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The roles of TM4SF5 in the promotion of fibrotic and tumorigenic potentials of hepatocytes

간섬유화 및 간암발병에 있어 TM4SF5 단백질의 역할

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강 민 경

A thesis of the Degree of Doctor of Philosophy

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The roles of TM4SF5 in the promotion of fibrotic and tumorigenic potentials of hepatocytes

by
Minkyung Kang

A thesis submitted to the Department of Biomedical Science in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biomedical Science at Seoul National University College of Medicine

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Approved by Thesis Committee:

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ABSTRACT

The roles of TM4SF5 in the promotion of fibrotic and tumorigenic potentials of hepatocytes

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Chronic injury and inflammation causes liver fibrosis, through a process involving epithelial–mesenchymal transition (EMT), which is characterized by excessive accumulation of extracellular matrix

proteins such as collagen [1-3]. Fibrosis can eventually lead to cirrhosis, liver failure, and the development of hepatocellular carcinoma (HCC).

TM4SF5 (transmembrane 4 L6 family member 5) is expressed at much higher levels in liver tumours than in normal hepatic tissues [4]. TM4SF5 is a transmembrane glycoprotein that has four transmembrane domains; its N- and C-terminal tails are located in the cytosol [5]. TM4SF5 expression causes morphological changes (with aberrant actin reorganization) and EMT [4], mediates aberrant growth in multilayers, accelerates G1-to-S phase progression [4,6] and enhances cellular migration and invasion [7]. In addition, TM4SF5 can form massive tetraspanin web structures (Teraspanin-enriched microdomain, TREM) by forming complexes with other tetraspanins or cell adhesion molecules, such as integrins, and can play a role in regulation of matastasis. However, it is not known TM4SF5 roles during liver fibrosis/cirrhosis and how TM4SF expression is regulated. Also, any hierarchy among teraspanins has not been reported.

In this study, I explored the mechanisms that induce TM4SF5 expression and whether impaired signalling pathways for TM4SF5 expression inhibit the acquisition of mesenchymal features, using human and mouse normal hepatocytes and animal model. And then, I examined the correlations between TM4SF5 and other teraspanins (CD151 and CD63) using TM4SF5- expressing and -none-expressing cells.

First, Using a CCl_4-mediated mouse liver in vivo model, I examined whether TM4SF5 is expressed during liver fibrosis mediated by CCl_4 administration and whether treatment with anti-TM4SF5 reagent blocks the fibrotic liver features. In the CCl_4-mediated mouse liver model, TM4SF5 was expressed during the liver fibrosis mediated by CCl_4 administration and correlated with α-smooth muscle actin expression and collagen I deposition in fibrotic septa regions. Interestingly, treatment with anti-TM4SF5 reagent blocked the TM4SF5-mediated liver fibrotic features. These results suggest that TM4SF5 expression is induced by fibrotic processes during chronic liver injuries. TM4SF5 is thus a candidate target for prevention of liver fibrosis following chronic liver injury.

I also explored the mechanisms that induce TM4SF5 expression and whether impaired signalling pathways for TM4SF5 expression inhibit the acquisition of mesenchymal cell features, using human and mouse normal hepatocytes. I found that TGFβ1-mediated Smad activation caused TM4SF5 expression and EMT, and activation of the EGFR pathway. Inhibition of EGFR activity following TGFβ1 treatment abolished acquisition of EMT, suggesting a link from Smads to EGFR for TM4SF5 expression. Further, TGFβ1-mediated EGFR activation and TM4SF5 expression were abolished by EGFR suppression or extracellular EGF depletion. Smad overexpression mediated EGFR activation and TM4SF5 expression in the absence of serum, and EGFR kinase inactivation or EGF depletion abolished Smad-overexpression-induced TM4SF5 and mesenchymal cell marker

expression. Inhibition of Smad, EGFR or TM4SF5 using Smad7 or small compounds also blocked TM4SF5 expression and/or EMT. These results indicate that TGFβ1- and growth factor-mediated signalling activities mediate TM4SF5 expression leading to acquisition of mesenchymal cell features.

Next, I investigated the relationship between TM4SF5-positive TERMs with liver fibrosis and tumorigenesis, using normal Chang hepatocytes that lack TM4SF5 expression and chronically TGFβ 1-treated Chang cells that express TM4SF5. TM4SF5 expression is positively correlated with tumorigenic CD151 expression, but is negatively correlated with tumorsuppressive CD63 expression in mouse fibrotic and human hepatic carcinoma tissues, indicating cooperative roles of the tetraspanins in liver malignancies. Although CD151 did not control the expression of TM4SF5, TM4SF5 appeared to control the expression levels of CD151 and CD63. TM4SF5 interacted with CD151, and caused the internalization of CD63 from the cell surface into late lysosomal membranes, presumably leading to terminating the tumor-suppressive functions of CD63. TM4SF5 could overcome the tumorigenic effects of CD151, especially cell migration and extracellular matrix (ECM)-degradation. Taken together, TM4SF5 appears to play a role in liver malignancy by controlling the levels of tetraspanins on the cell surface.

Altogether, this study reveals that TM4SF5 expression is induced by fibrotic processes during chronic liver injuries and TGFβ1- and growth factor-mediated signalling activities mediate TM4SF5

expression leading to acquisition of mesenchymal cell features. Thus, TM4SF5 induction may be involved in the development of liver pathologies. And TM4SF5 appears to play a role in liver malignancy by controlling the levels of tetraspanins (CD151 and CD63) on the cell surface. Taken together, TM4SF5 could provide a promising therapeutic target for prevention and treatment of liver malignancies.

Keywords : tetraspanin, TM4SF5, liver fibrosis, EMT, TGFβ1, anti-TM4SF5, cytokine, EGFR, tetraspanin web, CD151, CD63

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List of Abbreviations

BSA	Bovine Serum Albumin
BrdU	5-bromo-2-deoxyuridine
DAPI	4'6-diamidino-2-phenylindole
DIC	Differential Interference Contrast
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfixide
ECM	Extracellular Matrix
EDTA	Ethylnendiaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
EMT	Epithelial-mesenchymal transition
FAK	Focal adhesion kinase
FBS	Fetal bovine serum

FN	Fibronectin
H&E	Hematoxylin and eosin
HCC	Hepatocarcinoma
HGF	Hepatocyte Growth Factor
IP injection	Intraperitoneal injection
LN	Laminin
mAb	Monoclonal antibody
MMP	Matrix metalloproteinase
NP-40	Nonidet-P40
PBS	Phosphate buffered aline
Rho	Ras homolog
RIPA	Radioimmunoprecipitation assay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA	Smooth muscle α-actin
c-SRC	proto-oncogene tyrosine-protein kinase Src

TERM	Tetraspanin-enriched microdomain
TGFβ1	Tranforming growth factor β1
TIMP	Tissue inhibitors of metalloproteinase
TM4SF5	Four-transmembrane L6 family member 5
TSAHC	4'-(p-tuluenesulfonylamido)-4-hydroxychalcone
ZO1	Zonula occludens 1

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Introduction

Chronic injury and inflammation causes liver fibrosis, through a process involving epithelial-mesenchymal transition (EMT), which is characterized by excessive accumulation of extracellular matrix proteins such as collagen [1-3]. Fibrosis can eventually lead to cirrhosis, liver failure, and the development of hepatocellular carcinoma (HCC) [8]. Among the diverse mediators involved in liver fibrosis, transforming growth factor (TGF)β1 is a major cytokine that plays pivotal roles in initiating and promoting transdifferentiation of hepatic stellate cells (HSCs), hepatocytes and cholangiocytes [9] to myofibroblasts (i.e. EMT). TGFβ1 also functions as a potentially important link between fibrosis and neoplasia in the liver [10]. Many mediators of the EMT and fibrosis following chronic liver injuries have been investigated [11]. However, the protein (e.g. a membrane receptor) that is targeted by the cytokines and growth factors that surround injured liver cells and regulate cell behaviors, including EMT, proliferation and migration, during the development of liver malignancy has not been identified.

The EMT (epithelial–mesenchymal transition) is involved in diverse cellular functions in both homeostatic and pathological conditions [1]. During development and in certain disease states, the EMT process marks a transition from an epithelial to a mesenchymal cell type, resulting in altered features in terms of cellular polarity and cell–cell adhesion [2]. Epithelial cells are polarized with typical junctions along

cell-cell contacts where actin and intermediate filaments are linked [3]. After EMT occurs, cells lose polarity through disruption of cell-cell contacts, actin reorganization and suppression of cell-cell adhesion molecules [12]. There are three types of EMT: type 1 is involved in embryonic development; type 2 is involved in tissue damage, regeneration and organ fibrosis; and type 3 is involved in cancer progression and the dissemination of metastatic cancer cells from primary tumours [13]. EMT processes can cause alteration of cellular functions, including migration and proliferation, during either developmental or pathological processes [14]. The process of EMT involves different regulatory mechanisms supported by diverse extracellular signal-derived activities and gene regulation [15,16]. Extracellular cues include extracellular matrix components, HGF (hepatocyte growth factor), and TGFβ (transforming growth factor β) [13].

The plasma membrane is structurally important for signal transduction between the intracellular and extracellular environments. A diverse set of membrane proteins with specific membrane domains facilitates this signal transduction [17]. In addition to lipid rafts, which are small, dynamic, and heterogeneous membrane compartments enriched with sterol- and sphingolipids [18], tetraspanin-enriched microdomains (TERMs) are independent organizations of large protein complexes that include tetraspanins, integrins, and growth factor receptors contribute to adhesion, proliferation, and migration [19]. Tetraspanins are linked to the

progression of a variety of cancers [20]. Currently, 33 mammalian tetraspanins (TM4SFs) have been identified. These proteins weigh between 20 and 30 kDa and have variable sequence homology. However, all these proteins contain four common transmembrane domains, two cytosolic tails, a short extracellular loop (SEL), and a long extracellular loop (LEL) [17].

CD151 (Tspan24) was first identified as a promoter of metastasis [21]; its expression is increased in liver cancer, compared to normal cells [22]. CD151 functions in cellular migration, invasion, angiogenesis, and drug resistance by forming protein complexes with integrins [20,23,24].

CD63 (Tspan30) is a tumour suppressor expressed in endosomes and lysosomes and on the cell surface [25]. The trafficking of CD63 between the cell surface and the internal membranes occurs via AP2, clathrin-coated pit-mediated endocytosis, or caveolae-mediated endocytosis, and it requires specific amino acid motifs present in the CD63 protein [24]. The cell surface expression of CD63 is mediated by tumor-associated antigen L6, L6-Ag [25]. CD63 is abundantly expressed as a surface antigen in the early stage of melanoma, but its expression decreases with malignant progression [26], suggesting a negative correlation between CD63 surface levels and invasiveness.

Transmembrane 4 L6 family member 5 (TM4SF5) is highly expressed in pancreatic tumor, hepatocarcinoma, and colon carcinoma as compared with normal tissues. TM4SF5 is a homolog of tumour-associated antigen L6 (TM4SF1) and forms a 4-transmembrane L6

superfamily with L6, IL-TMP, and L6D.[5] TM4SF5 expression causes morphological changes (with aberrant actin reorganization) and EMT [4,27], mediates aberrant growth in multilayers, accelerates G1 - S phase progression [4,6] and enhances cellular migration and invasion [7]. Because more than 80% of HCC cases are associated with advanced fibrosis or cirrhosis [4,13], it is possible that TM4SF5 is involved in both liver fibrosis and tumorigenesis. Additionally, an anti-TM4SF5 reagent, 4-(*p*-toluenesulfonylamido)-4-hydroxychalcone (TSAHC), has been identified and shown to inhibit TM4SF5-mediated multilayer growth and migration [28]. TSAHC was originally screened as an a-glycosidase inhibitor [29], and appears to affect the structural integrity or N-glycosylation (at Asn138 / Asn155) of extracellular loop 2 (EC2) within TM4SF5, which appears to be important for multilayer growth and migration [28]. Considering its roles in multiple aspects of liver carcinogenesis, it is of interest to examine the regulatory mechanisms involved in TM4SF5 expression. Also, similar to tetraspanins, TM4SF5 cooperates with other membrane receptors such as integrins and growth factor receptors to modulate cell adhesion and migration [5]. But, any hierarchy among these tetraspan(in)s has not been reported.

In this study, I hypothesized that TM4SF5 might be induced by soluble factors and subsequently cause EMT. I examined how the expression of TM4SF5 and the resulting induction of EMT were regulated using cell and animal systems. I found that TM4SF5 was induced by CCl_4 administration, and appeared to correlate with TGFβ

1-mediated Smad signaling pathways, EMT marker expression, and collagen type I deposition. Induction of these fibrotic features by CCl_4 administration was attenuated by treatment with the anti-TM4SF5 reagent TSAHC. And, TM4SF5 was induced by crosstalk between TGFβ1-mediated Smads and EGFR (epidermal growth factor receptor) signalling and that this induction leads to the acquisition of mesenchymal cell features. Further I found that impaired TM4SF5 expression and function abolished the acquisition of mesenchymal cell features. In addition, I examined the correlations between TM4SF5, CD151, and CD63 expression using normal Chang hepatocytes that do not express TM4SF5, chronically TGFβ1- treated Chang cells that do express TM4SF5 [31], and other hepatocyte cells. I found that TM4SF5 expression could override CD151 functions, and TM4SF5 acted antagonistically to CD63 during liver fibrosis development and during hepatic migration/invasive extracellular matrix (ECM) -degradation.

Materials and Methods

Cell culture

Normal human Chang hepatocytes, murine AML12 hepatocytes, chronically TGFβ1-treated Chang cells (Chang-TGFβ1), hepatocellular carcinoma Huh7, Hep3B, SNU449 and non-small cell lung cancer (NSCLC) HCC827 cells were maintained in DMEM-H (Dulbecco's modified Eagle's medium, high glucose; WelGene) supplemented with 10% FBS (fetal bovine serum) and gentamycin/streptomycin (Invitrogen). Chang, SNU449, and HCC827 cells do not express TM4SF5, whereas Chang-TGFβ1, Huh7, and Hep3B cells express TM4SF5. Cells including stable Huh7-shScramble (TM4SF5-expressing) or Huh7-shTM4SF5 (TM4SF5-suppressed) cells were maintained in RPMI-1640 (WelGene, Daegu, Korea) containing 10% FBS and antibiotics (Invitrogen, Grand Island, NY, USA). LX2 cells (a gift from Dr Scott Friedman, Mount Sinai School of Medicine, New York, NY, U.S.A.) were cultured in DMEM-H containing 2% FBS, 1% glutamine and 25 mg/ml gentamycin (Invitrogen). Conditioned medium from LX2 cell cultures was prepared by incubating the cells with DMEM-H containing 0.2% FBS for 24 h.

Cell extract preparation

Cells were serum-deprived for 4 h, and then treated with different concentrations of TGFβ1 (BioSource International, Camarillo, CA, USA) or 50 ng/mL EGF (PeproTech, Rocky Hill, NJ, USA) in

serum-free medium or 10% FBS for the indicated times or for 24 h prior to whole cell lysate preparation. In some cases, cells were pretreated with dimethylsulfoxide (DMSO) or pharmacological inhibitors for 30 min or infected with adenovirus encoding Smads (tagged with FLAG) or LacZ (a negative control) for 20 h, before TGFβ1 treatment under serum-free conditions. Pre-treatment with 100 nM AG1478 (an EGFR kinase inhibitor) was also performed 30 min before TGFβ1 treatment. Control medium (DMEM-H containing 0.2% FBS as a negative control) or conditioned medium from LX2 cultures were added to AML12 cells for 12 or 24 h before whole cell lysate preparation. Chang cells were infected with FLAG - Smad4 for 24 h and then serum-starved for 4 h at 4℃ before TGFβ1 treatment (2.5 ng/ml for 24 h), or treated with cycloheximide (100 μg/ml) for 24 h in the absence of serum, prior to whole cell lysate harvest. In cases of extracellular antigen depletion or antibody neutralization, anti-TGFβ1 (20 μg/ml; R&D Systems) or anti-EGF neutralizing antibody (20 μg/ml; Millipore) was added to culture medium prior to harvest, TGFβ1 treatment or LX2-conditioned medium treatment. Cells were transfected with a control shRNA (small hairpin RNA) of a scramble sequence, TM4SF5 or EGFR (Santa Cruz Biotechnology) for 24 h, before TGFβ1 treatment (2.5 ng/ml) for an additional 24 h. Cells were transfected with pEGFP-control, shSmad2, shSmad3 or shSmad4 (Addgene) for 24 h, and infected with FLAG-tagged Smad4 adenovirus for 12 h before TGFβ1 treatment with or without AG1478 for an additional 24 h, and whole cell lysates were harvested. And,

cells were transiently transfected with control shRNA or shRNA against TM4SF5, CD151, or CD63) separately or in combination with each shRNA or cDNA plasmid encoding for FLAG-TM4SF5, Strep-TM4SF5, CD151, or CD63 for 48 h. Whole cell lysates and tissue extracts from human livers were prepared with RIPA buffer containing 0.1% SDS, 0.5% deoxycholate, and 1% NP-40.

Animal liver tissue extracts

Mice were housed in a specific pathogen-free room with controlled temperature and humidity. All animal procedures were performed in accordance with the procedures of the Seoul National University Laboratory Animal Maintenance Manual and institutional review board agreement. Mice were killed with ether, and the tissues were resected and frozen in liquid N2 until preparation of whole tissue extracts was performed, as described above.

Standard western blots

Cells or liver tissue extracts were normalized for protein concentrations and subjected to standard western blotting with antibodies against α-tubulin, p27Kip1 (BD Transduction Laboratories, Bedford, MA, USA), α-SMA or vimentin (Sigma, St Louis, MO, USA); phospho-Smad2, phospho- Smad3, pS10p27Kip1, snail2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Erk1/2, phospho- Erk1/2, PDGFRa (Cell Signaling Technology, Danvers, MA, USA), ZO1, Smad2, Smad3 (Zymed Laboratories, South San Francisco, CA, USA);

EGFR (Upstate Biotechnology, Billerica, MA, USA), pTyr992-EGFR, pTyr1068-EGFR (Cell Signaling Technology), pTyr1173-EGFR (Santa Cruz Biotechnology), collagen I (Chemicon, Billerica, MA, USA); anti-CD151, anti-CD63, anti-pTyr416c-Src, anti-FLAG (Cell Signaling Technol. Danvers, MA, USA), anti-FAK (BD Transduct. Lab., Bedford, MA, USA), anti-pTyr397FAK (Abcam, Cambridge, UK), anti-c-Src, anti-pTyr577-FAK (Santa Cruz Biotech., Santa Cruz, CA, USA), and anti-TM4SF5

TM4SF5 promoter transcriptional activity analysis

Cells were transiently cotransfected with pGL-human TM4SF5 promoter (−3.2 kb to +0.5 kb fragment in pGL3) and pBabe-β-galactosidase for 24hr. Control vector without any insert was also used to compansate the DNA amount. Luciferase activity was analyzed using a luminescence reader and normalized using β-galactosidase activity for the transfection efficiency between the experimental conditions.

Immunofluorescence

Cells were plated on to normal-culture-medium-precoated glass coverslips and incubated at 37℃ overnight to achieve typical cell adhesion and spreading prior to TGFβ1 treatment (2.5 ng/ml) for 24 h and immunostaining with anti-α-SMA, anti-vimentin, anti-β-catenin (Sigma), anti-E-cadherin (Zymed Laboratories) and anti-TRITC-conjugated mouse IgG antibody (Molecular Probes). Huh7

cells were transfected with shRNA of a control scrambled sequence or TM4SF5 sequence, and transfection-positive cells were enriched by G418 addition (500 μg/ml) for 1 week. Huh7 cells on serum-precoated coverslips were treated with either vehicle or HGF for 24 h, prior to immunostaining for β-catenin at cell - cell contact sites. Chang cells grown on glass coverslips without or with transient transfection with FLAG-TM4SF5 or CD151 for 48 h or cells on coverslips were immunostained using antibody against CD151, CD63, TM4SF5, and/or FLAG, in addition to DAPI staining for nucleus. In cases, LAMP2, a lysosomal marker, was immunostained using anti-LAMP2 (Abcam, Cambridge, UK). IF studies were performed according to standard protocol. Cells were fixed 4% paraformaldehyde or 100% methanol for 10 min and permeabilized with 0.1% Triton X-100 for 5min and blocked with 5% BSA. After blocking, cells were stained with each antibody. Immunofluorescent images were acquired on a fluorescent microscope (BX51TR, Tokyo, Olympus) or a confocal laser scanning microscope (Nikon C2, Nikon, Tokyo, Japan).

Flow Cytometry

Cells were analyzed by flow cytometry for tetraspanin expression profiles. Primary antibodies used included anti-CD151, anti-CD63, anti-CD9, and anti-TM4SF5 (Clone # 27, anti-TM4SF5 mAb)

Transwell Migration Assay

Cells transfected with diverse shRNA or plasmids were processed

for the Transwell migration assay using 8μm pore transwells (Corning, Corning, NY, USA). The migration assay was performed for 18 h with normal 10% FBS-containing media in the lower chambers. Migrating cells were stained and visualized using microscopy, and representative images were obtained. Mean values 6 standard deviation were evaluated from randomly saved images and were graphed.

ECM-Degradation Analysis

ECM-degradation by cells on coverslips precoated with Oregon Green 488-conjugated-gelatin (Invitrogen) was analyzed for 18 h.

