KLOTHO DEFICIENCY PROMOTES THE PROLIFERATION AND MIGRATION OF HUMAN AORTIC VASCULAR SMOOTH MUSCLE CELLS BY ACTIVATING THE WNT/β-CATENIN SIGNALING PATHWAY

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ABSTRACT

Objective: Arterial stiffness is a component of arterial aging and might be related to decreased expression of Klotho. We performed Klotho RNA interference in human aortic vascular smooth muscle cells (HA-VSMC) to assess the effects of Klotho silencing on cell proliferation, cell migration, and the Wnt signaling pathway. Methods: Klotho expression in HA-VSMC was reduced by RNA interference. Cell proliferation and migration were determined by Cell Counting Kit-8 (CCK-8) assay and transwell assay, respectively. Results: Decreased expression of Klotho promotes the proliferation and migration of HA-VSMC. Furthermore, we found that decreased expression of Klotho led to the activation of the Wnt/β-catenin signaling pathway, with increased expression of multiple Wnt protein isoforms and β-catenin accompanied by increased expression of TCF4 and Cyclin D1, which are downstream target proteins of the Wnt/β-catenin signaling pathway. Additionally, the Wnt signaling pathway inhibitor XAV939 significantly inhibited the proliferation of HA-VSMC treated with shKlotho in a dose-dependent manner. Conclusion: Klotho deficiency promotes HA-VSMC proliferation and migration, and the Wnt/β-catenin signaling pathway may play an important role in the process of arterial stiffness related to vascular aging.

Keywords: Arterial stiffness, human vascular smooth muscle cell, Klotho, Wnt signaling, XAV939
Significance Statement
Arterial stiffness is a component of arterial aging and might be related to decreased expression of Klotho. We performed Klotho RNA interference in human aortic vascular smooth muscle cells (HA-VSMC) to assess the effects of Klotho silencing on cell proliferation, cell migration, and the Wnt signaling pathway. Klotho expression in HA-VSMC was successfully reduced by RNA interference, and we found that decreased expression of Klotho promotes the proliferation and migration of HA-VSMC. Furthermore, we found that decreased expression of Klotho led to the activation of the Wnt/β-catenin signaling pathway, and the Wnt signaling pathway inhibitor XAV939 significantly inhibited the proliferation of HA-VSMC treated with shKlotho in a dose-dependent manner.

Introduction
Cardiovascular disease (CVD) is the leading cause of death in China and accounts for 40% of deaths in the Chinese population [1]. Among the risk factors of CVD, hypertension is the most critical risk factor in China; an estimated 43% of CVD events can be attributed to hypertension [2,3]. Indeed, the prevalence of hypertension in the Chinese population aged ≥18 years during October 2012 to December 2015 was 23.2%, accounting for about 240 million patients [4]. Aging is a significant risk factor for hypertension and CVD. Vascular aging, an essential aspect of human aging, leads to intimal and medial thickening as well as the gradual loss of arterial elasticity resulting in arterial stiffness. Furthermore, arterial stiffness is the primary cause of hypertension and pathophysiological processes related to aging [5]. Arterial stiffness may not only be a significant cause of hypertension but may also play a role in target organ damage [6,7]. One study has shown that severe arterial stiffness is the leading cause of poor efficacy of hypertension drugs. Most of the current antihypertensive drugs are neuroendocrine blocking agents, and there are no drugs that can effectively improve arterial elasticity [8].

Klotho is an anti-aging protein mainly expressed in the renal distal convoluted tubules and choroids [9, 10]. Klotho can exist as a membrane-bound protein that can be cleaved and released as a soluble protein in the blood, urine, and cerebrospinal fluid. Circulating Klotho declines with aging [10]. Previous studies have indicated that Klotho deficiency could promote medial calcification, intima hyperplasia, endothelial dysfunction, arterial stiffening, hypertension, and impaired angiogenesis and vasculogenesis, which are characteristics similar to those of aged human arteries [11-17]. However, the underlying mechanisms by which Klotho affects arterial stiffness are mostly unknown, and the mechanisms of Klotho deficiency in rat and human arterial stiffness might not be identical.

