약학석사학위논문

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TM4SF5를 발현하는 간암세포의 줄기세포 및 circulating tumor cell 기능에 대한 연구

2014년 2월

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이 논문을 약학석사학위논문으로 제출함

2013년 11월

서울대학교 대학원
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이두형의 석사학위논문을 인준함
2013년 12월

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부위원장 _____ (인)
위 원 _____ (인)

Abstract

Tetraspanin TM4SF5-mediated self-renewal stemness of hepatocellular carcinoma cells

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EMT is involved in tumor progression at the levels of dissemination, metastasis, drug resistance, and self-renewal. As a tetraspan(in), TM4SF5 is highly expressed in diverse cancers and causes EMT, metastasis of liver cancer cells, and gefitinib resistance of NSCLC. Here, the self-renewal by TM4SF5 was mechanistically explored using hepatocellular carcinoma cells with or without TM4SF5 expression. TM4SF5-positive cells well-formed spheroids without serum components and adhesive environments, whereas mutants of *N*-glycosylation residues (N138A/N155Q) or treatment of

anti-TM4SF5 reagent (TSAHC) that disturbs its *N*-glycosylation status resulted in no formation of spheroids. TM4SF5 expressing cells down-regulate CD24 and express CD44. Further, when another single pass type Ⅰ membrane protein CD44 was physically bound to TM4SF5, the cells formed spheroids; *N*-glycosylation mutant TM4SF5 did not bind to CD44 and not form spheroids and anti-TM4SF5 reagent had CD44 released free leading to no spheroid formation. Meanwhile, the spheroid formation capacity correlated with tumor formation in xenografts injected with 200 ~ 5000 cells per mouse and in serial xenografts. Moreover orthotopic injection of TM4SF5 expressing cells into mouse livers efficiently formed tumors and led the TM4SF5-positive cells to circulate in mouse peripheral blood even 4 weeks after the injection. TM4SF5 up-regulates twist1 through STAT3 signaling activation resulted in inducing EMT, and up-regulating bim1 expression. These observations suggest that TM4SF5 can render self-renewal stemness during HCC initiation and progression

Keywords : TM4SF5, Self-renewal, CD44, *N*-glycosylation, STAT3, Twist1, Bmi1

Student number : 2012-21607

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide in terms of numbers of cases and the third most common cause of mortality from cancer [1]. Hepatic resection and liver transplantation are the first - line treatment for HCC [2, 3]. However, the 5-year survival rate is mostly dependent on the stage of tumor at the time of diagnosis [4-6]. The second-line treatment that is the chemotherapy via either transarterial chemoembolization or a systemic route for HCC patient in advanced stages has limited efficacies [7-9]. Moreover, appearance of the cancer cell which is resistance to traditional chemotherapy leaves this disease with no chemopreventive effect.

It has demonstrated that the small portion of cells initiated the cancer. These small population of cells possess stem-like properties such as unlimited proliferating ability, a strong potential for self-renewal, and unlimited differentiation ability into cancer cell progeny [10]. These cells called as cancer stem cell (CSC) or tumor-initiating cell (T-IC). The idea of cancer stem cell was proposed over a decade ago, but the evidence of cancer stem cell has been found out in several types of solid tumor such as colon [11], pancreatic [12], brain [13], and prostate cancer [14] in recent years. Integrative comparative genomic analysis has provided molecular similarities between liver cancer stem cells and normal stem cells, implying the importance of liver cancer stem cell in hepatocarcinogenesis [15]. Recently, liver CSCs have been identified by a number of molecular markers including side population (SP) of cancer cell [10], CD133 [16, 17], CD90 [18], epithelial cell adhesion molecule (EpCAM) [19, 20], CD44 [17], CD13 [21], CD24 [22], OV6

[23], granulin-epithelin precursor (GEP) [24], Delta-like 1 homolog (DLK1) [25], and intercellular adhesion molecule 1 (ICAM-1) [26]. Lately, the evidence of origin of CSCs has demonstrated. Epithelial-Mesenchymal transition (EMT) that epithelial cell lose its polarity and gain mesenchyme traits in mammary epithelial models generates stem-cell-like cells [27].

As a tetraspan (in), TM4SF5 is highly expressed in diverse cancers and induces EMT, resulting in not only morphological elongation changes through actin reorganization, but also promotions of cell migration, invasion, and cell proliferation [28-31]. Moreover, gefitinib resistance of NSCLC depends on the TM4SF5-mediated EMT [32].

Since TM4SF5 induces EMT and EMT offers a satisfactory explanation for the origin of CSCs, we hypothesized that TM4SF5 could be involved in generating phenotypes of hepatic CSCs. In current study, we observed that TM4SF5 rendered stem-like-properties to HCC via a signaling linkage including STAT3, twist, and bmi-1.

Material & Methods.

1. Cell culture

Parental SNU449, Pooled clones (control Cp and TM4SF5-expressing pooled Tp cell clones), single cell-driven clones (TM4SF5-expressing T3, T7, T10, and T16) and TM4SF5 *N*-glycosylation mutant (N138A, N155Q, and NA/NQ; N138A/N155Q) of hepatocarcinoma SNU449 (Korean Cell Bank) cells, SNU761 and HepG2 cells were previously described [28,33,34]. Huh7 cell was cultured in RPMI-1640 (WelGene Inc.) containing 10% FBS and 1% penicillin/streptomycin (GenDEPOT Inc.) at 37° C in 5% CO2. Huh7 and SNU449T7 cells stably transfected with shRNA against TM4SF5 or Scramble with tGFP (Origene Inc.) were prepared by Puromycin (4 μg/ml for Huh7 or 7 μg/ml for SNU449T: Sigma) selection.

2. Drug sensitivity

SNU449 stable Cp and T7 Cells (2000 cells/well) were seeded in 96 well plates and 24 h later DMSO or paclitaxel was added at different concentrations (0 to 100 nM) for additional 48 h. Standard reading of MTT (Sigma) metabolites was performed for OD_{540} and mean± standard deviation values were graphed.

3. Spheroid formation assay

Cells were collected and washed by PBS 2 times to remove serum, then suspended in serum-free DMEM/F12 supplemented with 1% penicillin/streptomycin (GenDEPOT Inc.), 2% B27 supplement (Invitrogen). 25 ng/ml of hEGF and hbFGF

(Peprotech) were added every 2nd days. The cells were subsequently cultured in ultra low attachment 6-wellplates (Corning Inc. Corning, NY, USA) at a density of no more than $5x10^3$ cells/well.

4. RNA Isolation, Complementary DNA Synthesis, and Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated using TRIZOL Reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was synthesized using an amfiRivert Platinum cDNA synthesis master mix (GenDEPOT) according to manufacturer's instructions. cDNA was subject to reverse-transcription polymerase chain reaction with Dream Taq Green PCR master mix (Thermo scientific) and primers, *b-actin* forward : TGACGGGGTCACCCACACTGTGCCCATCTA and reverse : CTAGAAGCATTTGCGGTGGACGACGGAGGG, *TM4SF5* forward : CTGCCTCGTCTGCATTGTGG and reverse : CAGAAGACACCACTGGTCGCG, *CD24* forward : AACTAATGCCACCACCAAGG and reverse : CCTGTTTTTCCTTGCCACAT, *CD44* forward : CGGACACCATGGACAAGTTT and reverse : GAAAGCCTTGCAGAGGTCAG, *Twist* forward : GGAGTCCGCAGTCTTACGAG and reverse : TCTGGAGGACCTGGTAGAGG, *Bmi1* forward : GAGAAATCTAAGGAGGAGGTGAA and reverse : TGG AAATGTGAGGAAACTGTGG

5. Western Blots

Whole cell lysates were prepared by modified RIPA buffer then immunoblotted using antibodies against CD44 (biolegend), CD133 (miltenyi), STAT3 (Chemicon), phospho-Y705STAT3 (),phospho-Y397FAK (Abcam), phospho-Y416Src (Cell signaling), phospho-Y577FAK, pS10p27Kip1, c-Src, phosphor-S727STAT3 (SantaCruz Biotechnology), FAK, p27Kip1 (BD Transduction Laboratories), ZO-1 (Zymed Lab), α-tubulin (Sigma), TM4SF5 (is described [29]).

6. Coimmunoprecipitation

Whole cell extracts prepared as above were immunoprecipitated with biotin-precoated beads for 2 h (IBA, Germany), prior to immunoblotting for the indicated molecules.

