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Original Article

Fibroblast growth factor 21 delayed endothelial replicative senescence and protected cells from H_2O_2-induced premature senescence through SIRT1

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Abstract: Vascular aging is an independent risk factor for age-related diseases, including atherosclerosis. Fibroblast growth factor 21 (FGF21) has been widely recognized as a metabolic regulator that is elevated in response to caloric and nutritional restrictions. Recent studies have demonstrated its emerging role as a pro-longevity hormone, but its effects on the senescence of human umbilical vascular endothelial cells (HUVECs) remain unclear. In the present study, we explored the anti-senescence effects and underlying mechanism of FGF21 on HUVECs. Co-cultivation of HUVECs with 5 ng/mL FGF21 significantly attenuated the phenotype changes of cells during *in vitro* subculture, including increased senescent population, decreased proliferation rate, decreased SIRT1 and elevated P53 and P21 protein levels. FGF21 also protected HUVECs from H_2O_2-induced cell damage, including premature cell senescence, intracellular accumulation of reactive oxygen species, increased DNA damage, decreased SIRT1 protein level and elevated protein levels of VCAM-1, ICAM-1, P53 and P21. Transient knockdown of *SIRT1* in HUVECs significantly suppressed the protective effects of FGF21 for the rescue of H_2O_2-induced premature senescence and DNA damage, which suggests that the anti-senescence effect of FGF21 on HUVECs is SIRT1-dependent. These results support the potential of FGF21 as a therapeutic target for postponing vascular aging and preventing age-related vascular diseases.

Keywords: Fibroblast growth factor 21, HUVEC, replicative senescence, oxidative stress-induced premature senescence, SIRT1

Introduction

Fibroblast growth factor 21 (FGF21), which is a member of the FGF family, is mainly secreted by the liver, and it is also expressed in the adipocytes, skeletal muscle and pancreas [1-4]. Circulating FGF21 is elevated in an adaptive response to starvation and other nutritional restrictions [5-8] and promotes many beneficial physiological effects, including decreased glucose levels, increased insulin sensitivity, and improved lipid metabolism [9-14]. Recently, evidence has emerged indicating that the function of FGF21 is not limited to metabolic regulation but is also implicated in the anti-aging process. Chronic exposure to FGF21 markedly extends lifespans in mice [15] and provides similar benefits to those obtained by calorie restriction (CR), which is a confirmed enhancer of longevity [16]. Both CR [9] and nutritional restrictions, including protein deficiency [17] and methionine deficiency [18], have been reported to be sufficient to increase the serum FGF21 level and restore a younger phenotype in humans and mice. The anti-atherosclerosis [19], anti-inflammatory [20] and anti-oxidant [21-23] properties of FGF21 have also been reported. Thus, FGF21 has been called either a starvation or a pro-longevity hormone.

Vascular aging, which has been morphologically and functionally defined as increased vascular stiffness and pulse wave velocity, an enlarged vascular lumen, and decreased vascular elasticity, is considered to be an independent risk factor for age-related disease, especially for cardiovascular diseases such as atherosclerosis, hypertension and stroke [24, 25]. Vascular

cell senescence, particularly vascular endothelial cell (VEC) senescence, plays a critical role in vascular aging and in the initiation and advancement of vascular disease [24, 25]. The loss of proliferative potential during the *in vitro* culture of cells is a hallmark of replicative cell senescence, which results from the incompetency of VECs to repair the disrupted endothelial monolayer caused by increased vascular stiffness and other pathological stresses with aging [24, 26].

In humans, aging is also considered to be a state of chronic, systemic "oxidative stress" with the elevation of age-related oxidative stress. Aging vasculature generates excess reactive oxygen species (ROS), superoxides and hydrogen peroxide that compromise the vasodilatation activity of nitric oxide (NO) and facilitate the formation of deleterious radicals, peroxynitrite, etc. [27]. Vascular aging is also accompanied by an altered oxidative environment that can cause an increase in ROS levels in cells, including increased extra-/intra-cellular levels of oxidized low-density lipoprotein [28], increased hypoxia and ROS in the vascular peripheral tissues [29] and other stimuli, such as hyperglycemia, hyperlipidemia, hypertension, low-grade chronic inflammation, advanced glycation end-product accumulation, and angiotensin II [24, 25, 27, 30-32]. These oxidative stress-induced damages are a driving factor that causes stress-induced premature senescence of VECs and eventually endothelial injury, which leads to acceleration of vascular aging and development of age-related vascular diseases [24, 25, 33]

Although the pro-longevity properties of FGF21 have been reported, the effects of FGF21 on VEC senescence remain unclear. In this study, we explored the anti-senescence effects and the underlying mechanism of FGF21 in both replicative senescence and H_2O_2-induced premature senescence of human umbilical VECs (HUVECs).

