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Antibacterial periodontal ligament stem cells enhance periodontal regeneration and regulate the oral microbiome

Jiayi You[1,2,3], Qian Zhang[4], Linjue Qian[1,2,3], Zihan Shi[1,2,3], Xinyue Wang[1,2,3], Lu Jia[5] and Yang Xia[1,2,3*]

Abstract

Background The transplantation of periodontal ligament stem cells (PDLSCs) has been shown to enhance periodontal regeneration in animal models and clinical trials. However, it is not known whether PDLSCs are antibacterial and whether this affects oral microbiota and periodontal regeneration.

Methods We isolated human PDLSCs from periodontal ligament of extracted teeth. Rats' periodontal fenestration defects were prepared, and treated with PDLSC injections (Cell group), using saline injections (Saline group) as the control. The oral microbiota was explored by 16 S rDNA sequencing and compared with that before surgery (PRE group). The antibacterial property of PDLSCs and its underlying mechanism were tested in vitro.

Results Microbiome analyses reveal a decreased biodiversity, a changed community structure, and downregulated community functions of the oral microbiome in the Saline group. PDLSCs injections enhance periodontal regeneration, reverse the decrease in diversity, and increase the abundance of non-pathogenic bacterial *Bifidobacterium* sp. and *Lactobacillus* sp., making the oral microbiome similar to that of the PRE group. In vitro, PDLSCs inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Fusobacterium nucleatum*. The main mechanism of action is postulated to involve production of the cationic antimicrobial peptide LL-37.

Conclusions Our findings reveal that PDLSC injections enhance periodontal regeneration and regulate the oral microbiome to foster an oral cavity microenvironment conducive to symbiotic microbiota associated with health.

Keywords Periodontal ligament stem cell, Periodontal regeneration, Antibacterial, Antimicrobial peptides, LL-37, Oral microbiome

*Correspondence:
Yang Xia
xiayang@njmu.edu.cn
[1]The Affiliated Stomatological Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu, People's Republic of China
[2]State Key Laboratory Cultivation Base of Research, Prevention and Treatment for Oral Diseases, Nanjing Medical University, Nanjing 210029, Jiangsu, People's Republic of China
[3]Jiangsu Province Engineering Research Center of Stomatological Translational Medicine, Nanjing Medical University, Nanjing 210029, Jiangsu, People's Republic of China
[4]Suzhou Stomatological Hospital, Suzhou 215000, Jiangsu, People's Republic of China
[5]Department of Emergency General Dentistry, Hebei Key Laboratory of Stomatology, Hebei Medical University, Shijiazhuang 050017, Hebei, People's Republic of China

Background

The chronic noncommunicable inflammatory disease periodontitis is characterized by periodontal pathogen infection. It leads to irreversible damage to the teeth-supporting apparatus – mainly the periodontal ligaments and alveolar bone – and eventually to tooth loss. It is associated with the accumulation of dental plaque and systematic or behavioral factors such as diabetes and smoking, and it involves complex dynamic interactions among specific bacterial pathogens and destructive host immune responses [1]. Based on a National Health and Nutrition Examination Survey, periodontitis affects almost 50% of the adult population in the United States (≥30 years) [2]. Therefore, the treatment of periodontitis is an important focus in dentistry.

The first step of periodontitis treatment is to remove and clear pathogenic factors using mechanical methods such as scaling and root planning. After reversing dysbiosis of the oral microbiota and controlling inflammation, the second step is to restore the supporting function of damaged periodontal tissues, especially alveolar bone, by regenerative methods. The prevalent technique employed is guided tissue regeneration (GTR), which becoming a standard surgical treatment for periodontal regeneration. Although the histological and clinical outcome of GTR is typically positive, its ability to induce alveolar bone regeneration is limited [3]. Antibiotic agents can be used as an adjunct during periodontal regenerative therapy to prevent infection, which can prevent regeneration [4]. However, drug resistance becomes a concern if repeated drug application for long periods is needed. Incomplete alveolar bone repair may affect the treatment of periodontitis due to the re-formation of subgingival plaque at unreachable defect sites. Loose teeth due to insufficient bone support reduce the quality of patient life. Therefore, complete periodontal regeneration remains a clinical challenge.

Stem cells have multiple differentiation ability and can self-renew; thus, they have tremendous therapeutic potential. Mesenchymal stem cell (MSC) transplantation has been extensively studied as a means of tissue damage treatment. According to the results of various preclinical and clinical studies, the application of MSCs to periodontitis can enhance the regeneration of periodontal tissues [5, 6]. Therapies using stem cells can improve indexes such as clinical attachment level (CAL), pocket probing depth (PPD), and bone crest to bottom of defect (BC-BD) in follow-up periods [7]. Although cellfree therapeutic strategies using conditioned medium (CM) from MSCs (MSCCM) may have several advantages such as biological safety, it cannot guarantee similar effects to MSC-CM in every treatment because it is impossible to obtain MSC-CM containing similar secretomes for each treatment in clinics [8]. Therefore, the application of stem cells is still the main method for stem cell-based therapy.

Dental and nondental originated stem cells have been applied, including bone marrow MSCs (BMMSCs) and adipose derived stem cells (ADSCs), induced pluripotent stem cells, embryonic stem cells, periodontal ligament stem cells (PDLSCs), stem cells from human exfoliated deciduous teeth, dental pulp stem cells, gingival MSCs, stem cells from the apical papilla, and dental follicle cells. Among all these applications, PDLSCs are the most suitable for promoting the regeneration of periodontal defects [8]. PDLSCs show great differentiation ability, being able to differentiate into osteogenic, chondrogenic, adipogenic, neurogenic, and cardiomyogenic lineages. Furthermore, they can secrete bioactive components to maintain and regulate the in vivo microenvironment and in vitro cell performance by promoting proliferation, angiogenesis, and osteogenesis, as well as regulating inflammatory responses. Moreover, allogeneic PDLSCs can be used without immunological rejection due to their low immunogenicity [9].

For transplantation, methods like cell sheets and combination with scaffolds that can ensure the number and viability of MSCs in periodontal defects are commonly used. Comparatively, when MSCs are injected as a suspension, there is insufficient time for the cells to adhere, leading to low cell survival [10]. However, cell injection is minimally invasive, and it can be performed repeatedly to achieve the desired regeneration effects. Moreover, when injected, there is chance for MSCs to be exposed to the oral cavity, which may change the local environment, especially the oral microbiota, due to their biological activity and paracrine secretion functionality.

The mechanisms by which MSCs enhance periodontal regeneration are often explored in terms of their proliferation and differentiation potential, and they are increasingly attributed to paracrine secretion, particularly that of exosomes or small extracellular vesicles, through which MSCs exert immunomodulation and anti-inflammation properties as well as regulate intercellular communication and function [11]. More recently, it has been reported that the secretome of MSCs also contains some antimicrobial peptides (AMPs) such as hepcidin, lipocalin 2, β-defensin 2 (hBD2), and cathelicidin (also known as LL-37) [12–14]. These AMPs have shown great potential in the treatment of infectious diseases, including sepsis, cystic fibrosis, and *escherichia coli* pneumonia [15–17], partly due to the enhanced clearance of bacteria. Periodontitis is also an infectious disease, and infection prevention is the most important prerequisite during periodontal regeneration. However, little research has been done to explore whether PDLSCs are antibacterial or the effects of antibacterial PDLSCs on periodontal regeneration in the oral cavity, which harbors the

second-most abundant microbiota after the gastrointestinal tract.

