TUMORIGENESIS AND NEOPLASTIC PROGRESSION

CD133 Affects the Invasive Ability of HCT116 Cells by Regulating TIMP-2

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Accepted for publication
October 15, 2012.

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CD133 is widely expressed in colorectal cancer (CRC) tissues and cell lines. This protein has been used as a marker of CRC cancer stem cells, although the function and mechanism of CD133 in CRC invasion and metastasis remain unclear. In our study, we examined the role of CD133 in CRC invasion *in vitro* and investigated the mechanism involved in CD133-related invasion. CD133^{high} and CD133^{low} HCT116 cells were isolated, and the proliferation and invasive ability of these two subpopulations were tested. CD133^{high} HCT116 cells exhibited greater proliferation and invasion compared with CD133^{low} HCT116 cells. CD133 knockdown (using CD133 small-interfering [si]RNA) inhibited the invasive activity of CD133si-HCT116 cells. For the first time, we found that the expression of tissue inhibitor of matrix metalloproteinases-2 (TIMP-2) was down-regulated in CD133si-HCT116 cells. In addition, for the TIMP-2si-HCT116 cells (transfected with TIMP-2 siRNA), *in vitro* invasion was significantly decreased, whereas the expression of CD133 remained unchanged. Finally, the metalloproteinase 2 and MEK/ERK signaling pathways were examined, and no significant change was observed after the knockdown of CD133 or TIMP-2 in HCT116 cells. In conclusion, we demonstrated that CD133 plays an important role in HCT116 cell invasion, and for the first time, we found that CD133 knockdown significantly down-regulated TIMP-2 expression, which suggests that CD133 affects the invasive ability of HCT116 cells by regulating TIMP-2. (*Am J Pathol* 2013; 182: 565–576; <http://dx.doi.org/10.1016/j.ajpath.2012.10.015>)

Cancer is still a major public health problem in most parts of the world, although tremendous progress in cancer molecular biology and treatment has been achieved. Colorectal cancer (CRC) remains the third most common cause of cancer-related death, although the incidence and mortality of CRCs has continued to decrease due to early detection and treatment.¹ Radical colorectal resection in accordance with standardized guidelines² and the administration of adjuvant chemoradiotherapy (CRT) to improve survival have contributed to this advancement. The recurrence and metastasis of CRC, however, remains one of the most common causes of cancer-related death, which suggests that the available treatments are unable to eradicate all cancer cells,^{2,3} and a minority population of CRT-resistant CRC cells may remain.

Tumor cells are heterogeneous. The cancer stem cell hypothesis postulates that only a small subpopulation of cells within a tumor, cancer stem cells (CSCs), have the ability to proliferate extensively and form new tumors.^{4,5}

CD133 (prominin-1) is a five-transmembrane glycoprotein, with a molecular weight of 120 kDa.⁶ As a putative stem cell and CSC marker, CD133 was initially considered to be a marker of hematopoietic stem cells.⁷ A series of publications have demonstrated that CD133, however, can be used as a marker for the enrichment of CSCs in various solid tumors, including the brain,^{8,9} prostate,⁸ liver,⁹ and colon.^{10–12} Some authors, however, have denied the role of CD133 as a CSC marker for colon cancer because it has been found to be expressed by the full spectrum of undifferentiated and differentiated colonic epithelial cells.¹³

Recent investigations suggest that CD133 may be a useful marker for the clinical assessment of CRC. CD133⁺ CRC cells are more resistant to chemotherapy.¹⁴ A higher percentage of CD133⁺ cells was positively correlated with

Supported by the National Science and Technology Infrastructure Grant, China (H.F., X.B., Z.Y., B.G., and Y.L.).

M.Z. and Y.L. contributed equally to this work.

a poorer prognosis,^{15,16} adverse disease-free survival and overall survival,¹⁷ tumor size, histological grade, lymph node metastasis, distant metastasis, and TNM stage.¹⁸ Elevated CD133 may be a predictive marker of distant recurrence and poor survival after preoperative CRT in rectal cancer.¹⁹ The role and mechanism of CD133 in CRC invasion and metastasis, however, still remains to be elucidated.

Metalloproteinase 2 (MMP2) plays an important role in the migration of malignant cells because of its ability to degrade type IV collagen.²⁰ Tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) is an endogenous inhibitor for MMP2 and an adaptor molecule that is required for pro-MMP2 activation at the cell surface.²¹ Several studies have established that MMP2, TIMP-2, and MMP2/TIMP-2 complex are involved in the growth and progression of CRC.^{22–24} Even though previous studies revealed that TIMP-2 induced the stimulation of ERK signaling in human fibrosarcoma cancer cells in both the absence and presence of MMP2 activation,²⁵ and CD133-expressing hepatocellular carcinoma (HCC) cells require MMP2 activation to maintain their invasive ability,²⁶ the significance of the presence of CD133-expressing CRC cells requires more investigation.

