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Epigenetic Regulation of Bone cells

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Abstract

Bone is a major organ in the skeletal system that supports and protects muscle and other organs, facilitates movement and hematopoiesis, and forms a reservoir of minerals including calcium. The cells in the bone, such as osteoblasts, osteoclasts, and osteocytes, orchestrate sequential and balanced regulatory mechanisms to maintain bone and are capable of differentiating in bones. Bone development and remodeling require a precise regulation of gene expressions in bone cells, a process governed by epigenetic mechanisms such as histone modification, DNA methylation, and chromatin structure. Importantly, lineage-specific transcription factors can determine the epigenetic regulation of bone cells. Emerging data suggest that perturbation of epigenetic programs can affect function and activity of bone cells and contributes to pathogenesis of bone diseases, including osteoporosis. Thus, understanding epigenetic regulations in bone cells would be important for early diagnosis and future therapeutic approaches.

Keywords

epigenetic regulation; osteoclasts; osteoblasts; Histone modification; DNA methylation

Introduction

Epigenetics refers to the changes in gene expression or phenotype without alterations in DNA sequence [1]. Epigenetic regulation controls gene expression by chromatin regulators that bind to DNA, by modifications of chromatin such as histone modification and DNA methylation, or by non-coding RNAs. Increasing evidence suggests that epigenetic regulation plays an important role in physiological and pathological conditions. It has become clear that epigenetic regulation is a key molecular mechanism by which environmental influences and cues are imprinted on DNA/chromatin and determine patterns of gene expression, responses to environmental challenges, and disease causation and pathogenesis [2].

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Bone is a dynamic tissue that undergoes constant remodeling and controls skeletal development and regeneration. Bone remodeling is a complex process whereby old, mature bone is removed by bone resorption and is replaced by new bone formation. Bone remodeling is required for bone homeostasis including maintaining appropriate bone cell physiology and bone microarchitecture. It is tightly regulated by a balance between bone resorption by osteoclasts and bone formation by osteoblasts (Figure 1). Osteoblasts are bone-forming cells and are derived from mesenchymal stem cells (MSCs) [3]. Various extrinsic regulators and corresponding transcription factors important for osteogenic differentiation of MSCs have been well characterized. Osteoblasts not only play a central role in bone formation by synthesizing multiple bone matrix proteins, but they also regulate osteoclast maturation and bone resorption by secreting soluble factors and by direct cell-cell interactions. Osteoclasts are bone-resorbing cells important for bone homeostasis and pathological bone resorption [4–8]. Osteoclasts are differentiated from hematopoietic stem cells (HSCs) of the myeloid lineage under influence of M-CSF and RANKL. Various autocrine and paracrine factors modulate bone cell differentiation and function. One such factor is RANKL which is produced by several cell types, including osteoblasts, activated T cells, and osteocytes. RANKL binds to its receptor, RANK that is expressed in osteoclast-lineage cells and the RANKL-RANK interaction activates signaling pathways that induce NFATc1, a master transcription factor of osteoclast differentiation. OPG is secreted by osteoblasts and other cell types and functions as a decoy receptor by binding to RANKL, subsequently preventing the activation of RANK. Thus, OPG is a key negative regulator of osteoclastogenesis, a property that has already been therapeutically exploited. In addition to RANK/RANKL/OPG, cytokines from immune cells also influence osteoclast differentiation and function; and various proteins have been shown to control the bone integrity.

Over the past years, substantive progress in epigenetic research on osteoclasts, osteoblasts, and their differentiation from hematopoietic stem cells and mesenchymal stem cells (MSCs) respectively has been made, highlighting the importance of epigenetic regulation in bone remodeling. The impairment of bone remodeling is associated with many skeletal diseases such as osteoporosis and, to date, anti-resorptive or osteoanabolic treatments have been developed but all have significant side effects limiting their prolonged use. Investigating epigenetic regulations may provide novel treatment targets for promoting bone formation and suppressing excessive bone resorption. Understanding the epigenetic regulation during physiological bone remodeling further provides insight into clinical and mechanistic characterizations of bone metabolism and into pathophysiology of bone diseases. Although osteocytes are recognized as important regulators of bone turnover, this review summarizes current knowledge of epigenetic mechanisms in relation to the mechanisms regulating the differentiation of osteoclasts and osteoblasts, and how these regulations can be applied to therapies for bone diseases.

