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# Role of ferritin in the cytodifferentiation of periodontal ligament cells

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ABSTRACT

This study investigated the expression and functions of ferritin, which is involved in osteoblastogenesis, in the periodontal ligament (PDL). The PDL is one of the most important tissues for maintaining the homeostasis of teeth and tooth-supporting tissues. Real-time PCR analyses of the human PDL revealed abundant expression of *ferritin light polypeptide* (*FTL*) and *ferritin heavy polypeptide* (*FTH*), which encode the highly-conserved iron storage protein, ferritin. Immunohistochemical staining demonstrated predominant expression of FTL and FTH in mouse PDL tissues *in vivo*. In *in vitro*-maintained mouse PDL cells, FTL and FTH expressions were upregulated at both the mRNA and protein levels during the course of cytodifferentiation and mineralization. Interestingly, stimulation of PDL cells with exogenous apoferritin (iron-free ferritin) increased calcified nodule formation and alkaline phosphatase activity as well as the mRNA expressions of mineralization-related genes during the course of cytodifferentiation. On the other hand, RNA interference of *FTH* inhibited the mineralized nodule formation of PDL cells. This is the first report to demonstrate that ferritin is predominantly expressed in PDL tissues and positively regulates the cytodifferentiation and mineralization of PDL cells.



## 1. Introduction

The periodontal ligament (PDL) is a specialized connective tissue interposed between the roots of teeth and the inner wall of tooth-supporting bone (alveolar bone). The PDL links the teeth to the alveolar bone proper, thereby providing support, protection and sensory input for the masticatory system [1]. It has also been demonstrated that PDL tissues contain multipotent mesenchymal stem cells that can differentiate into mineralized tissue-forming cells, such as osteoblasts and cementoblasts [2,3]. Thus, the PDL is thought to play crucial roles for not only homeostasis of periodontal tissues but also bone remodeling, wound healing and tissue regeneration [1]. Recent reports have demonstrated that ferritin, a key molecule for controlling the iron concentration, regulates osteoblast differentiation [4,5]. Cytosolic ferritin is a ubiquitous and highly conserved iron storage molecule that is composed of ferritin light polypeptide (FTL) and ferritin heavy polypeptide (FTH) [6]. Twenty-four ferritin subunits assemble to form the apoferritin (iron-free ferritin) shell. The FTL and FTH subunits are encoded by separate genes and their functions are nonexchangeable [7,8].

In the present study, we investigated the expression of ferritin in the PDL to clarify the molecular characteristics of PDL cells. Interestingly, we found that FTL and FTH were highly expressed in PDL tissues *in vivo* and elucidated the function of ferritin for the cytodifferentiation of PDL cells *in vitro*.

## 2. Materials and methods

### 2.1. RNA extraction and real-time PCR analysis

The clinical study was performed with appropriate approval from the Institutional Ethics Committee, and informed consent was obtained from all patients. Total RNA was extracted from cultured cells and human PDL tissues freshly isolated from an extracted first premolar of patients undergoing tooth extraction using RNA Bee (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. Human tissue total RNAs (heart, skin, kidney, bone marrow, spleen, testis, lung, liver, skeletal muscle, brain and thymus) were purchased from Bio Chain (Hayward, CA). cDNA was synthesized from the purified total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. The obtained cDNA was mixed with Power PCR SYBR Master Mix (Applied Biosystems) and gene-specific primers (Takara Bio, Shiga, Japan). The nucleotide

*Abbreviations:* PDL, periodontal ligament; FTL, ferritin light polypeptide; FTH, ferritin heavy polypeptide; ALPase, alkaline phosphatase.

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sequences of the primers are shown in Table 1. Real-time PCR was performed using a 7300 Fast Real-time RT-PCR System (Applied Biosystems) according to the manufacturer's instructions. The amplification conditions consisted of an initial 15 min denaturation step at 95 °C, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. The dissociation curves were analyzed to ensure the amplification of a single PCR product in each case. The relative expression levels were calculated by normalization for the gene expression of *hypoxanthine phosphoribosyl transferase* (*HPRT*) for human genes and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) for mouse genes.