Animal experiments for liver fibrosis

Four-week-old mice (BALB/c) were purchased from Orient (Seungnam, Korea). Mice were housed in a specific pathogen- free room with controlled temperature and humidity. All animal procedures were performed in accordance with the procedures of the Seoul National University Laboratory Animal Maintenance Manual and IRB agreement. Mice aged 5 weeks (n = 5) were injected intraperitoneally with or without CCl_4 (Sigma; 1 m/kg)1) in 40% olive oil three times a week. For the TSAHC treatment experiments, TSAHC administration (intraperitoneally at 50 mg/kg)1 in 40% dimethylsulfoxide or orally at 250 mg/kg)1 in 5% carboxymethylcellulose) was performed on the day after each CCl_4 administration. After the indicated periods, mice were killed with

ether, the tissues were resected, and one piece of tissue was immediately frozen in liquid N2 and a second piece was embedded in paraffin. (SNU-100809-1, SNU-101229-1, 110503-1)

Tissue staining

Immunostaining of liver tissues was performed with primary antibody specific for phospho-Smad2/3 (Santa Cruz Biotechnology), α-SMA (Sigma), TGFβ1 (PeproTech), CD151, CD63 or TM4SF5. Alternatively, the tissues were processed for Masson's Trichrome and hematoxylin and eosin staining as previously described, or for double-immunofluorescence staining for TM4SF5 and either α-SMA, PDGFRα, CD151 or CD63. Incubation with primary antibody against TM4SF5 was followed by incubation with anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate, and incubation with antibody against α-SMA, PDGFRα, CD151 or CD63 was followed by incubation with anti-mouse IgG or anti-rabbit IgG conjugated with fluorescein isothiocyanate, respectively. In addition, the nucleus was stained with 4',6-diamidino-2- phenylindole (DAPI).

Statistical analysis

Student's *t*-tests were performed for statistical comparisons of mean values to determine significance. A *P* value less than 0.05 was considered statistically significant.

Results

1. TM4SF5 is expressed during CCl_4-mediated liver injury

TM4SF5 is involved in EMT and liver cancer [29,33]. Therefore, it is likely that TM4SF5 is involved in the development of liver fibrosis. To test this, I examined TM4SF5 expression and fibrotic features in the CCl_4-mediated mouse model of hepatic injury. Whereas vehicle-treated control mice showed no signs of hepatic injury, CCl4-treated mice developed severe liver injury and fibrosis, as shown by the prominent steatosis of hepatocytes and centrilobular bridging fibrosis (although the degree of fibrosis varied between individual mice) after 1-4 weeks of CCl_4 administration (n = 5; Fig. 1A). As expected, CCl_4-treated, but not vehicle-treated, livers also showed collagen type I deposition along the fibrotic septa that ran between the areas of centrilobular bridging fibrosis and at areas of centrilobular hepatic necrosteatosis (Fig. 1B). Because TGFβ1 is a well-known multifunctional cytokine that plays important roles in the development of liver diseases [10], I measured TGFβ1 expression in the livers after CCl_4-administration. TGFβ1 production in CCl_4-treated livers, but not in vehicle-treated controls, occurred in parallel with collagen I deposition around the fibrotic septa (Fig. 2A). Phospho-Smad2/3 was observed in the nuclei of cells in the fibrotic septa of CCl_4-treated livers, but not in vehicle-treated control livers

(Fig. 2B). Expression of α-SMA was also observed in parallel with collagen I and TGFβ1 staining in CCl_4-treated livers, but not in vehicle-treated control livers (Fig. 2C). Next, I examined whether the CCl_4-treated livers also expressed TM4SF5 by immunohistochemistry. Controls without the primary antibody or with normal IgG did not give any significant staining signals, whereas antibody against TM4SF5 gave specific positive staining along the fibrotic septa of CCl_4-treated livers. TM4SF5 expression was detected along the fibrotic septa soon after the start of CCl_4 administration, and later expanded to include the regenerative hepatic area around the septa, whereas vehicle-treated control livers did not show any significant expression of TM4SF5 (Fig. 2D). This concomitant expression of TGFβ1, collagen I, α-SMA and TM4SF5 along the fibrotic septa in CCl_4-treated livers, but not in vehicle-treated control livers, strongly suggests that TM4SF5 is involved in CCl_4-mediated mouse liver injury and fibrosis. When double-immunofluorescence staining was performed for TM4SF5 and α-SMA, the two proteins were generally colocalized in liver tissues from CCl_4-treated mice, but not in those from control vehicle- treated mice (Fig. 2E). Furthermore, in another double-immunostaining experiment, colocalization between TM4SF5 and the mesenchymal cell marker platelet-derived growth factor receptor-α (PDGFRα) was also obvious in liver tissues from CCl_4-treated mice, but not in those from control vehicle-treated mice (Fig. 2F). In addition, PDGFRα expression was increased in liver tissues from CCl_4-treated mice, but not in those from control mice

(Fig. 2G). These observations suggest that TM4SF5 expression was induced concomitantly with that of mesenchymal cell markers during the CCl_4-mediated development of liver fibrosis.

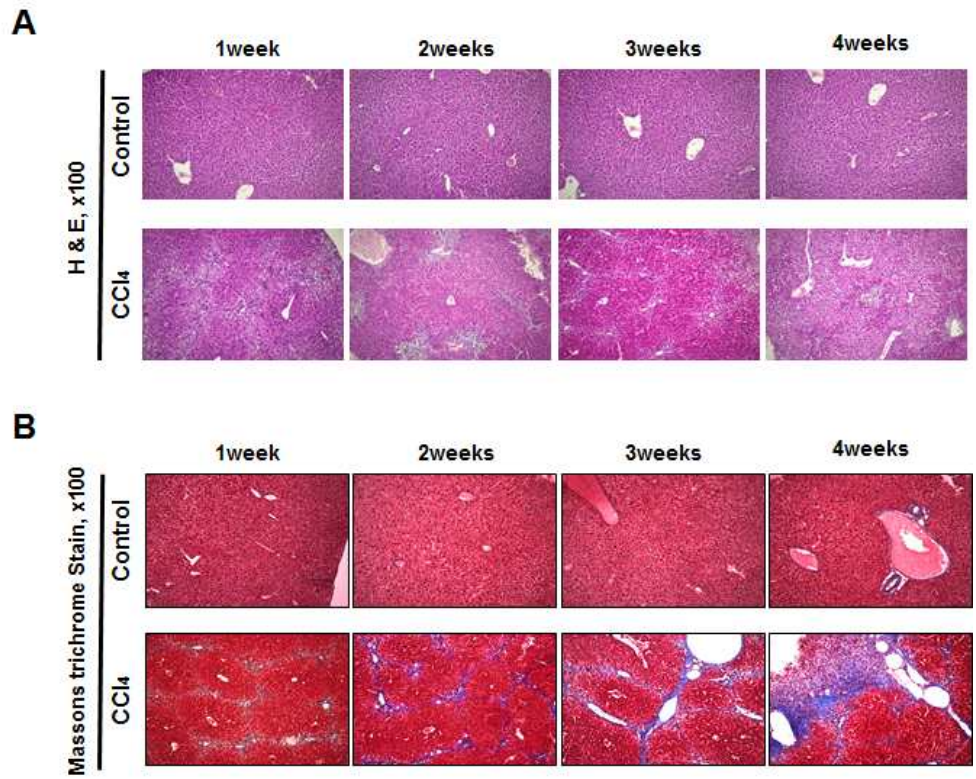
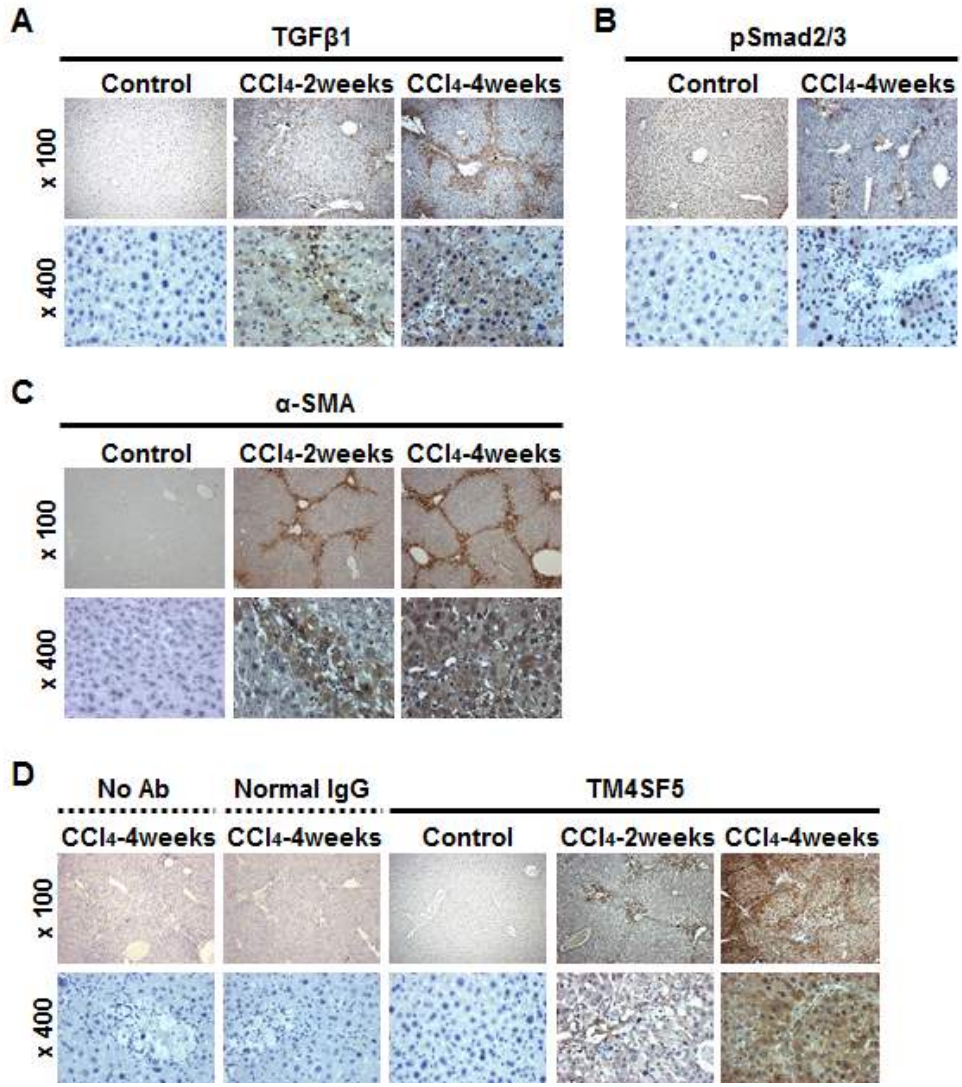


Fig 1. Liver injury and collagen I deposition in CCl_4-treated mice.

Livers from mice treated with vehicle (Control) or CCl_4 for the indicated times (1-4 weeks) were stained with hematoxylin and eosin (H&E) (A) or processed for Masson's Trichrome staining (B). Visualization of the representative images was performed at magnifications of x100.


A
TGFβ1
Control
CCl4-2weeks
CCl4-4weeks
x 100
x 400
B
pSmad2/3
Control
CCl4-4weeks
C
α-SMA
Control
CCl4-2weeks
CCl4-4weeks
x 100
x 400
D
No Ab
Normal IgG
TM4SF5
CCl4-4weeks
CCl4-4weeks
Control
CCl4-2weeks
CCl4-4weeks
x 100
x 400

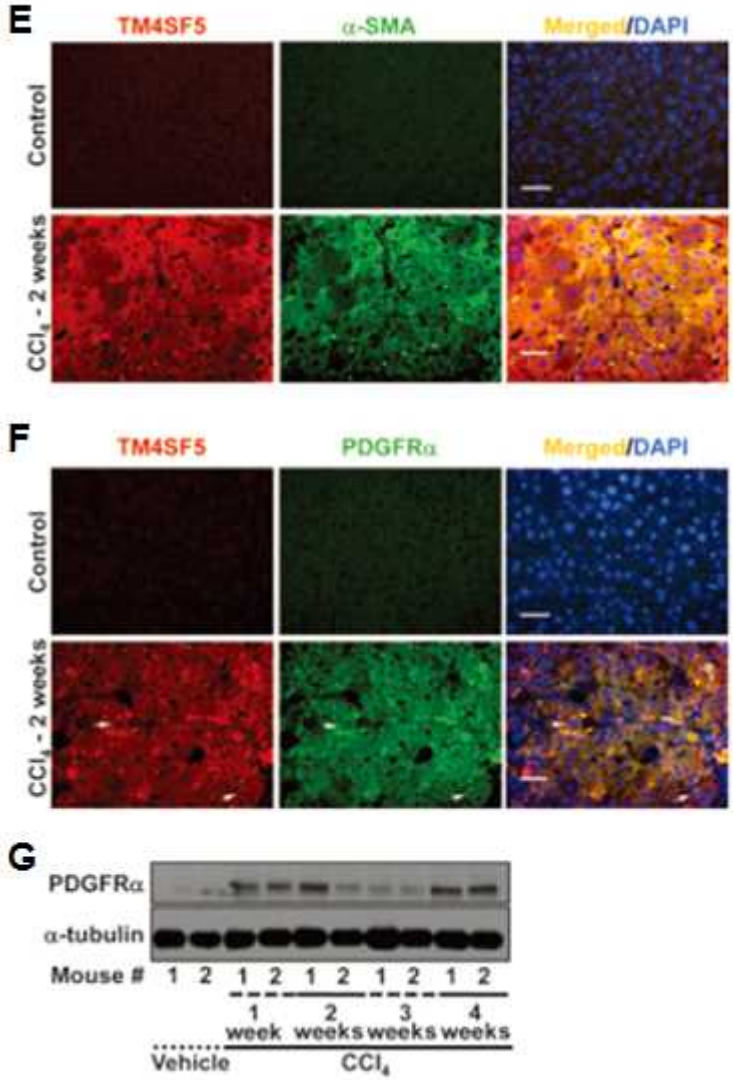

E
TM4SF5
α-SMA
Merged/DAPI
Control
CCl4 - 2 weeks
F
TM4SF5
PDGFRα
Merged/DAPI
Control
CCl4 - 2 weeks
G
PDGFRα
α-tubulin
Mouse # 1 2 1 2 1 2 1 2 1 2
1 week
2 weeks
3 weeks
4 weeks
Vehicle
CCl4

Fig 2. Induction of TM4SF5 expression in CCl_4-treated mouse liver.

(A to D) Livers from mice treated with vehicle (Control) or CCl_4 for the indicated times were processed for immunohistochemistry against TGFβ1 (A), phospho-Smad2/3 (B), α-SMA (C), or TM4SF5 (D). No Ab: no primary antibody. Normal IgG: normal rabbit IgG. Control liver: livers from mice treated with vehicle, but not with CCl_4. (E, F) Control or CCl_4-treated mouse liver tissues were processed for double-immunostaining of TM4SF5 and either α-SMA (C) or PDGFR α (F). Scale bars: 50 μm. (G) Vehicle-treated control or CCl_4-treated mouse liver tissues were processed for standard western blot analysis of PDGFRα and α-tubulin. The data represent three isolated experiments.

2. CCl_4-mediated liver fibrosis is attenuated by the anti-TM4SF5 compound TSAHC

Our laboratory previously reported that the anti-TM4SF5 reagent TSAHC could block TM4SF5-mediated multilayer growth and migration [28]. I therefore investigated whether TSAHC could also block liver injury, α-SMA expression and collagen I deposition in livers of the CCl_4-treated mice. Mice (n = 5) were treated with CCl_4 every other day for 1-4 weeks. Dimethyl sulfoxide vehicle, TSAHC or a control compound (4′-amino-4-hydroxychalcone, lacking functional groups) was orally or intraperitoneally administered to mice 1 day after each CCl_4 treatment. CCl_4 alone caused the formation of severe fibrotic septa between areas of centrilobular bridging fibrosis (Fig. 3A), and these areas of fibrosis showed collagen I expression and deposition (Fig. 3B,C), and α-SMA expression (Fig. 4A). Furthermore, expression of snail2 and vimentin mesenchymal markers was enhanced by CCl_4 administration, and was abolished by TSAHC but not by the control compound (Fig. 4B), indicating that TM4SF5-mediated functions during the development of EMT are also involved in CCl_4-mediated liver fibrosis, and that functional blocking of TM4SF5 can abolish the CCl_4-mediated mesenchymal features. Moreover, the dimethylsulfoxide vehicle and TSAHC alone did not induce any signs of fibrosis (Figs. 3A, 3B and 4A, upper panels). Importantly, oral or intraperitoneal administration of TSAHC (but not of the control compound) to mice treated with CCl_4 resulted in

reduced formation of fibrotic septa, which had less collagen I deposition and lower levels of α-SMA expression (Figs. 3A,B and 4A,B). Furthermore, biochemical determination of collagen I, snail2 and vimentin in the tissue samples gave similar results to the expression patterns shown by tissue staining (Figs. 3C and 4B). In a biochemically quantitative manner, the pattern of collagen I or α-SMA expression under diverse experimental conditions paralleled the expression levels determined by tissue staining (Figs. 3C and 4B). These results suggest that the TSAHC-mediated inhibition of TM4SF5 function can attenuate CCl_4-mediated liver fibrosis. Extracts from livers of CCl_4-treated mice with or without TSAHC treatment were then subjected to immunoblot analysis of molecules that are influenced by TM4SF5. TSAHC treatment did not reduce TM4SF5 expression, Smad phosphorylation or EGFR phosphorylation in livers of CCl_4-treated mice (Fig. 5A). This result was expected, because TSAHC appears to target EC2 of TM4SF5, where N-glycosylation residues important for protein - protein interactions and stabilization of cytosolic p27Kip1 for morphological changes are located [7,29], rather than regulate the expression of TM4SF5 (Fig. 5B). However, the enhancement of total p27Kip1 and pS10p27Kip1 levels for cytosolic stabilization induced by TM4SF5 [4] was reduced by treatment with TSAHC, but not by a control compound (Fig. 5A). Furthermore, CCl_4- mediated increases in α-SMA (Fig. 5A,C) and collagen I expression (Figs 3B,C and 5D) were abolished by TSAHC but not by the control compound. The TSAHC-mediated attenuation of α-SMA

expression and collagen I deposition provides further evidence that TM4SF5 can promote the fibrotic phenotypes observed in CCl_4-treated livers.

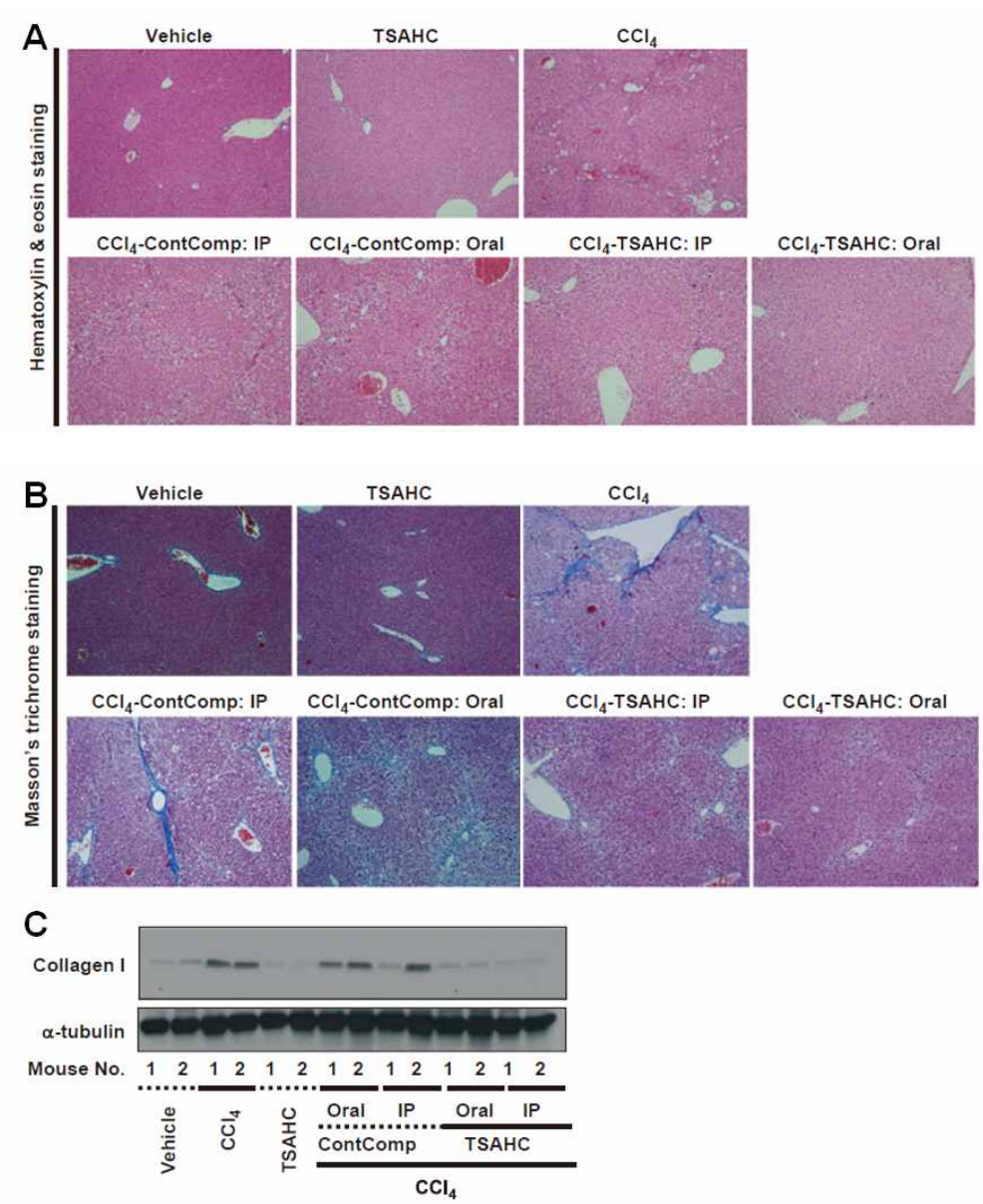


Fig 3. The anti-TM4SF5 reagent TSAHC attenuated liver injury and collagen I deposition.

