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Circ-sirt1 inhibits growth and invasion of gastric cancer by sponging miR-132-3p/miR-212-3p and upregulating sirt1 expression

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circRNAs have been considered as a rising factor in cancers. However, the roles and mechanisms of circ-sirt1 in gastric cancer (GC) remain largely unknown. In this study, we found that the expressions of sirt1 and circ-sirt1 are decreased in tissues or serums of GC patients by real-time quantitative PCR (RT-qPCR). The expressions of miR-132-3p/miR-212-3p showed an opposite tendency in these samples. The co-transfection of miR-132-3p/miR-212-3p mimics counteracted the enhancement of sirt1 expression induced by circ-sirt1. The results of cell colony-formation assay and transwell assays demonstrated that the proliferation, migration, and invasion activities of BGC-823 cells were inhibited by circ-sirt1 overexpression or miR-132-3p/miR-212-3p knockdown, respectively. The xenograft tumor model result indicated that the circ-sirt1 overexpression suppressed the tumor growth of BGC-823 cells. The regulation of miR-132-3p/miR-212-3p between circ-sirt1 and sirt1 was verified in the mice tumor tissues. Thus, circ-sirt1 inhibited tumor growth and invasion probably by sponging miR-132-3p/miR-212-3p and upregulating sirt1 expression in GC. These findings may provide a theoretical basis for the classification of GC and a novel therapeutic target for GC patients.

Key words: gastric cancer, circ-sirt1, sirt1, miR-132-3p, miR-212-3p, growth, invasion

Gastric cancer (GC) is an important disease worldwide, with over 1 million estimated new cases annually. Due to its frequently advanced stage at diagnosis, mortality from GC is high, making it the third reason in males and the fourth reason in females for cancer-related deaths globally in 2018 [1]. At present, the treatment methods for GC are mainly based on stages and types of the GC [2]. Although several classifications of GC have been developed to improve the patients' therapeutic effect, for each GC classification exist limitations in the diagnosis and treatment [3, 4]. Thus, the clarification of the molecular mechanism in GC development and the exploring of effective biomarkers of GC, to some extent, will improve the treatment of GC patients.

Evidence has shown that genetic mutations, epigenetic alterations, and aberrant molecular signaling pathways participated in the processes of tumorigenesis, spread, and metastasis of GC [5]. Recently, circRNAs have been considered as a rising factor in GC [6]. CircRNAs are a novel type of non-coding RNAs with covalently closed-loop structure, which possibly confer many unique characteristics to them, such as high abundance and stability, evolutionary species conservation, and tissues specific expression patterns. They have been demonstrated to serve as microRNA (miRNA) sponges involved in many human diseases including cancer, by regulating alternative splicing, binding to RNA binding proteins (RBPs) as well as encoding proteins [6]. CircRNAs have been reported to serve as oncogenes or antioncogenes in GC. For example, ciRS-7 has been demonstrated to act as an oncogenic circRNA by antagonizing miR-7-mediated PTEN/PI3K/AKT pathway in MGC-803 and HGC-27 GC cells [7]. CircYAP1 may serve as a potential suppressive factor for GC by regulating the miR-367-5p/p27 (Kip1) axis [8]. To date, several circRNA sequencing data have shown that circRNAs are abundant in GC tissues and many of them have aberrant expression levels, which could be GC-related circRNAs. Huang et al. have reported that 16 circRNAs were upregulated and 84 circRNAs were downregulated in GC tissues compared with the adjacent normal tissues [9]. In addition, circRNA-0026 may regulate RNA transcription, RNA metabolism, gene expression, gene silencing, and other biological

functions in GC [9]. Considering our previous study on circ-sirt1, which has been proved to be a regulator of vascular smooth muscle cells phenotype by upregulating sirtuin 1 (sirt1) [10], we wonder if circ-sirt1 executes a function and the similar regulation mechanism on GC development.

In the present study, we detected the expression of circ-sirt1 and sirt1, as well as miR-132-3p/miR-212-3p, in GC tissues and serums. The effects of circ-sirt1 overexpression on proliferative, migratory, and invasive activities of BGC-823 cells, as well as the expressions of sirt1 and miR-132-3p/miR-212-3p, were analyzed. Finally, we performed a tumor xenograft assay to confirm the exact roles of circ-sirt1 and the regulation mechanism in BGC-823 cells *in vivo*.

Patients and methods

Clinical specimens. The tissue and serum specimens from 20 gastric cancer (GC) patients who had not received chemotherapy or radiotherapy prior to radical resection were obtained from Tangshan People's Hospital between May and November 2017. The patients with GC were aged between 54 and 80 years and included male and female patients. The healthy serums were collected from 10 healthy subjects from the same hospital, between 41 and 73 years old, including males and females. The use of clinical specimens was approved by the Ethics Committee of Tangshan People's Hospital, and written informed consent was obtained from the individuals. All tissues and blood were stored at −80 °C for further study.

Cell culture and transfection. Human cell lines GES-1 cells and 293T cells (Saierbio, Tianjin, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, USA). AGS-1 and BGC-823 cells were cultured in 1640 medium (Gibco BRL, USA). All the culture mediums were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution in a humidified incubator containing 5% CO_2 at 37 °C.

Vector construction. To construct a circ-sirt1 overexpression plasmid, the fragment with 927 nt length of circ-sirt1 was cloned into pcDNA3.1 vector between the Kpn I and Xho I sites. The full length of circ-sirt1 was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The constructed plasmid was confirmed by 0.1% agarose gel electrophoresis and sequencing. The location and exons of circ-sirt1 are shown in Supplementary Figure S1, and the result of the sequencing is shown in Supplementary Figures S2A–S2D.

RNA extraction and quantitative PCR (qPCR). Total RNAs were extracted from cells, tissues, and serums with Trizol reagent (Invitrogen, American) and reverse converted into cDNA. The primers for reverse transcription assay, miR-132-3p: 5'-TCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACCGACCATG-3'; miR-212-3p: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACGCCGTGA-3'; U6: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGGAAC-3'. The qPCR was conducted using the SYBR Premix Ex Taq kit (Takara, Japan) and performed for the following genes: circ-sirt1, sirt1, miR-132-3p, and miR-212-3p. U6 and GAPDH were used as internal control. The primer sequences were as follows: circ-sirt1, forward: 5'-GCACTAATTCCAAATAACCTTC-3' and reverse: 5'-TCATATCATCCAACTAAGGT-3'; sirt1, forward: 5'-CCGGAAACAATACCTCCACC-3' and reverse: 5'-CACCCCAGCTCCAGTTAGAA-3'. GAPDH, forward: 5'-ACCACAGTCCATGCCATCAC-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3'; miR-212-3p, forward: 5'-TGCGGTAACAGTCTCCAGTCAC-3' and reverse 5'-CCAGTGCAGGGTCCGAGGT-3'; miR-132-3p, forward: 5'-TGCGGTAACAGTCTACAGCCATG-3' and reverse 5'-CCAGTGCAGGGTCCGAGGT-3'; U6, forward: 5'-TGCGGGTGCTCGCTTCGGCAGC-3' and reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'. All the primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Calculation of the mRNA levels was normalized to GAPDH (circ-sirt1) or U6 (miR-132-3p, miR-212-3p) relative to the control by the $2^{-\Delta\Delta Ct}$ method. Experiments were conducted in triplicate and repeated three times.