### 2.2. Tissue preparation and immunohistochemical staining

The animal experiments were approved by the Institutional Animal Care and Use Committee. Eight-week-old C57BL/6 mice were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg body weight). Next, intracardial perfusion was performed with physiological saline containing 5 U/ml heparin (Aventis Pharma, Tokyo, Japan) for 2–3 min, followed by perfusion with 5% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C for 15 min. Upper jaw samples containing teeth were excised, and most of the soft tissue was removed. All of the samples were further fixed by immersion in the above fixative overnight at 4 °C and then demineralized in buffered 10% EDTA at 4 °C under agitation for 7 days. The EDTA solution was changed daily. The samples were then embedded in OCT compound (Sakura Finetek USA, Torrance, CA) on dry ice. Serial 14 μm sections of the second molars were cut and mounted on aminopropylsilane-coated slides. Endogenous peroxidase activity was inactivated by incubation with 0.3% $H_2O_2$ in PBS containing 0.3% FBS (Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature. After blocking with 3% BSA (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in PBS overnight at 4 °C, immunohistochemical staining was carried out following standard procedures. A goat anti-FTL polyclonal antibody (1:1000) and a rabbit anti-FTH polyclonal antibody (1:1000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used as the primary antibodies. Normal goat IgG and rabbit IgG (Vector Laboratories, Burlingame, CA) served as controls for the primary antibodies. The incubations were carried out overnight at 4 °C. After washing, the sections were reacted with biotinylated anti-goat IgG and biotinylated anti-rabbit IgG secondary antibodies (Vector Laboratories) for 90 min at room temperature. A Vectastain Avidin–Biotin Complex Kit (Vector Laboratories) and DAB solution (20 mg DAB, 50 ml PBS, 4.5 μl $H_2O_2$) were used for signal detection. Counterstaining was performed with Mayer's Hematoxylin (Muto Pure Chemicals Ltd., Tokyo, Japan).

### 2.3. Cell culture and induction of PDL cell cytodifferentiation and mineralization

We established a mouse PDL cell line, MPDL22, and a mouse gingival fibroblast cell line, MG/B6. We maintained these cells as previously described [9]. A preosteoblastic cell line, KUSA-A1, was obtained from Riken Cell Bank (Tsukuba, Japan) [10]. For differentiation of these cells into hard tissue-forming cells *in vitro*, we cultured the cells in 12-well plates until they reached confluence. At this point, we replaced the standard medium with α-MEM supplemented with 10% FCS, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid (mineralization-inducing medium). Apoferritin (Sigma–Aldrich, St. Louis, MO) was added to the mineralization-inducing medium at various concentrations.

### 2.4. Western blotting analysis of FTL and FTH in PDL cells

MPDL22 cells cultured in the mineralization-inducing medium were lysed with RIPA lysis buffer (Millipore, Billerica, MA). The protein concentrations of the cell lysates were measured using a Bradford 595 assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Aliquots of the cell lysates (30 μg protein) were separated by 12% SDS–PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in TBST (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20) containing 5% (w/v) nonfat dried milk at 4 °C overnight. The above-described goat anti-FTL and rabbit anti-FTH polyclonal antibodies were used as the primary antibodies at a dilution of 1:1000. Briefly, after three washes in TBST, the membranes were incubated with the primary antibodies in TBST containing 5% milk overnight at 4 °C. After three further washes in TBST, the membranes were incubated with horseradish peroxidase-linked donkey anti-goat IgG and goat anti-rabbit IgG secondary antibodies (Research & Diagnostics Systems Inc., Minneapolis, MN) for 60 min at room temperature. The immunoreactive proteins were detected using an ECL Plus Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions.

### 2.5. Alizarin red staining, and determination of the alkaline phosphatase (ALPase) activity and cellular DNA content

Histochemical staining of $Ca^{2+}$ was performed by a modification of the method described by Dahl et al. [11]. ALPase activity was assessed according to the procedure of Bessay et al. and DNA content was measured using a modification of the method of Labarca and Pagien as previously described [12].