Epigenetic regulation

A notable feature of multicellular organisms is their capacity to create functionally unique cell types from the same genome sequence that is shared by all cells in the organism. This diversity results from the capacity of individual cell types to initiate and then maintain specific gene expression patterns during development. To achieve this, cellular signaling

events are thought to regulate the activity of cell type-specific DNA-binding transcription factors that function as master regulators of gene expression networks. Recently, epigenetic regulation has also been identified as an important factor that is involved in the regulation of gene expression and cell fate decision.

Due to their unique properties of self-renewal and their ability to undergo multilineage differentiation in response to appropriate signaling cues, pluripotent embryonic stem cells (ESCs) are widely used to study epigenetic mechanisms and show various characteristic epigenetic phenotypes based on prevalence of different histone markers [9]. A public research consortium named the Encyclopedia Of DNA Elements (ENCODE) [10] and the Epigenomics Roadmap [11] carried out a project to identify all functional elements in the human genome sequence and generated an epigenomic map. This data allows researchers to understand molecular and cellular processes of particular cell types and to elucidate fundamental processes of human diseases at a genomic level. Extensive epigenomic data sets of human osteoblasts are currently available in ENCODE database and the chromatin landscape of human osteoblasts has been shown by studies of ENCODE consortium.

Beyond the sequences of the genome, a large regulatory network controls cell specific identity and function of cells. Each cell type has its own unique chromatin structure and organization. Whereas mRNA expression profile indicates the current state of a cell, epigenomic regulation can give perspectives of history of cell states. Advanced high throughput sequencing makes the genome-wide analysis of chromatin profiling feasible, resulting in generating a profiling of the epigenome [12]. As chromatin patterns are related to underlying regulatory processes, chromatin profiling enables identification of the regulatory network in bone cells. Increasing knowledge of regulatory network in bone cells will reveal a cell-type specific chromatin structure and organization leading to affecting cell-specific gene regulation.

Transcription factor network

Transcription factors bind to specific DNA sequences (called binding motif) in promoter or enhancer regions to regulate gene expression. Transcription factor networks and transcriptional processes help shape epigenetic regulation as well as chromatin structure [13]. However, transcription factor binding to the promoter is insufficient to fully regulate transcription and the interaction between the promoter and the regulatory elements, called enhancers, provide another level of dynamic regulation of transcription [14]. Unlike other cells in mammals, interaction with bone and bone marrow environment would be important factors to control the gene expression profile in bone cells [15, 16].

Molecular and biochemical studies reveal principal regulators of transcription in bone cells, while ChIP-based assays have mapped the binding sites of many important transcription factors. During osteoblastogenesis, both the transcriptome and the epigenome of osteoblast lineage cells undergo dynamic changes [17–21]. Several transcription factors are known to control bone development and osteoblast differentiation [22]. Runt-related transcription factor 2 (RUNX2, Cbfa1) is a master transcription factor of osteoblasts and is indispensable in all stages of osteoblast differentiation [23]. RUNX2 ablation causes bone defects in

human and mouse [24–26]. RUNX2 regulates gene expression during osteoblastogenesis and detailed epigenetic regulation has been identified for RUNX2-mediated gene expression [27–29]. In MC3T3-E1 osteoblast-like cells, ChIP-sequencing demonstrated that Runx2 occupancy is actively regulated during osteoblast differentiation [19]. Other transcription factors have been identified to control osteoblast differentiation, including Osterix (OSX) [30], ATF4[31], twist homolog 1 (TWIST1)[32], and distal-less homeobox 5 (DLX5)[33].

Various transcription factors coordinate a broad spectrum of gene expression programs in osteoclastogenesis [34]. NFATc1 is a master transcriptional factor of osteoclastogenesis and drives the early stages of osteoclast differentiation [35–37]. NFATc1-deficient mice develop severe osteopetrosis *in vivo* [38], and NFATc1-deficient cells are incapable of differentiating into osteoclasts *in vitro* [36, 37, 39]. However, it has been shown that NFATc1 forms a complex with osterix and both are required for osteoblastic bone formation [40]. This data suggests that NFATc1 cooperatively regulates osteoclastogenesis by interacting with other transcription factors. c-Fos is another essential transcription factor for osteoclastogenesis. c-Fos belongs to AP-1 family and c-Fos-deficient cells can differentiate into macrophages but not into osteoclasts, suggesting the role of c-Fos in osteoclastogenesis [41, 42]. The cis-regulatory elements enriched with binding sites of transcription factor and the lineage determining transcription factors play an important role in pioneering activity of transcription. Following transcriptional program sets cell-specific gene expression program and establishes dynamic epigenetic states. Therefore, the complicated interaction between transcription factors and epigenetic regulation endow cell identify to bone cells.

