Sirt1 Regulates Aging and Resistance to Oxidative Stress in the Heart

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Abstract—Silent information regulator (Sir)2, a class III histone deacetylase, mediates lifespan extension in model organisms and prevents apoptosis in mammalian cells. However, beneficial functions of Sir2 remain to be shown in mammals in vivo at the organ level, such as in the heart. We addressed this issue by using transgenic mice with heart-specific overexpression of Sirt1, a mammalian homolog of Sir2. Sirt1 was significantly upregulated (4- to 8-fold) in response to pressure overload and oxidative stress in nontransgenic adult mouse hearts. Low (2.5-fold) to moderate (7.5-fold) overexpression of Sirt1 in transgenic mouse hearts attenuated age-dependent increases in cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers. In contrast, a high level (12.5-fold) of Sirt1 increased apoptosis and hypertrophy and decreased cardiac function, thereby stimulating the development of cardiomyopathy. Moderate overexpression of Sirt1 protected the heart from oxidative stress induced by pararquat, with increased expression of antioxidants, such as catalase, through forkhead box O (FoxO)-dependent mechanisms, whereas high levels of Sirt1 increased oxidative stress in the heart at baseline. Thus, mild to moderate expression of Sirt1 retards aging of the heart, whereas a high dose of Sirt1 induces cardiomyopathy. Furthermore, although high levels of Sirt1 increase oxidative stress, moderate expression of Sirt1 induces resistance to oxidative stress and apoptosis. These results suggest that Sirt1 could retard aging and confer stress resistance to the heart in vivo, but these beneficial effects can be observed only at low to moderate doses (up to 7.5-fold) of Sirt1. (Circ Res. 2007;100:1512-1521.)

Key Words: Sirt1 ■ aging ■ longevity factor ■ oxidative stress

Extrinsic and intrinsic factors cooperate in determining the rate of aging and the aging phenotype. Because aging reduces the function of organs and increases the risk of diseases, elucidating the mechanisms controlling aging has significant clinical implications.1 Increasing lines of evidence suggest that evolutionarily conserved molecular mechanisms are involved in the regulation of lifespan in animals.2 For example, caloric restriction prolongs the lifespan of organisms, from yeast to primates,3 possibly through silent information regulator 2 (Sir2)-dependent mechanisms.4,5 Genetic alterations causing metabolic effects similar to caloric restriction, including mutation/deletion of Cyr1/Sch9, also cause lifespan extension in yeast.6–8 Similarly, inhibition of calorie-unlike growth factor (IGF)-I signaling, including Daf-2 mutation in Caenorhabditis elegans, causes lifespan extension9 mediated by a loss of suppression of Daf-16 or forkhead box O (FoxO) transcription factors, which regulate antioxidant expression and DNA damage repair (GADD45).7,10,11 In mammals, Ames and Snell dwarf mice lacking GH/IGF-I signaling and IGF-I receptor heterozygous knockout mice have longer lifespans,12 and systemic overexpression of klotho, a hormone known to inhibit insulin/IGF-I signaling, extends lifespan in mice.13 Another important mechanism controlling aging is oxidative stress.1 Overexpression of antioxidant molecules, including mitochondrial catalase and thioredoxin,14,15 induces lifespan extension. Mice deficient in either mclk1 or p66shc16, both of which are involved in mitochondrial electron transfer, have longer lifespans.16,17 Importantly, these longevity factors also confer stress resistance to the organism,18 the accumulation of which leads to longer lifespan.

Yeast Sir2, an NAD+-dependent protein deacetylase and a founding member of the sirtuin family,19 functions in a wide array of cellular processes, including gene silencing, longevity, muscle differentiation, and DNA damage repair (reviewed elsewhere20). Stimulation of Sir2 by overexpression or sirtuin-activating compounds, such as resveratrol, is sufficient to induce prolonged lifespan in yeast, Caenorhabditis elegans, Drosophila melanogaster,21,22 and mice.23 Sirt1, a mammalian ortholog of Sir2, provides protection against apoptosis and plays an essential role in mediating survival of cardiac myocytes and neurons under stress in vitro.24–26
Sirt1-deficient mice rarely survive postnatally and exhibit developmental abnormalities in several organs, including the heart.27,28 Pancreatic β-cell–specific overexpression of Sirt1 enhances ATP production, thereby enhancing insulin secretion in response to glucose in transgenic mice, suggesting that Sirt1 potentially improves glucose metabolism.29 Judging from the generally cell-protective function of Sir2 in model organisms, Sirt1 may prevent aging and play a protective role in mammalian cells in vivo. Importantly, however, it remains to be shown, without relying on pharmacological interventions, that specific activation of Sirt1 confers antiaging and stress-resistance benefits to mammalian cells in vivo. In fact, recent evidence suggests that nonreplicating yeast cells without Sir2 exhibit greater stress resistance in extremely long-lived yeast mutants,30 suggesting that Sir2 could make cells more prone to stress under some conditions. Furthermore, overexpression of the Sir2 family proteins does not extend replicative lifespan in normal human fibroblasts or prostate epithelial cells31 but rather promotes replicative senescence in response to chronic cellular stress via a p19ARF-dependent mechanism in mouse embryonic fibroblasts.32 These reports suggest that the molecular mechanism mediating lifespan extension in lower organisms may not work as expected in higher organisms. Thus, it is important to examine whether Sir2 is able to retard in vivo aging of mammalian organs, such as the heart, the major component of which is terminally differentiated cardiac myocytes.

To elucidate the in vivo function of Sirt1 in the heart, we have recently generated transgenic mice with cardiac specific overexpression of Sirt1 (Tg-Sirt1). The central hypothesis in this study was that Sirt1 mediates antiaging and cell-protective effects in the heart in vivo.

