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Chapter 2

Epigenetic Mechanisms in Osteoarthritis

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1. Introduction

The hallmark of osteoarthritis (OA) is the progressive degeneration of articular cartilage, although bone and synovia are also involved in the development of the disease [1]. Chondrocytes are the unique cellular component of articular cartilage and, under physiological conditions, are responsible for a subtle balance between the synthesis of extracellular matrix (ECM) components, mainly type-II collagen and aggrecan [2], and its degradation by proteolytic enzymes such as the matrix metalloproteinases (MMP) [3] and A disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) [4]. In OA, there is an imbalance of this process driven by cytokines and the production of inflammatory mediators, resulting in an increase of the degradation process with respect to synthesis, and leading to articular cartilage loss [1, 5].

OA is considered a multifactorial disease in which genetics and environmental factors, such as aging, gender and obesity, among others, are strongly related with its development [6]. Primary OA possesses an important genetic component, and several genetic association studies have demonstrated that it is associated with different genes that encode molecules involved in a number of pathways, such as inflammation, Wnt signalling, bone morphogenetic proteins (BMPs), proteases and their inhibitors, and extracellular matrix proteins, among others [7, 8]. However, there has not always been consistency in the results, probably due to the low penetrance of the gene polymorphisms studied, or to different gene-gene interactions and gene-environment interactions. In this regard, epigenetics is one mechanism through which gene-environment interactions occur. Epigenetics refers to heritable changes in gene expression that occur without changes in DNA, and includes DNA methylation, histone modifications, chromatin remodelling and microRNAs (miRNAs), although debate continues concerning whether miRNA can be categorized as an epigenetic phenomenon [9]. Recent evidence has made it apparent that epigenetic changes alter the expression of genes that could participate in the pathogenesis of OA. This chapter does not intend to conduct a deep review of epigenetic modifications, but rather to review the main findings directly related with OA.

2. DNA methylation in osteoarthritis

DNA methylation is the most studied epigenetic mark in humans. DNA methylation involves the addition of a methyl group at CpG dinucleotides to convert cytosine into 5-methylcytosine. These CpG dinucleotides tend to cluster in regions termed 'islands', and approximately 70% of human gene promoters are associated with CpG, which are usually unmethylated. CpG-island methylation is associated with gene silencing due to the binding of methyl-CpG-binding proteins, which recruit proteins to the gene promoter, blocking its transcription. DNA methylation can also occur in CpG island shores, regions of lower CpG density that lie in close proximity to CpG islands (~2 kb); their methylation is associated with transcriptional inactivation. DNA methylation is mediated by the DNA methyltransferases (DNMTs) family of enzymes that catalyse the transfer of a methyl group to DNA. In mammals, five members of the DNMT family have been reported: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L, but only DNMT1, DNMT3a and DNMT3b possess methyltransferase activity. DNMTs are classified as *de novo* and maintenance enzymes. DNMT1 is the maintenance DNMT and has a preference for hemimethylated DNA; this is the most abundant DNMT in cells and is transcribed mostly during the S phase of the cell cycle. DNMT3A and DNMT3B are *de novo* DNMTs and are responsible for establishing the pattern of methylation during embryonic development [9-13].

Analysis of the overall methylation level of genomic DNA in the chondrocytes of degenerated OA cartilage shows no difference in comparison with normal chondrocytes. However, an inhibitor of cell proliferation, the cyclin-dependent kinase inhibitor 1 ($p21^{WAF1/CIP1}$) gene, which may mediate the re-initiation of cell proliferation in OA cartilage and which has demonstrated itself to be epigenetically regulated in neoplastic cells [14], is significantly downregulated in OA chondrocytes and does not exhibit hypermethylation in its promoter [15].

In chick embryos' chondrocytes, the *Col2a1* gene shows reduced methylation in comparison with other cells, such as fibroblasts and erythrocytes [16]. In humans, it remains unclear whether the methylation of DNA alters the regulation of cartilage matrix genes in OA. Aggrecan is reduced during aging and in OA cartilage; however, the aggrecan (*ACAN*) gene expression of normal aging and osteoarthritic articular human cartilage does not correlate with increased methylation of the *ACAN*-promoter CpG island [17]. Human articular chondrocytes remain negative for type X collagen, unless they become hypertrophic. In the latter cells, the gene methylation patterns and the expression of *COL10A1* and *COL2A1* have shown that the *COL10A1* promoter is methylated, which correlates with the suppression of hypertrophy observed in articular chondrocytes, and there is no evidence of *COL2A1* regulation at the methylation level, which demonstrates a low methylation rate [18].

The nuclear transcription factor SOX9, along with other SOX family members, is required for the control of the expression of ECM components. SOX9 is an important regulator of the chondrocyte phenotype and controls the expression of the *COL2A1*, *COL9A1*, *COL11A1* and *ACAN* genes. The SOX9 protein binds to its promoter elements and forms transactivating complexes with other proteins, such as SOX5/SOX6. The SOX family maintains the chondrocytic phenotypes, and is vital for chondrogenesis in embryonic development [19, 20]. In human

synovium-derived mesenchymal stem cells (MSCs) subjected to chondrogenesis the CpG island of *SOX9* is hypomethylated; as well as in other chondrogenesis related genes such as runt-related transcription factor 2 (*RUNX2)* and fibroblast growth factor receptor 3 (*FGFR3)* [21]. While in OA chondrocytes, *SOX9*-promoter is hypermethylated, which reduces the binding affinity of the transcription factors CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) and the cAMP response element binding (CREB) [22]. This means that the methylation of the *SOX9* promoter remains low during chondrogenesis, and in OA there is change in the epigenetic status of *SOX9*, including increased DNA methylation.

