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Metastasis associated in colon cancer 1 (MACC1) promotes growth and metastasis processes of colon cancer cells

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Abstract. – OBJECTIVE: Colon cancer (CC) is the third most common malignancy with high mortality rate in the world. The impacts of metastasis associated in colon cancer 1 (MACC1) on growth and metastasis processes of CC were investigated by overexpression and interference lentivirus infection.

MATERIALS AND METHODS: Firstly, overexpression and interference plasmids were constructed with GV115 vector and lentivirus were packaged using 293T cells. Human CC cell lines SW1116 and HCT116 were used and divided into four groups respectively, namely control group, blank group, MACC1 siRNA group (infected with interference lentivirus) and MACC1 group (infected with overexpression lentivirus). Then, cell proliferation and clone formation were examined. Besides, migration and invasion of cells were investigated by wound healing and transwell assays, respectively. Additionally, liver metastasis was evaluated in nude xenografts model.

RESULTS: Overexpression and interference lentivirus of MACC1 were successfully constructed. After infected with overexpression lentivirus, cell proliferation, clone formation number, cell invasion and migration capabilities of CC cells were significantly increased ($p < 0.05$). Furthermore, decreased cell proliferation, smaller and fewer clones, as well as weakened cell migratory and invasive capabilities were observed in SW1116 and HCT116 cells treated with interference lentivirus of MACC1 ($p < 0.05$). Additionally, liver metastasis rate was higher in SW1116 cells with a higher expression level of MACC1 than that in HCT116 cells with a lower MACC1 expression level.

CONCLUSIONS: MACC1 promotes the growth and metastasis processes of CC cells.

Key Words:
Colon cancer, Metastasis associated in colon cancer 1, Cell proliferation, Liver metastasis.

Introduction

Colon cancer (CC) is the third most common malignancy with high mortality rate worldwide[1]. Although considerable efforts have been made to investigate diagnosis and treatment for CC, advanced CC remains hardly curable[2-4]. About 50% of CC patients have been reported to develop distant metastases[5] and metastases could significantly reduce the 5-year-survival rates for patients[6]. Therefore, extending the understanding of molecular mechanisms of CC metastases and searching for new therapeutic targets are desirable and necessary.

Metastasis of primary tumor cells to other tissues is a dynamic process involving multi-metastatic genes and multi-linked processes. It has been reported that the metastatic process was closely associated with cancer stem cell and epithelial-mesenchymal transition formation[7]. HGF/Met signaling pathway can mediate a variety of biological functions, including tumor formation, differentiation, cell migration and invasion[8,9]. Metastasis-associated in colon cancer 1 (MACC1), located on chromosome 7, is identified through genome-wide expression analyses conducted on metastatic CC[5]. Increasing evidences have suggested that the overexpression of MACC1 was closely associated with the progression in multiple kinds of tumors[10,11]. MACC1 can transform into nucleus and bind to the promoter of the c-Met receptor, followed by promoting the transcription of the c-Met gene and increasing the related proteins expression in the membrane of CC cells[12-14]. Furthermore, overexpression of MACC1 significantly reduces the 5-year survival rate of CC patients and induces proliferation, migration and invasion, as well as promoting liver metastases in xenograft model[15]. MACC1 has been also identified as an independent prognostic factor for colorectal cancer metastasis[16]. Additionally, MACC1 is reported to be associated with distant metastasis in gastric cancer and lung cancer[16,17]. All of

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these studies certified that there is a certain role of MACC1 in various tumor metastases. However, a systemic study of the effects of MACC1 in CC is insufficient and needed and there are few studies to investigate the role of MACC1 in certain cancer in different cell lines with different expression levels of MACC1.

In order to further comprehensively investigate the role of MACC1 in CC, MACC1 overexpression and RNA interference lentiviral vectors were constructed to infect two different human CC cell lines, HAC116 and SW1116, to detect the impacts of MACC1 on growth and metastasis process of CC cell. Meanwhile, liver metastasis was also evaluated in a nude xenograft model. Our study might extend our understanding of the role of MACC1 in CC cells and suggest that some drugs might be developed to treat CC by targeting MACC1.