Western blot. BGC-823 cells or tissues samples were harvested and extracted using RIPA lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol), and equal amounts of cells extracts were separated on 10% SDS-PAGE gels and then the separated proteins were transferred samples onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA). After blocking with 5% Blotto for 2 h, the membrane was incubated at 4 °C overnight with the primary antibody, sirt1 (1:500, CY5023, Abways Technology, Inc.) and GAPDH (1:2000, SRP00849, Saierbio, Tianjin, China). HRP rabbit IgG secondary antibodies were added and incubated for 1.5 h at room temperature. The membranes were washed four times for 5 min with TBST buffer, then put the membranes into the Western Lightning™ Chemiluminescence Reagent for 30 s. Finally, blot signals were visualized by LabWorksTM gel imaging and analysis system.

Cell colony formation assay. For colony formation assay, the BGC-823 cells were seeded into a 6-well plate with a number 1×10^3/well at 24 h post-transfection. The medium was changed every 3 days. After 10 days, the cells were stained with crystal violet, and colonies including more than 50 cells were counted. The average number was used to evaluate the formation ability of GC cells.

Cell migration and invasion assays. The migration and invasion were analyzed by 24-well Boyden chambers with an 8-μm pore size polycarbonate membrane (Corning, Cambridge, MA). Briefly, 5×10^4/well of BGC-823 cells were resuspended in a culture medium without FBS and seeded in the upper chamber. Then the chamber was placed into a 24-well plate containing 600 μl of culture media with 20% FBS. The upper chamber was coated with 40 μl Matrigel (BD biosciences, USA) for invasion assay incubating for 36 h and without Matrigel for migration assay incubating for 24 h, both at 37 °C. After the cultured time, the cells were fixed

with 33% (v/v) acetic acid (glacial acetic acid: methyl alcohol, 1:3) and stained with 0.1% crystal violet (Solarbio Science & Technology Co., Ltd, Beijing, China). The cells that did not pass through the membrane were removed with the cotton stick. The number of migrated cells per field was counted with an Olympus IX 71 (Tokyo, Japan).

***In vivo* assay.** To assess tumor growth activity of BGC-823 cells with circ-sirt1 overexpression *in vivo*, stable cell lines were established. BGC-823 cells were resuspended with a number 5×10^6 (pcDNA3.1 or circ-sirt1) in 100 μl of serum-free RPMI-1640 medium and subcutaneously inoculated into the axillary fossae of BALB/c-nu (n=6, 6–8 weeks old), purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences, Beijing. The size of the tumor was observed once a week. When the tumors reached 50–100 mm^3, the mice were sacrificed, and their tumor tissues were harvested and stored at −80 °C. All the studies were performed according to the American Association for the Accreditation of Laboratory Animal Care guidelines.

Statistical analysis. Data analysis was performed using SPSS software version 17.0 (SPSS, Inc.). Each experiment was carried out at least in triplicate and all results were presented as the mean ± SD. Student's t-test was used to assess statistical significance between two groups. An ANOVA was performed to compare three groups. If a significant interaction was observed, Scheffe's post hoc testing was used to determine pairwise differences between mean; *p<0.05 and **p<0.01 were considered to indicate a statistically significant difference.

Results

The sirt1 and circ-sirt1 levels are decreased in tissues and serums of GC patients. To explore the role of circ-sirt1 in GC development, we first detected the expression of *sirt1* in GC tissues and the levels of circ-sirt1 in GC tissues and serum samples. The *sirt1* expression level was much lower in tumor tissues than that in non-tumor tissues, decreased by approximately 18.0% (**p<0.01, Figure 1A). In addition, the levels of circ-sirt1 in GC tissues (**p<0.01, Figure 1B) and the corresponding serum samples (***p<0.001, Figure 1C) were lower than those in the non-tumor tissues and healthy subjects, respectively. These results demonstrated that sirt1 and circ-sirt1 are both less expressed in GC tissues than in the noncancerous tissues, and the level of circ-sirt1 in serums from GC patients is lower than that noted from the healthy subjects. The data suggested that sirt1 and circ-sirt1 may be involved in the GC tumorigenesis and development.

The miR-132-3p/miR-212-3p levels are increased in GC tissues and serums of GC patients. Given that circ-sirt1 could bind and negatively regulate the miR-132-3p and miR-212-3p to promote sirt1 expression in vascular smooth muscle cells [10], we detected miR-132-3p/miR-212-3p levels in the GC tissues and serum samples. The levels of miR-132-3p were higher in GC tissues (**p<0.01, Figure 2A) and serums (***p<0.001, Figure 2B) with respect to the non-tumor tissues and healthy serums. The levels of miR-212-3p in GC tissues (**p<0.01, Figure 2C) and serums (**p<0.01, Figure 2D) indicated the similar changes as miR-132-3p. The levels of miR-132-3p and miR-212-3p were both increased in the tissues and serums of GC patients, which in contrast with the expression of circ-sirt1 and sirt1 in GC tissues. These results indicated that miR-132-3p/miR-212-3p might be the mediators between circ-sirt1 and sirt1 in GC.

Overexpression of circ-sirt1 inhibited cell proliferation, migration, and invasion of BGC-823 cells. Based on the above data, it was speculated that circ-sirt1 might be involved in tumorigenesis and the development of GC. To testify our speculation, we constructed the circ-sirt1 overexpression plasmid (Figure 3A) and validated the efficiency in 293T cells (Figure 3B). Then, the circ-sirt1 expressions in GES-1 cells, AGS-1 cells, and BGC-823 cells were detected by RT-qPCR. The BGC-823 cells were selected for further

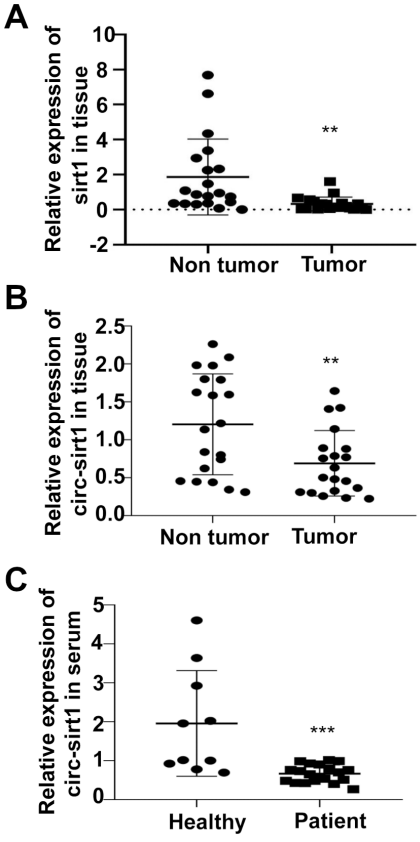

Figure 1. The expressions of sirt1 and circ-sirt1 in GC tissue and serum samples. (A) Relative mRNA levels of sirt1 in GC tissue (tumor) and adjacent non-tumor tissue (non-tumor) were detected by RT-qPCR, using GAPDH as an internal control. The circ-sirt1 levels in tumor and non-tumor (B), as well as in serum samples of gastric cancer patients (patient) and healthy subjects (healthy) (C) were analyzed by RT-qPCR. The GAPDH was used as an internal control. The results are expressed as the mean ± standard deviation (SD) ($n_{patients}$ = 20; $n_{healthy}$ = 10). **p<0.01; ***p<0.001

