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Original Article

Sirtuin6 protects renal cell carcinoma from hypoxic damage

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Abstract: Sirtuin6 (SIRT6) has been shown to suppress glycolysis and enhance DNA repair. As a tumor suppressor, SIRT6 can directly deacetylate hypoxia-inducible factors (HIFs). In this study, we examined the role of SIRT6 and HIFs in the progression of RCC (renal cell carcinoma). An MTT assay and Hoechst staining were performed to determine the viability and apoptosis of RCC cells, respectively. RT-qPCR and western blotting were used to determine the levels of SIRT6 and HIF1α proteins. SIRT6 was overexpressed using an adenovirus vector and was inhibited using a specific siRNA. SIRT6 protein levels were reduced, whereas the expression of HIF1α was increased in renal cell carcinoma (RCC) tissues and cell lines. The MTT assay showed that overexpression of SIRT6 could decrease 786-O cell viability. Moreover, SIRT6 overexpression decreased RCC cell colony formation and increased RCC cell apoptosis. Western blot analysis revealed that SIRT6 reduced HIF1α expression and downstream signaling in RCC cells. A ChIP assay demonstrated that SIRT6 overexpression significantly reduced H3K9 and H3K56 acetylation in the promoter regions of glycolytic genes. More importantly, HIF1α inhibition partially rescued the phenotype of SIRT6-silenced RCC cells. Taken together, these results demonstrated for the first time that SIRT6 inhibited RCC progression through the inhibition of HIF1α expression in vitro.

Keywords: SIRT6, RCC, HIF-1α, hypoxia damage

Introduction

Renal cell carcinoma (RCC) is one of the most common malignancies in both men and women [1]. Altered metabolism has been shown to be a major contributor to the pathogenesis of this disease [2]. Most studies have indicated that RCC may be associated with metabolic disorders [2].

SIRT6 plays a key role in cellular and systemic homeostasis [3]. SIRT6 belongs to class III histone deacetylases, which regulates aerobic glycolysis or the Warburg effect, a hallmark of cancer metabolism [4]. One of the major functions of SIRT6 is to deacetylate the histone residues acH3K9 and acH3K56 [5, 6]. Deacetylating H3 inhibits the activity of many transcription factors, including c-MYC, c-JUN, HIF1 and NF-κb [7]. In addition, SIRT6 enhances genomic DNA stability by regulating proteins involved in DNA repair, such as PAPR-1 and DNA-PK [8, 9]. And SIRT6 is also reported to function as a tumor suppressor, partially by inhibiting glycolysis-related proteins, such as glucose transporter1 (Glut1), lactate dehydrogenase A (Ldha) and pyruvate dehydrogenase kinase, isozyme 1 (Pdk1) [4]. The protein level of SIRT6 has been found to be dysregulated in many types of cancer, including prostate, breast and pancreatic cancers [10-12]. However, few studies have examined the role of SIRT6 in RCC.

The hypoxia-inducible factors (HIFs) HIF1, HIF2, and HIF3 are transcription factors that contribute to glycolysis and lactate production under limited oxygen availability [13]. The level of the α-subunit of HIF is dependent on oxygen concentration, but its β-subunit is stably expressed [13]. HIF1α was first identified as a ubiquitously expressed protein in 1995, whereas HIF2α was reported to be mainly expressed in epithelial cells [14, 15]. It has been reported that SIRT6 deficiency leads to enhanced HIF-1α protein

Table 1. PCR primers used for ChIP assay

Name	Sequence
LDHA-ChIP-5'	AGAGAGAGCGCTTCGCATAG
LDHA-ChIP-3'	GGCTGGATGAGACAAAGAGC
Bcl2-ChIP-5'	AGGGGGTGTGTGAAAACAAG
Bcl2-ChIP-3'	ATGGCTTGCCAGCTTACATC
Pkd1-ChIP-F	CTGTAGTCCCCCTTCCCTGT
Pdk1-ChIP-R	GAGCTTTTGGAGCAGACTGG
HIF1α-F	ACCTTCATCGGAAACTCCAAAG
HIF1α-R	CTGTTAGGCTGGGAAAAGTTAGG

synthesis and stability by reducing H3K9 and H3K56 acetylation in the promoters of downstream genes [5, 6].

Here, we demonstrated for the first time the expression of SIRT6 and HIF-1α in RCC carcinomas. We found that the overexpression of SIRT6 could inhibit the expression of HIF-1α, thereby enhancing glycolysis and reducing RCC progression.

Materials and methods

Clear cell renal cell carcinoma samples

Twenty-five clear cell renal cell carcinoma samples and four fresh-frozen normal kidney tissue samples were obtained from Department of Urology, School of Medicine, the Affiliated Hospital of Shanghai Jiaotong University. The use of patient-derived material was approved by the Research Ethics Committee of the Affiliated Hospital of Shanghai Jiaotong University, and written consent was obtained from all patients. Tissues were obtained at surgery on patients undergoing tumor resection, and the diagnosis of clear cell renal cell carcinoma was established post-operatively by histopathology. The tumor samples were examined according to the criteria provided by the International Union against Cancer [16].

