약학석사학위논문

Regulation of Cellular Functions by the C-terminus of Transmembrane 4 L Six Family Members

Transmembrane 4 L Six Family Member 들의 C 말단에 의한 세포 기능 조절 연구

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ABSTRACT

Regulation of Cellular Functions by the C-terminus of Transmembrane 4 L Six Family Members

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Transmembrane 4 L six family includes TM4SF1 (L6), TM4SF4 (IL-TMP), TM4SF5, and others, with sharing the sequence identity at 30 to 50% is similar to the tetraspanins. Since the C-terminus of TM4SF5 appeared important for the cellular invasion property, it is wondered whether the C-termini of the family members were also important for cellular functions or comparable for the invasiveness. Therefore, we have constructed deletion mutants or chimeras, where the C-terminus in each member was deleted or swapped one another to investigate their roles in regulation of cellular behaviors in 2D flat or 3D gel environment using colon cancer cells stably expressing diverse constructs. In 2D environment, the cells showed that the C-termini of the family members were comparable each other for proliferation, although deletion of the C-terminus in each member led to increased proliferation. However, in 3D spheroid within culture media, TM4SF5 and the C-terminus of TM4SF5 were superior to TM4SF1 and the C-terminus of TM4SF1,

respectively. As for migration, TM4SF5 wildtype is also superior to TM4SF1 or TM4SF4, and the replacement of the C-terminus of TM4SF5 for counterparts of TM4SF1 or TM4SF4 led to increased transwell migration and disseminative outgrowth and migration from spheroids embedded in 3D collagen I gels, which were relevant to and dependent of c-Src activity. Replacement of the extracellular loops and the intracellular loop and the C-terminus of TM4SF5 for the counterparts of TM4SF1 resulted in greater migration than TM4SF1 wildtype but less than the chimera that the C-terminus of TM4SF5 replaced the counter part of TM4SF1. Further, the chimera that the extracellular loops of TM4SF5 conjugated to the other parts of TM4SF1 nullified the migration to a basal level. Therefore, these observations suggest that the intracellular parts of TM4SF5 mediates signaling for c-Src and pro-migratory roles in 2D and 3D environment, but not proliferation.

Key words: TM4SF5, Transmembrane 4 L Six Family, Chimeric mutants, three-dimensional culture, cell migration, cell proliferation

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INTRODUCTION

The plasma membranes sense extracellular environmental cues to trigger the intracellular signaling activity to regulate cellular behaviors responding to their needs. On the membrane, there are suggested to exist diverse communities containing certain groups of membrane proteins to sense extracellular cues, where are focal adhesions, lipid rafts, or tetraspanin-enriched microdomains (TERMs) (Yanez-Mo et al., 2009). At these communities, membrane proteins form complexes with other membrane proteins (or receptors) and/or cytosolic proteins to transduce intracellular signaling activities upon extracellular stimulations (Detchokul et al., 2014). The signaling activity importantly regulates cell functions of proliferation, migration, gene expression, and so on.

The transmembrane 4 L six family, similar to the tetraspanins, includes TM4SF1 (L6, L6-Ag), TM4SF4 (IL-TMP), TM4SF5 (L6H), TM4SF18 (L6D), and TM4SF20 (Lee, 2015). They have 4 transmembrane domains, two extracellular loops (EC1 for short extracellular loop and EC2 for long extracellular loop), an intracellular loop (ICL), and two cytosolic termini (N- and C-termini). The EC2 of TM4SF5 is known to be *N*-glycosylated at N138 and N155, and its *N*-glycosylation status is important for the interaction with other receptors such as integrin α2 or CD44 (Lee et al., 2015; Lee et al., 2009a). Meanwhile, the ICL or the C-terminus of TM4SF5 interacts with focal adhesion kinase (FAK) or c-Src to direct cellular migration or invasive protrusion, respectively (Jung et al., 2012; Jung et al., 2013). The cytosolic part of TM4SF5 is also involved in the interaction with the cytosolic tail of integrin α5 to cause signaling for VEGF induction (Choi et al., 2009).

Meanwhile, the transmembrane domains of TM4SF1 contributes to targeting of TM4SF1 to late endocytic organelles and interaction of the C-terminus of TM4SF1 to cytosolic syntenin-2 is critical for its targeting to TERM (Lekishvili et al., 2008). Meanwhile, such roles of subdomains in other transmembrane 4 L six family members remained unknown. Among the transmembrane 4 L six family members of TM4SF1, TM4SF4, and TM4SF5 only, the sequence identity can be around 30-50% enough to share a common membrane topology. However, their C-termini show relatively differential sequences, so that certain unique aspects in regulation of cellular functions may be mediated by intracellular signaling pathway by the C-terminus of each member (Lee, 2015; Wright et al., 2000).

Here we have tried to investigate how the C-termini of TM4SF1, TM4SF4, and TM4SF5 may be differential for the member-mediated regulation of cellular behaviors. Using colon cancer cell lines that are minimally expressing them, stable cell lines with ectopic expression of each wildtype, C-terminally-deleted one, chimera that has another's C-terminus, or another chimeric form, have used in analyses of cellular functions on 2D flat or 3D culture environment. The observations suggested that the C-terminus of TM4SF5 might override other's C-termini for migration and invasive dissemination, although the C-termini were insignificantly distinguishable for cellular proliferation. The TM4SF5-mediated pro-migratory roles also involved the structural relay from the EC2 to the C-terminus.

Material and Methods

cDNAs: Mutant DNA constructs that the C-terminus in each transmembrane L six family member (TM4SF1, TM4SF4, or TM4SF5) was deleted or switched each other DNA sequences cloned from the wildtype cDNAs, using PCR approaches. Chimeric 5TM1 and 1EC5 DNA constructs were previously explained (Lekishvili et al., 2008). These DNA constructs including wildtype cDNAs, were comparably introduced to pBabe-HA2-puro retroviral vector.

***Cells*:** pBABE-HAII-puro retroviral vector containing each wildtype or mutant construct were cotransfected with packaging (G.P) and envelope(pDM.G) vectors into Phoenix retrovirus producing cells. Retrovirus was harvested from the supernatant at 24 hr postransfection. Polybrene was added in order to enhance infection efficiency. HCT-116, HT-29 (ATCC, Manassas, VA), or SNU-407 (Korean Cell Bank, Seoul National University, Seoul, Korea) were infected with the retrovirus and selected in 2 μg/ml puromycin for 2 days. Established stable cell lines were maintained in DMEM (Welgene Inc., Daegu, Korea) containing 10% FBS and 1% penicillin/streptomycin (GenDEPOT Inc.) at 37°C in 5% CO_2.

***Spheroid formation and embedding into 3D collagen type 1 gels*:** Spheroids were formed by seeding each stable cell line at 8 x 10^5 cells on non-adhesive petri dish in DMEM (Welgene Inc.) containing 10% FBS and 1% penicillin/streptomycin (GenDEPOT Inc.), and incubated on an orbital shaker within a CO_2 incubator at 37°C

and 5% CO_2, until collection of the spheroids.

***Sphere growth assay*:** Cells were collected and washed with PBS twice in order to remove serum, then suspended in serum-free DMEM/F12 supplemented with 1% penicillin/streptomycin (GenDEPOT Inc.) and 2% B27 supplement (Invitrogen). Twenty five ng/ml of hEGF and hbFGF (Peprotech) were added every other days. The cells were subsequently cultured for 7 days in Ultra Low Attachment 6-well plates (Corning Inc. Corning, NY, USA) at a density of 5 x 10^3/well.

***Proliferation assay*:** Each HCT116 stable cell line was seeded in 8 wells (5 x 10^3 cells/well) in two 96 well plates. After 24 or 48 hrs, cells were treated with 20 μM MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (Sigma) for 4 hrs at 37ºC and mixed with MTT solvent for 20 min, and then their optical densities were read at 540 nm.

***Time-lapse imaging of cells in 3D ECM gels*:** Time-lapse images of spheroids embedded in 3D collagen type 1 gels were collected every 30 min for 24~48 hrs using IX81-ZDC microscope (Olympus). The microscope was equipped with 48-well Chamlide Incubator System (Live Cell instrument, Seoul, Korea), and an environmental chamber was maintained at constant conditions of 37ºC, 5% CO_2, and 95% humidity. Scale bars depict 100 μm.

***RT-PCR*:** Total RNA was extracted from cells using TRIzol (Invitrogen), according to the manufacturer′s protocol. Total RNA (500 ng) was reversely transcribed using amfiRivert Platinum cDNA Synthesis master mix (GenDEPOT). Primers used for

PCR were indicated in the Table 1. Since 5TM1 and 1EC5 constructs share the same N-terminus and EC2, these regions were used to design primers for PCR (Table 1).

