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Original Article

Ganoderma lucidum polysaccharides improve renal aging through upregulating SIRT1 expression

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Abstract: *Ganoderma lucidum* polysaccharides (GL-PS) are characterized by antioxidant activity and a protective role in the immune system. However, it is unclear whether GL-PS can play a beneficial role in preventing aging-related renal diseases. First, the levels of α-smooth muscle actin (α-SMA) and zinc finger E-box binding homeobox 1 (Zeb1), P16, P21, and sirtuin 1 (SIRT1) were explored in renal tissues of senescence-accelerated-resistant (SAMR1) mice and (senescence-accelerated prone mouse) mice. Enhanced α-SMA, Zeb1, P16, and P21 expression was identified in aging mice compared with younger control mice, indicating increased epithelial-mesenchymal transition (EMT) and senescence. After GL-PS treatment, the corresponding protein levels were significantly suppressed. Furthermore, GL-PS treatment could enhance expression of SIRT1 in the kidneys of aging mice. To further determine whether HG-induced senescence and EMT were achieved via enhanced SIRT1 expression, a specific siRNA targeting SIRT1 was selected. Compared with NC, silencing of SIRT1 induced expression of P16, P21, Zeb1, and α-SMA in TCMK-1 cells occurred even with GL-PS pre-incubation. These data indicate that SIRT1 plays a key role in GL-PS regulated senescence and EMT of the aging kidney. In summary, these results indicate that GL-PS suppressed EMT and senescence in the renal tissues is mainly achieved via upregulating SIRT1.

Keywords: Kidney, senescence, *Ganoderma lucidum* polysaccharides, SIRT1

Introduction

The number of newly diagnosed cases of chronic kidney disease (CKD) is growing each year among the elderly [1]. CKD is characterized by decreased glomerular filtration rate, proteinuria, or structural kidney disease [2]. It is estimated that the average prevalence of CKD is three to five times higher than that of young and middle-aged populations [3]. Morphological and functional changes that accompany kidney aging include glomerulosclerosis, interstitial fibrosis, tubular atrophy, vascular sclerosis, and loss of renal function in the elderly [4]. The primary reason for kidney aging is attributed to cellular senescence, which is defined as the reduction of cell proliferation even in the presence of ample space, nutrients, and growth factors in the culture [5, 6]. Therefore, to elucidate the underlying mechanism by which cellular senescence and fibrosis is regulated during kidney aging is of great importance.

SIRT1, an oxidized form of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases and mono-ADP-ribosyltransferases, and it belongs to the mammalian sirtuin family [7]. Studies have indicated the important role of SIRT1 in stress response, metabolism, and lifespan regulation [7, 8]. It has been suggested that activation of SIRT1 protects mice from diet-induced obesity, metabolic disorders and oxidative injury [9]. In contrast, inactivation of SIRT1 results in increased cell injury and diabetic nephropathy [10, 11].

Ganoderma lucidum, a famous herbal medicine in China, is featured in traditional Chinese medicine for over 1,000 years [12, 13]. *G. lucidum* polysaccharides (GL-PS) are characterized by its antioxidant activity and protective role in the immune system [14, 15]. However, it is unclear whether GL-PS can play a beneficial role in preventing aging-related renal diseases. In this study, we provide novel evidence that GL-PS prevents aging-induced renal pathological dam-

age and cell senescence by activating SIRT1, thereby indicating the potential of GL-PS in the treatment of renal diseases.

Materials and methods

Animals

Sixty 3-month-old SAMP8 and fifteen 3-month-old senescence-accelerated-resistant (SAMR1) pathogen- and virus-free mice, which are considered to be the control-strain of SAMP8, were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). These SAMP8 mice were randomly divided into two groups: GL-PS groups and the blank control group. SAMR1 mice were considered as the control group. 98% purity GL-PS was purchased from Shaanxi Ciyuan Biotech Co., Ltd. (Xi'an, China). Briefly, GL-PS groups were intra-gastrically given 100 mg/kg of GL-PS every day for 30 days, respectively. The same volume of saline was provided to the model and control groups. The mice were euthanatized while the kidney were excised for immunohistochemistry staining, hematoxylin and eosin staining. Animal care and experimental procedures were implemented in accordance with the animal committee of Tianjin First Center Hospital.

