ORIGINAL RESEARCH

A peptide-CpG-DNA-liposome complex vaccine targeting TM4SF5 suppresses growth of pancreatic cancer in a mouse allograft model

Sangkyu Park[1,2,*]
Dongbum Kim[3,*]
Guang Wu[3,4]
Harry Jung[3]
Jeong-A Park[1,2]
Hyung-Joo Kwon[3,5]
Younghee Lee[1,2]

[1]Department of Biochemistry, College of Natural Sciences, Chungbuk National University, Cheongju, Chungbuk 28644, Republic of Korea; [2]Biotechnology Research Institute, Chungbuk National University, Cheongju, Chungbuk 28644, Republic of Korea; [3]Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 24252, Republic of Korea; [4]School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China; [5]Department of Microbiology, College of Medicine, Hallym University, Chuncheon 24252, Republic of Korea

*These authors contributed equally to this work

Correspondence: Younghee Lee
Department of Biochemistry, College of Natural Sciences, Chungbuk National University, 1, Chungdae-ro, Cheongju, Chungbuk 28644, Republic of Korea
Tel +82 43 261 3387
Fax +82 43 267 2306
Email yhl4177@cbnu.ac.kr

Hyung-Joo Kwon
Department of Microbiology, College of Medicine, Hallym University, 1 Hallymdaehak-gil, Chuncheon, Gangwon-do 24252, Republic of Korea
Tel +82 33 248 2635
Fax +82 33 241 3640
Email hjookwon@hallym.ac.kr

Background: Patients with pancreatic cancer have a poor prognosis and are usually diagnosed at a late stage. Because TM4SF5 is known to be overexpressed in hepatocellular carcinoma, colon cancer, and pancreatic cancer, it is considered as one of the candidate molecular targets for an anticancer strategies.

Purpose: The purpose of this study was to evaluate possible utility of TM4SF5 to treat pancreatic cancer using a mouse allograft model.

Materials and methods: We analyzed expression of TM4SF5 in pancreatic cancer tissues using immunohistochemistry. We established a mouse pancreatic cancer cell line stably expressing TM4SF5 and identified the effect of TM4SF5 expression in vitro. We used the CpG-DNA-peptide-liposome complex as a peptide vaccine and investigated antitumor effects of the vaccine in a mouse model with TM4SF5 expressing pancreatic cells. To investigate the function of produced antibody, we evaluated effects of the anti-TM4SF5 monoclonal antibody in vitro in terms of cell growth and migration properties.

Results: Immunohistochemical analysis showed that 36.4% of pancreatic cancer tissue samples expressed TM4SF5. Expression of TM4SF5 induced increased cell proliferation and motility in vitro. Injection of the TM4SF5 peptide vaccine induced the production of anti-hTM4SF5 antibodies and reduced the growth of pancreatic tumors in mice established by subcutaneous injection of the TM4SF5-expressing mouse pancreatic cancer cell line. The treatment of TM4SF5-expressing cells with the anti-hTM4SF5 monoclonal antibody reduced cell growth, modulated the expression of the epithelial–mesenchymal transition markers Vimentin and E-cadherin, and decreased cell motility in vitro.

Conclusion: Our results showed that the TM4SF5 peptide vaccine had a protective effect against pancreatic tumors expressing TM4SF5, and this effect was mediated, at least in part, by the production and suppressive function of the anti-TM4SF5 antibodies. Therefore, we suggest that targeting TM4SF5 could be a novel strategy to prevent or treat pancreatic cancer.

Keywords: transmembrane 4 superfamily member 5 protein, pancreatic cancer, peptide vaccine, antibody, targeting

Introduction

Pancreatic cancer has a poor prognosis, probably because it is diagnosed at an advanced stage in many cases. It is the eighth and ninth leading cause of death from cancer in men and women respectively throughout the world.[1] Approximately 90% of pancreatic cancers are pancreatic ductal adenocarcinoma (PDAC).[1] In pancreatic cancer, four genes are frequently mutated: KRAS, CDKN2A, SMAD4, and TP53.[2] KRAS is mutated in ~95% of PDAC patients, and the KRAS mutation triggers the genetic

progression of PDAC and drives PDAC development and maintenance.[3–5] However, despite a long period of attempts, anti-KRAS therapy has yet to be proven effective.[3] Recently, clinical trials based on therapeutic strategies targeting specific proteins and/or pathways have shown significant advances in many tumor types. However, in pancreatic cancer, these trials were proven not successful, and pancreatic cancer remains a lethal disease.[6–8]

Therapeutic strategies for cancer include surgery, chemotherapy, radiation therapy, immunotherapy, targeted therapy, etc. Peptide vaccine therapy is based on the stimulation of the immune system by immunization with peptides from defined tumor-associated antigens (TAAs) usually in combination with immune stimulators (including adjuvants).[9] Since MAGE-1 was identified as the first reported human TAA gene,[10] several other TAA genes have been identified and studied as targets of therapeutic peptide vaccines. These TAAs may be classified into several categories. Cancer/testis antigens are expressed in testis germ cells but not in adult somatic tissues, and differentiation antigens are specific for lineages or differentiation stage of various cell types and other tumor antigens.[9,11] Many clinical trials and research studies on peptide vaccines for cancer immunotherapy are currently exploring the efficacy of peptides that act as antigens in anticancer vaccines.[12–19]

A liposome is a vesicle used to deliver drugs or genetic materials into a cell.[20,21] Especially, cationic liposomes have advantages to efficiently bind with negatively charged DNA and cell membranes.[22] Non-cationic lipids are generally used as helpers for cationic liposomes. For example, dioleoyl phosphatidylethanolamine (DOPE) increases the endosomal escape ability of DNA after endocytosis of cationic liposome/DNA complex.[23,24] In a pancreatic cancer model, recent studies reported the use of a liposome complex to deliver shRNAs or anti-miRNA oligonucleotides for therapeutic purposes.[25,26] Liposomes are also used for efficient antigen delivery and enhanced immune response in developing vaccines.[27–29] Previously, we confirmed that natural phosphodiester bond CpG-DNA (specifically, MB-ODN 4531(O)) induced innate immune responses.[30] Immune stimulating activity of CpG-DNA was enhanced by encapsulation in a liposome complex consisting of DOPE: cholesterol hemisuccinate (CHEMS) (1:1 ratio). We named CpG-DNA co-encapsulated with DOPE:CHEMS "Lipoplex(O)".[31,32] Liposome complex consisting of B cell epitope and Lipoplex(O) successfully induced antibodies specifically recognizing viral proteins and cancer-associated proteins.[31,32]

TM4SF5, a membrane protein with four transmembrane domains, was first identified in 1998, and high expression of TM4SF5 was reported in hepatocellular carcinoma (HCC), colon cancer, soft tissue sarcoma, gastric cancer, esophageal cancer, and pancreatic cancer.[33–35] TM4SF5 expression enhances the motility and malignancy of hepatocytes by increased interaction with CD151, and TM4SF5 expression level is associated with cancer progression and poor survival of patients.[35–37] TM4SF5 regulates VEGF-mediated angiogenesis through cooperation with integrin and tumorigenic proliferation.[34,38] TM4SF5 reduces the RhoA activity by p27kip1 translocation and FAK phosphorylation, and RhoA inactivation induces the epithelial–mesenchymal transition (EMT) and G1/S phase progression.[39–42] TM4SF5-induced FAK phosphorylation promotes immune escape in HCC.[43] TM4SF5-induced growth and motility can be blocked by a synthetic compound 4′-(p-toluenesulfonyl-amide)-4-hydroxychalcone.[44] In our previous studies, we reported that immunization with the TM4SF5 peptide epitope-CpG-DNA-liposome complex is effective in the prevention and therapy of HCC and colon cancer.[45–47] We also confirmed that the TM4SF5-targeted monoclonal antibody inhibits the growth and metastasis of HCC and colon cancer.[39,48,49]

Considering that *TM4SF5* is also highly expressed in pancreatic cancer tissue,[33] it is likely that the TM4SF5 peptide vaccine may have preventive or therapeutic effects on pancreatic cancer. In this study, we established mouse pancreatic cancer cells expressing TM4SF5 and confirmed the preventive effects of a vaccination with the TM4SF5 peptide-CpG-DNA-liposome complex on TM4SF5-expressing mouse pancreatic tumors in a mouse allograft model.

Materials and methods
Tissue microarrays and immunohistochemistry
The formalin-fixed, paraffin-embedded AccuMax tissue array was obtained from ISUABXIS with the approval of the Institutional Review Board in Hallym University (approval number: HIRB-2014-114). The tissue array was analyzed by immunohistochemistry using the mouse anti-TM4SF5 monoclonal antibody[49] (mEC2-C, 1 µg/slide) and Histostain Plus kit (Thermo Fisher Scientific, Waltham, MA, USA) as previously described.[48] All images were examined using a Nikon Eclipse E-200 microscope. The percentages of cells expressing TM4SF5 were calculated as the number of TM4SF5-positive cells divided by the total number of cells in each tumor type.

