Cross-talk between TGFβ1 and EGFR signalling pathways induces TM4SF5 expression and epithelial–mesenchymal transition

Minkyung KANG*†, Suyong CHOI†, Soo-Jin JEONG‡, Sin-Ae LEE†, Tae Kyong KWAK†, Hyeonjung KIM†, Oisun JUNG§, Mi-Sook LEE†, Youra KO§, Jihye RYU†, Yoon-Ju CHOI†, Doyoung JEONG†, Hyo Jeong LEE‡, Sang-Kyu YE*, Sung-Hoon KIM† and Jung Weon LEE†‡§

*Department of Biomedical Sciences, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea; †Department of Pharmacy, Research Institute of Pharmaceutical Sciences, Tumor Microenvironment Global Core Research Center, Medicinal Biconvergence Research Center, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea; ‡Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea; and §Interdisciplinary Program in Genetic Engineering, Seoul National University, Seoul 151-742, Republic of Korea

INTRODUCTION

The EMT (epithelial–mesenchymal transition) is involved in fibrosis and cancer, and is regulated by different signalling pathways mediated through soluble factors, actin reorganization and transcription factor actions. Because the tetraspan (also called tetraspanin) TM4SF5 (transmembrane 4 L6 family member 5) is highly expressed in hepatocellular carcinoma and induces EMT, understanding how TM4SF5 expression in hepatocytes is regulated is important. We 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. We found that TGFβ1 (transforming growth factor β1)-mediated Smad activation caused TM4SF5 expression and EMT, and activation of the EGFR (EGF (epidermal growth factor) receptor) 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-induced 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, suggesting that TM4SF5 induction may be involved in the development of liver pathologies.

Key words: epidermal growth factor receptor (EGFR), epithelial–mesenchymal transition (EMT), gene induction, signalling cross-talk, tetraspanin, transforming growth factor β1 (TGFβ1).

Abbreviations used: ASAHC, 4′-(4-aminobenzensulfonylamido)-4-hydroxychalcone; Di-OH Chal, 4′,4′-dihydroxychalcone; DMEM-H, Dulbecco’s modified Eagle’s medium, high glucose; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial–mesenchymal transition; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; HGF, hepatocyte growth factor; HSC, hepatic stellate cell; α-SMA, α-smooth muscle actin; TGFβ, transforming growth factor β; TM4SF5, transmembrane 4 L6 family member 5; TSAHC, 4′-(p-toluenesulfonylamido)-4-hydroxychalcone; ZO1, zonula occludens protein 1.

1 Correspondence may be addressed to either of these authors (email sungkim7@khu.ac.kr or jwl@snu.ac.kr).
We examined how the expression of TM4SF5 and the resulting induction of EMT were regulated. We found that TM4SF5 was induced by cross-talk between TGFβ1-mediated Smads and EGFR [EGF (epidermal growth factor) receptor] signalling and that this induction leads to the acquisition of mesenchymal cell features. Further, we found that impaired TM4SF5 expression and function abolished the acquisition of mesenchymal cell features.

**EXPERIMENTAL**

**Cell culture**

Normal human Chang hepatocytes, murine AML12 hepatocytes and Huh7 hepatocarcinoma cells were maintained in DMEM-H (Dulbecco’s modified Eagle’s medium, high glucose; WelGene) supplemented with 10% FBS (fetal bovine serum) and 10 μg/ml gentamycin/100 μg/ml streptomycin (Invitrogen). 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**

Cell extracts were prepared as described previously [11]. Cells were serum-deprived for 4 h and treated with different concentrations of TGFβ1 (BioSource International) in serum-free conditions for the indicated times or for 24 h prior to whole cell lysate preparation. In some cases, cells were either pretreated with 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 °C 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), anti-EGF neutralizing antibody (20 μg/ml; Millipore) or anti-(human integrin β1) antibody 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 scrambled sequence, TM4SF5 [15] 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. Whole cell lysates or tissue extracts from human livers were prepared with RIPA buffer containing 0.1% SDS, 0.5% sodium deoxycholate and 1% NP-40 (Nonidet P40) [11].

