Cell Tissue Res (2016) 366:311–328
DOI 10.1007/s00441-016-2437-3

REGULAR ARTICLE

Effects of short-term inflammatory and/or hypoxic pretreatments on periodontal ligament stem cells: in vitro and in vivo studies

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Received: 25 November 2015 / Accepted: 28 May 2016 / Published online: 15 June 2016

Abstract In this study, we extensively screened the in vitro and in vivo effects of PDLSCs following short-term inflammatory and/or hypoxic pretreatments. We found that the 24-h hypoxic pretreatment of PDLSCs significantly enhanced cell migration and improved cell surface CXCR4 expression. In addition, hypoxia-pretreated PDLSCs exhibited improved cell colony formation and proliferation. Cells that were dually stimulated also formed more colonies compared to untreated cells but their proliferation did not increase. Importantly, the hypoxic pretreatment of PDLSCs enhanced cell differentiation as determined by elevated RUNX-2 and ALP protein expression. In this context, the inflammatory stimulus impaired cell OCN protein expression, while dual stimuli led to decreased *RUNX-2* and *OCN* mRNA levels. Although preconditioning PDLSCs with inflammatory and/or hypoxic pretreatments resulted in no differences in the production of matrix proteins, hypoxic pretreatment led to the generation of thicker cell sheets; the inflammatory stimulus weakened the ability of cells to form sheets. All the resultant cell sheets exhibited clear bone regeneration following ectopic transplantation as well as in periodontal defect models; the amount of new bone formed by hypoxia-preconditioned cells was significantly greater than that formed by inflammatory stimulus- or dual-stimuli-treated cells or by nonpreconditioned cells. The regeneration of new cementum and periodontal ligaments was only identified in the hypoxia-stimulus and no-stimulus cell groups. Our findings suggest that PDLSCs that undergo short-term hypoxic pretreatment show improved cellular behavior in vitro and enhanced regenerative potential in vivo. The preconditioning of PDLSCs via combined treatments or an inflammatory stimulus requires further investigation.

Keywords Cell pretreatment · Cell migration · Cell proliferation · Cell differentiation · Cell sheet engineering

Introduction

Periodontitis, characterized by progressive destruction of the tooth-supporting apparatus, is one of the most widespread infectious conditions and the most common cause of tooth loss in adults; this condition affects not only the oral health status but also the quality of life of individuals (Pihlstrom et al. 2005; Buset et al. 2016). More than 3 decades have passed since the introduction of bone grafting materials, bioactive agents and the guided tissue regeneration (GTR) technique to save teeth and regenerate lost/damaged periodontal hybrid tissues, including alveolar bone, cementum and the periodontal ligament (PDL) (McClain and Schallhorn 2000; Chen et al. 2009; Kim et al. 2014). Unfortunately, functional regeneration of the diseased periodontal structure has not been successful thus far (Larsson et al. 2016). Although this ambitious goal

This work was supported by the National Natural Science Foundation of China (No. 81530050, 81471791 and 81500853) and the Program for New Century Excellent Talents in University (NCET-12-1005).

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has been achieved for certain ideal clinical scenarios, it is often considered an unpredictable task (McClain and Schallhorn 2000; Pihlstrom et al. 2005). Along with concerted efforts to produce predictable and reliable approaches to augment bone regeneration in intrabony bone defects, the development of tissue engineering and regenerative medicine has provided new avenues to improve the clinical outcomes of periodontal regeneration using stem cell-based technologies (Chen and Jin 2010; Izumi et al. 2011; Chen et al. 2012; Lu et al. 2013; Monsarrat et al. 2014). In this context, stem cells derived from the PDL (PDLSCs) have been demonstrated to be effective in preclinical trials and have been suggested as the best cell type for periodontal tissue regeneration (Iwata et al. 2010; Dangaria et al. 2011; Tsumanuma et al. 2011; Dan et al. 2014). Despite several small-scale pilot/feasibility studies that have been performed in humans (Feng et al. 2010; Chen et al. 2016), the clinical use of PDLSCs for periodontal regeneration still faces numerous challenges (Hynes et al. 2012; Lu et al. 2013).

Teeth extracted due to impaction or orthodontic treatment are the most common resources for obtaining autologous PDLSCs for therapeutics (Chen et al. 2012; Lu et al. 2013; Monsarrat et al. 2014). Despite being a promising stem cell reservoir, the generation of sufficient amounts of stem cells from the limited PDL tissues remaining on the root surface of a removed tooth remains an elusive goal (Izumi et al. 2011; Tang et al. 2016); therefore, long-term ex vivo culture and expansion to reach the required population size are necessary. However, PDLSC proliferation remains slow under the current culture conditions. More importantly, these cells inevitably undergo replicative senescence, resulting in potential cellular phenotypic changes and/or loss of cell stemness (Kim et al. 2009; Jayaraman et al. 2016). Indeed, there is evidence that the in vitro lifespan of PDL-derived cells is shorter than that of other cell types in connective tissues (Sawa et al. 2000). Therefore, maintaining the stemness of PDLSCs, particularly with regard to their migration, proliferation and differentiation, has become a vital issue that must be carefully addressed before these cells enter the clinical testing stage (Wu et al. 2016). Clearly, the current in vitro system cannot replicate the desired in vivo environment, which consists in various factors, such as oxygen tension, biochemical molecules and mechanical forces (Ashton et al. 2011), generally leading to low-quality cellular products with impaired proliferation, early senescence and/or the loss of regenerative potential (Sawa et al. 2000; Kim et al. 2009; Jayaraman et al. 2016).

Many attempts have now been made to improve cell behavior during ex vivo expansion and to enhance the clinical success of stem cell-based therapies (Kono et al. 2013; Proksch et al. 2014; Teramatsu et al. 2014). Of the various available strategies, short-term pretreatment of cells with pharmaceuticals, such as cytokines, antiapoptotic molecules and growth factors, or the incubation of cells under particular conditions, such as hypoxic environments, appears to be a simple, practical and safe method to maintain, if not strengthen, the therapeutic potential of ex vivo-manipulated cell materials (Peng et al. 2013; Lee et al. 2015). There is substantial evidence that hypoxic pretreatment increases survival and migration (Liu et al. 2010; Beegle et al. 2015), supports osteogenic differentiation (Volkmer et al. 2010), promotes chondrogenesis (Kanichai et al. 2008) and enhances the adipogenic and osteogenic differentiation potentials (Valorani et al. 2012) of various mesenchymal stem cell (MSC) populations, leading to reliable strategies to increase the efficiency of cell-based therapeutics (Haque et al. 2013). Meanwhile, the preconditioning of MSCs with proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, has been found to enhance the capability of stem cells to proliferate in vitro (Yang et al. 2013) and to migrate after in vivo transplantation (Fan et al. 2012; Ziaei et al. 2014). Although IL-1β is one of the most basic inflammatory mediators, increasing evidence has revealed that IL-1β signaling activates immunosuppressive properties of MSCs, depending, at least partly, on eliciting their immunosuppressive capacity and enhancing their migratory ability (Groh et al. 2005). Moreover, IL-1β increases IL-6 secretion by MSCs derived from cord blood in a dose-dependent manner (0–30 ng/mL) (Liu and Hwang 2005). To determine pathways involved in inflammation-stimulated cell migration, the expression of chemokines and their receptors on cell surfaces has been demonstrated to play a crucial role in dictating the migration and activation of multiple cell types (Nagasawa 2014). In recent years, substantial evidence has demonstrated that the in vivo recruitment and efficacy of MSC therapy have been largely influenced by the expression of key homing receptors such as CXC chemokine receptor 4 (CXCR4) on the surfaces of MSCs (Marquez-Curtis and Janowska-Wieczorek 2013). Unfortunately, ex vivo-expanded MSCs show CXCR4 down-regulation, suggesting that approaches that increase the CXCR4 expression level in MSCs are necessary before their in vivo transplantation as therapeutics (Honczarenko et al. 2006; Shi et al. 2007; Liu et al. 2010; Ziaei et al. 2014). A comparison of TNF-α-treated and untreated cells indicated that the highest CXCR4 expression level was reached 24 h after treatment with TNF-α at a concentration of 1 or 10 ng/mL and that the production of *CXCR4* mRNA was upregulated 216- or 512-fold, respectively (Ziaei et al. 2014). Based on previously published data (Shi et al. 2007) as well as our endeavors (Yu et al. 2016), it is tempting to speculate that a combination of various cytokines or different pretreatments may shorten the incubation time and decrease the cytokine dose while eliciting a potential synergistic effect on target cells.

While substantial evidence suggests that either an inflammatory or hypoxic pretreatment can exert positive influences on cell behavior, the beneficial effects of these treatments on PDLSCs have gained less attention (Basciano et al. 2011; Glass et al. 2011; Fan et al. 2012; Beegle et al. 2015; Yu

et al. 2016). Indeed, there is primary evidence that the viability, relevant osteogenic marker expression and paracrine function of PDLSCs are significantly increased under hypoxic conditions (Wu et al. 2013). In the present study, the effects of short-term inflammatory and/or hypoxic pretreatments on PDLSCs were extensively screened using in vitro and in vivo models. Based on our previous work regarding preconditioning of bone marrow-derived MSCs (Yu et al. 2016), we established 4 experimental groups: cells were incubated under inflammatory conditions (inflammation), hypoxic conditions (hypoxia), or a combination of both (dual-stimuli) conditions, while cells without inflammatory or hypoxic stimuli served as the controls (no-stimulus). We selected the frequently used doses of O_2 (2 %) and of inflammatory factors (10 ng/mL TNF-α and 5 ng/mL IL-1β) to establish the hypoxic and/or inflammatory conditions for PDLSC preconditioning; the incubation time was 24 h, which is the most short-term pretreatment reported thus far. We attempted to provide experimental evidence of PDLSC pretreatments for the production of robust cell materials for clinical use in periodontal regenerative medicine.