***Western blots*:** The cells were grown in 6-well culture plates and harvested at 80% of confluency, before preparation of whole cell lysates with modified RIPA buffer (Lee et al., 2008). The spheroids cultured in 3D collagen type 1 environment were harvested as explained in previous study (Nam et al., 2015). Primary antibodies for immunoblot were used as follows: phospho-Y^{416} Src, (Cell Signaling); c-Src (Santa Cruz Biotechnology); FAK, phospho-Y^{397}FAK (BD Bioscience); anti-HA (BioLegend).

***Transwell migration assay*:** Stable cells were analyzed for migration using transwell Boyden chambers with 8.0 μm of pore size (Corning). Membrane was coated with ECM by incubating it with 10 μg/ml collagen type 1 in PBS solution at 37 °C for 2 hrs, and the control membrane was incubated with 5% BSA in DMEM culture medium at 37°C for 2 hrs. Prior to assay, stable cells were collected in the 1% BSA-DMEM and continuously agitated for 2 hrs in order to nullify their adhesion signals. Cells were then seeded at 1 x 10^5 cells per insert within 1% BSA-DMEM, and the wells were filled with the same culture media.

***Statistical Methods*:** A two-tailed unpaired Student′s *t*-test was performed to determine significance of difference between two groups. A *p*-value less than 0.05 was considered statistically significant

Results

1. The C-terminus of TM4SF5 was relatively comparable with the C-termini of TM4SF1 and TM4SF4 for cellular proliferation.

Although the amino acid sequence of TM4SF5 shares identifies of 40-50% with TM4SF1 and TM4SF4 (Fig. S1), their C-termini are very irrelative (Fig. 1A). To understand the significance of each C-terminus in diverse cellular functions, mutants that each C-terminus was deleted (i.e., ΔC), or chimera that one C-terminus was replaced with another′s C-terminus (i.e., 1C5 depicts the chimera where the C-terminus of TM4SF1 was replaced with the C-terminus of TM4SF5) (Fig. 1B). In addition, we have selected cell lines that minimally expressed either TM4SF1, TM4SF4, or TM4SF5 and used for the stable introductions of the constructs (Fig. 1C). The $(HA)_3$-tagged constructs were stably infected in HCT-116 cells and resulted in expression levels differential among each TM4SF backbone but comparable expression levels of different constructs within each backbone of either TM4SF1, TM4SF4, or TM4SF5 (Fig. 1D). While examination of the significance of each C-terminus in cell proliferation via MTT assay, WT TM4SF1 and TM4SF5 increased cell growth, but TM4SF4 cell slightly decreased cell growth, compared with control mock cells, whereas all deletion mutants increased growth, compared to those of own WT (Fig. 1E). More interestingly, exchanging of the C-terminus among them (i.e., chimeras) did not show different proliferation levels, compared to those of own WT (Fig. 1E).

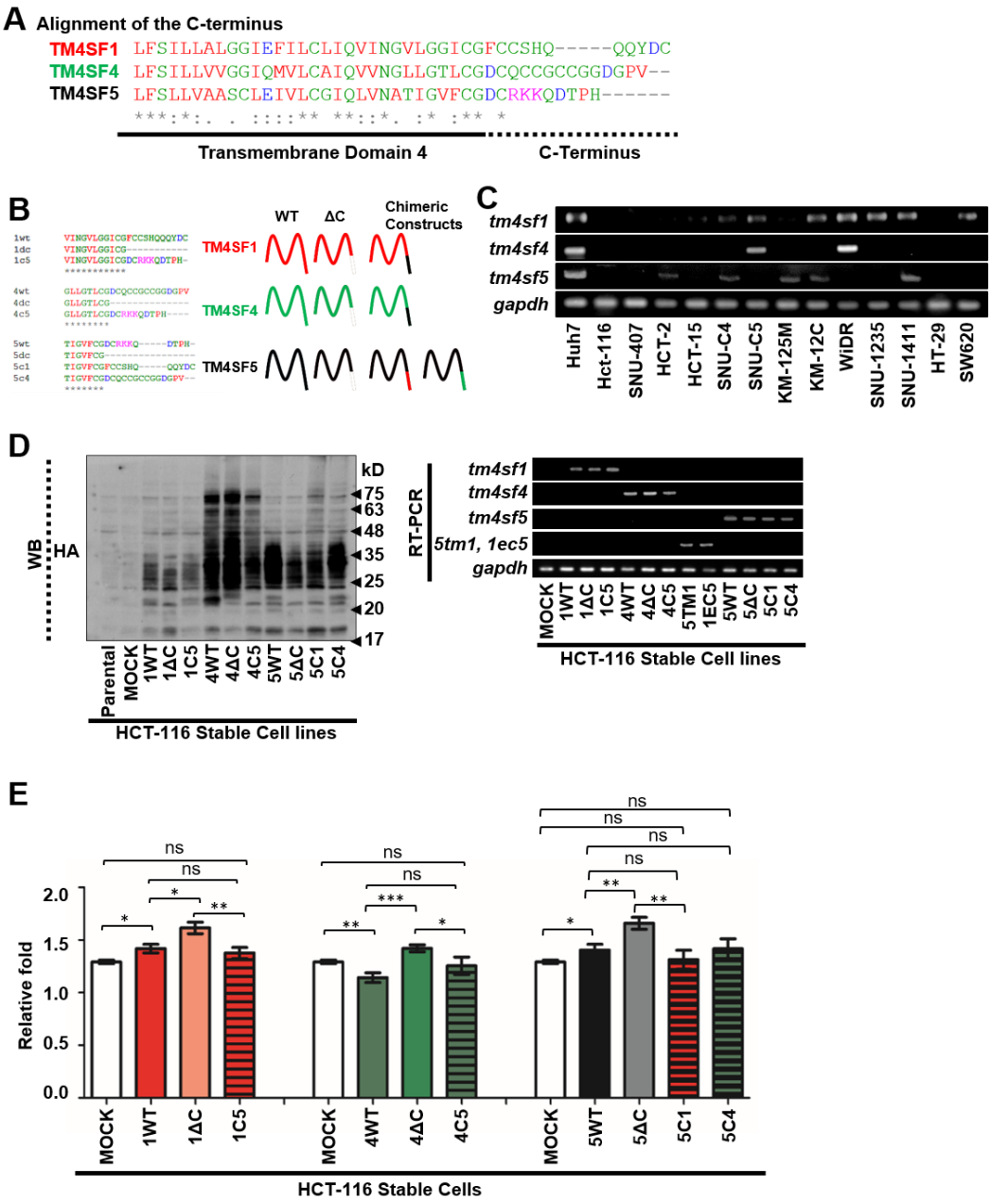

A
Alignment of the C-terminus
TM4SF1 LFSILLALGGIEFILCLIQVINGVLGGICGFCCSHQ-----QQYDC
TM4SF4 LFSILLVVGGIQMVLCAIQVVNGLLGTLCGDCQCCGCCGGDGPV--
TM4SF5 LFSLLVAASCLEIVLCGIQLVNATIGVFCGDCRKKQDTPH------
Transmembrane Domain 4
C-Terminus
B
WT
ΔC
Chimeric Constructs
TM4SF1
TM4SF4
TM4SF5
C
tm4sf1
tm4sf4
tm4sf5
gapdh
Huh7
Hct-116
SNU-407
HCT-2
HCT-15
SNU-C4
SNU-C5
KM-125M
KM-12C
WiDR
SNU-1235
SNU-1411
HT-29
SW620
D
WB
HA
kD
75
63
48
35
25
20
17
Parental
MOCK
1WT
1ΔC
1C5
4WT
4ΔC
4C5
5WT
5ΔC
5C1
5C4
HCT-116 Stable Cell lines
RT-PCR
tm4sf1
tm4sf4
tm4sf5
5tm1, 1ec5
gapdh
MOCK
1WT
1ΔC
1C5
4WT
4ΔC
4C5
5TM1
1EC5
5WT
5ΔC
5C1
5C4
HCT-116 Stable Cell lines
E
Relative fold
2.0
1.5
1.0
0.5
0.0
ns
*
**
***
MOCK
1WT
1ΔC
1C5
4WT
4ΔC
4C5
5WT
5ΔC
5C1
5C4
HCT-116 Stable Cells

Figure 1. The C-termini of transmembrane 4 L six family member 1, 4, and 5 played roles comparably in cell growth in 2D flat environment. (A) Alignment of the C-terminal sequences among TM4SF1, TM4SF4, and TM4SF5 showed differential sequences around their cytosolic tails.

