

RTEF-1 protects against oxidative damage induced by H₂O₂ in human umbilical vein endothelial cells through Klotho activation

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Abstract

Oxidative stress is a main risk factor of vascular aging, which may lead to age-associated diseases. Related transcriptional enhancer factor-1 (RTEF-1) has been suggested to regulate many genes expression which are involved in the endothelial angiogenesis and vasodilation. However, whether RTEF-1 has a direct role in anti-oxidation and what specific genes are involved in RTEF-1-driven anti-oxidation have not been elucidated. In this study, we found that overexpressing RTEF-1 in H₂O₂-treated human umbilical vein endothelial cells decreased senescence-associated- β -galactosidase (SA- β -gal)-positive cells and G0/G1 cells population. The expressions of p53 and p21 were decreased in H₂O₂-treated RTEF-1 o/e human umbilical vein endothelial cells. However, specific small interfering RNA of RTEF-1 totally reversed the anti-oxidation effect of RTEF-1 and inhibited RTEF-1-induced decreased p53 and p21 expressions. It demonstrated that RTEF-1 could protect cells from H₂O₂-induced oxidative damage. In addition, we demonstrated that RTEF-1 could up-regulate Klotho gene expression and activate its promoter. Furthermore, Klotho small interfering RNA significantly blocked RTEF-1-driven endothelial cell protection from H₂O₂-induced oxidative damage and increased p53 and p21 expressions. These results reveal that RTEF-1 is a potential anti-oxidation gene and can prevent H₂O₂-induced endothelial cell oxidative damage by activating Klotho.

Keywords: Related transcriptional enhancer factor-1, senescence, Klotho, human, umbilical vein endothelial cells, oxidative stress

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Introduction

Accumulation of excess reactive oxygen species (ROS), which includes superoxide anion and hydrogen peroxide (H₂O₂), can cause oxidative stress, leading to damage to proteins, nucleic acid, and cell membranes.¹ Then, ROS can participate in many biological processes, including programmed cell death and cellular senescence. Aging is considered to be an important risk factor for development of atherosclerosis and is also associated with endothelial senescence.² Senescence of endothelial cells has been proposed to be involved in endothelial dysfunction and atherogenesis. Increased numbers of senescent endothelial cells are found in atherosclerotic plaques of human aorta and coronary arteries and coronary vessels of patients with ischaemic heart disease.^{3,4} Vascular aging is associated with an increase in ROS and the endothelium is highly sensitive to injury caused by ROS.⁵

Senescent cultured cells often display enlargement, flattened morphology, elevated activity of senescence-associated- β -galactosidase (SA- β -gal) activity, and a

permanent cell-cycle arrest at G1.⁶ P53, known as a pro-apoptotic protein, regulates the transcription of genes involved in cell-cycle arrest and apoptosis and actually accelerates aging in certain settings, including DNA damage, oxidative stress, and hypoxia. When normal cells are not under stress, p53 levels and activity are very low.⁷ Cell cycle progression through the G1 phase into S is a major checkpoint for proliferating cells and can be mediated by p53. Then, increased p53 activity activates cyclin-dependent kinase inhibitor, such as p21, triggering growth arrest and senescence.⁸

Related transcriptional enhancer factor-1 (RTEF-1), also known as TEAD4, is a member of the transcriptional enhancer factor-1 family. RTEF-1 is a critical regulator of cardiac and smooth muscle-specific genes during cardiovascular development and cardiac disorders including cardiac hypertrophy.⁹ Endothelial cells are modulators of cardiovascular function. RTEF-1 expression can be up-regulated by hypoxia in endothelial cells. Downstream genes of RTEF-1 such as vascular endothelial growth factor (VEGF)

and connexin (Cx43) have been suggested to be involved in RTEF-1-induced angiogenesis.^{10,11} RTEF-1 also up-regulates hypoxia inducible factor-1 and endothelial differentiation gene-1 transcription and accelerates endothelial tube formation and enhanced cell aggregation in Matrigel models.^{12,13} In addition, RTEF-1 acts as a transcriptional stimulator of fibroblast growth factor receptor 1 and can stimulate microvascular relaxation via eNOS up-regulation.¹⁴

Overexpression of Klotho extends mouse lifespan by 20%, while a defect of Klotho gene expression results in a model for various phenotypes that resemble human aging, including a shortened lifespan, arteriosclerosis, osteoporosis, infertility, skin atrophy, pulmonary emphysema, and sub-cutaneous fat atrophy.^{15,16} Klotho expression is significantly decreased with age or in some age-related disease. Furthermore, Klotho gene has anti-apoptotic and anti-senescence effects on vascular endothelial cells and thus can protect against endothelial dysfunction in multiple risk factor.¹⁷ Klotho overexpression induces resistance to oxidative stress at the cellular and organismal levels in mammals.¹⁸ These findings suggest that Klotho plays an important role in anti-aging. However, transcription factors that regulate Klotho in the suppression of oxidative damage role have not been elucidated.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) and Human embryonic kidney cell line (HEK293 cells) were obtained from Tongji Medical College, China. HUVECs and HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in 5% CO₂.