Materials and Methods

Isolation and culture of human PDLSCs

The third molars (without caries, inflammation or periodontitis) used for cell isolation in this study were obtained from 3 systemically healthy donors (18–23 years) at the Dental Clinic of School of Stomatology, Fourth Military Medical University (FMMU), Xi'an, China. The experimental protocol was approved by the Institutional Review Board (IRB) of FMMU, School of Stomatology and an informed consent form agreeing to the contribution of their teeth for research purposes was signed by all donors. Human PDLSCs were isolated as previously described (Wu et al. 2015). Briefly, the PDL tissue, which was scratched from the middle part of the root surface of the extracted teeth, was cut into small blocks and then digested for 1 h. After the tissue was digested, the digested tissue suspension was transferred to 6-well plates containing complete medium, i.e., α-minimum essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials, Zhejiang, China), 0.292 mg/mL glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin (Gibco BRL) and 100 mg/mL streptomycin (Gibco BRL), for culturing the primary cells. Finally, the limiting dilution technique was used to purify stem cells from the primary cells. Cells at passages 3–5 (P3–P5) were used in the subsequent investigations.

Identification of isolated PDLSCs

First, flow cytometric analysis was performed to determine the expression of cell surface antigens. Briefly, aliquots of isolated PDLSCs (P2; $> 2 \times 10^5$ cells) were washed and resuspended for 40 min at 4 °C in the dark in sterile PBS containing saturating concentrations (1:100 dilution) of the following anti-human monoclonal antibodies (Abcam, Cambridge, UK): anti-CD29-fluorescein isothiocyanate (FITC), anti-CD31-phycoerythrin (PE), anti-CD34-PE, anti-CD44-PE, anti-CD90-PE, anti-CD105-FITC, anti-CD146-FITC and anti-STRO-1-FITC. The aliquots with corresponding dye-labeled isotype control antibodies served as the negative control. Then, the cell suspensions were washed twice, resuspended in PBS and analyzed using a Beckman Coulter Epics XL cytometer (Beckman Coulter, Fullerton, CA, USA). To further characterize the stem features of isolated PDLSCs, their proliferation ability was detected by the colony-forming assay and their differentiation potential was evaluated via osteogenic and adipogenic differentiation assays as described in our previous work (Wu et al. 2015; Yu et al. 2016).

Group design for cell incubation

Referring to our previous work (Yu et al. 2016), we designed three experimental groups to test the effects of short-term inflammatory and hypoxic pretreatments on cell behavior and regenerative potential: the cells were pretreated under (1) inflammatory (inflammation), (2) hypoxic (hypoxia), or (3) inflammatory plus hypoxic stimuli (dual-stimuli) conditions for 24 h. A culture without an inflammatory or hypoxic stimulus was used as the control group (no-stimulus). We applied complete medium supplemented with TNF-α (10 ng/mL) and IL-1β (5 ng/mL; both from Sigma-Aldrich, St. Louis, MO, USA) as the inflammatory stimulus medium. For the hypoxic stimulus, cultures were incubated in a humidified atmosphere containing 2 % O_2 instead of 20 % O_2, which is the general oxygen concentration within the incubator. The 'normal condition' described in this manuscript refers to cells cultured in complete medium and incubated in a humidified atmosphere containing 20 % O_2.

Effects of different pretreatments on cell migration

CXCR4 gene expression

The relative level of *CXCR4* mRNA expression in cells was determined via real-time PCR analysis. Briefly, 2×10^5 cells in each group were seeded in 6-well culture dishes and pre-incubated for 24 h as described in the group design. Total RNA was immediately isolated using TRIzol reagent (Invitrogen Life Technologies) and used as a template to synthesize cDNA using a RevertAid First Strand cDNA

Synthesis Kit (TaKaRa Bio, Otsu, Japan) via reverse transcription. *CXCR4* expression was analyzed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Amplification was performed under the following conditions: denaturation at 95 °C for 3 min, followed by 39 cycles at 95 °C for 15 s and 60 °C for 30 s. *GAPDH* served as an internal control. The primers employed in the present study are shown in Table 1.

CXCR4 protein expression

In parallel with the *CXCR4* gene analysis, total protein in the PDLSCs was immediately extracted in lysis buffer (Sigma-Aldrich) and the protein concentration was measured by western blot analysis using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Twenty micrograms of protein was separated in each lane of 12 % sodium dodecyl sulfate-polyacrylamide gels and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5 % bovine serum albumin (BSA; Sigma-Aldrich) for 2 h. Subsequently, the membranes were incubated with primary antibodies against CXCR4 (Abcam) and β-tubulin overnight at 4 °C and then further incubated with secondary antibodies against rabbit and mouse at 22 °C for 2 h. Immunodetection was performed using the Western-Light Chemiluminescent Detection System (Peiqing, Shanghai, China).

Tablet scratch experiment

The migratory ability of cells in the four groups was first evaluated by a tablet scratch experiment. Briefly, PDLSCs (P4) were plated in 6-well culture dishes at a density of 2×10^5 cells per well and cultured in complete medium until they reached 60 % confluence. Then, the cells were pre-incubated for 24 h as described in the group design, followed by the addition of 10 μg/mL mitomycin C (Sigma-Aldrich) to the medium to inhibit proliferation. After the cells were further cultured for 2 h, the cellular monolayer was scratched using a pipette tip (1000 μL) to generate a cell-free strip to record the baseline. After the cells were incubated under normal cell culture conditions (without inflammatory or hypoxic stimulus; 20 % O_2) for 24 h, the migrated cells in the four groups with respect to the baseline were photographed and counted under an inverted microscope (Olympus Optical, Tokyo, Japan).

Transwell assay

The ability of cells to migrate down a stromal-derived factor-1 (SDF-1) gradient was further evaluated using an 8-μm-pore Transwell membrane system (Zhou et al. 2013). Briefly, PDLSCs (P4) at a density of 1×10^5 cells per well were pre-incubated for 24 h as described in the group design and then transferred to the upper chamber; the medium in the lower chamber was complete medium (600 μL per well) containing 100 ng/mL SDF-1. After the cells were incubated for an additional 12 h, the system was fixed in 4 % paraformaldehyde for 20 min. Then, the nonmigrated cells were detached from the membrane of the Transwell insert. The migrated cells on the lower surface of the membrane were stained with 0.2 % crystal violet (Sigma-Aldrich) for 1 h and counted in 5 random fields (×100) for statistical analysis.

Effects of different pretreatments on cell proliferation

Colony-forming assay

PDLSCs (P3) were pre-incubated for 24 h as described in the group design and transferred to 10-cm-diameter culture dishes (1000 cells per dish). Then, the cells were cultured under

Table 1 Primer sequences used in the present study

Genes	Full name and Gene ID	Primers	Sequences (5'-3')
CXCR4	CXC chemokine receptor 4; ID: 7852	Forward	TCATCCTCATCCTGGCTTTC
		Reverse	CAAACTCACACCCTTGCTTG
Runx2	Runt-related transcription factor-2; ID: 860	Forward	CACTGGCGCTGCAACAAGA
		Reverse	CATTCCGGAGCTCAGCAGAATAA
ALP	Alkaline phosphatase; ID: 249	Forward	GGACCATTCCCACGTCTTCAC
		Reverse	CCTTGTAGCCAGGCCCATTG
OCN	Osteocalcin; ID: 632	Forward	CCCAGGCGCTACCTGTATCAA
		Reverse	GGTCAGCCAACTCGTCACAGTC
PPAR-γ	Peroxisome proliferator activated receptor gamma; ID: 5468	Forward	TCGAGGACACCGGAGAGG
		Reverse	CACGGAGCTGATCCCAAAGT
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase; ID: 2597	Forward	GGTGAAGGTCGGAGTCAACGGA
		Reverse	GAGGGATCTCGCTCCTGGAAGA

normal conditions (medium refreshed every 3 days). At day 14, the cells in the dishes were fixed in 4 % paraformaldehyde for 20 min and stained with 0.2 % crystal violet (Sigma-Aldrich) for 1 h. The cell colonies were subsequently observed under a stereomicroscope (Olympus).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

PDLSCs (P3) were pre-incubated for 24 h as described in the group design and transferred to 96-well plates (Corning) at a density of 2×10^3 cells per well. From the second day on, the tested wells were supplemented with 20 μL of 5 mg/mL MTT solution (Sigma-Aldrich) and incubated for 4 h at 37 °C. Then, the MTT solution was replaced with 150 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich). Finally, a microplate reader (ELx800; BioTek Instruments, Winooski, VT, USA) was used to measure the absorbance.

Effects of different pretreatments on cell differentiation

Cell expression of osteogenic and adipogenic genes

The expression of osteogenic genes (e.g., *RUNX-2*, *ALP* and *OCN*) and adipogenic genes (e.g., *PPAR-γ*) was detected by real-time PCR analysis. Briefly, 2×10^5 cells were pre-incubated for 24 h as described in the group design and then the medium was replaced with osteogenic medium (i.e., complete medium containing 50 μg/mL vitamin C, 5 mM β-glycerophosphate and 0.1 mM dexamethasone) or adipogenic medium (i.e., complete medium containing 200 μM indomethacin, 10 μM insulin, 0.5 mM 1-methyl-3-isobutylxanthine and 1 μM dexamethasone); all pharmaceuticals were obtained from Sigma-Aldrich. After cells were incubated for 7 days, cell samples were collected, and osteogenic (*RUNX-2*, *ALP* and *OCN*) and adipogenic gene (*PPAR-γ*) expression was detected using real-time PCR analysis (refer to *CXCR4* gene analysis). *GAPDH* was used as an internal control. The primers employed in the present study are shown in Table 1.

Expression of osteogenic and adipogenic proteins

In parallel to the gene analysis, the expression of osteogenic proteins (RUNX-2, ALP and OCN) and of an adipogenic protein (PPAR-γ) was detected by western blot analysis (refer to CXCR4 protein assay).

Effects of different pretreatments on cell sheet production

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining were used to evaluate sheet formation and extracellular matrix (ECM) production of PDLSCs. Briefly, the cells (P4) were seeded in 6-well culture dishes at a density of 2×10^5 cells per dish and pre-incubated for 24 h as described in the group design. Then, all the media were changed to a cell sheet-induction medium (complete medium supplemented with 50 μg/mL vitamin C) (Wu et al. 2015) for cell sheet production. After 14 days, the resulting cell sheets were harvested using sterile tweezers and fixed in 4 % paraformaldehyde for 12 h. The obtained specimens were embedded in paraffin blocks and then cut into 5-μm-thick sections. The thickness of the cell sheets was evaluated by H&E staining and analyzed by ImageJ software. For the IHC staining of COL-1, fibronectin and integrin β1, the sections were subjected to dewaxing, rehydration and antigen retrieval and then permeabilized with 0.2 % Triton X-100 for 10 min. Next, the sections were blocked in 3 % hydrogen peroxide solution for 10 min followed by blocking in 5 % BSA (Sigma) for another 30 min. The samples were subsequently incubated with primary antibodies, such as anti-COL-1, anti-fibronectin and anti-integrin β1 (all from Abcam), for 30 min and the staining was visualized using a System-HRP kit according to the manufacturer's instructions. Integrated optical density (IOD) measurements were calculated using Image-Pro Plus 6.0 software.

