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Ibandronate promotes osteogenic differentiation of periodontal ligament stem cells by regulating the expression of microRNAs

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ABSTRACT

Bisphosphonates (BPs) have a profound effect on bone resorption and are widely used to treat osteoclast-mediated bone diseases. They suppress bone resorption by inhibiting the activity of mature osteoclasts and/or the formation of new osteoclasts. Osteoblasts may be an alternative target for BPs. Periodontal ligament stem cells (PDLSCs) exhibit osteoblast-like features and are capable of differentiating into osteoblasts or cementoblasts. This study aimed to determine the effects of ibandronate, a nitrogen-containing BP, on the proliferation and the differentiation of PDLSCs and to identify the microRNAs (miRNAs) that mediate these effects. The PDLSCs were treated with ibandronate, and cell proliferation was measured using the MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay. The expression of genes and miRNAs involved in osteoblastic differentiation was assayed using quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). Ibandronate promoted the proliferation of PDLSCs and enhanced the expression of alkaline phosphatase (ALP), type I collagen (COL-1), osteoprotegerin (OPG), osteocalcin (OCN), and Runx2. The expression of miRNAs, including miR-18a, miR-133a, miR-141 and miR-19a, was significantly altered in the PDLSCs cultured with ibandronate. In PDLSCs, ibandronate regulates the expression of diverse bone formation-related genes via miRNAs. The exact mechanism underlying the role of ibandronate in osteoblasts has not been completely understood. Ibandronate may suppress the activity of osteoclasts while promoting the proliferation of osteoblasts by regulating the expression of miRNAs.

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1. Introduction

Bisphosphonates (BPs) are stable analogs of pyrophosphate with a potent inhibitory effect on the differentiation of osteoclast precursors, and they can induce apoptosis of osteoclasts and stimulate the release of the osteoclastic inhibitory factor from osteoblasts [1,2]. They are widely used as therapeutic agents for many bone disorders characterized by increased resorption, e.g., Paget's disease [3], tumoral bone disease, hypercalcemia of malignancy [4], and more recently, postmenopausal osteoporosis [5]. Ibandronate is a potent nitrogen-containing BP with an affinity for hydroxyapatite. Ibandronate inhibits osteoclast-mediated bone resorption and is effective in the treatment of estrogen-deficient osteoporosis [6]. Bisphosphonates have also been shown to interact with osteoblasts and enhance their proliferation and maturation [7–9] and to inhibit apoptosis [10]. These data suggest that BPs have an anabolic effect on osteoblasts and subsequently promote bone formation.

The periodontal ligament (PDL) is a type of non-mineralized connective tissue that attaches cementum to the inner wall of the alveolar bone socket, thus holding the tooth in place [11,12]. Human PDL contains stem cells that have the ability to differentiate into cementoblasts, osteoblasts, and adipogenic cells. These PDL stem cells (PDLSCs) play an important role in the maintenance and regeneration of periodontal tissues [13–15].

MicroRNAs (miRNAs) are a class of short (~22 nucleotides) RNAs that negatively regulate the expression of target genes [16,17]. They have been shown to promote mRNA degradation and inhibit mRNA translation by binding to the 3′-untranslated regions (3′-UTR) of specific target genes [18]. Moreover, miRNAs are involved in diverse biological processes, including cell proliferation, differentiation, and apoptosis during development, stress resistance, and fat metabolism. To date, hundreds of human miRNAs have been identified, and they are known to regulate up to 30% of the protein-coding

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genes in the human genome. However, little is known about the role of miRNAs in human PDLSCs (hPDLSCs) during osteogenic differentiation or in the regulation of osteogenesis [19].

To date, few studies have investigated the osteogenic effect of ibandronate on hPDLSCs. In this study, we aimed to analyze the bioactive effects of ibandronate on the osteogenic differentiation of hPDLSCs and to determine the differential expression of miRNAs and their potential target genes that are related to osteogenic differentiation.

