SHED Aggregate Derived Exosomes-shuttled miR-222 Promotes the Regenerative Properties of Periodontal Ligament Stem Cells

Feng Zhou  
Stomatology Hospital of Guangzhou Medical University

Jia Guo  
Fourth Military Medical University: Air Force Medical University

Fang Wang  
PLA Middle Military Command General Hospital

Wanmin Zhao  
Fourth Military Medical University: Air Force Medical University

Xiaoning He  
Fourth Military Medical University: Air Force Medical University

Meiling Wu  
Fourth Military Medical University: Air Force Medical University

Kun Ji  
Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital

Bei Li  
Fourth Military Medical University: Air Force Medical University

Yan Jin  
Fourth Military Medical University: Air Force Medical University

Miao Zhou  (zhm1000@gzhmu.edu.cn)  
Stomatology Hospital of Guangzhou Medical University

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Abstract

**Background:** Periodontal ligament stem cells (PDLSCs) aggregate is still limited in clinical application for lack of angiogenesis. This study aimed to investigate the effects and underlying mechanism of exosomes derived from stem cells from human exfoliated deciduous teeth (SHED) aggregate (SA-Exo) on the aggregate formation and angiogenic properties of PDLSCs.

**Methods:** SA-Exo were isolated by ultracentrifugation. The effect of SA-Exo on the aggregate formation and angiogenic differentiation of PDLSCs were evaluated by investigating extracellular matrix (ECM) deposition and tube formation assay. MicroRNA (miRNA) sequencing was employed to screen different miRNA expression. The effect of targeting miRNA on ECM deposition and angiogenesis of PDLSCs aggregate was investigated after overexpression and inhibition of miRNA. Periodontal bone defect rat models were established to evaluate the effect of the PDLSCs aggregate and SA-Exo combination on periodontal bone regeneration.

**Results:** SA-Exo could significantly enhance the ECM deposition and angiogenic ability of PDLSCs. The expression of ECM-associated proteins (COL-I, integrinβ1, and fibronectin), angiogenesis-related proteins (PDGF, ANG, TGFβRII), and related pathway (p-SMAD1/5 and p-SMAD2/3) were upregulated in PDLSCs aggregate with SA-Exo. Mechanistically, miR-222 was found relatively abundant in SA-Exo, which promoted ECM deposition and angiogenesis of PDLSCs. In vivo experiment further validated that combinational use of PDLSCs aggregate and SA-Exo promote more bone formation and neovascularization in rat’s periodontal bone defect.

**Conclusions:** SA-Exo-shuttled miR-222 contributes to PDLSCs aggregate engineering by promoting aggregate formation and angiogenesis, which might through activate the TGF-β/SMAD signaling pathway.

Background

Periodontal diseases comprise a wide variety of chronic inflammatory conditions which affect the supporting tissues of the teeth including the gingiva, alveolar bone and periodontal ligament, which could result in tooth loss and contribute to systemic inflammation [1]. Traditional treatment strategies for periodontal bone defects include guided bone regeneration (GBR), autologous or allogeneic bone transplantation, and biomaterials combined with genes/growth factors [2]. Recently, periodontal ligament stem cells (PDLSCs)-based cell sheet or aggregate therapy has emerged as a promising strategy in periodontal regeneration [3]. However, the therapeutic efficacy in animal and clinical studies are still controversial [4–6]. Lack of angiogenesis might be the leading cause of the defective periodontal tissue regeneration mediated by PDLSCs sheet or aggregate [7]. Therefore, improving angiogenesis of PDLSCs aggregate has become an urgent problem to be solved at present.

Exosomes are nanometer-sized extracellular vesicles, which contain a broad spectrum of bioactive molecules including proteins, microRNAs (miRNAs), mRNAs and lipids [8]. As indispensable mediators in
cell-cell communication, exosomes have been reported to participate in regulating angiogenesis [9]. Wu et al. documented that stem cells from human exfoliated deciduous teeth (SHED) derived exosomes promoted the angiogenic differentiation of human umbilical vein endothelial cells (HUVEC) and had potential value in alveolar bone regeneration [10]. We also found that SHED aggregate could regenerate plenty of blood vessels in dental pulp after implantation into injured teeth [11]. Additionally, a recent study investigated that exosomes from oral mucosal epithelial cell sheet showed pro-regenerative effects on skin wound healing [12]. These findings suggested that exosomes derived from SHED aggregate (SA-Exo) also might have an outstanding effect on angiogenesis and tissue regeneration. However, whether the SA-Exo promote angiogenic differentiation of PDLSCs aggregate in periodontal regeneration remain unclear.