7. Mouse and tumor xenografts

Three-or 4-week-old male BALB/c-nu/nu mice were purchased from Orient Co. Ltd. Mice were housed in a SPF room under controlled temperature and humidity. All animal procedures were performed in accordance with the procedures in the Seoul National University Laboratory Animal Maintenance Manual and with Institutional Review Board approval. $2x10^2$~$5x10^3$ viable SNU449Cp, SNU449N138A/N155Q, or SNU449T7cells resuspended in Matrigel(BDBiosciences) mixed with RPMI-1640 medium and 10%FBS were injected subcutaneously in the left-or right-flank of Mice age 4-5 weeks old. Tumor formation was monitored weekly for 6 weeks and tumor volumes were measured as described previously [29]. Briefly tumor volumes

were measured with a caliper and calculated using the following formula: volume = (a $\times b^2$)/2. For serial xenografts injection, tumors were minced into ~1-mm^3 pieces and incubated with Type Ⅱ collagenase (Gibco) and DNase I (Takara bio Inc.) for 0.5 ~ 1 hr at 37℃ under constant rotating conditions. A single cell suspension was obtained by filtering the supernatant through a 40 μm cell strainer (BD Biosciences), and for selection of TM4SF5 expressing cell, explant cells were maintained with culture medium with G418 (A.G. scientific Inc.).

8. Orthotopic mouse tumor model and blood collection

Male BALB/c nude mice (5 or 6 week old) were used for experiments. SNU449T7 cells were stably transfected with pGFP-V-RS shScramble (Origene Technologies, Inc), selected by puromycin (5 μg/ml). To generate orthotopic tumor model, SNU449T7 shScramble 5 $\times$ 10^5cells resuspended in PBS (50 μl) were injected orthotopically into left lobes of mouse liver. For studies of CTC, blood samples were collected by cardiac puncture at 4 weeks after cell injection. PMBCs in blood samples were purified by density gradient. GFP-positive PMBCs were sorted by FACSAria Ⅲ. Sorted GFP-positive cells were seeded in fibronectin (10 μg/ml)-coated cover glasses then immunofluorescence with tGFP (Origene Technologies, Inc) and DAPI.

9. Flow Cytometry

The cells were labeled with the following antibodies: CD24, CD44 (Santacruz), CD44 (Biolegend). Labeled cells were detected using a FACSCalibur (BD Biosciences). Only second antibody labeled cells were used as controls. A dead cell removal procedure was performed before antibody labeling and flow cytometry analysis.

10. ALDEFLUOR assay

ALDH activity of the SNU449 stable cells was measured using the ALDEFLUOR assay kit (StemCell Technologies) according to the manufacturer's protocol. Briefly, cells were harvested, placed in ALDEFLUOR assay buffer (6×10^5 cells/ml) and incubated with theALDEFLUOR substrate for 45 min. at 37° C to allow substrate conversion. As a negative control for all experiments, an aliquot of ALDEFLUOR-stained cells was immediately quenched with DEAB, a specific ALDH inhibitor. Cells were analysed using the green fluorescence channel (FL1) on a FACSCalibur.

11. Histology analysis

Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were processed for antigen retrieval by a standard boiling technique. Immunostaining of tumor nodules was incubated with rabbit anti-human TM4SF5 (prepared by our lab [29]) or with rat anti-CD44 (Biolegned). Alternatively, the tissues were processed for double-immunofluorescence staining for TM4SF5 and CD44. Incubation with primary antibody against TM4SF5 was followed by incubation with anti-rabbit IgG conjugated with fluorescein

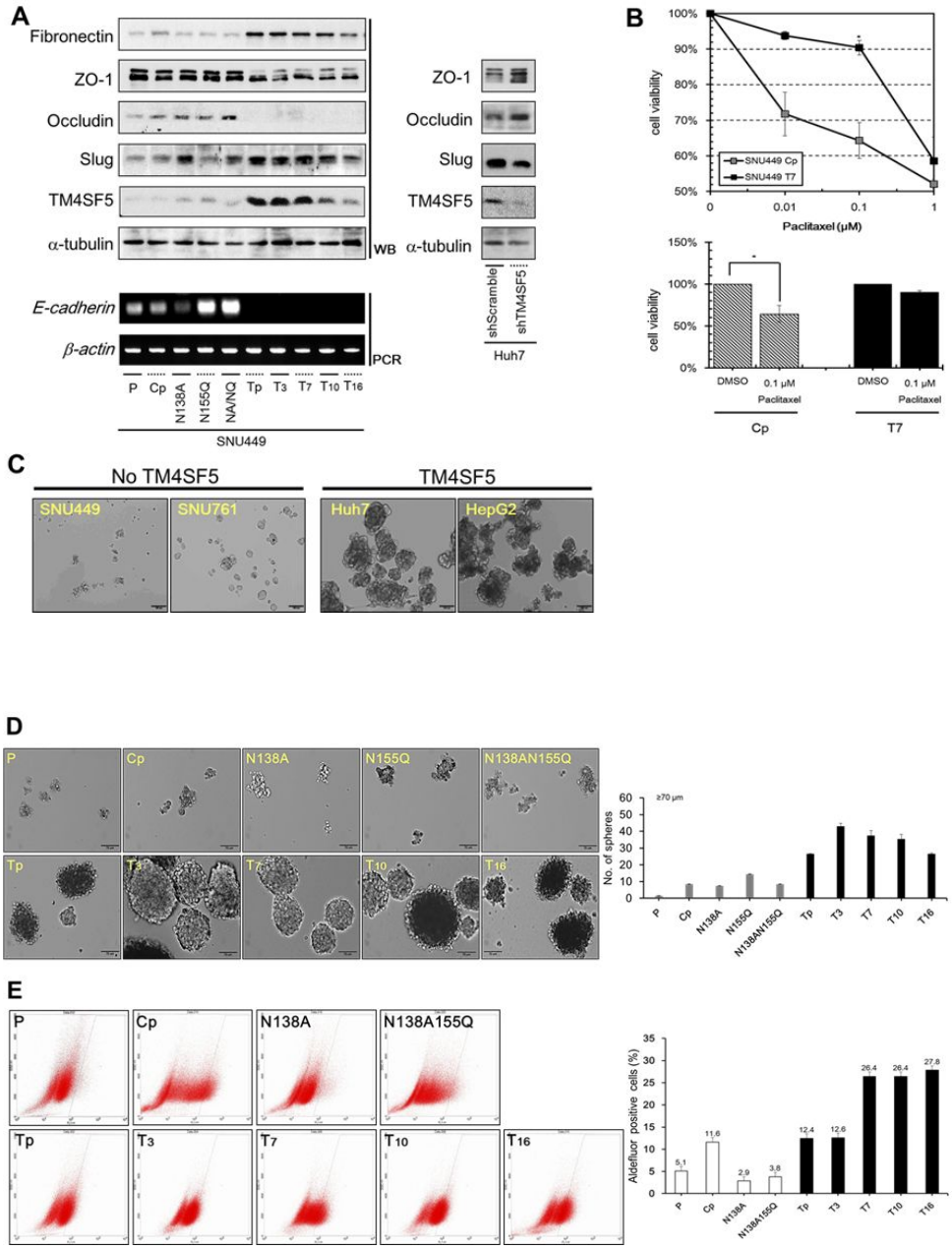
isothiocyanate, and incubation with antibody against CD44 was followed by incubation with anti-rat IgG conjugated with Alexa 546 respectively. In addition, the nucleus was stained with 4□,6-diamidino-2- phenylindole (DAPI).

Results

TM4SF5-mediated EMT rendered stem-like properties to HCC.

