Expression and antimicrobial character of cells transfected with human β-defensin-3 against periodontitis-associated microbiota *in vitro*

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Received July 1, 2016; Accepted April 27, 2017

DOI: 10.3892/mmr.2017.6913

Abstract. Periodontitis is an oral chronic inflammatory disease induced by microorganisms that can destroy tooth-supporting structures. Human β-defensin-3 (HBD-3) is a type of endogenous antimicrobial peptide that inhibits a broad spectrum of microorganisms. The objectives of the present study were to transfect human periodontal ligament cells (HPDLCs) and human bone marrow stromal cells (HBMSCs) with lentivirus containing the HBD-3 gene, determine the transfection efficiency, and investigate the antimicrobial activity of the experimental cells against periodontal pathogens. Fluorescence microscopy was used to calculated the transfection efficiency. Western blot analysis and ELISA were conducted to confirm the expression of HBD-3 at the protein level. The effect of the HBD-3 gene on the antimicrobial activity of the cells were demonstrated by antimicrobial tests. The results of the present study demonstrated that the transfected HPDLCs and HBMSCs stably expressed HBD-3. In addition, periodontal pathogens and caries-causing bacteria were susceptible to the antimicrobial activity of the cells. Both HPDLCs and HBMSCs hold potential for use as seeding cells in cell- and gene-based therapies for periodontal disease. The lentiviral vector containing HBD-3 resulted in broad-spectrum antimicrobial activity against a variety of oral organisms, and could potentially be applied in the treatment of oral infectious diseases, including periodontitis.

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Key words: human β-defensin-3, antimicrobial activity, transfection, periodontitis, human periodontal ligament cells, human bone marrow stromal cells

Introduction

Periodontitis is caused by a wide range of complex microorganisms and is the primary cause of alveolar bone absorption and eventual tooth loss in the adult population (1). This condition has become a major public health issue, and the development of effective therapies to treat this disease and regenerate periodontal tissue has become an important goal of current medicine. It is widely accepted that both the initiation of oral infectious diseases and the progression of these disease states are associated with increased diversity and richness of the microbiota. In contrast, oral health is associated with decreased diversity and richness within the microbial community. Furthermore, the immune response of the host to the oral microbiome should be considered with respect to the immunopathogenesis of periodontal disease and the immune defenses against caries (2). The inflammatory response fosters the growth of dysbiotic microbial communities, and the bacterial biomass of human periodontitis-associated biofilms has been shown to increase with the aggravation of periodontal inflammation (3). As periodontal tissue may continue to degrade even after being treated with conventional therapies (4), there is a need to develop an antimicrobial agent to protect regenerated periodontal tissue in infectious environments.

Human β-defensins (HBDs) are epithelial-derived antimicrobial peptides that contribute to the innate immune responses of eukaryotes (5). In addition to their microbicidal abilities, host defense peptides are multifunctional mediators of inflammation that have effects on cell proliferation, cytokine/chemokine production and chemotaxis in epithelial and inflammatory cells (6). The expression of three HBDs (namely HBD-1, -2 and -3) has been identified in oral mucosa, gingiva and salivary glands (7). Among these HBDs, HBD-3 is of particular interest for structural and functional studies, and for potential pharmaceutical applications. The broad-spectrum microbicidal activity of HBD-3 is effective against multiple organisms, including fungi, bacteria and viruses. Thus, HBD-3 plays an important role in the human body (8).

Recently, various approaches have been applied to regenerate periodontal tissue, including the use of osteoinductive

agents and biomaterials, guided tissue regeneration and cell therapy (9). Human periodontal ligament cells (HPDLCs) and human bone marrow stromal cells (HBMSCs) are useful seeding cells for periodontal cell therapies (10,11). However, these two cell types only secrete HBD-3 in trace amounts (12).

The present study aimed to construct a recombinant lentiviral vector with the HBD-3 gene, and to investigate the effects of the vector in an effort to develop a novel and suitable treatment for periodontal inflammation to promote periodontal tissue regeneration.

Materials and methods

Cell isolation and culture. HPDLCs and HBMSCs were received as gifts from the Shanghai Research Institute of Stomatology (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The medium was replaced the following day and subsequently every 3 days. The cells were passaged with 0.25% trypsin and 0.1% EDTA upon reaching confluence. Cells from passage three or four were used in the subsequent experiments.

