ARTICLE

Journal of Cellular Biochemistry 116:242–251 (2015)

Journal of Cellular Biochemistry

miR-124/ATF-6, A Novel Lifespan Extension Pathway of *Astragalus* Polysaccharide in *Caenorhabditis Elegans*

Ning Wang,[1] Jing Liu,[1] Fang Xie,[1,2] Xu Gao,[3] Jian-Han Ye,[1] Lu-Yao Sun,[1] Ran Wei,[1] and Jing Ai[1*]

[1]*Department of Pharmacology, Harbin Medical University, Harbin 150081, China*

[2]*Laboratory of Cardiovascular Medicine Research (Harbin Medical University), Ministry of Education, Harbin 150081, China*

[3]*Department of Biochemistry, Harbin Medical University, Harbin 150081, China*

ABSTRACT

MicroRNAs (miRNAs), especially evolutionarily conserved miRNAs play critical roles in regulating various biological process. However, the functions of conserved miRNAs in longevity are still largely unknown. *Astragalus* polysaccharide (APS) was recently shown to extend lifespan of *Caenorhabditis elegans*, but its molecular mechanisms have not been fully understood. In the present study, we characterize that microRNA mediated a novel longevity pathway of APS in *C. elegans*. We found that APS markedly extended the lifespan of *C. elegans* at the second and the fourth stages. A highly conserved miRNA *miR-124* was significantly upregulated in APS-treated *C. elegans*. Overexpression *miR-124* caused the lifespan extension of *C. elegans* and vice versa, indicating *miR-124* regulates the longevity of *C. elegans*. Using luciferase assay, *atf-6* was established as a target gene of *miR-124* which acting on three binding sites at *atf-6* 3′UTR. Consistently, agomir-*cel-miR-124* was also shown to inhibit ATF-6 expression in *C. elegans*. APS-treated *C. elegans* showed the down-regulation of *atf-6* at protein level. Furthermore, the knockdown of *atf-6* by RNAi extended the lifespan of *C. elegans*, indicating *atf-6* regulated by *miR-124* contributes to lifespan extension. Taken together, *miR-124* regulating ATF-6 is a new potential longevity signal pathway, which underlies the lifespan-extending effects of APS in *C. elegans*. J. Cell. Biochem. 116: 242–251, 2015. © 2014 Wiley Periodicals, Inc.

KEY WORDS: *ASTRAGALUS* POLYSACCHARIDES; LIFESPAN; *CAENORHABDITIS ELEGANS*; ATF-6; *miR*-124

MicroRNAs (miRNAs) are small non-coding RNA, with ~22 nucleotides in length, and function as post-transcriptional regulators of target genes in the processes of cell proliferation, apoptosis, differentiation and development. Especially, the evolutionarily conserved miRNAs play more important roles in these biological processes than unconserved miRNAs. However, whether conserved miRNAs function as longevity factors are still largely unclear. *C. elegans* has short lifespan but strong reproductive capacity. Many lifespan-associated genetic mutants have been well defined in *C. elegans*. Thus, *C. elegans* is suggested as a valuable and productive model for in vivo studies on aging and age-related disorders [Luo, 2004; Antebi, 2007; Braeckman and Vanfleteren, 2007]. In addition, 40% genes conserved with other species including human [Sternberg, 2001], leads *C. elegans* to be broadly used in identifying genes and developing drugs potentially prolonging the lifespan.

Astragalus membranaceus, a traditional herb medicine, has been widely used in China and East Asia for centuries, and now available in the markets of Europe and the USA as food supplement. *Astragalus* polysaccharide (APS), as a major active ingredient of

Ning Wang and Jing Liu contributed equally to this paper.

Conflict of interest: The authors declare no competing financial interest.

Grant sponsor: Key Project of National Science Foundation of China; Grant number: 81130088; Grant sponsor: Natural Science Foundation of China; Grant numbers: 81271207, 81070882, 31100825; Grant sponsor: Key Laboratory of Cardiovascular Medicine Research.

*Correspondence to: Jing Ai, Department of Pharmacology (the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, No.157 Baojian Road, Nangang District, Harbin 150081, China. E-mail: a.z.hrbmu@gmail.com

Manuscript Received: 5 December 2013; Manuscript Accepted: 29 August 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 September 2014

DOI 10.1002/jcb.24961 • © 2014 Wiley Periodicals, Inc.

A. membranaceus possesses multiple biological activities including immunoregulatory, antiviral, hypoglycemic, antioxidant, cardioprotection and antitumor [Shao et al., 2004; Mao et al., 2009; Fu et al., 2011; Li et al., 2011]. Previous studies have reported that APS could prevent diabetes and diabetic cardiomyopathy by restoring the imbalance of Th1/Th2 cytokines [Chen et al., 2008], suppressing the local cardiac chymase-Ang II system [Chen et al., 2010], and improving myocardial glucose and lipid metabolism [Chen et al., 2012]. Interestingly, APS could restore glucose homeostasis by affecting endoplasmic reticulum (ER) stress and insulin/IGF-1 signaling (IIS) pathways which is associated with the longevity [Mao et al., 2009; Narasimhan et al., 2009; Wang et al., 2009; Salminen and Kaarniranta, 2010]. Notably, it was lately reported that APS extended the lifespan of *C. elegans* independently of DAF-2, but dependently of DAF-16/FOXO transcription factor through inhibiting polyglutamine-mediated proteotoxicity [Zhang et al., 2012]. Despite of these findings, however, whether miRNAs underlie the molecular mechanisms of the lifespan-extending effects of APS has not been elucidated yet.

In the present study, we uncovered a new miRNA-mediated longevity pathway, and demonstrated that *miR-124* regulated the expression of *atf-6* via binding to its 3′UTR, which contributes to APS-induced lifespan extension in *C. elegans*.

MATERIALS AND METHODS

PREPARATION OF *ASTRAGALUS* POLYSACCHARIDES

Astragalus polysaccharides (APS) were prepared using the extraction procedure optimized by Jing-cheng Tang [Tang et al., 2006]. Briefly, the *A. membranaceus* roots (3.0 kg) were immersed in deionized water for 12 h, boiled for 1.5 h and extracted twice. The combined aqueous extracts were concentrated in vacuum and precipitated by 95% ethanol for 48 h at room temperature, and then filtered at reduced pressure. The residue was washed by ethanol for twice, redissolved in water and then filtered. The filtrate was precipitated with 85% ethanol (5 times in volume) for 4 h at 4°C. The precipitate was filtered and dried by lyophilization. We got a yield of 3.08% APS powder. All the crude APS have been deproteinated by the Sevag method, and intensively dialyzed for two days against distilled water (cut-off Mw 3500 Da). The solution of APS samples were scanned under UV-VIS spectrometer in the range from 190 to 400 nm, and there is no absorbance peak at 280 nm and 260 nm, implying that the protein and nucleic acid were absent in this polysaccharide. APS was diluted by ddH_2O in our study.

STRAINS AND HANDING PROCEDURES

N2 (wild-type) *C. elegans* strain and the *Escherichia coli* OP50 strain are gifts from the Academy of Military Sciences, China, which were ordered from the Caenorhabditis Genetics Center (CGC). The *C. elegans* strains were maintained and assayed at 20°C on nematode growth medium (NGM) with *E. coli* OP50 (OD600 = 1.2) as a food source according to previous experiments [Brenner, 1974]. Synchronization of worm culture was achieved by hypochlorite treatment of gravid hermaphrodites [Stiernagle, 2006]. The plates that had many gravid hermaphrodites were washed with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 L). Treated the eggs with M9 buffer 3.5 ml, 0.5 ml 5 N NaOH, and 1 ml bleach, followed by centrifuged, washed, and then transferred to NGM plates. Worms of each experiment were obtained from the same synchronization.

