Journal of
PERIODONTAL RESEARCH

J Periodont Res 2014; 49: 405–414

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12120

Periostin increases migration and proliferation of human periodontal ligament fibroblasts challenged by tumor necrosis factor-α and *Porphyromonas gingivalis* lipopolysaccharides

M. Padial-Molina[1,2]*, S. L. Volk[1]*, H. F. Rios[1,3]

[1]Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA, [2]Department of Oral Surgery and Implant Dentistry, School of Dentistry, University of Granada, Granada, Spain and [3]Michigan Center for Oral Health Research, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

Padial-Molina M, Volk SL, Rios HF. Periostin increases migration and proliferation of human periodontal ligament fibroblasts challenged by tumor necrosis factor-α and Porphyromonas gingivalis lipopolysaccharides. J Periodont Res 2014; 49: 405–414. © 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Background: In the chronic established periodontal lesion, the proliferation and migration potential of periodontal ligament (PDL) cells are significantly compromised. Thus, the progressive loss of tissue integrity is favored and normal healing and regeneration compromised. Periostin, a known PDL marker, modulates cell–matrix interactions, cell behavior, as well as the matrix biomechanics and PDL homeostasis.

Objective: To evaluate whether periostin restores the regenerative potential of PDL cells in terms of proliferation, migration, and activation of survival signaling pathways after being challenged by *Porphyromonas gingivalis* lipopolysaccharides and tumor necrosis factor alpha α.

Methods: Human PDL (hPDL) cells were cultured under different conditions: control, periostin (50 or 100 ng/mL), and fibroblast growth factor 2 (10 ng/mL) to evaluate cell proliferation (by Ki67), cell migration (by scratch assays) and PI3K/AKT/mTOR pathway activation (by western blot analyses of total AKT, phospho-AKT and PS6). A different set of cultures was challenged by adding tumor necrosis factor alpha α (10 ng/mL) and *P. gingivalis* lipopolysaccharides (200 ng/mL) to evaluate the effects of periostin as described above.

Results: Periostin significantly increased cell proliferation (twofold), migration (especially at earlier time points and low dose) and activation of survival signaling pathway (higher phosphorylation of AKT and PS6). Furthermore, periostin

Hector F. Rios, DDS, PhD, School of Dentistry, University of Michigan, 1011 North University Ave, Oce 3349, Ann Arbor, MI, 48109-1078, USA
Tel: +734 647 4326
Fax: +734 763 5503
e-mail address: hrios@umich.edu
*These authors contributed equally to this work.

Key words: cell migration; cell proliferation; periodontal disease; periodontal ligament; periostin

Accepted for publication June 17, 2013

promoted similar cellular effects even after being challenged with proinflammatory cytokines and bacterial virulence factors.

Conclusion: Periostin acts as an important modulator of hPDL cell–matrix dynamics. It modulates hPDL proliferation, migration and PI3K/AKT/mTOR pathway. It also helps in overcoming the altered biological phenotype that chronic exposure to periodontal pathogens and proinflammatory cytokines produce in hPDL cells.

The tooth-supporting apparatus (i.e. periodontium) includes the alveolar bone, periodontal ligament (PDL), cementum and gingiva. The periodontium is a highly specialized adaptive dynamic tissue able to sustain different microbiological, inflammatory and mechanical challenges through a number of complex molecular events involving growth factors, transcription factors and extracellular matrix (ECM) proteins (1,2).

In humans, microbially induced inflammatory periodontal diseases are the primary initiators that disrupt the functional and structural integrity of the periodontium. Different forms of periodontal diseases virtually affect the entire population (3,4), which constitutes a true public health concern. Moreover, once the periodontal breakdown occurs, the ideal restoration (i.e. regeneration) of its original structure, properties and function is a significant challenge in the clinical setting (5). Efforts have been made on regenerating lost alveolar bone. However, regeneration of the lost periodontium involves the formation of all tooth-supporting structures in a temporal and spatial progress (6). Although the exact cellular and molecular events are still not clear, specific cells must first migrate to the healing area and proliferate. This process is mediated and coordinated by soluble factors, other cells, ECM and matricellular proteins. Normal mechanical stimuli will increase and promote an organized ECM synthesis and organization as well as cementum and bone formation and maturation. Once those structures are established, PDL fibers are organized and oriented, connecting the tooth to the alveolar bone (7).

In this context, matricellular molecules within the PDL provide signals to the constituent cell populations to modulate their phenotype to maintain tissue homeostasis. Periostin (gene *POSTN*), a secreted adhesion molecule highly specific to the PDL, mainly secreted by fibroblasts (8), is essential for the periodontal tissue integrity and homeostasis and plays an important role in tooth development and eruption processes (2,9,10). Periostin is induced by biomechanical stimuli via transforming growth-ß1 signaling (2) and regulates collagen fibrillogenesis and tissue strength (11,12). However, it is reduced by the effect of inflammatory cytokines and bacterial virulence factors (13). Levels of periostin are inversely correlated to the extent of alveolar bone loss (14). Periostin has been suggested to modulate the connective tissue repair and remodeling in cardiac tissue (15) and skin (16) by the activation of the cell survival AKT/PKB pathway (17). It has also shown an increase in smooth muscle cell migration via αvβ3 and αvβ5-integrins and phosphorylation of FAK (18). However, the role of periostin in periodontal regeneration and PDL cells has still not been described.

Therefore, the aim of this study was to analyze the effects of recombinant periostin supplementation on the regenerative potential of human PDL (hPDL) cells in terms of cell proliferation and migration capacities as well as the activation of the PI3K/AKT/mTOR survival signaling pathway. The ability of periostin to recover cell activity after being challenged by proinflammatory cytokines and bacterial virulence factors was also analyzed.

Material and methods

Cell populations

Primary hPDL cells were characterized and used as previously reported (13). The use of human cells was approved by the University of Michigan Institutional Review Board. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), antifungal (1 : 1000 amphotericin B) and 2 mM glutamine, at 37°C in a humidified atmosphere of 95% air/5% CO_2. Cells were removed from the growth surface with a trypsin solution [0.25% trypsin, 0.1% glucose, citrate-saline buffer (pH 7.8); Gibco] and subsequently used for experiments. Cells were used between passages 5 and 8 for experiments.

Cell culture conditions

Cells were seeded in six-well plates. At 70% confluence, media was substituted with starving media (no fetal bovine serum). After 24 h, wells were assigned to four different groups and media changed and supplemented accordingly: C (control), P100 (recombinant human periostin, 100 ng/mL; R&D Systems Inc., Minneapolis, MN, USA), P50 (periostin, 50 ng/mL), and fibroblast growth factor (FGF)-2 as positive control (recombinant human FGF-2, 10 ng/mL; Gibco).

