Original Article

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Serum containing Buyang Huanwu decoction prevents age-associated migration and invasion of human vascular smooth muscle cells by up regulating SIRT1 expression

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Summary **The migration and invasion of vascular smooth muscle cells (VSMCs) caused by advanced aging play an important role in diffuse intimal thickening, facilitate adverse arterial remodeling and contribute to the initiation and progression of cardiovascular diseases. The inhibitory function of Buyang Huanwu decoction (BYHWD) has been found on aortic intimal hyperplasia and VSMC proliferation, but its effect on age-associated migration and invasion remains unknown. Here, we used an *in vitro* angiotensin II (Ang II)-induced senescence model to study the effects of serum containing BYHWD (BYHWS) on the migratory and invasive capacities, matrix metalloprotease type 2 (MMP-2) expression and modulation of sirtuin1 (SIRT1) signaling in human aorta VSMCs (HA-VAMCs). Our results showed that BYHWS was able to inhibit Ang II-induced migration and invasion, with down-regulation of MMP-2. In addition, manipulation of SIRT1 by either over-expression or siRNA knockdown ameliorated or promoted cellular migration and invasion, respectively. Moreover, BYHWS reversed senescence-mediated decrease of SIRT1 levels and SIRT1 was required for BYHWS regulation on migration and invasion of senescent HA-VAMCs. In summary, our data demonstrated that BYHWS suppressed the migration and invasion of age-associated VSMC *via* an increase of the SIRT1 level, which provides novel insights for the therapy of age-associated cardiovascular diseases.**

Keywords: Buyang Huanwu decoction, vascular smooth muscle cells, senescence, migration/invasion, matrix metalloprotease type 2, sirtuin1 (SIRT1)

1. Introduction

Vascular aging has been viewed as a specific risk factor of cardiovascular diseases (CVD), such as atherosclerosis and hypertension (*1-4*). A complex series of events are involved in remodeled arterial wall with advanced aging, including migration and invasion of abnormal vascular smooth muscle cells (VSMC), which significantly contribute to diffuse intimal thickening and the onset and progression of CVD (*2,3*). Previous data on interventions in age-associated VSMC migration and invasion indicated modulation of decrease and delay of the occurrence of severe CVD (*4*). New classes of drugs are currently being tested for CVD prevention, including glitazones and rimonabant. However, adverse effects such as heart failure and depression were found during the application of these treatments (*5*). Therefore, novel therapy remains to be developed.

It has been implicated that traditional Chinese Medicine (TCM) exerts a regulatory effect on the inhibition of aging, as well as the migration and invasion of VSMCs in diseased arteries (*6-9*). However, little is known about the effects of Buyang Huanwu decoction

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(BYHWD) on aging. BYHWD, a classic TCM formulation featured as Qi-tonifying, stasis-eliminating, and has been used for therapy of stroke for centuries (*10,11*). Interestingly, cumulative evidence revealed that one of the mechanisms of BYHWD on various diseases represents a target to vascularity (*10,11*). For example, BYHWD shows a protective effect on cerebral arteries, coronary arteries and pulmonary arteries, *etc.* (*11*), the underlying molecular mechanisms of which relies on its pharmacological role in anti-inflammation, anti-oxidative, anti-apoptosis and anti- angiotensin II (Ang II) (*12-14*). Of note, it has been shown that BYHWD could inhibit VSMC proliferation caused by injury- or platelet-derived growth factor (*15,16*). In this scenario, there is potential role for BYHWD to control age-associated migration and invasion of serum containing BYHWD (VSMCs).

Age-associated VSMC migration/invasion represents a complex process and is regulated by multiple factors. Ang II is a major inductor of VSMCs senescence, and has been shown to potently simulate VSMCs migration and invasion (*17-21*). In addition, inhibition of Ang II pathways has been shown to substantially reduce age-associated arterial remodeling (*3,4*). The basement membrane which surrounds VSMC is cleaved by matrix metalloprotease (MMP) and restructured for age-associated migration and invasion (*2,3,22*). Exposure of new VSMCs to Ang II *via* activation of MMP- 2 increases the invasive capacity of old cells whereas MMP inhibitor reverses this effect (*23,24*). Emerging evidence points to sirtuin1 (SIRT1) as a contributer to the regulation of health and lifespan (*25,26*). It has been demonstrated that over-expression of SIRT1 and an activator of SIRT1 (*e.g.* resveratrol) markedly inhibits VSMCs migration and invasion (*27,28*).

Serum pharmacology is generally accepted as a standardized experimental method for use of TCM in *in vitro* experiments. Serum containing BYHWD (BYHWS) has been developed and described as an *in vitro* model of BYHWD treatment (*29,30*). To determine the role of BYHWS in the regulation of age-associated VSMCs migration and invasion, we induced human VSMC senescence by Ang II treatment and cultured VSMC in the presence or absence of BYHWS. We then assessed the consequences of invasion and migration capacities, MMP-2 expression and change of SIRT1 signaling.

