

Original Article

SIRT1 counteracted the activation of STAT3 and NF- κ B to repress the gastric cancer growth

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Abstract: Sirtuin-1 (SIRT1) possesses apparently dual roles in regulation of tumor. Previous reports have documented the crosstalk between SIRT1 with signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa-B (NF- κ B) signaling in leukemia, lymphoma and myeloma. In this study, the purpose was to survey the regulatory effects of SIRT1 on gastric cancer (GC) cells (AGS and MKN-45) and the relationships between SIRT1 and activation of STAT3 and NF- κ B in GC cells. We found the SIRT1 activator (resveratrol RSV) contributed to the repression of viability and increase of senescence, which were rescued by SIRT1 inhibitor (nicotinamide NA) and SIRT1 depletion by CCK-8 assay and SA- β -gal assay respectively. Further study found SIRT1 activation (RSV supplement) not only inhibited the activation of STAT3 including STAT3 mRNA level, c-myc mRNA level phosphorylated STAT3 (pSTAT3) proteins and acetylated STAT3 (acSTAT3) proteins, but also repression of pNF- κ B p65 and acNF- κ B p65. NA reversed the effects of RSV. In addition, either RSV or NA application could not change the cellular viability and senescence in MKN-45 cells with STAT3 knockdown or NF- κ B knockdown. Overall, our findings suggested SIRT1 activation could induced the loss of viability and increases of senescence in GC in vitro. Moreover, our observations revealed SIRT1 displayed growth inhibitory activity in GC cells highly associated with causing repression of activation of STAT3 and NF- κ B proteins via deacetylation.

Keywords: Sirtuin-1, STAT3, NF- κ B, gastric cancer, acetylation, activation

Introduction

Gastric cancer (GC) results in 70 thousand people death every year and has been considered as the second leading mortality related to cancer diseases worldwide [1, 2]. Prevalence of GC is particularly serious in Asian countries [3]. Newly diagnosed GC cases from Asian countries accounted for about three-quarters of total cases in 2008 [4]. Up to date, the 5-year survival rate of GC patients is less than 15%, given without effective treatments and early identification for this disease [5]. Owing to the high mortality-to-incidence ratio, exploration of available management of GC is challenging and urgent, including the exploration of new therapeutic targets of GC.

Sirtuin-1 (SIRT1), a class III protein deacetylase, deacetylates proteins to exert diverse physiological functions containing DNA repair, cellular stress resistance, modulating the

threshold for cell death and metabolic regulation [6, 7]. SIRT1 exerts the potential effects on diabetes, inflammation, and neurodegeneration also [7]. In terms of cancer, SIRT1 possess apparently dual roles [8, 9]. On the one hand, loss of SIRT1 function protects from colorectal cancer [10]. Some data also illustrated promotion of cell survival could be directly concerned with tumorigenesis in the presence of stress conditions by SIRT1 [11, 12]. On the other hand, SIRT1 prevented DNA from damage and resisted the replicative life span [8, 13, 14]. It meant SIRT1 was tumor suppressor. Thereby, SIRT1 exploited elaborate network which was depended on cellular and molecular contexts to promote or inhibit tumorigenesis. Regarding of GC, recent reports revealed SIRT1 activator inhibits the growth of GC cells [15, 16]. Instead, SIRT1 promoted multidrug resistance in GC cells via binding to activating transcription factor 4 [17]. Moreover upregulation of SIRT1 facilitated murine GC growth promoted by diet-induced

obesity [18]. It was controversial whether augmenting SIRT1 levels/activity was in favor of physiological function or not in GC.

At present, numerous regulators have been identified as the substrates of SIRT1, such as ATG APE1, FOXO, Ku70 and so on referring to autophagy, DNA repair activity, oxidative stress and other regulatory mechanisms in cancer [19-22]. Besides, SIRT1 inhibited the signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa-B (NF- κ B) signaling to exhibit anticancer effects in leukemia, lymphoma and myeloma [23-25]. As our known, STAT3 and NF- κ B have intrinsic activator effects on cancer inflammation and regulators of the tumor microenvironment [26, 27]. All the evidences provided for the hypothesis SIRT1 could play regulatory roles in the important factors (STAT3 and NF- κ B) to influence the tumorigenesis of GC. Unfortunately, tumor regulatory functions of SIRT1 were unclear in GC and it is largely lacking whether the regulatory effects of SIRT1 on STAT3 and NF- κ B played dominant roles in physiological function of GC cells.