Materials and Methods
For an expanded Materials and Methods section, refer to the online data supplement at http://circres.ahajournals.org. Tg-Sirt1 mice were generated on an FVB background using the α-myosin heavy chain (α-MHC) promoter. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School. ATP content and citrate synthase (CS) activity were determined, whereas that of natriuretic factor, atrial natriuretic factor, and β-MHC were regulated, whereas that of α-MHC was significantly upregulated in Tg-Sirt1 line 39 or 40. In contrast, expression of β-MHC, atrial natriuretic factor, and α-skeletal actin was significantly upregulated, whereas that of α-MHC was significantly down-

Results
Sirt1 Is Upregulated in Response to Stress in the Heart
We have shown that expression of Sirt1 is upregulated in the dog heart during heart failure.24 We examined whether Sirt1 is upregulated in response to stresses. In mice, the level of Sirt1 in the heart was significantly upregulated after 2 and 4 weeks of pressure overload (5.5- and 8.8-fold) and by paraquat injection (4.3-fold), which induces oxidative stress33 in the heart (Figure 1A). Expression of Sirt1 in the heart was also significantly greater (2.9-fold) in old (20.8 years) than in young (6.1 years) monkeys (Figure 1B).

Figure 1. The effect of stress (A) and aging (B) on expression of Sirt1 in the heart. Heart homogenates were prepared from mice subjected to transverse aortic constriction (TAC) for 2 or 4 weeks or paraquat (PQ) injection for 2 weeks (A), or from young and old monkeys (B), and subjected to immunoblot analyses with anti-Sirt1 and anti-actin or anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. Results are representative of 3 to 5 experiments. In the bar graph, expression of Sirt1 normalized by actin without stress (A) or GAPDH (B) in young monkeys was expressed as 1. *P<0.05 vs control.

Basal Characterization of Tg-Sirt1
To examine the function of Sirt1 in the adult heart in vivo and to elucidate the role of Sirt1 upregulation during stress and aging, we generated Tg-Sirt1 using the α-MHC promoter. We generated 3 lines (lines 39, 40, and 53) of Tg-Sirt1 with different levels of Sirt1 in the heart (2.5-, 7.5-, and 12.5-fold increase more than nontransgenic [NTg] mice, respectively) (Figure 2A). Immunoblot analyses using homogenates from multiple organs confirmed that Sirt1 is overexpressed in a heart-specific manner (Figure 2B). Initial characterization of cardiac phenotype was conducted using mice aged 6 months old. Postmortem analyses showed that Tg-Sirt1 lines 39 and 40 had normal heart size, whereas the Tg-Sirt1 line 53 showed an enlarged left atrium and left ventricle (LV) (Figure 2C). LV enlargement in line 53 was confirmed by echocardiographic measurement of the LV end-diastolic dimension (LVEDD) (supplemental Table I). Baseline LV weight (LVW)/body weight and LVW/tibial length (LVW/TL) ratios, indices of cardiac hypertrophy, in Tg-Sirt1 lines 39 and 40 were normal, whereas those in the Tg-Sirt1 line 53 were significantly greater than those in NTg (supplemental Table II). There was no significant change in the level of hypertrophy-associated (“fetal type”) gene expression in Tg-Sirt1 line 39 or 40. In contrast, expression of α-MHC, atrial natriuretic factor, and α-skeletal actin was significantly upregulated, whereas that of α-MHC was significantly down-

Statistical analyses between groups were done by 1-way ANOVA, and when probability values were significant, differences among groups were evaluated using a post hoc test with Bonferroni’s correction. A probability value of <0.05 was considered significant.
regulated in Tg-Sirt1 line 53 (Figure 2D). These results indicate that high levels of Sirt1 overexpression induce cardiac hypertrophy in vivo.

To assess whether increased expression of Sirt1 alters baseline cardiac function, we performed echocardiographic analyses. Cardiac chamber size and cardiac function were normal in Tg-Sirt1 lines 39 and 40 (supplemental Table I). In Tg-Sirt1 line 53, however, the LV was significantly dilated and both LV ejection fraction (LVEF) and percentage fractional shortening were significantly reduced compared with those in NTg. Hemodynamic measurements indicated that LV end-diastolic pressure was significantly elevated, whereas the rates of contraction (dP/dt) and relaxation (−dP/dt) were significantly reduced in Tg-Sirt1 line 53 (supplemental Table III). The lung weight/body weight ratio was normal in Tg-Sirt1 lines 39 and 40, but significantly elevated, suggesting lung congestion, in line 53 (supplemental Table II). These results suggest that 2.5- to 7.5-fold overexpression of Sirt1 does not affect baseline cardiac chamber size or cardiac function, whereas 12.5-fold overexpression of Sirt1 induces LV chamber dilation, hypertrophy, and dysfunction, thereby mimicking cardiomyopathy.

Histological analyses of LV myocardial sections indicated that there was no significant fibrosis in Tg-Sirt1 line 39 or 40 at 6 months of age (Figure 3A and 3B). Tg-Sirt1 line 53 mice, however, showed a significantly increased level of LV fibrosis compared with NTg (Figure 3A and 3B). TUNEL analyses at 6 months of age showed that the frequency of TUNEL-positive nuclei was significantly lower in Tg-Sirt1 lines 39 and 40, whereas it was significantly elevated in Tg-Sirt1 line 53, compared with NTg, suggesting that myocardial apoptosis is suppressed by mild to modest overexpression of Sirt1 but is enhanced by a high level of Sirt1 overexpression (Figure 3C).

Figure 2. Baseline characterization of Tg-Sirt1 mice at 6 months of age (lines 39, 40 and 53). A, Representative immunoblots of heart homogenates with anti-Sirt1 and anti-actin antibodies. Note that endogenous Sirt1 in NTg mice was easily detected after longer exposure of the x-ray film. B, Tissue homogenates were prepared from multiple organs in Tg-Sirt1. Immunoblots with anti-Sirt1 and anti-GAPDH are shown. Sk muscle indicates skeletal muscle; LV, left ventricle. C, Gross morphology of the hearts. D, mRNA expression of β-MHC, atrial natriuretic factor (ANF) α-MHC, and α-skeletal actin (ASA). Expression of fetal type gene normalized by that of GAPDH in NTg was expressed as 1. Each column represents the mean from 5 mice.

