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Effects of DNA methyltransferase 2 (DNMT2) on gastric cancer cells proliferation and migration via regulation of structural maintenance of chromosomes 3 (SMC3)

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► ***Original article***

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ABSTRACT

Background: DNA methyltransferase 2 (DNMT2) is reported as an RNA modifier regulating the expression of oncogenes in cancers. This work explored the regulatory potential of DNMT2 in gastric cancer (GC) cell proliferation and migration. ***Materials and Methods:*** GC cells were induced with Transforming growth factor-beta1 (TGF-β1) for constructing DNMT2 overexpression model. GC cell proliferation was subject to Cell Counting Kit-8 (CCK-8) assay. GC cell migration was observed through wound scratch together with transwell migration assays. ***Results:*** The outcomes showed that the DNMT2 overexpression model was successfully built. Relative to the control, the levels of DNMT2 and SMC3 were apparently decreased by TGF-β1 stimulation, whereas E-cadherin, Smad2 as well as Vimentin expression was elevated by TGF-β1, and GC cell proliferation along with migration were significantly elevated. However, in comparison with the NC group, the DNMT2 overexpression group exhibited higher levels of DNMT2 and SMC3, significantly suppressed E-cadherin, Vimentin along with Smad2 expression, and significantly suppressed GC cell proliferation along with migration. ***Conclusion:*** Overexpression of DNMT2 inhibits GC cell proliferation along with migration. The level of SMC3 was also elevated by DNMT2 overexpression in GC cells. The findings of our study might provide the theoretical basis for the development of GC.

INTRODUCTION

Gastric cancer (GC) is the most common gastrointestinal tumor in China, with the highest mortality in malignancies [1,2]. Surgical resection combined with regional lymph node dissection is the only possible way to cure GC [3]. However, the clinical symptoms often appear at the advanced stage accompanied by distant metastasis, and the opportunity for surgical cure is lost. In recent years, biological therapy of cancer has become a hot topic. More and more studies have shown that the hypermethylation of some genes in the promoter region seriously affects gene expression, especially the inactivation of some oncogenes in tumorigenesis [4,5]. Specific DNA methyltransferases (DNMTs) are responsible for adding methyl groups to specific cytosine residues in the DNA promoter region, where DNMT2 plays a key role in maintaining DNA methylation in the adult cell genome [6]. Radiation treatment is revealed to be effective in GC treatment and the radiation treatment also induced the genomic changes such as DNMT2 [7-9]. Different from other DNMTs, the expression of DNMT2 is high in tumors, but low in GC [10,11]. DNMT2 is also reported to be involved in the recognition of DNA damage, DNA recombination, as well as mutation repair and is suggested as a promising target in cancer therapy [12,13]. This suggests that the high expression of DNMTs in tumors is not consistent, and whether the deregulation of DNMT2 is related to malignant development of GC needs further investigation.

Circular RNAs (CircRNAs) as a novel subtype of non-coding RNAs have been discovered for more than 40 years and attracted the increasing attention for their roles in cancer development [14]. The newly discovered CircRNA structural maintenance of chromosomes 3 (SMC3) is significantly elevated in GC cells, which may be closely linked to GC cell growth and metastasis [15,16]. SMC3 as a component of the cohesin complex is also demonstrated to be implicated in various cancer progression. For example, SMC3 methylation is identified in pancreatic ductal adenocarcinoma and high methylation level of SMC3 is related to unfavorable prognosis in pancreatic ductal adenocarcinoma [17,18]. SMC3 interacts with RIT1 and SMC3 knockdown inhibits the RIT1-mediated proliferation of hepatocellular

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carcinoma cells [19]. SMC3 is also required for the double-strand break repair and radiation can affect the copy number variation of SMC3 and may thereby impact cellular function [20]. Previous literature has revealed that epigenetic modification has a crucial role in affecting the immune microenvironment as well as regulating the occurrence of the immune microenvironment by regulating the expression of SMC3, thus promoting the cell proliferation and migration [21]. As one of the key enzymes of DNA methylation, DNA methyltransferase 2 (DNMT2) can modulate GC cell proliferation [11,22]. Whether SMC3 is mediated by DNMT2 in GC remains unclear. This work studied the DNMT2 expression pattern in GC cells and observed its influence on GC cells, which might deepen our understanding of the molecular basis in GC.

