Tumorigenic function of TMEPAI in cancer

(腫瘍におけるＴＭＥＰＡＩの機能解析)

２０１４

筑波大学大学院博士課程人間総合科学研究科
VO NGUYEN THANH THAO

ABSTRACT

[Purpose]

TMEPAI is a direct target gene of transforming growth factor-β (TGF-β)/Smad signaling, and participates in the negative feedback regulation to control the duration and intensity of TGF-β/Smad signaling. In this thesis work, I aimed to investigate the mechanism of enhanced TMEPAI expression in cancer cells and examined the cooperative roles of TGF-β and EGF signaling. In addition, I further aimed to investigate the tumorigenic activities of TMEPAI in lung cancer cells.

[Materials and methods]

We carried out experimental methods in molecular biology such as polymerase chain reaction (PCR), western-blotting, immunoprecipitation (IP), chromatin immunoprecipitation (ChIP) to examine the expression, phosphorylation and interactions of the proteins. The luciferase-reporter assays were used to examine the transcriptional activity of the target genes. TMEPAI was knockdown in lung cancer cells by short hairpin RNA, and sphere formation assay was used to examine oncogenic activities of these cells. For in vivo tumorigenic activity, two xenograft models, subcutaneous injection and tail vein injection were used.

[Results]

Lung adenocarcinoma cell lines Calu3, NCI-H23, and RERF-LC-KJ constitutively expressed TMEPAI and it was significantly suppressed by a TGF-β receptor kinase inhibitor SB208, suggesting that constitutive expression of TMEPAI in these cancer cells depends on autocrine TGF-β stimulation. Additionally, expression of TMEPAI in these cancer cells was significantly suppressed by MEK inhibitor U0126 too. TGF-β

and EGF signals cooperatively enhanced the transcription of TMEPAI, suggesting that the EGFR/Ras/MAPK pathway is also involved in the regulation of TMEPAI in cancer cells. There are ELK-1 binding sites in the first intron of the TMEPAI gene. EGF-induced activation of wild-type ELK-1, but not the mutant ELK-1S383A potentiated the TGF-β-induced transcription of the TMEPAI gene. Furthermore, the chromatin immunoprecipitation (ChIP) assay revealed that ELK-1 binds to the enhancer sequence in the first intron of the TMEPAI gene in response to EGF signaling.

Knockdown of TMEPAI in Calu3 cells enhanced levels of Smad2 phosphorylation and significantly suppressed cell proliferation in the presence of TGF-β. Furthermore, knockdown of TMEPAI in Calu3 cells and NCI-H23 cells suppressed sphere formation in vitro and tumor formation both in subcutaneous tissues and in lungs of NOD-SCID mice. Together, these results indicate that TMEPAI promotes tumorigenic activities in lung cancer cells.

Moreover, we recently identified that AktSer473 phosphorylation is suppressed in TMEPAI knockdown lung cancer cells, and the phosphatase working on this Akt residue PHLPP1 were increased in these TMEPAI knockdown cells.

[Discussion]

Cancer cells frequently have abnormal activation of EGF/Ras/MAPK signaling and TGF-β signaling. Deregulation of both signaling has been reported to contribute to cancer progression. Here, we showed that EGF/Ras/MEK and TGF-β signaling cooperatively control TMEPAI expression. On the other hand, knockdown of TMEPAI in lung cancer cells potentiated TGF-β-inducible Smad phosphorylation and growth inhibitory response to TGF-β. Consequently, the sphere-forming activity, subcutaneous tumor formation, and lung metastasis were suppressed. Moreover, we identified that AktSer473 phosphorylation is suppressed in TMEPAI knockdown lung cancer cells. Akt phosphatase PHLPP1 increased in these cells. These data suggest a possibility of novel function of TMEPAI, which is independent from the Smad regulator function, may also contribute to the oncogenic function of TMEPAI.

[Conclusion]

In this work, we revealed that the expression of TMEPAI is transcriptionally enhanced by the cooperation between TGF-β/Smad and EGF/Ras/MAPK signals. TMEPAI has tumorigenic activities in lung cancer cells and it might be exerted by suppression of the TGF-β/Smad signaling and activation of the AKT signaling.

TABLE OF CONTENTS

Abstract

CHAPTER 1: Introduction 1

1-1 Transforming growth factor β (TGF-β) signaling 1

1-2 TGF-β signaling and cancer 3

1-3 Transmembrance prostate androgen induced protein (TMEPAI) 4

1-4 Epidermal growth factor (EGF) signaling 7

1-4-1 EGF signaling 7

1-4-2 Ras/MAPK signaling 8

1-5 ELK-1 transcription factor 9

CHAPTER 2: Cooperative Induction of Transmembrane Prostate Androgen Induced protein-TMEPAI by Transforming Growth Factor-β and Epidermal Growth Factor signaling

2-1 Introduction 11

2-2 Materials and methods 12

2-2-1 Plasmid construction 12

2-2-2 Cell culture 12

2-2-3 Luciferase assay 13

2-2-4 Immunoprecipitation and western-blotting 13

2-2-5 Chromatin precipitation assay 15

2-3 Results 16

2-3-1 Cooperative enhancement of TMEPAI expression by EGF in the presence of TGF-β 16

2-3-2 MEK inhibitor suppresses TMEPAI expression enhanced by EGF 18

2-3-3 Identification of EGF responsiveness in the first intron of the TMEPAI gene 20
2-3-4 ELK-1 binding elements are required for the EGF-inducible enhancement of the TMEPAI gene expression 22
2-3-5 Binding of ELK-1 to Smad3 and ELK-1 binding sites in the first intron of the TMEPAI gene 24
2-4 Discussion 25

CHAPTER 3: TMEPAI enhances Tumorigenic Activities in Lung Cancer Cells

3-1 Introduction 29
3-2 Materials and methods 29
3-2-1 Cell culture 29
3-2-2 Luciferase assay 30
3-2-3 Western-blotting 31
3-2-4 Immunofluorescence staining 31
3-2-5 Cell proliferation assay 32
3-2-6 Sphere formation assay 32
3-2-7 In vivo tumor formation assay 32
3-2-8 In vivo lung metastasis assay 32
3-3 Results 33
3-3-1 Enhanced and constitutive expression of TMEPAI in lung cancer cell lines 33
3-3-2 TGF-β signaling mediates the enhanced expression of TMEPAI in lung cancer cells 35
3-3-3 Knockdown of TMEPAI enhances Smad phosphorylation and growth inhibitory responses of TGF-β 37
3-3-4 TMEPAI enhances sphere-forming activities 39
3-3-5 TMEPAI enhances in vivo tumor formation 40
3-3-6 TMEPAI enhances metastatic tumor formation in lungs 41
3-4 Discussion 43

CHAPTER 4: Perspectives 46
4-1 Oncogenic function of TMEPAI and its application in cancer therapy 46
4-2 Application of TMEPAI monoclonal antibody in cancer diagnosis 48

CHAPTER 5: Conclusions 50

References

Acknowledgements

CHAPTER 1: INTRODUCTION

1-1 Transforming growth factor β (TGF-β) signaling

TGF-β was identified almost 30 years ago as a factor that induced anchorage-independent growth of normal rat kidney fibroblasts. Over the past decades, 33 members of TGF-β family ligands have been identified in human comprising the TGF-βs, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), inhibin and activins (Roberts and Sporn et al., 1990; Massagué, 2000). All TGF-β family ligands are generated as dimeric precursor proteins and subsequently processed by proteases and secreted.

TGF-β signaling is initiated by ligand-induced heteromeric complex formation of specific type I and type II serine/threonine kinase receptors (TβRI and TβRII). TβRI (also termed activin receptor-like kinase-5; ALK5) is phosphorylated and activated by TβRII. Active TβRI catalyzes the C-terminal serine phosphorylation of receptor-regulated Smads (R-Smads). Among R-Smads, Smad2 and Smad3 act downstream of TGF-β, activin, and nodal type I receptors; whereas Smad1, Smad5, and Smad8 act downstream of bone morphogenetic protein (BMP) type I receptors. After phosphorylation, R-Smads form a ternary complex with common mediator Smad (Co-Smad, *i.e.*, Smad4), and translocate into the nucleus, where they regulate, together with other partner proteins, the transcription of specific target genes (Massagué, 2000, Shi and Massagué, 2003). Inhibitory Smads (I-Smads, *i.e.* Smad6 and Smad7) can inhibit the activation of R-Smads by competing with R-Smads for interaction with type I

receptors and by recruiting specific ubiquitin ligases or phosphatases to the activated receptor complex, thereby targeting it for proteosomal degradation or dephosphorylation, respectively (Heldin et al., 1997; Shi and Massagué, 2003).

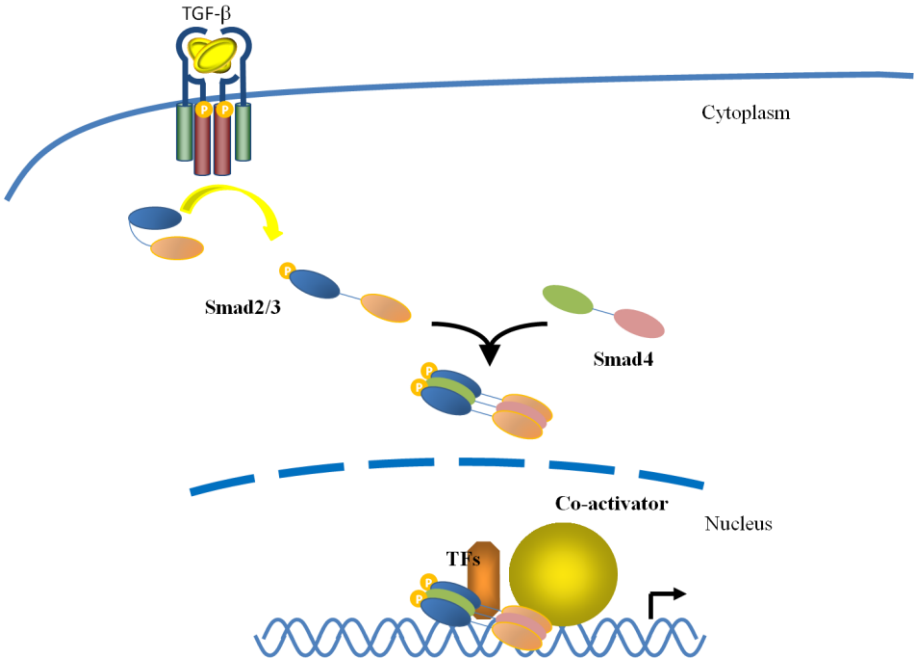


Figure 1: Transforming Growth Factor β (TGF-β) signaling. After ligand-induced heteromeric TGF-β receptor complex formation, R-Smads (*i.e.*, Smad2 and Smad3) are recruited to the activated TGF-β type I receptor and getting phosphorylated. Activated R-Smads form complexes with Smad4, which translocate to the nucleus, where they bind to the *cis*-elements of the target genes, together with transcription factor(s) (TF) and coactivator(s), and activate transcription of the various target genes.

The TGF-β family members have diverse effects on a wide variety of cellular activities, including cell cycle control, extracellular matrix formation, apoptosis, angiogenesis, immune function, and cancer progression (Whitman, 1998; Massagué et

al., 2000; Derynck et al., 2001). The multiple functions of the TGF-β family members require tight control of their activities (Massagué and Wotton, 2000). Both positive and negative regulators have been identified at nearly every step in the TGF-β family signaling cascade. For example, Smad anchor for receptor activation (SARA) has been shown to recruit R-Smads to the activated TGF-β receptor complex (Moustakas and Heldin, 2009). On the other hand, I-Smads (*i.e.*, Smad6 and Smad7), Smad ubiquitination regulatory factors (Smurfs), TG-interacting factor (TGIF), and ski-related novel protein N (SnoN) are direct target genes for TGF-β signaling and contribute to form negative feedback loops (Massagué et al., 2005; Itoh and ten Dijke, 2007). Misregulation of their functions has been implicated in the development of diseases such as cancer, fibrosis and autoimmune disease.

1-2 TGF-β signaling and cancer

There are numerous studies about the role of TGF-β signaling in human cancers. TGF-β1 has been reported that it is frequently upregulated in tumor cells, and the transgenic mice that produce a constitutively active form of TGF-β1 have been shown to be resistant to DMBA induced tumor formation. Mutation in *TGFBR1* and *TGFBR2* genes occur frequently in many cancers such as ovarian, breast, lung, esophageal, and prostate cancers (Derynck et al., 2001; Massagué, 2008). TGF-β can act as tumor suppressor in early stage and switch to tumor promoter in late stage (Pardali and Moustakas, 2007). During early stage of carcinogenesis, TGF-β acts as a tumor suppressor by inhibiting growth and promoting apoptosis. TGF-β downregulates c-Myc expression, which is required for the induction of p15 and p21 CDK inhibitors. TGF-β could also suppress the expression of ID1, which causes enhancement of Ras-driven

mammary tumorigenesis in mice, suggesting the tumor-suppressive role of TGF-β. Moreover, TGF-β can trigger apoptosis via inducing death-associated protein kinase DAPK, and Daxx adaptor protein through their ability to interact with TβRII (Massagué, 2008). In addition, the role of TGF-β in angiogenesis during embryonic development has been well-documented. Ablation of TβRI or TβRII genes results in embryonic lethality because of angiogenesis defection suggesting that TGF-β can induce angiogenesis (ten Dijke and Arthur, 2007). Nevertheless, TGF-β has been shown to be upregulated in many human cancers and associated with poor prognosis, which suggests the tumor promoter function of TGF-β in late stage. Loss of TβRII expression increases the invasiveness of lung cancer cells (Toonkey et al., 2010). Furthermore, TGF-β is known as a potent inducer of epithelial to mesenchymal transition (EMT), which is characterized by extensive changes in the expression of cell adhesion molecules such as E-cadherin, N-cadherin, ZO-1, *etc*. TGF-β promotes EMT by inducing the expression of Snail, Slug, Twist, and metalloproteinases MMP2, MMP9, which are implicated in the migratory and invasive processes (Davies et al., 2005; Miyazono, 2009). Taken together, TGF-β is strongly implicated in cancer progression by modulating multiple aspects of tumorigenesis, including cell growth, apoptosis, angiogenesis, invasion and metastasis.

