Supplementary information

Mitochondria derived from human embryonic stem cell-derived mesenchymal stem cells alleviate the inflammatory response in human gingival fibroblasts

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Materials and methods

1 Clinical subject

Gingival tissue of healthy control (HC) and human gingival fibroblasts (HGFs) used for cell culture were sourced from patients who underwent tooth extraction but had no history of chronic periodontitis. Gingival tissues of patients with chronic periodontitis (CP) were collected during the process of scaling. This study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (Approval No. IIT20221030A), and informed consents were obtained from the patients.

2 Cell isolation and cell culture

HGFs were isolated and cultured following previously established protocols (Bletsa et al., 2018). Specifically, these cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with low glucose (Gibco, USA) and supplemented with 15% fetal bovine serum (FBS) (Gibco, USA). hESC-MSCs were procured from Hangzhou Yuansheng Biotechnology Co., Ltd., and their surface markers were verified (Fig. 1). These hESC-MSCs were cultured in the designated hESC-MSC medium (Hangzhou Yuansheng Biotechnology Co., Ltd., China).

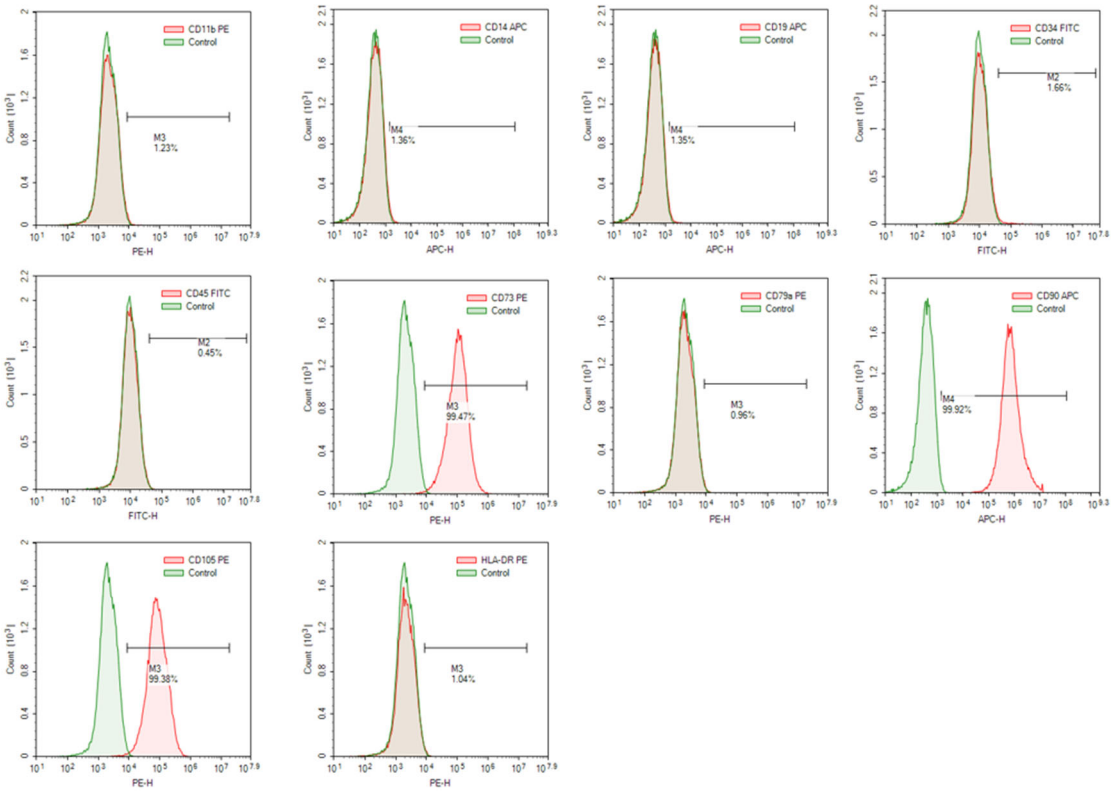

Fig. 1 Surface markers expression of hESC-MSCs.

All cell cultures were maintained in a controlled environment at 37 °C and a CO_2 concentration of 5% inside a cell incubator. Lipopolysaccharide (LPS; Beyotime, China) was diluted to 5 μg/mL with DMEM for cell culture experiments and was similarly diluted with PBS for animal studies.

