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SIRT1 prevents replicative senescence of normal human umbilical cord fibroblast through potentiating the transcription of human telomerase reverse transcriptase gene

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ABSTRACT

SIRT1, the mammalian homolog of sirtuins, has emerged as a mediator of the beneficial effects of calorie restriction. Among them, we focused on the SIRT1-induced prevention of cellular senescence, and tried to reveal the molecular mechanisms that define the effects of SIRT1. Firstly in this study, we observed that overexpression of SIRT1 resulted in the prevention of cellular senescence of normal human umbilical cord fibroblast HUC-F2 cells. Here, we focused on the human telomerase reverse transcriptase (hTERT) gene as a target of the SIRT1-induced prevention of cellular senescence. Results showed that SIRT1, SIRT1 activator, resveratrol, and SIRT1 activating condition, starved condition, increased the transcription of hTERT in HUC-F2 cells. Next, we found that SIRT1 increased hTERT transcription in a c-MYC-dependent manner, triggered the transcription of the c-MYC gene and increased the amount of c-MYC recruited to the hTERT promoter. Further, SIRT1 increased the transcriptional activation ability of c-MYC and correspondingly increased the amount of acetylated H4 histone at the hTERT promoter. All of these results indicated that SIRT1 activates hTERT transcription through the involvement of c-MYC, and suggested that this SIRT1-induced augmentation of hTERT transcription resulted in the extension of the cellular life span of HUC-F2 cells.

1. Introduction

Numerous studies have shown that a reduced-calorie diet, also known as calorie restriction (CR), promotes extension of the lifespan in various model organisms [1]. SIRT1, the mammalian homolog of sirtuins, has emerged as a mediator of the beneficial effects of CR in studies using genetically modified mice and SIRT1 activators [2]. Molecular mechanisms of SIRT1-induced effects have been studied extensively and many of the identified downstream mediators of SIRT1 were shown to exert profound effects on mammalian physiology and suppress diseases of aging [2]. In the present study, we focused on the SIRT1-induced prevention of cellular senescence.

Although there have been contradictory findings [3], researchers showed that SIRT1 can prevent cellular senescence in cell culture experiments [2], suggesting that SIRT1 have a growth-promoting activity. Actually, SIRT1 inhibition using sirtuin inhibitor, sirtinol, dominant negative type of SIRT1, SIRT1-HY, and small interfering RNA to SIRT1 mRNA induces premature senescence-like phenotypes [4,5], and overexpression of SIRT1 has been shown to promote cellular proliferation, reduce cellular senescence, and increase the cellular life span of human embryonic lung fibroblasts [6]. Further, researchers have reported that SIRT1-induced prevention of cellular senescence was provoked through the involvement of ERK/S6K1 or LKB1 [6,7]. In the present study, we tried to reveal the novel molecular mechanisms of SIRT1-induced prevention of cellular senescence.

2. Materials and methods

2.1. Cell line and a mammalian model of nutritional stress

Normal human umbilical cord fibroblasts (HUC-F2) were obtained from Riken Bioresource Center (Tsukuba, Japan). Cells were

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maintained in Dulbecco's Modified Eagle's Medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) (fed condition). Nutritional stress (starved condition) was induced by culturing cells in DMEM without glucose (D5030, Sigma, St Louis, MO) in the absence of FBS for 8 h prior to harvest unless otherwise noted, as reported previously [8]. Where indicated, cells were treated with 5 μM resveratrol (Sigma) for 12 h.

2.2. Retrovirus production and transduction

Viral supernatants were produced after transfection of 293T cells with pGag-pol, pVSV-G, and individual expression vector (pBABE-puro-SIRT1, pBABE-puro-SIRT1-HY, or mock) using the HilyMax reagent (Dojindo, Kumamoto, Japan). Cells were cultured at 37 °C in DMEM supplemented with 10% FBS for 24 h. The medium was replaced with DMEM supplemented with 2% FBS and incubated for an additional 24 h. Viral supernatant was collected and filtered using a 0.45-μm syringe filter, and then supplemented with 10% FBS and 10 μg/mL polybrene. Cells were infected with this viral supernatant for 24 h at 37 °C, and infection was repeated once to increase infection efficiency. After infection, cells were selected with 3 μg/mL puromycin for 3 days.

