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Sirt2 suppresses glioma cell growth through targeting NF-κB–miR-21 axis

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ABSTRACT

Sirtuins are NAD$^+$-dependent deacetylases that regulate numerous cellular processes including aging, DNA repair, cell cycle, metabolism, and survival under stress conditions. The roles of sirtuin family members are widely studied in carcinogenesis. However, their roles in glioma remain unclear. Here we report that Sir2 was under expressed in human glioma tissues and cell lines. We found that Sirt2 overexpression decreased cell proliferation and colony formation capacity. In addition, Sirt2 overexpression induced cellular apoptosis *via* up-regulating cleaved caspase 3 and Bax, and down-regulating anti-apoptotic protein Bcl-2. Sirt2 knockdown obtained opposing results. We showed that Sirt2 overexpression inhibited miR-21 expression, and Sirt2 was not sufficient to reduce cell proliferation and colony formation as well as to induce apoptosis when miR-21 was knocked down in glioma cells. Mechanically, we demonstrated that Sirt2 deacetylated p65 at K310 and blocked p65 binding to the promoter region of miR-21, thus regressing the transcription of miR-21. In summary, Sirt2 is critical in human glioma *via* NF-κB–miR-21 pathway and Sirt2 activator may serve as candidate drug for glioma therapy.

1. Introduction

Since its initial characterization in the yeast *Saccharomyces cerevisiae*, the *silent information regulator 2* (*Sir2*) protein has attracted increasing interest in the scientific community, mostly based on its putative ability to extend lifespan in several model organisms [1]. Despite some controversy concerning their roles in organisms longevity [2], it is generally assumed that members of this family of enzymes act as stress-response and survival genes, exerting beneficial effects against age-related diseases.

The sirtuins are a highly conserved family of NAD$^+$-dependent enzymes. Recently, the mammalian sirtuins have been connected to an widening circle of activities that encompass cellular stress resistance, inflammation, genomic stability, carcinogenesis, and energy metabolism [3]. The mammalian sirtuin family is consisted of seven members, from Sirt1 to Sirt7. Cell biological studies have demonstrated different subcellular compartments for each family member, with Sirt6 and Sirt7 being nuclear proteins, Sirt3, Sirt4, and Sirt5 mitochondrial proteins, and Sirt1 and Sirt2 being both in the nucleus and the cytoplasm, in a cell- and tissue-dependent context [4].

Of the seven known mammalian sirtuin isoforms, relatively little is known about the predominantly cytosolic family member Sirt2. Sirt2 seems to be overexpressed during mitosis, affecting mitotic exit, and has been shown to deacetylate tubulin and histone H4 Lys 16 during mitosis [5,6]. Sirt2 participates in oxidative stress and adipocyte differentiation through deacetylating FOXO3a and FOXO1 respectively [7,8]. Sirt2 inhibition with selective inhibitors achieves neuroprotection by decreasing sterol biosynthesis [9]. Recently, Sirt2 was reported to participate in necrosis *via* deacetylating RIP1 [10]. However, the role of Sirt2 in carcinogenesis is under debate, especially in glioma. Human Sirt2 is most predominantly expressed in the brain [11]. Sirt2 is located at 19q13.2, a region known to be frequently deleted in human glioma. One work reported that Sirt2 was down-regulated in glioma tissues and inhibited colony formation [12], while the other one showed that Sirt2 inhibition reduced apoptosis and growth of rate C6 cells [13]. Those confusing data strongly prompted us to investigate the true roles of Sirt2 in (human) glioma and the underling mechanisms.

Here in this study, we found that Sirt2 expression was decreased in human glioma tissues and cell lines and Sirt2 acted as a tumor suppressor through inhibiting NF-κB-dependent miR-21 expression.

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2. Materials and methods

2.1. Patients

All human tissue samples of normal brain and glioma were obtained from the Department of Neurosurgery, Changhai Hospital (Shanghai, China). All samples were classified according to the third edition of the histological grades of tumors of the nervous system published by the WHO in 2000. Informed consent for the use of samples was obtained from all patients before surgery and approval was obtained from the Medical Ethics Committee of the Changhai Hospital.

2.2. Cell lines and cell culture

Human glioma cell lines T98G, U87MG, A172, U251, and CCF-STTG1 were purchased from the ATCC and cultured according to the guidelines recommended by the ATCC. All cells were maintained at 37 °C with 5% CO_2. The NHA cell line was purchased from the Lonza group and cultured with Clonetics medium and reagents. The other HA cell line was purchased from ScienCell Research Laboratories and cultured with astrocyte medium.

