Activity of 25-Hydroxylase in Human Gingival Fibroblasts and Periodontal Ligament Cells

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Abstract

Background: We previously demonstrated that 25-hydroxyvitamin D concentrations in gingival crevicular fluid are 300 times higher than those in the plasma of patients with aggressive periodontitis. Here we explored whether 25-hydroxyvitamin D3 can be synthesized by periodontal soft tissue cells. We also investigated which of the two main kinds of hydroxylases, CYP27A1 and CYP2R1, is the key 25-hydroxylase in periodontal soft tissue cells.

Methodology/Principal Findings: Primary cultures of human gingival fibroblasts and periodontal ligament cells from 5 individual donors were established. CYP27A1 mRNA, CYP2R1 mRNA and CYP27A1 protein were detected in human gingival fibroblasts and periodontal ligament cells, whereas CYP2R1 protein was not. After incubation with the 25-hydroxylase substrate vitamin D3, human gingival fibroblasts and periodontal ligament cells generated detectable 25-hydroxyvitamin D3 that resulted in the production of 1α,25-dihydroxyvitamin D3. Specific knockdown of CYP27A1 in human gingival fibroblasts and periodontal ligament cells using siRNA resulted in a significant reduction in both 25-hydroxyvitamin D3 and 1α,25-dihydroxyvitamin D3 production. Knockdown of CYP2R1 did not significantly influence 25-hydroxyvitamin D3 synthesis. Sodium butyrate did not influence significantly CYP27A1 mRNA expression; however, interleukin-1β and Porphyromonas gingivalis lipopolysaccharide strongly induced CYP27A1 mRNA expression in human gingival fibroblasts and periodontal ligament cells.

Conclusions: The activity of 25-hydroxylase was verified in human gingival fibroblasts and periodontal ligament cells, and CYP27A1 was identified as the key 25-hydroxylase in these cells.
Results

CYP27A1 and CYP2R1 mRNA were detected in all the cells of the five donors, and no significant difference was found between the mRNA levels in hGF and hPDLC (Fig. 1). CYP27A1 protein was also detected in all cells of the five donors, whereas CYP2R1 was not detected, with the premise that anti-CYP2R1 antibody was able to recognize the protein in PC-3 cells, which were used as a positive control (Fig. 2). This indicated that CYP27A1 might be the key 25-hydroxylase in hGF and hPDLC.

After confirming the expression of 25-hydroxylase in hGF and hPDLC, the function of 25-hydroxylase was investigated. Whereas 1000 nM vitamin D₃ did not have a significant cytotoxic effect on any of the cells within 48 h, hGF and hPDLC generated 25OHD₃ in response to vitamin D₃ (Figs. 3A, B). The fact that extra- and intracellular 25OHD₃ was generated in the presence of vitamin D₃ provides direct and convincing evidence of the existence of 25-hydroxylase in hGF and hPDLC.

At all time points, there was no significant difference in the levels of intracellular and extracellular 25OHD₃ between the two cell types.

Additionally, exposure to vitamin D₃ also resulted in the synthesis of 1,25(OH)₂D₃ in hGF and hPDLC (Fig. 4). The observation that hGF and hPDLC could synthesize 1,25(OH)₂D₃ when exposed to 25OHD₃ [29] is further evidence of 25-hydroxylase activity in hGF and hPDLC.

Based on the above direct evidence for 25-hydroxylase activity in hGF and hPDLC, we examined the effect of 25-hydroxylase knockdown. The efficiency of RNA interference against both CYP27A1 and CYP2R1 was both over 70% (Fig. 5). The knockdown of the efficiency of RNA interference against both CYP27A1 and CYP2R1 was both over 70% (Fig. 5). The knockdown of CYP27A1 did not significantly influence 25OHD₃ generation by hGF (Figs. 6A, C), and only slightly influenced 25OHD₃ generation by hPDLC (Figs. 6B, D). These results suggest that CYP27A1 might be the key 25-hydroxylase in hGF and hPDLC. In addition, knockdown of CYP27A1 resulted in a significant reduction of 1,25(OH)₂D₃ generation (Figs. 7A–B).

