

Characterization of the Autocrine/Paracrine Function of Vitamin D in Human Gingival Fibroblasts and Periodontal Ligament Cells

Kaining Liu, Huanxin Meng*, Jianxia Hou

Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China

Abstract

Background: We previously demonstrated that 25-hydroxyvitamin D₃, the precursor of 1 α ,25-dihydroxyvitamin D₃, is abundant around periodontal soft tissues. Here we investigate whether 25-hydroxyvitamin D₃ is converted to 1 α ,25-dihydroxyvitamin D₃ in periodontal soft tissue cells and explore the possibility of an autocrine/paracrine function of 1 α ,25-dihydroxyvitamin D₃ in periodontal soft tissue cells.

Methodology/Principal Findings: We established primary cultures of human gingival fibroblasts and human periodontal ligament cells from 5 individual donors. We demonstrated that 1 α -hydroxylase was expressed in human gingival fibroblasts and periodontal ligament cells, as was cubilin. After incubation with the 1 α -hydroxylase substrate 25-hydroxyvitamin D₃, human gingival fibroblasts and periodontal ligament cells generated detectable 1 α ,25-dihydroxyvitamin D₃ that resulted in an up-regulation of CYP24A1 and RANKL mRNA. A specific knockdown of 1 α -hydroxylase in human gingival fibroblasts and periodontal ligament cells using siRNA resulted in a significant reduction in both 1 α ,25-dihydroxyvitamin D₃ production and mRNA expression of CYP24A1 and RANKL. The classical renal regulators of 1 α -hydroxylase (parathyroid hormone, calcium and 1 α ,25-dihydroxyvitamin D₃) and *Porphyromonas gingivalis* lipopolysaccharide did not influence 1 α -hydroxylase expression significantly, however, interleukin-1 β and sodium butyrate strongly induced 1 α -hydroxylase expression in human gingival fibroblasts and periodontal ligament cells.

Conclusions/Significance: In this study, the expression, activity and functionality of 1 α -hydroxylase were detected in human gingival fibroblasts and periodontal ligament cells, raising the possibility that vitamin D acts in an autocrine/paracrine manner in these cells.

Citation: Liu K, Meng H, Hou J (2012) Characterization of the Autocrine/Paracrine Function of Vitamin D in Human Gingival Fibroblasts and Periodontal Ligament Cells. PLoS ONE 7(6): e39878. doi:10.1371/journal.pone.0039878

Editor: Christopher R. Weber, University of Chicago, United States of America

Received: October 29, 2011; **Accepted:** June 2, 2012; **Published:** June 25, 2012

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Funding: This research was funded by National Natural Science Foundations of China (<http://www.nsf.gov.cn/Portal0/default152.htm>) (30772420, 81100749) and Beijing Natural Science Foundation (<http://www.bjkw.gov.cn/n1143/n1240/n1465/n2276/index.html>) (7102164). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kqhxmeng@bjmu.edu.cn

Introduction

Vitamin D₃ is a major component in the regulation of calcium and phosphorus metabolism. The function of vitamin D₃, via the active hormonal metabolite 1 α ,25-dihydroxyvitamin D₃ (1,25OH₂D₃), is to regulate the absorption of these essential minerals in the intestine, and their mobilization in bone tissues [1]. 1,25OH₂D₃ also plays an important role in the regulation of T cell function and immunological reaction [2]. The biological function of 1,25OH₂D₃ is orchestrated by vitamin D receptor (VDR). After binding with VDR, 1,25OH₂D₃ acts on the vitamin D responsive element (VDRE) located upstream of its target genes (eg. CYP24A1, RANKL, et al.) and up-regulates their expression which results in the corresponding biological effects [3,4,5,6,7].

1,25OH₂D₃ is formed from vitamin D₃ in a two-step hydroxylation: 25-hydroxyvitamin D₃ (25OHD₃) hydroxylated in the liver is transported to the kidney by vitamin D binding protein (DBP) and is metabolized to 1,25OH₂D₃ by the renal cytochrome P450 enzyme 25OHD₃-1 α -hydroxylase CYP27B1 [8].

Besides the kidney [9,10,11], there are extra-renal sites of 1,25OH₂D₃ synthesis, including the skin [12], prostate [13], activated monocytes/macrophages [14], bone [15,16] and sebocytes [17]. Furthermore, local production of 1,25OH₂D₃ in extra-renal tissues has been postulated to regulate parameters of cell growth and differentiation in an autocrine or paracrine fashion [15,18].

Periodontal tissues consist of two hard tissues, bone and cementum, and two soft tissues, gingiva and periodontal ligament. Although bone-derived cells, osteoblast-like cells and osteoblasts can synthesize 1,25OH₂D₃ [15,16], it is unknown whether this also occurs in periodontal soft tissues. Human gingival fibroblasts (hGF) and human periodontal ligament cells (hPDLC) are two kinds of periodontal fibroblasts and are important components of gingiva and periodontal ligament, respectively. Whether 1,25OH₂D₃ synthesis occurs in these cells warrants further investigation.

Our previous studies [19,20] demonstrated that levels of plasma 25OHD₃ of patients with aggressive periodontitis were signifi-

cantly higher than those of healthy controls; local 25OHD₃ levels in gingival crevicular fluids were 300 times higher than plasma levels in these patients. It is not known why the precursor of 1,25OH₂D₃ is abundant around periodontal soft tissues. Recently, McMahon et al. reported that 1,25OH₂D₃ could enhance the antibacterial defense of human gingival epithelial cells [21]. Thus, if 25OHD₃ is converted to 1,25OH₂D₃ by the cells of periodontal soft tissues, this conversion could enhance the innate immune defense in the oral cavity. Here we hypothesized that hGF and hPDLc have 1 α -hydroxylase activity and can convert 25OHD₃ to 1,25OH₂D₃. The objective of this study was to test the above hypothesis.

Results

1 α -hydroxylase mRNA was detected in all the cells of the five donors (Fig. 1 and 2). The 1 α -hydroxylase protein was detected with an antibody that detects 1 α -hydroxylase expression in human osteoblasts, indicating that the 1 α -hydroxylase in hGF and hPDLc could be the same CYP27B1 enzyme as that found in osteoblasts [15,16].

