Biochemical and Biophysical Research Communications 446 (2014) 364–369

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The histone deacetylase SIRT6 suppresses the expression of the RNA-binding protein PCBP2 in glioma

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ARTICLE INFO

Article history:
Received 22 February 2014
Available online 4 March 2014

Keywords:
SIRT6
PCBP2
H3K9ac
Glioma

ABSTRACT

More than 80% of tumors that occur in the brain are malignant gliomas. The prognosis of glioma patients is still poor, which makes glioma an urgent subject of cancer research. Previous evidence and our present data show that PCBP2 is over-expressed in human glioma tissues and predicts poor outcome. However, the mechanism by which PCBP2 is regulated in glioma remains elusive. We find that SIRT6, one of the NAD^+-dependent class III deacetylase SIRTUINs, is down-regulated in human glioma tissues and that the level of SIRT6 is negatively correlated with *PCBP2* level while H3K9ac enrichment on the promoter of *PCBP2* is positively correlated with *PCBP2* expression. Furthermore, we identify *PCBP2* as a target of SIRT6. We demonstrate that *PCBP2* expression is inhibited by SIRT6, which depends upon deacetylating H3K9ac. Finally, our results reveal that SIRT6 inhibits glioma cell proliferation and colony formation *in vitro* and glioma cell growth *in vivo* in a PCBP2 dependent manner. In summary, our findings implicate that SIRT6 inhibits *PCBP2* expression through deacetylating H3K9ac and SIRT6 acts as a tumor suppressor in human glioma.

1. Introduction

Eighty percent of tumors that develop in central neuron system are malignant gliomas, which are essentially incurable. Despite decades of concerted effort and advances in surgery, radiation, and chemotherapy, the overall 5-year survival rate of GBM remains less than 5% and is even worse for elderly patients [1]. This dismal clinical outcome makes glioma an urgent subject of cancer research, and identification of new therapeutic targets is critically important.

PCBP2 is a member of the poly(C)-binding protein (PCBP) family, which plays an important role in posttranscriptional and translational regulation by interacting with single-stranded poly(C) motifs in target mRNAs [2]. Several PCBP family members have been reported to be involved in human malignancies. In this family, PCBP2 is one of the least studied protein in human cancers among the PCBPs. Most of the reports on PCBP2 have focused on its posttranscriptional and translational controls in RNA viruses [3,4]. Recent findings have implicated that PCBP2 may be an orchestrator of tumor development. For instance, Molinaro et al. [5] showed that 2′, 5′-oligoadenylate synthetase (OAS) activation may occur in prostate cancer cells *in vivo* when stimulated by PCBP2. In leukemic blasts, PCBP2 expression is induced by BCR/ABL through constitutive activation of ERK1/2 [6]. Recently, Han et al. [7] found that PCBP2 was overexpressed in human glioma tissues and predicted adverse survival. They identified that four-and-a-half LIM domain 3 (FHL3), which was recently reported to be a tumor suppressor [8], as a PCBP2 target. Those findings suggested that PCBP2 may be a potential target for glioma therapy. However, it still remains elusive how PCBP2 is up-regulated in tumors, especially in glioma.

SIRT6 is a member of the NAD^+-dependent class III deacetylase SIRTUIN family. Current studies have revealed the role of SIRT6 in genome stability, metabolism, inflammation, longevity, and carcinogenesis [9–15]. SIRT6 regulates the development of various types of cancers, including liver cancer [16,17], breast cancer [18], pancreatic cancer and colon adenocarcinoma [15]. SIRT6 regulates tumor development by directly interacting with oncogenic proteins (e.g. MYC) or by modulating acetylation of histone 3 lysine 9 (H3K9ac) at the promoter of oncogenes [15–17]. However, the role of SIRT6 in glioma remains unknown. Our previous work and work form other groups have demonstrated that

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http://dx.doi.org/10.1016/j.bbrc.2014.02.116
0006-291X/

SIRT1 and SIRT2 critically participate in glioma development [19,20]. Therefore, we hypothesized that SIRT6 may be also involved in glioma biology.

