

Resveratrol attenuates senescence of adipose-derived mesenchymal stem cells and restores their paracrine effects on promoting insulin secretion of INS-1 cells through Pim-1

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Abstract. – OBJECTIVE: The paracrine effects of mesenchymal stem cells (MSCs) were weakened during aging. This study explored whether resveratrol can attenuate senescence of adipose-derived MSCs (ADMSCs) and whether Pim-1 is involved in resveratrol's effect on paracrine of ADMSCs and insulin secretion of INS-1 cells.

MATERIALS AND METHODS: CCK-8 assay and SA- β -gal assay were performed to test the protective effect of resveratrol on senescent models. QRT-PCR and western blot analysis were performed to analyze of senescence- and β -cell associated genes. QRT-PCR and ELISA analysis was performed to test telomere length and activity. Immunofluorescence and ELISA assay were performed to assess the paracrine effects on promoting insulin secretion of INS-1 cells.

RESULTS: Resveratrol could protect ADMSCs from H₂O₂ and D-glucose-induced senescence and also attenuate senescence in long-term cultured ADMSCs *in vitro*. In addition, resveratrol attenuated H₂O₂ induced higher expression of senescence-associated genes, including P53, P21, Cyclin D1, IL-6 and MMP1, but increased the expression of Sirt1, a well-known anti-senescence gene. Resveratrol significantly enhanced Pim-1 expression in aging ADMSCs through PI3K/AKT signal pathway. The conditioned medium (CM) of ADMSCs enhanced insulin secretion and expression of the key genes for β -cell function including TFAM, PDX1, GLUT2 and HNF-1 α via Pim-1. INS-1 cells with Pim-1 knock-down had decreased insulin secretion.

CONCLUSIONS: This study firstly reported that resveratrol has a protective effect on senescence of ADMSCs and can preserve the paracrine effect of the ADMSCs on promoting insulin secretion of INS-1 cells via Pim-1.

Therefore, it might be a promising adjuvant agent for future MSCs based therapy.

Key Words:

ADMSCs, Resveratrol, Senescence, Insulin.

Introduction

Type 1 diabetes is a chronic disease characterized by destruction of pancreatic islet β -cells and insufficient insulin secretion. Insulin replacement is still the main therapy for the patients¹. During the past years, transplantation of mesenchymal stem cells (MSCs) was tested as a strategy to improve the metabolic profiles in diabetic animal models². The transplanted MSCs can transdifferentiate into insulin-producing cells (IPCs) and exert a therapeutic effect. For example, human adipose derived MSCs (hADMSCs) can be used to generate insulin-secreting surrogate β -cells and show certain effect on controlling of hyperglycemia^{2,3}. In addition, the paracrine effect of the MSCs was also reported as a key mechanism contributing to the therapeutic effect. MSCs naturally secrete multiple cytokines and growth factors, promoting the survival of the surrounding cells. The cytokines and the growth factors, such as IL-6, M-CSF, IL-10, HGF, TGF- β and PGE2 contribute to preventing apoptosis of injured pancreatic β -cells and enhancing regeneration of endogenous progenitor cells via angiogenic, cytoprotective, anti-inflammatory, mitogenic and anti-apoptotic effects³⁻⁵.

However, the paracrine effects of MSCs were weakened during aging, thereby reduce their therapeutic effect. Therefore, it is quite necessary to explore how senescence of the MSCs can be alleviated. Resveratrol (3, 5, 4'-trihydroxystilbene) is a natural polyphenolic compound found in grape skins and various other plants^{6,7}. Previous studies⁸⁻¹¹ found that resveratrol has various health-benefiting effects, such as anti-inflammatory, anti-oxidative, anti-cancer, anti-aging and anti-senescence effects. Some recent studies showed that resveratrol can protect human endothelium from H₂O₂-induced oxidative stress and senescence via Sirt1 activation¹² and prevent oxidative stress-induced senescence in cultured primary human keratinocytes via activating AMPK-FOXO3 cascade¹⁰. Pim-1 has been recognized as a gene with pro-survival function and anti-apoptosis effect in MSCs^{13,14}.

In this study, we investigated whether resveratrol can attenuate senescence of ADMSCs and whether Pim-1 is involved in resveratrol's effect on paracrine of ADMSCs and insulin secretion of INS-1 cells.

Materials and Methods

Cell Culture

Rat insulinoma cell (INS-1) was purchased from Biohermes (Shanghai, China) and grown in RPMI 1640 medium supplemented with 10% FBS, 5.6 mM glucose, 25 mM HEPES, 2 mM L-glutamine, 50 μ M β -mercaptoethanol, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human ADMSCs were purchased from Invitrogen (R7788-110, Carlsbad, CA, USA) and cultured using the prepackaged MesenPRO RS™ Medium. All cells were cultured in an incubator with a humidified atmosphere and 5% CO₂ at 37°C. ADMSCs in passages 2-20 were used for experiments in this study, as described throughout the text. To collect condition medium (CM) from ADMSCs, 1 \times 10⁶ cells at the indicating passage were cultivated in 6-well plates with serum-free essential medium. 24 h after cultivation, CM were collected.

Cell Senescent Models

Two models of senescent ADMSCs including reagent-induced senescent ADMSCs and natural senescent ADMSCs were used in this study. To generate reagent-induced senescent ADMSCs, 1.5 \times 10³ passage 4 AMDSCs were treated with 25 μ M H₂O₂ or 5 mg/mL D-glucose.

