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Modulation of signaling between TM4SF5 and integrins in tumor microenvironment

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1. ABSTRACT

TM4SF5 is a transmembrane glycoprotein of the transmembrane 4 L six family, a branch of the tetraspanin family and highly expressed in many types of cancers. TM4SF5 induces epithelial-mesenchymal transition (EMT) by morphological changes resulting from inactivation of RhoA mediated by stabilized cytosolic $p27^{kip1}$. TM4SF5-mediated EMT can lead to loss of contact inhibition and enhanced migration/invasion, presumably depending on cross-talks between TM4SF5 and integrins. An anti-TM4SF5 agent appears to target the second extracellular domain of TM4SF5, which is important for cross-talk with integrins, leading to a blockade of TM4SF5-mediated multilayer growth and migration/invasion. In addition, TM4SF5 engages in cross-talk with integrin alpha5 to induce and secrete VEGF, which in turn causes activation of angiogenesis in endothelial cells. Therefore, TM4SF5 plays a central regulatory role in a wide variety of physiological processes through cross-talk with integrins. This review presents current knowledge from *in vitro* and *in vivo* observations of the roles of TM4SF5-integrin cooperation in hepatocellular carcinogenesis and discusses important areas for future investigation.

2. STRUCTURE OF TM4SF5

The *TM4SF5* gene is located on human chromosome 17q13.3. Translation from exon 1 to exon 5 results in a 197 amino acid protein, which has a membrane topology similar to a transmembrane 4 superfamily (TM4SF), also known as tetraspanins or tetraspans (1, 2). The structural features and general properties of the genuine tetraspanins and TM4SF5 are highlighted in Figure 1. TM4SF5 is homologous to tumor-associated antigen L6 (TM4SF1) and is a member of transmembrane 4 L six family along with L6, IL-TIMP, and L6D, which shares membrane topology but not other features found in other genuine TM4SF proteins (see below). All TM4SFs have four transmembrane domains (TM1 ~ TM4), short cytoplasmic domains at their N- and C-termini, and two extracellular loops (EC), a smaller loop domain (EC1) between transmembrane domains 1 and 2, and a larger loop domain (EC2) between transmembrane domains 3 and 4 (3, 4). The second extracellular loop domain of all tetraspanins has a highly conserved CCG motif and two additional cysteine residues that contribute to disulfide bonds within the EC2. However, transmembrane 4 L six family proteins do not have four conserved cysteine