To investigate the capacities of recombinant periostin to recover cell capacities in a challenged environment,

a similar set of cultures were subjected to chronic exposure (4 d) to TNF-α + *P. gingivalis* lipopolysaccharide (LPS) (T + L: 10 ng/mL TNF-α; Abcam Inc., Cambridge, MA, USA; 200 ng/mL *P. gingivalis* LPS), known to reduce periostin expression and extracellular deposition by hPDL cells (13). Then, cell cultures were subjected to starving media for 24 h and supplemented with periostin or FGF-2 as described above.

Three separate experiments with the different media supplementations were performed in triplicate for all primary hPDL populations for all assays.

Analysis of cell proliferation by Ki67

For Ki67 staining, cells were seeded into a 16-well Lab-Tek™ Chamber Slide™ System (Thermo Fisher Scientific, Waltham, MA, USA) at 50% confluence. After 24 h of starving, cells were subjected to the same conditions described above. Six hours later, the culture medium was removed, cells were washed twice with phosphate-buffered saline (PBS), and fixed in 100% cold methanol for 5 min. Cells were then washed again, and blocked in 3% bovine serum albumin (BSA)-PBS for 1 h. Immunofluorescence staining for Ki67 was performed using a mouse monoclonal antibody to human Ki67 overnight (1 : 100 dilution in 3% BSA-PBS; Dako North America, Inc., Carpinteria, CA, USA). The immunological reaction was visualized by using a secondary antibody to mouse conjugated with fluorescein isothiocyanate for 1 h (1 : 100 dilution in 3% BSA-PBS). Slides were then treated with an antifade agent containing DAPI (ProLong® Gold antifade reagent with DAPI; Invitrogen, Life Technologies Corporation) and covered with glass coverslips. All stained slides were imaged using an OLYMPUS Fluoview 500 confocal microscope (Olympus America Inc., Center Valley, PA, USA) using the same settings. Three images per well were captured. Three separate experiments with the different media supplementations in triplicate were performed for all primary hPDL populations. Ki67-positive cells (green) and total cell number (DAPI staining, blue) were counted to calculate the mean percentage of positive cells per group and normalized to the control.

Scratch migration assay

A scratch-simulated wound migration assay was performed to assess the effect of exogenous periostin supplementation. hPDL cells were seeded on to six well plates and allowed to grow close to 95% confluence. After 24 h of starving, a scratch was performed along the diameter of the well (19) and the scraped cells were removed by washing with PBS. Fresh culture media was added and supplemented with each one of the treatment groups as described above. After that, images from the same area were taken from 0 to 48 h after the scratch with a 6 h interval at 4×. Three separate experiments with the different media supplementations in triplicate were performed for all primary hPDL populations. The scraped area was measured by ImageJ software (National Institute of Health, USA; http://rsbweb.nih.gov/ij/). The wound area over time was measured considering the area at time 0 as 100%.

Survival pathway activation analysis

Six hours after media supplementation, supernatant was removed, cells were washed twice with PBS and collected by scraping. Cells suspended in PBS were spun at 3500 *g* for 10 min at 4°C to pellet the cells. Cell pellets were resuspended in lysis buffer (0.1 M Tris pH 6.8, 2% SDS, 1% β-mercaptoethanol, 1 : 100 protease inhibitor cocktail), vortexed for 5 min and incubated on ice for 30 min. The lysates were centrifuged again (14,000 *g* for 10 min at 4°C) and protein supernatants were collected.

Total protein concentration was quantified using the Quant-iT® Protein Assay Kit with the Qubit® Fluorometer (Invitrogen, Life Technologies Corporation). Ten μg of total protein from each solution were run on 10% SDS-PAGE gels (100 V, 2 h), electroblotted on to PVDF membranes (90 V, 90 min), blocked (5% milk in TBST pH 7.4, 1 h), and immunoprobed for phospho-S6 ribosomal protein [1 : 1000 in 5% milk, rabbit monoclonal to PS6 (Ser235/236) (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight; 1 : 4000 goat anti-rabbit IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), 1 h]. Membranes were stripped (25 mM glycine, 1% SDS, pH 2.0, for 2 h), blocked and immunoprobed for phospho-AKT [1 : 1000 in 5% milk, rabbit polyclonal to phospho-AKT (Ser473) (Cell Signaling Technology) overnight; 1 : 4000 goat antirabbit IgG-HRP (Santa Cruz Biotechnology), 1 h), stripped, blocked and immuno-probed for total-AKT (1 : 500 in 5% milk, rabbit polyclonal to pan-AKT (T308) (Abcam) overnight; 1 : 4000 goat antirabbit IgG-HRP (Santa Cruz Biotechnology), 1 h], and stripped, blocked, and immuno-probed again for GAPDH [1 : 4000 in 5% milk, goat polyclonal to GAPDH (R&D) overnight; 1 : 4000 donkey antigoat IgG-HRP (Santa Cruz Biotechnology), 1 h]. Immunopositive bands were detected by enhanced chemiluminescence (Thermo Fisher Scientific).

Western blot band signals were semi-quantified using ImageJ software (National Institute of Health). Immunopositive bands for each group were adjusted by GAPDH and the intensity fold changes normalized by assigning a relative value of 1 to the control group.

Statistical analysis

PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. All data are presented as the means ± SEM. The significance of the differences among groups was determined by using the ANOVA test ($\alpha = 0.05$). Fisher least significant difference test was used to define pairwise statistical differences ($\alpha = 0.05$).

Results

Periostin increases human periodontal ligament proliferation, migration and activates survival pathways

Under non-challenged conditions, immunofluorescence staining for Ki67 proliferation marker demonstrated that periostin 100 ng/mL (2.02 ± 0.21), 50 ng/mL (1.99 ± 0.18) and FGF-2 (1.92 ± 0.20) significantly increased hPDL cell proliferation by approximately twofold compared to control (1.00 ± 0.16) ($p < 0.004$; $p < 0.005$; $p < 0.008$, respectively) (Fig. 1; values of Ki67-positive cell percentages per group normalized to control).

hPDL cell migration analyzed by scratch assay was significantly accelerated by the addition of periostin or FGF-2 at every time point compared to the control group (Fig. 2). The highest concentration of periostin (100 ng/mL) showed a very similar effect as that achieved by the positive control FGF-2. Interestingly, the addition of 50 ng/mL of periostin showed a greater improvement, especially at earlier time points (up to 18 h), with statistically significant effects even when compared to FGF-2 ($p < 0.001$) and periostin 100 ng/mL ($p < 0.003$), as early as 6 h (Fig. 2B).