### 2.6. RNA interference

Short hairpin RNA (shRNA) plasmids for the mouse *FTH* gene (shFTH) and a negative control (shControl) were purchased from Santa Cruz Biotechnology. For stable transfection, we plated $2 \times 10^6$ MPDL22 cells/well in 6-well plates. After 12 h, the cells were transfected with the shFTH and shControl plasmids, respectively, using Nucleofector kit R reagent (Lonza, Basel, Switzerland) in accordance with the manufacturer's protocol. After 24 h, puromycin (2 μg/ml) was added to the culture medium to initiate drug selection. After the selection, *FTH* expression was evaluated by

**Table 1**
Nucleotide sequences of the primers used for real-time PCR analysis.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| Human *FTL* | 5′-ACCATGAGCTCCCAGATTCGTC-3′ | 5′-CACATCATCGCGGTCGAAATAG-3′ |
| Human *FTH* | 5′-CAGGTGCGCCAGAACTACCA-3′ | 5′-CCACATCATCGCGGTCAAAG-3′ |
| Human *HPRT* | 5′-GGCAGTATAATCCAAAGATGGTCAA-3′ | 5′-GTCAAGGGCATATCCTACAACAAAC-3′ |
| Mouse *FTL* | 5′-CCGTGCACTCTTCCAGGATGT-3′ | 5′-CCTTATCCAGATAGTGGCTTTCCAG-3′ |
| Mouse *FTH* | 5′-TGCGCCAGAACTACCACCAG-3′ | 5′-AGAGCCACATCATCTCGGTCAA-3′ |
| Mouse *Runx2* | 5′-CACTGGCGGTGCAACAAGA-3′ | 5′-TTTCATAACAGCGGAGGCATTTC-3′ |
| Mouse *type 1 collagen* | 5′-CAGGGTATTGCTGGACAACGTG-3′ | 5′-GGACCTTGTTTGCCAGGTTCA-3′ |
| Mouse *osteocalcin* | 5′-AGCAGCTTGGCCCAGACCTA-3′ | 5′-TAGCGCCGGAGTCTGTTCACTAC-3′ |
| Mouse *GAPDH* | 5′-TGTGTCCGTCGTGGATCTGA-3′ | 5′-TTGCTGTTGAAGTCGCAGGAG-3′ |

