RESEARCH ARTICLE

Adipose-derived stem cells regulate metabolic homeostasis and delay aging by promoting mitophagy

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Abstract
Tissues undergo a process of degeneration as the body ages. Mesenchymal stem cells (MSCs) have been found to have major potential in delaying the aging process in tissues and organs. However, the mechanism underlying the anti-aging effects of MSC is not clear which limits clinical applications. In this study, we used adipose-derived mesenchymal stem cells (ADSCs) to perform anti-aging treatments on senescent cells and progeroid animal models. Following intervention with ADSCs, replicative senescence was delayed and metabolic homeostasis was transformed from catabolism to anabolism. Metabolomic tests were used to analyze different metabolites. We found that ADSCs acted to accelerate mitophagy which eliminated intracellular ROS and improved the quality of mitochondria. These processes acted to regulate the cellular metabolic homeostasis and ultimately delayed the process of aging. Allogeneic stem cell therapy in a Progeria animal model (DNA polymerase gamma (POLG) knockin, mitochondrial dysfunction) also showed that ADSC therapy can improve alopecia and kyphosis by promoting mitophagy. Our research confirms for the first time that allogeneic stem cell therapy can improve aging-related symbols and phenotypes through mitochondrial quality control. These results are highly significant for the future applications of stem cells in aging-related diseases.

KEYWORDS
adipose-derived mesenchymal stem cells, aging, metabolism, mitophagy, stem cell transplantation

Abbreviations: ADSC, adipose tissue-derived stem cells; ECAR, extracellular acidification rate; ETC, electron transport chain; FC, fold change; MEF, mouse embryonic fibroblast; MSC, multipotent stromal cell; OCR, mitochondrial oxygen consumption; PCA, principal component analysis; PLS-DA, partial least squares discrimination analysis; QC, quality control; VIP, variable importance in the projection.

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1 | INTRODUCTION

Aging is characterized by the gradual loss of physiological integrity in tissues that results in an increased risk of functional impairment and death. This degenerative process is a major risk factor for human diseases such as cancer, diabetes, and cardiovascular and neurodegenerative diseases.\(^1\)\(^,\)\(^2\) Around 150,000 people die globally every day, of which about two-thirds die from age-related causes.\(^3\) As we age, various physiological functions weaken such as exercise capacity, sexual performance and cardiopulmonary function. These processes are not classified as diseases but can have major impacts on the quality of life for the elderly and have large socio-economic costs. Approaches to effectively maintain the homeostasis of tissues and delay the occurrence of aging-related phenotypes remain significant challenges in the clinic.

Mesenchymal stem cells, also known as multipotent stromal cells (MSCs), are therapeutic candidates for a variety of human diseases. The therapeutic potential of MSCs stems from their ability to maintain homeostasis by migrating to the site of tissue injury and to promote tissue repair.\(^4\)\(^,\)\(^5\) MSCs have shown great potential in the treatment of various models in vitro and in vivo of disease phenotypes including neurodegenerative and cardiovascular diseases, diabetes, acute kidney injury and female infertility.\(^6\)\(^-\)\(^10\)

In clinical studies, phase I and II randomized trials have evaluated the use of allogeneic human mesenchymal stem cells (allo-hMSC) to treat the signs and symptoms of aging frailty. The results showed that the administration of allo-hMSCs was effective in humans with preferential effects on the improvement of functional status, quality of life and sexual quality.\(^11\)\(^,\)\(^12\) These findings demonstrate the potential to use MSCs for improving a series of aging-related symptoms. However, the indicators in the study were relatively subjective and the molecular mechanisms of action remains to be determined.

Amongst the various types of MSCs, adipose tissue-derived stem cells (ADSCs) are relatively easy to obtain and harvest through subcutaneous fat aspiration and other methods. Compared with harvesting bone marrow stem cells, this process is less invasive and less controversial than the use of embryonic stem (ES) cells.\(^13\) ADSCs can self-renew and differentiate into adipocytes, chondrocytes, muscle cells, osteoblasts, nerve cells and other cell lineages. They have previously been used in clinical trials to treat a range of disease including diabetes, liver disease, corneal disease, and joint and skin diseases.\(^14\)\(^-\)\(^17\) Therefore, ADSCs are an ideal choice for the application of MSCs in clinical treatments.