In this study, we evaluated the malignant characteristics of the CD133-expressing human CRC cell line HCT116, and for the first time, we demonstrated that HCT116 CRC cells in which CD133 was knocked down by small-interfering RNA (siRNA) displayed a decreased invasive ability *in vitro* that was associated with the down-regulation of TIMP-2 expression. Activation of MMP2 and stimulation of ERK were also examined and were shown not to be involved. This study sheds light on the significance of CD133 expression in CRC cells and identifies a new target for developing treatments for human CRC.

Materials and Methods

Cell Culture

All of the human cell lines used in this study were from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College (Beijing, China) and were authenticated by Short Tandem Repeat (STR). The cells were cultured according to the recommended conditions provided by the manufacturer. The human colon carcinoma cell line HCT116 was maintained in Iscove's Modified Dulbecco's Medium containing 5% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD). All cell lines were grown in 5% CO₂ at 37°C.

Flow Cytometric Analysis and Fluorescence-Activated Cell Sorting

For the detection of CD133 in CRC cells, the cells were dissociated and washed twice with PBS containing 1% FBS. The suspended cells were incubated with an allophycocyanin-conjugated anti-CD133/2(293C3) antibody (Miltenyi Biotec,

Cologne, Germany) according to the manufacturer's recommendations.²⁷ Mouse IgG2b-allophycocyanin was used as the isotype control antibody. The cells were analyzed on an Accuri C6 Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ).

An FACSDiVa flow cytometer (Becton Dickinson) was used to sort subpopulations of HCT116 cells based on CD133/2 reactivity.

Cell Proliferation Assay

To evaluate the cell proliferation rate, cells were plated in 96-well plates at a density of 2×10^3 cells/well (for 6-day assays) or 1×10^4 cells/well (for 3-day assays) in a final volume of 100 μ L of culture medium per well. Cell proliferation was analyzed using the methyl thiazole tetrazolium assay (MTT assay, Sigma, Shanghai, China). In brief, 20 μ L of 5 g/L MTT was added to each well and incubated at 37°C for 4 hours. Then, 200 μ L dimethyl sulfoxide was added to dissolve the formazan crystals. Quantification of the viable cells was performed on a Synergy 4 reader (BioTek, Winooski, VT) by reading the absorbance of the supernatants at 570 nm.

In Vitro Invasion Assays

In vitro invasion was measured using an *in vitro* Transwell assay. The 24-well Transwell polycarbonate membrane inserts with 8.0- μ m pores (Costar, Cambridge, MA) were coated at the bottom with Matrigel (1:8, 70 μ L) (354277, BD Biosciences, San Jose, CA). Cells (7.5×10^4) were plated in the upper chamber in 0.2 mL serum-free Iscove's Modified Dulbecco's Medium, and the lower chamber was filled with 0.5 mL conditioned medium (serum-free supernatant of NIH/3T3 cells). Cells were incubated for 48 hours, and cells remaining on the upper side of the membrane were gently removed with wet cotton swabs. The invading cells on the lower surface of the membrane were fixed in 4% paraformaldehyde for 30 minutes and then stained with crystal violet staining solution (C0121, Beyotime Institution of Biotechnology, Hai Men, China) for 30 minutes before washing. The number of cells in nine randomly selected microscopic fields per well (20 $\times$) was counted under an Olympus IX51 microscope (Olympus, Miyazaki, Japan).

RNA Silencing

The siRNA method was used to knock down CD133 and TIMP-2 expressions in HCT116 cells. All siRNAs were obtained from the Ribobio Co. (Guangzhou, China). The sequences of the two CD133 siRNA duplexes were as follows: Si-h-CD133_002, 5'-GGAGAACAAUAAUAG-GAUAdTdT-3'; Si-h-CD133_003, 5'-GUGAACAUUCU-AACAUAAdTdT-3'. The sequences of the two TIMP-2 siRNA duplexes were as follows: si-h-TIMP-2_002, 5'-GG-AAAGAAGGAAUAUCUCAdTdT-3'; si-h-TIMP-2_003, 5'-GGAAGUGGACUCUGGAAACdTdT-3'. Ncontrol_05815 (Ribobio Co., Guangzhou, China) was used as negative

universal control (NCsi). Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two siRNAs for CD133 were mixed together as CD133si, and two siRNAs for TIMP-2 were mixed together as TIMP-2si.

