Supplementary material

Resveratrol counteracts bone loss via mitofilin-mediated osteogenic improvement of mesenchymal stem cells in senescence-accelerated mice

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Journal: Theranostics.

Supplementary material: 5 figures with their legends and 1 table.
Figure S1. Cell senescence of BMMSCs from SAMP6 mice. (A, B) Representative images of SA-β-gal staining (A) with quantification of positively stained cells (B) in BMMSCs from SAMR1 and SAMP6 mice at 4 months of age. Bars: 200 μm. (C) Representative images of cell colonies with flow cytometric analysis of cell cycle in BMMSCs from SAMR1 and SAMP6 mice at 4 months of age. Bars: 200 μm. (D) MTT analysis of cell proliferation in BMMSCs from SAMR1 and SAMP6 mice at 4 months of age. n = 3 per group. Data represent mean ± SD. *P < 0.05. Data were analyzed using the two-tailed Student’s t test.
Figure S2. Resveratrol rescues osteogenic decline of BMMSCs derived from natural aging mice. (A, B)
Representative images of SA-β-gal staining (A) with quantification of positively stained cells (B) in BMMSCs from C3H mice at 4 months and 22 months of age. Bars: 200 μm. (C) MTT analysis of cell proliferation in BMMSCs from C3H mice at 4 months and 22 months of age. (D, E) Representative images of ALP staining (D) with quantification of ALP activity (E) in osteogenic differentiation of BMMSCs. Bars: 5 mm. (F, G) Representative images of alizarin red staining (F) with quantification of mineralization (G) in osteogenic differentiation of BMMSCs. Bars: 5 mm. BMMSCs from C3H mice at 22 months of age were treated with either resveratrol (10 μM) or the DMSO (0.001%) solvent control. n = 3 per group. Data represent mean ± SD. *P <
0.05; NS, not significant \((P > 0.05)\). Data were analyzed using the two-tailed Student’s \(t\) test (B, C) or ANOVA followed by Newman-Keuls post-hoc tests (E, G).
Figure S3. General effects of resveratrol on mtDNA-encoded mitochondrial complex subunits in SAMP6 BMMSCs. qRT-PCR analysis of mRNA expression levels of mtDNA-encoded mitochondrial complex subunits in senescent BMMSCs (related to Figure 3H as supplements). BMMSCs from SAMP6 mice at 4 months of age were treated with either resveratrol (10 μM) or the DMSO (0.001%) solvent control. n = 3 per group. Data represent mean ± SD. *P < 0.05; NS, not significant (P > 0.05). Data were analyzed using the two-tailed Student’s t test.
Figure S4. Effects of resveratrol on Ampk and Pgc-1 pathways in SAMR1 and SAMP6 BMMSCs. (A) Western blot analysis of total and phosphorylated protein expression levels of Ampk in BMMSCs. (B) Western blot analysis of total and acetylated protein expression levels of Pgc-1 in BMMSCs. Acetylated Pgc-1 was immunoblotted with an anti-acetylated lysine antibody after immunoprecipitation of Pgc-1 from nuclear extracts. (C) qRT-PCR analysis of mRNA expression levels of Pgc-1 downstream targets in BMMSCs. BMMSCs from 4-month-old SAMR1 and SAMP6 mice were treated with either resveratrol (10 μM) or the DMSO (0.001%) solvent control. n = 3 per group. Data represent mean ± SD. *P < 0.05; NS, not significant (P > 0.05). Data were analyzed using ANOVA followed by Newman-Keuls post-hoc tests.
Figure S5. Knockdown of Mitofilin mimics senescence of BMMSCs and induces bone loss. (A, B) qRT-PCR analysis of mRNA expression (A) and western blot analysis of protein expression (B) levels of Immt (Mitofilin) after shRNA-mediated knockdown in BMMSCs from SAMR1 mice at 4 months of age. (C) qRT-PCR analysis of mRNA expression levels of nuclear-encoded mitochondrial complex subunit genes in BMMSCs (related to Figure 5J). (D, E) Representative images of SA-β-gal staining (D) with quantification of positively stained cells (E) in BMMSCs. Bars: 200 μm. (F) Flow cytometric analysis of cell cycle in BMMSCs. (G) MTT analysis of cell proliferation in BMMSCs. BMMSCs from SAMR1 mice at 4 months of age were transfected with either the shRNA for Immt (Mitofilin) or the negative control (a scrambled sequence, NC) by a lentiviral vector. (H-J) Representative 2D section and 3D reconstruction micro-CT images (H) and quantitative analysis of trabecular...
bone volume (I) and bone mineral density (J) in distal femora. Bars: 500 μm. SAMRI mice at 4 months of age were treated intra-bone marrow with either the shRNA for Immt (Mitofilin) or the negative control (a scrambled sequence, NC) in a lentiviral vector solution and were sacrificed at 6-month-old. \( n = 3 \) per group. Data represent mean ± SD. *\( P < 0.05 \); NS, not significant (\( P > 0.05 \)). Data were analyzed using the two-tailed Student’s \( t \) test.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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| β-actin | Forward: 5'-CATCCGTAAAGACCTCTATGCCAAC-3'  
Reverse: 5'-ATGGAGCCACCAGATCCCA-3' |
| Alp | Forward: 5'-CCTTGTAGCCAGGCCATTG-3'  
Reverse: 5'-GGACCATTCCACGTCTTCAC-3' |
| Atp5a | Forward: 5'-CATTTGTGATGGATTGGTCGC-3'  
Reverse: 5'-TCCCAACACGACAACCTCC-3' |
| Atp6 | Forward: 5'-TCCCAATCGTTGTAAGCCATC-3'  
Reverse: 5'-TGTTGGAAGAATGGACTGG-3' |
| Atp8 | Forward: 5'-GCCACAACCTAGATACTCAACATG-3'  
Reverse: 5'-TGTTTGTAGTTATTTGGTCAGG-3' |
| Cox1 | Forward: 5'-CCCAGATATAGCATCCACCAG-3'  
Reverse: 5'-ACTGTTCATCCTGTCTGC-3' |
| Cox2 | Forward: 5'-AGTTGATACCGAGTCCGCTTCTG-3'  
Reverse: 5'-CTGTGTGCTTGTATGTGTCGCC-3' |
| Cox3 | Forward: 5'-CGTGAAGGAACCTACCAAGG-3'  
Reverse: 5'-CGCTCAGAAGAATCTCCGAA-3' |
| Cox5b | Forward: 5'-ACCCTATCTTACTGCCTCG-3'  
Reverse: 5'-CAGCCAAAAACCGATGACAG-3' |
| Cytb | Forward: 5'-CCCACCCCAATATAACCAGG-3'  
Reverse: 5'-GAGGTATGGAAGGATTAGGGG-3' |
**Drp1**
Forward: 5’-ACTGGCCCCCGTCCTGCTTTAT-3’
Reverse: 5’-ATGGACCAGCTCCACACACCGT-3’