In this study, we used adipose tissue-derived stem cells (mADSCs) of mice to study the effects on aging cells and in an animal model. Under these interventions, the effects of delaying aging were observed at the cellular level and the tissue level in experimental animals. Also, for the first time, we demonstrated that ADSCs can affect metabolic homeostasis by promoting damaged mitochondrial clearance through mitophagy, thereby delaying aging. This research provides a theoretical basis for the treatment of aging-related diseases using MSCs and will facilitate clinical applications of these kinds of treatments.

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

Antibodies against PolG (EPR7296) were purchased from Abcam. p62/SQSTM1 (P0067) and β-actin (A1978) were purchased from Sigma. Tom20 (42406), LAMP1 (9091), Phospho-Histone H2AX (Ser139; 2577) and HSP60 (12165) were purchased from Cell Signaling Technology. TIM23 (NBP2-13432, 1:1,000 dilution) was purchased from NOVUS Biologicals. P16 (sc-1661), P53 (sc-126) and P21 (sc-6246) were purchased from Santa Cruz Biotechnology. ANTI-CD34-FITC, ANTI-CD29-PE, and ANTI-CD44-FITC were purchased from BD biosciences. ANTI-CD90 (ab33694) was purchased from Abcam.

DMEM/F12 medium, FBS, penicillin/streptomycin and trypsin were purchased from Invitrogen. Collagenase type I was purchased from Sigma. Osteogenic differentiation medium was purchased from OriCell™, Cyagen, USA. Complete adipogenic differentiation medium was purchased from MesenCult, Stem Cell Technologies, Canada. Oil red O was purchased from Cyagen, USA and alizarin red S was purchased from Solarbio, China.

2.2 | Isolation, culture and characterization of cells

ADSCs were isolated from the adipose tissues of C57BL/6 mice according to a previously reported method.\(^18\)\(^,\)\(^19\) Adipose tissue was dissected from the subcutaneous area and centrifuged at 1200 g for 5 minutes after washing with sterile phosphate-buffered saline (PBS) to remove red blood cells (RBC). This was repeated three times and then the tissue was digested with 0.2% type I collagenase at 37°C. DMEM/F12 containing 10% fetal bovine serum (FBS) was added to neutralize the enzyme activity. The tissue was then centrifuged for 5 minutes at 1200 g. The pellet was resuspended and filtered through a 100 μm mesh filter. Finally, the cells were seeded and incubated in a medium (DMEM/F12, 10% FBS, 1% penicillin-streptomycin solution) at 37°C, 5% CO\(_2\) and saturated humidity. Adherent cells were cultured and ADSCs were passaged after reaching 90% confluency, 0.25% Trypsin/0.02% EDTA at a ratio of 1:3 was used to dissociate...
the cells. Multi-lineage potential assays (osteogenic and adipogenic differentiation) and flow cytometry analysis (CD29, CD34, CD44) were conducted to identify the characteristics of the ADSCs as performed previously20 (Figure S1).

Wild-type (C57BL/6) mouse embryonic fibroblast (MEF) cells were obtained as follows: pregnant mice were euthanized to obtain embryos (days E12.5 to E14.5) and the limbs, head, tail and internal organs were removed. The remaining tissues were cut into small pieces which were digested using a pipette in 0.25% trypsin/0.02% EDTA. After 10 minutes, the mixture was neutralized with DMEM/F12 containing 10% FBS and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in 10% FBS DMEM/F12 and the cells were plated in a petri dish in a 5% CO₂ incubator at 37°C. Cells were passaged until they were 90% confluent, 0.25% Trypsin/0.02% EDTA at a ratio of 1:3 was used to dissociate the cells.

A co-culture system was performed using a Transwell Chamber (6-well plates with 0.4 micron membranes, Corning, 3412). P4 MEFs (1 × 10⁵) were seeded in the lower chamber while P3-mADSCs (1 × 10⁵) were seeded in the upper chambers. After 48 hours incubation, MEFs were collected for further analysis.

2.3 Western blot analysis

Cells were lysed with IP lysis buffer and a mixture of protease and phosphatase inhibitors was added for 30 minutes on ice. The sample was loaded onto a 8%-15% polyacrylamide gel, separated by SDS-PAGE and then transferred to a PVDF membrane at 80-120 V for 1.5-2.5 hours. After blocking with 5% BSA at room temperature, the membrane was incubated with a specific antibody overnight at 4°C. After washing 3 times with TBST, the membrane was incubated with secondary antibody at room temperature for 2 hours. Finally, the bands were analyzed by chemiluminescence detection (Tanon Technology Co., Ltd., Shanghai, China).