LIFESPAN ASSAYS IN S-COMPLETE MEDIUM

Lifespan assay in S-complete medium was performed according to previous study [Petrascheck et al., 2007]. Briefly, S-complete medium (0.1 μg/ml fungizone [Thermo], 50 μg/ml carbenicillin and 0.12/mM 5-fluoro-2′-deoxyuridine [Sigma]) containing freshly prepared *E. coli* OP50 150 μl in total volume were added in 96-well plates. 15–20 worms (synchronized 42–45 h) were transferred into each well. After worms were synchronized 60 h, APS (50 μg/ml, 100 μg/ml, and 200 μg/ml) were added into the S-complete medium. The number of survival animals was scored every other day by microscope (Nikon, Eclipse 80i). Before counting, we will gently shake each plate for 1–2 min. Died nematodes after internal hatch (a defect in egg laying) were excluded from total number.

LIFESPAN ASSAYS ON NEMATODE GROWTH MEDIUM (NGM) AGAR

The NGM plate was prepared as the protocol described on CGC [Stiernagle, 2006]. Briefly, approximately 30–40 L4 larvae (synchronized 48 h) were transferred to 35 mm NGM plates with or without APS (200 μg/ml, 400 μg/ml, 600 μg/ml). The lifespan assay was carried out as described in Pietsch et al. [2009] with minor modifications. The L4 stage was defined as day 1 in the lifespan assays. During the reproductive period (approximately the first 7 days), worms were transferred daily to new plates to avoid overcrowding with progeny, they were transferred every other day. Worms were counted twice every day until all nematodes died. Animals were defined as dead when no response to a gentle touch by the platinum wire. Nematodes which died for internal hatch or crawled off the NGM plate were not included in the lifespan counts. Lifespan assays were performed at least three times ($n > 90$ for each).

ANALYSES OF AGE-RELATED CHANGES IN PHYSIOLOGICAL PROCESSES

Self-fertile reproductive span, fast body movement span, fast pharyngeal pumping span and pharyngeal pumping were determined as described previously [Huang et al., 2004]. One synchronized L4 stage N2 *C. elegans* was placed on a 20 mm NGM plate with or without APS (200 μg/ml). Each animal was examined every day for the following phenotypes: (1) Self-fertile reproduction was assessed by the presence of self-progeny on the NGM plate; (2) Body movement was assessed by observation for 10 s. Worms were defined as fast movement if they exhibited continuous and well coordinated movement with a locomotion rate of >1 mm pre 10 s; (3) Pharyngeal pumping was assessed by observing the number of pharyngeal contraction during a 10 s interval. Worms that displayed 0, 1–24, and ≥25 pharyngeal contractions per 10 s were considered as no pumping, slow pumping, and fast pumping, respectively; and (4) Lifespan was defined from L4 stage to the last day of survival. All

indicators were observed from the same nematodes. The experiments were performed three times (n = 60 for each).

BACTERIAL GROWTH ASSAY

E. coli OP50 bacteria (OD600 = 0.2) were spiked into 5 ml LB broth (200 μg/ml streptomycin) with or without APS (200 μg/ml), and shaked at 200 rpm, 37°C until the OD600 in excess of 1.0.

ATTRACTION ASSAY

In accordance to Menzel et al. [2005], NGM plants (90 mm) were prepared with six alternating spots of *E. coli* OP50 bacteria. Three spots were treated with ddH_2O as control group, and the other three spots were treated with APS (200 μg/ml). One L4 stage worm was placed on each of the six spots and brought up for 72 h at 20°C. The number of the offsprings on each individual spot was evaluated.

POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from 60–80 worms at the 4th, 8th, and 12th day of adulthood by miRvana™ miRNA Isolation Kit (Ambion). cDNA synthesis used High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and used for PCR amplification (95°C 10 min [95°C 30 s, 55°C 30 s, 72°C 1 min, 30 cycles] and 72°C 10 min) with TransTaq™ HiFi PCR SuperMix II. (Beijing TransGen Biotech Co., Ltd.) The primer pairs are listed in Table I. Equal amount of each produce was analyzed by 1% agarose gels electrophoresis.

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

For qRT-PCR, the cDNA was assessed with SYBR Green PCR Master Mix Kit (Applied Biosystems) by 7500 FAST Real-time PCR system (Applied Biosystems). The PCR amplification program was 95°C 10 min, (95°C 15 s, 60°C 30 s, 72°C 30 s, 40 cycles) and followed by dissociation curve protocol (95°C 15 s, 60°C 1 min, 95°C 15 s, and 60°C 15 s). The U6 and act-1 were used as internal standards for miRNA and mRNA respectively. The primer pairs are listed in Table II. We determined the appropriate cycle threshold (Ct) using the automatic baseline determination feature and analyzed data by relative quantitative analysis method.

IMMUNOFLUORESCENCE STAINING

In accordance to Gil-Krzewska et al. [2010], worms adhered to the slides with poly-L-lysine were fixed in −20°C methanol for 15 min, then −20°C acetone for 15 min. After washed by PBST (PBS, 0.1% Tween 20) buffer, the worms were treated with primary antibodies: rabbit anti-ATF-6 (1:100; Novus), and incubated at 4°C over night, and secondary antibody (Goat anti-rabbit 594 (1:400; Santa)) for 2 h in room temperature. All images were obtained using fluorescence microscopes (Nikon Eclipse 80i). The protein level for each worm was quantified by testing the density of fluorescence in protein specific expression area (rectum, pharyngeal gland cell and vulva) by NIS-Elements BR 3.2 image software (Nikon).

TABLE I. RT-PCR Primers Sequence

Primers	Sequence
act-1-Forward	5′-CCAATCCAAGAGAGGTATCCTTAC-3′
act-1-Reverse	5′-GGTAAGGATCTTCATGAGGTAATCA-3′
atf-6-Forward	5′-GGCGGGAGTTTAGGAGATTC-3′
atf-6-Reverse	5′-TCTTGGCTCTTCGGACACTT-3′

TABLE II. qRT-PCR Primers Sequence

Primers	Sequence
U6-RT	5′-CGCTTCACGAATTTGCGTGTCAT-3′
Cel-miR-124-RT	5′-GTCGTATCCAGTGCGTGTCGTGGAGTC GGCAATTGCACTGGATACGACTGGCATT-3′
U6-Forward	5′-CGCTTCACGAATTTGCGTGTCAT-3′
U6-Reverse	5′-GCTTCGGCACATATACTAAAAT-3′
Cel-miR-124-Forward	5′-GTAAGGCACGCGGTGAATG-3′
Cel-miR-124-Reverse	5′-GTCGTGGAGTCGGCAATTG-3′
act-1-Forward	5′-CTGGCATCACACCTTCTAC-3′
act-1-Reverse	5′-AAGCGTAGAGGGAGAGGAC-3′
atf-6-Forward	5′-ATACCGCGTCAAGGAATCAC-3′
atf-6-Reverse	5′-TCTTGGCTCTTCGGACACTT-3′

RNA INTERFERENCE

The RNAi library and the RNAi feeding protocol are described by Ravi S. Kamath and Julie Ahringer [2003]. To generate *C. elegans* RNAi clones, the designated fragments were amplified by PCR (the primer pairs from CGC database: http://www.cbs.umn.edu/CGC/, Table III), and cloned into the L4440 feeding vector (pPD 129.36) [Timmons and Fire, 1998] The constructed plasmids were transformed into the HT115 (DE3) RNase III-deficient *E. coli* strain used as food source for RNAi by feeding. The HT115 bacteria with empty L4440 vector named "mock." The L4440 feeding vector and HT115 *E. coli* strain were obtained from Academy of Military Sciences which was ordered from CGC. We selected *dyn-1* and *myo-3* genes as positive controls of RNAi procedure.

