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Review article

Role of epigenetics in alveolar bone resorption and regeneration around periodontal and peri-implant tissues

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Periodontitis and peri-implantitis are multifactorial diseases characterized by alveolar bone destruction mediated by the host response to a microbial challenge. Alveolar bone resorption mediated by epigenetics could be one of the mechanisms responsible for this destruction of alveolar bone. The relationship between epigenetic modifications and bone metabolism has been thoroughly investigated in bone remodeling, cancer, and rheumatoid arthritis, but evidence is low regarding the relationship between epigenetic modifications and alveolar bone loss related to periodontal and peri-implant diseases. Therefore, we conducted a review of the pertinent literature based on *a priori*-formulated focused questions and a screening strategy, in an attempt to comprehend the role of different epigenetic mechanisms in alveolar bone loss and to determine the current state with respect to their possible therapeutic applications in regenerative medicine. The review showed that the roles of DNA methylation, histone modifications, and non-coding RNAs in bone loss have been investigated. The results indicate that epigenetic mechanisms can participate in periodontal and peri-implant alveolar bone breakdown, suggesting their potential as therapeutic targets in alveolar bone regeneration. However, there is still only preliminary information regarding the possible therapeutic utility of these epigenetic mechanisms, suggesting a need for basic and translational research to assess the potential of such mechanisms in promoting alveolar bone regeneration.

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Periodontitis and peri-implantitis are destructive diseases of tooth and implant-supporting tissues and are induced by bacterial biofilm (1, 2). This biofilm consists mainly of gram-negative, anaerobic, and micro-aerophilic bacteria that can colonize the subgingival tissues (3–5). The bacterial biofilm provokes an inflammatory host response, which is influenced by environmental, genetic, and epigenetic factors (6–8). Epigenetic changes refer to shifts in gene expression that are not encoded in the DNA sequence (9, 10); for example, chemical alterations of the DNA and its associated proteins (called histones) through the activity of specific enzymes. Such changes lead to remodeling of the chromatin, and activation or inactivation of a gene (11, 12). Figure 1 demonstrates these chemical alterations, namely DNA methylation and histone acetylation. Other epigenetic factors that regulate gene expression are non-coding RNAs, which include microRNAs (miRNAs) (11) and long non-coding RNAs (13).

Bacterial biofilm may trigger epigenetic changes in periodontal disease; indeed studies have indicated the existence of a number of different interactive mechanisms between dental plaque and epigenetic modifications. For example, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and specificity protein 1 (SP1) are activated by the binding of bacteria to toll-like receptors (TLRs) and the subsequent activation of mitogen-activated protein kinase (MAPK) pathways (14, 15). In addition to NF-κB and SP1, which can influence chromatin structure (16), signal transduction pathways, such as MAPK, are also able – directly or indirectly – to regulate the DNA-methylation enzymes (17). Furthermore, periodontal pathogens have been shown to influence chromatin modifications; Yin & Chung (18) demonstrated that stimulation with *Porphyromonas gingivalis*, a key pathogen in periodontal disease, resulted in decreased levels of the enzymes DNA methyltransferase 1 and histone deacetylases 1 and 2 that are responsible for the chemical alterations of DNA and histones in gingival epithelial cells. The interaction between other epigenetic mechanisms and *P. gingivalis* has also been studied; it was reported that miRNAs could probably mediate endotoxin tolerance through modulation of MAPK (19), increase the sensitivity of TLRs when exposed to bacterial lipopolysaccharide (LPS), or target

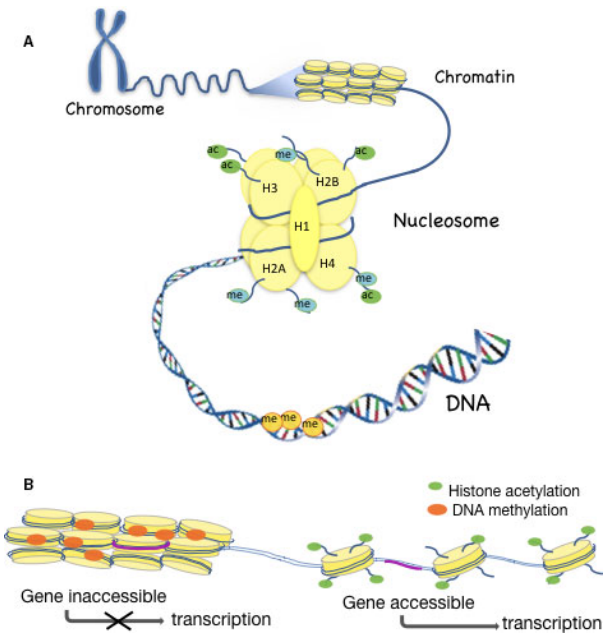

Fig. 1. Influence of epigenetic changes on gene transcription. (A) Chromatin structure. (B) Influence of DNA methylation and histone modifications on chromatin formation and gene expression. Ac, acetylation; Me, methylation. This figure was published in Larsson (12) and is reused under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

the NF-κB signaling pathway in response to bacterial stimuli (20–24). Yet, the relationship between periodontal pathogens and miRNAs is still poorly understood and requires further exploration and investigation (25). In addition, the relationship between long non-coding RNAs and bacterial biofilm needs to be investigated. Environmental and lifestyle factors, such as smoking and diet, also contribute to this complex network of interactions between an individual's immune response, bacterial biofilm (26), and epigenetics (27); these factors affect the host-defense system (28, 29), the inflammatory state in periodontal tissues (30, 31), the bacterial biofilm itself (32, 33), and the chromatin structure (27), because the epigenome is dynamic throughout life and may change in response to environmental factors (34).

Periodontal disease is characterized by alveolar bone loss (35). Interestingly, epigenetic regulation of bone tissues has been reported; epigenetic mechanisms were shown to regulate the receptor activator of nuclear factor κB ligand (RANKL) /osteoprotegerin (OPG) system and Runt-related transcription factor 2 (RUNX2) (36). In addition, a recent *in vitro* investigation demonstrated that specific epigenetic regulatory networks might represent a crucial factor for osteoblastic differentiation and thus a target for the development of preventive or therapeutic agents against osteogenic disorders (37, 38). Specifically, DNA methylation, histone modifications, and non-coding RNAs seem to play a pivotal role in alveolar bone changes, as shown by several preclinical trials (39–41). As the existing evidence on the influence of epigenetics on periodontal and peri-implant alveolar bone loss/regeneration is not yet well understood, we aimed to review this topic, based on the data in the current dental and medical literature, to gain a better understanding of this relationship and to obtain insights into the utilization of different gene-regulatory mechanisms that may provide more predictable outcomes of alveolar bone-regenerative procedures than achieved at present. Hence, epigenetic mechanisms are reversible and have therapeutic potential for improving individualized drug therapy (42).

Although we discuss both periodontal and peri-implant diseases, it must be borne in mind that both diseases differ in their histopathology and thus do not present a single disease entity.

Material and methods

This narrative review is based on preclinical and clinical studies identified with no restrictions regarding language or year of publication. We addressed the following focused questions:

(i) Which, if any, are the epigenetic mechanisms that play a role in periodontal and peri-implant tissue breakdown?
(ii) Which, if any, epigenetic mechanisms may have utility as potential therapeutic applications in alveolar bone regeneration?

Electronic and manual searches of the literature in several databases (namely MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials and Cochrane Oral Health Group Trials Register databases) were conducted by two independent reviewers (FA, LL) for articles up to June 2018 without language restriction. The search strategy is further described in Appendix S1. Three reviewers (FA, AM, LL) independently extracted the data from studies identified as relevant in the database search.

Additionally, a manual search of periodontics-related journals, namely *Journal of Dental Research, Journal of Clinical Periodontology, Journal of Periodontology*, and *The International Journal of Periodontics & Restorative Dentistry*, up to June 2018, was also performed to ensure a thorough screening process. The reference lists of articles identified were also screened for relevant articles.

Data were extracted from relevant articles and, if indicated, are presented in evidence tables. In the following, the overall findings are summarized in a narrative manner according to the mechanisms described in the literature retrieved.

