CB1 Promotes Osteogenic Differentiation Potential of Periodontal Ligament Stem Cells by Enhancing Mitochondrial Transfer of Bone Marrow Mesenchymal Stem Cells

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Objective: *To reveal the role and mechanism of cannabinoid receptor 1 (CB1) and mitochondria in promoting osteogenic differentiation of periodontal ligament stem cells (PDLSCs) in the inflammatory microenvironment.*
Methods: *Bidirectional mitochondrial transfer was performed in bone mesenchymal stem cells (BMSCs) and PDLSCs. Laser confocal microscopy and quantitative flow cytometry were used to observe the mitochondrial transfer and quantitative mitochondrial transfer efficiency. Real-time reverse transcription polymerase chain reaction (RT-PCR) was employed to detect gene expression. Alkaline phosphatase (ALP) activity, alizarin red staining (ARS) and quantitative calcium ion analysis were used to evaluate the degree of osteogenic differentiation of PDLSCs.*
Results: *Bidirectional mitochondrial transfer was observed between BMSCs and PDLSCs. The indirect co-culture system could simulate intercellular mitochondrial transfer. Compared with the conditioned medium (CM) for BMSCs, that for HA-CB1 BMSCs could significantly enhance the mineralisation ability of PDLSCs. The mineralisation ability of PDLSCs could not be enhanced after removing the mitochondria in CM for HA-CB1 BMSCs. The expression level of HO-1, PGC-1α, NRF-1, ND1 and HK2 was significantly increased in HA-CB1 BMSCs.*
Conclusion: *CM for HA-CB1 BMSCs could significantly enhance the damaged osteogenic differentiation ability of PDLSCs in the inflammatory microenvironment, and the mitochondria of CM played an important role. CB1 was related to the activation of the HO-1/PGC-1α/NRF-1 mitochondrial biogenesis pathway, and significantly increased the mitochondrial content in BMSCs.*
Keywords: *bone marrow mesenchymal stem cells, CB1, mitochondrial transfer, osteogenic differentiation, periodontal membrane stem cells,*
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Periodontitis is a multifactorial chronic infectious disease primarilly caused by bacteria. The main manifestations are gingival soft tissue inflammation and alveolar bone loss, which can eventually lead to tooth loss. Studies have shown that there is mitochondrial dysfunction in gingival fibroblasts of patients with chronic periodontitis, including increased production of reactive oxygen species (ROS), mitochondrial structure abnormality, mitochondrial DNA (mtDNA) reduction and mutation,[1,2] decreased mitochondrial membrane potential and increased oxygen consumption,[3] of which the latter is pathological. As a result, adenosine triphosphate (ATP) production is abnormally reduced whereas ROS production is increased, leading to a vicious cycle of continuous oxidative stress and aggravated tissue damage.[4]

It is well known that mitochondria produce most of the energy a cell needs through oxidative phosphorylation, and an increasing number of studies have shown that the energy supply conversion from glycolysis to aerobic metabolism is the key to osteogenic differentiation of mesenchymal stem cells.[5] Optimal periodontal tissue regeneration depends on mesenchymal stem cells, especially PDLSCs; however, inflammatory stimulation leads to decreased function of PDLSCs and impaired mitochondrial function, making it difficult for PDLSCs to meet the requirements of tissue regeneration. The physiological characteristics of healthy mitochondria, including replication, division, fusion, degradation, intracytoplasmic movement and intercellular transfer, make it possible to eliminate and replace damaged mitochondria in cells.[6,7] Thus, saving the damaged mitochondrial function of cells to promote the proliferation and differentiation of PDLSCs has become the key to periodontal tissue regeneration therapy.

CB1 is an important component of the endocannabinoid system and is involved in regulating the proliferation and differentiation of mesenchymal stem cells and hematopoietic stem cells.[8] Studies have shown that osteocyte derived 2-arachidonic glycerol (2-AG) activates CB1 receptor of bone sympathetic nerve endings and inhibits norepinephrine (NE) release, thus alleviating tonic inhibition of the sympathetic nervous system on bone formation and promoting bone formation.[9] In addition, recent studies have found that CB1 exists in the membrane of mouse neuron mitochondria, directly controlling cell respiration and energy production.[10] In addition, the present authors' previous research results also showed that CB1 can improve the oxidative phosphorylation function of mitochondria in BMSCs in the inflammatory environment simulated by tumour necrosis factor alpha (TNF-α), thus preserving the damaged osteogenic differentiation function of BMSCs in the inflammatory state.[11] This evidence suggested that CB1 may play an important role in regulating mitochondrial function in inflammatory environments. Thus, the mechanism by which CB1 regulates the osteogenic differentiation ability of mesenchymal stem cells by regulating mitochondrial function deserves further investigation.

In this study, 10 ng/ml TNF-α was used to simulate the inflammatory microenvironment, and conditioned medium (CM) was employed to simulate intercellular mitochondrial transfer in an indirect co-culture system. The mechanism of the effect of HA-CB1 BMSCs on osteoblast differentiation of PDLSCs through mitochondrial transfer was investigated, and the application prospect of mitochondrial transfer replacement therapy in periodontal tissue regeneration was explored.

Materials and methods

Cell culture

PDLSCs were derived from the impacted third molar or teeth that needed to be removed for orthodontic treatment after patients had provided informed consent and following the rules approved by the Beijing Stomatological Hospital, Capital Medical University (Ethics Committee Agreement, Beijing Stomatological Hospital Ethics Review CMUSH-IRB-KJ-PJ-2023-13). BMSCs were purchased from Cyagen Biosciences (Guangzhou, China). PDLSCs and BMSCs were stimulated with 10 ng/ml TNF-α to simulate the inflammatory environment.