2. Materials and Methods

2.1. *Animals and preparation of drug-containing serum*

Male Sprague-Dawley (SD) rats, aged 6-8 weeks, were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). All rats were caged under conditions of temperature control with water and food *ad libitum*. The experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Guangdong Pharmaceutical University.

BYHWD was composed of *Huangqi* (Radix Astragali seu Hedysari), *Danggui* (Radix Angelica sinensis), *Chishao* (Radix Paeoniae Rubra), *Chuanxiong* (Rhizoma Ligustici Chuanxiong), *Honghua* (Flos Carthami), *Taoren* (Semen Persicae) and *Dilong* (Pheretima). The components were purchased from the First Affiliated Hospital of Guangdong Pharmaceutical University (Guangzhou, China) and mixed in a ratio of 120:6:4.5:3:3:3:3 (dry weight). BYHWD was boiled and concentrated to a final concentration of 1 g/mL (equivalent to dry weight of raw materials). The 28 SD rats were then randomly divided into 2 groups: control group (n = 12) and BYHWD group (n = 16). BYHWD (18.5 g/Kg, equivalent to adult human dose) was intragastricly administrated for 7 days (twice per day) according to previous reports (*30,31*). Control group was treated with distilled water correspondingly. After the rat was fasted for 12 h and received the last administration of medicine for 90 min, abdominal aortic blood was collected. Blood samples were allowed to clot for 2 h at 4°C and then centrifuged at 1,500 ×g for 20 min. The serum was filtered and stored at -20°C after inactivation at 56°C for 30 min. Serum containing BYHWD and control was defined as BYHWS and CS.

2.2. *Cell culture*

Human aorta VSMCs (HA-VSMCs), obtained from ATCC (CRL-1999, Manassas, VA, USA), were cultured in F-12K medium (ATCC) containing 0.05 mg/mL ascorbic acid, 0.01 mg/mL insulin, 0.01 mg/mL transferrin, 10 ng/mL sodium selenite, 0.03 mg/mL endothelial cell growth supplement (ECGS), 10 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES) and 10 mM 2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid (TES), and supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% BYHWS or 10% CS. Cells were incubated in humidified 95% O_2 air and 5% CO_2 atmosphere at 37°C. Medium was renewed every 2 to 3 days. HA-VSMCs were cultured, passaged (less than passage 5) and treated with or without Ang II (100 nM, R&D research Inc., Minneapolis, MN, USA) for 72 h to induce senescence.

2.3. *SIRT1 siRNA silence*

HA-VSMCs were transfected with either si-SIRT1 (5 nM, AM 16708) or scrambled siRNA (Silencer Select SiRNA, 40 nM, AM 4635) (Ambion, Carlsbad, CA, USA) using Lipofectamine RNAiMAX Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 6 h following manufacturer's instructions. The final concentration of si-SIRT1 and scrambled siRNA were 50 nM.

2.4. *Lentiviral activation particles transduction*

HA-VSMCs were seeded at 1 × 10^5 cells per well in 6-well plates and incubated with complete medium (10% FBS) overnight at 37°C. Transduction was carried out with complete medium containing 3 μg/mL Polybrene (sc-134220, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Then 10 μL of SIRT1 lentiviral activation particles (sc-400085-LAC, Santa Cruz Biotechnology, Inc.) and 10 μL of control lentiviral activation particles (sc-437282, Santa Cruz Biotechnology, Inc.) were added to the culture, followed by incubation overnight. The culture medium was then removed and replaced with appropriate treatment.

2.5. *Senescence-associated β-galactosidase (SA β-gal) Assay*

HA-VSMCs in 6-well plates were washed twice in PBS, fixed for 5 min in 4% paraformaldehyde in PBS, and stained with SA β-gal (C0602, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The percentage of SA β-gal-expressing cells was examined in 4 randomly selected fields under the microscope (Leica Microsystems GmbH, Wetlzar, Germany).

2.6. *Wound healing migration*

HA-VSMCs were seeded into 24-well plates with inserts in wells (CytoSelect, CBA-120-T, Neobioscience Biotechnology, Shenzhen, China) and grown to confluence. The monolayer cells then generated a 0.9mm "wound field" by carefully removing inserts. The cells were allowed to heal for 48 h. Cell stain solution was added to each well for 15 min and the wounded areas were viewed through a microscope. Quantitative analysis of the cell percent closure was performed using ImageJ software version 1.37 (National Institutes of Health, Bethesda, MA, USA). The level of wound-healing percent closure was evaluated by calculating the percentage of the repopulated cell surface area divided by the cell free area at the initial state. Average data were from at least three independent experiments. Appropriate intervention was performed prior to seeding.

