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Proteasome Inhibition Causes Epithelial–Mesenchymal Transition upon TM4SF5 Expression

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ABSTRACT

Transmembrane 4 L six family member 5 (TM4SF5) is highly expressed in hepatocarcinoma and causes epithelial–mesenchymal transition (EMT) of hepatocytes. We found that TM4SF5-expressing cells showed lower mRNA levels but maintained normal protein levels in certain gene cases, indicating that TM4SF5 mediates stabilization of proteins. In this study, we explored whether regulation of proteasome activity and TM4SF5 expression led to EMT. We observed that TM4SF5 expression caused inhibition of proteasome activity and proteasome subunit expression, causing morphological changes and loss of cell–cell contacts. shRNA against TM4SF5 recovered proteasome expression, with leading to blockade of proteasome inactivation and EMT. Altogether, TM4SF5 expression appeared to cause loss of cell–cell adhesions via proteasome suppression and thereby proteasome inhibition, leading to repression of cell–cell adhesion molecules, such as E-cadherin. J. Cell. Biochem. 112: 782–792, 2011.

KEY WORDS: EPITHELIAL–MESENCHYMAL TRANSITION/PROTEASOME/TETRASPAN/TRANSCRIPTION

The epithelial monolayer is maintained by cell–cell contacts supported by homophilic interactions between cell adhesion molecules on each cell surface [Thiery, 2003; Thiery and Sleeman, 2006; Acloque et al., 2009]. Disruption of monolayer integrity can occur by the transition of epithelial cell types with well-established cell contacts to mesenchymal-like cells (i.e., elongated spindle-type cells) with few or no cell contacts, known as epithelial–mesenchymal transition (EMT) [Tse and Kalluri, 2007; Kalluri, 2009]. This disruption impairs normal epithelium function and can allow dissemination of metastatic cancer cells or cancer stem cells from primary tumor bodies [Thiery et al., 2009]. Tumor cell dissemination may facilitate cell migration and invasion leading to tumor metastasis [Gavert and Ben-Ze'ev, 2008; Guarino et al., 2007]. Therefore, it is of clinical importance to examine the mechanisms behind the loss of cell–cell contacts [Boyer et al., 2000].

EMT can be caused by many biochemical processes including aberrant actin bundling [Lee et al., 2008; Muschel and Gal, 2008]. Hepatocyte growth factor (HGF) and TGF-β1-mediated signaling pathways also induce epithelial cell scattering and motility [Wahab and Mason, 2006]. In addition, repression or mutation of cell adhesion molecules leads to EMT; EMT occurs either by transcriptional repression of epithelial phenotype genes (e.g., E-cadherin,

Abbreviations used: TM4SF5, transmembrane 4 L six family member 5; SNU449Cp, SNU449 hepatocytes stably infected with control retrovirus encoding pLNCX; SNU449Tp, SNU449 hepatocytes stably infected with retrovirus encoding pLNCX-TM4SF5.

Additional Supporting Information may be found in the online version of this article.

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claudins, occludins, desmoplakin, and desmoglein) or by transcriptional activation of genes related to functional myofibroblasts (e.g., FN-EDA$^+$, vimentin, α-SMA) [Rosivatz et al., 2002; Yang and Weinberg, 2008].

In eukaryotes, the ubiquitin–proteasome pathway performs selective protein degradation and is critically important for signal transduction, transcriptional regulation, response to stress, and control of receptor function [Adams, 2003; Adams, 2004]. Proteasome inhibition can cause cellular apoptosis by affecting the levels of various short-lived proteins [Rajkumar et al., 2005]. Up-regulation of the proteasome catalytic subunit was shown to be involved in neuronal differentiation [Klimaschewski et al., 2006]. Because cell adhesion molecules can be modulated by the proteasome, the ubiquitin–proteasome pathways are thought to be involved in regulation of cell–cell contacts. Interestingly, proteasome inhibition blocks HGF treatment-mediated EMT of MDCK cells [Tsukamoto and Nigam, 1999], and pharmacological inhibition of the proteasome inhibits the translocation of β-catenin into nuclei of cells with stabilized cell–cell contacts [Saitoh et al., 2009]. TGF-β1 signaling pathway is involved in EMT [Xu et al., 2009], and the ubiquitin–proteasome pathways regulate transcriptional activation by TGF-β1 pathway [Zhang and Laiho, 2003], increasing evidences for a link between proteasome activity and EMT.

In hepatocytes, we found that TM4SF5 expression resulted in greatly reduced mRNA but unaffected protein levels, indicating that TM4SF5 enhances stability of proteins. Since TM4SF5 expression also results in EMT [Lee et al., 2008], we examined whether proteasome activity was involved in EMT induction by TM4SF5 expression. We found that inhibition of proteasome activity or suppression of the proteasome subunit led to EMT and that TM4SF5 expression down-regulated proteasome expression and activity. HGF treatment-mediated EMT of hepatocytes expressing endogenous TM4SF5 involved proteasome suppression. Thus suppression of TM4SF5 resulted in recovery of proteasome expression and inhibition of EMT.

RESULTS

WILDTYPE TM4SF5 EXPRESSION REDUCED RNA SYNTHESIS BUT MAINTAINED PROTEIN LEVELS PRESUMABLY VIA DECREASED PROTEASOME ACTIVITY

While previous studies to understand effects of TM4SF5 expression on hepatic carcinogenesis using SNU449 cells stably expressing wildtype (WT) or functionally-inactive N138Q mutant TM4SF5 [Lee et al., 2008], we often found that total RNA was remarkably decreased in TM4SF5-expressing SNU449 cells compared with mock- or mutant TM4SF5-transfectants (Fig. 1A). In mock- and mutant-transfected cells, *GAPDH* and *β-actin* transcript levels were normal but they were much lower in TM4SF5 WT-expressing cells (Fig. S1). Interestingly, liver or breast cancer cells expressing TM4SF5 ectopically (i.e., SNU449-TM4SF5 or MDA-MB 231-TM4SF5, respectively) and endogenously (HepG2 and Huh7, [Choi et al., 2008]) showed relatively lower mRNAs than TM4SF5-null liver (SNU449) or breast (MDA-MB 231 and MDA-MB 453 [Choi et al., 2008]) cancer cells (Fig. 1B). Meanwhile, GADPH, β-actin, and α-tubulin protein levels were similar in all cells. Bcl-2 and p27^{Kip1} protein levels were increased in TM4SF5 WT-expressing cells (Fig. 1C, [Lee et al., 2008]). These observations suggest that TM4SF5 expression results in lower transcriptional activities of certain genes but sustains stability of their product proteins.