(B) Schemes of the constructs used in the study for wildtypes and deletion or chimeric mutants. (C) mRNA levels of *tm4sf1*, *tm4sf4*, and *tm4sf5* in different colon cancer cells. (D) Expression levels of the constructs (protein in left panel and mRNA in right panel) in HCT-116 stable cells. Within each backbone [i.e., TM4SF1 wildtype (1WT), C-terminal-deleted TM4SF1 (1ΔC), and a chimera with the TM4SF5 C-terminus linked to the other parts of TM4SF1 (1C5)], their expression levels were similar. As for TM4SF4 or TM4SF5 backbone, their expression levels are comparable within each backbone. (E) The stable cells were analyzed for growth (from day 1 to day 2 after seeding) via MTT assay, as described in the Materials and Methods. The graphic values were at mean ± standard deviation (SD). *, **, or *** depicts statistically significant difference at p <0.05, 0.005, or 0001, respectively, and ′ns′ depicts nonsignificant difference at $p \geq 0.05$. The data shown represent three independent experiments.

2. The growth of spheroid in 3D aqueous conditions increased by the C-terminus of TM4SF5 but not of TM4SF1

We next examined whether cell growth in 3D aqueous condition would be differentially regulated by the C-terminus of them. We have cultured cells on less adhesive culture dishes, leading to form spheroids for days (Fig. 2A). Then the spheroids for each cell lines were analyzed for the volumes using a software. TM4SF5 caused spheroids to aggressively grow to levels greater than TM4SF1 (Figs. 2A and 2B). More interestingly, a chimera of TM4SF1 whose C-terminus was replaced with the C-terminus of TM4SF5 (i.e., 1C5) showed growth greater than TM4SF1 WT, whereas 5C1 (i.e., an opposite chimera of TM4SF5 with the TM4SF1 C-terminus) decreased the growth, compared to TM4SF5 WT (Figs. 2A and 2B). These observations suggest that TM4SF5 might be superior to TM4SF1 and the C-terminus of TM4SF5 could be important for the positive effects on spheroid growth, in contrary to cell proliferation in 2D condition.

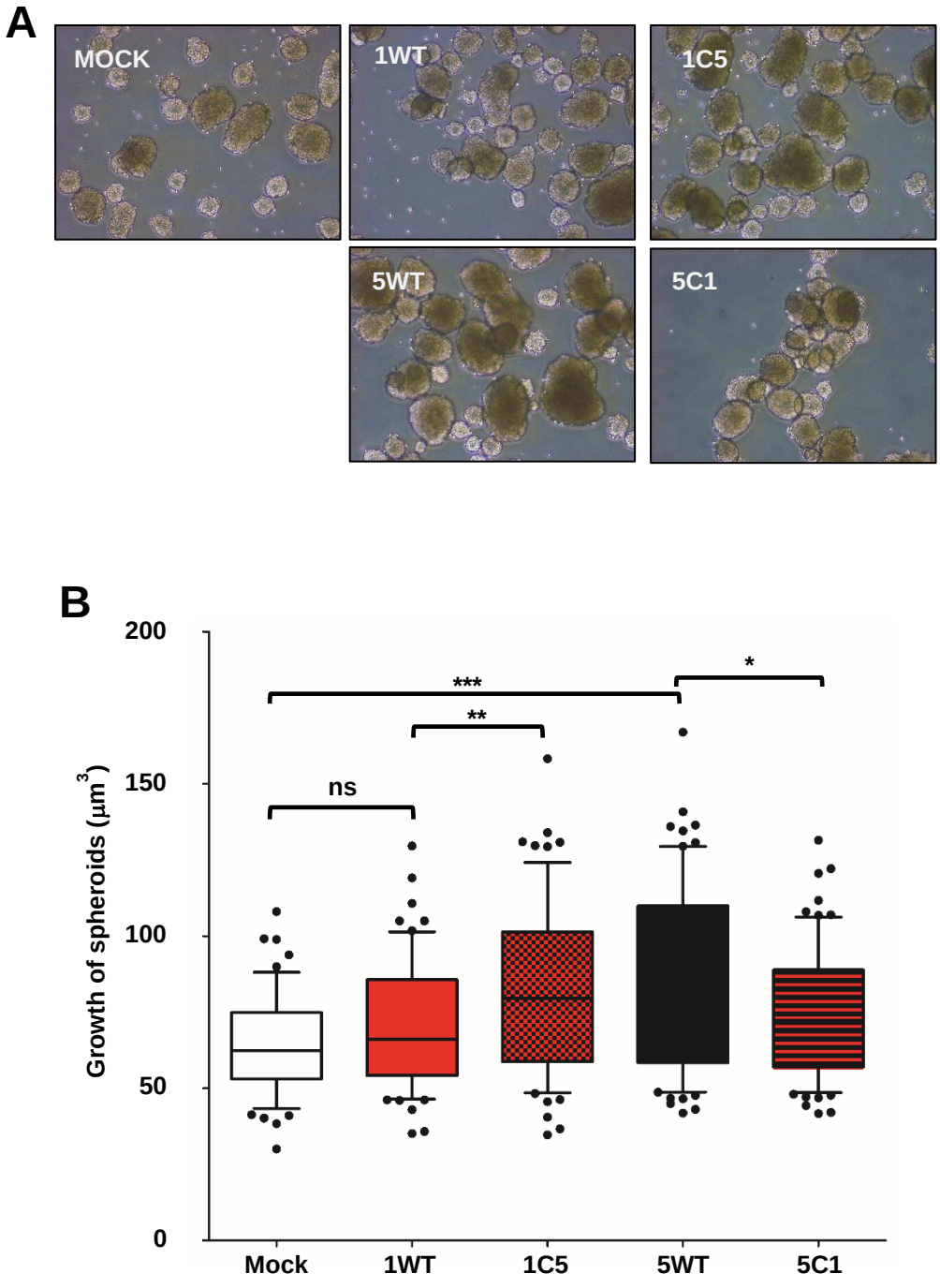
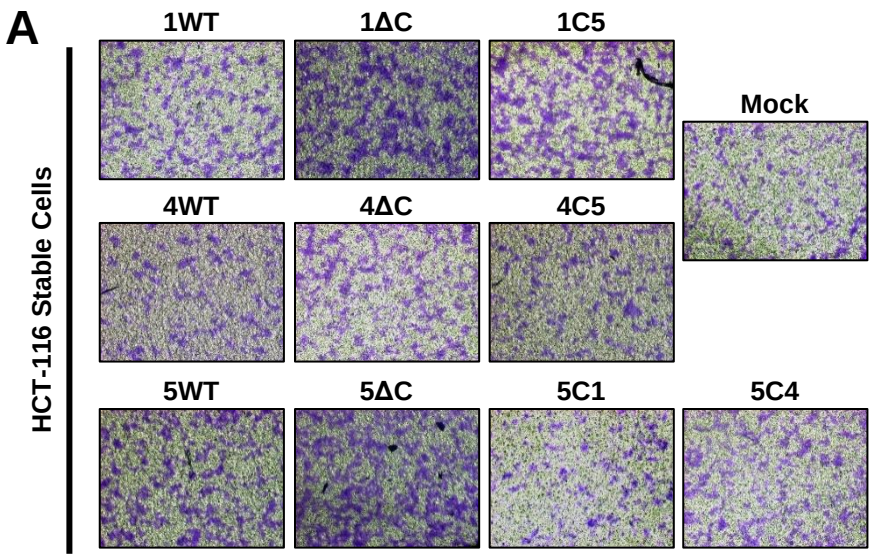

A
MOCK
1WT
1C5
5WT
5C1
B
Growth of spheroids (µm3)
200
150
100
50
0
***
**
*
ns
Mock
1WT
1C5
5WT
5C1

Figure 2. TM4SF5 and its C-terminus promoted sphere growth in 3D aqueous environment greater than TM4SF1 and its C-terminus. (A) The cells (5,000 cells/condition) were cultured in Ultra Low Attachment 6-well plates for 7 days, before saving the representative images. (B) The spheres in the wells were analyzed for their diameters using a software, before their volume calculations and graphic presentation at mean ± SD values. *, **, or *** depicts statistically significant difference at $p < 0.05$, 0.005, or 0001, respectively, and ′ns′ depicts nonsignificant difference at $p \geq 0.05$. The data shown represent three isolated experiments.