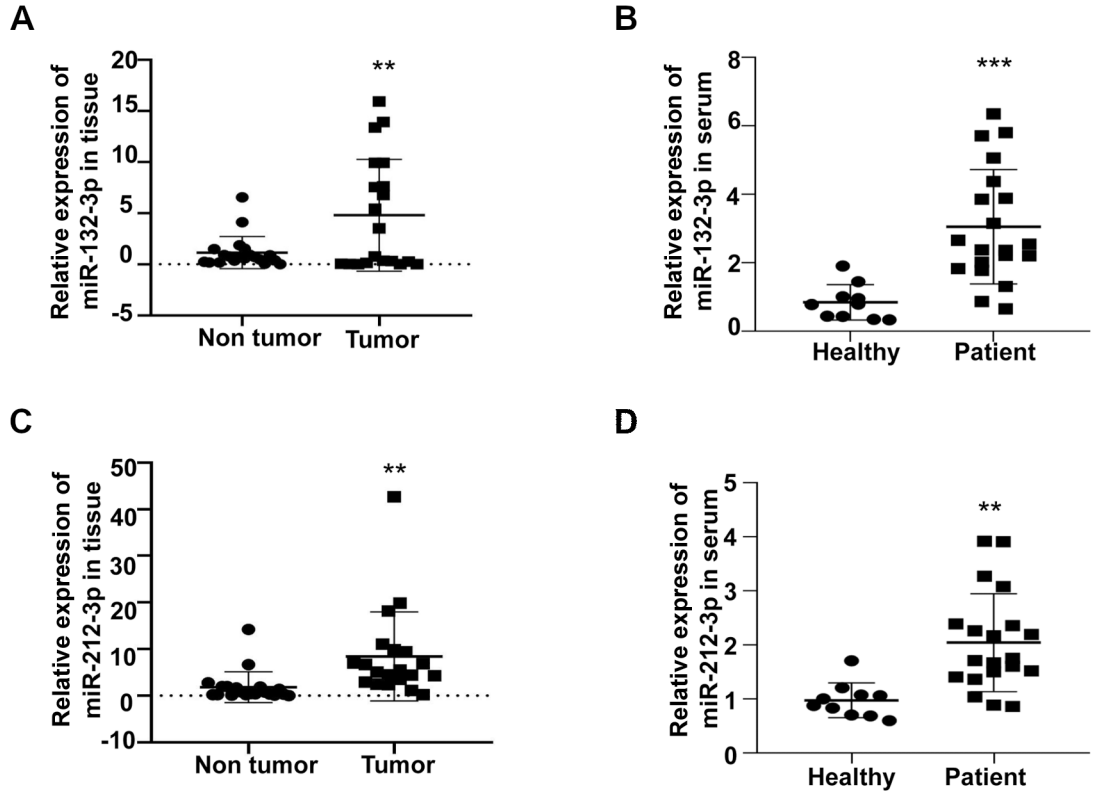

Figure 2. Relative levels of miR-132-3p and miR-212-3p in tissues and serums of GC patients. The miR-132-3p levels in tumor and non-tumor tissues (A), as well as in patients and healthy serums (B), were analyzed by RT-qPCR, using U6 as an internal control. The miR-212-3p levels in tumor and non-tumor tissues (C), as well as in patients and healthy serums (D), were detected by RT-qPCR, using U6 as an internal control. The results are expressed as the mean ± standard deviation (SD) ($n_{patients}$ = 20; $n_{healthy}$ = 10). *p<0.05; **p<0.01; ***p<0.001

experiment due to the lowest level of circ-sirt1 (*p<0.05, Figure 3C). BGC-823 cells transfected with pcDNA3/circ-sirt1 plasmid enhanced the mRNA expression of circ-sirt1 by nearly 3.7-fold (***p<0.001, Figure 3D), along with the expression of sirt1 at protein level (*p<0.05, Figure 3E). The cell colony formation assay indicated that the overexpression of circ-sirt1 reduced the proliferation activity of BGC-823 cells with respect to the control groups (*p<0.05, Figure 3F). The transwell migration and invasion assays demonstrated that circ-sirt1 overexpression decreased the migratory (*p<0.05) and invasive capabilities of BGC-823 cells (**p<0.01) (Figure 3G). All these results indicated that circ-sirt1 acts as a tumor inhibitor in GC tumorigenesis and development.

The miR-132-3p and miR-212-3p are mediators between circ-sirt1 and sirt1 and contribute to GC tumorigenesis and development. To confirm the cascade regulation relationship of circ-sirt1 on miR-132-3p/miR-212-3p and sirt1, we detected their expressions in BGC-823 cells with circ-sirt1 overexpression. Overexpression of circ-sirt1 in BGC-823 cells reduced the expression of miR-132-3p and miR-212-3p (**p<0.01, *p<0.05, Figure 4A), suggesting that circ-sirt1 negatively regulated these two miRs. Whereas cotransfected with circ-sirt1 plasmid and miR-132-3p/miR-212-3p mimics could counteract the hyperexpression of sirt1 induce by circ-sirt1 overexpression (**p<0.01, Figure 4B). Additionally, knockdown of miR-132-3p and miR-212-3p (**p<0.01, Figure 4C), the BGC-823 cells enhanced the expression of sirt1 at both mRNA and protein levels (**p<0.01, Figure 4D). These data demonstrated that miR-132-3p and miR-212-3p are negative regulated by circ-sirt1 and act as negative regulators of sirt1, that is, miR-132-3p and miR-212-3p are mediators between circ-sirt1 and sirt1 in BGC-823 cells.

Then, we evaluated the role of miR-132-3p and miR-212-3p in cell proliferation, colony formation, and migration of BGC-823 cells. Compared with the blank and negative control groups, the cell colony formation activity of BGC-823 cells with miR-132-3p and miR-212-3p knockdown was significantly declined by 43.9% (**p<0.01, Figure 4E). In addition, the migration and invasion abilities of BGC-823 cells with miR-132-3p and miR-212-3p knockdown were both reduced approximately 52.4% (***p<0.001) and 60.2% (**p<0.01) (Figure 4F). All these data hinted that the anti-tumor function of circ-sirt1 in GC cells probably mediated

