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Original Article

SIRT3 inhibits cell proliferation in human gastric cancer through down-regulation of Notch-1

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Abstract: SIRT3 is a member of the NAD$^+$-dependent class III deacetylase sirtuin family and plays pivotal roles in regulating cellular functions. Accumulating evidence has recently demonstrated that SIRT3 may function as either oncogene or tumor suppressor in a panel of cancers. However, the biological function of SIRT3 in gastric cancer has been poorly characterized. The present study revealed that the mRNA and protein levels of SIRT3 were significantly reduced in human gastric cancer tissues and cell lines. In addition, overexpression of SIRT3 dramatically suppressed the proliferation ability and colony formation number of gastric cancer cells. By contrast, SIRT3 knockdown using small interfering RNA enhanced tumor cell growth and colony formation. On the molecular level, we found that SIRT3 inhibited the expression of Notch-1 both at the mRNA and protein levels in gastric cancer cells. Furthermore, Notch-1 overexpression diminished the inhibitory effects of SIRT3 on tumor cells proliferation. Taken together, these results demonstrated that SIRT3 suppressed the proliferation gastric cancer cells via down-regulation of Notch-1, which might provide novel therapeutic targets in the gastric cancer therapy.

Keywords: Gastric cancer, cell proliferation, SIRT3, Notch-1

Introduction

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer-related death in the world [1]. Statistical analysis shows that the incidence rate of GC was higher in South American and Asian countries than the United States and Western Europe [2]. Currently, surgical resection, chemotherapy and radiotherapy remain to be the mainstay of GC treatment [3-5]. However, recent advances have begun to explore the molecular pathogenesis of the disease and developed various targeted therapies focused on epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-Met) and vascular endothelial growth factor (VEGF) related indications in advanced GC [6-8]. Although great progress has been achieved in the study of the GC in recent decades, the molecular mechanism of GC initiation and progression still remains elusive. Thus, it is urgent to develop novel therapeutic targets for GC treatment in the clinic.

The sirtuin family comprises seven members, which are NAD$^+$-dependent protein deacetylases and/or mono-[ADP-ribosyl] transferases [9, 10]. Studies demonstrated that these proteins diverge in localization and functions, with SIRT1, 2, 6, and 7 acting as critical modulators of epigenetic modifications, while SIRT3, 4 and 5 functioning mostly in the mitochondria [11, 12]. Human SIRT3 is a full-length 44-kD protein and has the capacity to activate amino-acid metabolism, fat oxidation, and electron transport [13, 14]. Recently, the association between SIRT3 and tumorigenesis has drawn much attention. It has been suggested that many types of cancers, such as breast cancer [15], hepatocellular carcinoma [16, 17], and head and neck squamous cell carcinoma [18], exhibited aberrant expression or deletion of SIRT3. However, its role in the pathogenesis of gastric cancer is still unclear. The present study detected the SIRT3 expression in tumor samples and cell lines. Furthermore, we performed the *in vitro* study to unfold the effects of SIRT3 on the proliferation of cultured GC cell lines.

Materials and methods

Patients

Twenty paired primary gastric carcinoma tissues and distant normal gastric tissues were collected from patients (age: 52.39±8.62 years) during routine therapeutic surgery in the Department of Oncological Surgery, Zhejiang Cancer Hospital, Hangzhou, China. Informed consent for the use of samples was obtained from all patients before surgery, and the study protocol was approved by the ethics committee of Zhejiang Cancer Hospital.

Cell culture

The human gastric cancer cell lines AGS, SGC-7901 and BGC-823 and the normal gastric epithelium cell line GES were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2 at 37°C.

Plasmid construction, siRNA and transfection

The cDNA fragment encoding SIRT3 and Notch-1 was isolated with Takara RNA PCR kit (Takara, Japan) using total RNAs from lung cancer cell line. The primers sequences were as following:

SIRT3 forward: 5'-ATCGATGGCGAATGAA-3' and reverse: 5'-ACATGCAGGAGGTATATAAGA-3'. Notch1 forward: 5'-ATGGCCTCGTTCCATCCATGATAAGAT-3' and reverse: 5'-AGAATGGCCAGCTCGGCTTCG-3'.