Recombinant plasmid construction. The expression vector pLV.Des3d.P/puro was purchased from Cyagen Biosciences (Guangzhou, China). *E. coli* Stbl3 was used as the host. The HBD-3 gene (code: MRIHYLLFALLFL FLVPVPGHGGIINTLQKYYCRVRGGRCAVLSCLPKEEQ IGKCSTRGRKCCRRKK) and the green fluorescent protein (eGFP) gene were cloned into the pLV.Des3d.P/puro vector. The recombinant plasmid pLV.EX3d.P/puro-EF1A-Humacalx-IRES/eGFP was constructed using Gateway Technology, as previously described (13). This technology was invented and commercialized by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and is a universal cloning method based on the site-specific recombination properties of bacteriophage λ. The recombinant plasmid pLV.EX3d.P/puro-EF1A-PTH-IRES/eGFP was constructed without HBD-3. The constructed expression plasmids were amplified in the *E. coli* strain Stbl3.

Transfection. The lentiviral vector containing HBD-3 was first transfected into 293T packaging cells to obtain high levels of lentiviral particles in the culture supernatant. The HPDLCs and HBMSCs were cultured in 25-cm^2 dishes until 80-90% confluence was reached. Transfection was performed by adding polybrene (8 μg/ml) and 20 μl each viral dilution to the cells, thoroughly and gently mixing the solutions, and incubating the cells in 5% CO_2 at 37˚C. After 18 h, the viral particles remaining in the supernatant were removed and the medium was replaced with fresh medium supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.). The cells were incubated in 5% CO_2 at 37˚C for an additional 72 h. The transfection efficiency was calculated using a fluorescence microscope.

Western blot analysis. The cells were collected from the culture dishes with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology) was added to the samples, and a Bicinchoninic Acid assay was used to determine the protein concentrations. The samples were boiled at 100˚C for 5 min. Total proteins (20 μg per lane) were separated by 5-15% SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk (5 g nonfat milk powder diluted in 100 ml PBS) for 1 h at room temperature and incubated overnight at 4˚C with the following corresponding primary antibodies: Anti-β-actin (cat. no. ab1801; 1:1,000; Abcam, Cambridge, UK) and anti-β-defensin-3 (cat. no. ab19270; 1:1,000; Abcam), followed by incubation with a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (cat. no. KC-RB-035; 1:5,000; KangChen Bio-tech, Inc., Shanghai, China) for 1 h at room temperature. After washing with TBS with Tween-20, the membranes were developed using an EZ-enhanced chemiluminescence detection kit according to the manufacturer's protocol (Biological Industries Beit Haemek Ltd., Israel) and were then imaged using a UVitec gel documentation system (UVitec Limited, Cambridge, UK).

Enzyme-linked immunosorbent assay (ELISA). The amount of secreted HBD-3 in the culture supernatant was detected using an HBD-3 ELISA kit (cat. no. JL19214; Shanghai Jiang Lai Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Absorbance at 490 nm was determined with a microplate reader (BioTek ELx800; Omega Bio-Tek, Inc., Norcross, GA, USA). Each sample was analyzed in triplicate.

Microbial strains. *Actinomyces viscosus* (ATCC 19246); *Candida albicans* (ATCC 10231); *Rothia dentocariosa* (ATCC 19426); *Porphyromonas gingivalis* (ATCC 33277) and *Streptococcus mutans* (UA 159) were used to test the antimicrobial activity of the cells. All the strains were received as gifts from the Shanghai Research Institute of Stomatology.

Antimicrobial activity as assessed by liquid growth inhibition assay. The antimicrobial activity of HBD-3 against the five microbial strains mentioned above was determined by a liquid growth inhibition assay. The purified HBD-3 peptide was serially diluted two-fold with 0.01% acetic acid and 0.2% bovine serum albumin (Thermo Fisher Scientific, Inc.). Aliquots (10 μl) from each dilution were transferred to a 96-well microplate, and each well was inoculated with 100 μl suspension of mid-log bacteria (10^6 CFU/ml) in brain heart infusion (BHI) broth (BBL, Cockeysville, USA). Medium alone (BHI broth) and untreated cells served as control groups. After the cultures were incubated at 37˚C for 24 h, microbial growth was assessed by measuring the optical density at a wavelength of 590 nm with a microplate reader. All experiments were performed in triplicate.

