**Sirt7 promotes gastric cancer growth and inhibits apoptosis by epigenetically inhibiting miR-34a**

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Gastric cancer is the fourth most common cancer worldwide, with a low 5-year survival rate. Epigenetic modification plays pivotal roles in gastric cancer development. However, the role of histone-modifying enzymes in gastric cancer remains largely unknown. Here we report that Sirt7, a NAD⁺-dependent class III histone deacetylase, is over-expressed in human gastric cancer tissues. Sirt7 level is significantly correlated with disease stage, metastasis, and survival. Knockdown of Sirt7 in gastric cancer cells inhibits cell proliferation and colony formation in vitro. In vivo subcutaneous xenograft results also show that Sirt7 knockdown can markedly repress gastric cancer cell growth. In addition, Sirt7 depletion induces apoptosis in gastric cancer cells via up-regulating expression of pro-apoptotic proteins and down-regulating anti-apoptotic proteins. Mechanically, Sirt7 binds to the promoter of miR-34a and deacetylases the H3K18ac, thus represses miR-34a expression. Reversely, depletion of miR-34a inhibits gastric cancer apoptosis induced by Sirt7 knockdown, and restores cellular capacity of proliferation and colony formation. miR-34a depletion reduces Sirt7-knockdown-induced arrest of gastric growth. Finally, miR-34a is tightly associated with survival of patients with gastric cancer.

Epigenetic alternation plays pivotal roles in the initiation and progression of human gastric cancers. DNA methylation of protein-coding and microRNA genes in gastric mucosa of gastric cancer patients is involved in the formation of epigenetic field defect. Aberrant methylation in gastric cancer is associated with the CpG island methylator phenotype. Methylation of CpG islands inactivates several tumor suppressor genes, including CHFR, PTEN, and RUNX3. Methylation-associated silencing of microRNAs is also involved in gastric cancer development. In addition to DNA methylation, histone modification is also important for the progress of gastric carcinogenesis. Expression of the enhancer of zeste homolog 2 (EZH2), a histone methyltransferase, is correlated with poor prognosis in human gastric cancer. In addition, trimethylation of H3K9 is positively correlated with tumor stage, lymphovascular invasion, cancer recurrence. However, whether and how histone acetyltransferases and deacetylases participate in gastric cancer are still largely unclear.

Sirtuins are a highly conserved family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylase and ADP-ribosyltransferase that play various roles in metabolism, stress response, and longevity. All the Sirtuin members are reported to play essential roles in carcinogenesis. However, the roles of Sirtuin family members in gastric cancer are largely unclear.

Here we show that the expression of Sirt7 is overexpressed in human gastric cancer tissues in addition to Sirt1. High expression of Sirt7 predicts poor survival. Further, we demonstrate that Sirt7 knockdown reduces gastric cancer growth in vitro and in vivo. Mechanically, Sirt7 prevents apoptosis of gastric cancer cells by epigenetically silencing miR-34a via deacetylating H3K18ac.

**Results**

**Sirt7 is overexpressed in human gastric cancer tissues and cell lines.** To investigate the roles of the Sirtuins in gastric cancer, we tested the mRNA levels of Sirtuins in gastric cancer tissues and non-cancer normal gastric mucosa (NGM) from healthy donors. The results showed that only two Sirtuins, Sirt1 and Sirt7 were overexpressed in human gastric cancer tissues (Fig. 1A). Next, we measured the mRNA level of Sirt7 in all non-cancer NGM and gastric cancer with different stages. We found that Sirt7 mRNA was significantly
up-regulated in gastric cancer tissues compared to non-cancer normal gastric mucosa (NGM), and the expression level was associated with disease stage (Fig. 1B and Table 1). Furthermore, we analyzed Sirt7 mRNA level in gastric cancer tissues and matched adjacent gastric mucosa (AGM). In consistent with the above findings, the expression of Sirt7 was up-regulated in gastric cancer compared with matched AGM (Fig. 1C). In detail, 78% of the cases overexpressed Sirt7, 19% did not change the expression of Sirt7 and 3% under-expressed Sirt7 in gastric cancer tissues compared with the AGM (Fig. 1D). In parallel with the mRNA expression alteration, the protein level of Sirt7 was also up-regulated in gastric cancer tissues and protein level was associated with disease stage (Fig. 1E–F). In addition, we used two normal gastric epithelial cell lines (CES-1 and HFE145) and six gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28) to analyze Sirt7 protein level in normal and cancer cells. The results showed that Sirt7 protein level was markedly overexpressed in gastric cancer cells in comparison with normal gastric epithelial cells (Fig. 1G).

