

Cinnamaldehyde Delays the Senescence of Mesenchymal Stem Cells by Maintaining Mitochondrial Homeostasis

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Background: Stem cell exhaustion is a primary factor in human aging. The ability to delay aging by maintaining the steady state of stem cells has emerged as a crucial area of concern. However, the regulatory impact of Cinnamaldehyde (Cina) on stem cell senescence remains unknown. Therefore, this study aimed to elucidate the regulatory effect of Cina on the senescence of mesenchymal stem cells (MSCs).

Methods: Physiological cell senescence model was established by cell subculture. Cell counting kit-8 (CCK-8) proliferation assay, continuous doubling experiment, Ki67 staining, and cell cycle assay were used to examine the impact of Cina on the proliferation of MSCs. Senescence-associated β -galactosidase (SA- β -gal) staining was used to evaluate the senescence of MSCs. Histone H3 trimethylated at lysine 9 (H3K9me3) staining was used to assess the stability of MSCs chromatin. Osteogenic and adipogenic induction tests were used to evaluate the differentiation potential of MSCs, and 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide (JC-1) staining was used to evaluate the mitochondrial membrane potential. Glutathione (GSH) assay was used to assess the intracellular redox status. Adenosine triphosphate (ATP) detection and the Seahorse test were used to detect the energy metabolism of cells. Additionally, real-time quantitative PCR (RT-PCR) was used to quantify the indexes associated with senescence, proliferation, and differentiation of MSCs. Moreover, changes in MSCs senescence-related signaling pathways were analyzed using transcriptome.

Results: Cina significantly promoted the proliferation of MSCs, maintained their proliferation rate in prolonged exposure, and delayed their senescence ($p < 0.05$). Cina reduced the number of SA- β -gal positive mycoplasma-free cells, decreased the levels of senescence markers such as cyclin dependent kinase inhibitor 2A (*P16*), cyclin dependent kinase inhibitor 1A (*P21*), interleukin 6 (*IL-6*), and *IL-8*, and increased the level of Recombinant lamin B1 (*LMNB1*). Furthermore, Cina treatment increased the expression of H3K9me3, increased the number of Ki67 positive cells, and reversed cell cycle arrest ($p < 0.05$). Additionally, Cina upregulated the expression of pluripotency-related genes and downregulated senescence-related signaling pathways, such as P53 and RAS-associated protein 1 (RAP1). In osteogenic and adipogenic experiments, Cina was found to promote the differentiation of MSCs ($p < 0.05$). Furthermore, we observed that Cina substantially protected mitochondrial membrane potential from damage caused by passage replication, maintained intracellular redox balance, and promoted mitochondrial ATP production ($p < 0.05$).

Conclusions: This study indicates that Cinnamaldehyde (Cina) can delay the senescence of MSCs by maintaining mitochondrial homeostasis, suggesting that Cina has a potential anti-aging effect.

Keywords: cinnamaldehyde; mesenchymal stem cells; aging; mitochondrial homeostasis

Introduction

With increasing age, the human body undergoes gradual aging. Macroscopically, bodily functions, including response time, memory, hormone regulation, and other crucial processes, decline and the defense ability of bacteria and viruses gradually decreases, increasing susceptibility to hypertension, hyperglycemia, hyperlipidemia, brain function decline, and other diseases [1,2]. Microscopically, the number of stem cells, which can differentiate into functional cells across various tissues and organs, gradually decreases, resulting in an insufficient supply of cells to meet

the body's needs. Moreover, the new cells generated by differentiation cannot completely replace or repair damaged or senescent cells [3,4]. Therefore, stem cell senescence is identified as the fundamental cause of aging. Hence, increasing the number of stem cells within the body to delay aging has emerged as a crucial area of concern [5].

Currently, several studies have investigated approaches to delay the senescence of stem cells by targeting specific genes. For example, Zhao *et al.* [6] successfully delayed the senescence of stem cells by inhibiting the apolipoprotein E (APOE) protein and maintaining chromatin stability. Wang *et al.* [7] reported that overexpressing

recombinant activating transcription factor 6 (ATF6) could maintain organelle homeostasis and thus delay mesenchymal stem cells (MSCs) senescence. Small-molecule drugs have made promising breakthroughs in disease treatment due to their ease of production, quality control, and administration. Many drugs have demonstrated potential anti-aging effects. These include rapamycin and torin1, which are mammalian target of rapamycin (mTOR) inhibitors, and trametinib, a mitogen-activated protein kinase (MEK) inhibitor [8–10]. These drugs may exert their anti-aging effects by modulating protein homeostasis [11]. These findings suggest that maintaining intracellular homeostasis is vital in delaying aging. Additionally, they indicated the significance of developing small-molecule anti-aging drugs in maintaining intracellular homeostasis.

Cinnamaldehyde (Cina) is the main component of the volatile oil extracted from Cassia twig or Cassia bark. It is an aldehyde organic compound with anti-inflammatory, antipyretic, analgesic, antitumor, antibacterial, hypoglycemic, antiobesity, and neuroprotective effects [12,13]. It exhibits specific preventive and therapeutic significance for the clinical conditions affecting the nervous system, cardiovascular system, tumors, diabetes, and other diseases [12,13]. Owing to these features, Cina holds substantial clinical and market potential as a natural medicine for prevention and health care. Recent studies have shown that Cina can reduce the senescence of nerve cells by maintaining redox homeostasis [14,15]. Furthermore, Cina has been demonstrated to delay endothelial cell senescence by activating NRF2 in keratinocytes and downregulating the senescence marker CDKN2A/p16INK4A [16]. These observations suggest that Cina may possess potential anti-aging effects, warranting the need for further investigation and identification as a potential anti-aging agent.

Stem cells are closely related to aging; however, the impact of Cina on the homeostasis of stem cells and anti-aging effects remains unclear. Therefore, we explored whether Cina plays a regulatory role in MSCs senescence. To achieve this, we constructed an MSC model of physiological senescence and evaluated senescence phenotypes, senescence markers, and mitochondrial homeostasis. We observed that Cina supplementation promotes the proliferation of MSCs by increasing the number of Ki67-positive cells, maintaining the balance of the cell proliferation cycle, maintaining the stability of chromatin by increasing the expression of histone H3 trimethylated at lysine 9 (H3K9me3), and ensuring energy generation by maintaining mitochondrial homeostasis to delay the physiological senescence of MSCs.

Materials and Methods

Cell lines: Human stem cells from human exfoliated deciduous teeth (SHEDs) passage 2 (p2) were obtained from Saliat Stem Cell Science and Technology Co., Ltd.,

Guangzhou, China (RDP000660403). Human adipose-derived stem cells (ADSCs) (p2) were obtained from Cyagen Biosciences, Guangzhou, China (RAD0001530401). These cells were cultured in serum-free MSC medium (#CM-SC01, Procell, Wuhan, China) and were maintained at 37 °C in humidified air containing 5% CO₂. After achieving the cell confluence of 70%–80%, they were washed with phosphate buffered saline (PBS) (P1010, Solarbio, Beijing, China) and passaged through 0.25% trypsin-EDTA (PB180224, Procell, Wuhan, China) digestion. The cell lines were authenticated through STR profiling and no mycoplasma contamination was detected.