As TM4SF5 induced EMT through RhoA inactivation in HCC [29] and epithelial-mesenchymal transition (EMT) had been to generate the cells with stem-like properties [27], so we determined that EMT, which is induced by TM4SF5, render stemness to HCC with hepatocarcinoma cells. TM4SF5 decreased epithelial markers such as zo-1, occuldin and increased mesencymal markers such as fibronectin, slug. Moreover, suppression of TM4SF5 led to undergo MET (Figure 1.A). Because cancer stem cell has been speculated to more resistance to chemotherapeutic drug, we next examined the drug sensitivity of TM4SF5 null- or expressing cells. SNU449T7 cell had enhanced drug resistance than control cell to paclitaxel (Figure 1.B). To determine whether TM4SF5 rendered self-renewal capacity to HCC, we examined the spheroid formation ability of endogenously TM4SF5 expressing hapatocarcinoma cell Huh7, HepG2 and TM4SF5 null-cell SNU449, SNU761. Cells were cultured in serum-free epidermal growth factor/basic fibroblast growth factor-supplemented medium. Huh7 and HepG2 cells had enhanced spheroid formation ability than TM4SF5 null-cell SNU449, SNU761 (Figure 1. C). Wild-type TM4SF5 expressing SNU449 stable cells (pooled clone Tp or single cell-driven clone T3, T7, T10, and T16) well-formed spheroid but *N*-glycosylation mutants of TM4SF5 (N138A, N155Q and N138A/N155Q) or control cell (parental and

pooled clone Cp) did not form spheroid (Figure 1. D). Recent studies have shown that ALDH1 is a CSCs marker and that strongly correlates with tumor malignancy and self-renewal properties of stem cells in many types of tumors, including breast, liver, colon, and lung cancer [35-38]. To determine whether expression of TM4SF5 is correlated with activity of aldehyde dehydrogenase (ALDH), we examined the activity of ALDH by flow cytometry analysis with SNU449 stable cells. TM4SF5 expressing cells had higher activity of ALDH compared with *N*-glycosylation mutants of TM4SF5 or control cells (Figure 1. E). Furthermore, when suppression of TM4SF5 in Huh7 led to decrease the TM4SF5-mediated spheroid formation ability (Figure 1. F). Thus, TM4SF5 induced EMT in HCC and TM4SF5-mediated EMT rendered self-renewal ability to HCC.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SNU449
shScramble
shTM4SF5
Huh7
B
cell viability
SNU449 Cp
SNU449 T7
Paclitaxel (μM)
DMSO
0.1 μM Paclitaxel
Cp
T7
C
No TM4SF5
TM4SF5
SNU449
SNU761
Huh7
HepG2
D
P
Cp
N138A
N155Q
N138AN155Q
Tp
T3
T7
T10
T16
No. of spheres
≥70 μm
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26.4
27.8

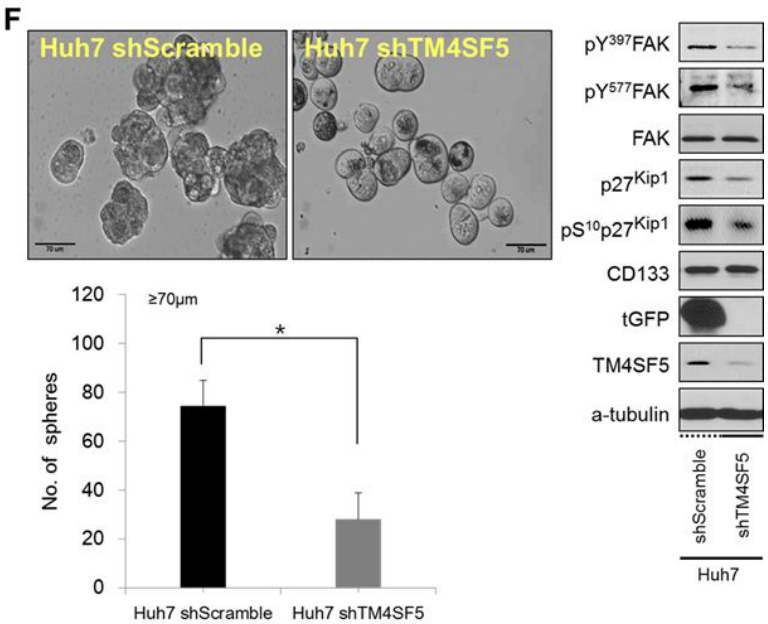


Figure 1. TM4SF5-mediated EMT rendered stem-like properties to HCC.

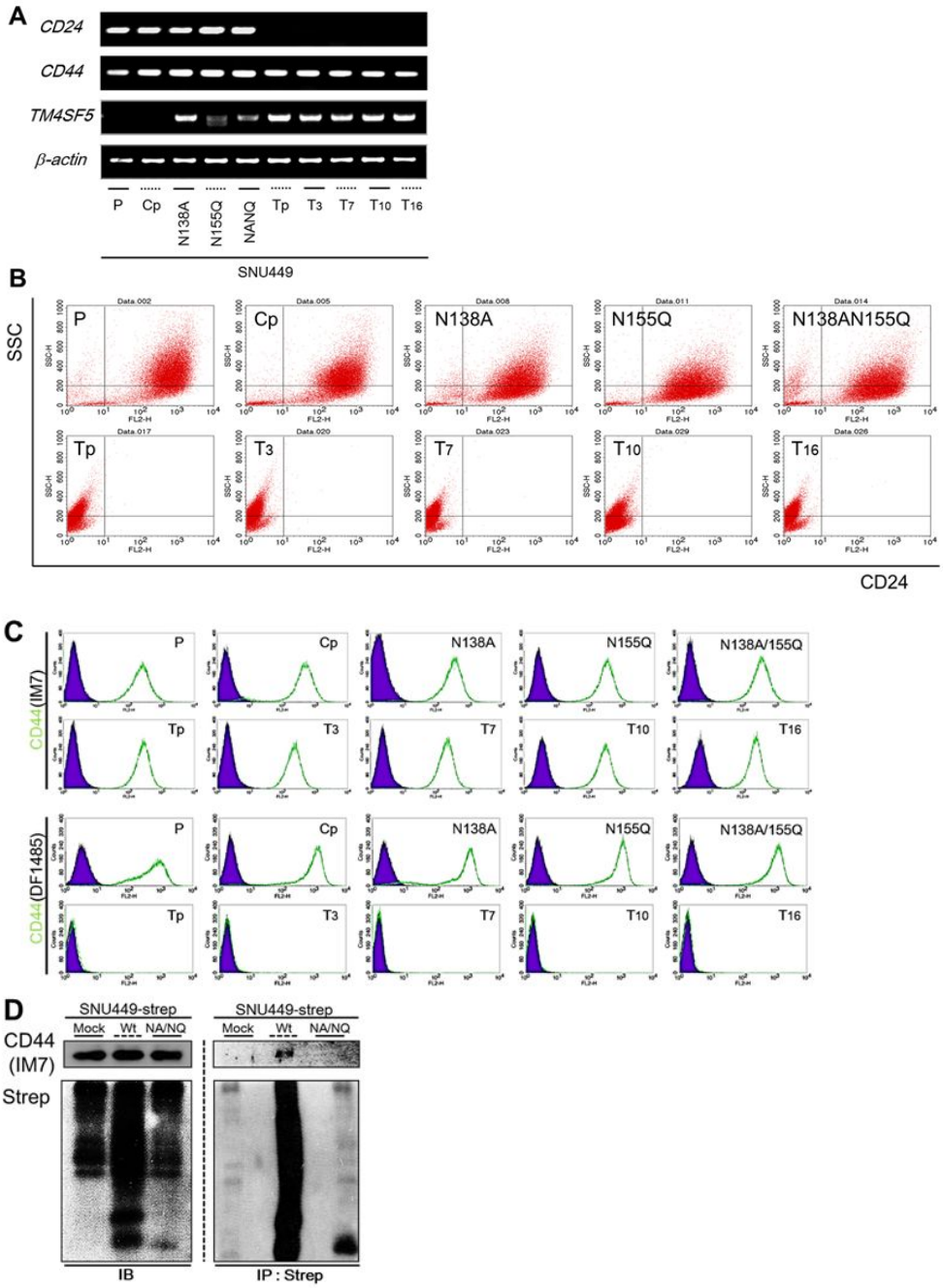
(A) TM4SF5 induced expression of mesenchymal markers and decreased epithelial markers in hepatocarcinoma cells. a-tubulin and b-actin are used as controls. (B) Expression of TM4SF5 increased a resistance to paclitaxel of SNU449T7, compared with TM4SF5-null SNU449Cp cells. $*p<0.05$ (C) Endogenously TM4SF5-expressing cells (Huh7, HepG2) possess greater spheroid formation abilities than TM4SF5-null cells (SNU449, SNU761). (D) Spheroid formation assays showed that TM4SF5-expressing cells exhibited enhanced sphere-forming capacities, compared with TM4SF5-lacking or *N*-glycosylation TM4SF5 mutant-expressing cells. (E) Aldefluor assays showed that TM4SF5-expressing cell clones (Tp, T3, T7, T10, and T16) exhibited higher aldefluor positivities, compared with TM4SF5-lacking (SNU449 parental cells or Cp control clone)

or *N*-glycosylation mutant (N138A or N138A/N155Q) TM4SF5-expressing cells. (**F**) Suppression of TM4SF5 decreased spheroid forming capacity, in paralleling with decreased TM4SF5-mediated downstream signaling activities. $*p=0.01$. Error bars represent the standard deviation (SD) of data obtained from at least 3 independent experiments.