Materials and methods

Cell isolation

Primary HUVECs were isolated from segments of fresh newborn human umbilical cords within 4 h of birth under approved guidelines set by the Ethics Committee for Human Experiments of Tongji Hospital, Huazhong University of Science and Technology. Written informed consent was obtained from all donors. Briefly, the inner wall of the segments of the newborn's umbilical cords were digested by 0.1% collagenase II (Worthington, USA) for 13 minutes at 37°C, as previously described [34]. Cells were precipitated and re-suspended in Medium 199 supplemented with 10% fetal bovine serum (FBS), low serum growth supplement (LSGS) (Gibco, USA), and 1% penicillin-streptomycin in a humid incubator at 37°C in 5% CO_2. Subcultures were obtained by treating cells with 0.05% trypsin ethylenediaminetetraacetic acid (EDTA) solution (Gibco, USA). The cell type was confirmed by the "cobblestone" cell morphology and by histochemical staining with a monoclonal anti-human vWF antibody (Boster, China).

Cell viability assay

Cell viability was assessed with the cell counting kit-8 (CCK-8, Boster, China) assay according to the manufacturer's guidelines. HUVECs were starved in 2% FBS for 12 h and then treated with different concentrations of FGF21 (ProSpec, Israel) for an additional 12 h. Cells were then treated with 10 μL CCK-8 solution for 3 h, and the absorbance was read at a 450-nm wavelength.

FGF21 treatment and cell senescence

FGF21 treatment was performed by supplementing the cell media with different concentrations of FGF21 as indicated in the figures. The replicative senescence of HUVECs was obtained by sustaining cell passage, as previously described [35, 36]. The oxidative stress-induced premature senescence of HUVECs was established by adding 50 μmol/L H_2O_2 [37] to the medium after FGF21 treatment, and the medium was changed every 36 h with medium containing 5 ng/mL FGF21.

Senescence-associated beta-galactosidase activity assay

Senescence-associated beta-galactosidase (SA-β-gal) activity was measured according to the manufacturer's protocol. Briefly, HUVECs were washed 3 times in phosphate-buffered saline (PBS), fixed for 15 min at room temperature by the fixative solution, and incubated overnight at 37°C with fresh SA-β-gal stain solution at pH 6.0 (Beyotime, China). The percentage of SA-β-gal was calculated by counting

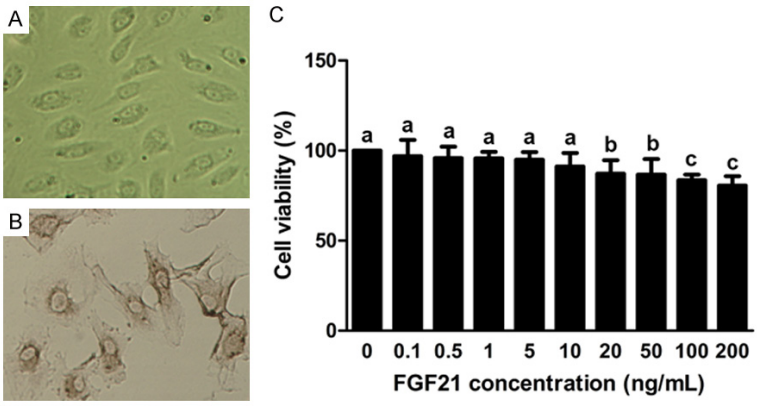

Figure 1. Cell identification and cell viability test. HUVECs were isolated by collagenase II digestion (A) and identified by vWF factors (× 100 magnification) (B). (C) Cell viability was detected with CCK-8. Different concentrations of FGF21 were added to the medium and absorbance was observed after 12 h. Values are the mean ± SD; n = 5 in each group. Different letters indicate differences between the treatment groups, $P < 0.05$.

the positively stained cells within a sample of 200 cells (× 100 magnification).