ChIPs and Q-RT-PCR

ACHN cells were grown to 60% confluence and transfected with ad-SIRT6 or ad-con for 48 h. The cells were washed with PBS three times for 5 min and fixed with 1% formaldehyde for 15 min at room temperature. The cells were then washed with PBS three times for 5 min and collected in RIPA buffer (Solarbio, Beijing, China). The samples were sonicated to generate DNA fragments (under 500 bp in length). Before immunoprecipitation, 1 mg of total protein lysates was pre-incubated with 30 μL of 50% G protein-Sepharose slurry for 2 h. The primary antibodies, anti-H3K9ac (#9649, 1:50; Cell Signaling Technology, Inc., Boston, MA, USA) and anti-H3K56ac (ab76307, 1:50; Abcam, Cambridge, UK) antibodies, were added to the samples and incubated overnight at 4°C in the presence of protein G beads. Anti-mouse IgM antibody was then added to capture the immune complexes. The complexes were washed twice with RIPA buffer and six times with ChIP wash buffer (Millipore Billerica, MA, USA). The immune complexes were washed with 1% SDS at 65°C for 10 min, and cross-linking was reversed with 200 mM NaCl at 65°C for 6 h. DNA was then purified and collected for analysis by RT-PCR. The primers used in this study are listed in **Table 1**, and GAPDH was used for normalization of gene expression. The ABI StepOne Plus (Applied Biosystems, Foster City, CA, USA) was used to perform the amplification reaction. Each experiment was performed in triplicate.

Immunofluorescence

ACHN cells were plated on 6-well chamber slides and fixed with 4% formaldehyde for 10 min at -20°C. The slides were washed with PBS three times for 5 min, blocked with 3% BSA and washed with PBS. The cells were incubated with a primary antibody against SIRT6 overnight at 4°C and washed again with PBS three times. FITC-conjugated anti-rabbit IgG antibody (1:100) was added to the slides and incubated for 1 h at RT. After washing with PBS three times, the slides were incubated with Hoechst 33258 dye (10 μg/mL) for 5 min. The slides were washed with PBS and mounted. Fluorescent cells were analyzed under a fluorescence microscope (Leica CM3000; Leica Microsystems GmbH, Germany).

MTT assay

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, St Louis, MO, USA) assay. Briefly, ACHN cells (5×10^4 cells per well) were seeded in 96-well tissue culture plates. At 70-80% confluence, the cells were incubated for 16 h in serum-free DMEM. After transfection of adenovirus vector or siRNAs,

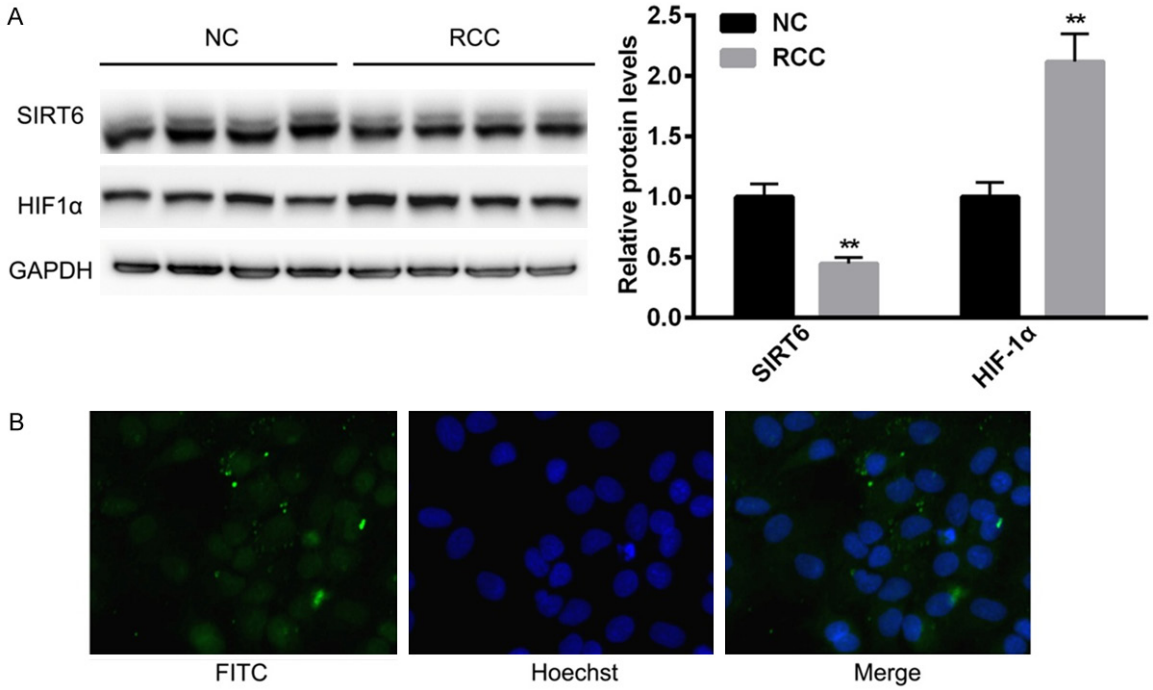

Figure 1. Decreased expression of SIRT6 and enhanced HIF1α expression in RCC tissues. A. Western blot analysis of SIRT6 and HIF1α in RCC tissues. B. Localization of SIRT was determined using immunofluorescence. *P<0.05, **P<0.01 versus control.

