“Do Epigenetic Marks Govern Bone Mass and Homeostasis?”

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Abstract

Bone is a specialized connective tissue with a calcified extracellular matrix in which cells are embedded. Besides providing the internal support of the body and protection for vital organs, bone also has several important metabolic functions, especially in mineral homeostasis. Far from being a passive tissue, it is continuously being resorbed and formed again throughout life, by a process known as bone remodeling. Bone development and remodeling are influenced by many factors, some of which may be modifiable in the early steps of life. Several studies have shown that environmental factors in uterus and in infancy may modify the skeletal growth pattern, influencing the risk of bone disease in later life. On the other hand, bone remodeling is a highly orchestrated multicellular process that requires the sequential and balanced events of osteoclast-mediated bone resorption and osteoblast-mediated bone formation. These processes are accompanied by specific gene expression patterns which are responsible for the differentiation of the mesenchymal and hematopoietic precursors of osteoblasts and osteoclasts, respectively, and the activity of differentiated bone cells. This review summarizes the current understanding of how epigenetic mechanisms influence these processes and their possible role in common skeletal diseases.

Key words: DNA methylation, histones, miRNA, osteoblasts, osteoclasts, gene expression

Introduction

The social and public health measures implemented in the last century in developed countries have decreased the societal impact of communicable and other acute diseases to a great extent. Therefore, chronic disorders represent nowadays a major component of disease burden. Many of them belong to the group of the so-called "complex disorders", which includes many prevalent disorders that are the final result of complex interactions of environmental and other acquired factors with genetic factors. Osteoporosis and osteoarthritis are the most prevalent skeletal disorders. In particular, osteoporosis has been estimated to affect about 30% of women and 12% of men above 50 years of age [1]. Osteoporosis is characterized by low bone mass and microarchitectural changes of bone tissue that result in a decreased bone resistance and susceptibility to fracture. Fractures in osteoporotic patients thus occur after low-impact trauma in the peripheral skeleton, particularly at the hip, the wrist and the humerus. Vertebral fractures are also very common and may appear even without any trauma, just as a consequence of the daily activities.

Osteoporosis is considered as a complex disorder. On the one hand, several acquired factors have been shown to be associated with decreased bone mass and/or increased risk of fractures, including low body weight, heavy alcohol and tobacco consumption, immobilization, corticoid use, etc. Nutritional factors are also important. Although some discrepant studies have been published, most researchers and clinicians agree that protein-energy malnutrition, low calcium intake and an inadequate supply of vitamin D increase the risk of osteoporosis. On the other hand, many epidemiological studies have provided strong evidence for a role of heredity in osteoporosis. In fact genetic factors have been estimated to account for 40-80% of bone mineral density (BMD) variance [2-4]. Although fractures depend not only on the intrinsic properties of bone, but also on other personal and environmental factors, including the propensity to fall, a hereditary component of fractures has also been demonstrated. Thus, in the Study of Osteoporosis Fractures, a maternal history of hip fracture doubled the risk of fracture and the increase in risk remained significant after adjustment for bone density [5]. In a meta-analysis of several cohorts, Kanis et al. estimated that a family history of hip fracture in parents increased the risk of hip fracture (relative risk 2.3) and all osteoporotic fractures (relative risk 1.5) [6].

BMD changes trough the life time of an individual. It accumulates during the growth period, reaches a peak by the third decade of life and then remains stable for some time. In later years, BMD begins to decrease progressively with aging. Therefore, osteoporosis may result from an inadequate peak bone mass attained in the early adulthood, from an accelerated loss of bone thereafter, or from a combination of both. The relative importance of peak bone mass and later losses on the development of osteoporosis probably varies among individuals, but the former is likely to be the most important. In fact, a 10% increase in peak BMD is predicted to delay the development of osteoporosis by 13 years, while a 10% change in the age at menopause or the rate of post-menopausal bone loss is predicted to delay osteoporosis by approximately 2 years, suggesting that peak BMD may be the single most important factor in the development of osteoporosis [7].

Theoretically, it could be anticipated that genetic factors have a more important influence on BMD in young individuals than in the elderly, as the relative contribution of environmental factors is likely stronger in the latter. Some studies support this notion. For example, Brown et al. studied 570 women from large Amish families and estimated that genes explained 58-88% of total variation in BMD in premenopausal women and 37-54% in the postmenopausal ones [8]. Likewise, in a twin study in Sweden, Michaelsson [9] estimated a greater heritability for hip fractures before the age of 69 years (0.68; 95% CI, 0.41-0.78) and between 69 and 79 years (0.47; 95% CI, 0.04-0.62) than for hip fractures after 79 years of age (0.03; 95% CI, 0.00-0.26).

From the studies mentioned above, and many others, the importance of genetic factors in osteoporosis is out of doubt. Some rare cases of osteoporosis due to single-gene mutations have been identified. They include mutations of the lipoprotein receptor related protein 5 (LRP5), collagen, aromatase and estrogen receptor genes [10]. However, most cases appear to be polygenic in nature, with multiple genes involved, each one having only a modest influence on the phenotype. In the past 15 years many linkage and association studies have been performed trying to identify the genes actually involved. Indeed, association signals have been reported and successfully replicated at various loci, using candidate gene or, more recently, genome-wide approaches.

