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Original Article

Original Article

ZNF3 regulates proliferation, migration and invasion through MMP1 and TWIST in colorectal cancer

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Abstract

Colorectal cancer (CRC) is a malignant tumor with a high incidence and mortality worldwide. Currently, the underlying molecular mechanisms of CRC are still unclear. Zinc finger protein 3 (ZNF3) is a zinc-finger transcription factor that has been reported as a candidate for breast cancer prognosis, suggesting its involvement in the regulation of tumorigenesis. However, the association between ZNF3 and CRC remains unknown. To investigate the role of ZNF3 in CRC, we first analyze the correlation between ZNF3 expression and CRC, and the results demonstrate that ZNF3 is highly expressed in CRC tissue and cells, which is associated with the age of CRC patients. *In vitro* studies show that ZNF3 overexpression promotes CRC cell migration. Compared to control cells, knockdown of *ZNF3* markedly suppresses CRC cell proliferation, migration and invasion and promotes G0/G1 phase cell cycle arrest. The expressions of the EMT-related markers TWIST and MMP1 are significantly decreased when *ZNF3* is silenced. Additionally, overexpression of MMP1 and TWIST exacerbates CRC cell proliferation, accelerates the S phase cell cycle in *ZNF3*-knockdown SW480 cells, and increases cell migration and invasion through Transwell chambers. These data suggest that ZNF3 is involved in cellular proliferation, migration and invasion by regulating MMP1 and TWIST in CRC cells.

Key words ZNF3, colorectal cancer, proliferation, migration, invasion

Introduction

Colorectal cancer (CRC) is a malignant tumor around the world, and the incidence and mortality of CRC rank in the top three according to Global Cancer Statistics 2020 [1]. Although there are some improvements in the rate of early diagnosis, the present surgery and chemoradiotherapy treatment are not ideal due to the pathological stage and invasion and metastasis of colon cancer cells which weaken the treatment effect.

Epithelial-mesenchymal transition (EMT) is a key step in the initiation of tumor metastasis [2]. EMT is characterized by loss of epithelial markers and increased levels of mesenchymal markers, which are regulated by various transcription factors. The TWIST family of basic helix-loop-helix (bHLH) transcription factors, including TWIST1 and TWIST2, is the major group of EMT-activating transcription factors [3]. TWIST can promote cell migration, invasion, and cancer metastasis and endows cancer cells with stem cell-like characteristics and therapeutic resistance [4]. TWIST1 has been considered as a proto-oncogene which is highly upregulated in a wide variety of human cancers. TWIST1 overexpression is closely related to tumor progression and meta-

static potential [5]. Additionally, matrix metalloproteinases (MMPs) are key factors which provide invasion and metastasis characteristics of malignant tumor cells by facilitating their infiltration and migration during EMT. The migration of cancer cells depends on the increased release and activation of MMPs, as well as their cell membrane expression, leading to the breakdown of the extracellular matrix (ECM) and favoring infiltration [6]. MMP-1 (or collagenase-1) has been revealed to play a significant role in the pathological progression of many cancers. A previous study showed that knockdown of *MMP1* inhibited the progression of colorectal cancer by suppressing the PI3K/Akt/cmyc signaling pathway [7]. Therefore, regulating the expressions of MMP1 and TWIST1 might be a potential strategy for suppressing CRC cell migration and invasion.

C2H2 (Cys2-His2)-type zinc finger protein 3 (ZNF3 or ZFP 3) is an important member of the ZFP family. ZFPs are a vital class of transcription factors that can bind with Zn^{2+} and self-fold to form "finger" domains and regulate the expression of target genes [8]. It has been reported that a substantial number of zinc finger transcription factors are involved in regulating the process of EMT [9]. A previous study showed that ZNF3 promoted cell fusion and pheromone production through a pathway parallel to and independent of the pheromone signaling cascade, indicating its key function in the sexual reproduction of *Cryptococcus neoformans* [10]. It had been revealed that the expression level of ZNF3 is higher in lung tissues of patients with moderate and severe emphysema than in normal lung tissues [11]. Notably, ZNF3 was identified as a prognostic biomarker for triple-negative breast cancer patients, but its high expression was associated with a better prognosis [12]. Moreover, the *ZNF3* gene harbors nonsynonymous mutations in head and neck squamous cell carcinoma [13]. Therefore, ZNF3 may be involved in tumorigenesis. However, the function of ZNF3 in tumors, especially in CRC, has not yet been elucidated.

In the present study, we first investigated the potential role of ZNF3 in CRC cell proliferation, migration, and invasion. Meanwhile, we evaluated whether EMT-related markers are involved in the biological activities of ZNF3 in the context of CRC. We found that ZNF3 was upregulated in CRC tissues and cells and that silencing of *ZNF3* impaired the proliferation, migration, and invasion of CRC cells by downregulating the expressions of TWIST and MMP1.