In this work, we investigated the efficacy of SA-Exo in angiogenic potential of PDLSCs. We found that SA-Exo promoted angiogenesis and aggregate formation of PDLSCs. Mechanistically, we revealed that miR-222 shuttled by SA-Exo promoted PDLSCs angiogenesis and aggregate formation. In addition, we explored the therapeutic effect of PDLSCs aggregate combined with SA-Exo on periodontal defects in Sprague-Dawley (SD) rats. Our data indicated that the combination of PDLSCs aggregate and SA-Exo could promote periodontal bone regeneration by enhancing angiogenesis, potentially providing a new strategy for repairing periodontal defects.

**Methods**

**Cell Isolation and Aggregate Culture**

Healthy human impacted third molars or extracted teeth for orthodontic reasons were collected from individuals aged 18-40 years. The standard of healthy periodontal tissues: no bleeding on probing, probing depth ≤ 4mm, alveolar bone loss ≤ 3mm. PDLSCs were isolated and cultured as previously described [13]. Briefly, the periodontal ligament was gently separated from the middle part of the root surface and enzymatically digested with type I collagenase (3mg/ml, Sigma-Aldrich, USA) for 2h at 37°C (suspended every 15 mins). Cells were then plated in 6-well plates in alpha modification of Eagle's medium (a-MEM; Gibco, USA) with 10% fetal bovine serum (FBS, Gibco, USA), 0.292 mg/ml L-glutamine (Invitrogen, USA), 100 units/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen) at 37 °C in 5% CO₂, cultured for 2 weeks and the medium was changed every 3 days. To further purify the stem cells, single-cell-derived colonies were obtained using the limiting dilution technique.

Normal exfoliated human deciduous incisors were collected from 7-8-year-old children. SHED was isolated and cultivated in accordance with the protocol previously described [14], that is, the pulp was separated from remnant crown and the digested with 3mg/ml type I collagenase (Sigma) and 4 mg/mL dispase (Sigma) for 1h at 37°C. The single suspensions were seeded into 6-well plates with regular cell culture medium mentioned above. SHED used were at passage 2-5, and the same passage were used for each experiment.
PDLSCs aggregates were prepared as described previously [15]. 1-2×10^5 PDLSCs per well were seeded in 6-well-plate with basal medium and changed for aggregate induction medium with SHED aggregate-derived exosomes at different concentrations when reaching 100% confluence. The induction medium was refreshed every 3-4 days. The aggregate was harvested after 10 days for the following experiments.

**Exosomes Isolation and Characterization**

SHED aggregates were prepared as described previously [11]. Then the SHED aggregate or SHED were cultured in the medium with exosome-deleted FBS for 48h. Exosomes-deleted FBS was prepared by ultracentrifuging for 16h at 100000g before use. Finally, the supernatants were collected and several centrifugates were performed for exosomes isolation as described previously [16]. Briefly, the supernatants were centrifuged at 300g for 10 min, 2000g for 10 min, and 16000g for 30min to remove dead cells and cellular debris, respectively. Then, the supernatants were ultra-centrifuged at 150000g for 70min, and additionally washed with PBS for another 70min at 150000g. All procedures were performed at 4°C. The final pellets were resuspended in sterile PBS and stored at -80°C for following experiments.

Western blotting was performed to detected exosome markers, including CD63 (ab217345, Abcam, USA), CD81 (sc-166029, Santa Cruz-Biotechnology, USA), CD9 (ab92726, Abcam). Cell extract was considered as control. Transmission electron microscopy (TEM) (Thermo Fisher, USA) was conducted to observe the morphology of exosomes. The exosomes were dropped onto carbon-coated copper grids and kept for 3-4 min, then dried with filter paper and stained with 1%phosphotungstic acid for 10s, finally washed with distilled water for 30s. Nanoparticle tracking analysis (NTA) was utilized to identify the particle size and distribution.

**Exosomes Uptake Assay**

Enriched exosomes were labeled with PKH67 (Sigma) according to the manufacturer’s protocol. PDLSCs were seeded in 24-well plates with climbing, and incubated with the labeled exosomes for 4 and 24h, respectively. Hoechst (33342, Sigma) was used for staining the nuclei. The uptake of exosomes was visualized by confocal fluorescence microscope (Nikon, Japan).

