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2 **Epigenetic dynamic during endochondral ossification and articular cartilage**
3 **development**

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Abstract

1
2 Within the last decade epigenetics has emerged as fundamental regulator of numerous cellular
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4 processes, including those orchestrating embryonic and fetal development. As such,
5
6 epigenetic factors play especially crucial roles in endochondral ossification, the process by
7
8 which bone tissue is created, as well during articular cartilage formation. In this review, we
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10 summarize the recent discoveries that characterize how DNA methylation, histone post-
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12 translational modifications and non-coding RNA (e.g., miRNA and lcnRNA) epigenetically
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14 regulate endochondral ossification and chondrogenesis.
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Epigenetic is the study of reversible changes in gene expression transmitted during cell divisions that do not result from DNA sequence modification. The epigenome includes DNA methylation, post-translational histone modifications which induce changes in chromatin structure, as well as non-coding RNA expression ⁽¹⁾. According to its condensation state, chromatin permits or prevents the access of the transcriptional machinery to DNA sequences. Epigenetic marks are established by proteins called “writers”, such as DNA methyltransferases (DNMT), histone acetyltransferases (HAT), and histone methyltransferases (HMT), whereas they are removed by proteins known as “erasers”, especially histone deacetylases (HDAC), histone demethylases (KDM) and DNA demethylases (TET). They are interpreted by transcription factors and cofactors referred to as “readers”. Non coding RNA (ncRNA) also act as epigenetic regulators by controlling DNA transcription, RNA maturation and translation. As developed in this review, many epigenetic actors and chromatin remodeling complexes regulate endochondral ossification as well as cartilage development. Epigenetic processes are also major mediators in joint disease development. However, this role in joint diseases has been well-documented elsewhere these last months ⁽²⁻⁵⁾.

Endochondral ossification

The mechanical integrity of the skeleton is imparted primarily by two tissues: bone and cartilage. During development, cells from different embryonic origins undergo a similar sequence of events to form fetal skeletal cartilage ⁽⁶⁾. The paraxial mesoderm gives rise to the sclerotome, a developmental structure that will pattern the vertebrae and ribs of the axial skeleton. In contrast, the lateral flank mesoderm generates the limb bud that patterns long bones of the limbs. Endochondral ossification contributes in part to formation of the craniofacial bones, including the cranial base, mandible and temporal bones.

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In each case, development of skeletal elements begins with cellular condensations. Cells within these condensations then undergo the complex process of chondrogenesis ⁽⁷⁾. During this process, mesenchymal chondroprogenitor cells aggregate. The expression of cell adhesion molecules increases. These changes in cell behavior are tightly regulated by activation of chondrocyte-selective transcription factors, particularly, the transcription factor Sex Determining Region Y-Box 9 (SOX9). Sox9 is expressed very early in the development and is the major regulator of the chondrogenic program ⁽⁸⁾.

Cells at the core of condensations change in morphology from fibroblast-like to more spherical, and increase the synthesis of specific extracellular molecules, including aggrecan and collagens type II, IX and XI. This is mediated through the transcription factor trio SOX5/6/9. At the same time, cells at the periphery of condensation retain a fibroblastic morphology and differentiate into perichondrial cells that continue to express type I collagen ⁽⁶⁾.

Chondrocytes at the core of the condensations quickly proliferate to promote limb extension and linear growth of the developing skeleton. Next, they stop to proliferate and undergo progressive hypertrophy. This final step of differentiation is a well-coordinated program that is governed, in part, by the transcription factor runt-related transcription factor 2 (RUNX2) ⁽⁸⁾. This ordered process of proliferation and differentiation leads to the formation of stratified zones of cells at different cell cycle stages ⁽⁷⁾ (figure 1). At the extremity of the elements, in the resting zone, chondrocytes exhibit a round cell morphology and are quiescent, whereas chondrocytes in the columnar or proliferative zone have a more flattened morphology and rapidly proliferate. Then, towards the core of the elements is a zone of post-mitotic cells that begin to enlarge and are characterized by the expression of Indian Hedgehog (IHH) and decreased expression of SOX9. This is the prehypertrophic zone. Finally, the hypertrophic zone represents terminal, enlarged chondrocytes that produce a unique mineralized

1 extracellular matrix (ECM) containing type X collagen. Hypertrophic chondrocytes also
2 produce matrix metalloproteinase 13 (MMP13) and vascular endothelial growth factor (VEGF)
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4 to facilitate matrix remodeling and vascular invasion. This invading vasculature permits the
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6 entry of osteoprogenitors that differentiate into osteoblasts. Hypertrophic chondrocytes
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8 undergo apoptosis ^(9,10); thereby, cartilage serves as a template and is replaced by bone during
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10 endochondral ossification.
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18 **Articular cartilage formation and maintenance**

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20 Articular cartilage plays an important role in withstanding mechanical stress associated with
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22 joint movement at the ends of long bones. It differs from growth plate cartilage in terms of
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24 extracellular matrix content, cellular organization and mechanical properties. Articular
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26 cartilage is comprised primarily of ECM, composed mainly of collagens and proteoglycans,
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28 populated sparsely with chondrocytes that perform matrix-generation and maintenance
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30 functions (figure 2). Mature articular cartilage has a zonal organization that is divided into a
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32 superficial layer, a mid layer, the deep layer, and the calcified layer, in order from the surface
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35 of articular cartilage toward the bone.
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39 During skeletal patterning, articular cartilage formation begins at the interzones, regions of
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41 undifferentiated mesenchyme that separate developing skeletal elements. The interzone is also
42
43 the presumptive joint formation site ⁽¹¹⁾. This suggests that articular “stable” chondrocytes
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45 may have a distinct embryonic origin compared to the “transient” chondrocytes that derive
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47 from the mesenchymal condensations forming the bulk of anlagen ⁽¹²⁾. Interzones first appear
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49 as densely cellular, homogenous regions. As joint development progresses, the interzone
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51 derives three layers: two chondrogenic layers that cover the articular surfaces of the
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53 developing opposed skeletal elements, and an intermediate layer that separates them. Some
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55 evidences suggests that the cells derived from this intermediate layer differentiate to become
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1 articular chondrocytes, whereas the outer layer chondrocytes are incorporated into the
2 growing epiphysis ^(11,13).
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4 Transient growth cartilage and articular cartilage differ in their collagen composition during
5 development. In embryonic growth cartilage, collagen type-IIA expression precedes
6 production of collagen type-IIB, which is not detectable at later stages of development. In
7 contrast, in articular cartilage collagen type-IIA protein is not produced within the interzone,
8 which reinforces the belief that the cartilage anlagen that forms individual skeletal elements is
9 not continuous ^(14,15).
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12 Other substantial differences exists between articular chondrocytes and transient
13 chondrocytes. Contrary to growth plate chondrocytes, articular chondrocytes maintain a
14 stable phenotype characterized by small cell size, and they do not undergo a sequence of
15 proliferation, maturation, hypertrophy, apoptosis and ossification ⁽¹¹⁾. Also, they maintain
16 joint function throughout life and do so by retaining a stable and permanent phenotype,
17 producing all the macromolecular components of articular cartilage including aggrecan and
18 collagen II, and providing the tissue with functional resilience.
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20 However, in pathological conditions, articular chondrocytes can potentially undergo
21 maturation and hypertrophy, and display traits of transient growth plate chondrocytes. This
22 shift from a permanent to a transient chondrocyte phenotype is seen for instance in the joints
23 of osteoarthritic patients ⁽¹²⁾. Traits expressed by such abnormal articular chondrocytes
24 include an enlarged hypertrophic cell size, increased production of metalloproteases (such as
25 MMP13), and expression of hypertrophic genes, such as alkaline phosphatase or type X
26 collagen. These aberrant characteristics are associated with joint dysfunction and disease.
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29 These processes of endochondral ossification and articular cartilage formation are highly
30 organized and tightly controlled. In particular, as we will discuss in this review, epigenetic
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1 regulations plays crucial roles in this orchestration. This includes DNA methylation, histone
2 post-translational modifications (acetylation, methylation) and non-coding RNA.
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6 7 **DNA methylation**