Plasmid construction and viral infection

According to previous methods used by the present authors,[11] the pQCXIN-HA-CB1 plasmid was constructed and infected with the virus. CB1 shRNA and control shRNA lentiviruses were purchased from GenePharma (Shanghai, China). Stable transfected HA-CB1 BMSCs and CB1sh BMSCs were obtained after transfection. MitoDsRed and mitoEGFP lentiviruses were purchased from Gikat Corporation (Shanghai, China) for localisation of intracellular mitochondria. Control LVCON525 and mitoDsRed and mitoEGFP viruses were transfected with BMSCs and PDLSCs, respectively. The stable transfected cells of mitoDsRed BMSCs, mitoEGFP PDLSCs, mitoEGFP BMSCs and mitoDsRed PDLSCs were confirmed under a fluorescence microscope.

Intracellular mitochondrial staining and cell staining

MitoDeepRed (Invitrogen, Waltham, MA, USA) was used to stain cells to locate intracellular mitochondria. 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) (Beyotime Biotechnology, Shanghai, China) was used to label cells. When the cells grew to 80% to 90% confluence, the present authors removed the supernatant and washed the cells twice with phosphate-buffered saline (PBS). The MitoDeepRed working dye or CFSE dye solution was added to the cells at 37°C and in a 5% CO_2 environment for 30 minutes. The efficiency of cell staining was confirmed by quantitative flow cytometry.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction, reverse transcription and real-time RT-PCR procedures were described previously.[12]

Table 1 Real-time RT-PCR primer sequence.

Gene	Primer sequence
GAPDH-FORWARD	5′-CGGACCAATACGACCAAATCCG-3′
GAPDH-REVERSE	5′-AGCCACATCGCTCAGACACC-3′
CB1-FORWARD	5′-CGGACCAATACGACCAAATCCG-3′
CB1-REVERSE	5′-AGCCACATCGCTCAGACACC-3′
HO-1-FORWARD	5′-TTCTTCACCTTCCCCAACATTG-3′
HO-1-REVERSE	5′-CAGCTCCTGCAACTCCTCAAA-3′
PGC-1A-FORWARD	5′-AGCCTCTTTGCCCAGATCTT-3′
PGC-1A-REVERSE	5′-GCAATCCGTCTTCATCCACC-3′
NRF1-FORWARD	5′-ACATACTCAACTCCACGGCA-3′
NRF1-REVERSE	5′-ATGTGGCTCTGAGTTTCCGA-3′
ND1-FORWARD	5′-CACACTAGCAGAGACCAACCGAAC-3′
ND1-REVERSE	5′-CGGCTATGAAGAATAGGGCGAAGG-3′
HK2-FORWARD	5′-GCCAGCCTCTCCTGATTTTAGTGT-3′
HK2-REVERSE	5′-GGGAACACAAAAGACCTCTTCTGG-3′

CB1, cannabinoid receptor I; HK2, hexokinase gene 2; HO-1, heme oxygenase-1; ND1, NADH dehydrogenase 1; NRF-1, nuclear respiratory factor-1; PGC-1α, PPARγ co-activator 1α.

The Icycler iQ multi-color real-time RT-PCR detection system was used, and the QuantiT ect SYBR Green PCR kit was employed for real-time RT-PCR reaction. Primer sequences for specific genes are shown in Table 1.

Establishing a cell co-culture system

The direct cell co-culture system was established by mixing cell suspension containing 1×10^5 BMSCs and 1×10^5 PDLSCs evenly and inoculating them in six-well plates or 35-mm laser confocal culture dishes.

The present authors established the indirect cell co-culture system based on the method described in the study by Hayakawa et al.[13] When BMSCs grew to a confluent degree of 80%, the complete medium was changed into DMEM containing 1% penicillin and streptomycin (P+S). The supernatant was collected 24 hours later, which was the CM containing mitochondria of BMSCs. CM was centrifuged at a high speed of 2000 g for 10 minutes, cell precipitation was removed and supernatant was collected. The mitochondria-depleted CM (mdCM) was obtained by filtering CM through a 0.22-μm filter. CM or mdCM and adherent PDLSCs were co-cultured for 24 hours. The experimental technique is shown in Fig S1 (provided on request).

Laser scanning confocal microscopy

After direct or indirect co-culture, the supernatant was discarded and washed with PBS three times. The cells were fixed with 4% paraformaldehyde at room temperature for 30 minutes, washed with PBS three times, and anti-fluorescence quenching sealing tablets (including DAPI) were added. The cells were observed and photographed under a laser confocal microscope.

Flow cytometry

The direction and efficiency of mitochondrial metastasis were analysed by quantitative flow cytometry. Adherent cells after 24 hours of direct or indirect co-culture were washed three times using PBS, followed by cell digestion using 0.25% ethylenediaminetetraacetic acid (EDTA)-free trypsin, and the cell suspension was collected and centrifuged at 1,100 rpm for 5 minutes. After the supernatant was discarded, the cells were resuspended in complete medium and analysed through flow cytometry. In order to perform single positive analysis of samples, the present authors used an FACS dot plot to gate mitoDsRed positive cells on APC channel. To perform double-positive analysis of samples, we gated mitoDsRed positive cells using FACS dot plots on APC channels and analysed mGFP or CFSE signals of cell populations using FACS dot plots on FITC channels.