Liposomal transduction, stable cell line generation, and small interfering RNA transfection

The coding sequence of RTEF-1 (NM_003213) is obtained from a pXJ40/RTEF-1 construct (gifted from Dr. Alexandre Stewart, University of Ottawa). The liposomal medium was used to transduce HUVECs, and stable transfectants were selected with G418 (500 µg/mL). Small interfering RNA (siRNA) encoding human RTEF-1 or Klotho (Genpharma Shanghai, China) at a final concentration of 50 nM was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and confirmed by real-time PCR and Western blotting. Two duplexes of RTEF-1 siRNA were 5'-GGG CAG ACC UCA ACA CCA ATT-3', 5'-UUG GUG UUG AGG UCU GCC CAG-3' and 5'-ACC CAA GAU GCU GUG UAU UTT-3', 5'-AAU ACA CAG CAU CUU GGG UTT-3'. Two duplexes of Klotho siRNA were 5'-GAU GAU GCC AAA UAU AUG UTT-3', 5'-ACA UAU AUU UGG CAU CAU CTT-3' and 5'-CUC CAG GAA AUG CAC GUU ATT-3', 5'-UAA CGU GCA UUU CCU GGA GTT-3'. A duplex of RNA (5'-UUC UCC GAA CGU GUC ACG UTT-3', 5'-ACG UGA CAC GUU CGG AGA ATT-3') that

is not targeted to any human gene was used as a negative control.

SA-β-gal staining

Cytochemical staining for SA-β-gal at pH 6.0 was performed using a senescence-β-galactosidase staining kit (Cell signaling technology). After staining, cells were imaged with a microscope using a camera. At least 200 cells in several fields were examined, and SA-β-gal-positive cells were counted.

Cell cycle analysis

Cells were washed with phosphate-buffered saline and fixed with ice-cold 75% ethanol at 4°C overnight. Samples were then washed with phosphate-buffered saline twice and stained with 100 µg/mL propidium iodide (Sigma) containing RNase A (Sigma) for 30 min at 37°C. Cell cycle distribution in different phases was determined using flow cytometry (Becton Dickinson).

Real-time qPCR analysis

Total RNA was isolated by Trizol (TAKARA Bio INC, Otsu, Shiga, Japan). Complementary DNA was synthesized by using PrimeScript RT Reagent Kit (TAKARA Bio INC, Otsu, Shiga, Japan) according to manufacturer's instructions. PCR was conducted using the Taq polymerase (TAKARA Bio INC, Otsu, Shiga, Japan). The oligonucleotide primers for RTEF-1, Klotho, p53, p21, and β-actin were as follows: RTEF-1 (NM_201443.2), forward 5'-CCACGAAGGTCT GCTCTTTC-3', reverse 5'-CTCACTGGCTGACACCTC AA-3'; Klotho (NM_004795.3), forward 5'-GTGTCCA TTGCCCTAAG-3', reverse 5'-CTCTCGGGATAGTCA CC-3'; p53 (NM_001126118.1) forward 5'-TGAGGTTGGCT CTGACTGTA-3', reverse 5'-TTCTCTTCCTCTGTGCGCC G-3'; p21 (NM_001291549.1), forward 5'-GGACCTGTCAC TGTCTTGTA-3', reverse 5'-CCTCTTGGAGAAGATCAG CCG-3'; and β-actin (NM_001101.3), forward 5'-GTCC ACCTTCCAGCAGATGT-3', reverse 5'-CACCTTCACCG TTCCAGTTT-3'.

Western blotting analysis

Protein samples (60 µg) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membrane (Invitrogen, Grand Island, NY, USA). Membranes were incubated with a 1:1000 dilution of rabbit monoclonal anti-RTEF-1 (Abcam, Cambridge, UK), 1:1000 dilution of rabbit monoclonal anti-Klotho (Abcam, Cambridge, UK), 1:1000 dilution of rabbit polyclonal anti-p53 (proteintech, Chicago, USA), 1:500 dilution of rabbit polyclonal anti-p21 (proteintech, Chicago, USA), and a 1:1000 dilution of mouse polyclonal anti-β-actin (AntGene Biotech, Wuhan, China) at 4°C for overnight, respectively. Membranes were then incubated with a 1:2000 dilution of HRP-conjugated secondary anti-rabbit and 1:2000 dilution of secondary anti-mouse (AntGene Biotech, Wuhan, China) at room temperature for 2 h, respectively. Finally, membranes were detected by enhanced chemiluminescence (ECL) method. For quantification, RTEF-1, Klotho, p53, and p21 protein

levels were normalized to the β -actin protein level, respectively.