MATERIALS AND METHODS

Cell source

Human GC SGC-790 cells were provided by the Shanghai Cell Bank of the Chinese Academy of Sciences.

Main reagents

Dulbecco's Modified Eagle Medium (DMEM) serum (Hylcone), Trypsin (Multicell), Transwell chamber (Coming), CCK-8 kit (Biosharp), Bicinchoninic Acid Assay (BCA) kit (Biyuntian), fetal bovine serum (FBS, Gibco,USA), TRIzol reagent (Invitrogen), Reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR) kit (Ecorui Bio), Rabbit primary antibodies glyceraldehyde-3-phosphate dehydrogenase (GAPDH), E-cadherin, Vimentin, Smad2 and SMC3 (Proteintech), Rabbit primary antibody DNMT2 (Abcam), Rabbit derived secondary antibody (Bioworld). The primer sequence was synthesized by Shanghai Bioengineering Company, as seen in table 1.

Table 1. Primer sequence in PCR

Primer	Forward	Reverse
DNMT2	5'-TGGCCACTGCT GCTTCCTCTTCTT-3'	5'-GGGGCCAGCTT CGTCATACTCCT-3'
E-cadherin	5'-GCAATGCTGA ATCGTCCCAC-3'	5'-CAGCACAGGC CCTCAAAAAC-3'
Smad2	5'-GGCATCCACG AAACCACCTA-3'	5'-GTATGCGTGTG ACGGCTCTA-3'
Vimentin	5'-GAGAACTACCTT CCGCTGTATCGC-3'	5'-GAGAACTACCTT CCGCTGTATCGC-3'
SMC3	5'-CTGGACTCATTG GACTGGCTGTG-3'	5'-AGGAAGGCGG TGGTGAGGATG-3'
GAPDH	5'-CTGGAGAAACC TGCCAAGTATG-3'	5'-GGTGGAAGAA TGGGAGTTGCT-3'

Main instruments

Cell culture ultra-clean workbench (Suzhou Purification), Autoclave (Boxun); Inverted fluorescence microscope (Zeiss), CO_2 Incubator (Thermo Feld), real-time fluorescence quantitative PCR instrument (Bio-Rad), enzyme labeling instrument (Varioskan LUX), Cryogenic centrifuge (Ebend, Germany), Electronic balance (Sartorius).

Cell culture

High-sugar DMEM including 10% fetal bovine serum was used for SGC-790 cell incubation at 37°C in a 5% CO_2 incubator until the bottom of the bottle was flat. Then the culture medium was passed and maintained for five generations and then the follow-up experimental study was carried out.

Transforming growth factor-beta1 (TGF-β1) induced GC cells

Human GC cells (SGC-7901) were induced by TGF-β1. SGC-7901 cells were plated into 6-well plates at 30% density and incubated overnight in a 5% CO_2 incubator at 37°C. On the second day, serum-free DMEM containing 5 μg/L TGF-β1 was added and cultured for another 24 hours. Cells were collected and RNA and total protein samples were extracted.

DNMT2 lentivirus transfection

The infection method and parameters were first verified by pre-experiment according to the Gemma gene lentivirus operation manual. GC cells at logarithmic phase were digested, counted, and then inoculated onto a 6-well plate. The plate was secured to the wall and then stimulated with 5 μg/L TGF-β1 for 24 hours to activate the infection. DNMT2 lentivirus was mixed and diluted with serum-free medium with a titer of 30 multiplicity of infection (MOI) and polycoagulant (5 mg/L) was supplemented to improve transfection efficiency. Meanwhile, a negative control group was established with the same virus titer and MOI. After a day, the fresh medium was provided and fluorescence was detected using a fluorescence microscopy 72 hours after infection. The results were used in the follow-up experiment.

Real-time reverse transcriptase-polymerase chain reaction (RT-qPCR)

After the cells were treated as described above, TRIzol reagent (1 mL) was added to each well to gradually extract RNA. RNA concentrations were examined at a wavelength of 260 nm and reverse-transcribed into cDNA via a kit (Acres) while keeping concentrations no higher than 50 mg/L. The liquid was mixed according to the requirements of the qPCR kit (Acres) and the mRNA was calculated by 2-M method normalized to GAPDH. The relative mRNA expression was measured by the same method.

