Saijo H, Hirohashi Y, Torigoe T, Kochin V, Takahashi H, Sato N.	Cytotoxic T lymphocytes: the future of cancer stem cell eradication?	Immunotherapy.	5(6)	549-51	2013
Morita R, Hirohashi Y, Nakatsugawa M, Kanaseki T, Torigoe T, Sato N.	Production of Multiple CTL Epitopes from Multiple Tumor-Associated Antigens.	Methods Mol Biol.	1139	345-355	2014
Tanaka T, Torigoe T, Hirohashi Y, Sato E, Honma I, Kitamura H, Masumori N, Tsukamoto T, Sato N.	Hypoxia-inducible factor (HIF)-independent expression mechanism and novel function of HIF prolyl hydroxylase-3 in renal cell carcinoma.	J Cancer Res Clin Oncol.	140	503-513	2014
Morita R, Nishizawa S, Torigoe T, Takahashi A, Tamura Y, Tsukahara T, Kanaseki T, Sokolovskaya A, Kochin V, Kondo T, Hashino S, Asaka M, Hara I, Hirohashi Y, Sato N.	Heat shock protein DNAJB8 is a novel target for immunotherapy of colon cancer-initiating cells.	Cancer Sci.	105 (4)	389-395	2014
廣橋良彦、鳥越俊彦、佐藤昇志	免疫療法の標的としてのがん細胞亜集団	実験医学	31	1904-1907	2013

雑誌（平田　公一）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Minatoya M, Kutomi G, Asakura S, Otokozawa S, Sugiyama Y, Nagata Y, Mori M, Hirata K.	Equol, Aiponectin, Insulin Levels and Risk of Breast Cancer.	Asian Pacific J Cancer Prev	14	2191-2199	2013
Kutomi G, Tamura Y, Tanaka T, Kajiwara T, Kukita K, Ohmura T, Shima H, Takamaru T, Satomi F, Suzuki Y, Torigoe T, Sato N, Hirata K.	Human Endoplasmic Reticulum Oxidoreductin 1-α (hERO1-α) is a Novel Predictor for Poor Prognosis of Breast Cancer.	Cancer Sci.	Aug; 104 (8)	1091-6	2013

Kyuno D, Kojima T, Yamaguchi H, Ito T, Kimura Y, Imamura M, Takasawa A, Murata M, Tanaka S, Hirata K, Sawada N.	Protein kinase Cα inhibitor protects against downregulation of claudin-1 during epithelial-mesenchymal transition of pancreatic cancer	Carcinogenesis.	Jun; 34(6)	1232-43	2013
Kiriyama K, Hirohashi Y, Torigoe T, Kubo T, Tamura Y, Kanaseki T, Takahashi A, Nakazawa E, Saka E, Ragnarsson C, Nakatsugawa M, Inoda S, Asanuma H, Takasu H, Hasegawa T, Yasoshima T, Hirata K, Sato N.	Expression and function of FERMT genes in colon carcinoma cells.	Anticancer Res.	Jan; 33(1)	167-73	2013

雑誌（釣田　義一郎）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamaguchi K, Yamaguchi R, Takahashi N, Ikenoue T, Fujii T, Shinozaki M, Tsurita G, Hata K, Niida A, Imoto S, Miyano S, Nakamura Y, Furukawa Y	Overexpression of cohesion establishment factor DSCC1 through E2F in colorectal cancer.	***PLoS One.***	9(1)	e85750	2014

雑誌（安井　寛）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shigematsu A, Kobayashi N, Yasui H, Shindo M, Kakinoki Y, Koda K, Iyama S, Kuroda H, Tsutsumi Y, Imamura M, Teshima T	High level of serum soluble interleukin-2 receptor at transplantation predicts poor outcome of allogeneic stem cell transplantation for adult T-cell leukemia	***Biol Blood Marrow Transplant***	-	in press	2014

Takeuchi M, Sato Y, Yasui H, Ozawa H, Ohno K, Takata K, Iwaki N, Orita Y, Asano N, Nakamura S, Swerdlow SH, Yoshino T.	Epstein-Barr virus-infected cells in IgG4-related lymphadenopathy with comparison to extranodal IgG4-related disease	***Am J Surg Pathol.***	-	in press	2014
Kunimoto Y, Yasui H, Touda N, Okazaki M, Nakata H, Noda N, Ikeda H, Hayashi T, Takahashi S, Shinomura Y, Ishida T, Miyamoto A.	Coadministration of tenofovir decreased atazanavir plasma concentration after unilateral nephrectomy	***J Infect Chemother.***	19(4)	750-3	2013

雑誌（瀬谷　司）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Okamoto M, Oshiumi H, Azuma M, Kato N, Matsumoto M, Seya T.	IPS-1 Is Essential for Type III IFN Production by Hepatocytes and Dendritic Cells in Response to Hepatitis C Virus Infection.	J Immunol.	in press		2014
Tatematsu M, Seya T, Matsumoto M.	Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses.	Biochem J.	458(2)	195-201	2014
Takaki H, Honda K, Atarashi K, Kobayashi F, Ebihara T, Oshiumi H, Matsumoto M, Shingai M, Seya T.	MAVS-dependent IRF3/7 bypass of interferon β-induction restricts the response to measles infection in CD150Tg mouse bone marrow-derived dendritic cells.	Mol Immunol.	57(2)	100-110	2014
Enokizono Y, Kumeta H, Funami K, Horiuchi M, Sarmiento J, Yamashita K, Standley DM, Matsumoto M, Seya T, Inagaki F.	Structures and interface mapping of the TIR domain-containing adaptor molecules involved in interferon signaling.	Proc Natl Acad Sci U S A.	110(49)	19908-19913	2013
Takaki H, Takeda M, Tahara M, Shingai M, Oshiumi H, Matsumoto M, Seya T.	The MyD88 pathway in plasmacytoid and CD4+ dendritic cells primarily triggers type I IFN production against measles virus in a mouse infection model.	J Immunol.	191(9)	4740-4747	2013

Oshiumi H, Miyashita M, Matsumoto M, Seya T.	A distinct role of Riplet-mediated K63-Linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses.	PLoS Pathog.	9(8)	e1003533	2013
Tanaka Y, Suenaga T, Matsumoto M, Seya T, Arase H.	Herpesvirus 6 glycoproteins B (gB), gH, gL, and gQ are necessary and sufficient for cell-to-cell fusion.	J Virol.	87(19)	10900-10903	2013
Nishitsuji H, Funami K, Shimizu Y, Ujino S, Sugiyama K, Seya T, Takaku H, Shimotohno K.	Hepatitis C virus infection induces inflammatory cytokines and chemokines mediated by the cross talk between hepatocytes and stellate cells.	J Virol.	87(14)	8169-8178	2013
Tatematsu M, Nishikawa F, Seya T, Matsumoto M.	Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA.	Nat Commun.	4	1833	2013
Seya T, Azuma M, Matsumoto M.	Targeting TLR3 with no RIG-I/MDA5 activation is effective in immunotherapy for cancer.	Expert Opin Ther Targets.	17(5)	533-544	2013
Oshiumi H, Funami K, Aly HH, Matsumoto M, Seya T.	Multi-step regulation of interferon induction by hepatitis C virus.	Arch Immunol Ther Exp (Warsz)	61(2)	127-138	2013
Toscano F, Estornes Y, Virard F, Garcia-Cattaneo A, Pierrot A, Vanbervliet B, Bonnin M, Ciancanelli MJ, Zhang SY, Funami K, Seya T, Matsumoto M, Pin JJ, Casanova JL, Renno T, Lebecque S.	Cleaved/associated TLR3 represents the primary form of the signaling receptor.	J Immunol.	190(2)	764-773	2013

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DNA methyltransferase 1 is essential for initiation of the colon cancers

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ABSTRACT

DNA methyltransferase 1 (Dnmt1) is essential for the maintenance of hematopoietic and somatic stem cells in mice; however, its roles in human cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are still elusive. In the present study, we investigated DNMT1 functions in the maintenance of human colon CSCs/CICs using the human colon cancer cell line HCT116 (HCT116 w/t) and its *DNMT1* knockout cell line (*DNMT1*$^{-/-}$). The rates of CSCs/CICs were evaluated by side population (SP) analysis, ALDEFLUOR assay and expression of CD44 and CD24. SP, ALDEFLUOR-positive (ALDEFLUOR^{+}) and CD44-positive and CD24-positive (CD44^{+}CD24^{+}) cell rates were lower in *DNMT1*$^{-/-}$ cells than in HCT116 w/t cells. Since CSCs/CICs have higher tumor-initiating ability than that of non-CSCs/CICs, the tumor-initiating abilities were addressed by injecting immune deficient (NOD/SCID) mice. *DNMT1*$^{-/-}$ cells showed less tumor-initiating ability than did HCT116 w/t cells, whereas the growing rate of *DNMT1*$^{-/-}$ cells showed no significant difference from that of HCT116 cells both *in vitro* and *in vivo*. Similar results were obtained for cells in which *DNMT1* had been transiently knocked-down using gene-specific siRNAs. Taken together, these results indicate that DNMT1 is essential for maintenance of colon CSCs/CICs and that short-term suppression of *DNMT1* might be sufficient to disrupt CSCs/CICs.

Introduction

Cancer tissues are composed from several heterogeneous cancer cells and small population of cancer cells are supposed to have higher tumor-initiating ability. These higher tumorigenic populations are named "cancer stem-like cells (CSCs)" or "cancer initiating cells (CICs)". CSCs/CICs are defined as small population of cancer cells which has (1) higher tumor-initiating ability, (2) self-renewal, (3) differentiation. (Clarke et al., 2006; Dalerba et al., 2007; Huang et al., 2009; O'Brien et, al., 2007; Ricci-Vitiani et al., 2007) CSCs/CICs have been reported to be resistant to chemotherapy, radiotherapy and certain molecular targeting therapies (Dean et al., 2005); thus, elucidation of the molecular mechanisms of the maintenance of CSCs/CICs should be useful for establishing efficient CSC/CIC targeting treatment.

Trowbridge et al. reported that hematopoietic stem cell self-renewal was abrogated by conditional gene knockout of *Dnmt1*, while the mature differentiated hematopoietic lineage was not affected. (Trowbridge et al., 2009) *Dnmt1* is essential for maintenance of the leukemia stem cells of bilinear myeloid-B lymphoid leukemia induced by transduction of *c-Myc* and *Bcl-2*. (Broske et al., 2009) *Dnmt1* was also shown to be essential for the self-renewal of skin progenitor cells. (Sen et al., 2010) Results of these studies indicate that DNMT1 is essential for self-renewal of progenitor or stem cells. However, no study has been mentioned the relation between *DNMT1* and CSCs/CICs in solid tumors including colon cancer.

