Downregulation of Msi1 suppresses the growth of human colon cancer by targeting *p21^{cip1}*

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Abstract. Musashi1 (Msi1), a member of the RNA-binding protein (RBP) family, is highly expressed in neural progenitor or stem cells for the maintenance of stemness as well as in various cancers. Emerging studies have demonstrated that it regulates cell processes by translational activation or suppresses specifically bound mRNA. In the present study, we initially reported remarkably increased expression of Msi1 in colon cancer tissues compared with adjacent non-tumor tissues. Knockdown of Msi1 significantly suppressed the proliferation, colony formation, tumorsphere formation and the progression of implanted colon cancers, and induced cell cycle attest at G_0/G_1 phase, along with the upregulated expression of p21^{cip1}. Reporter assays using a chimeric mRNA that combined luciferase and the 3'-UTR of *p21^{cip1}* revealed that Msi1 decreased the reporter activity through the specific motif. Thus, the current results suggested that downregulation of Msi1 could inhibit the growth of colon cancers and Msi1 may be a promising therapeutic target molecule for human colon cancers.

Introduction

Colon cancer is one of the most common causes of cancer related mortality worldwide (1). Its overall incidence is ~5% and the 5-year survival rate ranges from 40 to 60% (2). The mechanisms of colon cancer carcinogenesis and development have drawn much attention in recent years for its high incidence and poor prognosis. However, the etiology and pathogenesis of colon cancer remain unclear, although heterogeneous genetic alterations have been reported to be responsible. The most common genetic alterations include mutations and loss of heterozygosity of tumor suppressors, such as adenomatous polyposis coli (APC) (3). Several studies have demonstrated that colon cancers were involved in the activation of Wnt, Notch, BMP and Hedgehog signaling pathways, which accompany establishment of tumorigenic state (4). Msi1 has been found to activate the Notch and Wnt signaling pathways in several types of normal and cancerous cells (5,6). Therefore, we hypothesized that Msi1 maybe have association with the progression of colon cancer.

Msi1, located at chromosome 12q24, is a member of the Musashi (MSI) family of the RNA binding proteins, which are characterized by RNA recognition motif. It can be regulated by ELAV or HuR by maintaining the stabilization of its mRNA or accumulation of mRNA translation (7,8), as well as by tumor suppressor microRNAs (9). It was first found in *Drosophila* as a determinant of sensory organ development (10) and highly conserved across species (11) with essential roles in the stem cell maintenance, nervous system development and tumorigenesis (12,13). In mammals, Msi1 is highly expressed in the neural progenitor and stem cells (13) for maintenance of self-renewal and differentiation. Recent studies have demonstrated that Msi1 exists in many adult cancers, including gliomas (14), gastric cancer (15), hepatoma (16) and colorectal adenoma (17). Consistently, high level of Msi1 expression is considered to be associated with many malignancies (18) and predicts poor prognosis (19,20). All these finding suggest that Msi1 function as an oncogene. This notion is further supported by the findings that ablation of Msi1 in gliomas (6) or bladder carcinoma cells (21) suppressed the cell cycle, and induced apoptosis and showed severe decline in cell numbers. However, Msi1 has also been proposed to have no significant effect on cell proliferation in human intestinal epithelial cells (22).

We performed detailed analyses on the role of Msi1 in colon carcinoma, and found that Msi1 was upregulated. Knockdown of endogenous Msi1 protein inhibited the growth and tumor formation by activating the cell cycle suppressor *p21^{cip}*. Our findings support the hypothesis that Msi1 functions as an oncogene in colon cancer.

Materials and methods

Cell lines. Human colon cancer cell lines SW480, HCT116, SW620 and cervical cancer cell line HeLa were purchased

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from the American Type Culture Collection (ATCC), cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin. All cell lines were maintained at 37˚C in an atmosphere of 5% CO_2.

Tissue samples. Fresh frozen specimens of matched colon cancer tissues and adjacent non-tumor tissues were from 20 patients in the Department of General Surgery of the Fourth Hospital of Hebei Medical University, obtained between March 2011 and January 2013, and stored at -80˚C for further use. All of the tissues were from untreated patients who were undergoing surgery. Written informed consent was obtained from each patient before the surgery, and the study protocol was approved by the Institutional Research Ethics Committee.

Semi-quantitative real-time PCR analysis. Total RNA was isolated from colon cancer cell lines using TRIzol reagent (Invitrogen). Total cDNA was synthesized using the M-MLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania). The mRNA expression levels were measured by qRT-PCR using the 7500 Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA). Amplification was performed with the SYBR Premix Ex Taq™ II kit (code: DRR081A; Takara, Dalian, China) according to a 2-step cycle procedure consisting of 45 cycles of denaturation at 95˚C for 10 sec and annealing/elongation at 60˚C for 30 sec. We measured mRNA levels semi-quantitatively by the Δ/Δ threshold cycle (Ct) method. GAPDH was used as internal control. Fold-changes were calculated and normalized as relative to the parental cells. The primers were used as follows: *p21^{cip1}* F, 5'-AAATCGTCCAGCGACCTTCC-3' and *p21^{cip1}* R, 5'-GCCCTGTCCATAGCCTCTACT-3'; GAPDH F, 5'-CAAGGGCATCCTGGGCTACA-3', GAPDH R, 5'-AAGTGGTCGTTGAGGGCAAT-3'.