**Erra**
Forward: 5’-GGGGAGCATCGAGTACAGC-3’
Reverse: 5’-AGACGCACACCCTCCTTGA-3’

**Immt**
Forward: 5’-AAGGTCCAAGCAGCTCAGTCT-3’
Reverse: 5’-TGTTTCTCCAAGGCTAACGTGA-3’

**Mfn1**
Forward: 5’-TCGTTTTTCTGCTGTCG-3’
Reverse: 5’-TCGACGTAGGGGACGGAAT-3’

**Mfn2**
Forward: 5’-AAGCCCAAGGCGCATGCCG-3’
Reverse: 5’-TGGAGCTGCGATGTGCAGG-3’

**Nd1**
Forward: 5’-TGCACCTACCTATCACTCA-3’
Reverse: 5’-GGCTCATCTGATAGTACG-3’

**Nd2**
Forward: 5’-ATACTAGCAATTACTTCTTTTCATAGG-3’
Reverse: 5’-GAGGGATGCGTCGCTAGAAG-3’

**Nd3**
Forward: 5’-AAGCAATCCATATGACTTGCGG-3’
Reverse: 5’-GCTCATGCTGAGTGAAGTAGAAG-3’

**Nd4**
Forward: 5’-CATCACTCCTATTCTGCTAGC-3’
Reverse: 5’-CCAACCTCCATAAGCTCCATACC-3’

**Nd4l**
Forward: 5’-CCAACCTCCATAAGCTCCATACC-3’
Reverse: 5’-GATTTTGACGTAATCTGTCCCG-3’

**Nd5**
Forward: 5’-ACGAAAAATGACCCAGACCTC-3’
Reverse: 5’-GAGATGACAAATCCTGCAAGATG-3’
Nd6  Forward: 5’-TGTTGGAGTTATGTTGGAAGGAG-3’
      Reverse: 5’-CAAAGATCACCCAGCTACTACC-3’
Ndufs8 Forward: 5’-GTTTCATAGGGTCAGAGGTCAAG-3’
       Reverse: 5’-TCCATTAAGATGTCCTGTGCG-3’
Nrf1  Forward: 5’-AATGTCCGCAGTGATGTCC-3’
       Reverse: 5’-GCCTGAGTTTGTGTTTGCTG-3’
Ocn   Forward: 5’-TGACAAAGCGCTTCATGTCC-3’
       Reverse: 5’-GCGCCCGAGTCTGTTCACTA-3’
Opa1  Forward: 5’-ATAACTACCCGCCTGCGA-3’
       Reverse: 5’-TGCTTTTGGCGTGACCTGGCT-3’
Oxx   Forward: 5’-GGGCGTTCTACCTGCGACTG-3’
       Reverse: 5’-CGAAGCCTTGCCGTACACCT-3’
Phb   Forward: 5’-TGGCAGAAGAAGGCAGGGCA-3’
       Reverse: 5’-TCACACACACCTGCTTCCGCT-3’
Pink1 Forward: 5’-AAGCGCGTGTCTGACCCACT-3’
       Reverse: 5’-ACACAGCGGCGATTGCAACCCT-3’
Ppara Forward: 5’-AGGAAGCCTTCTGTGACAT-3’
       Reverse: 5’-GCCTGAGTTTGTGTTTGCTG-3’
Runx2 Forward: 5’-CCGCACGACAACCGCAACC-3’
       Reverse: 5’-CGCTCCGGCCCACAATCTC-3’
Sdhb  Forward: 5’-ACCCCTTCTCTGCTTACC-3’
       Reverse: 5’-TCCATTAAGATGTCCTGTGCG-3’
Uqrec1  Forward: 5’-ATCAAGGCACGTCCAAGG-3’

Reverse: 5’-TCATTTTCCTGCATCCCG-3’