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ORIGINAL ARTICLE

Effect of leptin on differentiation of human dental stem cells

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OBJECTIVES: Mesenchymal stem cells (MSCs) were identified in adult human periodontal ligament and dental pulp that are considered as potential stem cell sources for future clinical applications in dentistry. Leptin is known as an important regulator of mesenchymal differentiation. The objective of this study was to elucidate the role of leptin on proliferation and differentiation of dental MSCs.

MATERIALS AND METHODS: Enhancement of cemento/odontoblastic differentiation of dental stem cells by leptin was confirmed by alizarin red S staining and alkaline phosphatase activity staining. In contrast, leptin reduced adipogenesis in both dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) confirmed by oil red O staining and RT-PCR. The expression of adipogenic markers, lipoprotein lipase and proliferator-activated receptor γ2 (PPARγ2), were suppressed in PDLSCs incubated on media supplemented with leptin for 2 weeks.

RESULTS: Leptin had a relatively stronger osteogenesis promoting effect and adipogenesis suppressing effect in PDLSCs than in DPSCs.

CONCLUSIONS: Collectively, leptin had a relatively stronger promoting effect on cemento/odontoblastic differentiation and a suppressing effect on adipogenesis in PDLSCs than in DPSCs. This study has provided evidence that leptin acts as an important modulator of dental MSCs differentiation.

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Keywords: dental stem cell; periodontal ligament stem cell; dental pulp stem cell; leptin; differentiation

Introduction

Adult stem cells among various stem cells are found in various human tissue or organ. Adult stem cells consist of undifferentiated hematopoietic stem cells and non-hematopoietic stem cells (Morrison, 2001). Hematopoietic stem cells can give rise to several types of blood cells. On the other hand, mesenchymal stem cells (MSCs) called non-hematopoietic stem cells have a possibility of becoming differentiated cells that could produce bone, cartilage, and fat (Prockop, 1997; Watt and Hogan, 2000; Muguruma *et al*, 2006). Current research shows that MSCs are found in various sources such as bone marrow, umbilical cord blood (UCB), adipose tissue, and muscles (Minguell *et al*, 2001; Tocci and Forte, 2003; Chang *et al*, 2006). MSCs found in the various adult dental tissues are also considered as potential stem cell sources (Shi *et al*, 2005; Gay *et al*, 2007). Human MSCs show various differentiation potentials and characteristics when grown on the appropriate differentiation medium. Periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs) obtained from human teeth have self-renewal capacity and the potential to differentiate into osteoblasts, adipocytes, chondrocytes, and myoblasts (Shi *et al*, 2005; Chang *et al*, 2006). In the dental research field, PDLSCs and DPSCs are focused on as important cells that can be utilized to regenerate and repair damaged dental structures (Thesleff and Sharpe, 1997; Gay *et al*, 2007).

Leptin, a 16 kDa protein encoded by the *Ob* gene, is secreted as a peptide hormone by adipose tissues and is circulated in the blood vessels (Zhang *et al*, 1994; Rosenbaum *et al*, 1997). Leptin is known to be a modulator of food intake and a signal transmitter of energy expenditure in the hypothalamus (Ducy *et al*, 2000). According to a recent study, leptin plays the role of a regulator for functions of various differentiated cells such as myoblast (Liu *et al*, 1997), osteoblasts (Reseland *et al*, 2001), and adipocytes (Bai *et al*, 1996). Leptin is expressed and secreted from human osteoblasts during osteogenesis and promotes bone mineralization (Thomas *et al*, 1999; Ducy *et al*, 2000; Cornish *et al*, 2002; Chang *et al*, 2006; Scheller *et al*, 2010). Especially, leptin affects osteogenesis through the hypothalamus either directly or indirectly. Moreover, leptin acts on osteoblasts and osteoclasts directly to promote cell proliferation and differentiation (Ducy *et al*, 2000). Lots of leptin-related studies with bone marrow stem cells and cord blood stem cells were reported as they are

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considered the main sources of MSC from bone marrow and UCB (Hicok *et al*, 1998; Chang *et al*, 2006). It is known that leptin is more sensitive to bone marrow-derived MSCs than UCB-derived MSCs (Chang *et al*, 2006). Also, the effects of leptin on MSCs are various according to the origin of cells. Unlike bone marrow-derived and UCB-derived MSCs, it has been still unclear that the effect of leptin on tissue regeneration of PDLSCs and DPSCs. In this study, we hypothesized that leptin would promote the proliferation and differentiation of PDLSCs and DPSCs in the differentiation medium. To verify this hypothesis, we cultured PDLSCs and DPSCs treated with leptin that may have had an influence on cemento/odontoblastic differentiation and adipogenesis. In addition, we transplanted these cells into the back side of nude mice to identify the effect of leptin on dental MSCs.