In this study, human PDLSCs were harvested and repeatedly injected into a surgically created rat periodontal defect to explore whether PDLSCs are antibacterial and whether the antibacterial property of PDLSCs is beneficial for periodontal regeneration. In vitro bactericidal effects of PDLSCs on pathogenic bacteria and in vivo diversity changes of oral microbiota were explored. Periodontal regeneration was analyzed using radiography and histology. To explore the mechanism of their antibacterial property, the levels of LL-37 secreted by PDLSCs were measured. The results of the current study provide new insight into the mechanism for PDLSC-promoted periodontal regeneration.

Methods

Isolation and identification of PDLSCs

The isolation of PDLSCs was performed following our previous method [18, 19]. Normal premolars were collected from healthy individuals (aged 18–25 years) undergoing routine tooth extraction. Informed consent was obtained, and the plan was approved by the ethics committee of the Institute of Stomatology, Nanjing Medical University (NMU) (PJ2018-064-001). Cells surface markers were detected by flow cytometry. Cells from passages 3 to 6 were used.

Stem cell injection therapy

For stem cell injection therapy, 10^6 PDLSCs in 1 mL saline (Cell group) was compared with 1 mL saline (Saline group) ($n=8$). Cell viability was tested using Cell Viability/Cytotoxicity Assay Kits (Beyotime, China) and Cell Counting Kit-8 (CCK-8, Dojindo, Japan) at day 1.

The preparation of periodontal defects was performed following Padial-Molina et al. [20], and approved by the Institutional Animal Care and Use Committee of NMU (IACUC-1908036). The reporting of animal studies has been meticulously conducted in strict adherence to the ARRIVE guidelines 2.0. During the surgical procedures, Specific-pathogen-free (SPF) male Sprague–Dawley rats (SD rats, 180–200 g) underwent anesthesia via intraperitoneal injection of pentobarbital sodium at a dosage of 30 mg/kg. A skin incision was made along the inferior border of the mandible. Then, a mucoperiosteal flap was carefully elevated to expose the alveolar bone. To create experimental periodontal defects in the mesial region of the mandibular second molars, a surgical bur was used to precisely remove alveolar bone, resulting in a defect measuring 3.5×2×1.5 mm. Injections were performed as in Du et al. [21]. Briefly, the vehicle was injected into the middle of the defect slowly for 3–5 s. The tip of the needle was left for 5–10 s after injection. The injection was performed once a week until sacrifice.

Microbial diversity analysis

Two weeks after surgery, the microbiota of the defect region in the Cell and Saline groups was sampled using a cotton swab, rubbed gently for 15–20 s on the oral gingival mucosa of the periodontal defect area under anesthesia, with the pre-surgical microbiota serving as the PRE group control. DNA extraction was performed using a Mag-Bind Stool DNA 96 Kit (Omega, USA). The samples were frozen at −80 °C until transport. Amplification of the V4 region of the 16 S rRNA gene, cloning, and sequencing of the polymerase chain reaction products were performed at the laboratory of the BGI (Huada Gene Institute, China).

Micro-CT and histological staining

The animals were euthanized via carbon dioxide inhalation after two or four weeks of healing. The mandible was examined by micro-CT (SkyScan 1176, Germany). Indexes including BV/TV (Bone Volume/Tissue Volume) and residual defect area were quantified [22]. Then, the samples were prepared for hematoxylin-eosin (H&E) and Masson's trichrome staining. Indexes including width of new bone and new bone area fraction were analyzed.

Antibacterial tests

Antibacterial ability of PDLSCs was assessed by bacterial count analyses using classic and representative examples of Gram-positive and Gram-negative bacteria *Staphylococcus aureus* (*S. aureus*, ATCC25923) and *Escherichia coli* (*E. coli*, ATCC25923). To determine the antibacterial effect of the CM from PDLSCs (Cell CM), confocal microscopy, bacterial count test, SEM, and plate-crystal violet assay were performed against *S. aureus*, *E. coli*, and a typical periodontal pathogen *F. nucleatum*. α-MEM with 5% FBS was used as control.

CM was prepared following our previous method [18]. Briefly, cells in a 24-well plate (2×10^5 per well) were incubated in α-MEM medium with 5% FBS at 37 °C and 5% CO_2 for 24 h. The media were collected and centrifuged at 2000 rpm for 20 min. This process did not involve any additional concentration steps. The supernatant was filtered through a 0.22-μm membrane and stored at −80 ℃ before use.

To prepare the CM of cells stimulated with *F. nucleatum* (S-Cell CM), PDLSCs in a 24-well plate (2×10^5 per well) were infected with 5×10^6 CFU *F. nucleatum* and incubated for 24 h in a 37 °C humidified CO_2 incubator. The media was collected and *F. nucleatum* was removed with a 0.22-μm filter to eliminate any residual bacterial organisms. The filtered media was centrifuged at 2000 rpm for 20 min and frozen at −80 °C.

Detection of LL-37

LL-37 concentrations of the Cell CM, S-Cell CM, and I-Cell CM were measured using human enzyme linked immunosorbent assay (Elisa) kits (Elabscience, China) and compared with the CM from human gingival fibroblasts (hGFs). To determine LL-37 amount at the RNA level, quantitative real-time reverse transcription PCR was performed. The primer sequences are shown in Table S1. All experimental details are provided in the Supporting Material.

Statistical analysis

For all experiments, the results are expressed as mean±standard deviation (SD). SPSS statistics 21 was used. For two-group comparison, Student's t-test was used. For multiple comparisons, ANOVA (one-way) and post hoc least significant difference (LSD) was performed. $p<0.05$ was considered statistically significant.

Results

The isolated cells were identified to be PDLSCs

The cells grew out of the clone along the edge of the adherent tissue block in a radial row after seven days' culture. They showed uniform long fusiform shapes and uniform distribution at the 3rd generation (Fig. 1A). Immunophenotypic profiling was performed using markers commonly expressed by MSCs. Similarly to MSCs, the obtained cells highly expressed the specific surface markers CD29 (99.6%), CD73 (99.9%), CD90 (99.9%), and CD105 (96.9%), while the cells showed low expression of CD34 (0.023%) and CD45 (0.036%) (Fig. 1B). In combination with the tissue origin, the cells were identified as PDLSCs (Cell group).

PDLSCs injections promote periodontal regeneration

The viability of PDLSCs in saline for injection (I-Cell group) was compared with normal cells (PDLSCs are not subjected to the process of preparing a cell solution for injection). At one day, the Cell and I-Cell groups both present live cells with few dead ones, indicating excellent cell viability without inter-group difference ($p>0.05$, Fig. 1C and D). Quantitative detection by CCK-8 assay confirmed this result, showing no significant difference between the two groups ($p>0.05$, Fig. E).

The experimental design is schematically depicted in Fig. 2A. For all the animals, primary wound closure was achieved, and there were no signs of tumor formation or pathological growth in the surrounding tissues. The micro-computed tomography (micro-CT) section of the periodontal defect at day 0 is presented in Fig. 2B. Some bone formation was visible for the Saline group two weeks after surgery. More new bone was detected after four weeks healing. The new bone volume and bone fill for the Cell group were more than that in the Saline group at both time points, presenting as higher BV/TV values ($p<0.05$, Fig. 2C and D, and 2E). Conversely, the residual defect area was smaller in the Cell group than in the Saline group at two and four weeks ($p<0.05$, Fig. 2F).