Western Blot

Total protein was isolated using protein lysis buffer supplemented with phenylmethylsulfonyl fluoride (Beyotime Institution of Biotechnology) and protease inhibitor (Amresco, Solon, OH). Protein samples were separated by 10% SDS-PAGE (Beyotime Institution of Biotechnology, Haimen, China) and transferred onto nitrocellulose (NC) membranes (Pall Gelman Laboratory, Stevenage, England). For immunodetection, blots were incubated with primary antibodies (Table 1) overnight at 4°C and then washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG [H+L (heavy chain + light chain)] (1:5000, Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) or horseradish peroxidase-conjugated anti-rabbit IgG (H+L) (1:3000, Zhongshan Goldenbridge Biotechnology Co., Ltd.) for 1 hour. ECL Super Signal West Pico Chemiluminescent Substrate (Pierce, Appleton, WI) was applied to enable visualization of the bands. To assess the protein levels, the optical densities of the bands in each blot were analyzed using a Quantity One 1-D Analysis System (Bio-Rad Laboratories Inc., Hercules, CA). Details of the primary antibodies used for Western blot analysis are shown in Table 1.

RNA Isolation, Reverse Transcription, Real-Time PCR

Total RNA was extracted using the TRIzol RNA isolation reagent (Life Technologies Corporation, Carlsbad, CA). Reverse transcription was performed using a PrimeScript

RT Master Mix kit (TAKARA Biotechnology Co. Ltd., Dalian, China). The cDNA products were subjected to real-time PCR using a SYBR Premix Ex Taq™ II kit (TAKARA Biotechnology Co. Ltd.). Real-time PCR was performed in a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the following program: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Each sample was analyzed in triplicate. β -actin mRNA in each sample was quantified as an endogenous control. The primer sequences used in this study are shown in Table 2.

MMP2 Zymography

The proteolytic activity of the supernatants was detected by gelatin zymography. After transfection with siRNAs for 24 hours, cells were incubated in serum-free media for 48 hours. Serum-free supernatants were then subjected to electrophoresis on 10% SDS-polyacrylamide gels containing 0.1% gelatin. The gels were incubated for 45 minutes at room temperature in 2.5% Triton X-100 (Qxbio, Beijing, China) twice, then incubated in activation buffer (50 mmol/L Tris-Cl pH 8.0, 10 mmol/L CaCl₂, 50 mmol/L NaCl) for 42 hours at 37°C. The gels were stained with 0.05% Coomassie Brilliant Blue R-250 for 3 hours, and were then de-stained in wash buffer I (10% acetic acid, 30% methanol), II (10% acetic acid, 20% methanol), and III (5% acetic acid, 10% methanol) for 0.5 hour, 1 hour, and 2 hours, respectively. Gelatinolytic activity was detected as transparent bands on a blue background.

Statistical Analysis

The data are expressed as the mean $\pm$ SD. The statistical analyses comparing pairs of groups were performed using

Table 1 Details of the Primary Antibodies Used in Western Blot Analysis in This Study

Antibody	Supplier	Antibody type	Product code	Optimal dilution
CD133	Miltenyi Biotec (Cologne, Germany)	Mouse monoclonal	130-092-395	1:200
MMP2	Santa Cruz (Santa Cruz, CA)	Rabbit polyclonal	sc-10736	1:800
TIMP-2	Bioworld (St. Louis Park, MN)	Rabbit polyclonal	BS1366	1:800
ERK1/2	Bioworld	Rabbit polyclonal	BS1112	1:800
p-ERK1/2	Bioworld	Rabbit polyclonal	BS4621	1:800
MEK1/2	Bioworld	Rabbit polyclonal	BS3599	1:500
p-MEK1/2	Santa Cruz	Rabbit polyclonal	sc-10736	1:1500
MMP1	Proteintech Group (Chicago, IL)	Rabbit polyclonal	10371-2-AP	1:300
MMP3	Proteintech Group	Rabbit polyclonal	17873-1-AP	1:600
MMP7	Bioworld	Rabbit polyclonal	BS1239	1:500
MMP9	Bioworld	Rabbit polyclonal	BS1241	1:500
MMP13	Bioworld	Rabbit polyclonal	BS1231	1:500
MMP28	Proteintech Group	Rabbit polyclonal	18237-1-AP	1:1000
MT1-MMP	Bioworld	Rabbit polyclonal	BS1232	1:500
TIMP-1	Santa Cruz	Rabbit polyclonal	sc-5538	1:500
TIMP-4	Bioworld	Rabbit polyclonal	BS1477	1:800
GAPDH	Santa Cruz	Mouse monoclonal	sc-32233	1:3000
Actin	Zhongshan Goldenbridge (Beijing, China)	Mouse monoclonal	TA-09	1:1000

