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Review Article

Epigenetic regulation of chondrocyte differentiation

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Summary Chondrocytes play an essential role in endochondral bone development, which is requisite for mammalian skeletal development. During endochondral bone development, chondrocytes undergo well-organized stages of sequential differentiation, including proliferation and hypertrophy, a process harmoniously modulated by various transcription factors. Epigenetics, including DNA methylation and histone modification, has recently emerged as an essential regulatory system for gene expression, not only in physiological conditions, but also in human disease. During chondrocyte differentiation, transcriptional co-regulators, which form transcriptional protein complexes with specific transcription factors, are predominantly involved in this epigenetic process and cooperatively regulate chondrocyte gene expression. Importantly, several studies indicate that epigenetic regulators correlate with cartilage-related diseases, such as osteoarthritis, and are noted as a potential therapeutic target. Here, current studies of epigenetic regulation of chondrocyte differentiation are reviewed and novel aspects for the molecular mechanisms involved in chondrogenesis are introduced.

Contents

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1. Introduction

Epigenetics has recently emerged as an essential regulatory system for gene expression, not only in physiological conditions, but also in human disease. The word 'epigenetic' is defined as heritable changes in gene expression without changes in the DNA coding sequences [1]. Epigenetics is a developing area of research in many biological studies and has now become a potential therapeutic target for many human diseases, including cancer. One of the major epigenetic regulation processes is DNA methylation, into which a considerable number of studies have been made [2,3]. However, recent progress in the field of epigenetic research has revealed that various regulatory systems, including histone modification and dynamic chromatin structure, are involved in epigenetic regulation. In this article, the mechanism of chondrocyte differentiation is first introduced and then recent findings about epigenetic regulation of chondrocyte differentiation are reviewed, focusing on DNA methylation and histone modification. It is well established that miRNAs regulate diverse biological processes both post-transcriptionally and epigenetically. However, little is known about the direct association between miRNA and epigenetic programs in mammalian cells. Therefore, the role of miRNA in cartilage homeostasis and chondrocyte differentiation, through post-transcriptional regulation as a part of miRNA function, is also introduced.

2. Mechanism of chondrocyte differentiation

The majority of bones, including craniofacial bones, are formed by a unique biological event called endochondral bone formation [4,5]. Endochondral bone formation starts with mesenchymal cell condensation followed by differentiation into chondrocytes, which then form cartilage by producing an abundant extracellular matrix comprising, *Col2a1* (collagen type II, alpha 1) and *Acan* (aggrecan). From here, the chondrocytes undertake sequential steps of differentiation into proliferating, hypertrophic and terminal chondrocytes (Fig. 1) [4,5]. This process generates the chondrocyte differentiation layer of the growth plate. Finally, the chondrocytes die by apoptosis and are replaced by bone formation following blood vessel invasion. At each differentiation step, chondrocytes produce stage-specific marker genes. These include *Col2a1* and *Acan* in proliferating chondrocytes, *Ihh* (Indian hedgehog) and *Pth1r* (parathyroid hormone 1 receptor) in pre-hypertrophic chondrocytes, and *Col10a1* (collagen, type X, alpha 1) and *Mmp13* (matrix metalloproteinase 13) in hypertrophic chondrocytes (Fig. 1).

These well-organized chondrocyte differentiation processes are harmoniously regulated by several transcription factors in a temporal-spatial manner (Fig. 1). At the beginning of chondrocyte differentiation, *Sox9* (SRY-box containing gene 9) and its cofactors, *Sox5* (SRY-box containing gene 5) and *Sox6* (SRY-box containing gene 6), play essential roles in *Col2a1* gene expression [6,7] and chondrocyte-specific Sox9-deficient mice show severe defects in skeletal development [8]. In humans, mutation of the *SOX9* gene causes campomelic dysplasia, characterized by severe chondrodysplasia and autosomal sex reversal [9,10]. Moreover, disruption of the cis-regulatory element of *SOX9*, which leads to decreased *SOX9* transcription, causes Pierre Robin Sequence characterized by cleft palate [11]. During the process of chondrocyte hypertrophy, expression of *Mef2C* (myocyte-specific enhancer factor 2C), Forkhead box (FoxA) proteins, and Runx (runt-related transcription factor) family

