Abstract

Excessive reactive oxygen species (ROS) induce apoptosis and are associated with various diseases and with aging. SIRT1 (sirtuin-1), an NAD+ dependent protein deacetylase, decreases ROS levels and participates in cell survival under oxidative stress conditions. SIRT1 modulates the transcription factors p53, a tumor suppressor and inducer of apoptosis, and the forkhead O (FOXO) family, both of which play roles for cell survival and cell death. In this study, we aimed to know which is working greatly among p53 and FOXOs transcription factors in SIRT1’s cell protective functions under oxidative stress conditions. The antimycin A-induced increase in ROS levels and apoptosis was enhanced by SIRT1 inhibitors nicotinamide and splitomicin, whereas it was suppressed by a SIRT1 activator, resveratrol, and a SIRT1 cofactor, NAD+. SIRT1-siRNA abolished the effects of splitomicin and resveratrol. p53 knockdown experiment in C2C12 cells and experiment using p53-deficient HCT116 cells showed that splitomicin and resveratrol modulated apoptosis by p53-dependent and p53-independent pathways. In p53-independent cell protective pathway, we found that FOXO1, FOXO3a, and FOXO4 were involved in SOD2’s upregulation by resveratrol. The knockdown of these three FOXOs by siRNAs completely abolished the SOD2 induction, ROS reduction, and anti-apoptotic function of resveratrol. Our results indicate that FOXO1, FOXO3a and FOXO4, are indispensable for SIRT1-dependent cell survival against oxidative stress, although deacetylation of p53 has also some role for cell protective function of SIRT1.
indicating that SIRT1 mediates RSV’s cell survival-promoting effects [13,14,16]. In C2C12 cells, RSV increases the SOD2 levels and inhibits ROS-dependent apoptosis via SIRT1 [13], whereas SIRT1 knockdown increases the levels of NADPH oxidase (NOX) family members, which are membrane proteins that generate O$_2^-$ [14]. In fact, RSV administration increases the SOD2 level in the cardiomyocytes of TO-2 hamsters [13] and decreases the NOX family mRNAs in the skeletal muscle of mdx mice [14]. These results indicate that SIRT1 affects cellular ROS levels and cell survival via multiple pathways; however, how p53 and FOXOs participate in the SIRT1 signaling remains to be elucidated.

In this study, we focused on the roles of p53 and FOXOs in the anti-oxidative and anti-apoptotic function of SIRT1 in C2C12 cells treated with antimycin A, which increases and releases ROS from mitochondria by inhibiting mitochondrial respiratory chain complex III. We show that modulators of SIRT1 profoundly affect the cellular ROS levels and cell survival under oxidative stress. Whereas p53 was partly involved in the antimycin A-induced apoptosis of C2C12 cells, the knockdown of three members of the FOXO family, FOXO1, FOXO3a, and FOXO4, completely abolished RSV’s ROS-reducing and anti-apoptotic activities. These FOXOs contributed to SOD2’s induction by RSV. Thus, FOXO1, FOXO3a, and FOXO4 are indispensable for RSV’s ROS-reducing and anti-apoptotic activities in C2C12 cells.

Materials and Methods

Cell Culture and Treatment

C2C12 mouse myoblasts (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (Wako Pure Chemical Ind., Osaka, Japan) supplemented with 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto Japan) and 10% fetal bovine serum (MP Biomedicals, Aurora, OH, USA). HCT116 cells [17] were gifted by Dr. T. Tokino (Sapporo Medical University). Cells were pretreated with 10 or 30 μM RSV for 3 hrs followed by the addition of antimycin A at the indicated doses for 24 hrs. In experiments to analyze acetylation of p53 and FOXO protein, cell treatment was carried out in the presence of trichostatin A (TSA, 50 nM).