RCC cells were cultured in fresh medium containing 0.5 mg/ml MTT for an additional 4 h. The blue formazan products in the RCC cells were dissolved in DMSO and measured spectrophotometrically at a wavelength of 550 nm.

Hoechst 33258 staining

RCC cells (1×10^5 cells per well) were cultured in six-well tissue culture plates, and upon reaching 70-80% confluence, the cells were incubated for 16 h in serum-free DMEM. After drug treatment, the medium was removed, and the cells were rinsed once with cold PBS and fixed with 4% formaldehyde (Zhongshan Technology, Beijing, China) in PBS at 37°C for 15 min. The cells were washed three times with PBS, and the nuclei were stained with Hoechst 33258 (10 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 5 min before washing with PBS for three times (5min/time).

Apoptosis assay

Cells (50-60% confluent) were transfected with ad-SIRT6/ad-con or si-HIF1α/NC and washed with 1×PBS twice. Apoptosis was assessed using an Annexin-V FITC-PI Apoptosis Kit (Invitrogen, Carlsbad, CA). This assay employs fluorescein-labeled Annexin-V in concert with propidium iodide (PI) to detect cells undergoing apoptosis. Briefly, cells were washed with 1×PBS twice and suspended at 2-3 × 10^6 cells/mL in 1× Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, which were incubated at room temperature for 15 minutes in the dark. Cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 h of staining using the FL1 (FITC) and FL3 (PI) channels.

Colony formation assay

ACHN and 786-0 cells transfected with ad-SIRT6 or ad-con were plated in 6-well plates (10^6 cells per well) containing 700 μg/mL geneticin (G418, Sigma Aldrich, St Louis, MO, USA). Fourteen days later, the colonies were stained with 0.01% crystal violet. Each assay was performed in triplicate and in two independent experiments.