Promoter activity

A construct containing nucleotide fragment encompassing basal elements of the human Klotho promoter was obtained from Switchgear Genomics (Menlo Park, CA). HEK293 cells were transfected with the construct and different doses of pXJ40 RTEF-1 and pXJ40 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The addition of pXJ40 was used to maintain the same cDNA background. Luciferase activity was determined using the dual luciferase assay system (Galen, Beijing, China) 48 h after transfection.

Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation (SD). Direct comparisons between two groups were made using the Student's *t*-test. Data from more than two groups were available for ANOVA with repeated measures, followed by the Student-Newman-Keuls multiple comparison test. *P* values less than 0.05 were considered significant.

Results

RTEF-1 effectively suppresses cell senescence features and growth arrest in H₂O₂-treated HUVECs

To investigate the effect of RTEF-1 on H₂O₂-induced HUVECs senescence and growth arrest, we incubated the cells with 100 μ mol/L H₂O₂ for 48 h to induce senescence and growth arrest in HUVECs.¹⁸ As shown in Figure 1(a), the number of SA- β -gal-positive cells increased from $2.67 \pm 0.58\%$ in controls to $20 \pm 1.40\%$ in H₂O₂-treated HUVECs (mean $\pm$ SD, *p* < 0.05). Moreover, the proportion of HUVECs in the G0/G1 phase was increased and that in the S phase was decreased, which indicated the cell-cycle arrest (Figure 1(b)). To further understand the effect of RTEF-1 on H₂O₂-induced aging and growth arrest, we then detected the SA- β -gal staining and cell cycle in H₂O₂-treated RTEF-1 o/e HUVECs and RTEF-1 siRNA-transfected HUVECs. In the SA- β -gal staining, increases in SA- β -gal-positive cells induced by H₂O₂ were significantly attenuated in RTEF-1 o/e HUVECs, compared with negative controls (Figure 1(c)). However, RTEF-1 siRNA-transfected HUVECs in which endogenous RTEF-1 was knocked down increased the sum of SA- β -gal-positive cells (Figure 1(c)). Progression through the cell cycle is a critical cellular process and cell-cycle arrest during the G1 phase is a characteristic exhibited by senescent cells. As shown in Figure 1(d), treatment with H₂O₂ arrested RTEF-1 o/e HUVECs in the G0/G1 phase as the proportion of cells in the G0/G1 phase was 43.54% compared to 64.34% in the control group, while the proportion of cells of RTEF-1 siRNA-transfected HUVECs in the G0/G1 phase increased. These results indicated that RTEF-1 is protected against H₂O₂-induced HUVECs senescence and growth arrest.

RTEF-1 decreases the expression of p53 and p21 in H₂O₂-treated HUVECs

Since cell cycle regulatory pathways mediated by cyclin-dependent kinase inhibitors play a critical role in growth arrest and senescence induction, we examined their gene expression following RTEF-1 o/e and RTEF-1 siRNA treatment. As shown in Figure 2(a,c), p53 and p21 mRNA decreased to 0.47-fold and 0.30-fold in H₂O₂-treated RTEF-1 o/e HUVECs and increased to 1.23-, 1.27- and 1.20-, 1.23-fold in RTEF-1 knockdown HUVECs compared to their corresponding controls. The levels of p53 and p21 proteins were similar to that of p53 and p21 mRNA (Figure 2(b,d)).

RTEF-1 regulates Klotho gene expression

Klotho has been known as an anti-aging gene, which regulates the cellular lifespan of human cells. To determine whether Klotho was involved in RTEF-1-induced suppressing cell senescence and growth arrest features, the expression of Klotho was assayed by real-time qPCR and Western blotting. Figure 3(a) demonstrated that Klotho mRNA and protein levels increased in H₂O₂-treated RTEF-1 o/e HUVECs and decreased after using two specific sequences of RTEF-1 siRNA for knockdown of RTEF-1 in HUVECs.

To determine whether Klotho is regulated by RTEF-1 on a transcriptional level, the activities of a luciferase construct under the control of a Klotho promoter were measured. The Klotho promoter exhibited an RTEF-1 dose-dependent stimulating in activity, exhibiting a maximum of 3.98-fold increase (Figure 3(b)). Together, these data indicated that RTEF-1 regulates Klotho gene expression in endothelial cells.