Here in this study, we identified SIRT6 as an important tumor suppressor in human glioma by targeting PCBP2.

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 fourth edition of the histological grades of tumors of the nervous system published by the WHO in 2007 [21]. 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. Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to the manufacturer's instructions. In brief, chromatin from cells was cross-linked with 1% formaldehyde (10 min at 22 °C), sheared to an average size of ~500 bp and then immunoprecipitated with anti-SIRT6, anti-IgG or anti-H3K9ac antibodies. The ChIP-PCR primers listed in Supplementary Table 1 were used to amplify a proximal promoter region containing putative binding sites in the PCBP2 promoter identified by TFSEARCH.

2.4. 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.

2.5. Soft agar 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. The colony number in each well was calculated.

2.6. Xenograft mice experiment

Xenograft mice experiments were performed as described previously [7]. The tumor weight was evaluated at the terminal of experiments.

2.7. Statistical analysis

All values are expressed as the means ± SEM of at least three independent experiments if no additional information was indicated. Statistical differences among groups were determined using either Student's *t* test or one-way ANOVA. *p* values of less than 0.05 were considered statistically significant.

3. Results

3.1. PCBP2 is up-regulated in glioma and cell lines

As shown by Han et al. [7], PCBP2 level was up-regulated in human glioma tissues and cell lines. In this study, we explored the change in *PCBP2* expression in glioma tissues using a relative larger cohort of sample with 11 normal brain tissues and 31 glioma tissues. In consistence with the findings of Han et al., we found that *PCBP2* protein level and mRNA level were significantly up-regulated in human glioma tissues compared to those in normal brain tissues (Fig. 1A and B). In addition, the protein and mRNA levels of *PCBP2* were also increased in human glioma cell lines (Fig. 1C and D). In summary, *PCBP2* expression was up-regulated in glioma tissues and cell lines.

3.2. SIRT6 is down-regulated in glioma and correlates with PCBP2 expression

Because Han et al. [7] have demonstrated PCBP2 as a tumor supporter in glioma, we did not want to re-explore the role of PCBP2 in glioma. Instead, we wanted to know how PCBP2 is regulated in glioma. As SIRT6 has been reported to be a tumor repressor in several tumors, we hypothesized that SIRT6 may regulate the expression of PCBP2 in glioma. Firstly, we explored the expression level of SIRT6 in human glioma tissues. Our results showed that SIRT6 protein and mRNA levels were markedly down-regulated in human glioma tissues compared to those in normal brain tissues (Fig. 2A and B). Similar results were obtained in glioma cell lines (Data not shown). Next, we asked whether the expression of SIRT6 was correlated with expression of PCBP2. We performed linear regression analysis and found that the *SIRT6* mRNA level was negatively but significantly correlated with the *PCBP2* mRNA level (Fig. 2C), which implicated that SIRT6 may repress PCBP2 expression in glioma.

To further push this hypothesis, we performed ChIP assay in U251 cells and the results showed that SIRT6 can specifically bind to the promoter region of the *PCBP2* gene (Supplementary Fig. 1). We chose the region where SIRT6 abundance is the highest for further indicating SIRT6 binding ability. In addition, we also showed that SIRT6 bound to the promoter of *PCBP2* in glioma tissues (Fig. 2D). As SIRT6 regulates gene expression through deacetylating H3K9ac, we tested the H3K9ac level on the promoter region of the *PCBP2* gene using ChIP assay. We found that the H3K9ac level was significantly up-regulated on the promoter region of the *PCBP2* gene (Fig. 2E). Furthermore, our results showed that global H3K9ac level was also up-regulated (Fig. 2F), which is consistent with previous findings that SIRT6 regulated global H3K9ac level [22]. Accumulating data has demonstrated that H3K9ac is correlated with transcription activation. Therefore, we performed linear regression analysis to analyze the correlation between *PCBP2* mRNA level and H3K9ac enrichment on the *PCBP2* promoter. The results showed that H3K9ac enrichment was positively correlated with *PCBP2* mRNA level, implicating that H3K9ac enrichment on the PCBP2 promoter promotes its expression (Fig. 2G). Taken together, those findings revealed that SIRT6 expression was

