Overexpression of Sirt7 Exhibits Oncogenic Property and Serves as a Prognostic Factor in Colorectal Cancer

Hongyan Yu¹, Wen Ye¹, Jiangxue Wu¹, Xiangqi Meng¹, Ran-yi Liu¹, Xiaofang Ying¹, Yi Zhou¹, Hui Wang¹, Changchuan Pan², and Wenlin Huang¹,³

Abstract

**Purpose:** Sirtuins play an important role in cancer development. Sirt7, as a member of this family, is frequently overexpressed in certain carcinomas, but the oncogenic mechanism is seldom reported. In this study, Sirt7 was characterized for its role in colorectal cancer aggressiveness and underlying molecular mechanisms.

**Experimental Design:** Quantitative PCR, Western blotting, and immunohistochemistry were performed to study Sirt7 expression in a cohort of colorectal cancer tissues and non-tumor tissues and cells. A series of in vitro and in vivo assays was performed to elucidate the function of Sirt7 in colorectal cancer and its underlying mechanisms. Association between the Sirt7 signature and survival was examined using Kaplan–Meier analysis and log-rank tests.

**Results:** The Sirt7 protein level significantly correlated with tumor stage (P = 0.029), lymph node metastasis (P = 0.046), and poor patient survival (P < 0.05). Sirt7 knockdown significantly inhibited colorectal cancer cell proliferation, colony formation, and motility. Ectopic Sirt7 expression promoted colony formation, induced a more invasive phenotype, and accelerated cell growth both in vitro and in vivo. Moreover, Sirt7 enhanced MAPK pathway activity concomitantly with p-ERK and p-MEK upregulation. In Sirt7-overexpressing cells, the mesenchymal markers vimentin and fibronectin were upregulated, and the epithelial markers E-cadherin and β-catenin were downregulated, which was linked to enhanced invasion by colorectal cancer cells.

**Conclusion:** Our findings suggest that Sirt7 plays an important role in the development and progression of human colorectal cancer and functions as a valuable marker of colorectal cancer prognosis. *Clin Cancer Res*; 20(13); 3434–45. ©2014 AACR.

**Introduction**

Colorectal cancer is one of the three leading causes of cancer mortality worldwide (1). Despite the innovative therapeutic strategies applied in colorectal cancer, the prognosis has not significantly changed in the last 20 years. In past decades, studies have reported alternative factors involved in colorectal cancer pathogenesis, including genetic mutations in certain oncogenes or tumor suppressor genes (KRAS, APC, DCC, Smad-2, and Smad-4) and changes in the p53, β-catenin, TGF-β, and WNT transduction pathways (2, 3). These findings suggest that colorectal carcinogenesis results from an accumulation of genetic alterations. How these genetic changes precisely cause the clinical characteristics observed in individual patients with colorectal cancer is unclear; consequently, the mechanisms underlying colorectal cancer development and progression remain poorly understood. Sirtuins are homologous with yeast sirtuin silent information regulator 2 (Sir2), which was originally described as a transcriptional silencing regulator of mating-type loci, telomeres, and ribosomal DNA (4, 5) and extends the yeast lifespan (6). Sir2 was soon found to be an NAD-dependent histone deacetylase (HDAC)(7). Sirtuins, also designated as class III histone deacetylases, exhibit a broad spectrum of functions in mammalian cells. Sirt7, as a member of the sirtuin family, is frequently overexpressed in certain carcinomas, but the oncogenic mechanism is seldom reported. In this study, we characterized Sirt7 for its role in colorectal cancer aggressiveness and underlying molecular mechanisms.

**Experimental Design:** Quantitative PCR, Western blotting, and immunohistochemistry were performed to study Sirt7 expression in a cohort of colorectal cancer tissues and non-tumor tissues and cells. A series of in vitro and in vivo assays was performed to elucidate the function of Sirt7 in colorectal cancer and its underlying mechanisms. Association between the Sirt7 signature and survival was examined using Kaplan–Meier analysis and log-rank tests.

**Results:** The Sirt7 protein level significantly correlated with tumor stage (P = 0.029), lymph node metastasis (P = 0.046), and poor patient survival (P < 0.05). Sirt7 knockdown significantly inhibited colorectal cancer cell proliferation, colony formation, and motility. Ectopic Sirt7 expression promoted colony formation, induced a more invasive phenotype, and accelerated cell growth both in vitro and in vivo. Moreover, Sirt7 enhanced MAPK pathway activity concomitantly with p-ERK and p-MEK upregulation. In Sirt7-overexpressing cells, the mesenchymal markers vimentin and fibronectin were upregulated, and the epithelial markers E-cadherin and β-catenin were downregulated, which was linked to enhanced invasion by colorectal cancer cells.