In this research, SIRT1 activator resveratrol and inhibitor nicotinamide were applied to survey the effects of SIRT1 on viability and senescence of GC cells (AGS and MKN-45). Further study focused on the relationships between SIRT1 and activation of STAT3 and NF- κ B in GC cells.

Materials and methods

Cell lines, cell culture and treatments

GES-1 (human normal gastric epithelial cell line) was bought from Shanghai Institute of Cell Biology. Human GC cell lines (AGS and MKN-45) were acquired from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in DMEM (Gibco, Invitrogen, Carlsbad, USA) with 10% FBS in a humidified incubator containing 5% CO₂ at 37°C. Cells were subcultured after reached to 80% confluence. Cells were treated with indicated methods after confluence to about 90%. 10 mM resveratrol (RSV) and 5 mM nicotinamide (NA) (Sigma, St. Louis, USA) were performed to incubate cells [28]. SIRT1 knockdown (si) and NF- κ B p65 knockdown (NF- κ Bsi) were performed by using siRNA for SIRT1 and siRNA for NF- κ B p65 respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). STAT3-specific RNAi was used

to conduct the knockdown of human STAT3 (STAT3si) [29]. Non-Target shRNA Control was performed as negative control (si-control) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were incubated with RSV after SIRT1 knockdown. Total RNA or proteins were extracted after treatments for 24 h. All cellular transfections were conducted by Lipofectamine 2000 (Invitrogen, Carlsbad, USA).

RNA extraction and Quantitative real-time polymerase chain reaction (qRT-PCR) Total RNA was extracted from cells by RNeasy kit (Qiagen, Valencia, CA). qRT-PCR was conducted on ABI 7500 with SYBR green PCR kits (Applied Biosystems). GAPDH gene was named as endogenous control. The data were normalized by 2^{- $\Delta\Delta$ Ct} method as relative quantification. The primers were applied as follows. STAT3: F ggg tgg aga agg aca tca gcg gta a, R gcc gac aat act ttc cga atc c. c-Myc: F tag aat tgg att ggg gta aa, R cca acc aaa aat caa cat gaa t. GAPDH: F gga gtc aac gga ttt ggt c, R gga atc att gga aca tgt aaa c.

Viability assay and cell senescence assay

For viability assay, cells with indicated treatments were incubated in CCK-8 (DOJINDO, Kumamoto, Japan). The 450 nm absorbance of each well was assayed after visual color occurrence at indicated time points. All experiments were performed in triplicate. Data were expressed as the percentage of viability from normal cells. Cell senescence were measured by cellular senescence assay kit (Cell Biolabs, San Diego, CA) associated β -gal (SA- β -gal) assay as the manufacture's protocol. Briefly, cells were treated with RSV, NA or SIRT1 knockdown for 24 h. Then, cells were incubated with freshly prepared fluorometric substrate for 2 h at 37°C in darkness after washed and collected. The fluorescent intensity of each reaction mixture was determined and quantified by Image-J Software (NIH). The average fluorescence intensity was analyzed from five fields for each treatment. Data were expressed as the percentage of fluorescent intensity from normal cells. All experiments were performed in triplicate.

Western blot

Cells were lysed in lysis buffer (20 mM Tris HCL pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, protease and phosphatase inhibi-

