Expression of vitamin D 1α-hydroxylase in human gingival fibroblasts *in vivo*

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Background. Vitamin D 1α-hydroxylase CYP27B1 is the key factor in the vitamin D pathway. Previously, we analyzed the expression of CYP27B1 in human gingival fibroblasts *in vitro*. In the present study, we analyzed the gingival expression of CYP27B1 *in vivo*.
Methods. Forty-two patients with periodontitis Stage IV Grade C and 33 controls were recruited. All patients with periodontitis had unsalvageable teeth and part of the wall of the periodontal pocket was resected and obtained after tooth extraction. All controls needed crown-lengthening surgery, and samples of gingiva resected during surgery were also harvested. All the individuals' gingivae were used for immunohistochemistry and immunofluorescence. In addition, gingivae from seventeen subjects of the diseased group and twelve subjects of the control group were analyzed by real-time PCR.
Results. Expression of CYP27B1 was detected both in gingival epithelia and in gingival connective tissues, and the expression in connective tissues colocalized with vimentin, indicating that CYP27B1 protein is expressed in gingival fibroblasts. The expression of CYP27B1 mRNA in gingival connective tissues and the CYP27B1 staining scores in gingival fibroblasts in the diseased group were significantly higher than those in the control group.
Conclusions. Expression of CYP27B1 in human gingival tissues was detected, not only in the fibroblasts of gingival connective tissues, but also in the gingival epithelial cells, and might be positively correlated with periodontal inflammation.

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Abstract

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Results. Expression of CYP27B1 was detected both in gingival epithelia and in gingival connective tissues, and the expression in connective tissues colocalized with vimentin, indicating that CYP27B1 protein is expressed in gingival fibroblasts. The expression of CYP27B1 mRNA in gingival connective tissues and the CYP27B1 staining scores in gingival fibroblasts in the diseased group were significantly higher than those in the control group.

Conclusions. Expression of CYP27B1 in human gingival tissues was detected, not only in the fibroblasts of gingival connective tissues, but also in the gingival epithelial cells, and might be positively correlated with periodontal inflammation.

Introduction

Vitamin D_3 is of great importance in regulating calcium and phosphorus metabolism and immunological responses (Sassi et al., 2018; Medrano et al., 2018). The active hormonal metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3 ($1,25OH_2D_3$), is formed by two-step hydroxylations: ① from vitamin D_3 to 25-hydroxyvitamin D_3 ($25OHD_3$) by vitamin D 25-hydroxylase (Liu et al., 2012a), followed by ② from $25OHD_3$ to $1,25OH_2D_3$ by vitamin D 1α-hydroxylase (Liu et al., 2012b). Vitamin D 1α-hydroxylase CYP27B1 was first detected in the kidney (Nykjaer et al., 1999), but subsequently extra-renal sites of $1,25OH_2D_3$ synthesis were also verified, including the skin (Bikle & Christakos, 2020), prostate (Capiod et al., 2018), bone (van Driel et al., 2006), eye (Alsalem et al., 2014; Markiewicz et al., 2019), and blood vessels (Somjen et al., 2005; Zehnder et al., 2002).

We previously verified expression and activity of CYP27B1 in human gingival fibroblasts (hGFs) (Liu et al., 2012b), an important component of the gingiva. In our previous study (Gao et al., 2018), the vitamin D pathway, which might be involved in periodontal immune defense, was detected in hGFs, and CYP27B1 was demonstrated to be the key factor in the pathway. Our results suggested that CYP27B1 might be important in periodontal immune defense (Gao et al., 2018). However, the *in vivo* expression of CYP27B1 in hGFs has not been reported.

Although CYP27B1 expressed in hGFs *in vitro* was the same as that in kidney, its regulation was different: periodontitis-related inflammatory stimuli interleukin-1β (IL-1β), sodium butyrate and *Porphyromonas gingivalis* lipopolysaccharide (*Pg*-LPS) induced significant up-regulation of CYP27B1, while regulators of 1α-hydroxylase in kidney (parathyroid hormone, calcium and

1,25OH_2D_3) did not significantly influence the expression of CYP27B1 in hGFs *in vitro*. However, the actual situation *in vivo* is much more complicated than that simulated *in vitro* (Gao et al., 2018; Liu et al., 2012b). Previously, our group reported that IL-1β and butyric acid, which are both up-regulators of CYP27B1, could be detected in the gingival crevicular fluids of patients with periodontitis, and the concentrations were positively correlated with periodontal inflammation (Liu et al., 2010; Lu et al., 2014). Thus, based on our previous data, it might be hypothesized that CYP27B1 was expressed in hGFs *in vivo* and patients with periodontitis might have stronger expression. The aim of this study was to test the above hypothesis and to elucidate the features of CYP27B1 expression in hGFs *in vivo*. In addition, the expression of CYP27B1 in other cell types (such as gingival epithelial cells and endothelial cells) was also analyzed.

Materials & Methods

Tissue sampling

The institutional review board of Peking University School and Hospital of Stomatology approved the study protocol (PKUSSIRB-2011007). Written informed consent was obtained from each participant in accordance with the Declaration of Helsinki.

Forty-two patients with periodontitis Stage IV Grade C and 33 healthy controls were enrolled from the clinic of the Periodontology Department, Peking University School and Hospital of Stomatology. On the basis of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions (Tonetti et al., 2018; Lang & Bartold, 2018; Papapanou et al., 2018), diagnosis was made for each individual after complete periodontal examination. The actual inclusion criteria were as follows. Periodontitis: at least eight teeth with probing depth (PD) ≥ 7 mm and evidence of alveolar bone loss on radiographs; at least four teeth with mobility II or III; at least one hopeless tooth with mobility III and alveolar bone resorption close to the root apex, needing to be extracted. Healthy controls: no site with attachment loss (AL); no site with PD > 3 mm after supragingival scaling; no radiographic evidence of alveolar bone loss; less than 10% of sites with bleeding on probing (BOP); at least one tooth needing crown-lengthening

surgery. Any subjects with systemic diseases or pregnancy were excluded. All the 75 subjects enrolled were non-smokers.

PD of all the enrolled subjects' teeth and AL of each hopeless tooth or each control tooth needing crown-lengthening surgery were recorded at six sites (mesial, distal, and middle sites of facial and lingual sides). Bleeding index (BI) (Mazza et al., 1981) was also recorded for each tooth of each individual. The mean PD, AL and BI were calculated for each analyzed tooth. The percentage of surfaces (facial and lingual) with BOP was also calculated and recorded as BOP%.

All unsalvageable teeth were extracted before periodontal treatment. After extraction of the unsalvageable teeth, part of the wall of the periodontal pocket was resected and harvested. The gingiva resected during the crown-lengthening surgery of the controls was also collected. Gingival samples from 17 subjects of the diseased group and twelve subjects of the control group were divided into part 1 and part 2. Gingival connective and epithelial tissues were obtained from part 1 using sharp tissue scissors, and then were stored in RNAwait (Solarbio Science & Technology Co., Beijing, China) at -80°C until RNA extraction. Part 2 and the other subjects' gingival samples were dehydrated and embedded in paraffin and serial sections were cut with the microtome set at 5 μm. One section of each sample was examined after staining with hematoxylin and eosin (H&E).