Compound preparation: Cina (ST01700181, Nature Standard, Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO) (T795936, Macklin, Shanghai, China) to prepare a 100 mM stock solution, and was stored at –80 °C. Before the *in vitro* experiments, the Cina stock solution was diluted to the required final concentration in the medium, ensuring that the final DMSO volume fraction did not exceed 0.1%.

Cell viability assay: The cells were seeded into 96-well plates and subsequently treated with various concentrations of Cina. After 48 hours of incubation, the cell counting kit-8 (CCK-8) reagent (C0037, Beyotime, Shanghai, China) was added to each well, and the cells were incubated for 1 hour. The absorbance at 450 nm was assessed using an enzyme labeling instrument (A51119500C, Thermo, Lafayette, LA, USA). After subtracting the background signal from all wells, the cell survival rate was determined as a percentage relative to the control group.

Cumulative doubling curve of cells: Sixth-generation cells were inoculated into 6-well plates at a density of 7×10^5 cells/well, followed by the introduction of culture medium supplemented with either 10 μM Cina or control solvent. After 3 days, the number of cells was counted, and the extent of expansion was calculated. The cells were passaged continuously to the 16th generation, and the expansion times of the cells in each generation were documented.

Senescence-associated β-galactosidase (SA-β-gal) staining: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The cells were stained using the SA-β-gal staining kit (C0602, Beyotime, Beijing, China), following the manufacturer's instructions. The cells were washed 3 times with PBS, fixed for 20 minutes, and then incubated overnight at 37 °C. Random images were captured, and the number of SA-β-gal-positive cells was determined.

Cell cycle analysis: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The cells were collected and washed twice with PBS, followed by overnight fixation in 70% ethanol at –20 °C. After this, the cells were washed with PBS three times and stained with PI at room temperature following the protocol provided by the Cell Cycle and Apoptosis Analysis Kit (40301ES50, Yeasen, Shanghai, China). Finally, the cell cycle distri-

bution was assessed using Coulter CytoFLEX (Beckman Coulter CytoFlex S, Beckman, Brea, CA, USA) and analyzed through FlowJo software (v10.8.1, Dickinson & Company, Franklin Lakes, NJ, USA).

Immunofluorescence (IF) Assay: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The cells were seeded on 14 mm glass coverslips (BS-14-RC, Biosharp, Hefei, China), washed with PBS, and subsequently fixed with 4% paraformaldehyde (#P0099, Beyotime, Shanghai, China) for 15 minutes. Following three consecutive PBS washes, the cells were permeabilized with 1% Triton X-100 (P0096, Beyotime, Beijing, China) for 5 minutes. Subsequently, the cells were washed with PBS 3 times. After this, the membrane was blocked with 5% BSA for 30 minutes at room temperature and then washed with PBS 3 times for 5 minutes each. The membrane was incubated with primary antibodies against Ki67 (1:300, A21861, Abclonal, Wuhan, China) and H3K9me3 (1:250, 39285, Proteintech, Wuhan, China) at room temperature for 30 minutes, followed by washing with PBS 3 times (5 min each). After this, the membrane was incubated with a fluorescein isothiocyanate (FITC)-labeled (green) anti-rabbit secondary antibody (bs-0369M-FITC, Bioss, Beijing, China) at room temperature for 30 minutes and then washed with PBS 3 times for 5 min each.

Furthermore, the cells were transferred onto microscope slides and mounted with 4',6-diamidino-2-phenylindole (DAPI) (C1002, Beyotime, Shanghai, China) and examined using a fluorescence microscope (CX41, OLYMPUS, Tokyo, Japan).

RNA sequencing (RNA-seq) and Gene Set Enrichment Analysis (GSEA): SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The cells were collected and washed twice with PBS. The cells were incubated with total RNA extraction reagent (RK30129, Abclonal, Wuhan, China) for 5 minutes at room temperature before being transferred to eppendorf (EP) tubes. Subsequently, mRNA library construction and sequencing were performed through Applied Protein Technology Co., Ltd. (Shanghai, China). GSEA was performed through Broad GSEA software 4.0.2 (Broad Institute, Cambridge, MA, USA), using hallmark gene sets (h.all.v.7.4.symbols.gmt) from MSigDB for pathway annotation. Differentially expressed genes (DEGs) were defined as those with $|\log_2FC| > 2.0$ and $p\text{-adj} < 0.05$, as assessed by the R package edgeR (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>).

Real-time quantitative PCR (RT-PCR): SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The cells were collected and washed twice with PBS. Total RNA extraction reagent (RK30129, Abclonal, Wuhan, China) was added, and the samples were incubated at room temperature for 5 minutes before being transferred to EP tubes. Subsequently, 200 μL of chloroform was added and the mixture was shaken for 30 seconds.

The mixture was centrifuged at 12,000 rpm for 5 minutes, and the resultant supernatant was collected and transferred to new EP tubes. After this, the same volume of isopropanol was added, and the mixture was inverted and mixed well, followed by incubation at room temperature for 5 minutes. The mixture was centrifuged at 12,000 rpm for 5 minutes, and the upper layer was transferred to new EP tubes. Then, 500 μL of 75% ethanol was added to precipitate, the mixture was centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded, and RNA was dissolved in 10 μL of nuclease-free water. One microliter of RNA solution was added, and the RNA concentration was detected with an ultramicro spectrophotometer.

Furthermore, RNA was reverse transcribed into cDNA using ABScript III Reverse Transcriptase (RK30129, Abclonal, Wuhan, China) at 42 °C for 20 minutes, followed by denaturation at 82 °C for 1 minute. The resulting cDNA samples were stored at -80 °C for further analysis. After this, cDNA was amplified using real-time fluorescence quantitative PCR (Archimed R4, ROCGENE, Beijing, China). The 20 μL reaction mixture included 400 nM primers, 10 μL of SYBR Green Fast qPCR Mix (11201ES08, Yeasen, Shanghai, China), 2 μL of cDNA, and nuclease-free water. The fold changes of each target gene were determined using the $2^{-\Delta\Delta C_t}$ method relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *GAPDH* was used as the internal reference. The primers used in real-time quantitative PCR (RT-PCR) are shown in Table 1.

Osteogenic and adipogenic Assay: SHEDs were inoculated into a 6-well culture plate, and the induction medium was replaced after achieving cell confluence of 90% to 100%. Cina was added into the osteogenic and adipogenic induction media for SHED cell differentiation. During osteogenic differentiation, the osteogenic induction reagent (PD-007, Procell, Wuhan, China) was replaced every 3 days, while adipogenic differentiation was induced using a human-related stem cell adipogenic differentiation kit (PD-006, Procell, Wuhan, China). After 21 days of osteogenic and adipogenic induction, calcium nodules and lipid droplets stained with Alizarin Red S (PD-007, Procell, Wuhan, China) and Oil Red O (PD-006, Procell, Wuhan, China) were observed and imaged using a microscope.