In the present study, we investigated the functions of *DNMT1* in maintenance of colon CSCs/CICs. We investigated DNMT1 functions by using a *DNMT1*$^{-/-}$ cell line and also cells in which *DNMT1* was transiently knocked-down by siRNAs. Permanent DNMT1 gene knockout and transient *DNMT1* gene knock down by specific siRNAs reduced the population of CSCs/CICs. These results of this study suggest that *DNMT1* has an essential role in maintenance of CSCs/CICs, and transient inhibition of DNMT1 might be sufficient to eradicate CSCs/CICs.

Abbreviations: CSC, cancer stem-like cell; CIC, cancer-initiating cell; DNMT1, DNA methyltransferase 1; SP, side population; MP, main population.

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Materials and methods

Cell lines

The colon adenocarcinoma cell lines wild-type HCT116 and SW480 were kind gifts from Dr. K. Imai (Sapporo, Japan). A *DNMT1* knockout HCT116 cell line (*DNMT1*$^{-/-}$) that lacks exons 2–5 of *DNMT1* (Rhee et al., 2000) was a kind gift from Dr. B. Vogelstein (Baltimore, MD). HCT116 and *DNMT1*$^{-/-}$ cells were cultured in McCoy's 5A modified medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY). SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (SIGMA) supplemented with 10% FBS.

Xenograft transplantation

All mouse procedures were performed in accordance with institutional protocol guidelines at Sapporo Medical University School of Medicine. Serially diluted HCT116 w/t and *DNMT1*$^{-/-}$ cells were mixed with Matrigel (BD, Franklin Lakes, NJ) at a 1:1 volume and injected subcutaneously into the backs of 4–8-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size was assessed weekly.

RT-PCR analysis and quantitative real-time PCR analysis

RT-PCR analysis was performed as described previously. (Inoda et al., 2009) Primer pairs used for RT-PCR analysis were 5′-CATGATG GAGACGGAGCTGA-3′ and 5′-ACCCCGCTCGCCATGCTATT-3′ for *SOX2* with an expected PCR product size of 410 base pairs (bps), 5′-CC TGGGGCTGCTGCTGTTTATTA-3′ and 5′-TACCTGGTGATTTGCCACAA-3′ for *PROM1*(*CD133*) with an expected PCR product size of 208 bps, 5′-ATCGCCTCTCTCCGTTTGGTA-3′ and 5′-TGGACTCATCCGATTTGGCT-3′ for *DNMT-1* with an expected PCR product size of 757 bps, 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′ and 5′-GGCAGATGGTCGTTTG GCTGAATA-3′ for *POU5F1* with an expected PCR product size of 163 bps, 5′-CTCTTCCTCAAACCGTCTGC-3′ and 5′-GATCGGAGGCTAAG CAACTG-3′ for *LGR5* with an expected PCR product size of 181 bps and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCT GTA-3′ for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) with an expected product size of 452 bps. *GAPDH* was used as an internal control. PCR amplification was performed in 20 μl of PCR mixture containing 1 μl of cDNA mixture, 0.5 μl of Taq DNA polymerase (QIAGEN) and 4 pmol of primers. The PCR mixture was initially incubated at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s.

Side population (SP) analysis, ALDEFLUOR assay and flow cytometry

Side population (SP) analysis was performed as described previously with some modifications. (Goodell et al., 1996; Inoda et al., 2011) Hoechst 33342 (Lonza, Walkersville, MD) dye was used at the concentration of 1.25 μg/ml for HCT116 and *DNMT1*$^{-/-}$. Stained cells were analyzed by a BD FACS Aria II Cell-Sorting System (BD).

Aldehyde dehydrogenase (ALDH) activity was detected using an ALDEFLUOR assay kit (StemCell Technologies) according to the manufacturer's protocol. (Ginestier et al., 2007) Cells stained by bodipy-aminoacetaldehyde (BAAA) at 1.5 mM and incubated for 30 min at 37 °C were analyzed by a BD FACS Aria II and BD FACSDiva software ver. 6.1 (BD).

CD44 and CD24 were detected using anti-CD44-APC antibody and anti-CD24-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with these antibodies and analyzed using a FACS Aria II.

DNMT1 mRNA knockdown

A *DNMT1* gene knockdown experiment was performed using small interfering RNA (siRNA). DNMT1 siRNA duplexes were designed and synthesized using the BLOCK-it RNAi designer system (Life technologies). The oligonucleotide encoding DNMT1 siRNA i was 5′-AA AGATGGACAGCTTCTCATTTGTC-3′ and that encoding siRNA ii was 5′-AAATATGGGCGCATACTCGGGACTG-3′. Negative control siRNA was obtained from Invitrogen. Cells were seeded at 50% confluence, and transfections were carried out using Lipofectamine RNAi max (Life technologies) in Opti-MEM according to the manufacturer's instructions.

Bisulfite pyrosequencing

Genomic DNAs of SP and MP cells were modified with sodium bisulfite using an EpiTect Bisulfite kit (Qiagen), and bisulfite pyrosequencing analysis was done as described previously (Yamamoto et al., 2008). Primer sequences for LINE-1 methylation were the same as those described previously. (Yamamoto et al., 2008) Primer sequences for Alu Yb8 and centromeric satellite-α of chromosome 1 (Sat-α) were the same as those described previously (Igarashi et al., 2010).

Statistical analysis

In the xenograft model, cell growth *in vitro* and MIB-1 labeling index, samples were analyzed using Student's *t*-test, with $P<0.05$ conferring statistical significance.

Results

Lower rates of CSCs/CICs in DNMT1$^{-/-}$ cells

DNMT1 has been described to be essential for self-renewal of progenitor or stem cells. (Trowbridge et al., 2009; Broske et al., 2009; Sen et al., 2010). We therefore hypothesized that DNMT1 has also role in maintenance of human colon CSCs/CICs. CSC/CIC population in HCT116 *DNMT1*$^{-/-}$ cells and HCT116 w/t cells were analyzed by SP analysis, ALDEFLUOR assays and expression of cell surface CSC/CIC makers. Putative CSC/CIC including *SOX2*, *POU5F1*, *LGR5* and *PROM1* were analyzed by RT-PCR. (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Ben-Porath et al., 2008; Vermeulen et al., 2008) HCT116 w/t cells expressed *SOX2* and *PROM1* (*CD133*), representative markers for colon CSCs/CICs; however, SOX2 and PROM1 were undetectable in *DNMT1*$^{-/-}$ cells (Fig. 1A). The other CSCs/CICs markers including *POU5F1* and *LGR5* were not detectable in both HCT116 w/t and *DNMT1*$^{-/-}$ cells by RT-PCR (data not shown).

Since CSCs/CICs were enriched in SP cells in several malignancies (Inoda et al., 2011; Kondo et al., 2004), we evaluated HCT116 w/t cells and *DNMT1*$^{-/-}$ cells by SP analysis. The SP cell rates in HCT116 w/t cells was 1.2%, whereas the SP cell rates in *DNMT1*$^{-/-}$ cells were 0.1% (Fig. 1B). The SP population was completely inhibited by Verapamil an ABCG2 transporter inhibitor, indicating that the SP population was specific for ABCG2 transporter (Fig. 1B). Since HCT116 SP cells have not been described to be enriched with CSCs/CICs, we investigated the presence of CSCs/CICs in HCT116 SP cells by RT-PCR and tumorigenicity in NOD/SCID mice (Figs. 1C, D). HCT116 SP cells expressed higher levels of *SOX2* and *PROM1* than those in HCT116 MP cells (Fig. 1C) and HCT116 SP cells showed higher tumor-initiating ability than that of HCT116 MP cells (Fig. 1D).

We also performed other methods of isolating CSCs/CICs. It has been reported that colon CSCs/CICs could be detected using ALDEFLUOR assay and surface marker analysis such as CD24 and CD44. (Huang et al., 2009; Kemper et al., 2010; Vermeulen et al., 2008; Yeung et al., 2010) ALDEFLUOR-positive (ALDEFLUOR^{+}) cell rate was 2.6% in HCT116 w/t cells, and that population was inhibited by an ALDH inhibitor (Fig. 1E). On the other hand, ALDEFLUOR^{+} cell rate in *DNMT1*$^{-/-}$