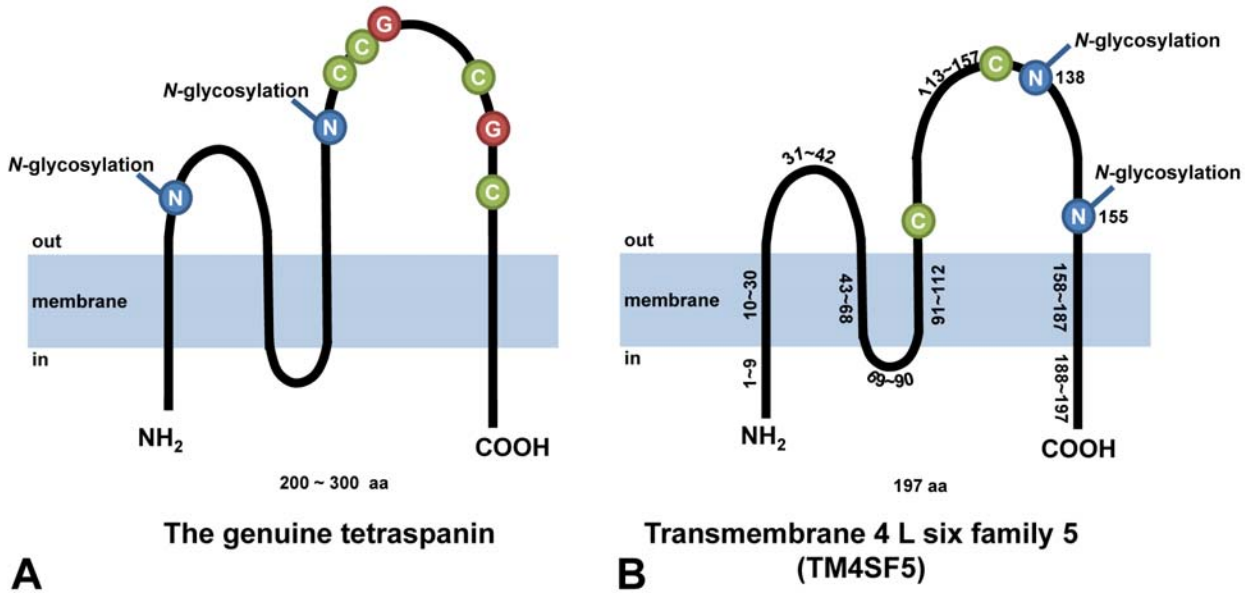


Figure 1. Different features in the genuine tetraspanin and transmembrane 4 L six family member 5 (TM4SF5). The genuine tetraspanins (or TM4SFs) including CD9, CD63, CD81, and CD151 (A) and transmembrane 4 L six family including TM4SF5, L6, IL-TIMP, and L6D have four transmembrane domains with the NH_2- and COOH-terminal tails in cytosol. The genuine tetraspanins have a highly conserved CCG (Cys-Cys-Gly) motif and two additional cysteine residues in the extracellular loop 2, whereas TM4SF5 has two cysteines irrelevantly to any structural motif in genuine tetraspanin.

residues but instead have two cysteine residues, neither of which occurs in the motifs found in the tetraspanins (2). The EC2 of most tetraspanins are N-glycosylated at one or more potential sites (5), and TM4SF5 has two *N*-glycosylation sites within the EC2 (6).

3. FUNCTIONS OF TM4SF5

3.1. TM4SF5-mediated actin reorganization

TM4SFs interact with integrins, other TM4SFs, and cell surface receptors to form a membrane receptor network, known as a 'tetraspanin web' or 'tetraspanin-enriched microdomain (TERM)' to collaboratively perform their biological functions such as morphogenesis (7-9). In fibroblast cells, TM4SF5 regulates actin organization and focal adhesion dynamics through phosphorylation of Tyr^{925} in FAK and Tyr^{118} in paxillin and the localizations of these molecules on peripheral cell boundaries in a serum-dependent manner. This evidence suggests that TM4SF5 cooperates with integrin-mediated signaling under growth factor receptor-mediated regulation (10). TM4SF5 causes morphological elongation in epithelial cells. Ectopic expression of TM4SF5 in human hepatocytes enhances FAK Tyr^{577} phosphorylation and the association between FAK and p190RhoGAP and GRAF (GAP for Rho associated with focal adhesions), leading to RhoA inactivation for morphological elongation (11). Recent studies indicate that $p27^{kip1}$ also inhibits RhoA by a direct interaction in cytosol (12). TM4SF5 expression induces stabilization of cytosolic $p27^{kip1}$, further inhibiting the RhoA pathway (11). Consequently, TM4SF5-mediated RhoA inactivation results in aberrant actin bundling and cellular elongation, leading to EMT.