The proteasome is the major proteolytic complex for intracellular proteins [Adams, 2003]. We rationalized that overall protein levels might be normal despite very low mRNA levels because the protein degradation system might be down-regulated. To confirm this, we first analyzed proteasome activities in different TM4SF5-negative or positive cell lines [Choi et al., 2008]. TM4SF5-negative SNU449 (mock), SNU668, and MDA MB-231 and MDA-MB 453 cells showed relatively higher proteasome activities, whereas TM4SF5-positive SNU449 (SNU449-TM4SF5), HepG2, and Huh7 cells showed lower activities (Fig. S2). We then analyzed proteasome activity in the absence or presence of the proteasome inhibitor MG-132. In TM4SF5-expressing cells without MG-132 treatment, the proteasome activity was 60% or less, compared with mock- or mutant-transfected cells (Fig. 1D). Further treatment of MG-132 at concentrations up to 0.5 μM reduced proteasome activity in both mock- and mutant-transfected cells (Fig. 1D). This MG-132-mediated reduction in proteasome activity was not due to altered cell proliferation or viability, since no significant changes in cell viability was observed by treatment of MG-132 at various concentrations (Fig. 1E). Whereas exogenously (i.e., SNU449-TM4SF5) or endogenously (i.e., Huh7) TM4SF5-positive cells showed insignificantly or slightly reduced proteasome activities on MG-132 treatment, TM4SF5-null cells (hepatic SNU449, gastric SNU668, and breast MDA-MB 231 cancer cells) showed significant decreases in proteasome activity on MG-132 treatment (Fig. 1F). In case of Huh7 with endogenous TM4SF5, the MG-132-mediated decrease in the activity was less in magnitude, compared to those in cases of TM4SF5-null SNU668 and MDA-MB 231 cells; basal activity was lower and MG-132-mediated decrease was slight (Fig. 1F). Stably-TM4SF5-expressing SNU449 cells showed an insignificant reduction in proteasome activity upon MG-132 treatment but endogenously TM4SF5-expressing Huh7 cells showed a certain but slight decrease in the activity. This discrepancy might be presumably because SNU449-TM4SF5 cells express more TM4SF5 than did Huh7 cells (Fig. 1B and [Choi et al., 2008]). Furthermore, ectopic expression of TM4SF5 in gastric SNU638 and SNU668 cancer cells resulted in reduced proteasome activities (Fig. 1G), indicating that the TM4SF5-mediated decrease in proteasome activity may occur in different cancer type cells. Therefore, the inhibitory effect of TM4SF5 expression on proteasome activity suggests that TM4SF5 expression may reduce proteasome function in a manner similar to MG-132 treatment.

TM4SF5 AFFECTED PROTEASOME SYNTHESIS BUT NOT UBIQUITINATION SYSTEM

The Ubiquitin (Ub)-proteasome system (UPS) functions in a multistep process involving the actions of E1 (Ub-activation enzyme), E2 (Ub-conjugation enzyme), E3 (Ub ligase), and a 26s proteasome complex [Dantuma and Lindsten, 2010]. UPS is initiated by the conjugation of a polyubiquitin chain to proteins destined for destruction. The polyubiquitin chain recruits the S19 regulatory cap of the proteasome, and the target protein is denatured and fed into the proteasome's

