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Original Article

ZNF516 suppresses stem cell-like characteristics by regulating the transcription of Sox2 in colorectal cancer

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Abstract: This study aimed to explore the biological function and the molecular mechanism of the action of zinc-finger protein 516 (ZNF516) in suppressing stem cell-like characteristics and tumor progression in colorectal cancer (CRC). The expression profiles of ZNF516 in clinical samples and from The Cancer Genome Atlas (TCGA) CRC database were analyzed. Cell transfection was used to overexpress and knockdown ZNF516 in CRC cells. Cell counting kit-8 (CCK8) assays, transwell assays and flow cytometry were used to study cell proliferation, invasion and stem cell-like characteristics, respectively. Cycloheximide (CHX) was used to examine the effect of ZNF516 expression on Sox2 degradation. Finally, the effects of ZNF516 on tumor growth and metastasis were tested on xenograft tumor models and lung metastasis models in immunocompromised mice. We found that the expression level of ZNF516 was lower in TCGA CRC tissue and clinical CRC samples compared with that in normal colorectal mucosal cells. Overexpression of ZNF516 in CRC cells inhibited cell proliferation, colony formation, migration and invasion, whereas ZNF516 knockdown showed the opposite effects. In addition, ZNF516 overexpression inhibited the sphere-forming ability of CRC cells and suppressed the expression of CD133, CD44 and Oct4 in CRC cells. ZNF516 decreased the stability of Sox2 through a mechanism mediated by EGFR. By *in vivo* experiments using mouse tumor models, we further confirmed that ZNF516 attenuated tumor growth and alleviated lung metastasis in mice. In conclusion, ZNF516 functions as a tumor suppressor by regulating the transcription of Sox2 to inhibit cell proliferation, invasion, and the development of stem cell-like characteristics in CRC cells.

Keywords: ZNF516, colorectal cancer, Sox2, stem cell-like characteristics, tumor progression

Introduction

Colorectal cancer (CRC) is a malignant tumor of the digestive tract; it is the third most commonly diagnosed malignant tumor in the world [1] and has the fifth highest mortality rate among malignant tumors. The aetiology of colon cancer is unclear; its pathogenesis is very complex, and no early-stage symptoms for this disease have been characterized [2, 3]. Surgical removal of the tumor is the main method of treatment and is highly effective in treating early-stage tumors, while tumors at late and advanced stage are difficult to treat and often relapse and metastasize after surgery [4, 5]. For patients who cannot be operated upon, comprehensive chemotherapy-based treatments are available. Several biomarkers for CRC have been reported, which are involved in tumorigenesis, tumor development, differentiation, metastasis and drug resistance [6, 7]. For example, TP53 (tumor protein p53), APC (adenomatous polyposis coli), GATA4 (GATA binding protein 4), HIC1 (hypermethylated in cancer 1), LTGC (long-tract gene conversion), SFRP1 (secreted frizzled-related protein 1), and SFRP2 (secreted frizzled related protein 2) are reported to be important biomarkers for the diagnosis of CRC [8, 9].

Previous studies have reported that there exit a small population of tumor cells with stem cell-like properties, known as cancer stem cells or cancer stem-like cells now, in cancer tissues [10, 11]. These cells can self-renew, differentiate, and have strong oncogenic potential; they are often considered to be the origin of new cancerous tissues during recurrence [12, 13].

Better understanding of the characteristics of these cancer stem cell should be beneficial for the development of novel cancer stem cell-targeting drugs. Since these cells were first isolated, cloned, and propagated *in vitro* from CRC, CRC cancer stem cells have been widely used in the studies for their stem cell-like or progenitor-like properties, which include sphere-forming, self-renewal and tumorigenic ability [14, 15].

Located on chromosome 18q23, ZNF516 was initially identified and studied for its function in chromosome instability in cervical cancer and was reported to have tumor suppressor activity [16]. It has also been reported that ZNF516 acts as a tumor suppressor in breast and cervical cancers [17, 18]. Ili *et al*. (2019) have reported that decreased ZNF516 expression could be considered as a early sign for cervical cancer [18]. Burrell *et al*. (2013) found that ZNF516 acted as a chromosomal instability suppressor and caused the loss of 18q, as the expression of ZNF516 was correlated with aneuploidy onset during adenoma-carcinoma transitions [19]. Furthermore, ZNF516 has been reported to suppress the transcription of epidermal growth factor (EGF), which is essential for the proliferation, angiogenesis, invasion, metastasis and apoptosis inhibition of breast cancer cells, by interacting with the CtBP/LSD1/CoREST complex [16, 20, 21]. Lv *et al*. (2020) have also discovered that EGFR was overexpressed in head and neck squamous cell carcinomas and enhanced the stemness and progression of oral cancer by inhibiting the autophagic degradation of Sox2 [22].

By analyzing The Cancer Genome Atlas (TCGA) data and performing a series of biochemical and cellular experiments, we found that, in CRC tissues, the expression of ZNF516 was lower than that in normal tissues. We also found that this difference in ZNF516 expression was related to the development of stem cell-like characteristics in CRC cells. Therefore, we hypothesize that ZNF516 can suppress tumor progression by regulating the transcription of Sox2 in CRC. Our study has determined the biological function and the molecular mechanism underlying the action of ZNF516 in suppressing the stem cell-like characteristics and tumor progression in CRC.

Materials and methods

Patient information and tissue collection

Between September 2017 and March 2020, a total of 95 CRC patients (56 male and 39 female patients aged from 42 to 76 years) who underwent resection surgeries at Changhai Hospital, were voluntarily enrolled in this study. Informed written consent from every patient and the approval of the Ethics Committee of Changhai Hospital were both obtained. All patients were diagnosed with CRC for the first time and had no prior history of cancer-related treatment at the time of enrolment. CRC tissues and matched normal tissues obtained from surgeries were immediately stored in liquid nitrogen.

Immunohistochemistry

Tissues were fixed in 4% formalin, dehydrated in an alcohol gradient, embedded in paraffin, and sliced into 4 μm thick sections. The sections were de-waxed, processed for antigen retrieval, and incubated in 5% goat serum for 10 min at room temperature. Subsequently, the sections were incubated with rabbit anti-ZNF516 (ab121486, Abcam, Cambridge, UK), anti-Ki67 (ab15580, Abcam), anti-EGFR (ab52894, Abcam), anti-Sox2 (ab92494, Abcam), and anti-CD133 (ab222782, Abcam) antibodies at 4°C overnight. Each section was washed twice in phosphate-buffered saline (PBS) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature. Following this, 3,3'-diaminobenzidine (DAB) staining was used to visualize ZNF516, Ki67, EGFR, Sox2, CD44, and CD133-expressing cells. The stained sections were observed under an optical microscope (Olympus, Tokyo, Japan).

Cell lines

CRC cell lines (T84, LoVo, SW480, HCT116 and SW620) and foetal human cells (FHCs) established from normal human foetal colorectal mucosal cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Science. FHCs, T84 and SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), while LoVo, HCT116 and SW620 were cultured in RPMI1640 medium, supplemented

with 10% foetal bovine serum (FBS) and penicillin G (100 U/mL) and streptomycin (100 μg/mL) at 37°C and 5% CO_2.

