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Preferential expression of cancer/testis genes in cancer stem-like cells: proposal of a novel sub-category, cancer/testis/stem gene

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Abstract

Cancer/testis (CT) antigens encoded by CT genes are immunogenic antigens, and the expression of CT gene is strictly restricted to only the testis among mature organs. Therefore, CT antigens are promising candidates for cancer immunotherapy. In a previous study, we identified a novel CT antigen, DNAJB8. DNAJB8 was found to be preferentially expressed in cancer stem-like cells (CSCs)/cancer-initiating cells (CICs), and it is thus a novel CSC antigen. In this study, we hypothesized that CT genes are preferentially expressed in CSCs/CICs rather than in non-CSCs/-CICs and we examined the expression of CT genes in CSCs/CICs. The expression of 74 CT genes was evaluated in side population (SP) cells (=CSC) and main population (MP) cells (=non-CSC) derived from LHK2 lung adenocarcinoma cells, SW480 colon adenocarcinoma cells and MCF7 breast adenocarcinoma cells by RT-PCR and real-time PCR. Eighteen genes (*MAGEA2, MAGEA3, MAGEA4, MAGEA6, MAGEA12, MAGEB2, GAGE1, GAGE8, SPANXA1, SPANXB1, SPANXC, XAGE2, SPA17, BORIS, PLU-1, SGY-1, TEX15* and *CT45A1*) showed higher expression levels in SP cells than in MP cells, whereas 10 genes (BAGE1, BAGE2, BAGE4, BAGE5, XAGE1, LIP1, D40, HCA661, TDRD1 and TPTE) showed similar expression levels in SP cells and MP cells. Thus, considerable numbers of CT genes showed preferential expression in CSCs/CICs. We therefore propose a novel sub-category of CT genes in this report: cancer/testis/stem (CTS) genes.

Introduction

Cancer treatment has been improved to some degree in the past several decades. However, cancer is still a biologically malignant disease that may result in death. It is expected that cancer immunotherapy will become an effective approach to treat cancer. Various tumor-associated antigens (TAAs) have been identified in the past several decades, and cancer immunotherapy targeting TAAs has become a reality (1, 2). TAAs can be classified into several categories according to their expression in cancer cells and normal cells (3). One of the major categories is cancer/testis (CT) genes, which are expressed in cancer cells and only in the testis among human matured organs (4). The testis does not express the major histocompatibility complex molecule, and CT antigens are not expressed on the surface of cells as antigenic peptides. The testis is therefore not accessible by cytotoxic T lymphocytes (CTLs), and the testis is thus called an 'immunological privileged' organ. Some proteins coded by CT genes are immunogenic to the cellular immune system and humoral immune system, and proteins coded by CT genes are therefore considered to be promising targets for cancer immunotherapy (4).

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are defined as a small population of cancer cells that have (i) high tumor-initiating ability, (ii) self-renewal ability and (iii) differentiation ability (5). CSCs/CICs are resistant to

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chemotherapy and radiotherapy and are therefore considered to be responsible for cancer recurrence and distant metastasis (6). As previous studies have shown that CSCs/CICs are sensitive to immune systems, cancer immunotherapy targeting them is a possible and promising approach (7–10). To achieve a CSC/CIC-specific immune reaction, it is essential to identify TAAs that are expressed in CSCs/CICs.

In our previous study, we identified a novel CT antigen, DNAJB8 (11). DNAJB8 has a role in the maintenance of CSCs/CICs and is expressed preferentially in CSCs/CICs than in non-CSCs/non-CICs. DNAJB8 is therefore a novel CSC antigen. Thus, DNAJB8 is a CT antigen and also a cancer stem antigen. In this study, we analyzed the expression profiles of known CT genes in CSCs/CICs and non-CSCs/non-CICs. Therefore we propose a novel sub-category of CT genes called the cancer/testis/stem (CTS) that are expressed in the testis and CSCs.

Materials and methods

Cell lines and isolation of side and main population cells

The LHK2 lung adenocarcinoma cell line was found by Hirohashi et al. (12). SW480 colon adenocarcinoma cells and MCF7 breast adenocarcinoma cells were purchased from ATCC. LHK2, SW480 and MCF7 cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Life Technologies, Grand Island, NY).

Side population (SP) cells were isolated as described previously using Hoechst 33342 dye (Lonza, Basel, Switzerland) with some modifications (13). Briefly, cells were resuspended at 1×10^6/ml in pre-warmed DMEM supplemented with 5% FBS. Hoechst 33342 dye was added at a final concentration of 2.5 μg/ml in the presence or absence of verapamil (50 μM; Sigma-Aldrich) and the cells were incubated at 37°C for 90 min with intermittent shaking. Analyses and sorting were performed with a FACSAria II cell sorter (BD Biosciences, San Jose, CA).

RT-PCR analysis

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described previously (14). Human Multiple Tissue cDNA PaneI II (Takara Bio, Otsu, Japan) was used as templates of normal adult testis tissue cDNA. PCR amplification was performed in 20 μl of PCR mixture containing 0.2 μl of the cDNA mixture, 0.5 μl of Taq DNA polymerase (QIAGEN, Duesseldorf, Germany) and 12 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 60°C for 30 s and extension at 68°C for 30 s. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.2% agarose gel. Primers used in experiments are summarized in the supplemental Table S1. Human testis cDNA was used as a positive control and water was used as a negative control.

Quantitative PCR (qPCR)

Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Life Technologies) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Life Technologies). Thermal cycling was performed using 40 cycles of denaturation at 95°C for 15 s followed by annealing at 60°C for 1 min. Each experiment was performed triplicate, with normalization to the *GAPDH* gene as an internal control.

Statistical analysis

Statistical analysis between two groups of real-time PCR was performed by Student's *t* test and that between two groups of X-CT genes was performed by the chi-squared test.

Results

Preferential expression of some CT genes in CSCs/CICs

SP cells derived from lung adenocarcinoma cells, LHK2, colon adenocarcinoma cells, SW480, and breast adenocarcinoma cells, MCF7, are enriched with CSCs/CICs, and we therefore used it as a source of CSCs/CICs and MP cells as a source of non-CSCs/non-CICs (9, 13, 15). SP cells and MP cells were isolated from LHK2 lung adenocarcinoma cells, SW480 colon adenocarcinoma cells and MCF7 breast adenocarcinoma cells. To evaluate the expression of known CT genes in CSCs/CICs, RT-PCR was performed using SP and MP cells. A total of 74 CT genes were evaluated (Figure 1).

MAGEA2, MAGEA3, MAGEA6 and *GAGE8* were preferentially expressed in SP cells derived from LHK2 and SW480 cells, whereas *MAGEA2*, *MAGEA3*, *MAGEA6* and *GAGE8* were not detectable in both SP and MP cells derived from MCF7 cells. *MAGEA4*, *MAGEB2*, *GAGE1, SPANXA1, SPANXB1, SPANXC* and *BORIS* were preferentially expressed in SP cells derived from SW480 cells, whereas *MAGEA4, MAGEB2, GAGE1, SPANXA1, SPANXB1, SPANXC* and *BORIS* were not detectable in both SP and MP cells derived from LHK2 cells and MCF7 cells. *XAGE2, SGY-1* and *CT45A1* were preferentially expressed in SP cells derived from LHK2 cells, whereas *XAGE2, SGY-1* and *CT45A1* were not detectable in SP and MP cells derived from SW480 and MCF7 cells. *MAGEA12* was preferentially expressed in SP cells derived from LHK2 and SW480 cells, whereas *MAGEA12* was expressed in both SP and MP cells derived from MCF7 cells at similar levels. *SPA17*, *PLU-1* and *TEX15* were preferentially expressed in SP cells derived from LHK2 cells, whereas *SPA17*, *PLU-1* and *TEX15* were expressed in both SP and MP cells derived from SW480

