Effect of KNDC1 overexpression on the senescence of human umbilical vein endothelial cells

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Abstract. Kinase non-catalytic C-lobe domain containing 1 (KNDC1) exists in dendrites, guanine nucleotide exchange factor complexes and neuronal cell bodies as a putative protein-protein interaction module that regulates a number of signaling pathways. Previous studies have demonstrated that the knockdown of KNDC1 delays human umbilical vein endothelial cell (HUVEC) senescence. However, the effect of KNDC1 overexpression on HUVEC function remains unclear. In the present study, an adenovirus vector carrying KNDC1 was constructed and then transfected into endothelial cells to observe cell senescence. Furthermore, the effect of KNDC1 overexpression on HUVEC senescence was investigated *in vitro* and the underlying molecular mechanism was investigated. Senescence-associated β-galactosidase staining was used to determine cellular senescence and reactive oxygen species (ROS) were monitored to detect the level of cell oxidative stress. The mRNA transcription level and protein expression were analyzed by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The results demonstrated that KNDC1 overexpression possibly inhibited HUVEC activity and function and promoted HUVEC senescence. Mechanistic studies demonstrated that KNDC1 triggered a p53-ROS positive feedback loop, which serves a crucial role in regulating senescence.

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In conclusion, to the best of the authors' knowledge, this is the first time that KNDC1-adenovirus vector inhibition of HUVEC proliferation by activating the p53 signaling pathway has been reported. Theoretically, the results of the present study also support KNDC1 as a therapeutic target for future anti-senescence.

Introduction

In a broad sense, cardiovascular diseases (CVD) are diseases associated with aging (1). A number of studies have indicated that a dysfunction in vascular endothelial cells is a key antecedent of vascular-associated diseases (2,3). Aging or cell senescence is partly caused by the dysfunction of vascular endothelial cells (4). The loss of replicative capacity in senescent endothelial cells destroys their cellular integrity and damages angiogenesis (5,6).

Kinase non-catalytic C-lobe domain containing 1 (KNDC1) exists in dendrites, guanine nucleotide exchange factor complexes and neuronal cell bodies as a putative protein-protein interaction module that regulates a signaling pathway in dendrites, which is suggested to serve an important role in the process of cell senescence (7). KNDC1 serves a key role in a number of signal transduction pathways that help in protein recognition and functional regulation. Previous studies demonstrated that the KNDC1 activity of Ras GEF by c-Jun N-terminal kinase 1 (JNK1) and/or extracellular signal regulated kinase (ERK) via the Ras-Raf-mitogen-activated protein (MAP) kinase pathway induced MAP2 phosphorylation and microtubule binding activity, thereby promoting an increase in the length of nerve cells (8,9). A previous study also demonstrated that the inhibition or knockdown of KNDC1 possibly delayed neuronal cell senescence and promoted dendritic growth. This suggests that it serves an important role in regulating neuronal dendrite development (10). Other results also suggest that KNDC1 regulates the development of neuronal dendrites via the Ras-Raf-MAP kinase signaling pathway (11). However, only a few reports regarding the regulatory functions and mechanisms of vascular endothelial cells exist.

Previous experiments demonstrated that the knockdown of KNDC1 may promote the proliferation of endothelial cells and delay their aging. However, the effect of KNDC1 overexpression remains unclear. Therefore, a KNDC1-adenovirus vector

was constructed and an endothelial cell model that expressed KNDC1 was designed to elucidate its role in HUVECs.

Materials and methods

Chemicals and reagents. Enriched Culture Medium (ECM; cat. no. 1001; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); KNDC1-adenovirus vector was constructed by GeneWay Co., Ltd., (Shanghai China); Senescence-associated β-galactosidase (SA-β-gal) staining kit (cat. no. C0602; Beyotime Institute of Biotechnology, Shanghai, China); TRIzol™ reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.); KNDC1 (cat. no. SC-90387; Santa Cruz Biotechnology Inc., Dallas, TX, USA); PrimeScript™ RT Master Mix kit (cat. no. D3720; Takara Bio Inc., Otsu, Japan); polyvinylidene difluoride (PVDF) membranes (cat. no. IPVH00010. Immobilon-P; EMD Millipore, Billerica, MA, USA); anti-p53, anti-phospho (p)-p53 and anti-GAPDH (cat. nos. 9282T, 9284T and 5174T, respectively. Cell Signaling Technology Inc., Danvers, MA, USA); superoxide dismutase (SOD) and glutathione peroxidase (GPx) assay kits (cat. no. A001-3, A006; Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Cells and culture. Endothelial cells were isolated from umbilical cord tissue obtained from women who underwent a full-term normal pregnancy, which was supplied by Beijing Hospital (Beijing, China). The umbilical cord samples for the present study were taken on 1st August 2013 (Beijing Hospital) from pregnant women without maternal hypertensive disorders, diabetes mellitus, or any other contagious diseases (n=5; aged 23-28-years-old). The umbilical cord of the newborn was cut under aseptic conditions and preserved in normal saline at 4˚C. As the primary culture is directly from the tissue or organ part of the organism, *in vitro* time is short; the genetic characteristics and functional structure of the primary culture are similar to the body. Therefore, the primary culture is suitable for the study of cell morphology, function and differentiation. Umbilical vein endothelial cells were isolated and cultured within 12 h. Endothelial cells were isolated using an enzyme perfusion digestion method (0.1% type I collagenase at 37˚C for 10-12 min) following primary passage (P0), inoculated in ECM containing 100 mg/ml streptomycin, 100 IU/ml penicillin, 40 μg/ml endothelial cell growth supplement and 20% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Endothelial cells were identified based on two aspects, morphology and immunohistochemistry, after they were grown to the second or third generation.

