

# TMEPAI/PMEPA1 enhances tumorigenic activities in lung cancer cells

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## Key words

Antibody, lung cancer, TMEPAI, TGF- $\beta$ , tumorigenesis

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The transforming growth factor- $\beta$  (TGF- $\beta$ ) family comprises 33 multifunctional polypeptide factors that include TGF- $\beta$ s, bone morphogenetic proteins, growth/differentiation factors, inhibin, and activins.<sup>(1,2)</sup> Transforming growth factor- $\beta$  signaling has essential effects on a wide variety of cellular activities, such as cell cycle control, cell adhesion, differentiation, ECM formation, apoptosis, angiogenesis, immune functions, and cancer progression.<sup>(2–6)</sup> Transforming growth factor- $\beta$  signaling is initiated by ligand-induced heteromeric complex formation of the specific type I and type II serine/threonine kinase receptors. Transforming growth factor- $\beta$  type I receptor (T $\beta$ RI), also termed activin receptor-like kinase-5, is phosphorylated and activated by TGF- $\beta$  type II receptor (T $\beta$ RII). Activated T $\beta$ RI catalyzes the C-terminal serine phosphorylation of receptor-regulated Smads (Smad2 and Smad3), and the activated receptor-regulated Smads form a ternary complex with the common mediator Smad (Smad4). The activated Smad complexes then translocate into the nucleus, where they regulate the transcription of specific target genes together with other transcription factors and cofactors.<sup>(7–10)</sup>

Transmembrane prostate androgen-induced RNA (TMEPAI), alternatively termed PMEPA1, STAG1, ERG1.2, or N4wbp4, was originally identified as a prostatic RNA, the synthesis of which is induced by testosterone or its derivatives.<sup>(11)</sup> In addition to androgen, epidermal growth

TMEPAI/PMEPA1 is a transmembrane protein that was originally identified as a prostatic RNA, the synthesis of which is induced by testosterone or its derivatives. We have recently identified TMEPAI as a direct target gene of transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad signaling that participates in negative feedback control of the duration and intensity of TGF- $\beta$ /Smad signaling. TMEPAI is constitutively and highly expressed in many types of cancer and is associated with poor prognosis. Here, we report that TMEPAI is highly expressed in the lung adenocarcinoma cell lines Calu3, NCI-H23, and RERF-LC-KJ. Expression of TMEPAI in these cancer cells was significantly suppressed by a TGF- $\beta$  receptor kinase antagonist, SB208, and by TGF- $\beta$  neutralizing antibodies. These results suggest that constitutive expression of TMEPAI in these cancer cells depends on autocrine TGF- $\beta$  stimulation. Knockdown of TMEPAI in Calu3 and NCI-H23 cells enhanced levels of Smad2 phosphorylation and significantly suppressed cell proliferation in the presence of TGF- $\beta$ , indicating that highly expressed TMEPAI suppresses levels of Smad phosphorylation in these cancer cells and reduces the growth inhibitory effects of TGF- $\beta$ /Smad signaling. Furthermore, knockdown of TMEPAI in Calu3 and NCI-H23 cells suppressed sphere formation *in vitro* and tumor formation in s.c. tissues and in lungs after tail vein injection in NOD-SCID mice *in vivo*. Together, these experiments indicate that TMEPAI promotes tumorigenic activities in lung cancer cells.

factor (EGF), activation of the RAS/ERK pathway, mutant p53, and TGF- $\beta$  have been reported to induce TMEPAI.<sup>(12–16)</sup> We have recently reported that TMEPAI is a direct target gene of TGF- $\beta$ /Smad signaling, which requires Smad3, Smad4, and TCF7L2 as cobinding transcription factors.<sup>(17)</sup> TMEPAI is a transmembrane protein containing two PY motifs that can interact with HECT-type E3 ubiquitin ligases.<sup>(18)</sup> TMEPAI has also been reported to mediate growth inhibition and p53-inducible apoptosis.<sup>(13,18)</sup> We have shown that TMEPAI can interact with Smad2 and Smad3 by way of its Smad interaction motif (SIM) to sequester Smads from TGF- $\beta$ /Smad signaling. Because of the competition with SARA for binding to Smads, TMEPAI participates in negative feedback regulation of the duration and intensity of TGF- $\beta$ /Smad signaling.<sup>(19)</sup>

High levels of TMEPAI expression have been reported in renal cell carcinoma, colon cancer, breast cancer, and ovarian cancer as well as in several cancer cell lines.<sup>(12,14,20)</sup> Genome-wide studies, which compared the gene expression levels of invasive cancer tissues with normal counterpart tissues or preinvasive cancers, suggested that TMEPAI is one of the most highly inducible genes in invasive cancers.<sup>(21,22)</sup> TMEPAI was further suggested as a “molecular switch” that converts TGF- $\beta$  signaling from a tumor suppressor to a tumor promoter.<sup>(23)</sup> These lines of evidence suggest an oncogenic function of TMEPAI in many

cancers. However, how TMEPAI regulates tumor progression remains largely unknown. In this study, we aimed to investigate the tumorigenic activities of TMEPAI in lung cancer cell lines.

