Online Supplemental Data

The Anti-aging Gene Klotho Regulates Proliferation and Differentiation of Adipose-derived Stem Cells

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Running title: Klotho effect on ADSCs

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Supplemental Figure S1. Flow cytometry was used to characterize ADSCs (P3) isolated from 129Sv mice. Cells with highly positive expression of surface markers CD44 and CD105 and negative for CD34 and CD45 were regarded as ADSCs.
Supplemental Figure S2. ADSCs were investigated for their in vitro multilineage differentiation capacity. ADSCs were stained by Oil Red O at day 7 of adipogenic induction (top row). Bar, 20μm. ADSCs were stained by Alizarin Red S at day 21 of osteogenic induction (middle row). Bar, 20μm. ADSCs were examined by α-SMA immunochemical staining (green) after 4 days of myofibroblast induction (bottom row). Bar, 50μm.

Supplemental Figure S3. The direct IP method was used to remove Klotho protein from fetal serum. A. Western blot analysis of SKL expression. B. Quantification of SKL expression. Data are means ± SEM (n=4 independent experiments). ** p< 0.01 vs. control serum.
Supplemental Figure S4. SKL distribution after Ni\(^+\)-column purification. A. Coomassie blue showed the protein distribution in different concentrations of elution buffer. B. Western blot showed SKL expression in different concentrations of elution buffer. The arrow indicates the SKL purified from the elution buffer that was used for the ADSC-treatment experiment.

Supplemental Figure S5. Klotho-deficient-serum treatment changed the shape of ADSCs from the spindle shape to the flattened shape, and the recombinant SKL protein maintained the spindle shape of ADSCs. The ADSCs were separately treated for 24 hours and 48 hours. Scale bar, 100 μm.
Supplemental Figure S6. rSKL rescued the Klotho deficiency-induced decrease in adipogenic differentiation in human ADSCs (hADSCs). A, Lipid formation assessed by Oil Red O staining after 7 days of adipogenic induction. B, Quantification of lipid-positive cells (lipid vacuoles stained with Oil Red O stain). Bar, 50μm. C, Quantification of lipid staining (density). *p<0.05, ***p<0.001 vs. the control group; #p<0.05, ###p<0.001 vs. the Klotho-deficient serum group. n=3 independent experiments.

Supplemental Figure S7. rSKL rescued the Klotho deficiency-induced decrease in adipogenic differentiation in mMSCs. A, Lipid formation assessed by Oil Red O staining after 7 days of adipogenic induction. Bar, 50μm. B, Quantification of lipid-positive cells. C, Quantification of lipid staining (density). *p<0.05, **p<0.01 vs. the control group; #p<0.05, ##p<0.01 vs. the Klotho-deficient serum group. n=3 independent experiments.
**Supplemental Figure S8.** Western blot analysis of Klotho expression in ADSCs (48 hours). Overexpression of the recombinant SKL gene increased SKL protein expression (65 kDa) in ADSCs.

**Supplemental Figure S9.** Western blot analysis of Oct-4, Nanog, and Sox-2 after SKL overexpression in ADSCs (48 hours). Overexpression of SKL protein did not increase the levels of transcription factors (Oct-4, Nanog, and Sox-2).
Supplemental Figure S10. rSKL protein reversed the inhibitory effect of TGFβ1 on adipogenic differentiation in ADSCs. A, Lipid formation assessed by Oil Red O staining after 7 days of adipogenic induction. B, Quantification of lipid-positive cells (lipid vacuoles stained with Oil Red O stain). C, Quantification of lipid accumulation (density). D, Western blot analysis of PPAR-γ expression in ADSCs. (+) adipogenic induction, (−) without adipogenic induction. Scale bar, 20 μm. *p<0.05, **p<0.01 vs. the control group; +p<0.05, ++p<0.01 vs. the TGFβ1(+) group.

Supplemental Figure S11. Recombinant SKL protein inhibited TGFβ signaling in ADSCs. A, Western blot analysis of TGF-β1, p-Smad2/3, and Smad2/3 expression in ADSCs. Quantification of TGF-β1 (B), p-Smad2/3 (C), and Smad2/3 (D) expression. Results were standardized to β-actin. *P<0.05 vs. the control group.
**Supplemental Figure S12.** rSKL protein did not increase expression of the transcription factors Oct-4, Sox-2, and Nanog. A, Western blot analysis of Oct-4, Sox-2, and Nanog expression in ADSCs. Quantification of Oct-4 (B), Sox-2 (C) and Nanog (D) expression in ADSCs.

**Supplemental Figure S13.** rSKL rescued the Klotho deficiency-induced decrease in osteogenic differentiation in ADSCs. A, ALP staining after 14 days of osteogenic differentiation. B, ALP activity after 14 days of osteogenic differentiation. *p<0.05 vs. the control group; #p<0.05 vs. the Klotho-deficient serum group. n=3 independent experiments.
Supplemental Figure S14. rSKL rescued the Klotho deficiency-induced decrease in myofibroblastic differentiation in ADSCs. A, Immunocytochemical analysis of α-SMA, a marker of myofibroblasts, in mADSCs after 14 days of myofibroblastic differentiation. Cells were subjected to immunofluorescence staining with antibody against α-SMA (green) and nuclear staining with DAPI (blue), respectively. Bar, 50μm. B, Western blot analysis of α-SMA protein expression in mADSCs. Results were standardized to β-actin. *p< 0.05 vs. the control group; #p<0.05 vs. the Klotho-deficient serum group. n=3 independent experiments.