**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**

Cell or tissue extracts were normalized for protein concentrations and subjected to standard Western blotting using antibodies against E-cadherin, ZO1 (zonula occludens protein 1), α-tubulin, p27Kip1 (BD Transduction Laboratories), vimentin, FLAG M2, β-catenin, α-SMA (α-smooth muscle actin) (Sigma), pTyr973-FAK (focal adhesion kinase; p is phospho-) (BioSource International), pSmad2/3, pSer10-p27Kip1, pTyr1173-EGFR (Santa Cruz Biotechnology), ERK (extracellular-signal-regulated-kinase) 1/2, pERK1/2, pTyr992-EGFR, pTyr1068-EGFR (Cell Signaling Technology), Smad2, Smad3, ZO1 (Zymed Laboratories), EGFR (Upstate Biotechnology) or TM4SF5 [11].

**TM4SF5 promoter transcriptional activity analysis**

The transcriptional activity of the TM4SF5 promoter region (−3.2 kb to +0.5 kb fragment in pGL3) was analysed as described previously [16].

**Immunofluorescence**

Cells were plated on to normal-culture-medium-pre-coated glass coverslips and incubated at 37 °C 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, anti-E-cadherin (Zymed Laboratories) and TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated anti-(mouse IgG) antibody (Molecular Probes) as described previously [11]. Huh7 cells were transfected with shRNA of a control scrambled sequence or TM4SF5 sequence [15], and transfection-positive cells were enriched by G418 addition (500 μg/ml) for 1 week. Huh7 cells on serum-pre-coated coverslips were treated with either vehicle or HGF for 24 h, prior to immunostaining for β-catenin at cell–cell contact sites.

**Statistical analysis**

All experiments were performed independently at least three times. Results are means ± S.D. Data were analysed using the Student’s t test; P ≤ 0.05 was considered significant.

**RESULTS**

**TGFβ1 induces TM4SF5 expression**

Since TM4SF5 is highly expressed in liver cancer [11] and TGFβ1 is a cytokine that plays important roles in both homeostatic and pathological processes in the liver [8], it is possible that TM4SF5 is regulated by TGFβ1-mediated signalling in liver malignancy. To examine this hypothesis, we firstly 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 (six out of nine) expressed higher levels of both TM4SF5 and Smad2/3 phosphorylation than did normal hepatic tissues (Figure 1A), 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 (Figure 1C) without causing significant apoptosis (results not shown). Furthermore, this TGFβ1-mediated induction of TM4SF5 expression was blocked by either the expression of inhibitory Smad7 (Figure 1D) or the introduction of shRNAs of TM4SF5 but not of a scrambled sequence (Figure 1E). Additionally, TGFβ1-mediated Smad activation was required for TM4SF5 expression in gastric carcinoma cells (Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/bj4430691add.htm), indicating that TGFβ1-mediated TM4SF5 expression is not limited to liver cells. These observations suggest that TM4SF5 expression is induced by TGFβ1-mediated signalling.

**Figure 1** 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. (E) AML12 cells were transiently transfected with shRNAs of either control scrambled (Scr) or TM4SF5 (shTM4SF5) sequence for 24 h and treated with (+) or without (−) TGFβ1 (2.5 ng/ml) for an additional 24 h. Standard Western blots were performed for the indicated molecules. Results are representative of three independent experiments.

**TM4SF5 expression causes EMT**

We 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 (Figures 2A and 2B). These TGFβ1-mediated effects also correlate with cell scattering (Figure 2C) and the increased expression levels of the mesenchymal markers α-SMA and vimentin (Figures 2A, 2B and 2D). In addition, localization of E-cadherin and β-catenin at the cell–cell contacts was diminished by TGFβ1 treatment of AML12 cells (Figure 2D). These observations suggest that TGFβ1-mediated induction of TM4SF5 expression leads to the scattering of cells that express mesenchymal cell markers (i.e. EMT).

