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REGULAR ARTICLE

Leptin and its receptor expression in dental and periodontal tissues of primates

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Abstract Leptin and its receptor (OBR) have attracted much attention since their discovery. They have been reported to play central roles in energy balance, the immune-inflammatory response and bone metabolism. Evidence indicates that leptin and OBR are associated with inflammatory diseases of dental and periodontal tissues. The first step for establishing this is to determine the expression of leptin and OBR in these tissues. Our study is the first to examine systematically the expression of leptin and OBR in dental and periodontal tissues of monkeys (*Macaca fascicularis*) by immunohistochemistry and in primary cultured cells, isolated from human dental and periodontal tissues, by reverse transcription plus the polymerase chain reaction and immunocytochemistry. Our results show that leptin and OBR are constitutively expressed and widely distributed in dental and periodontal tissues of primates. Their immunoreaction is especially strong in junctional epithelium, a unique front-line defense around teeth and in mineralizing areas of the dental pulp and periodontal ligament. The expression of the long and also functional form of OBR (OBRb) indicates that leptin has a direct effect on these cells. Thus, we can reasonably infer that leptin and OBR exert effects on defense, mineralization and angiogenesis in dental and periodontal tissues of primates.

Keywords Leptin · OBR · Dental and periodontal tissue · Immunohistochemistry · Primate · *Macaca fascicularis* · Human

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Introduction

Leptin is an adipocyte-derived hormone/cytokine reported to play a central role in energy balance (Friedman and Halaas 1998), the immune-inflammatory response (La Cava and Matarese 2004) and bone metabolism (Gimble 2011). As periodontitis has been demonstrated to be closely related to systemic inflammatory diseases, earlier studies on the action of leptin as an acute phase protein and its correlation with systemic inflammatory diseases such as myocardial infarction (Soderberg et al. 1999) and acute sepsis (Arnalich et al. 1999; Bornstein et al. 1998) have triggered the hypothesis that leptin might also be associated with periodontal inflammation. As expected, our group (Liu et al. 2013) and others (Gangadhar et al. 2011; Karthikeyan and Pradeep 2007a; Teles et al. 2011; Zimmermann et al. 2012) have found that serum levels of leptin are significantly higher in patients with periodontitis

compared with healthy controls and are correlated positively with leukocyte and neutrophil counts, serum interleukin (IL)-1β, IL-6 and tumor necrosis factor alpha (TNF-α) and with clinical markers of periodontal destruction, such as bleeding index (BI), probing depth (PD) and attachment loss (AL), whereas after periodontal treatment, serum leptin levels decrease significantly (Shimada et al. 2010). These findings indicate a role of leptin in periodontal diseases. Meanwhile, increased leptin levels in inflamed dental pulp (Martin-Gonzalez et al. 2012) and chronic periapical lesions (Kangarlou Haghighi et al. 2010) suggest a role of leptin in other inflammatory diseases of dental and periodontal tissues.

Since its discovery in 1994 (Zhang et al. 1994), appreciable attention has been directed to the determination of the location and distribution of leptin biosynthesis and secretion (Zhang et al. 2005). Research has shown that leptin is mainly produced by the adipose tissue and, at lower levels, by tissues such as the stomach, skeletal muscle and placenta (La Cava and Matarese 2004). Although leptin has been identified in gingival crevicular fluid (GCF; Bozkurt et al. 2006; Karthikeyan and Pradeep 2007a, 2007b; Zimmermann et al. 2012), gingival tissues (Johnson and Serio 2001), dental pulp (Martin-Gonzalez et al. 2012) and even chronic periapical lesions (Kangarlou Haghighi et al. 2010), no systematic study has been conducted to unveil the expression of leptin and its distribution in dental and periodontal tissues. Moreover, insufficient evidence has been obtained in order to determine whether leptin is a result of local production or is transported by circulation, let alone to pinpoint its cellular sources.

Leptin exerts its effects through binding to its specific receptors, OBR, which exist in alternatively spliced forms. Notably, only the long form of OBR (OBRb) has the full length of the intracellular domain and mediates most of the signaling of leptin (Bjorbaek et al. 1997; Murakami et al. 1997). OBRb has been reported to be primarily expressed by the hypothalamus in areas regulating appetite, body weight and bone mass (La Cava and Matarese 2004). However, endothelial cells, hematopoietic precursors, monocytes and T cells have also recently been found to express OBRb. This indicates a direct effect of leptin on these cells (Lord et al. 1998; Sanchez-Margalet et al. 2003; Sierra-Honigmann et al. 1998). Ay and colleagues (2011) have discovered OBR expression in gingival epithelium. However, the way in which OBR is expressed in other parts of dental and periodontal tissues and the types of cells that express OBRb need further determination.