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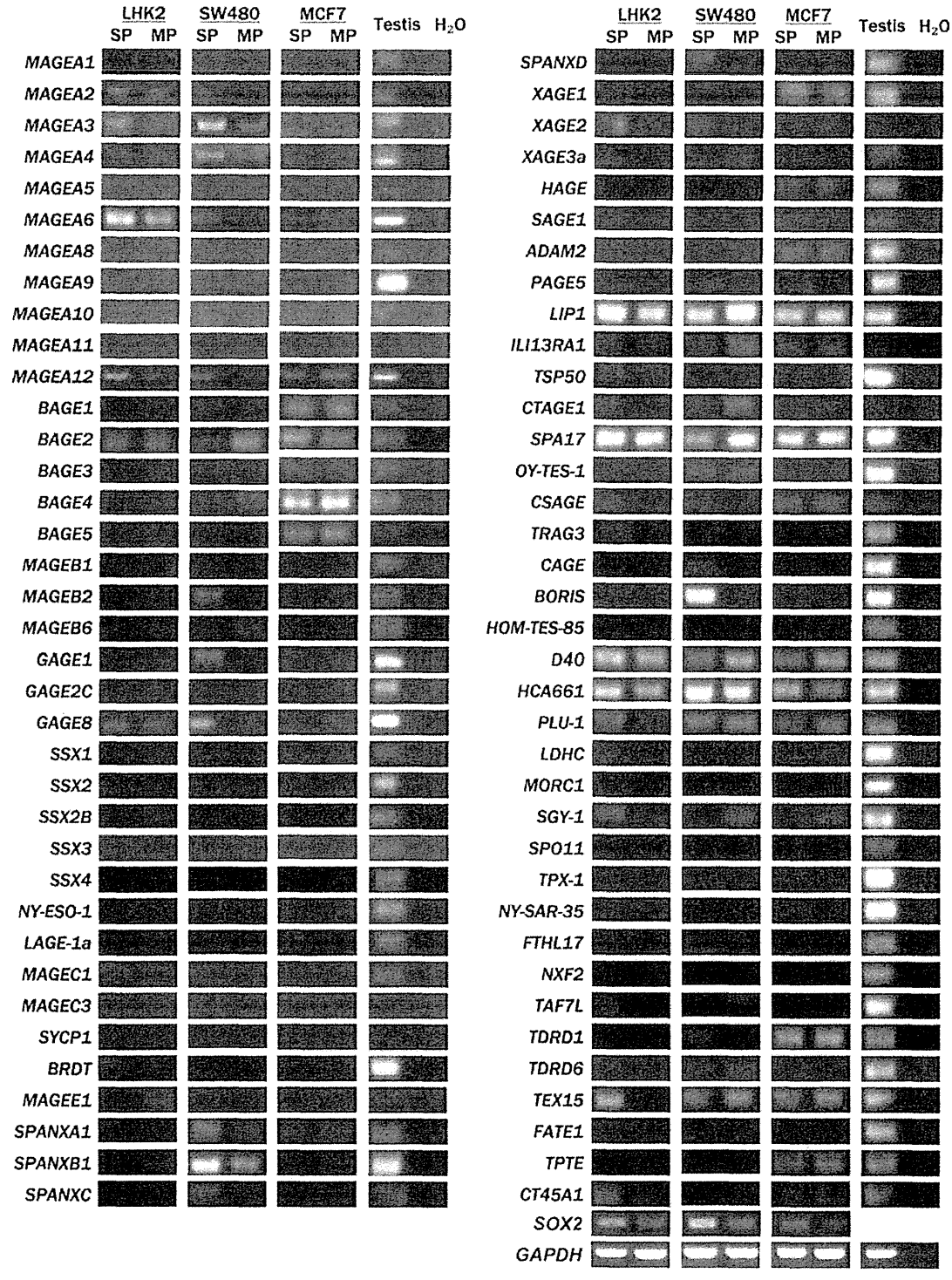

Figure 1 Expression of cancer/testis (CT) genes in cancer stem-like cell (CSCs)/cancer-initiating cell (CICs) and non-CSCs/CICs. The expression of CT genes in CSCs/CICs and non-CSCs/CICs was evaluated by RT-PCR. Side population (SP) cells were used as CSCs/CICs, and main population (MP) cells were used as non-CSCs/CICs. *SOX2* was used as a stem cell marker. Testis cDNA was used as a positive control and water was used as a negative control. *GAPDH* was included as a control for DNA quality.

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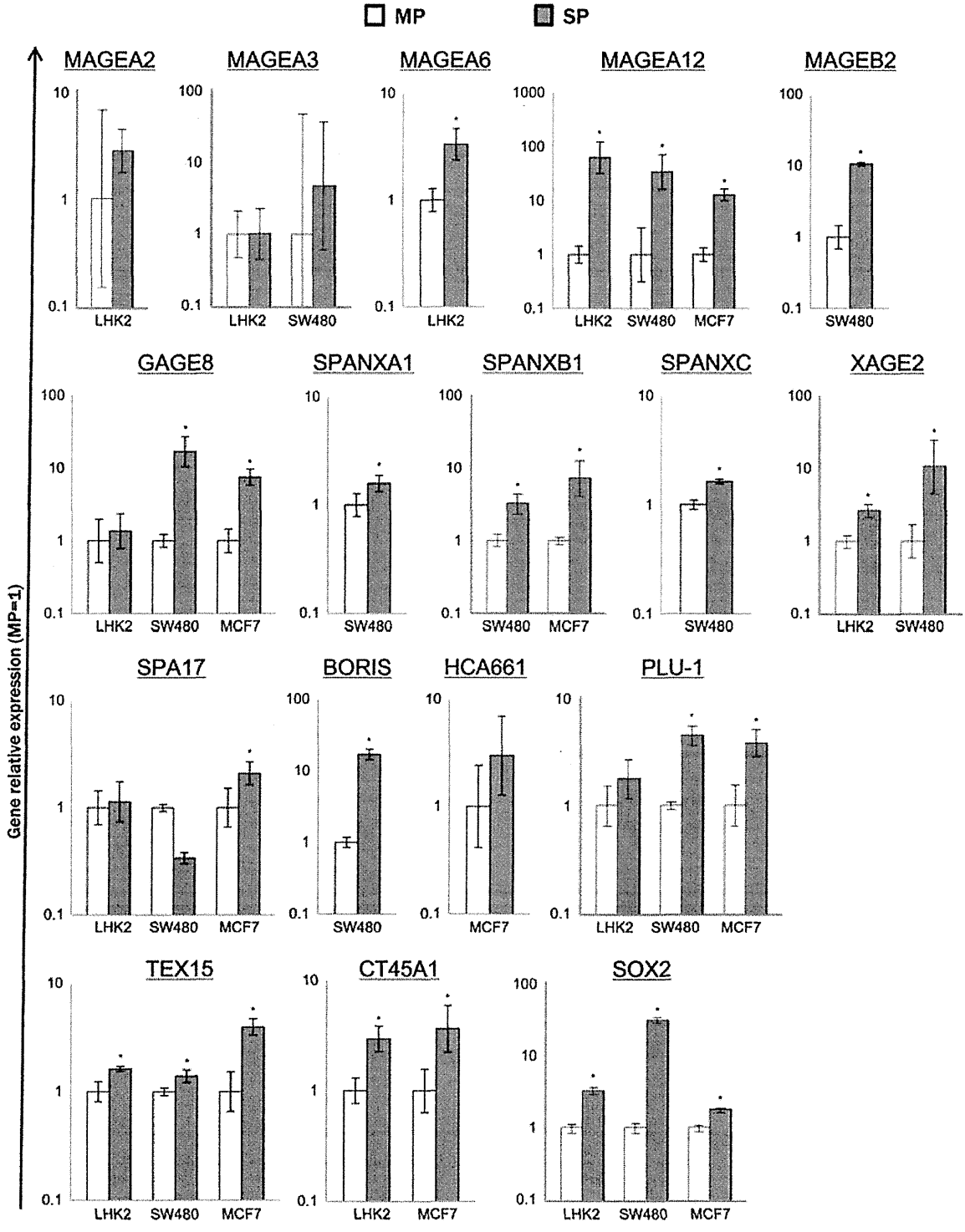

Figure 2 Real-time PCR of cancer/testis (CT) genes in cancer stem-like cell (CSCs)/cancer-initiating cell (CICs). The expression of CT genes in CSCs/CICs and non-CSCs/CICs was evaluated by real-time PCR. *SOX2* was used as a stem cell marker. Data represent means ± SD. Asterisks represent significant differences (*t*-test, $P < 0.05$).

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and MCF7 at similar levels. Therefore, *MAGEA2, MAGEA3, MAGEA4, MAGEA6, MAGEA12, MAGEB2, GAGE1, GAGE8, SPANXA1, SPANXB1, SPANXC, BORIS, XAGE2, SPA17, PLU-1, SGY-1, TEX15* and *CT45A1* were preferentially expressed in SP cells. On the other hand, *BAGE1, BAGE2, BAGE4, BAGE5, XAGE1, LIP1, D40, HCA661, TDRD1* and *TPTE* were expressed in both SP cells and MP cells at similar levels. The expression of *MAGEA1, MAGEA5, MAGEA8, MAGEA9, MAGEA10, MAGEA11, BAGE3, MAGEB1, MAGEB6, GAGE2C, SSX1, SSX2, SSX2B, SSX3, SSX4, NY-ESO-1, MAGEC1, MAGEC3, SYCP1, BRDT1, MAGEE1, XAGE3a, HAGE, SAGE1, ADAM2, PAGE5, IL13RA1, TSP50, CTAGE1, OY-TES-1, CSAGE, TRAG3, CAGE, HOM-TES-85, LDHC, MORC1, SPO11, TPX-1, NY-SAR-35, FTHL17, TDRD6* and *FATE1* was undetectable or very weak in SP and MP cells derived from LHK2, SW480 and MCF7 cells.

