SIRT1 promotes EMT, migration and invasion by regulating mTORC1/4E-BP1 in colorectal cancer

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Abstract

Background Metastasis, rather than primary tumors, was accounted for the most cases of cancer death in colorectal cancer(CRC). The understanding of the underlying mechanism associated with tumor metastasis would improve the patient's miserable fate. SIRT1 has been identified to play a role in tumorigenesis and progression of malignant tumors, especially in keeping the characteristics of cancer stem cells(CSCs) in CRC. This study was conducted to investigate the role of SIRT1 in the regulation of metastasis and the underlying mechanism in colorectal cancer.

Methods We detected the expression of SIRT1 in 42 metastatic CRC patients. The relationship between SIRT1 and time to metastasis was also analyzed. Then the SIRT1 activity was regulated to investigate the liver metastasis in BALB/c mice. SIRT1 was knocked down to evaluate the effect on migration and invasion. Besides, exogenetic SIRT1 to assess the motile ability by wound healing assay and transwell assay. We then further explored the underlying mechanism.

Results SIRT1 was overexpressed in 67% CRC metastatic patients and associated with reduced time to metastasis. High SIRT1 activity by resveratrol was companied with more liver metastasis in vivo. SIRT1 deficiency increased E-cadherin, while reduced Vimentin and Snail, attenuated migration and invasion significantly in CT26 and SW620 cells. Meanwhile, the exogenetic SIRT1 induced epithelial–mesenchymal transition (EMT) and elevated the migratory ability in SW480 cells. Further studies demonstrated that mTORC1 related genes were elevated while 4E-BP1 decayed by SIRT1 overexpression. The promotion of metastasis induced by SIRT1 overexpression could be abolished by mTOR inhibition, while the stemness of cells was not changed.

Conclusions Collectively, our findings illustrated that SIRT1 was a functional regulator in the promotion of metastasis in CRC via mTORC1-4E-BP1 axis. SIRT1 was a potential independent prognostic factor of CRC metastatic patients after tumor resection, which provided a promising treatment target in CRC.

Background

Colorectal cancer (CRC) was the second most common type of malignancy in the United States with nearly 140,000 new cases in 2018[1]. The death rates in individuals younger than 55 years elevated annually by 2% since mid-2000s[2]. Meanwhile, in Europe, the 5-year survival rate of CRC was still lower than 60%[3].

CRC often arose from a gradually accumulation of genetic changes and epigenetic alterations[4, 5]. During the progression of CRC, the cancer cells were given the invasive and metastatic properties, just like the ability to invade neighboring tissues or metastasize to distant organs[6, 7]. Actually, metastasis accounted for the majority of mortality related with CRC. As the second leading cause of carcinoma in the United States, CRC was the most common type of tumor to metastasize to the liver and lungs through portal vein and pulmonary artery[8].

The development of metastatic tumors consisted of a series of complex sequential steps, which finally established metastatic spot in distant organs[6]. Among these steps, the epithelial to mesenchymal transition (EMT) was considered to be a critical early program[9, 10]. EMT was a dynamic process to acquire the migratory capacity, with a reduction of epithelial markers, cell-cell connection and adhesive ability[11]. Cells with high EMT levels existed with elevated motility. Reports indicated that the increased EMT levels turned up the capacity of invasiveness in various cancers[12–14], including CRC[15]. Although it was clear that the alterations in cancer-causing genes had been proved to contribute to the progression of CRC, relative knowledge of the nature of molecular mechanisms associated with tumor metastasis was still incomplete.

SIRT1 was a highly conserved NAD^+ dependent class III deacetylase, which was related in numerous metabolic processes, such as cell survival, apoptosis, and differentiation[16, 17]. By the deacetylation of C-terminal lysine residues of p53, SIRT1 played an important role in cell apoptosis under oxidative stress[18, 19]. Meanwhile, SIRT1 influenced cell survival by deacetylating Ku70[20, 21], NFκB[22] and FOXO transcription factors[23]. Recent reports revealed that SIRT1 was overexpressed in many malignant carcinomas, such as prostate cancer[24], breast cancer[25], colorectal cancer[26] and leukemia lymphoblasts[27]. SIRT1 was implicated in tumorigenesis and progression of malignant tumors through its anti-apoptosis activity[28]. Besides, SIRT1 was found to maintain the properties of embryonic stem cells by the Oct4-SIRT1-p53 axis[29]. In many established malignancy, cancer stem cells (CSCs) were a minor population of neoplastic cells, which had the ability of self-renewing, initiating and maintaining tumor growth[30–32]. CSCs were reported to be responsible for the resistant of conventional therapy[33, 34], which were often followed by neoplasm recurrence and metastatic colonies establishment[35]. Our previous studies found that SIRT1 played a crucial role in keeping the characteristics of CSCs in CRC[36]. We wondered whether SIRT1 could influence the metastasis via the regulation of CSCs. In this study, we demonstrated, for the first time, a role of SIRT1 in the regulation of EMT, migration, invasion and metastasis in CRC. SIRT1 had an apparent effect on the promotion of migration, invasion and motility of CRC in vitro and vivo. Further studies explored the underlying mechanisms.

Methods

Ethics statement

The operation of human samples acquisition and experiments conducted in accordance with the 1964 Helsinki declaration and its subsequent amendments, and the protocol was approved by the Hospital Research Ethics Committee of Ren Ji Hospital. All animal studies were approved by the Animal Care Committee of Shanghai Jiao Tong University. All methods were carried out in accordance with the approved guidelines.

Clinical samples

CRC samples were collected from patients of Department of Gastrointestinal Surgery, Ren Ji Hospital, who were confirmed as CRC and were recruited for prospective follow-up from January 2006 to December

2013. The diagnosis and staging were performed according to the 2010 WHO classification. None of the cases had received tumor-related therapy before diagnosis. All participants signed informed consent form.

Cell lines and culture

The murine CRC cell line CT26, human CRC cell line SW480 and SW620 were obtained from the Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), where they were authenticated. SW480 and SW620 cells were cultured in L-15 (Invitrogen, Thermo Fisher, Cat.11415-064) with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher, Cat. 10100-147), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Gibco, Thermo Fisher, Cat.10378-016) at 37 °C in an atmosphere. CT26 cells were cultured in RPMI1640 (Gibco, Thermo Fisher, Cat.11875-093) with 10% FBS, penicillin, and streptomycin at 37 °C in an atmosphere containing 5% CO_2.

The activator resveratrol (Res, Sigma, Cat.R5010) and inhibitor nicotinamide (NAM, Sigma, Cat.72340) of SIRT1 were used at 10 μM and 20 mM. SIRT1 activity of cells was confirmed by SIRT1 activity assay kit (Abcam, Cat.ab156065) as the protocol. The inhibitors of mTOR, rapamycin (Sigma, Cat.V900930) and AZD8055 (Selleck, Cat.S1555) were used at 1 μM and 500 nM, respectively.

Animal

Male wild-type BALB/c mice (10–12 weeks, 25–30 g) were purchased from Shanghai SLAC Laboratory Animals Co.,Ltd (Shanghai, China). The mice were housed under specific pathogen-free conditions. All animal experiments were carried out in accordance with animal experimentation protocols approved by the Animal Care Committee of Shanghai Jiao Tong University. All efforts were made to minimize suffering.

Induction of liver metastasis in mice

The mice were divided into 4 groups: (1) sham group (n = 5), splenic injection of physiological saline only; (2) CT26 group (n = 5), splenic injection of CT26 cells; (3) CT26 + Res group (n = 5), splenic injection of CT26 cells treated with 10 μM Res; (4) CT26 + NAM group (n = 5), splenic injection of CT26 cells treated with 20 mM NAM.

The cultured cells were prepared in suspensions of single-cell type in physiological saline (10^6 cells /100 μl). The cell suspension was injected into the parenchyma of the spleen in 100 μl per mouse. After 3 minutes, the spleens were removed to prevent the intrasplenic tumor formation. Mice were reared for a further 14 days for the formation of liver metastasis.

Tumor analysis

Experimental mice were sacrificed at 14 days after injection. Livers were harvested and prepared for the further experiments. Total tumor volume per liver was detected. Tumor whose maximum diameter was larger than 0.5 cm was considered as one tumor spot. The tumor numbers per liver were counted.

Cell transfection and selection

CT26 and SW620 cells were transduced with lentivirus vectors expressing SIRT1 ShRNA. Cells were seeded in 6-well plate and cultured overnight, followed by the exposures to virus-containing supernatants (20 × PFU/cell) via polybrene. Cells were selected by puromycin (CT26 cells at 20 μg/ml, while SW620 cells at 2 μg/ml) (Sigma, Cat.P8833) 72 hours later. The knockdown efficiency of ShRNA was evaluated by real-time PCR and western blotting analysis.

ShRNA1-1 and ShRNA1-2 were the anti-SIRT1 ShRNA sequences. To wipe off the effect of ShRNA, a scrambled sequence was used as a control. ShRNA sequences were as follows:

ShSIRT1-1: GAAGTGCCTCAGATATTAA

ShSIRT1-2: GTTGACCTCCTCATTGTTA

Sh-Scramble: GCGCGCTTTGTAGGATTCG.