Alkaline phosphatase (ALP) activity assay and alizarin red detection

The osteogenic induction medium was prepared in a similar way to in the present authors' previous study,[14] which performed osteogenic induction of periodontal ligament stem cells. ALP activity was determined using an ALP activity kit. To detect the mineralisation potential of PDLSCs, the latter were fixed with 70% ethanol and

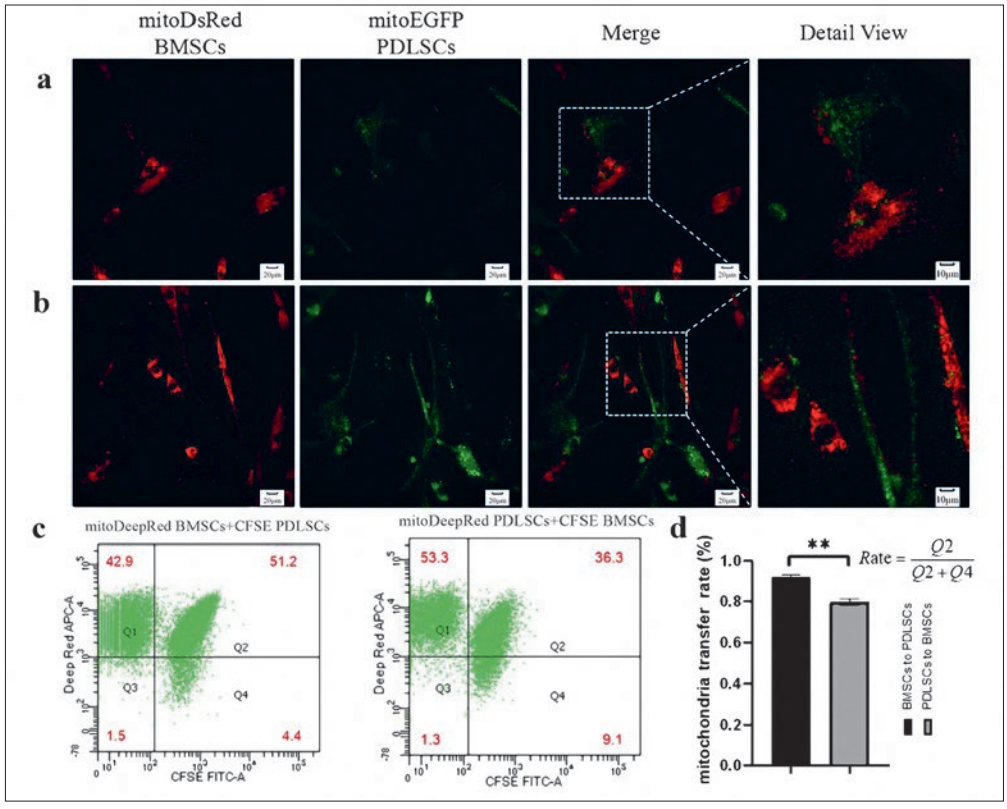

Fig 1a to d Direct co-culture of BMSCs and PDLSCs could induce mitochondrial transfer. Laser confocal microscope observed red and green fluorescence overlap between cells, bar = 20 μm **(a and b)**. The staining efficiency of mitoDeepRed and CFSE dyes was determined by quantitative flow cytometry, and the mitochondrial transfer efficiency of BMSCs to PDLSCs and PDLSCs to BMSCs was determined **(c)**. There were significant differences between the two groups. Error bars represent standard deviation (SD) (n = 3) **(d)**. **P* < 0.05; ***P* < 0.01.

stained with 2% alizarin red 2 weeks after osteogenesis induction. After scanning the staining results, calcium ion levels were measured using 10% w/v O-crephenolphthalein complex ketone (CPC) as described above.[14]

Statistical analysis

The relative expression content of each index was measured using SPSS 16.0 software (IBM, Chicago, IL, USA). A *t* test was used to compare the measurement data between the two groups, and a one-way analysis of variance was performed to compare the measurement data between multiple groups. The level of statistical significance was set at $P \leq 0.05$.

Results

Direct co-culture of BMSCs and PDLSCs could induce mitochondrial transfer

To confirm the presence of mitochondrial transfer between BMSCs and PDLSCs, mitoDsRed and mitoEGFP mitochondrial localisation lentivirus were used to transfect cells to obtain mitoDsRed BMSCs and mitoEGFP PDLSCs. The two cells were mixed in a ratio of 1:1 and inoculated in a confocal laser culture dish. The mixture was observed under a confocal laser microscope 24 hours later. Red and green fluorescence overlapping regions were observed between red fluorescent cells (mitoDsRed BMSCs) and green fluorescent cells (mitoEGFP PDLSCs), which revealed that mitochondria exchanged between cells (Fig 1a and b). Next, the present authors examined the direction and efficiency of mitochondrial transfer between cells. MitoDeepRed and CFSE dyes were used to label PDLSCs and BMSCs, respectively, to obtain mitoDeepRed PDLSCs, mitoDeepRed BMSCs, CFSE PDLSCs and CFSE BMSCs. MitoDeepRed BMSCs and CFSE PDLSCs, mitoDeepRed PDLSCs and CFSE BMSCs were mixed in a ratio of 1:1, respectively, and inoculated in the six-well plate. 0.25% EDTA free pancreatic enzyme was used to harvest cells 24 hours later, and the double positive cell rate of DeepRed and CFSE was analysed by quantitative flow cytometry. Flow cytometry (Fig 1c) showed that bidirectional mitochondrial transfer could occur between BMSCs and PDLSCs, and the mitochondrial transfer efficiency from BMSCs to PDLSCs (93%) was significantly higher than that from PDLSCs to BMSCs (79%) (Fig 1d). In addition, the present authors measured the rate of mitochondrial transfer