Reagents and Antibodies

NAD$^+$ (Oriental Yeast, Tokyo, Japan) and nicotinamide (Wako Pure Chemical Ind.) were dissolved in culture medium. Resveratrol (Wako Pure Chemical Ind.), splittomicin (Calbiochem-Millipore, Billerica, MA, USA), Ex527 (Toeris Bioscience, Ellisville, MO, USA), and antimycin A (Sigma Aldrich, St Louis, MO, USA) were dissolved in dimethyl sulfoxide to obtain stock solutions. H$_2$O$_2$ and Hoechst 33342 (1:1000 dilution) were combined and analyzed. Dead cells were also analyzed by Image-J software (NIH). The average fluorescence intensity was obtained under the same conditions, and the fluorescence was quantified by Image-J Software (NIH). The average fluorescence intensity was obtained from six fields of each treatment, and the data from three independent experiments were combined and analyzed.

Analysis of Intracellular ROS Levels

Intracellular ROS levels were monitored by the confocal laser microscopic analysis of cells stained with 3-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA) (Invitrogen). Cells attached to glass slides were incubated with CM-H$_2$DCFDA for 30 min at 37°C followed by two washes with PBS. After being treated with antimycin A or H$_2$O$_2$, the cells were fixed with 4% paraformaldehyde for 10 min, and washed with PBS. All images were captured under the same conditions, and the fluorescence was quantified by Image-J Software (NIH). The average fluorescence intensity was obtained from six fields for each treatment, and the data from three independent experiments were combined and analyzed.

Immunostaining

After fixation and washing, cells were blocked with PBS containing 3% BSA, 1% goat serum, and 0.1% Triton X-100 for 30 min. The cells were then incubated with antibodies against active caspase-3 (1:250 dilution), SOD2 (1:500 dilution), Bax (1:50 dilution), or acetyl-p53 (1:250 dilution) overnight at 4°C. The cells were then washed four times with PBS, incubated with secondary antibodies, Alexa Fluor 488 or 594 anti-rabbit IgG (Invitrogen), and washed with PBS. All images were captured under the same conditions, and the fluorescence was quantified by Image-J Software (NIH). The average fluorescence intensity was obtained from six fields of each treatment, and the data from three independent experiments were combined and analyzed.

Western Blotting

Cells were lysed in Celllytic M Cell Lysis Reagent (Sigma) with 1% protease inhibitor cocktail (Nacalai Tesque). For analyses of acetyl-p53 or acetyl-FOXO1, nicotinamide (10 nM) and trichostatin A (500 nM) were added to the lysis buffer. When phospho-AMPK and phospho-ACC were analyzed, phosphatase inhibitor cocktail (Nakalai Tesque) were added to the lysis buffer. The lysates were then sonicated and centrifuged to remove insoluble matter. The protein concentration of the supernatant was measured using

siRNA Transfection

siRNA against mouse SIRT1 (siTrio) was obtained from B-Bridge International as described previously [13]. siRNAs against mouse FOXO1, FOXO3a, and FOXO4 were obtained from Santa Cruz. A nonsense siRNA obtained from Sigma Genosys was used as the control for nonspecific effects on gene expression, as described previously [13,14,16]. Transfection was performed using a Nucleofector kit (Lonza, Walkersville). siRNAs (100 nM) were transfected twice into cells with an interval of 24 hours. Twenty-four hours after the second transfection, the cells were treated with antimycin A.
SIRT1 modulators show similar anti-oxidative and anti-apoptotic
effects on C2C12 cells treated with H2O2, we used H2O2 as an
oxidant (Figure 2). Again, SIRT1 inhibitors increased the ROS
levels (Figure 2A) and apoptosis (Figure 2B and 2C), while SIRT1 activators significantly inhibited the H2O2–
induced increase in ROS levels (Figure 2A) and apoptosis (Figure 2B and 2C).

To examine whether these SIRT1 modulators affect ROS levels
and cell survival via SIRT1, we transfected C2C12 cells with
SIRT1-siRNA. SIRT1 knockdown by siRNA (Figure 3A) abol-
ished RSV’s anti-oxidative (Figure 3B and 3C) and anti-apoptotic
functions (Figure 3D and 3E). In the presence of SIRT1-siRNA,
the antimycin A-induced apoptosis increased to the level seen in
SP-treated control-siRNA cells (Figure 3D and 3E), and SP did not
further enhance the apoptosis in cells transfected with SIRT1-
siRNA. In contrast, RSV decreased the cell death (Figure 3D).