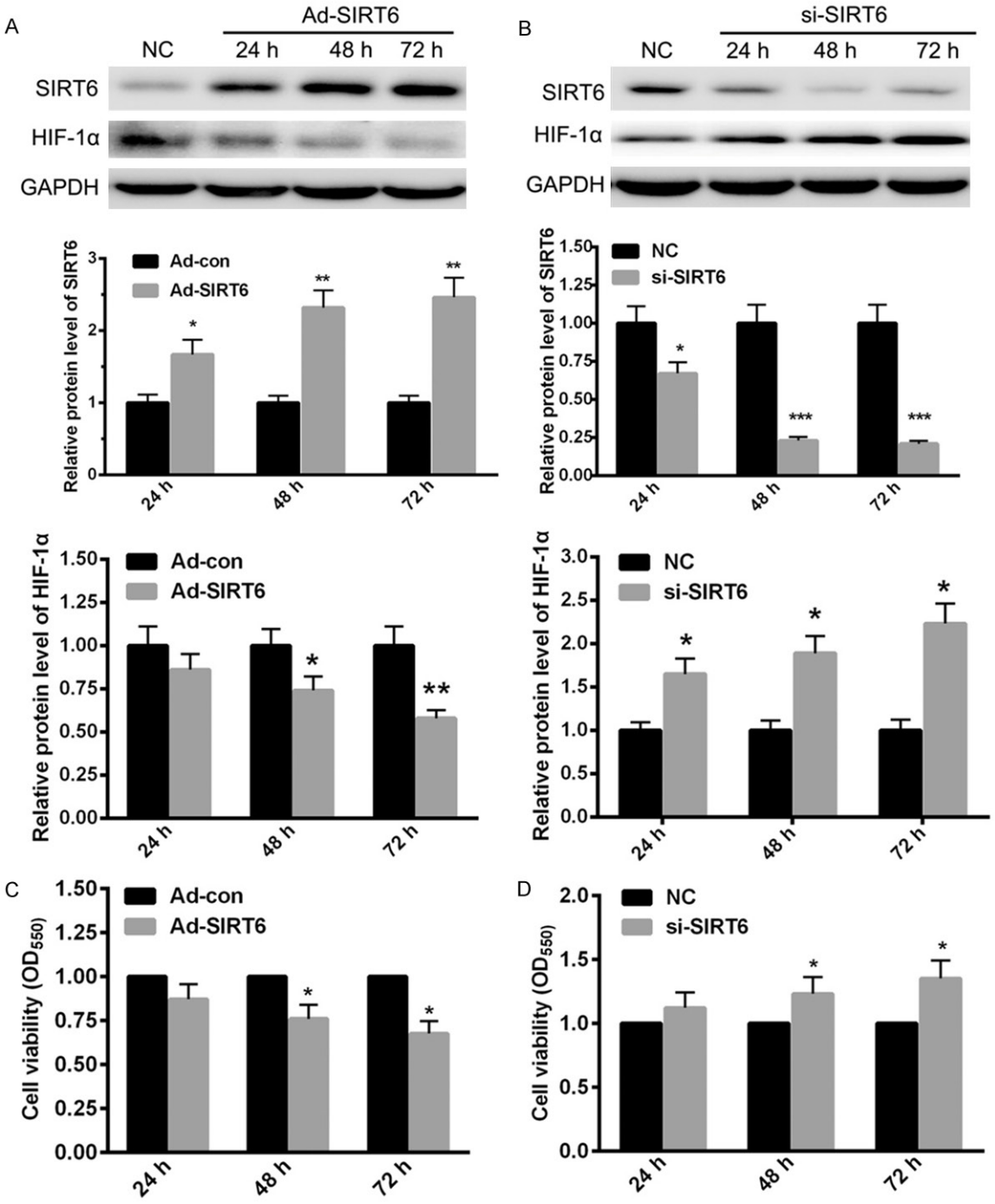

Figure 2. The inhibitory effect of SIRT6 on RCC cell viability was analyzed using an MTT assay. (A) Transfection with ad-SIRT6 significantly increased the protein level of SIRT6. (B) The expression of SIRT6 was significantly reduced using a specific siRNA targeting SIRT6. Cell viability was analyzed in cells transfected with ad-SIRT6/ad-con (C) or si-SIRT6/NC (D). *P<0.05, **P<0.01 versus control.

Statistical analysis

All experiments were performed three times. All data are presented as mean ± standard error. The data were analyzed by t-test (two-sided) and one-way ANOVA using SPSS 10.0 statistical software. P<0.05 was considered statistically significant.

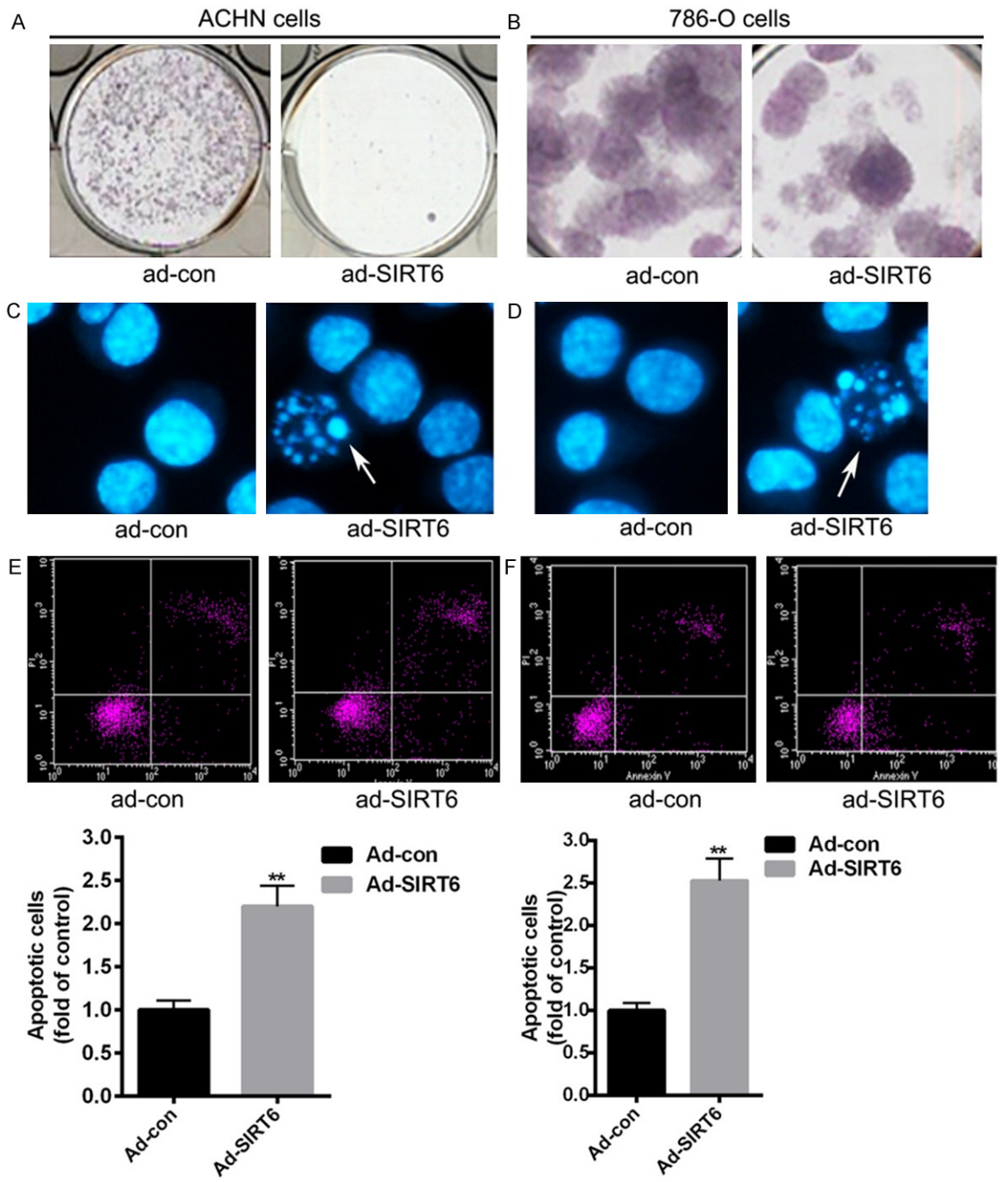

Figure 3. SIRT6 suppressed RCC cell colony formation and enhanced RCC cell apoptosis. SIRT6 inhibited colony formation in ACHN (A) and 786-O (B) cells. RCC cell proliferation was assessed in vitroin cells transiently transfected with ad-SIRT6 or ad-con for 48 h. Hoechst staining was applied to determine apoptosis in ACHN (C) or 786-O (D) cells. Flow cytometry was used to determine apoptosis in ACHN (E) or 786-O cells (F) transfected with ad-SIRT6 or ad-con. *P<0.05, **P<0.01 versus control.