2. Materials and methods

2.1. Cell culture and isolation of PDLSCs isolation

Human premolars were obtained from five healthy patients for orthodontic reasons after obtaining the patients' approval and informed consent (donor age: 10–12 years). Periodontal ligament tissues were gently scraped from the middle portion of the root surface, minced into 1 mm^3 cubes, and placed into 6-well culture dishes (Costar, Cambridge, MA). The explants were grown in α-minimum essential medium(α-MEM; Gibco BRL, Rockville, MD) supplemented with 10% (v/v) fetal bovine serum (FBS) 0.292 mg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. STRO-1^+ stem cells were prepared using immunomagnetic beads (Dynabeads; Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. After washing, bead-positive cells were segregated using a magnetic particle separator and subsequently seeded into 75-cm^2 culture flasks (Costar) at 37 °C in 5% CO_2. The PDLSCs from this passage were used for further study.

2.2. Cell proliferation assays

Human PDLSCs were seeded in 96-well plates at a density of 1×10^3 cells/well. After overnight incubation, the medium was changed and ibandronate was added at concentrations ranging from 10^{-2} to 10^{-12} M. In each experiments, in which treatments were done in triplicate, cells were allowed to grow for 5 days. Then, 20-μL Aliquots of 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) (Sigma, St. Louis, MO) in PBS were added to each well, and the cells were incubated for 4 h; this was followed by the addition of 150 μL DMSO. The absorbance at 490 nm was determined using a microplate reader (model 550; Bio-Rad, USA).

2.3. RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

The expression of the osteoblast differentiation genes alkaline phosphatase (*ALP*), type I collagen (*COL-1*), and osteocalcin (*OCN*) and the bone-specific transcription factor Runx2 in PDLSCs was measured using qRT-PCR. Cells were harvested using 1 mL of TRIzol reagent (Invitrogen, Carlsbad, California, USA). Total RNA was isolated according to the manufacturer's instructions and treated with RNase-free DNase. The reverse transcription reaction was performed using 1 μg of RNA purified with the RNeasy Mini Kit (Qiagen). A SYBER Premix Taq™ II Kit (Takara) was used for qRT-PCR. Relative transcript levels were measured in a 25-μL reaction volume by using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) following the recommended protocol for SYBR-Green, and normalized with GAPDH levels (Applied Biosystems, Foster City, CA, USA), following the recommended protocol for SYBR-Green, and were normalized to GAPDH levels (Applied Biosystems, Foster City, CA, USA). The primers used for amplification are shown in Table 1. The expression levels of miRNAs related to osteogenic differentiation were also detected by qRT-PCR with the primers listed in Table 2. The miRNA levels were normalized to U6, an internal control, and were measured using the comparative C_t (ΔC_t) method. The qRT-PCR was carried out for 45 cycles (95 °C for 10 s, 60 °C for 34 s) after an initial denaturation step (95 °C for 10 s).

Table 1
PCR primers and conditions for the specific amplification of human mRNA.

Gene	Forward (5′–3′)	Reverse (5′–3′)
Gapdh	TCAAGGGCATCCTGGGCTAC	TCCACCACCCTGTTGCTGTA
ALP	TTGTGCGAGAGAAAGGAGA	GTTTCAGGGCATTTTTCAAGGT
Osteocalcin	CTGACAAAGCCTTCATGTCCAA	GCGCCGGAGTCTGTTCACTA
Collagen 1	CCCAAGGAAAAGAAGCACGTC	AGGTCAGCTGGATAGCGACATC
Runx2	ACTTCCTGTGCTCGGTGCT	GACGGTTATGGTCAAGGTGAA
Ccn2	TGACTGCCCCTTCCCGAGAA	TCTTCCAGTCGGTAGGCAGCTAGG
SMAD1	CACAAACATGATGGCGCCT	CATAGTAGACAATAGAGCACCA GTGTTT
DLX5	AGAAAGTTCGTAAACCCAGGAC	TGTCAATCCCAGCGAGGC