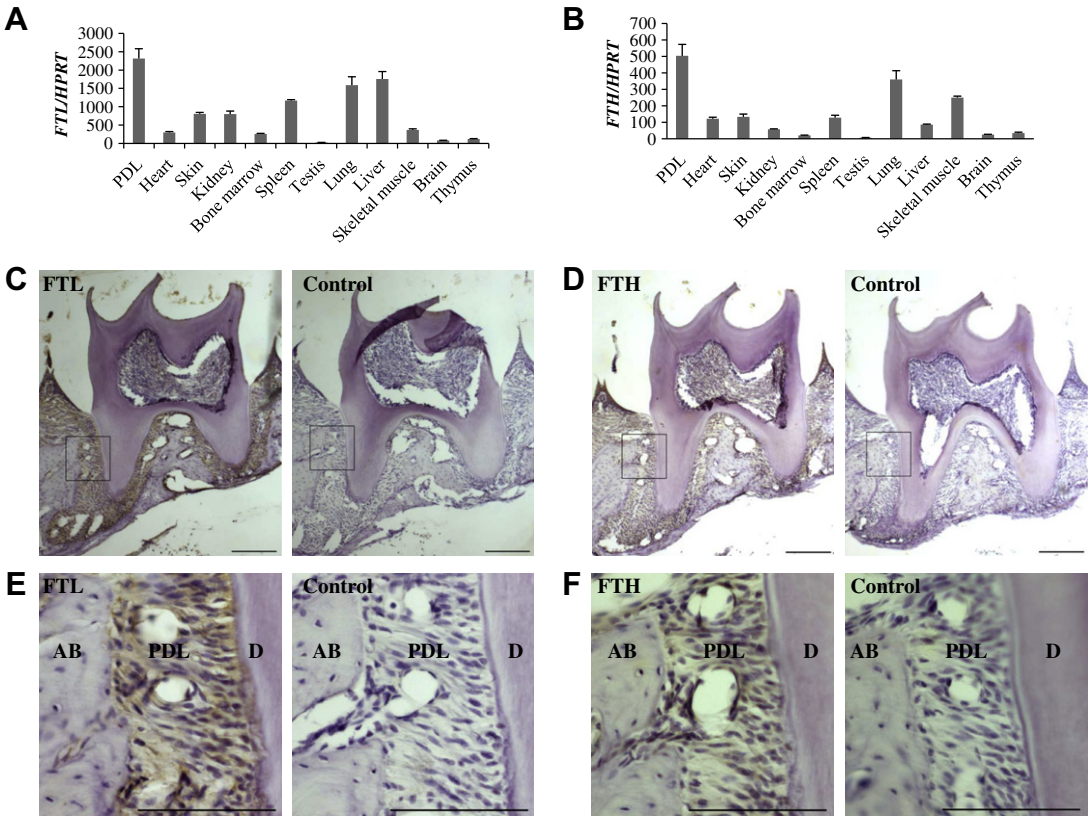


**Fig. 1.** Expression of FTL and FTH in human and mouse PDL tissues. (A, B) Real-time PCR was performed for the gene expression of *FTL* (A) and *FTH* (B) in various human tissues. The expressions of *FTH* and *FTL* were normalized by the expression of *HPRT*. The data represent the means ± SD of triplicate assays. (C, D) Immunohistochemical analyses of FTL (C) and FTH (D) in the maxilla of 8-week-old C57BL/6 mice. Scale bars, 200 μm. (E, F) Panels (E) and (F) show higher magnification images of the boxed areas in panels (C) and (D), respectively. Scale bars, 100 μm. AB, alveolar bone; PDL, periodontal ligament; D, dentin.

real-time PCR. Stable transfectants with *FTH* knockdown were then established.

### 2.7. Phosphate measurement

Cells were washed twice with PBS and solubilized with RIPA lysis buffer (Millipore). The Pi contents of the cell lysates were measured using a QuantiChrome Phosphate Assay Kit (BioAssay Systems, Hayward, CA). The phosphate contents of the cells were normalized by the protein contents and expressed as μM/mg cell protein.

### 2.8. Statistical analysis

The results were presented as means ± SD. Statistical analyses were carried out using Student's *t*-test for paired comparisons with the software Excel Statistics (SSRI, Tokyo, Japan). Values of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. FTL and FTH are highly expressed in human and mouse PDL tissues

First, we analyzed *FTL* and *FTH* expression in various human tissues. Real-time PCR analyses revealed markedly higher expression levels of *FTL* and *FTH* in the PDL than in the other human tissues examined (Fig. 1A and B). These findings prompted us to investigate the specific expression of FTL and FTH in periodontal tissues *in vivo*. We carried out immunohistochemical analyses of FTL and FTH expression in mouse maxilla specimens. FTL and FTH were both predominantly expressed in the PDL tissues (Fig. 1C–F). Expression of FTL and FTL was also observed in other soft tissues, such as the dental pulp and gingiva, but at much lower levels than in the PDL tissues.

### 3.2. FTL and FTH expressions are induced during PDL cell cytodifferentiation in vitro

To clarify a more complete expression pattern of FTL and FTH, we analyzed the FTL and FTH mRNA and protein expression levels during cytodifferentiation of the mouse PDL cell line MPDL22. Expression of *FTL* and *FTH* mRNAs and FTL and FTH proteins was induced during the course of MPDL22 cytodifferentiation and peaked at the early stage of the cytodifferentiation (Fig. 2). *GAPDH* mRNA expression was stable during the course of the MPDL22 cytodifferentiation.

### 3.3. Apoferritin enhances MPDL22 cytodifferentiation and mineralization

To examine the effects of ferritin on the cytodifferentiation and mineralization of PDL cells, we cultured MPDL22 cells in the mineralization-inducing medium in the presence of apoferritin, a kind of iron-free ferritin. First, we checked the effects of apoferritin on the mineralization of the preosteoblastic cell line KUSA-A1. In accordance with previous reports showing inhibitory effects of apoferritin on osteogenesis [4,5], we confirmed that apoferritin inhibited the mineralized nodule formation of KUSA-A1 cells in a dose-dependent manner (Fig. 3A). We also confirmed that apoferritin had no effects on the mouse gingival fibroblast cell line MG/