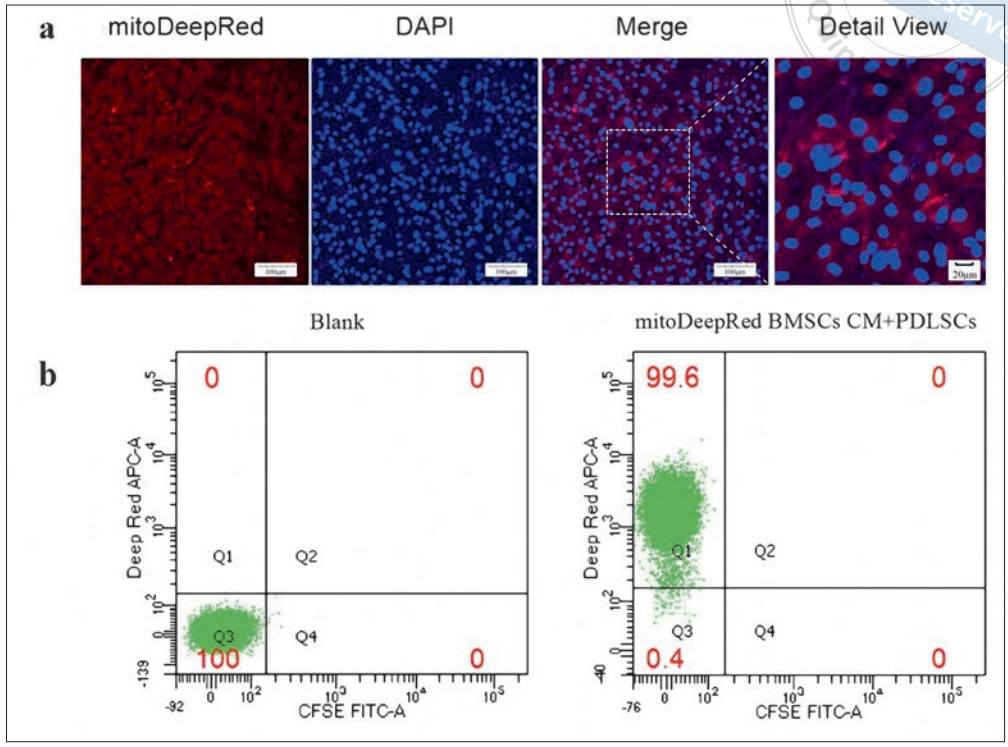

Fig 2a and b Mitochondrial transfer to PDLSCs could be achieved by CM. After MitoDeepRed BMSC CM and PDLSCs were co-cultured for 24 hours, red fluorescence was observed in the cells under laser confocal microscope, bar = 100 μm **(a)**. MitoDeepRed BMSCs CM and PDLSCs were co-cultured for 24 hours. The number of DeepRed-positive cells in the blank control group and indirect co-culture group was determined by quantitative flow cytometry. Error bars represent SD (n = 3) **(b)**. CM, conditioned medium.

between cells at 48 hours by quantitative flow cytometry, which showed that the rate of mitochondrial transfer from BMSCs to PDLSCs could be as high as 99%, and that from PDLSCs to BMSCs could be as high as 82% (Fig S2, provided on request). To test whether 10 ng/ml TNF-α could affect the mitochondrial transfer between cells, quantitative flow cytometry analysis was performed in the direct co-culture system under normal conditions and in the presence of 10 ng/ml TNF-α. The results showed that there was no significant difference in the mitochondrial transfer rate between the two groups, which was higher than 90%. The results are shown in Fig S3 (provided on request). This may be due to the fact that both BMSCs and PDLSCs have high stemness and a high level of mitochondrial transfer can occur under normal conditions.

The indirect co-culture system of CM and PDLSCs could simulate intercellular mitochondrial transfer

MitoDeepRed BMSCs CM was co-cultured with PDLSCs in confocal culture dishes for 24 hours to construct an indirect co-culture system of BMSCs and PDLSCs. Under laser confocal microscopy, adherent PDLSCs were found to capture red fluorescent-labelled BMSC mitochondria in CM (Fig 2a). PDLSCs were inoculated in six-well plates and co-cultured with CM for 24 hours. Cells were harvested and analysed by quantitative flow cytometry. The results showed that BMSCs CM could transfer mitochondria to PDLSCs, with an efficiency of up to 99.6% (Fig 2b). Comparing the mitochondrial transfer rate of the direct co-culture system with that of the indirect co-culture system, the present authors found that the indirect co-culture system had a higher mitochondrial transfer rate than the direct system, and there was a significant difference between the two groups, as shown in Fig S4 (provided on request).