1-3 Transmembrane prostate androgen induced protein (TMEPAI)

Transmembrane prostate androgen induced protein (TMEPAI), alternatively termed PMEPA1, STAG1, ERG1.2, or N4wbp4, is originally identified as a prostatic RNA, which is induced by testosterone or its derivatives (Xu et al., 2000, 2003; Giannini et al., 2003). Transcription of TMEPAI gene was later shown to be induced by TGF-β (Brunschwig et al., 2003; Itoh et al., 2003; Levy and Hill, 2005). TMEPAI is a

transmembrane protein containing two PY motifs that can interact with HECT-type E3 ubiquitin ligases (Li et al., 2008).

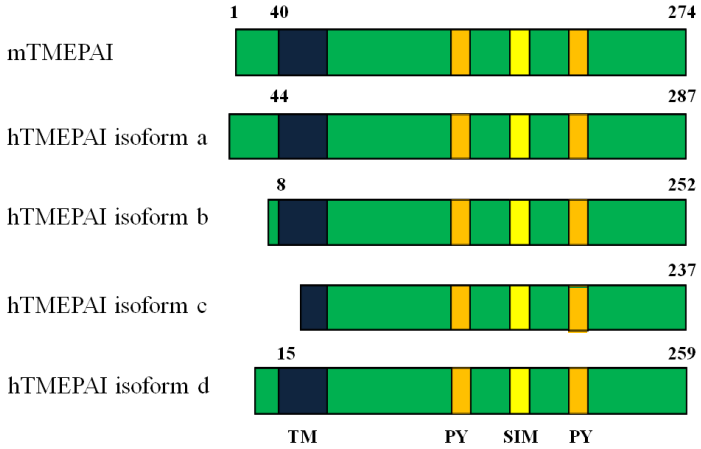


Figure 2: Schematic representation of TMEPAI. There are one isoform in mouse and four isoforms in human (isoform a, b, c, d), which is different in the N terminus. The TMEPAI protein contains transmembrane domain (TM) and one Smad interaction motif (SIM) locating between two PY motifs (PY).

TMEPAI has been reported to be involved in p53-mediated apoptosis and cell growth inhibition (Xu et al., 2003). A recent report indicated that TMEPAI is a direct target gene of TGF-β signaling, which can interact with Smad2 and Smad3 via its Smad interaction motif to sequester Smads from TGF-β/Smad signaling. Because of the competition with SARA for binding to Smads, TMEPAI participates in a negative feedback regulation to control the intensity and duration of TGF-β/Smad signaling (Watanabe et al., 2010). In addition to TGF-β stimulation, TMEPAI has been reported to be induced by treatment with androgen, introduction of mutant p53, or activation of the ERK pathway (Xu et al., 2000; Anazawa et al., 2004). TMEPAI expression was highly increased in breast cancer, colon cancer, and renal cell carcinoma tissues as well as in many cancer cell lines suggesting an oncogenic function of this molecule. Actually,

TMEPAI was indicated as a “molecular switch” that converts TGF-β signaling from a tumor suppressor to a tumor promoter (Prajial et al., 2010). A recent study also shown that TMEPAI is a downstream target of Wnt signaling, in which the Wnt/β-catenin/TCF7L2 pathway is preferentially able to activate the transcription of this gene (Nakano et al., 2010). Taken together, TMEPAI may play an important role in TGF-β signal regulation, highly expressed in cancer and contribute to cancer progression.

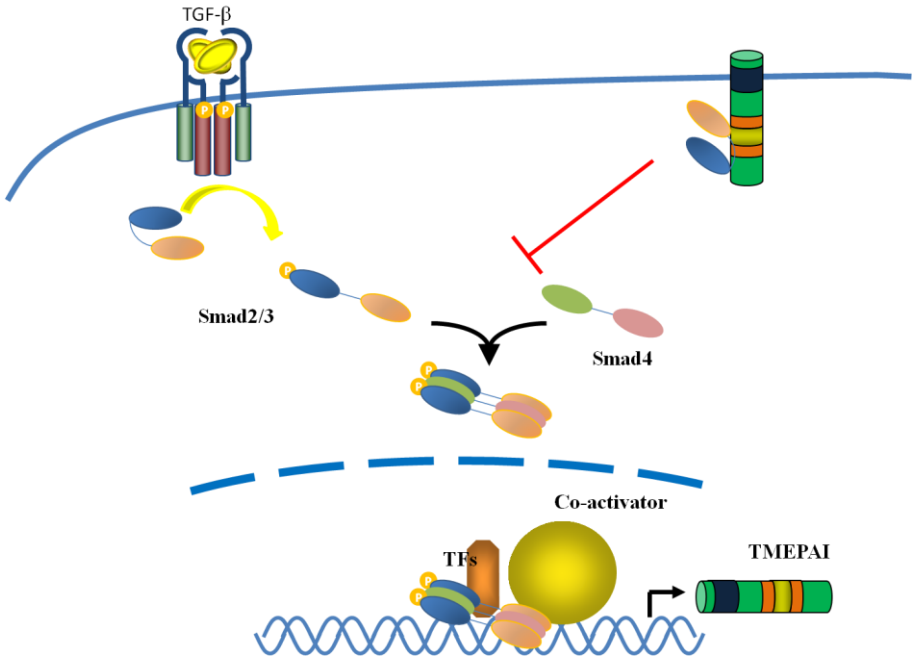


Figure 3: Scheme of TMEPAI regulatory action on TGF-β signaling. TMEPAI is a target gene of the TGF-β/Smad signaling. After induced by TGF-β, TMEPAI locates in the cell membrane and interact with R-Smads (Smad2/3). Thus, TMEPAI sequesters R-Smads from TGF-β/Smads signaling. Thereby, TMEPAI regulates the duration and intensity of TGF-β signaling.

1-4 Epidermal growth factor (EGF) signaling

1-4-1 Epidermal growth factor (EGF) signaling

Epidermal growth factor (EGF) is a key growth factor in regulating cell proliferation, cell survival, invasion and angiogenesis (Jorissen et al., 2003). Dysfunction of EGF signaling pathways is frequently observed in cancer and is involved in the pathogenesis and progression of cancers (Yarden, 2001; Henson and Gibson, 2006). Overexpression of EGFR has been occurred in many cancers and contributes to poor prognosis (Selvaggi et al., 2004; Lo et al., 2006). The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases (RTKs), which comprises four distinct receptors, EGFR (HER-1/ErbB1), HER-2 (ErbB2 or Neu), HER-3/ErbB3, and HER-4/ErbB4. Upon binding to their ligands, EGF receptors form either homodimers or heterodimers, and subsequent autophosphorylation by its intracellular tyrosine kinase activity. Receptor activation leads to the activation of downstream signaling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway, the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway, and the phosphotidylinositol 3-kinase/AKT (PI3K/AKT) pathway (Darnel et al., 1994; Jorissen et al., 2003; Quesnelle et al., 2007).

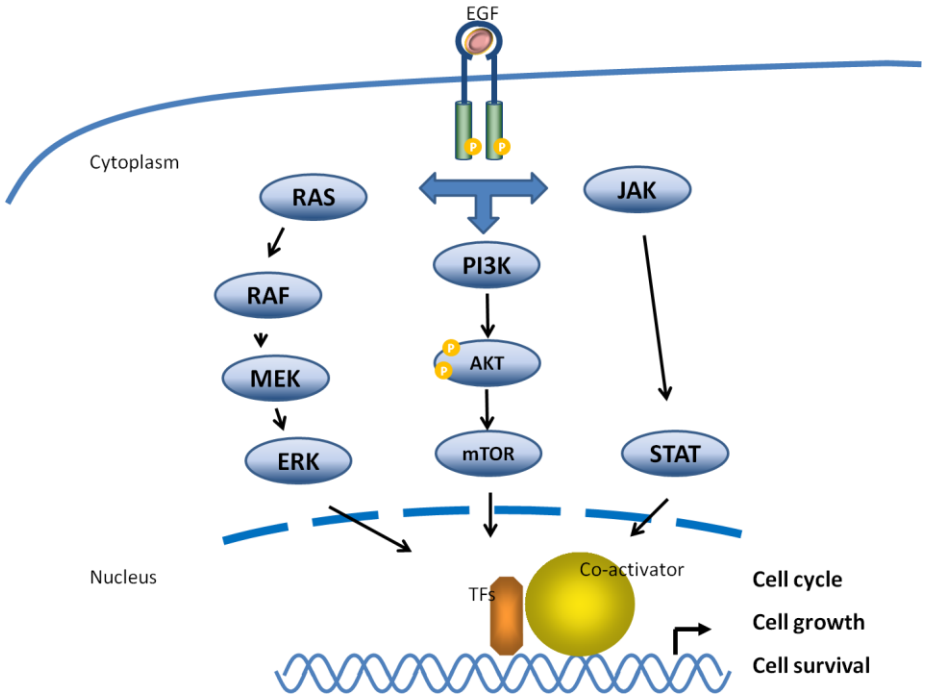


Figure 4: Epidermal Growth Factor (EGF) Signaling. Upon ligand-binding, the receptors form dimers and cause tyrosine phosphorylation by the intracellular tyrosine kinase domain. Receptor activation leads to activation of downstream signaling pathways, including Ras/Raf/MEK/ERK, Jak/STAT, and PI3K/AKT signaling pathways.

1-4-2 RAS/MAPK signaling

Mitogen-activated protein kinases (MAPKs) are a family of protein serine-threonine kinases that is activated by receptor tyrosine kinases (RTKs), including EGF receptor. MAPKs consists extracellular signal-regulated kinase (ERK), c-jun terminal kinase (JNK), and p38-mitogen activated protein kinase. ErbB receptor activates ERK through the binding of adaptor protein Grb2 and/or Shc, then activates small G protein Ras. Activated Ras then phosphorylates and activates their downstream cascades Raf, MEK, ERK, respectively, leading to the activation of a variety of transcription factors, such as c-Myc, Tal1, and ELK-1, and consequently increase expression of their target genes

(Shaw and Saxton, 2003). Mutations in Ras gene, causing activation of Ras/MAPK signaling pathways, have been reported in many human cancers, such as pancreas, lung adenocarcinoma, melanoma, thyroid carcinoma, and so on (Downward, 2003).

1-5 ELK-1 transcription factor

The E-twenty six (ETS) domain transcription factors including ELK-1, Net (ELK-3), and Sap-1 (ELK-4) belong to the ternary complex factor (TCFs) family of transcription factors to form a ternary nucleoprotein complex with the serum response factor (SRF) in regulating *c-fos* gene expression. The ETS domain transcription factors play a pivotal role in cell proliferation, differentiation, and development (Marais et al., 1993; Shaw and Saxton, 2003; Buchwalter et al., 2004). An ETS domain transcription factor ELK-1 is a direct substrate of MAPK. Activation of ELK-1 triggers the regulation of a subset of genes involved in transcription, splicing, and translation (Janknecht et al., 1993; Wasylyk et al., 1998). Activation of ELK-1 has been reported in T-cell activation and development. Moreover, knockdown ELK-1 inhibits *c-fos* induction in the forebrain formation in mice, but not in embryonic fibroblast (MEFs). In addition, a shorter isoform of ELK-1 (sELK-1) with a truncation of 54-amino-acid from the N-terminal, has been shown to play an opposite role to ELK-1 in regulation of neuronal cell differentiation (Araud et al., 2007; Rahim et al., 2012). ELK-1 shares four conserved binding domains with other TCF family proteins: the N-terminal ETS DNA binding domain, which binds specifically to the purine-rich sequence 5`-GGA(A/T)-3` ETS binding site (EBS), SRF binding domain (B-box) that is responsible for the ternary complex formation, a MAPKs induced-transcriptional activation domain in the C-

terminal end, and a MAPK binding domain. Moreover, a repressor domain (R motif), uniquely found in ELK-1, suppresses the transcriptional activity of ELK-1 and dampens its response to MAPK-inducible activation (Shore et al., 1995; Yang et al., 2002, 2003). Phosphorylation of ELK-1 by MAPK-1 triggers the conformational change resulting in enhancement of DNA binding activity and recruitment of coactivators such as CBP, p300, Srb, and consequently induces the regulation of the target genes.

Figure 5: Representative structure of ELK-1. ELK-1 is one of TCF subfamily members. ELK-1 contains DNA binding ETS domain (A box), SRF binding domain (B box), MAP kinase binding domain (D box and F box), transcriptional activation domain (C box), and repressor domain (R box).