Under an inverted phase contrast microscope (magnification, x200), the morphology of endothelial cells following passage were typically spherical, spindle or cobblestone; HUVECs were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS three times and blocked with 3% bovine serum albumin (BSA) in PBS (Shanghai Qiao Yu Biological Technology Co., Ltd., Shanghai, China) for 15 min at 37˚C. Subsequently, overnight incubation at 4˚C was conducted with primary antibodies diluted in 1% BSA PBS [cluster of differentiation (CD)31, CD34, von Willebrand factor, vascular endothelial growth factor (VEGF) receptor 1, VEGF receptor 2 (cat. nos. 3528S, 3569S, 65707S, 2893S, 9698S, respectively, Cell Signaling Technology Inc.), rewarmed 30 min at 37˚C and rinsed with PBS three times (5 min per wash). Then a fluorescein isothiocyanate-labeled rabbit secondary antibody (1:50; cat. no. sc-2359, Santa Cruz Biotechnology Inc.) was added and HUVECs were incubated for 1 h at room temperature, washed with PBS 3 times (3 min per wash); propidium iodide dye (Beijing Zhongsheng Ruitai Technology Co., Ltd. Beijing, China) was added for incubation for 5 min at room temperature. HUVECs were washed with PBS 3 times (3 min per wash), then immediately observed under a fluorescence microscope (535 nm excitation wavelength, magnification, x200). The cytoplasm of endothelial cells exhibited yellow-green fluorescent staining and the nucleus was dark green; the nucleus and the cell outline were clear. The purity of endothelial cells was close to 100% and expressed VEGF. Based on the data from the author's previous study (7), the purity of endothelial cells at the passage 6 (P6) generation was close to 100%. Endothelial cells at P6 were used for further experiments and were seeded into 6-well plates.

Transfections. KNDC1-adenovirus vector was constructed by GeneWay Co., Ltd. P6 endothelial cells ($7.5x10^5$) were selected to be transfected with the KNDC1-adenovirus vector and cultured for 24 h at 37˚C. A non-targeting control vector (NT-adenovirus vector; GeneWay Co., Ltd.) was also used [60 plaque forming units (pfu)/cell]. They were grouped as follows: i) Blank control (no treatment; C) group; ii) negative control group (A); HUVECs were transfected with NT-adenovirus vector; iii) HUVECs were transfected with 30/60/90 pfu/cell KNDC1-adenovirus vector (K30/60/90 experimental group, respectively). Finally, they were all cultured in ECM for 24 h as described above.

SA-β-gal staining. For anti-senescence experiments, human endothelial cells ($7.0x10^5$) were cultured in ECM using the aforementioned procedure. The endothelial cells were transfected with different doses of the KNDC1-adenovirus in the experimental group. A blank control group and a negative control group was also used.

Following a 2-day culture, the cell culture medium was removed and the cells were washed twice with PBS. They were then fixed for 15 min with PBS containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature. Following removal of the fixative solution, the cells were washed three times with PBS and then dyeing liquid was added. The cells were then incubated at 37˚C for 10 h in a staining solution of 40 mM citric acid, sodium phosphate, (pH 6.0), 1 mg/ml 5-brom o-4-chloro-3-isolyl-β-d-galactoside (X-gal; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$. Finally, they were observed under an inverted microscope (CKX31; Olympus Corporation, Beijing, China) at a magnification of x40. A blue stained cytoplasm indicated that the cells were aged. A total of five visual fields were randomly selected. A positive incidence of SA-β-gal was the percentage of positive cells in the total cell count. The experiment was repeated in triplicate.