2.3. Preparation of recombinant adenovirus, infection, and treatment of glioma cells

To prepare Sirt2-expressing adenovirus, the human Sirt2 cDNA was inserted into D-TOPO vector (Invitrogen). The D-TOPO–Sirt2 plasmid was cloned into the pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase (Invitrogen). The plasmid was linearized with PacI and was transfected into 293A cells for production of adenovirus. Ad–Sirt2–H187Y (Ad–Sirt2HY, without deacetylase activity) was created using the Stratagene QuikChange site-directed mutagenesis kit (Invitrogen). For Ad–GFP, the GFP cDNA was inserted into D-TOPO vector instead of Sirt2 cDNA. Control siRNA and specific siRNA targeting Sirt2 were purchased from Santa Cruz Biotechnology. All treatments of glioma cells with AK-1 (Calbiochem) and AGK2 (Sigma) were at 10 and 7.5 μM respectively for 24 h before cells were harvested. All treatments were performed in serum-free medium. All treatment of glioma cells with temozolomide (TMZ, Sigma) was 100 μM for 6 h before cells were harvested.

2.4. Western blot

Tissues and glioma cells were lysed with cell lysis buffer (Beyotime) supplemented with protease inhibitor mixture. Western blot was performed as previously described [14]. The following antibodies were used: ac-p65 (K310, Abcam), p65 (Santa Cruz), Gapdh (Santa Cruz), caspase 3 (CST), Bax (Abcam), Bcl-2(CST), and Sirt2 (Santa Cruz).

2.5. Cell proliferation assay

Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (R&D SYSTEMS) in according to the guidelines.

2.6. Cell colony formation assay

Glioma cells were suspended in 1.5 ml complete medium supplemented with 0.45% low melting point agarose (Invitrogen). The cells were placed in 35 mm tissue culture plates containing 1.5 ml complete medium and agarose (0.75%) on the bottom layer. The plates were incubated at 37 °C with 5% CO_2 for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

2.7. FACS analysis and TUNEL assays

Cells were washed twice with cold PBS and then re-suspended in 1 × binding buffer (BD Biosciences) at a concentration of 1×10^6 cells/ml. Then, 100 ml of the solution (1×10^5 cells) was transferred to a 5 ml culture tube and stained with 5 ml each of allophycocyanin–annexin V (BD Biosciences) and 50 mg/ml propidium iodide (Invitrogen). The cells were gently mixed and incubated at room temperature for 15 min. For assessment of apoptosis, 400 ml of 1 × binding buffer was then added to each tube and the samples were analyzed by flow cytometry using a LSRII instrument (Becton–Dickinson).

The induction of apoptosis was also monitored by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. The TUNEL assay was performed in according to the guidelines recommended of the TUNEL Apoptosis Kit (R&D SYSTEMS).

2.8. Statistical analysis

All values are expressed as the mean ± SEM of at least three independent experiments. Statistical differences among groups were determined using either Student's *t* test or one-way ANOVA. Linear regression was used to analyze the correlation between miR-21 and Sirt2 expression levels. *p* values of less than 0.05 were considered statistically significant. Additional information for materials and methods is given in the Supplementary Information.

3. Results

3.1. Sirt2 level decreases in human glioma tissues and glioma cell lines

To examine the expression pattern of Sirt2 in glioma, we tested Sirt2 protein and mRNA levels in 8 primary glioma samples, which contain two grade II, five grade III, and one grade IV glioma tissues, and four normal brain tissues. We also determined protein and mRNA levels of Sirt2 in five human glioma cell lines (T98G, U87MG, U251, A172, and CCF-STTG1) and two normal human astrocyte cell lines (NHA and HA). The results showed that the protein and mRNA levels of Sirt2 were down-regulated in both glioma tissues and cell lines compared with normal brain tissues or astrocyte cells (Fig. 1A–D).

3.2. Sirt2 regulates glioma cell proliferation and colony formation

Three (T98G, U87MG, and U251) of the five cell lines were used as models for further study of Sirt2 function in glioma. To investigate the role of Sirt2 in glioma development, we used adenovirus system to overexpress (rescue) Sirt2 or siRNA system to down-regulate Sirt2 in glioma cells. Adenovirus-mediated overexpression of Sirt2 significantly increased its protein level (Suppl. Fig. 1A). In contrast, the expression of Sirt2 was down-regulated when cells were transfected with si-Sirt2 (Suppl. Fig. 1B). Sirt2 overexpression reduced cell proliferation rate of glioma cells, whereas Sirt2 knockdown promoted glioma cell proliferation (Fig. 1E,F and Suppl. Fig. 2). We next probed the contribution of Sirt2 in the transformative properties of glioma cells. Sirt2-overexpressing cells possessed reduced colony-forming activity, while Sirt2 knockdown promotes colony-forming activity in glioma cells (Fig. 1G–I). Taken together, these results indicated that Sirt2 negatively contributed to the tumorigenic phenotype of glioma cells.