Ectopic bone regeneration assays

The use of animals for research was approved by the IRB of FMMU and the surgical procedures were performed according to the guidelines of the Animal Care Committee of FMMU. The in vivo bone formation capacity of PDLSC sheets derived from different pre-incubated cells, as described in the group design, was investigated based on an ectopic transplantation model (Gao et al. 2013). The transplants were composed of 15 mg of calcined bovine bone (CBB) wrapped in 4-fold sheets. A 1 % pentobarbital sodium solution (0.1 mL/100 g) was used for intraperitoneal anesthesia of the animals. The transplants were subcutaneously transplanted into the dorsal region of 6-week-old male nude mice (purchased from the FMMU Animal Center). Thirty-six animals were used in the present study, with 9 animals in each group. PDLSCs from 3 donors were used independently and the cell transplants derived from each cell line were transplanted into 3 animals in parallel. After 2 months, the mice were euthanized and the excised tissue samples were fixed in 4 % paraformaldehyde for 12 h and then scanned using a Siemens Inveon Microcomputed Tomography (Micro-CT) System (Siemens, Munich, Germany) with the following scanning parameters: 30 μm resolution, 80 kV and 500 μA. The obtained data were transferred to a workstation and then three-dimensional (3D) images were reconstructed by the system software. Mean bone density was assessed by built-in software. In addition to Micro-CT scanning, the samples

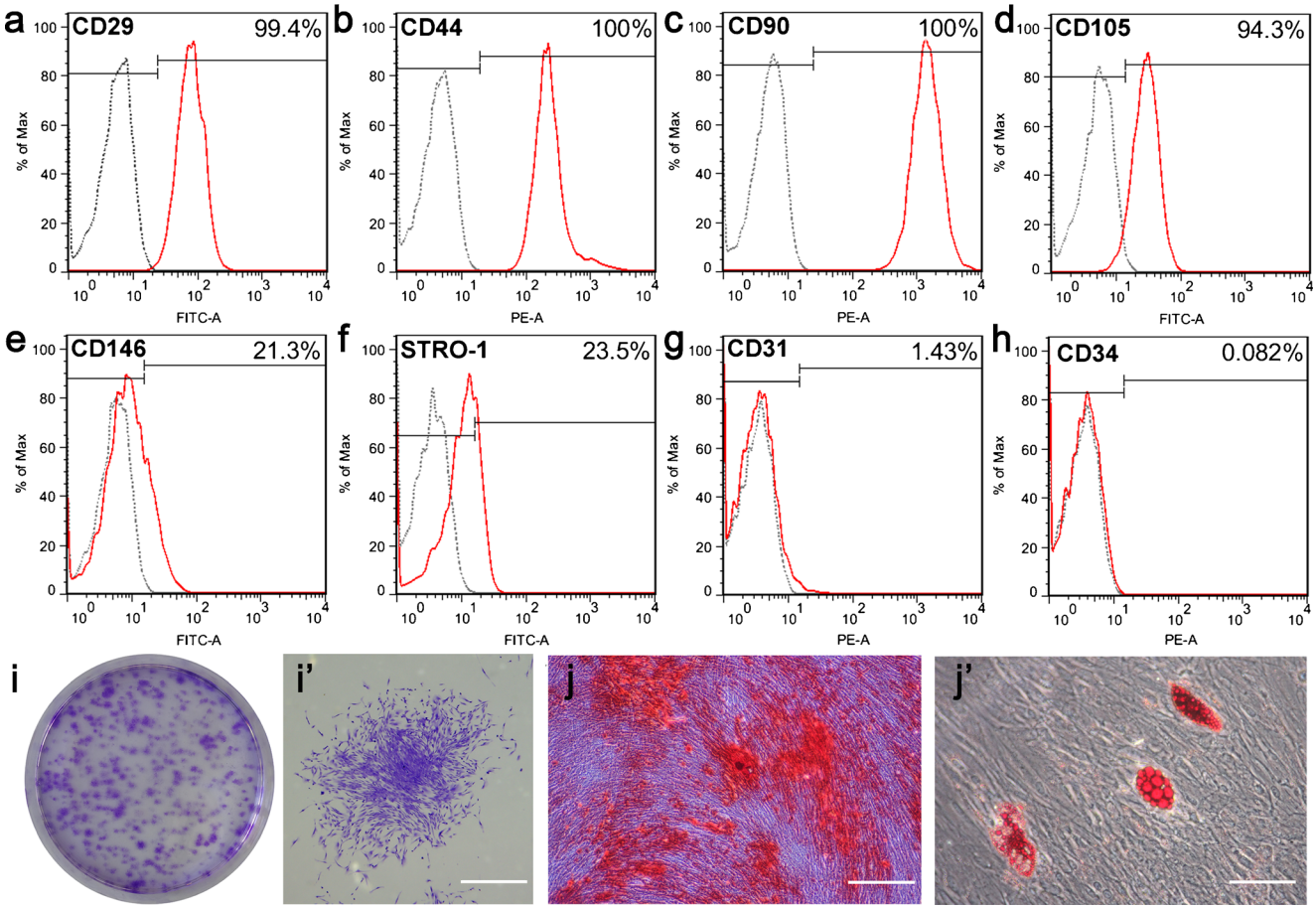


Fig. 1 Identification of human PDLSCs. **a**–**h** Cell surface markers identified by flow cytometric analysis. **i**, **i'** Representative images of colony-forming units (**i**) and a random single-cell clone (**i'**) on day 14. **j**, **j'** Representative images of mineralized cell nodules (**j**: histochemically stained with Alizarin Red) following a 4-week osteogenic induction period and of lipid droplets (**j'**: stained with Oil Red O) following a 3-week adipogenic induction period. *Bars* (**i'**) 1 mm , (**j**) 400 μm, (**j'**) 200 μm

were decalcified with 10 % ethylene diamine tetraacetic acid (EDTA) for 2 weeks. The obtained samples were also subjected to H&E and Masson's trichrome staining. The percentage of new bone area in the total area was calculated from 5 randomly selected fields from each specimen with the aid of Image-Pro Plus 6.0 software. Finally, the mean percentage of new bone area in the total area obtained from 9 animals (3 cell lines) in each treatment group was obtained for the statistical analysis.

In situ periodontal tissue regeneration

Creation of periodontal defects and surgical procedures

The creation of periodontal defects for tissue regeneration analysis was performed as described in previous studies (Yang et al. 2010; Dan et al. 2014). This animal experiment was performed with permission from the Animal Care Committee of FMMU. Briefly, the skin at the surgical site was shaved and disinfected with iodophor. An incision through the full thickness of the skin was made along the inferior border of the unilateral mandible and the masseter muscle and periosteum were removed to expose the buccal surface of the mandible. Finally, an inverted cone bur, supported with copious saline irrigation, was used to remove the alveolar bone and cementum covering the roots of the mandibular molars, resulting in a periodontal defect (approximately 3 mm × 1.5 mm). These defects were transplanted with sheet/CBB constructs (sheets obtained from 6-well culture dishes, folded 4 times and wrapped with 3 mg of CBB), where PDLSC sheets were derived from 4 different pre-incubated cells as described in the group design (no-stimulus, inflammation, hypoxia and dual-stimuli groups). Simultaneously, defects without transplants or those transplanted with only CBB served as the 'Blank' and 'CBB' groups, respectively. Sprague–Dawley (SD) male rats (weighing 220 ± 20 g) from the FMMU Animal Center were randomly divided into 6 groups. Fifty-four animals were used in the present study, with 9 animals in each group. PDLSCs from 3 donors were used independently and the cell transplants derived from each cell line were transplanted into 3 animals in parallel. After implantation, penicillin was

administered by muscular injection at 25,000 U/kg body weight. Rats were euthanized by excess anesthetic 4 weeks after surgery and the mandible samples were harvested and fixed in 4 % paraformaldehyde solution for 24 h.

Micro-CT analysis

A Siemens Inveon Micro-CT System was used to scan mandible samples with the following scan parameters: 30 μm resolution, 80 kV and 500 μA. The obtained data were transferred to a workstation and 3D images were reconstructed by the system software. For further data analysis, the areas of specific density (800–2800) were considered to be involved in new bone formation, while high-density areas (over 2800 density), considered CBB regions, were excluded from the statistical analysis. Within the defect region, the bone volume fraction (BV/TV), trabecular spacing (Tb. Sp) and trabecular bone volume (TBV) were assessed using built-in software.

