Check for updates

Received: 22 April 2020 | Revised: 3 September 2020 | Accepted: 20 October 2020

DOI: 10.1002/JPER.20-0225

JOURNAL OF Periodontology

ORIGINAL ARTICLE

Extending the vitamin D pathway to vitamin D_3 and CYP27A1 in periodontal ligament cells

Churen Zhang[1,2,3,4] | **Kaining Liu**[1,2,3,4] | **Jianxia Hou**[1,2,3,4]

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

[2] National Clinical Research Center for Oral Diseases, Beijing, China

[3] National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing, China

[4] Beijing Key Laboratory of Digital Stomatology, Beijing, China

Correspondence
Dr. Kaining Liu, Department of Periodontology, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Ave., Haidian District, Beijing 100081, P.R. China.
Email: liukainingbjmu@163.com
Dr. Jianxia Hou, Department of Periodontology, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Ave. Haidian District, Beijing 100081, P.R. China.
Email: jxhou@163.com

Funding information
National Natural Science Foundations of China, Grant/Award Numbers: 81470738, 81100749; GengYun Grant

Abstract

Background: In periodontal connective tissue cells, the vitamin D pathway has been elucidated, and vitamin D_3 in the main storage form, 25-hydroxy vitamin D_3 (25[OH]D_3), and the functional form, 1,25-dihydroxy vitamin D_3 (1,25[OH]$_2D_3$), have been found to induce the expression of human cationic antimicrobial protein (hCAP-18)/LL-37. Moreover, synergistic effects between Toll-like receptor agonists and 25(OH)D_3 have been reported. This research aimed at extending the vitamin D pathway to vitamin D_3 and CYP27A1 in human periodontal ligament cells (hPDLCs) to further explore its function in periodontal inflammatory reaction.

Methods: Vitamin D_3 was used to stimulate hPDLCs in the presence or absence of *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS). Conversely, CYP27A1 RNA interference was performed to further validate the findings. The mRNA expression of hCAP-18 was determined with real-time polymerase chain reaction. Monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8) were also detected. The cell supernatant levels of LL-37 were detected with enzyme-linked immunosorbent assay.

Results: Vitamin D_3 significantly enhanced the generation of hCAP-18/LL-37. A combination of *Pg*-LPS and vitamin D_3 significantly promoted hCAP-18/LL-37 expression. When the expression of CYP27A1 was knocked down with RNA interference, the induction of hCAP-18/LL-37 expression was significantly inhibited. Therefore, the mRNA levels of MCP-1 and IL-8 in hPDLCs were significantly decreased through the vitamin D pathway.

Conclusion: The vitamin D pathway from vitamin D_3 to hCAP-18/LL-37 exists in hPDLCs, and CYP27A1 might be involved in periodontal immune defense.

KEYWORDS
cholestanetriol 26-monooxygenase, periodontal ligament, vitamin D

1 | INTRODUCTION

In humans, vitamin D_3 is a secosteroid, a prohormonal precursor of biologically active hydroxyforms.[1] Vitamin D_3 is synthesized in the skin via the effects of ultraviolet B through two non-enzymatic steps: 7-dehydrocholesterol is first converted to pre-vitamin D_3, which is then converted to vitamin D_3.[1,2] Vitamin D_3 and its metabolites play important roles in the regulation of bone metabolism and the immune response.[3] Vitamin D_3 must be

© 2020 American Academy of Periodontology

metabolized through two-step hydroxylations to exert its biological functions. First, vitamin D_3 is hydroxylated by 25-hydroxylase, forming 25(OH)D_3. In human periodontal ligament cells (hPDLCs), which are of mesenchymal origin, CYP27A1 is the major 25-hydroxylase.[4] Second, 1,α-hydroxylase (CYP27B1) converts 25(OH)D_3 to 1,25$(OH)_2D_3$.[5] After binding the vitamin D receptor, 1,25$(OH)_2D_3$ exerts its biological functions.[6,7] Slominski et al.[8] have found another activation pathway of vitamin D_3 in dermal fibroblasts, characterized by CYP11A1, which hydroxylates vitamin D_3 and forms different biologically active products.

Liu et al.[9] first proposed that the vitamin D pathway includes a sequence of reactions from 25(OH)D_3 to the production of human cationic antimicrobial protein (hCAP-18)/LL-37, and reported that Toll-like receptor-2 (TLR2) agonists show synergistic effects with 25(OH)D_3 in the production of antimicrobial peptides in monocytes. Schauber et al.[10] found that TLR2 agonists induce the expression of genes encoding CYP27B1. 25(OH)D_3 is converted to 1,25$(OH)_2D_3$ by CYP27B1 and subsequently increases hCAP-18 expression in keratinocytes and monocytes. Khoo et al.[11] have reported enhancement of hCAP-18 gene expression after 1,25$(OH)_2D_3$ stimulation in peripheral blood mononuclear cells. Beyond hCAP-18, another class of human antimicrobial proteins, human β-defensin-3 is increased by 1,25$(OH)_2D_3$, thereby inhibiting the *P. gingivalis*-induced production of tumor necrosis factor-α, interleukin (IL)-8, and IL-12 in human periodontal ligament cells.[12]

hCAP-18) is the only antimicrobial protein from the cathelicidin family found in humans.[13] hCAP-18 includes a cathelin-like region in the amino-terminus and with 37 amino acid residues in the carboxy-terminus.[14] The predominant antimicrobial product, LL-37, must be cleaved by proteinases after release from cells.[15] LL-37 has a marked effect on resistance to microbes. Bactericidal effects are achieved through destruction of the bacterial cell wall.[16] Additionally, LL-37 helps human macrophages clear intracellular bacteria via binding specific receptors such as the P2 × 7 receptor.[17]