Role of epigenetics in periodontal tissue breakdown

DNA methylation

DNA methylation involves the covalent addition of methyl groups to the 5′ position at the base cytosine (5mC) and is regulated by DNA methyltransferases (9, 43).

Periodontal ligament cells have the potential to differentiate into osteoblasts via a process that involves *RUNX2*, a specific gene marker that plays a vital role in bone formation and osteoblast differentiation (44, 45). Human periodontal ligament cells that are stimulated with LPS

from *P. gingivalis* express high levels of DNA methyltransferase 1 and low levels of RUNX2 (46). This indicates that DNA hypermethylation of *RUNX2* might be involved in the inhibitory effect of LPS on osteoblastic differentiation in human periodontal ligament cells (46).

Regarding bone resorption, the RANKL–tumor necrosis factor receptor superfamily member 11A (RANK)–OPG pathway is the paramount factor in osteoclast differentiation and function (47). In studies on long bone, DNA methylation induced by DNA methyltransferase 3a has been shown to regulate osteoclastogenesis via epigenetic repression of anti-osteoclastogenic genes (48). Delgado-Calle *et al.* (36) reported lower DNA-methylation levels in cell lines in which RANKL was strongly expressed, relative to cells in which RANKL was expressed weakly. Similarly, cells with low expression of OPG showed high levels of DNA methylation. In line with the results found in cell culture, the authors reported low levels of DNA methylation in the downstream *RANKL* CpG island and in the *OPG* CpG island in bone tissue samples from patients with osteoarthritis and osteoporosis (36). In diseases characterized by localized bone loss, such as periodontitis and rheumatoid arthritis, there is an increase in expression of RANKL relative to its decoy receptor, OPG, which then leads to an increase in bone resorption (47). Therefore, the findings from studies on long bone could also apply to alveolar bone. Even so, further research is needed to explore the link between DNA methylation and alveolar bone loss through this specific pathway.

As the conversion of monocytes to osteoclasts represents a unique terminal differentiation process (49), DNA-methylation changes during the process of osteoclastogenesis have been further assessed across the entire genome of monocytes and osteoclasts. The results showed that 3,515 genes were differentially methylated; hypermethylation and hypomethylation changes took place in several thousand genes (50). Furthermore, DNA hypomethylation was related to genomic regions that are activated during osteoclastogenesis, while hypermethylation was associated with the silencing of alternative lineage genes that are not expressed in osteoclasts (50). The results presented in these studies indicate that bone resorption is highly regulated by DNA methylation and might thus offer a future therapeutic avenue in bone-destructive diseases, including those related to alveolar bone.

Histone acetylation and methylation

Histones, which form nucleosomes that are the building blocks of chromatin, can be acetylated or methylated. Acetylation of histones is regulated by histone acetyltransferases that add acetyl groups to histones and by histone deacetylase that remove the acetyl groups (51). On the other hand, histone methylation is regulated by histone methyltransferases and histone demethylases (52). Histone acetylation is linked to transcriptional activation (53), while histone methylation is associated with differential gene expression (i.e., active transcription or repression), depending on the location of the methyl lysine residue on a histone tail and the degree of methylation (52).

Interferon regulatory factor 8 (IRF-8) is key molecule for osteoclastogenesis that is regulated by epigenetic mechanisms. A negative regulator of osteoclast differentiation, IRF-8 plays an important role in decreasing bone resorption (54). Recently, Fang *et al.* (54) demonstrated that the enhancer of zeste homolog 2 (EZH2), a histone methyltransferase that epigenetically silences gene expression by histone methylation in osteoclastogenesis, was recruited to the *IRF8* promoter after stimulation with RANKL. This resulted in histone methylation (H3K27me3) and a subsequent decrease in expression of *IRF8*, which allowed osteoclastogenesis to proceed. In another study, inhibition of the histone demethylase, lysine-specific histone demethylase 1, was found to promote the osteogenic differentiation of human adipose-derived stem cells by blocking its catalytic activity, which epigenetically boosts osteogenic differentiation of these cells (55).

Even though histone methylation has been investigated in osteoclastogenesis, available data are still scarce, as most investigations have focused on histone acetylation. In a recent study, Dou *et al.* (56) demonstrated that histone deacetylase 2 is a key positive regulator of RANKL-induced osteoclastogenesis: bone marrow macrophages showed increased expression of histone deacetylase 2 during osteoclastogenesis, whereas deletion of histone deacetylase 2, through Lentivirus infection, suppressed bone resorption. By contrast, Stemig *et al.* (57) demonstrated that conditional deletion of histone deacetylase 7 in mouse osteoclasts leads to *in vivo* enhancement of osteoclast formation that is associated with increased bone resorption. Conditional deletion of histone deacetylase 7 has also been shown to promote the proliferation of osteoclast precursor cells and enhance RANKL-induced osteoclast differentiation (58). Similarly, histone deacetylase 9 was shown to inhibit the process of osteoclastogenesis as *ex-vivo* osteoclast differentiation was accelerated by deletion of histone deacetylase 9 but diminished by overexpression of histone deacetylase 9. Histone deacetylase 9 knockout mice exhibited elevated bone resorption and lowered bone mass compared with wild-type mice (59). These findings reflect different regulatory functions of different histone deacetylases in the osteoclastogenesis process.

Regarding the role of histone deacetylases in periodontal diseases, Cantley *et al.* (60) reported an increased expression of mRNA from several histone deacetylase genes in gingival tissues from patients with periodontitis relative to controls. Interestingly, of all the histone deacetylase proteins investigated, only cells positive for histone deacetylase 1 were proportionately more frequent in diseased tissues than in non-periodontitis tissues (60).

As for the relationship between histone deacetylases and osteoblasts, very few studies are available. In brief, *in vitro* studies have concluded that inhibition of histone deacetylases results in the acceleration of osteoblast maturation, matrix mineralization, and the expression of genes that are associated with osteoblast differentiation, such as collagen type I (*COL1*), bone sialoprotein (*BSP*), osteopontin (*OPN*), osteocalcin (*OCN*), osterix (*OSX*), and *RUNX2*, through the upregulation of *RUNX2* transcriptional activity and Wnt receptor genes (61). Findings regarding the relationship between histone deacetylases and osteoblasts differ depending on whether a study is conducted on animals or humans. In addition to potential differences between species, histone deacetylase inhibitors react to different structural alterations of histones and to different histones, which makes it difficult to compare the results of different studies. The exact function of histone deacetylase inhibitors used is not always well described in studies. This could be a result of the animal model, the presence of confounding factors in human studies, and the differences in specificity and affinity between different histone deacetylase inhibitors (61).

Use of DNA methyltransferase inhibitors and histone deacetylase inhibitors as potential therapeutics in alveolar bone regeneration

Epi-drugs is the term coined for epigenetic substances that target DNA-methylation and histone-acetylation processes with the aim of reversing epigenetic changes of the chromatin structures (11). Interestingly, epi-drugs (i.e., DNA methyltransferase inhibitors and histone deacetylase inhibitors) have been demonstrated to influence bone formation.

Cho *et al.* (62) demonstrated that treatment of human gingival fibroblasts with 5-aza-2′-deoxycytidine, a DNA methylation inhibitor, induces demethylation in the hypermethylated CpG islands of *RUNX2* and alkaline phosphatase (*ALP*), another osteogenic lineage marker gene. In another study, treatment of different cell lines with 5-aza-2′-deoxycytidine resulted in increased expression of RANKL and OPG, confirming that DNA methylation was indeed repressing expression of RANKL and OPG (36). These results suggest that this approach might represent a novel therapeutic modality for alveolar bone regeneration. In line with this suggestion, *in-vitro* application of 5-aza-2′-deoxycytidine to decrease DNA-methylation levels in human periodontal ligament stem cells was capable of rescuing the osteogenic capacity of these cells under high-glucose conditions (63). A subsequent animal experiment showed that diabetic rats with increased DNA-methylation levels in their periodontal ligaments exhibited reduced bone mass and bone density (63). It seems that suppression of DNA methylation could enhance the osteogenic capacity of human periodontal ligament stem cells even in pathological states, such as diabetes mellitus.