**Conclusion:** Our findings suggest that Sirt7 plays an important role in the development and progression of human colorectal cancer and functions as a valuable marker of colorectal cancer prognosis.
Sirt7 Promotes Colorectal Cancer Tumorigenesis

Translational Relevance
Colorectal cancer is one of the three leading causes of cancer mortality worldwide. Recurrence and metastasis are common lethal outcomes of colorectal cancer curative resection. Thus, a better understanding of the molecular mechanisms underlying colorectal cancer progression is conducive to the development of targeted therapy. In this study, we reveal a novel role of Sirtuin7 (Sirt7) in promoting colorectal cancer growth by activating the MAPK signal pathway and enhancing colorectal cancer invasiveness and metastasis by promoting epithelial–mesenchymal transition for the first time. We also find that overexpression of Sirt7 in colorectal cancer is associated with lymph node metastasis and TNM stage in patients with colorectal cancer. Taken together, these results implicate Sirt7 as a new effective therapeutic target for intervention of the progression of colorectal cancer.

deacetylases, are NAD$^+$-dependent deacetylases that target non-histone and histone proteins and are implicated in the control of a wide range of biologic processes, including apoptosis, stress responses, cell cycle, DNA repair, metabolism, and senescence (8).

The importance of sirtuins is demonstrated by their role in several major human pathologies, including cancer, cardiovascular disease, diabetes, and neurodegeneration (9). In mammals, the sirtuin family comprises 7 proteins (Sirt1–Sirt7) with considerably different functions and catalytic activities (10). The most studied sirtuin that is most closely related to yeast Sir2 is Sirt1. Little is known about the biologic function of the other 6 sirtuins (4, 11–13).

Sirt7, which is primarily located in the nucleus, binds to the ribosomal RNA (rRNA) gene and is relevant to the reactivation of rRNA transcription following mitosis (14). Sirt7 participates in the activation of RNA polymerase I transcription and may play a role in controlling rRNA expression (15). Furthermore, certain studies have reported that Sirt7 is overexpressed in thyroid and breast cancer tissues compared with normal tissues (16, 17), suggesting that Sirt7 has an oncogenic role. In addition, Sirt7 is located on chromosome 17q25.3, which is involved in various malignancies, including colorectal cancer (18). Barber and colleagues also found that Sirt7 mRNA expression was higher in colon tumors than in non-tumor tissues (19).

No study has assessed Sirt7 exact activity in colorectal cancer, which prompted our interest in investigating its biologic role in human colorectal cancer. In the present study, to elucidate the biologic roles of Sirt7 in colorectal tumorigenesis, we evaluated Sirt7 expression in a subset of human colorectal cancers using immunohistochemistry (IHC) and analyzed its mRNA levels in 18 patients with colorectal cancer. We showed that the frequent Sirt7 overexpression in primary colorectal cancer tumors correlated with shorter survival in early-stage patients. Our study also revealed the key functions of Sirt7 in activating and promoting colorectal cancer growth, epithelial–mesenchymal transition (EMT), which may have implications for colorectal cancer metastasis. Thus, we propose that Sirt7 is a promising target in colorectal cancer therapy.

Materials and Methods

Patients
Tumorous and adjacent non-tumorous colorectal tissues were collected from 131 patients (median age, 56 years; 55.6% male) at the Cancer Center of Sun Yat-sen University (Guangzhou, China) between September 1999 and December 2005. The samples contained matched tumors (percentage of tumor cells ≥ 70%) and corresponding normal mucosal tissue (>5 cm laterally from the edge of the cancerous region); all the patients who had a single primary lesion and no neoadjuvant therapy before operation were included in the study. The patients were followed-up once every 3 months during the first 2 years, once every 6 months during the third and fourth year, and once a year from the fifth year postoperatively, respectively. All patients were contacted by phone or with questionnaire to check upon their health status and the last censor date was May 1, 2012. The patients who did not have the followed-up information were excluded from this study. Informed consent was obtained from each patient, and the study protocol was approved by the Ethics Committee of the Sun Yat-sen University Cancer Center Institutional Board. Colorectal cancer diagnosis was confirmed by histologic examination. The TNM classification of our study is according to the sixth edition of AJCC/UICC; 15 patients were graded as stage I, 59 patients as stage II, 42 patients as stage III, and 15 patients as stage IV.

Cell culture
The human colorectal cancer cell lines HT29, SW480, DLD-1, SW620, and HCT116; the human embryonic kidney cell lines 293T; and the normal colon epithelial cell line FHC were obtained from the American Type Culture Collection. THC8307 and GP293 cells were obtained from our own laboratory collection. The colorectal cancer cell lines, the GP293 and 293T cells, were grown in DMEM, and the FHC cells were grown in DMEM:F12 (containing 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, and 100 ng/ml hydrocortisone) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were cultured in a humidified chamber with 5% CO2 at 37°C. The cells were tested regularly for mycoplasma (New MycoProbe Mycoplasma Detection Kit, R&D Systems).