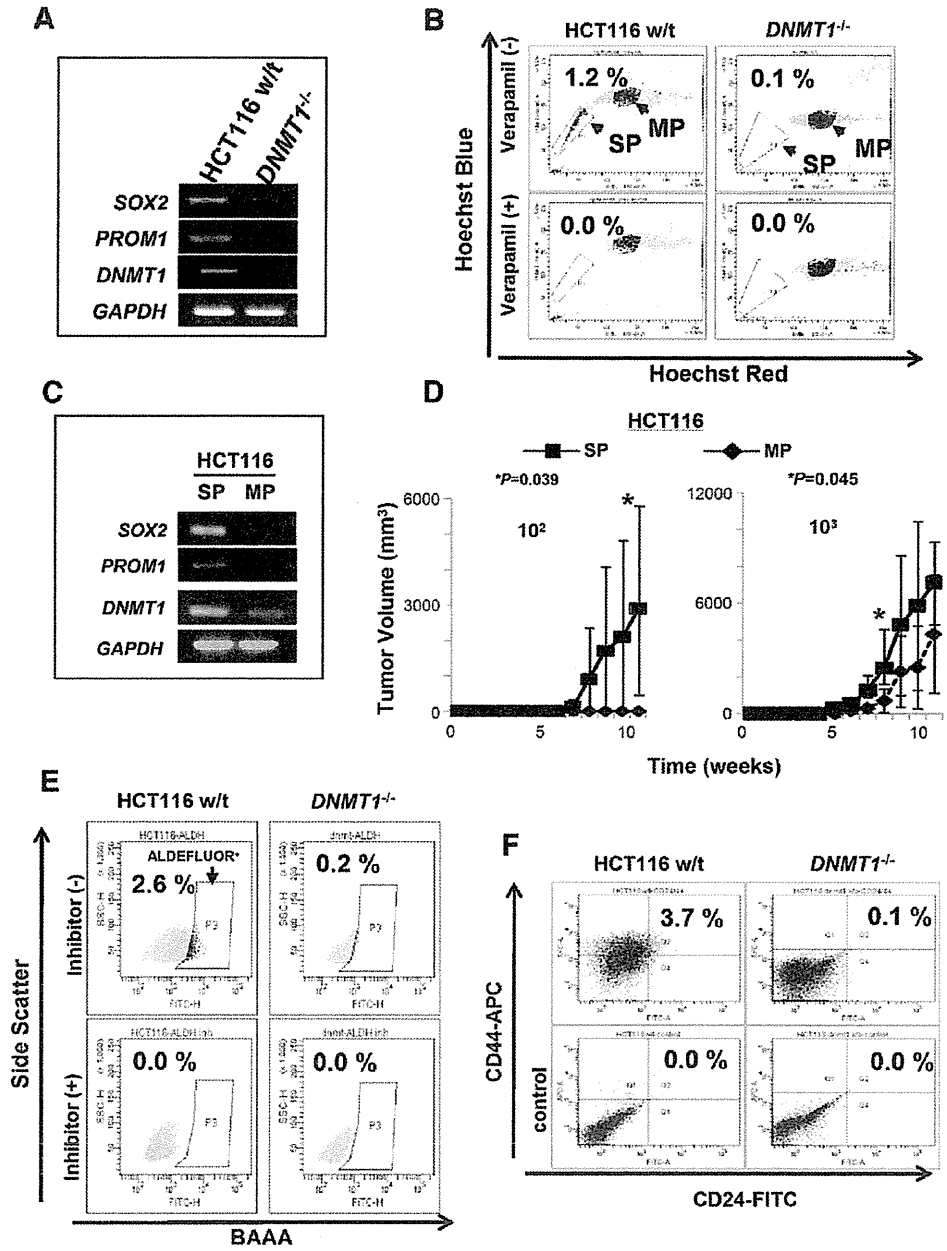

Fig. 1. *DNMT1*$^{-/-}$ cells contain lower CSCs/CICs than HCT116 w/t cells. A. RT-PCR of CSCs/CICs markers in HCT116 w/t and *DNMT1*$^{-/-}$ cells. B. Isolation of SP cells from colon cancer cell lines. HCT116 w/t and *DNMT1*$^{-/-}$ cells were stained with Hoechst 33342 dye and analyzed using a FACS Aria II cell sorter. SP: side population, MP: main population. C. RT-PCR of CSC/CIC markers in HCT116 SP and MP cells. D. Tumor growth of HCT116 SP and MP cells. 10^2 and 10^3 HCT116 SP and MP cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between HCT116 SP and MP cells were examined for statistical significance using Student's *t*-test. *P values. E. ALDEFLUOR Assay. ALDH activity was detected using the ALDEFLUOR assay kit as described in Materials and methods. A specific inhibitor of ALDH is diethylaminobenzaldehyde (DEAB). Stained cells were analyzed using a FACS Aria II cell sorter. F. Expression of CD44 and CD24. The numerical value in the dots plot graph is $CD24^+44^+$ cells rate. Stained cells were analyzed using a FACS Aria II cell sorter.

cells was 0.2%. Cell surface CD24 expression was decreased and CD44 expression greatly decreased in *DNMT1*$^{-/-}$ cells compared with that in HCT116 w/t cells (Fig. 1F). CD24/44-double positive population was 3.7% in HCT116 w/t cells and 0.1% in *DNMT1*$^{-/-}$ cells, respectively.

Lower tumor-initiating ability of DNMT1$^{-/-}$ *cells*

CSCs/CICs are known to have greater tumor-initiating ability than that of non-CSCs/CICs (Al-Hajj et al., 2003), we therefore evaluated the tumorigenicities of HCT116 w/t and *DNMT1*$^{-/-}$ cells. HCT116 w/t cells could initiate tumors with a minimum of 10^2 cells; however, 10^5 cells were needed to initiate tumors with *DNMT1*$^{-/-}$ cells (Table 1). Volumes of tumors derived from HCT116 w/t cells were also significantly higher than those of tumors derived from *DNMT1*$^{-/-}$ cells (Fig. 2A). The histology of tumors derived from each HCT116 w/t and *DNMT1*$^{-/-}$ cells showed high-grade poorly differentiated adenocarcinomas, and no significant histological difference was observed (Fig. 2B).

We investigated the growth rates of HCT116 w/t cells and *DNMT1*$^{-/-}$ cells. There was no significant difference between growth rates *in vitro* of HCT116 w/t cells and *DNMT1*$^{-/-}$ cells (Fig. 2C). No significant difference in MIB-1 labeling index, which represents cells undergoing the cell cycle, was observed in tumors derived from

Table 1
Tumor initiating ability in HCT116 and DNMT1 knockdown cells.

Cell lines	Percentage of SP cells	10^5	10^4	10^3	10^2	10^1
HCT116 w/t	1.2%	5/5	5/5*	3/5	2/5	n.d.
HCT116 *DNMT1*$^{-/-}$	0.1%	5/5	0/5	0/5	0/5	n.d.
HCT116 control siRNA	0.8%	n.d.	5/5*	5/5*	5/5*	0/5
DNMT1 siRNA	0.0%	n.d.	1/5	0/5	0/5	0/5
SW480 control siRNA	2.2%	n.d.	5/5	5/5*	1/5	0/5
DNMT1 siRNA	0.2%	n.d.	5/5	1/5	0/5	0/5

The analysis was completed 10 weeks following injection. Data are expressed as number of tumors formed/number of injections. Differences between HCT116 w/t cells and DNMT1$^{-/-}$ cells were examined for statistical significance using Student's *t*-test. *P<0.05, w/t: wild type, *DNMT1*$^{-/-}$: *DNMT1* knock out cell, n.d.: not done.

HCT116 w/t cells and *DNMT1*$^{-/-}$ cells (Fig. 2D). These observations indicate that *DNMT1*$^{-/-}$ cells sustain growth ability both *in vitro* and *in vivo*; however, they have very little tumor-initiating ability compared with that of HCT116 w/t cells.

DNMT1 gene knockdown experiments

DNMT1$^{-/-}$ cell phenotypes were confirmed by transient gene knockdown using gene-specific siRNAs. We designed two different siRNAs and confirmed suppression of *DNMT1* mRNA by RT-PCR (Fig. 3A). Transfection of siRNAs decreased the expression levels of *SOX2* and *PROM1* (Fig. 3A). Transfection of siRNAs drastically decreased SP cell ratios of HCT116 w/t cells (Fig. 3B). Furthermore, siRNA transfection decreased the ratio of ALDEFLUOR$^+$ cells in HCT116 w/t cells (Fig. 3C). CD24/44-double positive population was also decreased in HCT116 *DNMT1*-knockdown cells (Fig. 3D). We

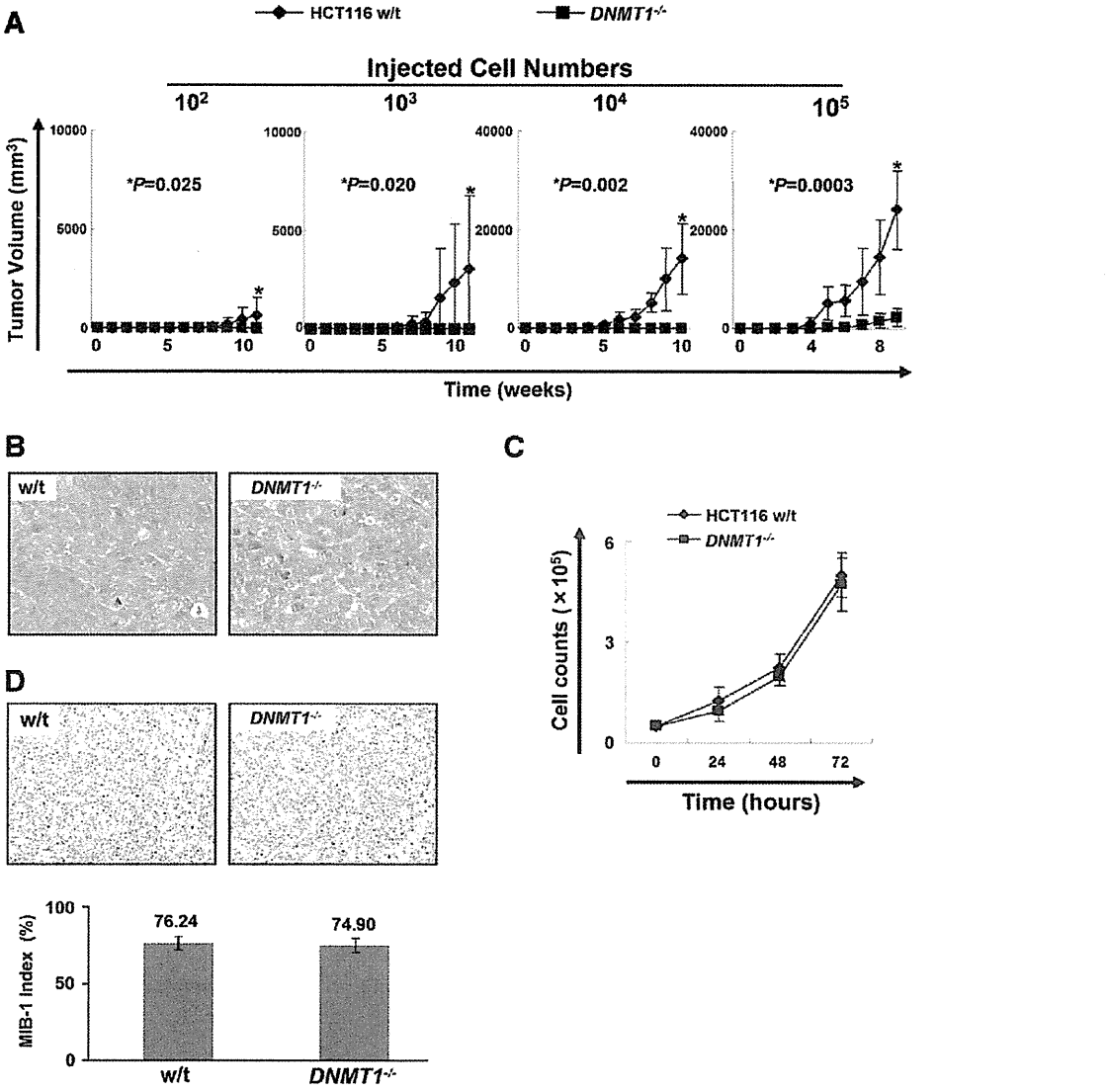

Fig. 2. Lower tumor-initiating ability of *DNMT1*$^{-/-}$ cells. A. Tumor growth of HCT116 w/t and *DNMT1*$^{-/-}$ cells. 10^2, 10^3, 10^4 or 10^5 HCT116 w/t cells and *DNMT1*$^{-/-}$ cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between HCT116 w/t and *DNMT1*$^{-/-}$ cells were examined for statistical significance using Student's *t*-test. *P values. B. Representative histology of tumors derived from HCT116 w/t and *DNMT1*$^{-/-}$ cells. Tumors derived from HCT116 w/t and DNMT1 −/− cells were stained with hematoxylin and eosin. Magnification, ×400. C. *In vitro* cell growth of HCT116 w/t and *DNMT1*$^{-/-}$ cells. 10^5 of HCT116 w/t and DNMT1 −/− cells were seeded into 6-well plates, and the cells were counted every 24 h. Data represent means ± SD. D. MIB-1 labeling index of HCT116 w/t and *DNMT1*$^{-/-}$ cells. Tumors derived from HCT116 w/t and DNMT1 −/− cells were stained with MIB-1. Magnification, ×100. The MIB-1 labeling indexes were calculated by the averages of MIB-1 positivities of 10 high power fields (H.P.F.). Data represent means ± SD.

operated xenograft model experiments using HCT116 *DNMT1*-knockdown cells and control cells. The engraftment of tumor derived from *DNMT1*-knockdown cells needed 10^4 cells (Table 1) and tumor grew slowly (Fig. 3E).