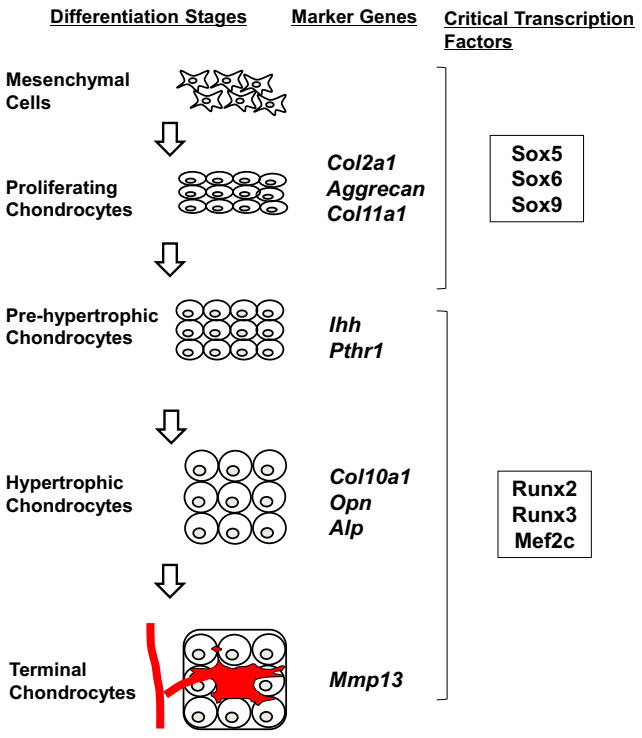

Figure 1 Schematic of chondrocyte differentiation steps during endochondral bone formation. Endochondral bone formation starts with mesenchymal condensation followed by the sequential differentiation steps as indicated in the figure. Morphological features, specific marker genes, and essential transcription factors for each differentiation step are shown.

proteins, including *Runx2* and *Runx3*, is critical [12—14]. *Runx2*- and *Runx3*-deficient mice show delayed chondrocyte hypertrophy and Runx2 regulates *Ihh* expression by direct binding to the *Ihh* gene promoter [12]. Interestingly, *Runx2* transgenic mice driven by the *Col2a1* gene promoter show accelerated chondrocyte hypertrophy in proliferating chondrocytes where hypertrophic change never occurs [15]. Furthermore, *Sox9* transgenic mice, using *Col2a1*-Cre mice, have delayed chondrocyte hypertrophy [16] suggesting a stage-specific function of Sox9 and Runx2 during endochondral bone formation. In addition, Mef2c activates the *Col10a1* gene promoter and heterozygous deletion of *Mef2c* has been show to result in abnormal chondrocyte hypertrophy and lack of ossification [13]. Chondrocyte-specific deletion of transcription factor *FoxA2* (Forkhead box protein A2) and *FoxA3* (Forkhead box protein A3) results in abnormal endochondral bone formation and dwarfism because of the reduction in hypertrophic gene expression, including *Col10a1*, *Alpl* (alkaline phosphatase), and *Mmp13* [14].

Gli transcription factors play essential roles in endochondral bone formation by activating Ihh signaling, a member of the hedgehog family. Among Gli family proteins, Gli2 is a major signaling molecule of Ihh-dependent chondrogenesis and directly regulates chondrocytic genes, including *PTHrP* (parathyroid hormone-related protein), *Ptch1* (patched homolog 1), *Col10a1*, and *Gli1* [17—20]. The deletion of both *Ihh* and *Gli2* causes severe skeletal defects with reduced chondrocyte proliferation and maturation [21—23].