Materials and Methods

Plasmid Construction and Lentivirus Packaging

The primers of 5'-GAGGATCCCCGGGTACCGGTCGCCACCATGCTAATCACTGAAAGAAAACATTTTCG-3' and 5'-TCCTTGTAGTCCATACCTACTTCCTCAGAAGT GGAGAATGCAG-3' were used to amplify the MACC1 from cDNA library. After predicated, the PCR product was inserted into GV115 vector (7.5 kb) using the AgeI/EcoRI (Shanghai Ji Kai Gene Chemical Technology Co., Ltd., Shanghai, China). The plasmids were then transfected into 293T cells using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). After 24 h, the transfection efficiency was assessed by observing the expression of fluorescence-marked gene (GFP). Once transfection efficiency was more than 70%, the cells were harvested after 36 h to screen effective RNAi vector using Western blotting assay. As a result, the effective fragment was as follows: 5'-CCGGTTCACCCTTCGTGGTAATAATCTCGAGATTATTACCACGAAGGGTGAATTTTT-3', 5'-AATTCAAAAATTCACCCTTCGTGGTAATAATCTCGAGATTATTACCACGAAGGGTGAA-3'.

Then the overexpression and effective interference lentivirus vectors were transfected into 293T cells combined with packaging plasmids (pMD2G and psPAX2). After 48 h, the supernatant containing lentiviral particles was harvested and centrifuged at the speed of 4,000 × g for 10 min. And then the lentivirus was filtered using 0.45 μm cellulose acetate filters and stored at -80°C until use.

Lentiviral Infection and Cell Grouping

SW1116 and HCT116, two human CC cell lines, were provided by Shanghai Institute of Digestive Disease (Shanghai, China). These two cell lines were cultured with RPMI 1640 medium containing 10% newborn calf serum (Gibco, Oklahoma USA) and placed at a 37°C incubator containing 5% CO_2. The cells were divided into four groups, namely control group (without lentiviral infection), blank group (infected with blank viral), MACC1 siRNA group (infected with interference lentivirus) and MACC1 group (infected with overexpression lentivirus). The lentiviral infection efficiency was observed using a fluorescence microscope after cultured for 3 days and the cells were collected for subsequent experiments after cultured for 5 days.

Western Blotting

SW1116 and HCT116 cells were washed with phosphate balanced solution (PBS) and transferred into a 1.5 ml EP tube. Then the cells were lysed using lysis buffer on ice for 10-15 min and then centrifuged at 4°C, 12000 × g for 15 min. The concentration of protein was detected and adjusted to a final concentration of 2 μg/μl. After mixed with an equal volume of 2 × loading buffer, the protein samples were denatured at 100°C for 5 min. The protein samples were separated using 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocked with 5% skim milk for 1 h, anti-MACC1 (1:1000; Abcam Inc., Cambridge, MA, USA) and anti-GAPDH (1:1000; Abcam) were added as primary antibodies for the specific protein for 2 h at room temperature, followed by incubation with the secondary antibodies anti-mouse IgG (1:5,000; Beijing Zhongshan Biotechnology Co., Ltd) at room temperature for 2 h. The immunoreactive bands were detected and visualized by electrochemiluminescent (ECL) detection system (Amersham Life Sciences, Inc., Arlington Heights, IL, USA). All the experiments were conducted for three times in several days.

Cell Proliferation

The cells were seeded in 96-well plates as the density of 2 × 10^3 cells/well and 3-5 wells for

each group. The cells were counted using Cellomics at 2-5 days. By adjusting the input parameters of Cellomics array scan, the number of each scan-well plates was accurately calculated with green fluorescent cells. All the experiments were repeated for three times.

Clone Formation Assay

Approx. 500 cells were seeded in each well of 96-well plates and 3-5 wells for each group to assess the clone formation for cells in different groups. The cell culture was exchanged every 3 days and continually cultured for about 15 days. The cellomics array scanned and photographs were taken for each well to analyze the clone size and cell number of clone when the cell numbers of vast majority clones were greater than 5 mm. All the experiments were repeated for three times.

Transwell Invasion Assay

Matrigel invasion chamber inserts (BD Biosciences, Franklin Lakes, NJ, USA) were equilibrated overnight at room temperature in 300 µl serum-free medium for 1-2 h. Cells were seeded in the upper chamber containing serum-free medium at a density of 1×10^4 cells/well. The lower chamber was added with 600 µl complete medium containing 10% fetal bovine serum (FBS) as a chemoattractant. After incubated for 24 h-72 h, cells in the upper chamber were softly removed with a cotton swab and cells on the bottom surface were fixed with 3% formaldehyde for 15 min. After washed with PBS, the cells were permeabilized using 1% Triton X-100 for 20 min, which was followed by 500 µl Giemsa (Sigma Chemical Co., St. Louis, MO, USA) staining for 30 min. The cells were visualized under a fluorescent microscope (MicroPublisher 3.3RTV; Olympus, Tokyo, Japan) and five random fields were captured at 100 magnification (n = 3). Image-pro Plus software (Media Cybernetics, Silver Springs, MD, USA) was utilized to count the number of cells.