The data by RT-PCR were confirmed by quantitative PCR (qPCR) (Figure 2). The expression levels of *MAGEA2, MAGEA3, MAGEA4, MAGEA6, MAGEA12, MAGEB2, GAGE1* and *GAGE8* were higher than those of MP cells by RT-PCR and so we analyzed the data by qPCR. The expression levels of *MAGEA6*, *MAGEA12*, *MAGEB2* and *GAGE8* in SP cells were significantly higher than that of MP cells, while the expressions of *MAGEA2* and *MAGEA3* were higher than that of MP cells, but the difference was not significant. *MAGEA4* and *GAGE1* were detectable in SP cells derived from SW480 cells, but they were not detectable in MP cells derived from SW480; thus we could not compare those expression levels. The expression levels of *SPANXA1*, *SPANXB1* and *SPANXC* in SP cells derived from SW480 cells were higher than those of MP cells by qPCR. *SPANXB2* mRNA was detectable also in MCF7 cells, and the expression level of *SPANXB1* in SP cells was higher than that of MP cells. The expression profiles of *XAGE2*, *BORIS*, *HCA661*, *PLU-1*, *TEX15* and *CT45A1* were confirmed by qPCR. The expression of *SGY-1* in LHK2 SP cells was higher than that of LHK2 MP cells, while we could not detect mRNA by qPCR. The expression of *SPA17* in LHK2 SP cells was same as LHK2 MP cells, and *SPA17* in SW480 SP cells was lower than that of SW480 MP cells and *SPA17* in MCF7 SP cells was higher than that of MCF7 MP cells.

As many CT genes showed preferential expression in SP cells rather than in MP cells, we propose a sub-category of CT genes: CTS genes that are expressed in the testis and CSCs (Figure 3). More than half of the CT genes are encoded by a gene located on X chromosome (X-CT genes). Twelve of the 18 CTS genes (67%) are located on the X chromosome, whereas only 2 of the 10 shared antigens (20%) are located on the X chromosome, and the difference is statistically significant ($P = 0.038$; Table 1).

Discussion

CT genes are expressed in several stages of spermatogenesis including spermatogonial stem cells, spermatogonium, spermatocytes, spermatids and spermatozoa (16). In some cases, CT genes are also expressed in the placenta and ovary. CT genes have a role in spermatogenesis, and some of them have been reported to have role in tumorigenesis. However, roles of most CT genes in cancer cells are still elusive (16). The expression of CT genes is controlled by epigenetic mechanisms of demethylation of promoter regions (17). More than half of the CT genes are located on the X chromosome. Sixty-seven percentage of CTS genes are located on the X chromosome, whereas only 20% of the shared CT genes are located on the X chromosome. The biological meaning of this difference is still elusive. X-CT genes are preferentially expressed in a more immature state of spermatogenesis (spermatogonial stem cells), whereas non-X-CT genes are preferentially expressed in more matured stages of spermatogenesis. We therefore hypothesize that the roles of X-CT genes in spermatogonial stem cells are also important in maintenance of CSCs/CICs.

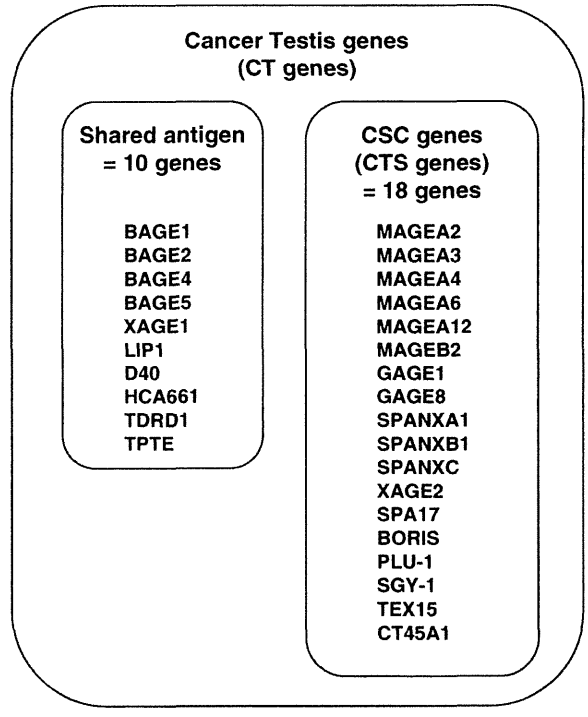

Figure 3 Categorization of cancer/testis (CT) genes.

Antigens coded by CT genes are expressed in a variety of malignant diseases, and humoral and cellular immune responses to the proteins coded by CT genes have been observed in patients with malignant disease (4, 18, 19). Thus, CT genes are considered to be ideal and promising targets for cancer immunotherapy. Indeed, MAGEA2, MAGEA3, MAGEA4, MAGEA6, MAGEA12 and GAGE1 have been shown to be recognized by CTLs (20, 21). On the other hand, MAGEB2, GAGE8, SPANXA1, SPANXB1, SPANXC,

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Table 1 Summary of cancer/testis genes

CT genes	Alias	Chromosomes[a]
Summary of CTS genes		
MAGEA2	CT1.2	Xq28
MAGEA3	CT1.3	Xq28
MAGEA4	CT1.4	Xq28
MAGEA6	CT1.6	Xq28
MAGEA12	CT1.12	Xq28
GAGE1	CT4.1	Xp11.23
GAGE8	CT4.8	Xp11
SPANXA1	CT11.1	Xq27.2
SPANXB1	CT11.2	Xq27.1
SPANXC	CT11.3, CTp11	Xq27.2
XAGE2	CT12.2	Xp11.22-p11.21
SPA17	CT22, SP17	11q24.2
CTCFL	CT27, BORIS	20Q13.31
KDM5B	CT31, PLU-1	1q32.1
DKKL1	CT34, SGY-1	19q13.3
TEX15	CT42	8p22
CT45A1	CT45.1	Xq26.1
Summary of shared genes		
BAGE	CT2.1, BAGE1	21p11.1
BAGE2	CT2.2	21p
BAGE4	CT2.4	21p11.1
BAGE5	CT2.5	13cen
XAGE1A	CT12.1a, XAGE1	Xp11.22
CASC5	CT29, D40	15q14
TFDP3	CT30, HCA661	Xq26.2
TDRD1	CT41.1	10p26.11
TPTE	CT44	21p11.1
CENPJ	LIP1, LAP, CPAP	13q12.12

CTS, cancer/testis/stem genes.
[a]The difference in frequencies of X-CT genes (localization in the X chromosome) was analyzed by the chi-squared test. $P = 0.038$.

XAGE2, SPA17, BORIS, PLU-1, SGY-1 TEX15 and CT45A1 have not been reported to be recognized by CTLs yet. In this study, we found that MAGEA12 and TEX15 are expressed in LHK2 lung carcinoma, SW480 colon carcinoma and MCF7 breast carcinoma CSCs/CICs; while the other genes exhibited different expression pattern. MAGEA4, MAGEA6, MAGEB2, SPANXA1, SPANXC and BORIS were preferentially expressed in SP cells derived from SW480 cells, but not expressed in LHK2 and MCF7 cells. These expression profiles indicate that MAGEA12 and TEX15 might be antigens that are expressed in several types of CSCs/CICs, while other antigens are expressed in CSCs/CICs derived from restricted organs. There are several potential CTL target peptides carrying HLA-binding anchor motif in amino acid sequences of these gene products (data not shown). Therefore, antigenic peptides derived from CTS antigens are promising targets for CSC/CIC-targeting immunotherapy, and the expression profile of this study will provide significant information.

In our recent review article, we classified TAAs into three groups according to the expression profiles in CSCs/CICs and non-CSCs/non-CICs: (i) CSC antigens, expressed preferentially in CSCs/CICs, (ii) shared antigens, expressed in both CSCs/CICs and non-CSCs/non-CICs and (iii) non-CSC antigens, expressed preferentially in non-CSCs/non-CICs (22). In subsequent works, we found that shared antigens are more potent than non-CSC antigens and that CSC antigens are more potent than shared antigens (11, 23). We therefore hypothesize that expression in only CSCs is important to achieve an efficient anti-tumor effect. Therefore, CTS genes might be promising targets among CT genes, and further analysis of CTS genes especially on immunogenicity to CTLs is expected.

In summary, we showed the preferential expression of CT genes in CSCs/CICs. We therefore propose a novel subcategory of CT genes: CTS genes. CTS genes might be promising targets for cancer immunotherapy.

Acknowledgments

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Conflict of interest

The authors have declared no conflicting interests.

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Supporting Information

The following supporting information is available for this article:

Table S1: List of primers.

