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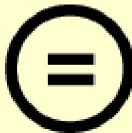
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약학석사 학위논문

Regulation of cellular behaviors via a cross-talk between TM4SF5 and protein tyrosine phosphatase receptor type-F (PTPRF) in liver cancer cells.

간암세포에서 TM4SF5와 단백질 타이로신 탈인산화 효소 (PTPRF)의 상호작용을 통한 세포 행동 양상 조절

2017년 8월

서울대학교 대학원

약학과 의약생명과학전공

조 창 연

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Abstract

Regulation of cellular behaviors via a cross-talk between TM4SF5 and protein tyrosine phosphatase receptor type-F (PTPRF) in liver cancer cells.

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Transmembrane 4 L six family member 5 (TM4SF5) is a glycoprotein on cell surface and highly expressed in various cancers including hepatocarcinoma. In previous studies, it is shown that TM4SF5 induces Epithelial-Mesenchymal Transition (EMT), as a regulation factor of cell morphological change, cell migration, invasion, and proliferation. In this study, I found protein tyrosine phosphatase receptor type-F (PTPRF) as a new binding partner of TM4SF5

through mass spectrometry. PTPRF is a transmembrane tyrosine phosphatase with one transmembrane domain. PTPRF is known to regulate cell proliferation, differentiation, and mitotic cell cycle, being associated with cell death-related protein activation. Here, I investigated how this interaction affected cellular behaviors using cell lines with overexpression or suppression of TM4SF5 and/or PTPRF. First, I could confirm their interaction by co-immunoprecipitation and immunofluorescence. Overexpression or down-regulation of either TM4SF5 or PTPRF did not affect their mRNA or protein levels. Further, the interaction between TM4SF5 and PTPRF decreased in the suspension state of the cells and recovered significantly when the cells became reattached to the extracellular matrix (ECM). I could thus rationalize that TM4SF5 and PTPRF interaction was correlated with and affect PTPRF's tyrosine-phosphatase activity. In a previous study, it was reported that TM4SF5 regulates activities of focal adhesion (FA) molecules and formation of FA enriched with paxillin. So, I focused on the influence of PTPRF expression on activities of FA molecules in the presence or absence of TM4SF5 expression. PTPRF co-localized with Paxillin during immunofluorescence studies, and overexpression of PTPRF led to dephosphorylation of FAK, Src, and Paxillin. When TM4SF5 and PTPRF were co-overexpressed, PTPRF's effects against phosphorylation of FAK, Src, and Paxillin were inhibited, indicating that TM4SF5 could antagonize PTPRF. To investigate such effects of PTPRF on cellular behaviors, I performed cell adhesion assay, cell migration assay, and sphere formation assay for the purposes of understanding the roles of the both in cancer metastasis. During the cell adhesion assay, cells

expressing PTPRF inhibited tyrosine phosphorylation of paxillin in the suspended state, whereas cells lacking PTPRF retained tyrosine phosphorylation of paxillin in the suspended state. When those cells were attached to ECM fibronectin, PTPRF-lacking cells formed FAs faster than control PTPRF-expressing cells. However, cells lacking both PTPRF and TM4SF5 showed paxillin phosphorylation higher than TM4SF5 alone-lacking cells. In the cell migration assay, migration ability of PTPRF-lacking cells increased but cells lacking both PTPRF and TM4SF5 showed reduced migration ability than PTPRF alone-lacking cells. In case of the spheroid formation assay, PTPRF-lacking cells increased sphere formation but cells lacking both PTPRF and TM4SF5 showed a lower sphere formation capacity than PTPRF-alone lacking cells.

Altogether, these observations suggest that the role of PTPRF in cells to inhibit molecules at FAs, such as FAK, c-Src, and Paxillin, would be controlled by TM4SF5, especially when the cells were attached to the ECMs. In HCC that TM4SF5 is overexpressed, however, TM4SF5 can promote tyrosine phosphorylation of FA molecules that would be negatively targeted by PTPRF. Thus, the coordinated cross-talks between TM4SF5 and PTPRF can play roles in successful cancer metastasis, which further can be a promising target to deals with TM4SF5-dependent metastasis.

Keywords : 3D cell culture, protein interaction, cellular metastasis, extracellular matrix, TM4SF5, PTPRF, focal adhesion, tumor microenvironment

Student number : 2015-21899

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INTRODUCTION

Interactions between proteins play an important role in the living cells. So, understanding physical and functional interactions between molecules in cells is important for the investigation of cellular signaling pathways in biology[1]. And these interactions utilized for clinical research. Proteins rarely act alone as their functions tend to be regulated. Many molecular processes within a cell are carried out by molecular machines that are built by many protein components organized by protein-protein interaction. These interactions properties are very diverse and influenced by cell environment and state. Protein-protein interactions are predictable through variety of experimental techniques.[1,2]

The function of proteins are regulated by many mechanisms and one of them is protein tyrosine phosphorylation. Addition of phosphate to proteins by protein tyrosine kinase (PTKs) and its removal by protein tyrosine phosphatases (PTPs) are collaborated in cellular regulation mechanisms. The protein tyrosine phosphatases (PTPs) have emerged as critical proteins in diverse cellular functions, such as proliferation, differentiation, adhesion, and metabolic homeostasis[3,4]. PTPs genetic alterations, such as mutation, deletion, duplication or translocation, may contribute to diverse cancer phenotypes. Genetic and epigenetic alterations in genes encoding PTPs can result in change to the equilibrium of kinase-phosphatase activity that might have deleterious effects, including abnormal cell proliferation, which could lead to cancer[5]. In this study, I focused on protein tyrosine phosphatase receptor type-F (PTPRF) as a

new binding partner of TM4SF5. The PTPRF is a transmembrane tyrosine phosphatase with one transmembrane domain. PTPRF is known to regulate cell proliferation, differentiation, and mitotic cell cycle, being associated with cell death-related protein activation[6,7]. However, the function of PTPRF in HCC is unclear but there are some researches that provide the relation between focal adhesion molecules and PTPRF[8]. And also clinical data shows, PTPRF was down-regulated in 42% HCC (37/89), 67% gastric cancer (27/40), and 100% colorectal cancer (40/40)[9].

Transmembrane 4 L six family member 5 (TM4SF5) is highly expressed in diverse clinical cancer tissues, and its overexpression causes aberrant proliferation, angiogenesis, migration, and invasion[10,11]. TM4SF5 is a membrane glycoprotein with four transmembrane domains, and its intracellular loop and N-and C-terminal tails are located in the cytosol. Also, TM4F5 localized at tetraspanin-enriched microdomain (TEM) and collaborates with integrins, growth factor receptors and other membrane proteins to regulate FA and cellular survival signals[12-16]. Overexpression of TM4SF5 derives cells to become more tumorigenic[15].

Here in the study, I found new binding partners of TM4SF5 through mass spectrometry and could find various candidates. Interestingly I could find protein tyrosine phosphatase receptor type-F (PTPRF) which is reported as a tumor suppressor. PTPRF is a receptor type protein tyrosine phosphatase that localize on the cell membrane and its role in cancer is unknown[17]. I tried to understand the meaning of interaction between TM4SF5 and PTPRF in HCC, I focused on the alteration of interaction level between TM4SF5 and PTPRF. This

findings suggest that interaction between TM4SF5 and PTPRF is related with regulation of cellular behaviors and another role of TM4SF5 in HCC as an inhibitor of protein tyrosine phosphatase receptor type-F (PTPRF).

Materials and Methods

1. Cell culture

Parental SNU449, SNU761 and Hep3B cells were cultured in RPMI-1630 (WelGene Inc.) containing 10% FBS and 1% penicillin/streptomycin (GenDEPOT Inc.) at 37℃ in5% CO_2 . Huh7 cell was cultured in DMEM (WelGene Inc.) under the same condition.