Histone modification

The basic unit of chromatin is the nucleosome[43]. Each nucleosome consists of a short (around 147 base pair) DNA segment wrapped around a histone octamer that comprises two copies each of core histones: H2A, H2B, H3 and H4, as shown figure 2A. The N-terminal tails of core histones are subject to various post-translational modifications by chromatin remodelers [44, 45]. More than eight different histone modifications are identified, including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation. A large-scale mapping of histone modifications and related chromatin structure enables the characterization and determination of functional consequences of changes in chromatin structure [46, 47]. Currently more than 14,000 data sets are deposited in public domain such as Gene Expression Omnibus (GEO), allowing computational integration of different data sets. These integrated approaches can identify novel interactions between histone modification and genomic elements and supports the idea that a different combination of histone modifications serves as a “switch” to fine-tune genomic elements [48, 49]. Unique patterns of histone modifications are found in different genomic elements such as promoters, gene bodies, enhancers, and chromatin insulators (boundary elements) and reflect the status of transcription (Figure 2B). For example, active promoters are enriched in histone H3 lysine 4 dimethylation (H3K4me2), H3 lysine trimethylation (H3K4me3), histone acetylation (H3Ac and H4 Ac), and H2A.Z.

Histone modifications are dynamically regulated and have an influence on chromatin structure and function [45]. Charting different combination of histone modifications in the

chromatin can predict the stage of transcription at a specific gene [49]. Histone modification is mediated by three components: writers, erasers, and readers. Histone acetylation is an important determinant in multiple chromatin-dependent processes, including gene expression, DNA replication, and DNA damage repair. Acetylation is generally associated with elevated gene expression and open chromatin by reducing a positive charge of histone. The N-ε-lysine acetylation and deacetylation of histones are also controlled by three groups of enzymes: histone acetyltransferase (HAT, reviewed [50]), histone deacetylase (HDAC, reviewed [51]), and histone acetylation readers (reviewed [52]). Histone acetylation by HATs plays a key role in transcriptional activation, whereas deacetylation of histones promotes transcriptional repression and silencing of genes. However, dysregulated acetylation of histone is often associated with the development of a wide variety of human cancers. An excessive level of histone acetylation induces apoptotic cell death, whereas extensive histone deacetylation has been linked to the repression of tumor regulatory genes promoting cancer pathologies. Recruitment of HATs and subsequent histone acetylation are dynamically regulated during the differentiation of bone cells [53, 54] and intervening histone acetylation by small molecule inhibitors results in altering the differentiation of bone cells.

Due to the importance of histone acetylation, various small molecule inhibitors which target HATs or HDACs have been developed and contain therapeutic potential for various diseases. HDACs (Histone deacetylases) function as “erasers” which remove an acetyl group from histones and also have activity on non-histone proteins [55]. HDACs are comprised of three classical classes (I, II, and IV) that have 11 HDACs and class III (sirtuins) that containing NAD-dependent catalytic sties. HDAC inhibitors (HDACi) cause an increase in the acetylated level of histones, which in turn promotes the re-expression of the silenced regulatory genes in cancer cells and reverse the malignant phenotype and thus HDAC inhibitors have recently emerged as potential cancer therapeutic agents. Two HDAC inhibitors, vorinostat and romidepsin, have received an approval from the US FDA for the treatment of cancer and certain neurological conditions.

Many studies testing the effect of HDAC inhibitors (HDACi; TSA, SAHA, MS-275, sodium butyrate, and valproic acid) on osteoblasts and osteoclasts have been documented, implicating that downregulation or deletion of HDACs may play a role in the differentiation of osteoclasts and osteoblasts. Histone acetylation of RUNX2 is one of key regulatory mechanisms for RUNX2-mediated gene programs [56]. HDAC inhibitors show positive effects on *in vitro* osteoblast differentiation and maturation, in part, mediated by promoting RUNX2 function. The treatment with a HDAC inhibitor promotes osteoblastogenesis [57–63] and also increases genome-wide distribution of H4 acetylation [64]. A conditional deletion of HDAC3 in osteochondroprogenitor cells results in osteopenia and increases marrow adiposity [65]. Resveratrol or isonicotinamide is an agonist of Sirtuin 1, class III HDAC, and blocks spontaneous adipocyte differentiation and thereby promotes osteoblast differentiation and bone mineralization [66].