TM4SF5 influenced expression of cancer stem cell markers, leading to spheroid formation

CD24 and CD44 are used to define cancer stem cells in multiple human epithelial cancers including liver and breast cancer [22,39]. We initially examined the expression of these markers in SNU449 stable cells. The expression of these markers were assessed at the messenger RNA (mRNA) and protein level. Transcription of CD24 was not detectable in TM4SF5 expressing cells, and it was further confirmed with Flow cytometry (Figure 2. A and B). In case of CD44, transcription level of CD44 was no difference between TM4SF5 expressing cells and *N*-glycosylation mutants of TM4SF5 or control cells. However when we examined protein level of CD44 in SNU449 cells by flow cytometry analysis with two different antibodies (IM7 and DF1485 clone), the signal of CD44 was not detectable in TM4SF5 expressing cells with DF1485 clone antibody (Figure 2. C). Tetraspanin is well known to recruit and complex with membrane proteins to form tetraspanin enriched microdomain (TERM) or tetraspanin web. We thought that tetraspanin, TM4SF5 complex with CD44 through interaction with each other. We then tested whether TM4SF5 may associated with CD44, we transfected SNU449 parental cell with strep-tagged mock, wild-type TM4SF5 (wt-TM4SF5) or *N*-glycosylation mutant of TM4SF5 and co-immunoprecipitated with CD44. Wt-TM4SF5 physically associated with CD44 however, *N*-glycosylation mutant TM4SF5 did not (Figure 2. D). Next, to investigate the impact of *N*-glycosylation of TM4SF5 to CD44 flow cytometry signal, we treated anti-TM4SF5 reagent TSAHC which disturbs

N-glycosylation of TM4SF5 [33]. Treatment of TSAHC to TM4SF5 expressing cells (Tp, T3, T7, T10 and T16) or suppression of TM4SF5 in Huh7 cell resulted in shifting of CD44 signals (Figure 2. E and F). This indicating that *N*-glycosylation of TM4SF5 might be important to interaction with CD44. Next we determined whether how TM4SF5 and CD44 interaction affect to TM4SF5-mediated self-renewal ability of HCC. Treatment of TSAHC to TM4SF5 expressing cell resulted in no spheroid formation (Figure 2. G). this indicating that TM4SF5 renders stemness to HCC through interaction with CD44.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CD24
CD44
TM4SF5
β-actin
P
Cp
N138A
N155Q
NANQ
Tp
T3
T7
T10
T16
SNU449
B
SSC
P
Cp
N138A
N155Q
N138AN155Q
Tp
T3
T7
T10
T16
CD24
C
CD44 (IM7)
CD44 (DF1485)
P
Cp
N138A
N155Q
N138A/155Q
Tp
T3
T7
T10
T16
D
SNU449-strep
Mock
Wt
NA/NQ
CD44 (IM7)
Strep
IB
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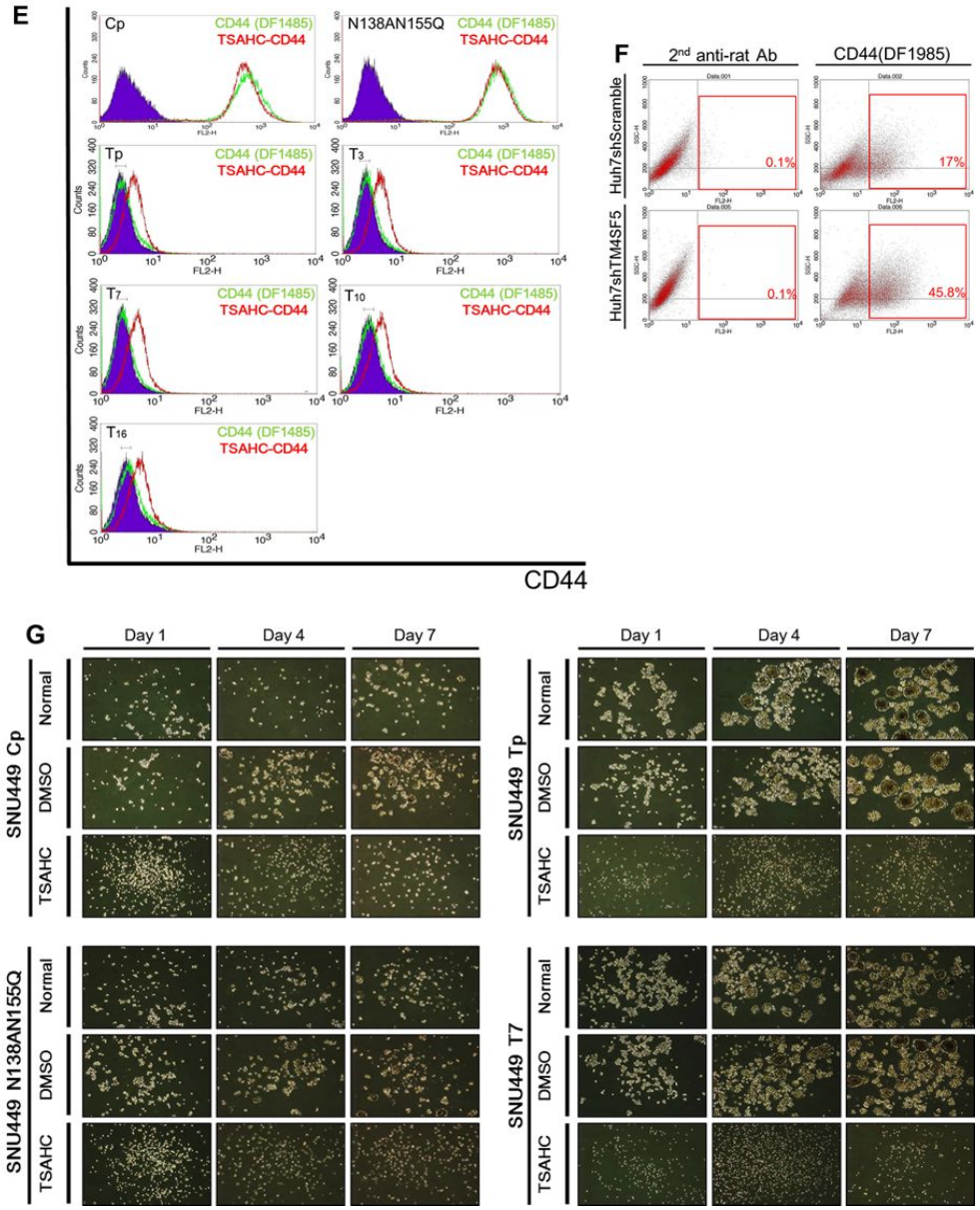


Figure 2. TM4SF5 influenced expression of cancer stem cell markers, leading to spheroid formation.

(A) *CD24, CD44* or *TM4SF5* mRNA levels were determined by

RT-PCR from mRNAs prepared from SNU449 parental (P), SNU449 control stable cells without TM4SF5 (Cp), TM4SF5 with *N*-glycosylation mutation (N138A, N155Q, NANQ) or TM4SF5 stable cell clones (Tp, T3, T7, T10, and T16). b-actin was used as a control. (B and C) Flow cytometry showing CD24 (**B**) or CD44 [**C**, upper panel by IM7 clone (Biolegend) and lower panel by DF1485 clone (Santa Cruz)] expression of different TM4SF5-null, -stably expressing, or -mutant expressing cells. (**D**)SNU449 parental cells were transfected with STrEP-tagged empty vector (Mock), TM4SF5 (WT), *N*-glycosylation mutant TM4SF5 (NA/NQ). After cell lysis, whole cell lysates were pull down by streptavidin-beads to eliminate potential other nonbinding proteins. Then, standard Western blots were performed against CD44 and anti-STrEP-tag. (**E**) Flow cytometry histograms showing CD44 expression of SNU449 control stable cells without TM4SF5 (Cp), TM4SF5 with *N*-glycosylation mutation (NANQ) or TM4SF5 stable cell clones (Tp, T3, T7, T10, and T16) in the absence or presence of TSAHC (20 mM, a specific anti-TM4SF5 reagent) treatment for 24 hr. DF1485 clone labeling was done for red. DMSO-treated (green) or no primary Ab (purple) samples were used as control. (**F**) Flow cytometry showing CD44 expression of Huh7 stably expressing shScramble(upper) or Huh7 shTM4SF5(lower).(**G**) Spheroid formation assay showing that TSAHC inhibits spheroid formation of TM4SF5-expressing cells. SNU449 control stable cells without TM4SF5 (Cp), TM4SF5 with *N*-glycosylation mutation (NANQ) or TM4SF5 stable cell clones (Tp, T7)) in the absence or presence of TSAHC (20 mM).