Western blot

After cell treatment, Radio Immunoprecipitation

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Assay (RIPA) lysis buffer was used for extracting protein and the protein concentration was assessed using BCA method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein electrophoresis was conducted, the membrane was transferred and blocked using nonfat milk. After washing, the membrane was incubated with primary antibodies at 4°C overnight. The membrane was removed and washed the next day, followed by incubation with the second antibody at room temperature for 1 h. Followed by washing, the membrane was developed by enhanced chemiluminescence (ECL) method. Results Image J software was used for gray value analysis normalized to GAPDH, and the experiment was repeated 3 times.

Cell proliferation measurement

The cell suspension was prepared by digesting logarithmic growth cells with trypsin. GC cells (2000 cells in each well) were inoculated with into 96-well plates and incubated at 37°C for 4 hours. After wall attachment, follow-up treatment was performed. Each group was equipped with 6 multiple wells and the edge holes were filled by sterile phosphate buffer saline (PBS). After lentivirus transfection, CCK-8 solution was supplemented into the plates (10 μL per well), and maintained in the incubator for 2 hours. After that, the absorbance at 450 nm was evaluated by enzymograph. The experiment was repeated 3 times.

Cell wound scratch assay

GC cells after indicated treatment were spread on a 6-well plate. After 48 hours, GC cells were scratched using a 200 μL sterile gun head on a vertical bottom parallel line. After washing by PBS thrice, cells were added in DMEM (serum-free) and placed in a 5% CO_2 incubator at 37°C. The variation of wound width at 0 h and 24 h was observed by inverted microscope and the scratch area was calculated by Image Mobility 1% = (initial scratch area - scratch area after 24 hours)/ initial scratch area ×100%, 3 fields of view were randomly selected for each hole and each hole was repeated 3 times.

Transwell migration assays

DMEM (serum free) was added 1 hour in advance in the lower chamber of Transwell for basement membrane hydration. The cells in each group were digested and centrifuged with pancreatic enzymes and then suspended in DMEM and diluted to 5×10^8/L. Next, 100 μL suspension was inoculated in the top chamber and complete culture media were supplemented into the bottom chamber, and incubated for 30-40 hours. After washing, cells were fastened using 4% paraformaldehyde at ambient temperature for 30 minutes, followed by dyeing using 0.1% crystal violet for 20 minutes. The cells on the upper ventricular membrane were cleared. After washing, 5 visual fields were randomly selected for observation. The counting analysis was carried out with ImageJ.

Statistical analysis

SPSS 21.0 software was adopted for analysis of data and results are shown as the (x ± s). One-way analysis of variance was adopted for analyzing the statistical differences among multiple groups, while SNK-q test was performed for further two-group comparisons. $P < 0.05$ indicated statistically significant differences.

RESULTS

DNMT2 expression in GC cells

After TGF-β1 stimulation on GC cells, the content of DNMT2 mRNA in GC cells decreased, as shown in figure 1. After 72 h infection with DNMT2 lentivirus with MOI value of 30, the results displayed that DNMT2 levels in the virus infection group was significantly enhanced at the mRNA and protein levels ($P < 0.01$), which indicated that the virus infection was successful and the DNMT2 overexpression model was successfully constructed.

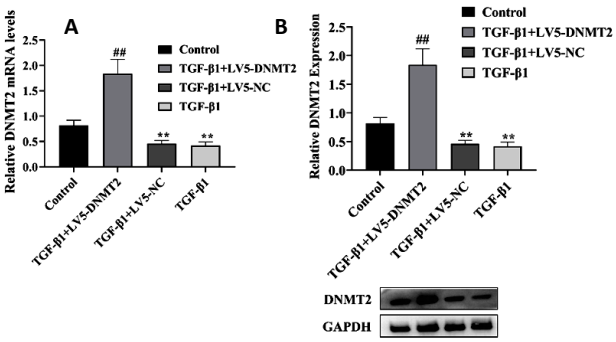

Figure 1. DNMT2 mRNA and protein expression after TGF-β1 treatment and lentivirus infection in GC cells (x% ± s, n = 3). **A:** qPCR measured the DNMT2 level in GC cells, **B:** Western blot detected DNMT2 protein expression, **P < 0.01 vs Control; ##P < 0.01 vs NC

DNMT2 regulates the E-cadherin, Vimentin and Smad2 mRNAs

The mRNA contents of E-cadherin, Smad2 as well as Vimentin were elevated after TGFβ1 stimulation relative to normal group in GC cells ($P < 0.05$). After DNMT2 lentivirus infection, the content of these mRNAs was significantly decreased in comparison with the NC ($P < 0.01$, figure 2).