Western blot analysis. Cells and clinical tissues were lysed on ice in lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 1% NP-40; and 0.1% sodium dodecyl sulfate SDS) containing freshly added protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Branchburg, NJ, USA). Aliquots of samples with the same amount of protein were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), and mixed with loading buffer (final concentrations of 62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 100 mM dithiothreitol and 0.005% bromophenol blue). Then the protein extracts were boiled and separated by SDS-PAGE and blotted to activated polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then the membranes were blocked with 5% fat-free milk and probed with first antibodies overnight. The first antibodies included the following: anti-Msi1 (1:500, sc-98845; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (1:500, sc-397; Santa Cruz Biotechnology), and anti-β-actin (1:500, sc-47778; Santa Cruz Biotechnology). The membranes were then washed four times in PBST and incubated with a secondary antibody coupled to horseradish peroxidase (Thermo Fisher Scientific, Inc., New York, NY, USA), followed by ECL detection (Millipore) and visualization on X-ray film. Relative quantitation was determined with the AlphaView system (Cell Biosciences, Santa Clara, CA, USA) using β-actin as the loading control.

Plasmid construction. To generate plasmids that express Msi1-specific small interfering RNA (siRNA) which targeting MSI1, the oligonucleotide inserts were designed using an online siRNA design tool (Ambion, Austin, TX, USA). The double oligonucleotides were annealed and cloned into the lentivirus GV248-GFP shRNA vector (Shanghai Genechem Co., Ltd., Shanghai, China) as per the manufacturer's introductions. Lentivirus was produced after GV248-GFP shRNA vector and two package plasmids were co-transfected to 293T cells with Lipofectamine 2000 transfection reagent (Invitrogen). Then colon cancer cells were infected by recombined lentivirus, and cell clones were selected to the amplification culture. Stably transfected clones were extracted and identified by western blotting.

A luciferase reporter vector (pMIR-REPORT; Ambion) was used to generate reporter constructs. Fragment of the 3' UTR of human *p21^{cip1}* was amplified from HCT116 cDNA by PCR using primers *p21^{cip1}* WT F/R and cloned into the pMIR-REPORT luciferase plasmid. The mutant *p21^{cip1}* 3' UTR was generated by using the *p21^{cip1}* WT F/MU R and *p21^{cip1}* MU F/ WT R primers. The following primers were used: shMsi1-1F, 5'-GATCCTGTTACATGGTGTTTCGAATTCAAGAGATTCGAAACACCATGTAACATCA-3' and shMsi1-1R, 5'-GACAATGTACCACACAAGCTTAAGTTGAGAAAGCTTTGTGGTACATTGTAGTTCGA-3'; shMsi1-2F, 5'-GATCCTCCTGTATCATATGTAAATTTCAAGAGAATTTACATATGATACTGGACGA-3' and shMsi1-2R, 5'-AGGACATAGTATACATTTAAAGTTCTCTTAAATGTATACTATGACCTGCTTTCGA-3'; *p21^{cip1}* WT F, 5'-ATTGAGCTCTAATCCGCCCACAGGAAG-3' and *p21^{cip1}* WT R, 5'-CTCAAGCTTACAAGTAAAGTCACTAAG-3'; *p21^{cip1}* MU F, 5'-TGGGAAGCAGTGTCTTTCCTGGCACTAACGTT-3' and *p21^{cip1}* MU R, 5'-AGACACTGCTTCCCAGCCCCATATGAGCCCAC-3'.

Cell proliferation and cell viability assays. Cells ($5x10^4$) were cultured in triplicate in 35-mm cell culture dishes. The cells were harvested longitudinally, and counted every day for one week using a hemocytometer under light microscopy.

Cell viability was assessed every other day using 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) dye according to the standard protocol. Approximately 1,000 cells/well were seeded in a 96-well plate. MTT solution (20 μl) was added to 200 μl of culture media and incubated for 4 h, and then cell growth was determined by measuring the absorbance at 490 nm.

Colony formation assay. Cells ($1x10^3$) of each type were cultured in 10-cm culture dishes and exposed to fresh media every 3 days. Colonies with diameters >0.2 mm were counted at day 14.

Tumorsphere formation assay. One hundred Msi1-KD or -control cells were seeded in 6-well plates in 2 ml of serum-free stem cell medium as described above. Fresh medium were added to each well every 3 days. The tumorspheres were analyzed on day 14 for tumorsphere forming ability.