Serial xenograft tumorigenicity assay using small numbers of TM4SF5-expressing SNU449T7 cells results in more aggressive tumors

We next inoculated nude mice subcutaneously with TM4SF5 null cells and *N*-glycosylation mutant of TM4SF5 or TM4SF5 expressing cells to investigate their tumorigenecity in vivo. A significant difference in tumor incidence was observed between the mice inoculated with TM4SF5 expressing cells and those inoculated with TM4SF5 null or *N*-glycosylation mutant of TM4SF5 cells (Table 1). 2.5 weeks after inoculation, as few as 500 TM4SF5 expressing cells were sufficient to generate tumors in nude mice, whereas the TM4SF5 null and *N*-glycosylation mutant of TM4SF5 cells did not induce tumor formation, even when 5 x 10^3cells were injected and 6weeks postinoculation (Figure3. A and Table 1). Tumor nodules that formed subcutaneously in the nude mice were stained with CD44 and TM4SF5 (Figure3.B). these data showed CD44 positive cells were enriched in small portion of tumor and furthermore where the CD44 positive cells were enriched, there were TM4SF5 and CD44 co-positive cells and CD44 only positive cells. this difference of cell population suggesting that TM4SF5 and CD44 co-positive cancer stem cell dedifferentiated heterogeneously. A most important property of cancer stem cells is their unique ability to self-renewal. One method to investigate whether TM4SF5 expressing cells have self-renewal capacity in vivo is to test their capability of serial passage, so we performed serial xenograft experiments from TM4SF5 expressing cell-derived tumors that grew from an initial injection of 5000 TM4SF5

expressing cells. 6weeks postinoculation, the xenograft tumor was excised from the primary mouse recipient, dissociated into single cell suspension, grown in culture for approximately 1 week with G418 culture medium (250 μg/mL of G418 in RPMI1640 medium). These explant cells were immunoblotted (Figure 3. C), or reinjected into secondary mouse recipients (Table 2). Mouse cells were excluded from this experiment because the G418 culture medium for selection of TM4SF5 expressing cells which we injected. The immunoblotting of explanted cells showed STAT3 signaling activation and twist expression were up-regulated, and moreover TM4SF5 downstream signaling proteins were up-regulated (Figure 3. C). Transplantation of explant cell to secondary mouse showed more tumorigenic (Table 2). Where 200 Explant cells injected developed tumors in 2 weeks after cell injection and all the mice had tumor nodules in 4 weeks postinoculation. The tumor volume was correlated with the number of cell injected (Figure 3. D). Cancer stem cells have been postulated to be present in the blood stream [40]. To demonstrated whether TM4SF5 expressing cells circulate in the peripheral blood stream, we orthotopic injected tGFP-positive TM4SF5 expressing cell to left lobes of nude mice liver for generating tumor model and 4weeks after cell injection, small volume (0.7-1 mL) blood samples were collected from each mouse by puncture of the left ventricle, then cells were sorted by FACSAria Ⅲ (Figure 3. E). Immunofluorescence analysis of GFP-positive sorted cells with tGFP and DAPI showed TM4SF5 expressing cells circulated in the mouse blood.

Table 1. In vivo tumor development experiments of TM4SF5 expressing cell lines in nude mice.

In Vivo tumor Development Experiments of TM4SF5 expressing cell lines in Nude mice

Cell line(or cell type)	Cell numbers injected[a]	No. of mice with tumor formation/ total No. of mice with cell SC. injection				
		2.5 weeks	3 weeks	4 weeks	5 weeks	6 weeks
SNU449 Cp / T7	$5x10^2$	$1^R/5$	$4^R/5$	$5^R/5$	$5^R/5$	$5^R/5$
	$2x10^3$	$2^R/5$	$3^R/5$	$4^R/5$	$4^R/5$	$4^R/5$
	$5x10^3$	$3^R/5$	$3^R/5$	$3^R/5$	$3^R/5$	$3^R/5$
SNU449 N138AN155Q / T7	$5x10^2$	$2^R/5$	$4^R/5$	$5^R/5$	$5^R/5$	$4^R/5$
	$2x10^3$	$1^R/5$	$3^R/5$	$3^R/5$	$3^R/5$	$4^R/5$
	$5x10^3$	$2^R/5$	$3^R/5$	$3^R/5$	$3^R/5$	$3^R/5$

[a]cells injected in left/right side flank of the same mice for avoiding individual difference (Left side-control cell line/Right side-test cell line)

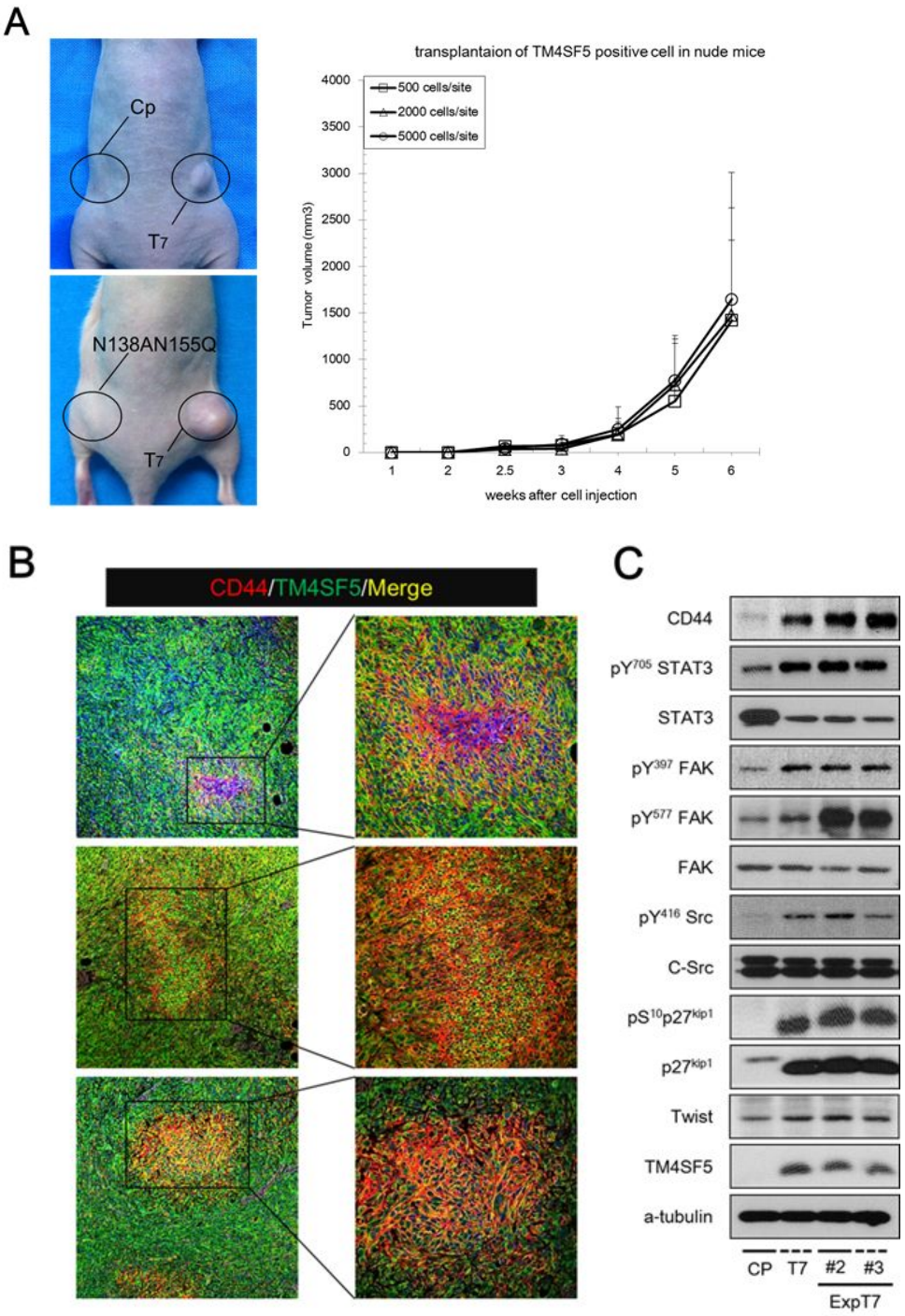
[R]tumor formation in right side flank of the mouse / [L]tumor formation in right side flank of the mouse

Table 2. serial xenograft of TM4SF5 expressing cell lines in nude mice.