Reverse Transcription and Polymerase Chain Reaction

Total RNA was isolated using an RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized using SuperScript III (Invitro-
gen). DNA amplification was performed by RT-PCR using Taq
DNA polymerase (New England Biolabs). The primer sequences
used for RT-PCR were as follows: p53 forward 5'-AGTCACAG-
CACATGCGAGGGT-3', reverse 5'-ATACACATGTACTTG-
TAATTTGAT-3'; GAPDH forward 5'-ACCACAGTCTCATGC-
CATCAC-3', reverse 5'-TCCACGCCCTTGTCCGTGTA;
FOXO1 forward 5'-CAGATTACATACAGGGCCATCC-3',
reverse 5'-TGCTGATGCCTGCCATCGCTGGCA-3'; FOXO3a
forward 5'-AAACAGGACGCGCCTTCTCT-3', reverse 5'-GCTGACAGAATTTGACAAGGCA-3'; FOXO4 forward 5'-ATACACCCAGCTTCTGCTGATG-3', reverse 5'-CCA-
GATCTGAGGATTTGCTCAT-3'. GAPDH served as a
control. For quantitative PCR, the cDNA amplification was
performed in StepOne (Applied Biosystems, Foster City, CA) using
TaqMan Universal Master Mix II, with UNG (Applied Biosys-
tems) and TaqMan Gene Expression Assays for SOD2
(Mm00449726_m1) or β-actin (Mm01205647_g1). β-actin served
as an internal control.

**Figure 1. Effects of SIRT1 modulators on antimycin A-induced ROS generation and cell death.** (A) Representative images (left) and
quantitative analysis (right) of CM-H2DCFDA (DCF) fluorescence in
C2C12 cells. Cells were treated with 200 μM antimycin A (AA) for 24
hours after their pretreatment with vehicle (Ctrl), 5 mM nicotinamide
(NA), 60 μM splotmicin (SP), 10 μM resveratrol (RSV), or 1 mM NAD+ for
3 hours. Data are from three independent experiments. Scale bar: 10 μm. (B) (Left) Representative images of nuclear staining with
Hoechst33342 in C2C12 cells treated as in (A). (Right) Quantification of cleaved caspase-3-positive cells. Data are from three independent experiments. Scale bar: 10 μm. (C) (Left) Representative images of immunostaining for cleaved (active) caspase-3 in C2C12 cells
treated as in (A). (Right) Quantification of cleaved caspase-3-positive
cells. Data are from three independent experiments. Scale bar: 10 μm. (D) Viability profiles (left) and quantification of dead cells (right) in
C2C12 cells treated for 9 hrs with 60 μM AA with or without
pretreatment with 10 mM NA or 30 μM RSV (N = 7). ***p<0.001,
**p<0.01, *p<0.05, n.s. = not significant. a.u. = arbitrary units.
doi:10.1371/journal.pone.0073875.g001

Results

**SIRT1 Modulators Affect the Intracellular ROS Levels and Cell Death Induced by Oxidative Stress**

We first analyzed the effect of SIRT1 modulators on the
apoptosis induced by antimycin A in C2C12 cells. Antimycin A
significantly elevated the intracellular ROS levels measured by
CM-H2DCFDA staining (Figure 1A) and increased the number of
apoptotic cells identified by nuclear condensation (Figure 1B)
and activated caspase-3 (Figure 1C). Pretreatment with a SIRT1
inhibitor nicotinamide (NA) or splotmicin (SP) augmented
antimycin A’s effects on both the increased ROS levels
(Figure 1A) and apoptosis (Figure 1B and 1C), whereas pretreat-
ment with the SIRT1 activator RSV or the SIRT1 cofactor NAD+ significantly decreased the ROS levels (Figure 1A) and inhibited
the apoptosis (Figure 1B and 1C) induced by antimycin A. Dead
cells were also analyzed by a Muse Cell Analyzer. NA augmented
late apoptotic/necrotic cell death induced by antimycin A,
whereas RSV decreased the cell death (Figure 1D).