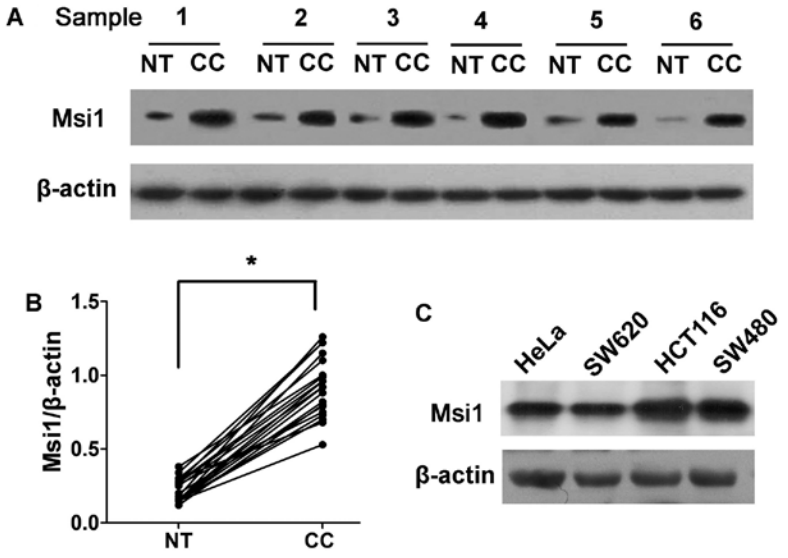

Figure 1. The expression of Msi1 is detected in colon cancer tissue, non-tumor colon tissues and colon cancer cell lines. (A) The representative western blot analyses show Msi1 expression in 20 pairs of matched colon cancer tissues (CC) and adjacent non-tumor tissues (NT). (B) Msi1 protein expression was compared between NT and CC tissues in 20 patients (P<0.05, paired t-test). (C) Msi1 expression is shown at the protein level by western blot analysis in human colon cancer cell lines. Cervical cancer cell line HeLa was used as positive control.

Animal and tumor xenograft assay. Exponentially growing cells collected from stable transfectants were bilaterally inoculated into subcutaneous sites of 4- to 6-week-old Balb/c nude mice. Tumor dimensions were measured with calipers once every week, and volumes (cm^3) were calculated according to the standard formula, length x $width^2/2$. At the end of the experiment, tumors were dissected out, and the net weight per mouse was measured. The experimental protocols used herein were evaluated and approved by the Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University.

Cell cycle analysis. Cells ($1x10^6$) were harvested and washed twice with PBS, followed by fixation with 75% cold ethanol at 4˚C overnight. After the cells were washed twice in PBS, the cells were suspended in PBS with 50 μg/ml propidium iodide (PI; Sigma) and 10 μg/ml RNaseA (Sigma). They were then incubated at 4˚C for 30 min in the dark. Cells were analyzed for DNA content by FACSCalibur (BD Biosciences) and the results of the cell cycle were analyzed by FlowJo software.

Luciferase assay. Cells were lysed in 100 μl of passive lysis buffer (Promega, Madison, WI, USA) at 48 h after transfection and assayed with a Dual-luciferase report assay kit according to the manufacturer's instructions. For each assay, cell extract (20 μl) was used and the reaction was started by injection of 50 μl of luciferase substrate. Each reaction was measured for 10 sec in the Luminometer. Luciferase activities were expressed as the ratio of firefly to *Renilla* luciferase activity.

Statistical analysis. Data are presented as the mean ± standard deviation (SD). The Student's t-test was performed using the SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was regarded as statistically significant.

Results

Msi1 expression in human colon cancer tissues and cell lines. To assess Msi1 expression in clinical patients, western blot analysis was conducted on 20 pairs of colon cancer tissues and matched adjacent non-tumor tissues (Fig. 1A). Msi1 expression in all 20 cancer tissues was markedly higher than that in the corresponding non-tumor tissues (Student's t-test, P<0.01; Fig. 1B), suggesting that the activation of Msi1 may contribute to the development and progression of colon cancer.

We also evaluated the expression of Msi1 in colon cancer cell lines by western blotting. We detected a high level of Msi1 expression in SW620, HCT116 and SW480 cells (Fig. 1C).

The knockdown of Msi1 inhibits the proliferation of colon cancer cells in vitro. To determine whether Msi1 regulated the proliferation of colon cancer cell lines, the endogenous Msi1 was knocked down by short hairpin RNA (shRNA) in HCT116 and SW480 cells (Fig. 2A). The Msi1 knockdown HCT116 and SW480 cells (HCT116-KD and SW480-KD) had much lower proliferation ability than the corresponding control cells (HCT116-control and SW480-control), as measured by both cell growth curve assay (P<0.01; Fig. 2B) and cell viability assay (P<0.01; Fig. 2C), indicating that the knockdown of Msi1 suppressed the growth of colon cancer cells *in vitro*.

Furthermore, colony formation assays showed that the efficiency of foci formation was dramatically decreased in Msi1 knockdown clones compared with control clones (P<0.01; Fig. 2D). These findings suggested that enforced inhibition of Msi1 led to retardation of colon cancer cell growth *in vitro*.