We 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-mediated expression of mesenchymal markers (i.e. vimentin and α-SMA) was lower than in control shRNA-transfected cells (Figure 3A). Furthermore, suppression of endogenous TM4SF5 expression in HuH7 hepatocarcinoma cells by shTM4SF5 resulted in a blockade of HGF-mediated loss of β-catenin expression at cell–cell contacts with cell scattering (Figure 3B), indicating that TM4SF5 expression is involved in EMT induction. Furthermore, TM4SF5-mediated functions, including multilayer growth and cell migration/invasion, are antagonized by the small synthetic compound TSAHC [4-(p-toluenesulfonylamido)-4-hydroxychalcone] [17]. Thus we 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 AML12 cells was inhibited by treatment with TSAHC, but not with a control compound 4′-NH4-4-OH-Chal (4′-amino-4-hydroxychalcone; Figure 3C). The TGFβ1-mediated increase in the expression of another mesenchymal marker, vimentin, was also blocked by TSAHC.
Figure 2  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 the 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 of three independent experiments.

EGFR activation correlates with TGFβ1-mediated TM4SF5 expression

Next, we examined the signalling molecules that are involved in the TGFβ1-mediated induction of TM4SF5 expression. When cells were either kept in suspension or reseeded on to fibronectin-pre-coated dishes in the absence of serum, TM4SF5 expression levels were higher in fibronectin-adherent cells than in suspended cells (Supplementary Figures S2 and S3 at http://www.BiochemJ.org/bj/443/bj4430691add.htm). AML12 cells showed adhesion-dependent TM4SF5 expression without a synergy by additional TGFβ1 treatment, whereas Chang cells showed a synergism between adhesion and TGFβ1 treatment in the induction of TM4SF5. Consistent with these findings, neutralization of integrin β1 by its antibody reduced TGFβ1-mediated EGFR phosphorylation and TM4SF5 expression, but not Smad2/3 phosphorylation (Supplementary Figure S3). In the absence of serum, TGFβ1 induced TM4SF5 expression in Chang cells in a dose-dependent manner (Figure 4A). Even under serum-free conditions, TGFβ1-mediated TM4SF5 expression correlated with EGFR phosphorylation and ERK1/2 activation (Figure 4A). Treatment with TGFβ1 for shorter durations (i.e. <2 h) caused R-Smad and EGFR phosphorylation, even after treatment for 30 min, as well as an increase in TM4SF5 expression (Figure 4B), 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 (Figure 4C). Similar results were observed in human Chang hepatocytes (Supplementary Figure S4 at http://www.BiochemJ.org/bj/443/bj4430691add.htm). 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

and its derivative ASAHC [4′-(4-aminobenzensulfonylamido)-4-hydroxychalcone] [18], but not by another control compound, Di-OH Chal (4′,4-dihydroxychalcone) (Figure 3D). These observations indicate that TGFβ1-induced TM4SF5 expression regulates the expression of mesenchymal markers during EMT.
Signalling from TGFβ1 to EGFR induces TM4SF5

Figure 3  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) Huh7 cells endogenously expressing TM4SF5 were transfected with control shRNA (a scrambled sequence, shControl) or TM4SF5 shRNA (shTM4SF5) and processed for G418-mediated enrichment for 1 week. The cells were reseeded on coverslips in the presence of 10% serum-containing medium overnight before vehicle (Veh) or HGF (100 ng/ml) treatment for 24 h followed by indirect immunostaining for β-catenin. Scale bar, 20 μm. (C and D) Cells were serum-deprived for 4 h, and cells were treated without (0 ng/ml) 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 (C) and (D) 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 of three independent experiments.

decreased TM4SF5 expression but not Tyr997 phosphorylation of FAK, which is important for cell survival (Figure 4D). Meanwhile, PI3K (phosphoinositide 3-kinase) inhibition decreased both TM4SF5 expression and pTyr997-FAK levels (Figure 4D), 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 TGFβ1-mediated induction of TM4SF5 expression.

HSCs (hepatic stellate cells) in the liver are the main cells that produce TGFβ1 [19]. Therefore we examined whether conditioned medium prepared from activated HSCs could induce TM4SF5 expression. The human HSC line LX2 is known to be activated [20]. Thus we 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 [21]) and TM4SF5 levels, although TM4SF5 levels after conditioned medium treatment were lower than those after TGFβ1 treatment (Figure 4E). Furthermore, pharmacological inhibition of EGFR kinase prior to LX2-conditioned medium treatment blocked EGFR/ERKs phosphorylation and TM4SF5 expression (Figure 4F). 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.