Mild to Moderate Overexpression of Sirt1 Retards Age-Dependent Changes in the Heart

Because Sirt1 is overexpressed in a heart-specific manner in Tg-Sirt1, overexpression of Sirt1 may not be sufficient to extend the lifespan of the animals. Nonetheless, we followed up the lifespan of Tg-Sirt1 mice for more than 600 days. Kaplan–Meier analysis indicated that there was no significant difference in mortality in line 39 or 40 compared with NTg mice. Tg-Sirt1 line 53 mice die significantly earlier than NTg and rarely survived more than 250 days (Figure I in the online data supplement).

Increases in hypertrophy, interstitial fibrosis, and apoptosis are commonly observed as age-dependent changes in the heart.34–36 To examine whether Sirt1 overexpression affects
the progression of aging in the heart, we conducted histopathological analyses by using all 3 lines of Tg-Sirt1 at mean ages of 2 to 3, 6 to 7, and 18 months. Cardiac myocyte size and LVW/TL in Tg-Sirt1 line 53 were significantly greater than those in NTg at 2 to 3 months, and both parameters in line 53 were even greater at 6 to 7 months. Tg-Sirt1 lines 39 and 40 showed smaller increases in cell size and LVW/TL by age, and their cell size at 18 months was significantly smaller than that of NTg (Figure 4A and supplemental Figure II). Similar results were obtained regarding age-dependent increases in fibrosis and myocyte apoptosis (Figure 4B and 4C and supplemental Figure II). The same trend was also observed in age-dependent decreases in cardiac function. LVEDD and LV end systolic dimension (LVESD) in Tg-Sirt1 line 53 were significantly greater than those in NTg at 6 to 7 months. LVEDD and LVESD in NTg were greater at 18 months than at 2 to 3 and 6 to 7 months, whereas LVEDD and LVESD in Tg-Sirt1 lines 39 and 40 were significantly smaller than those in NTg at 18 months (Figure 5A and 5B). Tg-Sirt1 line 53 mice showed significantly lower LVEF and percentage fractional shortening than NTg starting from 2 to 3 months. NTg mice showed significantly lower LVEF and percentage fractional shortening at 18 months than at 2 to 3 and 6 to 7 months, whereas lines 39 and 40 mice exhibited significantly greater LVEF and percentage fractional shortening than NTg at 18 months (Figure 5C and 5D). Taken together, although high levels of Sirt1 expression stimulate the development of cardiomyopathy, mild to moderate expression of Sirt1 retards aging-induced histological changes and LV dysfunction in the heart.

**Moderate Overexpression of Sirt1 Stimulates Cell-Protective Molecules and Inhibits Expression of Aging Markers**

Expression of the INK4/ARF family proteins, inducers of cellular senescence, increases with advancing age in many organs in rodents, including the heart. Protein levels of p15INKB and p19ARF were significantly lower in Tg-Sirt1 lines 39 and 40, but not in line 53, than in NTg at 2 to 3 and 8 to 12 months of age (Figure 6A and 6B). A similar result was obtained regarding expression of p53, another regulator of senescence (Figure 6C). Expression of Bcl-2 and Bcl-xL, both antiapoptotic molecules, was elevated in Tg-Sirt1 lines 39, 40, and 53, with the greatest expression found in line 40 (Figure 6D and data not shown).

To identify potential targets of Sirt1 that might mediate its antiaging effects, DNA microarray analyses were conducted. (The data have been deposited in the Gene Expression Omnibus [GEO] database, www.ncbi.nlm.nih.gov/geo; accession no. GSE7407.) Preliminary results indicated that some cell-protective molecules and potential regulators of aging are upregulated in Tg-Sirt1 line 40 mice. These
include heat shock protein (Hsp)40, Hsp70, Hsp90, telomere reverse transcriptase, telomere repeat binding factor2, Klotho, and Werner syndrome protein (supplemental Figure III).

Tg-Sirt1 Line 53 Mice Have Lower Cardiac ATP Content

Because Tg-Sirt1 line 53 exhibited reduced cardiac function at baseline, we examined potential mechanisms. Although the cardiac ATP content in Tg-Sirt1 line 40 was not significantly different from NTg, Tg-Sirt1 line 53 exhibited significantly lower cardiac ATP content than NTg at 2 to 3 months of age (Figure 7A). The ratio of phospho–5′-AMP-activated protein kinase (AMPK)/total AMPK was significantly elevated in Tg-Sirt1 line 53, but not in line 40, compared with NTg (Figure 7A), consistent with the notion that heart cells are under energy starvation in Tg-Sirt1 line 53. Tg-Sirt1 line 40 exhibited significantly higher, whereas line 53 showed significantly lower, citrate synthase activity than NTg, suggesting that mitochondrial function is depressed by high levels of Sirt1 (Figure 7B). Electron microscopic analyses indicated that the number of mitochondria is significantly smaller in Tg-Sirt1 line 53 than in NTg mice (Figure 7C), suggesting that mitochondrial biogenesis is reduced in Tg-Sirt1 line 53. Peroxisome proliferator-activated receptor γ coactivator (PGC)-1α is a transcriptional cofactor that plays an important role in mediating mitochondrial biogenesis. Interestingly, mRNA expression of PGC-1α was dose-dependently reduced by Sirt1 overexpression, where Tg-Sirt1 line 53 mice exhibited ∼50% reduction in PGC-1α expression in the heart (Figure 7D).