Cell transfection

Cells were trypsinized and cultured overnight to achieve 60-70% confluence before transfection, and then subjected to 6 h transient transfection using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cells were transfected with either pcDNA3.1-ZNF516 construct or empty pcDNA3.1 vector (GenePharma, Shanghai, China) for ZNF516 overexpression experiments. For ZNF516 knockdown experiments, cells were transfected with ZNF516-specific shRNA#1 (5'-GCGGGAACCTGTTTACAAACC-3'), shRNA#2 (5'-GCTAGCATGCCTAAGAATAAG-3'), or scramble negative control shRNA (NC: 5'-CGCCGATGCTGAATACATACG-3') (Genechem, Shanghai, China). For Sox2 overexpression experiments, HCT116 cells were co-transfected with pcDNA3.1 vector and Sox2 control vector (Control + Vector), pcDNA3.1-ZNF516 and Sox2 control vector (ZNF516 + Vector), pcDNA3.1-ZNF516 and pcDNA3.1-Sox2 (ZNF516 + Sox2). The efficiency of transfection was assessed at 48 h after transfection by western blotting.

Cell counting kit (CCK)-8 assay

After transfection, the viability of the ZNF516 and control vector transfected cells was determined by CCK-8 assays per the manufacturer's instruction (Dojindo, Tokyo, Japan). Briefly, 10^4 cells in 100 μL regular medium were plated in the well of 96-well plates and cultured for 1, 2, 3, and 4 days. Each group had 5 replicates, that is, cells from each group were plated in 5 wells. CCK-8 solution (10 μL) was added to each well 4 h before assay. The plates were then agitated on a shaker for 10 min to fully dissolve the formazan crystals formed. The optical density (OD) values at 450 nm were detected by plate reader.

Colony formation assay

Briefly, 24 h after transfection, SW480 and HCT116 cells were resuspended in regular DMEM and RPMI1640, respectively, and 2 ml of them (about 800 cells) were plated in 60 mm dishes and cultured for 10 days. The medium was changed every 2 days. At day 10, the cells were fixed with 4% polyformaldehyde for 10 min and stained with 0.1% crystal violet for 10 min. The number of colony of more than 50 cells was counted under a microscope.

Cell migration and invasion assay

Cell migration and invasion were assessed by Transwell assay in 24 well plates. Cells in serum-free medium at about 1×10^5 cells/200 ul were added to the upper chamber in Transwell. Complete medium of 500 μL was added to the lower chamber. After culture for 24 h, the remaining cells in the upper chamber were gently wiped with a cotton swab. The cells that migrated through the membrane to the lower surface of the chamber were fixed with methanol for 30 min and stained with crystal violet for 20 min. The chamber was placed under the microscope, and the number of cell migration was counted. For cell invasion assay, the upper chamber of Transwell was precoated with Matrigel matrix glue diluted with serum-free medium.

In vivo study of using xenograft tumor model

All animal experiments were approved by the Animal Ethics Committee of Changhai Hospital. Female nude mice (6 weeks old, 25-30 g) were obtained from SIPPR-BK Laboratory Animal Company (Shanghai, China). All mice were maintained in a sterile room at a constant temperature of 25°C with a light/dark cycle of 12 h and had free access to water and food. HCT116 cells (10^5 cells) transfected with either a ZNF516 overexpression construct or an empty vector were injected subcutaneously into the mice. For each group of transfected cells, 6 mice were used (n = 6). After cell injection, each mouse was injected at the original injection site with 30 μg of Lipofectamine 2000-encapsulated ZNF516 overexpression construct or empty vector, as used in the cell transfection, every 3 days [23, 24] (Chen et al., 2020; Liang et al., 2017). Tumor growth was monitored every 7 days for 56 days: the length (a) and breadth (b) of the subcutaneous tumors were measured with a vernier calliper. Tumor volume was calculated as $V = (ab^2 * 0.5)$. At day 56, the mice were euthanized by intraperitoneal injection of pentobarbital sodium. The tumors were dissected, weighed, and prepared for further study.

Isolation of CD133⁺ and CD44⁺ cells by FACS from CRC cell lines and manipulation of ZNF561in these cells by transfection

SW480 and HCT116 cells were resuspended and incubated with anti-CD133-FITC and anti-CD44-PI antibodies for 1 h at 37°C. After washing twice with PBS, the $CD133^+$ and $CD44^+$ cells were separated by flow cytometry and cultured at 37°C and 5% CO_2. In the logarithmic growth phase, these cells were collected and transfected by Lipofectamine 2000 with either ZNF516 overexpression or ZNF516 knockdown constructs. Forty-eight h after transfection, the total number of $CD133^+$ and $CD44^+$ cells in each group was counted by flow cytometry.

Sphere formation assay

Stable transfected SW480 and HCT116 cells were used in sphere formation assay. Overexpression of ZNF516, ZNF516 knockdown or ZNF516 + Sox2 was transfected into SW480 and HCT116 cells via lentiviral transfection. The transfection efficiency was examined by qRT-PCR. After stable transfection, SW480 and HCT116 were resuspended in DMEM/F12 medium supplemented with 2% B27, FGF (20 ng/mL), and EGF (20 ng/mL), and 1000 cells/mL were plated in 35 mm low attachment dishes and cultured in DMEM/F12 medium for 21 days at 37°C and 5% CO_2. The medium was changed every 3 days. The number of spheres >50 mm in diameter was counted under an inverted microscope.

qRT-PCR

Total RNA from tissues/cells was extracted by Trizol reagent (Invitrogen) according to the manufacturer's instructions and transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) per the manufacturer's instructions. The forward and reverse primers used for ZNF516, Sox2, OCT4, CD133 and CD44 were as follow: ZNF516 forward: 5'-GTCTGTGGGAATGCGTAGA-3', reverse: 5'-TGCAGAATGTAGCCCTGTT-3'; Sox2 forward: 5'-CATCCACACTCACGCAAA-3', reverse: 5'-CTCCCCAGGTTTTCTCTGT-3'; OCT4 forward: 5'-TGTCTCCGTCACCACTCTG-3', reverse: 5'-CACCCTTTGTGTTCCCAAT-3'; CD133 forward: 5'-CCGCAGGAGTGAATCTTTT-3', reverse: 5'-CTCGTTGCTGGTGAATTGT-3'; CD44 forward: 5'-CAAGAAGGTGGAGCAAACA-3', reverse: 5'-ACTGCAAGAATCAAAGCCA-3'. The qRT-PCR was performed on a TP800 Real-time PCR machine (Takara, Japan) using the two-step PCR amplification procedure: in the first step, pre-denaturation was carried out at 95°C for 30 s; in the second step, 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s and extension at 72°C for 30 s were performed. The relative expression levels of ZNF516, Sox2, OCT4, CD133 and CD44 in tissues/cells were calculated using the formula $2^{-\Delta\Delta Ct}$; β-actin was used as the internal reference.

Cycloheximide (CHX) treatment

To determine the stability of Sox2, SW480 and HCT116 cells transfected with either a ZNF516 overexpression construct, a ZNF516 knockdown vector, or ZNF516 shRNA#1, with or without gefitinib treatment (10 μg/mL), were incubated with the protein synthesis inhibitor CHX (10 μg/mL, Solarbio, Beijing, China) for 0, 2, 4, 6, and 8 h, and the expression level of Sox2 in these cells was evaluated by western blotting.

