

# Effects of deferoxamine on the osteogenic differentiation of human periodontal ligament cells

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Received January 1, 2017; Accepted August 17, 2017

DOI: 10.3892/mmr.2017.7810

**Abstract.** Hypoxia regulates a number of cell biological processes, including cell survival, development and differentiation. Deferoxamine (DFO), an oral chelator for blood transfusion patients, has been demonstrated to induce hypoxia and is frequently used as a hypoxia-mimicking agent. The purpose of the present study was to investigate the influence of DFO on the proliferation, migration and osteogenic differentiation of human periodontal ligament cells (hPDLCs). The effects of DFO on hPDLC viability and migration were measured using an MTT and wound healing assay. To characterize the hypoxia microenvironment, the expression of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in hPDLCs treated with DFO was quantified using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Subsequently, the osteogenic differentiation potential of DFO was determined by RT-qPCR of the mRNA of osteogenic markers (runt-related transcription factor 2 [Runx-2], osteopontin [OPN] and collagen type I [Col-1]). The alkaline phosphatase activity and mineral deposition were analyzed using alizarin red S staining. The MTT and wound healing assays demonstrated that low-concentrations of DFO had little impact on hPDLC viability and migration 48 h into the treatment. DFO upregulated the expression of hPDLC genes specific for osteogenic differentiation: HIF-1 $\alpha$ , Runx-2, OPN and Col-1. Furthermore, formation of mineralized nodules was enhanced by DFO. The present study suggests that DFO provided favorable culture

conditions to promote the osteogenic differentiation and mineralization of hPDLCs. The mechanism underlying these alterations remains to be elucidated.

## Introduction

Periodontal ligament (PDL) tissue, which originates from cranial neural-crest-derived ectomesenchymal cells (1), is a specialized fibrous connective tissue located between the cementum of the tooth root and the alveolar bone (2). PDL serves a role in anchoring teeth and maintaining the structural integrity of the periodontium (2). Human PDL cells (hPDLCs) form a heterogeneous mixture of cell types, including fibroblasts, cementoblasts, osteoblasts, epithelial cells, vascular endothelial cells, smooth muscle cells (SMCs) and certain types of neural cells (3). The presence of multiple cell types has led to a hypothesis that the PDL tissue may also contain stem cells that maintain PDL homeostasis and regenerate adjacent periodontal tissues. It has been confirmed that multipotent stem cells of hPDLCs have the ability to differentiate into osteoblastic/cementoblastic cells and generate cementum/PDL-like tissue *in vivo* (4). Therefore, the application of hPDLCs may be a promising therapeutic method for the reconstruction of tissues damaged by periodontal diseases.

Hypoxia is commonly associated with pathologies including tissue ischemia, inflammation and solid tumors (5-7). Additionally, hypoxic microenvironments form in embryos, and adults and often create specific niches which could regulate cellular differentiation and multi-gene expression in various cell types (8). Furthermore, hypoxia has been found to be a potent inducer of the expression of osteogenesis/mineralization genes (9). Cells have the ability to modulate their processes to adapt to specific hypoxic niches during development and tissue maintenance or repair (8).

Cells respond to hypoxic microenvironment by expressing a hypoxia-inducible heterodimeric transcription factor, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), which serves an essential role in sensing and responding to hypoxia-induced alterations

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**Key words:** deferoxamine, human periodontal ligament cells, hypoxia-inducible factor-1 $\alpha$ , osteogenic differentiation

in the cellular environment (10,11). HIF-1 $\alpha$  regulates numerous hypoxia-responsive genes which are involved in a variety of biological functions. Under normoxic conditions, HIF-1 $\alpha$  is targeted for proteasomal degradation by an iron-containing prolyl hydroxylase (PHD) enzyme (12). By contrast, under hypoxic conditions, HIF-1 $\alpha$  evades hydroxylation and translocates to the nucleus where it heterodimerizes with HIF-1 $\beta$  (13). Previous studies have demonstrated that HIF-1 $\alpha$  signaling pathway-mediated tissue hypoxia serves a fundamental role in the regulation of stem/progenitor cell recruitment, and the retention and differentiation of regenerating tissues (14,15). It has also been demonstrated that HIF-1 $\alpha$  mediates the angiogenic, and osteogenic phases of bone repair and regeneration, via genetic or pharmacological means (10,16).

Deferoxamine (DFO) iron chelators can artificially induce hypoxia by inhibiting PHD activity via chelation of iron in the PHD active sites, which is required for PHD function, including stabilization of HIF-1 $\alpha$  expression (11,17). Therefore, DFO has been extensively used as a hypoxia-mimetic agent in normoxia and the effects are comparable to those resulting from reduced atmospheric oxygen levels (17). Previous studies have demonstrated that DFO directly triggers the differentiation of marrow stromal cells (MSCs) (18), and increases bone formation by stimulating osteogenesis and angiogenesis through stabilization of HIF-1 $\alpha$  expression (10).

