약학석사학위논문

Cell-penetrating peptides containing TM4SF5 c-terminus suppress c-Src activation-dependent tumorigenic potential.

TM4SF5의 C말단을 포함하는 세포막 투과성 펩타이드의 c-Src 활성화 의존적 암화 기능 억제 연구

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ABSTRACT

Cell-penetrating peptides containing TM4SF5 c-terminus suppress c-Src activation-dependent tumorigenic potential.

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Transmembrane 4 L6 family member 5 (TM4SF5), a member of tetraspanin L6 superfamily, is a glycoprotein which has four transmembrane domains. TM4SF5 is highly expressed in various cancers including hepatocarcinoma and TM4SF5 has been reported to be closely involved in tumorigenesis, EMT (epithelial-mesenchymal transition). The c-Src non-receptor tyrosine kinase is overexpressed and activated in a large number of human malignancies and has linked to the development of cancer and progression to distant metastases. We previously reported that c-terminal tail of TM4SF5 bound c-Src and this binding induced c-Src-mediated invasion. However, it is remained unknown how TM4SF5 binds and regulates c-Src in details at the molecular levels.

Here, the details for c-Src to be regulated by a direct interaction with TM4SF5 were explored to understand how it occurs using SNU761 and SNU449 liver cancer cells without or with TM4SF5 expression. We first confirmed that the cytoplasmic part of TM4SF5 was associated with c-Src family kinases. To identify the region of c-Src to bind TM4SF5, coimmunoprecipitations from extracts of TM4SF5-expressing SNU761 or SNU449 cells transfected with diverse deletion constructs of c-Src were performed. The kinase domain of c-Src (250 ~ 536 amino acids, SH1 domain) bound to TM4SF5. Further interestingly, $SH1_{Y419F}$ bound greater than SH1 wild-type, and $SH1_{Y530F}$ did not bind. It is thus likely that

inactive c-Src form bound more efficiently to TM4SF5. In addition, $SH1_{K295M/Y530F}$ presumably being adapted in an opened form due to no possible phosphorylation at Y530, did not bind, whereas $SH1_{K295M}$ bound well to TM4SF5. Moreover, when various SH321 (91 ~ 536 amino acids) constructs with different point mutations were transfected together with TM4SF5 to SNU449 cells, we confirmed that $SH321_{Y419F}$, an inactive form, and $SH321_{Q531E/P532E/G533I}$, a closed form, constructs bound to TMSF5 more effectively than SH321 wild-type. By overexpression or silencing PTP1B (Protein Tyrosine Phosphatase-1B), regulated the preference of TM4SF5 to binding closed inactive c-Src confirmed again. From these data, we could conclude that TM4SF5 had preference to bind closed inactive c-Src, depending on PTP1B-mediated Tyr 530 phosphorylated in c-Src.

Further, treatment of cell-penetrating peptides of the cytosolic TM4SF5 tail into TM4SF5-expressing SNU449 and SNU761 cells resulted in inhibition of FAK/c-Src activations in cells within normal serum-containing media, but not cells adhered on extracellular matrix proteins without serum. When treating peptides to endogeneous TM4SF5 expressing cells or TM4SF5-null cells, cell-penetrating TAT peptides containing TM4SF5 c-terminus showed specific targeting against TM4SF5. The peptides reduced TM4SF5-mediated cell migration and invasion by interrupting interaction between TM4SF5 and c-Src SH1 domain.

Cell-penetrating peptides containing TM4SF5 c-terminus were treated to mice that were subcutaneous injected with TM4SF5-expressing SNU449 hepatic cancer cells resulted in inhibition of tumorigenesis. From *in vivo* lung metastasis model by tail vein injection of TM4SF5-expressing SNU449T7 cells, the peptides reduced lung metastasis caused by TM4SF5-positive cells. Altogether, this study suggests that the TM4SF5 c-terminal peptide can disrupt recruit of inactively closed c-Src to TM4SF5, leading to inhibition of TM4SF5-mediated c-Src activation and tumorigenic effects.

Keywords: TM4SF5, Protein-protein interaction, c-Src, SH1 (kinase) domain, cell-penetrating peptide, tumorigenesis, metastasis

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INTRODUCTION

The c-Src non-receptor tyrosine kinase is overexpressed and activated in a large number of human malignancies and has linked to the development of cancer and progression to distant metastases [1]. Members of the c-Src family play key roles in the regulation of cellular growth, division, adhesion, and motility through its activation and intracellular interaction. Phosphorylation of c-Src at tyrosine 530 induces a conformational change in the molecule that reduces its kinase activity [2]. Dephosphorylation of Tyr-530 opens the conformation of c-Src, thereby activating the tyrosine kinase activity. Interaction of proteins with the SH3 domain of c-Src also can lead to its activation [3]. Elevated growth factor signaling in human cancer leads to Src hyperactivation, promoting tumor progression through increased growth and invasive potential. Increased Src activity accomplishes this by promoting tumor cell migration, invadopodia formation, and matrix metalloproteinase (MMP) activity [4]. These attributes have resulted in the development of kinase-targeted Src inhibitory compounds that are currently being evaluated for efficacy as anti-tumor and -metastatic therapeutics [5].

Many biologically active compounds, including various large molecules, need to be delivered intracellularly to exert their therapeutic action inside cytoplasm or onto nucleus or other specific organelles, such as mitochondria. However, the lipophilic nature of the biological membranes restricts the direct intracellular delivery of such compounds. Although many compounds show a promising potential in vitro, thus, they cannot be used in vivo due to bioavailability problems. A novel approach to deliver such molecules involves tethering them to peptides that can translocate through the cellular membranes, thereby enhancing their delivery inside the cell [6]. These peptides have been used for intracellular delivery of various cargoes with molecular weights several times greater than their own [7]. This process of protein transduction was discovered first by Green and Frankel independently, who found that 86-mer trans-activating transcriptional activator (TAT) from HIV-1 was efficiently taken up by various cells, when added to the surrounding media [8], [9]. More precisely, the ability to translocate across the plasma membranes is

confined to short sequences of less than 20 amino acids, which are highly rich in basic residues. Such sequences are called “Protein Transduction Domains (PTDs)” or “Cell-Penetrating Peptides (CPPs)”. Cellular delivery using CPPs has several advantages over conventional techniques because it is efficient for a range of cell types, can be applied to cells en masse, and has a potential therapeutic application [10].

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 [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 [12]. Intracellular loop of TM4SF5 binds the F1 lobe of the FAK FERM domain during cell adhesion and results in FAK activation for location at the leading edges for a directional migration [13]. The C-terminus of TM4SF5 bound both inactive c-Src that might be sequestered to certain cellular areas and active c-Src that might form invasive protrusions. Wild-type (WT) TM4SF5 expression enhanced migration and invasive protrusion formation in a c-Src-dependent manner, compared with TM4SF5-null control hepatoma cell lines. However, tailless TM4SF5ΔC cells were more efficient than WT TM4SF5 cells, suggesting a negative regulatory role by the C-terminus. TM4SF5 modulates c-Src activity during TM4SF5-mediated invasion through a TM4SF5/c-Src/EGFR signaling pathway, differentially along the leading protrusive edges of an invasive cancer cell [14]. However, it is remained unknown how TM4SF5 regulates c-Src by interacting in details at the molecular levels.

Here in the study, we examined which domain of c-Src interacts with the c-terminal tail of TM4SF5 and how this interaction would occur more effectively. We found that closed inactive c-Src (SH1 domain) binds to TM4SF5 more strongly than open active c-Src and this interaction mediates downstream cellular signaling activation. To target this interaction and activation, we utilized cell-penetrating TAT peptide containing TM4SF5 c-terminus. The peptides which tranduced plasma membrane directly suppressed TM4SF5-mediated c-Src activation, tumorigenesis, metastatic potential by interrupting interaction between TM4SF5 and c-Src, which dependent on PTP1B-mediated Tyr 530 phosphorylation in c-Src.

MATERIALS AND METHODS

1. Cells

Control (SNU449, SNU761 parental, SNU449Cp, and SNU761-mock), TM4SF5 WT (SNU449Tp and SNU761-TM4SF5 WT), or mutant (SNU761-TM4SF5 ΔICL19 or ΔC) – expressing human hepatocellular carcinoma cells have been described previously [15]. Endogenously TM4SF5-expressing HepG2 cells were maintained DMEM (Welgene) containing 10% FBS and 1% penicillin/streptomycin (GenDEPOT Inc.) at 37℃ in 5% CO_2.

2. Peptides and DNA mutagenesis

Cell-penetrating peptides were TAT-Cscram (TCsr), TAT-Cter (TC), TAT-Caax-Cter (TcxC) (Anygen). The sequence of TAT is RKKRRQRRRP and the sequence of TM4SF5 c-terminus is GDCRKKQDTPH (187-197). The sequence of CAAX motif is CVIM, which conjugated between TAT and TM4SF5 c-terminal sequence. Peptides were diluted in PBS to 1mM and diluted in media containing 10% FBS when treating to cells with different conditions. C-Src point mutations (Y419F, Y530F, Y419F/Y530F, K298M, K298M/Y530F, Q531E/P532E/G533I, Y419F/Q531E/P532E/G533I) were engineered by *pfu* polymerase (Stratagene) and confirmed by direct sequence analyses.