Effects of BMSC CM, CB1sh BMSC CM and HA-CB1 BMSC CM on osteogenic differentiation of PDLSCs in the inflammatory microenvironment

After it was confirmed by laser confocal microscopy and quantitative flow cytometry that BMSC CM could transfer mitochondria to PDLSCs through the indirect co-culture system, the present authors began to investigate whether BMSC CM could improve the osteogenic differentiation ability of PDLSCs stimulated by 10 ng/ml TNF-α. First, 5 days after osteogenic induction, ALP activity assay results showed that 10 ng/ml TNF-α decreased the ALP activity in PDLSCs compared with the control group (Fig 3a). ARS and quantitative calcium ion analysis demonstrated that 10 ng/ml TNF-α stimulation significantly inhibited the mineralisation of PDLSCs in vitro compared with the

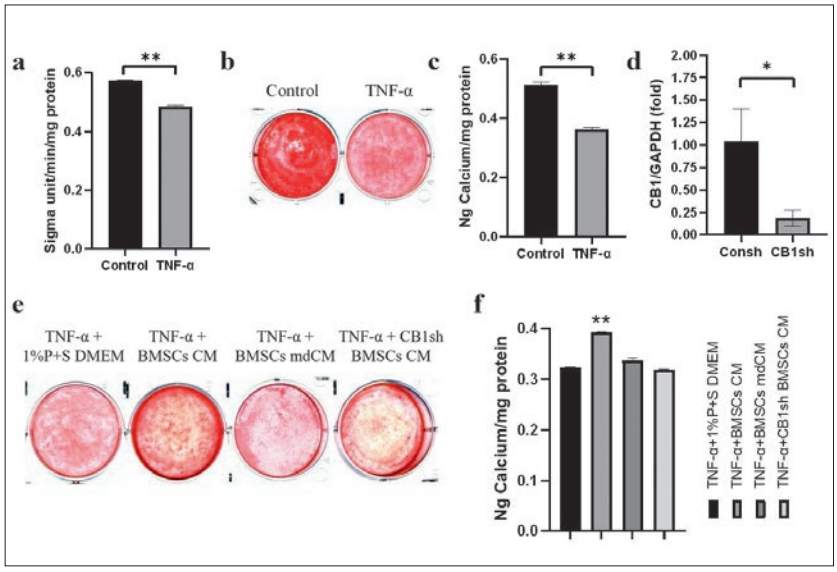

Fig 3a to f BMSCs CM promoted PDLSCs osteogenesis, but CB1sh BMSCs CM did not. After osteogenic induction, ALP activity **(a)**, ARS **(b)** and quantitative calcium ion analysis **(c)** were used to determine the effect of 10 ng/ml TNF-α on the osteogenic differentiation of PDLSCs. Real-time RT-PCR was used to detect the knockdown efficiency of CB1 in BMSCs **(d)**. ARS **(e)** and quantitative calcium ion analysis **(f)** were used to determine the effects of BMSC CM, BMSC mdCM and CB1sh BMSC CM on the osteogenic differentiation ability of PDLSCs stimulated by 10 ng/ml TNF-α. Error bars represent SD (n = 3). *P < 0.05; **P < 0.01. ALP, alkaline phosphatase activity; ARS, alizarin red staining; CB1, cannabinoid receptor I; CM, conditioned medium; P+S, penicillin and streptomycin; TNF-α, tumour necrosis factor alpha.

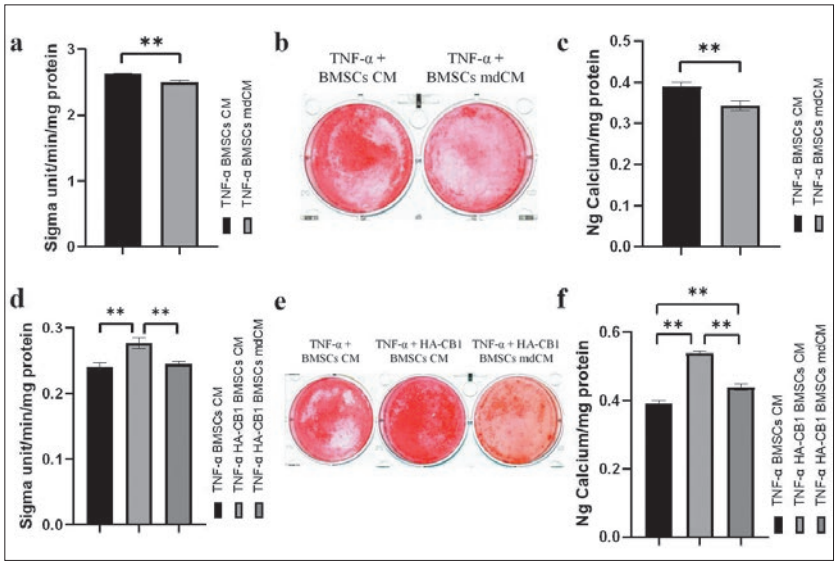

Fig 4a to f HA-CB1 BMSC CM significantly enhanced the osteogenic function of PDLSCs, but could not save the osteogenic ability of PDLSCs after mitochondrial filtration. ALP activity assay **(a)**, ARS **(b)** and quantitative calcium ion analysis **(c)** were used to determine the effects of BMSC CM and BMSC mdCM on the osteogenic differentiation ability of PDLSCs stimulated by 10 ng/ml TNF-α. ALP activity assay **(d)**, ARS **(e)** and quantitative calcium ion analysis **(f)** were used to detect the effects of BMSC CM, HA-CB1 BMSC CM and HA-CB1 BMSC mdCM on the osteogenic differentiation ability of PDLSCs under 10 ng/ml TNF-α stimulation. Error bars represent SD (n = 3). *P < 0.05; **P < 0.01. ALP, alkaline phosphatase activity; ARS, alizarin red staining; CB1, cannabinoid receptor 1; CM, conditioned medium; mdCM, mitochondria-depleted conditioned medium; TNF-α, tumour necrosis factor alpha.

