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Inflammation and epigenetic regulation in osteoarthritis

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Abstract

Osteoarthritis (OA) was once defined as a non-inflammatory arthropathy, but it is now well-recognized that there is a major inflammatory component to this disease. In addition to synovial cells, articular chondrocytes and other cells of diarthrodial joints are also known to express inflammatory mediators. It has been proposed that targeting inflammation pathways could be a promising strategy to treat OA. There have been many reports of cross-talk between inflammation and epigenetic factors in cartilage. Specifically, inflammatory mediators have been shown to regulate levels of enzymes that catalyze changes in DNA methylation and histone structure, as well as alter levels of non-coding RNAs. In addition, expression levels of a number of these epigenetic factors have been shown to be altered in OA, thereby suggesting potential interplay between inflammation and epigenetics in this disease. This review provides information on inflammatory pathways in arthritis and summarizes published research on how epigenetic regulators are affected by inflammation in chondrocytes. Furthermore, we discuss data showing how altered expression of some of these epigenetic factors can induce either catabolic or anti-catabolic effects in response to inflammatory signals. A better understanding of how inflammation affects epigenetic factors in OA may provide us with novel therapeutic strategies to treat this condition.

Keywords

DNA methylation; epigenetics; histone modification; inflammation; non-coding RNA; osteoarthritis

OA pathology

OA is a degenerative joint disease, characterized by articular cartilage degradation, subchondral bone sclerosis, inflammation, and osteophyte formation (1–7). Major clinical symptoms include chronic pain, joint instability, stiffness, and radiographic joint space narrowing (8). OA is the most common form of arthritis and is a leading cause of impaired

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Declaration of interest

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mobility among the elderly population. Aging, joint trauma, obesity, and genetic predisposition are some of the risk factors for developing OA (9,10). Although primarily affecting the elderly, sports-related traumatic injuries can also lead to post-traumatic OA (PTOA). It has been forecasted that 25% of the adult population, or more than 50 million people in the United States, will be affected by this disease by the year 2020, and it will be a major cause of morbidity and physical limitation among individuals over the age of 40 (11,12). Articular cartilage has no intrinsic repair capabilities, and there is an unmet clinical need to identify new therapeutic targets to slow down/stop cartilage degradation or to induce endogenous regeneration.

Many of the cellular and molecular changes known to occur in OA were identified in studies using mouse models of OA [e.g., destabilization of the medial meniscus (DMM); meniscal ligament injury (MLI)] (13,14) or from analysis of human cartilage/chondrocytes from OA patients (10). For example, alterations in TGF-β super-family, Wnt/β-catenin, Notch, and Indian Hedgehog (Ihh) pathways have been shown to contribute to OA development and progression by inducing primarily catabolic responses (15–22). Such responses include upregulation of inflammatory mediators that leads to cartilage extracellular matrix (ECM) degradation via increased expression of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) (23–27). Chondrocyte phenotype also changes during catabolism including cell clustering/proliferation (28), apoptosis, and/or hypertrophic differentiation with increased expression of markers such as *Col10a1*, *Runx2*, and *Mmp13* (29,30). A number of gene polymorphisms have been reported in human OA (e.g., *GDF5*, *SMAD3*) (31,32), and there are now several, robustly replicated, significant OA loci that have been identified by large-scale genome-wide association studies (GWASs) (33–35). However, many candidate gene studies for OA have identified false associations due to relatively small sample sizes. For example, one study carried out meta-analysis from nine GWAS and showed that only 2 out of 199 potential candidate genes (*COL11A1* and *VEGF*) were associated with OA in human patients (36). The OA loci discovered to date explain only a small fraction of the heritability of OA estimated by epidemiological studies. It is now becoming clear that some of the missing heritability may be explained by inheritance of epigenetic modifications of genomic DNA (37–39).

Epigenetic and inflammatory changes in OA

Epigenetics can be defined as changes in gene expression that occur without changes in the DNA sequence. In some cases, epigenetic modifications are stable and passed on to future generations, but in other instances they are dynamic and change in response to environmental stimuli, for example. Three main mechanisms are involved in epigenetic regulation: (1) post-translational modification of histones which alters chromatin conformation, (2) non-coding RNAs (i.e., microRNAs, long non-coding RNAs) acting both transcriptionally and post-transcriptionally in the regulation of mRNA expression, and (3) DNA methylation changes that covalently alter DNA structure (38,40–44). Recent methylome studies revealed differential DNA methylation signatures in OA patients (39), indicating that such epigenetic regulated changes in DNA structure could be an important factor in OA development and progression. The dynamic DNA methylation process is mediated by DNA methyltransferase (DNMT) enzymes and the demethylation enzymes, 10–