Discussion

In the present study, our hypothesis that hGF and hPDLC have 25-hydroxylase activity, and that they can synthesize 25OHD₃ was verified. Therefore, the origin of high 25OHD₃ concentrations in gingival crevicular fluid [27,28] might be hGF and hPDLC. Having demonstrated 1α-hydroxylase activity in hGF and hPDLC [29], we could consider that the conversion of vitamin D₃ to 1,25(OH)₂D₃ in hGF and hPDLC consisted of two steps: (i) from vitamin D₃ to 25OHD₃, under the action of 25-hydroxylase CYP27A1; (ii) from 25OHD₃ to 1,25(OH)₂D₃, under the action of 1α-hydroxylase CYP27B1. This two-step conversion is similar to that observed in human keratinocytes [7,19,30,31,32]. In addition, Slominski et al. reported an alternate pathway of vitamin D₃ metabolism by cytochrome P450scc (CYP11A1) [33,34,35,36]. P450scc activity in hGF and hPDLC is worth further investigation in our future study.

We can then calculate and compare the amount of 1,25(OH)₂D₃ synthesized from 1000 nM vitamin D₃ and from 1000 nM 25OHD₃. According to the present study, the amount of 1,25(OH)₂D₃ generated would be: (1) In hGF exposed to 1000 nM vitamin D₃ for 48 h, 9 fmol/10000 cells in supernatants +14 fmol/10000 cells in cell lysates = 23 fmol/10000 cells (Fig. 4). (2) In hPDLC exposed to 1000 nM vitamin D₃ for 48 h, 13 fmol/10000 cells in supernatants +16 fmol/10000 cells in cell lysates = 29 fmol/10000 cells (Fig. 4). According to our previous study [29], the amount of 1,25(OH)₂D₃ generated would be the following: (3) In hGF exposed to 1000 nM 25OHD₃ for 48 h, 5 fmol/10000 cells in supernatants +13 fmol/10000 cells in cell lysates = 18 fmol/10000 cells. (4) In hPDLC exposed to 1000 nM 25OHD₃ for 48 h, 13 fmol/10000 cells in supernatants +14 fmol/10000 cells in cell lysates = 27 fmol/10000 cells. It is interesting that 1000 nM vitamin D₃ could induce hGF and hPDLC to generate even more 1,25(OH)₂D₃ than 1000 nM 25OHD₃. Particular attention should be paid to the observation that after 1000 nM vitamin D₃ treatment, 25OHD₃ concentration in the cell supernatants of hGF and hPDLC were only about 45 nM–64 nM and 30 nM–50 nM respectively, much lower than the added 1000 nM vitamin D₃. So, why was less 25OHD₃ converted to more 1,25(OH)₂D₃? One reason might be that after vitamin D₃ treatment, 25OHD₃ is found not only in the supernatant, but also in the cell lysates, allowing intracellular 25OHD₃ to act directly as substrate of 1α-hydroxylase. On the other hand, exogenous 25OHD₃ should enter the cells before eliciting a response. Thus, the direct availability at the site of action might be of great importance.

After comprehensive verification of 25-hydroxylase activity and the demonstration of CYP27A1 as the key 25-hydroxylase in hGF
and hPDLC, the regulation of CYP27A1 in these cells was preliminarily investigated. IL-1β in gingival crevicular fluids of patients with periodontitis decreases significantly after initial periodontal therapy, indicating that IL-1β is associated with periodontitis [28]. Porphyromonas gingivalis is an important pathogen of periodontitis and butyrate is one of its metabolites [37]. It was demonstrated that the butyrate concentrations in gingival crevicular fluids of patients with periodontitis are significantly higher than those of healthy controls, and that butyrate concentrations in gingival crevicular fluids are significantly correlated with periodontal inflammation [38,39]. To investigate the regulation of CYP27A1 in hGF and hPDLC, IL-1β, Pg-LPS and sodium butyrate were chosen for the present study. It should be considered, however, that although stimuli with periodontal characteristics were used to simulate a periodontitis-like condition, this does not properly model the chronic disease situation in vivo, and can only help to investigate the regulation of CYP27A1 in hGF and hPDLC. The NF-κB activator, IL-1β, was demonstrated to be a potent up-regulator of CYP27A1 mRNA in hGF and hPDLC (Fig. 8). Pg-LPS could also up-regulate significantly the