Materials and methods

Samples and cell culture

Human extracted 3rd molar teeth were obtained from the Department of Oral and Maxillofacial Surgery at Seoul National University Dental Hospital. Adequate animal care was provided following the protocol cleared by the IRB at Seoul National University School of Dentistry (IRB No. S-D20080009). The periodontal ligament (PDL) was separated from the surface of normal impacted third molars (males, 20–24 years in ages). Dental Pulp (DP) was also isolated from the donors' teeth. This cell isolation and culture were followed previous research (Seo *et al*, 2004). Both cells were digested separately, in 3 mg ml^{-1} type 1 collagenase (BioBasic Inc., Toronto, Canada) and 4 mg ml^{-1} dispase (GIBCO BRL, Grand Island, NY, USA) for 1.5 h by shaking at 37°C in a 5% CO_2 incubator. Single-cell suspensions were obtained by passing the cells through a 70 μm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA). Primary cells from the PDL and DP samples were seeded into 10-cm culture dishes with alpha modification of Eagle's medium (GIBCO BRL) including 15% fetal bovine serum (GIBCO BRL), 100 μM of L-ascorbic acid (GIBCO BRL), 2 mM of L-glutamine (GIBCO BRL), and 100 U ml^{-1} of antibiotic-antimycotic (GIBCO BRL). After the cells were 80–90% confluent, primary cells were obtained and seeded for the consequent subculture. Cells were counted with a hemacytometer and 0.4% (w/v) trypan blue dye (GIBCO BRL). In all subsequent experiments, passage (P) 2–4 cells were used. To assay the effect of leptin on MSCs proliferation and differentiation in PDLSCs and DPSCs, human recombinant leptin (PEPROTech Inc., Rocky Hill, NJ, USA) was added to each culture medium at a concentration of 0.5 and 1.5 μg ml^{-1}.

Proliferation and differentiation

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effect of Leptin on the proliferation of PDLSCs and DPSCs as described previously (Zhang *et al*, 2009). Briefly, both cells were seeded at 10^4 cells per 96-well culture dish. After 24-h incubation, 5 mg ml^{-1} of MTT solution was added to wells and incubated at 37°C for 30 min. Formazan crystals were dissolved with dimethyl sulfoxide solution. The proliferation of cells was determined by colorimetric assays measuring the absorbance at 540 nm with a multi-well absorbance reader (FLUOstar OPTIMA ABS.; BMG labtech, Offenburg, Germany). PDLSCs and DPSCs were obtained and counted with a hemacytometer and 0.4% trypan blue (GIBCO BRL) for direct cell counting after 7 days. PDLSCs and DPSCs were seeded at about 5×10^4 cells/plates into six-well plates. To induce cemento/odontogenic/osteogenic differentiation, cells were incubated in alpha modification of Eagle's medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), 10 nM dexamethasone (Sigma-Aldrich Co., St. Louis, MO, USA), 5 mM glycerol phosphate (Sigma-Aldrich Co.), and 100 μM ascorbic acid (GIBCO BRL) for 2 weeks. Mineralized cells were washed with 1× PBS and fixed with ice-cold 70% ethanol at room temperature for 30 min. Calcium accumulation was detected by 40 mM of alizarin red S (Sigma-Aldrich Co.) solution. After washing with distilled water, images of the dried-culture dishes were taken by microscope (Olympus America Inc., Center Valley, PA, USA). With 10% cetylpyridinium chloride (Sigma-Aldrich Co.) in 10 mM sodium phosphate (pH 7.0), samples are destained and measured the absorbance at 562 nm. To measure the alkaline phosphatase (ALPase) activity, 5×10^4 cells of PDLSCs and DPSCs were seeded and cultured under mineralized medium. After 2 weeks, cells were washed and lysed in 0.5% Triton X-100 (Sigma-Aldrich Co.) in distilled water at room temperature. To the lysate, 1 mg ml^{-1} para-nitrophenylphosphate (p-NPP; Bioassay systems, Hayward, CA, USA) and 0.2 M diethanol amine (Bioassay systems) was added and incubated at 37°C for 30 min. The reaction was stopped by the addition of 60 μl of 0.3 N NaOH (Sigma-Aldrich Co.). The value for ALPase activity was determined by colorimetric assays measuring the absorbance at 405 nm with a multi-well absorbance reader (FLUOstar OPTIMA ABS.; BMG labtech). To induce adipogenesis, cells were incubated in adipogenesis differentiation medium (Invitrogen, Camarillo, CA, USA). To identify the adipogenesis, cells were fixed with 4% paraformaldehyde and stained with 1% oil red O (Chemicon, Temecula, CA, USA). The absorbance at 490 nm was measured with a multi-well absorbance reader (FLUOstar OPTIMA ABS.; BMG labtech).