CHAPTER 2:

Cooperative Induction of Transmembrane Prostate Androgen Induced protein-TMEPAI by Transforming Growth Factor-β and Epidermal Growth Factor signaling

2-1 Introduction

It has been well-known that the transforming growth factor β (TGF-β) keeps pivotal role in regulating tissue homeostasis. Deregulation of TGF-β signaling has been implicated in many diseases, including cancer (Massagué et al., 2000, 2008). On the other hand, the TGF-β signaling has been interwoven into a vast network of cell signaling (Kretzchmar et al., 1999; Lo et al., 2001; Derynck and Zhang, 2003; Guo and Wang, 2009, Moustakas and Heldin, 2009). The epidermal growth factor (EGF) signaling is involved in signaling crosstalk with TGF-β signaling, and is important pathway in cancer progression. Numerous reports have described the co-operation between EGF/Ras/MAPK pathway and TGF-β signaling (Mulder, 2000; Freytag et al., 2010). For instance, cells harboring oncogenic Ras mutations often confer resistance to growth inhibitory response to TGF-β. Activation of Ras via ERK/MAPK kinases causes linker phosphorylation of Smad2/3, leading to inhibit the nuclear translocation of Smads and their abilities to mediate TGF-β function (Mulder, 2000). Furthermore, Ras/MAPK pathway causes a decrease of Smad4 expression, inhibits TGF-β-induced complex formation between Smad2/3 and Smad4, and results in suppression of TGF-β signaling (Saha et al., 2001). In addition, Watanabe et al. reported that TGF-β-induced TMEPAI

participates in a negative feedback loop to regulate the activity of TGF-β signaling (Watanabe et al., 2010). Moreover the expression of TMEPAI is regulated not only by TGF-β signaling, but also by the other signaling pathways, for example EGF signaling and Wnt signaling (Giannini et al., 2003; Anazawa et al., 2004; Hirokawa et al., 2007, Nakano et al., 2010). However, the mechanism in which TMEPAI is regulated by EGF signaling still remains unclear. In this study, I aimed to investigate the regulation of TMEPAI by cooperation between TGF-β and EGF signaling.

2-2 Materials and methods

2-2-1 Plasmid construction. Human ELK-1 and the mutant ELK-1 S383A constructs were described previously (Araud et al., 2007). The luciferase constructs pGL3ti-1972-luc, pGL3ti-250, pGL3ti-850, and Flag-Smad3 were described previously (Nakano et al., 2010). The pGL3ti-250 mutants (pGL3ti-250-M1, pGL3ti-250-M2, pGL3ti-250-M123) were made by introducing the mutations to change the consensus sequence GGAT to TGCT by using corresponding mutant primers and pfx polymerase (Invitrogen). All plasmids were sequenced before use.

2-2-2 Cell culture. HaCaT cells (spontaneously immortalized human keratinocyte cell line)**,** HaCaT-mock, constitutively active H-Ras transformed HaCaT-RasG12V and COS7 cells (African green monkey kidney cells transformed by SV40) were cultured in Dulbecco`s modified Eagle's medium (Sigma) containing 10% fetal calf serum (FCS, Invitrogen) and penicillin streptomycin solution. HepG2 cells were cultured in

minimum essential medium (Sigma) containing 10% FCS, non-essential amino acids (NEAA, Invitrogen), penicillin streptomycin solution, and sodium pyruvate. A TGF-β receptor kinase inhibitor SB431542 (Sigma) and a MEK kinase inhibitor U0126 (Wako) were used to block TGF-β signaling and EGF/Ras/MAPK signaling, respectively.

2-2-3 Luciferase assay. HepG2 cells were seeded at 1.5 x 10^5 cells/ well in 12-well plate one day before transfection. The cells were transfected using FuGENE6 (Roche Applied Science). Where indicated, the cell were stimulated with TGF-β (0.1 ng/ml) and EGF (10 ng/ml) 24 hours after transfection, and cultured in the absence of FCS for 18 hours. Luciferase activities were determined by Luciferase Assay Systems (Promega) and normalized to β-galactosidase activity of co-transfected pCH110 (GE HealthCare). Each transfection was carried out in triplicate.

Lysis buffer : 2 mM DTT, 25 mM Tris-phosphate (pH 7.8),
2 mM trans-1,2-diaminocyclohexane-N,N,N`,N`-tetra-acetic
acid monohydrate, 10% glycerol, 1% Triton X-100

2x β-gal : 200 mM sodium phosphate buffer (pH 7.3),
100 mM β-mercaptoethanol,
1.33 mg/ml 2-nitrophentl-β-D-galactopyraniside

2-2-4 Immunoprecipitation and w**estern-blotting.** To detect the protein-protein interaction, the plasmids were transfected into COS7 cells (5 x 10^5 cells/ 6 cm dish) using FuGENE6. Thirty six hours after transfection, the cells were dissolved in 500 μl

of TNE lysis buffer. The cell lysates were precleared with protein G-Sepharose beads (GE Healthcare) for 30 minutes at 4^0C with end-over-end rotation and then precipitated with Flag-antibody for 2 hours at 4^0C. The immune complexes were precipitated by incubation with protein G-Sepharose beads for 30 minutes at 4^0C, followed by 3 washes with TNE buffer. The immunoprecipitated proteins and aliquots of the total cell lysates were subjected to western-blotting. The membranes were probed with different primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrate solution (Thermo Scientific).

TNE buffer : 10 mM Tris (pH 7.4), 150 mM NaCl, 10 mM EDTA,
1% NP-40, 1 mM PMSF, 20 mM β-glycerophosphate,
40 mM NaF, 100 units/ ml aprotinin, 5 µg/ml leupeptin.

Western-blotting:

SDS-PAGE : <Separating gel> distilled water 6.1ml,
polyacrylamide (10%) 30% acrylamide solution (Wako) 6.1 ml,
2 M Tris (pH 8.8) 4.2 ml, 10% SDS 64 µl, 10% APS 58 µl,
N-N-N-N-tetramethyl-ethylenediamine (Wako) 13 µl
<Stacking gel> 20% glycerol 3.5 ml
30% acrylamide (Wako) 700 µl, 0.5 M Tris (pH 6.8) 700 µl,
10% SDS 25 µl, 10% APS 25 µl,
N-N-N-N-tetramethyl-ethylenediamine (Wako) 5 µl

2x sample buffer : 4% SDS, 1.44 M β-mercaptoethanol, 20% glycerol,
125 mM Tris (pH 7.4), 0.002% bromophenol blue

TBST : 150 mM NaCl, 10 mM Tris (pH 7.4), 0.1% Tween20.

1st antibody : mouse anti-TMEPAI antibody (x 1000)
(homemade monoclonal antibody) (Vo Nguyen et al., 2014)
mouse anti-β-actin antibody (Sigma) (x 5000)
mouse anti-Erk 1/2 antibody (Zymed Ca) (x 1000)
mouse anti-phosphorylated Erk1/2 (pErk1/2) antibody
(Cell Signaling) (x 1000)
rabbit anti-Smad2/3 antibody (BD Bioscience) (x 1000)
rabbit anti-phosphorylated Smad2 (PS2) antibody
(Persson et al., 1998) (x 1000)
mouse anti-HA (3F10) antibody (Roche) (x 1000)
mouse anti-Flag M5 antibody (Sigma) (x 1000)

2nd antibody : anti mouse-IgG-(horseradish peroxidase; HRP)
(GE Healthcare) (x 10000)
anti rabbit-IgG-HRP (GE Healthcare) (x 10000)

2-2-5 Chromatin immunoprecipitation assay. HaCaT cells were stimulated with TGF-β (0.1 ng/ml) and EGF (10 ng/ml) as indicated for 1 hour, fixed by adding formaldehyde to the medium to a final concentration of 1% for 15 minutes at room temperature, and glycine was added to a final concentration of 125 mM. Then, the cells were washed with PBS once and collect into lysis buffer, and sonicated until the average length of input DNA became less than 500 bp in size. Then, the control IgG, anti-ELK-1, or anti-Smad3 was used for the precipitation of shared chromatins. The immunoprecipitated DNAs were purified and subjected to PCR amplification with

specific primers for detection of the TMEPAI promoter including ELK-1 binding elements.

Lysis buffer	: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA,
	1% SDS, 10 μg/ml leupeptin, 12.5 μg/ml aprotinin
Antibody	: rabbit anti-ELK-1 antibody (abcam)
	rabbit anti-Smad3 antibody (Cell Signaling)
	anti-rabbit IgG antibody
Primer sequence:	5`-TGA GCG TGT CCA TCT TTC TG- 3`
	5`-CAG TCC CAA ACA CAA ACA GC- 3`.

2-3 Results

2-3-1 Cooperative enhancement of TMEPAI expression by EGF in the presence of TGF-β. Human keratinocyte cell line HaCaT was stimulated with TGF-β, EGF, or both TGF-β and EGF. As shown in Fig. 6A, TMEPAI expression could be induced upon TGF-β stimulation, whereas there was no detectable level of TMEPAI upon EGF stimulation. However, co-stimulation with EGF clearly enhanced TMEPAI expression in the presence of TGF-β. Furthermore, HaCaT-RasG12V cells, in which constitutively active H-Ras was stably expressed, were used to confirm the contribution of EGF signaling on TMEPAI expression. Enhanced expression of TMEPAI was detected upon 8 hours of TGF-β stimulation in HaCaT-RasG12V cells approximately 5 fold higher than that in HaCaT-mock cells (Fig. 6B). These data suggest that EGFR/Ras/MAPK

signaling is involved in the regulation of TMEPAI expression in the presence of TGF-β signaling (Azami S., 2011).

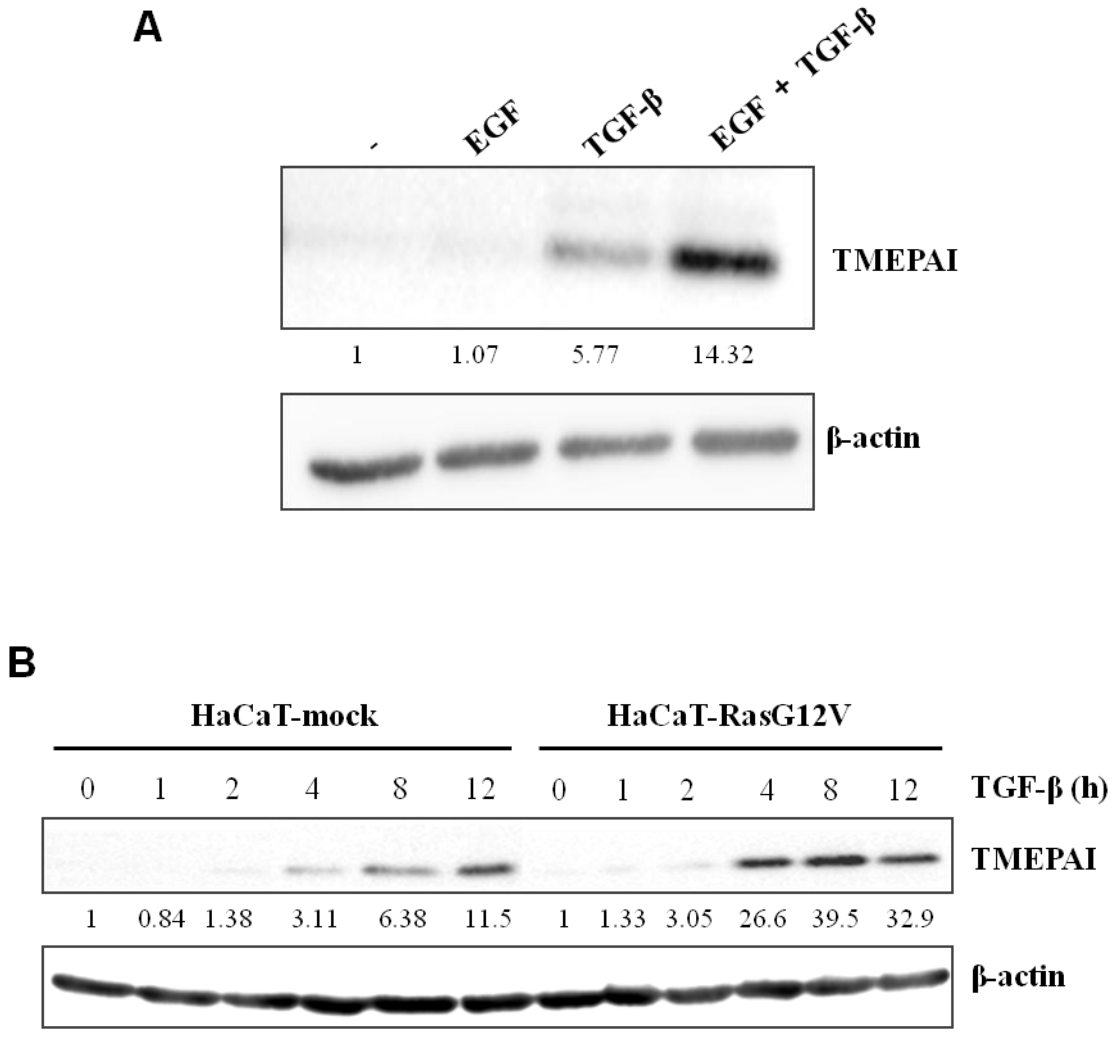
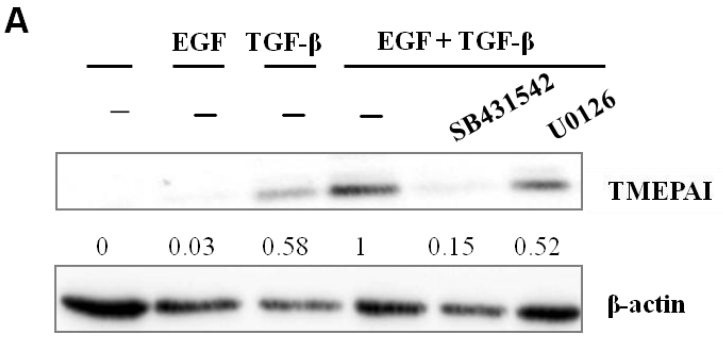


Figure 6: Cooperative Induction of TMEPAI by TGF-β and EGF signaling. (A) HaCaT cells were stimulated with TGF-β (0.5 ng/ml) and/or EGF (10 ng/ml) for 8 hours, as indicated. TMEPAI expression was detected by anti-TMEPAI antibody. β-actin was used as the loading control. (B) HaCaT-mock cells and HaCaT-RasG12V cells were stimulated with TGF-β (0.5 ng/ml) for indicated times. Total cell lysates were subjected to western-blotting using an anti-

TMEPAI antibody. β-actin was used as the loading control. Relative expression levels of TMEPAI/β-actin were detected by densitometry and indicated below the panels.
(This figure was done by Azami S.)