Western blot analysis

Cells were harvested and washed three times with cold PBS and lysed with cell lysis buffer on ice for 1 h. Clear supernatants were obtained by centrifugation of the cell lysates, and protein concentration of the supernatants was determined by bicinchoninic acid (BCA) (Beyotime Biotechnology, Shanghai, China). Proteins of 30 μg per sample were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% skim milk in PBS for 2 h at room temperature and incubated overnight with the following primary antibodies: anti-ZNF516 (1:800, YS-12238R, Y-J biological, Shanghai, China); anti-EGFR (1:1000, ab528-94, Abcam), anti-CD133 (1:1000, ab222782, Abcam), anti-Sox2 (1:1000, ab92494, Abcam), anti-CD44 (1:1000, ab189524, Abcam), anti-OCT4 (1:1000, ab200834, Abcam), anti-β-actin (1:1000, ab8226, Abcam) at 4°C. The membrane was washed thrice with TBST (Tris-buffered saline with Tween 20) and then incubated with HRP-conjugated secondary antibodies (Solarbio science & technology co., ltd., Beijing, China) for 2 h at room temperature, followed by washing thrice with TBST. Specific protein bands were visualised using enhanced chemiluminescence (ECL). The intensity of pro-

tein bands was analyzed using the Image J software (National Institutes of Health, Maryland, USA) with β-actin as the internal control.

Statistical analysis

Data from triplicate experiments were used for analysis. All statistical analyses for data in the form of mean ± SD were performed by using appropriate statistical tests in SPSS 19.0. *P-value* <0.05 indicated a statistically significant difference between groups. For comparison involving two groups, Student's t-test was performed; for comparison among 3 or more groups, analysis of variance (ANOVA) followed by a Tukey *post hoc* test was used.

Results

ZNF516 was downregulated in CRC tissue samples

Analysis of TCGA data indicated that the mRNA level of ZNF516 was significantly lower in tumor tissues (n = 275) than in normal tissues (n = 349) (**Figure 1A**, $P<0.05$). To confirm this database analysis result, we collected 95 CRC tissue samples and their matched normal tissue samples from patients. When the mRNA level of *ZNF516* in these samples was examined by qRT-PCR, we found that the expression of ZNF516 was lower in CRC tissues than in the matched normal tissues (**Figure 1B**, $P<0.01$). In addition, we also examined the ZNF516 protein level in these samples by western blotting and immunohistochemistry (IHC). As shown in **Figure 1C** and **1D**, the protein level of ZNF516 was lower in the tumor tissues than that in the normal tissues ($P<0.01$).

ZNF516 inhibited CRC cell proliferation and suppressed the mobility of CRC cells

The protein level of ZNF516 in five CRC cell lines (T84, LoVo, SW480, HCT116, and SW620) was compared with that in normal human colorectal mucosal cells (FHCs) by western blotting (**Figure 2A**). The results showed that CRC cell lines had lower expression levels of ZNF516 than FHCs. HCT116 and SW480 cells exhibited moderate expression level of ZNF516 among the five CRC cell lines examined and were used for our further overexpression and knockdown experiments. **Figure 2B** showed the western blotting results of ZNF516 overexpression and knockdown in HCT116 and SW480 cells by transfecting ZNF516 expression or ZNF516 shRNAs constructs (ZNF516 sh#1 and sh#2), respectively, ($P<0.01$). CCK-8 and colony formation assay revealed that ZNF516 overexpression inhibited cell proliferation and colony-forming ability, whereas ZNF516 knockdown promoted cell proliferation and colony-forming ability in both HCT116 and SW480 cells (**Figure 2C** and **2D**, $P<0.05$). Transwell assays showed that knockdown of ZNF516 enhanced the migration and the invasion of CRC cells, whereas ZNF516 overexpression had the opposite effects (**Figure 2E** and **2F**, $P<0.05$).

ZNF516 suppressed stem cell-like characteristics in CRC cells

For sphere formation assay, stable transfected SW480 and HCT116 cells by lentiviral transfection were obtained, and the transfection efficiency was examined by qRT-PCR (Figure S1A). As shown in **Figure 3A**, ZNF516 overexpression inhibited the sphere formation in HCT116 and SW480 ($P<0.01$), whereas ZNF516 knockdown promoted the sphere formation ($P<0.05$). **Figure 3B** showed that ZNF516 overexpression reduced the number of $CD133^+$ and $CD44^+$ cells, whereas ZNF516 knockdown by shRNA increased the number of $CD133^+$ and $CD44^+$ cells ($P<0.01$). In addition, we found that the protein level of CD133, CD44 and Oct4 was significantly reduced by ZNF516 overexpression, whereas the expression level of these proteins was significantly higher in cells with ZNF516 knockdown (**Figure 3C**, $P<0.01$).

ZNF516 counteracted the EGFR-mediated stability of Sox2

We further explored the molecular mechanism by which ZNF516 regulated the behavior of CRC cells. Particularly, we examined the effect of ZNF516 overexpression on the mRNA and protein expression profiles of EGFR and Sox2 in HCT116 and SW480 cells. We found that ZNF516 overexpression inhibited the transcription of EGFR, while ZNF516 knockdown promoted its transcription ($P<0.01$, **Figure 4A**). For the effect of ZNF516 knockdown on the mRNA and protein expression profiles of EGFR and Sox2, we used FHC cells transfected with Control, ZNF516 sh#1, or ZNF516 sh#2. The protein level of EGFR and Sox2 was examined

