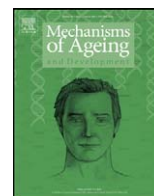




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High-affinity Na⁺-dependent dicarboxylate cotransporter promotes cellular senescence by inhibiting SIRT1

Weiping Liu¹, Quan Hong¹, Xue-Yuan Bai, Bo Fu, Yuansheng Xie, Xueguang Zhang, Jianjun Li, Suozhu Shi, Yang Lv, Xuefeng Sun, Xiangmei Chen^{*}

Department of Nephrology, Chinese PLA Institute of Nephrology & Key Lab, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, PR China

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ABSTRACT

High-affinity Na⁺-dependent dicarboxylate cotransporter (NaDC3) can transport Krebs cycle intermediates into cells. Our previous study has shown that NaDC3 promotes cellular senescence, but its mechanism is not clear. It is known that when the concentration of intermediates in Krebs cycle is increased, NAD⁺/NADH ratio will be decreased. NAD⁺-dependent histone deacetylase sirtuin1 (SIRT1) prolongs mammalian cellular lifespan. Therefore, we propose that NaDC3 accelerates cellular aging by inhibiting SIRT1. After NaDC3 was overexpressed in two human embryo lung fibroblastic cell lines, WI38 and MRC-5, we found that the cells displayed aging-related phenotypes in advance. Meanwhile, the level of SIRT1 activity was down-regulated. In WI38/hNaDC3 cells treated with the activators of SIRT1, aging-related phenotypes induced by NaDC3 were obviously improved. The NAD⁺/NADH ratio in WI38/hNaDC3 cells was also decreased. Further study found that enhanced intracellular NAD⁺ level could attenuate the aging phenotypes induced by NaDC3. Thus, NaDC3 promotes cellular senescence probably by inhibiting NAD⁺-dependent SIRT1.

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1. Introduction

Many studies have found that senescence is tightly related to energy metabolism. Caloric restriction (CR) has been confirmed to extend lifespan in various organisms (Fernandes et al., 1976; Maeda et al., 1985; Roth et al., 2001; Anderson et al., 2003; Howitz et al., 2003) and delay aging-related diseases (Yoshida et al., 1961; Pugh et al., 1999).

Na⁺-dependent dicarboxylate transporter (NaDC) is an organic anion transport protein family responsible for re-absorption or transport of tricarboxylic acid cycle (Krebs cycle) intermediates (Pajor, 1999) to participate in regulating cellular energy metabolism (Simpson, 1983; Chen et al., 1999). Based on their affinity for substrates, the family is classified into two types: low-affinity NaDC1 and high-affinity NaDC3 (Pajor, 2000). NaDC3 is mainly expressed in metabolic active tissues, including kidney, liver, placenta, and brain (Wang et al., 2003; Bai et al., 2006). Recent studies in our lab have demonstrated that overexpression of NaDC3 gene accelerates cellular senescence (Chen et al., 2005b), but the mechanism is yet unclear.

One bridge between energy metabolism and senescence is NAD⁺-dependent histone deacetylase Sir2 (Leonard and Frédéric,

2005). Sir2 (silent information regulator 2) is the key regulator of life span in a wide variety of organisms, including yeast, worms, and flies (Blander and Guarente, 2004). An increased dosage of Sir2 proteins extends life span in each of these organisms, while deletion or mutation of Sir2 shortens life span (Kaeberlein et al., 1999; Tissenbaum and Guarente, 2001; Rogina et al., 2000; Wood et al., 2004).

In mammals, SIRT1 is the closest ortholog of the yeast enzyme Sir2. It delays or reduces the incidence of aging-related disorders (Ramsey et al., 2008; Longo and Kennedy, 2006; Qin et al., 2006; Chu et al., 2005; Chen et al., 2005a). Moreover, studies demonstrated that SIRT1 plays an important role in the regulation of cell fate and stress response in mammalian cells. SIRT1 promotes cell survival by inhibiting cellular senescence or apoptosis induced by stress, including DNA damage and oxidative stress, and can reverse the induced aging in mouse embryonic fibroblasts and cardiac myocytes, etc. (Cohen et al., 2004; Alcendor et al., 2004; Langley et al., 2002). SIRT1 activity depends on NAD⁺, which is an important coenzyme in Krebs cycle (Imai et al., 2000). Therefore, SIRT1 is a junction point between senescence and energy metabolism (Leonard and Frédéric, 2005).

The increased concentration of Krebs cycle intermediates causes allosteric inhibition of cycle activity and down-regulation of NAD⁺/NADH ratio, which determines the intracellular redox state (Shawn et al., 2000). NaDC3 can transport Krebs cycle intermediates into cells and regulate cellular energy metabolism

^{*} Corresponding author. Tel.: +86 10 68211187; fax: +86 10 68130297.
E-mail addresses: xmchen301@126.com, liuwp1971@163.com (X. Chen).

¹ These authors contributed equally to this work.

(Murer et al., 1992), and SIRT1 links energy metabolism to senescence (Leonard and Frédéric, 2005). We propose a hypothesis that NaDC3 promotes aging by inhibiting NAD⁺-dependent SIRT1 pathway. To test this hypothesis, we firstly observed the change of SIRT1 activity and NaDC3 expression with aging in rat kidney tissue; investigated the change of SIRT1 and NAD⁺/NADH ratio after overexpression of NaDC3 gene in human embryonic lung diploid fibroblast (WI38 and MRC-5 cells), and studied the mechanism by which NaDC3 accelerated cellular senescence.

2. Materials and methods

2.1. Experimental animal

Eighteen male Wistar rats at the age of 3, 12 and 27 months, were obtained from the colony kept in the Experimental Animal Center of the General Hospital of PLA, which originated from the strain of National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The rats were housed two per cage (size 60 cm × 45 cm × 35 cm) in a climate-controlled quarter, where the relative humidity and ambient temperature were 45% and 23 °C, respectively, under a 12-h light/dark cycle. The rats were fed ad libitum water and standard laboratory chow with a composition of 25% protein, 5% fat, 56% carbohydrate, 8% ash, and 6% cellulose. SPF monitoring includes routine testing of the following. The agents specifically excluded from the Wistar rat colony included virus (Sendai virus, rat corona virus, rat parvovirus, etc.), bacteria (mycoplasma pulmonis, corynebacterium, clostridium, etc.), and parasites (endoparasites, tapeworms, protozoa, and ectoparasites). All animals were killed by exsanguination under general anesthesia. Meanwhile, bilateral kidneys were harvested, snap frozen in liquid nitrogen and stored at -80 °C for protein extraction and enzymatic activity detection.

2.2. Cell culture

Human embryonic lung diploid fibroblasts WI38 and MRC-5 were obtained from ATCC. Cells were cultured in Earle's Minimum Essential Medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 1 mM pyruvate (Invitrogen). PT67 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% bovine serum. All cells were grown in medium containing 100 µg/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37 °C in 5% CO₂.

2.3. Construction of retroviral vector and infection of WI38 and MRC-5 cells

Retroviral plasmid pLNCX2 was obtained from Clontech Inc. (CA, USA). pLNCX2-hNaDC3 was constructed by our laboratory (Chen et al., 2005b). pLNCX2-hNaDC3 and pLNCX2-neo were infected into PT67 cells (50–60% confluence) using Lipofectamine 2000 (Invitrogen). After infection for 48 h, the medium containing retroviruses was collected. Then the hNaDC3 gene was introduced into young human diploid fibroblast WI38 and MRC-5 cells (PD 20) (50–60% confluence) through infection with retroviral vectors. The transformants, after sustained selection by G418 with the concentration 300 µg/ml for 15 days, were termed as WI38/hNaDC3, WI38/neo and MRC-5/hNaDC3, MRC-5/neo, respectively.

2.4. WI38 cells were treated by resveratrol, niacinamide, CR serum or NAD⁺

Cells were treated, respectively, by resveratrol (Sigma Chemical) 200 µM (Borra et al., 2005), niacinamide (Sigma Chemical) 10 mM (Gerhart-Hines et al., 2007), CR serum (6-month-old rats that had been fed a CR diet (60% of AL) since weaning) or NAD⁺ (Roche) 0.1 mM (Araki et al., 2004).

2.5. Western blot analysis and densitometry

Western blots were performed as previously described (Sasaki et al., 2006). The following antibodies were used: rabbit anti-NaDC3 antibody against peptide (amino acid sequence 155–226 of human NaDC3, 1:100) (prepared by our laboratory); (Wang et al., 2003). Anti-β-actin (Sigma-Aldrich, 1:10,000).

Films were scanned with a Molecular Dynamics Densitometer (GE) and densitometry was performed using Alphaimager 2200. NaDC3 and actin signals of each sample were measured and NaDC3 levels of each sample were standardized to the levels of actin.

2.6. Measurement of SIRT1 deacetylase activity with fluorescent assay

Deacetylation was measured using the Fluor de Lys kit (AK-555; Biomol) using a fluorogenic peptide encompassing residues 379–382 of p53, acetylated on lysine 382 (KI-177; Biomol). The acetylated lysine residue was coupled to an amino-methylcoumarin moiety. The peptide was deacetylated by SIRT1, followed by the addition of a proteolytic developer that released the fluorescent amino-methylcoumarin. Briefly, enzyme preparations were incubated with 170 M NAD and 100 M p53 fluorogenic peptide for 45 min at 37 °C followed by incubation in

developer for 15 min at 37 °C. Fluorescence was measured by excitation at 360 nm and emission at 460 nm and enzymatic activity was expressed in relative fluorescence units (Solomon et al., 2006). Assays were performed in triplicate.

2.7. NAD⁺/NADH ratio determination

The NAD⁺/NADH Quantification Kit (BioVision) provided a convenient tool for sensitive detection of NAD⁺/NADH ratio. There is no need to purify NAD⁺/NADH from sample mix. The NAD⁺/NADH Quantification Kit was used according to manufacturer's instructions.

2.8. Senescence-associated beta-galactosidase staining

SA-beta-gal stained positive cell is one of the cellular senescent characteristics (Dimri et al., 1995). After being washed and fixed for 5 min at room temperature in 3% formaldehyde, cells were incubated overnight at 37 °C (without CO₂) with freshly prepared SA-beta-gal stain solution [1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂]. After washing with PBS, 100–200 cells in six fields were counted for the percentages of SA-beta-gal staining positive cells, which had blue color sedimentation in their cytoplasm under the microscope.