2. Cell lysate preparation and Western blots

The cells were grown in 6-well or 100 pi culture plate and harvested at 80% of confluency, before preparation of whole cell lysates sample. In some cases, cells were transiently transfected with TM4SF5 or PTPRF for 48 h by lipofectamin 3000 reagent (Thermo fisher scientific) or siPTPRF for 48h by RNAiMAX (Thermo fisher scientific). The cells were harvested using a RIPA buffer or lysis buffer containing 1% Brij58, 150 Mm NaCl, 20mM HEPES, pH 7.4, 2mM $MgCl_2$, and protease inhibitors for standard Western blots process. The samples were normalized for equal protein loading in standard immunoblots via α-tubulin or β-actin levels.

3. 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

fibronectin in PBS solution at 37℃ for overnight, and the control membrane was incubated with 5% BSA in RPMI culture medium at 37℃ for overnight. Prior to assay, stable cells were collected in the 1% BSA-RPMI and continuously agitated for 2 h in order to nullify their adhesion signals. Cells were then seeded at $1x10^5$ cells per insert within 1% BSA-RPMI, and the wells were filled with the same culture media.

4. Spheroid formation

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). 25ng/ml of hEGF and hbFGF (Peprotech) were added every 2nd days. The cells were subsequently cultured in ultra-low attachment 6-well plates (Corning Inc. Corning, NY, USA) at a density of no more than $2x10^3$ cells/well.

5. Antibodies and reagents

Primary antibodies for immunoblot were used as follows : PTPRF (R&D systems) c-Src (Santacruz), phospho-Y416 Src (Cell signaling), FAK (BD Bioscience), phospho-Y397 FAK (BD Bioscience), anti-HA (BioLegend), α-tubulin (Sigma-Aldrich), $pT^{473}Y^{473}$ERK (Cell signaling), ERK (BD Transduction Laboratory)

6. Immunofluorescence analysis

Cells were cultured with glass coverslip and fixed directly with 4% formaldehyde for 30 min at room temperature (RT). After PBS washing, cells were permeabilized for 30 min with 0.5% Triton X-100 at RT and blocked for 2 h with PBS in 3% BSA. Cells were stained with PTPRF antibody, HA antibody or Paxillin antibody at 4^{0}C overnight. Cells were then washed with washing buffer (PBS in 130mM NaCl, 13mM $Na_{10}HPO_4$, 3.5mM NaH_2PO_4, pH 7.4) and treated with fluorescence 488 or 555 labeled 2' antibody for 1 hr at RT. Nuclei were counterstained with DAPI (Molecular probes). Confocal images were captured using confocal microscope with a Nikon Plan-Apochromat and analyzed using the NIK software.

7. RT - PCR

Total RNA was extracted from cells using TRIzol (Invitrogen), according to the manufacturer's protocol. Total RNA (500ng) was reversely transcribed using amfiRivert Platinum cDNA Synthesis master mix (GenDEPOT). Primers used for PCR were indicated in the below. cDNA was subject to reverse-transcription polymerase chain reaction with Dream taq green PCR master mix (Thermo scientific).

TM4SF5 forward	CTGCCTCGTCTGCATTGTGG
TM4SF5 reverse	CAGAAGACACCACTGGTCGCG
PTPRF forward	CCAGCTTCCTGGATGGTT

PTPRF reverse GGTGGAATTGTGCTCCCA

8. Cell adhesion assay

Prior to assay, cells were transfected with DNA or siRNA incubated in 37℃ for 24 h and collected in the 1% BSA-RPMI. Cells were continuously agitated for 2 h in order to nullify their adhesion signals. Plate was coated with ECM by incubating it with 10 μg/ml fibronectin in PBS solution at 37℃ for overnight. Cells were then seeded at $1x10^5$ cells per plate within 1% BSA-RPMI for 90 min or 180 min. After then, plate washed by PBS and cells analyzed by cell counting or lysed for immunoblot.

RESULT

1. A new binding partner of TM4SF5 in hepatocarcinoma (HCC).

SNU 761 hepatocarcinoma (HCC) cell line with TM4SF5 overexpressed, break down with Brij 58 buffer and pull down with TM4SF5 and A/G beads. Beads were washed by Brij58 buffer to remove non-binding proteins and analyzed by Mass spectrometry to find TM4SF5's binding partners in HCC. I could identify a lot of potential candidates of TM4SF5 binding partner but I focused on protein tyrosine phosphatase receptor type-F (PTPRF) (Table 1.). To confirm the interaction between TM4SF5 and PTPRF that revealed by mass spectrometry, I used immunoprecipitation. SNU 449 parental cells transfected with empty vector and TM4SF5-HA. After cell lysed, whole cell lysates were incubated with HA antibody and pull down with A/G beads. After washing with lysis buffer to eliminate other non-binding proteins, western blots were performed against PTPRF and anti-HA. Data shown that TM4SF5 interact with PTPRF in vitro clearly (Fig. 1A). In addition, to confirm this interaction is an artificial or not, immunofluorescence assay performed and the interaction between TM4SF5 and PTPRF is identified in the cellular system (Fig. 1B).

number	Identified Proteins	Accession Number	Molecular Weight	Quantitative value	
				sample	control
1	Transferrin receptor protein 1	IPI00022462	85 kDa	22	0
2	Integrin beta-1	IPI00217563	88 kDa	23	0
3	Integrin alpha-3	IPI00215995	117 kDa	16	0
4	4F2 cell-surface antigen heavy chain	IPI00027493	58 kDa	13	0
5	CD44 antigen	IPI00827795	74 kDa	14	0
6	cDNA FLJ55574, highly similar to Calnexin	IPI00020984	72 kDa	13	0
7	Integrin alpha-2	IPI00013744	129 kDa	11	0
8	Ephrin type-B receptor 2	IPI00021275	117 kDa	9	0
9	Integrin alpha-V	IPI00027505	116 kDa	10	0
10	Receptor-type tyrosine-protein phosphatase F	IPI00107831	213 kDa	9	0
11	Solute carrier family 2, facilitated glucose transporter member 1	IPI00220194	54 kDa	8	0
12	Integrin alpha-5	IPI00306604	115 kDa	9	0
13	Cadherin-2	IPI00290085	100 kDa	8	0
14	Catenin alpha-1	IPI00215948	100 kDa	8	0
15	Plasma membrane calcium-transporting ATPase 4	IPI00012490	138 kDa	9	0
16	UDP-glucuronosyltransferase 1-6	IPI00451965	61 kDa	6	0
17	Integrin alpha-6	IPI00010697	127 kDa	6	0
18	annexin A4	IPI00793199	36 kDa	6	0
19	Neutral amino acid transporter B(0)	IPI00019472	57 kDa	6	0
20	Neuropilin-1	IPI00299594	103 kDa	6	0

Table 1. Mass spectrometry data of TM4SF5 binding partners in liver cancer cell.

Potential candidates of TM4SF5 binding partners in SNU 761. Quantitative value shows revealed as a TM4SF5 binding partner.