Histological analysis

After Micro-CT scanning, the obtained mandible samples were decalcified in 10 % EDTA solution for 3–4 weeks and then embedded in paraffin. The samples were sectioned along the vertical direction of the root axis every 5 μm, with every 10th slide stained by H&E and Masson's trichrome staining to locate the defect. The defect area was distinguished by the histologically visible cut edge of the cortical bone and root surface. Based on the observation of the defect area in all stained specimens, 3 specimens from each animal, which could reflect the regeneration status within the defect area, were selected for evaluation and statistical analysis. New bone formation was defined by H&E and Masson's trichrome staining within the defect area. The percentage of new bone area in the total area derived from 5 randomly selected fields for each specimen was averaged by Image-Pro Plus 6.0 software and then the mean values obtained from all specimens in each

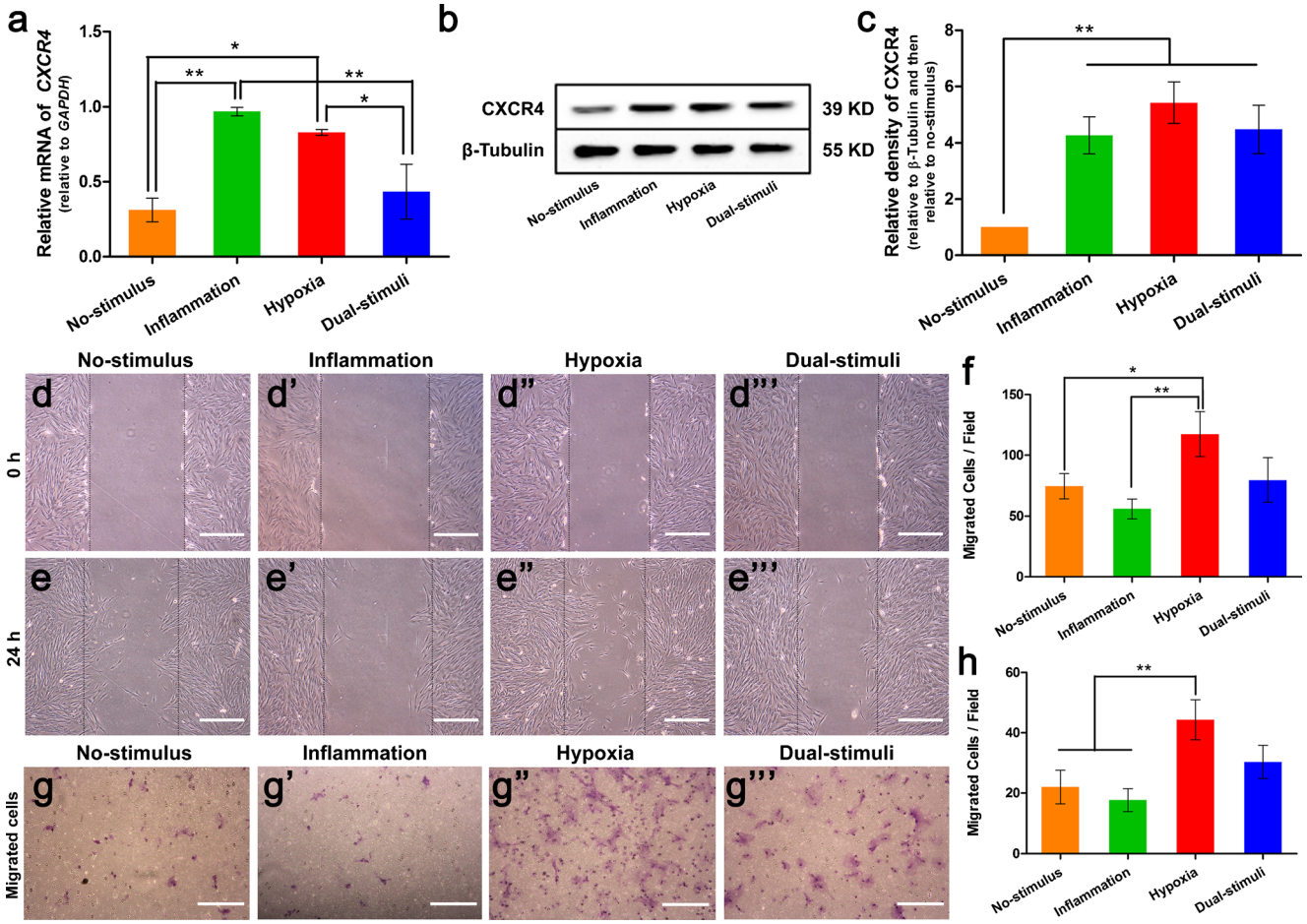


Fig. 2 Effects of 24-h inflammatory and/or hypoxic pretreatments, as described in the group design, on cell migration capacity. **a** Relative *CXCR4* gene expression level determined by real-time PCR analysis. **b**, **c** Representative images (**b**) and quantitative analysis (**c**) of CXCR4 protein expression by western blot analysis. **d**–**e**''' Representative images of cell migration 0 h (**d**–**d**''') and 24 h (**e**–**e**''') after scratching. **f** Statistical analysis of the number of migrated cells in the tablet scratch experiment. **g**–**g**''' Representative images of cell migration in response to SDF-1 (100 ng/mL) in the lower chamber of a Transwell system. **h** Statistical analysis of the number of migrated cells (SDF-1-induced chemotaxis) based on the Transwell system. $*P < 0.05$ and $**P < 0.01$ represent significant differences between the indicated columns. *Bars* (**d**–**e**''') 1 mm, (**g**–**g**''') 200 μm

treatment group were calculated for statistical analysis. New cementum was assessed as the mineralized tissue formed on the denuded root surface with collagen fiber insertion. The percentage of new cementum was calculated by dividing the length of the root surface covered in new cementum with the length of the whole denuded root surface. New PDL tissue was identified as collagenous fibers covering the denuded root surface, with the angle between the long axis of the fibers and the new cementum greater than 60°. The percentage of new PDL was calculated by dividing the length of the root surface covered with new collagenous fibers by the length of the entire denuded root surface.

Statistical analyses

All the data are presented as the means ± standard deviation (SD) from at least 3 independent experiments for each cell line. The obtained data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-test or two-way ANOVA using GraphPad Prism 5 software. $P < 0.05$ was considered statistically significant.

Results

Isolation and identification of human PDLSCs

Primary PDL cells were successfully isolated from the obtained teeth. PDLSCs derived from 3 donors exhibited long spindle-like morphology and were positive for the MSC markers CD29, CD44, CD90, CD105, CD146 and STRO-1 (Fig. 1a–f) but negative for the hematopoietic markers CD31 and CD34 (Fig. 1g, h). All cells derived from different individuals exhibited the ability to form colonies or to transdifferentiate into osteogenic and adipogenic lineages in vitro (Fig. 1i–j').

Effects of different pretreatments on cell migration

The effects of the 24-h inflammatory and/or hypoxic pretreatments on CXCR4 expression of PDLSCs were investigated by real-time PCR and western blot analyses. Real-time PCR showed that single inflammatory or hypoxic pretreatment resulted in significantly increased *CXCR4*

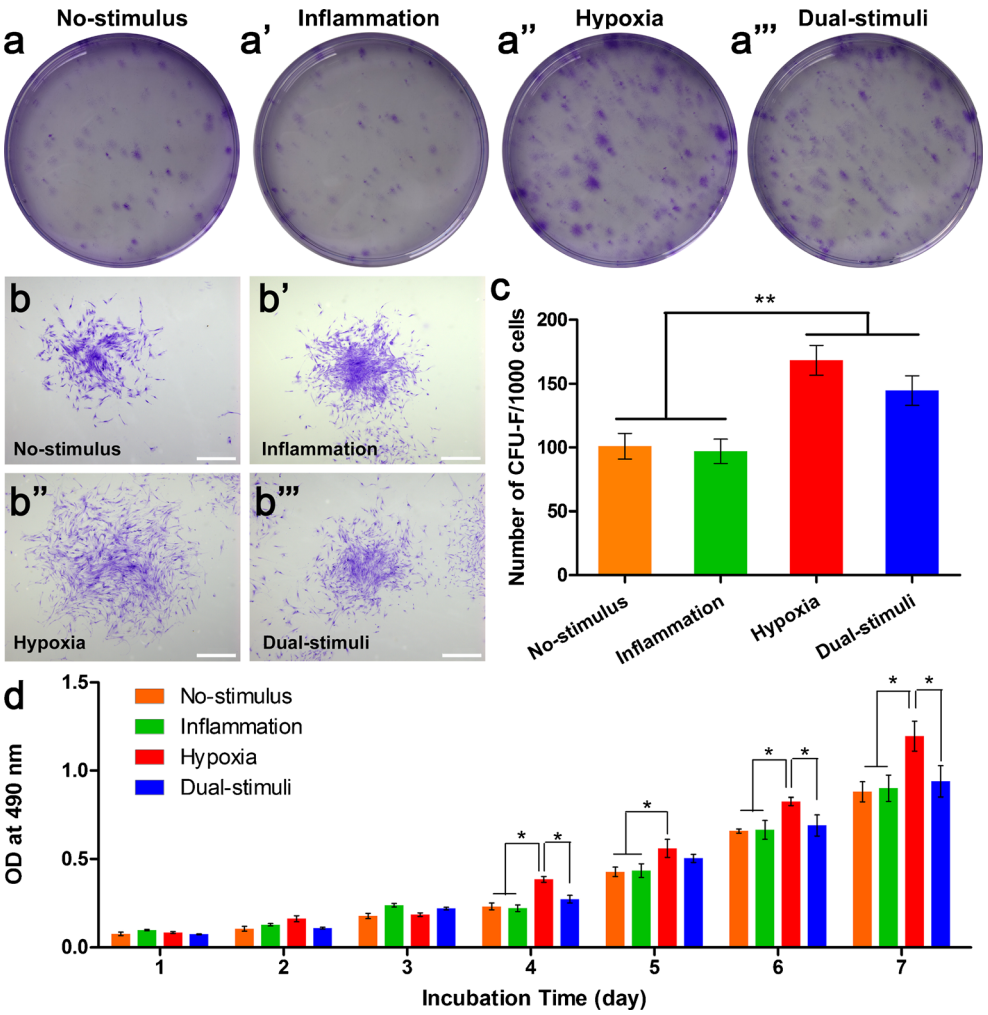


Fig. 3 Effects of 24-h inflammatory and hypoxic pretreatments, as described in the group design, on cell proliferation. **a**–**a'''** Representative images of cell colonies formed by cells from the 4 groups. **b**–**b'''** Representative images of a single colony-forming unit in each group. **c** Quantitative analysis of colony-forming units. **d** Proliferative capacity evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay during a 7-day incubation period. *$P < 0.05$ and **$P < 0.01$ represent significant differences between the indicated columns. *Bar* 1 mm

gene expression compared with non-pretreatment (no-stimulus; $P < 0.05$) or inflammatory plus hypoxic pretreatment (dual-stimuli; $P < 0.01$). Although inflammatory plus hypoxic pretreatment appeared to increase *CXCR4* expression, no significant difference was found between the no-stimulus group and the dual-stimuli group ($P > 0.05$; Fig. 2a). Additionally, CXCR4 protein expression in pretreated cells from the 3 testing groups was higher than that in cells from the no-stimulus group ($P < 0.01$; Fig. 2b, c). Based on tablet scratch experiments, the number of migrated cells from the hypoxia group was significantly higher than that from either the no-stimulus group or the inflammation group ($P < 0.05$ or $P < 0.01$). Contrary to speculation, the number of migrated cells in the inflammation group was less than that in the no-stimulus group, although there was no significant difference. In addition, there was no significant difference in cell migration between the no-stimulus group and the dual-stimuli group ($P > 0.05$) (Fig. 2d–f). Consistent with the results of the tablet scratch experiment, the results of the Transwell assay indicated that the cell migration toward SDF-1 was significantly enhanced following pretreatment with the hypoxic stimulus (hypoxia group) compared with no pretreatments (no-stimulus) or pretreatment with the inflammatory stimulus (inflammation) ($P < 0.01$; Fig. 2g, h). Similarly, dual stimuli did not result in enhanced cell migration compared to the control group (no-stimulus) ($P > 0.05$; Fig. 2g, h).