3. The C-terminus of TM4SF5 could improve transwell-migration of TM4SF1 and TM4SF4, but not *vice versa*.

We then explored how TM4SF1, TM4SF4, TM4SF5 WTs and their mutants influenced cell migration properties. During analysis of the migration capacities through transwell chambers, the migrated cells were stained and the stain intensity for each condition was analyzed using an image J software and compared each other. TM4SF5 and TM4SF1 increased migration more than Mock cells, whereas TM4SF4 decreased less than Mock cells (Figs. 3A and B). However, each C-terminus-deleted mutant rather increased migration, compared to that of each WT, indicating certain regulatory roles of the C-termini in transwell-migration (Figs. 3A and 3B). Further interestingly, replacement of the C-terminus of TM4SF1 or TM4SF4 with the C-terminus of TM4SF5 (i.e., 1C5 or 4C5, respectively) increased migration greater than each TM4SF1 or TM4SF4 WT. However, replacement of the C-terminus of TM4SF5 with the C-terminus of either TM4SF1 or TM4SF4 showed rather reduced migration, compared with that of TM4SF5 WT (Figs. 3A and B). These observations suggest again that the C-terminus of TM4SF5 could override the C-termini of other members for transwell-migration. In addition, the TM4SF5-mediated migration property was correlated with c-Src phosphorylation (Fig. 3B, blots).


A
1WT
1ΔC
1C5
Mock
4WT
4ΔC
4C5
5WT
5ΔC
5C1
5C4
HCT-116 Stable Cells

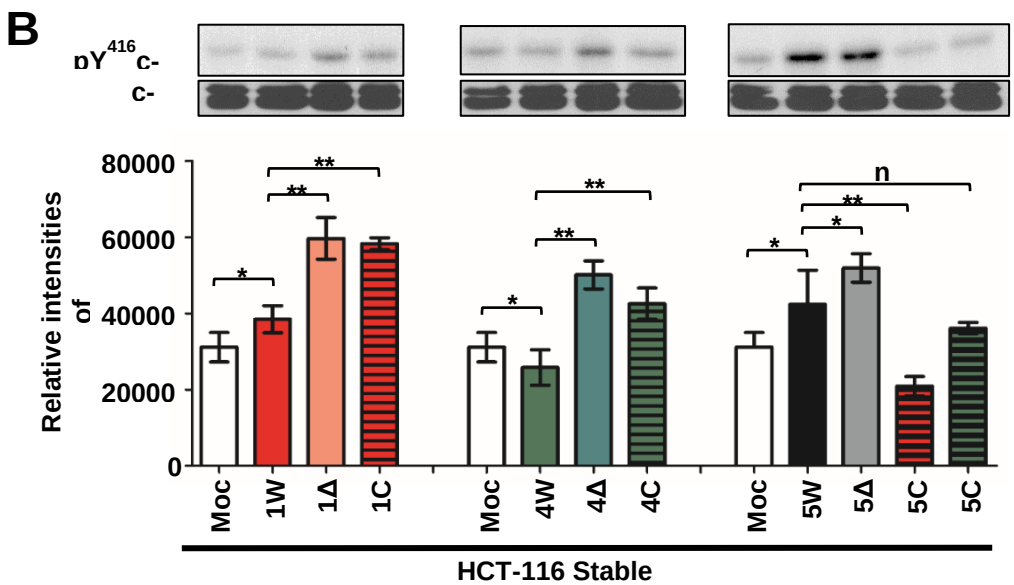

B
pY416c-
c-
Relative intensities of
80000
60000
40000
20000
0
Moc
1W
1Δ
1C
Moc
4W
4Δ
4C
Moc
5W
5Δ
5C
5C
HCT-116 Stable

Figure 3. TM4SF1 and TM4SF5 but not TM4SF4, promoted transwell migration and the TM4SF5 C-terminus overrode pro-migratory effect of the TM4SF1 C-terminus. (A) Cells were incubated with 1% BSA-DMEM for 2 h, before loadings to the transwell chambers. Insert cambers were precoated with collagen I (10 μg/ml), before cell loadings (10^5 cells/condition). Migrated cells through the transwell chambers for 10 hrs in each condition following loading of cells were stained with 5% crystal violet and randomly imaged and representative images were shown. (B) The migrated cells from 10 random images for experimental conditions were quantitated by using an Image J software for the intensities of the stains for a graphic presentation at mean ± SD values. The immunoblots for phospho-Tyr416 in c-Src and c-Src were performed by using the whole cell lysates from the stable cells. *, **, or *** depicts statistically significant difference at p <0.05, 0.005, or 0001, respectively, and ′ns′ depicts nonsignificant difference at $p \geq$ 0.05. The data shown represent three independent experiments.

4. Greater significance of the C-terminus over other parts of TM4SF5 in transwell migration

We next explored which part(s) or subdomain(s) of TM4SF5 would be important for its positive effects on migration in addition to the C-terminus. Thus, we have used chimera constructs where the N-terminus and the transmembrane domains of TM4SF1 was conjugated with the extracellular loops (ECLs) and the ICL and the C-terminus of TM4SF5 (i.e., 5TM1) or the ECLs of TM4SF5 were conjugated with other parts of TM4SF1 (i.e., 1EC5) (Fig. 4A). Their expressions following stable infections were comparable each other (Fig. 4B). However, transwell-migration capacities of the stable cells were differential. As shown in above, TM4SF5 WT caused transwell-migration greater than TM4SF1, and the chimera that the C-terminus of TM4SF5 replaced the counterpart of TM4SF1 (1C5) showed greater migration also than TM4SF1 WT, whereas the chimera with the ECLs of TM4SF5 replaced the counter parts of TM4SF1 (1EC5) abolished the migration to the level of control mock cells (Figs. 4C and 4D). Thus, the C-terminus of TM4SF5 promoted the migration, but the ECLs alone could not.

Interestingly, the TM4SF5 C-terminus-deleted mutant (5ΔC) showed much increased migration, compared with TM4SF5 WT, suggesting that the C-terminus alone might play negative regulatory roles in migration (Figs. 4C and 4D). It is not currently sure how the migratory capacities of 1C5 and 5ΔC showed migration greater than TM4SF1 and TM4SF5 WT, respectively, although the structural contexts next to the C-terminus of TM4SF5 might have effects on the migratory

capacities. Further, chimera of 5C1 and 5TM1 did not result in migration capacities comparable to TM4SF5 WT or 5ΔC, indicating that the C-terminus or TM domains of TM4SF1 could not promote the migration comparable to the C-terminus of TM4SF5, and that the TMs of TM4SF5 was also important for the migration (Figs. 4C and 4D). Such migratory effects in HCT-116 cells by the constructs were also valid in another colon cancer cells of SNU407 cells (Fig. 4E).