After confirming the expression of 1 α -hydroxylase in hGF and hPDLc, the function of 1 α -hydroxylase was investigated. While 1000 nM 25OHD₃ did not have a significant cytotoxic effect on any of the cells within 48 h. hGF and hPDLc generated 1,25OH₂D₃ in response to 25OHD₃ and the amount of 1,25OH₂D₃ increased with incubation time (Fig. 3A, B). The fact that extra- and intracellular 1,25OH₂D₃ was generated in the presence of 25OHD₃ provides the most convincing evidence of the existence of 1 α -hydroxylase in hGF and hPDLc. Although the total amounts of 1,25OH₂D₃ synthesized by hGF and hPDLc were not significantly different, more 1,25OH₂D₃ was released by hPDLc 12 h after adding 25OHD₃. At all other time points, there was no significant difference in the levels of intracellular and extracellular 1,25OH₂D₃ between the two cell types. Moreover, CYP27B1 mRNA expression peaked at 1 h and no difference was detected between 0 h and any other time point (Fig. 3C). CYP24A1 mRNA expression increased over time and was significant higher at 24 h and 48 h than at 0 h (Fig. 3D).

In addition, exposure to 25OHD₃ also resulted in an up-regulation of the 1,25OH₂D₃ responsive genes CYP24A1 and RANKL in hGF and hPDLc (Fig. 4A, B), which is further evidence of 1 α -hydroxylase activity in hGF and hPDLc.

Based on this direct evidence for 1 α -hydroxylase activity in hGF and hPDLc, we examined the effect of 1 α -hydroxylase knock-down. The efficiency of RNA interference against CYP27B1 was over 70% (Fig. 5). The generation of 1,25OH₂D₃ increased with increasing 25OHD₃ concentrations but dropped significantly when CYP27B1 was knocked down using specific siRNA (Fig. 6A, B).

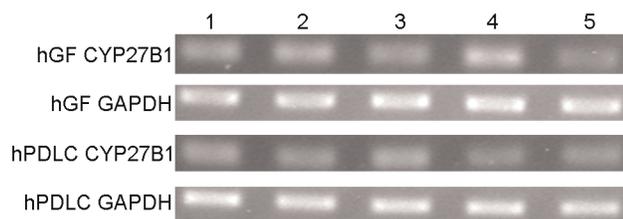


Figure 1. mRNA expression of CYP27B1 in hGF and hPDLc. mRNA expression of CYP27B1 was detected by RT-PCR in hGF and hPDLc from all five donors (each lane represents one donor). GAPDH was used as an internal control.

doi:10.1371/journal.pone.0039878.g001

Although 1,25OH₂D₃ is the major ligand of VDR, 25OHD₃ could bind VDR with lower affinity and have a biological effect [22]. To address this issue, we knocked down CYP27B1 and found that the expression of CYP24A1 and RANKL decreased significantly (Fig. 7A, B), which strongly suggests that the effect of 25OHD₃ in hGF and hPDLc occurs after its conversion to 1,25OH₂D₃. This is additional evidence for the activity of 1 α -hydroxylase in hGF and hPDLc.

After a comprehensive confirmation of 1 α -hydroxylase activity in hGF and hPDLc, the regulation of 1 α -hydroxylase in hGF and hPDLc was investigated. Parathyroid hormone (PTH), calcium, 1,25OH₂D₃ and *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) did not significantly influence CYP27B1 mRNA expression in hGF and hPDLc (Fig. 8A). In contrast, interleukin-1 β (IL-1 β) and sodium butyrate strongly induced CYP27B1 expression independently of 1,25OH₂D₃ (Fig. 8B). In addition, no significant differences in the regulation of CYP27B1 was observed between hGF and hPDLc.

Despite the detection of the two kinds of DBP receptor expression, megalin mRNA expression was not detected. Both mRNA and protein expression of cubilin were detected in hGF and hPDLc (Fig. 9, 10).

Discussion

Interestingly, the 1,25OH₂D₃ produced by hGF and hPDLc can influence vitamin D-responsive gene expression (Fig. 4, 7), an observation that provides a solid indication of an autocrine/paracrine action of vitamin D in periodontal fibroblasts. Because metabolism of 25OHD₃ and 1,25OH₂D₃ by CYP24A1 is very important in the autocrine/paracrine action of vitamin D, we investigated the time course of CYP27B1 and CYP24A1 mRNA expression. CYP27B1 was only up-regulated at 1 h, while CYP24A1 mRNA expression increased over time (Fig. 3C, D). The results indicated that as more 1,25OH₂D₃ was synthesized, CYP24A1 was more robustly expressed, which resulted in more conversion of 1,25OH₂D₃ to 1,24,25OH₃D₃ and 25OHD₃ to 24,25OH₂D₃, in turn resulting in less substrate for 1,25OH₂D₃ synthesis. Thus, hGF and hPDLc could regulate their own 1,25OH₂D₃ generation; this provided further evidence for autocrine/paracrine action of vitamin D.

Vitamin D binding protein (DBP) is the plasma carrier of vitamin D in humans [23]. Nykjaer et al. [24,25] confirmed that vitamin D enters cells by receptor-mediated endocytosis and via the endocytic receptors megalin and cubilin. In the present study, cubilin mRNA and protein were detected in hGF and hPDLc (Fig. 9, 10), however, megalin was not detected despite the use of two pairs of primers. Atkins et al. [16] reported that osteosarcoma cell lines and primary osteoblast-like cells express abundant cubilin mRNA but only osteosarcoma cell lines express megalin mRNA. The cells used in our study were primary cells and their expression of DBP receptors was similar to the DBP receptor expression in primary osteoblast-like cells. Due to the presence of DBP receptors, plentiful 25OHD₃ around periodontal fibroblasts [20] could be used as a substrate for 1,25OH₂D₃ synthesis. In addition, extracellular 1,25OH₂D₃ (Fig. 3A, 6A), generated by hGF and hPDLc, could enter the surrounding cells through cellular uptake associated with DBP, which suggests the possibility of paracrine action of vitamin D.