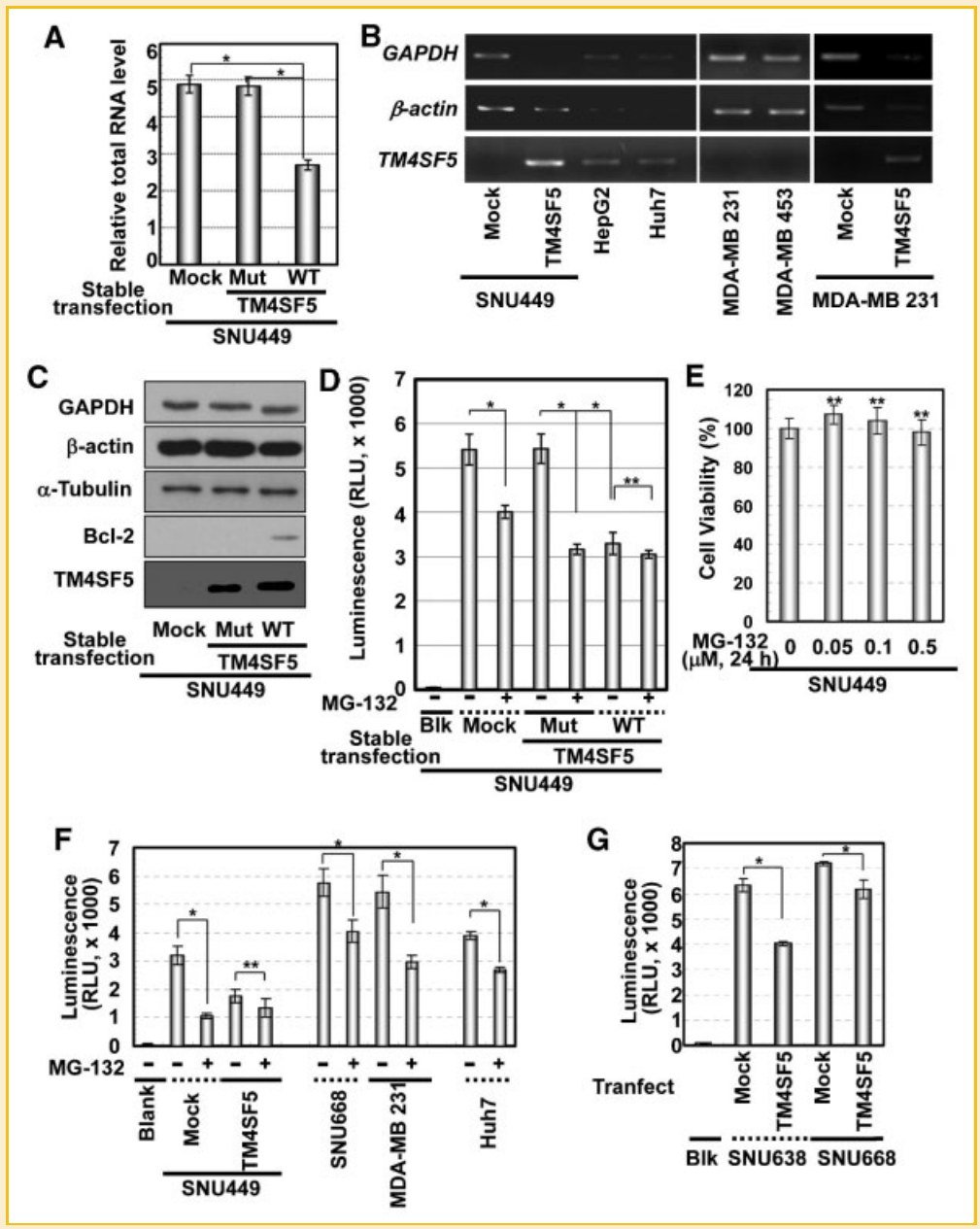

Fig. 1. TM4SF5 expression reduced RNA synthesis but not changed protein levels via decreased proteasome activity. (A) Decrease in total RNA in TM4SF5-expressing cells. SNU449 liver cancer cells stably transfected with mock (Mock), N138Q mutant, or wildtype TM4SF5 expression vector were cultured in 6 well culture plates and total RNA was isolated and quantitated by measuring absorbance at 260 nm. Values were at mean ± standard deviation from five independent experiments. (B) Stably transfected SNU449, endogenously TM4SF5-expressing HepG2 and Huh7 hepatic, and TM4SF5-null MDA-MB 231 transiently transfected with Mock or TM4SF5 plasmid or MDA-MB 453 breast cancer cells at subconfluent densities were used for RT-PCR for *TM4SF5*, *GAPDH*, and *β-actin* levels. mRNA of each gene was analyzed using specific primers, as explained in the Materials and Methods. TM4SF5-positive cell lines showed relatively lower levels of *GAPDH* and *β-actin*, compared to TM4SF5-negative cells. (C) Immunoblot analysis of cell extracts. Whole cell lysates of stably transfected SNU449 cells were prepared and immunoblotted for the indicated molecules, as described in the Materials and Methods. (D and F) TM4SF5-mediated down-regulation of proteasome activity. Stably transfected SNU449, TM4SF5-null SNU668 and MDA-MB 231, or endogenously TM4SF5-expressing Huh7 cells were treated with vehicle (−) or 0.5 μM MG-132 (+) for 24 h. Measurement of blank (Blk) with H_2O (instead of unknown samples as a negative control) was also performed and included. Proteasome activity was measured as described in the Material and Methods. (E) Subconfluent SNU449 parental cells were treated with MG-132 at diverse concentrations for 24 h, before cell viability analysis via MTT assay. (G) TM4SF5-null gastric SNU638 and SNU668 cancer cells [Choi et al., 2008] were transiently transfected with mock or TM4SF5 expression vector for 48 h before proteasome activity assay. * or ** depicts a statistic significance ($P \leq 0.05$) or insignificance ($P > 0.05$) by student *t*-test, respectively. Data shown represents at least three independent experiments.

proteolytic core [Pickart, 2001; Adams, 2003]. To confirm which step is altered in TM4SF5-expressing cells, we examined ubiquitination levels via Western blots using anti-ubiquitin antibody. Ubiquitination was dramatically increased in stably TM4SF5-transfected cells compared to mock- and mutant-transfected cells (Fig. 2A), despite low total mRNA levels in TM4SF5-expressing cells (Fig. 1A). These observations suggest that ubiquitinated proteins in TM4SF5-expressing cells were stabilized and not degraded. This evidence further suggests that the ubiquitination system (E1, E2, and E3) was not inhibited by TM4SF5 expression. To examine whether proteasome subunit expression was affected by TM4SF5 expression, we performed immunoblots using antibodies against the proteasome subunits S19 or S20. S19 and S20 proteasomes were detected in both mock and mutant-transfected cells but very slightly detected in TM4SF5 WT-expressing cells (Fig. 2B). To visualize the TM4SF5-dependent suppression of proteasome S19, we transiently-transfected TM4SF5 into SNU449 cells prior to immunofluorescence staining of S19. As shown in Figure 2C, the S19 proteasome levels (red, arrow) in TM4SF5-transfected cells (green, pEGFP-TM4SF5-positive) were much lower than those in neighboring non-transfected cells. Approximately 80% (80 ± 14.1 SEM) cells of pEGFP-TM4SF5-transfected cells showed a decreased S19 stain, compared to non-transfected neighboring cells (Fig. 2D). In addition, MG-132 treatment of mock or mutant TM4SF5-expressing cells did not alter S19 or S20 proteasome levels, although TM4SF5 WT expression itself decreased their expressions no matter whether MG-132 was added (Fig. 2E). An ectopic overexpression of TM4SF5 into a gastric SNU638 cancer cells also decreased S19 and S20 levels (Fig. 2F), indicating again that TM4SF5-mediated inhibition of proteasome expression may occur in various cancer type cells. These observations suggest that suppression or inhibition of the proteasome, but not the ubiquitination process, may be involved in TM4SF5-mediated effects of lower mRNA but normal protein levels.

PROTEASOME ACTIVITY AND EXPRESSION DEPEND ON TM4SF5 EXPRESSION

To confirm whether TM4SF5 expression inhibited proteasome activity, we transfected scrambled shRNA or shTM4SF5 into stably mock- or TM4SF5-transfected cells prior to analysis of proteasome activity. Proteasome activity of TM4SF5-expressing cells usually decreased to 50–60% of mock-transfected cells (Figs. 1D, E, and 3A), which was recovered by an introduction of shTM4SF5 (Fig. 3A and B). In addition, suppression of endogenous TM4SF5 in HepG2 and Huh7 cells significantly increased proteasome activities, as did suppression of TM4SF5 in SNU449-TM4SF5 cells (Fig. 3B).

We next examined whether the reduced proteasome activity might be due to a decrease in proteasome subunit synthesis. When RNA transcripts of proteasome β subunits, *PSMB1, PSMB2, PSMB3*, and *PSMB4* were examined by RT-PCR, we found that they were normally expressed in mock-transfected cells that were introduced with either scramble shRNA or shTM4SF5 (Fig. 3C, lanes 1 and 2). Compared to mock-transfected cells, TM4SF5-expressing cells showed greatly reduced proteasome subunit levels, except for non-altered *PSMB4* (Fig. 3C, lane 3). Furthermore, suppression of TM4SF5 by shTM4SF5 partially recovered *PSMB1, PSMB2*, and *PSMB3* levels (Fig. 3C, lanes 3 and 4). To confirm the recovery of proteasome subunit at the protein level, we performed immunoblotting and immunostaining using anti-S19 antibody. Suppression of exogenous or endogenous TM4SF5 in liver SNU449-TM4SF5 or lung H1975 cancer cells, respectively, increased S19 and S20 proteasome expressions (Fig. 3D). Cotransfection of shTM4SF5 with pEGFP into SNU449-TM4SF5 cells clearly showed that GFP-positive cells showed a brighter staining for S19, indicating a negative relationship between TM4SF5 and S19 expression (Fig. 3E). Approximately 75% (75 ± 12.0 SEM) cells of shTM4SF5-transfected cells showed increased S19 stains, compared to non-transfected neighboring cells (Fig. 3F). These results suggest that TM4SF5 negatively regulates proteasome expression.

