Review

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Epigenetic mechanisms in bone

Abstract: Epigenetics refers to the study of mechanisms able to influence gene expression in a stable and potentially heritable manner without altering the DNA sequence. These mechanisms include posttranslational histone modifications, miRNA-mediated post-transcriptional regulation and DNA methylation. The accumulation of molecular errors over time resulting, at least partly, in the alteration of normal epigenetic patterns is being widely associated with aging. Epigenetic processes are also considered important mechanisms through which environmental and stochastic stressors promote numerous pathologies in humans. It is, therefore, reasonable to expect that several complex multi-factorial late-onset disorders, like osteoporosis and osteoarthritis, could have a strong epigenetic component. The focal point of all skeletal pathologies is the deregulation of bone remodeling, mediated by bone-forming osteoblasts and bone-resorbing osteoclasts. In order to keep both processes in balance, the activity, differentiation and apoptosis of both cell types have to be tightly regulated. In particular, the differentiation of osteoblasts and osteoclasts is accompanied by profound changes in gene expression. It has been shown that histone deacetylation and DNA methylation negatively regulate the expression of several genes associated with different stages of osteoblast differentiation; however, several miRNAs promote osteoblastogenesis. Furthermore, inactivating mutations in the miRNA coding regions could be associated with the pathogenesis of osteoporosis. The aim of this review is to highlight the role of epigenetic mechanisms in bone remodeling and bone homeostasis, so as to implicate their diagnostic and therapeutic potential in skeletal diseases.

Keywords: bone remodeling; DNA methylation; miRNA; osteoarthritis; osteoporosis; posttranslational histone modifications.

Abbreviations: ACVR1B/2A, activin A receptor, type IB/IIA; ALPL, alkaline phosphatase; APC, adenomatous polyposis coli; ATF4, activating transcription factor 4; BAMBI, BMP and activin membrane-bound inhibitor; BMD, bone mineral density; BMP2, bone morphogenetic protein 2; C-FOS, activation of FBJ murine osteosarcoma viral oncogene homolog; CK1, casein kinase 1; COL1A1/4A2/5A3, collagen, type I, alpha 1/type IV, alpha 2/type V, alpha 3; CRIM1, cysteine rich transmembrane BMP regulator 1 (chordin-like); CSF1R, colony stimulating factor 1 receptor; CTNNBIP1, catenin, beta interacting protein 1; CX43, connexin 43; CXCL11, chemokine (C-X-C motif) ligand 11; CXCR3, chemokine (C-X-C motif) receptor 3; DKK1/2, Dickkopf WNT signaling pathway inhibitor 1/2; DLX5, distal-less homeobox 5; DNMT1/3a/3b, DNA methyltransferase 1/3a/3b; DUSP2, dual specificity phosphatase 2; E2, 17β-estradiol; ETS1, v-ets avian erythroblastosis virus E26 oncogene homolog 1; FAK, focal adhesion kinase; FASLG, Fas ligand; FGF2, fibroblast growth factor 2; FOX01, forkhead box O1; FZD1/4, frizzled family receptor 1/4; GALNT7, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyl transferase 7 (GalNAc-T7); GSK3β, glycogen synthase kinase 3β; GTP, guanosine triphosphate; HAT, histone acetyltransferase; HDAC, histone deacetyl transferase; HOXA2/10, homeobox A2/10; HSC, hematopoietic stem cell; IFNβ/γ, interferon β/γ; IL1/4/6/7/8/10/11/13/17/18/23, interleukin 1/4/6/7/8/10/11/13/17/18/23; JNK, c-Jun N-terminal kinase; KREMEN2, kringle containing transmembrane protein; LEP, leptin; LRPS/6, low density lipoprotein receptor-related protein 5/6; MAFB, v-maf musculoaponeurotic fibrosarcoma oncogene homolog B; MAPK, mitogen-activated protein kinase; MECP2, methyl CpG binding protein 2 (Rett syndrome); MITF, microphthalmia-associated transcription factor; MSC, mesenchymal stem cell; MYF5, myogenic factor 5; MYOD, myogenic differentiation; NFATC1, nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1; NFI-A, nuclear factor I-A; NFκB, nuclear factor κB; OPG, osteoprotegerin; OPN, osteopontin; OSCAR, osteoclast associated, immunoglobulin-like receptor; OSX, osterix; PBMC, peripheral blood mononuclear cell; PCP, planar cell planarity; PDCD4, programmed...
cell death 4; poly-miRTs, polymorphisms in miRNA target sites; PPARγ, peroxisome proliferator-activated receptor γ; PU.1, spleen focus forming virus (SFFV) proviral integration oncogene; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; RHO, ras homolog family; RISC, RNA-induced silencing complex; ROS, reactive oxygen species; RUNX2, runt-related transcription factor 2; SAHA, suberoylanilide hydroxamic acid; SATB2, SATB homeobox 2; SFRP1/2, secreted frizzled-related protein 1/2; SIRT1-7, sirtuin 1-7; SLC39A1, solute carrier family 39 (zinc transporter), member 1; SMAD1/5, SMAD family member 1/5; SNP, single nucleotide polymorphism; SOCS1, suppressor of cytokine signaling 1; SOST, sclerostin; SOX9, SRY (sex determining region Y)-box 9; SPARC, secreted protein, acidic, cysteine-rich (osteonectin); SPRY1, sprouty homolog 1, antagonist of FGF signaling (Drosophila); STUB1, STIP1 homology and U-box containing protein 1, E3 ubiquitin ligase; TBP, TATA-box binding protein; TCF7, transcription factor 7 (T cell-specific, HMG-box); TGFβ, transforming growth factor β; TGFβ, tumor necrosis factor α; TRAF6, tumor necrosis factor receptor activated factor 6; TSA, trichostatin A; WNT, Notch, Hedgehog and FGF [5]. By interacting with many transcriptional activators, repressors and other coregulatory proteins, RUNX2 can regulate the expression of several osteoblast-specific genes either positively or negatively. A further transcription factor necessary for osteoblast-specific gene expression, osteoblastogenesis and bone formation is OSX [6].

The differentiation factors responsible for osteoclast maturation are, however, much more diverse and include PU.1 and MITF, as well as CSF1R, C-FOS and RANK. Osteoclast bone-resorptive activity is mediated by RANKL through the activation of NFκB and NFATC1, two principal transcriptional mediators [7]. The multiple stages, together with key transcription factors and signaling pathways in osteoblast and osteoclast differentiation, are summarized in Figure 1.