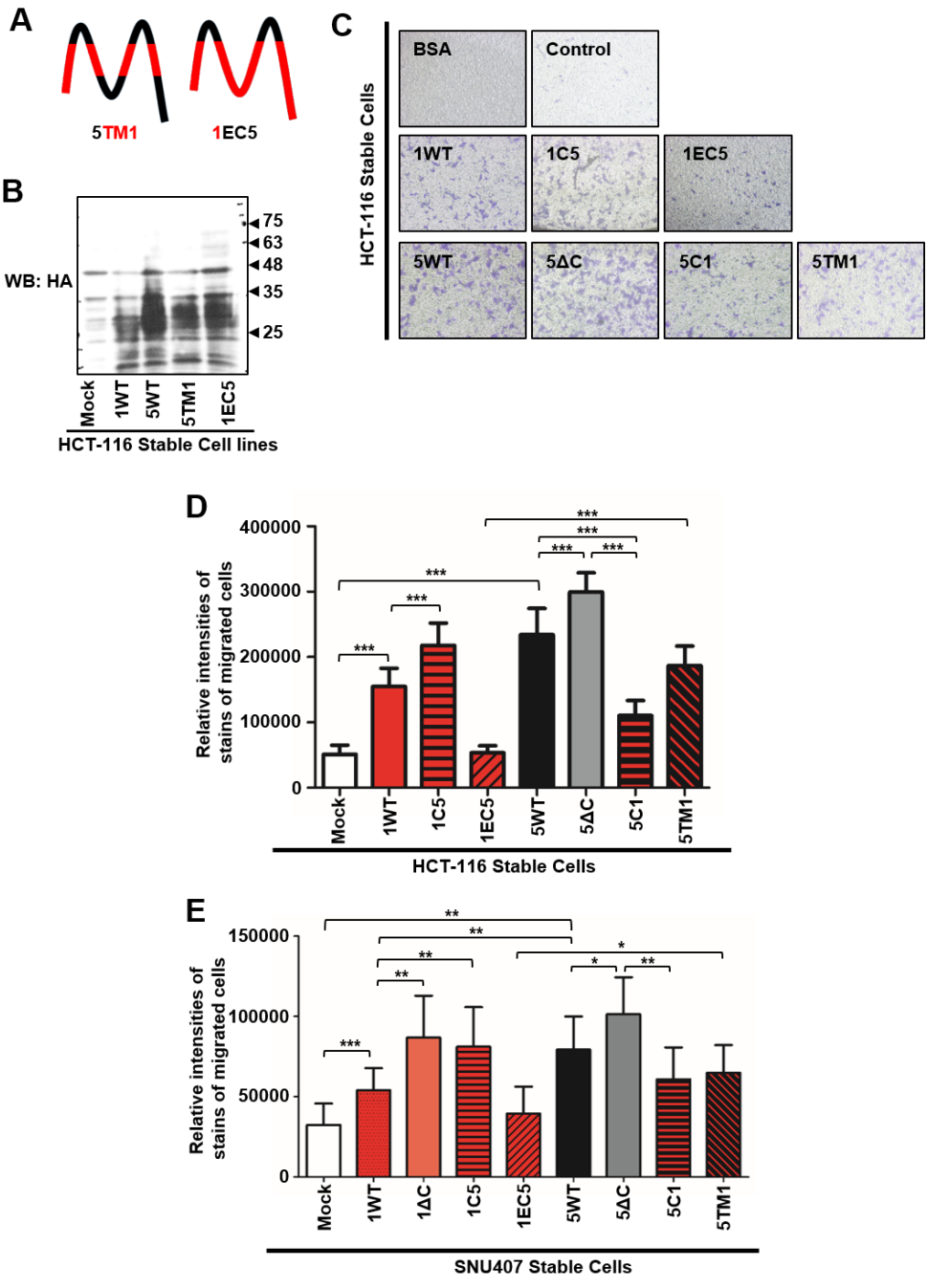

A
5TM1
1EC5
B
WB: HA
75
63
48
35
25
Mock
1WT
5WT
5TM1
1EC5
HCT-116 Stable Cell lines
C
HCT-116 Stable Cells
BSA
Control
1WT
1C5
1EC5
5WT
5ΔC
5C1
5TM1
D
Relative intensities of stains of migrated cells
400000
300000
200000
100000
0
***
Mock
1WT
1C5
1EC5
5WT
5ΔC
5C1
5TM1
HCT-116 Stable Cells
E
Relative intensities of stains of migrated cells
150000
100000
50000
0
**
*
***
Mock
1WT
1ΔC
1C5
1EC5
5WT
5ΔC
5C1
5TM1
SNU407 Stable Cells

Figure 4. The transmembrane domains of TM4SF5 but not the EC2 alone could support transwell migration. (A) The schemes of chimera that the transmembrane domains or the extracellular loops were exchanged between TM4SF1 and TM4SF5. (B) Stable cells were harvested for whole cell lysates before standard Western blots using anti-HA antibody. Expression levels of the wildtypes and chimeric mutants of TM4SF1 and TM4SF5. (C) Transwell migration assay using transwell inserts precoated with collagen I (10 μg/ml) was done for 10 hrs using HCT-116 stable cells (10^5 cells/condition), as explained in Figure 3. Representative images were shown among 10 random images for each experimental condition. (D and E) The stable HCT-116 (D) or SNU407 (E) colon cancer cells (10^5 cells/condition) were analyzed using transwell chambers for 13 hrs, as in Figure 3, and the migrated cells of 10 images for each experimental condition were stained and their intensities were measured by Image J for graphic presentations at mean ± SD values. *, **, or *** depicts statistically significant difference at p <0.05, 0.005, or 0001, respectively, and ′ns′ depicts nonsignificant difference at $p \geq 0.05$. The data shown represent three different experiments.

5. The C-terminus of TM4SF5 allowed cells to be dramatically disseminated from spheroids embedded in 3D collagen I gels.

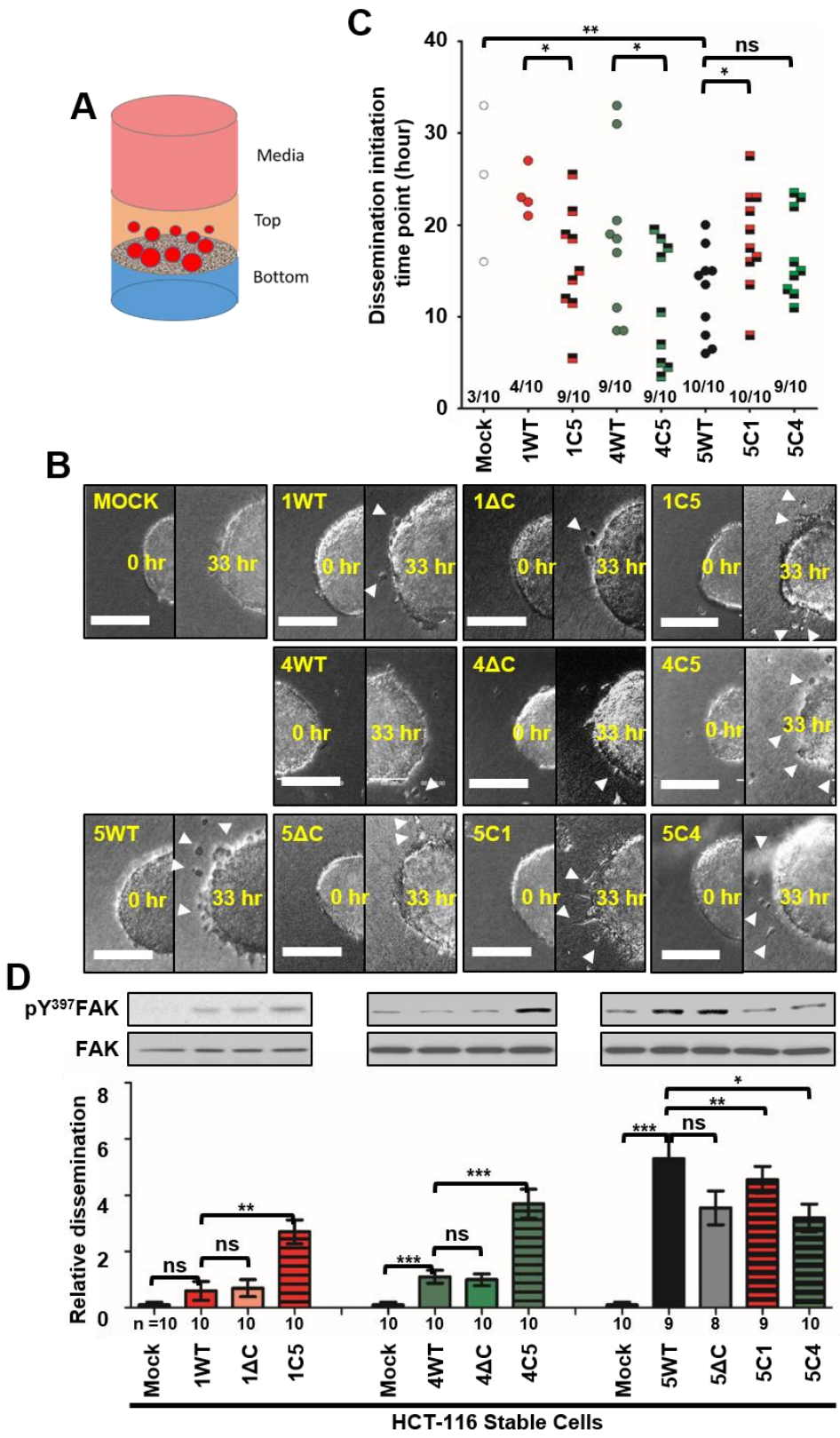
We next examined how the invasive migration properties could be regulated by them in 3D collagen I gel environment. First, we prepared spheroids from the stable cell lines using hanging-drop systems and selected them for spheroids with diameters from 70 to 100 μm, using sieves. The selected spheroids were embedded in to a 3D collagen I gels, where spheroids were put between a dense lower-layer and a soft top-layer (on-top system, Fig. 5A), before time-lapse imaging. When the times to initiate dissemination from the spheroids were analyzed, TM4SF5 cells showed much earlier dissemination than TM4SF1 or TM4SF4 cells (Figs. 5B and 5C). Further, TM4SF1 (1WT) or TM4SF4 (4WT) showed slower disseminations than the 1C5 or 4C5 cells that has the C-terminus of TM4SF5 conjugated to the other part of either TM4SF1 or TM4SF4, respectively (Figs. 5B and 5C). Interestingly, replacement of the C-terminus of TM4SF5 with that of TM4SF1 or TM4SF4 (leading to 5C1 or 5C4, respectively) delayed the dissemination initiation times (Figs. 5B and 5C). Meanwhile, when relative dissemination rates via counting the cells disseminated from the spheroids were counted, TM4SF1 or TM4SF4 cells were less significantly disseminated compared with TM4SF5 cells, and 1C5 or 4C5 cells increased the dissemination rates higher than those of TM4SF1 or TM4SF4 cells (Figs. 5B and 5D). Interestingly, deletion of the C-terminus of TM4SF5 or replacement of it with TM4SF1 or TM4SF4 C-terminus (leading to 5C1 or 5C4, respectively) showed lower dissemination rates, compared to TM4SF5 cells, which were still much higher than TM4SF1 or TM4SF4 (Figs. 5B and 5D). Thus, other

parts of TM4SF5 (rather than the C-terminus alone) might be involved in the TM4SF5-mediated dissemination. Furthermore, higher dissemination rates of 1C5, 4C5 and 5WT (TM4SF5 cells) were correlated with the higher Tyr397 phosphorylation in FAK (Fig. 5D, blots).