In terms of survival pathway activation under non-challenged conditions (Fig. 3; relative values of signal intensities compared to the control condition), there was an increase in detection of total AKT depending on the groups: 1 ± 0.55, 1.62 ± 0.67, 2.57 ± 1.28 and 6.75 ± 2.08 (control, periostin 100 ng/mL, 50 ng/mL and FGF-2, respectively; Fig. 3B). In terms of phosphorylation of AKT, there was an increase in periostin 50 ng/mL and FGF-2 groups compared to control and periostin 100 ng/mL: 1 ± 0.16, 0.47 ± 0.1, 1.57 ± 0.26 and 1.35 ± 0.13 (control, periostin 100 ng/mL, 50 ng/mL and FGF-2, respectively; Fig. 3C). The increase in periostin 50 ng/mL and FGF-2 groups was statistically significant compared to periostin 100 ng/mL ($p < 0.011$ for periostin 50 ng/mL; $p < 0.022$ for FGF-2). Similarly, PS-6 followed the same pattern: 1 ± 0.12, 0.1 ± 0.03, 3.52 ± 0.18 and 3.61 ± 0.06 (control, periostin 100 ng/mL, 50 ng/mL and FGF-2, respectively; Fig. 3D). Compared to control or periostin 100 ng/mL, phosphorylation of protein S6 was significantly higher when periostin 50 ng/mL ($p < 0.001$) or FGF-2 ($p < 0.001$) were added. Interestingly, phosphorylation of protein S6 was significantly lower when periostin 100 ng/mL was added ($p < 0.005$).

Periostin increases human periodontal ligament proliferation, migration and activates survival pathways under challenged conditions

Under challenged conditions, immunofluorescence staining for Ki67 proliferation marker showed that only the addition of periostin 100 ng/mL (1.59 ± 0.11) and FGF-2 (1.41 ± 0.12) showed statistically significant differences compared to the control group (1.00 ± 0.16; $p < 0.002$ and $p < 0.026$, respectively). However, and more interestingly, when compared to the TNF-α + *P. gingivalis* LPS group (0.85 ± 0.14), all groups, including the addition of periostin 50 ng/mL (1.31 ± 0.08), showed statistically significant differences ($p < 0.001$, $p < 0.008$ and $p < 0.003$; T + L + P100, T + L + P50 and T + L + FGF-2, respectively; Fig. 4) (values of Ki67-positive cell percentages per group normalized to control).

In terms of migration, the addition of periostin or FGF-2 improved the effects compared to TNF-α + *P. gingivalis* LPS alone (Fig. 5). Interestingly, the effects of periostin (50 and 100 ng/mL) were more noticeable at earlier time points (6 h), when the migration capacities were better than the positive control FGF-2 compared to TNF-α + *P. gingivalis* LPS ($p < 0.022$, $p < 0.045$ and $p = 0.224$; T + L + P100, T + L + P50 and T + L + FGF-2, respectively). Interestingly, the effects of periostin under challenged conditions improved migration to levels similar to those under non-challenged conditions. At later time points (more than 24 h), the differences between T + L + P100 and TNF-α + *P. gingivalis* LPS alone were not statistically significant. At those time points, only the addition of 50 ng/mL of periostin or FGF-2 showed significant improvements compared to TNF-α + *P. gingivalis* LPS alone; those two groups (T + L

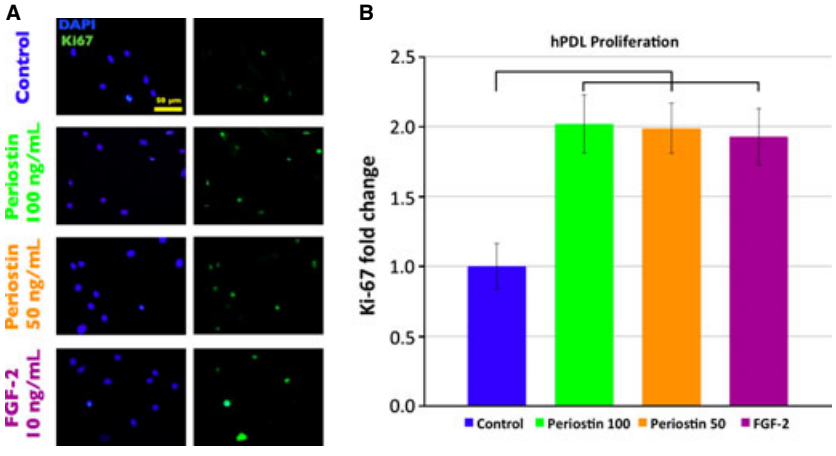

Fig. 1. Ki67 proliferation analysis under non-challenged conditions. (A) Representative image showing total cell number per image (DAPI in blue) and Ki67-positive cells (green). Original magnification: 20×. Scale bar = 50 μm. (B) Relative values of Ki67-positive cell percentages per group normalized to control (blue). Note the approximate twofold increase when adding periostin 100 ng/mL (green), 50 ng/mL (orange) and FGF-2 (purple). *p*-values compared to control: $p < 0.008$. $n = 216$ (four conditions, two cell lines, three experiments, triplicates per condition, three images per replicate). FGF, fibroblast growth factor; hPDL, human periodontal ligament.