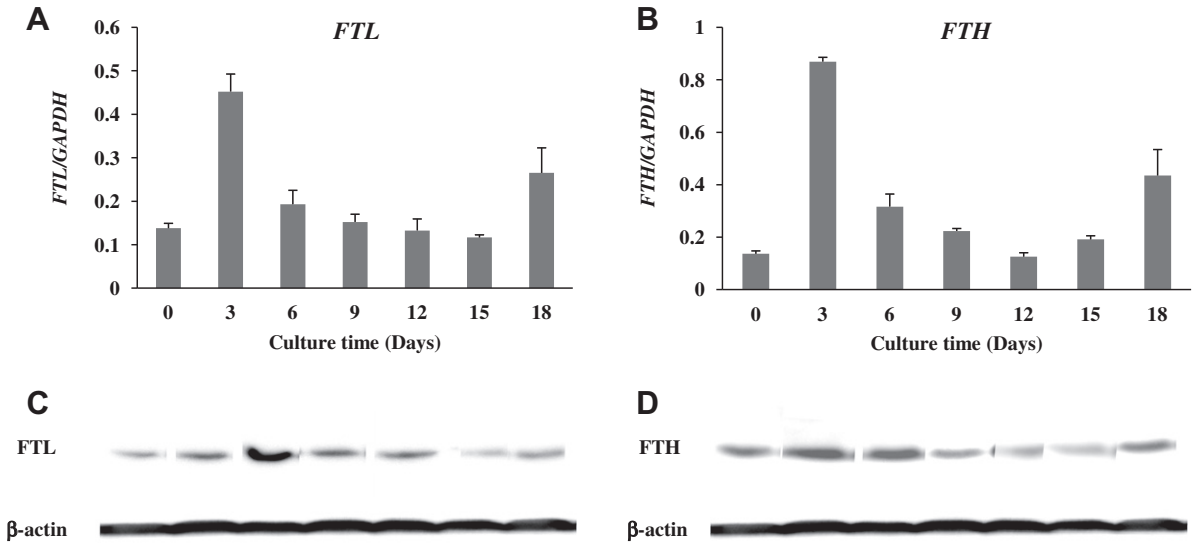


**Fig. 2.** Induction of FTL and FTH expression during cytodifferentiation of PDL cells. MPDL22 cells were cultured in the mineralization-inducing medium for 18 days. (A, B) Real-time PCR analyses for the gene expression of *FTL* (A) and *FTH* (B). The expression levels of *FTL* and *FTH* were normalized by the expression of *GAPDH*. The data represent the means ± SD of triplicate assays. (C, D) Western blotting analyses for FTL (C) and FTH (D) expression during the cytodifferentiation of MPDL22 cells.