For RNAi feeding, five synchronized hermaphrodite worms at adult stage were placed onto NGM plates containing seeded bacteria expressing dsRNA for each gene. Worms were allowed to lay eggs for 24 h before being removed. Progeny phenotypes were observed 3 days later. A gene was found to be positive for a given phenotype in at least two of three worms. At last, we selected the F2 generation of *atf-6*: RNAi worms to assess the lifespan with or without APS (200 μg/ml).

TRANSFECTION miRNA INTO *C. ELEGANS* BY FEEDING AND EXPOSURE

The agomir-*cel-miR-124* and antagomir-*cel-miR-124* (Guangzhou RiboBio Co., Ltd.), which were synthetic RNA duplexes/single-stranded RNA, developed for miRNA function studies in vivo, and chemically modified for greater stability and binding affinity to their targets [Krutzfeldt et al., 2005]. The agomir-*cel-miR-124* (50 nM) and antagomir-*cel-miR-124* (100 nM) were added to the OP50 bacterial, which were absorbed by the worms through feeding and exposure. Synchronized eggs were placed onto the NGM plate

TABLE III. RNAi PCR Primers Sequence

Primers	Sequence
dyn-1-Forward	5′-TCCCCCGGGGGAAACCGTGAGACTTGCCT-3′
dyn-1-Reverse	5′-GGGGTACCCCCTTCGCTGATTGTCCTTG-3′
myo-3-Forward	5′-TCCCCCGGGGGAGCTCATCGGCCTTAGTTTGG-3′
myo-3-Reverse	5′-CGGGGTACCCCGCGCATTGTTCTTGAGCCTTG-3′
atf-6-Forward	5′-GAAGATCTTCATACCGCGTCAAGGAATCAC-3′
atf-6-Reverse	5′-TCCCCCGGGGGAGATTTCTTTTGAACCGCCAA-3′

carrying agomir-*cel-miR-124* and antagomir-*cel-miR-124* OP50 bacterial lawn. Worms were collected after 72 h to test the expression of *miR-124* and ATF-6 protein as above description.

LUCIFERASE ASSAYS

We synthesized fragments of *atf-6* 3′UTR containing the exact target sites for *miR-124* or the mutated target sites using the Taq PCR amplification. The primers pairs are listed in Table IV. We insert the atf-6 3′UTR fragments into the multiple cloning sites (XhoI and NotI) of the psiCHECK^{TM} −2 luciferase miRNA expression reporter vector (Promega). The HEK293T cells (2×10^4 pre well) were transfected with *miR-124* mimics (20 μM) or *miR-124* inhibitor (20 μM) and 0.5 μg plasmid by lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Luciferase activities were measured 48 h after transfection with a dual luciferase reporter assay kit (Promega) on a luminometer (GloMax^{TM} 20/20, Promega).

DATA ANALYSIS

For the lifespan assay, worm survival was plotted using Kaplan–Meier survival curves and analyzed by Log-rank (Mantel-Cox) test and Gehan–Breslow–Wilcoxon test. Statistical comparisons among multiple groups were performed by Tukey's post hoc test and One-Way ANOVA. Significant differences between two groups were calculated by Student's *t* tests. All of the figures were plotted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ were considered as significantly different. The results were shown as the mean ± SEM.

RESULTS

APS EXTENDED THE LIFESPAN IN WIDE-TYPE (N2) *C. ELEGANS*

In the study, we found that APS markedly prolonged the lifespan of N2 *C. elegans* both in S-complete medium and on solid NGM medium (Figs. 1A and B, Supplementary Tabel S1 and S2). The mean lifespan was prolonged from 17.20 to 18.41, 21.13, 21.45 days and lifespan extension percentage about 7.03%, 22.84%, and 24.71% separately, after exposed to APS 50, 100, and 200 μg/ml in S-complete medium (Fig. 1A). Consistently, the mean lifespan was extended from 10.35–12.49, 12.38, 13.54 days and the lifespan extension about 20.68%, 19.61%, 30.82% by 200, 400, 600 μg/ml APS treatment in NGM medium (Fig. 1B). A significant extension of the mean lifespan by APS was observed in both two medium. Since 200 μg/ml APS effectively prolonged the lifespan in two kinds of growth medium, the dosage of 200 μg/ml was used in the following studies.

As *E. coli* OP50 is a good bacterial food source for *C. elegans*, and APS has anti-bacterial activity [Sinclair, 1998]. We investigated if APS led to the longevity through dietary restriction (DR) pathway by inhibiting the growth of OP50. The result showed that APS did not produce any effects on the growth of OP50 detected by bacterial growth assay (Fig. 1C). Then we performed the behavioral attraction assay to determine whether APS affected dietary preference of *C. elegans* [Menzel et al., 2005]. The result showed that total numbers of F2 offsprings had no difference of behavioral responses between control group and APS group ($P > 0.05$) (Fig. 1D), suggesting no dietary preference of *C. elegans* to APS.

C. elegans lifespan extension is often associated with reduced fecundity and delayed growth, which is evaluated by four stages [Huang et al., 2004]. In detail, it is defined as stage 1, from L4 stage to the end of the self-fertile reproductive span; stage 2, from the end of stage 1 to the end of the fast body movement span; stage 3, from the end of stage 2 to the end of the pharyngeal pumping span; stage 4, from the end of stage 3 to the end of the lifespan. Lifespan was defined from L4 stage to the last day of survival. The effects of APS on individual growth stages were then investigated. Although APS showed no effect on stage 1 and stage 3 as compared with control, it significantly extended spans of stage 2 and stage 4 (Fig. 1E). These results suggested that APS improved the health span as well as decrepit span, and further verified that APS could extend the lifespan of the *C. elegans*.

miR-124 MEDIATED APS-INDUCED LONGEVITY OF *C. ELEGANS*

As improtant regulators of most genes, miRNAs affect their protein expression in the post-transcriptional level [Kim et al., 2009]. We further explore if the conserved miRNAs among human, rat, mouse and *C. elegans* correspond to longevity of APS. We found that *cel-miR-124*, *cel-miR-1*, *cel-miR-34*, and *cel-let-7* had conserved sequences in 5′ seed regions (Fig. 2A). APS treatment significantly increased the expression of *cel-miR-124*, but not *cel-miR-1*, *cel-miR-34*, and *cel-let-7* in N2 *C. elegans* (Figs. 2B–E). Thus, we hypothesize that APS affects longevity through *cel-miR-124*. In order to clarify the role of *miR-124* in the longevity of *C. elegans*, we treated *C. elegans* with *miR-124* mimics and inhibitor by feeding and exposure. The successful uptake of agomir-*cel-miR-124*/antagomir-*cel-miR-124* into the *C. elegans* was verified by qRT-PCR (Fig. 2F). We observed that agomir-*cel-miR-124* significantly extended the lifespan of *C. elegans* (Fig. 2G). On the contrary, antagomir-*cel-miR-124* shortened the lifespan of *C. elegans*. It suggests that *miR-124* regulates the longevity of *C. elegans*.