PCR products were cloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). Scramble siRNA or siRNA SIRT3 were purchased from Invitrogen (Carlsbad, CA, USA). Cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the instruction.

CCK-8 assay

The proliferations of gastric cancer cells were determined using a CCK-8 assay (Beyotime, Jiangsu, China) according to the manufacturer's protocol. Briefly, the cells at a concentration of 2.0×10^3 cells /well were cultured in a 96-well plate. At 0, 24, 48 and 72 h after transfection, cells were incubated with 10 ml of CCK-8 solution at 37°C for 2 h. Absorbance was measured using BioRad microplate reader (FluoDia T70, Photon Technology International, Lawrenceville, NJ) at a wavelength of 450 nm.

Colony formation assay

Cell clone formation was assessed by colony formation assay. To be brief, tumor cells (1.0×10^3 cells/60 mm culture dish) were seeded in triplicate and incubated at 37°C for two weeks to form clones. The cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and stained with crystal violet (1% paraformaldehyde, 0.5% crystal violet, and 20% methanol in PBS) for 30 min. The clone number on each plate were counted to measure cell survival ability.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were isolated from tissues or cells by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Japan) according to the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Tokyo, Japan) on ABI 7500 system (Applied Biosystems, Foster, CA, USA). The oligonucleotide primers for human SIRT3 and GAPDH were as follows:

SIRT3 forward: 5'-ACCCAGTGGCATTCCAGAC-3'; reverse: 5'-GGCTTGGGGTTGTGAAAGAAG-3'; Notch-1 forward: 5'-AGCAGGTGCCATAGTCCAC-3'; reverse: 5'-GGTTGATGCTGACGAGATGAG-3'; GAPDH forward: 5'-GAGTCAACGGATTTGGTCGT-3'; reverse: 5'-GACAAGCTTCCCGTTCTCAG-3'. The gene expression level was normalized using GAPDH as an internal reference gene.

Western blot analysis

Cells were harvested by trypsinization, lysed in buffer and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After immunoblotting, the membranes were blocked in PBS/0.1% Tween-20 with 5% nonfat dry milk, and incubated with primary antibodies against SIRT3, Notch-1 and GAPDH (Santa Cruz, CA, USA). GAPDH was used as a loading control. The proteins were visualized by the enhanced chemiluminescence method and intensity of protein bands was quantified by densitometry.

Statistical analysis

All data were presented as mean ± SD and treated for statistics analysis by SPSS program. Statistical significance was calculated by one-