11 translocation methylcytosine dioxygenases (TETs), including TET1, 2, and 3 in mammals. Three DNMTs have been reported (DNMT1, 3A, 3B) and they function by catalyzing the addition of a methyl group (CH_3) to a cytosine located 5' of a guanine (CpG sites) to form methylated cytosine (5 mC). Subsequently, DNA methylation (whether in promoters, enhancers, or gene body regions) can significantly affect gene expression patterns. DNMT3A and DNMT3B act predominantly as *de novo* methyltransferases that establish DNA methylation patterns during development while DNMT1 functions to maintain these patterns during cell divisions (40,42,44–50). Deletion of these enzymes in mice results in embryonic (*Dnmt1*, *Dnmt3b*) or post-natal (*Dnmt3a*) lethality, confirming an essential role during development (51,52).

In addition to epigenetic changes, it is also clear that chronic and low-grade inflammation is involved in the progression of OA (53–56) that leads to catabolic responses in chondrocytes via upregulation of factors such as nuclear factor-kappa B (NF-κB) (57), MMPs, and markers of chondrocyte hypertrophy (e.g., *Col10a1*, *Mmp13*, *Runx2*, *Alp*). Recent investigations from human patients as well as animal models suggest that the entire synovial joint, including articular cartilage, subchondral bone, synovial tissue, ligament, and meniscus, contribute to the inflammation network. In aging and diabetic patients, conventional inflammatory factors, such as IL1β and TNFα, as well as chemokines were reported to contribute to systemic inflammation induced by NF-κB activation in both synovial cells and chondrocytes (58). Elevated systemic inflammation was also observed in obesity mice with DMM surgery, including cytokine and chemokine production, synovial tissue expansion, inflammatory cell infiltration, and NF-κB pathway activation (59), suggesting obesity plays a role during PTOA development. Indeed, recent transcriptomic analyses provide evidence that inflammatory signals contribute to OA pathogenesis through cytokine-induced MAP kinases, NF-κB activation, and oxidative phosphorylation (60).

Inflammation in disease

Inflammation is an immune response to pathogens and cellular aberrations that triggers the innate immune system. This system recognizes the undesired material and provides an acute first line of defense dominated by neutrophils. The immune system then utilizes specialized phagocytic, megakaryocyte and macrophage cells, and other cellular resources to resolve the pathology and restore homeostasis (61). However, unresolved chronic inflammation can result in detrimental effects and lead to tissue breakdown and degradation. These chronic conditions can develop into systemic diseases such as arthrosclerosis, neurodegenerative diseases, arthritis, inflammatory bowel disease, and cancer, just to name a few (62).

A large number of immune and proinflammatory cells mediate the inflammatory response, chiefly lymphocytes, dendritic cells, macrophages, leukocytes, neutrophils, and megakaryocytes (63). The function of these cells is guided and coordinated by a network of factors such as lipid mediators (eicosanoids, prostaglandins), chemokines, and proinflammatory cytokines, including TNFα, IL1β, and IL6, which activate signal transduction pathways, primarily NF-κB, which control the fate of inflammation.

NF-κB signaling and inflammation

The NF-κB transcription factor family is ubiquitously expressed in all cell types and regulates essential cellular responses including survival, differentiation, apoptosis, and autophagy. The NF-κB family includes homo- and heterodimers of P50 (P105/NF-κB1), P52 (P100/NF-κB2), P65 (RelA), RelB, and c-Rel proteins. These dimers are quiescently located in the cytoplasm bound to and sequestered by the inhibitory κB (IκB) proteins in a dynamic and tightly regulated equilibrium. At the center of this pathway and crucial for its activation is the kinase complex containing IKKα (IKK1), IKKβ (IKK2), and IKKγ/NF-κB Essential Modulator (NEMO). This complex is located in close proximity to distal receptor motifs (64–67). Upon stimulation, this complex is readily activated by most, if not all, inflammatory cytokines and factors including TNFα, IL1β, IL6, IL17, and more (66,68–71). In this regard, cytokine binding to cognate cell-surface receptor triggers receptor-mediated conformational changes that lead to recruitment of adaptor proteins and kinases in proximity to the cytoplasmic receptor motif. In the case of inflammatory cytokines, the recruited signaling molecules include TRAF proteins, the MAP kinase TGFβ activated kinase-1 (TAK1), NF-κB-inducing kinase (NIK), the tyrosine kinase c-Src, IKK1, IKK2, NEMO, and other adaptor proteins. Classical activation of NF-κB entails cytokine-induced activation of TAK1, which in turn phosphorylates IKK2 at specific serine residues located in its kinase activation loop. Active IKK2, in turn, phosphorylates IκBα, an event that leads to its subsequent ubiquitination and proteasome-mediated degradation. As a result, IκB-free NF-κB dimers translocate to the nucleus, bind to specific DNA sites and activate transcription of relevant genes. Alternative activation of NF-κB relies on NIK phosphorylation of IKK1, which then phosphorylates P100/NF-κB2, leading to proteosomal processing of its carboxy-terminus and production of P52. At the conclusion of this process, P52/RelB dimers form and translocate to the nucleus to activate transcription. Classical and alternative NF-κB pathways are activated in a cell- and signal-specific context and may play non-redundant roles under specific circumstances of physiologic and pathologic conditions, albeit overlapping functions were widely reported (65,66).