TM4SF5–MEDIATED PROTEASOME INHIBITION APPEARED TO BE INVOLVED IN EMT

In hepatocytes, TM4SF5 expression resulted in EMT via aberrant actin bundling and loss of E-cadherin expression [Lee et al., 2008; Muschel and Gal, 2008]. Proteasome inhibition resulted in changes to cell morphology and scattering similar to TM4SF5 expression (Fig. S3A). Therefore, it may be likely that proteasome inhibition may also lead to EMT. It was previously shown that proteasome pathways are involved in regulation of Snail1 to control EMT [Zhou et al., 2004]. Therefore, we wondered whether MG-132 treatment-mediated cell scattering in TM4SF5-negative cells involved upregulation of Snail1. Indeed, we found that TM4SF5-null SNU449 parental cells increased Snail1 on MG-132 treatment (Fig. S3B). We next examined whether TM4SF5-mediated regulation of proteasome expression and activity is involved in hepatocyte EMT. In hepatocytes that endogenously express TM4SF5 such as HepG2 and Huh7, hepatic growth factor (HGF) treatment caused cell scattering with no β-catenin at cell–cell contact sites (i.e., EMT). Depending on TM4SF5 expression, suppression of TM4SF5 via introduction of shTM4SF5 blocked EMT even after HGF treatment (Fig. 4A and B, left images, [Lee et al., 2008]). We concomitantly observed an increase in S19 and S20 proteasome expression upon suppression of TM4SF5 (Fig. 4A and B, right immunoblots, respectively). The proteasome activities of other hepatocytes such as SNU449 and PLC/PRF5 also decreased after HGF treatment (Fig. 5A). We next examined the effects of MG-132 treatment on cell–cell contacts by analyzing cell–cell adhesion-related molecule expression levels and by visualizing the adhesions. Expression of cell–cell adhesion molecules in mock- and mutant TM4SF5-expressing cells were clearly reduced after MG-132 treatment, and TM4SF5-expressing cells showed undetectable expression (Fig. 5B). MG-132 treatment of HepG2 cells decreased cell–cell adhesion molecules either in scrambled shRNA or shTM4SF5-transfected cells (Fig. 5C). When TM4SF5 in HepG2 cells was suppressed, the down-regulatory effects of MG-132 treatment on cell–cell adhesion molecules were more obvious than when scrambled shRNA was transfected (Fig. 5C). However, SNU449-TM4SF5 cells showed undetectable expressions of the cell–cell adhesion molecules even in the absence of MG-132 treatment so that additional MG-132 treatment did not cause any further decrease in them (Fig. 5B). No changes in cell–cell adhesion molecules on MG-132 treatment of SNU449-TM4SF5 cells, unlike HepG2 cells, might be due to different cell types and a higher TM4SF5 expression level

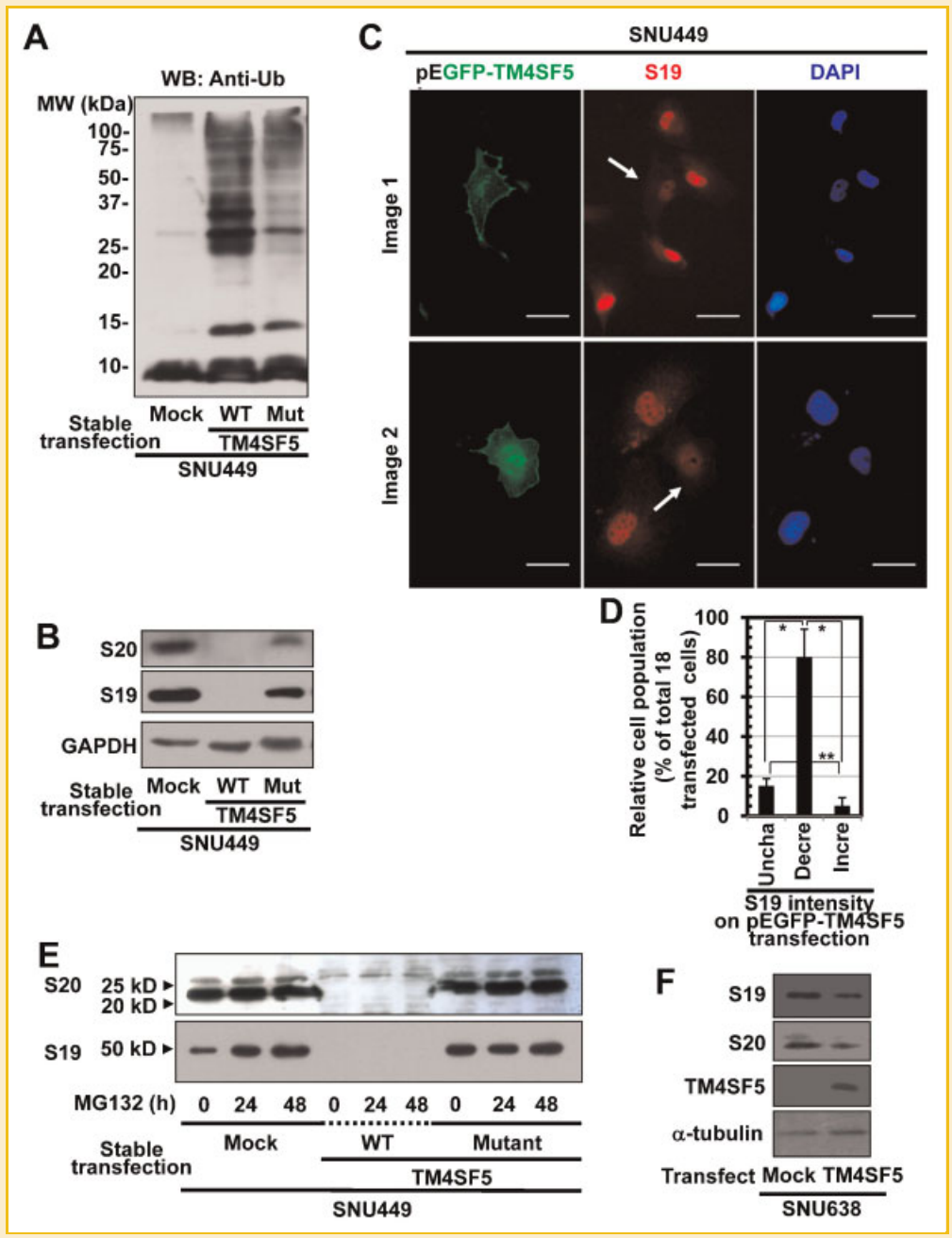

Fig. 2. TM4SF5 expression down-regulated proteasome expression. (A) Ubiquitination levels in stable SNU449 cells with mock, mutant, or wildtype TM4SF5 expression. Ubquitination levels were determined by immunoblotting of whole cell lysates using anti-ubiquitin antibody. (B) Loss of proteasome core in TM4SF5-expressing cells. Immunoblot analysis of whole cell lysates from stably transfected SNU449 cells was carried out using anti-S19 or -S20 proteasome antibody. (C and D) TM4SF5 expression down-regulated proteasome expression. Immunofluorescence microscopy was performed to detect S19 proteasome expression (red) in transiently pEGFP-TM4SF5-transfected SNU449 cells (green). Arrows indicate the cells which decreased S19 proteasome expression. Nuclei were also stained with 4′6-diamidino-2-phenylindole (DAPI, blue). Quantitation of pEGFP-TM4SF5-transfected cells ($n = 18$) with regard to changes in S19 stain intensity was performed for a graphic presentation at mean $\pm$ standard deviation. Uncha, Decr, and Incr depict unchanged, decreased, and increased, respectively (D). * or ** depicts a statistic significance ($P \leq 0.05$) or insignificance ($P > 0.05$) by student *t*-test. (E) SNU449 cells stably transfected with Mock, TM4SF5 wildtype (WT), or N138Q mutant plasmids were treated with 0.5 μM MG-132. After the indicated times, whole cell lysates were prepared and the lysates were subject to standard Western blot using anti-S19 or -S20 proteasome antibody. (F) TM4SF5-null gastric SNU638 cancer cells were transiently transfected with mock or TM4SF5 expression plasmid for 48 h before whole cell lysates preparation and standard Western blots for the indicated molecules. Data shown are representative in three different experiments.