2.4. Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA and the post hoc Student's two-tailed *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Ibandronate promotes proliferation of PDLSCs

To determine the effects of ibandronate on PDLSC growth, MTT assays were used. Ibandronate treatment differentially affected cell proliferation in a dose-dependent manner (Fig. 1). In particular, at a concentration of 10^{-3} M, ibandronate inhibited proliferation, whereas at concentrations ranging from 10^{-6} to 10^{-10} M, ibandronate incrementally promoted proliferation. The positive effect of ibandronate on proliferation peaked at a concentration of 10^{-8} M, which resulted in a 25% increase in cell proliferation. Ibandronate at 10^{-12} M did not significantly affect cell proliferation.

Table 2
qRT-PCR microRNA primers.

microRNAs	Primers (5′–3′)
miR-125b	tccctgagaccctaacttgtga
miR-130a	cagtgcaatgttaaaagggcat
miR-18a	taaggtgcatctagtgcagatag
miR-133a	tttggtccccttcaaccagctg
miR-133b	tttggtccccttcaaccagcta
miR-135b	tatggcttttcattcctatgtga
miR-141	taacactgtctggtaaagatgg
miR-148b	tcagtgcatcacagaactttgt
miR-19a	tgtgcaaatctatgcaaaactga
miR-26a	ttcaagtaatccaggataggct
miR-27a	ttcacagtggctaagttccgc
miR-29b	tagcaccatttgaaatcagtgtt
miR-196a	taggtagtttcatgttgttggg
miR-204	ttccctttgtcatcctatgcct
miR-211	ttccctttgtcatccttcgcct
miR-200a	taacactgtctggtaacgatgt
miR-200b	taatactgcctggtaatgatga
miR-489	gtgacatcacatatacggcagc

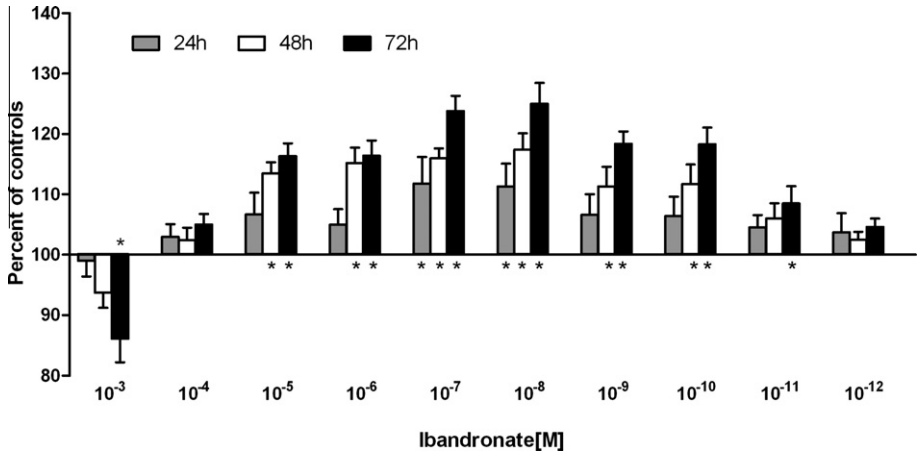

Fig. 1. Proliferation of PDLSCs treated with ibandronate. Proliferation was measured by MTT assay at different time intervals after treatment. The data are shown as the mean ± SEM, $n = 5$, $^{*}p < 0.05$, compared to the untreated control.