Although HDAC inhibitors suppress osteoclastogenesis [67], the deletion of individual HDAC shows differential effects on osteoclast differentiation depending on the class. The downregulation of HDAC3 by shRNA suppresses osteoclastogenesis [68]. However, Stemig

et al shows that a conditional deletion of HDAC7 in osteoclasts causes osteopenia [69], suggesting that HDAC7 functions as an inhibitor of osteoclastogenesis. Although 24% of 41 patients with epilepsy who received HDACi, Valproate, experienced osteopenia, there was no significant correlation between HDACi treatment and bone density, suggesting bone metabolism may not be significantly affected by valproate monotherapy [70]. Thus, a clinical association between HDACi and bone metabolism is not clear yet.

The readers of histone acetylation contain bromo domain [71], a tandem PHD domain [72] that recognizes acetylated lysines [52]. The readers of histone modification are regulated by non-coding RNAs, binding partners and conformational changes of readers. After recognizing modified histones, the readers function as chromatin architectural proteins, chromatin remodelers, and chromatin modifiers and recruit other components to regulate transcription, repair, recombination, replication, and RNA processing [52]. Bromodomain and extra terminal domain (BET) family proteins are readers of acetylated histones as well as other acetylated proteins such as NF- κ B and regulate gene expression [73]. BET proteins are often deregulated in cancers leading to an aberrant expression of proinflammatory cytokines and growth-promoting genes. Small molecule inhibitors of BET family proteins have demonstrated efficacy in treatment of cancer [73–79]. We and others reported that BET inhibitors, I-BET151 and JQ1, effectively suppress osteoclastogenesis, attenuate inflammatory bone loss, and protect mice from ovariectomy-induced bone loss as well as tumor metastasis induced osteolysis [54, 80]. Interestingly, BET inhibitors also suppress osteoblast differentiation but sensitivity to inhibitors of osteoblasts is lower than in osteoclasts [54, 80]. These results indicated that proper dosing of BET inhibitors can show beneficial effects on pathologic bone diseases, despite ubiquitous expression of BET proteins.

Histone methylation occurs on basic residues such as lysine and arginine and plays an important role in controlling gene expression. Histone methylation has been shown to display differential effects on transcription [81]. For example, trimethylation of H3K4, H3K36, and H3K79 positively control gene expression. In contrast, trimethylation of H3K27 and H3K9 and H4K20 is associated with repression. Protein methylation process was thought to be irreversible because the half-life of methylation is similar to that of histone. However, the discovery of histone demethylases had changed this paradigm to indicate that histone methylation is dynamically regulated. Two families of lysine demethylases are identified: the amine oxidases (LSD1[82]) and jumonji C (JmjC)-domain-containing family[83]. JMJD3 is a H3K27 demethylase [84]. Yasui et al has shown that RANKL stimulation removes trimethylation of H3K27, a repressive mark from the promoter of NFATC1 by, in part, inducing JMJD3, demonstrating the dynamic regulation of histone methylation during osteoclast differentiation [85]. JMJD3 expression is also induced during osteoblast differentiation and regulates the expression of bone-related genes including Runx2, osterix, osteopontin, bone sialoprotein (BSP), and osteocalcin (OCN) [86]. Consistently, *Jmjd3*^{-/-} embryos exhibited open fontanells, less-mineralized cranial bones and hypoplastic clavicles compared to wild type littermates at E18.5, suggesting that JMJD3 is required for osteoblast maturation during both intramembraneous and endochondral bone formation[87].

The recruitment of histone demethylases is mediated by specific DNA sequences such as PRE (polycomb responsive element), cofactors, and DNA-binding factors. Polycomb proteins are transcriptional repressors essential for maintaining tissue specific gene expression program and consist of two groups, polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) [88]. PRC1 and PRC2 target promoters and are responsible for methylation of H3K27 that is known as a repressive epigenetic mark and mediates transcriptional repression in cancer cells [89]. PRC1-type complexes also contain an ubiquitin ligase activity for monoubiquitination of histone H2A to promote H3K27 trimethylation [90]. The enhancer of zeste 2 (EZH2) is a methyltransferase and the catalytic subunit of the PRC2 and trimethylates H3K27 and plays an essential role in the epigenetic repression [89, 91]. Wei et al report that EZH2 suppresses mesenchymal stem cells (MSCs) from differentiating into osteoblasts [92]. Recently, Dudakovic et al demonstrate the expression profiles of epigenetic regulators during osteogenic differentiation of human mesenchymal cells derived from the stromal vascular fraction of adipose tissue (AMSCs) and show that EZH2 is downregulated during osteoblastogenesis [64]. Whereas EZH2 inhibition suppresses adipogenic differentiation, EZH2 functions as a repressor of osteoblastogenesis, supported by experiments using EZH2 inhibitor, siRNAs and knock-out mice. WD repeat domain 5 (WDR5) is another histone methyltransferase and is a component of the mixed lineage leukemia (MLL) complex, which methylates lysine 4 of histone H3. In contrast to EZH2, WDR5 accelerates osteoblast differentiation [93–95]. Osteoblasts with reduced expression of WDR5 exhibit persistent acceleration of osteoblast differentiation through the activation of the canonical Wnt pathway. Altogether, dynamic regulation of histone methylases and demethylases is required for skeletal development and histone lysine methylation plays an important role in bone remodeling by controlling the differentiation of both osteoclasts and osteoblasts although their specific function needs to be further clarified.