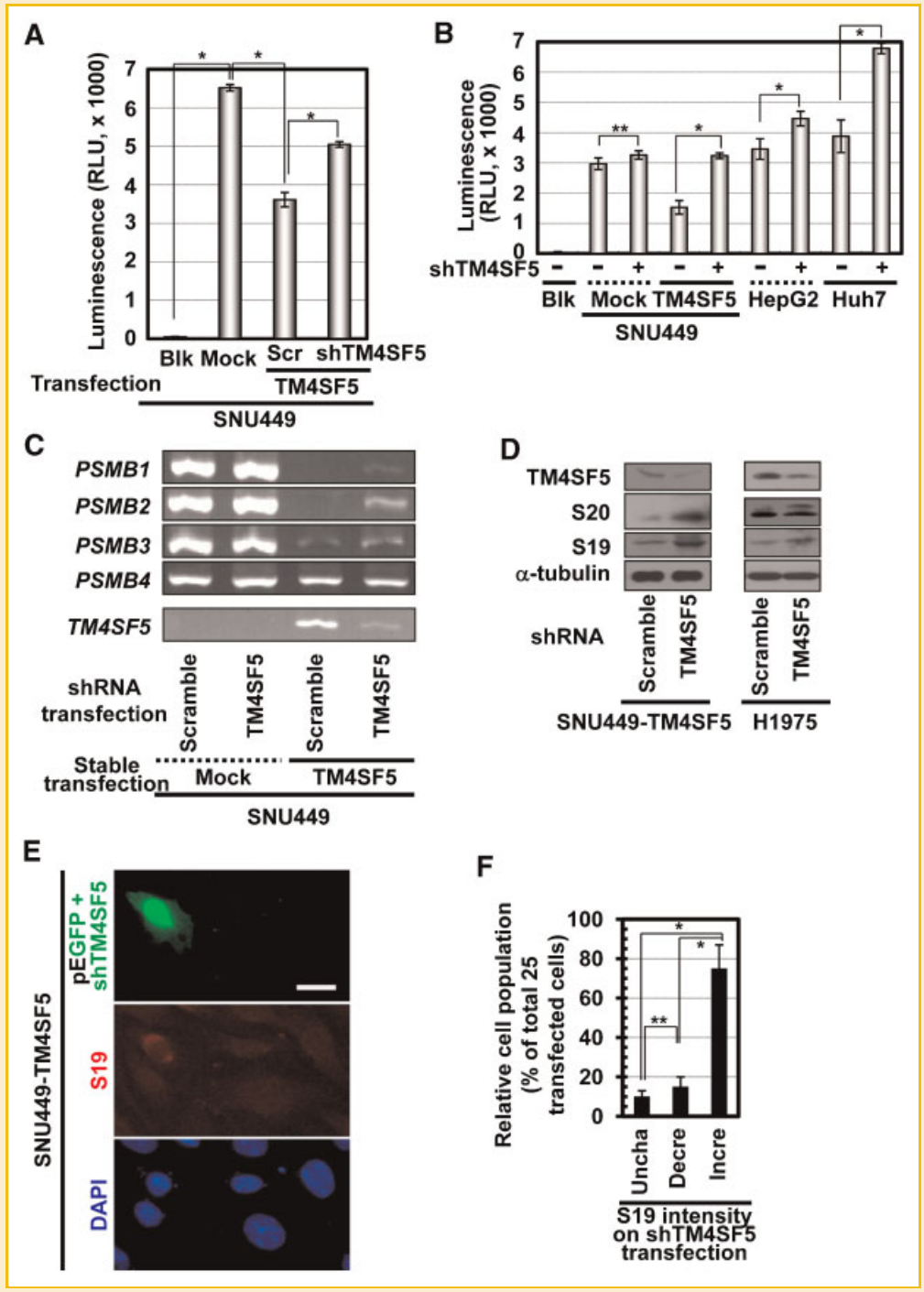

Fig. 3. Reciprocal relationship between TM4SF5 and proteasome expression. (A and B) TM4SF5 expression-dependent inhibition of proteasome activity. Proteasome activities were analyzed in SNU449 cells stably transfected with mock or TM4SF5 (A) and/or in endogenously TM4SF5-expressing HepG2 and Huh7 cells (B), which were transiently transfected with scrambled shRNA or shTM4SF5 and enriched by G418 application, as explained in the Materials and Methods. Measurement of blank (Blk) with H_2O was also performed and included. (C) TM4SF5 expression-dependent inhibition of proteasome subunit transcription. Stably Mock- or TM4SF5-expressing SNU449 cells were transiently transfected with shRNA against control (Scramble) or TM4SF5 sequence and enriched by G418 application, as described in the Materials and Methods. Semi-quantitative RT-PCR analyses for proteasome subunit genes (*PSMB1*, *2*, *3*, and *4*) and *TM4SF5* were performed. (D, E, and F) Suppression of TM4SF5 increased S19 and S20 proteasome expression. Scrambled shRNA or shRNA against TM4SF5 (shTM4SF5) (D) or pEGFP and shTM4SF5 (E) were transiently transfected into SNU449-TM4SF5 (D, E, and F) or lung H1975 cancer cells (D), before harvests of whole cell lysates and standard Western blots for the indicated molecules (D) or immunofluorescence microscopy using anti-S19 proteasome antibody (E and F). A cell positive for pEGFP (and thus shTM4SF5) showed increased S19 expression. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI, blue). (F) Quantitation of shTM4SF5-transfected cells ($n = 25$) with regard to changes in S19 stain intensity was performed for a graphic presentation at mean ± standard deviation. Uncha, Decr, and Incr depict unchanged, decreased, and increased, respectively. * or ** depicts a statistic significance ($P \leq 0.05$) or insignificance ($P > 0.05$) by student *t*-test. Data shown represent three independent experiments.