2.7. *Invasion assay*

Serum-induced invasion movement was assessed using modified Boyden chambers equipped with 8μm pore-size polycarbonate filters (PFA8, Neuro probe, Gaithursburg, MD, USA). The upper compartment was coated with BD Matrigel (356234, Solasrbio Science & Technology Co., Ltd., Shanghai, China) to form a matrix barrier. In total, 2 × 10^5 cells were suspended in serum-free F-12K complete medium following appropriate treatment and added to the upper chamber. The lower chamber was filled with F-12K complete medium containing 10% FBS, 10% BYHWS or 10% CS as chemoattractant. After a 4 h incubation period at 37°C, the cells that had crossed the basement membrane and migrated to the lower side of the filter were fixed and stained with hematoxylin and eosin (Solasrbio Science & Technology Co., Ltd.). Four random fields were counted at 400× magnification for each filter.

2.8. *Western blotting analysis*

Western blot was performed according to our previous method (*32*). Briefly, whole-cell lysates were prepared and quantified. Proteins were then separated by 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under reducing conditions and transferred onto polyvinylidene difluoride membranes. The transferred membranes were immunoblotted overnight at 4°C in phosphate buffer solution (PBS) containing primary antibodies to SIRT1 (04-1557, mouse anti-human monoclonal antibody, 1:1,000, EMD Millipore, Billerica, MA, USA), MMP-2 (AF902, goat anti-human polyclonal antibody, 1:500, R&D research Inc.), β-actin (cs-376421, mouse anti-human monoclonal antibody, 1:5,000, Santa Cruz Biotechnology, Inc.) after blocking nonpecific binding. The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse/goat immunoglobulin G (BA1050 and BA1060, 1:2,000, Wuhan Boster Biological Technology, Ltd., Wuhan, China) at room temperature for 2 h. The density of the visualized bands was quantified using an image analyzer (model GS-700, Bio-Rad Laboratories, Inc.). β-actin was used as the loading control.

2.9. *Gelatin zymography*

HA-VSMCs were treated and cell supernatant was resolved onto 10% Novex gelatin zymogram gels (Invitrogen; Thermo Fisher Scientific, Inc.), run at 90 V for 3 h at 4°C. The gels were incubated in Novex zymogram renature buffer (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 30 min and then transferred to Novex zymogram developing buffer (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C overnight. The gels were photographed after staining with 0.2% Coomassie blue and quantified using ImageJ (National Institutes of Health).

2.10. *Statistical Analysis*

All results were presented as mean ± standard error of the mean. All experiments were repeated independently 3 times. Statistical comparisons of multiple groups were made *via* an ANOVA, followed by Bonferroni post hoc test. The differences between two groups using Student's *t* tests were two-sided. All statistical analyses

were performed using GraphPad Prism version 4.00 for windows (GraphPad Software, San Deigo, CA, USA). $P < 0.05$ was defined as statistical significance.

3. Results

3.1. *BYHWS inhibited the migration and invasion of senescent HA-VSMC*

Previous studies have shown that Ang II is an inductive agent of VSMC premature senescence (*18,19*). In this study, SA β-gal activity was measured first in HA-VSMC grown in the presence of Ang II. Cellular premature senescence induced by Ang II is shown in Figure 1A, among which the percentage of SA β-gal-positive cells was significantly increased ($p < 0.05$). To determine whether BYHWS can affect age-associated migration and invasion of VSMC *in vitro*, wound-healing and invasion assays were performed in HA-VSMC cultured by treatment with BYHWS. In contrast to control cells, the administration of Ang II significantly promoted the migration of HA-VSMCs to the wounded surface (Figure 1B and 1C). In addition, the invasion of HA-VSMCs was significantly enhanced in the presence of Ang II compared to that of the control cells ($p < 0.05$) (Figure 1D). However, when HA-VSMCs in the presence of rat sera were analyzed, we found that the migration and invasion of Ang II-induced senescent cells cultured in 10% BYHWS were significantly decreased compared to those of cells grown in 10% CS ($p < 0.05$) (Figure 1B-1D), indicating that BYHWS inhibited migration and invasion of Ang II-induced senescent HA-VSMC.