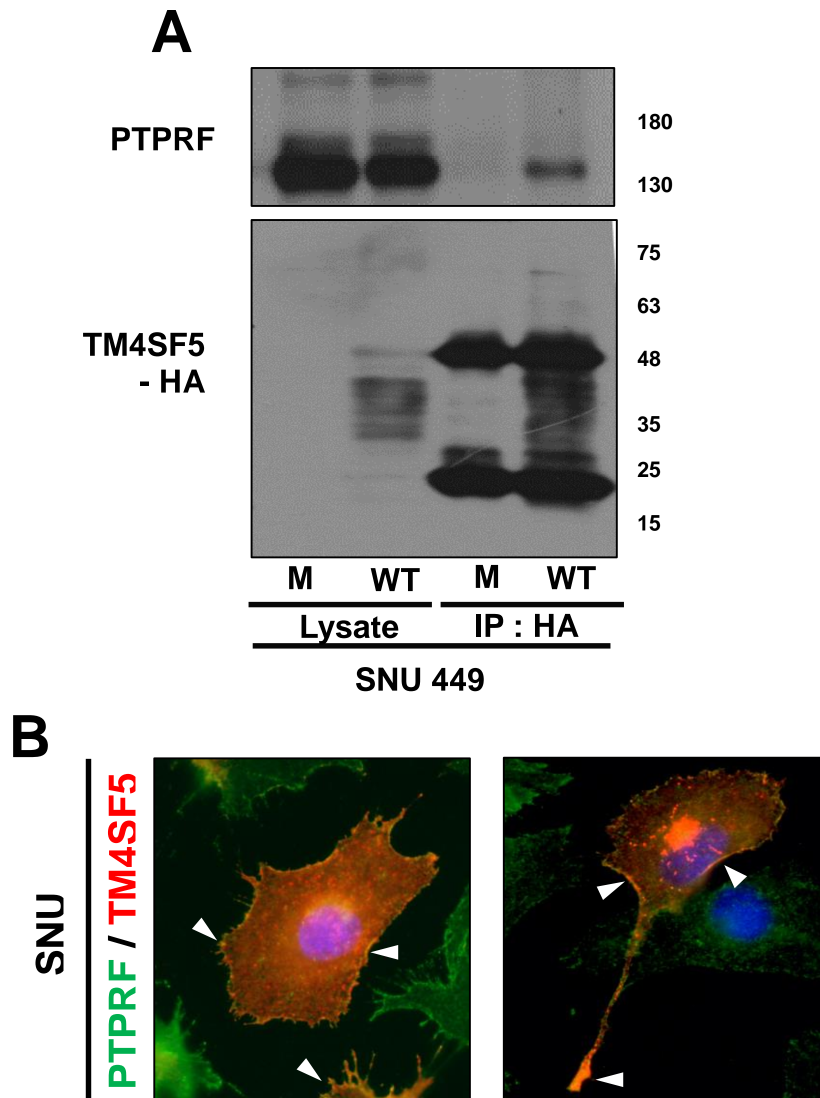


Figure 1. TM4SF5 interacts with PTPRF on cell surface.

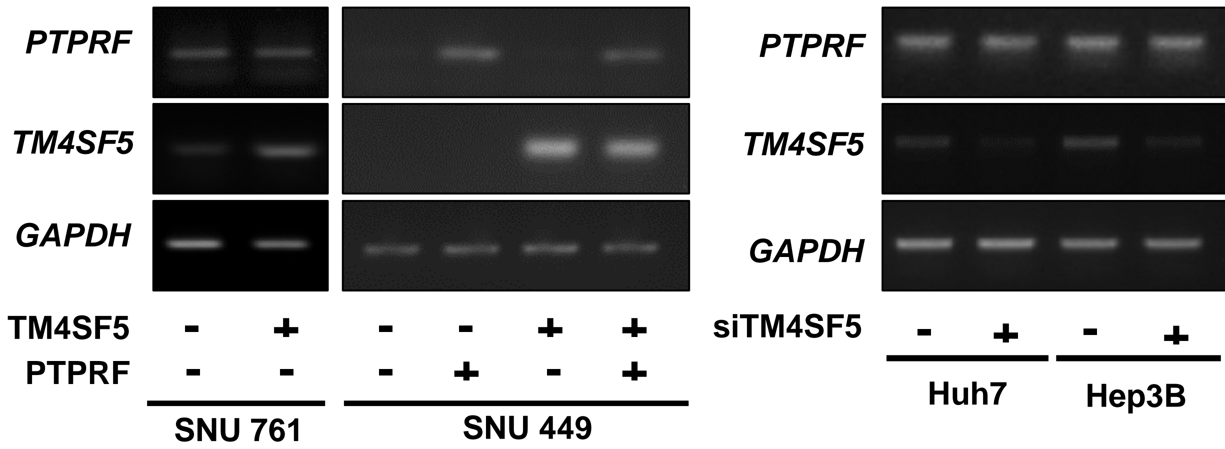
(A) SNU449 cells transiently transfected with TM4SF5-HA were harvested and immunoprecipitated with HA antibody, prior to immunobloting for PTPRF. From whole cell lysates, PTPRF and HA were immunoblotted. (B) SNU 761 cells transiently transfected with TM4SF5-HA. Immunofluorescence color shows PTPRF (green) and HA (red; TM4SF5) and co-localized spot shows as yellow color.

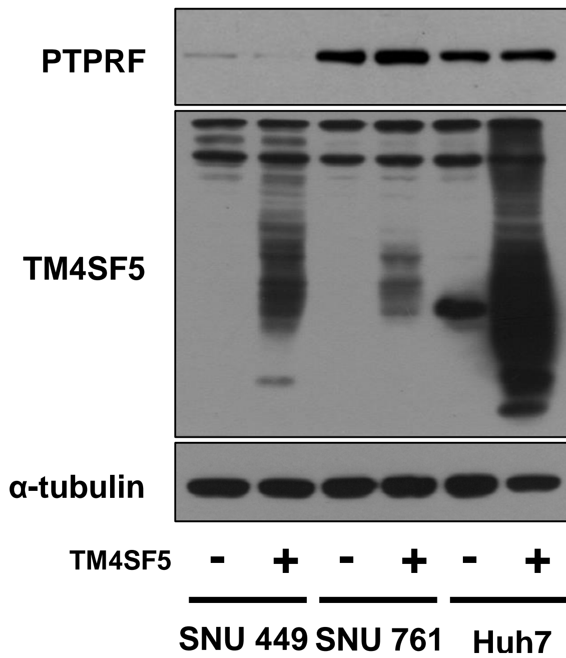
2. TM4SF5 inhibits the activity of PTPRF for focal adhesion molecules.

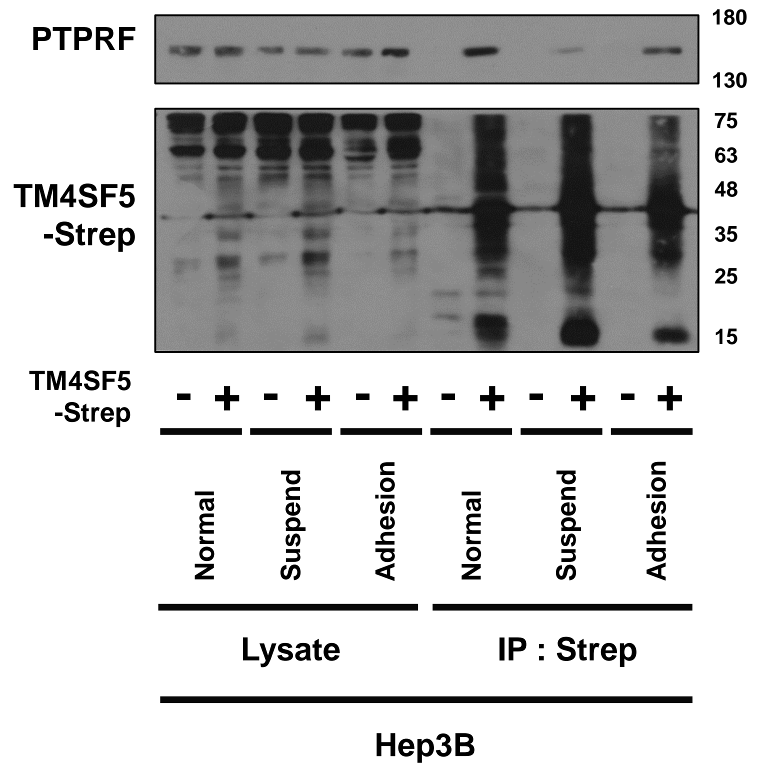
To understand the role of interaction between TM4SF5 and PTPRF in HCC, I performed RT-PCR and western blot to identify alteration of mRNA and protein level. SNU 761 and SNU 449 transfected with empty vector, TM4SF5-HA, PTPRF or TM4SF5 and PTPRF co-transfected. Huh7 and Hep3B transfected with siControl or siTM4SF5. Cells were extracted by TRIzol for RT-PCR or lysed by RIPA buffer for western blot. But there were any significant alteration of mRNA and protein level (Fig. 2A and 2B). So, I expect that the meaning of cross-talk between TM4SF5 and PTPRF is related with protein activity.