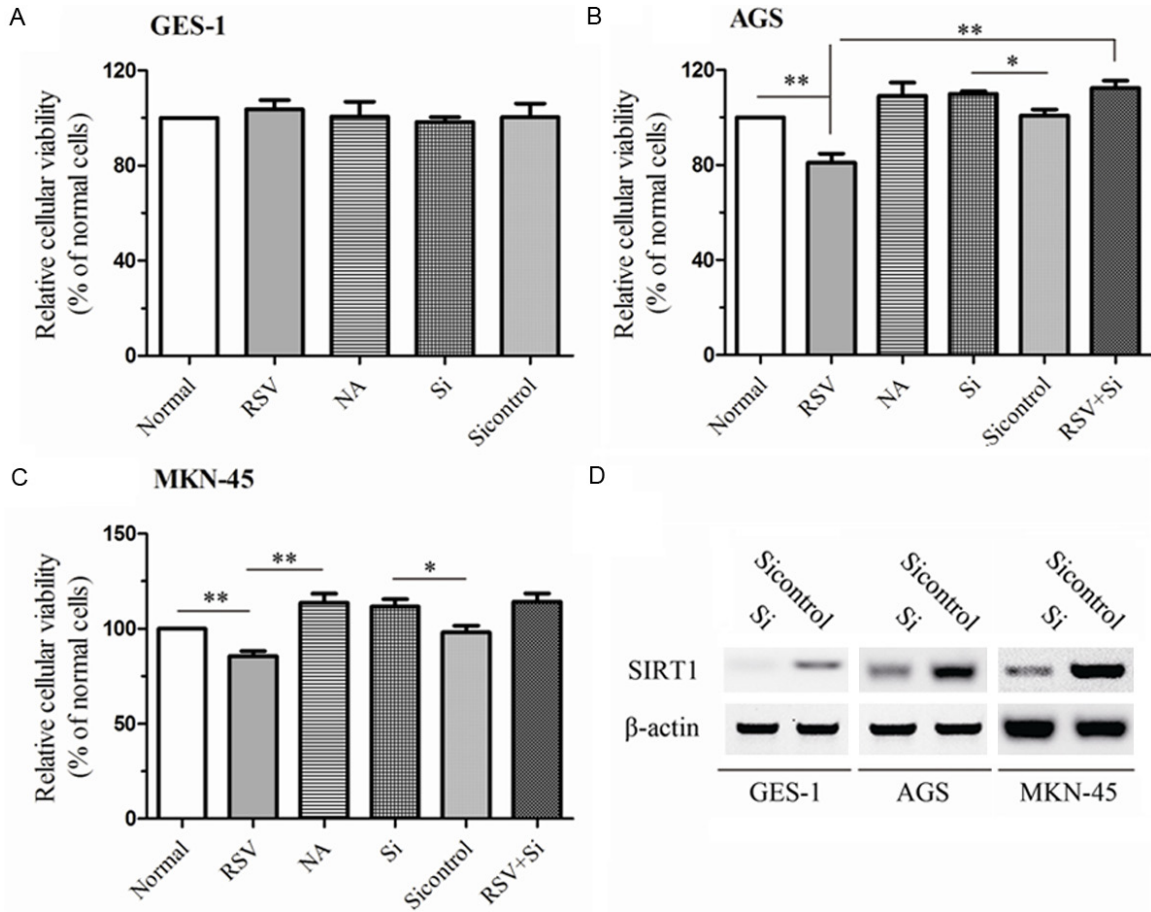


Figure 1. SIRT1 exerted the performances in influencing the cellular viability in normal gastric cells and in GC cells. Cells (GES-1, AGS and MKN-45) were silenced with siRNA for SIRT1 knockdown. The efficiency of knockdown was examined by western plot (D). Then, Cells or cells with silenced SIRT1 were preincubated with NA or RSV for 24 h. Normal, Si and Si control groups stood for the cells without treatment, cells with silenced SIRT1 and cells with Non-Target shRNA respectively. The viability of GES-1 (A), AGS (B) and MKN-45 (C) were measured by CCK-8 assay. All experiments were performed in triplicate. Data were expressed as the percentage of viability from normal cells. The data represented the mean \pm SEM. The statistical results are indicated by asterisks and **represents $P < 0.01$.

tors) followed by sonication on ice. Supernatants were fractionated by 10% SDS-PAGE gels and electro-transferred onto nitrocellulose membranes (Millipore) after centrifuged at 14,000 g for 30 min at 4°C. The membranes were incubated by primary antibodies. Then, the signals were acquired by using the ECL detection systems (Super Signal West Femto, Pierce) after incubated with horseradish-peroxidase-conjugated secondary antibodies (Pierce). Primary antibodies were used as follows: monoclonal STAT3 antibody (Minneapolis, Minnesota, USA), anti-phosphospecific STAT3 (Tyr705) (Cell Signaling Technology), anti-acetyl-STAT3 (Lys685) (Cell Signaling Technology), anti-acetyl-NF-κB p65 (Lys310), anti-phospho-NF-κB p65 (Ser536) (Cell Signaling Technology), Sirt1 primary

antibody (Sigma-Aldrich) and β-actin antibody (Sigma-Aldrich).