Flow cytometry

After trypsination of the cells, 10^5 cells were stained with 10 μl of allophycocyanin-conjugated mouse monoclonal anti-human leptin receptor antibody (R&D Systems, Minneapolis, MN, USA). Following incubation for 45 min at 4°C, cells are washed with 1× PBS (BioBasic Inc., Toronto, Canada). As a control, the washed cells were treated with allophycocyanin-conjugated Ig G antibody (R&D Systems). Cells expressing leptin receptor were determined by flow cytometric analysis

(FACSCAN; Becton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed with FACSDiva Software (Becton Dickinson).

RT-PCR

The RNA from each group was collected using RNeasy mini kit (Qiagen, Venlo, the Netherlands). Then, cDNA was synthesized with SuperScript III First-Strand kit (Invitrogen) using random hexamers. The primers for the RT-PCR included peroxisome proliferator-activated receptor γ2 (PPARγ2), lipoprotein lipase (LPL), glyceraldehydes-3-phophate dehydrogenase (GAPDH), alkaline phosphatase (ALP), Runx2, osteocalcin, and osteopontin as described previously (See Table 1). PCR was carried out as follows: denaturation at 94°C for 2 min; 35 cycles of 94°C for 30 s, 57–61°C for 30 s, and 72°C for 30 s (Seo *et al*, 2004).

In vivo transplantation

With 0.5 μg ml^{-1} of leptin treatment, P3 cells from PDLSCs and DPSCs were seeded at 10^4 cells per 15-cm^2 dishes for 14 days. Every 3 days, the leptin-containing media was changed after PBS washing. With 80% of confluent cells on the dish, the cells were collected with 1× Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA, GIBCO BRL). For *in vivo* transplantation, 5×10^6 cells were collected and incubated with 40 mg of hydroxyapatite/tricalcium phosphate particles (HA/TCP) (Zimmer, Warsaw, IN, USA) at 37°C in a 5% CO_2 incubator. After 1 h, the cells with HA/TCP were implanted into the dorsal surface of 6-week-old immunocompromised beige nude mice (NIH-*bg-nu-xid*, Harlan Sprague Dawley, Indianapolis, IN, USA).

Immunohistochemistry

After 6- and 12-week post-transplantation, transplants were harvested, fixed with 4% paraformaldehyde (Sigma-Aldrich Co.), decalcified with 10% EDTA (pH 8.0), and then embedded in paraffin. Sections were stained with hematoxylin and eosin staining (H&E staining) and immunohistochemistry (mouse anti-human mitochondria antibody (1:200), Chemicon, Temecula, CA, USA) with anti mouse HRP/DAB detection kit (Abcam, Cambridge, UK) was performed. To analyze hard tissue formation, the areas of the cementum-like and dentin-like structure from ten randomly-selected 10×-magnified sites from two different sections were calculated with TOMORO ScopeEye 6.0 program (Techsan Digital Imaging, Seoul, Korea). Animal experimentation followed the guidelines and care of the Dental Research Institute, Seoul National University, Korea.

Table 1 Primer sets used in reverse transcription polymerase chain reaction

Genes	*Sequence(5′ → 3′)*	*Tm* (°C)
GAPDH	F: AGC CGC ATC TTC TTT TGC GTC R: TCA TAT TTG GCA GGT TTT TCT	59
PPARγ2	F: CTC CTA TTG ACC CAG AAA GC R: GTA GAG CTG AGT CTT CTC AG	57
LPL	F: ATG GAG AGC AAA GCC CTG CTC R: GTT AGG TCC AGC TGG ATC GAG	61
Runx2	F: TCT GGC CTT CCA CTC TCA GT R: TAT GGA GTG CTG CTG GTC TG	59
ALP	F: TGG AGC TTC AGA AGC TCA ACA CCA R: ATC TCG TTG TCT GAG TAC CAG TCC	61
Osteocalcin	F: GTC CAA GCA GGA GGG CAG R: TTG AGC TCA CAC ACC TCC C	61

PPARγ2, peroxisome proliferator-activated receptor γ2; LPL, lipoprotein lipase; ALP, alkaline phosphatase.

Statistical analysis

The data were shown as means ± standard deviation of triplicate determinations. Statistical analyses of the data were determined using SPSS 18.0 package (SPSS Inc., Chicago, IL, USA). Statistical significance of the differences was assessed by one-way ANOVA followed by the Bonferroni method and Tukey HSD test. For the analyses of the data for area, statistical significance was determined with a two-tailed *t* test. The differences were significant at $P < 0.05$.