Metalloproteinase expression in normal cartilage is relatively low but is elevated in OA, resulting in ECM degradation [3, 23]. The altered synthesis of the cartilage-degrading enzymes in OA is the result of changes in the methylation status, as demonstrated by the analysis of the methylation of the promoter region of *MMP3*, *MMP9*, *MMP13* and *ADAMTS4* in the cartilage of patients with OA, in which the overall percentage of non-methylated sites increased in comparison with normal controls. However, not all CpG sites were equally susceptible to loss of methylation, and for each gene there was a specific site where OA demethylation was higher, namely: -635 for *MMP3*, -36 for *MMP9*, -110 for *MMP13*, and -753 for *ADAMTS4* [24]. This is interesting because it was generally thought that the methylation of many CpG sites was required to repress gene expression, and these findings suggest that methylation of a single site may be sufficient to affect gene expression. In agreement with this, demethylation of -110 in *MMP13* promoters and -299 in *IL1B* promoters is correlated with an increased gene expression. In addition, methylation of the -110 CpG site in *MMP13* decreases the hypoxia inducible factor 2α (HIF-2α), binding to the *MMP13* promoter. HIF-2α is a transcription factor that regulates *MMP13* expression [25]. On the other hand, demethylation of the -104 CpG region of the *MMP13* promoter correlates with increased gene expression and avoids the binding of the transcription factor CREB to its promoter [26]. ADAMTS5 is considered to be the major aggrecanase; however, ADAMTS4 also contributes to aggrecan degradation in OA. *ADAMTS4* is epigenetically regulated and, although methylation is lost in several promoter sites, the -753 site is that most consistently demethylated [27].

Nitric oxide (NO) is a multifunctional molecule that suppresses energy production by mitochondrial respiration. In OA, high amounts of NO are produced, a consequence of upregulation in the chondrocyte of the inducible NO synthase (iNOS) induced by inflammatory cytokines, such as interleukin 1-beta (IL-1β) and tumour necrosis factor alpha (TNFα), among others [28]. NO suppresses the synthesis of the cartilaginous matrix [29]. In culture, under-stimulated chondrocytes produce *iNOS*, and its promoter contains nuclear factor-kappa beta (NF-κB) binding sites; this regulates *iNOS* at the transcriptional level. NF-κB is a signalling factor activated by tissue damage and inflammation; its demethylation in specific enhancer elements favours the activation of *iNOS* in chondrocytes [30]. Interestingly, a study showed that glucosamine and an NF-kB inhibitor inhibit cytokine-induced demethylation at a specific site in the *IL1B* promoter, resulting in decreased gene and protein expression [31].

Leptin (*LEP*) is a cytokine-like peptide hormone secreted by white adipose tissue, which plays a key role in OA [32] because it has been shown that *LEP* exerts a detrimental effect on articular cartilage by promoting NO synthesis in chondrocytes [33]. In normal chondrocytes, *LEP* is

highly methylated and, in OA, it is demethylated and highly expressed. Additionally, *LEP* downregulation with small interference RNA (siRNA) decreases *MMP13* expression [34].

To date, it is well recognized that OA has an important inflammatory component in its development mediated by proinflammatory cytokines, such as IL-1β and TNFα [2, 32]. Healthy chondrocytes do not express *IL1B*, however, promoter demethylation increases the expression of the gene [35]. Suppressor of cytokine signalling (SOCS) proteins are inhibitors of cytokine signalling. There are eight SOCS proteins, including SOCS1-SOCS7 and cytokine-inducible SH2-domain-1 (CIS-1), with SOCS1, -2 and -3, and CIS-1 the best characterized. *SOCS2* and *CIS-1* expression is reduced in OA chondrocytes compared with normal chondrocytes, while *SOCS1* and *SOCS3* expression remains unchanged. In addition, the *SOCS2* promoter does not exhibit a change in its methylation status [36].

Bone morphogenetic protein-7 (BMP-7) – or osteogenic protein-1(OP-1) – is one of the most potent growth factors for cartilage maintenance and repair. It possesses a critical role in human cartilage homeostasis, regulating numerous metabolic pathways that are not only limited to its well-documented anabolic function but also to its anti-catabolic activity [37]. There is a positive correlation between age and the methylation of the *BMP7* promoter's status in aged chondrocytes; this age-related promoter methylation may contribute to a decrease in BMP7 production in cartilage, with the decreased expression of the insulin-like growth factor-1 (*IGF-1*) and the IGF-1 receptor (*IGF-1R*) genes, as well as the ECM component gene *ACAN* [38].

The growth differentiation factor 5 (*GDF5*) gene is a member of the transforming growth factor β (TGFβ) superfamily that is involved in chondrogenesis and chondrocyte proliferation [39]. The rs143383 C/T single nucleotide polymorphism (SNP) in the 5′ untranslated region (5′UTR) of the gene is associated with an increased risk of OA [40, 41]. This SNP is itself functional and exerts a joint-wide effect on *GDF5* expression, causing a significant reduction in the expression of the disease-associated T allele relative to the C allele in the cartilage and other joint tissues [42], a phenomenon known as 'differential allelic expression' (DAE). The transcriptional effect of rs143383 SNP is dependent on a second C-to-T SNP in the 5′UTR of *GDF5*, rs143384 C/T, with decreased expression of the T allele of rs143383 only observed in individuals which are compound-heterozygous for both SNPs. When the OA-protective C alleles are present at the rs143383 and rs143384 SNPs, they form CpG dinucleotides, which are potentially amenable to regulation by DNA methylation. In cell lines, *GDF5* is upregulated after demethylation, and methylation decreases transcriptional activity. Interestingly, CpG sites formed by the C alleles of both SNPs are methylated; however, their demethylation is associated with increased expression of the C allele of rs143383 relative to the T allele, which indicates that the OA-susceptibility conferred by rs143383 of the *GDF5* gene is regulated by methylation [43].