Histologically, there was a little new bone-like tissue in the defects for the Saline group, which were mainly filled with fibrous tissue. At four weeks, the new bone was more extensive than that at two weeks (Fig. 2G and H). No evidence of ongoing cementogenesis nor periodontal ligament (PDL) like structures was observed at two and four weeks for the Saline group (Fig. 2G and H). Comparatively, there was more newly regenerated bone for the Cell group than for the Saline group at two and four weeks. Moreover, the treated defects for the Cell group presented new cementum-like tissue (indicated by black arrows in Fig. 2H) extending buccally into the defect at four weeks. Ankylosis with obliteration of periodontal ligament space was not observed. The interfacial zone between new bone-like structures and new cementum-like tissue was obvious and connected with the original PDL (Fig. 2G and H). Quantitatively, higher values of width of new bone [23] and new bone area fraction were observed for the Cell group compared with the Saline group at two and four weeks ($p<0.05$, Fig. 2I and J).

PDLSCs injections reverse the unhealthy changes to the oral microbiome observed in saline-injection-treated periodontal defects in vivo

In the Venn diagram (Fig. 3A), there are 319, 88, and 154 operational taxonomic units (OTUs) identified for the PRE, Saline, and Cell groups, respectively. These groups have a total of 35 OTUs in common. 207, 21, and 24 unique OTUs were found individually in the PRE, Saline, and Cell groups. The species accumulation curves and the rank-abundance curves are shown in Fig. S1 and Fig. S2. For α diversity analysis, indexes including observed species, Chao, ACE, Shannon's diversity, and Simpson' diversity are different among the PRE, Cell, and Saline groups ($p<0.05$). However, only the Simpson index is different between the PRE and Cell groups ($p<0.05$), while only the Shannon index is similar between the PRE and Saline groups ($p>0.05$). These results indicate a decrease in the diversity of oral microbiota after surgery, while PDLSC injection reverses this condition by increasing the diversity (Fig. S3).

Principal coordinate analysis (species) indicated significant differences among the PRE, Cell, and Saline groups. PC1 explains 61.31% of the variation, while PC2 explains 15.42% (Fig. 3B). Cluster analysis of OTU based on the UPGMA method (Weighted unifrac) suggested that the Saline group is different from the PRE and Cell groups, while the PRE and Cell groups are similar (Fig. 3C). Moreover, the β-diversity values (Weighted unifrac) among the three groups are significantly different ($p<0.05$, Fig. 3D_1).

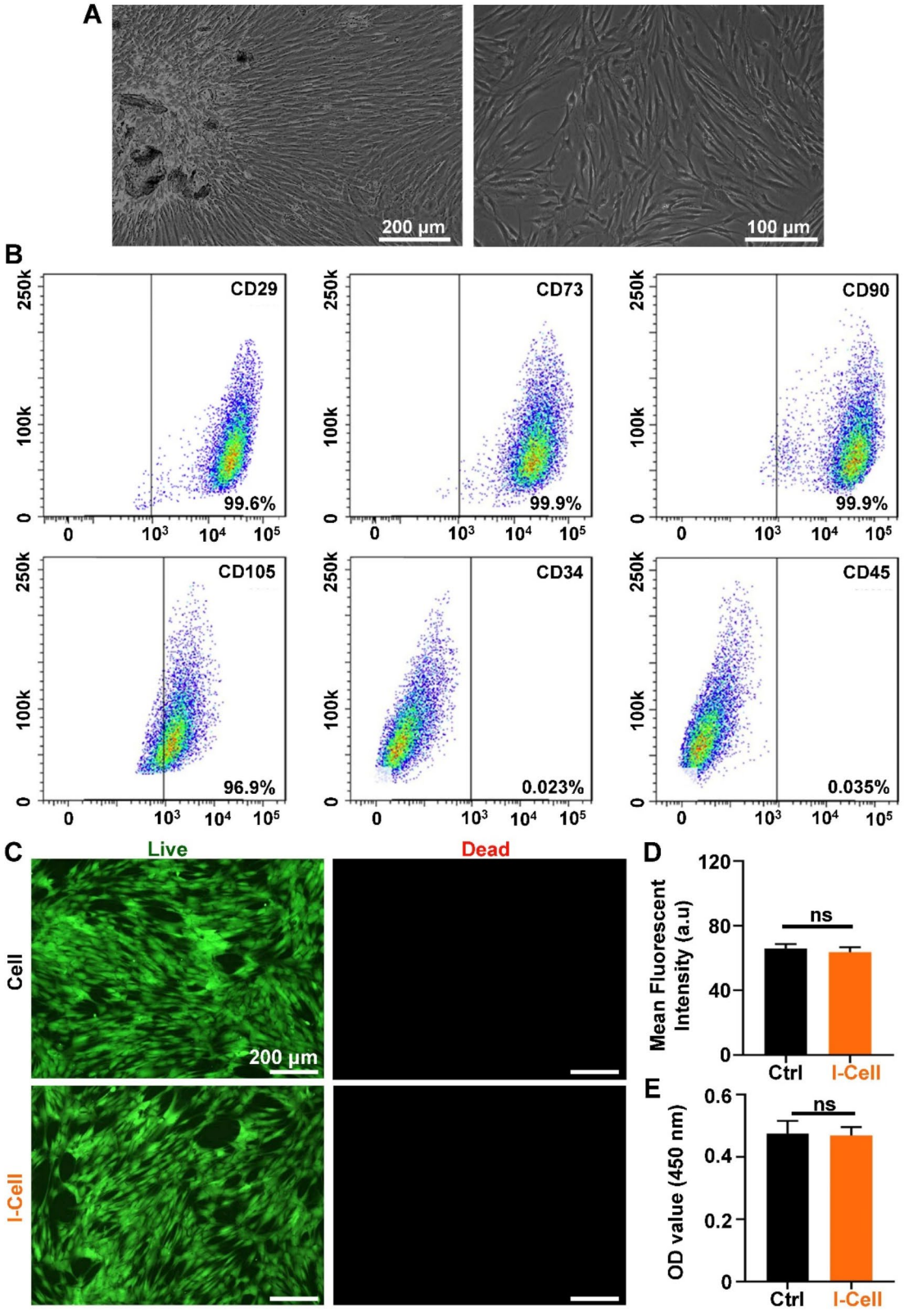

Fig. 1 Isolation and characterization of PDLSCs. (**A**) Isolation of PDLSCs. Spindle-shaped cells at passage 0 and 3; (**B**) Flow cytometric analyses of PDLSCs. They were positive for MSCs markers CD29, CD73, CD90 and CD105, while negative for hematopoietic markers CD34 and CD45. (**C**) Live/Dead staining images of PDLSCs (Cell) and PDLSCs for cell injection (I-Cell). (**D**) Quantitative analysis of fluorescent intensity at 24 h. (**E**) Cell viability by CCK-8 at 24. $n = 3$. $^*p < 0.05$