## Materials and Methods

**Monoclonal antibody.** We constructed a 117-bp DNA fragment coding a C-terminal peptide (249–287) of human TMEPAI isoform a, which is conjugated with a GST gene to produce a recombinant GST-TMEPAI (249–287) fusion protein for immunization (Fig. S1). TMEPAI-knockout mice were peritoneally immunized once a week for 3 weeks with purified GST-TMEPAI (249–287) mixed in Freund's adjuvant. Hybridoma cells were established and cloned essentially according to the methods described elsewhere.<sup>(24,25)</sup> The established clones were examined by ELISA, immunoblot analysis, immunoprecipitation, and immunofluorescence. Monoclonal antibodies from clone 9F10 were used for examination of lung cancer cells after large-scale preparation in nude mice ascites and purification using Protein-G columns.

**Plasmid construction.** The expression plasmid for human TMEPAI/V5 was previously described.<sup>(19)</sup> C18ORF1 cDNA was obtained by RT-PCR. The PCR product was inserted into the pcDNA3.1/V5 vector (Invitrogen, Carlsbad, CA, USA). Both TMEPAI and C18ORF1 constructs were connected to the V5-epitope tag at their C-terminus. All plasmids were sequenced before use.

**Cell culture.** HaCaT cells (spontaneously immortalized human keratinocyte cell line) and COS7 cells (African green monkey kidney cells transformed by SV40) were cultured in DMEM (Sigma) containing 10% FCS (Biowest, Rosenberg, TX, USA) and nonessential amino acids (Invitrogen). NCI-H23 and RERF-LC-KJ cells were cultured in RPMI-1640 medium containing 10% FCS. Calu3 cells and HepG2 cells were cultured in minimum essential medium (Sigma) containing 10% FCS. Non-targeting shRNA (SHC002), TMEPAI shRNA#9 (CCG GGA GCA AAG AGA AGG ATA AAC ACT CGA GTG TTT ATC CTT CTC TTT GCT CTT TTT), and TMEPAI shRNA#10 (CCG GGA GTT TGT TCA GAT CAT CAT CCT CGA GGA TGA TGA TCT GAA CAA ACT CTT TTT) ligated in a pSUPER RNAi system (Oligoengine, Seattle, WA, USA) were used for knockdown of TMEPAI. For the selection of stable TMEPAI-knockdown clones, Calu3 or NCI-H23 cells were cultured in the presence of 0.6 µg/mL or 1 µg/mL puromycin (Sigma), respectively. The TGF-β receptor kinase inhibitor SD208 (Tocris Bioscience, Bristol, UK) and anti-TGF-β neutralizing antibody (R&D Systems, Minneapolis, MI, USA) were used to block TGF-β signaling.

**Luciferase assay.** HepG2 cells were transfected with (CAGA)<sub>12</sub>-luc using FuGENE6 (Roche, Penzberg, Germany), treated with TGF-β or 50% v/v of heat-treated serum-free conditioned media (80°C, 10 min) after 24 h of incubation with HaCaT or lung cancer cell lines. Luciferase activities were determined by Luciferase Assay Systems (Promega, Madison, WI, USA) and normalized to β-galactosidase activity of co-transfected CH110 (GE Healthcare, Piscataway, NJ, USA).

**Immunoblot analysis and immunoprecipitation.** To examine the sensitivity and specificity of the established antibodies in immunoblot analysis and immunoprecipitation, the TMEPAI/V5 or C18ORF1/V5 expression plasmids were transfected into COS7 cells (5 × 10<sup>6</sup> cells/6-cm dish) using FuGENE 6 (Roche Applied Science). Thirty-six hours after the transfection, the cells were dissolved in 500 µL TNE buffer and the debris was removed by centrifugation as previously described.<sup>(19)</sup> For

immunoprecipitation, the lysate was precleared with protein G-Sepharose beads (GE Healthcare) for 30 min at 4°C with end-over-end rotation and then incubated with anti-V5 antibody (Sigma) or anti-TMEPAI antibodies for 2 h at 4°C. The immune complexes were precipitated by incubation with protein G-Sepharose beads for 30 min at 4°C followed by three washes with TNE buffer. The immunoprecipitated proteins and aliquots of the total cell lysate were separated by SDS-PAGE, and transferred to Hybond-C Extra membranes (GE Healthcare). The membranes were probed with different primary antibodies and then incubated with HRP-conjugated secondary antibodies and chemiluminescent substrate solution (Thermo Scientific, Waltham, MA, USA). Endogenously expressed TMEPAI in HaCaT or lung cancer cell lines was also detected by immunoblot analysis using total cell lysates with/without TGF-β stimulation.

**Immunofluorescence staining.** HaCaT and NCI-H23 cells were cultured on glass coverslips, fixed with 4% paraformaldehyde-PBS, permeabilized with 0.3% Triton X-100/PBS, and incubated with 1% BSA. Cells were then incubated with anti-TMEPAI 9F10 antibody and then with Alexa 488-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). The nuclei were stained with Hoechst 33342 (Sigma). Intracellular localization was then observed by fluorescence microscopy (Axiovert 200; Zeiss, Oberkochen, Germany).

**Cell proliferation assay.** Cells were seeded in 24-well plates, cultured for the indicated time periods, and counted with a hemocytometer.

**Sphere formation assay.** Spheres were cultured in DMEM/F12 serum-free medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/mL EGF (Sigma), and 20 ng/mL basic fibroblast growth factor (R&D Systems) in an ultra-low attachment culture dish (Corning, NY, USA).