Thus, based on the above, leptin and its receptor might play a role in dental and periodontal diseases. To verify this, the first step is to systematically examine the expression of leptin and its receptor in dental and periodontal tissues. In this study, our aim has been comprehensively to clarify the expression and distribution of leptin and its receptor in dental and periodontal tissues and to try to infer their possible roles.

Materials and methods

Experimental animals and tissue samples

Specimens from monkeys (*Macaca fascicularis*) were obtained from earlier studies of our group (Zhu et al. 2009, 2012). Briefly, three adult male monkeys (5.5–6.0 years old, weighing 5.1–5.5 kg, Laboratory Animal Center of Academy of Military Medical Sciences, Beijing, China) were killed by an overdose of ketamine hydrochloride. Mandibular posterior teeth with surrounding soft and hard tissues (gingiva and alveolar bones) were dissected and fixed in 10 % paraformaldehyde; they were then decalcified in EDTA, dehydrated and embedded in paraffin. Mesio-distal serial sections were cut parallel to the long axis of the teeth with the microtome set at 5 μm. At least one section from each sample was examined with hematoxylin and eosin (H&E). In total, six mandibular second premolar samples almost entirely filled with newly formed bone were selected for the experiments below. The study protocol was reviewed and approved by the Experimental Animal Welfare Ethical Branch of Peking University Biomedical Ethics Committee (LA2008-006).

Immunohistochemistry

Selected sections were transferred onto adhesive slides (Citotest labware manufacturing, Jiangsu, China). After deparaffinization with xylene and rehydration with descending concentrations of ethanol, endogenous peroxidase was blocked by treatment with 3 % H_2O_2 for 10 min at room temperature. Antigen retrieval was achieved by 1 mg/ml trypsin digestion at 37 °C for 10 min. After being blocked with 10 % normal goat serum at room temperature for 10 min, sections were incubated with rabbit anti-recombinant human leptin polyclonal antibody (1:400; Santa Cruz Biotechnology, Calif., USA; Dumond et al. 2003; Sanna et al. 2003; Ay et al. 2011) or rabbit anti-recombinant human OBR polyclonal antibody (1:50; Santa Cruz; Cammisotto et al. 2005) diluted in antibody diluent (Zhongshan Golden Bridge Biotechnology, Beijing, China) at 4 °C overnight. After being washed with phosphate-buffered saline (PBS, 0.01 M, pH 7.4), the location of leptin and OBR was visualized by using a PV-9001 kit and a 3,3′-diaminobenzidine (DAB) kit (Zhongshan Golden Bridge Biotechnology). Sections were finally counterstained with hematoxylin and mounted. The specificity of the immunoreaction was confirmed by incubation with normal rabbit IgG (Santa Cruz) and a preadsorption experiment (see Electronic Supplementary Material, File S1). Images were captured on a digital microscopic system (Olympus BX51/DP72, Tokyo, Japan).

Human cell isolation

To verify the results from dental and periodontal tissues of *Macaca fascicularis*, human periodontal ligament cells

(hPDLCs), gingival fibroblasts (hGFs) and gingival epithelial cells were isolated and passaged as described elsewhere (Diamond et al. 2010; Liu et al. 2012; Um et al. 2011). The study protocol was approved by the institutional review board of Peking University School and Hospital of Stomatology (PKUSSIRB-2011007). Primary cultured human dental pulp cells (hDPCs) were kindly donated by Dr. Wei Wang (Department of Prosthetics, Peking University School and Hospital of Stomatology).

