Adjudin delays cellular senescence through Sirt3-mediated attenuation of ROS production

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Abstract. Aging, marked by the physical and functional decline in numerous biological processes, is associated with multiple pathologies including cancer, neurodegenerative diseases and cardiocerebral vascular diseases. The accumulation of reactive oxygen species (ROS) production is considered one of the major causes of aging-associated diseases and a major therapeutic target. Hydroxyurea has been widely used for cellular senescence model. The expression level of cell cycle-related protein, ROS production and senescence-associated β-galactosidase are considered to be markers of cellular senescence. Strategies to slow senescence may be beneficial for various aging-associated diseases. The results of the current study indicated that adjudin, a multi-functional small molecule compound, delayed hydroxyurea-induced senescence in mouse embryo fibroblasts (MEFs). Adjudin reduced the proportion of senescence-associated β-galactosidase-positive cells and decreased the expression levels of senescence-associated markers, p16 and p21. Mechanistically, adjudin exerted its anti-senescence effect by elevating the expression level of sirtuin 3 (Sirt3), which attenuated ROS production through the regulation of forkhead box O3a and manganese superoxide dismutase expression. Furthermore, by comparing wild-type and Sirt3-knockout MEFs, it was demonstrated that Sirt3 mediated the anti-senescence effect of adjudin. Taken together, the findings indicated that adjudin has anti-aging properties that may be exploited to treat aging-associated diseases.

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Abbreviations: ROS, reactive oxygen species; HU, hydroxyurea; MEF, mouse embryo fibroblast; SA-β-gal, senescence-associated β-galactosidase; Foxo3a, forkhead box O3a; SOD2, manganese superoxide dismutase; CCK-8, cell counting kit-8

Key words: adjudin, cellular senescence, sirtuin 3, reactive oxygen species, manganese superoxide dismutase, forkhead box O3a

Introduction

Aging causes a progressive loss of tissue function processes (1) marked by numerous common hallmarks, including genome instability, deregulated nutrient sensing, molecular damage, telomere attrition, epigenetic and transcriptional changes, inflammation, cell death and senescence, and metabolic dysfunction (2,3). Cellular senescence refers to a process that imposes permanent proliferative arrest in response to various stressors, emerging as one of the most important contributors to age-associated disease and an attractive target for therapy (4,5). The mechanism underlying senescence remains largely unknown, which makes it challenging to determine the events involved in aging. An enduring potential explanation is oxidative stress. Excessive levels of intracellular reactive oxygen species (ROS) result in age-associated characteristics, including DNA damage, proteins oxidation, lipids degradation and increased ROS production, culminating in significant cellular injury (6). In the nervous system, accumulation of ROS contributes to the age-associated loss of cognitive, sensory and motor function (7).

Sirtuins are a conserved family of deacetylase proteins, which are among the first genes reported to extend lifespan (8,9). Sirtuin 3 (Sirt3), a mitochondrial deacetylase (10), has been reported to regulate major mitochondrial biological processes, including ATP generation, ROS detoxification, nutrient oxidation, mitochondrial dynamics and the unfolded protein response (11-15) by removing acetyl modifications from mitochondrial proteins (10,16) or other acyl modifications and histone crotonylation (17,18). A number of mitochondrial proteins involved in aging process are in an acetylated form (19) and are substrates of Sirt3 (10), suggesting that Sirt3 may mediate a broad spectrum of protection against ROS-induced aging.

Adjudin, formerly termed AF-2364 [1-(2,4-dichlorobenzyl) 1H-indazole-3-carbo-hydrazide] (20), exhibits reversible anti-spermatogenic activity through disruption of adherent junctions of premature sperm to seminiferous epithelium (21,22). We have previously reported that adjudin reduced ischemia-induced neuroinflammation through the nuclear factor-κB (NF-κB) pathway (23) and reduced loss of gentamycin-induced rodent cochlear hair cells through the Sirt3-ROS axis (24). The current study aimed to investigate whether adjudin has anti-aging effects and the underlying mechanisms involved.

Materials and methods

Reagents and animals. Hydroxyurea (HU) and reactive oxygen species (in dimethyl sulfoxide) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) and Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), respectively. Adjudin was provided by Mary M. Wohlford Laboratory (Population Council, New York, USA). Wild-type (WT) C57BL/129 mice and Sirt3-knockout (KO) C57BL/129 mice were acquired from the Jackson Laboratory (Sacramento, CA, USA). Animal procedures for this research were based on the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University (Shanghai, China).