Colony-forming assay. The suspensions of the tested microorganisms were separately cultured in BHI agar supplemented with 5% FBS at 37˚C for 48 h. Subsequently, the number of colony-forming units (CFUs) was counted.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS 19.0 software (IBM Corp., Armonk, NY,

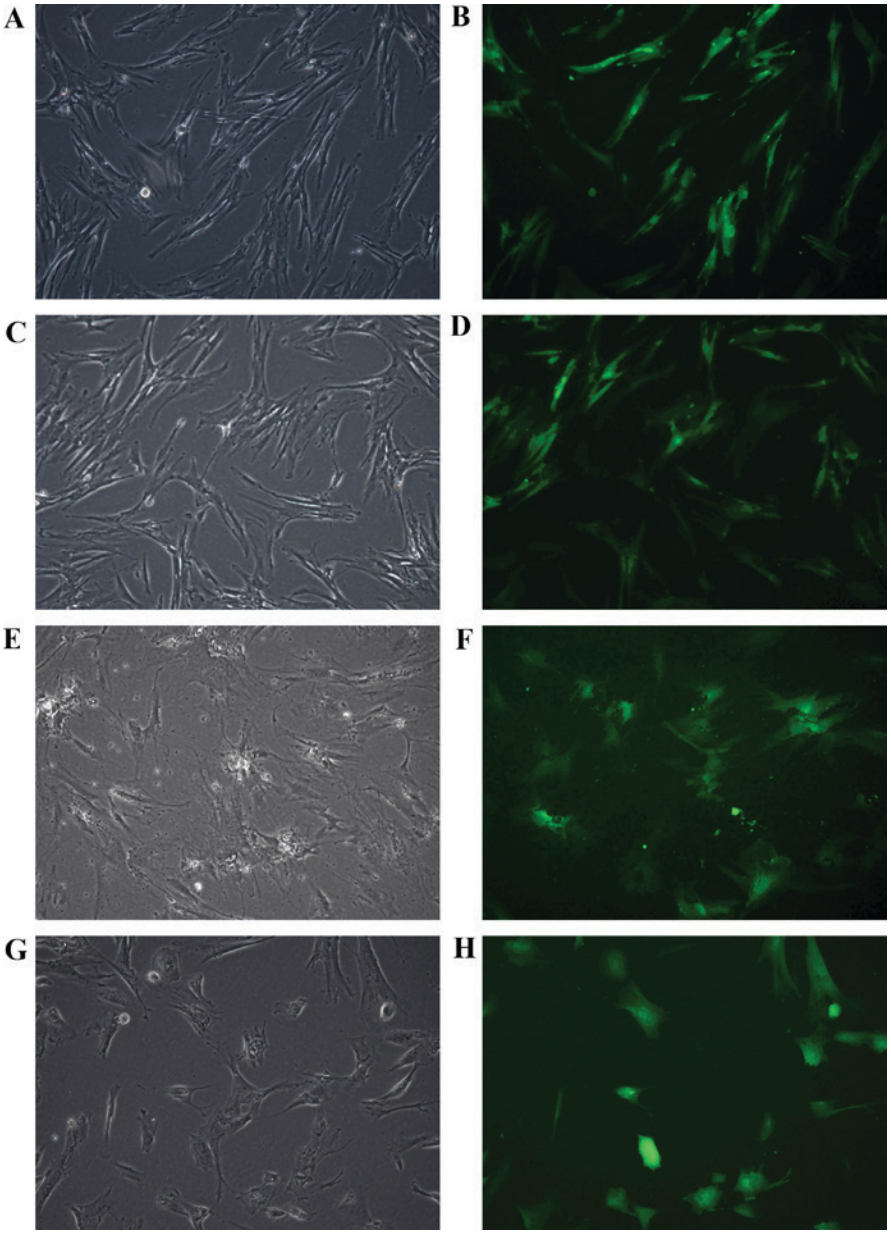

Figure 1. HPDLCs and HBMSCs transfected by the lentiviral vector containing the HBD-3 and GFP genes. (A) Optical and (B) fluorescence microscopy of HPDLCs transfected with the HBD-3 and GFP genes. (C) Optical and (D) fluorescence microscopy of HPDLCs transfected with the GFP gene. (E) Optical and (F) fluorescence microscopy of HBMSCs transfected with the HBD-3 and GFP genes. (G) Optical and (H) fluorescence microscopy of HBMSCs transfected with the GFP gene. Magnification, x400. HPDLCs, human periodontal ligament cells; GFP, green fluorescent protein; HMBSCs, human bone marrow stromal cells; HBD-3, human β-defensin-3.

USA) was used for all statistical analyses. Significant differences were calculated using one-way analysis of variance followed by Bonferroni or Tamhane post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Transfection efficiency. Fusiform