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9 DNA methylation is the addition of a methyl group to the DNA, at CpG dinucleotides to
10 convert cytosine to 5-methylcytosine (5mC). DNA methylation favors genomic integrity and
11 largely contributes to gene silencing ⁽¹⁶⁾.
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17 DNA methylation patterns are established and modified in response to environmental factors
18 by three DNA methyltransferases (DNMT), named DNMT1, DNMT3A and DNMT3B.
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20 DNMT1 is a maintenance methyltransferase that methylates hemi-methylated DNA (one
21 strand). It is therefore important for transferring patterns of methylation to a newly
22 synthesized strand after DNA replication. DNMT3A and DNMT3B are *de novo*
23 methyltransferases, which add methylation to unmethylated cytosines ⁽¹⁾.
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33 While embryonic regulation of genomic DNA methylation is crucial and relatively well
34 documented during the early stages of development, less is known about its involvement
35 during endochondral ossification and chondrogenesis. DNA methylation is an essential step
36 by which embryonic stem cells silence several key transcription factors, such as SOX2,
37 octamer-binding transcription factor 3/4 (OCT3/4), and Nanog homeobox (NANOG) ⁽¹⁷⁻¹⁹⁾,
38 and partial disruptions of the DNA methylation machinery by genetic mutations often cause
39 serious developmental defects ⁽²⁰⁻²³⁾; however, its role in cartilage and/or in chondrocytes is
40 unclear.
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52 An early study using chick embryos reported the possible role of DNA methylation in the
53 regulation of type I and II collagen genes during chondrocyte differentiation and
54 dedifferentiation ⁽²⁴⁾; however, the methylation status of promoter CpG islands was found to
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1 be preserved in critical genes, such as SOX9 and RUNX2, during chondrogenesis ⁽²⁵⁾. This
2 observation may indicate a retained capability of the mesenchymal progenitor cells to change
3 their differentiation status even after chondrogenesis, since similar observations about
4 adipogenesis of MSCs have been reported ⁽²⁶⁻²⁹⁾. In contrast, two CpG sites within
5 the COL10A1 promoter appear to be demethylated during chondrocyte differentiation from
6 mesenchymal stem cells correlating with COL10A1 expression ⁽³⁰⁾.

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15 Recently, using genetically modified murine models, DNMT3B has been identified as a
16 critical epigenetic factor in the regulation of articular chondrocyte differentiation and in the
17 maintenance of articular cartilage homeostasis ⁽³¹⁾, as well in the regulation of chondrocyte
18 hypertrophic differentiation ^(32,33).

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25 Additional insight has been demonstrated using potent demethylation agents, especially 5-
26 azacytidine (5-azaC) and 5-aza-2'-deoxycytidine (5-aza-dC). Pretreatment of mesenchymal
27 stem cells with 5-azaC facilitates osteogenic differentiation, causing hypomethylation of
28 genomic DNA and increased gene expression ^(34,35). Furthermore, 5-azaC promotes terminal
29 differentiation of articular chondrocytes ^(36,37) by acting as a modulator of chondrocyte
30 hypertrophy and maturation in cultured chick caudal region chondrocytes ⁽³⁸⁾.

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41 Methylated cytosines (5mC) can be further oxidized to 5-hydroxymethylcytosines (5hmC) by
42 Ten-eleven-translocation (TET) proteins ⁽³⁹⁻⁴¹⁾, leading to either a stable 5hmC gain, or
43 further oxidation to 5-formylcytosines (5fC) and 5-carboxylcytosines (5caC) and DNA
44 demethylation. 5hmC acquisition appears to play a major regulatory role in cartilage
45 differentiation and development ⁽⁴²⁾. Changes in 5hmC levels have been reported during limb
46 development, and global 5hmC levels increase during both *in vitro* and *in vivo*
47 chondrogenesis. Interestingly, stable gains in 5hmC were observed in specific subsets of
48 genes including genes associated with cartilage development and in chondrogenic lineage-
49 specific genes, such as SOX5, 6 and 9, as well as COL2A1, whereas minimal changes were
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1 observed in the housekeeping genes HPRT (Hypoxanthine guanine phosphoribosyl
2 transferase) and beta-actin ⁽⁴²⁾. Additionally, gene expression changes in Tet family
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4 members, TET 1, 2 and 3, demonstrate a similar pattern during chondrogenic differentiation,
5
6 with an initial increase followed by a decline in TET expression. Moreover, knockdown of
7
8 TET1 leads to both decreased 5hmC levels and impaired *in vitro* chondrogenic differentiation
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Histone acetylation

RUNX2 and SOX9 are often referred to as master regulators of osteoblast and chondrocyte lineage commitment, respectively. These transcription factors bind DNA and provide docking sites for transcriptional co-activators and repressors, including histone deacetylases (HDAC) and their enzymatic counterparts, histone acetyl transferases (HAT), permitting a tissue-specific gene expression regulation ⁽⁴³⁾.