Quantitative reverse transcription PCR
Total RNA was extracted using TRIzol reagent (DSBIO) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg total RNA using M-MLV Reverse Transcriptase (Promega) and amplified with AmpliTaq Gold DNA Polymerase (Applied Biosystems) and the following
gene-specific primers for Sirt7: 5'-CAGGGGATACCGTG-CGGGTGT-3' (forward) and 5'-TCCGGCCGGCCCTCC-AGTT-3' (reverse). GAPDH was used as an internal control.

Cell transfection

siSirt7 and negative control oligonucleotides were purchased from Santa Cruz Biotechnology. The coding sequences of the Sirt7 and Sirt7 mutations m1 (H187→Y) and m3 (H187→Y, N249→S) were amplified and cloned into the EcoRI and XhoI sites of pcDNA3.1 to generate pcDNA3.1-Sirt7−, pcDNA3.1-Sirt7 m1−, and pcDNA3.1-Sirt7 m3−expressing vectors; each resulting construct was confirmed by sequencing. For transient transfections, HCT116 and THCH307 cells were seeded into 24-well plates (at 4 × 10^3 and 3 × 10^3 cells per well, respectively) at 50% to 60% confluence. After 24 hours, the cells were transfected with oligonucleotides at a concentration of 20 nmol/L or with 0.8 μg plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 6 hours of incubation at 37°C, the transfection medium was replaced with 1 mL complete medium containing 10% FBS.

The cells were collected for real-time PCR, Western blotting, migration, and invasion assays at the indicated times.

Western blot analysis

Protein lysates were resolved on 8% and 12% SDS-PAGE gels and transferred electrophoretically onto a polyvinylidene fluoride membrane.

After blocking for nonspecific binding, the blots were incubated with specific antibodies against Sirt7 (1:1,000; Sigma-Aldrich); β-catenin, E-cadherin, vimentin, p-AKT, phospho-MEK1/2, and MEK1/2 (1:1,000; Cell Signaling Science and Technology); Raf-1 (1:1,000); ERK (1:2,000), fibronectin (1:1,000), and GAPDH (1:2,000; Santa Cruz Biotechnology). After incubation with a horseradish peroxidase–conjugated secondary antibody, protein bands were visualized using enhanced chemiluminescence detection (Perkin Elmer).

Immunohistochemical assay

Sirt7 expression in primary tumors and adjacent noncancerous colorectal mucosa was examined using IHC. Paraaffin-embedded blocks containing adjacent noncancerous colorectal mucosa and more than 70% primary tumor tissue were selected for immunohistochemical staining. Paraaffin sections were cut to a thickness of 4 μm and mounted on silanized slides and the slides were preserved at 4°C. The paraaffin sections were dewaxed, rehydrated, and blocked with 0.3% H2O2. Tissue antigens were retrieved with a microwave oven at 95°C for 25 minutes and cooled to room temperature in 10 mmol/L sodium citrate buffer (pH 6.0). Each slide was washed with PBS and incubated overnight at 4°C with Sirt7 (1:2,000, clone ABE103, Millipore). Primary antibodies were diluted with background-reducing components (S2022, Dako). Secondary antibody was applied using the Envision Detection Kit (Dako). Slides were stained for 2 minutes with 3,3′-diaminobenzidine (DAB) and then counterstained with hematoxylin. Tissue treated with antibody dilution solution was used as a negative control. All controls yielded satisfactory results. Sirt7 expression was evaluated using H-scores and dichotomized according to overall survival (OS) by an ROC curve. The positive rate’s cut-off value was the maximized sum of the sensitivity and specificity points (Supplementary Materials and Methods).

The H-scores consisted of an assessment of staining intensity and the percentage of the stained area with a given intensity. Only stained nuclei of malignant cells were assessed.

Cell viability assay

The 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay was used to detect cell proliferation. HCT116 and THCH307 cells were plated in 96-well plates at 1 × 10^4 cells per well. The absorbance of each sample was measured at 490 nm. The average of 3 repeated experiments was calculated.

For the colony formation assay, HT29 cells (8 × 10^4 cells per 6-well plate) overexpressing Sirt7 or control GFP and HCT116 cells (5 × 10^2 cells per 6-well plate) with siRNA-downregulated Sirt7 expression or a negative control were seeded in complete medium. The cells were cultured for 14 days at 37°C in 5% CO2 humidified air. Colony formation and growth were visualized using crystal violet staining. The numbers of colonies containing more than 50 cells were determined, and 12 fields were counted.