Controlled and balanced differentiation of osteoblasts and osteoclasts is a prerequisite of normal bone remodeling and skeletal health. Deregulation of these processes can lead to several metabolic bone diseases, including renal osteodystrophy, Paget’s disease, osteopetrosis, rickets and osteoporosis, among others. Osteoporosis, by far the most prevalent among them, is characterized by a decrease in bone mineral density (BMD) and deterioration in bone microarchitecture [1]. Osteoarthritis, however, is associated with degraded articular cartilage and subchondral bone sclerosis. Like osteoporosis, it is a highly prevalent disorder with a considerable amount of controversy surrounding the tissue of disease onset, some supporting the idea that

Introduction to bone biology

Before epigenetic mechanisms, just a short overview of bone remodeling, bone cell differentiation and their regulation will be given. Bone remodeling is tightly coordinated and requires the synchronized action of osteoclasts, osteoblasts, bone-lining cells and osteocytes, in order to ensure that there are no major net changes in bone mass or mechanical properties after each remodeling cycle [1]. It takes place in a microanatomical structure that is separated from the bone marrow cavity by a canopy of cells but accessible through microcapillaries [2]. The remodeling process starts with the retraction of bone-lining cells covering the bone surface and the recruitment of osteoclast precursors to this remodeling site. Mature osteoclasts – large, multinucleated, short-lived, highly active cells attached to the bone surface – are responsible for the dissolution of the minerals and enzymatic degradation of the remaining organic matrix. After osteoclast-mediated resorption is complete, collagen remnants are removed and the resorption lacunae is prepared for subsequent osteoblast-mediated bone formation in a process that is still poorly understood. Bone formation starts with the differentiation of osteoblasts and laying down of the organic osteoid, consisting mainly of collagen type I. It is completed after osteoblast-mediated mineralization of the organic matrix. Finally, the resting bone surface covered by bone-lining cells belonging to the osteoblast lineage is re-established [3]. Osteocytes – terminally differentiated osteoblasts entombed within the bone matrix that account for almost 95% of all cells in the mature bone tissue – form a network of canaliculi within the mineralized bone. These mechanosensing cells are thought to detect mechanical strain and associated bone microdamage and to respond by initiating bone resorption and the regulation of bone remodeling. Data show that hormonal stimuli also trigger bone remodeling via osteocytes [4].

The differentiation of osteoblasts and osteoclasts from their precursors is an inherent part of bone remodeling. The transcriptional regulator RUNX2 is indispensable in all stages of osteoblastogenesis and bone formation. It also represents an intersection of several signaling pathways regulating osteoblast differentiation, including BMP, WNT, Notch, Hedgehog and FGF [5]. By interacting with many transcriptional activators, repressors and other coregulatory proteins, RUNX2 can regulate the expression of several osteoblast-specific genes either positively or negatively. A further transcription factor necessary for osteoblast-specific gene expression, osteoblastogenesis and bone formation is OSX [6].

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it is a bone disease that also affects cartilage, and vice versa [10]. Due to the high prevalence of osteoporosis and osteoarthritis as well as the lack of studies in other metabolic bone disorders, the present review is focused on these two pathologies.

Similarly, since the WNT/β-catenin signaling pathway and RANK/RANKL/OPG system are, respectively, the most well described and perhaps the most significant stimulators of bone formation and bone resorption, only these two pathways are discussed, briefly, below.

**RANK/RANKL/OPG system in osteoclast regulation and bone resorption**

The RANK/RANKL/OPG system is one of the most important regulators of bone resorption and remodeling and, therefore, an obvious candidate for the studies of epigenetic modifications related to bone pathologies. RANK, located on the surface of osteoclasts and their precursors, and its ligand RANKL are essential for the formation, differentiation, activity and survival of osteoclasts. RANKL is produced by cells of the osteoblast lineage as well as other cell types in both soluble and membrane-bound forms. The binding of RANKL to RANK, results in the activation of transcription factors NFκB and NFATC1 and the expression of osteoclastogenic genes. In contrast, OPG, secreted by osteoblasts and a few other cell types, functions as a decoy receptor by binding to RANKL, thereby preventing the activation of RANK. This inhibition of RANKL, which leads to the rapid arrest of osteoclast formation, activation and survival, is crucial for the suppression of bone resorption and maintenance of bone mass [11]. Pro-inflammatory cytokines secreted by different immune cells also notably influence osteoclast activity and bone resorption by either targeting osteoclasts directly or modulating the RANK/RANKL/OPG system [12]. The importance of the RANK/RANKL/OPG system in maintaining bone homeostasis is further supported by the therapeutic success of denosumab, a monoclonal antibody directed against RANKL, used in patients with osteoporosis and cancer patients with treatment-induced bone loss or metastases [11, 13]. The RANK/RANKL/OPG system and its cytokine modulators are depicted in Figure 2.

**WNT/β-catenin signaling pathway in osteoblast regulation and bone formation**

The very precise regulation of the intensity, amplitude and duration of WNT/β-catenin signaling is necessary for proper skeletal development and bone remodeling, indicating a likely connection with the epigenetic control of gene and protein expression. There are several WNT signaling pathways, including the non-β-catenin PCP pathway, calcium ion signaling, RHO family GTPase pathways, and the JNK pathways; however, it is the WNT/β-catenin signaling pathway that is the most crucial for multiple stages of osteoblast differentiation, their proliferation and survival. In the latter, WNTs cause an increase in β-catenin levels and the activation of transcription factors TCF7 and LEF1. Their transcriptional targets include the aforementioned OPG and RUNX2, linking the WNT/β-catenin signaling pathway with the inhibition of osteoblast-mediated osteoclastogenesis and the stimulation of osteoblast proliferation and survival, respectively. The presence of several neutralizing extracellular factors that directly bind WNTs (e.g., SFRP1, WIF1) or their coreceptors (e.g., SOST, DKK1/2) increases the complexity of the WNT/β-catenin signaling pathway but, at the same time, represents an attractive opportunity for therapeutic
Figure 2  RANK/RANKL/OPG system.
The RANK/RANKL/OPG system is essential for the formation and differentiation of osteoclasts, their resorptive activity and survival. The binding of RANKL to RANK results in the recruitment of TRAF6, which activates various protein kinase pathways and transcription factors like NFκB. The activated NFκB up-regulates the expression of C-FOS, which subsequently interacts with NFATC1 to induce the expression of osteoclastogenic genes. Conversely, OPG prevents the activation of RANK by binding RANKL [11]. Pro-inflammatory cytokines secreted by different immune cells – including activated T cells, B cells, macrophages, mast cells and natural killer cells – also modulate the RANK/RANKL/OPG system. TNFα, IL1, IL6, IL8, IL11 and IL17 are osteoclastogenic cytokines promoting RANKL-mediated osteoclast differentiation and activity, while the anti-osteoclastogenic cytokines IFNβ, IFNγ, IL4, IL10, IL13 and IL18 inhibit osteoclasts through the RANK/RANKL/OPG system. Certain cytokines can exert opposite effects on osteoclasts (e.g., IL7 and IL23) [12].

intervention [14]. In particular, SOST has been identified as a key negative regulator of bone mass and formation. Its inhibition using a monoclonal antibody has a profound anabolic effect on the skeleton. Since it is secreted primarily in bone, specifically by osteocytes, and facilitates a novel anabolic approach, it represents a promising therapeutic target in the case of bone-related disorders such as osteoporosis [15, 16]. Due to the importance of WNT signaling pathways in osteoblast regulation and bone formation, it is not surprising that certain known bone-influencing stimuli exert their effect at least in part through WNT signaling pathways. These include estrogen and parathyroid hormone, as well as mechanical loading and pro-inflammatory cytokines, among others [17–19]. The WNT/β-catenin signaling pathway is schematically shown in Figure 3.