Detection of CYP27B1 expression

RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, US) and was reverse transcribed to cDNA using a reverse transcription kit (TOYOBO Life Science (Shanghai), Shanghai, China). Real-time PCR reactions were performed using Faststart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) in a real-time Thermocycler (Applied Biosystems, Foster City, CA, US). β-actin was used as an internal control (Forward primer: 5'-GCCGTGGTGGTGAAGCTGT-3' and reverse primer: 5'-ACCCACACTGTGCCCATCTA-3'). The forward primer for detection of CYP27B1 was 5'-ACGGTGTCCAACACGCTCT-3' and the reverse primer was 5'-AACAGTGGCTGAGGGGTAGG-3'. Data are presented as relative mRNA levels calculated by the equation $2^{-\Delta Ct}$ (ΔCt = Ct of target gene minus Ct of β-actin)

(Livak & Schmittgen, 2001).

Immunohistochemistry

Immunohistochemistry was performed according to previously-described methods (Li et al., 2017). Briefly, selected sections were transferred onto adhesive slides (Zhongshan Golden Bridge Biotechnology Co., Beijing, China), deparaffinized with xylene and rehydrated with descending concentrations of ethanol, then digested with 1 g/L trypsin at 37°C for 10 min for antigen retrieval. Endogenous peroxidase blocking was achieved by treatment with 3% H_2O_2 for 10 min at room temperature, then sections were incubated with primary sheep polyclonal antibody against CYP27B1 (1:100; The Binding Site Ltd., Birmingham, UK) at 4°C for 12 h. This was followed by incubation with an anti-sheep secondary antibody (1:500; EarthOx Life Sciences, San Francisco, CA, US) at 37°C for 30 min. The PV-9000 Polymer Detection System and a diaminobenzidine (DAB) kit (both from Zhongshan Golden Bridge Biotechnology Co., Beijing, China) were used for immunohistochemical staining of CYP27B1. The DAB staining time was 150 s for each section. Finally, sections were counterstained with hematoxylin.

Immunofluorescence

After deparaffinization of the sections, antigen retrieval was accomplished by boiling in citric acid–sodium citrate buffer (0.01 M, pH 6.0) for 15 min, and endogenous peroxidase blocking was performed using the same method as described earlier. Then sections were incubated with a working solution of primary rabbit anti-vimentin monoclonal antibody (ZA-0511; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) at 4°C for 12 h. Next, sections were incubated with an anti-rabbit secondary antibody (ZF-0511, diluted 1:400; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) at 37°C for 1 h. Then sections were incubated with primary mouse monoclonal antibody against CYP27B1 (sc-515903, diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, US) at 4°C for another 12 h. The anti-mouse secondary antibody (sc-516141, diluted 1:50; Santa Cruz Biotechnology, CA, US) was added and incubated at 37°C for 1 h, then nuclei were counterstained with DAPI (Neobioscience Biological Technology Co., Shenzhen, China), and sections were observed using immunofluorescence microscopy (Nikon, Tokyo, Japan).

158 **Image analysis**

159 Image evaluation of the immunohistochemical results was performed by two experienced
160 pathologists, who were unaware to which group the histological sections belonged. The
161 CYP27B1 staining of each sample was rated as one of the following four grades: negative (-),
162 weak (+), moderate (++) or strong (+++), translated as 0, 1, 2 and 3 for statistical analysis,
163 respectively. The staining intensity of each sample was the consensus of the opinions of the two
164 pathologists.

165 Each pathologist chose five non-overlapping 400× microscope fields of each section for
166 evaluation of hGFs. The total number of hGFs and the number of immunohistochemically
167 CYP27B1-positive ones in each chosen microscope field were recorded and staining of gingival
168 fibroblasts were rated as negative (-), weak (+), moderate (++) or strong (+++). The percentage
169 of +, ++ or +++ hGFs was calculated and CYP27B1 staining score was calculated using the
170 following formula: CYP27B1 staining score = (percentage of +++ cells) × 3 + (percentage of ++
171 cells) × 2 + (percentage of + cells). The method for calculating CYP27B1 staining score was
172 previously reported by Yoon *et al.* (Yoon et al., 2010) and a higher score indicated stronger
173 staining intensity. Cell counting and rating were performed by the two pathologists using a
174 multi-person sharing microscope (Olympus, Tokyo, Japan) at the same time. They calculated the
175 average CYP27B1 staining score of each section, and the mean of the two average CYP27B1
176 staining scores for each section was used for analysis.

177 Since immunofluorescence was usually used for qualitative analysis, the results of
178 immunofluorescence staining were only used for the colocalization of vimentin and CYP27B1 in
179 hGFs.

180 **Statistical methods**

181 The Mann-Whitney U Test was used to compare AL, BI, BOP% and the staining grades of the
182 two groups since normal distribution was not assumed. All the other comparisons between the
183 two groups were carried out using Independent-samples T Test. Paired-samples T Test was used
184 to compare mRNA expressions of CYP27B1 in gingival connective tissues and epithelia.

185 Statistical analyses were carried out using the SPSS 11.5 software package (SPSS Inc., Chicago,
186 IL, US). A P value < 0.05 was considered statistically significant.
187 All the parameters were used to calculate power values using PASS 2008 (NCSS Inc.,
188 Kaysville, UT, US) and each power value was over 0.99.
189
190 **Results**
191 The characteristics of the two groups are shown in Table 1. Significantly higher PD and AL were
192 observed in the periodontitis group than in the control group. BI and BOP% of all teeth analyzed
193 in the periodontitis group were 4 and 100%, respectively. BI of the teeth analyzed in the control
194 group was 0 or 1, so none had BOP.
195 The mRNA expressions of CYP27B1 in gingival connective tissues of patients with
196 periodontitis were significantly higher than those in the gingival connective tissues of controls
197 (Fig. 1A). In contrast, there was no significant difference in CYP27B1 mRNA expression
198 between the gingival epithelia of the two groups (Fig. 1B). As shown in Fig. 2, the mRNA
199 expressions of CYP27B1 in gingival epithelia were significantly lower than those in gingival
200 connective tissues.
201 The gingiva of one patient with periodontitis (Fig. 3A–C) and one control (Fig. 3D–F) are
202 shown. Negative controls are shown in Fig. 3 (G–I). The black frame indicates the epithelial
203 tissue, and the blue frame indicates the connective tissue. As shown in the figure, the gingival
204 connective tissues were CYP27B1 positive, and the expression of CYP27B1 was also detected in
205 gingival epithelia. In the periodontitis group, the expression of CYP27B1 was detected in all
206 epithelial layers, but expression was stronger in the superficial layer than in the deep layer of the
207 epithelia in the control group. As shown in Fig. 4(A–F), the expressions of CYP27B1 and
208 vimentin were colocalized, indicating that in gingival connective tissues, the cells positive for
209 CYP27B1 expression were hGFs. Statistical analysis indicated that CYP27B1 staining intensities
210 of the gingiva of patients with periodontitis [3.00, (3.00 to 3.00)] were significantly higher than
211 those of the controls [1.00, (1.00 to 2.00)] (Fig. 5A). In all the 40× microscopic fields chosen for