2.9. Senescence-associated heterochromatin foci (SAHF) analysis

SAHF formation is a novel specific biomarker of cellular senescence, because there is marked focal heterochromatin in the aged cells. To determine SAHF formation, cells were cultured directly on glass cover slips and then fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized with 0.2% Triton X-100/PBS for 10 min. DNA was visualized by DAPI (1 mg/ml) for 1 min, and then washed with PBS for twice. Cover slips were mounted in a 90% glycerol PBS solution. Cover slips were examined under Leica DMIRB inverted microscope.

2.10. Measurement of PD

Infected cell clones were selected with G418. The PD number of a clone grown to about 5 × 10⁵ cells is approximately 19 from the PD number at the beginning of the count. Therefore, the actual PD number of the infected cells should be increased by 19 PD when compared with the uninfected cells (Duan et al., 2001).

2.11. cell cycle analysis

Senescent human fibroblasts usually exhibit G1 cell cycle arrest (Ota et al., 2006). 1 × 10⁶ cells were collected in each sample with 0.25% trypsin, washed twice with cold PBS, and fixed with 70% alcohol in PBS for 12 h at 4 °C. Then the cells were stained for 30–60 min at 4 °C in 100 µg/ml propidium iodide solution (with 100 µg/ml RNase) after washing twice with PBS. Stained cells were analyzed in a flow cytometry (FACScan; BD Biosciences, San Jose, CA).

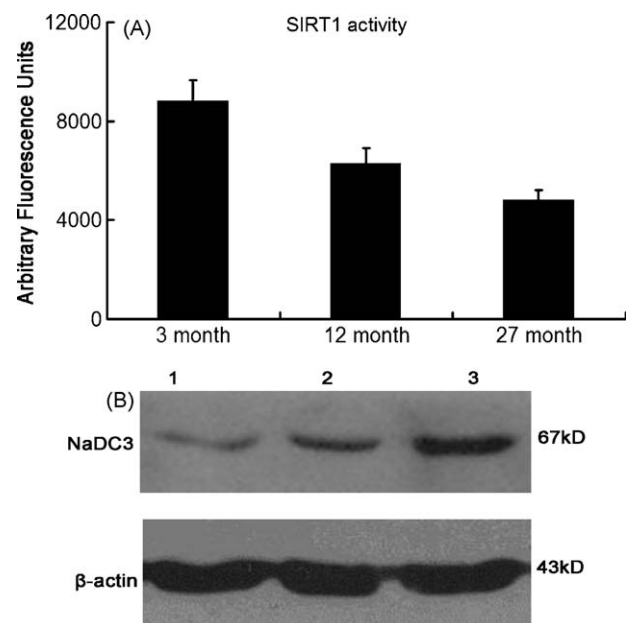


Fig. 1. The change of SIRT1 activity was detected by fluorescence and chemiluminescence (A). NaDC3 expression was detected by Western blot with aging in rat kidney (B). Lane 1: 3-month-old group; Lane 2: 12-month-old group; Lane 3: 27-month-old group.

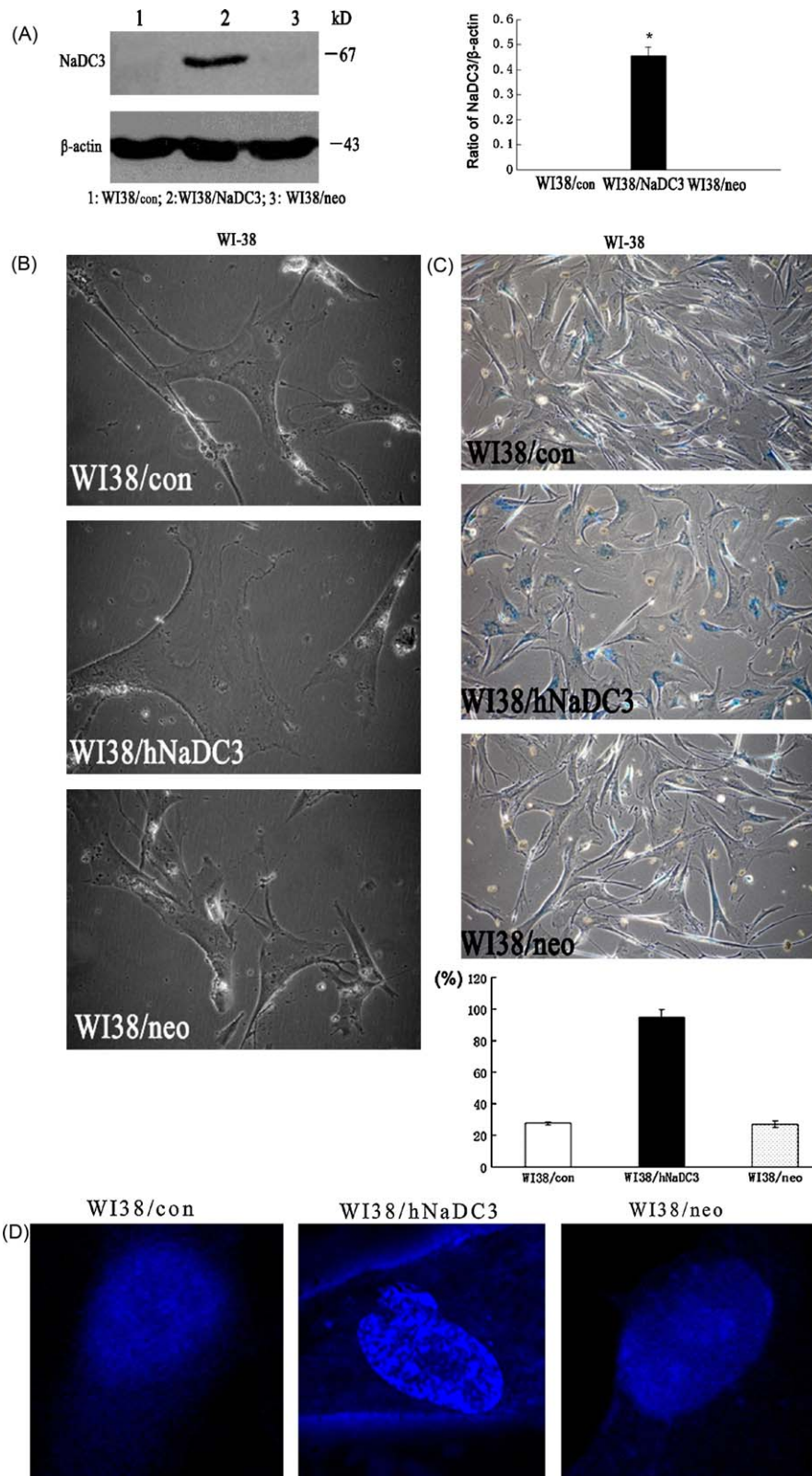


Fig. 2. NaDC3-induced senescence-related phenotypes in WI38 cells. (A) Efficiency of infection with NaDC3 was detected by Western blot. The WI38/hNaDC3 cells showed significantly high level of NaDC3, but no expression was found in control groups ($P < 0.05$) ($n = 6$). Empty vector groups had no difference compared to control ($P > 0.05$) ($n = 6$). Relative NaDC3 protein band density was normalized to β -actin. * $P < 0.05$ versus control group. (B) Morphology observation in the WI38 cells infected with NaDC3 vector. WI38/hNaDC3, WI38/neo and WI38/con cells cultured for 37PD were observed for morphology. (C) SA-beta-gal staining in the WI38 cells infected with NaDC3 retroviral vector. WI38 cells cultured for 37PD were stained for SA-beta-gal, which showed blue precipitation in the cytoplasm in senescent cells. Magnification, 100 \times . (D) Analysis of SAHF formation. The cells were stained with DAPI and heterochromatin foci were shown in the senescent cells. (E) Analysis of cell cycle by flow cytometry.