**Tube Formation Assay**

In vitro angiogenesis was determined by tube formation assay in Matrigel. PDLSCs (4×10^4) were seeded onto Matrigel (Sigma)-coated 96-well plate and cultured in FBS-free medium in the presence of exosomes at the indicated concentrations (0, 5, 10, 15, 30, and 60mg/ml) for 6h. The images of tube formation were acquired by inverted phase contrast microscope (Leica, Germany). The total tube length, number of junctions and number of nodes were calculated by randomly selecting five fields per well using ImageJ 1.53c (National Institutes of Health, USA). The same experimental approach was used in PDLSCs after transfection with mimics and inhibitor.

**Alkaline phosphatase (ALP) staining and Alizarin Red S (ARS) Staining**
PDLSCs were seeded in 12-well plate and cultured until the cells reached 80% confluence, then incubated with exosomes at different concentrations (0, 15, 30, and 60mg/ml) for 48h and further induced with osteogenic medium (basal medium supplemented with 50 mg/mL L-ascorbic-2-phosphate (MP Biomedicals, USA), 0.1 mM dexamethasone, and 5 mM b-glycerophosphate (Sigma)) for 7 days or 28 days for ALP staining or ARS staining, respectively. For ALP staining, the cells were rinsed with PBS, then nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Beyotime, China) were added for the staining. 30 min later, staining agent was discarded and cells were rinsed two times with PBS. For ARS staining, the cells were fixed with 60% isopropyl alcohol and stained with 2% ARS (Kermel, China). After rinsing with distilled water, stained calcium nodules were identified under microscope. Finally, the mineralized nodules were dissolved with hexadecyl pyridinium chloride, and absorbance was quantitatively measured at 570 nm for statistical analysis.

**Scanning Electron Microscopy (SEM)**

The cell aggregates from each group were washed with PBS three times, fixed with 2.5% glutaraldehyde at 4°C, dehydrated and dried in a critical-point dryer. Finally, the specimens were observed under SEM (Hitachi S-4300; EIKO Engineering, Tokyo, Japan).

**Western blot**

Total proteins were isolated from cell aggregates. Extracellular matrix (ECM)- and angiogenesis-related proteins were analyzed by western blot as previous described [17]. Primary antibodies employed in this study included fibronectin (ab2413, Abcam), integrinβ1 (ab52971, Abcam), collagen type I (COL-I) (ab34710, Abcam), angiogenin (ANG) (ab10600, Abcam), platelet derived growth factor (PDGF) (3174, Cell Signaling Technology), phosphate-SMAD1/5 (#9516, Cell signaling Technology), transforming growth factor-β receptor II (TGFβRII) (sc-400, Santa Cruz Biotechnology), phosphate-SMAD2/3 (sc-11769, Santa Cruz Biotechnology) and GAPDH (30201ES20, YEASEN). Secondary antibody was HRP conjugated to antibodies to rabbit and mouse.

**Small RNA sequencing**

Small RNA sequencing was performed by BGISEQ-2000 and the sequencing libraries were constructed in BGI online platform (Shenzhen, China). The miRNAs expression with significant differences between the SHED- and SHED aggregate- derived exosomes was shown by the heatmap.

**Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted with TRIzol reagent (Invitrogen) and converted to cDNA using Mir-X™ miRNA First- Stand Synthesis Kit (Takara, Japan). Then RT-PCR was conducted with SYBR® Premix Ex Taq™ II (Takara) using the quantitative PCR System (Bio-Rad, USA). The expression of miR-222 was normalized to that of U6 snRNA. The miR-222 primer was AGCUACAUCUGCUACUGGGU (RiboBio, China). The forward and reverser primer of U6 were included in the Mir-X™ miRNA First- Stand Synthesis Kit (Takara).
Cell transfection

miRNA mimics or inhibitors were transfected into PDLSCs using riboFECT™ CP (RiboBio, China) according to the manufacturer’s protocol. The miRNA mimics and inhibitor used were from Sangon Biotech (Shanghai, China).