Table 2 PCR Primers for Quantitative Real-Time PCR

Name	Sequence
CD133	Sense: 5'-AGTGGCATCGTGCAACCTG-3' Antisense: 5'-CTCCGAATCCATTCGACGATAGTA-3'
TIMP-2	Sense: 5'-AGATGTAGTGATCAGGGCCAAAG-3' Antisense: 5'-GCTTGATCTCATACTGGATCCTCTTG-3'
Hif-1 α	Sense: 5'-CCGAGGAAGAACTATGAA-3' Antisense: 5'-GTTGGTTACTGTTGGTATC-3'
Hif-1 β	Sense: 5'-AACAAGATGACAGCCTAC-3' Antisense: 5'-ATGCGTAAGATGGTTAGC-3'
Cdc42	Sense: 5'-AGGATTATGACAGATTACGA-3' Antisense: 5'-GGACAGTGGTGAGTTATC-3'
RhoA	Sense: 5'-TGCTTGCTCATAGTCTTC-3' Antisense: 5'-ATATCTGCCACATAGTTCTC-3'
Smad7	Sense: 5'-CCTTAGCCGACTCTGCGAACTA-3' Antisense: 5'-CCAGATAATTCTGTTCCCCTGT-3'
TIMP-1	Sense: 5'-AGTGGCACTCATGTCTGTG-3' Antisense: 5'-GCAGGATTCAGGCTATCTGG-3'
MMP1	Sense: 5'-AATGTGCTACACGGATAC-3' Antisense: 5'-TTATATTCATCATACCTCCAGTA-3'
MMP2	Sense: 5'-CTTCCAAGTCTGGAGCGATGT-3' Antisense: 5'-TACCGTCAAAGGGGTATCCAT-3'
MMP7	Sense: 5'-AGTTTAGAAGCCAACTCAAGG-3' Antisense: 5'-GCG GTA AGT CTC GAG TAT ATG-3'
MMP9	Sense: 5'-TACCGAGAGAAAGCCTATT-3' Antisense: 5'-CACCTGTTTCACTCACT-3'
MMP13	Sense: 5'-GCAGTCTTCTTCGGCTTAG-3' Antisense: 5'-GGAGTTACATCGGACCA AC-3'
MMP28	Sense: 5'-GAGGCATTCTTAGAGAAGTACG-3' Antisense: 5'-CTAGCAAACAAGTCACTGATCC-3'
MT1-MMP	Sense: 5'-CATGGGCAGCGATGAAGTCT-3' Antisense: 5'-CCAGTATTTGTTCCCCTGTAGAAGTA-3'
MT2-MMP	Sense: 5'-GACATCAGTGCTGCCTAC-3' Antisense: 5'-CTTCTCGAAAGAGCCAGTA-3'
TIMP-3	Sense: 5'-CCAGGACGCCTTCTGCAA-3' Antisense: 5'-CCCCTCCTTTACCAGCTTCTTC-3'
TIMP-4	Sense: 5'-GAAGCCAACAGCCAGAAG-3' Antisense: 5'-GCACAGATGGATGAAGACTT-3'
β -actin	Sense: 5'-AGAAATCTGGCACCACACC-3' Antisense: 5'-AGAGCGGTACAGGGATAGCA-3'

the Student's *t*-test or the two-sample *t*-test with the statistics software SPSS version 13.0 (Statistical Package for the Social Sciences). The threshold for statistical significance was defined as $P < 0.05$.

Results

CD133 Expression in CRC Cell Lines

The expression of CD133 was tested in six human CRC cell lines by flow cytometry (Figure 1A) and Western blot analysis (Figure 1B). As shown in Figure 1A, the percentage of cells that were CD133⁺ ranged from 1.5% to 98%. Western blot analysis shown in Figure 1B revealed that the CD133 protein was expressed in all six cell lines. The cell lines tested here could be categorized into three groups: i) HT29 and Caco-2 cells (more than 95% CD133⁺), ii) COLO201 and COLO320DM cells (less than 5% CD133⁺), and iii) HCT116 and COLO205 cells with a moderate level of CD133⁺ expression. Consistent with the flow cytometry

Figure 1 Expression of CD133 in human CRC cell lines. **A:** Human CRC cell lines were stained with an anti-CD133/2 antibody and analyzed by flow cytometry. Control IgG staining (black lines); CD133/2 staining (black filled); positive cells (arrows). **B:** Western blot analysis of CD133 (W6B3C1) in six human CRC cell lines.

results, CD133 protein expression in the HCT116, HT29, and Caco-2 cells was at a higher level. CD133 protein expression in COLO201 and COLO320DM CRC cells was lower and appears as dim bands in Figure 1B.

CD133^{high} HCT116 Cells Show Greater Proliferation and Invasion

The positive and negative CD133/2 HCT116 cells were sorted and collected by fluorescence-activated cell sorting (Figure 2A), and the biological behavior of the two sorted cell populations was tested by MTT (Figure 2B) and Transwell assays (Figure 2C).