Since phosphorylations of c-Src and FAK were correlated with transwell-migration and/or dissemination from spheroids in 3D collagen I gels, we have checked whether c-Src inhibition by specific inhibitor PP2 could block the TM4SF5- or its C-terminus-mediated dissemination. Whereas negative control inhibitor PP3 did not affect the dissemination, PP2 blocked dissemination of 1C5 and TM4SF5 spheroids (Fig. 5E). In addition, such disseminations of HCT-116 cells depending on TM4SF5 or its C-terminus, were also valid in another colon cancer HT29 cell line stably infected with those constructs (Figs. 5F and 5G). Compared to mock cells, TM4SF1 cells showed an accelerated dissemination, which was slower than TM4SF5 cells (Fig. 5G). Replacement of the C-terminus in TM4SF1 with the C-terminus of TM4SF5 (i.e., 1C5) or with the ECLs (consisting of EC1 and EC2) of TM4SF5 (i.e., 1EC5) led to differential effects; 1C5 significantly accelerated but 1EC5 significantly delayed dissemination, compared with TM4SF1 cells (Fig. 5G), suggesting that the extracellular loops alone might not contribute to the TM4SF5-mediated dissemination, but that the C-terminus of TM4SF5 was important for the effects. Since

Further, compared to TM4SF5 cells, cells expressing 5C1 (a chimera that the C-terminus of TM4SF5 was replaced with the C-terminus of TM4SF1) or 5TM1 [a chimera that the transmembrane domains (TMs) of TM4SF5 were replaced with

those of TM4SF1] showed insignificantly delayed disseminations, suggesting that the TMs of TM4SF5 could be replaced by others sufficient for the dissemination (Fig. 5G). All these observations suggest that the intracellular parts could be important for the TM4SF5-mediated c-Src activity and dissemination in 3D collagen I gels.


A
Media
Top
Bottom
C
Dissemination initiation time point (hour)
3/10
4/10
9/10
9/10
9/10
10/10
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9/10
Mock
1WT
1C5
4WT
4C5
5WT
5C1
5C4
B
MOCK
1WT
1ΔC
1C5
4WT
4ΔC
4C5
5WT
5ΔC
5C1
5C4
0 hr
33 hr
D
pY397FAK
FAK
Relative dissemination
n =10
HCT-116 Stable Cells

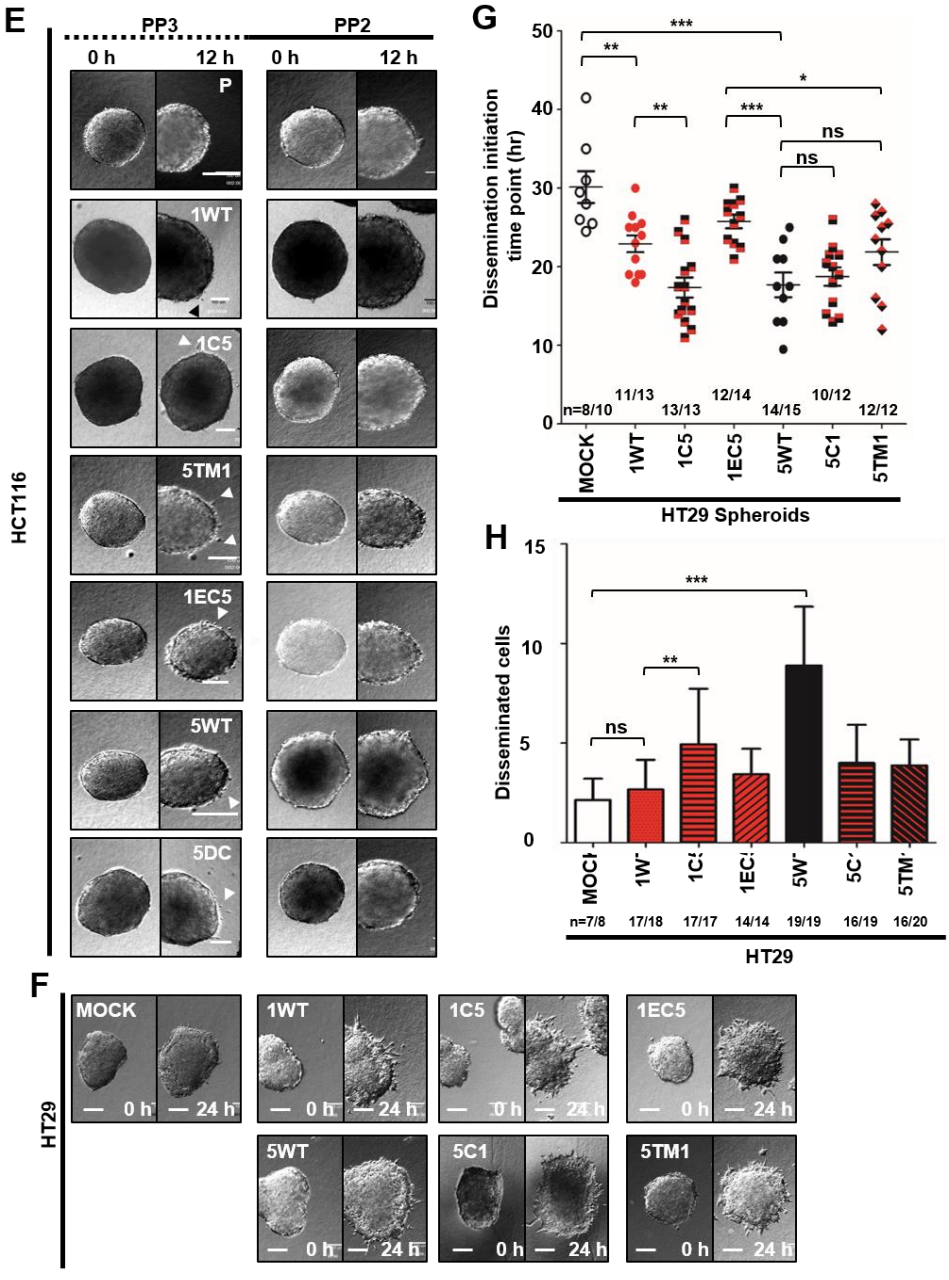

E
PP3
PP2
0 h
12 h
0 h
12 h
HCT116
P
1WT
1C5
5TM1
1EC5
5WT
5DC
G
Dissemination initiation time point (hr)
50
40
30
20
10
0
***
**
**
***
*
ns
ns
n=8/10
11/13
13/13
12/14
14/15
10/12
12/12
MOCK
1WT
1C5
1EC5
5WT
5C1
5TM1
HT29 Spheroids
H
Disseminated cells
15
10
5
0
***
**
ns
n=7/8
17/18
17/17
14/14
19/19
16/19
16/20
HT29
F
HT29
MOCK
1WT
1C5
1EC5
5WT
5C1
5TM1
0 h
24 h

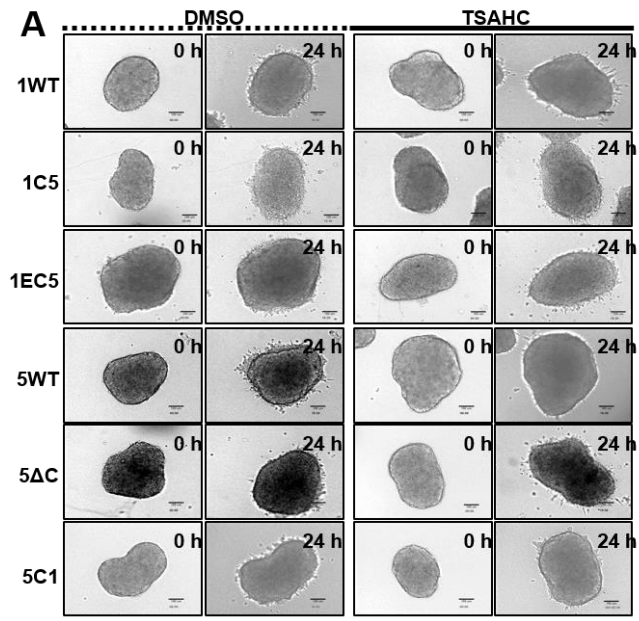
Figure 5. TM4SF5 and its C-terminus overwhelmed TM4SF1 and TM4SF4 to promote invasive dissemination from spheroids embedded in 3D collagen I gels. (A) Scheme of the ′on-top′ 3D ECM surrounded culture environment, where the cells (red) were mixed with a lower concentration (yellow-pink) of collagen I gel (2 mg/ml) and overlaid on the bottom dense part with collagen I gel at higher concentration (blue) that were previously solidified and top less dense part with before live imaging. (B to E) The representative images for the starting (each left) and ending (each right) points of the live-imaged spheroids in each condition were shown (B and E). The initiation times of dissemination (C) or numbers of disseminated cells (D) from the spheroids were measured during the live-imaging for 36 (C) or 24 hrs (D) and presented in the graph at mean ± SD values. White arrow heads depict the disseminative cells. The immunoblots for phospho-Tyr397 in FAK and FAK were performed by using the whole cell lysates from the spheroids in 3D collagen I gels. PP2 or PP3 (10 μM) were treated to the gels during the embedding processes, before live-imaging for 12 hrs. (F to H) Stable cells of HT29 were also processed for spheroids for live imaging as in (B). The starting (each left) and ending (each right) point of the dissemination process during the live imaging periods were presented (F). The initiation time point for each condition during live-imaging for 24 hrs (G) and numbers of the disseminative cells from spheroids embedded in collagen I gels (2 mg/ml) were presented in the graphs at mean ± SD values. The x/y values depict that dissemination process was shown spheroids of x number out of y spheroids we measured. *, **, or *** depicts