3. Cell lysate preparation and Western blots

Subconfluent cells in media containing 10% FBS were harvested, or cells transiently transfected with plasmid for 48h were either harvested for whole cell lysates. Whole cell lysates were prepared by using a lysis buffer containing 1% Brij58, 150 mM NaCl, 20 mM HEPES pH 7.4, 2mM $MgCl_2$, 2 mM CaCl, and protease and phosphatase inhibitors then immunoblotted using antibodies.

pY^{397}FAK, c-Src (abcam), pY^{577}FAK, pY^{861}FAK, pY^{925}FAK, FAK, pY^{118}Paxillin, pS^{10}p27Kip1, c-Src, PTP1B (SantaCruz Biotechnology), α-tubulin, α-sma (sigma), pY^{416}Src, pY^{527}Src, Paxillin (Cell signaling), p27Kip1 (BD Transduction Laboratories), TM4SF5 ([16]).

4. Coimmunoprecipitation

Whole cell extracts were immunoprecipitated with anti-FLAG or biotin-precoated beads overnight, prior to immunoblotting for the indicated molecules. Immunoprecipitated proteins were boiled within 2x SDS-PAGE sample buffer before standard western blots.

5. Transwell migration or invasion assay

Stable SNU449 cells treated peptides for 24h were analyzed for migration or invasion using transwell chambers with 8-μm pores (Corning), as explained previously [17]. The migration assay was performed for chemotaxis using 10% FBS.

6. Invasive protrusion analysis

Invasive protrusions of cells on Oregon Green® 488-conjugated-gelating (Invitorgen) were analyzed as described in previous report [17]. Briefly, Cells without or with expressing FLAG (mock), FLAG-TM4SF5 were analyzed for invasive protrusions by culturing cells on Oregon Green® 488-conjugated gelatin, prior to seeing the black spots following degradation of gelatin.

7. Mouse and tumor xenograft

4-week-old female or 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. 5 X 10^6 viable SNU449T7cells resuspended in sterile PBS were injected subcutaneously in the mice aged 5 weeks old. Tumor formation was monitored daily and treatment of peptides was begun when the average size of tumor volume reached approximately 100mm^3. Peptides were treated by intraperitoneal injection every day for a week and tumor volumes and weight were measured daily. Tumor volumes were measured as described previously [17].

8. *In vivo* lung metastasis analysis

SNU449T7 cells (5 X 10^6 cells/100μl sterile PBS) were injected in the lateral tail vein

of female BALB/c-nu/nu mice (n=4, Orient. Co. Ltd.). 100 or 500μg peptides were intraperitoneally injected every other day. All mice were sacrificed 6 weeks after cell injection, and their lungs were harvested. Metastatic colonies were counted macroscopically on the lung surface after staining with Bouin's solution (Sigma).

RESULTS

1. The C-terminus of TM4SF5 binds the SH1 domain of c-Src.

As intracellular loop of TM4SF5 bound the F1 lobe of the FAK FERM domain [18] and the c-terminal tail of TM4SF5 bound and regulated c-Src family kinases (SFKs) [15], we confirmed these binding in transiently transfected SNU449 cells and stably expressing SNU761 cells respectively (Figure 1.A). To determine the interacting region on c-Src, diverse c-Src constructs were used in co-immunoprecipitation experiments with SNU449 cells transiently co-transfected with Strep-Mock or TM4SF5 and c-Src WT, SH321, SH1 constructs. C-Src is a modular protein divided into four domains. Amino acids 1–90 contain the myristoylation site and unique domain, amino acids 91–150 contain the SH3 protein interaction domain, amino acids 151–249 contain the SH2 protein interaction domain, and amino acids 250–536 contain the SH1 (catalytic) domain (Figure 1.B) [19]. When all of the c-Src constructs were expressed well in both strep-Mock and strep-TM4SF5 transfected cells, they only bound to TM4SF5 in strep-TM4SF5 expressed cells. Also all of the constructs containing c-Src SH1 domain were bound to TM4SF5 as well as endogenous c-Src (Figure 1.C). When we used c-Src WT, 1-397, SH432 constructs, exogenous c-Src WT only bound to TM4SF5, whereas 1-397, SH432 constructs didn't bind to TM4SF5 (Figure 1.D). These observations suggest that the SH1 domain of c-Src binds to TM4SF5.

We next confirmed whether the SH1 domain of c-Src could bind to the c-terminal tail of TM4SF5. SNU761 cells stably expressing FLAG-TM4SF5 WT, ΔC, ΔICL19 cells transfected with c-Src domain constructs were immunoprecipitated with FLAG antibody-precoated agarose beads, prior to immunoblottings for c-Src. The SH1 domain of c-Src binds to WT, ΔICL19 cells, but didn't bind to ΔC cells (Figure 1.E-F). Further, we tested the ability of binding between TM4SF5 c-terminal tail including point mutant and the SH1 domain of c-Src. When $TM4SF5_{C189A}$ mutants were expressed, binding between TM4SF5 and the SH1 domain of c-Src reduced, as well as the binding of endogenous c-Src (Figure 1.G). Therefore, we concluded that the c-terminal tail of TM4SF5 binds to the SH1 domain of c-Src.

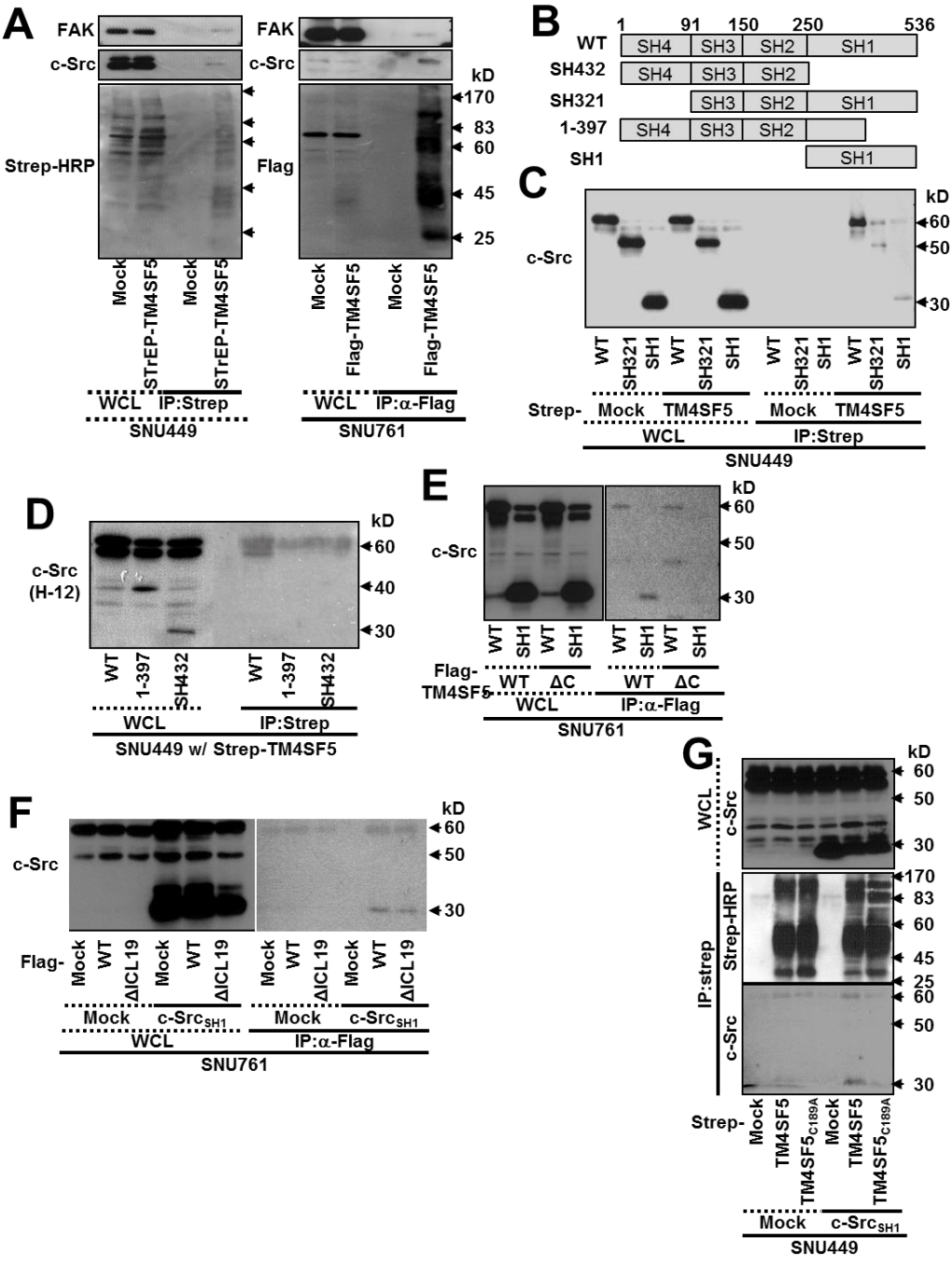


Figure 1. The C-terminus of TM4SF5 interacts with the c-Src SH1 domain.