Methylation analysis of 23,367 sites (corresponding to 13,463 genes) through a genome-wide methylation profile of bone from patients with OA and osteoporosis (OP) revealed an inverse relationship between methylation and gene expression in both groups, with 271 CpG sites being less methylated in OP than in OA. *In silico* pathway analysis revealed genes associated in glycoprotein metabolism or cell differentiation, particularly the homeobox superfamily of transcription factors such as homeobox A9 (*HOXA9*), Iroquois homeobox 2 (*IRX2*) and msh homeobox 2 (*MSX2*), which are involved in embryonic development [44].

3. Histone modification, chromatin remodelling and osteoarthritis

DNA is compacted by the tight weaving of approximately 147 base pairs around the proteins' denominated histones, forming a DNA-protein complex termed a 'nucleosome', the basic unit of chromatin. Each nucleosome consists of an octamer of two copies of the following four core histones: H2A, H2B, H3 and H4. The nucleosomes comprise a barrier to transcription that blocks the access of activators and transcription factors to their sites in DNA. The histones are subject to post-transcriptional modification; the most common are acetylation and methylation, although other modifications have been identified, including phosphorylation, ubiquitination, SUMOylation, citrullination, and adenosine diphosphate ribosylation. Histones acetylation occurs at lysine residues and is associated with DNA accessibility and transcriptional activity, whereas deacetylation is associated with transcriptional repression. Histone acetyltransferases (HATs) are enzymes that transfer the acetyl group onto the ε-amino group of the lysine residues within a histone tail; this is a reversible process, and the enzymes that remove the acetyl groups are known as 'histone deacetylases' (HDACs). Classical isoforms of HDACs comprise a total of 11, and are broadly divided into two classes: HDACs 1, -2, -3 and -8 are Class I HDACs, while Class II encompasses HDAC isoforms 4, -5, -6, -7, -9, -10 and -11. The newly characterized SIR2 family of HDACs (sirtuins), termed 'Class III', operate through a nicotinamide adenine dinucleotide (NAD+)-dependent mechanism. Histone methylation is another major modification that takes place in the ε-amino group of lysine residues; it is mediated by histone H3 N-lysine lysine methyltransferases (HKMTs). The effect of histone lysine methylation on gene regulation is highly complex, mediating either transcription repression or activation. Likewise, the methylation of arginine residues is catalysed by the protein arginine methyltransferase (PRMT) family. Regulation of gene transcription can also occur by chromatin remodelling. The SWItch/sucrose non-fermentable protein complex binds to the nucleosome and disconnects the DNA from the histones, creating a transient DNA loop and resulting in nucleosome repositioning, such that the transcription of targeted genes can be increased or decreased depending upon whether the gene is located in the open-chromatin or compacted chromatin region. Polycomb-group proteins are also involved in gene silencing through chromatin remodelling, repressing transcription by maintaining a heterochromatin state through particular histone modifications and DNA methylation [9-13].

Chondrocyte differentiation is controlled by transcription factors such as SOX9, among others. SOX9 requires other cofactors, such as the CREB binding protein, which activates SOX9-dependent transcription due to its intrinsic histone acetyl-transferase activity [45]. In human chondrocytes inducted by IL-1β and the fibroblast growth factor 2 (FGF2), there is increased expression of MMPs and ADAMTS, responsible for collagen and aggrecan loss, respectively. However, HDAC inhibitors (HDACi) block the induction of these enzymes at the mRNA and protein levels [46, 47]. Thus, HDACi also suppress IL-1β-induced NO and prostaglandin E_2 (PGE2) synthesis, which plays an important role in OA as well as in proteoglycan degradation [48].

Specific HDACs appear to be involved in different processes as well as targeting different chondrocyte-specific genes. In a murine model, *HDAC4* demonstrates the regulation of

chondrocyte hypertrophy and endochondral bone formation by interacting with Runx2 and inhibiting its activity [49]. In the chondrocytes of patients with OA, HDAC1 and HDAC2 proteins are elevated with the specific downregulation of *COL2A1* and *ACAN*, though not with other cartilage marker genes. This is because the snail transcription factor acts as a mediator of the HDAC1 and HDAC2 repression of *COL2A1* via its interaction with HDACs' carboxy-terminal domains [50]. *HDAC7* shows a significant increase in OA cartilage, while its knock-down by siRNA in a chondrosarcoma cell line suppress *MMP-13* expression [51].

The role of HDACi has been explored in OA. HDAC activity decreases during chondrocyte dedifferentiation, and the inhibition of HDAC with HDACi trichostatin suppresses type II collagen expression. This is because HDACi promotes the acetylation of Wnt-5a, increasing its expression, which is known to inhibit type II collagen [52]. Human chondrocytes under mechanical stress exhibit the downregulation of *COL2A1* and upregulation of *RUNX2*, *ADAMTS4* and *MMP3*; however, after treatment with HDACi there is an increase of *COL2A1* expression, the downregulation of *RUNX2*, *ADAMTS4* and *MMP3*, and an inhibition of the mechanical stress-induced phosphorylation of mitogen-activated protein kinase (MAPK) molecules after treatment with HDACi [53]. RUNX family members regulate the gene expression involved in cellular differentiation and cell cycle progression. *RUNX2* plays a key role in bone mineralization by stimulating osteoblast differentiation [54] and it contributes to OA pathogenesis through chondrocyte hypertrophy and matrix breakdown after the initiation of joint instability [55]. MAPK pathways play essential regulatory roles in early osteoblast differentiation in response to mechanical stress via the activation of *RUNX2* [56]. *In vivo,* the effects of HDACi trichostatin on cartilage degradation in a rabbit experimental model showed that HDACi decreases cartilage degradation as well as the expression of IL1 and MMPs, such as *MMP1*, *MMP3* and *MMP13* [57].