The overwhelming redundancy that governs NF-κB activation in different cell types by similar stimuli and in same cell by various stimuli overshadowed the intricate mechanisms by which this transcription factor assigns cell-specific functions. These cell- and signal-specific contexts are beginning to unravel and appear to be determined by cell-specific genetic, epigenetic, and post-translational signatures (72–75).

Homeostatic and pathologic NF-κB signaling in the skeletal system

Members of the NF-κB pathway regulate physiologic and pathologic responses in the skeleton and extensively cross-talk with other metabolic systems (65–67). Recent reports described an important role for NF-κB in joint health, owing to its position at the crossroads of complex signaling networks, including those activated by cytokines (e.g., IL1β, TNFα) and immune complexes (e.g., collagen or citrullinated antibodies) in responses to joint injury or immune system activation (76). As a result, NF-κB function impacts multiple cellular outcomes, including cell migration, proliferation, differentiation, and survival (77). While controlled NF-κB-mediated responses promote homeostasis, persistently elevated responses

of this pathway are associated with diseases such as rheumatoid arthritis (RA) and OA (60,78). Despite the large body of evidence on the role of TAK1-IKK2-NF-κB axis in these diseases, the mechanisms by which pathologic activity of NF-κB induces joint catabolism are largely unknown. Initial characterization of OA described the lesion as a non-inflammatory arthropathy owing to lack of robust infiltration of inflammatory cells to the joint. However, research advances in recent years have established contributions of environmental and inflammatory cues emanating from synovium and joint surrounding tissue in response to trauma, injury, mechanical stress, and other aberrations (79). More intriguingly, the intrinsic role of NF-κB as a homeostatic mechanism in chondrocytes and cartilage health is gaining significance. In this regard, baseline activity of NF-κB supports chondrocyte differentiation and survival. Further, evidence is emerging that chondrocytes directly respond to inflammatory cues and mount an NF-κB response, which under persisting pathologic conditions leads to expression of MMPs, cyclooxygenases (COXs), chemokines, and inflammatory cytokines (IL1, TNF, IL6), collectively accelerating catabolic changes in the cartilage and development of OA. In this regard, accumulating evidence suggest that NF-κB activity is elevated in chondrocytes during early stages of OA and mediates expression of proinflammatory cytokines and chemokines, such as TNFα, IL1β, IL6, and IL8. These and other NF-κB-mediated events, including production of nitric oxide, COX-2, and prostaglandin E2, facilitate the production of catabolic MMPs and aggrecanases including MMPs and ADAMTs, leading to articular cartilage degradation (80).

NF-κB also mediates the signal transduction of Toll-like receptors (TLRs) and receptor for advanced glycation end-products (RAGE) in response to syno-vial inflammation in chondrocytes and synoviocytes, leading to increased expression of MMPs, ADAMTs, reactive oxygen species, and inflammatory mediators (81). However, the precise intrinsic role of NF-κB during chondrogenesis and cartilage homeostasis awaits development of appropriate chondrocyte-specific NF-κB-transgenic mouse models.

Inflammation and epigenetics

The inflammatory response proceeds through distinct acute, adaptive, and chronic stages, which appear to be controlled by specific cell types, cytokines, and transcriptional signatures (82). It has been suggested that transcription factors, histone modifiers, and DNA-modifying enzymes alter chromatin landscapes to open foci allowing activation of gene expression or to close foci in tight formation to suppress gene expression (83). Hence, during short-lived inflammatory responses, sequential epi-genetic modifications of pro- and anti-inflammatory signatures occur, leading to acute inflammation at initial stages of the response followed by epigenetic changes that mute activation and promote anti-inflammatory action, and finally resolution of inflammation. These responses control a large number of genes highlighting the intricate nature of such epigenetic regulatory processes. Among these genes is NF-κB, which controls the expression of pro- and anti-inflammatory cytokines. During initial inflammatory responses, epigenetic landscape changes result in the activation of p65, which then assembles into a large activating complex in inflammatory cells. At later stages, epigenetic re-programming of p65 promoter ensues coinciding with chromatin remodeling of the proinflammatory *Tnfsf1α* and *Il1β* genes (72,83). Subsequently, histone methyltransferases, DNMTs, and other chromatin modifiers form a repressor complex to

attenuate the inflammatory response (84). Indeed, recent studies further clarified the relationship between inflammation and epigenetic alterations within the context of OA chondrocytes.

