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Transmembrane 4 L Six Family Member 5 (TM4SF5) Enhances Migration and Invasion of Hepatocytes for Effective Metastasis

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ABSTRACT

Overexpression of transmembrane 4 L six family member 5 (TM4SF5), a four-transmembrane L6 family member, causes aberrant cell proliferation and angiogenesis, but the roles of TM4SF5 in migration, invasion, and tumor metastasis remain unknown. Using in vitro hepatocarcinoma cells that ectopically or endogenously express TM4SF5 and in vivo mouse systems, roles of TM4SF5 in metastatic potentials were examined. We found that TM4SF5 expression facilitated migration, invadopodia formation, MMP activation, invasion, and eventually lung metastasis in nude mice, but suppression of TM4SF5 with its shRNA blocked the effects. Altogether, TM4SF5-mediated migration and invasion suggest that TM4SF5 may be therapeutically targeted to deal with TM4SF5-mediated hepatocellular cancers. J. Cell. Biochem. 111: 59–66, 2010.

KEY WORDS: INVADOPODIA; MIGRATION; INVASION; TM4SF5; MMP; METASTASIS

Tumor cells can undergo proliferation, survival, migration, and invasion facilitated via communication with micro-environment consisting of various extracellular matrices, soluble factors, and neighboring cells. The communication can be possible by actions of diverse membrane receptors [Liotta et al., 1986; Stamenkovic, 2003; Guo and Giancotti, 2004; Reddig and Juliano, 2005; Gavert and Ben-Ze'ev, 2008].

Tetraspanins (or TM4SFs) are a group of membrane receptors with four transmembrane domains, two extracellular loops, and two short cytoplasmic tails [Stipp et al., 2003]. Tetraspanins have homo- and heterophilic interactions that are supposed to organize the tetraspanin-enriched microdomain (TERM) on cell surfaces [Stipp et al., 2003]. They form complexes with integrins to collaboratively regulate cell motility [Berditchevski, 2001; Charrin et al., 2003].

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

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TM4SF5 is highly expressed in diverse tumor types [Muller-Pillasch et al., 1998; Pascual-Le Tallec et al., 2002] including hepatocarcinoma [Lee et al., 2008] and colon carcinoma (S.-A. Lee and J.W. Lee, unpublished work). Although TM4SF5 has four transmembrane domains like genuine tetraspanin or TM4SFs, it forms a transmembrane 4 L six family together with L6 (tumor-associated antigen TM4SF1 or L6-Ag), IL-TMP, and L6D [Wright et al., 2000]. The second extracellular loop (EC2) of TM4SF5 is relatively divergent and has two Cys residues, while the EC2 of genuine tetraspanins contains a variable region for specific protein–protein interactions, a conserved region for homodimerization, and four conserved Cys residues for disulfide bonding and correct folding [Wright et al., 2000; Stipp et al., 2003]. We recently found that TM4SF5 in fibroblasts can regulate focal adhesion turnover and actin reorganization depending on integrin and growth factor receptor activation [Lee et al., 2006], whereas TM4SF5 in epithelial cells causes morphological changes via RhoA inactivation, epithelial–mesenchymal transition (EMT), aberrant cell proliferation, multilayer growth [Lee et al., 2008], and VEGF induction/secretion leading to enhancement of angiogenic activity of (neighboring) endothelial cells [Choi et al., 2009]. It is highly expressed in the tumors from deceased breast cancer patients, compared to those from breast cancer patients survived for 10 years [Karlsson et al., 2008]. However, roles of TM4SF5 in tumor metastasis are not clear.

In this study, we explored the roles of TM4SF5 in cell migration/invasion and tumor metastasis. We found that TM4SF5 in hepatocarcinoma cells enhanced metastatic potentials of cellular migration and invasion via TM4SF5-enhanced MMP activation and invadopodia formation, which led to efficient metastasis to lung when TM4SF5-expressing cells were introduced into mouse tail veins. The observations thus suggest that TM4SF5 can be a promising target for hepatocarcinoma metastasis.

