

c-Myc maintains the self-renewal and chemoresistance properties of colon cancer stem cells

HUAN-LE ZHANG¹, PING WANG², MIAO-ZHEN LU¹, SAN-DIAN ZHANG¹ and LU ZHENG¹

¹Department of Oncology, Lihuili Hospital of Ningbo Medical Center; ²Department of Molecular Biology, Ningbo University School of Medicine, Ningbo, Zhejiang 315000, P.R. China

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Abstract. Cancer stem cells (CSCs) are responsible for cancer formation, recurrence and drug resistance. c-Myc, one of the core markers for stem cells, has recently been considered to serve as a link between malignancy and 'stemness'. However, the precise function of c-Myc in colon CSCs is still unclear. In the present study, a subpopulation of colon CSCs expressing a CD133 surface phenotype was isolated from the human HT-29 cell line, which possess greater tumor sphere-forming efficiency and have higher expression of 'stemness'-associated genes compared with CD133-negative cells. Furthermore, it was demonstrated that c-Myc was highly expressed in CD133⁺ colon CSCs. Knockdown of c-Myc expression with small interfering RNA in colon CSCs can significantly inhibit tumor sphere formation, reduce the invasive and migratory capacity of CD133⁺ cells *in vitro*, and suppress the tumorigenicity of colon CSCs *in vivo*. In addition, it was suggested that c-Myc silencing may sensitize colon CSCs to chemotherapy-induced cytotoxicity via the downregulation of ABCG2 and ABCB5. These findings support a central role for c-Myc in maintaining the self-renewing and chemoresistant properties of colon CSCs.

Introduction

Colon cancer was the third leading cause of malignancy-associated deaths in America in 2014, and represents a major therapeutic challenge (1). Despite advances in therapy, including surgery and chemotherapy, tumor recurrence and metastasis cannot yet be effectively prevented (2). Accumulating evidence suggests the presence of a small subpopulation of cells, known as cancer stem cells (CSCs), in colon cancer that exhibit stem-like features, and promote tumor formation, metastasis and resistance to therapy (3-6). Therefore, therapies targeting

colon CSCs may be a promising approach to eradicating colon cancer (7-9).

Higher expression of c-Myc has been found in colon cancer cells, relative to in normal mucosa, suggesting that c-Myc is involved in tumor development and progression (10). Recently, c-Myc has been recognized as an important regulator of stem cell biology (11); thus, it may serve as a link between malignancy and 'stemness'. Previous studies have shown that c-Myc has a key role in regulating the self-renewal and survival of glioma CSCs, and targeting c-Myc pathways may significantly improve brain tumor therapies (12). It is also reported that c-Myc can contribute to the multidrug resistance profile by dysregulating the transcription of specific ABC transporter genes in cancer cells (13,14). However, c-Myc gene regulation mechanisms underlying the maintenance of self-renewal and drug-resistant properties in colon CSCs are still unclear.

In a previous study (6), a subpopulation of colon CSCs expressing a CD133 surface phenotype were isolated from the human HT-29 colonic adenocarcinoma cell line using flow cytometry cell sorting (FACS). CD133⁺ cells possess a greater tumor sphere-forming efficiency *in vitro* and higher tumorigenic potential *in vivo*. Furthermore, CD133⁺ cells are endowed with stem cell-like properties, including the expression of 'stemness' genes, such as Wnt2, BMI1, Oct3/4, Notch1 and c-Myc, and the maintenance of self-renewal and differentiation capacities. The miRNA expression signature of colon CSCs was revealed in our previous study (6).

In the present study, it was further demonstrated that c-Myc was consistently overexpressed in colon CSCs. Additionally, it was shown that downregulation of c-Myc suppressed the self-renewal of colon CSCs and the growth of xenografts. Furthermore, it was suggested that the depletion of c-Myc may enhance the chemosensitivity of colon CSCs through the downregulation of ABCG2 and ABCB5 expression. The present study demonstrated that the expression of c-Myc has a crucial role in maintaining the self-renewal and chemoresistance of colon CSCs.