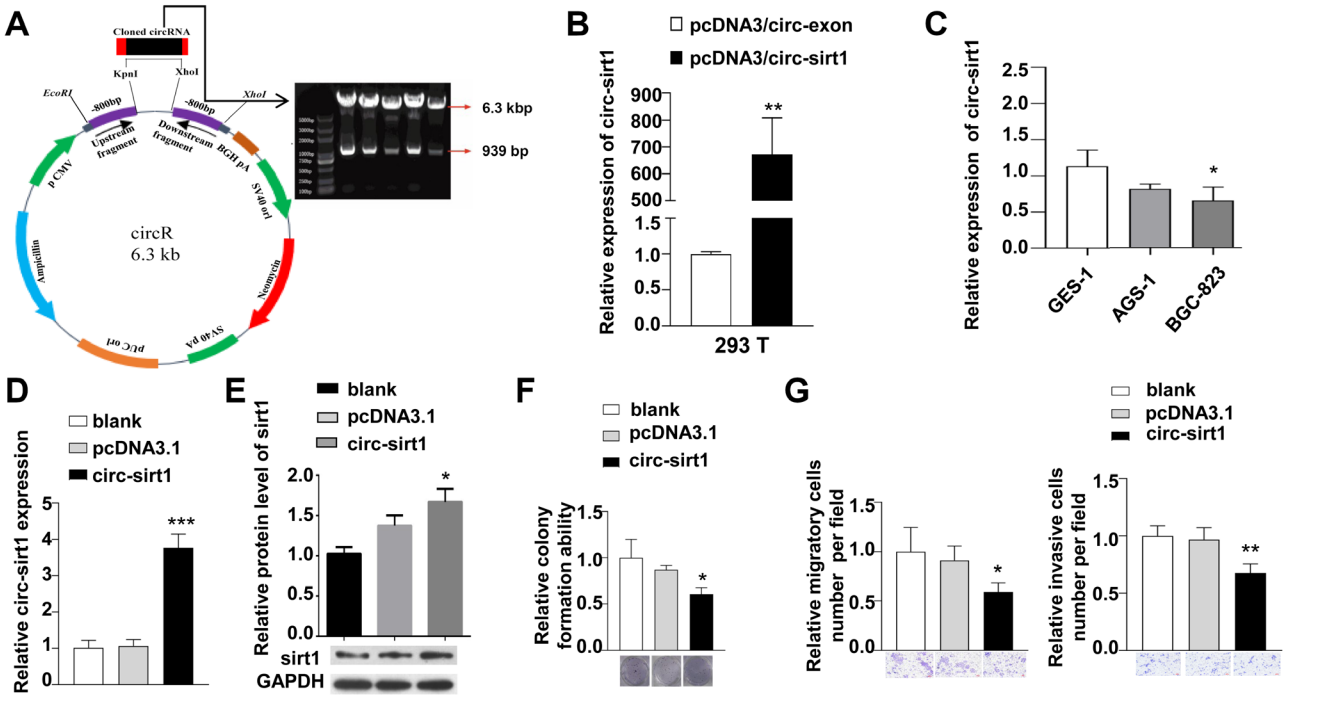

Figure 3. The affection of circ-sirt1 overexpression on proliferation, migration, and invasion abilities of BGC-823 cells. (A) The schematic diagram of pcDNA3.1 plasmid containing circ-sirt1 sequence (left panel). The pcDNA3/circ-sirt1 plasmid was confirmed by restrictive enzyme digestion (right panel). (B) The expression of circ-sirt1 in pcDNA3/circ-sirt1 plasmid in 293T cells was verified by RT-qPCR. (C) The circ-sirt1 expressions in GES-1, AGS-1, and BGC-823 cells were analyzed by RT-qPCR, with GAPDH as an internal control. (D) The levels of circ-sirt1 in BGC-823 cells transfected with circ-sirt1 plasmid or vector were detected by RT-qPCR. GAPDH was used as the housekeeping gene. (E) The protein levels of sirt1 were detected by western blot with GAPDH as an internal control. (F) Clone formation, (G) cell migration, and invasion assays of BGC-823 cells transfected with pcDNA3/circ-sirt1 plasmid or pcDNA3.1 after 48 h. Results are representative of three independent experiments. *p<0.05; **p<0.01; ***p<0.001

by miR-132-3p and miR-212-3p, as well as their negative target of sirt1.

Circ-sirt1 inhibited tumor growth and proliferation of BGC-823 cells in the mouse model by targeting miR-132-3p and miR-212-3p. To determine whether circ-sirt1 acts as a tumor suppressor by negatively regulating miR-132-3p and miR-212-3p *in vivo*, a xenograft tumor model in nude mice was used. The BGC-823 cells overexpressing circ-sirt1 were subcutaneously injected into the nude mice. After injection for one week, the tumor volume was calculated every two days to generate a growth curve. The growth curve showed that circ-sirt1 suppressed tumors derived from BGC-823 cells (*p<0.05, Figures 5A, 5B). The mice were sacrificed 21 days after injection, and the tumors were collected and weighed, separately. Overexpression of circ-sirt1 significantly reduced both the tumor volume and weight (*p<0.05, Figure 5C). These results indicated that circ-sirt1 inhibited the growth of tumors *in vivo*. In addition, the miR-132-3p/miR-212-3p levels were markedly decreased in the circ-sirt1 overexpression group compared with the control group (*p<0.05, **p<0.01, Figure 5D), while sirt1 expression was increased in the circ-sirt1 overexpression group (**p<0.01, Figure 5E). The data confirmed the anti-tumor function of circ-sirt1 and its downstream mechanism of miR-132-3p, miR-212-3p, and sirt1 in the mouse model.

Discussion

The present work demonstrates that circ-sirt1 and sirt1 levels were decreased in tissues and serums of patients with GC, while miR-132-3p/miR-212-3p expressions show an opposite tendency in the GC patients. The miR-132-3p and miR-212-3p were downregulated in GC cells with circ-sirt1 overexpression, while sirt1 was upregulated at both mRNA and protein levels. The co-transfection of miR-132-3p/miR-212-3p mimics counteracted the enhanced expression of sirt1 induced by circ-sirt1 overexpression in BGC-823 cells.

The circ-sirt1 was identified as a candidate from the sirt1 host gene in the human genome in our previous study, which expression is decreased during neointimal formation and acts as a novel biomarker of atherosclerosis. The circ-sirt1 could promote sirt1 expression mediating by miR-132/212. The present study found that circ-sirt1 acted as a suppressor