Results

Isolation and characterization of human dental stem cells

Mesenchymal stem cells from the periodontal ligament and dental pulp of the adult human tooth were grossly fibroblast-like in shape and had a spindle form in the microscopic view (Figure 1a). In the MTT assay, the absorbance of PDLSCs and DPSCs decreased with a short-term leptin treatment (Figure 1b). Similarly, BrdU labeling results parallel in MTT assay after 24 h with leptin treatment (Figure S1). Although the cell proliferation levels of PDLSCs decreased based on the direct cell counting method, DPSCs seemed not to be influenced by long-term leptin treatment (Figure 1c). There was no significant difference in direct cell counting in DPSCs after 7 days with leptin treatment.

Flow cytometric analysis showed that PDLSCs had a change in the expression of receptors on the cell surface. PDLSCs showed less frequent expression of leptin receptor after treatment with exogenous leptin. The decrease in leptin receptor positive cells was about 35%. However, DPSCs showed no distinct difference of leptin receptor expression after leptin treatment (Figures 1d and S2). The change in the expression of receptors was observed with same patterns in flow cytometry result after incubation with lower (1 and 100 ng ml^{-1}) concentrations (results not shown). This result indicates that PDLSCs may be more sensitive than DPSCs to exogenous leptin.

Cemento/odontoblastic differentiation of PDLSCs and DPSCs

Differentiation of PDLSCs and DPSCs into cemento/odontoblasts was influenced by leptin. Alizarin red S staining showed that mineral nodule accumulation of PDLSCs was much more abundant than that of DPSCs (Figure 2a). Higher concentrations (0.5 or 1.5 μg ml^{-1}) of leptin showed more accumulation of mineralized nodules that was analyzed by 10% cetylpyridinium