212 analysis, almost 100% of the hGFs were CYP27B1 positive and the CYP27B1 staining scores of
213 hGFs of patients with periodontitis (2.49 ± 0.08) were significantly higher than those of controls
214 (1.84 ± 0.12) (Fig. 5B).
215
216 **Discussion**
217 Our previous experiments *in vitro* verified that CYP27B1 is expressed in hGFs and that
218 expression is up-regulated by the inflammatory stimuli, IL-1β and sodium butyrate (Liu et al.,
219 2012b). In the present study, we demonstrated the expression of CYP27B1 in gingival
220 connective tissues *in vivo*. Since there are several types of cells in gingival connective tissues,
221 immunofluorescence experiments were performed and demonstrated that CYP27B1 colocalized
222 with vimentin, a marker of fibroblasts, indicating that the CYP27B1-positive cells in gingival
223 connective tissues were hGFs. However, it should be pointed out that endothelial cells also
224 express vimentin (Piera-Velazquez & Jimenez, 2019), as shown in Fig. 4. Additionally, as shown
225 in Fig. 3 and Fig. 4, endothelial cells were also found to be CYP27B1 positive, which was in line
226 with the results of Zehnder *et al.* (Zehnder et al., 2002). Because hGFs and endothelial cells can
227 easily be distinguished by pathologists, our analysis of hGFs was not influenced by endothelial
228 cells. Although the actual situation *in vivo* is much more complex than that simulated in the
229 laboratory, our observations that patients with periodontitis had higher mRNA expression of
230 CYP27B1 and higher CYP27B1 staining scores were in line with our results *in vitro*. Therefore,
231 our hypothesis that "CYP27B1 is expressed by hGFs *in vivo* and the expression might be
232 positively associated with periodontal inflammation" was verified.
233 Although we focused on hGFs, epithelial tissues were also observed in the present study, and
234 gingival epithelia were also found to be CYP27B1 positive in both groups, which was in line
235 with the findings of other researchers (McMahon et al., 2011; Menzel et al., 2019). What should
236 be pointed out is that the distribution of CYP27B1 expression differed between the two groups
237 (Fig. 3). When obtaining the gingival epithelial tissues for analysis of mRNA expression, it was
238 impossible to obtain the entire epithelium clinically. Therefore, only the superficial layer was

obtained to avoid contamination of connective tissues. Since CYP27B1 expression was relatively strong in the superficial layers in both groups, the lack of a significant difference in CYP27B1 expression between the epithelia of the two groups could be explained.

It has been demonstrated that a vitamin D pathway exists in hGFs (Gao et al., 2018) and gingival epithelial cells (McMahon et al., 2011; Menzel et al., 2019): $25OHD_3$ induces cathelicidin LL-37 generation. The pathway might be involved in periodontal immune defense for the following reasons. (1) In hGFs, the pathway could be activated by periodontal inflammatory stimulus *Pg*-LPS, and the pathway could suppress the expression of some inflammatory chemokines such as IL-8 and MCP-1 (Gao et al., 2018), directly indicating that the pathway might play a role in immune defense in periodontal soft tissues. (2) $25OHD_3$ is an important part of the vitamin D pathway, and higher $25OHD_3$ concentrations were detected in both the gingival crevicular fluids and the plasma of aggressive periodontitis patients compared to those of healthy controls (Liu et al., 2010; Liu et al., 2009). Moreover, after the periodontal inflammation was reduced by initial periodontal therapy, the $25OHD_3$ levels in gingival crevicular fluids and plasma significantly dropped, indicating that activity of the vitamin D pathway might be positively associated with periodontal inflammation. As the key factor in the pathway (Gao et al., 2018), CYP27B1 is worthy of further research. In the present study, the finding that the *in vivo* gingival CYP27B1 expression was higher in the periodontitis group than in the control group could provide new evidence of the involvement of the vitamin D pathway in periodontal immune defense. Thus, the present study is of biological significance, although more mechanisms via which the vitamin D pathway impacts gingival health in periodontitis still need to be elucidated.

Reasons for the higher expression of CYP27B1 in the periodontitis group might be as follows: (1) *Porphyromonas gingivalis*, the representative bacterium causing periodontitis (Potempa et al., 2017; Shibata, 2018), was abundant in subgingival plaque from Chinese patients with severe periodontitis (Li et al., 2015) and *Pg*-LPS was an up-regulator of CYP27B1 in hGFs (Gao et al., 2018); (2) Periodontal inflammation could result in the higher concentrations of IL-1β and

butyric acid in gingival crevicular fluids (Liu et al., 2010; Lu et al., 2014), which could also induce the expression of CYP27B1 in hGFs (Liu et al., 2012b).

In the present study, subjects matched by age and gender were included in the two groups and all were non-smokers, in order that the influences of potential confounding factors could be minimized. In order to analyze the typical inflammatory situation *in vivo*, all the patients enrolled were diagnosed with periodontitis Stage IV Grade C, the most severe periodontitis in the new classification scheme for periodontal diseases (Tonetti et al., 2018; Papapanou et al., 2018). In addition, all gingival tissues of patients with periodontitis were obtained around unsalvageable teeth, which had not received any periodontal therapy so that periodontal inflammation was serious enough and was not influenced by periodontal treatments. The PD and AL of the unsalvageable teeth analyzed were high and BOP was positive at all surfaces of the teeth. In contrast, all the teeth analyzed in the control group had PD less than 3 mm and had no AL or BOP, indicating that these teeth were clinically healthy. It should be pointed out that all the teeth analyzed in the control group needed crown-lengthening surgery because of excessive gingival display or subgingival location of fracture lines or carious lesions. When parts of the teeth were subgingival, accumulation of dental plaque was often detected. Thus, the BI of some teeth in the control group was 1 and mild inflammation of the gingiva could be detected. Similarly, it was reported that “healthy” gingiva might also harbor inflammatory cellular infiltrates, indicating that subclinical gingivitis might exist (Lang & Bartold, 2018). Thus, CYP27B1 staining intensities of two of the 33 teeth in the control group were strong (+++) and the mild inflammation of the gingiva might be the reason for high expression of CYP27B1 in the control group.

Immunohistochemistry is of course a highly subjective method. We tried to objectively evaluate CYP27B1 expression in gingiva *in vivo* by letting two experienced pathologists perform the evaluation in a blinded manner. However, the subjectivity of the evaluation was inevitable, which is a limitation of the present study.