2-3-2 MEK inhibitor suppresses TMEPAI expression enhanced by EGF. TGF-β receptor kinase inhibitor SB41542 totally suppressed the expression of TMEPAI induced by both TGF-β and EGF, on the other hand MEK inhibitor U0126 suppressed the expression of TMEPAI down to the level obtained by TGF-β alone (Fig. 7A). Treating HaCaT-RasG12V cells by U0126 in the presence of TGF-β stimulation resulted in the reduction of TGF-β-induced TMEPAI in a dose-dependent manner (Fig. 7B). These data indicate that EGF signaling through the EGFR/Ras/MEK pathway contributes to the enhanced expression of TMEPAI (Azami S., 2011).



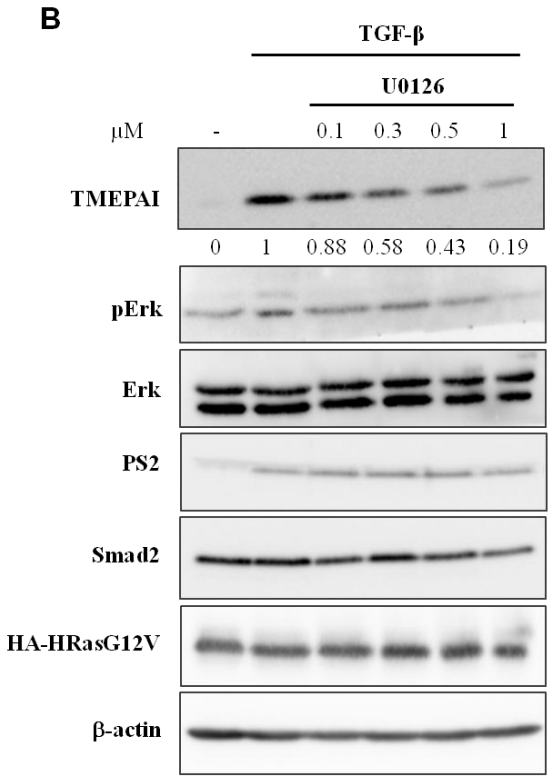
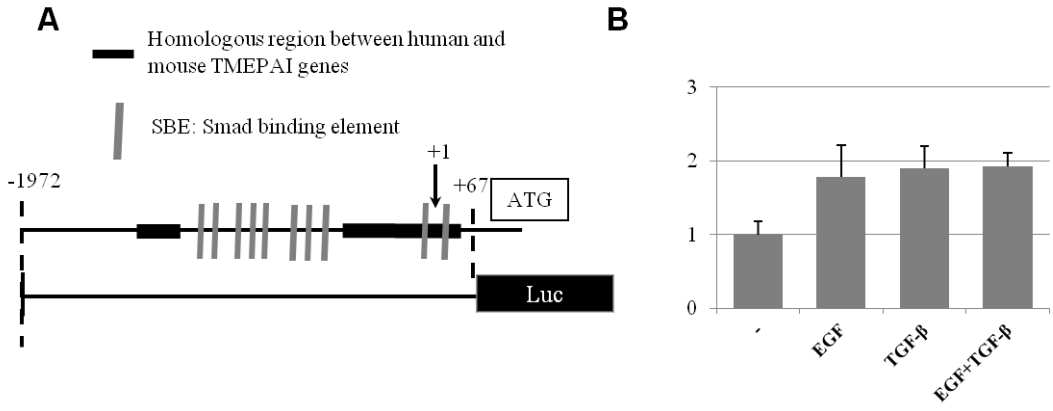


Figure 7: EGF/Ras/MAPK pathway enhances TGF-β induced-TMEPAI expression. (A) TGF-β receptor kinase inhibitor SB431542 (1 μM) or MEK kinase inhibitor U0126 (1 μM) was added 1 hour before stimulation with EGF (10 ng/ml), TGF-β (0.5 ng/ml), or both EGF (10 ng/ml) and TGF-β (0.5 ng/ml) for 8 hours, as indicated. The cell lysates were subjected to western-blotting. TMEPAI was detected by using anti-TMEPAI antibody. β-actin was used as the loading control. (B) HaCaT-HRasG12V cells were treated with MEK inhibitor U0126, as indicated 1 hour before TGF-β (0.5 ng/ml) stimulation for 8 hours. Cell lysates were subjected to western-blotting and detected with anti-TMEPAI antibody. The levels of phosphorylated ERK, total ERK, phosphorylated Smad2, Smad2 and HA-HRasG12V were also detected to examine the effects of TGF-β and U0126 treatments. β-actin was used as the loading control. (This figure was done by Azami S.)

2-3-3 Identification of EGF responsiveness in the first intron of the TMEPAI gene.

TMEPAI 5' promoter luciferase reporter, termed -1972TMEPAI-Luc (Fig. 8A), and a TMEPAI first intron luciferase reporter, termed pGL3ti-850-Luc (Fig. 8C) were used for the identification of the EGF responsive elements upon EGF, TGF-β, or both EGF and TGF-β stimulation. The activity of -1972TMEPAI-luc was marginally enhanced by these stimulation (Fig. 8B). On the other hand, the activity of the pGL3ti-850-Luc was highly activated by TGF-β, and that was further potentiated by the addition of EGF (Fig. 8D). Additionally, EGF potentiated the pGL3ti-850-Luc activities induced by TGF-β in dose-dependent manner (Fig. 8E). Furthermore, cotransfection of ELK-1, a transcription factor that is activated by EGFR/Ras/MEK pathway, significantly activated the pGL3ti-850 reporter but ELK-1(S383A), which cannot be phosphorylated by Ras/MAPK pathway, did not (Fig. 8F). These data suggest that EGF potentiates the enhancer activities in the first intron of the TMEPAI gene through the activation of ELK-1 (Azami S., 2011).



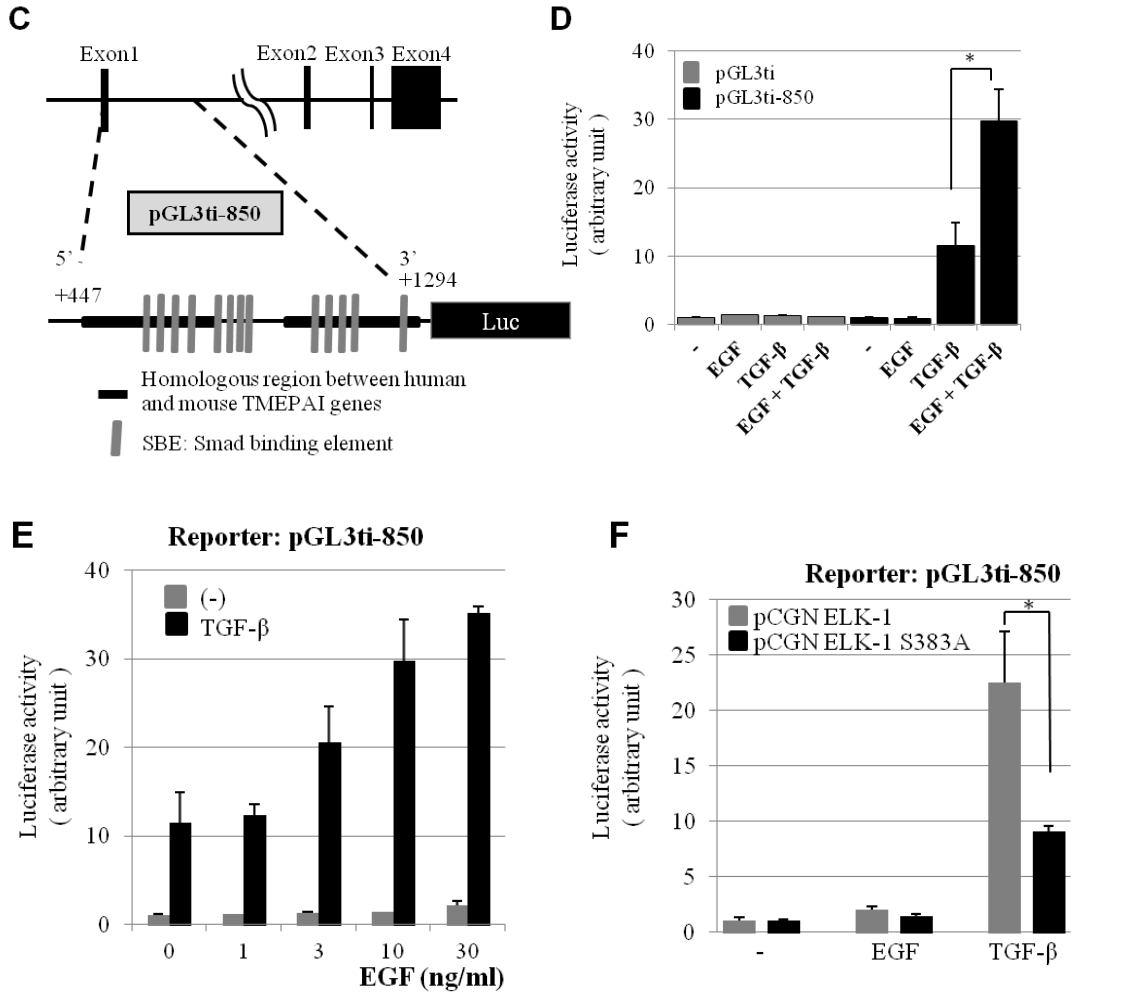


Figure 8: The first intron of TMEPAI gene contains the responsive sequences for TGF-β and EGF. (A) Schematic representation of the TMEPAI 5'-promoter luciferase reporter construct pGL3-TMEPAI (-1972/+67). The nucleotide numbers of mouse TMEPAI gene was shown with the transcriptional initiation site as +1. (B) Both EGF and TGF-β marginally enhanced the pGL3-TMEPAI(-1972/+67) luciferase activities in HepG2 cells. (C) Schematic representation of pGL3ti-850 reporter containing the +447 to +1294 sequence from the first intron of the TMEPAI gene. (D) HepG2 cells were transfected with pGL3ti or pGL3ti-850 and stimulated with EGF (10 ng/ml), TGF-β (0.1 ng/ml), or both EGF (10 ng/ml) and TGF-β (0.1 ng/ml) for 18 hours. (E) HepG2 cells were transfected with pGL3ti-850 and treated with EGF for 18 hours with indicated concentrations in the presence or absence of TGF-β (0.1 ng/ml) stimulation. (F) HepG2 cells were transfected with pGL3ti-850 together with ELK-1 or ELK-1(S383A), as indicated. Cells were stimulated with EGF (10 ng/ml) or TGF-β (0.1 ng/ml) for 18 hours. (*$P < 0.05$)

(This figure was done by Azami S.)

2-3-4 ELK-1 binding elements are required for the EGF-inducible enhancement of the TMEPAI gene expression. Stimulation by TGF-β increases the pGL3ti-850-Luc activity and co-stimulation by TGF-β and EGF made further activation of pGL3ti-850-Luc. In parallel, the shorter region pGL3ti-250 containing the 250 bp sequence from +1037 to +1294 responded to TGF-β and EGF as similar manner with pGL3ti-850 (Fig. 9A, 9B). I could find three ELK-1 binding consensus sequences (5`-GGAT-3` or 5`-GGAA-3`) in the 250 bp sequence of the first intron. Mutation for each of ELK-1 binding sites, or all three ELK-1 binding sites into pGL3ti-250-Luc reduced transcriptional responses upon EGF and TGF-β stimulation. Among them, the second mutant at position +1077 completely suppressed EGF-inducible enhancement of pGL3ti-250-Luc activity suggesting that this is the major binding element for ELK-1 in 250 bp sequence (from +1037 to +1294) in the first intron of TMEPAI gene (Fig. 9C).