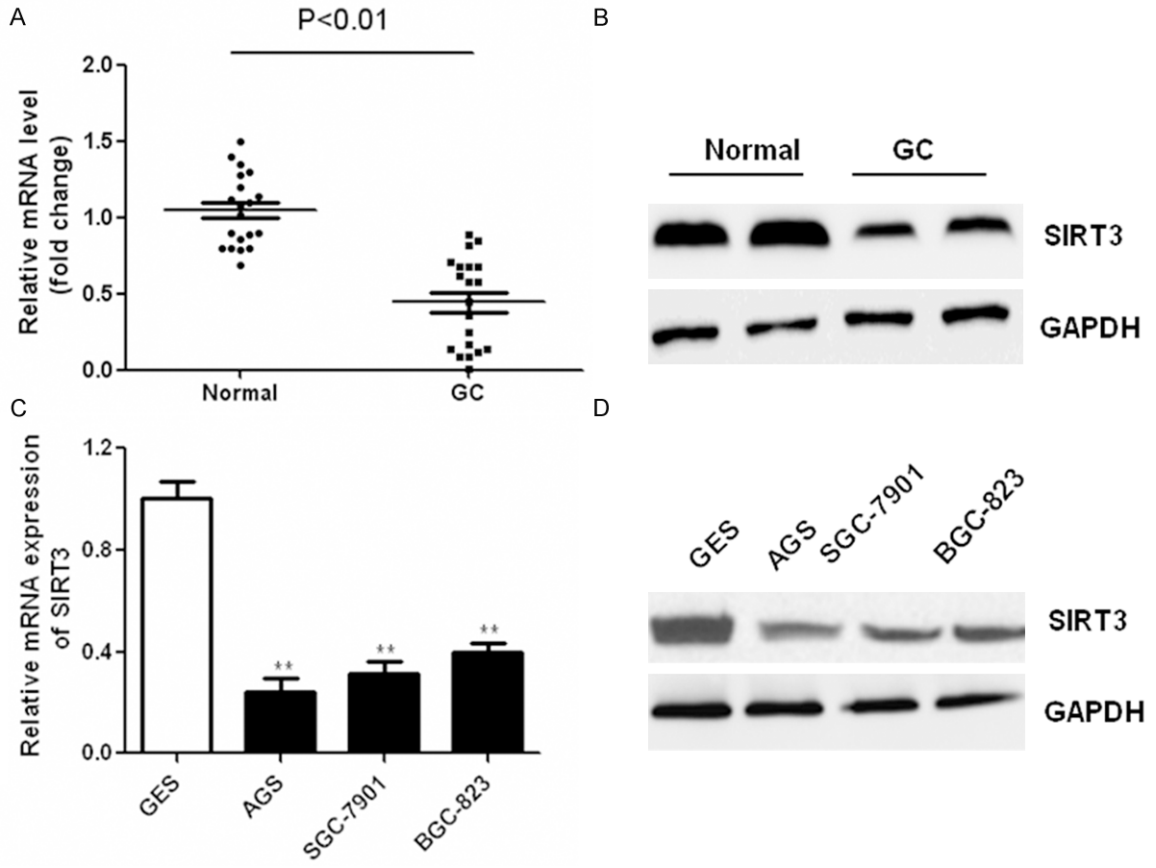

Figure 1. Downregulation of SIRT3 in human gastric cancer tissues and cell lines. The mRNA and protein levels of SIRT3 were measured by qRT-PCR (A) and western blot (B) in human gastric cancer tissues and normal tissues. qRT-PCR (C) and western blot (D) were performed to analyze the mRNA and protein expression of SIRT3 in gastric cancer cell lines, including AGS, SGC-7901 and BGC-823 cells and normal gastric epithelium cell line GES. $^{**}P<0.01$.

way analysis of variance (ANOVA) or by Student's t-test between the two groups. *P* values <0.05 were considered significant.

Results

SIRT3 expression was down-regulated in GC tissues and cell lines

Firstly, we analyzed the mRNA expression of SIRT3 in twenty paired gastric cancer and adjacent non-tumor normal tissues by qRT-PCR. Data showed that mRNA expression of SIRT3 in GC tissues was significantly down-regulated compared with the normal tissues (**Figure 1A**). Western blot analysis indicated that the SIRT3 protein expression was also reduced in GC tumor tissues (**Figure 1B**). Additionally, SIRT3 expression in several gastric cancer cell lines including AGS, SGC-7901 and BGC-823 cells were analyzed by qRT-PCR and western blot. We found that SIRT3 was dramatically decreased both at the mRNA (**Figure 1C**) and protein (**Figure 1D**) levels in three GC cell lines compared with normal gastric epithelium cell line GES. These results suggested that SIRT3 expression was down-regulated in both gastric cancer tissues and cell lines.

SIRT3 inhibited the proliferation of gastric cancer cells

To investigate the biological role of SIRT3 in gastric cancer, AGS cells were transfected with plasmids encoding SIRT3. qRT-PCR and western blot confirmed the upregulation of SIRT3 in AGS cells (**Figure 2A** and **2B**). The CCK-8 assay and colony formation assay showed that SIRT3 overexpression significantly inhibited the proliferation ability and colony formation numbers of AGS cells (**Figure 2C** and **2D**). In addition, we interfered with SIRT3 expression with small interfering RNA (siRNA) in AGS cells (**Figure 3A** and **3B**). Consequently, the growth curves showed that cell growth was obviously enhanced after SIRT3 knockdown in AGS cells