control group (Fig 3b and c). CB1 expression in BMSCs was knocked down using CB1 shRNA lentivirus, and the expression level of CB1 was detected by real-time RT-PCR, demonstrating decreased expression in BMSCs (Fig 3d). The BMSC and PDLSC CMs obtained were co-cultured under the stimulation of 10 ng/ml TNF-α, and osteogenic induction was performed 24 hours later. The results of ARS and calcium ion quantitative analysis showed that BMSC CM increased the osteogenic differentiation ability of PDLSCs damaged by 10 ng/ml TNF-α compared with the control group (Fig 3e and f), indicating that BMSC CM could save the damaged osteogenic differentiation ability of PDLSCs, but CB1sh BMSC CM could not. These results suggest that mitochondria might be essential to enhance the osteogenic differentiation of PDLSCs, and CB1 plays a role in this process.

Next, mitochondria in BMSC CM were removed using a 0.22-μm filter to obtain mdCM, which was co-cultured with adherent PDLSCs. ALP, ARS and quantitative calcium ion analysis showed that BMSC mdCM could not save the osteogenic differentiation ability of PDLSCs stimulated by 10 ng/ml TNF-α (Fig 4a to c). In addition, the present authors compared the results of osteogenesis experiments on PDLSCs after co-culturing with mdCM and CB1sh BMSC CM (Fig 3d to f). They found that CB1sh BMSC CM had no significant effect

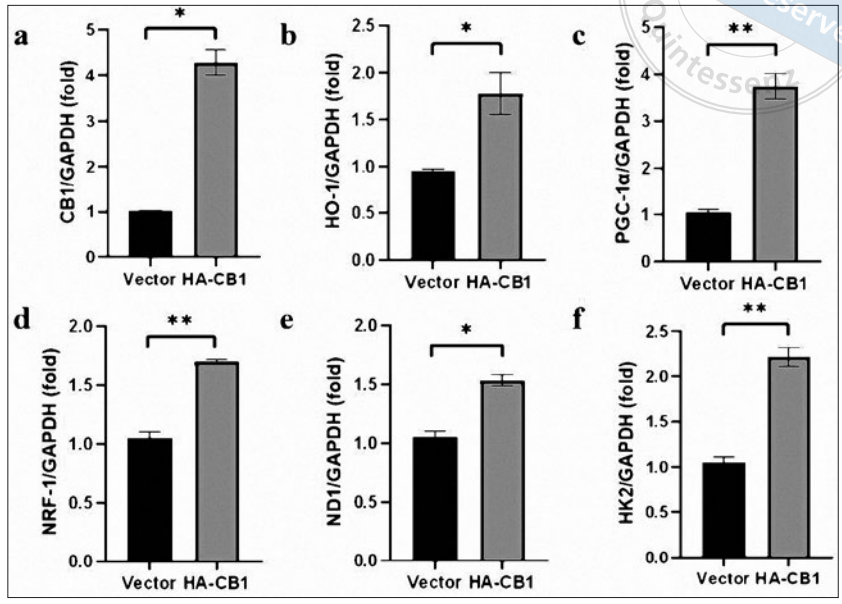

Fig 5a to f CB1 promoted the expression of mitochondrial biogenesis related factors and mitochondrial content marker genes in BMSCs. The overexpression efficiency of CB1 in BMSCs was detected by real-time RT-PCR **(a)**. The expression levels of HO-1, PGC-1α and NRF-1 in HA-CB1 BMSCs were detected by real-time RT-PCR **(b to d)**. The expression levels of ND1 and HK2 encoded by mitochondria in HA-CB1 BMSCs were detected by real-time RT-PCR **(e to f)**. Error bars represent SD (n = 3). **P* < 0.05; ***P* < 0.01. CB1, cannabinoid receptor I; HK2, hexokinase gene 2; HO-1, heme oxygenase-1; ND1, NADH dehydrogenase 1; NRF-1, nuclear respiratory factor-1; PGC-1α, PPARγ co-activator 1α.

on osteogenesis of PDLSCs, which was similar to the results of the control group using complete medium. There were no significant differences between the two groups; however, mdCM was least able to promote PDLSC osteogenesis compared with the other two groups, perhaps due to the 0.22-μm filter that can filter out mitochondria and vesicles containing various factors larger than 0.22 μm. To further verify the role and mechanism of HA-CB1 BMSC CM, the present authors formed three groups: BMSC CM and 10 ng/ml TNF-α PDLSCs, HA-CB1 BMSC CM and 10 ng/ml TNF-α PDLSCs, HA-CB1 BMSC mdCM and 10 ng/ml TNF-α PDLSCs. Osteogenesis induction was performed 24 hours after co-culture. ALP activity was performed 7 days later, whereas ARS and quantitative calcium ion analysis were performed 14 days later (Fig 4d to f). The results showed that compared with BMSC CM, HA-CB1 BMSC CM could significantly enhance the mineralisation ability of PDLSCs; however, that of PDLSCs could not be enhanced after removing the mitochondria in HA-CB1 BMSC CM.