Conclusions

In summary, CYP27B1 expression was detected in hGFs *in vivo*, and this expression might be induced by periodontal inflammation. These results validated our previous *in vitro* findings, and indicated the potential involvement of the vitamin D pathway in periodontal immune defense.

References

Alsalem, J.A., Patel, D., Susarla, R., Coca-Prados, M., Bland, R., Walker, E.A., Rauz, S., and Wallace, G.R. 2014. Characterization of vitamin D production by human ocular barrier cells. *Investigative Ophthalmology & Visual Science* 55:2140-2147. DOI 10.1167/iovs.13-13019

Bikle, D., and Christakos, S. 2020. New aspects of vitamin D metabolism and action - addressing the skin as source and target. *Nature Reviews. Endocrinology* 16:234-252. DOI 10.1038/s41574-019-0312-5

Capiod, T., Barry, D.N., Pigat, N., Souberbielle, J.C., and Goffin, V. 2018. Do dietary calcium and vitamin D matter in men with prostate cancer? *Nature Reviews. Urology* 15:453-461. DOI 10.1038/s41585-018-0015-z

Gao, Z., Liu, K., and Meng, H. 2018. Preliminary investigation of the vitamin D pathway in periodontal connective tissue cells. *Journal of Periodontology* 89:294-302. DOI 10.1002/JPER.17-0530

Lang, N.P., and Bartold, P.M. 2018. Periodontal health. *Journal of Clinical Periodontology* 45 Suppl 20:S9-S16. DOI 10.1111/jcpe.12936

Li, W., Zhu, W., Hou, J., and Meng, H. 2017. Vitamin D-binding protein expression in healthy tooth and periodontium: an experimental study both in monkeys in vivo and in humans in vitro. *Journal of Periodontal Research* 52:755-760. DOI 10.1111/jre.12445

Li, Y., Feng, X., Xu, L., Zhang, L., Lu, R., Shi, D., Wang, X., Chen, F., Li, J., and Meng, H. 2015. Oral microbiome in chinese patients with aggressive periodontitis and their family members. *Journal of Clinical Periodontology* 42:1015-1023. DOI 10.1111/jcpe.12463

Liu, K., Meng, H., Lu, R., Xu, L., Zhang, L., Chen, Z., Shi, D., Feng, X., and Tang, X. 2010. Initial periodontal therapy reduced systemic and local 25-hydroxy vitamin D(3) and

320 interleukin-1beta in patients with aggressive periodontitis. *Journal of Periodontology* 81:260-
321 266. DOI 10.1902/jop.2009.090355
322 Liu, K., Meng, H., Tang, X., Xu, L., Zhang, L., Chen, Z., Shi, D., Feng, X., and Lu, R. 2009.
323 Elevated plasma calcifediol is associated with aggressive periodontitis. *Journal of*
324 *Periodontology* 80:1114-1120. DOI 10.1902/jop.2009.080675
325 Liu, K., Meng, H., and Hou, J. 2012a. Activity of 25-hydroxylase in human gingival fibroblasts
326 and periodontal ligament cells. *PLoS One* 7:e52053. DOI 10.1371/journal.pone.0052053
327 Liu, K., Meng, H., and Hou, J. 2012b. Characterization of the autocrine/paracrine function of
328 vitamin d in human gingival fibroblasts and periodontal ligament cells. *PLoS One* 7:e39878.
329 DOI 10.1371/journal.pone.0039878
330 Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time
331 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408. DOI
332 10.1006/meth.2001.1262
333 Lu, R., Meng, H., Gao, X., Xu, L., and Feng, X. 2014. Effect of non-surgical periodontal
334 treatment on short chain fatty acid levels in gingival crevicular fluid of patients with
335 generalized aggressive periodontitis. *Journal of Periodontal Research* 49:574-583. DOI
336 10.1111/jre.12137
337 Markiewicz, A., Brozyna, A.A., Podgorska, E., Elas, M., Urbanska, K., Jetten, A.M., Slominski,
338 A.T., Jozwicki, W., Orlowska-Heitzman, J., Dyduch, G., and Romanowska-Dixon, B. 2019.
339 Vitamin D receptors (VDR), hydroxylases CYP27B1 and CYP24A1 and retinoid-related
340 orphan receptors (ROR) level in human uveal tract and ocular melanoma with different
341 melanization levels. *Scientific Reports* 9:9142. DOI 10.1038/s41598-019-45161-8
342 Mazza, J.E., Newman, M.G., and Sims, T.N. 1981. Clinical and antimicrobial effect of stannous
343 fluoride on periodontitis. *Journal of Clinical Periodontology* 8:203-212. DOI 10.1111/j.1600-
344 051x.1981.tb02031.x
345 McMahon, L., Schwartz, K., Yilmaz, O., Brown, E., Ryan, L.K., and Diamond, G. 2011.
346 Vitamin D-mediated induction of innate immunity in gingival epithelial cells. *Infection and*

Immunity 79:2250-2256. DOI 10.1128/IAI.00099-11

Medrano, M., Carrillo-Cruz, E., Montero, I., and Perez-Simon, J.A. 2018. Vitamin D: Effect on haematopoiesis and immune system and clinical applications. *International Journal of Molecular Sciences* 19:E2663. DOI 10.3390/ijms19092663

Menzel, L.P., Ruddick, W., Chowdhury, M.H., Brice, D.C., Clance, R., Porcelli, E., Ryan, L.K., Lee, J., Yilmaz, O., Kirkwood, K.L., McMahon, L., Tran, A., and Diamond, G. 2019. Activation of vitamin D in the gingival epithelium and its role in gingival inflammation and alveolar bone loss. *Journal of Periodontal Research* 54:444-452. DOI 10.1111/jre.12646

Nykjaer, A., Dragun, D., Walther, D., Vorum, H., Jacobsen, C., Herz, J., Melsen, F., Christensen, E.I., and Willnow, T.E. 1999. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* 96:507-515. DOI 10.1016/s0092-8674(00)80655-8

Papapanou, P.N., Sanz, M., Buduneli, N., Dietrich, T., Feres, M., Fine, D.H., Flemmig, T.F., Garcia, R., Giannobile, W.V., Graziani, F., Greenwell, H., Herrera, D., Kao, R.T., Kebschull, M., Kinane, D.F., Kirkwood, K.L., Kocher, T., Kornman, K.S., Kumar, P.S., Loos, B.G., Machtei, E., Meng, H., Mombelli, A., Needleman, I., Offenbacher, S., Seymour, G.J., Teles, R., and Tonetti, M.S. 2018. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *Journal of Clinical Periodontology* 45 Suppl 20:S162-S170. DOI 10.1111/jcpe.12946

Piera-Velazquez, S., and Jimenez, S.A. 2019. Endothelial to mesenchymal transition: role in physiology and in the pathogenesis of human diseases. *Physiological Reviews* 99:1281-1324. DOI 10.1152/physrev.00021.2018