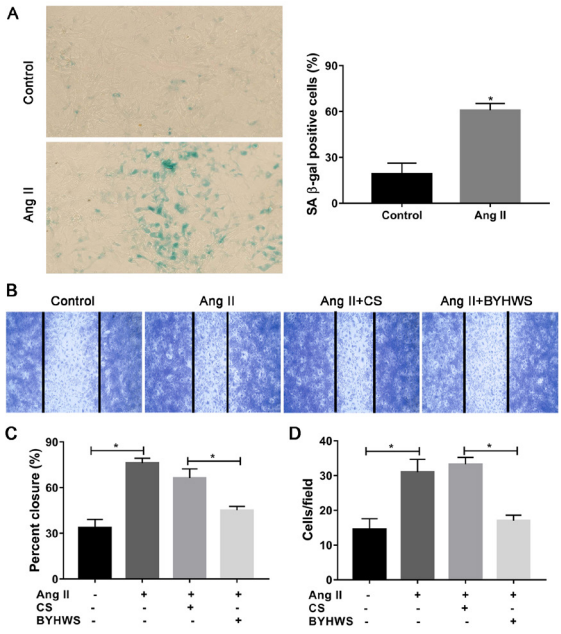

Figure 1. BYHWS inhibited the migration and invasion of senescent HA-VSMC. (A) Representative photomicrographs and average data of SA β-gal staining in HA-VSMC with and without Ang II treatment. Scale bar indicates 50μm. $*p < 0.05$ compared with control. **(B)** Representative photomicrographs of wound healing assay in HA-VSMC cultured without Ang II or with Ang II treatment in media containing 10% CS or 10% BYHWS. Original magnification ×40. **(C)** Average data of wound healing assay in HA-VSMC cultured without Ang II or with Ang II treatment in media containing 10% CS or 10% BYHWS. $*p < 0.05$ in comparison between two groups. **(D)** Average data of invasion analysis of HA-VSMC cultured without Ang II or with Ang II treatment in media containing 10% CS or 10% BYHWS. $*p < 0.05$ in comparison between two groups. Ang II, angiotensin II; CS, serum containing vehicle; BYHWS, serum containing Buyang Huanwu decoction.

3.2. *BYHWS reduced MMP-2 production and secretion in senescent HA-VSMC*

Since MMP-2 is a senescence marker and a key regulator in the migration and invasion of VSMCs (*2,3,22,25*), we conducted Western blotting and gelatin zymography detection to assess the effects of BYHWS on MMP-2 expression. As shown in Figure 2, Ang II treatment led to a significant increase of MMP-2 protein expression and induced its activation. Nevertheless, the treatment with serum containing BYHWD significantly reduced the expression and activation of MMP-2 protein, compared to that treated with medium containing 10% CS ($p < 0.05$) (Figure 2A and 2B).

3.3. *BYHWS delayed the down-regulation of SIRT1 protein in senescent HA-VSMCs*

A low SIRT1 level contributed to not only an acceleration of cellular senescence but also increased capacity for cellular migration and invasion (*25-28,33*). Thus we measured the levels of SIRT1 protein in the Ang II-induced HA-VSMCs. As shown in Figure 3, the amount of SIRT1 protein in HA-VSMCs progressively declined with Ang II treatment. Specifically, a 60% reduction in SIRT1 protein level was observed in Ang II-induced cells. Of note, in the Ang II-induced model,

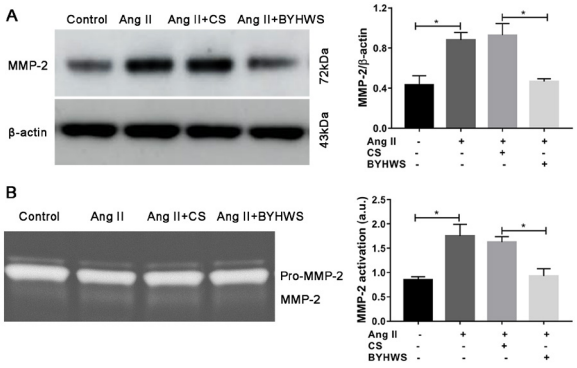

Figure 2. BYHWS reduced MMP-2 expression in senescent HA-VSMC. (A) Representative immunoblots and average data of MMP-2 protein from HA-VSMCs without Ang II or with Ang II treatment in media containing 10% CS or 10% BYHWS. $*p < 0.05$ in comparison between two groups. **(B)** Gelatin zymograms of cell supernatant from HA-VSMCs without Ang II or with Ang II treatment in media containing 10% CS or 10% BYHWS. $*p < 0.05$ in comparison between two groups. Ang II, angiotensin II; CS, serum containing vehicle; BYHWS, serum containing Buyang Huanwu decoction.

the SIRT1 protein levels in 10% BYHWS-treated cells were much higher than those found in 10% CS-treated cells. These results indicated that the administration of BYHWS significantly prevented the down-regulation of age-associated SIRT1 caused by Ang II.

