Effect of *STC2* gene silencing on colorectal cancer cells

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Abstract. Stanniocalcin 2 (STC2), a secretory glycoprotein hormone, regulates many biological processes including cell proliferation, apoptosis, tumorigenesis and atherosclerosis. However, the effect of *STC2* on proliferation, migration and epithelial-mesenchymal transition (EMT) progression in human colorectal cancer (CRC) cells remains poorly understood. The expression level of STC2 was determined by quantitative real-time polymerase chain reaction (qPCR) and western blot analysis. Cell Counting Kit-8 (CCK-8) was used to detect the viability of SW480 cells. The invasion and migration of cells were identified by wound healing and Transwell assays. The mRNA and protein expression levels of β-catenin, matrix metalloproteinase (MMP)-2, MMP-9, E-cadherin and vimentin were assessed by qPCR and western blot analysis. In the present study, it was demonstrated that *STC2* was highly expressed in the CRC cell lines. After silencing of *STC2*, the cell viability, migration and invasion were significantly reduced. Silencing of *STC2* in the CRC Sw480 cells increased the expression of E-cadherin and decreased the expression of vimentin, MMP-2 and MMP-9, compared to those in the normal and empty vector group. Furthermore, the expression of β-catenin in the *STC2* gene silenced group was suppressed, and the expression of β-catenin was reversed by Wnt activator, SB216763. These results demonstrated that *STC2* participates in the development and progression of CRC by promoting CRC cell proliferation, survival and migration and activating the Wnt/β-catenin signaling pathway.

Introduction

According to data released by GLOBOCAN in 2012, the incidence of colorectal cancer (CRC) was the third highest in regards to all male malignant tumors and the second highest in regards to female malignant tumors (1). Studies have shown that the progression of CRC is a multi-stage, multi-factor process (1-3). Effective inhibition of CRC metastasis is a major clinical issue. It has been found that the invasion and migration of tumors are closely related to epithelial-mesenchymal transition (EMT) (4).

EMT refers to the phenomenon that epithelial cells switch to mesenchymal cells under specific pathological conditions (4). During the evolution of tumor growth, EMT mainly includes changes in reconstruction of the cytoskeleton, cell polarity, loss of adhesion between cells, destruction of the tumor basement membrane and extracellular matrix, resulting in high migration and invasion capacity, anti-apoptosis and degradation of interstitial phenotypic characteristics such as the extracellular matrix (5). Studies have shown that matrix metalloproteinase (MMP) family genes, especially *MMP-2* and *MMP-9*, can alter the microenvironment of epithelial cells by remodeling of the matrix membrane and extracellular matrix, downregulating E-cadherin expression, upregulating vimentin expression and promoting EMT progression (6). This process is usually triggered by a variety of growth factors (such as epidermal growth factor) to activate the cascade of signal transduction pathways such as Wnt, resulting in enhanced anti-apoptotic and invasive capacities of tumor-initiating cells, ultimately leading to tumor development (7-9).

One of the most important signaling pathways for regulating EMT is the β-catenin-dependent classical Wnt signaling pathway (10). β-catenin is an intercellular adhesion molecule, mainly located in the cell membrane, which mediates intercellular adhesion and participates in gene expression. However, the adhesion activity will disappear once β-catenin is translocated to the cell nucleus or degraded (11). Overexpression of β-catenin is also a major manifestation of activation of signaling pathways (12). β-catenin is a key regulator of the Wnt/β-catenin signaling pathway. Wnt proteins bind to their receptors, which trigger intracellular signal transduction, and lead to β-catenin accumulation within the cell which translocates into the nucleus and interacts with transcription factors (such as TCF) to activate the transcription of the target genes such as *c-Myc* and *cyclin D1* (13,14). Research has confirmed that the Wnt signaling pathway activates genes related to the MMP family and leads to the development of EMT (15).

Stanniocalcin 2 (STC2) is a secreted glycoprotein that is expressed in a variety of tissues and organs and functions in an autocrine or paracrine manner (16). Recent experiments have shown that *STC2* plays an important role in the development

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of cancer including CRC (17,18). However, to the best of our knowledge, whether or not *STC2* regulates the Wnt signaling pathway in CRC has not yet been studied. In the present study, CRC cell line SW480 was used as the main research tool to investigate whether or not the effect of *STC2* on CRC proliferation, migration and EMT is related to the Wnt signaling pathway.