Potempa, J., Mydel, P., and Koziel, J. 2017. The case for periodontitis in the pathogenesis of rheumatoid arthritis. *Nature Reviews. Rheumatology* 13:606-620. DOI 10.1038/nrrheum.2017.132

Sassi, F., Tamone, C., and D'Amelio, P. 2018. Vitamin D: Nutrient, hormone, and immunomodulator. *Nutrients* 10:E1656. DOI 10.3390/nu10111656

Shibata, K. 2018. Historical aspects of studies on roles of the inflammasome in the pathogenesis

of periodontal diseases. *Molecular Oral Microbiology* 33:203-211. DOI 10.1111/omi.12217

Somjen, D., Weisman, Y., Kohen, F., Gayer, B., Limor, R., Sharon, O., Jaccard, N., Knoll, E., and Stern, N. 2005. 25-hydroxyvitamin D3-1alpha-hydroxylase is expressed in human vascular smooth muscle cells and is upregulated by parathyroid hormone and estrogenic compounds. *Circulation* 111:1666-1671. DOI 10.1161/01.CIR.0000160353.27927.70

Tonetti, M.S., Greenwell, H., and Kornman, K.S. 2018. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *Journal of Clinical Periodontology* 45 Suppl 20:S149-S161. DOI 10.1111/jcpe.12945

van Driel, M., Koedam, M., Buurman, C.J., Hewison, M., Chiba, H., Uitterlinden, A.G., Pols, H.A., and Van, L.J. 2006. Evidence for auto/paracrine actions of vitamin D in bone: 1alpha-hydroxylase expression and activity in human bone cells. *Faseb Journal* 20:2417-2419. DOI 10.1096/fj.06-6374fje

Yoon, N.K., Maresh, E.L., Shen, D., Elshimali, Y., Apple, S., Horvath, S., Mah, V., Bose, S., Chia, D., Chang, H.R., and Goodglick, L. 2010. Higher levels of GATA3 predict better survival in women with breast cancer. *Human Pathology* 41:1794-1801. DOI 10.1016/j.humpath.2010.06.010

Zehnder, D., Bland, R., Chana, R.S., Wheeler, D.C., Howie, A.J., Williams, M.C., Stewart, P.M., and Hewison, M. 2002. Synthesis of 1,25-dihydroxyvitamin D(3) by human endothelial cells is regulated by inflammatory cytokines: a novel autocrine determinant of vascular cell adhesion. *Journal of the American Society of Nephrology* 13:621-629.

Figure 1

Fig. 1 Expression of CYP27B1 mRNA in gingiva

(A) Expression of CYP27B1 mRNA in gingival connective tissues of the diseased group was significantly higher than in the control group. (B) Expression of CYP27B1 mRNA in gingival epithelia did not significantly differ between the two groups.

* Compared to the control group, $P < 0.05$.

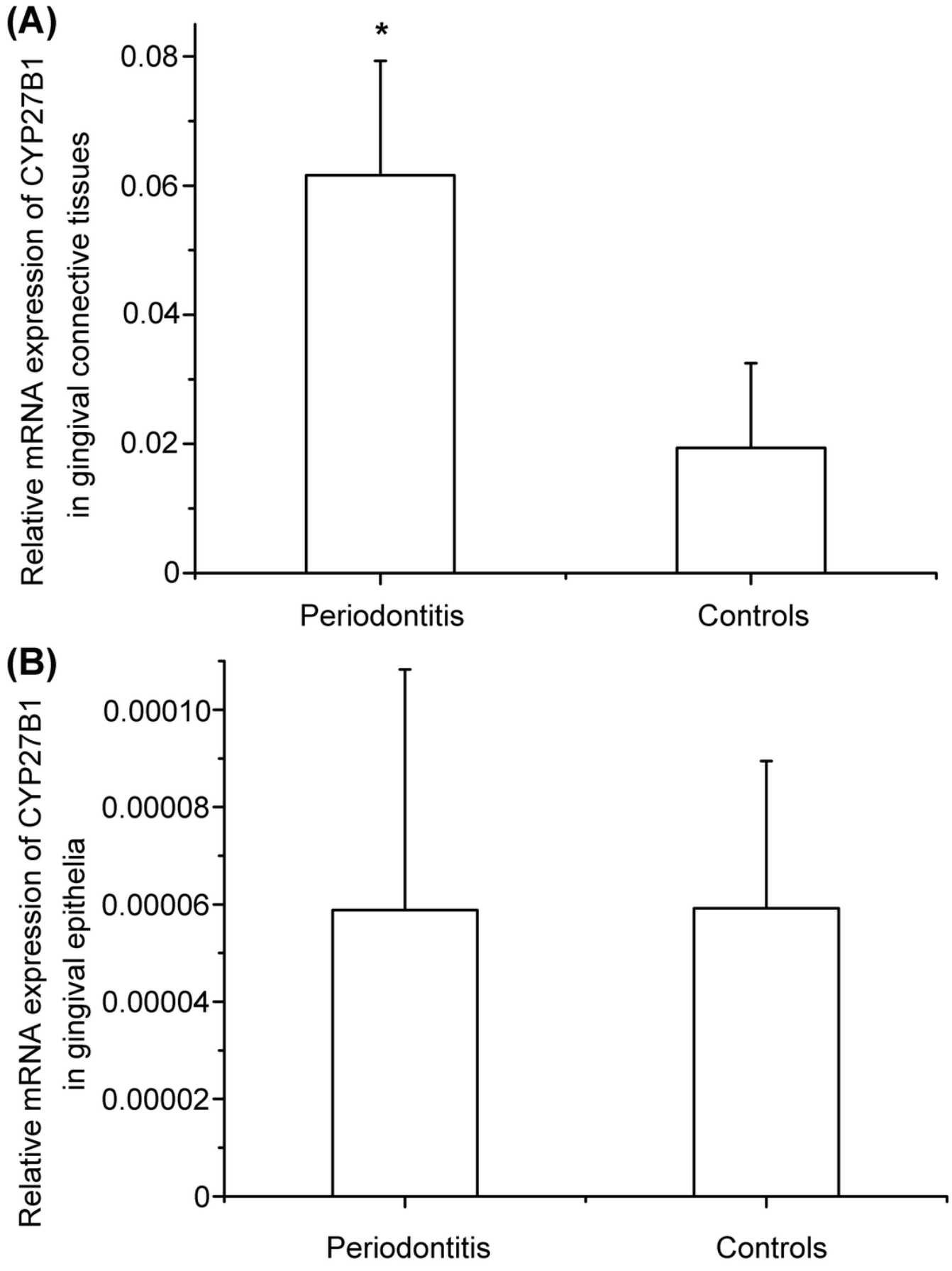

(A)
Relative mRNA expression of CYP27B1 in gingival connective tissues
0.08
0.06
0.04
0.02
0
*
Periodontitis
Controls
(B)
Relative mRNA expression of CYP27B1 in gingival epithelia
0.00010
0.00008
0.00006
0.00004
0.00002
0
Periodontitis
Controls

Figure 2

Fig.2 Comparison of CYP27B1 mRNA expressions in gingival connective tissues and epithelia

Expression of CYP27B1 mRNA in gingival connective tissues was significantly higher than in gingival epithelia.