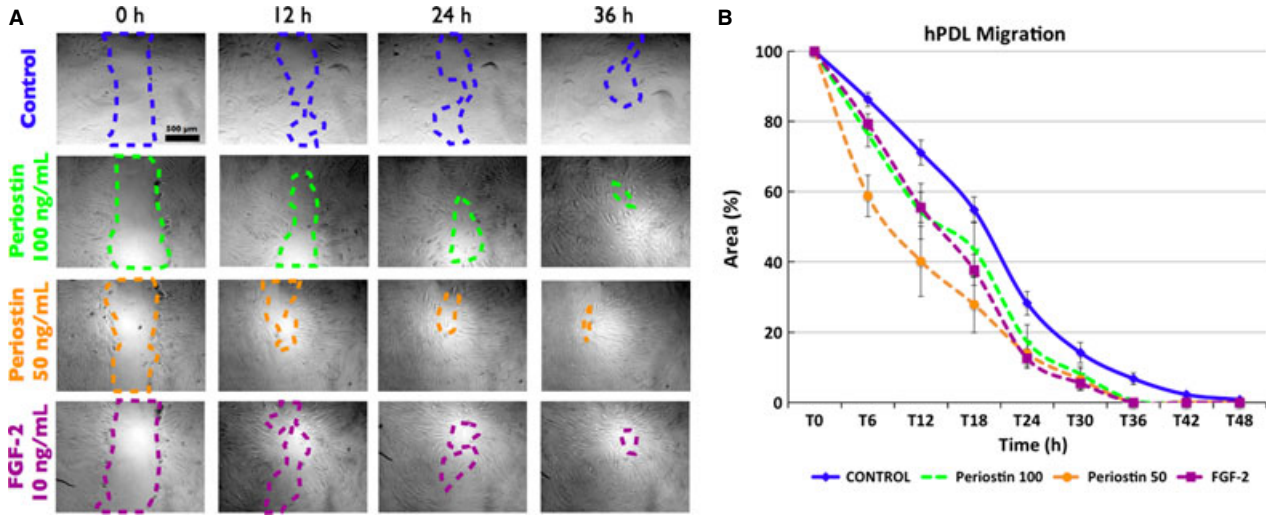

Fig. 2. Migration analysis under non-challenged conditions. (A) Representative images of hPDL migration under different conditions and time points. Original magnification: 4×. Scale bar: 500 µm. (B) Scratched area over time for the different culture conditions. Note the significantly faster area reduction compared to control (solid blue line) after treatment with periostin 100 ng/mL (dotted green line), FGF-2 (dotted purple line), and especially at earlier time points, with periostin 50 ng/mL (dotted orange line). $n = 216$ (four conditions, two cell lines, three experiments, triplicates per condition, three images per replicate). FGF, fibroblast growth factor; hPDL, human periodontal ligament.

+ P50 and T + L + FGF-2) were not different between themselves. Furthermore, the effects of periostin 50 ng/mL between 18 and 36 h were comparable to the non-challenged control group (Fig. 5B).

In terms of survival pathway activation under challenged conditions, previous observations were markedly evident (Fig. 6) (relative values of signal intensities compared to the control condition). In this scenario, even though total AKT was significantly higher in all groups compared to the control group (8.02 ± 1.42, 9.22 ± 0.02, 8.71 ± 0.85 and 14.5 ± 1.025; $p < 0.003$, $p < 0.001$, $p < 0.002$ and $p < 0.001$; T + L, T + L + P100, T + L + P50 and T + L + FGF-2, respectively; Fig. 6B), the addition of TNF-α + *P. gingivalis* LPS markedly decreased phosphorylation of this molecule compared to control, except when FGF-2 was present: 0.08 ± 0.01, 0.21 ± 0.08, 0.68 ± 0.07 and 0.81 ± 0.05 ($p < 0.001$, $p < 0.001$, $p < 0.046$ and $p = 0.184$; T + L, T + L + P100, T + L + P50 and T + L + FGF-2, respectively; Fig. 6C). When compared to TNF-α + *P. gingivalis* LPS, only the addition of periostin 50 ng/mL ($p < 0.005$) and FGF-2 ($p < 0.002$) significantly increased phosphorylation of AKT (Fig. 6C). Interestingly, phosphorylation of protein S6 was significantly higher than in the control group only with the addition of periostin or FGF-2: 1.3 ± 0.19, 2.71 ± 0.1, 4.68 ± 0.08 and 7.66 ± 0.3 ($p = 0.287$, $p < 0.001$, $p < 0.001$ and $p < 0.001$; T + L, T + L + P100, T + L + P50 and T + L + FGF-2, respectively; Fig. 6D). When compared to TNF-α + *P. gingivalis* LPS alone, the addition of periostin 100 ng/mL ($p < 0.002$), periostin 50 ng/mL ($p < 0.001$) and FGF-2 ($p < 0.001$) significantly increased phosphorylation of protein S6 (Fig. 6D).

Discussion

Regeneration of the tooth-supporting apparatus has become a major challenge in dentistry (20) as well as a highly demanded treatment due to the high prevalence and impact that periodontal diseases have in the daily periodontal practice (3). Periostin is primarily expressed by PDL fibroblasts (8). It is localized between the cytoplasmic processes of periodontal fibroblasts and cementoblasts and the adjacent collagen fibers (21). Periostin is required for the integrity and homeostasis of the PDL under normal physiological occlusal load (2,9,10). When not present or reduced, periodontal tissues break down, possibly due to the reduction on collagen fiber diameter and the subsequent tissue strength degradation (12). Within the context of periodontal disease, periostin is reduced by the effect of inflammatory cytokines and bacterial virulence factors (13) and it is inversely related to the extent of bone alveolar loss (14). Therefore, restoration of proper levels of this molecule in the PDL environment may have a key implication in the restoration of its original structure and function.

Periostin has been previously shown to increase cell survival and proliferation rates in different tissues, including skin (16) and heart (15,22,23). It plays a role in angiogenesis (24). Higher levels have been reported as key in cancer development, proliferation and metastasis (25,26). Such effects of periostin are mediated by integrins αvβ1, αvβ3 and αvβ5-integrins (17,27), which further activate the downstream cascade of the PI3K/AKT signaling pathway to induce cell proliferation, as well as phosphorylation of FAK to induce cell migration (18). Our results in