In the field of periodontal research, the existence of LL-37 has been confirmed in human gingival epithelial cells,[18–20] human gingival fibroblasts (hGFs),[21] hPDLCs,[21] and neutrophils.[22] LL-37 is present in gingival epithelia and connective tissues in humans, and people with periodontitis exhibit more LL-37 production than unaffected people.[19–21] In hGFs,[21] hPDLCs,[21] and gingival epithelial cells,[23] 25(OH)D_3 significantly enhances hCAP-18/LL-37 production. Our past research[21] indicated that the vitamin D pathway, from 25(OH)D_3 and 1,25$(OH)_2D_3$ to the production of LL-37, on which TLR2 agonist has a synergistic effect with 25(OH)D_3, exists in periodontal connective tissue cells. In another study from our group, stimulation of hPDLCs and hGFs with vitamin D_3 has been found to enhance 25(OH)D_3 and 1,25$(OH)_2D_3$ synthesis, and we detected CYP27A1 in both cell types. When higher concentrations of vitamin D_3 were used, the generation of 25(OH)D_3 and 1,25$(OH)_2D_3$ increased accordingly; however, their production clearly decreased after CYP27A1 silencing.[4] In hPDLCs, vitamin D_3 suppresses the level of lipopolysaccharide (LPS)-induced IL-6 by ≈ 70%,[24] and 1,25$(OH)_2D_3$ decreases *Pg*-LPS–induced mRNA expression of IL-8 and Monocyte chemotactic protein-1 (MCP-1).[25] Hence, we hypothesized that CYP27A1 and vitamin D_3 might function as part of vitamin D pathway in hPDLCs. To test this hypothesis, we performed in vitro studies exploring how vitamin D_3 influences hCAP-18/LL-37 production in inflammatory and non-inflammatory conditions.

2 | MATERIALS AND METHODS

2.1 | Culture of hPDLCs

This study plan was approved by the Institutional Review Board of Peking University School and Hospital of Stomatology (PKUSSIRB-2011007). The study was performed according to the 2013 revision of the Helsinki Declaration of 1975. Each participant signed an informed consent form.

Primary cell culture was conducted according to previously described methods.[26,27] Briefly, hPDLCs were acquired from removed wisdom teeth from eight healthy participants without periodontal inflammation. Periodontal ligament tissue was collected from the central section of the root by gently curetting and mincing. Twenty-four–well plates were used for inoculation of the isolated cells. hPDLCs were cultured in Dulbecco's modified Eagle's medium without phenol red,[*] with 1% penicillin-streptomycin and 10% fetal bovine serum[†] added. hPDLCs were placed in an incubator containing 5% CO2 with a temperature of 37°C. When the hPDLCs reached 90% confluence, cells were split with 0.25% trypsin and 0.02% EDTA. The fourth generation of cells was used in the following experiments. All experiments were conducted in triplicate.

2.2 | Stimulation with vitamin D_3 combined with *Pg*-LPS to detect the production of hCAP-18/LL-37

Six-well plates were used to inoculate cells obtained from three participants. The number of cells per well

[*] Sigma Aldrich, St. Louis, MO.
[†] PAA, Coelbe, Germany.

was ≈50,000. After reaching 80% to 90% confluence, hPDLCs were stimulated with 0.1% ethanol, vitamin D_3,[‡] 25(OH)D_3,[§] *Pg*-LPS,[**] vitamin D_3 combined with *Pg*-LPS, or 25(OH)D_3 combined with *Pg*-LPS. The concentrations of vitamin D_3 and 25(OH)D_3 were both 10^{-6} M. The concentration of *Pg*-LPS was 10 μg/mL. After 48 hours, RNA was obtained with cell lysis solution[††] according to the manufacturer's protocol. Simultaneously, supernatants were collected. With a kit,[‡‡] reverse transcription PCR was conducted to obtain cDNA. Next, real-time PCR was performed with SYBR Green[§§] with a real-time thermocycler.[***] The reference gene was GAPDH. The forward and reverse primer sequences for GAPDH were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′, respectively, and those for hCAP-18 were 5′-AGGATTGTGACTTCAAGA AGGACG-3′ and 5′-GTTTATTTCTCAGAGCCCAGAAGC-3′. The relative mRNA levels of hCAP-18 were computed with the equation, $2^{-\triangle\triangle Ct}$.[28]

2.3 | Stimulation with vitamin D_3 for various times

hPDLCs from two participants were inoculated on six-well plates. The number of cells per well was ≈50,000. After cells reached 80% to 90% confluence, hPDLCs were treated with 10^{-6} M vitamin D_3 for 24 hours, 48 hours, or 96 hours. Real-time PCR was used to detect hCAP-18 mRNA expression.

2.4 | Silencing of CYP27A1 RNA expression

Two participants' hPDLCs were inoculated on six-well plates. The number of cells per well was ≈150,000. After cells reached 80% to 90% confluence, small interfering RNA (20 nM)[†††] and transfection reagent[‡‡‡] were used to transfect the hPDLCs. One group had silenced CYP27A1 RNA expression, and the other group was a negative control without RNA silencing. The CYP27A1 siRNA's sequence was CACGCTGACATGGGCCCTGTA.

[‡] Sigma Aldrich, St. Louis, MO.
[§] Sigma Aldrich, St. Louis, MO.
[**] Invivogen, San Diego, CA.
[††] TRIzol, Thermo Fisher Scientific, Waltham, MA.
[‡‡] Rever Tra Ace qPCR RT Kit, Toyobo, Japan.
[§§] Roche, Indianapolis, IN.
[***] Applied Biosystems, Thermo Fisher Scientific, Waltham, MA.
[†††] CYP27A1 siRNA, Santa Cruz Biotechnology, Santa Cruz, CA.
[‡‡‡] Lipofectamine 3000, Thermo Fisher Scientific, Waltham, MA.

At 24 hours after transfection, the efficiency of RNA interference was verified by real-time PCR.

After confirmation of the RNA interference efficiency, hCAP-18/LL-37 production was detected. Immediately after the 24 hours of transfection, 10^{-6} M vitamin D_3 was used to stimulate the hPDLCs. After 48 hours, the cells were collected. Relative mRNA levels of hCAP-18 expression were determined by real-time PCR.

The forward and reverse primer sequences for CYP27A1 were 5′-GCTCTTGGAGCAAGTGATG-3′ and 5′-AGCATCCGTATAGAGCGC-3′, respectively.

2.5 | Stimulation by *Pg*-LPS with or without vitamin D_3

Cells from three donors were used. When cells seeded in six-well plates reached ≈90% confluence, they were divided into four groups treated with 0.1% ethanol, vitamin D_3, *Pg*-LPS alone, or vitamin D_3 with *Pg*-LPS. The concentration of *Pg*-LPS was 10 μg/mL. The concentrations of vitamin D_3[‡] were 10^{-6} M. After 48 hours of stimulation, real-time PCR was used to detect the mRNA levels of CYP27A1, IL-8, and MCP-1. The supernatant levels of IL-8 were detected with an ELISA kit.[§§§] The supernatant levels of MCP-1 were detected with an ELISA kit.[****] The forward and reverse primer sequences for MCP-1 were 5′-CAGCCAGATGCAATCAATGCC-3′ and 5′-TGGAATCCTGAACCCACTTCT-3′, respectively, and those for IL-8 were 5′-TTTTGCCAAGGAGTGCTAAAGA-3′ and 5′-AACCCTCTGCACCCAGTTTTC-3′, respectively.