3.2. TM4SF5-mediated epithelial-mesenchymal transition

TM4SF5-mediated cellular elongation leads to epithelial-mesenchymal transition (EMT) in human hepatocytes (11) (Figure 2A). Cells that overexpress TM4SF5 show down-regulation of the epithelial cell adhesion protein E-cadherin, actin-binding protein zonula occludens-1 (ZO-1), and upregulation of alpha-SMA, a mesenchymal cell marker. EMT is typically characterized by loss of cell-cell adhesion via hemophilic interactions of cell adhesion molecules including E-cadherin, and thus can be caused by transcriptional suppression of E-cadherin by Snails and others (13). However, unlike other EMT models, EMT is independent of Snail1 in TM4SF5-expressing cells. $p27^{kip1}$ suppression in TM4SF5-expressing cells results in reduction of alpha-SMA expression and inhibition of EMT mediated by TM4SF5 expression (11). In addition, knockdown of endogenous TM4SF5 in hepatic epithelial cells inhibits hepatocyte growth factor (HGF)-mediated loss of cell-cell adhesion, although alterations in $p27^{Kip1}$ expression and localization on HGF treatment of TM4SF5-suppressed cells are remained to be tested. In addition to cytosolic $p27^{Kip1}$ stabilization, TM4SF5 expression results in RhoA inactivation and its suppression recovers RhoA activity (11). Furthermore, cytosolic $p27^{Kip1}$ is well-known to inactive RhoA (12). These findings thus indicate that within certain signaling contexts, TM4SF5 causes EMT through regulation of cytosolic $p27^{kip1}$-stabilization and its effects on RhoA and subsequent morphological alterations (11).

3.3. TM4SF5-mediated cell proliferation

TM4SF5 causes loss of contact inhibition through EMT, leading to uncontrolled cell growth in a multilayered pattern. EMT may result from TM4SF5-