DNA methylation and demethylation in chondrocyte inflammation and OA

Epigenetic regulation is believed to play a significant role in OA development. Recent genome-wide methylation profiling has revealed differentially methylated loci in DNA from cells of OA cartilage and age-matched, non-diseased cartilage (85–87). It has also been showed that sub-groups of OA patients can be distinguished by differential methylation patterns (86,88), suggesting that DNMTs may play a significant role during OA pathogenesis. With respect to inflammation, we have identified an NF-κB-binding site in the murine *Dnmt3b* promoter (which is also present in the human *DNMT3B* promoter). Luciferase reporter assays showed functional utilization of the NF-κB-binding site following IL1β treatment of murine ATDC-5 cells; this effect was attenuated following mutation of the binding site. Chromatin immunoprecipitation assays showed that NF-κB could interact with the predicted binding site. Importantly, human primary chondrocytes from either OA patients or stimulated with IL1β results in decreased expression of *DNMT3B*. Taken together, we have potentially discovered a new important pathway, regulated by inflammation-mediated NF-κB signals, which affects epigenetic factors (Shen et al., unpublished data). Consistent with our findings, Nakano et al. found that IL1β and TNFα treatment of fibroblast-like synoviocytes resulted in decreased expression and activity of DNMT3A. Overall, their data suggest that proinflammatory cytokines such as IL1β can potentially imprint cells in chronic inflammatory conditions (89).

Furthermore, studies focusing on DNA methylation profiles of individual genes during OA uncovered that the promoter of *Col10a1* appeared to be hypo-methylated during chondrocyte hypertrophy and maturation which correlated with increased *Col10a1* expression (90). Similarly, the CpG sites within the promoter area of a number of metalloproteinases, including *MMP2*, *MMP9*, *MMP13*, and *ADAMTS4*, showed decreased methylation profiles in OA compared to normal cartilage, correlating with elevated gene expression and resulting in ECM degradation (91,92). In addition, Bui et al. analyzed the methylation status of the *MMP13* promoter and found that it was specifically demethylated in OA chondrocytes compared to healthy chondrocytes (93). Another study showed that *COL9A1* promoter was hypermethylated in OA chondrocytes and that such hypermethylation attenuated SOX9 binding to the *COL9A1* promoter (94). Changes in DNA methylation of the sclerostin (*SOST*) promoter were also identified in OA chondrocytes, thus explaining its upregulation in these cells (95).

Further evidence for epigenetic regulated changes in gene expression was found in the promoter region of the inflammatory chemokine, IL8. Here, Takahashi et al. showed that increased demethylation of the *IL8* promoter in OA chondrocytes correlated with enhanced IL8 expression and that expression was mediated by the activity of C/EBP, AP-1, and NF-κB (96). In addition, demethylation of an NF-κB-responsive enhancer was shown to increase the expression of inducible nitric oxide synthase (iNOS), a gene known to be dysregulated in OA (97). Recent analysis from methylation data of hip OA patients

identified that the promoter region of a subset of inflammation-associated genes including *IL1α* and *TNF* was hypo-methylated, which further led to increased *MMP13* expression in OA chondrocytes through zinc ZIP8-MTF 1 axis (88). Taken together, these studies suggest that DNA methylation changes are highly coordinated with the inflammation response and metalloproteinases activity within the context of OA progression, which is believed to contribute to catabolic responses in chondrocytes.

Besides DNA methylation, the DNA demethylation process has also been shown to be regulated by inflammation signals in chondrocytes. In mammals, 5-methylcytosine can be removed by the TET family of enzymes, including TET1, 2, and 3, which are normally involved in reducing CpG methyl groups and facilitate gene activation through several steps of oxidation of methyl groups to generate 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine (98). The end products, 5-formylcytosine and 5-carboxylcytosine, can be recognized and the cytosine methyl group is enzymatically excised. As a stable intermediate, DNA hydroxymethylation (5 hmC) has been recognized as a specific epigenetic mark, and recent studies have also revealed a significant increase in 5-hydroxymethylcytosine levels in OA chondrocytes (99,100). Although expression of TET1, 2, and 3 was found in human chondrocytes, TET1 was thought to be the major factor contributing to the 5hmC signature in chondrocytes since only *TET1* expression was significantly reduced by inflammatory factors, such as IL1β or TNFα in human chondrocytes (101). These data suggest that under inflammation conditions, DNA demethylation changes may occur in certain loci to alter chondrocyte homeostatic responses.