or polygonal transfected HPDLCs and HBMSCs were observed adhering to the bottom of the dishes by optical microscopy (Fig. 1). Transfection of the recombinant plasmids into HPDLCs and HBMSCs was assessed by detecting GFP expression using a fluorescence microscope. The transfected HPDLCs and HBMSCs exhibited similar GFP-positive expression throughout several repeated

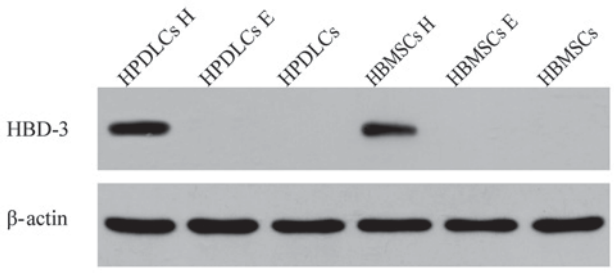

Figure 2. HBD-3 protein expression, detected by western blotting. Representative western blot image of protein expression levels of HBD-3. HBMSCs H and HPDLCs H represent the HBMSCs and HPDLCs transfected with the HBD-3 and GFP genes, respectively; HBMSCs E and HPDLCs E represent the HBMSCs and HPDLCs transfected with the GFP gene only. HBMSCs, human bone marrow stromal cells; HPDLCs, human periodontal ligament cells; HBD-3, human β-defensin-3; GFP, green fluorescent protein.

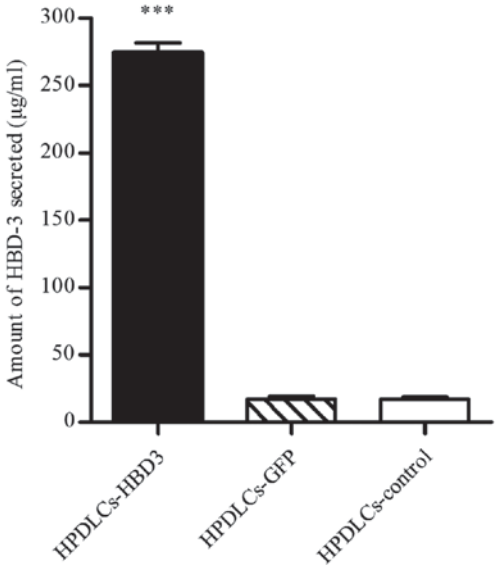

Figure 3. Amount of HBD-3 secreted by HPDLCs, as determined by ELISA. Data are expressed as the mean ± standard deviation. ***P<0.001 vs. control and GFP-transfected cells. HBD-3, human β-defensin-3; GFP, green fluorescent protein; HPDLCs, human periodontal ligament cells.

