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TMED10 Protein Interferes with Transforming Growth Factor (TGF)- β Signaling by Disrupting TGF- β Receptor Complex Formation^{*[S]}

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The intensity and duration of TGF- β signaling determine the cellular biological response. How this is negatively regulated is not well understood. Here, we identified a novel negative regulator of TGF- β signaling, transmembrane p24-trafficking protein 10 (TMED10). TMED10 disrupts the complex formation between TGF- β type I (also termed ALK5) and type II receptors (T β RII). Misexpression studies revealed that TMED10 attenuated TGF- β -mediated signaling. A 20-amino acid-long region from Thr⁹¹ to Glu¹¹⁰ within the extracellular region of TMED10 was found to be crucial for TMED10 interaction with both ALK5 and T β RII. Synthetic peptides corresponding to this region inhibit both TGF- β -induced Smad2 phosphorylation and Smad-dependent transcriptional reporter activity. In a xenograft cancer model, where previously TGF- β was shown to elicit tumor-promoting effects, gain-of-function and loss-of-function studies for TMED10 revealed a decrease and increase in the tumor size, respectively. Thus, we determined herein that TMED10 expression levels are the key determinant for efficiency of TGF- β receptor complex formation and signaling.

Transforming growth factor (TGF)- β is a secreted cytokine that modulates proliferation, differentiation, apoptosis, immune responses, and other cellular functions in many different cell types in a highly context-dependent manner (1–5). Other secreted proteins, including bone morphogenetic proteins (BMP)³ and activins, are similar to TGF- β in their protein structure and function; TGF- β -related proteins are collectively termed the TGF- β family (6). The TGF- β /Smad pathway is initiated by ligand binding to TGF- β type II receptor (T β RII), in which the intracellular domain has constitutive serine/threonine kinase activity (7). Subsequently, T β RII forms a heteromeric complex with TGF- β type I receptor (T β RI or activin receptor-like kinase 5 (ALK5)) and phosphorylates serine and threonine residues within the glycine-serine repeat (GS) domain and is present at the juxtamembrane region in ALK5 (8). Then, the intracellular serine/threonine kinase activity in ALK5 becomes active. The active ALK5 kinase catalyzes the phosphorylation of the two extreme C-terminal serine residues of certain receptor-regulated (R-Smad) proteins, *i.e.* Smad2 and Smad3 (7). These phosphorylated R-Smad form a binary or ternary complex with common-mediated Smad (Co-Smad, *i.e.* Smad4), which is followed by their translocation to the nucleus, where they transcriptionally regulate TGF- β target genes together with other transcriptional factors, coactivators, and corepressors (9–11). Besides the canonical Smad pathway, the TGF- β receptor complex can also initiate intracellular non-Smad pathways. Mitogen-activated protein kinases (MAPK) including Erk, JNK, p38, PI3K/Akt, RhoA/Rock1, mTORC, Par6, and Shc become activated by TGF- β contextually in certain cell types (12–16). In some cases, non-Smad pathways

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[S] This article contains supplemental Fig. 1.

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³ The abbreviations used are: BMP, bone morphogenetic protein; T β RI, TGF- β type I receptor; T β RII, TGF- β type II receptor; ALK, activin receptor-like kinase; ALK5ca, constitutively active ALK5; R-Smad, receptor-regulated Smad protein(s); kd, kinase-dead; ER, endoplasmic reticulum; SBE, Smad-binding element; BAMBI, BMP and activin membrane-bound inhibitor; pAb, polyclonal antibody; qPCR, quantitative real-time PCR; co-IP, coimmunoprecipitation.

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cooperate with the Smad pathway to adequately elicit TGF- β -dependent responsiveness in cells (17).

TGF- β signaling is of key importance in embryogenesis and tissue homeostasis; hence, dysregulation of the TGF- β signaling pathway invokes congenital abnormalities as well as a number of diseases including cancer, fibrosis, and vascular defects (18–22). Thus, TGF- β signaling needs to be intricately regulated. This intricate regulation occurs at all steps in the TGF- β signal transduction cascade, for example, in extracellular environments in which the bioavailability of the ligand is controlled by extracellular matrix ligand-binding proteins (23), ligand traps (2, 24), auxiliary coreceptors (2, 25), and decoy receptors (26). Frequently, these negative extracellular and intracellular regulators are induced by TGF- β , thereby exerting negative feedback functions. Consequently, cells are prohibited from being exposed by an excessive intensity and duration of TGF- β signaling (27, 28).

Transmembrane p24-trafficking protein 10 (TMED10), alternatively termed p23, TMP21 (transmembrane protein with type I topology 21), Tmp-21-I, and p24 δ , is a member of the EMP24 (endomembrane protein precursor of 24 kDa)/GP25L (glycoprotein 25L)/p24 family, which is involved in COP (coat protein) vesicle cargo receptors (29). TMED10, a type I transmembrane protein, is located to the plasma membrane, microsomal membranes, and zymogen granule membranes (30). Indeed, it has been reported that TMED10 contributes to the recruitment of the small GTPase ADP-ribosylation factor 1 (ARF1) to the Golgi apparatus owing to actin assembly (31–33). TMED10 has roles that extend beyond trafficking; for example, TMED10 interacts with presenilin complexes to modulate the activity of γ -secretase without any effect of ϵ -secretase in decreasing the secretion of amyloid- β (34, 35). Thus, TMED10 may be involved in Alzheimer disease (36). In addition, TMED10 can limit the activity of PKC δ via its association with PKC δ in the prostate cancer cell line LNCaP to inhibit apoptosis (37, 38) and activate the AMPK/mTOR (mammalian target of rapamycin) pathway to modulate cell growth (39). Furthermore, TMED10 preferentially binds to MHC I heavy chains that dissociate with β 2-microglobulin (40). Therefore, TMED10, dependent on the context, may act as a multipotent protein in cells.

In this study, we found that TMED10 attenuates TGF- β signaling via dissociation of the TGF- β type I/type II heteromeric receptor complex. In particular, the extracellular domain of TMED10 is required for TMED10 to bind to both T β R II and ALK5. Interestingly, the short peptide derived from the extracellular domain of TMED10 can antagonize TGF- β signaling. Thus, a low molecular weight mimetic based on the structure of this peptide might be therapeutically suitable for patients with diseases with underlying excessive TGF- β receptor signaling.

Results

Identification of ALK5-interacting Proteins—To elucidate the regulatory mechanisms that underlie TGF- β signaling via modulation of TGF- β receptor activity, we identified putative ALK5-interacting proteins by high throughput analysis of pro-

teins coimmunoprecipitated with epitope-tagged human constitutively active ALK5 (ALK5ca) in HEK293 cells by use of liquid chromatography-mass spectrometry and liquid chromatography (LC-MS/MS) analysis. One of the proteins we identified was TMED10. It was selected from among 13 candidates because TMED10 was always isolated using the above method in four independent experiments. To validate this finding, we analyzed whether TMED10 interacts with human ALK5ca when coexpressed in COS7 cells (Fig. 1A). We also tested whether the kinase activity of ALK5 influences its interaction with TMED10. We found that the interaction of TMED10 with ALK5ca was weaker than that with kinase-dead ALK5 (ALK5kd) (Fig. 1A). To investigate the specificity of the interaction of TMED10 with ALK5, we also investigated whether ALK6, a BMP type IB receptor, forms a complex with TMED10 upon their coexpression in COS7 cells. The interaction of TMED10 with either kinase-dead ALK6 (ALK6kd) or constitutively active (ALK6ca) was only marginally observed. In addition, TMED10 possessed the subtle ability to bind to T β R II and kinase-dead T β R II (T β R II kd) (Fig. 1, A and B). These results indicate that TMED10 has selectivity to interact with ALK5 when compared with other tested type I and type II receptors. Next, we performed affinity-labeling experiments using ^{125}I -TGF- β in COS1 cells transfected with TMED10, ALK5 (or its kinase-inactive mutant), T β R II (or its kinase-inactive mutant), or combinations thereof. The kinase activity of ALK5 was not required for ALK5 to interact with TMED10, whereas the loss of T β R II kinase activity made the interaction between T β R II and TMED10 weaker. The reason that the intensity of the band corresponding to T β R II covalently bound to ^{125}I -TGF- β becomes strong is that T β R II alone, but not ALK5 alone, can bind to TGF- β (Fig. 1C) (41, 42). On the other hand, we could not detect any interaction between TMED10 and ^{125}I -TGF- β when TMED10 alone was transfected in COS1 cells (Fig. 1D). Furthermore, we tried to show endogenous interaction between TGF- β receptors and TMED10 using ^{125}I -TGF- β in HaCaT cells. The immunoprecipitation of the receptor complex with a rabbit anti-TMED10 antibody could be detected, although rabbit anti-HA antibody as a control antibody could not isolate any receptor complex (Fig. 1E). Thus, TMED10 endogenously interacts with the TGF- β receptor complex on the cell membrane.

The physiological interaction between TMED10 and ALK5 prompted us to examine their subcellular colocalization. When human embryonic retinoblast 911 cells were transfected with both TMED10 and ALK5, both of them could be seen mainly in the cytosol (Fig. 1F, *upper panels*), because both proteins are synthesized in the rough endoplasmic reticulum (ER) and transported to the cell membrane via the Golgi apparatus as transmembrane proteins. However, the vertical image shows that their colocalization can be observed along the cell membrane in part (Fig. 1F, *insert in lower right panel*). These data suggest that TMED10 associates with ALK5 at the cell membrane.