Histone modification, inflammation, and OA

Histone modification, including acetylation, methylation, phosphorylation, and ubiquitination within the lysine residues of histone cores, is another epigentic landmark, which regulates the accessibility of transcriptional machinery to specific DNA loci (102,103). Histone acetylation mediated by histone acetyl transferase (HAT) is considered to be the major mechanism to de-condense the DNA structure, thereby permitting transcriptional networks to interact with DNA to initiate the gene expression. On the other hand, deacetylation mediated by histone deacetylase (HDAC) involves removing the acetylation marker from euchromation resulting in inhibition of gene expression (104). One study showed that protein levels of HDAC1 and HDAC2 were increased in chondrocytes from OA patients and that this was associated with down-regulation of some cartilage marker genes (e.g., type II collagen and aggrecan) (105). Mechanistically, this study showed that the carboxy-terminal domain of HDAC 1 and 2, via binding to the transcriptional repressor Snail 1, was critical in the suppression of *COL2A1*. Trichostatin (TSA) is an HDAC inhibitor that attenuates the induction of MMP expression mediated by IL1β stimulation, indicating that inflammation can increase HDAC expression and activity in OA chondrocytes (106). TSA was also shown to suppress synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model (107). Importantly, systematically administered TSA was shown to protect cartilage in the DMM model of OA in mice (108). In another study, TSA, as well as an additional HDAC inhibitor, butyric acid, were shown to suppress IL1β-induced nitric oxide and pros-taglandin E2 production in human chondrocytes (109). The HDAC inhibitor, vorinostat, was found to

induce anti-catabolic activites in human chondrocytes by blocking NF-κB nuclear translocation (110). Another study from human chondrocytes further demonstrated that inhibition of HDAC7 *in vitro* can attenuate IL1β-induced *MMP13* upregulation, indicating IL1β can increase *MMP13* expression in chondrocytes via HDAC7 (111). Interestingly, a correlation between elevated HDAC7 expresion and increased MMP13 expression in human OA cartilage further suggests a role for HDAC7 in OA progression (111).

Sirtuin deacetylases (SIRTs) are NAD+-dependent HDACs. A number of studies have been published on the role of sirtuin 1 (SIRT1) within the context of chondrocyte biology and OA (112). SIRT1 was found to be highly expressed in chondrocytes, while its expression decreased in OA cartilage (113,114). The reduction of SIRT1 expression was found to result in an increase in chondrocyte apoptosis in OA cartilage (115). SIRT1 has also been shown to initiate a gene-specific transcriptional repression program to terminate the inflammatory response by deacetylating the p65 subunit of NF-κB and blocking NF-κB binding to the DNA elements in chondrocytes (116,117). Other studies by Dvir-Ginzberg's group have shown that the 75kd form of SIRT1 (generated via cathepsin-B cleavage) could promote chondrocyte survival following exposure to proinflammatory cytokines (118). Recent studies showed that SIRT1 overexpression could inhibit the proinflammatory effects of IL1β induction in human chondrocytes (119) and that disruption of SIRT1 in chondrocytes caused accelerated progression of OA in mice (120). The benefits of SIRT1 function in chondrocytes were further highlighted in a study whereby intra-articular injection of the natural phenol resveratol following surgical induction of OA in mice attenuated OA progression by activating SIRT1 (121). Taken together, it is apparent that SIRT1 function offers chondroprotective functions during aging, inflammation, and OA.

Histone demethylases are a group of epigenetic regulatory enzymes that remove methyl groups from histones, thereby regulating the chromatin state at specific gene loci (122). One study showed increased demethylation mediated by the histone demethylase KDM1 (lysine-specific demethylase 1; LSD1) in OA chondrocytes (123). Specifically, IL1β was found to increase the expression of microsomal prostaglandin E synthase 1 (mPGES-1), a critical enzyme in the biosynthesis of PGE2. This increase in mPGES-1 expression correlated with decreased H3K9 levels and recruitment of LSD1 to the mPEGS-1 promoter. This study also showed that levels of LSD1 were elevated in OA compared to normal cartilage. Taken together, these results indicate that H3K9 demethylation by LSD1 contributes to IL1β-induced mPGES-1 expression and that this pathway could be potentially targeted as a means to treat OA.