Livers prepared from mice under different experimental conditions, as described in Experimental procedures, were processed for hematoxylin and eosin staining (A) or Masson's Trichrome staining for collagen I (B), or harvested for standard western blot analysis of collagen I and α-tubulin in whole tissue lysates (C). ContComp, control compound; IP, intraperitoneal injection; Oral, oral administration.

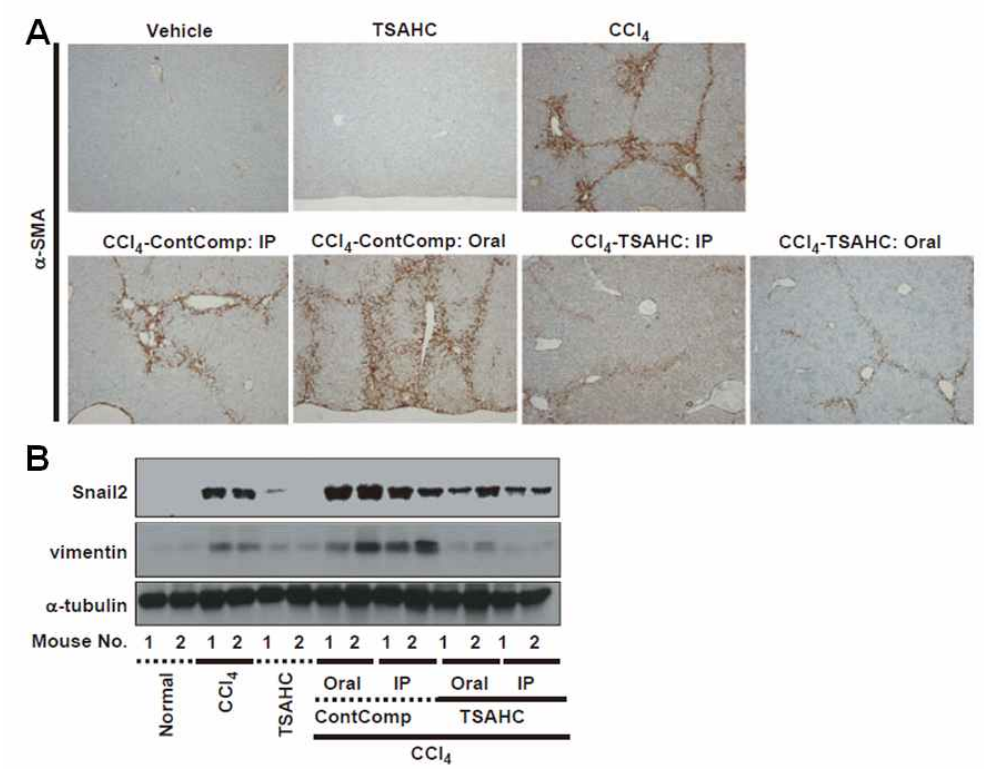


Fig 4. The anti-TM4SF5 reagent TSAHC attenuated α-SMA expression.

Livers prepared from mice under different experimental conditions were processed for immunohistochemistry for α-SMA (A) or harvested for whole tissue lysates for standard western blot analysis of snail2, vimentin, and α-tubulin (B).

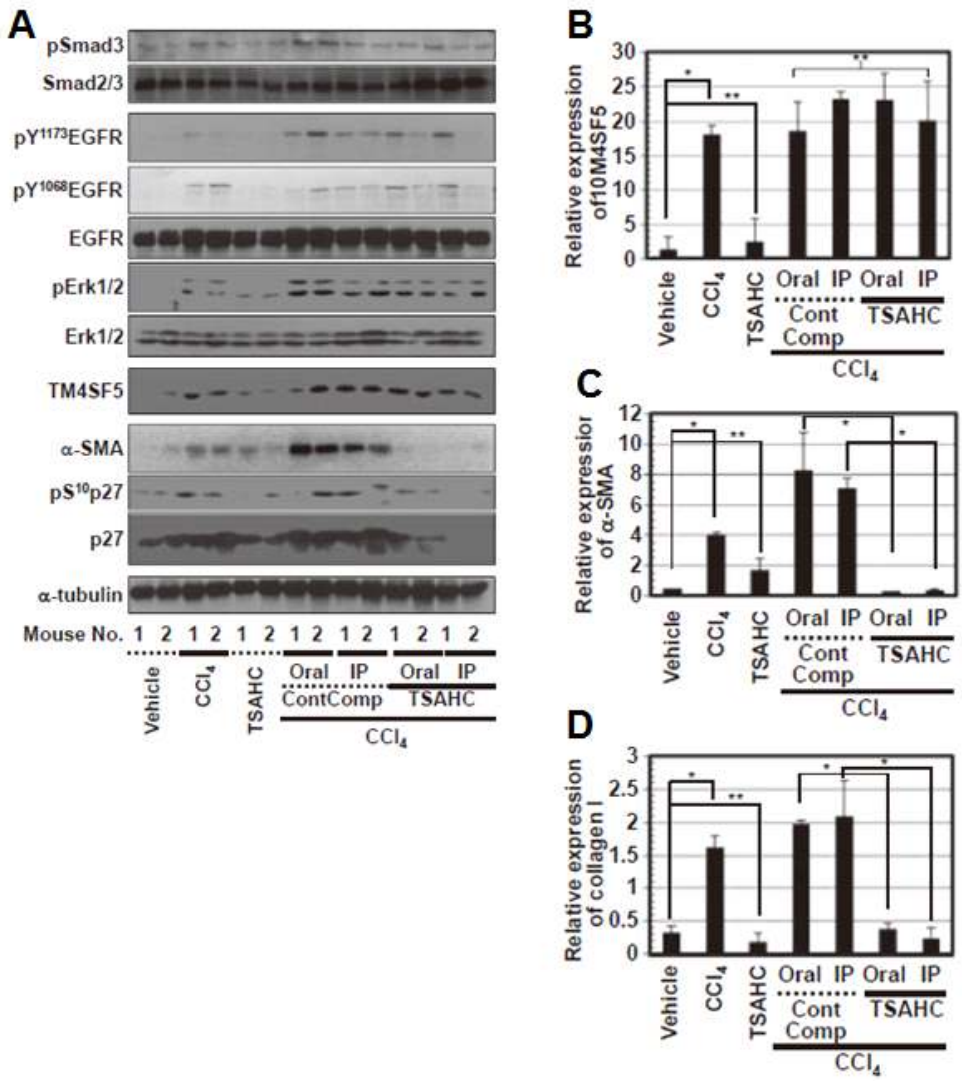

A
pSmad3
Smad2/3
pY1173EGFR
pY1068EGFR
EGFR
pErk1/2
Erk1/2
TM4SF5
α-SMA
pS10p27
p27
α-tubulin
Mouse No. 1 2 1 2 1 2 1 2 1 2 1 2 1 2
Vehicle
CCl4
TSAHC
Oral IP
Cont Comp
Oral IP
TSAHC
CCl4
B
Relative expression of10M4SF5
30
25
20
15
10
5
0
Vehicle
CCl4
TSAHC
Oral IP
Cont Comp
Oral IP
TSAHC
CCl4
C
Relative expressior of α-SMA
12
10
8
6
4
2
0
Vehicle
CCl4
TSAHC
Oral IP
Cont Comp
Oral IP
TSAHC
CCl4
D
Relative expression of collagen I
3
2.5
2
1.5
1
0.5
0
Vehicle
CCl4
TSAHC
Oral IP
Cont Comp
Oral IP
TSAHC
CCl4

Fig 5. TSAHC inhibited TM4SF5 function in EMT rather than its expression.

Liver extracts were prepared and immunoblotted for detection of the indicated molecules. Note that TSAHC did not alter TM4SF5 expression but blocked TM4SF5-mediated effects (A). The immunoblot bands for TM4SF5 (B), α-SMA (C) or collagen I (D) from liver tissues (n = 4) were quantitated to give mean ± standard deviation values for graphical presentations with IMAGEJ, although the immunoblots (A) included samples of n = 2, owing to the lane limitation of SDS/PAGE. *P-values $<$ 0.05, considered to be significant. **P-values $\geq$ 0.05, considered to be insignificant.

3. TGFβ1 induces TM4SF5 expression

Next, I investigated that how TM4SF5 expression is induced during liver injuries using cell system. TGFβ1 is a cytokine that plays important roles in both homoeostatic and pathological processes in the liver [10], it is possible that TM4SF5 is regulated by TGFβ 1-mediated signalling in liver malignancy. To examine this hypothesis, I first analysed the correlation between TGFβ1 signalling and TM4SF5 expression levels in hepatic cancer tissues. Liver tissues from hepatic carcinoma patients (n = 9) showed that certain tumours (6/9) expressed higher levels of both TM4SF5 and Smad2/3 phosphorylation than did normal hepatic tissues (Fig. 6A), suggesting a possible connection between TGFβ1 signalling and TM4SF5 expression in liver carcinogenesis. Transcriptional activation analysis of the *TM4SF5* promoter region (−3.2 kb to +0.5 kb) showed greater TGFβ1-mediated promoter activation (3-fold increase over basal levels) in hepatocarcinoma Huh7 cells (Figure 1B). TGFβ1-mediated activation of R-Smads (Smad2/3) was correlated with TM4SF5 induction in normal murine AML12 and human Chang hepatocytes upon TGFβ1 treatment, as TGFβ1 treatment induced TM4SF5 expression in a dose-dependent manner (Fig. 6C) without causing significant apoptosis (results not shown). Furthermore, this TGFβ 1-mediated induction of TM4SF5 expression was blocked by either the expression of inhibitory Smad7 (Fig. 6D). These observations suggest that TM4SF5 expression is induced by TGFβ1-mediated

signalling.

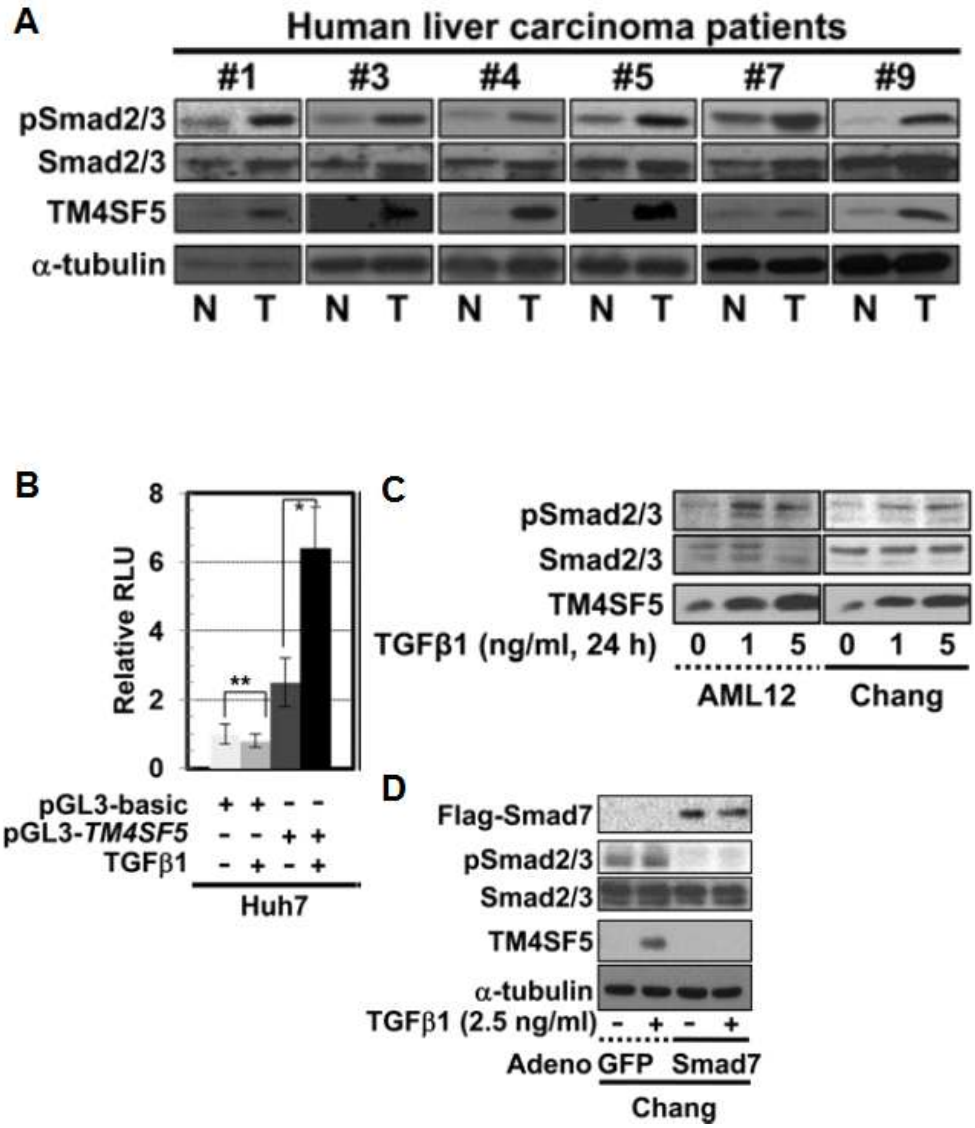

A
Human liver carcinoma patients
#1
#3
#4
#5
#7
#9
pSmad2/3
Smad2/3
TM4SF5
α-tubulin
N T N T N T N T N T N T
B
Relative RLU
8
6
4
2
0
*
**
pGL3-basic + + - -
pGL3-TM4SF5 - - + +
TGFβ1 - + - +
Huh7
C
pSmad2/3
Smad2/3
TM4SF5
TGFβ1 (ng/ml, 24 h) 0 1 5 0 1 5
AML12
Chang
D
Flag-Smad7
pSmad2/3
Smad2/3
TM4SF5
α-tubulin
TGFβ1 (2.5 ng/ml) - + - +
Adeno GFP Smad7
Chang

Fig 6. TGFβ1 signalling induces TM4SF5 expression.

(A) Extracts from human liver cancer and normal liver tissues were prepared and immunoblotted for the indicated molecules. (B) Assay for TM4SF5 promoter transcriptional activation in Huh7 cells was performed after TGFβ1 treatment in the absence of serum for 24 h. **P =0.423; *P =0.0002. (C) AML12 or Chang hepatocytes were serum-deprived for 4 h and treated with TGFβ1 (0-5 ng/ml) for 24 h prior to whole cell lysate preparation. (D) Chang cells were infected with adenovirus (Adeno) for either GFP or FLAG - Smad7 for 20 h and treated with (+) or without (-) TGFβ1 (2.5 ng/ml) in the absence of serum for an additional 24 h prior to harvests of whole cell lysates. Standard Western blots were performed for the indicated molecules. Results are representative for three independent experiments.

4. TGFβ1-induced TM4SF5 expression causes EMT

I next examined the biological effects of TGFβ1-mediated induction of TM4SF5 expression, especially with regard to EMT. When cells treated with TGFβ1 were examined for expression of cell-cell adhesion molecules and mesenchymal markers and compared with untreated cells,we found that TGFβ1-mediated TM4SF5 expression was accompanied by a loss of cell - cell adhesion molecules, including ZO1 and β-catenin in Chang cells and E-cadherin in AML12 cells (Figs. 7A and 7B). These TGFβ1-mediated effects also correlate with cell scattering (Figure 7C) and the increased expression levels of the mesenchymal markers α-SMA and vimentin (Figures 7A, 7B and 7D). In addition, localization of E-cadherin and β-catenin at the cell - cell contacts was diminished by TGFβ1 treatment of AML12 cells (Fig. 7D). These observations suggest that TGFβ1-mediated induction of TM4SF5 expression leads to the scattering of cells that express mesenchymal cell markers (i.e. EMT). I next examined whether impaired expression or function of TM4SF5 abolishes the induction of mesenchymal features. When TGFβ1 was administered to cells transfected with shRNA of a TM4SF5 sequence (shTM4SF5), the TGFβ1- induced expression of mesenchymal markers (i.e. vimentin and α-SMA) was lower than in control shRNA-transfected cells (Fig. 8A). Furthermore, TM4SF5-mediated functions, including multilayer growth and cell migration/invasion, are antagonized by the small synthetic compound TSAHC [29]. Thus I next tested whether

TSAHC could block the expression of the mesenchymal markers that correlate with TGFβ1-mediated TM4SF5 expression. α-SMA expression in TGFβ1-treated Chang cells was inhibited by treatment with TSAHC, but not with a control compound 4'-NH2-4-OH-Chal (4'-amino-4-hydroxychalcone; Fig. 8B). The TGFβ1-mediated increase in the expression of another mesenchymal marker, vimentin, was also blocked by TSAHC and its derivative ASAHC [4'-(4-aminobenzensulfonylamido)-4-hydroxychalcone], but not by another control compound 4',4-dihydroxychalcone (Di-OH chal., Fig. 8C). These observations indicate that TGFβ1-induced TM4SF5 expression regulates the expression of mesenchymal markers during EMT.

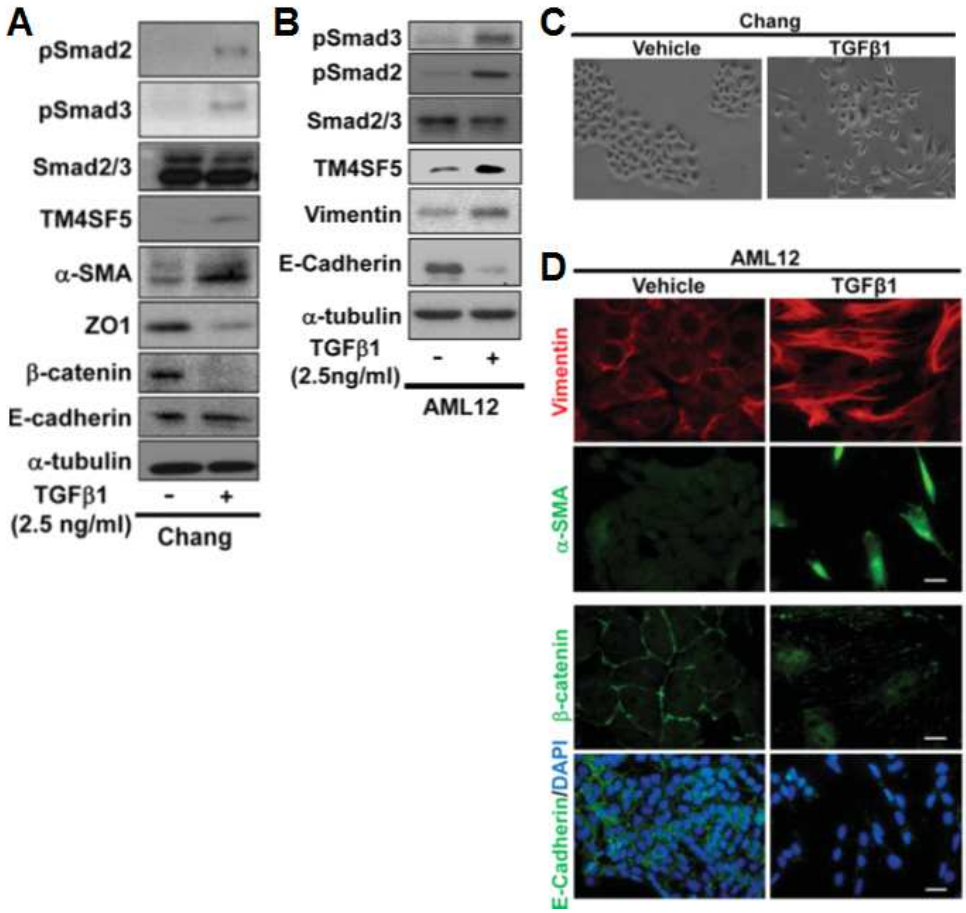

A
pSmad2
pSmad3
Smad2/3
TM4SF5
α-SMA
ZO1
β-catenin
E-cadherin
α-tubulin
TGFβ1
(2.5 ng/ml)
-
+
Chang
B
pSmad3
pSmad2
Smad2/3
TM4SF5
Vimentin
E-Cadherin
α-tubulin
TGFβ1
(2.5ng/ml)
-
+
AML12
C
Chang
Vehicle
TGFβ1
D
AML12
Vehicle
TGFβ1
Vimentin
α-SMA
β-catenin
E-Cadherin/DAPI

Fig 7. TGFβ1-mediated induction of TM4SF5 expression in hepatocytes results in acquisition of mesenchymal cell features.