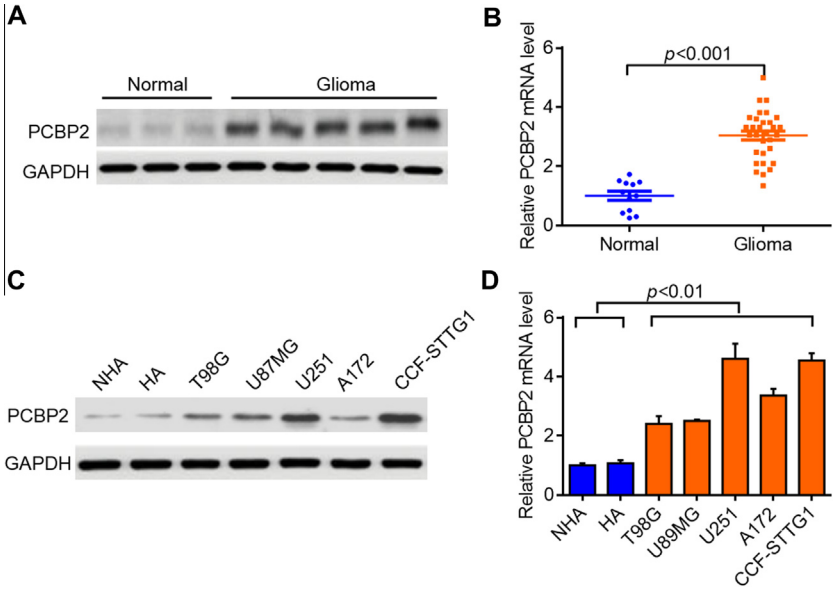

Fig. 1. PCBP2 is up-regulated in human glioma tissues and cell lines. (A and B) The protein and RNA were extracted from normal and glioma tissues and were subjected to Western blot and q-PCR analysis. (A) PCBP2 protein level is up-regulated in human glioma tissues. (B) *PCBP2* mRNA level is up-regulated in human glioma tissues. $N = 11$ in normal group and $n = 31$ in glioma group. (C and D) The protein and RNA were extracted from normal and glioma cell lines and were subjected to Western blot and q-PCR analysis. (C) PCBP2 protein level is up-regulated in glioma cell lines. (D) *PCBP2* mRNA level is up-regulated in glioma cell lines.