MicroRNAs regulated by inflammatory mediators in cartilage and OA

Another form of epigenetic regulation involves the small non-coding microRNAs (miRNAs). In general, miRNAs are generated from large primary precursors (pri-miRNAs) transcribed either from introns of protein coding genes or long non-coding RNA genes or from intergenic regions of the genome. Pri-miRNAs are processed in the nucleus by a complex consisting of the RNase III enzyme, Drosha, to form hairpin precursor miRNAs (pre-miRNAs). Pre-miRNAs are then further processed in the cytoplasm by the RNase III, Dicer, to generate short (~22 ntd) imperfect double-stranded mature miRNA duplexes. In the

majority of cases, either the 5p or the 3p strand of the miRNA duplex will enter the RNA-induced silencing complex (RISC) where it will bind (via its seed sequence) to a complementary region in the 3'UTR of specific target mRNAs. As a result, repression of target mRNAs then occurs via either inhibition of translation or mRNA degradation (124–126).

In the most current version of miRBase (http://www.mirbase.org/), 2588 mature miRNAs have been identified in humans and 1915 mature miRNAs identified in mouse. However, the number of human miRNAs that actually exist is thought to be much higher (127). From the vast amount of published reports on miRNAs, we now know that they are important regulators of many diverse cellular processes such as pluripotency control, differentiation, proliferation, metabolism, and apoptosis, for example (128). In many disease scenarios, miRNAs have been analyzed as potential biomarkers (129–132), and their small size renders them attractive therapeutic targets. A recent search for "microRNAs" on the clinical trials website, https://clinicaltrials.gov/, shows a number of studies (including active, recruiting, and completed) to determine miRNA expression profiling in blood, serum, or other tissues from patients with a wide range of diseases including various cancers, pathological conditions of the lung, heart, or nervous system, diabetes, and musculoskeletal diseases. Of note, a Phase I study is underway to test the effects of a double-stranded miR-34a mimic drug, MRX34, in patients with cancer (primary liver cancer, lymphoma, multiple myeloma, and others). miR-34a has been shown by many studies to inhibit multiple oncogenic pathways as well as stimulate anti-tumor responses to induce cancer cell death (133,134), thus rendering this miRNA a promising target for cancer therapy. Interestingly, increased expression of miR-34a has been reported in OA (135). Another study showed enhanced expression of miR-34a in chondrocytes following IL1β induction and that inhibition of miR-34a could attenuate the anti-anabolic effects of cytokine treatment in addition to preventing cell apoptosis (136). Therefore, it appears that in cancer situations, miR-34a overexpression could be a beneficial therapeutic approach, whereas silencing of miR-34a may in fact have a more favorable effect within the context of chondrocyte inflammation and OA. This points to the complexities of miRNA biology whereby the same miRNA may function differently depending on the cell/tissue type as well as disease status.

miR-146a, inflammation, and OA

A number of studies have reported miRNA expression changes between chondrocytes from OA cartilage and age-matched cartilage from patients with no sign of OA pathology (135,137–142). In general, the microarray data generated in these studies did not reveal extensive overlap in differential miRNA expression patterns. This could be due to the fact that diseased cartilage specimens can show considerable variability in chondrocyte activity depending on factors such as differences in stage of disease, patient body mass index (BMI), or sampling sites within the joint (i.e., lateral, medial, posterior or anterior regions of either tibial or femoral articular cartilage). Also, specimens classified as "normal control tissue" may also show considerable variability in chondrocyte gene/protein expression depending on parameters such as sampling site and patient BMI, for example.

From these miRNA expression array studies, one report showed decreased expression of miR-146a in human OA cartilage (135) and similar results were found by Yamasaki et al. in late-stage OA cartilage samples (143). However, the study by Yamasaki et al. also revealed that miR-146a expression is robustly upregulated following IL1β treatment of chondrocytes *in vitro* and that miR-146a levels were actually higher in low-grade OA cartilage compared to normal control cartilage. The higher expression levels of miR-146a in low-grade versus high-grade OA cartilage may be due to low-grade tissue containing more cytokine-induced chondrocytes. It may also be that the superficial layer of low-grade OA cartilage is retained to a greater degree than in late-stage OA specimens. This is important because Yamasaki et al. showed robust expression of miR-146a in chondrocytes in cartilage tissue sections, particularly in the superficial zone (143). In any case, the increase in miR-146a expression strongly suggests the involvement of inflammatory-mediated pathways in early-stage OA.