Vascular smooth muscle cells (VSMC) play a central role in the pathophysiologic process of vascular remodeling [18]. VSMC can perform both contractile and synthetic functions, which are characterized by differences in morphology, proliferation, and migration. VSMC play a key role in
many cardiovascular diseases such as atherosclerosis, restenosis, and hypertension [19]. Previous studies mainly studied Klotho deficiency or overexpression on animal VSMC calcification, proliferation, migration, and oxidative stress [20-22]. In kidney tissue of chronic kidney disease models, it has been shown that Klotho expression is inversely related to β-catenin expression, and that Klotho can inhibit Wnt-induced β-catenin activation, nuclear translocation, and target gene expression [23]. However, the effect of Klotho deficiency on the proliferation and migration of human VSMC and its underlying mechanisms are unclear.

In this study, we successfully reduced the expression of Klotho in human aortic VSMC (HA-VSMC) through RNA interference technology and investigated the effects of Klotho deficiency on the proliferation and migration of HA-VSMC. Specifically, we investigated whether the Wnt/β-catenin signaling pathway plays a key role in the response of HA-VSMC to Klotho deficiency, and used a small molecule inhibitor of the Wnt/β-catenin signaling pathway (XAV939) to confirm our results.

Methods
Materials
Human aortic smooth muscle cells (BeNa Culture Collection); Primer (Sangon Biotech); Protease inhibitor cocktail (Promega); Goat Anti-Mouse IgG and Goat Anti-Rabbit IgG (CWBio); β-catenin, Cyclin D1 and Klotho antibodies (Abcam); Wnt3A antibody (OriGene); Protease inhibitor cocktail (Promega); Cell Counting Kit-8 CCK-8 (Meilunbio); Transwell nesting chamber (6.5 mm transwell with 8.0 μm pore polycarbonate membrane, Corning); XAV939 (Selleck).

Cell Culture and Transfection
HA-VSMC were cultured in high glucose DMEM medium containing 15% FBS. The incubator temperature was 37°C and the CO2 concentration was 5%. Lentiviral shRNA (GenePharma) was used to interfere with Klotho expression. The Klotho-2073 shRNA (shKlotho-2073) target sequence was 5'-GAGCCGTATACAAAGGAATATG-3'; the Klotho-1021 target shRNA (shKlotho-1021) sequence was 5'-CCGAGAGCATGAAGAATAACC-3'; the negative control shRNA (shNC) target sequence was 5'-TTCTCCGAACGTGTCACGT-3'. The cells were seeded in a 6-well plate at 3×10^5/well, and the cells were treated with 1 μg/mL of polybrene and lentiviral suspension (MOI = 20) after 1 day of cell confluence. After 72 hours of transfection, the virus solution was discarded, and cells were used for experiments.

Proliferation Assay Using Cell Counting Kit-8
We inoculated the cells at 5×10^3 cells/well in 96-well plates, each of which was repeated 5 times while setting blank control wells. Cell Counting Kit-8 (CCK-8) was observed at 0 h, 24 h, 48 h, 72 h, 96 h, and 120 h. Before the test, 10 μl of CCK-8 solution was added to each well. The cells were incubated for 2 h, and the
absorbance (wavelength: 450 nm) was established on the enzyme marker. According to the instructions, 10 mg of XAV-939 was dissolved in 1 mL of DMSO, yielding a concentration of 10 mmol/L. The original solution was diluted into culture medium at concentrations of 0 μmol/L, 5 μmol/L, 10 μmol/L, 20 μmol/L, 50 μmol/L, and 100 μmol/L. We inoculated the cells at 5 ×10³ cells/well in 96-well plates, each of which was repeated 5 times while setting blank control wells. Cell Counting Kit-8 (CCK-8) was observed at 24 h and 48 h. Prior to testing, 10 μL of CCK-8 solution was added to each well. The cells were incubated for 2 hours, and absorbance was established on the enzyme label (wavelength: 450 nm).