2.6 | Detection of cell supernatant concentrations of LL-37

The supernatant levels of LL-37 were detected with an ELISA kit[††††] on the basis of the manufacturer's protocol.

2.7 | Statistical analyses

The Shapiro-Wilk test was used to demonstrate the distribution of the data. If the data were normally distributed, one-way ANOVA was used, and the data were presented as mean + SE. If the data were not normally distributed, the Kruskal–Wallis test was used, and the data were presented as median and interquartile range. The data analysis was

[§§§] Human IL-8 ELISA kit, Meimian, China.
[****] Human MCP-1 ELISA kit, Meimian, China.
[††††] Human LL-37 ELISA kit, Meimian, China.

performed using SPSS software.[‡‡‡‡] When the P value was <0.05, a significant statistical difference was confirmed.

3 | RESULTS

3.1 | Enhancement of hCAP-18/LL-37 after stimulation with vitamin D_3 or 25(OH)D_3 with or without *Pg*-LPS

The mRNA expression of hCAP-18 was detected in all hPDLCs. After a 48-hour stimulation with vitamin D_3 or 25(OH)D_3, the hCAP-18 mRNA clearly increased. When stimulated with vitamin D_3, the relative hCAP-18 mRNA level was ≈2- to 4-fold that in the ethanol group, and was ≈11 to 30 times higher when stimulated with 25(OH)D_3. Addition of *Pg*-LPS did not significantly influence the vitamin D_3-induced hCAP-18 mRNA expression. Treatment of hPDLCs with vitamin D_3 plus *Pg*-LPS increased the hCAP-18 mRNA expression to an average of 3.4-fold (Fig. 1A). The LL-37 protein level in cell supernatants of hPDLCs was 55.1 ± 7.4 pg/mL after treatment with ethanol, 67.5 (61.8, 101.6) pg/mL after treatment with vitamin D_3, 83.4 (69.9, 135.9) pg/mL after treatment with 25(OH)D_3, 55.6 ± 8.1 pg/mL after treatment with *Pg*-LPS, 75.1 ± 23.2 pg/mL after treatment with *Pg*-LPS plus vitamin D_3, and 78.1 (68.3, 96.6) pg/mL after treatment with *Pg*-LPS plus 25(OH)D_3.The LL-37 concentrations after treatment with vitamin D_3 or 25(OH)D_3 were significantly higher than those in the ethanol group. The LL-37 concentrations after treatment with *Pg*-LPS plus vitamin D_3 or 25(OH)D_3 were significantly higher than those in the *Pg*-LPS group (Fig. 1B).

3.2 | Increased hCAP-18 mRNA expression and LL-37 production with increasing vitamin D_3 stimulation time

Stimulation with vitamin D_3 increased the hCAP-18 mRNA expression and LL-37 production in a time-dependent manner. The increase in hCAP-18 mRNA expression after stimulation with vitamin D_3 for 24 hours, 48 hours, or 96 hours was ≈1.3-fold, 3.8-fold, and 9.1-fold that in the ethanol group, respectively (Fig. 2A). The LL-37 production was 55.1 ± 7.4 pg/mL in the ethanol group, 59.5 ± 6.5 pg/mL in the 24-hour stimulation group, 67.5 (61.8, 101.6) pg/mL in the 48-hour stimulation group, and 73.0 ± 6.4 pg/mL in the 96-hour stimulation group. The fold increase in LL-37 production from the ethanol group to the 48-hour or 96-hour stimulation group was significant (Fig. 2B).

[‡‡‡‡] IBM, Armonk, NY.

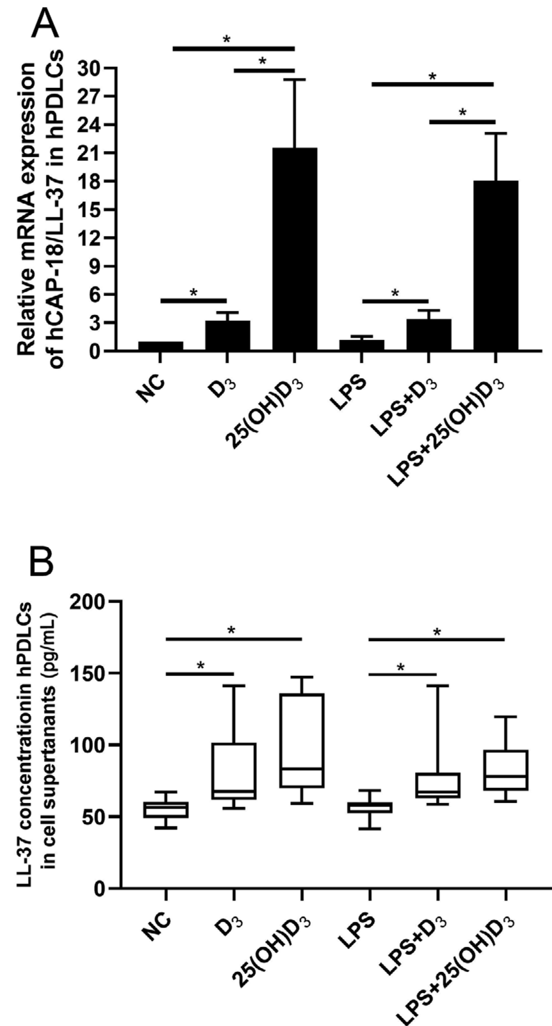

FIGURE 1 Increased generation of hCAP-18/LL-37 after vitamin D_3 and 25(OH)D_3 stimulation in hPDLCs. **A**) Relative expression of hCAP-18 mRNA. The cells were stimulated with ethanol; vitamin D_3; 25(OH)D_3; *Pg*-LPS; *Pg*-LPS plus vitamin D_3; or *Pg*-LPS plus 25(OH)D_3. **B**) Respective concentrations of LL-37 in the groups. The concentrations of vitamin D_3 and 25(OH)D_3 were both 10^{-6} M, and that of *Pg*-LPS was 10 μg/mL. Negative control (NC) represents ethanol; D_3, vitamin D_3. The data of relative mRNA expression are shown as mean + SE. The data of LL-37 concentrations are shown as median and interquartile range. Any two groups marked by the same "*" showed significantly different data