Cell culture

Mouse renal tubular epithelial (TCMK-1) cells was obtained from the cell bank of Shanghai Biology Institute, Chinese Academy of Science (Shanghai, China) and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 U/ml) at 37°C in a humidified atmosphere containing 5% CO_2.

High glucose or hydrogen peroxide treatment of endothelial cells

At 80% confluence, the cultures were switched to serum-free medium containing 0.1% bovine serum albumin (BSA) and treated with 25 mM glucose (HG) or 5 mM glucose (NG) media for 72 h.

Cell cycle analysis

TCMK-1 cells were plated onto 6-well plates. After treatment, cells were harvested by trypsinization without EDTA, washed 3 times with ice-cold PBS, and fixed with 70% ethanol overnight at 4°C. Cell cycle analysis was performed using RNaseA and PI staining by flow cytometry. Experiments were performed in triplicate. The percentage of cells in each cycle phase was assessed using FlowJo software.

RNA extraction

Total RNA was extracted from renal tissues using TRIzol reagent according to the manufacturers' instructions (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was reverse-transcribed using Takara microRNA Reverse Transcription Kit (Takara Bio, Inc., Otsu, Japan) with specific primers for SIRT6. Subsequently, the PCR amplification was performed. One mg of cDNA was used for qPCR using SYBR green Master mix (Roche Diagnostics, Basel, Switzerland) on a Roche Lightcycler 480 (Roche Diagnostics) at 95°C for 10 min followed by 50 cycles of 95°C for 10 sec, specific annealing temperature 55°C for 10 sec, 72°C for 5 sec; 99°C for 1 sec; 59°C for 15 sec; 95°C for 1 sec; then cooling to 40°C. Relative mRNA expression was normalized against the endogenous control, GAPDH, using the Δ-Δ Cq method [18].

Transient transfections

Shortly before transfection, 0.4-1.6×10^5 cells were seeded in per well of a 24-well plate with 0.5 ml of RPMI-1640 culture medium containing serum and antibiotics. At the same time, specific siRNA targeting SIRT1 or negative control (Genepharma) were pre-incubated with HiperFect transfection reagent (QIAGEN) at room temperature for 10 min. Then, the complex was transfected into TCMK-1 cells at a final concentration 5 nM. After transfection, the cells and complexes were incubated under normal growth conditions for 48 h.

Immunofluorescence

TCMK-1 cells were cultured on 6-well chamber slides and fixed with 4% paraformaldehyde for 10 minutes at -20°C. Then, the slides were washed in PBS for five minutes per time (three times). A polyclonal antibody against P21 or α-SMA was dropped on the slides and incubated for 2 hours at room temperature. After wash-