Cell culture

The mouse PDAC cell line PANC02 was provided by Professor Kyu Lim (Chungnam National University, Republic of Korea).[50] The cells were maintained in DMEM (Hyclone, Logan, UT, USA) with 10% FBS (Hyclone), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C under a humidified atmosphere of 5% CO_2. The use of the cell line was approved by the Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym 2015-55).

RT-PCR

Total RNA was isolated with TRI Reagent® according to the manufacturer's instructions (MRC, Cincinnati, OH, USA). Then, 2 µg of total RNA was reverse-transcribed in the first-strand synthesis buffer containing 6 µg/mL oligo(dT) primer, 50 U M-MLV reverse transcriptase, 2 mM dNTP, 10 mM DTT, and 40 U RNaseOUT™ recombinant ribonuclease inhibitor (Thermo Fisher Scientific). The reaction was done at 37°C for 50 minutes and heat inactivated at 70°C for 15 minutes. One microliter of synthetic cDNA was subjected to a standard PCR reaction of 25 or 30 cycles consisting of denaturation for 40 seconds at 95°C, annealing for 40 seconds at 58°C, and extension for 40 seconds at 72°C. The primer sequences used were as follows: GAPDH, 5′-TCC ACC ACC CTG TTG CTG TA-3′ (sense) and 5′-ACC ACA GTC CAT GCC ATC AC-3′ (anti-sense) (product size 452 bp); human TM4SF5, 5′-AGC TTG CAA GTC TGG CTC AT-3′ (sense) and 5′-GCT GGA TCC CAC ACA GTA CT-3′ (anti-sense) (product size 401 bp); mouse TM4SF5, 5′-CGC TTA CTT GCG AAA TGA CA-3′ (sense) and 5′-TTT CCT GCA ATC GCC ACA CA-3′ (anti-sense) (product size 174 bp).

Packaging and transduction of control and TM4SF5-encoding retroviruses

The human TM4SF5 cDNA was amplified from pcDNA3.1-hTM4SF5[49] by PCR using the following primer set: hTM4SF5 5′ primer, 5′-GAA TTC GCC ACC ATG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG GGT GCA ATG TGT ACG GGA AAA-3′ and hTM4SF5 3′ primer, 5′-CTC GAG TCA GTG AGG TGT GTC CTG-3′. The cDNA fragments were cloned into the expression vector pLXSN (Clontech, Mountain View, CA, USA) using the Xho I and EcoR I sites. GP2-293, a cell line derived from 293 cells, was obtained from Clontech and used as a packaging cell line for preparation of the retroviruses. GP2-293 cells were maintained in DMEM containing 10% FBS

in 5% CO_2 incubator at 37°C. Retroviral vectors pLXSN or pLXSN-hTM4SF5 along with pVSV-G (Clontech) encoding the pseudo-envelope protein gene were transfected into the cells by Lipofectamine 2000 (Thermo Fisher Scientific). Twelve hours later, the medium was exchanged with fresh culture medium supplemented with 10 mM sodium butyrate (Sigma-Aldrich Co., St Louis, MO, USA). After 48 hours, the supernatant of the culture medium was taken and filtered through a filter with a 0.45 µm pore size. The retrovirus supernatants were concentrated using Centricon centrifugal filters (EMD Millipore, Billerica, MA, USA) and stored at −80°C. The viral supernatant was applied to PANC02 cells along with 8 µg/mL of polybrene (Sigma-Aldrich Co.). Twenty-four hours later, G418 (Sigma-Aldrich Co.) was added at a concentration of 1 mg/mL, and the G418-resistant PANC02 cells were selected.

Western blot analysis

Harvested cells were lysed in a lysis buffer (pH 8.0, 20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 10 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, protease inhibitor cocktail, and phosphatase inhibitor). Proteins were resolved by SDS-PAGE and electro-transferred to polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% dry milk in PBS-Tween-20 ([PBST] 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.05% Tween-20) and probed with an appropriate primary antibody. The monoclonal anti-GAPDH antibody (cat no SC-32233, 1:1,000 dilution) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The anti-E-cadherin (cat no 3195, 1:1,000 dilution) and anti-Vimentin (cat no 5741, 1:1,000 dilution) polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Immunoreactive proteins were visualized by horseradish peroxidase (HRP)-conjugated anti-rabbit (cat no SC-2004) or anti-mouse (cat no SC-2005) secondary antibodies (1:5,000 dilution, Santa Cruz Biotechnology Inc.) and an enhanced chemiluminescence solution (ATTO, Tokyo, Japan).

Immunoprecipitation

Whole cell lysates were lysed in a lysis buffer. After the humanized anti-hTM4SF5 monoclonal antibody, hEC2-C-2,[49] was conjugated to protein A-agarose beads (Hoffman-La Roche Ltd., Basel, Switzerland), the whole cell lysates were immunoprecipitated with the antibody-conjugated agarose beads. Immunoprecipitated proteins were washed with PBS and processed for a standard Western blot analysis using the mouse

anti-hTM4SF5 monoclonal antibody (mEC2-CF, 1 µg/mL), which we previously reported,[51] and anti-Myc antibody (Cell Signaling Technology, cat no 2276, 1:1,000 dilution) which recognizes Myc-tag in the recombinant hTM4SF5.

Confocal microscopy

Cells were cultured on glass cover slips in 4-well plates (Thermo Fisher Scientific). The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Co.), and stained with humanized anti-hTM4SF5 monoclonal antibody (1 µg/mL) for 1 hour.[49] After extensive washing with PBS, the samples were incubated with Alexa Fluor 488-conjugated goat anti-human IgG (2 µg/mL, Thermo Fisher Scientific, cat no A11013) for 1 hour. The nuclei were stained with Hoechst 33258 (Sigma-Aldrich Co.), and the mounted samples were scanned with an LSM 710 (Carl Zeiss Meditec AG, Jena, Germany).

Cell proliferation assay

The cell proliferation ELISA, BrdU colorimetric kit (Hoffman-La Roche Ltd.), was used to measure the cell proliferation according to the manufacturer's instructions. Cells were treated with normal IgG or humanized anti-hTM4SF5 monoclonal antibody (10 µg/mL) for 3 days. The BrdU solution was added to each well, and then, the plates were incubated for 4 hours at 37°C. After fixation of the cells, anti-BrdU antibody conjugated with peroxidase was added to each well for 90 minutes at room temperature. A colorimetric assay was developed with a substrate solution, and the absorbance at 370 nm with a reference wavelength of 492 nm was measured using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Cell cycle analysis

Cell cycle status was determined by staining the cellular DNA with propidium iodide (PI). Cells were treated with normal IgG or the humanized anti-hTM4SF5 monoclonal antibody (10 µg/mL) for 3 days. For cycle detection, cells were treated with PI/RNase solution (BD Biosciences, San Jose, CA, USA) for 30 minutes after fixation overnight in 70% ethanol. The fluorescent signal was detected by flow cytometry using a FACSCalibur (BD Biosciences) and the cell cycle status was analyzed using FCS express program (De Novo Software, Glendale, CA, USA).

In vitro wound-healing assays

Cells were placed in a 6-well plate, cultured overnight to confluence in medium containing serum, and the monolayer was wounded with a pipette tip. Normal IgG or the humanized anti-hTM4SF5 monoclonal antibody (10 µg/mL) was added to the medium for the indicated periods. The cells were fixed with 4% paraformaldehyde (Biosesang, Seongnam, Republic of Korea) for 20 minutes and stained with 0.01% crystal violet (Sigma-Aldrich Co.) for 20 minutes. The wound-healing activity of cells was calculated by the following formula; the cells migrating into wound (%) = [(the wounded area at 0 day – cell-free space in the wounded area)/wounded area at 0-day ×100]. The percent ratio of migrated area to wounded area was measured in three wells per experimental treatment and three wounds per well under a microscope (Nikon Corporation, Tokyo, Japan).

RhoA activity assay

The RhoA Pull-down Activation Assay Biochem Kit (bead pull-down format) (Cytoskeleton, Denver, CO, USA) was used to detect the active form of RhoA according to the manufacturer's instructions. Cells were treated with normal IgG or the anti-hTM4SF5 monoclonal antibody for 6 hours. After the cells were lysed, the cell lysates were incubated with the beads conjugated with the RhoA binding domain of Rhotekin for 1 hour at 4°C. After extensive washing with PBS-T, the samples were processed for a standard Western blot analysis using RhoA-specific antibody.

In vitro cell migration and invasion assays

Transwell chambers with 8 µm porosity (Corning Incorporated, Corning, NY, USA) were used for these assays. For the migration assays, the lower side of the transwell chamber membranes was coated with gelatin (10 µg/well, Sigma-Aldrich Co.). For the invasion assays, a Matrigel invasion chamber (Corning Incorporated) was used. Cells were suspended in serum-free medium with normal IgG or the humanized anti-hTM4SF5 monoclonal antibody (10 µg/mL) and placed on the top of the transwell chamber. DMEM containing 10% FBS was placed in the lower chamber. After incubation for 48 hours, the cells that invaded to the lower surface of the filters were fixed, stained with crystal violet, and counted under a microscope (Nikon Corporation).