Periodontal Bone Defect Model

The use of Sprague-Dawley (SD) male rats for research was approved by the IRB of FMMU. The surgical procedures were based on the guidelines of the Animal Care Committee of FMMU. The periodontal defect model was created as previously described [15]. A periodontal bone defect of approximately 3×2×1 mm³ was created at the buccal alveolar bone of left mandibular molars. A total of 15 SD rats (8 weeks old) from the FMMU Animal Center were randomly divided into 3 groups: (1) a control group without cell and β-tricalcium phosphate (β-TCP) (provided by University of Extremadura, Spain) implantation (control, n=5); (2) a group treated with PDLSCs aggregate wrapping β-TCP (PDLSCA, n=5); and (3) a group treated with combination of PDLSCs aggregate and SA-Exo, which wrapping β-TCP (PDLSCA+SA-Exo, n=5). After 6 weeks, the rats’ mandible samples were harvested and fixed with 4% paraformaldehyde for 48h.

Micro-CT

The mandible samples were scanned using a Micron X-ray 3D Imaging System (YXLON, Germany) with 10mm resolution. Three-dimensional (3D) images were reconstructed and analyzed by the VG Studio 3.4 (VG, Germany). The ratio of new bone volume to tissue volume (BV/TV) was calculated.

Histological Analysis

After micro-CT analysis, the mandibles were decalcified with 17% ethylenediaminetetraacetic acid (EDTA) (MP Biomedicals) for 1 month and embedded in paraffin. Paraffin sections (3um thick) were stained using hematoxylin and eosin (HE) as described [18]. Photographs were taken using a microscope (OLYMPLUS, Japan). The percentage of new bone area in the total area was evaluated quantitatively from 3 randomly-selected sections by ImageJ 1.53c.

Masson’s Trichrome Staining

The paraffin sections were stained with Masson’s trichrome staining (Baso Diagnostic Inc., China) according to the manufacturer’s instructions. Photographs were taken using a microscope (OLYMPLUS).

Immunofluorescence Staining

The sections were deparaffinized and rehydrated, then subjected to antigen retrieval in boiled sodium citrate buffer solution (pH 6.0) for 10 minutes and cooled to room temperature. Slides were permeabilized with 1% TritonX-100 for 10 minutes and then blocked for 1h in blocking buffer (goat serum). After blocking, samples were incubated with primary antibody overnight at 4°C and then incubated with the
related fluorescence secondary antibodies for 1 hour at room temperature. Hoechst (MP Biomedicals) was used for counterstaining the nuclei. The immunofluorescent images were obtained using confocal fluorescence microscope (Nikon). Antibodies against fibronectin (ab2413, Abcam) and CD31 (ab212712, Abcam) were used in this study.

**Statistical Analysis**

All results were presented as the mean ± standard deviation (SD) of at least three independently experiments. Two-group comparisons were analyzed by Student’s t tests. Comparisons among three or four groups were evaluated by one-way ANOVA followed by LSD post hoc test. \( P \) value less than 0.05 was reflected to specify statistical significance.

**Results**

**SA-Exo promoted the angiogenic and osteogenic differentiation of PDLSCs**

Firstly, SHED and PDLSCs were isolated and characterized (Supplementary Figure S1). SA-Exo were isolated, which exhibited a cup-shaped morphology and had a bilayer membrane structure by TEM analysis (Fig. 1a). Western blotting analysis (Fig. 1b) demonstrated that SA-Exo expressed the exosome-specific markers CD9, CD63, CD81. In addition, the diameters of the exosomes ranged mainly from 90 to 200 nm was revealed by NTA. (Fig. 1c). To investigate whether the exosomes could be internalized into PDLSCs, SA-Exo labeled with PKH67 were incubated with PDLSCs for 4 and 24h, respectively. Confocal fluorescence microscopy analysis showed that PKH67-labeled exosomes (green) were gradually internalized by the PDLSCs from 4 to 24h (Fig. 1d).

The effects of SA-Exo at different concentrations (0, 5, 10, 15, 30, 60µg/ml) on PDLSCs angiogenesis were assessed by tube formation assay (Fig. 2a). In comparison with the control group (0µg/ml), the total length, number of junctions and number of nodes were significantly increased in high concentrations of exosomes group (10, 15, 30, 60 µg/ml) (Fig. 2b-d). To further investigate the dose effects of SA-Exo on PDLSCs osteogenesis, PDLSCs were cultured at different concentrations (0, 15, 30, 60µg/ml) of SA-Exo for 48h. After 7 days of osteogenic induction, ALP staining showed that 60µg/ml of exosomes obviously promoted ALP expression (Fig. 2e). Alizarin red staining on 28 days indicated that 30 and 60µg/ml of SA-Exo markedly enhanced the mineralization ability of PDLSCs (Fig. 2f, g). Thus, 60µg/ml of SA-Exo can significantly promote angiogenic and osteogenic differentiation of PDLSCs, and this dose was selected in the following experiments.