**Association of Sirt7 expression with clinicopathological factors.** To delineate the clinical significance of Sirt7, we analyzed the correlations between the Sirt7 level and clinicopathological factors in according to IHC results (Table 1). High expression of Sirt7 protein was significantly associated with liver or peritoneal metastasis ($p < 0.0001$), tumor size ($p = 0.0126$), extent of gastrostomy ($p = 0.0364$), depth of invasion ($p = 0.0113$), lymph node involvement ($p = 0.0014$) and TNM stage ($p < 0.0001$, Table 1). Further, we analyzed the correlations between Sirt7 level and overall or disease-free survival. Patients with high expression of Sirt7 had a markedly worse overall and disease-free survival compared to those with low Sirt7 level (Fig. 2A–B). Since we did not find significant difference of Sirt7 expression between intestinal and diffuse types of gastric cancer (Table 1). We analyzed the correlations between Sirt7 level and intestinal or diffuse types of gastric cancer respectively. The results demonstrated that Sirt7 level was markedly associated with overall and disease-free survival in patients with intestinal type of gastric cancer (Fig. 2C–D). Similar results were observed in diffuse type of gastric cancer (Fig. 2E–F).

**Sirt7 knockdown reduces gastric cancer growth.** We have demonstrated that overexpression of Sirt7 in human gastric cancer predicted poor survival. We next knocked down Sirt7 to investigate the role of Sirt7 in gastric cancer development (Fig. 3A). Sirt7 knockdown in MGC803 cells severely impaired cellular proliferation (Fig. 3B–C). Furthermore, colony formation capacity was also reduced by Sirt7 knockdown (Fig. 3D–E). In addition, Sirt7 knockdown also reduced cellular proliferation and colony formation in AGS and MKN-28 gastric cancer cells (Suppl. Fig. 1). Our data also indicated that Sirt7 overexpression promoted gastric cancer cell proliferation and colony formation (Suppl. Fig. 2A–C). Next, we examined the effects of Sirt7 on tumor growth using subcutaneous
Sirt7 knockdown promotes apoptosis of gastric cancer cells. To investigate whether Sirt7 plays a role in gastric cancer growth by regulating apoptosis, we performed FACS to detect Annexin V and propidium iodide (PI) positive cells in MGC803 cells with/without Sirt7 knockdown. Sirt7 depletion markedly increased Annexin V-positive cell numbers, indicating that Sirt7 knockdown promoted gastric cancer cell apoptosis (Fig. 4A–B). We also found that cleaved caspase-3 and its target cleaved PARP were significantly up-regulated when Sirt7 was knocked down (Fig. 4C and Suppl. Fig. 3A–B). Furthermore, we also detected the up-regulation of pro-apoptotic proteins (Bax and Bim) and down-regulation of anti-apoptotic proteins (Bcl-2 and Mcl-1) in Sirt7-deficient MGC803 cells (Fig. 4C). To determine whether DNA damage is involved in this process, we also performed TUNEL assay, and the results showed that Sirt7 knockdown increased TUNEL-positive cells (Fig. 4D), indicating that Sirt7 may reduce DNA damage. As previous work indicated that Sirt7 could affect cell cycle12, we therefore investigated whether cell cycle changed when Sirt7 was knocked down. We found in MGC803 cells that Sirt7 knockdown significantly promoted G2/M accumulation (Suppl. Fig. 4), indicating that Sirt7 also promoted cell cycle in MG803 cells.