Tg-Sirt1 Mice Are Protected Against Oxidative Stress

Because Tg-Sirt1 line 40 exhibited reduced levels of baseline apoptosis and aging markers, we further examined whether modest overexpression of Sirt1 is protective against stress. To this end, we treated mice with paraquat for 14 days. Paraquat treatment significantly reduced LV contraction in NTg controls but not in Tg-Sirt1 line 40 (supplemental Table IV and Figure IV). Furthermore, the extent of cardiac myocyte apoptosis after paraquat treatment was significantly greater in NTg than in Tg-Sirt1 (Figure 8A). Paraquat treatment significantly increased the extent of oxidative stress in NTg mouse hearts as determined by 8-hydroxy-
deoxyguanosine (8-OHdG) staining and malondialdehyde content, established markers of oxidative stress. However, the paraquat-induced increases in oxidative stress were abolished in Tg-Sirt1 line 40 (Figure 8B). These results suggest that increased oxidative stress and myocardial damage induced by paraquat were significantly attenuated by moderate Sirt1 overexpression. In addition, paraquat-induced increases in expression of catalase, an antioxidant, were greater in Tg-Sirt1 line 40 than in NTg hearts (Figure 8C). On the other hand, the tissue level of oxidative stress was significantly enhanced in Tg-Sirt1 line 53, even at baseline (Figure 8D), suggesting that high levels of Sirt1 overexpression rather enhance oxidative stress.

We examined the mechanism by which Sirt1 upregulates catalase by using cultured cardiac myocytes. In particular, we examined the role of FoxO in mediating the catalase upregulation, because Sirt1 stimulates resistance to oxidative stress through FoxO in fibroblasts.38 Overexpression of either Sirt1 or constitutively active FoxO1a in cultured cardiac myocytes stimulated expression of catalase, whereas upregulation of catalase was inhibited in the presence of dominant negative FoxO1a (supplemental Figure V). These results suggest that FoxO1a plays an important role in mediating Sirt1-induced upregulation of catalase, which may in part mediate suppression of myocardial damage caused by oxidative stress in Tg-Sirt1 line 40.

**Figure 6.** Expression of aging markers and cell-protective molecules. A through C, Expression levels of senescence markers, including p15 (A), p19 (B), and p53 (C) in Tg-Sirt1 and NTg hearts (n=4 to 6). D, Expression of Bcl-2 at 2 to 3 months old (n=5). In each graph, the mean value of NTg at 2 to 3 months old was expressed as 1.

**Discussion**

Aging hearts exhibit unique histological and biochemical features.35,36 Increases in apoptosis and necrosis, proliferation of myocyte nuclei, increased myocyte volume, and connective tissue accumulation are frequently observed in the myocardium of old animals.34 Aging also affects the level of expression of INK4/ARF family senescence factors.37 Induction of cell-protective mechanisms, such as antioxidants and heat shock proteins, in response to pathologic insults is attenuated in aging hearts.39 As a result, aging cardiac myocytes cannot be transcriptionally reprogrammed in response to increased workload.36,39 Optimal therapeutic interventions to antagonize aging should prevent cell death and accumulation of senescent myocytes,36 eventually leading to decreases in the occurrence of adult heart diseases.

Although mechanisms inducing extension of lifespan may slow down aging of the whole organism, whether or not each longevity factor can prevent the aging process of organs and cells in mammals remains to be tested. Heart-specific expression of a molecule known to induce lifespan extension (dPTEN or dFoxO) prevents the age-dependent decline of the heart function in *Drosophila*,40 indicating that the longevity factors may function autonomously to prevent aging of individual organs. Overexpression of catalase targeted to mitochondria retarded aging-induced cardiac damage in mice.15 Our results suggest that Sirt1 prevents aging of the heart when 2.5- to 7.5-fold overexpressed in the heart.
Although these results are consistent with the notion that stimulation of a longevity mechanism could prevent aging of the heart, more studies are needed to determine whether this is the property of the longevity mechanism in general or merely a molecule-specific effect. For example, considering the apparent cardioprotective effects of the IGF-I–Akt axis, at the least short term, whether or not inhibiting IGF-I signaling, which extends the lifespan of mice,12,13 prevents aging and aging-related diseases in the mammalian heart without impairing cardiac function remains to be tested. IGF-I may produce only a tradeoff between current benefits and later costs in senescence, like the relationship between inotropic agents and their exacerbation of heart failure long term.42 We have shown that cardiac specific overexpression of mammalian sterile 20 like kinase 1 (Mst1) in transgenic mice induces dilated cardiomyopathy.43 Interestingly, a homolog of Mst1 induces lifespan extension in Caenorhabditis elegans through phosphorylation/activation of DAF-16.44 Although this example may represent a difference in the mechanism mediating longevity/stress resistance between lower organisms and mammals, careful reevaluation of the effect of longevity factors on aging/stress resistance of the heart, with particular emphasis on dosages and its long-term effects, seems essential. The antiaging and stress-resistance effects of Sir1 could be mediated not only by well-established mechanisms of longevity, such as FoxO family transcription factors, but also by nonspecific upregulation of cytoprotective pathways. Further investigation is required to identify downstream targets of Sir1 in cardiac myocytes.