The HAT writers add a negatively charged acetyl groups (CH₃CO) to ε-amino groups of lysine residues in the N-terminal histone tails to neutralize the positively charged sidechains. This reduces the affinity of histones for negatively charged DNA, and thereby favors chromatin decompaction, facilitating the access of the transcriptional machinery to DNA target sequences. Conversely, by erasing acetyl groups, HDACs favor chromatin condensation and transcriptional repression. HDACs can also deacetylate lysine residues on non-histone proteins, including RUNX2, affecting their stability and/or cellular localization to influence gene expression programs ^(43,44).

In human, HDACs are subdivided into four classes. Classical HDACs, comprise class I, II and IV and have a zinc dependent activity, while class III (sirtuins, SIRT) are nicotinamide adenine dinucleotide (NAD⁺) dependent. Class II HDAC (HDAC4-9 and 10) demonstrate a

1 more tissue restricted expression pattern than class I HDAC (HDAC 1-3 and 8), making them
2 important contributors to cell-specific differentiation programs.
3

4 The roles of HDACs in the first step of endochondral ossification are difficult to ascertain
5 because many HDAC knock-out mice die during early embryogenesis (table 1). However,
6 HDAC2/4/5/6/8 and 9 do not appear to regulate mesenchymal condensation because
7 individual deletion of these enzymes allows early endochondral bone formation ⁽⁴⁵⁻⁴⁷⁾. But it
8 cannot be excluded that some redundancy exists amongst these HDACs in regulating the
9 endochondral ossification process, as is the case for HDAC5/9 that compensate during heart
10 development ⁽⁴⁵⁾.
11

12 More is known about the functions of HDACs within subsequent phases of endochondral
13 ossification. During commitment of mesenchymal precursors to the chondrocyte lineage,
14 HDAC1 associates with Nkx3.2, one of major transcriptional repressors governing cartilage
15 formation during endochondral ossification ^(48,49). This complex also represses Smad-
16 dependent signaling to control BMP2 responses ⁽⁵⁰⁾. During the following stages, HDAC1
17 regulates expression of chondrocyte specific matrix genes including aggrecan, collagens (type
18 II and IX) and COMP ⁽⁴³⁾. HDAC2, highly homologous to HDAC1, also regulates the
19 expression of cartilage ECM genes (aggrecan, collagen type II and XI) ⁽⁵¹⁾. A role for HDAC1
20 during bone formation was also supported by the differential expression of HDAC1 during
21 osteoblast differentiation. Indeed, its expression decreases during osteoblast differentiation,
22 whereas its knock-down stimulates osteoblastic differentiation ⁽⁵²⁾. Mechanistically, HDAC1
23 regulates osteoblast differentiation through a physical association with RUNX2 to decrease
24 transcriptional activity and to repress the stimulatory effects of p300 ⁽⁵²⁾.
25

26 HDAC3 and HDAC4 control chondrocyte hypertrophy. HDAC3 is highly expressed in resting
27 and prehypertrophic growth plate chondrocytes, as well as in articular chondrocytes
28 ⁽⁴³⁾. Conditional deletion of HDAC3 in osteo-chondroprogenitor cells decreases growth plate
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1 height, and expands the hypertrophic zone ⁽⁵³⁾. HDAC3-deficient chondrocytes enter in
2 hypertrophy sooner, exhibit increased expression of cytokine and matrix-degrading genes (IL-
3 6, MMP3, MMP13) and a reduced abundance of genes related to extracellular matrix
4 production, bone development, and ossification (aggrecan, collagen type II and X, IHH,
5 osteopontine) ^(54–56).
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7 The expression pattern of HDAC3 partially overlaps with that of HDAC4, which is also
8 required for proper endochondral ossification ⁽⁴⁷⁾. Both control chondrocyte hypertrophy
9 through their interaction with the key transcription factors RUNX2 and MEF2, as well as with
10 corepressor ZPF521 ^(47,53,57). However, unlike HDAC3, HDAC4 expression is maintained in
11 hypertrophic chondrocytes, and mice with germline deletion of HDAC4 function are viable,
12 but exhibit premature endochondral ossification ⁽⁴⁷⁾. Thus, genetic deletion of HDAC4 causes
13 premature ossification and accelerated chondrocyte hypertrophy, as evidenced by increased
14 expression of IHH, RUNX2 and collagen type X within the developing growth plate ⁽⁴⁷⁾. In
15 contrast, HDAC4 overexpression *in vivo* mimics the RUNX2 KO phenotype and results in
16 inhibition of endochondral ossification ⁽⁴⁷⁾. HDAC4 also regulates osteoblast maturation by
17 controlling expression of MMP13 through a RUNX2-dependent and PTH-sensitive
18 mechanism ⁽⁵⁸⁾. Also, BMP signaling facilitates nuclear export of HDAC4, impeding its
19 ability to bind and repress nuclear transcription factors like RUNX2 ^(59,60). Recently, it was
20 highlighted that mitogen-activated protein kinase p38 (p38 MAPK) increases caspase-
21 dependent HDAC4 degradation and consequently induces RUNX2 expression as well as
22 hypertrophic differentiation ⁽⁶¹⁾.
23

24 Less is known about the role of other HDACs in endochondral ossification, but numerous
25 HDACs regulate or interact with RUNX2. HDAC5, like HDAC4, is expressed in mature
26 osteoblasts ⁽⁶²⁾. It regulates TGF- β -dependent signaling and downstream gene repression in
27 osteoblasts through an interaction with a RUNX2/SMAD3 complex ⁽⁶²⁾. HDAC6 is expressed
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1 in differentiated osteoblasts, binds RUNX2 to repress gene expression, but is also rapidly
2 exported from the nucleus ^(60,63). HDAC6 deficiency results in a minor increase in trabecular
3 bone density ⁽⁶⁴⁾. HDAC7 also inhibits the activity of RUNX2 and slows osteoblast
4 maturation ⁽⁵⁹⁾. In addition, HDAC7, which is highly expressed in proliferating cells within the
5 growth plate, suppresses chondrocyte proliferation and β -catenin activity during endochondral
6 ossification ⁽⁶⁵⁾.