Sirt7 epigenetically regulates the expression of miR-34a. microRNAs participate essentially in the development of gastric cancer13,14, and previous study showed the important role of Sirt7 in regulation of RNA processing, splicing and metabolism15. We therefore wanted to know whether Sirt7 regulates gastric cancer by mediating microRNAs. We therefore knocked down Sirt7 and tested the level of several microRNAs that were reported in gastric cancer development. Among those microRNAs, the fold of change of miR-34a was the highest (Suppl. Fig. 5). miR-34 family is involved in cell-cycle arrest, senescence, and apoptosis in cancers. This family is consisted of miR-34a and miR-34b/c. miR-34b/c was reported to participate in gastric cancers14,15 whereas the function of miR-34a in gastric cancer remains largely unknown. We guess that miR-34a may be regulated by Sirt7 in gastric cancer. Here we found that Sirt7 knockdown markedly up-regulated the expression of miR-34a (Fig. 5A), and that miR-34a was down-regulated in human gastric cancer tissues (Fig. 5B). Furthermore, regression analysis showed that miR-34a expression was significantly but negatively correlated with Sirt7 mRNA and protein levels (Fig. 5C). Those findings demonstrated that Sirt7 could inhibit the expression of miR-34a. Next, we asked whether Sirt7 could regulate the expression of miR-34a directly. We found that Sirt7 selectively bond to the promoter of miR-34a in MGC803 cells (Fig. 5D). H3K18ac is a reported substrate of Sirt7 and plays important role in transactivation by regulating chromatin structure. We observed that H3K18ac could be recruited to the promoter of miR-34a in MGC803 cells (Fig. 5E). More importantly, we found that Sirt7 level was higher whereas H3K18ac level was lower at miR-34a promoter in fresh gastric tumor tissues compared to adjacent normal mucosa (Fig. 5F). Finally, we provided direct evidence that Sirt7 repress the promoter activity of miR-34a by using luciferase assay in 293T cells (Fig. 5G).

miR-34a knockdown neutralizes Sirt7 effects on gastric cancer cells. To explore whether miR-34a is critically essential for Sirt7 function in gastric cancer cells, we knocked down the expression of miR-34a by using specific LNA-antimiR-miR-34a (Fig. 6A). miR-34a knockdown markedly reduced gastric cancer cell apoptosis and DNA damage induced by Sirt7 knockdown (Fig. 6B–C, Suppl. Fig. 6 and 7). In consistent with this observation, we found that Sirt7 knockdown could not affect the cell proliferation and colony formation of MGC803 gastric cancer cells when miR-34a was interfered (Fig. 6D and Suppl. Fig. 8). Furthermore, our in vivo evidence also demonstrated that Sirt7 knockdown was not