Cell Transfection

Three candidate si-Pim-1 sequences were synthesized and purchased from RiBoBio (Shanghai, China). ADMSCs at indicating passage or INS-1 cells were transfected with 100 nM Pim-1 siRNAs using Hiperfect transfection reagent (QIAGEN, GmbH, Hilden, Germany) according to manufacturer's instruction. The effect of Pim-1 knockdown was verified 48 h after transfection using qRT-PCR and Western blot analysis. The siRNA sequence with the highest suppressing effect was used for following studies.

ADMSCs Preparation for Cell Viability Assay

To examine the protective effect of resveratrol on the viability of reagent induced senescent ADMSCs, passage 4 AMDSCs were treated with 25 μ M H₂O₂ or 5 mg/mL D-glucose alone or co-administrated with 50 μ M resveratrol up to 72 h. To examine the protective effect of resveratrol on the viability of natural senescent ADMSCs, ADMSCs at indicating passage were treated with 50 μ M resveratrol for 48 h.

ADMSCs Preparation for SA- β -gal Assay, qRT-PCR, ELISA Assay and Western blot Analysis

PI3K inhibitor LY294002 was purchased from EnoGene (New York, NY, USA). To prepare reagent-induced senescent ADMSCs for these assays, passage 4 AMDSCs with or without Pim-1 knockdown were treated with 25 μ M H₂O₂ or 5 mg/mL D-glucose alone or co-administrated with 50 μ M resveratrol with or without the presence of 20 μ M LY294002 for 48 h. To prepare natural senescent ADMSCs for these assays, ADMSCs at indicating passage (P2, P10, P15 and P20) were treated with 50 μ M resveratrol alone with co-administrated with 20 μ M LY294002 for 48 h. P15 or P20 ADMSCs with or without Pim-1 knockdown were treated with 50 μ M resveratrol alone with co-administrated with 20 μ M LY294002 for 48 h. Then, the cells were subjected to SA- β -gal assay, qRT-PCR and western blot analysis.

INS-1 Cell Treatment

INS-1 cells were treated with CMs collected from AMDSCs at indicating passage and with indicating treatment for 48 h. INS-1 cells treated with CMs from P20 ADMSCs with or without Pim-1 knockdown were additionally treated with 50 μ M resveratrol. Then, these cells were sub-

jected to immunofluorescence assay and ELISA assay of insulin secretion, as well as qRT-PCR analysis of β -cell associated genes. INS-1 cells with Pim-1 knockdown were treated with 50 μ M resveratrol for 48 h. Then these cells were subjected to ELISA assay of insulin secretion.

Cell Viability and SA- β -gal Assay

Cell viability at indicating time point was determined using WST-8 assay using Cell Counting Kit-8 (CCK-8; Dojindo, Gaithersburg, MD, USA) according to manufacturer's instruction. Cell senescence was measured by detection of SA- β -gal activity using the Senescence Detection Kit (ab65351, Abcam, Cambridge, MA, USA) according to manufacturer's protocol.

QRT-PCR Analysis of Senescence- and β -cell Associated Genes and Telomere length

Total RNAs of the cells were extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The first strand cDNA was reversely transcribed us-

ing the RevertAid first strand cDNA synthesis kit (Fermentas, Ontario, Canada) according to manufacturer's instructions. The expression of senescent related genes and β -islet cells associated genes were detected by using the gene-specific primers (Table I) and SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). GAPDH was used as the endogenous control gene. All qRT-PCR analysis was performed using an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA).

DNA of the ADMSCs was extracted by using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). The relative length of telomere of the ADMSCs at the indicated passage was measured using qRT-PCR, according to Cawthon's method¹⁵. This method measures the ratio of telomere repeat copy (T) and a single gene copy-albumin (S) in experimental DNA samples and reference DNA samples separately, assuming that both genes have similar amplification efficiency. The relative T/S ratios are proportional to average telomere length. Primers for telomere and albumin followed the author's recommendation¹⁵.

Table I. Primer Sequences for qRT-PCR.

Gene	Primer Sequences
SIRT1	F: 5'-CAGACCCTCAAGCCATGTTT-3' R: 5'-GATCCTTTGGATTCTTGAAA-3'
P53	F: 5'-GTTCCGAGAGCTGAATGAGG-3' R: 5'-TCTGAGTCAGGCCCTTCTGT-3'
P21	F: 5'-AGGCACCGAGGCACTCAGAG-3' R: 5'-AGTGGTAGAAATCTGTCATGCTG-3'
Cyclin D1	F: 5'-AAGGAGATTGTGCCATCCATGC-3' R: 5'-CGGCTCTTCTTCAGGGGCTCC-3'
IL-6	F: 5'-GGCACTGGCAGAAAACAACC-3' R: 5'-GCAAGTCTCCTCATTGAATCC-3'
PIM1	F: 5'-CAACAGTGGGGAAGAGTGAC-3' R: 5'-TGCTGGACACCTATGAGAAGAA-3'
TFAM	F: 5'-GCGTCCCCCTTCAGTTTTG-3' R: 5'-GTTTTGTCATCTGGGTTCTGAGC-3'
GLUT2	F: 5'-ATTGCTCCAACCGCTCTCA-3' R: 5'-CTGAAGGATGGCTCGCACACC-3'
PDX-1	F: 5'-GGCTTAACCTAAACGCCACA-3' R: 5'-AGAGTCCCAGAGGCAGACCT-3'
HNF-1 α	F: 5'-TTTTTCAAGCAGTAGGAATTGGA-3' R: 5'-GTGATGTAGTAGCTGCATGATCG-3'

Note: TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; BNP: brain sodium peptide; Group A: anterior/high lateral wall myocardial infarction; Group B: inferior/posterior wall myocardial infarction; Group C: right ventricle myocardial infarction; Group D: control group.