**In vivo tumor formation assay.** For cancer cell implantation, 10<sup>7</sup> cells were s.c. injected into 8-week-old female NOD-SCID mice. After 3 months, the mice were killed and the tumors weighed. All animal experiments were approved by the animal experiment committee of the University of Tsukuba (Tsukuba, Japan) and carried out in accordance with the university's animal experiment guidelines and the provisions of the Declaration of Helsinki in 1995.

**In vivo lung metastasis assay.** A suspension containing 10<sup>6</sup> cells in 0.2 mL PBS was injected into the lateral tail vein of 7-week-old NOD-SCID mice. After 8 weeks, the animals were killed and the removed lungs were fixed in 10% neutralized formalin solution, embedded in paraffin, sliced into 3-µm sections, and stained with H&E or immunostained with Ki-67 antibody (Novocastra, Newcastle Upon Tyne, UK). Three animals were used in each group. The tumor areas in a representative cut surface were measured with an Olympus Virtual Slide System and with ASW morphometry software (Olympus, Tokyo, Japan).

**Statistical analysis.** Statistical analyses of the data were carried out using a statics function in Microsoft Office (Microsoft, Redmond, WA, USA) and the *t*-test. Probability values of <0.05 were considered significant.

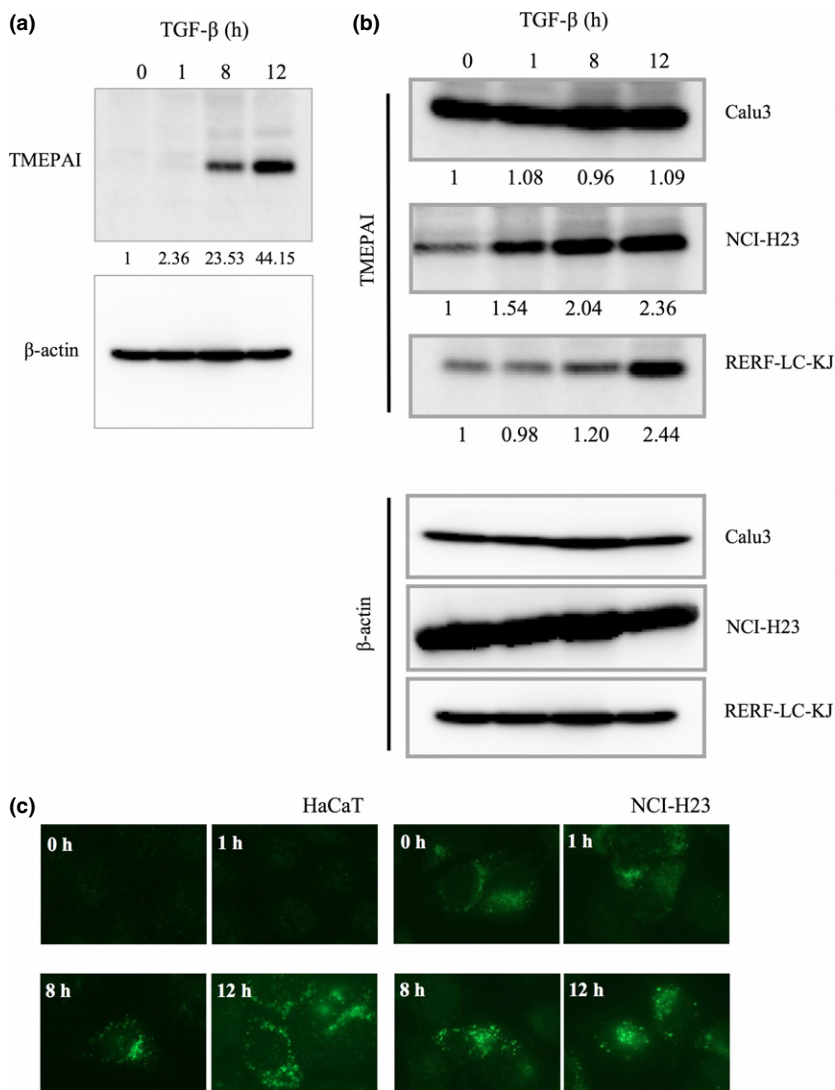
## Results

**Establishment and characterization of mAbs for TMEPAI.** To investigate the pathophysiological functions of TMEPAI, we first generated mAbs for TMEPAI. Antibody titers were checked by ELISA in the sample sera after each immunization. Consequently, the immunized mice with conspicuous increase of anti-TMEPAI activity in the sera were used for hybridoma

formation. After several screenings, we obtained six clones (4E5, 4C10, 4D11, 9F10, 1C4, and 6C7), which provided sensitive and specific detection of GST-TMEPAI in the first series of ELISA screening. The mAbs from these six clones were then prepared on a large scale and purified, and the isotypes of these antibodies were determined. The isotype of four of the clones (4E5, 4C10, 9F10, and 6C7) was IgG1, that of 4D11 was IgG2a, and that of 1C4 was IgG2b. All of the antibodies contained the kappa light chain except for 6C7, which contained the lambda light chain. Next, we examined the sensitivity of these antibodies by ELISA again. Four purified antibodies, 4E5, 4C10, 9F10, and 6C7, showed efficient and specific activity in the ELISA for TMEPAI at 0.4  $\mu\text{g}/\text{mL}$  antibody concentration (Fig. S2a). We next used the antibodies for immunoblot analysis using COS7 cell lysates transfected with TMEPAI/V5 or C18ORF1/V5 expression plasmids. Four monoclonal antibodies, 4E5, 4C10, 9F10, and 6C7, specifically recognized only TMEPAI, and three of them, 4E5, 4C10, and 9F10, could also efficiently work for the immunoprecipitation assay (Fig. S2b). Moreover, we characterized the ability of these TMEPAI antibodies in immunofluorescence staining. The TMEPAI/V5, C18ORF1/V5, or pcDNA3.1-alone vector was

transfected into 911 cells, and the subcellular localization of TMEPAI was detected at 24 h after transfection. Consistent with the previous finding of TMEPAI localization in early endosomes, anti-TMEPAI immunofluorescence gave cytoplasmic dot patterns. Three of the examined antibodies, 4E5, 9F10, and 6C7, specifically recognized TMEPAI and did not cross-react with C18ORF1 (Fig. S2c).