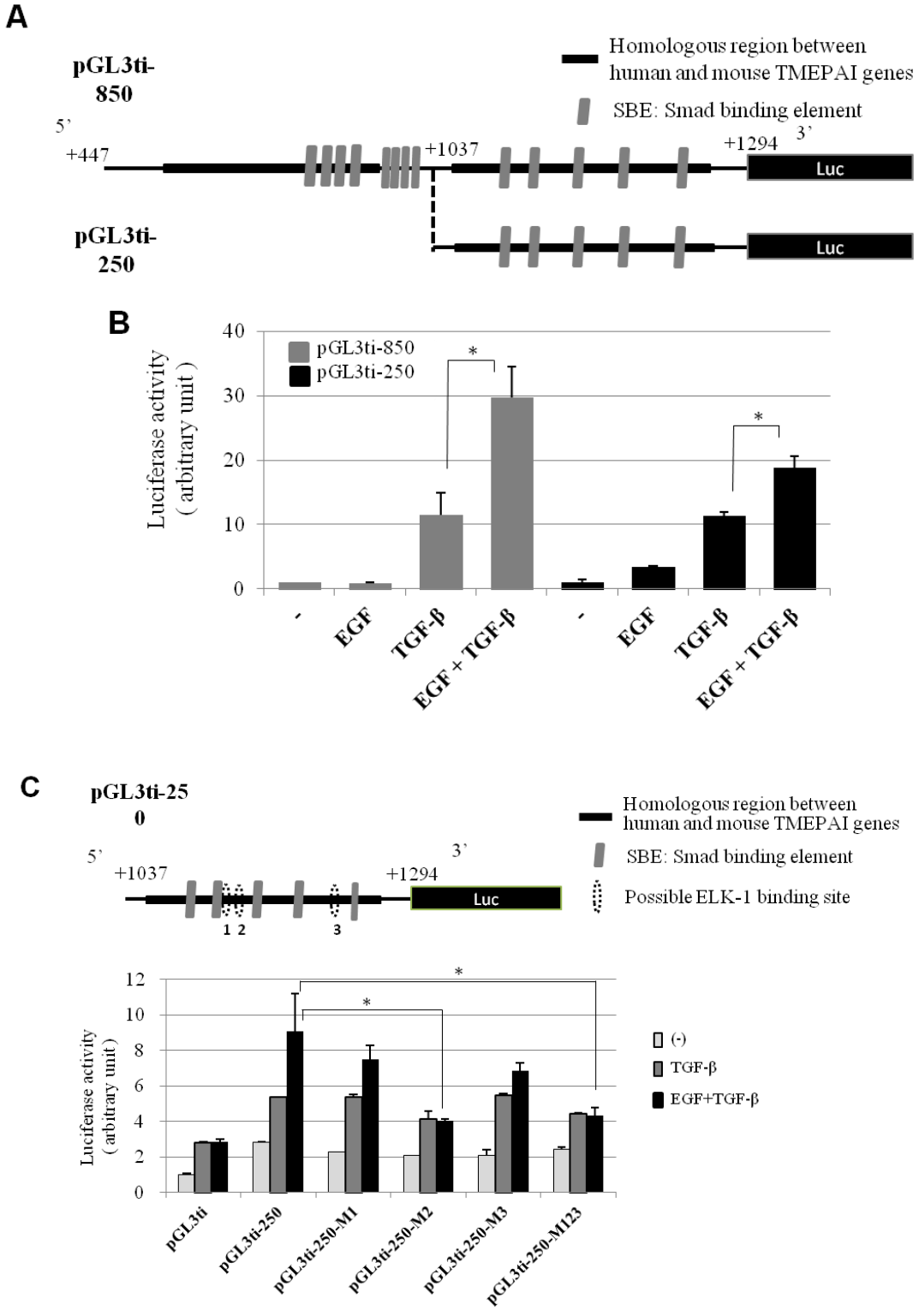

A
pGL3ti-850
Homologous region between human and mouse TMEPAI genes
SBE: Smad binding element
5'
+447
+1037
+1294
3'
Luc
pGL3ti-250
Luc
B
Luciferase activity (arbitrary unit)
pGL3ti-850
pGL3ti-250
40
30
20
10
0
*
*
-
EGF
TGF-β
EGF + TGF-β
-
EGF
TGF-β
EGF + TGF-β
C
pGL3ti-250
Homologous region between human and mouse TMEPAI genes
SBE: Smad binding element
Possible ELK-1 binding site
5'
3'
+1037
+1294
Luc
1 2
3
Luciferase activity (arbitrary unit)
12
10
8
6
4
2
0
*
*
(-)
TGF-β
EGF+TGF-β
pGL3ti
pGL3ti-250
pGL3ti-250-M1
pGL3ti-250-M2
pGL3ti-250-M3
pGL3ti-250-M123

Figure 9: ELK-1 binding elements in the 250 bp (+1037/+1294) region of the first intron of the TMEPAI gene is essential for the response to EGF. (A) Schematic representation of the luciferase reporters pGL3ti-850 (+447/+1294) and pGL3ti-250 (+1037/+1294) from the first intron of the TMEPAI gene. (B) HepG2 cells were transfected with pGL3ti-850 or pGL3ti-250, and stimulated with EGF (10 ng/ml), TGF-β (0.1 ng/ml), or both EGF (10 ng/ml) and TGF-β (0.1 ng/ml) for 18 hours. (C) Schematic representation of the pGL3ti-250 with/without mutations on ELK-1 binding elements. Dot-line circles indicate possible ELK-1 binding sites. HepG2 cells were transfected with the mutant pGL3ti-250 reporters, as indicated, and stimulated with TGF-β (0.1 ng/ml), or TGF-β (0.1 ng/ml) and EGF (10 ng/ml), as indicated. (*$P < 0.05$)

2-3-5 Binding of ELK-1 to Smad3 and ELK-1 binding sites in first intron of the TMEPAI gene. TGF-β-inducible target genes expression frequently requires the binding of Smad, which is activated by TGF-β, with coactivating transcription factors. I therefore examined the interaction between ELK-1 and Smad3 by coprecipitation assay, and revealed that ELK-1 actually interacted with Smad3 (Fig. 10A). I further investigated the binding of ELK-1 and Smad3 to the target sequence by chromatin immunoprecipitation (ChIP) assay using anti-ELK-1 and anti-Smad3 antibodies. The interaction of ELK-1 and Smad3 to the 250 bp sequence in the first intron of the TMEPAI gene could be detected upon EGF and TGF-β stimulation, respectively (Fig. 10B, 10C). Taken together, co-stimulation with EGF and TGF-β activates the binding of ELK-1 and Smad3 in the first intron of the TMEPAI gene and strongly activates the target gene expression in the presence of ELK-1-Smad3 interaction.

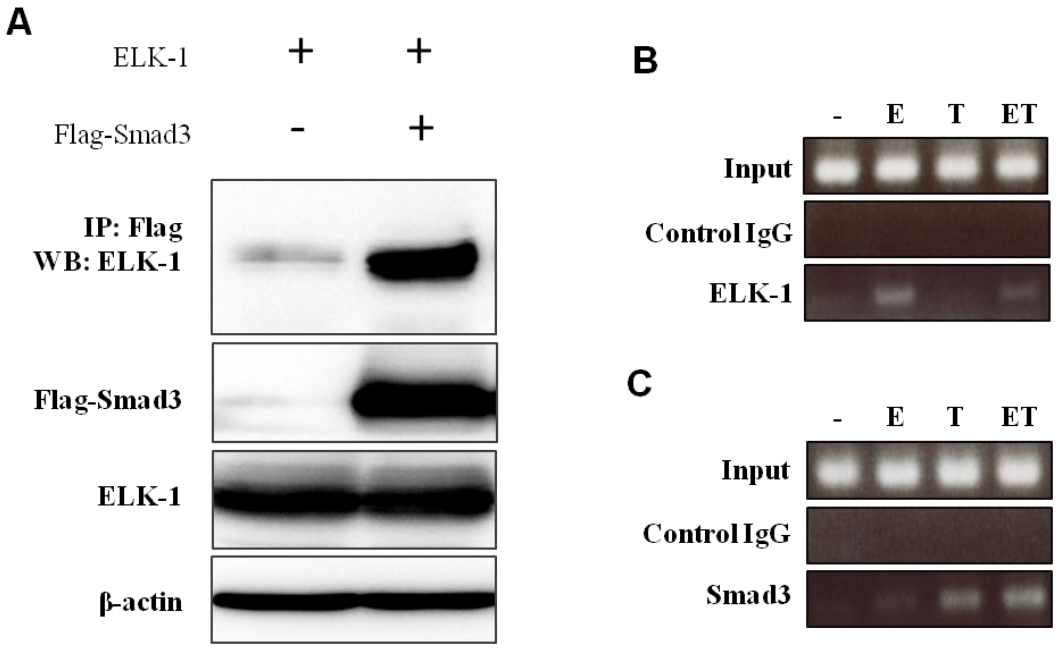


Figure 10: ELK-1 binds to the first intron of TMEPAI gene in response to EGF stimulation. (A) Interaction of ELK-1 with Smad3. Smad 3 was immunoprecipitated by anti FLAG antibody, and coimmunoprecipitated ELK-1 was detected by anti ELK-1 antibody (top panel), the whole cell lysates were subjected to western-blotting to detect the expression of Smad3, and ELK-1. β-actin was used as the loading control. (B, C) HaCaT cells were treated with EGF (10 ng/ml, E), TGF-β (0.1 ng/ml, T), or both EGF (10 ng/ml) and TGF-β (0.1 ng/ml) (ET) for 1 hour, as indicated.

2-4 Discussion

Since TGF-β signaling maintains a wide variety of cellular functions, un-controlled TGF-β signal results in many diseases and a prominent one being cancer. There is much evidence that TGF-β has functioned as both tumor suppressor by causing growth arrest and as a tumor promoter by promoting the invasion and migration of cancer cells via induction of EMT, recruiting new blood vessels to tumor sites (angiogenesis) and

suppression of the immune system (Masagué, 2008). The conflicting roles of TGF-β during tumor progression seem to occur in a tumor state-dependent manner. Nevertheless, much is still unknown on how TGF-β converts from being a tumor suppressor to a tumor promoter, stimulating research in this area.

On the other side, knockdown of TMEPAI results in tumor suppressive events including increased p27 expression, decreased HIF-1α, and VEGF expression via regulation of PI3K/PTEN/AKT pathway (Prajjal et al., 2010). Our previous study demonstrated that TMEPAI can interact with either Smad2 or Smad3 via its Smad interaction motif (SIM) in order to attenuate TGF-β/Smad pathway. Therefore, prolonged constitutive expression of TMEPAI would dampen TGF-β signaling and put cells at risk of aberrant growth.

Nakano et al. previously reported that TGF-β and Wnt signaling co-regulate the expression of TMEPAI via binding of Smads and TCF7L2 (transcription factor in Wnt signaling) to three Smad binding elements and a TGF-β-inducible TCF7L2 binding element in the first intron of TMEPAI gene (Nakano et al., 2010). However, this is merely a partial mechanism on constitutive expression of TMEPAI in cancer cells. Thus, it is of high importance to investigate deeper the mechanism of TMEPAI expression, for the targeted therapy in TMEPAI over-expressing cancers.

In the current study, I examined the role of EGF cross-regulation to TGF-β signaling on TMEPAI expression. Although by itself EGF signaling barely affects the expression of TMEPAI, EGF signaling strongly enhances the TGF-β induced TMEPAI expression, in comparison with HaCaT cells which were treated with TGF-β alone. Furthermore, HaCaT RasG12V transformed cells induced much higher level of TMEPAI protein and this was suppressed by EGFR inhibitor and MEK inhibitor. These data suggest that

EGFR/Ras/MEK pathway is essential for the expression of TMEPAI. The collaboration of EGF and TGF-β activates the pGL3ti-850 reporter which was constructed with mouse TMEPAI first intron (+447 to +1294) inserted upstream of the luciferase coding region. However, this signaling collaboration could not activate TMEPAI promoter (-1972 to +67) reporter. Since it was reported that the AP-1 transcription factors such as c-Jun and c-Fos contributes synergistically transcriptional activation of PAI-1 gene in response to TGF-β, I investigated the involvement of AP-1 family on TMEPAI transcriptional activation. But I could not see the cooperative effect of AP-1 and TGF-β signaling on pGL3ti-850 reporter.

In contrast, co-expression of transcription factor, ELK-1 (which was activated by MEK) could promote TGF-β induced-pGL3ti-850 reporter activation. Subsequently, the first intron sequence from +1037 to +1294 region was essential for its binding leading to cooperative activation by EGF and TGF-β signaling. The mutation of ELK-1 binding site totally abolished the enhancement of TGF-β-induced reporter activity by EGF. On the other hand, it was reported that EGF signaling directly phosphorylates and inhibits Smad functions. Therefore, I examined the effect of EGF on $(SBE)_4$-luc reporter that is directly activated by the TGF-β/Smad signaling pathway. As a result, I could not detect any influence of EGF signaling on $(SBE)_4$-luc activity (data not shown), suggesting that in this cell context EGF signaling does not inhibit R-Smads function. Thus, ELK-1 could be a partner of Smad and co-regulate TMEPAI transcription. Indeed, ELK-1 interacts with Smad3. Chromatin immunoprecipitation data showed that both ELK-1 and Smad3 bind to the first intron of TMEPAI gene in an EGF and TGF-β dependent manner for activation of TMEPAI transcription.

My proposed mechanism on TMEPAI regulation by EGF and TGF-β signaling may provide as part of the factors leading to dual roles of TGF-β in cancer progression. Taking lung cancer as an example whereby TGF-β signaling functions to suppress cell proliferation, this growth inhibitory mechanism could be circumvented by cancer cells by taking advantage of active EGF signaling and ELK-1 to alleviate restrictions on TMEPAI expression. High TMEPAI expression keeps TGF-β signaling to a minimum level that is insufficient for tumor growth inhibition while its autocrine signaling is still able to induce cell invasion and cancer cell dissemination.

In conclusion, both TGF-β and EGF signaling coordinately regulate the transcription of TMEPAI via activation of Smad3 and ELK-1 on the first intron of the TMEPAI gene.