Serial xenograft of TM4SF5 expressing cell lines in Nude mice

ExpT7[a] Cell numbers injected	No. of mice with tumor formation/ total No. of mice with cell SC. injection		
	2 weeks	3 weeks	4 weeks
$2x10^2$	2/7	6/7	7/7
$5x10^2$	6/7	7/7	7/7
$2x10^3$	7/7	7/7	7/7
$5x10^3$	7/7	7/7	7/7

[a] ExpT7 is the cells that were dissociated from the primary xenograft injected with SNU449T7 cells


A
Cp
T7
N138AN155Q
T7
transplantaion of TM4SF5 positive cell in nude mice
500 cells/site
2000 cells/site
5000 cells/site
Tumor volume (mm3)
4000
3500
3000
2500
2000
1500
1000
500
0
1
2
2.5
3
4
5
6
weeks after cell injection
B
CD44/TM4SF5/Merge
C
CD44
pY705 STAT3
STAT3
pY397 FAK
pY577 FAK
FAK
pY416 Src
C-Src
pS10p27kip1
p27kip1
Twist
TM4SF5
a-tubulin
CP
T7
#2
#3
ExpT7

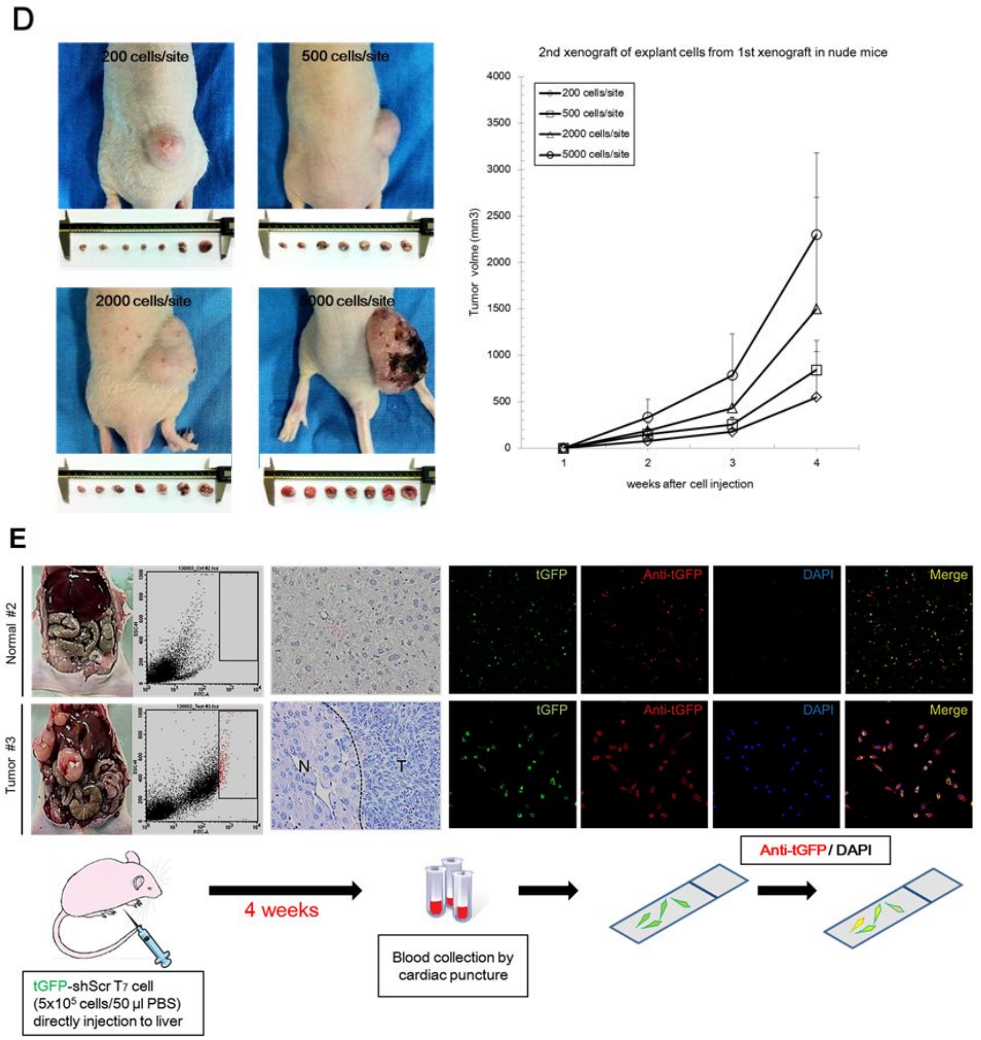


Figure 3. Serial xenograft tumorigenicity assay using small numbers of TM4SF5-expressing SNU449T7 cells results in more aggressive tumors

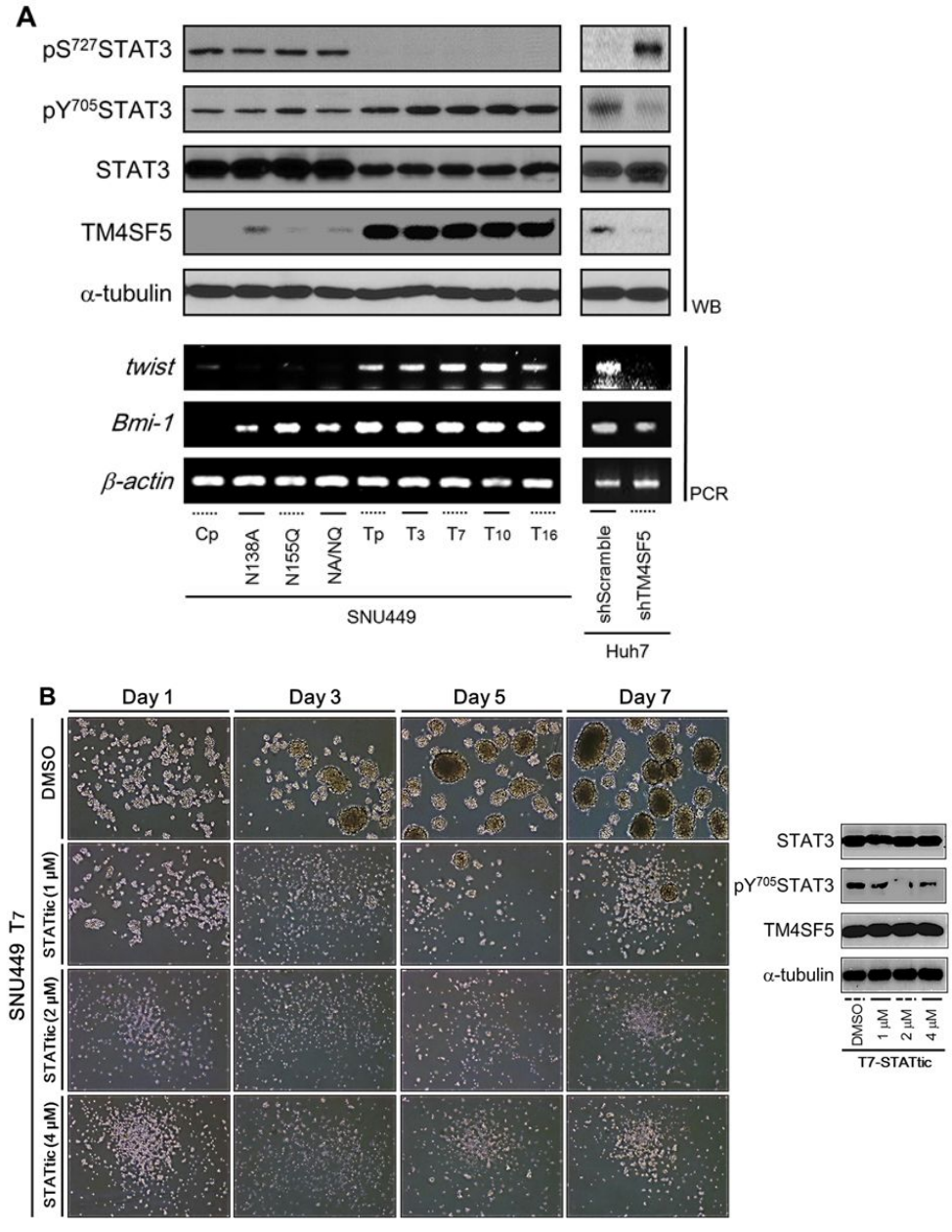
(A) Representative images (left) showing that SC injection of TM4SF5-expressing cells at numbers less than 5000 cells/mouse induced tumor formation. See also Table 1. The right flanks of mice were injected with TM4SF5-expressing