Cell culture and mouse embryo fibroblast (MEF) isolation. MEFs were isolated from embryonic 13.5 (E13.5) WT or Sirt3-KO mice. This study was approved by the Bioethics Committee of School of Biomedical Engineering, Shanghai Jiao Tong University. The embryos, excluding the head and visceral tissues, were washed with phosphate-buffered saline (PBS), minced with scissor and then transferred into 0.1 mM trypsin/1 mM EDTA solution. After incubation at 37˚C for 20 min, twice the amount of medium was added and cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (penicillin, 100 U/ml; 100 μg/ml, streptomycin) at 37˚C in a humidified incubator with 5% CO_2.

Cell viability assay. Cell viability was determined using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, ~10,000 MEFs were seeded into one well of 96-well plates. Different concentrations of adjudin were tested both with and without hydroxyurea (24 h, 6 mM: 0, 5, 10, 20 and 40 μM). The cells were pre-treaeted with adjudin for 1 h and then incubated with/without hydroxyurea for 24 h. Following treatment, CCK-8 solution was added to each well at the dilution of 1:10. After incubation for ~1.5 h, the absorbance at 450 nm was measured using a microplate reader (Synergy2; BioTek Instruments, Inc., Winooski, VT, USA).

Senescence-associated β-galactosidase (SA-β-gal) staining. Cellular senescence was determined by SA-β-gal staining with senescence-associated β-galactosidase staining kit (Beyotime Institute of Biotechnology, Haimen, China). Cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS three times. Subsequently, cells were incubated overnight at 37˚C in darkness with the working solution containing 0.05 mg/ml 5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside (X-gal).

Western blot analysis. Western blotting was performed as previously described (25). Cells were lysed in radioimmunoprecipitation assay lysis buffer (EMD Millipore, Billerica, MA, USA) containing Complete Protease Inhibitor Cocktail (1:100) and 2 mM phenylmethylsulfonyl fluoride. The protein concentration was quantified by bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Total protein (30 μg) was separated by 10% SDS-PAGE and then transferred to a 0.45 μm nitrocellulose membrane (EMD Millipore). Following the incubation with primary antibodies at 4˚C overnight, the membrane was hybridized with horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; 111-035-003; Jackson Laboratory) at room temperature for 1 h. Protein signals were visualized by enhanced chemiluminescence detection. The primary antibodies used were as follows: p16 (1:1,000 dilution; ab51243) and p21 (1:1,000 dilution; ab109199; both from Abcam, Shanghai, China); Sirt3 (1:1,000 dilution; 5490S; Cell Signaling Technology, Inc., Danvers, MA, USA); Sirt6 (1:1,000 dilution; ab191385; Abcam); MAPK family antibody [extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK); 9926; Cell Signaling Technology, Inc.]; phospho-MAPK family antibody [phospho-ERK, phospho-p38, phospho-JNK; 1:1,000 dilution; Cell Signaling Technology, Inc.)]; manganese superoxide dismutase (SOD2; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); Akt (1:1,000; ab8805), phospho-Akt (1:1,000; ab38449; both from Abcam); forkhead box O3a (Foxo3a; 1:1,000; 12829; Cell Signaling Technology, Inc.); and β-tubulin (1:1,000; ab6046; Abcam).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from MEFs with the use of RNAiso Plus (Takara Biotechnology Co., Ltd., Dalian, China), and first strand cDNA was synthesized from 1 μg of total RNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.). RT-qPCR was performed on an ABI 7900HT (Thermo Fisher Scientific, Inc.) by using SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) according to the following protocol: 95˚C for 30 sec; 40 cycles consisting of 95˚C for 5 sec and 60˚C for 30 sec, 95˚C for 15 sec and 60˚C for 1 min; and 95˚C for 15 sec. Primers used were as follows: mSirt1 (sense, 5'-TAGTCCTTCCTACCCCAA TTTCC-3' and antisense, 5'-TTGGTCCTTAGCCACTCC TTC-3'); mSirt2 (sense, 5'-GCCTGGGTTCCCAAA AGGAG-3' and antisense, 5'-GAGCGGAAGTCAGGG ATACC-3'); mSirt3 (sense, 5'-ATCCCGGACTTCAGA TCCCC-3' and antisense, 5'-CAACATGAAAAAGGGCTT GGG-3'); mSirt4 (sense, 5'-GTGGAAGAATAAGAATGA GCGGA-3' and antisense, 5'-GGCACAAATAACCCC GAGG-3'); mSirt5 (sense, 5'-CTCCGGGCCGATTCA TTTCC-3' and antisense, 5'-GCGTTCGCAAAACAC TTCCG-3'); mSirt6 (sense, 5'-ATG TCGGTGAATTATGCA GCA-3' and antisense, 5'-GCTGGAGGACTGCCA CATTA-3'); mSirt7 (sense, 5'-AGCATCACCCGTTTG CATGA-3' and antisense, 5'-GGCAGTACGCTCAGT CACAT-3'); mSOD2 (sense, 5'-CAGACCTGCCTTACGACT ATGG-3' and antisense, 5'-CTCGGTGGCGTTGAGATT GTT-3'); m ribosomal protein, large P0 (mRplp0; sense 5'-AGATTCGGGATATGCTGTTGGC-3' and antisense, 5'-TCGGGTCCTAGACCAGTGTTC-3'). RT-qPCR was performed out in triplicate and the results are presented as the Cq values. The mean Cq value was calculated, and the ΔCq value was determined as the mean Cq value for the target gene minus the mean Cq value for mRplp0. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Cq}$ method.