Effects of different pretreatments on cell proliferation

In both the colony-forming and MTT assays, the results showed that hypoxic pretreatment increased cell proliferation compared with either no pretreatment or inflammatory pretreatment ($P < 0.05$ or $P < 0.01$; Fig. 3). Although the cells pretreated with inflammatory plus hypoxic stimuli (dual-stimuli) also showed enhanced proliferation compared with those without any pretreatment in the colony-forming assay ($P < 0.01$; Fig. 3a–c), such enhancement was not observed in the MTT assay ($P > 0.05$; Fig. 3d). Compared to controls (no-stimulus), the inflammatory pretreatment had no obvious effect on cell proliferation ($P > 0.05$; Fig. 3a–d).

Effects of different pretreatments on cell differentiation

No significant difference in *RUNX-2*, *ALP* or *OCN* levels was observed between the hypoxia group and no-stimulus group as detected by real-time PCR analysis ($P > 0.05$; Fig. 4a). However, Western blot analysis showed increased RUNX-2 and ALP protein expression in the hypoxia group ($P < 0.05$; Fig. 4b, b'). Compared to controls, the gene expression levels of *RUNX-2* and *OCN* were significantly decreased in the dual-stimuli group ($P < 0.05$; Fig. 4a), while the protein expression of OCN decreased in the inflammation group ($P < 0.05$; Fig. 4b, b'). No significant effects of gene or protein levels on adipogenic differentiation following 24-h inflammatory

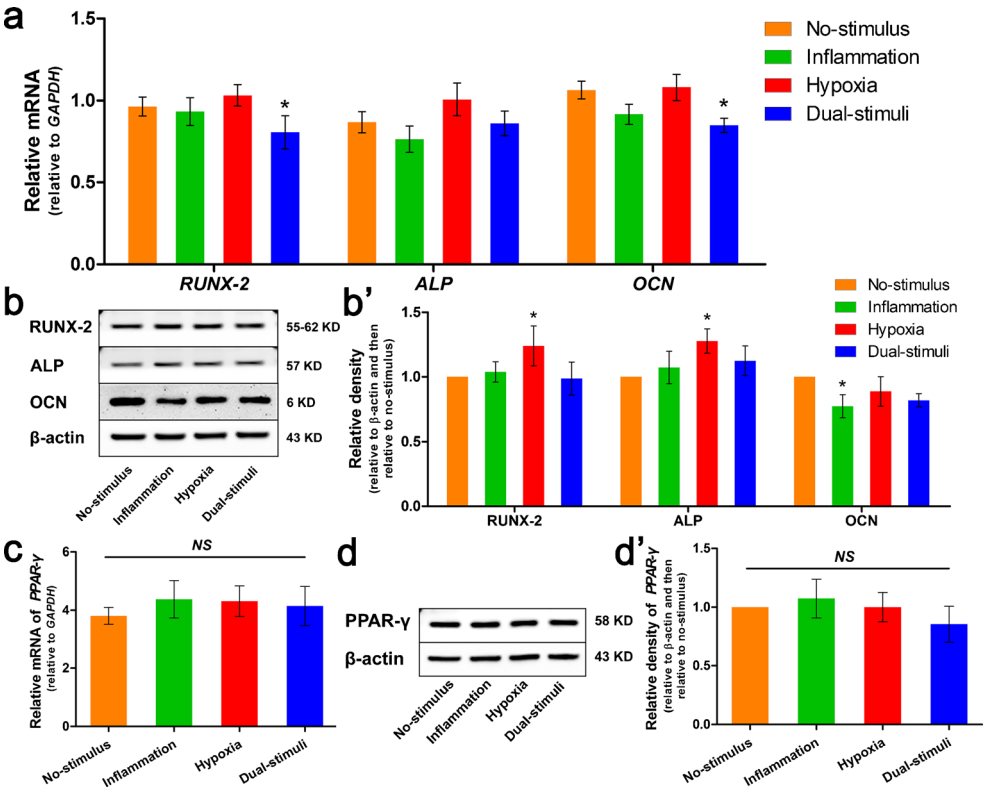


Fig. 4 Effects of 24-h inflammatory and hypoxic pretreatments, as described in the group design, on cell differentiation. **a** Quantitative analysis of *RUNX-2, ALP* and *OCN* gene levels of cells in the 4 groups by real-time PCR analysis. **b**, **b'** Representative images (**b**) and quantitative analysis (**b'**) of RUNX-2, ALP and OCN protein expression by western blot analysis. **c** Quantitative analysis of *PPAR-γ* gene levels. **d**, **d'** Representative images (**d**) and quantitative analysis (**d'**) of PPAR-γ protein expression. *$P < 0.05$ represents significant differences compared with the control (no-stimulus group). *NS* no significant differences between the indicated columns.

and hypoxic pretreatments were observed ($P > 0.05$; Fig. 4c–d').

Effects of different pretreatments on cell sheet production

After cell sheet induction, all pre-incubated PDLSCs formed complete cell sheets that could be detached from the plates by forceps (Fig. 5a–a"'). H&E staining revealed that the sheets in the hypoxia group were the thickest, while those in the inflammation group were the thinnest among the 4 groups; no significant difference in cell sheet thickness was observed between the no-stimulus group and the dual-stimuli group ($P > 0.05$) (Fig. 5b, c). Furthermore, we found that inflammatory and hypoxic pretreatments had no significant effects on ECM protein (COL-1, fibronectin and integrin β1) production in the cell sheets ($P > 0.05$; Fig. 5d–l).

Ectopic bone formation in vivo

In vivo ectopic bone formation of the 4 designed transplants was determined by Micro-CT and H&E/Masson's trichrome staining. Micro-CT scanning (Fig. 6a–a"') and a bone density analysis (Fig. 6b) revealed that the transplants in the hypoxia group displayed the most obvious bone formation among the 4 groups. Compared to the control group (no-stimulus), there was less hard tissue formed around the CBB in the inflammation group and the dual-stimuli group. Consistent with the Micro-CT findings, H&E and Masson's trichrome staining revealed that the transplants from the hypoxia group had larger new bone areas than those from the other 3 groups ($P < 0.05$ or $P < 0.01$; Fig. 6c–g). Although the new bone areas generated in the inflammation and dual-stimuli groups appeared to be smaller than those in the no-stimulus group, the differences were not statistically significant ($P > 0.05$; Fig. 6c–g).

In situ periodontal tissue regeneration

At 4 weeks after periodontal transplantation, the mandible samples were subjected to an analysis of new periodontal tissue regeneration. In general, cell-based transplants resulted in positive outcomes compared to the 2 negative controls, namely, defects without the placement of any transplants (Blank group) and defects transplanted with CBB only (CBB group). To avoid confusion, data generated from the 2 negative controls in this study were excluded from further analysis. Three-dimensional reconstruction images by Micro-CT scanning showed that the defects in the hypoxia group were almost completely covered by mineralized tissues. Although the defects in the no-stimulus group samples were still obvious, exposed roots were generally not observed. In comparison, at least partial surface exposure was observed in samples from the inflammation group and dual-stimuli group (Fig. 7a–a""'). Further analysis suggested that the hypoxia group generated more new bone among the 4 cell-based groups in terms of the BV/TV ratio (Fig. 7b) and trabecular spacing (Tb. Sp) (Fig. 7c). Although the TBV of the samples in the hypoxia group was larger than in those from the other 3 cell-based groups, a significant difference was found only between the hypoxia group and inflammation group ($P < 0.01$; Fig. 7d). Using H&E and Masson's trichrome staining (Fig. 7e–h""'), the hypoxia group revealed a significantly higher percentage of new bone coverage compared with the other 3 cell-based groups ($P < 0.01$; Fig. 7i). New cementum and new PDL tissue were only observed in the no-stimulus and hypoxia groups (Fig. 7e–h""'). Although the hypoxia group appeared to generate more new cementum and new functional PDL fibers than the no-stimulus group, the difference was not statistically significant ($P > 0.05$; Fig. 7j, k).

Discussion

Stem cell-based therapies offer tremendous potential in treating various degenerative diseases and have increasingly become the ideal therapeutic approach to cure periodontitis via periodontal tissue regeneration (Pihlstrom et al. 2005; Izumi et al. 2011; Chen et al. 2012; Lu et al. 2013; Monsarrat et al. 2014). Since the isolation and identification of multipotent postnatal stem cells from human PDL tissue 10 years ago (Seo et al. 2004), these cells, defined as PDLSCs, have become an interesting adult stem cell source for regenerative dentistry because of their ease of isolation from discarded or removed teeth, particularly those teeth extracted for impaction or orthodontic reasons (Chamila Prageeth Pandula et al. 2014; Bright et al. 2015). Furthermore, these cells can differentiate into osteoblasts, chondrocytes, neurons and muscle cells, among others. PDLSCs are derived from ectomesenchymal origin, are formed during tooth development and have been investigated for the repair of tissues not only within but also outside the mouth (Chen et al. 2012; Chamila Prageeth Pandula et al. 2014). In particular, many preclinical and clinical studies have suggested that PDLSCs represent an effective therapeutic tool for periodontal regeneration (Bright et al. 2015; Bassir et al. 2016). In parallel, methods for PDLSC isolation and production are developing very quickly, from rapid and safe cell propagation/expansion (Iwata et al. 2010; Jung et al. 2013; Trubiani et al. 2015; Wu et al. 2016) to optimal cell material production (Wei et al. 2012; Gao et al. 2013; Iwata et al. 2015). Nevertheless, major challenges still exist, particularly challenges related to producing large numbers of cells from clinically limited PDL tissues and manufacturing/banking robust cell materials, ideally in an off-the-shelf format (Izumi et al. 2011; Tang et al. 2016; Wu et al. 2016). Great efforts have been and are still being made in this arena to either avoid long-term ex vivo manipulation, maintain cell properties