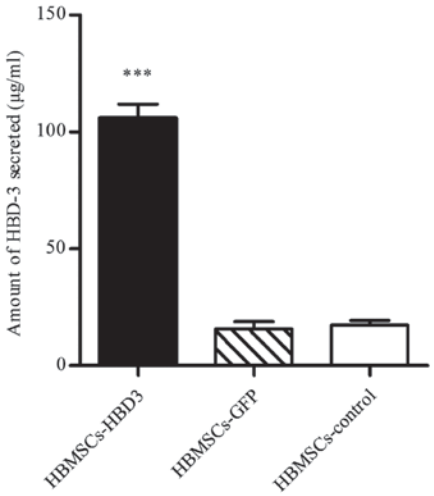

Figure 4. Amount of HBD-3 secreted by HBMSCs, as determined by ELISA. Data are expressed as the mean ± standard deviation. ***P<0.001 vs. control and GFP-transfected cells. HBD-3, human β-defensin-3; GFP, green fluorescent protein; HBMSCs, human bone marrow stromal cells.

experiments, as observed by fluorescence microscopy (Fig. 1). Transfection efficiency was calculated by counting the cells that fluoresced green. The results demonstrated that the rate of HPDLC and HBMSC transfection with the HBD-3 and GFP genes was 79.94 and 64.81%, respectively. The rate of HPDLC and HBMSC transfection with the GFP gene only was 75.98 and 53.71%, respectively (data not shown).

Western blot analysis. To examine the protein expression level of HBD-3 in the transfected HPDLCs and HBMSCs, western blot analysis was performed. Distinct positive bands were observed for HPDLCs and HBMSCs transfected with the HBD-3 and GFP genes. However, no positive bands were observed in the other two groups (Fig. 2).

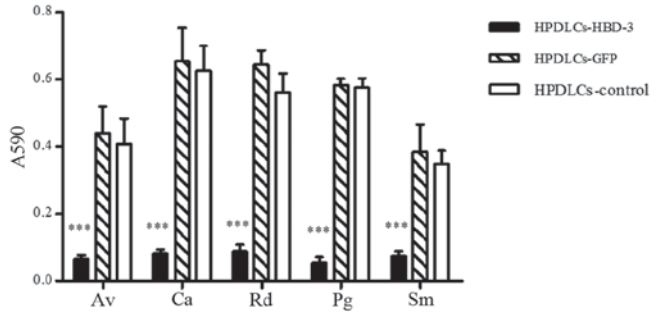

Figure 5. Antibacterial activity of HPDLCs transfected with HBD-3, as determined by liquid growth inhibition assay. Data are expressed as the mean ± standard deviation. ***P<0.001 vs. control and GFP-transfected cells. HBD-3, human β-defensin-3; GFP, green fluorescent protein; HPDLCs, human periodontal ligament cells; Av, *Actinomyces viscosus*; Ca, *Candida albicans*; Rd, *Rothia dentocariosa*; Pg, *Porphyromonas gingivalis*; Sm, *Streptococcus mutans*.

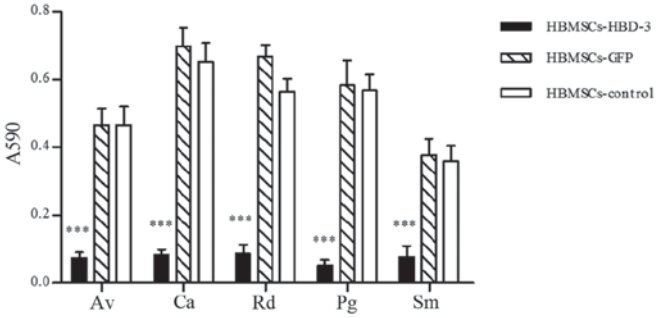

Figure 6. Antibacterial activity of HBMSCs transfected with HBD-3, as determined by liquid growth inhibition assay. Data are expressed as the mean ± standard deviation. ***P<0.001 vs. control and GFP-transfected cells. HBD-3, human β-defensin-3; GFP, green fluorescent protein; HBMSCs, human bone marrow stromal cells; Av, *Actinomyces viscosus*; Ca, *Candida albicans*; Rd, *Rothia dentocariosa*; Pg, *Porphyromonas gingivalis*; Sm, *Streptococcus mutans*.

ELISA. ELISA was performed to quantify the levels of HBD-3 in HPDLCs and HBMSCs transfected with the HBD-3 and GFP genes. The untreated cells served as a control. The results demonstrated that the concentration of secreted HBD-3 was 274.89±6.79 μg/ml and 106.11±5.67 μg/ml in the supernatants of transfected HPDLCs (Fig. 3) and HBMSCs (Fig. 4), respectively; these concentrations were significantly higher than those of the corresponding cells transfected with the GFP control vector, and that of the untreated control group (P<0.001).