RNA expression analysis. After the human endothelial cells (7.0×10^5) were treated with or without the KNDC1-adenovirus vector using the aforementioned procedure, total cellular RNA was extracted with TRIzol reagent according to the manufacturer's protocol. A 2 μl RNA sample was taken to determine its concentration and purity. The total RNA sample was reverse transcribed to cDNA according to the manufacturer's protocol of the PrimeScript™ RT Master Mix kit. The synthesized cDNA samples were subjected to reverse transcription (RT)-qPCR according to the protocol provided by the AceQ™ qPCR SYBR Green Master Mix kit (Vazyme, Piscataway, NJ, USA). GAPDH was used as a reference gene. The primer pairs used in the study are as follows: KNDC1, forward (FW): 5'-CAGGCTTCTTTCCCTACTGTTCGT-3', reverse (RV): 5'-CCGCTGCTTGTTTTGATAGTTCTC-3'; GAPDH (Shanghai Bioengineering Inc., Shanghai, China), FW: 5'-CGCTGAGTACGTCGTGGAGTC-3', RV: 5'-GCT GATGATCTTGAGGCTGTTGTC-3'. The PCR was run on an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The reaction conditions were as follows: 95°C for 5 min initial denaturation, followed by 40 cycles of 95°C for 10 sec and finally 60°C for 20 sec. The experimental results were analyzed by $2^{-\Delta\Delta Cq}$ method (12).

Western blotting. Endothelial cells (1.0×10^6) were collected with a cell scraper and 120 μl radio immunoprecipitation assay buffer was added to lyse the cells. Following lysis, the supernatant was collected and the sample buffer was added. Protein samples were prepared by boiling in water for 5-10 min. The supernatant collected following cell lysis was used to quantify protein concentration in the endothelial cells using standardized bicinchoninic acid protein. Based on the quantitative results, the protein samples (30 μg) were subjected to SDS-PAGE (10%) electrophoretic separation and the membrane reaction was carried out on the PVDF membrane (Immobilon-P; EMD Millipore). The PVDF membranes were then blocked with 5% non-fat milk for 30 min at room temperature, followed by diluted rabbit primary antibodies (1:1,000) as follows: Anti-p53, anti-phospho-p53 (Cell Signaling Technology Inc.). The membranes were incubated in diluted rabbit primary antibodies overnight at 4°C. Then diluted corresponding horseradish peroxidase-conjugated secondary antibodies (1:4,000; Cell Signaling Technology Inc.) were added for incubation for 1 h at room temperature. Chemiluminescence was performed in a gel imaging system and protein bands were visualized by incubating the membranes with high-performance autoradiography film (Fuji Film Co., Tokyo, Japan). The experimental results i.e., gray band analysis of protein bands with β-actin (cat. no. SC-8432, Santa Cruz Biotechnology Inc.) or GAPDH (cat. no. sc-47724, Santa Cruz Biotechnology Inc.) was carried out by using the ImageJ 2.1 software (National Institutes of Health, Bethesda, MD, USA). Values were calculated in terms of integrated optical density and expressed in arbitrary units.

Oxidative stress. The KNDC1-adenovirus vector was added to cells in the logarithmic growth phase followed by incubation for 24 h. Endothelial cells (5×10^5) from the KNDC1-adenovirus vector group, negative control group A and control group C were washed three times with PBS followed by digestion with 0.25% trypsin for 30 sec at room temperature. ECM containing FBS was added to terminate the digestion process. Next, the cell suspensions were collected, centrifuged at 1,000 x g for 5 min at room temperature and counted under an inverted phase contrast microscope (magnification, x200). Following cell density calculation and adjustment, the cells were seeded into Eppendorf tubes, each containing 1×10^4 cells, in 200 μl cell lysate solution [0.05 mmol/l EDTA, 1% Triton-X 100, (pH 8.0)]. The tubes were agitated to allow the lysate solution to completely lyse the cells, following which, the tubes were placed on ice for 20 min and finally centrifuged at 12,000 x g for 20 min at 4°C to isolate the supernatant. The SOD and GPx activities were detected by an ultraviolet spectrophotometer (excitation wavelength, 450 nm) (Bio-Rad Laboratories Inc.).

Statistical analysis. Statistical analysis was performed using SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL, USA). All the data are expressed as the mean ± standard deviation. The Student's t-test was used for comparisons between 2 groups. The 3 groups were compared using a single factor analysis of variance. The Student Newman-Keuls method was used to compare between each group if the difference was statistically significant. P<0.05 was considered to indicate a statistically significant difference.