Histone H3 lysine-4 (H3K4) methylation is associated with transcriptional activation, whereas H3K9 methylation correlates with transcriptional repression. In human osteoarthritic chondrocytes, the induction of iNOS and cyclooxygenase 2 (COX2) expressions by IL-1β are associated with H3K4 di and trimethylation at the *iNOS* and *COX2* promoters; these changes correlate with the recruitment of SET-1A, a HKMT. Furthermore, HKMT inhibition prevents the IL-1β induction of iNOS and COX2 [58].

Nfat1 is a nuclear factor of the activated T cells' transcription factor family and is a regulator of cytokine gene-expression. It has been reported that adult *Nfat1*-deficient mice display abnormal chondrocyte differentiation in their articular cartilage and develop several articular cartilage characteristics that resemble these changes in OA in humans. These OA-like changes appear at the adult stage and an increase in *Nfat1* expression in the chondrocytes is associated with increased H3K4 methylation, whereas a decrease of *Nfat1* is associated with an increase in H3K9 methylation, which demonstrates that *Nfat1* specifically regulates the function of adult articular chondrocytes through its age-dependent expression, mediated by dynamic histone methylation [59].

NAD-dependent class III HDACs consist of SIRT1-7. SIRT1 plays a key role in the regulation of metabolism, as well in regulating cell differentiation, proliferation, survival and longevity [60, 61]. SIRT1 increases cartilage-specific gene expression, such as *ACAN*, *COL2A1*, *COL9A1*

and *COMP*. SIRT1 deacetylate SOX9 enhances the transcription of *COL2A1* and, at least for the *COL2A1* gene promoter, also enhances the acetylation of critical histone core residues in the promoter through the recruitment of activator/co-activator proteins [62]. In human chondrocytes, SIRT1 inhibits NO-induced apoptosis caused, at least partially, by caspases 3 and 9 [63]. Another mechanism by means of which SIRT1 inhibits apoptosis in human chondrocytes is that of repressing protein tyrosine phosphatase 1B (PTP1B), a potent proapoptotic protein, and there is an inverse relationship in the expression patterns of SIRT1 and PTP1B in normal and OA cartilage. In contrast, SIRT1 levels are high and PTP1B levels are low in normal cartilage, while in OA SIRT1 levels are low and PTP1B levels are high [64]. In OA, the inhibition of SIRT1 induces OA-like gene expression changes with a downregulation of *ACAN* and upregulation of *COL10A1* and *ADAMTS5*, which suggests that SIRT1 expression decreases with the development of OA, favouring chondrocyte hypertrophy and cartilage matrix loss [65].

To date, OA is well-recognized as an inflammatory disease in which inflammatory cytokines play a central role. In human OA chondrocytes treated with TNFα there is an impaired SIRT1 activity due to cleavage mediated by cathepsin B, resulting in the upregulation of *MMP13* and *ADAMTS4* and reduced cartilage-specific gene expression, such as *COL2A1*, *COL11A1* and *ACAN* [66]. Null mice for SirT1 (SirT1 -/-) do not survive, and heterozygous mice for SirT1 (+/-) are smaller and exhibit a greater increase in OA changes than normal mice. In addition, in heterozygous mice, inflammatory cytokines are upregulated and demonstrate a marked increase in apoptosis, which suggests that SirT1 may prolong the viability of articular chondrocytes in adult mice [67]. In human chondrocytes, the overexpression of SIRT1 significantly inhibits the upregulation of genes caused by the pro-inflammatory cytokines IL-1β and TNF-α (*MMP1*, *-2*, *-9*, and *-13*, and *ADAMTS5*), while in the OA cartilage SIRT1 expression decreased while that of *MMP13* and *ADAMTS5* increased [68, 69]. Therefore, SIRT1 exerts an anti-inflammatory effect and prevents chondrocyte apoptosis.

4. microRNAs in chondrogenesis and osteoarthritis

MicroRNA (miRNA) are small noncoding RNAs of ~20–25 nucleotides (nt) in length that are transcribed in the nucleus by RNA polymerase II or III into a long precursor denominated primary-miRNA (pri-miRNA). This pri-miRNA is processed by the microprocessor Drosha-DGCR8 complex, an RNase III-type enzyme, to generate a precursor of ~70–100 nt, known as 'pre-miRNA', which is translocated into the cytoplasm via exportin 5. Pre-miRNA is processed by the ribonuclease dicer, generating a miRNA duplex of ~22 nt. Finally, one of the strands is incorporated into the RNA-induced silencing complex (RISC), where it is guided to its target mRNA. miRNA is involved in post-transcriptional gene-expression regulation, targeting 30% of the encoding genes through complementary base-pairing between the miRNA and the 3′-UTR of the messenger RNA (mRNA) target, resulting in the translational suppression or direct degradation of the mRNA [9-13].

4.1. Cartilage and miRNA

The influence of miRNAs in cartilage homeostasis and skeletal development has been demonstrated in recent years. Dicer is an essential enzyme for the generation of mature miRNA and for proper skeletal morphogenesis. In mouse models, the loss of *dicer1* leads to embryonic lethality, with animals surviving until embryonic day 7.5 [70] with limbs that are small in size due to the loss of processed miRNAs [71]. Dicer possesses an important function in cartilage as demonstrated in mice in which the gene was specifically deleted in cartilage. These mice exhibited a progressive reduction in the proliferating pool of chondrocytes in growth plates, leading to severe growth defects because of a decrease in proliferating chondrocytes and an accelerated differentiation into hypertrophic chondrocytes. The latter results may be explained by dicer loss having distinct functional effects at different stages of chondrocyte development [72].

miR-140 is the most studied miRNA in both cartilage and OA. This miRNA was originally identified as a cartilage-restricted miRNA in developing zebra fish, with its expression in the jaw, head and fins during embryonic development [73]. In mice, miR-140 is also expressed in cartilage during embryonic long- and flat-bone development [74]. In a murine model with a targeted deletion of miR-140, mice are borne with grossly normal skeletal development; however, postnatally they manifest skeletal deformities with short stature and craniofacial deformities, probably as a result of abnormal chondrocyte proliferation [75, 76]. miR-140 is encoded in an intronic region of the ubiquitin E3 ligase gene, *WWP2*, which plays an important role in cartilage biology. miR-140 is highly conserved among vertebrates and it is not present in invertebrates, which suggests that it plays an important role in skeletal development [77]. In mice, miR-140 is exclusively expressed in chondrocyte, is co-expressed with *Wwp2*, and is directly induced by the transcription factor SOX9. Sp1, the activator of the cell cycle regulator $p15^{INK4b}$, is a target of miR-140, suggesting that it regulates chondrogenic proliferation in part via the inhibition of Sp1 [78].