(A) SNU449 parental cells transfected with strep-tagged empty vector (Mock), TM4SF5 and SNU761 stably expressing either Flag-tagged empty vector (Mock) or

TM4SF5 were harvested. 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 FAK, c-Src and anti-strep-tag or anti-Flag. (B) Schematic representation of c-Src constructs (C) SNU449 cells were cotransfected with strep-Mock, strep-TM4SF5 and either pcDNA3 c-Src WT, SH321, SH1 were immunoprecipitated with strepavidin agarose beads, prior to immunoblotttings for c-Src. (D) Strep-TM4SF5 and either pcDNA3 c-Src WT, or SH432, 1-397 constructs were cotransfected to SNU449 cells before the analysis. (E) SNU761 cells stably expressing TM4SF5 WT and TM4SF5 ΔC transiently transfected with either c-Src WT or SH1 construct were harvested and immunoprecipitated with anti-Flag beads, prior to immunoblottings for c-Src. (F) SNU761 cells stably expressing Mock, TM4SF5 and TM4SF5 ΔICL19 transiently transfected with either pcDNA3 Mock or c-Src SH1 construct were harvested and immunoprecipitated with anti-Flag beads, prior to immunoblotting for c-Src. (G) SNU449 cells transiently cotransfected with strep-Mock, TM4SF5, $TM4SF5_{C189A}$ and either pcDNA3 mock, c-Src SH1 construct were harvested and immunoprecipitated with streptavidin agarose beads, prior to immunoblottings for anti-c-Src and anti-Strepatavidin.

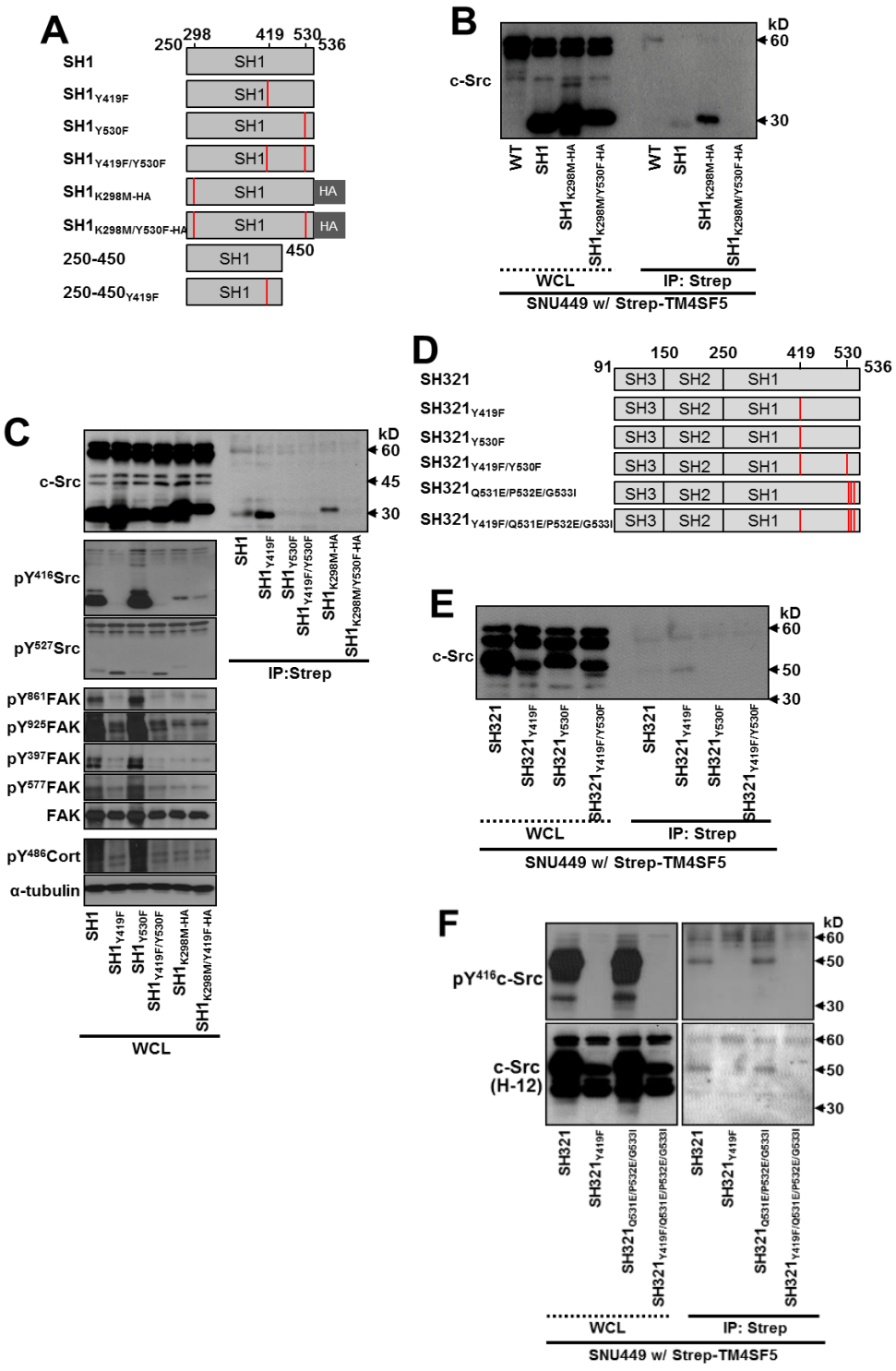
2. TM4SF5 prefers to bind inactively closed c-Src forms.

To understand the condition of binding vetween Tm4SF5 and c-Src SH1 domain, we generated various mutant constructs of c-Src SH1 domain. The SH1 kinase domain contains the autophosphorylation site and the Tyr527 (human Tyr530) residue which can bind to the SH2 domain when phosphorylated. The autophosphorylation site and the Tyr527 are important because it has been shown to be required for full Src activation [1]. Introduction of a kinase disabling mutation (K298M) into the c-Src (250–536) protein completely eliminated kinase activity [19].

We first tested the difference of binding by transfection of c-Src WT, SH1, $SH1_{K298M\text{-}HA}$, $SH1_{K298MY530F\text{-}HA}$ constructs in SNU449 cells expressing strep-TM4SF5. Interestingly, $SH1_{K298M\text{-}HA}$ construct binds more intensively to TM4SF5 more than WT and SH1 construct. Moreover, when c-Src SH1, $SH1_{Y419F}$, $SH1_{Y530F}$, $SH1_{Y419F/Y530F}$, $SH1_{K298M\text{-}HA}$, $SH1_{K298M/Y530F\text{-}HA}$ transfected respectively with strep-TM4SF5 to SNU449 cells, exogeneous c-Src $SH1_{Y419F}$ binds to TM4SF5 more strongly compared with the SH1 domain. But active c-Src SH1 containing $SH1_{Y530F}$, $SH1_{Y419F/Y530F,}$ $SH1_{K298M/Y530F\text{-}HA}$ did not bind to TM4SF5. Therefore, inactive c-Src SH1 binds to TM4SF5 more than active or intact c-Src SH1.

We next conducted experiments with various SH321 mutant constructs because of c-Src conformation. Crystallographic studies have shown that interactions between the c-terminus and the SH2 domain, and between the kinase domain and the SH3 domain, cause the c-Src molecule to assume a closed configuration that covers the kinase domain and reduces its potential for substrate interaction [20]. Mutational studies, primarily involving avian forms of Src, have clearly elucidated a closed, inactive conformation and an open, active state. When the c-terminal tyrosine is phosphorylated, SRC is inactive; when dephosphorylated, Src is active, with the potential for autophosphorylation and for downstream interactions with and phosphorylation of Src substrates [4]. When we transfected SH321, $SH321_{Y419F}$, $SH321_{Y530F}$, $SH321_{Y419F/Y530F}$ constructs in SNU449 cells transfected Strep-TM4SF5, $SH321_{Y419F}$ construct bound to TM4SF5 more than SH321 constructs, whereas $SH321_{Y530F}$, $SH321_{Y419F/Y530F}$ constructs did not bind.

Furthermore, we generated closed, repressed c-Src construct by giving mutation on Q531/P532/G533 of SH1 domain. Mutating the c-tail at residues Q528E, P529E, G530I, to mimic a high affinity c-Src SH2 ligand induces a constitutively closed state [21], as reported previously for the Src family member Hck [22]. When transfecting SH321, $SH321_{Y419F}$, $SH321_{Q531E/P532E/G533I}$ and $SH321_{Y419F/Q531E/P532E/G533I}$ constructs to SNU449 cells transfected Strep-TM4SF5 respectively, $SH321_{Q531E/P532E/G533I}$ construct bind to TM4SF5 more strongly than SH321 construct. These data suggested that inactive closed c-Src bind to TM4SF5 more than open or active c-Src.