RESULTS

TM4SF5 ENHANCES CELL MIGRATION

Although roles of TM4SF5 with regard to diverse cellular functions [Lee et al., 2006, 2008; Choi et al., 2009] were reported, roles of TM4SF5 in cellular migration/invasion and tumor metastasis remain unknown. Stable infectants of control- or TM4SF5-retrovirus using SNU449 hepatocytes lacking TM4SF5 (SNU449Cp or SNU449Tp, respectively, Fig. 1A, right), as previously described [Lee et al., 2008], were used in the transwell migration assay. We filled the bottom chamber with media containing 10% FBS and top chamber with cells. TM4SF5-expressing SNU449Tp cells showed a greater migration in a time-dependent manner than did control SNU449Cp cells (Fig. 1A). Time-lapse live imaging of migratory cells showed that SNU449Tp cells migrated more persistently for a longer distance (Fig. 1B) at an approximately twofold greater velocity than did SNU449Cp cells (Fig. 1C). To see whether suppression of TM4SF5 in SNU449Tp cells decreased migration, we performed transwell migration assay with SNU449Cp and SNU449Tp cells transfected with shRNA against GFP (shGFP as a control) or TM4SF5 (shTM4SF5). As TM4SF5 was suppressed by shTM4SF5 (Fig. 1D, top), migration of SNU449Tp cells decreased to a level similar to TM4SF5-null SNU449Cp cells (Fig. 1D, middle and bottom panels). As expected, SNU449Cp cell migration was not affected by transfection of shGFP or shTM4SF5 (Fig. 1D, middle). Furthermore, TM4SF5 suppression in Huh7 cells expressing endogenous TM4SF5 by shTM4SF5, but not by shGFP, reduced migration (Fig. 1E).

TM4SF5 EXPRESSION ENHANCES INVADOPODIA FORMATION AND INVASION

We next wondered whether TM4SF5 expression might affect cell invasion. To assess invasion using three-dimensional (3D) collagen I, we embedded Cytodex-3 microcarrier beads coated with cells into 3D collagen I gels in the presence of 10% FBS/RPMI-1640 and incubated them to see outgrowth (i.e., invasion) of cells from the beads through collagen I gel. While no outgrowth of SNU449Cp cells through collagen I gel was observed even after 3 days, outgrowth of SNU449Tp cells began on day 2 and was obvious on day 3 (Fig. 2A). Doubling times are approximately 24 and 20 h for SNU449Cp and SNU449Tp, respectively (data not shown), so that the TM4SF5-enhnaced outgrowth through 3D collagen I may involve a contribution from a facilitated proliferation for longer incubations. Therefore, invasion through 3D matrigel for a shorter time was also examined. Invasion through 3D matrigel for 16 h was also enhanced by TM4SF5 expression, which was blocked by shTM4SF5, but not shGFP, transfection (Fig. 2B). Also in case of Huh7 cells, TM4SF5 suppression by shTM4SF5 but not by shGFP decreased invasion through 3D matrigel for 16 h (Fig. 2C). These observations indicate that TM4SF5 facilitated invasion.

Tissue invasion involves motile and lytic events through action of prominently actin-enriched invadopodia structure [Gimona et al., 2008]. It is thus likely that TM4SF5-enhanced invasion may involve facilitated invadopodia formation. Formation of invadopodia with enriched actin was examined by analyzing degradation of Oregon Green® 488-conjugated gelatin beneath cells for 6 h via fluorescent microscope. Actin-enriched invadopodial structures in SNU449Tp but not in SNU449Cp cells were obviously observed and matched ECM-degraded spots (Fig. 2D). The invadopodia is shown to recruit membrane type 1 matrix metalloproteinase (MT1-MMP) [Hoshino et al., 2009; Poincloux et al., 2009; Yamaguchi et al., 2009], cortactin [Bowden et al., 1999], Arp2/3, N-WASP [Yamaguchi et al., 2005], and actin [Gimona et al., 2008]. Therefore, we examined whether the actin-enriched invadopodia structures in SNU449Tp cells might be colocalized with the invadopodia markers. In double immunofluorescence studies, actin-enriched spots in SNU449Tp cells were costained with MT1-MMP, Arp2, cortactin, and N-WASP (Fig. 2E). However, actin stains in SNU449Cp cells were not significantly located together with those for the markers (Supplementary Fig. SF1). These observations indicate that TM4SF5-expressing cells efficiently form invadopodia, compared to TM4SF5-null cells, presumably leading to enhanced invasion.