Real-time PCR

cDNA was synthesized by reverse transcription on ice according to the kit instructions (FastKing RT Kit With gDNase, TIANGEN). Real-time PCR amplification was performed using this cDNA as a template. The expression levels of Klotho, Wnt1, Wnt2, Wnt3, Wnt3A, Wnt4, Wnt5A, Wnt5B, Wnt7A, Wnt9A, Wnt9B, Wnt10B, TCF4, Cyclin D1, and β-catenin mRNA were detected, and GAPDH was used as an internal reference. The relative expression levels of each group were collected, analyzed, and expressed as 2⁻ΔΔCt values.

We treated cells with both XAV939 and Klotho RNA interference, extracted RNA, and determined the concentration to synthesize cDNA. Using this cDNA as a template, real-time PCR amplification was performed, and the relative expression of Wnt9A was detected, calculated, and statistically analyzed.

| Gene    | Primer sequence (5’-3’) | Product Size (bp) |
|---------|------------------------|-------------------|
| GAPDH   | Forward: TGCACCACCAACTGCTTAGC | 87bp |
|         | Reverse: GGCATGGACTGTGGTCATGAG | |
| Klotho  | Forward: CTGGGATGGTATCAATCTTTGCG | 90bp |
|         | Reverse: ATCTGCAGCATAACGATAGAG | |
| Wnt1    | Forward: CCGATGGTGGGGTATTTGTGA | 137bp |
|         | Reverse: TCCCCGGATTTTTGCGTAT | |
| Wnt2    | Forward: AGTCCGGAATCTGGCCTTTGTT | 124bp |
|         | Reverse: TGGCGCTTCCCATCTTCTTT | |
| Wnt3    | Forward: TTGTGAGCGCCAAACCAGAGA | 138bp |
|         | Reverse: TTTTCTTCCGCTTCTCCGT | |
| Gene   | Forward                  | Reverse                  | Length (bp) |
|--------|--------------------------|--------------------------|-------------|
| Wnt3A  | CTTTGCA GTGACACGCTCATG   | GAGACACC CATCCACAAACT    | 137         |
| Wnt4   | TTGTGGAT GTGCGGGAGAGAA   | AGCACGTCT TTTACCTCAGGG   | 156         |
| Wnt5A  | CCCACCACA CAAGACCTGGT    | ATGAGCTCG CAGCCATCCAT    | 134         |
| Wnt5B  | GTGCAGAGAC CCAGAGTGTTTA  | TTGGCTCCCT CCCCTATGTA    | 131         |
| Wnt7A  | CGTTCA CTACGCCATCATTG    | ACTGGCCTTG GTCCTTTGT     | 108         |
| Wnt9A  | CTCGTGGGT GTGAAGGTGAT    | TGCGCTTC TCACTTGTGCTT    | 150         |
| Wnt9B  | GCTGCACCTGT GATGACTCT    | GTTCCCTTG TTTCCCTCTTTGG  | 132         |
| Wnt10B | ATGGGAATGGGGTGCTGTAA     | GCATTTCCGCTTCAGGTTTTC    | 163         |
| TCF4   | TGCCCTCCT CTTTCCCTTCA    | GTTCTCAAC CCAAAGTGACTTG  | 128         |
| Cyclin D1 | A GGAGACCAC CGAGGAGTTTA | GATGGCAGCATGGA CTTTGGTC  | 167         |
| β-catenin | CCGACACCCAAGAAGCAGAGAT | AGTGGGATG GGTGGGTGAAGA  | 92          |
Western Blots