Based on these observations, the influence of DFO on hPDLCs was investigated and the preconditioning effect of DFO on hPDLC osteogenic differentiation was evaluated. The results of the present study may contribute to the efforts to design a cell therapy for periodontal disease.

## Materials and methods

**Cell culture.** All the participants of the present study signed informed consent forms and the experimental protocol was approved by the local Ethical Committee of The Second Affiliated Hospital of Harbin Medical University (Harbin, China). All hPDLCs were derived from PDL tissue which was obtained from a total of 10 clinically healthy premolar teeth extracted for orthodontic reasons (two males and three females aged 12-16 years) from April to June 2015 at The Second Affiliated Hospital of Harbin Medical University.

The extracted teeth were washed three times with PBS. Periodontal tissue was obtained from the middle third of the root using a sterile scalpel. The tissue was cut with scissors into small fragments following rinsing with growth media ( $\alpha$ -Minimum Essential Medium; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and placed in 25-cm<sup>2</sup> culture flasks containing Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), 4 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Periodontal tissue was incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide and 95% air. PDLCs were passaged until they reached 80% confluence and the cells from the third to fifth passages were used for the subsequent experiments.

For hypoxia treatment, hPDLCs were seeded on plates and incubated with various concentrations (0, 5, 10 and 20  $\mu$ M) of DFO (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

**Cell viability assay.** The cell viability of hPDLCs was measured using an MTT assay. hPDLCs were seeded in 96-well plates at an initial density of  $3.0 \times 10^3$  cells/well and cultured in DMEM with 10% FBS for 24 h. Subsequently, hPDLCs were starved overnight by changing DMEM to a serum-free medium to compensate for the effect of growth factors, and then incubated with 0, 5, 10 and 20  $\mu$ M DFO for 24, 48 or 72 h. At each time point, 20  $\mu$ l MTT (0.5 mg/ml final concentration; Sigma-Aldrich; Merck KGaA) solution was added into each well and hPDLCs were incubated at 37°C for 4 h. The supernatants were subsequently removed and replaced with 150  $\mu$ l dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) for 10 min to solubilize the formazan. The absorbance was measured at a wavelength of 490 nm with a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of three independent experiments were performed.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** To quantify the mRNA expression of HIF-1 $\alpha$ , runt-related transcription factor 2 (Runx-2), osteopontin (OPN), collagen type I (Col-1),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), confluent cells were incubated with DFO (0, 5, 10 or 20  $\mu$ M) for 2 days and total RNA was isolated with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cDNA was synthesized from 1  $\mu$ g total RNA using the PrimeScript<sup>TM</sup> RT Reagent kit (cat. no. RR047A; Takara Biotechnology Co. Ltd., Dalian, China), according to the manufacturer's protocol. The expression of HIF-1 $\alpha$  was detected using qPCR using SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> II (cat. no. RR820A; Takara Biotechnology Co. Ltd.). The Mx3005P qPCR System Thermal Cycler (Agilent Technologies, Inc., Santa Clara, USA) was used according to the manufacturer's protocol. A total of 1  $\mu$ l cDNA was used as a template in a 25- $\mu$ l reaction mixture. The mixture was initially heated at 95°C for 30 sec followed by 40 cycles of denaturation at 95°C for 5 sec and combined annealing/extension at 60°C for 30 sec. Quantities of the reaction products were normalized to the  $\beta$ -actin reference gene. All reactions were run in triplicate. The data was analyzed using the comparative threshold cycle ( $2^{-\Delta\Delta C_t}$ ) method (19). The primer sequences used for all qPCR reactions are listed in Table I.

**Wound healing assay.** The scratch-wound assay was performed to determine the effects of DFO on the migration of hPDLCs. The hPDLCs were seeded into 6-well plates at an initial density of  $5 \times 10^5$  cells/well and cultured to reach 80-90% confluence as a monolayer. In order capture images of the corresponding locations at each indicated time point, the outside surface of each well was marked prior to the test. The surface of the cellular monolayer was then gently and linearly scratched with a sterile pipette tip. Following washing and removal of the detached cells, the plates were incubated at 37°C with fresh medium containing 0, 5, 10 and 20  $\mu$ M DFO. Following 0, 24 and 48 h of incubation, phase contrast images of the wounds were visualized under a light microscope (Eclipse 80i; Nikon Corporation, Tokyo, Japan) and recorded using NIS-Elements Basic Research software (Nikon Corporation). The number of hPDLCs that migrated into the scratch area was counted to quantify the cell migration rates. Experiments were independently repeated three times.