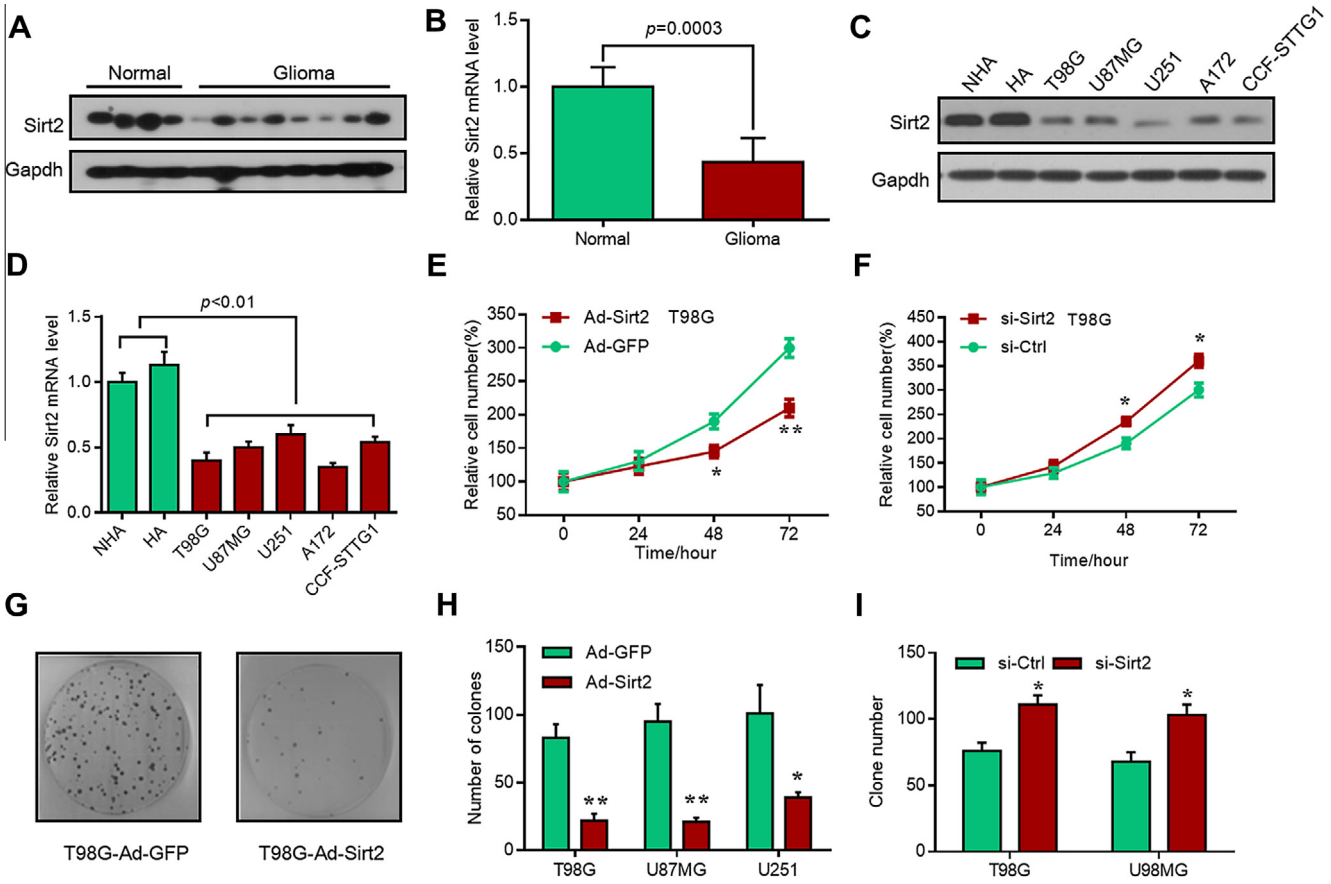

Fig. 1. Sirt2 decreases in glioma tissues and cell lines and regulates glioma cell growth. (A, B) Sirt2 protein and mRNA levels in normal human brain and glioma tissues. (C, D) Sirt2 protein and mRNA levels in normal human astrocyte cell lines (NHA and HA) and 5 glioma cell lines (T98G, A172, U251, U87MG, and CCF-STTG1). (E) Sirt2 overexpression reduces cell proliferation of T98G cells. (F) Sirt2 knockdown promotes cell proliferation of T98G cells. (G) Representative photos showing Sirt2 overexpression inhibits colony formation of T98G cells. (H) Sirt2 overexpression represses colony formation of T98G, U87MG, and U251 cells. (I) Sirt2 knockdown promotes colony formation of T98G and U87MG cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* Ad–GFP or si-Ctrl.