In the gene expression pattern in human articular chondrocytes and human MSC, miR-140 saw the largest differences in expression. During chondrogenesis, miR-140 increases in parallel with the expression of *SOX9* and *COL2A1*, and treating chondrocytes with IL-1β, suppresses miR-140 expression. On the other hand, transfection of chondrocytes with miR-140 down-regulate IL-1β-induced *ADAMTS5* expression and rescue the IL-1β-dependent repression of *ACAN* expression [79]. miR-140 has offered several targets: in mice it potentially suppresses *Hdac4*, a co-repressor of Runx2, the transcription factor essential for chondrocyte hypertrophy and osteoblast differentiation [74]. Other targets include the CXC group of chemokine ligand 12 (*Cxcl12*, also known as 'stromal-derived factor 1' (*SDF-1*)) [80] and *SMAD3* [81], both of which are implicated in chondrocyte differentiation. Interestingly, miR-140 was reported as suppressing *Dnpep*, an aspartyl aminopeptidase that catalyses the sequential removal of amino acids from the unblocked N termini of peptides and proteins, which antagonize BMP signalling downstream of *SMAD* activation [76].

In addition to miR-140, miR-455-3p expression is also restricted to the cartilage and perichondrium of the developing long bones in chicks and to the long bones and joints in mouse embryos, and it contributes to chondrogenesis in humans. miR-455-3p resides in an intron of

COL27A1, a collagen expressed in cartilage, and its expression is regulated by TGFβ ligands and miRNA-regulated TGFβ signalling. Activin receptor type IIB (*ACVR2B)*, *SMAD2*, and chordin-like protein (*CHRDL1*) are targets of miR-455-3p, and may mediate its functional impact on TGFβ signalling, suppressing the *SMAD2/3* pathway; therefore, its unincreased expression could exacerbate the OA process [82].

To study the miRNA-mediated regulation of chondrogenesis, the expression of 35 miRNAs in chondroblasts derived from MSC was analysed and it was found that miR-199a and miR-124a were strongly upregulated, while miR-96 was substantially suppressed. The potential targets of the miRNAs are the following transcriptional factors: HIF-1α for miR-199a, regulatory factor X1 (RFX1) for miR-124a, and SOX5 for miR-96, which demonstrate that miRNAs and transcription factors could fine-tune cellular differentiation [83].

In another miRNA microarray in MSC at four different stages of TGF-β3-induced chondrogenesis differentiation, eight significantly upregulated and five downregulated miRNA were observed. Two miRNA clusters, miR-143/145 and miR-132/212, were maintained on downregulation, while miR-140-3p was the most upregulated. By means of bioinformatics approaches, the following target genes were predicted: *ADAMTS5* for miR-140-3p; activin receptor 1B (*ACVR1B*) for miR143/145; *SOX6* for miR-132/212; and *RUNX2* for miR-30a [84]. Consistent with that finding, miR-145 decreased during TGF-β3-induced chondrogenic differentiation of murine MSC, and targeted the SRY-related high-mobility group-box gene 9 (SOX9), the key transcription factor for chondrogenesis. In cells overexpressing miR-145, the expression of chondrogenic markers was significantly decreased at the mRNA level, including *COL2A1*, *ACAN*, the cartilage oligomeric matrix protein (*COMP*), *COL9A2* and *COL11A1* [85], indicating that miR-145 comprises a key mediator of early chondrogenic differentiation attenuating SOX9 at the post-transcriptional level. In this way, it was reported that miR-145 is a direct regulator of SOX9 in normal human articular chondrocytes through binding to a non-conserved specific site in its human 3′-UTR. In addition, the increased expression of miR-145 in articular chondrocytes greatly reduced the expression *COL2A1* and *ACAN*, and critical cartilage ECM genes, and significantly increased the hypertrophic markers *RUNX2* and *MMP13*, responsible for the changes during the OA process [86].

miR-675 could also regulate *COL2A1* expression. miR-675 is processed from H19, a noncoding RNA, in healthy human chondrocytes. This miRNA is highly expressed and is regulated by SOX9 during chondrocyte differentiation, and upregulates the expression of *COL2A1.* The overexpression of miR-675 rescued COL2A1 levels in H19- or SOX9-depleted chondrocytes, which suggests that the regulation of *COL2A1* by SOX9/H19 is mediated specifically by miR-675. These data suggest that miR-675 may modulate cartilage homeostasis by suppressing a *COL2A1* transcriptional repressor [87].

A comparative miRNA array of approximately 380 miRNAs in C2C12 cells induced by BMP2 found that several miRNAs, including let-7e, miR-221, miR-199a-3p, miR-374 and miR-298 were positively regulated, while miR-125a, miR-210, miR-125b, miR-21, miR-145 and miR-143 were repressed. Among these, miR-199a-3p was the most significantly upregulated at 24 h following BMP2 induction in the C3H10T1/2 cells and in an *in vitro* cell model of chondrogenesis. When these cells were transfected with miRNA-199a-3p, they exhibited a significant

decrease in mRNA expression levels of the chondrogenic markers COL2A1 and COMP, suggesting that miR-199a-3p is an inhibitor of the early stages of chondrogenic differentiation. miRNA target-prediction demonstrated that the putative target genes are *SMAD1* and *SMAD5*, which are known downstream mediators of BMP signalling in osteochondroprogenitor cells, which suggests that miR-199a-3p is a BMP2 responsive microRNA that adversely regulates early chondrocyte differentiation via the direct targeting of the *SMAD1* transcription factor [88].