TM4SF5-MEDIATED INVASION INVOLVES MATRIX METALLOPROTEINASE INDUCTION AND ACTIVATION ENHANCED BY TM4SF5

MMPs are detected in invadopodial structures for cell invasion [Noel et al., 2008]. We therefore examined whether MMPs were also regulated by TM4SF5. We first examined the expression of MMPs in stably TM4SF5-null or -expressing cells. During RT-PCR (Fig. 3A)

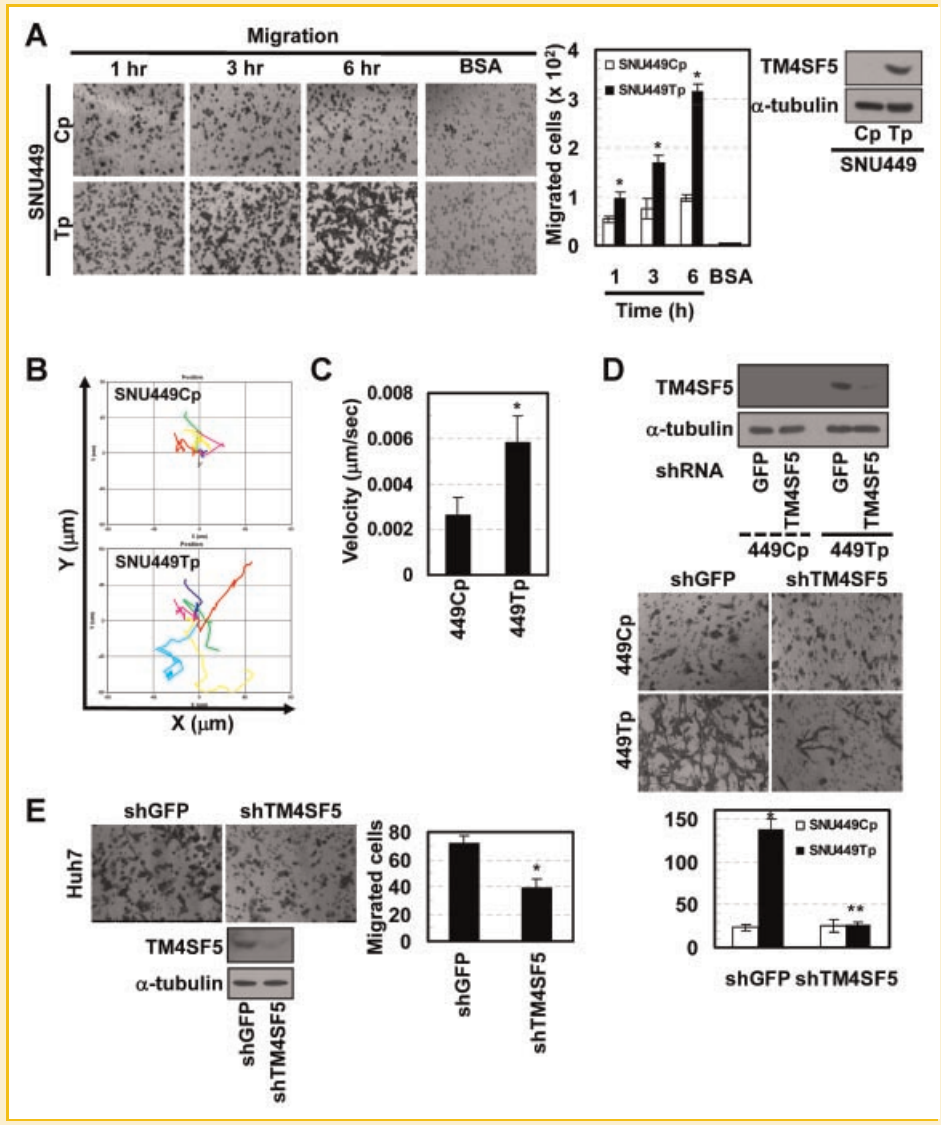

Fig. 1. TM4SF5-enhanced cell migration. A–C: TM4SF5-null SNU449Cp (449Cp) and TM4SF5-expressing SNU449Tp (449Tp) cells were analyzed for TM4SF5 expression using whole cell extracts (immunoblots) or for transwell migration. The bottom chamber was filled with 10% FBS/RPMI-1640 and cells were loaded to upper chamber. Migration was measured after incubation for the indicated times after adding cells. B,C: Tracking of individual cells and measurement of velocity (mean ± standard deviation) were performed for 5 h period using time-lapse live imaging, as explained in the Materials and Methods Section. D,E: SNU449Cp (449Cp) and SNU449Tp (449Tp) cells or endogenously TM4SF5-expressing Huh7 cells transfected with shRNA against either GFP (shGFP) or TM4SF5 (shTM4SF5) were analyzed for TM4SF5 expression (immunoblots) or for transwell migration (images and graphs). Graphs are with relative values at mean ± standard deviation values for numbers of cells migrated through the transwell filter. * or ** depicts *P*-values ≤0.05 or >0.05 considered significant or insignificant, respectively. Data shown are representative for three isolated experiments.