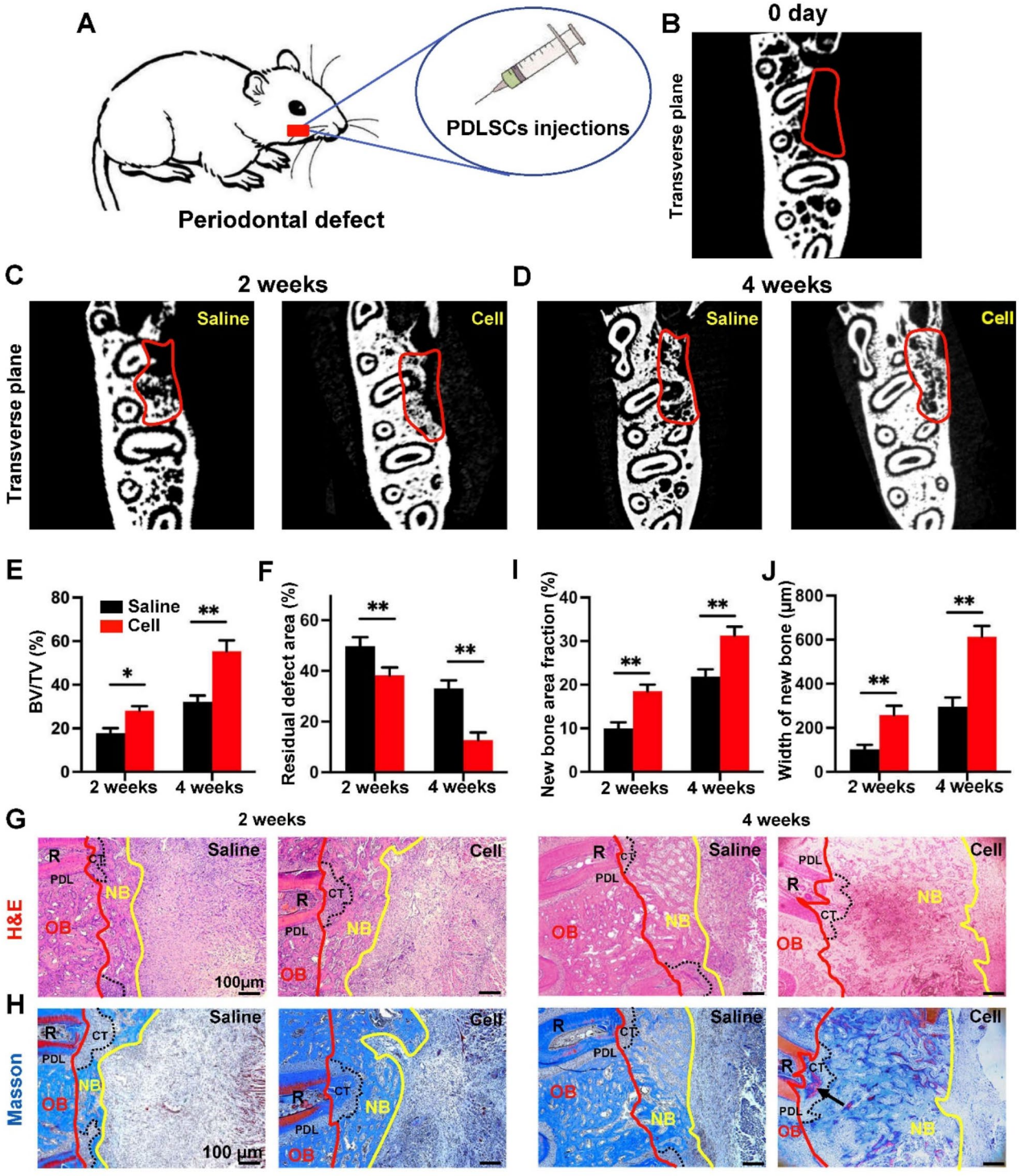

Fig. 2 In vivo bone regeneration. (**A**) Schematic illustration of the experimental design; (**B**) Micro-CT section of periodontal defect at 0 day; (**C**) Micro-CT sections of periodontal defect at 2 weeks; (**D**) Micro-CT sections of periodontal defect at 4 weeks; (**E**) Quantitative analysis of BV/TV; (**F**) Quantitative analysis of residual defect area; (**G**) H&E staining images; (**H**) Masson staining images; (**I**) Quantitative analysis of new bone area fraction; (**J**) Width of new bone, calculated between its lingual boundary marked as red dashed line and buccal boundary marked as yellow dashed line in the area of periodontal defect. OB: Original bone. NB: New bone. PDL: periodontal ligament. CT: connective tissue. R: root. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$

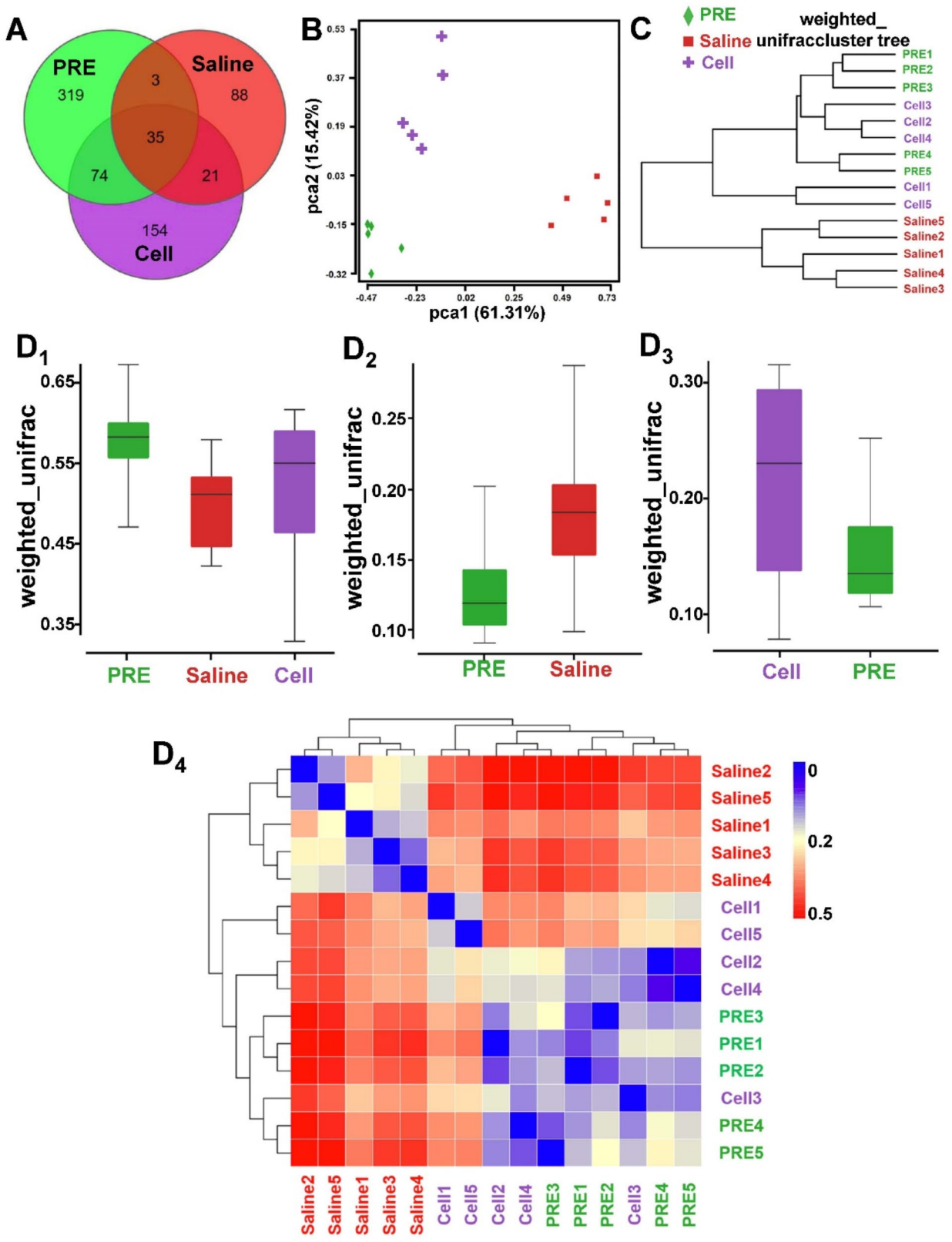

Fig. 3 Microbial diversity analysis. (**A**) Venn diagram; (**B**) Species PCA analysis; (**C**) UPGMA weighted_unifraccluster tree; (**D**) Analysis of beta diversity (weighted_unifraccluster). (**D**$_1$) PRE vs. Saline vs. Cell; (**D**$_2$) PRE vs. Saline; (**D**$_3$) Cell vs. PRE weighted_unifraccluster; (**D**$_4$) PRE vs. Saline vs. Cell heat map