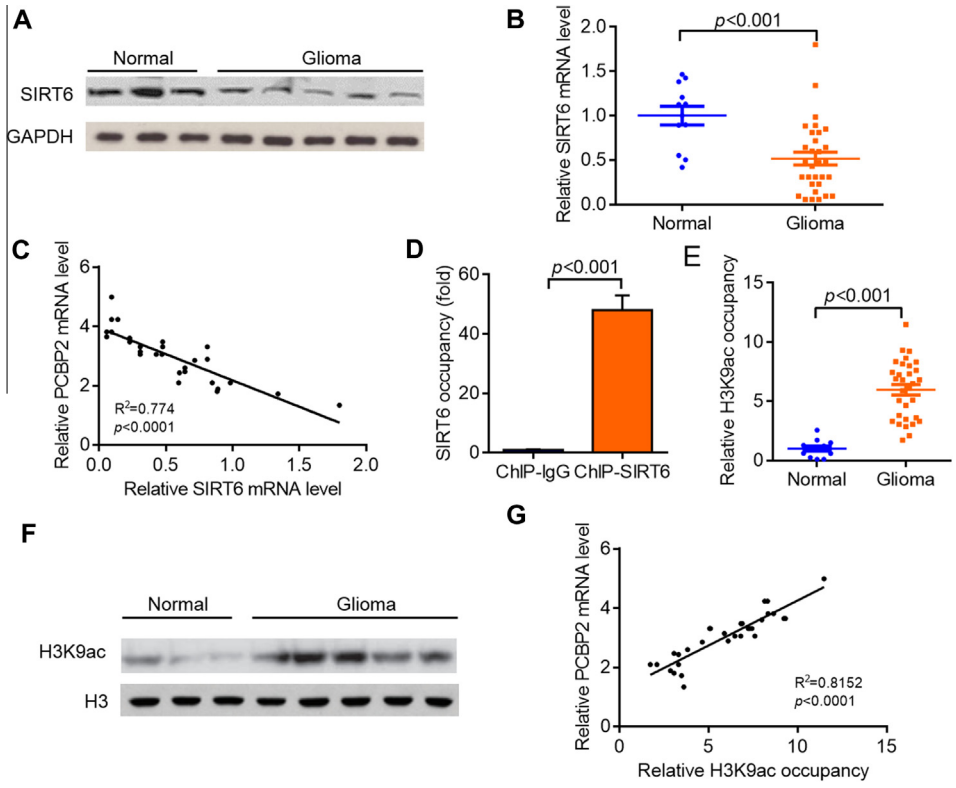

Fig. 2. SIRT6 is down-regulated in glioma and correlates with *PCBP2* expression. (A and B) The protein and RNA were extracted from normal and glioma tissues and were subjected to Western blot and q-PCR analysis. (A) SIRT6 protein level is down-regulated in glioma tissues. (B) *SIRT6* mRNA level is down-regulated in glioma tissues. $N = 11$ in normal group and $n = 31$ in glioma group. (C) *SIRT6* mRNA level is correlated with PCBP2 mRNA level. Linear regression analysis was performed to analyze the correlation between *PCBP2* mRNA level and SIRT6 *mRNA* level $N = 31$. (D and E) ChIP assay was performed using glioma tissues with anti-SIRT6, anti-H3K9ac or anti-IgG antibodies. The relative fold changes were shown. (D) SIRT6 can binds to the promoter region of *PCBP2* gene. (E) H3K9ac is enriched on the promoter region of *PCBP2* gene in glioma tissues compared to normal brain tissues. (F) Global H3K9ac level is decreased in glioma tissues. (G) H3K9ac enrichment is correlated with *PCBP2* mRNA level. Linear regression analysis was performed to analyze the correlation between H3K9ac enrichment level on the promoter region of *PCBP2* and *PCBP2* mRNA level. $N = 31$.

down-regulated in glioma tissues and was negatively correlated with *PCBP2* mRNA level, which may depend on H3K9ac.

3.3. SIRT6 down-regulates PCBP2 by deacetylating H3K9ac

To further confirm whether SIRT6 inhibits PCBP2 expression, we overexpressed or knocked-down SIRT6 in human U251 glioma cells. We found that SIRT6 overexpression down-regulated PCBP2 mRNA level, while SIRT6 knockdown up-regulated PCBP2 mRNA level (Fig. 3A and B). In fact, we found that four members (SIRT1, 2, 6, 7) of the SIRTUINs changed in glioma tissues (Supplementary Fig. 2). Interestingly, only SIRT6 could affect the expression of PCBP2 (Supplementary Fig. 3). Western Blot results also revealed that SIRT6 inhibited PCBP2 expression and down-regulated H3K9ac level (Fig. 3C). We next wanted to know whether the deacetylase activity of SIRT6 is essential for its effects on PCBP2. We generated two SIRT6 mutants (SIRT6S56Y and SIRT6H133Y) without H3K9ac deacetylation activity. The results showed that those two mutants were unable to change PCBP2 expression and H3K9ac level (Fig. 3D and E), indicating that SIRT6 regulated PCBP2 expression by targeting H3K9ac. Finally, our luciferase assay also confirmed that SIRT6 could inhibit *PCBP2* promoter activity and putatively subsequent *PCBP2* expression (Fig. 3F). In summary, SIRT6 inhibits PCBP2 expression by targeting H3K9ac.