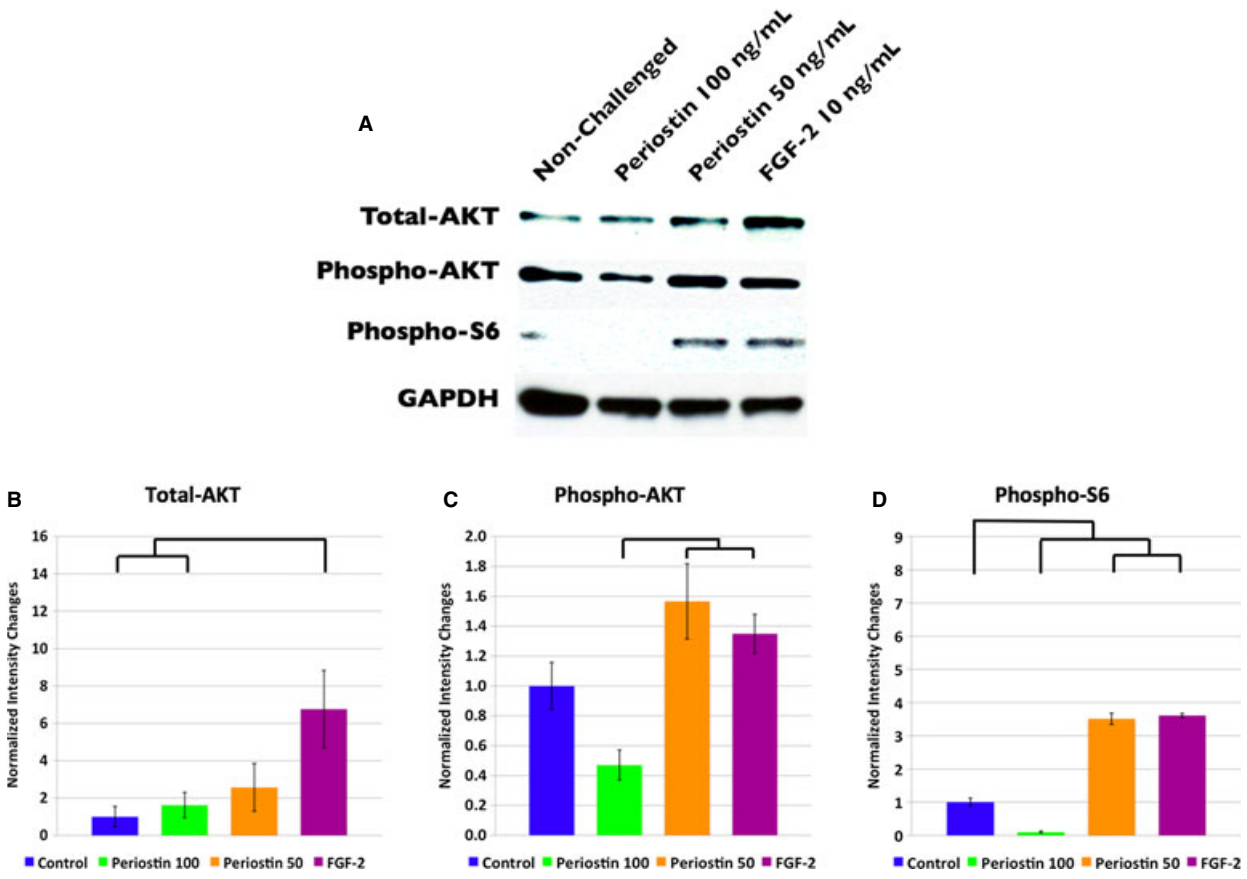

Fig. 3. Western blot evaluation of non-challenged conditions and relative values of signal intensities compared to the control condition. (A) Western blot images showing the activation of the PI3K/AKT/mTOR signaling pathway. (B) Total AKT semiquantification reflects an increase of this molecule in periostin and FGF-2 groups. $p < 0.049$. (C) Phosphorylation of AKT significantly increases in the presence of periostin 50 ng/mL ($p < 0.011$) and FGF-2 ($p < 0.022$). (D) Phosphorylation of protein S6 significantly increases in the presence of periostin 50 ng/mL or FGF-2, compared to either control or periostin 100 ng/mL ($p < 0.001$). FGF, fibroblast growth factor.

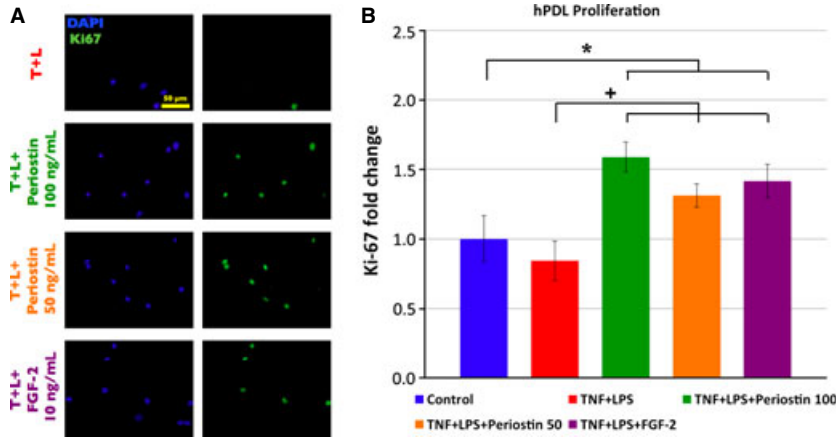

Fig. 4. Ki67 proliferation analysis under challenged conditions (addition of TNF-α + *P. gingivalis* LPS). (A) Representative image showing total cell number per image (DAPI in blue) and Ki67-positive cells (green). Original magnification: 20×. Scale bar = 50 μm. (B) Relative values of Ki67-positive cell percentages per group normalized to control (blue). Note the decrease after adding TNF-α + *P. gingivalis* LPS (red; not statistically significant) and the approximately 1.5-fold increase when adding periostin 100 ng/mL (green), 50 ng/mL (orange) and FGF-2 (purple). *p*-values compared to control: $^{*}p < 0.022$. *p*-values compared to TNF-α + *P. gingivalis* LPS: $^{+}p < 0.008$. $n = 216$ (four conditions, two cell lines, three experiments, triplicates per condition, three images per replicate). FGF, fibroblast growth factor; hPDL, human periodontal ligament; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

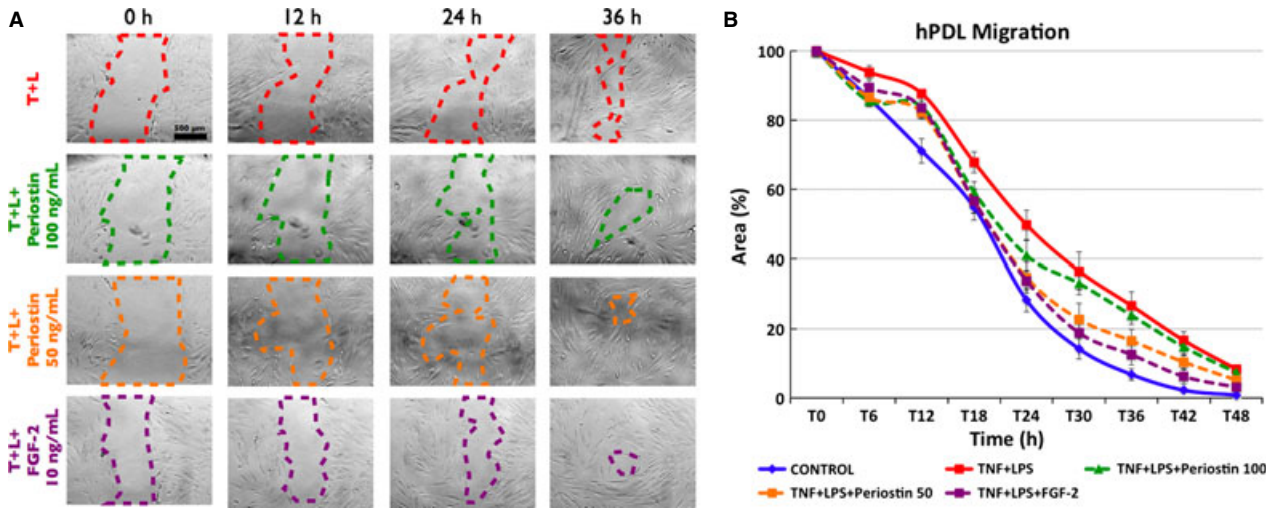

Fig. 5. Migration analysis under challenged conditions. (A) Representative images of hPDL migration under different conditions and time points. Original magnification: 4×. Scale bar: 500 μm. (B) Scratched area over time for the different culture conditions. Note the significantly slower area reduction caused by the combined effect of TNF-α+*P. gingivalis* LPS (solid red line) compared to non-challenged control (solid blue line). Also, note the significantly faster area reduction compared to TNF-α+*P. gingivalis* LPS after treatment with periostin 100 ng/mL (dotted green line), periostin 50 ng/mL (dotted orange line) and FGF-2 (dotted purple line). Area reduction was initially faster for both periostin treatments, but after 12–18 h only FGF-2 and periostin 50 ng/mL accelerate hPDL migration at a higher rate comparable to non-challenged control. $n = 216$ (four conditions, two cell lines, three experiments, triplicates per condition, three images per replicate). FGF, fibroblast growth factor; hPDL, human periodontal ligament; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