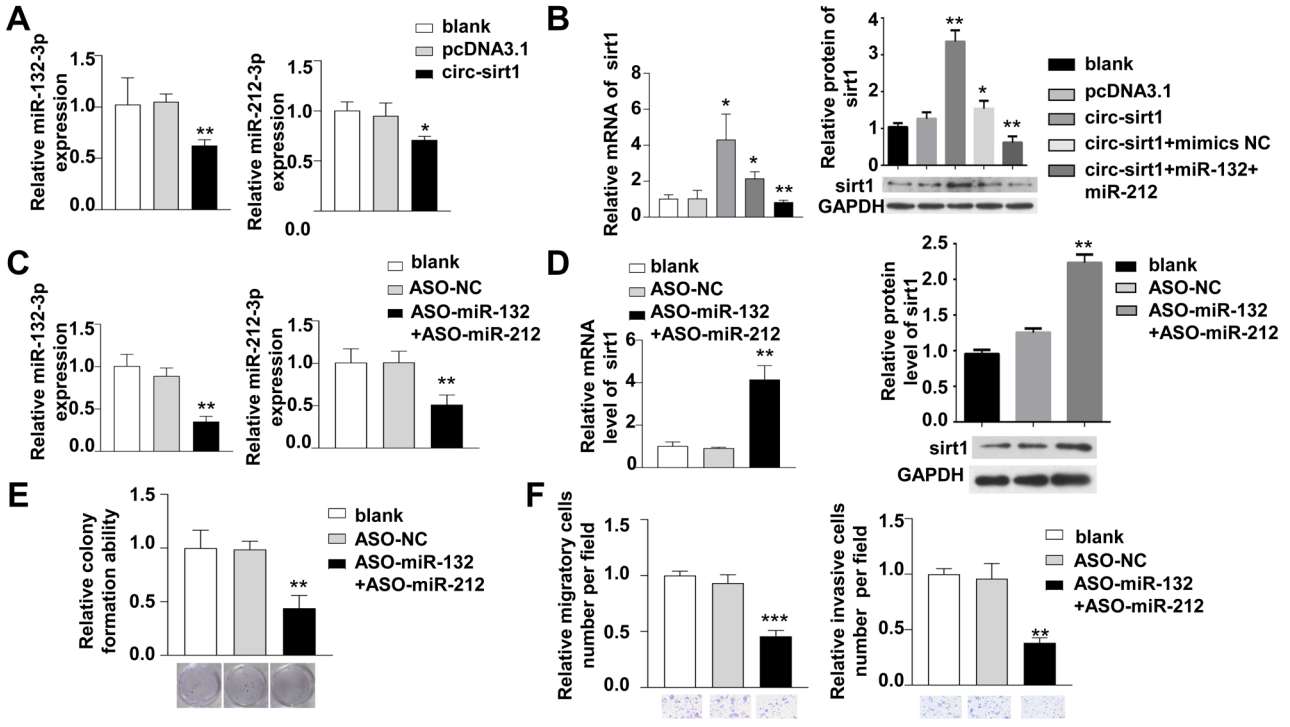

Figure 4. The function of miR-132-3p and miR-212-3p in circ-sirt1/sirt1 axis and in proliferation and development of GC cells. (A) The levels of miR-132-3p and miR-212-3p in BGC-823 cells in the indicated groups were detected by RT-qPCR. (B) The mRNA and protein levels of sirt1 in BGC-823 cells transfected with circ-sirt1, circ-sirt1+mimics NC, or circ-sirt1+miR-132-3p+miR-212-3p were analyzed by RT-qPCR and western blot. (C) The mRNA levels of miR-132-3p and miR-212-3p in BGC-823 cells cotransfected with ASO-miR-132 and ASO-miR-212 were detected by RT-qPCR. U6 was used as a housekeeping gene. (D) The expression levels of sirt1 in BGC-823 cells transfected with ASO NC or ASO-miR-132+ASO-miR-132 were detected by RT-qPCR and western blot. (E) Clone formation, (F) cell migration, and invasion assays of BGC-823 cells were performed at 48 h post-transfection with ASO NC or ASO-miR-132+ASO-miR-132. *p<0.05; **p<0.01; ***p<0.001

in proliferation, migration, and invasion in GC cells by sponging miR-132-3p/miR-212-3p to regulate the expression of sirt1. These suggested a similar function of circ-sirt1 in vascular smooth muscle cells and glandular epithelial cells of the stomach. However, whether there is a positive feedback loop between circ-sirt1 and sirt1 in GC, as well as the expression and role of circ-sirt1 in other cancers, still need to be clarified in future studies.

Sirt1 is a member of the class III histone deacetylases known as the sirtuins, widely recognized as a crucial epigenetic regulator involved in many biological processes, including metabolism [11], genomic stability maintenance [12, 13], reprogramming [14], and tumorigenesis [15]. The expression levels and roles of sirt1 in cancer seemed contradictory. Sirt1 was recognized as a tumor suppressor in oral squamous cell cancer [16] and colorectal cancer [17]. On the other hand, sirt1 has been reported to promote cell proliferation and metastasis of bladder cancer [18], breast cancer [19], and pancreatic cancer [20]. Thus, these reports indicated that the expression of sirt1 has obvious tissue specificity. Recent studies have confirmed the inhibited functions of sirt1 in malignant tumors, including in GC proliferation and metastasis [21, 22]. In our study, sirt1 was decreased in tumor tissues compared with the nontumor tissues of GC patients, suggesting sirt1 is an antioncogene in GC development. Whereas, previous evidence revealed that sirt1 was highly expressed in GC tissues and associated with poor prognosis [23]. Recently, Jiang et al. conducted a systematic meta-analysis study and declared that the expression of sirt1 is more likely found in elder, higher T/N stage and lower tumor differentiation patients [24]. The dual functions of sirt1 in GC in different studies might be attributed to advanced age, the complex pathological type, and stage.

In this study, miR-132-3p/miR-212-3p, functional variants, were confirmed to be overexpressed in cancer tissues compared with the adjacent nontumor tissues, suggesting that miR-132-3p/miR-212-3p possibly functioned as a tumor promoter in GC development. Wu et al. [25] pointed out miR-132-3p/miR-212-3p, inhibiting the expression of CD80 through binding with the CD80 3'-UTR, contributed to the occurrence of GC. These studies suggested that miR-132-3p/miR-212-3p were increased in GC tissues and regulated the occurrence of GC via binding different target genes. On the contrary, miR-132-3p was hypo-expressed in HepG2 and