and Western blot (Fig. 3B), MMP2 and MMP9 were shown to be more expressed in TM4SF5-expressing SNU449Tp (a pooled clone), T3, T7, and T16 (single-cell-driven clones) cells [Lee et al., 2008] rather than TM4SF5-null parental SNU449 (P) or control SNU449Cp cells, indicating that these MMP2 and MMP9 may account for the TM4SF5-mediated invasion. MT1-MMP protein was expressed similarly independent of TM4SF5 expression (Fig. 3B), indicating its role in the basal invasion independent of TM4SF5. Then we measured MMP activity using gelatin zymography with conditioned media prepared from TM4SF5-null or TM4SF5-expressing SNU449 cells. MMP2 and MMP9 activities were higher in TM4SF5-expressing SNU449 cells than in TM4SF5-null cells (Fig. 3C). In addition, antibody array revealed that tissue inhibitors of metalloproteinase 1 and 2 (TIMP1 and TIMP2), which are inhibitors of MMP2 and MMP9 [Chirco et al., 2006], were less abundant in conditioned media from SNU449Tp cells than from SNU449Cp cells (Fig. 3D). However, other cytokines were present at similar levels in these conditioned media, indicating no effect of TM4SF5 on them. Interestingly, suppression of TM4SF5 from Huh7 and HepG2 cells expressing endogenous TM4SF5 by shTM4SF5 but not by control shGFP decreased MMP2 and MMP9 activities (Fig. 3E). Furthermore, enhanced invasion of SNU449Tp cells through 3D matrigel for 16 h