**Enhanced and constitutive expression of TMEPAI in lung cancer cell lines.** Using anti-TMEPAI (9F10) antibody, we evaluated TMEPAI expression levels in the human lung adenocarcinoma cell lines Calu3, NCI-H23, and RERF-LC-KJ. HaCaT cells were used as a positive control. HaCaT cells expressed detectable levels of TMEPAI only in the presence of more than 8 h of TGF- $\beta$  stimulation; there were no detectable levels of TMEPAI without TGF- $\beta$  stimulation (Fig. 1a). In contrast, all three of the examined lung cancer cell lines expressed detectable levels of TMEPAI even in the absence of TGF- $\beta$  stimulation. Notably, Calu3 constitutively expressed high levels of TMEPAI, whereas NCI-H23 and RERF-LC-KJ expressed distinct but relatively low levels of TMEPAI and enhanced TMEPAI expression approximately twofold in response to TGF- $\beta$  (Fig. 1b). Endogenous TMEPAI could also be detected as



**Fig. 1.** Enhanced expression of TMEPAI in lung cancer cells. (a) Induction of TMEPAI by 5 ng/mL transforming growth factor- $\beta$  (TGF- $\beta$ ) in HaCaT cells. Endogenous TMEPAI was detected by a monoclonal anti-TMEPAI antibody (9F10).  $\beta$ -actin was used as the loading control. Relative amounts of TMEPAI (TMEPAI/ $\beta$ -actin) were measured by NIH Image and are shown below the panel. (b) Expression of TMEPAI in the lung cancer cell lines Calu3, RERF-LC-KJ, and NCI-H23 detected as in (a). (c) HaCaT or NCI-H23 cells were treated with 5 ng/mL TGF- $\beta$  as indicated. After treatment, the cells were subjected to fluorescence microscopy using anti-TMEPAI antibody.



cytoplasmic dot patterns in HaCaT and NCI-H23 cells by immunofluorescence staining (Fig. 1c).

**Transforming growth factor- $\beta$  signaling mediates enhanced expression of TMEPAI in lung cancer cells.** The high expression levels of TMEPAI in lung cancer cells prompted us to examine how TMEPAI expression is constitutively enhanced in these cells. To investigate the possibility that TGF- $\beta$  signaling is involved in TMEPAI expression, cells were treated with the TGF- $\beta$  receptor kinase inhibitor SD208 or anti-TGF- $\beta$  neutralizing antibodies. TMEPAI disappeared from all cell lines in the presence of SD208 (Fig. 2a). Although the effects of the TGF- $\beta$  neutralizing antibodies were not complete, they did cause the TMEPAI levels to significantly decrease to levels correlating with Smad2 phosphorylation levels in all three cell lines (Fig. 2b). We further examined TGF- $\beta$  activities in the conditioned media incubated 24 h with the lung cancer cells. Calu3 secreted abundant TGF- $\beta$  in the culture media and it had positive correlation with the levels of TMEPAI expression (Fig. 2c).