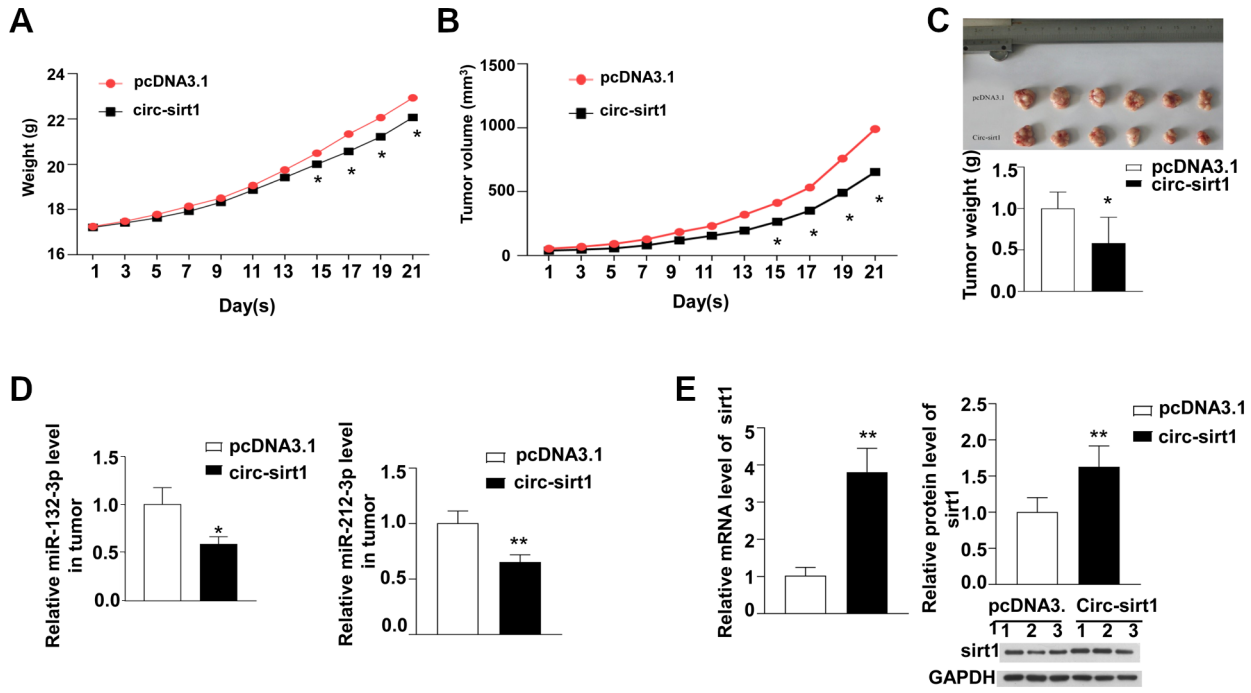

Figure 5. The effect of circ-sirt1 over-expression on tumor growth *in vivo*. (A) The growth curve and line chart of BALB/c nude mice. (B) The tumors of xenograft model mice inoculated with BGC-823 cells transfected with the circ-sirt1 and pcDNA3.1 vector. (C) The expression of miR-132-3p and miR-212-3p in tumor tissues were detected by RT-qPCR. (D) The mRNA and protein expressions of sirt1 in tumor tissues were detected by RT-qPCR and western blot (n=6). The data were presented as the mean ± SD. *p<0.05; **p<0.01

Huh7 human liver cancer cell lines compared with highly metastatic liver cancer HccLM3 cells. Overexpression of miR-132-3p resulted in significant inhibition of proliferation and induction of apoptosis in Langerhans cells (LC) [26]. Additionally, miR-132-3p was identified by integrated analysis of miRNAs and DNA methylation to be a tumor suppressor in lung adenocarcinoma [27]. For another variant, miR-212-3p has been reported to suppress cell proliferation in ovarian cancer and bladder cancer [28, 29]. Based on the controversial roles of miR-132-3p and miR-212-3p, the function of miR-132-3p/miR-212-3p on proliferation and invasion of GC cells needs to be further studied.

This study confirmed the function of the circ-sirt1/miR-132-3p/miR-212-3p/sirt1 axis in GC development. Taken together, we found that circ-sirt1 is downregulated not only in cancer tissues and serums from the patients with GC but also in GC cell lines. The reduced expression of sirt1 in GC tissues due to the enhancement of miR-132-3p/miR-212-3p induced by circ-sirt1. Overexpression circ-sirt1 suppressed the cell proliferation, migration, and invasion *in vitro*, and inhibited tumor growth *in vivo*. Our findings provide new sights into the signaling of circ-sirt1/miR-132-3p/miR-212-3p/ sirt1 in GC development.

Supplementary information is available in the online version of the paper.

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https://doi.org/10.4149/neo_2021_210218N222

Circ-sirt1 inhibits growth and invasion of gastric cancer by sponging miR-132-3p/miR-212-3p and upregulating sirt1 expression

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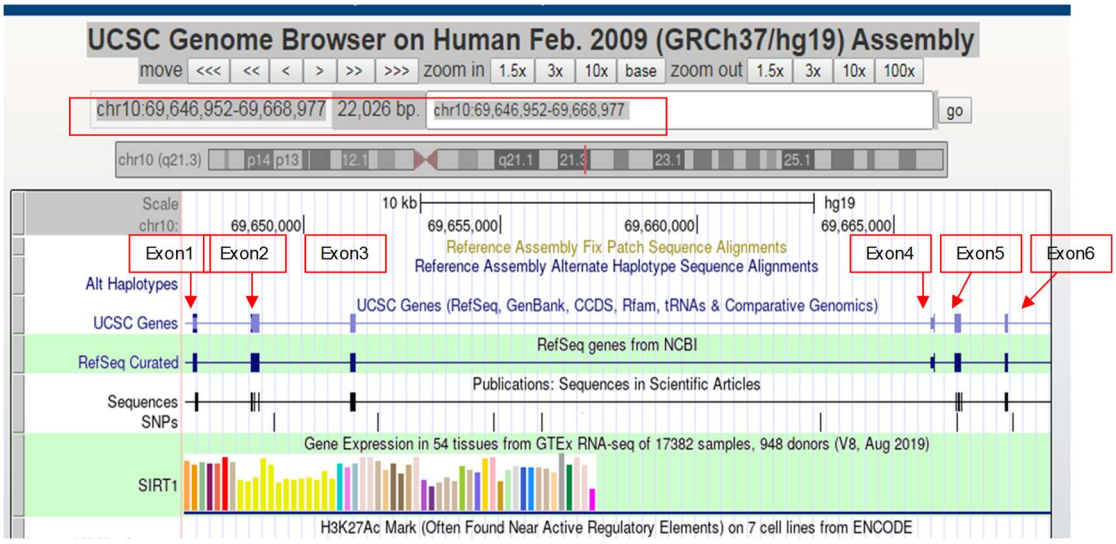
Supplementary Information



Supplementary Figure S1. Circ-sirt1 isoform is located at chr10:69647174-69669199 in human genome (hsa_circ_0093887). The six Exons of circ-sirt1 was shown from https://genome.ucsc.edu/. The location and exons of circ-sirt1 were shown in red boxes.

A

191108HE9847-7.YC2785F.CMV-F_F11：

CCATGGGAGTCAATAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGG
CTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAA
CTTAAGCTTGGTACCATAACCTTCTGTTCGGTGATGAAATTATCACTAATGGTTTTC
ATTCCTGTGAAAGTGATGAGGAGGATAGAGCCTCACATGCAAGCTCTAGTGACTG
GACTCCAAGGCCACGGATAGGTCCATATACTTTTGTTCAGCAACATCTTATGATTGG
CACAGATCCTCGAACAATTCTTAAAGATTTATTGCCGGAAACAATACCTCCACCTG
AGTTGGATGATATGACACTGTGGCAGATTGTTATTAATATCCTTTCAGAACCACCAA
AAAGGAAAAAAAGAAAAGATATTAATACAATTGAAGATGCTGTGAAATTACTGCA
AGAGTGCAAAAAAATTATAGTTCTAACTGGAGCTGGGGTGTCTGTTTCATGTGGAA
TACCTGACTTCAGGTCAAGGGATGGTATTTATGCTCGCCTTGCTGTAGACTTCCCAG
ATCTTCCAGATCCTCAAGCGATGTTTGATATTGAATATTTCAGAAAAGATCCAAGAC
CATTCTTCAAGTTTGCAAAGGAAATATATCCTGGACAATTCCAGCCATCTCTCTGTC
ACAAATTCATAGCCTTGTCAGATAAGGAAGGAAAAACTACTTCGCAACTATACCCA
GAACATAGACACGCTGGAACAGGTTGCGGGAATCCAAAGGATAATTCAGTGTCAT
GGTTTCCTTTGCAACAGCATCTTGCCTGATTTGTAAATACAAAGTTGACTGTGAAG
CTGTACGAGGAGATATTTTTAAATCAGTAGTTCCTCGATGTCCTAGTGCCCAGCTGA
TGGACGCTGGCTATCATGAAACAGAGATTGTATTTTTTGATGAAATTAACAGACCA
GGTTCATAGAAGTCATGCAGTATGAACAAAAAGAATGA