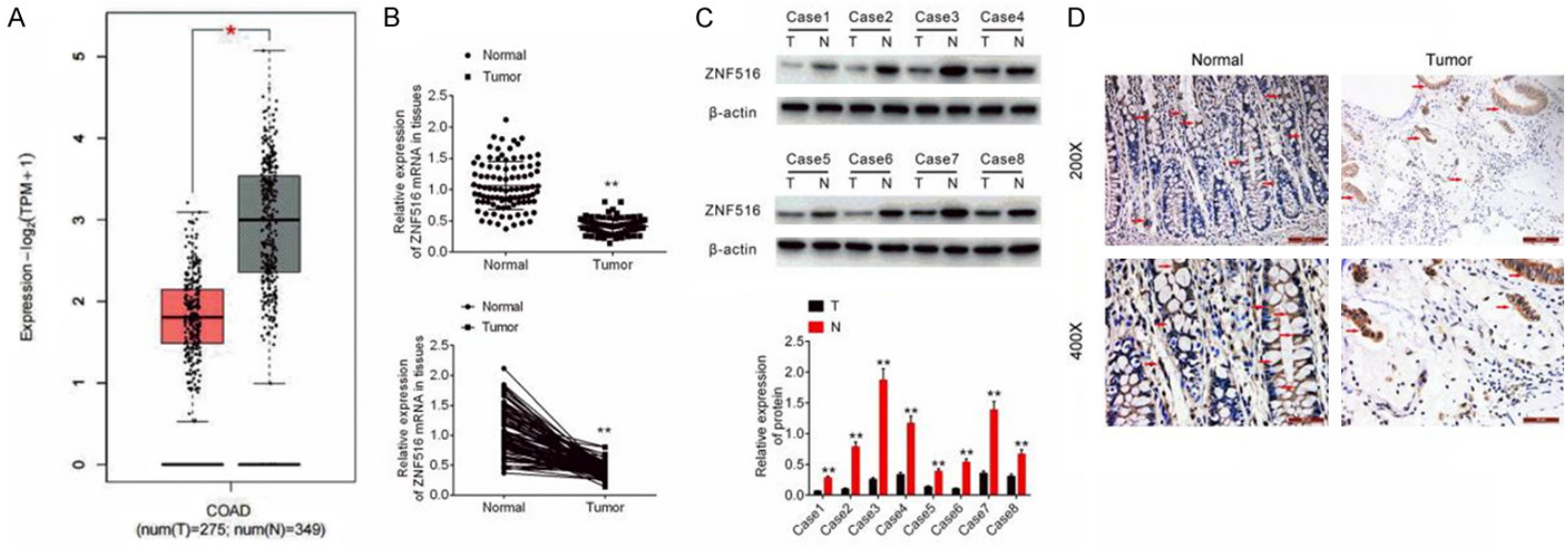

Figure 1. ZNF516 expression is lower in CRC tissues than in normal tissues. A. Analysis of ZNF516 expression level in TCGA CRC database [n (tumor) = 279, n (normal) = 349]. *P<0.05 vs Normal group. B. The mRNA level of ZNF516 in clinical CRC samples determined by qRT-PCR (n = 95). **P<0.01 vs Normal group. C. The protein expression profiles of ZNF516 in tumor and normal tissues determined by western blotting (n = 3). **P<0.01 vs Tumor group. D. The protein expression profiles of ZNF516 determined by immunohistochemistry (IHC) analysis (n = 2). Data are represented as mean ± SD of three independent experiments. Comparison between two groups was conducted by Student's t-test. **P<0.01 vs Normal group.

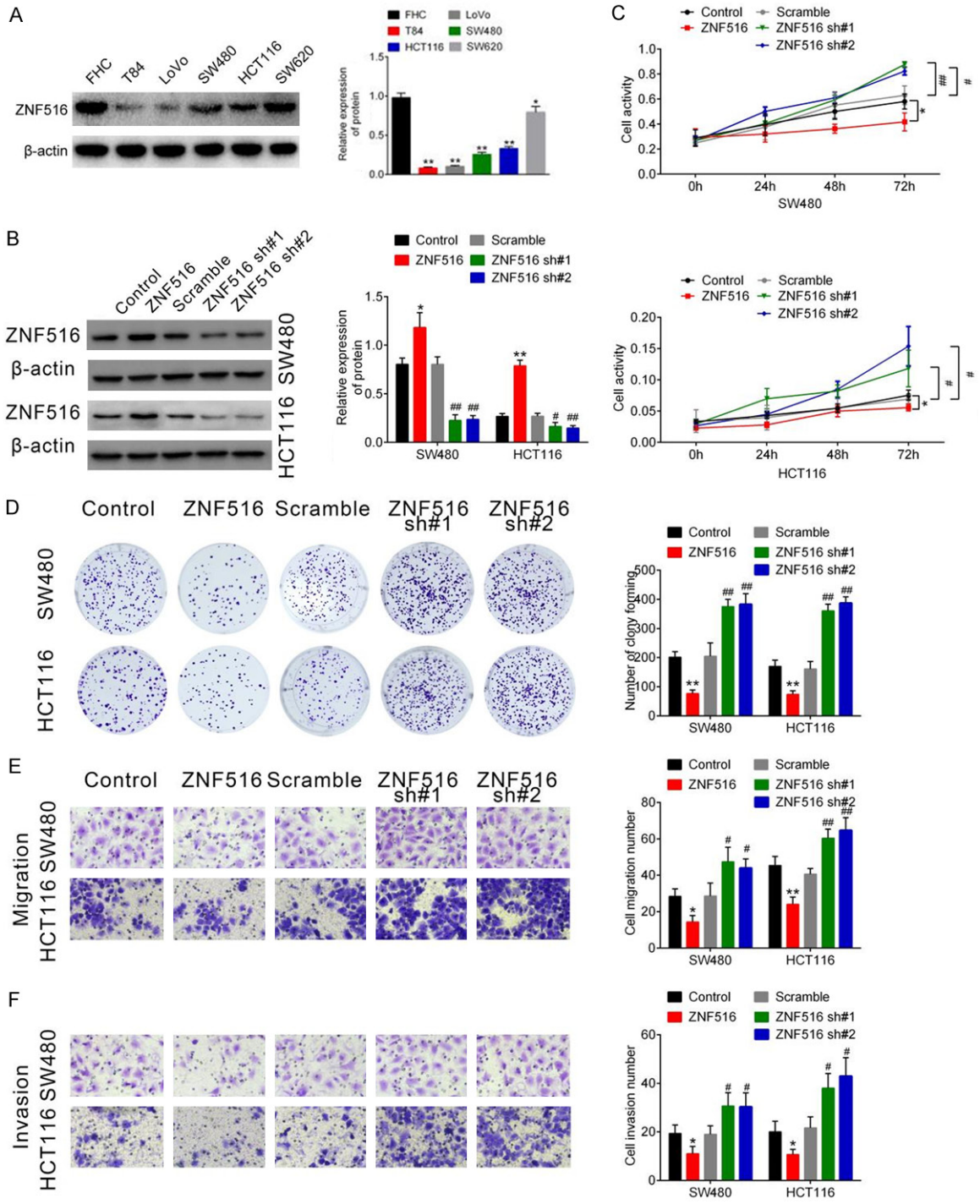

Figure 2. ZNF516 inhibits the proliferation, migration and invasion of CRC cells. A. ZNF516 protein expression level in five CRC cell lines (T84, LoVo, SW480, HCT116 and SW620) and normal human colorectal mucosal cells (FHC) determined by western blotting (n = 3). B. HCT116 and SW480 cells were transfected with ZNF516 overexpression vector and ZNF516 shRNAs (ZNF516 sh#1 and sh#2). Overexpression or knockdown of ZNF516 was confirmed by western blotting (n = 3). C and D. Cell viability and colony-forming ability were examined by CCK8 assay and crystal violet staining (n = 3). E and F. Transwell assays were used to measure the migration and invasion activity of CRC cells (n = 3). Data were represented as mean ± SD of three independent experiments. Comparison between two groups was conducted by Student's t-test. Comparison among multiple groups was conducted by one-way ANOVA with Tukey's *post hoc* test. **P*<0.05, ***P*<0.01 vs Control group; #*P*<0.05, ##*P*<0.01 vs Scramble group.