**SA-Exo enhanced ECM deposition and angiogenesis of PDLSCs aggregate**

After 10 days of culture with SA-Exo through the whole stage, PDLSCs formed complete aggregate that could be detached from the edge of the dishes. Cells in PDLSCs aggregate treated with SA-Exo (PDLSCA + SA-Exo group) exhibited more and better organization compared to those in PDLSCs aggregate (PDLSCA group) (Fig. 3a, first and second row). SEM was utilized to examine the micro-structure of
aggregate and showed a higher density network in PDLSCA + SA-Exo group (Fig. 3a). Moreover, PDLSCs aggregate combined with SA-Exo showed much more blue-stained collagen fibers compared to PDLSCs aggregate by Masson's trichrome staining (Fig. 3a). And then more expression of fibronectin, protein that mainly found in ECM, was observed in PDLSCA + SA-Exo group by immunofluorescence analysis (Fig. 3a). In addition, the expression of ECM-related proteins (fibronectin, integrinβ1, and COL-I) were also showed upregulated in PDLSCA + SA-Exo group compared to PDLSCA group by western blot (Fig. 3b). Meanwhile, we detected the expression of angiogenesis associated proteins and related pathways. The expression of PDGF, ANG, and TGFβRII and SMAD singling (phosphorylated SMAD2/3 and SMAD1/5) were upregulated after SA-Exo treatment (Fig. 3c). These outcomes suggested that SA-Exo can promote ECM deposition and angiogenesis of PDLSCs aggregate.

**MiR-222 contributed to SA-Exo mediated angiogenesis of PDLSCs**

Exosomes mediate biological activity by transforming cargos to the recipient cells. Accumulative evidences have demonstrated that exosomal miRNAs were important in regulation angiogenesis [9, 19, 20]. Therefore, to gain insights into how the angiogenesis of PDLSCs were promoted by SA-Exo, we analyzed the miRNAs expression profiles of SA-Exo. SHED-derived exosomes (S-Exo) were considered as control. The heat map of miRNA expression indicated that miR-222 was markedly upregulated in SA-Exo (Fig. 4a). The RT-PCR analysis also demonstrated that the expression of miR-222 in SA-Exo was significantly higher than that in S-Exo (Fig. 4b). Subsequently, in order to investigate whether miR-222 promote angiogenesis of PDLSCs, the tube formation assay was carried out after upregulation or downregulation of miR-222 (Fig. 4c). The expression of miR-222 in PDLSCs or SA-Exo were significantly overexpressed by pretreating PDLSCs or SHED aggregate with mimics, in contrast, miR-222 was suppressed by using the miR-222 inhibitor (Supplementary Figure S2). The total length, number of junctions and number of nodes were significantly increased in the miR-222 mimics group compared to the NC group. (Fig. 4d-f). In contrast, suppression results were found in the miR-222 inhibitor group (Fig. 4g-i). These data demonstrated that miR-222 contributed to SA-Exo mediated angiogenic differentiation of PDLSCs.

**MiR-222 contributed to SA-Exo mediated ECM deposition and angiogenesis of PDLSCs aggregate**

To further investigate whether miR-222 promote the ECM deposition and angiogenesis of PDLSCs aggregate, histological examination and associated protein expression were detected. When upregulating of miR-222 in PDLSCs aggregate, the more and better organization of cells in aggregate was observed by microscope and a higher density network was showed under SEM compared to the miR-222 mimics NC group (Fig. 5a and b, first and second row). Masson's trichrome staining also showed more collagen deposition and immunofluorescence analysis showed an upregulation of ECM-related protein fibronectin (Fig. 5c and d, first and second row). In contrast, opposite results were found after miR-222 was inhibited (Fig. 5a-d, third and fourth row). Additionally, the expression of ECM- and angiogenesis-associated protein were upregulated in the miR-222 mimics group and downregulated in the miR-222 inhibitor group (Fig. 5e-f). It has high light that TGF-β has a crucial role in the formation of ECM and
angiogenesis [21]. Thus, we detected the expression level of TGFβRII and related signaling (p-SMAD2/3 and p-SMAD1/5) by using western blot and found that overexpression of miR-222 activated the TGF-β signaling by upregulating p-SMAD2/3 and p-SMAD1/5 in PDLSCs aggregate compared to the NC group, and then these proteins were downregulated after miR-222 inhibition (Fig. 5f). Collectively, these findings suggested that SA-Exo shuttled miR-222 might activated TGF-β/SMAD signaling to promote ECM deposition and angiogenesis of PDLSCs aggregate.