CpG-DNA and peptide synthesis

CpG-DNA (MB-ODN 4531(O), AGCAGCGTTCGT-GTCGGCCT) was provided by Samchully Pharm (Seoul, Republic of Korea) as described previously.[30] The B cell epitope peptide of human TM4SF5 (hTM4SF5EC2, [131]TACAYLLNRTLWDRCEAPPRVVPWNCT[157]) was selected, and the cyclic form of the peptide (hTM4SF5EC2-C)

was synthesized by Peptron (Daejeon, Republic of Korea) as described previously.[49]

Preparation of the B cell epitope peptide and CpG-DNA co-encapsulated in DOPE:CHEMS complexes

The liposomes DOPE and CHEMS were obtained from Sigma-Aldrich Co. Liposome complexes consisting of peptide (50 μg) and CpG-DNA (50 μg) co-encapsulated with DOPE:CHEMS (at a 1:1 ratio) were formulated as reported previously.[31] Briefly, DOPE and CHEMS were mixed in 10% ethanol at a molar ratio of 1:1, evaporated with nitrogen gas to produce a solvent-free lipid film, and resuspended in a mixture containing equal volumes of water-soluble MB-ODN 4531(O) (50 μg) and peptide (50 μg), followed by vigorous stirring at room temperature for 30 minutes. After adjusting the pH to 7.0, the peptide and CpG-DNA co-encapsulated with the DOPE:CHEMS [Lipoplex(O)] complex were sonicated lightly for 30 seconds with a sonicator (Sonifier 450; Branson Ultrasonics, Danbury, CT, USA). The complex was filtered with a 0.22 μm filter and freeze-thawed three times with liquid nitrogen.

Mice and immunization

Four-week old female C57BL/6 mice (n=24; weight, 19–20 g) were obtained from Nara Biotech, Inc. (Seoul, Republic of Korea). The mice were maintained under specific pathogen-free conditions (20°C–25°C, 40%–45% humidity, 12-hour light/dark cycle; food and water access, ad libitum). All procedures for the animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea with the approval of the Institutional Animal Care and Use Committee of Hallym University (Permit Number: Hallym 2015-55). Mice were anesthetized under isoflurane (2%–3%) inhalation with a RC2-Rodent Circuit Controller (Lab Etc Inc., Clayton, MO, USA), and all efforts were made to minimize suffering. After letting them adapt for 1 week, the mice (weight, 20–21 g) were injected intraperitoneally with PBS or a complex of peptide and Lipoplex(O) three times at 10-day intervals. Ten days after the third immunization, the mice were injected subcutaneously in the dorsal right flank with PBS or 1×10^6 of PANC02-hTM4SF5 cells. The body weight and size of the tumors were measured every 7 days. Tumor volumes were evaluated as length × width^2/2. The mice were sacrificed 100 days after the first immunization, and the tumors were surgically excised and weighed. Mice were euthanized by CO_2 inhalation (all efforts were made

to minimize suffering) when the mice lost 20% of their adult body weight, tumor size reached 2,000 mm^3, observed evidence of debilitation, pain or distress such as a hunched posture, rough coat, reduced food consumption, emaciation, inactivity, difficulty ambulating, respiratory problems, and solid tumor growth. The CO_2 inhalation was performed with 100% CO_2 at a fill rate of 10%–30% of the chamber volume per minute.

ELISA

Mouse sera were obtained by retro-orbital bleeding under isoflurane (2%–3%) inhalation anesthesia with a RC2-Rodent Circuit Controller 10 days after the final injection. Ninety-six-well immunoplates (Nalgene Nunc International, Rochester, NY, USA) were coated with 5 μg/mL of the cyclic peptide hTM4SF5EC2-C and then blocked with PBST containing 1% BSA. Total IgG levels were measured as previously described.[31] Briefly, the sera were diluted to 1:50 with PBST and added to the wells of each plate. To measure the titers of total IgG, the sera were added to the top row of each plate, and serial 1:3 dilutions in PBST were then placed into the subsequent rows. The plates were incubated for 2 hours at room temperature and washed with PBST. Then, HRP-conjugated goat anti-mouse IgG (1:5,000, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, cat no 115-035-003) was added to the wells and incubated for 1 hour. A colorimetric assay was developed with a tetramethylbenzidine substrate solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), and we used a Spectra Max 250 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) to measure the absorbance at 405 nm.

Statistics

The results are shown as the mean±standard error of the mean (SEM) from at least three independent experiments. Statistical significance of the differences between two samples was evaluated using Student's *t*-test. $P<0.05$ was considered statistically significant.

Results
Expression of TM4SF5 in pancreatic cancer tissues

To confirm expression of TM4SF5 in pancreatic cancer, we analyzed human pancreatic cancer tissues by immunohistochemistry. Analysis of 33 pancreatic cancer specimens showed that 36.4% of pancreatic cancer tissue samples expressed TM4SF5 with staining in >11% of tumor cells (Figure 1 and Table 1). Therefore, we decided to investigate
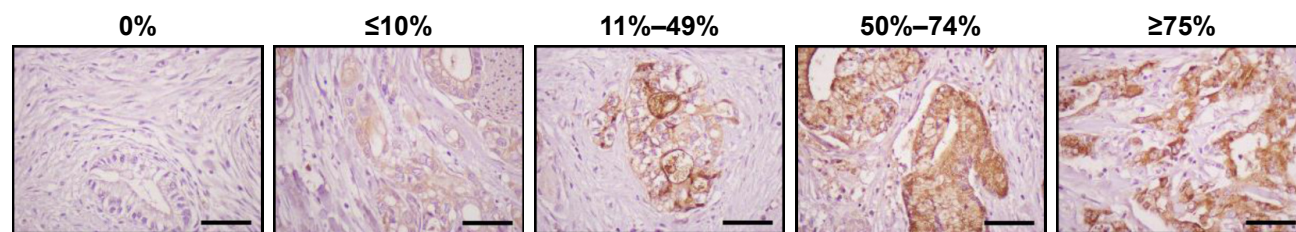
Figure 1 Expression of TM4SF5 in pancreatic cancer tissues.
Notes: An immunohistochemical analysis was performed with mouse anti-TM4SF5 monoclonal antibody (mEC2-C) and pancreatic cancer tissue array. These are examples of human pancreatic cancer tissues with 0%, ≤10%, 11%–49%, 50%–74%, and ≥75% of tumor cells expressing TM4SF5. Scale bars, 25 μm.

the effects of vaccination targeting TM4SF5 using a mouse allograft model.

Establishment of the TM4SF5-expressing mouse pancreatic cancer cells

The mouse PDAC cell line PANC02 is frequently used to form tumors in a mouse allograft model.[52–56] We first checked the expression of TM4SF5 in PANC02 cells, however expression of TM4SF5 was not detected in the PANC02 cells. Therefore, we then established a mouse PDAC cell line stably expressing TM4SF5. We cloned the human TM4SF5 gene into the retroviral vector pLXSN and prepared retrovirus particles using the packaging protocol with pVSV-G in GP2-293 cells. Then, we established control PANC02 cells harboring the pLXSN vector (PANC02-mock) and PANC02 cells expressing TM4SF5 (PANC02-hTM4SF5) by transduction. Expression of TM4SF5 at the mRNA level was detected in the PANC02-hTM4SF5 cells by RT-PCR (Figure 2A, right); however, there was no detected expression of TM4SF5 in the PANC02-mock cells. We also confirmed the expression of TM4SF5 at protein level by immunoprecipitation followed by Western blotting (Figure 2B). TM4SF5 protein was detected in the PANC02-hTM4SF5 cells but not in the PANC02-mock cells. Using confocal microscopy, TM4SF5 protein was detected in the PANC02-hTM4SF5 cells and hardly detected in the PANC02-mock cells (Figure 2C).

Effects of TM4SF5 expression in mouse pancreatic cancer cells

The tetraspanin superfamily, which includes TM4SF5, promotes tumor growth and metastasis by mediating activation of integrin signaling and/or modulating the immune response in other cancers.[39,48,49,57–59] To analyze the cellular consequences of TM4SF5 expression in a pancreatic cancer model, we checked the growth and motility of the PANC02-mock and PANC02-hTM4SF5 cells. First, we measured the change in cell proliferation using the BrdU incorporation assay. Expression of TM4SF5 increased the cell proliferation (Figure 3A). In addition, G1 phase was decreased and S phase was increased in PANC02-hTM4SF5 cells compared to PANC02-mock cells (Figure 3B and C). RhoA activity negatively regulates cell cycle progression through control of cyclin gene expression, and TM4SF5 promotes progression of G1/S phase by inhibition of RhoA activity in HCC.[42] In Figure 3D, the level of active RhoA (RhoA-GTP) was decreased by hTM4SF5 expression in PANC02 cells. Next, we investigated the effect of TM4SF5 on cell migration using the wound healing assay and found that the migration of PANC02-hTM4SF5 cells into the wounded area was increased compared to the PANC02-mock cells (Figure 3E).