Materials and methods

Reagents. Human CRC cell lines (SW480, SW620, HT29, LoVo and HCT116) and human normal colorectal mucosal FHC cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). SB216763 was purchased from Sigma-Aldrich (280744-09-4; HPLC >98%; Merck KGaA, Darmstadt, Germany). CCK-8 assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Transfection reagent Lipofectamine® 3000 was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell culture and transfection. Human CRC cell lines (SW480, SW620, HT29, LoVo and HCT116) and human normal colorectal mucosal cells (FHC) were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% (v/v) fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. The non-specific control small interference RNA (siRNA) and siSTC2 were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siSTC2 was inserted into the pSUPER vector (OligoEngine, Inc., Seattle, WA, USA), and the empty pSUPER vector was used as a negative control. Lipofectamine® (LFN) transfection (Thermo Fisher Scientific, Inc.) was performed according to the manufacturer's instructions. In brief, the lipid complex was prepared by combining 4 μl of the reagent LFN Plus with 2 μg of plasmid DNA suspended in 1 ml serum-free medium and incubated at room temperature for 15 min. The solution was then mixed with 40 μl of LFN in serum-free medium and further incubated at room temperature for 15 min. Lipid compounds will first be diluted in serum-free medium to produce a required concentration for 5 ml volume, and then incubated with the cells in an incubator with 5% CO_2 at 37˚C under 24 h for subsequent experimentation.

Cell viability assay. Having transferred the SW480 cells at 0, 12, 24 and 48 h post-culture, 10 μl of CCK-8 solution was added into the wells and the cells were incubated at 37˚C for 2 h in an incubator with 5% CO_2 in the dark. Subsequently, the optical density (OD) of each well in the different cell groups at an absorbance of 450 nm was determined using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was detected using the CCK-8 assay kit according to the manufacturer's protocol.

Wound healing assay. Having transferred the SW480 cells for 24 h, a straight gap was created using a 200-μl sterile tip in the middle of the well. The cells were washed with Dulbecco's modified Eagle's medium (DMEM) for 2 times for smoothing the edges of the scratch and removing floating cells. After being incubated in an incubator (37˚C, 5% CO_2) for 0 and 24 h, the images of the migrating cells were observed under a digital microscope (Keyence Corp., Osaka, Japan), and the distance of cell migration was measured by Image-Pro Plus Analysis software version 7 (Media Cybernetics, Inc., Rockville, MD, USA). The experiment was repeated 3 times and the results are presented using mean values.

Transwell assay. A Transwell chamber with 8-μm pores (3413; Corning Inc., Corning, NY, USA) was placed on a 24-well plate with a layer of 50 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated onto the Transwell chamber. The SW480 cells were cultured in serum-free medium for 12 h to eliminate the effects of the serum and then resuspended in DMEM containing bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) with free FBS. A total of 100 μl of the suspended cells was added to the Transwell chamber, and 400 μl of DMEM containing 20% FBS was added to the basolateral chamber. The cells were cultured for 24 h at 37˚C in an incubator with 5% CO_2. The Transwell chamber was removed and the culture solution in the Transwell was discarded and washed with calcium-free phosphate-buffered saline (PBS) for 2 times. Subsequently, the chamber was fixed in methanol solution for 30 min and stained with 0.1% crystal violet for 20 min at room temperature. Next, the chamber was washed several times with PBS, and the upper chamber liquid was aspirated. The non-migratory cells in the upper layer were gently wiped off using a cotton swab. The microporous membrane was carefully removed using small tweezers, dried with the bottom side up and then transferred to a glass slide and sealed using a neutral gum. Images were observed and collected by an inverted optical microscope (Keyence Corp.).

Western blot analysis. The SW480 cells were washed twice with PBS and added to protein lysis buffer (RIPA; Cell Signaling Technology, Inc., Danvers, MA, USA) for 2 h on ice, centrifuged at 12,000 x g for 30 min at 4˚C, and finally the supernatant was collected. The protein concentration was tested using the BCA protein kit (Bio-Rad Laboratories) and adjusted to a concentration of 5 μg/μl using 1X loading and diethylpyrocarbonate (DEPC) water. A total of 6 μl (at least 30 μg) of the samples were electrophoresed (80 V for 30 min and then transferred to 120 V for 1.5 h) on 10% running gels, which afterwards, was transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories) on ice for 110 min at 110 V. The membranes were blocked with 5% non-fat milk and eluted 3 times with TBS for 5 min each time. The bands were then incubated overnight with the corresponding primary antibody, washed with TBS 3 times for 15 min, incubated with secondary antibodies [horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit IgG (dilution 1:2,000; cat. nos. sc-516102 and sc-2357; Santa Cruz Biotechnology, Inc. Dallas, TX, USA)] for 2 h at room temperature, washed with TBS 3 times for 15 min each time, and furthermore washed once with TBS/0.1% Tween-20 (TBST) for 15 min. Development was carried out with a developer (EZ-ECL kit; Biological Industries Ltd., Kibbutz Beit Haemek, Israel), and the gray-scale value of the strips were analyzed and determined by ImageJ (version 5.0; Bio-Rad Laboratories). The antibodies used in the present study were as follows: Anti-GAPDH (mouse;