ROS assay. Following treatment, cells were washed with PBS. Dicholorofluorescein diacetate (DCF-DA; Beyotime Institute of Biotechnology) was diluted in FBS-free DMEM to 10 μM and then added to each well. After incubation for 0.5 h, cells were washed with PBS three times, and the fluorescence was

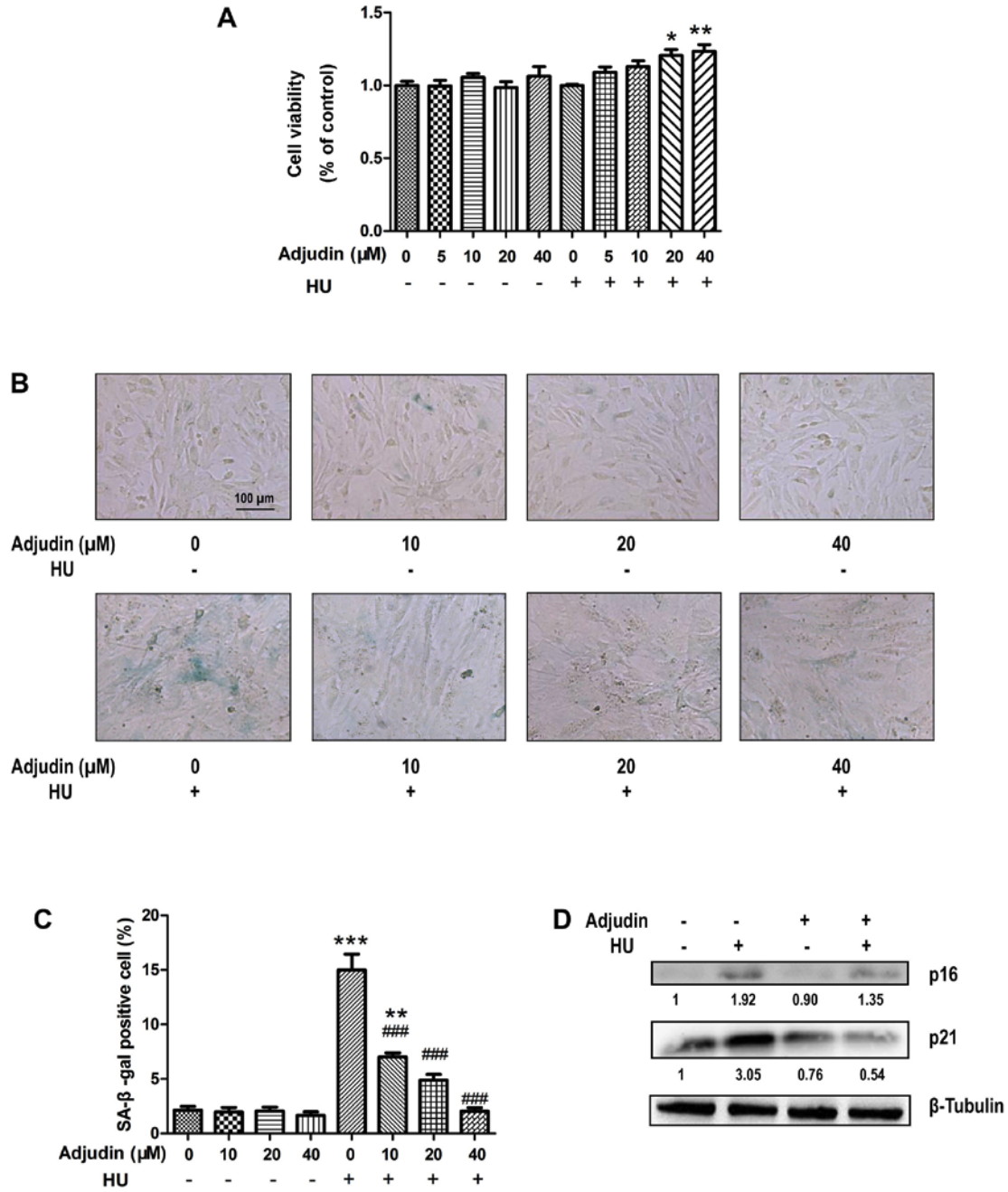

Figure 1. Adjudin attenuates HU-induced cell senescence. MEF cells were pretreated with indicated concentrations of adjudin for 1 h and then stimulated with 6 mM HU for a 24-h incubation period. (A) MEF cells were pretreated with adjudin at 5, 10, 20 and 40 μM in the absence or presence of HU and cell viability was determined by the cell counting kit-8 assay. (B) MEF cells were treated with adjudin at 10, 20 and 40 μM in the absence or presence of HU and stained for SA-β-gal. Scale bar, 100 μm. (C) Percentage of SA-β-gal activity positive cells was determined by counting blue cells counting 400 cells/well. (D) Cells were pretreated with adjudin at 40 μM. Cell lysates were analyzed by immunoblotting with antibodies specific to p16 and p21. β-tubulin was used as a reference. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. control treatment; $^{\#\#\#}$P<0.001 vs. HU alone treatment. HU, hydroxyurea; MEF, mouse embryo fibroblast; SA-β-gal, senescence-associated β-galactosidase.

detected using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 535 nm.

Statistical analysis. Data are presented as the mean ± standard deviation. Multiple comparisons were analyzed by one-way analysis of variance followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Adjudin delays HU-induced cellular senescence. Cells were treated with HU, which inhibits ribonucleotide reductase activity (26,27), to produce an *in vitro* senescence model. HU-induced senescence has been widely used to mimic cell aging (28-34). MEFs were pretreated with adjudin for 1 h, followed by a 24-h incubation period with 6 mM HU. As presented in Fig. 1A, cell viability following treatment with the indicated concentrations of adjudin was not significantly