We performed similar gene knockdown experiments using another human colon cancer cell line, SW480, to generalize the effects of gene knockdown of *DNMT1*. Expression levels of *SOX2* mRNA were reduced by DNMT1 mRNA knockdown in SW480 cells (Fig. 4A). The ratios of SP cells and ALDEFLUOR$^+$ cells and the CD24$^+$44$^+$ cells were reduced by *DNMT1* mRNA knockdown in SW480 cells (Figs. 4B, C and D). Both tumor-initiating ability and tumor-growing speed were similarly reduced (Fig. 4E).

Expression of DNMT3A and DNMT3B, and methylation status of LINE-1, Alu and Sat-alfa sequences in SP and MP cells

To address the genome methylation status are related to the maintenance of CSC/CIC, we investigated the expression of DNMT3A and DNMT3B, *de novo* DNA methyltransferases, and the methylation status of repetitive sequence of genome such as LINE-1, Alu and Sat-α genes as surrogate genome methylation markers by bisulfite pyrosequencing. The expression levels of *DNMT3A* and *DNMT3B* did not show any difference in SP and MP cells derived from HCT116 cells (Fig. 5A). The methylation status of LINE-1, Alu Sat-α did not show any significant difference in HCT116 SW480 SP cells and MP cells (Figs. 5B and C).

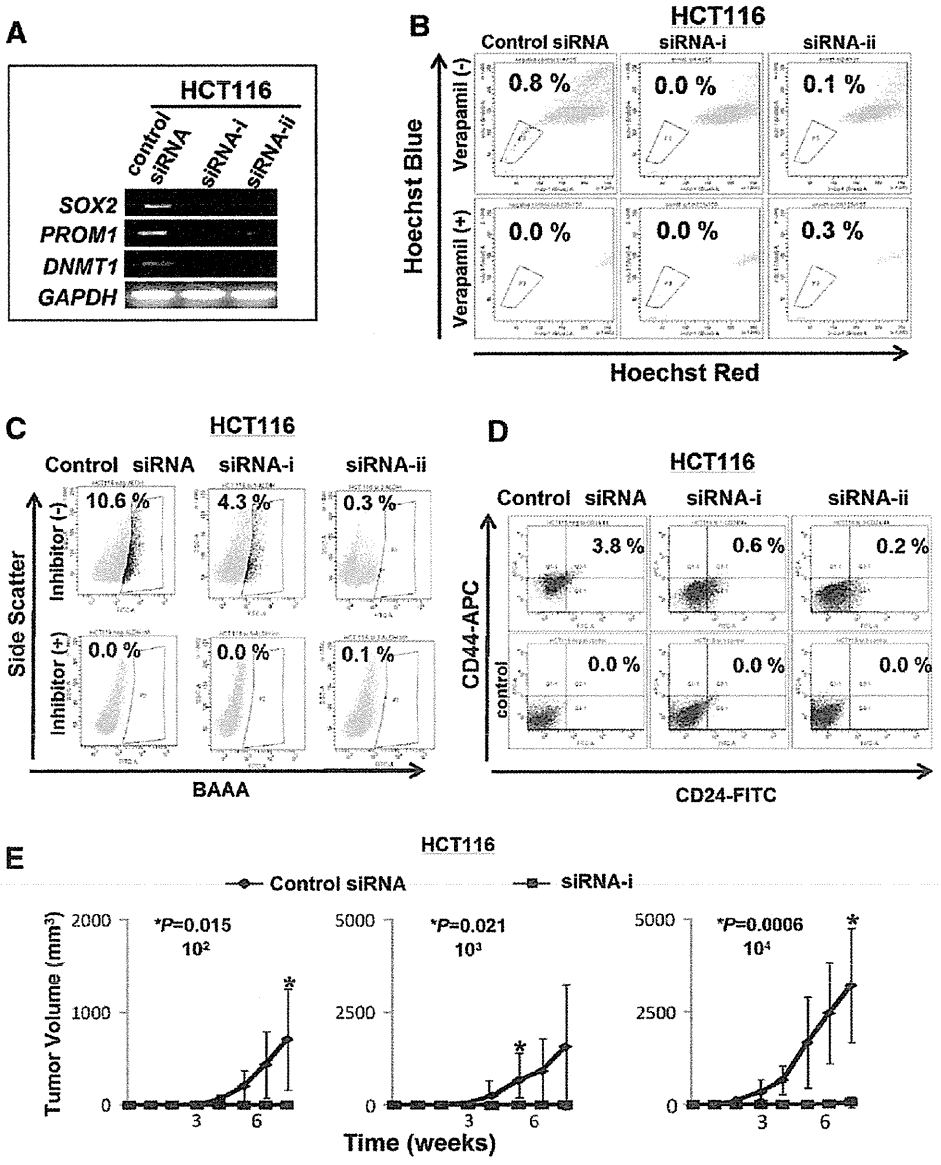

Fig. 3. *DNMT1* gene knockdown experiments in HCT116 cells. A. RT-PCR of *DNMT1* and CSCs/CICs markers. The mRNA expression levels of *DNMT1* and CSCs/CICs markers were evaluated by RT-PCR. Total RNAs were isolated 48 h after transfection of a negative control or *DNTM1*-specific siRNA i and siRNA ii. B. Isolation of SP cells from colon cancer cell lines. HCT116 *DNMT1*-knockdown cells and control cells were stained with Hoechst 33342 dye and analyzed. C. ALDEFLUOR Assay. ALDH activity was detected using the ALDEFLUOR assay kit 48 h after transfection of negative control or *DNMT1* specific siRNAs. D. Expression of CD44 and CD24. The numerical value in the dots plot graph is CD24$^+$44$^+$ cells rate. Stained cells were analyzed using a FACS Aria II cell sorter. E. Tumor growth of HCT116 *DNMT1*-knockdown cells and control cells. 10^2, 10^3 or 10^4 HCT116 *DNMT1*-knockdown cells and control cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between HCT116 *DNMT1*-knockdown cells and control cells were examined for statistical significance using Student's *t*-test. *P values.

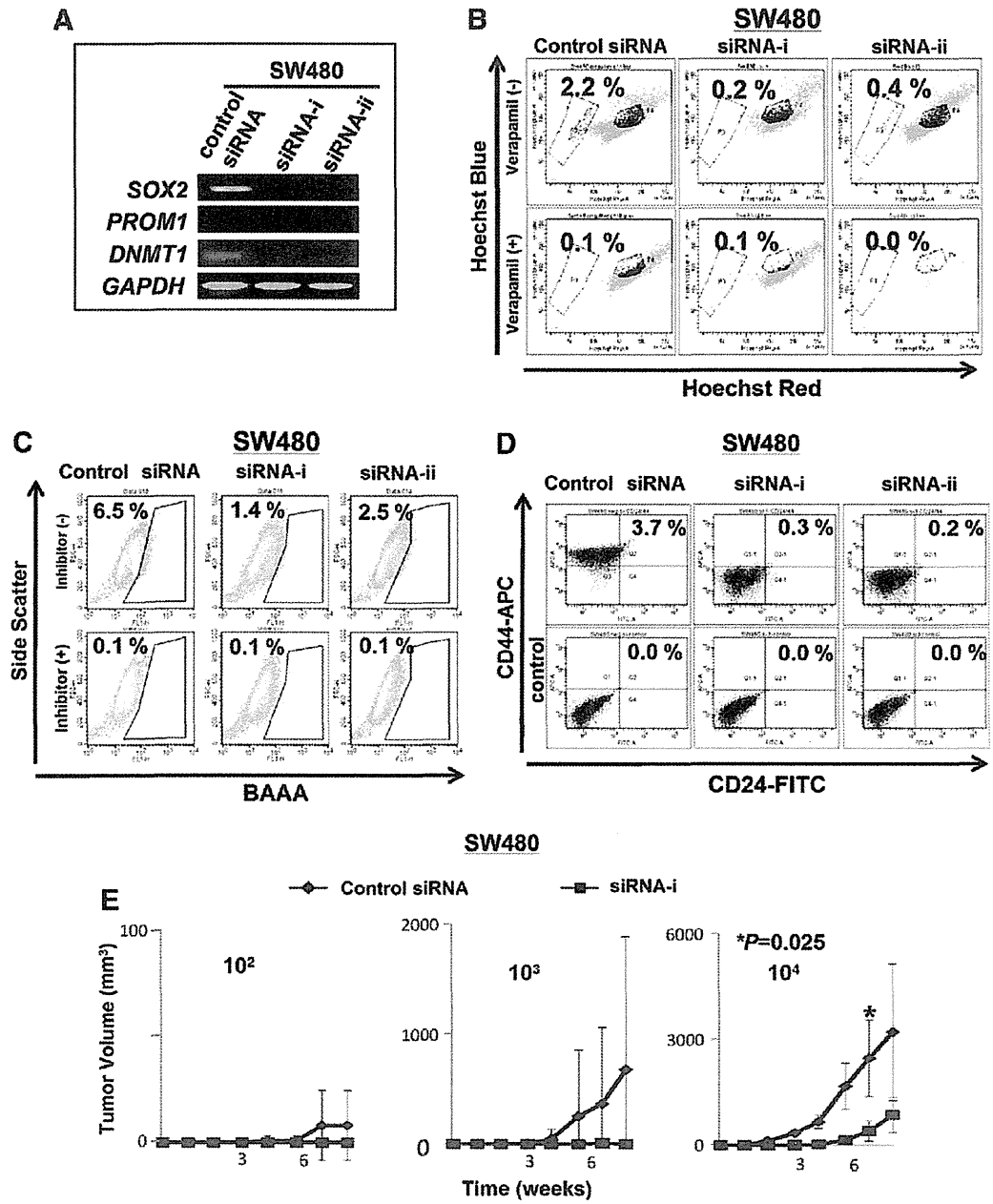

Fig. 4. *DNMT1* gene knockdown experiments in SW480 cells. A. RT-PCR of *DNMT1* and CSCs/CICs markers. The mRNA expression levels of *DNMT1* and CSCs/CICs markers were evaluated by RT-PCR. Total RNAs were isolated 48 h after transfection of a negative control or *DNTM1*-specific siRNA i and siRNA ii. B. Isolation of SP cells from colon cancer cell lines. SW480 *DNMT1*-knockdown cells and control cells were stained with Hoechst 33342 dye and analyzed. C. ALDEFLUOR Assay. ALDH activity was detected using the ALDEFLUOR assay kit 48 h after transfection of negative control or *DNMT1* specific siRNAs. D. Expression of CD44 and CD24. The numerical value in the dots plot graph is $CD24^{+}44^{+}$ cells rate. Stained cells were analyzed using a FACS Aria II cell sorter. E. Tumor growth of HCT116 *DNMT1*-knockdown cells and control cells. 10^2, 10^3 or 10^4 SW480 *DNMT1*-knockdown cells and control cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data represent means ± SD. Differences between SW480 *DNMT1*-knockdown cells and control cells were examined for statistical significance using Student's *t*-test. *P values.