7 HDAC class III members, called sirtuins, are structurally distinct from class I and II HDACs.
8 They are NAD⁺-dependent protein deacetylases that cleave off acetyl, as well as other acyl
9 groups, from the ϵ -amino group of lysines in histones, but also in other substrate proteins.
10 Seven sirtuin (SIRT1-7) isotypes have been identified in mammalian cells. Compared with
11 normal mice, Sirt1-null mice are smaller, and have cranio-facial abnormalities ^(66,67).
12 Additionally, SIRT1 KO mice exhibit delays in long bone mineralization ⁽⁶⁸⁾ and possess an
13 altered cartilage phenotype, characterized by low levels of type II collagen, aggrecan and
14 glycosaminoglycan (GAG) content and a high level of MMP-13 ⁽⁶⁹⁾. Additional observations
15 showed that, like heterozygous SIRT1 mice, SIRT1-null mice exhibit increased chondrocyte
16 apoptosis and OA severity with age, as compared to equivalent WT mice ^(69,70). SIRT1 also
17 promotes chondrocyte survival under various stress conditions ⁽⁷⁰⁻⁷⁴⁾, further supporting its
18 role in maintaining cartilage homeostasis. Furthermore, Sirtuin 6 (SIRT6) has been found
19 highly expressed in the growth plate preferentially in proliferating and prehypertrophic
20 chondrocytes. Homozygous SIRT6 null mice are smaller than their wild-type (WT)
21 littermates at birth and exhibit defects in proliferation and hypertrophic chondrocyte
22 differentiation, by interacting with the IHH signaling pathway ⁽⁷⁵⁾.

23 In contrast to deacetylation, various *in vitro* and *in vivo* studies demonstrate that histone
24 acetylation favors cartilage differentiation. In particular, the HAT p300 potentiates SOX9-

1 dependent transcription through histone hyperacetylation. Beside its HAT activity, p300 can
2 also acetylate non histone proteins. It can act as a coactivator to cartilage homeoprotein-1
3 (CART1), a protein expressed selectively in chondrocyte lineage during embryonic
4 development ⁽⁷⁶⁾, through acetylation of the conserved lysine residue adjacent to the
5 homeodomain ⁽⁷⁷⁾. During chondrogenesis, SOX9 also specifically interacts with Tat
6 interactive protein-60 (Tip60) to increase acetylation of SOX9 and subsequent enhancement
7 of its transcriptional activity ⁽⁷⁸⁾.
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19 **Histone methylation**

20 Besides acetylation, histones can undergo methylation. This post-tranlational modification is
21 driven by histone methyltransferases (HMT) which add a methyl group (-CH₃) to lysine or
22 arginine residues at the N-terminal tail of histones.
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29 The methylation of histone lysine residues is associated with either transcription activation or
30 repression depending on concerned amino acid. Indeed, lysine methylation is interpreted by
31 both positive and negative transcription regulators. For instance, histone H3 lysine 4
32 trimethylation (H3K4me₃) and H3K36me₃ are associated with gene expression, while
33 H3K27me₃ and H3K9me₃ are associated with transcriptional repression.
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42 Histone methylation is dynamically regulated during endochondral ossification by various
43 histone methyltransferases and demethylases. For instance, H3K9 methyltransferases
44 including PR domain zinc finger protein 2 (PRDM2), Suppressor of variegation 3-9,
45 drosophila homolog of 1 and 2 (SUV39H1, SUV39H2) G9a, GLP (G9a-like protein) and
46 ERG-associated protein with SET domain (ESET) are predominantly expressed in both
47 prehypertrophic and hypertrophic chondrocytes during mouse growth plate development.
48 These expression patterns overlap with the distributions of H3K9 HKM, especially H3K9me₁
49 and H3K9me₃. In addition, H3K9 methyltransferases and H3K9me₃ are enriched within
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1 trabecular bone ⁽⁷⁹⁾. ESET, a H3K9-specific histone methyltransferase also called Set domain
2 protein bifurcated 1 (SETDB1), is transiently upregulated in prehypertrophic chondrocytes in
3
4 newborn mice. ESET controls hypertrophic differentiation of growth plate chondrocytes and
5
6 formation of epiphyseal plates ⁽⁸⁰⁾, as well as the terminal differentiation of articular
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8 chondrocytes ⁽⁸¹⁾. ESET conditional deletion in mesenchymal cells causes acceleration of
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10 chondrocyte hypertrophy during embryonic and prenatal development, resulting in a defect in
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12 long bone growth and trabecular bone formation. This effect is associated with a significant
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14 reduction of IHH protein in pre- and early hypertrophic chondrocytes. One of mechanism
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16 explaining this phenotype is that ESET physically interacts with both RUNX2 and HDAC4 to
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18 repress the expression of RUNX2 target genes by a H3K9 methylation-dependent mechanism
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20 ⁽⁸⁰⁾. Conversely, the transcription factor AT-rich interactive domain 5b (ARID5b) facilitates
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22 chondrocyte differentiation and promotes expression of chondrogenic genes by recruiting the
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24 H3K9me2 demethylase PHD Finger Protein 2 (PHF2) to SOX9-regulated chondrogenic gene
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26 promoters ⁽⁸²⁾.

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29 H3K27 methylation plays also a crucial role in the regulation of chondrocyte proliferation and
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31 hypertrophy. H3K27 methylation is regulated by the histone methyltransferase Enhancer of
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33 zeste homolog 2 (EZH2). In contrast, H3K27 methylation is removed by the demethylases
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35 Jumonji domain containing-3 (JMJD3) and ubiquitously transcribed tetratricopeptide repeat
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37 gene on X chromosome (UTX), also called KDM6B and KDM6A, respectively. Loss of both
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39 EZH2 and its homolog EZH1 in cartilage induces severe growth retardation in mice,
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41 characterized by the alteration of chondrocyte proliferation due to an increase of cyclin-
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43 dependent kinase inhibitors CDKN2a/b, and changes in chondrocyte hypertrophy due to the
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45 inhibition of insulin-like growth factor (IGF) signaling by IGF-binding proteins (IGFBP).
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47 Both increases of CDKN2a/b and IGFBP are linked to a decrease of H3K27me3 on their
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49 respective promoter ⁽⁸³⁾. Also, conditional inactivation of EZH2 prevents craniofacial bone
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1 and cartilage formation, due to massive derepression of HOX genes in neural crest cells that
2 are usually devoid of HOX gene expression ⁽⁸⁴⁾.
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4 Conversely, JMJD3-deficient mice exhibit markedly reduced proliferation and hypertrophy of
5 chondrocytes and a severe delay of endochondral ossification. This histone demethylase is
6 highly expressed in prehypertrophic and hypertrophic chondrocytes during endochondral bone
7 formation. Genetic and biochemical analysis reveal that JMJD3 cooperates with RUNX2 to
8 promote chondrocyte proliferation and hypertrophy. JMJD3 acts as a RUNX2 co-activator
9 and this interaction not only induces H3K27me3-demethylation-dependent RUNX2
10 expression, but also recruits RUNX2 on the JMJD3 promoter ⁽⁸⁵⁾. JMJD3 expression increases
11 during TGF- β induced MSC chondrogenesis ⁽⁸⁶⁾. Moreover, incubation with the H3K27
12 demethylase inhibitor GSK-J4 during chondrogenesis inhibits MEC synthesis and
13 chondrogenic gene expression by MSCs ⁽⁸⁶⁾.
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28 The histone-lysine N-methyltransferase DOT1L likely also plays a role in chondrogenesis.
29 This H3 lysine-79 specific methyltransferase is strongly expressed within mouse developing
30 limbs during chondrogenesis. Furthermore knockdown studies in ATDC5 cells suggest that
31 DOT1L is involved in chondrogenic differentiation, presumably through the regulation of
32 canonical WNT-signaling ⁽⁸⁷⁾.
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43 **miRNA**