statistically significant difference at p <0.05, 0.005, or 0001, respectively, and ′ns′ depicts nonsignificant difference at $p \geq 0.05$. The data shown represent three independent experiments.

6. The (structural) relay from the EC2 to the C-terminus of TM4SF5 appeared important for the invasive dissemination.

An anti-TM4SF5 small synthetic compound [TSAHC; 4′-(*p*-toluenesulfonylamido)-4-hydroxychalcone] can block TM4SF5-mediated migration via interruption of *N*-glycosylation or structural integrity of the EC2 of TM4SF5 (Lee et al., 2009b). Although the IC_{50} of TSAHC against TM4SF5 was evaluated to be approximately 5 μM for 2D cell cultures and 0.3 μM for 3D aqueous spheroid culture systems (data not shown), we have treated 40 μM TSAHC to the spheroids embedded in 3D collagen I gels. Upon treatment of TSAHC to the spheroids before initiation of live imaging, TM4SF5 spheroids lost disseminative capacity but TM4SF1 spheroids did not (Fig. 6A). Interestingly, the C-terminus deleted mutant 5ΔC maintained the dissemination property even in the presence of TSAHC treatment, whereas the chimera 5C1 (TM4SF5 chimera with the TM4SF1 C-terminus) also showed sensitivity to TSAHC during the dissemination (Fig. 6A). As for the initiation time of dissemination, TSAHC treatment insignificantly affected TM4SF1 (1WT) or 5ΔC cells but significantly delayed the dissemination of TM4SF5 (5WT) or 5C1 chimera cells (Fig. 6B). Therefore, cells that have both the EC2 [presumably targeted by TSAHC (Lee et al., 2009b) and the C-terminus to recruit c-Src (Jung et al., 2013)] might be sensitive to TSAHC. However, a chimera (5C1) that have the C-terminus of TM4SF1 to replace the counterpart of TM4SF5 (i.e., 5C1 has the EC2 and the TM4SF1 C-terminus instead of the TM4SF5 C-terminus) showed a sensitivity to TSAHC, although the disseminations of 5C1 cells (before

and after TSAHC treatment) were less accelerated, compared to in TM4SF5 WT (Fig. 6B), suggesting that the C-terminus of TM4SF1 linked to the TMs and the EC2 of TM4SF5 might be still sensitive to the TSAHC.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0 h
24 h
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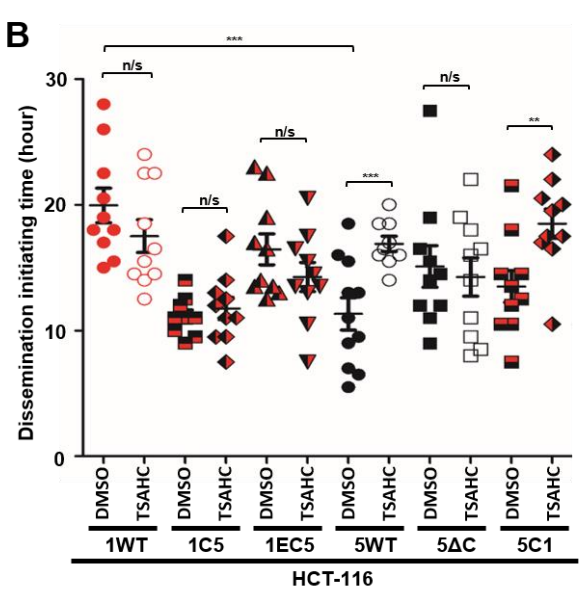
1C5
1EC5
5WT
5ΔC
5C1


B
***
n/s
n/s
n/s
***
n/s
**
Dissemination initiating time (hour)
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20
10
0
DMSO
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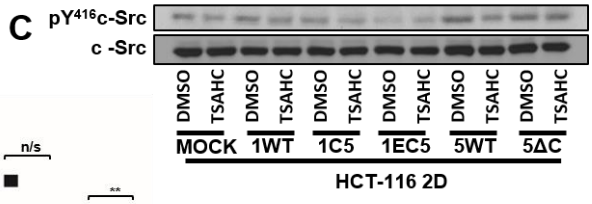
1C5
1EC5
5WT
5ΔC
5C1
HCT-116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pY416c-Src
c -Src
DMSO
TSAHC
MOCK
1WT
1C5
1EC5
5WT
5ΔC
HCT-116 2D

Figure 6. The structural relay from the EC2 to the C-terminus of TM4SF5 is important for the TM4SF5-mediated dissemination. (A) The spheroids were embedded into the 3D collagen I gels with DMSO or TSAHC (40 μM), before the live imaging for 24 hrs. The starting (0 h) or ending (24 h) time point of imaging were shown. (B) The initiation time of dissemination was measured for a graphic presentation at mean ± SD values. (C) Whole cell extracts were harvested from cells treated with TSAHC (20 μM) for 24 h on 2D flat conditions. Relative phospho-Tyr416 levels in c-Src were examined with standard Western blot and bands intensities among experimental conditions were measured and presented. The data shown represent three isolated experiment.

Discussion

This study shows that TM4SF5 could be superior to other isotype members of TM4SF1 or TM4SF4 for spheroid growth in 3D aqueous condition, transwell migration, and invasive dissemination from spheroids embedded in 3D collagen I gels, although they were comparable in growth in 2D flat environment. More specifically, the C-terminus of TM4SF5 could render the TM4SF5-mediated positive effects on cellular functions to TM4SF1 or TM4SF4 when either C-terminus was replaced with the TM4SF5 C-terminus. Such TM4SF5 (C-terminus) -mediated dissemination in 3D collagen I gels depended on c-Src activity, so that TM4SF5 or c-Src inhibitor blocked the TM4SF5-mediated effects. Therefore, these observations indicate that the C-terminus of TM4SF5 appears to be critical for the TM4SF5-mediated metastatic potentials and other isotypes′ C-terminus could not be comparable to the C-terminus of TM4SF5 for the metastatic functions including growth in 3D aqueous or ECM gels and migration and invasion in 3D ECM environment.

The tetraspanins (transmembrane 4 superfamily, TM4SF) and transmembrane 4 L six family can form TERMs (tetraspanin-enriched microdomains), where they can form protein-protein complexes in homophilic or heterophilic manners among tetraspanins, growth factor receptors, or integrins, leading to transducing unique intracellular signaling pathways (Hemler, 2005; Lee, 2014). Very similar to the tetraspanins, the transmembrane 4 L six family members include TM4SF1 (L6),

TM4SF4 (IL-TMP), TM4SF5 (L6H), TM4SF18, and TM4SF20. TM4SF5 shares sequence identity of 49.8% to TM4SF1 and 40.9%% to TM4SF4 (Lee, 2015). Further, among their C-termini, the sequence identity is 36.7% between TM4SF1 and TM4SF5 and 14.3% between TM4SF4 and TM4SF5 (Fig. 1A). Thus, the C-termini in the transmembrane 4 L six family members are relatively differential and may be critically involved in regulatory roles in cellular functions by each member.