Materials and Methods

Bioinformatic analysis

The expression data of ZNF3 in Pan-Cancer and CRC were obtained from the UCSC Xena dataset (https://xena. ucsc.edu/).

Cell culture

Immortal normal epithelial cells NCM460 and three types of human colorectal cancer cells (LoVo, HT-29, and SW480) were purchased from ATCC (American Type Culture Collection, Manassas, USA). All cells were cultured in complete medium containing 1% penicillin-streptomycin (HyClone, Logan, USA) according to the suggestions of ATCC. Cells were detached with trypsin (Corning, New York, USA) and then resuspended in complete medium without penicillin-streptomycin to perform further experiments.

Immunohistochemical (IHC) staining

ZNF3 immunohistochemistry was performed on tumor microarrays (TMAs) which were obtained from Xi'an Ding Guo Trading Co., Ltd (Xi'an, China). For antigen retrieval, tissue sections were boiled in 0.01 M citrate solution (pH 6.0) and incubated with primary antibody against ZNF3 (1:200; Proteintech, Wuhan, China). Tissue sections were observed with an automatic slice scanner (Beijing Una Technology, Beijing, China). The staining intensity and score of ZNF3 expression were negative (0 point), weak (1 point), moderate (2 points), or strong (3 points). The percentage and score of positive cells in each image ranged from 1%–25% (1 point), 26%–50% (2 points), 51%–75% (3 points), and 76%–100% (4 points). The final score of each tumor sample was calculated by multiplying the staining intensity score with the percentage score of positive cells, and the final score ranged from 0 to 12 points to determine the tumor as negative (—), 0 point; low expression (+), ≤4 points; medium expression (++), 5–8 points; and high expression (+++), ≥9 points.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cell lines using Trizol (Tiangen, Beijing, China) according to the manufacturer's instructions. The isolated RNA was then reverse transcribed to cDNA. SYBR Premix Ex *Taq* II (TaKaRa, Dalian, China) was used to conduct amplifications of the target genes on an ABI PRISM 7700 quantitative real-time PCR system (Applied Biosystems, Foster City, USA). PCR (a total volume of 25 μL) was performed through five reactions including pre-denaturation (95°C, 3 min), 35 cycles of denaturation (94°C, 30 s), annealing (60°C, 30 s) and extension (72°C, 30 s), and a final extension (72°C, 10 min). RT-PCR amplification was conducted in triplicate, and the expression levels of the target genes were calculated using the $2^{-\triangle\triangle Ct}$ method. The housekeeping gene *GAPDH* was used as the internal control. The specific primer sequences used in this study are shown in the Table 1.

Plasmids and transfection

Lentiviral infection and siRNA transfection were used to knock-

Table 1. Sequences of primers used in this study

Primer	Sequence (5′→3′)
ZNF3-F	CCCTCGAGATGGAAACTCAGGCTGATCTCG
ZNF3-R	GCGTCGACTCAGGTGGACTCTCTGATATTT
Vimentin-F	CAGCTCAACAGCTTCTTCGC
Vimentin-R	TTTGAAGCGGGGTTGGAACT
E-cadherin-F	GCTCGAGGATGCCAATACGA
E-cadherin-R	ACCACCGTTGTGTAGTCGTC
N-cadherin-F	CTGGAACGCAGTGTACAGAA
N-cadherin-R	GGTTTGACCACGGTGACTAA
TWIST-F	CCGTGGACAGTGATTCCCAG
TWIST-R	CCTTTCAGTGGCTGATTGGC
CK19-F	AGAATTGAACCGGGAGGTCG
CK19-R	CCTGATTCTGCCGCTCACTA
MMP1-F	AGAAAGAAGACAAAGGCAAGTTGA
MMP1-R	TTGTCCCGATGATCTCCCCT
ZEB2-F	AGCCTCTGTAGATGGTCCAGT
ZEB2-R	GGTCAGCAGTTGGGCAAAAG
GAPDH-F	GAAAGCCTGCCGGTGACTAA
GAPDH-R	TTCCCGTTCTCAGCCTTGAC

down *ZNF3*. For lentiviral infection, three ZNF3-shRNA plasmids and control plasmid were constructed and verified by sequencing and then transfected with HEK-293T cells for packaging of the lentivirus virus. Lentiviral particles were collected to infect SW480 cells, followed by puromycin selection to obtain stable *ZNF3*-knockdown cell lines. The sequences of ZNF3 shRNAs and siRNAs were as follows: siZNF3#1: 5′-GGAACCTCAGGCCCTGCTTGA-3′, siZNF3#2: 5′-GGAAGGAGTGGAAGCGTTTGG-3′, siZNF3 #3: 5′-GGTAATGCTGGCATGGCTTAC-3′, and control: 5′-TTCTCCGAACGTGTCACGT-3′. ZNF3 overexpression constructs were obtained by using the pEX-3 (pGCMV/MCS/Neo) vector (GenePharma, Shanghai, China), TWIST and MMP1 overexpression constructs were obtained by using the pcDNA3.1 vector (GenePharma), and an empty vector (EV) was used as the control. The Myc flag was provided by Dr. Guo Li at the Department of Biochemistry and Molecular Biology of Hainan Medical University (Haikou, China). Transfection was carried out using Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, USA).