Table I. Primers for qPCR.

Genes	Forward	Reverse
STC2	5'-ATGCTACCTCAAGCACGACC-3'	5'-TCTGCTCACACTGAACC TGC-3'
E-cadherin	5'-AAGGCACAGCCTGTCGAAGCA-3'	5'-ACGTTGTCCCGGGTGTCATCCT-3'
Vimentin	5'-TGCCCTTAAAGGAACCAATGAG-3'	5'-AGGCGGCCAATAGTGTCTTG-3'
MMP-2	5'-AGTTTCCATTCCGCTTCCAG-3'	5'-CGGTCGTAGTCCTCAGTGGT-3'
MMP-9	5'-GTCCACCCTTGTGCTCTTCC-3'	5'-GACTCTCCACGCATCTCTGC-3'
β-catenin	5'-ATGCGGCTGCTGTTCTATTC-3'	5'-ACCAATGTCCAGTCCGAGAT-3'
GAPDH	5'-ACTTTGGTATCGTGGAAGGACTCAT-3'	5'-GTTTTTCTAGACGGCAGGTCAGG-3'

qPCR, quantitative PCR; STC2, stanniocalcin 2.

dilution 1:1,000; cat. no. sc-47724; Santa Cruz Biotechnology), anti-STC2 (rabbit; dilution 1:1,000; cat. no. ab63057; Abcam, Cambridge, MA, USA), anti-E-cadherin (mouse; 1:1,000; ab1416; Abcam), anti-vimentin (rabbit; dilution 1:1,000; cat. no. ab92547; Abcam), anti-MMP-2 (rabbit; dilution 1:1,000; cat. no. ab37150; Abcam), anti-MMP-9 (rabbit; dilution 1:1,000; cat. no. ab73734; Abcam).

RNA isolation and real-time PCR. The cell culture medium in each well was aspirated as much as possible, and 1 ml of TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the SW480 cells. The cells were placed horizontally for a while and blown evenly. The cells containing the lysate were transferred to a 1.5-ml EP tube and allowed to stand at room temperature for 5 min. Chloroform (200 μl) was added to each tube and inverted for 15 sec. After emulsification, the cells were allowed to stand for 5 min. After being centrifuged at 12,000 x g for 15 min at 4˚C, the upper aqueous phase was pipetted into a new 1.5 ml of EP and an equal volume of isopropanol (~400 μl) was added to each tube and allowed to stay at room temperature for 10 min. After being centrifuged at 12,000 x g for 15 min at 4˚C, the supernatant was discarded and 1 ml of pre-cooled 75% ice ethanol was added. After being centrifuged at 7,500 x g for 10 min at 4˚C, the supernatant was discarded. An appropriate amount of DEPC (20 μl) was added to dissolve the RNA. The purity and concentration of RNA were tested at 260/280 nm using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). According to the program provided by the manufacturer (Thermo Fisher Scientific, Inc.), a reverse transcription cDNA kit was used to reverse transcribe 1 μg total RNA for synthesis of cDNA (at 42˚C for 60 min, at 70˚C for 5 min, at 4˚C for preservation). SYBR-Green PCR Master Mix (Roche Diagnostics, Basel, Switzerland) was used to perform quantitative real-time polymerase chain reaction (qPCR) experiment using Opticon Real-Time PCR Detection System (ABI 7500; Life Technologies; Thermo Fisher Scientific, Inc.). The PCR cycle was as follows: Pretreatment at 95˚C for 10 min; followed by 40 cycles at 94˚C for 15 sec, at 60˚C for 1 min, finally at 60˚C for 1 min and at 4˚C for preservation. The relative mRNA quantity was determined using comparative cycle threshold ($\Delta\Delta$Cq) method (19). GAPDH expression was used for normalization. The primer sequences used for RT-qPCR analysis are listed in Table I.