A
250
298
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SH1
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250-450Y419F
HA
450
B
c-Src
kD
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SH1
SH1K298M-HA
SH1K298M/Y530F-HA
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SNU449 w/ Strep-TM4SF5
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c-Src
kD
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pY416Src
pY527Src
pY861FAK
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WCL
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530
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SH321
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SH321Y419F/Y530F
SH321Q531E/P532E/G533I
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SH3
SH2
SH1
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c-Src
kD
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SH321
SH321Y419F
SH321Y530F
SH321Y419F/Y530F
WCL
IP: Strep
SNU449 w/ Strep-TM4SF5
F
pY416c-Src
c-Src
(H-12)
kD
60
50
30
SH321
SH321Y419F
SH321Q531E/P532E/G533I
SH321Y419F/Q531E/P532E/G533I
WCL
IP: Strep
SNU449 w/ Strep-TM4SF5

Figure 2. TM4SF5 prefers to bind to inactively closed c-Src forms.

(A) Schematic constructs of SH1 domain (B, C) SNU449 cells transiently cotransfected with strep-TM4SF5 and either c-Src various construct were harvested and immunoprecipitated with streptavidin agarose beads, prior to immunoblotting for anti c-Src. (B) WT, SH1, $SH1_{K298M\text{-}HA}$, and $SH1_{K298MY530F\text{-}HA}$ construct (C) SH1, $SH1_{Y419F}$, $SH1_{Y530F}$, $SH1_{Y419F/Y530F}$, $SH1_{K298M\text{-}HA}$, and $SH1_{K298M/Y530F\text{-}HA}$ construct (D) Schematic constructs of SH321 domain (E, F) SNU449 cells transiently cotransfected with strep-TM4SF5 and either c-Src SH321 construct were harvested and immunoprecipitated with streptavidin agarsoe beads, prior to immunoblotting for anti c-Src. (E) SH321, $SH321_{Y419F}$, $SH321_{Y530F}$, $SH321_{Y419F/Y530F}$ constructs (F) SH321, $SH321_{Y419F}$, $SH321_{Q531E/P532E/G533I}$ and $SH321_{Y419F/Q531E/P532E/G533I}$ constructs

3. Interaction between TM4SF5 and inactive c-Src was regulated by PTP1B.

To confirm the preference of c-Src to bind TM4SF5, we used PTP1B which phosphorylates Y530 of c-Src. Src kinase activity can be regulated by several protein tyrosine phosphatases (PTPs). PTPs such as PTPα, PTPγ, SHP-1 and -2, and PTP1B are able to dephosphorylate Src Tyr530 and are responsible for the regulation of its kinase activity [23]. Among them, PTP1B (also known as PTPN1) has been shown to activate Src in focal adhesions and integrin signaling [24], [25], [26] and in insulin signaling [27]. PTP1B is capable of both in vitro and in vivo activation of Src kinase activity as a result of its specificity towards tyrosine residues at the c-terminal tail [28].

We transfected with PTP1B with the condition of binding between c-Src SH321 and TM4SF5. When PTP1B transfected to SNU449 cells transiently cotransfected with c-Src SH321 and either Mock or TM4SF, the binding of TM4SF5 to c-Src reduced, whereas expressing mock cells were not influenced, From whole cell lysates, we confirmed that the level of pY^{527}Src was reduced following increased PTP1B expression. Furthermore, we conducted the experiments by using stably TM4SF5 expressing SNU761 cells. Consistently, interaction between TM4SF5 and c-Src reduced by PTP1B overexpression. Moreover, we conducted the experiment by using siRNA of PTP1B. When the expression of PTP1B was decreased well by siPTP1B, we confirmed that the interaction between TM4SF5 and c-Src SH321 enhanced. From these data, we could confirmed inactive closed c-Src binds TM4SF5 more preferential than open or active c-Src because PTP1B dephosphorylates phopho-Y530 and makes c-Src open and active.

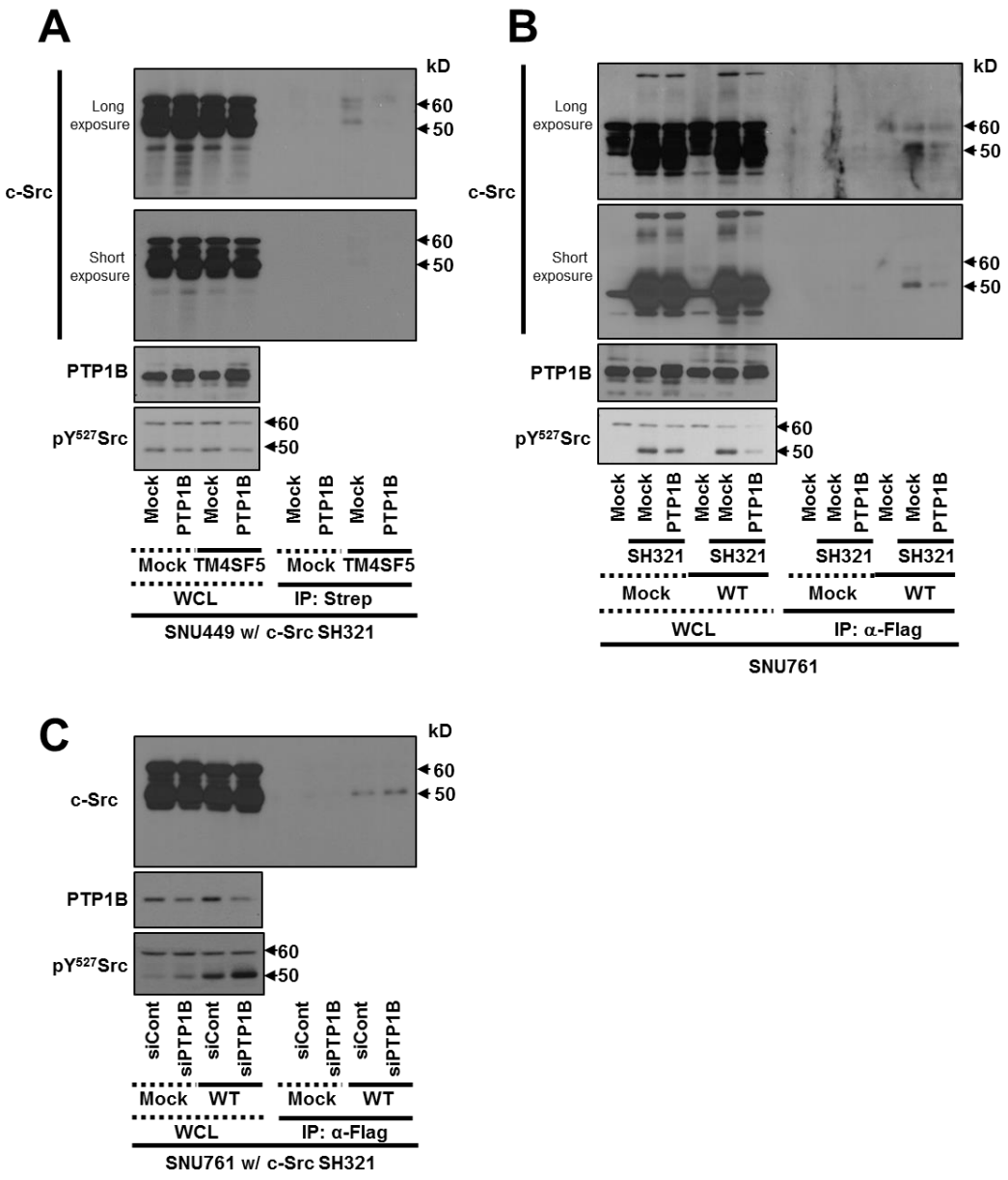


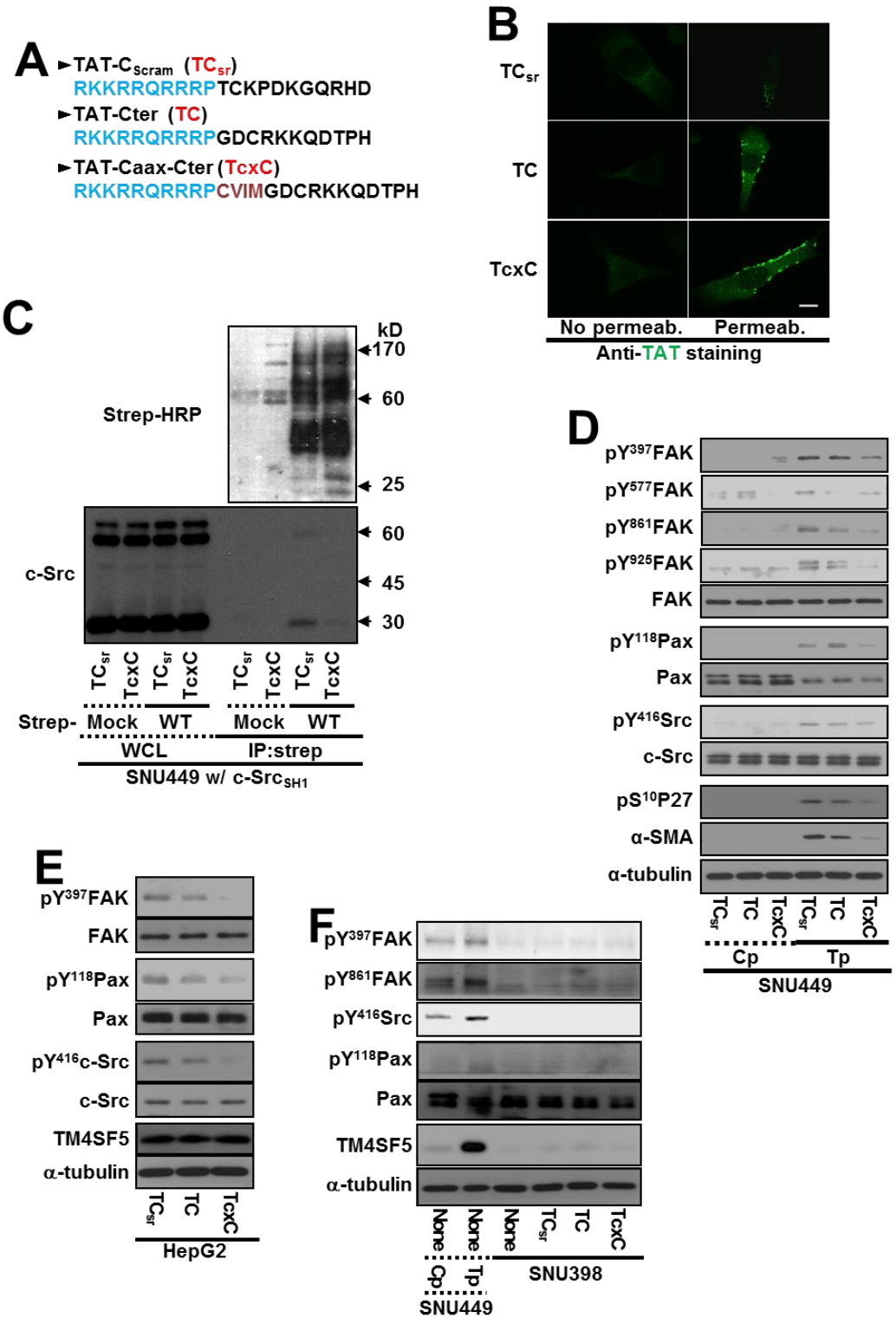
Figure 3. Interaction between TM4SF5 and inactive c-Src was regulated by PTP1B.