When human osteoclast precursors were treated with GSK126, a selective EZH2 inhibitor, recruitment of EZH2 to IRF-8 was inhibited, thereby suppressing osteoclastogenesis (54). This indicates a potential clinical application for GSK126 as a treatment modality for bone-resorptive diseases (54). This might be useful for treating alveolar bone resorption related to periodontal disease because IRF-8 plays an important role in attenuating LPS-induced inflammatory bone resorption and LPS-induced osteoclastogenesis (64).

Histone deacetylase inhibitors have been investigated as treatment modalities of bone-related diseases because they epigenetically regulate the expression of bone-related genes, affecting osteoclast differentiation, maturation, and activity [for review see Cantley *et al.* (65)]. In fact, histone deacetylase inhibitors make the chromatin more accessible for gene expression and increase histone acetylation, as removal of acetyl groups by histone deacetylases results in gene inactivation and a subsequent decrease in gene expression [for review see Bradley *et al.* (66)]. Several histone deacetylase inhibitors have been approved by the US Food and Drug Administration (FDA) as clinical therapeutic modalities and have shown good results in treatment of cancers (67). The role of histone deacetylase inhibitors has been assessed in bone regeneration therapy, specifically in human periodontal ligament cells. Huynh *et al.* (68) demonstrated that treatment of human periodontal ligament cells with trichostatin A, a histone deacetylase inhibitor, induced osteogenic differentiation and increased the expression of osteoblast-related genes. In addition, trichostatin A-induced hyperacetylation of histone H3 decreased expression of histone deacetylase 3 during osteogenic differentiation and promoted expression of RUNX2 in human periodontal ligament cells (68). A separate report showed that trichostatin A promoted the osteogenic differentiation of adipose-derived stem cells, enhanced histone H3 acetylation, and enhanced the expression of RUNX2 (69). From a periodontal point of view, enhancing the osteogenic differentiation of adipose-derived stem cells is of importance as these stem cells have already shown potential in periodontal tissue regeneration (70).

Cantley *et al.* (39, 40) reported that histone deacetylase inhibitors suppress bone loss in rheumatoid arthritis as well as in periodontitis, and they have therefore been suggested as potential treatment modalities for these diseases. The histone deacetylase inhibitor, 1179.4b, was shown to suppress alveolar bone loss but not gingival inflammation (39). Table 1 provides a summary of studies on histone deacetylase inhibitors and histone deacetylases in periodontal tissues.

The effect on osteoclastogenesis of MS-275, another histone deacetylase inhibitor, was assessed *in vitro* (71); the results showed that MS-275 inhibited osteoclast differentiation of bone marrow-derived macrophages by suppressing RANKL-induced expression of c-Fos and nuclear factor of activated T cells 1 (71). This inhibitory effect of MS-275 on RANKL-induced osteoclastogenesis was further supported by the findings of Dou *et al.* (56).

Inhibitors of bromodomain and extra-terminal motif (BET) proteins have also been studied in bone resorption. The BET proteins are regulatory molecules of the chromatin that binds to acetylated histones (72). Meng *et al.* (72) assessed the effect of JQ1, a BET inhibitor, in an experimental periodontitis model and found that JQ1 suppressed the transcription of LPS-stimulated inflammatory cytokines and RANKL-induced osteoclast markers. Even though several studies show promising results regarding the potential of histone deacetylase inhibitors in bone remodeling through inducing bone formation and limiting bone resorption, it has been suggested that histone deacetylase inhibitors could exert negative effects in the long term, perhaps even inducing an increase in bone resorption (66, 73). In periodontitis, numerous types of cells are present at the site of bone destruction, including inflammatory cells; thus, inhibition or knockdown of histone deacetylases might have an opposite effect on those cells, resulting in increased bone resorption or enhanced inflammatory responses. Further research is therefore needed to elucidate the combined effect of histone deacetylase inhibitors in the treatment of localized bone destruction.

MicroRNAs

Other epigenetic mechanisms that regulate gene expression through post-transcriptional modifications are the miRNAs (11). These molecules represent a group of small, non-coding RNAs, of about 22 bp in length (74), that regulate gene expression by binding to the 3′ untranslated region of a target mRNA. This leads to suppression of gene expression, either by degradation of a target mRNA or by preventing translation (75). Interestingly, one miRNA can control the expression of several genes, and the expression of a certain gene can be controlled by several miRNAs (76). MicroRNAs modulate cellular processes, such as cell growth, apoptosis and differentiation, and play key roles in inflammatory responses and in the development of diseases such as cancer and rheumatoid arthritis (77). As miRNAs are vital factors in osteoclastogenesis, osteogenesis, and osteoclast/osteoblast differentiation, they have been investigated in bone-related diseases and in bone-remodeling processes (78–82).

Table 1

Summary of studies that have investigated histone modifications of alveolar bone in periodontal disease

Study	Marker	Material	Method	Results
Cantley *et al.* (39)	Histone analysis	Experimental periodontitis mouse model	Micro-computed tomography Histologic analysis	HDACi suppressed bone loss. Differences were found for the different HDACis used
Cantley *et al.* (60)	Histone analysis, TNF-α	Gingival tissues from 21 patients with periodontitis and 19 patients without periodontitis	Real-time PCR Immunohistochemistry	HDACs 1, 5, 8, and 9 were upregulated in tissues with chronic periodontitis compared with those without periodontitis These HDACs could therefore be targeted with a specific HDACi
Meng *et al.* (72)	IL-1β, IL-6, TNF-α, IL-17, IL-10, and TGF-β	Experimental periodontitis mouse model	Histologic analysis Quantitative PCR	Alveolar bone loss was alleviated in JQ1-treated mice because of reduced osteoclast numbers in periodontal tissues

HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; IL, interleukin; PCR, polymerase chain reaction; TGF, transforming growth factor; TNF, tumor necrosis factor.

Specifically, miRNAs affect osteoclastogenesis and osteoclast differentiation either by directly regulating osteoclast activity, signaling intermediates, or through negative-feedback loops, while they control osteogenic lineage commitment of various stem cells through positive-feedback loops (83). Thus, the study of miRNAs provides new insights into the pathophysiology of diseases related to bone, and miRNAs may represent an important therapeutic target for regenerative medicine. Expression of miRNAs in tissues affected by periodontal disease has also been studied (19, 84–89)], and the results of these studies are summarized in Table 2. Although the findings suggest a relationship between miRNAs, development of periodontitis, and periodontal tissue hemostasis, their role in alveolar bone resorption is still largely unknown and needs to be elucidated. Therefore, we reviewed all the miRNAs that were reportedly expressed in periodontitis tissues and present here those that are involved in osteoclastogenesis, osteogenesis, and osteoclast/osteoblast differentiation. As the interaction of miRNAs with surrounding tissues is a complex process, we illustrate (in Fig. 2) their target key genes and proteins that are relevant to alveolar bone destruction. We also present a summary of the miRNAs discussed and their target genes/transcription factors/proteins (Table 3).

miRNA-20a. miRNA-20a promotes osteogenic differentiation by upregulating the bone morphogenetic protein (BMP)/RUNX2 signaling pathway (90). It stimulates the expression of BMPs 2 and 4, RUNX2, OSX, OCN, and OPN, which are osteoblastic regulators and markers, by targeting adipogenic markers such as peroxisome proliferator-activated receptor gamma (PPARγ), BMP, and activin membrane-bound inhibitor and cysteine rich transmembrane BMP regulator 1 (90). In gingival tissues affected by periodontitis, the expression of miRNA-20a was more than fivefold higher than that in healthy tissues (85).