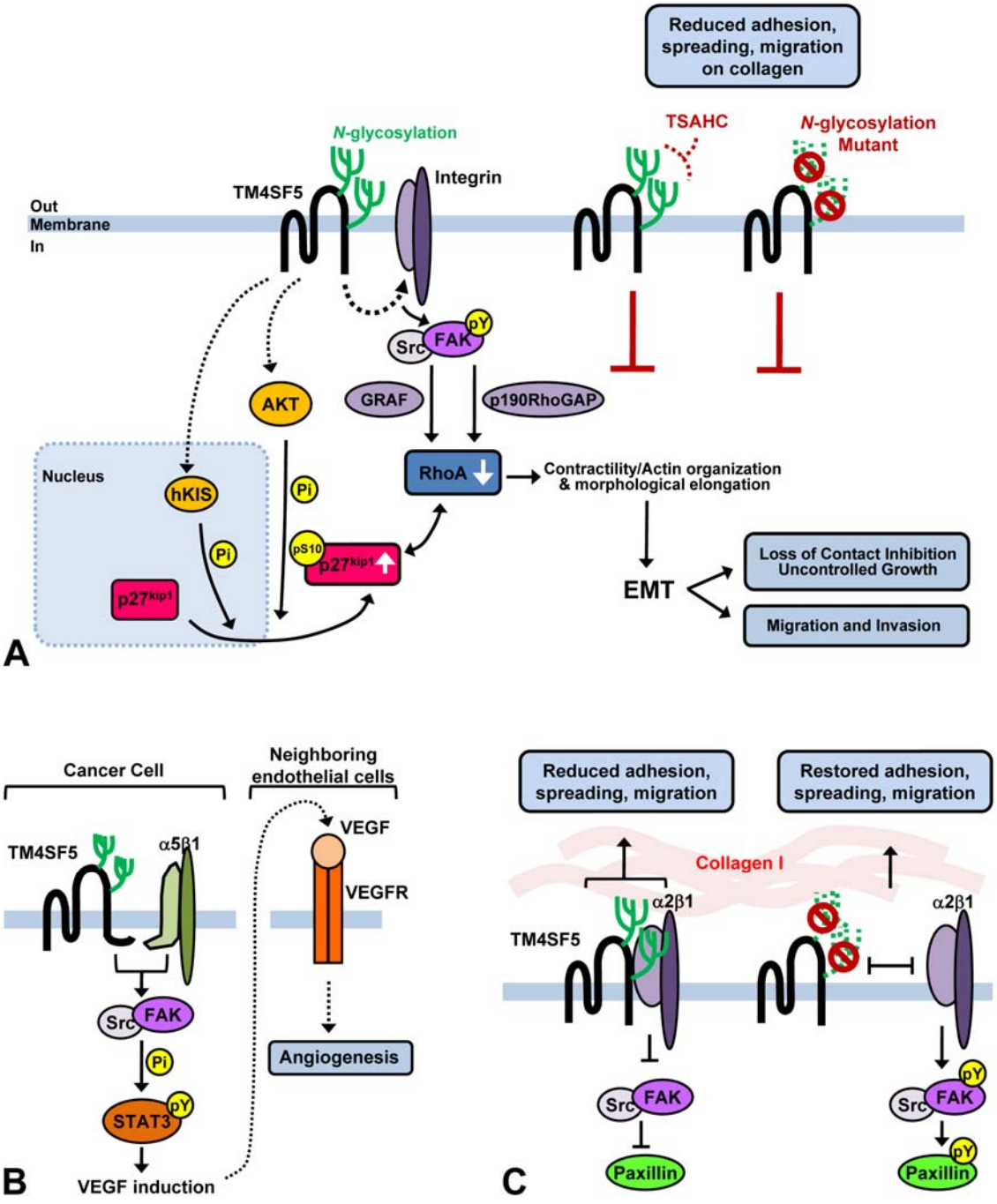


Figure 2. Regulation of cell functions through cross-talks between TM4SF5 and integrins. (A) Expression of TM4SF5 in epithelial cells results in stabilization of $p27^{Kip1}$ in cytosol, where it can bind to and inactivate RhoA. In addition, TM4SF5 can lead to activation of FAK, which can associate with RhoGAPs resulting in RhoA inactivation. Inactive RhoA can alter actin remodeling for morphological elongation, which can affect cell-cell adhesion leading to EMT. EMT in TM4SF5 expressing cells enhances migration and causes loss of contact inhibition leading to multilayer growth. Such TM4SF5-mediated growth in multilayer is inhibited by an anti-TM4SF5 reagent, TSAHC (see later) or point mutation in the *N*-glycosylation residues. (B) TM4SF5 on cancer cells collaborates with integrin alpha5 leading to activation c-Src/FAK/STAT3 pathway for VEGF induction and secretion. Secreted VEGF can influence the endothelial cells (in neighbor) to cause VEGF-enhanced angiogenic activities. (C) Especially in the environment with collagen I, TM4SF5-expressing cells shows a reduced adhesion, spreading and migration, presumably via a restricted function of integrin alpha2 that extracellularly binds to TM4SF5 (left). Disruption of the interaction between integrin alpha2 and TM4SF5 recovers the efficient integrin-mediated intracellular signaling activity, spreading, and migration (right).

positive cells being unable to sense neighboring cells. It is likely that TM4SF5-postive cells can maintain normal activity to progress through the cell cycle by overcoming contact inhibition. It is indeed observed that TM4SF5 expression accelerates G1 to S phase progression by shortening the G1 phase period (14). It was previously shown that active RhoA controls the timing of cyclin D1 expression to mid G1 (15). Cells overexpressing TM4SF5 show piled-up nuclei, normal S phase progression and continuous proliferation under confluent conditions, anchorage-independent growth, and tumor formation in nude mice (11). However, TM4SF5-mediated uncontrolled cell growth and tumor formation in nude mice are inhibited by silencing of TM4SF5 or $p27^{kip1}$, activation of RhoA, and re-expression of E-cadherin (11). These findings indicate that TM4SF5 plays a role in tumorigenesis by increasing cytosolic $p27^{kip1}$ stability and inactivating the RhoA pathway, inducing loss of contact inhibition through actin reorganization. TM4SF5 is upregulated in human hepatocarcinoma tissues (11), colon carcinoma (Lee SA and Lee JW, unpublished data), and various cancer types (16-18), providing evidence that TM4SF5 may function during tumorigenesis.

