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SIRT1 markedly extends replicative lifespan if the NAD+ salvage pathway is enhanced

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ABSTRACT

Sir2 mediates lifespan extension in lower eukaryotes but whether its mammalian homolog, sirtuin 1, silent mating type information regulation 2 homolog (SIRT1), is a longevity protein is controversial. We stably introduced the SIRT1 gene into human vascular smooth muscle cells (SMCs) and observed minimal extension of replicative lifespan. However, SIRT1 activity was found to be exquisitely dependent on nicotinamide phosphoribosyltransferase (Nampt) activity. Moreover, overexpression of Nampt converted SIRT1-overexpressing SMCs to senescence-resistant cells together with heightened SIRT1 activity, suppressed p21, and strikingly lengthened replicative lifespan. Thus, SIRT1 can markedly postpone SMC senescence, but this requires overcoming an otherwise vulnerable nicotinamide adenine dinucleotide salvage reaction in aging SMCs.
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1. Introduction

Silent information regulator 2 (*Sir2*) is a protein deacetylase that mediates the lifespan-extending actions of caloric restriction in yeast [1]. In addition, extra copies or increased expression of the *Sir2* gene extend the lifespan of yeast, worms, and flies [2–4]. The mammalian sirtuin with the highest sequence similarity to Sir2 is sirtuin 1, silent mating type information regulation 2 homolog (SIRT1), a predominantly nuclear enzyme that is essential for mammalian development [5]. SIRT1 can modify chromatin-associating proteins, transcription factors, and coregulators and, in so doing, controls the expression of key regulators of cell defense, metabolism, and survival [6]. Accordingly it might be expected that, like Sir2 in lower organisms, SIRT1 is a mammalian longevity protein. However, the capacity of SIRT1 to extend the replicative lifespan of mammalian cells is uncertain and there have been contradictory findings. In a seminal report, Langley et al. found that SIRT1 overexpression in mouse embryo fibroblasts inhibited promyelocytic leukemia protein-induced senescence [7]. Likewise, reduced SIRT1 activity in MCF-7 cells and endothelial cells has been reported to induce senescence-like growth arrest [8,9]. In contrast, Michishita and coworkers found that replicative lifespan of human fibroblasts was unaffected by SIRT1 overexpression [10] and SIRT1 has been found to suppress telomerase activity in telomerase-immortalized cells and hematopoietic stem cells [11]. Further confounding the question of a potential longevity role of SIRT1 is the observation that SIRT1-deficient mouse embryonic fibroblasts have extended, rather than shortened, replicative lifespan [12].

SIRT1 is a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase and is thus linked to NAD+ metabolism. Nicotinamide phosphoribosyltransferase (Nampt, also known as PBEF and visfatin) catalyzes a rate-limiting step in the NAD+ salvage pathway that generates nicotinamide mononucleotide from nicotinamide [13]. Interestingly, increased Nampt expression has been show to extend the lifespan of human smooth muscle cells (SMCs) by increasing SIRT1-dependent p53 deacetylation [13]. However, there are several pathways by which NAD+ can be made available in mammalian cells and whether intracellular Nampt is absolutely required for a given action of SIRT1 is uncertain. Moreover, if there is such a requirement, it is worth recognizing that Nampt is not an invariantly expressed enzyme. Instead, its abundance can be altered by serum factors, cytokines, and circadian cycles [14–16]. Furthermore, and particularly important to the question of lifespan, Nampt activity declines as cells approach senescence [13].

We report here that overexpressing SIRT1 alone in human vascular SMCs has minimal effect on replicative longevity but that SIRT1 can strikingly extend lifespan if the aging-related decline in the NAD+ salvage pathway can be overcome.

Abbreviations: NAD+, nicotinamide adenine dinucleotide; Nampt, nicotinamide phosphoribosyltransferase; SIRT1, sirtuin 1, silent mating type information regulation 2 homolog; SMC, smooth muscle cell; TSA, trichostatin A

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2. Methods

2.1. Cell culture

Experiments were performed using primary human SMCs and the HITC6 clonal SMC line, both derived from segments of human internal thoracic artery [17,18]. To quantify replication, cells were seeded at a density of 4500 cells/cm^2 and population doublings were calculated using the formula: Log_{10}[# cells harvested] − (Log_{10}[# cells seeded]/Log_{10}[2]).