Table I. Primer sequences for quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
HIF-1 $\alpha$	GTGTTATCTGTCGCTTTGAGTC	CTGGCTGCTGTAATAATGTTCC
$\alpha$ -SMA	CGGGACATCAAGGAGAACT	CCATCAGGCAACTCGTAACTC
TGF- $\beta$ 1	ACCTTGGGCACTGTTGAAGT	CTCTGGGCTTGTTCCTCAC
Runx2	TAGGCGCATTTTCAGGTGCTT	GGTGTGGTAGTGAGTGGTGG
OPN	AGCAGCTTTACAACAAATACCCAG	TTACTTGGAAGGGTCTGTGGG
Col-1	TGAACTTGTTGCTGAGGGC	GCAGGCGTGATGGCTTAT
$\beta$ -actin	TTGCCGACAGGATGCAGAA	CTCCTGCTTGCTGATCCACAT

HIF-1 $\alpha$ , hypoxia-inducible factor 1- $\alpha$ ;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; Runx2, runt-related transcription factor 2; OPN, osteopontin; Col-1, collagen type 1.

**Osteogenic induction.** Cells were seeded in 6-well plates at a density of  $7 \times 10^4$  cells/well. When confluent, the cells were treated with 0, 5, 10 or 20  $\mu$ M DFO for 2 days, and then changed to the osteogenic medium DMEM supplemented with 10% FBS, 10 mM sodium  $\beta$ -glycerophosphate, 100 nM dexamethasone and 50 mg/l ascorbic acid, to induce mineralization. Osteogenic medium was replaced every 3 days.

**Alkaline phosphatase activity assay.** Following 5 days of mineralization, ALP activity was measured using a p-nitrophenol phosphate assay (ALP assay kit; Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's protocol. Initially, hPDLCs were washed three times with PBS and lysed with 0.1% Triton X-100. Subsequently, the supernatant was collected into a 96-well plate, mixed with the substrates and p-nitrophenol from the ALP assay kit and incubated for 10 min at 37°C. Following the incubation, the absorbance of p-nitrophenol was measured using a microplate reader at a wavelength of 405 nm.

**Immunofluorescence analysis.** Immunofluorescence analysis of Col-1 in hPDLCs was performed on the 5th day of osteogenic induction. Cells were fixed in 4% paraformaldehyde at room temperature for 30 min. Following rinsing with PBS, cells were incubated with 3% bovine serum albumin (Beyotime Institute of Biotechnology) for 1 h at room temperature to bind nonspecific binding sites. Cells were then incubated overnight at 4°C with a rabbit anti-human Col-I polyclonal primary antibody (1:500; cat. no. ab34710; Abcam, Cambridge, UK). The fluorescein isothiocyanate-conjugated goat polyclonal anti-rabbit immunoglobulin G was used as the secondary antibody (1:160; cat. no. ZF-0314; OriGene Technologies, Inc., Rockville, MD, USA) and incubated with the membrane for 1 h at room temperature. Then, the samples were incubated with DAPI (Sigma-Aldrich; Merck KGaA) for 3 min at room temperature to allow for nucleus detection. Cell staining was observed using a fluorescence microscope (Nikon Corporation). Fluorescence quantification was achieved by comparing the integrated optical density/pixel value of Col-1 between the DFO group and the control group using ImageJ software (version 1.42q; National Institutes of Health, Bethesda, MD, USA).

**Alizarin red S (ARS) staining.** Following induction of mineralization for 14 days, mineral nodule formation was determined by 0.1% ARS staining (pH 4.2; Sigma-Aldrich; Merck KGaA). For each experiment, human hPDLC monolayers were washed twice with PBS and fixed with 95% ice-cold ethanol for 10 min and rinsed twice with distilled water. Fixed cells were stained with ARS for 30 min at room temperature and washed with distilled water once more. ARS staining images were captured using a phase-contrast microscope and calculated using the Matlab image analysis software (MathWorks, Natick, MA, USA).

**Statistical analysis.** Statistical analysis was performed using SPSS software for Windows (version 19.0.1; IBM Corp., Armonk, NY, USA). All experiments were performed in triplicate and results were expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed by one-way analysis of variance followed by the Dunnett's test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effects of DFO on cell viability.** MTT assays demonstrated a concentration-dependent effect of DFO on hPDLC viability (Fig. 1A). Following a 48 h treatment, no significant difference was observed between the 5 and 10  $\mu$ M DFO groups compared with the control group. At 72 h, the viability of hPDLCs decreased in all DFO groups compared with the control group (all  $P < 0.05$ ). When treated with high concentration of DFO (20  $\mu$ M), the viability of hPDLC significantly decreased as early as after 24 h.