In the present study, CYP24A1 and RANKL mRNA expression were chosen to test the downstream biological effects of 25OHD₃. It is striking that the biological effects caused by the locally produced 1,25OH₂D₃ after 1000 nM 25OHD₃ treatment were much stronger than those achieved with exogenous 10 nM

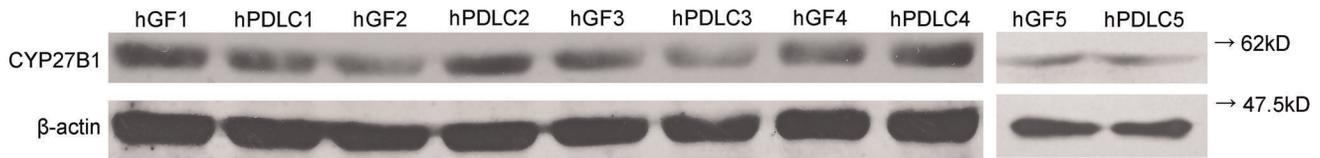


Figure 2. Protein expression of CYP27B1 in hGF and hPDLC. Protein expression of CYP27B1 was detected by Western blot in hGF and hPDLC from all five donors (donors are numbered 1–5). β -actin was used as an internal control.
doi:10.1371/journal.pone.0039878.g002

1,25OH₂D₃ (Fig. 4A, B), and those similarly observed in treatment of osteoblasts [15]. After 1000 nM 25OHD₃ treatment, 1,25OH₂D₃ concentration in the supernatants of hGF and hPDLC were 120 pM–360 pM and 120 pM–600 pM, respectively, much lower than 10 nM, but the biological effects were much stronger. One reason might be that after 25OHD₃ treatment, 1,25OH₂D₃ is found not only in the supernatant but also in the cell lysates, allowing intracellular 1,25OH₂D₃ to bind VDR and influence the function of these cells directly. On the other hand,

exogenous 1,25OH₂D₃ should enter the cells before eliciting a response. Thus, the direct availability at the site of action might be of great importance.

IL-1 β in gingival crevicular fluids of patients with periodontitis reduced significantly after initial periodontal therapy, indicating that IL-1 β is associated with periodontitis [20]. *Porphyromonas gingivalis* is an important pathogen of periodontitis and butyrate is its metabolite [26]. In our previous studies [27,28], we demonstrated that the butyrate concentrations in gingival crevicular fluids