Importantly, these transcription factors form large transcriptional complex machinery to regulate gene expression and do not induce transcription alone [24]. For instance, Sox9 physically interacts with various transcriptional co-regulators, including Arid5a (AT rich interactive domain 5a), p54nrb/NonO (non-POU domain containing, octamer-binding), Wwp2 (WW domain containing E3 ubiquitin protein ligase 2), Pgc1-alpha (PPAR-γ co-activator-1 alpha), Smad3 (Smad family member 3), and Znf219 (zinc finger protein 219) [25—30]. Regarding Gli2, we have recently reported that transcriptional activity of Gli2 is modulated by its transcriptional partner Foxc1 (Forkhead box C1) and the Ihh-Gli2 target gene expression was decreased in *Foxc1* inactivated mice [31]. These co-regulators are involved in multiple steps of the gene expression pathway, including mRNA splicing, chromatin organization, and mRNA maturation. Furthermore, different combinations of these transcriptional co-regulators modulate each step of the gene expression pathway. Of note, recent studies have demonstrated that transcriptional co-regulators significantly contribute to the epigenetic regulatory system, including DNA methylation and histone modification during gene expression.

3. DNA methylation

3.1. DNA methylation and chondrocyte differentiation

DNA methylation is one of the well-documented epigenetic regulatory systems for gene expression, and this system is conserved between species. This process occurs on the carbon 5 position of cytosine residues of CpG dinucleotides. The methylation of DNA is a reversible biological process and is regulated by DNMTs (DNA methyltransferases) [32]. When DNA methylation occurs in the promoter region of genes, binding of the specific transcription factors to their binding elements is inhibited, which in turn represses transcription. DNA methylation also causes the recruitment of transcriptional repressors to the methylated region of the gene promoter. In addition, several reports indicate that DNA methylation can control chromatin remodeling and histone modifications [33,34].

It is well established that DNA methylation is involved in various biological events, including genomic imprinting, cancer and X chromosome inactivation [35]. There are several reports regarding the association between chondrocyte gene expression and DNA methylation. Zimmermann et al. investigated the DNA methylation level of chondrocyte gene promoters using human articular cartilage chondrocytes, which exhibit high expression of *Col2a1* but not *Col10a1* [36]. They observed that the *Col10a1* gene promoter was highly methylated, whereas the transcriptional start site of the *Col2a1* gene promoter was unmethylated. DNA meythylation status is strongly associated with chondrocyte differentiation activity. Hiramatsu et al. generated chondrogenic cells directly from dermal fibroblasts and found that the promoter of the *Col1a1* gene was highly methylated, whereas the methylation level was low in the dermal fibroblasts [37]. These data indicate that control of DNA methylation in the chondrocyte gene promoter is important for successful repair of cartilage using tissue engineering approaches.

3.2. DNA methylation and cartilage disease

Several studies have reported a correlation between DNA methylation and cartilage destruction diseases, including osteoarthritis (OA) [38—40]. Interestingly, damaged chondrocytes in OA patients show an increased DNA methylation level in the *Sox9* gene promoter compared with that of normal cartilage [41], suggesting the epigenetically impaired chondrogenesis in OA is likely due to the decreased expression of *Sox9*. CpG sites in the *Col9a1* gene promoter are hypermethylated in the cartilage of OA patients compared with controls. Furthermore, impaired Sox9 binding to the *Col9a1* gene promoter has been observed in OA chondrocytes [42].

In addition to anabolic genes, a role for DNA methylation is also reported in catabolic gene expression. Roach et al. have shown that the methylation status of CpG sites in the promoter region of cartilage-degrading enzymes, including MMPs and a member of the ADAMTS (a distinegrin and metalloproteinase with thrombospondin motifs) family, ADAMTS-4, is decreased in chondrocytes from OA patients compared with normal chondrocytes. This suggests that epigenetically increased gene expression of these enzymes may contribute to the development of OA [43]. Bui et al. have reported that demethylation of CpG sites located in the proximal promoter of *MMP13* increases the binding of the transcription factor CREB, which in turn promotes *MMP13* gene expression. Importantly, they also found that the DNA methylation level of this CREB binding region is reduced in human chondrocytes obtained from OA patients [44]. Demethylation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated

B cells) responsive enhancer elements in human articular chondrocytes causes abnormal expression of inducible nitric oxide synthase in OA patients [45]. These reports indicate that epigenetic regulators that are able to modulate the DNA methylation level of cartilage destructive enzymes are potential therapeutic targets for cartilage diseases.