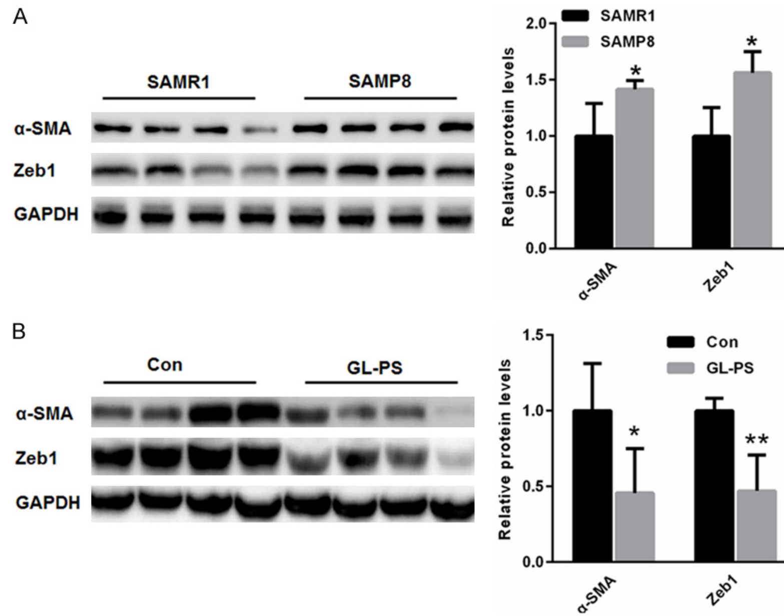

Figure 1. GL-PS treatment improved age-related EMT in the kidney of SAMP8 mice. A. Compared with that of SAMR1 mice, expression of α-SMA and Zeb1 was much higher in the kidneys of SAMP8 mice. B. After GL-PS treatment in SAMP8 mice, renal expression of both α-SMA and Zeb1 was lower than in the saline control group. *p<0.05, **p<0.01 vs. control.

ing with PBS for three times (five minutes per time), the slides were incubated with TRITC-conjugated anti-mouse IgG (1:100 diluted in PBS with 1% BSA) for 1 hour at room temperature. Three times after washing the slides in PBS, the slides were incubated with Hoechst 33258 (10 μg/mL) for 5 min. Then, the slides were washed again and examined using a fluorescence microscope.

Western blotting

Tissues were lysed in 1 mL of 1× RIPA Buffer containing 1 μL leupeptin (Amresco LLC, Solon, OH, USA), 1 μL aprotinin (Amresco), and 10 μL phenylmethylsufonyl fluoride (Amresco). A total of 60-100 μg of the extracted proteins was separated by 6-12% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and electrotransferred (DYCP-40C; Liuyi Instrument Factory, Beijing, China) onto nitrocellulose membranes (Merck Millipore, Billerica, MA, USA). The membranes were blocked with casein for 1 hour at room temperature and subsequently incubated with the following primary antibodies at 4°C overnight: anti-SIRT1 (Rabbit; Abcam, Cambridge, MA, USA) at 1:1000. After being washed with Tris-buffered saline containing 0.1% Tween (TBST) 20, the membranes were probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:1000 dilution; Beyotime Institute of Biotechnology, Shanghai, China). After washing again with TBST, the bands were visualized using an enhanced chemiluminescence system (DP2-BSW; Olympus, Tokyo, Japan) and densitometry was performed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

Cell cycle analysis

TCMK-1 cells were plated onto the 6-well plate, after treatments of nigericin with different concentrations for 48 hours, cells were harvested by trypsinization without EDTA, washed 3 times by ice-cold PBS, and fixed with 70% ethanol overnight at 4°C. Cell cycle analysis was performed using RNaseA and PI staining by flow cytometry. Experiments were done in triplicate. The percentage of cells in each cell cycle phase was assessed using FlowJo software.

Senescence-associated β-galactosidase (SA-β-gal) staining

Cryostat sections (4 μm) were fixed in 0.2% glutaraldehyde and 2% formaldehyde at room temperature for 15 minutes, and then washed in PBS and incubated in freshly prepared SA-β-gal staining solution (1 mg/mL X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$) at 37°C without CO_2 overnight. The tissue sections were counterstained with eosin and examined under a microscope.

Statistical analysis

All experiments were repeated at least three times. The results are expressed as the mean ± SD. Statistical analyses were performed using analysis of variance with SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA), and a