2.3. Senescence-associated β-galactosidase (SA-β-Gal) assay

HUC-F2 cells were seeded to a 60% confluency. The next day, cells were washed with 12 mM sodium phosphate (pH 7.2), 2.7 mM KCl, and 0.14 M NaCl solution (PBS) and fixed in 2% formaldehyde/0.2% glutaraldehyde at room temperature (RT) for 5 min. After washing with PBS, the cells were stained with 1 mg/mL X-gal in 40 mM sodium phosphate buffer, pH 6.0, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$, at 37 °C, and then observed with a phase-contrast microscope [9,10].

2.4. Promoter assay

The hTERT core promoter (−289 to −25) inserted into the firefly luciferase reporter vector pGL3-Basic (Promega, Madison, WI) was used as a reporter vector (phTERT-289) [10]. Two E-box elements in the hTERT core promoter region (−242 to −237 and −34 to −29) were changed from CACGTG to TTTGTG by site-directed mutagenesis, and inserted into pGL3-Basic (phTERTp-289EM). Luciferase reporter vector encompassing human c-MYC promoter (pDel-1-Luc) was obtained from Dr. Bert Vogelstein [11]. HUC-F2 (2.5×10^5 cells) cells were seeded onto a 24-well dish. The next day, reporter and effector constructs were transfected into the cells using the HilyMax reagent according to the manufacturer's protocol. After 48 h, luciferase assay was performed using Dual-Luciferase Reporter Assay System (Promega). To evaluate the transcriptional activation ability of c-MYC, we generated GAL4-c-MYC, which contains a full-length human c-*MYC* cDNA (amino acids 1–440) fused to the DNA-binding domain of the yeast transcription factor GAL4. Luciferase assays were performed by using this GAL4-c-MYC and pFR-Luc (Stratagene, La Jolla, CA), a luciferase reporter vector containing five GAL4-binding sites.

2.5. Adenovirus production and transduction

We produced recombinant adenovirus using the Adeno-X Expression System (Takara, Shiga, Japan) according to the manufacturer's protocol. Recombinant adenoviruses were prepared by disrupting HEK293 cells (JCRB9068; HSRRB, Osaka, Japan) transfected with recombinant adenoviral DNA including SIRT1 or SIRT1-HY cDNA. All viruses were titrated using a method that measures the