ALK5 and T β R II are present as homodimers at the cell surface (43). To examine whether this is also the case for TMED10, we transfected two differentially epitope-tagged TMED10 (TMED10/FLAG and TMED10/V5) (Fig. 1H) in COS7 cells was

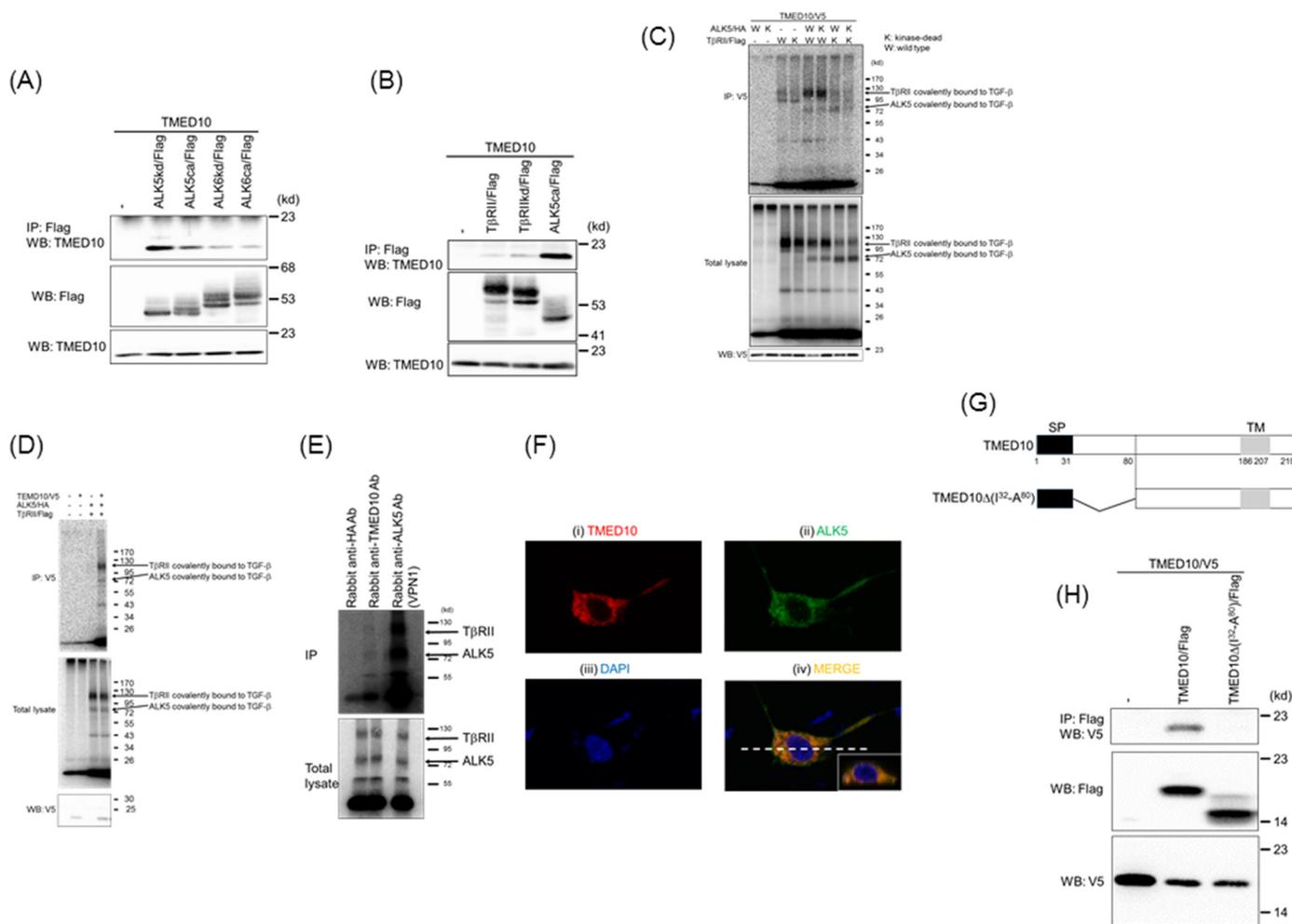


FIGURE 1. Interaction of TMED10 with TGF- β receptors. *A*, interaction of TMED10 with ALK5. COS7 cells were transfected with the indicated plasmids and harvested for coimmunoprecipitation (co-IP) experiments. The interaction between TMED10 and either ALK5 or ALK6 is shown in the *top panel*. The total expressions of FLAG-ALK and TMED10 are indicated in the *middle and bottom panels*, respectively. *B*, interaction of TMED10 with T β RII. COS7 cells were transfected with the indicated plasmids and harvested for the co-IP experiments. The interaction of TMED10 with T β RII is shown in the *top panel*. The total expressions of FLAG-T β RII and TMED10 are indicated in the *middle and bottom panels*, respectively. ALK5ca/FLAG was used as the positive control. *C*, association of TMED10 with the TGF- β receptor complex. COS1 cells were transfected with the indicated plasmids. Forty hours after transfection, the TGF- β receptors were covalently affinity-labeled with iodinated TGF- β . The cell lysates were subjected to immunoprecipitation with an anti-V5 antibody and analyzed by SDS-PAGE and a PhosphorImager (*top panel*). The expression of the receptors was determined by analyzing an aliquot of cell lysates by SDS-PAGE without immunoprecipitation (*middle panel*). The expression of TMED10 was detected by Western blotting analysis (WB) with an anti-V5 antibody using cell lysates without the addition of iodinated TGF- β (*bottom panel*). *D*, no direct interaction between TMED10 and TGF- β . COS1 cells were transfected with the indicated plasmids. Forty hours after transfection, iodinated TGF- β was added to the medium followed by covalent binding between cell surface molecules and iodinated TGF- β with disuccinimidyl suberate. The cell lysates were subjected to immunoprecipitation with an anti-V5 antibody and analyzed by SDS-PAGE and a PhosphorImager (*top panel*). The expression of receptors was determined by analyzing an aliquot of cell lysates by SDS-PAGE without immunoprecipitation (*middle panel*). The expression of TMED10 was detected by Western blotting analysis with an anti-V5 antibody using cell lysates without addition of iodinated TGF- β (*bottom panel*). *E*, endogenous interaction between TMED10 and TGF- β receptors. After iodinated TGF- β was added to the media in which HaCaT cells had been cultured, the cell lysates were prepared. The cell lysates were immunoprecipitated with anti-TMED10 antibody followed by SDS-PAGE. Expression of the receptors was determined by analyzing an aliquot of cell lysates by SDS-PAGE without immunoprecipitation. As the negative and positive controls, anti-HA (Y-11) and anti-ALK5 (VPN1) antibodies were used for immunoprecipitation. *F*, colocalization of TMED10 with ALK5. TMED10/V5 was transfected with ALK5/HA in 911 cells. Twenty-four hours after transfection, the cells were stimulated with 5 ng/ml TGF- β for 2 h. Subsequently, the cells were fixed and stained with mouse anti-V5 monoclonal and rabbit ALK5 (V-22) polyclonal antibodies. Then, Alexa 555-conjugated goat anti-mouse (i) and CF488A goat anti-rabbit IgG antibodies (ii) were used for visualization. Colocalization can be seen in the *merged panel* as the yellow portion (iv). Nuclear staining (blue) was carried out using DAPI (iii). The *insert* in *panel iv* shows the vertical image. *G*, schematic presentation of TMED10 Δ (I³²-A⁸⁰). SP, signal peptide; TM, transmembrane. *H*, homodimer formation of TMED10. COS7 cells were transfected with the indicated plasmids and harvested for the co-IP experiments. The homodimer formation of TMED10 is shown in the *top panel*. The total expressions of TMED10/FLAG or its mutant and TMED10/V5 are indicated in the *middle and bottom panels*, respectively.

followed by coimmunoprecipitation with an anti-FLAG antibody and Western blotting analysis with an anti-V5 antibody. The result showed evidence for homo-oligomerization of TMED10. On the other hand, a mutant TMED10 containing an internal deletion of TMED10 between Ile³² and Ala⁸⁰ (Fig. 1G, TMED10 Δ (I³²-A⁸⁰)) was unable to interact with wild-type TMED10 (Fig. 1H). Taken together, these results suggest that

the N-terminal region of TMED10 plays a key role in the homo-oligomerization of TMED10.

Negative Regulation of TGF- β Signaling by TMED10—To investigate whether TMED10 affects TGF- β signaling, we tested the effect of TMED10 on the TGF- β -induced Smad-driven transcriptional (SBE)₄-luc reporter (44), we and observed dose-dependent inhibition of TMED10 (Fig. 2A). Interestingly,

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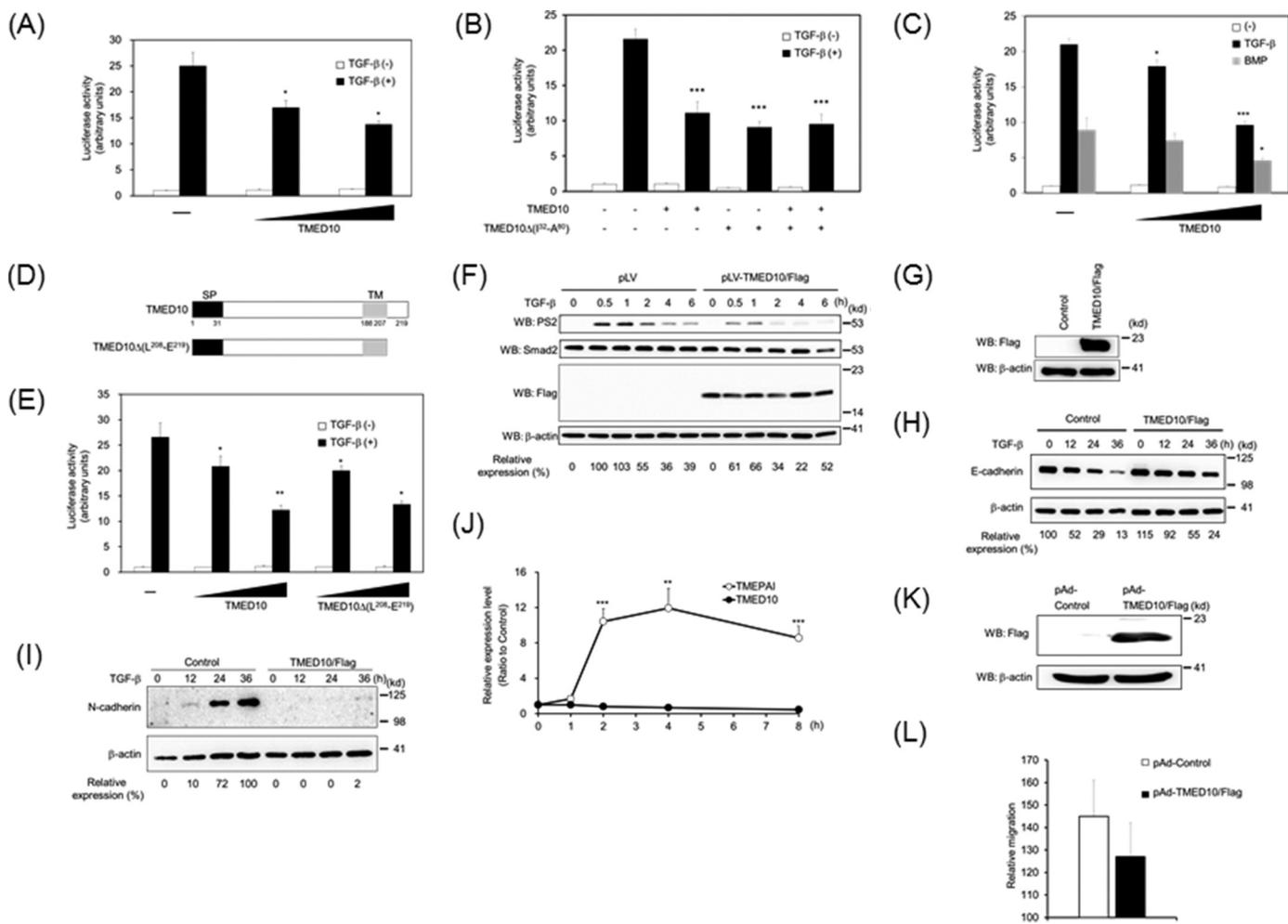