Klotho takes part in RTEF-1-driven endothelial cell anti-oxidation

Klotho expression was examined by qRT-PCR and Western blot in Klotho siRNA-treated RTEF-1 o/eHUVECs. A significant decrease of Klotho was observed (Figure 4(a)). To further examine whether RTEF-1 protects HUVECs against oxidative damage through Klotho, we transiently transfected Klotho siRNA into RTEF-1 o/e HUVECs and control HUVECs and observed endothelial cell senescence features. As shown in Figure 4(b,d), Klotho siRNA significantly increased the SA- β -gal-positive cells and G0/G1 cells population both in RTEF-1 o/e HUVECs and control HUVECs. Klotho siRNA up-regulated p53 and p21 expressions in control HUVECs but that was not observed in RTEF-1 o/e HUVECs (Figure 4(c,e)). These results suggested that Klotho participated in RTEF-1-stimulated anti-oxidation in endothelial cells.

Discussion

Recently, it has been demonstrated that an increased ROS production have been found in the senescent endothelial cells which can lead to endothelial dysfunction.¹⁹ Moreover, senescent endothelial cells contribute to age-related vascular disorders such as atherosclerosis and cardiovascular diseases.²⁰ Results from our current study

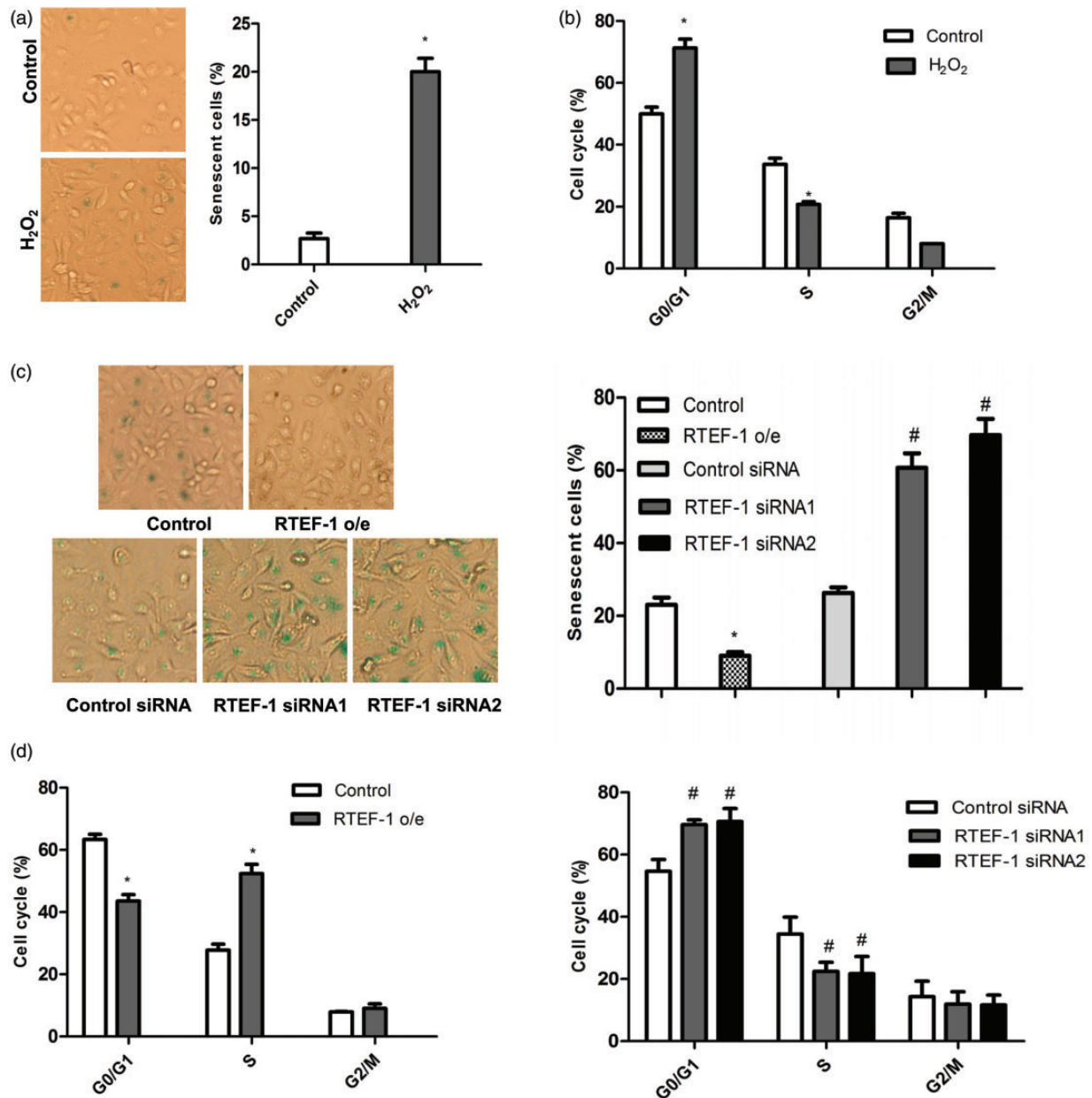


Figure 1 RTEF-1 protects human umbilical vein endothelial cells (HUVECs) from H₂O₂-induced senescence. HUVECs were incubated with 100 μ M H₂O₂ for 48 h. (a) Senescence-associated β -galactosidase (SA- β -gal)-positive HUVECs increased significantly following treatment with H₂O₂ (* p < 0.05 compared with control). (b) Cell cycle was analyzed by flow cytometry and cells arrested in G0/G1 phase were increased in H₂O₂-treated HUVECs (* p < 0.05 compared