However, as it is the case for other complex disorders, these studies have been somewhat disappointing because the combination of all the gene variants identified explains only a very small fraction of the disease risk. This suggest that, at the cellular level, those genetic variants have only a modest impact on gene expression [11]. The reasons explaining the missing heritability are disputed, but they are likely to include complex gene-gene and gene-environmental interactions. Indeed, as already mentioned, some environmental influences are well recognized risk factors for osteoporosis. Since the genome is highly

stable, in general environmental factors influence genome activity by mechanisms that do not involve DNA sequence modifications. Some of them may represent epigenetic mechanisms, which are heritable through generations or cell divisions. Thus, the epigenome of an individual is currently seen as the result of genetic factors, environmental influences and stochastic variations.
There are still very scarce data about the actual role of epigenetic mechanisms in bone disorders. However, several emerging lines of evidence suggest that they may be important in the biology of bone cells and the pathogenesis of osteoporosis. This review will summarize the current knowledge about these subjects.

Epigenetic regulation of gene expression

The group of well-known epigenetic mechanisms include DNA methylation, chromatin (in particular, histone) modifications and miRNAs [12]. However, the group is likely to be expanded in the future. For instance, the roles for hydroxymethylcytosine residues and CpG island shores have been recently proposed [13;14]. Among epigenetic mechanisms, DNA methylation and histone modification modulate gene transcription, whereas miRNAs act at the post-transcriptional level. The most widely studied epigenetic mark is DNA methylation, whose role in tumurogenesis has been clearly established. In humans DNA methylation consists in the addition of a methyl group in cytosines that precede guanines (CpGs), process catalyzed by DNA methyl transferases, using S-adenosyl-methionine as donor of methyl groups [15]. Interestingly, there are CpG-enriched areas in many gene promoters and their surrounding regions, known as CpG islands [16]. DNA methylation at these sites is usually associated with silencing of gene transcription. The exact mechanisms by which DNA methylation marks inhibit gene transcription are not fully understood yet. However, some data suggest that it can directly impair the binding of essential transcriptional factors to their target sites.
Histone modifications, including methylation, acetylation, phosporilation, SUMOylation and ubiquitination have been also largely studied [17;18]. The complexity of the histone tail modifications depends not only on the type of chemical groups added, but also on the aminoacid residue modified and the number of groups added. For example methylation may be in three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines [17]. In general, histone modifications can be divided into those that correlate with activation of transcription (mainly acetylation and phosphorilation) and those that correlate with repression (methylation, ubiquitination and sumoylation) [19].The term “histone code” is frequently used to describe a specific set of modifications for a given task.
The first miRNA were discovered in the early 90s [20] and their number is still growing. miRNAs are non coding RNAs of about 22 nucleotides. Approximately 1500 different miRNA have been identified in humans so far, and the number will probably increase in the future years [21]. Interestingly, bioinformatic predictions indicate that miRNAs may be involved in the regulation of 60% of the coding genes [22]. These small RNAs are initially transcribed as long primary transcripts, and then undergo specific cleavage driven by the Drosha and Dicer enzymes. The mature miRNA loss one strand, whereas the other, know as the complementary miRNA strand, is loaded into the RISC protein complex, which mediates the effect on gene expression [23]. miRNAs bind to the 3´or 5´UTR of messenger RNAs and induce mRNA cleavage or translational repression, depending on the degree of complementarity [24]. When there is extensive complementarity, RISC mediates the cleavage of the target mRNA, whereas in case of loose complementarity the complex will impair the advance of the ribosome complex repressing translation.
Although each mechanism by itself is capable of affecting gene expression, they also interact with each other in a cooperative manner, allowing the cells to activate or repress gene expression in a time and tissue-specific manner. It has been shown that DNA methylation and histone posttranslational modifications contribute to the establishment and maintenance of chromatin accessibility [25]. Indeed, methyl-CpG biding proteins, which recognize methylated CpGs, recruit HDACs to methylated DNA, promoting the gain of repressive marks in the histone tails [26]. Likewise, miRNAs may also influence DNA methylation or chromatin remodeling [27]. Furthermore, it has been recently suggested that the expression of certain miRNAs may be controlled by CpG methylation and histone modification [28;29].

The complex processes of bone formation and bone remodeling

Bone tissue consists in a heavily mineralized extracellular matrix, with collagen as the major protein component, and different cell types. Far from being a static organ, bone changes in structure and