DNMT2 regulates E-cadherin, Vimentin and Smad2 protein expression

The contents of E-cadherin, Vimentin as well as Smad2 proteins were reduced after TGF-β1 stimulation in comparison with the Control group ($P < 0.01$). After DNMT2 lentivirus infection, the levels of E-cadherin, Vimentin and Smad2 proteins were elevated relative to NC group ($P < 0.01$, figure 3).

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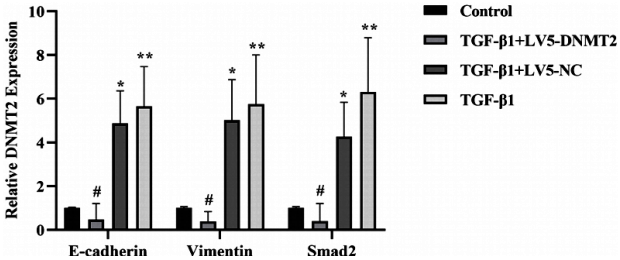

Figure 2. Regulation of DNMT2 on E-cadherin, Vimentin and Smad2 mRNA expression in TGF-β1-treated GC cells (x% ± s, n = 3). qPCR measured the mRNA level of E-cadherin, Vimentin and Smad2 in GC cells. *P < 0.05, **P < 0.01 relative to Control, #P < 0.05 relative to NC.

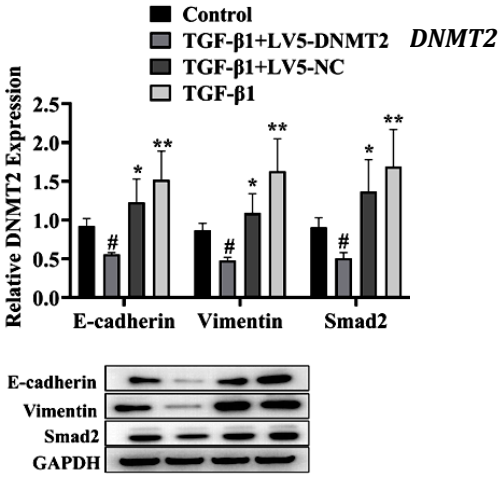

Figure 3. Regulation of DNMT2 on E-cadherin, Vimentin and Smad2 proteins in TGF-β1-treated GC cells (x% ± s, n = 3). Western blot measured the E-cadherin, Smad2 and Vimentin level in GC cells. *P < 0.05, **P < 0.01 relative to Control, #P < 0.05 relative to NC.

regulates SMC3 mRNA in GC cells

Then we investigate the potential regulation of DNMT2 on SMC3 in GC cells. As shown in figure 4, the content of SMC3 mRNA was decreased by TGF-β1 stimulation in GC cells (P < 0.01). However, DNMT2 lentivirus infection was revealed to significantly upregulate SMC3 mRNA content relative to NC group (P < 0.05).

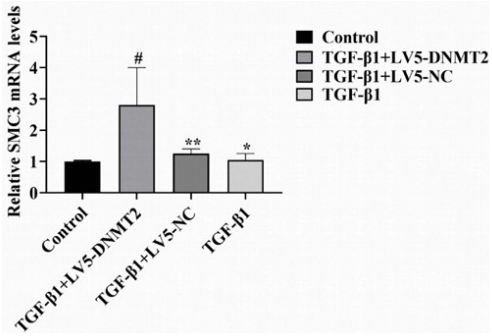

Figure 4. Regulation of DNMT2 on SMC3 mRNA expression in TGF-β1-treated GC cells (x% ± s, n = 3). qPCR measured the SMC3 mRNA level in GC cells. *P < 0.05, **P < 0.01 relative to Control, #P < 0.05 relative to NC.

DNMT2 regulates the expression of SMC3 protein

As shown in figure 5, SMC3 protein content was also decreased by TGF-β1 treatment in GC cells (P < 0.01). After DNMT2 lentivirus infection, SMC3 protein content was also elevated by overexpressing DNMT2 in GC cells (P < 0.01).