3 Cell viability assay

Cell viability was measured by cell counting kit-8 assay (CCK8; Yeasen Biotech Co., Ltd., China). HGFs were seeded in 96-well plates. After 24 h, cells were treated with LPS (at 0, 0.1, 0.5, 1, 5, and 10 μg/mL) for 24 h. CCK8 (10 μL) was added to each well and incubated for 1 h. The optical density at 450 nm was measured with a full-wavelength Microplate reader (Epoch2, BioTek, USA).

4 Indirect co-culture and direct co-culture

HGFs were pre-treated with 5 μg/mL of LPS for 24 h. Subsequently, the HGFs were seeded at a density of 37 500 cells/cm², and an equal number of hESC-MSCs were co-cultured with them. In the experimental group designated as “IC,” HGFs were indirectly co-cultured with hESC-MSCs for 24 h using Transwells (JET BIOFIL, China) equipped with a polycarbonate membrane featuring an 8 μm pore size. In the experimental group labeled as “DC,” HGFs were directly co-cultured with hESC-MSCs for 24h. In the experimental group labeled as “SUP,” HGFs were cultured with a mixture of conditioned supernatant medium of hESC-MSCs and an equal volume of culture medium (Fig. 2) for 24 h. All the co-culture cells were collected after 24 h for detection.

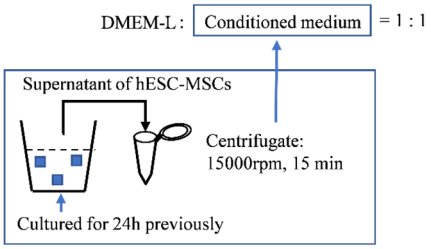

Fig. 2 Diagram of obtaining conditioned medium process.

5 Fluorescence staining of cells and mitochondria

To visualize the cells, HGFs were stained with Dil (Beyotime, Wuhan, China) for 5 min, while hESC-MSCs were stained with MitoTracker Green (Beyotime, Wuhan, China) for 30 min. Following staining, the cells were rinsed twice with 1× PBS and resuspended in DMEM. Cells in the lower chamber were examined using a fluorescence microscope (Olympus IX83, Japan). For direct co-culture, HGFs and hESC-MSCs were placed in a confocal dish (Biosharp, China) for 24 h and observed under a Confocal Laser Scanning Microscope (Olympus FV3000, Japan).

Flow cytometry was performed using fluorescein isothiocyanate (FITC) and polyethylene (PE) channels to evaluate mitochondrial transfer efficiency. The percentage of $FITC^+/PE^+$ cells served as the metric for mitochondrial transfer efficiency. Additionally, cells from each group were harvested for quantitative polymerase chain reaction (qPCR).

6 Mitochondrial isolation

Mitochondria were isolated from hESC-MSCs using the Cell Mitochondria Isolation Kit (Beyotime, Wuhan, China), according to the manufacturer's instructions. Mitochondrial concentration was determined based on protein content, measured using a bicinchoninic acid protein assay kit (Solarbio, Beijing, China).

HGFs were co-cultured with isolated mitochondria at the concentration of 5 μg in mitochondrial storage buffer per 10^5 HGFs. An equal volume of mitochondrial storage buffer was added in Group CTRL (Caicedo et al., 2015; Kim et al., 2018). This co-culture was maintained for 24 h, following centrifugation at 1500*g* for 5 min at 4 °C.

After co-culturing, cells were stained with a fluorescent dye and observed under a Confocal Laser Scanning Microscope (Olympus FV3000, Japan). Additionally, cells from each duplicate group were harvested for qPCR analysis post-co-culture.

7 Detection of mitochondrial membrane potential

The mitochondrial membrane potential was assessed using an enhanced mitochondrial membrane potential assay kit featuring a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) assay (Beyotime, Wuhan, China). The dye solution was prepared according to the manufacturer's instructions. Cells were incubated with the staining solution for 20 min and subsequently washed twice with 1× PBS. JC-1 aggregates fluorescence was detected using a PE channel, and monomers were detected using a FITC channel on a flow cytometer. The fluorescence ratio of aggregates to monomers was analyzed to reflect the mitochondrial membrane potential, as a higher ratio indicated a higher mitochondrial membrane potential.

8 Reactive oxygen species

Reactive oxygen species (ROS) were detected using a ROS assay kit (Beyotime, Wuhan, China). DCFH-DA was diluted to 5 μmol/L using a serum-free medium. Cells were treated with the staining solution for 1 h in an incubator and subsequently washed twice with 1× PBS. ROS levels in the FITC channel were analyzed using flow cytometry.