composition from birth to adulthood. Modeling and remodeling are the processes by which bone adapts to external influences. Bone modeling is responsible for the gain in skeletal mass and the changes in skeletal size and shape taking place during the growth period, whereas bone remodeling replaces old bone by new bone tissue, throughout life, and specifically in the adult skeleton, to maintain bone mass, repair bone microfractures and allow the adaptation to external physical requirements [30].
The most important cells in bone homeostasis belong to two separate families: the osteoclastic and the osteoblastic lineages. Osteoclasts derive from hematopoietic precursors and are responsible for bone resorption. On the other hand, osteoblasts derive from local mesenchymal precursors and are responsible for bone formation. There are several types of cells within the osteoblastic lineage, with different gene repertoires, shapes and functions. The bone forming osteoblasts are cuboidal cells that synthesize alkaline phosphatase, collagen and other constituents of the bone matrix. The osteocytes are stellated cells that derive from osteoblasts that become embedded in the cell matrix they have formed. Finally, the so-called lining cells are flat-shaped cells that cover the inactive bone surfaces. However, cells in the osteoblastic lineage not only form bone, but have other roles in bone metabolism. They modulate the proliferation and differentiation of osteoclast precursors, and regulate osteoclast activity [31;32].
During bone modeling, resorption and bone formation occur on separates surfaces, whereas during remodeling, formation and resorption are coupled. The process of bone remodeling begins when a group of osteoclasts resorb a small volume of bone tissue (Figure 1). When this phase finishes, osteoblasts arrive to the area and fill with new bone the cavity eroded by osteoclasts. Therefore, the processes of bone resorption and bone formation are critical determinants of bone mass and strength. In fact, the decreased bone mass that characterizes osteoporosis at the tissue level represents the consequence of an imbalance between osteoclast and osteoblast function at the cellular level [33;34].
Maintaining skeletal properties and functionality depends on the organized action of all cell types present in this tissue. In fact, there are complex interaction networks between osteoblasts, osteoclasts and osteocytes. In particular, osteocytes are emerging as critical elements in the regulation of skeletal homeostasis. They may act as mechanosensors, mark the sites where a remodeling cycle must be initiated and secrete a number of factors that influence the activity of other cells in the osteoblastic as well as the osteoclastic lineages [35]. This interplay between cells promotes changes of the expression level of target genes, resulting in variations of cell activity. In addition, since bone remodeling takes place in a time and site-coordinated way, the differentiation of osteoblast and osteoclast precursors must also be controlled in a time and site-specific manner. The differentiation programs of these cells promote marked changes in gene expression, translated into different morphologies and activities, thus allowing the mature cells to achieve its expected function. Therefore, mechanisms regulating the transcriptional activity of those genes play a critical role in bone homeostasis and bone disease.

Epigenetic marks in the osteoblastic lineage differentiation

Osteoblasts and osteocytes originate from mesenchymal stem cells (MSCs). Interestingly, not only bone cells derive from these precursors, but also adipocytes and myogenic cells, as well as chondrocytes, share the same progenitor [36]. This highlights the need for mechanisms regulating lineage-specific differentiation of MSCs and then maintaining the mature phenotypes. Regarding the bone tissue, MSCs differentiate into osteoblasts and these will eventually evolve into osteocytes and lining cells, through a complex process that involves transcription factors and also modifications of the epigenetic marks (Figure 2) [37;38]. During osteogenic differentiation, MSCs undergo a dramatic transformation, at gene expression, functional and morphological levels [39]. Thus, cell shape changes from the polygonal bone forming osteoblasts to the dendrite-rich stellate osteocytes The podoplanin gene appears to be involved in this process, and its expression may be regulated by a cooperative crosstalk between DNA methylation and histone modification in osteoblastic cells [40]. On the other hand, the change in cell shape between bone forming osteoblasts and osteocytes is accompanied by different gene expression profiles. It is known that alkaline phosphatase activity, an enzyme critical for bone mineralization, is high in osteoblasts, the unique bone forming cell, whereas it is reduced in osteocytes, which do not produce bone [41]. In line with this, we recently demonstrated that osteoblasts and osteocytes have opposite DNA methylation profiles in the alkaline phosphatase (ALPL) promoter, which is hypomethylated in osteoblasts and hypermethylated in osteocytes, suggesting that DNA methylation is inhibiting ALPL expression in the latter [42]. The opposite is the case of SOST, which is actively expressed in osteocytes, but not in osteoblasts [39]. SOST is the gene encoding sclerostin, a peptide that tends to impair osteoblast activity by inhibiting Wnt signaling [43]. We have recently demonstrated that DNA methylation may be responsible for the repression of SOST expression in osteoblasts. Furthermore, we observed that DNA demethylation occur during osteoblast-osteocyte transition, allowing osteocytes to express SOST [44]. In