Open Chromatin

In order for transcription factors to access and interact with genomic regulatory elements, local chromatin needs to undergo rearrangement. Nucleosomes are able to change their location and are depleted in highly active regulatory regions, called “open chromatin” [96]. DNA in open chromatin regions has higher accessibility to proteins including transcription factors and open chromatin has a signature of active histone modification. Combined with next-generation sequencing, methods for searching genome-wide DNA accessibility have proven extremely effective in identifying regulatory elements across a variety of cell types [96, 97] and quantifying changes that lead to both activation or repression of gene expression. Open chromatin has been first identified by its sensitivity to cleavage by non-specific nucleases such as deoxyribonuclease I (DNase I) and micrococcal nuclease (MNase) at a genome-wide level [98]. Several methods to map open chromatin are developed including formaldehyde-assisted isolation of regulatory elements (FAIRE) [99], and sonication of cross-linked chromatin sequencing (Sono-seq) [100]. Assay for transposase-accessible chromatin using sequencing (ATAC-seq) is a newly developed method to detect open chromatin faster and with a higher sensitivity and applying the preferential integration of transposons into nucleosome-depleted region to interrogate

chromatin structure using Tn5 transposase[101]. Measuring open chromatin is a powerful tool for identifying regulatory elements including transcription factors and enhancers. Binding of proteins, such as transcription factors to genomic DNA, protects the underlying sequences from cleavage by DNase I. DNase I footprinting assay allows identification of transcription factor-DNA interaction at single base pair resolution and provide information on regulatory elements [102–104]. Identification of open chromatin regions further allows predicting cell-type specific function by patterning of chromatin accessibility [96].

Open chromatin regions have been identified in osteoblasts and osteoclasts. Tai et al performed DNase seq analysis during osteoblast differentiation including growth phase, matrix deposition stage, and mineralization stage (GEO55046) and identified a role of a long-range interaction between bone specific RUNX2 P1 promoter-Supt3h (suppressor of Ty 3 homolog) promoters in murine pre-osteoblastic MC3T3-E1 cells using DNase seq in combination with the ENCODE data set [105, 106]. Although these interactions are detected in cells in which RUNX2 is silent, the interaction frequency between RUNX2-Supt3h is significantly increased during osteoblastogenesis as shown by chromosome conformation capture (3C) analysis, suggesting Supt3h as a potential regulator of bone specific RUNX2 P1 promoter. DNase-seq analysis also detected that RANKL facilitates the dynamic changes of chromatin accessibility in RAW 264.7 cells, a murine monocytic cell line that is capable of differentiating into osteoclasts upon RANKL stimulation [107]. Using motif discovery analysis, Inoue et al further profiled the transcription motifs in open chromatin regions and identified novel players for osteoclastogenesis in addition to other well-defined osteoclastogenic transcription factors. Therefore, linking DNase seq footprinting analysis with cell-specific gene expression profiles will expand our knowledge of a complicated transcriptomic network of bone cells.

DNA methylation

DNA methylation is a reversible and covalent modification of the 5'-carbon of cytosine residue with addition of methyl group from SAM and occurs mostly within CpG dinucleotides that cluster as a region called CpG islands in mammal [108, 109]. DNA methylation can be mapped at base-pair resolution with bisulfite sequencing [110]. In general, DNA methylation establishes the pattern of a repressive state of gene expression by maintaining a stable condensed chromatin configuration. DNA methylation is preserved during DNA replication and mitosis and thus a repressive state of genome can be inherited into the next generation. Local deposition and removal of DNA methylation is tightly coupled with occupancy of transcription factors [111]. It has been suggested that DNA methylation-mediated silencing plays a role in the expression of important osteogenic transcriptional factors. During osteoblast differentiation, osteocalcin expression is increased but methylation in the promoter of osteocalcin is significantly decreased. However, CpG methylation of osteocalcin promoter does not alter binding of RUNX2, basal transcriptional activity, or vitamin-D mediated enhancement of OC genes, supporting an indirect association between OC promoter and CpG methylation [112]. Hagh et al has shown that the methylation status of promoters of RUNX2, DLX5 and BSP are not changed during osteoblastic differentiation. In contrast, OSX promoter showed a dynamic change in its methylation pattern [113]. DNA methylation is also not overlapped with active and open

chromatin architecture, supporting a negative correlation between H3K4me3 and DNA methylation [114].