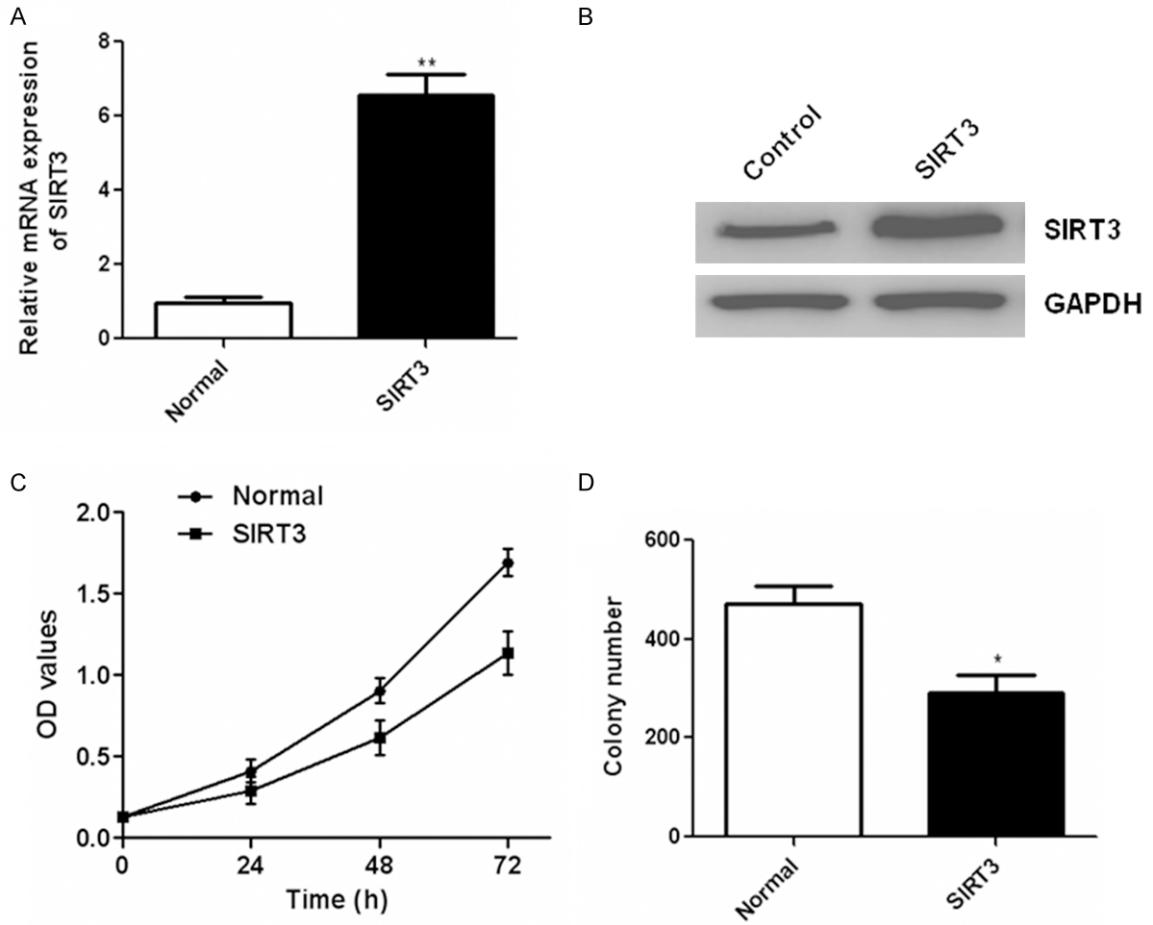

Figure 2. Overexpression of SIRT3 inhibited AGS cells proliferation and colony formation. SIRT3 expression was determined by qRT-PCR (A) and western blot (B) in AGS cells transfected with empty vector or plasmids encoding SIRT3. CCK-8 assay (C) and colony formation assay (D) were used to measure the proliferation and colony number of tumor cells after transfection with plasmids encoding SIRT3. $^{*}P<0.05$; $^{**}P<0.01$.

(**Figure 3C**). The colony formation assay displayed a dramatic increase in colony number when AGS cells were transfected with the SIRT3 siRNA (**Figure 3D**). In addition, similar results were also observed in SGC-7901 cells (data not shown). Taken together, our results demonstrated that SIRT3 might be a growth suppressor in gastric cancer cells.