| Variable | No. of Patients (n = 262) | Sirt7 low expression (n = 97) | Sirt7 high expression (n = 165) | p       |
|----------------|-----------------------------|-----------------------------|-----------------------------|---------|
| Age <60       | 95                          | 40                          | 55                          | 0.2313  |
| ≥60           | 167                         | 57                          | 110                         | 0.7022  |
| Sex Male      | 126                         | 45                          | 81                          | <0.0001 |
| Female        | 136                         | 52                          | 84                          |         |
| Liver or peritoneal metastases Present | 119 | 34 | 85 |         |
| Absent        | 143                         | 79                          | 64                          |         |
| Tumor size    |                             |                             |                             |         |
| <3 cm         | 178                         | 75                          | 103                         | 0.0126  |
| ≥3 cm         | 84                          | 22                          | 62                          |         |
| Radicaty of resection |             |                             |                             |         |
| RO            | 181                         | 60                          | 121                         | 0.0522  |
| R1/2          | 81                          | 37                          | 44                          |         |
| Extent of gastrectomy |       |                             |                             |         |
| Partial       | 107                         | 37                          | 44                          |         |
| Total         | 65                          | 17                          | 48                          |         |
| Lymphadenectomy |                             |                             |                             |         |
| D1            | 98                          | 37                          | 51                          | 0.3958  |
| D2/3          | 164                         | 60                          | 104                         |         |
| Lauren histotype |                             |                             |                             |         |
| Intestinal    | 117                         | 47                          | 70                          | 0.3432  |
| Diffuse       | 145                         | 50                          | 95                          | 0.0113  |
| Depth of invasion |                             |                             |                             |         |
| pT1           | 17                          | 12                          | 5                           |         |
| pT2           | 89                          | 31                          | 58                          |         |
| pT3-4         | 158                         | 54                          | 104                         |         |
| Lymph node involvement |         |                             |                             |         |
| Absent (pN0)  | 87                          | 54                          | 33                          | 0.0014  |
| Present (pN+) | 175                         | 72                          | 103                         |         |
| TNM Stage     |                             |                             |                             |         |
| I             | 54                          | 33                          | 21                          | <0.0001 |
| II            | 45                          | 21                          | 24                          |         |
| III           | 104                         | 37                          | 67                          |         |
| IV            | 59                          | 6                           | 53                          |         |
| GC familial aggregation |         |                             |                             |         |
| Positive      | 98                          | 36                          | 62                          | 0.9405  |
| Negative      | 164                         | 61                          | 103                         |         |
sufficient to reduce gastric cancer cell growth when miR-34a was stably knocked down (Fig. 6E and Suppl. Fig. 9). We did not observe any toxicity in Sirt7 shRNA and LNA-miR-34a or placebo treated mice (data not shown). To further confirm the role of miR-34a in the function of Sirt7 in gastric cancer, we then pre-treated MG803 cells with miR-34a mimic followed by Sirt7 overexpression. Treatment with miR-34a mimic inhibited MG803 cell growth and colony formation. Interestingly, Sirt7 was unable to promote MG803 cells proliferation and colony formation when cells were pre-treated with miR-34a mimic (Suppl. Fig. 10). Those observations suggested that miR-34a down-regulation played a pivotal role in Sirt7-mediated effects on gastric cancer. Finally, we showed that low level of miR-34a was significantly associated with worse survival (Fig. 6F and Suppl. Table 3).

Discussion

Here in the present work, we identified the novel role of Sirt7 in human gastric cancer. Sirt7 is up-regulated in human gastric cancer tissues and high expression of Sirt7 predicts poor survival. We demonstrate that Sirt7 promotes gastric cancer cell growth by using in vitro and in vivo evidence. We also find that Sirt7 maintains gastric cancer cell survival. The mechanism analysis indicates that Sirt7 reduces miR-34a expression by deacetylating H3K18ac, and miR-34a silence blocks the effects of Sirt7. Finally, we show that miR-34a low expression predicts poor prognosis.

Sirtuins are a family of NAD+–dependent protein deacetylases involved in stress resistance, metabolic homeostasis and carcinogenesis. Only Sirt1 was reported to be involved in human gastric cancer until now16,17. Frame shift mutation of SIRT1 gene in gastric carcinomas was reported to be associated with microsatellite instability18. Up-regulation of Sirt1 is essential for ATF4-facilitated multidrug resistance19. In addition, Sirt1 is critical for murine gastric cancer growth mediated by diet-induced obesity20. Herein we found that Sirt7 was another Sirtuin family member that was overexpressed in human gastric cancer tissues (Fig. 1), and that Sirt7 was significantly correlated with tumor size, metastasis, disease stage and patients prognostic independent of the histological types (Fig. 2 and Table 1). Among the seven Sirtuins, the function of Sirt7 is the least well understood. Recently, Barber et al.12 discovered a specific target of Sirt7 and identified a crucial role for Sirt7 in the maintenance of cancer phenotype and transformation. Sirt7 is specific for a single histone mark, H3K18Ac, directly linked to control of gene

Figure 2 | Kaplan-Meier plot of survival durations in gastric cancer patients with different Sirt7 expression. (A) Overall survival and (B) disease-free survival duration are poor in gastric cancer patients with higher Sirt7 expression. (C) Overall survival and (D) disease-free survival duration are poor in intestinal gastric cancer patients with higher Sirt7 expression. (E) Overall survival and (F) disease-free survival duration are poor in diffuse gastric cancer patients with higher Sirt7 expression.
expression. Sirt7 was overexpressed in several cancer types. In addition, analysis of Sirt7-occupied genes revealed a clear correlation with factors whose expression is altered in various cancers, including Bladder cancer, Leukemia, prostate cancer, breast cancer and gastric cancer. Together with our data, their results implicate that Sirt7 may have certain roles in human gastric cancer. Our in vitro and in vivo evidence demonstrated that Sirt7 significantly facilitated human gastric cancer cell growth and transformation (Fig. 3 and Suppl. Fig. 1).

