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FULL LENGTH ARTICLE

Extracellular vesicles derived from human dental mesenchymal stem cells stimulated with low-intensity pulsed ultrasound alleviate inflammation-induced bone loss in a mouse model of periodontitis

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Abstract Extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) have emerged as a new mode of intercellular crosstalk and are responsible for many of the therapeutic effects of MSCs. To promote the application of MSC-EVs, recent studies have focused on the manipulation of MSCs to improve the production of EVs and EV-mediated activities. The current paper details an optimization method using non-invasive low-intensity pulsed ultrasound (LIPUS) as the stimulation for improving oral MSC-EV production and effectiveness. Stem cells from apical papilla (SCAP), a type of oral mesenchymal stem cell, displayed intensity-dependent pro-osteogenic and anti-inflammatory responses to LIPUS without significant cytotoxicity or apoptosis. The stimuli increased the secretion of EVs by promoting the expression of neutral sphingomyelinases in SCAP. In addition, EVs from LIPUS-induced SCAP exhibited stronger efficacy in promoting the osteogenic differentiation and anti-inflammation of periodontal ligament cells *in vitro* and alleviating oral inflammatory bone loss *in vivo*. In addition, LIPUS stimulation affected the physical characteristics and miRNA cargo of SCAP-EVs. Further investigations indicated that miR-935 is an important mediator of the pro-osteogenic and anti-inflammatory capabilities of LIPUS-induced SCAP-EVs. Taken together, these findings

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demonstrate that LIPUS is a simple and effective physical method to optimize SCAP-EV production and efficacy.

Introduction

Bone loss and defects in the oral cavity caused by inflammation and trauma have become a major public health issue, producing significant economic and health burdens on the broad population.[1] Periodontitis, in particular, is a globally prevalent inflammatory condition that leads to the destruction of oral bone; it is the main cause of tooth loss in adults.[2] Bone reconstruction, especially for severe periodontal intrabony defects, has been a challenge for clinicians throughout the history of periodontal therapy.[3] Strategies for promoting oral bone regeneration include guided tissue regeneration (GTR), autogenous bone transplantation, bone allograft transplantation, and tissue engineering.[4] However, GTR cannot achieve satisfactory restoration,[5] and autogenous bone and bone allograft transplantation are limited to donor sources. Compared with these traditional methods, tissue engineering, which combines bone graft materials with biological mediators, can achieve predictable tissue repair and regeneration.[6] Enamel matrix derivative (EMD) and platelet-derived growth factor-BB (PDGF-BB) are two commercially available tissue engineering biological mediators that have been shown to be effective for infrabony defects.[7–9] However, the regeneration effects of a single factor are still limited and unstable. The achievement of maximum regeneration will require the application of multiple growth factors.

Due to their self-renewal, differentiation pluripotency, and immunomodulatory properties, mesenchymal stem cells (MSCs) offer a new strategy for tissue engineering and immunotherapy.[10,11] At present, it is believed that the effects of MSCs are due to the release of extracellular vesicles (EVs) and soluble factors, rather than the direct regeneration of engraftment cells.[12] EVs are a heterogeneous population of cell-derived small membranous spheres that carry proteins, nucleic acids, and lipids.[13] In particular, MSC-derived EVs can transmit multiple signals for tissue repair to the recipient cells. This better mimics the function of their parent cells, addresses the limited effects of a single factor, and avoids the disadvantages of direct MSC transplantation, such as contamination, immune rejection, and tumorigenic potential.[14,15]

Before the clinical application of MSC-EVs, there must be careful consideration of the cell sources and capability of derived EVs. Stem cells from apical papilla (SCAP) are a type of oral mesenchymal stem cell isolated from the developing tissue at the apex of a tooth root, termed the apical papilla. Compared with bone marrow MSCs, SCAP are easily available by extracting the growing third molar, and show better anti-inflammation, angiogenesis, and osteogenesis effects.[16,17] Due to their embryonic-like properties and homology with cells in oral tissue, SCAP are a promising cell source for MSC-based therapy for oral bone loss.[18–20] SCAP-derived EVs have also become an attractive cell-free treatment for oral tissue repair. Our preliminary data indicated that SCAP-derived EVs have the best multi-differentiation promotion potential as compared to other oral MSC-EVs (unpublished results). SCAP-derived EVs have been found to be beneficial for the regeneration of craniofacial soft tissue and dentine–pulp complex.[21,22] However, their application in oral bone loss requires further investigation.

In addition, the biogenesis, cargo, and function of EVs are highly related to the cellular culture environment.[23] Recently, intense efforts have been devoted to improving the production and therapeutic efficacy of EVs.[24] For EV-based therapy for bone loss, most published studies to date have relied on isolating EVs from cells induced by osteogenic induction medium.[25] Despite enhancement of the osteogenic capacity of EVs, these methods are time-intensive and costly. In addition, oral tissue healing and regeneration require the control of tissue inflammation due to the complicated oral environment.[26] Thus, there is a need to identify more convenient and effective ways to promote the production and function of MSV-EVs and their osteogenic induction and anti-inflammation effects. In this work, low-intensity pulsed ultrasound (LIPUS) was found to be a promising avenue of optimization. LIPUS is a non-invasive mechanical stimulus with a power density much lower (below 0.1 W/cm^2) than traditional ultrasound (0.1–1 W/cm^2). It has been approved for use as clinical adjuvant therapy for fresh bone fracture healing.[27] In our previous studies, LIPUS was found to promote oral osteogenesis and inhibit periodontal inflammation both *in vitro* and *in vivo*.[28–30] The aim of the current study was to investigate whether the effects of LIPUS on osteogenesis and anti-inflammation in previous studies can be partly explained by its effects on cell-derived EVs. Further, the specific influences of LIPUS on the physical characteristics, production, and cargo of EVs must still be elucidated before this approach can be adopted in the clinical setting.

Based on these considerations, in this study, we identified the optimal intensities of LIPUS for SCAP induction, meticulously examined the physical characteristics and yields of EVs derived from LIPUS-induced SCAP and uninduced SCAP, and compared their biological functions *in vitro* and the efficacy for inflammation-induced oral bone loss *in vivo*. Furthermore, we compared the microRNA (miRNA) expression profiles of EVs isolated from LIPUS-induced SCAP versus uninduced SCAP, and attempted to identify which differentially expressed miRNA caused the functional differences. The results indicate that LIPUS is a new physical stimulation approach that can promote the production of SCAP-EVs and their efficacy for oral bone loss.

LIPUS has the potential for application to other mesenchymal cells.

Materials and methods

In vivo study

Animals

All animal experiments in this study were performed in accordance with the Declaration of Helsinki and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Medical Ethics Committee of the School of Stomatology, Chongqing Medical University [No: 2019 (LsNo89)]. Two-month-old male C57BL/6 mice were purchased from the Animal Experimental Center of Chongqing Medical University. All animals were housed in temperature- and moisture-controlled specific pathogen-free facilities under a 12-h light/dark cycle and had access to water and food ad libitum. Twenty-four C57BL/6 mice were randomly divided into four groups ($n = 6$/group): control group (without any treatment), periodontitis + phosphate buffered saline (PBS) group (induced experimental periodontitis by a ligature and injected with PBS), periodontitis + Con-EV group (induced experimental periodontitis by a ligature and injected with Con-EVs (EVs from the uninduced SCAP)), and periodontitis + LIPUS-EV group (induced experimental periodontitis by a ligature and injected with LIPUS-EVs (EVs from the LIPUS-induced SCAP)). A flowchart of the full experimental procedure is provided in Figure 5A.