**DFO promotes the expression of HIF-1 $\alpha$  in hPDLCs.** As presented in Fig. 1B, the expression of HIF-1 $\alpha$  mRNA in hPDLCs was increased by DFO. Compared with the control group, at the 48 h time point, the expression levels of HIF-1 $\alpha$  mRNA were increased 1.4-fold and 1.6-fold following incubation with 5 and 10  $\mu$ M DFO, respectively (both  $P < 0.05$ ).

**Scratch wound healing assay.** The results of the scratch-wound assay are presented in Fig. 2A. On the 2nd day DFO

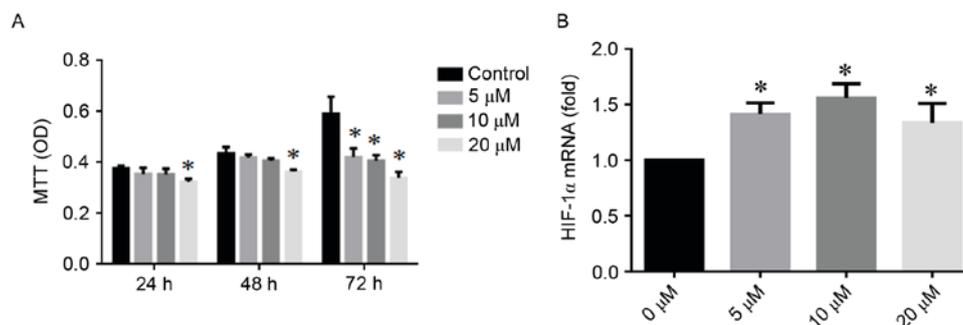


Figure 1. Effects of DFO on hPDLCs viability and mRNA expression of HIF-1 $\alpha$ . (A) Cellular viability assayed by MTT. Histograms depict a concentration-dependent decrease in hPDLC proliferation following treatment with different concentrations of DFO (0, 5, 10 and 20  $\mu$ M). (B) Relative gene expression values for HIF-1 $\alpha$  evaluated following hPDLC stimulation with DFO for 2 days, using the quantitative polymerase chain reaction. \* $P$ <0.05 vs. the respective control group. hPDLCs, human periodontal ligament cells; OD, optical density; DFO, deferoxamine; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ .

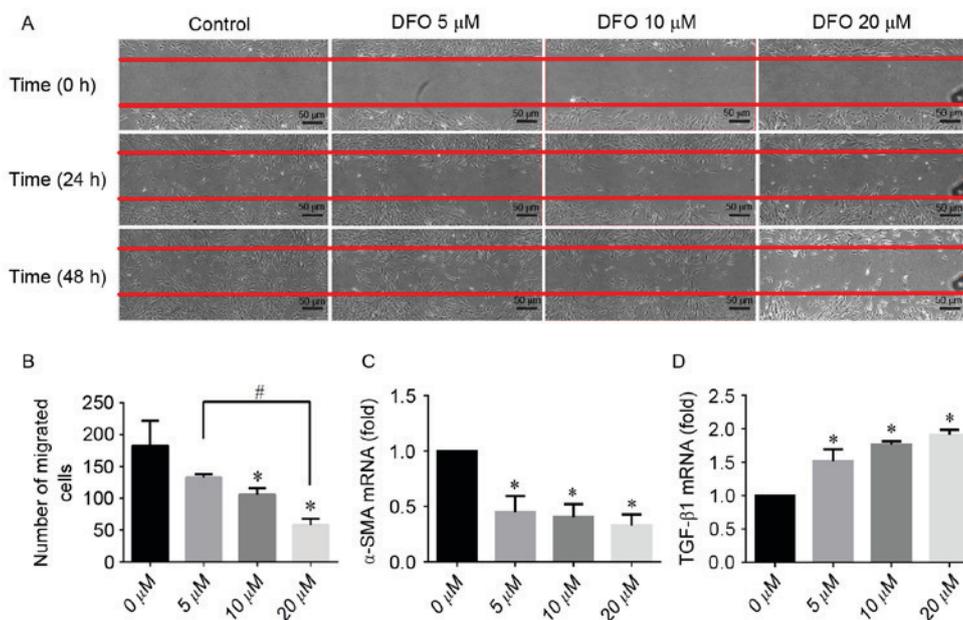


Figure 2. Effects of DFO on cell migration and mRNA expression of  $\alpha$ -SMA and TGF- $\beta$ 1. (A) Wound healing migration assay of DFO-treated and untreated human periodontal ligament cells. (B) Quantitative analysis of the number of cells crossing the edge of wound at 48 h following introduction of the scratch. (C)  $\alpha$ -SMA RNA expression 48 h following treatment with DFO compared with the control group. (D) TGF- $\beta$ 1 RNA expression 48 h following treatment with DFO compared with the control group. \* $P$ <0.05 vs. the control group; # $P$ <0.05.  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; DFO, deferoxamine.