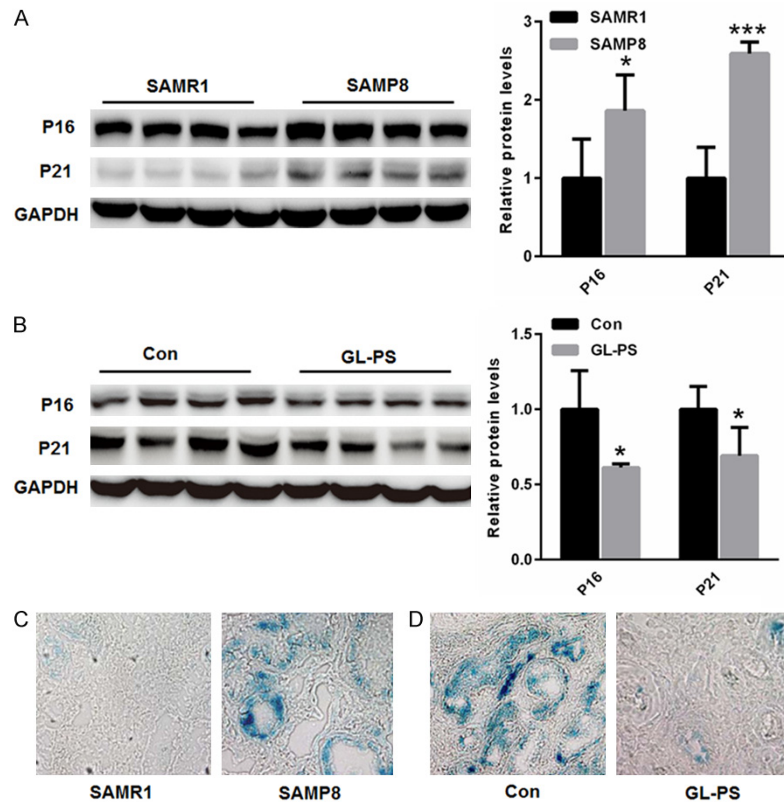

Figure 2. GL-PS treatment ameliorated renal cell senescence in aging kidneys. (A) Western blot analysis indicated that renal expression of P16 and P21 was increased in SAMP8 mice than that of SAMR1 mice. (B) Renal expression of P16 and P21 was significantly reduced in GL-PS group than in saline group of SAMP8 mice. (C) SA-β-gal staining showed that more senescence cells were observed in the kidneys SAMP8 mice than that of SAMR1 mice, (D) but GL-PS treatment decreased senescence cells in SAMP8 mice. *p<0.05, **p<0.01, ***p<0.001 vs. control.

level of $p<0.05$ was considered statistically significant.

Results

GL-PS treatment improved age-related EMT in the kidney of SAMP8 mice

First, we explored expression of α-SMA, a specific marker of mesenchymal fibroblasts, as well as the transcription factor Zeb1, an inducer of EMT [16]. Compared with that of SAMR1 mice, expression of α-SMA and Zeb1 was much higher in the kidneys of SAMP8 mice (**Figure 1A**). After GL-PS treatment, renal expression of both α-SMA and Zeb1 was lower than in the control group (**Figure 1B**). These results showed that EMT was evident in the kidneys of the older rats than those of younger control, and GL-PS treatment improved EMT process in the kidneys of the SAMP8 mice.

GL-PS treatment ameliorated renal cell senescence in aging kidneys

Next, we evaluated whether cell senescence is mitigated by GL-PS treatment in aging kidneys. It is well known that P16 and P21, two markers of cell cycle arrest in the process of cell senescence [17]. Western blot analysis indicated that renal expression of P16 and P21 was increased in SAMP8 mice than that of SAMR1 mice (**Figure 2A**). However, renal expression of P16 and P21 was significantly reduced in GL-PS group than in the saline group of SAMP8 mice (**Figure 2B**). Similar results were identified through SA-β-gal staining. As shown in **Figure 2C** and **2D**, more senescence cells were observed in the kidneys SAMP8 mice than that of SAMR1 mice, but GL-PS treatment decreased senescence cells in SAMP8 mice. Our data suggested that GL-PS remediates cellular senescence in aging kidney.

GL-PS treatment enhanced SIRT1 expression

Western blot analysis indicated that the expression of SIRT1 was decreased in the renal tissues of SAMP8 mice than that of SAMR1 mice (**Figure 3A**). However, GL-PS treatment increased the expression of SIRT1 in the kidneys than that of control (**Figure 3B**).