44 Non coding RNA (ncRNA), particularly miRNA, are important epigenetic regulators of
45 physiological and pathological processes.
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51 MiRNA are evolutionarily conserved non-coding RNA measuring approximately 22
52 nucleotides that control translation and/or mRNA degradation ⁽⁸⁸⁾. Biogenesis of miRNA
53 (figure 3) begins in the nucleus by their transcription as primary miRNA (pri-miRNA). Next,
54 the pri-miRNA undergoes a first cleavage catalyzed by a complex formed by DROSHA and
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1 DiGeorge syndrome critical region 8 (DGCR8), to produce a miRNA precursor (pre-miRNA).
2 This pre-miRNA is then exported to the cytoplasm by Exportine-5-RAS-related nuclear
3 protein guanosine 5'-triphosphate (Exportine-5-RAN-GTP). The maturation continues with a
4 second cleavage by the RNase III DICER, which produces a miRNA duplex. The miRNA
5 guide strand is next loaded into miRNA-induced silencing complex (miRISC) containing
6 DICER1 and Argonaute (AGO) proteins to mediate gene extinction ⁽⁸⁹⁾.
7
8 Interestingly, germline deletion of DICER limits growth plate chondrocyte proliferation and
9 accelerates differentiation into post-mitotic hypertrophic chondrocytes, leading to severe
10 skeletal growth defects ⁽⁹⁰⁾. In addition, *in vitro* knockdown of DICER inhibits chondrogenic
11 differentiation, showing that miRNA machinery is also crucial for chondrogenesis ⁽⁹¹⁾.
12
13 MiRNA microarray analysis highlight that miR-193b, miR-199a-3p/hsa-miR-199b-3p, miR-
14 455-3p, miR-381, miR-92a, miR-320c, and miR-136 miR-23b, miR-140, miR-181 and miR-
15 210 are upregulated during MSCs differentiation into chondrocytes, while miR-490-5p, miR-
16 4287, miR-BART8, and miR-US25-1 are downregulated ^(92,93). The roles of the majority of
17 these miRNA are still largely unknown, but several studies demonstrate the crucial role of
18 miR-410. Mice deficient in miR-140 present an impairment of longitudinal bone growth and
19 an acceleration of hypertrophic differentiation ⁽⁹⁴⁾. Among others, miR-140 targets p38
20 MAPK signaling pathway, which promotes activity and expression of mads box transcription
21 enhancer factor 2, polypeptide C (MEF2C), a transcription factor that controls chondrocyte
22 hypertrophy and bone development ^(61,94). Furthermore, *in vitro*, miR-140 expression has been
23 found to increase in parallel with SOX9 and COL2A1 during MSC chondrogenic
24 differentiation *in vitro* ⁽⁹⁵⁾; thus, miR-140 may promote chondrogenesis by enhancing WNT
25 signaling ⁽⁹¹⁾. Moreover, this miRNA consistently decreases during chondrocyte
26 dedifferentiation and its expression is recovered during re-differentiation in a 3D culture with
27 TGF- β 1 ⁽⁹⁶⁾. miR-23b was also described as a miRNA promoting MSC chondrogenic
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1 differentiation ⁽⁹⁷⁾. miR-574-3p, which is regulated by direct binding of SOX9 on its
2 promoter, is likewise involved in the commitment of MSCs towards chondrocytes ⁽⁹⁸⁾. On the
3
4 other hand, some miRNAs, such as miR-495 and miR-194, directly target mRNA of SOX9 or
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6 SOX5, inhibiting MSC differentiation into chondrocytes ^(99,100). Additionally, miR-146a
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8 affects skeletal cell differentiation by down-regulation of SMAD2 and SMAD3 protein
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10 translation ⁽¹⁰¹⁾. Furthermore, decreased production of miR-221 induces an increase of
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12 chondrogenic markers, including collagen type II (COL2A1), and SOX9 ⁽¹⁰²⁾. Interestingly,
13
14 miR-221 silencing promotes MSCs differentiation towards chondrocytes *in vitro* in the
15
16 absence of TGFβ, and strongly enhanced *in vivo* cartilage repair ⁽¹⁰³⁾.
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24 **Long non-coding RNAs**

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26 Long non-coding RNA (lncRNA) are a class of RNA transcripts longer than 200 nucleotides
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28 lacking open reading frames. They are important regulators of a variety of biological
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30 processes, including transcriptional regulation, mRNA processing and stability, chromatin
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32 organization and scaffolding for multi-protein complexes ⁽¹⁰⁴⁾. Whereas a vast number of
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34 lncRNA are expressed in human cells, their physiological functions remain mostly unknown.
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36 Even fewer studies have investigated their role in endochondral ossification and/or cartilage
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38 development and maintenance ⁽¹⁰⁵⁾: however, one of the best-studied lncRNA is HOTAIR.
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40 Among its multiple roles, it is now clear that this lncRNA regulates skeletal element
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42 development. Indeed, mice lacking HOTAIR exhibit homeotic transformation of the spine and
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44 malformation of metacarpal-carpal bones ⁽¹⁰⁶⁾. Mechanistically, HOTAIR recruits PRC2 to the
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46 HOXD cluster, where it mediates H3K27 methylation to repress the expression of several 5'
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48 HOXD genes ⁽¹⁰⁷⁾, namely HOXD11 and HOXD13 ⁽¹⁰⁶⁾.
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56 The lncRNA HOTTIP functions as an enhancer to regulate the expression of 5' HOXA genes
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58 and control the growth and elongation of zeugopod and autopod skeletal elements ⁽¹⁰⁸⁾. At
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1 molecular level, HOTTIP modulates gene expression by chromosomal looping, placing the
2 recruited Trithorax/WDR5/MLL protein complexes proximal to 5' HOXA genes to facilitate
3 H3K4me3 and gene expression ^(108,109). Additionally, HOTTIP function is associated with
4 endochondral ossification ⁽¹¹⁰⁾. Also, lncRNA-HIT was identified within the HOXA locus
5 ⁽¹¹¹⁾. This lncRNA is expressed in mice embryo limb mesenchyme just before cartilaginous
6 differentiation ⁽¹⁰⁵⁾. lncRNA-HIT associates with chondrogenic genes and promotes their
7 expression by recruiting p100 and the histone acetyltransferase CREB-binding protein (CBP)
8 ⁽¹⁰⁵⁾.