Antimycin A releases a superoxide radical from mitochondria,
which is dismutated into oxygen and hydrogen peroxide (H2O2)
by endogenous superoxide dismutases (SODs). To examine whether
SIRT1 modulators show similar anti-oxidative and anti-apoptotic
effects on C2C12 cells treated with H2O2, we used H2O2 as an
oxidant (Figure 2). Again, SIRT1 inhibitors increased the ROS
levels (Figure 2A) and the number of apoptotic cells (Figure 2B and
2C), while SIRT1 activators significantly inhibited the H2O2–
induced increase in ROS levels (Figure 2A) and apoptosis (Figure 2B and 2C).

To examine whether these SIRT1 modulators affect ROS levels
and cell survival via SIRT1, we transfected C2C12 cells with
SIRT1-siRNA. SIRT1 knockdown by siRNA (Figure 3A) abol-
ished RSV’s anti-oxidative (Figure 3B and 3C) and anti-apoptotic
functions (Figure 3D and 3E). In the presence of SIRT1-siRNA,
the antimycin A-induced apoptosis increased to the level seen in
SP-treated control-siRNA cells (Figure 3D and 3E), and SP did not
further enhance the apoptosis in cells transfected with SIRT1-
siRNA (Figure 3D and 3E). These results indicate that the SIRT1 modulators affected the cellular ROS levels and apoptosis via SIRT1 in C2C12 cells.

SIRT1 Modulators Affect Both p53-dependent and p53-independent Apoptosis Induced by Antimycin A

The transcription factor p53 is deacetylated and inactivated by SIRT1 [7,8]. Since antimycin A activates p53 [19], we examined whether p53 mediates the apoptosis induced by antimycin A in C2C12 cells. p53 knockdown itself did not affect cell death in vehicle-treated C2C12 cells (Figure 4A and 4B). In the presence of antimycin A, however, p53 knockdown significantly decreased the number of apoptotic cells but failed to completely suppress apoptosis (Figure 4B), indicating that both p53-dependent and p53-independent mechanisms regulated the antimycin A-induced apoptosis of C2C12 cells. Transfection with p53-siRNA partially inhibited the apoptosis-promoting activity of the SIRT1 inhibitor SP (Figure 4C), suggesting that the p53-dependent pathway is involved in the SP-induced enhancement of apoptosis. However, SP still enhanced the antimycin A-induced apoptosis in p53-knockdown cells (Figure 4C). Furthermore, in the presence of p53-siRNA, RSV still reduced the number of apoptotic cells (Figure 4D).

To exclude the possibility that residual p53 contributed to the apoptosis in cells transfected with p53-siRNA, we examined effects of SIRT1 modulators on p53 acetylation level. Western blotting and immunocytochemistry showed that antimycin A increased acetyl-p53 level. Treatment of cells with RSV reduced acetyl-p53 level, whereas a specific SIRT1 modulator, resveratrol (RSV), significantly decreased the acetyl-p53 level (Figure 5A and 5B). These findings indicate that both p53-dependent and p53-independent mechanisms regulated the apoptosis triggered by oxidative stress in C2C12 cells and HCT116 cells, and that SIRT1 modulators affected both mechanisms to regulate cell death.

Since SIRT1 inhibits p53 activity by promoting p53 deacetylation [7,8], we examined effects of SIRT1 modulators on p53 acetylation level. Western blotting and immunocytochemistry showed that antimycin A increased acetyl-p53 level. Treatment of cells with RSV reduced acetyl-p53 level, whereas a specific SIRT1 modulator, resveratrol (RSV), significantly decreased the acetyl-p53 level (Figure 5A and 5B). These findings indicate that both p53-dependent and p53-independent mechanisms regulated the apoptosis triggered by oxidative stress in C2C12 cells and HCT116 cells, and that SIRT1 modulators affected both mechanisms to regulate cell death.
family consists of FOXO1, FOXO3a, FOXO4, and FOXO6. FOXO1, FOXO3a, and FOXO4 are widely expressed in various tissues, whereas FOXO6 is predominantly expressed in the brain [20]. We therefore analyzed FOXO1, FOXO3a, and FOXO4 in the following experiments. Because all three of these FOXOs were expressed in C2C12 cells, we examined which ones are involved in SOD2’s induction by RSV under antimycin A treatment, by using siRNAs for each FOXO (Figure 5C). We found that the individual knockdown of FOXO1, FOXO3a, or FOXO4 significantly reduced the expression of SOD2 (Figure 5C and 5D). When the C2C12 cells were transfected with a mixture of siRNAs (FOXOs-siRNA), against FOXO1, FOXO3a, and FOXO4, SOD2’s induction by RSV was abolished (Figure 5E). Furthermore, monitoring the intracellular ROS levels by CM-H2DCFDA showed that the decrease in ROS levels by RSV was blocked by the FOXOs-siRNA (Figure 5F and 5G).