9 Measurements of mitochondrial respiration

An XFe24 analyzer (Seahorse Bioscience, Agilent Technologies, USA) was used to assess the oxygen consumption rate (OCR) of the cells, in accordance with the manufacturer's instructions. All consumable materials and reagents were sourced from Agilent Technologies. HGFs were pre-treated with LPS (5 μg/mL). Stimulated HGFs were then seeded at a density of 40 000 cells/well. The mitochondrial stress test was conducted using the following final concentrations of inhibitors: 1.5 μmol/L oligomycin, 4.0 μmol/L carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and 0.5 μmol/L Rotenone/Antimycin-A (Rot/AA). Parameters for the mitochondrial stress test in the mitochondrial stress test medium were calculated using Wave software (Version 2.6) (Agilent Technologies) as per the manufacturer's guidelines.

10 Animals

Six-week-old male C57 mice were acquired from Hangzhou Medical College. The mice were housed under specific pathogen-free conditions in the animal facility of The First Affiliated Hospital, College of Medicine, Zhejiang University. All animal experiments were approved by the Animal Ethics Committee of The First Affiliated Hospital, College of Medicine, Zhejiang University (No. 2022-1601).

The experimental animal procedure is depicted in Fig. 5a (in main text). Eight mice were used for the experiment. The sample size and LPS-induced periodontitis model were based on a previous study (Li et al., 2020). To establish an experimental periodontitis model, we injected LPS into the palatal gingival sulcus of the maxillary first molars bilaterally every 2 d. Prior to injection, the mice were anesthetized with 1% sodium pentobarbital (80 mg/kg body weight). Each injection site received 2 μL LPS (1 mg/mL). After seven injections, the mice were treated with isolated mitochondria. Both sides of the palatal gingival tissues of the maxillary first molars from each mouse were compared.

The experimental side selection was determined by random number grouping. The left side of the palatal gingival tissues of the maxillary first molars was for the Group MITO and the right side was for the Group CTRL. For the left maxillary first molar, 5 μL of isolated mitochondria (5 mg/mL) in mitochondrial storage buffer was injected into the palatal gingival sulcus. The corresponding position on the right received an injection of mitochondrial storage liquid. Three days later, the mice were euthanized using an overdose of the anesthetic agent. Palatal gingival tissues from both sides of the maxillary first molars were collected for qPCR analysis, transmission electron microscopy (TEM) observation, and pathological section. Five mice were used for qPCR, which was noted in Fig. 5c (in main text). Gingival tissues from three mice were divided into two parts, used for TEM and pathological section. For TEM observation, the mitochondrial count, length/width ratio, and area were calculated (n=3). For TUNEL observation, six photographs of staining slices from three mice were randomly selected to analyze the percentage of TUNEL-positive cells (n=6) (Fig. 3b).

11 Transmission electron microscopy (TEM)

Fresh tissues were washed with 1× PBS and fixed in 2.5% glutaraldehyde at room temperature for 4 h. Mitochondria were isolated as previously described. The supernatant was carefully removed, and the cellular pellet was fixed in 2.5% glutaraldehyde at room temperature for an additional 4 h. The samples were then post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at room temperature and washed three times with phosphate buffer. Dehydration was performed using a graded series of alcohol concentrations (50%, 70%, 80%, 90%, 95%, 100% (twice)) followed by two 15 min washes with 100% acetone. The samples were treated with a 1:1 mixture of acetone and 812 embedding medium for 2–4 h and then infiltrated with a 1:3 mixture of acetone and 812 embedding medium overnight. The samples were transferred into embedding molds filled with pure 812 embedding medium and polymerized overnight at 37 °C. Polymerization was completed at 60 °C over 48 h. Ultrathin slices (60–80 nm) were obtained using an ultramicrotome. The slices were then stained with uranium-lead double stain and allowed to dry overnight at room temperature. Finally, the slices were examined using transmission electron microscopy, and images were captured for subsequent analysis. The mitochondrial count, length-to-width ratio, and area were analyzed in three images from each group, all at the same magnification (22 000×).