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Query  128  ATAACCTTCTGTTCGGTGATGAAATTATCACTAATGGTTTTCATTCCTGTGAAAGTGATG  187
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATAACCTTCTGTTCGGTGATGAAATTATCACTAATGGTTTTCATTCCTGTGAAAGTGATG  60

Query  188  AGGAGGATAGAGCCTCACATGCAAGCTCTAGTGACTGGACTCCAAGGCCACGGATAGGTC  247
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AGGAGGATAGAGCCTCACATGCAAGCTCTAGTGACTGGACTCCAAGGCCACGGATAGGTC  120

Query  248  CATATACTTTTGTTCAGCAACATCTTATGATTGGCACAGATCCTCGAACAATTCTTAAAG  307
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CATATACTTTTGTTCAGCAACATCTTATGATTGGCACAGATCCTCGAACAATTCTTAAAG  180

Query  308  ATTTATTGCCGGAAACAATACCTCCACCTGAGTTGGATGATATGACACTGTGGCAGATTG  367
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Sbjct  181  ATTTATTGCCGGAAACAATACCTCCACCTGAGTTGGATGATATGACACTGTGGCAGATTG  240

Query  368  TTATTAATATCCTTTCAGAACCACCaaaaaggaaaaaaagaaaaGATATTAATACAATTG  427
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TTATTAATATCCTTTCAGAACCACCAAAAAGGAAAAAAAGAAAAGATATTAATACAATTG  300

Query  428  AAGATGCTGTGAAATTACTGCAAGAGTGCaaaaaaaTTATAGTTCTAACTGGAGCTGGGG  487
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  AAGATGCTGTGAAATTACTGCAAGAGTGCAAAAAAATTATAGTTCTAACTGGAGCTGGGG  360

Query  488  TGTCTGTTTCATGTGGAATACCTGACTTCAGGTCAAGGGATGGTATTTATGCTCGCCTTG  547
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGTCTGTTTCATGTGGAATACCTGACTTCAGGTCAAGGGATGGTATTTATGCTCGCCTTG  420

Query  548  CTGTAGACTTCCCAGATCTTCCAGATCCTCAAGCGATGTTTGATATTGAATATTTCAGAA  607
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CTGTAGACTTCCCAGATCTTCCAGATCCTCAAGCGATGTTTGATATTGAATATTTCAGAA  480

Query  608  AAGATCCAAGACCATTCTTCAAGTTTGCAAAGGAAATATATCCTGGACAATTCCAGCCAT  667
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  AAGATCCAAGACCATTCTTCAAGTTTGCAAAGGAAATATATCCTGGACAATTCCAGCCAT  540

Query  668  CTCTCTGTCACAAATTCATAGCCTTGTCAGATAAGGAAGGAAAAACTACTTCGCAACTAT  727
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  541  CTCTCTGTCACAAATTCATAGCCTTGTCAGATAAGGAAGG-AAAACTACTTCGCAACTAT  599

Query  728  ACCCAGAACATAGACACGCTGGAACAGGTTGCGGGAATCCAAAGGATAATTCAGTGTCAT  787
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Sbjct  600  ACCCAGAACATAGACACGCTGGAACAGGTTGCGGGAATCCAAAGGATAATTCAGTGTCAT  659

Query  788  GGTTTCCTTTGCAACAGCATCTTGCCTGATTTGTAAATACAAAGTTGACTGTGAAGCTGT  847
             || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660  GG-TTCCTTTGCAACAGCATCTTGCCTGATTTGTAAATACAAAGTTGACTGTGAAGCTGT  718

Query  848  ACGAGGAGATATTTTTAAATCA-GTAGTTCCTCGATGTCCTA-GTGCCCAGCTGATGGA-  904
             |||||||||||||||||||| ||| |||||||||||||||| |||||||||||||||| |
Sbjct  719  ACGAGGAGATATTTTTAA-TCAGGTAGTTCCTCGATGTCCTAGGTGCCCAGCTGATGAAC  777

Query  905  CGCTGGCTATCATGAAA-CAGAGATTGTATTTTTTGATGAAA-TTAA-CAGACCAGGTTC  961
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Sbjct  778  CGCTTGCTATCATGAAACCAGAGATTGTGTTTTTTGGTGAAAATTTACCAGAACAGTTTC  837

Query  962  ATAGAAGTCATGCAGTATGAACAAA  986
             |||| || |||| |||||||| ||||
Sbjct  838  ATAG-AGCCATGAAGTATGA-CAAA  860
```

Supplementary Figure S2. A) The sequence of the pcDNA3.1 plasmid containing circ-sirt1 from 5' to 3'.

B

191108HE9847-7.YC2785F.PMP9002-_G11：
TGGATGACGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGC
AAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCAGCGGGTTTA
AACGGGCCCTCTAGACTCGAGTTGGAATTAGTGCTACTGGTCTTACTTTGAGGGAA
GACCCAATAACAATGAGGAGGTCAACTTCATCTTTGTCATACTTCATGGCTCTATGA
AACTGTTCTGGTAAATTTTCACCAAAAAACACAATCTCTGGTTTCATGATAGCAAG
CGGTTCATCAGCTGGGCACCTAGGACATCGAGGAACTACCTGATTAAAAATATCTC
CTCGTACAGCTTCACAGTCAACTTTGTATTTACAAATCAGGCAAGATGCTGTTGCA
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TATGTTCTGGGTATAGTTGCGAAGTAGTTTTCCTTCCTTATCTGACAAGGCTATGAA
TTTGTGACAGAGAGATGGCTGGAATTGTCCAGGATATATTTCCTTTGCAAACTTGA
AGAATGGTCTTGGATCTTTTCTGAAATATTCAATATCAAACATCGCTTGAGGATCTG
GAAGATCTGGGAAGTCTACAGCAAGGCGAGCATAAATACCATCCCTTGACCTGAA
GTCAGGTATTCCACATGAAACAGACACCCCAGCTCCAGTTAGAACTATAATTTTTTT
GCACTCTTGCAGTAATTTCACAGCATCTTCAATTGTATTAATATCTTTTCTTTTTTTC
CTTTTTGGTGGTTCTGAAAGGATATTAATAACAATCTGCCACAGTGTCATATCATCC
AACTCAGTGGGAGGTATTGTTTCCGGCATAAATCTTTAGATTGTTTCGAGGATCTGT
GCAATCATAGATGTGCTGACAAGTATATGGGAACTATCGTGGCTTGGAGTCCAGTC
ACTAGAGCTTGCATGGTGAAGGC

Score	Expect	Identities	Gaps	Strand
1474 bits(798)	0.0	842/860(98%)	15/860(1%)	Plus/Min