SIRT3 inhibited Notch-1 expression in NSCLC cells

We furthermore investigated the molecular mechanism underlying the inhibitory effects of SIRT3 on gastric cancer cells. Surprisingly, over-expression of SIRT3 reduced the mRNA expression of Notch-1 in AGS cells (**Figure 4A**). In addition, western blot analysis also showed that Notch-1 protein expression was significantly decreased in AGS cells overexpressing SIRT3 (**Figure 4B** and **4C**). By contrast, down-regulation of SIRT3 with siRNA obviously elevated the Notch-1 expression both at mRNA (**Figure 4D**) and protein levels (**Figure 4E** and **4F**). These results showed that SIRT3 negatively regulated Notch-1 expression in AGS cells.

Inhibitory effects of SIRT3 were mediated by up-regulation of Notch-1

In order to explore how SIRT3 exhibited an inhibitory effect on gastric cancer cells growth, the expression of Notch-1 was up-regulated after transfection with plasmids encoding Notch-1 as shown by real time PCR and western blot (**Figure 5A** and **5B**). As a result, the inhibitory effects of SIRT3 on cell proliferation and colony formation were partially reversed after overexpression of Notch-1 in AGS cells (**Figure 5C** and **5D**). Taken together, these data showed

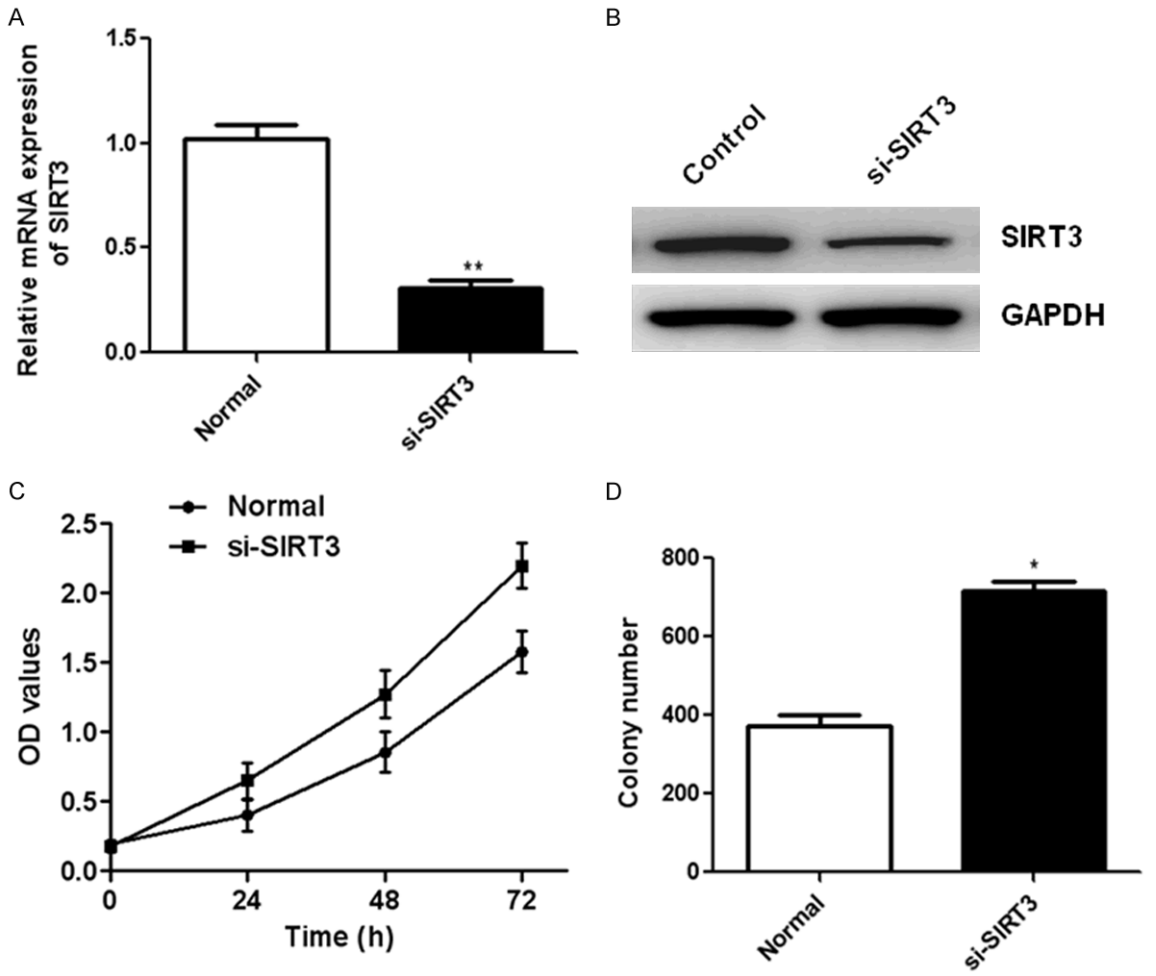

Figure 3. Downregulation of SIRT3 promoted AGS cells proliferation and colony formation. qRT-PCR (A) and western blot (B) were used to measure SIRT3 expression in AGS cells after transfection with siRNA oligos. The proliferation and colony number of tumor cells were analyzed by CCK-8 assay (C) and colony formation assay (D) after transfection with siRNA oligos. $^{*}P<0.05$; $^{**}P<0.01$.