Data analysis

All data were calculated as Mean \pm SEM. Student t-test was used to compare two groups. Comparison among multiple samples was made by ANOVA. The SPSS 18.0 was used for statistical analysis. $P < 0.05$ was considered as statistically significant difference.

Results

SIRT1 reduced the viability of GC cells

Two GC cell lines (AGS and MKN-45) and human normal gastric epithelial cell line (GES-1) were

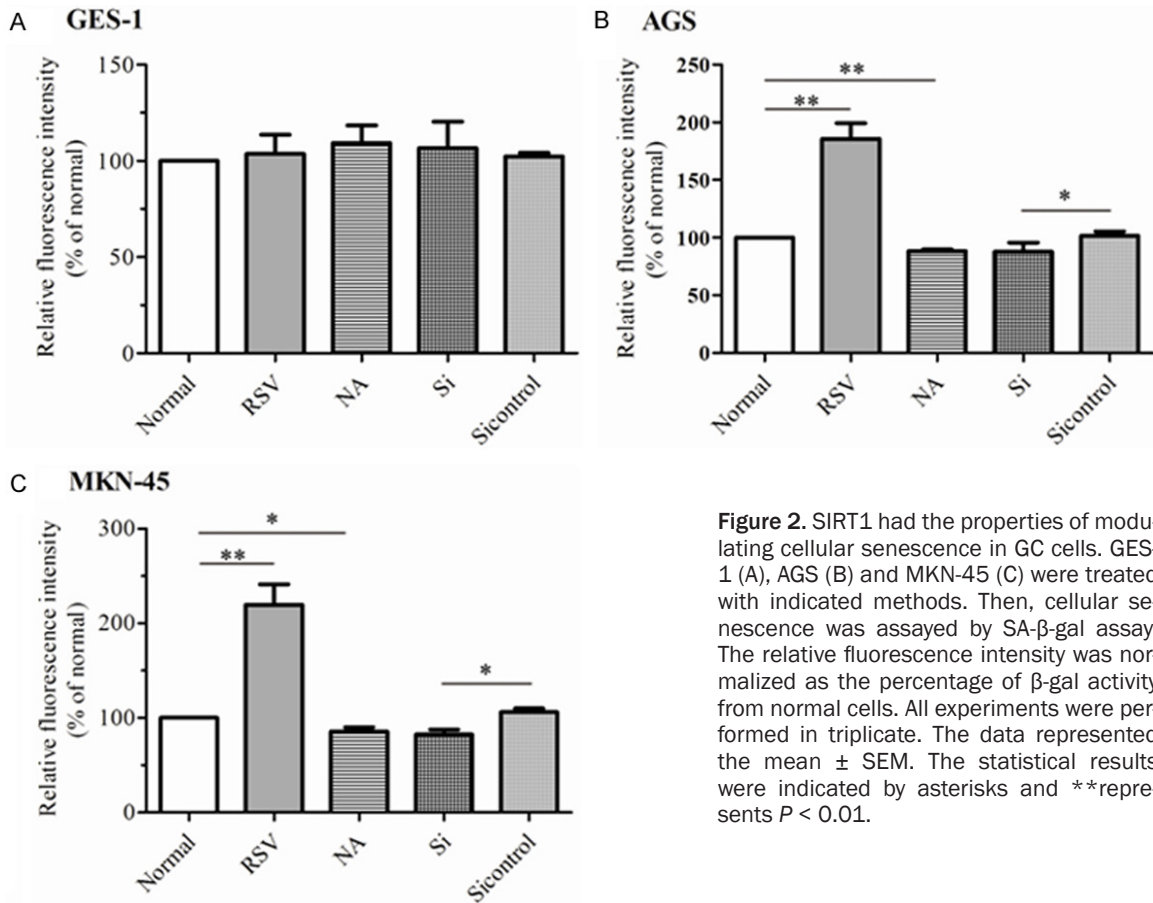