Migration and invasion assays

Transfected HCT116 and THCH307 cells (1 × 10^5 each) were serum starved in medium overnight and then seeded into 24-well plates with or without Matrigel-coated inserts (8-mm pore; BD Falcon). After 18 to 24 hours, the cells attached to the lower surface of the insert filter were counted after crystal violet staining.

Overexpression retrovirus packaging and transduction

Sirt7 and control sequences were amplified from human gDNA and cloned into the BglII and SalI sites of the retroviral vector pLNCX2Virus (Clontech). Packaging was performed in GP293 cells cultured in DMEM with 10% FBS in a 37°C incubator with 5% CO2. Forty-eight hours after transfection, the supernatant was harvested and cleared by centrifugation at 1,000 × g for 10 minutes. HT29 cells were then transduced with the retrovirus containing Sirt7 or control plasmids. Forty-eight hours after infection, 1000 μg/mL G418 was added to the medium for 2 weeks to select retrovirus-infected cells. Western blotting was performed to evaluate Sirt7 expression in the 2 stable cell lines.

Sirt7 shRNA retrovirus packaging and transduction

Virus packaging was performed in 293T cells. p-SUPER-Sirt7 shRNAs or p-SUPER-control and the p-SUPER PIK Expression Packaging plasmids (OligoEngine) were cotransfected with the transfection reagent according to the manufacturer’s instructions. Viral particles were harvested
48 hours after transfection. HCT116 cells were transduced with the retrovirus containing Sirt7 shRNA or control shRNA. Forty-eight hours after infection, 1 μg/mL puromycin was added to the medium for 2 weeks to select retrovirus-infected cells. Western blotting was performed to detect Sirt7 expression in the 2 stable cell lines as described above.

PD98059 treatment

Cells were grown in medium containing different PD98059 concentrations (Sigma-Aldrich) for the indicated times. Cellular proteins were then extracted and subjected to different analyses.

Immunofluorescence analysis

Cells grown on glass coverslips were fixed in 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, and incubated with primary antibody against E-cadherin (1:50) or vimentin (1:100). The cells were then incubated with rhodamine-conjugated or FITC-conjugated goat anti-rabbit or anti-mouse IgG. The coverslips were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) and imaged with a confocal laser scanning microscope (Olympus FV1000). Data were processed with Adobe Photoshop 7.0 software.

In vivo proliferation assays

Female athymic 4- to 5-week-old BALB/c nude mice were purchased from the Medical Experimental Animal Center of Guangdong (PR China). All animal studies were conducted in accordance with the NIH animal use guidelines and current Chinese regulations and standards for laboratory animal use.

To determine the impact of Sirt7 on colorectal cancer cell line proliferation in vivo, 1 × 10⁶ cells were injected subcutaneously into nude mice (HCT116, n = 9; HT29, n = 7), and tumor progression was studied over time. Four weeks post-implantation, the mice were sacrificed, and the tumors were removed. The tumor volume was evaluated using the following formula: tumor volume = 4π/3 × (width/2)² × (length/2).
**In vivo metastasis assays**

We chose the HCT116 and HT29 cell lines for the lung and hepatic metastasis models, respectively, based on preliminary experiments.

**Hepatic metastasis model**

Mice were randomly assigned to 2 groups (8 per group). Both groups were anesthetized with isoflurane and subjected to laparotomy. The spleen was removed through a 1-cm incision in the upper left lateral abdomen, and \( \times 10^6 \) cells (NC-HCT116 or siSirt7-HCT116) in 20 \( \mu \)L PBS were injected into the spleen’s distal tip using an insulin syringe. The spleen was then replaced in the abdomen, and the abdominal cavity was closed with staples. After 6 weeks, the mice were euthanized, and the spleens and livers were removed for pathologic examination. Transverse sections (5 \( \mu \)m) of the liver were prepared at 10 different levels to cover the entire liver, and the sections were stained with hematoxylin and eosin (H&E). Metastatic nodules were counted in a double-blind manner under a microscope.