FIGURE 2. Inhibitory action of TMED10 on TGF- β signaling. A, dose-dependent inhibition of TGF- β -driven reporter activity by TMED10. HepG2 cells were transfected with (SBE)₄-luc, which can be activated upon not only by TGF- β but also BMP signals (44), pCH110, and the indicated plasmids at two different doses. Twenty-four hours later, the cells were stimulated with 5 ng/ml TGF- β for 18 h. Significant differences from the control in the presence of TGF- β are indicated with *asterisks*. B, effect of TMED10 Δ (I³²-A⁸⁰) on the activity of TGF- β -driven transcriptional reporter. The experiments were performed as described in A. The combined total amount of TMED10 with TMED10 Δ (I³²-A⁸⁰) was the same in all columns. Significant differences from the control in the presence of TGF- β are indicated with *asterisks*. C, inhibitory ability of TMED10 on BMP signaling. The experiments were performed as described in A except for the addition of 25 ng/ml BMP-6. Significant differences from the control in the presence of TGF- β or BMP are indicated with *asterisks*. D, illustration of C-terminal deletion of TMED10. SP, signal peptide; TM, transmembrane E, dispensation of the C-terminal end of TMED10 for its inhibitory action. The experiments were performed as described in A. Significant differences from the control in the presence of TGF- β are indicated with *asterisks*. F, inhibition of Smad2 phosphorylation by TMED10 in HaCaT cells. HaCaT cells carrying TMED10/FLAG by the method of lentiviral gene transfer were stimulated with 0.5 ng/ml TGF- β for the indicated times. After preparation of the cell lysates, anti-phosphorylated Smad2 (PS2) (top panel), anti-Smad2 (second panel), anti-FLAG (third panel), and anti- β -actin antibodies (bottom panel) were used for Western blotting analyses (WB). The expression of phosphorylated Smad2 upon TGF- β stimulation was normalized using the intensity of the band corresponding to Smad2. Relative expression was calculated relative to the value for pLV-CMV-IRES-Puro-infected cells in the absence of TGF- β . G, overexpression of TMED10/FLAG by adenoviral transfer. NMuMG cells were infected with TMED10/FLAG-expressing adenovirus. After preparation of the cell lysates, anti-FLAG (top panel) or anti- β -actin antibody (bottom panel) was used. H, extension of E-cadherin expression in NMuMG cells expressing TMED10/FLAG upon TGF- β stimulation. TMED10 were introduced into NMuMG cells by an adenoviral gene transfer system as described in G. Forty hours after infection, the cells were stimulated with 0.5 ng/ml TGF- β for the indicated times. After preparation of the cell lysates, anti-E-cadherin (top panel) and anti- β -actin antibodies (bottom panel) were used for Western blotting analyses. The expression of E-cadherin upon TGF- β stimulation was normalized using the intensity of the band corresponding to β -actin. Relative expression was calculated relative to the value for control cells in the absence of TGF- β . I, inhibition of N-cadherin expression by TMED10 in NMuMG cells. After gene transfer of TMED10/FLAG by adenovirus, the cells were cultured for 40 h. Subsequently, the cells were stimulated with 0.5 ng/ml TGF- β for the indicated times. After preparation of the cell lysates, anti-N-cadherin (top panel) and anti- β -actin antibodies (bottom panel) were used for Western blotting analyses. The expression of N-cadherin upon TGF- β stimulation was normalized using the intensity of the band corresponding to β -actin. Relative expression was calculated relative to the value for control cells in the absence of TGF- β . J, expression of TMED10 mRNA upon TGF- β stimulation. HepG2 cells were stimulated with 5 ng/ml TGF- β at the different time points. After preparation of total RNA from the cells, PCR was carried out using specific primer sets. As the positive control, the *TMEPAI* gene, which is well known as a TGF- β target gene, was used. Before qPCR, the amplified PCR product using each primer set could be seen in the agarose gel as a single band. Significant differences from the cells without the treatment of TGF- β are indicated with *asterisks*. K, overexpression of TMED10/FLAG in A549 cells by adenoviral gene transfer. The experiment was performed as described in G. L, inhibition of TGF- β -induced cell migration by TMED10. After adenoviral infection as described in K, A549 cells were seeded on the upper membrane of the Boyden chamber. Subsequently, 5 ng/ml TGF- β was added to the lower chamber for 18 h. The cells were then stained with hematoxylin/eosin solution, and the number of transmigrated cells was counted. Probability values below 0.05, 0.01, and 0.001 were considered significant: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

TMED10 Δ (I³²-A⁸⁰) could also inhibit TGF- β signaling like TMED10. Thus, the oligomerization of TMED10 is not required for the inhibition of TGF- β signaling (Fig. 2B).

TMED10 also has the ability to interfere with BMP signaling, albeit with relatively weak inhibition (Fig. 2C). Thus, we focused on the inhibitory effect of TMED10 on TGF- β

signaling in the subsequent experiments. To examine the possibility that the short C-terminal intracellular tail of TMED10 plays a role in the perturbation of TGF- β signaling, TMED10 Δ (L²⁰⁸-E²¹⁹) was constructed (Fig. 2D) and then transfected and subsequently stimulated with TGF- β in HepG2 cells. As seen in Fig. 2E, TMED10 Δ (L²⁰⁸-E²¹⁹) had an inhibitory action comparable to that of wild-type TMED10. We further examined whether TMED10 had an ability to hamper TGF- β -mediated Smad2 phosphorylation. Ectopic expression of TMED10 in human keratinocyte HaCaT (Fig. 2F), 293T (data not shown), and mouse mammary gland epithelial NMuMG cells (data not shown) inhibited phosphorylation of endogenous Smad2 upon TGF- β stimulation. It is well known that TGF- β potentiates the epithelial-mesenchymal transition (EMT) in NMuMG cells (45). During this process, a switch from E-cadherin to N-cadherin expression takes place (46). Adenoviral infection of TMED10 in NMuMG cells blocked TGF- β -mediated suppression of E-cadherin expression (Fig. 2, G and H) and TGF- β -mediated induction of N-cadherin expression (Fig. 2I).

Negative regulators of TGF- β signaling such as *Smad7*, *SnoN*, *TGIF*, *Smurf1*, and *TM6PA1* are known to be direct target genes of TGF- β signaling (28). However, TGF- β did not influence the expression of TMED10 mRNA, whereas it did influence the expression of *TM6PA1* mRNA (Fig. 2J).

Because cell motility is potentiated in some epithelial cells by TGF- β , we tried to confirm that TMED10 inhibits cell migration upon TGF- β stimulation. In the Transwell assay, we observed a trend for the inhibition by TMED10 on TGF- β -induced cell migration when TMED10 was overexpressed by adenoviral gene transfer (Fig. 2, K and L).

Enhancement of TGF- β Signaling due to Knockdown of TMED10—Because of the inhibitory effect of TMED10 on TGF- β signaling, we investigated whether loss-of-function of TMED10 enhances TGF- β signaling. When we reduced the expression of TMED10 in HaCaT cells by lentiviral transfer of TMED10-specific shRNA (Fig. 3A), an extension of TGF- β -induced Smad2 phosphorylation could be observed (Fig. 3B). Consistently, E-cadherin expression in HaCaT cells carrying shTMED10 decreased more quickly than in HaCaT cells carrying control shRNA when the cells were stimulated with TGF- β (Fig. 3C). We also confirmed the enhancement of stress fiber formation in HaCaT cells carrying TMED10 shRNA (Fig. 3D). Next we explored the effect of TMED10 depletion on cell motility. As seen in Fig. 3, E and F, not only the cell migration (using a Boyden chamber) but also the migration speed, measured by a wound healing assay, was raised, albeit weakly, in TMED10 knocked-down cells when cells received TGF- β signaling. Taken together, these results indicate that TMED10 interferes with TGF- β /Smad signaling.

Disruption of the TGF- β Receptor Complex by TMED10—Having shown that TMED10 can interact not only with ALK5 but also with T β R2, we hypothesized that TMED10 could dissociate the complex between ALK5 and T β R2. Fig. 4A revealed that TMED10 disrupts the TGF- β receptor complex between ALK5 and T β R2 when both receptors are overexpressed together with TMED10 in COS7 cells. After TGF- β binds to T β R2, T β R2 recruits ALK5 to phosphorylate serine and thre-

onine residues in its GS domain close to the juxtamembrane region (47). When ALK5/HA was transfected into 911 cells, we could observe the phosphorylation of serine residues in ALK5 1h after TGF- β stimulation. Co-transfection with TMED10 decreased the level of serine phosphorylation in ALK5 (Fig. 4B). We also checked whether TMED10 could disrupt ALK5 or T β R2 homodimerization. Besides the assembly of heteromeric complexes between ALK5 and T β R2, both ALK5 and T β R2 homodimer formations were attenuated upon introduction of TMED10 into cells (Fig. 4, C and D). Thus, TMED10 inhibits not only ligand-independent homomeric but also TGF- β -induced heteromeric complex formation between ALK5 and T β R2. Because TMED10 blocks TGF- β receptor complex formation, there is a possibility that TMED10 also interferes with non-Smad pathways upon TGF- β receptor activation (12–16). As expected, the TGF- β -mediated JNK and p38 pathways could be inhibited by TMED10 (Fig. 4, E and F).

Determination of the Interaction Domain of TMED10 with TGF- β Receptors—To determine the binding region of TMED10 with the TGF- β receptor, we made several TMED10 mutants, as seen in Fig. 5A. Upon deletion of TMED10 from Gly⁸¹ to Val¹³⁰ (TMED10 Δ (G⁸¹-V¹³⁰)), the interaction between ALK5ca and TMED10 was lost (Fig. 5B). Although TMED10 Δ (V¹⁸⁶-E²¹⁹), which lacks a C-terminal region, became a secretory protein (Fig. 5C, bottom panel), ALK5ca could associate with TMED10 Δ (V¹⁸⁶-E²¹⁹). Thus, the transmembrane and intracellular domains of TMED10 were not required for TMED10 to interact with ALK5 (Fig. 5C). Similarly, T β R2 also required the same 50-amino acid-long region from Gly⁸¹ to Val¹³⁰ in TMED10 to interact with T β R2 (Fig. 5D). To further narrow the binding region of TMED10 with the TGF- β receptor, we performed a pulldown assay using three GST fusion proteins (Fig. 5E). As observed in Fig. 5F, the 30-amino acid-long peptide composed of the region between Gly⁸¹ and Glu¹¹⁰ in TMED10 was sufficient for TMED10 to interact with ALK5. We next divided this region into two fragments, GST-TMED10(81–100) and GST-TMED10(91–110) (Fig. 5G). GST-TMED10(91–110), but not GST-TMED10(81–100) associated with ALK5 (Fig. 5H) as well as with T β R2 (Fig. 5I).