Wound Healing Assay

Cells were seeded in a 24-well plate at a density of approx. 2×10^5 cells/well. The cells were denuded through the center of the plate with a plastic pipette tip. The plate was softly washed with PBS to remove the floating cells, followed by adding a fresh serum-free medium. The new cells moved into the scratch were observed and captured at 0, 6, 12 and 24 h. All experiments were performed in triplicates.

Tumor Xenografts

A total of 40 health male BALB/c (nu/nu) nudes (4 weeks, 15-18 g)[18] were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences and fed in SPF Animal Laboratory of Shanghai Fudan University with sterile water and food. After on diet for 12 h, the nudes were anesthetized by intraperitoneal injection of 1 % sodium pentobarbital at the doze of 45 mg/kg. Then the nudes were fixed on the operating table in the supine position, with 75% alcohol to disinfect the skin. A 0.5-1.0 cm oblique incision was made on the left back. Then 200 µl, 5×10^6/ml SW1116 or HCT116 cells (blank groups) were injected into the spleen of nudes. After 10 minutes, the spleen was removed and the wounds were continuously sutured using 3-0 absorbable sutures. The nudes were sacrificed on 35 days after injection and the distribution of CC cells in various organs was observed by using fluorescence dissecting microscope. Liver metastasis rate was calculated with a percentage of the fluorescent area accounts on the liver surface.

All the experiments were strictly accordant with the care and use guidelines of experimental animal and approved by the Animal Protection Committee.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD) and analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Unpaired *t*-test and one-way Analysis of Variance (ANOVA) followed by LSD test were performed for the differences between different groups. A value of $p < 0.05$ was considered as statistically significant difference.

Results

The MACC1 Expression in SW1116 and HCT116 Cells After Lentiviral Infection

After 3 days of lentiviral infection, GFP expression was found in the blank group, MACC1 siRNA and MACC1 groups, but not in the control group, indicating similar infection efficiencies of different lentivirus in SW1116 and HCT116 cells (Figure 1 A). Then MACC1 protein expression was examined after 5 days of lentiviral infection (Figure 1 B) and the differences were reflected clearly with the relative protein expression (Figure 1 C). As the results, no significant difference in MACC1 protein expression was found between