5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide (JC-1) assay: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. SHEDs passage 15 (P15) were seeded into 12-well plates and incubated in the presence of 5% CO₂ at 37 °C. Once adhered to the walls, the cells were stained using the JC-1 staining kit (#C2003S, Beyotime, Shanghai, China) following the manufacturer's instructions. After staining, the cells were immediately examined under a CX41 fluorescence microscope (CX41, OLYMPUS, Tokyo, Japan).

Table 1. The list of primers used in RT-PCR.

Gene	Forward primer	Reverse primer
<i>P16</i>	ATGGAGCCGCGCGGGGA	TCAATCGGGGATGTCTGAGGGACC
<i>P21</i>	ATGTCAGAACCGGCTGGG	TTAGGGCTTCTCTTGGAGAA
<i>IL-6</i>	ATGTGTGAAAGCAGCAAAGAGG	CTACATTTGCCGAAGAGCC
<i>IL-8</i>	ATGACTTCCAAGCTGGCC	TTATGAATTCTCAGCCCTCTTC
<i>LMNB1</i>	ATGTATGAAGAGGAGATTAACGAGACC	TTACATAATTGCACAGCTTCTATTGGATG
<i>CDK1</i>	ATGGAAGATTATACAAAATAGAGAAAA	TTAAGCTTTTACCTTAACAAGTG
<i>CDK2</i>	ATGGAGAACTTCCAAAAGGTGG	TCAGAGTCGAAGATGGGGTA
<i>CDK4</i>	ATGGCTACCTCTCGATATGAG	TCACTCCGGATTACCTTCATC
<i>CDK6</i>	ATGGAGAAGGACGGCCTGT	TCAGGCTGTATTAGCTCC
<i>CCND1</i>	ATGGAACACCAGCTCCTG	TCAGATGTCCACGTCCCG
<i>BMP4</i>	ATGATTCTTGTAACCGAATGCTG	TCAGCGGCACCCACATCC
<i>RUNX2</i>	ATGGCATCAAACAGCCTCTTC	TCAATATGGTCGCCAACAGATTC
<i>SPP1</i>	ATGAGAATTGCAGTGATTTGCTTTTGC	TTAATTGACCTCAGAAGATGCACTATC
<i>AQP1</i>	ATGGCCAGCGAGTTCAAGAA	TTACGGTAGAATCCCAGG
<i>EBF2</i>	ATGTTTGAATTCAAGATACTTTAGG	TTACATCGGGGTACAACAAGT
<i>AdipoQ</i>	ATGCTGTTGCTGGGAGCTG	TCAGTTGGTGTGATGGTAGAG
<i>GAPDH</i>	ATGGTTTACATGTTCCAATATGATTCC	TTACTCCTTGGAGGCCATGT

RT-PCR, real-time quantitative PCR; *P16*, cyclin dependent kinase inhibitor 2A; *P21*, cyclin dependent kinase inhibitor 1A; *IL-6*, interleukin 6; *LMNB1*, lamin B1; *CDK1*, cyclin dependent kinase 1; *CCND1*, cyclin D1; *BMP4*, bone morphogenetic protein 4; *RUNX2*, runt-related transcription factor 2; *SPP1*, secreted phosphoprotein 1; *AQP1*, Aquaporin 1; *EBF2*, Early B-cell factor 2; *AdipoQ*, Adiponectin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Glutathione/oxidized glutathione (GSH/GSSG) detection: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. Samples were prepared following the protocol provided by the GSH/GSSG ratio test kit (#50120ES70, Yeasen, Shanghai, China). GSH standard dilutions were prepared and measured. After this, a thiol green indicator reaction mixture was added to each sample and incubated at room temperature for 60 minutes. Finally, the cells were observed using a multifunction microplate reader (Synergy2, BioTek, Winooski, VT, USA), and the fluorescence was measured at excitation/emission (Ex/Em) = 490/520 nm.

Adenosine triphosphate (ATP) detection: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The ATP levels in the supernatant were determined using an ATP assay kit (S0027, Beyotime, Shanghai, China).

Cellular mitochondrial stress assay: SHEDs (p6) were continuously cultured with Cina for 15 passages before testing. The cells were seeded into Seahorse 96-well plates (Seahorse XFe96, Agilent, Santa Clara, CA, USA) at a density of 4×10^4 cells/well. The cells were pretreated with drug-containing MSC serum-free medium for 12 hours, and mitochondrial respiration-related indices were determined using a Seahorse XFe96 analyzer (Agilent) following the instructions provided by the cellular mitochondrial stress assay kits (ALS22011, Alicelligent, Beijing, China).

Quantification and statistical analysis: Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad

Software, La Jolla, CA, USA). The data were presented as the means \pm standard deviations (SDs). The differences among multiple groups were compared using one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparisons test. Statistical significance was defined at a *p*-value < 0.05.

Results

Cina Can Maintain the Proliferation of MSCs

A cell proliferation assay, commonly employed to evaluate the viability of cells, was used to assess the senescence of stem cells. Therefore, we explored the impact of Cina on the viability of young SHEDs and ADSCs using a CCK-8 assay (Fig. 1). We observed that Cina significantly promoted SHED and ADSC proliferation at low doses (10–50 μ M) (*p* < 0.05), but did not significantly inhibit proliferation at high doses (200 μ M) (Fig. 1B,C). This suggests that Cina not only promotes the proliferation of MSCs but also exhibits good safety. Furthermore, in the continuous doubling experiments, the MSCs cultured with Cina maintained higher proliferation activity compared to the control group, indicating that Cina could maintain the proliferation of MSCs and possess an anti-aging effect (Fig. 1C,D).

Cina Delays SHED Senescence and Increases Chromatin Stability

We investigated the impact of Cina on the physiological senescence of SHEDs using SA- β -gal staining. Our