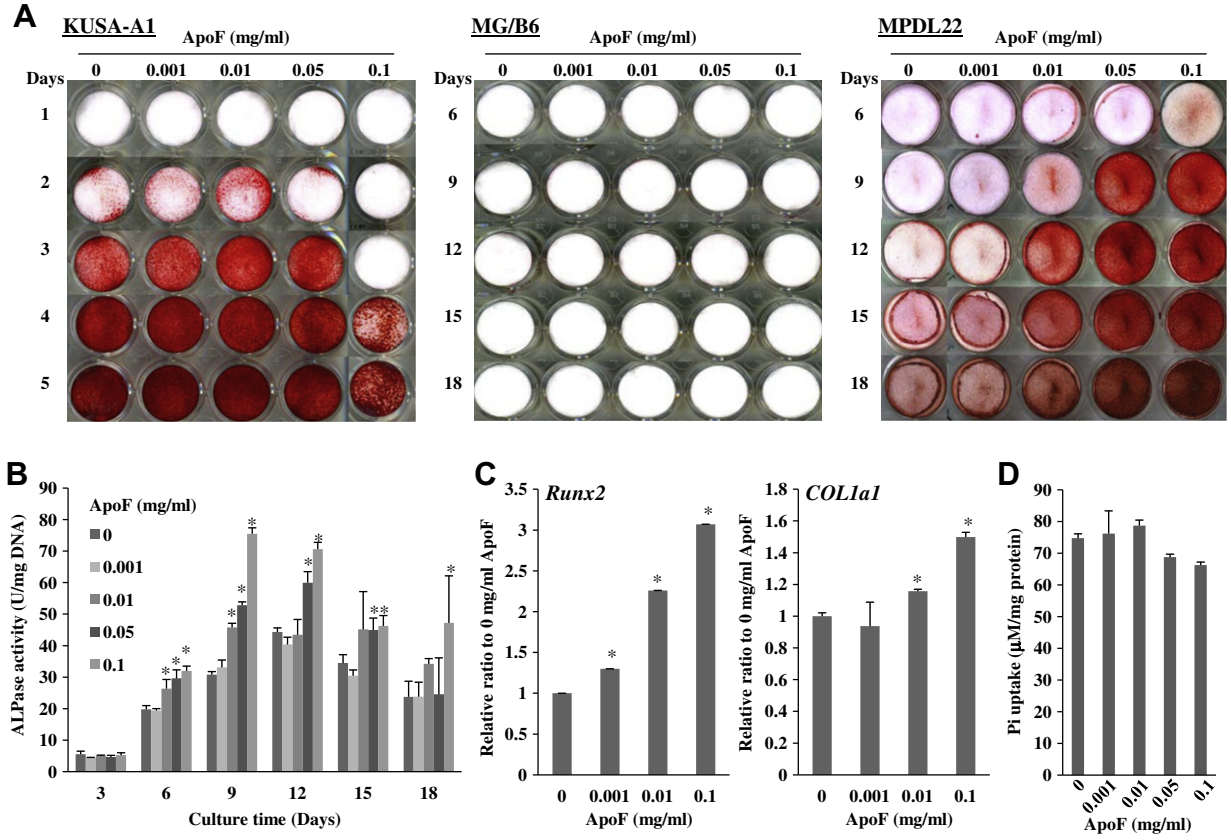


**Fig. 3.** Apoferritin enhances the cytodifferentiation and mineralization of MPDL22 cells. Cells were cultured in the mineralization-inducing medium alone or supplemented with apoferritin at 0.001, 0.01, 0.05 and 0.1 mg/ml for the indicated days. (A) Apoferritin enhances the mineralized nodule formation of MPDL22 cells in a dose- and time-dependent manner, as indicated by alizarin red staining. On the other hand, apoferritin inhibits the mineralized nodule formation of KUSA-A1 cells. Apoferritin has no effects on the mouse gingival fibroblast cell line MG/B6. (B) Apoferritin increases the ALPase activity of MPDL22 cells in a dose-dependent manner. (C) Real-time PCR was performed for the expression of cytodifferentiation- and mineralization-related genes, namely *Runx2* and *type I collagen*, after stimulation with exogenous apoferritin on day 15. The expression of the indicated genes was normalized by the expression of *GAPDH*. The data represent the means ± SD of triplicate assays. $^{*}P < 0.05$, *vs.* 0 mg/ml apoferritin. (D) Intracellular Pi concentrations are not affected by apoferritin. MPDL22 cells were cultured in the mineralization-inducing medium alone or supplemented with apoferritin at the indicated concentrations for 48 h. Cell lysates were used to measure the Pi levels. The data represent the means ± SD of triplicate assays. ApoF, apoferritin; *COL1a1*, *type I collagen*.