As shown in Figure 2A, the CD133/2 HCT116 cells with the highest (top 8%) and lowest (bottom 8%) expression were sorted and collected by fluorescence-activated cell sorting and designated as CD133^{high} and CD133^{low} cells, respectively. Consistent expression levels of CD133 mRNA and protein in these two populations were confirmed by real-time PCR and Western blot assays. MTT assays showed that compared with the CD133^{low} cells, the CD133^{high} HCT116 cells grew faster (Figure 2B) *in vitro*. The CD133^{high} HCT116 cells also showed a higher invasive ability *in vitro* (Figure 2C).

Figure 2 Isolation and characterization of CD133^{high} and CD133^{low} HCT116 cells. **A:** Flow diagram shows the gating of CD133^{high} (P2) and CD133^{low} (P3) HCT116 cells (left panel), real-time PCR (middle panel), and Western blot analysis (right panel) for isolated CD133^{high} and CD133^{low} HCT116 cells. **B:** The proliferation of isolated CD133^{high} and CD133^{low} HCT116 cells was evaluated by MTT assay. **C:** The *in vitro* invasion of isolated CD133^{high} and CD133^{low} HCT116 cells was assayed using a Transwell assay. Representative photomicrographs of invading cells (upper panels) and the number of invading cells (lower panel). ***P* < 0.01, ****P* < 0.001.

CD133 Knockdown by siRNA Reduces Invasion by HCT116 Cells *in Vitro*

We proposed that high expression of *CD133* would be associated with an important function for CD133. To elucidate this function, HCT116 cells were transfected with CD133 and control siRNAs. The transfected cells were designated CD133si-HCT116 and NCsi-HCT116, respectively. The expression of CD133 within 72 hours after transfection is shown in Figure 3A and Supplemental Figure S1. As shown in Figure 3A, the expression of CD133 mRNA and protein in CD133si-HCT116 cells was inhibited, and the percentage of CD133-positive HCT116 cells decreased by 60%, as indicated by flow cytometry. The proliferation (MTT) and invasion (Transwell assay) of the two groups is shown in Figure 3, B and C. The proliferation of the CD133si-HCT116 cells was similar to that of the NCsi-HCT116 cells (Figure 3B). The CD133si-HCT116 cells showed a lower invasive ability compared with the NCsi-HCT116 cells (Figure 3C). Therefore, knockdown of *CD133* affected the invasive ability of HCT116 cells.

TIMP-2 Is Most Significantly Affected by Knockdown of *CD133*

Various pathways may be involved in the invasion of CRC. We examined changes in the mRNA expression of Hif-1 α , Hif-1 β , cdc42, RhoA, Smad7, TIMP-1, and TIMP-2 caused by the knockdown of *CD133*. We found that only TIMP-2 expression was significantly decreased; other mRNAs were not significantly affected (Supplemental Figure S2). Therefore, we examined the relationship between CD133 and TIMP-2.

Previous studies have demonstrated that MMP2 could be regulated by CD133 in HCC cells,²⁶ but the relationship between TIMP-2 and CD133 in CRC was not documented. Our data, shown in Figure 4, indicates that the expression of TIMP-2 in CD133^{high} HCT116 cells is similar to that of CD133^{low} HCT116 cells (Figure 4A), whereas the expression of TIMP-2 in CD133si-HCT116 cells decreases compared with NCsi-HCT116 cells (Figure 4B). For the first time, we observed that TIMP-2 was significantly down-regulated after the knockdown of *CD133* in HCT116 cells.

Figure 3 *CD133* knockdown by siRNA reduces invasion by HCT116 cells *in vitro*. **A:** HCT116 cells were transfected with NCsi and CD133si. **Upper panels:** Flow cytometric analysis of CD133/2 expression on HCT116 cells at different time points after transfection (staining of IgG2b control, gray lines; CD133/2 staining of NCsi groups, black lines; CD133/2 staining of CD133si groups, black filled). **Arrows** indicate positive cell rates. **Lower panels:** Real-time PCR (left panel) and Western blot analysis (right panel) shows the mRNA and protein expression of CD133 in HCT116 cells after transfection with NCsi and CD133si. **B:** The proliferation of HCT116 cells after transfection with NCsi and CD133si was evaluated by MTT assay. **C:** The *in vitro* invasive ability of HCT116 cells after transfection with NCsi and CD133si were assayed using a Transwell assay. Representative photomicrographs of invading cells (upper panel), and the quantification of the number of invading cells (lower panel). *** $P < 0.001$.