Materials and methods

Cell culture and colon sphere formation. The human HT-29 colonic adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was maintained in McCoy's 5A medium

Correspondence to: Dr Huan-Le Zhang, Department of Oncology, Lihuili Hospital of Ningbo Medical Center, 57 Xingning Road, Ningbo, Zhejiang 315000, P.R. China
E-mail: zhl520@163.com

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(Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO₂, and the medium was changed every 3 days. Cells were passaged at 80% confluence and seeded at 20% confluence to keep them at optimal proliferating conditions.

For colon sphere formation, single-cell suspensions were cultured in a DMEM/F-12 basal serum-free medium (Gibco; Thermo Fisher Scientific, Inc.), containing 2 mM L-glutamine, 1 mg/ml NaHCO₃, 4 µg/ml heparin, 100 µg/ml transferrin, 25 µg/ml insulin, 30 nM sodium selenite anhydrous and 20 nM progesterone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and supplemented with 20 ng/ml pro-epidermal growth factor (EGF; R&D Systems China Co., Ltd., Shanghai, China) and 10 ng/ml fibroblast growth factor 2 (FGF-2; R&D Systems China Co.).

In chemoresistance experiments, colon CSCs and adherent HT-29 cells were exposed to 50 µM 5-FU (Sigma-Aldrich; Merck KGaA) or 1.25 µM oxaliplatin (Sigma-Aldrich; Merck KGaA) or FOLFOX (50 µM 5-FU plus 1.25 µM oxaliplatin) for 72 h, and then observed by inverted phase-contrast microscope.

FACS. For the isolation of CD133⁺ and CD133⁻ populations, single-cell suspensions were incubated with phycoerythrin (PE)-conjugated anti-human CD133/1 (1:1,000; 130-108, AC133 clone; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and FcR blocking reagent (Miltenyi Biotec GmbH) in staining solution containing 1% BSA and 2 mM EDTA for 10 min at 4°C. Isotype-matched mouse immunoglobulin served as the control. Samples were analyzed and sorted with a FACS Aria flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For the positive and negative populations, only the top 10% most brightly stained cells or the bottom 10% most dimly stained cells were selected, respectively. Following cytofluorimetric sorting, viability was assessed using trypan blue exclusion; cell purity was controlled via flow cytometry with an antibody against CD133/2-phycoerythrin (1:1,000; 130-090-853, 293C3 clone; Miltenyi Biotec GmbH) in staining solution containing 1% BSA and 2 mM EDTA for 10 min at 4°C.

Small interfering RNA (siRNA) transfection. siRNA specific to c-Myc (sc-29227; sense, 5'-CAGAAAGUCCUGAGCAAUUU-3' and antisense, 5'-AUUGCUCAGGACAUUUCU GUU-3') and a scrambled siRNA (sc-37007; sense, 5'-GACUUCAUAAGGCGCAUGCUU-3' and antisense, 5'-GCAUGC GCCUUAUGAAGUCUU-3') were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Sorted CD133⁺ HT-29 cells (5 × 10⁵) were seeded into 6-well plates in SFM. After 24 h, siRNA was transfected into CD133⁺ cells at a final concentration of 100 nM using LipofectamineTM RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The cells were collected for a series of experiments at 48 h after transfection.

In vivo tumorigenicity. Transfected cells (5 × 10⁵) were resuspended in 50 µl PBS, and cell aliquots were diluted 1:1 with growth factor-reduced Matrigel matrix (BD Biosciences) before injection. A total of 10 BALB/c nude female mice

(6 weeks old), weighing 18-22 g, were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). The mice were maintained under a 12 h light/dark cycle at an ambient temperature of 24 ± 1°C in a humidity-controlled environment in a specific pathogen-free animal facility at the Center for Animal Experimentation, Medical Institute of Shanghai Jiao Tong University (Shanghai, China). Sterilized water and γ-irradiated diet were provided to animals *ad libitum*. Mice were subcutaneously injected with post-transfected cells, and the mice were observed every 2 weeks. The duration of the experiments was 8 weeks following transplantation, after which all mice were anesthetized with 1% pentobarbital sodium (40 mg/kg) and euthanized by cervical dislocation. Every mouse had one tumor, which was excised and measured. Each group consisted of 5 mice.