ELISA Analysis of Telomere Activity and Insulin Expression

Telomerase activity in cell lysates was determined using the TeloTAGGG Telomerase PCR ELISA (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. Briefly, 1×10^4 cells were used per measurement and absorbance at 450 nm were normalized to a standard cell lysate supplied with the kit. For INS-1 assays, after indicating treatment, the medium was removed and cells were washed three times with warm Krebs buffer. Then, the cells were incubated with Krebs solution for one hour at 37°C and, at the end of incubation, cells were stimulated with Krebs solution supplemented with a 20 mM glucose for 30 min at 37°C. Then, the supernatants were collected and insulin secretion was measured using a commercially available human insulin ELISA kit (Merckodia AB, Uppsala, Sweden), according to manufacturer's specifications.

Western Blot Analysis

Cells were lysed using a lysis buffer (Beyotime, Shanghai, China) and protein concentrations of the lysates were measured by BCA protein assay kit (Beyotime, Shanghai, China). The lysates were separated on 10% SDS-PAGE and then transferred to a PVDF membrane. After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies (anti-Pim-1, 1:1000, ab117110; anti-AKT, 1:2000, ab185633; anti-phosphorylated AKT, ab18206, 1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C. Membranes were washed and incubated with corresponding HRP-labeled secondary antibodies. The blot signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA). The signal intensity was quantified using ImageQuant 5.2 (GE Healthcare, Piscataway, NJ, USA). To clearly demonstrate the difference, the relative gray-scale value of target protein vs. GAPDH of the control group was set as 1.

Immunofluorescence Assay

INS-1 cells after indicating treatments were fixed for 20 min in fresh 4% paraformaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) and washed three times with phosphate buffered saline (PBS). Then the cells were permeabilized using PBS containing 0.5% Triton-X 100 (Sigma-Aldrich) for 1 hour and blocked using PBS supplemented with 4% bovine serum albumin

(BSA) and 0.3% Triton-X 100 for another hour. Cells were incubated with anti-insulin (1:100, ab7842, Abcam) over night at 4°C. Cells were then washed three times with PBS and incubated with secondary antibodies for 1 hour at room temperature. After washing, nuclei were stained in PBS containing 1 µg/ml Hoechst 33342 (Sigma-Aldrich). Digital images were obtained using an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Data analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were represented as mean ± standard deviation (SD). Group difference was assessed using Student's *t*-test. $p < 0.05$ was considered as statistically significant. * and # indicate $p < 0.05$, while ** and ## indicate $p < 0.01$.

Results

Resveratrol Protects ADMSCs from H₂O₂ and D-glucose Induced Senescence *in vitro*

By performing CCK-8 analysis of cell proliferation, we found that resveratrol substantially counteracted the H₂O₂ (Figure 1A) or D-glucose (Figure 1D) induced suppression of cell proliferation. In addition, co-administration with resveratrol also reduced the ratio of β-gal positive cells induced by H₂O₂ (Figure 1B) or D-glucose (Figure 1E), suggesting a potential protective effect of resveratrol on senescence. To continue to study the effects of resveratrol on senescence, the expression of senescence-associated genes were detected using qRT-PCR. Results showed that H₂O₂ (Figure 1C) or D-glucose (Figure 1F) significantly decreased Sirt1 expression, but increased the expression of P53, P21, Cyclin D1 and IL-6, the key genes modulating apoptosis¹⁶. Supplementation of resveratrol significantly weakened these effects (Figure 1C and F). These results suggest that resveratrol protects ADMSCs from H₂O₂ and D-glucose induced senescence *in vitro*.

Resveratrol Exerts Anti-senescence Effects on long-term cultured ADMSCs *in vitro*

Since resveratrol has protective effects on H₂O₂ and D-glucose induced senescence, we further explored whether it has anti-senescence effects on aging cells. With the increasing of passage num-