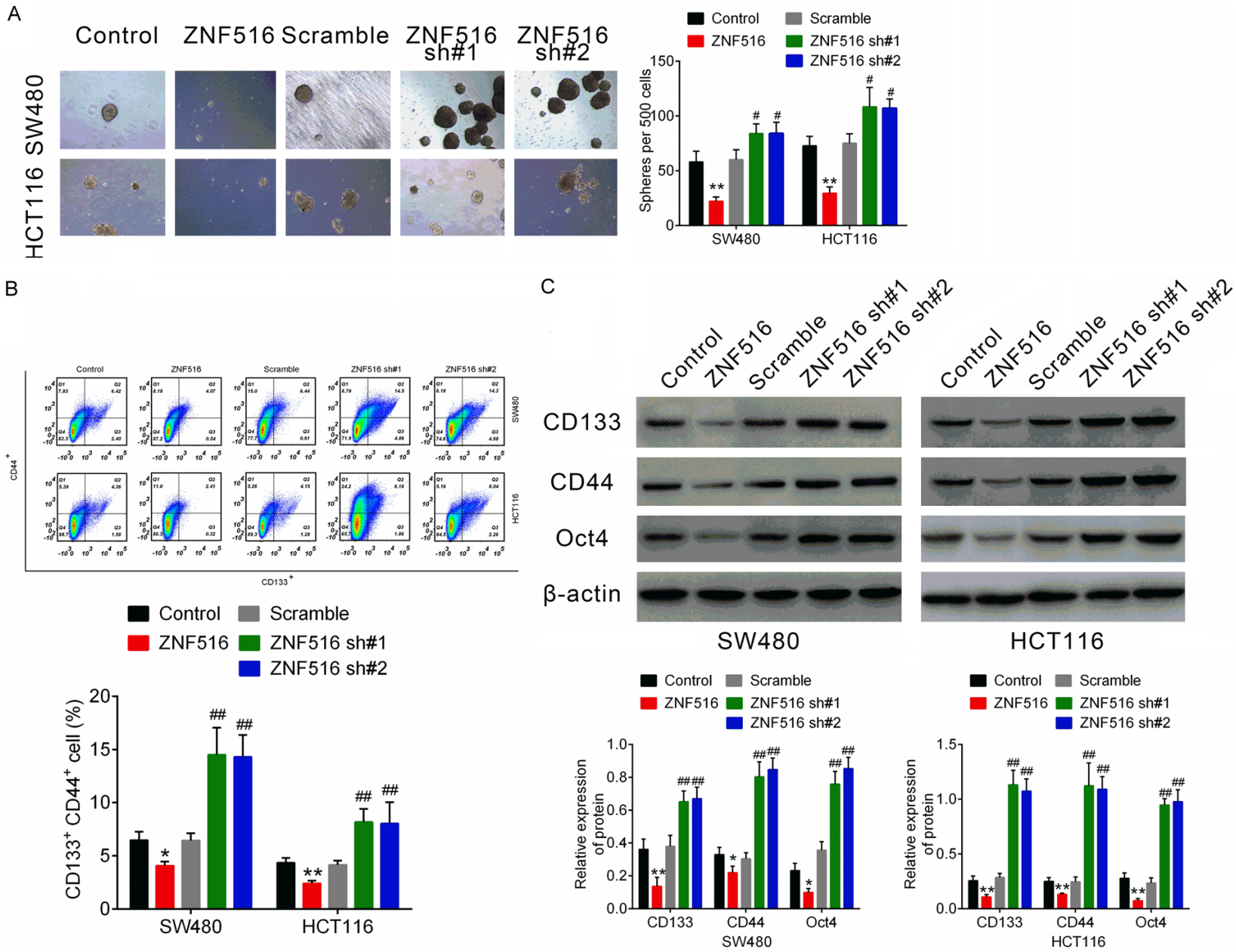
A
Control
ZNF516
Scramble
ZNF516 sh#1
ZNF516 sh#2
HCT116
SW480
Spheres per 500 cells
SW480
HCT116
B
CD44+
CD133+
SW480
HCT116
CD133+ CD44+ cell (%)
C
CD133
CD44
Oct4
β-actin
SW480
HCT116
Relative expression of protein

Figure 3. ZNF516 suppressed the stem cell-like characteristics in CRC cells. A. Sphere forming ability of CRC cells (n = 3). B. Flow cytometry was used to examine the proportion of CD133 and CD44 positive cells (n = 3). C. The expression levels of CD133, CD44 and Oct4 in CRC cells were examined by western blotting (n = 3). Data were represented as mean ± SD of three independent experiments. Comparison between two groups was conducted by Student's t-test. Comparison among multiple groups was conducted by one-way ANOVA with Tukey's *post hoc* test. **P*<0.05, ***P*<0.01 vs Control group; #*P*<0.05, #*P*<0.01 vs Scramble group.

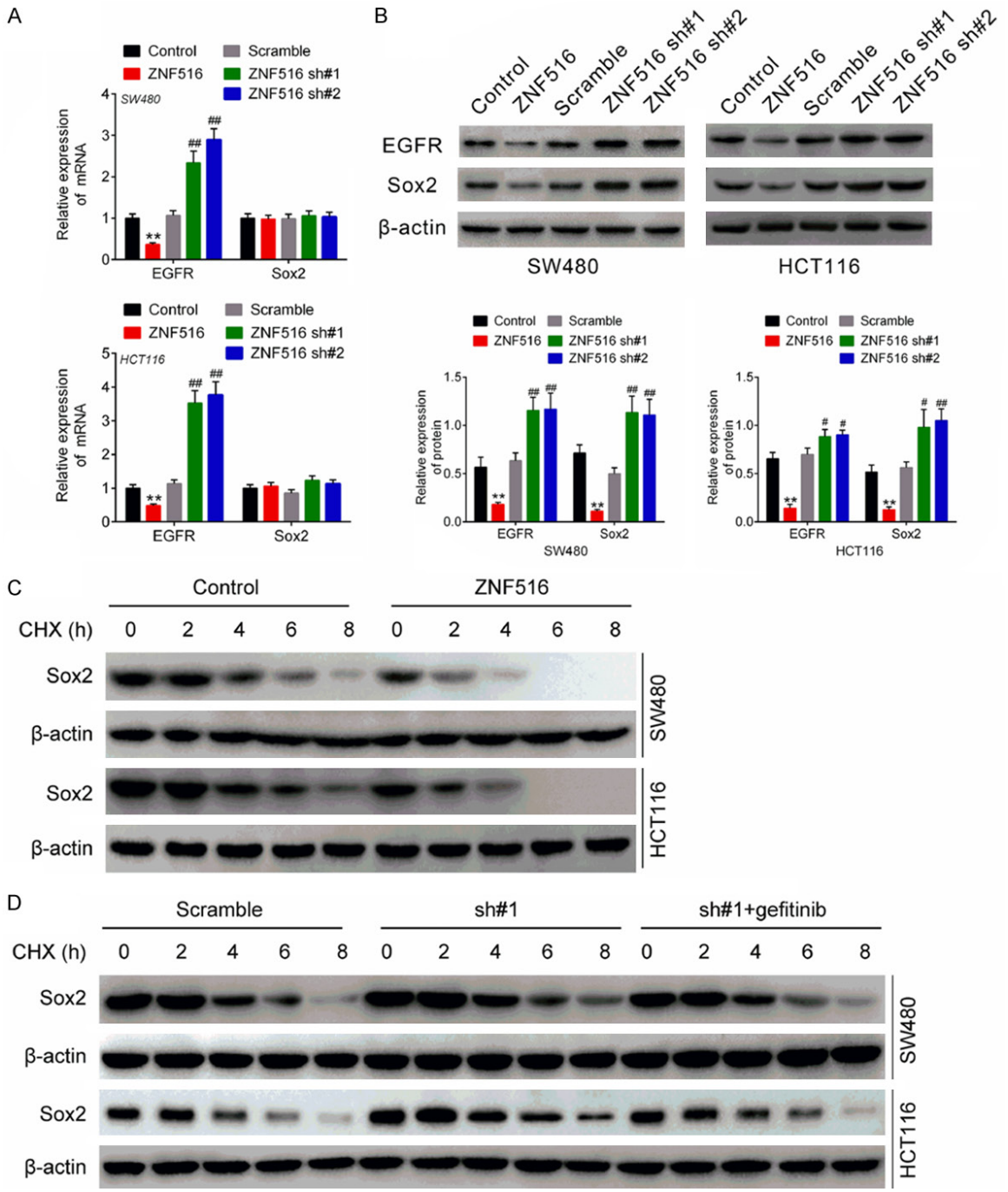

Figure 4. ZNF516 overexpression decreased the EGFR-mediated stability of Sox2. A. Overexpression or knockdown of ZNF516 affected the mRNA levels of EGFR and Sox2 in HCT116 and SW480 cells, as determined by qRT-PCR (n = 3). B. The protein levels of EGFR and Sox2 in HCT116 and SW480 cells with either overexpression or knockdown of ZNF516, as examined by western blotting (n = 3). C. Western blot analysis of Sox2 protein level in ZNF516-overexpressing HCT116 and SW480 cells treated with the protein synthesis inhibitor cycloheximide (CHX, 10 μg/mL) (n =