Western blotting. Protein was extracted with protein extraction kit (MinuteTM Protein Extraction Kit; SD-001; Invent Biotechnologies, Inc., Plymouth, MN, USA), and the protein concentration was detected using the bicinchoninic acid (BCA) Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Subsequently, 20 µg protein was separated via 10% SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, and blocked in 5% skimmed milk at 20°C for 1 h. The following primary antibodies were added to membranes at a dilution of 1:1,000 at 4°C for 24 h: Bmi1 (sc-13519; Santa Cruz Biotechnology, Inc.), ABCG2 (sc-13519; Santa Cruz Biotechnology, Inc.), ABCB5 (sc-517565; Santa Cruz Biotechnology, Inc.), Sox2 (sc-365964; Santa Cruz Biotechnology, Inc.), Wnt2 (sc-5208; Santa Cruz Biotechnology, Inc.), Oct3/4 (sc-5279; Santa Cruz Biotechnology, Inc.), CD133 (130-108-062, AC133 clone; Miltenyi Biotec GmbH), c-Myc (ab32072; Abcam, Cambridge, MA, USA), β-actin (ab1801; Abcam) and GAPDH (ab8245; Abcam). The membrane was then incubated with a horseradish peroxidase-labeled secondary antibody (1:2,000; A0216; Beyotime Institute of Biotechnology) at room temperature for 1 h. The membrane was visualized using an enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and analyzed using a gel analyzer (Bio-Rad Laboratories, Inc.).

Chemo-sensitivity assays. Chemosensitivity assays were conducted using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Sorted CD133⁺ HT-29 cells were seeded into 96-well plates at a density of 4 × 10³ cells/well in 100 µl SFM overnight, and then transfected with 100 µM of an indicated siRNA. For the chemosensitivity assay, post-transfected (48 h) cells were exposed to 5-FU (50 µM), oxaliplatin (1.25 µM) and FOLFOX (50 µM 5-FU plus 1.25 µM oxaliplatin). After incubation for 72 h, a CCK-8 assay was performed, and the survival rate of cells was calculated as follows: OD treatment/OD control × 100. Experiments were performed in triplicate.

Migration and invasion assays. Migration and invasion assays were performed in 24-well Transwell chambers with 8-mm pore polycarbonate filter inserts (Corning Inc., Corning, NY, USA). A total of 5 × 10⁴ post-transfected cells were seeded on uncoated or Matrigel-coated inserts in 500 µl serum-free