2.2. Western blot analysis

Protein expression was assessed by immunoblot analysis of lysates of confluent SMCs, as described [19]. Primary antibodies included mouse monoclonal antibodies against human p53 (DO-1, Santa Cruz Biotechnology), human p21 (DCS-60, Santa Cruz Biotechnology), and α-tubulin (B512, Sigma), and polyclonal antibodies raised against human SIRT1 (ab13749, Abcam) and human Nampt (BL2122, Bethyl Laboratories). Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG Fab (Amersham Biosciences).

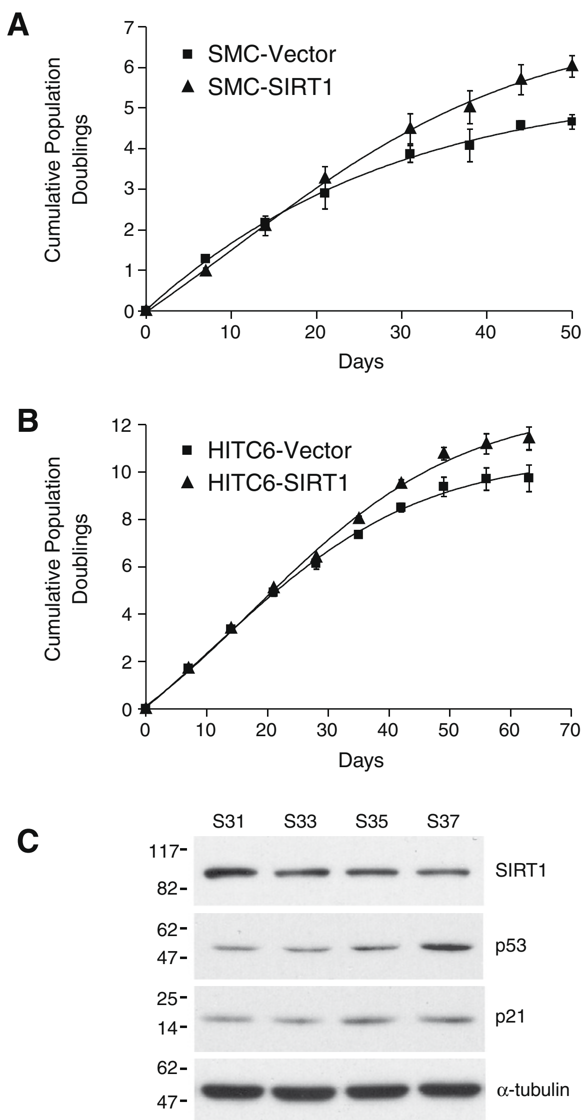

Fig. 1. SIRT1 minimally extends human SMC lifespan. Cumulative population doublings of primary SMCs (A) and HITC6 SMCs (B) infected with retrovirus containing vector (pQCXIP) or vector with cDNA encoding human SIRT1. Graphs depict the averaged results from three longevity assessments and curves were fit using non-linear regression. (C) Western blots showing decreasing SIRT1 abundance and increasing p53 and p21 expression in HITC6 SMCs serially subcultured to senescence (S = subculture).

2.3. Overexpression of SIRT1 and Nampt in human SMCs

Stable overexpression of SIRT1 and Nampt in SMCs was carried out using retrovirus-mediated gene delivery, as previously described [19,20]. Retrovirus containing pQCXIP-Nampt-IRES-PURO or pQCXIP-IRES-PURO (Clontech Laboratories) was generated by calcium phosphate-mediated transfection of the Phoenix amphotropic retrovirus packaging cell line (ATCC). Stable transductants were selected with 3 μg/ml puromycin. SIRT1 was similarly transduced using the pQCXIN retroviral expression vector and selected with 400 μg/ml G418.

2.4. Senescence-associated beta-galactosidase activity

Senescence-associated β-galactosidase (SA-β-gal) activity was determined in SMCs fixed with 2% formaldehyde/0.2% glutaraldehyde for 3 min and by staining for β-galactosidase activity at pH 6.0, as described [21]. SMCs were counterstained with 2.5 μg/ml Hoechst33258 and the proportion of SA-β-gal activity-positive cells was quantified using light and fluorescence microscopy.