miRNA-21. Several studies have shown that miRNA-21 may play a key role in osteoclast differentiation and thus is a positive regulator of osteoclastogenesis. Sugatani *et al.* (91) demonstrated that RANKL-induced c-Fos resulted in the upregulation of miRNA-21, which downregulated the level of programmed cell death 4 protein, a negative regulator of osteoclastogenesis (91). In another study from the same group, estrogen was found to decrease the expression of mouse bone marrow-derived monocyte/macrophage (BMM) precursors, resulting in increased transcription of Fas ligand and subsequently in osteoclastic apoptosis (92). In concordance, Kagiya & Nakamura (93) reported that treatment with tumor necrosis factor-alpha (TNF-α) + RANKL increased expression of miRNA-21 by 3- to 4.5-fold relative to the expression in untreated RAW264.7 cells and BMMs. However, expression of miRNA-21 in osteoclastogenesis may be induced by TNF-α signaling, rather than by RANKL signaling, as no increase in miRNA-21 expression was observed in either RAW264.7 cells or BMMs following treatment with RANKL (93). In periodontal disease, Lee *et al.* (84) reported overexpression of miRNA-21 in tissues affected by periodontitis.

miRNA-29b. There are conflicting reports from the literature regarding the regulatory role of miRNA-29b in osteoclastogenesis and osteogenesis. Rossi *et al.* (94) concluded that miRNA-29b negatively regulates human osteoclastic cell differentiation and function by suppressing c-Fos and matrix metalloproteinase-2. Furthermore, miRNA-29b was able to downregulate certain inhibitors of osteoblast differentiation in mice, thus promoting osteogenesis (95). By contrast, Franceschetti *et al.* (96) reported that expression of all miRNA-29 family members (a, b, and c) not only increased during osteoclast differentiation of BMMs in mice, but also were able to target genes that are important in osteoclast differentiation and function. The reason for these conflicting reports regarding the regulatory role of miRNA-29b in osteoclastogenesis between humans and mice is still unclear. One possible interpretation could be that miRNA-29 promotes the initial osteoclast lineage commitment but inhibits osteoclast maturation as cells with higher stage of differentation were used in the human study (81). In line with these findings, Kagiya & Nakamura (93) reported upregulation of miRNA-29b expression in cells treated with TNF-α + RANKL compared with cells treated only with RANKL. In tissues affected by periodontitis, miRNA-29b was reportedly overexpressed (84).

Table 2

Summary of studies that have investigated microRNAs (miRNAs) in periodontal tissues, showing the regulation of expression of bone-related miRNAs in subjects with periodontitis compared with periodontally healthy subjects

Study	Marker	Material	Method	Bone-related miRNA expression in gingival tissues of patients with periodontitis*
Na *et al.* (19)	miRNA profiling of samples	Gingival tissues from three healthy individuals and five patients with chronic periodontitis	miRNA PCR array system	Upregulated: miRNA-34a
Lee *et al.* (84)	miRNA profiling of samples	Gingival biopsies from patients with chronic periodontitis and individuals with clinically healthy gingiva (the number of subjects in each group was not mentioned)	miRNA PCR array system	Upregulated: miRNA-21, miRNA-29b, miRNA-34a, miRNA-125a
Xie *et al.* (85)	miRNA profiling of samples	Gingival tissues from 10 patients with periodontitis and 10 healthy individuals	Microarray confirmed by real-time PCR	Upregulated: miRNA-20a, miRNA-31, miRNA-141, miRNA-146a Downregulated: miRNA-155, miRNA-182, miRNA-200a
Stoecklin-wasmer *et al.* (86)	miRNA profiling of samples	Gingival tissues from 86 patients with periodontitis and 40 healthy individuals	Microarray confirmed by real-time PCR	Upregulated: miRNA-148a, miRNA-155, miRNA-223, Downregulated: miRNA-31, miRNA-141, miRNA-200a, miRNA-210
Ogata *et al.* (88)	miRNA profiling of samples	Gingival tissue samples from three patients with chronic periodontitis (inflamed gingiva) and three patients with edentulous residual ridges (non-inflamed gingiva)	Microarray confirmed by real-time PCR	Upregulated: miRNA-150, miRNA-223 Downregulated: miRNA-34a
Motedayyen *et al.* (89)	miRNA-146a, TNF-α, IL-1β, and IL-6	Gingival tissues from 20 patients with chronic periodontitis and 10 healthy individuals	Real-time PCR	Upregulated: miRNA-146a, which also was accompanied by a significant reduction in TNF-α and IL-6

*Only results of bone-related miRNAs are presented.
IL, interleukin; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

miRNA-31. Studies have shown that miRNA-31 acts as a positive regulator of osteoclastogenesis. Mizoguchi *et al.* (97) reported upregulation of miRNA-31 expression during osteoclast differentiation under RANKL stimulation and that miRNA-31 controls osteoclast formation by targeting the ras homolog family member A gene, which plays a fundamental role in actin ring formation in osteoclasts. Inhibition of miRNA-31 expression was shown to strongly impair the formation of an actin ring in osteoclasts (97). This ring is an important structure for tight osteoclast adhesion to the bone surface (98). Moreover, miRNA-31 suppresses osteogenic differentiation of bone marrow-derived mesenchymal stem cells by targeting OSX (99). In periodontal disease, miRNA-31 was either overexpressed (85) or underexpressed in affected tissues (86).

miRNA-34a. Krzeszinski *et al.* (100) reported that miRNA-34a is a critical suppressor of osteoclastogenesis because it was downregulated during differentiation of both mouse bone marrow precursors and human peripheral blood mononuclear cells to osteoclasts. Expression of miRNA-34a inhibited osteoclastogenesis through the suppression of transforming growth factor B induced factor 2. Although miRNA-34a expression was reportedly upregulated during differentiation of human stromal stem cells to osteoblasts (101), its 'overexpression' inhibited early commitment and late differentiation of human stromal stem cells to osteoblasts *in vitro* (101). Therefore, it seems that miRNA-34a exhibits unique dual regulatory effects by controlling both human stromal stem cell proliferation and osteoblastic differentiation (101). Jagged 1 was identified as an miRNA-34a target during osteoblast differentiation (101). Regarding expression of miRNA-34a in periodontal disease, conflicting findings are reported in the literature; decreased expression was reported in periodontitis tissues examined from Japanese patients (88), while overexpression was found in inflamed gingival tissues in other studies (19, 84).

miRNA-125a. Conflicting results have been reported regarding the regulatory role of miRNA-125a in bone tissues. For example, Guo *et al.* (102) suggested that miRNA-125a negatively regulates osteoclastogenesis by inhibiting osteoclast differentiation of $CD14^+$ peripheral blood mononuclear cells through TNF-receptor-associated factor 6, while expression of miRNA-125a was downregulated during macrophage colony-stimulating factor 1 (M-CSF) + RANKL-induced osteoclastogenesis. Moreover, overexpression of miRNA-125a in $CD14^+$ peripheral blood mononuclear cells inhibited osteoclastogenesis (102). By contrast, de la Rica *et al.* (49) demonstrated the rapid upregulation of two miRNA clusters – miRNA-212/132 and miRNA-99b/let-7e/125a – in the osteoclastic differentiation of human primary monocytes by targeting monocyte-specific and immunomodulatory genes, such as TNF alpha-induced protein 3, insulin-like growth factor 1 receptor, and interleukin-15. Lee *et al.* (84) reported overexpression of miRNA-125a in periodontitis-affected tissues compared with healthy tissues.

miRNA-141 and miRNA-200a. Both miRNA-141 and miRNA-200a inhibit osteoblast differentiation through repression of the distal-less homeobox 5 (*DLX5*) gene, which produces an osteogeneic transcription factor that