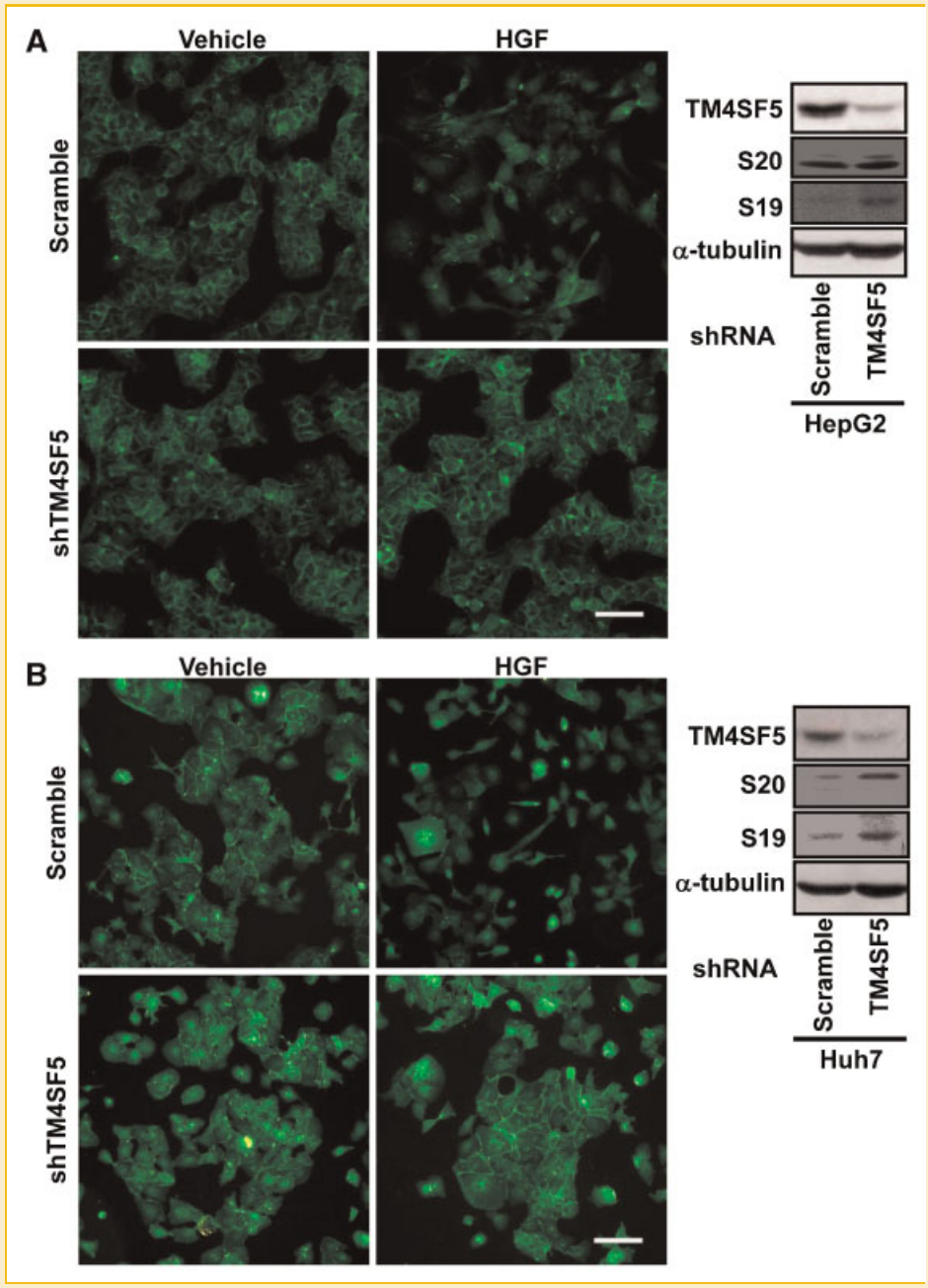

Fig. 4. Suppression of TM4SF5 caused blockade of HGF-mediated EMT and induced expression of proteasome subunits. HepG2 (A) or Huh7 (B) cells stably transfected with scrambled shRNA or shTM4SF5 were treated with vehicle or 100 ng/ml HGF, prior to immunofluorescence staining of β-catenin (images) or harvests of whole cell lysates for standard Western blots for the indicated molecules (immunoblots). Data shown represent three independent experiments.

than HepG2 cells (Fig. 1B and [Choi et al., 2008]). These observations suggest that MG-132 treatment or TM4SF5 expression similarly affects cell–cell adhesions, presumably leading to EMT. We visualized molecules at cell–cell contact sites via immunofluorescence staining to see whether proteasome inhibition might cause loss of cell–cell contacts. Cell–cell adhesions of mock- and mutant-transfected cells were abolished by MG-132 treatment and showed elongated morphologies similar to those of TM4SF5-expressing cells (Fig. 5D). These results suggest that TM4SF5 expression decreases proteasome activity and expression, which in turn presumably leads to morphological changes and EMT.

DISCUSSION

We previously reported that TM4SF5 expression in hepatocytes causes EMT by aberrant actin bundling and loss of E-cadherin expression, resulting in uncontrolled growth of S-phase transition under confluent or anchorage-independent conditions and tumor