addition, Cohen-Kfir et al. suggested that sirtuin 1, a histone deacetylase, directly regulates SOST expression [45].
It has been shown that DNA demethylation induced by chemical compounds facilitates osteogenic gene expression and differentiation [46]. Likewise, it has been reported that reduced DNA methylation of other CpG islands in the promoter regions of osteocalcin (BGLAP) and osteopontin genes is associated with osteogenic differentiation [47;48]. On the other hand, high DNA methylation at the promoter of Brachyury transcription factor may be required for this process [49]. Other genes influencing osteogenesis, such as osterix, the osteogenic protein Dlx-5, aromatase and the estrogen receptor are also regulated by DNA methylation [50-53]. Not only DNA methylation is critical for osteogenic differentiation, but also chromatin remodeling plays an important role. It has been shown that different transcription factors induce chromatin remodeling at target promoters [54]. In fact, histone modifications are associated with BGLAP expression [55]. H3K4 and H3K6 methylation is associated with HOXa-10 and AP-2α expression respectively, and determine the advance of the osteogenic differentiation [56;57]. Other important genes regulated by histone modifications are Runx2, AP-1, ATF4 and Smads [58].
The analysis of miRNA arrays have identified several miRNAs whose expression changes during MSC differentiation, affecting target gene translation [59], and thus suggesting that miRNAs expression is actively involved in the regulation of this process (table 1). It has been shown that miRNAs regulate the expression of pivotal osteogenic transcription factors, such as Runx2 or Smads. Runx2 is required for determination of the osteoblast lineage. This transcription factor induces the differentiation of multipotent MSCs into immature osteoblasts modulating the expression of key genes during the early stages of osteoblast differentiation, such us collagen type 1 and 2, BGLAP, fibronectin, sclerostin or osteoprotegerin [60;61]. miR-204 and its homologue miR-211, as well as miR-133 and miR-135b, inhibit Runx2 [62-64]. On its turn, Runx2 may also regulate the expression of some miRNAs involved in the osteogenic process [65].
miRNAs are actively involved in the regulation of Smad protein levels. Smads are intracellular proteins that transmit signals originating from the interaction of bone morphogenetic proteins (BMPs) and transforming growth factors (TGFs) with their receptors at the cell membrane. It is know that BMP/TGF and Runx2 pathways converge for the transcriptional control of bone formation. Thus, Smad proteins are recruited to Runx2 regulatory complexes and collaborate to modulate gene expression [66]. miR-26a has been shown to negatively regulate Smad1, resulting in a decreased expression of various bone markers, such as ALPL, BGLAP, osteopontin, and COL2A1 [67]. miR-135 regulates other member of the Smad family, Smad5 [64]. miR-141 and miR-200a, through Homebox Distal-Less-5 (Dlx5) decreased the expression, among others, of BMP-2 [68]. Recent studies demonstrated that miR-206 inhibit connexin 43 expression and tend to impair osteoblast differentiation [69]. The miR-23a ~ 27a ~ 24-2 complex inhibits osteoblastogenesis by negative regulation of SATB2 [65]. miR-29a and miR-29c are involved in the regulation of Wnt pathway and inhibit the expression of osteonectin [70]. Finally, miR-125b inhibits Erb-2 and negatively regulates osteoblast proliferation [71].
Contrary to the negative role of various miRNAs, it has been described that some miRNAs promote osteogenesis. miR-29b, miR208 and miR-210 modulate BMP/TGF/Activin signaling [72]. miR-218 negatively regulates Erb1 (TOB1) and sclerostin (SOST) [73]. miR-196 targets Hoxc8 (a Smad1 negative regulator) impairing adipogenesis and promoting osteogenesis [74]. Finally, miR-335-5p attenuates Dkk1, an inhibitor of the Wnt pathway, and consequently increase Wnt pathway activity and tends to facilitate osteoblast formation [75]. The exact role of these mechanisms in the pathogenesis of bone disorders remains to be elucidated. Nevertheless, whether they are involved in skeletal disorders or not, those studies suggest that the modulation of epigenetic mechanisms may be used to improve bone tissue engineering and in general in bone regenerative medicine, specifically when there is a need to form new bone to heal a local skeletal defect.

Epigenetic changes during osteoclastogenesis: control of remodeling

Activation of osteoclastogenesis is required for remodeling. Although not definitively proved in humans, some experimental data suggest that osteocytes start the cascade of events for osteoclast differentiation, presumably releasing cytokines and other factors that modulate the activity and differentiation of cells in both the osteoblastic and osteoclastic lineages [35;39]. On the other hand, there is extensive evidence for a role of cells of the osteoblastic lineage in the regulation of osteoclastogenesis. In fact, different molecules produced by osteoblastic cells, including stromal derived factor (SDF), the monocyte chemotactic protein type 1 (MCP-1) or the macrophage colony-stimulating factor (M-CSF), have been shown to either attract osteoclast precursors to the sites of bone remodeling or stimulate their proliferation [76-78]. Other critical factor in osteoclastogenesis is the Receptor activator of nuclear factor Kappa-B ligand (RANKL) which initiates the cascade of events for osteoclast maturation. RANKL is produced by

many cell types, such as immune, vascular and stromal cells. However, it is well accepted that osteoblasts and probably osteocytes, are the major sources of this cytokine in bone tissue. Interestingly, Kitazawa et al. demonstrated that DNA methylation at the proximal promoter of the RANKL gene inhibits its expression in a murine system, which results in impaired osteoclastogenesis [79]. Our group has recently reported a similar regulation of RANKL expression by the methylation/demethylation of a CpG island located in the vicinity of the RANKL promoter in human cells [80].

RANKL acts by binding to its receptor RANK located in the membrane of osteoclast precursors [81;82]. This induces a cascade of molecular events that leads to the activation and nuclear translocation of the nuclear factor of activated T cells (NFATc1) [83] which, in turn, induces the expression of a variety of target genes, thus promoting osteoclast differentiation. Recent reports suggest that NFATc1 activity may be controlled by Jumonji, a histone demethylase, as well as by miR-146a [84;85].

It is important to note that osteoblasts also synthesize and secrete osteoprotegerin (OPG), a soluble decoy receptor of RANKL that inhibits its interaction with RANK [86;87]. Together with RANKL and RANK, these three factors constitute the RANKL-RANK-OPG system. The RANKL/OPG balance at the bone microenvironment is considered a major determinant of bone mass. Indeed, RANKL and RANK knockout mice show a marked increase in bone mass, mainly due to a decrease in osteoclast numbers, whereas OPG knockout mice show the opposite phenotype [88-90]. Therefore a precise control of the expression of these genes is required for normal bone homeostasis, as well as for bone adaptation to environmental factors. Not only RANKL and NFATc1, but also other genes related to this signaling system are epigenetically regulated (Figure 3). Indeed, it has been shown that DNA methylation and histone modifications cooperate to regulate OPG expression in nasopharyngeal carcinoma tumors [91]. Data from our laboratory suggest that the methylation of CpG-rich regions in the OPG gene may also regulate OPG levels in human osteoblastic cells and non-neoplastic bone tissue [80]. Furthermore, results from in vitro experiments using histone deacetylase inhibitors suggest that osteoclast activity is modulated by these enzymes [92].