Because SIRT1 regulates FOXO activity via dacetylation, we assessed the effect of RSV on acetyl-FOXO1 level (Figure 5H). In the presence of antimycin A, RSV significantly reduced acetyl-FOXO1 level (Figure 5H), indicating that RSV enhanced the deacetylation of FOXO1. SIRT1 inhibitor NA increased acetyl-FOXO1 level (Figure 5H). Unexpectedly, we found that treatment of C2C12 cells with antimycin A decreased FOXO1 protein level, which was not affected by RSV. FOXO3a and FOXO4 protein levels were also downregulated by antimycin A treatment (Figure 5I). Down regulation of FOXO proteins by antimycin A made us difficult to detect acetylated FOXOs by immunoprecipitation experiments (data not shown).

Knockdown of FOXO1, FOXO3a, and FOXO4 Completely Inhibited the Cell-protective Function of Resveratrol in C2C12 Cells

Finally, we examined whether the knockdown of all three FOXO mRNAs affects RSV’s cytoprotective function in C2C12 cells treated with antimycin A. RSV significantly inhibited the cell death induced by antimycin A in C2C12 cells transfected with control-siRNAs, whereas its cytoprotective effect was completely abolished by the expression of the three FOXO-siRNAs (Figure 6A). The antimycin A-induced accumulation of BAX, an early-stage indicator of apoptosis, was also inhibited by RSV pretreatment of C2C12 cells transfected with control-siRNA (Figure 6B). This suppression of BAX accumulation by RSV was inhibited by knockdown of the three FOXO mRNAs (Figure 6B). In addition, the FOXOs-siRNA significantly increased the number of antimycin A-induced apoptotic cells, compared with cells transfected with control-siRNA (Figure 6A and 6B). These findings indicate that the FOXOs were indispensable for the cellular protective mechanism elicited by RSV against oxidative stress. To exclude the possibility that the FOXOs-siRNAs affected the deacetylation status of p53, we immunostained C2C12 cells for acetyl-p53. All cells were treated in the presence of 50 nM of trichostatin A. Representative images of immunostaining for acetyl-p53 (Lys379) in C2C12 cells treated as in A. Scale bar: 20 μm.

doi:10.1371/journal.pone.0073875.g004

inhibitor Ex527 strongly augmented p53 acetylation (Figure 4I and 4J).

FOXO1, FOXO3a, and FOXO4 are Involved in the Resveratrol-dependent Induction of SOD2

We next examined whether FOXO transcription factors played a role in the p53-independent regulation of the antimycin A-induced apoptosis by SIRT1 modulators, especially RSV. FOXOs have dual roles in response to oxidative stress, in which they both promote apoptosis and induce ROS-detoxifying enzymes such as SOD2. As previously reported [13,16], RSV induced SOD2 expression in C2C12 cells and neonatal rat cardiomyocytes (Figure 5A and 5B). In mammals, the FOXO

RSV did not Modulate AMPK Activity in C2C12 Cells

Recent reports have demonstrated that RSV activates AMPK, which contributes to protective effects of RSV [21,22]. We examined whether RSV modulates AMPK activity in our model. Activated AMPK was monitored by levels of phosphorylated AMPKα and phosphorylated acetyl-CoA carboxylase (ACC), a

Knockdown of FOXO1, FOXO3a, and FOXO4 Completely Inhibited the Cell-protective Function of Resveratrol in C2C12 Cells