that SIRT3 could suppress NSCLC cell proliferation by down-regulation of Notch-1.

Discussion

It has been reported that sirtuin family members (SIRT 1-7) are pivotal modulators in the initiation and progression of cancers [19, 20]. SIRT3 is the only member that has been associated with longevity in human and has been identified as a cell survival factor protecting cells from genotoxic stress [21]. Association of SIRT3 with breast, oral, and lung adenocarcinoma has been previously reported [15, 18, 22]. But the biological function of SIRT3 in gastric cancer cells has not been investigated. The present study is to address the role of SIRT3 in gastric cancer cell lines along with the molecular mechanism underlying these effects.

Sirtuins are protein deacetylases/ADP ribosyltransferases which target a wide range of cellular proteins in the mitochondria, cytoplasm, and nucleus for post-translational regulation by ADP ribosylation or acetylation, therefore modulating the expression levels of many genes [23]. Accumulating evidences show that the aberrant expression of sirtuins is closely associated with tumor initiation and development [24].

The sirtuin family member SIRT3 is universally expressed in metabolically active tissues and located in the mitochondria [25]. Recently, SIRT3 has been drawn much attention due to its association with tumorigenesis. It is demonstrated that the expression of SIRT3 is reduced in a panel of human tumors, such as breast carcinoma, hepatocellular carcinoma, ovarian car-