Mouse model of ligature-induced periodontitis

A mouse model of ligature-induced periodontitis was constructed according to a previous report.[31] A 5-0 silk ligature was inserted between the maxillary left first and second molar (M1—M2), and knots were tied on both ends to secure the ligature. On day 0, mice were subjected to ligature insertion. Then, EVs (5 μg/time) or PBS control were injected into the surface of the periodontal region on the ligature-inserted area every three days from day 0 to day 12 (the success of injection was confirmed by blanching of the periodontal tissue around the ligature-inserted area). The ligatures were also examined every three days to ensure that they remained in place during the experimental period. Periodontal inflammation and bone loss in this model are initiated by massive local accumulation of bacteria on the ligature-treated molars. The controls included mice that were not treated with ligatures on either side and not injected with anything. The mice were euthanized, and the tissues were harvested to examine inflammation and alveolar bone loss 14 days after placement of the ligatures.

In vitro study

Cell culture

Human impacted third molars with immature roots were collected from 10 healthy patients (18 years old) who were referred for third molar extraction at the Affiliated Hospital of Stomatology, Chongqing Medical University (Chongqing, China). The study was approved by the Ethics Committee of Chongqing Medical University [No: 2019 (LsNo89)]. All patients provided informed consent before participation. Briefly, the teeth were washed in PBS (Hyclone, USA) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone). The apical papillae and periodontal ligament tissue were gently separated from the tooth root and then digested in Type I collagenase (3 mg/mL). The digested tissue was dispersed in a culture flask. Alpha-minimum essential medium (Hyclone) containing 10% fetal bovine serum (FBS; Biological Industries, South America) was added, and the flask was turned upside down after 12 h. In all cases, the cells were maintained at 37°C in a humidified incubator containing 5% carbon dioxide. The culture medium was replaced every two days. SCAP and periodontal ligament cells (PDLCs) were obtained at the 3rd passage. Cells at passages 4—6 were used for EV isolation and other experiments. 293 T cells were maintained in DMEM with 10% FBS at 37°C in a humidified incubator containing 5% carbon dioxide.

Cell treatment

In order to establish an inflammatory environment, lipopolysaccharide (LPS) from *E. coli* (10 μg/mL) (Sigma, USA) was adopted to treat cells in FBS-free medium. For osteogenic induction, cells were seeded into six-well plates at a density of 1.2×10^6 cells/well. When the cells reached 70% confluency, the medium was replaced with osteogenic differentiation medium [5 mM β-glycerophosphate (Sigma, USA), 50 μg/mL ascorbic acid (Sigma, USA), and 10 nM dexamethasone (Sigma, USA)] made with EV-free FBS. The medium was changed every two days and osteogenesis was induced for seven days.

For LIPUS treatment, low-intensity pulsed ultrasound (1.5 MHz, 200-μs burst sine waves; National Engineering Research Center of Ultrasound Medicine, Chongqing, China) at 90 mW/cm^2 was applied to stimulate SCAP for 30 min in a water bath at 37°C. The transducer was excited at a frequency of 1.5 MHz, a pulse duty cycle of 1:4, and a pulse repetition frequency of 1.0 kHz.

EV isolation

EVs were isolated and purified based on an established protocol (Fig. 3A). After the cell density reached 70%—80%, SCAP were washed with PBS three times and changed to EV-free medium (bovine serum EVs were removed by 16 h centrifugation at 110,000 × *g*). The culture medium was collected after 48 h, and the EVs were isolated at 4°C by sequential centrifugation. The collected medium was centrifuged at 300 × *g* for 10 min to remove cell debris. The supernatant was centrifuged at 2000 × *g* for 10 min and then at 10,000 × *g* for 30 min. The resulting supernatant was filtered through a 0.22-μm filter, and EVs were pelleted by ultracentrifugation at 100,000 × *g* (Beckman Coulter Optima L-100 XP, SW 32 Ti) for 90 min. The EV pellet was washed in cold PBS and collected by ultracentrifugation again at 110,000×*g* for 90 min. Finally, the EV pellet was resuspended in PBS and stored at −80°C. The EV preparation is pooled for this study. The culture medium was mixed from different batch of cells to avoid the influences from different donors and different cell passages.