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Prostate cancer stem-like cells/cancer-initiating cells have an autocrine system of hepatocyte growth factor

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Prostate cancer cells include a small population of cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) that have roles in initiation and progression of the cancer. Recently, we isolated prostate CSCs/CICs as aldehyde dehydrogenase 1-highh (ALDH1^{high}) cells using the ALDEFLUOR assay; however, the molecular mechanisms of prostate CSCs/CICs are still elusive. Prostate CSCs/CICs were isolated as ALDH1^{high} cells using the ALDEFLUOR assay, and the gene expression profiles were analyzed using a cDNA microarray and RT-PCR. We found that prostate CSCs/CICs expressed higher levels of growth factors including hepatocyte growth factor (HGF). Hepatocyte growth factor protein expression was confirmed by enzyme linked immunosorbent assay and Western blotting. On the other hand, c-MET HGF receptor was expressed in both CSCs/CICs and non-CSCs/CICs at similar levels. Hepatocyte growth factor and the supernatant of myofibloblasts derived from the prostate augmented prostasphere formation *in vitro*, and prostasphere formation was inhibited by an anti-HGF antibody. Furthermore, c-MET gene knockdown by siRNA inhibited the prostasphere-forming ability *in vitro* and tumor-initiating ability *in vivo*. Taken together, the results indicate that HGF secreted by prostate CSCs/CICs and prostate myofibroblasts has a role in the maintenance of prostate CSCs/CICs in an autocrine and paracrine fashion. (*Cancer Sci* 2013; 104: 431–436)

Prostate cancer is one of the common and lethal cancers in males. Although there are some curative treatments for early-stage prostate cancer, there is no effective treatment for advanced metastatic prostate cancer. Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) have high tumor-initiating ability and are resistant to chemotherapy and radiotherapy, and CSCs/CICs are therefore thought to be responsible for cancer recurrence after treatment and for distant metastasis.[1,2]

Prostate cancer contains a small population of CSCs/CICs, and we previously described the successful isolation of prostate CSCs/CICs from the human prostate carcinoma cell line 22Rv1 by using aldehyde dehydrogenase (ALDH) activity.[3] In addition, we isolated prostate CSC/CIC-specific genes, including *HGF* and *IGF1*, by microarray screening and RT-PCR analysis. In a physiological condition, hepatocyte growth factor (HGF) is secreted by mesenchymal cells and promotes epithelial cell growth in a paracrine fashion. However, signaling of HGF and its receptor c-MET is activated in cancer cells and is related to cancer cell growth, cell motility and matrix invasion, and HGF/c-MET signaling can therefore be a reasonable target of cancer therapy.[4] In this study, we confirmed HGF protein secretion from prostate CSCs/CICs, and we investigated the HGF/c-MET signaling function in maintenance of prostate CSCs/CICs.

Materials and Methods

Cell culture and ALDEFLUOR assay. The human prostate cancer cell line 22Rv1 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and the cells were cultured in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS. Prostate myofibroblast cells (WPMY-1) were purchased from ATCC and were cultured in DMEM supplemented with 5% of FCS. The cells were kept in a 37°C incubator with humidified air and 5% CO_2. The culture medium was changed twice a week. ALDEFLUOR assay was performed as described previously.[3] ALDH1^{high} and ALDH1^{low} cells were defined as those in the previous report.[3]

RNA preparation and reverse transcription-polymerase chain reaction. Isolation of total RNA and RT-PCR analysis were performed as described previously.[3] Primer pairs used for RT-PCR analysis were 5′-GGGCTGAAAAGATTGGATCA-3′ and 5′-TTGTATTGGTGGGTGCTTCA-3′ for *HGF* with an expected PCR product size of 245 base pairs (bp), 5′-CAGGC AGTGCAGCATGTAGT-3′ and 5′-GATGATTCCCTCGGTCA GAA-3′ for *MET* (hepatocyte growth factor receptor) with an expected PCR product size of 201 bp, and 5′-ACCACAGTC CATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) with an expected product size of 452 bp.

Enzyme-linked immunosorbent assay. Sorted ALDH1^{high} and ALDH1^{low} cells (10 000 cells each) were incubated in 150 μL DMEM + 10% FCS media in each well of a 96-well plate for 48 h. Enzyme linked immunosorbent assay was performed for detection of HGF in each cell culture supernatant (HGF Human ELISA Kit, Abcam, Cambridge, UK). The absorbance was measured at 450 nm. Data were obtained from seven independent samples, and the data were statistically analyzed.

Western blotting and immunohistochemical staining. Western blotting was performed as described previously.[5] Briefly, 5×10^4 of ALDH1^{high} cells and ALDH1^{low} cells were lysed in 100 μL of SDS sample buffer. Anti-ALDH1 mouse monoclonal antibody (clone: 44/ALDH; BD Biosciences, San Jose, CA, USA) was used at 1000-times dilution. Anti-HGF goat polyclonal antibody (R&D Systems, Minneapolis, MN, USA) and anti-MET goat polyclonal antibody (R&D Systems) were used at 1000-times dilution. Anti-β-Actin mouse monoclonal antibody (Sigma, St. Louis, MO, USA) was used at 2000-times dilution. Anti-mouse IgG + IgM and anti-goat IgG and IgM second antibodies (KPL) were uses at 2000-times dilution. The membrane was visualized with Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) according to the manu-

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facturer's protocol, and pictures were taken by Odyssey Fc Imaging System (LI-COR, Lincoln, NE, USA).

Immunohistochemical staining using formalin-fixed paraffin-embedded sections of surgically resected prostate carcinoma was performed as described previously.[6] Anti-ALDH1 mouse antibody was used at 250-times dilution. Anti-HGF mouse monoclonal antibody (Abcam) was used at 10 μg/mL. Anti-SOX2 rabbit polyclonal antibody (Invitrogen, Palo Alto, CA, USA) was used at 250-times dilution. Peroxidase-labeled goat anti-rabbit polyclonal antibody (Nichirei, Tokyo, Japan) was used as manufacturer's protocol and visualized by 3,3′-diaminobenzidine tetrachloride (DAB). Alkaline phosphatase-labeled goat anti-mouse polyclonal antibody (Nichirei) was used according to the manufacturer's protocol and visualized by New Fuchsin (Nichirei). Nucleus brown staining was judged as positive staining for SOX2, and cytoplasm red staining was judged as positive staining for ALDH1 and HGF.

Prostasphere formation assay. Isolated $ALDH1^{high}$ and $ALDH1^{low}$ cells were seeded into an Ultra-Low Attachment Surface culture six-well plate (CORNING, Tewksbury, MA, USA) at a concentration of 8×10^3/well and cultured in DMEM/F12 medium (Invitrogen, San Diego, CA, USA) supplemented with 20 ng/mL of epidermal growth factor (EGF; Sigma-Aldrich, St. Louis, MO, USA), 10 ng/mL of basic fibroblast growth factor (bFGF; Sigma-Aldrich), 10 ng/mL of HGF (Sigma-Aldrich) and 10 ng/mL IGF-1 (Sigma-Aldrich). The morphology of the cells was assayed and pictures were taken under a light microscope every day. For inhibition of HGF, anti-HGF antibody was supplemented at a concentration of 20 ng/mL. Anti-HLA class I antibody (clone: W6/32) was used as a negative control. Prostate myofibroblast culture supernatant was obtained from myofibroblasts cultured in serum-free DMEM media for 2 days. The myofibroblast supernatant was supplemented in a sphere culture condition at 50% (v/v).

***MET* mRNA knockdown by siRNA.** A *MET* gene knockdown experiment was performed using small interfering RNA (siRNA). MET siRNA duplex was obtained from Validated Stealth RNAi siRNA systems (Oligo ID; HSS106479; Invitrogen). Negative control siRNA was obtained from Invitrogen. 22Rv1 cells were seeded into a 24-well plate, and transfections were carried out using Lipofectamine RNAi max (Invitrogen) in Opti-MEM according to the manufacturer's instructions.

Analysis of cell growth. Negative control cells and MET knocked-down 22Rv1 cells were each seeded into a six-well plate at 5×10^4 cells per well. After incubation for 24, 48 and 72 h, the cells were removed by trypsin and viable cell numbers were determined using Countess (Life Technologies).

Xenograft transplantation into NOD/SCID mice. Experiments using animals were done in accordance with the institutional guidelines for the use of laboratory animals. Bulk and MET knocked-down cells were re-suspended at concentrations of 1×10^3 cells in 100 μL of PBS and Matrigel (BD Biosciences) mixture (1:1). The cells were injected subcutaneously into the right and left mid-back areas of anesthetized 6-week-old male non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. The progression of cancer cell growth was monitored weekly, and mice underwent autopsy at 50 days after cell injection.

Statistical analysis. Data are presented as means ± SD from at least three independent experiments. Statistical analysis of the data was performed by Student's *t*-test. *P*-values of ≤ 0.05 were considered statistically significant.