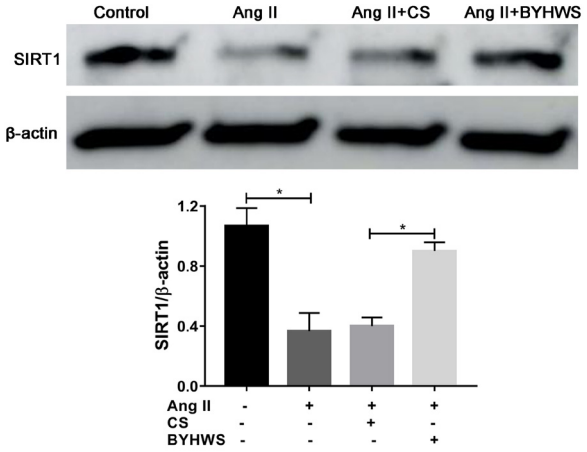

Figure 3. SIRT1 protein decreased in HA-VSMCs with Ang II-induced senescence and BYHWS retarded this effect. Representative immunoblots and average data of SIRT1 protein from HA-VSMCs without Ang II or with Ang II treatment in media containing 10% CS or 10% BYHWS. *$p < 0.05$ in comparison between two groups. Ang II, angiotensin II; CS, serum containing vehicle; BYHWS, serum containing Buyang Huanwu decoction.

3.4. *SIRT1 over-expression decreased the capacity of cell migration and invasion, as well as MMP-2 activation in senescent HA-VSMCs*

To investigate the effects of SIRT1 on VSMC migration and invasion, and MMP-2 secretion, SIRT1 over-expression was conducted by transduction, and SIRT1 levels were significantly increased in SIRT1 over-expressed HA-VSMCs compared to negative lentiviral and controls ($p < 0.05$) (Figure 4A). Remarkably, the over expression of SIRT1 in senescent HA-VAMCs significantly reduced migratory and invasive capacity and MMP-2 activation compared to those of control cells ($p < 0.05$) (Figure 4B-4D).

3.5. *SIRT1 was required for BYHWS's effects on the migration and invasion of senescent HA-VSMCs*

We finally investigated the involvement of SIRT1 in the protective effect of BYHWS towards senescent HA-VSMC. Western blot analysis showed that after transfection of HA-VSMCs with si-SIRT1, the expression of SIRT1 protein was statistically down-regulated by approximately 45% compared to SIRT1 levels in cells