demonstrated an inhibitory effect on hPDLC migration in a concentration-dependent manner. There was no significant difference in the number of cells that migrated into the wound area between the low concentration (5  $\mu$ M) DFO group and the control group (Fig. 2B). In the 20- $\mu$ M DFO group, an increased amount of interspersed non-viable cells could be observed under the light microscope, compared with the control group. Subsequently, the  $\alpha$ -SMA and TGF- $\beta$ 1 mRNA expression during scratch wound healing was examined. Results from qPCR demonstrated that DFO downregulated the expression of  $\alpha$ -SMA (Fig. 2C). Contrastingly, the level of TGF- $\beta$ 1 mRNA was upregulated by DFO in a concentration-dependent manner (all  $P$ <0.05; Fig. 2D).

#### Effects of DFO on osteogenic differentiation

**ALP activity assay.** ALP activity assays demonstrated that DFO significantly promoted ALP activity in hPDLCs.

Particularly, 5  $\mu$ M DFO demonstrated an increased activating potential compared with the control group ( $P$ <0.05; Fig. 3A).

**DFO induces the expression of osteogenic-associated genes *in vitro*.** qPCR demonstrated that DFO upregulated the mRNA expression of hPDLC-osteogenic markers after 5 days of mineralization (Fig. 3B-D). Similarly to the results of the ALP assay, 5  $\mu$ M DFO exhibited an increased ability to stimulate osteogenic differentiation compared with 10  $\mu$ M DFO. Compared with the control group, the mRNA expression of Runx2 increased to nearly 3.7- and 2.3-fold when hPDLCs were treated with 5 and 10  $\mu$ M DFO, respectively (Fig. 3B). The expression level of OPN increased to >5- and 3-fold when treated with 5 and 10  $\mu$ M DFO, compared with the control group (Fig. 3C). The Col-1 expression quadrupled and doubled compared with the control group when 5 and 10  $\mu$ M DFO

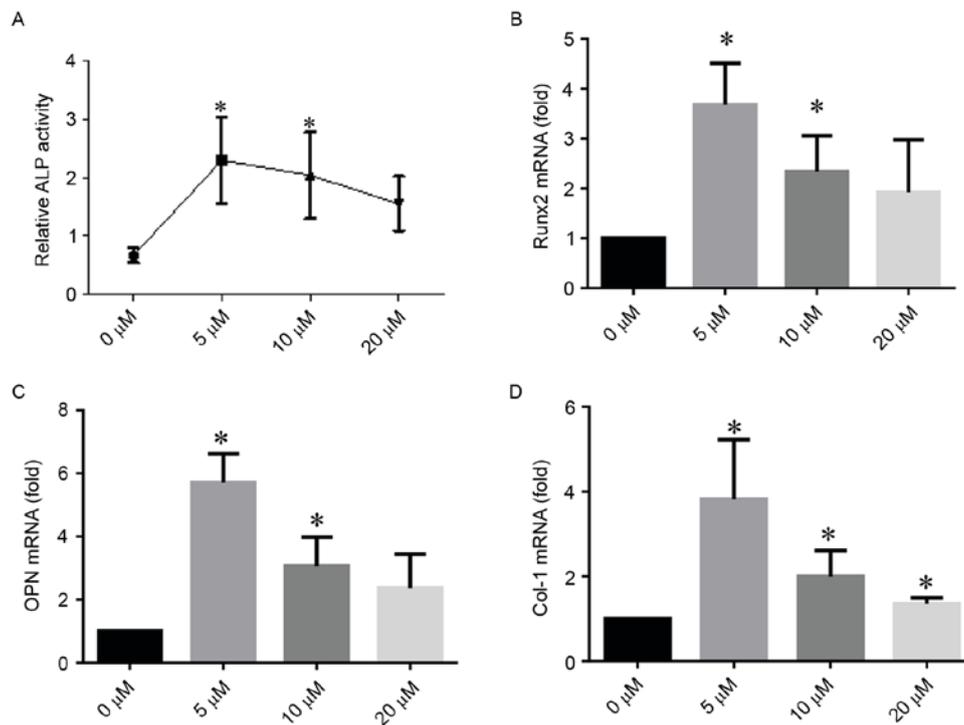


Figure 3. ALP activity assay and expressions of osteogenic-marker genes following induction of mineralization. (A) Quantitative ALP assay. (B) mRNA expressions of Runx2 following treatment with DFO. (C) mRNA expressions of OPN following treatment with DFO. (D) mRNA expressions of Col-1 following treatment with DFO. \*P<0.05 vs. the control group. Runx2, runt-related transcription factor 2; OPN, osteopontin; Col-1, collagen type I; ALP, alkaline phosphatase; DFO, deferoxamine.

were applied, respectively (Fig. 3D). The 20 μM treatment with DFO also induced significant changes in Col-1 expression (P<0.05).