Statistical analyses. Data are presented as the means ± standard deviation (SD). Differences between the multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Differences were analyzed by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and all comparisons with a $P<0.05$ were considered statistically significant.

Results

STC2 is highly expressed in CRC cell lines. To select a suitable CRC cell line, qPCR (Fig. 1A) and western blot analysis (Fig. 1B) were performed to analyze the expression level of STC2 in the cell lines. We discovered that STC2 was highly expressed in CRC cell lines (SW480, SW620, HT29, LoVo and HCT116), particularly in SW480 cells. Thus, SW480 cells were selected for subsequent experiments.

Viability, invasion and migration potentials of the CRC cells are suppressed by siSTC2. In the present study, the efficiency of siSTC2 transfection was confirmed by qPCR and western blot analysis. We found that STC2 was silenced by plasmid transfection (Fig. 2A and B). Subsequently, we examined the viability, invasion and migration of the cells after plasmid transfection, and found that the cell viability was lower in the siSTC2 group than that in the control or NC group after 24 and 48 h of plasmid transfection (Fig. 2C), and that the abilities of migration (Fig. 2D) and invasion (Fig. 2E) were decreased in the siSTC2 group, compared to the control or NC group. The wound closure gap in the siSTC2 group was significantly wider than that in the control and NC group at 24 h indicating reduced migration ability (Fig. 2F). As shown in Fig. 2G, the rate of invasion in the siSTC2 group was significantly lower than that in the control and NC group.

siSTC2 downregulates the expression of vimentin, MMP-2 and MMP-9 and upregulates the expression of E-cadherin in CRC cells. It has been reported that EMT is an important marker of CRC development (20). The results showed a significant increase in the expression of E-cadherin (Fig. 3A) and a reduction in the expressions of vimentin (Fig. 3B), *MMP-2* (Fig. 3C) and *MMP-9* (Fig. 3D) in the SW480 cells following silencing of STC2. The protein level of E-cadherin (Fig. 3E) was upregulated and vimentin (Fig. 3E), MMP-2 (Fig. 3E) and

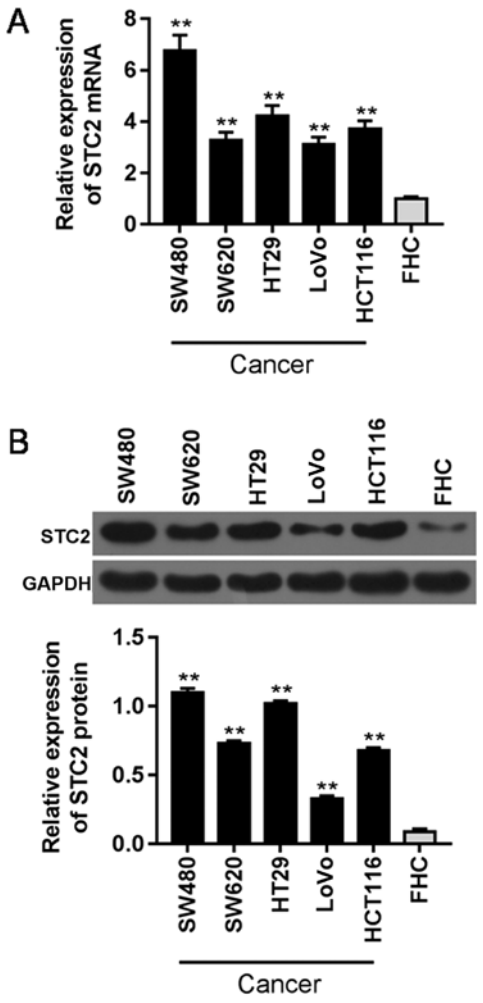

Figure 1. *STC2* is highly expressed in CRC cell lines. (A) Quantitative real-time polymerase chain reaction (qPCR) was used to detect the mRNA level of STC2 in CRC cell lines. (B) The protein level of *STC2* in CRC cells lines was assessed by western blot analysis. All data are expressed as means ± SEM. **P<0.01 vs. the FHC cell line. *STC2*, stanniocalcin 2; CRC, colorectal cancer.

MMP-9 (Fig. 3E) were downregulated in the siSTC2 group, compared to control group.