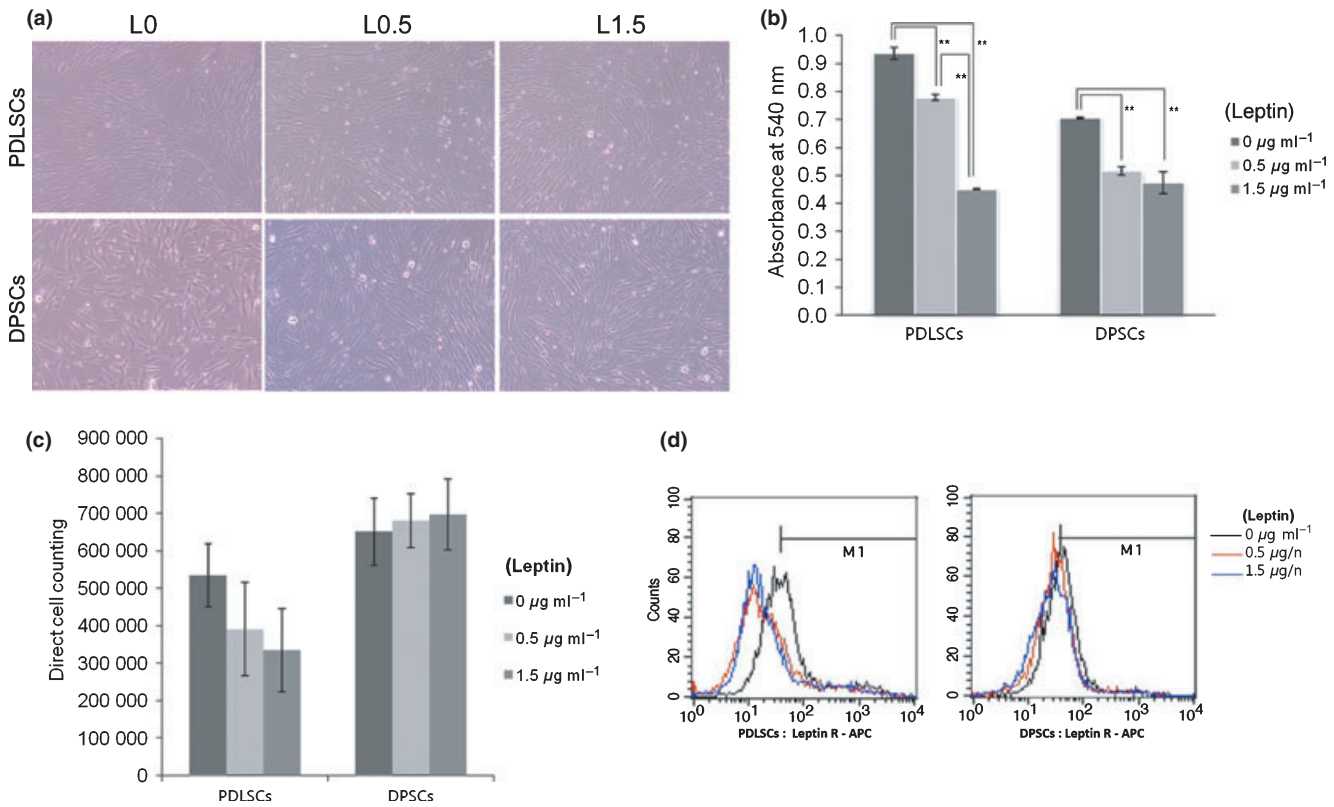

Figure 1 Isolation and proliferation of cultured mesenchymal stem cells from adult human periodontal ligament and dental pulp. (**a**) Periodontal ligament stem cells (PDLSCs) and dentl pulp stem cells (DPSCs) at 7 days incubation showed a fibroblast-like spindle shape in the microscopic view. (×100) (**b**) Effect of leptin on proliferation of PDLSCs and DPSCs using MTT assay after 24 h of leptin treatment showed decreased cell proliferation in leptin-treated groups. (**c**) Direct cell counting after 7 days of leptin treatment. Leptin inhibited the proliferation of PDLSCs whereas did not inhibit that of DPSCs. The data are shown as the mean ± SD of triplicate determinations. (**d**) Flow cytometric analysis: PDLSCs treated with leptin for 7 days show low expression levels of leptin receptor. On the other hand, DPSCs has no distinct change in the expression of leptin receptors. $*P < 0.05$; $**P < 0.01$

chloride destaining (Figure 2b). ALPase activity of PDLSCs and DPSCs on various concentrations of leptin showed that higher concentrations of leptin induced higher ALPase activity in both cell lines (Figure 2c). Cementoblastic differentiation was significantly enhanced by leptin treatment in PDLSCs. However, DPSCs showed increased ALPase activity only with a higher concentration of leptin. DPSCs grown in 0.5 μg ml^{-1} leptin-treated medium showed no difference compared with the control. The expression of differentiation markers, including osteocalcin, ALP, Runx2, increased with the treatment of leptin (Figure 2d). PDLSC expressed higher ALP and Runx2 than DPSC in 2 weeks cemento/odonto/osteoblastic differentiation. Leptin promoted the cemento/odontoblastic differentiation of PDLSCs rather than DPSCs.

Adipogenic differentiation of PDLSCs and DPSCs

Leptin had an inhibitory effect on adipogenic differentiation of PDLSCs and DPSCs *in vitro*. Oil red O staining showed that both PDLSCs and DPSCs differentiated into oil red O-positive lipid-laden cells. Compared with DPSCs, leptin had a greater inhibitory effect on adipogenesis in PDLSCs (Figure 3a). The expression of two adipogenic markers, PPARγ2 and LPL, were observed in leptin-treated adipogenic differentiation compared with the control (Figure 3b). The gene expression of PPARγ2 and LPL decreased with the treatment of leptin in PDLSCs. Higher concentrations of leptin decreased adipogenesis in both cell lines. However, DPSCs had less expression of both adipogenic markers compared with PDLSCs. In particular, PPARγ2 expression was much less in DPSCs than in PDLSCs. In this result, leptin seems to have more of an inhibitory action on adipogenesis in PDLSCs compared with DPSCs. From the mineralization and adipogenesis results of PDLSCs and DPSCs grown in leptin-treated media, the results indicate that PDLSCs are more sensitive to leptin than DPSCs. Collectively, leptin has more of a suppressive effect on adipogenesis and a promotive effect on mineralization in PDLSCs compared with DPSCs.

In vivo *transplantation with leptin treatment*

In vivo transplantation of leptin-treated PDLSCs and DPSCs confirmed that leptin enhanced hard tissue formation. After 12 weeks transplantation on the backside of nude mice, PDLSC and leptin-treated PDLSCs had differentiated into cementoblast-like cells that formed a cementum-like structure on the surface of