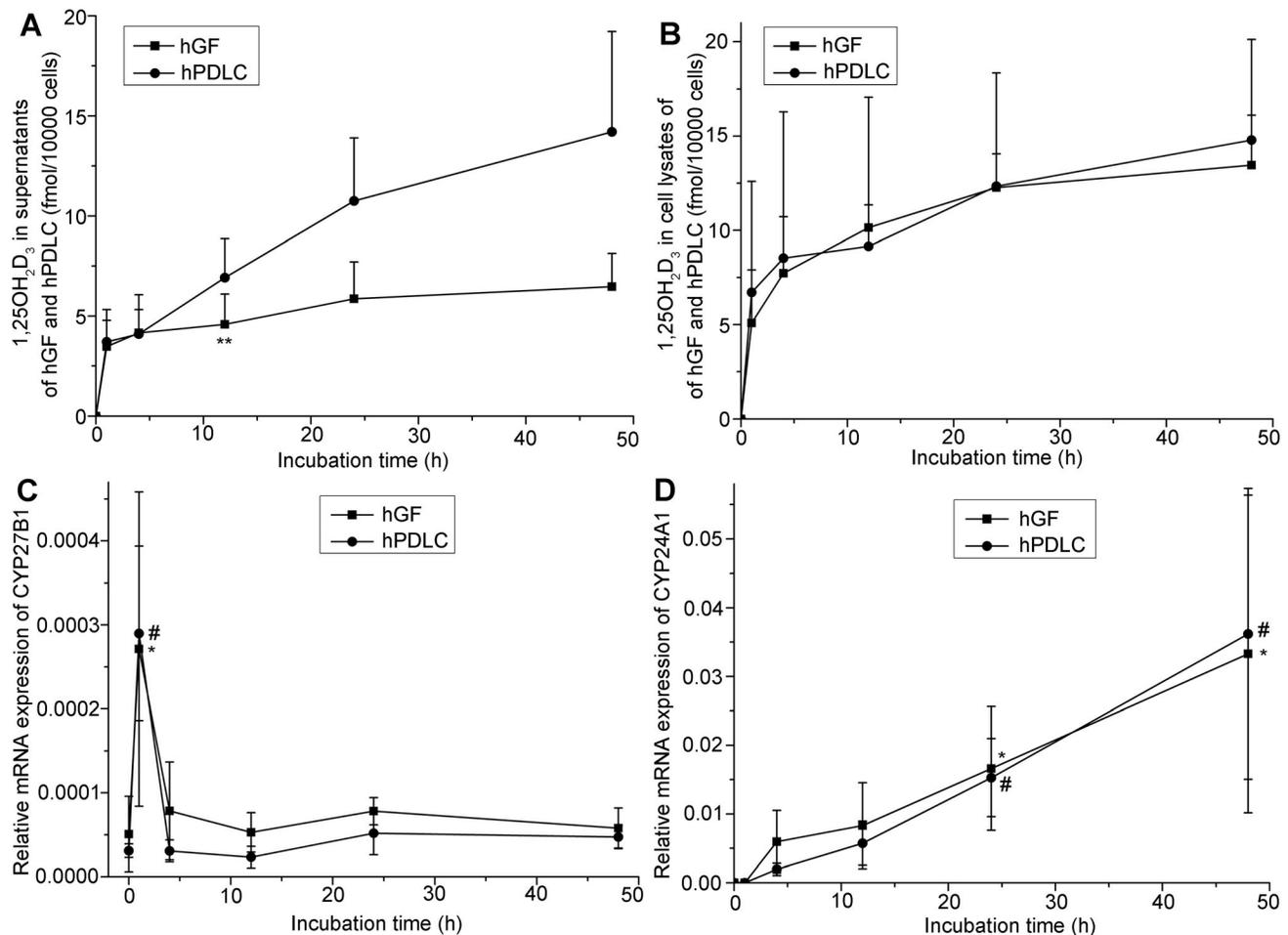


Figure 3. Activity of 1 α -hydroxylase in hGF and hPDLC. hGF and hPDLC from donors 2, 3 and 5 were incubated with 1000 nM 25OHD₃ for the times indicated and the production of 1,25OH₂D₃ was determined in (A) supernatants and (B) cell lysates. After prolonged incubation, production of 1,25OH₂D₃ increased. hPDLC released more 1,25OH₂D₃ than hGF 12 h after incubation with 25OHD₃. The data is presented as the mean \pm SE. ** hGF generated significantly less 1,25OH₂D₃ than hPDLC at the same time point ($p < 0.05$). The time course of CYP27B1 (C) and CYP24A1 (D) mRNA expression is also presented. CYP27B1 mRNA expression peaked at 1 h and no difference was detected between 0 h and any other time point. CYP24A1 mRNA expression was significantly higher at 24 h and 48 h than 0 h. * CYP27B1 or CYP24A1 mRNA expression at the time point was significantly different from that at 0 h in hGF ($p < 0.05$). # CYP27B1 or CYP24A1 mRNA expression at the time point was significantly different from that at 0 h in hPDLC ($p < 0.05$).
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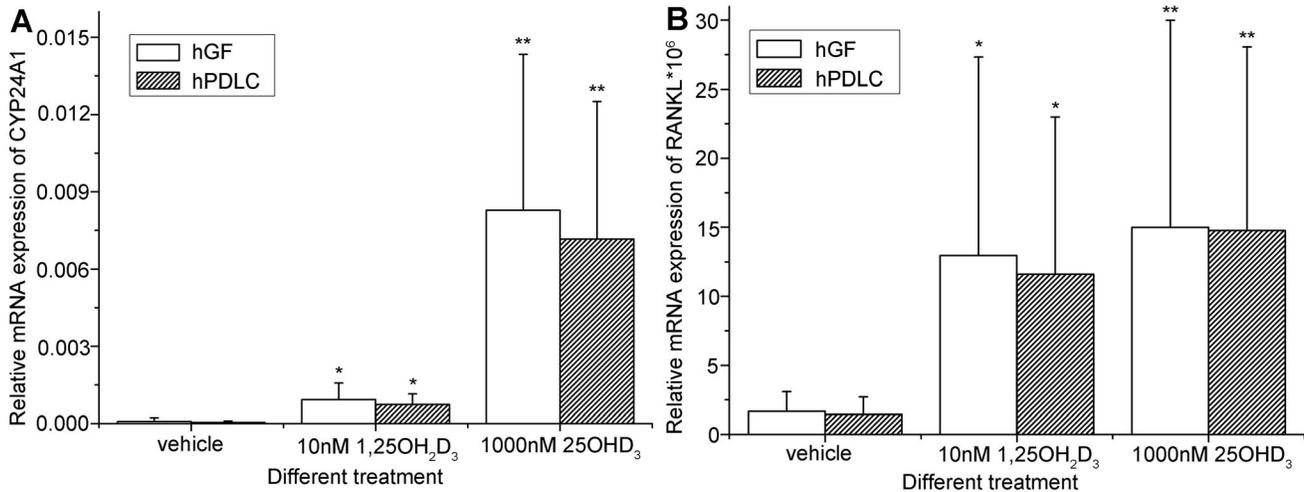


Figure 4. Effect of 25OHD₃ incubation on gene expression in hGF and hPDLC. hGF and hPDLC from all five donors were treated with 1000 nM 25OHD₃, 10 nM 1,25OH₂D₃ or vehicle for 48 h and mRNA expression of (A) CYP24A1 and (B) RANKL was examined by real-time PCR. The up-regulation observed after 1000 nM 25OHD₃ treatment was significantly stronger than that observed after 10 nM 1,25OH₂D₃ treatment. The data are presented as the mean ± SE. * denotes difference from vehicle ($p < 0.05$). ** denotes difference from vehicle and 1,25OH₂D₃ ($p < 0.05$). doi:10.1371/journal.pone.0039878.g004

of patients with periodontitis were significantly higher than those of healthy controls and butyrate levels in gingival crevicular fluids were significantly correlated with periodontal inflammation. To investigate the regulation of 1α -hydroxylase in hGF and hPDLC, IL-1 β , *Pg*-LPS and sodium butyrate were chosen for the current study. However, although stimuli with periodontal characteristics were used here to simulate a periodontitis condition, this does not properly model a chronic disease situation in vivo and can only help to investigate the regulation of CYP27B1 in hGF and hPDLC. We found the NF- κ B activator IL-1 β to be a potent up-regulator of CYP27B1 mRNA in hGF and hPDLC (Fig. 8), which is in line with the observation in osteoblasts [15]. We also found that sodium butyrate could significantly up-regulate the expression

of CYP27B1 while *Pg*-LPS could not, an observation which requires further study. Classical renal regulators of 1α -hydroxylase are parathyroid hormone, calcium and 1,25OH₂D₃ [10,29]. Renal 1,25OH₂D₃ synthesis is primarily stimulated by low serum calcium, and consequently by parathyroid hormone that up-regulates CYP27B1 expression while 1,25OH₂D₃ itself down-regulates CYP27B1 by negative feedback [30]. In contrast to this, we found that 1α -hydroxylase expression in hGF and hPDLC was less sensitive to a regulation by these agents (Fig. 8). In addition, 1,25OH₂D₃ did not significantly change the up-regulation of CYP27B1 by IL-1 β and sodium butyrate. A possible explanation might be that the induction of 1α -hydroxylase in hGF and hPDLC involves NF- κ B pathways that are different from those involved in renal, cAMP-mediated protein kinase A signaling [31,32] and negative vitamin D response element [33,34]. This concept is similar to that of Hewison [35].

In the present study, each donor supplied both hGF and hPDLC and the CYP27B1 activity of hGF and hPDLC was compared. We demonstrated that hPDLC released more 1,25OH₂D₃ than hGF 12 h after adding 25OHD₃. In the study of van D M et al. [15], osteoblasts incubated with 1000 nM 25OHD₃ also generated 1,25OH₂D₃ and the concentration of 1,25OH₂D₃ in the supernatant was about 400–800 pM. In the present study, 1,25OH₂D₃ concentrations in the supernatants of hGF and hPDLC were 120 pM–360 pM and 120 pM–600 pM, respectively. Thus, osteoblasts might release more 1,25OH₂D₃ than hGF and hPDLC. hGF and hPDLC are two different types of fibroblasts of periodontal soft tissues. It has previously been demonstrated that hPDLC has a higher degree of similarity with osteoblasts than hGF [36,37,38]. This might be the reason for the higher release of 1,25OH₂D₃ by hPDLC than by hGF.