CHAPTER 3:

TMEPAI enhances Tumorigenic Activities in Lung Cancer Cells

3-1 Introduction

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 (Rae et al., 2001; Giannini et al., 2003; Brunschwig et al., 2003). Genomewide 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 (Saadi et al., 2010; Rajkumar et al., 2010). TMEPAI was further suggested as a "molecular switch" that converts TGF-β signaling from a tumor suppressor to a tumor promoter (Rae et al., 2001). 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, I aimed to investigate the tumorigenic activities of TMEPAI in lung cancer cell lines.

3-2 Materials and methods

3-2-1 Cell culture. HaCaT cells (spontaneously immortalized human keratinocyte cell line) and COS7 cells (African green monkey kidney cells transformed by SV40) were cultured in Dulbecco`s modified Eagle's medium (Sigma) containing 10% fetal calf serum (FCS, Invitrogen), penicillin streptomycin solution. NCI-H23 and RERF-LC-KJ

cells were cultured in RPMI 1640 medium containing 10% FCS, penicillin streptomycin solution. Calu3 cells and HepG2 cells were cultured in minimum essential medium (Sigma) containing 10% FCS, penicillin streptomycin solution. Nontargeting shRNA (SHC002), TMEPAI shRNA#9, and TMEPAI shRNA#10 ligated in a pSUPER RNAi system (Oligoengine) 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 of puromycin (Sigma), respectively. The TGF-β receptor kinase inhibitor SD208 (Bioscience) and anti-TGF-β neutralizing antibody (R&D Systems) were used to block TGF-β signaling.

shRNA	Sequence
Non-targeting	SHC002 (Sigma)
sh RNA # 9	CCG GGA GCA AAG AGA AGG ATA AAC ACT CGA GTG TTT ATC CTT CTC TTT GCT CTT TTT
sh RNA # 10	CCG GGA GTT TGT TCA GAT CAT CAT CCT CGA GGA TGA TGA TCT GAA CAA ACT CTT TTT

3-2-2 Luciferase assay. HepG2 cells were transfected with $(CAGA)_{12}$-luc using FuGENE6 (Roche Diagnostics), treated with TGF-β or 50% v/v of heat-treated serum-free conditioned media (80^0C, 10 minutes) after 24 hours incubation with HaCaT or lung cancer cell lines. Luciferase activities were determined as described in [2-2-3].

3-2-3 Western-blotting. Endogenously expressed TMEPAI in HaCaT or lung cancer cell lines was detected by western-blotting using total cell lysates with/without TGF-β stimulation. The assay was carried out as described in [2-2-4]. Antibodies were used as follow:

1st antibody : mouse anti-TMEPAI antibody (x 1000)
(homemade monoclonal antibody) (Vo Nguyen et al., 2014)
rabbit anti-Smad2/3 antibody (BD Bioscience) (x 1000)
rabbit anti-phosphorylated Smad2 (PS2) antibody
(Persson et al., 1998) (x 1000)
mouse anti-β-actin antibody (Sigma) (x 5000)

2nd antibody : anti mouse-IgG-(horseradish peroxidase; HRP)
(GE Healthcare) (x 10000)
anti rabbit-IgG-HRP (GE Healthcare) (x 10000)

3-2-4 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% bovine serum albumin. Cells were then incubated with antibodies, and the nuclei were stained with Hoechst 33342 (Sigma). Intracellular localization was then observed by fluorescence microscopy (Axiovert 200; Zeiss).

1st antibody : mouse anti-TMEPAI antibody (x 250)
(homemade monoclonal antibody) (Vo Nguyen et al., 2014)

2nd antibody : goat Alexa888 anti-mouse IgG- (Molecular Probes) (x 250)

3-2-5 Cell proliferation assay. Cells were seeded in 24-well plates, cultured for the indicated time periods, and counted with a hematocytometer.

3-2-6 Sphere formation assay. Spheres were cultured in DMEM/F12 serum-free medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml of EGF (Sigma), and 20 ng/ml of bFGF (R&D Systems) in an ultra-low attachment culture dish (Corning).

3-2-7 In vivo tumor formation assay. For cancer cell implantation, 10^7 cells were subcutaneously injected into 8-week-old female nonobese diabetic/severe combined immunodeficiency mice (NOD-SCID mice). After 3 months, the mice were sacrificed and the tumors were weighed. All animal experiments were approved by the animal experiment committee of the University of Tsukuba and performed in accordance with the university's animal experiment guidelines and the provisions of the Declaration of Helsinki in 1995.

3-3-8 In vivo lung metastasis assay. A suspension containing 10^6 cells in 0.2 ml of PBS was injected into the lateral tail vein of 7-week-old NOD-SCID mice. After 8 weeks, the animals were sacrificed and the removed lungs were fixed in 10% neutralized formalin solution, embedded in paraffin, sliced into 3-μm sections, and stained with hematoxylin and eosin or immunostained with Ki-67 antibody (Novocastra). 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).

3-3 Results

3-3-1 Enhanced and constitutive expression of TMEPAI in lung cancer cell lines. I 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 hours of TGF-β stimulation; there were no detectable levels of TMEPAI without TGF-β stimulation (Fig. 11A). In contrast, all 3 of the examined lung cancer cell lines expressed detectable levels of TMEPAI even in the absence of TGF-β 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 about twofold in response to TGF-β (Fig. 11B). Endogenous TMEPAI could also be detected as cytoplasmic dot patterns in HaCaT and NCI-H23 cells by immunofluorescence staining (Fig. 11C).

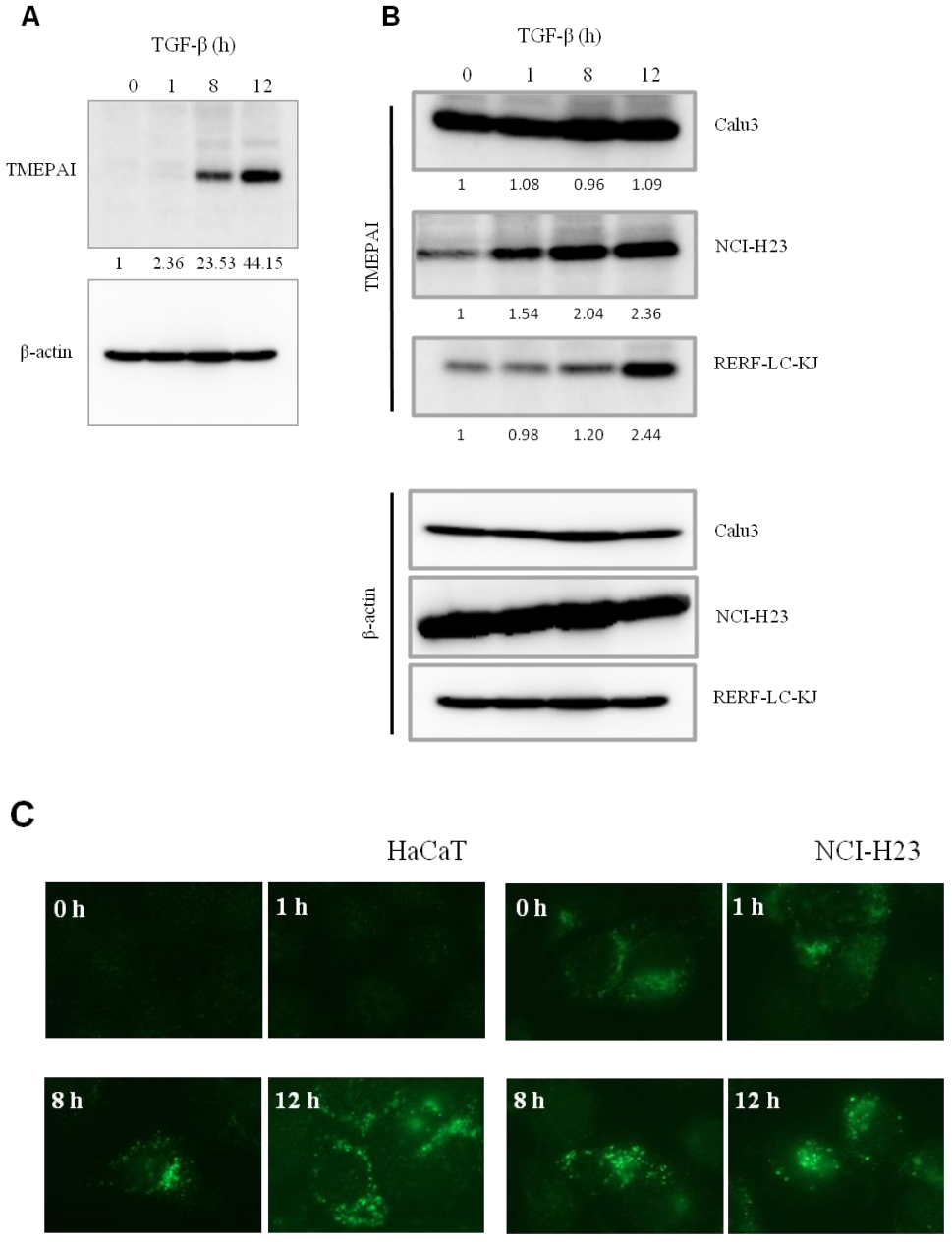
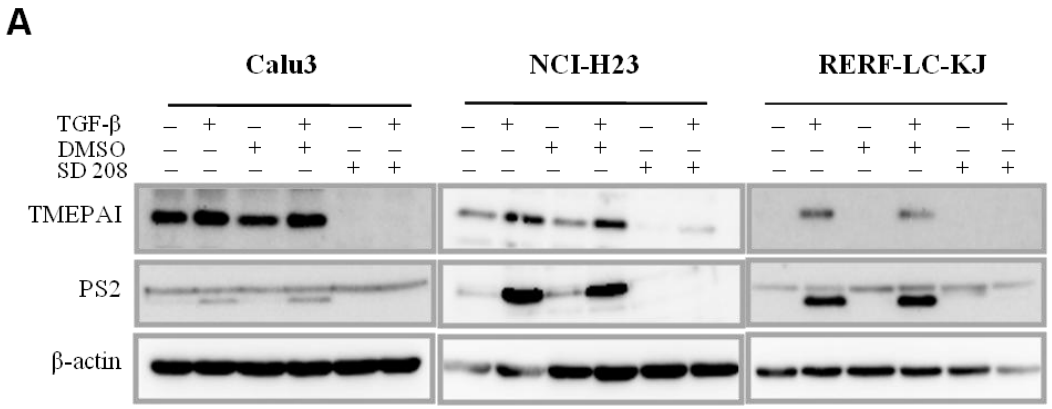


Figure 11: Enhanced expression of TMEPAI in lung cancer cells**.** (A) Induction of TMEPAI by 5 ng/ml of TGF-β in HaCaT cells. Endogenous TMEPAI was detected by a monoclonal anti-TMEPAI antibody. β-actin was used as the loading control. Relative expression levels of TMEPAI/β-actin were detected by densitometry and indicated below the panels. (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-B23 cells were treated with 5 ng/ml of TGF-β as indicated. After treatment, the cells were subjected to fluorescence microscopy using anti-TMEPAI antibody.

3-3-2 TGF-β signaling mediates the 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-β signaling is involved in TMEPAI expression, cells were treated with the TGF-β receptor kinase inhibitor SD208 or anti-TGF-β neutralizing antibodies. TMEPAI disappeared from all cell lines in the presence of SD208 (Fig. 12A). While the effects of the TGF-β neutralizing antibodies were not complete, they did cause the TMEPAI levels to significantly decrease to levels correlating with Smad2 phosphorylation levels in all 3 cell lines (Fig. 12B). I further examined TGF-β activities in the conditioned media incubated 24 hours with the lung cancer cells. Calu3 secreted abundant TGF-β in the culture media and it had positive correlation with the levels of TMEPAI expression (Fig. 12C)