3.3 | Decreased levels of hCAP-18/LL-37 after CYP27A1 RNA interference

The knockdown efficiency of RNA interference against CYP27A1 reached >80% (Fig. 3A). After effective

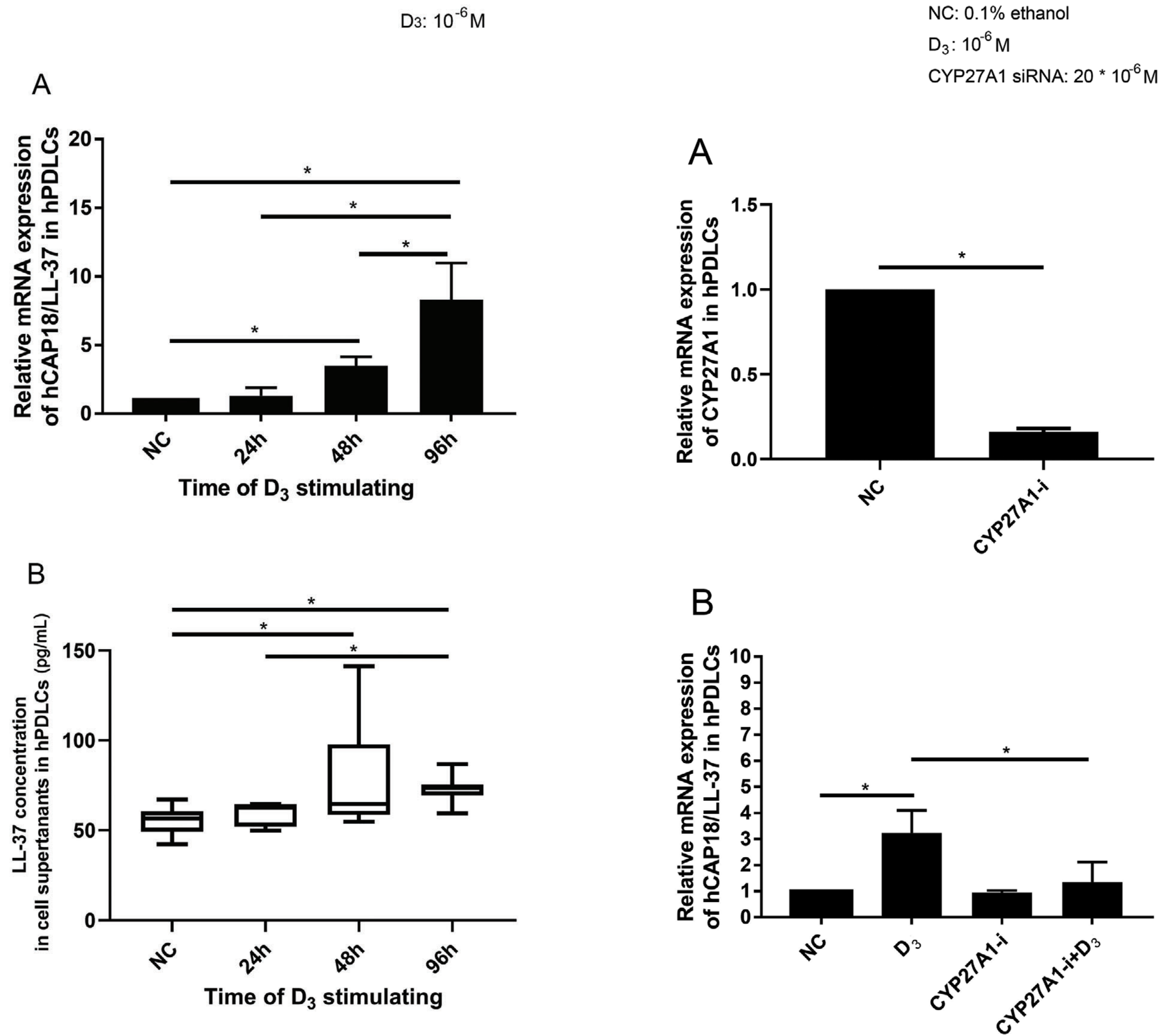

FIGURE 2 Increased generation of hCAP-18/LL-37 after different vitamin D_3 stimulation times. **A**) Relative hCAP-18 mRNA expression. The cells were treated with ethanol; vitamin D_3; *Pg*-LPS; or *Pg*-LPS plus vitamin D_3. **B**) LL-37 protein production. The data of relative mRNA expression are shown as mean + SE. The data of LL-37 concentrations are shown as median and interquartile range. Any two groups marked by the same "*" show significantly different data

knockdown of CYP27A1 and stimulation with vitamin D_3, the relative hCAP-18 mRNA levels significantly decreased ≈60%, to a level close to that in the ethanol group (Fig. 3). The LL-37 protein level in the CYP27A1 RNA interference group remained at 59.2 ± 3.2 pg/mL, which was close to the 55.1 ± 7.4 pg/mL in the ethanol group. After CYP27A1 RNA interference, the vitamin D_3-induced LL-37 production decreased significantly from 67.5 (61.8, 101.6) pg/mL to 55.78 (50.6, 59.2) pg/mL (Fig. 3C).

FIGURE 3 Vitamin D_3 treatment after CYP27A1 silencing inhibits hCAP-18/LL-37 production in hPDLCs. **A**) Relative CYP27A1 mRNA expression after CYP27A1 RNA interference. The

3.4 | Unchanged CYP27A1 mRNA expression after stimulation with vitamin D_3 or *Pg*-LPS

Compared with stimulation with ethanol, stimulation with vitamin D_3 or *Pg*-LPS did not markedly influence the CYP27A1 mRNA expression (Fig. 4).