Western blot analysis

Cells were collected and washed twice with PBS and then lysed on ice for 30 min in RIPA buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitor. The protein concentrations of the lysates were determined using a BCA protein assay reagent kit (Beyotime Biotechnology). Approximately 30 μg of total protein lysate was subject to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and was then transferred to PVDF membranes (Millipore, Billerica, USA) for subsequent incubation with primary antibodies, which was followed by incubation with rabbit and mouse horseradish peroxidase-coupled secondary antibodies for 1 h. Specific bands were detected on automatic chemiluminescence fluorescence gel imaging analysis system (Beijing Sage Creation, Beijing, China). The antibodies used in the study were as follows: anti-ZNF3 (1:1000; Proteintech), anti-TWIST (1:1000; Affinity Biosciences, Nanjing, China), anti-MMP1 (1:1000; Abcam, Cambridge, UK), anti-Vimentin (1:1000; CST, Beverly, USA), anti-E-cadherin (1:1000; CST), anti-N-cadherin (1:1000; CST), anti-CK19 (1:20,000; Abcam), anti-ZEB2 (1:1000; CST), anti-GAPDH (1:10,00; Servicebio, Wuhan, China), anti-rabbit IgG (1:120000; Sigma, St Louis, USA), and anti-mouse IgG (1:120000; Sigma).

Cell proliferation assay

Cell proliferation was measured using Cell Counting Kit-8 (Beyotime Biotechnology). Cells were inoculated into a 96-well plate at a density of 1000 cells/well with 6 replicates after 24 h of transfection. To detect the cell proliferative ability, 10 μL CCK-8 solution and 90 μL complete medium were mixed and then added into each well. Subsequently, the cells were incubated at 37°C for 4 h. After that, the optical density was detected at a wavelength of 450 nm. The number of viable cells was detected for 4 continuous days.

Flow cytometry analysis

Cells were collected and washed twice with PBS at 48 h after transfection. For cell cycle analysis, the cells were fixed with 70% ethanol overnight at 4°C, washed with PBS, resuspended in 500 μL buffer and then incubated with 100 μg/mL RNase A and 50 μg/mL propidium iodide (PI; Beyotime Biotechnology) for 30 min at 37°C. After incubation, the cells were subject to DNA content analysis by flow cytometry using a FACSCalibur (NovoCyte; Agilent Biosciences, Santa Clara, USA), and the results were analyzed with Summit v4.3 software.

Migration and invasion assays

Transwell chambers (Corning) were used to assess cell migration and invasion ability. For the cell migration assay, LoVo cells or SW480 cells were suspended in serum-free medium and then inoculated into the upper chamber of a 24-well plate at a density of 10^6 cells/mL and a volume of 100 μL. Simultaneously, 800 μL of medium containing 10% FBS was added to the lower chamber. After incubation at 37°C for 48 h, the nonmigratory cells were wiped away from the upper chambers with cotton swabs. The migrated cells in the lower chamber were fixed with 4% paraformaldehyde solution for 30 min and then stained with 0.1% crystal violet. The number of migrated cells was counted under a microscope at a magnification of 100 × . Three fields were randomly selected in each group, and this assay was independently conducted three times. The method of the cell invasion assay was similar to that of the cell migration assay. Before cell inoculation, Matrigel (BD Biosciences, Bedford, USA) was mixed with the culture medium at a ratio of 1:8, and 50 μL was added into the upper chamber. Then cells were inoculated into the upper chamber after the Matrigel solidified at 37°C. Other steps were the same as those in the cell migration assay.

Statistical analysis

Data are shown as the mean ± standard deviation (SD) and were analysed by GraphPad Prism 6 (GraphPad, Chicago, USA). Nonpaired *t* tests and one-way ANOVA were used to compare the differences among groups. A P value < 0.05 was considered statistically significant.

Results

ZNF3 is upregulated in Pan-Cancer and CRC

To explore the functions of ZNF3 in CRC development, the expression of ZNF3 in Pan-Cancer and CRC was analyzed by the UCSC Xena database. Compared with those in normal tissues, ZNF3 expression levels were significantly elevated in Pan-Cancer tissues ($P < 0.0001$; Figure 1A). Notably, ZNF3 was highly expressed in CRC tissue compared with that in normal tissue ($P < 0.0001$; Figure 1B). Subsequently, the associations between ZNF3 expression levels and potential clinical characteristics were evaluated in a cohort of CRC tissues by IHC staining. ZNF3 expression level was significantly higher in colon cancer tissue than in normal tissue ($P < 0.01$; Figure 1C,D). Moreover, compared with patients younger than 55 years, patients older than 55 years had markedly elevated expression of ZNF3 ($P < 0.05$; Supplementary Figure S1B). There was no correlation between ZNF3 and other factors, including sex, T stage, N stage or M stage (Supplementary Figure S1A,C–E). For the endogenous expression analysis in cell lines, the expression levels of ZNF3 mRNA were upregulated in colon cancer cell lines (LoVo, HT-29, SW480, and HCT116) compared with that in the normal intestinal cell line NCM460 (Figure 1E). These results suggest that the expression of ZNF3 is elevated in CRC.