β-catenin is suppressed by siCST2, and SB216763 treatment reverses this effect. In the present study, the expression of β-catenin was significantly decreased by siSTC2 at the mRNA (Fig. 4A) and protein level (Fig. 4B). SB216763 (10 μM), a Wnt activator, reversed the inhibitory effect of siSTC2 on β-catenin at the mRNA (Fig. 4C) and protein levels (Fig. 4D), and it also reversed the inhibitory effect of siSTC2 on cell proliferation (Fig. 4E).

Discussion

One of the most common malignancies in the clinic is colon cancer, the incidence of which is the third highest worldwide (21). It is highly important to identify new colorectal cancer (CRC) treatment targets. It has been reported that stanniocalcin 2 (STC2) is involved in the progression of various types of cancer, such as gastric and breast cancer (22,23). The differential expression levels of *STC2* have certain guiding significance for the prediction, metastasis and prognosis of various malignant tumors (24). Studies have shown that *STC2* is involved in the development of CRC (25,26). However, its mechanism of action is not well understood. Therefore, the present study aimed to further explore the mechanism of action of *STC2* in CRC.

Studies have found that the expression level of *STC2* in CRC patients is higher than that in normal tissues, and that the expression level of *STC2* is related to tumor size and pathological grade (25,27,28). In the present study, it was demonstrated that *STC2* was obviously increased in CRC cell lines. The results indicated that *STC2* may play a vital role in the occurrence and development of CRC. Studies have shown that abnormal proliferation, invasion and migration of tumor cells are common conditions in malignant tumors (29-32). Therefore, the ability of tumor invasion and migration is a detrimental biological feature of malignant tumors, and it is also the most prominent clinical manifestation of malignant tumors (33). It has been found that overexpression of *STC2* promotes CRC tumorigenesis by activating the AKT/ERK signaling pathway (26). In the present study, we found that silencing of STC2 reduced the cell viability and the migration and invasion abilities, when compared to the control or NC group. These results suggested that STC2 silencing may inhibit the progression of CRC. One study has shown that epithelial-mesenchymal transition (EMT) plays a vital role in tumor invasion and migration (34). Therefore, we explored the effect of *STC2* on EMT.

Previous studies on EMT have focused on cancer metastasis profiles (35,36). In fact, the progression of adenocarcinoma from normal intestinal mucosa to adenoma (adenoma mucosa) and eventually to CRC is closely correlated with the EMT process, and the expression changes in a series of genes, such as E-cadherin and vimentin (37,38). MMPs are a group of Zn^{2+}-dependent endopeptidase that can degrade the extracellular matrix (ECM), and promote tumor invasion and migration, proliferation, differentiation and apoptosis of tumor cells (39). Degradation of the extracellular matrix mainly involves the matrix metalloproteinase family, particularly *MMP-2* and *MMP-9* (40-42). It has been reported that *MMP-2* and *MMP-9* are vital for the metastasis of CRC (43). We hypothesized that knockdown of STC2 would inhibit EMT. Therefore, we investigated the biomarkers of human colon mucosal epithelial cells. Chen *et al* found that STC2 overexpression could promote the occurrence of EMT in CRC *in vitro* and *in vivo* (26). Similarly, we found that silencing of STC2 upregulated E-cadherin expression and downregulated vimentin and MMP-2/-9 expression. Our findings confirmed that the EMT process mediated by *STC2* was associated with the occurrence and development of colon cancer.

Studies have shown that the main signaling pathways involved in the process of EMT are the Wnt/β-catenin signaling pathway (44,45). As the Wnt/β-catenin signaling pathway is widely involved in cell proliferation, differentiation and metabolism (46), we explored the STC2-mediated Wnt/β-catenin molecular signaling pathway and found that the expression of β-catenin was decreased in the STC2-silenced SW480 cells. β-catenin is a central molecule of the Wnt signaling pathway. In the presence of Wnt signal stimulation,