**Lung metastasis model**

Tumor formation and Sirt7-overexpressing and control cancer cell metastasis were examined following intravenous injection into the tail veins of nude mice. A modified transplantation through subcutaneous injection was

| Table 1. Clinicopathologic findings and correlation with Sirt7 expression |
|---------------------------------|-------|----------|----------|------|
| **Variable**                    | **n (%)** | **Sirt7-low (%)** | **Sirt7-high (%)** | **P** |
| Total cases                     | 131    | 27 (20.6) | 104 (79.4) |      |
| Age, y                          |        |          |          |      |
| \( \leq 65 \)                   | 96 (73.3) | 21 (21.9) | 75 (78.1) | 0.633 |
| \( >65 \)                       | 35 (26.7) | 6 (17.1)  | 29 (82.9) |      |
| Gender                          |        |          |          |      |
| Male                            | 74 (56.5) | 15 (20.3) | 59 (79.7) | 1.000 |
| Female                          | 57 (43.5) | 12 (21.1) | 45 (78.9) |      |
| Tumor location                  |        |          |          |      |
| Colon                           | 71 (54.2) | 15 (21.1) | 56 (78.9) | 1.000 |
| Rectum                          | 60 (45.8) | 12 (20.0) | 48 (80.0) |      |
| Tumor size, \( \text{cm} \)     |        |          |          |      |
| \( \leq 5 \)                    | 69 (53.1) | 12 (17.4) | 57 (82.6) | 0.388 |
| \( >5 \)                        | 61 (46.9) | 15 (24.6) | 46 (75.4) |      |
| Histology                       |        |          |          |      |
| Adenocarcinoma                  | 117 (89.3) | 23 (19.7) | 94 (80.3) | 0.486 |
| Mucinous                        | 14 (10.7)  | 4 (28.6)  | 10 (71.4) |      |
| Tumor invasive depth            |        |          |          |      |
| T1–T2                           | 18 (13.7)  | 4 (22.2)  | 14 (77.8) | 1.000 |
| T3–T4                           | 113 (86.3) | 23 (20.4) | 90 (79.6) |      |
| Lymph node status               |        |          |          |      |
| N0 (\( n = 0 \))                | 80 (61.1)  | 13 (16.3) | 67 (83.8) | 0.046 |
| N1 (\( n \leq 3 \))             | 35 (26.7)  | 7 (20.0)  | 28 (80.0) |      |
| N2 (\( n > 3 \))                | 16 (12.2)  | 7 (43.8)  | 9 (56.3)  |      |
| Preoperative CEA, \( \text{ng/mL} \) |        |          |          |      |
| \( <5 \)                        | 62 (56.4)  | 12 (19.4) | 50 (80.6) | 0.288 |
| \( \geq 5 \)                    | 48 (43.6)  | 5 (10.4)  | 43 (89.6) |      |
| AJCC/TNM stage                  |        |          |          |      |
| I–II                            | 74 (56.5)  | 10 (13.5) | 64 (86.5) | 0.029 |
| III–IV                          | 57 (34.5)  | 17 (29.8) | 40 (70.2) |      |
| Preoperative CA199, \( \text{ng/mL} \) |        |          |          |      |
| \( \leq 35 \)                   | 90 (81.8)  | 14 (15.6) | 76 (84.4) | 1.000 |
| \( >35 \)                       | 20 (18.2)  | 3 (15.0)  | 17 (85.0) |      |

NOTE: The numbers in parentheses indicate the percentages of tumors with a specific clinical or pathologic feature for a given Sirt7 subtype.

Abbreviation: CA199, carbohydrate antigen 19–9.

\( ^a \)Analysis of this parameter was available for 130 cases.

\( ^b \)Analysis of this parameter was available for 110 cases.

\( ^c \)Statistically significant.
reported by Quintana and colleagues (20). Briefly, mice were injected with cells (10^6 per mouse) and sacrificed 6 weeks after injection. The lungs were then removed for pathologic examination. Transverse sections (5 μm) of whole lungs were prepared and stained with H&E. Metastatic nodules were counted as described previously.

**Statistical analysis**

Statistical analysis was performed using SPSS standard version 16.0 (SPSS Inc.). Significant associations between Sirt7 expression and clinicopathologic parameters were assessed using the χ² test. Kaplan–Meier and log-rank tests were used to compare patient survival and to create survival curves based on high and low Sirt7 IHC scores (as defined by the ROC curve). Multivariate survival analysis was performed for all parameters that were significant in univariate analyses using the Cox regression model. The data were assessed with Student 2-tailed t test. P < 0.05 was considered statistically significant.

**Results**

**Sirt7 overexpression is frequently detected in colorectal cancer tissues and cell lines**

Sirt7 expression was first examined using real-time PCR in 18 pairs of colorectal cancers and adjacent non-tumor colorectal tissues and using Western blotting in 6 colorectal cell lines. Sirt7 mRNA was overexpressed in 15 of 18 pairs (83.3%, Fig. 1A), and all 5 colorectal cancer cell lines (i.e.,
HCT116, DLD1, THC8307, HT29, and SW620) demonstrated higher Sirt7 expression than the normal colorectal cell line FHC (Fig. 1B).