4. Histone modification and chondrocyte differentiation

Because the dynamic organization of the chromatin structure is influenced by histones, which make up the nucleosome, histone modification largely affects the gene expression machinery at various levels. Recent progress in the epigenetic field has identified and characterized eight types of histone modification, such as acetylation, methylation, phosphorylation, and SUMOylation [46]. There is crosstalk between histone modification and combinations of histone modification, which affects transcription. In this section, the role of histone acetylation and methylation during chondrocyte differentiation is reviewed.

4.1. Histone acetylation

Histone acetylation is a well-studied histone modification, with a considerable number of studies to date reporting on its functional role in gene expression [47]. The N-tail of histone H3 contains positively charged lysine residues and thereby strongly binds to the negatively charged DNA. Lysine acetylation causes neutralization of the positive charge of the histone tail, which reduces the binding affinity of histone H3 toward DNA. This decreased electrostatic bond strength between histone and DNA makes the nucleosomal DNA more accessible and allows transcriptional regulator proteins, including tissue-specific transcription factors and RNA polymerase, to bind to the transcription sites [48]. Based on this mechanism, histone acetylation increases transcriptional activity whereas histone deacetylation inhibits transcription (Fig. 2). Histone acetylases (HATs) and histone deacetylases (HDACs) physically and functionally interact with the proteins that constitute the transcriptional machinery and regulate gene expression [48,49].

During chondrocyte differentiation, several HAT enzymes are reported to positively regulate chondrocyte gene expression with Sox9. For instance, p300/CBP physically interacts with Sox9 and increases the acetylation level of the Sox9 target gene promoter, including *Col2a1* [50]. Moreover, Hattori et al. searched for the Sox9 binding protein using a yeast two-hybrid assay and identified Tip60 (Tat interactive protein-60), which belongs to the MYST family of the HAT enzymes [51]. They also demonstrated that Tip60 is involved in chromatin organization in association with Sox9 and Sox5.

In addition to HATs, several studies have revealed the role of HDACs in chondrocyte differentiation. Hang et al. have reported that HDAC1 and HDAC2 decrease the expression of *Col2a1* and *aggrecan* gene expression [52] and Huh et al. showed that HDAC inhibitor increases *Col2a1* gene expression in articular chondrocytes [53]. NAD-dependent protein deacetylase, Sirtuin1, is also involved in the regulation of *Col2a1* gene expression by binding to the chromatin region of the *Col2a1* gene promoter and enhancer [54].