Blockage of TGF- β Signaling by Short Peptides Derived from TMED10—Because the 20-amino acid-long peptide derived from TMED10 is critical for TMED10 to interact with TGF- β receptors, it is possible that the peptides within these 20 amino acids disrupt TGF- β signaling. To test this possibility, three peptides were synthesized to investigate whether they could perturb TGF- β signaling (Fig. 6A). All three peptides inhibited TGF- β -mediated luciferase reporter activity to a certain extent. However, the addition of peptide in the media without Xfect protein transfection reagent did not significantly inhibit this response. Although we do not know the exact reasons, one possibility is that peptides incorporated into the Golgi apparatus and/or ER bind to immature biosynthetic intermediate TGF- β receptors (ALK5 and/or T β R2), thereby leading to decreased homodimer and/or heterodimer formations of TGF- β receptors at the cell surface. Among the three peptides, the peptide composed of 15 amino acids (Ala⁹⁶-Glu¹¹⁰) was the most effective inhibitor (Fig. 6B). We do not know why Thr⁹¹-Glu¹¹⁰ has the weakest inhibitory activity among the three

Inhibition of TGF- β Signaling by TMED10

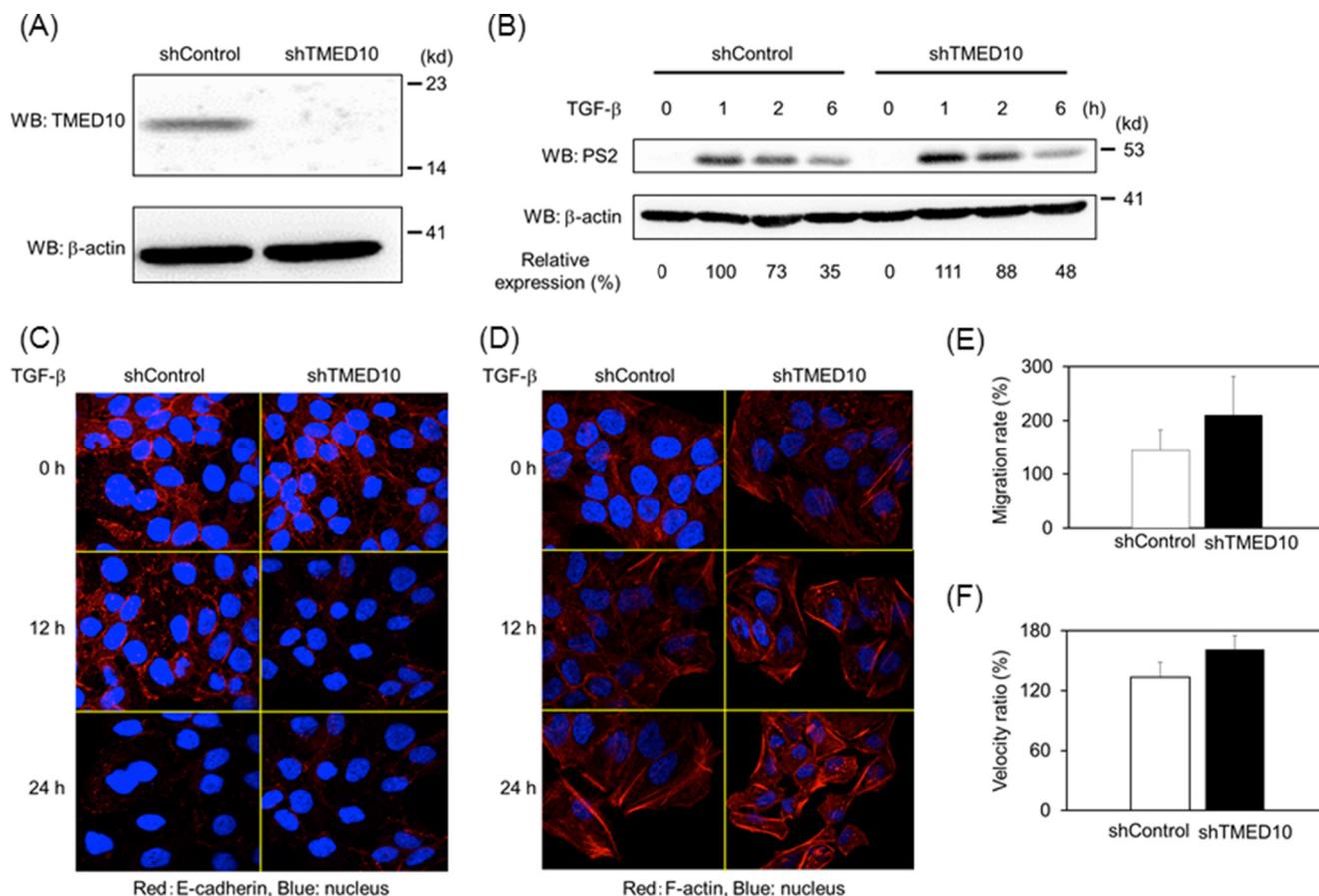


FIGURE 3. Loss-of-function analyses for TMED10. *A*, the expression of TMED10 in HaCaT cells carrying shTMED10 by lentiviral gene transfer. After establishment of shTMED10-expressing HaCaT cells by puromycin selection, the total cell lysates were prepared. Western blotting analyses (WB) were carried out using anti-TMED10 (*top panel*) and anti- β -actin (*bottom panel*) antibodies. *B*, extension of TGF- β -induced Smad2 phosphorylation in shTMED10-expressing HaCaT cells. The cells were stimulated with TGF- β for the indicated times. After cell lysis, Western blotting analysis was performed as described in the legend for Fig. 2*F*. *shControl*, HaCaT cells carrying the empty pLKO.1 vector. The expression of phosphorylated Smad2 upon TGF- β stimulation was normalized using the intensity of the band corresponding to β -actin. Relative expression was calculated relative to the value for HaCaT cells carrying the empty pLKO.1 vector in the absence of TGF- β . *C*, E-cadherin expression by immunofluorescence staining in shTMED10-expressing HaCaT cells. The cells were stimulated with 0.5 ng/ml TGF- β for the indicated times. Subsequently, the cells were fixed and stained with anti-E-cadherin antibody. Then Alexa 555-conjugated goat anti-mouse antibody was used for visualization. Nuclear staining (*blue*) was carried out using DAPI. *D*, stress fiber formation upon TGF- β stimulation in HaCaT cells carrying shTMED10. The experiments were done as described in *D* above, except for the use of Alexa 555-conjugated phalloidin instead of anti-E-cadherin and Alexa 555-conjugated goat anti-mouse antibodies. *E*, TGF- β -mediated chemotaxis in shTMED10-expressing HaCaT cells. Experiments were performed as described in the legend for Fig. 2*L*. The value derived by dividing the cell number in the presence of TGF- β by that in the absence of TGF- β is shown. *F*, TGF- β -induced cell migration in shTMED10-expressing HaCaT cells. Cells were seeded confluent in 12-well plates. After cell scratching, 0.5 ng/ml TGF- β was added to the media. The cell movement was then measured by microscopy. The velocity of the cell movement was calculated. Then the value derived by dividing the velocity in the presence of TGF- β by that in the absence of TGF- β was shown.

peptides. One possibility is that the active site (Ala⁹⁶–Phe¹⁰⁵) might be hidden because of the steric structure of Thr⁹¹–Glu¹¹⁰. Further experiments will be needed to confirm this possibility. When Ala⁹⁶–Glu¹¹⁰ was added to the cells and then stimulated with TGF- β , Smad2 phosphorylation was inhibited (Fig. 6*C*).

Decreased and Enhanced Tumor Formation of JygMC(A) Cells with Gain and Loss of TMED10 Expression in Nude Mice—Mouse mammary carcinoma JygMC(A) cells are known to proliferate in the presence of TGF- β under low serum conditions (48). Additionally, the inhibition of TGF- β signaling in JygMC(A) cells by gene transfer of Smad7 was reported to suppress metastasis *in vivo* as well as in epithelial-mesenchymal transition and cell motility *in vitro* (49). These lines of evidence encouraged us to investigate whether increased and reduced expression of TMED10 in JygMC(A) cells would result in decreased and increased tumorigenicity *in vivo*, respectively.

When we implanted two TMED10-expressing JygMC(A) cells (TMED10#16 and TMED10#18) (Fig. 7*A*) into the mammary fat pad of each nude mouse, the primary tumors from TMED10#16 and TMED10#18 were smaller than those from the control (Fig. 7*B* and Table 1). Next, we established two cell lines with descending levels of TMED10 expression (TMED10#1 and TMED10#2). Then, low expression of TMED10 could be observed in the TMED10#1 cells when compared with the control cells (Fig. 7*C*). Consistently, the TGF- β -induced duration of Smad2 phosphorylation was extended in the TMED10#1 cells (Fig. 7*D*). Subsequently, we injected JygMC(A) cells carrying shTMED10#1 or shControl cells according to the method described in the legend for Fig. 7*B*. The primary tumors in the mammary glands from mice injected with JygMC(A) cells carrying shTMED10#1 were bigger than those carrying control shRNA (Fig. 7*E* and Table 2). These lines of evidence demonstrated that TMED10 is implicated as a negative reg-