* Compared to gingival epithelia, $P < 0.05$.

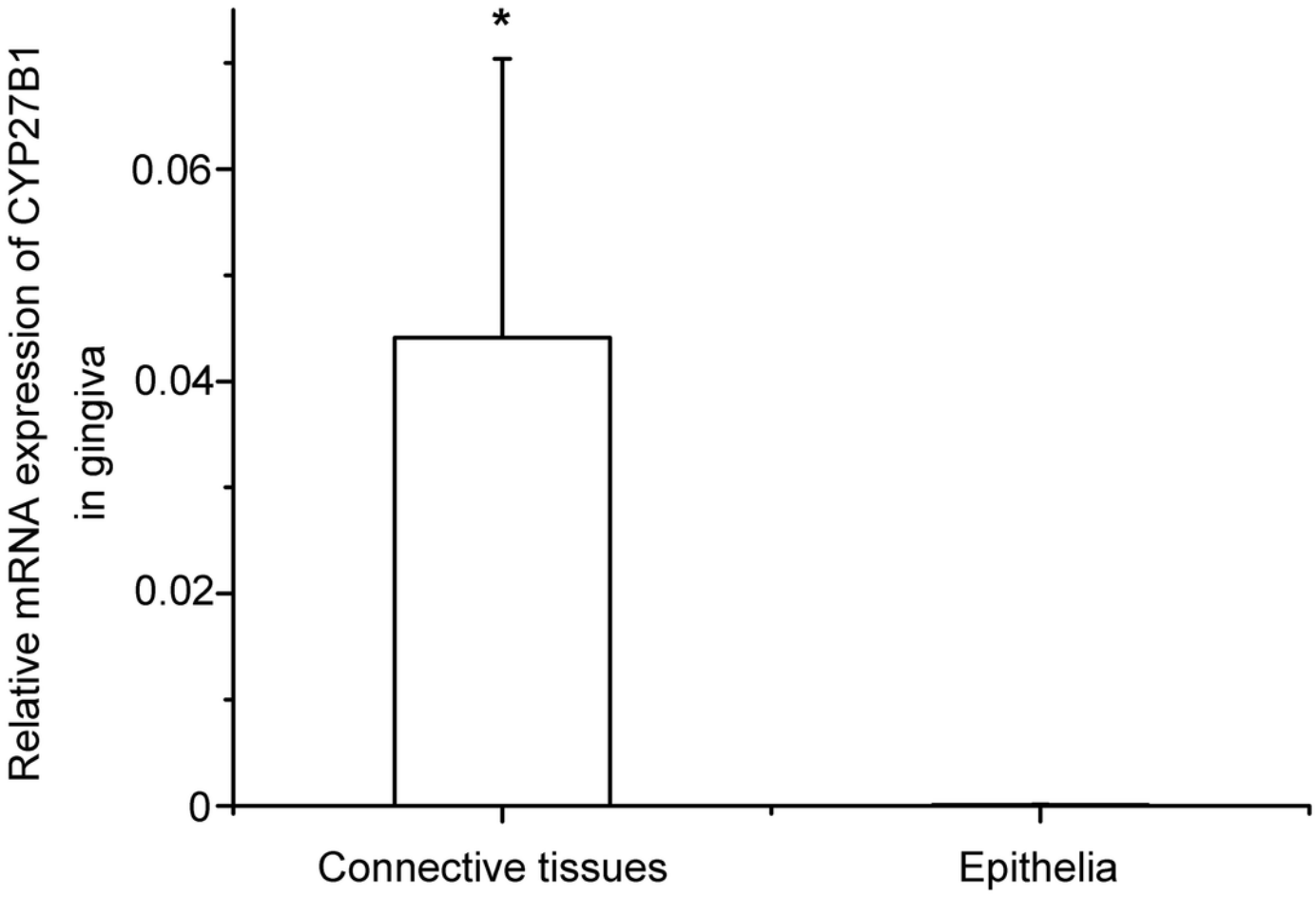


Figure 3

Fig.3 Detection of CYP27B1 in gingiva by immunohistochemistry

Panels (A)–(C) and (D)–(F) show immunohistochemical staining of CYP27B1 in the gingiva of one patient with periodontitis and one control, respectively. Panels (G)–(I) are negative controls. The black frame indicates the epithelial tissue (Panels B, E, H), while the blue frame shows the connective tissue (Panels C, F, I).

Magnification: (A), (D) and (G) 20×; all others 180×.

