythmatosus [82-85]. Similar changes may also be involved in other complex diseases [68,86,87].

5.2 Do epigenetic changes occur in OA?

Could altered DNA methylation play a role in OA? If so, one would predict DNA demethylation in the promoter regions of those genes that are expressed *de novo* in OA (e.g., the proteases) and high promoter methylation for those genes (e.g., cartilage collagens or aggrecan) that are silenced in OA chondrocytes. Of the few studies of epigenetic gene regulation in OA, the study that by Roach *et al.* [19] was the first to investigate whether demethylation or 'unsilencing' could explain the abnormal

Table 1. Promoter CpG methylation for four cartilage-degrading enzymes in controls (#NOF patients) and OA patients.

	MMP-3	MMP-9	MMP-13	ADAMTS-4	Overall mean
#NOF	70 ± 7	53 ± 11	96 ± 2	100	80 ± 4
OA	41 ± 6	19 ± 5	80 ± 4	50 ± 6	51 ± 3
p-Value	0.007	0.015	0.012	< 0.001	< 0.001

DNA was extracted from the articular cartilage from the deep zone of #NOF patients, whose chondrocytes did not express the proteases, and from the surface zones and around the weight-bearing regions in OA patients, because only those regions contained protease-expressing 'degradative' chondrocytes. The numbers show the means (and SD) of the percentage of CpG sites that were methylated in 10 #NOF patients and 16 OA patients. These were derived from the data presented in [17].

CpG: Cytosine-phosphate-guanine; #NOF: Fracture of the neck of femur; OA: Osteoarthritis.

gene expression of four key proteases involved in OA, namely MMP-3, MMP-9, MMP-13 and ADAMTS-4. The promoters of all four proteases contain relatively few CpG sites, the so-called sparse CpG promoters [19,88], in contrast to genes that contain many closely spaced CpG sites, the so-called CpG island promoters. Investigation of the DNA methylation status of CpG sites revealed that in chondrocytes from control (#NOF) patients most CpG sites were methylated [19], consistent with the silencing of four proteases in these chondrocytes. However, significant loss of DNA methylation had occurred at specific CpG sites in the degradative chondrocytes of OA patients (cf. **Table 1** and [19]). Another example is the abnormal expression of leptin in OA, which is also the result of an epigenetic loss of DNA methylation in OA chondrocytes [89]. These two examples provide 'proof of principle' in as much as abnormal gene expression by 'degradative' OA chondrocytes in both conditions is associated with epigenetic loss of DNA methylation.

Poschl *et al.* [90] have investigated whether the loss of aggrecan expression in OA was linked to high DNA methylation of the promoter. Although the aggrecan promoter contains a CpG island, increased DNA methylation was ruled out as cause for silencing this cartilage-specific gene. Data from the Human Epigenome Project [91] may provide an explanation. This Project studied the DNA methylation status of 1.8 million CpG sites in 873 genes located on chromosomes 6, 20 and 22 in 12 different tissues and showed that 88% of CpG island promoters are un-methylated, irrespective of expression, whereas at least 50% of sparse promoters are have a high level of DNA methylation in non-expressing cells. The findings of Poschl *et al.* [90] suggest that the silencing of aggrecan in OA is not owing to hypermethylation of the CpG island but may involve other mechanisms, perhaps histone modification or repressor activation. The absence of hypermethylation in a gene silenced in OA thus contrasts with the well-documented silencing by hypermethylation in tumor suppressor genes [92], where epigenetic therapy based on reversing the hypermethylation has been approved for the treatment of certain types of cancer [80,93].

6. Expert opinion: Is OA an epigenetic disease?

It is the author's hypothesis that OA is a disease in which abnormal genes are activated, because of epigenetic unsilencing. These genes include the typical cartilage-degrading enzymes, that is, aggrecanases and MMPs, and also numerous other genes, including IL-1β. The epigenetic unsilencing is responsible for the phenotypic change from normal to 'degradative' chondrocytes. As yet, we do not know what induces unsilencing. Excess mechanical forces, joint instability or episodes of synovial inflammation (**Figure 4**) are possible initiating factors that affect chondrocytes in the superficial zone to set in motion the phenotypic changes to degradative chondrocytes. An important observation is that not all chondrocytes are affected simultaneously in OA, as would be the case if OA was predominantly a genetic disease. Rather the phenotypic changes start in a few cells in the superficial zone of the weight-bearing regions, then propagate over a period of 10 – 30 years to cells of the intermediate and deep zones and to non-weight-bearing regions (**Figure 4**). Epigenetic changes in progressively more chondrocytes may thus explain the progressive nature of OA. There is no doubt that genetic predisposition is important in OA [94,95], but, in the author's view, it is the epigenetic changes that actually lead to cartilage erosion in a particular patient.