Specifically, the PRE and Saline groups exhibit statistical difference ($p<0.05$, Fig. 3D$_2$), while the PRE and Cell groups are similar ($p>0.05$, Fig. 3D$_3$). The β-diversity heatmap is consistent with the cluster analysis results, indicating that the microbiota of the PRE and Cell groups are similar, while they are both different from that of the Saline group (Fig. 3D$_4$).

The relative abundance of oral microbiota was assessed at the genus level. According to the heatmap, genus *Bifidobacterium*, *Brachybacterium*, *Escherichia-Shigella*, *Staphylococcus*, *Aerococcus*, *Jeotgalicoccus*, *Enterococcus*, *Lactobacillus*, *Psychrobacter*, *Corynebacterium*, and *Streptococcus* were more relatively abundant in Cell group, while *Corynebacterium*, *Rodentibacter*, *Streptococcus* were more relatively abundant in the Saline group. *Rothia*, which was the most abundant Genus in the PRE group, was less in the Saline group ($p<0.05$), but not in the Cell group ($p>0.05$, Fig. 4A). The histogram representing the TOP 13 core microbiome at the genus level of the three groups (Fig. 4B) is consistent with the heatmap results, both showing an obvious increase for the Genus *Lactobacillus* in the Cell group and an obvious increase for the Genus *Rodentibacter* in the Saline group. A more significant increase for *Bifidobacterium* in the Cell group than in the PRE and Saline groups was detected using species difference analysis at the genus level ($p<0.05$, Fig. 4C). Linear discriminant analysis of effect size analyses (Fig. 4D and Fig. S4) revealed that the Cell group harbors significantly higher proportions of *Firmicutes*, *Bacilli*, and *Lactobacillales* (LDA=5.07, $p=0.012$); *Actinobacteriota*, *Actinobacteria*, *Bifidobacteriales*, *Bifidobacteriaceae*, and *Bifidobacterium* (LDA=3.21, $p=0.013$); and *Verrucomicrobiota*, *Verrucomicrobiae*, *Verrucomicrobiales*, *Akkermansiaceae*, and *Akkermansia* (LDA=2.51, $p=0.035$) compared with the PRE and Saline groups. *Actinobacteriota* (LDA=5.36, $p=0.005$) was dominant in the PRE group, while *Proteobacteria* (LDA=5.30, $p=0.007$) is dominant in the Saline group.

The function of oral microbial communities was analyzed using the KEGG database. At level 1, the most abundant cellular function in each sample is Metabolism (Fig. 5A). Functional analysis at level 2 and level 3 are provided as Fig. S5 and S6. Significant differences are detected between the PRE and Saline groups among all the five functions ($p<0.05$, Fig. 5B), with the downregulation of Metabolism and the upregulation of the other five. No differences are observed between the PRE and Cell groups ($p>0.05$, Fig. 5C). Relative abundance of differential functions of the three groups are presented in Table S2. According to these results, functional differences are inferred to be significant between the Saline and Cell groups.

Therefore, these results indicate that periodontal defect therapy by injections of PDLSCs reverses the negative shifts in oral microbiota caused by periodontal surgery back towards normal and balanced healthy conditions.

Human PDLSCs inhibit pathogenic bacterial growth in vitro

Bacterial count tests showed that human PDLSCs significantly inhibit the growth of *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) compared with the control medium (α-MEM) ($p<0.05$, Fig. 6A and B). To determine if the observed antibacterial effect was associated with soluble secreted factors, we assessed the ability of the CM to inhibit bacterial growth by incubation with *S. aureus*, *E. coli*, and the periodontal pathogenic bacteria *Fusobacterium nucleatum* (*F. nucleatum*).

Live/dead staining showed that the intensity of red fluorescence representing dead bacteria is increased while the intensity of green fluorescence representing live bacteria is decreased in the Cell CM group compared with the Control group ($p<0.05$, Fig. 6C and D for *S. aureus*, Fig. 6E and F for *E. coli*, Fig. 6G and H for *F. nucleatum*). These evidence the bactericidal effects of Cell CM. Consistently, the colony counting results show that the number of bacteria colonies for the Cell CM group is less than that for the Control group ($p<0.05$, Fig. 6I for *S. aureus*, Fig. 6J for *E. coli*, Fig. 6K for *F. nucleatum*). Using plate-crystal violet assays, the OD value of the biofilms for the Cell CM group is much lower than that for the Control group ($p<0.05$, Fig. 6L for *S. aureus*, Fig. 6M for *E. coli*, Fig. 6N for *F. nucleatum*).

Under SEM observation, the bacteria in the Control group show intact and smooth surfaces, and their morphology is regular and with good cellular aspects. Comparatively, the bacteria morphology is changed in the Cell CM group, the cell surface shows irregular shrinkage and is deformed to large extent. Structures with poorly defined shapes and sizes indicate cell lysis, with extravasation of the intracellular contents (Fig. 6O for *S. aureus*, Fig. 6P for *E. coli*, Fig. 6Q for *F. nucleatum*). The above results confirm the inhibitive effect of Cell CM on *S. aureus*, *E. coli*, and *F. nucleatum*.

Interestingly, a significant effect of CM from cells stimulated with *F. nucleatum* (S-Cell CM) on *F. nucleatum* growth in comparison with control medium or Cell CM is observed ($p<0.05$, Fig. 6K and N, and 6Q).