Besides DNA methylation and histone post-translational modifications, growing evidence supports the notion that osteoclastogenesis may be regulated by miRNAs, acting in both a positive and negative ways (Figure 3C). In support of this notion, it has been shown that specific ablation of the Dicer enzyme in osteoclastic cells suppresses bone resorption [93]. Additionally, over-expression of miR-223 inhibits osteoclastogenesis [94], whereas, on the contrary, two miRNAs, miR-21 and miR-155, have been reported to exert a permissive role on osteoclast differentiation, decreasing the levels of some inhibitory genes [95;96]. As an obvious consequence of those studies, it is tempting to speculate that the inhibition of miRNA facilitating osteoclastogenesis might represent an attractive new approach to treat disorders characterized by an accelerated bone resorption. Interestingly, it has been suggested that not only osteoblasts regulate osteoclast activity, but osteoclasts may also, in turn, influence osteoblast differentiation. Several mechanisms may be involved, including changes in the expression levels of some miRNAs [97].

The role of epigenetic marks in Osteoporosis

The role of epigenetics in osteoporosis is just starting to be studied. However, it is gradually being postulated as a key concept, because epigenetic mechanisms are involved in the interactions between the genome and the environment, and, on the other hand, they drive the differentiation programs for cell fate, which, as already mentioned, play a critical role in remodeling of bone tissue. Although the exact relationship between epigenotypes and disease phenotypes is still to be elucidated, it is known that epigenetic marks change during aging, including a global decrease in the abundance of 5-methylcytosines and some histone modifications [98]. Since osteoporosis is an age-related disease, it could be speculated that those age-related changes in epigenetic marks participate in the pathophysiology of the disease. However, at the present time this remains merely speculative.

There is evidence that the environment has a strong influence on bone mass, even during the gestation period. Indeed, the intra-uterus environment has been shown to affect the development of the fetal skeleton with effects not only evident at birth, but also persisting later in life (recently reviewed by Holroyd et al.) [99]. For instance, the velocity of fetal femur length growth has been associated with the skeletal size at 4 years of age [100]. Maternal factors that have been associated with neonatal bone mass include body weight, fat stores, physical activity and smoking. Likewise, vitamin D and calcium availability during pregnancy have been shown to influence both fetal skeletal development and childhood bone mass [101]. Furthermore, poor growth in uterus and during the first years of life seems to be associated with thinner bones and the risk of fractures in the adulthood [102].

Overall, these and other data strongly suggest that, besides genetic factors, environmental influences during the early phases of development and growth influence the peak bone mass, and consequently the

risk of osteoporosis. The mechanisms involved remain to be elucidated, but some data indeed suggest that they may include epigenetic changes. Most data come from animal studies. For instance, feeding pregnant rats with a low protein diet resulted in a reduced bone mass in the offspring that persisted up to 75 weeks of age and was accompanied by an impaired proliferation and differentiation of bone marrow stromal cells, a population including osteoblast precursors [103;104]. It has been shown that maternal dietary restriction in rats induces changes in the methylation status of the genes coding for the glucocorticoid receptor and the peroxisomal proliferator-activated receptor alpha (PPARα) which persist after weaning and are transmitted to future generations [105-107]. There is also indirect evidence for an influence of early life conditions on the gene methylation status in humans. Thus, Dutch subjects exposed prenatally to famine in 1944 have been reported to have an increased risk of a variety of metabolic and neurological disorders, along an abnormal methylation status of various gene promoters, including IGF2 and other genes related to tissue growth and metabolism [108;109]. However, a direct proof of a link between in uterus environment, DNA methylation and bone mass in humans is not available yet.

Besides DNA methylation, the roles of miRNAs and histone modifications in osteoporosis are being actively studied. In a recent report, three DNA polymorphisms have been found to alter the binding affinity of specific miRNAs that regulate the levels of the FGF2 gene and contribute to determine the susceptibility to osteoporosis [110]. A study by Li et al. represents an interesting example of interaction between two epigenetic mechanisms, miRNA and histone code. They described a homozygous mutation in pre-miR-2861 that impaired the formation of the mature miRNA and caused a decrease of bone mass, mainly by targeting HDAC5 [111]. Eskildsen aet al. suggested that miR-138 could be involved in the reduced levels of the focal adhesion kinase gene observed in osteoporotic and osteoarthritic patients [112].

On the other hand, a decreased expression of heparanase (HPSE) has been observed in osteoblasts of osteoporotic patients, in comparison with those isolated from healthy individuals. HPSE is presumably involved in histone phosphorylation [113].

Some drugs used or postulated as treatments for osteoporotic patients have been suggested to induce epigenetic changes. This is the case of the parathyroid hormone, which modulates histone deacetylase activity in osteoblasts [114]. Although not approved as an anti-osteoporotic drug, resveratrol, a polyphenolic phytoestrogen, has been shown to exhibit potent bone anti-catabolic properties, and presumably acts influencing the activity of sirtuin 1, an histone deacetylase [115]. Bisphosphonates are the most widely drugs used to treat osteoporotic patients. They are potent inhibitors of bone resorption by targeting the mevalonate pathway in the osteoclasts. Moreover, some data suggest that they may also have effects on bone formation. In line with this, it has been reported that bisphosphonates modulate the expression of some miRNAs, including miR-18a, miR-133a, miR-141 and miR-19a, that influence osteoblast proliferation [116]. Further studies are needed to elucidate whether it is central to bisphosphonate action or just an epiphenomenon associated with their osteoclast inhibitory effects.