3.4. TM4SF5-mediated cell migration and invasion

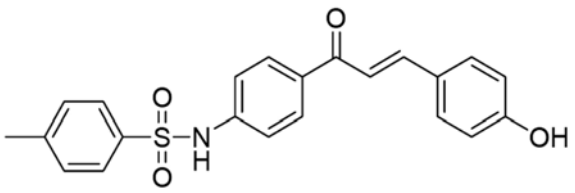
Metastasis is a complex, multistep cascade of cellular events including tumor cell migration through the surrounding stroma, entry into the circulatory system and finally arrest, extravasation and growth at a distant secondary site (19). These processes are involved in cell motility. TM4SFs form complexes with integrins to collaboratively regulate cell motility (7, 9). The genuine tetraspanin CD151 modulates integrin-dependent cell spreading, migration, and signaling, and strengthens adhesion by linking laminin-binding integrins such as alpha3beta1 and alpha6beta4 (20-22). In hepatocytes, TM4SF5 expression enhances chemotactic migration with enhanced directionality and speed of migration. TM4SF5 expression allows enhanced invasion through 3D collagen I and matrigel environments by MMP activation and invadopodia formation, which are actin-enriched structural units that degrade ECM (23). Furthermore, TM4SF5 appears to recruit or co-localize with Arp2/3, N-WASP, and cortactin in lammelipodia presumably to mediate actin organization, and in invadopodia to enhance invasive protrusions (24). Arp2/3 has been shown to interact with FAK (25) and we have also observed that TM4SF5 expression leads to FAK activation (11). Therefore, it is likely that TM4SF5-mediated migration/invasion can involve dynamic actin organization also by FAK-mediated signaling pathways.

3.5. TM4SF5-mediated angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is crucial for many physiological and pathological situations such as tumor growth (26, 27). Angiogenesis involves coordinated endothelial-cell proliferation, migration, and tube formation, which depend on roles of integrin adhesion receptors (28). TM4SFs are involved in angiogenesis and tumorigenesis. TM4SF1 (known as L6) interacts with integrin subunits in an endothelial cell activation-dependent manner and affects cell motility, and TM4SF1 suppression inhibits maturation of $VEGF\text{-}A^{164}$-induced angiogenesis (29). The genuine tetraspanin CD151 affects endothelial cell spreading, migration, and invasion, and its ablation shows impaired pathological angiogenesis through disrupted association between laminin-binding integrins and other tetraspanins (30). Similarly, TM4SF5 overexpression in hepatocytes results in enhanced surface retention of integrin alpha5 through intracellular association between the TM4SF5 and integrin alpha5 cytoplasmic tails, and activation of FAK/c-Src and signal transducer and activator of transcription 3 (STAT3). Eventually, TM4SF5 induces VEGF expression and secretion, leading to enhanced angiogenic activities of (presumably neighboring) endothelial cells, including endothelial cell survival, growth, and tube formation (Figure 2B). TM4SF5 overexpression correlates with enhanced vessel formation in clinical hepatocarcinoma samples, suggesting that TM4SF5 is pro-angiogenic (31).

3.6. Cross-talks between TM4SF5 and integrins

TM4SFs cooperate with integrins by physical association (7). Various integrins, including alpha3beta1, alpha6beta1, alpha4beta1, alpha2beta1, alpha5beta1, alphaLbeta2, and alphaIIbbeta3, may associate with one or more TM4SF proteins, including CD9, CD53, CD63, CD81, CD82, CD151, and NAG-2 (8, 32). Many studies clearly demonstrate that the function of integrin-TM4SF protein complexes is particularly relevant to cell adhesion, spreading, and migration. For example, CD151 affects integrin-dependent cell spreading and morphogenesis on matrigel basement by interacting with integrin alpha6beta1 (33). In melanoma cells, CD63 suppresses cell motility on various ECM substrates (34). Similarly, monoclonal antibodies to CD9, CD53, CD81, and CD82 inhibit integrin-mediated cell migration (35-37). Many previous reports suggest that integrin-TM4SF protein associations appear to depend on detergents included in the lysis buffer during cell extract preparations. TM4SF5 interacts with the cytoplasmic tail of integrin alpha5 when SNU449 hepatocyte extracts were prepared by using a Brij58-containing lysis buffer (31). However, TM4SF5 associates with integrin alpha2beta1 in hepatocytes but not with alpha3, alpha5, or alpha6 in cells lysed with SDS-containing buffer (38). Through the extracellular interactions between the EC2 of TM4SF5 and integrin alpha2, TM4SF5 in hepatocytes inhibits integrin alpha2-mediated adhesion and spreading on and migration toward collagen I (Figure 2C). This interaction is affected by status of *N*-glycosylation within EC2 or structural integrity of EC2 of TM4SF5 (38). Disturbing the association between TM4SF5 and integrin alpha2 using a peptide, a pseudo-binding partner since it is a part of the EC2, results in recoveries in spreading on and migration toward collagen I (38). The interaction between TM4SF5 and integrin alpha2also occurs in Cos7 fibroblasts. TM4SF5 regulates actin remodeling and focal adhesion dynamics through cross-talk with integrin alpha2, which is negatively controlled by serum treatment (10). These studies suggest that TM4SF5 is also capable of modulating the signaling properties of integrins through physical association.