with control). (c) The percentage of SA- β -gal-positive HUVECs was significantly decreased in RTEF-1 o/e HUVECs and increased in RTEF-1 knockdown HUVECs (* p < 0.05 compared with control, # p < 0.05 compared with control siRNA). (d) Cell cycle analysis in RTEF-1 o/e HUVECs and control HUVECs (left) and in RTEF-1 siRNA HUVECs and control siRNA HUVECs (right) (* p < 0.05 compared with control, # p < 0.05 compared with control siRNA). Data represent the mean $\pm$ SD from three independent experiments (n = 3). (A color version of this figure is available in the online journal.)

revealed that RTEF-1 could prevent H₂O₂-induced endothelial cell oxidative damage and changed cell cycle through modulation of Klotho activity. First, we determined the rate of the senescent endothelial cells with the SA- β -gal staining and cell cycle analysis and found that RTEF-1 could effectively overcome H₂O₂-induced endothelial cell senescence and cell-cycle arrest. RTEF-1 down-regulated the expressions of p53 and p21, which mediated cell cycle in H₂O₂-treated HUVECs. Second, our result showed that RTEF-1 could up-regulate Klotho gene expression and activate its

promoter. In addition, specific siRNA of Klotho significantly blocked RTEF-1-driven endothelial cell protection from H₂O₂-induced cell-cycle arrest and up-regulated p53 and p21 expressions. These data indicated that Klotho might be involved in the anti-oxidation effects of RTEF-1 in H₂O₂-treated HUVECs.

It is widely accepted that several ROS are involved in the genesis of vascular damage in the aging process. In response to oxidative stress, cellular proliferation can be inhibited, via transient cell-cycle arrest or senescence.²¹