To examine Sirt7 protein expression, IHC was performed. Sirt7 was upregulated in tumors compared with paired adjacent non-tumor tissues (Fig. 1C and D; Supplementary Fig. S1).

Sirt7 overexpression is associated with decreased survival in colorectal cancer

We then determined the association between Sirt7 expression and the clinicopathologic characteristics of 131 colorectal cancer tumor tissue samples with the associated clinical details (Table 1). Regression–correlation analysis showed that Sirt7 overexpression was significantly
associated with both the TNM stage $(P = 0.029)$ and the lymph node status $(P = 0.046)$.

Kaplan–Meier survival analysis showed that patients at TNM stages I and II with high Sirt7 expression had decreased OS $(P = 0.006, \text{log-rank test}; \text{Fig. 1E})$ and disease-free survival $(P = 0.011, \text{log-rank test}; \text{Fig. 1F})$. High Sirt7 expression tended to be associated with decreased OS in a multivariate Cox proportional hazards model adjusted for age, carcinoembryonic antigen (CEA), and sex [HR, 6.358; 95% confidence interval (CI), 1.37–29.51; [Supplementary Tables S1 and S2]. However, Kaplan–Meier analysis showed no significant association between stages I–IV regarding OS or disease-free survival and Sirt7 expression $(P > 0.05, \text{data not shown})$.

**Sirt7 promotes colorectal cancer cell line growth, migration, and invasion in vitro**

The above observations prompted us to explore the potential biologic function of Sirt7 in colorectal cancer tumorigenesis and/or progression. First, we investigated the effects of Sirt7 manipulation (by gain-of-function and loss-of-function) on cancer cell proliferation. We knocked down Sirt7 in the HCT116 cell line, which has high endogenous expression, and evaluated the impact of Sirt7 on cellular proliferation using MTT and clonogenic assays. Sirt7 knockdown inhibited proliferation in transiently transfected HCT116 cells. Sirt7 knockdown had a greater negative effect on proliferation in the control group, whereas Sirt7 overexpression promoted proliferation in stable HT29 cell lines (Fig. 2B). The clonogenic assay showed that Sirt7 knockdown significantly reduced the number of HCT116 colonies formed after 14 days of culture compared with the controls $(P < 0.0001)$, whereas Sirt7 upregulation increased the number of HT29 colonies compared with the controls (Fig. 2C).

The effects of Sirt7 on cell invasion were observed using Transwell and Matrigel invasion assays. Sirt7 depletion in both the HCT116 and the SW620 lines significantly reduced cell invasion and migration (Fig. 2D; [Supplementary Fig. S2]). Supporting the role of Sirt7 in promoting cell motility, ectopic expression of pcDNA3.1-Sirt7 in THC8307 cells increased invasion and migration by >50% $(P = 0.017; \text{Fig. 2D})$.

**Sirt7 promotes colorectal cancer growth and metastasis in vivo**

To investigate the role of Sirt7 in vivo, we constructed a retroviral vector to mediate Sirt7 expression and established 4 stable cell lines named NC-HCT116, shSirt7-HCT116, ctrl-HT29, and Sirt7-HT29. These 4 cell lines were injected subcutaneously into the flanks of nude mice. The volume and weight of the tumors resulting from shSirt7-HCT116 cell injection were significantly lower than those from NC-HCT116 cell injection; conversely, the tumors resulting...
from Sirt7-HT29 cell injection were larger and heavier than those from ctrl-HT29 cell injection (Fig. 3A; Supplementary Fig. S3A).

We next evaluated the in vivo effects of Sirt7 on metastasis using 2 experimental metastasis assays. The ctrl-HT29 and Sirt7-HT29 stable cell lines were implanted into the tail veins of nude mice, and the number and size of metastatic tumor nodules in the lungs were examined by microscopy. The number of pulmonary metastatic nodules was clearly more in the Sirt7-HT29 group than in the ctrl-HT29 group (P = 0.045; Fig. 3B). More interestingly, tumors from 5 mice also metastasized to the skin in the Sirt7-HT29 group, but not in the ctrl-HT29 group (Supplementary Fig. S3B). When shSirt7-HCT116 and NC-HCT116 cells were injected into the spleens of nude mice, most mice in the NC-HCT116 group developed liver metastases, in contrast to the siSirt7-HCT116 group (Fig. 3C). Collectively, these findings are consistent with the in vitro results, indicating that Sirt7 promotes colorectal cancer growth and metastasis in vivo.

Sirt7 induces ERK1/2 phosphorylation and activates the Raf–MEK–ERK pathway

Several reports have shown that the MAPK–ERK signaling pathway enhances epithelial cancer cell growth, particularly in lung and colon cancers (21). Therefore, we examined whether Sirt7 expression influences ERK1/2 activation. Sirt7 overexpression was associated with increased phosphorylated ERK1/2 and cyclin D1 levels (Fig. 4A). In contrast, Sirt7 siRNA decreased p-ERK1/2 and cyclin D1 levels, but AKT and p-AKT levels were not significantly altered.