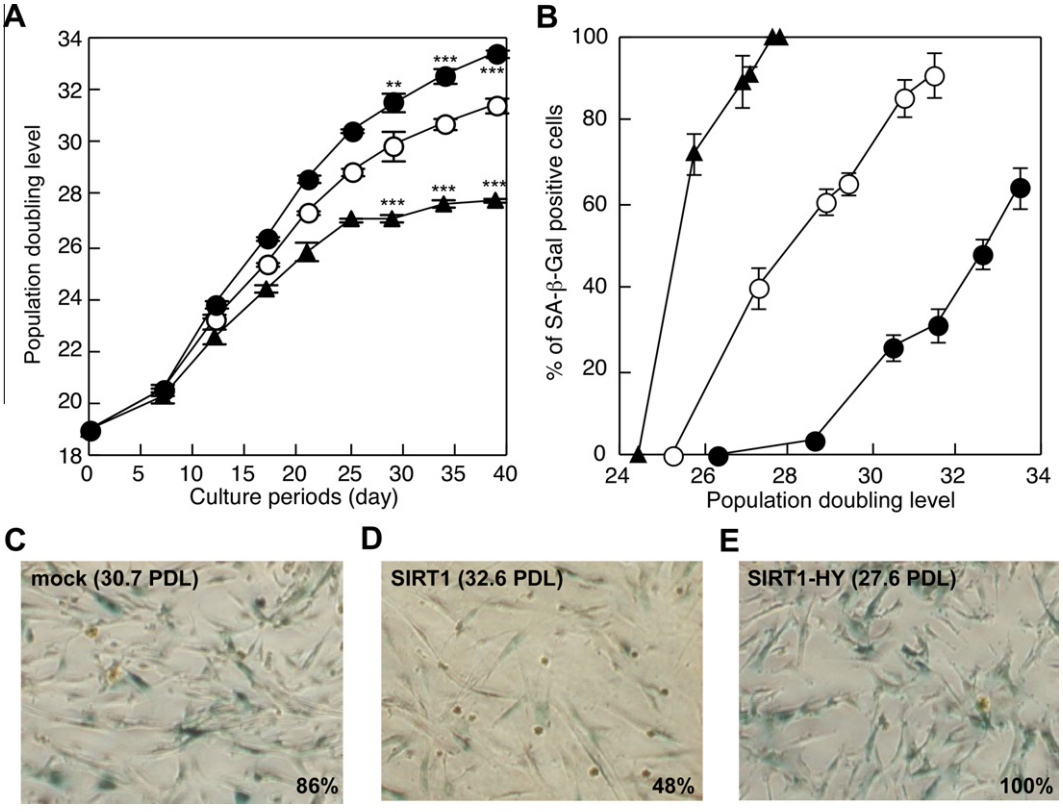

Fig. 1. Effects of SIRT1 on replicative senescence of HUC-F2 cells. After HUC-F2 cells were transduced with recombinant retrovirus for SIRT1 (●), SIRT1-HY (♦) or mock (○), proliferative potential (A) and SA-β-Gal activity (B) were monitored. After HUC-F2 cells were transduced with recombinant retrovirus for SIRT1 (D), SIRT1-HY (E) or mock (C) and cultured for 35 days, % of SA-β-Gal positive cells was assessed.

50% tissue culture infectious dose ($TCID_{50}$). HUC-F2 cells underwent adenoviral transduction after a 1-h infection at a multiplicity of infection of 50.

2.6. Quantitative RT-PCR (qRT-PCR)

RNA was prepared using the FastPure RNA kit (Takara). cDNA was prepared as described [10]. qRT-PCR was performed using SYBR Premix Ex Taq (Takara) and Thermal Cycler Dice Real Time System TP-800 instrument (Takara). PCR amplification began with a 10-s denaturation step at 95 °C and then 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 20 s, and extension at 72 °C for 20 s. The samples were analyzed in triplicate, and the hTERT, SIRT1, and c-MYC levels were normalized to the corresponding β-actin levels. The PCR primer sequences used were as follows: hTERT forward primer CGTACAGGTTTCAC GCATGTG and back primer ATGACGCGCAGGAAAAATG; human SIRT1 forward primer GCCTCACATGCAAGCTCTAGTGAC and back primer TTCGA GGATCTGTGCCAATCATAA; human c-MYC forward primer CGGAT TCTCTGCTCTCCTCGAC and back primer CCTCCAGCAGAAGGTG ATCCA; human β-actin forward primer TGGCACCCAGCACAATGAA and back primer CTAAGTCATAGTCCGCCTAGAAGCA.

2.7. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the ChIP assay kit (Millipore, Bedford, MA) as described previously [10]. In brief, cells were cross-linked using 1% formaldehyde for 10 min at RT. After washing with PBS, the cell pellet was resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL aprotinin, and 1 μg/mL pepstatin A) and incubated on ice for 10 min. Next, 100 μL of the supernatant of the sonicated chromatin solution was diluted with 900 μL of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.1), 167 mM NaCl, 1 mM PMSF, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A) and precleared by incubating with 80 μL salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4 °C. The supernatant was incubated overnight at 4 °C either with anti-c-MYC antibody (N-262; Santa Cruz Biotech., Santa Cruz, CA) or anti-acetyl-histone H4 antibody (06-866; Millipore). The immunecomplexes thus formed were collected using 60 μL of salmon sperm DNA/protein A agarose-50% slurry and eluted two times with 250 μL elution buffer (1% SDS and 0.1 M $NaHCO_3$) after extensive washings, and the crosslinkage was reversed by heating at 65 °C for 6 h, followed by treatment with 80 μg/mL proteinase K at 45 °C for 60 min. The DNA was recovered by ethanol precipitation and used as a template for PCR to amplify the target site in the hTERT promoter as described previously [10]. The target site and the corresponding primer sequences are as follows: 5′-GGCCGGGCTC CCAGTGGATTCG-3′ (−293 to −272) and 5′-CAGCGGGGAGC GCGC GGCATCG-3’ (+20 to −2). The PCR products were subjected to electrophoresis in 2% agarose gel.

2.8. Data analysis

All the experiments shown were performed independently at least three times with comparable results. All the data represented in the graphs are expressed as means ± standard error of mean. The statistical difference was determined by two-sided Student’s *t*-test. The differences were considered to be significant at $P < 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3. Results and discussion

Firstly, we evaluated the ability of SIRT1 to prevent cellular senescence by using normal human umbilical cord fibroblasts