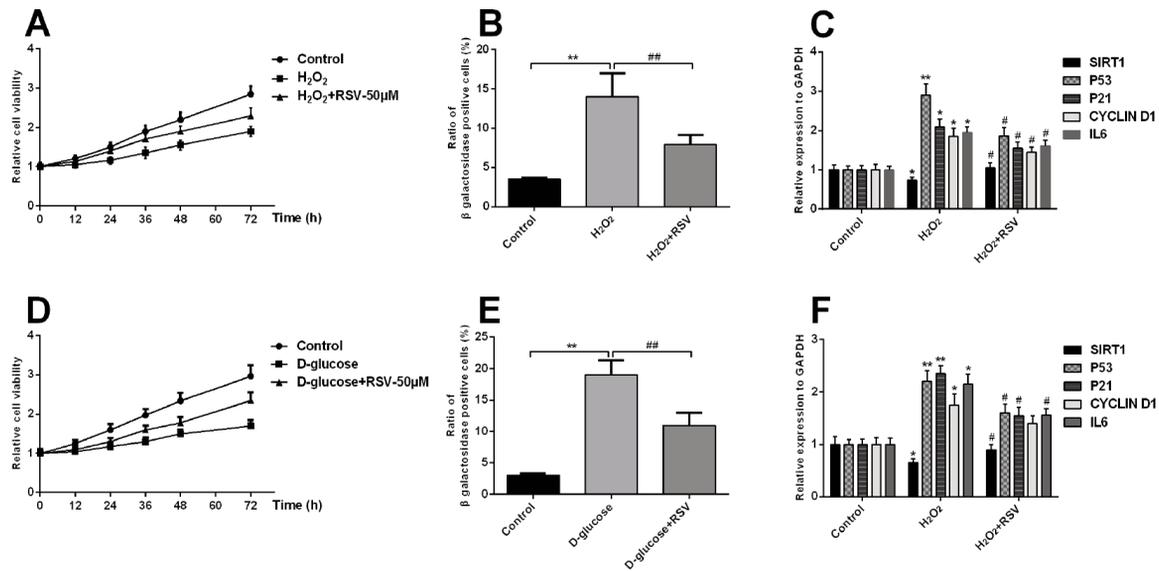


Figure 1. Resveratrol protects ADMSCs from H₂O₂ and D-glucose induced senescence *in vitro*. **(A-D)** CCK-8 analysis was performed to measure the cell viability of ADMSCs treated with H₂O₂ **(A)** and D-glucose **(D)** or co-administrated with 50 μM resveratrol for 72 h. **(B-E)** 25 μM H₂O₂ **(B)** and 5 g/L D-glucose **(E)** induced senescent cells for 48 h were evaluated by the number of SA-β-gal-positive cells in randomized three microscopic field. **(C-F)** Senescence-associated genes expression levels of ADMSCs were detected using Quantitative RT-PCR method. Values are means ± SD of 3 independent experiments with each performed in triplicate. **p*<0.05, ***p*<0.01 vs. control, #*p*<0.05, ##*p*<0.01 vs. H₂O₂ or D-glucose group.

ber, the ADMSCs showed a lower proliferation rate (Figure 2A) and a higher senescent rate (Figure 2B). Administration of resveratrol partly reversed these trends (Figure 2A and B). Then, we examined the telomere length and activity, two indicators of senescence. QRT-PCR showed that the telomere length (Figure 2C) and activity (Figure 2D) decreased with the increasing of passage number. Administration of resveratrol significantly slowed these processes (Figure 2C and D). Then, we quantified the change of senescence-associated genes in the ADMSCs with different passage numbers and how resveratrol affected the changes. With the increasing of passage number, the cells had decreased Sirt1 expression and increased expression of P53, P21, Cyclin D1, IL-6 and MMP1 (Figure 2E). Although supplementation of resveratrol could not reverse these changes, it slowed them (Figure 2E).

Resveratrol Reverses the Downregulation of Pim-1 Expression at Both mRNA and Protein Level Through PI3K/AKT Signal pathway.

Since resveratrol exerts a strong anti-senescence effect on ADMSCs, we decided to study the underlying mechanism. Pim-1 is a gene mainly involved in cell cycle progression and apopto-

sis. One recent study found senescent characteristics, such as decrease in proliferation, telomere length, survival, and increased expression of senescent markers of human cardiac progenitor cells (hCPCs) are ameliorated by Pim-1 kinase¹⁷. In this study we observed that resveratrol significantly promoted Pim-1 expression at both mRNA and protein levels in aging ADMSCs, in conjunction with increased p-AKT and unchanged AKT (Figure 3A and B). However, this effect was largely abrogated by LY294002, a PI3K inhibitor. LY294002 significantly reduced p-AKT and Pim-1 expression (Figure 3A and B). Similarly, in H₂O₂ (Figure 3 C and D) and D-glucose (Figure 3 E and F) induced senescent models, resveratrol partly restored Pim-1 expression, with increased p-AKT and unchanged AKT. LY294002 significantly decreased the effect of resveratrol.

The Anti-Senescence Function of Resveratrol can be Partially Abrogated by Knockdown of Endogenous Pim-1

Through screening of three candidate Pim-1 siRNA in passage 4 ADMSCs, we identified two siRNAs with relatively high suppressing effect (Figure 4 A and B). Knockdown of endogenous Pim-1, similar as LY294002 substantially abrogated resveratrol's effect on reducing H₂O₂ (Fig-