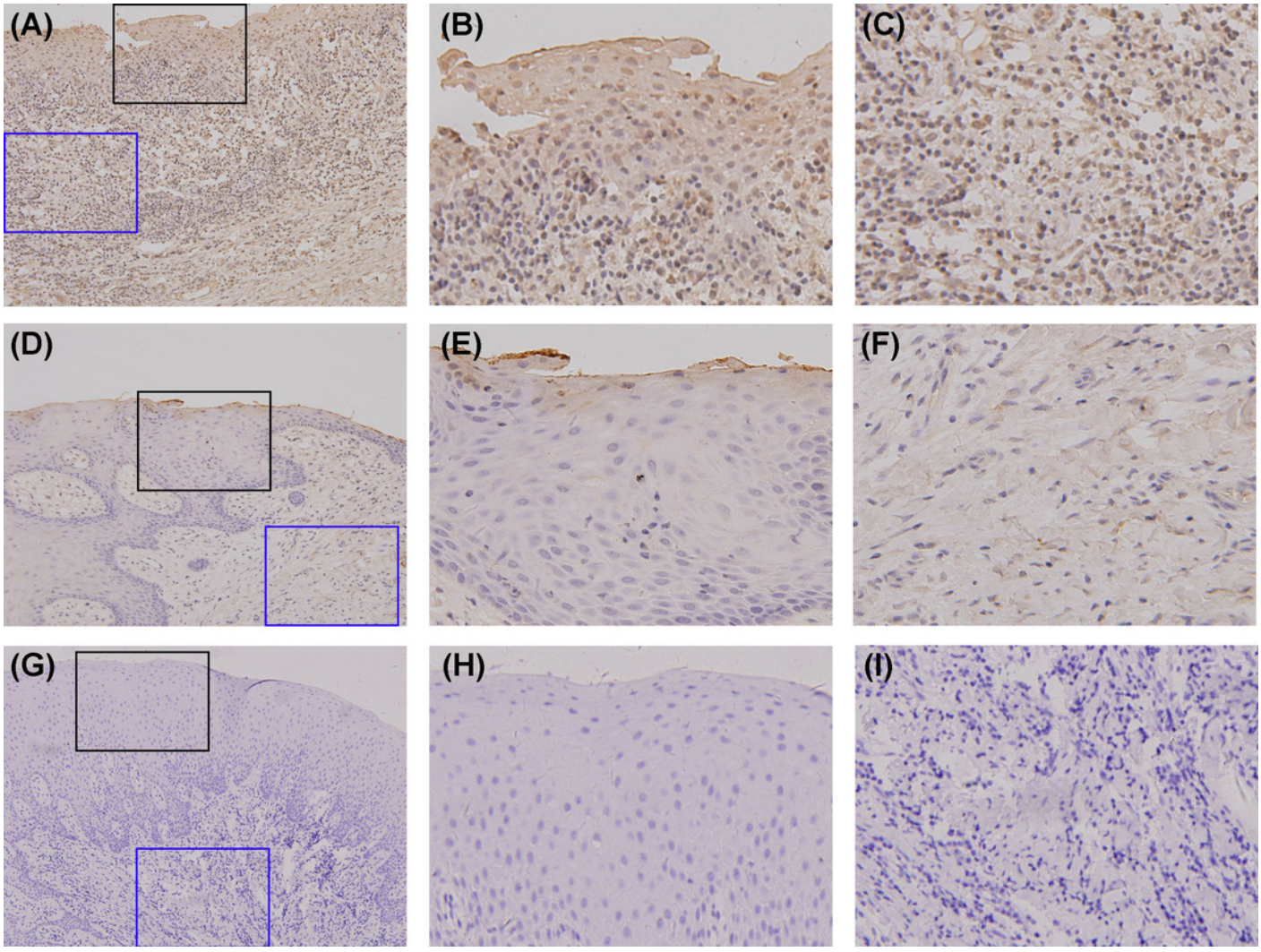


Figure 4

Fig.4 Colocalization of CYP27B1 and vimentin in hGFs

Panels (A)–(C) show immunofluorescence staining of samples from a periodontitis patient, and Panels (D)–(F) show the corresponding results from a control. Panels (A) and (D): CYP27B1; Panels (B) and (E): vimentin; Panels (C) and (F): combination.

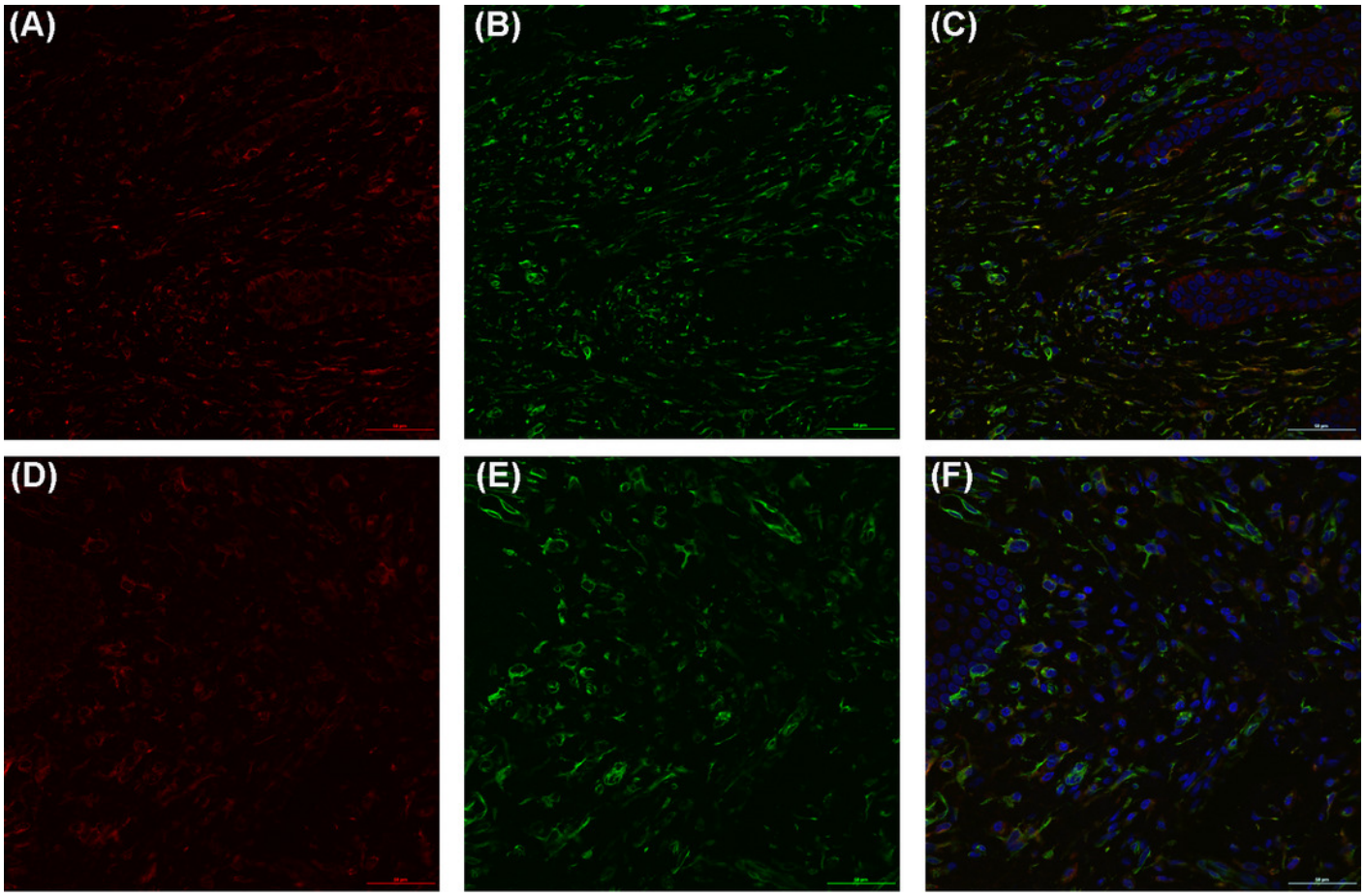
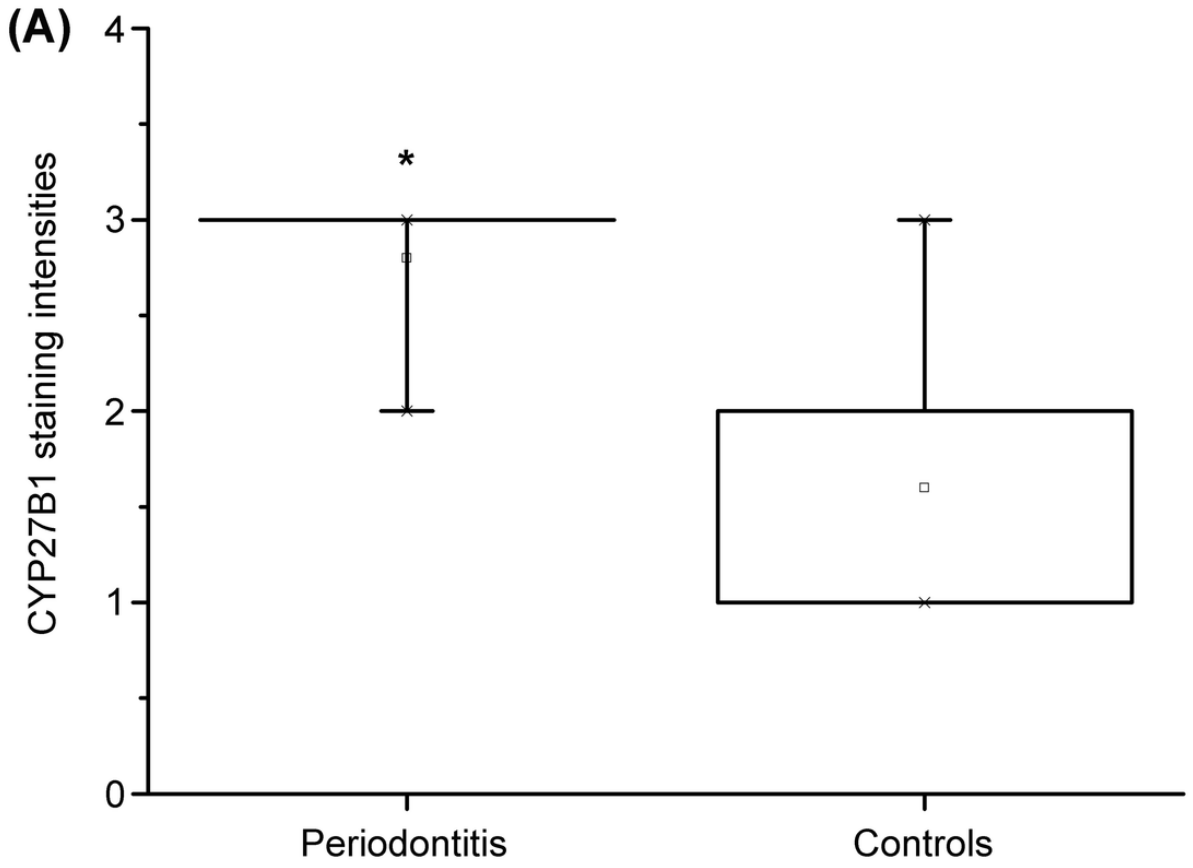