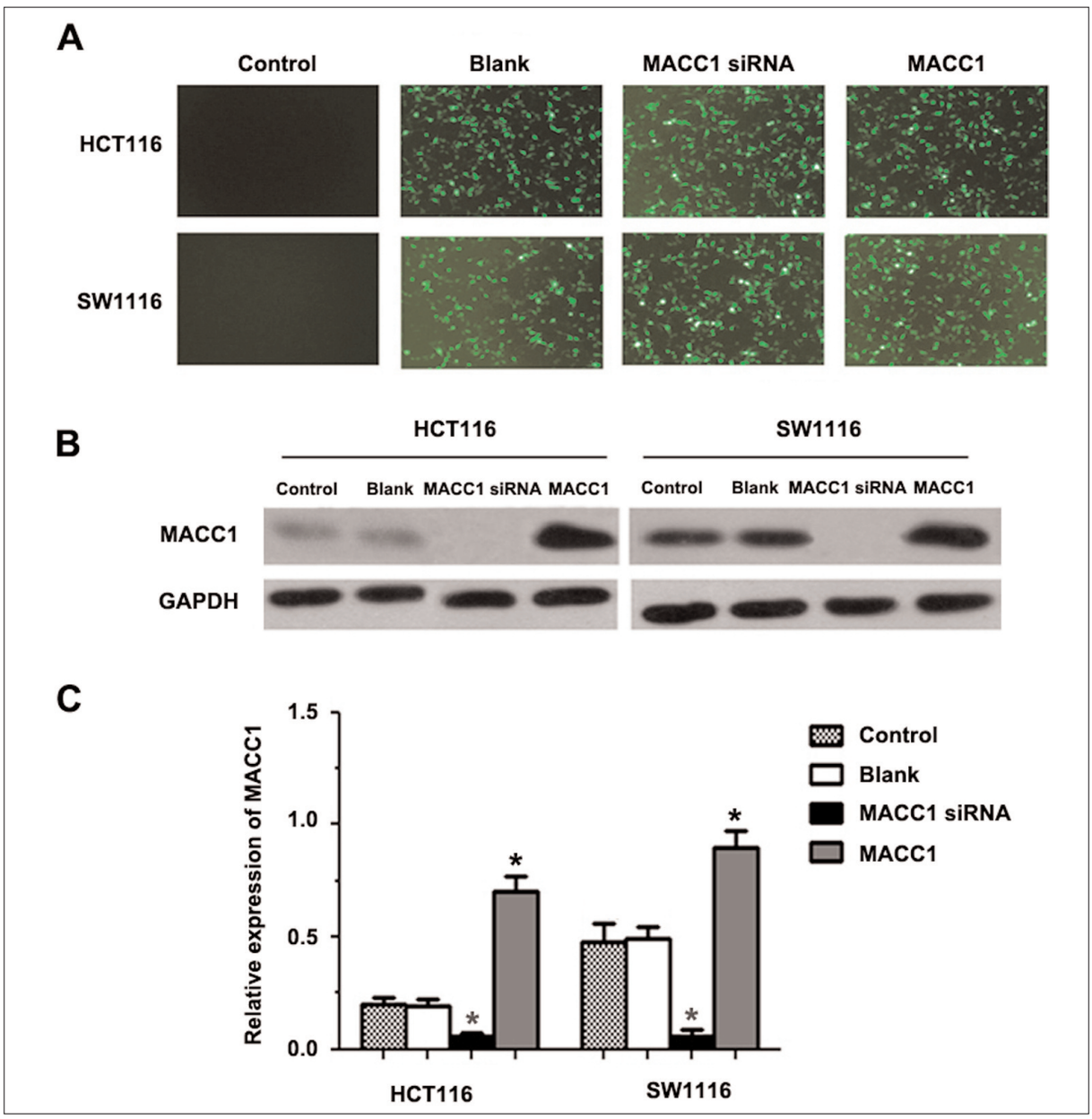

Figure 1. The MACC1 expression in SW1116 and HCT116 cells after lentiviral infection. ***A,*** Infection efficiency was observed under a fluorescence microscope for HCT116 and SW1116 cells after 3 days of lentiviral infection (100 ×); ***B,*** MACC1 expression of SW1116 and HCT116 cells in each group by western blot assay; ***C,*** The relative protein expression of MACC1; Columns, mean (n = 3); bars, SD; *$p < 0.05$ *vs*. control group.

control and blank groups both in these two cell lines ($p > 0.05$). Meanwhile, MACC1 protein expression was scarcely detected in MACC1 siRNA groups. After infected with overexpression lentivirus, the protein levels of MACC1 were significantly increased compared with those in the blank group ($p < 0.05$).

MACC1 Promotes the Colon Tumor Cell Growth and Development

To study the role of MACC1 in CC, colon tumor cell growth (Figure 2) and development (Figure 3) were detected in SW1116 and HCT116 after transfected with overexpression or interference lentivirus using Cellomics method.

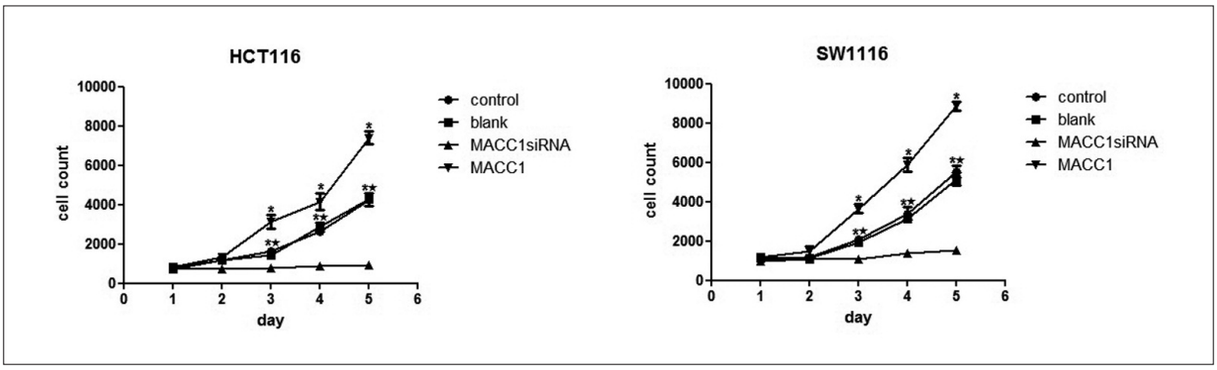

Figure 2. MACC1 enhances cell proliferation rate of colon cancer. Colon tumor cell growth were detected in SW1116 and HCT116 after infected with overexpression or interference lentivirus using Cellomics method (n=3). *$p < 0.05$ *vs*. control group.