ROS level was measured by flow cytometry

ROS level was measured by 2',7'-dichlorofluorescein diacetate (DCHFDA). Briefly, HUVECs were seeded in 6-well plates and treated with or without 5 ng/mL FGF21 for 12 h before they were treated with 50 μM H_2O_2 for 48 h. The cells were harvested and washed, then incubated with 10 μM DCHFDA for 30 min at 37°C and detected with flow cytometry.

DNA damage detected with γ-H2AX immunofluorescence

DNA double-strand breaks in chromatin were detected using γ-H2AX immunofluorescence [38]. Briefly, cells were blocked with a specific blocking buffer for 1 h at room temperature in a humidified chamber, and then the cells were incubated with the primary antibodies anti-γ-H2AX (1:200 dilution, Santa Cruz, USA) overnight at 4°C. After washing the cells with phosphate-buffered saline containing 0.15% Triton X-100, the cells were incubated for 1 h at room temperature with fluorescently tagged secondary antibodies (1:1000 dilution, Promotor, China), followed by a 5-min incubation with DAPI to identify nuclei.

siRNA silencing and RT-PCR

The small-interfering RNA (siRNA) utilized in this study targeted the following sequences of the human *NAD-dependent deacetylase sirtuin-1* (*SIRT1)* gene: sense, 5'-GCTAAGAATTTCAGGATTA-3' (Ribobio, China). HUVECs were transfected with 100 nmol human *SIRT1* siRNA using a LipoJet transfection kit (SignaGen, USA) and opti-medium supplemented with 20% FBS overnight according to the manufacturer's instructions.

Seventy-two hours after siRNA transfection, the cells were harvested. Total RNA was extracted with anHI Pure RNA extract Kit (Magen, China) and converted to cDNA using a ReverTra Ace qPCR RT kit (TOYOBO, Japan). The silencing efficiency was detected by RT-PCR performed on the ABI Step One Plus (Applied Biosystems, USA) with SYBR green PCR mix (TOYOBO, Japan) according to the manufacturer's instructions. The specific oligos used in the study were as follows: *SIRT1* (forward): 5'-TCAGTGGCTGGAACAGTGAG-3', *SIRT1* (reverse): 5'-ACTGATTACCATCAAGCCGC-3'; *GAPDH* (forward): 5'-TCCAAAATCAAGTGGGGCGA-3', *GAPDH* (reverse): 5'-AAATGAGCCCCAGCCTTCTC-3'. The fold change of relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Protein lysates were resolved by gel electrophoresis and transferred onto polypropylene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked using 5% nonfat dried milk in TBST for 1 h at room temperature and subsequently probed with the specific primary antibodies anti-SIRT1, anti-P53, anti-VCAM-1, anti-ICAM-1 (1:1000 dilution, Cell Signaling Technology, USA), anti-P21 (1:1000 dilution, Santa Cruz, USA), and anti-GAPDH (1:1000 dilution, Promotor, China) overnight at 4°C. The membranes were then washed and incubated again with a peroxidase-conjugated secondary antibody (1:5000 dilution, Promotor, China) at room temperature for 1h before they were visualized using an enhanced chemiluminescence detection method (Boster, China) and a UVP imaging system (UVP, USA).