Detection of leptin and its receptor mRNA expression

Total RNA was extracted from the above cultured cells by using TRIzol reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. Approximately 2 μg total RNA was converted to cDNA by reverse transcription (RT) with a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA). Polymerase chain reactions (PCR) were performed by using the *Taq* PCR MasterMix (Beijing Solarbio Science & Technology, China) in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The primer sequences used were as follows: D-glyceraldehyde-3-phosphate dehydrogenase, forward: CGACAGTCAGCCGCATCTT, reverse: CCAATACGAC CAAATCCGTTG; leptin, forward: TCCTGGGCTCCAC CCCATCC, reverse: CCCAGGGCAAGTGGCAGCTC; OBR, forward: TGGAAGGAGTGGGAAAACCAA, reverse: TAAGTCCTTGTGCCCAGGAA; OBRb, forward: CAAGAATTGTTCCTGGGCACA, reverse: TCAGGCTCC AAAAGAAGAGGA. The products were confirmed by agarose gel electrophoresis and nucleotide sequencing.

Detection of leptin and its receptor protein expression

The protein expression of leptin and its receptor was detected by immunocytochemistry. The above cultured cells were seeded on glass slides. After 8 h of culture, cells on glass slides were fixed with 95 % ethanol at room temperature for 30 min. Then, endogenous peroxidase was blocked by treatment with 3 % H_2O_2 for 10 min at room temperature. After being blocked with 10 % normal goat serum at room temperature for 10 min, cells were incubated with the same antibodies mentioned above at 4 °C overnight. After being washed with PBS, the localization of leptin and OBR was visualized by using a PV-9001 kit and a DAB kit (Zhongshan Golden Bridge Biotechnology) followed by hematoxylin staining. The primary antibody was replaced with normal rabbit IgG for negative controls.

Results

In situ expression of leptin and its receptor in dental and periodontal tissues of monkeys

H&E staining is shown in Fig. 1a. Leptin expression was detected in gingival epithelium, dental pulp, periodontal ligament and bone marrow (Fig. 1b). To be specific, in junctional epithelium, a unique epithelial seal around teeth, leptin was strongly positive in every layer (Fig. 2c). In the oral epithelium and sulcular epithelium (from another sample), leptin appeared to be evenly positive from the stratum basale to the stratum granulosum, whereas in the stratum corneum of the oral epithelium with parakeratinization, leptin immunoreaction was much weaker (Fig. 3c, f). In gingival connective tissue, only some gingival fibroblasts expressed leptin and the staining was weak (Figs. 2c, 3c, f). Some mononuclear inflammatory cells, most likely to be lymphocytes (Bartold et al. 2000; see Electronic Supplementary Material, File S1, Fig. S1) beneath the sulcular epithelium also expressed leptin (Fig. 3f). In dental pulp (Fig. 1b), cells in the pulp core were weakly positive for leptin, whereas odontoblasts (Fig. 2g) lining the outermost layer of pulp were significantly positive for leptin. Even some cytoplasmic processes of odontoblasts, extending into the