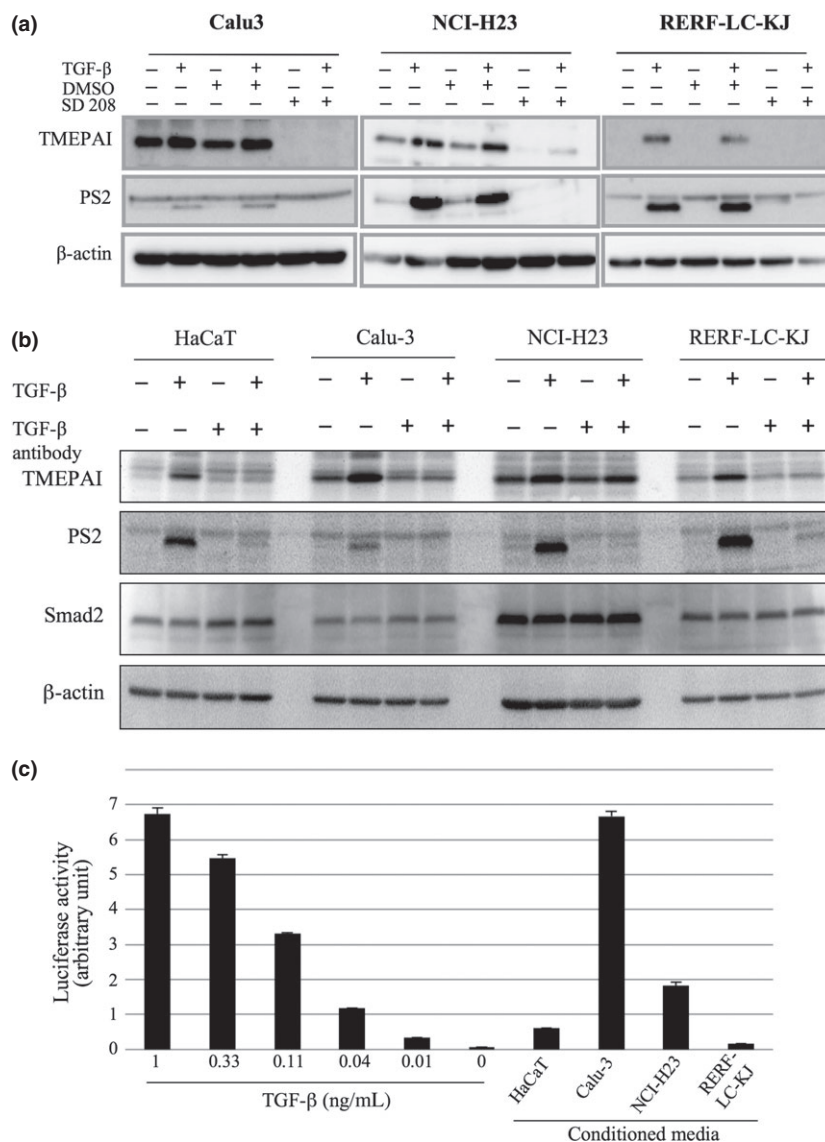
**Knockdown of TMEPAI enhances Smad phosphorylation and growth inhibitory responses of TGF- $\beta$ .** We next engineered stable knockdown of TMEPAI by two individual shRNAs (shTMEPAI#9 and shTMEPAI#10). These shRNAs significantly reduced TMEPAI expression in Calu3 cells

(Calu3-sh#9 and Calu3-sh#10; Fig. 3a). As a result, phosphorylated Smad2 levels were clearly enhanced (Fig. 3b). Significantly stronger cell growth inhibition was obtained in Calu3-sh#9 and Calu3-sh#10 cells in the presence of 0.1 ng/mL TGF- $\beta$ , and it was recovered by anti-TGF- $\beta$  neutralizing antibodies (Fig. 3c,d). Similar results were obtained with NCI-H23 cells (Fig. S3a-c).

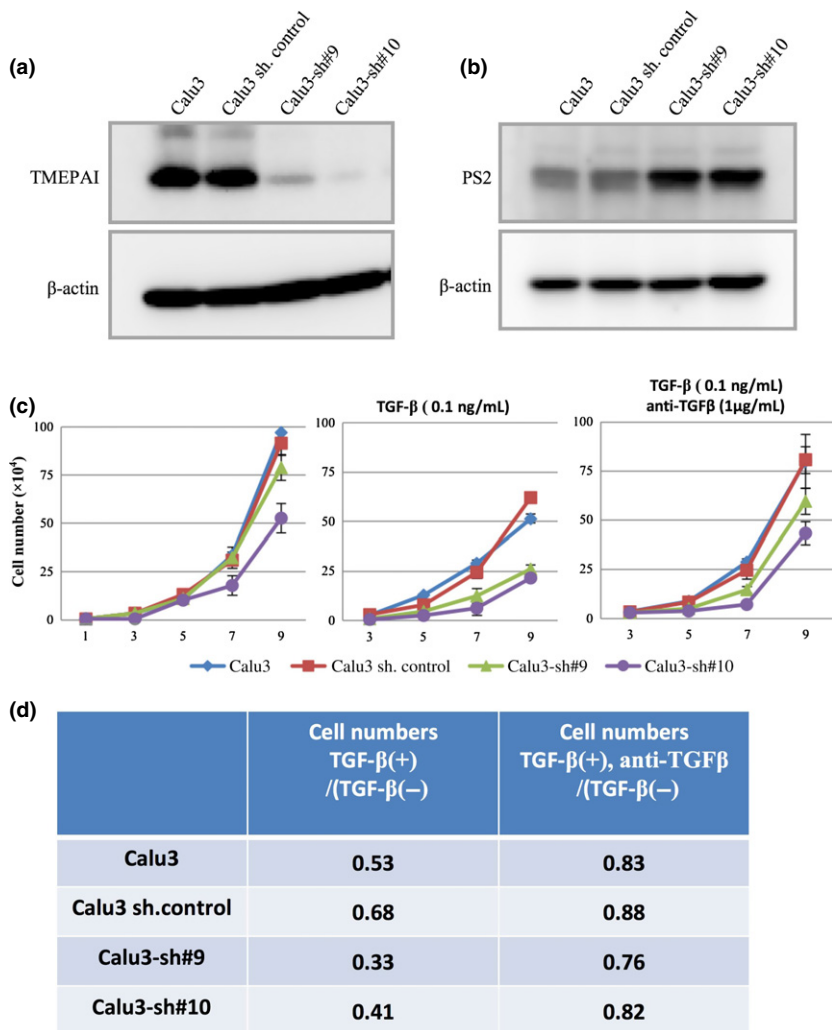
**Sphere-forming activities enhanced by TMEPAI.** We further investigated the functional significance of TMEPAI in sphere-forming activities. The sphere-forming activities of TMEPAI knockdown cells were significantly reduced both in Calu3 (Fig. 4a,b) and in NCI-H23 cells (Fig. S3d,e).