Figure 2. SIRT1 had the properties of modulating cellular senescence in GC cells. GES-1 (A), AGS (B) and MKN-45 (C) were treated with indicated methods. Then, cellular senescence was assayed by SA- β -gal assay. The relative fluorescence intensity was normalized as the percentage of β -gal activity from normal cells. All experiments were performed in triplicate. The data represented the mean \pm SEM. The statistical results were indicated by asterisks and **represents $P < 0.01$.

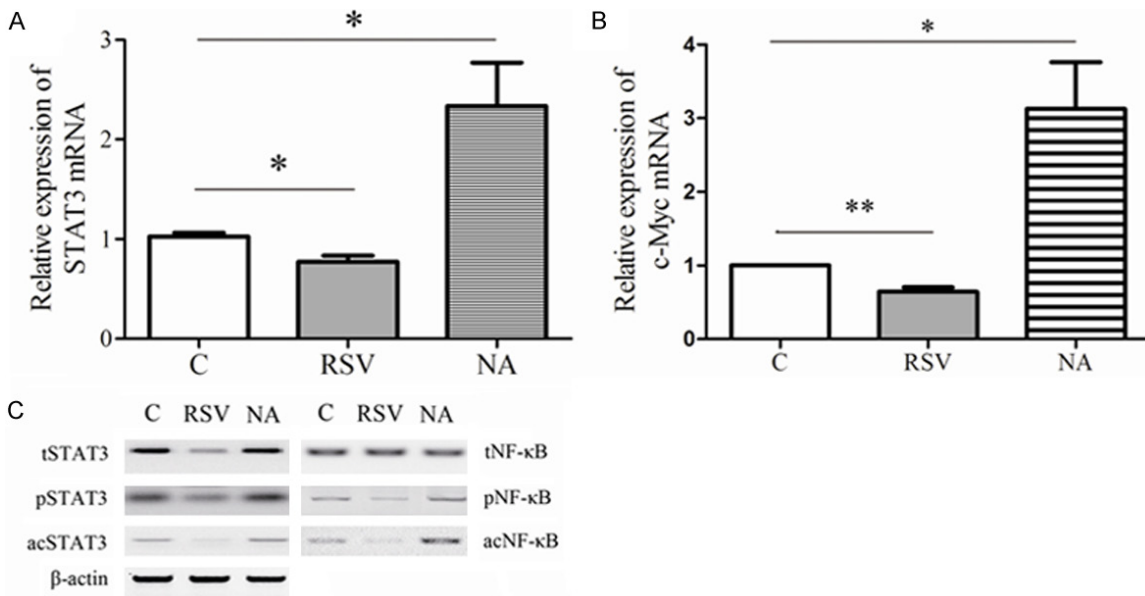
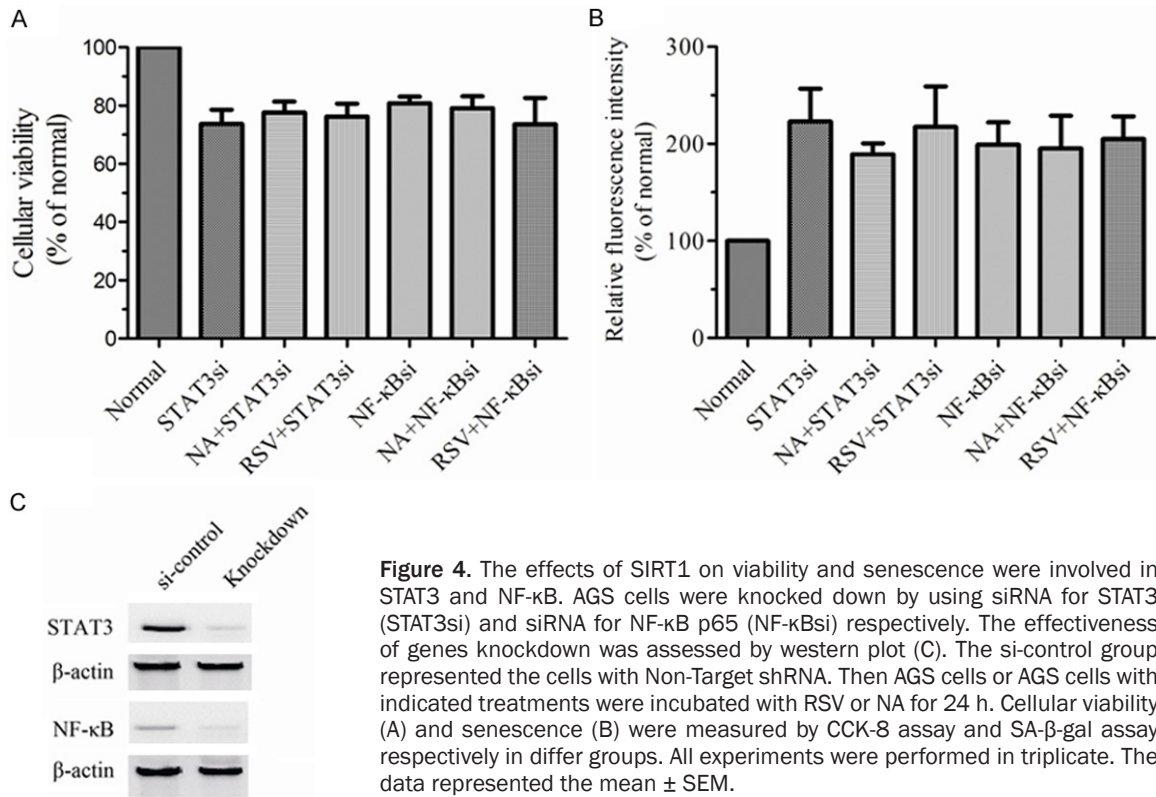