9 The lncRNA DANCR may also promote chondrogenic differentiation of synovium-derived
10 MSCs (SMSC) in concert with SOX4 ⁽¹¹²⁾, by interaction with SMAD3, and STAT3 mRNA
11 to regulate their stability ⁽¹¹³⁾. DANCR favors also SMSC proliferation through MYC
12 regulation ⁽¹¹³⁾. Other Sox transcription factors interact also with lncRNA. For instance,
13 ROCR, which is induced during chondrogenic differentiation, plays an important role in the
14 induction of SOX9 and cartilage gene expression ⁽¹¹⁴⁾. Similarly, UCA1 plays an important
15 role in the skeletal development ⁽¹¹⁵⁾. UCA1 is expressed in normal human chondrocytes and
16 favors chondrocyte differentiation of these cells, but UCA1 is not detected in human bone
17 marrow mesenchymal stem cells (hBMSCs).

18 H19 is also a new player in musculoskeletal system. H19 could promote osteogenic
19 differentiation by suppressing the TGF- β /pSmad3/histone deacetylase 4/5 (HDAC4/5)
20 signaling pathway, and enhancing the expression of osteogenic marker genes such as RUNX2
21 and osteocalcine (OCN), and by serving as a competing endogenous RNA for both miR-141
22 and miR-22, which are the negative regulators of WNT/ β -catenin signaling. H19 could also
23 promote chondrogenic differentiation, in particular H19 may regulate the expression of
24 collagen type 2 through miR-675 ⁽¹¹⁶⁾.

Conclusion and perspectives

1
2 Chondrogenesis during both MSC chondrogenic differentiation and endochondral ossification
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4 is intrinsically controlled by epigenetic mechanisms, including DNA methylation, histone
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6 post-translational modification and non-coding RNA. All these regulatory processes are
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8 strongly interconnected together.
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11 Investigations of their involvement during endochondral ossification as well as cartilage
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13 differentiation and stability could be useful in cartilage tissue-engineering applications. For
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15 instance, inhibition of DNA methylation or effective miRNA-based treatment modalities that
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17 should have minimal or no off-target side effects, could provide promising approaches to
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19 maintain the chondrocyte phenotype. A better understanding of epigenetic regulation during
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21 chondrogenesis and chondrocyte terminal differentiation and hypertrophy may open new
22
23 strategies to decelerate chondrocyte dedifferentiation and hypertrophy observed during
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25 autologous chondrocyte implantation, or to promote chondrogenesis of stem cells.
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27 Consequently, more information regarding how epigenetic regulations established and
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29 maintains the chondrocyte phenotype is required and new “epidrugs” need to be developed
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31 improve the outcome of cartilage cell therapy.
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53 Figure legends

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56 **Figure 1: Endochondral ossification.** (A) Mesenchymal cells condensation. (B) Fetal hyaline
57 cartilage model develops. (C) Cartilage calcifies and a periosteal bone collar forms around diaphysis.
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(D) Primary ossification center forms in the diaphysis. (E) Secondary ossification center forms in epiphysis.

Figure 2: Components of cartilage. Cartilage is composed of specialized cells called chondrocytes that produce an abundant extracellular matrix. Three main protein classes are present in articular cartilage: collagens (mainly type II collagen), proteoglycans (mainly aggrecan), and non-collagenous proteins (including link protein, fibronectin and cartilage oligomeric matrix protein, COMP). Other proteoglycans are present such as decorin and biglycan.

Figure 3: miRNA biogenesis. miRNAs are transcribed by RNA polymerase II into primary transcripts (pri-miRNA). Then, pri-miRNA are cleaved into precursor miRNA (pre-miRNA) by the microprocessor complex formed by the ribonuclease DROSHA and the DNA binding protein DiGeorge syndrome critical region 8 (DGCR8). Next, the pre-miRNA are exported to the cytoplasm by Ran-GTP-dependent dsRNA-binding protein, Exportin-5. In the cytoplasm, DICER, an RNAase III-type endonuclease, cleaves pre-miRNA to produce a duplex. Finally, the guide miRNA generated by DICER is loaded onto the RNA-induced silencing complex (RISC) and consequently binds to the 3'UTR of target genes, inducing degradation or translational inhibition of the target mRNA.

Table 1:

Component	Genetic modification	Cell affected	Results	References
<i>Histone deacetylases</i>				
HDAC1	KO	All (Germline deletion)	Embryonic lethal	[1,2]
HDAC2	KO	All (Germline deletion)	Reduced body size (reduced vertebrae/pelvis size)	[3]
HDAC3	KO	All (Germline deletion)	Embryonic lethal	[4,5]
HDAC3	cKO	Osteochondral lineage (<i>Osx:Cre</i> deletion)	Severe osteopenia due to abnormal maturation of osteoblasts. Reduced body size. Cortical bone loss. Trabecular bone loss. Decreased bone formation rate. Increased bone marrow adipocyte differentiation	[6]
HDAC4	KO	All (Germline deletion)	Premature ossification of developing bones due to ectopic and early onset chondrocyte hypertrophy	[7]
HDAC4	Tg	Chondrocytes (<i>Col2a1</i> promoter driven)	No endochondral ossification	[7]
HDAC5	Tg	All (antagonization of Hdac5 repressor)	Trabecular bone loss. Decreased cortical bone strength. Decreased bone formation rate	[8]
HDAC6	KO	All (Germline deletion)	Increased trabecular bone mineral density	[9]
HDAC7	ciKO	Early chondrocytes (tamoxifen-inducible <i>Col2a1:Cre</i> deletion)	Increased proliferation and β -catenin levels in growth plate chondrocytes. Expanded proliferative zone	[10]
HDAC8	KO	All (Germline deletion)	Ossification defects in frontal and interparietal bones	[11]
HDAC8	cKO	Pre-osteoblasts (<i>Twist1:Cre</i> deletion)	No effect	[11]
HDAC8	cKO	Osteoblasts (<i>Col1a1:Cre</i> deletion)	No effect	[11]
HDAC8	cKO	Chondrocytes (<i>Col2a1:Cre</i> deletion)	No effect	[11]
HDAC8	cKO	Neural crest cells	Ossification defects in frontal and interparietal bones	[11]