Enzymes responsible for DNA methylation are DNA methyltransferases (DNMTs), which consist of DNMT1, DNMT3a, and DNMT3b. DNMT3a and 3b are de novo DNA methyltransferases and transfer methyl groups to unmethylated sites. Mice with DNMT deficiencies are embryonic lethal with reduced DNA methylation [115, 116]. These studies reveal that DNA methylation is essential for mammalian development, suggesting methylation patterns are pre-deposited during development. Genome-wide sequencing in wild-type mouse ESCs reveals that most methylated genes are differentiation-associated genes, which are repressed in mouse ESCs. The cell-specific roles of DNMT3a and 3b have been investigated, showing that DNMTs have a large impact on bone cells and the importance of DNMTs in skeletal biology. Recently, Nishikawa et al has demonstrated that DNMT3a is needed for osteoclast differentiation and bone resorption by suppressing IRF8, a negative regulator of osteoclast differentiation. DNMT3a suppresses IRF8 via increased methylation on distal regulatory elements of IRF8; methylation was promoted by increased S-Adenosyl methionine (SAM) concentration. Both conditional osteoclast-specific DNMT3a deletion and treatment of mice with TF-3, a DNMT3 inhibitor, protected mice from ovariectomy-induced bone loss, supporting a potential role of epigenetic therapy in bone diseases. 5-aza-cytidine, an another DNMT inhibitor, inhibits differentiation of MSCs into osteoblasts, adipocytes, and chondrocytes [117], suggesting DNMT regulates the differentiation potential of MSCs. Consistently, oxidized adenosine (ADOX), an inhibitor of SAM-dependent methyltransferases suppresses osteoblast differentiation[118]. Bone mineral density and body weight decrease with aging by reduced DNMT1 activity [119] and differentially methylated regions are enriched in genes associated with cell differentiation and skeletal embryogenesis in bone samples from patients with osteoporotic hip fracture [120]. These data suggest that DNA methylation plays an important role in aging [117] and changes of DNA methylation can be linked to disease pathogenesis of age-associated bone diseases. In addition to DNMTs, the Ten-Eleven Translocation (TET) proteins are recently discovered 5-methylcytosine hydroxylases that convert 5-methylcytosine (5mc) to (5hmC). It has been suggested that 5-hydroxymethylcytosine (5hmC) plays a role as an early intermediate in active DNA demethylation [121]. Although there is no direct evidence of association of TET proteins with bone cells, the increase in AP activity and matrix mineralization in Ad-MSCs has been shown to be associated with an increased presence of 5hmC as well as with an increased TET2/3 gene expression [122]. DNA methylation changes during osteoclast differentiation are also highly associated with TET2-mediated demethylation and DNMT3b-mediated methylation [123]. Although DNA methylation is important for the differentiation of bone cells, the role of DNA methylation in bone cells remains elusive and needs further investigation.

Non-coding RNAs

Only 1–2% of the human genome is translated into protein [124, 125] and the remaining transcribed RNAs are not annotated into protein-coding genes. The transcripts from non-coding regions of genomes are called non-coding RNAs (ncRNAs)[126, 127]. Non-coding RNAs have essential roles in regulating gene expression as well as other ncRNAs both in cis