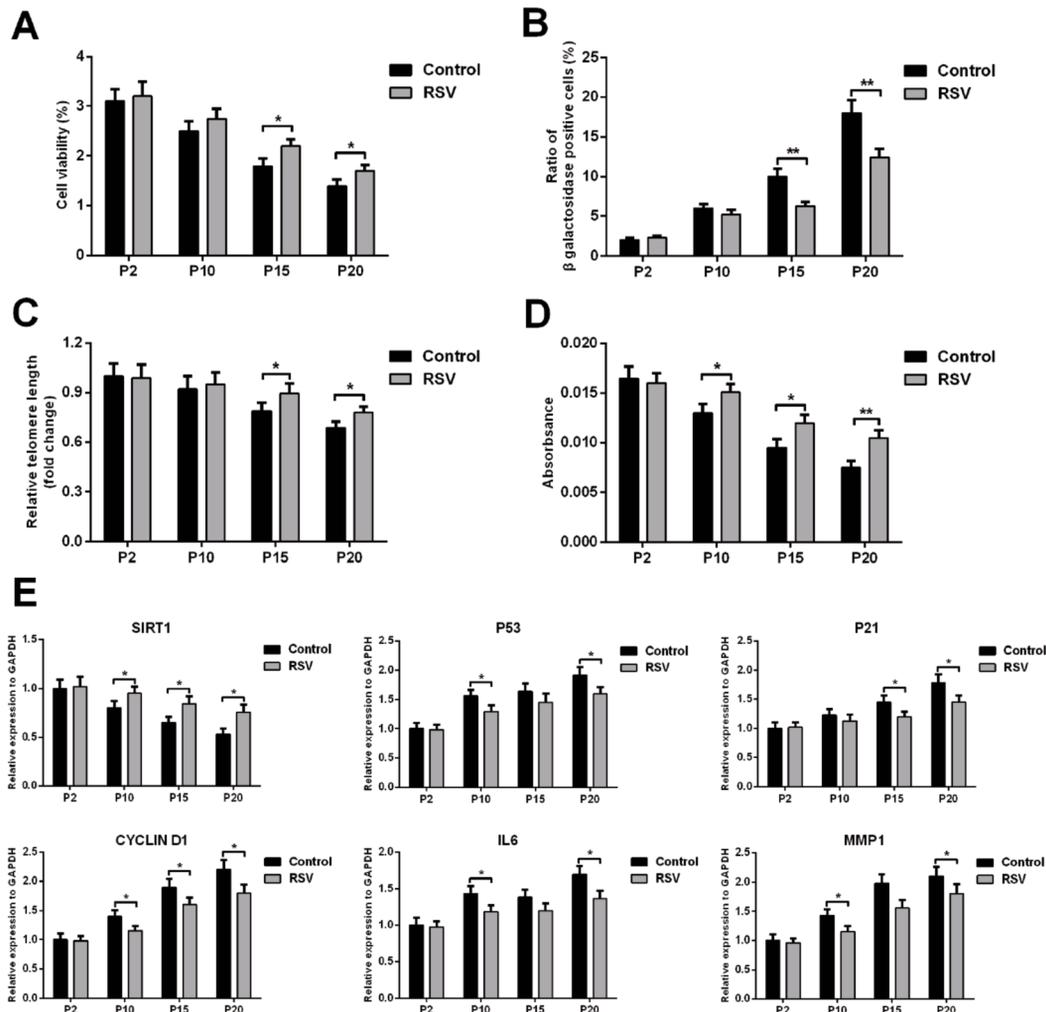


Figure 2. Resveratrol exerts anti-senescence effects in long term cultured ADMSCs *in vitro*. Serial parameters were compared between control and resveratrol treated cells after 48h in different passages (P2, P5, P10 and P20). **(A)** Cell viability was measured by CCK-8 analysis. **(B)** The number of SA-β-gal-positive cells in randomized three microscopic fields was counted. **(C)** Telomere length measurement was performed by qRT-PCR methods. **(D)** Telomerase activity was measured using ELISA methods. **(E)** Quantitative RT-PCR analysis was used to determine senescence-associated genes expression levels of ADMSCs. Values are means ± SD of 3 independent experiments with each performed in triplicate. * $p < 0.05$, ** $p < 0.01$. P: passage.

ure 4C) and D-glucose (Figure 4D) induced cell senescence. In the cells with relatively late passages (P15 and P20), knockdown of endogenous Pim-1, similar as LY294002, abrogated resveratrol's protective effect on senescence (Figure 4E). These results suggest that the anti-senescence effect of resveratrol can be partially abrogated by knockdown of endogenous Pim-1.

Resveratrol Restores the Paracrine Effects of Senescent ADMSCs and Promotes Insulin Secretion of INS-1 Cells Through Pim-1

The paracrine effect of ADMSCs is quite important in modulating microenvironment, which

indirectly affects the biological function of cells nearby. To explore how ADMSCs affect insulin secretion of islet cells, the CM of ADMSCs was used to treat INS-1 cells. Immunofluorescence analysis showed that the CM of P2 ADMSCs had substantiality stronger effect than that from P20 ADMSCs on promoting insulin secretion of INS-1 cells (Figure 5A). However, knockdown of Pim-1 substantially weakened the effect (Figure 5A). Resveratrol partly rescued the insulin inducing effect of the senescent ADMSCs (P20). But it had little effect on the senescent ADMSCs with Pim-1 knockdown (Figure 5A). Following ELISA assay also confirmed these tendencies