Results

Preferential growth factor gene expressions in prostate cancer stem cells. We previously reported the successful isolation of prostate CSCs/CICs from the prostate carcinoma cell line 22Rv1 by the ALDEFLUOR assay as $ALDH^{high}$ cells, and we found that more than 200 genes were overexpressed in prostate CSCs/CICs compared with expression levels in non-CSCs/CICs.[3] The overexpressed genes included the growth factors *IGF1* and *HGF*. Activation of c-MET, the receptor for HGF, is related to a stem-like phenotype in prostate cancer cells,[7] and we therefore further analyzed the function of HGF in this study. Upregulation of the expression of *IGF1* and *HGF* was confirmed by RT-PCR analysis, whereas the expression levels of *MET* showed no significant difference in $ALDH^{high}$ and $ALDH^{low}$ cells (Fig. 1A). Hepatocyte growth factor protein expression level was higher in $ALDH1^{high}$ cells than in $ALDH1^{low}$ cells, whereas MET protein expression level were similar in $ALDH1^{high}$ cells and $ALDH1^{low}$ cells (Fig. 1B). Stem cell markers including SOX2, POU5F1 and NANOG were described to be overexpressed in prostate CSCs/CICs.[8] To address expressions of ALDH1 and HGF in stem cells of prostate cancer tissue, we performed double immunohistochemical staining using anti-ALDH1 antibody, anti-HGF antibody and anti-SOX2 antibody as a prostate CSC/CIC marker. Part of SOX2-positive prostate adenocarcinoma cells exhibited positive expression of ALDH1 and HGF (Fig. 1C). Thus ALDH1 and HGF proteins were expressed in prostate CSCs/CICs in primary prostate cancer tissue.

Prostate cancer stem cells secrete a growth factor, HGF. Hepatocyte growth factor protein secretion by $ALDH^{high}$ cells was investigated by ELISA. The mean HGF level in $ALDH^{high}$ cell culture medium was 7.499 pg/mL, whereas HGF level in $ALDH^{low}$ cell culture medium was 1.082 pg/mL (Fig. 1D), and the difference was statistically significant ($P < 0.05$).

Growth factors enhance prostasphere formation. Non-adherent sphere-forming assays are increasingly being used to evaluate stem cell phenotypes in normal tissues as well as putative CSCs/CICs. We previously reported that $ALDH1^{high}$ cells have higher sphere-forming ability than that of $ALDH1^{low}$ cells using EGF and bFGF.[3] In the present study, we found that growth factors (HGF and IGF1) are overexpressed in $ALDH1^{high}$ cells compared with the expression in $ALDH1^{low}$ cells, and we therefore investigated the effects of growth factors on sphere-forming ability of prostate carcinoma cells. Eight thousand prostate carcinoma cells were incubated in ultra-low attachment surface culture dishes in serum-free culture media with or without additional growth factors (IGF1 and HGF). The addition of HGF onto EGF and bFGF enhanced sphere formation, whereas the addition of IGF1 onto EGF and bFGF did not enhance sphere formation. The addition of both HGF and IGF1 resulted in maximum sphere formation of $ALDH1^{high}$ cells (Fig. 2A). Growth factors increased sphere formation of both $ALDH1^{high}$ and $ALDH1^{low}$ cells, and $ALDH1^{high}$ cells cultured with growth factors showed the highest sphere formation efficiency (Fig. 2B,C).

Since $ALDH1^{high}$ cells secrete HGF, we hypothesized that HGF secreted from $ALDH1^{high}$ cells has a role in sphere formation of $ALDH1^{high}$ cells in an autocrine fashion. We therefore added anti-HGF mAb to the sphere culture supplemented with only EGF and bFGF. The anti-HGF mAb significantly suppressed sphere formation of $ALDH1^{high}$ cells (Fig. 3).

Since HGF is secreted from mesenchymal cells, we hypothesized that HGF secreted from stromal cells also has a role in the maintenance of prostate CSCs/CICs. To address this question, myofibroblasts derived from the prostate were cultured, and the culture supernatant was added to $ALDH1^{high}$ cells. The myofibloblast supernatant increased the sphere formation of $ALDH1^{high}$ cells, which was inhibited by anti-HGF mAb (Fig. 4). These results indicate that HGF derived from both $ALDH1^{high}$ cells and stromal cells has a role in the maintenance of prostate CSCs/CICs.

c-MET has a role in the maintenance of prostate cancer stem cells both *in vitro* and *in vivo*. To confirm the roles of HGF in

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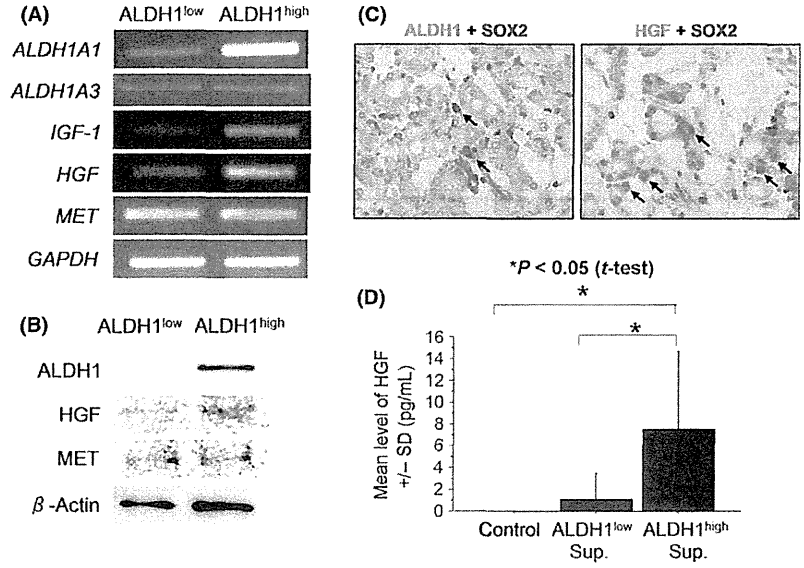

Fig. 1. Expression of growth factors and receptors in prostate cancer stem-like cells (CSCs)/cancer-initiating cells (CICs). (A) Reverse transcription-polymerase chain reaction (RT-PCR) of *HGF*, *IGF1* and *MET* in ALDH1^{high} cells and ALDH1^{low} cells. *HGF*, *IGF1* and *MET* mRNA expression was evaluated by RT-PCR. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as a positive control. (B) Western blot analysis of ALDH1, hepatocyte growth factor (HGF) and c-MET. Western blot analysis using 5×10^4 ALDH1^{high} cells and ALDH1^{low} cells was performed. Anti-β-Actin was used as an internal control. (C) ALDH1 and HGF expression in primary prostate CSCs/CICs. Prostate adenocarcinoma tissue was stained by anti-SOX2 and anti-ALDH1 (left panel) and anti-SOX2 and anti-HGF (right panel). Nucleus brown staining was judged as positive staining for SOX2, and cytoplasm red staining was judged as positive staining for ALDH1 and HGF. Arrows indicate SOX2 and ALDH1 double positive cells and SOX2 and HGF double positive cells. Magnification, ×400. (D) Hepatocyte growth factor secretion by ALDH1^{high} cells and ALDH1^{low} cells. Isolated ALDH1^{high} cells and ALDH1^{low} cells were incubated in a 96-well plate for 2 days. Then HGF was evaluated by enzyme linked immunosorbent assay (ELISA). Control represents only culture medium. Asterisks represents statistically significant difference ($P < 0.05$, *t*-test).