Our experiments found that the two shRNA sequences could have silencing effects in both mice and human cell lines. The efficiencies of interference were all above 85%. The ShRNA were synthesized by Genechem Co., Ltd (Shanghai, China).

Adenoviral vector encoding the human SIRT1 gene (Ad-SIRT1-GFP) and negative control (Ad-GFP) was constructed, packaged, purified, and titrated at Genechem Co., Ltd. For adenovirus-mediated gene transfer, Ad-SIRT1-GFP or Ad-GFP was transfected into SW480 cells at a final concentration of 50 × PFU/cell for 72 hours. After 72 hours, the overexpression efficiency of Ad-SIRT1-GFP was evaluated by western blotting analysis.

Western blotting analysis

Cells were lysed and boiled in the loading buffer supplemented with β-mercaptoethanol (Sigma, Cat.M6250) for 15 minutes. Proteins were separated in 8%, 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose NC membrane (0.22 μM, Whatman, Cat.10401396). Membranes were blocked with 5% BSA and incubated in primary antibody and secondary antibody. The primary antibodies used in this research included E-cadherin (E-cad, Sigma, Cat.SAB5500022), β-catenin (CST, Cat.8480), Snail (CST, Cat.3879), Vimentin (CST, Cat.5741), SIRT1 (CST, Cat.9475), phospho-mTOR (p-mTOR, Ser2448, CST, Cat.5536), mTOR (CST, Cat.2983), Raptor (Abcam, Cat.ab40768), S6K (Abcam, Cat.ab32529), GβL (CST, Cat.3274), phospho-AKT (p-AKT, Ser473, CST, Cat.9271), AKT (CST, Cat.9272), phospho-4E-BP1 (4E-BP1, CST, Cat.2855) and β-actin (Hang Zhou HuaAn Biotechnology Co.,Ltd, Cat.R1207-1). The secondary antibody was fluorescently labeled secondary antibody (LI-COR, Cat.926-32211). Protein levels were measured by gray value with Odyssey 3.0 software. All experiments were carried out in triplicate.

Wound healing assay

Cells were seeded in 24-well plate at 1×10^5 cells/well. After the cells reached 90% confluence, artificial wounds were created by sterile 200 μl pipette tips. Photographs were taken immediately (as time 0 h) and at 12 or 24 hours under an inverted microscope (Leica, DMI3000B). The number of migrant cells was calculated.

Transswell assay

BD uncoated (Corning, Cat.353097) and matrigel-coated invasion chambers (Corning, Cat. 354480) were used. Cells were seeded in 200 μl serum-free medium in the upper chamber, while the lower chamber was filled with 500 μl culture medium with 10% FBS. CT26 cells were seeded at a concentration of 5×10^4 cells/chamber and incubated for 24 hours. SW620 cell lines and SW480 cell lines were seeded at a concentration of 1×10^5 cells/chamber and incubated for 72 hours. The infiltrated cells were fixed with 4% paraformaldehyde (Beyotime, Cat.P0099) and stained with crystal violet solution (Beyotime, Cat.C0121) for 30 minutes. The infiltrated cells were counted and photographed (Leica, DMI3000B).

Immunofluorescence staining

Cells were seeded on sterile glass coverslips overnight. Then cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 ((Beyotime, Cat.P0096), blocked with 5% goat serum (Beyotime, Cat.C0265) and sequentially incubated in primary antibody against β-catenin (CST, Cat.8480) and Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG secondary antibody (Invitrogen, Thermo Fisher, Cat.A11008). Cells were washed with PBS for three times and stained with DAPI (Sigma, Cat.D9542). Finally, the slides of cells were examined under a confocal microscope (Leica, TCS_SP5). All experiments were carried out in triplicate.

Immunohistochemistry analysis

The clinical chips were deparaffinized with xylene and rehydrated through graded alcohol series. Tissue slices were treated in citra solution with microwave for 15 minutes. 3% H_2O_2 (treated for 20 minutes) were used to block the endogenous peroxidase. Tissue sections were blocked and incubated in 5% goat serum (Beyotime, Cat.C0265) and primary antibodies sequentially. Primary antibodies against SIRT1 (Rabbit monoclonal to SIRT1, Sigma, Cat.HPA006295) (dilution 1:300) and 4E-BP1 (Rabbit monoclonal to 4E-BP1, CST, Cat.2855) (dilution 1:1000) were applied and maintained overnight at 4 °C. Secondary antibodies anti-rabbit IgG (Gene tech Co.,Ltd, GP021729) (dilution 1:100) were incubated for 30 minutes at 37 °C. The slides were visualized by DAB (Dako, Cat.K3468) and hematoxylin counterstain (Dako, Cat.S202084). All experiments were carried out in triplicate.

Evaluation of immunohistochemistry

The level of SIRT1 and 4E-BP1 expression was evaluated on entire tissue sections of CRC specimens. Three fields of every section were viewed. Every specimen was given a score according to both the percentage of protein expression and the intensity of staining. Strong was defined as a score of ≥ 50% protein expression. Score of < 50% was considered to be weak. Immunohistochemistry results were evaluated by two skilled pathologists independently, blinded from other data.

Statistics

All values of independent experiments were demonstrated as the mean ± SEM. Student's t-test analysis and One-way ANOVA were performed to calculate the statistical significance (P value) using GraphPad Prism software (Version 6.0). Log-rank test was performed for categorical data of metastasis progression analysis. The Chi-square test and multivariate analysis were conducted on the Cox regression model using SPSS software (Version 19.0). Statistical significance was set at P value < 0.05.

Results

Strong SIRT1 expression of tumor tissues correlated with high metastasis in CRC patients

Data from 42 patients of CRC with metastasis were evaluated. The clinicopathologic features were summarized in Table 1. Immunohistochemical staining of SIRT1 and 4E-BP1 were shown in Figure 1. The results revealed that SIRT1 had a nuclear localization while 4E-BP1 was found in cytoplasm (Figure 1a, 1b and 1c). According to the score of immunohistochemistry, strong expression of SIRT1 was seen in 67% (28 of 42) in 42 metastatic CRC patients, while the percentage of strong 4E-BP1 expression was 38% (16 of 42). It was obvious that SIRT1 and 4E-BP1 had an inverse expression relationship in CRC tissues with metastasis (Figure 1a, 1b and 1c).

According to the immunohistochemistry score, CRC specimens with weak and strong SIRT1 expression were 14 and 28 samples, respectively. Chi-square test indicated that SIRT1 had no significant correlation to clinicopathological features such as Age (P=0.661), Gender (P=0.513), Tumor location (P=0.578) and Serum CEA (P=0.657). On the other hand, the altered SIRT1 expression was significantly correlated with Tumor size ($P<0.001$) and Clinical stage ($P<0.001$). By using univariate Cox proportional hazard analysis, the variable and its relationship to metastasis were shown in Table 2. Strong SIRT1 expression was significantly related to the occurrence of metastasis, which displayed an independent relative risk of 2.517 (95% CI: 1.217-5.203) compared with the weak group (P=0.013, Table 2). However, the Age, Gender, Tumor location, Serum CEA, Tumor size and Clinical stage did not predict the occurrence of metastasis. Besides, multivariate analysis was conducted using 42 patients with complete information of all variables (Table 2). From the multivariate analysis, only the SIRT1 expression was independent factors significantly associated with the occurrence of metastasis. Patients with strong SIRT1 expression had a 3.035-fold (95% CI: 1.392-6.618) greater risk for metastasis (P=0.005, Table 2).

Meanwhile, applying Kaplan-Meier analysis, we found that strong SIRT1 expression had an obvious association with the time to metastasis (Log-rank test, $P<0.01$, Figure 1d). Patients with weak SIRT1 expression had a higher percentage of free progression survival ($P<0.01$, Figure 1e). All these data indicated that SIRT1 represented as an independent prognostic factor, which related to the occurrence of metastasis of CRC patients.

The pharmacological regulation of SIRT1 affected the liver metastasis of CRC in vivo

In order to identify the role of SIRT1 in the metastasis, we chose two molecules to regulate the SIRT1 activity. Resveratrol (Res) was identified as an activator of SIRT1[37-39]. Meanwhile, recent studies exposed that nicotinamide (NAM), as a product of deacetylation, noncompetitively inhibited SIRT1[40, 41]. In this study, CT26 cells were pre-treated with Res at 10 µM or the inhibitor NAM at 20 mM for 24 hours. The SIRT1 activity were regulated obviously and confirmed by SIRT1 activity assay kit. Then equal numbers of cells were injected into the mice. Results showed that numerous metastatic tumors appeared in the livers of mice injected with Res treated CT26 cells. The metastatic spots occupied almost the entire liver (Figure 2a). H-E stain showed that the Res treated group had a larger percentage of liver tissue replaced by tumor (Figure 2b). The Res group exhibited obvious increase of tumor volumes and tumor numbers, while the NAM group revealed an opposing situation (Figure 2c and 2d). These observations indicated the regulation of SIRT1 by the pharmacological molecules affected the liver metastasis of CRC in vivo.