TIMP-2 Knockdown by siRNA Reduces the Invasion of HCT116 Cells *in Vitro* Without Affecting CD133 Expression

To further elucidate the regulation of *TIMP-2* by CD133 and the role of *TIMP-2* in HCT116 cells, *TIMP-2*si-HCT116

and NCsi-HCT116 cell lines were established by transfection with *TIMP-2*si and control siRNAs, respectively. The mRNA and protein expression of *TIMP-2* decreased more than 90% within 72 hours after transfection (Figure 5A). CD133 expression was not affected at either the mRNA or protein expression level after the knockdown of *TIMP-2* in HCT116

Figure 4 Expression of TIMP-2 in CD133^{high} and CD133^{low} HCT116 cells, *CD133*si- and NCsi-HCT116 cells. **A:** In CD133^{high} and CD133^{low} HCT116 cells, TIMP-2 mRNA expression was detected by real-time PCR (left panel), and the protein expression of TIMP-2 was detected by Western blot analysis (middle panel) and quantified by densitometry (right panel). **B:** In *CD133*si- and NCsi-HCT116 cells, TIMP-2 mRNA expression was detected by real-time PCR (left panel), and the protein level of TIMP-2 was detected by Western blot analysis (middle panel) and quantified by densitometry (right panel). ***P* < 0.01, ****P* < 0.001.

cells (Figure 5B). Invasion by *TIMP*-2si-HCT116 cells, as measured by using a Transwell assay was lower compared with the NCsi-HCT116 cells (Figure 5D), whereas the proliferation of *TIMP*-2si-HCT116 cells by MTT was similar to that of NCsi-HCT116 cells (Figure 5C). These results demonstrated that the CD133-expressing HCT116 CRC cells had higher invasive activity, and invasion by *CD133*si-HCT116 cells was reduced after the down-regulation of *TIMP*-2.

The levels of other MMPs and TIMPs, including MMP1, MMP2, MMP7, MMP9, MMP13, MMP28, MT1-MMP, MT2-MMP, TIMP-1, TIMP-3, and TIMP-4, were also measured following the knockdown of *CD133* (Supplemental Figure S3). MMP3 and MMP7 were upregulated, and MMP9 and MMP28 were down-regulated, after *CD133* knockdown. These data suggest that CD133 could also regulate other members of the MMP and TIMP families and that the complete profile of MMPs and TIMPs regulated by CD133 may also play an important role in invasion by HCT116 cells.

The Role of CD133 in Invasion by HCT116 Cells Is Not Due to the Activation of Either MMP2 or ERK Signaling

Many studies have demonstrated that TIMP-2 is involved in the activation of MMP2²¹ or ERK signaling and that CD133 is involved in the activation of the MAPK/ERK pathway in U87MG human glioblastoma cells.²⁸ The role of MMP2 or ERK signaling activation in CD133-expressing CRC cells was also investigated in this study. *CD133* knockdown and *TIMP*-2 knockdown in HCT116 cells had no obvious effect on the expression of MMP2 (pro-form and active

form) (Figure 6A), MEK1/2, pMEK1/2, ERK1/2, and p-ERK1/2 (Figure 6B), and no significant difference was detected in the proteolytic activity of MMP2 (Figure 6A). These data indicate that *CD133* knockdown and *TIMP*-2 knockdown did not affect MMP2 activity or ERK signaling.

Discussion

Expression of CD133 in CRC Cell Lines

The five-transmembrane glycoprotein CD133 has been used as a marker for CRC stem cells.^{12–14} Recent investigations have demonstrated that CD133 is expressed by the full spectrum of undifferentiated and differentiated colonic epithelial cells.¹³ Thus, the role of CD133 as a CSC marker has been challenged. The expression of CD133 has been tested in CRC tissues^{16,19,29} and various CRC cell lines,^{29–33} and the CD133/1 (AC133) antibody against CD133 epitopes has been widely used.^{29–31,33,34} In this study, we measured the percentage of CD133⁺ cells in 6 human CRC cell lines by flow cytometry using an antibody to CD133/2 (293C3, which recognizes epitope 2 and differs from clone AC133, which recognizes epitope 1). Among these cell lines, the percentage of CD133⁺ cells ranged from 1.5% to 98%. This is consistent with our previous results obtained with the antibody to CD133/1 (AC133).²⁷ The same antibody was used by Chen et al,³² although our data showed a different level of CD133 expression in the HCT116 cell line. The reason for this difference is unknown. The passage number, culture medium, and technique differences may provide some explanation. The *in vitro* serum deprivation is commonly used to mimic

Figure 5 *TIMP-2* knockdown by siRNA reduces invasion by HCT116 cells *in vitro* without affecting CD133 expression. HCT116 cells were transfected with NCsi and *TIMP-2*si. **A:** Real-time PCR (left panel) and Western blot analysis (right panel) showed the mRNA and protein expression, respectively, of NCsi-HCT116 cells and *CD133*si-HCT116 cells. **B:** The proliferation of the NCsi-HCT116 and *CD133*si-HCT116 cells were evaluated by MTT assay. **C:** The *in vitro* invasion ability of NCsi-HCT116 cells and *CD133*si-HCT116 cells were assayed using a Transwell assay. **D:** Representative photomicrographs of invading cells (upper panels), and the number of invading cells (lower panel). *** $P < 0.001$.

cellular stress, and this increases the surface expression of CD133³⁵; in our study, HCT116 cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 5% FBS, whereas the HCT116 cells in Chen et al's³² study were cultured in McCoy's 5A supplemented with 10% FBS.