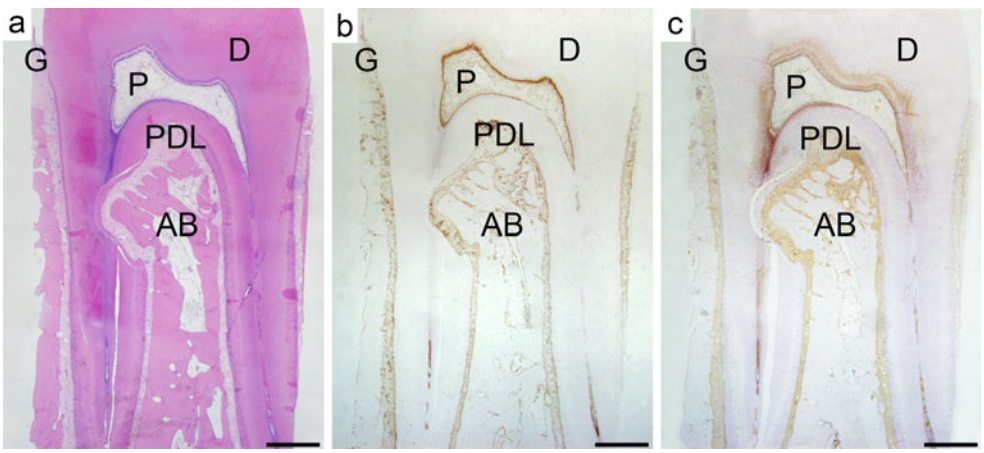
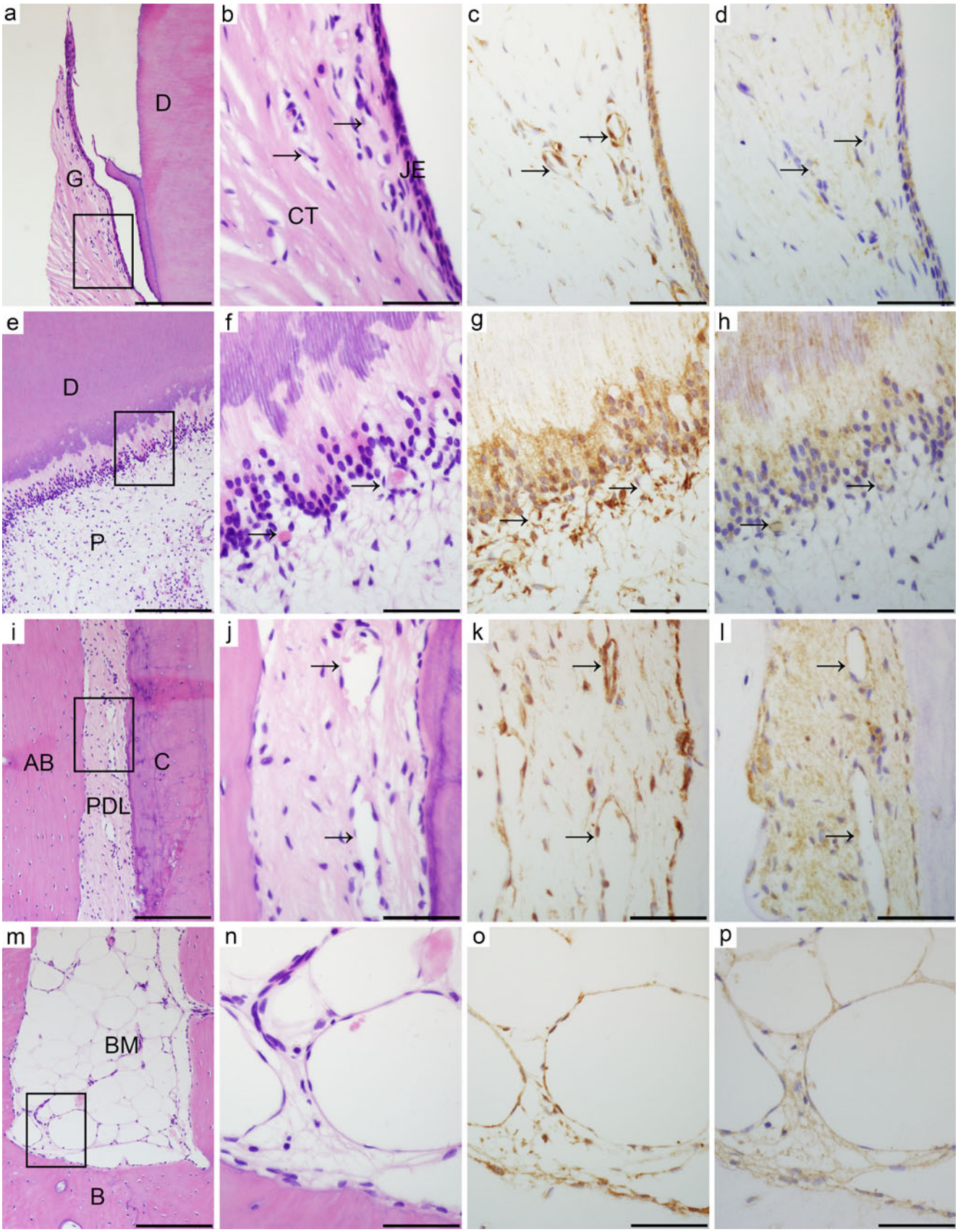


Fig. 1 Hematoxylin and eosin (H&E) staining (**a**) and leptin (**b**) and its receptor OBR (**c**) immunohistochemistry of dental and periodontal tissue samples of the studied monkeys, *Macaca fascicularis* (*G* gingiva, *D* dentin, *P* pulp, *PDL* periodontal ligament, *AB* alveolar bone). *Bars* 1 mm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◀ **Fig. 2** **a**, **e**, **i**, **m** H&E staining of gingiva, pulp, periodontal ligament and alveolar bone. **b**, **f**, **j**, **n** Higher magnification of the *boxed areas* in **a**, **e**, **i**, **m**, respectively. **c**, **g**, **k**, **o** Leptin immunoreactivity in the same areas of **b**, **f**, **j**, **n**, respectively. **d**, **h**, **l**, **p** OBR immunoreactivity in the same areas of **b**, **f**, **j**, **n**, respectively (*arrows* endothelial cells lining the capillaries, *G* gingiva, *D* dentin, *JE* junctional epithelium, *CT* connective tissue, *P* pulp, *AB* alveolar bone, *C* cementum, *PDL* periodontal ligament, *BM* bone marrow, *B* bone). *Bars* 200 μm (**a**, **e**, **i**, **m**), 50 μm (**b–d**, **f–h**, **j–l**, **n–p**)