Perspectives

As reviewed above, both epidemiological and experimental data suggest that epigenetic mechanisms influence skeletal development and the risk of bone disorders. Nevertheless, further research is required to elucidate the exact role of these mechanisms in the phenotypic changes responsible for skeletal diseases. In particular, the specific changes in DNA methylation, histone modifications and miRNA expression involved in the pathogenesis of osteoporosis remain to be identified. Although animal models provide a useful resource for the investigation of these mechanisms, so far it is unclear to what extent the results can be extrapolated to humans. Therefore, studies in human systems are of special interest.

Genome-wide studies have identified a number of genes associated with bone mass. However, they only explain a small fraction of the bone mass variation observed in the population [117]. The explanations for this unexpected result are unclear, but may include the interaction between genetic and epigenetic mechanisms. In fact, epigenetic changes regulate gene transcription, but there is also growing evidence for an influence of genetic (i.e., DNA sequence) variants on DNA methylation and miRNA-dependent mechanisms (“genetical epigenomics”) [118].

New technologies such as epigenetic microarrays and ultra-high throughput sequencing may help to establish a complete epigenetic landscape of normal bone and skeletal diseases on a genome-wide basis. For instance, the comparison of cytosine methylation in subjects with different bone phenotypes may lead to the identification of differentially methylated regions involved in disease pathogenesis. It is worth mentioning that those studies present more difficulties than genome studies, due to the cell/tissue specificity of epigenetic marks and their instability over time. Since bone is a very heterogeneous tissue, composed of a variety of cells in bone itself and bone marrow, the results obtained in bone tissue samples may be hard to interpret. Nevertheless, the identification of disease-specific patterns of DNA methylation

or miRNA expression may give valuable information. First, it may help to identify genes and metabolic pathways involved in the disease and consequently lead to discover new targets for disease therapy. On the other hand, the identification of disease-specific epigenetic marks may be helpful for both diagnosis and prevention. DNA, either methylated or unmethylated, and miRNA are more stable than RNA [119], which may be an advantage for clinical use. Of course, to this respect, markers in accessible samples, especially those present in circulating blood and body fluids, will be much more feasible to be used as diagnostic tools [120].

Studies of bone epigenetics may not only point to certain genes as new targets for therapy, but may represent the foundation of new therapies based on the control of epigenetic mechanisms. Some demethylating agents are already used to treat neoplastic disorders [121;122] . It would be interesting to study their effects on "bone genes". However, their potential utility is limited by their widespread effects. Agents able to modulate specifically the epigenetic control of genes in a given pathway would be much more useful. In line with this, therapies based on small RNAs, mimicking or interfering with miRNAs, might be more specific and promising [123;124]. Both DNA-methylation- and miRNA-based treatments may be also useful in regenerative medicine. For instance, one could envision a future in which the demethylation of the promoters of certain genes driving osteoblast proliferation in a limited region of the skeleton might be used to fill bone defects and enhance the consolidation of delayed-union fractures.

Another priority research line in this field may be the study of the potential influence of current therapies on the epigenetic regulation of bone factors. Of particular interest, the effect of anabolic agents, such as parathyroid hormone and bone morphogenetic proteins on the methylation of CpG-rich regions of genes involved in the regulation of bone remodeling should be investigated. If these drugs indeed modify DNA methylation patterns, prior knowledge of the patient methylation profile might help to individualize therapy according to his epigenome ("pharmacoepigenomics").

Overall, although data are still scarce, studies highlighted in this review invite to think that epigenetics will be an important subject in the bone research field in the next years.

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Figure 1

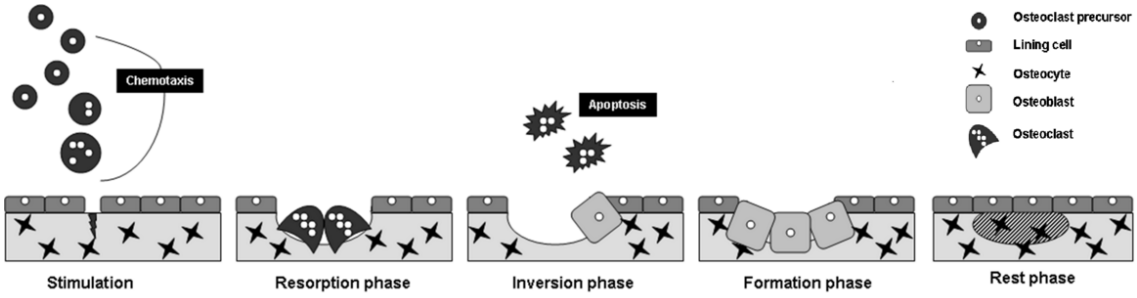
Chemotaxis
Apoptosis
Osteoclast precursor
Lining cell
Osteocyte
Osteoblast
Osteoclast
Stimulation
Resorption phase
Inversion phase
Formation phase
Rest phase

