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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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'-ACTGGATTCTTCTTTGCTCTT-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

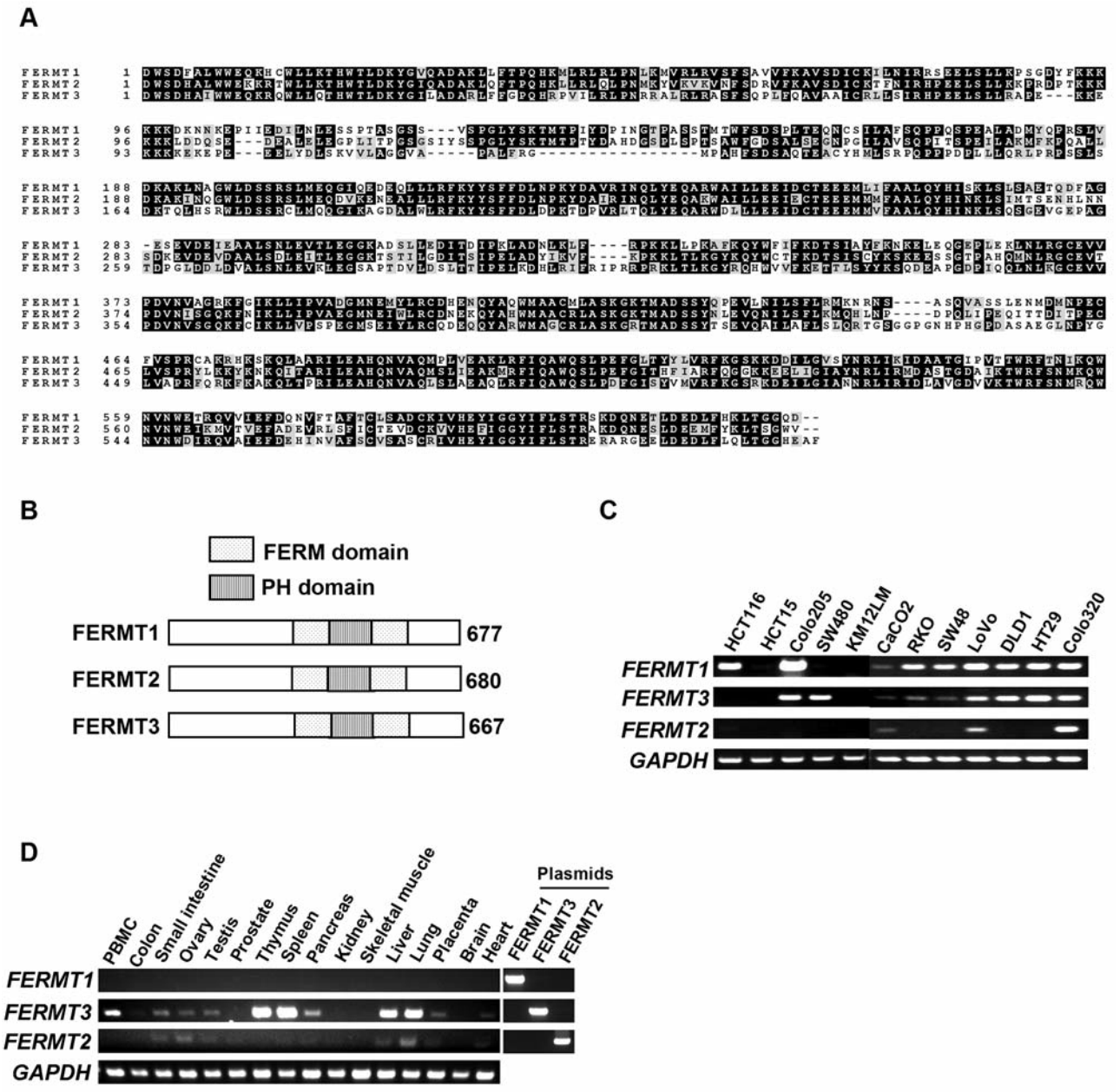

Figure 1. *Expression profiles of fermitin family member (FERMT) family genes. A: Sequence alignment of FERMT proteins. FERMT1, FERMT2 and FERMT3 amino acid sequences are shown. A black box indicates the same alignment, a gray box indicates similar alignment. B: Molecular structure of FERMT family proteins.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