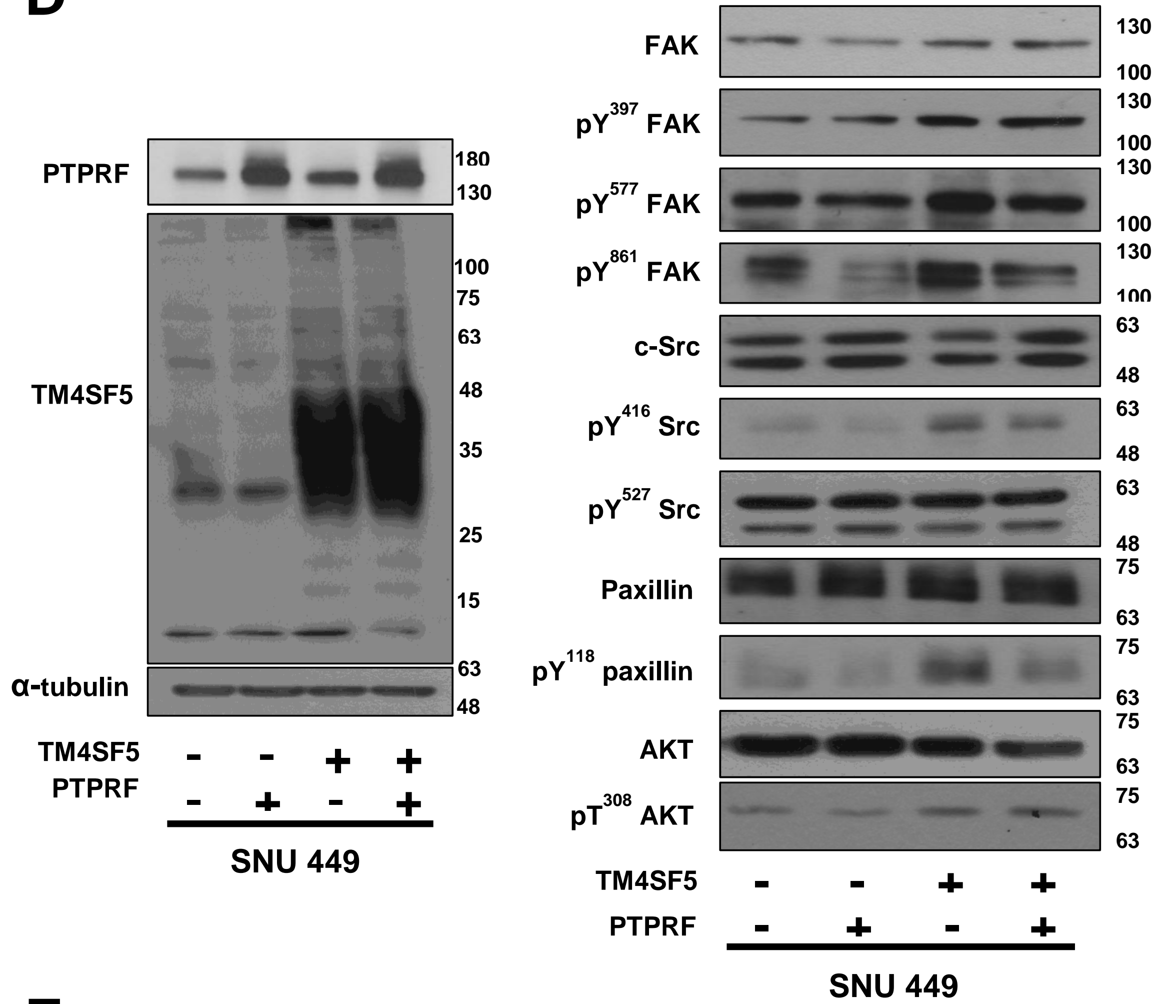
In previous studies, TM4SF5 promote focal adhesion molecules activation and led cells to tumorigenic[18]. Regarding this points, to identify the relation in cross-talk between TM4SF5 and PTPRF with FAs, I designed the cellular adhesion assay to mimic the situation that cells form focal adhesion complexes. When cells were suspended, the interaction between TM4SF5 and PTPRF decreased. Otherwise, after the cells attached to ECM fibronectin coated plate, the interaction between TM4SF5 and PTPRF was recovered. According to this results, the interaction between TM4SF5 and PTPRF is related in focal adhesion complex formation (Fig. 2C). So, first I identified the activity PTPRF against FA molecules. When PTPRF is overexpressed, the tyrosine phosphorylations of FAK (pY861), c-Src (pY416), and Paxillin (pY118) reduced. But when both TM4SF5 and PTPRF are overexpressed, tyrosine phosphorylation of FAK (pY861), c-Src (pY416), Paxillin (pY118) recovered, which suggest that overexpression of PTPRF regulates the focal adhesion molecules activation but TM4SF5

inhibits the PTPRF dependent tyrosine-dephosphorylate activity for TM4SF5 dependent FAs (Fig. 2D). And immunofluorescence data supports the relation between PTPRF and focal adhesion molecules by co-localization of PTPRF and Paxillin (Fig. 2E)[4].


A
PTPRF
TM4SF5
GAPDH
TM4SF5 - + - - + +
PTPRF - - - + - +
SNU 761
SNU 449
PTPRF
TM4SF5
GAPDH
siTM4SF5 - + - +
Huh7
Hep3B


B
PTPRF
TM4SF5
α-tubulin
TM4SF5 - + - + - +
SNU 449
SNU 761
Huh7


C
PTPRF
TM4SF5
-Strep
180
130
75
63
48
35
25
15
TM4SF5
-Strep
- + - + - + - + - + - +
Normal
Suspend
Adhesion
Normal
Suspend
Adhesion
Lysate
IP : Strep
Hep3B


D
PTPRF
TM4SF5
α-tubulin
180
130
100
75
63
48
35
25
15
63
48
TM4SF5 - - + +
PTPRF - + - +
SNU 449
FAK
pY397 FAK
pY577 FAK
pY861 FAK
c-Src
pY416 Src
pY527 Src
Paxillin
pY118 paxillin
AKT
pT308 AKT
TM4SF5 - - + +
PTPRF - + - +
SNU 449

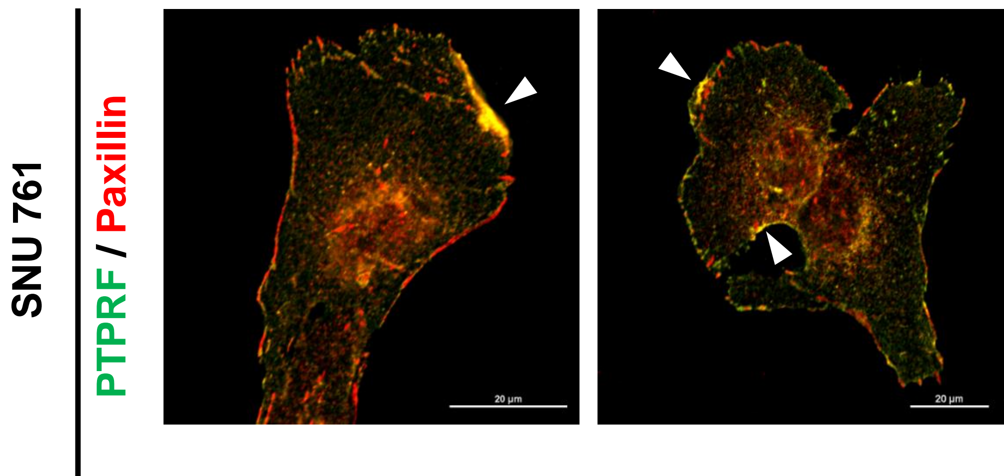

E
SNU 761
PTPRF / Paxillin
20 μm
20 μm

Figure 2. TM4SF5 inhibits the tyrosine-dephosphorylation activity of PTPRF..