Next, we postulated that if Sirt7 affects proliferation by activating MEK–ERK, MEK–ERK inhibition should abrogate Sirt7-induced growth. Indeed, PD98059 treatment completely abrogated Sirt7-induced growth (Fig. 4B). In addition, forced overexpression of mutant Sirt7 (m1 and m3) did not induce ERK1/2 or p-ERK1/2 (Fig. 4C). To confirm that the effects were directly dependent on Sirt7 expression, we treated Sirt7-overexpressing cells with plasmid-resistant versions of Sirt7 siRNA or negative control siRNA (Supplementary Fig. S4A). The viability increase induced by Sirt7 was reduced by the expression of Sirt7 siRNA (Supplementary Fig. S4B). This approach demonstrated that Sirt7 directly induces proliferation.

The observation that Sirt7 induces ERK1/2 activation led us to evaluate the RAS–RAF–MEK–ERK pathway upon Sirt7 overexpression. We observed robust RAF–MEK–ERK pathway activation upon Sirt7 overexpression, whereas Sirt7 knockdown inhibited the RAF–MEK–ERK pathway (Fig. 5A and B).

Immunohistochemical staining of tumor nodules originating from the in vivo demonstrated that Sirt7-HT29 cells had increased p-ERK1/2 level compared with that from ctrl-HT29 cells, whereas p-ERK1/2 level was downregulated in shSirt7-HCT116 group compared with the NC-HCT116 group (Fig. 5C).

Sirt7 promotes colorectal cancer cell motility through EMT induction

Given that upregulated Sirt7 correlated with enhanced colorectal cancer cell migration, we examined whether EMT is an underlying mechanism. In Sirt7 siRNA–treated cells, Western blotting revealed upregulation of the cohesive epithelial markers E-cadherin and β-catenin and a corresponding downregulation of the mesenchymal markers fibronectin and vimentin. In Sirt7-overexpressing cells, fibronectin and vimentin expression was elevated compared with expression in vector-transfected cells, whereas E-cadherin and β-catenin were...
downregulated (Fig. 6A). In addition, the involvement of EMT was further supported by immunofluorescence (Fig. 6C).

In our SCID mouse metastasis model, further immunohistochemical staining demonstrated that the metastatic tumor nodules originating from shSirt7-HCT116 cells had increased the expression of E-cadherin and β-catenin, compared with that from NC-HCT116 cells (Fig. 6B).

Discussion
Sirtuins have received significant attention since the discovery of yeast Sir2, described as a transcriptional silencer of mating-type loci, telomeres, and ribosomal DNA (4, 5). Sirt7 overexpression has been detected in several malignancies, including thyroid, breast, and hepatocellular cancers (16, 17). A recent breakthrough showed that Sirt7 maintains human cancer cell oncogenic transformation by deacetylating lysine 18 of histone H3 (19). However, no detailed analysis of the biologic roles of Sirt7 in colorectal cancer has been conducted. In our study, Sirt7 upregulation was frequently observed in colorectal cancer tissues. Sirt7 overexpression was a strong independent predictor of OS in patients with early-stage colorectal cancers. In functional studies, Sirt7 reintroduction dramatically promoted colorectal cancer cell colony formation, migration, and invasion in vitro and tumor metastasis in vivo. Thus, Sirt7 plays a critical role in colorectal cancer growth, invasive, and/or metastatic potential, but the detailed mechanisms remain unclear.

MEK and ERK are central to cell growth because they increase the synthesis of pyrimidine nucleotides and increase the activity of transcription factors that enhance gene expression and cyclin D1 activity (22–26). We postulated that Sirt7 was important to the subsequent activation of this pathway. In overexpression and knockdown assays, Sirt7 enhanced proliferation, ERK activation, and cyclin D1 levels. These findings suggest that ERK activation is responsible for the proliferative influence and downstream effects on cell growth of Sirt7. Beyond ERK, MEK directs growth factor and G-protein–coupled receptor signals to their intracellular targets, which regulate cellular processes, including proliferation, differentiation, cell morphology, and oncogenesis. MEK1/2 activation in mitogen-stimulated cells is directly mediated by MAPKs, including Raf-1 kinase.
which phosphorylates 2 serine residues (S218, S222) in the activation loop of MEK. In turn, MEK activates ERK1/2 (26). Indeed, Sirt7 overexpression in colorectal cancer cells significantly increased MEK1/2 phosphorylation and Raf-1 levels, whereas Sirt7 knockdown had the opposite effect. MEK activation is the seminal signaling event that activates ERK and causes cell proliferation. Taken together, these findings demonstrate that Sirt7 is an integral mediator of cell proliferation via MEK–ERK MAPK pathway activation, which is known to be dysregulated in many cancers (27). We failed to identify the proteins directly activated by Sirt7 in the MAPK pathway; thus, further research using co-immunoprecipitation and direct binding assays will be necessary.