Secretion of LL-37 by PDLSCs may be the main mechanism of their antibacterial action

The above results suggests that the antimicrobial mechanism of PDLSCs is associated with a secreted product, which can be induced with previous bacterial challenge. To investigate this potential mechanism, Cell CM, S-Cell CM, and I-Cell CM were analyzed for the presence of LL-37. The levels of LL-37 protein secreted for the Cell and I-Cell groups are higher compared with that for the

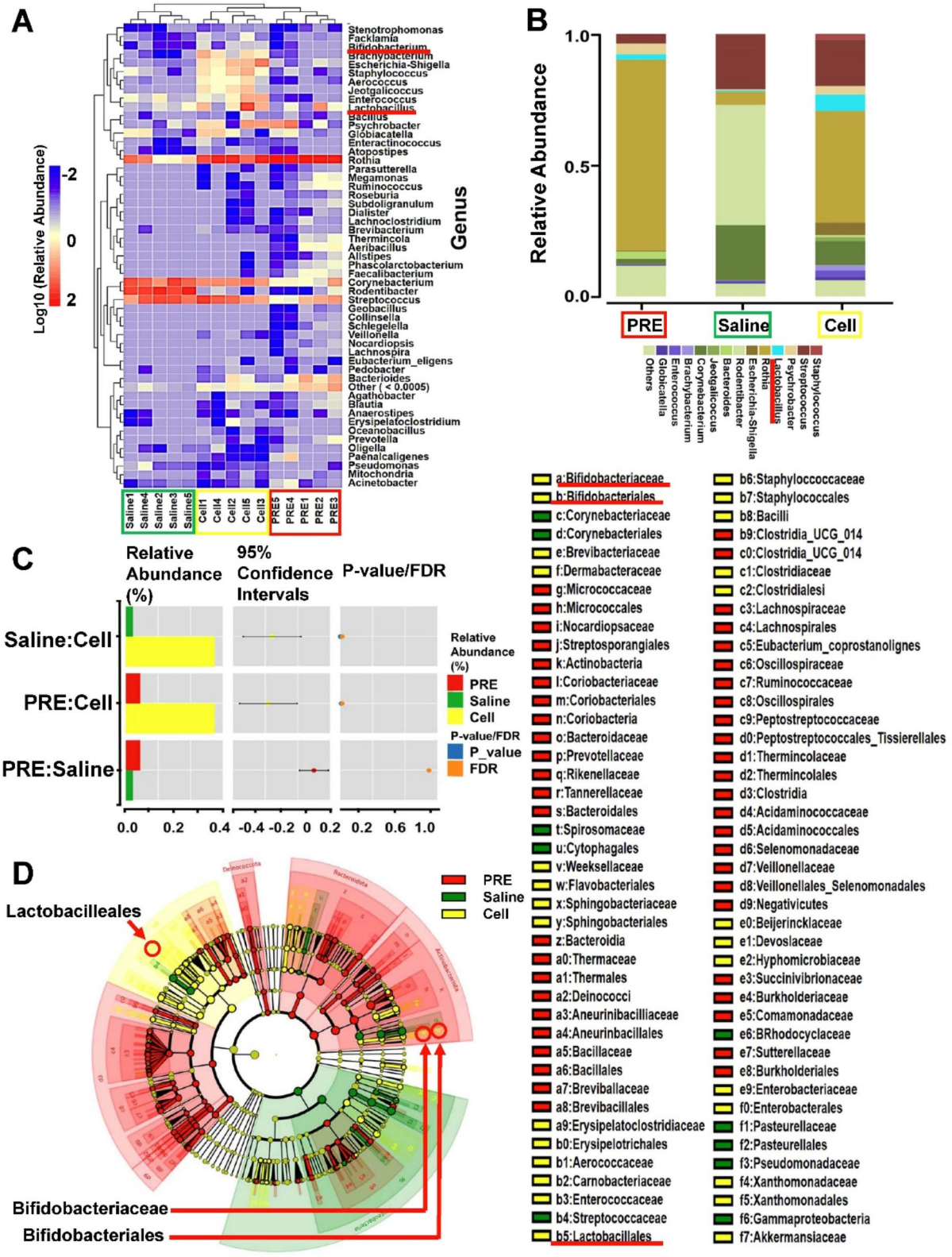

Fig. 4 Variations in the microbiota communities. (**A**) Heat map of species abundance at the genus level; (**B**) Species abundance analysis at the genus level; (**C**) Species difference analysis at the genus level (Bifidobacterium); (**D**) Linear discriminant analysis of effect size analyses (LEfSe) comparison

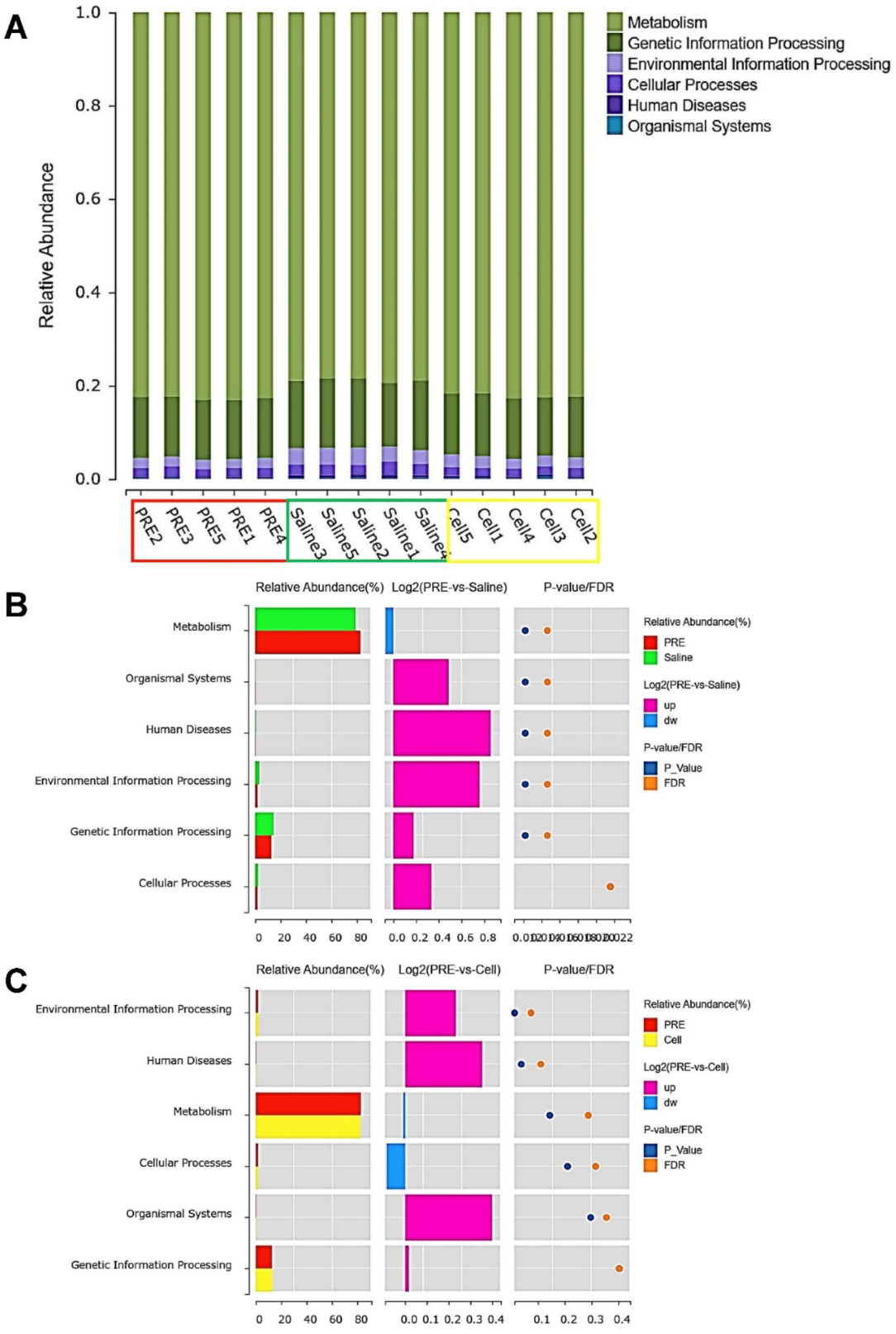

Fig. 5 Functional difference analysis. (**A**) Functional analysis at level 1 using the KEGG database; (**B**) Functional difference analysis between PRE group and Saline group at level 1 using the KEGG database; (**C**) Functional difference analysis between PRE group and Cell group at level 1 using the KEGG database