3.3. Sirt2 orchestrates glioma cell apoptosis

To investigate whether Sirt2 regulates glioma cell proliferation and colony formation by orchestrating apoptosis, we performed FACS to detect apoptotic cells. We found that Sirt2 overexpression significantly induced apoptosis in glioma cells (Fig. 2A). A quantitative analysis of the FACS results revealed a five–sevenfold increase in apoptosis in glioma cells infected with Sirt2 (Fig. 2B). As Sirt2 knockdown alone did not affect glioma cells survival (data not shown). We used TMZ, a clinical anti-glioma drug, to induce apoptosis in glioma cells. We found that Sirt2 knockdown desensitized glioma cells to TMZ treatment and reduced apoptosis and necrosis (Fig. 2C,D). To confirm those observations, we performed TUNEL assay and found increased DNA damage and fragmentation in glioma cells infected with Ad-Sirt2. A quantitative analysis also revealed a four–eightfold increase in apoptosis in glioma cells infected with Ad–Sirt2 (Suppl. Fig. 3A). In contrast, Sirt2 knockdown reduced percentage of TUNEL-positive cells induced by TMZ (Suppl. Fig. 3B). In addition, we extracted total proteins from T98G cells and analyzed the apoptotic proteins. The results showed that Sirt2 overexpression significantly activated pro-apoptotic proteins caspase 3 and Bax, and inhibited the anti-apoptotic protein Bcl-2 (Fig. 2E) and Sirt2 knockdown obtained opposing results in the presence of TMZ (Fig. 2F).

3.4. Sirt2 regulates miR-21 expression

miR-21 is widely accepted as an oncogenic microRNA and its role has been deeply investigated in human glioma. miR-21 was reported to be an important anti-apoptotic factor and regulates drug resistance in human glioma [15,16]. We confirmed here that miR-21 was overexpressed in human glioma tissues (Fig. 3A). To explore whether miR-21 level is correlated with Sirt2 mRNA level in human glioma tissues, we performed linear regression analysis and found that miR-21 level was significantly and negatively correlated with Sirt2 mRNA level (Fig. 3B). What is more, we also detected the overexpression of miR-21 in glioma cells compared to normal human astrocyte cells (Fig. 3C). Next, we wanted to know whether Sirt2 regulates the expression of miR-21. We found that miR-21 level was significantly decreased in T98G cells infected with Ad–Sirt2 (Fig. 3D). Our luciferase assay showed that Sirt2 overexpression reduced the promoter activity of miR-21 (Fig. 3E), confirming that Sirt2 can decrease the transcription of miR-21.

3.5. miR-21 is essential for Sirt2-overexpression-induced growth regression and apoptosis

As we have demonstrated that Sirt2 regressed the expression of miR-21, we wanted to know whether miR-21 was essential for the function of Sirt2. We knocked down miR-21 in T98G cells using specific shRNA. shRNA-mediated knockdown of miR-21 reduced its level by at least 80% (Suppl. Fig. 4). miR-21 knockdown markedly increased proliferation rate of glioma cells. However, when miR-21 was knocked down, Sirt2 overexpression could not affect the proliferation and colony formation of glioma cells (Fig. 3F,G). Interestingly, Sirt2 overexpression was also unable to induce apoptosis in shRNA–miR-21 transduced glioma cells (Fig. 3H,I). This was