Figure 3. SIRT1 modulated activation of STAT3 and NF- κ B by deacetylation. AGS cells were incubated with RSV (RSV group) or NA (NA group) for 24 h. C group represented the AGS cells without treatment. Then, STAT3 mRNA (A) and c-Myc mRNA (B) were isolated from cells and measured by qRT-PCR. The data were normalized by $2^{-\Delta\Delta Ct}$ method as relative quantification and expressed as mean \pm SEM. The statistical results were indicated by asterisks and **represents $P < 0.01$. Total STAT3 proteins (tSTAT3), phosphorylated STAT3 proteins (pSTAT3), acetylated STAT3 proteins (acSTAT3), tNF- κ B, pNF- κ B and acNF- κ B were assessed by western plot (C) in three groups.

SIRT1 inhibited GC via STAT3 and NF-κB



applied in our study. The SIRT1 activator resveratrol (RSV 100 μM) and SIRT1 inhibitor nicotinamide (NA 5 mM) were performed to analyze the influences of SIRT1 on viability of GC cells. Treatment with RSV or NA could not significantly change the viability of GES-1 (**Figure 1A**). Interestingly, RSV remarkably inhibited viability of AGS cells as well as MKN-45 cells (80.96% for AGS, $P = 0.007$ and 85.52% for MKN-45, $P = 0.006$, respectively), when compared to the C group, as shown in **Figure 1B** and **1C**. In two GC cell lines, the presence of SIRT1 inhibitor NA augmented the amount of viability, by compared to normal cells (109.02% ± 9.90% for AGS and MKN-45, $P = 0.049$). We also silenced the expression of SIRT1 to further examine the effects of SIRT1. The efficiency of SIRT1 expression was confirmed by western plot (**Figure 1D**). The results displayed that the effects of SIRT1 knockdown were similar to the NA ($P > 0.05$) and RSV did not repressed viability in cells with SIRT1 knockdown, when compared to Si groups (112.37% ± 5.42% VS 109.93% ± 1.95% for AGS and 114.14% ± 7.57% vs. 111.71% ± 6.60% for MKN-45) as shown in **Figure 1B** and **1C**. These data demonstrated that SIRT1 activator contributed to the repression of viability,

which were rescued by SIRT1 inhibitor and SIRT1 depletion.