High glucose-induced senescence and EMT was accompanied by reduced SIRT1 expression in TCMK-1 cells

Furthermore, we established *in vitro* TCMK-1 cellular senescence models by 25 mM high glucose treatment. Compared with NG, high glucose treatment induced the expression of P16, P21, Zeb1, and α-SMA in TCMK-1 cells. Additionally, HG suppressed expression of SIRT1 in TCMK-1 cells to less than that of NG (**Figure 4A**). Comparable results with respect to α-SMA

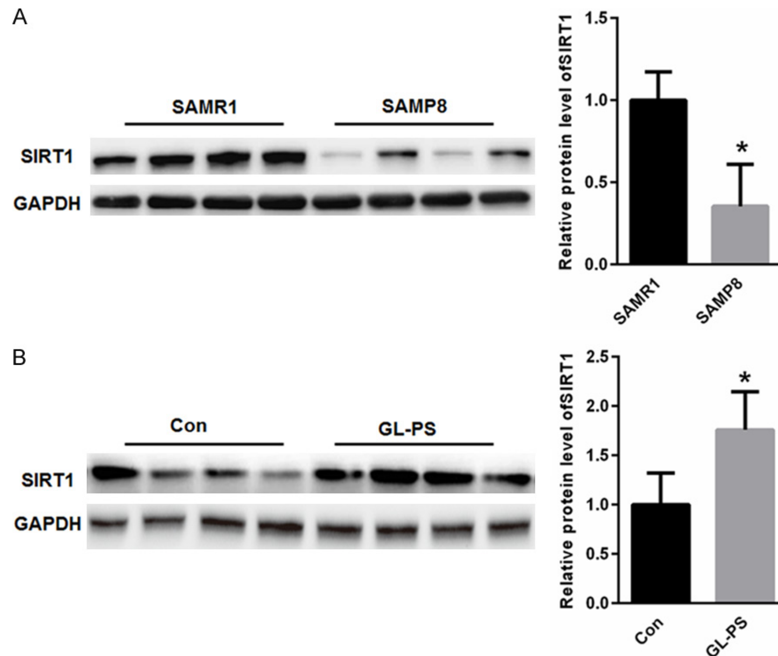

Figure 3. GL-PS treatment enhanced SIRT1 expression. A. Western blot analysis indicates that the expression of SIRT1 was decreased in the renal tissues of SAMP8 mice than that of SAMR1 mice. B. GL-PS treatment increased the expression of SIRT1 in the kidneys than that of control. *p<0.05, **p<0.01 vs. control.

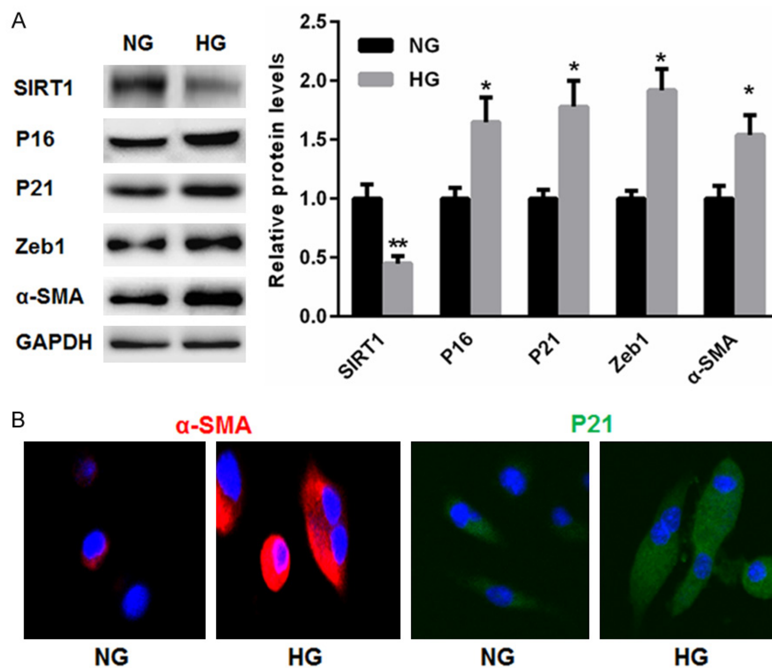

Figure 4. High glucose-induced senescence and EMT was accompanied by reduced SIRT1 expression in TCMK-1 cells. A. Compared with NG, high glucose treatment induced expression of P16, P21, Zeb1, and α-SMA, but suppressed SIRT1 expression in TCMK-1 cells. B. Comparable results with respect to α-SMA and P21 in HG group were obtained with immunofluorescence staining. *p<0.05, **p<0.01 vs. control.

and P21 in HG group were obtained with immunofluorescence staining (**Figure 4B**).