3). D. Western blot analysis of Sox2 protein level in ZNF516 knocked down HCT116 and SW480 cells treated by CHX followed by EGFR inhibitor, gefitinib (10 μmol/L), treatment (n = 3). Data were represented as mean ± SD of three independent experiments. Comparison between two groups was conducted by Student's t-test. Comparison among multiple groups was conducted by one-way ANOVA with Tukey's *post hoc* test. **P*<0.05, ***P*<0.01 vs Control group; #*P*<0.05, #*P*<0.01 vs Scramble group.

by western blotting. The results shown in Figure S1B indicated that down-regulation of ZNF516 upregulated the protein level of EGFR and Sox2 (*P*<0.05). Furthermore, as shown in **Figure 4B**, ZNF516 overexpression in HCT116 and SW480 cells also led to a lower EGFR and Sox2 protein level, whereas ZNF516 knockdown led to a higher level of EGFR and Sox2 proteins (*P*<0.01). In addition, when we examined the effects of ZNF516 knockdown on the protein level of EGFR and Sox2 in FHC cells, we found that these cells had higher EGFR and Sox2 protein levels than control cells (Figure S1B, *P*<0.05).

When ZNF516-overexpressing HCT116 and SW480 cells were treated with the protein synthesis inhibitor CHX, the Sox2 protein level was lower than vector-expressing cells, suggesting that overexpression of ZNF516 probably accelerated Sox2 degradation in HCT116 and SW480 cells (**Figure 4C**). To determine whether ZNF516 accelerated Sox2 degradation by regulating EGFR signaling, the EGFR inhibitor, gefitinib, was used to treat ZNF516 knocked-down HCT116 and SW480 cells in combination with CHX. As shown in **Figure 4D**, ZNF516 knockdown slowed the degradation of Sox2, while gefitinib treatment reversed the effect of ZNF516 knockdown on Sox2 degradation.

ZNF516 suppressed stem cell-like characteristics in CRC cells by reducing Sox2 protein level

To determine whether ZNF516 suppresses stem cell-like characteristics in CRC cells by inhibiting the action of Sox2, HCT116 cells were co-transfected with ZNF516 and Sox2 expression vectors. We found that the protein level of ZNF516 was not affected by Sox2 co-expression in HCT116 cells, as determined by western blotting (**Figure 5A**). When examining the effect of ZNF516 and Sox2 co-expression on the proliferation, migration, invasion, and stem cell-like characteristics of cells, we found that up-regulation of ZNF516 suppressed the proliferation, migration and invasion of HCT116 cells, whereas Sox2 co-overexpression counteracted the effects of ZNF516 on these cellular processes (**Figure 5B** and **5C**, *P*<0.05). Furthermore, after obtaining the stable transfected HCT116 cells (Figure S1A), we found that ZNF516 overexpression reduced the sphere-forming ability of HCT116 cells, while Sox2 co-overexpression restored this ability of HCT116 cells (**Figure 5D**, *P*<0.01). When the protein expression profiles of CD133, CD44, Sox2 and Oct4 were evaluated by western blotting, we found that compared with the Control + Vector transfected cells, the ZNF516-overexpression + Vector cells showed a lower expression level of CD133, CD44, Sox2 and Oct4 (**Figure 5E**, *P*<0.05). However, Sox2 overexpression rescued the effects of ZNF516 overexpression on the protein expression profiles of CD133, CD44, Sox2 and Oct4 (**Figure 5E**, *P*<0.05).

ZNF516 delayed CRC tumor growth and tumor metastasis in vivo

Using a mouse xenograft tumor model, we evaluated the effect of ZNF516 on CRC tumor growth *in vivo*. HCT116 cells transfected with empty vector or ZNF516 expression construct were implanted subcutaneously into nude mice. Western blotting experiment indicated that the expression of Sox2 was decreased in the tumor of ZNF516-overexpressing mice (**Figure 6A**, *P*<0.01). Overexpression of ZNF516 significantly delayed the tumor growth in the nude mice (**Figure 6B**, *P*<0.01). At 8 weeks post-implantation, the mice were sacrificed, and the tumors were collected and weighed. ZNF516 overexpression significantly decreased the tumor size and weight (**Figure 6C**, *P*<0.01). Subsequently, the expression level of ZNF516, Ki67, EGFR, Sox2, CD133 and CD44 proteins was determined in the tumor samples by IHC. The tumor samples derived from ZNF516-overexpressing cells had significantly lower levels of Ki67, EGFR, Sox2 and CD133 than the samples derived from empty vector-transfected cells (**Figure 6D**, *P*<0.01). We also found that ZNF516 overexpression significantly reduced the number of pulmonary metastases