SIRT1 promoted the senescence of GC cells

Previous study had identified SIRT1 led to G1-phase arrest rather than apoptosis in GC cells [16]. As SIRT1 had the important antioxidative effects on combating oxidative stress and senescence, further we were interested in whether SIRT1 could reduce the senescence in GC cells. In GES-1 cells, SIRT1 could not change the cellular senescence obviously (**Figure 2A**). Of note, supplement of RSV induced senescence rather than repressed senescence in AGS cells as well as in MKN-45 cells, when compared with C groups (185.70% ± 23.65% for AGS and 219.68% ± 37.53% for MKN-45, $P < 0.001$) as shown in **Figure 2B** and **2C**. Moreover, addition of 5 mM NA could abolish the increase of β-gal activity induced by RSV in AGS cells, when compared to RSV groups ($P < 0.001$). The effects of SIRT1 knockdown were similar to NA and SIRT1 knockdown blocked the cellular senescence induced by RSV in AGS cells, by contrast to RSV group ($P < 0.001$) (**Figure 2B**). Similar results were acquired from

MKN-45 cells (**Figure 2C**). These data illustrated SIRT1 promoted the senescence of GC cells.

SIRT1 counteracted the activation of STAT3 and NF- κ B in GC in vitro.

STAT3 and NF- κ B have been considered as the substrates of SIRT1, which is a class III protein deacetylase [23-25]. Acetylation of NF- κ B was necessary to exploit its transcriptional activity [30]. Moreover, SIRT1 activation had been reported to have anti-myeloma, anti-lymphoma, and anti-leukemia effects which was involved in STAT3 and NF- κ B activity [31-33]. Thereby, we preferred to know whether SIRT1 was concerned with activation of STAT3 and NF- κ B in GC. The AGS cells were applied in these experiments. First, the relative expression of STAT3 and was examined by qRT-PCR. RSV significantly downregulated the STAT3 mRNA, compared to the C group ($P = 0.024$). NA increased the expression of total STAT3 mRNA dramatically, by contrast to C group ($P = 0.041$) as shown in **Figure 3A**. Moreover, c-myc mRNA level was compared to analyze the activation of STAT3 and NF- κ B as the c-myc gene has been identified as co-dependent target of STAT3 and NF- κ B [34]. RSV inhibited the expression of c-Myc mRNA (0.65 ± 0.10 , $P = 0.004$) and there was significant difference in expression of c-Myc mRNA expression between in NA group and C group (3.13 ± 1.10 , $P = 0.028$) as shown in **Figure 3B**. Following, we examined the proteins of total STAT3 (tSTAT3), phosphorylated STAT3 (pSTAT3), total NF- κ B p65, phosphorylated NF- κ B (pNF- κ B), acetylated NF- κ B (acNF- κ B) and acetylated STAT3 (acSTAT3) in various groups by western plot as shown in **Figure 3C**. RSV repressed the expression of total STAT3 proteins and pSTAT3 proteins as well as acSTAT3 proteins. Whereas, NA preincubation promoted expression of three kinds of proteins, including tSTAT3, pSTAT3 and acSTAT3. Excitingly, SIRT1 activation inhibited the expression of pNF- κ B and acNF- κ B, also.

To reconfirm the crosstalk between SIRT1 with STAT3 and NF- κ B, the cells were treated with STAT3 knockdown or NF- κ B p65 knockdown. **Figure 4C** showed the efficiency of knockdown. Following, the cellular viability and senescence were assessed (**Figure 4A, 4B**). According to application of RSV and NA, we found either RSV or NA could not change the cellular viability and senescence in MKN-45 cells with STAT3 knock-

down or NF- κ B knockdown. All the data revealed two mechanisms. The one was deacetylation inhibited the activation of STAT3 and NF- κ B in AGS cells. The other was SIRT1 counteracted the activation of STAT3 and NF- κ B in GC in vitro.