Results

Decreased expression of SIRT6 and enhanced HIF1α in RCC tissues

A previous study has indicated that SIRT6 may function as a corepressor of the transcription of HIF1α [17]. In the present study, we first demonstrated the level of SIRT6 and HIF1α in RCC tissues. Western blot analysis revealed that the protein level of SIRT6 was reduced and the expression of HIF1α was increased (**Figure 1A**). Additionally, immunofluoresence demon-

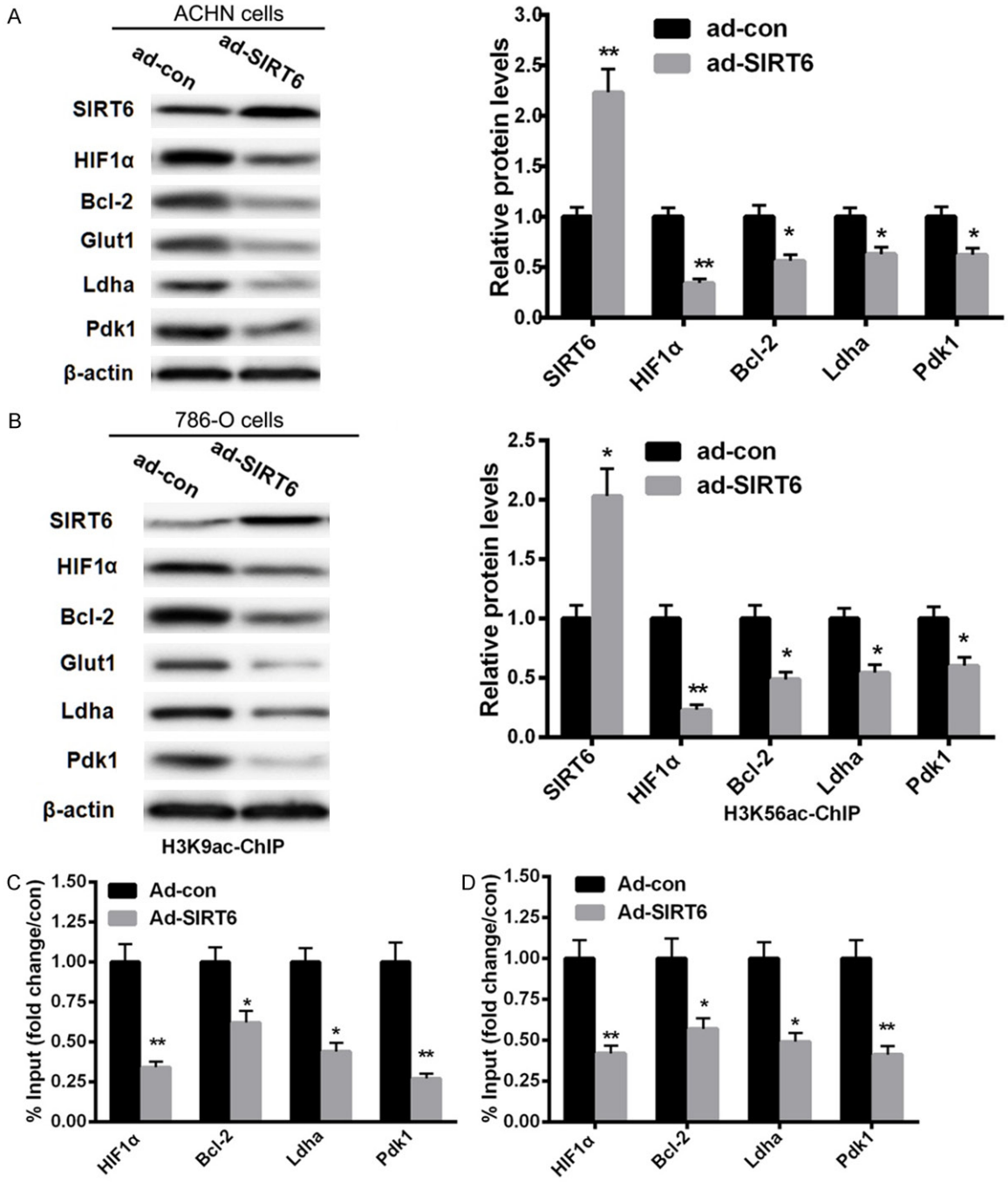

Figure 4. SIRT6 suppresses the RCC cancer progression, in part through inhibition of HIF1α. Overexpression of SIRT6 significantly reduced HIF1α expression and downstream signaling in ACHN (A) and 786-O (B) cells. ChIP assay was conducted to characterize the mechanism of SIRT6-dependent regulationusing anti-H3K9ac (C) and anti-H3K56ac antibodies (D). *P<0.05, **P<0.01 versus control.

strated that SIRT6 was located in the nuclei of ACHN cells (**Figure 1B**).