CB1 contributed to mitochondrial biogenesis of BMSCs and increased the number of mitochondria in cells

To further explore the mechanism of CB1 affecting intracellular mitochondrial function, the present authors used real-time RT-PCR to detect the expression levels of mitochondrial biogenesis related factors, including heme oxygenase-1 (HO-1), PPARγ co-activator 1α (PGC-1α) and nuclear respiratory factor-1 (NRF-1), and detected the expression levels of mitochondrial coding genes NADH dehydrogenase 1 (ND1) and hexokinase gene 2 (HK2) to quantitatively determine intracellular mitochondrial content. Real-time RT-PCR results showed that BMSCs with stable overexpression of CB1 were successfully constructed (Fig 5a). Meanwhile, real-time RT-PCR results showed that, compared with the control group, the expression of HO-1, PGC-1α and NRF-1 in HA-CB1 BMSCs was significantly increased (Fig 5b to d), and the expression of marker genes ND1 and HK2 of mitochondrial content were also increased in the HA-CB1 group (Fig 5e and f). These results suggest that CB1 might promote BMSC mitochondrial biogenesis to increase mitochondrial content.

Discussion

Studies have shown that during bone formation induction of BMSCs, proteins involved in mitochondrial biogenesis are increased, accompanied by the development of mitochondrial ridge, the expression level of constituent subunits and the activity of the respiratory enzyme complex. Mitochondrial membrane potential, cellular respiration rate and intracellular ATP content are also increased. This evidence indicates that mitochondrial oxidative metabolism is upregulated during BMSC differentiation, and biological energy changes occur in cells from glycolysis to aerobic metabolism,[5] indicating that mitochondria play a vital role in osteogenic differentiation of BMSCs. However, many studies have shown that the periodontal inflammatory microenvironment impels the mitochondrial function of periodontal cells, and some have confirmed that bacterial lipopolysaccha-

rids (LPS)-mediated mitochondrial dysfunction may be the initial cause of oxidative stress in patients with periodontitis. Insufficient energy supply, massive release of ROS and an imbalance of calcium ions lead to mitochondrial damage, and aggravate apoptosis and necrosis of cells around the damaged area.[15,16] This suggests that mitochondrial dysfunction may be a key factor in the onset and progression of periodontitis.

In the inflammatory microenvironment, the accumulation of TNF-α is involved in the alveolar bone resorption process, which is crucial in the occurrence and development of periodontitis.[17,18] Studies have shown that the osteogenesis and regeneration abilities of PDLSCs in periodontitis patients are impaired in inflammatory environment, and such impaired characteristics can be simulated in vitro by treating healthy PDLSCs with 10 ng/ml TNF-α.[19] In the previous study conducted by the present authors, it was found that 10 ng/ml TNF-α reduced the oxygen consumption rate (OCR) and membrane potential in BMSCs, and inhibited mitochondrial synthesis.[11] These results indicated that the mitochondrial function of MSCs was impaired under 10 ng/ml TNF-α stimulation.

Many studies have proved that stem cells can replace damaged mitochondria by transferring mitochondria to recipient cells and restore their oxidative metabolism function,[12,20] which provides therapeutic ideas for saving mitochondrial dysfunction of damaged cells.[21] Thus, the present authors hypothesised that BMSCs can also improve the function of PDLSCs in inflammatory injury through mitochondrial transfer, and observed mitochondrial exchange between mitoDsRed BMSCs and mitoEGFP PDLSCs under laser confocal microscopy. In addition, the results of quantitative flow cytometry proved the efficiency of mitochondrial transfer from BMSCs to PDLSCs was up to 93%. In the study by Hayakawa et al,[13] astrocyte-derived CM was co-cultured with adherent neuron cells to achieve mitochondrial transfer to neurons, improve neuron vitality and promote the repair of the nervous system after stroke. These results indicated that mitochondrial transfer can be carried out by non-cellular contact. Therefore, to examine the effect of mitochondria from BMSCs on PDLSCs, the present authors constructed an indirect co-culture system for experiments, and laser confocal microscopy showed that PDLSCs could obtain free mitochondria from the conditioned medium. Flow cytometry also proved that up to 99.6% of PDLSCs could internalise exogenous mitochondria.

Based on these results, the present authors used the indirect co-culture system to further explore the role of mitochondria in saving the osteogenic differentiation ability of damaged cells in an inflammatory microenvironment. ALP activity results showed that BMSC CM could significantly enhance the osteogenic ability of PDLSCs damaged by 10 ng/ml TNF-α. ARS and quantitative calcium ion analysis results also demonstrated that the mineralisation ability of PDLSCs was significantly enhanced. In the present authors' previous study,[11] CB1 was found to promote the mitochondrial energy metabolism of BMSCs, including OCR and MMP, and recover the impaired osteogenic differentiation potential in the case of 10 ng/ml TNF-α stimulation. This suggested that CB1 activation promoted osteogenic differentiation of BMSCs by rescuing mitochondrial metabolism under inflammatory conditions. Therefore, in the present study, the authors continued to explore whether mitochondria are the key to the effect of CB1 on osteogenic differentiation by the indirect co-culture system. ALP, ARS and quantitative calcium analysis showed that BMSC CM could enhance the osteogenic differentiation ability of PDLSCs; however, the effect of CB1-knockdown BMSC-derived CM was significantly reduced. Next, the present authors removed mitochondria from CM for experiments and found that BMSC mdCM did not save the osteogenic differentiation ability of PDLSCs stimulated by 10 ng/ml TNF-α. In order to verify whether CB1 affects the impact of CM on the osteogenic ability of PDLSCs by influencing the mitochondrial function of BMSCs, the authors conducted three osteogenesis experiments. The results of ALP, ARS and quantitative calcium ion analysis showed that CM obtaining from CB1 over-expressed BMSCs had a stronger promoting effect on osteogenic differentiation of PDLSCs than the vector group; however, this effect was cancelled after removing mitochondria from HA-CB1 BMSC CM. This indicated that HA-CB1 BMSC CM can significantly enhance the osteogenic differentiation potential of PDLSCs damaged by 10 ng/ml TNF-α, and the core of this enhancement mechanism might be providing mitochondria to PDLSCs.