Silencing of Msi1 inhibits the tumor formation of colon cancer cells in vivo and in vitro. As Msi1 modulates the proliferation *in vitro*, we explored whether it alters tumorigenic potential of colon cancer cells *in vivo*. Xenograft assays were created with nude mice, and the development and growth of solid tumors

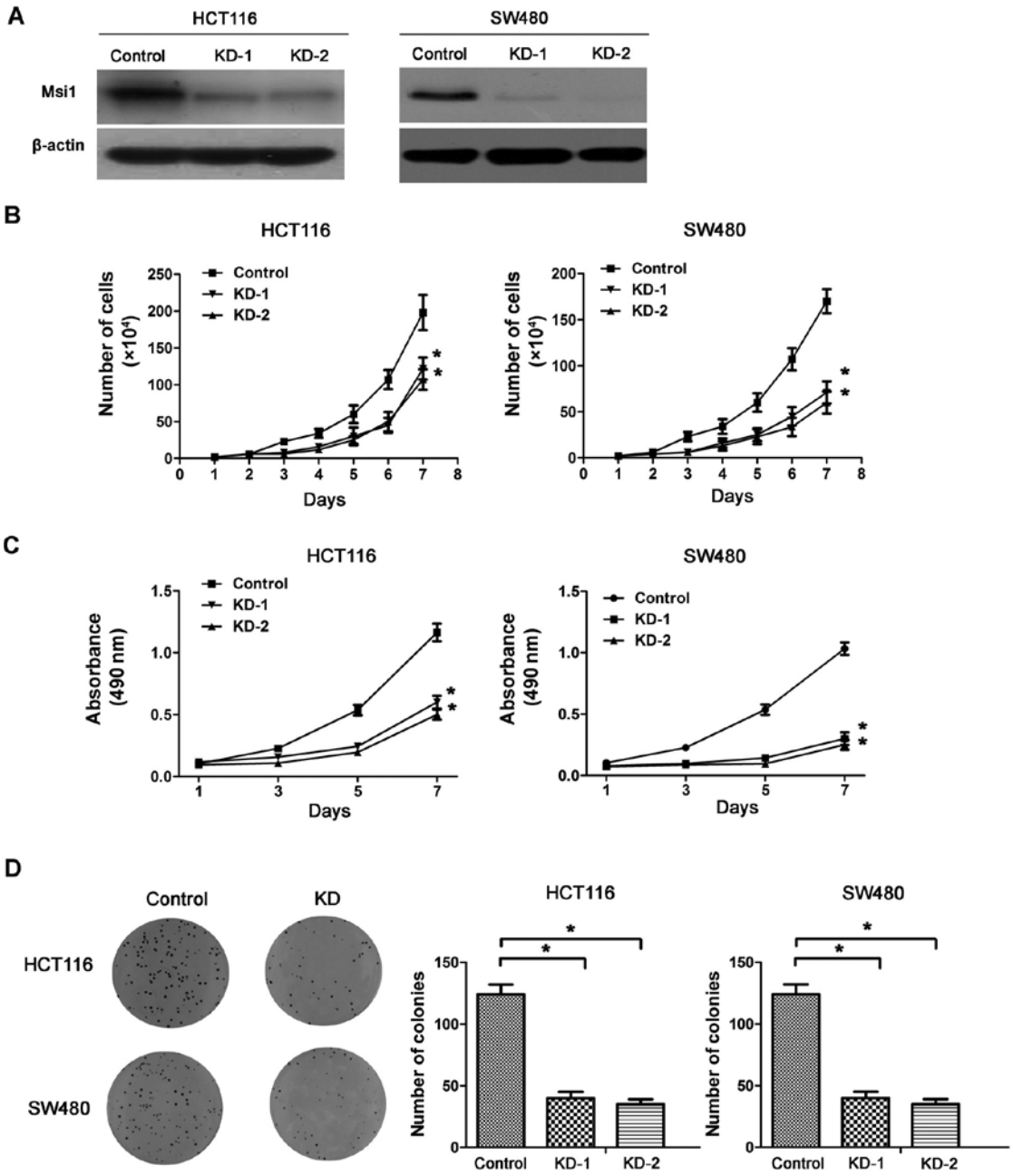

Figure 2. Knockdown of Msi1 inhibits the proliferation of colon cancer cells. (A) Western blot analysis of the expression of Msi1 in HCT116 and SW480 cells with Msi1 knockdown (KD) by two shRNAs. (B) The proliferation of Msi1-KD and control cells was evaluated by counting cells longitudinally. (C) Cell viability assay was conducted for Msi1-KD and the control cells. (D) These photomicrographs show colony formation assays and the graph illustrates quantitative analysis. Data are shown as the means standard error for representative images from 3 separate experiments. P-values were determined with the Student's t-test. *P<0.05.

were monitored every week. The palpable tumor formation of HCT116-KD and HCT116-control cells occurred at the same time after inoculation. However, tumor development with HCT116-KD cells was significantly delayed (P<0.01; Fig. 3A). The tumor weights generated from HCT116-KD were reduced significantly (P<0.01; Fig. 3B). Similar results were observed from SW480 cells (Fig. 3A and B), suggesting that the silencing of Msi1 induces the suppression of the tumor formation and development of colon cancer, and this inhibition may have a close relationship with the decreased cell proliferation. Together, these data indicated that knockdown of Msi1 expression inhibited the growth of colon cancers.