A discrepancy between the results of the Western blot and flow cytometry has been shown in previous studies. Dittfeld et al³¹ reported a negative result for CD133 expression by Western blot analysis in DLD1 and HCC2998 cells, which had a larger CD133⁺ subpopulation, according to flow cytometry. In our study, the cell-surface expression pattern of

CD133 was confirmed by Western blot analysis. The protein levels of CD133 were in accordance with the percentages of CD133⁺ cells detected by flow cytometry. The use of different antibodies may account for the contradiction because in the study by Dittfeld et al,³¹ the anti-human CD133 antibody Ab19898 (polyclonal antibody) was obtained from Abcam, whereas ours was from Miltenyi. This suggests that antibody selection is pivotal for some studies.

As a canonical marker of CRC stem cells, it was suggested that CD133 was associated with the differentiation of CRC cells in our previous research²⁷. In this article, we tried

Figure 6 Expression and activation of the MMP2 and MEK/ERK signaling pathway in NCsi-HCT116, CD133si-HCT116 and TIMP-2 si-HCT116 cells. **A:** Western blot analysis and Zymography assay tested the protein expression (upper panel) and proteolytic activity (lower panel) of MMP2 in the three groups. **B:** Western blot analysis examined the expression of MEK1/2, p-MEK1/2, ERK1/2, and p-ERK1/2 in the three groups.

to focus on the role of CD133 in the CRC invasion process, which has not yet been completely elucidated. Recently, ALDH1 was found to be a new marker for CRC stem cells and to play an important role in the prognosis of CRC.³⁶ The roles of these stem cell markers are important area for investigation, and the comprehensive study of these CSC markers will improve our understanding of the mechanism of metastasis.

The Role of CD133 in the Invasion and Proliferation of HCT116 CRC Cells

Because of the high and wide-range expression of CD133 in CRC, its role needs to be studied further. Clinically, CD133

showed strong prognostic significance for CRT^{14,37} and was correlated with lymphovascular invasion¹⁷ and a lower 5-year survival rate.¹⁵ For CRC, recurrence and metastasis is one of the most common causes of cancer-related death, although the role of CD133 in CRC remains unclear. Previous studies demonstrated that the metastatic subpopulation was highly enriched in CD133⁺CD44⁺ HCT116 cells,³² and the *in vitro* invasion and *in vivo* tumor growth of CD133⁺ HCT116 cells were enhanced in the presence of CD10⁺ fibroblasts.³³ These results suggest that CD133 may be involved in CRC invasion.

Because of their high level of CD133 expression, HCT116 cells were used for the remainder of this study. We separated CD133^{high} and CD133^{low} populations of HCT116 cells by fluorescence-activated cell sorting, and the biological behavior of these two sorted cell populations was examined using MTT and Transwell assays. CD133^{high} HCT116 cells showed higher levels of *in vitro* proliferation and higher invasive ability compared with the CD133^{low} HCT116 cells. CD133si-HCT116 and NCsi-HCT116 cell lines were established after transfection with CD133 and control siRNAs. As with the CD133^{low} HCT116 cells, CD133si-HCT116 cells showed a lower invasive ability than NCsi-HCT116 cells. CD133^{high} HCT116 cells displayed a higher level of *in vitro* proliferation compared with of the CD133^{low} HCT116 cells. The proliferation of the CD133si-HCT116 cells, however, was similar to that of the NCsi-HCT116 cells, which was consistent with a previous study.²⁷ The reason for this discrepancy is still unclear, but the same discrepancy existed in another study of HCC cells.²⁶ This phenomenon suggests that CD133 may not functionally participate in the proliferation of CRC cells.