To investigate the miRNA expression pattern involved in the chondrocyte dedifferentiation process, a microarray analysis was performed. Several miRNA were deregulated, including 13 upregulated and 12 downregulated miRNA in differentiated as compared with dedifferentiated chondrocytes. The most notable changes were for miR-491-3p, miR-140-3p, miR-140-5p and let-7d, which were upregulated, and for miR-548e, miR-342-5p, miR-1248 and miR-146a, which were downregulated. Bioinformatics analysis revealed 21 microRNA–gene target pairs potentially involved in chondrocyte dedifferentiation. Among these, miR-548e-*SOX9*, miR-1248-*ACAN*, miR-18a-*IGF1*, miR-193b-*SOX5*, miR-631-*RUNX1*, miR-335-*CRTAP*, miR-153-*MATN2* and miR-26a-*COL1A2* are involved in ECM proteins and homeostasis in chondrocytes, miR-365-*BCL2* in the apoptosis mechanism, and let-7a, d, f- *ITGB3*, miR-320d-*DBN1*, miR-1260-*LAMC2* and miR-222-*ITGB3* are involved in cytoskeleton organization [89]. Human primary articular chondrocytes allow the initiation of proliferation to produce ECM molecules similar to embryological chondroblasts, and are called 'chondroblast-like' cells. The miRNA expression profile in these cells showed five differentially expressed miRNA clusters. Among these, one cluster consisted of miR-451, four miRNA included miR-140-3p, another cluster had five miRNA including miR-221 and miR-222, and the last cluster consisted of 11 miRNA, including miR-143 and miR-145, all of these being upregulated at different differentiation stages that might inhibit the molecules from participating in the dedifferentiation process [90].

miR-194 decreased during chondrogenic differentiation in adipose-derived stem cells (ASCs) (which are capable of differentiating into cartilage lineages *in vitro*). This downregulation increases its direct target gene, *SOX5*, resulting in the chondrogenic differentiation of ASC; thus, miR-194 may mediate chondrogenic differentiation via the suppression of the transcription factor *SOX5* [91]. In a rat model, miR-337 is associated with chondrogenesis by repressing transforming growth factor-beta type II receptor (TGFBR2) expression and modulating the expression of cartilage-specific genes, such as *ACAN*, in chondrocytes [92].

During the chondrogenesis differentiation of chick-limb mesenchymal cells, miR-221 may be involved in chondrocyte apoptosis; its inhibition reverses the chondro-inhibitory actions of a Jun N-terminal protein kinase (JNK) inhibitor in the proliferation and migration of chondrogenic progenitors. A target for miR-221 is Mmd2, an oncoprotein that has been shown to inhibit the activity of the p53 tumour suppressor protein with E3 ubiquitin ligase activity, and downregulation of Mmd2 prevents the degradation of the slug protein, which negatively regulates the proliferation of chondroprogenitors. The slug protein is a snail family member – it controls the developmental process by regulating the genes involved in cell adhesion and migration [93]. In the same model, miR-34a is a negative modulator of chondrogenesis and

affects MSC migration but not proliferation. EphA5, a receptor in Eph/ephrin signalling that mediates cell-to-cell interaction, is a miR-34a target [94]. Moreover, miR-34a regulates RhoA/Rac1 cross-talk and negatively modulates the reorganization of the actin cytoskeleton, which is one of the essential processes in establishing chondrocyte morphology [95]. Table 1 depicts the miRNAs implicated in chondrogenesis.

microRNA	Expression	Target		Ref.
miR-140	↑	*HDAC4* *Sp1* *ADAMTS5* *CXCL12* *SMAD3* *DNPEP*	Histone Deacetylase 4 Transcription Factor Sp1 A Disintegrin-Like And Metalloproteinase With Thrombospondin Type 1 Motif, 5 Chemokine, Cxc Motif, Ligand 12 Mothers against decapentaplegic homolog 3 Aspartyl aminopeptidase	74,76,78-81
miR-96	↓	*SOX5*	SRY-Related HMG-Box gene 5	83
miR-199a	↑	*HIF1α*	Hypoxia-inducible factor 1-alpha	83
miR-124a	↑	*RFX1*	Regulatory factor x 1	83
miR-199a-3p	↑	*Smad1* *Smad5*	Mothers against decapentaplegic homolog 1 Mothers against decapentaplegic homolog 5	88
miR-221		*Mdm2*	p53-binding protein Mdm	93
miR-675	↑	*COL2A1*	Type II collagen	87
miR-143/145	↓	*ACVR1B*	Activin a receptor, Type IB	84
miR-140-3p	↑	*ADAMTS5*		84
miR-132/212	↓	*SOX6*	SRY-Related HMG-Box gene 6	84
miR-30a	↑	*RUNX2*	Runt-related transcription factor 2	84
miR-145	↑	*SOX9*	SRY-Related HMG-Box gene 9	85,86
miR-let-7a,d, f	↑	*ITGB3*	Integrin, beta 3	89
miR-631		*RUNX2*		89
miR-548e	↓	*SOX9*	SRY-Related HMG-Box gene 9	89
miR-365	↓	*BCL2*	B-Cell CLL	89
miR-342-5p	↓	*IGFBP5*	Insulin-Like Growth Factor-Binding Protein 5	89
miR-335	↓	*CRTAP*	Cartilage-Associated Protein	89
miR-320d	↑	*DBN1*	Drebrin 1	89
miR-26a	↑	*COL2A1*	Type II collagen	89
miR-222	↑	*ITGB3*	Integrin, beta 3	89
miR-193b	↓	*SOX5*	SRY-Related HMG-Box gene 5	89