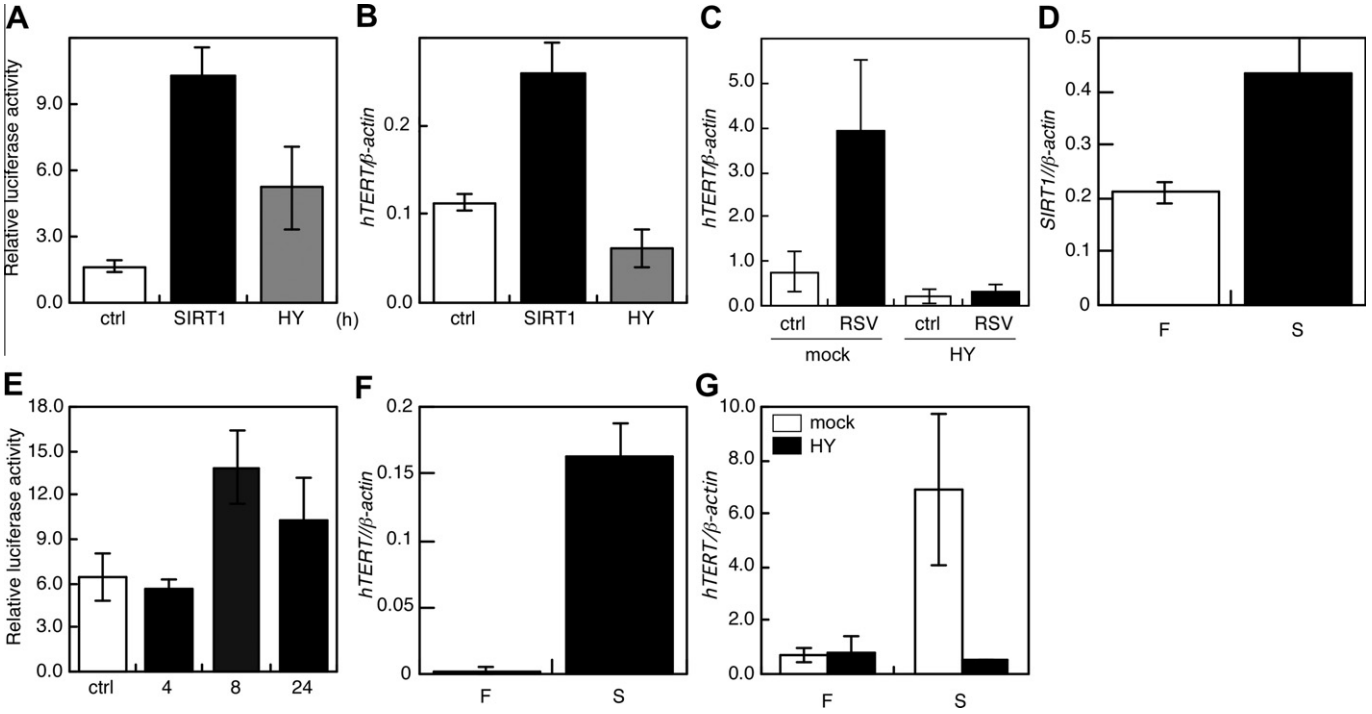

Fig. 2. SIRT1 potentiates transcription of the hTERT gene. Transcriptional activation of the hTERT gene by SIRT1 was determined by normalized luciferase activity under the control of the hTERT core promoter (A) and by qRT-PCR analysis of hTERT mRNA (B). Resveratrol (RSV) activates the transcription of hTERT gene dependently on SIRT1. HUC-F2 cells transduced with recombinant adenovirus for SIRT1-HY or mock were treated with 5 mM of RSV for 12 h. Transcription activation of hTERT gene was determined by qRT-PCR (C). HUC-F2 cells were cultured under starved condition for 8 h, and qRT-PCR analysis of SIRT1 mRNA isolated from HUC-F2 cells under fed (F) or starved (S) conditions was done (D). HUC-F2 cells were cultured under starved condition for the periods indicated (E) and for 8 h (F), normalized luciferase activity under the control of the hTERT core promoter (phTERTp-289) (E) and qRT-PCR analysis of hTERT mRNA (F) were done. (G) HUC-F2 cells transduced with recombinant adenovirus for SIRT1-HY (black bar) or mock (white bar) were cultured under fed (F) or starved (S) conditions, and qRT-PCR analysis of hTERT mRNA was carried out.