**SA-Exo promoted PDLSCs aggregate to repair the periodontal bone defect in animal model**

In order to confirm the therapeutic effect of SA-Exo on PDLSCs aggregate, we carried out in vivo experiments of periodontal bone defect in SD rat. As shown by micro-CT, obvious bone regeneration was showed in PDLSCA + SA-Exo group when compared with the blank control and PDLSCA group (Fig. 6a, b). Meanwhile, an increased new bone formation in PDLSCA + SA-Exo group was observed by HE staining (Fig. 6c, d). Then Masson's trichrome staining showed more collagen deposition in the SA-Exo treated group (Fig. 6e). Additionally, the expression of angiogenesis-related protein CD31 was upregulated obviously in PDLSCA + SA-Exo group compared to the blank control and PDLSCA group (Fig. 6f). Therefore, the results indicated that SA-Exo promoted the PDLSCs aggregate to repair the periodontal bone defect by upregulating angiogenesis.

**Discussion**

Stem cell-based tissue engineering is recognized as a prospective approach for periodontal regeneration. Recently, cell sheet/aggregate technology served as an important strategy has been extensively used in periodontal regeneration, which could deliver high-density stem cells and preserve abundant ECM thereby mimicking natural microenvironments to improve cell survival and function [5, 18, 22]. However, the therapeutic effect of PDLSCs aggregate is still controversial because lack of vascular and mineral tissue formation in tissue regeneration [7]. In the present study, our results demonstrated that SA-Exo promoted ECM deposition and angiogenesis of PDLSCs aggregate by shuttling miR-222, which might activate TGF-β/SMAD signaling. Compared with PDLSCs aggregate, the combinational use of PDLSCs aggregate and SA-Exo enhanced bone repair in periodontal bone defect rat models, which might provide an alternative option for optimizing the therapeutic effect of PDLSCs aggregate-based periodontal regeneration.

Periodontal regeneration is a complex process, and angiogenesis considered as a fundamental element plays an important role in tissue regeneration due to neovascularization provides sufficient oxygen, nutrients and as well as discharges the metabolite [23]. Our previous study has reported that SHED aggregate regenerate plenty of blood vessels in dental pulp after implantation into immature permanent teeth, which suggested that the aggregate had powerful angiogenic ability [11]. Additionally, other than directly participating in tissue regeneration, reports also suggest that the extracellular vesicles (EVs) contribute to the regenerative effect of stem cells [24]. Exosomes are important components of EVs and abundant in ECM, which participate in matrix organization and regulation of cells within it [25]. Previous study has indicated that exosomes derived from clinical-grade oral mucosal epithelial cell sheets showed
pro-regenerative effects on skin wound healing [12]. Here, we found that exosomes derived from SHED aggregates would promote PDLSCs angiogenesis. Furthermore, the combinational use of PDLSCs aggregate and SA-Exo would significantly promote ECM deposition and angiogenesis.

Exosomes are important mediators of intercellular communication by shuttling a wide range of functional cargos. It has reported that exosomes contain multiple bioactive molecules involved in angiogenesis, including protein like vascular endothelial growth factor [26], fibroblast growth factor [27], PDGF [28], as well as miRNAs like miR-126, miR-26 [9]. In this study, we investigated the exact exosomal cargos that have a positive effect on angiogenesis of PDLSCs. We analyzed the miRNA expression profile of SA-Exo and found that miR-222 was markedly upregulated in SA-Exo compared to S-Exo. It has been demonstrated that miR-222 plays different roles in different cells and can positively and negatively modulate angiogenesis. Previous researches have indicated that miR-222 plays anti-angiogenic effect in endothelial cells, while has effects of pro-proliferation and pro-migration in vascular smooth muscle cells (VSMCs) [29]. In contrast, the high level of the miR-222 in many tumor cells as well as in many inflammation-based diseases does not fit with the view and exhibits promoting angiogenesis [30, 31]. Ribeiro-Rodrigues et al. reported that exosomes released by ischemic cardiomyocytes were enriched in miR-222 and promote angiogenesis. Simultaneously, miR-222 overexpression induced an increase in tubulation and sprouting by HUVECs [31]. Here, we observed PDLSCs overexpressed miR-222 exhibited increase of angiogenic differentiation, while decrease of this was showed when miR-222 inhibition. These findings imply that exosome-mediated miR-222 plays an important role in the angiogenesis. However, the exact target gene on angiogenesis should be investigated in future studies.