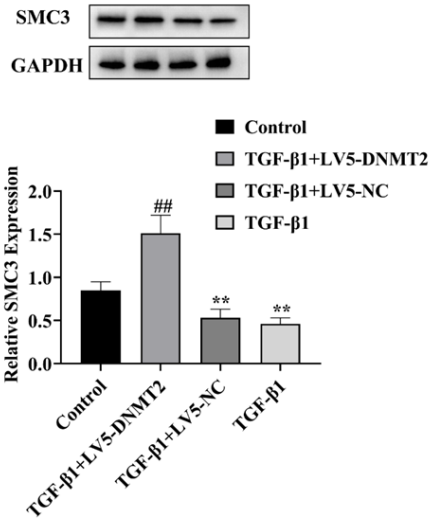

Figure 5. Regulation of DNMT2 on expression of SMC3 protein in TGF-β1-treated GC cells (x% ± s, n = 3). Western blot was performed to measure the protein levels of SMC3 in GC cells. **P < 0.01 relative to Control, ##P < 0.01 relative to NC.

DNMT2 regulates GC cell proliferation

We then investigated the function of DNMT2 in GC cells. Based on the results of CCK-8 assays, GC cell proliferation was significantly enhanced by TGF-β1 stimulation (P < 0.01). After DNMT2 lentivirus infection, the cell proliferation activity was evidently reduced when comparing with the NC (P < 0.01), suggesting that GC cell proliferation activity was inhibited after DNMT2 overexpression.

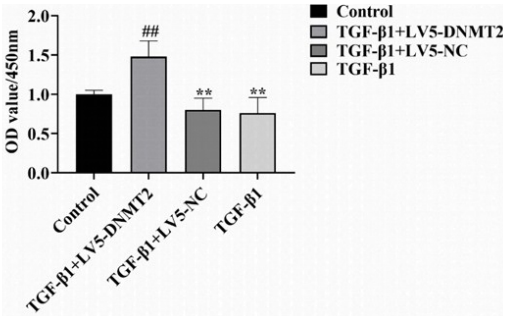

Figure 6. DNMT2 inhibits the proliferation of TGF-β1-treated SGC-790 cells (x% ± s, n = 6). CCK-8 assays evaluated the proliferation capacity of SGC-790 cells after TGF-β1 stimulation and lentivirus infection. **P < 0.01 relative to Control, ##P < 0.01 relative to NC.

DNMT2 regulates GC cell migration

According to the outcomes in Transwell assays, GC cell migration ability was elevated followed by TGF-β1 stimulation relative to normal group (P < 0.01). However, the number of migrated TGF-β1-stimulated GC cells was significantly reduced after DNMT2 lentivirus infection, when comparing with NC group (P < 0.01), suggesting that cell migration ability was

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also inhibited after DNMT2 overexpression (figure 7A). Consistently, scratch experiments also confirmed that the wound healing rate of GC cells increased by TGF-β1 stimulation, which was also reversed by DNMT2 lentivirus infection ($P < 0.01$, figure 7B).

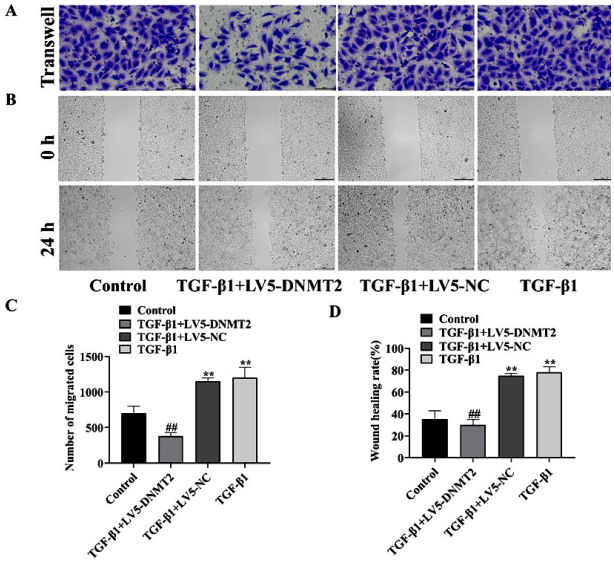

Figure 7. Regulation of DNMT2 on the migration ability of TGF-β1-treated SGC-790 cells (x% ± s, n = 5). **A:** Representative results of transwell migration (× 200). **B:** Representative results of wound healing (× 200). **C:** Statistical analysis of the influence of DNMT2 on SGC-790 migration ability in the Transwell assay. **D:** Statistical analysis of the influence of DNMT2 on SGC-790 on the migration in the wound healing assays. **P<0.01 relative to Control, ##P<0.01 relative to NC.