Results

KNDC1 expression increases with aging of HUVECs. In a previous study (7), it was observed that with increasing passage number, the number of aging HUVEC cells increases, as demonstrated by SA-β-gal staining. In the 1-2nd generation of the endothelial cells cell morphology was observed to be polygonal, intercalated with transparent cytoplasm, a blurred cell outline and a unilateral cobblestone arrangement. With an increase in generation number, cell morphological alterations occurred, including a flattened morphology, greater volume, lower proliferative rates and slower growth. The 4-5th generation of the endothelial cells were not of uniform size, exhibited cytoplasm turbidity, a clear cell outline, an increased volume, slow growth, a sparse arrangement and difficulty forming a monolayer of cells (Fig. 1A) (7). The present study investigated whether KNDC1 was associated with the senescence of normal cells. The level of KNDC1 transcription and expression in the HUVECs was examined at different passages by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The results demonstrated that the transcription and expression levels of KNDC1 increased gradually with the aging of the HUVECs with the mRNA level significantly increasing at P5 whereas the protein expression level was significantly increased from P3 (P<0.05; Fig. 1B and C) (7).

KNDC1-adenovirus vector increases KNDC1 mRNA and protein expression levels. In order to investigate the association between KNDC1 and the HUVEC aging process, the expression levels of KNDC1 were upregulated in P6 HUVECs by transfecting them with the KNDC1-adenovirus vector (Fig. 2). As exhibited in Fig. 2C, transfection with the KNDC1-adenovirus vector resulted in a 239-344% increase in KNDC1 mRNA levels. Similarly, KNDC1 protein levels
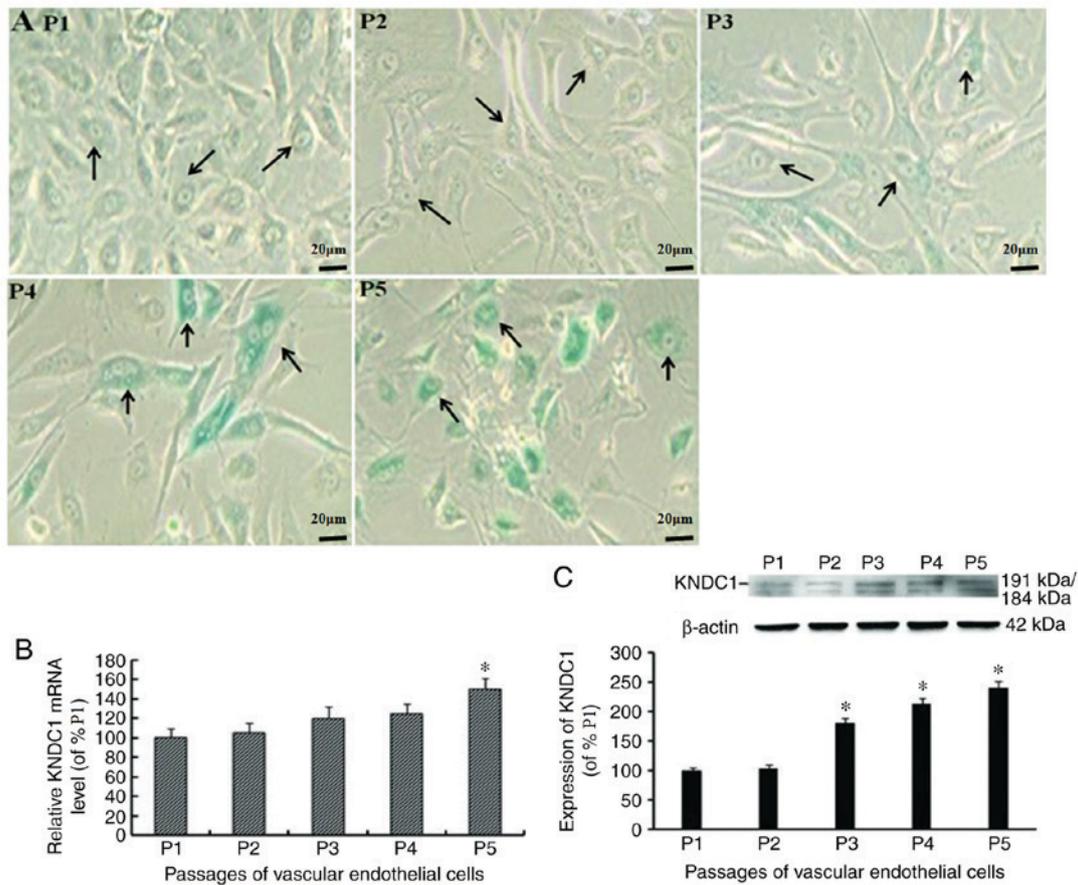
Figure 1. KNDC1 expression in HUVECs of differing passages. (A) Expression of KNDC1 and senescence in different of generations of human umbilical vein endothelial cells *in vitro* (adapted from Mol Med Rep 10: 82-88, 2014, with permission from Zhang *et al*) (7). Senescence associated-β-galactosidase staining of cell senescence at different passages (magnification, x200); Black arrows indicate the generation of the typical morphological characteristics of cell senescence. (B) mRNA and (C) protein levels of KNDC1 detected by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Data are presented as the mean ± standard deviation. *P<0.05 vs. P1. KNDC1, kinase noncatalytic C-lobe domain containing 1; P, passage.

among the groups also demonstrated a statistically significant increase in a dose-dependent manner (Fig. 2B).