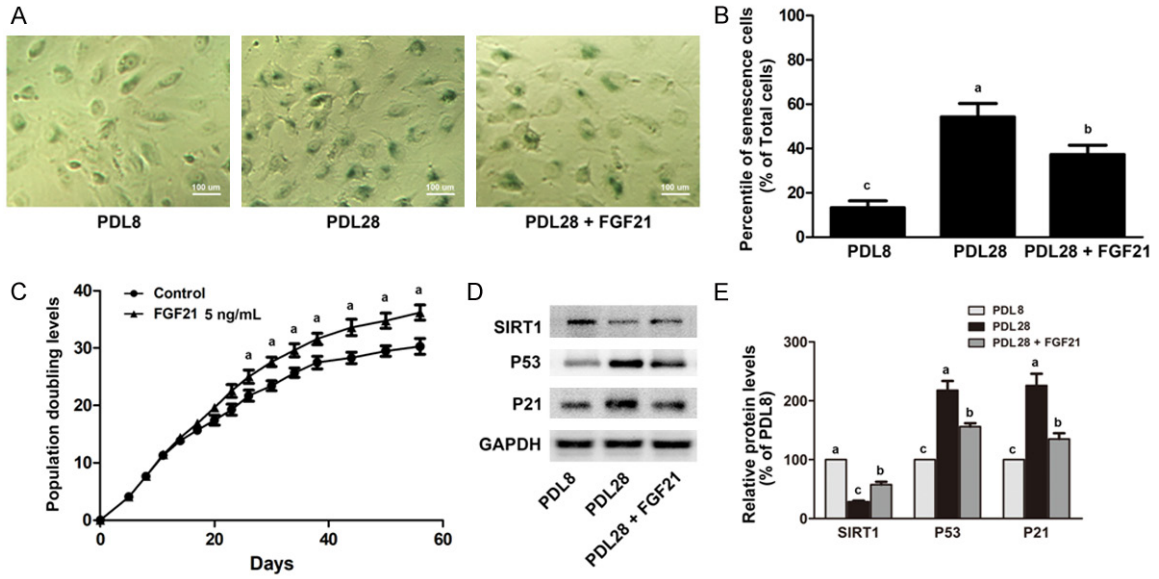

Figure 2. FGF21 intervention delayed HUVEC replicative senescence. A representative picture (A) and the histogram (B) of the SA-β-gal staining (× 100 magnification) of HUVECs from earlier passages (PDL8), later passages (PDL28) and the later passage cells supplemented with 5 ng/mL FGF21 during subculture (PDL28 + FGF21). (C) The replicative potential of HUVECs (Control) and cells co-cultivated with 5 ng/mL FGF21 (FGF21) were expressed as the population doubling time (population doubling levels vs times). The representative picture (D) and the histogram (E) of levels of proteins SIRT1, P53, P21 detected by western blot analysis with protein GAPDH as an internal reference. Values are the mean ± SD; n = 3 in each group. Different letters indicate differences between the treatment groups, $P < 0.05$.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) for the number of replicates indicated. Statistical analysis was performed by using an unpaired Student's t-test using SPSS 19.0 software (SPSS Inc., USA). *P* values < 0.05 were considered statistically significant.

Results

The effects of FGF21 on cell viability

Primary HUVECs isolated from the fetal umbilical cords were identified and confirmed by vWF factors (**Figure 1A**, **1B**). The purity of HUVECs was more than 98%, and the cells were used in the following experiments. Toxicity of FGF21 to HUVECs was evaluated by CCK-8 (**Figure 1C**), and the results indicated that FGF21 at a low concentration (0-10 ng/mL) had no significant effect on the cell viability of HUVECs (P > 0.05), whereas FGF21 at concentrations higher than 20 ng/mL slightly decreased cell viability in a dose-dependent manner (P < 0.05). The 5 ng/mL concentration was then used in the following experiments that required FGF21 treatment.

FGF21 intervention delayed HUVEC replicative senescence

Cell senescence was evaluated by SA-β-gal staining, and the protein levels of SIRT1, P53, P21 were assessed by western blotting at the indicated population doubling level (PDL). As shown in **Figure 2A**, **2B**, during *in vitro* subculture, the percentage of positive SA-β-gal stained cells significantly increased in the senescent cells (54.3% ± 6.0% in the later-passage cells, ~PDL28) compared to the younger cells (13.3% ± 3.1% in the earlier-passage cells, ~PDL8) (P < 0.05), whereas the cells co-incubated with 5 ng/mL FGF21 to PDL28 (PDL28 + FGF21) had significantly fewer senescent cells (47.3% ± 4.2%) compared to PDL28 in the absence of FGF21 (P < 0.05). The proliferation rate of HUVECs under our experimental conditions, which was evaluated as the population doubling level at a time, was significantly higher (P < 0.05 from the day 27) in the medium sup-