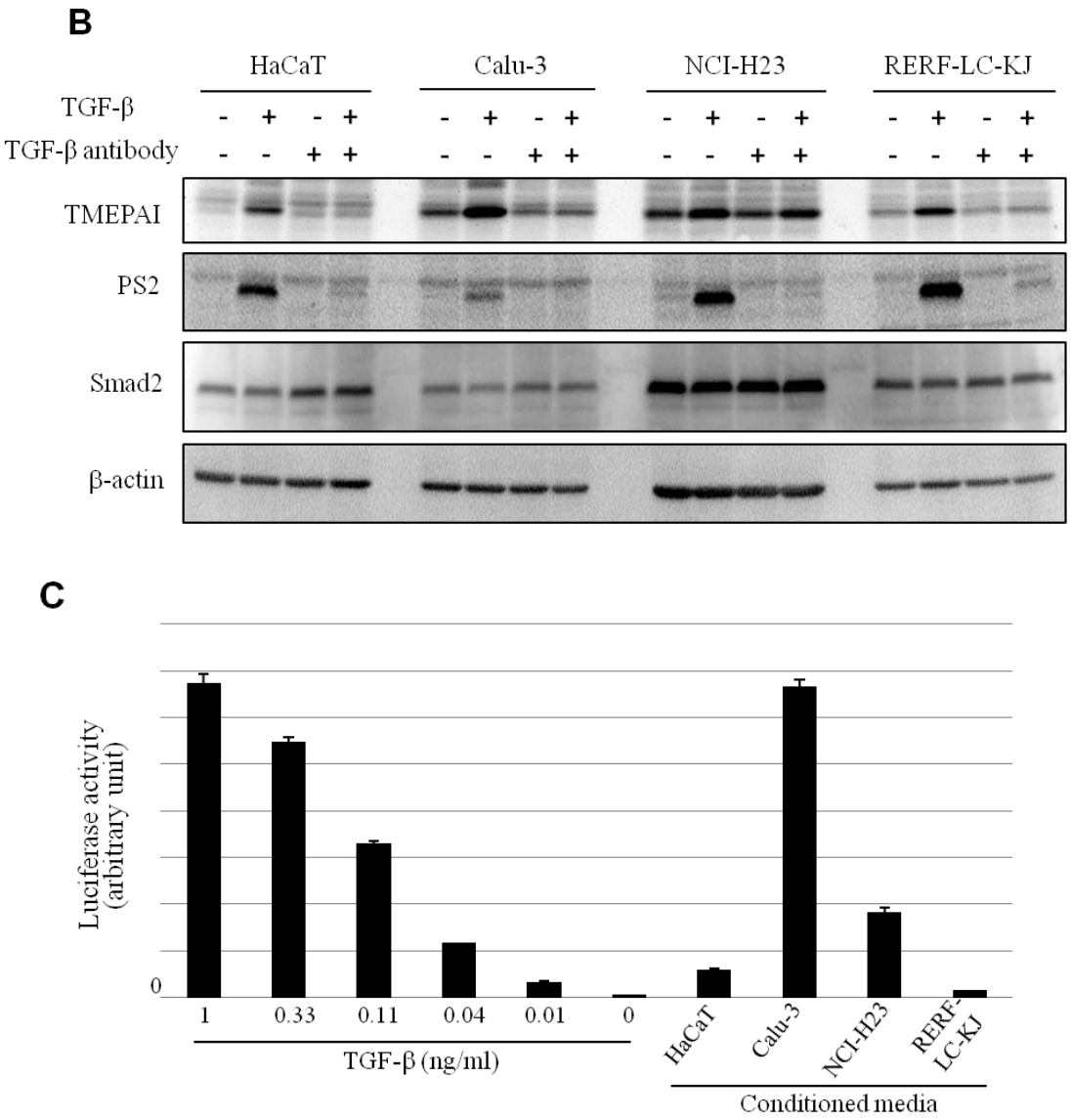
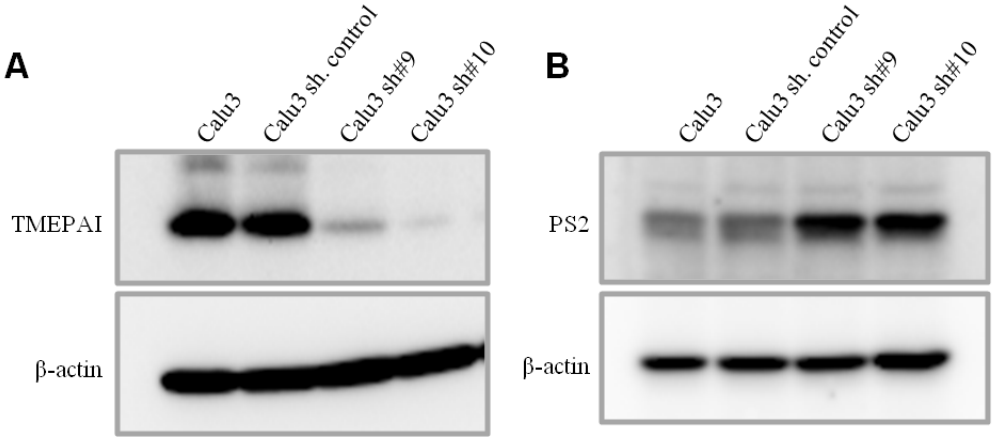
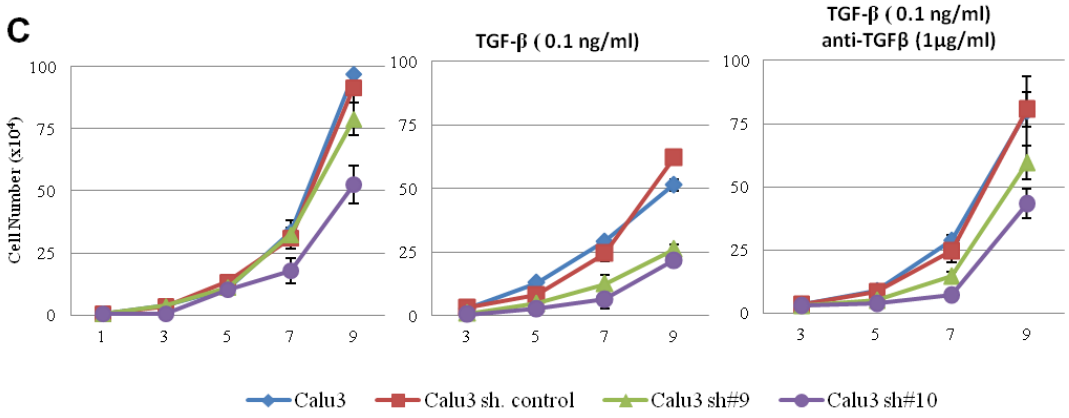


Figure 12: TGF-β signaling mediates the expression of TMEPAI in lung cancer cell lines Calu3, NCI-H23, and RERF-LC-KJ. (A) The TGF-β receptor kinase inhibitor SD208 (1 μM) or (B) a TGF-β neutralizing antibody (5 μg/ml) were added 1 hour before stimulation with 5 ng/ml of TGF-β for 8 hours as indicated. Then, the cell lysates were subjected to immunoblot analysis. TMEPAI was detected by using anti-TMEPAI antibody and the levels of phosphorylated Smad2 were detected with anti-phosphorylated Smad2 antibody (PS2). β-actin was used as the loading control. (C) TGF-β activities in the serum-free conditioned media after 24 hours incubation with the indicated cells were measured by $(CAGA)_{12}$-luc reporter assay. The means ± SDs are shown.

3-3-3 Knockdown of TMEPAI enhances Smad phosphorylation and growth inhibitory responses of TGF-β. I 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. 13A). As a result, phosphorylated Smad2 levels were clearly enhanced (Fig. 13B). Significantly stronger cell growth inhibition was obtained in Calu3-sh#9 and Calu3-sh#10 cells in the presence of 0.1 ng/ml of TGF-β, and it was recovered by anti-TGF-β neutralizing antibodies (Fig. 13C, 13D).



C



D

	Cell numbers TGF-β(+) /(TGF-β(-)	Cell numbers TGF-β(+), anti-TGFβ /(TGF-β(-)
Calu3	0.53	0.83
Calu3 sh.control	0.68	0.88
Calu3 sh#9	0.33	0.76
Calu3 sh#10	0.41	0.82

Figure 13: Calu3-shTMEPAI cells have increased sensitivity to 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 hours, and endogenous TMEPAI was detected by western-blotting. β-actin was used as the loading control. (B) Cells were stimulated with TGF-β (1 ng/ml) for 1 hour and phosphorylated Smad2 was detected with anti-phosphorylated Smad2 antibody (PS2). β-actin was used as the loading control. (C, D) Cell proliferation assay. (C) TMEPAI-knockdown Calu3 cells (Calu3 sh#9 and Calu3 sh#10) were cultured in 12-well plate 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 % growth inhibition by TGF-β (0.1 ng/ml) and the reversal by the inclusion of TGF-β neutralizing antibody (1 ng/ml) was calculated on day 9 in each cell lines.

3-3-4 TMEPAI enhances sphere-forming activities. I further investigated the functional significance of TMEPAI in the sphere-forming activities. The sphere-forming activities of TMEPAI-knockdown cells were significantly reduced in Calu3 cells (Fig. 14A, 14B).

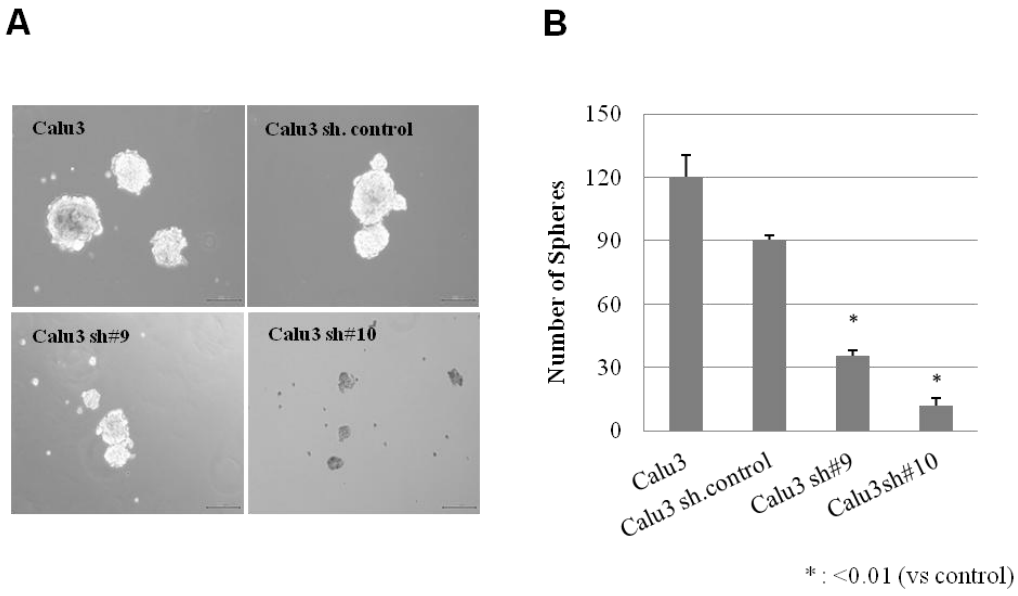


Figure 14: Calu3-shTMEPAI cells have decreased sphere-forming activities. (A) Control and TMEPAI-knockdown Calu3 cells (Calu3 sh#9 and Calu3 sh#10) were cultured in sphere formation medium for 12 days. Typical photographs are shown. (B) The numbers of spheres larger than 100 μm in diameter were counted. The means ± SDs are shown. **P* < 0.01 (vs control).

3-3-5 TMEPAI enhances *in vivo* tumor formation. In addition, I performed xenograft assays in NOD-SCID mice. Calu3, Calu3-shcontrol, Calu3-sh#9 and Calu3-sh#10 cells were subcutaneously injected into NOD-SCID mice. Three months after injection, the tumors were collected and weighed. As shown in Fig. 15, the tumors of the Calu3-sh#9 and -sh#10 cells were significantly smaller than those of the control cells in NOD-SCID mice.

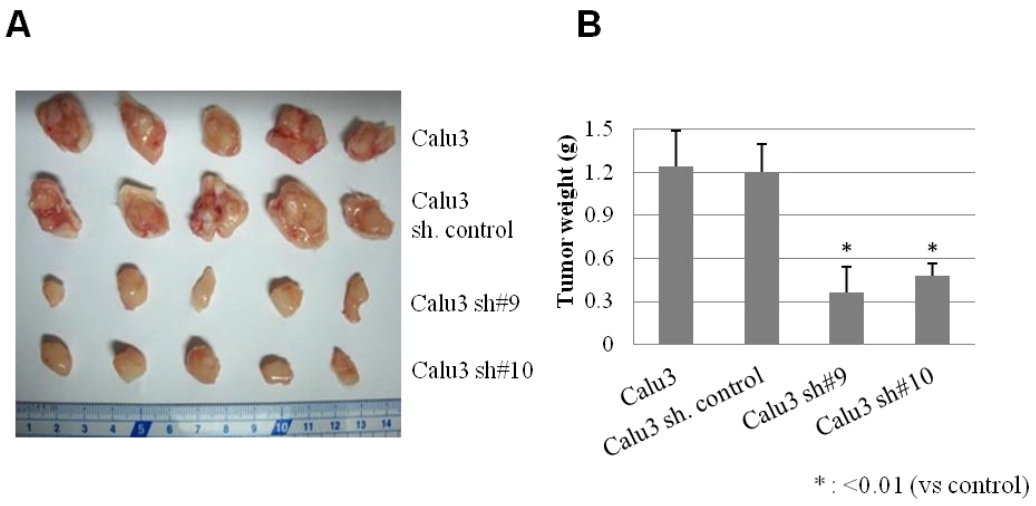
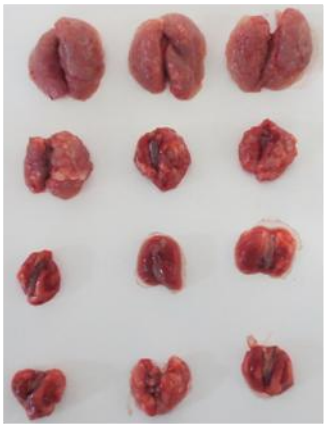


Figure 15: Calu3-shTMEPAI cells have decreased tumor-forming activities. (A) Calu3, Calu3-shcontrol and TMEPAI-knockdown Calu3 cells (Calu3-sh#9 and Calu3-sh#10) were subcutaneously injected into NOD-SCID mice with Matrigel supplement. After 3 months, the tumors were harvested and photographed. (B) The tumors were weighed. The means ± SDs are shown. *$P < 0.01$ (vs control).

3-3-6 TMEPAI enhances metastatic tumor formation in lungs. To examine the effects of TMEPAI on the metastatic potential in lungs, I injected Calu3, Calu3-shcontrol, Calu3-sh#9 and -sh#10 cells into the tail veins of NOD-SCID mice. Eight weeks after injection, mice were sacrificed and the collected lungs were examined histopathologically with hematoxylin and eosin staining. I also stained for Ki-67 to detect proliferating cancer cells. Using morphometric software, I 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 is size and numbers than those of the control Calu3 cells (Fig. 16A-D). These results indicated that knockdown of TMEPAI suppresses the ability of lung cancer cells to develop metastatic tumors in lungs.