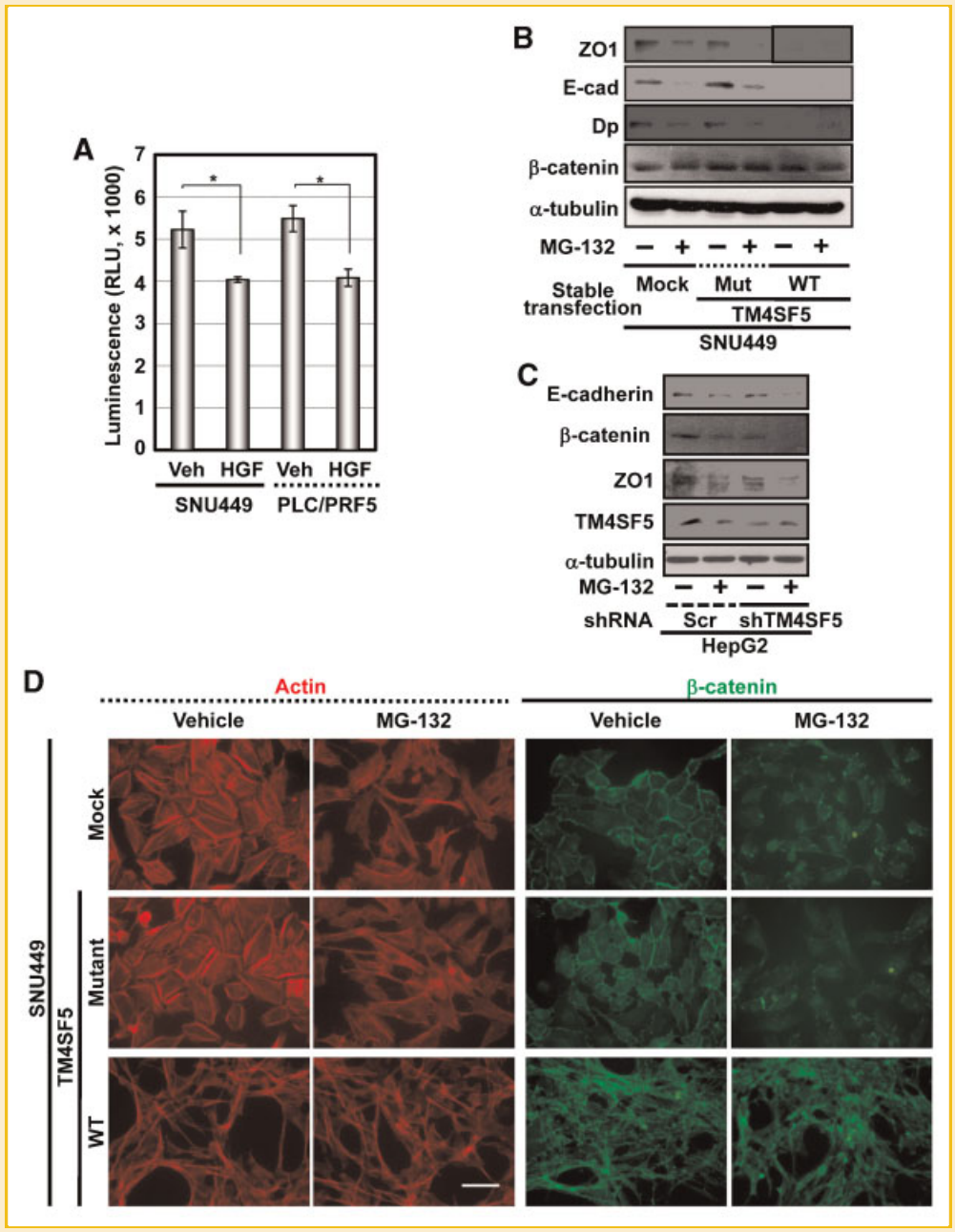

Fig. 5. TM4SF5 expression–mediated EMT involved proteasome suppression and inhibition. (A) SNU449 or PLC/PRF5 hepatocytes were treated with vehicle (Veh) or 50 ng/ml HGF for 16 h, prior to proteasome activity analysis. Measurement of blank (Blk) with H_2O was also performed and included, as described in the Materials and Methods. (B and D) SNU449 cells stably transfected with either mock, N138Q mutant, or wildtype (WT) TM4SF5 were treated with vehicle (−) or MG-132 treatment (+) for 24 h, before standard Western blots using antibodies against the indicated molecules (B) or actin staining using phalloidin–conjugated with TRITC or immunostaining using anti-β-catenin antibody (D). (C) HepG2 cells transfected with control shRNA (scrambled, Scr) or shTM4SF5 and enriched with G418 application, were treated without (−) or with (+) MG-132 for 24 h and harvested for immunoblots using antibodies against the indicated molecules. * depicts a statistic significance of $P \leq 0.05$ by student *t*-test. Data shown represent three independent experiments.

formation in nude mice [Lee et al., 2008; Muschel and Gal, 2008]. In this study, we provide more details of how TM4SF5 causes EMT in a protein metabolism-dependent manner. We observed that inhibition of proteasome activity or suppression of proteasome subunits was caused by TM4SF5, presumably leading to cell scattering. HGF treatment-mediated EMT of hepatocytes was blocked by suppression of TM4SF5, which also concomitantly increased (or recovered) S19 and S20 expression. Inhibition of proteasome activity with MG-132 also caused cell elongation and loss of cell–cell contacts, as did TM4SF5 expression itself.

Previous evidence suggests that UPSs are intimately involved in transcriptional control [Muratani and Tansey, 2003]. Total RNA, proteasome subunits transcription, and proteasome activity were decreased in TM4SF5 expressing cells. Ubiquitin-labeled protein levels were more intensive in TM4SF5-expressing cells than in mock-transfected cells, but proteasome expression levels were hardly detected. The accumulation of ubiquitylated proteins causes dysfunction of transcriptional machinery [Ferdous et al., 2001; Daulny et al., 2008], and results in inhibited biosynthesis of RNAs leading to lower RNA levels in cells. However, housekeeping proteins in TM4SF5-expressing cells were maintained at levels similar to mock- or mutant TM4SF5-expressing cells, presumably due to a decreased proteasome activity. *PSMB1, PSMB2,* and *PSMB3* (proteasome subunit β type 1, 2, and 3, respectively) mRNA levels were negatively regulated by TM4SF5 expressions. Since it was shown that PSMB2 functions as a core protein in the proteasome assembly [Murata et al., 2009], TM4SF5 expression-mediated inhibition of proteasome might lead to efficient stabilizations of certain proteins.

The ubiquitin-proteasome degradation system is involved in diverse biological processes, including cell-cycle progression, DNA repair, apoptosis, immune response, signal transduction, transcription, metabolism, and development [Adams, 2003; Adams, 2004; Dantuma and Lindsten, 2010]. Although E-cadherin was reduced in TM4SF5-expressing cells, TM4SF5 expression did not lead to an increase in Snail1 expression; rather Snail1 protein was reduced by TM4SF5 expression [Lee et al., 2008]. Down-regulation of proteasome activity during EMT has not been reported to date. Interestingly, this study provides evidence that reduced proteasome activity and/or subunit expression may lead to EMT. When TM4SF5 is not expressed, MG-132 treatment could efficiently increase expression of Snail1 (Fig. S3B) and decrease cell–cell adhesion molecule expressions (Fig. 5C), leading to delocalization of β-catenin from and loss of cell–cell contacts (i.e., EMT, Fig. 5D). In case when TM4SF5 is expressed, the effect of MG-132 treatment appears to be complicate. The effects of MG-132 treatment on cell adhesion molecule expression of stably TM4SF5-expressing SNU449-TM4SF5 cells showed insignificant changes due to undetectable levels, whereas endogenously TM4SF5-expressing HepG2 cells showed MG-132-mediated decrease in cell–cell adhesion molecules expressions (Fig. 5C, lanes 1 and 2). Probably this discrepancy of MG-132 effects on cell–cell adhesion molecule expression might attribute to differential expression levels of TM4SF5 between SNU449-TM4SF5 and HepG2 cells. The different TM4SF5 expression in the two cell lines caused also differential changes in the proteasome activity upon MG-132 treatment, as shown in Figure 1F. Furthermore, a partial suppression of TM4SF5 by shTM4SF5 (and thus still a certain expression level of TM4SF5) resulted in MG-132-mediated decreases in cell–cell adhesion molecule expression (Fig. 5C, lanes 3 and 4). It is thus likely that proteasome suppression and/or inactivation may be downstream of TM4SF5 during EMT.