Vimentin, one of the mesenchymal markers, contributes to tumorigenesis and the EMT mediated by the organization of cytoskeletons and loss of an epithelial junction protein E-cadherin.[60] The expression level of E-cadherin is reduced during the EMT in tumor progression and inversely correlated with tumor malignancy.[61] Because the TM4SF5 expression increased the cell motility, we further checked the expression levels of Vimentin and E-cadherin. As shown in Figure 3F, the expression levels of Vimentin and E-cadherin were increased and decreased, respectively, in the PANC02-hTM4SF5 cells compared to the PANC02-mock cells. Therefore, we confirmed that TM4SF5 expression induced cell growth and reinforced mesenchymal properties in mouse PDAC cells.

Table 1 Immunohistochemical analysis of TM4SF5 expression in pancreatic cancer tissues

Pancreatic cancer tissue sections (AccuMax Array)	n	TM4SF5 positive (%)	Number (%) of cases expressing TM4SF5				
			≤10%	11%–49%	50%–74%	≥75%	Negative
A207(IV)	33	36.4	10 (30.3)	5 (15.2)	4 (12.1)	3 (9.1)	11 (33.3)

Note: Percentages in parentheses were calculated as the number of TM4SF5-positive samples for each quartile, divided by the total number of samples in each tumor type.
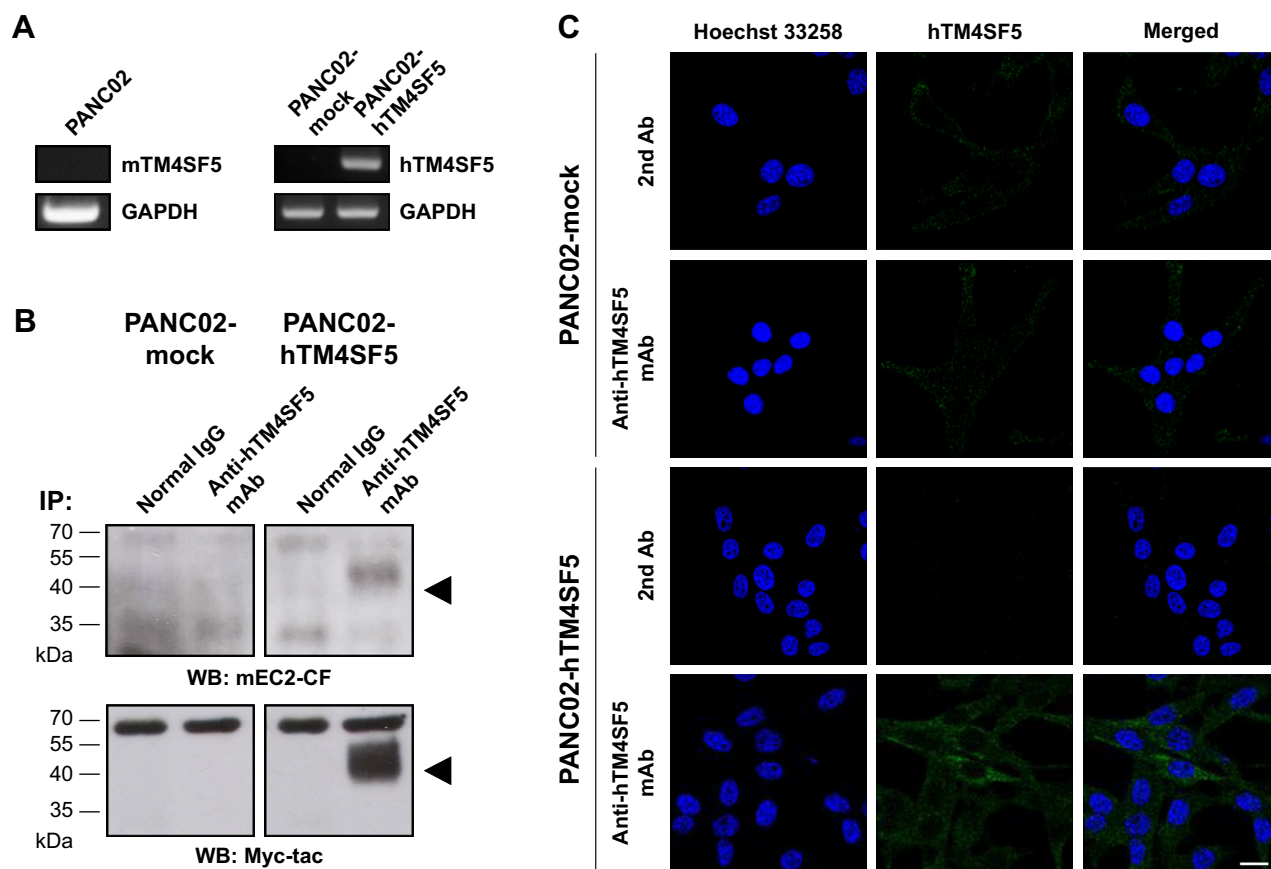
Figure 2 Establishment of mouse pancreatic cancer cells expressing human TM4SF5.
Notes: (**A**) Expression of TM4SF5 mRNA in parental PANC02, PANC02-mock, and PANC02-hTM4SF5 cells was determined by RT-PCR analysis. (**B**, **C**) Expression of the TM4SF5 protein in PANC02-hTM4SF5 cells was verified by immunoprecipitation using the humanized anti-hTM4SF5 monoclonal antibody and then Western blot analysis using mouse anti-hTM4SF5 monoclonal antibody (mEC2-CF) or anti-Myc-tag antibody (**B**), followed by confocal microscopy using the humanized anti-hTM4SF5 monoclonal antibody (**C**). Scale bar, 20 μm. Anti-hTM4SF5 mAb: the humanized anti-hTM4SF5 monoclonal antibody; 2nd Ab: secondary antibody only.
Abbreviations: WB, Western blotting; IP, immunoprecipitation.

Suppression of tumor growth by vaccination with a complex of TM4SF5 peptide and Lipoplex(O) in a mouse pancreatic tumor model established with the TM4SF5-expressing PDAC cells

To evaluate the prophylactic efficacy of the TM4SF5-targeting vaccine with regards to tumor growth, we immunized C57BL/6 mice with the peptide hTM4SF5EC2-C in combination with Lipoplex(O),[45] and then, PANC02-hTM4SF5 cells were implanted into the mice (Figure 4A). The formula consisting of the cyclic peptide hTM4SF5EC2-C, corresponding to the amino acids 131–157 in the extracellular domain 2 of human TM4SF5, and Lipoplex(O) were previously proven to successfully induce immune responses targeting cell membrane TM4SF5 in mice.[49] After the third vaccination, we checked the titers of TM4SF5-specific IgG in the mouse sera using ELISA plates coated with the cyclic peptide hTM4SF5EC2-C and confirmed that the immunized

mice had a large amount of anti-hTM4SF5 antibodies in their sera (Figure 4B). In contrast, the control mice injected with PBS had no production of TM4SF5-reactive antibodies. The PANC02-hTM4SF5 cells implanted into C57BL6 mice grew continuously and formed a tumor mass, whereas tumor development was significantly inhibited by the vaccination with the TM4SF5 cyclic peptide with Lipoplex(O) (Figure 4C–E); tumor volumes (Figure 4C and D); and tumor weight (Figure 4E) were reduced in the vaccinated mice compared to the control mice. The vaccination is not likely to induce significant side effects because there was no difference during the experiment in the body weights of the immunized mice compared to the control group (Figure 4F). On the other hand, when PANC02 cells were implanted into C57BL/6 mice, the volumes and weights of the tumors were not reduced by vaccination with Lipoplex(O) alone or a complex of the TM4SF5 cyclic peptide in combination with Lipoplex(O) (Figure S1). Therefore, we conclude that the TM4SF5 cyclic peptide and Lipoplex(O) complex induced
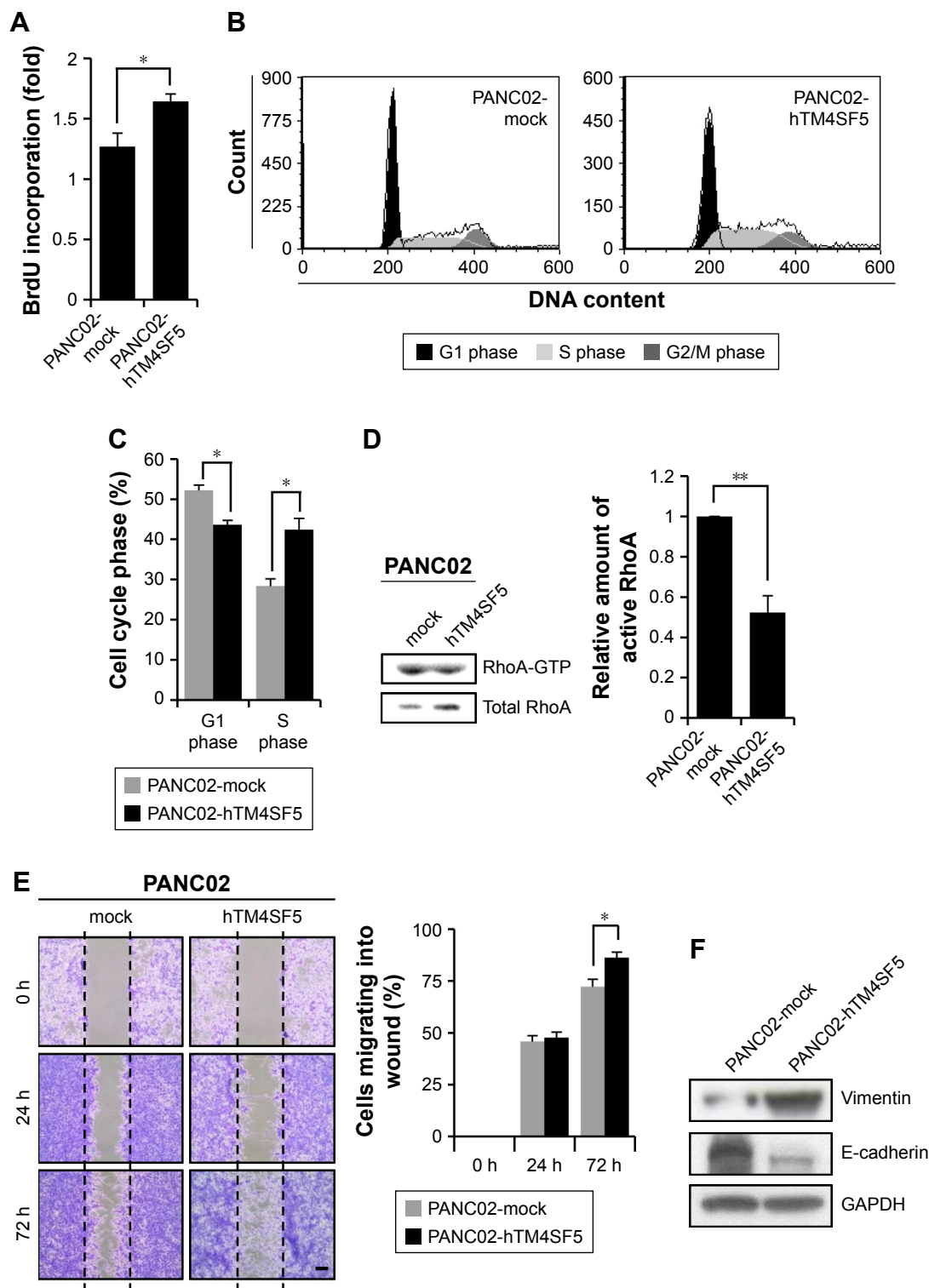
Figure 3 Effects of hTM4SF5 expression in mouse pancreatic cancer cells.