microRNA	Expression	Target		Ref.
miR-18a	↓	*IGF1*	Insulin-Like Growth Factor I	89
miR-153		*MATN2*	Matrilin-2	89
miR-1248	↓	*ACAN*	Aggrecan	89
miR-142-3p		*ADAM9*	A Disintegrin And Metalloproteinase Domain 9	95
miR-455-3p	↑	*ACVR2B* *SMAD2* *CHRDL1*	Activin a receptor, Type IIB Chordin-Like 1	82
miR-194	↓	*SOX5*	Chondrogenic differentiation	91
miR-337	↑	*TGFBR2*	Transforming Growth Factor-Beta Receptor, Type II	92
miR-181b	↓	*MMP13*	Matrix Metalloproteinase 13	96

Table 1. microRNAs implicated in chondrogenesis

4.2. Osteoarthritis and miRNAs

The effects of miRNA deregulation on OA are evident in studies comparing the expression of miRNAs between OA tissue specimens and their normal articular counterparts. A study tested 365 miRNA expression in articular cartilage obtained from patients with OA undergoing total joint replacement surgery as well as from normal controls. The study identified 16 miRNA differentially expressed in OA cartilage, of which nine were upregulated and seven downregulated. Through an *in silico* analysis, miRNA-gene targets were potentially involved in cartilage homeostasis and its structure (*miR-377-CART1, miR-140-ADAMTS5, miR-483-ACAN, miR-23b-CRTAP, miR-16-TPM2, miR-223-GDF5, miR-509-SOX9* and *miR-26a-ASPN*), in biomechanical pathways (*miR-25-ITGA5*), in apoptotic mechanisms (*miR-373-CASP6* and *miR-210-CASP10*), and in lipid metabolism pathways (*miR-22-PPARA, miR-22-BMP7, miR-103-ACOX1, miR-337-RETN* and *miR-29a-LEP*). The comparison of the molecular and clinical data revealed that miR-22 and miR-103 were highly correlated with body mass index (BMI) [97]. In another study, the expression profiling of 157 miRNA in chondrocytes obtained from OA cartilage identified 17 miRNAs that showed differential expression in comparison with normal controls. The most notable changes were observed for miR-9, miR-25 and miR-98, which were upregulated, and for miR-146 and miR-149, which were downregulated. A bioinformatics analysis performed to identify the potential gene targets suggested that a significant number of genes involved in inflammation were related with miR-9, miR-98 and miR-146. In overexpression experiments involving these miRNAs, miR-9, miR-98 and miR-146 were implicated in the control of TNFα, and miR-9 was implicated in MMP13 regulation [98]. To identify and characterize the expression profile of miRNA in the chondrocytes of III and IV grade OA, 723 miRNA were analysed and seven exhibited differential expression, of which one was upregulated and six were downregulated. In the bioinformatics prediction for knowing potential target genes regulated by these miRNA, it was found that the genes were involved in TGF-β, Wnt, MAPK and the mammalian target of rapamycin (mTOR) signalling pathways, as well in

focal adhesion, cytoskeleton regulation, ubiquitin-mediated proteolysis and the cell cycle. Interestingly, TGF-β and Wnt both played a role in OA [99].

Some miRNAs were examined as potential biomarkers of patients with OA of the knee, and miR-16, miR-132, miR-146a and miR-22 were significantly lower in the synovial fluid than in plasma. miR-132 in plasma exhibited a number of miRNAs in plasma, some of which were found at different levels between patients with rheumatoid arthritis (RA) and with OA. Concentrations of miR-16, miR-132, miR-146a and miR-223 are reduced in the synovial fluid of individuals suffering from OA compared with healthy controls [100]. More recently a profiling of 384 miRNAs was developed in the plasma of patients with radiographic OA of the knee, and 12 miRNAs were found to be differentially expressed with a clear differentiation of OA samples from those of healthy controls. *In silico* analysis revealed that potential miRNA targets belonged to OA-related pathways, such as those of chondrocyte maintenance, osteocyte modulation, inflammation, proteases, extracellular matrix (ECM) molecules and signalling pathways. Interestingly, some specific target genes are also involved in OA development, such as fibroblast growth factor receptor 1 (*FGFR1*), histone deacetylase 4 (*HDAC4*), (*FGF2*), vascular endothelial growth factor A (*VEGFA*), the insulin-like growth factor I receptor (*IGF1R*), A disintegrin-like and metalloproteinase with thrombospondin type 1 motif-5 (*ADAMTS5*), tissue inhibitor of metalloproteinase 2 (*TIMP2*), and *WNT1*-inducible signalling pathway protein 1 (*WISP1*) [101].

miR-140 has also been implicated in OA pathogenesis. In mice, the disruption of miR-140 *in vivo* induced the early onset of spontaneous OA-like changes in articular cartilage, in part due to elevated ADAMTS5, characterized by proteoglycan loss and articular cartilage fibrillation in the age-related model, and more severe OA-like changes in the surgical model [75]. In OA cartilage chondrocytes, *COL2A1* expression is low and *ADAMTS5* is increased. In response to IL-1β stimulation, miR-140 expression decreases while that of *ADAMTS5* and *MMP13* increases. These results demonstrate that miR-140 regulates cartilage-specific genes, playing an important role in regulating the balance between ECM synthesis and degradation [79]. Cartilage degradation in OA is due to factors such as MMP and to insulin-like growth factor protein 5 (IGFBP-5). In human OA chondrocytes, miR-140 significantly decreases *IGFBP5* expression, and anti-miR-140 exerts the contrary effect; therefore, these data suggest that *IGFBP5* is a direct target of miR-140 [102]. miR-140 was shown to mediate *MMP13* expression directly *in vitro*. In C28/I2 cells, a model cell of OA, stimulation by IL-1β increases the expression of *MMP13* [103].