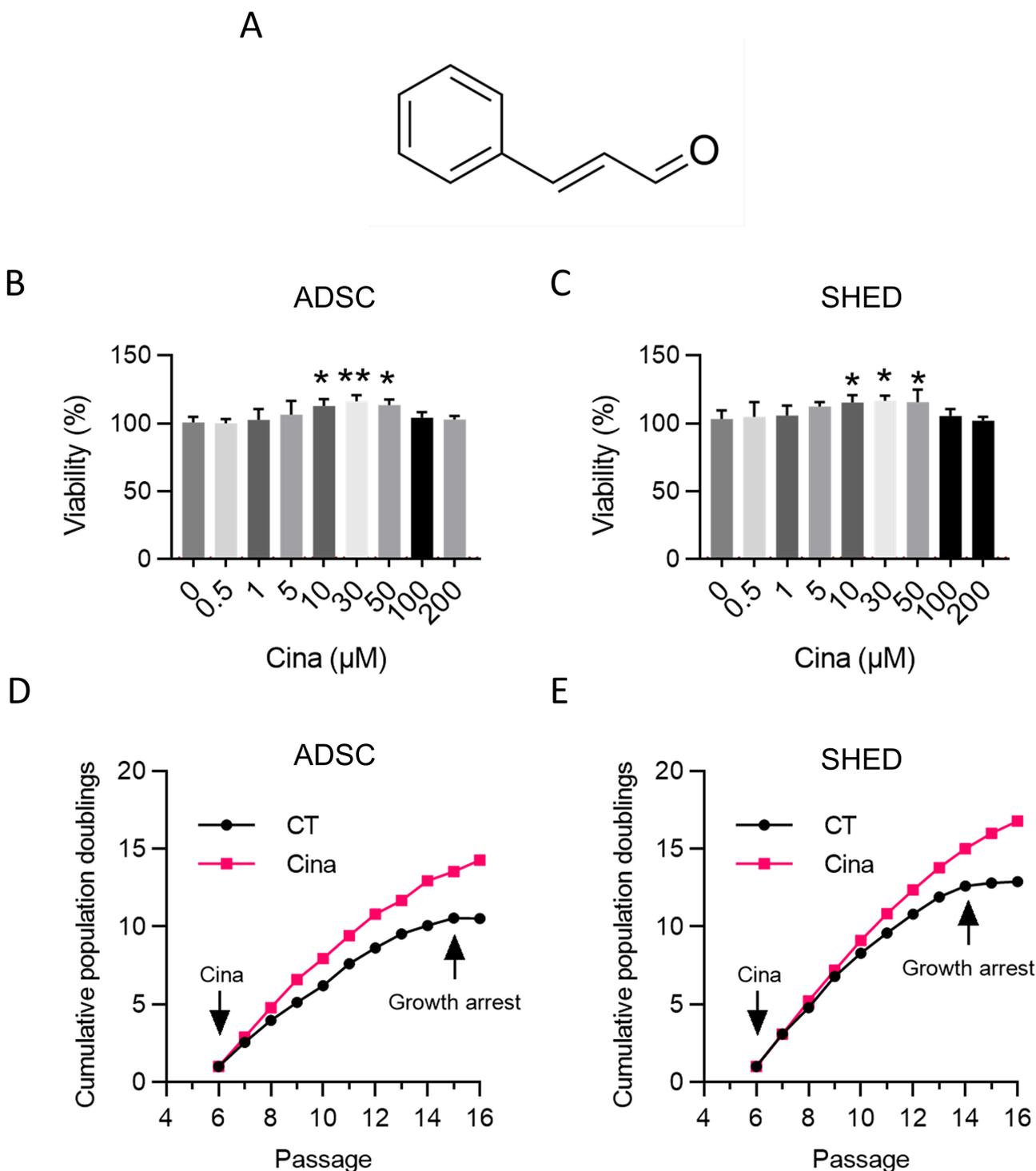


Fig. 1. Cina promotes the proliferation of MSCs in passage 8 (p8). (A) Molecular structure of Cina. (B,C) A cell counting kit-8 (CCK-8) assay was used to examine the impact of Cina on the proliferation of MSCs ($n = 3$). (D,E) The impact of Cina on the proliferation of MSCs was assessed through continuous subculture. The data were presented as the means \pm standard deviations (SDs). * $p < 0.05$; ** $p < 0.001$ compared to the control group. Cina, Cinnamaldehyde; MSCs, mesenchymal stem cells; SHED, stem cell from human exfoliated deciduous teeth; ADSC, adipose derived stem cell.

study revealed that long-term cultivation of Cina significantly reduced the percentage of SA- β -gal-positive cells compared to the control group ($p < 0.05$, Fig. 2A,B), de-

creased the mRNA levels of the senescence-related markers, such as cyclin dependent kinase inhibitor 2A (*P16*), cyclin dependent kinase inhibitor 1A (*P21*), interleukin 6 (*IL*-

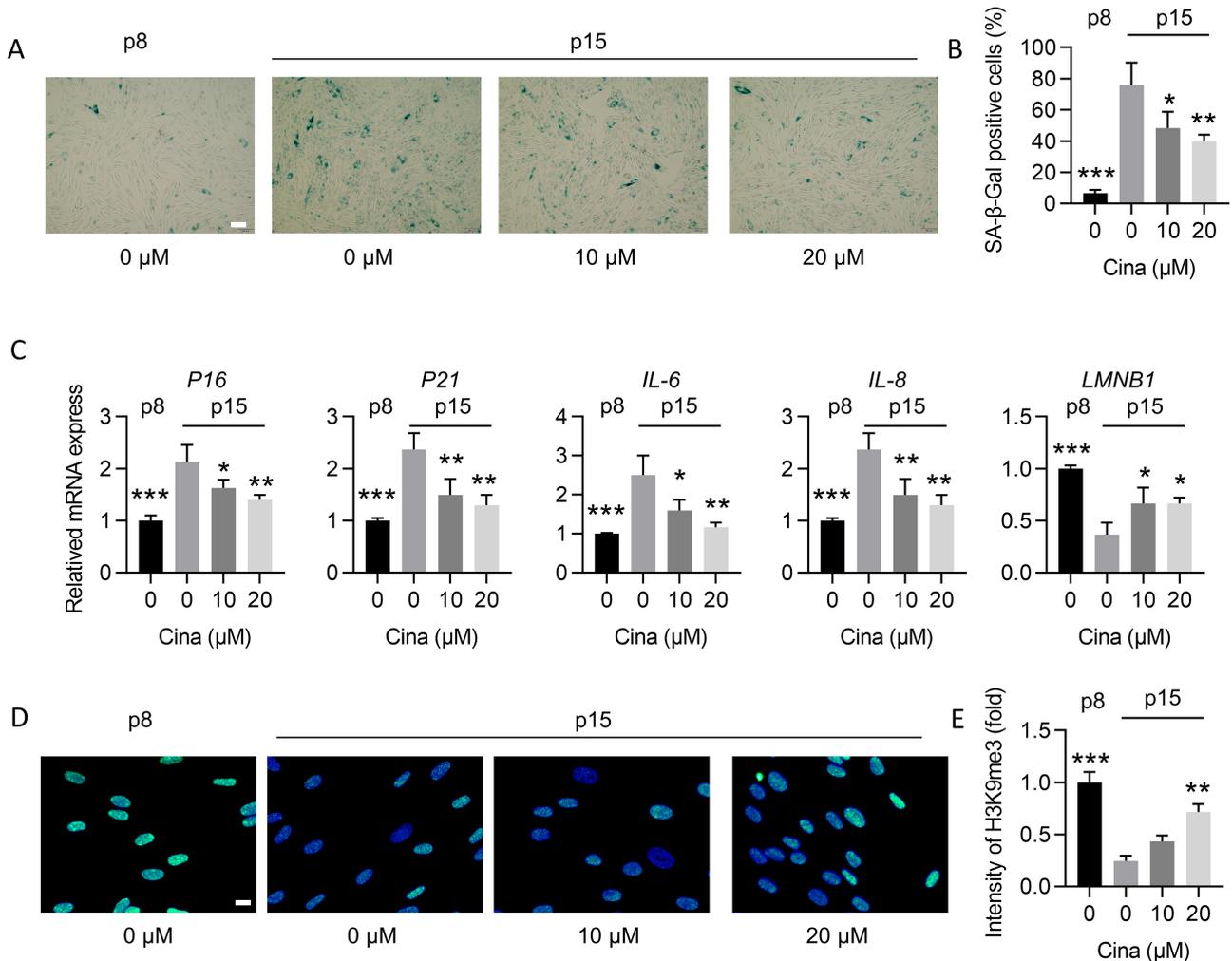


Fig. 2. Cina reverses D-Gal-induced SHED senescence and maintains chromatin homeostasis. (A,B) Senescence-associated β -galactosidase (SA- β -gal) staining was used to assess the impact of Cina on SHED senescence, $n = 6$. Scale bars = 50 μm . (C) The mRNA levels of senescence-related markers were evaluated using real-time quantitative PCR (RT-PCR), $n = 3$. (D,E) The effect of Cina on histone H3 trimethylated at lysine 9 (H3K9me3) expression was examined using an immunofluorescence assay. $n = 6$. Scale bars = 50 μm . The data were presented as the means \pm SDs. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to SHEDs at passage 15 (P15) in the 0 μM Cina group.