Several studies have recently indicated the existence of CSCs (cancer stem cells) in various cancer types (23-25), and CSCs are critical for the maintenance of tumor growth, progression and resistance to chemotherapy or radiotherapy, as well as recurrence and metastasis (26). It has been demonstrated that Msi1 regulates the proliferation and differentiation of CSCs. In the present study, we used a tumorsphere culture system to conduct the latent role of Msi1 on tumorsphere formation. Knockdown of Msi1 inhibited the tumorsphere formation and growth of HCT116-KD and SW480-KD cell lines, as demonstrated by the significantly reduced numbers of tumorspheres compared with the numbers in control cells (P<0.01; Fig. 3C).

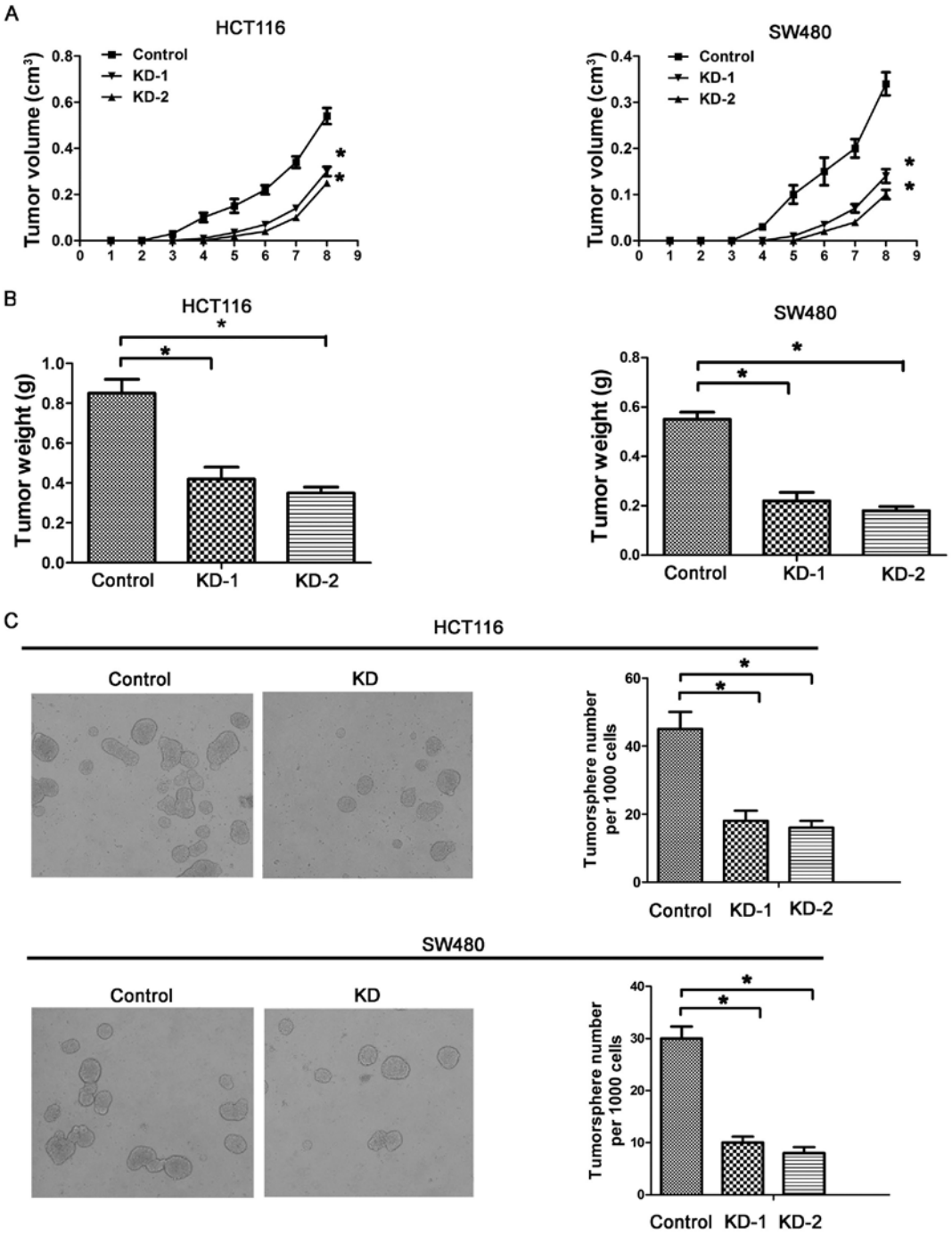

Figure 3. Knockdown of Msi1 suppresses colon cancer formation *in vitro* and *in vivo*. (A) Tumor growth curves of nude mice with Msi1-KD and the control colon cancer cells. (B) The tumor net weights were measured at 8 weeks post-implantation. (C) The impact of Msi KD on the formation of tumorspheres was determined by tumorsphere formation assays. The formed tumorspheres were imaged and counted. P-values were determined with the Student's t-test. *P<0.05.