2.5. Assessment of SIRT1 and Nampt activity

SIRT1 deacetylase activity was quantified in live cells as reported [22] using 25 μM Fluor-de-Lys-SIRT1 fluorogenic substrate (Biomol) and quantifying reaction product in the media by spectrofluorometry and normalizing to cell protein content. Nampt activity was determined in SMC lysates in 10 mM NaH_2PO_4/Na_2HPO_4, pH 8.8, by quantifying the conversion of [carbonyl-^{14}C]nicotinamide to acetone-precipitable nicotinamide mononucleotide, as described [23].

2.6. Quantitative real-time RT-PCR

Transcript abundance was quantified by real-time RT-PCR. A TaqMan-based primer/probe set (Applied Biosystems) was used to detect human p21 (Hs00355782_m1) and a primer/probe set for p16 was customized using the exon 1–2 boundary sequence. Values were normalized to the endogenous reference GAPDH (Hs00266705_g1).

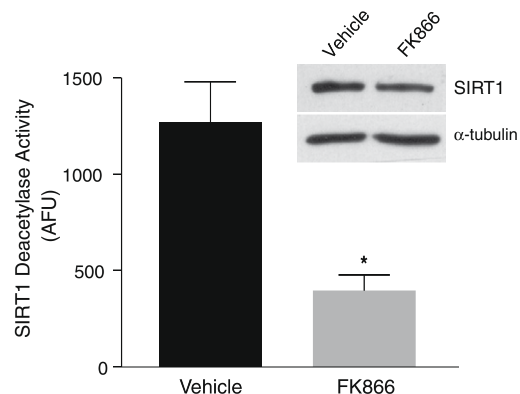

Fig. 2. SIRT1 deacetylase activity in SMCs is dependent on Nampt. SIRT1 activity in HITC6 SMCs incubated with the Nampt antagonist FK866 (10 nM) or vehicle for 48 h was assessed based on NAD^+-dependent deacetylation of an acetylated peptide substrate in the presence of 1 μM trichostatin A. SIRT1 protein expression was unaffected (inset) (*P < 0.005 vs. vehicle).

3. Results

3.1. Introducing SIRT1 into human SMCs trivially extends replicative lifespan

To determine if the replicative lifespan of human vascular SMCs was impacted by enhanced expression of SIRT1, we infected proliferating human SMCs with retrovirus containing SIRT1 cDNA. Population doublings of stable transductants were tracked until proliferation ceased, a transition concurrent with accumulation of flattened and enlarged SMCs indicative of senescence. In primary cultures of adult SMCs, which are generally short-lived, there was a modest (1.30 ± 0.93-fold, $P < 0.01$) increase in cumulative population doublings in SIRT1-overexpressing cells (SIRT1–SMCs) compared to vector-infected SMCs (Fig. 1A). In a clonal line of non-immortal but longer-lived human SMCs [14,17,18], popula-