TABLE IV. PCR Primers Sequence of *atf-6* 3′UTR and *atf-6* 3′UTR Mutants

Primers	Sequence
atf-6 XhoI-Forward	5′-CCGCTCGAG TACTGATATTGATCAACAACTACG-3′
atf-6 NotI-Reverse	5′-ATAAGAATGCGGCCGCTAATTATTGACAAGAAAAATGC-3′
mut atf-6-1 Forward	5′-ATTCCCACTTTTCTCAATTTCGCACGGAATTTTATTCACCCACTTTACCT-3′
mut atf-6-1 Reverse	5′-AGGTAAAGTGGGTGAATAAAATTCCGTGCGAAATTGAGAAAAGTGGGAAT-3′
mut atf-6-2 Forward	5′-TTGTTGTACCCGTTTTCGCTCGCAGAGACACTTTACATCAATCATATTC-3′
mut atf-6-2 Reverse	5′-GAATATGATTGATGTAAAGTGTCTCTGCGAGCGAAAACGGGTACAACAA-3′
mut atf-6-3 Forward	5′-CTATTCAAATTCTAATTATAACACGGATTTTATCTGGGATTTTTGCAT-3′
mut atf-6-3 Reverse	5′-ATGCAAAAATCCCAGATAAAATCCGTGTTATAATTAGAATTTGAATAG-3′

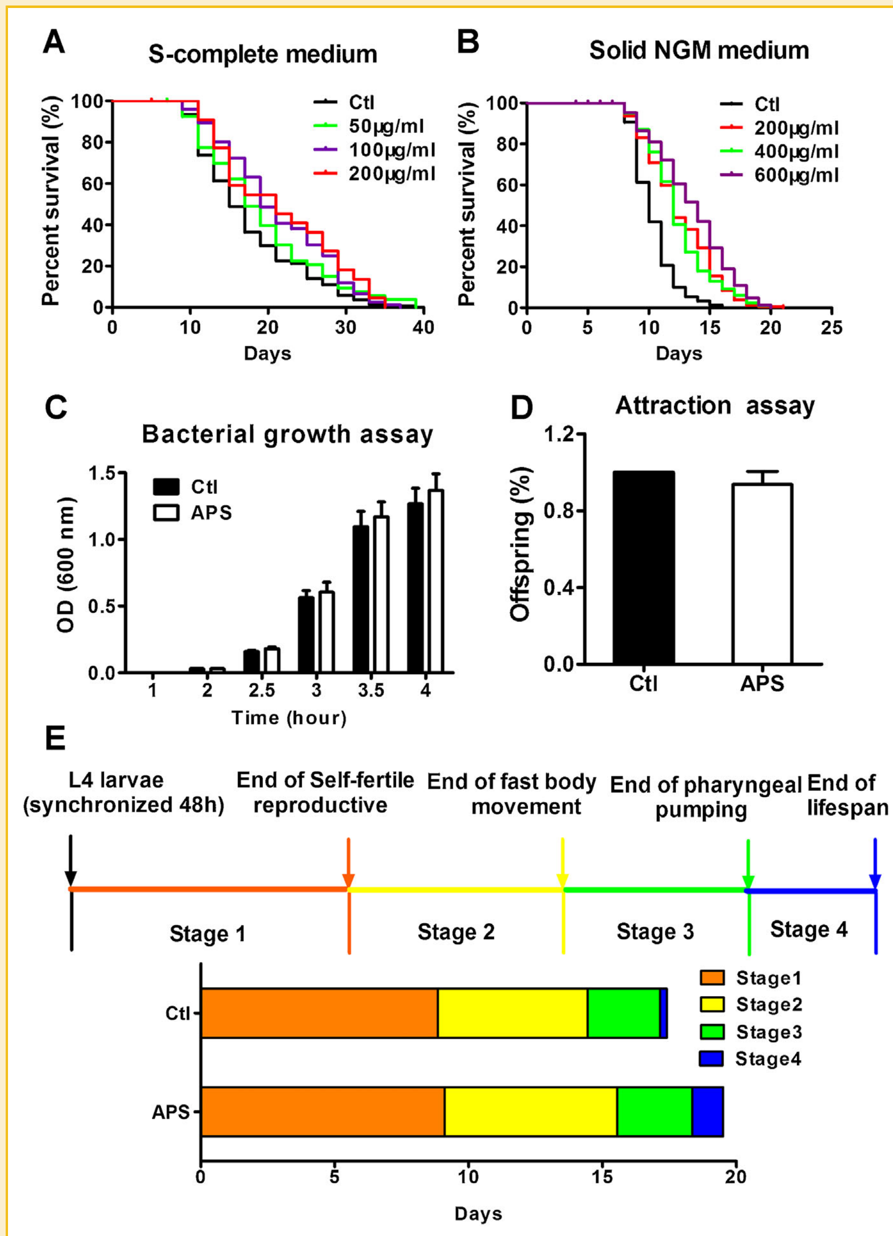

Fig. 1. Lifespan-extension effect of APS on wild-type (N2) *C. elegans*. (A) Effect of APS on the lifespan of N2 *C. elegans* in S-complete medium. Ctl (ddH_2O): n = 135, m = 17.20. APS (50 μg/ml): n = 51, m = 18.41. APS (100 μg/ml): n = 76, m = 21.13, $^{**}P < 0.01$ versus Ctl (ddH_2O). APS (200 μg/ml): n = 62, m = 21.45, $^{**}P < 0.01$ versus Ctl (ddH_2O). (B) Effect of APS on the lifespan of N2 *C. elegans* on solid NGM medium. Ctl (ddH_2O): n = 150, m = 10.35. APS (200 μg/ml): n = 154, m = 12.49, $^{***}P < 0.001$ versus Ctl (ddH_2O). APS (400 μg/ml): n = 162, m = 12.38, $^{***}P < 0.001$ versus Ctl (ddH_2O). APS (600 μg/ml): n = 147, m = 13.54, $^{***}P < 0.001$ versus Ctl (ddH_2O). All experiments were repeated at least twice. n: number of animals observed in each experiment; m: mean adult lifespan (days). (C) APS did not inhibit the growth of OP50. OP50 containing sterile deionized water (0 μg/ml, control) or OP50 with 200 μg/ml APS. D: Behavioral assay in N2 *C. elegans* response to APS. n = 18 nematodes in each treatment. E: The schematic of stages of *C. elegans* lifespan cycle and effect of APS on stages of N2 *C. elegans* lifespan cycle.

miR-124 TARGETED AT ATF-6 GENE

Then, we aimed to find out *cel-miR-124* target genes. Based on computation predication using miRNAs database, we found that *cel-miR-124* had three binding sites in the 3′UTR of *atf-6* mRNA, a key regulatory protein of ER stress in *C. elegans* (Fig. 3A). To observe whether *miR-124* regulates the expression of ATF-6 in *C. elegans*, we cloned full length 3′UTR of *atf-6* containing the *miR-124* binding sites into the luciferase reporter vector to determine the effects of *miR-124* on *atf-6* luciferase reporter activities in HEK293T cells. As shown in Figure 3B, overexpression of *miR-124* produced a significant decrease in luciferase activity, while antagomir *miR-124* transfection eliminated the silencing function of *miR-124* on *atf-6* luciferase reporter activity. Interestingly, *miR-124* overexpression also decreased the activity of luciferase reporter with