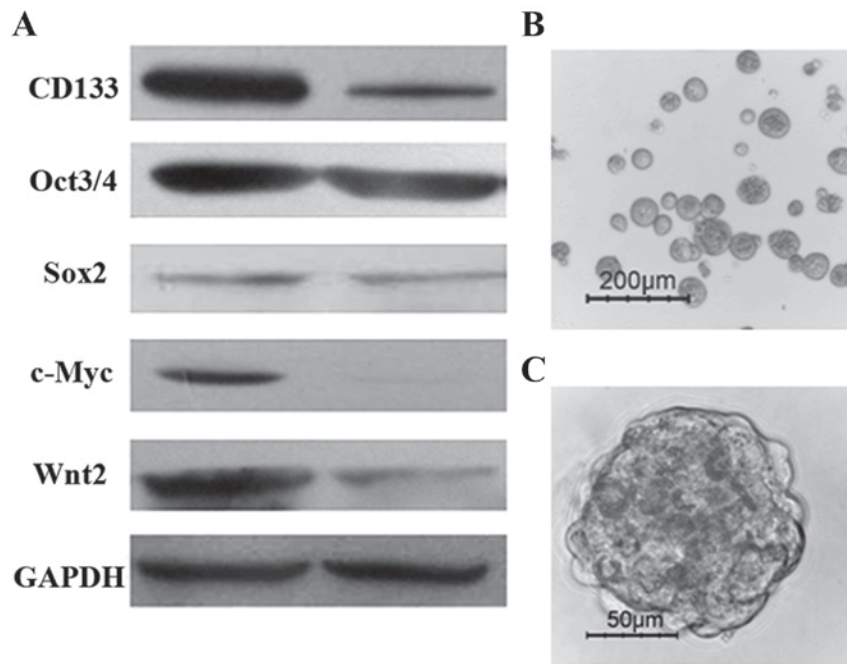


Figure 1. Isolated CD133⁺ HT-29 cells exhibit stem-like features *in vitro*. (A) 'Stemness' genes, c-Myc, Sox2, Oct4, CD133 and Wnt2, were highly expressed in CD133⁺ cells, as assessed by western blotting; lane 1, CD133⁺ cells; lane 2, CD133⁻ cells. (B and C) CD133⁺ colon cancer cells were expanded *in vitro* as undifferentiated spheres in serum-free medium, as observed by inverted phase contrast microscopy after 2 weeks of culture.

medium for migration or invasion assays, respectively. The lower chambers were filled with 500 µl 10% FBS-supplemented DMEM/F12 medium. After 48 h, cells on the upper side of the filter were removed, and the cells on the lower surface of the insert were fixed with 4% paraformaldehyde (500 µl) at room temperature for 20 min, washed twice with PBS (500 µl) and stained with crystal violet (400 µl) at 37°C for 20 min. The number of stained cells was counted in 3 randomly selected fields of view under a light microscope using a x10 objective (Nikon Corporation, Tokyo, Japan). Assays were performed in triplicate.

Statistical analysis. SPSS 19.00 software (IBM Corp., Armonk, NY, USA) was used to analyze the data in the present study. Data are presented as the mean ± standard deviation and differences between 2 groups were compared using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CD133⁺ cells exhibit stem-like features *in vitro*. It was previously proven that CD133 is a powerful marker for detecting colon CSC subpopulations in the human HT-29 colonic adenocarcinoma cell line (6). In the present study, CD133⁺ and CD133⁻ cells were purified from the HT-29 cell line using FACS. To explore the molecular features of colon CSCs, the expression of genes involved in stem cell-associated pathways was detected by western blotting. As shown in Fig. 1A, the expression of 'stemness' genes, such as c-Myc, Sox2, Oct4, CD133 and Wnt2, was higher in CD133⁺ cells compared with that in CD133⁻ cells. As has been reported previously (6), isolated CD133⁺ cells were able to generate tumor spheres when cultured for 10-14 days

in serum-free stem cell medium supplemented with 20 ng/ml EGF and 10 ng/ml FGF-2. Floating sphere-like cellular aggregates were formed, as shown in Fig. 1B and C. Additionally, colon cancer spheres had a larger volume and more compact sphere structure following the third cell-culture passage (data not shown). The results demonstrated that CD133⁺ HT-29 cells exhibit stem-like properties, including the expression of 'stemness' genes and self-renewal capacity.

Depletion of c-Myc attenuates tumor sphere formation among colon CSCs. Tumor sphere generation *in vitro* is indicative of self-renewal potential (5,9). To investigate whether c-Myc regulates the self-renewal capacity of colon CSCs, c-Myc expression was downregulated in CD133⁺ colon CSCs using siRNA, and the results showed that c-Myc-siRNA cells formed smaller and fewer tumor spheres than the scramble-siRNA control and CD133⁺ counterparts when cultured in serum-free stem cell medium (Fig. 2A). On the other hand, the c-Myc-siRNA group demonstrated lower Bmi1 expression levels. These data reveal that c-Myc serves an important role in regulating the self-renewal ability of colon CSCs cells, partly through regulating Bmi1.