**In vivo tumor formation enhanced by TMEPAI.** In addition, we carried out xenograft assays in NOD-SCID mice. Calu3-sh#9 and Calu3-sh#10 cells were s.c. injected into NOD-SCID mice. Three months after injection, the tumors were collected and weighed. As shown in Figure 5, the tumors of the Calu3-sh#9 and Calu3-sh#10 cells were significantly smaller than those of the control cells in NOD-SCID mice. The results of the NCI-H23 cells were essentially the same (Fig. S4).

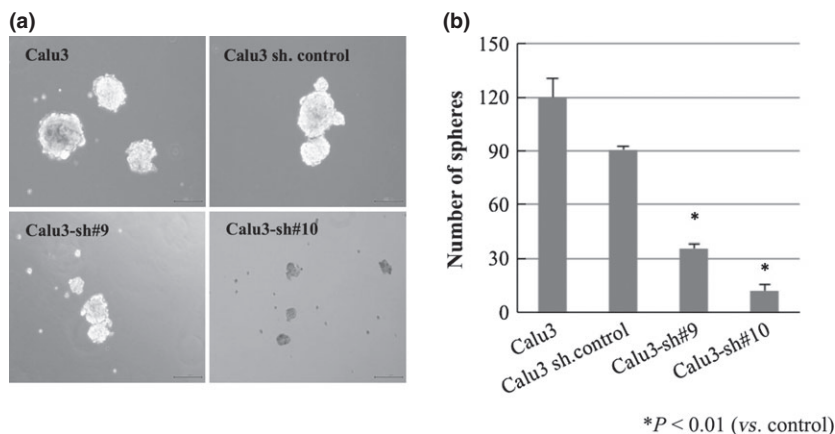
**Metastatic tumor formation in lungs enhanced by TMEPAI.** To examine the effects of TMEPAI on the metastatic potential in lungs, we injected Calu3-sh#9 and Calu3-sh#10 cells into



**Fig. 2.** Transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling mediates the expression of TMEPAI in lung cancer cell lines Calu3, NCI-H23, and RERF-LC-KJ. (a) The TGF- $\beta$  receptor kinase inhibitor SD208 (1  $\mu$ M/mL) or (b) a TGF- $\beta$  neutralizing antibody (5  $\mu$ g/mL) were added 1 h before stimulation with 5 ng/mL TGF- $\beta$  for 8 h as indicated. The cell lysates were then subjected to immunoblot analysis. TMEPAI was detected by using anti-TMEPAI antibody and the levels of phosphorylated Smad2 were detected with anti-phospho Smad2 antibody (PS2).  $\beta$ -actin was used as the loading control. (c) TGF- $\beta$  activities in serum-free conditioned media after 24 h of incubation with the indicated cells were measured by (CAGA)<sub>12</sub>-luc reporter assay. The means  $\pm$  SDs are shown.



**Fig. 3.** Calu3-shTMEPAI cells have increased sensitivity to transforming growth factor-β (TGF-β). (a) Generation of TMEPAI-knockdown Calu3 cell lines. The expression of TMEPAI was significantly suppressed by stable expression of two independent shRNAs (#9 and #10) targeting TMEPAI mRNA. Cells were treated with TGF-β for 8 h, and endogenous TMEPAI was detected by immunoblot analysis. β-actin was used as the loading control. (b) Cells were stimulated with TGF-β (1 ng/mL) for 1 h and phosphorylated Smad2 was detected with anti-phospho Smad2 antibody (PS2). β-actin was used as the loading control. (c,d) Cell proliferation assays. (c) TMEPAI-knockdown Calu3 cells (Calu3-sh#9 and Calu3-sh#10) were cultured in 12-well plates without TGF-β (left), with TGF-β (0.1 ng/mL) (middle) or with TGF-β (0.1 ng/mL) and TGF-β neutralizing antibody (1 ng/mL) (right), as indicated. The cell numbers were counted every second day. The means ± SDs are shown. (d) Mean percentage growth inhibition by TGF-β (0.1 ng/mL) and the reversal by inclusion of TGF-β neutralizing antibody (1 ng/mL) was calculated on day 9 in each cell line. Calu3 sh. control, Calu3 sh. control expresses non-targeting shRNA (SHC002, Sigma) in pLKO.1-puro vector.



**Fig. 4.** Calu3-shTMEPAI cells have decreased sphere-forming activities. (a) Control and TMEPAI-knockdown Calu3 cells (Calu3-sh#9 and Calu3-sh#10) were cultured in sphere formation medium for 12 days. Typical photographs are shown. (b) The numbers of spheres larger than 100 μm in diameter were counted. The means ± SDs are shown. \*P < 0.01 (vs control). Calu3 sh. control, Calu3 sh. control expresses non-targeting shRNA (SHC002, Sigma) in pLKO.1-puro vector.