Overexpressing KNDC1 enhances senescent phenotype. Furthermore, KNDC1 overexpression notably promoted the senescent phenotype of HUVECs. The number of positive SA-β-Gal staining observed in the HUVECs following the transfection of the KNDC1-adenovirus vector significantly increased when compared with that in the control group (Fig. 3A). The positive rate of SA-β-gal staining in the HUVECs also increased in a dose-dependent manner over a 24 h incubation period i.e., with increasing transfection doses of the KNDC1-adenovirus vector from 30-60 pfu/cell. However, the morphological difference of endothelial cells between the groups with different transfection doses was not obvious, only the positive rate of SA-β-gal staining increased. Morphological alterations may require long-term observation. The positive rate of SA-β-gal staining in each group (Fig. 3A) was group C 6.2±1.3%, group A 8.2±0.3%, K30 26.3±2.5%, K60, 62.9±2.8% and K90 55.3±5.8%. No significant difference (P=0.054) was observed between the positive rates in SA-β-gal staining at the K60 and K90 doses (Fig. 3B), and the 60 pfu/cell resulted in the highest level of positively stained cells. Therefore, transfection was carried out at 60 pfu/cell in the experimental group for further studies.

Phosphorylated (p)p53 expression increases if KNDC1 is overexpressed. Furthermore, the genes associated with HUVEC senescence were investigated by examining the activation of the phosphorylated forms of their proteins by western blot analysis. The results demonstrated no significant difference in p53 (an important cell cycle inhibitory factor) expression in the experimental HUVEC group compared with the blank and negative control groups. However, a significant increase in the expression of p-p53 (Fig. 4A) was observed in HUVECs that overexpressed KNDC1 when compared with the NT-adenovirus-transfected control cells (244.3±19.5 vs. 113.5±9.0; Fig. 4B), which suggests that the p53 signaling pathway probably serves an important role in the senescence process of the HUVECs.

ROS activity increases with KNDC1 overexpression. The author's previous study demonstrated that KNDC1 knockdown delayed HUVEC senescence by decreasing intracellular ROS (7). In the present study, this alteration was further verified. After 24 h of transfection with KNDC1, the activity of antioxidant enzymes in HUVECs significantly decreased. The antioxidase content in the
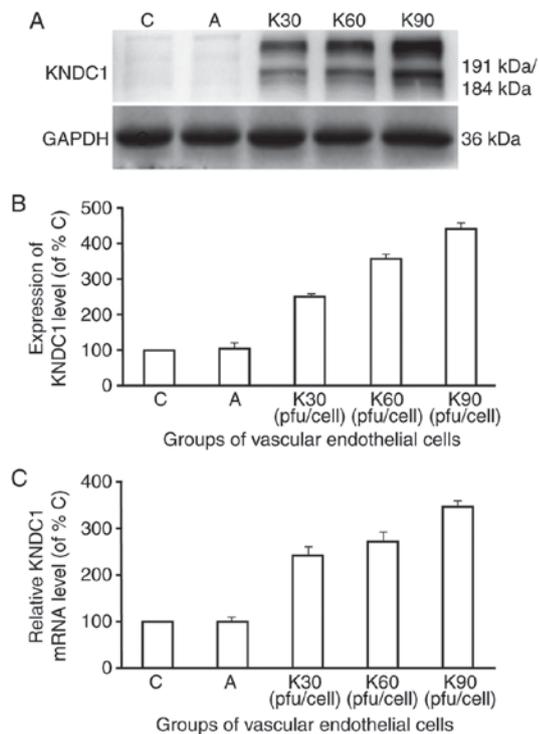
Figure 2. Expression levels of KNDC1. (A) Representative image of western blots. GAPDH served as loading control. (B) Quantified results of KNDC1 expression analyzed by western blotting. (C) KNDC1 transcription was analyzed by reverse transcription-quantitative polymerase chain reaction (n=3). Data are mean of three replicate experiments (n=3) ± standard deviation. Bars having different letters are significantly different (*P<0.05 vs. C and A groups). C, blank control group; A, negative control group, HUVECs were transfected with adenovirus; K30/60/90, experimental group, HUVECs were transfected with 30/60/90 pfu/cell KNDC1-adenovirus. KNDC1, kinase noncatalytic C-lobe domain containing 1; HUVECs, human umbilical vein endothelial cells; pfu, plaque forming units.