3.4. SIRT6 suppresses glioma growth in vitro and in vivo

Since SIRT6 have been demonstrated to participate in liver cancer, breast cancer, pancreatic cancer and colon adenocarcinoma, and PCBP2 was shown to be a tumor driver, we wanted to know whether SIRT6 regulates glioma growth. We overexpressed SIRT6 in U251 glioma cells and found that SIRT6 overexpression reduced glioma cell proliferation and colony formation (Fig. 4A and C). In contrast, SIRT6 knockdown promoted glioma cell proliferation and colony formation (Fig. 4B and D). To further demonstrate the role of SIRT6 in glioma, we performed xenograft mice experiments with SIRT6-overexpressed or SIRT6-depleted U251 cells. The results showed that SIRT6 overexpression repressed glioma cell growth *in vivo*, while SIRT6 knockdown facilitated glioma cell growth *in vivo* (Fig. 4E and F).

However, it's still unknown whether PCBP2 is critically essential for the function of SIRT6 in glioma cells. Therefore, we knocked down PCBP2 or double-knocked down PCBP2 and SIRT6 in U251 cells. If SIRT6 functions in glioma cells independent of PCBP2, SIRT6 knockdown could induce additional up-regulation of proliferation rate and colony formation in glioma cells with PCBP2 knockdown. Interestingly, we found that SIRT6 knockdown was unable to change proliferation rate and colony formation of glioma cells when PCBP2 was knocked down (Fig. 4G and H), indicating that PCBP2 is critically important for the function of SIRT6 in regulating glioma cell proliferation and colony formation. Taken together, those results demonstrated that SIRT6 regressed glioma cell growth in a PCBP2 dependent manner.

4. Discussion

Previous evidence and our present findings showed that PCBP2 was up-regulated in human glioma and predicted poor survival. Those facts prompted us to explore the underling mechanism by which PCBP2 was regulated in human glioma. We found that SIRT6 was down-regulated in human glioma tissues and was negatively correlated with the expression of PCBP2. In addition, we demonstrated that H3K9ac enrichment on the promoter of *PCBP2* was up-regulated, and that H3K9 enrichment was correlated with PCBP2 expression level. Further, we showed that SIRT6 inhibited PCBP2 expression, which depends on the deacetylation of