Patients with colorectal cancers frequently develop lymph node metastases during the early stages of disease. At the advanced stages, most patients also develop liver, lung, or peritoneal metastases (28). Therefore, we used 2 models (hepatic and lung metastasis) to simulate colorectal cancer metastasis (29). Sirt7 was a significant metastasis facilitator in human colorectal cancers. Surprisingly, Sirt7 overexpression promoted HT29 metastasis not only to the lungs but also to the skin. We hypothesized that Sirt7 promoted the colorectal cancer cells primarily via the lymph nodes metastasis to the skin.

We found that Sirt7 was a prognostic factor only in early-stage colorectal cancers and that Sirt7 has the lowest expression in stage VI. It is possible that Sirt7 plays an oncogenic role in early colorectal cancer development and that during the advanced stage, Sirt7 is corrupted by other proteins, although the pathways and molecules downstream of Sirt7 are already activated, further promoting colorectal cancer development.

The two most important hallmarks of malignant tumors, invasion and metastasis, are most associated with fatality in human cancer. Therefore, identifying the factors involved in invasion and/or metastasis and understanding the underlying molecular mechanisms involved in tumor progression are critical (30). Accumulating evidence indicates that EMT mediates tumor progression, including local invasion, dissemination from the primary tumor, intravasation into the circulation, and metastasis (31,32). EMT is a complex process requiring extensive changes in cell adhesion and morphology and signaling pathway activation, and our findings suggest that Sirt7 represents an upstream molecule that induces this transition. We found that Sirt7 re-introduction into colorectal cancer cells promoted EMT, as shown by the decreased expression of the epithelial markers E-cadherin and β-catenin and the enhanced expression of the mesenchymal markers fibronectin and vimentin. The ERK signaling pathway has been shown to upregulate Snail and slug, leading to the downregulation of E-cadherin (29,33). To test whether Sirt7 promotes EMT through the MAPK pathway, we used the ERK inhibitor PD98059 in migration assays. PD98059 dose independently inhibited the Sirt7 overexpression-induced migration of colorectal cancer cells (Supplementary Fig. S5). Although ERK activation occurs, it is not the only mechanism responsible for EMT induction, the exact mechanisms remain unclear.

In summary, we investigated Sirt7 expression in human colorectal cancers and found that Sirt7 overexpression may be important in the aggressive phenotype of colorectal cancers. Furthermore, a functional analysis showed that Sirt7 plays a critical role in colorectal cancer cell proliferation and metastasis by regulating MAPK signaling and EMT. Thus, our data suggest that Sirt7 could be used as a new prognostic marker and/or an effective therapeutic target in colorectal cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
All of the authors planned and implemented the investigation.
Conception and design: H. Yu, J. Wu, C. Pan, W. Huang
Development of methodology: H. Yu, R.-y. Liu, Y. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Yu, W. Wu, J. Wu, Y. Zhou, W. Huang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Yu, J. Wu, R.-y. Liu, X. Ying, Y. Zhou, H. Wang
Writing, review, and/or revision of the manuscript: H. Yu, J. Wu, X. Meng, H. Wang, C. Pan, W. Huang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Yu, J. Wu, Y. Zhou, W. Huang
Study supervision: J. Wu, Y. Zhou, W. Huang
Principal investigator, managed the research fund, designed project, organized experimental materials: W. Huang

Acknowledgments
The authors thank Jinmin Chen and Ling Zhou (Sun Yat-sen University) for their technical assistance.

Grant Support
This work was supported by grants from the National High Technology Research and Development Program of China (project 863, No. 2012AA02A204, 2012AA020803) and the National Basic Research Program of China (973 Program, No 2011CB504805, 2010CB912201).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 26, 2013; revised March 5, 2014; accepted April 5, 2014; published OnlineFirst April 25, 2014.

References
Sirt7 Promotes Colorectal Cancer Tumorigenesis

Overexpression of Sirt7 Exhibits Oncogenic Property and Serves as a Prognostic Factor in Colorectal Cancer

Hongyan Yu, Wen Ye, Jiangxue Wu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-2952

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/04/29/1078-0432.CCR-13-2952.DC1

Cited articles
This article cites 33 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/13/3434.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/20/13/3434.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/20/13/3434.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.