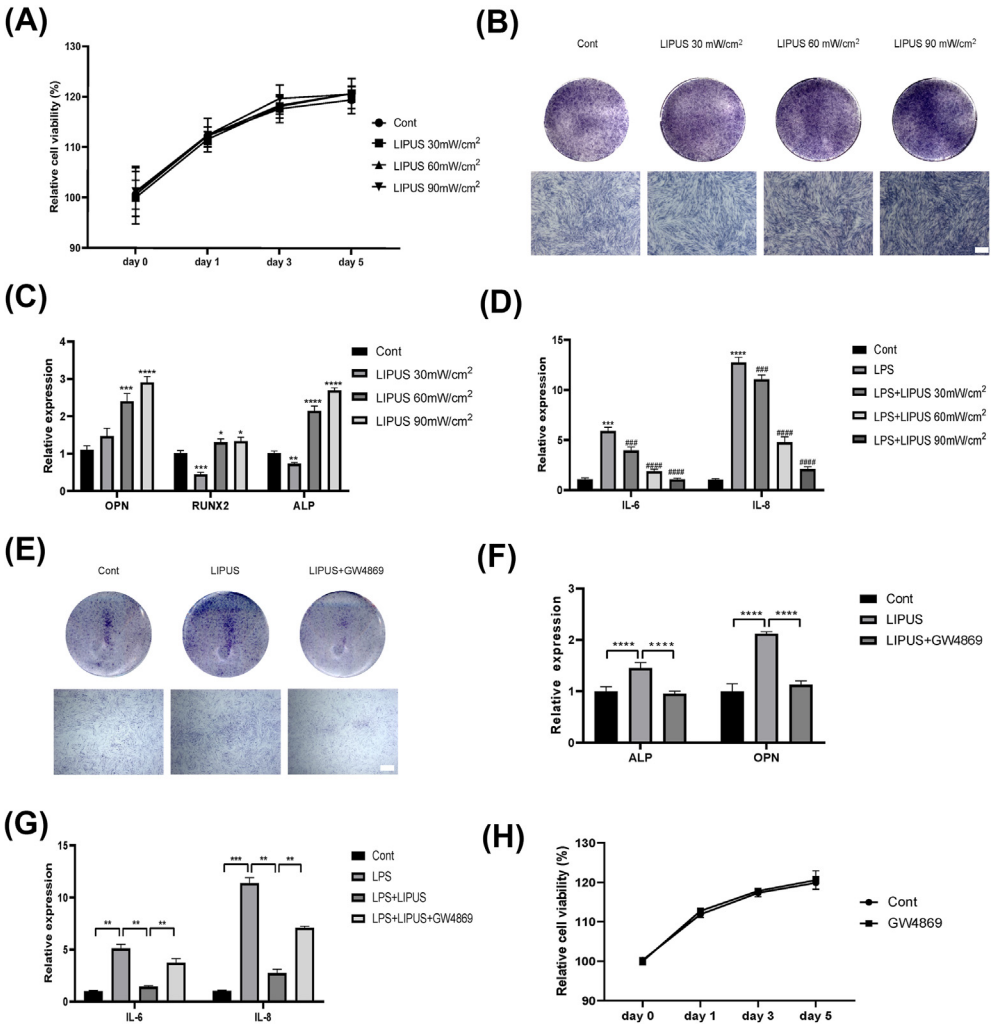

Figure 1 GW4869 impedes the promoting effects of LIPUS on SCAP. **(A—D)** The effects of LIPUS on SCAP. (A) SCAP were treated with or without LIPUS of different intensities for 30 min per day. Cell viability was analyzed with the CCK8 assay. (B) After osteogenic induction for 7 days, osteogenic differentiation was detected by ALP staining. Scale bar = 500 μm. (C) The expression levels of *ALP*, *OPN*, and *RUNX2* mRNA were assessed by qPCR. (D) SCAP were pretreated with 10 μg/mL LPS for 2 h and then treated with or without LIPUS of different intensities for 30 min. Cells were examined 10 h after the LIPUS treatment. The expression levels of *IL-6* and *IL*-8 mRNA were assessed by qPCR. Data are presented as the mean ± SEM of three independent experiments. $^{*}P < 0.05$ *vs*. Control group, $^{**}P < 0.01$ *vs*. Control group, $^{***}P < 0.001$ *vs*. Control group, $^{****}P < 0.0001$ *vs*. Control group. $^{\#\#\#}P < 0.001$ *vs*. LPS group, $^{\#\#\#\#}P < 0.0001$ *vs*. LPS group. **(E—H)** GW4869 impedes the promoting effects of LIPUS. (E) SCAP were pretreated with 10 μM GW4869 and then treated with or without LIPUS (90 mW/cm^2) for 30 min per day. After osteogenic induction for 7 days, osteogenic differentiation was detected by ALP staining. Scale bar = 500 μm. (F) The expression levels of *ALP* and *OPN* mRNA were assessed by qPCR (G) SCAP were treated with 10 μM GW4869 and 10 μg/mL LPS for 2 h and then treated with or without LIPUS of different intensities for 30 min. Cells were examined 10 h after the LIPUS treatment. The expression levels of *IL-6* and *IL*-8 mRNA were assessed by qPCR. (H) Cell viability was analyzed with the CCK8 assay. Data are presented as the mean ± SEM of three independent experiments. **, $P < 0.01$; ****, $P < 0.0001$.

Statistical analysis

All data in this study are expressed as the mean ± SEM and were analyzed by student's *t*-tests or one-way ANOVA. $P < 0.05$ was considered statistically significant. GraphPad Prism 8.0 software was used to analyze the statistical significance of the differences.

Detailed materials and methods are included in the Appendix. The original data are available from the corresponding author upon reasonable request.

Results

Isolation and characterization of stem cells from apical papilla

SCAP from the human third molar with developing roots were isolated and purified (Fig. S1A). The differentiation capacity of the SCAP was confirmed by inducing osteogenesis, adipogenesis, and chondrogenesis (Fig. S1B—D). Flow cytometry showed that SCAP positively expressed

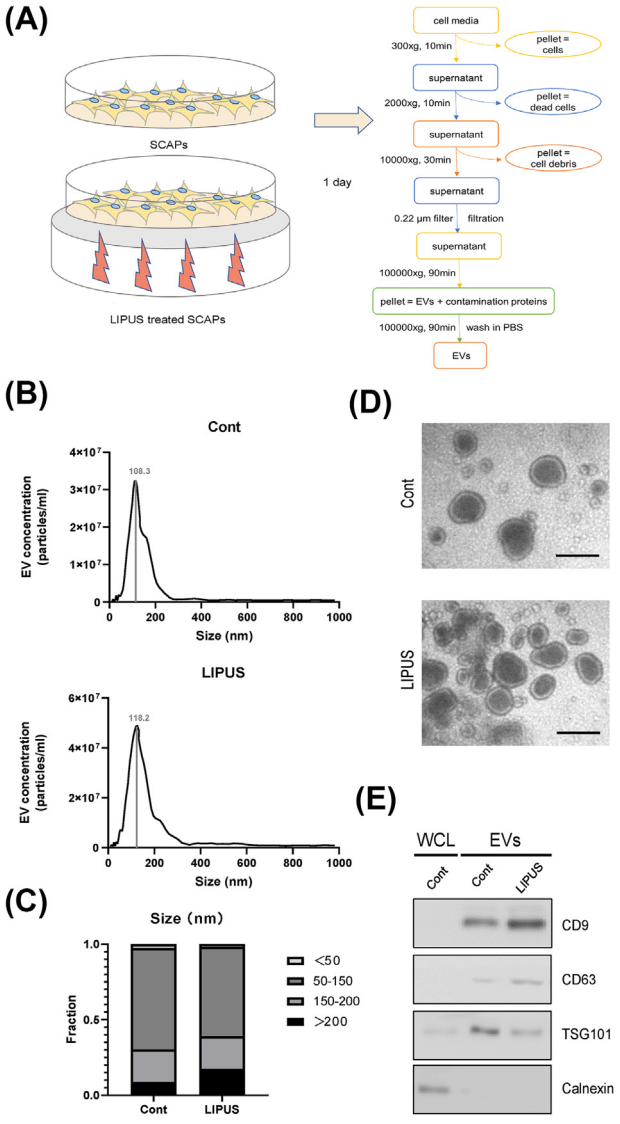

Figure 2 Physical characterization of EVs isolated from SCAP treated with or without LIPUS. **(A)** The procedure to isolate EVs from SCAP treated with or without LIPUS. SCAP were treated with or without LIPUS (90 mW/cm^2) for 30 min. Culture medium was collected 1 day after the LIPUS treatment. EVs were purified as indicated. **(B)** The representative EV size distribution by NTA. **(C)** Statistical analysis of the fraction of EV particle diameters. **(D)** Representative TEM images of EVs. Scale bar = 200 nm. **(E)** Western blot analysis of cells and matched EVs. Cell lysates and EVs were assessed with anti-CD9, anti-CD63, anti-TSG101, and anti-Calnexin. Data are presented as the mean ± SEM of three independent experiments.

CD90 and CD105, and negatively expressed CD45 and CD31. This indicates that SCAP have the characteristics of mesenchymal cells (Fig. S1E).

Identification of the applicable intensities for ultrasonic stimulation of SCAP

The biological properties of EVs primarily depend on their cellular origin.[32] To obtain better SCAP-EVs for oral bone loss, it is important to select an applicable ultrasound power density that exhibits the better promotion of features potentially important for bone repair. We first selected ultrasound signals with intensities of 30 mW/cm^2, 60 mW/cm^2, and 90 mW/cm^2; these are the most frequently used densities in clinical and *in vitro* studies.[33–35] Then, the effects of LIPUS on the cell functions of SCAP, including proliferation, osteogenesis, and anti-inflammation, were compared. SCAP were exposed to LIPUS of different intensities for 30 min per day. Cell proliferation was not significantly altered after LIPUS stimulation, as revealed by the Cell Counting Kit 8 (CCK8) assay (Fig. 1A). Next, the effects of LIPUS on osteogenesis were investigated. Compared with untreated control cells, the 60 mW/cm^2 and 90 mW/cm^2 groups showed improved osteogenic potential while there was a significant decrease in the 30 mW/cm^2 group, as evidenced by alkaline phosphatase (ALP) staining (Fig. 1B) and the expression of the osteogenic genes *OPN*, *Runx 2*, and *ALP* (Fig. 1C). Moreover, analysis of the inflammatory genes *IL-6* and *IL-8* following LPS treatment showed an increased anti-inflammatory capacity in all of the LIPUS-treated groups (Fig. 1D). These results suggest that LIPUS is effective at promoting osteogenesis and anti-inflammation of SCAP. In particular, the 90 mW/cm^2 group exhibited the most significant therapeutic potential. Therefore, 90 mW/cm^2 was chosen as the optimal stimulation intensity and was used in the subsequent SCAP experiments.