GL-PS alleviated HG-induced senescence and EMT via upregulating SIRT1 expression

To further determine whether HG-induced senescence and EMT is achieved via enhancing SIRT1 expression, a specific siRNA targeting SIRT1 was selected. Compared with NC, silencing of SIRT1 induced the expression of P16, P21, Zeb1, and α-SMA in TCMK-1 cells even with GL-PS pre-incubation (**Figure 5A**). Furthermore, flow cytometry analysis indicated enhanced G0-G1 cell cycle arrest in TCMK-1 cells transfected with si-SIRT1 compared with that of NC, even with GL-PS pre-incubation (**Figure 5B**). These data indicate that SIRT1 plays a key role in GL-PS regulated senescence and EMT.

Discussion

EMT is the process in which differentiated epithelial cells are converted into matrix-producing fibroblasts [18]. Recent studies have indicated that EMT is a key contributor in the process of kidney fibrosis and decreased renal function [19]. Multiple factors, including transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), are considered to result in tissue homeostasis, thereby triggering EMT and progressive fibrosis [20]. Based on the above

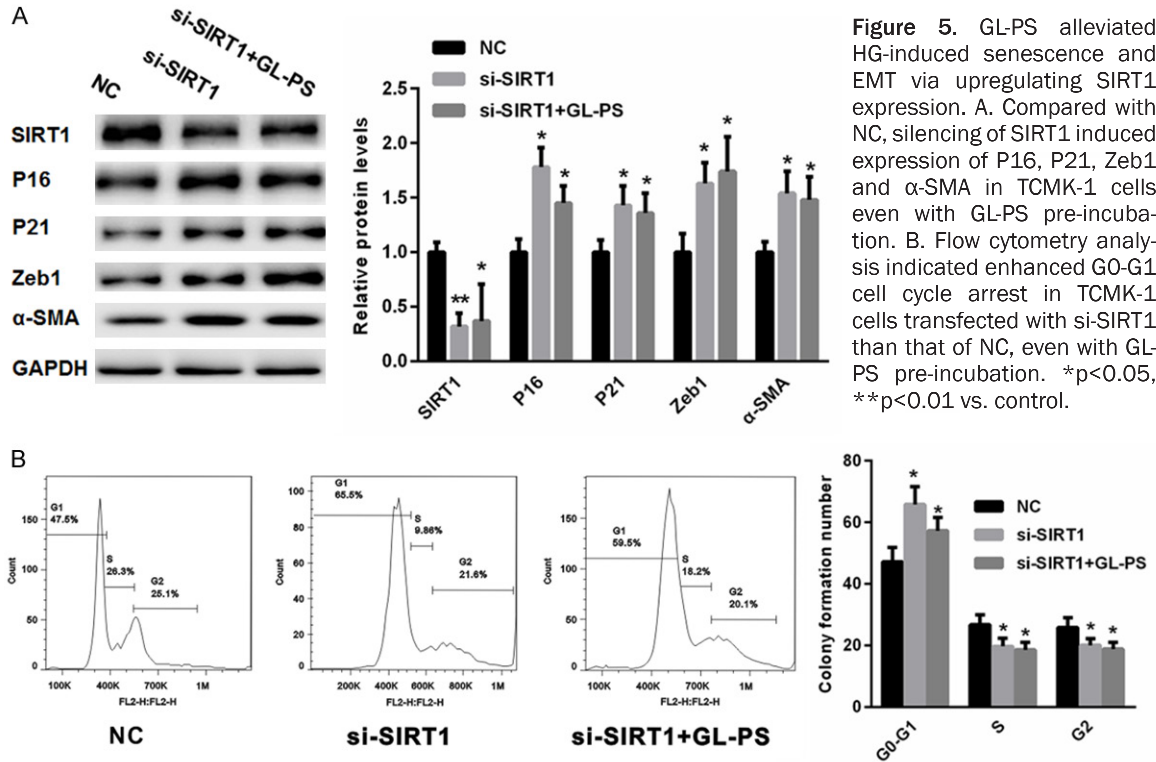

Figure 5. GL-PS alleviated HG-induced senescence and EMT via upregulating SIRT1 expression. A. Compared with NC, silencing of SIRT1 induced expression of P16, P21, Zeb1 and α-SMA in TCMK-1 cells even with GL-PS pre-incubation. B. Flow cytometry analysis indicated enhanced G0-G1 cell cycle arrest in TCMK-1 cells transfected with si-SIRT1 than that of NC, even with GL-PS pre-incubation. *p<0.05, **p<0.01 vs. control.

studies, early therapeutic interventions may be helpful in the conversation of cellular senescence and EMT process. In line with previous studies, our data showed that EMT was enhanced in the kidneys of older rats than that of the younger rats, indicating that EMT is increased along with the senescence as a function of age. Given that EMT is attributed to the decline in renal function with age, we investigated the effect of GL-PS on age-related EMT in our rat models. We found that GL-PS reduced the number senescent cells in the kidneys and improved age-related EMT process, indicating a protective role of GL-PS in kidney aging.