The present authors then investigated the mechanism of CB1 affecting mitochondrial function. Mitochondria, the centre of cellular metabolism and energy production, are produced by phagocytosis of α-protein bacillus by eukaryotic progenitor cells.[22] When mitochondria are damaged, cells will not regenerate them, but complete the dynamic regulation of the cellular mitochondrial system through a mitochondrial biogenesis process to degrade damaged mitochondria and promote the proliferation of healthy mitochondria.[23] Mitochondrial biogenesis is regulated by a dual genomic program of nuclear coding genes and mitochondrial coding genes, including nuclear coding mitochondrial pro-

teins that control mtDNA transcription and replication. This process requires the induction of mitochondrial DNA polymerase, mitochondrial transcription factor A (TFAM) and TFB2M.[24,25] Many nuclear coding genes of mitochondrial proteins contain NRF-1 and NRF-2, and PGC-1α is the central transcription coactivator of NRF-1, NRF-2 and PPARγ. It can promote the transcription of mitochondrial coding genes such as TFAM and participate in the physiological integration of mitochondrial biosynthesis and oxidative metabolism.[26,27] When the energy demand of cells increases, the NAD+/NADH ratio increases and activates Sirtuin-1 (SIRT1), leading to PGC-1α deacetylation and activation of NRF-1 and other transcription factors involved in mitochondrial biogenesis, promoting the transcription of target genes and stimulating mitochondrial biogenesis.[28] In addition, studies have confirmed that HO-1 also plays an initiating role in mitochondrial biogenesis[29]; it controls the degradation of free heme and produces CO, iron and biliverdin.[30] Elevated endogenous CO levels stimulate the expression of antioxidant enzyme SOD2 and the production of mitochondrial H_2O_2 in Complex III, leading to the activation of Akt/PKB and the inactivation of glycogen synthetase kinase-3. Activation of this signalling pathway greatly stimulates mitochondrial biogenesis by allowing nuclear translocation of NRF-2 and occupying antioxidant response elements (AREs) in the NRF-1 promoter.[31,32] In the present authors' previous study, the results showed that overexpression of CB1 could significantly increase the expression levels of HO-1, PGC-1α and NRF-1 in BMSCs compared with the control group.[11] This result is consistent with the results of our study conducted in BMSCs. In addition, mtDNA levels were detected to measure intracellular mitochondrial content, and the results showed that the expressions of nicotinamide adenine dinucleotide ND1 and HK2 encoded by mitochondria were significantly higher in HA-CB1 BMSCs. This suggested that CB1 may be associated with mitochondrial biogenesis of BMSCs by activating the HO-1/PGC-1α/NRF-1 pathway and increase intracellular mitochondrial content.

The present results suggested that supplying mitochondria to damaged cells in an in vitro inflammatory microenvironment is an effective way to save the osteoblast ability of cells. Compared with stem cells, the outer membrane of mitochondria lacks surface antigens and has low immunogenicity, and one high-functioning cell can contain hundreds of mitochondria. In addition to directly replacing the damaged mitochondria, some studies have shown that exogenous mitochondria can colocate with the original mitochondria after entering the recipient cells, suggesting that they can fuse with them,[33] indicating that exogenous mitochondria may also have the ability to rescue the damaged mitochondria in the recipient cells to promote the recovery of cell function. Thus, mitochondria replacement therapy has a wider range of sources, and autogenous, allogeneic and even xenogeneic tissues are expected to become donor sources for mitochondrial replacement therapy. However, further studies are required on the acquisition of mitochondria with high purity concentration, the realisation of mitochondrial transfer in the vivo model of periodontitis, and the changes and effects of the mitochondrial energy metabolism system after mitochondria enter recipient cells.

Conclusion

In summary, the authors conclude that mitochondrial transfer exists between BMSCs and PDLSCs, which can be simulated by the CM indirect co-culture system; in an 10 ng/ml TNF-α simulated inflammatory microenvironment, HA-CB1 BMSC CM could enhance the osteogenic differentiation ability of PDLSCs, and mitochondria in CM played an important role. CB1 was related to the activation of the HO-1/PGC-1α/NRF-1 mitochondrial biogenesis pathway, and significantly increased the content of mitochondria in BMSCs, which might be why HA-CB1 BMSC CM had stronger osteogenic differentiation ability of PDLSCs than BMSC CM. These findings have identified the role of mitochondria in restoring the osteogenic differentiation function of PDLSCs damaged by 10 ng/ml TNF-α, which established the research basis for the application of mitochondrial therapy in periodontal tissue regeneration.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Lan LUO, Wan Hao YAN, Feng Qiu ZHANG and Zhi Peng FAN contributed to the research concept; Dr Lan LUO developed the protocol, extracted the data, analysed the findings and drafted and critically revised the manuscript; Drs Wan Hao YAN, Feng Qiu ZHANG, and Zhi Peng FAN reviewed and revised the manuscript. All the authors approved the manuscript.

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