Knockdown of c-Myc inhibits the invasion and migration potential of colon CSCs. Invasive and migratory abilities are critical features of CSCs; thus, it was examined whether c-Myc affected these features in colon cancer. Here, *in vitro* assays were conducted to evaluate migratory and invasive capacities. A Transwell assay found that c-Myc-siRNA cells display a significant decrease in cell motility relative to their scramble-siRNA counterparts ($P < 0.01$, Fig. 2B). Additionally, significantly fewer c-Myc-siRNA cells were able to invade Matrigel-coated inserts in the Transwell migration chambers

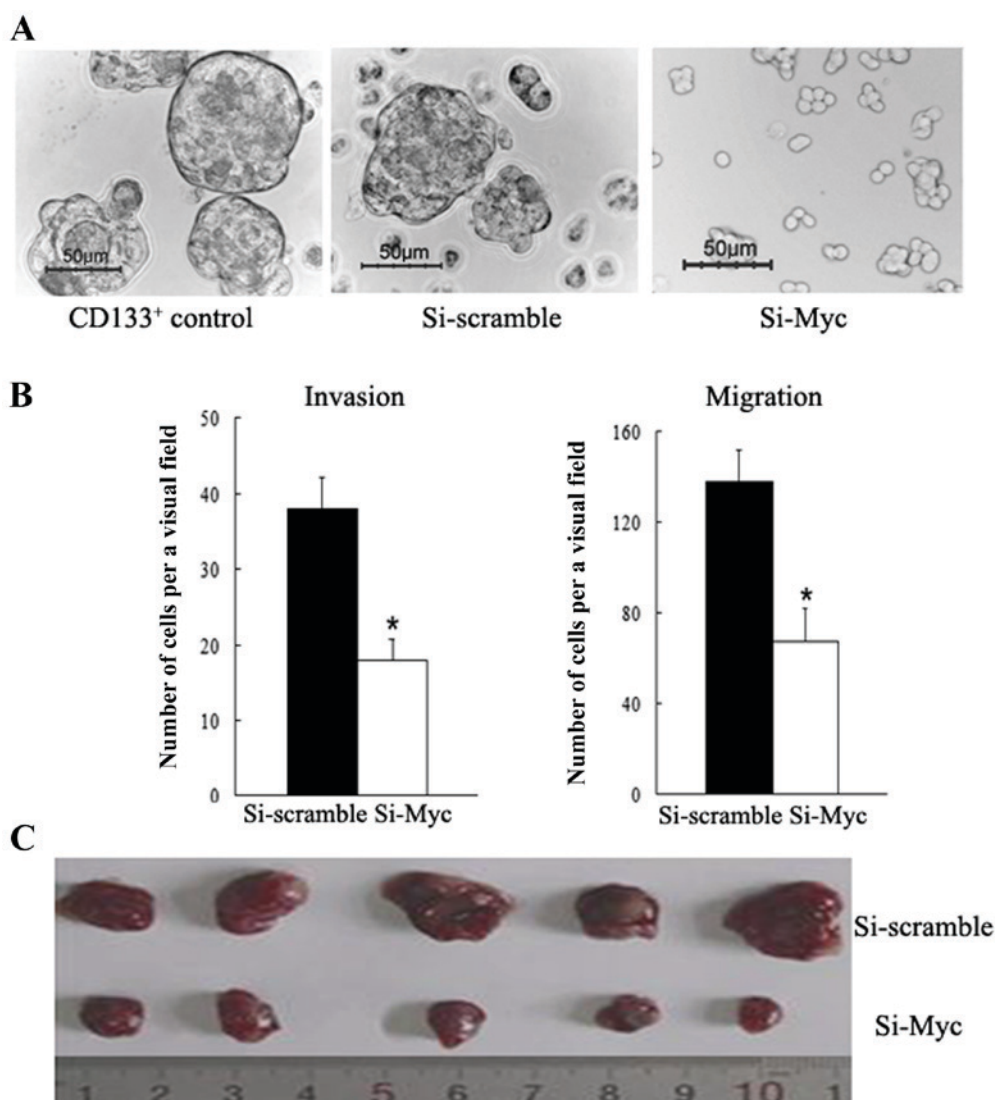


Figure 2. Knockdown of c-Myc in colon CSCs attenuates sphere formation and cell mobility *in vitro*, and inhibits tumorigenicity *in vivo*. (A) c-Myc-siRNA was transfected into CD133⁺ cells, which formed smaller and fewer tumor spheres than the scramble-siRNA control and CD133⁺ counterpart groups cultured in serum-free stem cell medium. Representative images of colon cell spheres observed by the inverted phase contrast microscope are shown (bars, 50 μ m). (B) Knockdown of c-Myc inhibits the invasion and migration potential of CD133⁺ cells (all assays were performed in triplicate; data are presented as the mean \pm standard deviation; * P <0.05, compared with the control). (C) c-Myc-siRNA-transfected CD133⁺ cells generated smaller xenograft tumors when compared with the scramble-siRNA group; the grafts were excised at 8 weeks after transplantation *in vivo*. siRNA, small interfering RNA; CSC, cancer stem cell.

than with the NC counterparts (P <0.01, Fig. 2B). These results indicate that knockdown of c-Myc inhibits the invasion and migration potential of colon CSCs, promoting a functional phenotype associated with tumor aggressiveness.