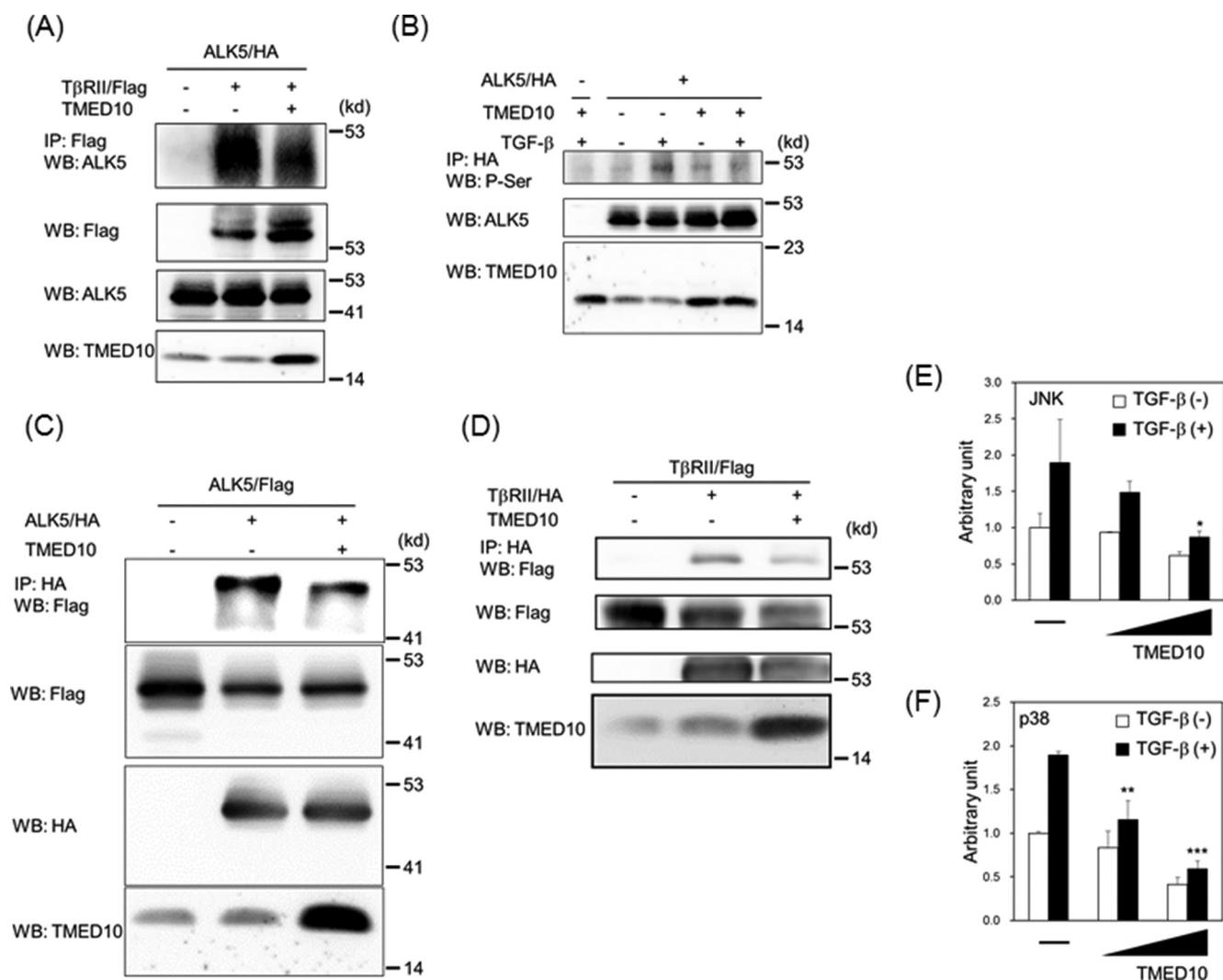


FIGURE 4. Disruption of the TGF- β receptor complex by TMED10. *A*, disruption of the receptor complex between ALK5 and T β RII by TMED10. COS7 cells were transfected with the indicated plasmids and harvested for co-IP experiments using anti-FLAG antibody. The interaction between ALK5 and T β RII is shown in the *top panel*. The total expressions of T β RII/FLAG, ALK5/HA, and TMED10 are indicated in the *second, third, and bottom panels*, respectively. The bands corresponding to TMED10 in the *lane* without overexpression of TMED10 seem to be endogenous TMED10. *B*, inhibition of TGF- β -induced ALK5 phosphorylation by TMED10. The 911 cells were transfected with the indicated plasmids. After the cells had been stimulated with 5 ng/ml TGF- β for 1 h, they were harvested for immunoprecipitation using anti-HA3F10 antibody followed by Western blotting analysis (WB) using anti-pSer antibody (*top panel*). Total expressions of ALK5 (*middle panel*) and TMED10 (*bottom panel*) were detected with anti-ALK5 (V-22) and anti-TMED10 antibodies, respectively. *C*, hampering of homo-oligomer formation of ALK5 by TMED10. The COS7 cells were transfected with the indicated plasmids and harvested for the co-IP experiments using anti-HA3F10 antibody. The homodimer formation of ALK5 is shown in the *top panel*. Total expressions of ALK5/FLAG, ALK5/HA, and TMED10 are indicated in the *second, third, and bottom panel*, respectively. *D*, interference of T β RII homo-oligomer formation by TMED10. The COS7 cells were transfected with the indicated plasmids and harvested for the co-IP experiments using anti-HA3F10 antibody. The homodimer formation of T β RII is shown in the *top panel*. Total expressions of T β RII/FLAG, T β RII/HA, and TMED10 are indicated in the *second, third, and bottom panel*, respectively. *E* and *F*, dose-dependent inhibition of TGF- β -mediated JNK and p38 activities by TMED10. *E*, HepG2 cells were transfected with pFR-luc, pFA2-cJun, and pCH110 (17) together with two different doses of TMED10 expression plasmids. Twenty-four hours later, the cells were stimulated with 5 ng/ml TGF- β for 18 h. *F*, HepG2 cells were transfected with pFR-luc, pFA-CHOP (17), and pCH110 together with two different doses of TMED10 expression plasmids. Twenty-four hours later, the cells were stimulated with 5 ng/ml TGF- β for 18 h. Significant differences from the control in the presence of TGF- β are indicated with asterisks. Probability values below 0.05, 0.01, and 0.001 were considered significant: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

ulator of TGF- β -induced pro-oncogenic signaling in these breast cancer cells.

Discussion

During the more than two decades of study of Smads since they were first discovered, Smad signaling has been characterized as intricately regulated by a great number of molecules (4, 11, 50, 51). Because not only hyper-TGF- β signaling but also hypo-TGF- β signaling is known to be involved in various malformations and severe diseases in humans, it is important to

understand overall TGF- β signaling from the extracellular microenvironment to the nucleus.

Until now, it has been acknowledged that negative regulators of TGF- β family signaling act mainly at the Smad level, including phosphatases, ubiquitin ligases, inhibitory Smads (I-Smads), TMEPAI, c-Ski, and SnoN (27, 28). Only a few molecules, such as BAMBI (BMP and activin membrane-bound inhibitor) (26) and ETV6-NTRK3 (52), have been implicated in the disruption of TGF- β receptor complexes. Therefore, we tried to identify novel interactors for ALK5 through

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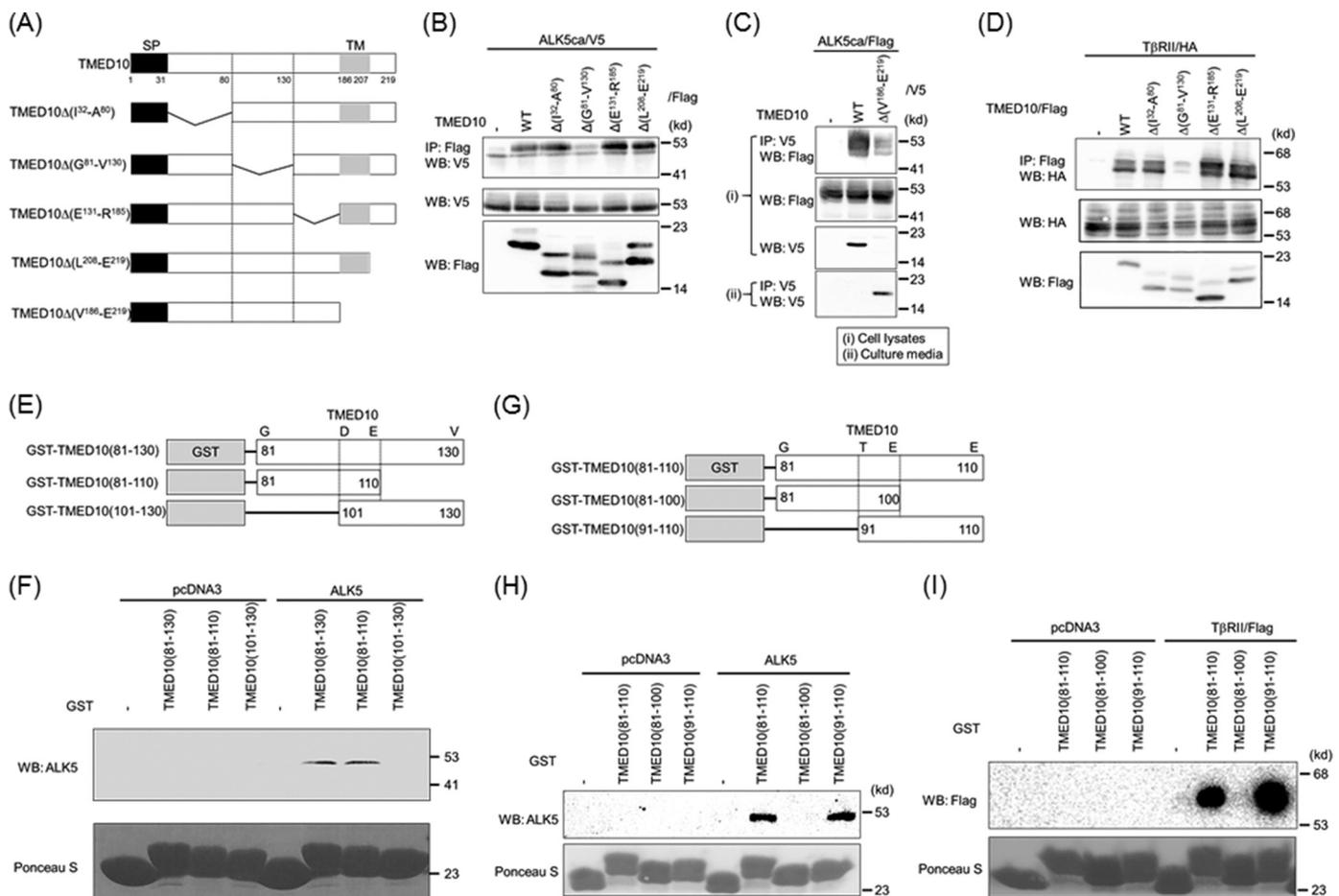


FIGURE 5. Determination of the TGF- β receptor-interacting domain in TMED10. *A*, illustration of TMED10 and its mutants. *SP*, signal peptide; *TM*, transmembrane. *B*, interaction of TMED10 or its mutants with ALK5. COS7 cells were transfected with the indicated plasmids and harvested for co-IP experiments. The interaction between ALK5 and either TMED10 or its mutants is shown in the *top panel*. Total expressions of ALK5ca/V5 and TMED10/FLAG (or its mutants) are shown in the *middle and bottom panel*, respectively. *C*, non-necessity of both the transmembrane and the intracellular domains of TMED10 to interact with ALK5. The COS7 cells were transfected with the indicated plasmids. *Section I*, after cell lysis, anti-V5 antibodies were used for immunoprecipitation. The immunoprecipitates were separated by SDS-PAGE followed by Western blotting analysis (*WB*) using anti-FLAG antibody to detect their interaction (*top panel*). Total expressions of ALK5ca/FLAG and TMED10/V5 (or its mutants) in cells are shown in the *second and third panel*, respectively. *Section II*, because TMED10 Δ (V¹⁸⁶-E²¹⁹) seemed to be a secretory protein, immunoprecipitation of TMED10 Δ (V¹⁸⁶-E²¹⁹) into the media in which the cells were cultured was performed to detect secretory TMED10 Δ (V¹⁸⁶-E²¹⁹) (*bottom panel*). *D*, interaction of TMED10 or its mutants with T β RII. Co-IP was performed as described in *B* above. The interaction between T β RII and TMED10 or its mutants is shown in the *top panel*. Total expressions of T β RII and TMED10 or its mutants were detected using anti-HA12CA5 (*middle panel*) and anti-FLAG antibodies (*bottom panel*), respectively. *E*, schematic presentation of GST-TMED10 fusion proteins. The region from Gly⁸¹ to Val¹³⁰ in TMED10 was split into two pieces to make GST fusion proteins. *F*, requirement of a 30-amino acid region consisting of Gly⁸¹ to Glu¹¹⁰ within TMED10 to interact with ALK5. A GST pull-down assay was performed using the GST fusion proteins described in *E* above. ALK5 ectopically expressed in COS7 cells was mixed with GST fusion protein. After loading the protein(s) bound to GST-Sepharose 4B in SDS-PAGE, Western blotting analysis was performed using anti-ALK5 (V-22) antibody (*top panel*). The cell lysates from COS7 cells transfected with pcDNA3 were used as the negative control. As loading controls, GST and GST fusion proteins were visible with Ponceau S staining (*bottom panel*). *G*, illustration of GST-TMED10 fusion proteins. The region from Gly⁸¹ to Glu¹¹⁰ in TMED10 was divided into two pieces to make GST fusion proteins. *H*, determination of the ALK5-binding region in TMED10. A GST pull-down assay was carried out as described in *F* above. The *top and bottom panels* show the interaction of ALK5 with GST fusion proteins and the loading controls with Ponceau S staining. *I*, requirement of a 20-amino acid-long region in TMED10 to associate with T β RII. A GST pull-down assay was carried out as described in *F* above. The *upper and lower panels* show the interaction of T β RII with GST fusion proteins and the loading controls with Ponceau S staining.