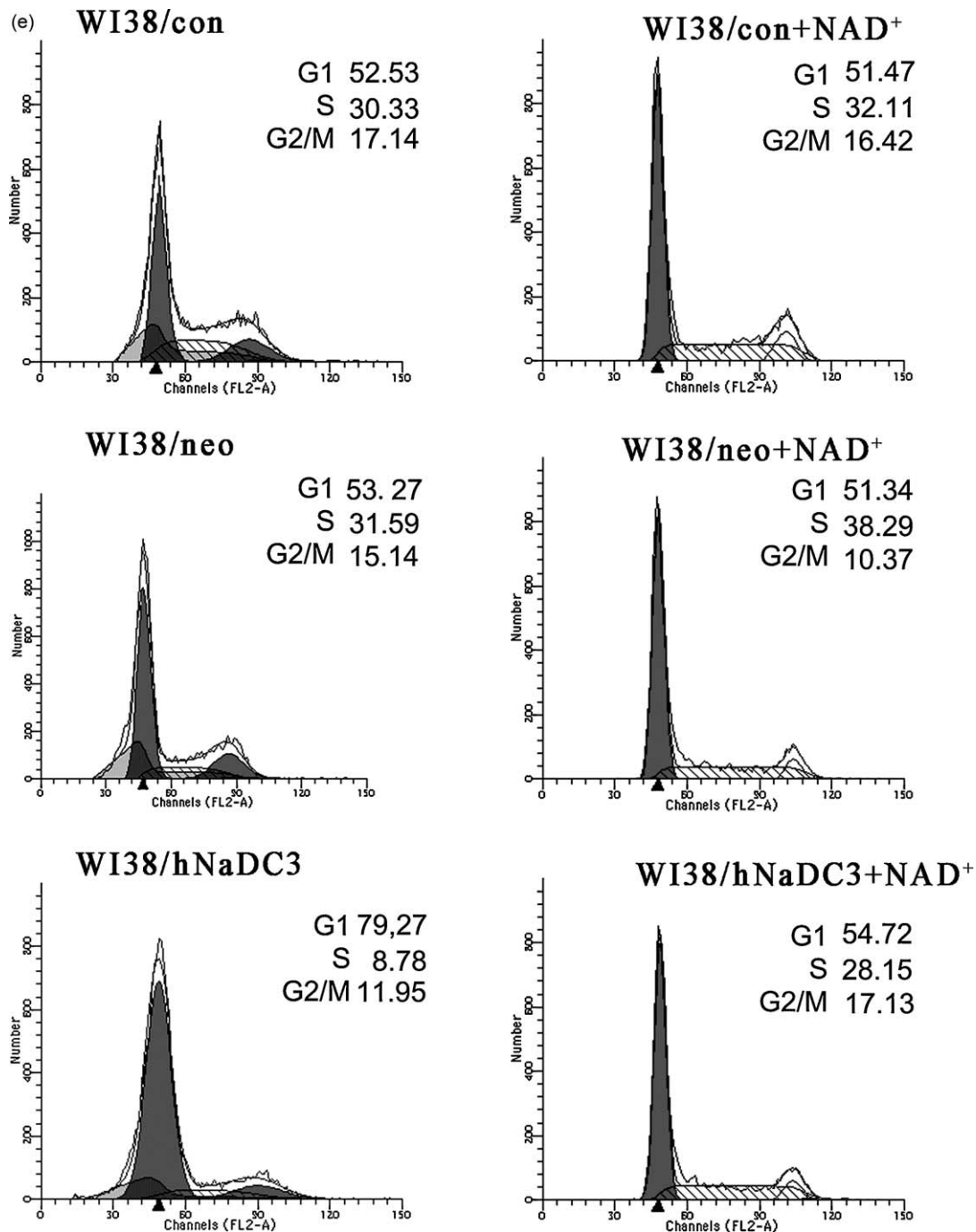


Fig. 2. (Continued).

2.12. Statistical analysis

Data was expressed as mean \pm SEM unless otherwise stated. Statistical differences were evaluated with ANOVA method. *P* value less than 0.05 was regarded as statistically significant.

3. Results

3.1. The change of SIRT1 activity and NaDC3 expression with aging in rat kidney

We detected the change of SIRT1 enzymatic activity and NaDC3 protein expression with aging in rat kidney. The results showed that the level of SIRT1 activity decreased gradually with aging in 3-,

12- and 27-month-old rats ($P < 0.05$) (Fig. 1A). Meanwhile, NaDC3 protein expression increased slowly ($P < 0.05$) (Fig. 1B).

3.2. NaDC3-induced cellular premature senescence in WI38 and MRC-5 cells

Because it is difficult to transfect genes efficiently into WI38 cells using plasmids, the recombinant NaDC3 retrovirus vector was produced by packaging PT67 cells, and WI38 cells were effectively infected. The results of Western blot showed that in WI38/hNaDC3 group, the level of NaDC3 was greatly increased, while the control groups (WI38/con) and empty vectors (WI38/neo) had no expression of NaDC3 (Fig. 2A).

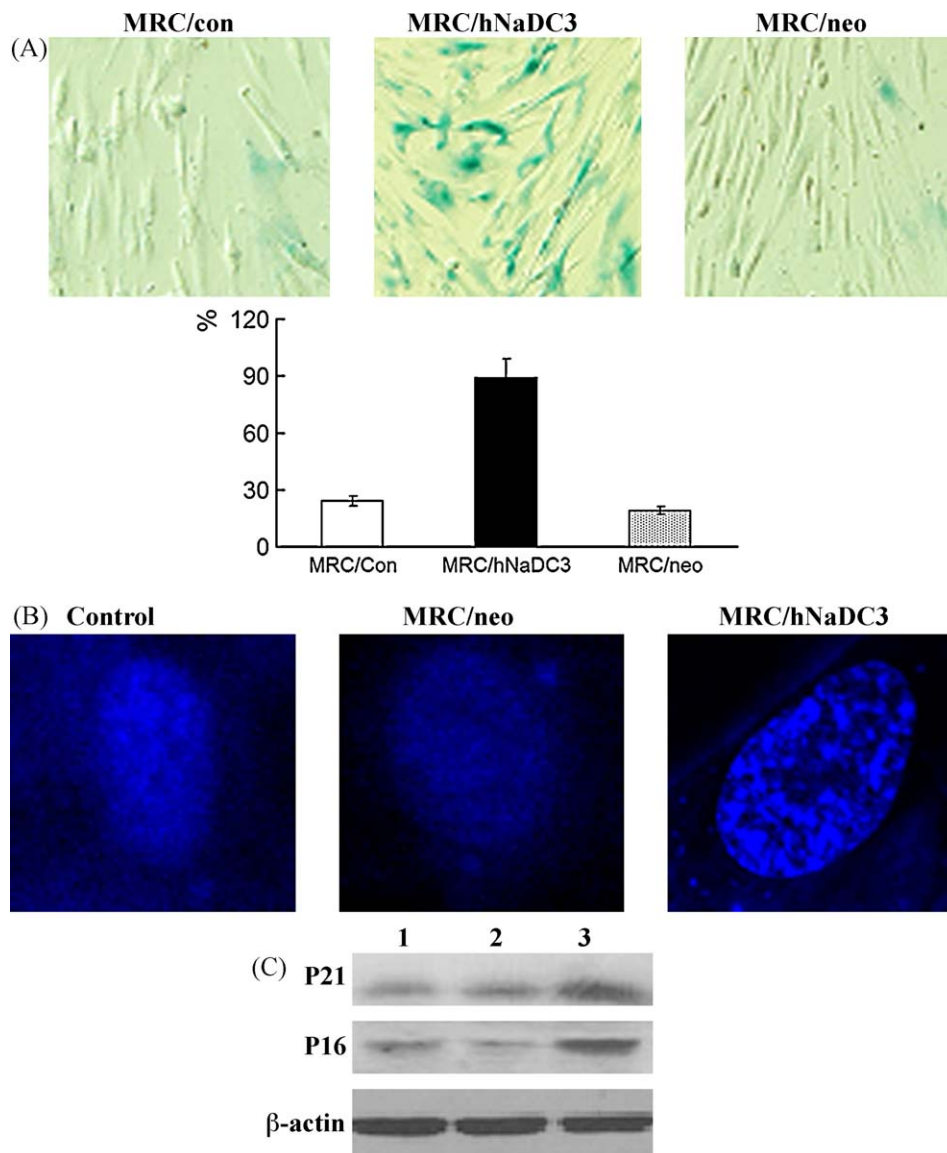


Fig. 3. NaDC3-induced senescence-related phenotypes in MRC-5 cells. (A) SA-beta-gal staining in the MRC-5 cells infected with NaDC3 retroviral vector showed blue precipitation in the cytoplasm in senescent cells. (B) Analysis of SAHF formation. The cells were stained with DAPI and prominent heterochromatin foci in nuclei were shown in the senescent cells. (C) Expression of cell cycle inhibitory proteins p16 and p21 was analyzed by Western blot. 1: The MRC-5 cells uninfected; 2: the MRC-5 cells infected with neo vector; 3: the MRC-5 cells infected with NaDC3 retroviral vector.

We first studied the effect of NaDC3 on the aging process of WI38 cells, which entered replicative senescence after completing a finite number of divisions (50 ± 10 population doublings, PD) and lost replicative ability. The results showed that WI38/hNaDC3 cells entered premature senescence after reaching PD 37–40, while the replicative lifespan of control groups and empty vector groups were PD 48–51. In addition, the volume of WI38/hNaDC3 cells in PD37 showed hypertrophy compared to that of control and empty vector groups (Fig. 2B). The SA-beta-gal (a biomarker of cell senescence) staining results showed that the percentage of staining positive cells in WI38/hNaDC3 group ($98.5 \pm 5.6\%$) was significantly greater than that in the WI38/con ($22.3 \pm 2.4\%$) and WI38/neo group ($23.4 \pm 2.18\%$) ($P < 0.05$) in 37 PD (Fig. 2C).

The accumulation of senescence-associated heterochromatin foci (SAHF) is another specific biomarker of senescent cells. As shown in Fig. 2D, senescence induced by hNaDC3 displayed pronounced punctuated DNA foci which were visualized by DAPI staining. In contrast, the cells in the WI38/con and WI38/neo group displayed a relatively uniform DAPI staining pattern.

We analyzed cell cycle with flow cytometry, as aging cells will enter permanent growth arrest at G1 phase after completing a finite number of divisions. The results showed that the percentages of the cells in WI38/hNaDC3 group at G1 phase ($79.14 \pm 0.58\%$) were significantly higher than that in the WI38/con ($53.54 \pm 3.19\%$) and WI38/neo ($54.21 \pm 5.04\%$) groups ($P < 0.05$) (Fig. 2E).

At the same time, MRC-5 cells were also infected with recombinant NaDC3 retrovirus vector. The SA-beta-gal staining and SAHF results showed that NaDC3 also induced cellular premature senescence of MRC-5 cells (Fig. 3A and B). Western blot analysis demonstrated that expression level of cell cycle regulatory proteins p16 and p21 (they also are marker of cellular senescence) was significantly increased in the MRC/hNaDC3 cells compared with those in the MRC/con and MRC/neo cells (Fig. 3C).