The Invasion Mechanism of CD133-Expressing HCT116 CRC Cells

Various pathways may be involved in the *in vitro* invasion. We examined key factors in several pathways, including the hypoxia (Hif-1 α , Hif-1 β), cell motility (cdc42, RhoA), transforming growth factor β receptor signaling (Smad7), and tissue inhibitor of metalloproteinase (TIMP-1, TIMP-2) pathways. No changes were observed except in the TIMP-2 pathway. Activation of the Akt and MAPK pathways enhances the tumorigenicity of CD133⁺ primary colon cancer cells,³⁸ and a functional relationship between CD133, END-1, and NR4A2 expression existed in Caco2 CRC cells.²⁹ No evidence, however, supported the involvement of any of these pathways or factors in CD133-related CRC invasion. Previous studies demonstrated that MMP2 was involved in HCC invasion,²⁶ and TIMP-2 is a potent inhibitor of MMP2. Several studies have established that MMP2, TIMP-2, and the MMP2/TIMP-2 complex play a role in the growth and progression of CRC.^{22–24} Hence, the expression of TIMP-2 was further investigated.

CD133 knockdown by siRNA transfection reduced TIMP-2 expression, suggesting that CD133 could regulate

the expression of TIMP-2. The expression of TIMP-2 in CD133^{high} HCT116 cells, however, was similar to that in CD133^{low} HCT116 cells. Gaiser et al³⁹ showed that there are many genes differentially expressed in these two subpopulations. These differentially expressed genes may also affect the expression of TIMP-2 and obscure the relationship between CD133 and TIMP-2. These results suggest that the function of the CD133 molecule itself (functional role) is different from its role as a CSC marker (marker role). Next, a TIMP-2si-HCT116 cell line was established by transfection with TIMP-2 siRNA. We found that even though the mRNA and protein expression of CD133 were not changed after the knockdown of *TIMP-2*, the invasive ability of the TIMP-2si-HCT116 cells sharply decreased. These data suggest that *in vitro* invasion by CD133si-HCT116 cells decreased through the down-regulation of TIMP-2 and that the relationship between CD133 and TIMP-2 was involved in tumor invasion.

The MMPs and TIMPs play significant roles in tumor invasion and metastasis.^{22,40–42} Murray et al⁴³ demonstrated that MMP1 is associated with poor prognosis in CRC. Curran et al⁴⁴ and Lyall et al⁴⁵ demonstrated that the complete marker profile of MMPs and TIMPs was the most significant independent prognostic factor for CRC. Therefore, in addition to TIMP-2 and MMP2, we examined other MMPs and TIMPs. MMP3 and MMP7 were upregulated, and MMP9 and MMP28 were down-regulated after the knockdown of *CD133*. These data suggested that *CD133* knockdown triggered changes in several members of the MMP and TIMP families. The decreased invasion could be the result of all these changes combined. The role of single genes needs further elucidation.

On the mechanism of metastatic evolution, as Coghlin and Murray⁴⁶ reviewed, there are two distinct models early model and late model. The early model emphasizes the heterogeneity of the primary tumor and therefore predicts a much earlier metastasis than that of the late model. In this study, among the CRC cell lines we tested, the expression of CD133 showed high heterogeneity. We also demonstrated that highly expressed CD133 affects the invasive ability of the cells via important invasion-related genes. We argue, therefore, that the function of CD133 could be consistent with the early model theory of metastasis.

MMP2 and MEK/ERK Signaling Are Not Involved in the Effects of *CD133* Knockdown in HCT116 Cells

To further elucidate the mechanism by which CD133 regulates invasion, additional molecules were evaluated. TIMP-2 has dual effects on MMP2, such as the activation of pro-MMP2⁴⁷ and the inhibition of active MMP2.⁴⁸ Our data showed that both the protein expression and proteolytic activity of MMP2 remained unchanged after the knockdown of *CD133* or *TIMP-2*. Controlling its expression or proteolytic activity with a pharmaceutical agent or MMP2 inhibitor, therefore, may not be an effective therapeutic

strategy for inhibiting the invasion of CD133^{high} HCT116 cells.

In brain tumors, activation of the pro-migratory ERK signaling cascade through TIMP-2 was associated with poor prognosis.²⁵ We found no significant difference in the activation of the MEK/ERK signaling pathway in CRC. Tissue specificity may partially account for this difference. CD133 overexpression significantly activated Erk in U87MG human glioblastoma cells,²⁸ and transforming growth factor- β -induced apoptosis in CD133⁺ cells was blocked by the activation of MEK/ERK signaling in tumorigenic liver stem cells from *Mat1a*^{-/-} mice.⁴⁹ No direct relationship between CD133 and MEK/ERK signaling was observed in CRC.

In conclusion, we demonstrated that CD133^{high} HCT116 cells possessed a greater invasive ability than CD133^{low} cells and that the knockdown of *CD133* in this cell line decreased invasion. For the first time, we observed that *CD133* knockdown significantly decreased TIMP-2 expression, and *TIMP-2* knockdown decreased invasion, which suggests that CD133 affects invasion by regulating TIMP-2 expression. Elucidation of the function and mechanism of CD133 may be critical for the development of new therapeutic strategies for treating CRC.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2012.10.015>.

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