Cells were serum-deprived for 4 h and TGFβ1 (2.5 ng/ml) was added in the absence of serum for 24 h prior to harvest of cell lysates from Chang or AML12 hepatocytes. Whole cell lysates were used for immunoblots for the indicated molecules (A and B), or cells were imaged (C) or processed by indirect immunofluorescence for vimentin, α-SMA, β-catenin, and E-cadherin (D). In case of E-cadherin, 4,6-diamidino-2-phenylindole (DAPI) staining for nuclei was performed in parallel. Note that TGFβ1-treated cells showed an accumulation of β-catenin in the nucleus, compared with localization at cell-cell contacts in untreated cells, and loss of E-cadherin at cell-cell contacts. Results are representative for three independent experiments.

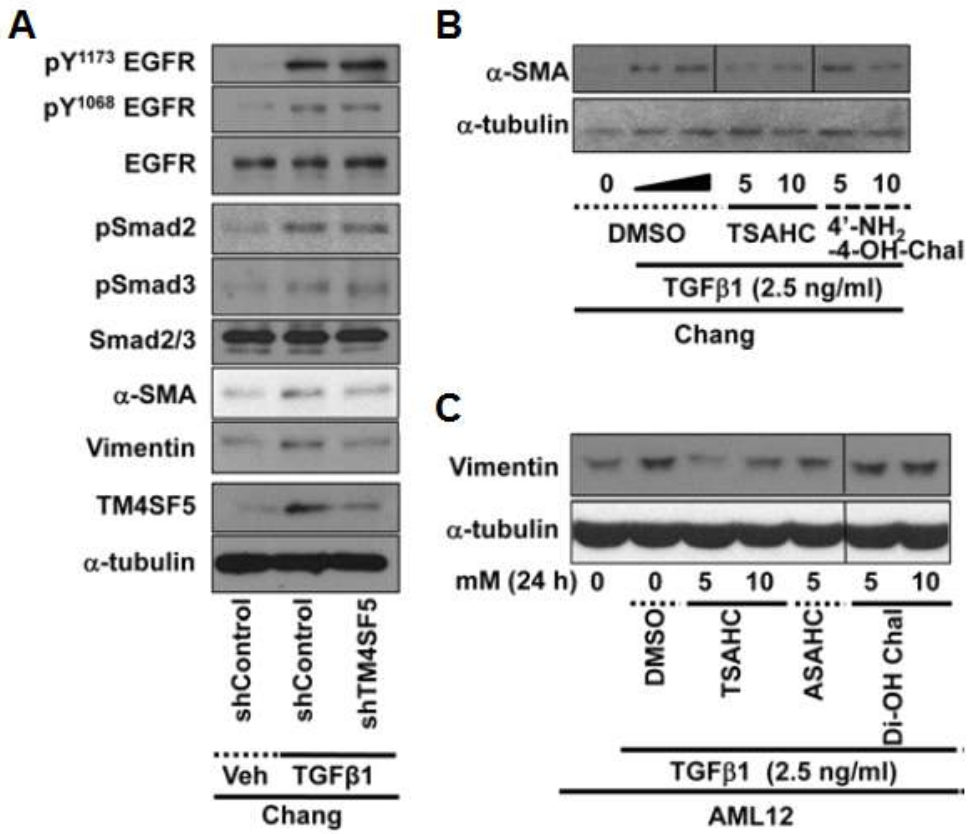


Fig 8 . Impaired TM4SF5 expression or function in hepatocytes blocks EMT.

(A) Chang cells were transiently transfected with shRNA of either a scrambled control sequence (shControl) or a TM4SF5 sequence (shTM4SF5) for 24 h, and either TGFβ1 or vehicle was added to the culture medium for an additional 24 h prior to harvests of whole cell lysates and standard Western blotting for the indicated molecules. (B and C) Cells were serum-deprived for 4 h, and cells were treated without (-) or with TGFβ1 (2.5 ng/ml, +) in the absence of serum for 24 h together with vehicle DMSO, control synthetic compounds

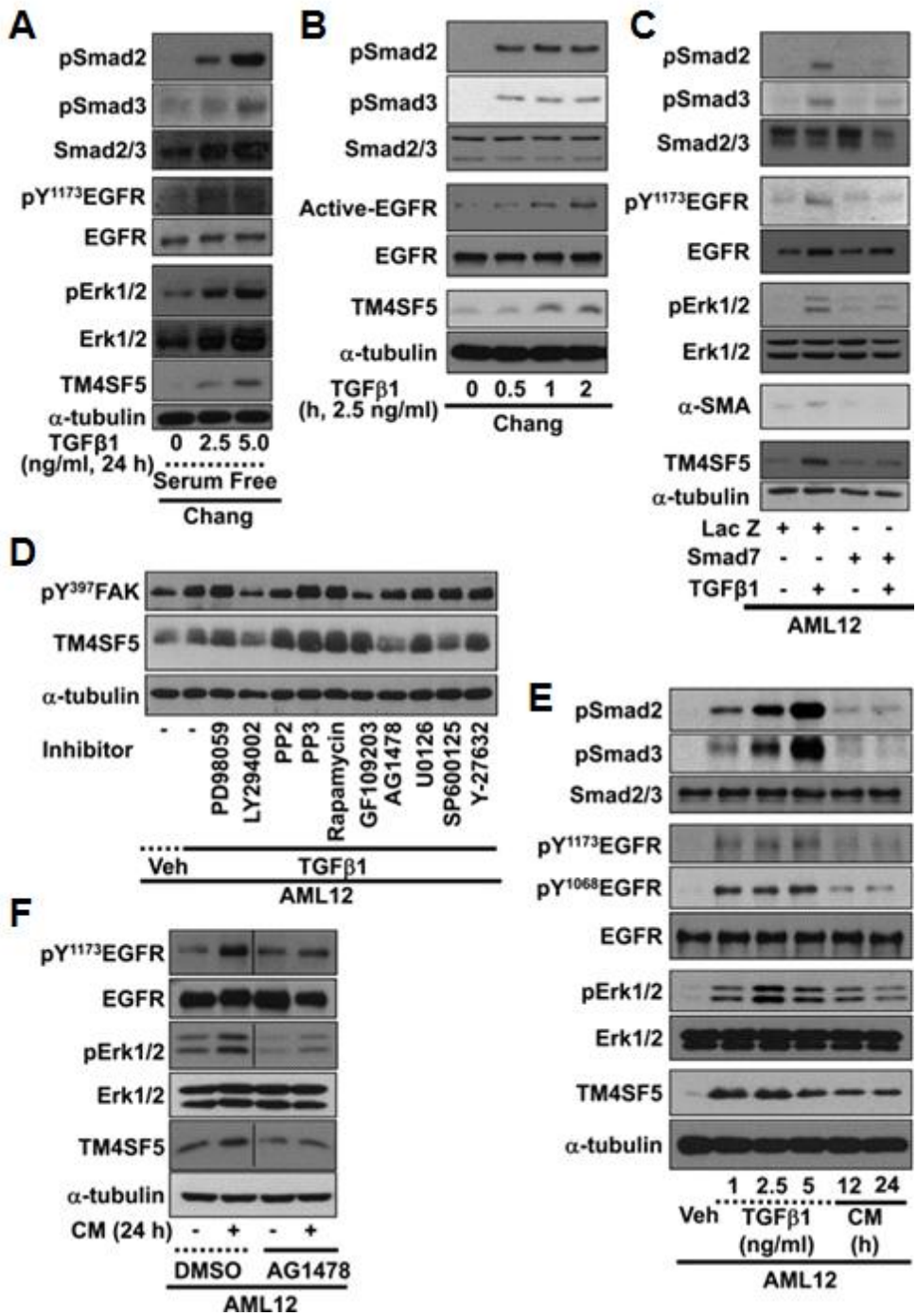
[4-NH2-4-OH-Chal or Di-OH Chal in (B) and (C) respectively] or anti-TM4SF5 compounds (TSAHC or ASAHC) at the indicated concentrations prior to harvest of whole cell lysates for standard Western blots. Results are representative for three independent experiments.

5. EGFR activation correlates with TGFβ1-mediated TM4SF5 expression

Next, I examined the signalling molecules that are involved in the TGFβ1- mediated induction of TM4SF5 expression. In the absence of serum, TGFβ1 induced TM4SF5 expression in Chang cells in a dose-dependent manner (Fig. 9A). Even under serum-free conditions, TGFβ1-mediated TM4SF5 expression correlated with EGFR phosphorylation and ERK1/2 activation (Fig.9A). Treatment with TGF β1 for shorter durations (i.e. <2 h) caused R-Smad and EGFR phosphorylation (i.e. 30 min) as well as an increase in TM4SF5 expression (Figure 9B), indicating that a signal was rapidly transduced from the TGFβ1 pathway to the EGFR signalling pathway during induction of TM4SF5 expression. EGFR signalling activation following TGFβ1 treatment in AML12 cells was abolished by inhibition of R-Smads through Smad7 expression as was the

expression of both TM4SF5 and α-SMA (Fig. 9C). The pharmacological inhibition of diverse signalling molecules was performed prior to TGFβ1 treatment of AML12 cells to evaluate the effects on TGFβ1-mediated induction of TM4SF5 expression. EGFR activity appeared to be important because EGFR kinase inhibition decreased TM4SF5 expression but not Tyr397 phosphorylation of FAK, which is important for cell survival (Fig. 9D). Meanwhile, PI3K inhibition decreased both TM4SF5 expression and pTyr397-FAK levels (Fig. 9D), suggesting that reduced TM4SF5 expression levels due to PI3K inhibition may be the result of global cytotoxicity. These data suggest that EGFR activation could be involved in TGFβ 1-mediated induction of TM4SF5 expression. HSCs (hepatic stellate cells) in the liver are the main cells that produce TGFβ1 [35]. Therefore I examined whether conditioned medium prepared from activated HSCs could induce TM4SF5 expression. The human HSC line LX2 is known to be activated [36]. Thus I treated AML12 cells with either TGFβ1 or conditioned medium prepared from LX2 cell cultures and analysed TM4SF5 expression levels. Compared with control non-conditioned medium (vehicle), LX2-CM treatment increased R-Smads and EGFR phosphorylation (e.g. pTyr1068 or pTyr1173 eventually leading to ERK1/2 activation [35]) and TM4SF5 levels, although TM4SF5 levels after conditioned medium treatment were lower than those after TGFβ1 treatment (Fig. 9E). Furthermore, pharmacological inhibition of EGFR kinase prior to LX2-conditioned medium treatment blocked EGFR/ERKs phosphorylation and TM4SF5

expression (Figure 9F). These LX2-conditioned medium studies suggest that TM4SF5 expression is biologically involved in liver pathology, as TM4SF5 expression can be induced by soluble factors such as TGFβ1 in the liver, leading to EMT.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pSmad3
Smad2/3
pY1173EGFR
EGFR
pErk1/2
Erk1/2
TM4SF5
α-tubulin
TGFβ1 (ng/ml, 24 h)
0 2.5 5.0
Serum Free
Chang
B
pSmad2
pSmad3
Smad2/3
Active-EGFR
EGFR
TM4SF5
α-tubulin
TGFβ1 (h, 2.5 ng/ml)
0 0.5 1 2
Chang
C
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pSmad3
Smad2/3
pY1173EGFR
EGFR
pErk1/2
Erk1/2
α-SMA
TM4SF5
α-tubulin
Lac Z + + - -
Smad7 - - + +
TGFβ1 - + - +
AML12
D
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TM4SF5
α-tubulin
Inhibitor
- -
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TGFβ1 (ng/ml)
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AML12
F
pY1173EGFR
EGFR
pErk1/2
Erk1/2
TM4SF5
α-tubulin
CM (24 h) - + - +
DMSO
AG1478
AML12

Fig 9. TGFβ1 treatment-mediated induction of TM4SF5 expression correlates with EGFR/ERKs signalling activation.

(A and B) Chang cells were serum-deprived for 4 h and TGFβ1 [0, 2.5 or 5.0 ng/ml in (A) or 2.5 ng/ml in (B)] was added in the absence of serum for 24 h or the indicated times (B) prior to harvests. (C) Cells were infected with control LacZ or FLAG - Smad7 adenovirus for 20 h and treated with TGFβ1 (2.5 ng/ml) for another 24 h prior to harvest. (D) Cells were serum-starved for 4 h and treated with TGFβ1 (2.5 ng/ml) and either vehicle (Veh) DMSO or different pharmacological inhibitors (pre-treated 30 min before TGFβ1 addition) for 24 h before harvest. (E) Cells were serum-deprived for 4 h and treated with TGFβ1 at the indicated concentrations for 24 h, control medium (Veh; 0.2% FBS-containing medium) or conditional medium (CM) from LX2 cell culture (24 h of culture in 0.2% FBS-containing medium) for 12 or 24 h before harvest. (F) Cells were treated with control medium (-) or conditioned medium (CM; +) from LX2 cell cultures and either DMSO or AG1478 (100 nM) for 24 h before whole cell lysate harvest. Results are representative for three independent experiments.

6. Signal transduction from TGFβ1-activated Smads to EGFR leads to elevated TM4SF5 expression and EMT

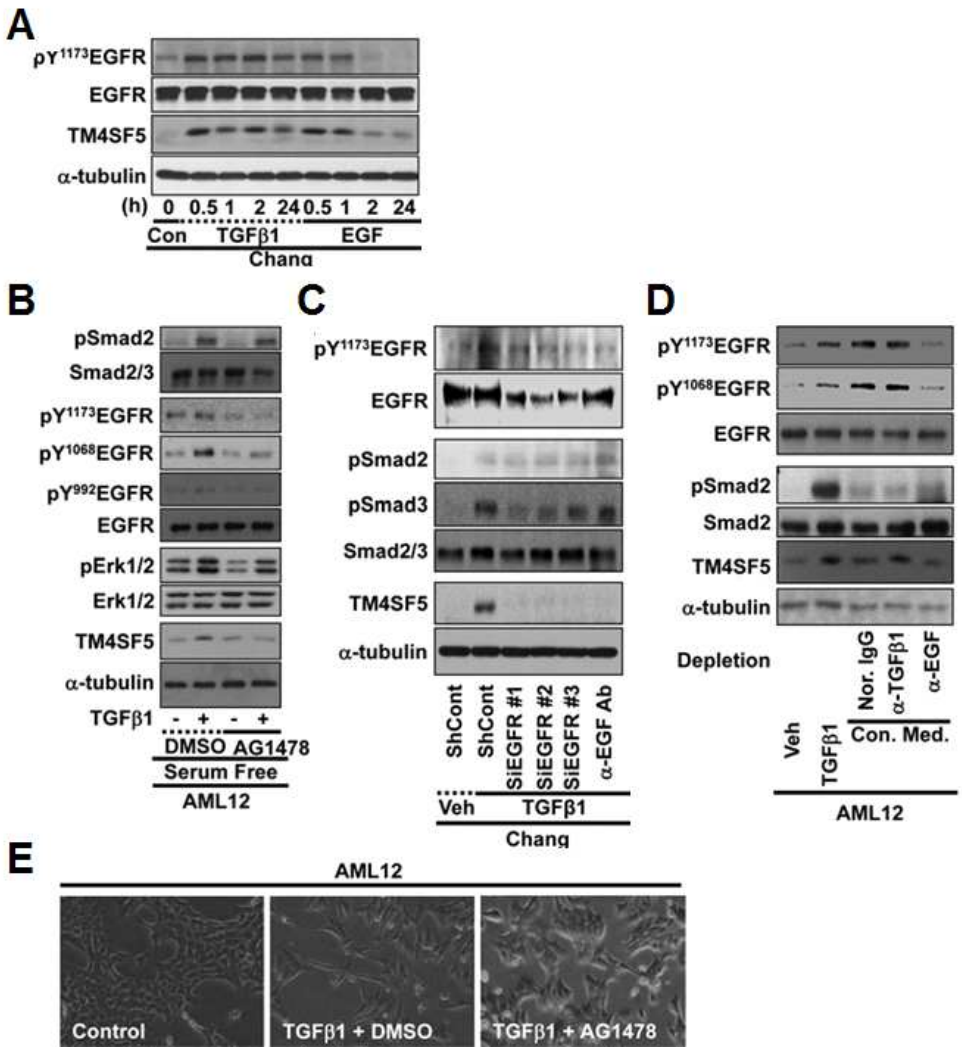
Since a signalling link was observed between TGFβ1-mediated Smads and EGFR activation, I wished to explore the mechanism involved. First, I examined whether either TGFβ1 or EGF could induce TM4SF5 expression. Treatment of Chang cells with TGFβ1 alone for different durations that ranged from 30 min to 24 h caused EGFR/ERKs activation and TM4SF5 expression (Fig. 10A). However, treatment with EGF alone only induced a transient (with a peak within 1 h after EGF treatment) expression of TM4SF5; when treatment was extended to 24 h, there was no significant increase in either EGFR phosphorylation or TM4SF5 expression, indicating a rapid termination of signal for EGF-mediated TM4SF5 induction (Fig.10A). Consistent with these results, treatment with EGF alone at concentrations ranging from 0 to 100 ng/ml for 4 h showed a very slight activation of EGFR/ERKs and induction of TM4SF5 expression (Fig. 10B). Furthermore, EGF and TGFβ1 co-treatment for 24 h resulted in a slight increase in TM4SF5 expression compared with TGFβ1 treatment alone (Fig. 10C). Interestingly, TGFβ1 and HGF co-treatment resulted in slightly increased TM4SF5 expression levels compared with TGFβ1 treatment alone, but co-treatment of TGFβ1 and platelet-derived growth factor had no effect (Fig. 10C). Moreover, the pharmacological inhibition of EGFR activity in murine AML12 cells treated with TGFβ1 abolished EGFR/Erk1/2 activity and

TM4SF5 expression, but Smad2 phosphorylation was unaltered (Fig. 10D). These observations indicate that TM4SF5 expression requires EGFR/Erks activation after TGFβ1-mediated R-Smad activation. Moreover, TGFβ1-mediated scattering of AML12 cells was blocked by the pharmacological inhibition of EGFR kinase activity (Fig. 10E). Furthermore, suppression of EGFR through siRNA or functional blockade of EGFR by anti-EGF antibody-mediated depletion of EGF after TGFβ1 treatment of Chang cells abolished TGFβ1-mediated EGFR phosphorylation and TM4SF5 expression (Fig. 10F), indicating that TGFβ1-mediated TM4SF5 induction might involve EGF secretion and EGFR activation. In addition, LX2-conditioned-medium induced EGFR phosphorylation and TM4SF5 expression were blocked by EGF depletion in conditioned medium, whereas application of anti-TGFβ1 antibody or normal IgG did not have any effect (Fig. 10G). These observations suggest that a signalling link between TGFβ1-mediated R-Smads and EGFR activation in which the signal is transduced from R-Smads to the EGFR is involved in the induction of TM4SF5 expression and the acquisition of mesenchymal cell features. To understand this link in more detail, exogenous Smads were introduced into cells, the cells were treated with TGFβ1 in the absence of serum, and both TM4SF5 expression and EGFR activation were analysed. Compared with control- virus infected AML12 cells, AML12 cells infected with FLAG - Smad2 adenovirus showed enhanced TGFβ 1-dependent TM4SF5 expression, whereas FLAG - Smad3 overexpression did not alter TM4SF5 levels (Fig. 11A, lanes 1 - 6),

indicating that Smad2 is the major R-Smad involved in TGFβ 1-mediated induction of TM4SF5 expression in AML12 cells, when Smads were overexpressed. Interestingly, overexpression of FLAG-Smad4 dramatically enhanced basal TM4SF5 expression to the extent that the TGFβ1-mediated induction of TM4SF5 expression was no longer obvious (Fig. 11A, lanes 7 and 8). In addition, EGFR phosphorylation levels correlated with TM4SF5 expression levels, even under serum-free conditions (Figure 11A). Since Smad4 overexpression alone (i.e. without TGFβ1 treatment) resulted in a dramatic induction of TM4SF5 expression (Fig. 11A, lane 7), I next examined the significance of each Smad in TGFβ1-mediated TM4SF5 expression with or without Smad4 overexpression. Chang cells were transfected with pEGFP-control, shSmad2, shSmad3 or shSmad4 for 24 h, and infected with FLAG-tagged Smad4 adenovirus for 12 h, prior to TGFβ1 treatment for an additional 24 h. Smad2 suppression enhanced both Smad3 phosphorylation and TGFβ1-mediated induction of TM4SF5 expression under both basal and Smad4- overexpression conditions (Fig. 11B, lanes 1-6). Additionally, Smad3 suppression slightly suppressed TGFβ1/Smad4-mediated induction of TM4SF5 expression without affecting EGFR phosphorylation, indicating that Smad3 might be involved in TGFβ1/Smad4-mediated induction of TM4SF5 expression in an EGFR-independent manner (Fig. 11B). More interestingly, suppression of Smad4 abolished TGFβ 1/Smad4-mediated EGFR phosphorylation and induction of TM4SF5 expression (Fig. 11B), suggesting a link from Smad4 expression to

EGFR activation. I examined whether FLAG-Smad-overexpression-mediated TM4SF5 expression could be blocked by EGFR kinase inhibitors. Under serum-free conditions, AG1478 pre-treatment decreased TGFβ1-mediated induction of TM4SF5 expression in both control cells and cells that overexpress either FLAG-Smad2 or FLAG-Smad4 (Fig. 11C). Therefore it is likely that the mechanism of TGFβ1-mediated TM4SF5 expression involves EGFR activation. Therefore I next investigated whether Smad4-overexpression-mediated EGFR phosphorylation and induction of TM4SF5 expression might be affected by depletion of extracellular EGF using anti-EGF antibody. Interestingly, Smad4 overexpression alone caused EGFR phosphorylation and TM4SF5 induction (Fig. 11A, lane 7), which were decreased by extracellular EGF depletion during cultures (Fig. 11D), suggesting that Smad4 expression might cause EGF secretion and EGFR activation, thus leading to TM4SF5 expression. Although there were no increases in the levels of EGFR after TGFβ1 treatment or Smad infection (Figs. 11A, 11A and 11C), TGFβ1 treatment of FLAG-Smad4-infected cells was performed at 4℃ to determine whether EGFR activation following TGFβ1 treatment involves the recycling or trafficking of EGFR between the cell surface and intracellular membranes. Even with TGFβ1 treatment at 4℃, Smad4 overexpression resulted in a very slight activation of EGFR/Erks and the induction of TM4SF5 expression with no increase in EGFR expression levels (Fig. 11E). To see whether *de novo* synthesis of EGFR (and rapid internalization, leading to unchanged

levels of EGFR) was achieved by Smad4 overexpression and was involved in basal TM4SF5 expression, cells were infected with FLAG - Smad4 adenovirus and treated with cycloheximide for 24h prior to harvest of cell extracts. Cycloheximide treatment maintained similar EGFR levels both before and after Smad4 overexpression, but blocked Smad4-induced TM4SF5 expression (Fig. 11F). These observations suggest that *de novo* EGFR synthesis is not involved in EGFR/Erks phosphorylation due to Smad4 overexpression.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pY1173EGFR
EGFR
TM4SF5
α-tubulin
(h) 0 0.5 1 2 24 0.5 1 2 24
Con TGFβ1 EGF
Chang
B
pSmad2
Smad2/3
pY1173EGFR
pY1068EGFR
pY992EGFR
EGFR
pErk1/2
Erk1/2
TM4SF5
α-tubulin
TGFβ1 - + - +
DMSO AG1478
Serum Free
AML12
C
pY1173EGFR
EGFR
pSmad2
pSmad3
Smad2/3
TM4SF5
α-tubulin
ShCont
ShCont
SiEGFR #1
SiEGFR #2
SiEGFR #3
α-EGF Ab
Veh
TGFβ1
Chang
D
pY1173EGFR
pY1068EGFR
EGFR
pSmad2
Smad2
TM4SF5
α-tubulin
Depletion
Nor. IgG
α-TGFβ1
α-EGF
Veh
TGFβ1
Con. Med.
AML12
E
AML12
Control
TGFβ1 + DMSO
TGFβ1 + AG1478

Fig 10. Signalling linkage from TGFβ1 to the EGFR signalling pathway is involved in TM4SF5 expression and EMT.