Notes: (**A**) The cell proliferation of the PANC02-mock and PANC02-hTM4SF5 cells was measured by the BrdU incorporation assay. (**B**, **C**) The cells were stained with PI and analyzed using FACS. The DNA content was calculated using FCS Express software. (**D**) RhoA activity was determined by RhoA-GTP pull-down assay. The active RhoA was captured by RhoA-GTP binding domain of Rhotekin and detected by RhoA-specific antibody. The relative intensities of RhoA-GTP bands are shown as a graph after normalization with the levels of total RhoA. (**E**) Wound healing activity. A monolayer culture of PANC02-mock and PANC02-hTM4SF5 was wounded with a pipette tip, and migration of the cells into the wound area was examined at the indicated time points. Scale bar, 200 μm. (**F**) Expression levels of Vimentin and E-cadherin in PANC02-mock and PANC02-hTM4SF5 cells were examined by Western blot analysis. The amounts of GAPDH protein are shown as a loading control. Values are the mean±SEM. *$P<0.05$, **$P<0.01$ vs PANC02-mock or normal IgG control.

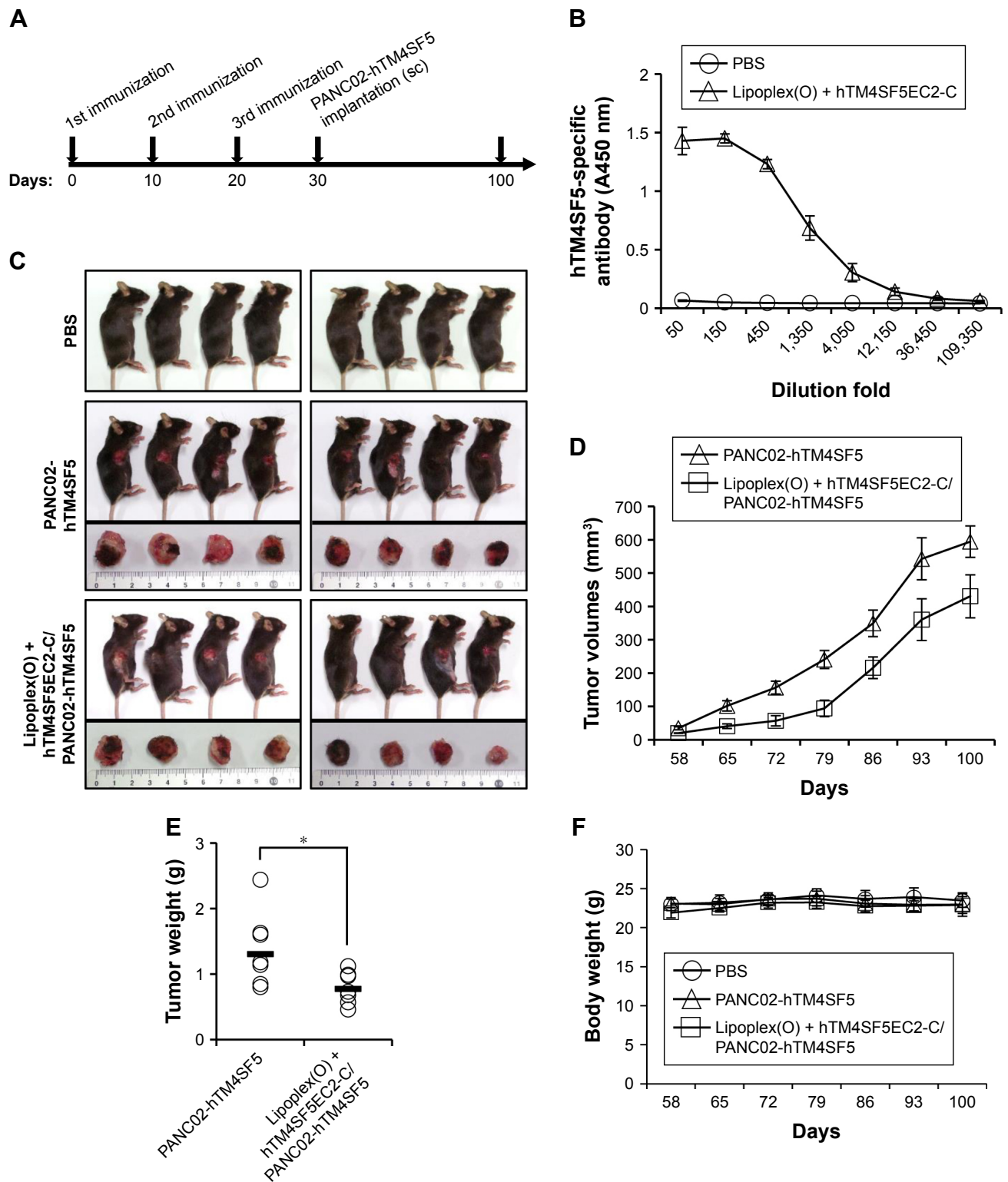**Abbreviations:** PI, propidium iodide; SEM, standard error of mean.

Figure 4 Prophylactic efficacy of a vaccine containing a complex of hTM4SF5EC2-C peptide and Lipoplex(O) in a mouse pancreatic cancer model established by implantation of the PANC02-hTM4SF5 cells.

Notes: (**A**) Experimental schedule. C57BL/6 mice were immunized at a 10-day interval (n=8/group). After the third immunization, the mice were challenged with PANC02-hTM4SF5 cells, and their condition monitored. (**B**) Production of hTM4SF5-reactive IgG in the immunized mice. The IgG titers in the serum after the third immunization were measured with an ELISA kit. (**C**) Macroscopic appearance of pancreatic tumor tissues derived from the PANC02-hTM4SF5 cells. (**D**) Tumor volumes were calculated as length × width²/2. (**E**) Tumor growth was measured by tumor weight. (**F**) Body weights were measured at the indicated time intervals. Values are the mean±SEM. *$P < 0.05$ vs non-vaccinated control.

Abbreviations: sc, subcutaneously; SEM, standard error of the mean.

a protective immune response and inhibited the growth of TM4SF5-expressing tumor in vivo.