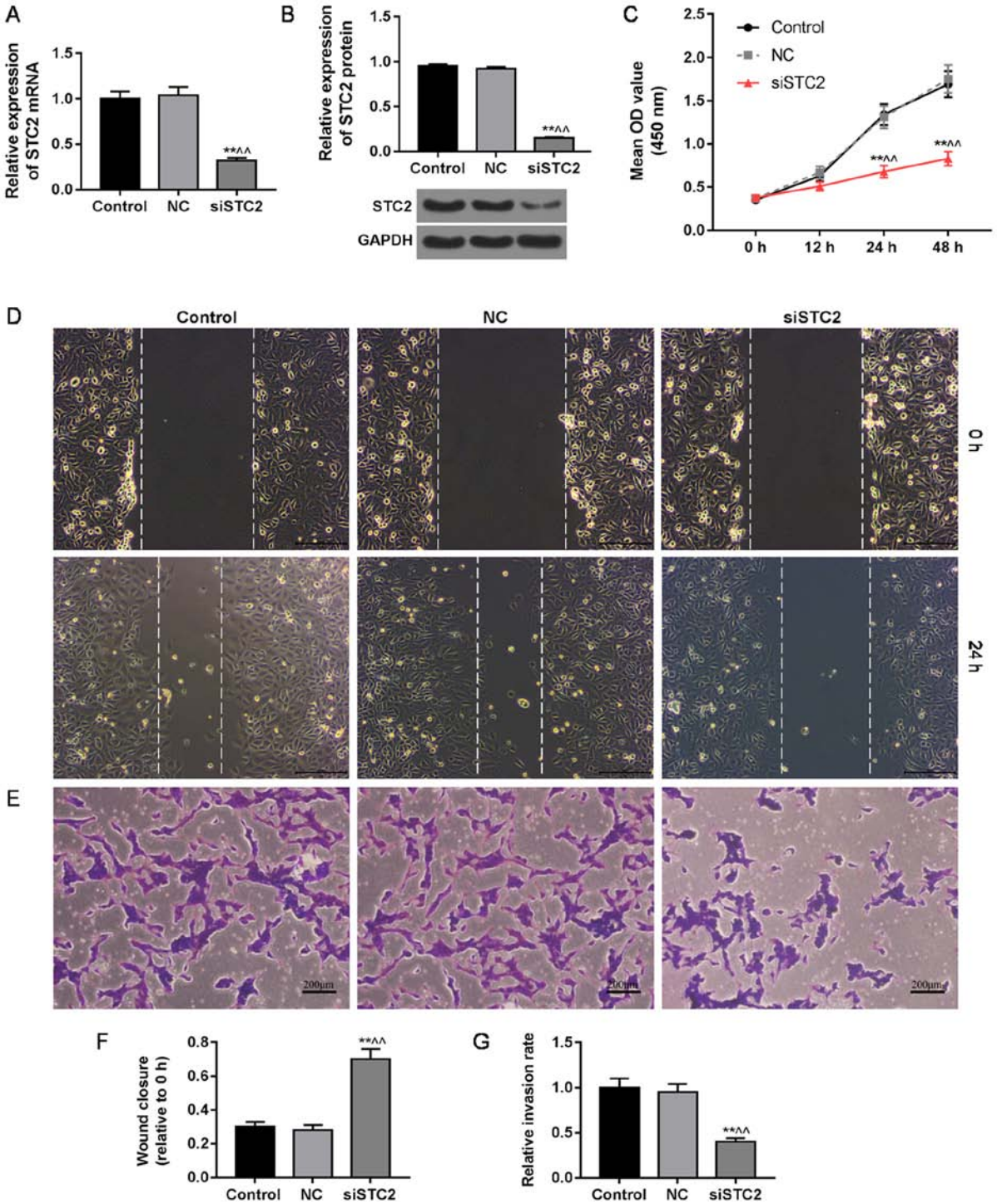

Figure 2. Silencing of *STC2* displays an anticancer effect on CRC SW480 cells. (A) Quantitative real-time polymerase chain reaction (qPCR) and (B) western blot analysis were used to evaluate the efficiency of siSTC2 transfection. (C) The cell viability was identified by CCK-8 assay. The migration (D) and invasive (E) abilities were assessed by wound healing and Transwell assays. (F) The relative wound closure distance in the control, NC and siSTC2 groups. (G) The invasion rate of the control, NC and siSTC2 groups. All data are expressed as means ± SEM. **P<0.01 vs. control; $^{\wedge\wedge}$P<0.01 vs. NC. *STC2*, stanniocalcin 2; CCK-8, Cell Counting Kit-8.