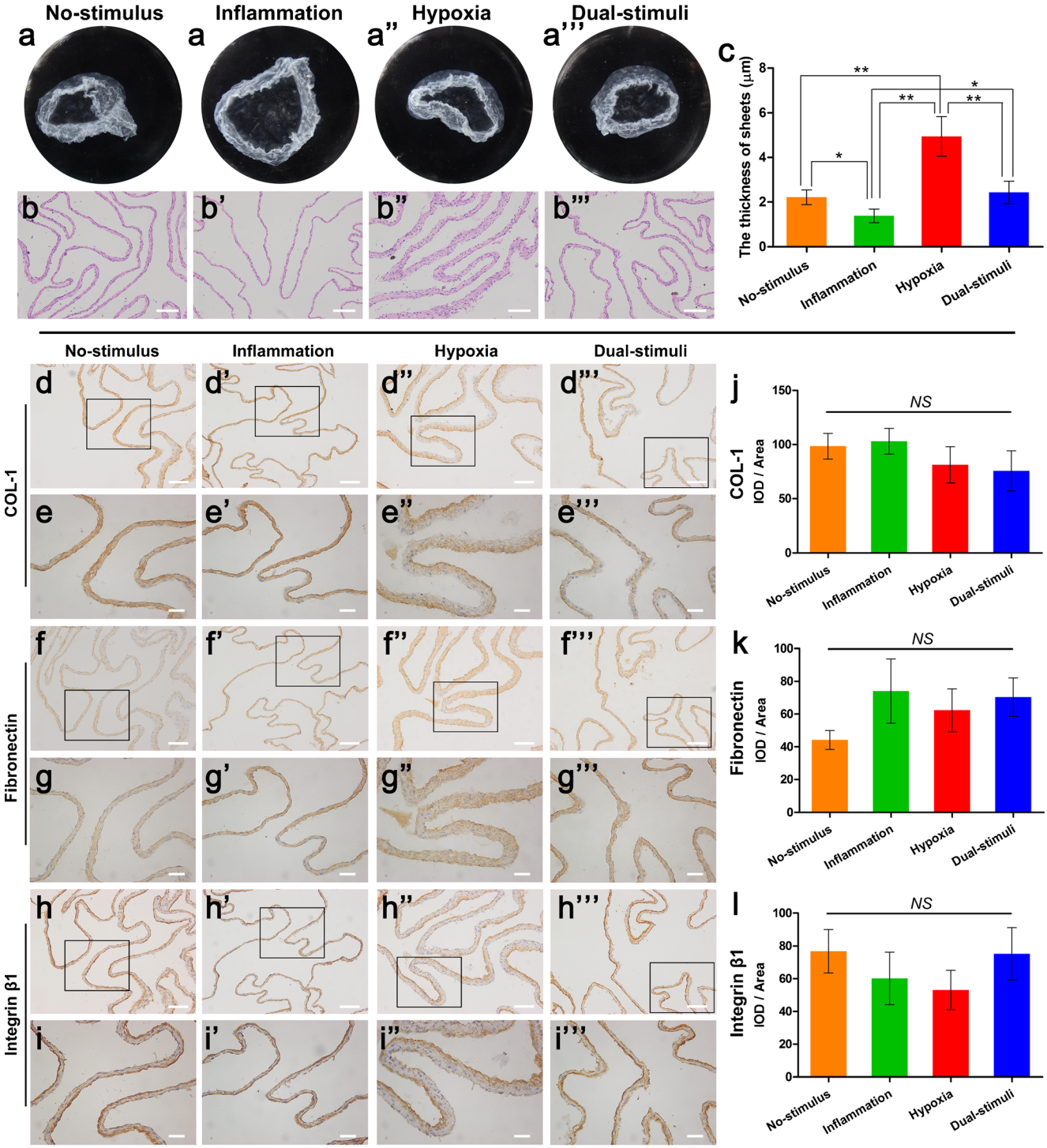


Fig. 5 Effects of 24-h inflammatory and/or hypoxic pretreatments, as described in the group design, on cell sheet production. **a**–**a**”’ Representative images of PDLSC sheets in the plates (general view). **b**–**b**”’ Representative images of PDLSC sheets observed by hematoxylin and eosin (H&E) staining. **c** Analysis of the thickness of the PDLSC sheets. **d**–**i**”’ Immunohistochemical (IHC) staining of COL-1 (**d**–**e**”’), fibronectin (**f**–**g**”’) and integrin β1 (**h**–**i**”’) proteins in the cell sheets. **j**–**l** Statistical expression intensity of COL-1 (**j**), fibronectin (**k**) and integrin β1 (**l**) via Image-Pro Plus 6.0 software. $*P < 0.05$ and $**P < 0.01$ represent significant differences between the indicated columns. *NS* no significant differences between the indicated columns. *Bars* (**b**–**b**”’, **d**–**d**”’, **f**–**f**”’, **h**–**h**”’) 50 μm, (**e**–**e**”’, **g**–**g**”’, **i**–**i**”’) 20 μm

during extensive ex vivo handling and/or modify/engineer cell behavior toward a target purpose (Sharma et al. 2011; Thirumala et al. 2013). Based on the data published to date, short-term pretreatment of cells is the most utilized method (Peng et al. 2013; Lee et al. 2015). Although mounting evidence has demonstrated the beneficial effects of short-term inflammatory or hypoxic pretreatment on the behavior of several MSC types (Basciano et al. 2011; Glass et al. 2011; Fan

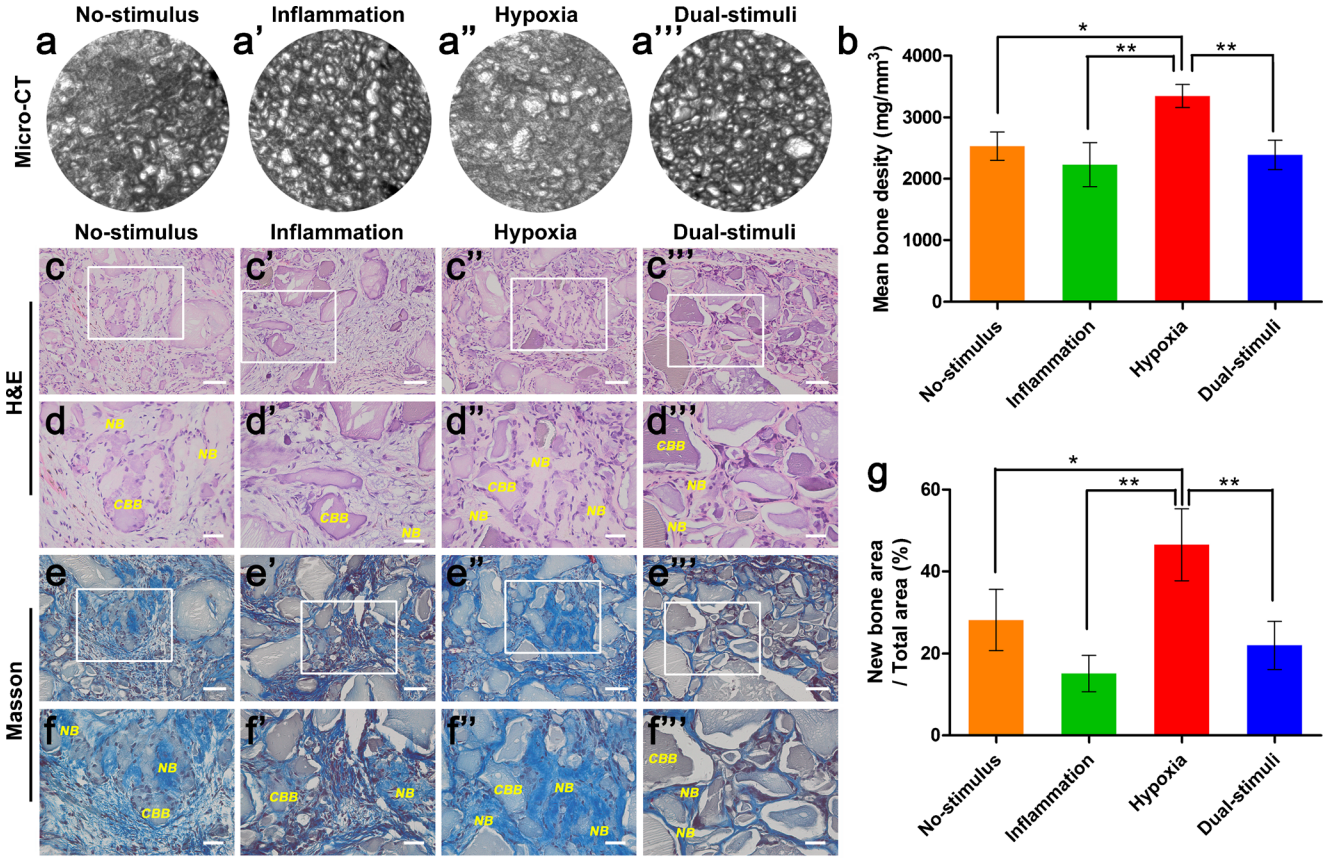
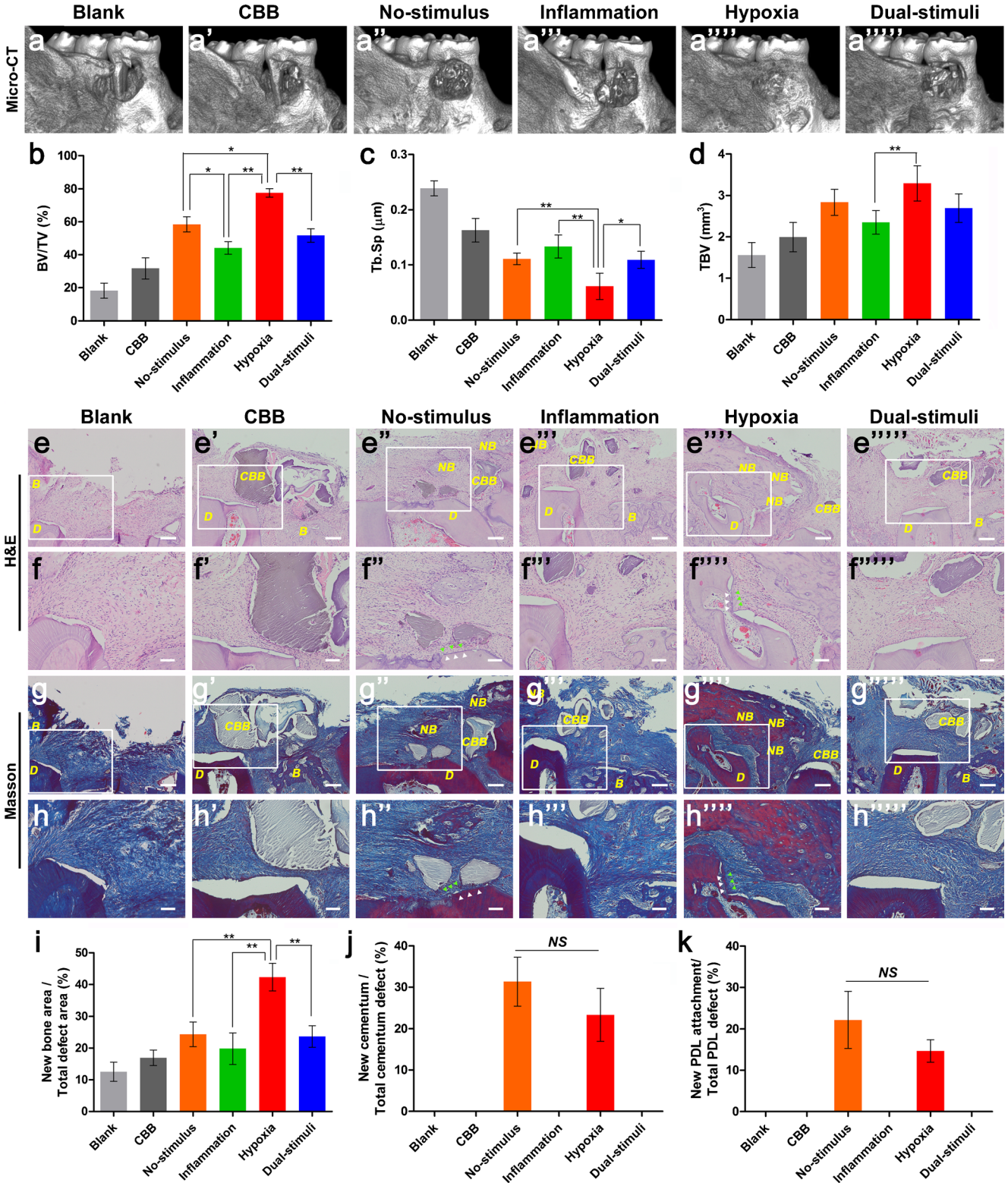