Suppression of tumor cell growth by the humanized anti-hTM4SF5 antibody in vitro

Because we confirmed that the TM4SF5 peptide vaccination induced TM4SF5-specific antibody production and suppressed the growth of TM4SF5-expressing pancreatic tumors in vivo (Figure 4), we checked the effects of the anti-hTM4SF5 antibody on the growth of control and TM4SF5-expressing

pancreatic cancer cells in vitro. Here, we used the humanized anti-hTM4SF5 monoclonal antibody (hEC2-C-2) that we established with the cyclic peptide hTM4SF5EC2-C as an antigen.[49] When we measured the proliferation rate of the PANC02-mock and PANC02-hTM4SF5 cells using the BrdU incorporation assay, the proliferative activity of the PANC02-hTM4SF5 cells was significantly decreased by the humanized anti-hTM4SF5 monoclonal antibody compared to the control PANC02-mock cells in a dose-dependent manner (Figure 5A). When we checked cell cycle status after treatment with the humanized anti-hTM4SF5 monoclonal
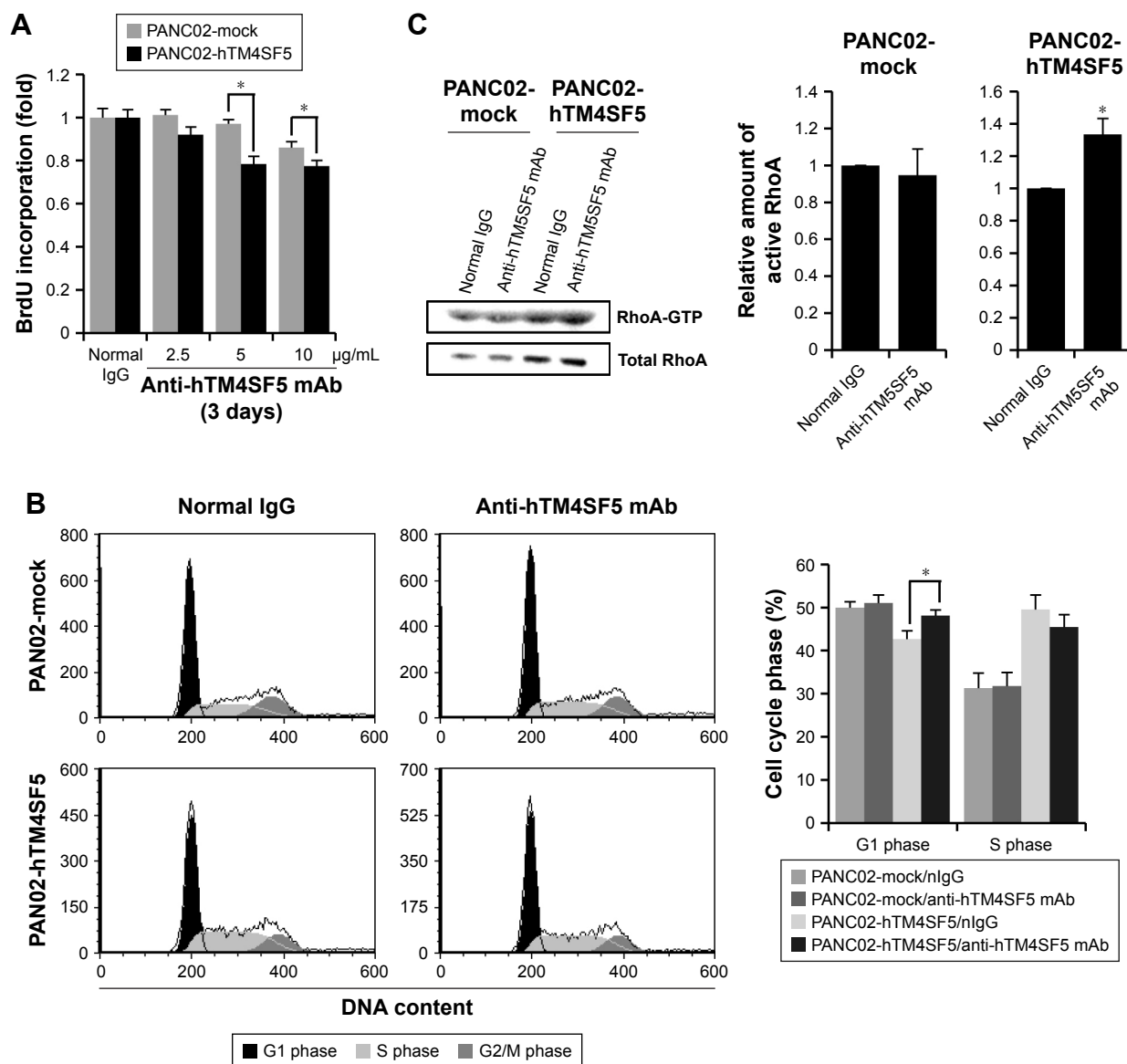

Figure 5 Effect of the normal IgG and the humanized anti-hTM4SF5 monoclonal antibody on the proliferation of the control and TM4SF5-expressing pancreatic cancer cells.
Notes: (**A**) The proliferative activities of the PANC02-mock and PANC02-hTM4SF5 cells were measured by the BrdU incorporation assay. (**B**) The cells were stained with PI and analyzed using FACS. The DNA content was calculated using FCS Express software. (**C**) The RhoA activity was detected by RhoA-GTP pull-down assay. The relative intensities of RhoA-GTP bands are shown as a graph after normalization with the levels of total RhoA. The normal IgG and the humanized anti-hTM4SF5 monoclonal antibody (anti-hTM4SF5 mAb) were treated for 3 days. Values are the mean±SEM. *$P<0.05$ vs each PANC02-mock or normal IgG control.
Abbreviations: PI, propidium iodide; SEM, standard error of the mean.

antibody, cell population in the G1 phase was increased and S phase population was slightly decreased in the PANC02-hTM4SF5 cells (Figure 5B). In addition, the level of active RhoA was increased by the humanized anti-hTM4SF5 monoclonal antibody in the PANC02-hTM4SF5 cells (Figure 5C). In the PANC02-mock cells, the cell cycle status and RhoA activity were not changed by the humanized anti-hTM4SF5 monoclonal antibody (Figure 5B and C). These data suggest that the targeting of TM4SF5 with the antibody decreased cell proliferation via regulation of cell cycle.

Suppression of tumor cell motility by the humanized anti-hTM4SF5 antibody

Because the motility of pancreatic cells was increased when TM4SF5 was expressed (Figure 3E), we investigated the effects of the humanized anti-hTM4SF5 monoclonal antibody on cell migration and invasion using the PANC02-mock and PANC02-hTM4SF5 cells. As shown by the wound-healing assay (Figure 6A), the migration of PANC02-hTM4SF5 cells into the wounded area was significantly reduced by treatment with the humanized anti-hTM4SF5 monoclonal antibody compared to the normal IgG whereas there was no effect in the control PANC02-mock cells. In addition, we measured the migration/invasion using a transwell migration/invasion chamber. The humanized anti-hTM4SF5 monoclonal antibody treatment, but not normal IgG, inhibited the migration and invasion of the PANC02-hTM4SF5 cells (Figure 6B and C). In contrast, the humanized anti-hTM4SF5 monoclonal antibody had no effect on the migration/invasion of the PANC02-mock cells. These results show that targeting of TM4SF5 with the antibody reduced the motility of TM4SF5-expressing pancreatic cancer cells in vitro.

Molecular change in the EMT markers by the humanized anti-hTM4SF5 antibody

Because we previously found that the anti-hTM4SF5 antibody (clone #2D4-18) treatment modulated EMT molecules in HCC and colon cancer,[39,48] and here we confirmed the reduced cell motility of the PANC02-hTM4SF5 cells by treatment with the humanized anti-hTM4SF5 monoclonal antibody (Figure 6), we checked the expression levels of Vimentin and E-cadherin after the antibody treatment. In the PANC02-hTM4SF5 cells, the expression level of Vimentin was decreased and that of E-cadherin was increased at 3 and 5 days after the humanized anti-hTM4SF5 monoclonal antibody treatment compared to the normal IgG treatment (Figure 7B). In contrast, the expression levels of Vimentin and E-cadherin during the culture were similar when the

normal IgG or the humanized anti-hTM4SF5 monoclonal antibody was treated in the PANC02-mock cells (Figure 7A). These findings suggest that the TM4SF5-targeting antibody can reduce EMT during tumor progression in TM4SF5-expressing pancreatic cancer cells.

Discussion

High expression of TM4SF5 has been reported in several cancers including pancreatic cancer.[33–35] Therefore, TM4SF5 has gained much attention as a target for anticancer strategies against those cancers. Previously, we found that immunization with a TM4SF5 peptide-CpG-DNA-liposome complex has preventive and therapeutic effects on tumor growth in a mouse HCC and colon cancer model.[31,45–49] In this study, we showed that the peptide vaccine targeting TM4SF5 can induce the production of anti-hTM4SF5 antibodies and suppress the growth of TM4SF5-expressing pancreatic cancer in a mouse allograft model.

Almost all pancreatic cancers are PDAC.[1] When we examined expression of TM4SF5 in the pancreatic tissues prepared from cancer patients, TM4SF5 was expressed in 36% of the samples. Even though the percentage is relatively low, 58% of the positive samples do have expression of TM4SF5 in 50% or more of tumor cells. Therefore, to investigate whether TM4SF5 could be a potential target for an anticancer strategy in pancreatic cancer, we searched for a TM4SF5-expressing mouse PDAC cell line as a model. However, we could not find a commercially or privately available one. Therefore, we established TM4SF5-expressing mouse PDAC cells (PANC02-hTM4SF5) and control cells (PANC02-mock) using a retroviral system. The PANC02-hTM4SF5 cells showed higher cancerous properties in the context of growth, migration, and mesenchymal marker expression compared to the control cells, supporting that TM4SF5 can act as an oncogenic factor. On the other hand, the proliferative activity of the PANC02-hTM4SF5 cells was significantly reduced by the anti-hTM4SF5 antibody compared to the normal IgG. Migration and EMT marker expression were also changed by the anti-hTM4SF5 antibody in PANC02-hTM4SF5. Therefore, we believe that suppression of TM4SF5 could be a strategy to treat pancreatic cancer expressing TM4SF5.