SIRT1 silencing inhibited the mesenchymal morphology, migration, invasion and the EMT in CRC cells

To investigate the function of SIRT1 in CRC metastasis, we constructed CT26 cell derivatives whose SIRT1 was knocked down by shRNA constitutively (ShSIRT1-1 and ShSIRT1-2). The efficiency of interference was proved by western blotting assay (Figure 3f). CT26 cells were murine CRC cells with strong abilities of motility and invasion, which were always spindle-shaped or more elongated structures. As shown in Figure 3a, SIRT1 silencing resulted in a change in the cell morphology with a spheroid-like characteristic.

Migration and invasion were critical steps in the initial progression of cancer which facilitated metastasis. Wound healing assay was conducted firstly. After the scratch, cells were cultured for 12 hours. Strikingly, SIRT1 knockdown reduced the numbers of migrant cells by 2-3 folds (Figure 3b and 3d). Then the chambers pre-treated with matrigel were used to evaluate the capacity of invasion. Consistent with the result of wound healing, transwell assay results showed that SIRT1 silencing significantly decreased the invasion of CT26 cells (Figure 3c and 3e). The ratio of cells was about (5-7): 1.

EMT was a dynamic process to acquire cell motility, which was thought to be a key step of cancer metastasis[10, 14]. According to the shift of morphology, we wondered whether there was the occurrence of EMT. Our western blotting results illustrated that E-cadherin, a critical marker of EMT, was elevated when SIRT1 was silenced (Figure 3f). In contrast, the expression of mesenchymal marker Vimentin reduced along with the decrease of SIRT1. Meanwhile, a major transcription factor in dominating EMT, Snail, was proved to decline (Figure 3f). Our results also revealed that β-catenin was increased with the localization shifted from the nucleus to the cytoplasm when SIRT1 was silenced (Figure 3f and 3g). The Wnt/β-catenin pathway was always be activated in CRC, which resulted in a nuclear accumulation of β-catenin[42, 43]. The nuclear accumulation of β-catenin was thought to have a connection with EMT rearrangement[44]. Taken together, these data suggested that SIRT1 was an important regulator of the mesenchymal morphology, migration, invasion and the EMT in CT26 cells.

To further certify the regulation of SIRT1 in the metastasis of CRC, we then assessed the effect of SIRT1 on motility in human CRC SW620 cells with high metastasis. SW620 cells were transfected with Scr-ShRNA or SIRT1-targeted ShRNAs, and then the scratch assay and transwell analysis were performed as mentioned above.

As similar with CT26 cells, SIRT1 silencing cells exhibited rare migration after 24 hours in SW620 cells (Figure 4a and 4c). The numbers of infiltrated cells were obviously reduced by SIRT1 silencing after 72 hours incubation (13.00 ± 2.309 vs. 73.33 ± 3.333, 14.67 ± 1.764 vs. 73.33 ± 3.333) (Figure 4b and 4d). Meanwhile, E-cadherin and β-catenin expressions were elevated, while Vimentin and Snail were reduced coupled with SIRT1 silencing (Figure 4e). Unlike the obvious morphological change in CT26 cells, there was no distinct alteration in the appearance in SW620 cells. Collectively, these results declared that knockdown of SIRT1 significantly inhibited the migration, invasion and EMT in SW620 cells.

Overexpression of SIRT1 promoted migration and motility in SW480 cells

Finding that SIRT1 knockdown repressed metastasis in high metastatic CT26 and SW620 cells, we wondered whether stable SIRT1 overexpression could accelerate the metastatic potential. SW480 cells were thought to be a non-metastatic cell line with a lower expression of SIRT1 originally[36, 45]. SW480 cells were transfected with exogenetic SIRT1. After puromycin selection, derivates of stable SIRT1 overexpression were verified by western blotting analysis (Figure 5f). Overexpression of SIRT1 in SW480 cells led to an outstanding morphological change. Cells with overexpressed SIRT1 displayed an appearance of fibroblast-like cell shape with a more elongated structure (Figure 5a). As shown in Figure 5b and 5d, SIRT1 overexpression appeared to increase the number of migrant cells with a ratio of 4-fold compared with the control. Moreover, non-matrigel-coated transwell assay showed that the elevation of SIRT1 significantly increased the number of infiltrated cells after the incubation of 72 hours (Figure 5c and 5e). Transwell assay with matrigel-coated showed that cells with exogenetic SIRT1 still had no invasive ability after incubation of 72 hours (Data were no shown). Meanwhile, the overexpressed SIRT1 increased the Vimentin and Snail expression (Figure 5f). The E-cadherin was markedly reduced after the SIRT1 overexpression, while β-catenin had no significant change (Figure 5f). Collectively, SIRT1 overexpression in SW480 cells strongly promoted the migration, motility and EMT, which supported an important role of SIRT1 in promoting the progression of CRC.

SIRT1 regulated the mTOR signal pathway via the mTORC1/4E-BP1 axis

To explore the molecular mechanism by which SIRT1 promoted the malignant progression, we examined several promising signal pathways. Results showed that SIRT1 knockdown in both CT26 cells and SW620 cells led to an inhibition of mTOR signal pathway (Figure 6a, 6b, 6e and 6f). The p-mTOR and mTOR levels were both reduced along with SIRT1 silencing. The mTOR was an important serine/threonine kinase existed in two complexes: mTORC1 and mTORC2. Raptor, as an important member of mTORC1, was also found to decrease after the silencing of SIRT1. As shown in Figure 6a and 6b, p-AKT and AKT were both attenuated. However, 4E-BP1, a downstream component of mTORC1, was

inversely expressed with SIRT1 in both CT26 and SW620 cells (Figure 6a, 6b, 6e and 6f). It suggested that mTOR signal pathway attenuated while SIRT1 was knocked down.

Meanwhile, overexpressed SIRT1 could elevate the p-mTOR, mTOR and Raptor in SW480 cells, while the 4E-BP1 was reduced (Figure 6c and 6d). Nevertheless, in contrast with the CT26 cells and SW620 cells, the levels of p-AKT and AKT had no significant difference after the SIRT1 overexpression in SW480 cells. Taken together, these data illustrated that SIRT1 affected the mTOR signal pathway via mTORC1/4E-BP1 axis.

mTOR inhibition withdrew the promotion effect of metastasis caused by SIRT1 overexpression

To further confirm that SIRT1 promoted metastasis by activating the mTOR signal pathway, two pharmacological inhibitors of mTOR, rapamycin and AZD8055 were used. Rapamycin was partially reported to only inhibit mTORC1, while AZD8055 as a competitive inhibitor, was proved to have an inhibition effect on both mTORC1 and mTORC2[46]. SW480 cells were additionally express SIRT1 firstly. Then the processed cells were treated with the mTOR inhibitors. As shown in Figure 7e and 7f, the levels of p-mTOR and mTOR were reduced significantly. Meanwhile, Raptor, S6K and GβL, the main components of mTORC1 were all decreased after the treatment, while 4E-BP1 was increased compared with the SIRT1 overexpressed group. Interestingly, the mTOR inhibition also decreased the expression of SIRT1, which implied that there was a regulated loop between mTOR signal pathway and SIRT1 (Figure 7e and 7f). Moreover, the wound healing data displayed that the mTOR inhibition could erase the promotion of migration caused by SIRT1 overexpression (Figure 7a and 7c). The similar results were obtained by transwell analysis. The inhibitor treatment revoked the increase of motility caused by SIRT1 overexpression (Figure 7b and 7d). These results demonstrated that the promotion effect of metastasis induced by SIRT1 overexpression could be abolished by the mTOR inhibition, which implied mTORC1/4E-BP1 was critical to the promotion effect of SIRT1 in metastasis.

mTOR inhibition failed in reversing the promotion of stemness caused by SIRT1 overexpression

According to the previous studies, we wondered whether SIRT1 regulated the metastasis by the influence of stemness in CRC. As shown in Figure 8a, the sphere formation ability of cells was kept the same after the mTOR inhibition compared with exogenetic SIRT1 cells. Meanwhile, the colony formation assay had no distinct difference between the mTOR inhibited cells and exogenetic SIRT1 cells (Figure 8b). We then detected the expression of markers of stemness, such as CD133, CXCR4 and LGR5. Results of flow cytometry indicated that there were no difference in the expression of CD133, CXCR4 and LGR5 after mTOR inhibitors treatment (Figure 8c, 8d and 8e). It demonstrated that mTOR inhibition failed in reversing the promotion of stemness caused by SIRT1 overexpression. All these data suggested that the promotion of metastasis caused by SIRT1 through the mTORC1/4E-BP1 axis was not attributed to the influence of stemness in CRC.

Discussion

CRC was the second leading cause of cancer death,with a 5-year survival rate was only 11.7% in advanced stage[47]. In most cases, cancer death was not caused by the primary tumor, but rather the metastasis. About half of CRC patients developed synchronous or metachronous liver metastases[48]. Though intensive studies had indicated the correlation between the mutations and tumorigenesis, the worldwide sequencing result between metastatic nidus and primary CRCs still showed that there was barely metastasis-specific mutations[49]. Therefore, understanding the mechanism of metastasis was critical for the effective treatment of CRC.