ZNF3 enhances CRC cell proliferation

LoVo and SW480 cells were selected as the low and high ZNF3 expression cell models, respectively, for subsequent functional experiments. To further examine the role of ZNF3 in CRC, we

overexpressed ZNF3 by transfecting the ZNF3 plasmid and ZNF3-Myc fusion plasmid into LoVo cells and silenced its expression by transfecting the lentivirus-mediated ZNF3-shRNAs into SW480 cells. The transfection efficiency was validated by western blot analysis (Figure 2A,B and Supplementary Figure S2A). The effect of ZNF3 on the proliferative ability of CRC cells was investigated by CCK-8 assay. The results in Figure 2C demonstrate no significant change in the cell proliferation rate in ZNF3-overexpressing LoVo cells compared with that in the control cells ($P > 0.05$). Meanwhile, *ZNF3* knockdown led to notable proliferation inhibition in SW480 cells ($P < 0.001$; Figure 2D). These findings were confirmed by marked G0/G1 phase cell cycle arrest when ZNF3 was knocked down (Figure 2E). Therefore, the above data indicate that ZNF3 promotes CRC cell proliferation.

ZNF3 promotes CRC cell migration and invasion

To further establish the potential role of ZNF3 in CRC cell migration and invasion. Cell migration and invasion were analyzed by

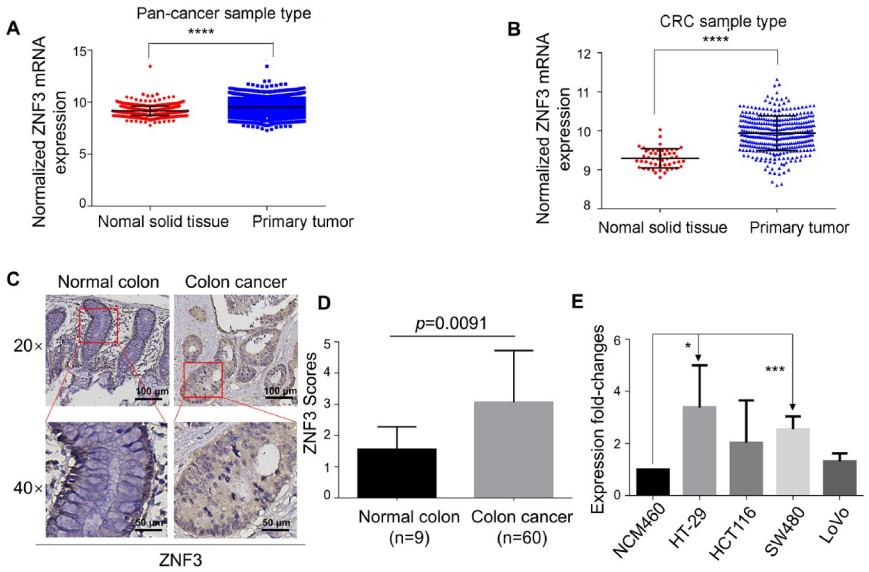

Figure 1. ZNF3 is highly expressed in CRC tissues and cell lines (A) Data from the UCSC Xena database show the mRNA expression levels of *ZNF3* in 9701 Pan-Cancer tissues and in 738 para-carcinoma tissues. (B) mRNA expression levels of *ZNF3* in 51 para-carcinoma tissues and 380 CRC tissues from the UCSC Xena database. (C) Immunohistochemical analysis of ZNF3 in 10 normal colon tissues and 70 colon cancer tissues at 20 × and 40 × magnification. Scale bar = 100 μm (up) and scale bar = 50 μm (down). (D) The quantitative analysis results of C. (E) RT-qRCR analysis of *ZNF3* transcriptional expression levels in human normal colon epithelial cells (NCM460) and CRC cell lines (HT-29, HCT116, SW480 and LoVo). Data were analyzed by independent samples *t* test. $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