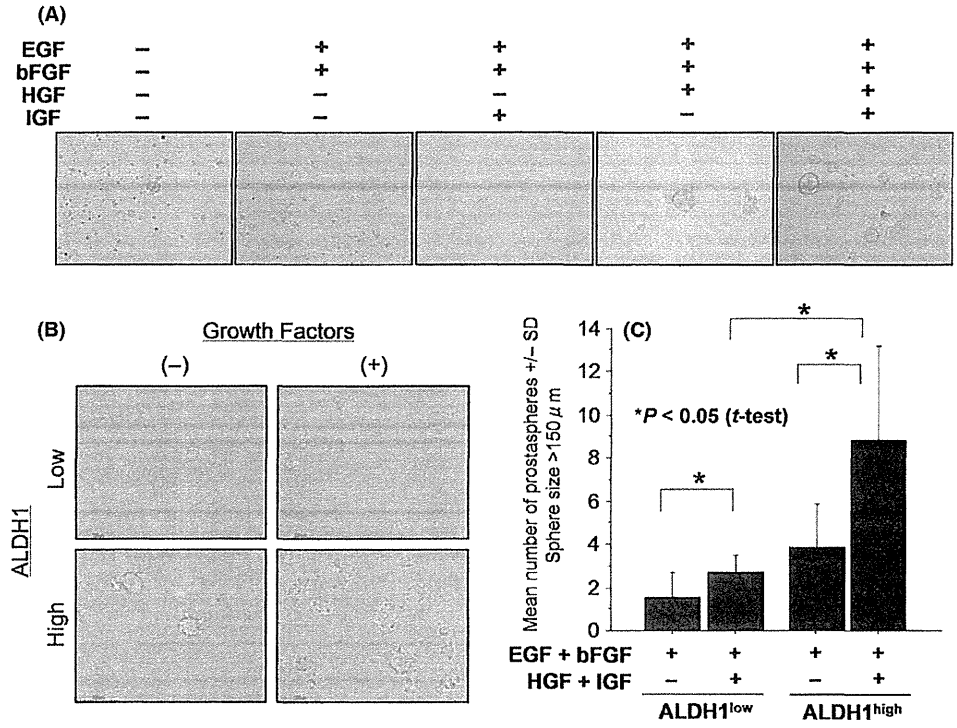

Fig. 2. Growth factors enhance prostasphere formation *in vitro*. (A) Prostasphere formation was increased by hepatocyte growth factor (HGF). Prostasphere formation was performed in serum-free Dulbecco's modified eagle medium (DMEM)/F12 media. Growth factors were added to the prostasphere culture. Representative pictures of prostaspheres are shown. Magnification, ×100. (B) Representative pictures of prostaspheres formed by ALDH1^{high} cells and ALDH1^{low} cells. ALDH1^{high} cells and ALDH1^{low} cells were cultured with or without HGF and IGF1. Representative pictures are shown. Magnification ×100. (C) Prostasphere formation of ALDH1^{high} cells and ALDH1^{low} cells with or without growth factors (HGF and IGF1). ALDH1^{high} cells and ALDH1^{low} cells were cultured in serum-free media with or without HGF and IGF1. The numbers of prostaspheres were evaluated at day 3. Asterisks represents statistically significant difference ($P < 0.05$, *t*-test).

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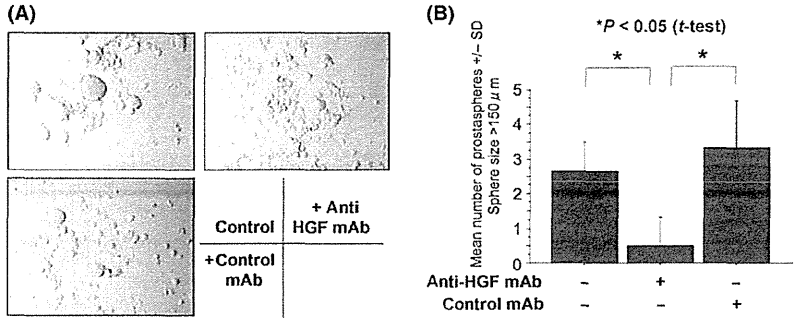

Fig. 3. Prostasphere formation was inhibited by anti-hepatocyte growth factor (HGF) antibody. ALDH1^{high} cells were cultured in Dulbecco's modified eagle medium (DMEM)/F12 medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Anti-HGF antibody and control antibody were added to the culture. Representative pictures are shown (A). Magnification, ×100. The numbers of prostaspheres were evaluated at day 3 (B). Asterisks represents statistically significant difference ($P < 0.05$, *t*-test).

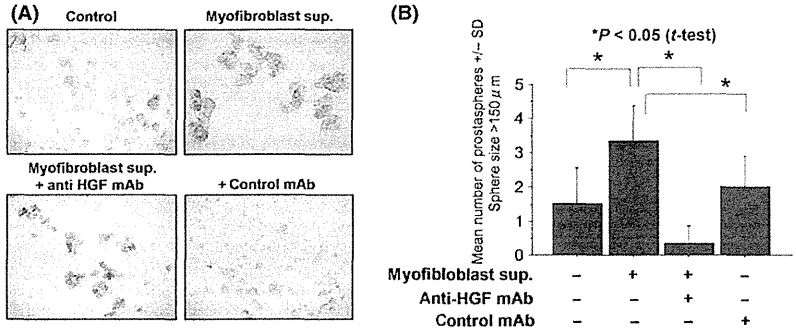

Fig. 4. Prostasphere formation was increased by supernatant of prostate myofibroblasts. ALDH1^{high} cells were cultured in Dulbecco's modified eagle medium (DMEM)/F12 medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Fifty percent (v/v) of myofibroblast culture supernatant was added to culture. Anti-HGF antibody and control antibody were added to the culture. Representative pictures are shown (A). Magnification, ×100. The numbers of prostaspheres were evaluated at day 3 (B). Asterisks represents statistically significant difference ($P < 0.05$, *t*-test).

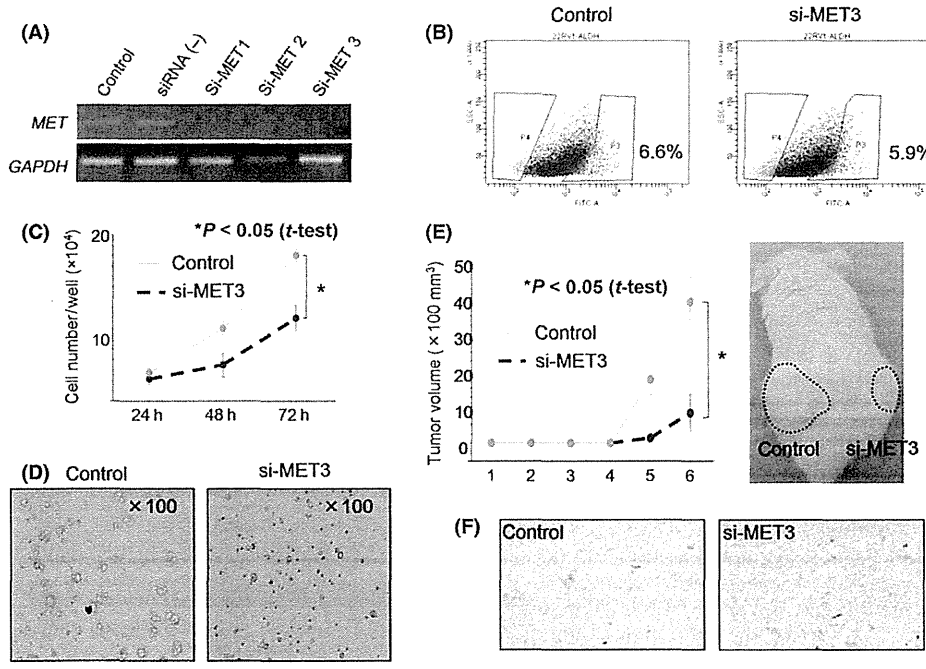

Fig. 5. c-MET has a role in the maintenance of prostate cancer stem-like cells (CSCs)/cancer-initiating cells (CICs). (A) Reverse transcription-polymerase chain reaction (RT-PCR) of MET knockdown cells. MET-specific siRNA and control siRNA were transfected into 22Rv1 cells. Two days after transfection, total RNA was purified and analyzed by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. (B) ALDEFLUOR assay of MET knockdown cells. MET siRNA3- and control siRNA-transfected 22Rv1 cells were analyzed by ALDEFLUOR assay. Percentages indicate rates of ALDH1^{high} cells. (C) MET gene knockdown inhibited cell growth *in vitro*. MET siRNA3- and control siRNA-transfected 22Rv1 cells were seeded into a six-well plate, and cell numbers were counted at 24, 48 and 72 h. An asterisk represents a statistically significant difference ($P < 0.05$, *t*-test). (D) MET gene knockdown inhibited prostasphere formation *in vitro*. MET siRNA3- and control siRNA-transfected 22Rv1 cells were seeded into an Ultra-Low Attachment plate and cultured for 3 days. (E) MET gene knockdown inhibited tumor initiation *in vivo*. MET siRNA3- and control siRNA-transfected 22Rv1 cells were transplanted into male NOD/SCID mice subcutaneously. Tumor growth was measured every week. Tumor growth curve and a representative picture are shown. An asterisk represents a statistically significant difference ($P < 0.05$, *t*-test). (F) ALDH1 expression in MET knockdown tumor. ALDH1 expression was investigated by immunohistochemical staining. Representative pictures are shown. Magnification, ×100.

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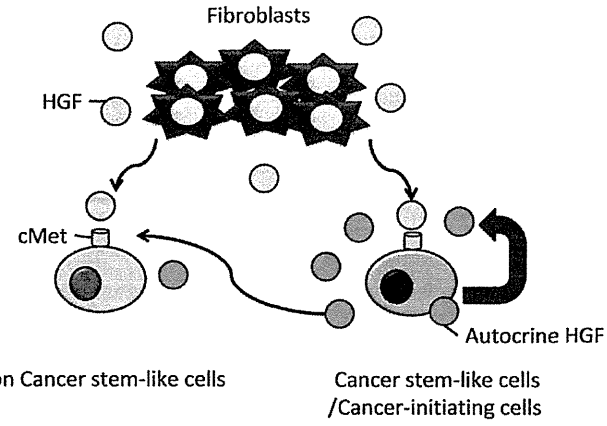

Fig. 6. Model of the maintenance of prostate cancer stem-like cells (CSCs)/cancer-initiating cells (CICs)through hepatocyte growth factor (HGF)/c-MET signaling.

cell growth and tumorigenicity, we performed gene-knockdown studies using siRNA of c-MET, the HGF receptor (Fig. 5A). Gene knockdown of c-MET mRNA did not affect the rate of $ALDH1^{high}$ cells, whereas it suppressed cell growth *in vitro* and sphere formation (Fig. 5B–D).