All of the aforementioned signaling pathways play an important role in the regulation of bone metabolism by fine tuning the expression of thousands of target genes. Since posttranslational histone modifications, microRNAs (miRNAs) and DNA methylation act as important regulators of gene expression, they could also interfere significantly with bone homeostasis. Furthermore, these epigenetic mechanisms are increasingly linked to aging, providing additional support for their possible association with late-onset disorders like osteoporosis and osteoarthritis.

Following a brief overview of epigenetic mechanisms, the complex intertwining of epigenetic processes and bone remodeling will be discussed, revealing potential new diagnostic and therapeutic targets in skeletal diseases. The articles included in the review have been critically selected from the results of a PubMed database search using combinations of the keywords osteoblast differentiation, histone acetylation, HDAC, SIRT1, miRNA, DNA methylation, osteoporosis and osteoarthritis.

Epigenetic mechanisms controlling gene expression in bone cells

Posttranslational histone modifications in regulation of gene expression

Of the three epigenetic mechanisms, posttranslational histone modifications and accompanying histone-modifying enzymes form probably the biggest and most complex
regulatory entity. As far as is presently known, an octamer of four different canonical histone molecules (H2A, H2B, H3, and H4) wrapped by a 147-bp segment of DNA forms a nucleosome, the basic unit of chromatin. Flexible N-terminal tails of core histones that protrude from the nucleosome are subjected to various posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination and a non-covalent proline isomerization [20]. It has been shown that euchromatin, a more relaxed, actively transcribed state of DNA, is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79, while low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation are indicative of a more condensed, transcriptionally inactive heterochromatin [21]. Most histone post-translational modifications are dynamic and regulated by families of enzymes that promote or reverse specific modifications. For example, histone acetyltransferases (HATs) add, while histone deacetyl transferases (HDACs) remove, acetylation marks. HATs are classified as belonging to one of two classes, HAT A and HAT B, and several subclasses, depending on the mechanism of their action and cellular localization. HDACs are divided into four groups based on their structural and functional similarities; class I includes HDAC 1, 2, 3 and 8, class II HDAC 4–7, 9 and 10, class III SIRT 1–7 and class IV solely HDAC11. Due to the fact that many transcriptional coactivators and corepressors possess HAT or HDAC activity, respectively, or associate with such enzymes, the balance between acetylation and deacetylation of histones that harbor target genes significantly influences their expression [20, 22].

miRNAs are small – approximately 21 nucleotides long – non-coding RNA molecules. Mature miRNAs are incorporated into the RISC in order to facilitate binding to the target mRNA, usually within its 3′ UTR region. A perfect match between miRNA and the target results in the degradation of the targeted transcript. More commonly, miRNAs bind imperfectly to their targets, causing translational repression without the cleavage of mRNA [23]. In general, miRNAs are considered predominantly as negative regulators of gene expression involved in physiological and pathological processes, even though some of them have been shown to activate translation under certain conditions [24].

DNA methylation is a reversible, covalent modification of the 5′-carbon of a cytosine residue, which results in the production of 5-methyl-cytosine. In mammals, cytosine methylation is restricted to residues located 5′ to a guanosine (commonly referred to as CpGs), which occur more frequently in regions known as CpG islands. The methylation of DNA can either inhibit or facilitate the binding of proteins to the DNA molecule. In general, DNA methylation is associated with gene repression. As DNA methylation patterns can be preserved during DNA replication and mitosis, the associated repressed state can be inherited. Enzymes responsible for the transfer of a methyl group from S-adenosyl methionine to DNA include DNMT1, DNMT3a and DNMT3b. DNMT1 is the so-called maintenance DNMT, copying the DNA methylation pattern of the parent DNA strand onto the daughter
The role of histone-modifying enzymes in bone remodeling

As the large number of histone marks and their combinations increase the complexity of transcriptional regulation through histone-modifying enzymes, we will concentrate only on the importance of histone acetylation in bone remodeling.

Histone acetylation in osteoblast differentiation

Several studies have used HDAC inhibitors to elucidate the influence of general histone hyperacetylation on osteoblast differentiation and gene expression. There are many different HDAC inhibitors, including TSA, SAHA, MS-275, sodium butyrate, and valproic acid; some of these, in addition to being in use with regard to certain neurological conditions, also have great potential as anti-cancer drugs and therapeutics for inflammatory and cardiac diseases. TSA and SAHA are potent inhibitors of class I and II HDACs, MS-275 inhibits class I HDACs and HDAC9 – with the exception of HDAC8 and a preference for HDAC1 – while sodium butyrate and valproic acid also inhibit class I HDACs, albeit with somewhat lower potency, especially towards HDAC8 [25, 26]. In vitro studies have revealed that the inhibition of either class I and II HDACs or class I HDACs alone accelerates osteoblast maturation, matrix mineralization and the expression of genes associated with osteoblast differentiation (e.g., type I collagen, bone sialoprotein, osteopontin, osteocalcin, ALPL, OSX and RUNX2). This was achieved, at least in part, through the up-regulation of RUNX2 transcriptional activity. Early changes in gene expression also included two WNT receptor genes, FZD1 and FZD4. TSA-induced minimal cytotoxicity in osteoblasts at low concentrations still sufficient to cause histone hyperacetylation, while MS-275 and sodium butyrate enhanced osteoblast viability and proliferation. Valproic acid was also shown to enhance the viability and proliferation of osteoblasts while inhibiting the proliferation of mesenchymal stem cells (MSCs) without affecting their survival [27–29]. Interestingly, RANKL expression was also enhanced by the TSA-mediated increase in the acetylation of histones H4 and H3 at the RANKL promoter [30]. In vivo studies in animal models of bone loss revealed stimulatory effects on bone regeneration after MS-275 treatment, while studies using healthy animals resulted in bone loss during treatment with SAHA or valproate [31–33]. Furthermore, human epidemiological studies in patients with different neurological conditions treated with valproate tend to provide support for there being negative effects of HDAC inhibitors on bone, as evidenced by decreased BMD and increased fracture risk in children and adults [34]. It has been suggested that this discrepancy could be due to various HDAC inhibitors targeting individual HDACs with different specificity and affinity as well as their broad range of action, which can include the inhibition of other enzymes besides HDACs. In the case of studies involving animals, the selection of animal models could also play an important part, as certain mice strains were shown to be resistant to bone effects caused by HDAC-inhibitor treatment [33]. Human studies also featured many confounding factors, since they involved very different patient cohorts, including patients with decreased physical activity [34].