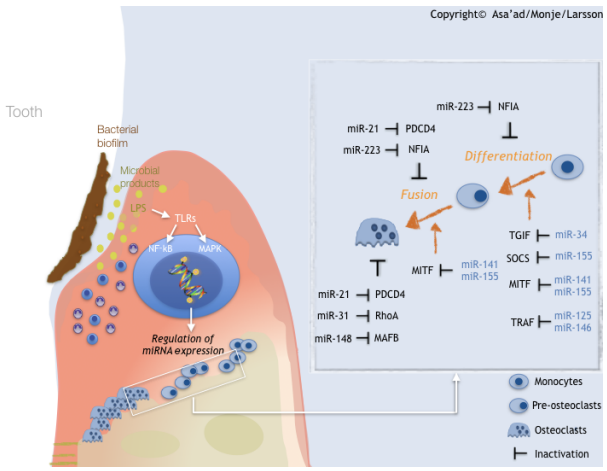

Fig. 2. MicroRNAs (miRNAs) regulating osteoclastogenesis in periodontitis-affected tissues, and the targets of these miRNAs in alveolar bone. The illustration briefly demonstrates the influence of bacterial biofilm on the expression of miRNAs. When tissues are exposed to bacterial lipopolysaccharide (LPS), expressed miRNAs can increase the sensitivity of toll-like receptors (TLRs) or can target nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway or can mediate endotoxin tolerance through modulation of mitogen-activated protein kinase (MAPK). miRNAs marked in black are positive regulators of osteoclastogenesis and osteoclastic differentiation. miRNAs marked in blue are negative regulators of osteoclastogenesis and osteoclastic differentiation. MAFB, MAF BZIP transcription factor B; MITF, microphthalmia-associated transcription factor; NFIA, nuclear factor I/A; PDCD4, programmed cell death 4 protein; RhoA, ras homolog family member A; SOCS, suppressor of cytokine signaling-1; TGIF, transforming growth factor B induced factor; TRAF, TNF-receptor-associated factor.

participates in pre-osteoblast differentiation (103). Both miRNAs were found to be underexpressed in periodontal disease (86), while Xie *et al.* (85) reported increased expression of miRNA-141 and decreased expression of miRNA-200a in periodontitis-affected gingival tissues. Interestingly, miRNA-141 might also act as a negative regulator of osteoclastogenesis, in contrast to what could be expected; Ell *et al.* (80) demonstrated that miRNA-141 downregulates osteoclastogenesis by targeting microphthalmia-associated transcription factor and human calcitonin receptor gene, and that its expression strongly inhibits osteoclastic differentiation. These findings show that a single miRNA might target different pathways with different functions. Therefore, only pathway-specific miRNAs should be considered for use as therapeutic modalities.

miRNA-146a. Nakasa *et al.* (78) reported that overexpression of miRNA-146a inhibits osteoclast formation and thus acts as a negative regulator of osteoclastogenesis. The direct target gene of miRNA-146a was identified as TNF-receptor-associated factor 6 (104), one of the major factors in osteoclastogenesis (78). Interestingly, Kagiya & Nakamura (93) reported that expression of miRNA-146a increased during TNF-α-regulated osteoclast differentiation. Taken together, it was suggested that miRNA-146a expression, induced by treatment with TNF-α + RANKL, might serve as a negative feedback regulator of osteoclastogenesis (93). Different studies have reported overexpression of miRNA-146a in gingival tissues of patients with periodontitis (85, 89).

miRNA-148a. Studies have shown that miRNA-148a acts as a positive regulator of osteoclastogenesis and promotes osteoclast differentiation by targeting the MAF BZIP transcription factor B gene (105). Under normal conditions, MAF BZIP transcription factor B prevents osteoclast differentiation in response to RANKL-induced osteoclastogenesis. Overexpression of miR-148a in $CD14^+$ peripheral blood mononuclear cells promoted osteoclastogenesis, whereas inhibition of miR-148a suppressed it (105). Stoecklin-Wasmer *et al.* (86) found that miRNA-148a was overexpressed in gingival tissues affected by periodontal disease.

miRNA-150. Results of studies of miRNA-150 suggest that it acts as a negative regulator of osteoclastogenesis. Dong *et al.* (106) demonstrated that the endogenous levels of miRNA-150 supported the osteoblastic phenotype expression of MC3T3-E1 cells. In addition, Choi *et al.* (107) reported that osteoclast differentiation was accompanied by downregulation of miRNA-150 expression, and that miRNA-150 knockout mice exhibited decreased bone mass and an increased number of osteoclasts (107). The knockout mice had significantly lower serum levels of OPG, which may induce B-cell expansion and subsequently increase the serum levels of immunoglobulins that, in turn, activate osteoclast differentiation (107). miRNA-150 was among the most highly expressed miRNAs in tissues from Japanese subjects with periodontitis (88).

miRNA-155. Several studies have suggested miRNA-155 as a negative regulator of osteoclastogenesis. In an *in vitro* investigation, miRNA-155 partly mediated the inhibitory effect of interferon-β, a suppressor of osteoclast differentiation, on osteoclastogenesis (108). Moreover, expression of miRNA-155 in BMMs from mice suppressed osteoclast differentiation by targeting two positive regulators of osteoclastogenesis: suppressor of cytokine signaling-1; and microphthalmia-associated transcription factor (108). Likewise, Mann *et al.* (109) reported that miRNA-155 suppressed osteoclast formation by targeting microphthalmia-associated transcription factor and transcription factor PU.1, and that its expression decreased during osteoclast differentiation. However, different results were reported by Blüml *et al.* (110) who demonstrated reduced differentiation of BMMs into osteoclasts in miRNA-155 knockout mice in a rheumatoid arthritis model. Similarly, Kagiya & Nakamura (93) reported upregulation of miRNA-155 in murine BMMs following treatment with TNF-α+RANKL+M-CSF, but downregulation of miRNA-155 following treatment with RANKL+M-CSF. By contrast, Mizoguchi *et al.* (111) reported that RANKL treatment did not alter miRNA-155 expression in wild-type mice. Based on these results, it was suggested that downregulation of miRNA-155 may not be necessary for osteoclastogenesis (41, 93). Regarding periodontal disease, conflicting findings are reported in the literature; miRNA-155 expression has been found to be either decreased (85) or increased (86) in tissues affected by periodontal disease.

miRNA-182. Recent studies confirmed that miRNA-182 is a positive regulator of osteoclastogenesis; Miller *et al.* (112) demonstrated that the upregulation of miRNA-182

Table 3

Summary of osteoclastogenesis and osteogenesis-related microRNAs (miRNAs) and their target

miRNAs	Target(s)	Function(s)	Reference
miRNA-20a	PPARγ, BAMBI, CRIM1	Positive regulator of osteogenesis	Zhang *et al.* (90)
miRNA-21	PDCD4, FasL	Positive regulator of osteoclastogenesis	Sugatani *et al.* (91), Sugatani & Hurska (92), Kagiya & Nakamura (93)
miRNA-29b	c-Fos and MMP-2	Positive and negative regulator of osteoclastogenesis	Rossi *et al.* (94), Franceschetti *et al.* (96)
miRNA-31	*RhoA*, OSX	Positive regulator of osteoclastogenesis and negative regulator of osteogenesis	Mizoguchi *et al.* (97), Baglìo *et al.* (99)
miRNA-34a	*TGIF2*, *JAG1*	Negative regulator of osteoclastogenesis and positive regulator of osteogenesis	Krzeszinski *et al.* (100), Chen *et al.* (101)
miRNA-125a	*TRAF6*, *TNFAIP3*, *IGF1R*, *IL15*	Negative or positive regulator of osteoclastogenesis	Guo *et al.* (102)
miRNA-141	*MITF*, *CALCR*, *DLX5*	Negative regulator of osteoclastogenesis Negative regulator of osteogenesis	Ell *et al.* (80), Itoh *et al.* (103)
miRNA-146a	*TRAF6*	Negative regulator of osteoclastogenesis	Nakasa *et al.* (78)
miRNA-148a	*MAFB*	Positive regulator of osteoclastogenesis	Cheng *et al.* (105)
miRNA-150	OPG	Negative regulator of osteoclastogenesis	Choi *et al.* (107)
miRNA-155	*MITF*, *SOCS1*, PU.1	Negative regulator of osteoclastogenesis	Zhang *et al.* (108)
miRNA-200a	*DLX5*	Negative regulator of osteogenesis	Itoh *et al.* (103)
miRNA-182	*FOXO3*, *MAML1*, PKR	Positive regulator of osteoclastogenesis	Miller *et al.* (112), Inoue *et al.* (113)
miRNA-210	*ACVR1B*	Positive regulator of osteogenesis	Mizuno *et al.* (114)
miRNA-223	*NFIA*	Positive and negative regulator of osteoclastogenesis	Sugatani & Hruska (115), M'Baya-Moutoula *et al.* (116)