As a result, overexpression of MACC1 enhanced the cell growth both in SW1116 and HCT116 cells. After silencing MACC1, the cell count was significantly decreased ($p < 0.05$), indicating that the growth of tumor cells was influenced by MACC1 (Figure 2).

Similarly, more colonies appeared in SW1116 and HCT116 cells treated with overexpression lentivirus than those in controls ($p < 0.05$). In contrast, only a few small colonies formatted in the plate after knocking down MACC1 with specific shRNA lentivirus ($p < 0.05$, Figure 3).

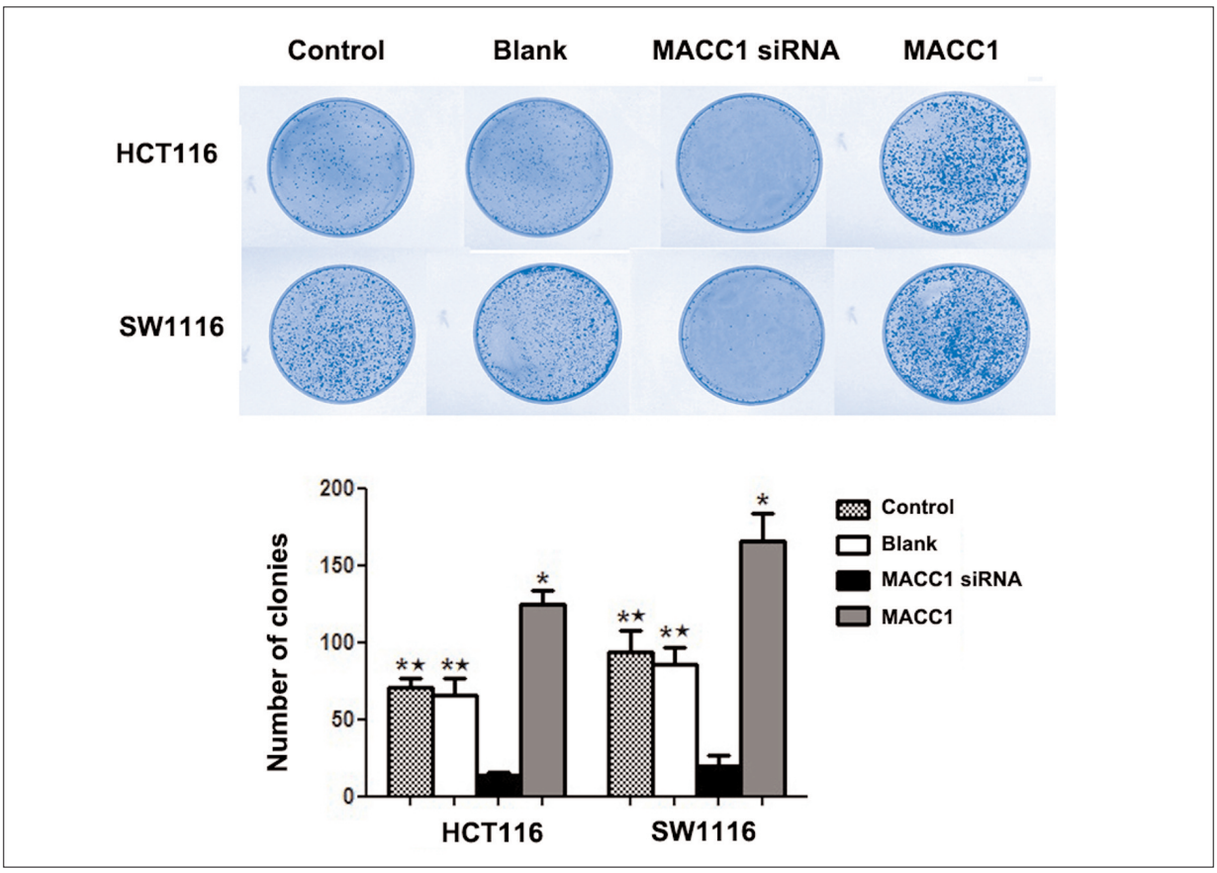

Figure 3. MACC1 promotes cell colony formation of colon cancer. After transfected with overexpression or interference lentivirus, the ability of colony formation was detected in SW1116 and HCT116 cells. Columns, mean (n=3); bars, SD; *$p < 0.05$ *vs*. control group.