12 Detection of protein expression and histological stain

The cells were lysed using RIPA Lysis Buffer (Solarbio, Beijing, China) containing 1% cocktail (Beyotime, China) for 15 min. The supernatant was collected following centrifugation and heated in a 5× loading buffer. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Millipore, HATF00010). These membranes were incubated with primary and secondary antibodies before being treated with the WesternBright ECL kit for chemiluminescence detection on a Bio-Rad system. Antibodies used for western blot are as follows: Caspase 1 (3866 T, Cell Signaling Technology, USA), NLRP3 (DF7438, Affinity, China), IL-1β (ab216995, abcam, China), and GSDMD-N (DF12275, Affinity, China). The content of IL-6 was detected with the human Th1/Th2/Th17 subsets detection kit (Cellgene, China) with flow cytometry according to the manufacturer's instructions.

Fresh gingival tissues were fixed in 4% paraformaldehyde (Servicebio, China) at room temperature and observed under an optical microscope. According to the manufacturer's instructions, sections were stained with hematoxylin and eosin (H&E), 4',6-diamidino-2-phenylindole (DAPI), or TdT-mediated dUTP nick end labeling (TUNEL). For TUNEL observation, six photographs of staining slices from three samples were randomly selected to analyze the percentage of TUNEL-positive cells (n=6) (Fig. 3).

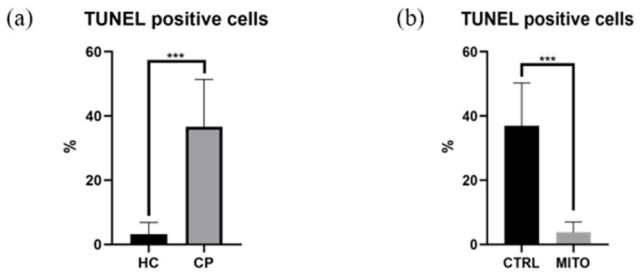

Fig. 3 TUNEL-positive cells of clinical samples between Group HC and Group CP (a) and experimental animal samples between Group CTRL and Group MITO (b) were analyzed. Six photographs of staining slices were randomly selected to analyze the percentage of TUNEL-positive cells (*n*=6).

13 RNA isolation and quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (TaKaRa, Japan) in accordance with the manufacturer's instructions. The purity and quantity of the total RNA samples were assessed using a NanoDrop ND-1000 instrument (Thermo Fisher, Shanghai, China). Total RNA was then reverse-transcribed to cDNA using HiScript Q RT SuperMix for qPCR (Vazyme, Nanjing, China), following the manufacturer's guidelines. Real-time quantitative PCR was conducted using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), also in accordance with the manufacturer's instructions. Relative gene expression levels were analyzed using the $2^{-\Delta\Delta C_T}$ method and normalized to GAPDH. The primers used are listed in Table 1.

Table 1 Following primers used for RT-qPCR

Gene name		Primer sequence (5' to 3')
Human *NLRP3*	Forward	AAAGGAAGTGGACTGCGAGA
	Reverse	TTCAAACGACTCCCTGGAAC
Human *IL-6*	Forward	ACAACCACGGCCTTCCCTAC
	Reverse	TCTCATTTCCACGATTTCCCAG
Human *IL-1β*	Forward	CCACAGACCTTCCAGGAGAA
	Reverse	GTGATCGTACAGGTGCATCG
Human *TNF-α*	Forward	CGAGTGACAAGCCTGTAGCCC
	Reverse	GTCTTTGAGATCCATGCCGTTG
Human *GAPDH*	Forward	GGACTCATGACCACAGTCCAT
	Reverse	CAGGGATGATGTTCTGGAGAG
Mouse *Nlrp3*	Forward	ATTACCCGCCCGAGAAAGG
	Reverse	TCGCAGCAAAGATCCACACAG
Mouse *Il-6*	Forward	TAGTCCTTCCTACCCCAATTTCC
	Reverse	TTGGTCCTTAGCCACTCCTTC
Mouse *Il-1β*	Forward	GAAATGCCACCTTTTGACAGTG
	Reverse	TGGATGCTCTCATCAGGACAG
Mouse *Tnf-α*	Forward	CCCTCACACTCAGATCATCTTCT
	Reverse	GCTACGACGTGGGCTACAG
Mouse *Gapdh*	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	GGGGTCGTTGATGGCAACA

14 Statistical analysis

Data analysis was conducted using GraphPad Prism software version 8.4 (San Diego, CA, USA). Data were presented as mean±standard error of the mean (SEM). Unpaired *t*-tests were employed for statistical analysis. Statistical significance was set at $P<0.05$.

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