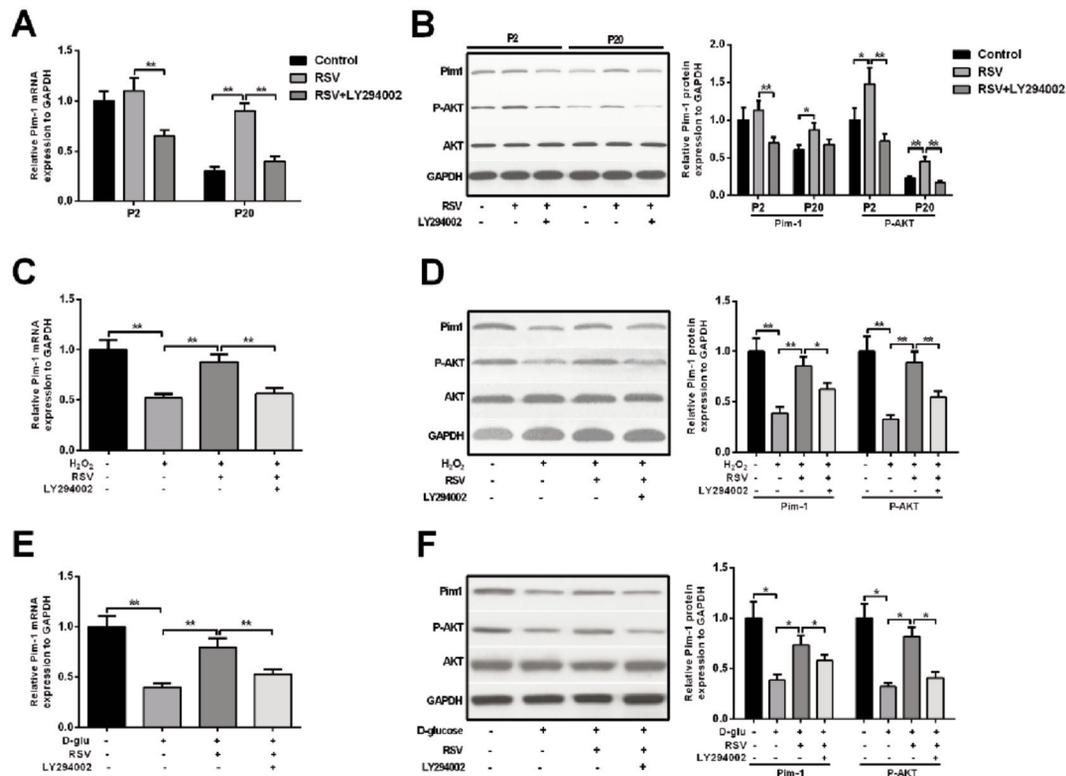


Figure 3. Resveratrol reverses the downregulation of Pim-1 expression both in mRNA and protein level through PI3K/AKT signal pathway. The effects of resveratrol and PI3K inhibitor, LY294002 on the expression of Pim-1 was evaluated using qRT-PCR and Western blot methods. **(A, C and E)** qRT-PCR analysis was performed to detect the mRNA levels of Pim-1 in three senescent models, long culture condition **(A)**, H₂O₂ **(C)** and D-glucose **(E)** induced models. Values are means \pm SD of 3 independent experiments with each performed in triplicate. **(B, D and F)** In three different senescent models, the protein levels of Pim-1, AKT and phosphorylated AKT were determined using Western blot method. Corresponding histograms were showed as calculations of relative fold change of Pim-1 and phosphorylated AKT to control group. The data was showed as representative of three independent experiments. * p <0.05, ** p <0.01.

(Figure 5B). Then, we detected β -islet cells associated genes expression of INS-1 cells treated with different CMs. QRT-PCR results showed that the CM of P2 ADMSCs up-regulated of key genes associated with β -cell function, including TFAM, PDX1, GLUT2 and HNF-1 α in INS-1 cells (Figure 5C). Knockdown of Pim-1 significantly abrogated the effects (Figure 5C). Although the CM of P20 ADMSCs had significantly weaker inducing effect, resveratrol treatment substantially rescued its inducing effect (Figure 5C). However, resveratrol had little influence on the CM of P20 ADMSCs with Pim-1knockdown (Figure 5C). These results suggest that the paracrine effects of the ADMSCs are weakened during senescence and is at least partly due to reduced Pim-1 expression. Since Pim-1 has an important role in modulating senescence, we further explored its involvement in insulin secretion of INS-1 cells. INS-1 were firstly transfected with

si-Pim-1 (Figure 5D). The cells with Pim-1 knockdown had significantly inhibited secretion of insulin, which was partly rescued by resveratrol (Figure 5E). These results suggested that resveratrol restores the paracrine effects of senescent ADMSCs and promotes insulin secretion of INS-1 cells through Pim-1

Discussion

Transplantation of MSC has been considered a promising regenerative strategy for several diseases such as heart diseases, ovarian damage, liver cirrhosis, stroke and diabetes mellitus¹⁸⁻²⁰. Although the MSCs can be transdifferentiated into insulin secreting cells, recent studies showed that the implantation of MSCs decreases glucose level mainly through paracrine effects rather than through direct transdifferentia-