To evaluate the role of HGF in tumorigenicity, 1×10^3 negative control and siRNA-transfected 22Rv1 cells were injected into the backs of NOD/SCID mice ($n = 4$). The tumors derived from siRNA-transfected 22Rv1 cells were significantly smaller than those derived from negative control cells ($P < 0.05$, Fig. 5E), whereas ALDH1 expression was not suppressed by c-MET siRNA (Fig. 5F). These results indicate that MET signaling has a role in the maintenance of prostate CSCs/CICs, whereas it has no role in the expression of ALDH1.

Discussion

Hepatocyte growth factor is a paracrine factor produced by cells of mesenchymal origin, while the HGF receptor, c-MET, is expressed by epithelial and endothelial cells.[9] Hepatocyte growth factor is a heterodimeric protein comprised of a 55–60-kDa α chain and a 32–34-kDa β chain linked by a single disulfide bond. c-MET is a tyrosine kinase receptor with a single transmembrane spanning region and a conserved tyrosine kinase domain. c-MET is translated as a single polypeptide chain that is proteolytically cleaved to form an approximately 145-kDa β heavy chain and an approximately 35-kDa α light chain linked by a single disulfide bond. Signal transduction by HGF leads to a variety of biological responses including proliferation, migration and morphogenesis. Hepatocyte growth factor plays important roles in the progression of many invasive and metastatic cancers.[10] The interaction between tumor cells and their surrounding stromal environment remains a crucial factor governing tumor invasion and metastasis.

c-MET receptor tyrosine kinase had been shown to be involved in tumor proliferation and progression, and overexpression of c-MET has been shown to be associated with advanced prostate cancer.[11–15] These findings suggested that the strategy of inhibiting the activation of both HGF/c-MET and androgen receptor (AR) signaling pathways should be considered in the treatment of advanced prostate cancer. It has recently been reported that c-MET inhibitors demonstrated anti-proliferative efficacy when combined with androgen ablation therapy for advanced prostate cancer.[16]

It has been reported that c-MET was overexpressed in immature DU145 prostate carcinoma cells and that c-MET signaling has a role in induction of a stem-like cell phenotype.[5] On the other hand, c-MET was expressed in both $ALDH1^{high}$ cells and $ALDH1^{low}$ cells derived from 22Rv1 prostate carcinoma cells at similar levels, whereas its ligand HGF was overexpressed in $ALDH1^{high}$ cells.[3] $ALDH1^{high}$ cells secrete a larger amount of HGF than do $ALDH1^{low}$ cells, and HGF together with IGF1 enhanced the efficiency of sphere formation *in vitro*. Gene knockdown of c-MET inhibited cell growth *in vitro* and abrogated the sphere-forming ability and tumor-initiating ability in NOD/SCID mice. Therefore, our results indicate that activation of c-MET signaling has a role in the maintenance of prostate CSCs/CICs and that c-MET activation might be caused by HGF secretion from prostate CSCs/CICs, not by overexpression of c-MET.

Hepatocyte growth factor/c-MET signaling is related to epithelial-mesenchymal transition (EMT), anoikis and dissemination in gastric carcinoma cells, and high expression levels of HGF and c-MET in gastric carcinoma specimens are related to poor prognosis.[17] In colon carcinoma cells, HGF secreted by stromal fibroblasts is required for nuclear localization of β-catenin, which is essential for maintenance of colon CSCs/CICs.[18] Cancer-associated fibroblasts (CAFs) are a candidate of HGF secretion, and we actually confirmed that the culture supernatant of myofibroblasts derived from the prostate enhanced prostasphere formation. Therefore, we hypothesize that both prostate CSCs/CICs and fibroblasts secrete HGF and that HGF has a role in maintenance of prostate CSCs/CICs in autocrine and paracrine fashions. Hepatocyte growth factor may also enable non-CSCs/CICs to obtain malignant phenotypes including stemness in a paracrine fashion through activating the Wnt/β-catenin signal (Fig. 6).

In summary, HGF/c-MET signaling has a major role in the maintenance of prostate CSCs/CICs. Prostate CSCs/CICs secrete HGF, and HGF may stimulate c-MET expressed on prostate CSCs/CICs in an autocrine fashion. Blocking HGF/c-MET autocrine activation might be a promising approach to target prostate CSCs/CICs.

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Disclosure Statement

The authors have no conflict of interest.

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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.

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

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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

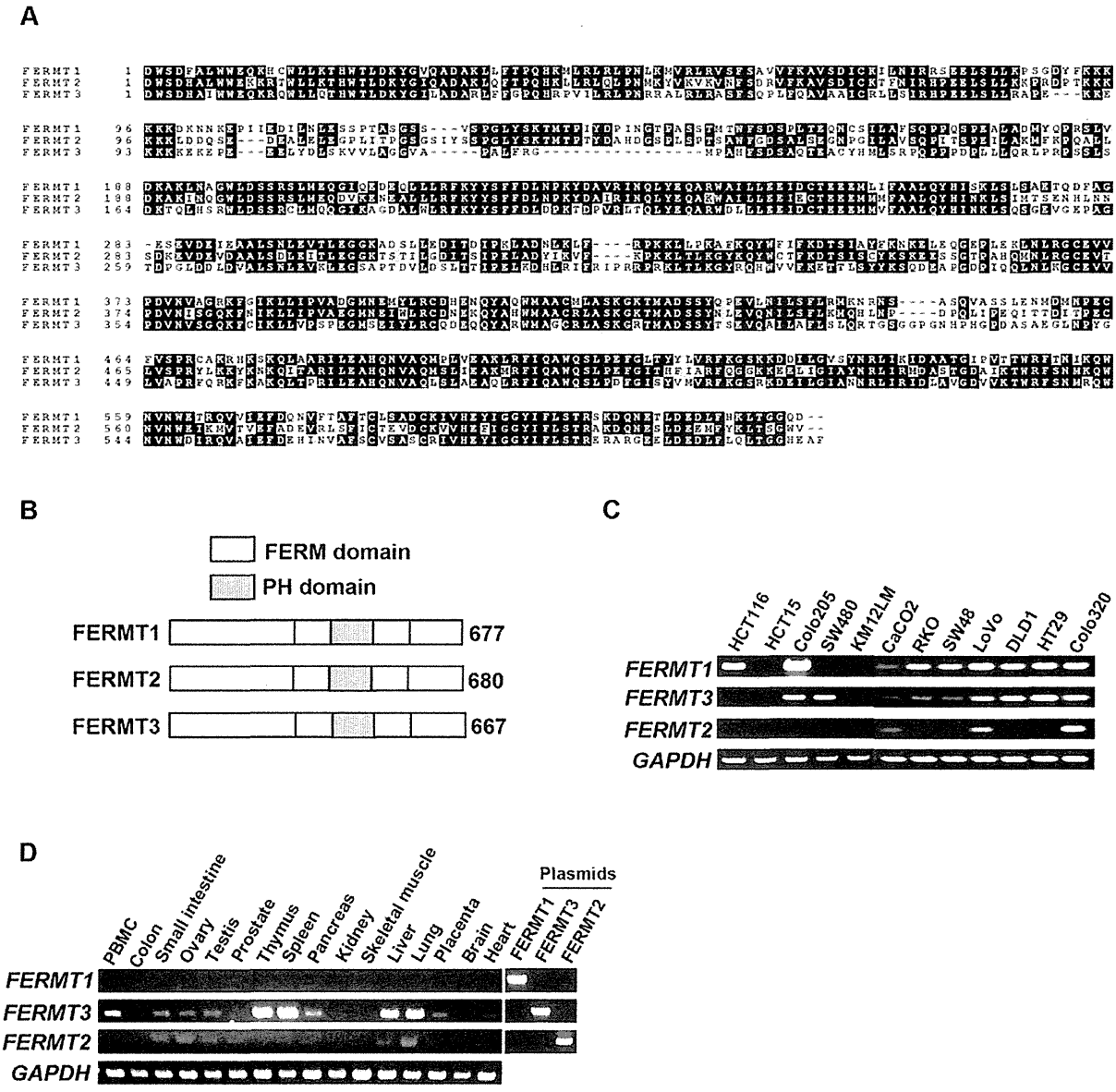

Figure 1. *Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins. 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