6), and *IL-8*, and increased the lamin B1 (*LMNB1*) level ($p < 0.05$, Fig. 2C). H3K9me3 is a marker of chromatin stability. Moreover, our study revealed that 20 μM Cina significantly increased the level of H3K9me3 ($p < 0.05$, Fig. 2D,E). These findings indicate that Cina can maintain chromatin stability and delay senescence in SHEDs.

Cina Can Reverse the Cell Cycle Arrest Induced by Physiological Senescence

Considering the Cina's ability to increase the proliferation of MSCs and maintain the doubling rate (Fig. 1B–D), we further investigated its underlying mechanism. As shown in Fig. 3A,B, Cina treatment resulted in a dose-dependent increase in the number of Ki67-positive cells compared to the control group. Furthermore, cell cycle analysis revealed that senescent cells exhibited G1 cell cy-

cle arrest, and 20 μM Cina supplementation significantly reduced the proportion of G1 cells and reversed cell cycle arrest ($p < 0.001$, Fig. 3C,D). However, research has demonstrated that cell cycle proteins, such as cyclin dependent kinase 2 (CDK2) and CDK4, can act on the G1 phase of the cell cycle, initiate DNA replication, and induce mitosis [17,18]. Therefore, evaluating cell cycle-related proteins (Fig. 3E) indicated that Cina treatment significantly increased the transcription of the cell cycle-related proteins, including cyclin dependent kinase 1 (CDK1), CDK2, CDK4, CDK6, and cyclin D1 (*CCND1*), compared to those in the control group ($p < 0.05$).

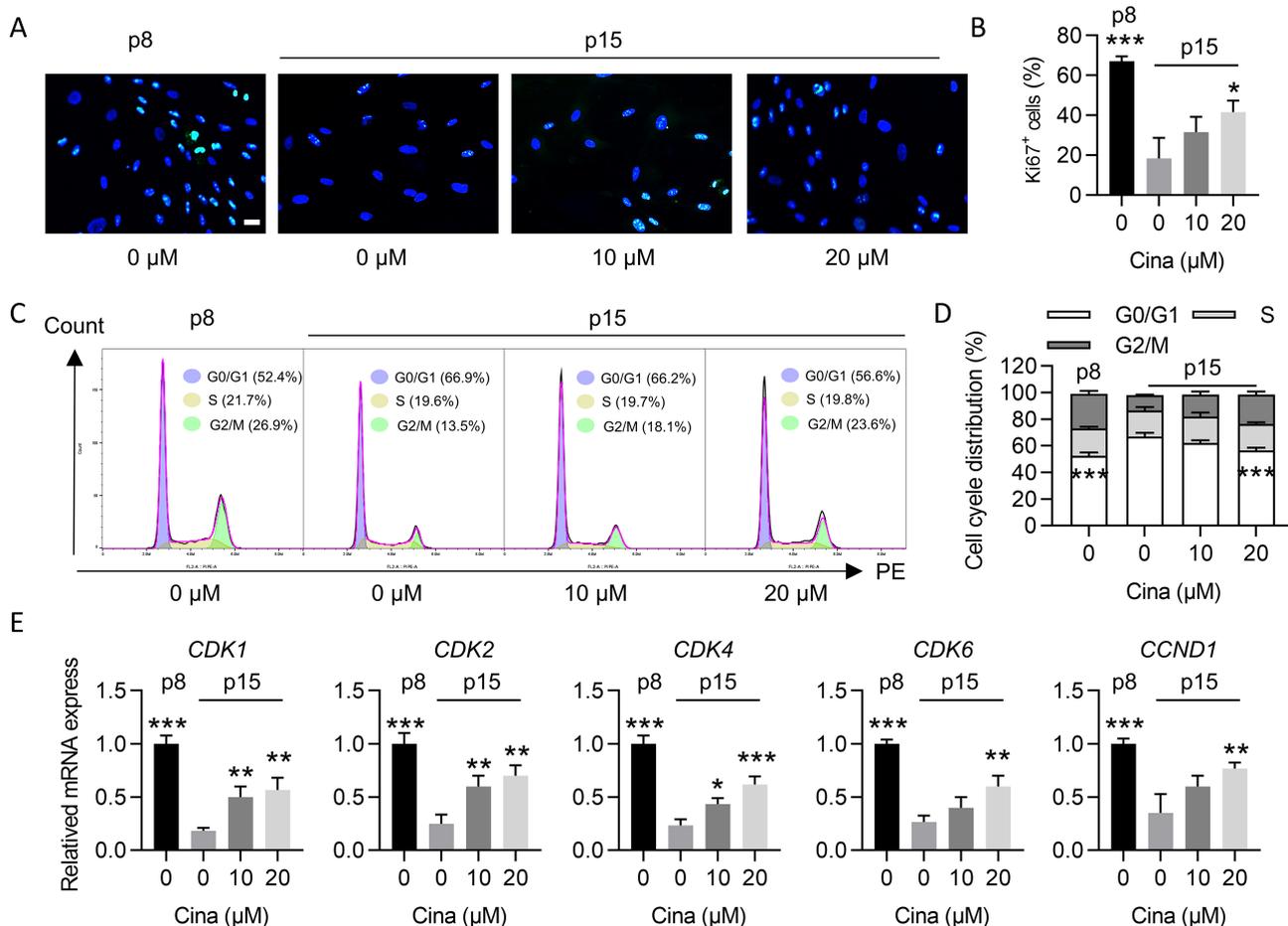


Fig. 3. Cina reverses passage replication-induced cell cycle arrest in SHEDs. (A,B) The impact of Cina on Ki67 expression was detected using immunofluorescence analysis. $n = 6$. Scale bars = 100 μm . (C,D) The effect of Cina on the cell cycle of senescent SHEDs was analyzed using flow cytometry, $n = 3$. (E) The mRNA levels of cell cycle-related markers were assessed using RT-PCR, $n = 3$. The data were presented as the means \pm SDs; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to SHEDs at P15 in the 0 μM Cina treatment group.