ACVR1B, activin A receptor type 1B; BAMBI, BMP and activin membrane-bound inhibitor; *CALCR*, human calcitonin receptor; CRIM1, cysteine rich transmembrane BMP regulator 1; c-Fos, a proto-oncogene; *DLX5*, distal-less homeobox 5; FasL, Fas ligand; *FOXO3,* forkhead box O3; *IGF1R*, insulin-like growth factor 1 receptor; *IL15*, interleukin-15; *JAG1*, jagged 1; *MAFB*, MAF BZIP transcription factor B; *MAML1*, mastermind-like 1; *MITF*, microphthalmia-associated transcription factor; MMP-2, matrix metalloproteinase-2; *NFIA*, nuclear factor I/A; OPG, osteoprotegerin; OSX, osterix; PDCD4, programmed cell death 4 protein; PKR, double-stranded RNA-dependent protein kinase; PPARγ, peroxisome proliferator-activated receptor γ; PU.1, transcription factor PU.1; *RhoA*, ras homolog family member A; *SOCS1*, suppressor of cytokine signaling-1; *TGIF2*, transforming growth factor B induced factor 2; *TNFAIP3*, TNF alpha induced protein 3; *TRAF6*, TNF-receptor-associated factor 6.

resulted in the activation of osteoclastogenesis by TNF-α in mouse bone marrow-derived precursor cells. The forkhead box O3 and mastermind-like 1 genes were both identified as the direct targets for miRNA-182 that are suppressors of osteoclastogenesis. In another investigation (113), double-stranded RNA-dependent protein kinase (PKR) was identified as the new and essential miRNA-182 target. It was suggested that PKR is a novel inhibitor of osteoclastogenesis via regulation of the endogenous interferon (IFN)-β-mediated autocrine feedback loop. These findings revealed a previously unrecognized regulatory network mediated by the miR-182/PKR/IFN-β axis in osteoclastogenesis, which significantly correlated with the osteoclastogenic capacity of monocytes in rheumatoid arthritis (113). Interestingly, the expression of miRNA-182 was decreased in tissues affected by periodontitis (85).

miRNA-210. Findings reported in the literature suggest that miRNA-210 acts as a positive regulator for osteoblastic differentiation; miRNA-210 promoted osteoblastic differentiation of marrow-derived ST2 stromal cells through preventing transcription of the activin A receptor type 1B gene, resulting in the inhibition of the transforming growth factor-β/activin signaling pathway (114). Interestingly, an *in vitro* study concluded that miRNA-210 was upregulated in cells treated with TNF-α/RANKL in comparison with cells treated with RANKL only (93). In periodontal disease, miRNA-210 was reportedly underexpressed by twofold in gingiva affected by periodontitis (86).

miRNA-223. There are conflicting findings regarding the role of miRNA-223 in osteoclastogenesis; miRNA-223 expression was decreased during murine osteoclastogenesis (93, 115), but promoted osteoclastogenesis in humans (116). In fact, miRNA-223 facilitates osteoclast differentiation by downregulating nuclear factor I/A (117). Expression of miRNA-223 was also investigated in rheumatoid arthritis, which shares pathophysiology with periodontal disease. Fulci *et al.* (118) reported overexpression of miRNA-223 in $CD4^+$ naive T-lymphocytes from patients with rheumatoid arthritis compared with those from healthy individuals, suggesting that miRNA-223 expression in this cell type could contribute to the etiology of the disease. In another study, Shibuya *et al.* (119) reported miRNA-223 overexpression in synovium of patients with rheumatoid arthritis, which resulted in the downregulation of osteoclastogenesis marker genes. In an *in vitro* study focused on TNF-α-regulated osteoclast differentiation, miRNA-223 was downregulated during osteoclast formation (93). In a recent study in Japanese subjects, miRNA-223 was among the miRNAs that were most overexpressed in periodontitis tissues (88), which was in concordance with previous findings (86). Accordingly, Irwandi &

Vacharaska (120) suggested that miRNA-223 might be a key player in alveolar bone loss caused by periodontitis, having shown a consistent pattern of expression in gingival tissues isolated from individuals with periodontal disease.

The expression levels observed in the different periodontal tissues of some of the mentioned miRNAs contradict what has been reported regarding bone. However, miRNA expression in periodontal tissues might not be representative of that in bone, as periodontal tissues are composed of mixed cell populations. Hence, the cellular origin of miRNAs in gingival biopsies is impossible to define as the tissues analyzed contain epithelium, fibroblasts, and infiltrating inflammatory cells (86). Nevertheless, miRNA expression still reflects their involvement in periodontal disease considering that they might be transported to and from osteoclasts through exosomes (121, 122). In fact, it was suggested that the highly expressed miRNAs in periodontitis tissues are likely to be miRNAs important in osteoclastogenesis and osteoclastic differentiation (41). Regarding the discrepancy across studies of the results for expression patterns of the same miRNA in tissues from subjects with periodontitis, this might be explained by variability as a result of limited sample sizes and different methods applied in miRNA profiling. As epigenetic modifications might be influenced by genetic ancestry and environmental exposure, these factors might also add to the discrepancy in results. However, the genetic ancestry of subjects was clearly mentioned in only one study and the influence on the results therefore remains speculative.

Use of miRNAs as potential therapeutics in alveolar bone regeneration

MicroRNAs have been shown to play an important role in the differentiation of periodontal ligament stem cells into an osteogenic lineage. An *in vitro* study demonstrated that a specific type of miRNA promotes osteogenic differentiation of periodontal ligament stem cells; miRNA-218 acted as a positive regulator of osteogenesis in periodontal ligament stem cells and its ectopic expression promoted the differentiation of periodontal ligament stem cells (38). Furthermore, miRNA-218 directly targeted secreted frizzled-related protein 2, which is a Wnt signaling pathway antagonist. From these findings, it seems that miRNA-218 is an important mediator of osteoblast differentiation, thus offering a new target for the development of therapeutic agents against osteogenic disorders (38). In another *in vitro* investigation, miRNA-146a promoted the differentiation of periodontal ligament cells through the downregulation of NF-κB signaling (123), showing that certain miRNAs might be necessary for the osteogenic differentiation of periodontal ligament cells. It must be underlined that the microenvironment tends to determine the role of miRNAs in the regulation of osteoblast differentiation; microenvironment or stromal cells may activate different signaling pathways that, in turn, could be regulated by miRNAs. Liu *et al.* (124) found that miRNA-17 either inhibits or stimulates osteoblast differentiation, depending on the microenvironment. Overexpression of miRNA-17 in mesenchymal stem cells isolated from periodontitis-affected periodontal ligament tissue promoted osteogenic differentiation by increasing expression of *ALP*, *OCN*, and *RUNX2* genes through suppression of SMAD specific E3 ubiquitin protein ligase 1. By contrast, expression of *ALP*, *OCN*, and *RUNX2* genes was decreased as a result of overexpression of miRNA-17 in mesenchymal stem cells isolated from healthy human periodontal ligament tissue. These findings indicate that the administration of miRNA-17 to mesenchymal stem cells may improve bone regeneration and repair in inflammatory diseases in the case of stem cell malfunction. In line with these findings, it is reasonable to suggest that miRNAs are sensitive to specific microenvironments. Among the promising miRNAs in bone-regenerative treatment is miRNA-21, which plays a key role in osteoclast differentiation and thus acts a positive regulator of osteoclastogenesis. As such, suppression of miRNA-21 expression could serve as a potential therapeutic tool in treating bone-destructive diseases. In fact, Wei *et al.* (125) demonstrated that miRNA-21 significantly inhibited the osteogenesis of human periodontal ligament cells, while inhibition of miRNA-21 promoted osteogenesis. Taken together, inhibition of miRNA-21 expression might be a viable therapeutic approach in arresting the alveolar bone destruction caused by periodontal diseases, especially because there are marked differences between tissues affected by periodontitis and healthy tissues in their expression of miRNA-21. Another miRNA that might be a future therapeutic target is miRNA-182. Inuoe *et al.* (113) demonstrated that pharmacological treatment of ovariectomy-induced osteoporosis and inflammatory arthritis with miRNA-182 inhibitors completely suppressed pathologic bone erosion, suggesting that a miRNA-182 inhibitor may represent an effective regulator to control inflammatory osteoclastogenesis and bone resorption.