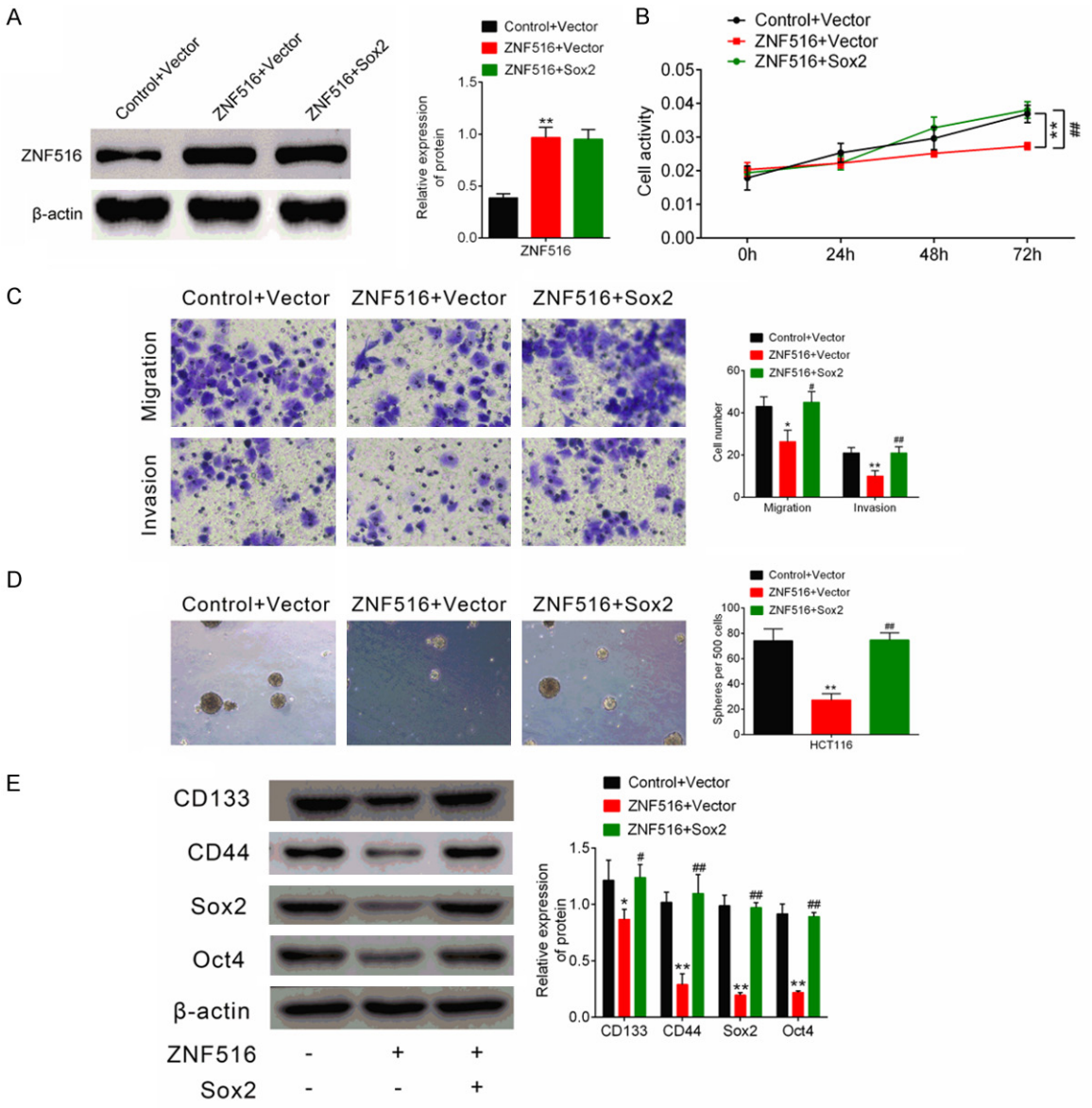

Figure 5. ZNF516 suppresses the stem cell-like characteristics of CRC cells by attenuating the expression of Sox2. HCT116 cells were co-transfected with the overexpression vectors of ZNF516 and Sox2. ZNF516 protein level in these cells was examined by Western blotting (n = 3) (A). The proliferation (B), migration and invasion (C), and stem cell characteristics (D and E) of these cells were also deternined (n = 3). Data were represented as mean ± SD of three independent experiments. Comparison between two groups was conducted by Student's t-test. Comparison among multiple groups was conducted by one-way ANOVA with Tukey's *post hoc* test. **P*<0.05, ***P*<0.01 vs Control + Vector group; #*P*<0.05, ##*P*<0.01 vs ZNF516 + Vector group.

and pulmonary nodules formed by the implanted HCT116 cells (**Figure 6E**).

Discussion

CRC is a group of heterogeneous tumors with complex molecular mechanisms of onset, progression and metastasis. In recent years, many studies have focused on identifying the potential molecular markers of CRC to develop better treatment options [1]. Of these markers, ZNF516, a regulatory protein in several other cancers such as cervical carcinoma, breast cancer and lung adenocarcinoma [25], was identified as a possible biomarker for CRC. In this study, we focused on the role of ZNF516 in suppressing CRC progression, as the loss of ZNF516 expression has been reported to cause

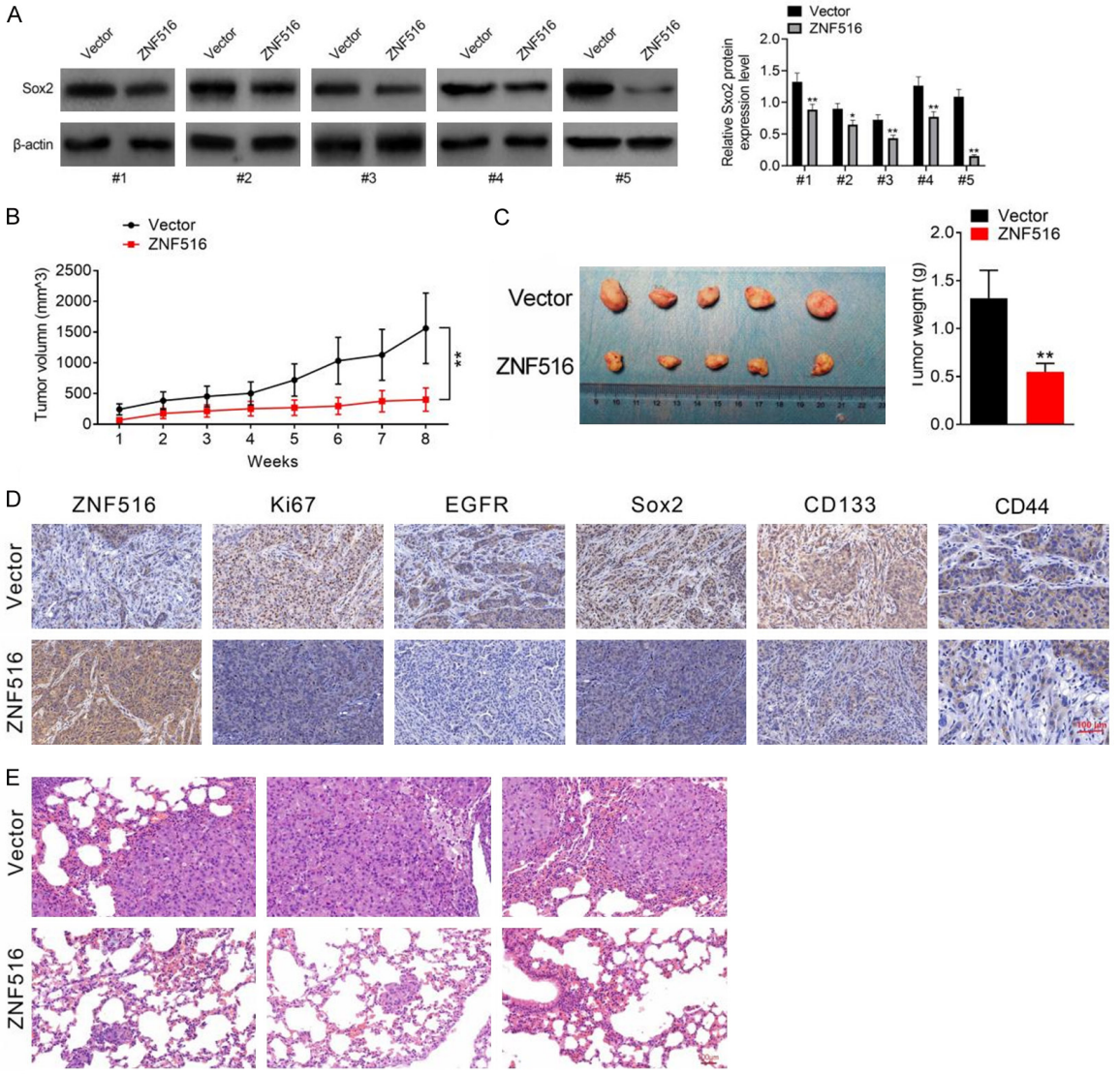

Figure 6. ZNF516 delays CRC tumor growth and metastasis *in vivo*. A. The Sox2 protein expression in tumor tissues was examine by western blot analysis. B, C. HCT116 cells transfected with empty vector or ZNF516 expression vector were implanted subcutaneously into nude mice (n = 6/group). Tumor growth was monitered every 7 days. At 8 weeks post-implantation, the mice were sacrificed, and the tumors were collected and weighed. D. The expression levels of ZNF516, Ki67, EGFR, Sox2, CD133 and CD44 were determined in xenograft tumors by immunohistochemistry (IHC). E. The number of pulmonary metastases and nodules was examined by haematoxylin-eosin (HE) staining. Data were represented as mean ± SD of three independent experiments. Comparison between two groups was conducted by Student's t-test. $^{**}P<0.01$ vs Vector group.