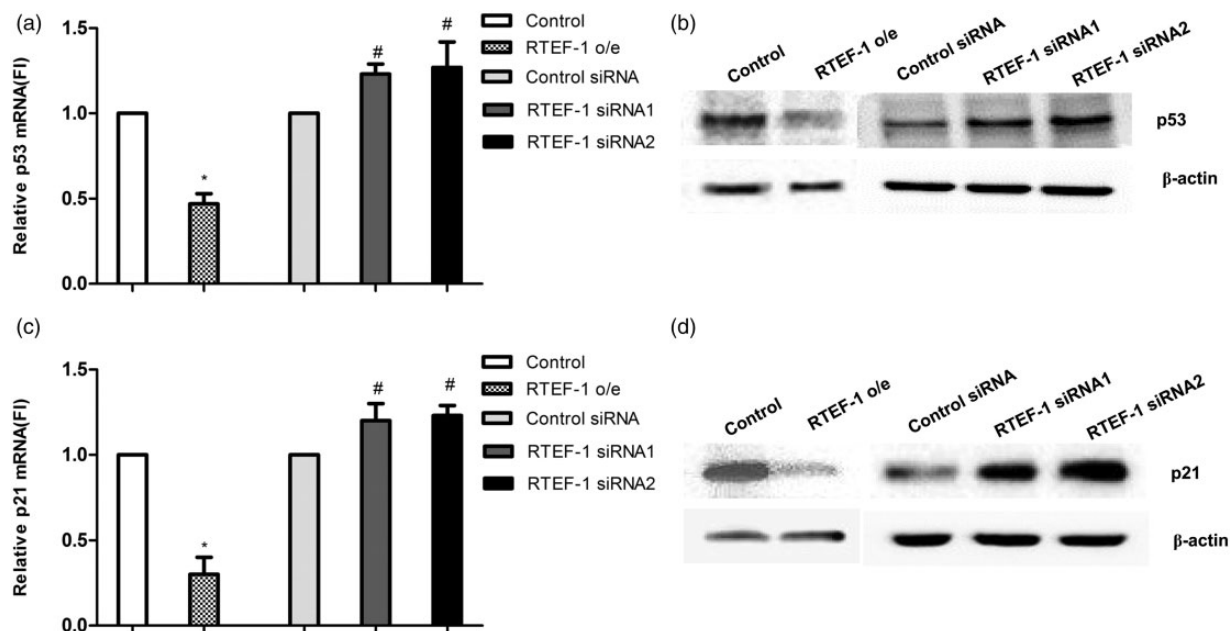


Figure 2 RTEF-1 down-regulated p53 and p21 expressions in H_2O_2 -treated HUVECs. HUVECs were incubated with $100 \mu\text{mol/l}$ H_2O_2 for 48 h. (aA) p53 mRNA levels were significantly decreased in RTEF-1 o/e HUVECs and increased in RTEF-1 knockdown HUVECs (* $p < 0.05$ compared with control, # $p < 0.05$ compared with control siRNA). (bB) p53 protein levels showed similar results to mRNA levels when RTEF-1 was overexpressed or knocked down. (cC) p21 mRNA levels were significantly decreased in RTEF-1 o/e HUVECs and increased in RTEF-1 knockdown HUVECs (* $p < 0.05$ compared with control, # $p < 0.05$ compared with control siRNA). (dD) p21 protein levels showed similar results to mRNA levels when RTEF-1 was overexpressed or knocked down. Results are expressed as fold (FI) of control (control = 1 arbitrary unit). Data represent the mean $\pm$ SD from three independent experiments ($n = 3$)

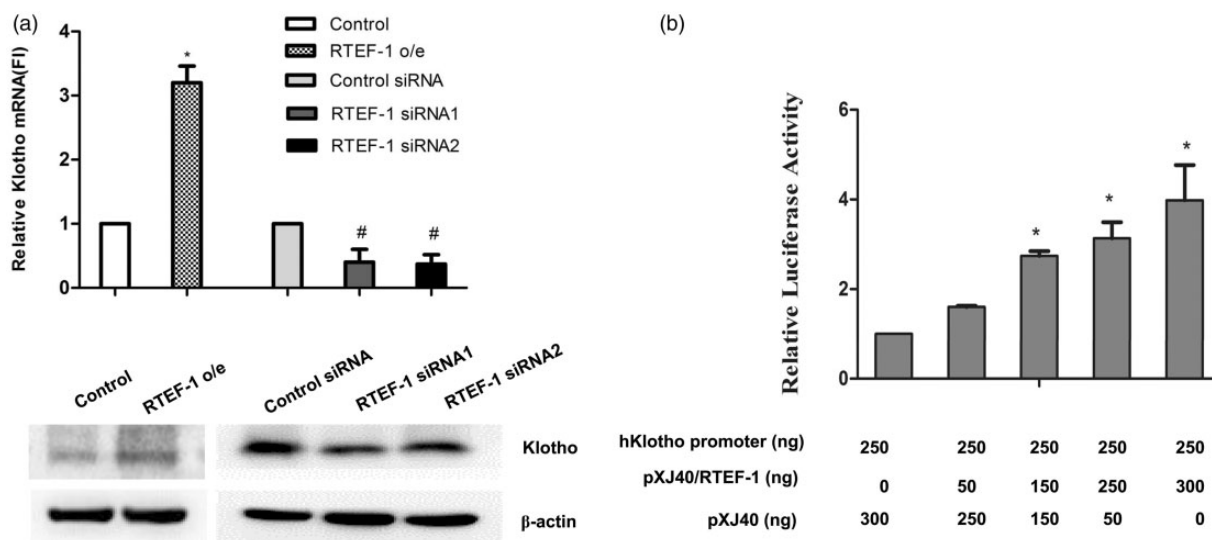


Figure 3 Klotho is a potential target gene of RTEF-1. (aA) Klotho mRNA levels and protein levels were significantly increased in RTEF-1 o/e HUVECs and decreased in RTEF-1 knockdown HUVECs (* $p < 0.05$ compared with control, # $p < 0.05$ compared with control siRNA). (bB) Klotho full length promoter was transiently co-transfected with different concentrations of RTEF-1, and luciferase activity was examined. Klotho promoter activity was shown to be up-regulated in an RTEF-1 dose-dependent manner (* $p < 0.05$). Results are expressed as fold (FI) of control (control = 1 arbitrary unit). Data represent the mean $\pm$ SD from three independent experiments ($n = 3$)