Finally, we examined whether the knockdown of all three FOXO mRNAs affects RSV’s cytoprotective function in C2C12 cells treated with antimycin A. RSV significantly inhibited the cell death induced by antimycin A in C2C12 cells transfected with control-siRNAs, whereas its cytoprotective effect was completely abolished by the expression of the three FOXO-siRNAs (Figure 6A). The antimycin A-induced accumulation of BAX, an early-stage indicator of apoptosis, was also inhibited by RSV pretreatment of C2C12 cells transfected with control-siRNA (Figure 6B). This suppression of BAX accumulation by RSV was inhibited by knockdown of the three FOXO mRNAs (Figure 6B). In addition, the FOXOs-siRNA significantly increased the number of antimycin A-induced apoptotic cells, compared with cells transfected with control-siRNA (Figure 6A and 6B). These findings indicate that the FOXOs were indispensable for the cellular protective mechanism elicited by RSV against oxidative stress. To exclude the possibility that the FOXOs-siRNAs affected the deacetylation status of p53, we immunostained C2C12 cells for acetyl-p53. All cells were treated in the presence of 50 nM of trichostatin A. Representative images of immunostaining for acetyl-p53 (Lys379) in C2C12 cells treated as in A. Scale bar: 20 μm.
downstream enzyme of AMPK. Neither phospho-AMPKα nor phospho-ACC was changed by RSV treatment alone. Antimycin A markedly induced AMPKα and ACC phosphorylation, which was not promoted by RSV (Figure 7A and 7B). These findings indicate that RSV did not activate AMPK in our experimental setting.

**Discussion**

In this study we examined whether p53 and/or FOXOs participate in the effect of SIRT1 modulators in C2C12 cells exposed to a high level of oxidative stress. Antimycin A was used to induce and liberate ROS from mitochondria. Although the modulation of p53 by SIRT1 partially inhibited the oxidative stress-induced cell death, the knockdown of FOXO1, FOXO3a, and FOXO4 completely abolished the cell-protective effect of the
A (AA, 50 μM under conditions of high oxidative stress. FOXO1, FOXO3a, and FOXO4 are essential for cell survival via the FOXOs' activity is modulated by acetylation and deacetylation [9]. Whereas SIRT1 activation enhances the transcriptional activity of FOXO1 [24], FOXO3a [25], and FOXO4 [26] on ROS-detoxifying enzymes by deacetylation, the CBP/p300-mediated acetylation of FOXOs impairs their DNA-binding activity [9]. We recently showed that the deacetylation of p300 by SIRT1 promotes p300's ubiquitination and downregulation, and that RSV reduces the p300 protein level [15]. Thus, p300’s downregulation by SIRT1 could also increase the deacetylated FOXOs levels. In addition, RSV is reported to promote FOXO1's nuclear retention via SIRT1-mediated deacetylation [27], which may increase its transcriptional activity. necessary for cell survival. The knockdown of FOXO1, FOXO3a, and FOXO4 inhibited SOD2's induction by RSV, but failed to abolish the baseline expression of SOD2 (Figure 5E). This may indicate that the upregulation of ROS-detoxifying enzymes including SOD2 is important for RSV's cell-protective roles against high ROS levels. SOD2 catalyzes the dismutation of O$_2^-$ to H$_2$O$_2$, which is further metabolized to H$_2$O and O$_2$ by catalase and peroxidases. Because FOXOs can induce catalase and some peroxidases [9,23] and because SIRT1 modulators exerted similar effects on H$_2$O$_2$-induced cell death as on antimycin A-induced apoptosis (Figures 1 and 2), RSV's cell-survival effect via FOXOs depends not only on SOD2's induction but also on the increase in downstream ROS-detoxifying enzymes.

In the absence of antimycin A, the expression of the three FOXO-siRNAs unexpectedly increased the cellular ROS levels and the number of apoptotic cells, compared with cells transfected with control-siRNA (Figures 5F and 6). Thus, in the absence of FOXOs, the ROS produced by cellular metabolism, such as mitochondrial oxidative phosphorylation, may trigger apoptosis, and FOXOs may constantly protect cells by reducing the cellular ROS levels.