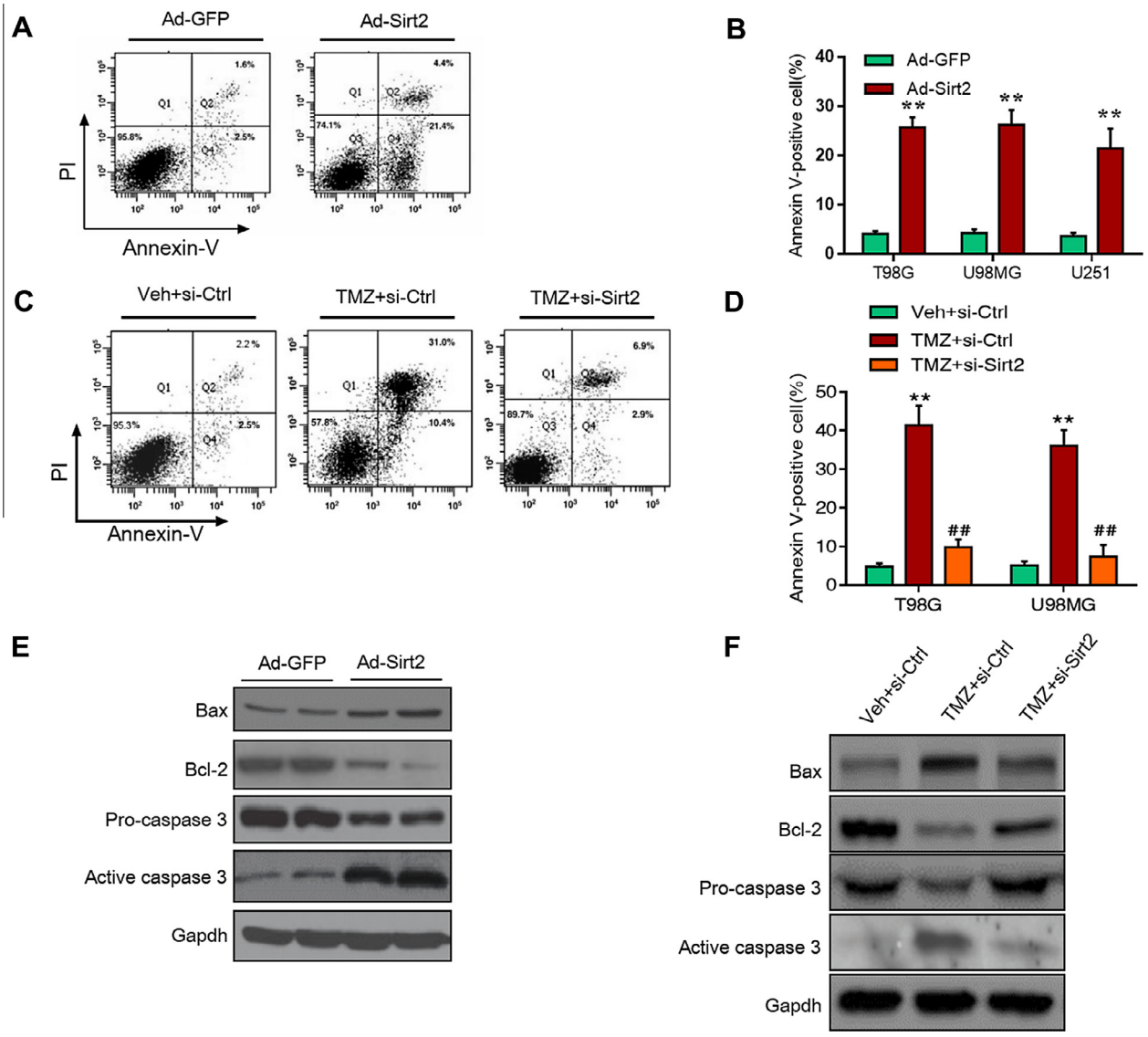

Fig. 2. Sirt2 orchestrates apoptosis in glioma cells. (A) Representative FACS analysis of annexin V and propidium iodide (PI) staining of T98G cells infected with Ad–GFP or Ad–Sirt2. (B) Sirt2 overexpression promotes apoptosis (Annexin V-positive) of T98G, U87MG, and U251 glioma cells. $^{**}p < 0.01$ *vs.* Ad–GFP. (C) Representative FACS analysis of annexin V and PI staining showing the effects of Sirt2 knockdown on TMZ-induced apoptosis of T98G cells. (D) Sirt2 knockdown promotes apoptosis of T98G and U87MG cells. $^{**}p < 0.01$ *vs.* Veh + si-Ctrl; $^{\#\#}p < 0.01$*vs.* TMZ + si-Ctrl. (E) Representative western blot showing Sirt2 overexpression activates caspase 3, Bax, and inhibits Bcl-2 in T98G cells. (F) Sirt2 knockdown represses TMZ-induced activation of caspase 3, Bax, and inhibits TMZ-induced inhibition of Bcl-2 in T98G cells.

in consistent with previous report that miR-21 protected human glioblastoma U87MG cells from TMZ-induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase 3 activity [17].

3.6. Sirt2 deacetylates p65 to regulates miR-21 expression

Since Sirt2 can reduce the expression of miR-21, we next wanted to investigate the underling mechanism. In consistence with previous reports in other systems [10,18], we showed in glioma cells that Sirt2 reduced p65 acetylation level, which depended on the deacetylation activity of Sirt2 (Fig. 4A,B). What is more, Sirt2 inhibitors, AK1, and AGK2, also blocked the deacetylation of p65 (Fig. 4C). Using ChIP assay, we showed that p65 could binding to the promoter region of miR-21 gene, which was blocked by Sirt2 (Fig. 4D). Further, we confirmed that the acetylation of K310 of p65 promoted its binding to miR-21 promoter, but Sirt2 could not reduce binding of p65K310Q (lysine–glutamine mutant, mimic of acetylated p65 at amino acid 310) to miR-21 promoter (Fig. 4E). Finally, we showed that p65 knockdown with sh-RNA markedly reduced the expression of miR-21 and Sirt2 was unable to inhibit miR-21 expression when p65 was knocked down (Fig. 4F).