GW4869 inhibits the effects of LIPUS on SCAP

To verify whether the promoting effects of LIPUS are partially dependent on SCAP-EVs, SCAP-EV formation and release were inhibited using an EV inhibitor, GW4869.[36] SCAP were treated with 10 μM GW4869 and then stimulated with LIPUS (90 mW/cm^2). GW4869 impeded the promoting effects of LIPUS on osteogenic differentiation (Fig. 1E, F) and anti-inflammation of SCAP (Fig. 1G). Further, 10 μM GW4869 was confirmed to be non-toxic to SCAP by CCK8 assays (Fig. 1H).

LIPUS influences the physical characteristics of SCAP-derived EVs

To assess the dimensions in which LIPUS affects SCAP-EVs, SCAP-EVs were first isolated, and their physical characteristics were evaluated. SCAP-EVs were purified using the standard ultracentrifugation steps displayed in Figure 2A.[37] Nanoparticle-tracking analysis showed that the majority of vesicles in both groups were of the size generally considered to be small EVs (*i.e.*, <200 nm) (Fig. 2B). The LIPUS stimulation induced higher representation of medium vesicles (*i.e.*, >200 nm) and lower representation of vesicles smaller than 50 nm (Fig. 2C). Transmission electron microscopy analysis did not reveal differences in morphology between the LIPUS-treated and the untreated groups (Fig. 2E). Western blot (WB) analysis revealed the enrichment of classical EV protein markers, TSG101, CD9, and CD63, and the absence of the cytosolic contaminant marker Calnexin in EVs compared to cells

(A)

SCAPs	cell numbers (x 10^8)	particles/ml (x 10^{11})	protein concentration (mg/ml)
Cont	3.33±0.13	12.87± 0.56	1.07±0.04
LIPUS	3.54±0.07	20.33± 0.67	1.90±0.03
P value	> 0.05	< 0.05	< 0.05

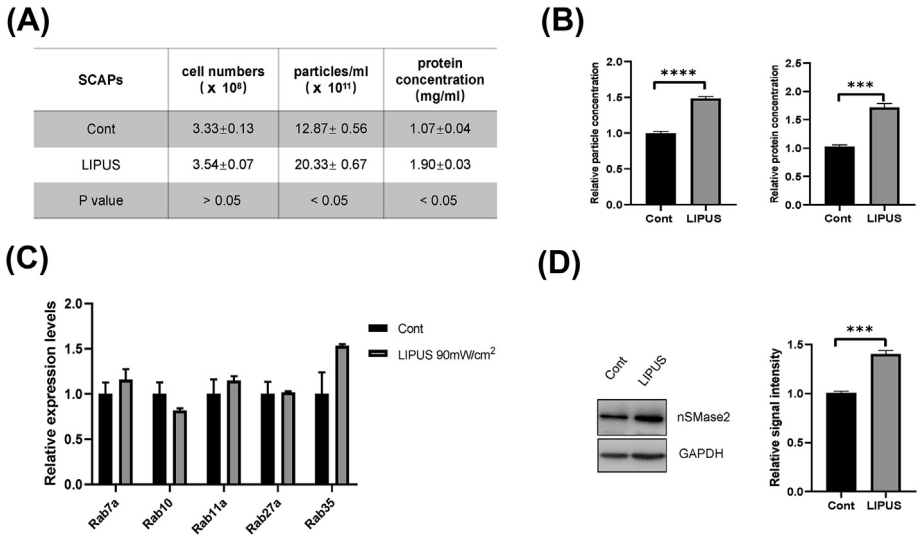

Figure 3 LIPUS promotes EV secretion and the expression of nSMase 2 in SCAP. **(A)** Comparisons of the cell numbers, the particle concentrations of EVs, and the protein concentrations of EVs in the control and LIPUS (90 mW/cm^2) groups at the time of EV isolation. **(B)** The relative particle concentrations and protein concentrations of EVs normalized to the control group. **(C)** The expression levels of *Rab7a*, *Rab 10*, *Rab11a*, *Rab27a*, and *Rab 3*5 mRNA were assessed by qPCR. **(D)** The expression of nSMase2 was assessed by Western blot analysis. The expression of nSMase2 relative to GAPDH was measured by ImageJ software and quantified. Data are presented as the mean ± SEM of three independent experiments. ***, $P < 0.001$; ****, $P < 0.0001$.

(Fig. 2E). Further, the expressions of CD9 and CD63 were increased and TSG101 was decreased after LIPUS treatment.

LIPUS promotes EV secretion and the expression of nSMase 2 in SCAP

Whether LIPUS stimulation can benefit the secretion of SCAP-EVs is a key point that deserves attention. To address this question, almost the same number of living cells was plated in both groups and the cell number was counted when the culture medium was collected for EV purification. To avoid the saturation of EVs, cells were cultured for only one day after LIPUS stimulation. EV yields were determined by bicinchoninic acid (BCA) and nanoparticle tracking analysis (NTA) and then quantified by normalizing the number of cells. LIPUS stimulation was found to significantly promote SCAP-EV yields (Fig. 3A, B). To avoid interference by apoptotic bodies, cell apoptosis was detected by flow cytometry. Few apoptotic cells were observed in both groups on the day of culture medium collection (Fig. S2A). Rab GTPases and neutral sphingomyelinases (nSMases) proteins play crucial roles in EV secretion.[38,39] The expressions of the Rab GTPases family genes *Rab7a*, *Rab 10*, *Rab11a*, *Rab27a*, and *Rab 35* were first examined and no significant differences were observed between the two groups (Fig. 3C). Next, the expression of nSMase 2 was assessed and the results revealed that LIPUS increased nSMase2 expression at the protein level in SCAP (Fig. 3D, E).

We also examined whether the promotion effect on EV secretion occurs in other cell types. 293 T cells, a human embryonic kidney cell line, and human primary PDLCs were chosen for this study. Interestingly, increased EV secretion was observed in PDLCs (Fig. S3A) but not in 293 T cells (Fig. S3B). The results suggest that different cell types respond differently to LIPUS stimulation.