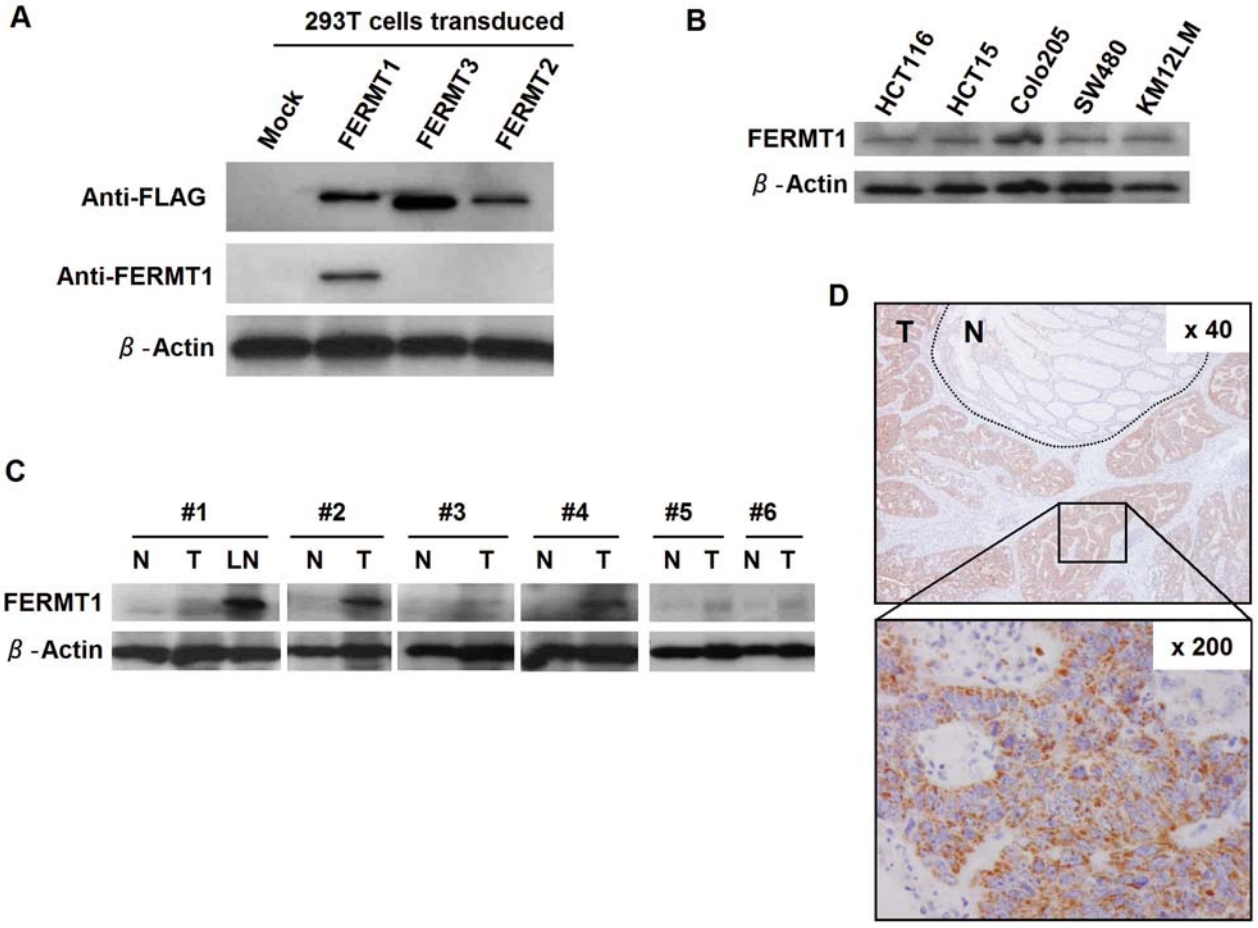

Figure 2. *Fermitin family member 1 (FERMT1) protein expression in colonic carcinomas. A: Western blotting using monoclonal antibody (mAb) against FERMT1. 293T cells were transfected with FERMT1, FERMT2 and FERMT3 plasmids. Western blotting using anti-FLAG mAb and anti-FERMT1 mAb was performed. Anti-FLAG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