(A) and (B) mRNA and protein levels of TM4SF5, PTPRF and GAPDH in different liver cells which are TM4SF5 and PTPRF overexpressed or depleted. (C) SNU449 cells transiently transfected with TM4SF5-Strep used for cell adhesion assay. Normal culture, suspended, adhesion condition treated cells were harvested and immunoprecipitated with HA antibody, prior to immunobloting for PTPRF. From whole cell lysates, PTPRF and Strep were immunoblotted. (D) Expression and phosphorylation levels of focal adhesion molecules in PTPRF, TM4SF5 overexpressed HCC. (E) Immunofluorescence with PTPRF (green) and Paxillin (red).

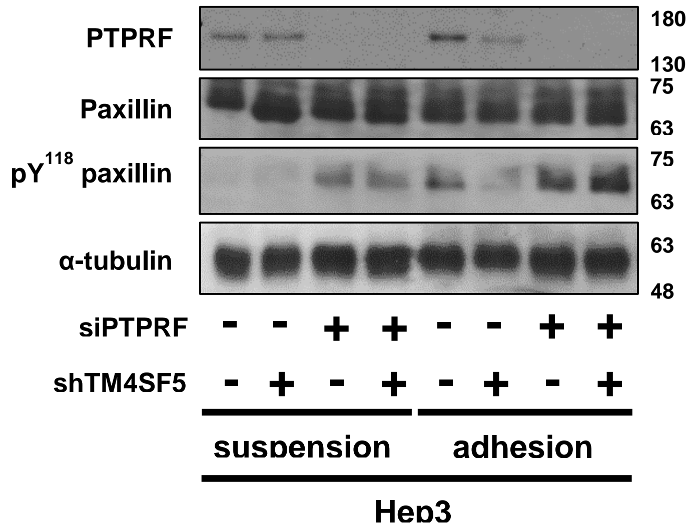
3. TM4SF5 and PTPRF regulate the adhesion and migration of HCC.

I explored the alteration of cellular behaviors derived by the cross-talk between TM4SF5 and PTPRF. Previous data showed TM4SF5 and PTPRF regulate the FA signals, I analyze cellular adhesion and cell migration properties influenced by TM4SF5 and PTPRF. For adhesion assay, Hep3B cells were infected by lenti-virus with shTM4SF5 to down regulate the TM4SF5 and PTPRF down regulated by siPTPRF. Prepared cells were agitated to nullify focal adhesion signals and seeded on the plate after agitation. The adhesion assay result shows that PTPRF depleted cell retained phosphorylation level of paxillin during agitation and after attachment, their focal adhesion signals activated faster than control. Otherwise, TM4SF5 down regulated cells activation of paxillin delayed compare with control (Fig. 3A). Through cell attachment, less TM4SF5 depleted cells attached to ECM and more PTPRF depleted cells attached to ECM than control Hep3B cells. Interestingly both TM4SF5 and PTPRF-lacking cells showed reduced number of attached cells than alone PTPRF-lacking cells (Fig. 3B).

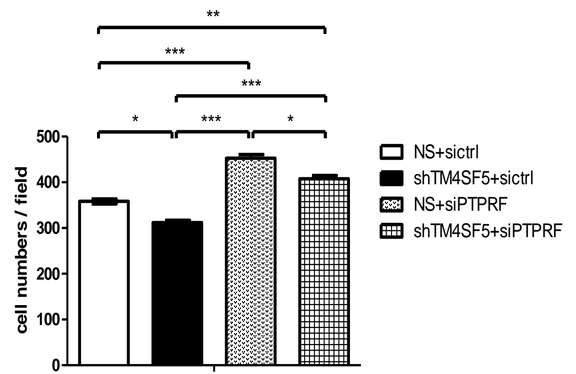
Migration assay were performed as like schematic figure (Fig. 3C), Hep3B cells were infected by lenti-virus with shTM4SF5 to suppress the TM4SF5 and PTPRF suppressed by siPTPRF. Prepared cells were agitated to nullify FA signals and inserted in upper chamber of transwell and led to migrate for 12 h. Comparing with control cells, TM4SF5 depleted cells showed that reduced migration ability and PTPRF depleted cells showed that increased migration ability. Whereas, TM4SF5 and PTPRF both depleted cells showed no significant difference between control (Fig. 3D and E). These observation suggest that molecules related

with cell adhesion and migration are activated by PTPRF down-regulation but this signaling activation requires TM4SF5 in HCC[19,20].

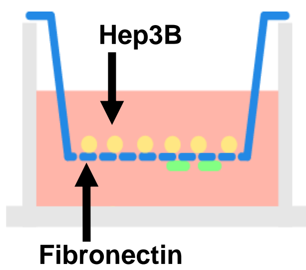
A



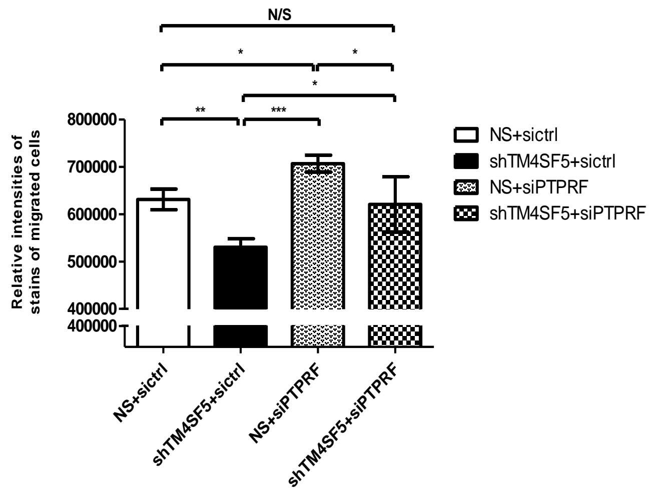
B



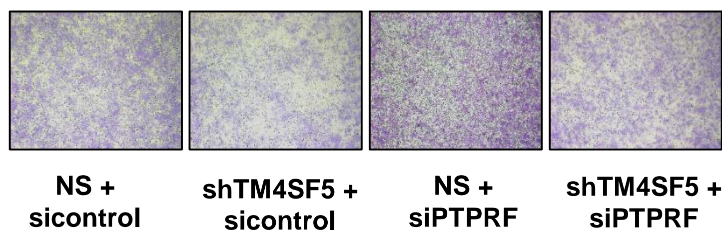
C



D



E



NS + sicontrol | shTM4SF5 + sicontrol | NS + siPTPRF | shTM4SF5 + siPTPRF

Figure 3. TM4SF5 recovers cellular adhesion and migration that was inhibited by PTPRF.

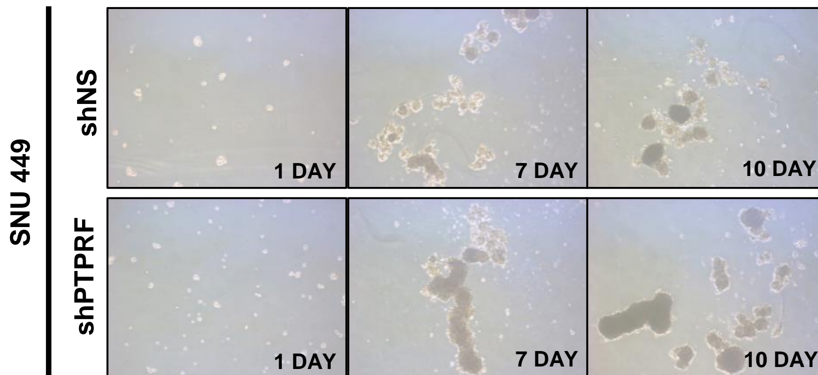
(A) Suspension cells were incubated with 1% BSA-RPMI for 2 h. Adhesion cells were incubated with 1% BSA-RPMI for 2 h before seeding on the fibronectin coated plate. After 2 h for attachment, suspension cells and adhesion cells were collected for western blot. (B) Adhesion cells washed by PBS and randomly imaged. Through the images, cells were counted and analyzed. (D) Cells were incubated with 1% BSA-RPMI for 2 h, before cell loading in insert (10^5 cells/condition). Migrated cells through the transwell chambers for 12 h in each condition following loading of cells were stained with 5% crystal violet and analyzed by image J software. (E) Transwell migrated cells randomly imaged and representative images were shown. *, ** or *** depicts statically significant difference at $p < 0.05$, 0.005, 0.001, respectively, and 'ns' depicts non-significant difference at $p \geq 0.05$.