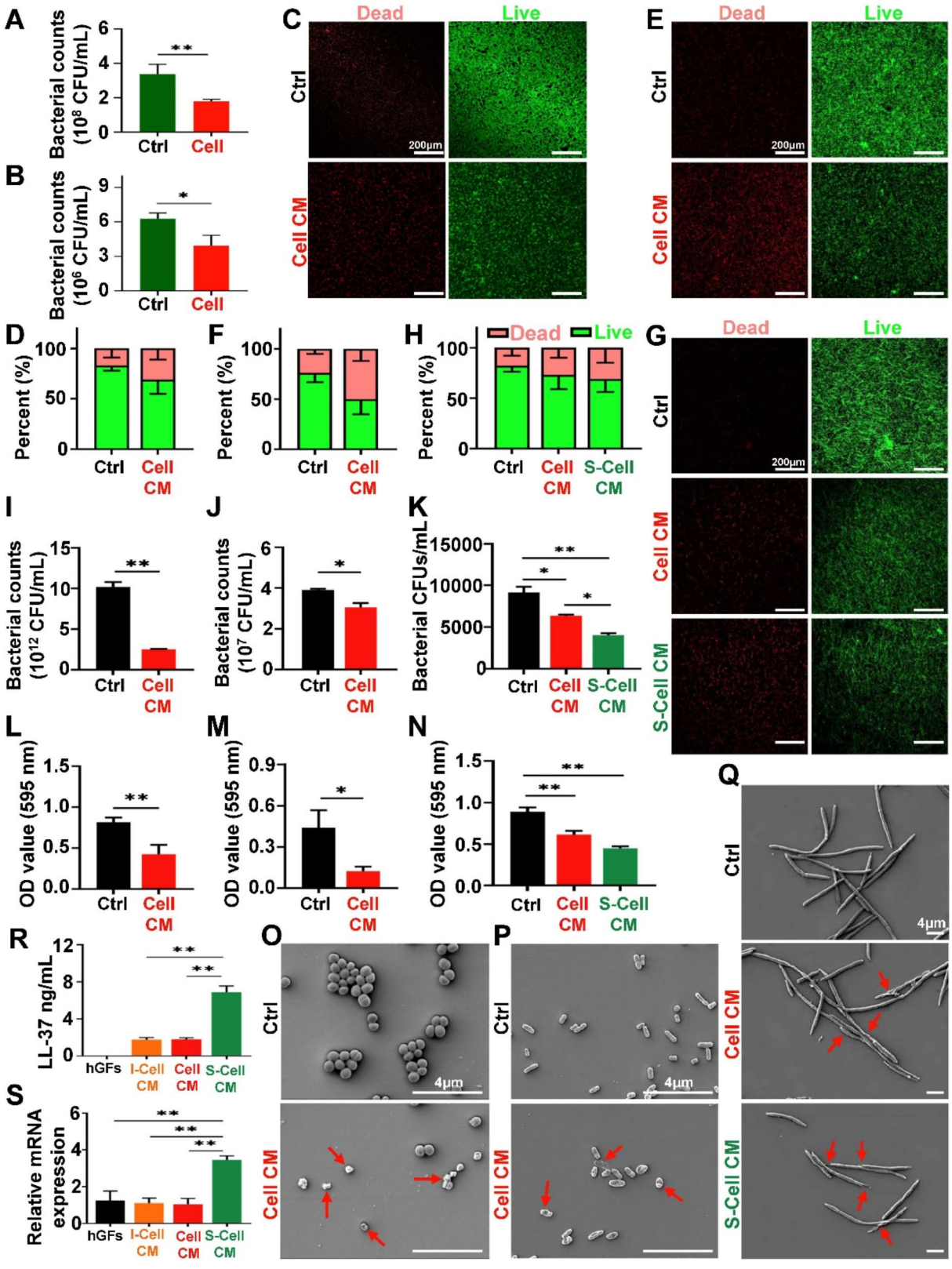

Fig. 6 (See legend on next page.)

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Fig. 6 The antibacterial effects of PDLSCs and PDLSCs CM in vitro. (**A**) The antibacterial effects of PDLSCs on *S. aureus*. (**B**) The antibacterial effects of PDLSCs on *E. coli*. (**C**) Live/dead bacteria staining images for *S. aureus* biofilms by PDLSCs CM; (**D**) Quantitative analysis of fluorescent intensity (*S. aureus*); (**E**) Live/dead bacteria staining images for *E. coli* biofilms by PDLSCs CM; (**F**) Quantitative analysis of fluorescent intensity (*E. coli*); (**G**) Live/dead bacteria staining images for *F. nucleatum* biofilms by PDLSCs CM; (**H**) Quantitative analysis of fluorescent intensity (*F. nucleatum*); (**I**) Colony formation test by ager plate (*S. aureus*); (**J**) Colony formation test by ager plate (*E. coli*); (**K**) Colony formation test by ager plate (*F. nucleatum*); (**L**) Biofilm test (*S. aureus*); (**M**) Biofilm test (*E. coli*); (**N**) Biofilm test (*F. nucleatum*); (**O**) SEM images (*S. aureus*); (**P**) SEM images (*E. coli*); (**Q**) SEM images (*F. nucleatum*); (**R**) Protein levels of LL-37 by ELISA; (**S**) mRNA expression levels of LL-37. $n=3$. $^{*}p<0.05$, $^{**}p<0.01$

control hGFs ($p<0.05$, Fig. 6R). Moreover, S-Cell CM shows the highest level of LL-37 ($p<0.05$). At the mRNA level, the amounts of LL-37 expressed for the Cell and I-Cell groups are similar to that for hGFs, all at a low baseline level ($p>0.05$). However, it is increased significantly for the S-Cell group ($p<0.05$, Fig. 6S).

Discussion

PDLSCs have been applied to enhance periodontal regeneration, using their whole cells, conditioned media, and exosomes [24–26]. Results from animal studies and clinical trials show that the use of PDLSCs can be expected to achieve a beneficial outcome for periodontal regeneration, although the transplanted cells disappear instead of showing drastic engraftment [25]. The efficacy of stem cell transplantation relies on their modulatory effects rather than their own proliferation and differentiation ability. Previous studies focused on the immune cells in the oral cavity while ignoring its microbiome. In this study, the effects of PDLSCs on the microbiome were explored by assessing the inhibition of the growth and virulence of pathogenic bacterial in vitro as well as diversity changes in the oral microbiota during the treatment of periodontal defects by PDLSC injection in vivo. The overall aim was to explore new strategies for PDLSC-promoted periodontal regeneration.

The Saline group represents the diseased periodontal condition with vehicle treatment. It showed a significant shift in microbiome composition and the abundances of core species, mainly decreased microbial diversity, as detected by α and β-diversity metrics. There have been many studies on changes to the oral microbiome in sufferers of conditions such as periodontitis, oral cancer, and diabetes compared with those of healthy subjects [27–29]. Healthy oral microbiomes have been reported to exhibit high levels of inter-individual variability. Although subject to external influences from the host and the environment as a result of the host's behavior, the oral microbiome may achieve a resilient state as a result of the influence of external factors [30]. However, it is difficult for a disease-affected microbiota to return to a healthy state. Interestingly, PDLSC injections help to reverse the unhealthy changes of the oral microbiome towards that of the PRE group, which represents a normal and healthy oral condition. Meanwhile, PDLSC injections enhance the regeneration of periodontal defects, as confirmed by micro-CT and histological analyses.