experimental group was decreased compared with the control group (SOD: 83.3±5.1 vs. 98.0±3.0; GPx: 83.0±5.6 vs. 95.0±6.0; *P=NS*; Fig. 5). These results suggested that KNDC1 overexpression accelerated the senescence process in cells by inhibiting the antioxidants (SOD and GPx) and activating ROS generation.

Discussion

Aging or cell senescence is an important risk factor for CVD. The development of vascular endothelial cellular senescence serves an important role in several biological processes (13). Senescent cells exhibit several characteristic features including positive staining for SA-β-gal, enlarged and flat cellular morphology and specific gene expression patterns (14-16).

As a novel Ras GEF protein, a study described KNDC1 as one of the genes associated with senescent cells (17). The preliminary study also demonstrated that the inhibition and/or knockdown of KNDC1 expression promotes cerebellar granule cells and hippocampal neuron dendritic growth in cultured cells, suggesting it is a signaling molecule involved in the development, regulation, and restriction of cell growth (18,19). However, there are few studies in this field, which are insufficient to confirm the mechanism of KNDC1 in promoting the aging of endothelial cells. The regulatory functions and mechanisms of KNDC1 on vascular endothelial

cells have not been reported. HUVECs are frequently used as biological senescent models due to their limited capacity to divide when cultured *in vitro* (20,21). Therefore, in the present study, a biological senescence model was constructed by culturing HUVECs *in vitro* and then the effect of KNDC1 on cell senescence was investigated. In the present study KNDC1-adenovirus-transfected HUVECs were demonstrated to undergo senescence over a short time-period (24 h). It was also demonstrated that with an increased number of passages, SA-β-gal staining demonstrated a gradual increase in the number of senescent endothelial cells and that the transcription and expression levels of KNDC1 also increased gradually.

To further investigate the effect of KNDC1 on endothelial cell senescence, gene transfection techniques were used. The recombinant KNDC1-adenovirus was constructed through a homologous recombination of cells, followed by the KNDC1 gene being transfected into HUVECs by the adenovirus vector. On comparing the positive rates of KNDC1-adenovirus transfection at different doses, the group dosed with 60 pfu/cell exhibited SA-β-gal staining similar to the group dosed with 90 pfu/cell, however increased activity compared with the group dosed with 30 pfu/cell. RT-qPCR and western blotting results further demonstrated that the mRNA and protein expression levels in the KNDC1-adenovirus vector group were increased compared with the empty vector and blank group following a 24 h incubation. Typical morphological alterations including cell enlargement, multinucleation and the activity of the lysosomal enzyme β-galactosidase were also observed to be increased in HUVECs where the expression of KNDC1 was upregulated with the KNDC1-adenovirus vector when compared with the NT-adenovirus-transfected HUVECs of the same passage.

Following this, the mechanism of the functional alterations in HUVECs following transfection with the KNDC1-adenovirus vector was investigated. Consistent with previous results, the overexpression of KNDC1 led to alterations in the expression of specific genes closely associated with cell growth regulation (p-p53) in the present study (22). Previous studies have confirmed p53 as a regulator of the DNA damage response and p53/p21 are thought to serve a role in hydrogen peroxide induced cell growth, inactivity, and replicative senescence (23,24). Transcription factor p53 is one of the most important proteins that inhibits the aging process and its multiple isoforms also serve a role in maintaining genomic integrity (25). p53 performs specific biological functions ranging from transient or permanent cell cycle arrest to cell death (26,27). In the present study, the results of the western blotting experiments demonstrated that although the p53 expression did not markedly alter in the endothelial cells transfected with the KNDC1-adenovirus vector, p53 phosphorylation and p-p53 expression increased. It was reported that p53 and its binding partners may be modified (e.g., via phosphorylation) to induce a conformational change in their protein structure or to directly interfere with their interactive process (28). Therefore, the increase in p53 phosphorylation indicates that KNDC1 exerts an aging effect by regulating p53 protein modification, increasing the expression of functional p53 and then activating downstream genes (29).