Generally, cyclic peptides have high biological activity compared to linear peptides due to the conformational structure.[62,63] The rigidity of cyclic peptides reduces the entropy, therefore enabling increased binding or receptor selectivity. In addition, a cyclic structure has resistance to hydrolysis by exopeptidase.[64] Cyclic peptides have been investigated for their utility as an antitumor drug.
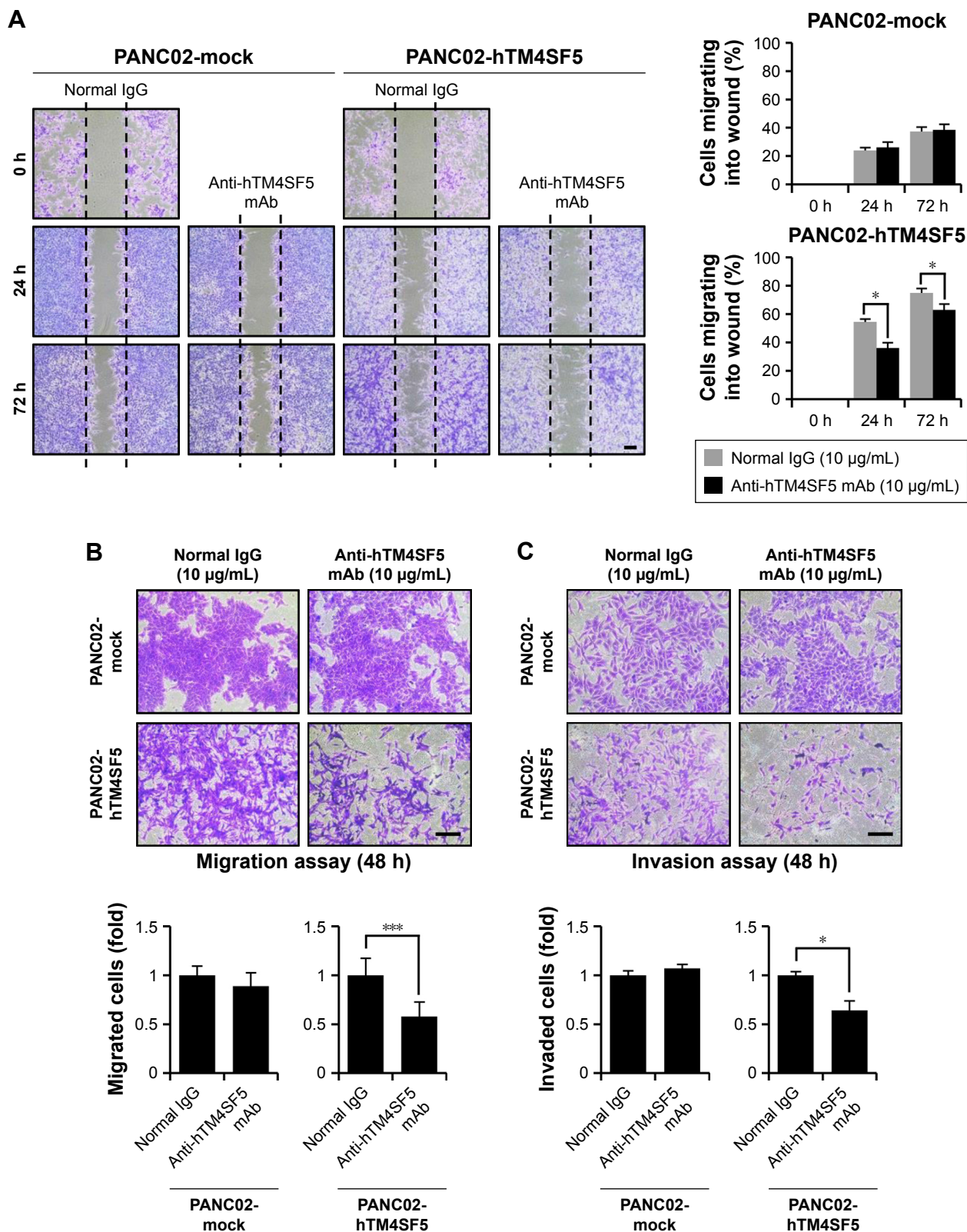
Figure 6 Changes in motility of the TM4SF5-expressing pancreatic cancer cells after the humanized anti-hTM4SF5 monoclonal antibody treatment.
Notes: PANC02-mock and PANC02-hTM4SF5 cells were treated with normal IgG control or humanized anti-hTM4SF5 monoclonal antibody (anti-hTM4SF5 mAb), and motility was examined. (**A**) Wound healing activity was examined at the indicated time points. Scale bar, 200 μm. (**B**, **C**) The migratory and invasive properties. To measure the migration (**B**) and invasion (**C**) activity, the migrated and invaded cells on the lower sides of the transwell chambers were counted after incubation with the indicated materials. Scale bar, 100 μm. The percentage of cells migrated into wound, and the numbers of migrated or invaded cells were measured and compared (graphs). Values are the mean±SEM. *$P<0.05$, ***$P<0.005$ vs normal IgG control.
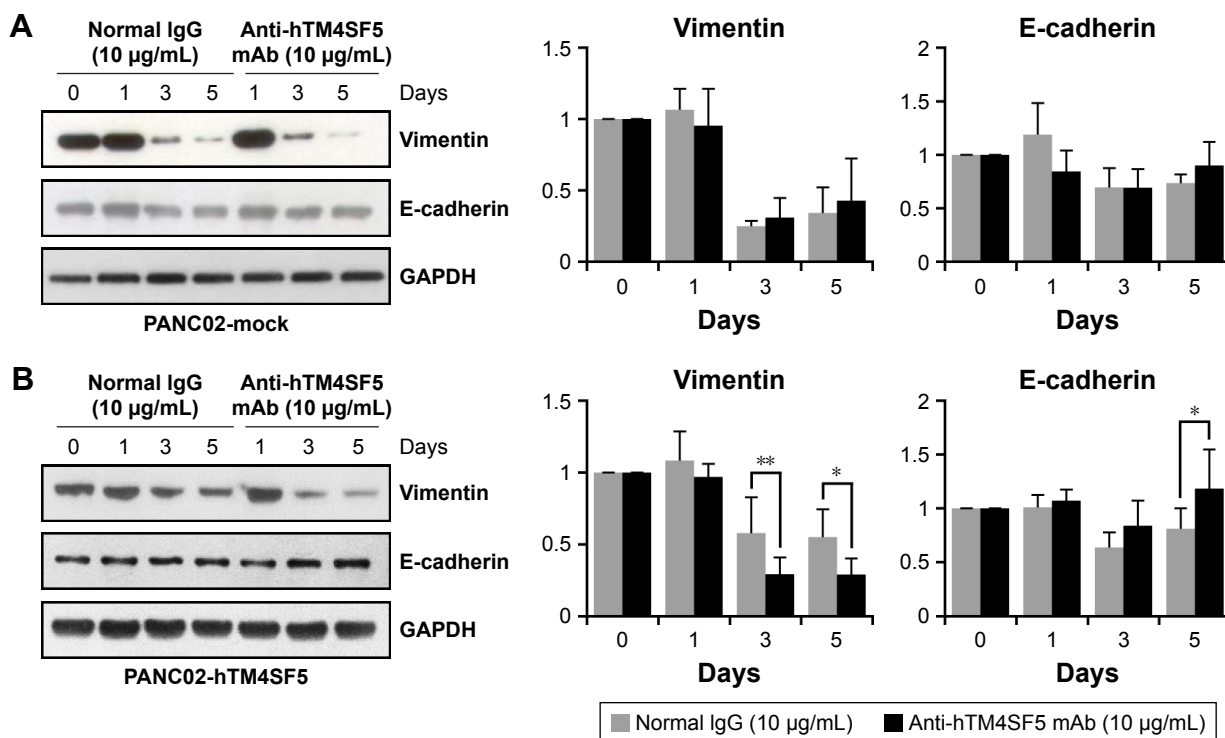**Abbreviation:** SEM, standard error of the mean.

Figure 7 Molecular change of the EMT markers in the TM4SF5-expressing pancreatic cancer cells induced by the humanized anti-hTM4SF5 monoclonal antibody.
Notes: (**A**, **B**) Expression levels of Vimentin and E-cadherin in the PANC02-mock (**A**) and PANC02-hTM4SF5 (**B**) cells were examined by Western blot analysis after treatment with normal IgG or humanized anti-hTM4SF5 monoclonal antibody (anti-hTM4SF5 mAb) for the indicated periods. The relative intensities of E-cadherin and Vimentin bands are shown as a graph after normalization with the levels of GAPDH. P-values were evaluated using ratio paired t-test. Values are the mean±SEM. *$P < 0.05$, **$P < 0.01$ vs normal IgG control.
Abbreviations: EMT, epithelial–mesenchymal transition; SEM, standard error of the mean.