MACC1 Promotes the Colon Tumor Cell Invasion and Migration

Transwell assay was conducted to examine the effect of MACC1 on the invasion of SW1116 and HCT116 cells (Figure 4 A). Compared with control group, no obvious changes of cell invasion capacity were found in cells infected by control lentivirus ($p > 0.05$). The numbers of invaded cells were significantly higher in MACC1 group and significantly lower in MACC1 siRNA group compared with those in control and blank groups ($p < 0.05$). The migration capacity was measured by "wound healing" assay and the results are shown in Figure 4 B. As it was shown, the cells migration capacity was improved by overexpressing MACC1 and suppressed by silencing MACC1.

MACC1 Promotes Colon Tumor Metastasis to Liver

SW1116 and HCT116 cells were two different human CC cell lines with different MACC1 expression levels. As shown in Figure 5 A, MACC1 expression level was higher in SW1116 cells than that in HCT116 cells ($p < 0.05$), similar with the results of Figure 1 B and C. After 5 weeks, 7/20 and 6/20 mice injected with SW1116 and HCT116 cells (blank group) developed liver tumor metastasis, respectively (data were not shown). The tumor volume was significantly larger in mice injected with SW1116 cells than that in mice injected with HCT116 cells ($p < 0.05$, Figure 5 B and C).

Discussion

MACC1 is identified in various cancers and plays pivotal roles in cancer-related progressions. MACC1 expression is more frequent in hepatocellular carcinoma (HCC) with vascular invasion[19] and might be served as a new indicator to stratify the prognosis of TNM stage I HCC patients[20]. A significant correlation is found between MACC1 expression and peritoneal dissemination of gastric carcinoma[16]. Meng et al[21] demonstrated that MACC1 expression was significantly higher in nasopharyngeal carcinoma cells than that in normal nasopharyngeal epithelial cells. In this study, two kinds of CC cell lines, SW1116 and HCT116, were used to detect the expression of MACC1 and MACC1 expression was significantly higher in SW1116 cells than that in HCT116 cells. Furthermore, the expression level of MACC1 was significantly associated with the growth and metastases of CC cells.

MACC1 is considered as a key regulator of HGF-MET signaling in CC, and participated in several cancer-related processes by activating of HGF/Met signaling pathway[5,22,23]. Increasing evidences proved that MACC1 could influence the proliferation and invasion in multiple cancers. Downregulation of MACC1 inhibits proliferation in tongue squamous cell carcinoma and gallbladder cancer[24,25]. MACC1 is reported to play an important role in tumor development in adenoid cystic carcinoma[26]. Zhang et al[27] demonstrated that MACC1 regulated proliferation and colony formation, of osteosarcoma through Akt signaling pathway. In the present study, overexpression and interference lentivirus of MACC1 were transfected into SW1116 and HCT116 to investigate the effects of MACC1 on cell proliferation, clone formation, invasion and migration in CC. Consistent with the previous studies, our results showed that overexpression of MACC1 promoted cell proliferation, the colony-forming ability as well as invasion and migration capacities in both SW1116 and HCT116 cell lines[28]. Further, these results were certified by interference lentivirus assay, and reduced proliferation, weaken colony-forming ability as well as low attenuated invasion and migration capacities were observed in these two human colon cell lines after silencing MACC1.

CC is the most common gastrointestinal malignancy frequently metastasizing to other tissues, including liver, lung, bone, adrenal gland, brain as well as ovary, and liver metastasis is the most common situation which accounts for about 30%-70% of all metastases[29]. The metastatic process is characterized by increased motility, dysregulated adhesion, enhanced invasiveness, changed microenvironment and proliferate, and resistance to apoptosis[30]. Unfortunately, a large number of CC patients with liver metastasis are incompatible with the indications of resection and can't accept metastasis resection owing to liver dysfunction, incomplete capsule and vein thrombosis, and then the prognosis of these patients is often very poor with a high 2-year recurrence rate of approximately 75%[31,32]. MACC1 expression has also been reported to predict the postoperative recurrence for lung adenocarcinoma patients undergoing surgery[17,33]. Overexpression of MACC1 was significantly correlated with poor overall patient survival[24]. MACC1 is consid-