Overexpression of SIRT6 decreased 786-O cell line viability

To examine the role of SIRT6 on RCC cell viability, an MTT assay was conducted. As shown in **Figure 2A**, transfection with ad-SIRT6 significantly increased the level of SIRT6 at 24 h, 48 h and 72 h. In contrast, the expression of SIRT6 was significantly reduced by transfection with a specific siRNA targeting SIRT6 at 48 h and 72 h (**Figure 2B**). As shown in **Figure 2C**, overexpression of SIRT6 decreased 786-O cell viability by

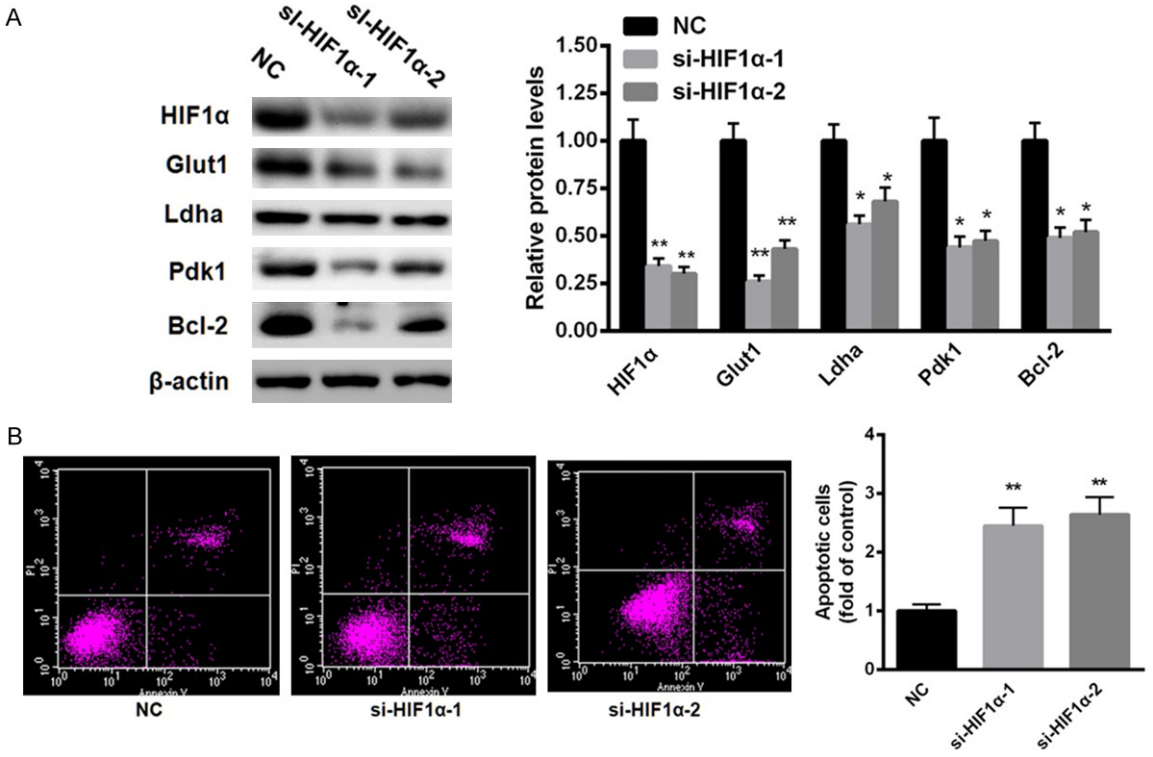

Figure 5. Knockdown of HIF1α reduced the glycolysis genes and enhanced cell apoptosis. A. Western blot was performed to determine the protein levels of HIF1α as well as the downstream genes in ACHN cells transfected with si-HIF1α. B. Cell apoptosis was analyzed in ACHN cells transfected with si-HIF1α. *P<0.05, **P<0.01 versus control.

23% and 32.5% at 48 h and 72 h, respectively (**Figure 2C**). In comparison, knockdown of SIRT6 clearly enhanced cell viability in a time-dependent manner (**Figure 2D**), suggesting the inhibitory effect of SIRT6 on RCC cell viability.

SIRT6 suppressed RCC cell colony formation and enhanced RCC cell apoptosis

Fourteen days after transfection of ad-SIRT6 or ad-con into ACHN and 786-O cell lines, the colony formation capability was analyzed. As shown in **Figure 3A** and **3B**, cell colony formation was markedly reduced in cells transfected with ad-SIRT6 compared with ad-con. We also evaluated the role of SIRT6 on 786-O cell apoptosis when SIRT6 was overexpressed. Hoechst staining indicated that cell apoptosis was enhanced in ACHN and 786-O cells transfected with ad-SIRT6 (**Figure 3C** and **3D**). Moreover, flow cytometry analysis revealed that overexpression of SIRT6 enhanced the apoptosis of ACHN and 786-O cells by ~1.2-fold or ~1.5-fold, respectively (**Figure 3E** and **3F**). These data indicated the tumor suppressor role of SIRT6 in ACHN and 786-O cells.