comprehensive screening using mass spectrometry analysis. Among the positive candidates in the primary screening, TMED10 was further characterized in our current study as a potent negative regulator of TGF- β signaling by acting on TGF- β receptors. It is possible that stoichiometric conversion of ALK5 by T β RII kinase is involved in the interaction between ALK5 and TMED10. Although it was curious how one molecule of TMED10 could simultaneously associate with two receptors using the same 20-amino acid-long region of TMED10, we obtained evidence that TMED10 could oligomerize through its N-terminal region. Besides ALK5, TMED10 also interacted with ALK6, despite its relatively weaker interaction

with ALK6 than with ALK5. BAMBI, which is closely related to BMP receptor type I without its C-terminal kinase domain, is known to bind not only to ALK5 but also to ALK6 (26). Like BAMBI, TMED10 tends to interact with other type I and type II receptors of the TGF- β family via its N-terminal extracellular region (data not shown), although its inhibitory action on TGF- β signaling seems to be stronger than that on BMP signaling. Thus, TMED10 interrupts TGF- β receptor complex formation without sequestering the TGF- β ligand. Because the protein structure of TMED10 resembles that of the EMP24/GP25L/p24 family, TMED10 may be localized in the Golgi apparatus or the ER (53, 54). However, Blum and Lepier (55)

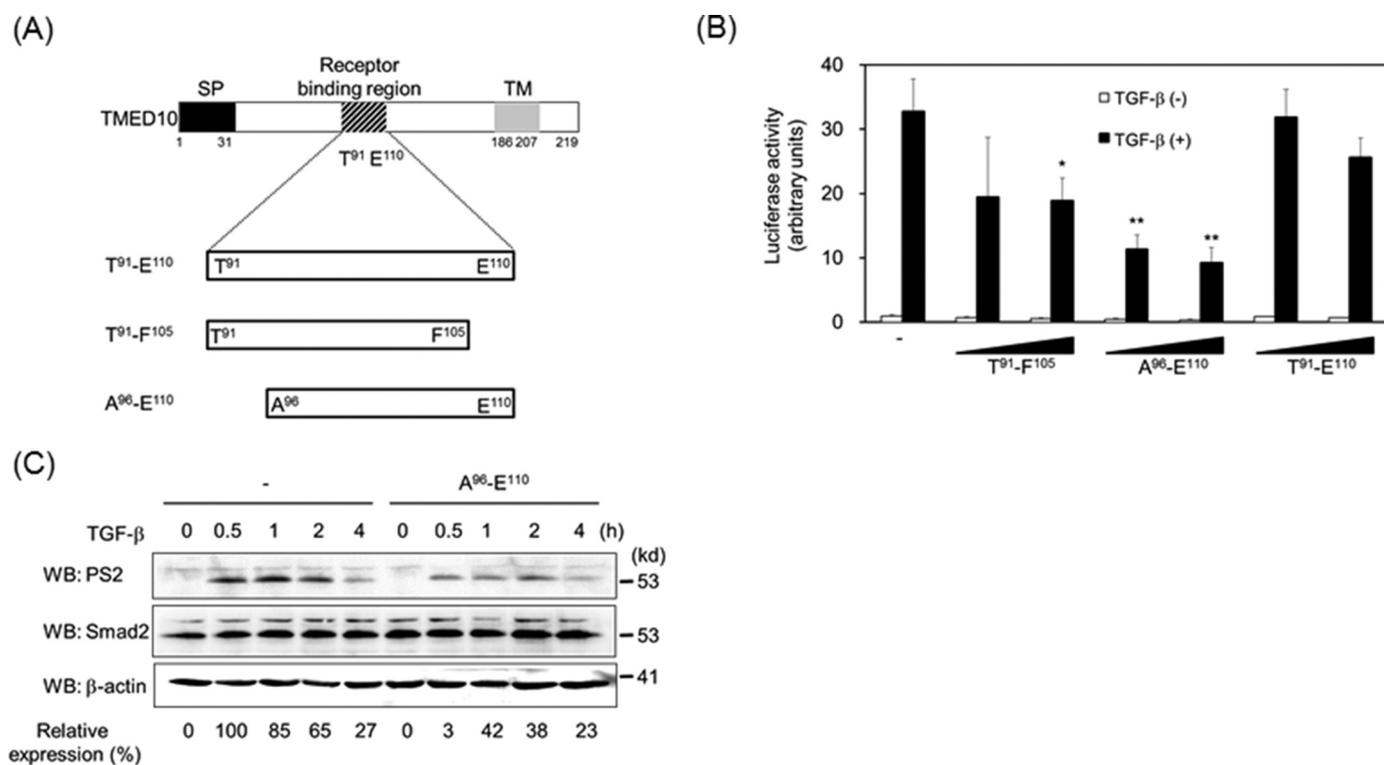


FIGURE 6. Fifteen-amino acid-long peptide as a unique inhibitor of TGF- β signaling. *A*, illustration of the synthesized peptides. *SP*, signal peptide; *TM*, transmembrane. *B*, inhibition of TGF- β -driven reporter activity by the synthesized peptides. The experiments were performed as described in Fig. 2*A* except for the addition of 200 nM or 1 μ M peptide 2 h prior to TGF- β (5 ng/ml) stimulation. Significant differences from the control in the presence of TGF- β are indicated with asterisks. *C*, inhibition of TGF- β -induced Smad2 phosphorylation by peptides from Ala⁹⁶ to Glu¹¹⁰. Two hours after the addition of 1 μ M peptide, HepG2 cells were stimulated with 0.5 ng/ml TGF- β for the indicated times. Western blotting analyses (WB) were performed as described in Fig. 2*F*. The top, middle, and bottom panels show the expressions of phosphorylated Smad2, Smad2, and β -actin, respectively. The expression of phosphorylated Smad2 upon TGF- β stimulation was normalized using the intensity of the band corresponding to Smad2. Relative expression was calculated relative to the value without peptide in the absence of TGF- β . Probability values below 0.01 and 0.001 were considered significant: **, $p < 0.01$; ***, $p < 0.001$.

reported that two different domains in TMED10 determine the destination of TMED10 in cells: either the ER/Golgi apparatus or the plasma membrane. Our experiments using confocal microscopy also supported their conclusion that parts of TMED10 were localized in the cell membrane and the remaining parts were present in the cytosol, although we did not identify the organelles associated with TMED10.

Blum and Lepier (55) show that the extracellular domain and extreme C terminus including KKLIE in TMED10 are involved in the retention of TMED10 in the plasma membrane and the ER, respectively. Mutation analyses of TMED10 demonstrated that the N-terminal domain following the signal peptide and the middle domain from Thr⁹¹ to Glu¹¹⁰ was needed for TMED10 to make a homo-oligomer and a hetero-oligomer with TGF- β receptors, respectively. In addition, a secreted form of TMED10 lacking a transmembrane and an intracellular domain could interfere with TGF- β signaling because of the presence of the region between Thr⁹¹ to Glu¹¹⁰. Therefore, TMED10 can be classified into three parts at least: a homo-oligomeric domain from Ile³² to Ala⁸⁰, a hetero-oligomeric domain from Thr⁹¹ to Glu¹¹⁰, and a C-terminal short region including KKLIE. After the receptor-binding domain was narrowed down, the 15-amino acid-long peptide was evaluated as an antagonist for TGF- β signaling. Therefore, TMED10 probably reveals its inhibitory action through its N-terminal extracellular domain, which has at least three functional domains: homodimer formation, heterodimer formation of TGF- β re-

ceptors, and retention of the plasma membrane. As TMED10 is not induced by TGF- β , we supposed that TMED10 could serve as a way to set up a threshold for activating TGF- β signaling in cells.

To examine the antagonistic effect of TMED10 on tumor growth, JygMC(A) cells, which are known to proliferate in the presence of TGF- β under low serum conditions (48), were implanted into murine mammary glands after TMED10 expression had been increased and reduced by gain-of-function or loss-of-function methods, respectively. Expectedly, the increase and the decrease in TMED10 expression suppressed and promoted tumor growth *in vivo*, respectively. Thus, high expression of TMED10 in JygMC(A) cells, in which TGF- β acts as a tumor-promoting factor, suppressed tumorigenicity. In contrast, the Human Protein Atlas Web database reveals that most tumors show high expression of TMED10. Consistently, TMED10 was expressed at a relatively high level in MCF10CA1a, characterized as a model of high grade breast carcinoma, whereas the expression of TMED10 was low in MCF10A1, characterized as a model of normal breast epithelium, and MCF10AT1, categorized as a premalignant epithelium (supplemental Fig. 1). However, TGF- β signaling is known to act context-dependently in the human body (11). These lines of evidence may support the notion that most tumors probably overcome TGF- β -mediated growth arrest through high expression of TMED10. It is possible that cancer cells themselves