*DFO increases Col-1 synthesis in hPDLCs.* Immunofluorescence staining for Col-1 demonstrated a significant increase in staining intensity following treatment with 5 and 10 μM DFO, compared with the control group (both P<0.05; Fig. 4).

*ARS staining.* Mineralized nodule formation was analyzed using ARS staining following 14 days of osteogenic induction (Fig. 5). The results were consistent with the data obtained from the ALP activity assay and qPCR. There was an increased calcium deposition in DFO groups compared with the control group, and the intensity of ARS staining with 5 and 10 μM DFO was increased 2.977 and 2.562-fold compared with the control group, respectively. However, due to low cell viability, no mineralization was observed when hPDLCs were treated with 20 μM DFO (data not shown).

## Discussion

Periodontitis is one of the most common inflammatory diseases resulting from a complex interplay between bacterial infection and host responses, and is characterized by destruction of the attachment apparatus and the supporting bone of teeth (20). Periodontitis is the most prevalent cause of tooth loss and is difficult to heal (20). Searching for an effective method for periodontium remodeling is an important part of the effort to develop periodontitis therapeutic strategies.

PDL cells form a heterogeneous cell population and have a potential to regenerate periodontal tissue under certain conditions (4). Previous *in vitro* and *in vivo* studies demonstrated that under hypoxic conditions the proportion of osteogenesis cells and their differentiation potential are increased compared with under normoxic conditions (9,21). In the present study, genetics-based evidence that the DFO-induced hypoxia has the potency to induce osteogenic differentiation in hPDLCs was presented.

Initially, the effect of DFO on human PDL cell viability was investigated using the MTT method. It was demonstrated that proliferation of hPDLCs was inhibited by DFO in a concentration-dependent manner, while low concentrations of DFO exhibited no apparent inhibitory effect on hPDLCs. One of the reasons for the negative effect of DFO on hPDL proliferation may be associated with the removal of iron, which serves a role in metabolic pathways. It has been previously suggested that through chelating the intracellular iron pool, DFO can inhibit the activity of ribonucleotide reductase, which is required for the synthesis of deoxynucleotides and results in G1/S cell-cycle arrest (22).

The MTT results were consistent with those previously reported in the literature. The results demonstrated that DFO-induced hypoxia could downregulate the growth of osteoblast through inhibition of Wnt signaling pathways via activation of HIF-1α (23). The proliferation of umbilical cord-derived human mesenchymal stem cells is also significantly inhibited by DFO through influencing the cell cycle, attenuating the intercellular signal transmission and altering cell morphology/ultrastructure (24). Previous experimental *in vivo* and *in vitro* research, as well as several initial clinical

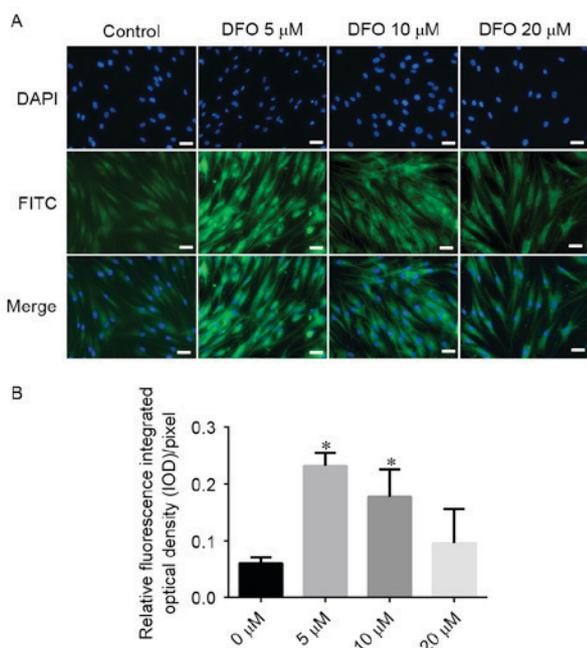


Figure 4. Immunofluorescent analysis of Col-1 using FITC in hPDLCs treated with DFO. (A) hPDLCs were incubated with anti-Col-1 antibody (green), and nuclei were stained with DAPI (blue). Scale bar, 200  $\mu$ m. (B) Fluorescence quantification analysis performed by comparing the IOD/pixel value of Col-1 in DFO with the control group. \* $P < 0.05$  vs. the control group. Col-1, collagen type 1; FITC, fluorescein isothiocyanate; hPDLCs, human periodontal ligament cells; IOD, integrated optical density; DFO, deferoxamine.