3.3. SIRT1 delayed replicative senescence in WI38 cells

We studied the effects of SIRT1 on the aging process by regulating SIRT1 activity. Resveratrol, a polyphenol found in

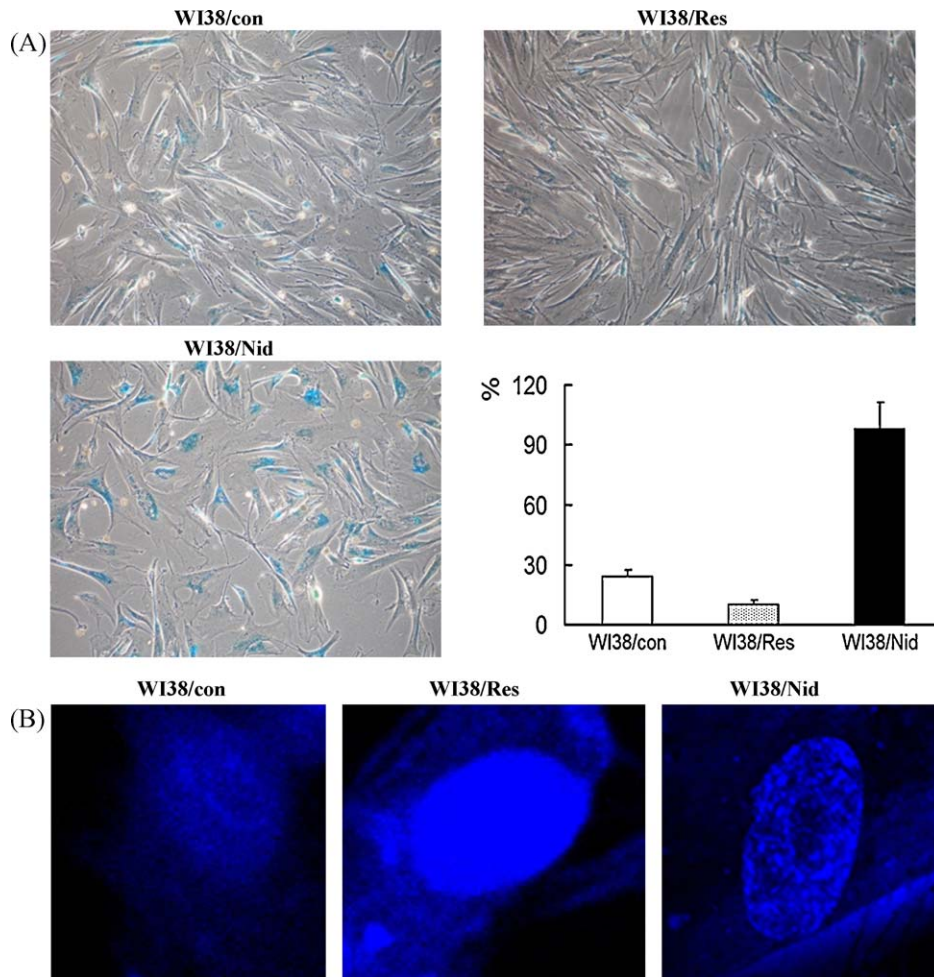


Fig. 4. SIRT1 inhibited Senescence-related phenotypes in WI38 cells. The SA-beta-gal staining and DAPI staining in WI38 cells were observed. The SA-beta-gal activity (A) and SAHF formation (B) were observed in the WI38 cells treated with resveratrol or niacinamide.

wines and thought to harbor major health benefits, is the specific agonist of SIRT1 enzymes. Studies have shown if expression of SIRT1 is suppressed by siRNA silencing or knock-out method, resveratrol will no longer play a biological function (Zhang et al., 2009). Niacinamide is a specific inhibitor of SIRT1 enzymes. SIRT1 activity will increase in the absence of nicotinamide (Liu et al., 2009; Wang et al., 2005; Chong et al., 2005). The WI38 cells treated with niacinamide (PD 37) showed increased enlargement and flattened inclusions similar to those of the senescent WI38 cells (PD 48). However, the morphology of the WI38 cells treated with resveratrol (PD 37) was similar to that of the young WI38 cells (PD 30). Moreover, we found that

the replicative life span of WI38 cells treated with resveratrol (PD 55–60) was longer than that of the control cells (PD 48–51). In contrast, those cells treated with niacinamide ceased cell division (PD 35–38) earlier than the control group. SA-beta-gal staining indicated that the percentage of positive cells was 25% in control cells (PD 37), whereas almost all of the WI38 cells treated with niacinamide were positively stained, just like the senescent cells (PD 48). But only sporadic SA-beta-gal-positive cells were seen in WI38 cells with resveratrol, similar to that of the young WI38 cells (PD 30) (Fig. 4A). DAPI staining results also shown that resveratrol treatment can retard the aging of WI38; niacinamide can accelerate the aging of WI38 (Fig. 4B).

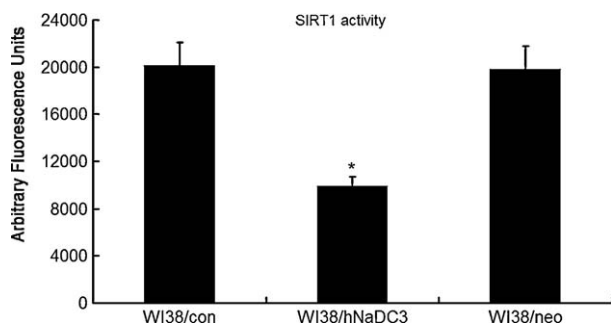


Fig. 5. NaDC3 inhibited SIRT1 Activity in WI38 cells. SIRT1 activity of WI38/hNaDC3 cells was inhibited dramatically, compared with those in control and WI38/neo groups ($P < 0.05$). * $P < 0.05$ versus control group ($n = 6$).

3.4. NaDC3 inhibited SIRT1 deacetylase activity in WI38 cells

To determine whether NaDC3 induces aging through inhibiting SIRT1 pathway, SIRT1 deacetylase activity was detected by fluorescent assay. The results showed that the activity was down-regulated in the WI38/hNaDC3 group (9256 ± 675), compared with that in the control ($20,569 \pm 1035$) and the WI38/neo groups ($18,972 \pm 1158$) ($P < 0.05$), (Fig. 5).

3.5. SIRT1 activators delayed cellular premature senescence induced by NaDC3

To elucidate whether the decreased level of SIRT1 activity was directly caused by NaDC3, or only as an accompanying

phenomenon in the aging process induced by NaDC3, we treated the WI38/hNaDC3 cells with resveratrol or CR serum, which are activators of SIRT1, and studied the change of aging-related phenotypes. The results indicated that CR serum and resveratrol

could elevate the level of SIRT1 activity of WI38/hNaDC3 cells (Fig. 6A).

The life span of WI38/hNaDC3 cells treated with CR serum (PD 47–52) or resveratrol (PD 46–51) was similar to that of the control

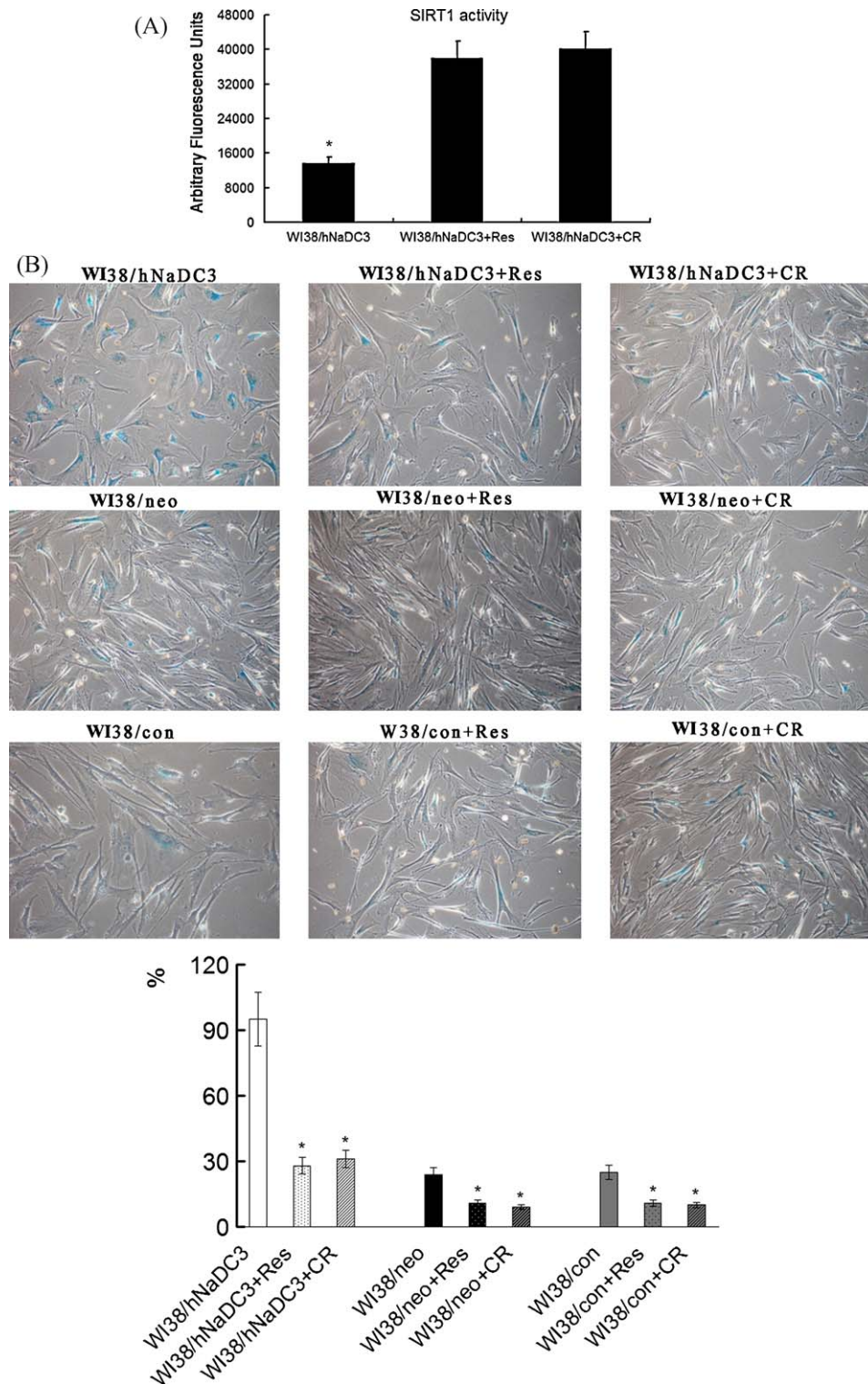


Fig. 6. Elevation of SIRT1 activity reduced senescence-related phenotypes induced by NaDC3. (A) SIRT1 activity was measured using the Fluor de Lys kit in WI38/hNaDC3 cells in the presence of CR serum or resveratrol cultured for 37PD. WI38/hNaDC3 cells in the presence of CR serum or resveratrol showed significantly higher levels of SIRT1 than that in WI38/hNaDC3 group ($P < 0.05$). * $P < 0.05$ versus WI38/hNaDC3 group ($n = 6$). (B) Effect of elevating SIRT1 activity on morphology and SA-beta-gal staining of WI38/hNaDC3 cells. WI38/hNaDC3 cells cultured in the presence CR serum or resveratrol for 37 PD were stained for SA-beta-gal. The percentage of SA-beta-gal stained positive cells. For each group, 100–200 cells were counted. Comparison among groups was conducted with ANOVA. * $P < 0.05$ versus WI38/hNaDC3 group. Magnification, 100 \times . (C) SAHG formations were observe in the WI38/hNaDC3 cells treated with resveratrol or CR serum. (D) Analysis of cell cycle by flow cytometry in the WI38/hNaDC3 cells treated with resveratrol or CR serum.

cells (PD 48–51). Meanwhile, the life span of WI38/con and WI38/neo cells have also been increased slightly treated with CR serum or resveratrol (PD 52–55), respectively. It was also found that cellular hypertrophy was ameliorated after WI38/hNaDC3 cells were treated with resveratrol or CR serum. Meanwhile, the change of the cellular shape in WI38/con and WI38/neo groups has not been found.