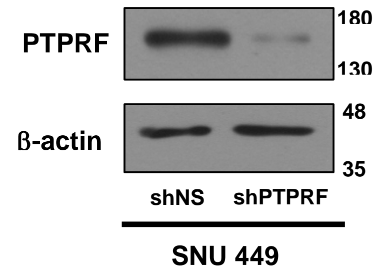
4. Sphere formation promoted by PTPRF depletion requires TM4SF5.

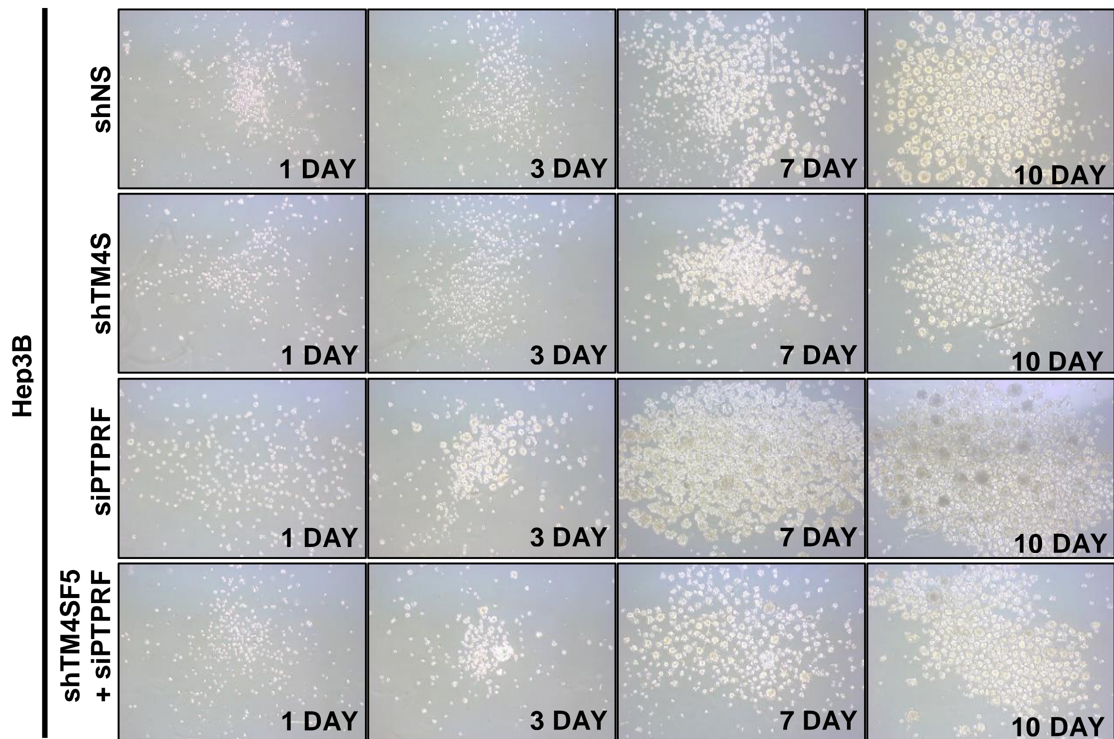
Next, I investigated whether PTPRF down-regulation is associated with sphere forming ability in 3D aqueous condition. Hep3B cells transfected with lenti-virus with shRNA and selected by puromycin. Targeted protein (TM4SF5 and PTPRF) stably down regulated in Hep3B. Control cells, PTPRF down regulated cells, TM4SF5 down regulated cell and TM4SF5 and PTPRF both down regulated cells plated in low attachment 6-well culture plate, leading sphere formation after 10 days. The results showed that PTPRF down regulated cells formed more and bigger spheres (Fig. 4A). Western blot data shows the level of PTPRF (Fig. 4B). Next, I investigate that how the interaction between TM4SF5 and PTPRF influence sphere forming. TM4SF5 down regulated cells formed less and smaller spheres compare with control cells. Otherwise, TM4SF5 and PTPRF both down regulated cells formed less and smaller spheres than PTPRF down regulated cells (Fig. 4C). These spheres were collected and extracted proteins to confirm the signals related with cell proliferation. PTPRF down regulated cells phosphor-ERK is increased but when TM4SF5 is down regulated together, the level of phosphor-ERK is recovered (Fig. 4D).

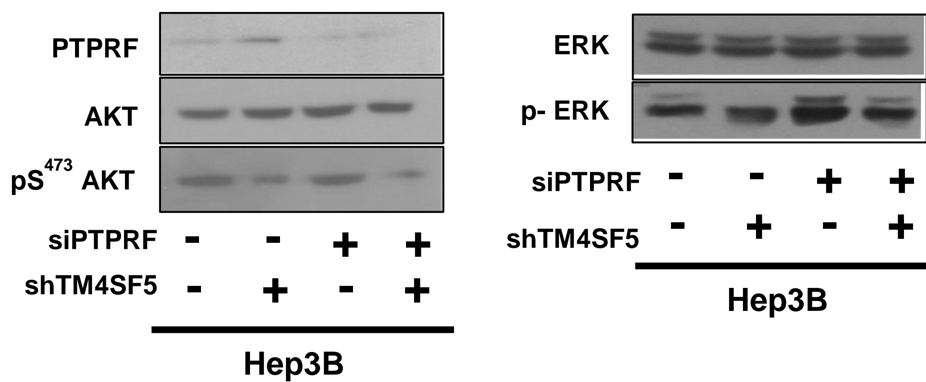
In previous study, It was reported that expression of TM4SF5 is regulated by CD133 and enhance sphere growth and survival in 3D aqueous condition. So, I perform sphere formation assay with CD133 down regulated cells and CD133 and PTPRF both down regulated Huh7 cells. The results showed that CD133 down regulated cells formed less and smaller spheres compare with control cells. Otherwise, CD133 and PTPRF both down regulated cells

formed less and smaller spheres than only PTPRF down regulated cells (Fig. 4E). By these results, I suggesting that TM4SF5 is required for the activation of sphere forming and growth signals derived by PTPRF down-rgulation[21].