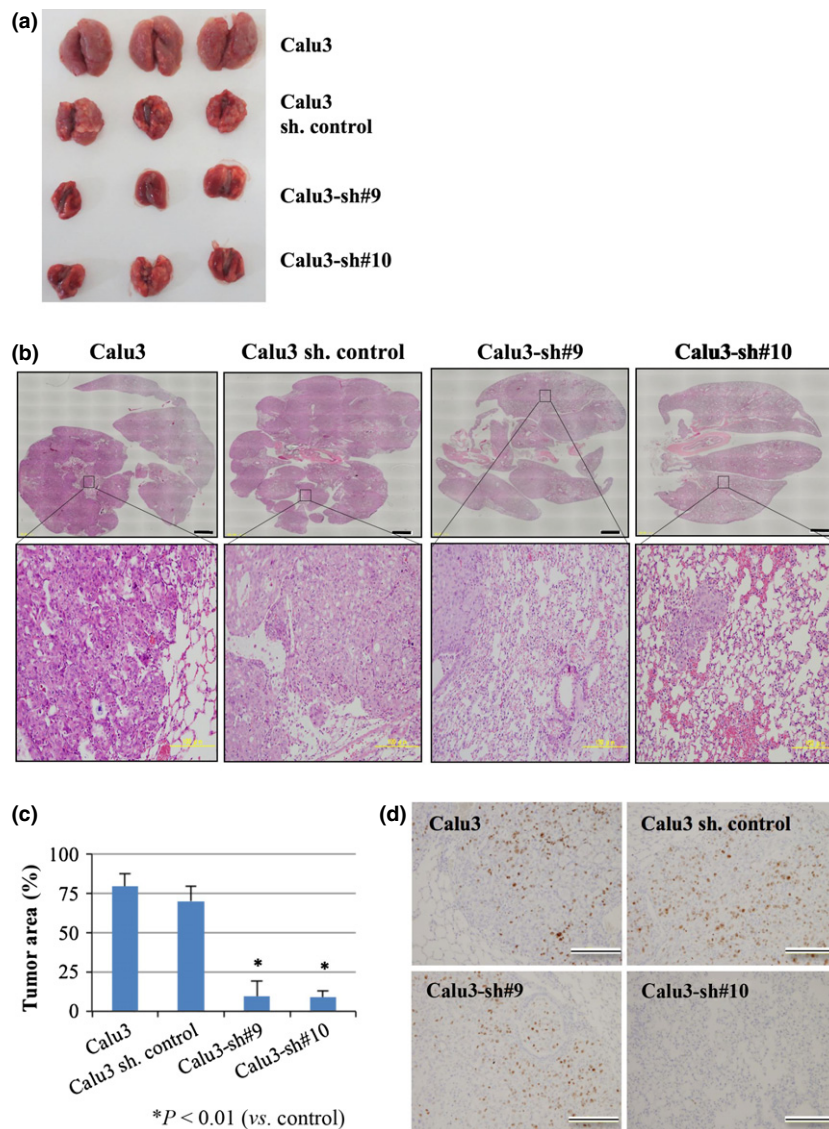
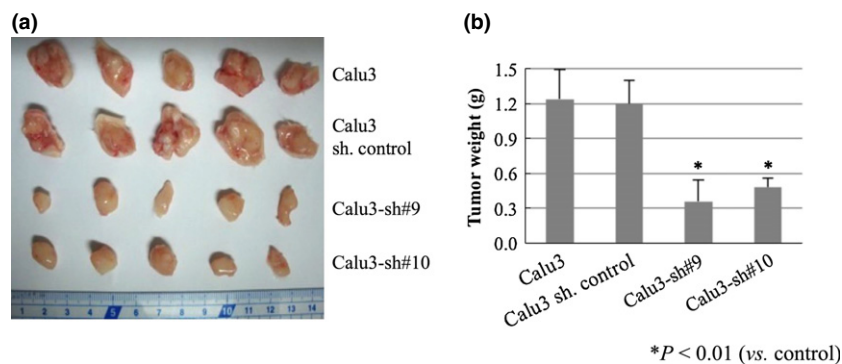
the tail veins of NOD-SCID mice. Eight weeks after injection, mice were killed and the collected lungs were examined histopathologically with H&E staining. We also stained for Ki-67 to detect proliferating cancer cells. Using morphometric software, we calculated the percentage of the representative tumor areas in the total lung areas. Calu3-sh#9 and Calu3-sh#10 cells formed significantly smaller tumors both in size and numbers than those of the control Calu3 cells

(Fig. 6). These results indicated that knockdown of TMEPAI suppresses the ability of lung cancer cells to develop metastatic tumors in lungs.

### Discussion

Multiple positive and negative regulators have critical roles in the regulation of the TGF-β/Smad signaling pathway.<sup>(26,27)</sup>

**Fig. 5.** Calu3-shTMEPAI cells have decreased tumor-forming activities. (a) TMEPAI-knockdown Calu3 cells (Calu3-sh#9 and Calu3-sh#10) were injected s.c. into NOD-SCID mice with Matrigel supplement. After 3 months, the tumors were harvested and photographed. (b) The tumors were weighed. The means  $\pm$  SDs are shown. \* $P < 0.01$  (vs control). Calu3 sh. control, Calu3 sh. control expresses non-targeting shRNA (SHC002, Sigma) in pLKO.1-puro vector.



**Fig. 6.** Calu3-shTMEPAI cells have decreased tumorigenic activity in the lungs. TMEPAI-knockdown Calu3 cells (Calu3-sh#9 and Calu3-sh#10) were injected into the tail vein of NOD-SCID mice ( $10^6$  cells in 200  $\mu$ L PBS/mice). (a) After 8 weeks, the lungs were collected and photographed. (b) The thin sections of lungs were stained with H&E. Scale bar = 1 mm. Higher magnifications show a typical metastatic area. (c) The percentages of tumor areas were measured by image analyses. The means  $\pm$  SDs are shown. \* $P < 0.01$  (vs control). (d) The same lung sections as those in (b) were stained with anti-Ki67 antibodies. The nuclei of proliferating cancer cells showed positive staining. Scale bar = 100  $\mu$ m. Calu3 sh. control, Calu3 sh. control expresses non-targeting shRNA (SHC002, Sigma) in pLKO.1-puro vector.