hPDL cells show high similarities with previous reports. The present study confirms a PI3K/AKT-dependent migration and proliferation signaling cascade activated by periostin toward phosphorylation of the ribosomal protein S6, the final step of the mTORC1 pathway after activation of p70-S6 Kinase 1 (S6K1), especially with lower dose (50 ng/mL) (Fig. 3) (28). In fact, in terms of cell proliferation, as analyzed by Ki67 immunofluorescence staining, periostin was able to double the proliferation rate as similar to that achieved by the positive control FGF-2 (Fig. 1); in terms of cell migration, periostin was able to accelerate wound closure even faster than the positive control FGF-2 (Fig. 2).

On the other hand, the effects of *P. gingivalis* LPS and TNF-α have been also studied. A summative effect between both factors has been extensively studied (29–32). LPS can directly induce cell death or apoptosis in many cell types, including macrophages, vascular endothelial cells, hepatocytes and myocytes. In contrast, LPS has an anti-apoptotic effect on neutrophils both *in vivo* and *in vitro* (31). In a similar way, it is commonly accepted that members of the TNF superfamily mediate either proliferation, survival or apoptosis of cells (33). Furthermore, during the inflammatory process, TNF-α, mediated or not by LPS, also stimulates the release of matrix metalloproteinases and other products that enhance tissue destruction (31). Previous studies have also shown an indirect effect of bacterial LPS on apoptosis of PDL cells through the induction of TNF-α production (31) and TNFR1 (34).

Within the context of the present study, we have observed a reduction in proliferation by the effects of *P. gingivalis* LPS and TNF-α combined, although the differences were not statistically significant. This apparent discrepancy might be due to specific characteristics of the experiments to detect Ki67 in terms of cell confluence and duration of the exposure to that challenge. Interestingly, and important within the context of periodontal regeneration, in a challenged situation, the addition of periostin promoted cell proliferation to a similar level as FGF-2 (Fig. 4), but not as much as the effects achieved when *P. gingivalis* LPS and TNF-α were not present (Fig. 1); the effect seems to be reduced (possible competition with TNF-α/LPS), but not completely cancelled.

It is interesting that phosphorylation of AKT and protein S6 with the addition of periostin 100 ng/mL is actually decreased under non-challenged conditions. The reason for this is not clear at this moment but we can speculate that higher doses of periostin may actually exert a limiting or inhibitory effect, as seen with other molecules and growth factors such as platelet-derived growth factor (35,36). The fact that we observed other effects with that dose (Ki-67 and migration), rather than contradict this plausible explanation, suggests a possible supplementary mechanism different than that involving AKT and PS6.

However, to achieve true periodontal regeneration, not only proliferation is needed. Migration of the appropriate cells into the healing area is key during the initial phases. PDL cells are capable of differentiating into cementogenic or osteogenic lineage cells (37–39). Therefore, PDL