Fig. 6 Effects of 24-h inflammatory and hypoxic pretreatments, as described in the group design, on cell-based ectopic bone formation in vivo. **a**–**a**”’ Representative Micro-CT reconstruction images showing new bone formation around calcined bovine bone (CBB) in the 4 groups. **b** Quantitative analysis of the mean bone density by Micro-CT software. **c**–**f**”’ Representative images of hematoxylin and eosin (H&E) (**c**–**d**”) and Masson's trichrome staining (**e**–**f**”’) (*NB* new bone, *CBB* calcined bovine bone). **g** Quantitative analysis of the percentage of new bone area in the total area by Image-Pro Plus 6.0 software. $*P < 0.05$ and $**P < 0.01$ represent significant differences between the indicated columns. *Bars* (**c**–**c**”’, **e**–**e**”’) 50 μm, (**d**–**d**”’, **f**–**f**”’) 20 μm

et al. 2012; Beegle et al. 2015; Yu et al. 2016), this study is the first to investigate the cellular responses of PDLSCs to short-term inflammatory and hypoxic pretreatments and the influences of such pretreatments on subsequent cell regenerative potential.

The ability of cells to migrate and home to a site of injury is an important issue for cellular therapy. Currently, 3–6 weeks are generally required for ex vivo expansion to obtain enough cells for transplantation; most of those culture-expanded cells were found to lose their migration potential, at least to a certain degree (Maijenburg et al. 2012). Given the finding that CXCR4, which is expressed on the surface of MSCs, plays an important role in cell migration via bonding to SDF-1 (Nagasawa 2014), we first investigated the effects of inflammatory and/or hypoxic pretreatments on the CXCR4 expression of PDLSCs in the current study. Consistent with previous studies (Shi et al. 2007; Liu et al. 2010; Ziaei et al. 2014), increased CXCR4 expression was found on cells in response to inflammatory or hypoxic pretreatments but dual stimuli did not result in a synergistic effect (Fig. 2a–c). However, based on the tablet scratch experiment and Transwell assays, only hypoxic pretreatment induced a significant increase in cell migration irrespective of the chemotactic effect of SDF-1;

Fig. 7 Effects of 24-h inflammatory and hypoxic pretreatments, as described in the group design, on cell-based in situ periodontal tissue regeneration, where defects without placement of any transplants and defects transplanted with only CBB were marked as the *Blank* group and the *CBB* group, respectively. **a**–**a**””’ Representative Micro-CT reconstruction images of periodontal defect areas at 4 weeks. **b**–**d** Quantitative analysis of the bone volume fraction (*BV/TV*) ratio (**b**), trabecular spacing (*Tb. Sp*) (**c**) and trabecular bone volume (*TBV*) (**d**) within defect areas. **e**–**h**””’ Representative images of hematoxylin and eosin (H&E) (**e**–**f**””’) and Masson's trichrome staining (**g**–**h**””’) within the defect areas (*NB* new bone, *CBB* calcined bovine bone). White arrows show the new cementum formed on the denuded root surface; green arrows show new periodontal ligaments attached to the new cementum. **i** Quantitative analysis of the percentage of new bone area in the total defect area. **j** Percentage of the length of the root surface covered by new cementum within the length of the entire denuded root surface. **k** Percentage of the length of the root surface covered by new collagenous fibers within the length of the entire denuded root surface. $*P < 0.05$ and $**P < 0.01$ represent significant differences between the indicated columns. *NS* no significant differences between the indicated columns. *Bars* (**e**–**e**””’, **g**–**g**””’) 50 μm, (**f**–**f**””’, **h**–**h**””’) 20 μm



inflammatory pretreatment did not exhibit a positive effect (Fig. 2d–h). We suspect the reason for this phenomenon is that PDLSCs are very sensitive to the inflammatory stimulus. In this study, based on our previous work (Yang et al. 2010; Yu et al. 2016) as well as that of many others (Liu and Hwang 2005; Ziaei et al. 2014), 10 ng/mL TNF-α and 5 ng/mL IL-1β were used to establish the inflammatory stimulus environment. Although up to 30 ng/mL IL-1β (Liu and Hwang 2005) and up to 10 ng/mL TNF-α (Ziaei et al. 2014) were demonstrated to be safe for cell modification, the dose for their

combined use is still unknown. The use of high concentrations of combined cytokines (10 ng/mL TNF-α and 5 ng/mL IL-1β) has the potential to impair the activity of PDLSCs to migrate. In this case, although increased CXCR4 expression was found in the inflammation group and in the dual-stimuli group, significant enhancement of cell migration could not be observed. Although further studies are required to identify the underlying reason, there is evidence that short-term stimulation of human bone marrow-derived MSCs with a cocktail of cytokines upregulates both cell surface and intracellular CXCR4 expression and increases in vitro migration to SDF-1 and homing to the bone marrow of irradiated NOD/SCID mice (Shi et al. 2007). This finding suggests that if properly designed, a combination of TNF-α and IL-1β may also lead to a synergistic effect. Given possible synergistic inflammatory responses, the concentration of TNF-α and IL-1β used in combination would need to be further decreased.

The cell proliferation rate in cultures is critical for obtaining sufficient cell populations in an acceptable period. In this regard, accumulating evidence indicates that the proliferation of MSCs increases under hypoxic conditions (1 % O_2 to 5 % O_2) (Nekanti et al. 2010; Basciano et al. 2011; Estrada et al. 2012). Grayson and colleagues reported that human MSCs could maintain their proliferative capacity under hypoxic conditions (2 % O_2) for long-term incubation (Grayson et al. 2007). Recently, the proliferation rate of PDLSCs was found to increase significantly after exposure to a 2 % hypoxic atmosphere (Zhang et al. 2014). Based on the colony-forming and MTT assays, our data also confirmed that cells pretreated with a hypoxic stimulus (2 % O_2) exhibited a stronger capacity for self-renewal compared to the controls (Fig. 3). This finding is not consistent with a previous report, where the proliferation of human MSCs was significantly reduced in 1 % or less O_2 culture conditions (Holzwarth et al. 2010). The combined data suggest that cell responses to a hypoxic stimulus may vary from cell type to cell type. While previous work indicated that an inflammatory stimulus causes an increased proliferation rate of PDLSCs (Yang et al. 2013; Tang et al. 2016; Zheng et al. 2015), such an increase has not been detected in this particular research; our data suggest that the 24-h inflammatory pretreatment has no obvious effect on PDLSC colony formation and proliferation (Fig. 3). We further found that, although PDLSCs pretreated with inflammatory plus hypoxic stimuli showed enhanced proliferation compared to those without pretreatment by colony-forming assay (Fig. 3a–c), such enhancement was not observed in the MTT assay (Fig. 3d). In the colony-forming assay, only aggregates of 50 or more cells were scored as colonies for the statistical analysis. However, the cell number in each aggregate may change from 50 to hundreds or even more (Fig. 3b–b"'). In contrast, in the MTT assay, the OD value reflects the exact cell number. This difference may partially explain the discrepancy in terms of colony-forming and MTT assays. Indeed, the OD values in the dual-stimuli group from days 3 to 6 were quantitatively higher than those in no-stimulus group but this difference was not statistically significant (Fig. 3d).