In recent years, increasing attention has been paid to the relationship between vitamin D and periodontitis. Dietrich et al. [39] reported that serum 25OHD₃ concentrations are significantly and inversely associated with attachment loss in people aged over 50 years, however, there is no significant association between serum 25OHD₃ concentrations and attachment loss in patients younger than 50 years. Dietrich et al. [40] also reported that subjects with high serum 25OHD₃ concentrations were less likely to have bleeding on probing compared to subjects with low serum

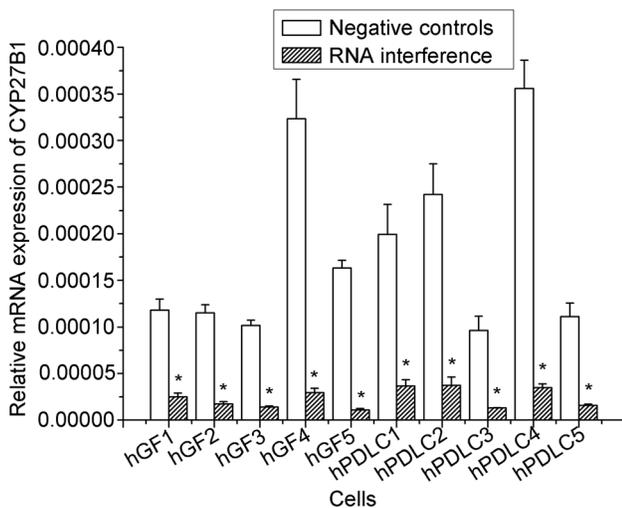


Figure 5. Efficiency of RNA interference against CYP27B1. All cells were transfected with either a siRNA oligonucleotide for CYP27B1 or a non-silencing control. Using real-time PCR as a measure, the efficiency of RNA interference against CYP27B1 was over 70% in hGF and hPDLC from all 5 donors. Donors are numbered 1–5. The data are presented as the mean ± SD. * denotes difference from vehicle ($p < 0.05$). doi:10.1371/journal.pone.0039878.g005

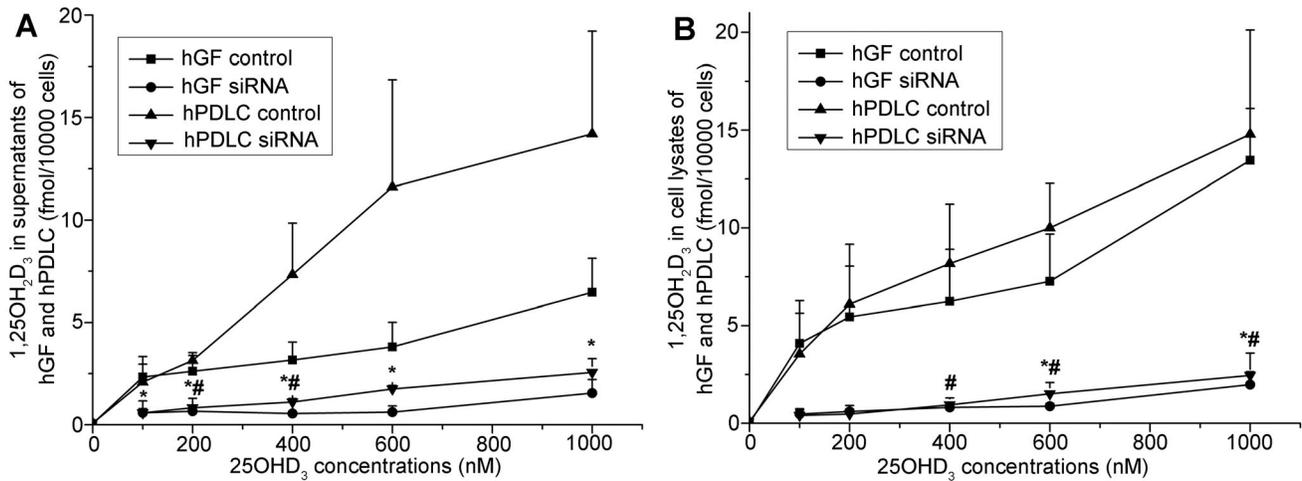


Figure 6. Effect of CYP27B1 silencing on 1,25OH₂D₃ generation. hGF and hPDLc from donors 2, 3 and 5 were treated with 25OHD₃ at various concentrations indicated in the figure for 48 h after transfection with the siRNA oligonucleotide for CYP27B1 or a non-silencing control and 1,25OH₂D₃ production was measured in (A) supernatants and (B) cell lysates. When CYP27B1 was not silenced, production of 1,25OH₂D₃ increased with increasing concentration of 25OHD₃. When CYP27B1 was silenced, the generation of 1,25OH₂D₃ decreased significantly compared with when CYP27B1 was not silenced. The data are presented as the mean ± SE. * hGF generated significantly less 1,25OH₂D₃ with the same amount of 25OHD₃ when CYP27B1 was knocked down ($p < 0.05$). # hPDLc generated significantly less 1,25OH₂D₃ (with the same amount of added 25OHD₃) when CYP27B1 was knocked down ($p < 0.05$). doi:10.1371/journal.pone.0039878.g006

25OHD₃ concentrations. However, periodontally healthy subjects were not distinguishable from patients with either aggressive periodontitis or chronic periodontitis, and local 25OHD₃ levels in gingival crevicular fluids were not detected in these studies. Thus, we designed our previous studies and found that patients with aggressive periodontitis had higher plasma 25OHD₃ concentrations than healthy controls and that after periodontal therapy the higher plasma 25OHD₃ concentrations were significantly reduced, indicating that 25OHD₃ might be involved in periodontal inflammation [19,20]. In addition, local 25OHD₃ levels in gingival crevicular fluids were 300 times higher than plasma levels in patients with aggressive periodontitis [20]. Because 1,25OH₂D₃

may enhance the antibacterial defense of human gingival epithelial cells [21], the confirmation of 1 α -hydroxylase activity in hGF and hPDLc implies that local 25OHD₃ in gingival crevicular fluids might be metabolized to 1,25OH₂D₃ by hGF and hPDLc and be involved in the innate immune defense in the oral cavity. From this perspective, 1 α -hydroxylase activity in hGF and hPDLc may benefit periodontal health. Recently, it was reported that calcium and vitamin D supplementation have a positive effect on periodontal health [41,42]. However, topical application of vitamin D has not been reported. Since hGF and hPDLc have the ability to synthesize 1,25OH₂D₃, a topical application of 25OHD₃ might fulfill the function of 1,25OH₂D₃. Thus, our data