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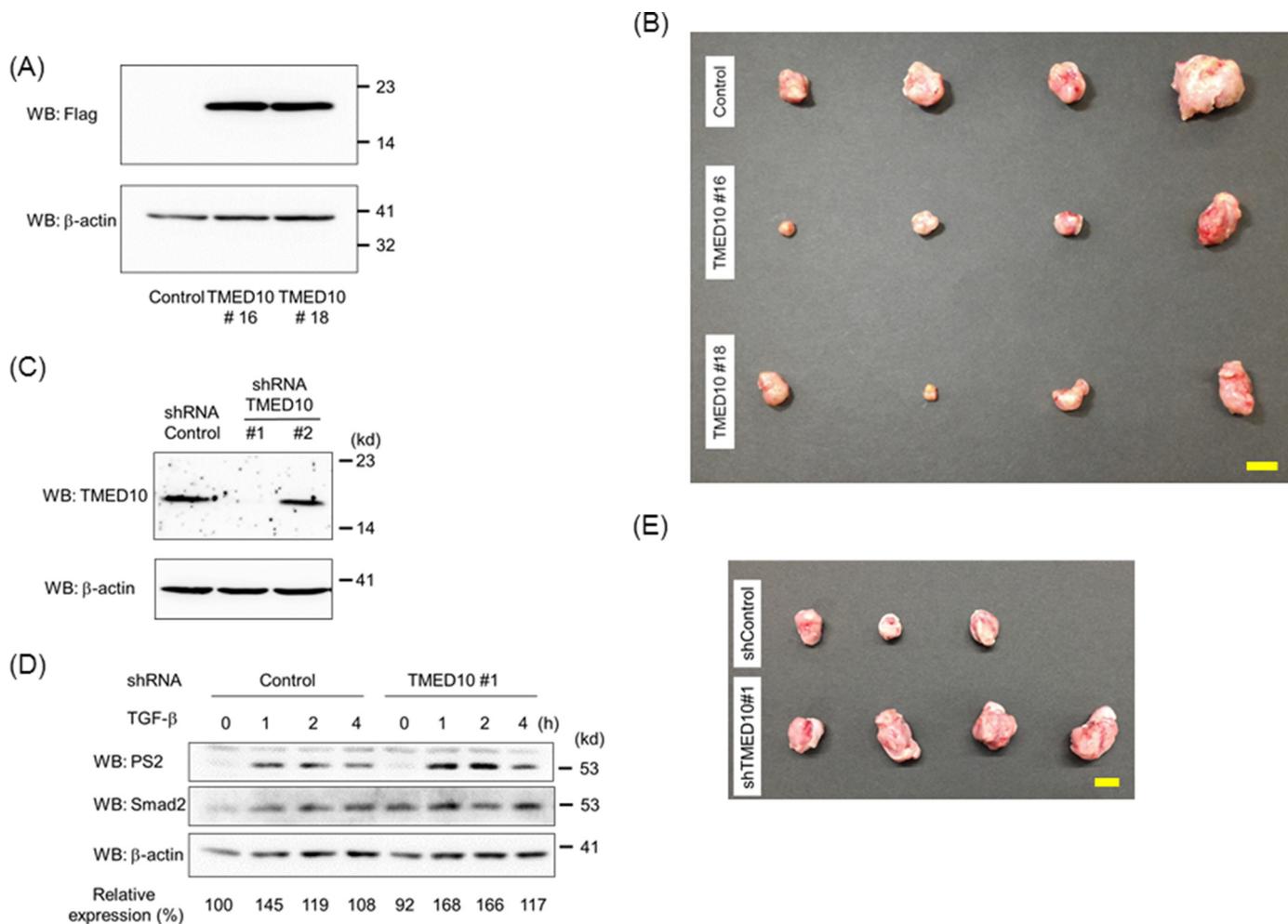


FIGURE 7. Xenograft implantation of mammary tumors carrying TMED10/FLAG or TMED10 shRNA. *A*, expression of TMED10/FLAG in JygMC(A) cells with TMED10. After JygMC(A) cells carrying control, TMED10#16, or TMED10#18 were established, the cell lysates were prepared. Western blotting analyses (WB) were then carried out using anti-FLAG (*top panel*) and anti- β -actin (*bottom panel*) antibodies. *B*, effect of TMED10 expression in JygMC(A) cells on primary tumors. Photos of tumors derived from JygMC(A) cells carrying control, TMED10#16, or TMED10#18 are shown. *Scale bar*: 10 mm. *C*, expression of TMED10 in JygMC(A) cells with the introduced TMED10 shRNA. After JygMC(A) cells carrying control, shTMED10#1, or shTMED10#2 were established, the cell lysates were prepared. Western blotting analyses were then carried out using anti-TMED10 (*top panel*) and anti- β -actin (*bottom panel*) antibodies. *D*, extension of TGF- β -induced Smad2 phosphorylation in JygMC(A) cells in which the TMED10 expression was reduced. Cells were stimulated with 0.25 ng/ml TGF- β for the indicated times. Western blotting analyses were performed as described in Fig. 2*F*. The *top*, *middle*, and *bottom panels* show the expressions of phosphorylated Smad2, Smad2, and β -actin, respectively. The expression of phosphorylated Smad2 upon TGF- β stimulation was normalized using the intensity of the band corresponding to Smad2. Relative expression was calculated relative to the value for control cells in the absence of TGF- β . *E*, effect of shTMED10 expression in JygMC(A) cells on primary tumors. Photos of tumors derived from JygMC(A) cells carrying control shRNA or shTMED10#1 are shown. *Scale bar*: 10 mm.

TABLE 1

Quantification of volumes and weights for primary tumors after inoculation of JygMC(A) cells carrying TMED10

The data represent means \pm S.D. ($n = 4$). Primary tumors in mammary glands were enucleated 5 weeks after orthotopic transplantation. Probability values below 0.05 were considered significant: *, $p < 0.05$.

Cell line	Average tumor volume <i>mm</i> ³	Average tumor weight <i>g</i>
Control	1631.1 \pm 1191.4	2.0 \pm 1.5
pAd-TMED10#16	403.3 \pm 286.9*	0.7 \pm 0.7
pAd-TMED10#18	450.8 \pm 290.9	0.5 \pm 0.4

alter the expression of TMED10 depending on the way in which TGF- β signaling in cancer cells acts on their survival or death.

In conclusion, TMED10 is a novel antagonist of TGF- β signaling that preferentially disrupts ALK5 and T β RII. Thus, TMED10 can limit the duration of Smad phosphorylation followed by transcriptional regulation of direct target genes for TGF- β signaling. Because the short peptide derived from

TABLE 2

Quantification of volumes and weights for primary tumors after inoculation of JygMC(A) cells carrying TMED10 shRNA

The data represent means \pm S.D. ($n = 3$ or 4). Primary tumors in mammary glands were enucleated 5 weeks after xenograft transplantation.

shRNA	Average tumor volume <i>mm</i> ³	Average tumor weight <i>g</i>
shControl	533.0 \pm 94	0.88 \pm 0.25
shTMED10#1	2967.5 \pm 1198	3.02 \pm 0.70

the N-terminal extracellular domain of TMED10 could interfere with TGF- β signaling, this peptide or its derivatives might become a potential anti-tumor therapeutic agent.

Experimental Procedures

Antibodies—Antibodies were obtained from the following sources: anti-FLAG M2 mouse monoclonal antibody (mAb) (F3165-1MG, Sigma); anti-DYKDDDDK tag mAb (catalog No.

040-30953, Wako), corresponding to anti-FLAG antibody; anti-V5 tag mouse mAb (catalog No. 011-23594, Wako); anti-HA12CA5 mouse mAb (catalog No. 11583816001, Roche Applied Science); anti-HA3F10 rat mAb (catalog No. 11867423001, Roche Applied Science); anti- β -actin mouse mAb (AC-15) (catalog No. sc-69879, Santa Cruz Biotechnology); anti-ALK5 (V-22) rabbit polyclonal antibody (pAb) (catalog No. sc-398, Santa Cruz Biotechnology), corresponding to anti-ALK5 antibody; anti-E-cadherin mouse mAb (catalog No. 610920, BD Biosciences); anti-HA rabbit pAb Y-11 (catalog No. sc-805, Santa Cruz); anti-N-cadherin mouse mAb (catalog No. 610921, BD Biosciences); anti-phosphoserine mouse mAb (clone 4A4) (catalog No. 05-1000, Merck); anti-mouse IgG HRP-linked sheep pAb (catalog No. NA931-1ML, GE Healthcare); and anti-rabbit IgG HRP-linked donkey F(ab)₂ fragment (catalog No. NA9340-1ML, GE Healthcare).

To generate anti-TMED10 rabbit pAb, synthetic peptides (Asn⁹⁷-Met¹¹⁴) were used to immunize rabbits (Sigma). After the sera were obtained, they were purified using a protein A-IgG purification kit (catalog No. 44557, Thermo Fisher Scientific). Anti-phosphorylated Smad2 (PS2), anti-Smad2, and anti-ALK5 (VPN) rabbit pAb were homemade (42, 56, 57).

Cell Culture—NMuMG, HaCaT, 911, HEK293T, 293A, A549, COS1, and COS7 cells were cultured in Dulbecco's modified Eagle's medium (Nacalai Tesque or Sigma) containing 10% fetal calf serum (FCS) (Invitrogen). JygMC(A) cells provided by RIKEN BioResource Center were also maintained in DMEM/10% FCS. HepG2 cells were maintained in minimum essential medium (Wako) containing 10% FCS, nonessential amino acids (Nacalai Tesque), and sodium pyruvate (Nacalai Tesque). The expression of TMED10 in the cell lines used is shown in supplemental Fig. 1.

Expression Plasmids—Human TMED10 cDNA was cloned by RT-PCR. All of the TMED10 mutants were made using PrimeStar HS DNA polymerase (Takara). TMED10 and its mutants were inserted into pcDNA3.1-V5-His-A (Invitrogen), pcDNA3-HA, or pcDNA3-FLAG (58). Subsequently, cDNA fused with each tag sequence were excised to ligate them to a pcDEF3 vector (59). All TMED10 constructs possessed the FLAG-, HA-, or V5 epitope tag at their C terminus. Additionally, TMED10 or its mutants without the tags were constructed using pcDEF3. Adenoviruses expressing TMED10/FLAG were generated using the pAd/CMV/V5-DEST vector (Thermo Fisher Scientific). The resulting plasmids were transfected into 293T cells, and the adenoviruses were amplified. A lentiviral expression vector for TMED10/FLAG was made with the pLV-CMV-IRES-Puro vector (60) and then co-transfected with VSV, GAG, and REV expression vectors in 293T cells to obtain viral particles. The other constructs have been described previously (17, 44, 61–63).