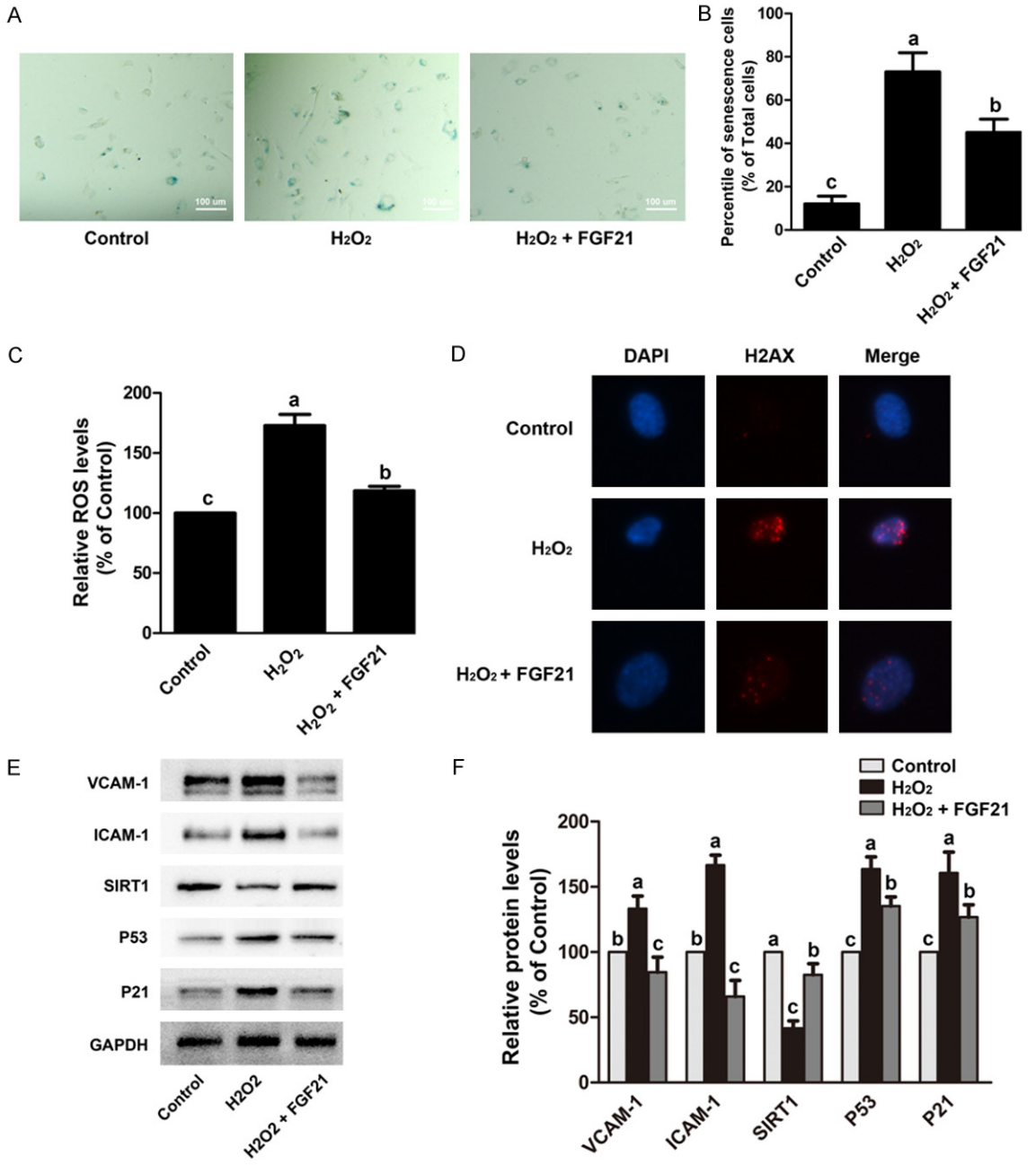

Figure 3. FGF21 delayed H_2O_2-induced HUVEC senescence. A representative picture (× 100 magnification) (A) and histogram (B) of SA-β-gal staining of HUVECs at PDL8 (Control), cells treated with 50 μmol/L H_2O_2 (H_2O_2), and cells pre-cultivated with 5 ng/mL FGF21 for 12 h before H_2O_2 treatment (H_2O_2 + FGF21). (C) The relative ROS level of each group compared to the control group. (D) The γ-H2AX immunofluorescence detection of DNA damage (× 200 magnification) with red showing γ-H2AX foci and blue showed nuclei stained with DAPI. A representative picture (E) and histogram (F) of the levels of proteins VCAM-1, ICAM-1, SIRT1, P53, P21 detected by western blot analysis with the protein GAPDH as a reference. Values are the mean ± SD; n = 3 in each group. Different letters indicate differences between the treatment groups, $P < 0.05$.