dentinal tubules showed leptin immunoreativity (Fig. 2g). In periodontal ligament, leptin staining seemed to be more obvious in PDLCs lying next to the alveolar bone and cementum (Figs. 1b, 2k). In alveolar bone, osteoblasts lining the bone marrow cavities and fat cells in bone marrow were leptin-positive (Figs. 1b, 2o). Moreover, observed endothelial cells were positive for leptin (arrows in Figs. 2c, g, k, 3c, f). Overall, leptin staining was most obvious in junctional epithelium, the outermost layer of pulp and periodontal ligament, followed by oral and sulcular epithelium, alveolar bone and the central part of the pulp, with the lowest staining in gingival connective tissue.

Localization of OBR staining was similar to that of leptin in dental and periodontal tissues. OBR expression was also demonstrated in gingival epithelium, dental pulp, periodontal ligament and alveolar bone (Fig. 1c). However, in inflammatory zone subepithelially, OBR immunoreaction was weak (Fig. 3g). Results of the negative controls confirmed the specificity of the immunoreaction (see Electronic Supplementary Material, File S1, Fig. S2, Fig. S3).

Leptin and its receptor expression in human cells isolated from dental and periodontal tissues in vitro

RT-PCR analysis of total RNA from hPDLCs, hGFs, hDPCs and human gingival epithelial cells revealed the presence of leptin, OBR and OBRb mRNA transcripts in all these cells (Fig. 4). Immunocytochemical staining of leptin and OBR protein confirmed the result of RT-PCR. Leptin and OBR (the primary antibody was raised against amino acids mapping within the internal domain) immunostaining was localized in the cytoplasm of PDLCs, DPCs, gingival epithelial cells and some GFs (Fig. 5). Taken together, leptin was most abundant in gingival epithelial cells and PDLCs, followed by DPCs and was the lowest in GFs. In contrast, OBR and OBRb were most abundant in gingival epithelial cells, followed by PDLCs, DPCs and GFs. The results were almost consistent with those observed in dental and periodontal tissues of *Macaca fascicularis* in vivo.

Discussion

To our knowledge, we are the first to identify systematically the expression of leptin and its receptor in situ in dental and periodontal tissues in vivo. Compared with other species, non-human primates are ideal experimental animal models for human diseases, as their physiology, pathology, immunology and anatomy show high similarities with those of humans (Schou et al. 1993). In our study, the expression of leptin