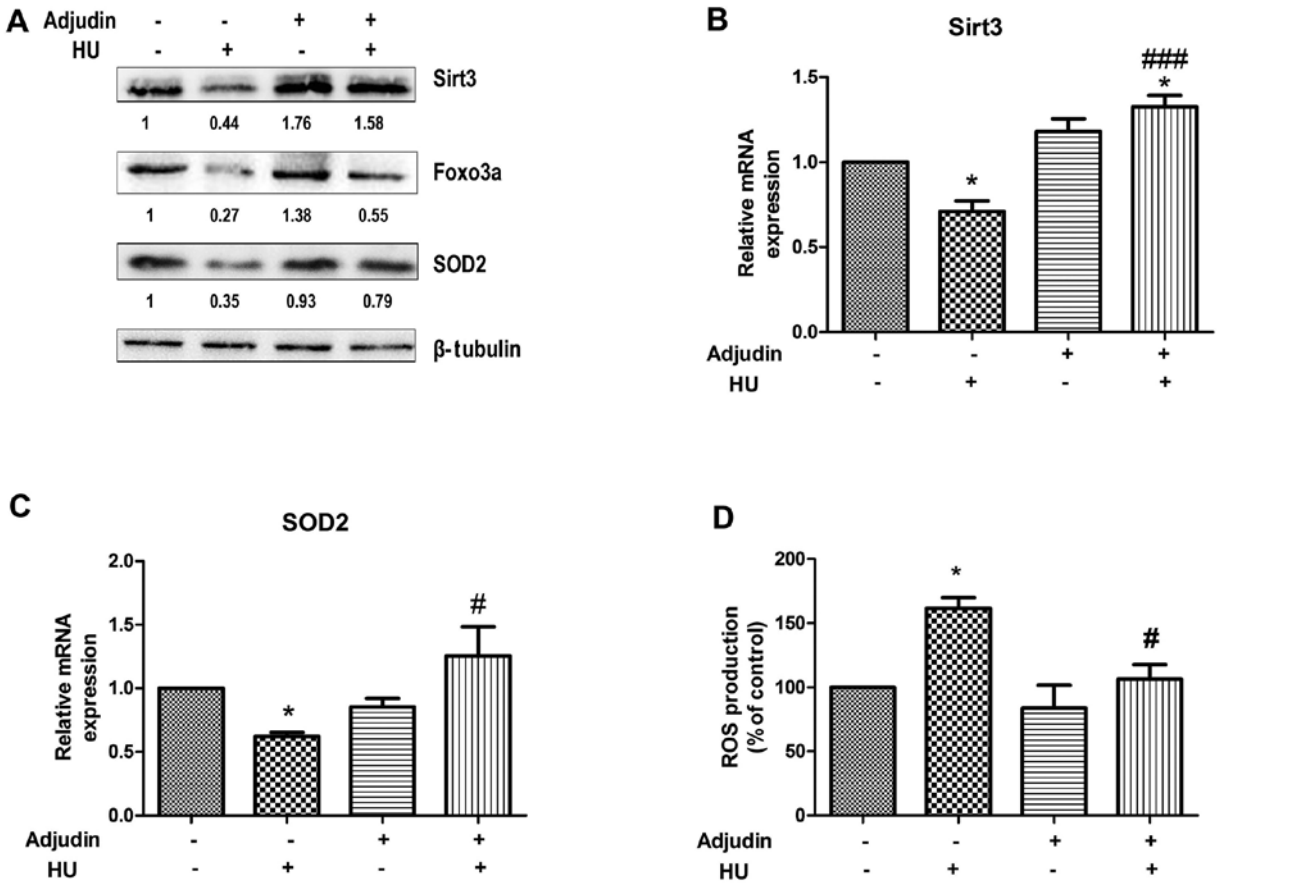

Figure 2. Adjudin upregulates Sirt3 and reduces ROS production. Cells were pretreated with 40 μM of adjudin for 1 h and then stimulated with 6 mM HU for 24 h. (A) Cell lysates were analyzed by immunoblotting with antibodies specific to Sirt3, Foxo3a and SOD2. Equal loading of proteins was estimated by β-tubulin. (B) Sirt3 and (C) SOD2 mRNA expression was evaluated by quantitative polymerase chain reaction. Data were normalized to ribosomal protein, large P0. (D) Cells were stimulated with HU for 24 h, and the total ROS level was determined using dicholorofluorescein diacetate. Data are presented as the mean ± standard of three independent experiments and are presented relative to control. *P<0.05 vs. control treatment. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. HU alone treatment. HU, hydroxyurea; Sirt3, sirtuin 3; Foxo3a, forkhead box O3a; SOD2, manganese superoxide dismutase 2; ROS, reactive oxygen species.

different from the control group in the absence of HU, while a slight elevation in cell survival was observed when treated with 20 and 40 μM adjudin in the presence of HU.

Subsequently, whether adjudin could affect cellular senescence was determined. The results demonstrated that pretreatment with adjudin (10, 20 and 40 μM) for 1 h reduced the percentage of HU-induced SA-β-gal-positive cells at a dose-dependent manner (Fig. 1B and C). Furthermore, levels of p16 and p21, two cyclin-dependent kinase inhibitors considered as senescence markers (35,36), were detected by western blot. HU increased the expression of p16 and p21, which was notably decreased by pretreatment with 40 μM adjudin compared with HU alone (Fig. 1D).