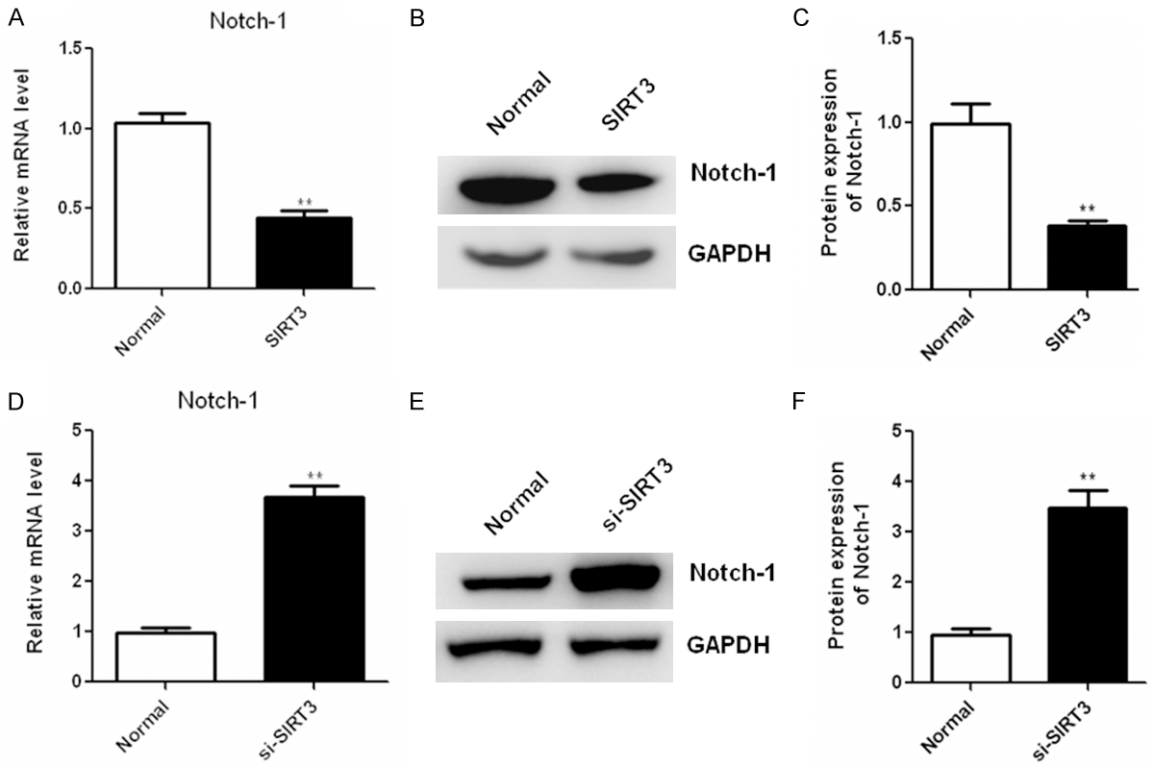

Figure 4. SIRT3 inhibited the expression of Notch-1. qRT-PCR and western blot were applied to measure the mRNA (A) and protein (B and C) expression of Notch-1 in AGS cells over-expressing SIRT3. (B and C) Western blot was performed to determine the Notch-1 protein expression in AGS cells over-expressing SIRT3. AGS cells were transfected with scramble siRNA or si-SIRT3, then the mRNA (D) and protein (E and F) levels of Notch-1 were determined by qRT-PCR and western blot. Relative band intensities of each protein were quantified by densitometry. $^{*}P<0.05$; $^{**}P<0.01$.

cinoma, and medulloblastoma, suggesting that SIRT3 might function as a mitochondrial tumor suppressor [26]. For example, SIRT3 inhibits hepatocellular carcinoma cell growth through reducing Mdm2-mediated p53 degradation [17]. However, SIRT3 also seems to function as an oncogene in several tumors such as oral carcinoma; SIRT3 was up-regulated in oral squamous cell carcinoma cell lines and down-regulation of SIRT3 inhibited cells growth and proliferation [26].

In patients with gastric cancer, the expression level of SIRT3 has been shown to be decreased and inversely correlated with clinicopathological variable, including tumor differentiation, tumor stage, tumor infiltration, and 5-year survival of these patients [27, 28]. Consistently, our study showed that both the mRNA and protein expression of SIRT3 were significantly reduced in GC tissues. We also demonstrated that SIRT3 expression was decreased in several gastric cancer cell lines. These results suggested that SIRT3 might function as a tumor suppressor in GC. Furthermore, we up-or down-regulated the expression of SIRT3 in GC cells to investigate its biological roles. Results showed that over-expression of SIRT3 significantly reduced the proliferation rate and colony formation number, whereas SIRT3 knockdown promoted cells growth and colony formation ability, implicating that SIRT3 acted as a negative regulator of growth in GC cells.

The Notch family proteins play critical roles in cell behavior including cell proliferation, differentiation and apoptosis [29, 30]. Notch-1 encodes a member of the Notch family and shares structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple, different domain types [31]. Studies have shown that Notch-1 is overexpressed in many types of cancers, suggesting that Notch-1 may play a critical role in tumorigenesis [32, 33]. Previous