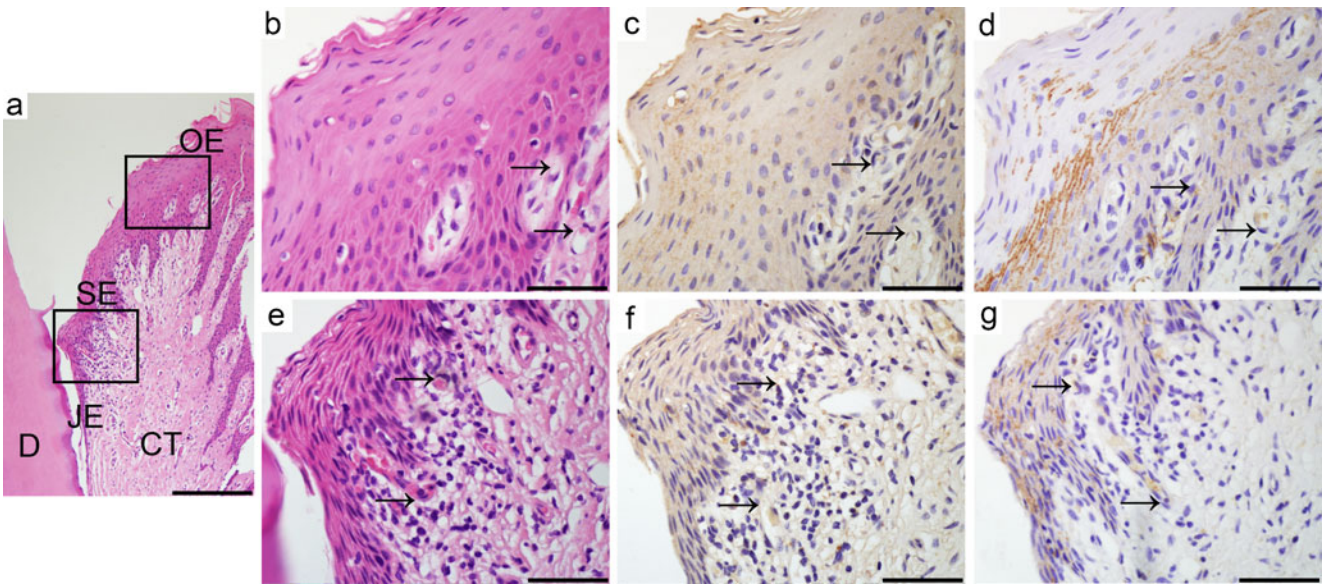

Fig. 3 **a** H&E staining of gingiva. **b**, **e** Higher magnification of the *boxed areas* in oral epithelium (*OE*) and sulcular epithelium (*SE*), respectively. **c**, **f** Leptin immunoreactivity in the same areas of **b**, **e**, respectively. **d**, **g** OBR immunoreactivity in the same areas of **b**, **e**, respectively (*arrows* endothelial cells lining the capillaries, *OE* oral epithelium, *SE* sulcular epithelium, *JE* junctional epithelium, *D* dentin, *CT* connective tissue. *Bar* 200 μm (**a**), 50 μm (**b–g**)

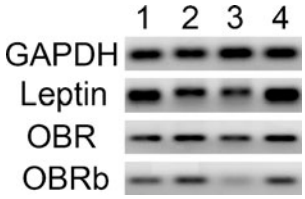

Fig. 4 Messenger RNA expression of leptin, OBR and the long form of OBR (*OBRb*) in primary cultured human periodontal ligament cells (*lane 1*), gingival fibroblasts (*lane 2*), dental pulp cells (*lane 3*) and gingival epithelial cells (*lane 4*). D-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control

and its receptor and their distribution in the gingiva and dental pulp were consistent with those found in previous human studies (Martin-Gonzalez et al. 2012; Ay et al. 2011). Moreover, the investigation of human primary cultured cells from dental and periodontal tissues in vitro further confirmed the reliability of our data. In addition to generally known bone marrow adipocytes (Laharrague et al. 1998), major cell components of dental and periodontal tissues all constitutively express leptin and its receptor to some extent. Notably, the expression levels of leptin in the periodontal ligament and dental pulp are equivalent to or even higher than that in bone marrow adipose tissue. Combined with our previous results showing that leptin levels in GCF are significantly higher than that in serum, both in healthy controls and in patients with periodontitis (Liu et al. 2013), our findings indicate that dental and periodontal tissues represent another important source of leptin not only locally but also systemically. Furthermore, as the short and soluble forms of OBR seem to mediate the transport and degradation of leptin, the widely expressed OBR shown by immunohistochemistry indicates that dental and periodontal tissues are active places for leptin transport and degradation. The observation that primary cultured human PDLCs, GFs and gingival epithelial cells all express OBRb suggests a role for leptin in the direct regulation of these cells.

Leptin is now recognized as a critical factor in host defense mechanisms (La Cava and Matarese 2004; Matarese et al. 2005). In intestinal epithelial cells, leptin has been demonstrated to protect cells from a challenge with *Entamoeba histolytica* by inhibiting host-cell apoptosis, promoting proliferation and differentiation and inducing the expression of anti-microbial peptides and chemokines (Mackey-Lawrence and Petri 2012). As shown in our immunohistochemical results, leptin in gingival epithelium (including oral, sulcular and junctional epithelium) might share the same mechanisms in mediating resistance to microbial pathogens. The epithelia are now recognized not to be "passive bystanders" but rather to be metabolically active and capable of reacting to external stimuli. Among them, the junctional epithelium is unique for serving many more roles in regulating tissue health. One remarkable character is its shorter cellular turnover time, which in monkeys is estimated to be 4–6 days, i.e., 50–100 times shorter than that for the oral epithelium (Shimono et al. 2003). Moreover, throughout the junctional epithelium, numerous migrating polymorphonuclear leukocytes and lymphocytes (particularly T lymphocytes) are evident, even in healthy tissues (Bartold et al. 2000). Finally, intercellular adhesion molecule-1 (ICAM-1) is selectively expressed by the junctional epithelium (Bartold et al. 2000; Rummel et al. 2010). All the underlying mechanisms are unknown, among which the highly expressed leptin might account for one possible mechanism.