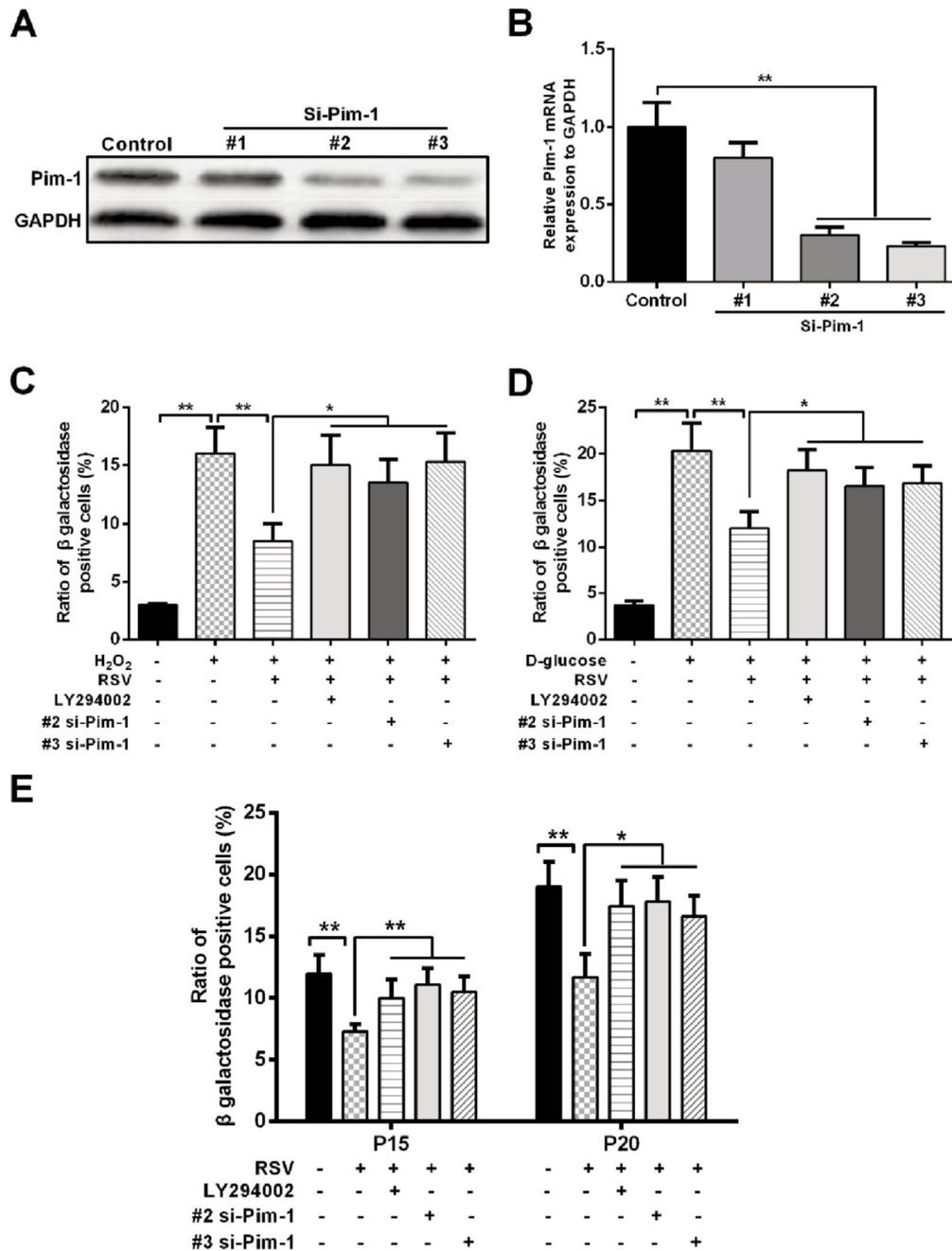


Figure 4. Anti-senescence function of resveratrol can be partially abrogated by knockdown of endogenous Pim-1. Upon inhibition of Pim-1 expression using siRNA method in ADMSCs, Anti-senescence function of resveratrol was evaluated in three senescence models. **(A)** Western blot analysis was used to check the inhibited expression of Pim-1 protein. **(B)** The quantitative results of figure A were showed in histogram form. **(C, D and E)** The number of SA-β-gal-positive cells was counted in three microscopic fields in random in order to evaluate the senescence in the three models including H₂O₂ **(C)**, D-glucose **(D)** and long-time culture condition **(E)**, respectively. Values are means ± SD of three independent experiments with each performed in triplicate. **p*<0.05, ***p*<0.01.

tion into insulin-producing cells⁵. Therefore, their paracrine effect directly and largely determines the therapeutic effect.

Senescence results in a series of changes of the

cells. One recent study explored the influence of senescence on the therapeutic potential of human MSCs in the lethal endotoxemia model. The authors found that senescence induces extensive

phenotypic changes in hMSCs and results in impaired migratory capacity in response to proinflammatory signals²¹. In addition, the senescent cells have inhibited AP-1 pathway, which mediates the response to a variety of extracellular stimuli and regulates differentiation, proliferation, and migration of the cells²¹. Another study observed that the ability of MSCs to repair damaged organs is severely compromised with advanced age²². Conditioned medium of senescent MSCs even contains a set of secreted factors that are able to induce a full senescence response in young cells²³. These results suggest that the paracrine effect of MSCs was impaired due to senescence. Therefore, to alleviate the negative impact of senescence is critical for therapeutic use of MSCs. In this study, we demonstrated that resveratrol could protect ADMSCs from H₂O₂ and D-glucose induced senescence and also attenuate senescence in long-term cultured ADMSCs *in vitro*. In addition, resveratrol also reduced the expression of senescence associated genes, including P53, P21, Cyclin D1, IL-6 and MMP1, but increased the expression of Sirt1, a well-known anti-senescence gene. Aging is a progressive degenerative process that is closely associated with inflammation. Senescent cells also develop a senescence-associated secretory phenotype (SASP), which is characterized as high level secretion of the cytokines,

IL-6 and IL-8, the key mediators of inflammation²⁴. Therefore, the resveratrol induced lower IL-6 expression might represent a rejuvenation of the senescent ADMSCs.