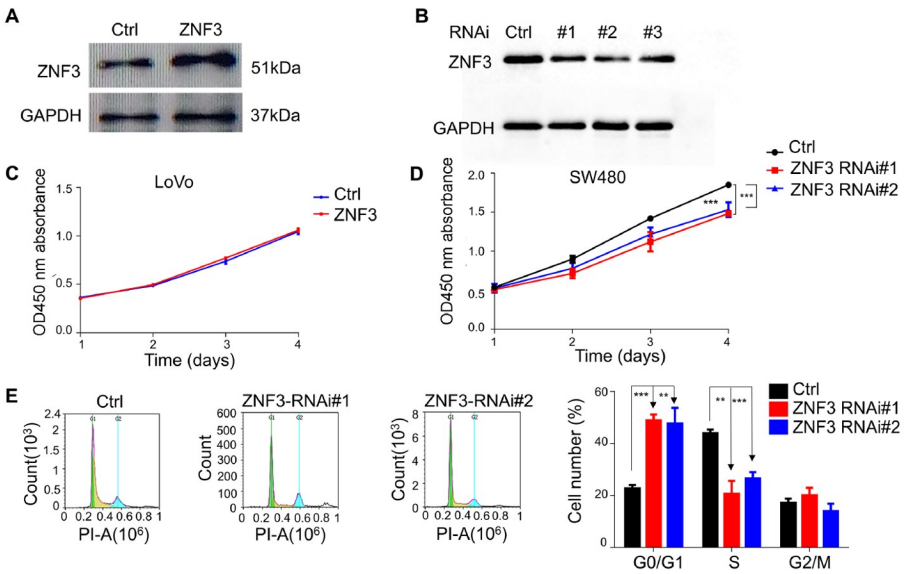

Figure 2. ZNF3 knockdown inhibits CRC cell proliferation (A,B) Western blot analysis was carried out to verify the efficacy of ZNF3 overexpression in LoVo cells and *ZNF3* knockdown in SW480 cells. (C) ZNF3 overexpression does not affect the proliferation of LoVo cells. (D) ZNF3 silencing reduced the proliferation of SW480 cells. (E) Cell cycle analysis in *ZNF3*-knockdown SW480 cells. Three independent experiments were conducted. Data are shown as the mean ± SD. $^{**}P < 0.01$, and $^{***}P < 0.001$.

Transwell assays. The results revealed that *ZNF3*-transfected LoVo cells showed an apparent increase in the number of migrated cells compared with control cells (Figure 3A,B). However, *ZNF3* knockdown by two shRNAs significantly reduced cell migration and invasion in SW480 cells (Figure 3C,D). Therefore, these results demonstrate that ZNF3 promotes CRC cell migration and invasion.

ZNF3 increases cell proliferation, migration, and invasion through EMT

To establish whether ZNF3 promotes CRC progression through EMT, the mRNA expression levels of EMT-related markers (*N-cadherin*, *Vimentin*, *TWIST*, *CK19*, *MMP1*, and *ZEB2*) were evaluated in ZNF3-transfected LoVo cells and ZNF3-knockdown SW480 cells. ZNF3 overexpression increased the *ZEB2* and *MMP1* expression levels in LoVo cells; however, no significant change was detected in the expressions of *Vimentin*, *N-cadherin* and *CK19* (Supplementary Figure S2B). The mRNA expression levels of *Vimentin*, *TWIST*, and *MMP1* were suppressed following *ZNF3* knockdown, and no difference was shown in *CK19* and *ZEB2* expression levels (Figure 4A). The changes of TWIST and MMP1 protein expression levels were consistent with the changes of their mRNA levels when *ZNF3* was silenced (Figure 4B). However, the protein expressions of other EMT markers (Vimentin, E-cadherin, CK19, and ZEB2) did not change after ZNF3 overexpression or knockdown (Figure 4B and Supplementary Figure 2C,D). These data indicate that ZNF3 regulates EMT progression possibly through TWIST and MMP1.

To further elucidate whether TWIST and MMP1 are associated with ZNF3-mediated CRC cell proliferation, migration and invasion, TWIST and MMP1 plasmids were transfected into *ZNF3*-knockdown SW480 cells. The TWIST and MMP1 overexpression efficiency was verified by western blot analysis (Figure 4C,D). Overexpression of MMP1 or TWIST not only promoted the proliferation of *ZNF3*-knockdown SW480 cells (Figure 5A–D) but also markedly accelerated the S phase cell cycle (Figure 5E,F). Moreover, in SW480 cells with ZNF3-RNAi#1 knockdown, only MMP1 transfection could promote cell invasion, while in SW480 cells with ZNF3-RNAi #2 knockdown, both TWIST and MMP1 overexpression reversed the ZNF3 knockdown-mediated ability of cell migration and invasion (Figure 6). These data indicate that ZNF3 functions as a positive regulator of TWIST and MMP1, which consequently stimulates CRC cell proliferation, migration and invasion.