2.4 The respiratory (OCR) and glycolytic (extracellular acidification rate) ability of living cells

We examined the OCR and extracellular acidification rate (ECAR) with a Seahorse Bioscience Extracellular Flux Analyzer according to the manufacturer's instructions (Seahorse Bioscience, Billerica, MA, USA). One day before the assay, cells were plated in cell culture microplates (Hippocampal Biosciences). The Seahorse buffer consisted of DMEM, phenol red, 25 mM glucose, 2 mM sodium pyruvate and 2 mM glutamine. When ECAR values were measured, 10 mM glucose, 1 μM oligomycin and 100 mM 2-deoxyglucose were added automatically. After monitoring baseline respiration, 1 μM oligomycin, 1 M FCCP and 1 μM rotenone were automatically injected into cell culture microplates to measure OCR. ECAR and OCR measurements was normalized to total protein content and reported as pH/min. Each sample was determined in triplicate.

2.5 Immunofluorescence staining

MEF were treated in the co-culture system for 48h and then seeded into 24-well plates with glass coverslips. The analyses were carried out after 12 hours adhere. The cells were washed twice with PBS and fixed in 4% paraformaldehyde at room temperature for 15 minutes. The cells were then permeabilized with 0.1% Triton X-100. The cells were blocked with 5% BSA in PBS for 1 hour at room temperature and then incubated with primary antibody overnight at 4°C. Cells were then incubated with 488 goat anti-mouse IgG (H + L) or Alexafluor 594 goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific) at room temperature for 1 hour and imaged using a fluorescence microscope (Nikon, Ti-E, DS-Ri2). Confocal images were obtained using a 60× oil objective lens on an inverted fluorescence microscope (Nikon, Ti-E, DS-Ri2, NY USA).

2.6 Senescence-associated β-galactosidase (SA-β-gal) staining

According to the manufacturer’s protocol (Beyotime, C0602), SA-β-gal staining was used to evaluate senescence in MEF. MEFs at different conditions were seeded in 6-well culture plates. Cells were then washed with PBS, fixed for 30 minutes and stained with SA-β-gal staining solution overnight at 37°C (no CO 2). After washing three times with PBS, the cells were randomly photographed.

2.7 Measurement of mitochondrial ROS production and membrane potential

The level of mitochondrial ROS in MEF was measured based on MitoSOX staining. MEFs were cultured in 24-well plates before treatment. MEFs were then washed with PBS and incubated with 10 μM MitoSOX (Invitrogen, M36008) in the dark at 37°C for 10 minutes. Cells were then washed again with PBS and the fluorescent signal was photographed in a randomly selected area using a motorized inverted microscope (Olympus, Hamburg, Germany). A mitochondrial membrane potential measurement kit with JC-1 was used according to the manufacturer's instructions (Beyotime Institute of Biotechnology, China). In high-potential cells, J-aggregates form a red fluorescence signal, whilst in low-potential cells, JC-1 monomers form a green fluorescence signal.
The production of ROS was evaluated using the 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) (Beyotime Biotechnology, Shanghai, China) assay by flow cytometry to quantitatively analyze cells under the same conditions.

2.8 Extraction of metabolites and data analysis

To ascertain the effect of mADSC secretion on aging, P3 MEF, P4 MEF and co-cultured P4 MEF were collected and immediately frozen in liquid nitrogen. The cell homogenate was pre-cooled with 80% methanol and mixed with 0.1% formic acid by vortexing. The homogenate was incubated on ice for 5 minutes and then centrifuged at 15 000 rpm and 4°C for 5 minutes. Some of the supernatant was diluted with LC-MS grade water to a final concentration of 53% methanol. The sample was then transferred to a fresh Eppendorf tube and centrifuged at 15 000 g at 4°C for 10 minutes. Finally, the supernatant was injected into the LC-MS/MS system for analysis. The Vanquish UHPLC system (Thermo Fisher, Germany) was combined with the Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher, Germany) of Novogene Co., Ltd. (Beijing, China) for UHPLC-MS/MS analysis.

The Compound Finder 3.1 (CD3.1, Thermo Fisher) software was used to process the raw data files generated by UHPLC-MS/MS to allow peak comparison, peak selection and quantification for each metabolite. The peaks of metabolites were matched with mzVault, mzCloud (https://www.mzcloud.org/) and MassList databases to acquire precise and relative quantitative results. The statistical software packages R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS version 6.6) were used for statistical analysis. When the data were not normally distributed, an area normalization was performed using a normal conversion method.