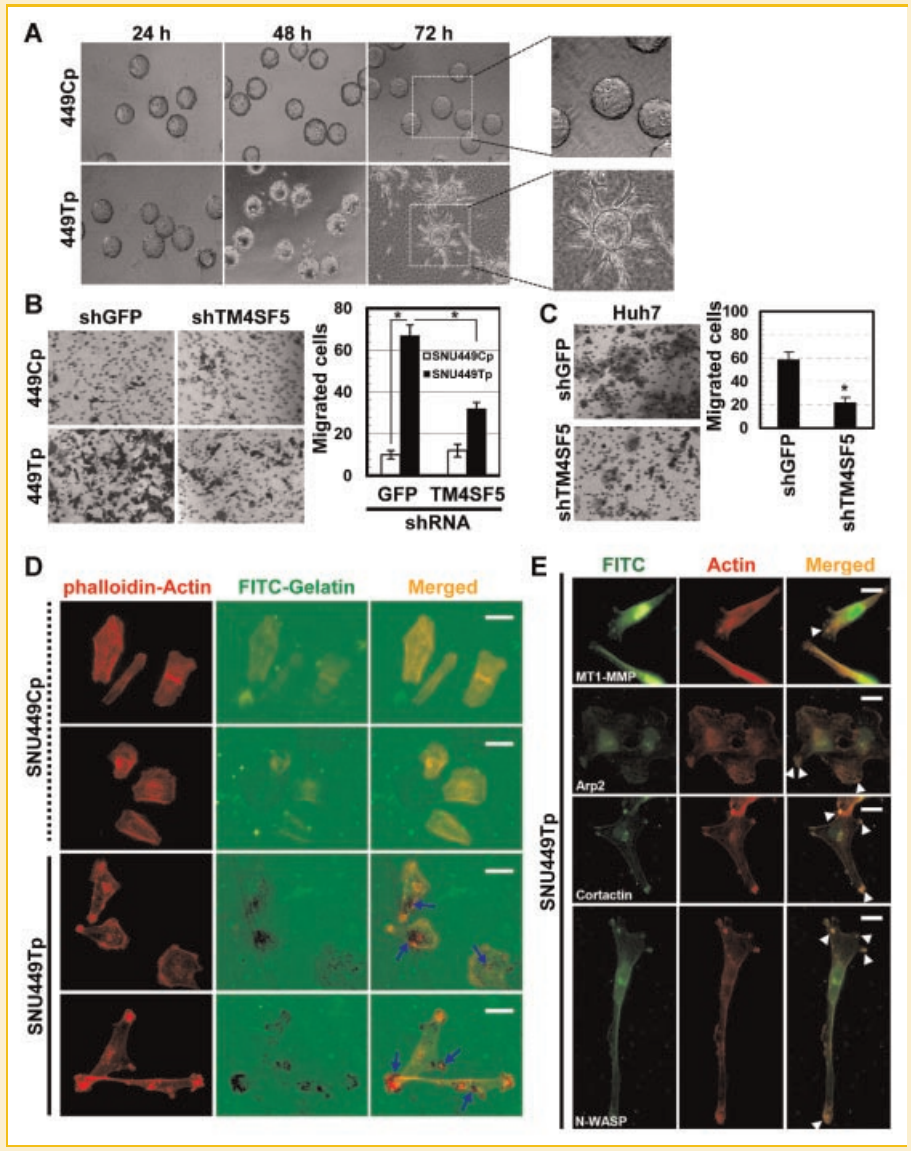

Fig. 2. TM4SF5 expression facilitated invadopodia formation and invasion. A: SNU449Cp (449Cp) and SNU449Tp (449Tp) cells were analyzed for invasion through 3D collagen I, and invaded cells through the 3D gel for the indicated times were imaged by a phase-contrast microscope. Regions in rectangle were scaled up for more obvious presentations. B,C: SNU449Cp (449Cp) and SNU449Tp (449Tp) cells or endogenously TM4SF5-expressing Huh7 cells transfected with either shRNA against GFP (shGFP) or TM4SF5 (shTM4SF5) were analyzed for transwell invasion with 3D matrigel. Graphs are with relative values at mean ± standard deviation values for numbers of invaded cells. * depicts *P*-values $\leq$0.05 considered significant. D: SNU449Cp and SNU449Tp cells were reseeded on coverglasses precoated with Oregon Green® 488-conjugated gelatin and incubated for 6 h, before staining actin and visualizing fluorescent-gelatin degradation. Arrows indicate actin-enriched spots where ECM is degraded. E: SNU449Tp cells coverglasses were stained for actin (red) using phalloidin-TRITC and immunostained for the indicated molecules (FITC). Arrowheads indicate stains to show actin-enriched spots costained with the indicated invadopodia marker. Scale bars, 20 μm. Data shown represent three independent experiments.

was blocked by pharmacological inhibition of either MMP2 or MMP9, whereas invasion of SNU449Cp cells were too insignificant to show changes by MMP inhibitions under the experimental condition (Fig. 3F). Interestingly, both MMP2 and MMP9 appear to be important for TM4SF5-mediated invasion, since TM4SF5-enhanced invasion was blocked partially by inhibition of either MMP2 or MMP9 (Fig. 3F). These observations indicate that TM4SF5 expression enhances MMPs induction and activation and causes TIMPs suppression, which may lead to an enhanced invadopodia formation and invasion.

TM4SF5 MEDIATES IN VIVO LUNG METASTASIS

It is likely that TM4SF5-enhanced migration and invasion of cells in vitro may correlate to effective metastasis of TM4SF5-expressing cells in mice in vivo. We thus injected SNU449Cp or SNU449Tp cells into the tail vein of nude mice to see if the cells could metastasize into lung or other organs. Mice were sacrificed 4 weeks after the injection and their organs were examined. Mice injected with SNU449Tp cells had three- to fourfold larger lungs than did mice injected with SNU449Cp cells (Fig. 4A,B). More importantly, mice injected with SNU449Tp cells had more nodules on their lung