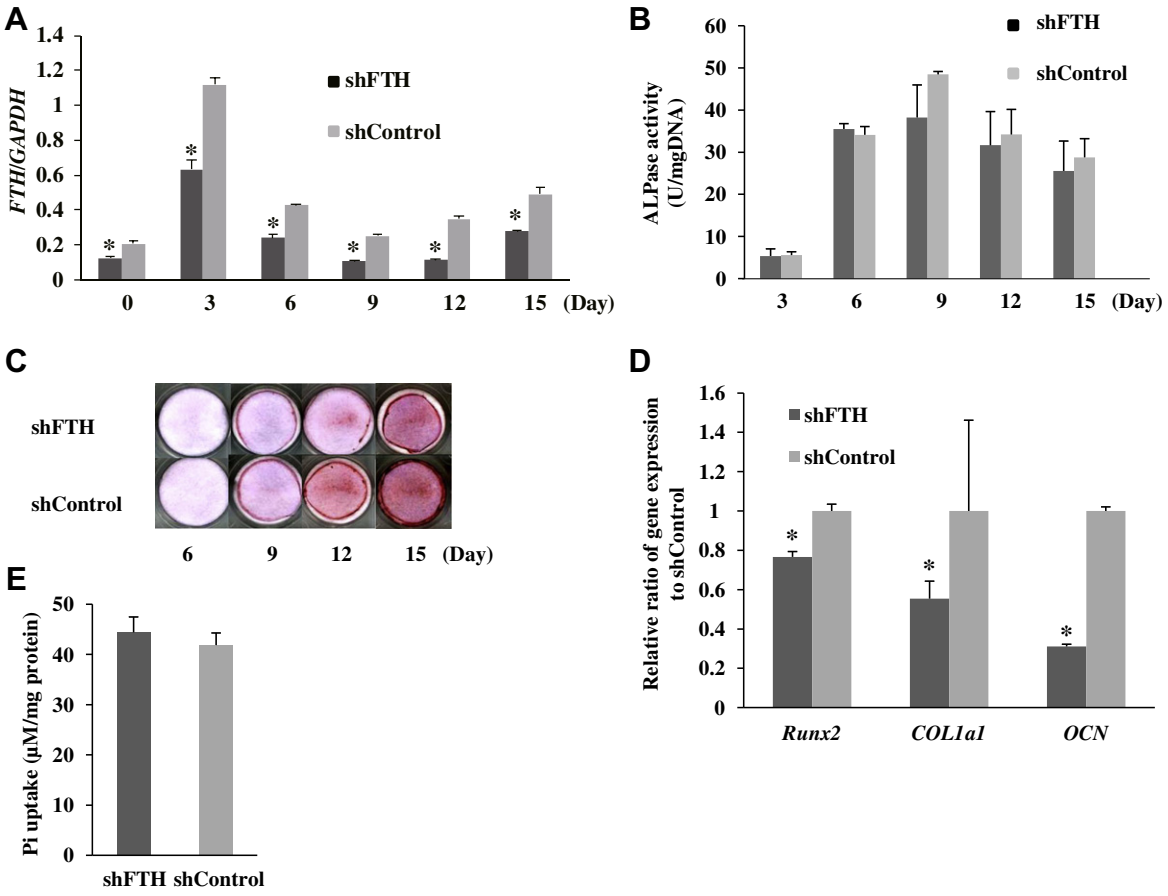


**Fig. 4.** Stable knockdown of *FTH* suppresses the cytodifferentiation of MPDL22 cells. Transfected MPDL22 cells were cultured in the mineralization-inducing medium for 15 days. (A) Confirmation of stable knockdown of *FTH* by real-time PCR analysis. (B) ALPase activities during the cytodifferentiation course. The data represent the means ± SD of triplicate assays. $^{*}P < 0.05$, *vs.* shControl transfectants. (C) Suppression of mineralized nodule formation in the *FTH* shRNA transfectants indicated by alizarin red staining. (D) Real-time PCR reveals strong inhibition of cytodifferentiation- and mineralization-inducing gene expression in the *FTH* shRNA transfectants on day 12. The data represent the means ± SD of triplicate assays. $^{*}P < 0.05$, *vs.* shControl transfectants. (E) No difference in the intracellular Pi concentrations between the *FTH* shRNA and shControl transfectants. The transfectant cells were cultured in the mineralization-inducing medium and cell lysates were used to measure the Pi levels on day 6. The data represent the means ± SD of triplicate assays. *COL1a1*, *type I collagen*; *OCN*, *osteocalcin*.

B6, which has no ability to undergo cytodifferentiation and mineralization (Fig. 3A). On the other hand, apoferritin clearly increased the mineralized nodule formation during the course of MPDL22 cytodifferentiation in a time- and dose-dependent manner (Fig. 3A). Moreover, apoferritin significantly increased the ALPase activity in a dose-dependent manner (Fig. 3B). In addition, real-time PCR analyses demonstrated that apoferritin significantly upregulated the gene expressions of *Runx2* and *type I collagen* during MPDL22 cytodifferentiation (Fig. 3C). We confirmed that the intracellular Pi concentrations of MPDL cells were not affected by apoferritin during culture for 48 h (Fig. 3D).

### 3.4. RNA interference of FTH downregulates MDPL22 cytodifferentiation and mineralization

It has been reported that FTH, which has ferroxidase activity, regulates osteoblast differentiation [4]. Thus, we established MPDL22 cells transfected with a shRNA for the *FTH* gene to examine the effects of *FTH* knockdown on the cytodifferentiation and mineralization of PDL cells. First, we confirmed that the transfectants showed reduced *FTH* expression (Fig. 4A). We then cultured the transfectants in the mineralization-inducing medium and analyzed the ALPase activity and mineralized nodule formation. The *FTH* shRNA transfectants did not show significant decreases in the ALPase activity (Fig. 4B). However, the mineralized nodule formation of the *FTH* shRNA transfectants was significantly inhibited compared with the shControl transfectants (Fig. 4C). In addition, the *FTH* shRNA transfectants showed strong downregulation of *Runx2*, *type I collagen* and *osteocalcin* expression, compared with the shControl transfectants (Fig. 4D). There was no difference in the intracellular Pi concentrations between the *FTH* shRNA and shControl transfectants (Fig. 4E).