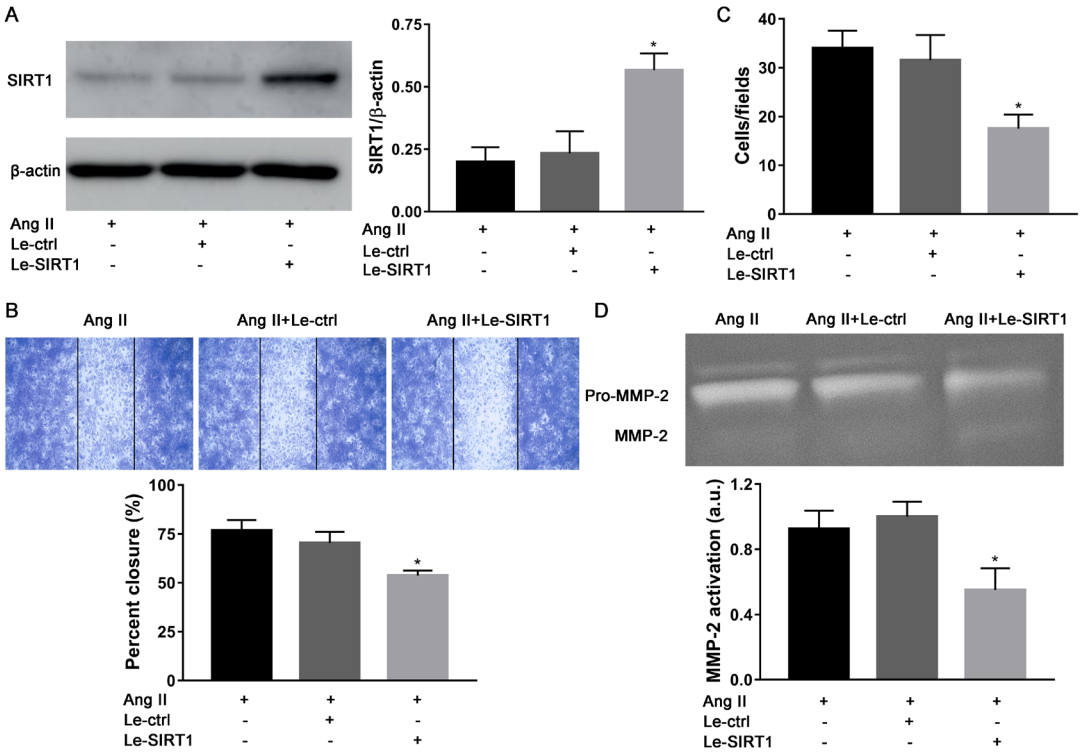

Figure 4. Over-expression of SIRT1 decreased cellular invasion and MMP-2 activation in HA-VSMCs with Ang II-induced senescence. (A) Representative immunoblots and average data of SIRT1 protein in senescent HA-VSMC transduced with Le-SIRT1 or Le-ctrl. *$p < 0.05$ compared with Ang II control. **(B)** Representative average invasion analysis of senescent HA-VSMC transduced with Le-SIRT1 or Le-ctrl. *$p < 0.05$ compared with Ang II control. **(C)** Representative average invasion analysis of HA-VSMC transduced with Le-SIRT1 or Le-ctrl. *$p < 0.05$ compared with Ang II control. **(D)** Gelatin zymograms of cell supernatant from senescent HA-VSMCs transduced with Le-SIRT1 or Le-ctrl. *$p < 0.05$ compared with Ang II control group. Ang II, angiotensin II; Le-ctrl, control lentiviral activation particles; Le-SIRT1, SIRT1 lentiviral activation particles. Ang II control group, AngII+, Le-ctrl-, Le-SIRT1-.

transfected with a negative control siRNA ($p < 0.05$) (Figure 5A). In wound healing and Boyden chamber assays, the decrease of SIRT1 expression dramatically increased the capacity of migration and invasion of senescent HA-VSMCs (Figure 5B and 5C). Moreover, si-SIRT1 also partially counteracted the inhibitory effect of BYHWS on cell migration and invasion (Figure 5B-5D). Similarly, transfection of si-SIRT1 in senescent HA-VAMCs further significantly increased MMP-2 protein and activation, and statistically abolished the protective effects of BYHWS with inhibition of MMP-2 expression ($p < 0.05$) (Figure 5E and 5F). These data suggested that BYHWS inhibited the migration and invasion of HA-VSMCs, *via* SIRT1 expression in Ang II-treated HA-VSMCs.

4. Discussion

The study of cellular processes of age-associated migration and invasion in organisms subjected to BYHWD regimens is experimentally challenging. Here, we aimed to determine the effects of BYHWS