Liquid growth inhibition assay. A liquid growth inhibition assay was performed, and the optical density of each stock suspension was measured at 590 nm to evaluate the approximate numbers of microbes present. As presented in Fig. 5, the HPDLCs transfected with the HBD-3 gene and cultured with *Actinomyces viscosus*, *Candida albicans*, *Rothia dentocariosa*, *Porphyromonas gingivalis* and *Streptococcus mutans* yielded small optical density (OD) values of 0.065±0.012, 0.081±0.013, 0.088±0.020, 0.054±0.017 and 0.073±0.016, respectively, which were significantly reduced compared with those of the other groups (P<0.001). A similar effect

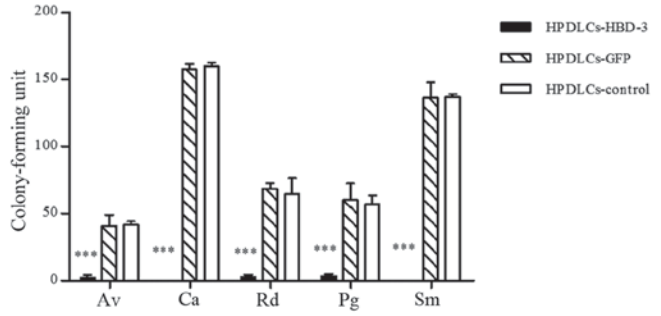

Figure 7. Antimicrobial testing for HPDLCs transfected with HBD-3, as determined by colony-forming assay. Data are expressed as the mean ± standard deviation. *P<0.05, ***P<0.001 vs. control and GFP-transfected cells. HBD-3, human β-defensin-3; GFP, green fluorescent protein; HPDLCs, human periodontal ligament cells; Av, *Actinomyces viscosus*; Ca, *Candida albicans*; Rd, *Rothia dentocariosa*; Pg, *Porphyromonas gingivalis*; Sm, *Streptococcus mutans*.

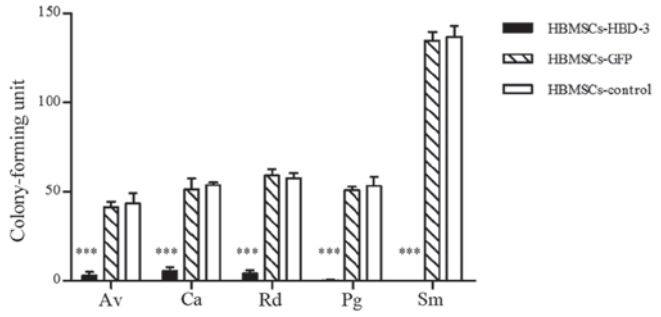

Figure 8. Antimicrobial testing for HBMSCs transfected with HBD-3, as determined by colony-forming assay. Data are expressed as the mean ± standard deviation. *P<0.05, ***P<0.001 vs. control and GFP-transfected cells. HBD-3, human β-defensin-3; GFP, green fluorescent protein; HBMSCs, human bone marrow stromal cells; Av, *Actinomyces viscosus*; Ca, *Candida albicans*; Rd, *Rothia dentocariosa*; Pg, *Porphyromonas gingivalis*; Sm, *Streptococcus mutans*.

was observed for HBMSCs transfected with the HBD-3 gene and cultured with the microbes; corresponding to the order listed above, the cultures yielded OD values of 0.073±0.017, 0.082±0.016, 0.086±0.026, 0.052±0.017 and 0.076±0.033, respectively (P<0.001; Fig. 6).

Colony-forming assay. A colony-forming assay was performed to evaluate the effects of HBD-3 on the important antimicrobial activity of the cells against the tested bacteria. The HPDLCs transfected with the HBD-3 gene demonstrated significantly reduced colony counts compared with control and GFP-transfected HPDLCs (P<0.001; Fig. 7). Similar results were observed in HBMSCs (P<0.001; Fig. 8).