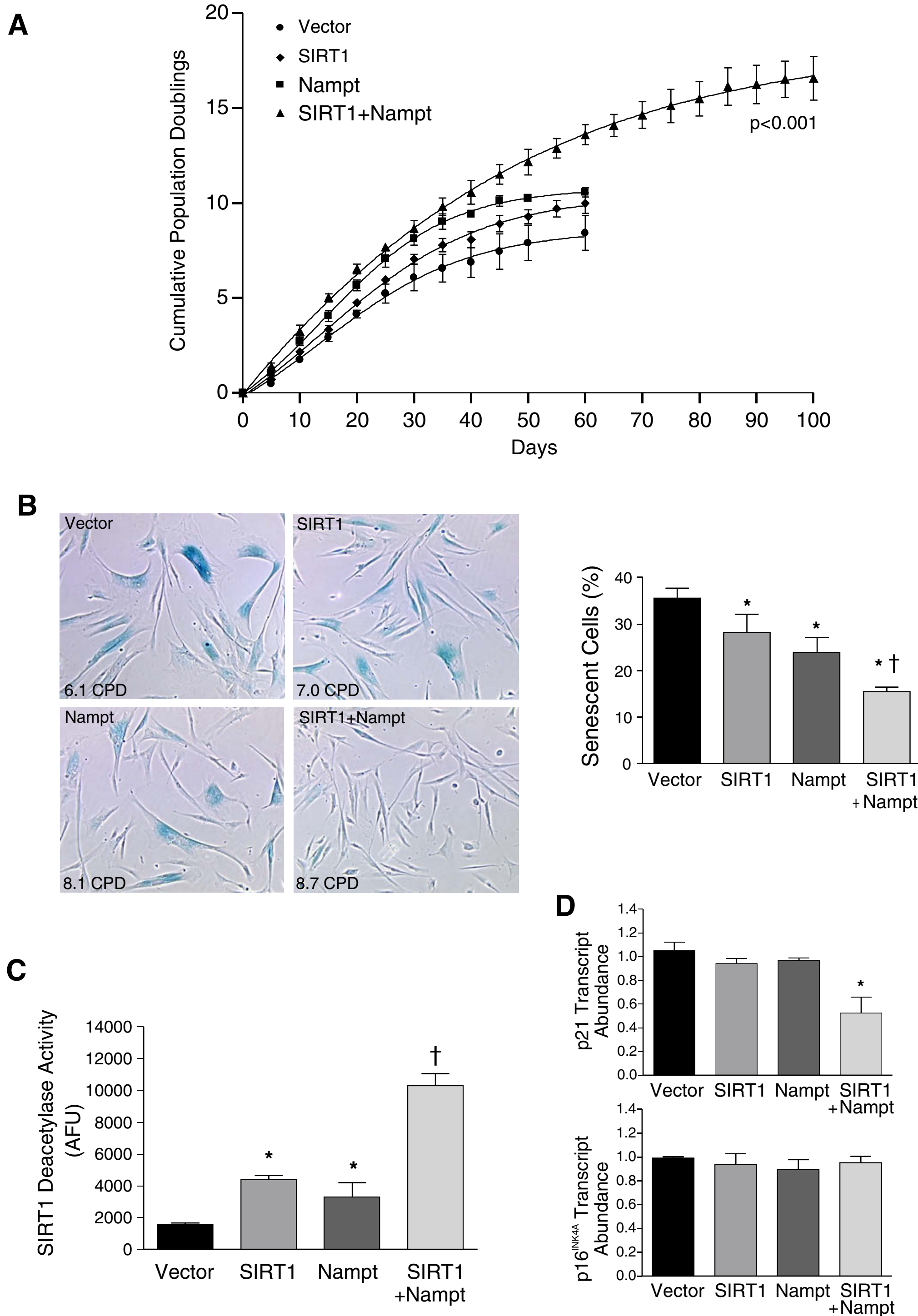

Fig. 3. Marked extension of human SMC lifespan by SIRT1 and Nampt. (A) Population doubling of HITC6 SMCs transduced with retrovirus containing control vectors, or cDNA encoding SIRT1, Nampt, or both SIRT1 and Nampt. The pronounced increase in replicative lifespan in SIRT1–Nampt SMCs corresponds to a substantial decrease in SMC senescence (B), determined after 35 days by staining for SA-β-gal activity at pH 6.0 ($^*P < 0.05$ vs. vector, $^{\dagger}P < 0.05$ vs. SIRT1 and Nampt). (C) Graph showing amplification of SIRT1 activity by Nampt ($^*P < 0.05$ vs. vector, $^{\dagger}P < 0.001$ vs. vector, SIRT1, and Nampt). (D) Graphs depicting abundance of p21 and $p16^{INK4A}$ mRNA in human SMCs, assessed by quantitative real-time RT-PCR ($^*P < 0.05$ vs. vector, SIRT1, and Nampt).

tion doubling of SIRT1–SMCs also increased trivially (1.17 ± 0.03-fold, $P < 0.05$) (Fig 1B). To determine if the reason for the minimal effect on replicative lifespan was simply because endogenous SIRT1 was already abundant, we serially tracked SIRT1 expression in replicating, non-transduced SMCs. In fact, over six subcultures SIRT1 content fell by 44 ± 13%, with a reciprocal rise in p53 and p21, two pro-senescence proteins whose expression is repressed by SIRT1-mediated deacetylation (Fig. 1C) [24]. Thus in aging SMCs with otherwise declining SIRT1 content, introducing the SIRT1 gene had little effect on replicative longevity.

3.2. SIRT1 activity in SMCs depends on Nampt

The negligible effect of overexpressing SIRT1 on SMC longevity could indicate either that SIRT1 is not a longevity protein for SMCs or that the metabolic conditions for its activity were suboptimal. Thus, we next determined if SIRT1 activity was dependent on Nampt, an enzyme that increases NAD^+ and clears nicotinamide, using the specific Nampt antagonist, FK866 [25]. FK866 reduced Nampt activity to 0.11 ± 0.05 of basal level ($P < 0.001$). This had no effect on SIRT1 expression. However, SIRT1 activity fell to 28% of baseline (Fig. 2). Thus, endogenous SIRT1 activity in SMCs is highly dependent on Nampt activity.