Figure 2

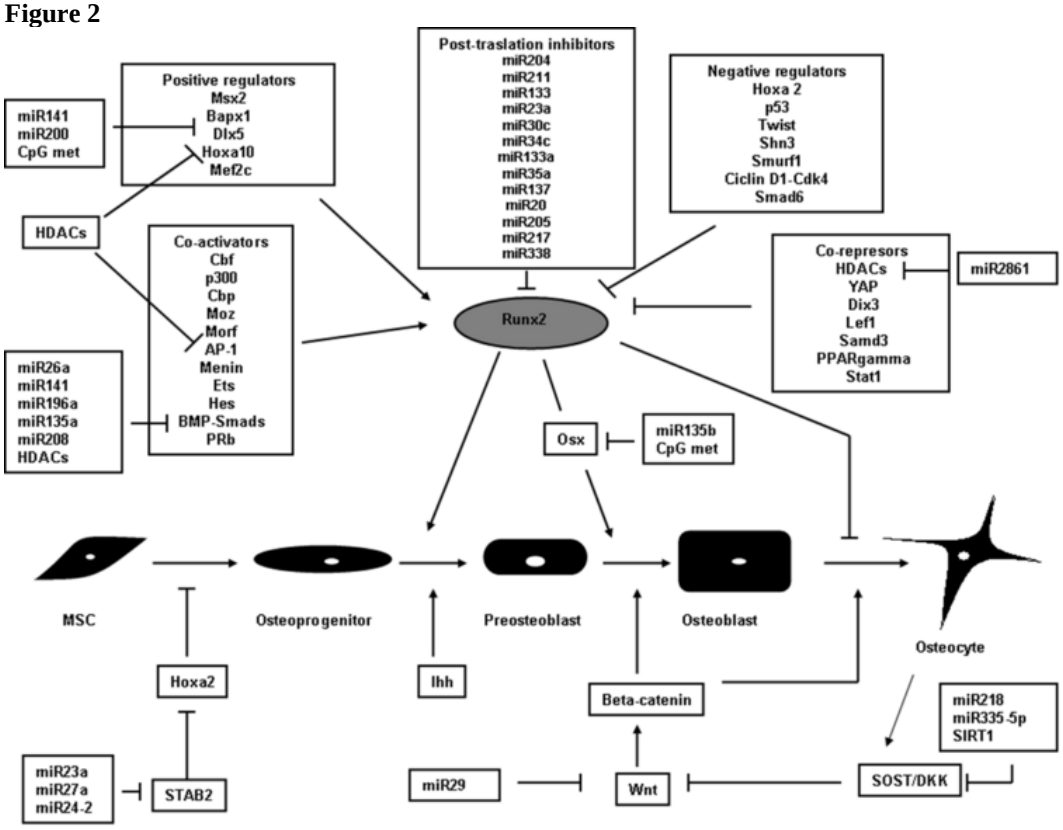
Post-traslation inhibitors
miR204
miR211
miR133
miR23a
miR30c
miR34c
miR133a
miR35a
miR137
miR20
miR205
miR217
miR338
Positive regulators
Msx2
Bapx1
Dlx5
Hoxa10
Mef2c
miR141
miR200
CpG met
HDACs
Negative regulators
Hoxa 2
p53
Twist
Shn3
Smurf1
Ciclin D1-Cdk4
Smad6
Co-activators
Cbf
p300
Cbp
Moz
Morf
AP-1
Menin
Ets
Hes
BMP-Smads
PRb
miR26a
miR141
miR196a
miR135a
miR208
HDACs
Runx2
Co-represors
HDACs
YAP
Dix3
Lef1
Samd3
PPARgamma
Stat1
miR2861
Osx
miR135b
CpG met
MSC
Osteoprogenitor
Preosteoblast
Osteoblast
Osteocyte
Hoxa2
Ihh
Beta-catenin
miR218
miR335-5p
SIRT1
miR23a
miR27a
miR24-2
STAB2
miR29
Wnt
SOST/DKK