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, we wished to explore the mechanism involved. First, we examined whether either TGFβ1 or EGF could induce TM4SF5 expression. Treatment of Chang cells with 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 [21]) and TM4SF5 levels, although TM4SF5 levels after conditioned medium treatment were lower than those after TGFβ1 treatment (Figure 4E). Furthermore, pharmacological inhibition of EGFR kinase prior to LX2-conditioned medium treatment blocked EGFR/ERKs phosphorylation and TM4SF5 expression (Figure 4F). 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.
in slightly increased TM4SF5 expression levels compared with TGFβ1 treatment alone, but co-treatment of TGFβ1 and platelet-derived growth factor had no effect (Figure 5C). 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 (Figure 5D). These observations indicate that TM4SF5 expression requires EGFR/ERK activation after TGFβ1-mediated R-Smad activation. Moreover, TGFβ1-mediated scattering of AML12 cells was blocked by the pharmacological inhibition of EGFR kinase activity (Figure 5E). 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 (Figure 5F), 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 (Figure 5G). 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 TGFβ1-dependent TM4SF5 expression, whereas FLAG–Smad3 overexpression did not alter TM4SF5 levels (Figure 6A, lanes 1–6), indicating that Smad2 is the major R-Smad involved in TGFβ1-mediated...
Signalling from TGFβ1 to EGFR induces TM4SF5

In the presence of serum, 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) prior to harvest. (B) Chang cells were serum-deprived for 4 h and treated without (−) or with (+) TGFβ1 (2.5 ng/ml) and the indicated growth factors (50 ng/ml) in the absence of serum for 24 h. *P ≤ 0.05 or ***P ≥ 0.5 indicates a significant or insignificant difference respectively from serum-free control conditions; **P ≤ 0.05 indicates significant differences from TGFβ1 treatment alone. (D) 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 (D) or cell images were recorded (E). (F) Chang cells were transfected with control shRNAs of either a scrambled sequence (ShCont) or EGFR (ShEGFR) for 24 h, and infected with FLAG-tagged Smad4 adenovirus for 12 h, prior to TGFβ1 treatment for an additional 24 h. Whole cell lysates were prepared and used for standard Western blot analysis. Results are representative of three independent experiments. Veh, vehicle.

Figure 5  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) 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) prior to harvest. (C) Cells were serum-deprived for 4 h and treated without (−) or with (+) TGFβ1 (2.5 ng/ml) and the indicated growth factors (50 ng/ml) in the absence of serum for 24 h. *P ≤ 0.05 or ***P ≥ 0.5 indicates a significant or insignificant difference respectively from serum-free control conditions; **P ≤ 0.05 indicates significant differences from TGFβ1 treatment alone. (D) 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 (D) or cell images were recorded (E). (F) Chang cells were transfected with control shRNAs of either a scrambled sequence (ShCont) or EGFR (ShEGFR) for 24 h, and infected with FLAG-tagged Smad4 adenovirus for 12 h, prior to TGFβ1 treatment for an additional 24 h. Whole cell lysates were prepared and used for standard Western blot analysis. Results are representative of three independent experiments.
Figure 6  Smad-mediated EGFR activation correlates with TM4SF5 expression

(A and B) Cells were infected with adenovirus (Ad) encoding either LacZ (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 (for 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 lysis 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°C 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 of three independent experiments. exp/exp., exposure.

in both control cells and cells that overexpress either FLAG–Smad2 or FLAG–Smad4 (Figure 6C). Therefore it is likely that the mechanism of TGFβ1-mediated TM4SF5 expression involves EGFR activation. Therefore we 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 (Figure 6A, lane 7), which were decreased by extracellular EGF depletion during cultures (Figure 6D), 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 (Figures 5A, 6A and 6C), TGFβ1 treatment of FLAG–Smad4-infected cells was performed at 4°C 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°C, Smad4 overexpression resulted in a very slight activation of EGFR/ERKs and the induction of TM4SF5 expression with no increase in EGFR
expression levels (Figure 6E). 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 24 h prior to harvest of cell extracts. Cycloheximide treatment maintained similar EGFR levels both before and after Smad4 overexpression, but blocked Smad4-induced TM4SF5 expression (Figure 6F). These observations suggest that de novo EGFR synthesis is not involved in EGFR/ERK phosphorylation due to Smad4 overexpression.