In other systems, miR-146a has been identified as a regulator of inflammatory mediators: it has been shown to directly target IL-1 receptor-associated kinase I (*Irak1*) and TNF receptor-associated factor 6 (*Traf6*), which are upstream regulators of NF-κB (144). In fact, Jones et al. showed that overexpression of miR-146a in chondrocytes could reduce IL1β induced production of TNFα (135). Another study has shown induction of miR-146a in IL1β-treated rat primary chondrocytes as well as in cartilage following surgically induced instability of the rat knee joint (145). Wang et al. demonstrated that HDAC inhibitors could enhance NF-κB binding to a region of the miR-146a promoter, thereby increasing miR-146a induction in OA fibroblast-like synoviocytes (146). While this work agrees with other studies showing attenuation of IL1β-induced effects as a result of miR-146a upregulation, it also suggests that miR-146a itself can be epigenetically regulated. Interestingly, increased levels of miR-146a have been detected in circulating peripheral blood mononuclear cells (147) or in plasma (148) of OA patients compared to controls. Taken together, these findings suggest that miR-146a could be a promising OA biomarker as well as a potential therapeutic target to regulate inflammatory/catabolic effects in chondrocytes and synoviocytes.

Other miRNAs associated with inflammatory pathways in OA

miR-140 is relatively specific to cartilage and has been well-studied in this tissue. Loss of miR-140 in mice has been shown to result in a mild skeletal growth phenotype and, in post-natal articular cartilage, results in accelerated OA following aging or surgical destabilization of knee joints (149,150). This post-natal phenotype may be explained by other reports that miR-140 can function to suppress IL1β-induced *Mmp13* expression (151) or *Adamts5* expression (139).

Recently, a number of other miRNAs have been identified that also appear to function in attenuating the pro-catabolic gene expression patterns induced by IL1β in chondrocytes including miR-502-5p (152), miR-320 (153), miR-149 (154), miR-558 (155), and miR-199a* (156). A recent study by Xie et al. utilized an obese mouse model (i.e., C57BL/6 male mice fed a high fat diet for 12 weeks) and showed not only that the high-fat diet group had increased plasma concentrations of proinflammatory cytokines (IL-1β, IL-6, TNF-α), but also that the plasma level of a specific miRNA, miR-26b, was decreased (157). To determine the potential mechanism of miR-26a, non-esterified fatty acids (NEFAs) were

used to treat chondrocytes *in vitro* to mimic the *in vivo* obesity-induced inflammatory effects. The authors found that miR-26a overexpression could attenuate NEFA-induced activation of NF-κB (p65) and production of proinflammatory cytokines in murine primary chondrocytes. It was then shown that NF-κB could inhibit miR-26a production by binding to a region in the miR-26a promoter. To attempt to correlate these findings with the human condition, they found that plasma NEFA, cartilage p65 activity, and TNFα levels were positively correlated with BMI in patients with OA, whereas expression of miR-26a in chondrocytes from OA cartilage showed the opposite effect.

Another study by Zhang et al. utilized both *in vitro* and *in vivo* approaches to show potential chondroprotective effects of miR-210 (158). Overexpression of miR-210 via mimics was found to inhibit lipopolysaccharide-induced proinflammatory cytokines and cell death in primary rat chondrocytes and that one of its potential targets was death receptor 6 (DR6) mRNA. Transection of the anterior cruciate ligament was then performed to destabilize the knee joint of rats as a means to induce OA. At the time of surgery, one group was administered with miR-210-expressing lentivirus via intra-articular injection. However, no control groups were injected with lentivirus only or lentivirus expressing a scrambled miRNA. Twenty days following surgery, knee joints from treatment and control saline-injected groups were harvested and cartilage from the medial tibial plateaus was extracted for gene and protein expression analysis. Although seemingly challenging to be able to extract sufficient amounts of RNA and protein from one region of skeletally mature rat articular cartilage for downstream polymerase chain reaction (PCR) and Western blotting, the authors apparently showed decreased DR6, decreased p65, and increased IκBα protein expression in the surgically induced OA cartilage overexpressing miR-210 compared to cartilage from the saline-injected OA group. However, this study did not perform histological analysis of knee joints at later time points after surgeries, and so the longer-term effects of miR-210 overexpression in potentially ameliorating OA progression remains to be shown.

Clearly, many miRNAs have now been reported to induce anti-inflammatory effects in chondrocytes via regulating different target genes and signaling pathways. With this knowledge, a potentially fruitful strategy moving forward could be to explore a combination miRNA approach to attempt to further inhibit catabolic events and hence stop or slow down OA progression.