Cina Enhances the Pluripotency of MSCs and Inhibits Senescence-Associated Gene Expression

Transcriptome sequencing of SHEDs treated with Cina for 15 generations revealed that Cina exerts a regulatory impact on various genes in stem cells (Fig. 4A). Furthermore, gene enrichment analysis indicated that Cina significantly upregulated genes related to stem cell pluripotency but significantly downregulated multiple genes associated with the P53 pathway and cell senescence (Fig. 4B,C). Interestingly, Gene Set Enrichment Analysis (GSEA) revealed that Cina significantly downregulated the RAS-associated protein 1 (RAP1) signaling pathway, which is associated with telomere length (Fig. 4D). Finally, assessing molecular markers of senescence demonstrated that Cina significantly decreased the levels of senescence-associated markers such as P53 and cyclin-dependent kinase inhibitor 1A (CDKN1A), and increased the levels of the cycle-related proteins including CDK1, CDK2, and CCND1 (Fig. 4E).

Cina Maintains the Pluripotency of MSCs and Promotes Osteogenic and Adipogenic Differentiation

Transcriptome data indicated that Cina maintains pluripotency in SHEDs (Fig. 4B). Therefore, we examined the impact of Cina on the osteogenic and adipogenic differentiation of SHEDs. After long-term induction of differentiation, we observed that compared to young SHED cells, P15 SHED cells exhibited significantly reduced osteogenic and adipogenic differentiation capacities ($p < 0.05$, Fig. 5A,B,D,E). Additionally, Cina treatment significantly enhanced osteogenic and adipogenic differentiation compared to senescent SHEDs ($p < 0.05$, Fig. 5A,B,D,E). Moreover, we examined the impact of Cina on osteogenic and adipogenic differentiation-related transcription in SHEDs, indicating that Cina significantly increased osteogenic differentiation-related bone morphogenetic protein 4 (*BMP4*), runt-related transcription factor 2 (*RUNX2*), and secreted phosphoprotein 1 (*SPPI1*) mRNA levels, and adipogenic differentiation-related Aquaporin 1

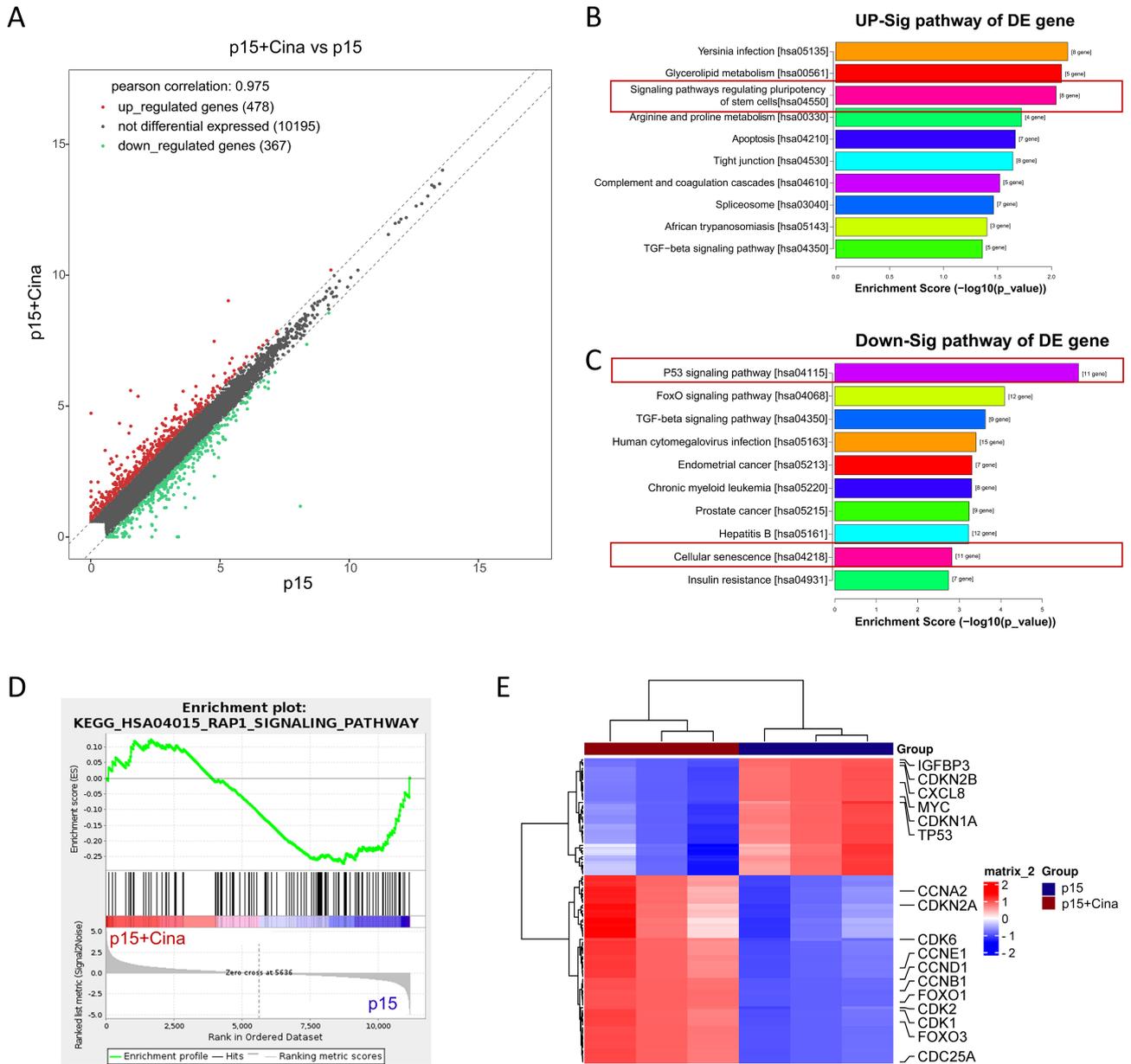


Fig. 4. Cina maintains stem cell pluripotency and downregulates senescence-associated signaling pathways. (A) Volcano diagram of the effect of Cina on the transcriptome of SHED-derived cells. (B,C) Functional enrichment of differentially expressed genes. (D) GSEA of Cina in SHEDs. (E) Differential genes associated with senescence according to the transcriptome data. GSEA, Gene Set Enrichment Analysis.

(*AQP1*), Early B-cell factor 2 (*EBF2*), and Adiponectin (*AdipoQ*) mRNA levels compared to senescent SHEDs ($p < 0.05$, Fig. 5C,F).

Cina Maintains Mitochondrial Homeostasis and Promotes Mitochondrial Energy Metabolism

Furthermore, we examined the impact of Cina on mitochondrial function. Our study revealed that after SHEDs senescence, the mitochondrial membrane potential and ATP production decreased, and redox imbalance increased compared to p8 SHEDs ($p < 0.05$, Fig. 6A–C). Furthermore, when compared to senescence SHEDs, Cina significantly

reversed the membrane potential damage induced by senescence, restoring redox equilibrium and ATP production ($p < 0.05$, Fig. 6A–C). Moreover, the results of Seahorse mitochondrial energy metabolism demonstrated that Cina significantly reversed the decrease in mitochondrial function induced by senescence, maintaining SHED mitochondrial homeostasis and energy production ($p < 0.01$, Fig. 6D–H).