Sirt7 performs critically in oxidative and genotoxic stress response. Homozygous knockout of Sirt7 in mice causes diminished lifespan and leads to heart hypertrophy and inflammatory cardiopathy. Cardiomyocytes derived from these mice show increased apoptosis as well as hypersensitivity to oxidative and genotoxic stress. In consistent with that finding, Sirt7 knockdown induced apoptosis of human gastric cancer cell growth and transformation (Fig. 3 and Suppl. Fig. 1).

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Figure 3 | Sirt7 knockdown inhibits gastric cancer growth in vitro and in vivo. (A) Representative western blot showing Sirt7 knockdown in MGC803 cells. (B-C) Sirt7 knockdown represses MGC803 cells proliferation. (B) Representative image by phasecontrast microscopy. Bar = 30 μm. (C) MTT analysis of cellular proliferation ability at the indicated time points. * p < 0.05 and ** p < 0.01 vs. sh-Ctrl. (D-E) Sirt7 knockdown inhibits colony formation of MGC803 cells. (D) Representative image showing cellular colonies of MGC803 cells with/without Sirt7 knockdown. (E) Quantitative results of colony numbers of different groups. (F-I) Sirt7 knockdown inhibits MGC803 cancer cells growth in vivo. (F) Representative image of subcutaneous tumors isolated from nude mice. (G) Quantitative analysis of tumor weights. n = 10. (H) Representative image of in vivo bioluminescence imaging indicating tumor size. (I) Quantitative analysis of total photon flux values. n = 10. MGC803 cells expressing luciferase were used in this experiment.

In summary, Sirt7 is overexpressed in human gastric cancers. Expression of Sirt7 is markedly correlated with tumor size, metastasis, disease stage and prognosis. Mechanically, Sirt7 prevents...
Figure 4 | Sirt7 knockdown promotes gastric cancer apoptosis. (A-B) Apoptotic cells were detected by fluorescence-activated cell sorting (FACS) using Annexin V and propidium iodide (PI). (A) Representative FACS analysis of annexin V and PI staining of MGC803 cells infected with retrovirus expressing either control or Sirt7 knockdown induces expression of pro-apoptotic proteins and inhibits expression of anti-apoptotic proteins in MGC803 cells. (C) Sirt7 knockdown induces expression of pro-apoptotic proteins and inhibits expression of anti-apoptotic proteins in MGC803 cells. (D) Sirt7 knockdown increases TUNEL-positive cells. Cells expressing either control or Sirt7 knockdown vectors were subjected to TUNEL analysis.

Methods

Patients and tissue specimens. 262 cases of gastric cancer patients with full case history between January 1997 and December 2005 were selected and included in the present study. Fresh and paraffin-embedded tissues were obtained, and fresh tissues were stored at −80 °C before use. The patients were recruited at Ningbo No.2 Hospital (Ningbo, Zhejiang). The patients included 126 (48.1%) males and 136 (51.9%) females with a mean age 61 years. Tumors were histologically classified into 117 (47.7%) intestinal gastric cancer and 145 (52.3%) diffuse gastric cancer (Table 1). The diagnosis of gastric cancer was established using World Health Organization (WHO) morphological criteria. The adjacent gastric mucosa (AGM) was also collected from cancer patients under surgery. For the non-cancer normal gastric samples (NGM), 23 biopsy-tissue specimens were obtained from the antrum and the body of the normal stomach separated by a distance of 5 cm. A written form of informed consent was obtained from all patients and donors. The study was approved by the Clinical Research Ethics Committee of Ningbo University. The methods were carried out in accordance with the approved guidelines.