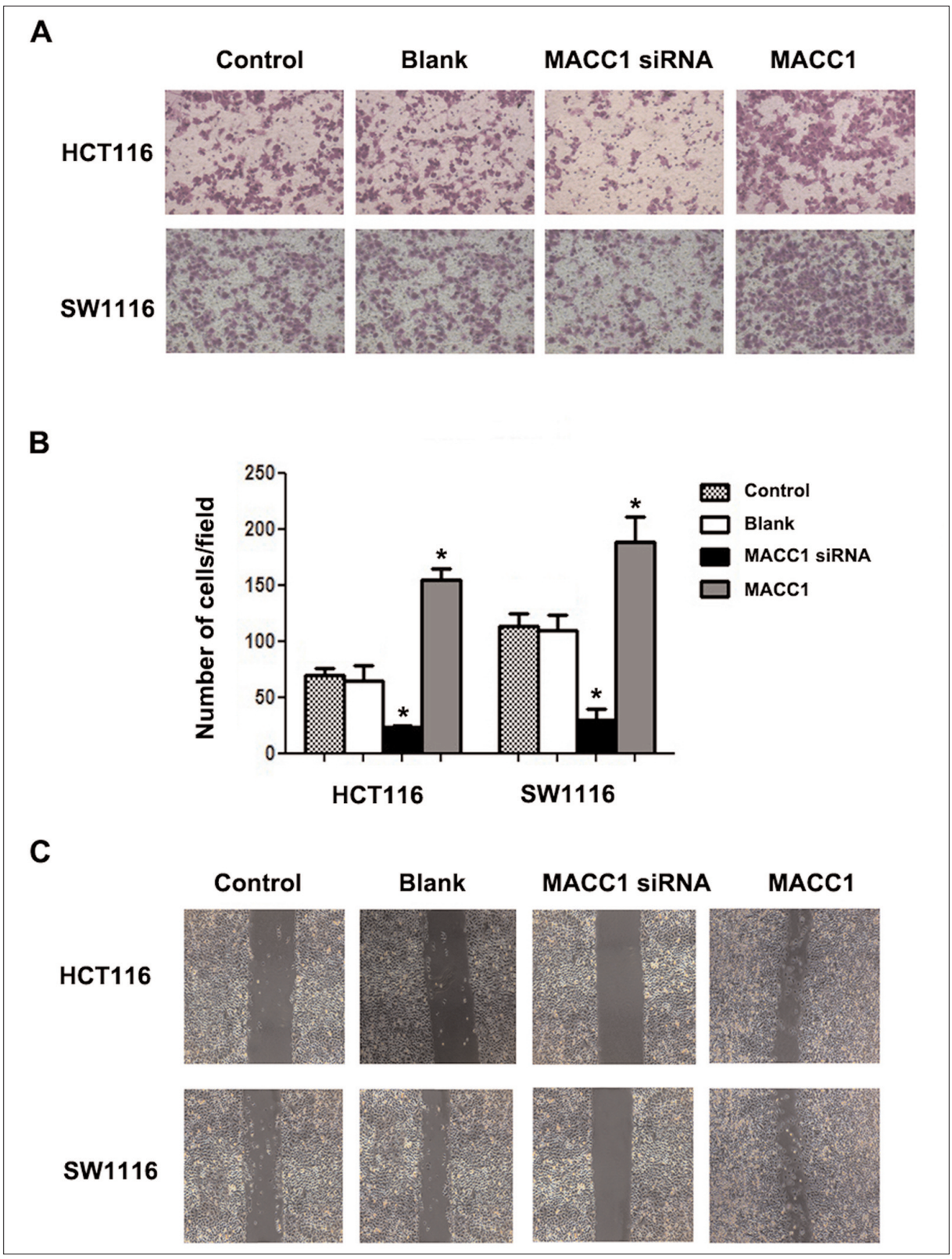

Figure 4. MACC1 affects cell invasion and migration of colon cancer. ***A,*** Cellular invasion through Matrigel-coated Transwells is shown for each group (100 ×). ***B,*** The counts of cellular invasion cells in each group; Columns, mean (n=3); bars, SD; *$p < 0.05$ *vs*. control group; C. Representative images for the wound healing assays are shown for each group (50 ×).