Discussion

Based on the GeneCards database, ZNF3 is characterized as a kind of C2H2-type zinc finger protein with a KRAB domain. One previous study showed that most KRAB-ZNFs were commonly upregulated in cancer samples, and the expressions of cancer-associated KRAB-ZNFs were associated with patient survival, tumor histology, and

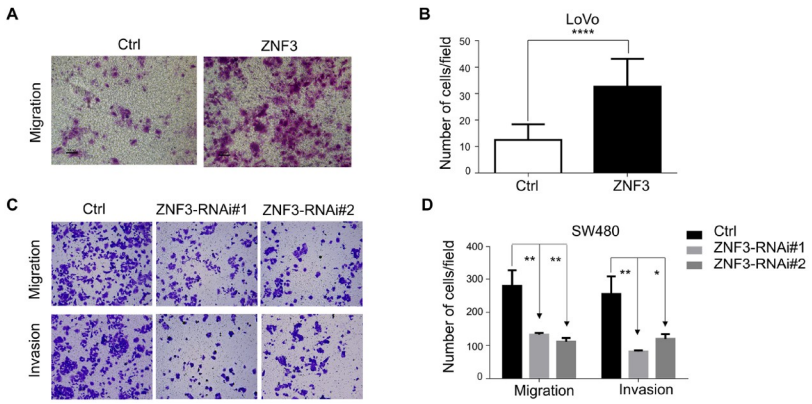

Figure 3. ZNF3 promotes CRC cell migration and invasion (A) Migration of LoVo cells was detected by Transwell assay (magnification, 100 ×). (B) Quantitative analysis of migrated cells is shown as the mean ± SD. (C) Migration and invasion of SW480 cells was detected by Transwell assay (magnification, 100 ×). (D) Quantitative analysis of migrated cells is shown as the mean ± SD. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$.

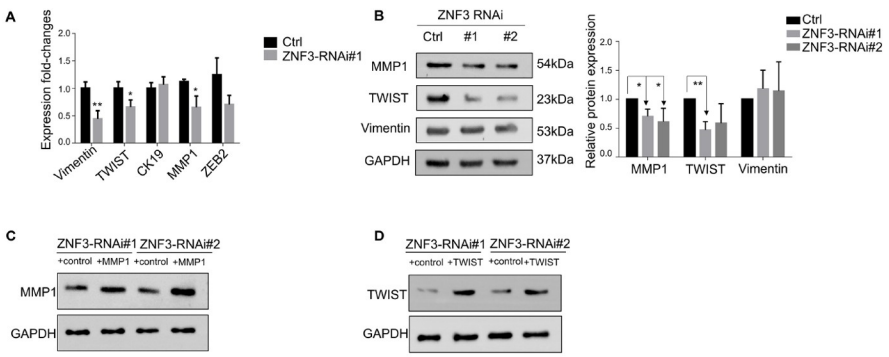

Figure 4. ZNF3 regulates the expressions of EMT-related markers (A) The mRNA expression levels of EMT-related markers (*Vimentin*, *TWIST*, *CK19*, *MMP1*, and *ZEB2*) were decreased in *ZNF3*-silenced SW480 cells. (B) The protein expression levels of EMT-related markers (Vimentin, TWIST, and MMP1) were decreased in *ZNF3*-silenced SW480 cells. (C) MMP1 was overexpressed in *ZNF3*-silenced SW480 cells. (D) TWIST was overexpressed in *ZNF3*-silenced SW480 cells. *$P < 0.05$, and **$P < 0.01$.

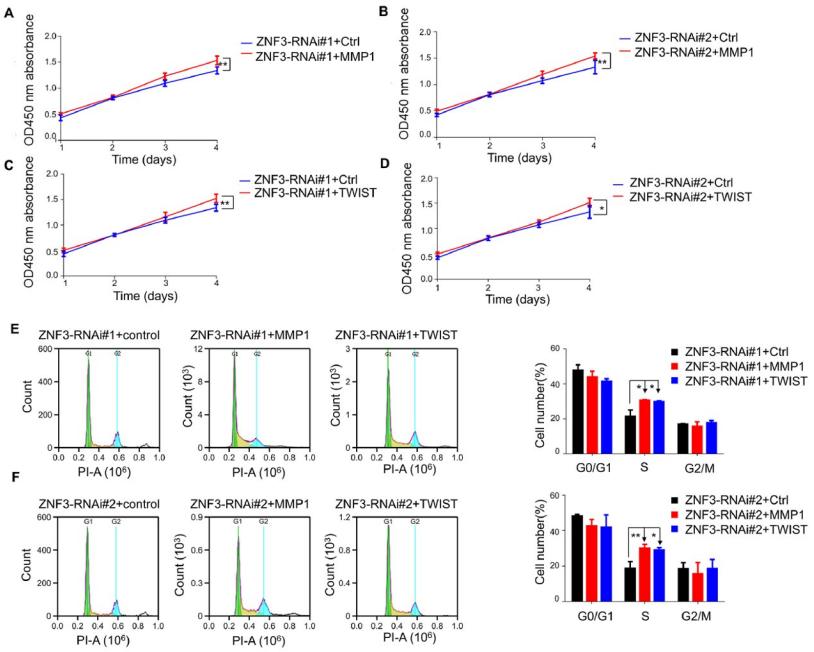

Figure 5. Overexpression of MMP1 and TWIST promotes the proliferation of *ZNF3*-knockdown SW480 cells (A,B) Effect of MMP1 on the proliferation of *ZNF3*-knockdown SW480 cells. (C,D) Effect of TWIST on the proliferation of *ZNF3*-knockdown SW480 cells. (E,F) Effect of MMP1 and TWIST overexpression on the cell cycle of *ZNF3*-knockdown SW480 cells. **P* < 0.05, and ***P* < 0.01.