SA-beta-gal staining showed that in the WI38/hNaDC3 group treated with resveratrol or CR serum, the positive rate was 24% and 23%, respectively, and that in the WI38/hNaDC3 cells without resveratrol or CR serum was 98.5% (Fig. 6B). Meanwhile, the positive rate of SA-beta-gal staining in WI38/con and WI38/neo groups was 20% and similar to those treated with resveratrol or CR serum. DAPI staining results also shown similar results as SA-beta-gal staining (Fig. 6C).

The results of cell cycle with flow cytometry showed that in WI38/hNaDC3 cells treated with resveratrol or CR serum, the percentage of cells at G1 phase was significantly lower than in the WI38/NaDC3 without resveratrol or CR serum, and was nearly equal to that in control and WI38/neo cells, consistent with the above results. Meanwhile, in WI38/con and WI38/neo groups treated with resveratrol or CR serum, the percentage of cells at G1 phase also have a slight decrease, respectively (Fig. 6D).

3.6. NaDC3 lowered NAD^+ /NADH ratio in WI38/hNaDC3 cells

To study the effect of NaDC3 on NAD^+ /NADH ratio, the ratio was tested in the cytoplasm of WI38/hNaDC3 cells. Our results indicated that intracellular NAD^+ /NADH ratio was lower in the WI38/hNaDC3 group (0.53 ± 0.01), compared to WI38/con (1.12 ± 0.05) and WI38/neo groups (1.18 ± 0.1) ($P < 0.05$) (Fig. 7A).

3.7. The increase of NAD^+ /NADH ratio by adding exogenous NAD delayed cellular premature senescence induced by NaDC3

To investigate whether NaDC3 promoted premature cellular senescence by directly down-regulating NAD^+ /NADH ratio to inhibit the activity of SIRT1, we further added exogenous NAD^+ to up-regulate the NAD^+ /NADH ratio in 3 groups (Fig. 7A) and observed the change of premature senescence-related phenotypes induced by NaDC3. The results indicated that exogenous NAD^+ can elevate the level of SIRT1 activity (Fig. 7B), and decrease the positive rate of beta-gal staining (Fig. 7C) and the prominent heterochromatin foci in nuclei in the WI38/hNaDC3 cells (Fig. 7D), and the number of arresting cells at G1 phase in 3 groups (Fig. 2E). The life span of WI38/hNaDC3 cells treated with NAD^+ (PD 45–50) was similar to that of the control cells (PD 48–51). Meanwhile, in WI38/con and WI38/neo groups

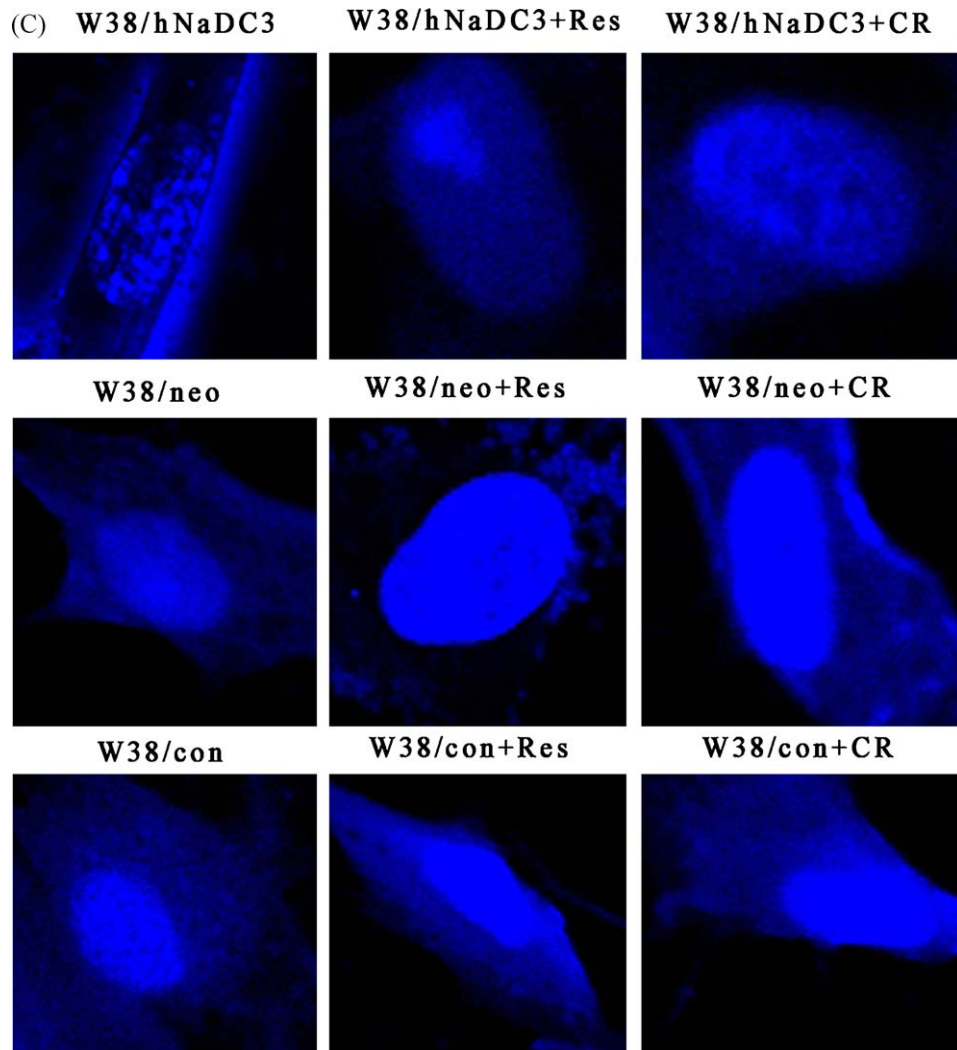


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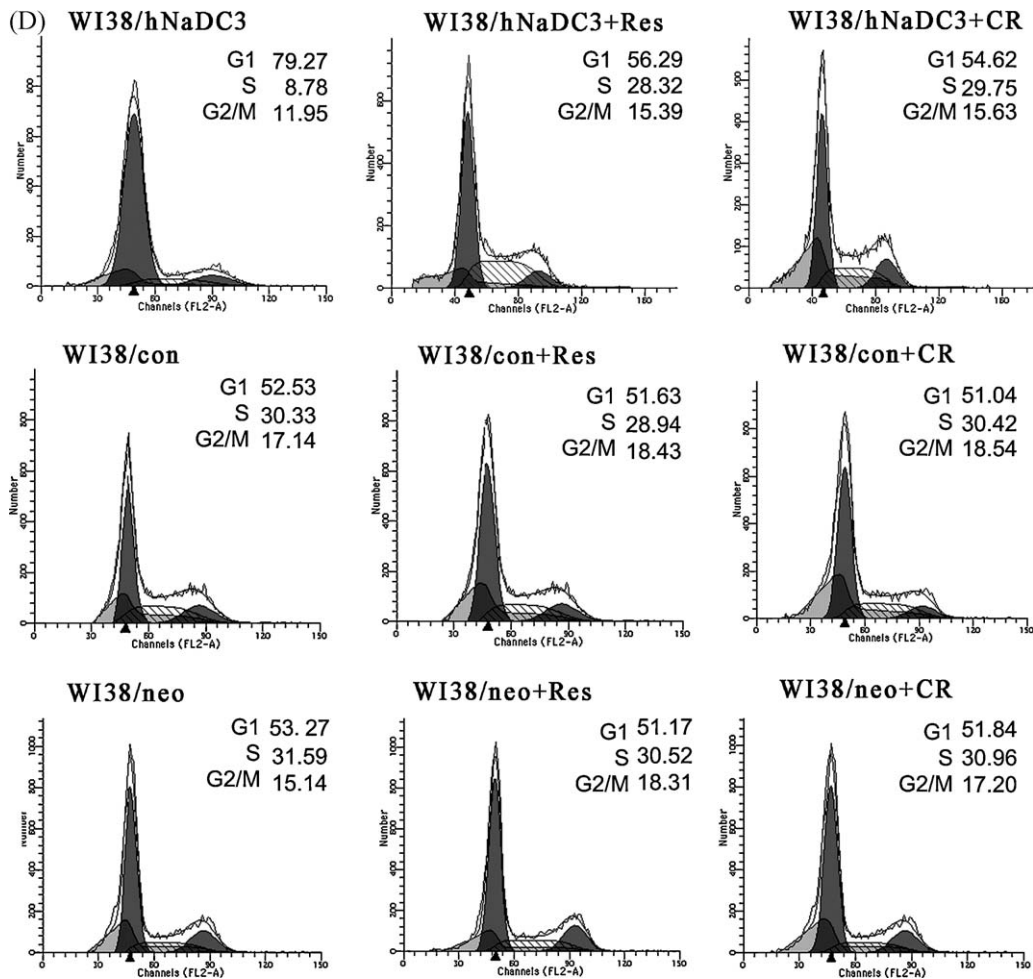


Fig. 6. (Continued).

treated with NAD⁺, The life span also have a slight increase, respectively (PD 52–55).