Discussion

In the present study, we found that *DNMT1*$^{-/-}$ cells show much lower tumor-initiating ability than that of HCT116 w/t cells *in vivo*, whereas the growth rate of *DNMT1*$^{-/-}$ cells *in vitro* was not affected compared to that of HCT116 w/t cells, being consistent with a previous report. (Rhee et al., 2002) To address this controversial phenomenon of tumor-initiating ability and cell growth potential, we hypothesized that the CSC/CIC population is reduced in *DNMT1*$^{-/-}$ cells. We evaluated *DNMT1*$^{-/-}$ cells by SP analysis and clarified that *DNMT1*$^{-/-}$ cells contain a very small CSCs/CICs population, compared with HCT116 w/t cells. *DNMT1*$^{-/-}$ cells still have a very low tumor-initiating ability.

The *DNMT1*$^{-/-}$ cells we used in this study lack exons 2–5 of the *DNMT1* gene (Rhee et al., 2000), and *DNMT1*$^{-/-}$ cell has been proved to be a hypomorph that express a truncated form of the DNMT1 C-terminal DNA methyltransferase catalytic domain at a low level. (Egger et al., 2006). Therefore, a low level DNMT1 expression might be sufficient for cell viability. On the other hand, a low expression level might not be sufficient for maintenance of CSCs/CICs. In previous studies, human cancers have been found to overexpress DNMT1. (De

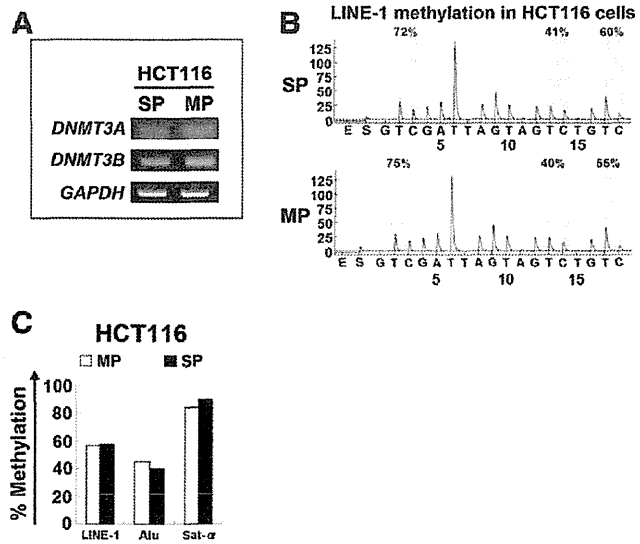

Fig. 5. Expression of DNMT3A and DNMT3B and methylation status of repetitive sequence in SP and MP cells. A. Expression of *DNMT3A* and *DNMT3B* mRNA in SP and MP cells derived from HCT116 cells. *DNMT3A* and *DNMT3B* mRNA expressions were evaluated in SP cells and MP cells derived from HCT116 cells. B. Quantitative pyrosequencing analysis of LINE-1 in SP and MP cells. Quantitative pyrosequencing analysis was performed using SP cells and MP cells derived from HCT116 cells. Representative pyrograms of SP cells and MP cells. Gray columns represent C-to-T polymorphic sites. C. Methylation status of LINE-1, Alu and Sat-alfa in SP cells and MP cells. SP cells and MP cells derived from HCT116 cells were analyzed. Data represent means. Open bars represent MP cells and closed bars represent SP cells.

Marzo et al., 1999; Mizuno et al., 2001; Girault et al., 2003; Saito et al., 2003; Etoh et al., 2004; Lin et al. (2007, 2010)) DNTM1 overexpression is correlated with poorer tumor differentiation in gastric carcinomas (Etoh et al., 2004), and it is correlated with poorer prognosis in hepatocellular and lung carcinomas. (Saito et al., 2003; Lin et al., 2010) Since CSCs/CICs are related to poorer prognosis and also poorer differentiation, these observations suggest that poorer differentiation and poorer prognosis might be caused by a high ratio of CSCs/CICs that is maintained by a high expression level of DNMT1.

We also investigated several functioning CSCs/CICs markers including ALDH1 enzymatic activity and the expression of CD44. ALDH1 enzyme identifies the cells that are resistant to alkylating agents, and gives these cells cytoprotective effects. ALDH1 members catalyze the final step in the conversion of retinol to retinoic acid that concerns with differentiation and self-renewal. (Kemper et al., 2010; Gires, 2011) This enzyme plays an important role in the maintenance of CSCs/CICs. CD44 also has a functional role in CSCs/CICs, such as survival, growth, differentiation and chemotherapy-resistance. (Kemper et al., 2010; Zeilstra et al., 2008) In addition, CD44 works as the adhesion molecule related in migration. (Cho et al., 2012) In this study, the cells that had high ALDH1 activity were detected only 0.2% in *DNMT1*$^{-/-}$ cells and CD44 expression greatly decreased in *DNMT1*$^{-/-}$ cells. Although we could not reveal the exact mechanisms of how DNMT1 controls the maintenance of CSCs/CICs, deletion of DNMT1 decreases CSCs/CICs and reduces the expressions of these functioning molecules. Therefore, we suppose that DNMT1 might be essential for initiating of the colon cancers. Actually, we observed that DNMT1 positive rates were significantly correlated with SOX2, that was reported to be as transcription factor in embryonic stem cells (Masui et al., 2007) and to highly express in CSCs/CICs of lung cancer (Nakatsugawa et al., 2011), positive rates in the immunohistochemical staining of primary colon cancer (data not shown). Although further analyses are required, this might be a clue that elucidates the role of DNMT1 in CSC/CICs.

Previous report described that there are no difference in genomic methylation status in both HCT116 cells and *DNMT1*$^{-/-}$ cells (Rhee et al., 2000), and we showed there are also no difference in genomic methylation status in SP and MP cells. Taken together, these results indicate that the methylation status of genome does not matter for the maintenance of CSCs/CICs.

In conclusion, we showed for the first time that *DNMT1* is essential for maintenance of human colon CSCs/CICs. Transient suppression of *DNMT1* is sufficient to exhaust CSCs/CICs. Our observations indicate the possibility that transient systemic or local gene suppression of *DNMT1* is an effective approach for eradicating CSCs/CICs, which will make disease more treatable by chemotherapy or radiotherapy.

Declaration of financial disclosure

The authors have no financial conflict of interest.

Acknowledgments

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References

Al-Hajj, M., et al., 2003. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 100, 3983–3988.
Ben-Porath, I., et al., 2008. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nature Genetics 40, 499–507.
Broske, A.M., et al., 2009. DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction. Nature Genetics 41, 1207–1215.
Cho, S.H., et al., 2012. CD44 enhances the epithelial–mesenchymal transition in association with colon cancer invasion. International Journal of Oncology 41, 211–218.
Clarke, M.F., et al., 2006. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Research 66, 9339–9344.
Dalerba, P., et al., 2007. Phenotypic characterization of human colorectal cancer stem cells. Proceedings of the National Academy of Sciences of the United States of America 104, 10158–10163.
De Marzo, A.M., et al., 1999. Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Research 59, 3855–3860.
Dean, M., et al., 2005. Tumour stem cells and drug resistance. Nature Reviews Cancer 5, 275–284.
Egger, G., et al., 2006. Identification of DNMT1 (DNA methyltransferase 1) hypomorphs in somatic knockouts suggests an essential role for DNMT1 in cell survival. Proceedings of the National Academy of Sciences of the United States of America 103, 14080–14085.
Etoh, T., et al., 2004. Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. American Journal of Pathology 164, 689–699.
Ginestier, C., et al., 2007. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 1, 555–567.
Girault, I., et al., 2003. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clinical Cancer Research 9, 4415–4422.
Gires, O., 2011. Lessons from common markers of tumor-initiating cells in solid cancers. Cellular and Molecular Life Sciences 68, 4009–4022.
Goodell, M.A., et al., 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. The Journal of Experimental Medicine 183, 1797–1806.
Huang, E.H., et al., 2009. Aldehyde dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Research 69, 3382–3389.
Igarashi, S., et al., 2010. A novel correlation between LINE-1 hypomethylation and the malignancy of gastrointestinal stromal tumors. Clinical Cancer Research 16, 5114–5123.
Inoda, S., et al., 2009. Cep55/c10orf3, a tumor antigen derived from a centrosome residing protein in breast carcinoma. Journal of Immunotherapy 32, 474–485.

Inoda, S., et al., 2011. Cytotoxic T lymphocytes efficiently recognize human colon cancer stem-like cells. American Journal of Pathology 178, 1805–1813.
Kemper, K., et al., 2010. Molecular identification and targeting of colorectal cancer stem cells. Oncotarget 1, 387–395.
Kondo, T., et al., 2004. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proceedings of the National Academy of Sciences of the United States of America 101, 781–786.
Lin, R.K., et al., 2007. Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer. Lung Cancer 55, 205–213.
Lin, R.K., et al., 2010. Dysregulation of p53/Sp1 control leads to DNA methyltransferase-1 overexpression in lung cancer. Cancer Research 70, 5807–5817.
Masui, S., et al., 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nature Cell Biology 9, 625–635.
Mizuno, S., et al., 2001. Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood 97, 1172–1179.
Nakatsugawa, M., et al., 2011. SOX2 is overexpressed in stem-like cells of human lung adenocarcinoma and augments the tumorigenicity. Laboratory Investigation 91, 1796–1804.
O'Brien, C.A., et al., 2007. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 445, 106–110.
Rhee, I., et al., 2000. CpG methylation is maintained in human cancer cells lacking DNMT1. Nature 404, 1003–1007.
Rhee, I., et al., 2002. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 416, 552–556.
Ricci-Vitiani, L., et al., 2007. Identification and expansion of human colon-cancer-initiating cells. Nature 445, 111–115.
Saito, Y., et al., 2003. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. International Journal of Cancer 105, 527–532.
Sen, G.L., et al., 2010. DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature 463, 563–567.
Trowbridge, J.J., et al., 2009. DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. Cell Stem Cell 5, 442–449.
Vermeulen, L., et al., 2008. Single-cell cloning of colon cancer stem cells reveals a multilineage differentiation capacity. Proceedings of the National Academy of Sciences of the United States of America 105, 13427–13432.
Yamamoto, E., et al., 2008. LINE-1 hypomethylation is associated with increased CpG island methylation in *Helicobacter pylori*-related enlarged-fold gastritis. Cancer Epidemiology Biomarkers & Prevention 17, 2555–2564.
Yeung, T.M., et al., 2010. Cancer stem cells from colorectal cancer-derived cell lines. Proceedings of the National Academy of Sciences of the United States of America 107, 3722–3727.
Zeilstra, J., et al., 2008. Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. Cancer Research 68, 3655–3661.