3.5 | Decreased IL-8 and MCP-1 mRNA expression and protein production after stimulation with *Pg*-LPS plus vitamin D_3, compared with stimulation with *Pg*-LPS

Compared with IL-8 and MCP-1 mRNA expression in the ethanol group, stimulation with vitamin D_3 did not clearly alter IL-8 and MCP-1 mRNA expression or protein production. In contrast to those in the *Pg*-LPS group, the IL-8 and MCP-1 mRNA expression levels decreased significantly after treatment with vitamin D_3 combined with *Pg*-LPS. The IL-8 protein concentrations decreased significantly from 1,821.1 ± 188.7 pg/mL to 1,490.8 ± 134.3 pg/mL, and the MCP-1 protein concentrations decreased significantly from 220.8 ± 40.4 pg/mL to 168.4 ± 26.4 pg/mL (Fig. 5).

control group was the non-silenced group without any stimulation. **B**) Relative expression of hCAP-18 mRNA. The cells were treated with ethanol; vitamin D_3; CYP27A1 RNA interference; or CYP27A1 RNA interference plus vitamin D_3. **C**) Respective concentrations of LL-37. The concentration of vitamin D_3 was 10^{-6} M. Negative control (NC) represents ethanol; D_3, represents vitamin D_3. The data of relative mRNA expression are shown as mean + SE. The data of LL-37 concentrations are shown as median and interquartile range. Any two groups marked by the same "*" showed significantly different data

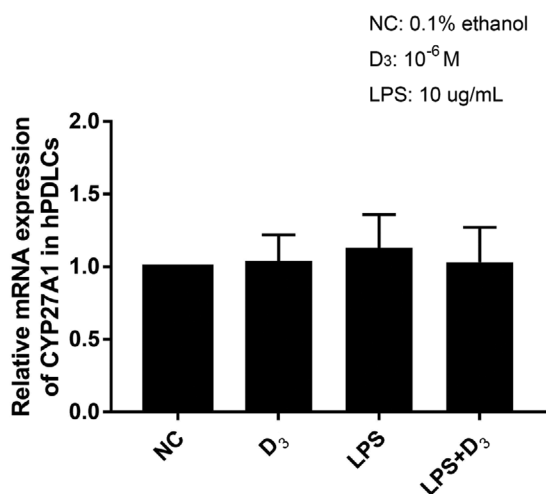

FIGURE 4 Relative CYP27A1 mRNA expression after stimulation with vitamin D_3 and *Pg*-LPS. The concentration of vitamin D_3 was 10^{-6} M, and that of *Pg*-LPS was 10 μg/mL. D_3 represents vitamin D_3. The data of relative mRNA expression are shown as mean + SE

4 | DISCUSSION

This in vitro research indicated that vitamin D_3 induces hCAP-18/LL-37 production in hPDLCs. In contrast, knockdown of CYP27A1 expression resulted in 60% decrease in the level of hCAP-18/LL-37. These findings provided evidences that in hPDLCs, vitamin D_3 and CYP27A1 are components of the vitamin D pathway. Previous studies[6,9,29,30] on the vitamin D pathway have focused on the key factors CYP27B1, VDR, but CYP27A1 was not investigated. To our knowledge, this study provides the first evidence extending the vitamin D pathway to vitamin D_3 and CYP27A1. In the present study, vitamin D_3 was found to activate the vitamin D pathway in hPDLCs, however, this activation effect was not as strong as that of 25(OH)D_3. Vitamin D_3 must be metabolized by CYP27A1 to 25(OH)D_3 to further exert its biological roles. Not all vitamin D_3 can be transformed to 25(OH)D_3. Thus, stimulation with the same concentration of vitamin D_3 or 25(OH)D_3 resulted in a difference in hCAP-18/LL-37 upregulation.

The vitamin D pathway also exists in human gingival epithelial cells (hGECs),[31,32] and the production of hCAP-18/LL-37 has been reported to increase 75-fold after stimulation with 1,25(OH)$_2D_3$ compared with the negative control in 24 hours.[20] However, in the present study, the upregulation of hCAP-18 RNA expression after stimulation with vitamin D_3 was only 2- to 4-fold in hPDLCs. In periodontal tissues, hGECs are the first line of defense against external stimulation, and they play a more important role in periodontal immune defense than hPDLCs. Accordingly, hGECs have a more active vitamin D pathway than hPDLCs.[33] After the hGEC-induced immune defense becomes insufficient to stop the inflammation from moving from the surface to deep periodontal soft tissues, subepithelial connective tissue cells are activated and participate in the immune defense. Through the vitamin D pathway, hPDLCs produce hCAP-18/LL-37, thereby exerting anti-infection and immune regulation functions. Therefore, although the vitamin D pathway in hPDLCs is relatively less active, the biological importance of the vitamin D pathway in hPDLCs remains worthy of study.

Our previous study has indicated that the plasma 25(OH)D_3 levels in people with aggressive periodontitis are significantly higher than those in uninfected individuals, whereas the plasma 25(OH)D_3 level in people with chronic periodontitis do not significantly differ between these groups.[34] Further studies have shown that the plasma and