Another group of studies focused on the expression of osteocalcin in order to elucidate the role of histone acetylation in osteoblastogenesis. Osteocalcin is a very abundant bone-specific protein that is able to bind calcium and, consequently, hydroxyapatite, and is essential for proper bone mineralization. Serum osteocalcin levels are used as a bone formation marker, while its expression in cell cultures enables osteoblast differentiation status and activity to be determined. These studies showed that the osteocalcin promoter is associated with the acetylated histones H3 and H4 when transcriptionally active, while very low levels of acetylated H3 and H4 accompany an inactive osteocalcin gene [35, 36]. It was also shown that the transcriptional coactivator and HAT p300 interacted with RUNX2 at the osteocalcin promoter in such a way as to stimulate its expression, although it was not responsible for the increased levels of acetylation. It appears that other proteins that contain HAT activity and associate with p300, e.g., PCAF, could potentially increase H3 and H4 acetylation [37]. Given that the active expression of other bone-related genes was also reflected in...
the presence of acetylated H3 and H4 at their respective loci, it is plausible that the acetylation of histones associated with bone-related gene promoters is functionally coupled to the chromatin remodeling events that mediate the regulation of gene expression during osteoblast differentiation [38]. Accordingly, decreased histone acetylation was associated with the negative regulation of osteoblast differentiation and concomitant repression of osteocalcin expression. Elevated levels of NFATC1, e.g., interacted with HDAC3 at the osteocalcin promoter and prevented the acetylation of both H3 and H4 [39]. In addition, HDAC3 was also shown to repress activation of the osteocalcin promoter by interacting with RUNX2, thereby antagonizing its transcriptional activity [40, 41]. Lamour et al. confirmed the suppressive role of HDAC3 by showing it is also highly likely to be responsible for the deacetylation of H3 at the bone sialoprotein promoter, thus preventing its gene expression [42]. Interestingly, the conditional knockout of HDAC3 in mice had no effect on osteocalcin levels. Furthermore, it reduced trabecular and cortical bone mass due to impaired osteoblast function associated with increased DNA damage [43, 44]. This large discrepancy between the results of in vitro and in vivo studies of HDAC3 action is probably due to differences in duration and the extent of HDAC3 regulation as well as the maturity of the osteoblasts used. However, TGFβ, a well-known negative regulator of bone formation, inhibited osteoblast differentiation and osteocalcin expression by recruiting HDAC4 and 5 to RUNX2, resulting in the deacetylation of H4 at the osteocalcin promoter [45]. Interestingly, HDAC4 and 5 could also directly decrease and p300 directly increase RUNX2 acetylation, thereby reducing or enhancing, respectively, its protein stability and transcriptional activity [46]. Besides HDAC3, 4 and 5, HDAC1 was also identified as an important regulator of osteoblast differentiation. Lee et al. discovered that the hyperacytlation of H3 and H4 at promoters of OSX and osteocalcin in differentiated osteoblasts was due to a decrease in the recruitment of HDAC1 and the enhanced association of p300 at those promoters. Furthermore, the knockdown of HDAC1 alone was enough to promote the expression of osteogenic genes and induce osteogenesis [47].

**Sirtuin 1 as an important regulator of bone homeostasis**

SIRT1 is probably one of the most studied HDACs due to its proposed role in linking calorie restriction and lifespan extension. It also turned out to be a major regulator of bone mass. Cohen-Kfir et al. demonstrated that SIRT1 repressed the expression of SOST by deacetylating H3K9 at the SOST promoter. Accordingly, SIRT1 haplo-insufficient female mice exhibited substantially increased SOST levels and reduced bone formation and mass. In addition, differentiated MSCs derived from SIRT1 haplo-insufficient mice showed reduced osteoblast activity, while the same cells displayed increased adipogenesis and PPARγ expression when adipogenesis was induced [48]. A reciprocal relationship between the level of SIRT1, on one hand, and adipocyte differentiation and PPARγ expression, on the other, has also been observed by others in vitro and in vivo [49–51]. It is important to note that SIRT1 action is not limited to epigenetic mechanisms as it exerts its multiple activities by interacting not only with histones but also with numerous transcription factors, enzymes and other protein species [52]. Additional studies, for example, have shown that SIRT1 promotes the activation of Runx2 and the suppression of NFkB signaling, thus stimulating osteoblastogenesis while also inhibiting osteoclastogenesis; this indicates that it plays an important role in the coupling of bone formation and resorption with a possible contribution to bone quality and a reduction in bone frailty (Figure 4) [51, 53–55].

**Histone acetylation in osteoclast differentiation**

In contrast to osteoblasts, the inhibition of either class I and II HDACs or class I HDACs alone suppressed osteoclast differentiation in vitro [56–59]. Class I HDAC inhibitors targeting predominantly HDAC1 and HDAC2 also decreased bone destruction in animal models of bone loss in vivo [57, 59]. Osteoclasts exposed to TSA, SAHA or sodium butyrate treatment exhibited enhanced apoptosis and reduced activation of NFκB and MAPK signaling [56, 58]. In addition, a reduction in the expression of transcription factors C-FOS and NFATC1, osteoclast-specific genes cathepsin K, calcitonin receptor and OSCAR, and even the induction of IFNβ, an inhibitor of osteoclastogenesis, were observed after the inhibition of class I and II HDACs or class I HDACs alone [56, 57, 59, 60]. In support of this, Pham et al. were able to inhibit osteoclast differentiation in vitro by suppressing the expression of HDAC3 using appropriate short hairpin RNAs. Conversely, the suppression of HDAC7 resulted in enhanced osteoclast formation. This was due to the fact that HDAC7 was able to inhibit osteoclast differentiation by repressing the MITF transcription factor, in all likelihood by means of a deacetylation-independent mechanism [61]. Furthermore, RANKL was able to increase the transcriptional activity of NFATC1 by stimulating the
Vrtačnik et al.: Epigenetic mechanisms in bone discussed hereinafter, placed HDAC5 downstream from a miRNA associated with primary osteoporosis in adolescents [63, 64]. Nevertheless, little is known about the involvement of HATs and HDACs and their acetylation marks in the pathogenesis of bone disorders. When taking into account all the possible posttranslational modifications potentially present at individual nucleosomes, there is great potential for the discovery of biomarkers associated with bone pathologies.

The role of miRNA in bone remodeling

Various cancers often exhibit unique miRNA-expression profiles of up- and down-regulated miRNAs, opening up the possibility of using them as biomarkers for cancer diagnosis, prognosis, and response to therapy [65, 66]. The most promising epigenetic biomarkers in lung, colorectal and prostate cancers have recently been reviewed by Sandoval et al. [67]. Increasing evidence indicates a similar role for miRNA in metabolic bone diseases.

miRNAs in osteoblast differentiation

In recent years, more than 30 papers have investigated the role of miRNA in osteoblast differentiation. They have identified several miRNAs – namely, miR-23a, miR-30a–d, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-211, miR-217, miR-335, miR-338, miR-433 and miR-3077–5p – that impede osteoblast differentiation by directly targeting the master osteogenic transcription factor RUNX2 with differences in efficacy and quantity depending on the stage of osteoblast lineage progression [68–76]. Conversely, miR-2861 and miR-3960 have been shown to be able to indirectly stimulate RUNX2 expression by suppressing the translation of its inhibitors HDAC5 and HOXA2, respectively, thus promoting osteoblast differentiation [64, 77]. Furthermore, RUNX2 was shown to positively and negatively regulate the expression of particular miRNAs [74, 77]. To a certain extent, OSX is also under miRNA control, forming a unique autoregulatory feedback loop with miR-93. It has been shown that miR-93 inhibited osteoblast mineralization by directly targeting OSX [78].