3.2. Ibandronate promotes the osteoblastic differentiation of PDLSCs

The expression of genes involved in osteoblastic differentiation was determined by qRT-PCR. The expression of the osteoblast-related genes *ALP*, *COL-1*, osteoprotegerin (*OPG*), *OC* and *Runx2* is indicative of the osteoblastic differentiation status of PDLSCs. Our results show that the expression of these genes was modified by ibandronate, ALP expression is an early marker of osteoblastic differentiation. We measured its expression at 4, 8, 12, and 24 h after ibnadronate (10^{-8} M) treatment. The results showed that ALP levels were upregulated 24 h after treatment with 10^{-8} M of ibandronate and reached a peak 8 h later (Fig. 2). The expression of the other four genes reflecting PDLSC differentiation status was also assessed after treatment with ibandronate (10^{-8} M) for 12, 24, and 48 h. Consequently, the mRNA levels of COL-1, OPG, OC, and Runx2 were also found to be upregulated by ibnadronate (Fig. 2).

3.3. Ibandronate promotes the expression of several miRNAs

Several miRNAs, such as miR-125b, miR-141, and miR-135, are involved in the regulation of osteoblastic differentiation (Table 3). To measure the expression of these miRNAs in PDLSCs with or without ibandronate treatment (10^{-8} M) for 24 h, qRT-PCR analysis was used. While miR-18a and miR-130a are upregulated after ibandronate treatment, the levels of all other miRNAs tested decreased after ibandronate treatment (Fig. 3).

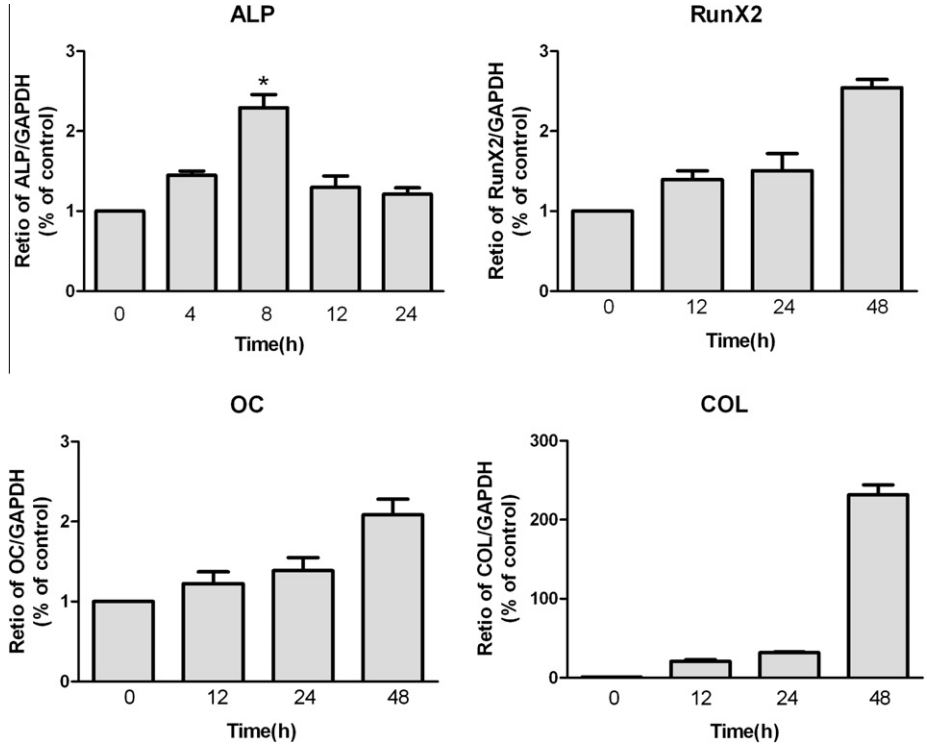

Fig. 2. Effects of ibandronate on the mRNA expression of osteogenic genes and ALP activity of PDLSCs. Cells were incubated with ibandronate for different time intervals as indicated and the mRNA levels of ALP, OC, OPG, COL-1, and Runx2 were assayed by real-time PCR. The relative mRNA expression was calculated from the threshold cycle (C_t) value of each PCR product and was normalized to GAPDH using the comparative C_t method. Each bar represents the mean ± SEM of five reactions from replicated cultures. Data from cultures treated with ibandronate for different time intervals at the same dose were compared and evaluated by one-way ANOVA.