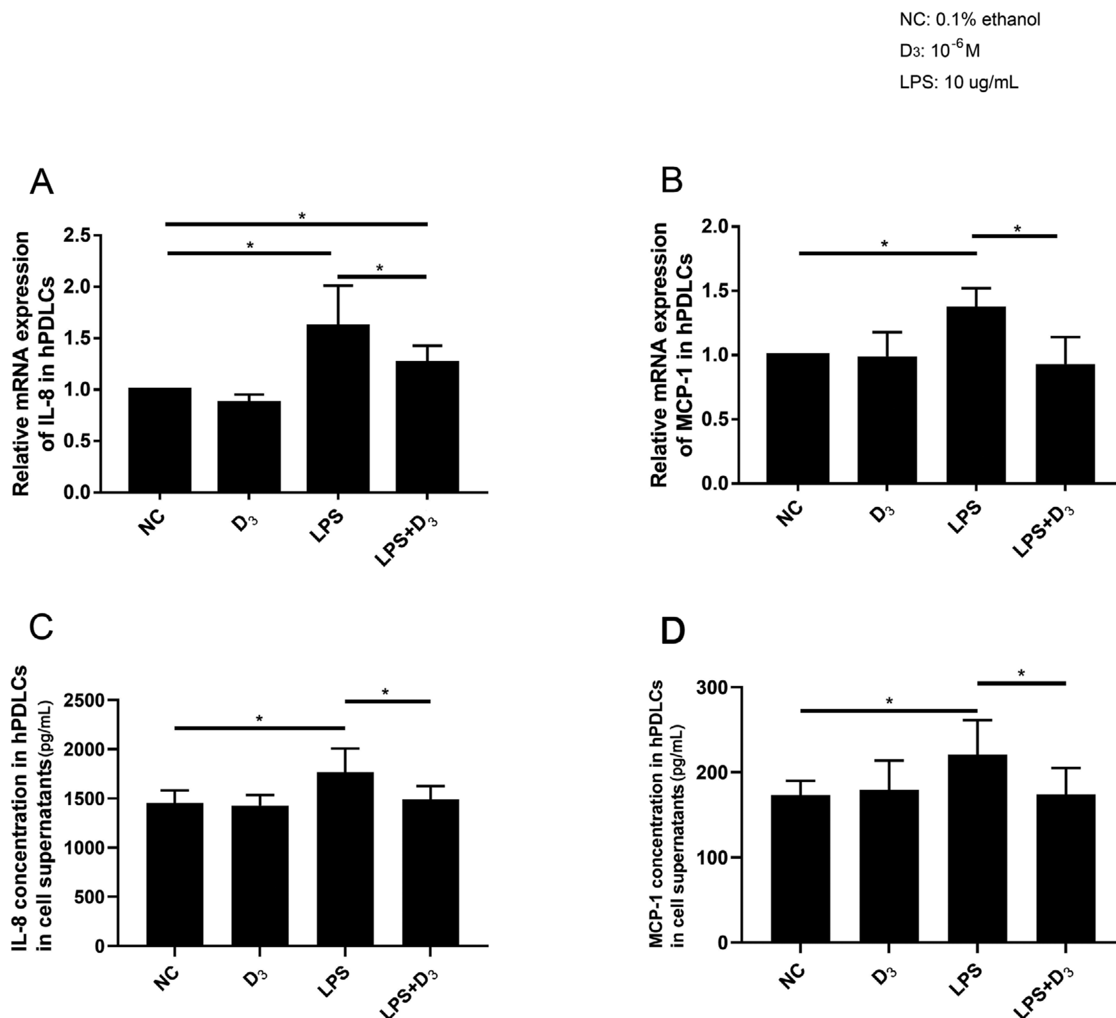

FIGURE 5 Modulation of MCP-1 and IL-8 after stimulation with *Pg*-LPS combined with vitamin D_3. **A**) Relative levels of IL-8 mRNA. **B**) Relative levels of MCP-1 mRNA. **C**) IL-8 protein production. **D**) MCP-1 protein production. The cells were treated with ethanol; vitamin D_3; *Pg*-LPS; or *Pg*-LPS plus vitamin D_3. B) IL-8 and MCP-1 protein production. D_3 represents vitamin D_3. All data are shown as mean + SE. Any two groups marked by the same "*" showed significantly different data

gingival crevicular fluid 25(OH)D_3 levels in people with aggressive periodontitis decreases after periodontal initial therapy.[35] Most of the people with aggressive periodontitis and the unaffected individuals whom we studied were aged <30 years. However, the third American Health and Nutrition Survey found that plasma 25(OH)D_3 levels are negatively correlated with attachment loss in people aged >50 years.[36] Two other large longitudinal studies[37,38] have found that plasma 25(OH)D_3 levels and tooth loss are negatively correlated, thus, indicating that the higher the 25(OH)D_3 level in the plasma, the lower the possibility of tooth loss. However, the participants in those two studies were 40 to 75 years, and the average age was about 50 years, an age range that does not overlap with that of the population in our previous study. Therefore, the relationship between plasma 25(OH)D_3 levels and periodontitis in young people might possibly have different characteristics. In addition, another large cross-sectional study[39] found no correlation between plasma 25(OH)D_3 levels and periodontal health. The age of the participants in that study was 30 to 49 years, which also differs from the ages of our previous research participants. Therefore, not all studies support a positive correlation between higher plasma 25(OH)D_3 levels and better periodontal health.

According to the classification of periodontitis in 1999,[40] aggressive periodontitis is characterized by severe periodontal inflammation in young people. It has particular characteristics in terms of periodontal disease gene expression and response to periodontal pathogens. The prevalence in the population is very low. In our previous study, periodontal inflammation in people with aggressive periodontitis was found to be more severe than that in all the other participants. People with chronic periodontitis had much more pronounced periodontal inflammation than

JOURNAL OF Periodontology

unaffected individuals. According to the new classification in 2018, most people with aggressive periodontitis can be diagnosed with periodontitis to stage IV, grade C periodontitis, which still accounts for a relatively low proportion of the population. In studies investigating the relationship between 25(OH)D_3 levels and periodontal health in large samples,[36–39,41] the participants were from the general population. The population characteristics differed from those of people with stage IV, grade C periodontitis in our previous study. In this special group, the question of whether a high plasma 25(OH)D_3 level is the cause or the result of severe periodontal inflammation remains unclear. The results of our previous study[21] suggest that 25(OH)D_3 promotes the activity of the vitamin D pathway and results in further production of LL-37, which participates in antibacterial and anti-inflammatory functions. Therefore, owing to extensive periodontal inflammation in stage IV, grade C periodontitis, more LL-37 might potentially be needed for antibacterial and anti-inflammatory function. Thus, the more active the vitamin D pathway is, the more 25(OH)D_3 is necessary. Our present study on the vitamin D pathway should aid in further understanding of the relationship between vitamin D and periodontitis, and should provide a scientific basis for explaining this causal relationship. Because CYP27A1 is the key enzyme in the periodontal production of 25(OH)D_3,[4] CYP27A1 expression might potentially be associated with periodontitis. The present study revealed that vitamin D_3 and CYP27A1 induce the production of the anti-inflammatory bio-factor LL-37, and decrease the levels of MCP-1 and IL-8, thus, indicating a role in periodontal anti-inflammatory function.

In this study, vitamin D_3 enhanced hCAP-18/LL-37 production. In a periodontal clinical study, Gao et al.[42] reported that daily vitamin D supplementation for 3 months results in an apparent increase in serum 25(OH)D_3 concentrations and improvements in attachment loss and probing depth in people with periodontitis. Bashutski et al.[43] have shown that people lacking vitamin D exhibit poor treatment outcomes after periodontal surgery. Hiremath et al.[44] demonstrated that supplementation with vitamin D inhibits the inflammation in gingivitis. However, the principles underlying the periodontal benefits of vitamin D remain to be thoroughly clarified. According to the function of vitamin D_3 in the vitamin D pathway demonstrated above, vitamin D supplementation might potentially increase the generation of LL-37 via the vitamin D pathway in periodontal soft tissues, and this response might be beneficial to periodontal immune defense. This study may provide theoretical support for periodontal clinical application of vitamin D supplementation. However, further investigation remains necessary.