The metabolites were annotated using the KEGG (https://www.genome.jp/kegg/pathway.html), HMDB (https://hmdb.ca/metabolites) and LIPID Maps database (http://www.lipidmaps.org/). Univariate analysis (t-test) was performed to calculate the statistical significance (P-value). Metabolites with VIP > 1 and P-value < .05 and fold change ≥ 2 or FC ≤ .5 were considered to be differential metabolites (VIP, Variable important in projection, which can be used to measure the impact strength and explanatory power of the difference in accumulation of various metabolites on the classification of each group of samples).

2.9 Animal experiments

PolG+/− mice were a kind gift from Nils-Göran Larsson. Mice were housed in a 12 hours light-12 hours dark cycle in a 21-23°C facility and were fed with a standard chow diet (LabDiet, 5C02C). PolG+/+ and PolGmut/mut mice were generated by mating POLG+/− males and females. Genotyping was performed by standard PCR (Figure S2). A total of 30 mice (10 WT and 20 KO, all male) were used in the experiment. When the mice grew up to 8 weeks of age, 1 × 10⁶ mADSCs suspended in physiological saline solution were administered to the KOC group (n = 10) via the lateral tail vein. The control group was injected with physiological saline. The cell injection was repeated every 4 weeks for a total of three injections. A total of 3 injections were given (every 4 weeks, 1 × 10⁶ cells each time). The experiment was terminated at 24 weeks and the mice were killed by CO₂ asphyxiation.

Mice were kept under specific pathogen-free conditions and all animal experiments were carried out under the guidance of the Institutional Animal Care and Use Committee (IACUC) of China Medical University.

2.10 Statistical analysis

Three independent experimental values were expressed as the mean ± standard deviation. Student's t-test or one-way ANOVA followed by Tukey’s post hoc tests were used to evaluate differences between or among groups. All statistical analyses were performed using SPSS 17.0 and Prism 5.0 software. A value of P < .05 was defined as statistically significant.

3 RESULTS

3.1 ADSCs postpone MEF replicative senescence

To determine the impact of ADSCs on aging, we used the cells as a cell model of replicative senescence. mADSCs and MEFs were co-cultured in a Transwell Chamber system to observe any exocrine effects of ADSCs on delaying MEF senescence with increasing passage number.

Cell senescence was determined by the activity of SA-β-gal in these cells. Our results showed that with increasing passage of MEF, the staining activity of SA-β-gal increased (P4 > P3). The positive rate of P4 cells in the mADSC cocultured group was significantly lower than that in the normal P4 group (Figure 1A). We performed γH2AX fluorescent staining on the same cells to detect the level of DNA damage and showed similar results (Figure 1B). Quantitative analysis was performed to determine the delaying effect of mADSCs on MEF senescence and DNA damage.

It has been shown that senescence is accompanied with the accumulation of p16, p53, p21. 1 To characterize the nature of the senescence-delayed effect of mADSCs on MEF,
we examined the expression of these proteins in MEF in P4 and co-cultured P4 by Western blotting. The expression of p16, p21, p53 gradually decreased in co-cultured P4 MEF compared to P4 cells (Figure 1C). The cell viability of MEF was also evaluated and CCK8 (Cell counting kit 8) levels suggested that the cell viability of co-cultured P4 MEF was significantly higher (Figure 1D).

In general, under the effects of mADSCs, the replicative senescence of MEF was effectively delayed and the overall viability of the cells also increased.

### 3.2 ADSCs regulate energy metabolism homeostasis in MEF

As aging progress, the metabolic homeostasis of cells and the body is difficult to maintain. The metabolic balance is more inclined to transform from anabolism to non-toxic catabolism to produce large amounts of adenosine 5-triphosphate (ATP) to ensure effective nutrient utilization and resistance to external stimulation.

We found that ADSCs can delay the senescence of MEF and improve the viability and then further analyzed energy metabolism in MEF. The mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XFe96 analyzer (Seahorse Bioscience, 103020-100). We found that with the increasing passage of MEF (P3 vs P4), the OCR and ECAR of cells both showed an upward trend. Under the effects of mADSCs (co-cultured P4 MEF), OCR and ECAR both decreased (Figure 2A,B), and the basic level of cellular respiration and ATP production decreased (Figure 2C,D). These trends are typical of cell youthfulness, suggesting that under the effects of ADSCs, the metabolic balance of MEF changed from...
catabolism to anabolism which is more conducive to cell division and growth.