A previous study demonstrated that p53 exerts its antiproliferative function via the regulation of energy metabolism and oxidative stress (30). One of the stress factors closely
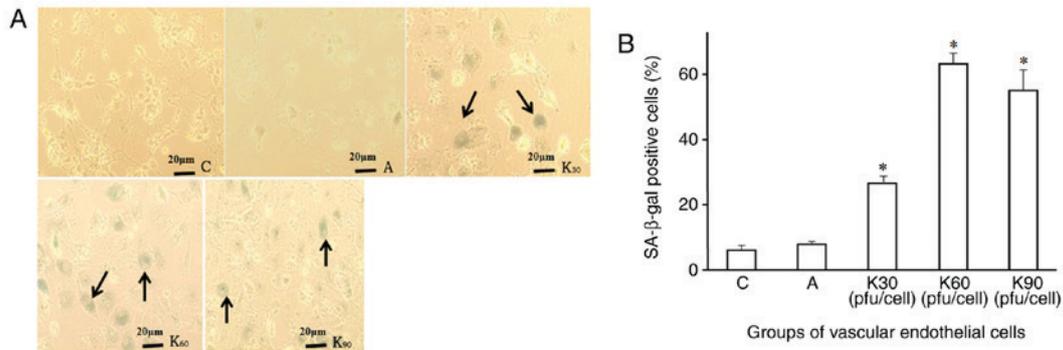
Figure 3. SA-β-gal activity was induced by transfection of KNDC1-adenovirus vector. (A) Representative images. Black arrows indicate the generation of the typical morphological characteristics of cell senescence. (B) Senescence level of the HUVECs was evaluated by the ratio of SA-β-Gal-positive to total cells. Data are presented as the mean of three replicate experiments (n=3) ± standard deviation. Bars having different letters are significantly different (*P<0.05 vs. C and A groups). C, blank control group; A, negative control group, HUVECs were transfected with adenovirus; K30/60/90, experimental group, HUVECs were transfected with 30/60/90 pfu/cell KNDC1-adenovirus. KNDC1, kinase noncatalytic C-lobe domain containing 1; HUVECs, human umbilical vein endothelial cells; pfu, plaque forming units; SA-β-Gal, senescence-associated β-galactosidase.
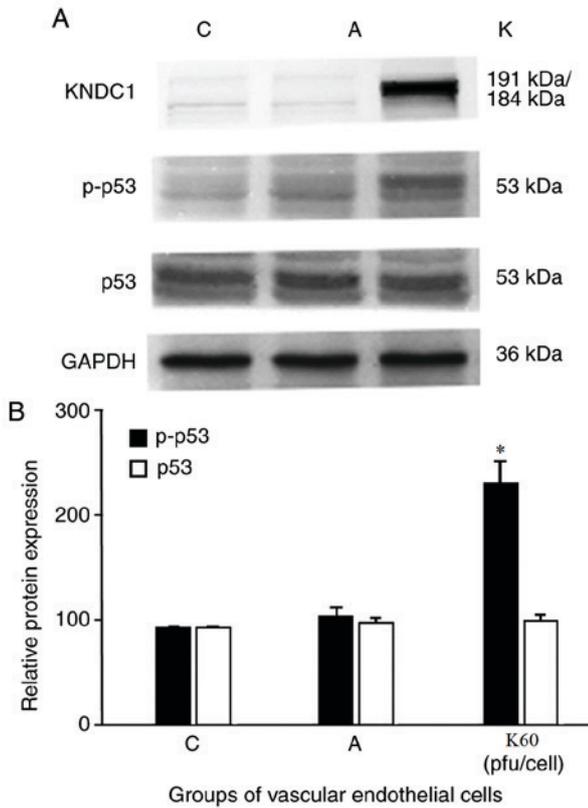
Figure 4. Effect of KNDC1 overexpression on p53 and p-p53 activity in HUVECs. (A) Typical western blots for total p53, p-p53 are shown. GAPDH served as the loading control. (B) Summary of quantification of densitometric measurement of the immunoblot data. Data are presented as the mean of three replicate determinations (n=3) ± standard deviation. Bars having different letters are significantly different (*P<0.05 vs. C and A groups). C, blank control group; A, negative control group, HUVECs were transfected with the adenovirus; K60, experimental group, HUVECs were transfected with 60 pfu/cell KNDC1-adenovirus. KNDC1, kinase noncatalytic C-lobe domain containing 1; HUVECs, human umbilical vein endothelial cells; pfu, plaque forming units; p, phosphorylated.
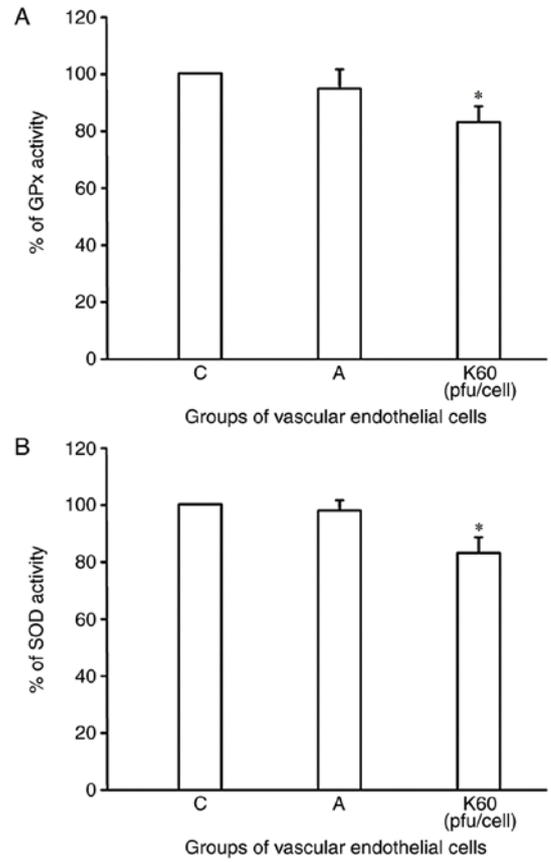
Figure 5. Effect of KNDC1 overexpression on antioxidant activity. (A) SOD and (B) GPx activity in HUVECs. Data are presented as the mean of three replicate determinations (n=3) ± standard deviation. Bars having different letters are significantly different (*P<0.05 vs. C and A groups). C, blank control group; A, negative control group, HUVECs were transfected with adenovirus; K60, experimental group, HUVECs were transfected with 60 pfu/cell KNDC1-adenovirus. KNDC1, kinase noncatalytic C-lobe domain containing 1; HUVECs, human umbilical vein endothelial cells; pfu, plaque forming units; SOD, superoxide dismutase; GPx, glutathione peroxidase.