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Expression and Function of *FERMT* Genes in Colon Carcinoma Cells

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Abstract. *Invasion into the matrix is one of hallmarks of malignant diseases and is the first step for tumor metastasis. Thus, analysis of the molecular mechanisms of invasion is essential to overcome tumor cell invasion. In the present study, we screened for colon carcinoma-specific genes using a cDNA microarray database of colon carcinoma tissues and normal colon tissues, and we found that fermitin family member-1 (FERMT1) is overexpressed in colon carcinoma cells. FRRMT1, FERMT2 and FERMT3 expression was investigated in colon carcinoma cells. Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that only FERMT1 had cancer cell-specific expression. Protein expression of FERMT1 was confirmed by western blotting and immunohistochemical staining. To address the molecular functions of FERMT genes in colon carcinoma cells, we established FERMT1-, FERMT2- and FERMT3-overexpressing colon carcinoma cells. FERMT1-overexpressing cells exhibited greater invasive ability than did FERMT2- and FERMT3-overexpressing cells. On the other hand, FERMT1-, FERMT2- and FERMT3-overexpressing cells exhibited enhancement of cell growth. Taken together, the results of this study indicate that FERMT1 is expressed specifically in colon carcinoma cells, and has roles in matrix invasion and cell growth. These findings indicate that FERMT1 is a potential molecular target for cancer therapy.*

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Key Words: Colon carcinoma, invasion, *FERMT1*, DNA microarray, fermitin family.

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Colon carcinoma is a major malignancy, with a high mortality rate. In the process of tumorigenesis, tumor cells undergo multiple steps of genetic events (1), and multiple steps are also required for the cells to obtain several different phenotypes. Tissue invasion and metastasis are hallmarks that distinguish malignant from benign diseases (2). Several classes of proteins are involved in the process of tissue invasion; however, the exact molecular mechanisms of invasion remain unclear.

Fermitin family member (*FERMT*) genes include *FERMT1*, *FERMT2* and *FERMT3*, and these genes have been reported to be mammalian homologs of the *Caenorhabditis elegans* gene (3,4). The *unc-112* gene mutant had a phenotype similar to that of *unc-52* (perlecan), *pat-2* (α-integrin) and *pat-3* (β-integrin) mutants, and *unc-112* has been described as a novel matrix-associated protein (3). In subsequent studies, *FERMT2* was found to be related to invasion in MCF-7 breast carcinoma cells (5). *FERMT1* has been reported to be overexpressed in lung carcinoma cells and colon carcinoma cells (4), and has been reported to be related to invasion of breast carcinoma cells (6). However, the molecular functions of *FERMT1* in colon carcinoma cells remain elusive.

In this study, we screened a gene expression database of carcinoma tissues to analyze the molecular mechanisms of colon carcinoma, and we isolated *FERMT1* as a gene overexpressed in colon carcinoma tissues. We then analyzed the molecular functions of *FERMT* genes in colon carcinoma cells.

Materials and Methods

Cell lines, culture, cell growth assay and gene transfer. Colon adenocarcinoma cell lines HCT116, HCT15, Colo205, SW480, CaCO2, RTK, SW48, LoVo, DLD1, HT29 and Colo320 were kind gifts from Dr. K. Imai (Sapporo, Japan), and the KM12LM cell line was a kind gift from Dr. K. Itoh (Kurume, Japan). All cell lines were

cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Japan, Tokyo, Japan).

For cell growth assay, 1×10^5 cells were seeded in a 6-well plate, and total cell numbers were counted every day by using Countess™ (Life Technologies).

A retrovirus system was used for gene transfer, as described previously (7). Briefly, a pMXs-puro retroviral vector was transfected into PLAT-A amphotropic packaging cells (kind gift from Dr. T. Kitamura), and then HCT116 and SW480 cells were infected with the retrovirus. Puromycin was added at 5 μg/ml for establishment of stable transformants.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of FERMT genes in normal tissues and colon carcinoma cells. RT-PCR analysis was performed as described previously (8). Primer pairs used for RT-PCR analysis were 5'-GTCTGCTGAAACACAGGATTT-3' and 5'-GTTTTTCTAGTGGTTCTCCTT-3' for *FERMT1*, with an expected PCR product size of 272 base pairs (bps); 5'-CATGACATCAGAGAATCATTT-3' and 5'-ACTGGATTCTTCTTT GCTCTT-3' for *FERMT2*, with an expected PCR product size of 256 bps; 5'-AAAGTTCAAGGCCAAGCAGCT-3' and 5'-TGAAGGCCA CATTGATGTGTT-3' for *FERMT3* with an expected PCR product size of 326 bps; and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) with an expected product size of 452 bps. *GAPDH* was used as an internal control. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.2% agarose gel. Nucleotide sequences of the PCR products were confirmed by direct sequencing.

Construction of plasmids and transfection. Full-length *FERMT1*, *FRERMT2* and *FERMT3* cDNAs were amplified from cDNA of LoVo cells with PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The primer pairs were 5'-CGGGGTACCATGCTGTCATCC ACTGACTTT-3' as a forward primer and 5'-CCGCTCGAGATCCTG ACCGCCGGTCAATTT-3' as a reverse primer (underlines indicating *KpnI* and *XhoI* recognition sites, respectively) for *FERMT1*, 5'-CGGGGTACCGCCACCATGGCTCTGGACGGGATAAGG-3' as a forward primer and 5'-CCGCTCGAGCACCCAACCACTGGTA AGTTT-3' as a reverse primer for *FERMT2*, and 5'-CGGGGTACC GCCACCATGGCGGGGATGAAGACAGCC-3' as a forward primer and 5'-CCGCTCGAGGAAGGCCTCATGGCCCCCGGT-3' as a reverse primer for *FERMT3*. The PCR product was inserted into the pcDNA3.1 expression vector (Life Technologies) fused with a FLAG-tag. The cDNA sequences were confirmed by direct sequencing, and proved to be identical as reported previously (4). The inserts were then sub-cloned into a pMXs-puro retrovirus vector (kind gift from Dr. T. Kitamura, Tokyo, Japan). For the construct of protein expression, a *BglII* and *XhoI*-digested deletion mutant of *FERMT1* cDNA that was amplified by PCR using the primer pair 5'-GAAGATCTATGCT GTCATCCACTGACTTT-3' and 5'-CCGCTCGAGATCCTGACCGC CGGTCAATTT-3' (underlines indicating *BglII* and *XhoI* recognition sites, respectively) was inserted into a *BamHI* and *XhoI*-digested pQE30 (Qiagen Japan, Tokyo, Japan) vector.

FERMT1 recombinant protein production and establishment of a monoclonal antibody (mAb). A pQE30-*FERMT1* deletion mutant construct was transformed into Escherichia coli strain M15 (Qiagane Japan, Tokyo, Japan), and His6 tag-fused FERMT1 protein was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30°C. Cells were lysed in lysis buffer [6 M guanidine hydrochloride, 20 mM HEPES (pH 8.0), 50 mM NaCl], and recombinant FERMT1 protein was purified using Ni-NTA resin (Qiagen Japan).

The FERMT1 recombinant protein (100 μg) was used for immunization of BALB/c mice (CHARLES RIVER LABORATORIES JAPAN, INC., Yokohama, Japan) by intraperitoneal (*i.p.*) injection four times at two-week intervals. One week after the last injection, splenic cells were collected and fused with the NS-1 mouse myeloma cell line (ATCC, Manassas, VA, USA) at a 4:1 ratio. FERMT1 protein-specific hybrydomas were screened with enzyme-linked immunosorbent assay (ELISA) and western blotting using recombinant FERMT1 protein.

Immunohistochemical staining and western blotting. Immunohistochemical staining was performed with a colon carcinoma tissue microarray established from formalin-fixed surgically-resected tumor specimens of colon carcinoma at Sapporo the Medical University Hospital, as described previously (8). Anti-FERMT1 antibody was used at a 10-fold dilution with the anti-FERMT1-specific hybridoma culture supernatant. Western blotting of colon carcinoma tissues and colon carcinoma cells was performed as described previously (8). Anti-FERMT1 antibody was used at a 10-fold dilution with hybridoma culture supernatant.

Matrigel invasion assay. BD BioCoat Matrigel Invasion Chambers (Discovery Labware, Bedford, MA, USA) and polyethylene terephthalate (PET) track-etched membranes with pore sizes of 8.0 μm (Becton Dickinson, San Diego, CA, USA) were used for the invasion assay, according to the protocol of the manufacturer. HCT116- and SW480-transformant cells (2.5×10^4 cells/500 ml) were plated in the top chamber in DMEM, and culture medium with 10% FBS was used in the bottom chamber as a chemoattractant. Twenty-four hours later, cells were fixed and stained using a HEMA 3 STAT Pack (Fisher Scientific Japan, Tokyo, Japan). Cell numbers were counted on microphotographs taken in ten areas of the membrane.

Statistical analysis. In cell growth assays and invasion assays, samples were analyzed using Student's *t*-test, with $p<0.05$ conferring statistical significance.