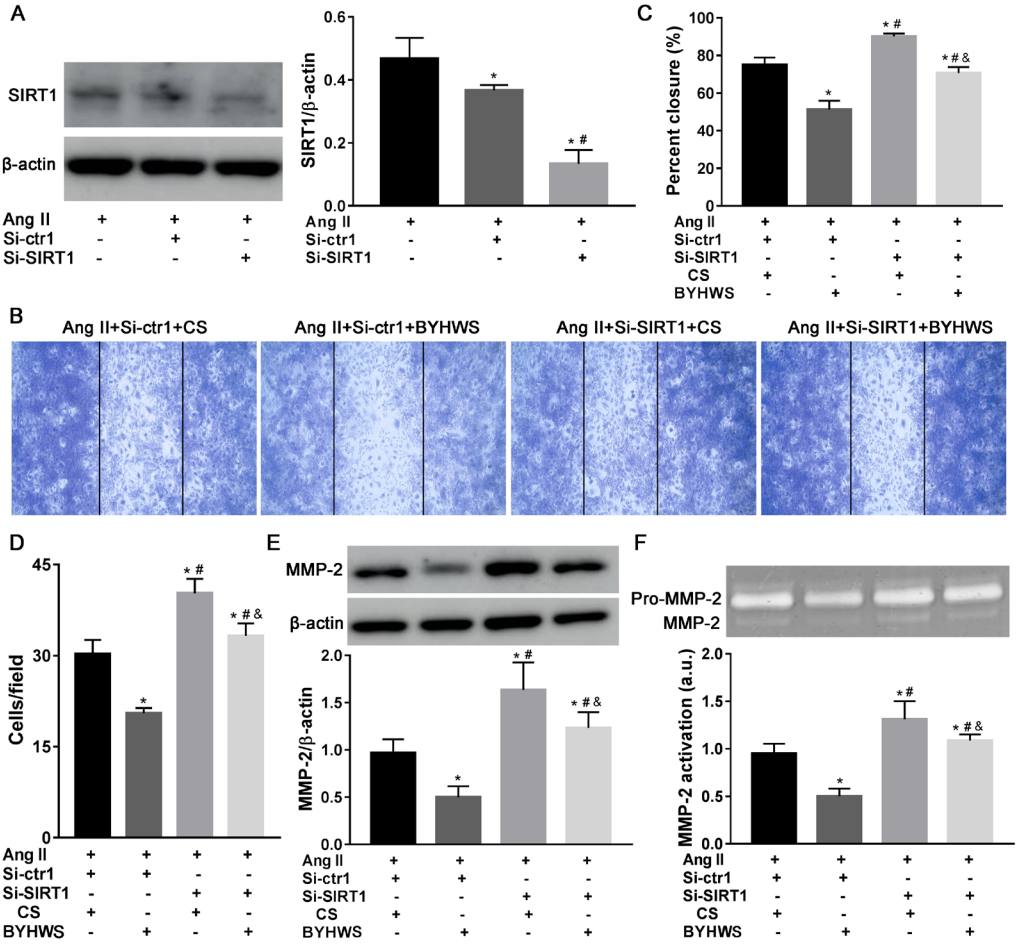

Figure 5. BYHWS modulated HA-VSMCs migration and invasion in a SIRT1-dependent manner. (A) Representative immunoblots and average data of SIRT1 protein in senescent HA-VSMC transfected with si-SIRT1 or si-ctrl. *$p < 0.05$ compared with Ang II control group. *$p < 0.05$ compared with Si-ctrl group. **(B)** Representative photomicrographs and average data of wound healing assay in HA-VSMC transfected with si-SIRT1 or si-ctrl, and then treated with Ang II in media containing 10% CS or 10% BYHWS . Original magnification ×40. *$p < 0.05$ compared with 10% CS control group. #$p < 0.05$ compared with 10% BYHWS group. &$p < 0.05$ compared with 10% CS + Si-SIRT1 group. **(C)** Representative average invasion analysis of HA-VSMC transfected with si-SIRT1 or si-ctrl, and then treated with Ang II in media containing 10% CS or 10% BYHWS. *$p < 0.05$ compared with 10% CS control group. #$p < 0.05$ compared with 10% BYHWS group. &$p < 0.05$ compared with 10% CS + Si-SIRT1 group. **(D)** Representative immunoblots and average data of MMP-2 protein from HA-VSMCs transfected with si-SIRT1 or si-ctrl, and then treated with Ang II in media containing 10% CS or 10% BYHWS. *$p < 0.05$ compared with 10% CS control group. #$p < 0.05$ compared with 10% BYHWS group. &$p < 0.05$ compared with 10% CS + Si-SIRT1 group. **(E)** Representative immunoblots and average data of MMP-2 protein from HA-VSMCs transfected with si-SIRT1 or si-ctrl, and then treated with Ang II in media containing 10% CS or 10% BYHWS. *$p < 0.05$ compared with 10% CS control group. #$p < 0.05$ compared with 10% BYHWS group. &$p < 0.05$ compared with 10% CS + Si-SIRT1 group. **(F)** Gelatin zymograms of cell supernatant from HA-VSMCs transfected with si-SIRT1 or si-ctrl, and then treated with Ang II in media containing 10% CS or 10% BYHWS. *$p < 0.05$ compared with 10% CS control group. #$p < 0.05$ compared with 10% BYHWS group. &$p < 0.05$ compared with 10% CS + Si-SIRT1 group. Ang II, angiotensin II; CS, serum containing vehicle; BYHWS, serum containing Buyang Huanwu decoction; si-ctrl, scrambled siRNA silence; si-SIRT1, SIRT1 siRNA silence. Ang II control group, Ang II+, Si-ctrl-, Si-SIRT1-. Si-ctrl group, Ang II+, Si-ctrl+, Si-SIRT1-. 10% CS control group, AngII+, Si-ctrl+, Si-SIRT1-, 10% CS+, 10% BYHWS-. 10% BYHWS group, AngII+, Si-ctrl+, Si-SIRT1-, 10% CS-, 10% BYHWS+. 10% CS + si SIRT1 group, AngII+, Si-ctrl-, Si-SIRT1+, 10% CS+, 10% BYHWS-.

on this cellular process *in vitro* using Ang II-induced senescent HA-VSMCs. Our results clearly showed that BYHWS could significantly delay age-associated increased migration and invasion of these cells, which was accompanied with reduced MMP-2 expression. In addition, we also identified SIRT1 as a pivotal factor in modulating BYHWS's effects on age-associated HA-VSMC responses.

Arterial aging is a cornerstone of systemic aging (*2,3,17*). The increase of intima thickness is nearly linearly associated with age mainly due to the migration and invasion of VSMCs from the arterial media to the intimal (*2,3,34*). While cumulative studies demonstrated that the capacity of migration and invasion was decreased in senescent VSMCs (*35,36*). Here, we used a model of HA-VSMC senescence induced by Ang II and verified that senescent HA-VSMCs enhanced migration and invasion, which was in agreement with previous reports regarding increasing migratory and invasive capacities in senescent rat VSMCs (*24,37*). Furthermore, it has been shown that Ang II can stimulate VSMC migration and invasion measured by a wound healing approach and Boyden chamber assay (*20,21*).