associated with aging is an increase in intracellular ROS levels (31). In the present study, it was demonstrated that increased phosphorylated p53 levels compared with the control led to an increased production of ROS in a dose-dependent manner. Therefore, the activation of the p53 pathway may be in response to the ROS-induced DNA damage (32). p53 may downregulate the expression of antioxidant enzymes including SOD and GPx under physiological conditions

when acutely stressed. Furthermore, p53 exhibits pro-oxidant activity and may exacerbate oxidative stress responses to cell sustained stressors (33). ROS may also activate p53 and initiate p53-dependent stress-response programs including cell cycle arrest, senescence, and apoptosis (34,35). Therefore, the positive feedback loops of p53 and ROS transform stressed cells into apoptotic or senescent cells. Additionally, p53 activation initiates a series of downstream transcriptional events that lead to cell growth inhibition and senescence. These results suggest that KNDC1 may serve an important role in the aging process of HUVECs and that the overexpression of KNDC1 promoted the aging process.

In conclusion, the overexpression of KNDC1 inhibited proliferation and promoted senescence of the HUVECs. With respect to the mechanism of action, p53 proved to be an essential inducer of KNDC1-mediated senescence. Furthermore, it was demonstrated that the overexpression of KNDC1 increased the expression of ROS by activating the p53 signaling pathway. The p53-ROS positive feedback loop probably served an important role in the regulation of KNDC1-induced cell senescence. KNDC1 also promoted the activity of p53, the amplification of the p53-ROS feedback loop and then served a catalytic role in the cell senescence process by increasing the expression of the phosphorylated p53 protein. The present study differs from previous research (17-19) as it further demonstrated the role of KNDC1 overexpression in promoting endothelial cell senescence and identified a novel molecular mechanism (p53-ROS positive feedback loop). To the best of the authors' knowledge this study is the first time that KNDC1-adenovirus vector inhibition of HUVEC proliferation by activating the p53 signaling pathway has been reported. Elucidating the biological function and molecular mechanism of KNDC1 will provide theoretical support for the study of cell senescence mechanism. The authors' next step will be to carry out experiments *in vivo*, to further invest the mechanism of the high expression of KNDC1 induced senescence of endothelial cells and potentially provide novel targets for anti-aging treatment. Finally, the results depict KNDC1 as a promising candidate since it delays the aging process and prolongs human life.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JJ and ZH designed the present study, conducted experiments, analysis and interpretation of data, and drafting of manuscripts. HL and YJL reviewed the concepts of the experiment. YL, JL, CM and BL were involved in experimental analysis and data acquisition. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study had obtained human research ethics approval from the Ethics Committee of Beijing Hospital on April 15th, 2013. The informed consent of the subjects was obtained at May 2nd, 2013. Human umbilical vein endothelial cells (HUVECs) used in this study were isolated by Dr. Lin in 1st August 2013 (Beijing Hospital).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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