The use of miRNAs as part of therapeutic 'delivery systems' has also been investigated. The inhibitory function of miRNA-146a in osteoclastogenesis was demonstrated in a novel miRNA delivery system based on virus-like particles of bacteriophage MS2, which transported miRNA-146a into peripheral blood mononuclear cells (126). In fact, miRNA delivery systems have been strongly proposed for the treatment of osteoporosis (82) and it seems that miRNA-146a is a valuable candidate in the treatment of diseases related to bone destruction. Nakasa *et al.* (78) reported that administration of miRNA-146a prevented joint destruction in arthritic mice and thus might be a principal therapeutic strategy for such bone pathologies. In a different investigation, Liu *et al.* (127) established an efficient delivery system of miRNA-148a antagonist to reduce bone resorption in resorbed surfaces, with minimal off-target effects. Similarly, Dong *et al.* (106) showed that miRNA-150 supported the osteoblastic phenotype related to osteoblast function and bone mineralization and thus can be used as a therapeutic target to promote bone formation.

Although miRNA-223 has been proposed as a feasible therapeutic option in the case of excessive osteoclastic activity (115), there is a lack of consensus in the literature regarding the use of this particular miRNA as a result of different findings in murine models and humans (128), and therefore further *in vitro* studies on human cells are needed to verify the effect shown in human studies. It must be highlighted that cell culture studies can be of importance in understanding the treatment potentials, by providing information on cell interactions because cells in culture represent cells *in vivo* but without the complex *in vivo* environment (129).

Long non-coding RNAs

Other non-coding RNAs that participate in epigenetic modifications are the long non-coding RNAs (lncRNAs). These molecules are more than 200 nucleotides in length (130) and have been shown to have various biologic functions; they tend to regulate gene expression by binding to

chromatin regulatory proteins, thus controlling access of proteins binding to the DNA enhancer regions (131–133). From an epigenetic point of view, many lncRNAs have been shown to compete for miRNA-binding sites. They therefore act as miRNA target-site decoys and affect the miRNAs target genes and the related biological processes (134, 135). In other words, lncRNAs form a regulatory network with miRNAs that regulate target genes of miRNAs (13), and both tend to suppress each other as competing endogenous RNAs (13).

Table 4 provides a summary of the studies investigating the role of lncRNAs in periodontal disease. Despite the limited data on lncRNAs and periodontal disease, their role has been implied in periodontitis and in the osteogenic differentiation of periodontal ligament stem cells. Advances in high-throughput sequencing technologies and bioinformatic analysis have enabled the identification of key lncRNAs involved in osteogenic differentiation of mesenchymal cells (136). Recently, Wang *et al.* (130) performed a comprehensive lncRNA profiling analysis using microarray technology and were able to identify osteogenesis impairment-related lncRNA (lncRNA-POIR) in periodontal mesenchymal stem cells from patients with periodontitis. The lncRNA-POIR was under-expressed in periodontal ligament stem cells, but positively regulated osteogenic differentiation of periodontal stem cells from healthy individuals and periodontal ligament stem cells was observed in both *in vitro* and *in vivo* models. Furthermore, lncRNA-POIR acted as a competing endogenous RNA for miRNA-182, leading to re-expression of its target gene. These findings provide evidence that specific lncRNA–miRNA regulatory networks, in an inflammatory microenvironment, play a significant role in osteogenic differentiation of periodontal ligament stem cells. In concordance, He *et al.* (137) demonstrated that taurine up-regulated 1 (TUG1), an lncRNA present in abundance, was significantly upregulated in osteogenically induced periodontal ligament stem cells, and that expression of TUG1 was positively correlated with the osteogenic differentiation of periodontal ligament stem cells. Similarly, Jia *et al.* (138) demonstrated that the downregulation of antidifferentiation noncoding RNA (lncRNA-ANCR), a *RUNX2* inhibitor, promoted osteogenic differentiation of periodontal ligament stem cells and might be involved in the canonical Wnt signaling pathway. The important role played by lncRNAs in the osteogenic differentiation of periodontal ligament stem cells was further confirmed by Qu *et al.* (139), by demonstrating that lncRNAs could regulate the expression of relevant mRNAs. Nonetheless, no statistically significant difference in lncRNA-ANCR expression was observed, and the Wnt signaling pathway was not detected in pathway analysis. The discrepancy between these results and those reported by Jia *et al.* (138) could be attributed to the isolation of cell lines from different populations in these studies. In a recent bioinformatics study, three species of lncRNAs were identified as targets for miRNA-125a and miRNA-142 in the competing endogenous RNA network, namely metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), TUG1, and FYVE RhoGEF/PH domain containing 5-antisense RNA 1 (FGD5-AS1) (140). Those three lncRNAs regulate periodontitis-associated pathways and genes as follows: (i) MALAT1 regulates LPS-induced inflammatory responses by interacting with NF-κB (141), which mediates RANKL-induced osteoclastogenesis and causes destruction of alveolar bone in periodontitis (142); (ii) TUG1 inhibits cell proliferation by inducing transforming growth factor-β (143), which is involved in the interaction between fibroblasts and epithelial cells in periodontitis (144); and (iii) FGD5-AS1 expands the vascular network associated with the progression of periodontal inflammation (145) by mediating the proangiogenic action of vascular endothelial growth factor in endothelial cells (146). Therefore, it may be suggested that these three lncRNAs are involved in the lncRNA-associated competing endogenous RNA (ceRNA) network of periodontitis that could further explain the pathogenic mechanisms in periodontal disease and uncover potential therapeutic targets. Despite the lack of data on the potential of lncRNAs as therapeutic targets in bone regeneration, it is definitely important to explore their role in this respect as they form regulatory networks with miRNAs.

Based on our observations on epigenetics and periodontal tissue breakdown, we conclude the following answers to the focused questions:

(i) DNA methylation, histone modifications, and noncoding RNAs might be associated with alveolar bone loss during the course of periodontitis.
(ii) Epi-drugs (i.e., DNA methyltransferase inhibitors and histone deacetylase inhibitors) can be used to counteract bone resorption. Delivery of osteogenic miRNAs or the inhibition of osteoclastogenic miRNAs can be utilized in bone regeneration and in the treatment of bone resorption.

Role of epigenetics in peri-implant tissue breakdown

Studies on the relationship between epigenetic mechanisms and peri-implantitis are scarce and limited to DNA methylation and miRNAs.

Recent evidence suggests that site-specific DNA methylation may change around dental implants as a result of the presence of dissolved titanium particles, which could influence the epigenetic patterns (147). Global DNA methylation was more pronounced in peri-implant crevicular fluid of patients with peri-implantitis than in controls. Although the concentration of titanium particles was associated with the global methylation levels, this was actually independent of the peri-implantitis status. While these findings suggest that hypermethylation could be a potential mechanism in peri-implantitis and that dissolved titanium particles could be a local environmental factor that induces epigenetic change, further investigations are needed to determine if these associations are causal or ecological in nature. Moreover, future studies should provide a clear definition of peri-implantitis status and how the diagnosis of peri-implantitis was established, as this was not stated by the authors (147).