4'-(*p*-toluenesulfonylamido)-4-hydroxychalcone (**TSAHC**)

Figure 3. Structure of an anti-TM4SF5 compound, TSAHC. TSAHC depicts 4'-(*p*-toluenesulfonylamido)-4-hydroxychalcone**.**

3.7. TM4SF5-mediated communication with tumor microenvironment

Tumor initiation and progression involve complex communications between tumor cells and their microenvironment including cytokines, ECM, growth factors, and neighboring cells (39). The communication is transmitted by membrane receptors including integrins, growth factor receptors, and tetraspanins. Numerous studies provide evidence that TM4SF proteins cooperate with integrins for communication with the microenvironment. For example, when MDA-MB-231 breast cancer cells were cocultured with human endothelial cells, CD151 contributed to tumorigenesis through the interaction with integrin alpha3beta1 and alpha6beta4 on epithelial cells, indicating that CD151 sensitizes tumor cells to soluble factors secreted by endothelial cells (40). In keratinocytes, CD9 associates with alpha6beta4 to regulate integrin function, affecting keratinocyte motility on collagen (41). Similarly, TM4SF5 inhibits cell spreading and migration on collagen I through its interaction with alpha2beta1. In TM4SF5, putative *N*-glycosylation sites are presumably important for interaction with other membrane proteins; *N*-glycosylation mutants do not inhibit the function of integrin alpha2beta1 to ligand collagen I (38) (Figure 2C). TM4SF5 causes retention of integrin alpha5beta1 on the cell surface, and mediates FAK/c-Src/STAT3 signaling to induce and secrete VEGF, which in turn promotes angiogenic activities in neighboring endothelial cells. Blocking of cell surface integrin alpha5 using a monoclonal antibody abolished TM4SF5-mediated activation of VEGF transcription and secretion. Furthermore, antibodies blocking integrin alpha5 or VEGF inhibits tumor growth into nude mice xenografts (31). TM4SF5 on fibroblasts regulates integrin-mediated signaling through association with integrin alpha2, and the association is negatively regulated by growth factor receptor signaling (10). TM4SF5 can thus facilitate efficient communication between cancer cells and their extracellular environments by regulating membrane receptors on the cell surface (Figure 2B).

4. INHIBITION OF TM4SF5

4.1. TSAHC, an anti-TM4SF5 reagent

Chalcones are common precursors to the flavonoid family, which have been investigated as anticancer agents (42-44). Certain novel chalcones, chalcone derivatives or chalcone analogues, shows different biological activities on sensitive cancerous cells (45-47). Recently, our group identified a synthetic chalcone compound 4'-(*p*-toluenesulfonylamido)-4-hydroxychalcone (TSAHC, Figure 3) that functions as an anti-TM4SF5 reagent (6). TSAHC blocks TM4SF5-mediated EMT, aberrant growth, G1/S phase progression, migration/invasion, and tumor growth in nude mice (6). TSAHC is a chalcone functionalized with a 4'- *p*-toloenesulfonylamido group that is critical for its anti-TM4SF5 activity. In a structure-activity relationship study, chalcones lacking the *p*-toluenesulfonylamido group and/or 4-hydroxy group do not inhibit TM4SF5-mediated effects, implying that these structural features are important. TM4SF5-mediated effects depend on the state of EC2 *N*-glycosylation, which is involved in protein-protein interaction (6) or structural integrity of the EC2. TSAHC may target *N*-glycosylation or disturb the structure of TM4SF5 EC2 (6, 38). TSAHC altered the sensitivity of the *N*-glycosylated moiety of TM4SF5 to treatment with exogenous endoglycosidase and that mutation of the putative *N*-glycosylation residues abolished TM4SF5-mediated effects. TSAHC thus functions as a specific anti-TM4SF5 agent and can be a potential therapeutic agent for tumors overexpressing TM4SF5.