cells (SNU449T7, n=10), whereas the left flanks were injected with TM4SF5-null control cells (SNU449Cp, n=5), or *N*-glycosylation TM4SF5 mutant-expressing cells (SNU449 N138A/N155Q, n=5). Graphic presentation (right) of tumor volumes at mean " } SD after TM4SF5-expressing cell injection. (**B**) Tumor sections after TM4SF5-expressing cells injection were stained with DAPI (blue), and immunostained for CD44 (red) and TM4SF5 (green). (**C**) SNU449 control stable cells without TM4SF5 (Cp), TM4SF5 stable cell clone (T7) and ExpT7 cells (#2 and #3) were harvested for standard Western blots for STAT3, pY^{705}STAT3, FAK, pY^{577}FAK, pY^{397}FAK, c-Src, pY^{416}Src, $p27^{kip1}$, $pS^{10}p27^{kip1}$, CD44, Twist or TM4SF5. *a*-tubulin was used as control. (**D**) Representative images (left) showing that TM4SF5-expressing explants from the 1^{st} injection formed secondly tumors in serial xenograft. See also Table 2. Graphic presentation (right) of tumor volumes at mean " } SD after the TM4SF5-expressing explant cells injection. (**E**) Representative images showing the tGFP positive TM4SF5 expressing cell formed tumor nodules in the liver of nude mice. GFP-positive cells were sorted by FACSAria, then stained with DAPI (Blue), and immunostained for tGFP (red)

TM4SF5 rendered stemness to HCC through STAT3-Twist-Bmi1 signaling pathway

Having documented that TM4SF5 was a potential cancer stem cell marker, we sought to elucidate the major downstream mediator of TM4SF5 in tumor initiation and self-renewal. Because Bmi1 was reported to be involved in the self-renewal of neuronal, hematopoietic and intestinal cells by promoting epigenetic changes favoring chromatin condensation [41-43] and its overexpression is frequently observed in many cancer stem cells [44,45]. Recently, Bmi1 is directly regulated by Twist1, the EMT regulator has reported [46]. Interestingly, Twist1 had up-regulated in explant T7 cells from primary xenograft experiments of SNU449T7 cells (Figure 3. C), and furthermore it has well documented that transcription of Twist1 is induced by activation of STAT3 signaling [47]. We previously reported that TM4SF5 interaction with integrin a5 to transduce signaling through FAK-c-Src complex activation and STAT3 activation [48]. We therefore hypothesized that TM4SF5 generated cancer stemness through regulating expression of Twist1 and Bmi1 by STAT3 signaling activation. To test this hypothesis, we first examined the expression levels of phospho-STAT3, Twsit1, and Bmi1 in SNU449 stable cells (Figure 4. A). Phosphorylated STAT3 (Y705) and expression of Twist1 and Bmi1 also were up-regulated in TM4SF5 expressing cells compare with TM4SF5 null- or *N*-glycosylation mutant of TM4SF5 cells and when suppression of TM4SF5 in Huh7, these findings were confirmed. We next examined the TM4SF5-mediated cancer stemness through STAT3 signaling activation. When STAT3 signaling

activation was disturbed by stattic, STAT3 inhibitor, spheroid formation of TM4SF5 expressing cells was decreased (Figure 4. B) and furthermore, treatment of anti-TM4SF5 reagent TSAHC resulted in suppression of phosphorylated STAT3 (Y705) and decreasing expression level of Twist1 and Bmi1 (Figure 4. C). thus these findings demonstrate that TM4SF5 render cancer stemness to HCC through signaling pathway linkage via STAT3/Twist1/Bmi1.


A
pS727STAT3
pY705STAT3
STAT3
TM4SF5
α-tubulin
WB
twist
Bmi-1
β-actin
PCR
Cp
N138A
N155Q
NA/NQ
Tp
T3
T7
T10
T16
SNU449
shScramble
shTM4SF5
Huh7
B
Day 1
Day 3
Day 5
Day 7
SNU449 T7
DMSO
STATtic (1 μM)
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1 μM
2 μM
4 μM
T7-STATtic

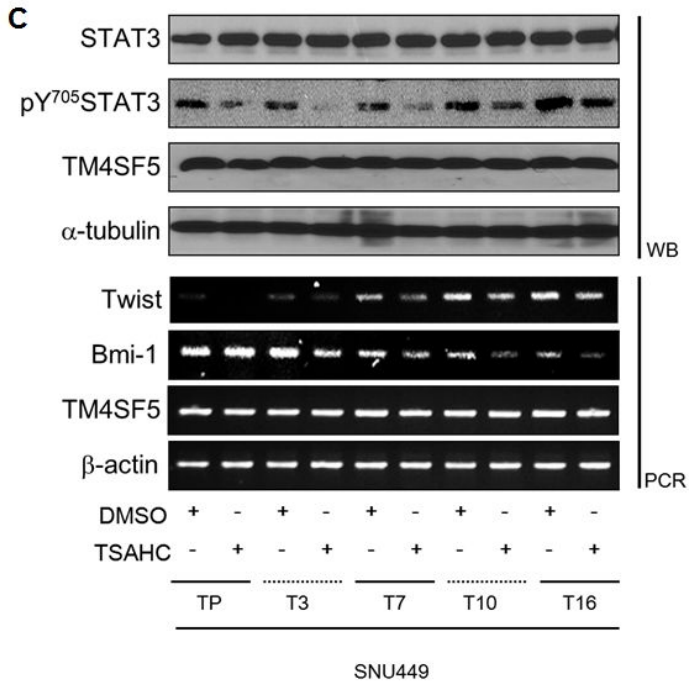

Figure 4. TM4SF5 rendered stemness to HCC through STAT3-Twist-Bmi-1 signaling pathway.

(A) SNU449 control stable cells without TM4SF5 (Cp), TM4SF5 with *N*-glycosylation mutation (N138A, N155Q, NANQ) or TM4SF5 stable cell clones (Tp, T3, T7, T10, and T16), Huh7-sHScramble, and Huh7-shTM4SF5 stable cells were harvested for standard Western blots for STAT3, pY^{705}STAT3, pY^{727}STAT3, or TM4SF5, or were processed for RT-PCR for *Twist* and *Bmi-1*. *a*-tubulin and *β-actin* were used as controls. (B) Representative images showing that inhibition of STAT3 activity decreased the spheroid formation ability. (C) SNU449 stable cell clones with TM4SF5 expression (Tp, T3, T7, T10, and T16) in the absence or presence of TSAHC (20 mM)

treatment for 24 hr were harvested for standard Western blots for STAT3, pY^{705}STAT3, and TM4SF5, or were processed for RT-PCR for *Twist* and *Bmi-1*. *a*-tubulin and *β-actin* were used as controls.

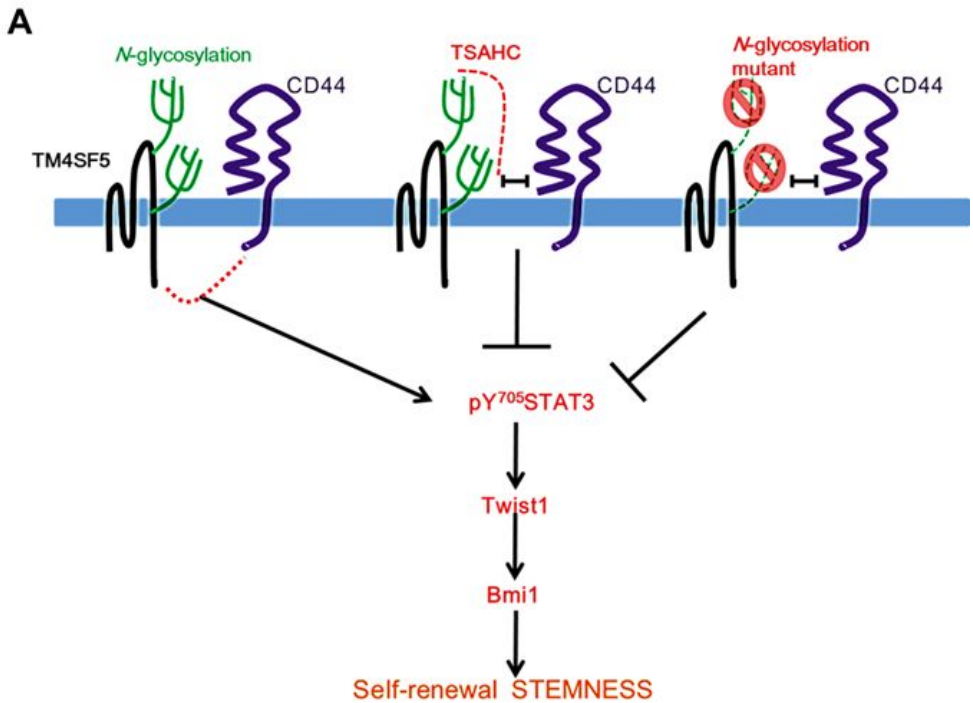

Figure 5. The working model

(A) Cooperation between TM4SF5 and CD44 results in generating stem-like properties ; Physical association between TM4SF5 and the CD44 may be render cancer stemness to HCC. Activation of STAT3 signaling induced by cooperation between TM4SF5 and CD44 regulated transcription of Twist1 and resulted in inducing EMT. Up-regulation of Twsit1 up-regulates Bmi1 expression in the cell, consequently promoting cancer stemness, including drug resistance, high aldehyde dehydrogenase activity, and self-renewal capacity.