Regarding the relationship between peri-implantitis and miRNAs, evidence is only available from a few preclinical studies. In a canine ligature-induced peri-implantitis model (148), quantitative real-time PCR revealed that let-7g, miRNA-27a, and miRNA-145 influenced the onset and progression of peri-implantitis. This highlights the potential biological effects of the differentially expressed miRNAs and the specific enrichment of target genes involved in the MAPK signaling pathway. In a subsequent investigation, delivery of miRNA-27a was utilized in the regenerative treatment of bone defects in the same canine peri-

Table 4

Summary of studies that have investigated long non-coding RNAs (lncRNAs) in periodontal disease

Study	Marker	Material	Method	Results
Wang *et al.* (130)	lncRNA	Primary PDL cell culture of gingival tissues from 10 patients with periodontitis and 10 participants without periodontitis	Microarray analysis Quantitative real time PCR	Identification of a novel lncRNA (lncRNA-POIR) that plays a role in osteogenic differentiation of PDL cells
Jia *et al.* (138)	lncRNA-ANCR	Cell culture of PDL tissues isolated from healthy human impacted third molars	Quantitative real-time PCR	lncRNA-ANCR promoted osteogenic differentiation of periodontal ligament stem cells and might be involved in the canonical Wnt signaling pathway, thus offering a new target for oral stem-cell differentiation that could facilitate oral tissue engineering
Qu *et al.* (139)	lncRNA mRNA	PDL tissues isolated from healthy impacted third molars from 15 individuals	Microarray analysis Quantitative real-time PCR	After osteoinduction, 3557 mRNAs and 2171 lncRNAs were differentially expressed lncRNAs and mRNAs from the experimental and control groups belonged to different clusters The coding-non-coding gene coexpression (CNC) network indicated that 393 lncRNAs were closely related to osteogenesis-related mRNAs
Li *et al.* (140)	lncRNA	Bioinformatics study	High-throughput RNA sequencing Microarray data	Three lncRNAs (MALAT1, TUG1, FGD5-AS1) might be involved in the lncRNA-associated ceRNA network of periodontitis

ceRNA, competing endogenous RNA; FGD5-AS1, FYVE RhoGEF/PH domain containing 5-antisense RNA 1; lncRNA-ANCR, antidifferentiation noncoding RNA; lncRNA-POIR, osteogenesis impairment-related lncRNA; MALAT1, metastasis associated lung adenocarcinoma transcript 1; PCR, polymerase chain reaction; PDL, periodontal ligament; TUG1, taurine up-regulated 1.

implantitis model (149). It must be underlined that miRNA-27a was chosen for the regenerative treatment as a result of its twofold downregulation in peri-implantitis tissue relative to healthy tissue around implants (148). The findings of this *in vivo* study showed that delivery of miRNA-27a resulted in new bone formation and reosseointegration *in vivo*, by targeting dickkopf-related protein 2 and secreted frizzled-related protein 1, which are inhibitors of the Wnt signaling pathway. Targeting Wnt signaling inhibitors activates the osteogenic and angiogenic capacity of Wnt signaling and ameliorates the TNF-α-mediated inhibition of bone formation (149).

From *in vitro* experiments, it can be extrapolated that the implant surface could affect osseointegration through the differential expression of miRNAs. For instance, zirconium oxide results in a downregulation of osteogenic genes via an increase in the production of miRNAs, leading to downregulation of the production of BMPs 4 and 7 in comparison with machined titanium surfaces (150). Moreover, sandblasted or acid-etched titanium surfaces were shown to upregulate osteogenic genes more than machined and zirconia implants via downregulation of miRNAs that influence osteoprogenitor cells (150, 151). It might therefore be suggested that miRNAs represent a very dynamic epigenetic mechanism around dental implants that could participate in the development/progression of peri-implantitis.

Research on the relationship between peri-implantitis and epigenetics is still in its early stages. In order to understand this link more clearly, factors associated with peri-implantitis need to be investigated from an epigenetic standpoint, which could be of value in explaining certain observations stated in the literature. Periodontitis is a crucial confounder for peri-implantitis (1, 152–159). Accordingly, it might be speculated that patients with peri-implantitis exhibit the same epigenetic profile as when they had periodontitis (i.e., prior to tooth extraction). In fact, a recent pilot study has shown that DNA methylation levels were sustained in gingival tissues affected by periodontitis, despite periodontal therapy (160). Observations from DNA methylation could be also applied for miRNAs, as patients with peri-implantitis could have the same miRNA profile as when they had periodontitis. This hypothesis might, in fact, explain why completely edentulous subjects with dental implants present higher rates of peri-implantitis (161, 162) despite the fact that there is a significant reduction in periodontopathogens after full-mouth extraction (163, 164). In other words, epigenetic cues could increase susceptibility to peri-implantitis via sustained DNA methylation levels and the upregulation of pro-inflammatory cytokines through differential miRNA expression in patients previously exposed to periodontitis (165). Understanding these epigenetic cues and how they are related to peri-implantitis can be important in utilizing epigenetics as therapeutic approaches, which can improve the treatment outcomes of peri-implantitis, especially given that anti-infective treatment of peri-implantitis has shown unsuccessful outcomes with substantial recurrence at 5 years of follow-up, even in subjects enrolled in regular peri-implant maintenance programs (166). As this is merely a speculation, future studies should investigate

epigenetic biomarkers from peri-implant crevicular fluid to identify patients highly susceptible to peri-implant tissue breakdown, with a view to elucidate if epigenetic events might provoke peri-implant tissue breakdown.

Another important risk factor associated with peri-implantitis is smoking; this factor plays a fundamental role in the early stages of healing around implants, and it tends to have a significant effect on estrogen production and bone metabolism in the late stages of healing, resulting in poor bone density and downregulated expression of genes that encode bone matrix proteins (167). Although the effect of smoking on epigenetic changes has been assessed in periodontitis (168, 169), there is a lack of knowledge on how smoking induces epigenetic mechanisms related to alveolar bone resorption and compromises osseointegration; accordingly, alteration of bone responses to smoking via epigenetic cues should be further explored.

Based on our observations on epigenetics and peri-implant tissue breakdown, we conclude the following answers to the focused questions:

(i) DNA methylation and miRNAs might be associated with alveolar bone loss in peri-implantitis. Currently, no evidence is available on the relationship between histone modifications, lncRNAs, and peri-implantitis.
(ii) Delivery of miRNAs is the only therapeutic approach studied to date in the treatment of peri-implantitis.

Concluding remarks

Based on the observations reviewed in preceding sections, we conclude that:

(i) Epigenetic modifications might represent a viable therapeutic target in alveolar bone regeneration and in the treatment of local alveolar bone destruction resulting from periodontal disease.
(ii) MicroRNAs have promising therapeutic potential in that local delivery of miRNAs can be used to promote bone regeneration and to treat local bone loss. However, as a single miRNA might have different target genes, future investigations should ensure that miRNA manipulation does not interfere with unwanted target gene pathways, thus preventing adverse biologic events.
(iii) Targeting DNA methylation and histone modifications may affect unwanted targets and thus have side effects. As periodontitis and peri-implantitis tissues comprise several different cell types and as the presence of bacteria can further influence the configuration of chromatin in cells, the enhancement in knowledge of how these substances affect different cells and their signaling pathways may assist in the primary prevention of these conditions.
(iv) Epigenetic modifications might represent a novel component in the regenerative medicine paradigm; findings of a recently published review suggested that combining epigenetic therapy with scaffolds might optimize the results of tissue regeneration (170). As known, scaffolds are now being widely investigated in periodontal tissue regeneration, with different biomaterials and designs being currently studied (171). Hence, the optimal scaffold design and biomaterial aimed for the regeneration/repair of periodontal and peri-implant damaged/lost tissues must be further explored.
(v) Epigenetic modifications can be used to treat stem cells and help them differentiate into the desired lineage *in vitro*, which can then be seeded into the scaffold and eventually implanted into the area where bone regeneration is desired.
(vi) lncRNAs form a regulatory network with miRNAs; thus, exploring their role further will be helpful in unveiling their potential as future therapeutic modalities.
(vii) Factors associated with peri-implantitis, such as a history of periodontitis, might increase susceptibility of certain individuals to develop peri-implantitis via sustained DNA-methylation levels or differential miRNA expression. Therefore, further investigations on how these epigenetic cues are related to peri-implantitis in susceptible individuals are still needed.
(viii) Knowledge on the role of epigenetics in peri-implantitis is still in its early stages and therefore further investigations are still needed in this field and also for unstudied mechanisms (i.e., histone modifications, lncRNA).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Search strategy.