4. Discussion

NaDC3 is a transmembrane protein that is responsible for the transport of intermediates of the Krebs cycle (Simpson, 1983; Chen et al., 1999; Bai et al., 2007), and is related to energy metabolism (Pajor, 2000). The increase of the concentration of intracellular intermediates causes the decrease of NAD⁺/NADH ratio (Shawn et al., 2000). NAD⁺ or NAD⁺/NADH ratio determines SIRT1 deacetylase activity (Imai et al., 2000). Recently it was reported that SIRT1 could delay mammalian cellular senescence (Ota et al., 2006; Ota et al., 2007; Huang et al., 2008). In this study, we have confirmed that SIRT1 can delay senescence of WI38 cells, which are generally accepted in cell senescence studies, and found that NaDC3 induces cellular premature senescence through inhibiting NAD⁺-dependent SIRT1 pathway (Fig. 8).

4.1. NaDC3 induces cellular premature senescence

We have found that WI38/hNaDC3 cells show the phenotypes of premature senescence, including decrease in proliferative ability, presence of hypertrophic cellular volume, and increase in the positive staining percentage of SA-beta-gal and cell number arrested in the G1 phase. These results indicate that over-expression of NaDC3 induces aging-related phenotypes. This confirms our previous results (Chen et al., 2005b).

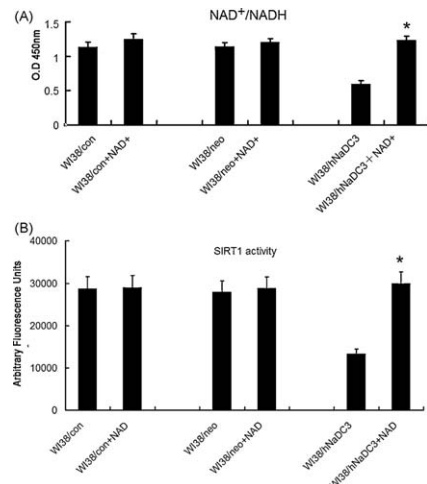


Fig. 7. Exogenous NAD⁺ delayed cellular premature senescence induced by NaDC3. (A) NAD⁺/NADH ratio of WI38/hNaDC3 cells was down-regulated. But WI38/hNaDC3 + NAD cells were noted NAD⁺/NADH ratio was enhanced, compared with WI38/hNaDC3 cells in 37 PD ($P < 0.05$). * $P < 0.05$ versus WI38/hNaDC3 group. (B) SIRT1 activity was measured in WI38/hNaDC3 + NAD⁺ cells. NAD⁺ enhanced SIRT1 activity of WI38/hNaDC3 cells, compared with WI38/hNaDC3 cells without NAD⁺ in 37 PD ($P < 0.05$). * $P < 0.05$ versus WI38/hNaDC3 group. The data shown is one representative experiment out of a series of five with similar results. (C) The change of morphology and the percentage of SA-beta-gal stained positive cells. For each group in 37 PD, 100–200 cells were counted. Comparison among groups was conducted with ANOVA. * $P < 0.05$ versus WI38/hNaDC3 group. Magnification, 100 \times . (D) SAHF formations were observed in the WI38/hNaDC3 cells treated with NAD⁺.

Rogina et al. found that mutation of the *Indy* (I'm not dead yet) gene, the homolog of NaDC in the fruit fly, extends the mean life span (Rogina et al., 2000). Knockdown of *ceNaDC2* gene from *Caenorhabditis elegans*, the counterpart of hNaDC3, leads to a significant increase in the average life span (Fei et al., 2003). This supports that the NaDC3 can accelerate cellular premature senescence.

4.2. SIRT1 delays replicative senescence in WI38 cells

Sir2, an NAD⁺-dependent protein deacetylase, extends the lifespan in diverse species from yeast to flies. Sir2 is remarkably conserved in evolution from archaeobacteria to eukaryotes. Mammals have seven homologs of Sir2, including SIRT1–7, which affect aging and metabolism. We found that SIRT1 level decreases

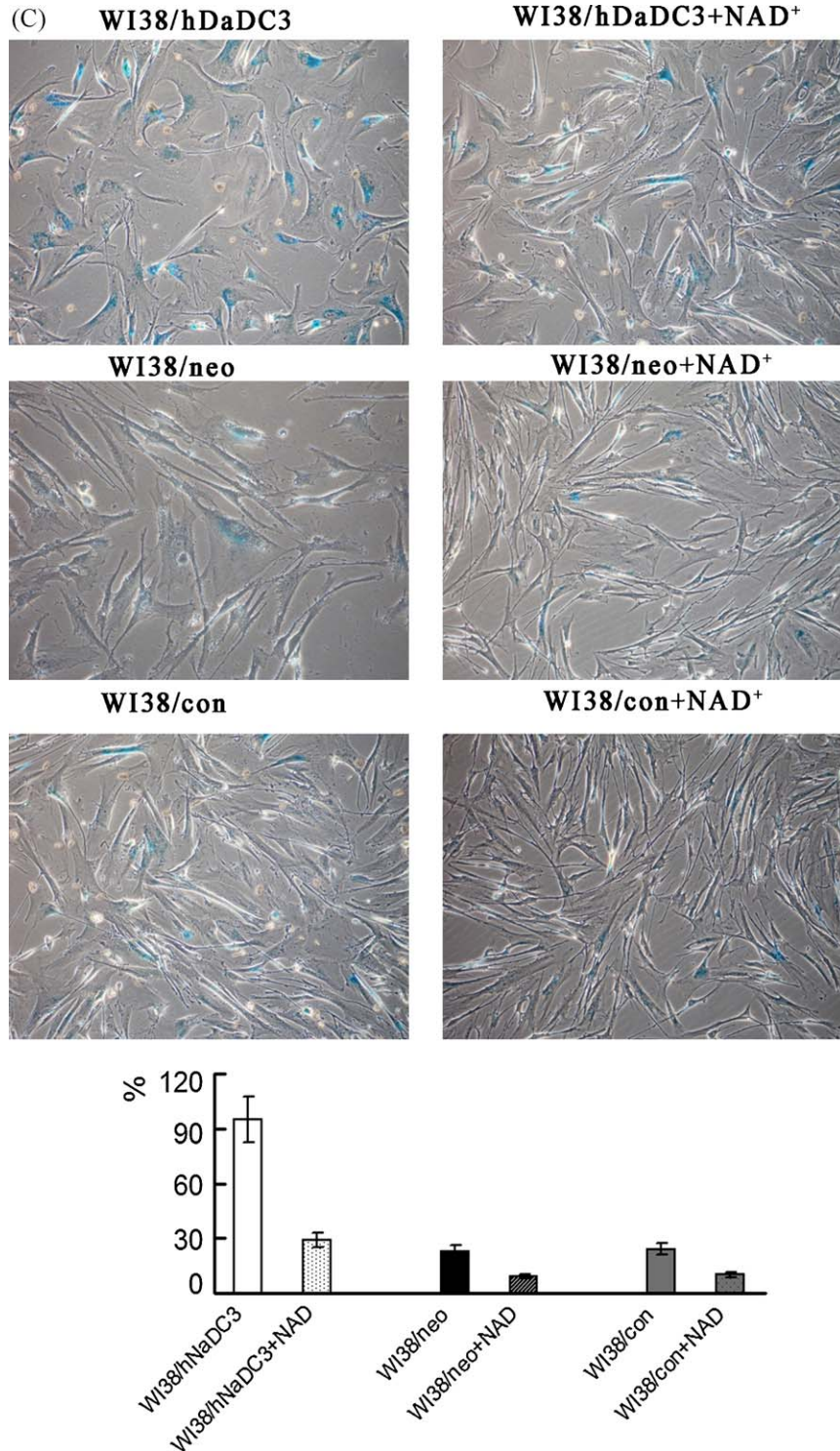


Fig. 7. (Continued).

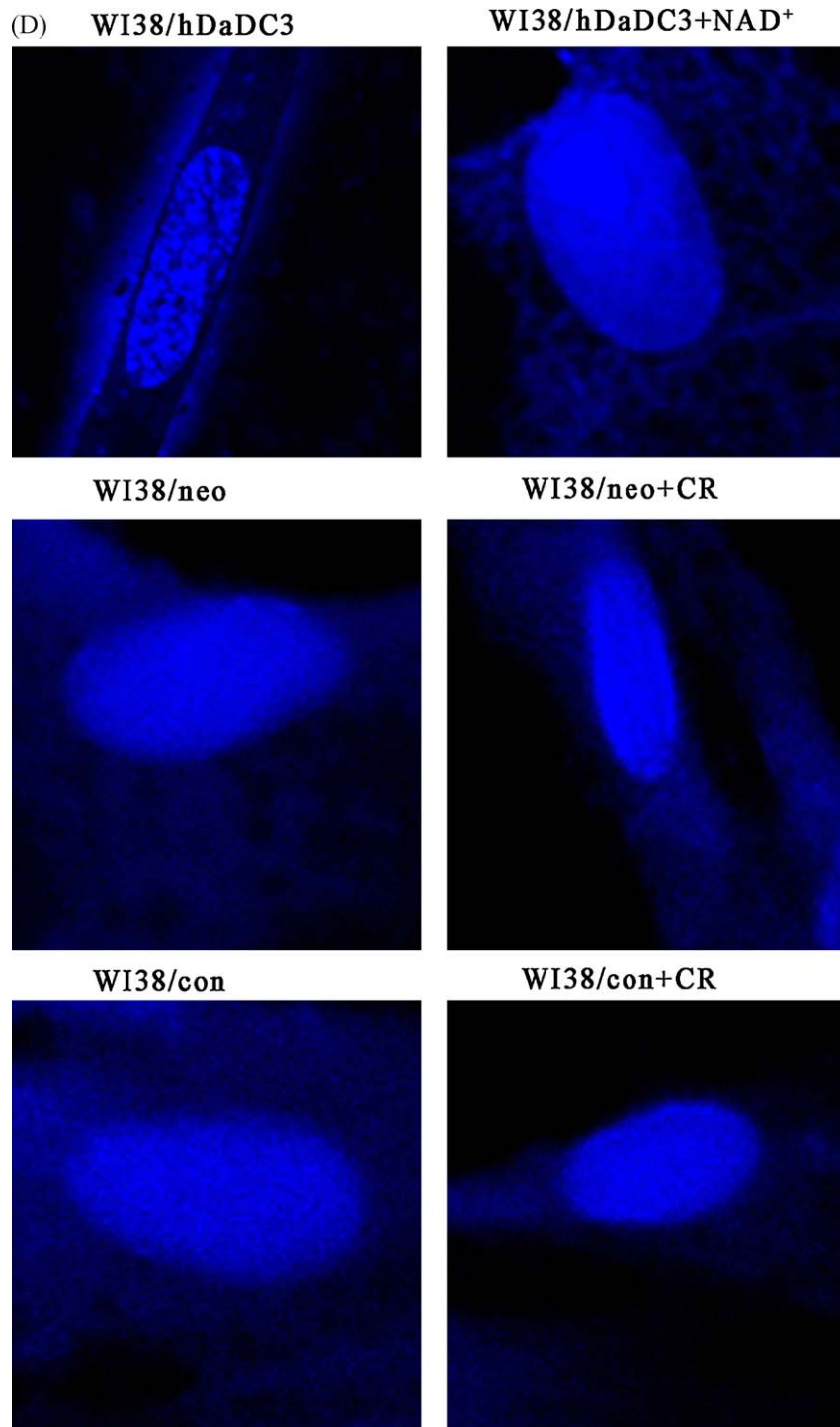


Fig. 7. (Continued).

with ageing in rat kidney tissue and WI38 cells. We also found that up-regulation of SIRT1 activity delays cellular senescence, and down-regulation of SIRT1 activity promotes senescence. Therefore, SIRT1 plays an important role in the senescent progress.