(A) Chang cells were serum-starved for 4 h and untreated (Con) or treated with either 2.5 ng/ml TGFβ1 or 50 ng/ml EGF for the indicated times (h) in the absence of serum before harvest. (B and E) Cells were pre-treated with DMSO or the EGFR kinase inhibitor AG1478 (100 nM) for 30 min before vehicle (control, -) or TGFβ1 treatment (2.5 ng/ml, +) for 24 h, and lysates were prepared for Western blotting (B) or cell images were recorded (E). (C) Chang cells were transfected with control shRNAs of either a scramble sequence (ShCont) or EGFR (SiEGFR) for 24 h, before TGFβ1 treatment (2.5 ng/ml) for an additional 24 h in the absence or presence of anti-EGF antibody (Ab) pre-treatment. Whole cell lysates were prepared for standard Western blots for the indicated molecules. (D) AML12 cells were treated with vehicle (0.2%-FBS-containing DMEM-H), TGFβ1 or LX2-conditioned medium (with 0.2%-FBS-containing DMEM-H, Con. Med.) for 24 h. The conditioned medium had been pre-treated without or with normal IgG (Nor. IgG) or antibody against TGFβ1 (α-TGFβ1) or EGF (α-EGF). Whole cell lysates were prepared and used for standard Western blot analysis. Data represent three independent experiments. Veh, vehicle.

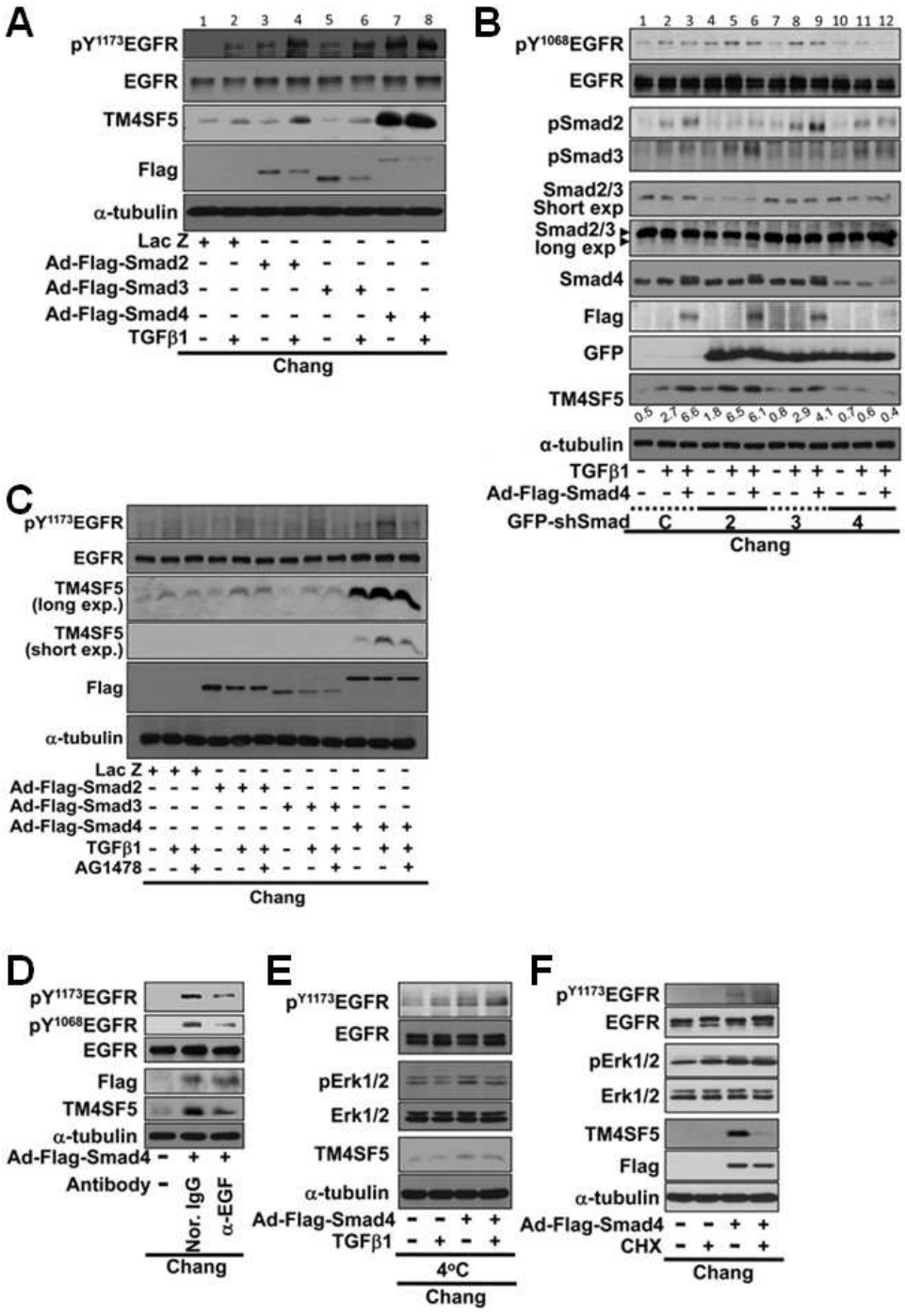

A
1 2 3 4 5 6 7 8
pY1173EGFR
EGFR
TM4SF5
Flag
α-tubulin
Lac Z + + - - - - - -
Ad-Flag-Smad2 - - + + - - - -
Ad-Flag-Smad3 - - - - + + - -
Ad-Flag-Smad4 - - - - - - + +
TGFβ1 - + - + - + - +
Chang
B
1 2 3 4 5 6 7 8 9 10 11 12
pY1068EGFR
EGFR
pSmad2
pSmad3
Smad2/3 Short exp
Smad2/3 long exp
Smad4
Flag
GFP
TM4SF5
0.5 2.7 6.6 1.8 6.5 6.1 0.8 2.9 4.1 0.7 0.6 0.4
α-tubulin
TGFβ1 - + + - + + - + + - + +
Ad-Flag-Smad4 - - + - - + - - + - - +
GFP-shSmad C 2 3 4
Chang
C
pY1173EGFR
EGFR
TM4SF5 (long exp.)
TM4SF5 (short exp.)
Flag
α-tubulin
Lac Z + + + - - - - - - - - -
Ad-Flag-Smad2 - - - + + + - - - - - -
Ad-Flag-Smad3 - - - - - - + + + - - -
Ad-Flag-Smad4 - - - - - - - - - + + +
TGFβ1 - + + - + + - + + - + +
AG1478 - - + - - + - - + - - +
Chang
D
pY1173EGFR
pY1068EGFR
EGFR
Flag
TM4SF5
α-tubulin
Ad-Flag-Smad4 - + +
Antibody - Nor. IgG α-EGF
Chang
E
pY1173EGFR
EGFR
pErk1/2
Erk1/2
TM4SF5
α-tubulin
Ad-Flag-Smad4 - - + +
TGFβ1 - + - +
4°C
Chang
F
pY1173EGFR
EGFR
pErk1/2
Erk1/2
TM4SF5
Flag
α-tubulin
Ad-Flag-Smad4 - - + +
CHX - + - +
Chang

Fig 11. Smad-mediated EGFR activation correlates with TM4SF5 expression.

(A and B) Cells were infected with adenovirus (Ad) encoding either Lac Z (control) or FLAG-tagged Smad2, 3 or 4 for 20 h before treatment with vehicle (-) or TGFβ1 (2.5 ng/ml) for an additional 24 h in the absence (A) or presence (B) of AG1478 (100 nM) pre-treatment (30 min before TGFβ1 treatment). Whole cell lysates were prepared for standard Western blot analysis. (C) Cells were transfected with pEGFP-control, shSmad2, shSmad3 or shSmad4 for 24 h, and infected with FLAG-tagged Smad4 adenovirus for 12 h, before TGFβ1 treatment without or with AG1478 for an additional 24 h. Whole cell lysates were prepared for standard Western blot analysis for the indicated molecules. (D) Cells were infected with FLAG-tagged Smad4 adenovirus for 12 h and untreated or treated with normal IgG (Nor. IgG, 20 μg/ml) or anti-EGF antibody (α-EGF, 20 μg/ml) for 24 h, before whole cell lysate preparation and standard Western blot analysis for the indicated molecules. (E) Chang cells were infected with adenovirus for control (-) or FLAG-Smad4 (+) for 20 h and serum-starved at 4℃ for 4 h. TGFβ1 was added at 4○C for 24 h prior to harvest. (F) Chang cells were infected with adenovirus for control (-) or FLAG - Smad4 (+) for 24 h and treated with DMSO (-) or cycloheximide (100 μg/ml, +) for 24 h in the absence of serum prior to harvest and standard Western blotting. Results are representative for three independent experiments.

7. Correlations between TM4SF5, CD151, and CD63 expression levels

To determine the inter-relationships between the tetraspanins and TM4SF5, I examined the expression profiles of certain tetraspanins (i.e., CD9, CD63, CD82, CD105, CD117, and CD151) and TM4SF5 using normal human liver hepatocyte Chang cells and Chang-TGFβ1 cells that were derived by chronic culturing in TGFβ1-containing media, leading to TM4SF5 expression [14]. Flow cytometry analyses showed that Chang-TGFβ1 cells expressed TM4SF5, whereas the control parental Chang cells did not; additionally, the Chang-TGFβ1 cells expressed more CD151 but less CD63 on the cell surface. However, levels of CD9, CD82, CD105, and CD117 did not change (Fig. 12A and data not shown). Therefore, a positive correlation was found between TM4SF5 and CD151, and a negative correlation was found between TM4SF5 and CD63 at both the mRNA (Fig. 12B) and protein levels (Fig. 12C). In addition to increased TM4SF5 expression, Chang-TGFβ1 cells also showed increased FAK activity (i.e., pTyr577FAK). Since the intracellular loop (ICL) of TM4SF5 binds to and activates FAK to direct persistent migration [37].

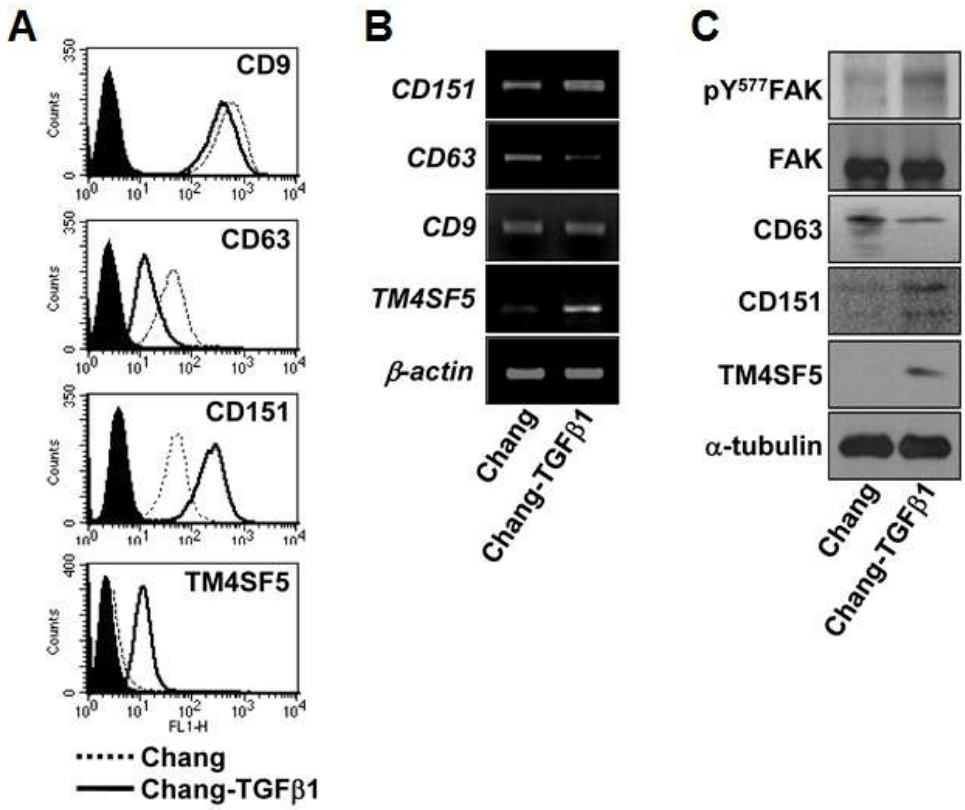


Fig 12. Differential relationships of TM4SF5, CD151, and CD63 expression.

(A to C) TM4SF5-null Chang (normal hepatocyte) and TM4SF5-expressing Chang-TGFβ1 (Chang cells chronically treated with TGFβ1) cells were analyzed for CD9, CD63, CD151, and TM4SF5 expression by flow cytometry (A), by RT-PCR (B), and by Western blot (C). Data represent three independent experiments.

8. The relationships between TM4SF5, CD151, and CD63 were maintained during the development of liver malignancies

I examined the relationships of the tetraspanins in control mouse livers and CCl_4-treated mouse fibrotic livers. A positive correlation between TM4SF5 and CD151 mRNA levels was more obvious in the CCl_4-treated mouse livers, than in the control livers (Fig. 13A). A positive relationship between CD151 and TM4SF5 and a concomitant negative relationship between CD63 and TM4SF5 were observed in the CCl_4-administered mouse livers but not in the control livers (Fig. 13B). Further, whole liver extracts prepared from CCl_4-treated mice showed generally higher c-Src activities, compared with those from control mice (Fig. 13B), as expected from the previous study showing that c-Src activity is downstream of TM4SF5 for cellular invasion [18]. In the CCl_4-treated mouse livers, collagen I was deposited along the fibrotic septa, as observed after Masson's Trichrome staining (Fig. 13C), and the co-localization of CD151 and TM4SF5 was observed after immunofluorescent double-staining (Fig. 13D). I next examined the relationship between CD151, CD63, and TM4SF5 in human liver cancer tissues. Liver tumor tissues with high levels of TM4SF5 expression also showed higher levels of CD151 expression, but very lower levels of CD63, compared with those in normal liver tissues (Fig. 13E). These observations demonstrated that a positive relationship existed between TM4SF5 and CD151 and that a negative relationship existed between CD63 and TM4SF5; this correlationship

in expression could be involved in liver malignancy.

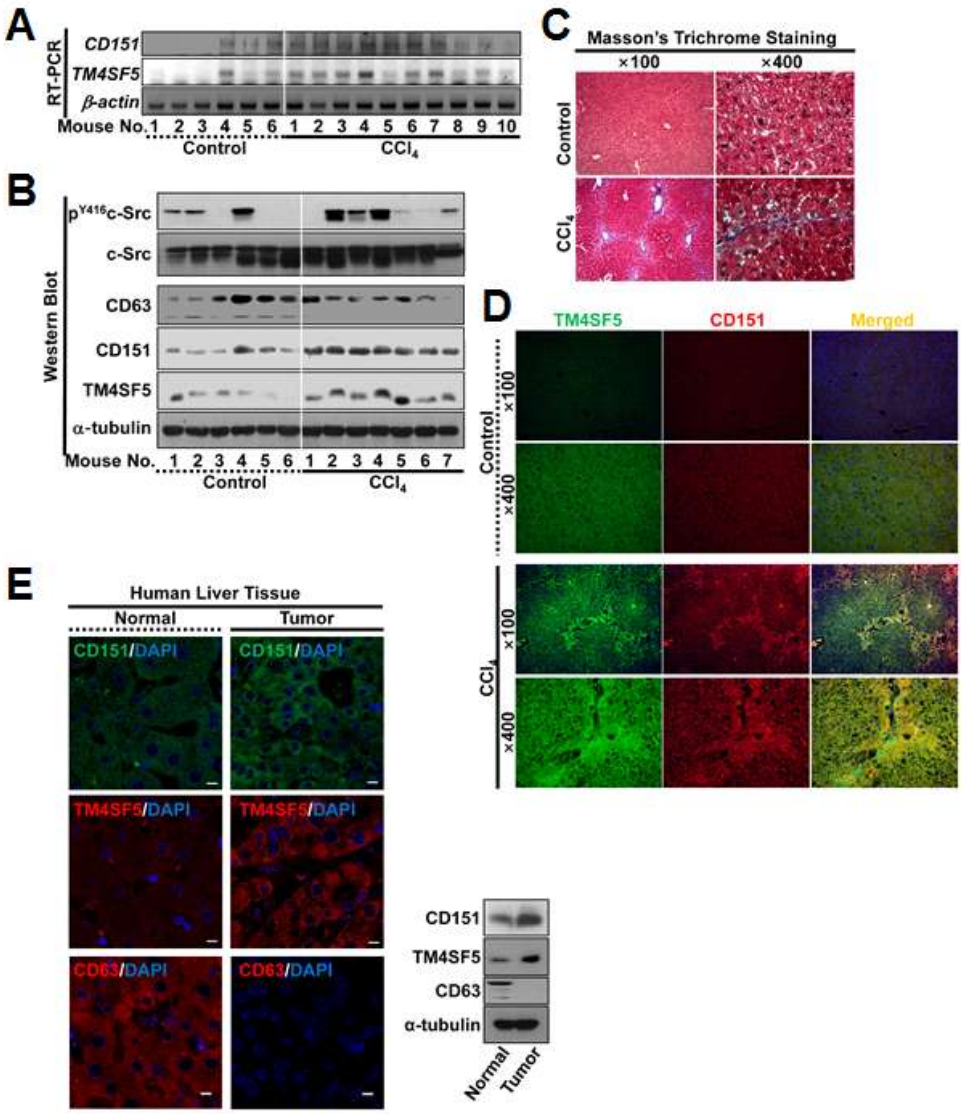


Fig 13. The relationships of TM4SF5, CD151, and CD63 in the development of murine liver fibrosis and liver cancer.

(A to D) Liver tissues from mice administrated with control vehicle or CCl_4 every other day for 2 weeks were used for RT-PCR analysis (A), harvested for whole extracts, prior to Western blots for the indicated proteins (B), used for Masson's trichrome staining to determine collagen I expression (C), or processed for immunohistochemistry with double-staining for TM4SF5 and CD151 (D). (E) Human normal or liver tumor tissues were processed for Western blots or immunohistochemistry to identify CD151 (top), TM4SF5 (middle), and CD63 (bottom) and the nuclei were stained using DAPI. Scale bars depict 10 mm. Data represent three independent experiments.