The FOXOs' activity is modulated by acetylation and deacetylation [9]. Whereas SIRT1 activation enhances the transcriptional activity of FOXO1 [24], FOXO3a [25], and FOXO4 [26] on ROS-detoxifying enzymes by deacetylation, the CBP/p300-mediated acetylation of FOXOs impairs their DNA-binding activity [9]. We recently showed that the deacetylation of p300 by SIRT1 promotes p300’s ubiquitination and downregulation, and that RSV reduces the p300 protein level [15]. Thus, p300’s downregulation by SIRT1 could also increase the deacetylated FOXOs levels. In addition, RSV is reported to promote FOXO1’s nuclear retention via SIRT1-mediated deacetylation [27], which may increase its transcriptional activity.

**Figure 7. The effect of resveratrol on AMPK activity in C2C12 cells.** (A) Representative immunoblots of phospho-AMPK (Thr172), total AMPK, phospho-acetyl-CoA carboxylase (ACC) (Ser79), total ACC, and GAPDH in C2C12 cells treated with vehicle or 60 μM antimycin A (AA) with or without 30 μM resveratrol (RSV). (B) Quantitation of phospho-AMPK (Thr172) level normalized to GAPDH (N = 5). (C) Quantitation of phospho-ACC (Ser79) level normalized to GAPDH (N = 5). n.s. = not significant. doi:10.1371/journal.pone.0073875.g007

**SIRT1 activator RSV.** Moreover, unexpectedly, we found that FOXO1, FOXO3a, and FOXO4 are essential for cell survival under conditions of high oxidative stress.

**Cell Survival via the Modulation of FOXOs Activity**

In mammals, there are four FOXO transcription factor members, whose functions appear to overlap with each other [10]. In fact, here we observed that SOD2’s induction by RSV in the presence of antimycin A was significantly inhibited by the individual knockdown of FOXO1, FOXO3a, or FOXO4 (Figure 5D). Under oxidative stress conditions, the SIRT1 activator RSV increased the SOD2 level (Figure 5E), reduced the ROS levels (Figure 5F and 5G), and promoted cell survival (Figure 6A and 6B) via the FOXOs.

We previously showed that SOD2 knockdown completely inhibits RSV’s anti-apoptotic function against antimycin A [13]. Taken together, the results indicate that the increase in SOD2 expression by RSV under antimycin A-induced oxidative stress is
also regulated by SIRT1 modulators, which is consistent with SIRT1’s inhibitory effect on p33’s activity under ionizing radiation, etoposide, and H2O2 treatment [7,8].

SIRT1 Inhibitors as Inducers of Apoptosis

We showed here that the inhibition of SIRT1 enhances the apoptosis elicited by oxidative stress (Figure 1 and 2). Although SIRT1 regulates both p33-dependent cell death and FOXO-dependent cell survival signals under oxidative stress conditions, our findings suggest that the FOXOs-dependent cell-protective mechanism, which induces the expression of ROS-detoxifying enzymes, is more important for the cell-fate decision. More than 50% of human tumors contain a mutation or deficiency in p53 [28]. Our results suggest that SIRT1 inhibition enhances the cell-death effect of ROS-generating anti-cancer drugs, even in p53-deficient or p53-mutated cancer cells. Thus, SIRT1 inhibition may contribute to the treatment of cancer patients in combination with ROS-producing cancer therapies such as ionizing radiation and alkylating antineoplastic agents. In addition, FOXO inhibitors may be alternative agents for enhancing cancer chemotherapy.

Acknowledgments

We thank T. Tokino for the HCT 116 cells and M. Tanno for technical advice.

Author Contributions

Conceived and designed the experiments: YSH AK YH. Performed the experiments: YSH AK YH. Analyzed the data: YSH AK YH. Wrote the paper: YSH AK YH.