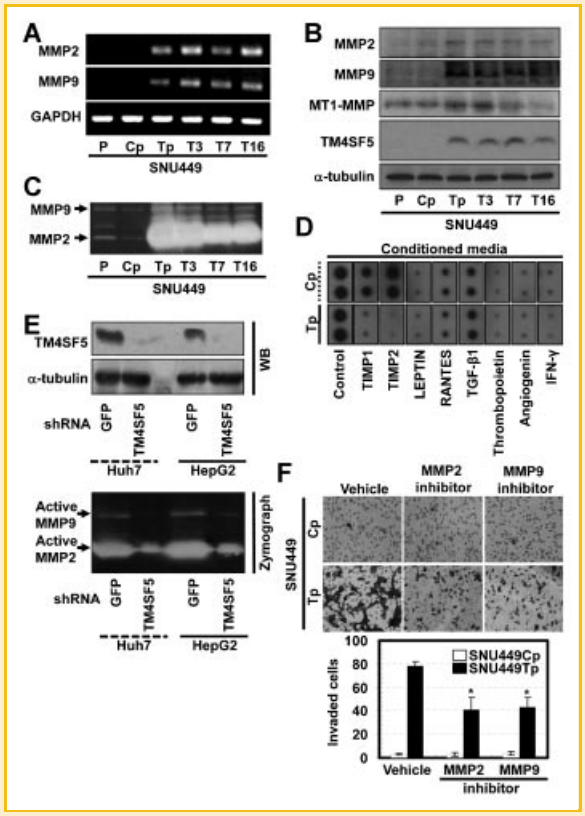

Fig. 3. TM4SF5-mediated invasion involved induction and activation of MMP2 and MMP9 and no TIMPs secretion. A: RT-PCR for MMP2 or MMP9 in diverse cell lines was performed, as described in the Materials and Methods Section. SNU449 cell clones of TM4SF5-null (P; parental SNU449, Cp; SNU449Cp) or -expressing cells (Tp; SNU449Tp, T3, T7, and T16; SNU449T3, T7, and T16 clones) [Lee et al., 2008] were analyzed. B: Diverse SNU449 cell lines in subconfluent conditions were harvested before standard Western blots for the indicated molecules. C: Conditioned media were prepared from subconfluent SNU449 cell clones serum-starved for 24 h, before analysis for MMP2 and MMP9 activities by gelatin zymography. D: Subconfluent SNU449Cp (Cp) and SNU449Tp (Tp) cells were serum-free for 24 h to collect conditioned media. Diverse factors including TIMP1 and 2 in the conditioned media were analyzed using RayBiotech® human antibody array. E: Whole cell extracts or conditioned media from Huh7 and HepG2 cells stably transfected with shGFP or shTM4SF5 were analyzed for TM4SF5 expression (WB, top) or for MMP2 and MMP9 activities (zymograph, bottom). F: SNU449Cp (Cp) or SNU449Tp (Tp) cells were analyzed for invasion through 3D matrigel for 16 h in the presence of vehicle, MMP2 or MMP9 inhibitor, as explained in the Materials and Methods Section. Invaded cells were imaged and counted from five random images to obtain mean ± standard deviations for graphs. * depicts *P*-values ≤0.05 considered significant. Data shown are representative for three different experiments.

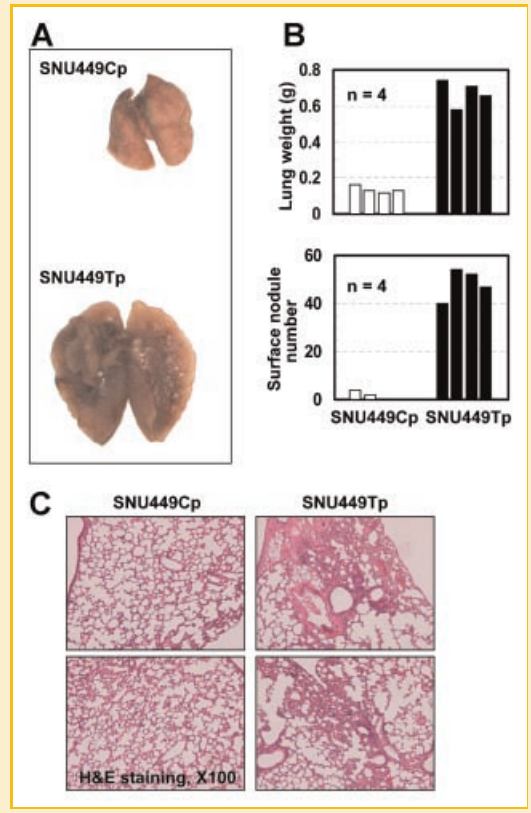

Fig. 4. TM4SF5 expression caused lung metastasis in vivo. A–C: SNU449Cp (Cp) and SNU449Tp (Tp) cells were injected into the tail veins of mice (10^7 cells/100 μl/mice, n = 4). After normal maintenance for 4 weeks, mice were sacrificed to examine in vivo metastasis. A: One representative lung image in each condition was shown. Note that the lung from SNU449Tp cell-injected mouse showed nodules on lung surface. B: Weights of mice lungs were graphed (top) and surface nodules were counted and graphed (bottom). C: The slice of paraffin blocks of the mice lungs were stained with hematoxylin and eosin, and visualized at 100× magnification using a microscope.

surfaces than did mice injected with SNU449Cp cells (Fig. 4A,B, lower), which appeared to be infiltrated with metastatic SNU449Tp cells (Fig. 4C).

DISCUSSION

TM4SF5 gene and its protein product TM4SF5 were highly expressed in different tumor types, compared to normal counterparts [Muller-Pillasch et al., 1998; Lee et al., 2006, 2008; Karlsson et al., 2008]. Although the roles of TM4SF5 in actin reorganization [Lee et al., 2006], abnormal proliferation [Lee et al., 2008], and angiogenesis [Choi et al., 2009] were reported, the roles of TM4SF5 in cellular migration/invasion and tumor metastasis have been unknown. We observed in this study that TM4SF5 expression in hepatocytes could enhance MMPs expression and activation, invadopodia formation, invasion through 3D ECMs in vitro, and thereby effective lung metastasis in vivo. Arp2, N-WASP, cortactin, and MT1-MMP, which are already-known invadopodia markers [Bowden et al., 1999; Yamaguchi et al., 2005; Hoshino et al., 2009], obviously located at actin-enriched invadopodia in TM4SF5-expressing cells but not in TM4SF5-null cells, indicating that TM4SF5 efficiently mediates formation of invadopodia to degrade ECM during invasion.