SIRT6 suppressed RCC cancer progression by inhibiting HIF1α

To determine the specific mechanism by which SIRT6 regulates RCC progression, we overexpressed SIRT6 in ACHN and 786-O cells, respectively. Western blot analysis revealed that overexpression of SIRT6 significantly reduced HIF1α expression and the downstream signaling pathways in ACHN (**Figure 4A**) and 786-O (**Figure 4B**) cells, including the anti-apoptotic protein Bcl-2 and glycolysis-related proteins, such as Glut1, Ldha and Pdk1. To characterize the mechanism of SIRT6-dependent regulation, anti-H3K9ac and anti-H3K56ac antibodies were used. As shown in **Figure 4**, we found that overexpression of SIRT6 significantly reduced H3K9 and H3K56 acetylation in the promoters of these glycolytic genes and HIF1α as demonstrated by ChIP analysis (**Figure 4C** and **4D**). These data indicated that SIRT6 suppresses RCC cancer progression partially by

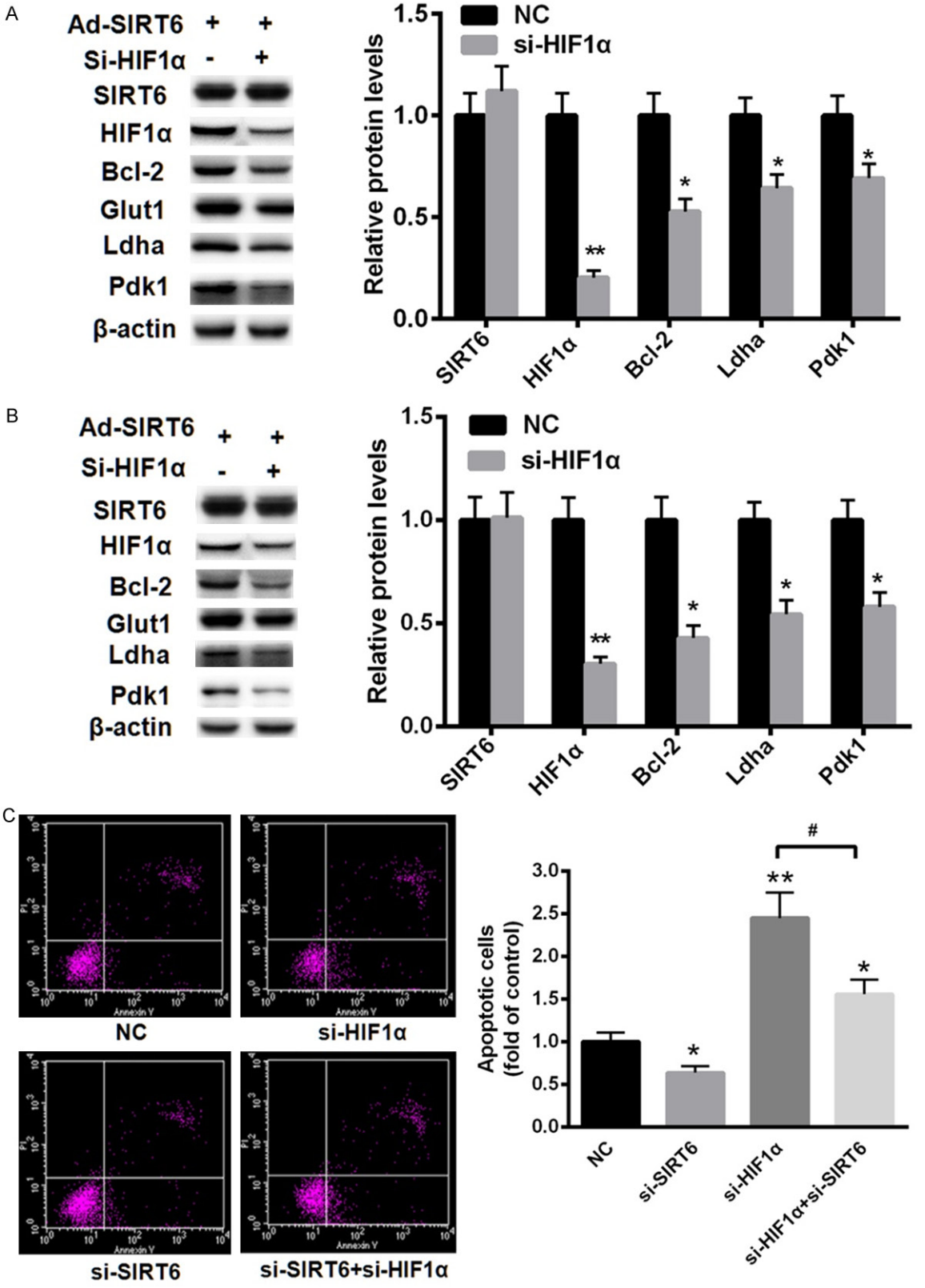

Figure 6. Regulation of RCC progression by SIRT6 depends on HIF1α in vitro. Knockdown of HIF1α significantly reversed the protein levels of Bcl-2, Glut1, Ldha and Pdk1 in ACHN (A) and 786-O cells (B) transfected with si-SIRT6. (C) Cell apoptosis was analyzed in ACHNand 786-O cells transfected with si-SIRT6 or si-HIF1α or NC. *P<0.05, **P<0.01 versus control, #P<0.05 versus si-HIF1α.

inhibiting HIF1α through the SIRT6-dependent regulation.