Multiple research studies have indicated that common hallmarks of aging kidney include cellular senescence, upregulation of P16 and P21, and enhanced SA-β-gal activity [21]. To understand age-related changes in kidney function, it is important to explore the expression of P16 and P21 in the renal tissues. Compared to young rats, increases in expression of P16 and P21 were observed in the older rats. In contrast, treatment with GL-PS significantly decreased the levels of P16 and P21 in the kidneys of SAMP8 mice.

These above observations have led us to the underlying mechanism in which GL-PS improves EMT and cellular senescence in aging kidneys. Here, we mainly focused on SIRT1, a key longevity gene [22]. Previous studies have suggested that SIRT1 protects kidney cell injury from various cellular stresses [23, 24]. Furthermore, the podocyte-specific loss of SIRT1 aggravates diabetic kidney injury [7]. However, whether SIRT1 is involved in GL-PS-induced improvement of aging kidney has never been explored. Therefore, in this study, we sought to determine the effects of SIRT1 in age-induced kidney injury after GL-PS treatment. In line with previous studies, renal reduction of SIRT1 was identified in the kidneys of aging mice. Not surprisingly, GL-PS treatment increased SIRT1 expression in the kidney of aging mice. We propose that increased levels of SIRT1 may improve EMT and cellular senescence in the renal tissues of aging mice. This is, to our knowledge, for the first study to demonstrate an effect of GL-PS on SIRT1 expression in the aging kidney.

In the *in vivo* experiments, we showed that SIRT1 was upregulated by GL-PS treatment in the aging mice. To verify whether it is the major contributor of GL-PS-improved renal function, EMT and cellular senescence of TCMK-1 cells *in vitro* was induced with high glucose [25, 26]. We found that exposure of TCMK-1 cells to high

glucose for 48 hours results in significant EMT, cellular senescence, and reduced levels of SIRT1 expression. In contrast, pre-incubation with GL-PS alleviated high glucose-induced EMT and cellular senescence, and enhanced SIRT1 expression. Moreover, silencing of SIRT1 could induce EMT and senescence even in GL-PS treated cells. These results indicate that GL-PS suppresses EMT and senescence in the renal tissues mainly via upregulating SIRT1.

In conclusion, this study shows the protective effects of GL-PS on renal senescence and aging-related EMT. Therefore, GL-PS could be used as an early prevention and treatment in the protection of kidney function among the elderly.

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