EVs derived from SCAP stimulated with LIPUS exhibit stronger inhibition of bone resorption in periodontitis

Periodontitis is a chronic inflammatory disease; it is the most common cause of bone loss in the oral cavity.[40] Since LIPUS was found to promote osteogenesis and anti-inflammation of SCAP, and the effects were partially dependent on SCAP-EVs, it was important to investigate whether LIPUS stimulation promotes the therapeutic effects of SCAP-EVs. To address this question, a murine experimental model of ligature-induced periodontitis was utilized.[41] Ligatures were inserted to induce periodontitis, EVs were injected, and then the periodontal tissues were harvested, as illustrated in Figure 4A and B. Based on the nicroscopic computed tomography (microCT) results, quantitative analyses of the distance between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC), as well as the remaining alveolar bone volume fraction, were performed. The results indicated that ligature insertion induced significant bone loss in the mouse oral cavity, as demonstrated by an increase in the CEJ-ABC distance and a decrease in the alveolar bone volume (Fig. 4C—E). Both SCAP-EVs from the LIPUS-induced (LIPUS-EVs) and uninduced (Con-EVs) groups efficiently suppressed bone resorption, with LIPUS-EVs being more potent than Con-EVs. SCAP-EVs also decreased the expression of the inflammatory cytokines *Tnf-α* and *IL-8* in the periodontal tissue, where the LIPUS-EVs showed more apparent inhibitory effects (Fig. 4F, G). Meanwhile, to confirm the uptake of EVs by periodontal

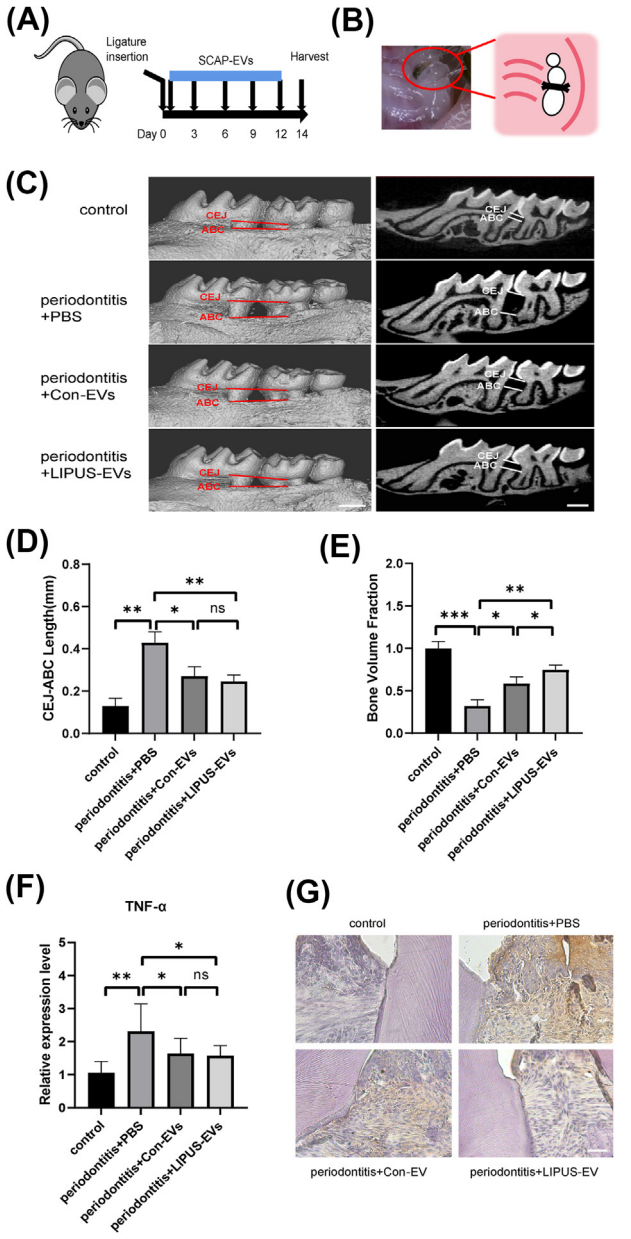

Figure 4 EVs derived from SCAP stimulated with LIPUS exhibit stronger inhibition of bone resorption in periodontitis. **(A)** The treatment regimen for EV application and ligature-induced periodontitis. On day 0, mice were subjected to ligature insertion. Then, EVs or PBS control was injected to the surface of the periodontal region on the ligature-inserted area every three days from day 0 to day 12. Mice were harvested on day 14 for analysis. **(B)** A ligature was inserted in between the first and second mouse left maxillary molars, as imaged. **(C–E)** Three-dimensional images of representative molar areas (C) (scale bar = 500 μm). CEJ-ABC distance (D) and bone volume fraction (E) were determined by microCT analysis. $N = 6$ per group. **(F)** Periodontal soft tissues were surgically removed for RNA extraction. *Tnf-α* mRNA expression was assessed by qPCR. $N = 3$ per group. **(G)** Maxillae were fixed for decalcification and histologic analysis. The expression of TNF-α was assessed by DAB staining via an anti-TNF-α antibody. Scale bar = 50 μm, $N = 3$ per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tissue, PKH67-labeled SCAP-EVs were injected, and the periodontal tissue was harvested after 4 h. As seen in Figure S4, the EVs had accumulated in the periodontal tissue.

EVs derived from SCAP stimulated with LIPUS exhibit increased promotion of osteogenic differentiation and anti-inflammation *in vitro*

PDLCs are vital for alveolar bone remodeling.[42,43] Figure S4 showed the uptake of SCAP-EVs is mostly in the periodontal ligaments. To further explore whether LIPUS stimulation promotes the therapeutic effects of SCAP-EVs *in vitro*, functional assays with human PDLCs exposed to EVs derived from untreated and LIPUS-treated SCAP were performed. To induce osteogenic differentiation, human PDLCs were cultured in an osteogenic medium containing EV-free FBS. Seven days after exposure to PBS, Con-EVs, and LIPUS-EVs, the LIPUS-EVs group exhibited the greatest increase in ALP activity, as evidenced by ALP staining (Fig. 5A). The same pattern of results was found in the osteogenic gene expression levels (Fig. 5B). Next, the influences of Con-EVs and LIPUS-EVs on anti-inflammation were compared. Human PDLCs were cultured in FBS-free medium, pre-treated with LPS, and then treated with PBS or EVs derived from untreated and LIPUS-treated SCAP. The expression levels of the inflammatory genes *IL-6* and *IL-8* were decreased most in the LIPUS-EVs group compared with the PBS group (Fig. 5C).

LIPUS stimulation alters the miRNA expression profile of SCAP-derived EVs with up-regulation of miR-935

The next question to be addressed was whether LIPUS stimulation alters the contents of SCAP-EVs. To address this question, next-generation sequencing (NGS) was performed to track the differences in SCAP-EV miRNAs. Based on miRBase 22.1, a total of 461 miRNAs were identified, 438 of which were found in both Con-EVs and LIPUS-EVs, 9 of which were specific to Con-EVs, and 14 of which were found only in LIPUS-EVs (Fig. 6A and Table S1). Compared to the miRNA expression in Con-EVs, 29 up-regulated and 23 down-regulated miRNAs were detected in LIPUS-EVs ($|\text{Fold change}| \geq 2$, $P < 0.05$ and $q < 0.05$) (Fig. 6B–E). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses identified the signaling pathways with significant enrichment of differential miRNA targets (Fig. S5 and Table S2), including miRNAs implicated in the PI3K-Akt signaling pathway, the MAPK signaling pathway, and the TNF signaling pathway, which are associated with regulation of the inflammatory or cell differentiation processes. Of note, miR-935 was found to be markedly up-regulated in LIPUS-EVs with the highest statistical significance and expression abundance, compared with Con-EVs. The increased miR-935 expression in LIPUS-EVs compared with Con-Evs was confirmed by qPCR analysis (Fig. 6F). Human PDLCs treated with 10 μg/mL LIPUS-EVs also showed increased miR-935 expression compared with those treated with Con-EVs (Fig. 6G).