c-Myc-siRNA suppresses the tumorigenicity of colon CSCs in vivo. To assess the function of c-Myc with respect to tumorigenicity *in vivo*, tumor development experiments were carried out. Nude mice were subcutaneously injected with c-Myc-siRNA transfected CD133⁺ cells or scramble-siRNA counterparts. As shown in Fig. 2C, mice in the c-Myc-siRNA group developed much smaller subcutaneous tumors than those in the scramble-siRNA group. The tumor diameters of the c-Myc-siRNA group and the scramble-siRNA group were 1.07 ± 0.18 and 0.43 ± 0.13 cm, respectively. *In vivo* experiment results illustrated that c-Myc-siRNA attenuates the tumorigenicity of CD133⁺ colon CSCs.

Depletion of c-Myc enhances the chemosensitivity in colon CSCs through the downregulation of ABCG2 and ABCB5 expression. Previous studies have reported that CSCs are widely resistant to chemotherapeutic drugs (7,8). Herein, colon CSCs and adherent cells were exposed to 5-FU (50 μ M) or oxaliplatin (1.25 μ M) or FOLFOX (50 μ M 5-FU plus 1.25 μ M oxaliplatin) for 72 h; as expected, the chemotherapy of HT-29 adherent cells resulted in a significant increase in cell death and disintegration compared with colon CSCs, as observed via inverted phase-contrast microscopy (Fig. 3A). Furthermore, to evaluate the effect of c-Myc on the drug resistance of colon CSCs, a chemosensitivity assay was conducted. Transfected cells were treated with the same chemotherapy strategy as aforementioned. After incubation for further 72 h, the CCK-8 assay results demonstrated that the survival rates of c-Myc-siRNA-transfected cells were significantly reduced compared with those of the scramble-siRNA group (Fig. 3B).