The improved state of the oral microbiome should be stable to prevent the recurrence of the disease. It is known that periodontal therapy can affect the composition of subgingival microbiota. Treatments ameliorate oral microbiome conditions, resulting in oral disease remission. However, patients with poor treatment outcomes also present limited microbiome shifts. This indicates the inefficient elimination of virulent species in these patients, the failure of commensal species to be established following treatment, and potential disease reemergence [31]. In the prevention and clinical management of many diseases, restoration of oral eubiosis represent a true revolution in the long term, especially for periodontitis.

Increased bacterial diversity was detected in rats receiving PDLSC injections compared with that in saline-treated rats. Consistently, an increase in commensal flora was revealed by species analyses. Collectively, beneficial oral taxa were more effectively enriched by PDLSC treatment compared with vehicle treatment. Further supporting this notion, *Bifidobacterium* sp. and *Lactobacillus* sp. were detected as biomarkers in PDLSC-treated rats. These results suggest that a microbial microenvironment beneficial for the regeneration of periodontal tissue is supported by PDLSC treatment. Although 16 S rDNA sequencing provides valuable insights, it has a limitation in annotating only up to the genus level, thus obscuring strain-level variations. For a more nuanced understanding of strain changes in PDLSCs relevant to periodontal defects, macrogenomic sequencing represents a promising approach for future experiments.

Changes in the oral microbiome due to the antibacterial activity of PDLSCs may play a positive role in periodontal regeneration. Periodontopathic bacteria such as *P. gingivalis* and *(A) actinomycetemcomitans* have been shown to be inhibited by many *lactobacilli* and *streptococcal* strains in vitro [32, 33]. The antibacterial activity of *Lactobacilli* can also present as the regulation of periodontal pathogen-induced inflammatory response that affects the host immunological reactivity. In an animal study based on a rat periodontitis model, the application of *Lactobacillus reuteri* resulted in a reduction in bone loss, a controlled local inflammatory response, and an enhanced the periodontal tissue repair [34]. The beneficial effect of *Bifidobacterium* on periodontal tissues is not a common trait of this genus. *(B) breve* 110^{1A} induced an inflammatory profile in gingival tissues and did not

prevent alveolar bone loss, while *B. bifidum* 162^{2A} shows the potential to control periodontitis [35].

Different from probiotics, *Bifidobacterium* sp. and *Lactobacillus* sp. are biomarkers of commensal microbiota in PDLSC-treated rats. An important function of commensal oral microbiota is to maintain oral health. Commensal-microbiota-derived ligands signaling at pattern-recognition receptor-expressing host cells stimulate the host's immunity throughout life [36]. Clinical isolates from individuals with good oral health contain strains of *Streptococcus*, *Actinomyces*, and *Bifidobacterium*, all of which have been shown to inhibit the growth of key periodontal pathogen *P. gingivalis*. Nisin produced by a commensal of the oral microbiota *Lactococcus lactis* is a bacteriocin. It can inhibit oral-tumor formation and lengthen the life span of mice with tumors [37]. Therefore, the commensal oral microbiome plays important role in maintaining oral health and promoting systemic health.

In vivo microbiome diversity changes could be attributed to the antibacterial properties of PDLSCs. We previously reported that SPION-coated scaffolds enhance bone regeneration, which was partly related to alteration of the oral microbiota by the antibacterial effects of the SPIONs [38]. As confirmed by in vitro bacterial tests, PDLSCs inhibit bacteria growth and disrupt the integrity of bacterial cell membrane structure. MSCs that have been reported to be antibacterial include BMMSCs, umbilical cord mesenchymal stem cells, ADSCs, and menstrual-derived mesenchymal stem cells. The most well-characterized AMP is LL-37, the main antibacterial mechanism of which involves bacterial membrane disruption. Compared with hGFs, PDLSCs express more LL-37. Levels of AMPs are reported to be positively correlated with the concentration of MSCs and preconditioning treatment such as stimulation by bacteria, IFNγ, IL-1β, and IL-12 [39, 40]. Our finding that *F.-nucleatum*-pretreated PDLSCs secrete more LL-37 is consistent with previous findings, i.e., that MSCs secrete more antimicrobial peptides after being stimulated.

LL-37 can also inhibit biofilm formation, eradicate preformed bacterial biofilms, and act against fungi, viruses, and parasites [41–44]. Furthermore, LL-37 has immunomodulatory functionality, induces the proliferation of endothelial cells, and promotes angiogenesis through FPRL1 signaling [45]. Whether these LL-37 effects are involved in PDSLC-treated periodontal regeneration needs further study.

During more complex bone grafting periodontal surgeries, practitioners are likely to prescribe antibiotics. The most common rationale is that it decreases the chances of developing an infection [46]. However, clinical evidence has failed to show that the use of adjunct systemic antibiotics in regenerative periodontal surgery achieves more favorable clinical outcomes [47, 48]. Moreover, the application of antibiotics causes many adverse effects such as type 1 hypersensitivity reactions, taste alteration, and burning sensation [49]. Due to their antibacterial activity, the potential of MSCs has gained attention for the treatment of a variety of immune-mediated disorders and conditions. As the antibacterial mechanisms of MSCs are different from those of conventional antibiotics, they show potential as a novel therapeutic approach combatting multidrug resistant pathogens.

Within the limitations of the current study, it is not known whether other antimicrobial factors are induced by PDLSCs, as only the level of LL-37 was explored. An LL-37-blocking antibody could be used in future studies to confirm whether the antimicrobial activity of PDLSCs is due to LL-37. The regulatory effects of PDLSCs on immune cells to enhance cellular behaviors such as phagocytic activity will also be explored.

Conclusions

In summary, our data explicitly revealed that PDLSCs have antibacterial properties and that this activity is beneficial for periodontal regeneration. In vitro, it presents as the inhibition of pathogenic bacteria growth, while in vivo, it is shown as regulation of the diseased microbiome to restore health. The main mechanism of action is postulated to involve production of LL-37. These findings shed new light on the mechanisms for MSC-promoted periodontal regeneration.

Supplementary Information

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Supplementary Material 1

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Author contributions

JYY contributed to the study design, performed all experiments, performed data extraction and analysis. QZ performed bacterial assay and prepared the manuscript. LJQ performed tissue procurement, data generation, interpretation, and intellectual contribution. ZHS assisted with animal experiments, results interpretation and artwork. XYW provided intellectual contribution and critically appraised themanuscript. LJ assisted with formal analysis, and revised manuscript. YX contributed to the conception and design of the study, obtained grant funding for the study, analyze the data, revised the manuscript and made the final quality control. All authors have read and approved the final submitted manuscript.

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Data availability
Not applicable.

Declarations

Ethics approval and consent to participate
For animal experiments, (1) Title of the approved project: Periodontal regeneration by stem cell injections; (2) Name of the institutional approval committee or unit: Institutional Animal Care and Use Committee of Nanjing Medical University; (3) Approval number: IACUC-1908036; (4) Date of approval: Jan 25, 2019. For PDLSCs isolation, (1) Title of the approved project: Isolation of PDLSCs; (2) Name of the institutional approval committee or unit: Ethics committee of the Institute of Stomatology, Nanjing Medical University; (3) Approval number: PJ2018-064-001; (4) Date of approval: Sep 5, 2018.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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