Given the arthritogenic effects of IL-1β/TNF-α, it would be of interest to know whether these cytokines demethylate specific CpGs in the promoters of their target genes. There is evidence that demethylation (in combination with histone acetylation) is the mechanism by which IL-1β exerts its effects [96]. The author's group has preliminary evidence that IL-1β or TNF-α causes loss of DNA methylation at specific CpG sites in specific promoters *in vitro*. A model in which loss of DNA methylation can be induced is in current use to test whether substances that are known to protect chondrocytes from the arthritogenic effects of IL-1β, such as glucosamine [97] or hyaluronan [98], exert their effects by preventing demethylation. The system can also be

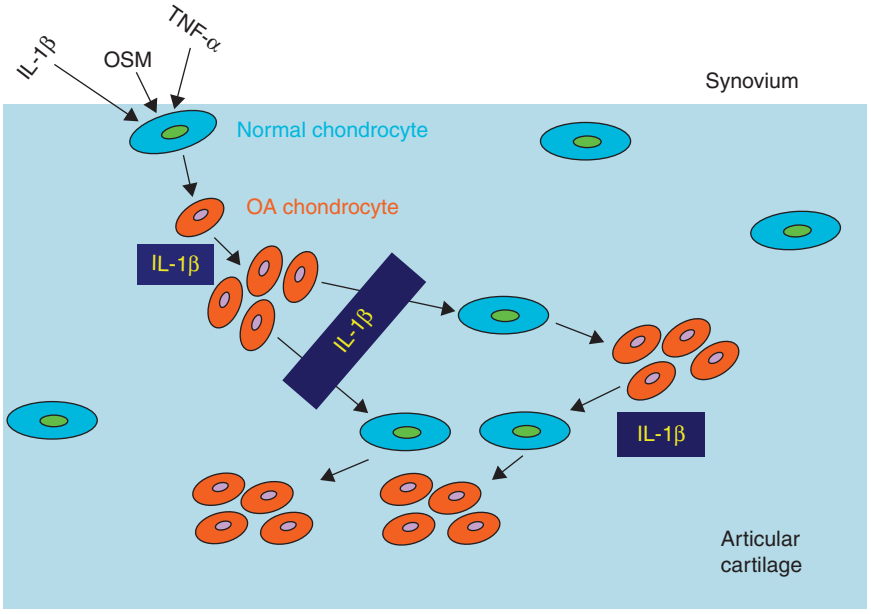

Figure 4. Epigenetic changes and OA progression. Inflammatory cytokines from the synovium cause normal articular chondrocytes (blue) in the superficial zone to change to 'degradative' OA chondrocytes (orange). This phenotypic change includes loss of DNA methylation in the promoters of many genes, which can result in aberrant gene expression, providing the required transcription factors are present. IL-1β (and other cytokines) are induced as part of the phenotypic change. Degradative chondrocytes proliferate and transmit the aberrant gene expression to daughter cells. IL-1β (and may be other cytokines) diffuse to adjacent, still healthy, chondrocytes and causes these to change phenotype to degradative cells, thus, propagating the abnormal phenotype.

used to test other compounds and may therefore become a first screening test for epigenetic therapy.

To date most epigenetic therapy is based on preventing or reversing hypermethylation or chromatin compaction of tumor suppressor genes by applying non-specific DNA methyltransferase inhibitors (e.g., 5-aza-deoxycytidine) or histone deacetylase inhibitors (e.g., trichostatin A) [99]. For OA, the opposite approach would be required, namely inhibiting the loss of DNA methylation in the promoters of proteases and/or cytokines would prevent or slow down cartilage erosion. A drug capable of achieving this would indeed be a disease-modifying drug. In addition, if the genes expressing the proteases and cytokines could be silenced by site-specific DNA methylation, this would constitute a cure for OA. Both strategies depend on defining the epigenetic differences between control and degradative OA chondrocytes, and on understanding the mechanism of DNA demethylation in articular chondrocytes. At present, the questions far exceed the answers. We need to study the mechanisms of DNA methylation loss, how histone modifications are involved, why only some CpGs in some genes are demethylated, whereas in others the methylation status remains unchanged even when exposed to the same stimuli. In particular, we need to understand the specific nature of DNA methylation for a given cell type.

Preventing DNA demethylation might be effective in the early stages of the disease. A far more difficult undertaking would be to reinduce epigenetic gene silencing by epigenetic means in OA chondrocytes with already unsilenced genes. One way to accomplish this is to engineer zinc-finger proteins (ZFP) to bind to specific sequences on the DNA. In yeast cells, where no DNA methylation is present, a zinc-finger fused with the DNA methylase M.SssI has been shown to target methylation to CpG sites within 353bp of the ZFP binding site [100]. Greater specificity can be achieved by using several ZFP in combination. A construct consisting of eight ZFP linked to the repressor domain KRAB, together with the corepressor KAP1, can effect the necessary co-ordination to bring about gene silencing at the site selected by the zinc fingers in NIH2T3 cells [74], which includes DNA methylation. Engineered transcription factors, linked to an effector domain that functions to normalize disrupted DNA methylation status, are a promising tool to treat and possibly cure the defective DNA methylation that characterizes several diseases [101]. Although remethylation at specific sites can be accomplished in yeast or cultured cells, but we do not know how to translate this to the human situation, because chondrocytes are present within lacunae, which are separated by large amounts of ECM.

Much time, expertise and funding are needed to reach the point when it is reasonable to proceed with the development of drugs for epigenetic therapy of OA. Yet, the holy grail of disease-modifying drugs for OA now seems to be within sight, a prospect that may benefit millions of elderly patients in the future.

636 ## Declaration of interest

639 Research in the author's lab has been supported by grants from Wessex Medical Research (Southampton).

Acknowledgement 640

F Bronner, University of Connecticut Health Center, Farmington, CT, USA, has helped in preparing the manuscript. 643

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