Treatment with mice on a high-calorie diet with resveratrol improves health and survival, which is assumed to be mediated by stimulation of Sir1.23 Whether or not stimulation of Sir1 is sufficient to extend the lifespan of mammals remains to be tested. Our results suggest that heart-specific overexpression is not sufficient to induce lifespan extension in mice. Sir2 increases replicative lifespan, but actually inhibits chronological lifespan under extreme stresses in yeast.30 Increasing lines of evidence suggest that the heart may be a regenerative organ whose aging could be defined by the capacity for self-renewal in cardiac stem cells.45 In this context, Sir2 overexpression in transgenic mice leads to increased cardiomyocyte regeneration.46-48

Figure 7. All experiments were conducted using NTg and Tg-Sirt1 at 2 to 3 months of age. A (left), Relative cardiac ATP content in lines 40 and 53 mice (n=4 to 5). A (right), Immunoblot analyses of phospho-AMPK/total AMPK (n=6). B, Relative citrate synthase activity in the hearts from lines 40 and 53 (n=4 to 5). C, Electron microscopic analyses of cardiac mitochondria in line 53 mice (n=3 each). The number of mitochondria was determined from 8 sections. D, mRNA expression of PGC-1α in line 39, 40, and 53 mouse hearts (n=4). In each graph, the mean value of NTg was expressed as 1.
regard, Sirt1 may protect the heart from aging even if it selectively affects the replicative lifespan of mammalian cells.

Importantly, a very high level of Sirt1 is not protective for the heart. Tg-Sirt1 line 53, with 12.5-fold overexpression of Sirt1, spontaneously developed heart failure, accompanied by hypertrophy, increases in apoptosis and fibrosis, and oxidative stress. ATP content, citrate synthase activity, and mitochondrial biogenesis were all reduced in line 53, suggesting that high levels of Sirt1 induce mitochondrial dysfunction, which may in turn cause increases in oxidative stress. Interestingly, Sirt1 significantly reduced protein expression of PGC-1α, a master regulator of mitochondrial biogenesis and fatty acid oxidation. Downregulation of PGC-1α could be the result rather than the cause of heart failure. Thus, the causative role of PGC-1α downregulation in mediating mitochondrial dysfunction in Tg-Sirt1 line 53 mice remains to be elucidated. High levels of Sirt1 may also consume NAD+, thereby causing depletion of NAD+. Such dose-dependent adverse effects have been documented in poly ADP-ribose polymerase-1 (PARP-1), another NAD+-dependent enzyme implicated in DNA repair, where high doses of PARP-1 could lead to NAD+ depletion. Because NAD+ is required for mitochondrial respiration, depletion of NAD+ could lead to deficiency in ATP and, consequently, cellular dysfunction and eventual cell death.

Expression of Sirt1 is upregulated 3- to 9-fold in response to stresses (see also Figure 1). Similarly, Sirt1 was upregulated 2.9-fold in old monkey hearts. Such modest upregulation of Sirt1 is likely to be a compensatory mechanism and is expected to retard aging and inhibit apoptosis without causing mitochondrial dysfunction or NAD+ depletion. It would be important to keep the extent of Sirt1 overexpression modest, however, if upregulation of Sirt1 is to be considered as a therapeutic option.

Resistance to intrinsic and extrinsic stressors is strongly correlated with lifespan in many species. This positive correlation between stress resistance and longevity is also observed in mice with genetic mutations, as well as in animals pretreated with sublethal doses of stress, a phenomenon known as hormesis. According to the hormesis hypothesis, the longevity factors could be upregulated in re-
spose to a low grade of stress and confer stress resistance to the organism.\textsuperscript{49} Expression of Sir2t is upregulated in failing hearts\textsuperscript{24} as well as in animals subjected to caloric restriction.\textsuperscript{48} Here we demonstrated that Tg-Sirt1 exhibited resistance to oxidative stress. We have previously shown that expression of thioredoxin 1, an antioxidant, is upregulated in response to pressure overload and acts as an antihypertrophic factor, as well as a stimulator of mitochondrial function.\textsuperscript{50} Thioredoxin 1 is among the few antioxidants that prolong the lifespan of mice when overexpressed systemically.\textsuperscript{49} This raises a possibility that stimulation of the known longevity mechanisms could be a new modality of heart failure treatment, by increasing resistance of the heart to pathologic insults.

In summary, 2.5- to 7.5-fold overexpression of Sir2t has antiaging and stress-resistance effects, whereas higher levels of Sir2t induces cardiomyopathy, possibly through induction of mitochondrial dysfunction in the heart in vivo. Although stimulation of Sir2t may be considered as an antiaging therapy for the heart, careful evaluation regarding the dosage seems essential to best use the therapeutic potential of Sir2t.

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Disclosures
None.

References


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Expanded Methods

Materials

Anti-Sirt1 antibody was purchased from Upstate. Anti-p15 and p19 antibodies were purchased from Santa Cruz. Anti-Bcl2, Bcl-xL, phospho-AMPK and total AMPK antibodies were purchased from Cell Signaling Technology. Anti–8-OHdG antibody was purchased from Oxis International. Anti-catalase antibody and paraquat were obtained from Sigma.

Transgenic mice

Tg-Sirt1 mice were generated on an FVB background using the α-myosin heavy chain promoter (courtesy of J. Robbins, University of Cincinnati). All experiments involving animals were approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

RT-PCR

Total tissue mRNA was prepared using the RNeasy fibrous tissue kit (QIAGEN). Methods of quantitative RT-PCR have been described. PCR primers for rat atrial natriuretic factor (ANF), α-MHC, β-MHC, α-skeletal actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were selected using Vector NTI (Invitrogen).

The following primer pairs were used.

ANF,
Sense ATGGGCTCCTTCTCCATCAC
Anti-sense ATCTTCGGTACCGGAAGCTG
α-MHC,
Sense GGAAGAGTGAGCGGAGGCTCAG

Anti-sense CTGCTGGAGAGGTTATTCCTCG
β-MHC,
Sense GCCAACACCAACCTGTCCAAGTTC
Anti-sense TGCAAAGGCTCCAGGTCTGAGGGC
α-skeletal actin,
Sense TATTCCTTCGTGACCACAGCTGAACGT
Anti-sense CGCGAACGCAGACGCGAGTGCGC
GAPDH,
Sense AGCCAAAAGGGTCATCATCT
Anti-sense GGGGCCATCCACAGTCTTCT

First-strand cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen). Real-time PCR was then carried out using the DyNAmo hot start SYBR Green qPCR kit (Finnzymes) and a DNA Engine Opticon 2 system (Biorad).