SIRT1	KO	(<i>Wnt1:Cre</i> deletion) All (Germline deletion)	Reduced body size. Cranio-facial abnormalities. Trabecular bone loss. Altered cartilage phenotype. Increased apoptotic chondrocytes and OA severity with age	[12,13]
SIRT6	KO	All (Germline deletion)	Reduced body size. Defects in proliferation and hypertrophic chondrocyte differentiation	[14]

Histone methyltransferases

ESET	cKO	Mesenchymal cells (<i>Prx1:Cre</i> deletion)	Accelerated chondrocyte hypertrophy. Defect in long bone growth. Defect in trabecular bone formation	[15,16]
EZH2	KO	All (Germline deletion)	Embryonic lethal	[17]
EZH2/EZH1	cKO	<i>Col2-cre Ezh1^{-/-} Ezh2^{fl/fl}</i>	Decreased overall body growth and longitudinal bone growth. Insufficient growth of the rib cage and by the abnormal vertebral column. Decreased chondrocyte proliferation and hypertrophy	[17]
EZH2	cKO	Neural crest cells (<i>Wnt1:Cre</i> deletion)	Defect in craniofacial bone and cartilage formation	[18]

Histone demethylase

JMJD3	KO	All (Germline deletion)	Reduced proliferation and hypertrophy of chondrocytes. Delayed endochondral ossification	[19]
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miRNA process

DICER	cKO	Chondrocytes (<i>Col2a1:Cre</i> deletion)	Accelerated differentiation into postmitotic hypertrophic chondrocytes. Defects in skeletal growth	[20]
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miR-140 KO All (Germline deletion) Defects in growth of endochondral bones. Dwarfism and craniofacial deformities. [21]

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Figure(s)