The osteogenic differentiation of PDLSCs into osteoblasts is a prerequisite for subsequent bone formation. Thus far, there is no unanimous agreement regarding the effects of inflammatory or hypoxic stimuli on the osteogenic and adipogenic differentiation potentials of MSCs. Zhang and colleagues reported that PDLSCs induced under an atmosphere of 5 % CO_2 and 2 % O_2 exhibited a higher osteogenic differentiation potential in vitro and that those cells differentiated into osteoblasts most rapidly in vivo (Zhang et al. 2014). Although this outcome has been supported by most, if not all, previous studies using other MSC types (Grayson et al. 2007; Nekanti et al. 2010; Valorani et al. 2012), the opposite finding for PDLSCs has also been reported, whereby cells exhibited a reduced osteogenic potential when maintained and induced in a 5 % O_2 concentration (Wu et al. 2013). However, the previous researchers exposed cells to hypoxic conditions for prolonged periods, while the effects of a short-term hypoxic stimulus on the osteogenic potential of PDLSCs were not investigated. As for the inflammatory stimulus, TNF-α and IL-β have been reported to inhibit cell osteogenic differentiation and subsequent bone formation (Schett 2011; Zhao et al. 2012). However, several other reports support the finding that TNF-α promotes the osteogenic differentiation of human MSCs (Hess et al. 2009; Glass et al. 2011) and there is evidence that IL-1β elicits biphasic effects on in vitro cell osteogenic differentiation (Lin et al. 2010). Alone or in combination, variations in the inflammatory condition design (e.g., cytokine dose and combination), cell incubation duration and differences in cell sources and donors (e.g., age and gender) may contribute to such disparities. From a careful review of the literature, we soon realized that many more questions remain to be elucidated in this field. When the in vitro osteogenic and adipogenic differentiation potentials of PDLSCs in response to 24-h inflammatory or hypoxic pretreatment were investigated in the present study, we found that hypoxic pretreatment resulted in increased protein expression of RUNX-2 and ALP, while inflammatory pretreatment led to decreased protein expression of OCN (Fig. 4a–b'). Furthermore, the gene expression of *RUNX-2* and *OCN* was significantly decreased when cells were pretreated with inflammatory plus hypoxic pretreatments (Fig. 4a–b'). Inflammatory and/or hypoxic pretreatments had no significant effects on the in vitro adipogenic differentiation of PDLSCs at both the gene and protein levels (Fig. 4c–d'). Because different osteogenic genes and proteins play their respective roles at different stages of osteogenesis, their expression levels could differ in response to inflammatory and/or hypoxic stimuli. In addition, the expression levels of the same gene and protein could also differ because of the intended sequence of gene transcription and protein translation. This possibility may partially explain why the western

blot data in the present study were not similar for all groups and why there were also some differences between real-time PCR and western blot analyses. From the data obtained in this particular experimental design, we can only conclude that short-term hypoxic incubation appears to be a useful strategy for the modification of PDLSC osteogenic differentiation. Nevertheless, none of the designed pretreatments induced any observed adverse effects on PDLSC differentiation.

Given the poor engraftment following cell transplantation (Chen et al. 2012), cell sheet engineering has been widely employed as an effective method for cell delivery for periodontal regeneration (Iwata et al. 2015). The use of cell sheets can improve the survival rate of therapeutic cells and allows them to easily attach to host tissues. Moreover, the cell–matrix and cell–cell interactions in an intact sheet play an essential role in maintaining their cell genomic stability as 'building blocks' and their biochemical signaling and modulation of stem cell behavior toward therapeutic regeneration (Patel and Zhang 2014; Wu et al. 2015). Most importantly, cell sheets make delivering and securing PDLSCs onto the tooth root surface possible, which is a key to PDL tissue regeneration (Dan et al. 2014). Therefore, the identification of an appropriate technique for enhancing sheet production from PDLSCs has attracted growing interest (Wei et al. 2012; Gao et al. 2013). In contrast, the ability of cells to generate robust cell sheets has become a key parameter for evaluating the properties of a target cell population (Tang et al. 2016; Wu et al. 2015). In the present study, we attempted to identify whether 24-h inflammatory and hypoxic pretreatments could facilitate sheet formation of PDLSCs and improve their performance in therapeutic applications. Cell sheets generated by cells pretreated with a hypoxic stimulus were thicker than those generated by cells without pretreatment, whereas pretreatment with an inflammatory environment weakened their ability to form sheets, as demonstrated by the thinner sheets produced by the cells in this group. Meanwhile, inflammatory plus hypoxic pretreatment had no obvious effect on the thickness of the resulting cell sheet (Fig. 5a–c). In addition, components of the ECM and their levels also play a pivotal role in the overall therapeutic outcome. Therefore, we further evaluated the representative ECM protein production (i.e., COL-1, fibronectin and integrin β1) of cell sheets using IHC staining (Fig. 5d–i"'). Quantitative analysis revealed no significant difference in ECM contents among the 4 groups (Fig. 5j–l), suggesting that inflammatory and hypoxic pretreatments do not harm PDLSCs in terms of their protein production.

When the sheet/CBB transplants were subcutaneously placed into the dorsal region of male nude mice, all transplants led to significant bone regeneration as measured by Micro-CT and H&E/Masson's trichrome staining (Fig. 6a–a"', c–f"'). Statistical analysis of the mean bone density (Fig. 6b) and the percentage of new bone area (Fig. 6g) showed that sheets formed by cells following hypoxic pretreatment generated more bone compared to sheets obtained from the other 3 designed groups; inflammatory alone or inflammatory plus hypoxic pretreatment appeared to have a negative effect on the bone-forming capabilities of PDLSC sheets compared to the control, although there were no significant differences (Fig. 6b, g). These data are generally consistent with the in vitro analysis of cell osteogenic marker expression. The overall findings imply that 24-h hypoxic pretreatment of PDLSCs promotes their osteogenic potential both in vitro and in vivo. Although we could not provide conclusive information on the outcomes of other pretreatments from this study, the preconditioned cells were able to maintain their osteogenic and bone regenerative potentials.

Another important objective of this study was to evaluate the potential of transplants to synchronously generate alveolar bone, cementum and PDL in a periodontal defect model, wherein the sheets were placed directly facing the denuded root to increase the number of PDLSCs attached to the root surfaces. Defects without the placement of any transplants (Blank group) and defects transplanted with only CBB (CBB group) were used to ensure the success of the model but the data generated from these 2 negative controls were excluded from further statistical analysis. We found that all the cell sheets exhibited significant mineralization potentials (Fig. 7a–a"'). In general, cell sheets derived from cells following hypoxic pretreatment formed more mineralized tissue (Fig. 7b–d). Moreover, the red area in slices of Masson's trichrome staining, which indicates new bone formation, could be largely observed in the 4 designed groups (Fig. 7e–h"'), with the highest value identified in the hypoxic group (Fig. 7i). All these data suggest that hypoxic pretreatment of PDLSCs enhances their potential to form hard tissue but other pretreatments were found to either have no impact or slightly weaken this potential. Unfortunately, new cementum formation and PDL fibers with an angle of greater than 60° inserted into the newly deposited cementum were only identified in the no-stimulus and hypoxic groups (Fig. 7j, k). The reason underlying this finding requires further investigation.

Taken together, our results show that short-term hypoxic pretreatment efficiently improves the migration, proliferation and differentiation of PDLSCs and promotes in vivo ectopic bone/periodontal tissue formation. Long-term hypoxic stimulus might negatively affect cells via up-regulation of HIF-1 expression. Additionally, there is mounting evidence that short-term hypoxic stimulus positively modifies cell behavior (Liu et al. 2010; Beegle et al. 2015; Yu et al. 2016). Thus far, no studies have reported the response of PDLSCs to a short-term hypoxic stimulus. In this study, we found that the short-term (24 h) hypoxic stimulus improved PDLSC proliferation, migration and differentiation potentials. Although the underlying mechanism remains unknown, short-term hypoxic stimulus might produce a state of stress in the cells. In this case, most cell activities could be improved. Although an

inflammatory stimulus, at least in certain selected situations, has also been suggested as a useful technique for cell pretreatment, no enhancement of PDLSC proliferation and migration in response to inflammatory factors (10 ng/mL TNF-α and 5 ng/mL IL-1β) was observed in this work. From a clinical point of view, incubation of PDLSCs in an inflammatory environment may impair their potency and regenerative potential, as demonstrated by those cells derived from teeth extracted due to periodontal disease (Tang et al. 2016). Because no harm resulted from the synergistic effect of the combined pretreatments, we believe that the inflammatory plus hypoxic pretreatments for PDLSCs before their use is a promising choice, as inflammatory pretreatment enhances cell proliferation and migration (Ziaei et al. 2014; Zheng et al. 2015), while hypoxic pretreatment maintains cell genomic stability and enhances regenerative potential (Estrada et al. 2012; Zhou et al. 2014). Clearly, such a hypothesis warrants further in-depth investigation.

Conclusions

The ability to isolate and manipulate stem cells from human medical wastes, such as dental stem cells including but not limited to PDLSCs, has been a significant advancement in tissue engineering and new approaches are continuing to be developed. In this context, PDLSCs hold great promise for developing effective cellular therapeutics and recent work on clinical trials employing PDLSCs has raised hopes for functional periodontal regeneration. Exploiting the full potential of PDLSCs requires the development of cost-effective techniques for cell production, manufacturing, cryopreservation and banking. Current trends indicate that this demand will continue to increase. Currently, producing large numbers of cells necessitates extensive ex vivo cell expansion, which subsequently impairs cell potency. Our findings from this study indicate that short-term hypoxic pretreatment of PDLSCs improves their in vitro cellular behavior and enhances their in vivo regenerative potential; however, the preconditioning of PDLSCs using medium containing 10 ng/mL TNF-α and 5 ng/mL IL-1β did not lead to a positive response, suggesting that further studies are required to identify both the essential inflammatory mediator(s) and the criteria to establish an effective dosing. The preconditioning of PDLSCs via combined treatment (i.e., hypoxic and inflammatory stimuli) did not lead to a synergistic effect; in contrast, it more commonly had an inhibitory effect compared to hypoxic pretreatment alone, suggesting that a rough combination of different cell-processing strategies is not necessarily practical. For therapeutic use, the production of robust cell materials of consistent quality from PDLSCs requires further investigation. Future studies should focus on the choice of an optimal combination of inflammatory mediators and on the control of stimulus variables during the overall manufacturing process.

Compliance with ethical standards

Conflicts of interest The authors indicate no potential conflicts of interest.

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