9. Different hepatocytes exhibited differential relationships between TM4SF5, CD151, and CD63 expression at the transcriptional level

I also examined whether CD151 and CD63 levels were altered when TM4SF5 expression was suppressed in Chang-TGFβ1 cells and Huh7 cells that endogenously express TM4SF5. In contrast to the control shRNA transfected cells, Chang-TGFβ1 cells transfected with shTM4SF5 had decreased levels of CD151 and concomitantly increased levels of CD63 mRNA and proteins (Figs. 14A and B). These relationships were also observed in Huh7 cells (Fig. 14C). Additionally, FAK and c-Src phosphorylation in the Chang-TGFβ1 and Huh7 cells decreased upon TM4SF5 suppression (Figs. 14B and 14C). TM4SF5 interacts with and activates FAK and c-Src, resulting in enhanced migration and invasion [37,38]. When TM4SF5 was ectopically expressed in Chang or SNU449 cells, CD151 mRNA and protein levels increased along with FAK and c-Src activities, whereas CD63 levels decreased (Figs. 14D, 14E, and 14F). I next examined whether the modulation of CD151 expression might affect the expression of TM4SF5 or CD63. CD151 suppression in Chang-TGFβ1 cells did not change the mRNA or protein levels of TM4SF5, but suppression did increase CD63 expression levels (Figs. 14A and 14B). However, c-Src and FAK activities were slightly reduced, presumably because CD151 is also important for their activation, as shown in melanoma cells [39]. Overexpression of CD151 in Chang cells

decreased the levels of CD63 mRNA and protein, although the endogenous (usually null) levels of TM4SF5 mRNA and protein were not altered (Figs. 14C and 14D). The lack of change in TM4SF5 expression after alteration of CD151 expression supports the hypothesis that TM4SF5 is upstream of CD151. I further examined if the modulation of CD63 levels affected TM4SF5 or CD151 levels. Overexpression of CD63 in Chang-TGFβ1 cells decreased TM4SF5 and CD151 mRNA and protein levels and consequently decreased c-Src activity; however, FAK activity was unchanged (Fig. 15A). This relationship was also observed in Huh7 cells transfected with CD63 (Fig. 15B). Further, TGFβ1 treatment resulted in an enhanced transcriptional activity of TM4SF5 promoter (Fig. 15C), which was declined to the basal level by CD63 introduction (Fig. 15C), indicating an antagonistic effect of CD63 on TM4SF5 transcription.

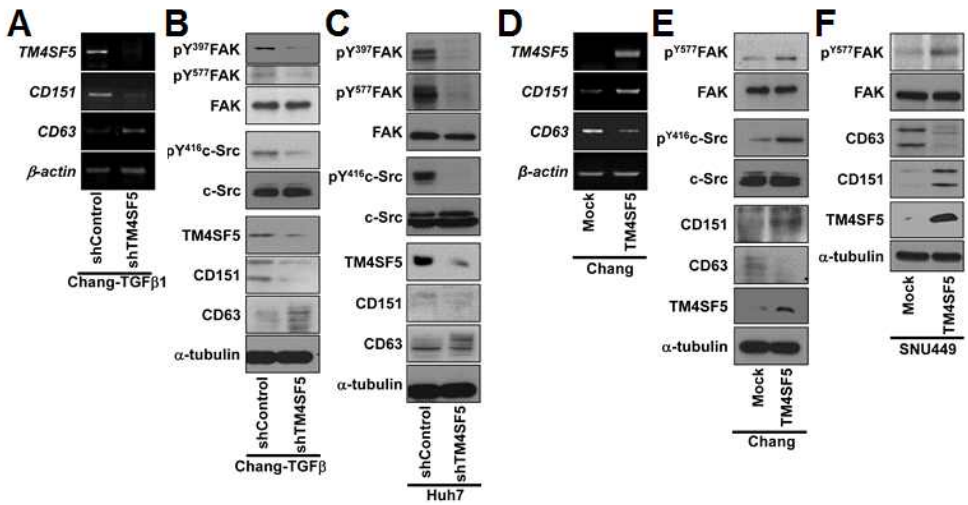


Fig 14. TM4SF5 expression positively or negatively regulated CD151 and CD63 expression levels, respectively, and altered migratory signaling activity.

(A to C) TM4SF5-expressing Chang-TGFβ1 (A and B) or Huh7 cells (C) transfected with shRNA against TM4SF5 (shTM4SF5) or a control-scrambled sequence (shControl) were processed for RT-PCR (A) or Western blots (B and C) against the indicated molecules. (D to F) TM4SF5-null Chang (D and E) or SNU449 (F) cells transfected with TM4SF5 cDNA or control plasmids (Mock) were processed for RT-PCR (D) or Western Blots (E and F). Note that CD63 immunoblots showed multiple bands, presumably due to 3 possible isoforms with N-glycosylations. Data represent three different experiments.

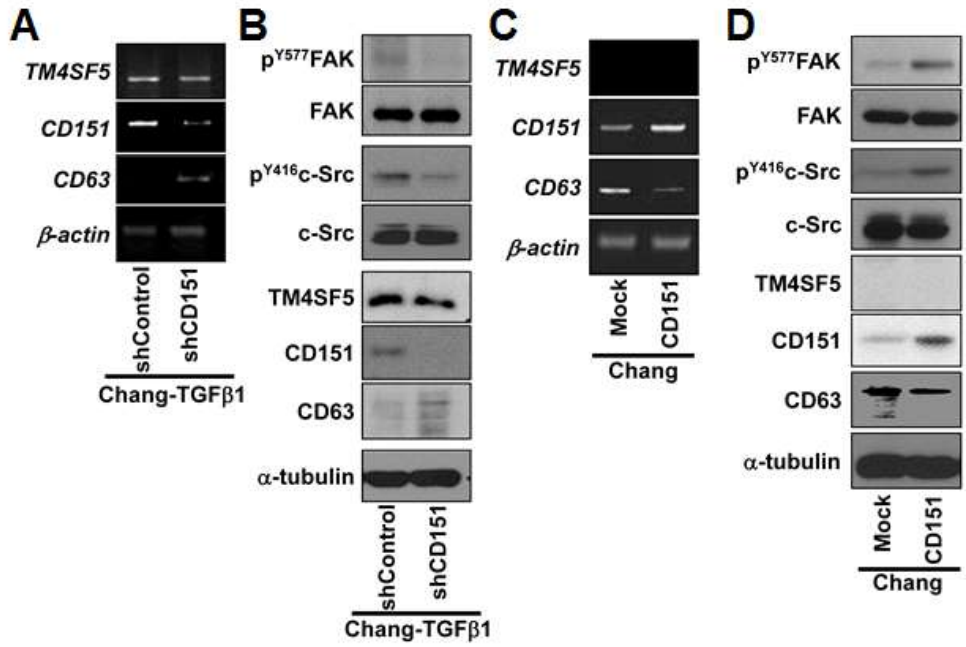


Fig 15. CD151 regulated CD63 expression, but not TM4SF5 expression.

Chang-TGFβ1 cells were transiently transfected with shRNA against CD151 (shCD151) or a control-scrambled sequence (shControl) for 48 h (A and B). Chang cells were transiently transfected with CD151 cDNA or a control plasmid (Mock) for 48 h (C and D). The cells were then processed for RT-PCR (A and C) or for standard Western blots (B and D) for the indicated molecules. Data represent three different experiments.

10. TM4SF5 and CD151 interact at the membrane surface, and TM4SF5 mediates the exclusion of CD63 from the membrane surface

I next examined the expression of TM4SF5 in Chang or Chang-TGFβ1 cells via immunofluorescence microscopy. Whereas TM4SF5 was very minimally detected in Chang cells, it was obviously shown on the plasma membranes and in cytosolic compartments of Chang-TGFβ1 cells (Fig. 16D). Further, when CD63 and CD151 were double immunostained, Chang cells showed CD63 both on plasma membrane and in cytosol but showed a hardly detectable expression level of CD151 (Fig. 5E, upper panels). However, Chang-TGFβ1 cells showed CD151 throughout a cell and CD63 mostly in cytosolic area, without any co-localization between CD151 and CD63 in cytosol (Fig. 16E, lower panels). In addition, when endogenous TM4SF5 and either CD151 or CD63 in Chang-TGF β1 cells were immunostained, TM4SF5 were partially co-localized with CD151 around protrusive tips but not with CD63 that was mostly in cytosol compartments (Fig. 16F). I then examined whether the localization of CD63 might be influenced by TM4SF5. Immunostaining of CD63 in Chang cells transfected with FLAG-TM4SF5 revealed the translocation of CD63 to endosomal regions around the nucleus (Fig. 16G, left panels), whereas the effects of transfection with CD151 on CD63 localization were obviously insignificant since both CD151- transfected and -untransfected

(neighboring) cells showed a similar pattern in CD63 localization (Fig. 16G, right panels). Therefore, cytosolic localization of CD63 in Chang-TGFβ1 cells (Fig. 16E, lower panel) could be due to TM4SF5 overexpression in those cells. Interestingly, when TM4SF5 was exogenously expressed in Chang cells, CD63 was mostly co-localized with LAMP2, a lysosome marker (Fig. 16H). These observations indicate that TM4SF5 increased CD151 expression levels (Figs. 14D and 14E) leading to enhanced tumorigenic roles of CD151, and at the same time caused the translocation of CD63 from the membrane surface to the lysosomal membranes, possibly leading to the inhibition of the tumor-suppressive roles of CD63. I next examined the relationship between TM4SF5 and CD151 at the membrane surface. CD151 coimmunoprecipitated strep-tagged TM4SF5 in Chang cells, whereas CD63 did not (Fig. 17A); neither protein coimmunoprecipitated with TM4SF5 in the mock reaction, indicating a possible physical interaction between TM4SF5 and CD151. This interaction was also observed in HCC827 non-small lung cancer cells transfected with FLAG-TM4SF5 but not with mock plasmids (Fig. 17B). In addition, endogenous TM4SF5 in Chang-TGFβ1 cells was shown to be coimmunoprecipitated with CD151 (Fig. 17C). Furthermore, confocal immunofluorescent images indicated that TM4SF5 and CD151 co-localized at the membrane boundaries. However, this colocalization was not always observed, so that either TM4SF5 or CD151 alone could also localize to the membrane boundaries and intracellular areas near the membrane surfaces (Fig.

17D).

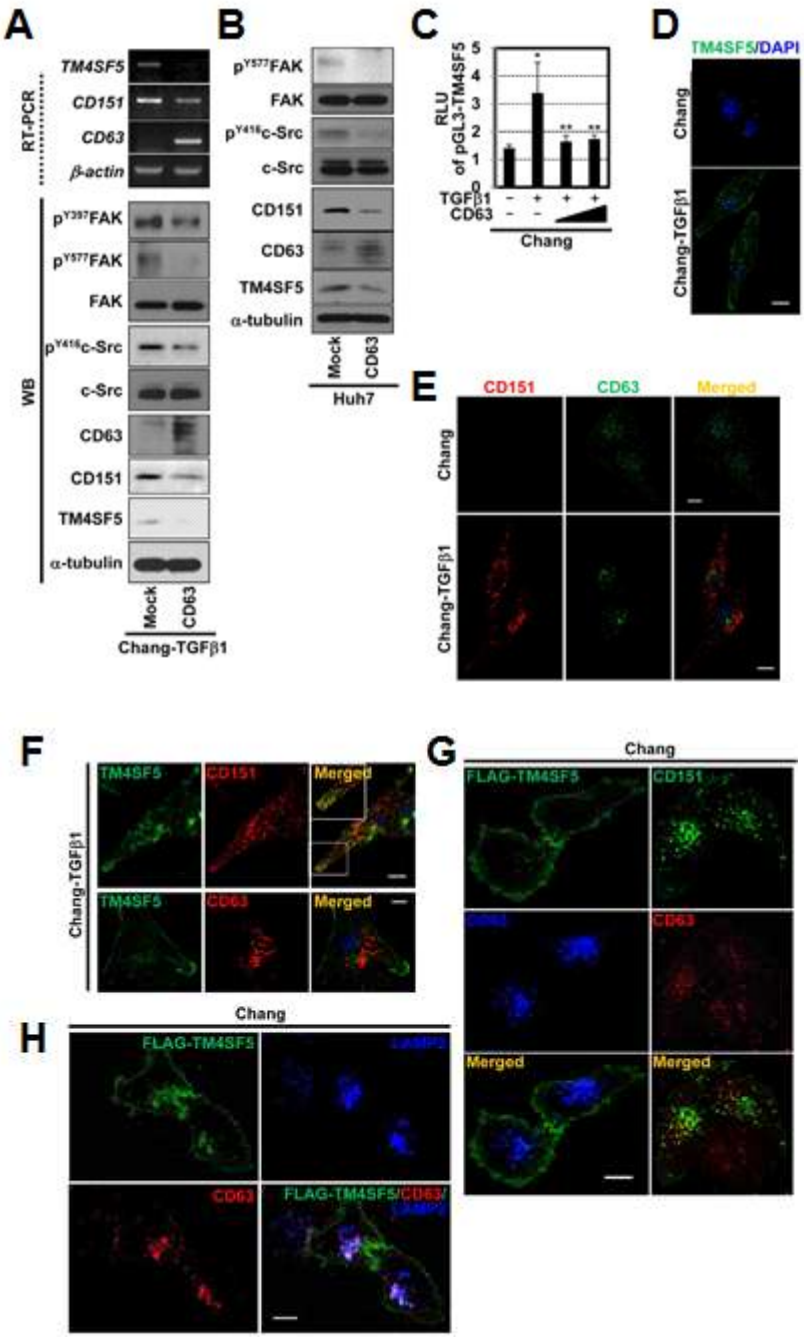

A
RT-PCR
TM4SF5
CD151
CD63
β-actin
WB
pY397FAK
pY577FAK
FAK
pY416c-Src
c-Src
CD63
CD151
TM4SF5
α-tubulin
Mock
CD63
Chang-TGFβ1
B
pY577FAK
FAK
pY416c-Src
c-Src
CD151
CD63
TM4SF5
α-tubulin
Mock
CD63
Huh7
C
RLU of pGL3-TM4SF5
TGFβ1
CD63
Chang
D
TM4SF5/DAPI
Chang
Chang-TGFβ1
E
CD151
CD63
Merged
Chang
Chang-TGFβ1
F
Chang-TGFβ1
TM4SF5
CD151
Merged
TM4SF5
CD63
Merged
G
Chang
FLAG-TM4SF5
CD151
CD63
Merged
Merged
H
Chang
FLAG-TM4SF5
CD63
FLAG-TM4SF5/CD63/

Fig 16. CD63 was antagonistic to TM4SF5 and CD151.

(A and B) Chang-TGFβ1 cells (A) or Huh7 cells (B) transfected with CD63 cDNA or a control plasmid (Mock) were processed for RT-PCR or Western blots. (C) Chang cells transfected with pGL3-TM4SF5 promoter DNA without or with CD63 cDNA for 24 h were treated with vehicle or TGFβ1 for additional 24 h, prior to luciferase reporter gene assay. Each value was shown at mean 6 standard deviation. *depicts statistical significance ($p<0.05$) and ** depicts insignificance ($p\geq0.05$). (D and E) Chang and Chang-TGFβ1 cells were immunostained for TM4SF5 (green, D), CD63 (green, E), or CD151 (red, E) in addition to nuclear< staining using DAPI. (F) Chang-TGFβ1 cells were immunostained for TM4SF5 (green) and either CD151 (top panel) or CD63 (bottom panel) in addition to DAPI staining. White box depicts area for an enlarged insert. (G) Chang cells transiently transfected with FLAG-TM4SF5 or CD151 were immunostained for CD63 (blue or red) and either FLAG-TM4SF5 (green) or CD151 (green) prior to visualization by confocal microscopy. (H) Chang cells transfected with FLAG-TM4SF5 for 48 h were immunostained using anti-FLAG (green), anti-LAMP2 (a lysosomal marker, blue), and anti-CD63 (red) antibody, prior to visualizations using confocal microscopy. Scale bars depict 10 mm. Data represent three independent experiments.

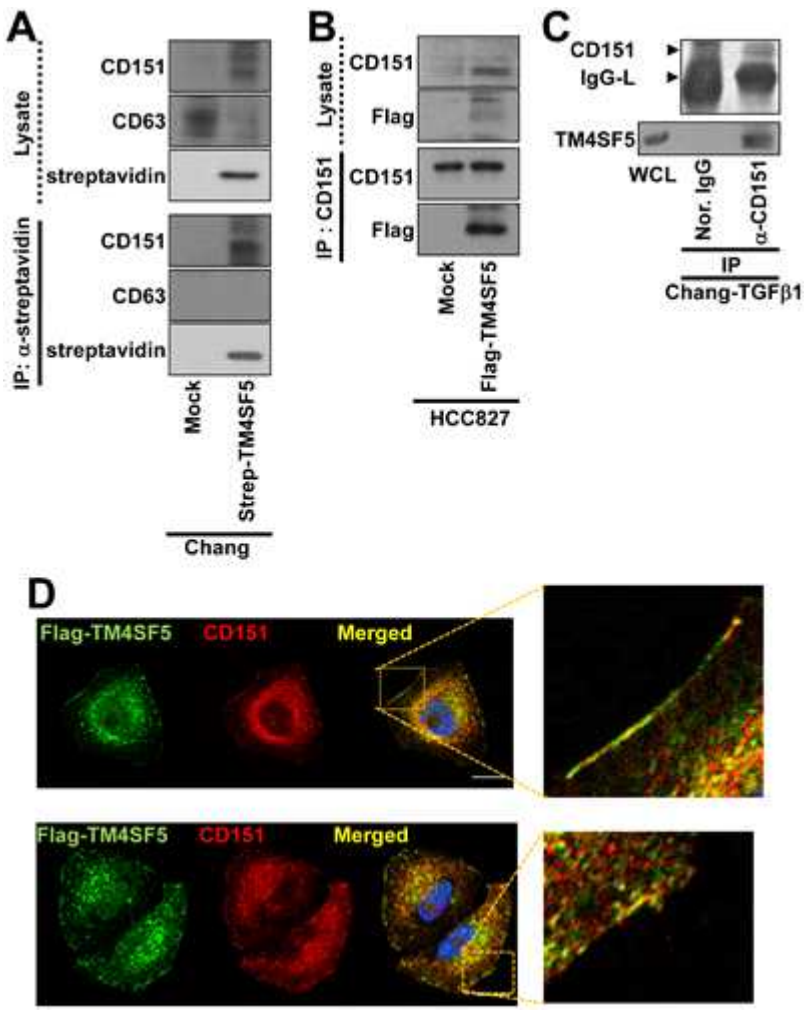

A
Lysate
CD151
CD63
streptavidin
IP: α-streptavidin
CD151
CD63
streptavidin
Mock
Strep-TM4SF5
Chang
B
Lysate
CD151
Flag
IP : CD151
CD151
Flag
Mock
Flag-TM4SF5
HCC827
C
CD151
IgG-L
TM4SF5
WCL
Nor. IgG
α-CD151
IP
Chang-TGFβ1
D
Flag-TM4SF5
CD151
Merged
Flag-TM4SF5
CD151
Merged

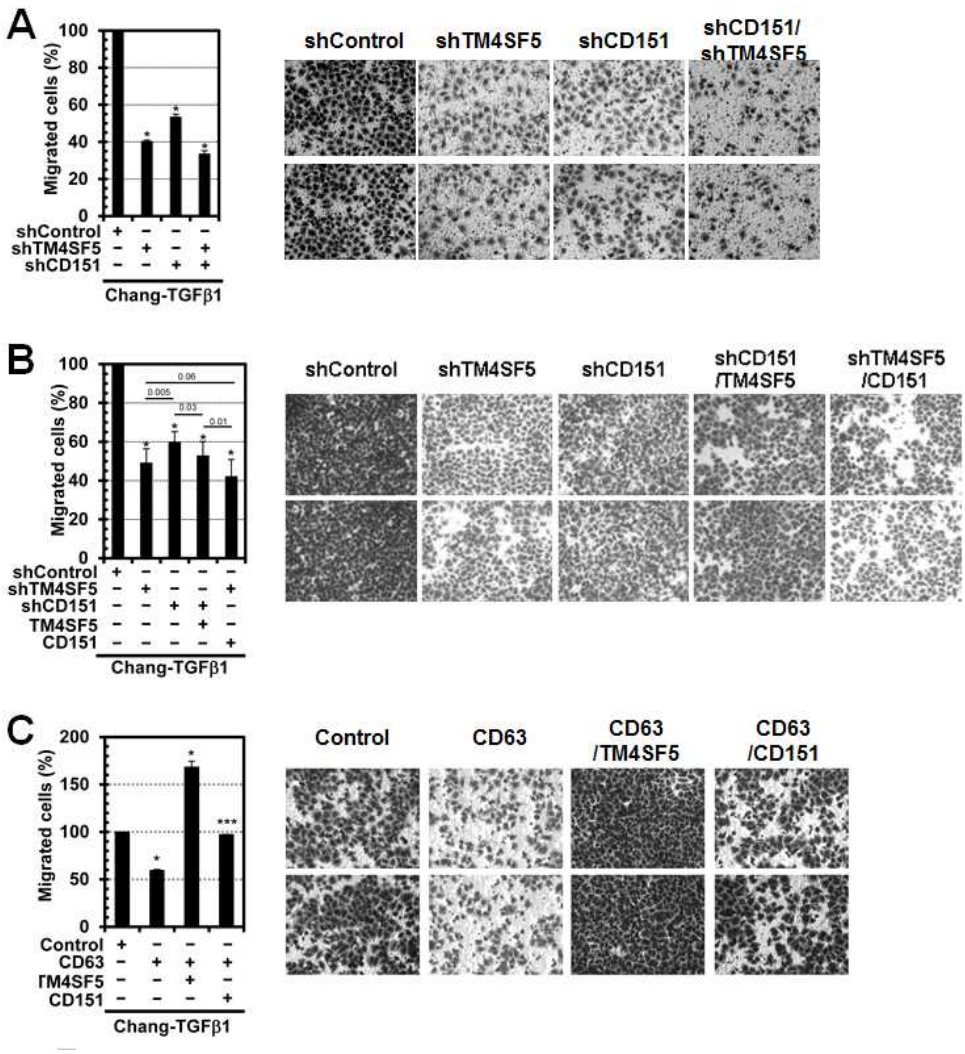
Fig 17. TM4SF5 coimmunoprecipitated with CD151.