Results

Isolation of the colon carcinoma-related gene FERMT1. We screened a gene expression database of approximately 700 normal organ tissues and about 4000 carcinoma tissues using the Affymetrix GeneChip Human Genome U133 Array Set that contains approximately 39,000 genes. One of the genes that was overexpressed in colon carcinoma tissues was shown to be *FERMT1*, a member of the *FERMT* gene family. In a previous study, *FERMT1* was shown to be overexpressed in lung carcinoma cells and colon carcinoma cells (4). FERMT1 is member of a family of highly homologous gene products including FERMT2 and FERMT3 (Figure 1A). *FERMT1*, *FERMT2* and *FERMT3* share a FERM domain and a Pleckstrin homology domain (PH) domain, which are a cytoskeletal-associated domain and phosphatidylinositol

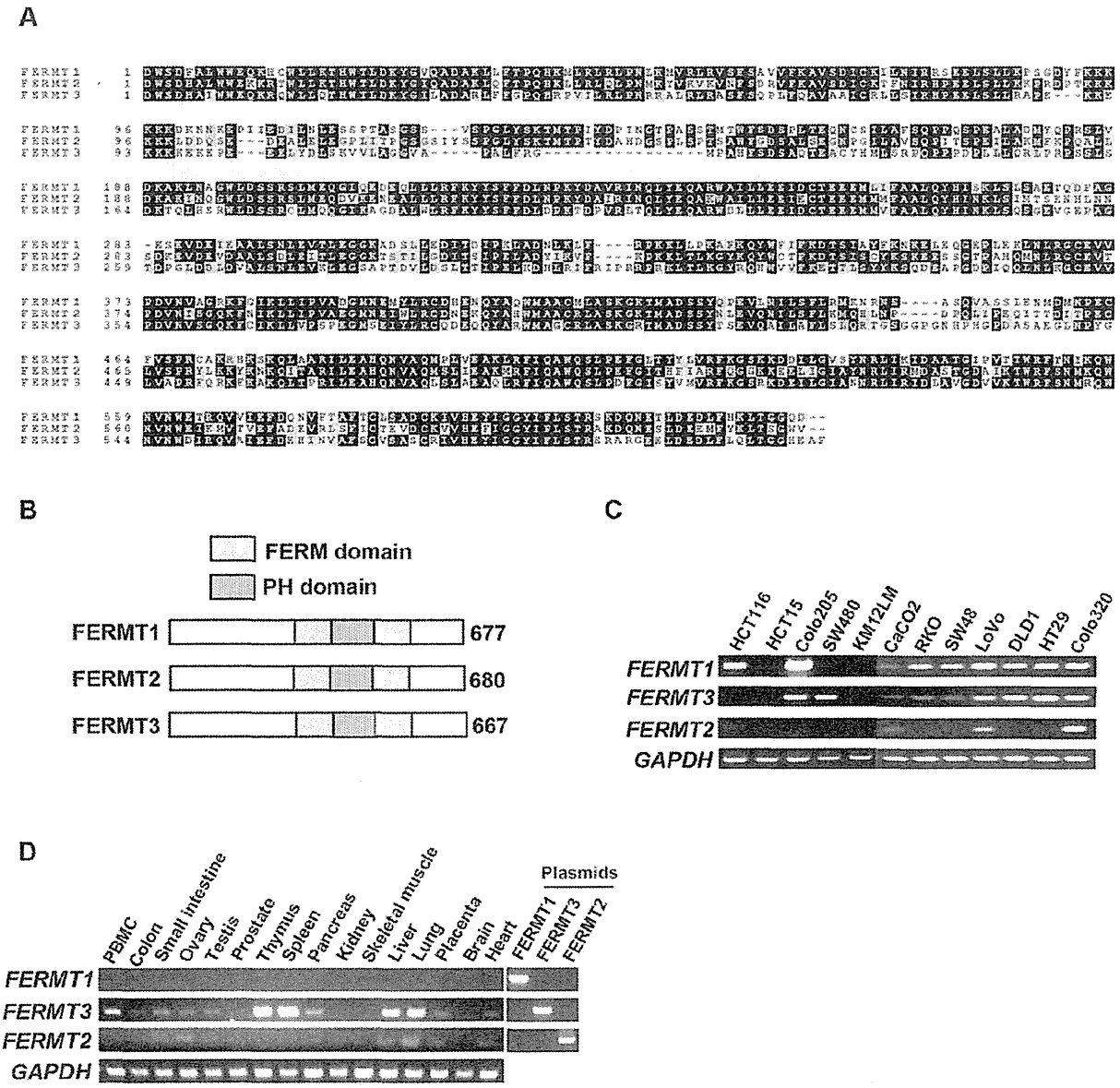

Figure 1. *Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins. A dotted box indicates the FERMT domain, cytoskeletal-associated domain, a lined box indicates the Pleckstrin homology domain (PH) domain, phosphatidylinositol lipid association domain. C: Reverse transcription-polymerase chain reaction (RT-PCR) of FERMT family in colon carcinoma cells. FERMT1, FERMT2 and FERMT3 expression in colon carcinoma cells was evaluated by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal positive control. D: RT-PCR of FERMT family genes in normal organ tissues. FERMT1, FERMT2 and FERMT3 expression in normal organ tissues was evaluated by RT-PCR. FERMT1, FERMT2 and FERMT3 plasmids were used as positive controls. GAPDH was used as an internal positive control.*

lipids association domain, respectively (Figure 1B). Since *FERMT1*, *FERMT2* and *FERMT3* show high homology with each other, we evaluated the expressions of these genes in colon carcinoma cells and also in normal organ tissues by RT-PCR. *FERMT1* was expressed in 9 (75%) out of 12 colon carcinoma line cells, and *FERMT3* was expressed in 9 (75%) out of 12 colon carcinoma line cells and *FERMT2* was expressed in 3 (25%) out of 12 colon carcinoma line cells

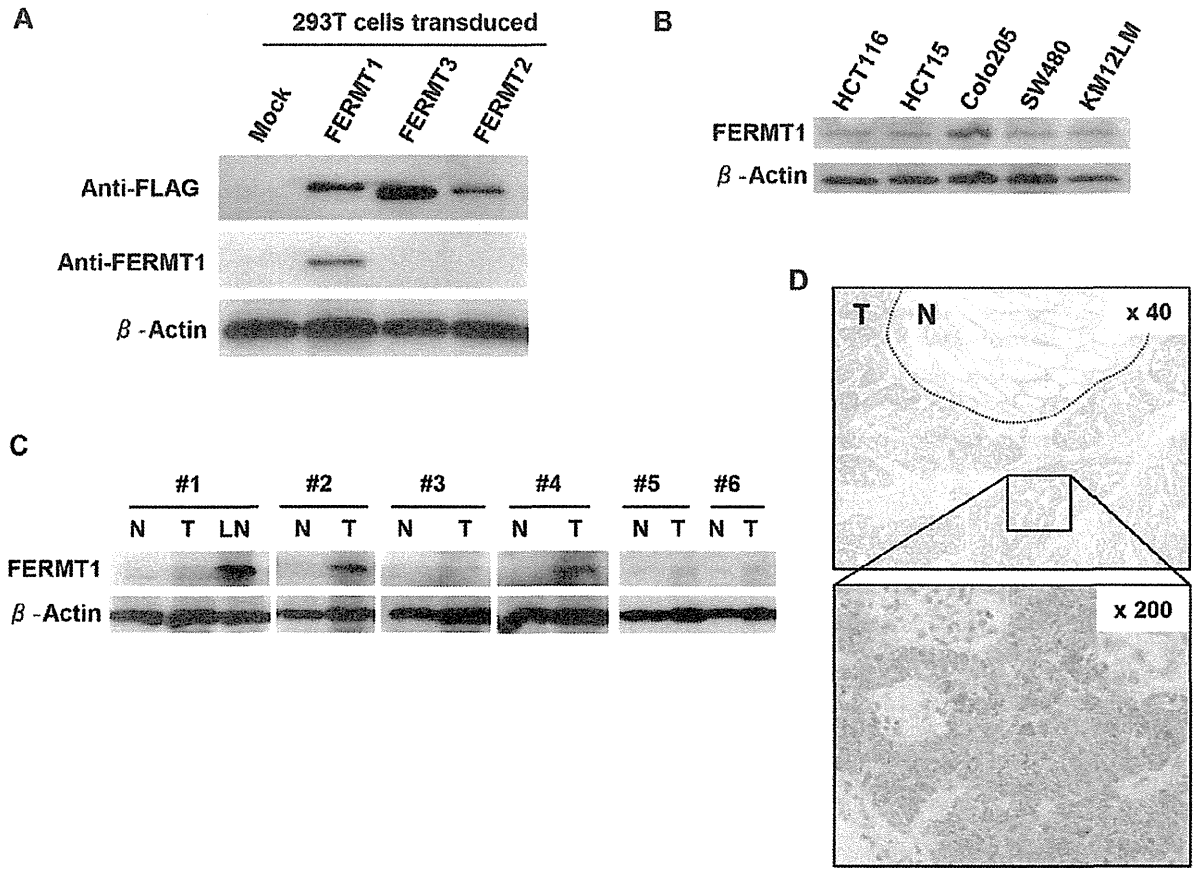

Figure 2. *Fermitin family member 1 (FERMT1) protein expression in colonic carcinomas. A: Western blotting using monoclonal antibody (mAb) against FERMT1. 293T cells were transfected with FERMT1, FERMT2 and FERMT3 plasmids. Western blotting using anti-FLAG mAb and anti-FERMT1 mAb was performed. Anti-FLAG mAb was used as a positive control. β-Actin was used as an internal positive control. B: Western blotting of colonic carcinoma cells. Western blotting using anti-FERMT1 mAb was performed. β-Actin was used as an internal positive control. C: Western blot of colon carcinoma tissues. Protein expression of FERMT1 in primary human colonic carcinoma cases (#1-#6) was evaluated by western blotting using an anti-FERMT1 mAb. T, Tumoral part of colonic carcinoma tissue; N, adjacent normal colonic mucosa tissue; LN, lymph node metastatic tissue of the corresponding case. β-Actin was used as an internal positive control. D: Immunohistochemical staining of FERMT1. Representative images of immunohistochemical staining of colonic carcinoma tissues using anti-FERMT1 mAb are shown. Brown indicates positive staining. Dotted line indicates normal colonic mucosa cells. N, Normal colon mucosa tissue; T, colonic carcinoma tissue.*

(Figure 1C). *FERMT1* was not expressed in normal organ tissues, whereas *FERMT3* and *FERMT2* were expressed ubiquitously in normal organ tissues. Only *FERMT1* exhibits colon carcinoma cell-specific expression. We therefore focused on *FERMT1* for further analysis.

Protein expression of FERMT1 in colon carcinoma cells and tissues. To address FERMT1 protein expression, we established a novel anti-FERMT1 mAb. Since FERMT1, FERMT2 and FERMT3 have similar protein structures, we evaluated the specificity of the mAb to FERMT1. FERMT1 mAb showed reactivity for 293T cells transfected with a FERMT1 expression vector, whereas it did not react to 293T cells transfected with a FERMT2 or FERMT3 vector, as shown in western blot analysis (Figure 2A), indicating that the mAb against FERMT1 mAb is specific for FERMT1. Western blot analysis revealed positive FERMT1 protein expression in all five colon carcinoma lines tested (Figure 2B).