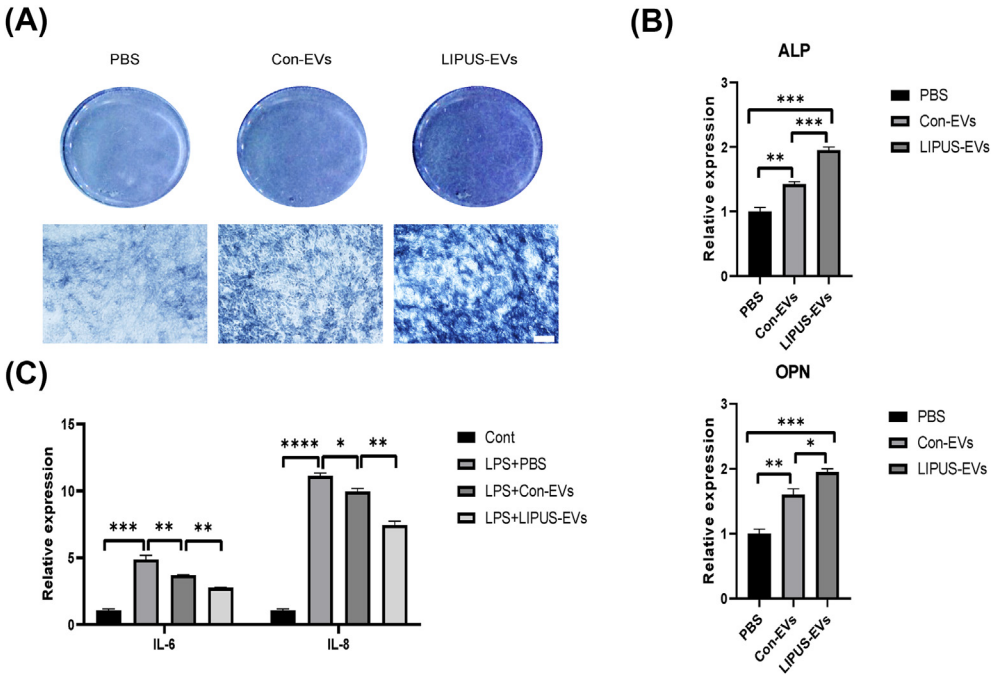

Figure 5 EVs derived from SCAP stimulated with LIPUS exhibit increased promotion of osteogenic differentiation and anti-inflammation *in vitro*. **(A, B)** Human PDLCs cultured in EV-free osteogenic differentiation medium were treated with PBS or EVs (10 μg/mL) derived from the untreated and LIPUS-treated SCAP for seven days. Cell osteogenic differentiation was detected by ALP staining (A). The expression levels of *ALP* and *OPN* mRNA were assessed by qPCR (B). **(C)** Human PDLCs cultured in EV-free medium were pretreated with 10 μg/mL LPS for 2 h and then treated with PBS or EVs (10 μg/mL) derived from the untreated and LIPUS-treated SCAP for 10 h. The expression levels of *IL-6* and *IL*-8 mRNA were assessed by qPCR. Data are presented as the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

MiR-935 promotes osteogenic differentiation and anti-inflammation in human PDLCs

Given the better osteogenic and anti-inflammatory effects of LIPUS-EVs and the significant up-regulation of miR-935 in LIPUS-EVs, we sought to test whether miR-935 mimics LIPUS-EV-induced osteogenesis and anti-inflammation in human PDLCs. To this end, human PDLCs were transfected with the miR-935 mimic or miR-935 mimic control. Remarkably, transfection of the miR-935 mimic resulted in a significant increase in the promotion of osteogenesis of human PDLCs compared with the miR-935 mimic control (Fig. 7A, C). Furthermore, transfection of the miR-935 mimic also significantly promoted anti-inflammation in human PDLCs following LPS treatment, as compared with the miR-935 mimic control (Fig. 7E). On the contrary, transfection of the miR-935 inhibitor produced inhibition of both osteogenesis and anti-inflammation in human PDLCs, as compared with the miR-935 inhibitor control (Fig. 7B, D, E).

Discussion

This study provides both experimental evidence and mechanistic data indicating that LIPUS is capable of promoting the release of SCAP-EVs and the efficacy of SCAP-EVs in treating inflammatory bone loss in the oral cavity. The findings provide insight into the function of SCAP-EVs in pro-osteogenic differentiation and anti-inflammation. This study provides a potential new strategy for the optimization of SCAP-EV production and function.

Regeneration of oral bone defects using conventional therapies has produced limited and variable clinical outcomes.[44] Even so, a recent 10-year follow-up of a randomized controlled clinical trial demonstrated that periodontal regeneration costs less and achieves similar survival compared to tooth extraction and replacement.[45] Therefore, more efforts are required to exploit novel, efficient regeneration strategies. In recent years, MSC-derived EVs have been recognized as the key paracrine factor responsible for the intrinsic regenerative and immunomodulatory properties of MSCs.[15] In our study, EVs derived from SCAP were found to improve osteogenesis and inhibit inflammation *in vitro*, and decrease oral inflammatory bone loss *in vivo*, with similar therapeutic potential to SCAP. Compared with MSCs, MSC-derived EVs are less immunogenic because they inhibit the immune response by promoting the secretion of anti-inflammatory factors and they contain fewer membrane proteins, such as major histocompatibility complex.[25] The results also demonstrated that SCAP-EVs inhibited inflammation by decreasing the secretion of pro-inflammatory factors. Together, these findings suggest that SCAP-EVs could be a potential cell-free strategy for treating oral bone defects caused by inflammation.

The production technology for MSC-EVs is the main issue to be addressed to enable the application of MSC-EVs as regenerative effectors. MSCs are influenced by a variety of physical, biological, and chemical stimuli, such as