trials, demonstrated that DFO could effectively inhibit certain neoplasm growths and induce cell differentiation (25). Additionally, certain experiments demonstrated that inhibition of growth is an important transition point in the initiation of differentiation and mineralization of proliferating cells (26). The mechanism of DFO activity on PDL cells is complex and it is unclear whether hPDLC proliferation inhibition is associated with differentiation.

The migration of hPDLCs towards the site of injury is necessary for periodontium healing. In the present study, scratch-wound assays were performed to observe the effects of DFO on the migration of hPDLCs. The results indicated that low concentrations of DFO exhibited little inhibition on migration within 48 h; however, the high concentration exhibited a concentration-dependent inhibitory effect. In agreement with the present study, a previous study reported that stress-fiber formation was inhibited through cellular iron depletion mediated by DFO, which could lead to inhibition of cellular migration (27). A previous study on the inhibitory effect of DFO was also performed in the context of anticancer treatments (28).

The mRNA expression of  $\alpha$ -SMA and TGF- $\beta$ 1, which are involved in scratch wound healing, was also examined. In wound contraction,  $\alpha$ -SMA serves a crucial role by increasing contractile activity of stress fibers and the abundance of this protein is positively associated with wound contraction efficiency *in vivo* (29). The results from qPCR demonstrated that the expression of  $\alpha$ -SMA was downregulated by DFO in a concentration-dependent manner, consistent with the wound healing assay results. Downregulated expression of  $\alpha$ -SMA may be partly due to a low cellular oxygen tension in hPDLCs resulting from upregulated expression of HIF-1 $\alpha$  induced

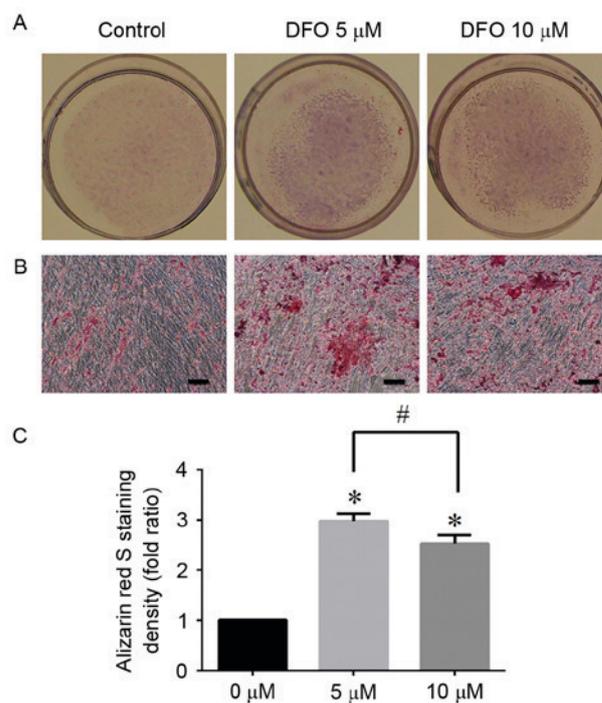


Figure 5. Mineral deposition assayed by ARS staining. (A) Whole cell-culture dishes. (B) Mineralized deposition in hPDLCs. Scale bar, 250  $\mu$ m. (C) The ratio of ARS staining density compared with the control. \* $P < 0.05$  vs. control group; # $P < 0.05$ . ARS, alizarin red S; hPDLCs, human periodontal ligament cells; DFO, deferoxamine.

by DFO. Previous studies have demonstrated that reduction of extra- and intra-cellular tension leads to the removal of  $\alpha$ -SMA from stress fibers within h; under continued low stress, expression levels of  $\alpha$ -SMA are reduced (29,30). The results of the present study were in agreement with previous evidence that dermal fibroblasts exhibit low expression of  $\alpha$ -SMA, and decreased migration and proliferation under hypoxic conditions (31). In the future, DFO may serve a role in the treatment of liver fibrosis by decreasing the expression of  $\alpha$ -SMA in hepatic stellate cells (32).