Further evaluation of FERMT1 protein expression in primary colon carcinoma tissues was performed. Six colon carcinoma primary tumor tissues exhibited higher levels of FERMT1 protein expression than those in adjacent normal colonic mucosa tissues (Figure 2C). Of note, stronger FERMT1 protein expression was detected in tissue from lymph node metastasis of case #1 than in primary colonic tumor tissue and normal colonic mucosa of the same case.

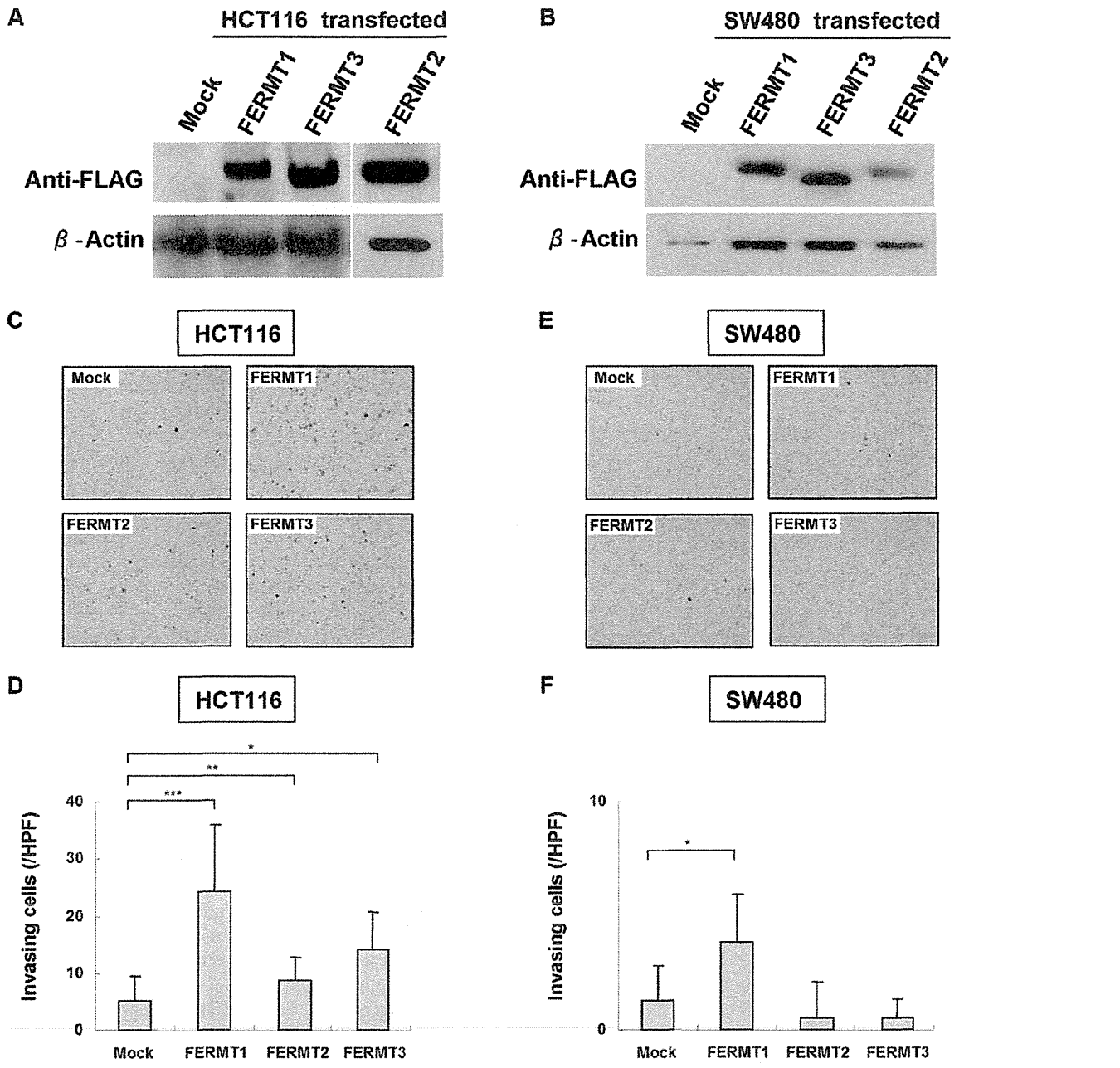

Figure 3. *Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control. B: Western blotting using a monoclonal antibody (mAb) to FLAG-tag. SW480 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using a mAb to FLAG-tag. β-Actin was used as an internal positive control. C: Invasion assay of FERMT family-overexpressing HCT116 cells. Representative images of invasion assay using FERMT family cDNA-overexpressing HCT116 cells. Purple cells indicate HCT116 cells that have invaded through the Matrigel. D: Invasion assay of FERMT family-overexpressing HCT116 cells. Invading cells were counted in 10 high power fields (HPFs). Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 cells were examined for statistical significance using the Student's t-test. *p=0.03, **p=0.001, ***p<0.0001. E: Invasion assay of FERMT family-overexpressing SW480 cells. Representative images of invasion assay using FERMT family cDNA-overexpressing SW480 cells. Purple cells indicate SW480 cells that have invaded through the Matrigel. F: Invasion assay of FERMT family-overexpressing SW480 cells. Invaded cells were counted in 10 HPF. Data represent means+SD. Differences between FERMT family-overexpressing SW480 cells and mock-transfected SW480 cells were examined for statistical significance using Student's t-test. *p=0.04.*

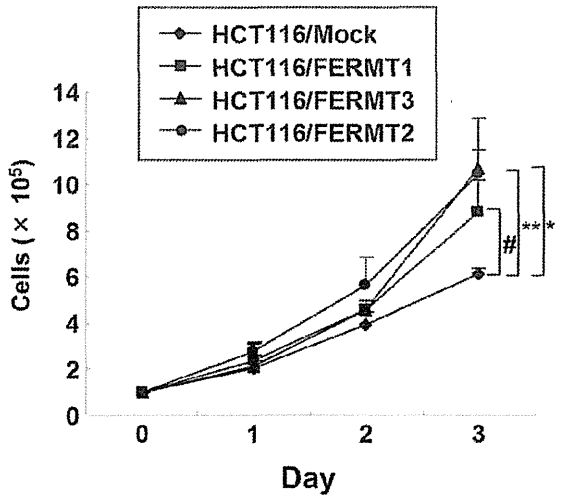

Figure 4. *Cell growth of FERMT famliy-overexpressing HCT116 cells. FERMT family cDNA-overexpressing HCT116 cells were seeded in a 6-well plate, and the cell growth rate was recorded daily. Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 cells were examined for statistical significance using Student's t-test. *p=0.015, #p=0.012, **p=0.001.*

Immunohistochemical staining of primary colonic carcinoma tissues also revealed FERMT1 protein expression in carcinoma cells but not in normal epithelial cells (Figure 2D). The positive immunohistochemical staining rate of FERMT1 protein in colon carcinoma tissues was 95% (38 out of 40 cases).

Role of FERMT1 in invasion and cell growth. Since western blot analysis revealed a high level of FERMT1 protein expression in lymph node metastasis tissue, we hypothesized that FERMT1 is related to the invasion of colonic carcinoma cells. In order to analyze the functions of *FERMT* genes, we established *FERMT1*-, *FERMT2*- and *FERMT3*-overexpressing HCT116 cells and SW480 cells. Protein expression of FERMT1, FERMT2 and FERMT3 was confirmed by western blot analysis, using an anti-FLAG antibody (Figure 3A and 3B). Invasion assays using Matrigel were performed, and FERMT1-overexpressing HCT116 cells exhibited greater invasive ability than mock vector-transformed HCT116 cells ($p<0.001$) (Figure 3C and 3D). *FERMT1*-overexpressing SW480 cells also exhibited greater invasive ability than did mock-transfected SW480 cells (Figure 3E and 3F). *FERMT2* and *FERMT3* had the ability to enhance the invasion of HCT116 cells, whereas they had no effect on SW480 cells. Cell growth ability was evaluated by a cell growth assay. *FERMT1*-, *FERMT2*- and *FERMT3*-overexpressing HCT116 cells showed greater growth *in vitro* than non-transfected cells, indicating that *FERMT1*, *FERMT2* and *FERMT3* have roles in cell growth (Figure 4).

Discussion

During cancer progression, cells gain multiple abilities allowing them to become malignant cells. Malignant diseases are defined by invasion into adjacent organs and distant metastasis, and invasion is thus a prominent ability of malignant cells. In this study, we identified *FERMT1* as a colon carcinoma-related gene by screening of a gene database. *FERMT1* was reported to be overexpressed in lung carcinoma cells and colonic carcinoma cells (4). However, the molecular functions of *FERMT1* in colonic carcinoma cells have not been elucidated. In another study, *FERMT1* was shown to be overexpressed in lung metastasis of breast carcinoma (9). The same research group reported that *FERMT1* has a role in epithelial mesenchymal transition through activation of transforming growth factor-β (TGFβ) signaling (6). However, the molecular functions of *FERMT1* have remained elusive, and we therefore analyzed *FERMT1* function in colon carcinoma cells.

FERMT1 has 80% homology with *FERMT2* and 72% homology with *FERMT3*. The three molecules have similar domain structures (Figure 1B), suggesting similar molecular functions. However, the expression profiles of *FERMT1*, *FERMT2* and *FERMT3* in normal organ tissues exhibited significant differences, and only *FERMT1* showed carcinoma cell-specific expression. In this study, we did not address the expression of *FERMT1* in skin tissue; however, previous studies showed that *FERMT1* is expressed in skin keratinocytes and that gene mutation in *FERMT1* is related to Kindler syndrome (10-12). *FERMT2* was shown to have invasion ability in MCF7 breast carcinoma cells (5). *FERMT3* was reported to be expressed in leukocytes and to have a role in the activation of integrin signals (13, 14); however, there has been no report describing the relationship between *FERMT3* and invasion. In our study, *FERMT1*, *FERMT2* and *FRMT3* were all shown to have roles in invasion, indicating that they may have similar functions. *FERMT1* and *FERMT2* have been reported to share some molecular functions in skin keratinocytes (15, 16). These observations indicate that *FERMT1*, *FERMT2* and *FERMT3* may have similar molecular functions and that the difference in expression defines the role of each molecule. Of note, *FERMT1* is ectopically and specifically overexpressed in carcinoma cells and *FERMT1* is thus the most suitable target for future cancer therapy.

In summary, to our knowledge this is the first report on *FERMT1* functions in colon carcinoma cells. While *FERMT1*, *FERMT2* and *FERMT3* are expressed in colon carcinoma cells, only *FERMT1* exhibites cancer cell-specific expression. FERMT1 also has a role in invasion and growth of colonic carcinoma cells. The results indicate that *FERMT1* is a possible target for cancer therapy.