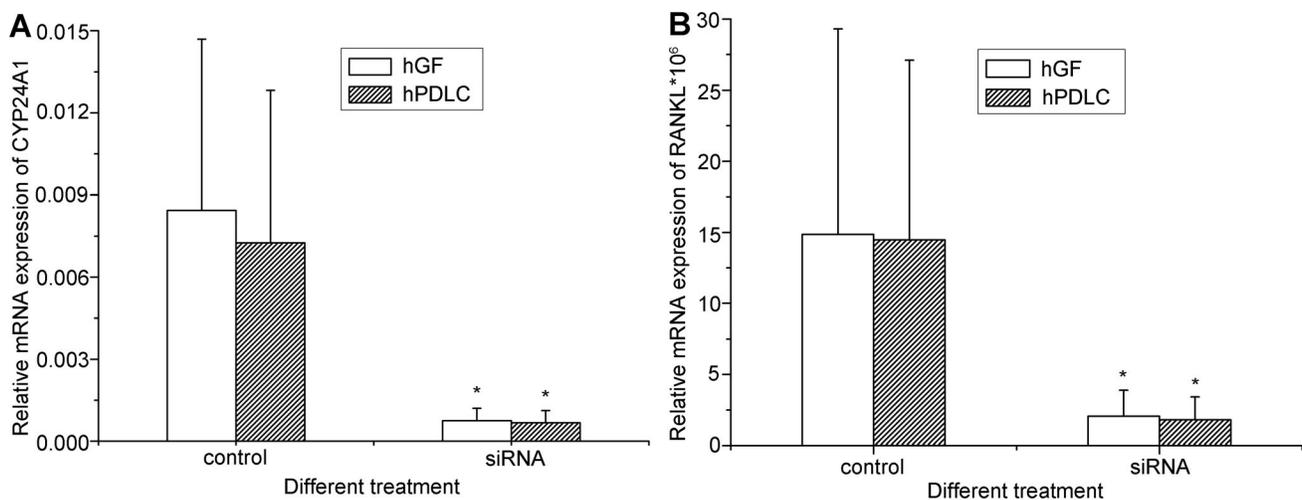


Figure 7. Effect of CYP27B1 silencing on gene up-regulation by 25OHD₃. hGF and hPDLc from all five donors were treated with 1000 nM 25OHD₃ for 48 h after transfection with the siRNA oligonucleotide for CYP27B1 or a non-silencing control and mRNA expression of (A) CYP24A1 and (B) RANKL was determined by real-time PCR. Compared with the transfection with a non-silencing control, transfection with the siRNA oligonucleotide for CYP27B1 resulted in a significantly weaker up-regulation of CYP24A1 and RANKL in both hGF and hPDLc. The data are presented as the mean ± SE. * denotes difference from control ($p < 0.05$). doi:10.1371/journal.pone.0039878.g007

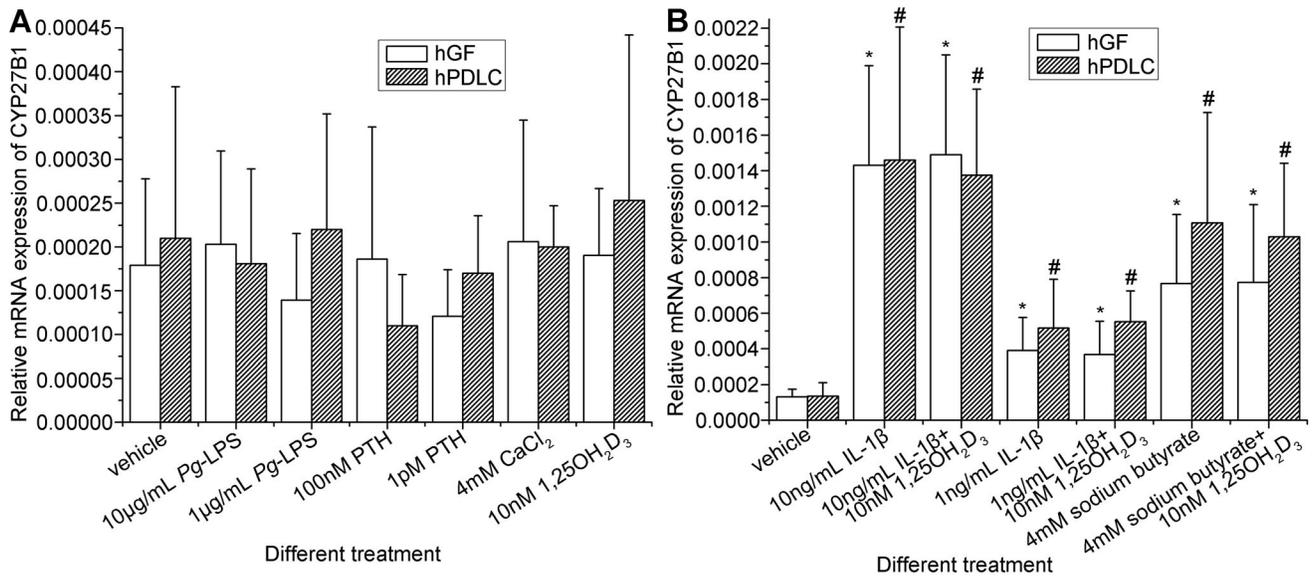


Figure 8. Preliminary investigation of CYP27B1 regulation in hGF and hPDLC. hGF and hPDLC from donors 2, 3, 4 and 5 were stimulated with different treatments indicated in the figure for 24 h and CYP27B1 expression was determined by real-time PCR. (A) *Pg-LPS*, parathyroid hormone, CaCl_2 and $1,25\text{OH}_2\text{D}_3$ did not significantly influence CYP27B1 mRNA expression. (B) IL-1 β and sodium butyrate significantly up-regulated CYP27B1 mRNA expression independently of $1,25\text{OH}_2\text{D}_3$. Additionally, the characteristics of CYP27B1 regulation in hGF and hPDLC were not significantly different. The data are presented as the mean \pm SE. * CYP27B1 mRNA expression was significantly different from that of the vehicle group in hGF ($p < 0.05$). # CYP27B1 mRNA expression was significantly different from that of the vehicle group in hPDLC ($p < 0.05$). IL-1 β : interleukin-1 β . *Pg-LPS*: *Porphyromonas gingivalis* lipopolysaccharide. PTH: parathyroid hormone. doi:10.1371/journal.pone.0039878.g008

suggest a potential benefit of topical application of $25\text{OH}_2\text{D}_3$ in periodontal therapy.

Because no established cell lines of hGF or hPDLC are available, the cells used in the present study were all obtained from primary culture. Although all data were obtained from cell from at least 3 donors, the existence of individual differences among the cells from primary culture was the limitation of this study.

In conclusion, the results in the current study have identified for the first time hGF and hPDLC as new sites of extrarenal synthesis of $1,25\text{OH}_2\text{D}_3$. Similar to other extrarenal 1α -hydroxylases, periodontal 1α -hydroxylase appears to fulfill an autocrine/paracrine function.