In human OA chondrocytes, miR-27a expression is decreased in OA and treatment with anti-miR-27a increases the expression of *IGFBP5* and *MMP-13*, which suggests that miR-27a may indirectly regulate the levels of both genes by targeting upstream positive effectors of both genes [102]. In agreement with these results, miR-27b is downregulated in IL-1β-stimulated OA chondrocytes stimulated with an inverse correlation of MMP-13 expression [104].

Members of the miR-34 family are induced by p53, leading to apoptosis, cell cycle arrest and senescence through targeting E2F3, cyclin E2 and CDK6, etc. [105]. miR-34a is the most significantly induced miRNA after the activation of p53. In chondrocytes of a rat model of OA,

miR-34a is upregulated after IL-1β stimulation and its silencing prevents the IL-1β-induced upregulation of iNOS and the downregulation of Col2a1 [106].

miR-146a/b has been described as a key molecule in the inflammatory response [107] and is expressed in all layers of human articular cartilage, especially in the superficial zone. In grade I OA, the expression of miR-146a and COL2A1 is significantly increased, and is decreased in grades II and III, along with MMP13. Thus, their expression gradually decreased with progressive tissue degeneration [108]. In a rat model with surgically induced OA, miR-146a is upregulated in articular chondrocytes in response to treatment with IL-1β. *SMAD4* is a direct target of miR-146a, and the inhibition of *SMAD4* results in the upregulation of the vascular endothelial growth factor (VEGF) and apoptosis in chondrocytes. This VEGF induction by miR-146a may affect angiogenesis and inflammation during OA pathogenesis [109]. Interestingly, miR-146a has been implicated in the control of knee joint homeostasis and OA-associated algesia by balancing the inflammatory response in cartilage and synovia with pain-related factors in glial cells [110], because miR-146a is induced by joint stability resulting from medial collateral ligament transaction and medial meniscal tearing in the knee joints of an OA mouse model, suggesting that miR-146a might be a mechano-responsive miRNA in articular cartilage [109]. Previously, miR-365 was described as a mechano-responsive miRNA in chicken primary proliferative chondrocytes under mechanical stimulation. This miRNA stimulates chondrocyte proliferation and differentiation, and increases the expression of the hypertrophic marker COL10A1. Additionally, it targets *HDAC4*, which modulates cell growth and inhibits chondrocyte hypertrophy and endochondral bone formation by inhibiting Runx2 [111]. Table 2 depicts the miRNAs implicated in OA.

microRNA	Expression	Target		Ref.
miR-140	↓	*ADAMTS5* *MMP13* *IGFBP5*	A Disintegrin-Like And Metalloproteinase With Thrombospondin Type 1 Motif, 5 Matrix Metalloproteinase 13 Insulin-Like Growth Factor-Binding Protein 5	75,79,97,98,102, 103
miR-146	↓	*IRAK1* *TRAF6*	Interleukin 1 Receptor-Associated Kinase 1 TNF Receptor-Associated Factor 6	98
miR-146a	↑	*MMP13* *SMAD4*	Matrix Metalloproteinase 13 Mothers against decapentaplegic homolog 4	108, 109
miR-103	↑	*ACOX1*	AcyL-CoA Oxidase 1	97
miR-16	↑	*TIPM2*	Tissue Inhibitor Of Metalloproteinase 2	97
miR-210	↓	*CASP10*	Caspase 10	97
miR-22	↑	*PPAR* *BMP7*	Peroxisome Proliferator-Activated Receptor Bone Morphogenetic Protein 7	97
miR-223	↑	*GDF5*	Growth/Differentiation Factor 5	97
miR-23b	↑	*CRTAP*	Cartilage-Associated Protein	97

microRNA	Expression	Target		Ref.
miR-25	↓	*ITGA5*	Integrin, Alpha 5	97
miR-26a	↓	*ASPN*	Asporin	97
miR-29a	↓	*LEP*	Leptin	97
miR-337	↓	*RETN*	Resistin	97
miR-373	↓	*CASP6*	Caspase 6	97
miR-377	↑	*CART1*	TNF Receptor-Associated Factor 4	97
miR-483	↑	*ACAN*	Aggrecan	97
miR-509	↑	*SOX9*	SRY-Related HMG-Box gene 9	97
miR-9	↑	*MMP13*	Matrix Metalloproteinase 13	98
miR-27a	↓	*MMP13* *IGFBP5*	Matrix Metalloproteinase 13 Insulin-Like Growth Factor-Binding Protein 5	102
miR-27b	↓	*MMP13*	Matrix Metalloproteinase 13	104
miR-365	↑	*HDAC4*	Histone Deacetylase 4	111
miR-488	↓	*ZIP8*	Solute carrier family 39 (zinc transporter), member 8	112

Table 2. microRNAs implicated in Osteoarthritis

5. Conclusions

At present, there is increasing progress in the description of epigenetic mechanisms under normal conditions, as well in disease, and much of the current knowledge has focused on cancer. Yet epigenetics research has provided new insights in relation to other entities, such as neurological and autoimmune diseases. Epigenetics research into OA continues to be developed, but could shed light on its pathological mechanisms. One promising field is related with OA treatment, such as HDACi or miRNAs. However, although HDACi and miRNAs could inhibit several genes related with its development, they also inhibit ECM genes. To date, there is no appropriate biomarker for OA. Epigenetics marks in OA have been associated with the condition, and even with its progression, and could be biomarkers of disease and progression, as miRNAs determined in plasma. However, research into epigenetics continues to be required.

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To Ivonne, Ivana, and Romina, with love
To my father, Antonio Miranda-Novoa, in loving memory.

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