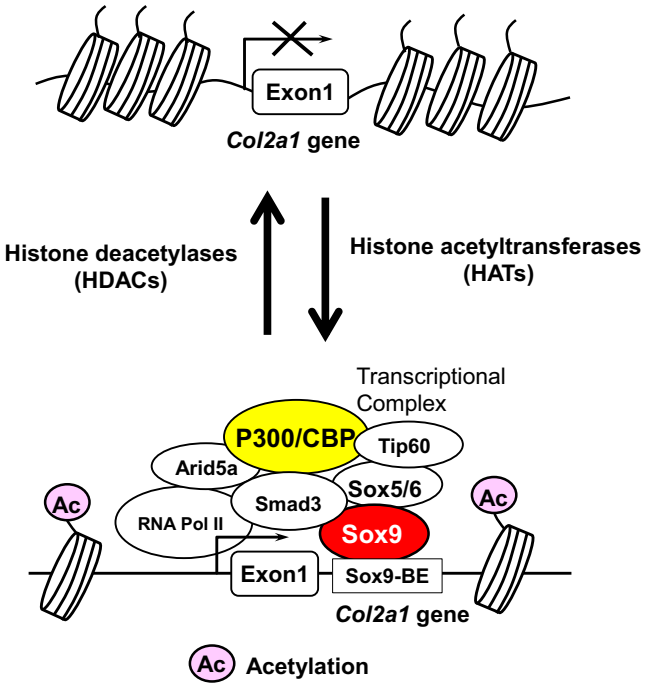

Figure 2 Schematic of histone acetylation and deacetylation during chondrocyte differentiation.
When histone is deacetylated, chromatin becomes condensed and inactive, therefore Sox9 is unable to bind to the Col2a1 gene enhancer. In contrast, histone acetylation causes loose packing of chromatin, which makes Sox9 more accessible and allows it to bind to the Col2a1 enhancer region. During transcription of *Col2a1*, various transcriptional co-regulators for *Sox9* are recruited and co-operatively induce gene expression.

HDAC4, expressed in pre-hypertrophic chondrocytes, controls chondrocyte hypertrophy by inhibiting Runx2 function [55]. Moreover, HDACs, HDAC-1, -2, -3, and -7, regulate *Mmp13* expression. Inhibition of these HDACs protects the cartilage destruction induced by IL-1 (interleukin-1), suggesting the potential therapeutic use of HDAC inhibitors for cytokine-induced cartilage degradation observed in OA [56,57].

4.2. Histone methylation

Methylation has been recognized as a common protein modification for a long time. However, very recently, histone methylation/demethylation has emerged as a critical step for transcription. Similar to acetylation, lysine residues are also methylated, but there are three types of methyl-lysine molecular structures including mono-methyl (me1), di-methyl (me2) and tri-methyl (me3). As described in the previous section, histone acetylation reduces the positive charge of histone proteins, whereas histone methylation does not affect their charge. However, histone methylation/demethylation results in both transcriptional repression and activation, depending on the target site. Methylation at H3K9 and H3K27 inactivates gene expression, while H3K4 and H3K36 methylation activates gene expression [58—60]. Recent studies of histone methylation have revealed the physiologic and pathologic significance

of histone methylation in many cellular events, including differentiation and proliferation [61,62]. Rodova et al. reported that the promoter region of the transcription factor NFAT1 (nuclear factor of activated T cells 1) gene, which is involved in OA progression, showed an increased methylation level of H3K4me2 but decreased methylation level of H3K9me2 [63]. They also reported that lysine-specific demethylase-1 (Lsd1) and Jmjc-containing histone demethylase-2a (Jhdm2a) are involved in the regulation of lysine methylation. Herlofsen et al. have demonstrated that the methylation level of H3K4me3 and H3K36me3 is largely associated with chondrocyte differentiation of human mesenchymal stem cells, assessed by Chip-Seq analysis [64]. Setdb1 (SET domain, bifurcated1) regulates chondrocyte hypertrophy by inhibiting Runx2 activity through H3K9 methylation [65,66]. However, despite its significance in gene expression pathways, only the role of histone acetylation has been predominantly studied in chondrogenesis, with little attention given to the regulatory mechanism of histone methylation/demethylation during chondrocyte differentiation.

In light of this, we have previously attempted to uncover the physiological significance of histone methylation during chondrocyte differentiation [67]. We identified Arid5b (AT rich interactive domain 5b) as a transcriptional co-regulator of Sox9, by performing gene expression profiling between chondrogenic cells and fibroblasts, the latter of which do not show chondrogenic activity. Arid5b was highly expressed in cartilage tissue and synergistically induced chondrocyte gene expression through physical and functional interactions with Sox9. Moreover, we showed that Arid5b recruits the histone demethylase, Phf2, to chondrocyte gene promoters, including *Col2a1*, and decreases the methylation level of H3K9me2, a repressive marker of transcription. These data collectively indicated that Arid5b promotes chondrogenesis by facilitating a relationship between Sox9 and Phf2-mediated histone demethylation of chondrogenic gene promoters and establishes a novel epigenomic mechanism of endochondral bone development [67].