plemented with 5 ng/mL FGF21 compared to the control medium (**Figure 2C**). The level of protein SIRT1 decreased during HUVEC senescence and could be partially recovered when cells were co-incubated with FGF21, while the levels of proteins P53 and P21 increased during cell senescence and diminished in response to the FGF21 treatment (**Figure 2D**, **2E**).

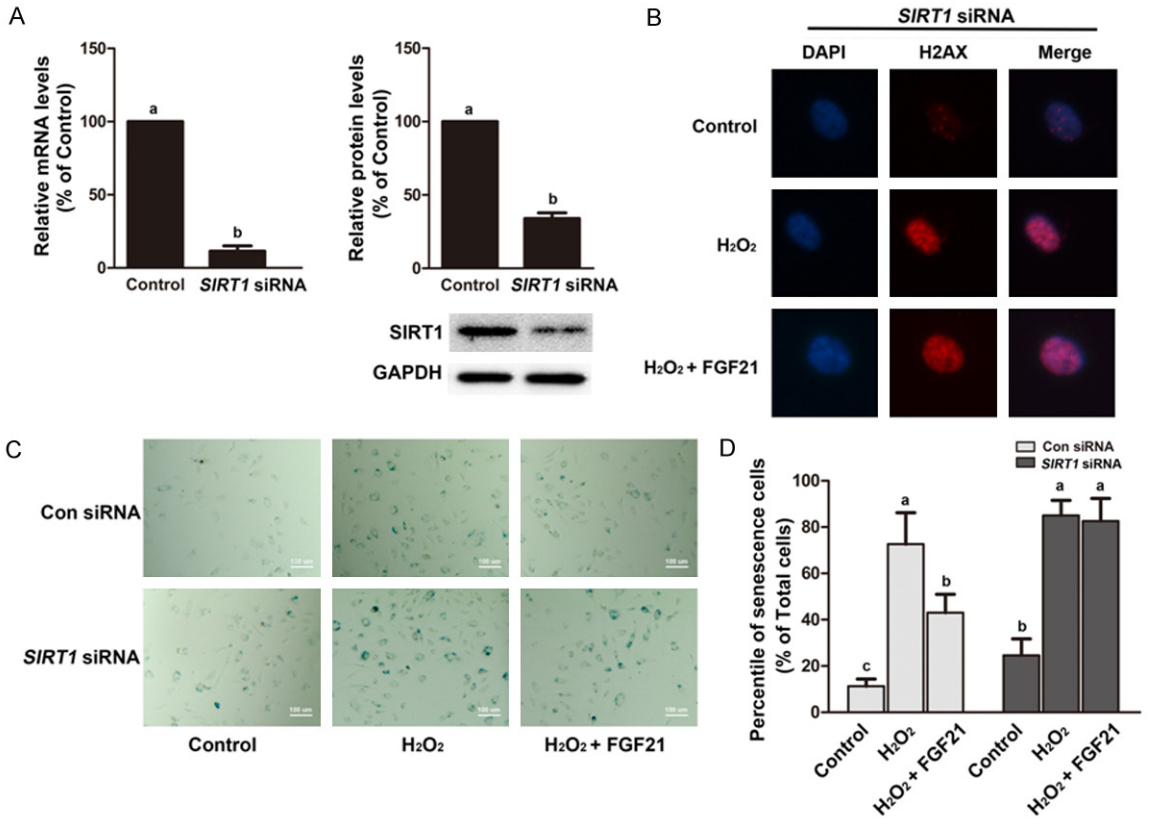

Figure 4. Transient transfection of *SIRT1* siRNA abrogated the anti-senescence effects of FGF21 in HUVECs. (A) The efficiency of *SIRT1* siRNA transfection was detected by RT-PCR and western blot. The γ-H2AX immunofluorescence detection of DNA damage (× 200 magnification) (B), a representative picture (× 100 magnification) (C) and histogram (D) of SA-β-gal staining of *SIRT1* siRNA transfected-HUVECs after FGF21 and H_2O_2 treatment. Values are the mean ± SD; n = 3 in each group. Different letters indicate differences between the treatment groups, $P < 0.05$.