Discussion

The senescence phenotype results from cellular senescence due to failures in intracellular signaling homeosta-

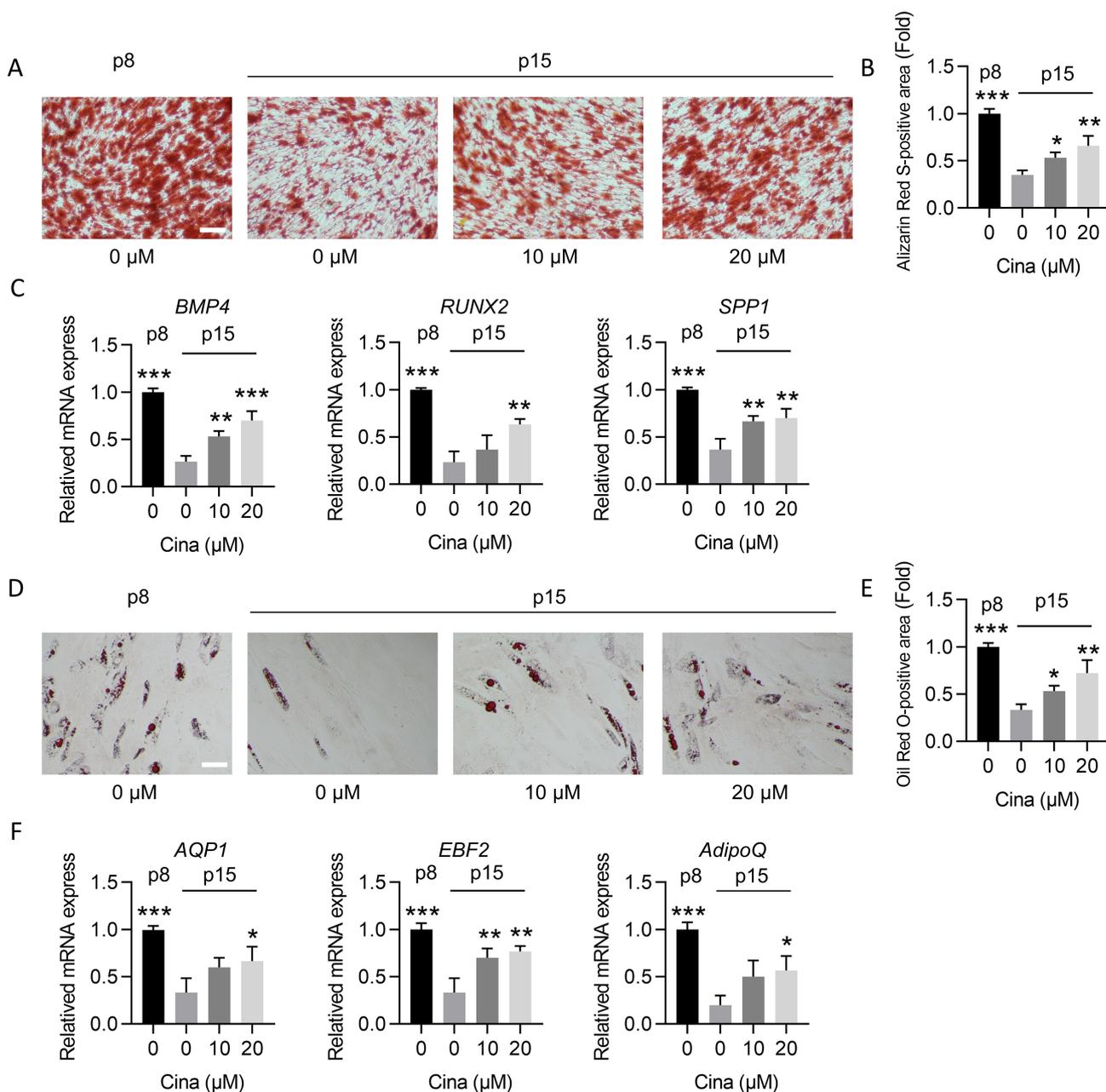


Fig. 5. Cina promotes the osteogenic and adipogenic differentiation of SHEDs. (A,B) Alizarin red S staining was used to examine the impact of Cina on the osteogenic differentiation ability of SHEDs, $n = 3$. Scale bars = 100 μm . (C) RT-PCR was used to assess the expression levels of osteogenic differentiation-related mRNAs, $n = 3$. (D,E) Oil red O staining was used to evaluate the impact of Cina on the adipogenic differentiation ability of SHEDs, $n = 3$. Scale bars = 100 μm . (F) RT-PCR was used to assess the mRNA expression levels of genes related to adipogenic differentiation, $n = 3$. The data were presented as the means \pm SDs; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to SHEDs at P15 in the 0 μM Cina treatment group.

sis. Cell senescence and stem cell depletion are the core mechanisms of aging, increasing tissue senescence and decreasing the regenerative potential of stem cells, representing major features of aging [3]. Therefore, the regeneration of stem cells can reverse age-related phenotypes [5]. MSCs are progenitors of mesodermal origin that have a fibroblast-like morphology, express a specific set of surface CD markers and are capable of differentiating into bone

cells, adipocytes, and chondrocytes [4]. Since Friedenstein's discovery of MSCs in 1970, scientists have investigated various activities associated with their immunomodulatory features and therapeutic uses [19]. Furthermore, numerous studies have elucidated the efficacy of MSCs *in vitro* and *in vivo*, aiming to develop approaches to prevent senescence in MSCs. Overcoming senescence of MSCs has become a critical concern of research. In this study,