It appears that TM4SF5 on cell surface may collaborate with cell adhesion receptors, integrins, to transduce signaling activity to FAK and/or Rho GTPases leading to regulation of actin reorganization [Lee et al., 2006, 2008]. TM4SF5-mediated RhoA regulation led to alteration in cellular morphology and consequently EMT [Lee et al.,

2008], which may facilitate cell migration. It has recently been reported that angiotensin II mediates MMP2 activation through FAK/c-Src and JNK signaling activities [Jimenez et al., 2009]. FAK activation by adhesion onto fibronectin leads to an enhanced MMP9 expression and invasion [Meng et al., 2009]. We also observed that TM4SF5 could transduce intracellular signaling to activate FAK/c-Src (O.S. Jung and J.W. Lee, unpublished work). It is thus likely that TM4SF5-mediated activation of FAK/c-Src may lead to activation of MMPs. In addition to activation, we also observed that TM4SF5 expression led to induction of MMP2 and MMP9. These observations indicate that MMP2 and MMP9 can play important roles in TM4SF5-enhanced invasion. It would be interesting to see whether MMP2 and MMP9 also locate at invadopodia of TM4SF5-expressing cells, which we are currently pursuing, although MMP2 [Chen and Wang, 1999] and MMP9 [Nascimento et al., 2010] are shown to be at invadopodia. Meanwhile, MT1-MMP was expressed both in TM4SF5-null and -expressing cells. MT1-MMP in TM4SF5-expressing cells but not in TM4SF5-null cells located at invadopodia. Since TM4SF5-null control cells did not form invadopodia under our experimental conditions, MT1-MMP in TM4SF5-null cells may thus locate at podosome, as also shown previously [Sato et al., 1997], which may account for a basal migration and invasion activity independent on TM4SF5 expression. Since we found actin and other invadopodia markers co-localized in TM4SF5-expressing cells, it may be likely that TM4SF5 expression appeared to facilitate to have Arp2/3, N-WASP, and cortactin involved in actin reorganization for invadopodia formation, which correlated with an enhanced invasion of TM4SF5-expressing cells. Around the TM4SF5-enhanced invadopodia, TM4SF5-mediated MMP2 and MMP9 activities may critically function to degrade surrounding ECMs [Linder, 2007]. This TM4SF5-enhnaced invasion capacity of cells in vitro was further correlated with an enhanced metastasis to mouse lung in vivo. Therefore, TM4SF5 can be a promising therapeutic target for hepatocarcinoma metastasis.

MATERIALS AND METHODS

CELLS

Parental SNU449 hepatocellular carcinoma cells lacking TM4SF5 (Korean Cell Bank) and stable SNU449Cp or SNU449Tp, T3, T7, and T16 cell clones infected with control or TM4SF5 retrovirus, respectively, were previously described [Lee et al., 2008]; SNU449Cp is control virus infected cells, whereas SNU449Tp (a pooled clone), T3, T7, and T16 (single cell-driven clones) are cells stably infected with TM4SF5 retrovirus. Huh7 and HepG2 hepatocytes expressing endogenous TM4SF5 [Choi et al., 2008] were transfected with shRNA against GFP (shGFP as a control) or TM4SF5 (shTM4SF5), and transfection-positive cells were enriched with G418 (200 μg/ml) for 1 week before the experiments.

WESTERN BLOTS

Subconfluent cells in normal serum-containing culture media were harvested, as previously described [Lee et al., 2008]. Western blots were done with the whole cell lysates using anti-MMP2 (Calbiochem), -MMP9, -MT1-MMP (Santa Cruz Biotech.), -TM4SF5 [Lee et al., 2008], and -α-tubulin (Sigma) antibody.