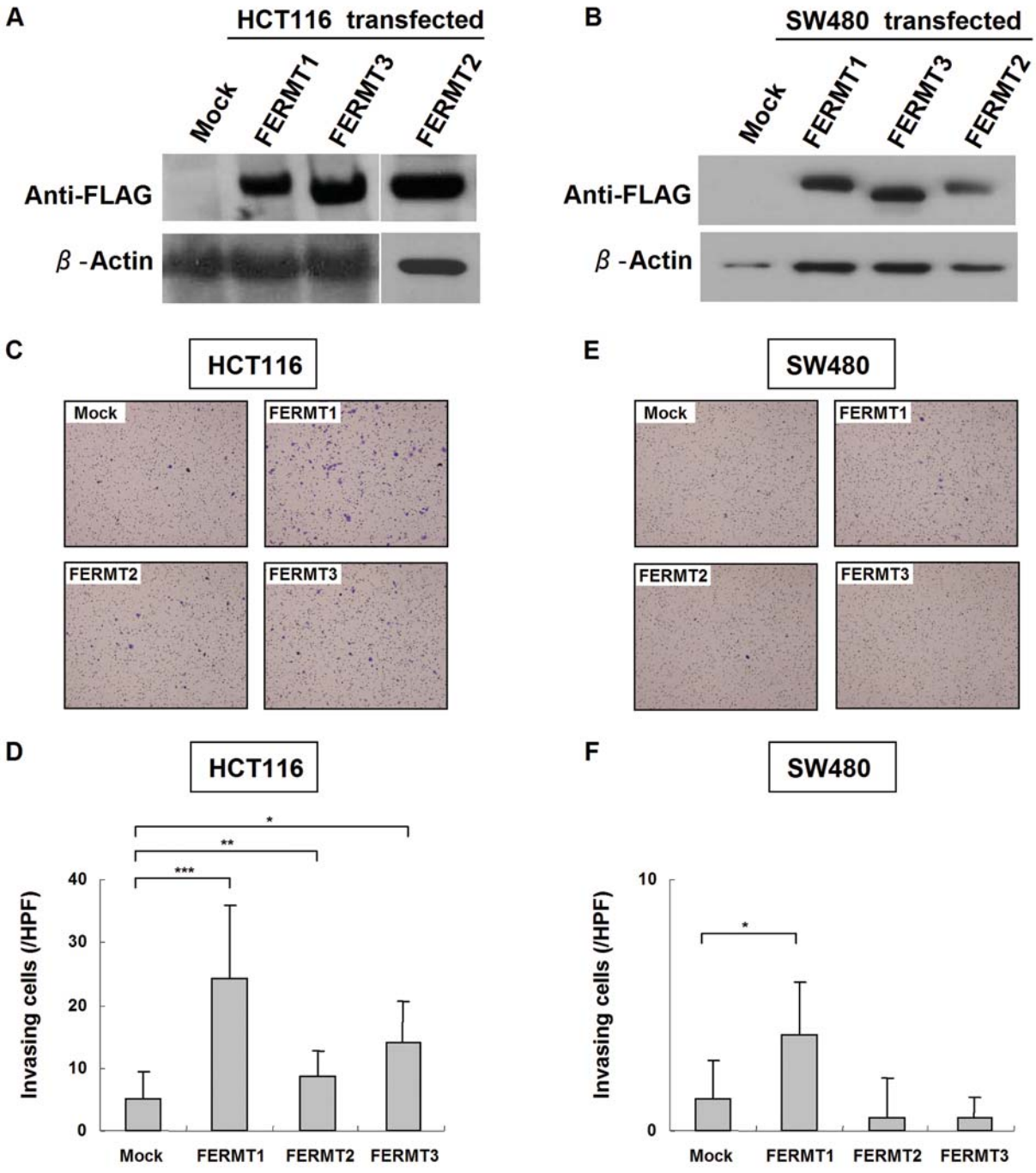

Figure 3. *Molecular function of FERMT1 in colon carcinoma cells. A: Western blotting using monoclonal antibody (mAb) to FLAG-tag. HCT116 cells were transfected with FREMT1, FERMT3, FERMT2 plasmids, and analyzed by western blot using mAb to FLAG-tag. β-Actin was used as an internal positive control.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

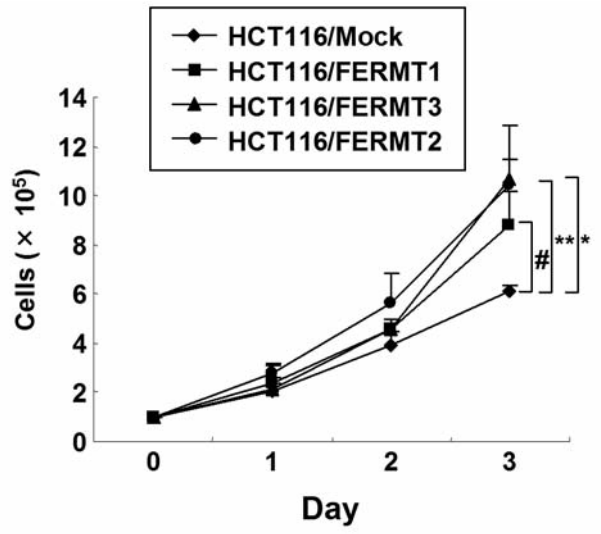

Figure 4. *Cell growth of FERMT famliy-overexpressing HCT116 cells. FERMT family cDNA-overexpressing HCT116 cells were seeded in a 6-well plate, and the cell growth rate was recorded daily. Data represent means±SD. Differences between FERMT family-overexpressing HCT116 cells and mock-transfected HCT116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

Declaration of Financial Disclosure

Hideo Takasu is an employee of Dainippon Sumitomo Pharma Co., Ltd.

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