3.3 | Metabolomic profile of ADSC-induced MEF

To more fully understand the mechanisms of the anti-aging effect of ADSCs on MEF, P3, P4 and co-cultured P4 MEF were collected and analyzed in the UHPLC-MS/MS system. Our analysis identified 851 structurally named compounds in a wide array of biochemical pathways including amino acids, carbohydrates, lipids and nucleotides. In total, 167 and 279 metabolites showed differential abundance in the P3 vs P4 groups and co-cultured P4 vs P4 groups, respectively. Heatmap and hierarchical clustering by individual metabolites revealed that the metabolic profile of co-cultured P4 MEF appeared markedly distinct from P4 MEF. The differences between the groups were very similar to those between P3 and P4 (Figure 3A). Further analysis showed an overlap that contained 60 common metabolites between the different groups (Figure 3B). These differential metabolites were widely related to the metabolism of various cellular nutrients of which lipid and carbohydrate metabolism were the most closely related (Figure 3C).

3.4 | Anti-aging effects of ADSCs may be related to the regulation of mitochondrial function

To further explore the regulation effects of ADSCs on MEF metabolism, we screened the differential metabolites between the two groups. A total of 45 differential metabolites in the co-cultured P4 vs P4 group were identified that were consistent with the changing trend of P3 vs P4 (Table S1). These 45 compounds were analyzed using STITCH (http://stitch.embl.de/) online analysis for chemical compounds, proteins and gene correlation interaction analysis (only related metabolites were shown) (Figure 4A).

A KEGG pathway analysis was performed and implied that besides of the Metabolic Pathway, those nucleotide-related pathways were highly related (Figure 4B). To identify the potential biological functions of these related genes, we performed GO enrichment analyses including CC, BP and MF by DAVID. According to statistically significant GO analysis results (P values < .05), we found that mitochondrial and organic compound synthesis-related functions were mainly affected (Figure 4C).

Through cell experiments and data analysis, we found that under the effects of ADSCs, the material synthesis and
cell viability of MEF were significantly enhanced. Also, the energy production used to maintain cell homeostasis was reduced. These data indicate that ADSCs maintain MEF in a more youthful and energetic state that may be attributed to ADSCs speeding up the removal of harmful substances in MEF. Combined with the reduction of ATP and the enrichment of mitochondrial-related genes, we hypothesize that ADSCs promote the accumulation of damaged mitochondria in MEF and eliminate ROS produced by these mitochondria, thereby causing the effect of anti-aging.

3.5 | ADSCs delay cell senescence by promoting mitophagy

To explore the changes in the quantity and quality of mitochondria in MEF under the effects of ADSCs, we conducted the following experiments. Firstly, we performed WB to detect several types of mitochondrial marker proteins in MEF. It was found that under the effects of ADSCs, the markers of mitochondrial membrane protein (Tom20, Tim23) and cytoplasmic protein (HSP60) decreased, showing that the number of mitochondria in MEF was reduced (Figure 5A).
To determine impacts on mitochondrial homeostasis by ADSCs in MEF, the levels of mitochondrial reactive oxygen species (ROS) were analyzed using the MitoSOX Red Mitochondrial Superoxide Indicator. As shown in Figure 5B, ADSCs decreased mitochondrial ROS generation in MEF cells. We also detected a decrease in the ROS content of MEF after ADSC co-culture by flow cytometry (Figure 5C).

We then used JC-1 staining assays to evaluate mitochondrial quality in the presence or absence of ADSCs. Compared to the control group, ADSCs caused a decrease in the number of damaged mitochondria with low potential (JC-1 monomer with green fluorescence) (Figure 5D). Furthermore, flow cytometry was used to quantitatively analyze the ratio of monomer to aggregates of JC-1 (Figure 5E) and confirmed that under the effects of ADSCs, the mitochondrial quality of MEF was significantly improved.