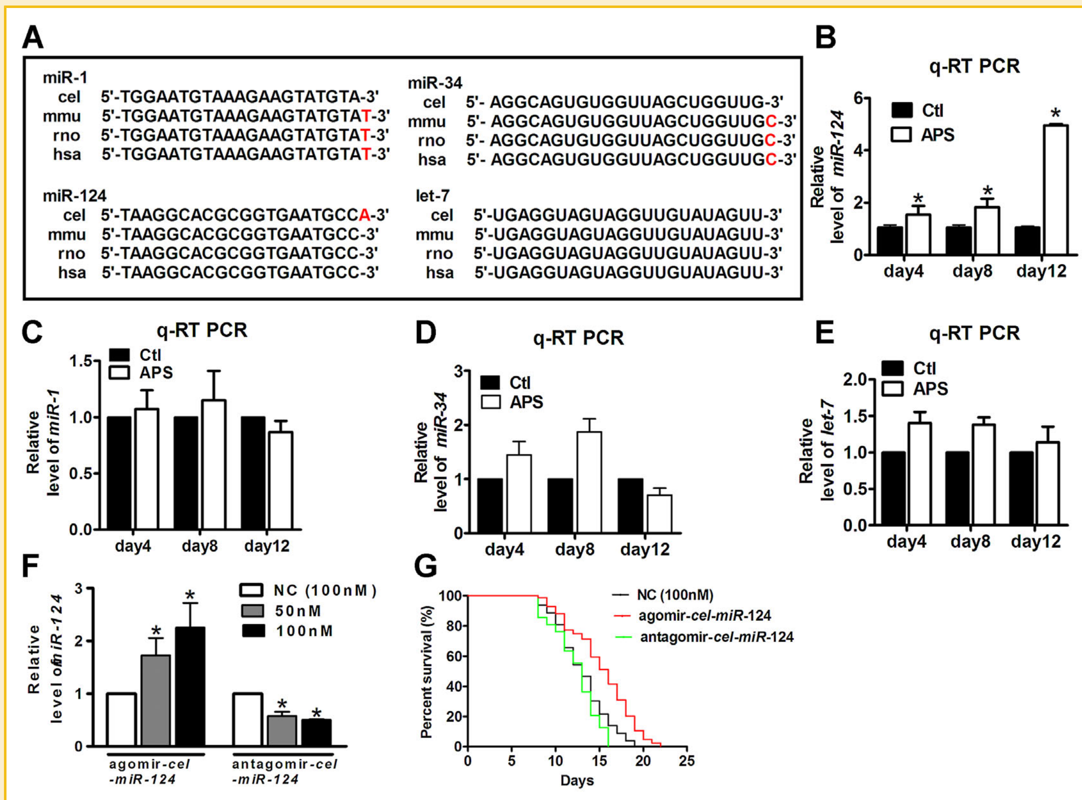

Fig. 2. *miR-124* contributed to the longevity of wild-type *C. elegans*. A: The highly conserved *miRNA* sequences from *C. elegans* to human. B: *MiR-124* level was increased in N2 *C. elegans* with APS treatment. n = 30, *$P < 0.05$ versus Ctl. C: Effect of APS on *miR-1* expression in N2 *C. elegans*. D: Effect of APS on *miR-34* expression in N2 *C. elegans*. E: Effect of APS on *let-7* expression in N2 *C. elegans*. F: The levels of *miR-124* after transfection with agomir-*cel-miR-124* or antagomir-*cel-miR-124* for 72 h in N2 *C. elegans*. *$P < 0.05$ versus Ctl. G: The agomir-*cel-miR-124* (50 nM) and antagomir-*cel-miR-124* (50 nM) affect lifespan of N2 *C. elegans*. Ctl: n = 79, m = 13.09. agomir-*cel-miR-124*: n = 84, m = 15.25 ***$P < 0.001$ versus Ctl. antagomir-*cel-miR-124*: n = 63, m = 12.32. *$P < 0.05$ versus Ctl. n, number of animals observed in each experiment. m, mean adult lifespan (days).

single mutation of three binding sites. But *miR-124* failed to decrease the *atf-6* luciferase reporter activity when all three binding sites in the 3′UTR of *atf-6* were mutated (Fig. 3C). The results suggested that *miR-124* regulated ATF-6 expression via acting on at least two binding sites of *atf-6* 3′UTR. Consistently, immunofluorescence assay showed that agomir-*cel-miR-124* inhibited the expression of ATF-6 protein, and antagomir-*cel-miR-124* increased the protein level of ATF-6 in *C. elegans* (Fig. 3D–E).

THE ROLE OF ATF-6 IN THE LIFESPAN EXTENSION OF *C. ELEGANS*

ATF-6 is an ER stress-regulated transmembrane transcription factor. Though we have found that ATF-6 expression was affected by *miR-124*, but the role of ATF-6 in the longevity remains uncertain. Thus, we further investigated if ATF-6 plays a determinant role in the life-extending effect of APS in *C. elegans*. We found the mRNA level of *atf-6* in N2*C. elegans* was not significantly changed after treatment with APS for 4, 8, and 12 days (Fig. 4A). But, the expression of ATF-6 protein was significantly decreased at 8 and 12 days after APS administration (Fig. 4B). To further verify the role of ATF-6 in the lifespan extension of APS, the RNAi technique was used to generate the knockdown model of *atf-6* in *C. elegans*. The successful establishment of *atf-6*: RNAi model in *C. elegans* was identified by *myo-3*: RNAi and *dyn-1*: RNAi phenotype (Fig. 4C). RNAi against *myo-3* in N2*C. elegans* showed dyskinesia phenotype, and the body can not bend as ‘S’ shape. N2*C. elegans* with *dyn-1* gene interference could not ovulate normally and their offsprings hatched in the parent body. The lifespan of *atf-6*: RNAi *C. elegans* was longer than that in N2*C. elegans* (Fig. 4D). The result showed that *atf-6* deletion induced the lifespan extension in *C. elegans*, suggesting that ATF-6 is essential for the life-extending effect of APS.

DISCUSSION

Here we revealed, for the first time that *miR-124* suppressing *atf-6* expression was a new longevity pathway and contributed to the lifespan-extending effect of APS on *C. elegans*. This study extends our understanding about the molecular mechanisms underlying the lifespan extension of APS in *C. elegans*.

Considering the highly conserved biochemical pathway cross species, *C. elegans* is an excellent model system for evaluating the