Figure 2. Protein expression of CYP27A1 and CYP2R1 in hGF and hPDLC. Protein expression of CYP27A1 was detected by Western blot in hGF and hPDLC from all five donors (donors are numbered 1–5). Protein expression of CYP2R1 was detected by Western blot in PC-3 cells, which were used as a positive control, but was not detected in hGF and hPDLC. β-actin was used as an internal control.
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Figure 3. Activity of 25-hydroxylases in hGF and hPDLC. hGF and hPDLC from donors 2, 4 and 5 were incubated with 1000 nM vitamin D3 for the times indicated, and the production of 25OHD3 was determined in supernatants(A) and cell lysates (B). After incubation, the amount of 25OHD3 generated was not significantly different between hGF and hPDLC. The data are presented as the mean ± SE.
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Figure 4. 1,25OH2D3 generation by hGF and hPDLC. hGF and hPDLC from donors 2, 4 and 5 were incubated with 1000 nM vitamin D3 for 48 h, and the production of 1,25OH2D3 was determined in supernatants and cell lysates. The amount of 1,25OH2D3 generated was not significantly different between hGF and hPDLC. The data are presented as the mean ± SE.
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expression of CYP27A1 mRNA, whereas sodium butyrate could not. It was reported that Pg-LPS is the ligand of Toll-like receptor 2 (TLR2) and TLR4 [40,41] and that both hGF and hPDLC expressed TLR2 and TLR4 [42]. Upon ligand binding, TLR2 or TLR4-mediated signaling could activate signal transduction, leading to NF-κB activation [43,44]. Thus, NF-κB might be involved in the regulation of CYP27A1 expression, an observation that warrants further investigation.

Each donor supplied both hGF and hPDLC in the present study. Although hGF and hPDLC are two different kinds of cells, they shared many features in 25-hydroxylase expression, activity and regulation, and only subtle differences were detected. As shown in Fig. 6, when CYP2R1 was knocked down, 25OHD₃ generation by hGF was not changed significantly, whereas 25OHD₃ generation by hPDLC was affected slightly. However, the difference did not affect our conclusion that CYP27A1 might be the key 25-hydroxylase in hGF and hPDLC.

Since 1,25(OH)₂D₃ may enhance the antibacterial defense of human gingival epithelial cells [45] and hGF and hPDLC could synthesize 1,25(OH)₂D₃ with 25OHD₃ [29], the confirmation of 25-hydroxylase activity in hGF and hPDLC implies that these cells could generate 25OHD₃ as a substrate for 1,25(OH)₂D₃. From this perspective, 25-hydroxylase activity in hGF and hPDLC may be involved in the innate immune defense of the oral cavity. Recently, it was reported that oral calcium and vitamin D supplementation have a positive effect on periodontal health [46,47]. However, topical application of vitamin D has not been reported. Since hGF and hPDLC have the ability to synthesize 25OHD₃ and then to synthesize 1,25(OH)₂D₃, the topical application of vitamin D₃ might fulfill the function of 1,25(OH)₂D₃. Thus, our data suggest a potential benefit of topical application of vitamin D₃ in periodontal therapy.

In conclusion, hGF and hPDLC were identified as new extra-hepatic sites of 25OHD₃ synthesis for the first time, and CYP27A1 might be the key 25-hydroxylase in these cells.

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

Cell Culture

Primary culture of hGF and hPDLC was carried out according to our previous methods [29]. In brief, hPDLC were obtained from extracted third molars of 5 young healthy volunteers, and hGF was isolated from the gingiva of the same 5 donors. The periodontal ligament tissues attached to the middle third of the roots were curedtted gently by a surgical scalpel, minced and placed in 24-well plates. Gingivae were also minced and transferred into 24-well plates. Tissue explants were cultured 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 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% (v/v) CO₂ at 37°C. After reaching 80% confluence, hGF and hPDLC were digested with a mixture of 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% (v/v) dextran-coated, charcoal-stripped FBS (DCC-FBS; TBD, Tianjin, China) and hGF and hPDLC of passage 4 were used in all the following experiments. All experiments were conducted in triplicate.

The prostate cancer cell line, PC-3 (American Type Culture Collection, Rockville, MD, USA), was cultured in RPMI 1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) FBS (PAA, Coelbe, Germany), 100 U/mL penicillin G and 100 μg/mL streptomycin. Cultures were maintained in a humidified atmosphere of 5% (v/v) CO₂ at 37°C. After reaching 80% confluence, hGF and hPDLC were digested with a mixture of 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% (v/v) dextran-coated, charcoal-stripped FBS (DCC-FBS; TBD, Tianjin, China) and hGF and hPDLC of passage 4 were used in all the following experiments. All experiments were conducted in triplicate.

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Cytotoxicity Test of Vitamin D₃

hGF and hPDLC of three donors were used in the cytotoxicity test. hGF and hPDLC in their logarithmic growth phase were plated into 96-well plates at a density of 3000 cells/well in DMEM with 10% DCC-FBS, and the medium was replaced by DMEM without DCC-FBS after 24 h. After another 24 h, the medium...
was changed to DMEM with 10% DCC-FBS, and supplemented with 1000 nM vitamin D3 or vehicle, respectively. The cytotoxicity test was carried out according to the Cell Counting Kit-8 protocol (CCK-8; Dojindo, Kumamoto, Japan). 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 detected at 490 nm with a microplate reader (Bio-Rad Model 550, Hercules, CA, USA).