4. Discussion

In the present study, we found that Sirt2 expression was markedly down-regulated in glioma tissues and glioma cell lines. Our *in vitro* proliferation and colony formation experiments strongly demonstrated that Sirt2 reduced glioma cell proliferation and colony formation. In addition, using FACS and TNUEL assay as well as western blot we showed that Sirt2 regulated cellular apoptosis. We found that miR-21 was up-regulated in human glioma tissues and cell lines, and its expression level was significantly and negatively correlated with Sirt2 mRNA level. When Sirt2 was overexpressed, the expression of miR-21 was significantly down-regulated in glioma cells. Interestingly, Sirt2 was not sufficient to enhance glioma cell proliferation, colony formation as well as cellular apoptosis

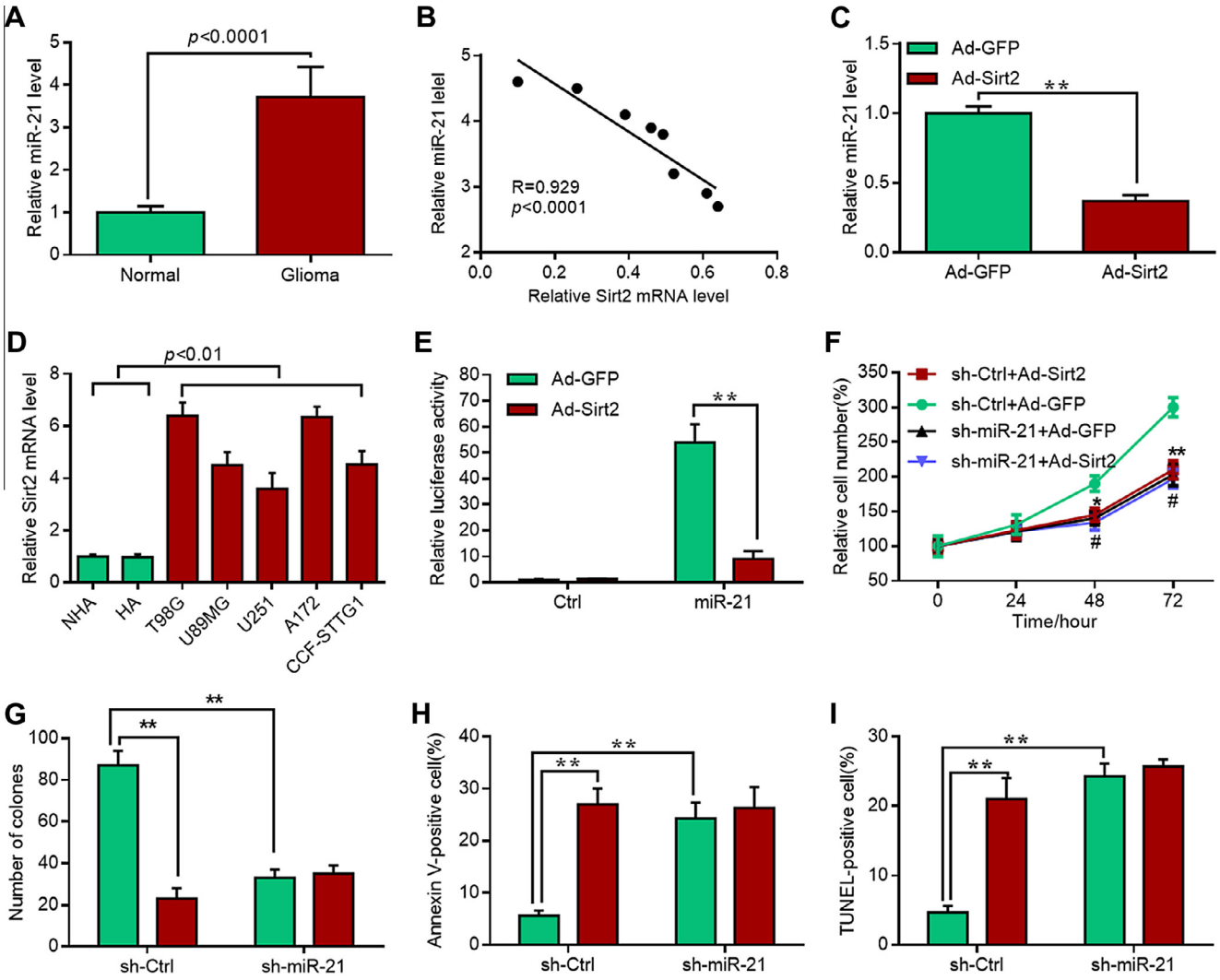

Fig. 3. miR-21 is essential for Sirt2 function (A) miR-21 level increases in human glioma tissues. (B) miR-21 level negatively correlates with the mRNA level of Sirt2. (C) miR-21 overexpresses in human glioma cell lines compared with normal human astrocyte cell lines. $^{**}p < 0.01$. (D) Sirt2 overexpression inhibits the expression of miR-21 in T98G cells. (E) Sirt2 overexpression decreases luciferase activity of miR-21 promoter. (F) Sirt2 overexpression cannot affect cell proliferation of T98G cell when miR-21 was knocked down with specific sh-RNA. $^{*}p < 0.05$ and $^{**}p < 0.01$ sh-Ctrl + Ad–Sirt2 *vs.* sh-Ctrl + Ad–GFP. $^{\#}p < 0.05$ sh-Ctrl + Ad–GFP *vs.* sh-miR-21 + Ad–GFP. (G) Sirt2 overexpression cannot affect colony formation of T98G cells when miR-21 was knocked down. (H, I) Sirt2 overexpression cannot affect the percentage of Annexin V-positive or TUNEL-positive cells of T98G cells when miR-21 was knocked down. $^{*}p < 0.05$ and $^{**}p < 0.01$.