(A) Whole cell lysates from Chang cells transfected with control Strep or Strep-TM4SF5 plasmid were immunoprecipitated (IP) with anti-streptavidin-coated agarose beads before immunoblotting using anti-CD151, anti-CD63, or antistreptavidin. (B) HCC827 lung carcinoma cells transfected with FLAG-mock or FLAG-TM4SF5 were harvested for whole cell extracts. The extracts were immunoprecipitated (IP) with anti-CD151 antibody prior to immunoblotting for FLAG and CD151, in parallel with lysates. (C) Whole cells extracts from Chang-TGFβ1 cells were immunoprecipitated with normal (Nor.) IgG or anti-CD151 (α-CD151) antibody, prior to standard Western blots for CD151 or TM4SF5, in parallel with whole cell lysates (WCL). (D) Chang cells transfected with FLAG-TM4SF5 were immunostained for CD151 (red) and FLAG (green), prior to visualization using a confocal microscope. Note that some TM4SF5 co-localized with CD151 at the membrane surface and internally in endosomal regions, but other TM4SF5 localized independently of CD151 even on the membrane surface. The images were presented in an independent duplicate for the same condition. Data represent three independent experiments.

11. Cross-talks between TM4SF5, CD151, and CD63 regulates cell migration and invasive ECM degradation

I next investigated the biological functions of these relationships in the regulation of migration and invasive ECM-degradation. The cells were first analyzed using a Transwell migration assay in which normal culture media including 10% serum was added to the lower chamber. When Chang-TGFβ1 cells were transfected with shTM4SF5 or shCD151, either suppression of TM4SF5 or CD151 alone or in combination significantly decreased cellular migration. However, TM4SF5 suppression alone decreased the cells' migration capacity more than CD151 suppression alone (Fig. 18A). The decreased migration, caused by either TM4SF5 or CD151 suppression, did not recover after the addition of CD151 or TM4SF5, but was instead slightly-reduced (Fig. 18B). This observation suggests that there might be specific relationships between the TM4SF5- and CD151-mediated migrations, by which the migration could become minimal by suppression of either of both molecules. Overexpression of CD63 in Chang-TGFβ1 cells decreased the migratory capacity of these cells, but the addition of CD151 allowed cells to recover to their basal migratory level. On top of the CD63 overexpression, the addition of TM4SF5 increased the cells' migratory capacity above the basal levels (Fig. 18C). This result indicates that the effects of TM4SF5 were stronger, compared to CD63 and CD151, and both CD151 and TM4SF5 antagonized CD63 during migration. Next, the

effect of each molecule on invasive ECM degradation was analyzed. Chang cells were not able to degrade the ECM under our experimental conditions (data not shown), whereas Chang-TGFβ1 cells could (Figs. 18D). TM4SF5 suppression in Chang-TGFβ1 cells almost completely abolished the ECM degradation capacity, whereas CD151 suppression only slightly inhibited ECM-degradation, compared to the shControl- transfected cells (Figs. 18D). Furthermore, the suppression of both TM4SF5 and CD151 significantly inhibited ECM-degradation (Figs. 18D), suggesting a dominant effect of TM4SF5 over CD151 in ECM degradation. When TM4SF5 was expressed in CD151-depleted cells, ECM degradation was enhanced and occurred at a level higher than the level in shControl-transfected cells (Figs. 18E). However, the expression of CD151 in TM4SF5-depleted cells did not change the level of ECM degradation, which remained almost completely inhibited (Figs. 18E). CD63 overexpression in Chang-TGFβ1 cells completely abolished ECM-degradation, which was recovered to a rather enhanced level by the addition of TM4SF5, but not by the addition of CD151 (Figs. 18E and 18F). This observation indicates that TM4SF5 plays major roles in invasive ECM degradation, and can overwhelm the effects caused by CD151 and CD63.


A
Migrated cells (%)
100
80
60
40
20
0
shControl + − − −
shTM4SF5 − + − +
shCD151 − − + +
Chang-TGFβ1
shControl
shTM4SF5
shCD151
shCD151/
shTM4SF5
B
Migrated cells (%)
0.06
0.005
0.03
0.01
shControl + − − − −
shTM4SF5 − + − − +
shCD151 − − + + −
TM4SF5 − − − + −
CD151 − − − − +
Chang-TGFβ1
shControl
shTM4SF5
shCD151
shCD151
/TM4SF5
shTM4SF5
/CD151
C
Migrated cells (%)
200
150
100
50
0
***
Control + − − −
CD63 − + + +
ΓM4SF5 − − + −
CD151 − − − +
Chang-TGFβ1
Control
CD63
CD63
/TM4SF5
CD63
/CD151

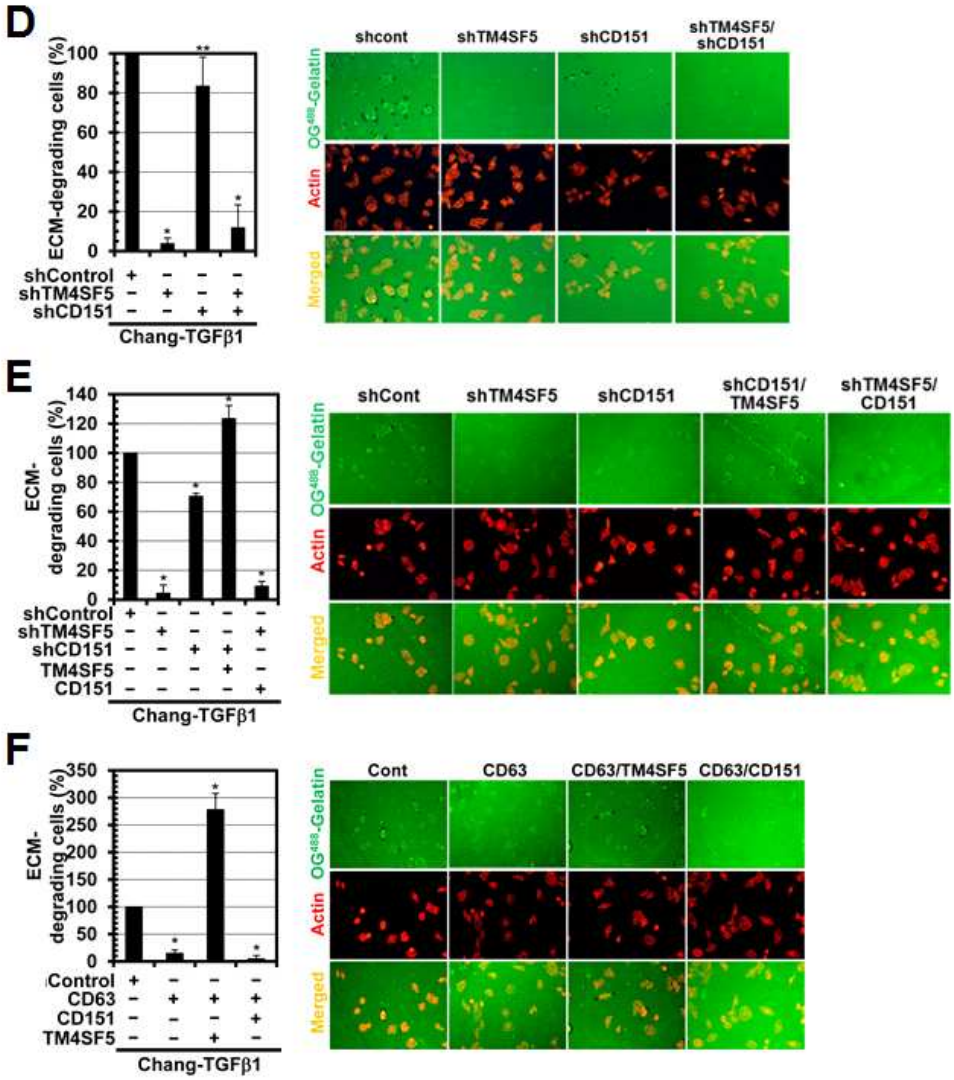

D
ECM-degrading cells (%)
100
80
60
40
20
0
shControl + − − −
shTM4SF5 − + − +
shCD151 − − + +
Chang-TGFβ1
shcont
shTM4SF5
shCD151
shTM4SF5/ shCD151
OG488-Gelatin
Actin
Merged
E
ECM-degrading cells (%)
140
120
100
80
60
40
20
0
shControl + − − − −
shTM4SF5 − + − − +
shCD151 − − + + −
TM4SF5 − − − + −
CD151 − − − − +
Chang-TGFβ1
shCont
shTM4SF5
shCD151
shCD151/ TM4SF5
shTM4SF5/ CD151
OG488-Gelatin
Actin
Merged
F
ECM-degrading cells (%)
350
300
250
200
150
100
50
0
Control + − − −
CD63 − + + +
CD151 − − − +
TM4SF5 − − + −
Chang-TGFβ1
Cont
CD63
CD63/TM4SF5
CD63/CD151
OG488-Gelatin
Actin
Merged

Fig 18. Different collaborative effects of TM4SF5, CD151, and CD63 on migration and invasive ECM degradation.

Transwell migration analyses (A to C) or ECM-degradation analyses (D to F) were performed using Chang-TGFβ1 cells transfected with the indicated shRNAs or plasmids. (A to C) The bottom chamber was filled with 10% FBS/DMEM-H. After 18 h, cells migrated to the bottom surface of the filter were stained and imaged. Representative images (at least 5 images) were counted to determine migration in each experimental condition. Mean ± standard deviation were graphed and representative images of were shown (D to F) Chang-TGFβ1 cells transfected with shRNA or plasmids were reseeded onto coverslips precoated with Oregon Green 488-conjugated gelatin and incubated for 18 h in a CO2 incubator. The dark-spotted ECM-degraded areas from more than 5 random images were counted for graphic presentations using mean ± standard deviation.

* or ** depict P values less than 0.001 or 0.05 for statistical significance, respectively, whereas *** depicts P values greater than 0.05 for insignificance, and numbers in (B) represent the P values by Student's t-tests. Data represent three independent experiments.

Discussion

This study shows that TM4SF5 expression is induced during CCl_4-mediated mouse liver injury together with fibrotic phenotypes, including expression of α-SMA (a marker for activated myofibroblasts that have undergone EMT) and collagen I deposition. TM4SF5 expression appeared to correlate with α-SMA expression in livers of CCl_4-treated mice, which may indicate an EMT process and /or HSC activation [40]. Liver injury and fibrotic phenotypes in CCl_4-treated mice were attenuated by further injection of the anti-TM4SF5 reagent TSAHC. Therefore, it is likely that TM4SF5 is an important regulatable player in the activation of myofibroblasts that is mediated by TGFβ1 and soluble factors during the development of liver fibrosis. During liver fibrosis, TGFβ1-mediated EMT results in activation of HSCs and myofibroblasts, which are characterized by enhanced α-SMA expression [2,34]. Several previous studies have suggested that myofibroblasts can be generated from diverse sources, such as resident mesenchymal, epithelial and endothelial cells [41,42]. Activated HSCs (or myofibroblasts) showed increased secretion of TGFβ1 and other growth soluble factors, such as platelet-derived growth factor and EGF [3]. These soluble factors allow enhanced proliferation of the activated HSCs and myofibroblasts, leading to induction and accumulation of ECM components, such as collagens, outside the myofibroblasts. Therefore,

activation of HSCs or myofibroblasts via the EMT appears to be critical for the development of liver fibrosis [2]. Furthermore, TM4SF5-mediated EMT appears to play an important role in CCl_4-mediated liver fibrotic malignancy *in vivo*. CCl_4-treated livers showed TM4SF5 expression along the fibrotic septa, where expression of collagen I and α-SMA was obvious. This observation supports the idea that TM4SF5 may be involved in the activation of HSCs or myofibroblasts, which eventually produce and deposit collagen I for fibrosis. Furthermore, the expression of signaling molecules such as TGFβ1, phosphorylated Smad2 and α-SMA correlated with TM4SF5 expression and collagen I accumulation along the fibrotic septa in livers of CCl_4-treated mice. But, The anti-TM4SF5 reagent TSAHC and its derivative attenuated CCl_4-mediated collagen I deposition, α-SMA expression and fibrotic septa formation *in vivo*. This observation indicates that TM4SF5 expression is indeed important for the development of liver fibrotic phenotypes, and that blockade of TM4SF5 function may be an effective approach to inhibit or retard the development of liver fibrotic malignacy. Because TSAHC affects structural aspects and/or N-glycosylation of EC2 of TM4SF5 [29], it is reasonable that TSAHC treatment did not lead to alteration of TM4SF5 expression, but instead inhibited TM4SF5- mediated EMT, α-SMA expression, and collagen I deposition. Also, this study shows that TGFβ1 induces TM4SF5 expression and the consequent acquisition of mesenchymal features in TM4SF5-expressing hepatocytes through activated

Smad-mediated crosstalk with the EGFR/Erks pathway. The TM4SF5-induced mesenchymal cell features could be abolished by the inhibition of TGFβ1-mediated R-Smad activity, EGFR activity or TM4SF5 function. Therefore it is likely that TM4SF5 expression in liver cells is regulated by a signalling link between the TGFβ1 and EGFR signalling pathways. It is interesting to understand how TM4SF5 expression is regulated by Smads downstream of TGFβ1, although there are no known Smad-responsive elements in the promoter region (~ −5 kb) of *TM4SF5*. In the system in which each Smad type was overexpressed, Smad2 appeared to be involved in the TGFβ1-mediated TM4SF5 expression, whereas during suppression experiments Smad3 appeared to be involved in TGFβ 1/Smad4-mediated induction of TM4SF5 expression in an EGFR-independent manner. In this study, I observed a link between activated Smads and EGFR that results in TM4SF5 induction of TM4SF5 expression, reminiscent of a signalling link between serine/threonine kinase and tyrosine kinase signalling pathways. I found that Smad4 overexpression alone caused dramatic EGFR phosphorylation and TM4SF5 expression under serum-free conditions. This link could occur through either the induction of new transcription or the action of an additional mediator. However, TGFβ1 treatment of hepatocytes for 30 min resulted in Smad2/3 phosphorylation followed by EGFR activation (within 1 h) that was not accompanied by any increase in the expression levels of EGFR. This TGFβ1- mediated signalling also resulted in enhanced TM4SF5

expression within a very short treatment period (i.e. 1 h). In addition, Smad4 overexpression in Chang cells did not increase EGFR expression levels, although Smad4 overexpression enhanced EGFR/Erk2 phosphorylation and TM4SF5 expression even without TGFβ1 treatment. Furthermore, both TGFβ1-mediated EGFR phosphorylation and TM4SF5 expression were blocked by either suppression of EGFR by siRNAs or depletion of extracellular EGF using anti-EGF antibodies. However, depletion of HB-EGF via application of neutralizing antibody did not affect the TGFβ 1-mediated EGFR phosphorylation and TM4SF5 expression (results not shown). It is thus likely that activated Smad-mediated EGFR activation does not involve additional transcription of the *EGFR* gene but, rather, increased EGF activity. Consistent with this hypothesis, the livers of TM4SF5-overexpressing transgenic mice showed enhanced Smad2/3 and EGFR phosphorylation, indicating a close connection between TGFβ1 and EGFR signalling that is relevant to TM4SF5 expression (results not shown). Similar results have found that TGFβ1 enhances EGFR surface expression on NRK fibroblasts [43], Smad4 mediates EGFR expression in K-251 rat hepatoma cells [44] and TGFβ1 transactivates EGFR to stimulate actin reorganization in FaO rat hepatoma cells [10]. However, the present study showed that TM4SF5 expression following TGFβ1 treatment involved neither *de novo* synthesis of EGFR nor the recycling of internalized EGFR. The metalloproteinase TACE [TNFα (tumour necrosis factor *α*)-converting enzyme]/ ADAM 17 (disintegrin and metalloproteinase

domain-containing protein 17) may be stimulated by TGFβ1 treatment to rapidly induce the shedding of an EGF family member(s) [45]. Interestingly, I showed that Smad4-overexpression-mediated EGFR phosphorylation and induction of TM4SF5 expression was blocked by depletion of extracellular EGF through the addition of anti-EGF antibody to the culture medium. Similarly, depletion of EGF in LX2 conditioned medium blocked conditioned-medium-mediated TM4SF5 expression, although the application of anti-TGFβ1 or normal IgG had no effect, presumably due to an insignificant level of TGFβ1 in the conditionedmedium. More importantly, TGFβ1-mediated EGFR phosphorylation leading to induction of TM4SF5 expression was blocked using anti-EGF antibodies. Alternatively, it cannot be ruled out that an additional molecule(s) may directly transduce a signal from activated Smads to EGFR via protein-protein interactions. The results of the present study indicate the biological significance of TM4SF5. TGFβ1-mediated induction of TM4SF5 expression in normal AML12 and Chang hepatocytes correlates with EMT, including the loss of cell-cell adhesion molecules such as E-cadherin and β-catenin, the induction of vimentin and α-SMA, and cell scattering, as previously shown in hepatocarcinoma cells [29]. Furthermore, the suppression of TGFβ1-mediated induction of TM4SF5 expression in Chang cells results in the blockade of TGFβ 1-mediated induction of vimentin and α-SMA, and suppression of endogenous TM4SF5 expression in Huh7 hepatocarcinoma cells blocks HGF-mediated cell scattering and β-catenin localization at cell - cell

contacts. Therefore, TGFβ1-mediated induction of TM4SF5 expression might cause EMT in hepatocytes. TM4SF5 plays several protumorigenic roles [32], all of which can be inhibited by TM4SF5 suppression or administration of anti-TM4SF5 reagents, such as TSAHC and its derivatives, which also inhibited TGFβ1- mediated α-SMA expression *in vitro*, although these compounds did not alter TM4SF5 expression levels. I speculate that TM4SF5 plays several roles in the development of liver diseases due to the following: (1) TGFβ1 has diverse activities and functions in chronic liver injury and inflammation and has been shown to induce TM4SF5 expression; (2) EMT involved in liver disease can be induced by TM4SF5 expression, and can be blocked by TM4SF5 suppression or treatment with an anti-TM4SF5 reagent; and (3) more than 80% of hepatocellular carcinomas are associated with advanced fibrosis or cirrhosis [46,47]. In addition, it is worthwhile to note that TSAHC and its derivatives are promising therapeutic agents for the treatment of liver malignancy.