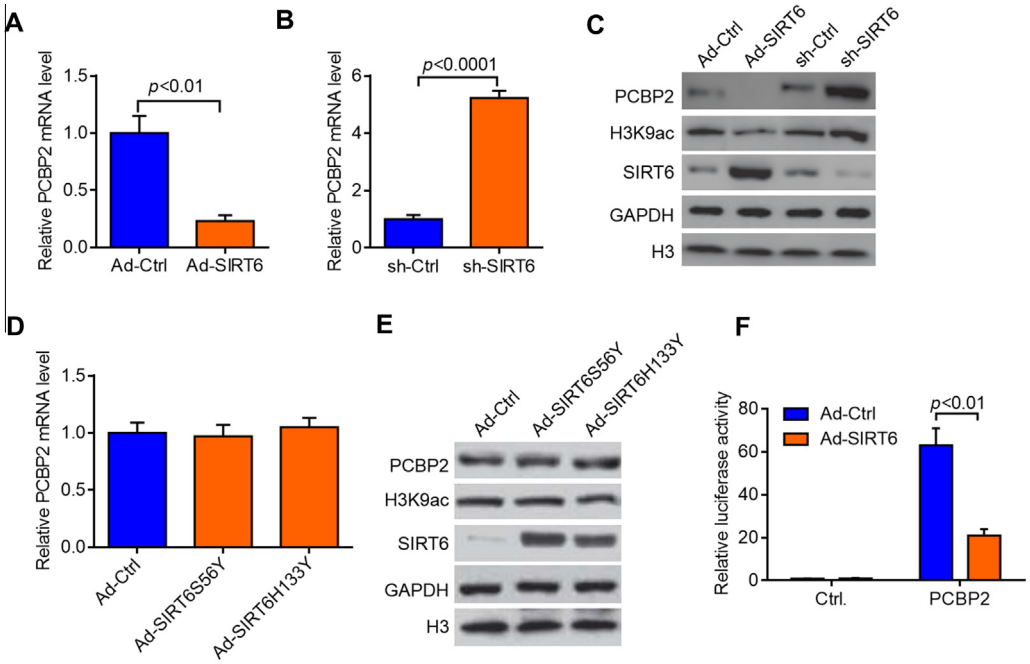

Fig. 3. SIRT6 inhibits the expression of *PCBP2* by targeting H3K9ac. (A) SIRT6 overexpression decreases *PCBP2* mRNA level in U251 glioma cell lines. U251 glioma cells were infected with adenovirus expressing ctrl GFP (Ad-Ctrl) or SIRT6 (Ad-SIRT6). 48 h later, cells were harvested for RNA extraction and the samples were subjected to q-PCR analysis. (B) SIRT6 knockdown increases *PCBP2* mRNA level in U251 glioma cell lines. U251 glioma cells were infected with retrovirus expressing ctrl shRNA (sh-Ctrl) or sh-SIRT6. 48 h later, cells were harvested for RNA extraction and the samples were subjected to q-PCR analysis. (C) SIRT6 overexpression decreased H3K9ac and *PCBP2* protein level, while SIRT6 knockdown increased H3K9ac and *PCBP2* protein level. U251 glioma cells were treated as in (A) and (B). 48 h later cells were harvested for protein extraction and the samples were subjected to Western blot analysis with indicated antibodies. (D and E) SIRT6 regulates *PCBP2* depending upon H3K9ac. U251 glioma cells were infected with adenovirus expressing ctrl GFP (Ad-Ctrl) or SIRT6 mutants (Ad-SIRT6S56Y, Ad-SIRT6H133Y, without deacetylase activity). 48 h later, protein and RNA were extracted and subjected to Western blot and q-PCR analysis, respectively. (F) Luciferase assay showing that SIRT6 overexpression decreases the activity of *PCBP2* promoter.