The CSCs theory posited that there was a fraction of cancer cells with the abilities of self-renewal and differentiation. CSCs were thought to play a crucial role in tumor formation, especially the tumor recurrence and metastasis in many kinds of malignancies. Patrick H et al. found that, in pancreatic adenocarcinoma, a group of $CD133^+$ CSCs with $CXCR4^+$ expression was proved to determine the metastatic phenotype. The remove of the CSC pool obviously abolished the lymph node metastasis[50]. Meanwhile, the subpopulation $CD44^+/CD24^-$ breast cancer cells with potential CSCs properties was indicated to have a higher pro-invasive genes expression and increased invasive properties[51]. Recent studies also reported that CSCs existed in CRC with an important role in the regulation of metastatic capacity. Pang R et al. illustrated that a subpopulation of $CD26^+$ CSCs led to development of distant metastasis associated with enhanced invasiveness and chemoresistance[52]. Meanwhile, SIRT1, a NAD^+ dependent deacetylase, was discovered to play an important role in the maintenance of self-renewal and tumorigenesis of CSCs. Limei L demonstrated that SIRT1 was responsible for the self-renewal and stemness maintenance by the transcriptional regulation of SOX2 in liver CSCs[53]. Simultaneously, SIRT1, as a mediator of NAMPT, activated stem cells and tumorigenic properties in human colon cancer[54]. Our previous study demonstrated that SIRT1 played an important role in the tumorigenesis and stemness maintenance in CRC[36]. SIRT1 was elevated in CRC tissues compared with corresponding pericarcinomatous tissues. The Chi-square test of 102 CRC patients showed that SIRT1 had a significant correlation with the metastasis (P = 0.02). SIRT1 was higher expressed in the advanced stage CRC cells (SW620) compared with SW480 cells. All these results implied that SIRT1 may as a pivotal candidate in regulating the progression and metastasis of CRC.

SIRT1 had been discovered to up-regulate in many cancers, including breast cancer[25], prostate cancer[24] and colorectal cancer[26]. SIRT1 overexpression always accompanied with poor prognosis, which implied SIRT1 correlated with the progression of cancer, especially the advanced progression[36, 55]. Recently, more and more emphasis was focused on the correlation between SIRT1 and tumor progression. In prostate cancer, Byles V and his colleagues illustrated that SIRT1 had a positive effect in the regulation of EMT[56]. SIRT1 induced cell migration and tumor metastasis by cooperating with the EMT-inducing transcription factor ZEB1 to attenuate E-cadherin. The authors demonstrated that SIRT1 silencing reduced the ZEB1 expression. Meanwhile, SIRT1 was found to be recruited to the promoter of E-cadherin to deacetylate histone H3 and inhibit E-cadherin transcription[56]. Moreover, SIRT1 was reported to coordinate regulate the acetylation level of cortactin with p300. The deacetylation of cortactin induced by SIRT1 significantly increased cell migration in ovarian and breast cancer cells[57]. Besides, SIRT1 was

also certified to have a promotion in the aggressiveness of ovarian carcinoma cells by elevating a cluster of antioxidant pathways to inhibit oxidative stress[58].

Our clinic analysis with 42 metastatic CRC samples showed that SIRT1 expression had a correlation with the time to metastasis. Patients with strong SIRT1 expression had a greater risk for metastasis. According to these speculations and results, a series of studies were conducted in this study. Firstly, the activity of SIRT1 was regulated by pharmaceutical molecules. The activation of SIRT1 resulted in more and larger liver metastatic tumor spots distinctly, while the suppression had a totally different outcome. Secondly, the silencing of SIRT1 used by ShRNAs apparently attenuated the migrant and invasive abilities in CT26 cells and SW620 cells. SIRT1 silencing reduced the expression of mesenchymal cell markers, which down regulated EMT. Moreover, the exogenetic SIRT1 overexpression could elevate the migrant and motile capacities significantly in SW480 cells which had a low SIRT1 expression originally. All these data illustrated that SIRT1 played a crucial role in the regulation of motility and metastasis in CRC.

mTOR was a conservative regulator in cell proliferation, cell cycle and autophagy[59]. It worked in two distinct kinase complexes, mTORC1 and mTORC2. mTORC2 was reported to regulate the actin cytoskeleton rearrangement[60]. Meanwhile, evidences certified that mTORC2 was a critical component in the regulation of cell proliferation and metastasis by the effect of autophagy[61]. Besides, Pat G et al. proved that the increased mTORC1 and mTORC2 activity induced EMT, motility and metastasis through RhoA and Rac1 pathway in CRC[62]. Recently, increasing articles reported that activation of mTORC1-mediated S6K1 and 4E-BP1 could be accounted for cell migration[63–65]. Consistent with these discoveries, our further studies indicated that SIRT1 inhibition resulted in the reduction of p-mTOR, mTOR, Raptor, p-AKT and AKT accompanied with the increase of 4E-BP1 in CRC cells. Synchronously, the overexpressed SIRT1 induced the up-regulation of p-mTOR, mTOR, Raptor with the decreased 4E-BP1, while p-AKT and AKT kept the same. The S6K had no difference. All these data implied that SIRT1 regulated the mTOR signal pathway through the mTORC1/4E-BP1 axis. To further prove the above hypothesis, cells with lower original SIRT1 expression was transfected with exogenous SIRT1 firstly. Then two inhibitors of mTOR were applied. The migration and motility analysis demonstrated that mTOR inhibition withdrew the promotion of metastasis caused by SIRT1 overexpression. However, the analysis of colony formation and sphere formation showed that there was no difference after the mTOR inhibition compared with the SIRT1 overexpressed group. The markers of stemness of CRC, CD133, CXCR4 and LGR5 were all kept the same after the mTOR inhibitor treatment. These data indicated that mTOR inhibition had no obvious effect on the regulation of stemness of CSCs after SIRT1 overexpression in CRC. The regulation of metastasis caused by SIRT1 was not attributed to the influence of stemness in CRC. The mechanism of SIRT1 on metastasis needed further study.

In our research, we found that Snail, a critical transcriptional factor which was well-studied in promoting EMT[14, 66], had a correlation with the expression of SIRT1. SIRT1 silencing reduced the level of Snail in CT26 cells and sw620 cells. The reconstitution of SIRT1 in SW480 cells elevated Snail. Simultaneously, V Byles demonstrated that SIRT1 silencing down-regulated the expression of some EMT transcription factors including Snail in DU145 and PC3 cells[56]. The authors did not discover a physical interaction

between SIRT1 and Snail by Co-IP experiment. Nonetheless, they overexpressed Snail in DU145 cells with SIRT1 silencing and proved that Snail overexpression could repeal the epithelial morphology caused by SIRT1 silencing[56]. It indicated that SIRT1 had a functional regulation in Snail expression. Meanwhile, Snail as a downstream molecular could be affected by mTORC1 through the effect of GSK3β nuclear localization. Bautista SJ et al. identified that the down-regulation of mTORC1 resulted in elevated GSK3β nuclear localization accompanied by the increased GSK3β-dependent degradation of Snail[67]. Treatment of rapamycin gave rise to a reduction of Snail in RPE cells and breast cancer cells[67]. Besides, targeted inhibition of Snail was reported to increase the 4E-BP1 levels[68]. Snail played as a strong repressor of 4E-BP1, but also a positive feedback loop of sustaining translation of Snail via the 4E-BP1 inhibition[68]. Previous articles also demonstrated that 4E-BP1 deficiency induced EMT, motility, invasion and be linked to cancer progression[69]. Our study discovered that SIRT1 had an effect on the activity of mTORC1/4E-BP1 accompanied with a change of Snail expression. The increase of migration and motility ability induced by overexpressed SIRT1 could be abolished by mTOR inhibition. All these findings indicated that SIRT1 may affect the EMT, migration and invasion in CRC by Snail through the regulation of mTORC1/GSK3β nuclear localization or the regulation of 4E-BP1. However, the underlying mechanisms needed further exploration.

Conclusions

Our data showed that high SIRT1 expression was associated with a reduced time-to-metastasis after tumor resection in CRC patients. SIRT1 deficiency reduced the EMT ability, migration, invasion and liver metastasis, while the overexpression of SIRT1 elevated the capacity of migration and motility of CRC cells. Additionally, the promotion of metastasis induced by SIRT1 overexpression could be abolished by mTOR inhibition. All these results indicated that SIRT1 was a critical regulator in metastasis of CRC patients by mTORC1/4E-BP1 axis, and illustrated a promising treatment target in CRC.

Abbreviations

CRC colorectal carcinoma

EMT epithelial−mesenchymal transition

SIRT1 Silent information regulator 1

mTORC1 mammalian target of rapamycin complex 1

4E-BP1 eukaryotic initiation factor 4E binding protein-1

CSCs cancer stem cells

Res resveratrol

NAM nicotinamide

CEA carcinoembryonic antigen

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Xiaojing Chen, Ming Zhong and Peifeng Liu conceived and designed the study. Yan Zhou and Hongmei Liu performed animal experiments. Shaolan Qin and Yang Luo collected the colorectal cancer samples and conducted the immunohistochemistry analysis. Bo Yu performed the statistical analysis of clinical samples. Xiaojing Chen and Shaolan Qin wrote the draft manuscript. Ming Zhong and Peifeng Liu finalized the manuscript. All authors read and approve the final manuscript.

Availability of data and materials

All data generated or analyzed in this study are included in this published article.