To the best of our knowledge, there are no publication that definitively show the stimulatory effects of miR-222 on ECM production. However, it has reported that miR-222 is closely related to atherosclerosis and considered as a biomarker for early diagnosis [32]. miR-222 promoted VSMCs proliferation and migration, which secrete diverse cytokines in atherosclerotic lesions, including interferon-γ, macrophage inhibitory factor, and TGF-β, which triggering the ECM remodeling or synthesis/deposition [33]. Its high light that TGF-β executes the production of matrix molecules and represses matrix degradation by stimulation of protease inhibitor synthesis and inhibition of proteases synthesis [21]. TGF-β binding to the TβRII results in phosphorylating and activating activin receptor-like kinase 5 (ALK5), then phosphorylate SMAD2/3 to increase ECM deposition [34]. In addition, high concentration of TGF-β was able to activate p-SMAD2/3 and p-SMAD1/5 induce a pro-angiogenic state [34, 35]. Here, we found that miR-222 upregulated ECM associated proteins in PDLSCs aggregate and downregulated with inhibition. Moreover, both p-SMAD2/3 and p-SMAD1/5 was upregulated in PDLSCs aggregate combined with SA-Exo via shuttling miR-222, which can be downregulated by miR-222 inhibition. Collectively, these results might suggest that miR-222 mediated ECM deposition and angiogenic differentiation in PDLSCs might through TGF-β/SMAD signaling pathway.

We further evaluate the function of PDLSCs aggregate and SA-Exo combined implantation in periodontal defect models. Consistent with the in vitro results, micro-CT and histological analyses revealed more new bone formation after treatment with SA-Exo than that in the without exosomes group in the rat
periodontal bone defects. Angiogenesis is an essential step during bone regeneration, as restoration of blood flow provides nutrients and renewable autologous cells to heal the defect. In vivo, we found that the addition of the SA-Exo resulted in generating more new blood vessels by immunofluorescence staining for CD31 which is common marker of neovascularization. Thus, our data suggested that combination of PDLSCs aggregate and SA-Exo could be considered as a potential strategy for periodontal bone regeneration. However, there were some limitations in our study. Periodontal defects are inflammation-mediated microenvironments and more complex than the defect that we established in this study. To better evaluate the therapeutic effect of SA-Exo on PDLSCs aggregate regeneration in periodontitis-induced alveolar bone defects, the immunoregulatory role should be further investigated in large animal periodontitis models.

**Conclusions**

Our study firstly showed that exosomes derived from SHED aggregate promote PDLSCs aggregate formation and angiogenesis, which contribute to periodontal bone defects reparation. Moreover, the exosomes transfer miR-222 to PDLSCs and might activate TGF-β/SMAD pathways to increase ECM deposition and angiogenesis. Our study provides a new potential strategy to improve the clinical therapy of PDLSCs aggregate in periodontal regenerative medicine.

**Abbreviations**

PDLSCs: Periodontal ligament stem cells

SHED: Stem cells from human exfoliated deciduous teeth

GBR: Guided bone regeneration

HUVECs: Human umbilical vein endothelial cells

SA-Exo: Exosomes derived from SHED aggregate

S-Exo: exosomes derived from SHED

ALP: Alkaline phosphatase

ARS: Alizarin Red Staining

NBT: Nitro blue tetrazolium

BCIP: 5-bromo-4-choro-3-indolyl phosphate

SEM: Scanning Electron Microscopy

ECM: Extracellular matrix
COL-I: Collagen type I

ANG: Angiogenin

PDGF: Platelet derived growth factor

TGFβRII: Transforming growth factor-β type II receptor

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

β-TCP: β-tricalcium phosphate

EDTA: Ethylenediaminetetraacetic acid

VSMCs: Vascular smooth muscle cells

EVs: Extracellular vesicles

Declarations

Ethics approval and consent to participate

The experimental protocols were reviewed and approved by the Institutional Review Board and the Ethics Committee of the school of stomatology, Fourth Military Medical University (FMMU). Consent forms were provided before conducting the research project.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

ZM and JY performed the design of the work and provided financial aid; LB and ZF performed this study and was a major contributor in writing the manuscript; GJ helped cell collection and data analysis; WF performed animal studies and results analysis; ZWM, HXN, WLM and JK helped exosomes collection. All authors read and approved the final manuscript.