References

1. Konstantinidis K, Whelan RS, Kinis RN (2012) Mechanisms of cell death in heart disease. Annu Rev Thromb Haemost 32: 1552–1562.
2. Mattson MP (2000) Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol 1: 120–129.
3. Tidball JG, Wehling-Henricks M (2007) The role of free radicals in the pathophysiology of muscular dystrophy. J Appl Physiol 102: 1677–1616.
4. Hori Y, Hayashi T, Kuno A, Konanoto R (2011) Cellular and molecular effects of sirtuins in health and disease. Clin Sci (Lond) 121: 191–203.
5. Achanta G, Huang P (2004) Role of p53 in sensing oxidative DNA damage in response to reactive oxygen species-generating agents. Cancer Res 64: 6233–6239.
6. Vousden KH (2000) p53: death star. Cell 103: 691–694.
7. Luo J, Nikolaeff AY, Imai S, Chen D, Su F, et al. (2001) Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell 107: 137–149.
8. Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, et al. (2001) Resveratrol activates Sirt1, a NAD-dependent histone deacetylase. Science 293: 1354–1357.
9. Huang H, Tindall DJ (2007) Dynamic FoxO transcription factors. J Cell Sci 120: 2479–2487.
10. Greer EL, Brunet A (2005) FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24: 7410–7425.
11. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, et al. (2003) Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 425: 191–196.
12. Bau B, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5: 495–506.
13. Tanno M, Kuno A, Yano T, Miura T, Hisahara S, et al. (2010) Induction of manganese superoxide dismutase by nuclear translocation and activation of SIRT1 promotes cell survival in chronic heart failure. J Biol Chem 285: 8375–8382.
14. Hori YS, Kuno A, Hosoda R, Tanno M, Miura T, et al. (2011) Resveratrol ameliorates muscular pathology in the dystrophic mdx mouse, a model for Duchenne muscular dystrophy. J Pharmacol Exp Ther 338: 784–794.
15. Kuno A, Hori YS, Hosoda R, Tanno M, Miura T, et al. (2011) Resveratrol improves cardiomyopathy in dystrophy-deficient mice through SIRT1 protein-mediated modulation of p300 protein. J Biol Chem 286: 5963–5972.
16. Hosoda R, Kuno A, Horii YS, Ohnoki K, Nakamuro Y, et al. (2013) Resveratrol Elicits Cell Survival via FOXOs -trihydroxystilbene)
17. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, et al. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 282: 1497–1501.
18. Sakamoto J, Miura T, Shimamoto K, Horio Y (2004) Predominant expression of Sir2alpha, an NAD-dependent histone deacetylase, in the embryonic mouse heart and brain. FEBS Lett 556: 281–286.
19. Khromonenko AA, Rosolko VV, Chernyak BV, Vartapetian AB, Chumakov PM, et al. (2011) Pyrimidine biosynthesis links mitochondrial respiration to the p53 pathway. Proc Natl Acad Sci U S A 107: 12828–12833.
20. Salih DA, Brunet A (2006) FOXO transcription factors in the maintenance of cellular homeostasis during aging.Curr Opin Cell Biol 20: 126–136.
21. Price NL, Gomes AP, Ding AJ, Duarte FV, Martin-Montalvo A, et al. (2012) SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab 15: 675–690.
22. Shin SM, Cho IJ, Koo SG (2009) Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycolysis. J Biol Chem 284: 149–159.
23. Marinkovic D, Zhang X, Yalcin S, Luciano JP, Brugnara C, et al. (2007) Foxo3a expression is required for the regulation of oxidative stress in erythropoiesis. J Clin Invest 117: 2133–2144.
24. Daitoku H, Hatta M, Matsuzaki H, Aratani S, Ohshima T, et al. (2004) Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Proc Natl Acad Sci U S A 101: 10042–10047.
25. Brunet A, Sweeney LJ, Sturgill JF, Chua KF, Greer PL, et al. (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 303: 2011–2015.
26. van der Horst A, Tertoolen LG, de Vries-Smits LM, Frye RA, Medema RH, et al. (2004) FOXOs are acetylated upon oxidative stress and deacetylated by the longevity protein Sir2(SIRT1).J Biol Chem 279: 20873–20879.
27. Frescas D, Valenti L, Acott D (2005) Nuclear trapping of the forkhead information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. Mol Cell Biol 1: 120–129.
28. Mustonen O, Notenstein LG, de Vries-Smits LM, Frye RA, Medema RH, et al. (2004) FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein Sir2(SIRT1). J Biol Chem 279: 20873–20879.
29. Frosch D, Valenti L, Acott D (2005) Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucocorticoid genes. J Biol Chem 280: 20589–20595.
30. Pollen N, Dobrzykowski D, Volegelbein B, Harris CC (1991) p53 mutations in human cancers. Science 253: 49–53.