Ethics approval and consent to participate

The operation of human samples acquisition and experiments conducted in accordance with the 1964 Helsinki declaration and its subsequent amendments, and the protocol was approved by the Hospital Research Ethics Committee of Ren Ji Hospital. All participants signed informed consent form.

Consent for publication

All authors approved the publication of this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Comparison of clinical characteristics between patients with weak and strong SIRT1 expression.

Variable	SIRT1 expression		
	Weak	Strong	PA
	n=14	n=28	
Age(years)			
≤65	7	12	0.661
>65	7	16	
Gender	8	13	0.513
male	6	15	
female			
Tumor location			
rectum	12	22	0.578
colon	2	6	
Serum CEA			
≤5 ng/ml	9	16	0.657
>5 ng/ml	5	12	
Tumor size(cm)			**0.000**
≤5	8	13	
>5	6	15	
Clinical stage			**0.000**
III	8	11	
IV	6	17	

A: Chi-square test. The bold number represents the *P*-values with significant differences.

Table 2. Univariate and multivariate analyses showing the metastases in colorectal cancer.

Variable	Univariate analysis			multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
SIRT1 (high versus low)	2.517	1.217-5.203	**0.013**	3.035	1.392-6.618	**0.005**
Age (>65 versus ≤65)	1.375	1.004-1.882	0.783	1.146	0.572-2.294	0.701
Gender (male versus female)	0.855	0.455-1.607	0.627	1.137	0.562-2.304	0.721
Tumor Location (colon versus rectum)	1.575	0.711-3.486	0.263	0.995	0.396-2.501	0.991
Serum CEA (>5ng/mL versus ≤5 ng/mL)	1.677	0.882-3.189	0.115	1.990	0.862-4.594	0.107
Tumor size (>5cm versus ≤5 cm)	1.104	0.592-2.058	0.755	0.773	0.382-1.567	0.476
Clinical stage (IV versus III)	1.339	0.718-2.498	0.359	1.225	0.578-2.596	0.597

HR: Hazard ratio; CI: Confidence interval. The bold number represents the *P*-values with significant differences.

Figures
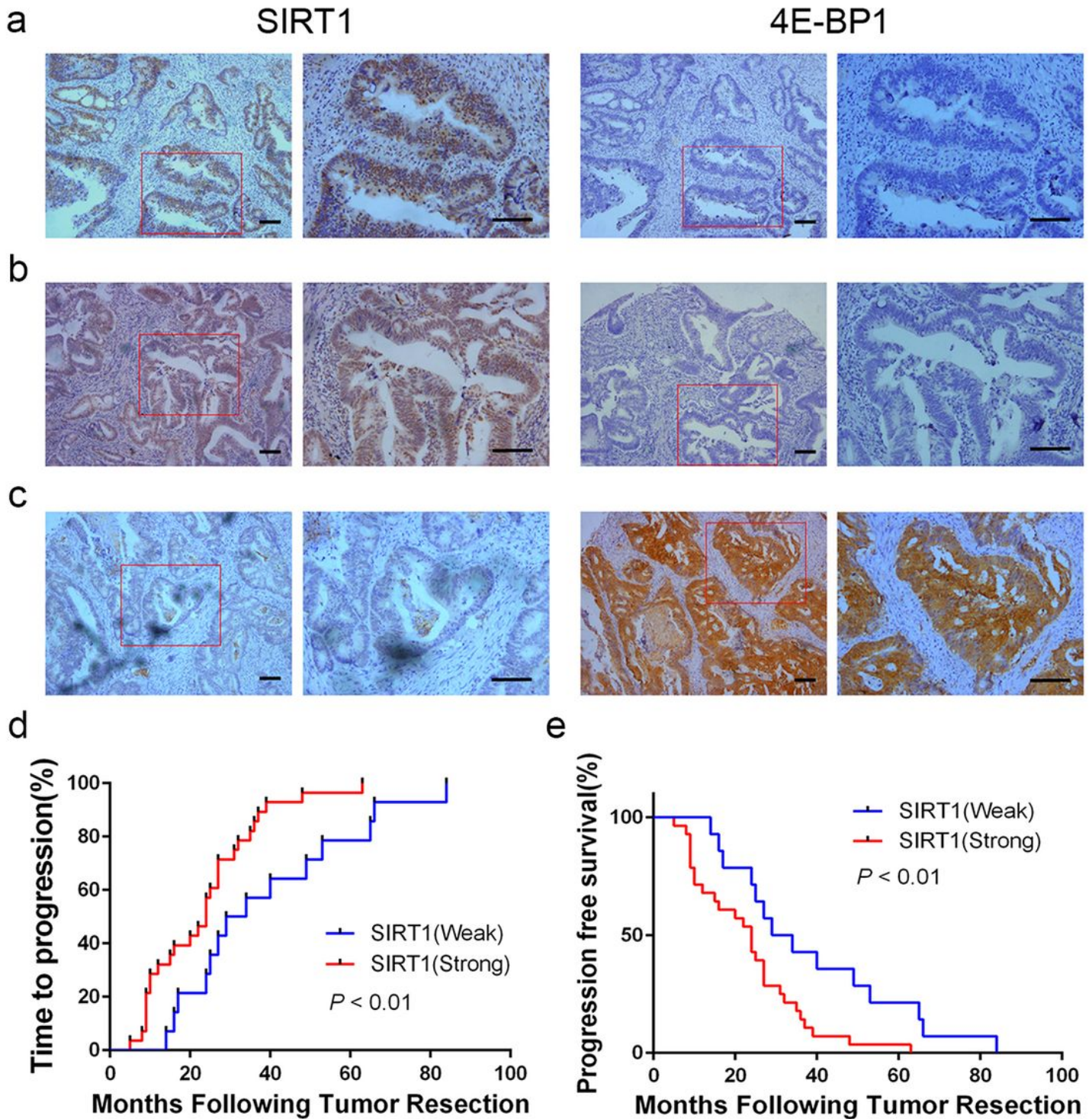
Figure 1

SIRT1 expression in CRC tissue correlated with the progression of metastasis. a-c, Immunohistochemical analysis of SIRT1 and 4E-BP1 was conducted in CRC patients. The scale bars were 100 μm. d, Time to progression curve showed that patients with strong SIRT1 expression had a higher proportion of progression (P < 0.01). e, Free progression survival curve indicated that patients with weak SIRT1 expression had a higher percentage of free progression survival (P < 0.01).
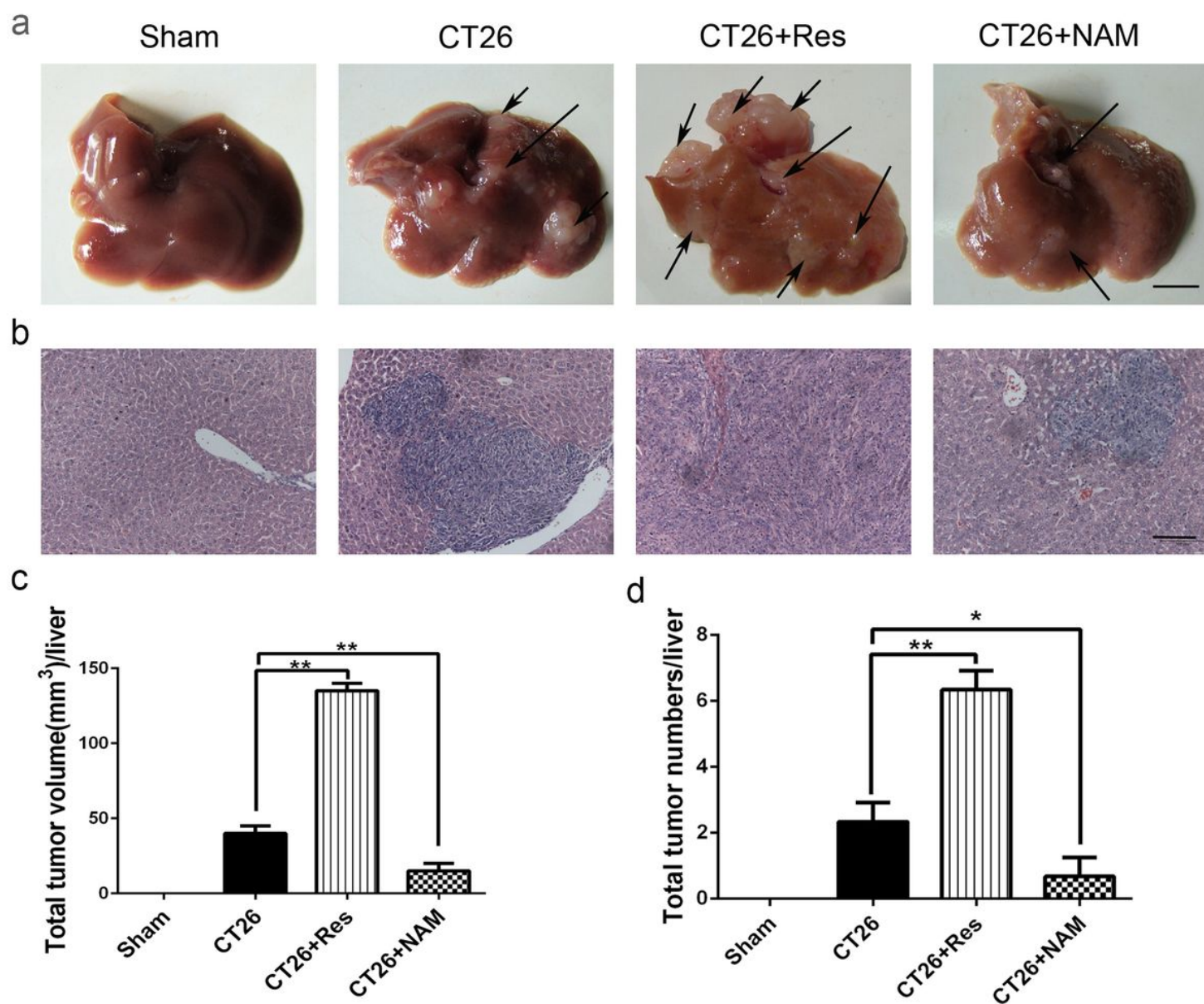
Figure 2