Both TM4SF1 and TM4SF5 show enhanced expression levels in many different cancer types including hepatocarcinoma, compared to those in normal tissues (Lee et al., 2008; Xu et al., 2000). Further, during zebrafish developmental stages, the mRNA levels of both *tm4sf1* and *tm4sf5* are similarly altered enough to indicate their similarity in the roles in development, and TM4SF5 promotes muscle development via cross-talks with integrin α5 signaling during somitogenesis in zebrafish (Choi et al., 2014b). TM4SF1 and TM4SF5 play roles not only in cell proliferation leading to HCC but also in migration and invasion for cancer metastasis (Jung et al., 2013; Lee et al., 2008; Lekishvili et al., 2008). In case of TM4SF1, the transmembrane domains are sufficient to direct TM4SF1 to the late endosomal organelles or membranes, and the cytosolic parts of TM4SF1 (the N-terminus, the intracellular loop, and the C-terminus) appear to be important for targeting to the TERM via an interaction with a PDZ-domain containing syntenin2, leading to an efficient migration ability (Lekishvili et al., 2008). Meanwhile, TM4SF5 can traffic between plasma membranes and late endosomal membranes depending on metabolic status of the cells (JW Jung and JW Lee, unpublished data), and further the intracellular loop and the C-terminus of TM4SF5 interact with FAK and SFKs (c-Src family

kinases) to regulate migration and invasion, respectively (Jung et al., 2012; Jung et al., 2013). Therefore, the C-terminus of either TM4SF1 or TM4SF5 can associate with cytosolic proteins to modulate invasive migratory capacity. Given the promotive roles of the C-termini in the invasive migration, it could be interests to understand how the both can be different. This current study revealed that the C-terminus of TM4SF5 could play pro-migratory or pro-metastatic roles in transwell migration and invasive migration/dissemination from spheroids embedded in 3D collagen I gels superior to the C-terminus of TM4SF1, although the their C-termini played comparable roles in proliferation in 2D flat environment. This tendency was observed when the C-terminus of TM4SF5 replaced the C-terminus of TM4SF1 and vice versa, in addition to each wildtype-expressing cells.

In contrast to TM4SF1 and TM4SF5 that slightly increased cell proliferation in 2D, TM4SF4 slightly decreased cell growth in this study. Similarly, TM4SF4 wildtype cells showed transwell migration lower than mock control, TM4SF1 wildtype, or TM4SF5 wildtype cells. However, as for the invasive dissemination from spheroids in 3D gels, TM4SF4 wildtype cells showed dissemination greater than mock cells, similar to that of TM4SF1 wildtype cells, but much less than TM4SF5 wildtype cells. TM4SF4 is shown to be overexpressed in the regenerating liver after two-thirds hepatectomy in rats (Liu et al., 2001). TM4SF4 is also involved in pancreas development in Nkx2.2$^{-/-}$ mice (Anderson et al., 2011). TM4SF4 is upregulated in injured liver of CCl_4-administrated mice (Qiu et al., 2007), similar to TM4SF5 (Kang et al., 2012). Further similar to TM4SF5, TM4SF4 is overexpressed in liver cancer tissues (Lee et al., 2008; Li et al., 2012) and correlates with directional migration (Anderson et al., 2011; Jung et al., 2012). TM4SF4 has been controversial

for its effect on cell growth. TM4SF4 expression correlates with increased growth and migration via IGF1R activation following NFkB-mediated IGF1 induction in A549 cells (Choi et al., 2014a). Meanwhile, forced expression of TM4SF4 in HeLa cells causes cell-density-related inhibition of proliferation depending on its *N*-Glycosylation (Wice and Gordon, 1995). Therefore, TM4SF5 shares certain regulatory roles with TM4SF4.

Although TM4SF1, TM4SF4, and TM4SF5 share a common membrane topology and regulatory roles in certain cellular function with a limited sequence identity for their C-termini, TM4SF5 and the C-terminus of TM4SF5 appeared to be superior to other members and their C-termini. It would thus be interesting to understand how the C-terminus of TM4SF5 could play pro-migratory roles superior to that of TM4SF1 or TM4SF4. Anti-TM4SF5 reagent, a small synthetic compound TSAHC (Lee et al., 2009b) could block the pro-migratory effects mediated by TM4SF5 wildtype but not by the C-terminus-deleted TM4SF5 (i.e., 5ΔC), indicating that the (allosteric) structural aspects to relay from the EC2 to the C-terminus should allow the functional fulfillment of the EC2 to be transferred to the C-terminus that recruits c-Src (Jung et al., 2013). Unlike the EC2 of TM4SF5, the application of TSAHC to TM4SF1 wildtype (-positive cells) did not have any influence, indicating that the EC2 of TM4SF5 but not of TM4SF1 was responsive to TSAHC that causes dissemination to be delayed, as expected from the fact that TSAHC does not have any effects to TM4SF5-null cells (Lee et al., 2011). To be consistent, cells with chimeric TM4SF1 with the C-terminus (i.e., 1C5) or with the EC2 of TM4SF5 (i.e., 1EC5) did not respond to TSAHC treatment, leading to comparable dissemination

initiation time and disseminated-cell numbers between DMSO- and TSAHC-treated cells. Meanwhile, to the cells where the C-terminus of TM4SF1 replaced that of TM4SF5 (i.e., 5C1), the treatment of TSAHC retarded the dissemination initiation time and disseminated-cell numbers of 5C1 cells, compared with those of DMSO-treated 5C1 cells. This suggests that the C-terminus of TM4SF1 might play roles partially similar to the C-terminus of TM4SF5 for the sensitivity to TSAHC, as long as the EC2 and the TMs of TM4SF5 were available for a structural relay. Therefore, the structural relay from the EC2 containing the *N*-glycosylation residues (at N138 and N155) to the C-terminus through the TM4 may be critical for the TM4SF5-mediated invasive dissemination from the colon cancer spheroids in 3D collagen I gels.

Altogether, the current study showed that the structural axis from the EC2 and the C-terminus of TM4SF5 played promotive roles in cellular functions in 2D and/or 3D environment, superior to other family members including TM4SF1 and TM4SF4. Thus, it is reasonable to target the signaling activity of the TM4SF5 C-terminus that would be supported by the structural axis from the EC2 to the C-terminus.

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국문초록

TM4SF1 (L6)와 TM4SF4 (IL-TMP), TM4SF5 (L6H) 등을 포함하는 transmembrane 4 L six family는 서로 30~50%의 서열 동일성을 가지며, 다른 tetraspanin들과도 유사하다. TM4SF5의 C 말단 부위가 세포 침윤성에 중요하다고 보여진 바가 있기에, family member들의 C 말단 부위 역시 세포 기능에 어떻게 중요한 역할을 담당하는지, 혹은 세포 침윤성 측면에서 서로 유사한지 의문을 가지게 되었다. 이에 나는 C 말단 결손 돌연변이와 C 말단을 서로 교차시킨 키메라 돌연변이를 각 family member 별로 제작하여 안정적으로 발현하는 대장암 세포주들을 만들었고, 2차원적 평면 환경과 3차원적 겔 환경에서 각각의 C 말단이 세포 행동을 어떻게 조절하는지 조사하였다.

2차원적 환경에서 각 family member들의 C 말단이 제거되었을 때는 세포 증식이 빨라졌고, 세포 증식에 있어서 서로 대체될 수 있도록 비슷한 역할을 하는 것으로 보였다. 하지만 배양액에서 스페로이드 형태로 3차원 환경에서 배양하였을 때 TM4SF5와 그것의 C말단이 TM4SF1과 그것의 C 말단 각각에 대비해서 스페로이드 형성능력이 높게 나타났다.

트랜스웰 이동 분석 시스템을 이용하자 세포 이동성 면에서 TM4SF5는 TM4SF1과 TM4SF4보다 우세하였고, TM4SF5의 C 말단을 TM4SF1 혹은 TM4SF4 의 C 말단과 교체 하였을 때,

트렌스웰 이동성이 증가하였다. 제1형 콜라겐 3차원 겔 환경에서는 스페로이드의 침윤적 이탈현상과 이동성이 증가하였다. 이러한 현상은 c-Src 활성과 긍정적으로 관련 있었다.

TM4SF5의 세포외고리(extraelluar loop)와 세포내고리(intracellular loop) 그리고 C 말단으로 TM4SF1의 해당 부위를 교체하자 TM4SF1보다는 높지만, TM4SF5의 C말단으로 교체한 TM4SF1 보다는 낮은 이동성을 나타냈다. 그리고 TM4SF5의 세포외고리만 TM4SF1의 해당 부위에 교체하자 이동성을 기저 수준으로 무효화 하였다.

이러한 관찰 결과들은 TM4SF5의 세포내 부위들이 세포 증식을 제외하고, c-Src 관련 신호전달과 2차원과 3차원 환경에서의 이동성 촉진 역할을 중재한다는 것을 시사한다.

주요어: TM4SF5, Transmembrane 4 L Six Family, 키메라 돌연변이, 3차원적 배양, 세포이동, 세포성장

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