Long non-coding RNAs regulated by inflammation in chondrocytes

A number of large-scale transcriptome analyses have revealed that a huge proportion of the non-coding genome is transcribed, including the epigenetic regulators known as long non-coding RNAs (lncRNAs) (159,160). lncRNAs are generally defined as transcripts of ~200 nucleotides or more in length that do not encode proteins. Similar to mRNAs, they are primarily transcribed by RNA polymerase II and can be post-transcriptionally processed (i.e., intron removal, alternatively spliced, addition of poly A tails etc.). Generally, lncRNAs have been found to be more restricted to specific tissue types, their expression levels to be significantly lower than those of protein-coding transcripts, and they are less frequently conserved between species (161–163). However, lack of conservation does not necessarily

mean lack of function; some reports suggest that RNA structure is an important feature of lncRNA function (164).

LncRNAs can be transcribed from various locations in the genome: (1) antisense lncRNAs are transcribed in the opposite direction within a protein-coding gene and overlaps with coding exons; (2) intronic lncRNAs initiate within the intron of a protein-coding gene in either direction and does not overlap with coding exons; (3) bidirectional lncRNAs can be transcribed in a divergent manner from a promoter within a coding gene; (4) intergenic lncRNAs (large intervening noncoding RNAs; lincRNAs) are transcribed from their own transcription unit and are generally located ~5kb from protein coding genes (159,165). Studies have shown that lncRNAs are more likely to be localized to the nucleus where they are involved in various epigenetic mechanisms to control gene expression (160,166). For more in-depth information on lncRNA function, particularly in the context of skeletal biology, please refer to the review by Nguyen et al. in this Epigenetics Special Edition of Connective Tissue Research (PMID: 27254479).

For the purpose of this review, we will highlight one recent study showing lncRNA expression changes in chondrocytes following inflammatory cytokine induction (167). Specifically, short-term treatment of human primary hip OA chondrocytes with IL1β followed by RNA-Seq analysis revealed a number of lncRNAs expressed in chondrocytes as well as differentially expressed lncRNAs between treated and untreated chondrocytes (their RNA-Seq data is publically available in the GEO data repository: GSE74220). Three lincRNAs were found to be more highly expressed in IL1β-treated cells and were pursued further in this study: *PACER* (p5-associated COX2-extragenic RNA) and two novel chondrocyte inflammation-associated lincRNAs (*CILinc01* and *CILinc02*). Interestingly, qPCR analysis showed that expression of these lincRNAs was lower in OA cartilage compared to control cartilage. This result may indicate that the inflammatory status (and hence lncRNA expression) between chondrocytes embedded within late-stage OA cartilage versus cultured chondrocytes isolated from cartilage following treatment with IL-1β does not correlate well. In any case, this report has provided the first set of lncRNA expression data within the context of cytokine-treated chondrocytes and will be a useful resource for future studies designed to understand how inflammatory factors may affect the epigenetic functions of specific lncRNAs. Overall, we anticipate that the field of lncRNAs in cartilage and bone-related research will increase exponentially over the coming years.

Conclusions and future studies

Clearly, epigenetic switches induced by inflammation can play an important role in regulating chondrocyte catabolic processes in cartilage tissue. In the cancer field, inflammation is thought to induce many of the epigenetic changes observed in these diseases (168). Therefore, given the involvement of inflammation in OA pathology, the methylome changes that have been identified in OA chondrocytes, and the growing evidence that inflammation can regulate epigenetic factors, we propose that cross-talk between inflammation and epigenetic regulators (e.g., TET1, DNMT3B, HDACs, SIRT1) can contribute toward the initiation and/or progression of OA. More research is needed to further investigate the functional roles of DNMTs and TETs not only in response to inflammatory

cytokines, but also in regulating other chondrocyte homeostatic responses. In addition, modulation of DNMTs or TETs *in vitro* or *in vivo* followed by RNA-Seq and Methyl-Seq analysis could provide important information on novel genes/pathways that could be therapeutically targeted. Therapies to potentially mimic epigenetic enzymes that decrease with age or are negatively regulated in an inflammatory setting (e.g., TET1, SIRT1) could also be promising future strategies to treat OA. Analysis of HDAC inhibitors to treat inflammatory joint disease is another area of research worthy of further study given the current published data. With respect to non-coding RNAs, a lot of information is now available on the expression of miRNAs in OA as well as their potential role in attenuating inflammatory signaling. Given all of the findings to date, it may be that no one individual miRNA could be targeted to effectively attenuate catabolic responses in OA, but perhaps a combination therapy approach would be more useful in future studies. To date, epigenetic functions of lncRNAs in response to inflammation and in potentially regulating chondrocyte homeostasis are completely unknown; it is anticipated that research interests in this field will significantly increase over the coming years and provide us with novel therapeutic targets to treat cartilage degeneration.

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