DNA replication stress, structural chromosome abnormalities and chromosome mis-segregation [19]. We examined the transcription level of ZNF516 in CRC tissues deposited in TCGA database, and more importantly, we used our CRC patient samples to validate the conclusion from database analysis. The level of ZNF516 protein in CRC tissues and normal tissues was also compared. Our results showed that both mRNA and protein levels of ZNF516 were lower in CRC tissues than in normal tissues. Furthermore, we also found that ZNF516 expression was downregulated in the CRC cell lines we tested: T84, LoVo, SW480, HCT116 and SW480. This suggested that ZNF516 might function as a tumor suppressor in CRC.

When we examined the effect of ZNF516 overexpression and knockdown on the proliferation, colony formation, migration and invasion of CRC cells, we found that ZNF516 overexpression significantly suppressed these cellular pro-

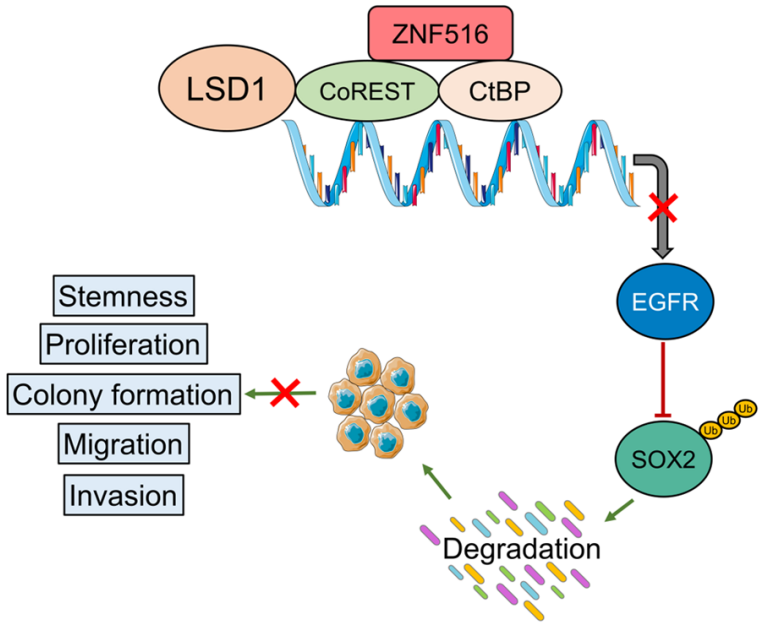

Figure 7. Schematic representation of the mechanism by which ZNF516 regulates transcription of Sox2 in colorectal cancer cells to suppress the development of stem cell-like characteristics and tumor progression.

cesses, whereas ZNF516 knockdown promoted these processes, confirming the growth and invasion-suppressive function of ZNF516.

Emerging evidence has confirmed that a small population of tumor cells with stem cell-like properties, known as cancer stem cells or cancer stem-like cells, exist in cancer tissues. These cells can self-renew, differentiate, and have strong oncogenic potential; such cells are thought to be the origin of tumor formation during recurrence. Thus, cancer stem cell-targeted therapy would improve the prognosis of patients with CRC, where such recurrence occurs often. When the effects of ZNF516 on the stem cell-like characteristics of CRC cells, such as sphere formation, and the expression of CD133, CD44 and Oct4 (which are important biomarkers of cancer stem cells [26, 27]) were examined, we found that ZNF516 overexpression inhibited sphere formation and decreased the number of cells expressing CD133 and CD44. CRC stem cells are often characterized by the expression of genes associated with stemness, such as CD133, CD44 and Oct4, all of which play key roles in maintaining the pluripotency of embryonic stem cells [28, 29]. We also examined the protein level of these marker molecules, and the results showed that ZNF516 reduced their expression levels. Conversely, ZNF516 knockdown enhanced the expressions of stem cell-like characteristics in CRC cells, including increased expression of CD133, CD44 and Oct4. These results suggested that ZNF516 expression likely contributes to the suppression of stem-like properties in CRC cells.

To explore the mechanisms underlying ZNF516-mediated stemness-suppressing effects, we evaluated the effect of ZNF516 expression on EGFR and Sox2 expression. We found that ZNF516 overexpression inhibited the protein level of EGFR and Sox2, and ZNF516 accelerated Sox2 degradation, likely via regulating EGFR expression. Although Li *et al*. (2017) have reported that ZNF516 suppresses EGFR transcription by interacting with the CtBP/LSD1/CoREST complex, the mechanism of how this occurs needs to be further explored. To verify this hypothesis, we performed rescue experiments in which HCT116 cells were co-transfected with ZNF516 and Sox2 overexpression vectors. While overexpression of ZNF516 suppressed cell viability, migration, invasion, and the development of stem cell-like characteristics in HCT116 cells, Sox2 overexpression counteracted the effect of ZNF516 overexpression in these cells. Finally, when we evaluated the effect of ZNF516 dysregulation on CRC tumor growth and metastasis *in vivo* by using nude mice, we found that tumors derived from ZNF516 overexpressing cells were smaller and weighed less with fewer pulmonary metastases and nodules compared with tumors derived from cells without ZNF516 overexpression. These data further demonstrate the growth inhibitory effects of ZNF516.

In summary (**Figure 7**), our present studies indicated that ZNF516 functions as a carcinostatic gene in CRC cells by inhibiting cell proliferation, migration and invasion as well as suppressing the development of stem cell-like characteristics. Mechanistically, ZNF516 functions by reducing the stability of Sox2 in CRC cancer cells. Given its importance in suppressing can development, ZNF516 may be an important therapeutic target for novel CRC treatment.

Disclosure of conflict of interest

None.

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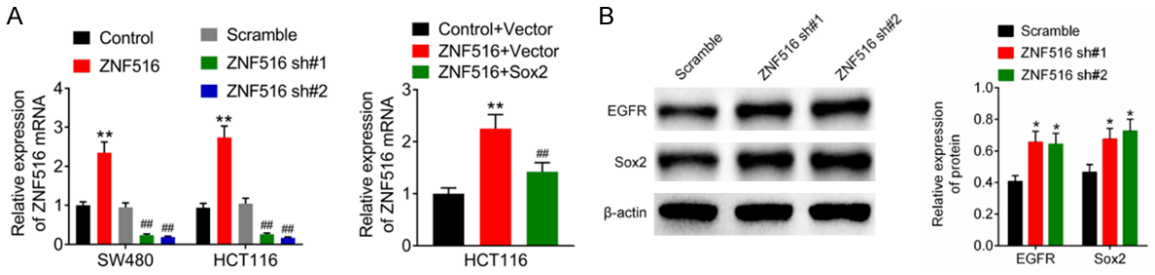

Figure S1. The lentivirus transfection efficiency and EGFR and Sox2 expression in human FHC cells. A. The identification of lentivirus transfection efficiency was performed by qRT-PCR in HCT116 and SW480 cells, $^{**}P<0.01$ vs Control or Control + Vector, $^{\#\#}P<0.01$ vs Scramble or Scramble + Vector. B. The expression of EGFR and Sox2 in normal human colorectal mucosal cells (FHC) cells with ZNF516 knockdown, $^{*}P<0.05$ vs Scramble.