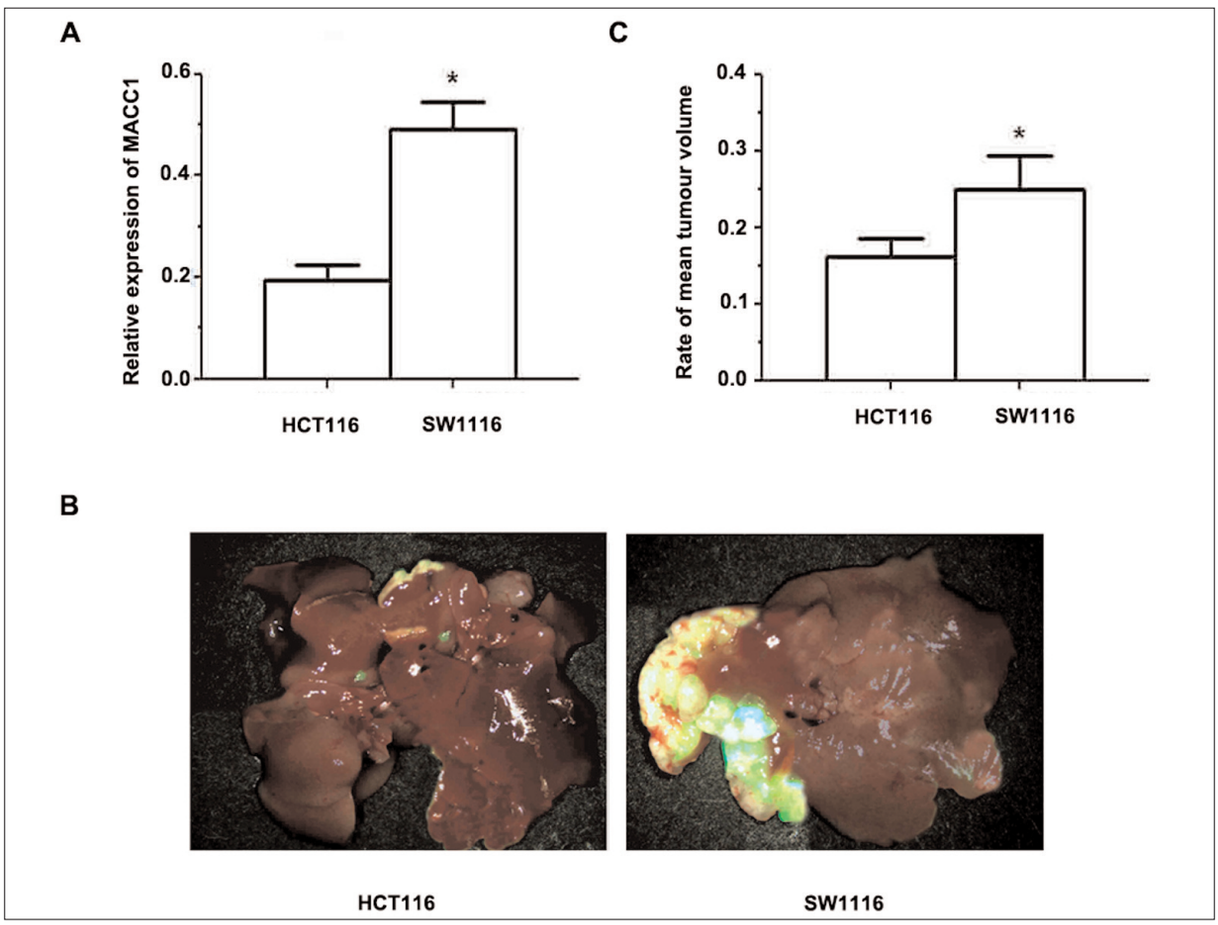

Figure 5. Liver metastasis rates in nudes injected with SW1116 and HCT116 cells. ***A,*** MACC1 protein expression was higher in SW1116 than that in HCT116 cells; ***B,*** Representative images for the liver metastases in nudes injected with HCT116 or SW1116 cells; ***C,*** Mean tumor volume of liver metastasis in nudes injected with SW1116 cells was larger than that of HCT116 cells. Columns, mean (n=3); bars, SD; $*p < 0.05$ vs. HCT116.

ered as an early prognostic indicator for CC and the 5 years survival of patients with high MACC1 levels was 80% compared to 15% in those with low MACC1 levels[34]. In mice xenograft model of our study, tumor size of liver metastases was smaller small in HCT116 cells with low MACC1 expression. Therefore, MACC1 might enhance the liver metastasis of CC, accordingly leading to a poor outcome. These results suggested that targeting MACC1 might be a promising strategy for preventing colon tumor metastasis[28,35].

Conclusions

Our study reflected that MACC1 had several effects on cell proliferation, colony-forming, invasion and migration capacity, as well as liver metastasis of CC. Our study provided a systematic certification for the role of MACC1 in CC and proves that different development and metastasis capabilities of different CC cell lines might be related with the expression levels of MACC1. MACC1 might be considered as a candidate target for CC treatment and prognostic indicator. However, confirmatory studies are still needed to confirm these preliminary findings.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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