The WNT/β-catenin signaling pathway in human osteoblasts regulates, while also being under the control of, specific miRNAs. By directly targeting known WNT antagonists (e.g., DKK1, DKK2, SFRP2 and SOST), miR-29a,
miR-218 and miR-335-5p were able to enhance the WNT/β-catenin signaling pathway and potentiate osteoblast differentiation [79–81]. Activation of the WNT/β-catenin signaling pathway was also observed following the suppression of the expression of APC, an integral part of the β-catenin destruction complex, by miR-27 and miR-142-3p [82, 83]. Similarly, miR-29b was indicated to target CTNNBIP1, an inhibitor of β-catenin-mediated transcription, resulting in enhanced osteogenesis [84]. Together with those previously described, additional positive and negative miRNA regulators of osteoblast differentiation and their respective targets are presented in Table 1.

In the described differentiation experiments, researchers used both mesenchymal and more committed preosteoblast cell lines; these were predominantly of mouse origin, often supported by primary mouse- or human bone marrow-derived MSCs or mouse calvarial osteoblasts. When also taking into account the variability of the differentiation media used, the diversity of RNA isolation methods and differences in miRNA screening procedures, it is not surprising that there is little overlap between the results of the aforementioned studies. However, each miRNA targets several different transcripts and most mRNAs are regulated by a number of miRNAs. In this setting, it is expected that a vast network of miRNAs takes part in the precise regulation of the complex process that is osteoblast differentiation.

miRNAs in osteoclast differentiation

In comparison to osteoblast differentiation, the involvement of miRNAs in osteoclast differentiation is markedly less researched (Table 2). Sugatani et al. showed that both the overexpression of pre-miR-223 and antisense inhibitors of miR-223 suppressed osteoclastogenesis, suggesting that expression levels of miR-223 that were either too high or too low were not appropriate for efficient osteoclast differentiation. They proposed a mechanism by which miR-223 suppressed NFI-A, an indirect inhibitor of CSF1R, thus stimulating osteoclast differentiation and the expression of transcription factors such as MITF, C-FOS and PU.1 [103, 105]. In addition, miR-21 was shown to promote osteoclast differentiation and protect osteoclasts from apoptosis. By targeting PDCD4, miR-21 derepressed C-FOS, resulting in increased osteoclastogenesis and miR-21 expression via a positive feedback mechanism [99]. Protection against estrogen-induced apoptosis during osteoclastogenesis was, however, achieved by down-regulating the inducer of apoptosis, Fas ligand, another miR-21 target; this suggests that the anti-osteoclastic activity of estrogen could be counteracted by miR-21 overexpression [100]. Conversely, miR-155 efficiently blocked the activation of the osteoclast transcriptional program and repressed osteoclastogenesis by directly targeting MITF [104]. The stimulatory effects of miR-127, miR-136 [An et al., Unpublished manuscript], miR-133a [101] and miR-148a [102], as well as the inhibitory effects of miR-503 [Chen et al., Unpublished manuscript], on osteoclastogenesis were also observed and are described in the following section.

miRNAs associated with osteoporosis

Most studies analyzing the associations between metabolic bone diseases and epigenetic changes have concentrated on differences in miRNA expression and function. Among these, studies using bone tissue as their main study material have focused almost entirely on osteoporosis, with the exception of one on osteoarthritis. The latter identified 30 miRNAs differentially expressed in osteoarthritic bone when compared to normal tissue, with miR-27a, miR-34b, miR-98 and miR-330 showing the greatest fold change. Interestingly, miR-9 and miR-98 were up-regulated in both osteoarthritic bone and cartilage tissue and could be functionally linked to inflammation [106].

Profiling of bone tissue and bone marrow-derived MSCs from ovariectomized mice, however, revealed increased expression of miR-127, miR-133a, miR-133b, miR-136, miR-206, and miR-378a, together with decreased expression of miR-204 in bone tissue, while MSCs showed overexpression of miR-705 and miR-3077-5p and the suppression of miR-21 after ovariectomy [76, 86, An et al., Unpublished manuscript]. The high expression of miR-133a, miR-133b, miR-206 and miR-378a is in line with the already described studies; however, this is not the case for miR-204, since it has previously been shown to inhibit osteoblast differentiation. The discrepancy in this instance could be due to differences in the starting material, with one study using osteoblastic cell lines, the other bone tissue. Based on the predicted targets of identified miRNAs and the associated mRNA expression profile, it was suggested that the PPARγ and CREB pathways were important mediators of change in bone tissue after ovariectomy. In addition, miR-127 and miR-136 were shown to suppress osteoblast differentiation and osteocyte function and survival while at the same time promoting osteoclast differentiation [An et al., Unpublished manuscript]. Similarly, miR-705 and miR-3077-5p were shown to inhibit osteogenic differentiation and promote the adipogenic differentiation of MSCs by directly targeting HOXA10 and RUNX2, respectively. Both target genes are involved in the
Table 1 miRNAs involved in the regulation of osteoblast differentiation.

### Positive miRNA regulators of osteoblast differentiation

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target mRNA symbol</th>
<th>Target mRNA name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-20a</td>
<td>PPARγ; Bambi; CTRM1</td>
<td>Peroxisome proliferator-activated receptor γ; BMP and activin membrane-bound inhibitor; cysteine-rich transmembrane BMP regulator 1 (chordin-like)</td>
<td>[85]</td>
</tr>
<tr>
<td>miR-21</td>
<td>SPRY1</td>
<td>Sprouty homolog 1, antagonist of FGF signaling (Drosophila)</td>
<td>[86]</td>
</tr>
<tr>
<td>miR-27; miR-142-3p</td>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
<td>[82, 83]</td>
</tr>
<tr>
<td>miR-29a</td>
<td>DKK1; KREMEN2; SFRP2; SPARC</td>
<td>Dickkopf WNT signaling pathway inhibitor 1; kringle containing transmembrane protein 2; secreted frizzled-related protein 2; secreted protein, acidic, cysteine-rich (osteonectin)</td>
<td>[79, 87]</td>
</tr>
<tr>
<td>miR-29b</td>
<td>COL1A1; COL5A3; COL4A2; HDAC4; TGFβ3; ACVR2A; CTNNB1; DUSP2</td>
<td>Collagen, type I, alpha 1; collagen, type V, alpha 3; collagen, type IV, alpha 2; histone deacetyl transferase 4; transforming growth factor β3; activin A receptor, type IIA; catenin, beta interacting protein 1; dual specificity phosphatase 2</td>
<td>[84]</td>
</tr>
<tr>
<td>miR-29c</td>
<td>SPARC</td>
<td>Secreted protein, acidic, cysteine-rich (osteonectin)</td>
<td>[87]</td>
</tr>
<tr>
<td>miR-210</td>
<td>ACVR1B</td>
<td>Activin A receptor, type IB</td>
<td>[88]</td>
</tr>
<tr>
<td>miR-218</td>
<td>SOST; DKK2; SFRP2</td>
<td>Sclerostin; Dickkopf WNT signaling pathway inhibitor 2; secreted frizzled-related protein 2</td>
<td>[80]</td>
</tr>
<tr>
<td>miR-335-5p</td>
<td>DKK1</td>
<td>Dickkopf WNT signaling pathway inhibitor 1</td>
<td>[81]</td>
</tr>
<tr>
<td>miR-764-5p</td>
<td>STUB1</td>
<td>STIP1 homology and U-box containing protein 1, E3 ubiquitin protein ligase</td>
<td>[89]</td>
</tr>
<tr>
<td>miR-2861</td>
<td>HDAC5</td>
<td>Histone deacetyl transferase 5</td>
<td>[64]</td>
</tr>
<tr>
<td>miR-3960</td>
<td>HOXA2</td>
<td>Homeobox A2</td>
<td>[77]</td>
</tr>
</tbody>
</table>