(HUC-F2). HUC-F2 cells were transduced either with recombinant retrovirus expressing SIRT1, SIRT1-HY, a dominant negative SIRT1 or with control retrovirus, and cultured for 40 days. Proliferative potential and senescence-associated β-galactosidase (SA-β-Gal) activity were monitored. Results clearly showed that SIRT1 significantly promoted proliferation and prevented replicative senescence in HUC-F2 cells (Fig. 1), as reported previously in other cell lines [6,12]. Transduction of SIRT1-HY mutant failed to exert anti-senescence function or rather inhibited the growth and induced senescence in HUC-F2 cells, indicating that the effect of SIRT1 on the prevention of replicative senescence in HUC-F2 cells was dependent on its deacetylase activity, and suggest that endogenous SIRT1 would exert anti-senescence function in HUC-F2 cells. In this study, we tried to clarify the molecular mechanisms of the SIRT1-induced prevention of replicative senescence in HUC-F2 cells. We then focused on human telomerase reverse transcriptase (hTERT) that functions in the maintenance of telomeres, and investigated whether SIRT1 extends cellular lifespan through the regulation of hTERT.

Results showed that SIRT1, but not SIRT1-HY, increased hTERT transcription, as evidenced by the promoter assay and quantitative RT-PCR (qRT-PCR) (Fig. 2A and B). Further, resveratrol (RSV), a SIRT1 activator, increased the hTERT transcription, which was alleviated by the transduction of SIRT1-HY (Fig. 2C). Next, we adopted a mammalian model of nutritional stress to activate endogenous SIRT1 by culture conditions [8], then examined the effects of nutritional withdrawal on the transcription of SIRT1 and hTERT in HUC-F2 cells. Reportedly, level of SIRT1 mRNA increased under starved condition (Fig. 2D). Further, when HUC-F2 cells were starved of both serum and glucose, hTERT promoter activity and hTERT mRNA level both increased compared with those of cells maintained in complete medium (Fig. 2E and F). In addition, the starvation-induced increase in hTERT completely disappeared following transduction with dominant negative-SIRT1 (SIRT1-HY) (Fig. 2G). All these results indicated that SIRT1 potentiates the transcription of hTERT gene in HUC-F2 cells.

We next tried to identify the molecular mechanisms of the SIRT1-induced increase in hTERT transcription. We first investigated the involvement of c-MYC, a dominant transcriptional activator for the hTERT promoter, in this increase with the promoter assay using a wild type hTERT promoter reporter vector (phTERTp-289; wt) and a mutant hTERT promoter reporter vector with disrupted E-boxes (phTERTp-289EM; mt). The results clearly showed that the SIRT1-induced increase in hTERT transcription completely disappeared with the disruption of E-boxes (Fig. 3A), thus was considered to be dependent upon c-MYC/E-box. We then investigated whether SIRT1 regulates the expression and activity of c-MYC. The results indicated that SIRT1, as well as starvation conditions, increased the promoter activity of c-MYC [11] (Fig. 3B) and the transcription of c-MYC gene (Fig. 3C). Further, we observed the increased amount of c-MYC recruited to the hTERT promoter (Fig. 3D). Next, we examined the effects of SIRT1 on the activity of c-MYC, and examined the transcriptional activation ability of c-MYC using a vector expressing c-MYC fused with the GAL4 DNA-binding domain (GAL4-c-MYC)