Integrins-recognition sequence Arg-Gly-Asp (RGD) inhibits tumor cell growth and metastasis, and a cyclic peptide with the RGD sequence is more potent than a linear peptide with the RGD sequence.[65–67] The use of cyclic RGD peptides in drug delivery systems has also been studied.[68] Antibodies produced by cyclic peptide immunization bind more strongly to the recombinant whole protein than antibodies produced by linear peptide immunization.[69] Here, we found that immunization with the cyclic peptide epitope in combination with the CpG-DNA-liposome complex induced the successful production of anti-hTM4SF5 antibodies in mice. Furthermore, the growth of the tumor was suppressed in the vaccinated mice. Therefore, we confirmed that the peptide vaccine targeting TM4SF5 had a preventive effect against pancreatic tumor expressing TM4SF5 in an allograft model. Considering that the cancerous properties of PANC02-hTM4SF5 were suppressed by the treatment with the humanized anti-hTM4SF5 monoclonal antibody in vitro, antibodies produced by the vaccination clearly contributed to the suppressive effect of the peptide vaccine targeting TM4SF5 in vivo. Given the adjuvant effect of CpG-DNA inducing various immunostimulatory effects, other humoral and cellular effects

in concert with the anti-hTM4SF5 antibodies could surely contribute to the defense against the pancreatic tumors in the vaccinated mice.

For the treatment of pancreatic cancer, investigators have tried various therapeutic strategies.[70] Treatment with gemcitabine (dFdC: 2′,2′-difluorodeoxycytidine) has been recommended as a first-line therapy for advanced pancreatic cancer patients since 1997; however, gemcitabine treatment provides only a slight improvement in survival compared to no treatment. The overall survival of gemcitabine-treated patients is approximately 6 months.[71] Pancreatic cancer cells are more sensitive to gemcitabine than other anticancer drugs; however, most patients get resistance within weeks after gemcitabine treatment.[72] To improve the survival rate of gemcitabine monotherapy, combination therapy has been attempted with other cytotoxic agents such as capecitabine, oxaliplatin, and erlotinib.[73–77] However, the improvement was very weak and limited. Therefore, many researchers have investigated better therapeutic strategies including targeted therapy.[78,79] Because EGFR is overexpressed in 40%–60% of pancreatic cancers, lapatinib, an EGFR and HER-2 inhibitor, in combination with capecitabine, which is converted to

5-flurouracil, was tested in clinical trials to treat pancreatic cancer.[80] The combination of gemcitabine with nimotuzumab, an anti-EGFR monoclonal antibody, was also tried.[80,81] For targeting the angiogenesis pathway, aflibercept, a VEGF antagonist, or sorafenib, a VEGF-R2/3 kinase repressor, was combined with gemcitabine in pancreatic cancer patients. However, these combination trials did not significantly improve the therapeutic effects compared to gemcitabine monotherapy.[82,83] PI3K/Akt is important in cell growth, survival, and apoptosis of various tumors, such as PDAC.[84] Akt is activated in approximately 45% of PDAC patients, and the level of activated Akt is related with the survival rate after surgery.[85] The alkylphospholipid perifosine, a suppressor of PI3K and Akt phosphorylation, in combination with gemcitabine showed a synergetic effect in pancreatic cancer cells expressing a high level of activated Akt.[86,87] Despite these efforts, there has been no significant clinical outcome yet in pancreatic cancer, and it remains the most lethal among solid tumors. Therefore, we believe that our trial, to verify the effect of targeting and suppressing TM4SF5, is meaningful to pursue. Because our results suggest that targeting TM4SF5 could be a novel strategy to prevent or treat pancreatic cancer, further study on the effects of the humanized anti-hTM4SF5 monoclonal antibody in combination with other anticancer reagents may provide more useful information.

Conclusion

This study suggests that the targeting of TM4SF5 could be a novel strategy to prevent or treat pancreatic cancer. Vaccination targeting TM4SF5 induced production of anti-TM4SF5 antibodies and suppressed growth of TM4SF5-expressing pancreatic cancer in a mouse model. The cell growth and motility were decreased by treatment with the anti-hTM4SF5 monoclonal antibody in TM4SF5-expressing mouse pancreatic cancer cells in vitro. However, a strategy to strengthen the therapeutic effect of TM4SF5-targeting has to be investigated in the future.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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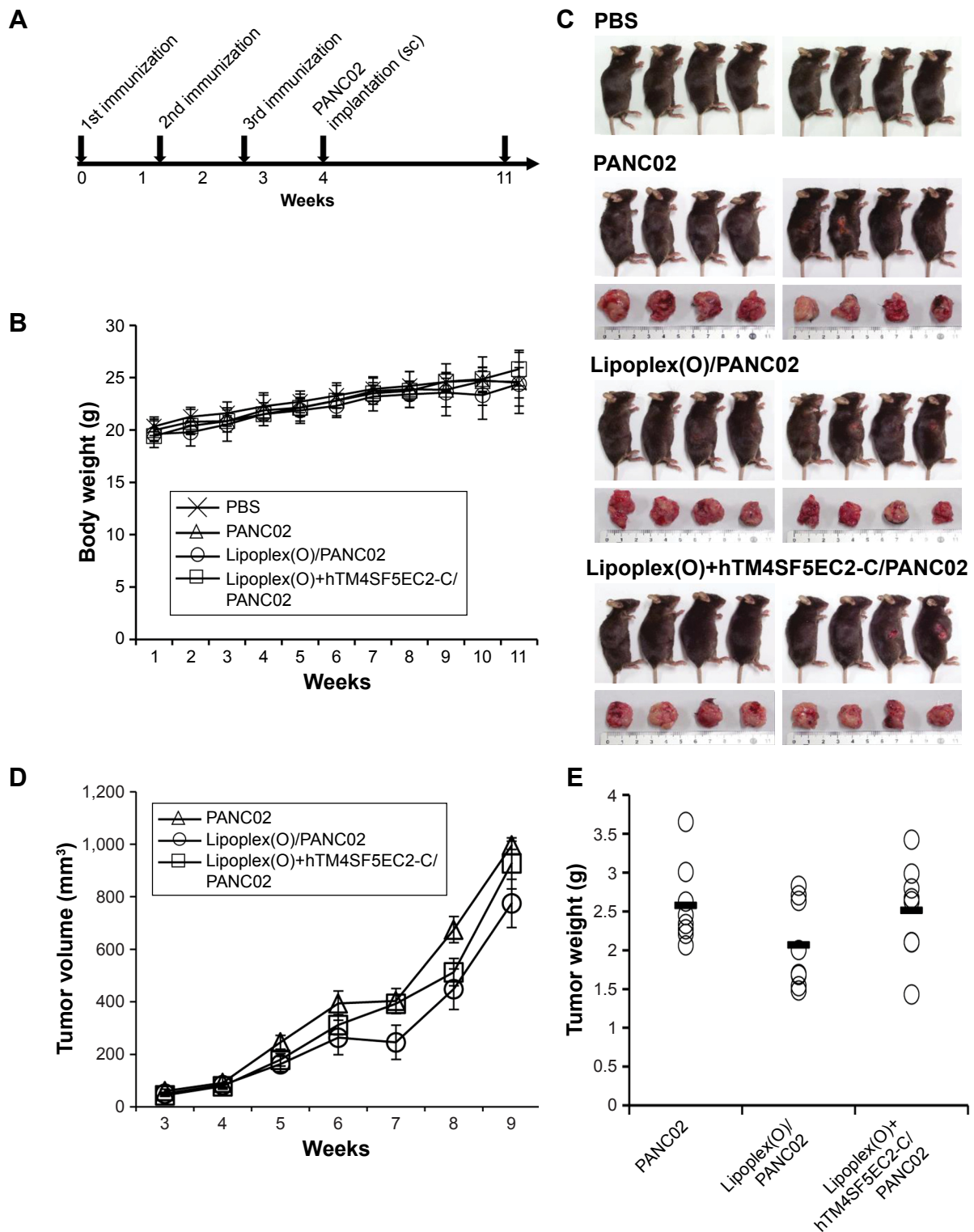
Supplementary material

Figure S1 Vaccination with a complex of hTM4SF5EC2-C peptide and Lipoplex(O) had no prophylactic efficacy in a mouse pancreatic cancer model established by implantation of PANC02 cells.

Notes: (**A**) Experimental schedule. C57BL/6 mice were immunized with PBS, Lipoplex(O), or a complex of hTM4SF5EC2-C peptide and Lipoplex(O) at 10-day intervals (n=8/group). After the third immunization, the mice were challenged with PANC02, and their condition monitored. (**B**) Body weights were measured at the indicated time intervals. (**C**) Macroscopic appearance of pancreatic tumor tissues derived from PANC02 cells. (**D**) Tumor volumes were calculated as length × width^2/2. (**E**) Tumor growth was measured by tumor weight. Values are the mean±SEM.

Abbreviations: sc, subcutaneously; SEM, standard error of the mean.