Table 3
Differential expressed microRNAs involved in esteogenic differentiation.

Potential microRNA	Supporting observations	Target (gene)	Cell line
mir-125b	miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation [33]		Mouse mesenchymal stem cells, ST2
18a	Our present study revealed a regulatory role for miR-18a in chondrocytic differentiation through CCN2 [32]	Ccn2	Human chondrocytic HCS-2/8 and HeLa cells and chicken sternum chondrocytes
mir-133	miR-133 directly targets Runx2, an early BMP response gene essential for bone formation, and miR-135 targets Smad5, a key transducer of the BMP2 osteogenic signal, controlled through their 3′UTR sequences [34]	Runx2	C2C12 mesenchymal cells
mir-135b	This finding suggests that hsa-miR-135b may control osteoblastic differentiation of USSCs by regulating expression of bone-related genes [35]	Smad5	Unrestricted Somatic Stem Cells (USSCs)
mir-141	Thus, we have observed for the first time that miR-141 and -200a are involved in pre-osteoblast differentiation in part by regulating the expression of Dlx5 [36]	Dlx5	Human adipose tissue-derived stem cells, mouse mesenchymal ST2 stem cells and mouse premyogenic C2C12 cells.
mir-26a	MicroRNA-26a modulated late osteoblasts differentiation by targeting the SMAD1 transcription factor [19] down-regulated [37]	Smad1	Human adipose tissue-derived stem cells (hADSCs) osteoblast-like cell line (MG-63)
mir-29b	miR-29bpromotes osteogenesis by directly down-regulating known inhibitors of osteoblast differentiation [38]		Primary fetal rat calvaria osteoblasts
mir-196a	Our data indicate that miR-196a plays a role in hASC osteogenic differentiation and proliferation, which may be mediated through its predicted target, HOXC8 [39]		Human adipose tissue-derived mesenchymal stem cells (hASCs)
mir-204	miR-204/211 act as important endogenous negative regulators of Runx2, which inhibit osteogenesis and promote adipogenesis of mesenchymal progenitor cells and BMSCs [40]		(Mesenchymal progenitor cell lines, bone marrow stromal cell BMSCs)
mir-211	miR-204's homolog		
mir-148 mir-27a mir-489	Play a critical role in osteogenesis [41]		
mir-337, mir-377 mir-9 mir-516 mir-515-3p mir-496 mir-200b mir-489 mir-25 mir-423 mir26a mir30d	miRNAs whose expression was significantly modified in an osteoblast-like cell line (MG-63) cultured with BO vs PG [37]		Human multipotent mesenchymal stromal cells (hMSC) osteoblast-like cell line (MG-63)
mir-337 mir-200b mir-377 mir-130a mir-214 mir-27a mir-93	By using miRNA microarrays containing 329probes designed from human miRNA sequences, we investigated miRNAs whose expression was significantly modified in an osteoblast-like cell line (MG-63) cultured with BO vs PG [42]		Osteoblast-like cell line (MG-63)