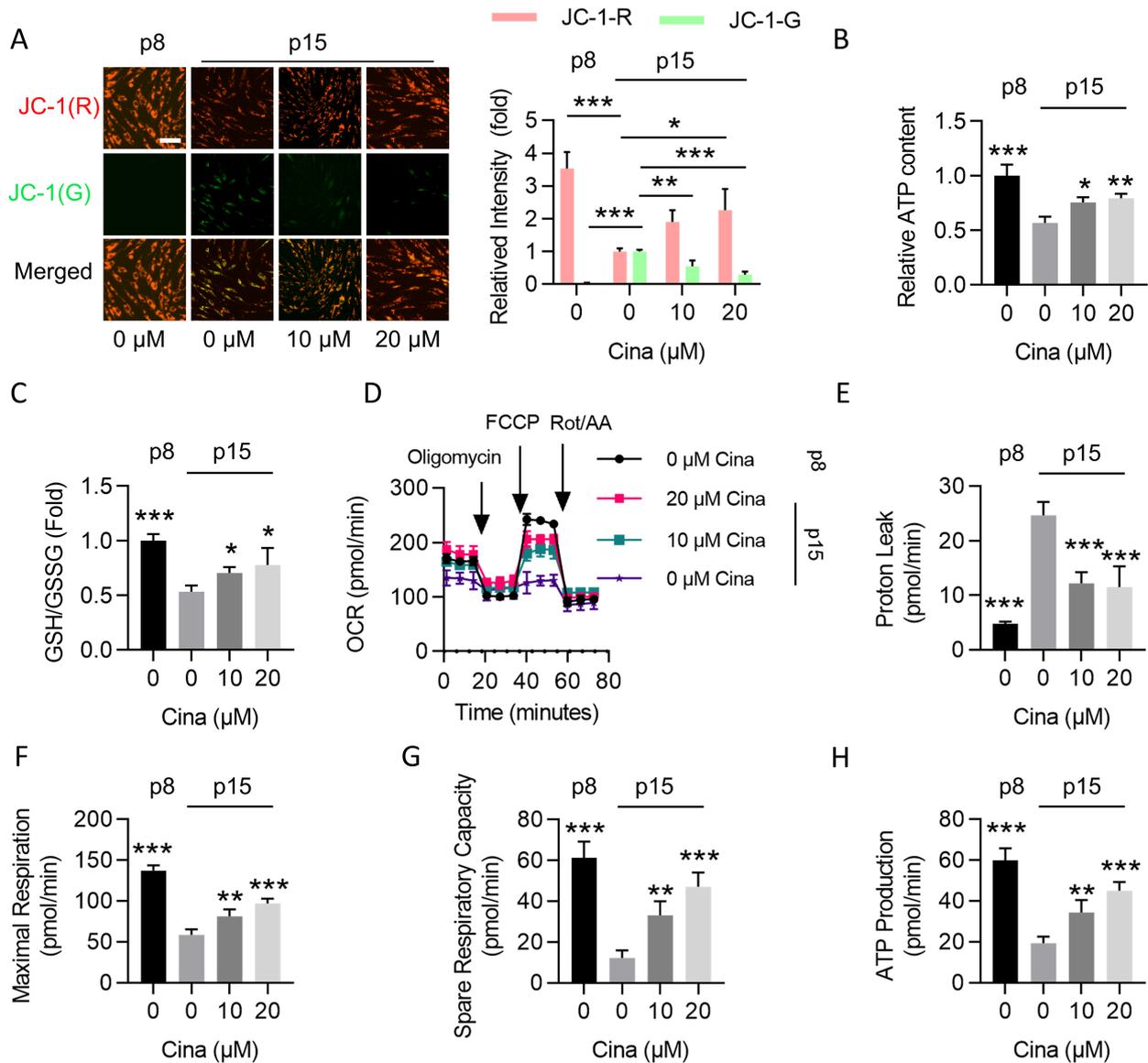


Fig. 6. Cina maintains mitochondrial homeostasis. (A) 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) staining was used to examine the impact of Cina on the membrane potential of aged SHEDs. Scale bars = 50 μ m. (B) Impact of Cina on ATP production in aged SHEDs, $n = 3$. (C) Impact of Cina on glutathione/oxidized glutathione (GSH/GSSG) ratio in aged SHEDs, $n = 3$. (D–H) Seahorse energy metabolism was used to assess the impact of Cina on mitochondrial respiration in senescence SHEDs, $n = 6$. The data were presented as the means \pm SDs; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to SHEDs at P15 in the 0 μ M Cina treatment group. ATP, Adenosine triphosphate.

we found that Cina significantly promotes proliferation of MSCs. Furthermore, continuous exposure of MSCs to Cina maintains good proliferation activity, reduces levels of senescence markers such as *P16*, *P21*, *IL-6*, and *IL-8*, and significantly delays senescence of MSCs. Ki67 serves as an indicator of cellular proliferation potential. Our study revealed that Cina substantially increases the number of Ki67-positive cells and reverses cell cycle arrest caused by senescence. This effect may be related to increased transcription of cycle-associated proteins, including CDK1, CDK2, CDK4, CDK6, and CCND1.

Studies have indicated that human mesenchymal progenitor cell senescence is often associated with genomic instability [20,21]. Human MSC senescence can be delayed by enhancing the stability of heterochromatin, suggesting that regulating chromatin stability could be a strategy for stem cell regeneration [22,23]. H3K9me3 is a common histone modification associated with heterochromatin formation and can be used as a marker of heterochromatin stability [24]. Similarly, our study revealed that the physiological senescence process of MSCs is accompanied by a decrease in H3K9me3, and supplementation with Cina significantly

restores the expression of H3K9me3. This finding suggests that Cina increases the stability of heterochromatin. Furthermore, the transcriptomic analysis revealed that Cina downregulates several signaling pathways associated with senescence. Studies have shown that P53 is the primary factor controlling cell senescence, with increased P53 being a cause of cell senescence [25–27]. As a major component of the DNA damage response (DDR) pathway, the P53 tumor suppressor protein also plays a pivotal role in regulating the cell cycle. The accumulation of phosphorylated P53 stimulates the activation of cyclin-dependent kinase inhibitors, ultimately leading to cell cycle arrest [28]. Our study indicated that Cina significantly downregulates the P53 signaling pathway, which may be linked to Cina's enhancement of genomic stability through H3K9me3 promotion. The RAP1 signaling pathway is closely associated with telomere length. Research has demonstrated that RAP1-deficient MSCs exhibit a delayed senescence process compared to the genotypic controls [29], which is consistent with our experimental results. Similarly, our study revealed that Cina significantly downregulates the RAP1 signaling pathway. Furthermore, transcriptome analysis suggested that Cina significantly elevates the expression of multiple genes associated with stem cell pluripotency. Therefore, we assessed the ability of stem cells to differentiate and found that Cina substantially enhances the osteogenic and lipogenic differentiation ability of MSCs, accompanied by increased transcription of osteogenic and lipogenic factors.

In 2013, López-Otín *C et al.* [3] published a review on senescence markers in cells, describing nine common characteristics of senescence across various species. In addition to extensively studied factors of stem cell depletion, telomere wear, cell senescence, and insulin-like growth factor/mTOR/AMPK/sirtuin signaling inactivation, the feature of “mitochondrial dysfunction” has also been proposed. As cellular engines, mitochondria undergo tricarboxylic acid cycle (TCA) reactions under aerobic conditions as well as oxidative phosphorylation to break down nutrients into carbon dioxide, water, and ATP. TCA, the most common metabolic pathway in aerobic organisms, is an essential hub of material and energy metabolism. Many studies have shown that TCA-circulating substrates can affect ATP production and cell fate and have various biological functions, such as lipid and protein synthesis, epigenetic regulation, support for stem cell and immune cell functions, adaptation to oxygen changes, inflammation regulation, and other essential roles [30–32]. With increasing research, many researchers have recognized that the TCA cycle is a critical target for intervention in senescence [33]. Our results showed that Cina can maintain normal ATP production by maintaining mitochondrial redox homeostasis and retard senescence by maintaining heterochromatin homeostasis and mitochondrial homeostasis.

Conclusions

In summary, our study revealed the role of Cina in delaying the senescence of MSCs. Cina promotes the proliferation of MSCs, maintains their proliferation activity, and maintains their pluripotency. During this process, Cina maintains the homeostasis of MSCs by maintaining heterochromatin stability and mitochondrial homeostasis, thereby delaying senescence. Cina, a naturally active small molecule with low cost, strong pharmacological activity, and low toxicity, holds promise as a potential anti-aging drug to help delay aging and treat age-related diseases.

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting the first author if needed.

Author Contributions

FS, JML and HRH: designed the study. DLH and CL: performed the research. PQW, RX, and QS: formal analysis and data curation. JML: drafted this manuscript. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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