Adjudin elevates Sirt3 expression and suppresses ROS level. Previous studies have implicated sirtuins as key mediators of caloric restriction, which is known to inhibit senescence (37). The effect of adjudin on sirtuin expression was examined, and adjudin significantly upregulated mRNA levels of Sirt3 and Sirt6, but there was no change in Sirt6 protein level between different groups (data not shown).

Adjudin has been reported to protect cochlear hair cell via Sirt3-ROS pathway (24) and Sirt3 mediates antioxidant defense and metabolic adaptation that greatly influences mammalian lifespan (37), so we speculate whether adjudin delays senescence through Sirt3-mediated inhibition of ROS production. Indeed, Sirt3 protein level exhibited a 56% decline following HU treatment. In addition, Foxo3a and SOD2, two target proteins of Sirt3 closely associated with ROS, also presented a 73 and 65% decline in senescent cells, relatively. However, pretreatment with 40 μM adjudin significantly counteracted those changes in the presence of HU in MEFs (Fig. 2A). Similar results were also observed at the mRNA level for Sirt3 and SOD2 (Fig. 2B and C).

Subsequently, it was examined whether adjudin could reduce ROS production by using the DCF-DA method. HU stimulation resulted in accumulation of intracellular ROS, which was alleviated by adjudin treatment (Fig. 2D).

Sirt3 mediates anti-senescence effect of adjudin. To validate the role of Sirt3 in the anti-senescence effects of adjudin, MEFs from WT and Sirt3-KO mice were isolated. Both WT and Sirt3-KO MEFs exhibited strong SA-β-gal activity following exposure to HU. Notably, adjudin co-treatment with HU caused a 30% reduction in SA-β-gal-positive cells in Sirt3-KO MEFs compared to a 60% reduction in SA-β-gal-positive cells in WT MEFs, indicating that Sirt3 may be involved in the anti-aging property of adjudin (Fig. 3A and B).