A
SNU 449
shNS
shPTPRF
1 DAY
7 DAY
10 DAY


B
PTPRF
β-actin
180
130
48
35
shNS
shPTPRF
SNU 449


C
Hep3B
shNS
shTM4S
siPTPRF
shTM4SF5 + siPTPRF
1 DAY
3 DAY
7 DAY
10 DAY


D
PTPRF
AKT
pS473 AKT
siPTPRF
shTM4SF5
Hep3B
ERK
p- ERK

E

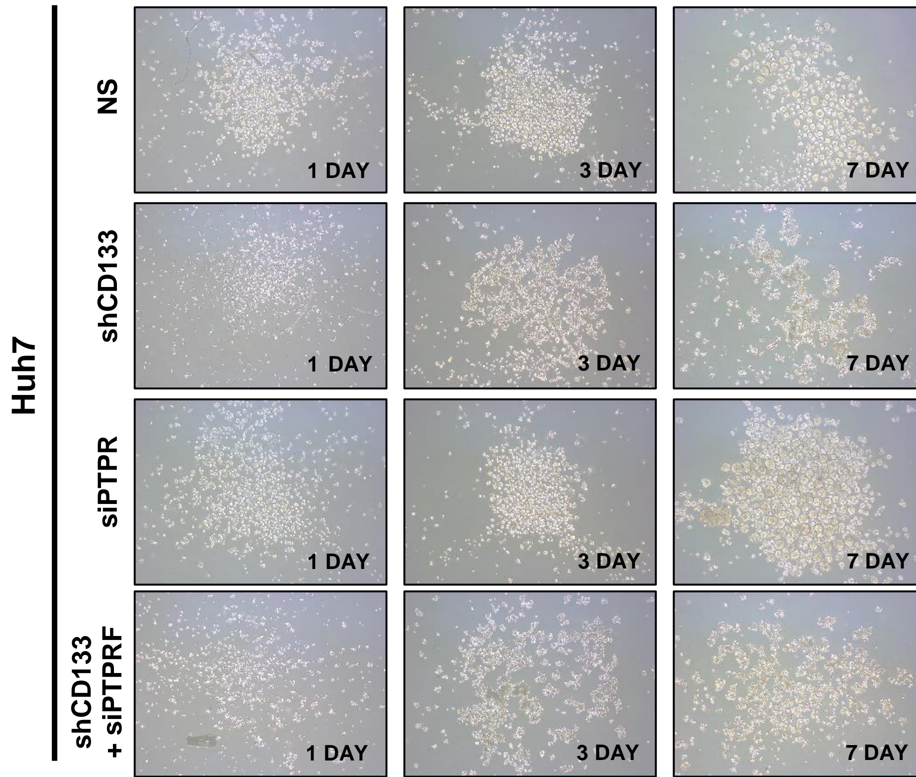


Figure 4. Sphere formation was positively and negatively regulated by TM4SF5 and PTPRF, respectively. Sphere growth. Cells (2 X 10^3 cells in each well) were cultured for 7 or 10 days with DMEM/F12 with supplements and image of spheres were obtained. Representative images are shown. (A) PTPRF down regulated cells form more spheres compared to control cells. (B) Expression level of PTPRF in Spheres in (A). (C) TM4SF5 down regulated cells form less spheres compared to control cells and PTPRF down regulated cells form more spheres compared to control cells. Otherwise, both down regulated cells form less spheres than only PTPRF down regulated cells. (D) Spheres in (C) are collected for western blot to identify growth signals and expression level of PTPRF. (E) CD133 down regulated cells form less spheres compared to control cells and PTPRF down regulated cells form more spheres compared to control cells. Otherwise, both down regulated cells form less spheres than only PTPRF down regulated cells.

5. Summary of the present study.

I found PTPRF as a new binding partners of TM4SF5 in HCC by mass spectrometry. The function of PTPRF in cancer was unclear but results showed that the overexpression of PTPRF induced inactivation of focal adhesion signals. Whereas, down regulation of PTPRF induced alteration of cell behaviors as increasing of cell adhesion, migration and sphere formation. So I suggest that the role of PTPRF is regulation of focal adhesion molecules via FAK, c-Src and Paxillin. But these effects in HCC were controlled by TM4SF5. In TM4SF5 overexpressed cells, focal adhesion signals were recovered even if PTPRF overexpressed. And without TM4SF5, there was any alteration of cellular behaviors and cell proliferation signals that induced by lacking of PTPRF. Therefore, results suggests that the role of PTPRF is tumor suppressor that regulates the focal adhesion molecules activation and another role of TM4SF5 is disrupting PTPRF.

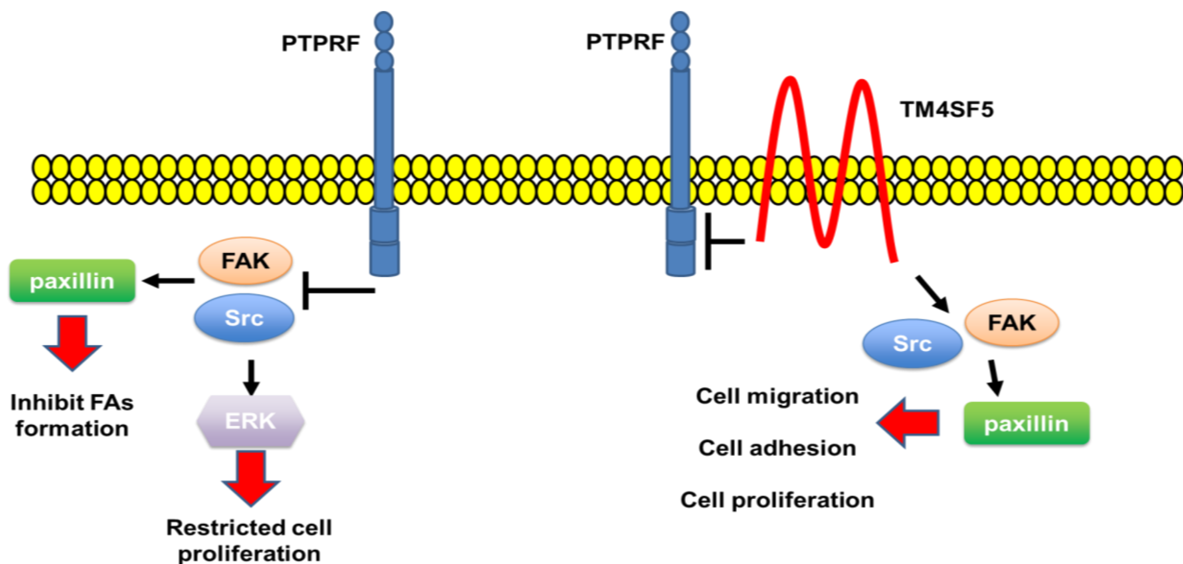


Figure 5. The scheme to summarize the observation of the present study.

Discussion

In this study, I could find the interaction between TM4SF5 and PTPRF in HCC, but the function of PTPRF in HCC was unclear. I could find the role of PTPRF in HCC as a regulator of the FAs, such as FAK, c-Src, and Paxillin. In HCC that TM4SF5 is overexpressed, however, TM4SF5 can promote tyrosine phosphorylation of FA molecules that would be negatively targeted by PTPRF. Thus, the coordinated cross-talks between TM4SF5 and PTPRF can play roles in successful cancer metastasis, which further can be a promising target to deals with TM4SF5-dependent metastasis. Further, regulatory function of PTPRF against cell growth observed by sphere formation assay in 3D aqueous condition[22]. PTPRF-lacking cells could form more and bigger sphere but cells lacking both PTPRF and TM4SF5 formed less sphere than PTPRF-alone lacking cells.

According to previous studies, TM4SF5 interacts with FAs, and the adhesion-dependent binding of TM4SF5 causes a structural alteration of FAK and c-Src and their activation at the cell's leading edge, to promote migration/invasion and in vivo metastasis[10,16,23]. Also, while TM4SF5 modulates c-Src activity during TM4SF5 mediated cell migration and invasion, TM4SF5 influence c-Src / EGFR signaling pathway. Whereas it was reported that enhanced EGFR signal down regulates the PTPRF and its activity [24,25]. And according to clinical data, TM4SF5 is overexpressed but PTPRF is down regulated in many types of cancer[9]. So I could expect there is a relation between TM4SF5 and PTPRF in HCC and it was demonstrated by mass spectrometry and experiments

The cross-talk between TM4SF5 and PTPRF is important to explain the TM4SF5-dependent alteration of cellular behaviors that enhanced metastasis, cell growth, and angiogenesis. Of primary importance in the prognosis of cancer patients is the sequence of events leading to the development of tumor cell invasion and metastasis[26]. The course of tumor metastasis entails a series of stages that lead to the formation of secondary tumors in distant organs and is, largely, responsible for the mortality of cancer. Cell adhesion molecules play a significant role in cancer progression and metastasis[27]. Activation of FA molecules at leading edge of cell membrane induces rapid turn-over of focal adhesion structure contribute to cancer cells adhesion, extravasation, and metastasis.