Materials and Methods

Ethics Statement

The study protocol was approved by the institutional review board of Peking University School and Hospital of Stomatology (PKUSSIRB-2011007) and written informed consent was ob-

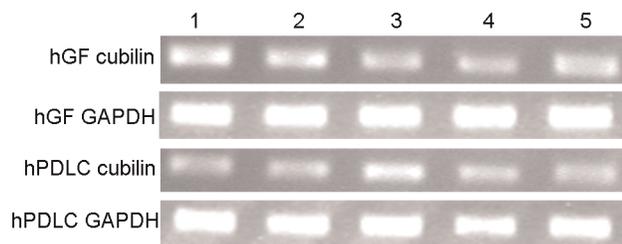


Figure 9. mRNA expression of cubilin in hGF and hPDLC. mRNA expression of cubilin was determined by RT-PCR in hGF and hPDLC from all five donors (each lane represents one donor). GAPDH was used as an internal control. doi:10.1371/journal.pone.0039878.g009

tained from each participant in accordance with the Declaration of Helsinki.

Cell culture

Primary culture of hGF and hPDLC was carried out according to previously described methods [7,26,36,43]. Briefly, hPDLC were prepared from extracted third molars of 5 young healthy volunteers and hGF were obtained from gingiva of the same 5 donors. The periodontal ligament tissues attached to the middle third of the roots were gently curetted by a surgical scalpel, minced and inoculated into 24-well plates. Gingiva from different donors was also minced and inoculated into 24-well plates. Tissue explants were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; PAA, Coelbe, Germany), 100 U/ml penicillin G and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cultures were maintained in a humidified atmosphere of 5% (v/v) CO_2 at 37°C . After reaching 80% confluence, hGF and hPDLC were digested with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA and subcultured at a 1:3 ratio.

DMEM without phenol red (Sigma, St. Louis, MO, USA), 10% dextran-coated charcoal stripped FBS (DCC-FBS; TBD, Tianjin, China) and cells of passage 4 were used in all of the following experiments. Experiments were all conducted in triplicate.

Cytotoxicity test of $25\text{OH}_2\text{D}_3$

hGF and hPDLC from three donors were used in the cytotoxicity test. hGF and hPDLC in their logarithmic phase were plated into 96-well plates at a density of 3000 cells/well in DMEM with 10% DCC-FBS. 24 h later, the medium was replaced by DMEM without DCC-FBS. After another 24 h, the medium was changed to DMEM with 10% DCC-FBS and supplemented with 1000 nM $25\text{OH}_2\text{D}_3$ or vehicle, respectively. The cytotoxicity test was carried out according to the Cell

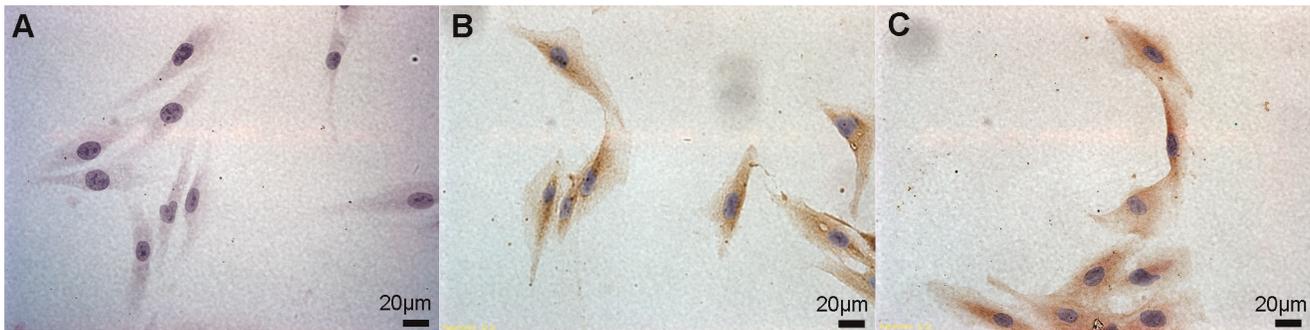


Figure 10. Protein expression of cubilin in hGF and hPDL. hGF and hPDL from all five donors were used for immunocytochemical staining of cubilin; expression of cubilin was detected in all the cells examined. Panel A is the negative control for the immunocytochemical staining of cubilin (400×). Panel B and C contain images of the hGF and hPDL, respectively, from donor 2 (400×). The primary antibody was replaced with PBS for the negative control.

doi:10.1371/journal.pone.0039878.g010

Counting Kit-8 protocol (CCK-8; Dojindo, Kumamoto, Japan). Briefly, at hours 0, 24 and 48, cells were incubated with CCK-8 for the last 3 h of the culture period after which the optical density values (OD values) were determined at 490 nm with a microplate reader (Bio-Rad Model 550, Hercules, CA, USA).

Detection of CYP27B1 expression

Cells from all five donor were seeded into 6-well plates at a density of 5000/cm² in DMEM supplemented with 10% DCC-FBS. 4 days later, a portion of the cells were harvested using Trizol reagent (Dongsheng Biotech, Guangzhou, China) for RT-PCR. RNA was extracted using Trizol according to the manufacturer's instructions and was reverse transcribed to cDNA using a reverse transcription kit (Bio-Rad, Hercules, CA, USA). PCR reactions were performed using the Taq PCR MasterMix (Tiangen Biotech, Beijing, China) in a Hybaid PCR Thermal Cycler (Thermo Scientific, Boston, Mass, USA). The primers used are listed in Table 1.

The remaining cells were harvested using lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate and 2 mM Na₃VO₄ supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany)] [43] for Western blotting. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Applygen, Beijing, China). 20 µg of total protein from each sample was loaded onto a gel comprising a 5% (w/v) stacking gel and a 10% (w/v) running gel. At the end of the electrophoresis, samples were transferred onto nitrocellulose blotting membranes (HybondTM; Amersham

Pharmacia, Little Chalfont, UK). Blots were probed with a sheep polyclonal antibody to CYP27B1 (diluted 1:200; The Binding Site Ltd., Birmingham, UK) or a mouse monoclonal antibody to β-actin (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, blots were incubated with horseradish peroxidase-linked secondary antibody. The secondary antibody against sheep (Kirkegaard & Perry Laboratories, Inc., Maryland, USA) and mouse (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) were both diluted 1:2500. Antigen-antibody complexes were detected using the Enhanced Chemiluminescence reagent (Applygen, Beijing, China).