Lentiviral shRNAs—Short double-stranded DNA were inserted into the pLKO.1-TRC vector (64) purchased from Addgene. Lentiviral vectors expressing shTMED10 were transfected into 293A cells together with psPAX2 and pMD2.G. After 48 h of transfection, the media were collected as a source of lentiviruses. The lentiviruses were simultaneously incubated in DMEM containing 8 μ g/ml Polybrene (Sigma) for 2 h and then added to the cell culture dishes. Twelve hours after infec-

tion, the cells were washed and cultured in medium. Infected HaCaT and JygMC(A) cells, which became puromycin-resistant, were used for the experiments. The short double-stranded DNA used here were as follows: 5'-CCGCGCTTCT-TCAAGGCCAAGAACTCGAGTTTCTTGGCCTTGAA-GAAGCGTTTTTG-3'/3'-GCGAAGAAGTTCGGTTCT-TTGAGCTCAAAGAACCAGAACTTCTTCGCAAAAAC-TTAA-5' for human shTMED10; 5'-CCGGAAGTCTCG-CAAGTGTCTCCGACTCGAGTCGGAGACACTTGCGAG-AGTTTTTTTTG-3'/TTGAGAGCGTTCACAGAGGCTGAG-CTCAGCCTCTGTGAACGCTCTCAAAAAAAGTTAA-5' for mouse shTMED10#1; and 5'-CCGGAAGATCACAGATT-CTGCTGGCCTCGAGGCCAGCAGAATCTGTGATCTTTT-TTTG-3'/3'-TTCTAGTGTCTAAGACGACCGGAGCTCCG-GTCGTCTTAGACTAGAAAAAAGTTAA-5' for mouse shTMED10#2.

Protein Identification by LC/MS Analysis—FLAG-tagged human ALK5ca was expressed in HEK 293T cells, and the associated proteins were recovered from the cell extracts by immunoprecipitation with anti-FLAG M2 antibody. The ALK5ca-associated protein complexes were digested with lysyl endopeptidase (Lys-C, Wako), and the resulting peptides were analyzed using a direct nanoflow LC-MS/MS system as described previously (65).

Transcriptional Reporter Assays—One day before transfection, HepG2 cells were seeded at 1.2×10^5 cells/well in 12-well plates. The cells were transfected with a reporter gene, *pCHI10* (GE Healthcare), and the indicated plasmids by use of polyethylenimine (Polysciences). Where indicated, 5 ng/ml TGF- β or 25 ng/ml BMP-6 was added to the wells 24 h after transfection. Subsequently, the cells were cultured in the absence of FCS for 18 h. In all experiments, β -galactosidase activity was measured to normalize for transfection efficiency. Each transfection was carried out in triplicate and repeated at least twice.

Immunoprecipitation and Western Blotting Analysis—To detect interactions among the proteins, plasmids were transfected into COS7 cells (5×10^5 cells/6-cm dish) by use of polyethylenimine. Forty hours after the transfection, the cells were lysed in 500 μ l of TNE buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 5 μ g/ml leupeptin, 100 units/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF, and 20 mM β -glycerophosphate). The cell lysates were precleared with protein G-Sepharose beads (GE Healthcare) for 30 min at 4 $^{\circ}$ C and then incubated with the indicated antibodies for 2 h at 4 $^{\circ}$ C. The protein complexes were immunoprecipitated by incubation with protein G-Sepharose beads for 30 min at 4 $^{\circ}$ C and then washed three times with TNE buffer. The immunoprecipitated proteins and aliquots of the total cell lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to either Hybond-C Extra membrane (GE Healthcare) or UltraCruz nitrocellulose pure transfer membrane (Santa Cruz Biotechnology). The membranes were probed with the primary antibodies, which were detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrate (Western BLoT Quant HRP substrate, Takara). The protein expression in the total cell lysates was evaluated by Western blotting analysis.

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Iodination of Ligands and Affinity Cross-linking—TGF- β was iodinated using the chloramine T method according to Frolík *et al.* (66). For the ectopic expression system, COS1 cells were transfected with the indicated plasmids 40 h prior to incubation with ^{125}I -TGF- β . Cross-linking was performed as described previously (67). Complexes of TMED10 and affinity-labeled receptors were immunoprecipitated with an anti-V5 or anti-TMED10 antibody. To investigate the expression levels of the receptors, aliquots of the cell lysates were analyzed directly by SDS-polyacrylamide gel electrophoresis.

GST Pulldown Assay—TMED10 mutants were subcloned in pGEX4T-1 (GE Healthcare). GST fusion proteins from *Escherichia coli* were purified according to the manufacturer's instructions (GE Healthcare). Cell lysates were prepared from COS7 cells transfected with ALK5, T β R2/FLAG, or an empty vector precleared with GST immobilized to GSH-Sepharose 4B (GE Healthcare) for 30 min at 4 °C. Subsequently, the above cell lysates were incubated with GST-TMED10 mutants immobilized to GSH-Sepharose 4B for 2 h at 4 °C and washed three times with 50 mM Tris (pH 7.4) containing 100 mM NaCl, 2 mM MgCl₂, 10% glycerol, 1% Nonidet P-40, 1 mM PMSF, 5 $\mu\text{g}/\text{ml}$ leupeptin, 20 units/ml aprotinin, and 5 mM benzamide. After the samples had been loaded on SDS-PAGE, the proteins were blotted on the nitrocellulose membrane and detected with either an anti-ALK5 (V-22) or an anti-FLAG antibody using a chemiluminescent substrate (Takara). To show the quantity of GST fusion proteins in each sample, Ponceau S staining was performed after blotting.

Immunofluorescence Staining—Immunofluorescence staining for detection of colocalization was performed as described previously (68). Briefly, 911 cells grown on cover glasses were transfected with the indicated plasmids. After 24 h of transfection, the cells were stimulated with TGF- β for 2 h. Then, the cover glasses were washed once with PBS, fixed for 10 min with 4% paraformaldehyde, washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and again washed three times with PBS. The cover glasses were blocked with 5% bovine serum albumin (BSA) in PBS at 37 °C for 1 h and incubated with 5% BSA in PBS containing the indicated antibodies at 4 °C overnight. The cover glasses were then washed three times with PBS, incubated with 5% BSA in PBS containing both Alexa Fluor 555-conjugated goat anti-mouse IgG antibody (diluted 1:250; A-21422, Thermo Fisher Scientific) and CF488A-conjugated goat anti-rabbit IgG antibody (diluted 1:250; catalog No. 20012, Biotium, Inc.) at room temperature for 1 h, and washed three times with PBS. The nuclei were stained with 4',6-diamidino-2-phenylidole (DAPI). To visualize the fluorescence, a confocal microscope (A1Rsi, Nikon) was used. For E-cadherin expression, we performed the same experiments except using the anti-E-cadherin antibody as the primary antibody and the Alexa Fluor 555-conjugated goat anti-mouse IgG antibody (A21422, Thermo Fisher Scientific) as the secondary antibody instead of the above combination of primary and second antibodies. To detect stress fiber formation, we carried out the same experiment except for using Alexa Fluor 555-phalloidin (A34055, Thermo Fisher Scientific).

RNA Preparation and Quantitative Real-time PCR (qPCR) Analysis—Total RNA from HepG2 cells was extracted using a ReliaPrep RNA cell miniprep system (Promega). Reverse transcription was performed with a high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific). qPCR was performed with Fast-Start SYBR Green Master Mix (Roche Applied Science). All reactions were carried out on a StepOnePlus system (Applied Biosystems Inc.). Each sample was analyzed in triplicate at least twice for each PCR measurement. Melting curves were checked to ensure specificity. The relative quantification of mRNA expression was calculated using the standard curve method with the β -actin level. Before qPCR, the DNA fragment amplified using each primer set was detected to be a single band with the correct size by agarose gel electrophoresis. The following primer sets were used to amplify TMED10, TMEPAI, and β -actin cDNA: 5'-GAGATGCGTGATACCAACGA-3' and 5'-TTCTTGGCCTTGAAGAAGCG-3' for human TMED10; 5'-TGTCCTCAGAAGGATGCCTG-3' and 5'-CACTGTCG-AAGATGGTTCTG-3' for human TMEPAI; and 5'-caagagatggccacggctgct-3' and 5'-tccttctgcatcctgtcggca-3' for human β -actin.

Migration Assay—Cell migration assays were performed using a Boyden chamber. Costar nucleopore filters (8- μm pore diameter) were coated with 10 $\mu\text{g}/\text{ml}$ fibronectin (Sigma) overnight at 4 °C. The chambers were washed three times with PBS. Cells cultured for 12 h without FCS were added to the top of each migration chamber at a density of 1.5×10^5 cells/chamber in 150 μl of DMEM with 0.5% FCS. The cells were allowed to migrate to the underside of the chamber in the presence or absence of 1 ng/ml TGF- β in the lower chamber. After 24 h, the cells were fixed in 4% paraformaldehyde and stained with hematoxylin (Leica) and eosin (Muto Chemicals). The upper surface was wiped with cotton swabs to remove nonmigrating cells. The cells present on the lower surface were counted. Each experiment was carried out in triplicate and repeated more than twice.

Scratch Assay—Cells were seeded in 12-well plates, grown until confluent, and wounded with a 200- μl tip. After wounding, 0.5 ng/ml TGF- β was added. Photographs were then taken under a microscope (Nikon) for 24 h. The wound distance from each well was measured in duplicate at three randomly defined wound gap locations per frame recorded per experiment, and at least three independent scratch assays were used for the calculation. Each experiment was carried out in triplicate and repeated several times.

Introduction of Peptides—Three peptides (Thr⁹¹-Glu¹¹⁰, TKGKFAFTTEDYDMFEVCFE; Thr⁹¹-Phe¹⁰⁵, TKGKFAFTTEDYDMF; and Ala⁹⁶-Glu¹¹⁰, AFTTEDYDMFEVCFE) were synthesized (Bio-Synthesis or CS Bio Co.). We mixed 200 nM or 1 μM peptides and Xfect protein transfection reagent (Clontech) for 30 min. After the cells had been incubated with the peptides for 1 h, the cell media were refreshed. The cells were then cultured for 2 h followed by stimulation of the cells with TGF- β for the indicated times.

Xenograft Model—Six-week-old female BALB/c nu/nu mice were purchased from Japan SLC Inc. JyMC(A) cells (1×10^7 cells) carrying TMED10/FLAG or TMED10 shRNA were implanted into the mammary fat pad of each mouse. Five weeks

later, the primary tumors and lungs were removed after the mice had been sacrificed. Tumor volumes were calculated using the following formula: $0.4 \times (\text{longest axis}) \times (\text{shortest perpendicular axis})^2$ (48). The mice were housed in the animal facilities of Showa Pharmaceutical University under specific pathogen-free conditions at a constant temperature and humidity and fed a standard diet. They were treated in accordance with the institutional guidelines of the Animal Care and Use Program of Showa Pharmaceutical University.

Statistical Analysis—Data were expressed as means \pm SD unless otherwise mentioned. Significance was assessed using the *t* test. Probability values below 0.05, 0.01, and 0.001 were considered significant: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Author Contributions—N. N., Y. T., K. K., K. U., S. Ikeno, E. O., M. S., A. N., N. S., F. I., and M. v. D. performed the *in vitro* experiments as well as the cell-based experiments. K. S., E. O., and Y. N. carried out the *in vivo* xenograft tumor model. S. Iemura identified ALK5-interacting proteins by TOF-MASS. T. N., P. t. D., and S. Itoh designed most aspects of the research, interpreted the data, and drafted the manuscript.

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