Mitophagy is the most common process of removing damaged mitochondria from cells. After co-culture with ADSC, the quantity and quality of mitochondria in MEF were altered, accompanied by the elimination of overall ROS. We considered that ADSCs may stimulate the occurrence of mitophagy in MEF. Therefore, we studied the factors relating to autophagy and mitophagy. WB found that
FIGURE 5  ADSCs delay cell senescence by promoting mitophagy. A, Western blot analysis of Tom20, Tim23, HSP60, P62 in P4 and co-cultured P4 MEFs. Quantification is shown as the mean ± SEM of n ≥ 3 technical replicates and are representative of three independent experiments, ***P < .001; **P < .01; *P < .05. B, The levels of mitochondrial ROS were detected using the MitoSox Red mitochondrial superoxide indicator by fluorescence microscope, Scale bar = 100 μm. C, The production of ROS was evaluated by DCFH-DA through flow cytometry, data are presented as the mean ± SD, n = 3, ***P < .001. The mitochondrial membrane potential with JC-1 detected by fluorescence microscope (D, Scale bar = 100 μm) and flow cytometry (E). F, Immunofluorescence co-localization analysis of LAMP1 and HSP60 in MEF, Scale bar = 20 μm. Quantifications are shown as the mean ± SEM of n ≥ 3 technical replicates and are representative of three independent experiments, **P < .01
the autophagy marker protein P62 was reduced suggesting the activation of autophagy flux in co-cultured MEFs (Figure 5A). Also, we performed immunofluorescence co-localization analysis of the lysosomal membrane marker (LAMP1) and the mitochondrial plasma marker (HSP60) in MEF. Our results showed that the fluorescence

FIGURE 6  ADSCs delay the aging of PolG knockin mice. A, Bodyweight curves for WT, KO, KOC mice (n = 10) at different time points. KO mouse had obvious alopecia. Blue arrows indicate the ADSCs injection. B, and kyphosis (C) which was significantly improved in the KOC group. D, Western blot analysis of Tom20 and P62 in the brain and heart muscle of WT, KO and KOC mice. Quantifications are shown as the mean ± SEM of n ≥ 3 technical replicates and are representative of three independent experiments, ***P < .001; **P < .01. E, Immunohistochemical staining of LC3 in the heart muscle from WT, KO and KOC mice, Scale bar = 100/50 μm. Blue arrows indicate the positive expression of LC3. F, SA-β-gal activity test of the heart muscle from WT, KO and KOC mice, Scale bar = 100/50 μm. Blue arrows indicate blue stained of the SA-β-gal
co-localization signal in the co-culture group was enhanced indicating the activation of mitophagy (Figure 5F). Taken together, these data suggest that ADSCs promote mitophagy and maintain mitochondrial quality, thereby exerting the effect of anti-aging.

3.6  **ADSCs delay aging in mice with mitochondrial dysfunction**

To explore the effects of ADSCs on delaying aging by removing damaged mitochondria, we used PolG knockin (PolG mut/mut) mice as the animal model of progeria. PolG is the main polymerase of mitochondrial DNA. PolG affects the mitochondrial DNA stability and interferes with the expression of proteins synthesized by mitochondrial DNA transcription, thereby affecting the homeostasis of mitochondria.

PolGmut/mut mice had a normal appearance until the age of 4-6 months. As the animals aged, kyphosis was marked and they showed varying degrees of alopecia. Studies have shown that mitophagy is inhibited in PolGmut/mut mice. Therefore, mADSC treatment would be more likely to demonstrate this effect of promoting mitophagy and delaying aging-related symptoms in this model.

We injected mADSC regularly into mice in the KOC group (1 × 10⁶ per four weeks, 3 times). With growth, the body weights of KO mice were significantly lower than that of WT mice while in the KOC group they were slightly increased (Figure 6A). At 24 weeks, KO mice had obvious alopecia and kyphosis which were significantly improved in the KOC group (Figure 6B,C).

We then extracted the heart and brain tissues of mice. WB analysis indicated that expression of Tom20 and P62 were decreased, indicating that the number of tissue mitochondria in the KOC group decreased and autophagy increased (Figure 6D). Immunohistochemical detection of myocardia tissue implied that the expression of LC3 in the KOC group was significantly higher than in the WT and KO groups (Figure 6E). The SA-β-gal activity test showed that the SA-β-gal activity of myocardial tissue in the KO group was significantly stronger than that in the WT group, while the KOC group returned to normal levels (Figure 6F). The aging symptoms of mice in the KOC group were partially rescued.