4.3. NaDC3 inhibits the activity of SIRT1 deacetylase in WI38 cells

Caloric absorption has a significant effect on the maximum lifespan of animals, in that lifespan is shortened when food amount consumed exceeds the proper levels (Weindruch and

Walford, 1982). Disruption of the function of ceNaDC2 in *C. elegans* leads to decreased availability of dicarboxylate to produce metabolic energy, thus creating a biologic state similar to that of caloric restriction, and consequently leading to life span extension (Chen et al., 2005a). So it is probable that acceleration of cellular senescence after overexpression of NaDC3 is involved in loaded energy metabolism (Chen et al., 2005b). NAD⁺-dependent SIRT1 is a bridge between Energy metabolism and senescence (Leonard, 2000). We found that overexpression of NaDC3 can down-regulate the level of SIRT1

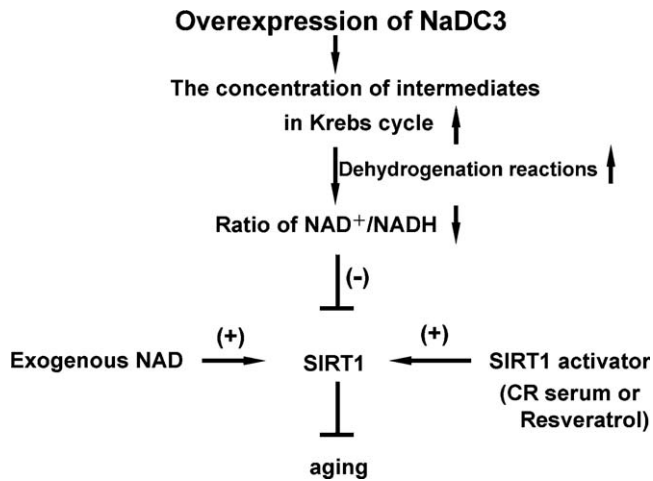


Fig. 8. A proposed signaling pathway involved in NaDC3-induced aging in the WI38 cells.

activity, which supports the hypothesis that NaDC3 inhibits SIRT1 pathway.

4.4. The increase of SIRT1 activity delays cellular premature senescence induced by NaDC3

Serum from CR rat and resveratrol are activators of SIRT1 enzymes *in vivo* and *in vitro* (Howitz et al., 2003; Borra et al., 2005). Our results indicate that CR serum or resveratrol can elevate the level of SIRT1 activity. The senescence-related phenotypes of WI38/hNaDC3 cells in the presence of CR serum or resveratrol are significantly improved compared with that of WI38/hNaDC3 cells in the absence of CR serum or resveratrol, as demonstrated by SA-beta-gal staining, population doublings, cell cycle analysis, proliferation capability and cell morphology. In other words, cellular senescence-related phenotypes induced by NaDC3 are reversed if the level of SIRT1 activity is enhanced. Therefore, it is reasonable to suggest that NaDC3 promotes cellular premature senescence by inhibiting the SIRT1 pathway.

4.5. NaDC3 lowers NAD⁺/NADH ratio in WI38 cells

NaDC3 is expressed in cellular membrane, while SIRT1 is expressed in cellular nucleus. How does NaDC3 cause the decreased level of SIRT1 activity? To answer the question, we determined the intracellular NAD⁺/NADH ratio in WI38/hNaDC3 cells and found that this ratio was decreased. In a previous study, we also found that overexpression of NaDC3 can transport more intermediates into cells for cellular energy metabolism (He et al., 2004). It is known that there are five dehydrogenation reactions in the Krebs cycle, and four of the resulting hydrogens are accepted by NAD⁺, and NAD⁺ is reduced to NADH. Thus, the increase of concentration in Krebs cycle intermediates causes the decrease of NAD⁺/NADH ratio (Shawn et al., 2000).

Sirt2 is an NAD⁺-dependent deacetylase. The NAD⁺-dependent deacetylation occurs via a two-step mechanism. The first step is the cleavage of the high energy glycosidic bond that joins the ADP ribose moiety of NAD⁺ to nicotinamide. Upon cleavage, Sir2 then catalyzes the transfer of an acetyl group to ADP ribose (Min et al., 2001; Zhao et al., 2004). When NAD⁺ exists or NAD⁺/NADH ratio increases, deacetylation by Sir2 occurs. But when NAD⁺ is omitted or NAD⁺/NADH ratio decreases, Sir2 will lose deacetylation (Lin et al., 2004). NaDC3 probably promotes cellular premature senescence through down-regulating the NAD⁺/NADH ratio, then inhibiting the level of SIRT1 activity. Our results confirm this hypothesis that NaDC3

inhibits SIRT1 activity to promote premature senescence, accompanied by the decreased level of NAD⁺/NADH ratio.

4.6. The increase of NAD⁺/NADH ratio delays cellular premature senescence induced by NaDC3

SIRT1 enzymatic activity has revealed a strict and unique requirement for NAD⁺. The requirement for NAD⁺ as a co-substrate suggested that SIRT1 can evolve as a sensor of cellular energy, and its redox states is coupled to the metabolic status of the cell by NAD⁺ (Blander and Guarente, 2004). If the level of NAD⁺ decreases 60% or mutation of NAD⁺ biosynthetic enzyme occurs, SIRT1 will lose activity even if given CR condition (Lin et al., 2004; Smith et al., 2000). Perturbations of NAD⁺ metabolism alter SIRT1 catalytic activity in yeast and human cells (Cohen et al., 2004).

To investigate that NaDC3 promotes cellular premature senescence through down-regulation of NAD⁺/NADH ratio to inhibit SIRT1 activity, we further elevate NAD⁺ level by adding exogenous NAD⁺ in culture system to up-regulate NAD⁺/NADH ratio. Exogenous NAD⁺ could enter the cell freely (Araki et al., 2004) or be transported across the plasma membrane (Bruzzone et al., 2001). The data indicates that NaDC3-induced aging-associated phenotypes are attenuated when WI38/hNaDC3 cells are treated with NAD⁺. These results suggest that NaDC3 decreases NAD⁺/NADH ratio, and subsequently inhibits the SIRT1 pathway to promote cellular premature senescence. In this report, we attempt to determine whether NaDC3 induces premature senescence through the SIRT1 pathway. Our results show that NaDC3 inhibits SIRT1 enzymatic activity by down-regulating NAD⁺/NADH ratio in the process of cellular senescence. This mechanism will provide new pathways and targets for the intervention of cellular senescence (Fig. 8).

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References

- Alcendor, R.R., Kirshenbaum, L.A., Imai, S., Vatner, S.F., Sadoshima, J., 2004. Silent information regulator- α , a longevity factor and class III histone deacetylase, is an essential endogenous apoptosis inhibitor in cardiac myocytes. *Circ. Res.* 95, 971–980.
- Anderson, R.M., Bitterman, K.J., Wood, J.G., Medvedik, O., Sinclair, D.A., 2003. Nicotinamide and PNC1 govern lifespan extension by calorie restriction in *Saccharomyces cerevisiae*. *Nature* 423, 181–185.
- Araki, T., Sasaki, Y., Milbrandt, J., 2004. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 305, 1010–1013.
- Bai, X., Chen, X.M., Feng, Z., Hou, K., Zhang, P., Fu, B., Shi, S., 2006. Identification of basolateral membrane targeting signal of human sodium-dependent dicarboxylate transporter 3. *J. Cell. Physiol.* 206, 821–830.
- Bai, X.Y., Chen, X., Sun, A.Q., Feng, Z., Hou, K., Fu, B., 2007. Membrane topology structure of human high-affinity, sodium-dependent dicarboxylate transporter. *FASEB J.* 21, 2409–2417.
- Borra, M.T., Smith, B.C., Denu, J.M., 2005. Mechanism of human SIRT1 activation by resveratrol. *J. Biol. Chem.* 280, 17187–17195.
- Bruzzone, S., Franco, L., Guida, L., Zocchi, E., Contini, P., Bisso, A., Usai, C., De Flora, A., 2001. A self-restricted CD38-connexin 43 cross-talk affects NAD⁺ and cyclic ADP-ribose metabolism and regulates intracellular calcium in 3T3 fibroblasts. *J. Biol. Chem.* 276, 48300–48308.
- Blander, G., Guarente, L., 2004. The Sir2 family of protein deacetylases. *Annu. Rev. Biochem.* 73, 417–435.
- Chen, J., Zhou, Y., Mueller-Steiner, S., Chen, L.F., Kwon, H., Yi, S., Mucke, L., Gan, L., 2005a. SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF- κ B signaling. *J. Biol. Chem.* 280, 40364–40374.
- Chen, X.M., Cao, D.W., Wang, J.Z., Yuan, L., Feng, Z., Fu, B., Hong, Q., Zhang, X.J., Bai, X.Y., Lu, Y., Ding, R., 2005b. Effects of human Na/Dicarboxylate cotransporter 3 on the replicative senescence of human embryonic lung diploid fibroblasts. *J. Gerontol. A: Biol.* 60A, 709–714.