Cell culture and retroviral transduction. Gastric epithelial cell lines (GES-1 and MEE145) and gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28) were acquired from the American Type Culture Collection (ATCC). These cells were cultured in RPMI1640 (Invitrogen) supplemented with penicillin-streptomycin (Invitrogen), GlutaMAX-1 (Invitrogen) and 10% fetal bovine serum (FBS, Gibco). Sirt7 and ctrl shRNA retroviral particles were purchased from Santa Cruz Biotechnology. The shRNA sequences targeting Sirt7 is shown in Supplementary Table 1. Retrovirus expressing human Sirt7 was generated by sub-cloning Sirt7 cDNA from pcDNA4-Sirt7 plasmid. For retroviral packaging, 293T cells were co-transfected with the retroviral particles. miR-34a was knocked down with the locked nucleic acid (LNA)-antisense. For transduction, cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 hours, infected cells were selected for 72 hours with puromycin (2 mg/ml) or hygromycin (200 mg/ml). For miR-34a treatment, MGC803 cells were transfected with miR-34a mimic and miRNA mimic negative control (Ambion, Life Technologies Grand Island) at a final concentration of 200 nM for 96 h using combiMAgnetofection (OZ BIOSCIENCES) in accordance with manufacturer’s procedure.

Cellular apoptosis by epigenetically down-regulating miR-34a via deacetylating H3K18ac. The novel Sirt7/miR-34a regulatory pathway characterized here provides new insight into the mechanisms underlying gastric carcinogenesis. Restoration of miR-34a expression or inhibition of Sirt7 may be a potential therapeutic strategy for the treatment of gastric cancer.

Immunohistochemical assay. Tissues were fixed with 4% neutral formalin. Paraffin sections were subjected to high-temperature antigen retrieval, 3 min in a pressure cooker in 0.01 M citrate buffer pH 6.0. Slides were treated with 3% H2O2 for 15 min, blocked in 5% normal horse serum in PBS for 20 min, and stained with anti-Sirt7 antibody in 5% normal goat serum overnight at 4 °C. Secondary antibody was used to amplify the Sirt7/H3K18ac-binding region of the miR-34a promoter (forward, 5'-CAGCTTGGAGGAGGATCGA-3'; reverse, 5'-TCCAAAGGCCCCCAATCT-3').

Chromatin immunoprecipitation (ChiP). ChiP assays were performed as described by the Upstate protocol with some modifications. Cells were cross-linked by adding formaldehyde to a final concentration of 1% at room temperature for 10 min. After washing four times with 20 ml PBS in 50 ml conical tubes, cells were scraped and swelled in hypotonic swelling buffer (25 mM HEPES (pH 7.8), 1.5 mM MgCl2, 10 mM KCl, 0.1% NP-40, protease inhibitor cocktail from Sigma) and incubated on ice for 10 min. Following centrifugation at 2 000 rpm for 5 min, the nuclei were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris (pH 8.1)) and sonicated with Branson 150 sonicator. Antibodies against H3K18Ac (Abcam) and Sirt7 antibody (Santa Cruz) were used for IP. q-PCR was carried out with specific primers to amplify the Sirt7/H3K18Ac-binding region of the miR-34a promoter (forward, 5'-CAGCTTGGAGGAGGATCGA-3'; reverse, 5'-TCCAAAGGCCCCCAATCT-3').

Western blotting. Gastric epithelial cells, gastric cancer cells, normal and cancer gastric tissues were lysed with cell lysis buffer (Beyotime) supplemented with protease inhibitor mixture. Protein concentrations of the extracts were measured with bichinonic acid assay (Pierce). 40 µg cell proteins were applied to 12–15% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, which were then blocked in 5% fish-free milk for 1 hour. The membranes were probed with primary antibody for Sirt7 (Santa Cruz), Caspase-3 (Santa Cruz), PARP (Abcam), Bax (Abcam), Bcl (Abcam), Bcl-2(Santa Cruz), Mcl-1 (Abcam), H3(Santa Cruz), H3K18ac (Santa Cruz) or β-actin (Abcam) at 4 °C overnight, and then the membranes were washed and incubated with

Quantitative RT-PCR (q-PCR). Total RNA was extracted from cells or tissues with TRIzol and cDNA was synthesized from 1 µg of RNA with One Step RT-PCR Kit (TAKARA). q-PCR was performed with the SYBR Green (TAKARA) on an ABI-7500 RT-PCR system (Applied Biosystems). The primers were listed in Supplemental table 2.
HRP-conjugated secondary antibody (in 5% fat-free milk) for 2 hours and finally visualized using Chemiluminescent ECL reagent (Beyotime).