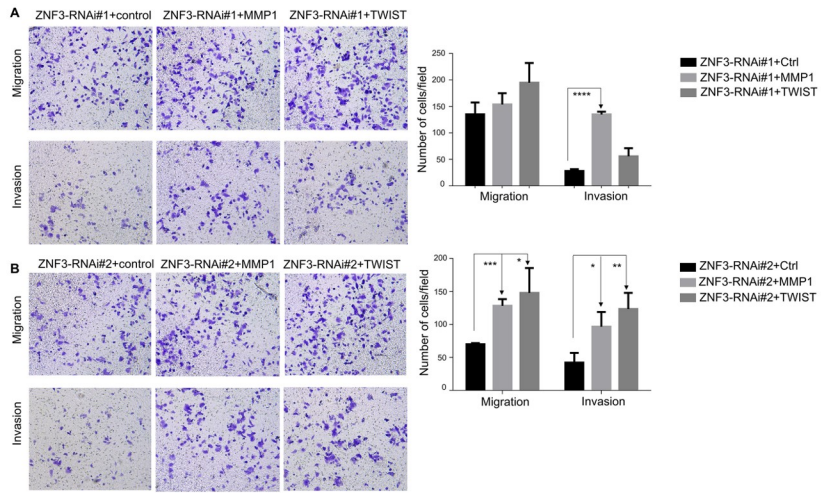

Figure 6. Overexpression of MMP1 and TWIST increases the migration and invasion of *ZNF3*-knockdown SW480 cells (A) Effects of MMP1 and TWIST on the migration and invasion of ZNF3-RNAi#1 SW480 cells (magnification, 100 ×). (B) Effects of MMP1 and TWIST on the migration and invasion of ZNF3-RNAi#2 SW480 cells (magnification, 100 ×). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

molecular subtyping [14]. Research on ZNF3 is limited. A previous study indicated that the expression of ZNF3 was related to the prognosis of breast cancer [12]. However, to date, no research has been conducted to investigate the association between ZNF3 and CRC. In this study, we first used *in vitro* experiments to evaluate the relationships between ZNF3 and CRC. ZNF3 was significantly upregulated in Pan-Cancer and CRC tissues based on the UCSC Xena database. IHC of tissue microarrays further revealed that the expression of ZNF3 was significantly higher in CRC tissues than in normal tissues, and the expression of ZNF3 was associated with the age of patients. Moreover, ZNF3 was highly expressed in CRC cells compared with that in normal intestinal cells. This observation is similar to the previous finding that the expression of ZNF3 was higher in the lung tissue of patients with moderate and severe emphysema than in normal lung tissue [11]. These results suggest that ZNF3 may be associated with CRC.

Numerous studies have demonstrated that zinc finger proteins regulate the occurrence and development of cancer [15]. Therefore, we hypothesized that ZNF3 may also be involved in the progression of CRC. Cell proliferation, migration and invasion are the three most important characteristics of cancer cell behavior [16]. Then, we instantaneously transfected LoVo cells with ZNF3 plasmid and stably knocked down *ZNF3* in SW480 cells using a lentivirus-mediated shRNA targeting assay. The results suggested that LoVo

cell proliferation capacity did not change after ZNF3 overexpression, while SW480 cell proliferation capacity was markedly decreased following *ZNF3* knockdown. We further detected the cell cycle by flow cytometry and found that SW480 cells showed G0/G1 phase arrest after *ZNF3* silencing. Therefore, these results imply that the inhibition of CRC cell proliferation capacity by *ZNF3* knockdown may be caused by G0/G1 phase arrest.

Metastasis is the main reason for cancer-related deaths [17]. EMT has been well studied in cancer metastasis. The regulation of EMT requires a strong transcription mechanism, consisting of a variety of transcription factors that orchestrate epithelial and mesenchymal markers [9]. Therefore, we hypothesized that ZNF3 could regulate the expressions of EMT-related markers. E-cadherin is an important epithelial marker, and its downregulation is a key feature of EMT, leading to unstable adherens junctions. The expression of E-cadherin is regulated at various levels, including transcriptional repression [18], promoter methylation [19], and protein phosphorylation and degradation [20], in response to the induction of various signals. Loss of E-cadherin expression is related to a poor prognosis in stage III CRC [21]. Another epithelial marker, cytokeratin 19 (CK19), is well acknowledged as a biliary/progenitor cell marker and a marker of tumor stem cells [22]. However, we found that E-cadherin and CK19 expression levels were not altered by ZNF3 overexpression or knockdown. As a key mesenchymal marker of EMT, the activation of Vimentin induces mesenchymal adhesion, thus promoting EMT development [23]. Here, our data suggested that *ZNF3* silencing significantly suppressed Vimentin expression at the transcription level but not at the translation level. The zinc finger E-box binding homeobox (ZEB) family of transcription factors, including ZEB1/ZEB2, TWIST1 and TWIST2, are important regulators of EMT [3,24]. Our results implied that *ZNF3* knockdown significantly reduced the expression of TWIST but not ZEB2. Additionally, some enzymes play an important role in EMT progression. MMPs are zinc-containing endopeptidases with extensive substrate specificity that degrade various components of extracellular matrix proteins and promote the growth and migration of cancer cells [25]. In this study, we found that MMP1 expression was markedly decreased following *ZNF3* knockdown. Therefore, we speculate that TWIST and MMP1 may be involved in ZNF3-promoted CRC migration and invasion.