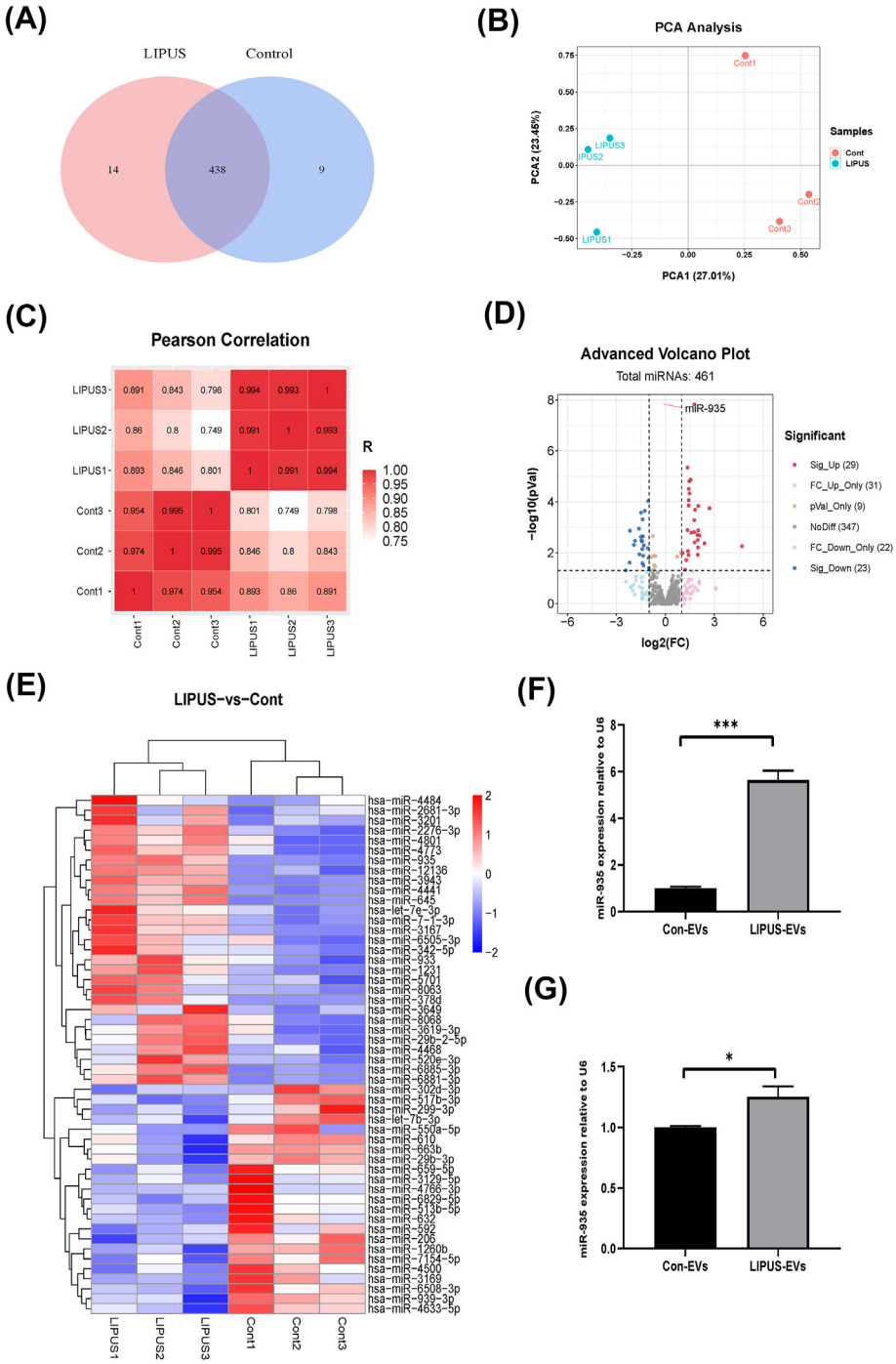

Figure 6 LIPUS stimulation alters the miRNA expression profile of SCAP-derived EVs with up-regulation of miR-935. **(A—C)** Expression profiling analysis of the miRNA in EVs derived from SCAP was treated with or without LIPUS. Venn diagram showed the overlap of the miRNAs in the two groups (A). PCA analysis (B) and Pearson correlation analysis (C) of the miRNAs in the two groups were conducted. **(D, E)** Volcano analysis (D) and heatmap (E) showed the expressions of the differentially expressed miRNAs between the two groups ($|\text{Fold change}| \geq 2$, $P < 0.05$, $q < 0.05$). **(F)** The expression levels of miR-935 in LIPUS-EVs and Con-Evs were assessed by qPCR. **(G)** The expression levels of miR-935 in human PDLCs treated with 10 μg/mL LIPUS-EVs or Con-EVs were assessed by qPCR. Data are presented as the mean ± SEM of three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

electromagnetic field exposure,[46] hypoxia,[47] lipopolysaccharides,[48] and cytokines.[49] These environmental changes can influence MSC secretion profiles. Thus, in order to improve the secretion of EVs, the use of different stimuli to manipulate MSCs attracted our attention. In previous studies, LIPUS has been found to be a physical stimulation that alters mesenchymal cell functions. Here, LIPUS was found to be capable of promoting the EV yield of SCAP.