Western blot analysis was performed to study protein expression in HA-VSMC. The total HA-VSMC protein content was isolated using a western blotting lysis kit (Protease inhibitor cocktail, Promega) and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto a polyvinyl difluoride (PVDF) membrane and blocked with 5% dried milk for 2 h. The primary antibody (1:1000 Tubulin, 1:1000 anti-klotho, 1:1000 anti-Wnt3A, 1:5000 anti-β-catenin and 1:1000 anti-Cyclin D1) was diluted in TBS-T containing 5% skim milk, and was then added to the membrane and incubated at 4°C overnight. After three washes with TBS-T, the secondary antibody conjugated to horseradish peroxidase (1:2000 Goat Anti-Mouse IgG, 1:2000 Goat Anti-Rabbit IgG) was added to the membrane and incubated at room temperature for 2 h. Immunoblots were developed on films using the Bio-Rad gel imaging system. The optical density analysis of the bands was determined using Image J and assayed with Tubulin correction for relative amount analysis.

Transwell migration assay

Cells were digested, centrifuged, and washed with PBS once. Then, cell pellets were suspended in DMEM medium without serum, and cells were counted and the cell concentration was adjusted. We added 200 μL cell suspension (2×10^5 cells) to the upper chamber of each Transwell plate, and added 500 μl medium containing 15% FBS into the lower chamber of each Transwell plate. Plates were incubated in a 37°C and 5% CO_2 incubator. After 48 h, 72 h, and 96 h of incubation, plates were fixed, stained, and dried. Ten different visual fields were randomly selected to calculate the number of transmembrane cells.

Statistical Analysis

Data analysis was performed using SPSS 23.0 software. Measurement data are expressed as mean ± standard deviation, and two sets of data were compared using two independent sample t-tests; multiple samples were compared using analysis of variance. When P < 0.05, the difference was considered statistically significant.

Results

Our fluorescence microscopy and quantitative fluorescence PCR experiments showed that the expression of Klotho was significantly inhibited by shKlotho-2073. The knockdown efficiency was 86.4% (Fig. 1A). Thus, shKlotho-2073 (shKlotho) was chosen as the interference vector. The results were further verified by Western blot (Fig. 1B, 1C).

The CCK-8 tests showed that the proliferation of shKlotho-transfected HA-VSMC was significantly higher than that of the control group, and the difference was more obvious with increasing experimental time (Fig. 2A). The comparison of each time point from 24 h was statistically significant (P < 0.001). Throughout the four-day-long Transwell migration experiment, the migration ability of shKlotho-transfected HA-VSMC was significantly increased. The results show that the shKlotho group has a statistically
higher number of transmembrane cells after 72 h compared with the shNC group (P < 0.01) (Fig. 2B).

After the transfection of HA-VSMC with shKlotho, we further investigated whether the Wnt/β-catenin signaling pathway was affected. Using RT-PCR and Western blot analyses, we found that the relative mRNA expression of Wnt1, Wnt2, Wnt3, Wnt3A, Wnt4, Wnt5A, Wnt5B, Wnt7A, Wnt10B, and especially Wnt9A and Wnt9B was increased significantly (Fig. 3A, 3B). The results of Western blot analyses of Wnt3A (Fig. 3D, 3G) indicated that its expression in the shKlotho group was significantly higher than that in the shNC group (n = 3, 2.441 vs. 1.870, P=0.0003). The relative mRNA (n = 3, Fig. 3C) and protein expression (n = 3, Fig. 3F, 3I) of β-catenin was significantly higher in the shKlotho group than in the shNC group. These results indicate that Klotho deficiency can activate the Wnt/β-catenin signaling pathway. In addition, after transfection with shKlotho, we detected a significant increase in TCF4 expression at the mRNA level (Fig. 3C). RT-PCR and Western blot also confirmed that the expression of Cyclin D1 was increased significantly (Fig. 3C, 3E, 3H).