```
Query  131  TTGGAATTAGTGCTACTGGTCTTACTTTGAGGGAAGACCCAATAACAATGAGGAGGTCAA  190
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  927  TTGGAATTAGTGCTACTGGTCTTACTTTGAGGGAAGACCCAATAACAATGAGGAGGTCAA  868

Query  191  CTTCATCTTTGTCATACTTCATGGCTCTATGAAACTGTTCTGGTAAATTTTCACCAAAAA  250
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  867  CTTCATCTTTGTCATACTTCATGGCTCTATGAAACTGTTCTGGTAAATTTTCACCAAAAA  808

Query  251  ACACAATCTCTGGTTTCATGATAGCAAGCGGTTCATCAGCTGGGCACCTAGGACATCGAG  310
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Sbjct  807  ACACAATCTCTGGTTTCATGATAGCAAGCGGTTCATCAGCTGGGCACCTAGGACATCGAG  748

Query  311  GAACTACCTGATTAAAAATATCTCCTCGTACAGCTTCACAGTCAACTTTGTATTTACAAA  370
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  747  GAACTACCTGATTAAAAATATCTCCTCGTACAGCTTCACAGTCAACTTTGTATTTACAAA  688

Query  371  TCAGGCAAGATGCTGTTGCAAAGGAACCATGACACTGAATTATCCTTTGGATTCCCGCAA  430
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  687  TCAGGCAAGATGCTGTTGCAAAGGAACCATGACACTGAATTATCCTTTGGATTCCCGCAA  628

Query  431  CCTGTTCCAGCGTGTCTATGTTCTGGGTATAGTTGCGAAGTAGTTTTCCTTCCTTATCTG  490
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  627  CCTGTTCCAGCGTGTCTATGTTCTGGGTATAGTTGCGAAGTAGTTTTCCTTCCTTATCTG  568

Query  491  ACAAGGCTATGAATTTGTGACAGAGAGATGGCTGGAATTGTCCAGGATATATTTCCTTTG  550
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  567  ACAAGGCTATGAATTTGTGACAGAGAGATGGCTGGAATTGTCCAGGATATATTTCCTTTG  508
```

Supplementary Figure S2. B) The sequence of the pcDNA3.1 plasmid from 3' to 5'.

C

GGGCTGGTTAGCTTAGTGACAGTGGAGATTGTACAGTTTTTTCCTCGATTTGTCAG
GATTTTTTTTTTTGACGGAGTTTAACTTCTTGTCTCCCAGGTAGGAAGTGCAGTGG
CGTAATCCCGGCTCACTACAACCTCCACCTCCTGGGTTCAAGCGTTTCTCCTGCCT
CAGCTTTCCGAGTAGCTGGGATTACAGGCGCCTGCCACCATGCCCTGCTGACTTTT
GTATTTTTAGTAGAGACGGGGTTTCACCATGTTGGCCAGGCTGGTCTTGAACTCCT
GACCGCAGGCGATTGGCCTGCCTCGGCCTCCCAAAGTGCTGAGATTACAGGCGTG
AGCCACCACCCCCGGCCTCAGGAGCGTTCTGATAGTGCCTCGATGTGCTGCCTCCT
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TTTATACTTCAGGAGGTACCATAACCTTCTGTTCGGTGATGAAATTATCACTAATGG
TTTTCATTCCTGTGAAAGTGATGAGGAGGATAGAGCCTCACATGCAAGCTCTAGTG
ACTGGACTCCAAGGCCACGGATAGGTCCATATACTTTTGTTCAGCAACATCTTATGA
TTGGCACAGATCCTCGAACAATTCTTAAAGATTTATTGCCGGAAACAATACCTCCA
CCTGAGTTGGATGATATGACACTGTGGCAGATTGTTATTAATATCCTTTCAGAACCA
CCAAAAAGGAAAAAAAGAAAAGATATTAATACAATTGAAGATGCTGTGAAATTAC
TGCAAGAGTGCAAAAAAATTATAGTTCTAACTGGAGCTGGGGTGTCTGTTTCATGT
GGAATACCTGACTTCAGGTCAAGGGATGGTATTTATGCTCGCCTTGCTGTAGACTTC
CCAGATCTTCCAGATCCTCAAGCGATGTTTGATATTGAATATTTCAGAAAAGATCCA
AGACCATTCTTCAAGTTTGCAAAGGAAATATATCCTGGACAATTCCAGCCATCTCTC
TGTCCCAATTCATAGCCTTGTCAGATAGGAAGGAAACTACTTCCAACTTTCCCAGA
ACTTAAACCGCTGGAAAAGTTGGGGAACCAAGGATATTCATGT

Supplementary Figure S2. C) The sequence of the circ-sirt1 from 5' to 3'.

D

```
Query  551  CAAACTTGAAGAATGGTCTTGGATCTTTTCTGAAATATTCAATATCAAACATCGCTTGAG  610
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  507  CAAACTTGAAGAATGGTCTTGGATCTTTTCTGAAATATTCAATATCAAACATCGCTTGAG  448

Query  611  GATCTGGAAGATCTGGGAAGTCTACAGCAAGGCGAGCATAAATACCATCCCTTGACCTGA  670
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  447  GATCTGGAAGATCTGGGAAGTCTACAGCAAGGCGAGCATAAATACCATCCCTTGACCTGA  388

Query  671  AGTCAGGTATTCCACATGAAACAGACACCCCAGCTCCAGTTAGAACTATAAttttttGC  730
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Sbjct  387  AGTCAGGTATTCCACATGAAACAGACACCCCAGCTCCAGTTAGAACTATAATTTTTTTGC  328

Query  731  ACTCTTGCAGTAATTTCACAGCATCTTCAATTGTATTAATATCttttctttttttccttt  790
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  ACTCTTGCAGTAATTTCACAGCATCTTCAATTGTATTAATATCTTTTCTTTTTTTCCTTT  268

Query  791  ttGGTGGTTCTGAAAGGATATTAATAACAATCTGCCACAGTGTCATATCATCCAACTCAG  850
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  267  TTGGTGGTTCTGAAAGGATATTAATAACAATCTGCCACAGTGTCATATCATCCAACTCAG  208

Query  851  TGGGAGGTATTGTTTCCGGCA-TAAATCTTTA-GA-TTGTTTCGAGGATCTGTGC-AATC  906
               ||||||||||||||||||| |||||||||| || ||| |||||||||||||| ||||
Sbjct  207  GTGGAGGTATTGTTTCCGGCAATAAATCTTTAAGAATTG-TTCGAGGATCTGTGCCAATC  149

Query  907  ATA-GATGT-GCTGA-CAA--GTATATGGGAACTATC-GTGG-CTTGGAGTCCAGTCACT  959
             ||| ||||| ||||| |||  |||||||| | |||||| |||| ||||||||||||||||
Sbjct  148  ATAAGATGTTGCTGAACAAAAGTATATGG-ACCTATCCGTGGCCTTGGAGTCCAGTCACT  90

Query  960  AGAGCTTGCATGGTGAAGGC  979
             |||||||||||| ||| |||
Sbjct  89   AGAGCTTGCATG-TGA-GGC  72
```

Supplementary Figure S2. D) The sequences of 0001_31219120401535_(circ-sirt1-2)_[T7].