Discussion

It was controversial whether activation or inhibition of SIRT1 possessed more anticancer activity. SIRT1 might develop elaborate network to promote or inhibit tumorigenesis depending on cellular and molecular contexts [8, 9]. It was largely unknown whether SIRT1 had tumor regulatory functions in GC. In order to investigate the functions of SIRT1, we supplied resveratrol or nicotinamide for SIRT1 activation or its inhibition [28]. Cellular viability possesses key effects on mechanism for neoplastic progression [35]. In this study, we illustrated SIRT1 activator contributed to the repression of viability, which were rescued by SIRT1 inhibitor and Sirt1 depletion in GC cells (AGS and MKN-45) (**Figure 1B-D**). We found there was no remarkable change in viability of normal gastric epithelial cells (GES-1) in the presence of SIRT1 activation or its inhibition (**Figure 1A and 1D**) as well. The process of cellular senescence stands for an important barrier which combats cancer initiation and development [36, 37]. In our report, NA could abolish the increase of β -gal activity induced by RSV in GC cells (**Figure 2B and 2C**). It demonstrated SIRT1 accelerated the senescence of GC cells. Our data not only supported the reports of Yang et al about the anti-tumor activity of RSV [15], but also further improve the understanding that SIRT1 inhibitor and SIRT1 depletion reversed the effects of RSV on anti-GC.

STAT3 activation is characterized by STAT3 phosphorylation, which causes upregulation of its gene and regulation of its downstream genes to take intrinsic and extrinsic functions [26]. Constitutive STAT3 activation promotes gastric tumorigenesis in both human adenocarcinomas and mouse models [38]. It has been confirmed acetylation is critical and active for STAT3 phosphorylation. In addition, acetylation of NF- κ B p65 at Lys-310 exhibited enhanced phosphorylation and transcriptional activity [39]. Activation of deacetylase SIRT1 inhibited the STAT3 and NF- κ B activity to exert the anti-myeloma, anti-lymphoma, and anti-leukemia effects [23-25]. So we were interest-

ed in whether SIRT1 activator and inhibitor could influence activity of STAT3 and NF- κ B by deacetylation and acetylation in AGS cell line. Our data illustrated RSV induced the repression of the STAT3 and NF- κ B transcriptional activity in protein levels, as well as reduction of acSTAT3 and acNF- κ B. The NA displayed adverse effects of RSV (**Figure 3C**). The c-myc gene was the target and downstream gene of STAT3 activation [26, 40]. STAT3 and NF- κ B were interdependent. pro-oncogenic transcription factor c-myc gene was the target of STAT3 activation as well as the downstream factor of NF- κ B [34]. Thus, we assayed the expression of c-myc to assess the transcriptional activity of STAT3 and NF- κ B. It found RSV significantly reduced the c-myc mRNA level and NA abolished the reduction of c-myc mRNA level induced by RSV in AGS (**Figure 3B**). These results demonstrated SIRT1 counteracted the activation of STAT3 and NF- κ B by deacetylation. Deacetylation of SIRT1 was ubiquitination [8, 9, 41]. It meant SIRT1 had deacetylase activity affecting lots of transcription factors, besides STAT3 and NF- κ B. Previous researches verified SIRT1 had developed elaborate network to take diverse activities. So, whether the regulation of STAT3 and NF- κ B mediated by SIRT1 were dominant in physiological function of GC cells needed to be further investigated. According to silenced STAT3 and NF- κ B p65 genes (**Figure 4C**), we found either STAT3 knockdown or NF- κ B p65 knockdown could significant induce the loss of viability, but supplements of RSV and NA didn't change the effects of STAT3 or NF- κ B P65 knockdown significantly in AGS (**Figure 4A and 4B**). These data further supported SIRT1 counteracted the activation of STAT3 and NF- κ B to repress the GC growth.

Above all, our findings suggested SIRT1 activation could induced the loss of viability and increases of senescence in GC in vitro. Moreover, our observations revealed SIRT1 displayed growth inhibitory activity in GC cells highly associated with causing repression of activation of STAT3 and NF- κ B proteins via deacetylation. Our work provided detailed evidences for the anti-tumor mechanism of SIRT1 in GC. We proposed application of SIRT1 activators might be new therapeutic targets of GC.

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Disclosure of conflict of interest

None.

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