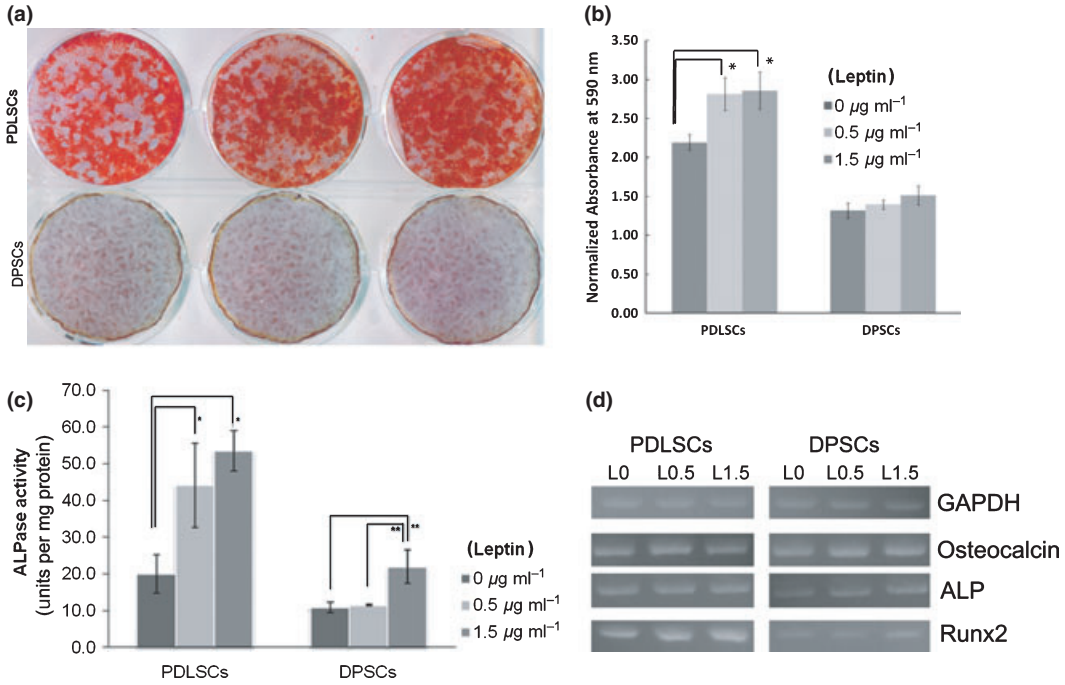

Figure 2 Effect of leptin on cemento/odontoblastic differentiation of periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs). (**a**) Alizarin Red S staining shows that mineral nodule accumulation of PDLSCs was more abundant than the ones in DPSCs. (**b**) Higher concentration (0.5 or 1.5 μg ml^{-1}) of leptin showed little more accumulation of mineralized nodules in both PDLSCs and DPSCs with no statistical significance. (**c**) ALPase activity of PDLSCs and DPSCs on various concentrations of leptin showed that a higher concentration of leptin had a higher ALPase activity in both cell lines. (**d**) Leptin enhanced the expression of differentiation markers; osteocalcin, ALP, Runx2. Leptin promoted cemento/odontoblastic differentiation of both PDLSCs and DPSCs. The data are shown as the mean ± SD of triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$

the HA-TCP carrier (Figure 4a, b). With anti-human mitochondrial antibody staining, cementoblast-like cells stained positive (Figure 4c, d). Also, DPSCs and leptin-treated DPSCs differentiated into odontoblast-like cells that formed a dentin-like structure on the HA-TCP (Figure 4e, f). Also, DPSCs and leptin-treated DPSCs were positive for anti-human mitochondrial antibody and generated a dentin-like structure (Figure 4g, h). As a negative control, PDLSCs and DPSCs were reacted with anti-IgG antibody that had not been positively identified (Figure S3). These results indicate that transplanted human cells supplemented with leptin can form cementum-like and dentin-like structures. Six weeks after transplantation, both PDLSCs and DPSCs showed no big difference in the hard tissue area. By comparing hard tissue formation on PDLSCs and DPSCs, leptin-treated PDLSCs and DPSCs had a higher degree of hard tissue formation at 12 weeks after transplantation (Figure S3).

Discussion

Mesenchymal stem cells isolated from adult human dental pulp and periodontal ligament have been known to have the potential to differentiate into odontoblasts, cementoblasts, adipocytes, chondrocytes, and myoblasts (Seo *et al*, 2004; Shi *et al*, 2005; Chang *et al*, 2006). These multipotent human dental stem cells indicate that dental stem cells *in vitro* and *in vivo* could proliferate and differentiate into several adult cells with differentiation medium (Shi *et al*, 2005). Recent reports showed that osteoblastic lineages from human stromal cells (Thomas *et al*, 1999) and MSCs (Reseland *et al*, 2001; Cornish *et al*, 2002) were influenced by leptin either directly or indirectly.

Leptin, secreted by white adipose tissue, has been known to be a key regulator of body fat, body mass, and bone formation (Cinti *et al*, 1997; Couce *et al*, 1997; Rosenbaum *et al*, 1997). Leptin is known to promote proliferation and differentiation of osteoblasts and suppress adipocytes. Current studies indicated that leptin is an important regulator of osteogenesis and adipogenesis. Some of the studies showed that osteoblasts cells isolated from bone marrow were suppressed in early osteogenesis and proliferated and synthesized collagen with leptin expression in late osteoblasts (Gordeladze *et al*, 2002). However, the metabolism of leptin in the bone for osteogenesis still remains to be elucidated. Several previous studies demonstrated leptin signaling pathways via complex integration of transcription factors and mechanisms in the ventral hypothalamus (Takeda *et al*, 2002; Harada and Rodan, 2003; Thomas, 2004). Ducy *et al* (2000) suggested that leptin suppressed bone formation via a hypothalamus relay. In their findings, *ob/ob* mice and *db/db* mice, which lacked leptin and leptin receptor, showed a higher trabecular bone formation. This data conflicts with the *fa/fa* rats, which has an inactive