Our results indicate that Msi1 is a key regulator of the growth of tumorspheres.

Msi1 regulates the cell cycle of colon cancer cells in vitro. To further investigate the potential mechanism by which silence of Msi1 regulates growth of colon cancer cells, we characterized cell cycles by FACS analysis. As shown in Fig. 4A, the proportion of HCT116-KD cells in G_0/G_1 phase increased markedly to 69.3%, while proportion in S phase decreased to 19.0%. The ratio of G_1 phase to S phase of HCT116-KD cells was much higher than that of HCT116-control cells. A similar effect was observed in SW480-KD cells (P<0.01; Fig. 4B), indicating that the knockdown of Msi1 expression blocked G_1/S phase transition of cell cycle.

Msi1 negatively regulates p21^{cip1} expression. It has been reported that Msi1 blocked the G_1/S phase transition of cell cycle by negative regulation of cyclin-dependent kinase (CDK) inhibitor *p21^{cip1}* in bladder carcinoma (21) and breast cancer (19). To test whether it also occurs in colon cancer cells, *p21^{cip1}* mRNA and protein levels were examined by qRT-PCR and western blot analysis. Significantly, increased

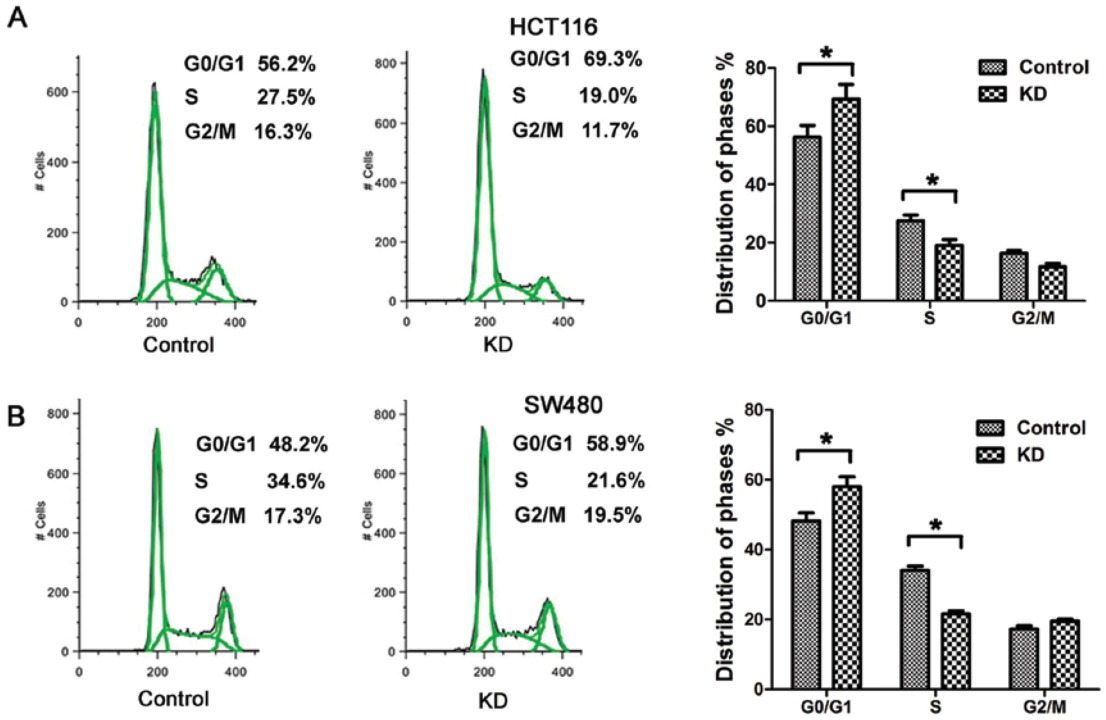

Figure 4. Knockdown of Msi1 blocks the cell cycle at G_0/G_1 phase in colon cancer cells. The cell cycle was monitored with fluorescence-activated cell sorting (FACS) analysis (left) and quantitative analysis for (A) HCT116 and (B) SW480 cells transfected with Msi1-KD or the control vector. Error bars represent the SD; *P<0.05, vs. the corresponding controls.

levels of $p21^{cip1}$ protein were observed in the lysates of both HCT116-KD and SW480-KD cells, but there was little change in $p21^{cip1}$ mRNA levels, suggesting that Msi1 may reduce $p21^{cip1}$ protein levels by inhibiting translation.