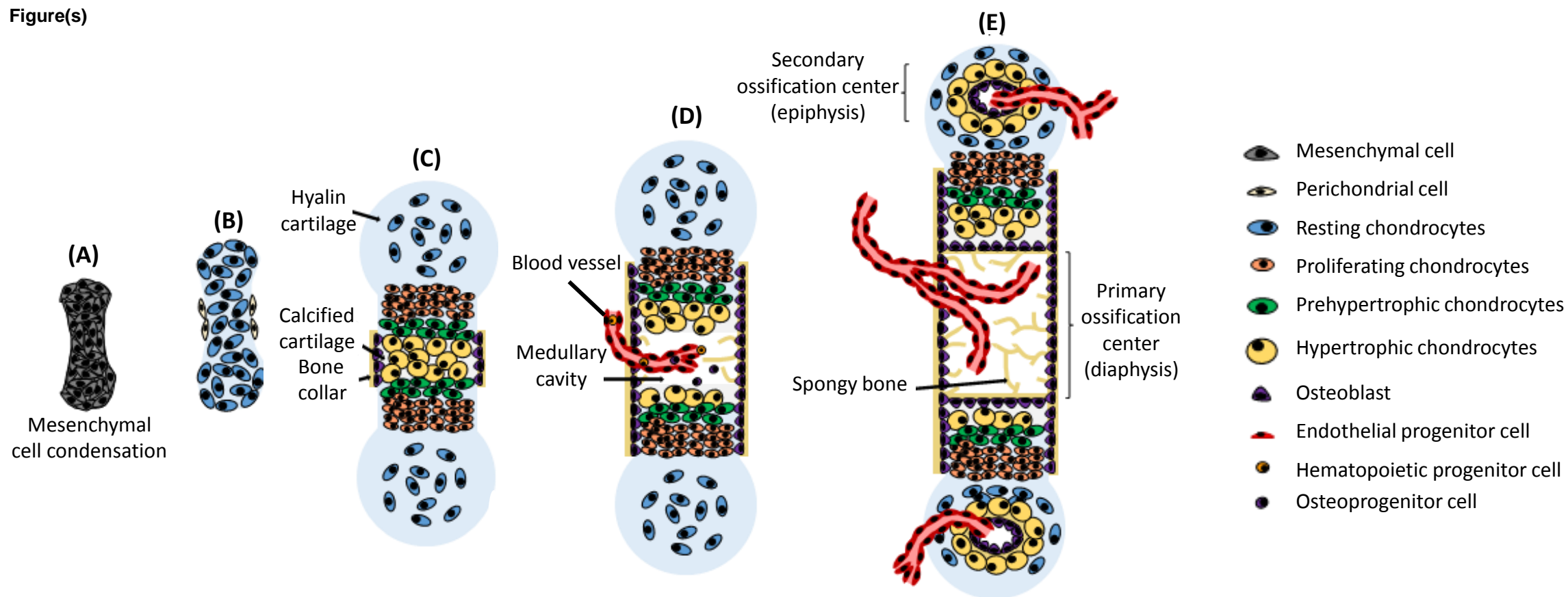


Figure 1

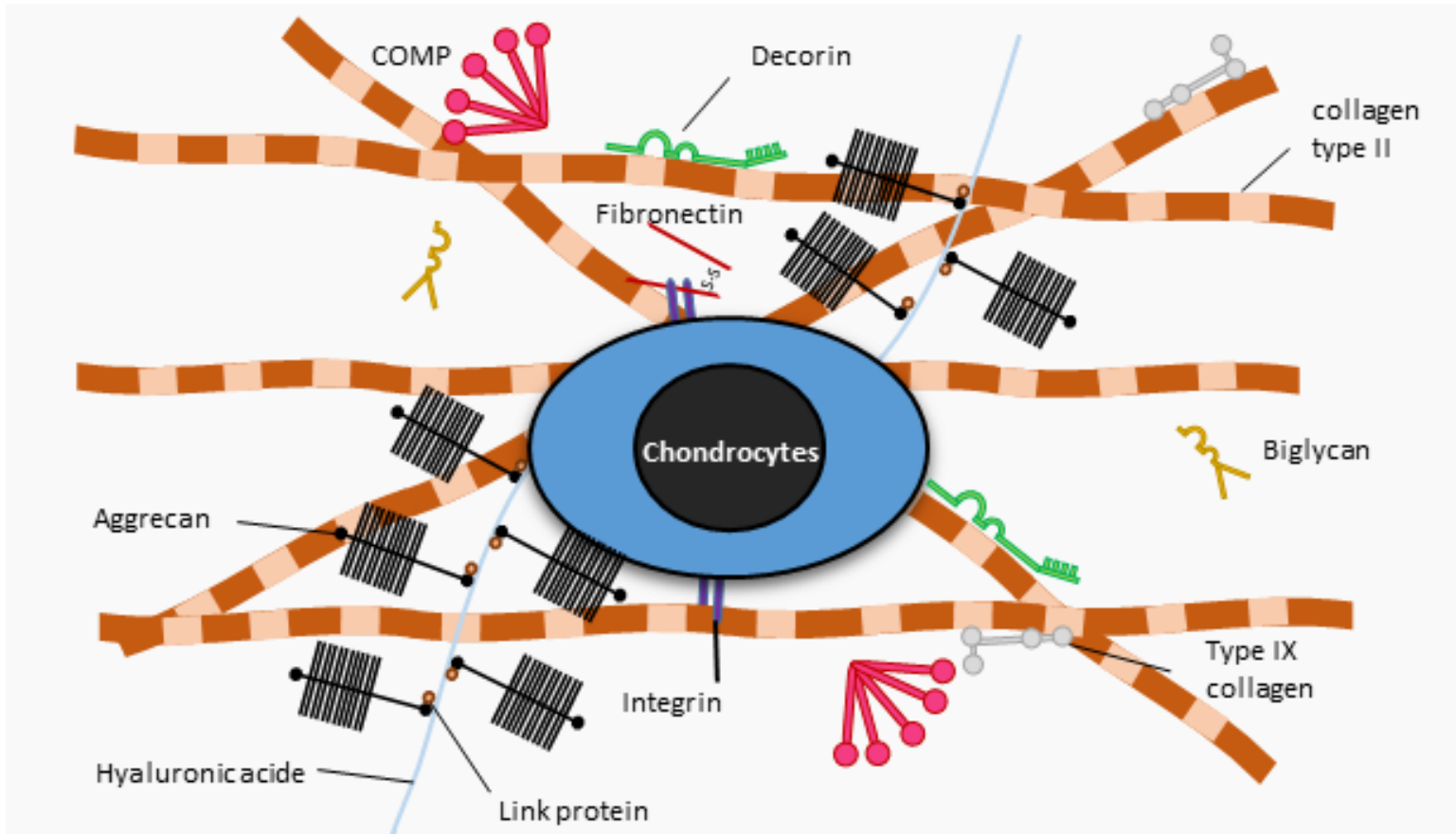


Figure 2

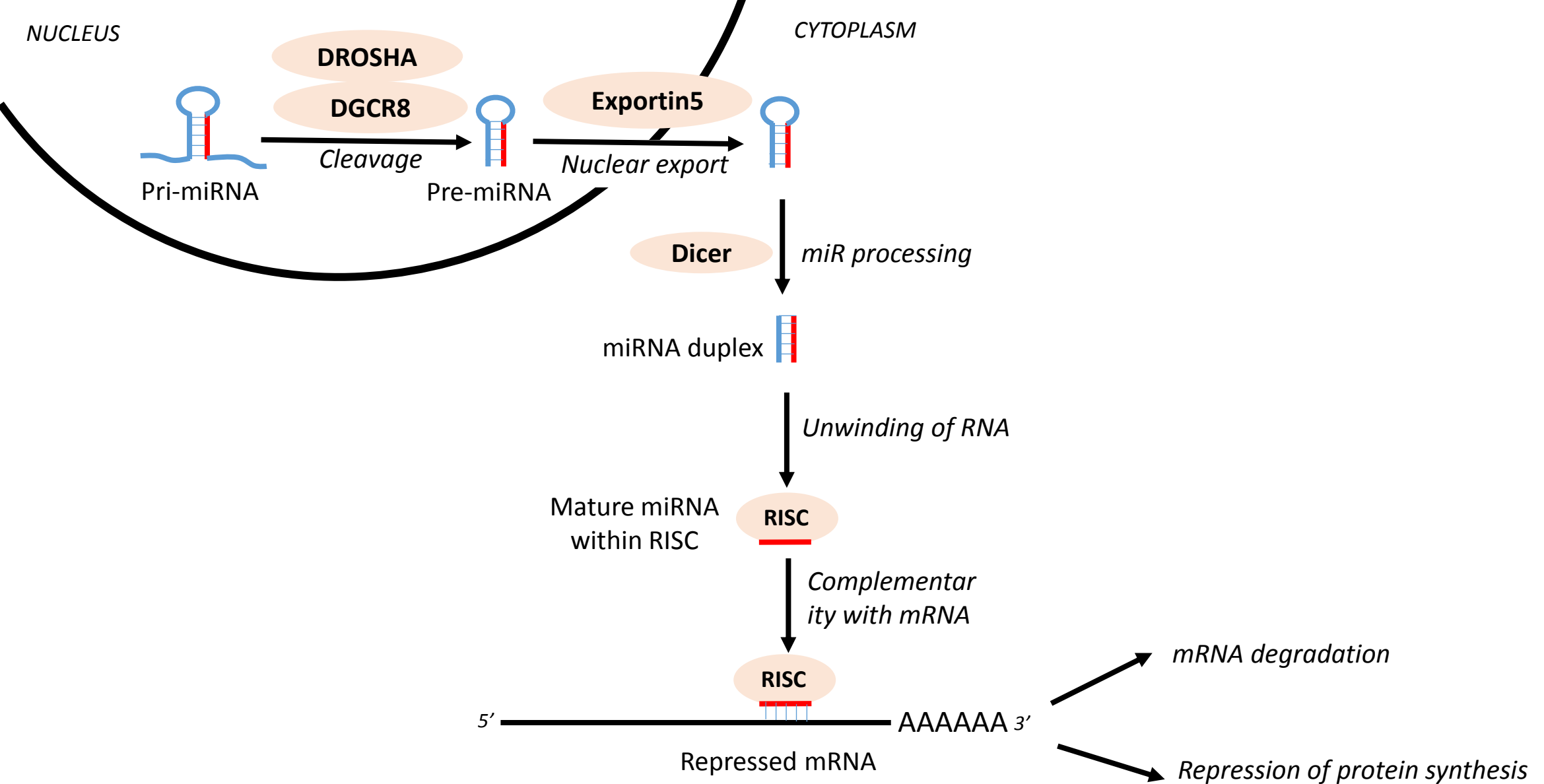


Figure 3