DISSCUSION

DNMT2 is a member of the DNMT family, and it has both DNA methyltransferase activity [23] and tRNA aspartic acid methyltransferase 1 (TRDMT1) effect [24], thus its biological function is complicated and controversial. As the former, its mediated DNA methylation may play a role in silencing retrotransposons [25]. As the latter, its mediated tRNA methylation may be implicated in modulating plant and animal growth [26,27] and also cellular stress tolerance [28]. However, in general, the expression pattern and function of DNMT2 are rarely studied, especially in tumors. In our work, we demonstrated the down-regulation of DNMT2 in TGF-β1-treated GC cells and revealed the anti-tumor potential of DNMT2 on GC cell malignancy.

Previous studies of our research group have found that DNA methylation is closely linked to GC cell proliferation and can be implicated in modulating the GC cell proliferation activation and proliferation [29]. Additionally, numerous studies have indicated that TGF-β1 can induce GC cell migration potential as well as the epithelial-mesenchymal transition (EMT) [30-33]. Consistently, in this study, E-cadherin, Vimentin along with Smad2 expression was elevated by TGF-β1 stimulation in GC (SGC-790) cells (figure 3A), and the proliferative ability as well as the migrating capacity of GC cells were enhanced by TGF-β1 (figures 6-7), which indicated that the activation and proliferation model of GC cells was successfully induced by TGF-β1. Previous research has also revealed that DNMT2 is related to cell proliferation and migration via diverse mechanisms and is a promising target for limiting cancer cell growth [29,34]. Additionally, DNMT2 silencing is involved in the regulation of mRNA methylation and suppresses the migration and invasion of HEK293 cells [35]. Different from previous findings, we found that DNMT2 was lowly expressed in response to TGF-β1 stimulation in GC cells (figure 1A-B), and its overexpression inhibited GC cells proliferation, migration as well as the EMT process (figures 6-7). These outcomes suggest that DNMT2 may have a crucial role in activated GC cells.

Xia *et al.* [21] have reported that CircSMC3 can be adopted to be a new potential circulating biomarker for the detection of GC, offering new ideas for GC treatment. Increasing evidence has demonstrated the involvement of SMC3 in various cancers. For example, downregulation of SMC3 induced by hydrogen gas suppresses lung cancer cell proliferation, migration and invasion. SMC3 is reported with prognostic value in hepatocellular carcinoma [36]. SMC3 is indicated to be upregulated in colorectal cancer and overexpression of SMC3 is related to the elevated expression of oncogenic mediators in 293 cells [37]. Recent studies have shown that DNA methylation regulation of the microenvironment has a crucial role in GC, but whether DNMT2 regulates the SMC3-mediated tumor microenvironment to accelerate the activation and proliferation of GC cells has not been reported. In order to further unveil the relationship between DNMT2 and SMC3, in this work, after lentivirus infection with overexpression of DNMT2, the content of SMC3 was also decreased in TGF-β1-treated GC cells, and this reduction was significantly reversed by DNMT2 lentivirus infection (figure 4-5). Contrary to the previous studies, these findings also suggested the anti-tumor activity of SMC3 in GC. It is suggested that overexpression of DNMT2 can increase SMC3 expression, and effectively reduce GC cells proliferation along with migration. The outcomes suggest that DNMT2 has a critical role in modulating SMC3 expression, and DNMT2 may repress GC cells proliferation along with migration by influencing SMC3.

In conclusion, DNMT2 inhibits the proliferative, migrating abilities and EMT in TGF-β1-treated GC cells by modulating the SMC3, thus suppressing the development of GC, which might provide the theoretical basis for gene targeted therapy of GC.

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Ethical consideration: Not applicable.

Author contribution: T.X. and Z.Z.: conception and design, performed the experiments and data analysis, draft the manuscript; X.G.: conception, data analysis, revision. All authors have read and approved the final version of the manuscript.

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