MIGRATION AND INVASION ANALYSIS

Transwell chambers (8 μm porosity; Corning Costar) were used in the migration assay. The bottom chamber was filled with 10% FBS/RPMI-1640. Cells (5×10^5/0.2 ml serum-containing media) were placed in the upper chamber of a transwell and incubated for the indicated time at 37°C in 5% CO_2. After incubation, unmigrated cells in the upper chamber were removed and migrated cells on the bottom side of filter surface were fixed and stained with Diff-Quik solution (Scientific Products) before imaging [Lee et al., 2006]. Cells from at least five random fields were counted and mean ± standard deviation values were calculated for the graphic presentations. Cell invasion was evaluated by invasion through 3D matrigel for 16 h in the absence or presence of MMP inhibitor (Calbiochem; MMP2 inhibitor I at 10 μM and MMP9 inhibitor I at 50 nM) or by outgrowth through 3D collagen I gel (PureCol™, INAMED) from Cytodex-3 microcarrier beads (Sigma) for the indicated times in spinner flasks at 37°C and 5% CO_2 via recording images by phase-contrast microscope (Olympus) [Nehls and Drenckhahn, 1995]. Cells from at least five random fields were counted for the graphic presentations at mean ± standard deviation values.

TIME-LAPSE LIVE CELL IMAGING

SNU449 cells were replated on glass chambers (LabTekII; Thermo Fisher Scientific) in 10% FBS/RPMI-1640 and placed in a temperature- and CO_2-controlled chamber of a microscope (Olympus IX81-ZDC). Images were collected at 10 min intervals over 5 h with a Coolsnap HQ/QL cold CCD digital camera (model of CoolSNAP-HQ2). Cells were tracked using the position of centroids with MetaMorph image software (MDS Analytical Tech.) and their velocities were calculated for mean ± standard deviations.

INVADOPODIA FORMATION ASSAY

Cells were cultured on Oregon Green® 488-conjugated gelatin (10 μg/ml; Molecular Probes)-coated glasses for 6 h, before actin staining. Cells were double-stained for MT1-MMP, Arp2, N-WASP (Santa Cruz Biotech.), or cortactin (Cell Signaling Tech.) together with actin, before visualization using a fluorescent microscope (BX51; Olympus).

IN VIVO METASTASIS ANALYSIS

Cells (10^7 cells/100 μl RPMI-1640) were IV injected into the lateral tail vein of female BALB/c-n/n mice (Orient Co. Ltd). All animal procedures were performed according to the procedures in the Seoul National University Laboratory Animal Maintenance Manual and IRB agreement. All mice were sacrificed 4 weeks after tumor cell injection and their lungs were weighed and harvested. Metastatic colonies were counted macroscopically on the lung surface after staining with Bouin's solution (Sigma). The formalin-fixed lungs were stained for hematoxylin and eosin, prior to visualization at 100× using a microscope (BX51; Olympus).

REVERSE TRANSCRIPTASE-PCR

Total RNA from cells was isolated using TRIzol reagent (Invitrogen) and used for synthesis of cDNAs using Superscript III (Invitrogen). PCR amplification for MMP2 or MMP9 was performed using Taq DNA polymerase (New England Biolabs, Inc.). The primers used in the reactions were as follows: MMP2: 5′-CGGCCGCAGTGACGG-AAA-3′ and 5′-CATCCTGGGACAGACGGAAG-3′; MMP9: 5′-GAC-GCAGACATCGTCATCCAGTTT-3′ and 5′-GCCGCGCCATCTGCGTTT-3′ [Wegiel et al., 2008].

GELATIN ZYMOGRAPHY

Conditioned media prepared from cells in the absence of serum for 24 h were concentrated with a Microcon YM-30 centrifugal filter device (Millipore). The concentrated media were mixed with gel sample buffer [40% (v/v) glycerol, 0.25 M Tris–HCl, pH 6.8, and 0.1% bromophenol blue] at 3:1 (v/v) ratio, and loaded without boiling onto SDS–PAGE containing type 1 gelatin (1.5 mg/ml). The electrophoresed gel was soaked in 2.5% Triton X-100 with shaking (two times × 30 min), incubated for 24 h at 37°C in a substrate buffer (50 mM Tris–HCl, pH 7.5, 5 mM $CaCl_2$, and 0.02% NaN_3), stained with 0.05% Coomassie Brilliant Blue G-250 (Sigma), and destained in 10% acetic acid and 20% methanol.

ANTIBODY ARRAY

Subconfluent SNU449Cp and SNU449Tp cells were serum-deprived for 24 h. Conditioned media were collected from each cell culture. Soluble factors in the conditioned media were determined using RayBiotech® Human Angiogenesis Antibody Array, following the manufacturer's protocol.

STATISTICAL METHODS

Student's *t*-tests were performed for comparisons of mean values to see if the difference is significant. *P*-values ≤ 0.05 were considered significant.

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