### Negative miRNA regulators of osteoblast differentiation

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target mRNA symbol</th>
<th>Target mRNA name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-23a</td>
<td>RUNX2; SATB2</td>
<td>Runt-related transcription factor 2; SATB homeobox 2</td>
<td>[68, 70, 74]</td>
</tr>
<tr>
<td>miR-24-2; miR-27a</td>
<td>SATB2</td>
<td>SATB homeobox 2</td>
<td>[74]</td>
</tr>
<tr>
<td>miR-30a–d</td>
<td>RUNX2; SMAD1</td>
<td>Runt-related transcription factor 2; SMAD family member 1</td>
<td>[68, 70, 72]</td>
</tr>
<tr>
<td>miR-34c; miR-133a; miR-137; miR-204; miR-205; miR-211; miR-217; miR-335; miR-338; miR-433; miR-3077-5p</td>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
<td>[68–71, 73, 75, 76]</td>
</tr>
<tr>
<td>miR-93</td>
<td>OSX</td>
<td>Osterix</td>
<td>[78]</td>
</tr>
<tr>
<td>miR-135a</td>
<td>RUNX2; SMAD5</td>
<td>Runt-related transcription factor 2; SMAD family member 5</td>
<td>[68–70]</td>
</tr>
<tr>
<td>miR-138</td>
<td>FAK</td>
<td>Focal adhesion kinase</td>
<td>[90]</td>
</tr>
<tr>
<td>miR-141; miR-200a</td>
<td>DLX5</td>
<td>Distal-less homeobox 5</td>
<td>[91]</td>
</tr>
<tr>
<td>miR-155</td>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
<td>[92]</td>
</tr>
<tr>
<td>miR-182</td>
<td>FOXO1</td>
<td>Forkhead box O1</td>
<td>[93]</td>
</tr>
<tr>
<td>miR-206</td>
<td>CX43</td>
<td>Connexin 43</td>
<td>[94]</td>
</tr>
<tr>
<td>miR-208</td>
<td>ETS1</td>
<td>v-ets avian erythroblastosis virus E26 oncogene homolog 1</td>
<td>[95]</td>
</tr>
<tr>
<td>miR-214</td>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
<td>[96]</td>
</tr>
<tr>
<td>miR-370</td>
<td>BMP2; ETS1</td>
<td>Bone morphogenetic protein 2; v-ets avian erythroblastosis virus E26 oncogene homolog 1</td>
<td>[97]</td>
</tr>
<tr>
<td>miR-378</td>
<td>GALNT7</td>
<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 7 (GalNAc-T7)</td>
<td>[98]</td>
</tr>
<tr>
<td>miR-705</td>
<td>HOXA10</td>
<td>Homeobox A10</td>
<td>[76]</td>
</tr>
</tbody>
</table>
Table 2 miRNAs involved in the regulation of osteoclast differentiation.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target mRNA symbol</th>
<th>Target mRNA name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>PDCD4; FASLG</td>
<td>Programmed cell death 4; Fas ligand</td>
<td>[99, 100]</td>
</tr>
<tr>
<td>miR-127; miR-136</td>
<td>Not reported</td>
<td>Not reported</td>
<td>[An et al., Unpublished manuscript]</td>
</tr>
<tr>
<td>miR-133a</td>
<td>CXCL11; CXCR3; SLC39A1 (in silico predicted)</td>
<td>Chemokine (C-X-C motif) ligand 11; chemokine (C-X-C motif) receptor 3; solute carrier family 39 (zinc transporter), member 1</td>
<td>[101]</td>
</tr>
<tr>
<td>miR-148a</td>
<td>MAFB</td>
<td>v-maf musculoaponeurotic fibrosarcoma oncogene homolog B</td>
<td>[102]</td>
</tr>
<tr>
<td>miR-223</td>
<td>NFI-A</td>
<td>Nuclear factor I-A</td>
<td>[103]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Target mRNA symbol</th>
<th>Target mRNA name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-155</td>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
<td>[104]</td>
</tr>
<tr>
<td>miR-223</td>
<td>Not reported</td>
<td>Not reported</td>
<td>[105]</td>
</tr>
<tr>
<td>miR-503</td>
<td>RANK</td>
<td>Receptor activator of nuclear factor xB</td>
<td>[Chen et al., Unpublished manuscript]</td>
</tr>
</tbody>
</table>

BMP signaling pathway, potentially providing a synergistic effect on MSC differentiation. Furthermore, increased levels of TNFα and reactive oxygen species (ROS), associated with an estrogen deficiency, significantly increased the expression of miR-705 and miR-3077-5p through the activation of NFκB pathway. The shift of cell lineage commitment in MSCs from osteoblasts towards adipocytes was suggested as being an important event in the pathogenesis of osteoporosis [76]. Conversely, miR-21 was shown to directly target SPRY1, a key negative regulator of FGF and MAPK signaling pathways, resulting in enhanced osteoblast differentiation and bone formation. Decreased miR-21 in MSCs from osteoporosis patients and ovariectomized mice was also most likely to be due to high levels of TNFα, associated with estrogen deficiency (Figure 5) [86].

A different study identified the repressed expression of miR-2861 due to a mutation in pre-miR-2861 as a contributing factor in primary osteoporosis in two related adolescents. It was shown that miR-2861 promoted osteoblast differentiation by directly targeting HDAC5, which is responsible for reducing RUNX2 protein stability and transcriptional activity. This was confirmed in vivo in a mouse model where the absence of miR-2861 significantly reduced BMD, bone formation, osteoblast number and RUNX2 protein levels while increasing HDAC5 protein expression. Bone resorption and osteoclast activity were unaffected. According to the study’s results, the discovered mutation in pre-miR-2861 is, in all likelihood, a rare variant and thus unlikely to be associated with postmenopausal osteoporosis [64].