**Apoptosis assay.** Apoptosis analysis was conducted with an Annexin V-FITC Apoptosis Detection Kit (Becton Dickinson) according to the manufacturer’s protocol. A FACSCalibur flow cytometer was used for data analysis. TUNEL assay was performed with a TUNEL Labeling Kits (R & D systems) according to the manufacturer’s protocol.

**Tumor xenograft experiments.** Equal numbers of MGC803 cells stably expressing luciferase and either control or Sirt7 knockdown or Sirt7 plus miR-34a knockdown vectors (5 × 10^6) in 100 μl of a 1:1 mixture of culture medium and growth factor-reduced Matrigel were implanted subcutaneously into the forelegs of 4- to 5-week-old male BALB/c athymic nu/nu mice (Vital River). When the tumors reached approximately 7 to 10 mm in diameter, they were prepared to form a brei and then injected subcutaneously into nude mice. Tumor growth was monitored using calipers and visualized with a bioluminescence-based IVIS system (Caliper LifeSciences). The study was approved by the Animal Research Ethics Committee of Ningbo University. The methods were carried out in accordance with the approved guidelines.

**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 accordance with the guidelines.

**Colony formation.** Gastric cancer 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% CO₂ for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

**Luciferase assays.** To generate the luciferase reporter vectors, an IRES was amplified from pMSCV-PIG and cloned into the BglII site of pGL3-Basic (Promega). miR-34a promoter fragments were amplified from human genomic DNA and cloned into the XhoI site. 24 hours before transfection, 7 × 10^4 cells were plated per well in a 24-well plate. pGL3 constructs (100 ng) plus 1 ng of the Renilla luciferase plasmid pRL-SV40 (Promega) were transfected using FuGENE 6 (Roche). 24 hours after transfection, luciferase assays were performed using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla Sirt7-mediated transactivation of miR-34a luciferase activity for each transfected well.

**Statistics.** Statistical differences between two groups were determined using Student’s t test. The correlations of Sirt7 immunoreactivity and miR-34a level with patients’ clinicopathological variables were analyzed by the χ² test or Fisher’s exact test. The Kaplan-Meier method was used to estimate overall and disease-free survival. Survival differences according to Sirt7 or miR-34a expression were analyzed by the log-rank test.

**Figure 5| Sirt7 knockdown up-regulates miR-34a expression.** (A) Sirt7 knockdown up-regulates the expression of miR-34a. (B) miR-34a is down-regulated in human gastric cancer tissues compared with adjacent gastric mucosa (AGM; n = 46 in each group). (C) Regression analysis showing the level of miR-34a is negatively correlated with the mRNA and protein levels of Sirt7 in human gastric cancer tissues (n = 46). The protein score was obtained from the IHC results, which indicates the percentage of Sirt7-positive area. (D) ChIP-qPCR data showing Sirt7 binds to the promoter region of miR-34a. ** p < 0.01 vs. IgG-ChIP; ### p < 0.01 vs. sh-Ctrl. (E) ChIP-qPCR data showing Sirt7 knockdown enhances H3K18 acetylation level at the promoter region of miR-34a. ** p < 0.01 vs. IgG-ChIP; ## p < 0.01 vs. sh-Ctrl. (F) Sirt7 and H3K18ac levels at miR-34a promoters in AGM and gastric cancer tissues. (G) Luciferase assay showing Sirt7 knockdown up-regulates the transcriptional activity of miR-34a.
test. Linear regression analysis was performed to analyze the relation between Sirt7 and miR-34a expression in human gastric cancers. p values of less than 0.05 were considered statistically significant. Values were expressed as Mean ± SEM of at least three independent experiments.

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Author contributions
S.Z., P.C. and T.C. designed this study. S.Z. and P.C. performed most of the experiments. Z.H. and X.H. collected the tumor tissues and performed clinical analysis. M.C. and S.H. performed in vivo tumor growth analysis. Y.H. performed statistical analysis, and T.C. wrote the manuscript. All authors reviewed the manuscript.

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