## 4. Discussion

Our present data demonstrate for the first time that ferritin is predominantly expressed in the PDL and enhances the cytodifferentiation and mineralization of PDL cells. Considering the fact that the PDL plays crucial roles in the homeostasis, remodeling and regeneration of periodontal tissues, including the alveolar bone and cementum, we speculate that ferritin may be one of the key molecules involved in regulating the specific functions of the PDL.

Iron is essential for many important cellular functions and processes, including the cell cycle, reductive conversion of ribonucleotides to deoxyribonucleotides and electron transport. Ferritin plays a key role in maintaining iron homeostasis by binding and regulating excess intracellular iron. Cytosolic ferritin is a highly conserved

three-dimensional iron storage molecule that can capture up to 4500 $Fe^{3+}$ ions. Each hollow apoferritin (iron-free ferritin) shell is made up of 24 FTL and FTH polypeptide chains [13,14]. Ferritin possesses two well-studied properties as follows: iron incorporation to maintain the balance of cellular iron and ferroxidase activity, an inherent feature of FTH, to promote iron incorporation and oxidize $Fe^{2+}$ into the safer $Fe^{3+}$ form. FTL is associated with iron nucleation, mineralization and long-term iron storage [14,15]. The expression of ferritin is under delicate control at both the transcriptional and posttranscriptional levels [16,17]. Both FTL and FTH are critical for maintaining iron homeostasis. It is well known that tight regulation of iron homeostasis is crucial for not only maintaining normal cellular functions but also preventing iron-mediated oxidative stress [18]. Reactive oxygen species induced by oxidative stress tightly regulate ferritin expression [19]. Hypoxia is also involved in the translational regulation of ferritin [18]. The PDL constitutively receives mechanical stress, such as occlusal pressure, that reduces the blood flow and leads to local hypoxia and reoxygenation [20]. This *in vivo* situation may regulate the expression and function of FTL and FTH, especially in the PDL.

It is well known that iron overload leads to both osteoporosis and osteopenia *via* direct effects on osteoblast activity. Recently, ferritin, especially FTH with ferroxidase activity, has been reported to negatively modulate Pi-mediated calcification and osteoblastic differentiation of human smooth muscle cells and osteosarcoma cells mainly *via* the ferroxidase activity of ferritin [4,5]. The opposing effects of ferritin on PDL cells and other cell lines may arise through different mineralization-inducing environments as well as different cell properties in nature. PDL cells have been reported to differentiate into either osteoblasts or cementoblasts depending on the need and the environment. *In vitro*-maintained PDL cells from rats were found to form mineralized nodules that differed from those formed by osteoblasts [21].

To clarify the role of FTH in PDL cells during the course of their cytodifferentiation and mineralization, we established MPDL22 transfectants with *FTH* knockdown. Downregulation of the cytodifferentiation and mineralization-related genes *Runx2*, *type I collagen* and *osteocalcin* were observed in the *FTH* shRNA transfectants. The *FTH* shRNA also inhibited mineralized nodule formation. We further found that ceruloplasmin, which possesses ferroxidase activity, enhanced the mineralized nodule formation of PDL cells (data not shown). These findings clearly suggest that ferritin, probably through the ferroxidase activity of FTH, regulates the cytodifferentiation and mineralization of PDL cells.

In conclusion, we have demonstrated for the first time that FTL and FTH are predominantly expressed in PDL tissues. Ferritin enhanced the cytodifferentiation and mineralization of PDL cells, at least in part *via* FTH, which possesses ferroxidase activity. These findings suggest that ferritin is involved in the homeostasis, remodeling and regeneration of periodontal tissues. Further characterization of the roles of ferritin in the cytodifferentiation of cells may uncover novel functions for this highly conserved protein.

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