4.2. Therapeutic approaches targeting TM4SF5-positive tumor

In 1998, *TM4SF5* was first identified as a homologue to tumor associated antigen L6 that is an overexpressed in a number of tumor types. Several studies suggest that TM4SF5 is highly expressed in liver, pancreatic, gastric, colon, papilla vateri carcinoma, soft-tissue sarcoma, nonendocrine lung, and ACTH (corticotropin)-negative bronchial carcinoid tumors (1, 16-18). A clinical study reported that *TM4SF5* is highly expressed in tumors from deceased breast cancer patients, compared to those from 10-year breast cancer survivors (48), suggesting that TM4SF5 overexpression correlates with poor prognosis. Using an *in vivo* experimental model, mice bearing liver injected with TM4SF5-overexpressing cancer cells showed reduced survival time, compared to mice with TM4SF5-null cancer cell-injected liver. TSAHC-administrated mice with TM4SF5-positive cancer cell-injected liver survived approximately 2-fold longer than mice without TSAHC treatment (6). TSAHC was also shown to block diverse TM4SF5-mediated tumorigenic effects *in vitro*. In addition to TSAHC compound, a peptide corresponding to a part of the EC2 in TM4SF5 also affects the cross-talk between TM4SF5 and integrin alpha2beta1, eventually leading to regulation of cell spreading on, migration toward, and invasion through collagen I (38). Therefore, TM4SF5 is a very good candidate for a prognostic and diagnostic marker. A reagent that modulates *N*-glycosylation or other structural aspects of the EC2 in TM4SF5, such as TSAHC and a peptide (38), can be promising for the development of therapeutic reagents against TM4SF5-mediated tumors. Because cells lacking TM4SF5 are insensitive to TSAHC, this approach can be expected as a promising targeting approach for the treatment of TM4SF5-positive tumors.

5. FUTURE PERSPECTIVE

Since the original identification of TM4SF5 as a homologue of tumor antigen L6 (TM4SF1), significant

progress has been made towards understanding the cellular functions of TM4SF5 and its role in tumorigenesis. Evidence suggests that TM4SF5 plays a significant role in EMT and loss of contact inhibition, and it can regulate integrin-mediated signaling pathways through physical interaction. We are currently exploring the detailed structure of the EC2 and of TM4SF5 to clarify the mechanisms to activate FAK (11) signaling, cross-talk with other membrane receptors, and TSAHC-mediated antagonism. In addition, we are exploring the conditions that induce expression of TM4SF5, during pathological situations. Understanding induction of TM4SF5 and its functions will enable us to develop future therapeutic reagents against TM4SF5-positive cancers.

6. ACKNOWLEDGEMENTS

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Abbreviations: Arp: actin-related protein; CD: cluster of differentiation; EC2: the 2nd extracellular loop; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; FAK: focal adhesion kinase; GRAF: GAP for Rho associated with focal adhesions; HGF: hepatocyte growth factor; MMP: matrix metalloproteinase; N-WASP: neuronal Wiskott-Aldrich syndrome protein; SDS: sodium dodecyl sulfate; alpha-SMA: alpha-smooth muscle actin; STAT3: signal transducer and activator of transcription 3; TERM: tetraspanin-enriched microdomain; TM4SF: transmembrane 4 superfamily; TM4SF5: transmembrane 4 L six family member 5; TSAHC: 4'-(*p*-toluenesulfonylamido)-4-hydroxychalcone; VEGF: vascular endothelial growth factor; ZO1: zonula occludens-1

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