The results of the CCK-8 experiments showed that the Wnt signaling pathway inhibitor XAV939 significantly inhibited the proliferation of normal HA-VSMC in a dose-dependent manner (Fig. 4A). After shKlotho treatment, XAV939 also significantly inhibited the proliferation of HA-VSMC in a dose-dependent manner (Fig. 4B, 4C). After treating HA-VSMC with shKlotho, cells were cultured for 48 hours in normal medium, media containing 50 μmol/L XAV939, and media containing 100 μmol/L XAV939. We found that XAV939 significantly inhibited the expression of Wnt9A at the mRNA level in HA-VSMC treated with shKlotho (Fig. 4D).

**Discussion**

Arterial stiffness and hypertension are aging-related cardiovascular disorders. It is important to investigate how vascular aging, a process characterized by gradual Klotho deficiency, participates in arterial stiffness and hypertension. It is well known that excessive VSMC proliferation and migration are primary mechanisms of vascular remodeling. In the present study, we systematically assessed the effect of Klotho deficiency on the Wnt/β-catenin signaling pathway and VSMC proliferation and migration. We found that shKlotho intervention significantly reduced the expression of Klotho, and subsequently increased proliferation and migration of VSMCs via activating the Wnt/β-catenin pathway. Previous studies have shown that the anti-aging protein Klotho plays an important role in vascular endothelial dysfunction, VSMC proliferation and migration, monocyte chemotaxis, and vascular tissue inflammation [24,25]. Klotho is involved in vascular protection via inhibition of oxidative stress, regulation of inflammation, and reduction of vascular calcification [12,26-28]. The results of the present study are consistent with those of previous studies. In rat VSMC, recombinant
exogenous Klotho regulates BAG3 expression through the NF-κB p65 pathway and inhibits the proliferation and migration of VSMC induced by Angiotensin II (Ang II) [29]. Yu et al. [21] found that Ang II can promote the proliferation and migration of VSMC, reduce SM22a, and promote the expression of PCNA, suggesting that Ang II can regulate the phenotype of VSMCs. Co-treatment with Klotho increased the expression of contractile proteins, reduced the expression of proliferating proteins, and changed the cytoskeleton by inhibiting the NF-κB, p65, Akt, and ERK signaling pathways. Therefore, Klotho can effectively inhibit Ang II-induced VSMC proliferation, migration, and phenotypic regulation [21]. In this study, we confirmed that low expression of Klotho can promote the proliferation and migration of HA-VSMC, which may be involved in the occurrence and development of arteriosclerosis.

Fig 1. Klotho silencing in HA-VSMC. (A) Detection of Klotho transfection efficiency at the mRNA level. (B, C) Changes in protein expression after shKlotho interference in HA-VSMC. *P<0.05, **P<0.01, ***P<0.001.
Fig 2. shKlotho interference significantly promotes the proliferation and migration of HA-VSMC. (A) CCK-8 detected the proliferation of shKlotho-treated HA-VSMC. (B) Changes in migration at different time points after shKlotho treatment in HA-VSMC. *P <0.05, **P<0.01, ***P<0.001.
Fig 3. shKlotho interference on HA-VSMC activates the Wnt signaling pathway. (A, B) Changes in Wnt family protein expression after shKlotho transfection. (C) β-catenin, TCF-4 and cyclin D1 expression after shKlotho transfection. (D, G) Wnt3A protein expression after shKlotho transfection. (E, H) Cyclin D1 protein expression after shKlotho transfection. (F, I) β-catenin protein expression after shKlotho transfection. *P<0.05, **P<0.01, ***P<0.001.
Fig 4. Treatment of HA-VSMC with XAV939 inhibits proliferation and Wnt9A expression. (A) CCK-8 experiments showing that XAV939 significantly inhibits the proliferation of normal HA-VSMC in a dose-dependent manner. (B, C) Proliferation of HA-VSMC after shKlotho and XAV939 treatment. (D) RT-PCR measurement of Wnt9a mRNA expression after shKlotho and XAV939 treatment. *P<0.05, **P<0.01, ***P<0.001.