Activation of vitamin D_3 hydroxylated by CYP27A1 and CYP27B1 constitutes the classical activation pathway. Slominski et al.[45–47] proposed a new metabolic pathway for vitamin D_3 in the placenta, adrenal glands and epidermal keratinocytes, in which serial hydroxylation by CYP11A1 forms 20,22(OH)D_3, 20,23(OH)D_3, and 17,20,23(OH)D_3, which, like 1,25$(OH)_2D_3$, have anti-fibrosis, pro-differentiation and anticancer biological activities. In addition, different receptors exist for different forms of vitamin D. These secosteroids can act as negative agonists of the retinoic acid orphan receptors α and γ.[48] 20,23(OH)D_3 mainly acts on the aryl hydrocarbon receptor.[49] Whether the newly described metabolic pathway of vitamin D_3 exists in hPDLCs requires further study.

In this study, hPDLCs were obtained from eight different participants and cultured. One limitation of this study was the presence of individual differences between primary cultured cells.

5 | CONCLUSIONS

In hPDLCs, vitamin D_3 and CYP27A1 are likely to play a role in the vitamin D pathway and might be involved in periodontal anti-inflammatory function. This study expands knowledge of the periodontal vitamin D pathway and may provide theoretical support for clinical periodontal vitamin D supplementation in the future.

ACKNOWLEDGMENTS

Funding was provided by National Natural Science Foundations of China (81470738, 81100749) and GengYun Grant (Beijing). The authors report no conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS

Churen Zhang conducted experiments, data analyses, production of figures, and composed the document. Kaining Liu and Jianxia Hou gave suggestions in primary cell culture, proposed research protocol, and modified the document.

ORCID

Churen Zhang https://orcid.org/0000-0001-9070-7138

Jianxia Hou https://orcid.org/0000-0003-2414-0895

REFERENCES

1. Bikle D, Christakos S. New aspects of vitamin D metabolism and action – addressing the skin as source and target. *Nat Rev Endocrinol*. 2020;16:234-252.

2. Holick MF. Vitamin D: a millenium perspective. *J Cell Biochem*. 2003;88:296-307.
3. Bikle D. Vitamin D Assays. *Front Horm Res*. 2018;50:14-30.
4. Liu K, Meng H, Hou J. Activity of 25-hydroxylase in human gingival fibroblasts and periodontal ligament cells. *PLoS One*. 2012;7:e52053.
5. Liu K, Meng H, Hou J. Characterization of the autocrine/paracrine function of vitamin D in human gingival fibroblasts and periodontal ligament cells. *PLoS One*. 2012;7:e39878.
6. Yuan C, Renfro L, Ambadwar PB, et al. Influence of genetic variation in the vitamin D pathway on plasma 25-hydroxyvitamin D3 levels and survival among patients with metastatic colorectal cancer. *Cancer Causes Control*. 2019;30:757-765.
7. Yang S, Li A, Wang J, et al. Vitamin D receptor: a novel therapeutic target for kidney diseases. *Curr Med Chem*. 2018;25: 3256-3271.
8. Slominski AT, Kim TK, Li W, Tuckey RC. Classical and non-classical metabolic transformation of vitamin D in dermal fibroblasts. *Exp Dermatol*. 2016;25:231-232.
9. Liu PT, Stenger S, Li H, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science*. 2006;311:1770-1773.
10. Schauber J, Dorschner RA, Coda AB, et al. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J Clin Invest*. 2007;117:803-811.
11. Khoo AL, Chai LY, Koenen HJ, et al. Vitamin D(3) down-regulates proinflammatory cytokine response to Mycobacterium tuberculosis through pattern recognition receptors while inducing protective cathelicidin production. *Cytokine*. 2011;55:294-300.
12. De Filippis A, Fiorentino M, Guida L, Annunziata M, Nastri L, Rizzo A. Vitamin D reduces the inflammatory response by *Porphyromonas gingivalis* infection by modulating human beta-defensin-3 in human gingival epithelium and periodontal ligament cells. *Int Immunopharmacol*. 2017;47:106-117.
13. Pinheiro da Silva F, Machado MC. The dual role of cathelicidins in systemic inflammation. *Immunol Lett*. 2017;182:57-60.
14. Fabisiak A, Murawska N, Fichna J. LL-37: cathelicidin-related antimicrobial peptide with pleiotropic activity. *Pharmacol Rep*. 2016;68:802-808.
15. Xie F, Zan Y, Zhang Y, et al. The cysteine protease ApdS from Streptococcus suis promotes evasion of innate immune defenses by cleaving the antimicrobial peptide cathelicidin LL-37. *J Biol Chem*. 2019;294:17962-17977.
16. Sierra JM, Fuste E, Rabanal F, Vinuesa T, Vinas M. An overview of antimicrobial peptides and the latest advances in their development. *Expert Opin Biol Ther*. 2017;17:663-676.
17. Tang X, Basavarajappa D, Haeggstrom JZ, Wan M. P2X7 receptor regulates internalization of antimicrobial peptide LL-37 by human macrophages that promotes intracellular pathogen clearance. *J Immunol*. 2015;195:1191-1201.
18. Dale BA, Kimball JR, Krisanaprakornkit S, et al. Localized antimicrobial peptide expression in human gingiva. *J Periodontal Res*. 2001;36:285-294.
19. Hosokawa I, Hosokawa Y, Komatsuzawa H, et al. Innate immune peptide LL-37 displays distinct expression pattern from beta-defensins in inflamed gingival tissue. *Clin Exp Immunol*. 2006;146:218-225.
20. Tada H, Shimizu T, Nagaoka I, Takada H. Vitamin D3 analog maxacalcitol (OCT) induces hCAP-18/LL-37 production in human oral epithelial cells. *Biomed Res*. 2016;37:199-205.
21. Gao Z, Liu K, Meng H. Preliminary investigation of the vitamin D pathway in periodontal connective tissue cells. *J Periodontol*. 2018;89:294-302.
22. Turkoglu O, Kandiloglu G, Berdeli A, Emingil G, Atilla G. Antimicrobial peptide hCAP-18/LL-37 protein and mRNA expressions in different periodontal diseases. *Oral Dis*. 2011;17:60-67.
23. Svensson D, Nebel D, Nilsson BO. Vitamin D3 modulates the innate immune response through regulation of the hCAP-18/LL-37 gene expression and cytokine production. *Inflamm Res*. 2016;65:25-32.
24. Nebel D, Svensson D, Arosenius K, Larsson E, Jonsson D, Nilsson BO. 1alpha,25-dihydroxyvitamin D3 promotes osteogenic activity and downregulates proinflammatory cytokine expression in human periodontal ligament cells. *J Periodontal Res*. 2015;50:666-673.
25. Tang X, Pan Y, Zhao Y. Vitamin D inhibits the expression of interleukin-8 in human periodontal ligament cells stimulated with *Porphyromonas gingivalis*. *Arch Oral Biol*. 2013;58:397-407.
26. Liu K, Han B, Hou J, Meng H. Preliminary investigation on the molecular mechanisms underlying the correlation between VDR-FokI genotype and periodontitis. *J Periodontol*. 2020;91:403-412.
27. Liu K, Han B, Meng H, Hou J. Influence of rs2228570 on transcriptional activation by the vitamin D receptor in human gingival fibroblasts and periodontal ligament cells. *J Periodontol*. 2017;88:915-925.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25:402-408.
29. Jamali N, Sorenson CM, Sheibani N. Vitamin D and regulation of vascular cell function. *Am J Physiol Heart Circ Physiol*. 2018;314:H753-H765.
30. Tebben PJ, Singh RJ, Kumar R. Vitamin D-mediated hypercalcemia: mechanisms, diagnosis, and treatment. *Endocr Rev*. 2016;37:521-547.
31. Menzel LP, Ruddick W, Chowdhury MH, et al. Activation of vitamin D in the gingival epithelium and its role in gingival inflammation and alveolar bone loss. *J Periodontal Res*. 2019;54:444-452.
32. McMahon L, Schwartz K, Yilmaz O, Brown E, Ryan LK, Diamond G. Vitamin D-mediated induction of innate immunity in gingival epithelial cells. *Infect Immun*. 2011;79:2250-2256.
33. Greer A, Zenobia C, Darveau RP. Defensins and LL-37: a review of function in the gingival epithelium. *Periodontol 2000*. 2013;63:67-79.
34. Liu K, Meng H, Tang X, et al. Elevated plasma calcifediol is associated with aggressive periodontitis. *J Periodontol*. 2009;80:1114-1120.
35. Liu K, Meng H, Lu R, et al. Initial periodontal therapy reduced systemic and local 25-hydroxy vitamin D(3) and interleukin-1beta in patients with aggressive periodontitis. *J Periodontol*. 2010;81:260-266.