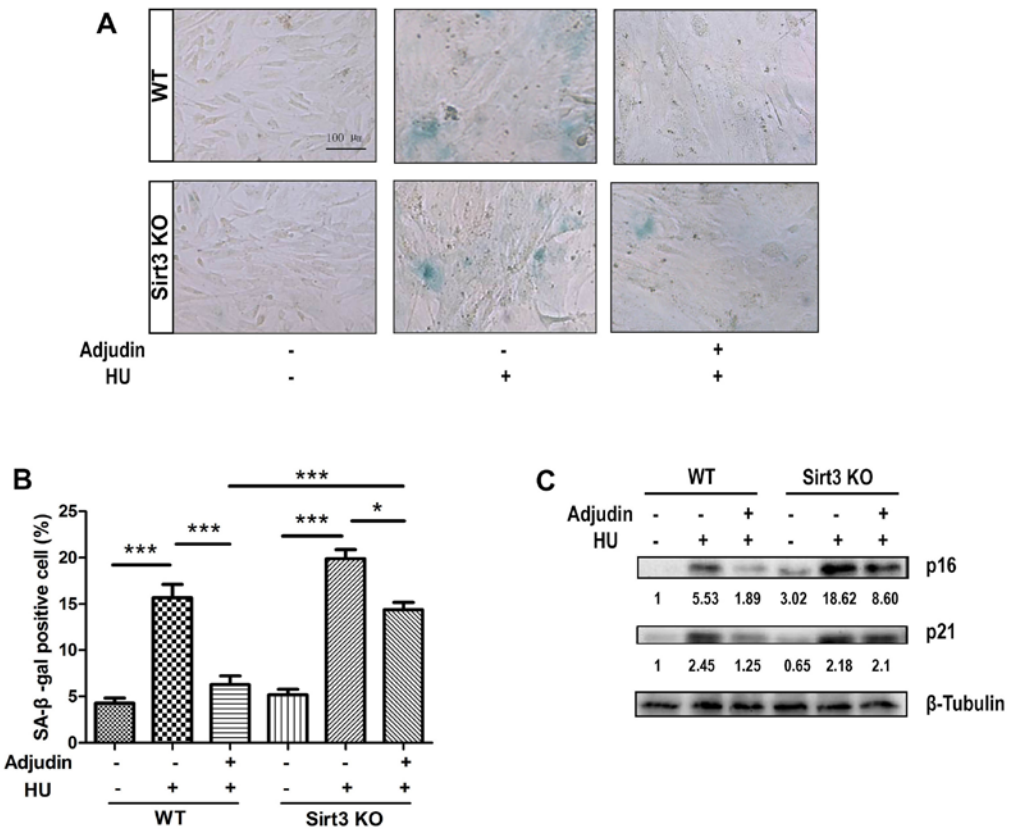

Figure 3. Anti-aging effect of adjudin is mediated by Sirt3. Cells were pretreated with 40 μM of adjudin for 1 h and then stimulated with 6 mM HU for a 24-h incubation period. (A) Cells were stained for SA-β-gal. Scale bar, 100 μm. (B) Percentage of SA-β-gal activity positive cells was determined by counting 400 cells/well. (C) Cell lysates were analyzed by immunoblotting with antibodies specific to p16 and p21. β-tubulin was used as a reference for equal loading. Data are presented as the mean ± standard of three independent experiments. *P<0.05 and ***P<0.001. WT, wild-type; Sirt3, sirtuin; KO knockout; HU, hydroxyurea.