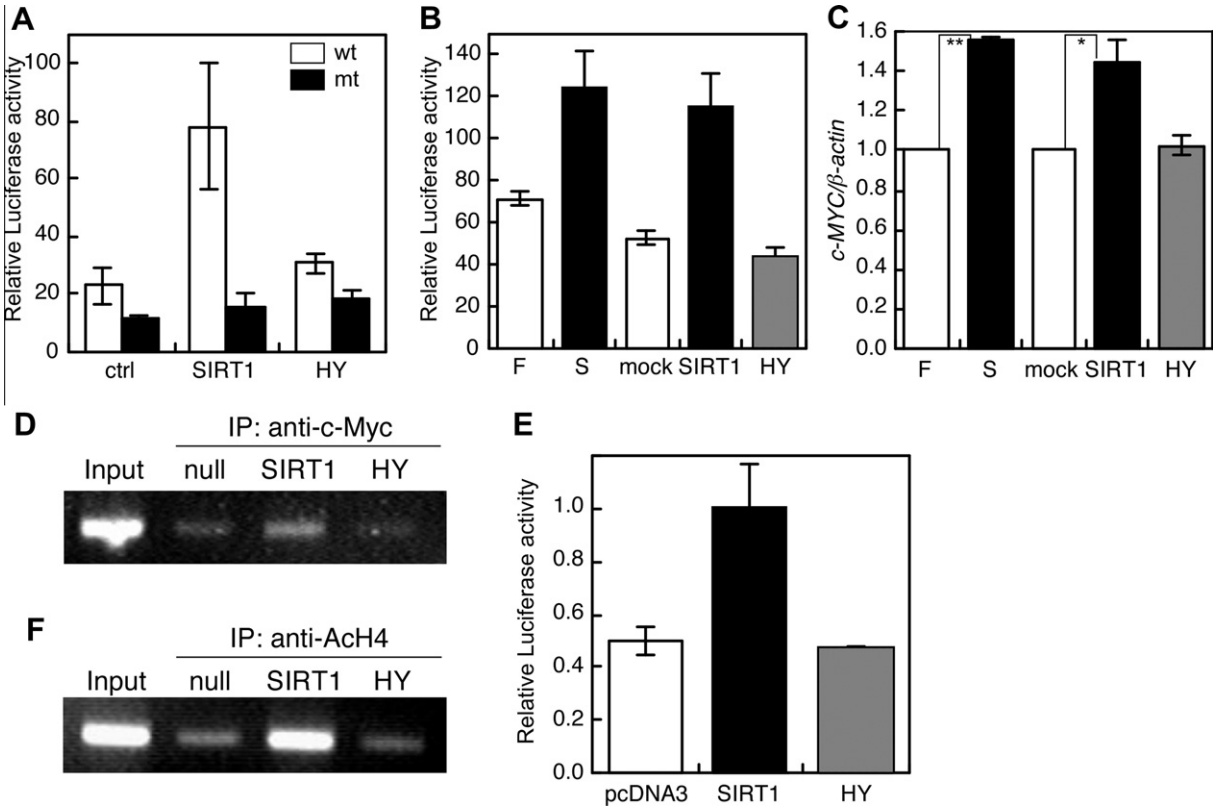

Fig. 3. Molecular mechanism for the SIRT1-induced increase in hTERT transcription. (A) c-MYC/E-box dependency of the SIRT1-induced increase in hTERT was assessed using luciferase reporters bearing a wild-type hTERT core promoter (phTERTp-289, wt; white bar) or a mutant hTERT core promoter (phTERTp-289EM, mt; black bar). (B) SIRT1 activates c-MYC transcription. HUC-F2 cells were cultured under starved condition (S) or transfected with SIRT1 or SIRT1-HY, and normalized luciferase activity under the control of the c-MYC promoter was assessed using pDel-1-Luc (He et al. [11]). (C) HUC-F2 cells were cultured under starved condition (S) or transduced with recombinant adenovirus for SIRT1 or SIRT1-HY, and qRT-PCR analysis of c-MYC mRNA was done. (D) Increased amounts of c-MYC were recruited to the hTERT core promoter. HUC-F2 cells were transduced with recombinant adenovirus for SIRT1 or SIRT1-HY. The association of c-MYC with the hTERT core promoter was assessed by the ChIP assay. (E) SIRT1 activates the transcriptional activation ability of c-MYC. GAL4-c-MYC, pFR-Luc, and the expression vector for SIRT1 or SIRT1-HY were co-transfected into HUC-F2 cells, and normalized luciferase activity was assessed. (F) SIRT1-induced histone H4 acetylation at the hTERT core promoter. HUC-F2 cells were transduced with recombinant adenovirus for SIRT1 or SIRT1-HY. The ChIP assay was performed to identify the acetylation status of histone H4 at the hTERT promoter.

in the promoter assay. The results showed that SIRT1 significantly increased the transcriptional activation ability of c-MYC (Fig. 3E). Corresponding to this result, a significant increase in the amount of acetylated H4 histone was noted at the hTERT promoter on transduction with SIRT1 (Fig. 3F). These results suggested that SIRT1 activates hTERT transcription through increasing the amount of c-MYC recruited to the hTERT core promoter and activating the transcriptional activation ability of c-MYC.

Several studies have shown using cell culture experiments that increased SIRT1 expression in human fibroblasts promotes cellular proliferation, reduces cellular senescence, and increases the lifespan of human embryonic lung fibroblasts [6]. Although several causes have been attributed to this SIRT1-induced phenomenon, our study is the first to reveal that SIRT1 potentiates transcription of the hTERT gene, which might be a mechanism of SIRT1-induced prevention of cellular senescence and extension of the cellular lifespan of HUC-F2. However, there exist contradictory reports about the effects of SIRT1 on the cellular senescence, suggesting that may be associated with cell-type-specific context and different molecular mechanisms involved. We would like to clarify the molecular basis causing the differential effects of SIRT1 in the future study.

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