Figure 3

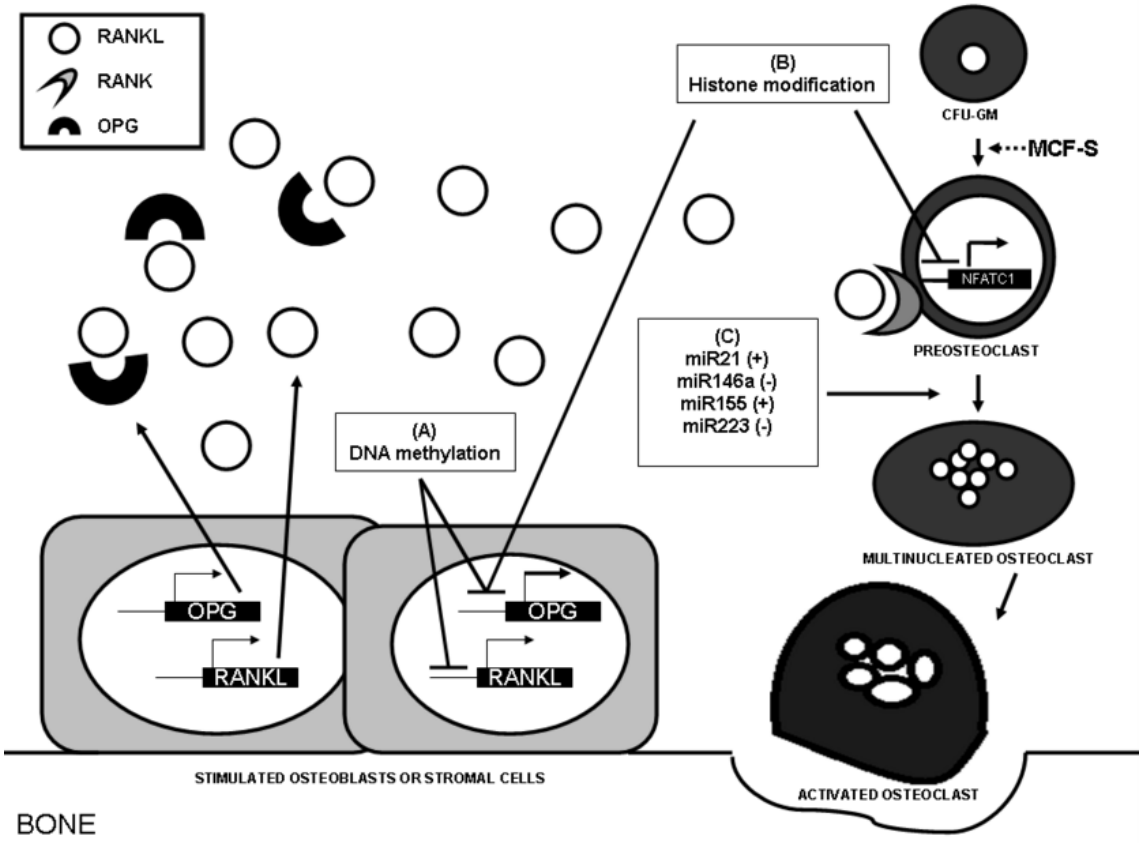
RANKL
RANK
OPG
(B)
Histone modification
CFU-GM
MCF-S
NFATC1
PREOSTEOCLAST
(C)
miR21 (+)
miR146a (-)
miR155 (+)
miR223 (-)
(A)
DNA methylation
MULTINUCLEATED OSTEOCLAST
OPG
RANKL
OPG
RANKL
STIMULATED OSTEOBLASTS OR STROMAL CELLS
ACTIVATED OSTEOCLAST
BONE

Figure legends

Figure 1.The bone remodeling cycle. The start of bone remodeling is probably driven by osteocytes (Stimulation). Osteoclasts are recruited to the bone surface by chemoatraction and remove a discrete packet of bone (Resorption). After a brief reversal phase in which osteoclast stop to resorbe bone and undergo apoptosis, osteoblasts arrive to the region and secrete bone matrix, filling the cavity (Formation). Note that some osteoblasts are buried within the new matrix, becoming osteocytes. The resorption phase last only a few weeks, whereas bone formation takes several months. Once the cavity is completely restored, bone enters the resting phase.

Figure 2. The osteoblastic lineage differentiation. A complex network drives the osteogenic differentiation of the mesenchymal stem cells (MSCs). Transcription factor Runx2 controls the process inducing the expression of key genes. Its expression is tightly regulated by a wide variety of mechanisms, including positive and negative modulators, co- activators and co-repressors, as well as epigenetic marks. Likewise, some of these mechanisms are also epigenetically regulated.

Figure 3. Mechanisms involved in the epigenetic regulation of osteoclastogenesis. A) DNA methylation at CpG-rich areas within the RANKL and OPG promoters blocks gene transcription, impairing osteoclastogenesis. B) Histone post-translational modifications have been shown to directly modulate NFATc1 activity and OPG transcription. C) miRNAs have both positive and negative effects on the progression of osteoclast precursor differentiation. miR-21 and miR-155 have a positive effect by decreasing the levels of inhibitory genes, whereas miR-146a and miR-223 tend to impair osteoclast differentiation.

Table 1. Some studies identifying miRNAs involved in the regulation of osteoblast differentiation

miR # ID	Observation	Ref.
miR-125b	Inhibits proliferation and impairs osteoblast differentiation	[71]
miR-133/135-a	Target Runx2 and Smad 5, impairing osteoblast differentiation	[64]
miR-135b	Targets sialoprotein, osterix, osteocalcin and Runx2	[63]
miR-141/200a	Target Dlx5, impairing osteoblast differentiation	[68]
miR-196a	Target Hoxc8, enhancing osteogenic differentiation	[74]
miR-204/211	Target Runx2 impairing osteoblast differentiation	[62]
miR-206	Targets connexin 43, impairs osteoblast differentiation	[69]
miR-208	Indirectly upregulates BMPs	[125]
miR-210	Targets AcvR1b, inducing osteogenesis.	[126]
miR-218	Decreases SOST and TOB1 expression	[73]
miR-23a/27a/24-2	Regulated by Runx2; target SATB2 and Runx2.	[65]
miR-26a	Targets Smad1, impairing osteoblast differentiation	[67]
miR-2861	Targets HDAC5, inducing osteogenic differentiation	[111]
miR-29/29c	Modulate Wnt pathway; target osteonectin	[70]
miR-29b	Promotes osteogenic differentiation	[72]
miR-30c/34c/133a/135a/137/204/205/217/338	Target Runx2 and impair osteoblast differentiation	[127]
miR-31/106a/148a/424/30c/15b	Differentially expressed during MSC osteogenic differentiation	[59]
miR-335-5p	Negative regulation of DKK1, increasing Wnt signaling	[75]