Moreover, endothelial cell angiogenesis is damaged by ROS and pro-inflammatory cytokines during aging.²² Previous studies have suggested that aged endothelial cells show impaired proliferation and migration in response to cytokines such as platelet-derived growth factor. The deficiency of pivotal cytokine VEGF is a major responsibility for

impaired angiogenesis.^{23,24} The deterioration in angiogenesis may be in turn linked with the age-related endothelial dysfunction. RTEF-1 has demonstrated an obvious proangiogenesis effect, inducing endothelial cell growth, migration, and capillary network formation through its downstream genes VEGF, hypoxia inducible factor-1 α ,

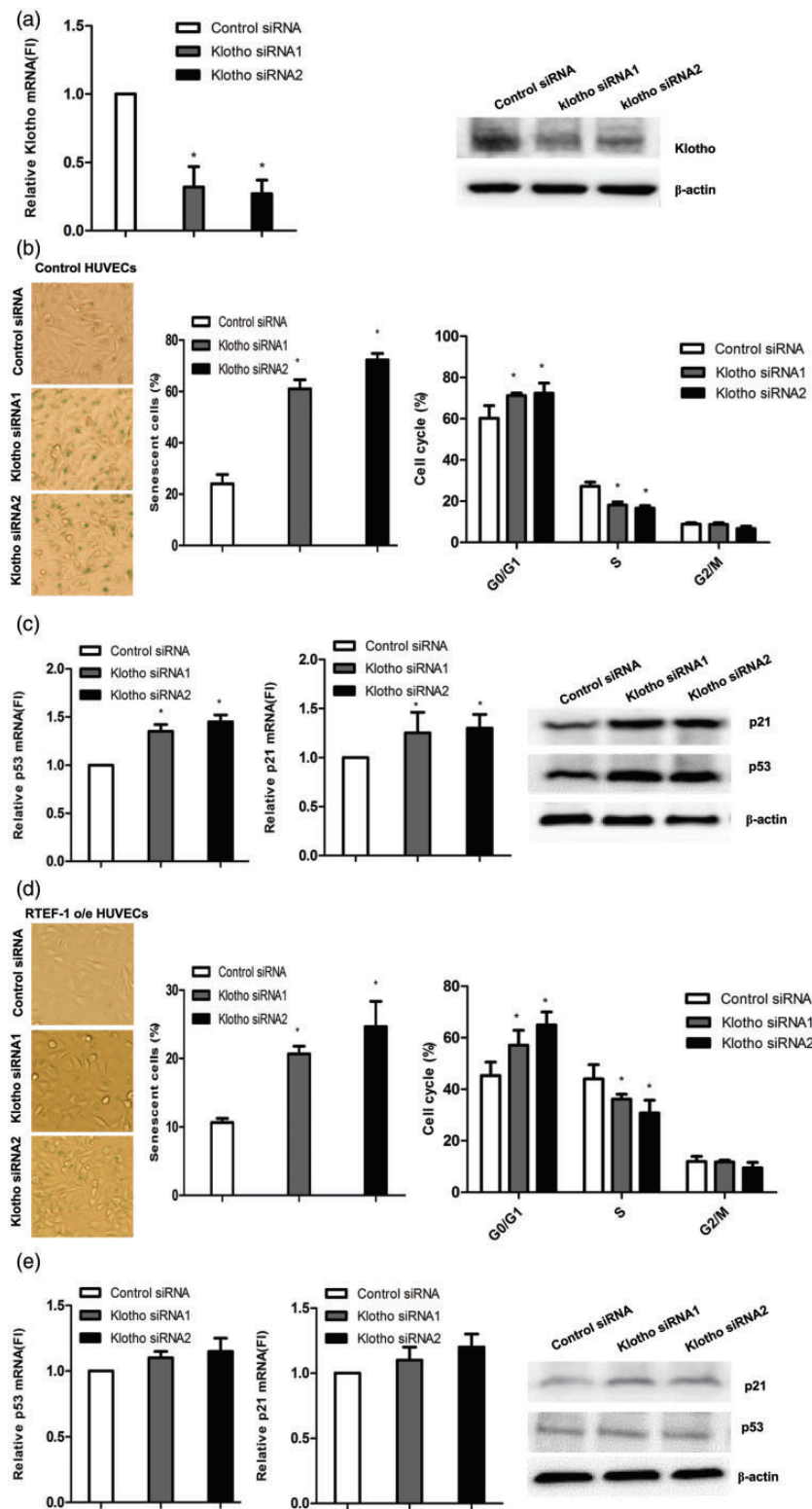


Figure 4 Klotho is involved in RTEF-1-driven anti-oxidation effect in endothelial cells. HUVECs were incubated with 100 μ mol/l H₂O₂ for 48 h. (a) Klotho expression was knocked down by Klotho siRNA in RTEF-1 o/e HUVECs ($p < 0.05$ compared with control siRNA). (b) Klotho siRNA-induced control HUVECs senescence. SA- β -gal-positive cells and G0/G1 cells population were increased in Klotho knockdown control HUVECs ($p < 0.05$ compared with control siRNA-treated control HUVECs). (c) The expressions of p53 and p21 were up-regulated by Klotho siRNA in control HUVECs ($p < 0.05$ compared with control siRNA-treated control HUVECs; left: p53 mRNA; middle: p21 mRNA; right: p53 and p21 protein). (d) Treatment with Klotho siRNA increased SA- β -gal-positive cells and G0/G1 cells population in RTEF-1 o/e HUVECs ($p < 0.05$ compared with control siRNA-treated RTEF-1 o/e HUVECs). (e) The expressions of p53 and p21 were not significantly increased in Klotho siRNA-treated RTEF-1 o/e HUVECs ($p > 0.05$; left: p53 mRNA; middle: p21 mRNA; right: p53 and p21 protein). Results are expressed as fold (FI) of control (control = 1 arbitrary unit). Data represent the mean $\pm$ SD from three independent experiments ($n = 3$). (A color version of this figure is available in the online journal.).

fibroblast growth factor receptor 1, and endothelial differentiation gene-1.^{10–14} Thus, we hypothesize that RTEF-1 might play a role in anti-oxidation. Interestingly, we found that stable overexpression of RTEF-1 cDNA in endothelial cells led to a decrease in H₂O₂-treated endothelial cell growth arrest and knockdown of RTEF-1 expression by siRNA showed an increase in cell growth arrest induced by H₂O₂. In addition, our results showed that overexpression of RTEF-1 down-regulated p53 and p21 expressions and specific siRNA of RTEF-1 increased the expressions of p53 and p21. These changes of p53 and p21 expressions were consistent with the fact that RTEF-1 decreased G0/G1 cells population in cell cycle analysis. It revealed that RTEF-1 participated in anti-oxidation process. However, genes which may be involved in RTEF-1-driven anti-oxidation effect in endothelial cells have not been elucidated.