To confirm the direct interaction between Msi1 and its putative target site, the human $p21^{cip1}$ 3' UTR containing the wild-type and mutant Msi1 binding sequence was cloned downstream from the luciferase report element (Fig. 5E). The relative luciferase activity of wild-type $p21^{cip1}$ 3' UTR reporter was increased by 48% in HCT116-KD cells relative to controls, while the augmentation was diminished in cells transfected with the mutant reporter (P<0.01; Fig. 5F). Similarly, knockdown of Msi1 enhanced the relative luciferase activity of the wild-type in SW480 cells (P<0.01; Fig. 5F). Mutation of the Msi1 binding site abrogated the elevate effect, demonstrating the specificity of the Msi1 target sequence.

Discussion

Msi1, a multifunction RNA binding protein of MSI family, is present in many types of normal cells (27-29) and is involved in CNS stem/progenitor cell fate (13,30), inner ear development (31), repair of small-intestinal and stomach injury (32,33), and maturation of photoreceptor and oocyte (34,35), intestinal metaplasia (36), atherosclerotic arteries (37) and Alzheimer disease and Pick disease (38). Its overexpression in tumor cells promotes tumor growth, invasion and metastasis, inhibits apoptosis and predicts poor prognosis. The pathophysiological functions involved have been found in several types of cancers, such as glioma (6), medulloblastoma (39) and breast cancer (19).

In the present study, we detected the expression of Msi1 in colon cancer tissues. Msi1 protein was abundant in colon cancer tissues. Low levels of Msi1 can be detected in matched adjacent non-tumor tissues. Nishimura and co-workers (40) found that Msi1 were located in the human normal colon crypt cells, indicating that it may be a possible stem cell marker of human colon epithelium. The low levels of Msi1 in normal colon tissues suggests that Msi1 may participate in the physiological function of the colon, and may be involved in the proliferation and initiation of the cells. In addition, colon cancer tissues have a significantly higher expression than normal tissues, indicating Msi1 plays important roles in colon cancers. Thus, we hypothesized that Msi1 was associated with colon cancer stem cells.

To further explore how Msi1 is involved in colon carcinogenesis, functional characterization was conducted by downregulation of Msi1 in colon cancer cell lines. We found that knockdown of Msi1 significantly inhibited cell proliferation and colony formation, induced cell cycle arrest in G_0/G_1 phase, demonstrating that Msi1 related signaling is a crucial regulator of the development and growth of colon cancers. Our data are in agreement with previous findings in other types of tumors (6,19,39), suggesting that Msi1 related signaling may have a similar regulatory effect on the growth of different types of human malignancies.

Several studies have recently suggested the existence of CSC in colon cancers (41,42). Cancer stem cells are a rare cell population, which have the ability for self-renewal, differentiation and tumorigenesis (43). Recent research has found that cancer stem cells are rich in tumorspheres and give rise to tumors (44). Indeed, it has been reported that Msi1 related signaling regulates the proliferation of normal and cancer