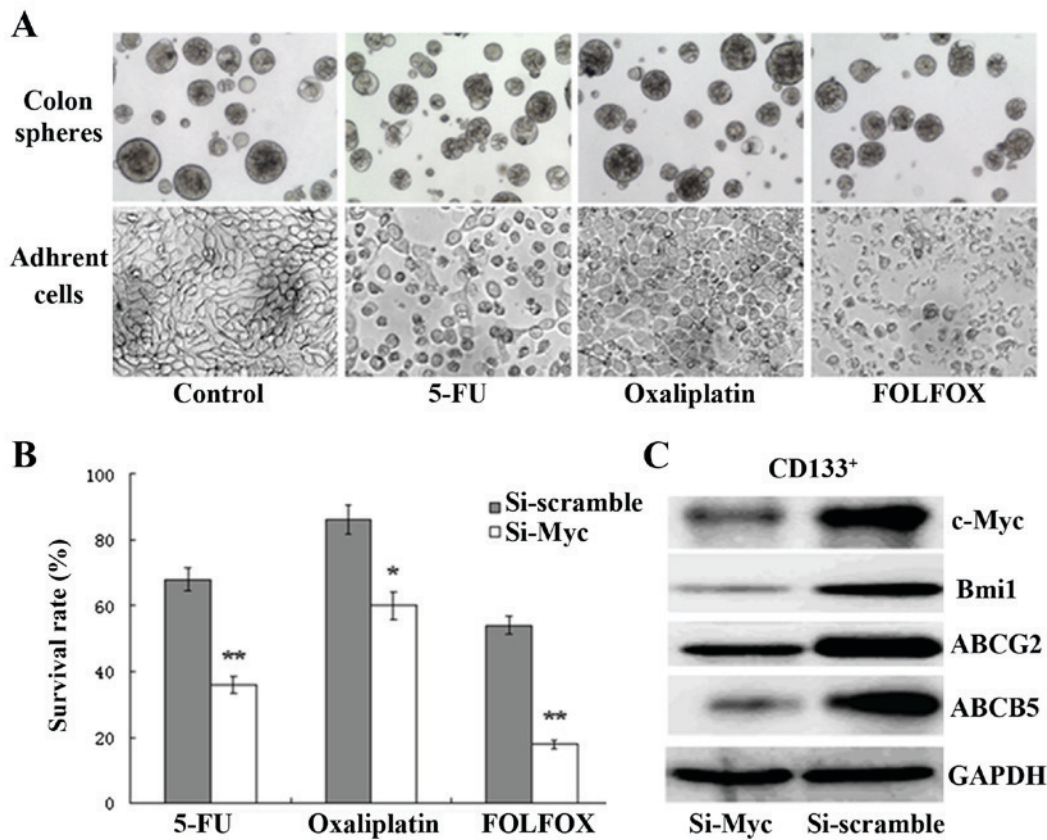


Figure 3. Depletion of c-Myc enhances the chemosensitivity of colon CSCs through the regulation of ABCG2 and ABCB5 expression. (A) Chemotherapy treatment of HT-29 adherent cells resulted in a significant increase in cell death and disintegration, compared with colon CSCs, as observed by inverted phase contrast microscopy (bars, 50 μ m). (B) The survival rates of c-Myc-siRNA-transfected CD133⁺ cells were significantly reduced compared with the scramble-siRNA group following treatment with 5-FU, oxaliplatin or FOLFOX (* P <0.05, ** P <0.01). (C) c-Myc, Bmi1, ABCG-2 and ABCB5 were all down-regulated in CD133⁺ colon CSCs, as determined by western blotting, following treatment with c-Myc siRNA. siRNA, small interfering RNA; CSC, cancer stem cell; FOLFOX, 5-FU plus oxaliplatin.

High expression of the ATP-binding cassette and multidrug resistance protein is essential for CSC chemoresistance (15-18). In the present study, a strong decrease was found in ABCG2 and ABCB5 expression upon c-Myc silencing (Fig. 3C). The results show that c-Myc silencing enhances the chemosensitivity of colon CSCs through the downregulation of ABCG2 and ABCB5 expression, thus representing a valid approach for sensitizing colon CSCs to conventional treatment.

Discussion

There is accumulating evidence supporting the fact that tumors contain a small subpopulation of CSCs, which have a self-renewing capacity and are responsible for tumor maintenance and metastasis. In colon cancer, CD133 is regarded as a specific marker for the isolation and identification of CSCs in primary colon cancer, and in colon cancer cell lines (3-9). In the present study, we purified CD133⁺ colon CSCs from HT-29 cell line by FACS. CD133⁺ cells have high expression of 'stemness' genes, including CD133, Sox2, Oct4, c-Myc and Wnt2; many of these molecular markers have been previously reported to be involved in the maintenance of stemness in human CSCs (19). Additionally, it was found that isolated CD133⁺ cells were able to generate tumor spheres when cultivated in serum-free stem cell medium supplemented with 20 ng/ml EGF and 10 ng/ml FGF-2, which sustained the cells in an undifferentiated state

as sphere-like cellular aggregates. In addition, it was determined that the secondary passage tumor spheres have a larger volume and a more compact sphere structure (data not shown), suggesting that they retained capacity for self-renewal. The results of the present study demonstrated that CD133⁺ cells are endowed with stem cell-like properties.