Fratl mediates glycogen synthetase kinase 3β (GSK-3β) dissociation from Axin, and prevents the phosphorylation of β-catenin. β-catenin is not degraded and accumulates in the cytoplasm. Inhibition of the Wnt/β-catenin signaling pathway in colon cancer has been found to alleviate the degree of deterioration of colon cancer (47-49). In the present study, with the activation of the Wnt/β-catenin signal pathway, the Wnt activator SB2617763 was found to reverse the inhibitory effect of siSTC2 on Wnt/β-catenin and cell proliferation. SB216763 has been shown to specifically inhibit GSK-3α and GSK-3β, thereby increasing the level of dephosphorylation (active) of β-catenin (50,51). Briefly, these findings suggest that Wnt/β-catenin may be involved in the regulation of CRC following silencing of STC2.

In conclusion, it was demonstrated that *STC2* was highly expressed in CRC cell lines, particularly SW480 cells. STC2

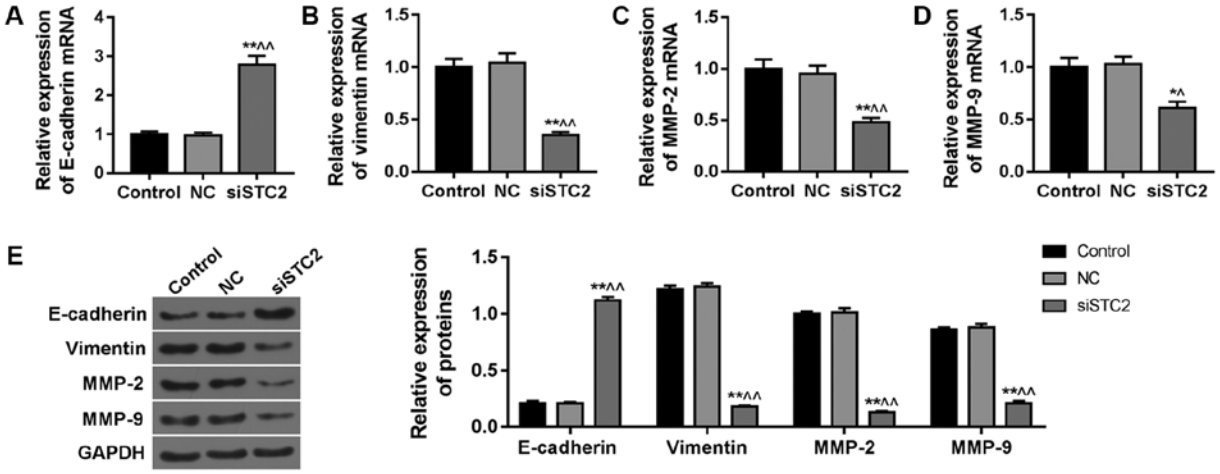

Figure 3. EMT and MMP-2/-9 are suppressed by siSCT2. (A) E-cadherin was increased, while (B) vimentin, (C) *MMP-2* and (D) *MMP-9* were decreased in the siSTC2 group, compared to control or NC group as detected by quantitative real-time polymerase chain reaction (qPCR). (E) The proteins levels of E-cadherin, vimentin, MMP-2 and MMP-9 were assessed by normalization to GAPDH. All data are expressed as means ± SEM. *P<0.05, **P<0.01 vs. control; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 vs. NC. EMT, epithelial-mesenchymal transition.