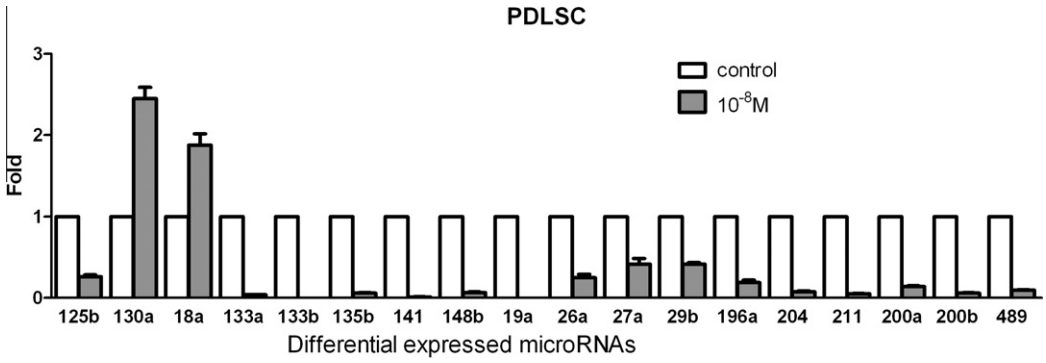

Fig. 3. Expression of microRNAs related to osteogenic differentiation in PDLSCs treated with ibandronate (10^{-8} M) for 24 h. qRT-PCR was used to determine the expression of these microRNAs.

3.4. The expression of ibandronate-regulated miRNA target genes

Several previous studies had identified putative target genes of differentially expressed miRNAs that were involved in osteogenic differentiation. To measure the expression of these osteogenic differentiation-related genes in PDLSCs, qRT-PCR was performed. These genes include *Smad1*, *Ccn2*, Distal-less homeobox 5 (*Dlx5*), and *Smad5*, which are the putative targets of miR-26a, miR-18a, miR-141, and miR-200b, respectively. The mRNA levels of these genes were upregulated in PDLSCs treated with ibandronate

(10^{-8} M) in a time-dependent manner. Upregulation was inversely correlated with the expression of the corresponding targeting miRNAs (Fig. 4).

4. Discussion

Clinical periodontitis is a common disease caused by systemic disorders and frequently results in tooth loss in adults. Generally, the disease is characterized by symptoms such as bleeding upon probing, marked bone loss, deep periodontal pocket, or loose teeth [20]. Improving the regeneration of periodontium (PDL, cementum, and alveolar bone) has proven effective in the treatment of periodontitis [21,22]. Human PDL contains a novel population of multipotent postnatal stem cells that have the capacity to develop into cells with diverse phenotypes and therefore provide a unique reservoir of stem cells [23–27]. The PDLSCs have the potential to regenerate PDL attachment; this finding supports the notion that PDLSCs are a unique population of postnatal stem cells. Therefore, tissue regeneration mediated by human PDLSCs has the potential for use as a practical cell-based treatment for periodontal diseases [28–30].

Previous studies have reported that BPs enhance osteoblast proliferation and differentiation in bone marrow-derived mesenchymal stem cells (MSCs) and osteoblasts [7,8,31]. Our results showed that, unlike the control, ibandronate at a concentration of 10^{-8} M significantly promoted the expression of ALP in human PDLSCs, and the expression peaked after 8 h of treatment. Markers for osteogenic differentiation were assessed by qRT-PCR. Significant upregulation of *COL-1*, *Runx2*, *OC*, and *OPG* was detected in the PDLSCs treated with ibandronate. These data support the idea that ibandronate promotes the maturation of PDLSCs by modifying the expression of esteogenic genes. However, further studies are required to elucidate the underlying mechanism. MicroRNAs are non-coding RNAs (20–24 nucleotides in length) that negatively regulate the expression of target genes post-transcriptionally and/or translationally depending on the degree of complementarity between the miRNA and the target. Thus, miRNAs may be involved in almost all biological events, including esteogenic differentiation in eukaryotes. According to previous reports, there are several miRNAs that participate as regulators in osteogenic differentiation (Table 3). In our study, some of these miRNAs were differentially expressed in hPDLSCs after treatment with ibandronate. In particular, miR-18a and miR-133a were upregulated whereas miR-141 and miR-19a were downregulated by ibandronate. These findings provide clues to the role of miRNAs in mediating ibandronate-induced esteogenic differentiation; however, the exact mechanism has not been elucidated.

One of the differentially expressed miRNAs downregulated by ibandronate in hPDLSCs is miR-26a. Luzi et al. reported that miR-26a promotes the osteogenic differentiation of human adipose tissue-derived stem cells (hADSCs) by targeting SMAD1 [19]. SMAD1 is downstream of bone morphogenetic protein (BMP) signaling and plays a significant role in osteoblastic differentiation. Here, we found that treatment of PDLSCs with ibandronate significantly suppressed miR-26a expression and consequently upregulated SMAD1. This finding suggested that ibandronate promotes osteogenic differentiation of PDLSCs via a mechanism similar to the differentiation of hADSCs into osteogenic cells.