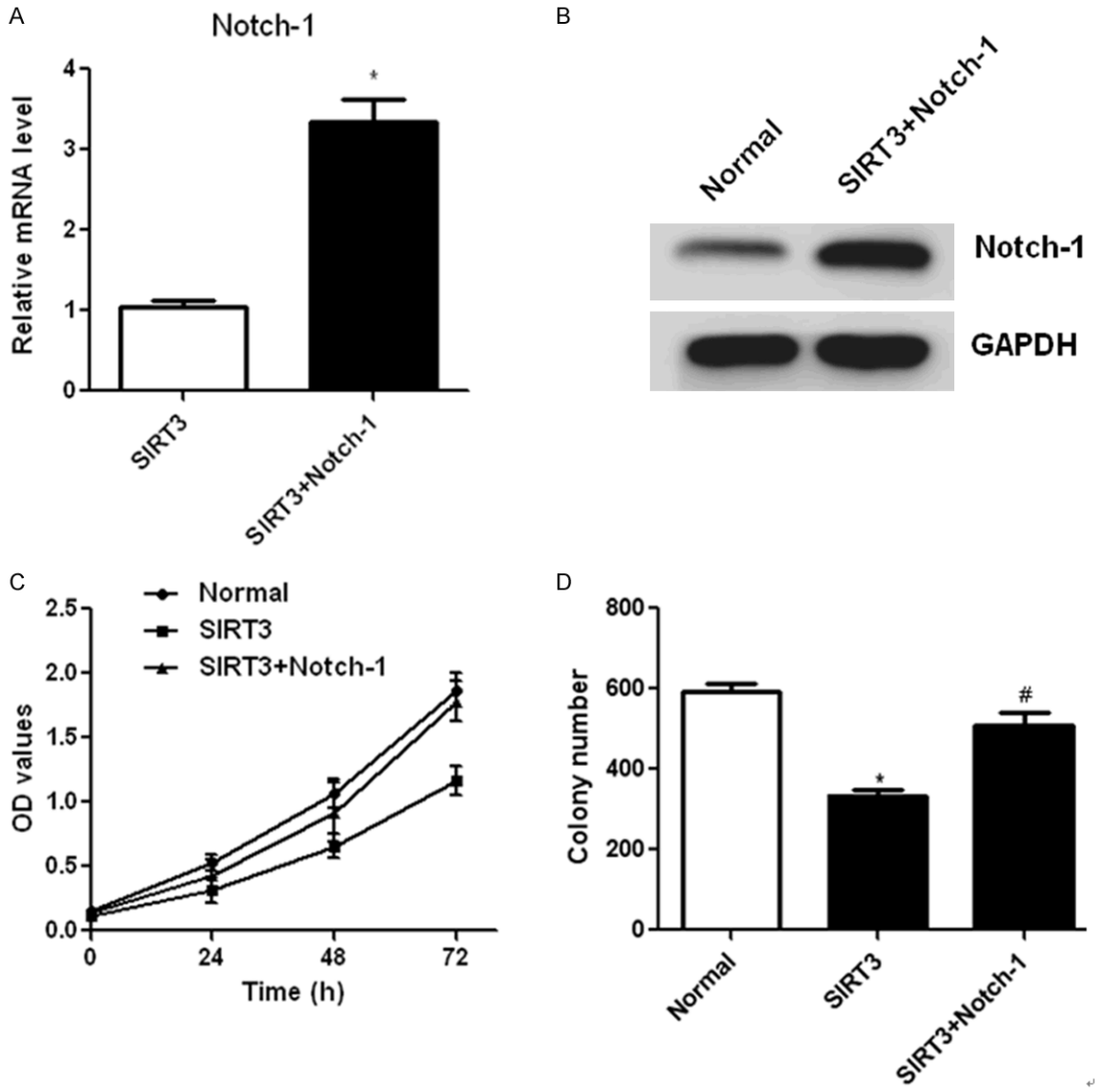

Figure 5. Inhibitory effects of SIRT3 on gastric cancer cell growth was modulated by Notch-1. AGS cells over-expressing SIRT3 were transfected with plasmids encoding Notch-1. Then, the expression of Notch-1 at the mRNA (A) and protein (B) levels was detected by qRT-PCR and western blot. CCK-8 assay (C) and colony formation assay (D) were used to measure the proliferation and clone formation of AGS cells over-expressing SIRT3 and Notch-1. *P<0.05, compared to normal group; #P<0.05, compared to SIRT3 group.

study reported that activation of Notch-1 reduced the TNFα-induced growth suppression and apoptosis via inhibition of caspase-3 in gastric cancer cells [34]. In addition, it has been reported that Notch-1 expression is inversely correlated with the survival time of GC patients [35]. These results indicate that Notch-1 might be a novel therapeutic target in the treatment of gastric caner. In the present study, we found that SIRT3 significantly suppressed the expression of Notch-1. Furthermore, over-expression of Notch-1 diminished the inhibitory effect on cells proliferation and colony formation, indicating that the anti-proliferative ability of SIRT3 was mediated by Notch-1.

In conclusion, our results demonstrated that SIRT3 suppresses the proliferation of gastric cancer cells via inhibition of Notch-1 expression and might provide novel therapeutic targets in the gastric cancer treatment.

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Disclosure of conflict of interest

None.

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