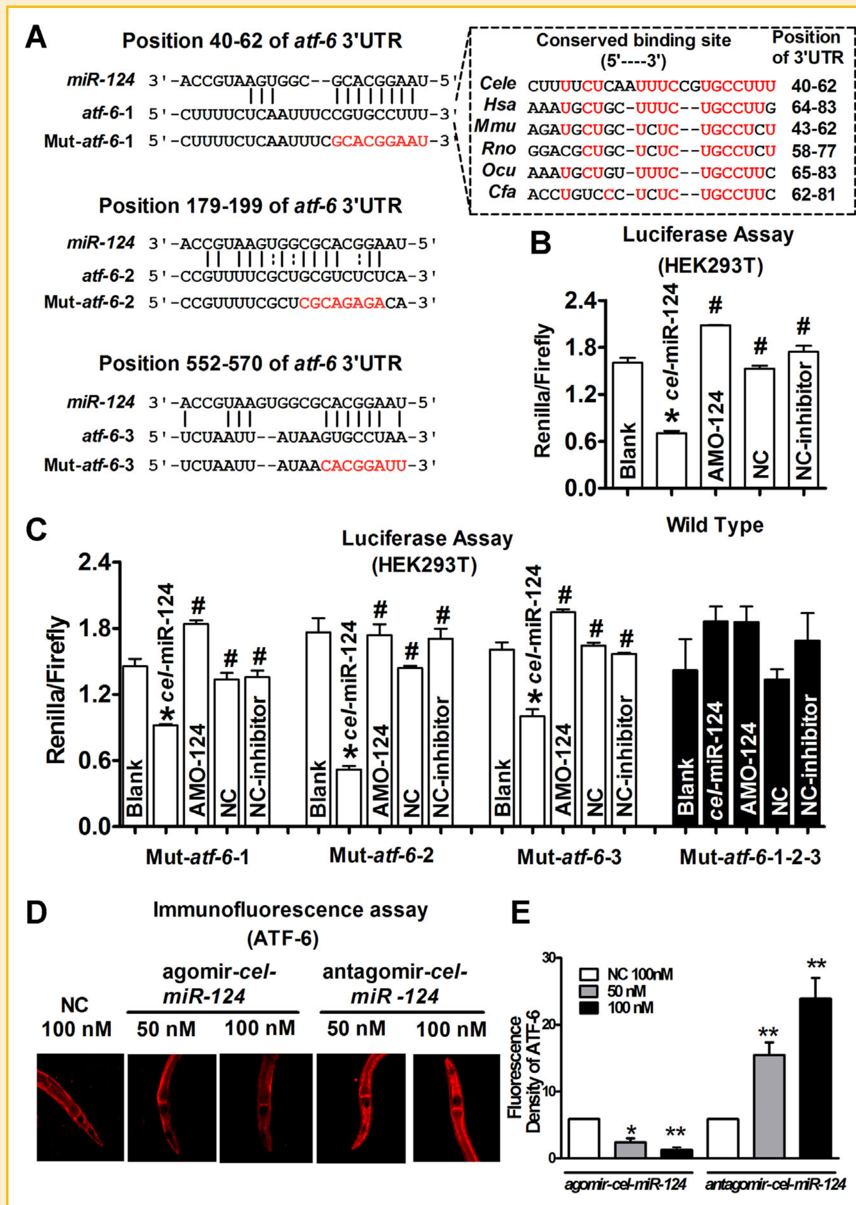

Fig. 3. *miR-124* targeted at *atf-6* gene . A: *MiR-124* binding sites at 3′UTR region of *atf-6* gene. The sequences showed the three binding sites of miRNA:mRNA complementarity between *miR-124* and 3′UTR region of *atf-6* gene in *C. elegans* (Cele). The binding site-1 is highly conserved across mammals including human (Hsa), mouse (Mmu), rat (Rno), rabbit (Ocu), and dog (Cfa). The matched base pairs are connected by a vertical line and the G:U/U:G wobble is connected by dots. The positions of the target sites are numbered. The red color represents the mutant base. B: Effects of *miR-124* on luciferase activity of WT *atf-6* 3′UTR reporter. C: Regulation of luciferase activity by *miR-124* on mutant *atf-6* 3′UTR reporter. Transfection was performed with lipofectamine 2000 and measurements were made 48 h after transfection. $^{*}P < 0.05$ versus Blank; $^{\#}P < 0.05$ versus *cel-miR-124* alone; n = 3 for each group. D: Immunofluorescence assay of ATF-6 protein. N2 *C. elegans* (eggs) were treated with OP50 containing 100 nM unrelated sequence as negative control (NC) or OP50 containing 50 nM, 100 nM agomir-*cel-miR-124*, or 50 nM, 100 nM antagomir-*cel-miR-124*. E: The quantification of ATF-6 fluorescence signal in N2 *C. elegans* treated OP50 containing NC or agomir-*cel-miR-124* or antagomir-*cel-miR-124*. $^{*}P < 0.05$ versus Ctl, $^{**}P < 0.01$ versus Ctl.

efficacy of life-extending medicine. It was recently suggested that APS extended the lifespan of *C. elegans* in S-complete medium [Zhang et al., 2012]. Consistently, we also observed the longevity effects of APS on *C. elegans* in S-complete medium. Some studies showed that the life-extending effects in S-complete medium were not verified on solid NGM growth medium [Collins et al., 2008; Zarse and Ristow, 2008]. Therefore, we observed the life-extending effect of APS on *C. elegans* on solid NGM medium, and found APS significantly extended mean lifespan. Since the collected data from NGM plate was more consistent than that in S-complete medium, we used the NGM plate for the following study. Then, we found APS had no effect on stage 1 (self-fertile reproduction) and stage 3 (pharyngeal pumping), but significantly prolonged stage 2 (fast body movement), and stage 4 (lifespan) of the lifespan cycle in *C. elegans*. These results suggested that APS improved the health span as well as decrepit span of *C. elegans*, and further verified the