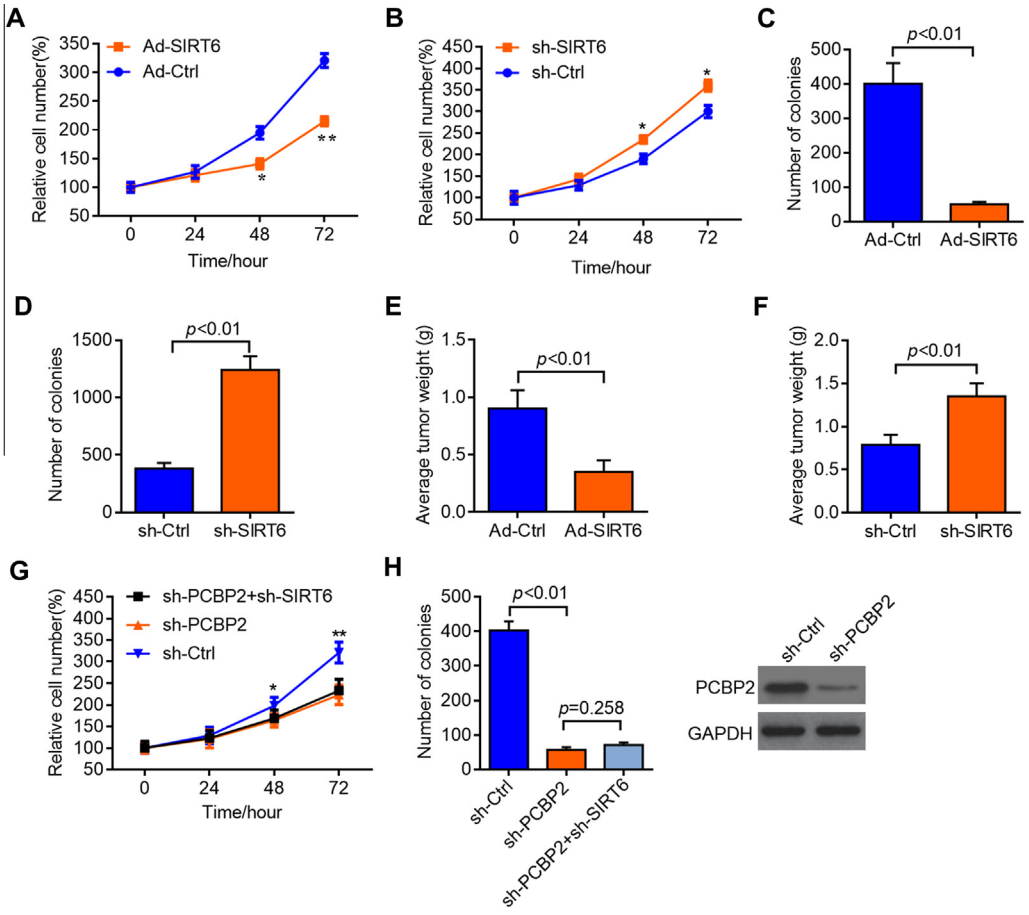

Fig. 4. SIRT6 suppresses glioma growth *in vitro* and *in vivo*. (A and B) U251 glioma cells were infected with adenovirus expressing ctrl GFP (Ad-Ctrl) or SIRT6 (Ad-SIRT6), and retrovirus expressing sh-Ctrl or sh-SIRT6. Relative cell numbers were evaluated at the indicated time points using MTT method. (A) SIRT6 overexpression reduces U251 glioma cell growth. (B) SIRT6 knockdown promotes glioma cell growth. $^{*}p < 0.05$ and $^{**}p < 0.01$. (C and D) U251 glioma cells infected with Ad-Ctrl, Ad-SIRT6, retro-sh-Ctrl, or retro-sh-SIRT6 were subjected to soft sugar colony formation assay. The colony number in each well was evaluated 2 weeks later. (C) SIRT6 overexpression reduces colony formation of glioma cells. (D) SIRT6 knockdown increases colony formation of glioma cells. (E and F) U251 glioma cells with SIRT6 overexpression or knockdown were subjected to xenograft mice experiment. At the terminal of the experiment, the tumors were harvested and their weights were evaluated. (E) SIRT6 overexpression reduces glioma growth *in vivo*. (F) SIRT6 knockdown promotes glioma growth *in vivo*. $N = 10$ in each group. (G and H) U251 glioma cells were infected with retrovirus expressing Ctrl shRNA, PCBP2 shRNA or PCBP2 shRNA plus SIRT6 shRNA. Cell proliferation rate and colony formation capacity were assessed. (G) PCBP2 knockdown blocks the effects of SIRT6 on glioma cell proliferation. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate sh-Ctrl vs sh-Nrf2. (H) PCBP2 knockdown blocks the effects of SIRT6 on glioma cell colony formation.

H3K9ac, by using loss-of-function and gain-of-function experiments. Finally, we demonstrated that SIRT6 inhibited glioma cell growth and colony formation *in vitro* and glioma growth *in vivo*. In summary, our findings reveal that SIRT6 is tumor suppresser in human glioma.