Other microRNAs regulated by ibandronate in PDLSCs are miR-18a and miR-141. Ohgawara et al. revealed that miR-18a regulates chondrocytic differentiation through the CCN family protein 2/connective tissue growth factor (CCN2/CTGF), which plays a pivotal role in endochondral bone formation and chondrocytic ossification [32]. Previous studies have reported that miR-141 modulated the BMP-2-induced pre-osteoblast differentiation through the translational repression of Dlx5, which is a bone-generating transcription factor expressed during pre-osteoblast differentiation. We found that the expression of these miRNAs and their targets were altered by ibandronate. This finding was consistent with that of previous studies which reported that miRNAs have regulatory roles in osteogenic differentiation and bone formation.

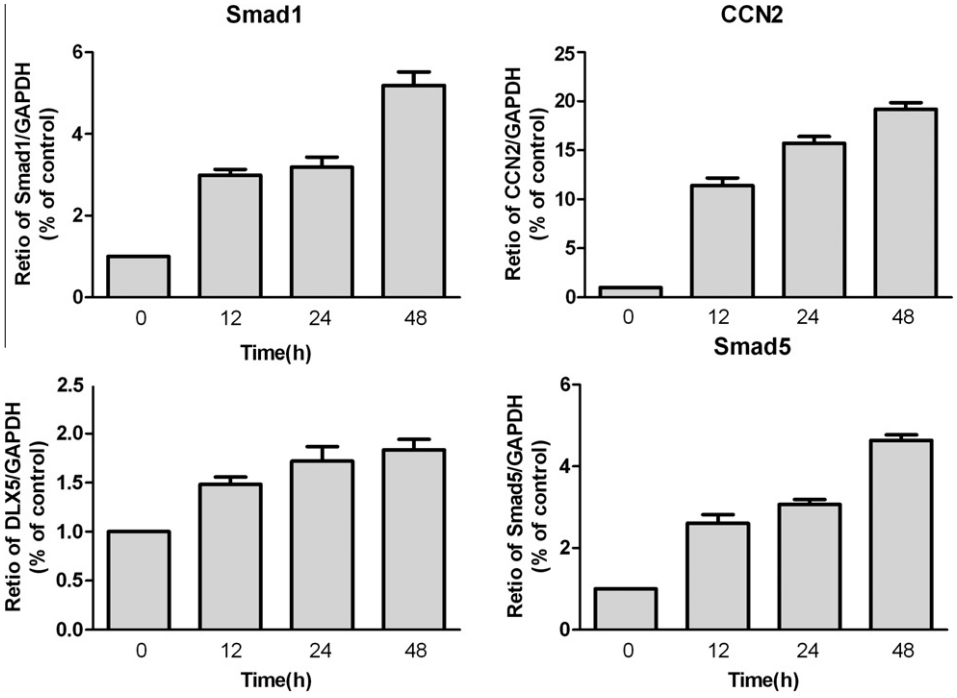

Fig. 4. The expression of the target genes of differential expressed microRNAs in cells treated with ibandronate. qRT-PCR was used to measure the expression of genes related to PDLSC osteogenic differentiation including Smad1, Ccn2, Dlx5 and Smad5, which are putative targets of miR-26a, miR-18a, miR-141, and miR-200b, respectively.

In summary, our study is the first to demonstrate that ibandronate treatment promotes the osteogenic differentiation of hPDLSCs by modulating the expression of miRNAs, which subsequently regulate the expression of diverse target genes involved in bone metabolism. Our findings provide insights into the molecular mechanisms underlying the complex osteoblastic functions of hPDLSCs.

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