A different DNA-based study conducted an association analysis between selected polymorphisms in miRNA target sites (poly-miRTSs) and osteoporosis, identifying three poly-miRTSs in the FGF2 gene significantly associated with femoral neck BMD. These three single nucleotide polymorphisms (SNPs) in the 3′-UTR of the FGF2 gene reside within predicted binding sites for nine miRNAs (miR-146a, miR-146b, miR-545, miR-25, miR-32, miR-92, miR-363, miR-367 and miR-92b) and possibly alter the binding affinity between FGF2 transcripts and the identified miRNAs. Theoretically, an unfavorable allele would reduce the efficiency of miRNA binding to its target site, resulting in higher levels of target-protein expression. In the case of FGF2, such levels would stimulate osteoclastogenesis, enhance bone resorption and reduce BMD [107]. Even though this study lacks extensive functional confirmation, it raises an interesting point that encourages re-analysis of genome-wide association data, with the emphasis on miRNA target sites and pri-miRNA coding sequences.
miR-705, miR-3077-5p and miR-214 were shown to suppress osteogenic differentiation by directly inhibiting their respective targets, thus, in all likelihood, contributing to bone loss associated with osteoporosis. Conversely, miR-2861 and miR-21 directly target negative regulators of osteoblastogenesis resulting in enhanced osteoblast differentiation and bone formation. The increased expression of miR-705 and miR-3077-5p and decreased levels of miR-21 were linked to high levels of TNFα and ROS, associated with estrogen deficiency. However, it was suggested that miR-503 and miRNAs targeting the FGF2 gene reduce osteoclastogenesis, suppress bone resorption and increase bone mass. Conversely, miR-148a and miR-133a promoted osteoclastogenesis and bone resorption by directly targeting negative regulators of osteoclast differentiation. Full lines represent established or experimentally proven associations while dotted lines represent hypothetical or in silico predicted connections.

Two studies have profiled the expression of miRNAs in peripheral blood mononuclear cells (PBMCs) from postmenopausal women with low and high BMD, while a third study focused on premenopausal women with systemic lupus erythematosus and low BMD. They found that miR-503 was markedly reduced and miR-133a significantly increased in osteoporosis patients [101, Chen et al., Unpublished manuscript]. Substantially raised levels of miR-148a were associated with lower BMD in lupus patients [102]. RANK was identified as a direct target of miR-503 and in vivo experiments in an ovariectomized mouse model showed that silencing of miR-503 increased RANK protein levels and osteoclastogenesis, promoted bone resorption and decreased bone mass [Chen et al., Unpublished manuscript]. Both in vitro and in vivo experiments also confirmed that miR-148a promoted osteoclastogenesis and bone resorption by directly targeting MAFB, a negative regulator of NFATC1, C-FOS and MITF [102]. Functional testing of miR-133a was not carried out, but in silico testing predicted that osteoclast-related target genes include CXCL11, CXCR3 and SLC39A1 (Figure 5) [101]. Since all three miRNAs are present in PBMCs, they represent easily accessible potential biomarkers associated with postmenopausal osteoporosis and increased bone resorption. Of these, miR-503 and miR-148a are especially promising as it has been experimentally proven that they play a significant role in regulating bone

![Diagram](image_url)
resorption and turnover. However, their status as putative therapeutic targets is more questionable, as it has been shown that the stimulation of miR-503 and inhibition of miR-148a expression suppressed both bone resorption and formation [102, Chen et al., Unpublished manuscript].

Bone is a highly vascularized tissue with a close relationship between osteogenesis and angiogenesis as well as bone remodeling and vascularization [108]. Furthermore, bone remodeling takes place within the bone-remodeling unit tightly linked to bone marrow capillaries; this is in order to provide necessary precursor cells and nutrients to this otherwise isolated environment [2]. Since bone tissue samples are rarely obtainable, the fact that miRNAs are present in serum, plasma, saliva, urine and other body fluids gives hope that miRNAs could also be used as potential non-invasive biomarkers for skeletal disorders [109].

**The role of DNA methylation in bone remodeling**

All three types of epigenetic marks represent novel diagnostic and therapeutic opportunities; however, DNA methylation and DNMTs are currently showing the most promising results as adjuvant treatment for certain malignancies. Given that DNA methylation is also relevant in considerations of aging and age-related chronic diseases, future applications in osteoporosis and other bone-related pathologies are expected. An aberrant DNA methylation pattern that includes both global DNA hypomethylation and site-specific DNA hypermethylation seems to be one of the hallmarks of cancer. Several articles discussing DNA methylation in the context of clinical laboratory cancer management and neurodegenerative disorder research have been published recently in Clinical Chemistry and Laboratory Medicine [110–113].

**DNA methylation in osteoblast differentiation**

Like posttranslational histone modifications and miRNAs, DNA methylation also plays a part in regulating gene expression in bone tissue cells (Table 3). Several studies have shown that methylation of the gene encoding *ALPL* is a major controller of its expression, since DNA demethylating agents repeatedly increased *ALPL* expression and activity in osteoblasts under osteogenic and non-osteogenic conditions [114–117]. More specifically, the degree of methylation in the CpG island located in the proximal region of the *ALPL* gene has been shown to be inversely associated with *ALPL* transcriptional levels. Accordingly, *ALPL*-expressing osteoblasts displayed the least methylated *ALPL* gene, while it was hypermethylated in osteocytes, which do not express *ALPL*. Bone lining cells, which represent a differentiation stage somewhere between osteoblasts and osteocytes, exhibited an intermediate methylation status; this suggests that *ALPL* methylation may increase progressively during osteoblastic differentiation, thus contributing notably to the phenotypic change associated with this process [117]. Interestingly, the transition from osteoblasts towards osteocytes was accompanied by a progressive decrease in levels of the *SOST* promoter methylation, enabling *SOST* to be expressed in an osteocyte-specific manner [118].

Genes shown to be coregulated by DNA methylation in osteoblasts also include transcription factors *OSX* and *DLX5* [119], estrogen receptor α [120] and osteopontin [121]. Interestingly, mechanical stimulation, essential for maintaining bone homeostasis, was able to reduce DNA methylation at the osteopontin promoter, thus increasing its expression. This indicates that epigenetic mechanisms could represent the means through which mechanical signals are able to regulate target gene expression and cell differentiation [121].

Both the expression of *RANKL* in osteoblasts and the concomitant stimulation of osteoclastogenesis were also shown to be regulated, at least in part, by DNA methylation. Methylation of the *RANKL* gene promoter suppressed its transcriptional activity and responsiveness to vitamin D₃ in osteoblasts, this probably being due to the binding of MECP2 to the methylated *RANKL* gene promoter, which, in turn, prevented occupancy by TBP [122, 123]. Interestingly, unlike in the cases of *ALPL* and *SOST*, the DNA methylation pattern of *RANKL* appeared to be established early in osteoblast precursors and did not undergo significant changes during osteoblastic differentiation [121].