To verify the above hypothesis, we further transfected TWIST and MMP1 vectors into *ZNF3*-knockdown SW480 cells, and the results showed that the migration and invasion capacity of SW480 cells were increased following TWIST and MMP1 overexpression. Interestingly, the proliferation ability of SW480 cells was significantly improved, and the cell cycle arrest of G0/G1 was relieved by TWIST and MMP1 overexpression. The possible reason is that MMP1 and TWIST not only play a role in tumor migration and invasion but also can promote tumor cell proliferation when they are overexpressed. Previous studies have shown that MMP1 derived from tumor-associated macrophages (TAMs) markedly facilitates colon cancer cell proliferation by accelerating the cell cycle transition from G0/G1 to S and G2/M phases [26]. The knockdown of *MMP1* in CRC cells (SW620 cells) resulted in reduced cell viability and migration [27]. In addition to its role in promoting CRC cell proliferation, MMP1 has also been shown to accelerate cell proliferation in other tumors. MMP1 is highly expressed in cutaneous squamous cell carcinoma (CSCC) cells, and *MMP1* silencing inhibits cell proliferation and invasion in CSCC. MMP1 is regulated by the NEAT1/miR-361-5p axis, which promotes CSCC malignant behaviors by Wnt pathway activation [28]. The transcription factor TWIST was reported to be correlated with multiple human malignancies. TWIST overexpression causes various biochemical changes, such as increased hepatic cancer cell proliferation, reduced apoptosis, cell cycle deregulation, hepatic cancer stem cell-like traits, and in some cases, drug resistance. These changes cause various physiological changes, such as angiogenesis, cellular migration and invasion, and vasculogenic mimicry, which ultimately leads to hepatocellular metastasis [29]. Additionally, the TWIST protein was highly expressed in gastric cancer samples. Silencing of TWIST significantly induced apoptosis, cell cycle arrest at G0/G1 phase, and proliferation inhibition and reduced migration and invasion in human gastric cancer cells [30]. TWIST gene silencing blocked IL-1β-induced proliferation and migration of gallbladder cancer (GBC) cells [31]. Therefore, our findings suggest that ZNF3 knockdown may suppress CRC proliferation, migration and invasion by downregulating the EMT-related markers TWIST and MMP1.

To further clarify the roles of ZNF3 in the occurrence and development of CRC, further experiments are still needed to elucidate the underlying mechanisms. In further studies, we will collect a larger set of CRC samples to clarify the expression conditions in CRC and analyze the relationship between ZNF3 expression and CRC metastasis and prognosis. More importantly, it is necessary to reveal the molecular mechanism by which ZNF3 promotes CRC cell malignant behaviors such as proliferation, migration and invasion. ZNF3 is a C2H2-type transcription factor. Transcription factors usually bind to some transcription cofactors to collectively regulate the expressions of downstream target genes [32,33]. Therefore, we will use chromatin immunoprecipitation followed by sequencing (ChIP-seq) technology combined with RNA sequencing (RNA-seq) to find the downstream target genes of ZNF3 and systematically explore the mechanism of the carcinogenic effect of ZNF3 in CRC through *in vivo* and *in vitro* experiments.

In summary, our study confirmed that ZNF3 was overexpressed in CRC tissues and cells. ZNF3 silencing inhibited the proliferation, migration and invasion of CRC cells by downregulating the EMT-related markers TWIST and MMP1 (Figure 7). Our findings imply that ZNF3 is an important transcription factor associated with CRC and may serve as a potential therapeutic target for CRC. Further studies should be carried out to verify these conclusions.

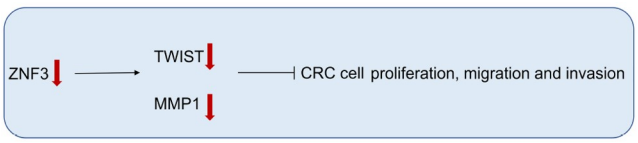

Figure 7. Schematic overview of ZNF3-mediated cellular malignant behaviors in CRC

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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