The precise roles mediating aging and age-associated events related with BYHWD are not fully understood. TCM of Qi-tonifying and stasis- eliminating may play a role in delaying aging, mainly due to the well-known theory that "Qi deficiency and blood stasis" is essential for human aging (*38*). In terms of vascular aging, it has been proved that TCM based on invigorating Qi and activating blood had effects on delaying VSMC senescence and lowering age-associated increase of VSMC proliferation (*6*). As a classic TCM formulation of invigorating Qi and activating blood, BYHWD can be used to treat many disorders with Qi deficiency and blood stasis, which is likely to target the specific pathogenesis of vascular diseases (*11*). BYHWD was previously shown to inhibit aortic intimal hyperplasia and cellular proliferation in cultured VSMC (*15,16*). In the current study we demonstrated for the first time that BYHWS treatment inhibited the migration and invasion of senescent HA-VSMCs. However, similar inhibition was not observed in CS-treated senescent HA-VSMCs. Other beneficial effects of BYHWD appear to promote the migration of neural precursor cells to ischemic brain areas, thus facilitating its neuroprotective effect (*39*). Under this condition, cellular migration is a nonpathogenic process for tissue repair in response to ischemic injury, which is different than the age-increased pathological process.

Uncontrolled MMPs, potently activated by Ang II, can cleave both cellular basement membrane and elastin fibers around VSMCs, which results in degradation of extracellular matrix, enabling VSMCs to migration, invasion and proliferation with advanced aging. In cultured VSMCs, both MMP-2 and MMP-9 are related to the migration and invasion of VSMCs (*2,3,22*). It has been shown that MMP-9 is less important than MMP-2 in triggering intimal thickening in coronary artery rings *ex vivo* (*40*). Moreover, MMP-2 expression becomes increased within aging arterial walls, particularly in the thickened intima of different species including humans (*2,3,22,23*). Here, we showed that the administration of Ang II in HA-VSMCs enhanced MMP-2 expression whereas BYHWS inhibited this promoting effect. These results indicate that BYHWS treatment might retard age-associated migration and invasion through inhibition of MMP-2 in HA-VSMCs.

SIRT1 (member of the sirtuin family) is a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase that removes acetyl groups from various proteins. It has been implicated that Sirt1 can impact a wide array of proteins, such as peroxisome proliferator-activated receptor-gamma and its coactivator-1alpha, forkhead transcriptional factors, AMP-activated protein kinase, NF-kappaB and protein tyrosine phosphatase involved in cardiovascular and metabolic diseases, through regulating metabolic and physiologic processes including stress resistance, metabolism, apoptosis and energy balance (*41-43*). Notably, in the cardiovascular system, activation of SIRT1 can not only protect against oxidative stress at the cellular level, but can also elevate survival at the systemic level to reduce the risk of coronary heart disease and cerebrovascular disease (*42*). In the present study, SIRT1 levels *in vitro* as HA-VSMCs were decreased and senescence was induced. This is consistent with previous studies concerning the reduction of SIRT1 levels in senescent human VSMCs, endothelial cells and fibroblasts (*25,44,45*). Interestingly, when these senescent HA-VSMCs were cultured in the presence of BYHWS, much higher SIRT1 was induced than the same cells cultured in CS. Therefore, BYHWS protects cells against the decrease of SIRT1 associated with the senescence process. Although a study has reported that BYHWS is involved in a neuron protective role through suppression of the P53 pathway (*46*), to our knowledge, no previous studies have investigated whether BYHWS directly influences SIRT1 expression. It has been demonstrated that SIRT1's predominate function is in delaying aging and suppressing arterial remodeling (*26,27,33*). Our further experiments revealed that down regulation of SIRT1 levels by siRNA-knockdown elevated cellular migration/invasion and MMP-2, leading to enhancement in senescent HA-VSMCs. Of note, the suppression of SIRT1 reversed BYHWS-mediated inhibition of MMP-2 levels and the migration/invasion in senescent HA-VSMCs. Conversely, over-expression of SIRT1 in senescent HA-VSMCs resulted in reduced expression of MMP2 and was also consistent with concomitant decreases in cellular migration and invasion. Cumulative evidence unraveled that SIRT1 plays a pivotal role in the treatment of cardiovascular disease, through which, hydroxytyrosol, mitochondrial aldehyde dehydrogenase (ALDH2), and sulforaphane (SFN)

showed great promise for providing protection (47-50). Our data suggest that BYHWS regulation of cellular migration/invasion and MMP-2 expression in HA-VSMCs is dependent on activation of SIRT1. However, *in vitro* experiments are still required to investigate the effect of BYHWS in clinical practice, and the therapeutic value of combined use of BYHWS, ALDH2, SFN, *etc*. for instance, and requires further evaluation.

5. Conclusion

This study demonstrated that treatment with BYHWS in senescent HA-VSMCs resulted in *i*) reduced capacity of migration and invasion, *ii*) decreased MMP-2 expression, and *iii*) sustained the increase of SIRT1 levels. Our data also showed that modulation of SIRT1 levels by either knockdown or over-expression of this protein promoted or inhibited age-associated VSMC migration and invasion. Moreover, BYHWS has an effect on age-associated VSMC responses in a SIRT1-dependent manner.

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