and in trans. It has been shown that non-coding RNAs affect various biological processes during development and in some pathological conditions. Importantly, ncRNAs are expressed in a stage specific and cell specific manner and provide another view of transcriptional circuit. The major classes of ncRNAs are micro RNAs (miRNAs) and long-non-coding RNAs (lncRNAs). lncRNAs are non-coding transcripts length over 200 nucleotides long [127] and are involved in many biological functions [128]. More than 28,000 distinct lncRNA transcripts are currently identified and lncRNAs regulate gene expression by various mechanisms [129]. Large intergenic non-coding RNAs (lincRNAs) are the largest class of lncRNAs and other classes of lncRNAs are intronic lncRNA, sense lncRNA, and antisense lncRNA. Tong et al recently reported that lncRNA-DANCR expression is unregulated in circulating monocytes in patients with low-BMD and DANCR promotes the expression of inflammatory cytokines such as IL-6 and TNF [130]. lncRNA-DANCR directly interacts with EZH2 and suppresses RUNX2 expression and osteoblast differentiation [131]. Although lncRNAs have been shown to engage in various biological processes, the identity of lncRNAs in skeletal biology is not well known [132]. However, many studies about the function and regulation of miRNAs in skeletal biology have been reported (reviewed in [133, 134]). Thus, this review briefly discusses the recent prospective view of miRNAs in bone diseases.

MicroRNAs (miRNAs) are a class of short non-coding RNAs that mediate post-transcriptional silencing and are important for cell differentiation, embryonic organ development, and apoptosis [135–138]. miRNAs control the expression of more than 30% of genes and have profound effects on physiological and pathological conditions. miRNAs are transcribed as primary miRNAs (pri-miRNAs) containing a secondary loop structure, a 5-cap structure and a poly(A) tail [139]. Pri-mRNAs are processed to pre-miRNA by Drosha (nuclear RNase III) and cofactor, DGCR8. Dicer (cytosolic RNase III) sequentially processes pre-miRNAs to a mature form. miRNAs are regulated during bone development [140] and Dicer inactivation, which results in defects on miRNAs cleavage, regulates bone mass, suggesting that Dicer-generated miRNAs play an important role in osteoblastogenesis and osteoclastogenesis [141–144]. Whereas DICER deficiency inhibits both *in vitro* and *in vivo* osteoclastogenesis, CD11b-specific DICER null mice exhibit mild osteopetrosis [144]. Mizoguchi et al has shown that osteoclast-specific DICER deletion by cathepsin K cre also increases bone mass and, surprisingly, decreased osteoblastogenesis in addition to inhibition of osteoclastogenesis [143]. These studies point to the potential functions of miRNAs from osteoclasts on osteoblast differentiation. Indeed, miRNAs secreted from osteoclasts are delivered into osteoblasts and regulate osteoblast differentiation. miRNAs are regulated by important signaling pathways such as WNT, RANKL, and BMP in osteoclasts and osteoblasts and inversely control and target multiple signaling pathways in osteoclasts and osteoblasts. In addition to the *in vitro* studies that identified the regulation and function of miRNAs in osteoblast and osteoclast differentiation [145–148], several studies have examined the expression of miRNAs during bone development *in vivo* and skeletal diseases. Extracellular miRNAs have recently been found in a variety of body fluids including synovial fluids, plasma, serum, saliva, and urine. Emerging evidence shows that miRNAs can be used a diagnostic tool to detect skeletal diseases such as osteoporosis. Osteoporosis is characterized by low bone mass that results from imbalanced bone remodeling: high

osteoclast activity and low osteoblast activity [149]. Bone fractures associated with osteoporosis can be predicted by measuring bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA) and clinical history [150, 151]. However, current methods have a low detection rate and thus improved fracture risk assessment methods are required. miRNAs can be extruded into extracellular space, supporting the idea that miRNAs may function as biomarkers. Several miRNAs are detected in the blood of osteoporosis patients, although the function of individual miRNAs is not well characterized in the pathogenesis of osteoporosis. A recent study shows that nine cell free miRNAs in the serum are significantly upregulated in patients with osteoporotic fractures, opening up a possibility of using miRNAs as a biomarker for diagnosing osteoporotic fracture [152]. In addition, abnormal miRNA expressions are often found in several diseases such as cancer, leading to the first miRNA mimics reaching the clinical phase I trial in patients with primary liver cancer in 2013 [153]. Since one miRNA can interact with many genes simultaneously, miRNA replacement therapy using synthetic miRNA mimics would be an option to treat diseases, if it can avoid off-target effects.

Conclusions

The crucial role of bone cells in bone homeostasis and in skeletal diseases makes them potential therapeutic targets. For example, understanding the osteogenic potential of MSCs can be used to treat bone defect repair and bone diseases[154]. Suppressing osteoclast activity or differentiation allows one to block pathological bone resorption. Epigenetics regulations were implicated in skeletal diseases by a few studies [120, 152], suggesting that epigenetic changes can be predisposed or used as a signature of skeletal diseases. Epigenetic regulation affects differentiation and activity of bone cells and the response of bone cells to exogenous stimulus. Understanding bone cells-specific epigenetic regulation using genome-wide sequencing technology will provide the foundation for cell-specific therapeutic approaches. Recent drug development targeting epigenetic mechanisms shows a promising prospect for the treatment of cancers and inflammatory conditions. The development of epigenetic drugs or therapies that changes epigenetic states will contribute to such treatments. In this review, we provide a brief overview of epigenetic regulations in bone cells but the understanding of chromatin landscapes in bone cell is still incomplete.