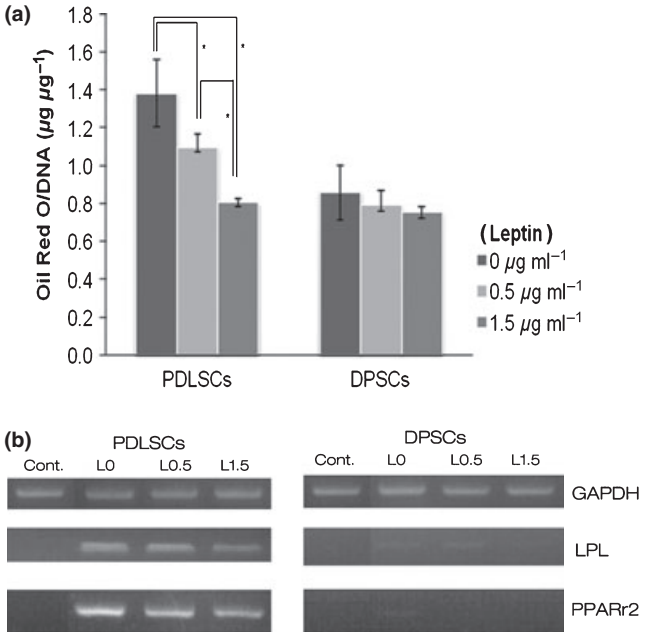

Figure 3 Effect of leptin on adipogenic differentiation of periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs). (**a**) Oil red O staining shows that both PDLSCs and DPSCs developed into oil red O-positive lipid-laden cells. Compared with adipogenic-induced DPLSCs, leptin inhibited adipogenesis in PDLSCs more. The data are shown as the mean ± SD of triplicate determinations. (**b**) Two adipogenic markers, peroxisome proliferator-activated receptor γ2 (PPARγ2) and lipoprotein lipase (LPL), were observed in leptin-treated adipogenic differentiation induction compared with the non-induced control (cont.). Both PPARγ2 and LPL gene expressions decreased with the treatment of leptin in PDLSCs. However, DPSCs showed that both adipogenic markers were expressed to a lower degree than in PDLSCs. $^*P < 0.05$ (cont. = no induction, L0 = adipogenic induction but no leptin treatment, L0.5 = adipogenic induction + 0.5 μg ml^{-1} leptin, L1.5 = adipogenic induction + 1.5 μg ml^{-1} leptin)

leptin receptor. Foldes *et al* (1992) found that *fa/fa* rats had a lower degree of bone formation and Picherit *et al* (2003) showed that *fa/fa* rats had a lower degree of bone mineralization, bone formation, and a lower level of osteocalcin, which makes osteoblasts. Gordeladze *et al* suggested that leptin enhanced human osteoblasts proliferation, de novo collagen synthesis, and mineralization (Gordeladze *et al*, 2002; Scheller *et al*, 2010).

The *in vitro* data in this study supports the conclusion that leptin enhances cemento/odontoblastic activity. In our experiment, we used two kinds of MSCs from different origins, periodontal ligament, and dental pulp from human third molars. Both PDLSCs and DPSCs showed cell proliferation to varying degrees. The results showed that leptin had a greater suppressive effect on proliferation in PDLSCs than in DPSCs. Also for cemento/odontoblastic differentiation of the two cell lines, leptin had a stronger inductive effect on PDLSCs than on DPSCs. Alizarin red S staining and ALPase activity showed that leptin promoted cementoblastic differentiation of the PDLSCs compared with odontoblastic differentiation of the DPSCs.

In vivo transplantation showed significant increases in the degree of hard tissue formation in the leptin-treated group 12 weeks after transplantation. With the HA/TCP carrier, both PDLSCs and DPSCs formed more abundant hard tissue compared with the non-treated control. A possible explanation for this difference caused by leptin is that PDLSCs and DPSCs could have a different sensitivity to leptin. This relative sensitivity to leptin may be related to the changes in the expression of leptin receptors identified by flow cytometry analysis when treated with exogenous leptin. Treating both cell lines with leptin, PDLSCs displayed a different level of expression for leptin receptors based on the signal intensity from staining with monoclonal anti-human leptin receptor antibodies. There are several studies about leptin receptor forms and functions in peripheral pathways (Lee *et al*, 1996; Bjorbaek *et al*, 1997; Tartaglia, 1997; Margetic *et al*, 2002). Unlike the central nervous system, the different types of isoforms have a role in signal transduction in peripheral tissues. Also, particular isoforms of leptin receptors are expressed in various cells in peripheral tissues. In this study, the expression of leptin receptors in PDLSCs was affected by leptin treatment. However, DPSCs showed no difference in the expression of leptin receptors after leptin treatment. Therefore, PDLSCs and DPSCs may have a different sensitivity to leptin because of the different isoforms of the leptin receptor. Further investigation is needed to elucidate the interaction between leptin and the leptin receptor in MSC differentiation.