Measurement of 1,25OH₂D₃ production

Cells from 3 donors were treated with 1000 nM 25OHD₃ (Sigma, St. Louis, MO, USA) for 1 h, 4 h, 12 h, 24 h or 48 h, after which supernatants were collected and a portion of cells were scraped in PBS containing 0.2% Triton X-100 and stored at -80°C. Prior to use, cell lysates were sonicated on ice in a sonifier cell disrupter for 2×15 s. 1,25OH₂D₃ levels in cell supernatants and cell lysates were detected using a 1,25OH₂D₃ radioimmunoassay kit (DiaSorin, Stillwater, MN) according to the manufacturer's instructions. The cross-reactivity with 25OHD₃ was less than 0.01%.

In addition, a portion of the cells were harvested using Trizol reagent for the detection of the time course of CYP27B1 and CYP24A1 mRNA expression using real-time PCR. Real-time PCR reactions were performed using SYBR[®] Premix Ex Taq[™] II (TaKaRa Biotechnology, Dalian, China) in an ABI 7500 real-time Thermocycler (Applied Biosystems, Foster city, CA, USA).

Table 1. Primer sequences used for PCR or real-time PCR.

Target genes	Forward primer (5'→3')	Reverse primer (5'→3')	Products (bp)
CYP27B1	GAAGTGCTAAGACTGTACCCTGT	CCTTGAAGTGGCATAGTGACAC	126
RANKL	GACATCCCATCTGGTTCCCA	CCCAACCCCGATCATGGTA	61
CYP24A1	GACTACCGCAAAGAAGGCTAC	CATCACTTCCCCTGGTTTCATTA	105
megalin	GCCGGCCAGTGGCCAAGAA	ACAGCGCAGCCAATTTTCATCC	129
megalin	AAATTGAGCACAGCACCTTTGA	TCTGCTTCTGACTCGAATAATG	151
cubilin	TTCTTACGGGGTCTGCTCAAA	TGCAAGCCTTGGAAATTTCTCTC	226
GAPDH	GAAGGTGAAGTCTGGAGTC	GAAGATGGTATGGGATTC	226

doi:10.1371/journal.pone.0039878.t001

The primers used are listed in Table 1. The data were analyzed using the SDS software according to the manufacturer's instructions. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal control. The data are presented as relative mRNA levels calculated by the equation $2^{-\Delta Ct}$ ($\Delta Ct = Ct$ of target gene minus Ct of GAPDH) [44].

Detection of CYP24A1 and RANKL mRNA expression

Cells from five donors were incubated with 10 nM 1,25OH₂D₃ (Sigma, St. Louis, MO, USA), 1000 nM 25OHD₃ or vehicle for 48 h before harvesting. Then RNA and cDNA were obtained and real-time PCR reactions were performed as described previously.

RNA interference of CYP27B1

To confirm the dependence of 25OHD₃ conversion into 1,25OH₂D₃ on CYP27B1, the highly specific technique of RNA interference was utilized. Cells were seeded at a density of 15000/cm² in 6-well plates. 8 h later, cells were transfected with either CYP27B1 siRNA (10 nM) or non-silencing control siRNA using HiPerfectTM transfection reagent (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. The target sequence of CYP27B1 siRNA was 5'-CTGGTTTACGGTTTCTTATAA-3' and the non-silencing control was a non-homologous, scrambled sequence equivalent.

60 h after transfection, cells were harvested to confirm the effect of RNAi using real-time PCR. RNA and cDNA were obtained and real-time PCR was performed as described earlier.

After verifying the effect of RNAi, 1,25OH₂D₃ production after RNAi was determined. Cells were first transfected with CYP27B1 siRNA (10 nM) or non-silencing control siRNA and 12 h after transfection, these cells were treated with 200 nM, 400 nM, 600 nM or 1000 nM 25OHD₃ (Sigma, St. Louis, MO, USA) for another 48 h. Then, the 1,25OH₂D₃ concentrations in the cell supernatants and cell lysates were determined as described earlier.

CYP24A1 and RANKL mRNA expression was also examined after RNAi. Cells were first transfected with CYP27B1 siRNA (10 nM) or non-silencing control siRNA and 12 h after transfection, these cells were treated with 1000 nM 25OHD₃ for another 48 h. Then, CYP24A1 and RANKL mRNA expression was determined by real-time PCR.

Regulation of CYP27B1 in hGF and hPDL

A portion of the cells from four donors were incubated with IL-1 β (PeproTech, London, UK; 1 ng/mL and 10 ng/mL), parathyroid hormone (PTH; SinoBio, Shanghai, China; 100 nM and

1 pM), P β -LPS (Invivogen, San Diego, CA, USA; 1 μ g/mL and 10 μ g/mL), sodium butyrate (SCRC, Shanghai, China; 4 mM), CaCl₂ (SCRC, Shanghai, China; 4 mM) and 1,25OH₂D₃ (10 nM) for 24 h. The other portion of the donor cells were incubated with IL-1 β (1 ng/mL or 10 ng/mL) and 1,25OH₂D₃ (10 nM) or sodium butyrate (4 mM) and 1,25OH₂D₃ (10 nM) for 24 h.

Detection of vitamin D binding protein receptor expression

The mRNA expression of megalin and cubilin were detected by RT-PCR as described earlier and the primers used are listed in Table 1.

Protein expression of cubilin was detected by immunocytochemistry. Cells from five donors were seeded on glass slides at a density of 20000/cm² and 8 h later cells on glass slides were incubated with primary antibodies against cubilin (diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The PV-9000 Polymer Detection System (Zhongshan Golden Bridge Biotechnology, Beijing, China) was used for immunocytochemical staining and a diaminobenzidine (DAB) kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) was used to develop the colour followed by haematoxylin staining. The primary antibody was replaced by PBS for negative controls.

Statistical Methods

The Shapiro-Wilk test was used to determinate the distribution of the variants. The Wilcoxon test and Friedman test were used to compare differences between the mRNA expression levels of CYP24A1 and RANKL in different groups. The effect of RNA interference and the effect of 25OHD₃ exposure on CYP27B1 mRNA expression was analyzed using the paired samples t-test. Comparison of 1,25OH₂D₃ generation by hGF and hPDL, comparison of 1,25OH₂D₃ generation with and without knock-down of CYP27B1 and comparison of CYP27B1 up-regulation in hGF and hPDL were all performed using a paired samples t-test.

Statistical analyses were carried out using the SPSS 11.5 software package (SPSS Inc., Chicago, IL, USA). A *p* value < 0.05 was considered statistically significant.

Author Contributions

Conceived and designed the experiments: HXM KNL JXH. Performed the experiments: KNL. Analyzed the data: KNL JXH. Contributed reagents/materials/analysis tools: HXM JXH. Wrote the paper: KNL HXM JXH.

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