Discussion

Periodontal diseases are highly prevalent and affect <90% of the population worldwide (1). Pathogenic bacteria are widely recognized to be a major cause of periodontal tissue destruction, and the ultimate goals of periodontal treatments are to support good oral hygiene and regenerate tissue integrity, which may have been damaged by the inflammatory process (14). Currently, periodontal regeneration is shifting towards cell- and gene-based therapies (15). The present study constructed a recombinant lentiviral HBD-3 expression vector and investigated the effects of an antimicrobial peptide using a combination of gene- and cell-based therapies.

Firstly, the efficiency of HBD-3 transfection into HPDLCs and HBMSCs was determined, and transfection was validated using western blotting. HBD-3 protein levels in the transfected HPDLCs and HBMSCs were sustained and were significantly higher than those of the control group. Similar results were also obtained from the ELISA analyses. Certain studies have reported that HPDLCs have the capacity to function as osteoblasts or cementoblasts under regenerative conditions, suggesting that they are the best candidates for regeneration applications (16,17). In addition, HBMSCs are the most widely investigated mesenchymal stem cells, which have tremendous potential in regenerative medicine because of their multipotency and capability of forming a variety of tissues, including the periodontium (18). The crucial steps of gene therapy include the efficient transfer and appropriate expression of the target gene. Currently, the lentiviral vector is one of the most useful methods for treating periodontal disease by virtue of its high transduction efficiency (15). The studies mentioned above indicated that HPDLCs and HBMSCs are promising for use as seeding cells for cell- and gene-based therapies for periodontal disease. In addition, the lentiviral vector with eGFP is an appropriate expression vector system.

Furthermore, the present study detected the antimicrobial activity of the HPDLCs and HBMSCs transfected by a lentivirus containing the HBD-3 gene, using liquid growth inhibition and colony-forming assays. HBD-3 is an endogenous antibiotic and is active against both gram-positive and gram-negative bacteria. Its ability to act against multidrug-resistant clinical isolates of *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Acinetobacter baumannii* has been confirmed (19). In the present study, the numbers of bacteria were significantly lower in the experimental group than in the control group. The periodontal pathogens *Actinomyces viscosus, Porphyromonas gingivalis*, *Rothia dentocariosa* and *Candida albicans* (20-22) were demonstrated to be susceptible to the cells containing Humacalx-IRES compared with those in the control and untreated groups. The results indicated that HBD-3 has the capacity to inhibit microbial activity *in vitro*, which is consistent with the results of our previous study (23) and with other research (24). As *Candida albicans* is the most common opportunistic fungal pathogen of humans, and can cause superficial epithelial infections and life-threatening systemic infections, HBD-3 also demonstrated beneficial antifungal effects. Notably, the caries-causing bacteria *Streptococcus mutans* (2) was also susceptible to HBD-3 in both experimental groups. The results of the present study demonstrated the multifunctional, broad-spectrum activity of HBD-3 against a collection of oral microorganisms; this activity could be applied in the treatment of oral infectious diseases.

Cells of several human tissue types can secrete HBD-3. Previous studies have demonstrated that HBD is susceptible to degradation and inactivation by both host and bacterial

proteases. It has also been reported that inflamed gingival tissues express lower levels of HBD-3 mRNA than healthy tissues (7,25). Brancatisano *et al* (26) detected HBD-3 using ELISA, and demonstrated that its levels were inversely correlated with the severity of the disease and with the degree of colonization by combinations of bacterial species having elevated periodontopathogenic potential. Based on this information, it is reasonable to hypothesize that aggressive inflammation and tissue destruction occur when the HBD-3 peptide cannot counteract the antimicrobial activity. However, appropriate expression of HBD peptides in states of health and disease may contribute to the maintenance of periodontal homeostasis, potentially via the antimicrobial effects of HBD-3 and the promotion of adaptive immune responses (27). Therefore, the transfection of HPDLCs and HBMSCs with HBD-3 may have favorable effects on antimicrobial activity by complementing the low levels of HBD-3 in aggressive periodontitis and other oral infectious diseases.

In conclusion, application of the lentiviral vector containing HBD-3 has great potential as a safe and efficient gene therapy for antimicrobial activity in periodontitis. Further research will be conducted to investigate the influence of HBD-3 transfection on HPDLCs and HBMSCs in periodontal tissue regeneration.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81271157).

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