When this program was undergoing, Han et al. [7] have published their data that PCBP2 was up-regulated in glioma tissues and glioma cell lines. Their data also showed that PCBP2 facilitated glioma growth *in vitro* and *in vivo* partly by targeting the FHL3, which has recently been reported to act as a tumor suppressor. Therefore, we did not investigate the participation of PCBP2 in glioma, instead, we focused on the underling mechanism by which PCBP2 is regulated and whether modulating the regulator for PCBP2 can reduce glioma growth.

SIRT6 is a member of the NAD^{+}-dependent class III deacetylase SIRTUIN family. During the past several years, the role of SIRT6 have been deeply investigated. Current evidence show that SIRT6 essentially participates in genome stability, metabolism, inflammation, heart failure, longevity and carcinogenesis [9–15]. The physiological SIRT6 substrates include H3K9ac [22], H3K56ac [23], and none histones, including CtIP and GCN5 [24,25]. SIRT6 also acts as co-repressor of transcription factors, such as MYC and HIF1α [11,15].

SIRT6 controls cancer cell growth in different manners. SIRT6 acts as a tumor suppressor that regulates aerobic glycolysis in cancer cells. Loss of SIRT6 leads to tumor formation without activation of known oncogenes, whereas transformed SIRT6-deficient cells display elevated glycolysis and tumor growth, suggesting that SIRT6 plays a role in both establishment and maintenance of cancer. SIRT6 also functions as a regulator of ribosome metabolism by co-repressing MYC transcriptional activity [15]. SIRT6 overexpression induces massive apoptosis in a variety of cancer cell lines but not in normal, non-transformed cells. This cell death requires the mono-ADP-ribosyltransferase but not the deacetylase activity of SIRT6 and is mediated by the activation of both the p53 and p73 apoptotic signaling cascades in cancer cells by SIRT6 [26]. In liver cancer, c-Fos induces SIRT6 transcription, which represses survivin by reducing histone H3K9 acetylation and NF-κB activation. Importantly, increasing the level of SIRT6 or targeting the anti-apoptotic activity of survivin at the initiation stage markedly impairs cancer development [17]. In pancreatic cancer cells, SIRT6 enhances the expression of pro-inflammatory cytokines and chemokines, such as IL8 and TNF, and promotes cell migration by enhancing Ca^{2+} responses. via its enzymatic activity, SIRT6 increases the intracellular levels of ADP-ribose, an activator of the Ca^{2+} channel TRPM2. In turn, TRPM2 and Ca^{2+} are shown to

be involved in SIRT6-induced TNF and IL8 expression. SIRT6 increases the nuclear levels of the Ca^{2+}-dependent transcription factor, nuclear factor of activated T cells (NFAT), and cyclosporin A, a calcineurin inhibitor that reduces NFAT activity, reduces TNF and IL8 expression in SIRT6-overexpressing cells [27]. Here we identified a novel mechanism by which SIRT6 regulates glioma cell growth. We showed that SIRT6 controls PCBP2, which was previously reported to overexpress in glioma tissues and facilitated glioma growth [7]. The activity of SIRT6 depends upon its deacetylation activation on H3K9ac on the promoter of *PCBP2* gene.

In summary, our data and previous report show that PCBP2 is overexpressed in human glioma tissues, which is regulated by the histone deacetylase SIRT6. SIRT6 binds to *PCBP2* promoter region and deacetylates H3K9ac, resulting in transcription regression. Finally, we show that SIRT6 acts as a tumor suppressor in human glioma cells using *in vitro* and *in vivo* evidence. Those findings indicate that PCBP2 inhibitors and SIRT6 activators may serve as potential drug candidates for glioma therapy.

Conflict of interest

None.

Acknowledgments

This work was supported by Science and Technology Commission of Shanghai Municipality (12nm0501201; 12140901802).

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.2014.02.116.

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