Disruption of the balance between these positive and negative regulators can lead to various diseases.<sup>(28–30)</sup> We have recently identified TMEPAI as a direct target gene of TGF- $\beta$  signaling that participates in negative feedback regulation of the duration and intensity of TGF- $\beta$ /Smad signaling.<sup>(17,19)</sup> TMEPAI has a family molecule, C18ORF1; we established several mAbs specific for TMEPAI without cross-reactivity to C18ORF1.

Previous studies indicated that TMEPAI is highly expressed in various cancers such as renal cell carcinoma, colon cancer,

breast cancer, and ovarian cancer.<sup>(12,14,20)</sup> Genome-wide studies, which compared the gene expression levels of invasive cancer tissues with normal counterpart tissues or preinvasive cancers, suggested that TMEPAI is one of the most highly inducible genes in invasive cancers.<sup>(21,22)</sup> Furthermore, dysregulation of TGF- $\beta$  signaling was identified as an important mediator of lung cancer invasion.<sup>(30)</sup> Therefore, we examined the expression of TMEPAI in lung cancer cell lines. Both immunoblot and immunofluorescent analyses clearly detected



high levels of TMEPAI expression in all three examined lung cancer cell lines (Fig. 1b).

The mechanism of enhanced TMEPAI expression in cancer cells is an important issue to be elucidated. Oncogenes can be activated by gene amplification and other mechanisms. Human chromosomal region 20q13, on which the *TMEPAI* gene is located, is frequently amplified in breast cancers.<sup>(31,32)</sup> Expression of TMEPAI in lung cancer cells disappeared in the presence of a TGF- $\beta$  receptor kinase inhibitor and was significantly suppressed by anti-TGF- $\beta$  neutralizing antibodies (Fig. 2a,b). Although Calu3 cells expressed high levels of TMEPAI in the absence of detectable Smad2 phosphorylation, expression of TMEPAI was significantly suppressed by a TGF- $\beta$  receptor kinase inhibitor (Fig. 2a). Our previous findings indicated that Smad3 and Smad4 are essential for the TGF- $\beta$ -inducible expression of TMEPAI.<sup>(17)</sup> However, it is also known that the expression of TMEPAI is enhanced by Wnt, EGFR/Ras/MAPK, androgen, and mutant p53 as shown by us and others.<sup>(11–13,17,33)</sup> The effects of SD208 (Fig. 2a) indicate that activity of type I TGF- $\beta$  receptor kinase is required for the stable expression of TMEPAI in lung adenocarcinoma cells. Many of these cells usually have activated EGFR/RAS/MAPK signaling, even if Smad2 phosphorylation is undetectable in immunoblot analysis. These results suggest that cancer cells can maintain TMEPAI expression by multiple oncogenic signaling to suppress Smad phosphorylation down to undetectable levels but even in these cases TGF- $\beta$  receptor kinase activity is required to support TMEPAI expression. The contributions of synergistic effects of multiple oncogenic signaling, including the role of the TGF- $\beta$  non-Smad signaling pathway in cancer cells, must be elucidated in the future.

The functions of TMEPAI in cancer cells are the next important issue to be examined. Recent reports indicated that knockdown of TMEPAI suppresses the tumorigenic activities of breast cancer cells and androgen receptor-negative prostatic cancer cells.<sup>(23,34)</sup> Here, we showed that knockdown of TMEPAI in lung cancer cells potentiates TGF- $\beta$ -inducible Smad

phosphorylation and growth inhibitory responses to TGF- $\beta$  (Fig. 3). Consequently, the sphere-forming activity *in vitro*, s.c. tumor formation, and lung metastasis were significantly suppressed in NOD-SCID mice (Figs 4–6, S3, S4). Liu *et al.*<sup>(34)</sup> reported that knockdown of PMP1/TMEPAI suppresses the Smad3/4-cMyc-p21<sup>Cip1</sup> pathway in androgen receptor-negative prostatic cancer cells. These effects can be explained by the suppressive function of TMEPAI on Smad signaling through its SIM domain. However, Prajjal *et al.*<sup>(23)</sup> identified suppression of Akt phosphorylation and expression of hypoxia-inducible factor-1 $\beta$  and vascular endothelial growth factor in TMEPAI-knockdown xenograft breast tumors. These effects may be independent from the Smad regulatory function of TMEPAI. Both TMEPAI and its family molecule C18ORF1 share tandem PY motifs interposing a SIM domain. The possibility of another TMEPAI function such as that indicated by Prajjal *et al.* may explain the reason for TMEPAI's involvement among multiple regulators of Smad signaling in outstandingly divergent cancers. Further studies will be required to reveal the overall functions of TMEPAI in cancer development.

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### Disclosure Statement

The authors have no conflict of interest.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Fig. S1.** Protein sequences of TMEPAI and C18ORF1.

**Fig. S2.** Establishment of monoclonal anti-TMEPAI antibodies.

**Fig. S3.** NCI-H23-shTMEPAI cells have increased sensitivity to transforming growth factor- $\beta$  (TGF- $\beta$ ) and decreased sphere-forming activities.

**Fig. S4.** NCI-H23-shTMEPAI cells have decreased tumor-forming activities.