c-Myc has been proven as one of 4 major factors that render the reprogramming capability of adult cells into germ-line-competent-induced pluripotent stem cells, suggesting that c-Myc is an important regulator of stem cell biology (11). Previous studies showed that c-Myc expression may be implicated in tumorigenesis via activating its downstream target genes (10). These results suggested that c-Myc may serve as a link connecting malignancy and 'stemness'. Herein, it was also demonstrated that c-Myc expression was upregulated in CD133⁺ colon CSCs (Fig. 1A). c-Myc expression was down-regulated in CD133⁺ colon CSCs by siRNA and examined to discern whether it would affect the cells. First, it was observed that treatment with c-Myc siRNA can block sphere formation (Fig. 2A) inhibit the invasion and migration potential of CD133⁺ colon CSCs *in vitro* (Fig. 2B). In addition, tumor growth *in vivo* was suppressed by c-Myc siRNA (Fig. 2C), and it was found that the Bmi1, which controls many diverse biological cancer processes such as differentiation, proliferation, migration, and tumorigenesis (20), was downregulated in the c-Myc siRNA group (Fig. 3C). These data show that c-Myc

is essential for survival, and that colon CSC self-renewal is consistent with the findings in brain CSCs (12).

Curative treatment failure in patients with cancer often occurs as a result of intrinsic or acquired resistance of tumors to chemotherapeutic agents (7). 5-FU, or 5-FU plus oxaliplatin (FOLFOX), which remains the backbone of colorectal cancer chemotherapeutics, still shows limited success (2). As previously reported, CSCs show resistance to conventional therapies, which may explain why it is difficult to completely eradicate cancer and why recurrence is an ever-present threat (7-9). Consistent with these hypotheses, colon cell spheres were significantly resistant to 5-FU and oxaliplatin compared with HT-29 adherent cells; even treatment combined with FOLFOX failed to cause death among treated colon cell spheres (Fig. 3A). Therapeutic strategies that specifically target colon CSCs are likely to be effective in eradicating tumors (21-24). To overcome the resistance to chemotherapy of colon CSCs, treatment with c-Myc siRNA was used, and the results showed that c-Myc knockdown can significantly improve the anti-cancer effects in single or combination-treated CD133⁺ colon CSCs *in vitro* (Fig. 3B).

Understanding how chemoresistance develops, and eventually how it can be prevented, is crucial to fighting cancer effectively (23,24). Recent studies have revealed that the ABC transporter family are involved in multidrug resistance (15-18). Xie *et al* (15) observed that 'side population' CSCs in colon cancer expressed high levels of ABCG2 and had a greater capacity to expel cytotoxic drugs. Furthermore, Porro *et al* (14) showed that c-Myc may contribute to the multidrug resistance profile and malignant progression of myeloid tumors by dysregulating the transcription of specific ABC transporter genes. Similarly, Kugimiya *et al* (18) reported that c-Myc confers resistance to 5-FU through regulating ABCB5 expression in human colon cancer cells. Accordingly, in the present study, it was found that CD133⁺ colon CSCs were highly co-expressed with ABCG2 and ABCB5 (data not shown). Furthermore, it was observed that there was a significant downregulation of ABCG2 and ABCB5 expression in CD133⁺ colon CSCs after c-Myc siRNA treatment was administered (Fig. 3C), thus, enhancing the chemosensitivity of colon CSCs (Fig. 3B). These findings indicate that c-Myc silencing sensitizes colon CSCs to chemotherapy-induced cytotoxicity, at least in part via the downregulation of ABCG2 and ABCB5, although the molecular mechanisms remain to be fully clarified; our group is currently planning further study into this.

In conclusion, the data suggest that c-Myc knockdown suppresses the self-renewal, tumorigenicity, invasion and drug resistance of colon CSCs. c-Myc is, therefore, indispensable for the maintenance of colon CSCs, and targeting c-Myc may be an effective therapeutic strategy for eliminating colon cancer.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HLZ and PW performed all experiments in the present study. LZ and SDZ analyzed the data, and HLZ and MZL designed all experiments in the present study.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Lihuili Hospital of Ningbo Medical Center (Ningbo, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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