Contrary to this study, previous reports have shown that proteasome inhibition results in stabilization of adherence junctions. MG-132 or TGF-β treatment to NMuMG cells reduces E-cadherin expression, but MG-132 treatment together with TGF-β stabilizes E-cadherin localization at cell–cell contacts [Saitoh et al., 2009], indicating that regulatory effects of proteasome activity on EMT may be differential depending on cell signaling contexts. The proteasome inhibitor NPI-0052 repressed Snail1 via inhibition of NF-κB in LNCaP cells, leading to blockade of EMT [Baritaki et al., 2009]. Meanwhile, our observation in this study showed that MG-132 treatment reduced β-catenin at cell–cell contact sites and increased Snail1, leading to EMT. This discrepancy between previous and current studies may attribute to different cell types and cell signaling pathways underlying proteasome inhibition. Taken together, TM4SF5 expression appears to cause not only aberrant actin bundling, resulting in morphological changes enough to physically disturb cell–cell contacts [Lee et al., 2008; Muschel and Gal, 2008], but also proteasome suppression and inhibition leading to EMT.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

SNU449 (Korean Cell Bank, Seoul, Korea) and PLC/PRF-5 (ATCC) cells were cultured in RPMI with 10% fetal bovine serum (GIBCO). Huh7, HepG2, MDA-MB 231, and MDA-MB 453, H1975 (ATCC), SNU638, and SNU668 (Korean Cell Bank) cells were cultured in DMEM-H with 10% fetal bovine serum. SNU449 cells transfected with Myc-$(His)_6$-pcDNA 3.1-TM4SF5 wildtype or N138Q mutant [Lee et al., 2009] were selected by G418 (A.G., Scientifics) after transfection using Lipofectamin-Plus (Invitrogen) according to manufacturer's protocols. Stably mock-, mutant- or wildtype TM4SF5-expressing SNU449 cells or endogenously TM4SF5-expressing Huh7, HepG2, or H1975 cells were transfected with scrambled shRNA (control shRNA) or shRNA against TM4SF5 (shTM4SF5), as previously described [Lee et al., 2008], and transfection-positive cells were enriched with 500 μg/ml G418 treatment for 1 week. SNU638 and SNU668 cells were transiently transfected with pcDNA3.1-TM4SF5 plasmid for 48 h before analysis. In some cases, HGF (eBioscience) was added at the indicated concentration to induce EMT. In cases, cells were treated with DMSO or MG-132 for 24 h and imaged using a phase-contrast microscope (BX41, Olympus).

WESTERN BLOTS AND ANTIBODIES

Cells transfected with indicated plasmids or treated with MG-132 were twice washed with ice-cold PBS and harvested in RIPA buffer (50 mM Tris-HCl pH 8.1, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors). Total protein amounts were measured using the bicinchoninc acid (BCA) assay (Pierce). Proteins in samples were separated on SDS–polyacrylamide gels, transferred to PVDF membrane (Whatman), probed with specific primary antibodies, washed, and probed with secondary antibody. Signals were detected using an enhanced chemiluminescent substrate (WEST-ZOL, iNtRON Biotechnology Inc, Seoul, Korea). The primary antibodies used include anti-proteasome S19, -proteasome S20 (Abcam, Cambridge, UK), -GAPDH (Cell Signaling Technology), -ubiquitin, -β-catenin, -β-actin, Snail1 (Santa Cruz Biotechnology), -α-tubulin (Bio Legend), -E-cadherin, -ZO1 (BD Biosciences), and -desmoplakin (DP, Abd Serotec, Oxford,

UK). In some cases, cells were treated with MG-132 (Calbiochem), a specific proteasome inhibitor, at the indicated concentrations.

PROTEASOME ACTIVITY ASSAY

For proteasome activity analysis using Proteasome-Glo™ Chymotrypsine-Like Cell-Based reagent (Promega), reagent treatments were done in growth media containing 5% FBS. Cells were seeded (0.5×10^5 cells/well of a 24 well plate) in triplicate, and then 0.5 μM MG-132 or 100 ng/ml HGF was added for 24 or 16 h at 37°C, respectively, before collection of cells using trypsin. Cells (10^4 cells/ml) were then seeded 100 μl/well of a 96-well plate, before addition of 100 μl/well of Proteasome-Glo™ Chymotrypsine-Like Cell-Based reagent and incubation for 5 min. Luminescence was measured with a luminometer (BMS). Relative light units (RLU) of luminescence were graphed at mean ± standard error of the mean (SEM).

RT-PCR ANALYSIS

Total RNA was isolated from cells using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was carried out using Reverse transcription system (Promega). PCR primers for amplification of PSMB1: Forward 5′-GCAGCCGTGCGATGTTGTCC-3′, Reverse 5′-GAGCACACAGATCAGTCCTTCC-3′; PSMB2: Forward 5′-TCGTGCTGTGTCGGACCTGC-3′, Reverse 5′-GTTCCCTGGCAAGTGGGAGG-3′; PSMB3: Forward 5′-GAGGGGTCCTAGTACACCGC-3′, Reverse 5′-CAGGGTTAGTCCATTCGGGC-3′; PSMB4: Forward 5′-GCTACCGTGACTAAGATGGAAGC-3′, Reverse 5′- TCAAAGCCACTGATCATGTGGGC-3′; GAPDH: Forward 5′-GAAGGTGAAGGTCGGAGTC-3′, Reverse 5′-GAAGATGGTGATGGGATTTC-3′; β-actin: Forward 5′-CTTCCTGGGCATGGAGTC-3′, Reverse 5′ –GCCAGGGTACATGGTGGT-3′; TM4SF5: Forward 5′-AGATCTCGAGCCATGTGCCCGCTG-3, Reverse 5′-TGCAGAATTCGTGAGGTGTGTCCTG -3′. PCR was performed using Taq polymerase (iNtRON Biotechnology Inc, Seoul, Korea). PCR was started with 5 min at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C, and then 1 min elongation at 72°C. TM4SF5 PCR conditions was with 5 min at 95°C, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Samples were analyzed by electrophoresis in 1% agarose gels containing 0.002% Nucleic acid staining solution (RedSafe™, Biotechnology Inc, Seoul, Korea).

MTT ASSAY

Cells (3000 cells/well of a 96 well plate) in triplicates were seeded and 24 h later MG-132 within DMSO was treated at different concentrations from 0 to 0.5 μM for additional 24 h. Standard reading of MTT (Sigma) metabolites was performed for OD_{540} and mean ± standard deviation values were graphed.

IMMUNOFLUORESCENCE MICROSCOPY

Cells were seeded in normal culture media-precoated cover glasses (Corning). Cells were washed with ice-cold PBS, fixed in 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and washed three times with PBS for 10 min. Cells were then blocked with 5% BSA in PBS, incubated with primary antibody for 1 h, washed with PBS, and then incubated with secondary antibody-conjugated FITC or TRITC (Chemicon) in a dark for 1 h. The primary antibodies include anti-β-catenin (Santa Cruz Biotech.) and -proteasome S19 (Abcam Inc). In cases, cell nuclei were stained with DAPI (Sigma). Cells were then washed with PBS (3 times x 10 min) and once with pure H_2O. Cells were mounted in a mounting solution (DakoCytomation, Germany) and visualized by a fluorescence microscopy (BX51, Olympus).

STATISTICAL ANALYSIS

All experiments were independently done at least three times. Data are presented as values at means ± standard deviation or SEM. Data were analyzed using student *t*-test. *P*-values ≤ 0.05 are considered significant.

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