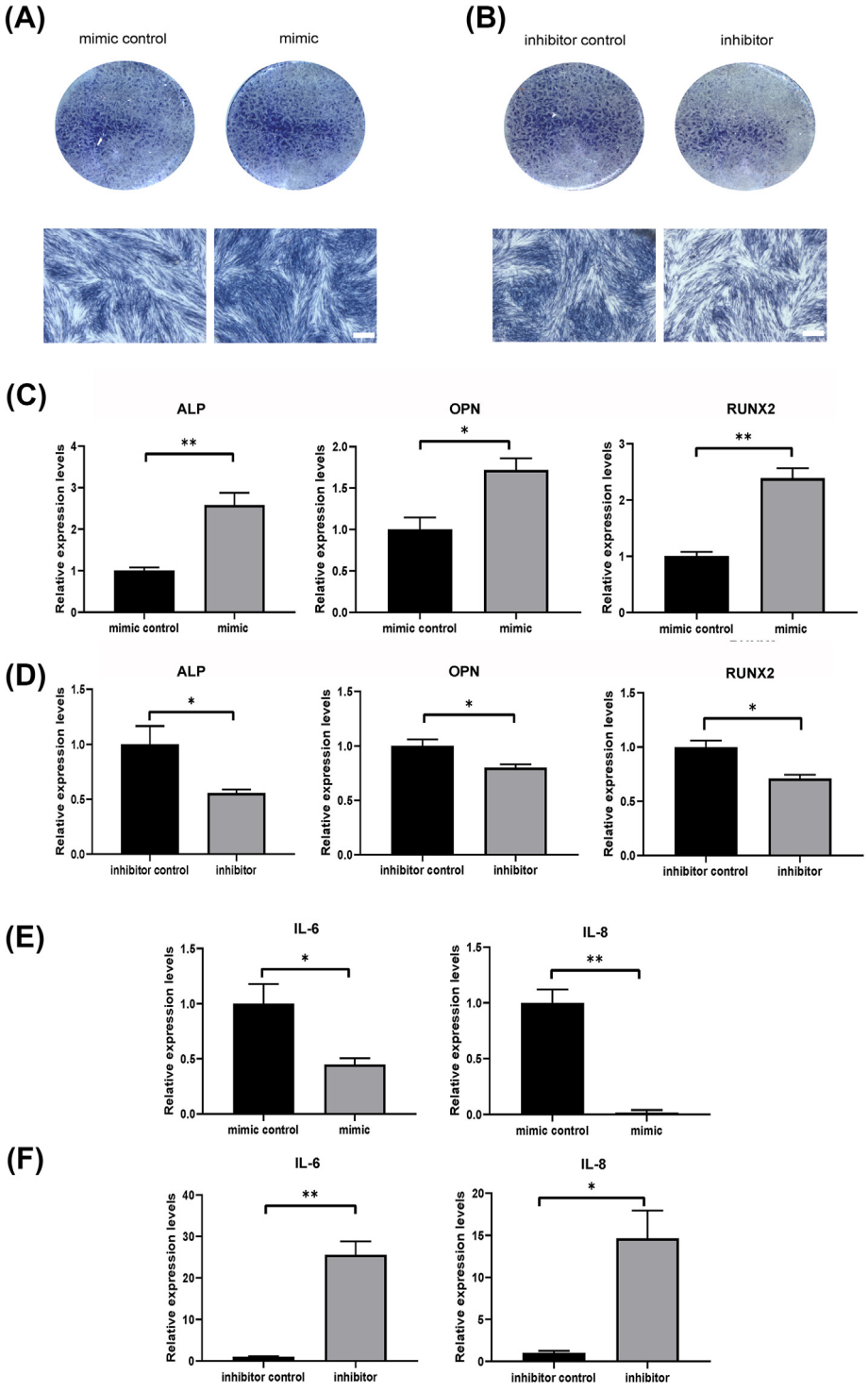

Figure 7 MiR-935 promotes osteogenic differentiation and anti-inflammation in human PDLCs. **(A, C)** Human PDLCs transfected with the miR-935 mimic or the miR-935 mimic control were cultured in osteogenic differentiation medium for seven days. Cell osteogenic differentiation was detected by ALP staining (A). The expression levels of *ALP, OPN*, and *RUNX2* mRNA were assessed by qPCR (C). **(B, D)** Human PDLCs transfected with the miR-935 inhibitor or miR-935 inhibitor control were cultured in osteogenic differentiation medium for seven days. Cell osteogenic differentiation was detected by ALP staining (B). The expression levels of *ALP*, *OPN*, and *RUNX2* mRNA were assessed by qPCR (D). **(E)** Human PDLCs transfected with the miR-935 mimic or miR-935 mimic control were treated with 10 μg/mL LPS for 12 h. The expression levels of *IL-6* and *IL*-8 mRNA were assessed by qPCR. **(F)** Human PDLCs transfected with the miR-935 inhibitor or miR-935 inhibitor control were treated with 10 μg/mL LPS for 12 h. The expression levels of *IL-6* and *IL*-8 mRNA were assessed by qPCR. Data are presented as the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Cavitation is one of the mechanisms by which LIPUS induces micromechanical stress in cells.[50] A recent study also revealed that cavitation improves the production of neutrophil-EVs by 16-fold.[51] Further, LIPUS can enhance the concentration of Ca^{2+} in cells.[52] An increase in intracellular Ca^{2+} levels has also been shown to increase the EV release process.[53] These possible mechanisms support the current findings. EV production is mediated by nSMase 2 and Rab family members.[38,39] The current results revealed that LIPUS promotes the expression of nSMase2 but not Rab family members in SCAP, which suggests that LIPUS can promote SCAP-EV release by nSMase2. Whether cavitation or the Ca^{2+} signaling pathway is involved in the process still requires further validation. Interestingly, in terms of EV production, LIPUS is not effective for all kinds of cells. The enhancement of EV production was observed in human PDLCs but not in HEK293T in the current study. It is hypothesized that mesenchymal cells and normal cell lines might have different responses to LIPUS. We plan to further explore the mechanisms underlying the different regulatory effects of LIPUS in the future in order to expand its application.

Another critical issue worth exploring is how to improve the therapeutic effects of MSC-EVs. The current results demonstrated that LIPUS stimulation promoteed the effects of SCAP-EVs on pro-osteogenic differentiation and anti-inflammation *in vitro* and the inhibition of oral inflammatory bone loss *in vivo*. One recent study also reported that EVs from LIPUS-treated dendritic cells could inhibit endothelial inflammation.[54] To elucidate the mechanism underlying the effects of LIPUS-induced SCAP-EVs, the current study focused on differential miRNA expression between the Con-EVs and the LIPUS-EVs. Extracellular miRNAs carried by EVs have recently been discovered to play principal roles in intercellular communication.[55] Upon profiling SCAP-EV miRNAs, the current results revealed that a set of miRNAs was significantly altered by LIPUS stimulation, as compared with the control group. Specifically, a significant increase in miR-935 expression was identified in the LIPUS-induced SCAP-EVs. The sequence of miR-935 is conserved between humans and mice according to miRBase. MiR-935 has been demonstrated to be highly expressed in porcine-induced pluripotent stem cells and can promote osteoblast differentiation.[56,57] In addition, miR-935 can inhibit inflammation by inducing a change in macrophage polarization to an M2-like phenotype.[58] The current results confirmed that the expression of miR-935 was about six-fold higher in LIPUS-EVs than in Con-EVs, and miR-935 was found to mimic the function of LIPUS-EVs in terms of pro-osteogenic differentiation and anti-inflammation, as evidenced by transfection of the miR-935 mimic and inhibitor. According to the bioinformatics analysis, the predicted target candidates of miR-935, which have been confirmed by other studies, include STAT1 and HMGB1.[57,59] Overexpression of miR-935 in bone marrow mesenchymal stem cells (BMSCs) has been found to promote the effects of BMSC-EVs on osteoblast proliferation and differentiation by inhibiting STAT1.[57] HMGB1 has been detected in the gingival tissue of patients with periodontitis, and it has been shown that secreted HMGB1 induces pro-inflammatory cytokine expression. This indicates that miR-935 can decrease the inflammation associated with periodontitis via inhibition of HMGB1.[60] Taken together, these findings suggest that miR-935 might contribute to the enhanced therapeutic effects of LIPUS-EVs on inflammatory oral bone loss. Recent studies have utilized various other methods to enhance the therapeutic effects of MSC-EVs on bone diseases. For example, MSCs pre-differentiated for 10 and 15 days were found to lead to the production of osteogenic EVs.[25] Further, overexpressing osteogenic proteins or miRNAs in MSCs has been found to promote the effects of their EVs.[61] Compared with these approaches, the LIPUS technique has a number of advantages, especially with regard to its simplicity, speed, and stability, which makes it easily scalable for clinical applications.

Conclusions

In conclusion, LIPUS was found to promote the secretion of EVs from SCAP with enhanced pro-osteogenic and anti-inflammatory capabilities. GW4869 inhibited the promoting effects of LIPUS on SCAP. LIPUS stimulation affected the physical characteristics of EVs. EVs from LIPUS-induced SCAP exhibited stronger efficacy in alleviating oral inflammatory bone loss *in vivo*. *In vitro*, EVs from LIPUS-induced SCAP were also stronger promotors of the osteogenic differentiation and anti-inflammation of human PDLCs. The analysis of EV miRNA cargo revealed that the expression of miR-935 was significantly elevated in LIPUS-induced SCAP-EVs. The functions of miR-935 in promoting the osteogenic differentiation and anti-inflammation of human PDLCs were confirmed and a potential mechanism underlying these functions is discussed. Further validation in other cell types would be valuable and would provide a better understanding of the regulatory effect of LIPUS on EV production. The current study demonstrates that LIPUS is a new potential optimization approach to promote the production and efficacy of SCAP-EVs for oral inflammatory bone loss. This approach may be applicable for the optimization of other MSC-EVs.

Author contributions

T. Zhang and Z. Chen contributed to conception, design, data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript; M. Zhu, X. Jing and X. Xu contributed to data acquisition and critically revised the manuscript; X. Yuan, M. Zhou contributed to data acquisition, analysis, and interpretation, and critically revised the manuscript; Y. Zhang, M. Lu, D. Chen contributed to data acquisition and analysis, and critically revised the manuscript; S. Xu contributed to design and data analysis, and critically revised the manuscript; J. Song contributed to conception, design, data acquisition, analysis, and interpretation, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Conflict of interests

The authors declare no potential conflicts of interest with respect to the authorship and/or the publication of this article.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.06.009.

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