(A) SNU449 cells transiently cotransfected with c-Src SH321 construct and either Mock or PTP1B, Strep-Mock or Strep-TM4SF5 were harvested and immunoprecipitated

with streptavidin agarose beads, prior to immunoblotting for anti c-Src. From whole cell lysates, PTP1B and pY^{527}Src were immunoblotted. (B) SNU761 cells stably expressing Mock, Flag-TM4SF5 transiently cotrasnfected with either Mock or c-Src SH321 and either Mock or PTP1B were harvested and immunoprecipitated with Flag-precoated beads, prior to immunoblotting for anti c-Src. From whole cell lysates, PTP1B and pY^{527}Src were immunoblotted. (C) SNU761 cells stably expressing Mock, Flag-TM4SF5 transiently were cotrasnfected with either siCont or siPTP1B and c-Src SH321 construct. After cell lysis, immunoprecipitating with Flag-precoated bead and immunoblotting for anti c-Src, PTP1B and pY^{527}Src were conducted.

4. Cell-penetrating TM4SF5 c-terminal peptides regulate TM4SF5-mediated c-Src activation by interrupting the interaction between TM4SF5 and the c-Src SH1 domain.

To target the c-terminal tail of TM4SF5 and regulate TM4SF5 c-terminus-mediated c-Src activation and metastatic potential, we synthesized cell-penetrating peptides containing TM4SF5 c-terminus. TAT sequence has a characteristic of penetrating cell plasma membrane directly with conjugating cargo, so we conjugated the sequence of TM4SF5 c-terminus to TAT sequence (Figure 4.A). Then, we conducted indirect immunofluorescence to confirm the ability to transduce cell membrane of peptides by using anti-TAT antibody. When permeating with Triton X-100, the localization of all the cell-penetrating peptides (TCsr, TC, TcxC) were confirmed in cells (Figure 4.B). To understand how cell-penetrating peptides function, SNU449 cells cotransfected with Strep-Mock or TM4SF5 and pcDNA3 c-Src SH1 domain construct were treated 10μM TCsr and TcxC peptides for 24 hour and were immunoprecipitated and immunoblotted c-Src. The specific binding between TM4SF5 and c-Src SH1 domain as well as endogeneous c-Src were interrupted by TcxC peptide, whereas TCsr peptides were not (Figure 4.C). When 10μM cell-penetrating peptides treated to SNU449Cp and SNU449Tp cells, only SNU449Tp cells were targeted by peptides and TM4SF5-mediated activation of various cellular molecules was suppressed by TM4SF5-targeting peptides (TC, TcxC) (Figure 4.D). Furthermore, using endogeneously TM4SF5-expressing HepG2 cells and TM4SF5-null SNU398 cells, we found that cell-penetrating targeting peptides (TC, TcxC) only targeted TM4SF5 specifically and inhibited TM4SF5-mediated activation of cellular molecules including c-Src. Comparing TC peptides, TcxC peptides which conjugating CAAX motif between TAT and c-terminus of TM4SF5 sequence were more effective in reducing TM4SF5-mediated activation. These data indicated cell-penetrating peptides containing TM4SF5 c-terminus suppressed TM4SF5-mediated activation of cellular molecules by interrupting binding between TM4SF5 and c-Src.


A
►TAT-C$_{Scram}$ (TC$_{sr}$)
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►TAT-Cter (TC)
RKKRRQRRRPGDCRKKQDTPH
►TAT-Caax-Cter (TcxC)
RKKRRQRRRPCVIMGDCRKKQDTPH
B
TC$_{sr}$
TC
TcxC
No permeab.
Permeab.
Anti-TAT staining
C
kD
170
60
25
60
45
30
Strep-HRP
c-Src
TC$_{sr}$ TcxC TC$_{sr}$ TcxC TC$_{sr}$ TcxC TC$_{sr}$ TcxC
Strep- Mock WT Mock WT
WCL
IP:strep
SNU449 w/ c-Src$_{SH1}$
D
pY^{397}FAK
pY^{577}FAK
pY^{861}FAK
pY^{925}FAK
FAK
pY^{118}Pax
Pax
pY^{416}Src
c-Src
pS^{10}P27
α-SMA
α-tubulin
TC$_{sr}$ TC TcxC TC$_{sr}$ TC TcxC
Cp
Tp
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FAK
pY^{118}Pax
Pax
pY^{416}c-Src
c-Src
TM4SF5
α-tubulin
TC$_{sr}$ TC TcxC
HepG2
F
pY^{397}FAK
pY^{861}FAK
pY^{416}Src
pY^{118}Pax
Pax
TM4SF5
α-tubulin
None None None TC$_{sr}$ TC TcxC
Cp
Tp
SNU449
SNU398

Figure 4. Cell-penetrating TM4SF5 c-terminal peptides regulate TM4SF5-mediated c-Src activation by interrupting the interaction between TM4SF5 and the c-Src SH1 domain.

(A) The Sequence of cell-penetrating peptides containing TAT and TM4SF5 c-terminus (B) SNU449 cells were treated with cell-penetrating peptides, prior to processing indirect immunofluorescence using anti-TAT (green). (C) SNU449 cells transiently cotransfected with Strep-Mock, TM4SF5, and pcDNA3 c-Src SH1 construct were harvested after treated peptides for 24h and immunoprecipitated with streptavidin agarose beads, prior to immunoblottings for anti-c-Src and anti-Strepatavidin. (D) Control SNU449Cp (Cp) or ectopically TM4SF5-expressing SNU449Tp (Tp) cells treated cell-penetrating peptides were harvested for immunoblottings. (E) HepG2 cells expressing endogeneously TM4SF5 treated cell-penetrating peptides were harvested for immunoblottings. (F) TM4SF5-null SNU398 cells were treated with cell-penetrating peptides and were harvested for immunoblottings (SNU449Cp, SNU449Tp cells are control of expressing TM4SF5).

5. Cell-penetrating TM4SF5 c-terminal peptides suppress TM4SF5-mediated metastatic potentials.

To confirm the effect of cell-penetrating TM4SF5 c-terminal peptides in TM4SF5-mediated metastatic potential, we first conducted indirect immunofluorescence by staining pY^{397}FAK, pY^{861}FAK. When peptides treated to SNU449Cp cells, the formation of focal adhesion spots was not changed. But when targeting peptides (TC, TcxC) were treated to SNU449Tp cells, focal adhesion spots disappeared, different from control peptides (TCsr) (Figure 5.A). We next confirmed the effect of peptides in TM4SF5-mediated invasion and migration by ECM degradation assay and transwell migration assay. We analyzed whether cell-penetrating peptides might regulate invasive feature such as invasive protrusions with activities to degrade the ECM. ECM degradation was visualized via microscopic observation of dark spots after the gelatin underneath cells was degraded by culturing cells on fluorophore-conjugated gelatin [2]. We examined invasive protrusions between SNU761 Mock, and WT cells treated each peptides. When SNU761 WT cells were examined, stably expressing TM4SF5 SNU761 WT cells enhanced invasive protrusion formations, compared with TM4SF5-null SNU761 Mock cells. Enhanced invasive protrusion formations by TM4SF5 decreased by treating cell-penetrating TM4SF5 targeting peptides (TC, TcxC), whereas SNU761 mock cells were not influenced (Figure 5.B). SNU449Cp or SNU449Tp, as previously described [29], were used in the transwell migration assay. We filled the bottom chamber with media containing 10% FBS and top chamber with cells treated 10μM peptides for 24 hour. TM4SF5-expressing SNU449Tp cells showed a greater migration than did control SNU449Cp cells and moreover a greater migration of SNU449Tp cells was inhibited by treatment of TC and TcxC peptides (Figure 5.C).