TGF- $\beta$ 1, which is highly expressed in the entirety of periodontal ligament tissue, is a multifunctional cytokine that regulates various cellular activities, including cell differentiation, synthesis of extracellular matrix proteins and wound repair (33,34). TGF- $\beta$ 1 induces  $\alpha$ -SMA expression in myofibroblasts and is a key regulator of type I collagen (25). In the present study, the expression level of TGF- $\beta$ 1 in DFO-treated hPDLCs increased markedly, which is consistent with the previous experiments demonstrating increased levels of active and total TGF- $\beta$ 1 in response to hypoxic conditions (30,35).  $\alpha$ -SMA expression decreased in hypoxic conditions, suggesting that hypoxia desensitized the expression of  $\alpha$ -SMA in the presence of TGF- $\beta$ 1. This phenomenon may be in part due to concomitant downregulation of the expression of the TGF- $\beta$ 1 receptor subunit, TGF $\beta$ -RII, possibly by a negative TGF- $\beta$ 1 feedback loop (30). This mechanism was proposed in a previous study, which indicated significantly reduced expression of  $\alpha$ -SMA and wound contraction despite a high level of TGF- $\beta$ 1 when skin myofibroblasts were cultured in hypoxic condition for 5 days (30). In addition, an increased level of TGF- $\beta$ 1 could further increase the expression of HIF-1 $\alpha$  (36)

and stabilize hypoxia in hPDLCs. In the future, thorough studies focused on this issue shall be conducted.

Hard tissue regeneration is the most important part in periodontium reconstruction. It is accepted that hPDLCs demonstrate specific osteoblast-like properties, have osteogenic characteristics (37), and can differentiate into cementoblasts and osteoblasts, which are necessary for cementum and alveolar bone formation, respectively. The present study revealed that preconditioning of hPDLCs with DFO could significantly stimulate ALP activity, upregulate the expression of osteogenesis/cementogenesis-associated genes and induce mineralization.

ALP, a membrane-associated enzyme, serves a key role in connective tissue calcification and mineral deposition (38). Furthermore, ALP is also an early marker for osteoblastic differentiation, and an indicator of the presence of osteoblasts and new bone formation (39). hPDLCs exhibit increased ALP activity compared with gingival fibroblasts, suggesting that hPDLCs are crucial in the development of osteogenic characteristics (38). In the present study, DFO significantly increased ALP activity in hPDLCs and 5  $\mu$ M DFO had the most prominent effect.

Runx2, OPN and Col-1 are osteogenic-specific genes in hPDLCs. Runx2, the osteoblast-specific transcription factor, is described as the master regulator in the commitment of osteoblastogenesis (40) and regulates the expression of a number of genes associated with osteoblasts (41). OPN produced by osteoblasts at various stages of differentiation is one of the vital non-collagenous bone matrix proteins. High OPN calcium-binding potential can promote calcium adhesion, thus influencing mineralization events (42). Col-1, an early marker of osteogenic differentiation, is a major extracellular matrix component of periodontal tissues that promotes osteoblast differentiation and mineralization (43). The qPCR conducted in the present study further demonstrated that DFO could enhance osteogenic differentiation of hPDLCs by markedly accelerating the expression of the osteogenic-specific genes.

To verify the role of DFO in the mineralization of hPDLCs, a mineralization assay using ARS staining was performed. An enhanced calcium deposition was observed in DFO groups compared with the control group, indicating that DFO could promote the mineralization properties of hPDLCs.

From the qPCR and ARS staining results it was inferred that 5  $\mu$ M DFO is more efficient than 10  $\mu$ M for mineralization induction under normoxic condition. The adverse effect of high DFO level on osteogenic ability was partly due to an excessive depletion of iron in hPDLCs, which could also lead to decreased cell viability. These results reinforce the previous findings that osteogenesis is dependent on iron homeostasis in a biphasic manner: Mild iron deficiency promotes osteogenesis, while severe iron deficiency can inhibit this process (44). The results are further reinforced by the evidence that a higher concentration of 20  $\mu$ M DFO completely abolished mineralization due to its toxicity on hPDLCs. Therefore, it is vital to determine a suitable DFO concentration in order to achieve the optimal osteogenic efficacy.

To conclude, the data gathered in the present study indicate that by affecting osteogenic-associated factors, DFO could enhance osteogenic differentiation and mineralization in hPDLCs. These results provide a reference for further research on the molecular mechanisms behind these processes. The

results may also have clinical implications since preconditioning of hPDLCs with DFO may improve the efficacy of cell therapy and DFO is a promising candidate for improving hard tissue regenerative potential in periodontal disease. The mechanism underlying periodontal hard tissue formation by hPDLCs treated with DFO *in vivo* remains to be elucidated.

### Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81570951) and the Special Foundation for Sino-Russian Translational Medicine Research Center of Harbin Medical University (grant no. CR201412).

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