Over the past years, the study of histone post-translational modifications (PTMs) has made progress to show the association of epigenetic modifications with fundamental biological processes and pathological conditions. During the differentiation of bone cells, histone PTMs are dynamically regulated. Recent advances in developing small molecules to modulate pharmacologically epigenetic enzymes and interfere with these biochemical mechanisms offer great promise for therapy of bone diseases. In this article, we provide an overview of modulating histone PTMs by small molecule inhibitors including HDAC inhibitor and BET inhibitor which provides elucidation of the basic epigenetic regulation of bone cells and devising future epigenetic therapy for bone diseases.

Recent progress in genome-wide technologies using small cell numbers have allowed researchers to capture transcriptional and epigenetic snapshots of cells from small or rare biological samples. These new methods allow identifying single-cell transcriptional and

epigenomic variability across several cell types and suggests that controlling single-cell variance is a fundamental characteristic of different biological states [155]. Heterogeneity within cellular populations has been obvious since the first microscopic observation of individual cells. Dissecting single-cell based epigenetic heterogeneity and linked cis- and trans-effectors to variability in chromatin accessibility provides new opportunities for studying multiple different layers of regulation and advances our understanding of physiological development and the changes in pathological conditions and aging. Much effort has been made to identify osteoclast-specific or osteoblast-specific precursors from stem cells. Specific populations have been sorted using flow cytometry techniques. However, single cell ChIP sequencing or RNA sequencing would provide an advantage over isolating cells by flow cytometry and would allow investigation of a larger number of genes or epigenetic regulation in a single cell which has the potential for differentiation into bone cells. Thus, single cell sequencing techniques will allow for identifying bone-cell specific epigenetic mechanisms and for detailed new information about cellular differentiation or disease in different biological contexts.

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Figure 1. The process of bone remodeling and epigenetic factors

Bone is maintained by bone remodeling which is a continuous cycle of bone resorption and bone formation. Bone remodeling is a coupled and balanced process and takes place in basic multicellular units. In a resting stage, the non-remodeling bone surface is covered by bone lining cells. Osteocytes sense bone damages and recruit osteoclast precursor cells (OCPs), which further differentiate into mature osteoclasts. Osteoclasts resorb a damaged or old bone matrix and subsequently osteoblasts are recruited into resorbed sites and form new bone. Osteoclasts are differentiated from hematopoietic stem cells (HSCs). M-CSF and RANKL are key drivers of osteoclastogenesis. M-CSF enables HSCs to differentiate into OCPs which express RANK, a receptor for RANKL. RANKL further promotes OCPs into mature osteoclasts. Mesenchymal stem cells (MSCs) differentiate into osteoblasts and classical coupling factors, which are released from resorbed bone matrix or produced by osteoclasts, promote osteogenic differentiation through the osteoblast lineage. Key transcription factors (orange boxes), epigenetic enzymes that participate in DNA methylation (blue boxes), and histone deacetylases (yellow boxes) are indicated and described in the text.

A

B

Figure 2.

A. The core proteins of nucleosomes are two copies of H2A, H2B, H3, and H4 and 146 base pairs of DNA wraps around these core histone octamer. Core histones are highly conserved and have amino-terminal tails which are subject to various post-translational modifications. Histone modifications, such as methylation and acetylation, play an important role in gene expression and active promoter regions are distinguished by specific histone modifications including H3K4me3 and H3K27Ac. Nucleosomes are depleted in highly active regions, called “open chromatin”. Transcription factors and lineage determining factors such as NFAT and RUNX2 bind to a specific binding motif in promoters or enhancers. Ac, acetylation; Me, methylation.

B. Histone modifications of functional elements including promoters, enhancers, and insulators. Insulator is a genetic boundary element that blocks the interaction between enhancers and promoters and is defined by CCCTC-binding factor (CTCF) binding although CTCF has dual effects on enhancers; either blocking or activating. Active promoters are enriched for H3K4me3 and H3/H4 acetylation. H3K9 me_{2/3} and H3K27me3 are associated with repressed promoter regions. Active enhancers are marked by H3Kme1 and H3K27Ac.