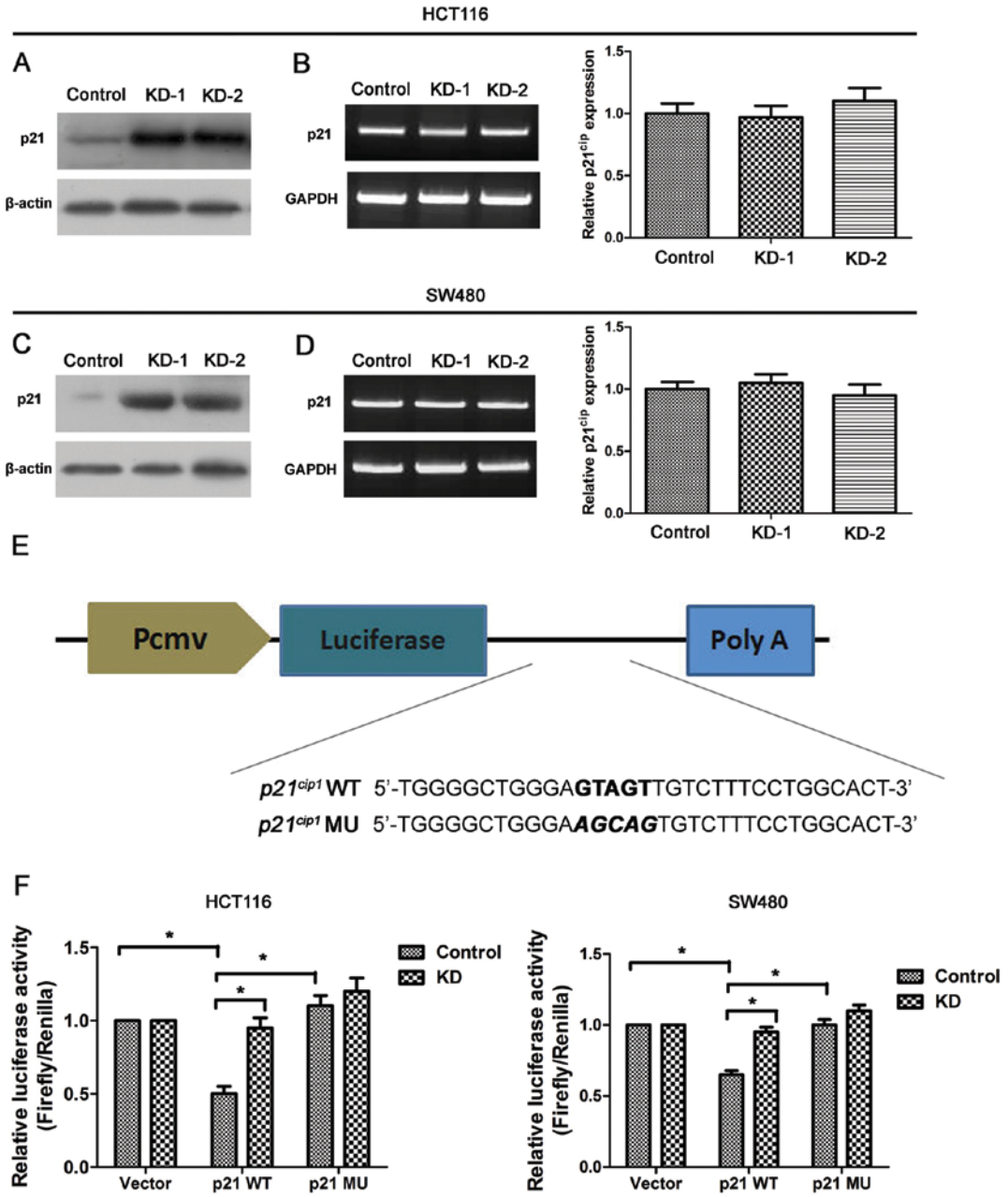

Figure 5. Knockdown of Msi1 increases the protein level of the predicted target $p21^{cip1}$ without altering its mRNA levels. (A and C) Western blot analysis showing the expression of the endogenous protein levels of $p21^{cip1}$ in (A) HCT116 and (C) SW480 cells after Msi1-KD or the control vector transfection. β-actin served as the loading control. (B and D) Quantitative analysis of $p21^{cip1}$ mRNA levels by qRT-PCR in Msi1-KD or the control vector transfected HCT116 (B) and SW480 (D) cells as compared with control cells. (E) Schematic representation of the reporter construct containing the firefly luciferase coding sequence fused to the $p21^{cip1}$ 3' UTR. Predicted pairing regions within the wild-type $p21^{cip1}$ 3' UTR are noted in bold, and the residues altered in the $p21^{cip1}$ 3' UTR mutant construct are italic bold. (F) Luciferase reporter assays for $p21^{cip1}$ targeted by Msi1 in colon cancer cells. The histogram shows the ratio of firefly to *Renilla* luciferase activity normalized to empty vector transfected cells. Error bars represent the SD; $^{*}P<0.05$, vs. the corresponding controls.

stem cells (12,30). We characterized the role of Msi1 in the proliferation of colon CSC by using tumor xenograft and tumorsphere formation assays and observed that the knockdown of Msi1 expression significantly suppressed the tumor formation *in vivo* and remarkably decreased the number of tumorspheres formed in culture conditions *in vitro* that allowed the proliferation of only CSC. Therefore, it is possible that Msi1 related signaling may regulate the growth of human colon cancers by promoting the proliferation of both CSC and cancer cells.

Several lines of evidence suggest that Msi1 is a positive regulator of cell proliferation (45). Msi1 promotes cell proliferation through downregulation of the inhibitors of proliferation (6). In the present study, we also found that downregulation of Msi1 inhibited the cell cycle by blocking the G_1/S transition in 2 colon cancer cell lines. Furthermore, we found that knockdown of Msi1 upregulated the expression of $p21^{cip1}$. Luciferase assay revealed that Msi1 suppressed $p21^{cip1}$ expression by directly binding to the consensus sequence of $p21^{cip1}$ 3'UTR in colon cancer cells. These results are consistent with

the findings in bladder carcinoma (21) and breast cancer (19). $p21^{cip1}$ is a universal cyclin dependent kinase (CDK) inhibitor that directly inhibits the activity of cyclin-CDK complexes, resulting in cell cycle arrest at G_0/G_1 phase. Therefore, our data suggested that cell growth retardation induced by downregulation of Msi1 in colon cancer is probably through activation of $p21^{cip1}$.

In summary, we showed that Msi1 was upregulated in colon cancers, and knockdown of Msi1 inhibited the proliferation involved the coordinated direct activation of CDK inhibitor $p21^{cip1}$. The present study indicates that downregulating the expression or restoring the expression of $p21^{cip1}$ is a possible method to treat certain types of colon cancers.

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