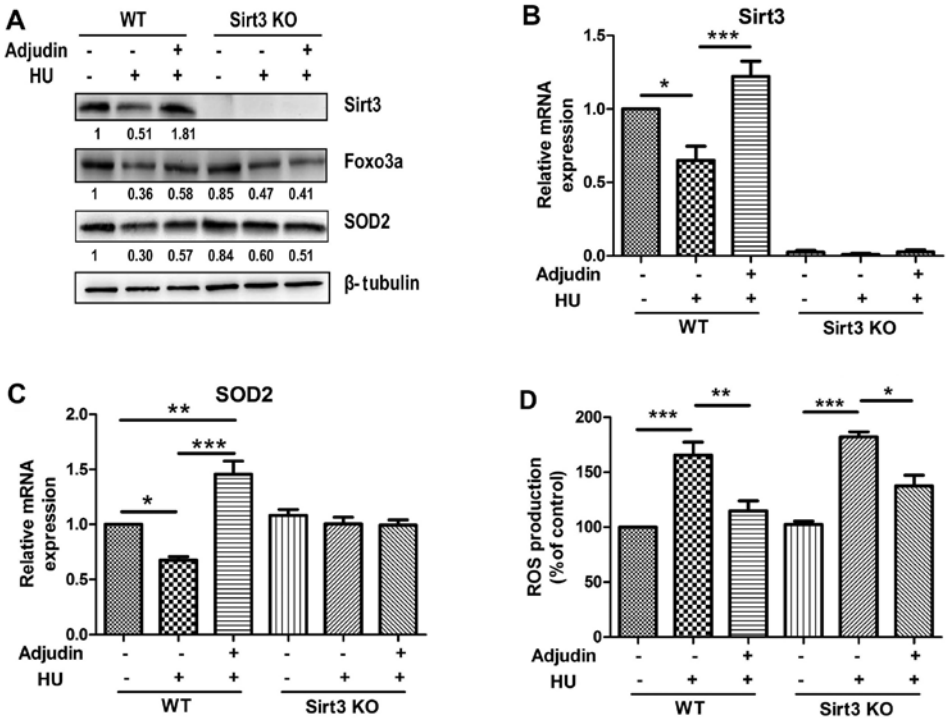

Figure 4. Adjudin exerts anti-aging effects via a Sirt3/ROS pathway. Cells were pretreated with 40 μM of adjudin for 1 h and then stimulated with 6 mM HU for 24 h. (A) Cell lysates were analyzed by immunoblotting with antibodies specific to Sirt3, Foxo3a and SOD2. β-tubulin was used as internal control for proteins. (B) Sirt3 and (C) SOD2 mRNA expression was evaluated by quantitative polymerase chain reaction. (D) The total ROS level was determined using dicholorofluorescein diacetate. Data were normalized to ribosomal protein large P0. Data are presented as the mean ± standard deviation of three independent experiments and are presented relative to the control. *P<0.05, **P<0.01 and ***P<0.001. WT, wild-type; Sirt3, sirtuin; KO knockout; HU hydroxyurea; Foxo3a, forkhead box O3a; SOD2, manganese superoxide dismutase 2; ROS, reactive oxygen species.

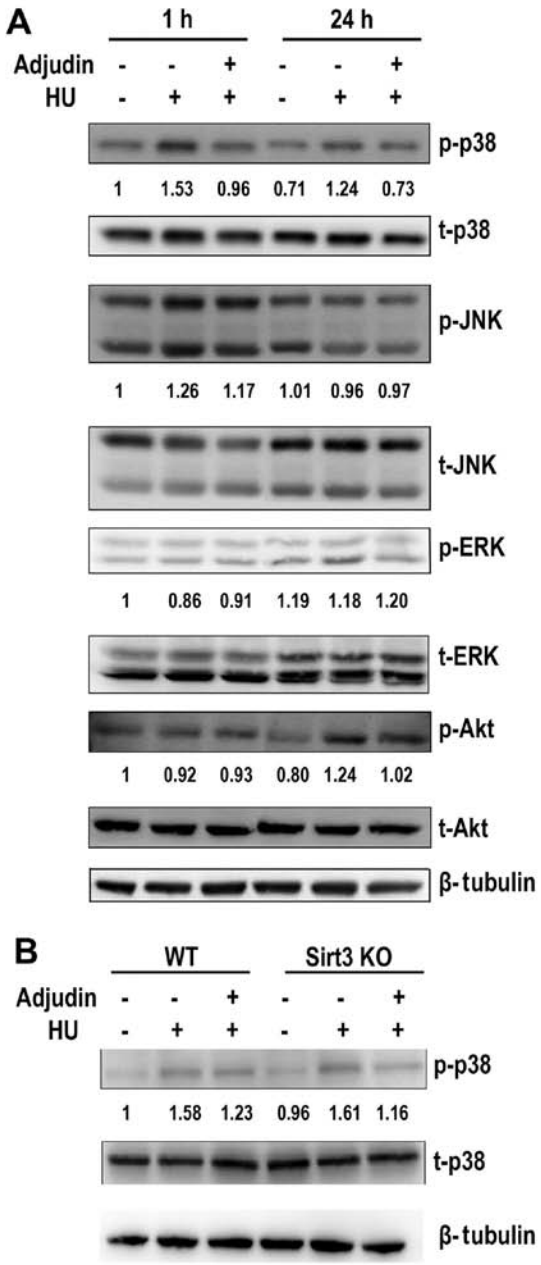

Figure 5. Adjudin suppress the phosphorylation of p38. (A) Cells were treated with HU for 1 or 24 h. Cell lysates were analyzed by immunoblotting with antibodies specific to p-p38 and p38, p-JNK and JNK, p-Akt and Akt, p-ERK1/2 and ERK1/2, and β-tubulin. (B) WT and Sirt3 KO cells were treated with HU for 24 h. Western blot analysis of p-p38 and p38 were performed. HU hydroxyurea; p-, phospho; t-, total; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; WT, wild-type; Sirt3, sirtuin; KO knockout.

In agreement with the SA-β-gal staining results, p16 and p21 levels were also induced by HU in WT and Sirt3-KO MEFs. Adjudin treatment suppressed the upregulation of p16 and p21 expression by HU in WT MEFs, while the effect was not as potent in Sirt3-KO MEFs, demonstrating that Sirt3 mediated, at least partially, the anti-senescence effect of adjudin (Fig. 3C).