5. miRNAs and chondrocyte differentiation

miRNAs are small non-coding RNAs comprising about 22 nucleotides. miRNA is first synthesized as primary miRNA and subsequently truncated into the pre-miRNA by ribonuclease. Pre-miRNA is then transported into the cytoplasm and converted into the functional and mature miRNA by Dicer, which is an essential player in the biogenesis of miRNAs. Finally, miRNA forms a protein complex called RISC (RNA-induced silencing complex), which destroys the target mRNAs. miRNAs regulate target gene expression by directly binding to the sequence-specific elements located in their target RNAs. Thus, miRNA controls gene expression at the post-transcriptional stage and just one miRNA targets hundreds of genes.

The important roles of miRNA in chondrocyte differentiation were first described by Kobayashi et al. by generating chondrocyte-specific Dicer-deficient mice, resulting in the global deletion of miRNAs in chondrocytes. Dicer-deficient mice show severe defects in skeletal development and a reduction in chondrocyte proliferation and differentiation [68]. These data suggest that miRNAs play some roles in cartilage development and homeostasis.

It is well established that miRNA modulates gene expression both post-transcriptionally and epigenetically. Although, there has been no evidence which shows the direct association between miRNA and epigenetic regulation during chondrocyte differentiation, miRNA can act as an epigenetic regulator during chondrogenesis. Previous studies have indicated that epigenetic modification enzymes, such as DNMTs, are controlled by miRNAs [69,70]. Interestingly, several miRNAs can recruit chromatin-modifying proteins, including histone methyltransferases, through physical interaction with Ago2 (Argonaute2), which increases the chromatin accesibility and transcription [71]. These reports strongly indicate that miRNA epigenetically regulates chondrocyte gene expression. To date however, chromatin remodeling, DNA methylation and histone modification by miRNA in mammalian cells is largely unreported. Moreover, most studies focus on the miRNA-dependent post-transcriptional regulation. Post-transcriptional regulation by miRNA is not epigenetic in the strict sense but regulates diverse biological processes. Thus, it is impossible to ignore the role of miRNA in order to better understand the molecular mechanisms underlying chondrocyte differentiation. Indeed, accumulating evidence indicates the important roles of miRNA in cartilage homeostasis and chondrocyte differentiation. In the last section of this review, the role of miRNA as a part of miRNA function is introduced, focusing on miR-140.

5.1. miR-140 and chondrocyte differentiation

Although almost 1000 miRNAs are reported, miR-140 is one of the chondrocyte-specific miRNAs. Previous reports indicate that miR-140 is specifically expressed in cartilage tissue in both zebrafish and mice [72,73]. In accordance with the specific expression of miR-140 in chondrocytes, miR-140 is reported to be associated with Sox9 function and expression. For example, miR-140 is biosynthesized from intron 16 of the *Wwp2* gene, which is the direct target gene of Sox9 [74,75]. Yamashita et al. found a Sox9 response element located in the upstream of miR-140 and reported that Sox9-dependent miR-140 expression is further increased by addition of Sox5 and Sox6 [76]. In addition to Sox9, miR-140 also regulates the expression of various genes involved in chondrogenesis, including *Smad3* and *Rala* [77,78]. In particular, several groups have reported that *HDAC4* is the direct target gene of miR140 [72,79]. *HDAC4* is expressed in proliferating chondrocytes and inhibits chondrocyte hypertrophy by decreasing Runx2 transcriptional activity [55]. These reports indicate that miR-140 indirectly inhibits chondrocyte hypertrophy, which maintains chondrocyte proliferation. Moreover, Nakamura et al. reported that Dnpep, which regulates BMP signaling, is the target gene of miR-140 in chondrocytes [80]. These reports collectively indicate the multifunctional role of miR-140 during chondrocyte differentiation and further analysis of the miR-140 target gene would contribute to a better understanding of chondrocyte differentiation (Fig. 3).