The next study revealed that TM4SF5 levels were positively correlated with CD151 expression but were negatively with CD63 expression during liver fibrosis and tumorigenesis. TM4SF5 collaborated with CD151 for migration but played a more important role than CD151 in invasive ECM-degradation. TM4SF5 appeared to bind CD151 on the membrane surface for roles in cell migration. However, CD63 was excluded from the membrane surface, where it plays a tumor-suppressive role, by the expression of TM4SF5 and/or

CD151. Therefore, it is likely that TM4SF5 may collaborate with CD151 to regulate cell migration, but TM4SF5 overrides CD151 during invasion by promoting the internalization of CD63 from the membrane surface to the lysosomes. This action may decrease the tumor-suppressive functions by CD63, during TM4SF5-mediated liver malignancies. Tetraspanins are located in TERMs and are involved in cellular adhesion, migration, and invasion via homophilic and/or heterophilic interactions among the tetraspanins, integrins, and growth factor receptors [8]. TM4SF5 interacts with integrins α2β1 [31] and α5 [48], and with EGFR [49]. CD151 interacts with laminin-binding integrins tetraspanins [20], and CD63 also interacts with integrins and tetraspanins [25]. The present study revealed an interaction between TM4SF5 and CD151, however this interaction might further be associated to other membrane proteins, including tetraspanins, integrins α2β1 and/or α5β1, and growth factor receptors. In a putative TM4SF5-enriched microdomain (i.e., T_5EM) containing TM4SF5, interactions among the membrane proteins including CD151 could transduce intracellular signaling and regulate cellular functions. A mechanistic evaluation of the composition of the T_5EMs and their roles in directional migration is currently underway, although TM4SF5 is not a member of but rather related to tetraspanins. Although TM4SF5, CD151, and CD63 can localize to the membrane surface and within the endosome system, their localizations could play critical roles in tumor progression and fibrotic phenotype development in the livers. Fibrosis and tumorigenesis commonly involve cell

migration and EMT processes [50]. TM4SF5 regulates RhoA/Rac1 activity during EMT [4], binds through its intracellular loop and activates FAK for a directional migration [37], and binds through its cytosolic C-terminus to facilitate c-Src activation and invasion [38]. CD151 expression also causes FAK activation [51], and engagement of laminin-binding integrins in CD151-expressing cells to activate the RhoA GTPase family during cell motility [52]. I found that a certain population of TM4SF5 protein on the membrane surface is bound to CD151. Thus, at the membrane edges TM4SF5 and CD151 might synergistically function in cell migration. However, the collaborations between TM4SF5 and CD151 appeared limited. Some amount of each protein was separately localized on the membrane boundary, and cell migration inhibited by either TM4SF5 or CD151 suppression was not restored by the addition of CD151 or TM4SF5, respectively. Thus, TM4SF5 or CD151 alone might be separately involved in cell migration, as previously reported [37,53]. CD63 is localized either on the plasma membrane or on intracellular vesicles co-localized with the markers for late lysosomal compartments, and CD63 localization at the membrane surface increases by suppression of TM4SF1 [26]. CD63 at the membrane surface associates with TM4SF1 (L6-Ag) to enact its cell migration effects [26]. TM4SF1 is another member of transmembrane 4 L6 family and shares approximately 50% sequence identity with TM4SF5 [5]. Similar to TM4SF1, TM4SF5 expression resulted in the internalization of CD63 from the cell surface to the lysosomes, thus decreasing CD63 level on the membrane surface and

reducing its tumor suppressive actions. Thus, it is likely that the regulation of one $T_{(5)}$EM component controls other components of the $T_{(5)}$EM, leading to the regulation of cell motility. I observed that cell migration was significantly blocked by CD63 expression. However, the addition of TM4SF5 into CD63-transfected cells resulted in enhanced migration at levels higher than observed in the controls, in addition to the recovery of CD63-inhibited migration. The addition of CD151 into CD63-transfected cells only resulted in recovery of the CD63-induced inhibition of migration. CD63 expression completely decreased ECM-degradation; the ECM degradation was dramatically enhanced by the addition of TM4SF5. However, this inhibition was not overcome by the overexpression of CD151. Furthermore, CD151 suppression slightly inhibited ECM-degradation, compared with the stronger inhibition caused by TM4SF5 suppression. Overexpression of TM4SF5 greatly enhanced ECM degradation beyond the basal levels, in addition to its capacity to overcome the inhibition of ECM degradation caused by CD151 suppression- or CD63 overexpression. Interestingly, the CD63-mediated inhibition of migration or ECM-degradation was recovered or unaltered by CD151 overexpression, respectively, suggesting that CD151's effects were stronger than CD63's effects on migration but had no effect on invasive ECM-degradation. This study suggests a hierarchical relationship among the tetraspanins of TM4SF5-containing TERMs (i.e., T_5EMs), and suggests that these TERMs are involved in cell migration and invasion. Therefore, the components of these TERMs

may be promising target(s) for future treatments of liver malignancy.

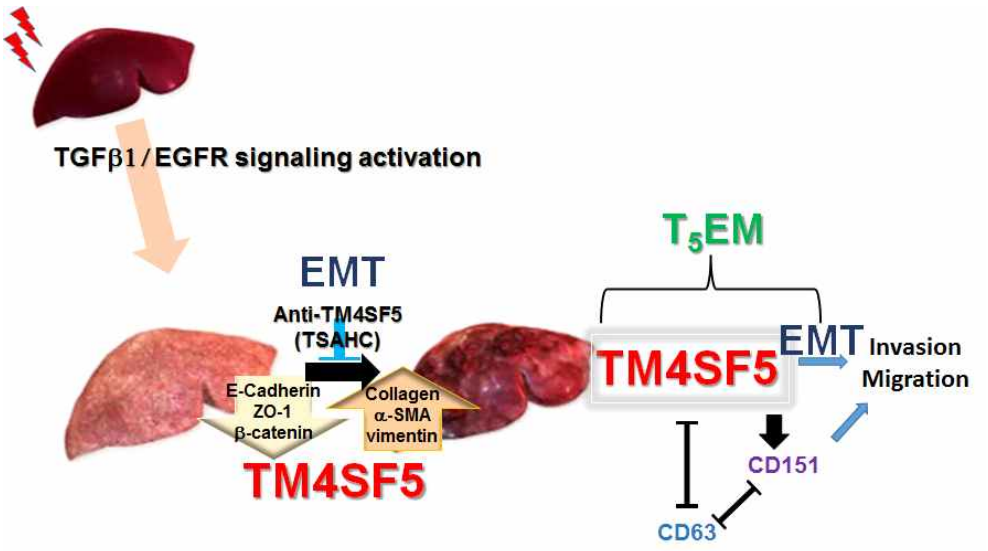


Fig 19. Mechanism of TM4SF5 expression and its functions in fibrotic / tumorigenic liver.

TGFβ1/EGFR signalling-induced TM4SF5 expression causes EMT and regulates invasion/migration by forming TERM (T_5EM) with CD151 and CD63 in fibrotic/tumorigenic liver.

References

1.Kalluri R & Neilson EG (2003) Epithelial–mesenchymal transition and its implications for fibrosis. J Clin Invest 112, 1776-1784.

2.Gressner AM & Weiskirchen R (2006) Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-β as major players and therapeutic targets. J Cell Mol Med 10, 76-99.

3.Jarcuska P, Janicko M, Veseliny E & Skladany L (2010) Circulating markers of liver fibrosis progression. Clin Chim Acta 411, 1009-1017.

4.Lee SA, Lee SY, Cho IH, Oh MA, Kang ES, Kim YB, et al. (2008) Tetraspanin TM4SF5 mediates loss of contact inhibition through epithelial–mesenchymal transition in human hepatocarcinoma. J Clin Invest 118, 1354-1366.

5.Wright, M. D., Ni, J. and Rudy, G. B. (2000) The L6 membrane proteins- a new four-transmembrane superfamily. Protein Sci 9, 1594-1600.

6.Kim, H., Kang, M., Lee, S. A., Kwak, T. K., Jung, O., Lee, H. J., et al. (2010) TM4SF5 accelerates G1/S phase progression via cytosolic p27Kip1 expression and RhoA activity. Biochim. Biophys. Acta 1803, 975-982.

7.Lee, S. A., Kim, T. Y., Kwak, T. K., Kim, H., Kim, S., Lee, H. J., et al. (2010) Transmembrane 4 L six family member 5 (TM4SF5) enhances migration and invasion of hepatocytes for effective metastasis. J. Cell. Biochem. 111, 59-66.

8.Severi T, van Malenstein H, Verslype C & van Pelt JF (2010) Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets. Acta Pharmacol Sin 31, 1409-1420.

9.Choi SS & Diehl AM (2009) Epithelial-to-mesenchymal transitions in the liver. Hepatology 50, 2007-2013.

10.Caja L, Ortiz C, Bertran E, Murillo MM, Miro-Obradors MJ, Palacios E & Fabregat I (2007) Differential intracellular signalling induced by TGF-β in rat adult hepatocytes and hepatoma cells: implications in liver carcinogenesis. Cell Signal 19, 683-694.

11.Bataller R & Brenner DA (2005) Liver fibrosis. J Clin Invest 115, 209-218.

12.Acloque, H., Adams, M. S., Fishwick, K., Bronner-Fraser, M. and Nieto, M. A. (2009) Epithelial - mesenchymal transitions: the importance of changing cell state in development and disease. J. Clin. Invest. 119, 1438-1449.

13.Cannito, S., Novo, E., di Bonzo, L. V., Busletta, C., Colombatto, S. and Parola, M. (2010) Epithelial–mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. Antioxid. Redox Signal. 12, 1383-1430.

14.Revenu, C. and Gilmour, D. (2009) EMT 2.0: shaping epithelia through collective migration. Curr. Opin. Genet. Dev. 19, 338-342.

15.Shook, D. and Keller, R. (2003) Mechanisms, mechanics and function of epithelial–mesenchymal transitions in early development. Mech. Dev. 120, 1351-1383.

16.Kang, Y. and Massague, J. (2004) Epithelial–mesenchymal transitions: twist in development and metastasis. Cell 118, 277-279.

17.Yanez-Mo M, Barreiro O, Gordon-Alonso M, Sala-Valdes M, Sanchez-Madrid F (2009) Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. Trends Cell Biol 19: 434-446.

18.Patra SK (2008) Dissecting lipid raft facilitated cell signaling pathways in cancer. Biochim Biophys Acta 1785: 182-206.

19.Detchokul S, Williams ED, Parker MW, Frauman AG (2013) Tetraspanins as regulators of the tumour microenvironment:

implications for metastasis and therapeutic strategies. Br J Pharmacol. 171: 5462-5490

20.Sala-Valdes M, Ailane N, Greco C, Rubinstein E, Boucheix C (2012) Targeting tetraspanins in cancer. Expert Opin Ther Targets 16: 985-997.

21.Testa JE, Brooks PC, Lin JM, Quigley JP (1999) Eukaryotic expression cloning with an antimetastatic monoclonal antibody identifies a tetraspanin (PETA-3/CD151) as an effector of human tumor cell migration and metastasis. Cancer Res 59: 3812-3820.

22.Sincock PM, Mayrhofer G, Ashman LK (1997) Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and a5b1 integrin. J Histochem Cytochem 45: 515-525.

23.Takeda Y, Kazarov AR, Butterfield CE, Hopkins BD, Benjamin LE, et al. (2007) Deletion of tetraspanin CD151 results in decreased pathologic angiogenesis in vivo and in vitro. Blood 109: 1524-1532.

24.Yang XH, Flores LM, Li Q, Zhou P, Xu F, et al. (2010) Disruption of laminin integrin- CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. Cancer Res 70: 2256-2263.

25.Pols MS, Klumperman J (2009) Trafficking and function of the tetraspanin CD63. Exp Cell Res 315: 1584-1592.

26.Lekishvili T, Fromm E, Mujoomdar M, Berditchevski F (2008) The tumourassociated antigen L6 (L6-Ag) is recruited to the tetraspanin-enriched microdomains: implication for tumour cell motility. J Cell Sci 121: 685-694.

27.Radford KJ, Thorne RF, Hersey P (1997) Regulation of tumor cell motility and migration by CD63 in a human melanoma cell line. J Immunol 158: 3353-3358.

28.Muschel RJ & Gal A (2008) Tetraspanin in oncogenic epithelial - mesenchymal transition. J Clin Invest 118, 1347-1350.

29.Lee SA, Ryu HW, Kim YM, Choi S, Lee MJ, Kwak TK, et al. (2009) Blockade of four-transmembrane L6 family member 5 (TM4SF5)-mediated tumorigenicity in hepatocytes by a synthetic chalcone derivative. Hepatology 49, 1316-1325.

30.Seo WD, Kim JH, Kang JE, Ryu HW, Curtis-Long MJ, Lee HS, Yang MS & Park KH (2005) Sulfonamide chalcone as a new class of alpha-glucosidase inhibitors. Bioorg Med Chem Lett 15, 5514-5516.

31..Lee, S. Y., Kim, Y. T., Lee, M. S., Kim, Y. B., Chung, E., Kim, S.

and Lee, J. W. (2006) Focal adhesion and actin organization by a cross-talk of TM4SF5 with integrin α2 are regulated by serum treatment. Exp. Cell Res. 312, 2983-2999.

32.Lee SA, Park KH & Lee JW (2011) Modulation of signaling between TM4SF5 and integrins in tumor microenvironment. Front Biosci 16, 1752-1758.

33. Lee, S. A., Lee, M. S., Ryu, H. W., Kwak, T. K., Kim, H., Kang, M., et al. (2011) Differential inhibition of transmembrane 4 L six family member 5 (TM4SF5)-mediated tumorigenesis by TSAHC and sorafenib. Cancer Biol.Ther. 11, 330-336.

34.Meindl-Beinker, N. M. and Dooley, S. (2008) Transforming growth factor-β and hepatocyte transdifferentiation in liver fibrogenesis. J. Gastroenterol. Hepatol. **23**,S122-S127.

35.Xu, L., Hui, A. Y., Albanis, E., Arthur, M. J., O'Byrne, S. M., Blaner, W. S., et al. (2005) Human hepatic stellate cell lines, LX-1 and LX-2:new tools for analysis of hepatic fibrosis. Gut **54**, 142-151

36.Olayioye, M. A., Neve, R. M., Lane, H. A. and Hynes, N. E. (2000) The ErbB signalingnetwork: receptor heterodimerization in development and cancer. EMBO J. **19**, 3159-3167.

37.Jung O, Choi S, Jang SB, Lee SA, Lim ST, et al. (2012) Tetraspan TM4SF5-dependent direct activation of FAK and metastatic potential of hepatocarcinoma cells. J Cell Sci 125: 5960-5973.

38.Jung O, Choi YJ, Kwak TK, Kang M, Lee MS, et al. (2013) The COOH terminus of TM4SF5 in hepatoma cell lines regulates c-Src to form invasive protrusions via EGFR Tyr845 phosphorylation. Biochim Biophys Acta 1833: 629-642.

39.Hong IK, Jin YJ, Byun HJ, Jeoung DI, Kim YM, et al. (2006) Homophilic interactions of Tetraspanin CD151 up-regulate motility and matrix metalloproteinase-9 expression of human melanoma cells through adhesion-dependent c- Jun activation signaling pathways. J Biol Chem 281: 24279-24292.

40.Zhong W, Shen WF, Ning BF, Hu PF, Lin Y, Yue HY, et al. (2009) Inhibition of extracellular signal-regulated kinase 1 by adenovirus mediated small interfering RNA attenuates hepatic fibrosis in rats. Hepatology 50, 1524-1536.

41.Meindl-Beinker NM & Dooley S (2008) Transforming growth factor-b and hepatocyte transdifferentiation in liver fibrogenesis. J Gastroenterol Hepatol 23(Suppl 1), S122-S127.

42.Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J

Pathol 214, 199-210.

43.Beaussier M, Wendum D, Schiffer E, Dumont S, Rey C, Lienhart A & Housset C (2007) Prominent contribution of portal mesenchymal cells to liver fibrosis in ischemic and obstructive cholestatic injuries. Lab Invest 87, 292-303.

44.Assoian, R. K., Frolik, C. A., Roberts, A. B., Miller, D. M. and Sporn, M. B. (1984) Transforming growth factor-β controls receptor levels for epidermal growth factor in NRK fibroblasts. Cell 36, 35-41.

45.Nishimura, T., Azuma, T., Yokoyama, A., Ochiai, H., Saito, H. and Hibi, T. (2009) New mechanism of transforming growth factor-β signaling in hepatoma: dramatic up-regulation of tumor initiating cells and epidermal growth factor receptor expression. Hepatol. Res. 39, 501-509.

46.Murillo, M. M., del Castillo, G., Sanchez, A., Fernandez, M. and Fabregat, I. (2005) Involvement of EGF receptor and c-Src in the survival signals induced by TGF-β1 in hepatocytes. Oncogene 24, 4580-4587.

47.Sangiovanni, A., Del Ninno, E., Fasani, P., De Fazio, C., Ronchi, G., Romeo, R., Morabito, A., et al. (2004) Increased survival of cirrhotic patients with a hepatocellular carcinoma detected during surveillance.

Gastroenterology 126, 1005-1014.

48.Severi, T., van Malenstein, H., Verslype, C. and van Pelt, J. F. (2010) Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets. Acta Pharmacol. Sin. 31, 1409-1420.

49.Hemler ME (2005) Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 6: 801-811.

50.Choi S, Lee SA, Kwak TK, Kim HJ, Lee MJ, et al. (2009) Cooperation between integrin α5 and tetraspan TM4SF5 regulates VEGF-mediated angiogenic activity. Blood 113: 1845-1855.

51.Lee MS, Kim HP, Kim TY, Lee JW (2012) Gefitinib resistance of cancer cellscorrelated with TM4SF5-mediated epithelial-mesenchymal transition. Biochim Biophys Acta 1823: 514-523.

52.Radisky DC, Kenny PA, Bissell MJ (2007) Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? J Cell Biochem 101: 830-839.

53.Hong IK, Jeoung DI, Ha KS, Kim YM, Lee H (2012) Tetraspanin CD151 stimulates adhesion-dependent activation of Ras, Rac, and Cdc42 by facilitating molecular association between b1 integrins and

small GTPases. J Biol Chem 287: 32027-32039.

Abstract in Korean (국문 초록)

간섬유화 및 간암발병에 있어 TM4SF5 단백질의 역할

강 민 경

만성적인 질환과 염증은 콜라겐같은 세포외 매트릭스 단백질들이 과도하게 축적되는 상피세포-중배엽 세포로의 전이과정 (EMT ,epithelial-mesenchymal transition) 을 통해 간섬유화를 일으키며, 간섬유화는 궁극적으로 간경화와 간암으로 발전된다. EMT는 여러 soluble factors, 액틴 재구성 그리고 전사인자의 활동에 의한 여러 신호전달 체계를 통해 나타나며, 대표적으로 TGFβ1이 주 역할을 하는 것으로 보고되어진다.

정상 간조직에서 보다 간암조직에서 높게 발현되는 것으로 보고된 TM4SF5는 N- 과 C-터미널이 세포질에 위치하며 세포막을 4번 통과하는 glycoprotein으로, 액틴의 재구성과 EMT, 비정상적인 세포증식을 일으키며 세포내 이동과 침투에 관여한다. 또한 TM4SF5를 포함한 tetrapanin 단백질들은 여러 receptor 단백질들과 세포표면에서 'Tetraspanin-enriched microdomain (TERM)'이라 불리는 네트워크를 형성하여, 여러 신호전달을 통해 세포의 기능을 조절하는 것으로 알려져

있다.

따라서, 본 연구는 간암조직에서 높게 발현되고 EMT를 일으키는 것으로 알려진 TM4SF5 단백질이 간섬유화 > 간경화 > 간암으로 이어지는 간질병단계에서의 역할을 알아보고자 하였고, 이러한 역할을 하는 TM4SF5 단백질의 발현에 관여하는 세포내 신호전달체계를 밝히고자 하였다. 또한 간질병단계에서 TM4SF5를 포함한 TERM을 구성하는 tetraspanin 단백질들에 대하여 알아보고자 하였다.

먼저 CCl_4를 이용한 간섬유화 동물모델을 이용하여, 간섬유화에서 TM4SF5의 발현여부를 확인하고, TM4SF5의 저해제를 사용하여 간섬유화과정에서 TM4SF5의 역할을 알아보고자 하였다. 그 결과, TM4SF5가 CCl_4를 통한 간섬유화 동물모델에서 발현되는 것을 알 수 있었고, TM4SF5의 저해제에 의해 간섬유화의 특징인 α-smooth muscle actin과 콜라겐 I 의 발현이 약화되는 현상을 관찰할 수 있었다. 이에 따라, 간섬유화에서 TM4SF5의 역할을 확인하고, TM4SF5 단백질이 간섬유화 예방을 위한 타겟 단백질이 될 수 있음을 제시할 수 있다.

다음으로, 정상 간세포주를 이용하여 TM4SF5의 발현메카니즘을 알아보고자 하였다. 그 결과, TGFβ1에 의한 Smad 시그널링의 활성화에 의해 EGFR 시그널링이 활성화되어 TM4SF5의 발현이 유도되는 것을 알 수 있었다. Smad의 발현저해에 의해 EGFR 시그널링의 활성화가 저해되고 TM4SF5의 발현이 억제되는 것과 TGFβ1 시그널링의 활성화상태에서 EGFR 시그널링 inhibitor와 세포외 EGF 제거에 의해 TM4SF5 발현저해와 EMT 현상이 나타나지 않는 것으로 TM4SF5의 발현에 있어서 Smad와 EGFR 신호전달체계 간의 interaction을 알 수 있다. 결과적으로, TGFβ1 와 EGFR 시그널링의 활성화에 의해 TM4SF5가 발현되고 중배엽세포 특징을 갖게 되는 것을 확인하였다.

마지막으로, 초기 간질환인 간섬유화과정에서 간암까지의 발전에 역할을 할 것으로 보여지는 TM4SF5가 어떠한 tetraspanin 단백질들과 TERM을 형성하는지 알아보고자 하였다. 세포주를 이용한 실험과 쥐 간섬유화 조직, 사람의 간암조직을 이용한 실험을 통해, TM4SF5는 CD151과 상호작용하여 간질병화에 역할을 하는 것을 확인하였고, CD63은 반대되는 역할을 하는 것을 확인하였다. 비록, CD151은 TM4SF5의 발현을 조절하지 못하지만, TM4SF5는 CD151과 CD63의 발현을 조절하는 것으로 나타났다. TM4SF5는 CD151과는 상호작용을 가지면서 CD63의 세포표면의 발현을 라이소좀 멤브레인으로 이동시킴으로써, CD63의 암억제작용을 억제하는 것으로 보여진다. 이로써, TM4SF5는 세포표면에서 CD151과 CD63의 발현을 조절함으로써 간질병화에서의 역할을 하는 것을 알 수 있으며, 간질병화의 치료를 위한 타겟 단백질이 될 수 있음을 제시할 수 있다.

위 내용들을 종합하였을 때, TM4SF5는 TGFβ1과 EGFR 신호전달체계를 통해 발현이 유도되며, 간암 뿐 아니라 간질병 초기단계인 간섬유화과정에서 발현되어 EMT를 유도하고, 암화와 암억제에 관련된 tetraspanin 단백질들의 발현과 기능을 조절함으로써, 간섬유화의 예방과 치료를 포함한 전반적인 간질환의 타겟 단백질이 될 수 있음을 제시한다.

주요어 : tetraspanin, TM4SF5, liver fibrosis, EMT, TGFβ1, anti-TM4SF5, cytokine, EGFR, tetraspanin web, CD151, CD63

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