Detection of 25-hydroxylase Expression

hGF and hPDLC from all five donors were seeded into six-well plates at a density of 5000 cm$^{-2}$ in DMEM supplemented with 10% DCC-FBS. Four days later, a portion of the cells were harvested using Trizol agent (Dongsheng Biotech, Guangzhou, China). 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). Real-time PCR reactions were accomplished using SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology, Dalian, China) in an ABI 7500 real-time Thermocycler (Applied Biosystems, Foster City, CA, USA). 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. Data were presented as relative mRNA levels calculated by the equation $2^{-\Delta\Delta C_t}$ ($\Delta C_t = C_t$ of target gene minus $C_t$ of GAPDH) [48]. The primers used are listed in Table 1.

Figure 6. Effect of knockdown of 25-hydroxylases on 25OHD$_3$ generation. hGF and hPDLC from donors 2, 4 and 5 were treated with vitamin D$_3$ at various concentrations indicated in the figure for 12 h after transfection with a siRNA oligonucleotide for CYP27A1, a siRNA oligonucleotide for CYP2R1, or a non-silencing control. 25OHD$_3$ production was measured in supernatants of hGF (A), supernatants of hPDLC (B), cell lysates of hGF (C), and cell lysates of hPDLC (D). When CYP27A1 or CYP2R1 was not knocked down, the production of 25OHD$_3$ increased with an increasing concentration of 25OHD$_3$. When CYP27A1 was knocked down in hGF and hPDLC, the generation of 25OHD$_3$ decreased significantly compared to when CYP27A1 was not knocked down. When CYP2R1 was knocked down in hGF (A, C), the generation of 25OHD$_3$ was not significantly different from that when CYP2R1 was not knocked down. When CYP2R1 was knocked down in hPDLC (B, D), the generation of 25OHD$_3$ was only slightly different at some time points from that when CYP2R1 was not knocked down. The data are presented as the mean ± SE. * hGF or hPDLC generated significantly less 25OHD$_3$ with the same amount of added vitamin D$_3$ when CYP27A1 or CYP2R1 was knocked down ($p<0.05$). # hGF or hPDLC generated significantly more 25OHD$_3$ with the same amount of added vitamin D$_3$ when CYP27A1 or CYP2R1 was knocked down ($p<0.05$). doi:10.1371/journal.pone.0052053.g006
concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Applygen, Beijing, China). Twenty micrograms of total protein from each sample were 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 goat polyclonal antibody to CYP27A1 (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse polyclonal antibody to CYP2R1 (diluted 1:500; ABCAM, Cambridge, 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 antibodies against sheep (Kirkegaard & Perry Laboratories, Inc., Maryland, USA) and mouse (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) IgG were both diluted 1:2500. Antigen-antibody complexes were detected using the Enhanced Chemiluminescence reagent (Applygen, Beijing, China).

Figure 7. The effect of 25-hydroxylase knockdown on 1,25OH₂D₃ generation. hGF and hPDLC from donors 2, 4 and 5 were treated with 1000 nM vitamin D₃ for 48 h after transfection with a siRNA oligonucleotide for CYP27A1 or a non-silencing control, and 1,25OH₂D₃ production was measured in supernatants (A) and cell lysates (B). When CYP27A1 was knocked down, the generation of 1,25OH₂D₃ decreased significantly compared to when CYP27A1 was not knocked down. The data are presented as the mean ± SE. * hGF or hPDLC generated significantly less 1,25OH₂D₃ with 1000 nM vitamin D₃ when CYP27A1 was knocked down (p<0.05).

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Figure 8. Preliminary investigation of CYP27A1 regulation by inflammatory stimuli 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 CYP27A1 mRNA expression was determined by real-time PCR. IL-1β and Pg-LPS significantly up-regulated CYP27A1 mRNA expression and the higher dose of IL-1β or Pg-LPS raised higher CYP27A1 mRNA up-regulation in both hGF and hPDLC. Sodium butyrate did not significantly influence CYP27A1 mRNA expression. Additionally, the characteristics of CYP27A1 regulation in hGF and hPDLC were not significantly different. The data are presented as the mean ± SE. * CYP27A1 mRNA expression was significantly different from that of the vehicle group (p<0.05). # CYP27A1 mRNA expression was significantly different from that of the vehicle group (p<0.05). * CYP27A1 mRNA expression was significantly different from that of the 1 ng/mL IL-1β group (p<0.05). CYP27A1 mRNA expression was significantly different from that of the 1 μg/mL Pg-LPS group (p<0.05). IL-1β: interleukin-1β. Pg-LPS: Porphyromonas gingivalis lipopolysaccharide.