Adjudin exerts anti-senescence effects via Sirt3/ROS. Sirt3 deficiency prevented adjudin from elevating Foxo3a and SOD2 levels with HU treatment compared with the effects in WT MEFs (Fig. 4A-C). To determine whether Sirt3 influences ROS production, experiments were performed using DCF-DA. Intracellular ROS levels were higher in Sirt3-KO MEFs than in WT MEFs following treatment with HU and adjudin, suggesting that Sirt3 is, at least partially, required for adjudin to perform antioxidant activity (Fig. 4D). This may suggest that other signaling pathways may be involved in this process.

Adjudin decreases phosphorylation of p38 mitogen-activated protein kinase (MAPK). ROS generation activates the MAPK pathway, thus, it was determined whether adjudin affects the MAPK pathway. As presented in Fig. 5A and B, stimulation with HU induced phosphorylation of p38 at the early time point (1 h), which was suppressed by adjudin in WT and Sirt3-KO MEFs, indicating that Sirt3 and p38 play independent roles. However, levels of phosphorylated ERK or JNK were not affected by treatment with adjudin (Fig. 5A). Phosphorylation of Akt was not affected either (Fig. 5A).

Discussion

The current study identified a new role of adjudin in delaying cellular senescence, demonstrated by reduced SA-β-gal-positive cells, and p16 and p21 levels. The anti-aging property of adjudin was demonstrated associated with Sirt3-mediated upregulation of Foxo3a and SOD2 expression and attenuation of ROS production, which was validated using Sirt3-KO MEFs.

Mammalian sirtuins (Sirt1-7) are a family of highly conserved NAD^+-dependent deacetylases, which are involved in numerous fundamental cellular processes, including metabolic regulation, genomic stability maintenance, DNA repair and stress responses (12). Previous studies have revealed that Sirt3 KO mice spontaneously develop or have accelerated progression of multiple age-associated pathologies, including metabolic syndrome, cancer, cardiovascular diseases, and neurodegenerative diseases (38). Sirt3 has been reported to block aging-associated tissue fibrosis (39) and prevent hearing loss during caloric restriction by stimulating isocitrate dehydrogenase 2 to convert NADP to NADPH in mitochondria, leading to decreased ROS production (40). The results of the current study illustrated that adjudin may upregulate Sirt3 expression in MEFs and in turn counteract premature senescence induced by HU, which supports with the role of Sirt3 in aging and metabolism.

Foxo3a and SOD2 are the two well-established substrates of Sirt3. Foxo3a forms a physical interaction with Sirt3 in mitochondria, and overexpression of Sirt3 increases DNA-binding activity and targeted gene expression of Foxo3a (41). Sirt3 deacetylates Foxo3 at K271 and K290, leading to upregulation of a set of genes that are essential for mitochondrial homeostasis. Consequently, mitochondrial reserve capacity is ensured in response to oxidative damage (42). In addition, Sirt3 deacetylates two important lysine residues on SOD2, thus promoting its antioxidant capacity to reduce oxidative stress damage and extend life span during caloric restriction (43,44). The findings demonstrated that adjudin increased the expression of Foxo3a and SOD2 in WT MEFs; however, this effect was not observed in Sirt3-KO MEFs, indicating Sirt3 is required for the regulation of Foxo3a and SOD2 by adjudin.

Excessive accumulation of ROS and oxidative stress damage are the main causes of age-associated diseases (45,46). Mitochondrial ROS is linked to the elevation of pro-inflammatory mediators and susceptibility to pathological conditions (47). Thus, the antioxidant property of adjudin in HU-stimulated MEFs was examined. DCF-DA signals revealed lower intracellular ROS levels in adjudin-treated cells, implying contribution to delayed cellular senescence driven by the antioxidant property of adjudin.

In the current study, adjudin also suppressed HU-induced phosphorylation of p38, and may have roles independent of Sirt3, as lack of Sirt3 in the KO cells did not affect phosphorylation of p38, nor completely abolish the anti-senescence effect of adjudin. It has been reported that p53 restrains constitutive activation of p38 MAPK, preventing the senescence-associated phenotype (48). Another possibility is the MAPK-nuclear factor-κB (NF-κB) cascade. Our previous work demonstrated that adjudin inhibited neuroinflammation via attenuation of NF-κB signaling pathway (23), which functions as a regulator of redox-sensitive gene expression (49,50).

In conclusion, the results of the current study demonstrated that adjudin upregulated Sirt3 expression, thus raising Foxo3a and SOD2 levels and reducing ROS production to delay cellular senescence. Further investigation on the role of adjudin in animal models is necessary to confirm that adjudin may be a promising therapeutic option to age-associated diseases.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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