- Chen, X.M., Tsukaguchi, H., Chen, X.Z., Berger, U.V., Hediger, M.A., 1999. Molecular and functional analysis of SDC2, a novel rat sodium-dependent dicarboxylate transporter. *J. Clin. Invest.* 103, 1159–1168.
- Chong, Z.Z., Lin, S.H., Li, F.Q., Maiese, K., 2005. The sirtuin inhibitor nicotinamide enhances neuronal cell survival during acute anoxic injury through AKT, BAD, PARP, and mitochondrial associated “anti-apoptotic” pathways. *Curr. Neurovasc. Res.* 2, 271–285.
- Chu, F., Chou, P.M., Zheng, X., Mirkin, B.L., Rebbaa, A., 2005. Control of multidrug resistance gene *mdr1* and cancer resistance to chemotherapy by the longevity gene *sirt1*. *Cancer Res.* 65, 10183–10187.
- Cohen, H.Y., Miller, C., Bitterman, K.J., Cohen, H.Y., Miller, C., Bitterman, K.J., Wall, N.R., Hekking, B., Kessler, B., Howitz, K.T., Gorospe, M., de Cabo, R., Sinclair, D.A., 2004. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305, 390–392.
- Dimri, G.P., Lee, X., Basile, G., Acosta, M., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9363–9367.
- Duan, J., Zhang, Z., Tong, T., 2001. Senescence delay of human diploid fibroblast induced by anti-sense p16INK4a expression. *J. Biol. Chem.* 276, 48325–48331.
- Fei, Y.J., Inoue, K., Ganapathy, V., 2003. Structural and functional characteristics of two sodium-coupled dicarboxylate transporters (ceNaDC1 and ceNaDC2) from *Caenorhabditis elegans* and their relevance to life span. *J. Biol. Chem.* 278, 6136–6144.
- Fernandes, G., Yunis, E.J., Good, R.A., 1976. Influence of diet on survival of mice. *Proc. Natl. Acad. Sci. U.S.A.* 73, 1279–1283.
- Gerhart-Hines, Z., Rodgers, J.T., Bare, O., Lerin, C., Kim, S.H., Mostoslavsky, R., Alt, F.W., Wu, Z., Puigserver, P., 2007. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1 α . *EMBO J.* 26, 1913–1923.
- He, J.Y., Chen, X.M., Bai, X.Y., Wang, J.Z., 2004. The effects of human Na⁺-dependent high-affinity dicarboxylate cotransporter3 and glucose on the transport of succinate in human proximal tubular epithelial cells. *Med. J. Chin. PLA* 29, 600–602.
- Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.L., Scherer, B., Sinclair, D.A., 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425, 191–196.
- Huang, J., Gan, Q.N., Han, L.M., Li, J., Zhang, H., Sun, Y., Zhang, Z.Y., Tong, T.J., 2008. SIRT1 overexpression antagonizes cellular senescence with activated ERK/S6k1 signaling in human diploid fibroblasts. *PLOS One* 3, e1710.
- Imai, S., Armstrong, C.M., Kaeberlein, M., Guarente, L., 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 403, 795–800.
- Kaeberlein, M., McVey, M., Guarente, L., 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Gene Dev.* 13, 2570–2580.
- Langley, E., Pearson, M., Faretta, M., Bauer, U.M., Frye, R.A., Minucci, S., Pelicci, P.G., Kouzarides, T., 2002. Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J.* 21, 2383–2396.
- Leonard, G., Frédéric, P., 2005. Calorie restriction—the SIR2 connection. *Cell* 120, 473–482.
- Leonard, G., 2000. Sir2 links chromatin silencing, metabolism, and aging. *Gene Dev.* 14, 1021–1026.
- Lin, S.J., Ethan, F., Marcia, H., Liszt, G., Guarente, L., 2004. Calorie restriction extends yeast life span by lowering the level of NADH. *Gene Dev.* 18, 12–16.
- Liu, D., Gharavi, R., Pitta, M., Gleichmann, M., Mattson, M.P., 2009. Nicotinamide prevents NAD⁺ depletion and protects neurons against excitotoxicity and cerebral ischemia: NAD⁺ consumption by SIRT1 may endanger energetically compromised neurons. *Neuromol. Med.* 11, 28–42.
- Longo, V.D., Kennedy, B.K., 2006. Sirtuins in aging and age-related disease. *Cell* 126, 257–268.
- Maeda, H., Gleiser, C.A., Masoro, E.J., Murata, I., McMahan, C.A., Yu, B.P., 1985. Nutritional influences on aging of Fischer 344 rats. II. Pathology. *J. Gerontol.* 40, 671–688.
- Min, J., Landry, J., Sternglanz, R., Xu, R.M., 2001. Crystal structure of a SIR2 homolog-NAD complex. *Cell* 105, 269–279.
- Murer, H., Manganel, M., Roch-Ramel, F., 1992. Tubular transport of monocarboxylates, Krebs cycle intermediates, and inorganic sulfate. In: Windhager, E.E. (Ed.), *Handbook of Physiology: Renal Physiology*. American Physiological Society, Bethesda, MD, pp. 2165–2188.
- Ota, H., Akishita, M., Eto, M., Iijima, K., Kaneki, M., Ouchi, Y., 2007. Sirt1 modulates premature senescence-like phenotype in human endothelial cells. *J. Mol. Cell. Cardiol.* 43, 571–579.
- Ota, H., Tokunaga, E., Chang, K., Hikasa, M., Iijima, K., Eto, M., Kozaki, K., Akishita, M., Ouchi, Y., Kaneki, M., 2006. Sirt1 inhibitor, Sirtinol, induces senescence-like growth arrest with attenuated Ras-MAPK signaling in human cancer cells. *Oncogene* 25, 176–185.
- Pajor, A.M., 2000. Molecular properties of sodium/dicarboxylate cotransporters. *J. Membr. Biol.* 175, 1–8.
- Pajor, A.M., 1999. Sodium-coupled transporters for Krebs cycle intermediates. *Annu. Rev. Physiol.* 61, 663–682.
- Pugh, T.D., Oberley, T.D., Weindruch, R., 1999. Dietary intervention at middle age: caloric restriction but not dehydroepiandrosterone sulfate increases lifespan and lifetime cancer incidence in mice. *Cancer Res.* 59, 1642–1648.
- Qin, W., Yang, T., Ho, L., Zhao, Z., Wang, J., Chen, L., Zhao, W., Thiyagarajan, M., MacGrogan, D., Rodgers, J.T., Puigserver, P., Sadoshima, J., Deng, H., Pedrini, S., Gandy, S., Sauve, A.A., Pasinetti, G.M., 2006. Neuronal SIRT1 activation as a novel mechanism underlying the prevention of Alzheimer disease amyloid neuropathology by calorie restriction. *J. Biol. Chem.* 281, 21745–21754.
- Ramsey, K.M., Mills, K.F., Satoh, A., Imai, S., 2008. Age-associated loss of Sirt1-mediated enhancement of glucose stimulated insulin secretion in beta cell-specific Sirt1-overexpressing (BESTO) mice. *Aging Cell.* 7, 78–88.
- Rogina, B., Reenan, R.A., Nilsen, S.P., Helfand, S.L., 2000. Extended life-span conferred by cotransporter gene mutations in *Drosophila*. *Science* 290, 2137–2140.
- Roth, G.S., Ingram, D.K., Lane, M.A., 2001. Caloric restriction in primates and relevance to humans. *Ann. N. Y. Acad. Sci.* 928, 305–315.
- Sasaki, T., Maier, B., Bartke, A., Scoble, H., 2006. Progressive loss of SIRT1 with cell cycle withdrawal. *Aging Cell.* 5, 413–422.
- Burgess, S.C., Hausler, N., Merritt, M., Jeffrey, F.M., Storey, C., Milde, A., Koshy, S., Lindner, J., Magnuson, M.A., Malloy, C.R., Sherry, A.D., 2000. Impaired tricarboxylic acid cycle activity in mouse livers lacking cytosolic phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* 279, 48941–48949.
- Simpson, D.P., 1983. Citrate excretion: a window on renal metabolism. *Am. J. Physiol.* 244, F223–F234.
- Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C., et al., 2000. A phylogenetically conserved NAD⁺-dependent protein deacetylase activity in the Sir2 protein family. *Proc. Natl. Acad. Sci.* 97, 6658–6663.
- Solomon, J.M., Pasupuleti, R., Xu, L., McDonagh, T., Curtis, R., DiStefano, P.S., Huber, L.J., 2006. Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol. Cell. Biol.* 26, 28–38.
- Tissenbaum, H.A., Guarente, L., 2001. Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* 410, 227–230.
- Wang, J., Chen, X., Zhu, H., Peng, L., Hong, Q., 2003. Relationship between aging and renal high-affinity sodium-dependent dicarboxylate cotransporter-3 expression characterized with antifusion protein antibody. *J. Gerontol. A: Biol. Sci. Med. Sci.* 58, B879–888.
- Wang, J., Zhai, Q.W., Chen, Y., Lin, E., Gu, W., McBurney, M.W., He, Z.G., 2005. A local mechanism mediates NAD-dependent protection of axon degeneration. *J. Cell. Biol.* 170, 349–355.
- Weindruch, R., Walford, R.L., 1982. Dietary restriction in mice beginning at one year of age: effect on life-span and spontaneous cancer incidence. *Science* 215, 1415–1418.
- Wood, J.G., Rogina, B., Lavu, S., Howitz, K., Helfand, S.L., Tatar, M., Sinclair, D., 2004. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 430, 686–689.
- Yoshida, A., Ashida, K., Harper, A.E., 1961. Prevention of fatty liver due to threonine deficiency by moderate caloric restriction. *Nature* 189, 917–918.
- Zhang, H.S., Zhou, Y., Wu, M.R., Zhou, H.S., Xu, F., 2009. Resveratrol inhibited Tat-induced HIV-1 LTR transactivation via NAD(+)-dependent SIRT1 activity. *Life Sci.* 85, 484–489.
- Zhao, K., Harshaw, R., Chai, X., Marmorstein, R., 2004. Structural basis for nicotinamide cleavage and ADP-ribose transfer by NAD⁺-dependent Sir2 histone/protein deacetylases. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8563–8568.