**Cell lysate and tissue homogenates**

Whole-cell lysates were obtained using Lysis Buffer A, containing 150 mmol/L NaCl, 50 mmol/L Tris, pH 7.5, 0.1 mmol/L Na₃VO₄, 1 mmol/L NaF, 0.5 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 1% IGEPAL, 0.1% sodium dodecyl sulfate, 0.5% deoxycholic acid, 0.5 μg/mL leupeptin, 0.5 μg/mL aprotinin. Cardiac tissue homogenates were made in RIPA Buffer containing 1% IGEPAL, 0.25% Na-deoxycholate, 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, 1 mmol/L AEBSF, 0.5 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin.
**Histological analysis and immunohistochemistry**

Histological analyses of heart sections were conducted as described. Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6-μm thickness. Interstitial fibrosis was evaluated by picric acid Sirius red (PASR) staining. The positively stained fibrotic area was measured and expressed as a percentage of total area. Myocyte cross sectional area was measured from images captured from PASR-stained sections. Suitable cross sectional area was defined as having nearly circular capillary profiles and circular-to-oval myocyte sections. The outline of 100-200 myocytes was traced in each section, using Image-Pro Plus software (Media Cybernetics). The number of myocyte in the sample area was measured from 6 random fields of DAPI stained myocytes.

**Evaluation of apoptosis in tissue sections**

DNA fragmentation was detected *in situ* using the TUNEL assay system (Roche Diagnostics). The total number of nuclei was determined by manually counting DAPI-stained nuclei in 6 fields of each section, as described.

**Echocardiography**

Mice were anesthetized using 12 μL/g BW of 2.5% avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions) as previously described.
**Hemodynamic measurements**

Mice were anesthetized as described above, and a 1.4-French (Millar Instruments) catheter-tip micromanometer catheter was inserted through the right carotid artery into the aorta and then into the LV where pressures, dp/dt, and –dp/dt were recorded as described\(^3\).

**Transverse aortic constriction (TAC)**

The method of imposing pressure overload in mice has been described\(^3\). Mice were anesthetized with a mixture of ketamine (0.065 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g) and mechanically ventilated. The left chest was opened at the second intercostal space. Aortic constriction was performed by ligation of the transverse thoracic aorta between the innominate artery and left common carotid artery with a 28-gauge needle using a 7-0 braided polyester suture. Sham operation was performed without constricting the aorta.

**Oxidative stress and mitochondrial function**

In order to induce oxidative stress in the heart, mice were treated with paraquat (10 mg/kg) or vehicle by intraperitoneal injection once a week for 2 weeks. Tissue homogenates were prepared using 20 mmol/L phosphate buffer (pH 7.4) with 5 mmol/L butylated hydroxytoluene. Tissue levels of malondialdehyde (MDA) were determined using a Bioxytech LPO-586 kit (Oxis International)\(^3\). Staining with anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) antibody has been described\(^3\). ATP content and citrate synthase activity were measured using assay kits from Sigma.

**Electron microscopy**
Conventional electron microscopy was performed as described previously\textsuperscript{4}. In brief, cardiac myocytes were fixed in Karnofsky’s fixative, and then postfixed in 1% osmium tetraoxide, dehydrated in a graded series of acetone concentrations, and embedded in Sparr resin. Sections of 98 nm thickness were placed on copper grids that were double-stained with uranyl acetate and lead citrate. Discs were examined with a JEOL 1200 electron microscope.

**Cultured cardiac myocytes**

Primary cultures of cardiac ventricular myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats as described previously\textsuperscript{3}. Cell suspensions were applied on a discontinuous Percoll gradient as described\textsuperscript{3}. We obtained cultures in which more than 95% of cells were myocytes. Culture media were changed to serum-free at 24 h. Myocytes were cultured under serum-free conditions for 48 hours before experiments.

**Adenoviral vectors**

Adenovirus harboring Sirt1 (Ad-Sirt1) has been described\textsuperscript{5}. shRNA targeting Sirt1 was designed and cloned into pSilencer 1.0 (Ambion) to generate an adenovirus vector harboring shRNA targeting Sirt1 (Ad-shRNA-Sirt1), using the AdMax adenovirus construction kit (Microbix). Adenovirus vectors harboring constitutively active FoxO1a and dominant negative FoxO1a were a generous gift from Dr. Accili (Columbia University, New York, NY)

**DNA microarray analysis**
In order to identify potential targets of Sirt1 which might mediate its anti-aging effects, DNA microarray analyses were conducted. The results have been submitted to NCBI GEO database. The series ID for this work is GSE7407. Our microarray experiments were conducted using the following experimental conditions.

**Experimental design:**

Experimental goal: To examine the effect of moderate Sirt1 overexpression upon gene expression profile in the mouse heart.

Brief description of the experiment: We harvested the heart from transgenic mice with cardiac specific overexpression of Sirt1 (Tg-Sirt1) and non-transgenic (NTg) control littermate at 3 months of age and then microarray analyses were conducted.

Key words: Sirt1, longevity factor, stress resistance.