Discussion

Liver cancer stem cells (CSCs) have been identified in HCC with some markers in recent years [10,16-26]. Although it has been little reported about mechanisms of generating CSC. Lately, several evidences suggest that the process of EMT generates cells with stem-like properties [27,49,50]. The EMT process in tumor cells results in cells becoming more aggressive in the tumor progression. The CSC is the small population of the cells residing in a tumor mass that are chemo-resistant. These CSCs have the ability of self-renewal and forming secondary tumors, as described as tumor initiating ability [51].

We previously reported that TM4SF5 increased p27Kip1 expression and cytosolic stabilization in JNK activity dependent manner [34]. The TM4SF5-mediated cytosolic p27Kip1 stabilization resulted in RhoA inactivation leading to EMT that involved E-cadherin suppression in a snail1-independent manner, increased α-smooth muscle actin (α-SMA) expression resulted in morphological elongation, continuous uncontrolled growth in multilayer through loss of contact inhibition, uncontrolled S-phase progression, and anchorage-independent growth [52]. The CSCs has high resistant chemotherapy in several solid tumor [53], and this high drug resistance ability of CSCs is associated with EMT process [54]. Our group recently reported TM4SF5-mediated EMT is correlated with gefitinib resistance of NSCLC. Therefore, we could hypothesized that TM4SF5 and its ability of inducing EMT might be involved in generating hepatic CSCs in HCC.

In this study, we showed that TM4SF5 expressing cells underwent EMT. Moreover, these cells were capable of initiating tumors in vivo and forming spheroids in vitro, whereas TM4SF5 null cell were not. These observations indicating that the TM4SF5 expressing cells possessed CSC properties and that TM4SF5 could be a potential marker for liver CSCs. Moreover wild-type TM4SF5 physically bound to CD44, whereas *N*-glycosylation mutant of TM4SF5 did not. Intriguingly, *N*-glycosylation mutant of TM4SF5 expressing cells were not able of initiating tumor in vivo and forming spheroid in vitro and treatment of anti-TM4SF5 reagent TSAHC that disturbed *N*-glycosylation residues of TM4SF5 had CD44 released to be unbound form resulted in no spheroid formation. These observations indicated that TM4SF5 binding to CD44 via its *N*-glycosylation could be important to stemness of HCC and be a therapeutic target.

In the present study, we identified that TM4SF5 expressing cells expressed gene that important for the self-renewal and proliferation such as Bmi1. Emerging studies have demonstrated the general expression of stemness-related genes, including Bmi1, in CSCs isolated based on their CSC markers [16,45]. Although twist1 is reported to directly regulate transcription of Bmi1 [46], the relationship of these potential CSC markers with the expressed stemness-related genes and the underlying mechanism have been unclear. In this study, we demonstrated that TM4SF5, a potential hepatic CSC marker, regulated the expression of Bmi1 through signaling linkage via STAT3/Twist (Figure 5. A). Intriguingly, TM4SF5 is reported to crosstalk with integrin $\alpha 5$ results in activating STAT3 signaling through

activation of FAK/c-Src complex [48]. Activated STAT3 signaling induced up-regulation of Twist and Bmi1 under our experimental conditions. However another phosphorylation residue of STAT3 (S727) was phosphorylated in TM4SF5 null and *N*-glycosylation mutant of TM4SF5 cell (Figure 4. A). it has been reported that STAT3 serine residue (S727) phosphorylation negatively regulates STAT3 tyrosine residue (Y705) phosphorylation via ERK-dependent or -independent manner [55], so this suggest that STAT3 serine phosphorylation may reside between TM4SF5-mediated FAK/c-Src complex activation and tyrosine phosphorylation of STAT3. In the present study, TM4SF5 expression in HCC cells is not overlapped in expression of CD24, although it is a well-known hepatic CSC marker via its activation of the Nanog promoter in a STAT3 dependent manner [22].

These data suggest that hepatic CSCs has distinct subpopulation, additionally even CSCs isolated from the same tumor can express several different surface markers and exhibit different cellular behaviors indicates that tumors may contain various subpopulation of CSCs [56].

In summary, this study identified TM4SF5 as a CSC marker and TM4SF5-mediated EMT rendered "stemness" to HCC, through signaling linkage including STAT3/Twist/Bmi1 depending on *N*-glycosylation status of TM4SF5. We investigated that expression of TM4SF5 in HCC resulted in spheroid formation in vitro and tumor incidence in vivo, and TM4SF5/CD44 co-positive cancer stem cell dedifferentiated heterogeneously. Moreover TM4SF5 expressing cell circulated in blood for 1 month in

orthotopic injection tumor model supporting that TM4SF5+ circulating tumor cells could possessed tumor stem cell properties. Our results suggested that TM4SF5 might be used as a potential hepatic CSC marker and therapeutic target.

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요 약

EMT (Epithelial-Mesenchymal transition) 은 종양 발달과정 중 종양 세포의 이동, 전이, 항암제 저항성, 자가복제 등에 관여한다. TM4SF5 단백질은 다양한 종류의 암에서 높게 발현되며 EMT, 전이, 그리고 폐암세포주의 항암제 gefitinib에 대한 저항을 유발한다. 그러나 아직 TM4SF5 단백질에 의한 종양 세포의 자기재생에 관한 연구는 이루어지지 않았다. 이에 본 연구는 TM4SF5에 의한 자가복제능력 유도 기작이 가능한지를 TM4SF5 단백질의 발현을 조절한 간암세포주를 통해 알아보았다. TM4SF5 단백질을 발현하는 세포는 혈청 성분과 부착 환경이 없는 상황에서 spheroids를 잘 형성한 반면에, TM4SF5 단백질의 발현을 억제시킨 세포와, TM4SF5의 아미노산 서열 138, 155번 위치의 asparagine을 각각 glycine, glutamine으로 치환한 *N*-glycosylation의 mutant TM4SF5 단백질을 발현하는 세포에서는 spheroids를 형성 하지 못하였고, TM4SF5 단백질을 발현하는 세포에 anti-TM4SF5 reagent인 TSAHC를 처리하였을 때에도 역시 spheroids를 형성 하지 못하는 것을 확인하였다. TM4SF5 단백질을 발현하는 세포에서는 CD24 단백질의 발현은 감소하였고, CD44 단백질을 발현하는 것을 확인하였다. 이 때, single pass type I membrane protein 인 CD44 단백질이 TM4SF5 단백질과 결합한 상태에서는 spheroids를 형성하는 것으로 보아, *N*-glycosylation mutants TM4SF5 단백질은 CD44 단백질과 결합을 하지 못하기에, spheroids를 형성하지 못하는 것으로 확인되었다. 또한, anti-TM4SF5 reagent인 TSAHC를 처리하였을 때, CD44 단백질이 TM4SF5 단백질 으로부터 유리되어지고 spheroids형성이 되지 않는 것으로 보아, TM4SF5 단백질과 CD44 단백질의 결합이 spheroids 형성에 중요함을 확인할 수 있었다. 한편 이러한 spheroids 형성 능력은 마우스에서의 종양 형성 능력과 관련이 있음을 5000개 이하의 세포를 피하 주입한 serial xenograft를 통하여 알아내었다. 이러한 TM4SF5 단백질은 STAT3

signaling의 활성화를 통해 twist1 단백질의 발현을 증가함으로써, EMT 현상을 유발하고 최종적으로 종양세포의 자가복제능력에 중요하다고 알려진 bmi1 유전자의 발현을 증가시켰다. 즉 이와 같은 결과들은, TM4SF5 단백질이 간암세포의 자가복제능력 획득에 기여한다는 것을 제시한다.

주 요 어 : TM4SF5, self-renewal, CD44, *N*-glycosylation, STAT3, Twist1, Bmi1

학 번 : 2012-21607