Although deletion of miR-140 in mice results in a slightly dwarf phenotype with shortened limbs, these mice

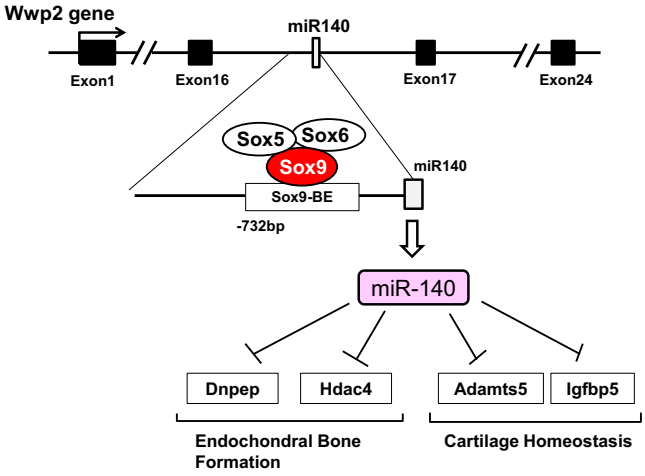

Figure 3 The role of miR-140 in chondrocyte differentiation. miR-140 is generated from intron 16 of the *Wwp2* gene and is directly regulated by Sox9 and its cofactors Sox5/6. miR140 plays important roles in endochondral bone formation and cartilage homeostasis through regulating Dnpep, Hdac4, Adamts5 and Igfbp5.

exhibit an age-dependent OA phenotype [81]. This suggests involvement of miR-140 not only in the physiological condition, but also in the pathological condition for the maintenance of cartilage tissue. miR-140 has also been demonstrated to directly regulate ADAMTS5 expression, which is strongly associated with OA progression [81,82]. Tardif et al. found that the expression of *IGFBP5* (insulin growth factor binding protein-5) in human OA chondrocytes was significantly lower than that of controls and that IGFBP5 is the direct target of miR-140 [83]. These data suggest that miR-140 is essential for cartilage homeostasis in both developing and adult stages and a candidate therapeutic target for OA treatment (Fig. 3).

5.2. Other miRNAs and chondrocyte differentiation

In addition to miR-140, other miRNAs have been identified as regulators of chondrocyte differentiation. Several groups have shown that the expression of miR-145 decreases during chondrocyte differentiation of mesenchymal cells and *Sox9* is the direct target gene of miR-145 [84,85]. Overexpression of miR-145 inhibits chondrocyte differentiation by the reduction of chondrocyte gene expression, including *Col2a1* and *aggrecan*, whereas inhibition of miR-145 promotes chondrocyte differentiation [84]. Furthermore, miR-495, miR-101, and miR1247 are also reported to directly regulate *Sox9* expression and inhibit chondrocyte differentiation [86—88]. In contrast, *Sox9* up-regulates miR-574-3p, which inhibits chondrocyte differentiation [89]. Overexpression of miR-675 increases *Col2a1* expression in human chondrocytes and BMP2-responsive miR-199a controls chondrocyte differentiation though regulating Smad1 [90,91]. Although many miRNAs have been identified as regulators of chondrocyte differentiation, the significance of these *in vivo* remains to be elucidated.

6. Conclusions

Epigenetic regulation is now noted as a critical biological process and is well documented in many research fields, however little is known about the role of epigenetics in skeletal development. Recent studies have shown remarkable progress in epigenetic research owing to advanced technologies, including deep sequencing and bioinformatics. In addition to basic research, epigenetic regulators are recognized as an effective therapeutic target for many diseases [92] and regenerative medicine using induced pluripotent stem cells [93]. However, histone acetylation and methylation are not the only processes for histone modification, and various combinations may affect the gene expression. Although little is known about the direct association between miRNA and chromatin modification in mammals, regulatory mechanisms underlying miRNA-dependent epigenetic regulation during chondrocyte differentiation are of interest. It should be noted that miRNA is a critical regulator for post-transcriptional gene expression and represents a promising therapeutic target. Further advances in epigenetic regulation of chondrocyte differentiation would contribute to the development of treatments and therapies for cartilage diseases such as OA.

Conflict of interest statement

None declared.

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