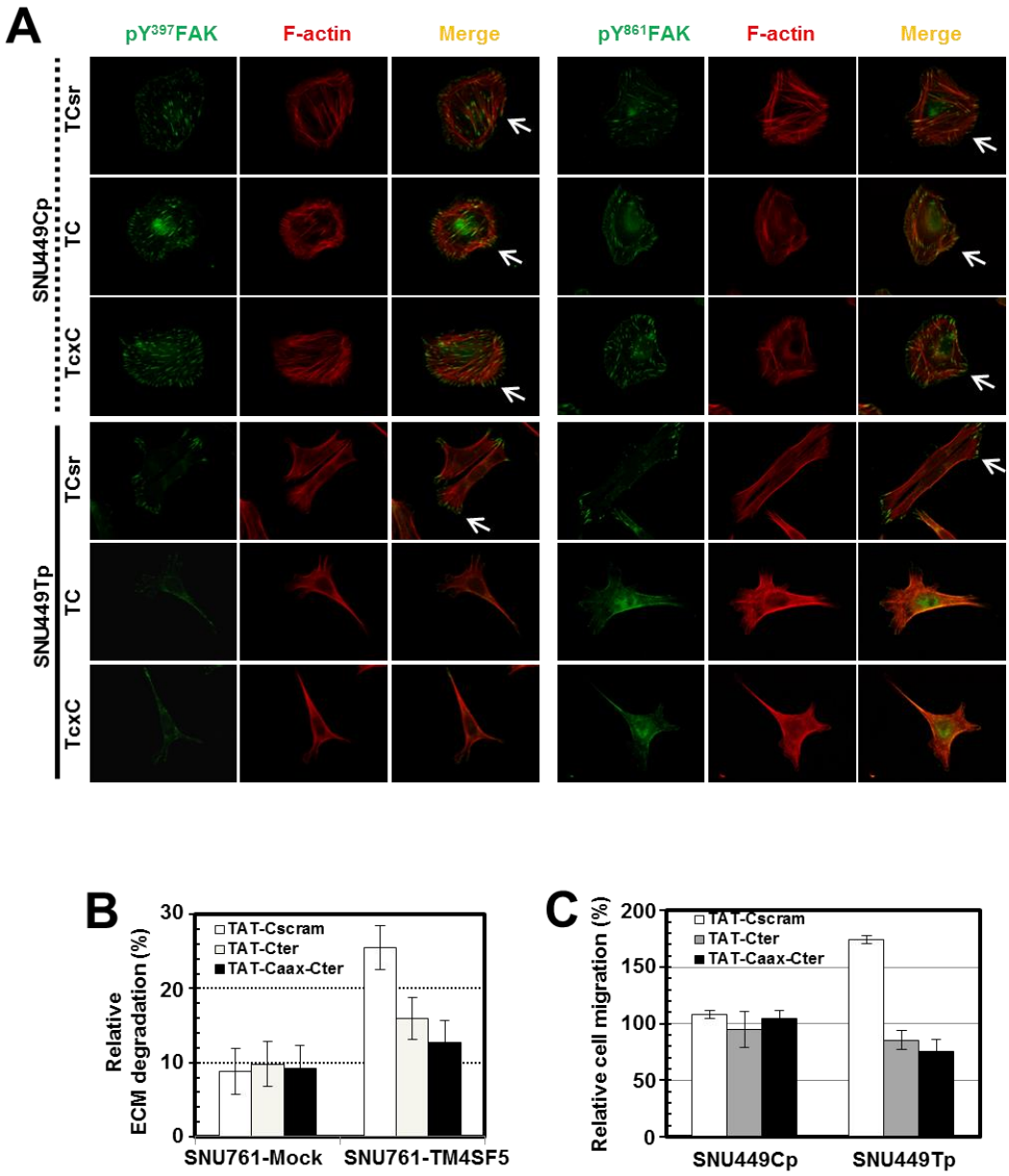
A
pY397FAK
F-actin
Merge
pY861FAK
F-actin
Merge
SNU449Cp
TCsr
TC
TcxC
SNU449Tp
TCsr
TC
TcxC
B
Relative ECM degradation (%)
30
20
10
0
TAT-Cscram
TAT-Cter
TAT-Caax-Cter
SNU761-Mock
SNU761-TM4SF5
C
Relative cell migration (%)
200
150
100
50
0
TAT-Cscram
TAT-Cter
TAT-Caax-Cter
SNU449Cp
SNU449Tp

Figure 5. Cell-penetrating TM4SF5 c-terminal peptides suppress TM4SF5-mediated focal adhesion formation, migration, and invasive ECM degradation.

(A) SNU449Cp (Cp, a stable control) cells without TM4SF5 expression, but SNU449Tp (Tp, a stably pooled clone) cells were treated cell-penetrating TAT peptides, prior to processing to indirect immunofluorescence for pY^{397}FAK or pY^{861}FAK (green) and phalloidin (red) antibodies. White arrows indicate focal adhesion spots. (B) Degradation of Oregon Green® 488-conjugated gelatin by SNU761 cells stably expressing Mock, TM4SF5 treated cell-penetrating peptides were analyzed in graph. (C) TM4SF5-null SNU449Cp (Cp) and TM4SF5-expressing SNU449Tp (Tp) cells were analyzed for transwell migration. The bottom chamber was filled with 10% FBS/RPMI-1640 and cells treated cell-penetrating peptides were loaded to upper chamber. Migration was measured after incubation for the indicated times after adding cells.

6. Cell-penetrating TM4FS5 c-terminal peptides suppress TM4SF5-mediated tumorigenesis and metastasis *in vivo*.

To examine TM4SF5-mediated tumor formation in nude mice, we previously conducted the experiments that SNU449 cells were subcutaneously injected into mice. Whereas SNU449Cp cells did not form substantial tumors, TM4SF5-expressing SNU449T16 cells formed tumors growing continuously. Furthermore, all mice showed normal increases in body weight [29]. So, we generated xenograft mouse models by injecting SNU449T7 cells subcutaneously to nude mice to investigate the effect of the peptides *in vivo*. When tumor size of mice reached approximately 100mm^3, we intraperitoneally injected 600μg TCsr peptides, 200μg TcxC peptides and 600μg TcxC peptides to five mice respectively. Peptides were injected to each mouse every day for a week and tumor size and weight were measured daily. Through all mice injected peptides showed normal increases in body weight, we noticed that all peptides were non-toxic to mouse. Also TcxC peptides had significant effect of inhibiting proliferation of tumor dependent on concentration (Figure 6.A). We next conducted immunoblotting by tumor tissue extracted from each mouse (Figure 6.B). Consistent with the result of in vitro, TM4SF5-mediated activation of c-Src and FAK was decreased by TcxC peptides.

We previously injected SNU449Cp or SNU449Tp cells into the tail vein of nude mice to see if the cells could metastasize into lung or other organs. Mice were sacrificed 4 weeks after the injection and their organs were examined. Mice injected with SNU449Tp cells had three- to fourfold larger lungs than did mice injected with SNU449Cp cells. More importantly, mice injected with SNU449Tp cells had more nodules on their lung surfaces than did mice injected with SNU449Cp cells, which appeared to be infiltrated with metastatic SNU449Tp cells [30]. So, we generated metastasis model by tail-vein injection of cells to mouse. SNU449T7 cells were injected to nude mice and 2 weeks later, we injected peptides intraperitoneally to mice every other day for 2 weeks. After that, we sacrificed all of mice and measured each lung. Mice treated TcxC peptides had a less tumor nodules in lung dependently on concentration, compared with those 500μg TCsr peptides (Figure 6.C). From these data, we suggest that cell-penetrating peptides containing TM4SF5 c-terminus can reduce TM4SF5-mediated tumor proliferation and metastasis.