Recently, MSCs have showed the potential to become the adipocytes. With adipogenic inductive medium, PDLSCs, DPSCs, bone marrow stem cells, and UCB stem cells have adipogenesis-related gene expression for PPARγ, acylCoA synthetase (ACS), and LPL (Sekiya *et al*, 2004; Seo *et al*, 2004; Chang *et al*, 2006). Also, lipid clusters were detected with oil red O staining (Seo *et al*, 2004). As a modulator of fat tissue, leptin acts on human marrow stromal cells to inhibit adipogenesis (Thomas *et al*, 1999). In this study, the gene expression of PPARγ2 and LPL in dental MSCs during the adipogenesis was checked. Both PPARγ and LPL gene expression decreased with leptin treatment in PDLSCs. This inhibition of adipogenesis was shown with oil red O staining. However, DPSCs had a lower gene expression for adipocytes. Leptin affected both cell lines for adipogenic gene expression during adipogenesis to different degrees. From Oil red O staining, DPSCs showed a lower level of lipid clusters. Even though DPSCs has a low potential to become adipocytes, the adipogenic differentiation of MSCs was suppressed by leptin.

In summary, this study shows that leptin acts on PDLSCs and DPSCs to enhance cementogenic/odontogenic potential and to inhibit adipogenesis. We have also found that leptin significantly reduced proliferation in both DPSCs and PDLSCs. Our study has provided evidence that leptin acts as an important modulator of dental MSCs' differentiation.

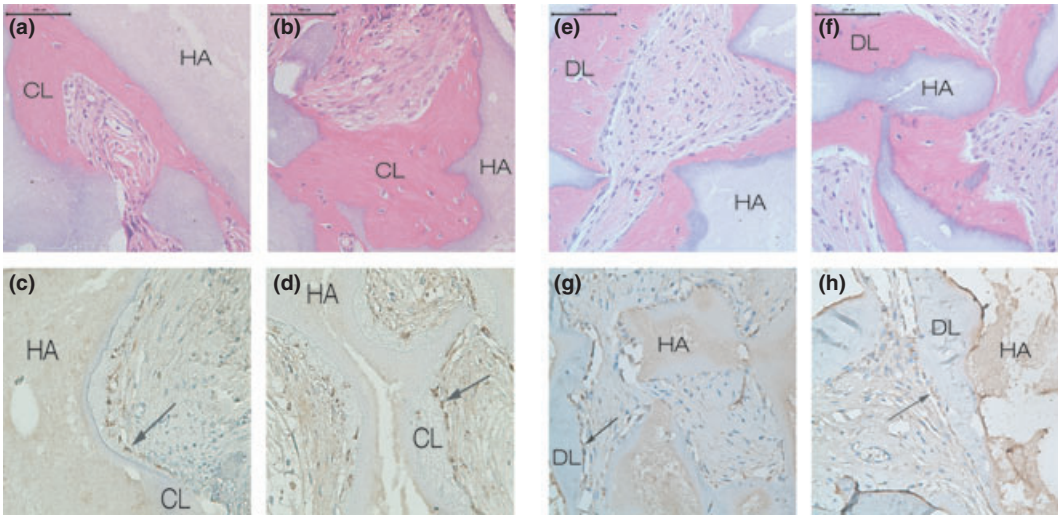

Figure 4 *In vivo* hard tissue formation by leptin-treated periodontal ligament stem cells (PDLSCs) and dental pulp stem cells (DPSCs). After 12 weeks of *in vivo* transplantation, (**a**) PDLSCs and (**b**) leptin-treated PDLSCs differentiated into cementoblast-like cells that formed a cementum-like structure (CL) on the surface of hydroxyapatite tricalcium phosphate (HA) carrier. Immunohistochemical staining with anti-human mitochondria antibodies showed that (**c**) PDLSCs and (**d**) leptin-treated PDLSCs were positive and generated a cementum-like structure from cementoblast-like cell (arrow). (**e**) DPSCs and (**f**) leptin-treated DPSCs differentiated into dentin-like cells that formed a dentin-like structure (DL) on the surface of the HA/TCP carrier. (**g**) DPSCs and (**h**) leptin-treated DPSCs were positive and generated a dentin-like structure from odontoblast-like cells (arrow) (Bar = 100 μm, magnification ×400)

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Author contributions

S. Um produced and analyzed data, drafted paper. J.-R. Choi initiated study, produced data. J.-H. Lee help acquiring data. Q. Zhang help acquiring data. B.M. Seo designed study, analyzed data, drafted paper.

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Supporting Information

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

Figure S1 BrdU labeling for cell proliferation.

Figure S2 Flow cytometric analysis.

Figure S3 *In vivo* hard tissue formation by leptin-treated PDLSCs and DPSCs after12 weeks.

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