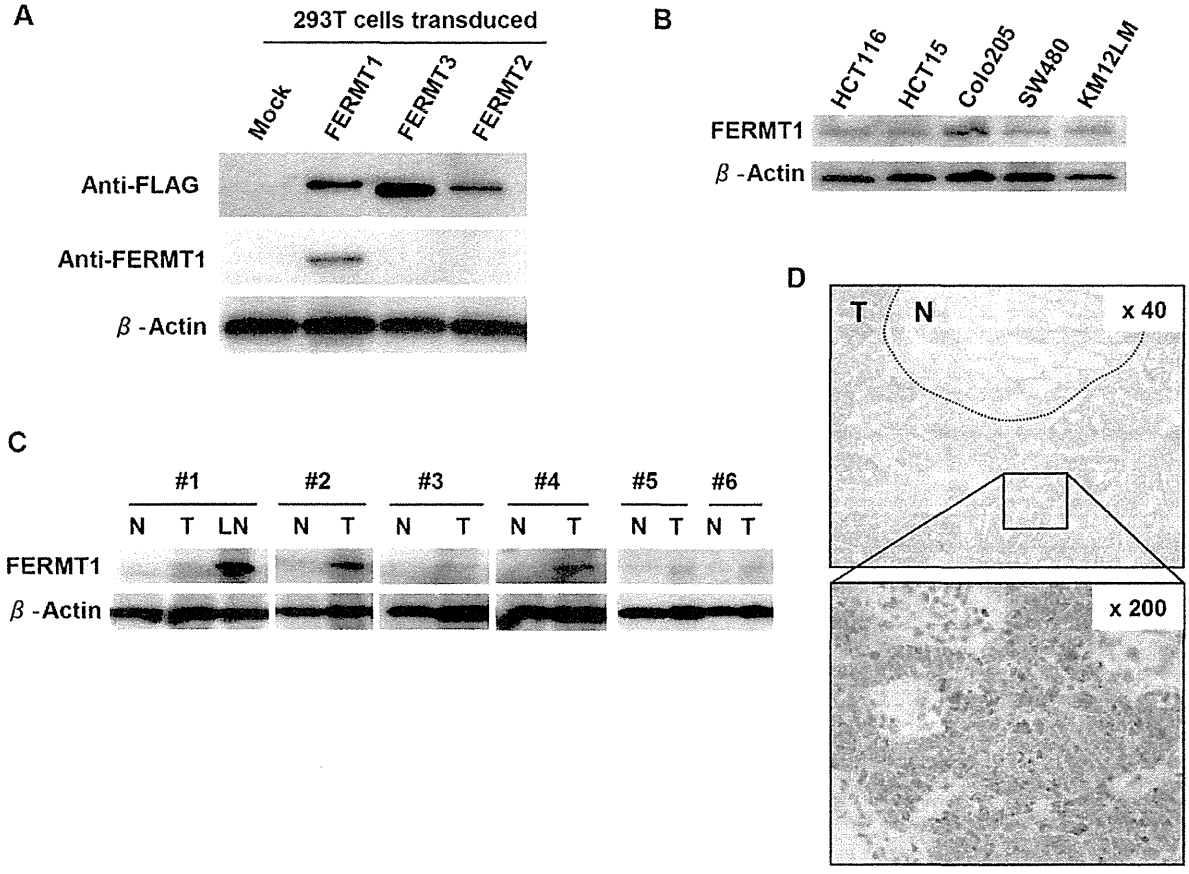

Figure 2. *Fermitin family member 1 (FERMT1) protein expression in colonic carcinomas. A: Western blotting using monoclonal antibody (mAb) against FERMT1. 293T cells were transfected with FERMT1, FERMT2 and FERMT3 plasmids. Western blotting using anti-FLAG mAb and anti-FERMT1 mAb was performed. Anti-FLAG 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.

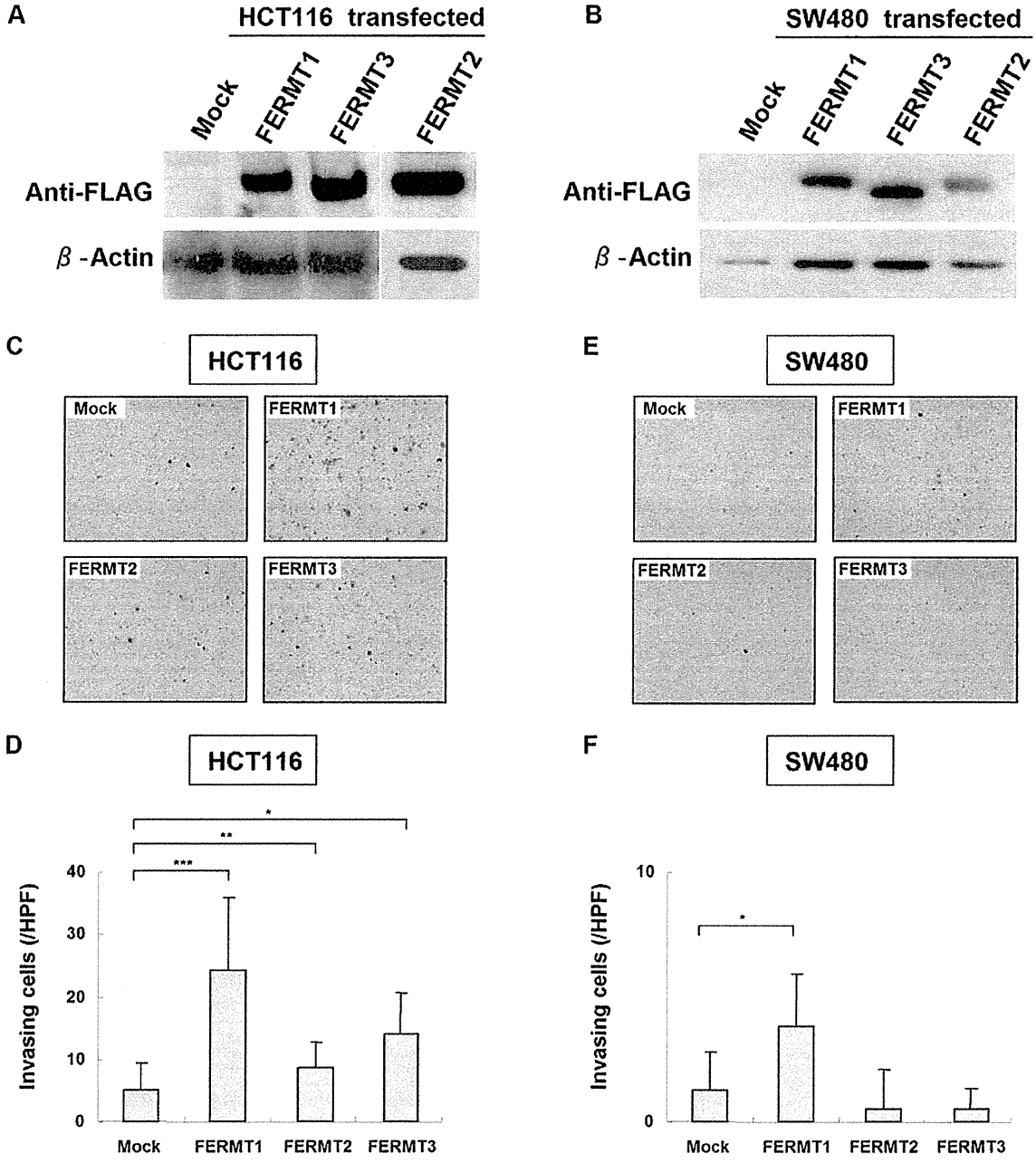

Figure 3. *Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control. 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.*

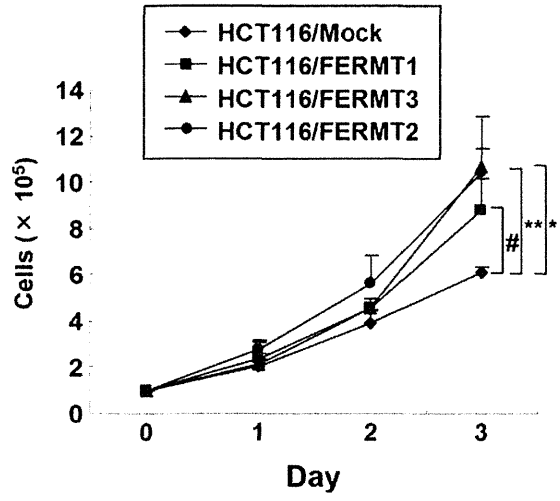

Figure 4. *Cell growth of FERMT famliy-overexpressing HCT116 cells. FERMT family cDNA-overexpressing HCT116 cells were seeded in a 6-well plate, and the cell growth rate was recorded daily. Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116 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.