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Zhongshan Golden Bridge Biotechnology, Beijing, China) IgG were both diluted 1:2500. Antigen-antibody complexes were detected using the Enhanced Chemiluminescence reagent (Applygen, Beijing, China).
Detection of 25OHD₃ Production

Cells from 3 donors were treated with 1000 nM vitamin D₃ (Sigma, St. Louis, MO, USA) for 1, 4, 12, 24 or 48 h, after which supernatants were collected, and the 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 x 15 s. The levels of 25OHD₃ in cell supernatants and cell lysates were determined using a 25OHD₃ radioimmunoassay kit (DiaSorin, Stillwater, MN, USA) with a sensitivity of 1.5 ng/mL.

Detection of 1,25(OH)₂D₃ Production

Cells from 3 donors were treated with 1000 nM vitamin D₃ (Sigma, St. Louis, MO, USA) for 48 h and then supernatants were collected and 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 x 15 s. The levels of 1,25(OH)₂D₃ in cell supernatants and cell lysates were determined using a 1,25(OH)₂D₃ radioimmunoassay kit (DiaSorin, Stillwater, MN, USA) with a sensitivity of 2.0 pg/mL.

RNA Interference of 25-hydroxylase

To confirm the dependence of vitamin D₃ conversion to 25OHD₃ on 25-hydroxylase, the highly specific technique of RNA interference was utilized. Cells were seeded at a density of 15000 cm⁻² in six-well plates. Eight hours later, the cells were transfected with either CYP27A1 siRNA (10 nM) or CYP2R1 siRNA (10 nM), or a non-silencing control siRNA using Hiperfect™ transfection reagent (Qiagen, Duesseldorf, Germany), according to the manufacturer’s instructions. The target sequence of CYP27A1 siRNA was 5'- CACGCTGACATGGGCCCTGTA -3', the target sequence of CYP2R1 siRNA was 5'- TGGGTGATCACAGACGATTA -3', and the non-silencing control was a non-homologous, scrambled sequence equivalent. Sixty hours after transfection, cells were harvested, RNA and cDNA were obtained, and real-time PCR was performed as described earlier to test the effect of RNAi.

After confirming the effect of RNAi, 25OHD₃ production after RNAi was determined. Cells were first transfected with CYP27A1 siRNA (10 nM) or CYP2R1 siRNA (10 nM), or non-silencing control siRNA. Twelve hours after transfection, these cells were treated with 100 nM, 200 nM, 400 nM, 600 nM or 1000 nM vitamin D₃ (Sigma, St. Louis, MO, USA) for another 12 h. Then, the 25OHD₃ concentrations in the cell supernatants and cell lysates were determined as described earlier.

Some other cells were first transfected with CYP27A1 siRNA (10 nM), or non-silencing control siRNA, and 12 h after transfection, these cells were treated with 1000 nM vitamin D₃ (Sigma, St. Louis, MO, USA) for another 48 h. Then, the 1,25(OH)₂D₃ concentrations in the cell supernatants and cell lysates were detected as described earlier.

Regulation of CYP27A1 in hGF and hPDLC

Cells from four donors were seeded into six-well plates at a density of 5000 cm⁻² in DMEM supplemented with 10% DCC-FBS. Four days later, cells were incubated with IL-1β (PeproTech, London, UK; 1 ng/mL and 10 ng/mL), P2-LPS (Invivogen, San Diego, CA, USA; 1 μg/mL and 10 μg/mL) or sodium butyrate (SCRC, Shanghai, China; 4 μM) for 24 h. Then mRNA expression was detected by real-time PCR as described previously.

Statistical Methods

The Shapiro-Wilk test was used to determine the distribution of the variants. The paired samples t-test was used to compare differences of the mRNA expression levels of CYP27A1 and CYP2R1 between hGF and hPDLC, differences of 25OHD₃ generation by hGF and hPDLC, and the effect of RNA interference. Comparison of 25OHD₃ generation with and without knockdown of 25-hydroxylase, and 1,25(OH)₂D₃ generation with and without knockdown of CYP27A1 were also performed using a paired samples t-test. The impact of stimulation on CYP27A1 mRNA expression was analyzed using a paired-samples t-test, and the difference between CYP27A1 regulation in hGF and hPDLC was analyzed using a Wilcoxon test.

Statistical analyses were accomplished 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: KNL HXM JXH. Performed the experiments: KNL. Analyzed the data: KNL HXM. Contributed reagents/materials/analysis tools: KNL JXH. Wrote the paper: KNL HXM JXH.

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