JOURNAL OF Periodontology

36. Dietrich T, Joshipura KJ, Dawson-Hughes B, Bischoff-Ferrari HA. Association between serum concentrations of 25-hydroxyvitamin D3 and periodontal disease in the US population. *Am J Clin Nutr*. 2004;80:108-113.
37. Jimenez M, Giovannucci E, Krall Kaye E, Joshipura KJ, Dietrich T. Predicted vitamin D status and incidence of tooth loss and periodontitis. *Public Health Nutr*. 2014;17:844-852.
38. Zhan Y, Samietz S, Holtfreter B, et al. Prospective Study of Serum 25-hydroxy vitamin D and tooth loss. *J Dent Res*. 2014;93:639-644.
39. Antonoglou GN, Suominen AL, Knuuttila M, et al. Associations between serum 25-hydroxyvitamin d and periodontal pocketing and gingival bleeding: results of a study in a non-smoking population in Finland. *J Periodontol*. 2015;86:755-765.
40. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol*. 1999;4:1-6.
41. Dietrich T, Nunn M, Dawson-Hughes B, Bischoff-Ferrari HA. Association between serum concentrations of 25-hydroxyvitamin D and gingival inflammation. *Am J Clin Nutr*. 2005;82:575-580.
42. Gao W, Tang H, Wang D, Zhou X, Song Y, Wang Z. Effect of short-term vitamin D supplementation after nonsurgical periodontal treatment: A randomized, double-masked, placebo-controlled clinical trial. *J Periodontal Res*. 2020;55:354-362.
43. Bashutski JD, Eber RM, Kinney JS, et al. The impact of vitamin D status on periodontal surgery outcomes. *J Dent Res*. 2011;90:1007-1012.
44. Hiremath VP, Rao CB, Naik V, Prasad KV. Anti-inflammatory effect of vitamin D on gingivitis: a dose-response randomised control trial. *Oral Health and Preventive Dentistry*. 2013;11:61-69.
45. Slominski AT, Kim TK, Shehabi HZ, et al. In vivo evidence for a novel pathway of vitamin D(3) metabolism initiated by P450scc and modified by CYP27B1. *FASEB J*. 2012;26:3901-3915.
46. Slominski AT, Li W, Kim TK, et al. Novel activities of CYP11A1 and their potential physiological significance. *J Steroid Biochem Mol Biol*. 2015;151:25-37.
47. Slominski AT, Kim TK, Shehabi HZ, et al. In vivo production of novel vitamin D2 hydroxy-derivatives by human placentas, epidermal keratinocytes, Caco-2 colon cells and the adrenal gland. *Mol Cell Endocrinol*. 2014;383:181-192.
48. Slominski AT, Kim TK, Hobrath JV, et al. Endogenously produced nonclassical vitamin D hydroxy-metabolites act as “biased” agonists on VDR and inverse agonists on RORalpha and RORgamma. *J Steroid Biochem Mol Biol*. 2017;173:42-56.
49. Slominski AT, Kim TK, Janjetovic Z, et al. Differential and Overlapping Effects of 20,23(OH)(2)D3 and 1,25(OH)(2)D3 on Gene Expression in Human Epidermal Keratinocytes: identification of AhR as an Alternative Receptor for 20,23(OH)(2)D3. *Int J Mol Sci*. 2018;19:3072.

How to cite this article: Zhang C, Liu K, Hou J. Extending the vitamin D pathway to vitamin D_3 and CYP27A1 in periodontal ligament cells. *J Periodontol*. 2021;92:e44-e53.
https://doi.org/10.1002/JPER.20-0225