**DISCUSSION**

The present study shows that TGFβ1 induces TM4SF5 expression and the consequent acquisition of mesenchymal features in TM4SF5-expressing hepatocytes through activated Smad-mediated cross-talk 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 TGFβ1-mediated EGFR phosphorylation 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 β1, 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 TGFβ1/Smad4-mediated induction of TM4SF5 expression in an EGFR-independent manner. In the present study, we 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; we 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 TGFβ1-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 (Supplementary Figures S5 and S6 at http://www.BiochemJ.org/bj/443/bj4430691add.htm). Similar results have found that TGFβ1 enhances EGFR surface expression on NRK fibroblasts [22], Smad4 mediates EGFR expression in K-251 rat hepatoma cells [23] and TGFβ1 transactivates EGFR to stimulate actin reorganization in FaO rat hepatoma cells [8]. 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) [24]. Interestingly, we showed that Smad4-overexpression-mediated EGFR phosphorylation and induction of TM4SF5 expression were 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 conditioned medium. 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 [17]. Furthermore, the suppression of TGFβ1-mediated induction of TM4SF5 expression in Chang cells results in the blockade of TGFβ1-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 pro-tumorigenic roles [25], all of which can be inhibited by TM4SF5 suppression or administration of anti-TM4SF5 reagents, such as TSAHC and its derivatives [17,18], which also inhibited TGFβ1-mediated α-SMA expression in the present study, although these compounds did not alter TM4SF5 expression levels (results not shown, and [17]). As TSAHC appears to affect the structural aspects and/or N-glycosylation of extracellular loop 2 of TM4SF5 [17], it is reasonable to assume that TSAHC treatment antagonizes TM4SF5-mediated α-SMA expression, but not TM4SF5 expression itself. We speculate that TM4SF5 plays several roles in the development of liver diseases due to the following: (i) TGFβ1 has diverse activities and functions in chronic liver injury and inflammation, and has been shown to induce TM4SF5 expression in the present study; (ii) 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 (iii) more than 80 % of hepatocellular carcinomas are associated with advanced fibrosis or cirrhosis [26,27]. In addition, it is worthwhile to note that TSAHC and its derivatives are promising therapeutic agents for the treatment of liver malignancy.

**AUTHOR CONTRIBUTION**

Jung Weon Lee and Sung-Hoon Kim conceived and designed the experiments, and Jung Weon Lee wrote the paper. Minkyung Kang and Suyong Choi performed experiments, and...
ACKNOWLEDGEMENTS

We thank Dr Scott Friedman (Mount Sinai School of Medicine, New York, NY, U.S.A.) for providing the LX2 cells.

FUNDING

This work was supported by the National Research Foundation (NRF) of Korea funded by the Ministry of Education, Science and Technology of Korea (MEST) [grant numbers 2010-0063466 (to S.-H.K.) and 2011-0001160 (to J.W.L.)]; the Tumor Microenvironment Research Center (GCRC) [grant number 2011-0030678]; the NRF support for senior researchers programme (Leap research) [grant number NRF-M1AXA002-2011-0028411]; and the Korean Health researchers programme (Leap research) [grant number 2011-0016446]; the NRF Global Frontier Project [grant number NRF-M1AXA002-2011-0028411]; and the Korean Health Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Korea [grant number A100727 (to J.W.L.)].