Leptin expression has been reported in primary cultured human osteoblasts in the mineralization and/or the osteocyte transition period (Reseland et al. 2001). PDLCs and DPCs both have high mineralization ability and have some characteristics of osteoblasts (Lallier et al. 2005; Mori et al. 2011). During mineralization, PDLCs and DPCs might also express high levels of leptin, as evidenced by the immunohistochemical results, with leptin staining being more obvious in odontoblasts lining predentin and PDLCs lining alveolar bones and the precementum. However, more understandably, leptin is a potent stimulator of bone formation peripherally (Turner et al. 2012) and has been established to promote osteogenesis in PDLCs (Um et al. 2011) and bone marrow mesenchymal stem cells (BMSCs; Thomas et al. 1999) in vitro. Thus, leptin might form an important autocrine and/or paracrine loop in the local environment, promoting mineralization homeostasis in healthy tissues and enhancing osteogenesis after destruction. In another aspect, GFs and PDLCs are two populations of fibroblasts residing in the periodontal tissue. However, because of their

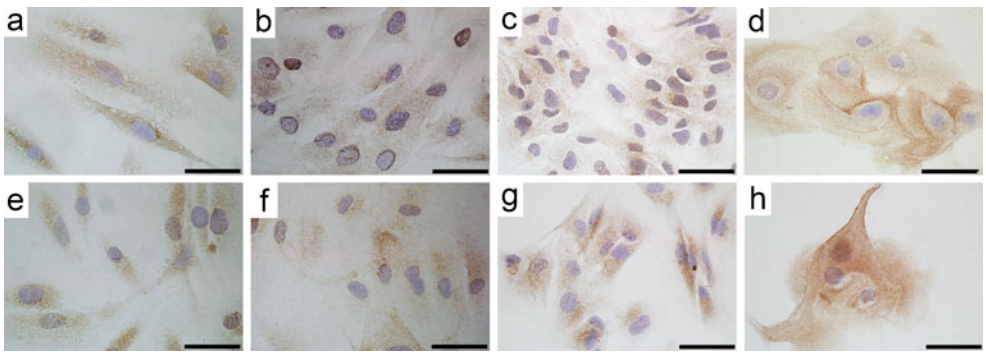

Fig. 5 Protein expression of leptin and OBR in primarily cultured human periodontal ligament cells (**a**, **e**), gingival fibroblasts (**b**, **f**), dental pulp cells (**c**, **g**) and gingival epithelial cells (**d**, **h**), respectively. *Bar* 50 μm

distinct localization, GFs show unique characteristics regarding proliferation, protein expression profiles and their significantly lower osteogenic ability in comparison with PDLCs (Giannopoulou and Cimasoni 1996; Hou and Yaeger 1993; Mariotti and Cochran 1990; Somerman et al. 1988). The obvious divergence of leptin levels shown by in vivo immunohistochemical results might further explain the functional differences between GFs and PDLCs.

Furthermore, leptin has proangiogenic effects, including the induction of neovascularization and the formation of capillary-like structures (Bouloumie et al. 1998; Sierra-Honigmann et al. 1998). The dental pulp, gingiva, periodontium and alveolar bone are all highly vascularized connective tissues with good healing potential after injury and inflammation. In part, this good healing potential is related to the ability of local cells to secrete factors that are essential for cell differentiation and neovascularization (Schroder 1985). Thus, the local, abundantly expressed leptin might in part contribute to the great healing potential of dental pulp and periodontal tissues.

From the above data taken together, leptin can generally be assumed to be an inherent component in dental and periodontal tissues and to take an active part in local metabolism, defense and regeneration. The high levels of leptin might stimulate the immune system (Gainsford et al. 1996), protect the host from inflammation and infection and maintain bone levels (Dilsiz et al. 2010). Further investigation of its function in dental pulp and periodontal tissues is imperative for understanding the role of leptin and its receptor in the initiation and progression of local diseases.

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