Figure 5

Fig.5 Evaluation of CYP27B1 protein expressions in gingiva

(A) CYP27B1 staining intensities of gingival connective tissues of the diseased group were significantly higher than those of the control group. (B) CYP27B1 staining scores of gingival fibroblasts of the diseased group were significantly higher than those of the control group.

* Compared to the control group, $P < 0.05$.


(A)
CYP27B1 staining intensities
4
3
2
1
0
*
Periodontitis
Controls

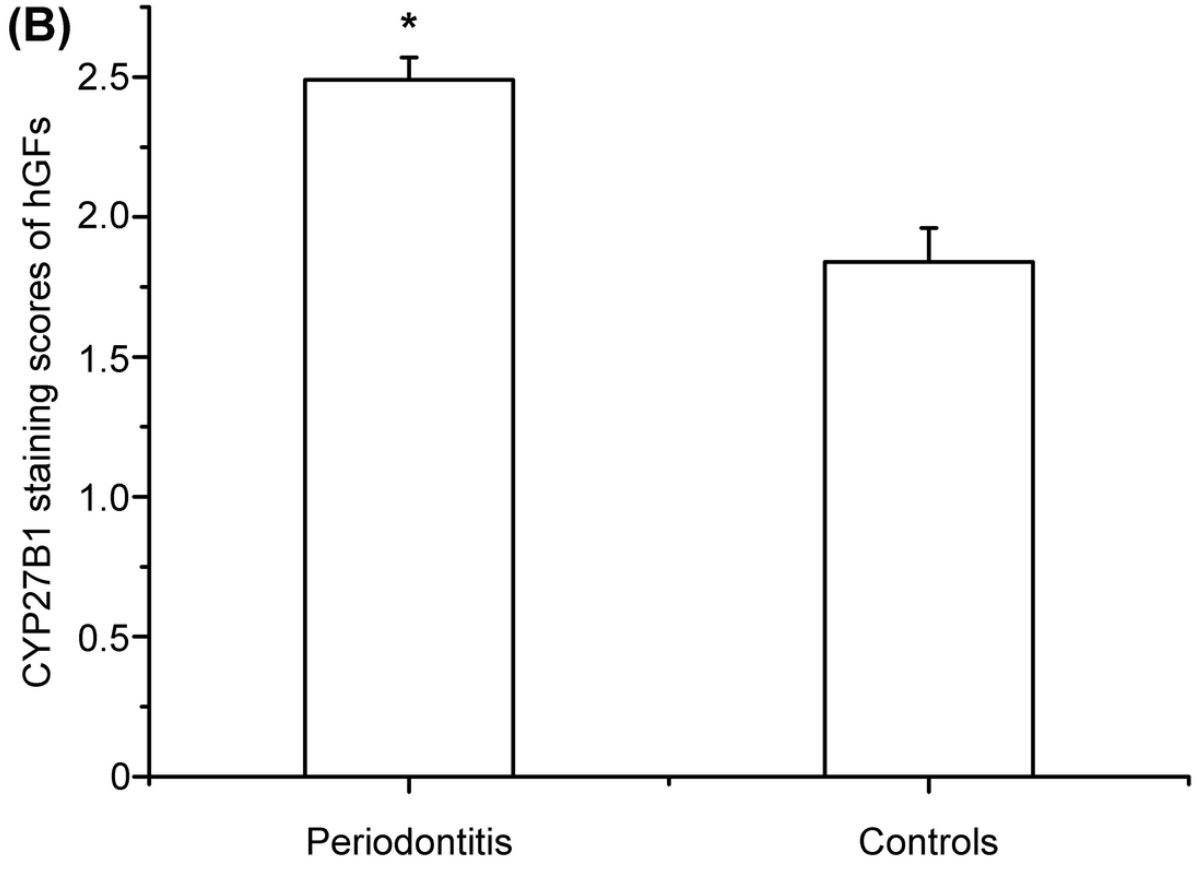

(B)
CYP27B1 staining scores of hGFs
2.5
2.0
1.5
1.0
0.5
0
*
Periodontitis
Controls

Table 1(on next page)

Table 1 Demographic data and clinical parameters of the two groups

Table 1 Demographic data and clinical parameters of the two groups

Parameters	Periodontitis (n = 42)	Controls (n = 33)
Age (years)	33.5 ± 7.8	30.0 ± 9.2
Gender (male/female)	20/22	16/17
PD (mm)	7.3 ± 0.4*	1.9 ± 0.5
AL (mm)	5.9 ± 0.6*	0
BI	4*	0 (0 to 0.5)
BOP%	100%*	0

Data are presented as mean ± SD, median (lower to upper quartile), or number of subjects, as indicated

* Compared to the control group ($P < 0.05$)