when miR-21 was knocked down. Mechanically, we found that Sirt2 deacetylated p65 and blocked p65 binding to the miR-21 promoter, thus regressing the transcription of miR-21.

Hiratsuka et al. [12] reported that Sirt2 decreased in human glioma tissues and cell lines and Sirt2 overexpression reduced colony formation of human glioma cells. Interestingly, another work contributed by He et al. [13] last yeast obtained opposing conclusion using rate glioma cell line C6. In the work of Hiratsuka et al., they did not show whether Sirt2 was important for glioma growth and how Sirt2 participated in glioma. What is more, they only used one cell line for the colony formation study. He et al. used Sirt2 inhibitor AGK2 in most of their experiments. However, they failed to show the expression level of Sirt2 in C6 cells, and no data of the effects of AGK2 of on Sirt2 activity in their system was given. They could not exclude the possibility that AGK2 itself induced apoptosis, but not through inhibiting the activity of Sirt2. What is more, they did not provide strong evidence to support their conclusion that Sirt2 knockdown induced C6 cell apoptosis. Finally, C6 is a rat glioma cell line. This cell line does not represent the situation in human glioma. In the present work, we systemically studied the expression pattern of Sirt2 in human glioma tissues and five cell lines, and explored the effects of Sirt2 on cellular proliferation and colony formation in three cell lines by gain-of-function and lose-of-function. Those data strongly indicated Sirt2 acts as a tumor suppressor in human glioma (Fig. 1).

Sirt2 plays a complex role in apoptosis and necrosis. Sirt2 enhances cell death induced by H_2O_2 and staurosporine by up-regulating the apoptotic protein Bim in NIH3T3 cells [8]. Sirt2 deacetylates FoXO3a, increases RNA and protein levels of Bim, and as a result, enhances apoptosis in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson disease [19]. Sirt2 functions to moderate cellular stress–tolerance, in part, by modulating the levels of 14-3-3ζ with the concordant control of BAD subcellular localization [20]. Sirt2-deficience protects MEFs from TNFα-induced apoptosis. However, opposing evidence show that Sirt2 absence sensitizes cells to apoptotic stimuli *via* regulating p53 [21,22]. Recently, Sirt2 was evidenced in the process of necrosis *via* binding to RIP3 and deacetylating RIP1 [23]. Those works also strongly challenge the work of He et al. [13]. In the present study, we demonstrated that Sirt2 is a pro-apoptotic factor in