Klotho is an endogenous inhibitor of the Wnt/β-catenin signaling pathway, which is widely involved in the regulation of blood pressure, kidney injury, hypertensive heart disease, and type 2 cardiorenal syndrome [30-33]. Depending on the Wnt protein and
the downstream receptor protein, the Wnt signaling pathway is divided into a classical signaling pathway (Wnt/β-catenin signaling pathway) and a non-canonical signal (the planar cell polarity pathway and Wnt/Ca²⁺ signaling pathway) [10]. The classical Wnt signaling pathway is dependent on intracellular β-catenin. When the Wnt protein interacts with the transmembrane Frizzled receptor Frizzled (Fzd) and the co-receptor, low-density lipoprotein (LDL) receptor-associated protein (LRP5/6), a series of downstream signaling events are induced [34]. Eventually, β-catenin is translocated into the nucleus and binds to the nuclear transcription factor, T cell factor (TCF)/lymphoid enhancer-binding factor (LEF), to stimulate transcription of the Wnt target gene. Zhou et al. found that Klotho expression was negatively correlated with β-catenin in kidney tissues of animal models of chronic kidney disease [35]. In vitro experiments showed that either membrane Klotho or secretory Klotho could bind to Wnt proteins and inhibit Wnt-induced β-catenin activation, nuclear translocation, and target gene expression. When the Wnt signal is absent, β-catenin in the cytoplasm is degraded by the proteasome. The activation of the Wnt signaling pathway will result in the accumulation of β-catenin in the cytoplasm and then translocation into the nucleus. β-catenin interacts with transcription factors such as those in the T-cytokines (TCF) family to activate target genes such as TCF4 and Cyclin D1 and lead to abnormal cell proliferation. It has been found that the extracellular segment of Klotho can bind to some Wnt ligands and block the binding of Wnt to its corresponding cell surface receptors, thus inhibiting the Wnt signaling pathway [36]. In the present study, we investigated the regulatory effect of Klotho on the Wnt/β-catenin signaling pathway in HA-VSMC. Our results showed that silencing of Klotho enhanced the activation of the Wnt/β-catenin signaling pathway, suggesting that Klotho could activate the Wnt/β-catenin signaling pathway in arterial stiffness. However, we do not know whether the HA-VSMC response to Klotho silencing depends solely on the Wnt/β-catenin signaling pathway or if other signaling pathways are involved. Further study is needed.

A variety of diseases are currently associated with abnormal activation of the Wnt signaling pathway, and targeted therapy for Wnt signaling has become a research hotspot [37]. Chen et al. treated rat VSMCs with XAV939, and found that XAV939 inhibits the formation of intima, which is manifested by the loss of intimal area and the I/M ratio, as well as by weakened proliferation and migration capabilities, increased ROS production, and promotion of cell cycle arrest in VSMCs. They therefore confirmed that XAV939 attenuates the formation of the intima by inhibiting the Wnt signaling pathway and inhibiting VSMC proliferation, migration, and apoptosis [38]. Studies have shown that Klotho significantly inhibits Ang II-induced cardiac hypertrophy and β-catenin pathway activation. The use of
XAV939 can inhibit Ang II-induced β-catenin activity, expression of the β-catenin target genes c-myc and Cyclin D1, and Ang II-induced cardiac hypertrophy [39].

To our knowledge, this is the first study to systematically demonstrate Klotho deficiency promotes proliferation and migration in HA-VSMC via Wnt/β-catenin signaling pathway activation. Our finding is significant because it points to a new direction for understanding the pathogenesis of aging-related arterial stiffness. Our study may offer novel insights for using the antiaging protein Klotho or small molecule Wnt/β-catenin inhibitors as new therapeutic agents to treat arterial stiffness and hypertension.

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