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Figures
Characterization and internalization of SHED aggregate derived exosomes. (a) TEM observation of SA-Exo morphology. Scale bar: 200nm. (b) Western blot analysis of the exosomal surface markers (CD9, CD63, CD81) and a cytosolic marker ($\beta$-actin). (c) Particle size distribution and concentration of SA-Exo measured by NTA. (d) Confocal fluorescence analysis showed that SA-Exo were taken up by PDLSCs after 4h and 24h. Exosomes were labeled with PKH-67 (green), and Hoechst was used to stain the cell nuclei (blue). Scale bar: 50µm.

Figure 1
Figure 2

SA-Exo promoted the angiogenic and osteogenic differentiation of PDLSCs. (a) Tube formation assay showed angiogenic ability of PDLSCs with different concentrations (0, 5, 10, 15, 30, 60 µg/ml). Scale bar: 100µm. (b) Relative total tube lengths. (c) Relative number of junctions. (d) Relative number of nodes. (e) Representative images of ALP staining in PDLSCs pretreated by medium with different concentrations of exosomes (0, 15, 30, 60 µg/ml). (f) Representative images of Alizarin Red S staining. (g) Quantitative
analysis of the calcium deposition in PDLSCs. *P < 0.05, ** P < 0.01, *** P < 0.001 represent significant differences compared with the matched control group.

Figure 3

SA-Exo enhanced the ECM Deposition and angiogenesis of PDLSCs aggregate. (a) Microscopic images of PDLSCs aggregate (PDLSCA) and PDLSCs aggregate with 60 µg/ml exosomes (PDLSCA+SA-Exo); SEM images of PDLSCA and PDLSCA+SA-Exo. Scale bar: 200nm; Masson's trichrome staining showed that the aggregate was rich in collagen fibers; Immunofluorescence analysis revealed the expression of
ECM protein fibronectin in aggregate. Scale bar: 50µm. (b) Western blot analysis of ECM proteins fibronectin, integrinβ1 and COL-I. (c) Western blot analysis of angiogenic proteins (PDGF, ANG, and TGFβRII) and related signaling pathway (p-SMAD2/3 and p-SMAD1/5). GAPDH served as internal control.

**Figure 4**

MiR-222 contributed to SA-Exo mediated angiogenesis of PDLSCs. (a) Heatmap of the miRNAs expression with significant differences between the SA- and S-Exo. (b) RT-PCR showed the different
expression of miR-222 in SA- and S-Exo. (c) Tube formation analysis showed angiogenic ability of PDLSCs with upregulation and downregulation of miR-222. (d-f) Relative of total tube lengths (d), number of junctions (e) and number of nodes (f) when upregulation of miR-222. (g-i) Relative of total tube lengths (g), number of junctions (h) and number of nodes (i) when downregulation of miR-222. *P < 0.05, *** P < 0.001, **** P < 0.0001 represent significant differences compared with the matched control group.

Figure 5
MiR-222 contributed to SA-Exo Mediated ECM deposition and angiogenesis of PDLSCs aggregate. After upregulation and downregulation of miR-222 in PDLSCs aggregate, (a) Microscopic images of PDLSCs aggregates. (b) Microstructures of PDLSCs aggregates were observed by SEM. Scale bar: 200nm. (c) Masson’s trichrome staining showed the aggregates were rich in collagen fibers. (d) Immunofluorescence analysis revealed the expression of ECM protein fibronectin in aggregates. Scale bar: 50µm. (e) Western blot analysis of ECM proteins. (f) Western blot analysis of angiogenic proteins (PDGF, ANG, and TGFβRII) and related signaling pathway (p-SMAD2/3 and p-SMAD1/5). GAPDH served as internal control.
Figure 6

SA-Exo promoted PDLSCs aggregate to repair the periodontal bone defect in animal model. (a) micro-CT vertical and cross section images of the control, PDLSCA/β-TCP, and PDLSCA+SA-Exo/β-TCP groups. The periodontal bone defect area was marked with a red line. (b) Quantitative analysis of bone volume/total volume. (c) Quantitative analysis of the new bone area by HE staining sections. (d, e) HE staining and Masson's trichrome staining of cross sections. Scale bar: 250µm. (f) Immunofluorescence staining of CD 31 (green), nucleus (blue). The periodontal bone defect area was marked with a black or white dotted line. AB, alveolar bone; R, root. *P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 represent significant differences compared with the matched control group.

Supplementary Files

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