Pharmacological regulation of SIRT1 affected the liver metastasis of CRC in mice. a, Tumors were indicated by arrows. The scale bar was 1 cm. b, H-E stain results of liver sections. The scale bar was 500 µm. c and d, Tumor volumes and numbers per mouse. Data were presented as mean ± SEM (n=5). Significance: ** P < 0.01, * P < 0.05.
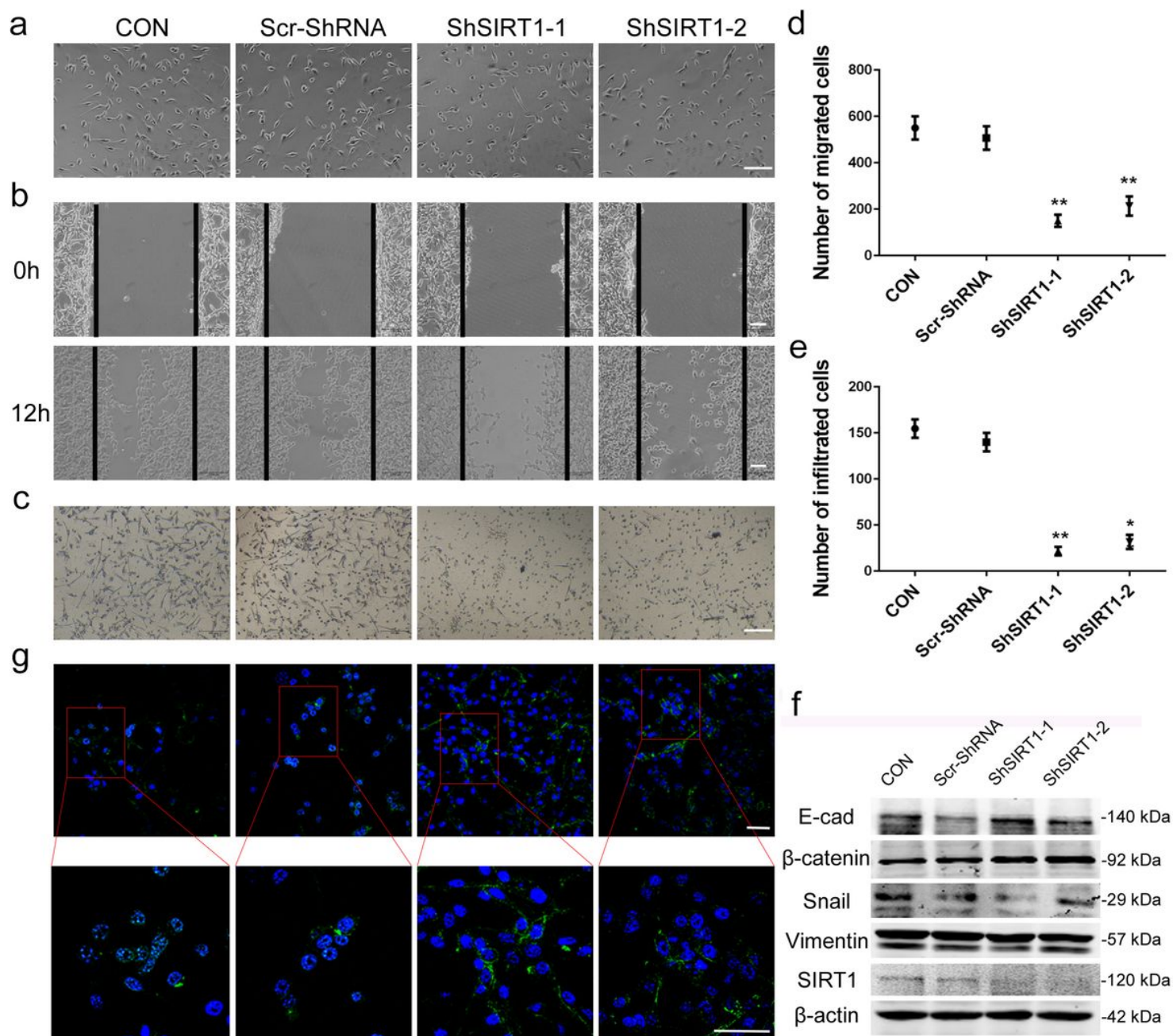
Figure 3

SIRT1 deficiency restrained the mesenchymal morphology, migration, invasion and EMT in CT26 cells. a, CT26 cells had spheroid-like characteristics after the treatment. b, The wound healing assay. The margins of the wound were denoted by black solid lines. c, The transwell assay. The scale bars of A, B and C were 500 μm. d, The numbers of cells that migrated to close the wounded area after 12 hours. e, The numbers of cells that infiltrated from the upper chamber to the lower after 24 hours. f, Western blotting analysis of EMT typical markers, SIRT1 and β-actin. g, Immunofluorescence staining of the location of β-catenin. The scale bar was 50 μm. Data were presented as mean ± SEM. Significance: ** P < 0.01, * P < 0.05.
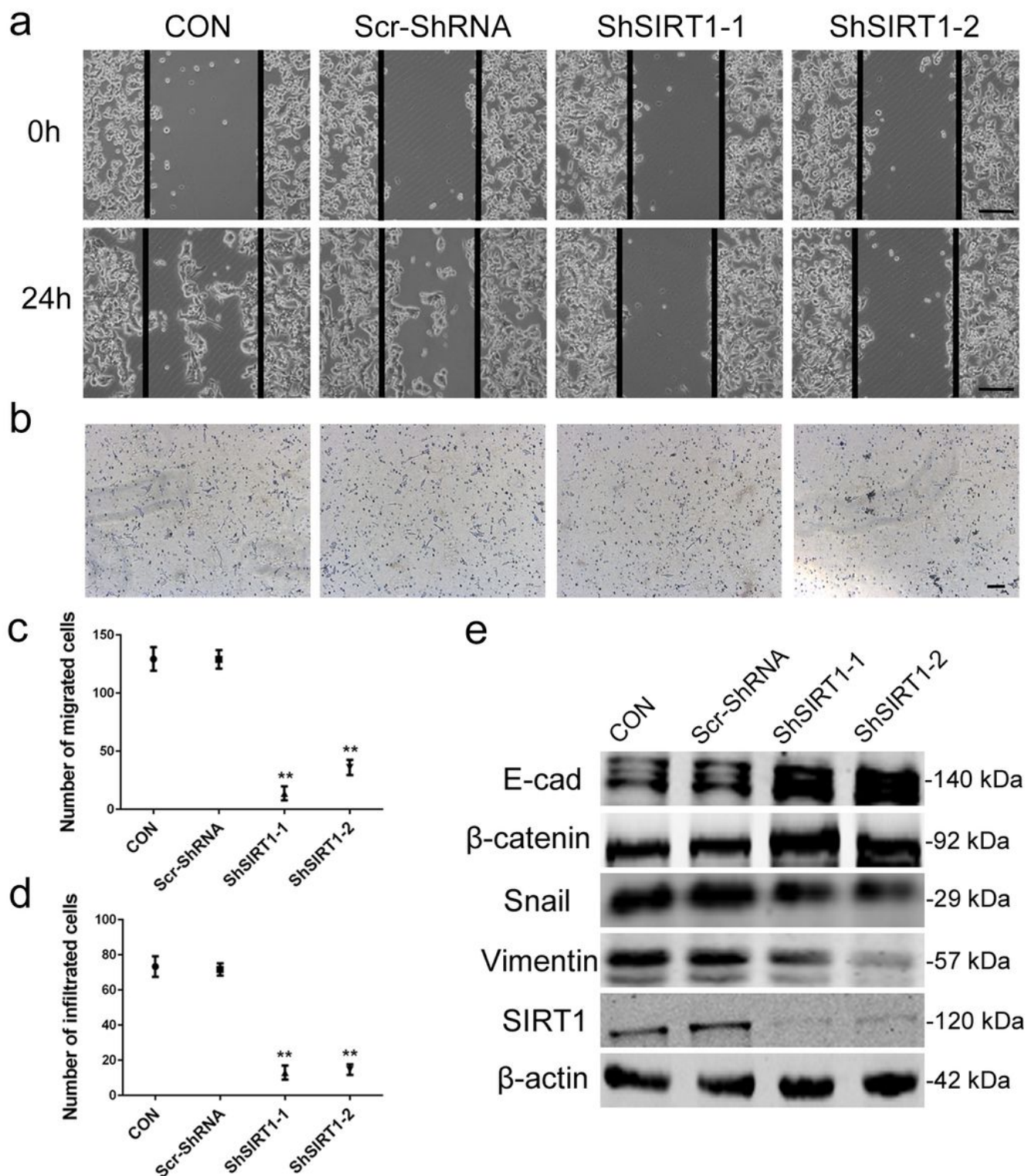
Figure 4