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ALPL</em></td>
<td>Alkaline phosphatase</td>
<td>[114–117]</td>
</tr>
<tr>
<td><em>SOST</em></td>
<td>Sclerostin</td>
<td>[118]</td>
</tr>
<tr>
<td><em>OSX</em></td>
<td>Osterix</td>
<td>[119]</td>
</tr>
<tr>
<td><em>DLX5</em></td>
<td>Distal-less homeobox 5</td>
<td>[119]</td>
</tr>
<tr>
<td><em>ESR1</em></td>
<td>Estrogen receptor 1 (estrogen receptor α)</td>
<td>[120]</td>
</tr>
<tr>
<td><em>OPN</em></td>
<td>Osteopontin</td>
<td>[121]</td>
</tr>
<tr>
<td><em>RANKL</em></td>
<td>Receptor activator of nuclear factor κB ligand</td>
<td>[122–124]</td>
</tr>
<tr>
<td><em>OPG</em></td>
<td>Osteoprotegerin</td>
<td>[124]</td>
</tr>
<tr>
<td><em>SFRP1</em></td>
<td>Secreted frizzled-related protein 1</td>
<td>[125]</td>
</tr>
<tr>
<td><em>LEP</em></td>
<td>Leptin</td>
<td>[125]</td>
</tr>
</tbody>
</table>
changes during osteoblast differentiation. In addition to RANKL, a similar epigenetic regulation was also suggested for the expression of OPG. Even though DNA methylation-based regulation of RANKL and OPG expression was clearly shown at the cellular level, methylation analysis of RANKL and OPG genes in osteoporotic and osteoarthritic bone tissue samples revealed hypomethylated RANKL and OPG CpG islands without differences between the two pathologies, even though the expression of RANKL and the RANKL:OPG transcript ratio differed significantly. This could be due to the heterogeneity of bone tissue composed of cells of several different osteoblast and osteoclast differentiation stages compared to the homogeneity of individual cell lines. However, there is also the possibility that other mechanisms independent of DNA methylation are responsible for the increased RANKL:OPG ratio in osteoporotic patients [124]. In addition to analyses of individual genes, a genome-wide methylation profiling of bone tissue samples from patients with osteoporosis and osteoarthritis was also conducted, which revealed differences in the methylation of genes generally associated with the fate of less differentiated cells and with cell-matrix interactions that tend to be involved in skeletal development. The homeobox group of genes was particularly overrepresented among various differently methylated genes that, with the exceptions of SFRP1, a WNT-related inhibitor, and leptin, did not include the classic bone candidate genes discussed throughout this review. Even though this study has identified some differently methylated regions in osteoporosis and osteoarthritis and suggested a developmental component associated with these disorders, it is necessary to keep in mind that DNA methylation is only one of the many factors influencing gene expression that can change during aging or under environmental stimuli. The involvement of aberrant DNA methylation in the pathogenesis of osteoporosis and osteoarthritis is therefore still inconclusive [125].

DNA methylation plays an undeniable regulatory role in osteoblasts that, through the control of RANKL gene expression, extends to osteoclasts and bone remodeling. Nevertheless, little is known about osteoclast-specific methylation patterns and about DNA methylation upstream regulatory elements in general. Revealing the nature of the regulatory role DNA methylation plays in gene expression is difficult because methylation patterns are cell specific and because only some of the numerous CpGs present in the human genome take part in the process of gene transcription. Even though there are bioinformatic prediction tools available, it is still necessary to confirm experimentally the involvement of individual CpGs. Unlike in the case of SNPs, the analysis of single CpGs is not usually enough, which necessitates the use of sequencing techniques. Similarly to that which is the case with miRNAs, DNA methylation biomarkers can be detected in several different non-invasive biological samples like serum, plasma, blood, PBMCs, saliva, sputum, oral rinses, bronchial washings and even exhaled breath. This is especially important in the context of metabolic bone diseases where tissue samples are seldom used. In addition, DNA is the most stable biological macromolecule and its methylation represents a covalent modification that is chemically inert and resistant to sample processing perturbations [111]. Therefore, DNA methylation biomarkers possess great potential that is yet to be fully explored and utilized. It is likely that many aberrantly methylated genes will never reach wider clinical significance as biomarkers in their own right; however, when assembled into sets of several genes there is a much greater probability of them becoming useful clinical tools. We have shown that DNA methylation is an important coregulator of bone homeostasis and it is reasonable to expect that deregulated methylation patterns could be associated with certain bone disorders. Unlike in oncology, where methylation tests have already reached clinical application, we are still waiting for a bone-associated DNA methylation biomarker.

Analysis of posttranslational histone modifications still presents many challenges, ranging from the isolation of histones with intact posttranslational modifications to the simultaneous identification of numerous histones, histone variants and posttranslational histone modifications in a single sample (the latter only possible using mass spectroscopy-based proteomics). The analysis of miRNA expression and DNA methylation is perhaps less challenging, utilizing predominantly various PCR-, microarray- or sequencing-based methodologies, although their isolation from body fluids has its difficulties [67, 126].

Conclusions

The precise control of gene expression is essential for proper development, differentiation, function and homeostasis. Epigenetic mechanisms are important regulators of gene expression and are capable of establishing stable, long-term expression patterns passed on during mitosis [20]. DNA methylation, miRNAs and posttranslational histone modifications thus represent additional layers of information-focusing and, in a way, help tailor genetic information to suit each cell in accordance with its location and purpose. It therefore makes sense to look at all the complementary layers of information, genetic and
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epigenetic, in order to understand both physiological and pathological processes at the molecular level. This is even more obvious when we look at how these layers interact with each other; e.g., SNPs in miRNA coding and target regions can change the efficiency of miRNA signaling, miRNAs can down-regulate the expression of many genes, including those coding for DNA-methylating and histone-modifying enzymes, while DNA methylation can suppress miRNA expression and so on. As epigenetic mechanisms are so essential to cell biology, they are associated with all cells of the body, all biological processes and, probably, all pathologies. This last is true even though there is usually no known causal relationship. Various environmental stimuli, such as physical and mental stresses, an imbalance of nutrients, infections of bacterial or viral origin, or contact with pollutants and chemical entities, can influence the organism through epigenetic changes [113]. Epigenetic mechanisms can also be targeted by different pharmacological agents, an approach successfully adopted in the treatment of certain cancers and neurological disorders. As mentioned earlier, broad-acting HDAC inhibitors showed great osteogenic potential in vitro, but in vivo observations have indicated negative skeletal consequences [34]. However, resveratrol, a SIRT1-activating plant polyphenol, promotes osteogenesis in vitro and prevents bone loss in vivo [49, 51, Zhao et al., Unpublished manuscript]. There are also a number of miRNAs that may represent appealing pharmacological targets. The in vivo inhibition of bone resorption by overexpressing miR-503 with a specific agomir is one such example [Chen et al., Unpublished manuscript]. Hypomethylating agents are also available, although their skeletal effects are yet to be well characterized and their potential utility is limited by their widespread mechanism of action. Manipulation of epigenetic mechanisms holds great promise for the future treatment of metabolic bone diseases but a better understanding of epigenetic regulation and increased specificity of pharmacological agents are needed first. The presence of epigenetic marks in various body fluids also makes them very attractive biomarkers, especially in pathologies associated with tissues and organs normally impossible to sample. This is also the case in bone disorders, where imaging techniques still represent the main diagnostic tools, while biochemical markers have failed to reach wider significance and tissue biopsies are only seldom obtained. Epigenetic marks represent a new generation of potential biochemical markers that will hopefully enable patients at risk of bone loss to be screened before proceeding with imaging techniques, be able to identify small changes in the progress of a given disease and response to therapy beyond the capabilities of current methods, and will facilitate the personalized therapy of skeletal diseases.

Conflict of interest statement

Authors’ conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: This work was supported by the Slovenian Research Agency (Grants ARMR19, P3-298 and J3-551).

Employment or leadership: None declared.

Honorarium: None declared.

Received September 13, 2013; accepted November 19, 2013; previously published online December 18, 2013

References


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