The anti-senescence effect of resveratrol in normal cells has been reported in recent studies. For example, one study finds resveratrol significantly abolished the oxidative effects of H₂O₂ on human endothelium cell line HUVEC via Sirt1 activation¹². In cultured primary human keratinocytes, resveratrol also can prevent oxidative stress-induced senescence and proliferative dysfunction by activating the AMPK-FOXO3 cascade¹⁰. In aging heart, resveratrol mitigates proapoptotic signaling through a deacetylation mechanism of Sirt1, thereby suppressing the Foxo1-Bim-associated pro-apoptotic signaling axis²⁵. Therefore, it seems that resveratrol exerts an anti-aging effect through multiple signaling pathways.

Some recent studies reported that Pim-1 kinase exerts a pro-survival function via preserving mitochondrial integrity¹³ and antagonizing intrinsic apoptotic cascades¹⁴. Another recent study finds that Pim-1 prevents MSC apoptosis induced by hypoxia or serum deprivation via enhancing mitochondria integrity¹⁴. Furthermore, Pim-1 preserves telomere length and telomerase activity, giving cells youthful cellular phenotype²⁶. These results suggest that Pim-1 is an im-

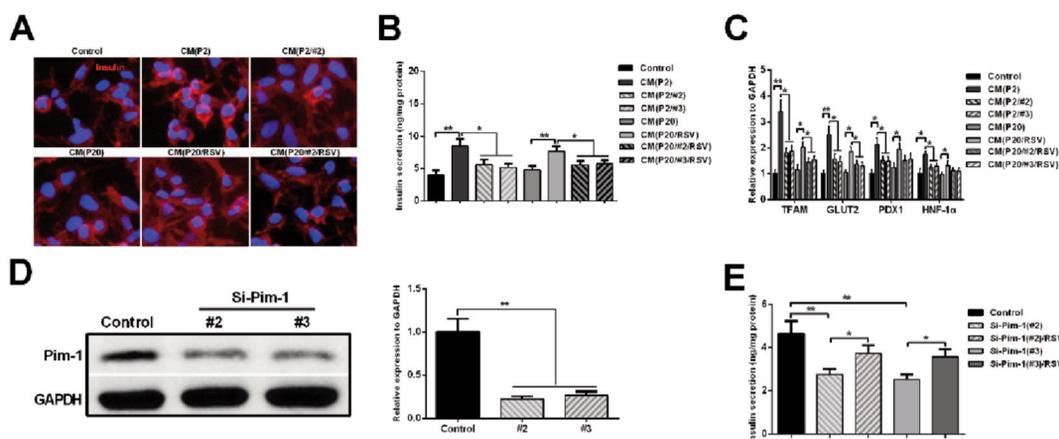


Figure 5. Resveratrol restores the paracrine effects of senescent ADMSCs and promotes insulin secretion of INS-1 cells through Pim-1. **(A)** INS-1 cells were treated with different condition medium for 48 h, immunofluorescence analysis was performed to visualize insulin content. Nuclei are stained with Hoechst (blue). **(B)** INS-1 cells were treated with different condition medium for 48 h, the insulin release was measured using ELISA method over a 30-min incubation period at stimulatory 20 mM glucose. **(C)** QRT-PCR was performed to detect the beta-islet cells associated genes expression of INS-1 cells in different culture conditions. **(D)** Inhibition of endogenous Pim-1 gene expression using siRNA method was confirmed by Western blot analysis. **(E)** ELISA method was used to determine the effects of endogenous Pim-1 and Resveratrol on the insulin secretion of INS-1 cells. Values are means \pm SD of three independent experiments, with each performed in triplicate. * $p < 0.05$, ** $p < 0.01$. CM: Condition medium. P: Passage.

portant gene regulating cell senescence. In this study, we explored whether Pim-1 is involved in regulation of resveratrol's protective effect on senescence. The results showed that resveratrol significantly promoted Pim-1 expression via PI3K/AKT signaling pathway. By quantifying several key genes for β -cell function, we found resveratrol significantly restored the expression of TFAM (a nuclear-encoded transcription factor required for stability and transcriptional activity of the mitochondrial genome)²⁷, PDX1 (a necessary transcription factor for β -cell differentiation)²⁷, GLUT2 (a bidirectional glucose transporter which enables the cells to accurately gauge the serum glucose levels)²⁸ and HNF-1 α (required for GLUT2 transcription in differentiated insulin-producing cells)²⁹. These results suggest that resveratrol rejuvenated senescent ADMSCs and restored their paracrine that promotes insulin secretion of INS-1 cells.

Since Pim-1 exerts a strong anti-aging effect, we tested whether the aging status of INS-1 is related to insulin secretion. INS-1 cells with Pim-1 knockdown had significantly reduced insulin secretion. But the reduction was partly restored by resveratrol treatment. Therefore, we hypothesized that Pim-1 knockdown might facilitate senescence of INS-1 cells, which directly or indirectly resulted in reduced insulin secretion. However, further study is required to confirm it.

ADMSCs is viewed as the most promising MSCs in future clinical use due to strong differentiation capability and relatively easy approach to harvest³⁰. But most of the preclinical and animal studies use the cells from passage 3 to passage 5^{30,31}. The potent anti-senescence effect of resveratrol might support to use the relatively late passage MSC. However, more detailed research is required in the future.

Conclusions

Resveratrol has a protective effect on senescence of ADMSCs and can preserve the paracrine effect of the ADMSCs on promoting insulin secretion of INS-1 cells. Therefore, it might be a promising adjuvant agent for future MSCs based therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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