4  **DISCUSSION**

Aging is a multi-system process characterized by the gradual loss of physiological integrity that results in an increased risk of functional impairment and death. This deterioration is a major risk factor for major human diseases. In the past few decades, many studies have reported on the molecular mechanisms underlying the aging process that is now characterized by nine

![FIGURE 7](image-url)  **Experimental mechanism and schematic diagram**
specific signs of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. In our research, two models of aging were selected: one involves MEF cells, which represent cell senescence due to replication, the other is the PolG knockin progeroid mouse model, which represents animal aging due to mitochondrial dysfunction. After ADSCs treatment, both models showed obvious anti-aging effects. Through the analysis of cell energy metabolism and differential metabolites, we found that under the action of ADSCs, the balance of cell metabolism changed from catabolism to anabolism, and the synthesis of biological macromolecules increased, which is consistent with the youthful state of the cells. At the same time, the biological function analysis of the differential metabolites found that the pathways related to nucleotide metabolism, and genes related to mitochondria, were enriched. This suggests that the anti-aging effect of ADSCs may be related to mitochondrial function (Figure 7).

Mitochondria are the main producer of power in eukaryotic cells. In addition to acting as the generators of ATP, mitochondria also act as a signal hub for programmed cell death, they regulate calcium homeostasis and are necessary for the synthesis of cholesterol, nucleotides and amino acids. The accumulation of abnormal mitochondria caused by disturbances in mitochondrial quality control is crucial in the regulation of many age-related diseases such as cardiac disease and neurodegeneration. In our research, for the first time, we found that, under the effect of ADSCs, the number of cell mitochondria decreased, accompanied by a decrease in cellular ROS and an increase in mitochondrial membrane potential. This means that ADSCs improve the quality and dynamics of target cell mitochondria, which ultimately leads to increased cell macromolecule synthesis and cell proliferation. Homeostasis in this environment tends to occur earlier.

Treatments targeting mitochondrial-related pathologies are of great significance in aging-related diseases. Many studies have explored related mechanisms which are roughly divided into three categories. The first mechanism is ROS-related processes. Studies have confirmed that drugs or lifestyle adjustments can reduce the production of ROS or accelerate ROS clearance and can be used to reduce mitochondrial dysfunction due to oxidative stress. The second mechanism is mitochondrial transmission. Studies have confirmed that healthy mitochondria can be delivered into different types of damaged cells through intercellular structures such as tunnel nanotubes (TNT) or extracellular vesicles and improve target cell mitochondrial respiration and ATP levels. The third mechanism is mitophagy. Autophagy allows macromolecules and cytoplasmic components to be digested by cells. In this way, cells can recycle components to rejuvenate or obtain energy under stress conditions. Since damaged mitochondria can activate the apoptotic response, the elimination of abnormal mitochondria by mitophagy can be regarded as an adaptive survival mechanism. It can avoid the cell death response while at the same time providing sufficient opportunity to replenish healthy mitochondria that act to maintain energy production and cell survival. Therefore, mitochondrial quality control based on mitophagy is particularly important in maintaining homeostasis for cell survival.

Most previous studies showed that mitochondrial function of damaged cells by stem cells can be regulated through mitochondrial transfer. Excessive mitochondria are transferred from the stem cells to the dysfunctional mitochondria in damaged cells to improve the impaired cell metabolism. In our study, we found that the number of mitochondria and the ATP production of senescent cells decreased. We confirmed for the first time that ADSCs can promote the activation of mitophagy and ensure the quality of the remaining mitochondria. We found that ADSCs can improve mitochondrial function comprehensively. ADSCs first promote the occurrence of autophagy. This process not only removes damaged mitochondria and reduces ROS in the body, but also provides many raw materials for the synthesis of macromolecules. Meanwhile, healthy mitochondria may be spread to severely damaged cells to maintain their survival.

Our research proposes, for the first time, that MSCs can postpone the aging process and regulate the homeostasis of cellular metabolism by promoting mitophagy. In the future, we will further explore how MSCs selectively regulate cell mitochondrial functions through different mechanisms. It is expected that this method can be used for the treatment of aging-related diseases and symptoms in the future.

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**CONFLICT OF INTEREST**

The authors report no conflict of interest.

**AUTHOR CONTRIBUTIONS**

M. Lv carried out all the experiments; S. Cao and B. Jiang contribute to data collection and analysis; S. Zhang and Y. Dong contributed to animal experiment; S. Guo and L. Cao performed experimental guidance. All authors read and approved the final manuscript.

**DATA AVAILABILITY STATEMENT**

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