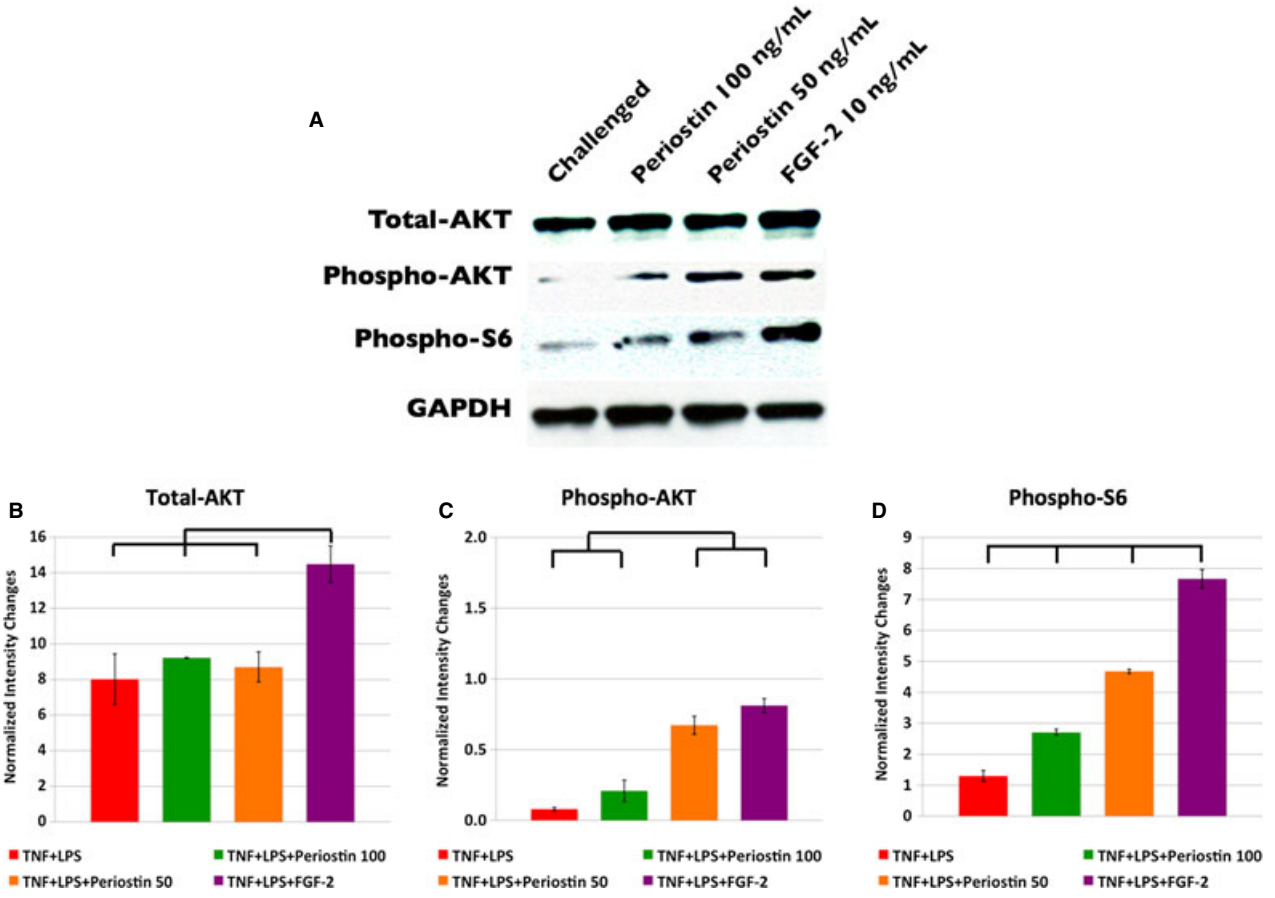

Fig. 6. Western blot evaluation of challenged conditions and relative values of signal intensities compared to the control condition. (A) Western blot images showing the activation of the PI3K/AKT/mTOR signaling pathway. (B) Total AKT semiquantification reflects an increase of this molecule for all groups compared to control ($p < 0.003$). C) Compared to control, phosphorylation of AKT is significantly lower in the presence of TNF-α and *P. gingivalis* LPS ($p < 0.001$) even with the addition of periostin 100 ng/mL ($p < 0.001$) or periostin 50 ng/mL ($p < 0.046$). Compared to TNF-α and *P. gingivalis* LPS, phosphorylation of AKT is significantly higher in the presence of periostin 50 ng/mL ($p < 0.005$) or FGF-2 ($p < 0.002$). D) Phosphorylation of protein S6 significantly increases only in the presence of periostin or FGF-2, compared to either control or TNF-α and *P. gingivalis* LPS ($p < 0.001$). FGF, fibroblast growth factor; hPDL, human periodontal ligament; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

cells are essential not only for the maintenance but also for regeneration of the periodontium (40). hPDL cell migration in the present study was significantly increased by periostin at 50 ng/mL, even at very early time points (Fig. 2). Furthermore, in a context of disease-simulated conditions, with the addition of TNF-α and *P. gingivalis* LPS, hPDL migration was significantly reduced (Fig. 5). In this challenged scenario, periostin recovered the migration potential of hPDL cells to levels not significantly different as those achieved with the addition of the positive control FGF-2 or the non-challenged control group. These are key mechanisms, as occupying the space with this specific cell population with multipotent capacities (PDL cells) could lead to the regeneration of the area reducing the chances of formation of reparative tissues.

FGF-2, used in this study as positive control, has been used, among other applications, in clinical studies for periodontal tissue regeneration (41,42) due to the activation of a plethora of cellular events: proliferative responses of PDL cells through the extracellular signal-regulated kinase (ERK) 1/2 signaling molecule (43,44), cell migration via PI3K/AKT signaling (44,45), and regulatory effects of ECM production and PDL cell differentiation (46). FGF-2 seems to act on immature PDL cells to stimulate proliferation while suppressing differentiation into hard tissue-forming cells. During the subsequent healing processes, PDL cells begin to differentiate, inducing periodontal tissue regeneration (46). In contrast, different studies have shown that FGF-stimulated effects are partially inhibited by TNF-α (47).

The effects achieved in this study with the application of FGF-2 are comparable to those obtained with periostin. Therefore, periostin may become a promising agent in periodontal regeneration with some important advantages as it integrates a spectrum of key events in matrix–cellular interactions: accelerates hPDL cell migration, favors hPDL cell

proliferation, promotes angiogenesis and, finally and decisively, will promote a mechanically stable ECM suitable to support periodontal tissue stability with a higher specificity for the PDL.

Migration and proliferation, although related, are different processes. Studying these processes *in vitro* is complicated. Therefore, further studies to confirm the optimum concentration for each process and the combination of both are needed as well as *in vivo* studies to confirm these observations.

Conclusion

Periostin, a key factor for periodontal integrity, is involved in important cellular events, such as cell proliferation, migration and activation of the survival signaling pathway PI3K/AKT/mTOR. Such effects may support the use of periostin as a novel biological agent for periodontal regeneration purposes. Further studies of the specific mechanisms, protein concentrations, timely assessment and the regenerative value of periostin in animal studies are needed.

Acknowledgements

This study was supported by the NIH/NIDCR grant DE019872 (HFR), and the Talentia Scholarship Program (Junta de Andalucía, Spain) (MPM). The authors report no conflict of interest related to this study.