Links to the publication:


**Samples used, extract preparation and labeling:**

The origin of samples: We used line #40 Tg-Sirt1 mice, which have 7.5 fold overexpression of Sirt1 in the heart. These mice do not have obvious cardiac phenotype at 3 months of age. We used male mice for these experiments. Cardiac phenotype of Tg-Sirt1 and NTg has been described in reference 2.
Manipulation of biological samples and protocols used: These mice were bred and maintained using standard conditions. Mice were euthanized using pentobarbital 120 mg/kg. Harvested heart tissue was flash frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated using the Rneasy Fibrous Tissue Midi Kit (QIAGEN) according to the manufacturer’s instructions. Double-stranded cDNA was then synthesized from total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) with minor modifications to the manufacturer’s instructions. Briefly, 5 μg total RNA was annealed to T7-(dT)24 primer for 10 min at 70°C and chilled on ice. First strand synthesis was accomplished by incubation with DTT, dNTP mix and SuperScript II RT enzyme at 42°C for 1 hr. Second strand cDNA synthesis was accomplished by the addition of buffer, dNTP mix, DNA ligase, DNA polymerase I and RNase H and incubation at 16°C for 2 hr, followed by incubation with T4 DNA polymerase at 16°C for 5 min. The double-stranded cDNA was cleaned up by phenol:chloroform extraction followed by ethanol precipitation. Biotin-labeled RNA transcripts were produced by in vitro transcription using the BioArray HighYield RNA Transcript Labeling Kit (Enzo) according to the manufacturer’s instructions. The resultant cRNA was cleaned up using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s protocol for RNA cleanup. The cRNA was eluted with RNase-free water, concentrated by ethanol precipitation and stored at -80°C until use.

Hybridization protocol:

Hybridization, and processing of Affymetrix arrays were performed according to Affymetrix’s standard protocol.

Measurement data specifications:
Intensity values of all probe sets (45,101 total), from each array, were normalized by the Robust Multichip Analysis (RMA) method using the Affymetrix® Expression Console™ Software.

**Array Design:**

Affymetrix GeneChip® Mouse Genome 430 2.0.

**Statistical analyses**

Data are reported as mean ± SEM. Statistical analyses between groups were done by 1-way ANOVA, and when p values were significant, differences among group means were evaluated using t test with Bonferroni’s correction. Survival rates were monitored and compared using Kaplan-Meier survival analysis and the log-rank test. A p value of less than 0.05 was considered significant.
Fig. S1 The Kaplan-Meier survival curve shows no significant difference in survival of Tg-Sirt1 line 39 (n=34) and line 40 (n=22) and significantly reduced survival of Tg-Sirt1 line 53 mice (n=16) compared to their WT littermates (n=42, 18 and 26, respectively) studied anterospectively from birth to death.
Fig. S2 (A) Age-associated histopathological phenotype is attenuated in Tg-Sirt1 lines 39 and 40 mice. LV myocardial sections were obtained at mean ages of 2-3, 6-7, and 18 months old. Postmortem measurement of LV weight/tibial length (LVW/TL) is shown. n=5-16 in each group. Mean value in non-transgenic (NTg) at a mean age of 2-3 months old was expressed as 1. (B) Representative pictures of cardiac fibrosis determined by PASR staining. The LV sections were obtained from Tg-Sirt1 lines 39 and 40 and NTg at a mean age of 18 months old.
Fig. S3 Expression of anti-aging and stress resistance mechanisms in Tg-Sirt1 mice. All experiments were conducted using lines 40 and 53 at 6 months old. (A) Protein expression of TERT and Trf2. n=5. (B-D) Protein expression of Heat shock proteins, including HSP40 (B), HSP70 (C) and HSP90 (D). Bar graphs indicate relative expression levels of each protein. n = 4. (E) mRNA expression of klotho determined by RT-PCR (n = 3). In all cases, values are presented as means ± SEM.
Fig. S4 Tg-Sirt1 mice are more resistant to oxidative stress. NTg (n = 4) and Tg-Sirt1 (n = 6) mice from line 40 were treated with ip injection of 10 mg/kg of paraquat (PQ) for 2 weeks. LVEF (A) and %FS (B) of Tg-Sirt1 and NTg mice after PQ treatment.
**Fig. S5** The role of FoxO1a in mediating Sirt1-induced upregulation of catalase in cultured cardiac myocytes. Immunoblot analyses of catalase were conducted, using cardiac myocytes transduced with adenovirus vectors harboring constitutively actively FoxO1a (F-CA), dominant negative FoxO1a (F-DN), or Sirt1. n = 3-4.
Supplementary tables

Abbreviations used in supplemental Tables

HW   heart weight
BW   body weight
LVW  left ventricular weight
LUW  lung weight
LIW  liver weight
KIW  kidney weight
TL   tibial length
DSEP WT  end-diastolic septal wall thickness
LVEDD  LV end-diastolic dimension
DPW WT  end-diastolic posterior wall thickness
SSEP WT  end-systolic septal wall thickness
LVESD  LV end-systolic dimension
SPW WT  end-systolic posterior wall thickness
EF   ejection fraction
FS   fractional shortening
HR   heart rate
LVSP  LV systolic pressure
LVEDP  LV end-diastolic pressure
SBP  systolic blood pressure
DBP  diastolic blood pressure
MBP  mean blood pressure
**Table S1**