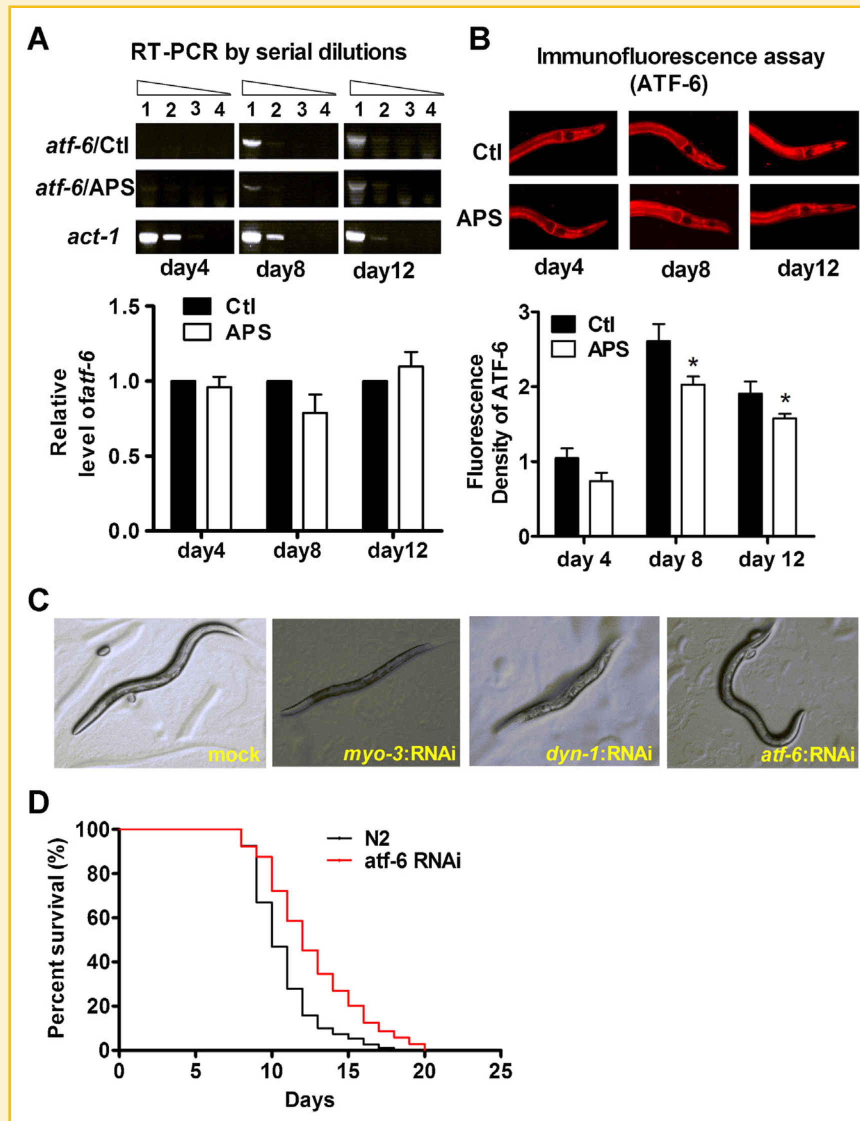

Fig. 4. ATF-6 was a critical regulator of the longevity of *C. elegans*. A: The mRNA level of *atf-6* in N2 *C. elegans* treated with APS for 4, 8, and 12 days. Top panel is the agarose gel electrophoresis of RT-PCR products, and lanes 1–4 are generated from stepwise 10-fold serially diluted cDNA and amplified using *atf-6* gene-specific primers. Bottom panel is the qRT-PCR result of *atf-6* mRNA expression. B: The expression of ATF-6 protein was decreased at 8 and 12 days after APS administration. Fluorescence microscopy revealed red fluorescence in ATF-6 expression. Bottom panel shows quantification of ATF-6 fluorescence signal in N2 *C. elegans* treated with or without APS. *$P < 0.05$ versus Ctl. C: The phenotypes of *C. elegans* by RNAi. *Myo-3*: RNAi in N2 appeared dyskinesia phenotype. *Dyn-1*: RNAi in N2 could not ovulation normally. *Atf-6*: RNAi and mock were normal as N2. D: Comparison of the lifespan of N2 *C. elegans* and *atf-6*: RNAi *C. elegans* cultured in solid NGM medium. N2: n = 190, m = 10.76, *atf-6*: RNAi: n = 104, m = 12.67, ***$P < 0.001$ versus N2. n: number of animals observed in each experiment. m: mean adult lifespan (days).

longevity of APS on *C. elegans*. However, its corresponding mechanisms remain to be elucidated.

We first presumed that APS might extend the lifespan of *C. elegans* by affecting the diet (OP50). However, the result showed that APS treatment did not affect the growth ratio of OP50 and diet preference of *C. elegans*. We also considered whether APS was metabolized by OP50. Because APS mixed in the NGM medium while the plaque of *E. coli* OP50 bacteria was placed on the surface of plate. Hence, OP50 did not show any impact on the metabolism of APS in this experiment. In addition, it was reported that dead (dry) OP50 could extend the lifespan of *C. elegans* [Garigan et al., 2002]. Using nonliving OP50 can not entirely clear the life-extending role of APS. Therefore, we provided adequate fresh OP50 in each batch of the experiment. The results suggest that the life-extending activity of APS is not associated with its nature.

It is well known that miRNAs play important roles in a wide range of biological process such as cell proliferation, apoptosis and differentiation. Compared with unconserved miRNAs, the miRNAs conserved in various species have more important functions [Lee et al., 1993; Lim et al., 2003]. So, we investigate the potential roles of highly conserved miRNAs (*miR-1*, *miR-124*, *miR-34*, *let-7*) in this study, and found only *miR-124* level was significantly increased in

C. elegans with APS treatment. Gain of function study showed that exogenous overexpression of *cel-miR-124* extended the lifespan of *C. elegans*. The down-regulation of *cel-miR-124* shortened the lifespan of *C. elegans*. Therefore, we hypothesize that the lifespan of *C. elegans* were affected by the exogenous *miR-124*. Then we treated *C. elegans* with agomir/antagomir by exposure and eating. Even though we have verified the agomir/antagomir has been successful delivered into *C. elegans* in a dose–dependent manner, but due to the limitation of our delivery method, there is only twofold increase in *miR-124* in transfected worms. In addition, many studies have shown that bacteria-induced gene knockdown via feeding was effective to reduce the expression of target proteins [Murphy et al., 2003; Grishok et al., 2005; Burton et al., 2011]. Moreover, it has been reported that the transfected RNA will not be degraded after feeding to worms [Timmons et al., 2001; Kamath and Ahringer, 2003], because the bacteria such as *Escherischia coli* are deficient in RNaseIII, an enzyme that normally degrades a majority of RNA in the bacterial cell. On this basis, the gain of function study showed that exogenous overexpression of *cel-miR-124* extended the lifespan of *C. elegans*. The down-regulation of *cel-miR-124* shortened the lifespan of *C. elegans*. In the present study, we did not use the antagomir-*cel-miR-124* to evaluate the lifespan extension effect of APS, because the lifespan extension effect of APS was shown at least partially associated with daf-16 [Zhang et al., 2012]. Even if we used the antagonist of *miR-124* to test the lifespan extension of APS, it only partly inhibits the lifespan extending effects of APS and cannot completely block APS activities. We also identified the lifespan extension effect of APS on daf-16 mutant worm daf-16(mu86). As shown in supplementary Figure S1, the lifespan was shorten in daf-16(mu86) *C. elegans* than in N2, but APS treatment rescued the lifespan shortening in absence of daf-16, indicating that APS cause lifespan extension via another extension pathway in addition to daf-16 pathway. In addition, antagomir-*cel-miR-124* only caused a partial shortening of lifespan in *C. elegans*. We think antagomir-*cel-miR-124* is an inhibitor of *miR-124*. It was tranfected to *C. elegans* by exposure and feeding, which induced the knockdown but not knockout of endogenous *miR*-124 in *C. elegans*. In previous studies, *miR-124* was shown mainly expressed in sensory neurons and functioned on cell-type-specific gene activity by inhibiting different set of co-expressed genes in *C. elegans* [Lim et al., 2003; Clark et al., 2010]. To our best knowledge, this is the first study to show the lifespan-extending role of *miR-124*.

Computational predication based on MiRBase, TargetScan, and PicTA microRNA database suggested *atf-6* as the target gene of miR-124 because it has three binding sites with 8 mers in its "seed" region in the 3′UTR of *atf-6* in *C. elegans*, and one of them is highly conserved across mammals including human, mouse, rat, rabbit, and dog. Then, luciferase reporter assay and immunochemistry assay confirmed that *miR-124* inhibited the expression of ATF-6 by targeting at the 3′UTR of *atf-6* gene.

The activation of ATF-6 plays an important role in ER stress-mediated the longevity and aging [Jager et al., 2012]. APS was able to inhibit ER stress-induced activation of ATF-6 in rat model of type 2 diabetes [Wang et al., 2009]. However, whether APS extends *C. elegans* lifespan by acting on ATF-6 is largely unknown. Our study showed that the lifespan of *C. elegans* with *atf-6* knockdown was longer than normal *C. elegans*, implying that *atf-6* is critical for the longevity of *C. elegans*. It indicates that *miR-124* mediating ATF-6 expression is essential for APS life-span extension. We did not demonstrate whether *atf-6* overexpression will cancel the lifespan extension effect by APS in *C. elegans*. Because APS treatment induced lifespan extension of *C. elegans* via at least two pathways such as *daf-16* and *miR-124*/ATF6, that means the blockage or activation of only one pathway will not completely reverse the lifespan extension of *C. elegans* induced by APS. Because *miR-124* and *atf-6* are highly conservative in mammalian animals, their effects and the molecular mechanism of *miR-124* on the lifespan in *C. elegans* might be also suitable for other species.