SIRT1 silencing suppressed the migration, invasion and EMT in SW620 cells. a, The wound healing assay of cells. The margins of the wound were denoted by black solid lines. b, The transwell assay of cells. The scale bars of A and B were 500 μm. c, The numbers of cells that migrated to close the wounded area after 24 hours were counted. d, The numbers of cells that travelled from the upper chamber to the lower after

72 hours. e, Western blotting analysis of EMT typical markers, SIRT1 and β-actin. Data were presented as mean ± SEM. Significance: ** P < 0.01, * P < 0.05.
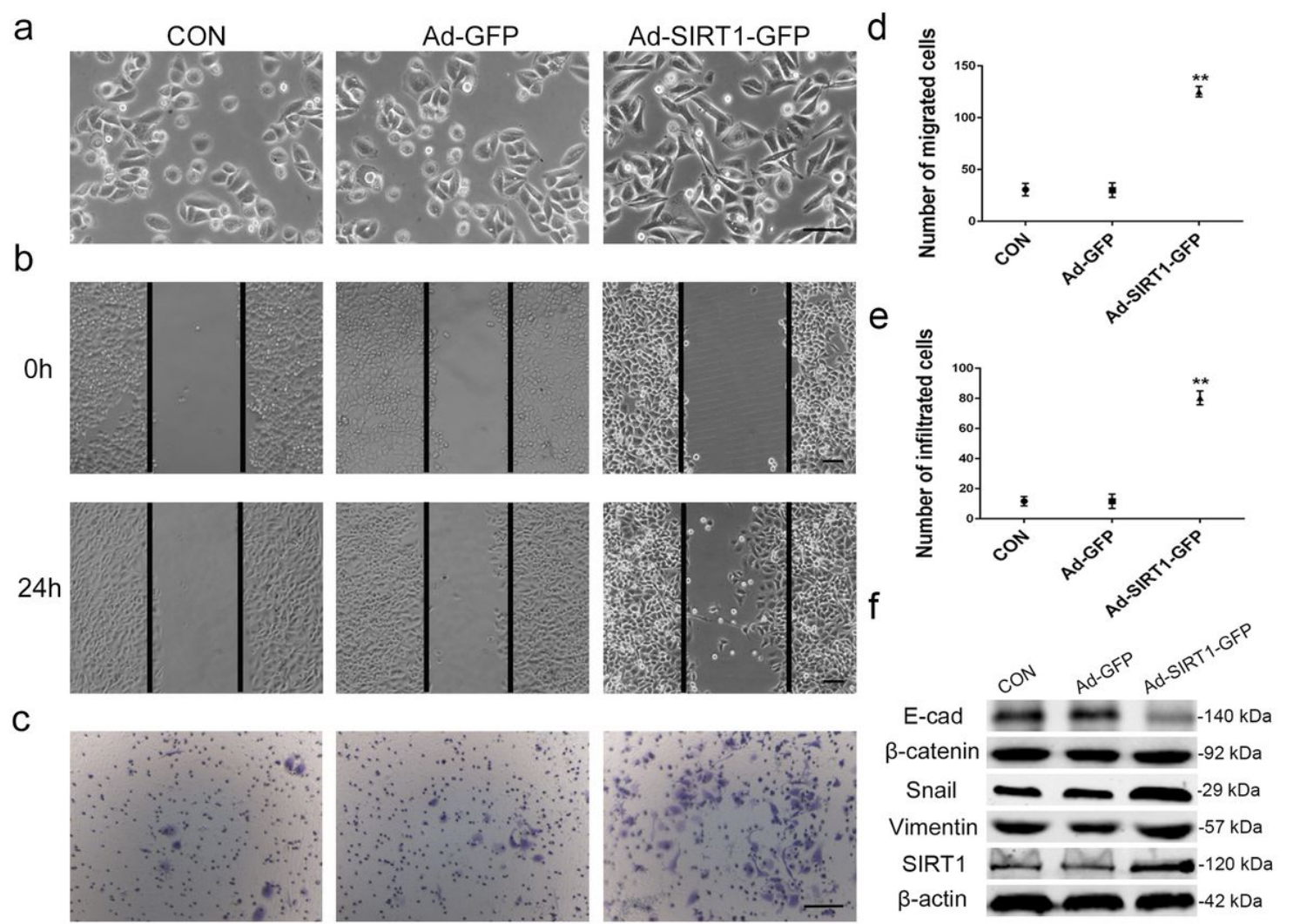
Figure 5

SIRT1 overexpression improved the migration, motility and EMT ability in SW480 cells. a, The morphology of cells showed a more elongated structure after SIRT1 overexpression. b, The wound healing assay of cells. The margins of the wound were denoted by black solid lines. c, The uncoated transwell assay of cells. The scale bars of A, B and C were 500 μm. d, The numbers of cells that migrated to close the wounded area after 24 hours. e, The numbers of cells that travelled from the upper chamber to the lower after 72 hours. f, Western blotting analysis of EMT typical markers, SIRT1 and β-actin. Data were presented as mean ± SEM. Significance: ** P < 0.01, * P < 0.05.
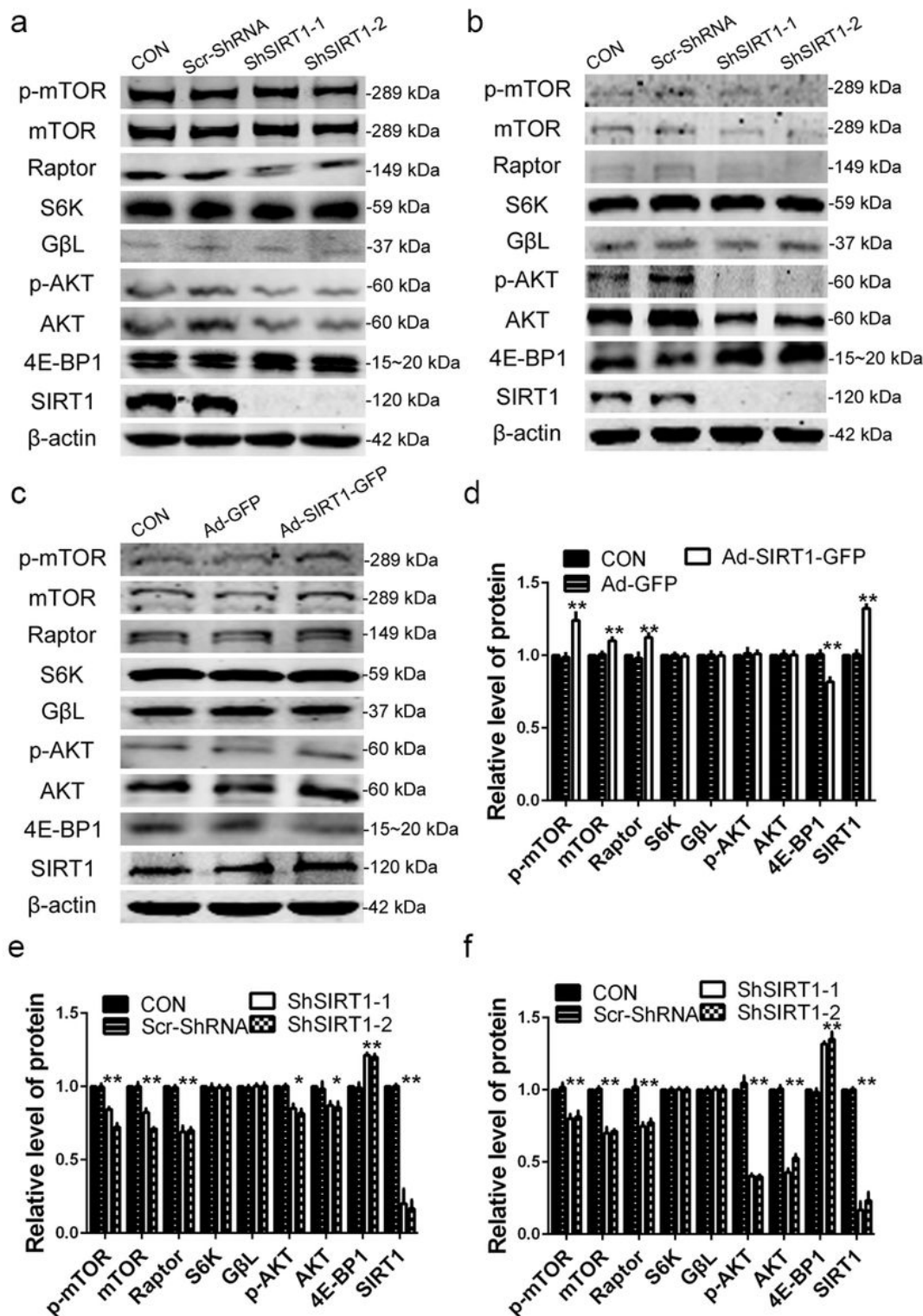
Figure 6