Knockdown of HIF1α reduced the expression of glycolysis genes and enhanced cell apoptosis

We further evaluated the role of HIF1α on glycolysis genes with two siRNAs targeting HIF1α. As shown in **Figure 5A**, the two siRNAs markedly decreased the protein level of HIF1α in ACHN cells as well as the downstream genes, including Bcl-2, Glut1, Pdk1, and Ldha. In addition, flow cytometry analysis revealed that apoptosis was significantly enhanced when HIF1α was inhibited in ACHN cells (**Figure 5B**). These data suggested an anti-apoptotic role of HIF1α.

HIF1α inhibition rescued the phenotype of SIRT6-silenced RCC cells

To test whether SIRT6 exerts its function partially through HIF1α, we treated RCC cells with specific siRNAs targeting HIF1α. Strikingly, transfection with si-HIF1α significantly reversed the protein levels of Bcl-2, Glut1, Ldha and Pdk1 in ACHN (**Figure 6A**) and 786-O cells (**Figure 6B**) previously transfected with si-SIRT6. In addition, we assessed apoptosis in ACHN and 786-O cells transfected with si-SIRT6, si-HIF1α or NC (**Figure 6C**). We found that knockdown of HIF1α could markedly enhance cell apoptosis even in ACHN cells transfected with si-SIRT6. These results indicated that regulation of RCC progression by SIRT6 was dependent on HIF1α in vitro.

Discussion

Adaption to stress plays a key role in the maintenance of homeostatic balance [18]. Previous studies have indicated that the chromatin-bound protein SIRT6 is located in the nucleus [19]. SIRT 6 deficiency resulted in a severe metabolic imbalance. SIRT6 is also found to improvethe repair of DNA damage caused by oxidative stress in cancer cells [19]. In this study, we first examined the expression of SIRT6 in RCC tissues and found that it was significantly reduced. More importantly, we found that overexpression of SIRT6 suppressed RCC cell colony formation and enhanced RCC cell apoptosis.

In the normal oxygen environment, glucose is converted into pyruvate and enters the mitochondria, where itis transformed into acetyl coenzyme A and further generates adequate amounts of ATP. However, in the absence of oxygen, cell metabolism is shifted from aerobic to anaerobic pathways and glucose is converted into lactate [20]. During the period of stress, ATP is less efficiently produced to compensate for the metabolic demands [21]. The hypoxia-inducible transcription factor, Hif1α, is an important regulator of the cellular adaptation to nutrient and oxygen deficiencies [22]. The activity of HIF1α is strictly regulated under normoxia or oxygen-deficient environmental conditions [21]. In the absence of oxygen, prolyl-hydroxylase-domain (PHD) proteins are inactivated and HIF1α expression is stabilized. We now presented data that HIF1α expression was significantly increased in RCC tissues. More importantly, we found that SIRT6 acted as a tumor suppressor in RCC cells, in part by inhibiting HIF1α.

In this study, we found that the expression of HIF1α was significantly reduced in ACHN and 786-O cells. As shown in **Figure 4A** and **4B**, overexpression of SIRT6 significantly inhibited HIF1α expression and downstream signaling pathways in ACHN and 786-Ocells. In comparison, knockdown of HIF1α markedly increased the level of HIF1α protein (**Figure 4C** and **4D**). More importantly, overexpression of SIRT6 was found to reduce the expression of glycolysis-related genes, indicating a nutrient sufficient status. Consistent with a previous study, reduction and enhancement of nutrients in cancer cells can function as a positive regulator of HIF1α synthesis and stability [23].

An enhanced glycolytic capacity was identified in SIRT6-silenced cell lines, which is similar to the “Warburg effect” described by Otto Warburg [24]. The Warburg effect refers to the observation that cancer cell proliferation mainly depends on aerobic glycolysis rather than respiration to meet the cellular energy and metabolic requirements [20]. Consistent with our observations, inhibition of SIRT6 enhanced glycolysis mediated by HIF1α. More importantly, we also found that HIF1α contributed to enhanced RCC cell proliferation and reduced cell apoptosis. These data indicated that reduced SIRT6 expression in RCC cells may contribute to abnormal tumor growth.

In conclusion, our data have suggested an important role of SIRT6 in controlling RCC progression, in part, by HIF1α in renal cell carcinoma. Knockdown of SIRT6 caused phenotypes with severe metabolic insufficiency, indicating a predominant role of SIRT6 in regulating energy balance of RCC.

Disclosure of conflict of interest

None.

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