Taken together, in the present study, our data uncovered that APS prolonged the lifespan of *C. elegans* by increasing *miR-124* level and in turn decreasing the expression of ATF-6. This study provides new insights into mechanisms of the life-extending actions of APS, and also identified a novel longevity pathway, which will be helpful to develop a series of longevity products.

ACKNOWLEDGMENTS

We thank Chang-hong Ren for gifting *C. elegans* and technical guidance. This work was supported by Key Project of National Science Foundation of China (81130088), Natural Science Foundation of China (81271207, 81070882, and 31100825), and the funding from Key Laboratory of Cardiovascular Medicine Research (Harbin Medical University), Ministry of Education.

REFERENCES

Antebi A. 2007. Ageing: When less is more. Nature 447:536–537.

Braeckman BP, Vanfleteren JR. 2007. Genetic control of longevity in C. elegans. Exp Gerontol 42:90–98.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

Burton NO, Burkhart KB, Kennedy S. 2011. Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 108:19683–19688.

Chen W, Li Y, Yu M. 2008. *Astragalus* polysaccharides: An effective treatment for diabetes prevention in NOD mice. Exp Clin Endocrinol Diabetes 116:468–474.

Chen W, Li YM, Yu MH. 2010. *Astragalus* polysaccharides inhibited diabetic cardiomyopathy in hamsters depending on suppression of heart chymase activation. J Diabetes Complications 24:199–208.

Chen W, Xia YP, Chen WJ, Yu MH, Li YM, Ye HY. 2012. Improvement of myocardial glycolipid metabolic disorder in diabetic hamster with *Astragalus* polysaccharides treatment. Mol Biol Rep 39:7609–7615.

Clark AM, Goldstein LD, Tevlin M, Tavare S, Shaham S, Miska EA. 2010. The microRNA miR-124 controls gene expression in the sensory nervous system of *Caenorhabditis elegans*. Nucleic Acids Res 38:3780–3793.

Collins JJ, Evason K, Pickett CL, Schneider DL, Kornfeld K. 2008. The anticonvulsant ethosuximide disrupts sensory function to extend *C. elegans* lifespan. PLoS Genet 4:e1000230.

Fu S, Zhang J, Menniti-Ippolito F, Gao X, Galeotti F, Massari M, Hu L, Zhang B, Ferrelli R, Fauci A, Firenzuoli F, Shang H, Guerra R, Raschetti R. 2011. Huangqi injection (a traditional Chinese patent medicine) for chronic heart failure: A systematic review. PloS ONE. 6:e19604.

Garigan D, Hsu AL, Fraser AG, Kamath RS, Ahringer J, Kenyon C. 2002. Genetic analysis of tissue aging in *Caenorhabditis elegans*: A role for heat-shock factor and bacterial proliferation. Genetics 161:1101–1112.

Gil-Krzewska AJ, Farber E, Buttner EA, Hunter CP. 2010. Regulators of the actin cytoskeleton mediate lethality in a *Caenorhabditis elegans* dhc-1 mutant. Mol Biol Cell 21:2707–2720.

Grishok A, Sinskey JL, Sharp PA. 2005. Transcriptional silencing of a transgene by RNAi in the soma of *C. elegans*. Genes Dev 19:683–696.

Huang C, Xiong C, Kornfeld K. 2004. Measurements of age-related changes of physiological processes that predict lifespan of *Caenorhabditis elegans*. Proc Natl Acad Sci U S A 101:8084–8089.

Jager R, Bertrand MJ, Gorman AM, Vandenabeele P, Samali A. 2012. The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. Biol Cell 104:259–270.

Kamath RS, Ahringer J. 2003. Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods 30:313–321.

Kim VN, Han J, Siomi MC. 2009. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Bio 10:126–139.

Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. 2005. Silencing of microRNAs in vivo with 'antagomirs'. Nature 438:685–689.

Lee RC, Feinbaum RL, Ambros V. 1993. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854.

Li M, Wang W, Xue J, Gu Y, Lin S. 2011. Meta-analysis of the clinical value of *Astragalus membranaceus* in diabetic nephropathy. J Ethnopharmacology 133:412–419.

Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP. 2003. The microRNAs of *Caenorhabditis elegans*. Genes Dev 17:991–1008.

Luo Y. 2004. Long-lived worms and aging. Redox Rep 9:65–69.

Mao XQ, Yu F, Wang N, Wu Y, Zou F, Wu K, Liu M, Ouyang JP. 2009. Hypoglycemic effect of polysaccharide enriched extract of *Astragalus membranaceus* in diet induced insulin resistant C57BL/6J mice and its potential mechanism. Phytomedicine 16:416–425.

Menzel R, Sturzenbaum S, Barenwaldt A, Kulas J, Steinberg CE. 2005. Humic material induces behavioral and global transcriptional responses in the nematode *Caenorhabditis elegans*. Environ Sci Technol 39:8324–8332.

Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C. 2003. Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. Nature 424:277–283.

Narasimhan SD, Yen Tissenbaum KHA. 2009. Converging pathways in lifespan regulation. Curr Biol 19:R657–R666.

Petrascheck M, Ye X, Buck LB. 2007. An antidepressant that extends lifespan in adult *Caenorhabditis elegans*. Nature 450:553–556.

Pietsch K, Saul N, Menzel R, Sturzenbaum SR, Steinberg CE. 2009. Quercetin mediated lifespan extension in *Caenorhabditis elegans* is modulated by age-1, daf-2, sek-1 and unc-43. Biogerontology 10:565–578.

Salminen A, Kaarniranta K. 2010. ER stress and hormetic regulation of the aging process. Ageing Res Rev 9:211–217.

Shao BM, Xu W, Dai H, Tu P, Li Z, Gao XM. 2004. A study on the immune receptors for polysaccharides from the roots of *Astragalus membranaceus*, a Chinese medicinal herb. Biochem Biophys Res Commun 320:1103–1111.

Sinclair S. 1998. Chinese herbs: A clinical review of *Astragalus*, *Ligusticum*, and *Schizandrae*. Altern Med Rev 3:338–344.

Sternberg PW. 2001. Working in the post-genomic *C. elegans* world. Cell 105:173–176.

Stiernagle T. 2006. Maintenance of *C. elegans*. WormBook 1–11.

Tang JC, Zhang JN, Wu YT, Li ZX. 2006. Effect of the water extract and ethanol extract from traditional Chinese medicines *Angelica sinensis* (Oliv.) *Diels*, *Ligusticum chuanxiong* Hort and *Rheum palmatum* L. on rat liver cytochrome P450 activity. Phytother Res 20:1046–1051.

Timmons L, Court DL, Fire A. 2001. Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263:103–112.

Timmons L, Fire A. 1998. Specific interference by ingested dsRNA. Nature 395:854.

Wang N, Zhang D, Mao X, Zou F, Jin H, Ouyang J. 2009. *Astragalus* polysaccharides decreased the expression of PTP1B through relieving ER stress induced activation of ATF6 in a rat model of type 2 diabetes. Mol Cell Endocrinol 307:89–98.

Zarse K, Ristow M. 2008. Antidepressants of the serotonin-antagonist type increase body fat and decrease lifespan of adult *Caenorhabditis elegans*. PloS ONE 3:e4062.

Zhang H, Pan N, Xiong S, Zou S, Li H, Xiao L, Cao Z, Tunnacliffe A, Huang Z. 2012. Inhibition of polyglutamine-mediated proteotoxicity by *Astragalus membranaceus* polysaccharide through the DAF-16/FOXO transcription factor in *Caenorhabditis elegans*. Biochem J 441:417–424.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.