Echocardiographic measurements of 6 month old Tg-Sirt1 mice and NTg

<table>
<thead>
<tr>
<th></th>
<th>NTg</th>
<th>Tg</th>
<th>NTg</th>
<th>Tg</th>
<th>NTg</th>
<th>Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSEP WT(mm)</td>
<td>0.86±0.03</td>
<td>0.92±0.04</td>
<td>0.93±0.04</td>
<td>0.88±0.04</td>
<td>0.92±0.02</td>
<td>0.89±0.03</td>
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<tr>
<td>LVEDD(mm)</td>
<td>3.79±0.07</td>
<td>3.85±0.12</td>
<td>3.72±0.10</td>
<td>3.96±0.17</td>
<td>3.78±0.05</td>
<td>4.70±0.12*</td>
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<td>DPW WT(mm)</td>
<td>0.84±0.03</td>
<td>0.87±0.04</td>
<td>0.86±0.04</td>
<td>0.86±0.06</td>
<td>0.87±0.02</td>
<td>0.85±0.03</td>
</tr>
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<td>SSEP WT(mm)</td>
<td>1.24±0.03</td>
<td>1.23±0.06</td>
<td>1.28±0.05</td>
<td>1.33±0.10</td>
<td>1.27±0.03</td>
<td>1.17±0.05</td>
</tr>
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<td>LVEVD(mm)</td>
<td>2.55±0.05</td>
<td>2.60±0.09</td>
<td>2.56±0.07</td>
<td>2.62±0.14</td>
<td>2.51±0.03</td>
<td>3.80±0.17*</td>
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<td>SPW WT(mm)</td>
<td>1.10±0.04</td>
<td>1.10±0.05</td>
<td>1.13±0.05</td>
<td>1.12±0.09</td>
<td>1.18±0.04</td>
<td>1.07±0.03*</td>
</tr>
<tr>
<td>%FS</td>
<td>32.9±0.4</td>
<td>32.4±0.4</td>
<td>32.9±1.2</td>
<td>33.9±1.5</td>
<td>33.4±0.5</td>
<td>19.8±1.9*</td>
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<tr>
<td>HR(bpm)</td>
<td>420±16</td>
<td>416±13</td>
<td>433±13</td>
<td>413±30</td>
<td>422±14</td>
<td>422±21</td>
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<tr>
<td>BW(g)</td>
<td>30.8±1.4</td>
<td>35.1±1.9</td>
<td>31.3±1.1</td>
<td>32.9±2.0</td>
<td>29.5±1.0</td>
<td>28.3±1.3</td>
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<tr>
<td>n</td>
<td>6</td>
<td>7</td>
<td>9</td>
<td>8</td>
<td>21</td>
<td>18</td>
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* P<0.05 compared with NTg.
Table S2
Organ weight ratios of 6 month old Tg-Sirt1 mice and NTg

<table>
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<th>Line 39</th>
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<tr>
<td></td>
<td>NTg</td>
<td>Tg</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>3.94±0.11</td>
<td>3.99±0.18</td>
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<tr>
<td>LVW/BW (mg/g)</td>
<td>2.92±0.08</td>
<td>2.85±0.13</td>
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<tr>
<td>LIW/BW (mg/g)</td>
<td>7.30±0.67</td>
<td>8.34±0.56</td>
</tr>
<tr>
<td>KW/BW (mg/g)</td>
<td>49.9±2.1</td>
<td>46.7±2.1</td>
</tr>
<tr>
<td>HW/TL (mg/mm)</td>
<td>15.7±0.4</td>
<td>14.2±0.6</td>
</tr>
<tr>
<td>LVW/TL (mg/mm)</td>
<td>7.68±0.40</td>
<td>6.53±0.32</td>
</tr>
<tr>
<td>LIW/TL (mg/mm)</td>
<td>5.70±0.27</td>
<td>4.68±1.25</td>
</tr>
<tr>
<td>KW/TL (mg/mm)</td>
<td>13.4±1.5</td>
<td>13.5±0.3</td>
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<tr>
<td>LIW/BW (mg/mm)</td>
<td>96.8±2.2</td>
<td>77.2±6.3</td>
</tr>
<tr>
<td>KIW/TL (mg/mm)</td>
<td>30.9±1.5</td>
<td>23.7±2.3</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

* P<0.05 compared with NTg.
Table S3  Hemodynamic measurements of Tg-Sirt1 lines 40 and 53 mice and NTg

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<tbody>
<tr>
<td>HR (bpm)</td>
<td>473 ± 22</td>
<td>420 ± 16</td>
<td>488 ± 19</td>
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<tr>
<td>LVSP (mmHg)</td>
<td>83.3 ± 3.2</td>
<td>90.0 ± 1.0</td>
<td>79.0 ± 0.9</td>
</tr>
<tr>
<td>LV+ dP/dt (mmHg/s)</td>
<td>8667 ± 937</td>
<td>8125 ± 569</td>
<td>4250 ± 375*</td>
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<tr>
<td>LV- dP/dt (mmHg/s)</td>
<td>7500 ± 730</td>
<td>6625 ± 370</td>
<td>4125 ± 480*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>2.67 ± 0.42</td>
<td>2.50 ± 0.43</td>
<td>12.5 ± 3.0*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>82.7 ± 3.5</td>
<td>90.0 ± 3.0</td>
<td>80.0 ± 2.8</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>57.3 ± 2.7</td>
<td>56.0 ± 1.4</td>
<td>56.0 ± 3.2</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
<td>65.8 ± 2.9</td>
<td>67.3 ± 1.9</td>
<td>64.0 ± 2.8</td>
</tr>
</tbody>
</table>

* P<0.05 compared with NTG
Table S4
Echocardiographic data of Tg-Sirt1 line 40 and NTg hearts after paraquat treatment

<table>
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<tr>
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<th>NTg</th>
<th>Tg-40</th>
</tr>
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<tbody>
<tr>
<td>DSEP WT (mm)</td>
<td>0.78±0.04</td>
<td>0.89±0.02*</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.79±0.07</td>
<td>3.72±0.10</td>
</tr>
<tr>
<td>DPW WT (mm)</td>
<td>0.77±0.03</td>
<td>0.88±0.03*</td>
</tr>
<tr>
<td>SSEP WT (mm)</td>
<td>1.00±0.03</td>
<td>1.24±0.06*</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.73±0.07</td>
<td>2.50±0.07*</td>
</tr>
<tr>
<td>SPW WT (mm)</td>
<td>0.90±0.03</td>
<td>1.08±0.03*</td>
</tr>
<tr>
<td>EF</td>
<td>0.63±0.02</td>
<td>0.70±0.01*</td>
</tr>
<tr>
<td>%FS</td>
<td>28.1±1.1</td>
<td>32.9±1.0*</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>500±17</td>
<td>457±20</td>
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<tr>
<td>BW(g)</td>
<td>26.8±0.9</td>
<td>26.7±2.0</td>
</tr>
</tbody>
</table>

n 7 6

* P<0.05 compared with NTG.
References