3.3. Pronounced extension of human SMC longevity by paired overexpression of Nampt and SIRT1

Given this, we next determined whether the only modest longevity effect of overexpressed SIRT1 would still be the case if Nampt was also overexpressed. As shown in Fig. 3A, the effect of combined overexpression was striking – replicative lifespan of Nampt/SIRT1-SMCs was double that of control SMCs (1.97 ± 0.24-fold increase) and 66% greater (1.66 ± 0.20-fold) that of SMCs overexpressing SIRT1 alone ($P < 0.001$). To verify that the altered lifespan was related to senescence inhibition, cells were stained for SA-β-gal activity. This revealed that the proportion of senescent cells in cultures double-overexpressing SIRT1 and Nampt was significantly lower than that for either SIRT1–SMCs or Nampt–SMCs ($P < 0.05$) (Fig. 3B). In addition, whereas SIRT1–SMCs had a 2.8-fold increase in SIRT1 activity over baseline, double transductants had a 7.0-fold increase SIRT1 activity (Fig. 3C). When non-transduced SMCs were supplemented with 50 μM nicotinamide mononucleotide, a product of Nampt action and immediate precursor to NAD, SIRT1 activity increased by 2.1-fold, supporting NAD^+ content as a limiting factor for SIRT1 activity in SMCs. We also found that Nampt-SIRT1 SMCs expressed significantly less p21, a downstream effector of SIRT1 deacetylase activity (Fig 3D, $P < 0.05$). Interestingly, expression of $p16^{INK4A}$ was not affected by Nampt/SIRT1 overexpression, suggesting that a Nampt-SIRT1 axis does not regulate expression of this particular longevity regulator.

4. Discussion

The findings reported here establish that increasing expression of SIRT1 in aging SMCs has little effect on SMC lifespan, despite an otherwise aging-related decline in SIRT1 expression. However, replicative lifespan of SMCs can be extended markedly by SIRT1 if Nampt, the rate-limiting enzyme for NAD^+ salvage, is also overexpressed. This striking synergy is related to the dependence of SIRT1 on Nampt for its deacetylase activity. Therefore, SIRT1 can be considered a lifespan regulator for SMCs but this action may ultimately depend on the metabolic milieu in which SIRT1 operates.

NAD^+ is essential for SIRT1 activity because it supplies the ADP ribose moiety necessary to accept the acetyl group from the SIRT1-targeted protein [26]. Whether NAD^+-generating enzymes besides Nampt are equally important to SIRT1 activity, particularly when SIRT1 is overexpressed, is not known. However, the linkage between Nampt and SIRT1 may be particularly important given that Nampt both generates NAD^+ and clears nicotinamide, the latter an inhibitor of SIRT1 that is hydrolytically released from NAD^+ as part of the deacetylation reaction. Recent evidence has also shown that the downstream consequences of different NAD^+ biosynthetic enzymes can differ, even for enzymes in the same linear pathway, a finding that could reflect differential localization or microdomains [27]. Regardless, the findings that SIRT1 in SMCs requires Nampt, and that Nampt can be a vulnerable enzyme that declines as SMCs age [13], highlight the importance of considering Nampt activity when studying SIRT1-mediated responses.

Our findings may explain the apparently discrepant published results regarding SIRT1 and mammalian cell longevity with reports indicating that SIRT1 inhibits senescence [7–9], promotes senescence [11,12], or has no effect on senescence at all [10]. We propose that these variable findings may, at least in part, reflect the vulnerability of Nampt and differences in the efficiency with which nicotinamide is converted to NAD^+. Augmenting SIRT1 expression in cells with limited capacity to salvage NAD^+ from nicotinamide may have little, if any, effect on SIRT1-mediated processes. In contrast, there may be a strong effect if endogenous Nampt activity remains robust.

In summary, these data establish that the actions of SIRT1 must be considered in the context of the prevailing, and aging-sensitive, metabolic pathways that regulate the biosynthesis of NAD^+. This may be particularly important as therapies designed to exogenously activate SIRT1 are pursued. Senescent SMCs can destabilize the artery wall because they are proinflammatory and lose their capacity to proliferate [28]. Strategies that enhance both SIRT1 content and Nampt activity may hold promise for stabilizing diseased arteries and other aging tissues.

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