One of the main characteristic of cancer stem cell is the sphere forming capacity with low adhesion environment[28]. Usually, when sphere is become bigger, cell-cell contact also increased and it generates the signals to restrict cell growth. In this events, many kind of PTPs and RTKs are participated[9]. However, during sphere forming in cancer cells, cell-cell contact is increasing either but there are some mechanisms to avoid the cell growth inhibition signals. Our previous study showed that CD133 up-regulate the expression of TM4SF5 in HCC and promotes the TM4SF5-dependent signals and sphere forming in a non-adhesion condition[21]. In the present study, I could find another TM4SF5-dependent mechanism to down regulate the growth inhibitory signals during sphere forming in a non-adhesion 3D aqueous condition. Altogether, the cross-talk between TM4SF5and PTPRF is that mechanism. Here data showed that sphere forming assay with PTPRF depleted cells and/or TM4SF5 depleted cells, even if

cell-cell contact increased, PTPRF depleted cells form more and bigger spheres as like as TM4SF5 overexpressed[29]. This result proof that PTPRF is a tumor suppressor. PTPRF depleted cells cannot generate enough signals to inhibit the cell growth, but disrupted cell growth signals leads cells to form more and bigger spheres. Otherwise, both TM4SF5 and PTPRF depleted cells formed less and smaller spheres than only PTPRF depleted cells. This result suggesting that activation of molecules that derived by PTPRF down-regulation require TM4SF5. So, if there are TM4SF5 is overexpressed in the cell, TM4SF5 blocks the regulatory function of PTPRF that inhibits the cell growth and retains growing. Finally, TM4SF5 promotes the sphere formation.

Altogether, data showed TM4SF5 regulates the activity of PTPRF in HCC but exact mechanism is unclear. There are several mechanisms to regulate protein activity. Transcriptional regulation, translational regulation, protein phosphorylation, protein-protein interaction regulatory subunit and ETC. But interestingly, TM4SF5 has any enzymatic activity so TM4SF5 cannot use PTPRF as a substrate for degradation or phosphorylation. And also data showed there are any transcriptional and translational alteration by regulation of TM4SF5 and PTPRF. The presumption is that TM4SF5 trans-localization of PTPRF or blocks PTPRF to meet its substrate or other proteins because TM4SF5 is localized on cell membrane but also lysosomal vesicles.

In conclusion, this study demonstrated the PTPRF as a new binding partner of TM4SF5 which has tumor suppressor role. TM4SF5 has PTPRF inhibitory function in HCC

and promotes cell adhesion, migration and growth via FAK, c-Src, Paxillin. I suggest that drugs or therapeutic treatment to disrupt the role of TM4SF5 for PTPRF in HCC, it leads to activation of PTPRF as a tumor suppressor and inhibits the cell growth and metastasis of cancer cells successfully.

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국문 초록

Transmembrane 4 L6 family member 5 (TM4SF5) 는 tetraspanin superfamily 에 속하는 막 단백질로서, 간암을 비롯해 다양한 암 종에서 높게 발현되며 EMT(epithelial-mesenchymal transition, 상피-중배엽 세포 전이)를 유발함으로써 세포의 형태 변화뿐만 아니라 이동과 침윤, 증식을 촉진하는 세포내 신호전달을 조절하는 인자로 선행 연구를 통해 밝혀졌다. 본 연구에서는 Mass spectrometry를 통해 TM4SF5의 새로운 결합 단백질인 Protein Tyrosine Phosphatase Receptor type-F (PTPRF)를 찾아내고 TM4SF5 와의 세포내 상호작용이 세포의 이동과 침윤, 증식에 어떠한 영향을 미치는지 조사하였다. PTPRF 는 세포막에 존재하는 타이로신 탈인산화 효소로 세포의 증식, 분화, 세포주기 조절에 영향을 미치는 것으로 알려져 있으며 암세포에서 세포 사멸 활성화, 세포 성장 저해, 암세포 이동 및 전이 능력 억제 단백질로 보고된 바 있다.

TM4SF5 를 발현하지 않거나 발현하는 SNU449 및 SNU761 간암 세포주를 이용하여 면역 침강 실험과 면역 형광 실험을 통해 TM4SF5 와 PTPRF 의 in vitro/in vivo 에서의 상호작용을 확인하였다. TM4SF5 와 PTPRF 의 과발현이나 결여는 서로의 RNA 및 단백질 발현에 영향을 주지 않았고 이를 통해 TM4SF5 가 PTPRF 의 활성에 영향을 줄 것이라 생각하였다. 앞선 연구를 통해 TM4SF5 가 국부 부착 단백질들의 활성화를 조절하는 것을 확인하였기에 PTPRF 가 이러한 단백질들에 영향을 미치는지 확인하였고 세포내에서 PTPRF 와 Paxillin 이 함께 위치하고 PTPRF 의 과발현이 FAK, c-Src, Paxillin 의 탈인산화를 유도하지만 TM4SF5 가 PTPRF 와 함께 과발현 된 경우 PTPRF 의 국부 부착 단백질의

탈인산화 활성이 억제되는 것을 확인하였다.

세포의 여러 상황에서 면역 침강 반응 실험을 통해 TM4SF5 와 PTPRF 의 상호작용이 세포가 부유된 상태에서는 감소하며 세포가 ECM (Extra Cellular Matrix) 에 부착되었을 때 다시 증가하는 결과를 통해 세포의 부유 상태에서 세포의 국부 부착 구조의 형성이 억제되고 관련 단백질들의 타이로신 인산화가 저해되는 현상이 두 단백질과 관련 있을 것이라는 가설을 세우게 되었다. PTPRF 가 발현되는 세포는 부유 상태일 때 Paxillin 의 타이로신 인산화가 저해되었지만 PTPRF 를 결여시킨 세포는 부유 상태에서 Paxillin 의 타이로신 인산화가 유지되었다. 이를 통해 세포의 부유 상태에서 TM4SF5 와 PTPRF 의 상호작용 감소는 PTPRF 를 활성화하고 이는 부착 단백질의 활성을 억제하며 세포의 부착상태에서 TM4SF5 와 PTPRF 의 상호작용 증가는 PTPRF 의 활성을 억제하여 국부 부착 단백질의 활성화한다고 볼 수 있다. 이러한 TM4SF5와 PTPRF의 상호작용의 변화가 세포의 부착능력과 전이능력에 영향을 미치는지 세포 부착 분석과 트랜스웰 이동 분석을 하였다. TM4SF5가 결여된 세포는 부착능력이 감소하고 PTPRF 가 결여된 세포는 부착능력이 증가하는 반면 TM4SF5와 PTPRF가 함께 결여된 세포는 PTPRF 만 결여된 세포에 비해 부착능력이 감소하였는데 이는 PTPRF 의 결여로 활성화되는 단백질의 활성화에 TM4SF5가 필요함을 보여준다. 또한 TM4SF5가 결여된 경우 세포의 이동 능력이 감소되었고 PTPRF 가 함께 결여 된 경우 세포의 이동능력이 회복되었다. 이를 통해 TM4SF5 에 의한 세포 이동 능력 조절은 TM4SF5 에 의한 PTPRF 억제임을 확인하였다. 나아가 spheroid 형성 분석기법을 통해 TM4SF5 가 결여된 세포는 spheroid 의 형성이 감소하며 PTPRF 가 결여된 세포는 spheroid 형성이 증가하는 반면

TM4SF5, PTPRF 가 함께 결여된 세포는 대조군과 비슷한 정도의 spheroid 형성 능력을 보여주었다. 이는 PTPRF 의 결여에 의한 spheroid 형성 및 증식 신호전달에 있어 TM4SF5가 필요하다는 것을 보여준다.

이와 같은 결과들을 종합할 때, TM4SF5 는 세포의 부착상태에서 PTPRF 를 억제하여 PTPRF 에 의한 FAK, c-Src, Paxillin 과 같은 국부 부착 단백질들의 탈인산화를 저해하고 국부 부착 구조의 형성을 촉진시킨다. 또한 이는 세포의 이동 및 전이를 촉진하며 결과적으로 간암세포의 종양형성내지는 circulatory 종양세포로서의 특성을 더 가지도록 한다는 것을 보여주고 있다.

Keywords : 3D cell culture, protein interaction, cellular metastasis, extracellular matrix, TM4SF5, PTPRF, focal adhesion, tumor microenvironment

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