SIRT1 regulated key molecules of mTOR signal pathway. a-c, Western blotting analysis of key molecules of mTOR signal pathway, SIRT1 and β-actin in CT26 cells, SW620 cells, SW480 cells and their derivates. d-f, Quantitative analysis of relative proteins in SW480 cells, CT26 cells, SW620 cells and their derivates. Data were presented as mean ± SEM. Significance: ** P < 0.01, * P < 0.05.
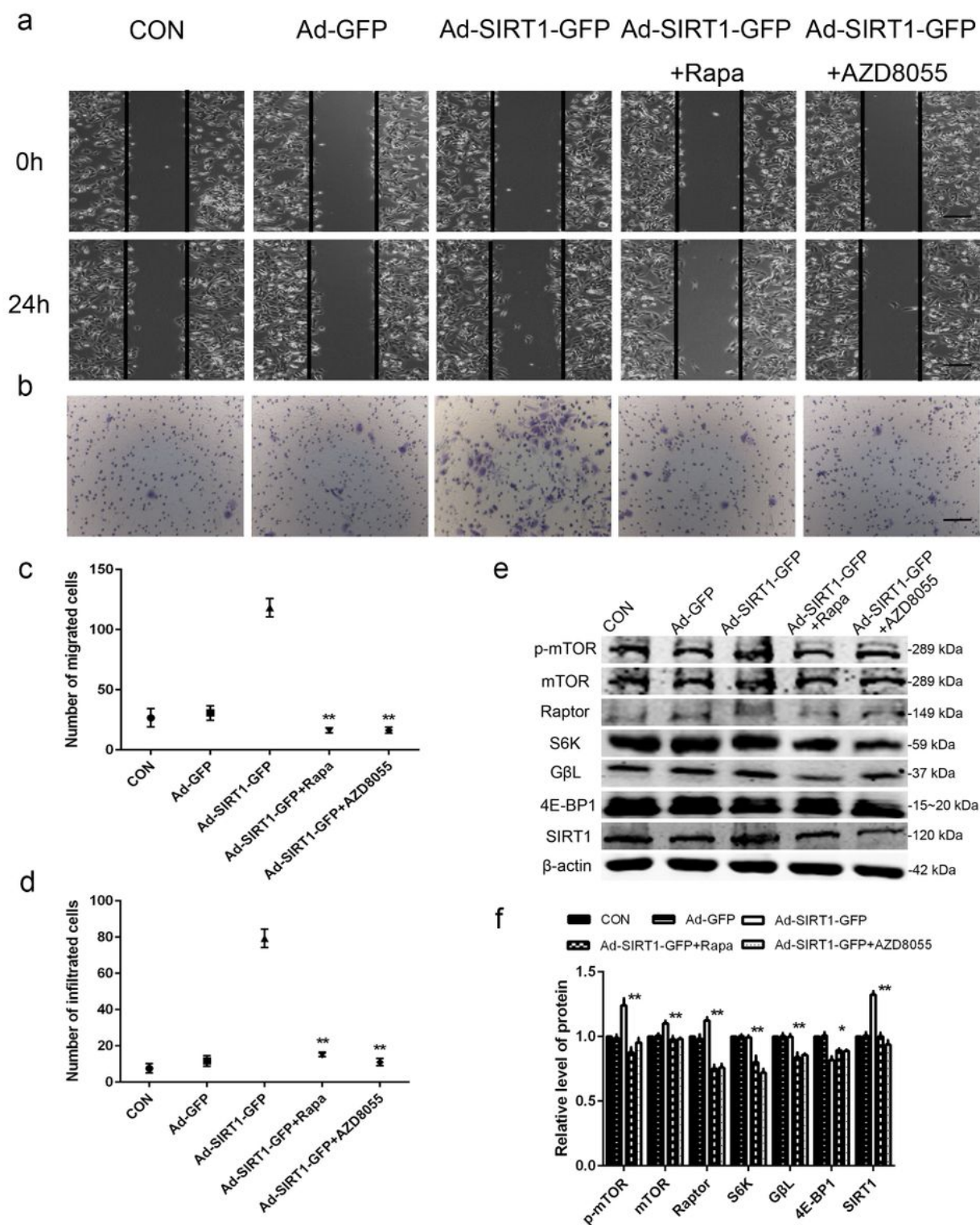
Figure 7

mTOR inhibition repealed the promotion of migration and motility caused by SIRT1 overexpression in SW480 cells. a, The wound healing assay of cells. b, The uncoated transwell assay of cells. The scale bars of A and B were 500 μm. c, The numbers of cells that migrated to close the wounded area after 24 hours. d, The numbers of cells that travelled from the upper chamber to the lower after 72 hours. e, Western blotting analysis of key molecules of mTOR signal pathway, SIRT1 and β-actin. f, Quantitative

analysis of relative proteins in SW480 cells. Data were presented as mean ± SEM. Significance: ** $P < 0.01$, * $P < 0.05$.
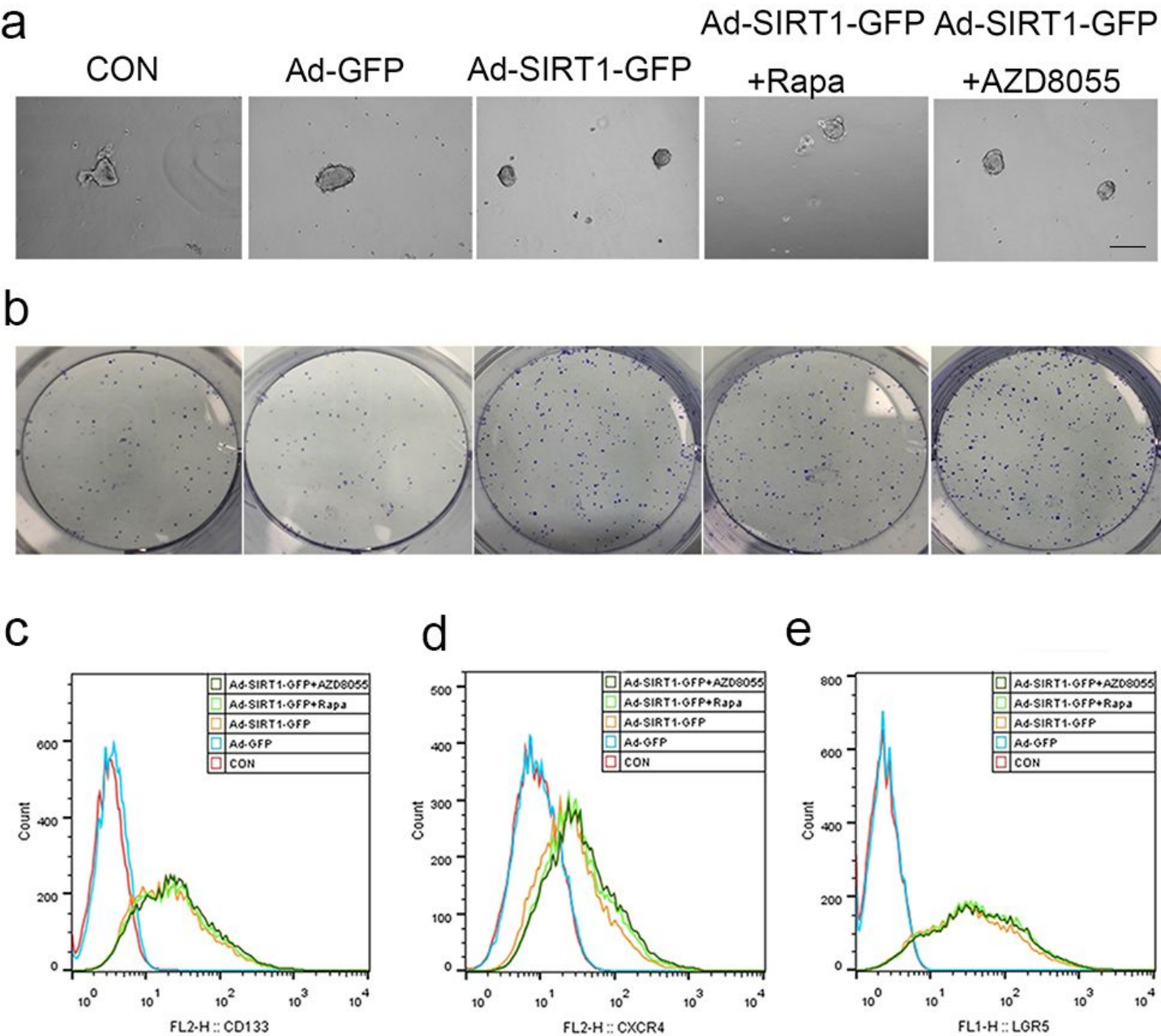

Figure 8

mTOR inhibition failed in reversing the promotion of stemness caused by SIRT1 overexpression. a, The sphere formation assay of the treated and untreated SW480 cells for 14 days. The scale bar was 1000 µm b, The colony formation analysis of the treated and untreated SW480 cells for 14 days. c-e, The expression of CD133, CXCR4 and LGR5 of the treated and untreated SW480 cells detected by flow cytometry.