Klotho, known as an aging suppressor gene, can protect against endothelial dysfunction by increasing endothelial-derived NO production and exhibit significant resistance to oxidative stress through inhibiting insulin-like signaling. Recent observations also demonstrated that Klotho can control fibroblast growth factors signalling and stimulate proliferation.^{25,26} Our results revealed with convincing evidence that Klotho is a target gene of RTEF-1. First, we disclosed that the expression of Klotho was in a higher level in RTEF-1 o/e HUVECs, while the expression was decreased by RTEF-1 siRNA. Second, RTEF-1 could activate Klotho promoter and demonstrate a significant dose-dependent stimulating effect.

Whether Klotho takes part in RTEF-1-enhanced anti-oxidation effect in endothelial cells is largely unknown. Previous study has shown that Klotho normally regulates cellular senescence by repressing the p53/p21 pathway and this may be associated with increased signaling through the insulin/IGF-1 pathway.²⁷ The results from our study revealed that SA- β -gal-positive cells and G0/G1 cells population were increased by Klotho siRNA in H₂O₂-treated RTEF-1 o/e HUVECs and control HUVECs. It also showed that knockdown of Klotho expression by its siRNA increased expressions of p53 and p21 in control HUVECs. However, Klotho siRNA did not show a statistically significant increase of p53 and p21 expressions in RTEF-1 o/e HUVECs. We hypothesize that there may be two reasons. First, knockdown of Klotho by siRNA may retard the effect of RTEF-1 on cell cycle but cannot overcome it. Second, we speculate that there may be other intracellular signaling pathways involved in the effect of RTEF-1 on cell cycle of endothelial cells. These findings elucidate that Klotho is participated in RTEF-1-enhanced anti-oxidation effect in endothelial cells.

In summary, we demonstrate that RTEF-1 can prevent H₂O₂-induced cell-cycle arrest, and repress the expressions of p53 and p21 in endothelial cells. In addition, Klotho is a new target gene of RTEF-1 involved in anti-oxidation effect. Previous study has shown that RTEF-1 play an important role in cardiovascular system. Our study provides a novel role for RTEF-1, which protects against endothelial cell oxidative damage.

Author contributions: SS designed and performed the study, analyzed, and wrote the paper. SS, PGS, XHW, and QQW conducted the experiments. PH and BC assisted in experimental design and data analysis, and wrote the paper. SS and BC contributed equally to this study.

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