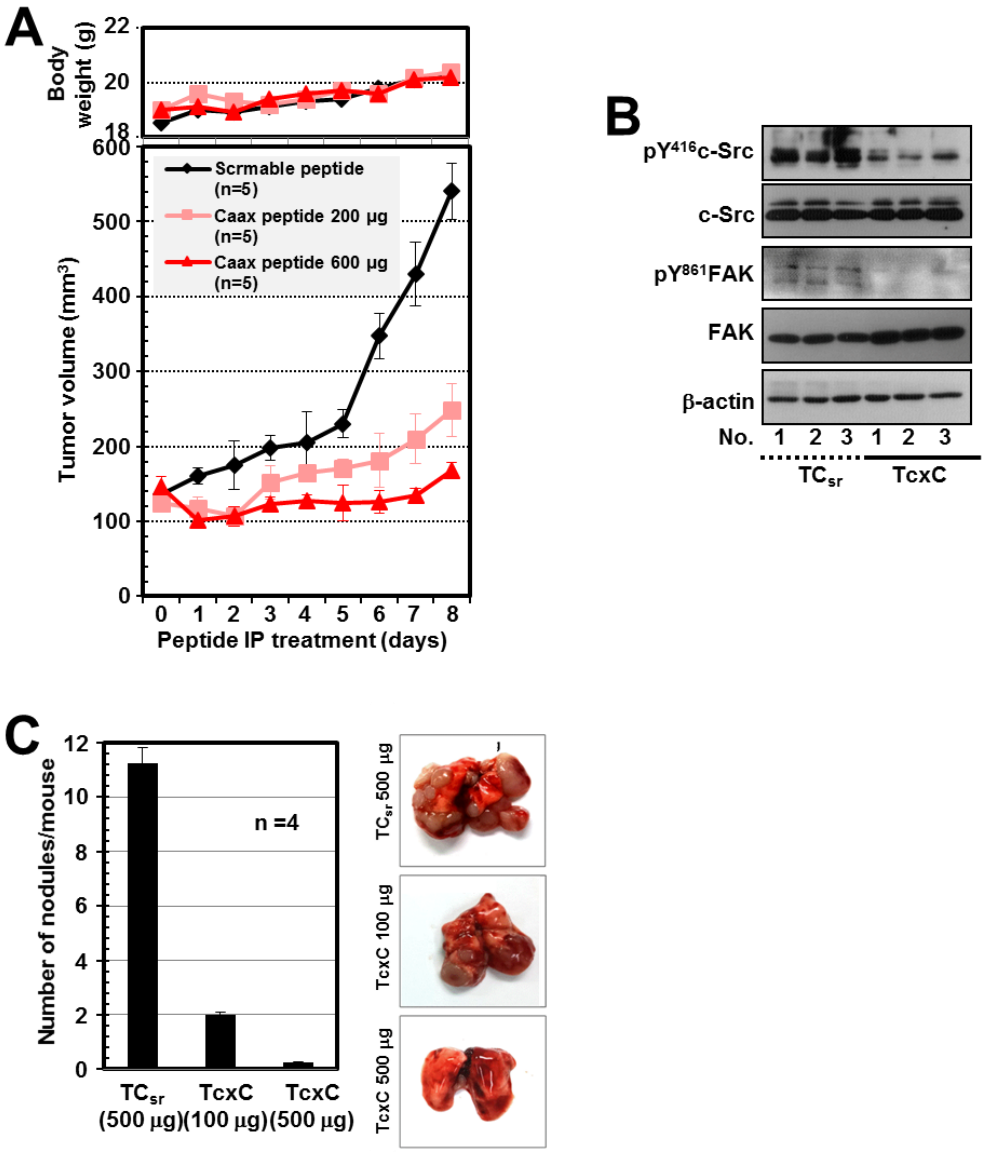

Figure 6. Cell-penetrating TM4FS5 c-terminal peptides suppress TM4SF5-mediated tumorigenesis and metastasis *in vivo*.

(A) SNU449T7 cells were (A, B) injected subcutaneously into nude mice (5 X 10^6 cells/mouse). Cell-penetrating scramble or caax peptide administration at 200 or 600μg was performed every day for a week after TM4SF5-mediated tumors had reached about 100 mm^3. Tumor volumes were calculated and body weights were measured, as explained in Materials and Methods. (B) Immunoblots of tumor tissue extracts (C) SNU449T7 cells

were injected into the tail veins of mice (5 X 10^6 cells/100 ml/mice). After normal maintenance for 2 weeks, cell-penetrating scramble or caax peptide administration at 100 or 500μg was performed every other day for 7 days. After administration for 2 weeks, mice were sacrificed to examine *in vivo* metastasis. Representative lung images in each condition were shown. Surface nodules were counted and graphed.

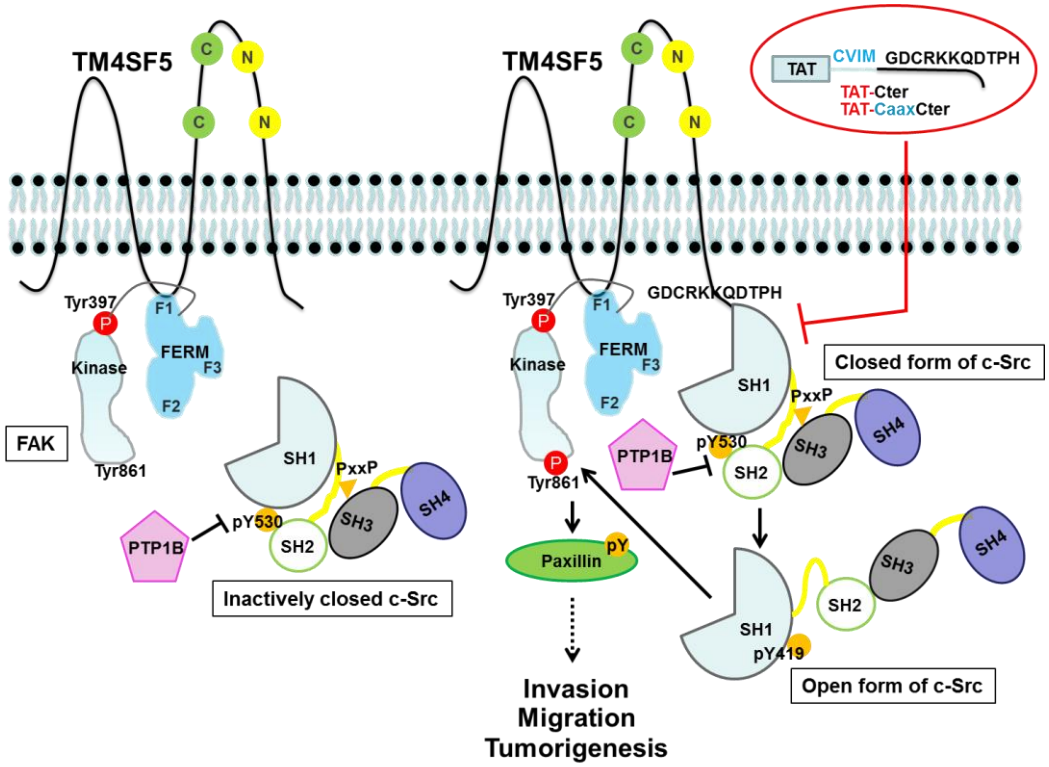

Figure 7. The working model

TM4SF5 whose the intercellular loop interacts the F1 lobe of FAK directly prefers to bind to inactively closed c-Src. After binding, TM4SF5 mediates c-Src activation, by further recruits PTP1B to dephosphorylate Tyr 530 in c-Src, which c-Src became open in conformation. Then the activated c-Src can phosphorylate neighboring FAK Tyr 861, which makes the downstream signaling molecules activated leading to TM4SF5-medated invasion, migration and tumorigenesis. Cell-penetrating peptides containing TM4SF5 c-terminus can suppress TM4SF5-mediated c-Src activation, tumorigenesis and metastatic potential by interrupting the interaction between TM4FS5 and the c-Src SH1 domain.

DISCUSSION

Here we show that the c-terminal tail of TM4SF5 which consists of 11 amino acids without a specific protein subdomain interacts with c-Src via the SH1 domain. C-Src is a modular protein divided into four domains. Amino acids 1–90 contain the myristoylation site and unique domain, amino acids 91–150 contain the SH3 protein interaction domain, amino acids 151–249 contain the SH2 protein interaction domain, and amino acids 250–536 contain the SH1 (catalytic) domain [19]. Although the catalytic domain of c-Src is clearly important, as it contains the enzymatic activity of the protein, very little is known about protein interactions involving this domain. The transforming gene from polyomavirus, middle T antigen, does interact with the kinase domain of c-Src [31], [32], and a major interaction also exists between β-arrestin1 and the catalytic or kinase domain (SH1) of c-Src [33]. The finding that TM4SF5 can specifically bind to the kinase domain of c-Src suggests that the isolated kinase domain may be a useful reagent to further study the functional consequences of the TM4SF5/c-Src interaction. Interestingly, the isolated c-Src catalytic domain contains significant kinase activity compared with wild-type c-Src. This finding is somewhat surprising and indicates that the isolated kinase domain folds properly and is therefore a useful reagent in these studies.

The C-terminus of TM4SF5 bound more efficiently to inactive or closed c-Src than active or open active c-Src, although active c-Src could still bind TM4SF5. The preferred binding between the c-terminus of TM4SF5 and inactive c-Src was confirmed with various c-Src constructs and SNU449 and SNU761 liver cancer cells without or with TM4SF5 expression. SH1 domain has three important residue for activation, which are lysine 298, tyrosine 419 and tyrosine 530. So we generated point mutation constructs by using these residues; K298M, Y419F, Y530F. K298M construct contains a point mutation in the ATP binding site, which means inactive state. Also Y419F construct is unable of autophosphorylation, it is inactive state. Because Y530F construct is unable of phosphorylation by intercellular machinery including Csk, its homologue CHK and protein tyrosine phosphatases, it remains always open conformation. Therefore, we can suggest

that inactive closed c-Src interacts TM4SF5 more strongly.