REFERENCES

SUPPLEMENTARY ONLINE DATA

Cross-talk between TGFβ1 and EGFR signalling pathways induces TM4SF5 expression and epithelial–mesenchymal transition

Minkyung KANG*†, Suyong CHO†, Soo-Jin JEONG‡, Sin-Ae LEE†, Tae Kyoung KWAK†, Hyeonjung KIM†, Oisun JUNG§, Mi-Sook LEE†, Youra KO§, Jihye RYU†, Yoon-Ju CHOI†, Doyoung JEONG†, Hyo Jeong LEE‡, Sang-Kyu YE*, Sung-Hoon KIM‡ and Jung Weon LEE†§1

*Department of Biomedical Sciences, College of Medicine, Seoul National University, Seoul 110-799, Republic of Korea, †Department of Pharmacy, Research Institute of Pharmaceutical Sciences, Tumor Microenvironment Global Core Research Center, Medicinal Bioconvergence Research Center, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea, ‡Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea, and §Interdisciplinary Program in Genetic Engineering, Seoul National University, Seoul 151-742, Republic of Korea

Figure S1 TGFβ1-mediated TM4SF5 expression is inhibited by Smad7 overexpression

SNU16mAd gastric carcinoma cells were treated with TGFβ1 (2.5 ng/ml) for 24 h prior to whole cell lysate preparation and standard Western blots for the indicated molecules. Results are representative of three independent experiments. Adeno, adenovirus.

Figure S2 TGFβ1 and cell adhesion induces TM4SF5 expression

Cells were kept in suspension (Sus) or newly reseeded on to fibronectin (FN; 10 μg/ml)-pre-coated culture dishes in the absence of serum for 2 h without (−) or with (+) treatment of TGFβ1 (5 ng/ml) or pre-treatment of functional blocking antibody against human integrin β1 (anti-β1 Ab; 20 μg/ml). Cells were harvested and processed for Western blots using antibodies against the indicated molecules.

Figure S3 TGFβ1-mediated TM4SF5 expression is blocked by functional blocking of integrin β1

Chang cells were kept in suspension or newly reseeded on to fibronectin (10 μg/ml)-pre-coated culture dishes in the absence of serum for 2 h without (−) or with (+) treatment of TGFβ1 (5 ng/ml) or pre-treatment of functional blocking antibody against human integrin β1 (anti-β1 Ab; 20 μg/ml). Cells were harvested and processed for Western blots using antibodies against the indicated molecules.

1 Correspondence may be addressed to either of these authors (email sungkim7@khu.ac.kr or jwl@snu.ac.kr).
Figure S4  TGFβ1-mediated TM4SF5 expression in Chang hepatocytes is inhibited by Smad7 overexpression

Cells were infected with adenovirus encoding LacZ (as a negative control) or FLAG–Smad7 for 24 h, serum-deprived for 4 h and then treated without (−) or with TGFβ1 (2.5 ng/mL, +) for 24 h, before cell harvests for Western blots using antibodies against the indicated molecules.

Figure S5  TM4SF5 expression-related signalling activities in livers of transgenic TM4SF5 mice

Liver extracts from TM4SF5-overexpressing transgenic (Tg) mice were prepared and immunobotted for the indicated molecules.

Figure S6  Generation of TM4SF5-transgenic mice and confirmation of TM4SF5 expression

pcDNA3-FLAG-TM4SF5 was digested with HincII, DraIII and the fragment consisting of the CMV (cytomegalovirus) promoter, whole TM4SF5–FLAG sequence and BGH (bovine growth hormone, BGH) poly(A) region was purified and microinjected into fertilized eggs from C57BL/6N mice according to standard procedures (Macrogen). Founders (2 weeks old) were screened by PCR using the primer set for CMV-F1 (forward primer, 5’-CGCTATTACCATGGTGATGCG-3’) and TM4SF5-R1 (reverse primer, 5’-AGACACCGAGAGGCAGTAGAT-3’). Upper panel: DNA fragment for microinjection to generate a TM4SF5-transgenic mouse. Lower panel: PCR screening for the TM4SF5 genotype was performed using primers CMV-F1 and TM4SF5-R1. In addition to negative controls, normal mouse genomic DNA or non-transgenic mice did not have the PCR constructs of 555 bp covering a part of the CMV promoter and a part of FLAG–TM4SF5.

Received 31 August 2011/9 January 2012; accepted 31 January 2012
Published as BJ Immediate Publication 31 January 2012, doi:10.1042/BJ20111584