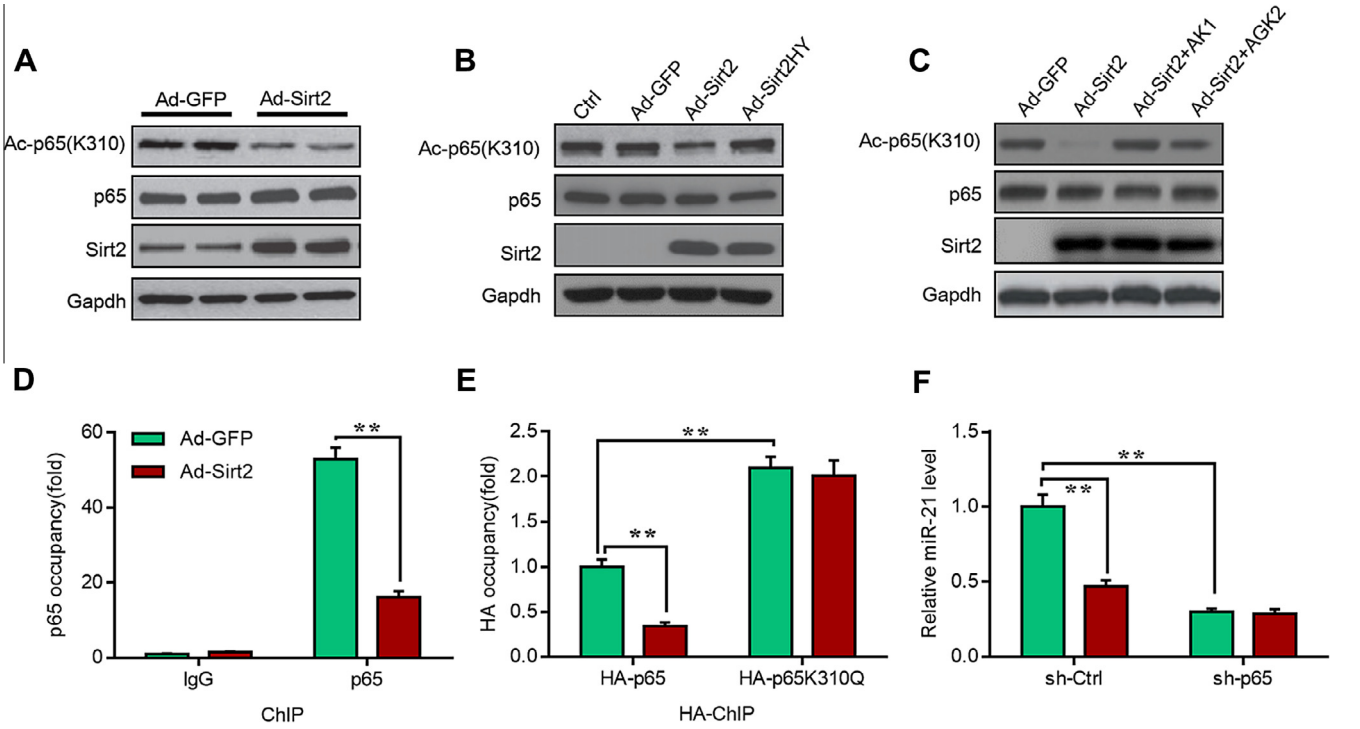

Fig. 4. Sirt2 deacetylates p65 to regulate miR-21 expression (A) Sirt2 overexpression decreases acetylation level of p65 in T98G cells. (B) Sirt2H187Y mutant cannot affect the acetylation level of p65. (C) Sirt2 inhibitors block p65 deacetylation by Sirt2. (D) Sirt2 overexpression inhibits p65 binding to miR-21 promoter. (E) Sirt2 cannot block p65K310Q binding to miR-21 promoter. (F) Sirt2 cannot affect the expression of miR-21 when p65 was knocked down with specific sh-RNA. The knockdown efficacy of p65 is shown in Suppl. Fig. 5. $^{**}p < 0.01$.

glioma cells. We found that Sirt2 orchestrates cellular apoptosis through activating pro-apoptotic proteins caspase 3, Bax, and down-regulating the anti-apoptotic protein Bcl-2. Those results were consistent in the three glioma cell lines used in our study, indicating Sirt2 is a pro-apoptotic factor in glioma cells (Fig. 2).

miR-21 acts as an oncogenic microRNA and its participation has been described in many tumors, such as glioma. miR-21 was demonstrated to be an anti-apoptotic factor in human glioblasma [15]. Reduction of miR-21 induced glioma cell apoptosis *via* activating caspase 9 and 3 [24]. miR-21 knockdown disrupted glioma growth *in vivo* and displayed synergistic cytotoxicity with neural precursor cell-delivered S-TRAL in human glioma [16]. Down-regulation of miR-21 in glioblastoma cells leads to repression of growth, increased apoptosis, and cell cycle arrest. miR-21 protected U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax/Bcl-2 ratio and caspase 3 activity [17]. However, how miR-21 is regulated is still not fully understood. Here we found that miR-21 expression level was negatively correlated with Sirt2 mRNA level in human glioma and Sirt2 inhibited the miR-21 expression. When miR-21 was knocked down, the overexpression of Sirt2 was not sufficient to induce cell proliferation arrest and to reduce colony formation. In addition, Sirt2 cannot induce cell apoptosis in miR-21 knockdown cells (Fig. 3). Those findings identified a novel upstream of miR-21 and miR-21 is essential for Sirt2 function in glioma cells.

Activation of NF-κB is sufficient to activate the expression of miR-21 [25]. Binding of NF-κB p65 subunit to the promoter is involved in transactivation of miR-21 [26]. Sirt2 regulates NF-κB-dependent gene expression through deacetylation of p65 on K310 [10]. When this paper was under preparation, Paris et al. [18] also reported that Sirt2 deacetylated NF-κB in the brain and regulates microglial activation and brain inflammation. Here we also found that Sirt2 can deacetylate p65 at K310 in glioma cells. Acetylation of p65 at K310 is essential for its binding to miR-21 promoter. Deacetylation of p65 blocked its binding to miR-21 promoter and regressed miR-21 expression (Fig. 4). This finding uncovered the underling mechanism by which Sirt2 regulated miR-21 expression.

In summary, we conclude that Sirt2 is down-regulated in glioma and regresses glioma growth *via* a NF-κB–p21-apoptosis axis. Those findings indicate that Sirt2 is a tumor suppressor gene in human glioma and Sirt2 activator may serve as candidate drug for therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.077.

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