FGF21 protected HUVECs from H_2O_2-induced premature senescence

HUVECs at PDL8 exposed to 50 μmol/L H_2O_2 for 48 h showed a significant increase in the SA-β-gal activity that corresponded to an increase in the senescent population from 12.0% ± 3.6% in the control to 73.0% ± 8.9% in the presence of H_2O_2 ($P < 0.05$), whereas addition of 5 ng/mL FGF21 could significantly suppress the H_2O_2-induced senescent cells to 45.0% ± 6.2% of the total population ($P < 0.05$) (**Figure 3A**, **3B**). The ROS level in HUVECs increased to 172.9% ± 9.3% of the basal level after H_2O_2 treatment ($P < 0.05$), whereas the ROS level was attenuated to 118.4% ± 3.8% of the basal level ($P > 0.05$) when the cells were co-incubated with FGF21 (**Figure 3C**). DNA damage was visualized using γ-H2AX immunofluorescence, and FGF21 attenuated the H_2O_2-induced DNA damage in the nuclei of HUVECs (**Figure 3D**). As shown in **Figure 3E**, **3F**, H_2O_2 treatment significantly increased the protein levels of VCAM-1, ICAM-1, P53 and P21 as well as decreased the protein level of SIRT1, while co-cultivation with 5 ng/mL FGF21 partially reversed the H_2O_2-induced changes of protein expression.

Silencing of SIRT1 abrogated the anti-senescence effect of FGF21

SIRT1 was transiently knocked-down by siRNA in HUVECs, and the anti-senescence effects of FGF21 on HUVECs were assessed by SA-β-gal staining and a DNA damage assay. *SIRT1* mRNA and protein levels in the siRNA transfected-HUVECs were reduced to 12.2% ± 2.4% ($P < 0.05$) and 33.9% ± 3.8% ($P < 0.05$) (**Figure 4A**), respectively, compared to untransfected control cells. Increased DNA damage and SA-β-gal activity in HUVECs were observed after knockdown of *SIRT1* and were further aggra-

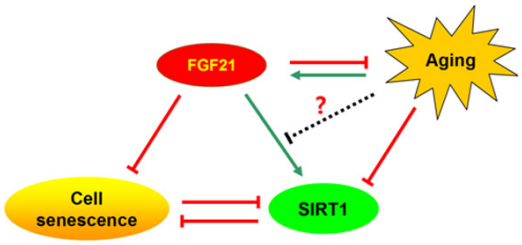

Figure 5. A proposed model of exogenous FGF21 in delaying senescence of HUVECs through up-regulation of SIRT1. Combined with the aging-associated increase in circulating FGF21 [1, 43] and decrease in SIRT1 expression [25, 42], it was suggested that the efficiency of FGF21 for up-regulation of SIRT1 was compromised during aging.

vated after H_2O_2 treatment, whereas addition of FGF21 had no significant effects on the rescue of H_2O_2-induced cell premature senescence and DNA damage in HUVECs (**Figure 4B-D**).

Discussion

Anti-senescence effects of FGF21 in HUVECs

The progressive loss of proliferation potential and increased SA-β-gal positive staining are the hallmarks of morphological alterations during replicative senescence of HUVECs [24, 39]. In our study, we demonstrated for the first time that the addition of exogenous FGF21 resulted in reduced SA-β-gal activity, a higher proliferation rate, higher levels of the proteins P53 and P21, and a lower SIRT1 protein level in the later-passage HUVECs (PDL28) compared to cells from earlier passages (PLD8) (**Figure 2**), which confirmed the role of FGF21 as a pro-longevity hormone for HUVECs by delaying cell senescence through mechanisms that may involve P53, P21 and SIRT1.