References

1. Bartold PM, Narayanan AS. Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontol 2000* 2006;**40**:29–49.
2. Rios HF, Ma D, Xie Y *et al*. Periostin is essential for the integrity and function of the periodontal ligament during occlusal loading in mice. *J Periodontol* 2008;**79**:1480–1490.
3. Papapanou PN. The prevalence of periodontitis in the US: forget what you were told. *J Dent Res* 2012;**91**:907–908.
4. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res* 2012;**91**:914–920.
5. Bartold PM, Shi S, Gronthos S. Stem cells and periodontal regeneration. *Periodontol 2000* 2006;**40**:164–172.
6. Chen FM, An Y, Zhang R, Zhang M. New insights into and novel applications of release technology for periodontal reconstructive therapies. *J Control Release* 2011;**149**:92–110.
7. Padial-Molina M, Marchesan JT, Taut AD, Jin Q, Giannobile WV, Rios HF. Methods to validate tooth-supporting regenerative therapies. *Methods Mol Biol* 2012;**887**:135–148.
8. Saito Y, Yoshizawa T, Takizawa F *et al*. A cell line with characteristics of the periodontal ligament fibroblasts is negatively regulated for mineralization and Runx2/Cbfa1/Osf2 activity, part of which can be overcome by bone morphogenetic protein-2. *J Cell Sci* 2002;**115**:4191–4200.
9. Ma D, Zhang R, Sun Y *et al*. A novel role of periostin in postnatal tooth formation and mineralization. *J Biol Chem* 2011;**286**:4302–4309.
10. Rios H, Koushik SV, Wang H *et al*. Periostin null mice exhibit dwarfism, incisor enamel defects, and an early-onset periodontal disease-like phenotype. *Mol Cell Biol* 2005;**25**:11131–11144.
11. Hamilton DW. Functional role of periostin in development and wound repair: implications for connective tissue disease. *J Cell Commun Signal* 2008;**2**:9–17.
12. Norris RA, Damon B, Mironov V *et al*. Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. *J Cell Biochem* 2007;**101**:695–711.
13. Padial-Molina M, Volk SL, Rodriguez JC, Marchesan JT, Galindo-Moreno P, Rios HF. TNF-alpha and *P. gingivalis* lipopolysaccharides decrease periostin in human PDL fibroblasts. *J Periodontol* 2012;**84**:694–703.
14. Padial-Molina M, Volk SL, Taut AD, Giannobile WV, Rios HF. Periostin is down-regulated during periodontal inflammation. *J Dent Res* 2012;**91**:1078–1084.
15. Kuhn B, del Monte F, Hajjar RJ *et al*. Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. *Nat Med* 2007;**13**:962–969.
16. Nishiyama T, Kii I, Kashima TG *et al*. Delayed re-epithelialization in periostin-deficient mice during cutaneous wound healing. *PLoS ONE* 2011;**6**:e18410.
17. Bao S, Ouyang G, Bai X *et al*. Periostin potently promotes metastatic growth of colon cancer by augmenting cell survival via the Akt/PKB pathway. *Cancer Cell* 2004;**5**:329–339.
18. Li G, Jin R, Norris RA *et al*. Periostin mediates vascular smooth muscle cell migration through the integrins alphavbeta3 and alphavbeta5 and focal adhesion kinase (FAK) pathway. *Atherosclerosis* 2010;**208**:358–365.
19. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2007;**2**:329–333.
20. Park CH, Rios HF, Jin Q *et al*. Tissue engineering bone-ligament complexes using fiber-guiding scaffolds. *Biomaterials* 2012;**33**:137–145.
21. Suzuki H, Amizuka N, Kii I *et al*. Immunohistochemical localization of periostin in tooth and its surrounding tissues in mouse mandibles during development. *Anat Rec A Discov Mol Cell Evol Biol* 2004;**281**:1264–1275.
22. Dorn GW 2nd. Periostin and myocardial repair, regeneration, and recovery. *N Engl J Med* 2007;**357**:1552–1554.
23. Polizzotti BD, Arab S, Kuhn B. Intrapericardial delivery of gelfoam enables the targeted delivery of periostin peptide after myocardial infarction by inducing fibrin clot formation. *PLoS ONE* 2012;**7**:e36788.
24. Zhu M, Fejzo MS, Anderson L *et al*. Periostin promotes ovarian cancer angiogenesis and metastasis. *Gynecol Oncol* 2010;**119**:337–344.
25. Wang Z, Ouyang G. Periostin: a bridge between cancer stem cells and their metastatic niche. *Cell Stem Cell* 2012;**10**:111–112.
26. Malanchi I, Santamaria-Martinez A, Susanto E *et al*. Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature* 2012;**481**:85–89.
27. Utispan K, Sonongbua J, Thuwajit P *et al*. Periostin activates integrin alpha5-beta1 through a PI3K/AKTdependent pathway in invasion of cholangiocarcinoma. *Int J Oncol* 2012;**41**:1110–1118.
28. Saitoh M, Pullen N, Brennan P, Cantrell D, Dennis PB, Thomas G. Regulation of an activated S6 kinase 1 variant reveals a novel mammalian target of rapamycin phosphorylation site. *J Biol Chem* 2002;**277**:20104–20112.
29. Hosokawa Y, Hosokawa I, Ozaki K *et al*. CXCL12 and CXCR4 expression by human gingival fibroblasts in periodontal disease. *Clin Exp Immunol* 2005;**141**:467–474.
30. Quintero JC, Piesco NP, Langkamp HH, Bowen L, Agarwal S. LPS responsiveness in periodontal ligament cells is regulated by tumor necrosis factor-alpha. *J Dent Res* 1995;**74**:1802–1811.
31. Thammasitboon K, Goldring SR, Boch JA. Role of macrophages in LPS-induced osteoblast and PDL cell apoptosis. *Bone* 2006;**38**:845–852.
32. Wang PL, Ohura K. Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts-CD14 and toll-like

receptors. *Crit Rev Oral Biol Med* 2002;**13**:132–142.
33. Gaur U, Aggarwal BB. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem Pharmacol* 2003;**66**:1403–1408.
34. Alikhani M, Alikhani Z, Graves DT. Apoptotic effects of LPS on fibroblasts are indirectly mediated through TNFR1. *J Dent Res* 2004;**83**:671–676.
35. Nevins M, Giannobile WV, McGuire MK *et al*. Platelet-derived growth factor stimulates bone fill and rate of attachment level gain: results of a large multicenter randomized controlled trial. *J Periodontol* 2005;**76**:2205–2215.
36. Nevins M, Kao RT, McGuire MK *et al*. Platelet-derived growth factor promotes periodontal regeneration in localized osseous defects: 36-month extension results from a randomized, controlled, double-masked clinical trial. *J Periodontol* 2013;**84**:456–464.
37. Ivanovski S, Gronthos S, Shi S, Bartold PM. Stem cells in the periodontal ligament. *Oral Dis* 2006;**12**:358–363.
38. Nagatomo K, Komaki M, Sekiya I *et al*. Stem cell properties of human periodontal ligament cells. *J Periodontal Res* 2006;**41**:303–310.
39. Somerman MJ, Young MF, Foster RA, Moehring JM, Imm G, Sauk JJ. Characteristics of human periodontal ligament cells in vitro. *Arch Oral Biol* 1990;**35**: 241–247.
40. Pitaru S, McCulloch CA, Narayanan SA. Cellular origins and differentiation control mechanisms during periodontal development and wound healing. *J Periodontal Res* 1994;**29**:81–94.
41. Kitamura M, Nakashima K, Kowashi Y *et al*. Periodontal tissue regeneration using fibroblast growth factor-2: randomized controlled phase II clinical trial. *PLoS ONE* 2008;**3**:e2611.
42. Kitamura M, Akamatsu M, Machigashira M *et al*. FGF-2 stimulates periodontal regeneration: results of a multi-center randomized clinical trial. *J Dent Res* 2011;**90**:35–40.
43. Takayama S, Yoshida J, Hirano H, Okada H, Murakami S. Effects of basic fibroblast growth factor on human gingival epithelial cells. *J Periodontol* 2002;**73**:1467–1473.
44. Boilly B, Vercoutter-Edouart AS, Hondermarck H, Nurcombe V, Le Bourhis X. FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev* 2000;**11**: 295–302.
45. Shimabukuro Y, Terashima H, Takedachi M *et al*. Fibroblast growth factor-2 stimulates directed migration of periodontal ligament cells via PI3K/AKT signaling and CD44/hyaluronan interaction. *J Cell Physiol* 2011;**226**:809–821.
46. Murakami S. Periodontal tissue regeneration by signaling molecule(s): what role does basic fibroblast growth factor (FGF-2) have in periodontal therapy? *Periodontol 2000* 2011;**56**:188–208.
47. Frater-Schroder M, Risau W, Hallmann R, Gautschi P, Bohlen P. Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc Natl Acad Sci USA* 1987;**84**:5277–5281.