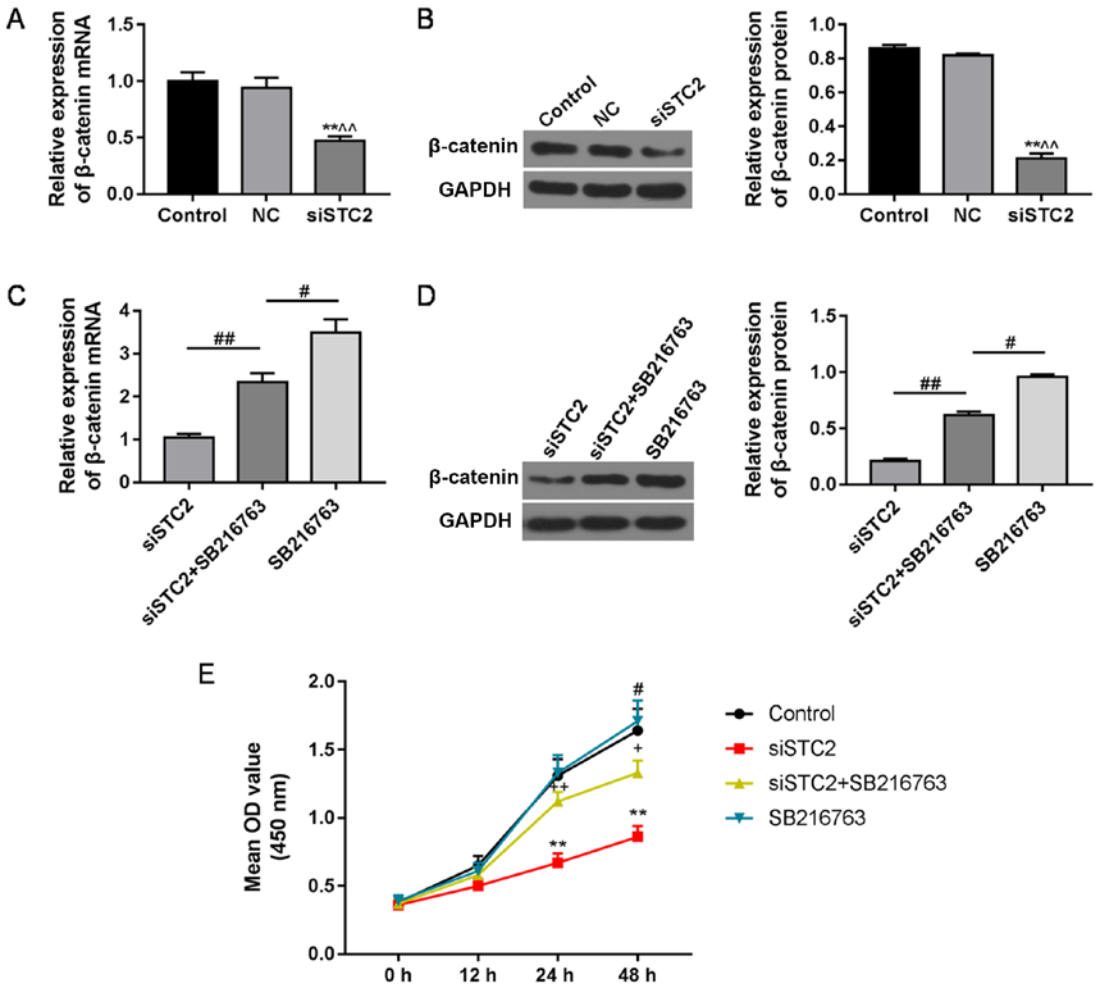

Figure 4. Wnt/β-catenin is suppressed by siCST2, and SB216763 reverses the inhibitory effect of siSTC2. β-catenin was tested by quantitative real-time polymerase chain reaction (qPCR) and western blot analysis at the (A) mRNA and (B) protein levels. SB216763, a Wnt activator, reversed the inhibitory effect of siSTC2 on the Wnt/β-catenin pathway at the (C) mRNA and (D) protein levels. (E) SB216763 reversed the inhibitory effect of siSTC2 on cell proliferation. All data are expressed as means ± SEM. **P<0.01 vs. control; $^{\wedge\wedge}$P<0.01 vs. NC; $^{\#}$P<0.05 vs. siSTC2+SB216763; $^{\#\#}$P<0.01 vs. siSTC2; $^{+}$P<0.05, $^{++}$P<0.01 vs. siSTC2.

silencing inhibited cell viability, invasion and migration, compared to the control cells. Meanwhile, E-cadherin was increased, while vimentin, *MMP-2* and *MMP-9* were decreased in the CRC cells by siSTC2. Unsurprisingly, β-catenin was

suppressed by siSTC2, however, SB216763 reversed the level of β-catenin. Taken together, these data showed that siSTC2 treatment conferred a strong protective effect on CRC. This study suggests that STC2 silencing suppresses the migration of CRC cells and the occurrence of EMT. In addition, the effect of *STC2* on CRC may be related to the Wnt/β-catenin signaling pathway. These results provide novel insight into the identification of targeted therapeutic strategies for CRC. However, the present study also has some limitations. Due to the limited experimental funds, we only explored the effect of *STC2* on the proliferation, migration and invasion of the CRC SW480 cell line. Whether or not STC2 has the same effect on other CRC cell lines remains to be studied, and we will further conduct gain-of-function experiments in regards to *STC2* in future studies. In addition, it is necessary to investigate the role of the Wnt/β-catenin pathway in the STC2-mediated effects, so as to better understand the mechanism of *STC2* in CRC.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QL substantially contributed to the conception and design of the study. XZ and ZF acquired the data, analyzed and interpreted the data. QL and ZP drafted the manuscript or critically revised it for important intellectual content. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No human or animals were involved in this research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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