Increases in endothelial permeability have been considered to be the main cause of various vascular diseases, including atherosclerosis [26], and the replicative potential of HUVECs is critical for maintaining the integrity of endothelial monolayers by replacing adjacent senescent VECs and repairing disrupted endothelial permeability caused by aging and various pathogenic environments [24, 26]. FGF21 had a significant effect on maintaining the proliferation potential of HUVECs during subculture under our experimental conditions (**Figure 2C**), which suggests that FGF21 could postpone vascular aging by preserving endothelial integrity.

An increase in intracellular protein levels of P53 and P21 have been considered functional markers for endothelial cell senescence [24, 25], which is caused by P53-mediated cell cycle arrest through increasing the expression of P21 in senescence-associated DNA damage and telomere dysfunction [40, 41]. FGF21 lowered the protein levels of P53 and P21 in HUVECs during subculture under our experimental conditions, which suggests that FGF21 could delay the senescence of HUVECs by protecting cells from DNA damage and telomere dysfunction.

H_2O_2 treatment increased the intracellular ROS level of cells accompanied by increased SA-β-gal activity, DNA damage, higher levels of the proteins P53, P21, VCAM-1, and ICAM-1 as well as a lower SIRT1 protein level. The addition of exogenous FGF21 attenuated H_2O_2-induced phenotype changes in HUVECs, which indicated that FGF21 could protect HUVECs from oxidative damage-associated premature senescence and suggested that there was a protective role of FGF21 in vascular aging and diseases caused by an altered oxidative environment.

Anti-senescence effect of FGF21 is SIRT1-dependent

SIRT1 is an extensively studied and well-established anti-aging/senescence protein that plays an essential role in preventing vascular aging and atherosclerosis through various mechanisms, such as increasing endothelial nitric oxide production, decreasing inflammation and oxidative stress and inducing autophagy [42]. The protein level of SIRT1 decreased in HUVECs during subculture under our experimental conditions (**Figure 2D**, **2E**), which is consistent with previous findings of decreased SIRT1 expression/activation in vascular cells/tissue undergoing senescence/aging [25, 42]. The addition of FGF21 significantly recovered the protein level of SIRT1 and restored younger phenotypes in the HUVECs of later passages. Furthermore, FGF21 could also restore the protein level of SIRT1 that was repressed after H_2O_2 treatment in HUVECs, which occurred concurrently with a reverse in other phenotype changes caused by H_2O_2 treatment. Both observations suggested that FGF21 delayed both replicative senescence and oxidative stress-induced premature senescence of HUVECs through the SIRT1 pathway. To verify

this hypothesis, *SIRT1* was transiently knocked down (**Figure 4**); then the addition of FGF21 became ineffective for the rescue of H_2O_2-induced premature senescence and DNA damage of HUVECs, indicating that the anti-senescence effect of FGF21 was SIRT1-dependent.

Is FGF21-mediated SIRT1 activation compromised during aging?

Interestingly, recent studies have shown that circulating levels of FGF21 were generally elevated with age among healthy individuals [43] as well as in patients with metabolic syndromes, including obesity, hyperlipidemia, and type II diabetes [1]. To explain the supraphysiological levels of FGF21 in healthy elderly individuals and patients with these metabolic syndromes, a hypothesis for the presence of FGF21 resistance or compensatory responses to the underlying metabolic stress was proposed. In the present study, we found that the anti-senescence effects of FGF21 in HUVECs were SIRT1-dependent, and when these effects are combined with previous findings of decreased SIRT1 expression/activation in vascular cells/tissue undergoing senescence/aging [25, 42], it is likely that the efficiency of FGF21 for the upregulation of SIRT1 is compromised during aging and in patients with metabolic syndromes (**Figure 5**). Determining the underlying mechanism will require further investigation.

In conclusion, our study demonstrated that FGF21 can delay the replicative senescence of HUVECs and protect HUVECs from H_2O_2-induced premature senescence in a SIRT1-dependent manner (**Figure 5**), which supports the potential of FGF21 to function as a therapeutic target for postponing vascular aging and preventing age-related vascular diseases, including atherosclerosis.

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Disclosure of conflict of interest

None.

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