Furthermore, we confirmed the preference of the interaction by overexpressing or silencing PTP1B. PTP1B is a non-receptor protein tyrosine phosphatase bound to the cytosolic face of the endoplasmic reticulum (ER) through a hydrophobic C-terminal tail [34]. PTP1B is present in complexes of β1- and β3-integrin [35], [36], and interacts with the adaptor protein p130Cas, which in part localizes at focal adhesions [37]. As the catalytic domain of PTP1B faces the cytosol, it has the potential for substrate dephosphorylation throughout the extensive branching network occupied by the ER [38]. Indeed, PTP1B has been shown to dephosphorylate plasma membrane receptors [39], [40], [41], [42], protein adaptors [43], and cytosolic tyrosine kinases such as Src [35],[27]. The observations in the current study may support a role for PTP1B to dephosphorylate phopho-Y530 of c-Src and preferential binding to C-terminus of TM4SF5 is confirmed.

Because TM4SF5 is highly expressed in hepatocarcinoma and plays roles in aberrant cell proliferation and enhanced metastatic potential [11], understanding of TM4SF5-mediated c-Src activation and binding to each other would be important for development of therapeutic reagents against tumorigenesis mediated by TM4SF5 or -related signaling molecule(s). The present study suggests that cell-penetrating peptides containing TM4SF5 c-terminus can suppress TM4SF5-mediated activation of intercellular molecules by interrupting interaction between TM4SF5 and c-Src SH1 domain. Moreover, the peptides had effects in reducing tumor cell proliferation and metastasis. We confirmed these effects by treating the peptides to xenograft model and *in vivo* metastatic model. It suggests that the peptides can be the therapeutic reagent for treatment or prevention of liver cancer specifically targeting TM4SF5. Membranes of eucaryotic cells and organelles, as well as the cell wall and membrane of pathogenic microorganisms, constitute a serious barrier for the access of hydrophilic drugs to their target molecules inside the cell structures. To overcome problems of conventional and gene drug delivery, various techniques have been developed. A conventional procedure for delivering genetic material is to use viral vectors, but treating genetic disorders with this method has met with only limited success [44]. Alternative non-viral methods, such as electroporation, microinjection, and the use of liposomes, have been developed for conventional and gene drug delivery. These methods

have been proved to be effective in vitro and for research purposes, but show limited potential for delivery in vivo due to toxicity, cell damage, and immunogenicity. They are also technically demanding in their application, lack tissue and cell specificity, and can deliver material to only a limited amount of cells. In view of these considerations, it has been found over the last 10 years that certain peptides and proteins can penetrate the cell membrane and enter the cell. A variety of cargo molecules can be attached to these peptides and proteins and translocated into the cell. Carrier peptides and proteins thus constitute a new class of potential drug delivery vectors. Currently, about hundred such peptides and a few proteins are known [45]. TAT is a transcription activating factor of HIV-1 and the cluster of basic amino acids (Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) is thought to be crucial for TAT uptake. Cellular delivery using TAT has several advantages over conventional techniques because it is efficient for a range of cell types, can be applied to cells en masse, and has a potential therapeutic application [10]. We synthesized cell-penetrating TAT peptides containing TM4SF5 by conjugating the sequence of c-terminal tail of TM4SF5 to TAT sequence. With TAT which can penetrates plasma cell membrane directly without any help of specific receptors, the peptides of TM4SF5 c-terminus could localize in the cell, especially near the cell membrane which TM4SF5 localizes. Furthermore, we conjugated CAAX motif between TAT and TM4SF5 c-terminus sequence for enhancing the ability to target TM4SF5 and c-Src. CAAX sequence that serves as a substrate for three enzymes that sequentially modify the sequence to create a lipidated, hydrophobic domain that mediates association with cellular membranes [46]. We found that CAAX conjugated cell-penetrating TAT peptides containing TM4SF5 c-terminus is more effective to suppressing TM4SF5-mediated activation of cellular molecules. So we used this CAAX peptides in mouse experiments.

In conclusion, the present study demonstrated that the c-terminal tail of TM4SF5 interacted with c-Src via SH1 domain. When c-Src binds TM4SF5, closed inactive c-Src prefers to bind TM4SF5 than open or active c-Src. Cell-penetrating TAT peptides containing TM4SF5 c-terminus can suppress TM4SF5-mediated c-Src activation, migration and invasion when treating to the cells. We found that CAAX motif-conjugated cell-penetrating TAT peptides are more effective in suppression of TM4SF5 and it can

interrupt the interaction between TM4SF5 and c-Src SH1 domain. Moreover, Xenograft injected with TM4SF5-expressing SNU449 hepatic cancer cells resulted in inhibition of tumorigenesis by the peptide. From *in vivo* lung metastasis model by tail vein injection of TM4SF5-expressing SNU449T7 cells, peptides reduced lung metastasis. Altogether, this study suggests that the peptide could disrupt the recruit of the inactive closed c-Src by TM4SF5 to activate it, leading to inhibition of TM4SF5-mediated tumorigenic effects.

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요 약

Transmembrane 4 L6 family member 5 (TM4SF5) 는 tetraspanin superfamily 에 속하는 막 단백질로서, 세포막을 네 번 통과하는 구조를 가진 당단백질이다. TM4SF5는 간암을 포함한 다양한 암종에서 높게 발현되며, EMT (epithelial-mesenchymal transition, 상피-중배엽 세포 전이)를 유발함으로써 세포의 형태 변화뿐 아니라 이동과 침윤, 증식을 촉진하는 역할을 하고 있음이 밝혀졌다. 한편, c-Src는 세포 내에서 다양한 역할을 수행하는 신호 전달 인자로서, 선행 연구를 통해 TM4SF5의 C-말단과 결합하여 TM4SF5에 의해 매개 되는 세포의 침윤을 조절한다는 것이 밝혀졌다. 이에 본 연구는 TM4SF5와 c-Src 와의 결합에 의한 자세한 분자적 수준의 c-Src 활성화 메커니즘과 그것을 억제할 수 있는 세포막 투과성 펩타이드를 개발함으로써 그것의 항암제 효과를 발견하였다.

TM4SF5 를 발현하지 않거나 발현하는 SNU449 및 SNU761 간암 세포주를 이용하여 c-Src 의 다양한 construct 와의 면역 침강 실험을 통해 c-Src 의 kinase 도메인 (250-536 amino acids, SH1 domain)이 TM4SF5 에 특이적으로 결합한다는 것을 확인하였다. 더욱이, $SH1_{Y419F}$ 이 SH1 의 wild-type 보다 TM4SF5 와 더 잘 결합하며, $SH1_{Y530F}$ 와는 결합하지 않았다. 이러한 결과는 SH321, $SH321_{Y419F}$, $SH321_{Y530F}$ construct 를 이용한 면역 침강 실험 결과에서도 동일하게 확인할 수 있었으며, 더불어 c-Src 의 타이로신 530번을 탈인산화시키는 단백질인 PTP1B 를 과발현시키거나 억제시킨 조건에서의 실험 결과를 통해서도 TM4SF5 가 c-Src 의 닫힌 구조의 비활성화 형태와의 결합을 더욱 선호한다는 것을 확인할 수 있었다.

세포막을 특별한 수용체의 도움 없이 통과할 수 있는 세포막 투과성 펩타이드의 서열 뒤에 TM4SF5 의 C-말단 서열을 접합시켜 만든 펩타이드는

TM4SF5 와 c-Src 의 SH1 도메인과의 결합을 방해함으로써 TM4SF5 에 의해 매개된 FAK/c-Src 의 활성을 억제하였다. 이는 TM4SF5 를 과발현시킨 세포와 형질감염을 통해 일시적으로 TM4SF5 를 발현시킨 세포, 내재적으로 TM4SF5 를 발현하는 세포 모두에서 serum 에 의존적으로 그 효과가 나타남을 확인함으로써 알 수 있었다. 나아가, 이러한 세포막 투과성 TM4SF5 C-말단 펩타이드는 TM4SF5 특이적으로 그것에 의해 매개된 세포의 전이와 침윤을 억제함은 물론, 누드 마우스에서 TM4SF5 에 의해 형성된 종양의 부피를 독성 없이 유의적으로 감소시켰다. 또한, 누드 마우스의 꼬리 정맥에 TM4SF5 가 과발현된 종양 세포를 주입시켜 폐로 전이시킨 후 세포막 투과성 TM4SF5 C-말단 펩타이드를 복강 투여하였을 때에도 대조군에 비해 폐에 형성된 종양의 수가 유의적으로 감소하였다.

이러한 결과를 통해 TM4SF5 가 c-Src 의 구조 상태에 따른 선호성을 가지며 c-Src의 kinase 도메인과 특이적으로 결합함을 알 수 있었다. TM4SF5 의 C-말단과 c-Src 의 SH1 도메인의 결합은 세포막을 투과할 수 있는 TM4SF5 C-말단 펩타이드를 처리함으로써 억제될 수 있으며, 이 펩타이드는 TM4SF5 특이적으로 세포 내의 FAK/c-Src 의 세포 활성을 조절하여 TM4SF5에 의해 매개된 세포의 전이와 침윤, 누드마우스에서의 종양 증식, 전이를 억제함으로써 항암제의 역할을 할 수 있을 것으로 기대된다.

주요어: TM4SF5, 단백질-단백질 결합, c-Src, 세포막 투과성 펩타이드, 종양의 증식 및 전이

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