A



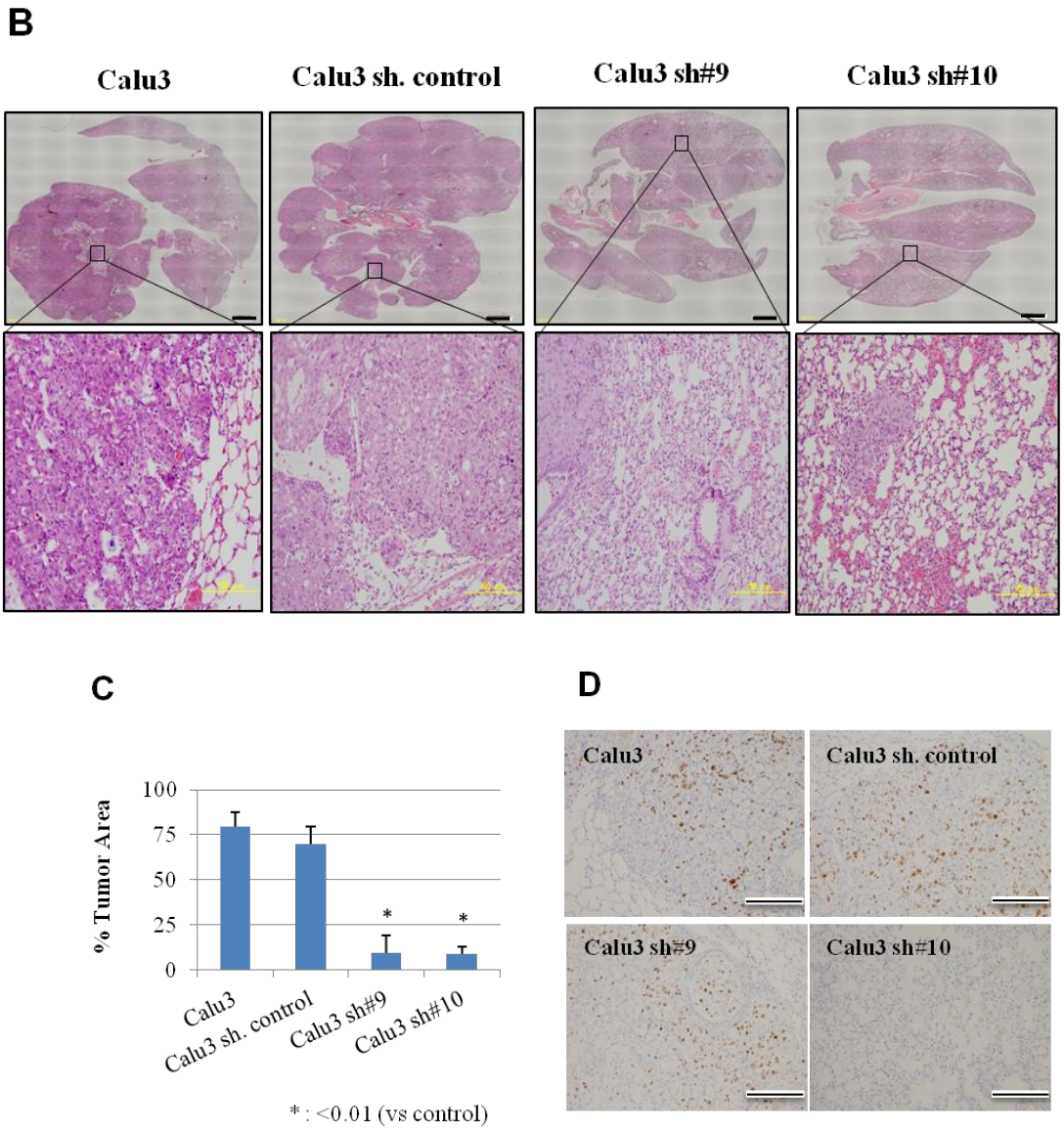


Figure 16: Calu3-shTMEPAI cells have decreased tumorigenic activity in the lungs. Calu3, Calu3-shcontrol and TMEPAI-knockdown Calu3 cells (Calu3-sh#9 and Calu3-sh#10) were injected into the tail vein of NOD-SCID mice (10^6 cells in 200 µl PBS/ mice). (A) After 8 weeks, the lungs were collected and photographed. (B) The thin sections of lungs were stained with hematoxylin and eosin. Scale bars = 1 mm. Higher magnifications show a typical metastatic area. (C) The percentages of tumor areas were measured by image analyses as described in the Materials and Methods. The means ± 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 bars = 100 µm.

3-4 Discussion

Transmenbrance prostate androgen induced protein (TMEPAI) is one of responsible protein of TGF-β signaling upon induction. Previous studies demonstrated that TMEPAI is highly expressed in various cancers such as renal cell carcinoma, colon cancer, breast cancer, and ovarian cancer (Rae et al., 2001; Giannini et al., 2003; Brunschwig et al., 2003). Genomewide 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 (Saadi et al., 2010; Rajkumar et al., 2010). Furthermore, dysregulation of TGF-β signaling was identified as an important mediator of lung cancer invasion (Toonkey et al., 2010). Therefore, I examined the expression of TMEPAI in lung cancer cell lines. Both immunoblot and immunofluorescent analyses clearly detected high levels of TMEPAI expression in all 3 examined lung cancer cell lines.

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 (Tanner et al., 1994; Courjal et al., 1996). Expression of TMEPAI in lung cancer cells disappeared in the presence of a TGF-β receptor kinase inhibitor and was significantly suppressed by anti-TGF-β neutralizing antibodies. Although Calu3 cells expressed high levels of TMEPAI in the absence of detectable Smad2 phosphorylation, expression of TMEPAI was significantly suppressed by a TGF-β receptor kinase inhibitor. Nakano et al. reported that Smad3 and Smad4 are essential for the TGF-β-inducible expression of TMEPAI (Nakano et al., 2010). On the other hand, 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 (Xu et al., 2000; Giannini et al., 2003; Anazawa et al., 2004; Muñoz et al., 2006; Nakano et al., 2010). The effects of SD208 in Fig. 12A indicate that activity of type I TGF-β receptor kinase is required for the stable expression of TMEPAI in lung adenocarcinoma cells. Moreover, many of these cells usually have activated EGFR/RAS/MAPK signaling, even if Smad2 phosphorylation is underdetectable levels in immunoblot analysis. These results suggest that cancer cells can maintain TMEPAI expression by multiple oncogenic signaling to suppress Smad phosphorylation down to under detectable levels but even in these cases TGF-β receptor kinase activity is required to support TMEPAI expression. The contributions of synergistic effects of multiple oncogenic signaling including the role of TGF-β 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 (Prajjal et al., 2010; Liu et al., 2011). Here, I showed that knockdown of TMEPAI in lung cancer cells potentiates TGF-β-inducible Smad phosphorylation and growth inhibitory responses to TGF-β (Fig. 13). Consequently, the sphere-forming activity in vitro, subcutaneous tumor formation, and lung metastasis were significantly suppressed in NOD-SCID mice (Fig. 15, 16). Liu et al. reported that knockdown of PMEPA1/TMEPAI suppresses the Smad3/4-cMyc-$p21^{Cip1}$ pathway in AR-negative prostatic cancer cells. These effects can be explained by the suppressive function of TMEPAI on Smad signaling via its SIM domain (Liu et al., 2011). However, Prajjal et al. identified suppression of Akt phosphorylation and expression of HIF1β and VEGF in

TMEPAI-knockdown xenograft breast tumors (Prajjal et al., 2010). 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.

CHAPTER 4: PERSPECTIVES

4-1 Oncogenic function of TMEPAI and its application in cancer therapy.

TGF-β-induced TMEPAI is highly expressed in many cases of human cancer and TMEPAI participates in the negative feedback regulation of TGF-β/Smad signaling. There are several other negative feedback regulators of TGF-β/Smad pathway, such as Smad7, smurf2 and SnoN. Among these regulators, TMEPAI has strong and direct correlation with carcinogenic activities (Watanabe et al., 2010). TMEPAI has been shown to be highly expressed in many cases of cancer and knockdown of TMEPAI efficiently suppresses tumorigenic activities. Therefore, I speculated that TMEPAI may have additional function other than the suppression of TGF-β/Smad signaling, which might cause oncogenic signaling. I recently found that the phosphorylation of AKT serine 473 was strongly reduced in TMEPAI knockdown Calu3 cells, whereas, the other phosphorylation site threonine 308 did not (Fig. 17). It`s well-documented that the fully activation of AKT required the phosphorylation of two important amino acid residues threonine 308 and serine 473, which is negatively regulated by phosphatases PTEN and PHLPP1, respectively (Manning and Cantley, 2007; Brognard and Hunter, 2011; Liu et al., 2009, 2011). The AKT signaling regulates cell growth, proliferation and survival, angiogenensis, cell motilities, and glucose metabolism. The frequent hyperactivation of AKT signaling is observed in many human cancer, and aberrant regulation of these processes are considered the hallmarks of cancer (Chin and Toker, 1996; Shayesteh et al., 1999; Ma et al., 2000; Vivanco and Sawyers, 2002; Altomare and Testa, 2005). My preliminary data showed that knockdown TMEPAI suppressed AKT phosphorylation

leads to the idea that TMEPAI possibly affect PI3K/AKT signaling. Indeed, I found that the endogenous PHLPP1 was relatively low in Calu3 cells, but high in TMEPAI knockdown Calu3 cells (data not shown), indicated that TMEPAI might reduce the protein levels of PHLPP1, and could be involved in the regulation of AKT phosphorylation at serine 473. There is a possibility that TMEPAI promotes PHLPP1 degradation via its two PY motifs, which can interact with HECT type E3 ubiquitin ligases such as NEDD4. Furthermore, the transcription factors Sp1 and Smad3 have been reported to bind to the PHLPP1 promoter sequence and increase expression of the PHLPP1 gene (Dong et al., 2013; ÓNeilll et al., 2013). TMEPAI negatively regulates TGF-β; therefore, TMEPAI might be involved in the transcriptional regulation of PHLPP1 through the suppression of TGF-β/Smad3 signaling. Further studies will be required to reveal the novel function of TMEPAI on regulating AKT signaling. Targeting on TMEPAI could be considered as a potential molecularly targeted strategy in cancer therapy.

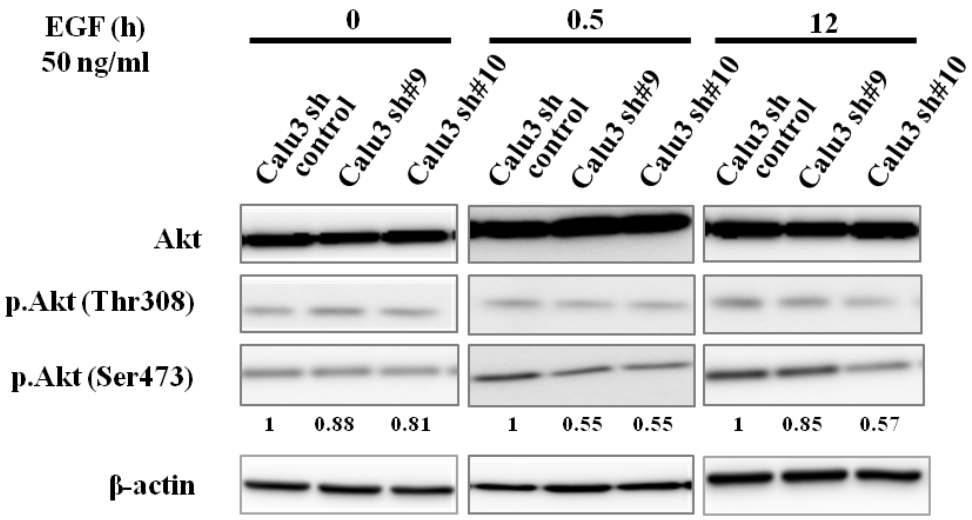


Figure 17: Knockdown of TMEPAI supresses AKT phosphorylation at Serine 473. Control and TMEPAI knockdown Calu3 lung cancer cells, as indicated, were treated with EGF (50 ng/ml) for 0.5 or 12 hours. Then the cell lysates were subjected to western-blotting detecting Akt, phosphorylated Akt at Thr308, and phosphorylated Akt at Ser473. β-actin was used as the loading control. Results of densitometry p-Akt (ser473)/β-actin was indicated below the corresponding panels (arbitrary units; control = 1).

4-2 Application of TMEPAI Monoclonal antibody in cancer diagnosis.

Previous studies demonstrated that TMEPAI was highly expressed in various cancers such as colon cancer, breast cancer and ovarian cancer. Here we showed that lung cancer cell lines also give high levels of expression. High levels of TMEPAI expression lead us an idea to use TMEPAI as a tumor marker for cancer diagnosis. In general, tumor markers are secreted or flew out from cancer cells, and the serum concentration detected by "blood test" would increase with cancer progression. Several proteins and sugar chains were already being used as tumor markers; for example carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), CA 125, or prostate-specific antigen (PSA). Genome wide studies, which compared the gene expression

levels between cancer tissues and normal counterparts, suggested that TMEPAI is one of the highest inducible genes in cancer. On the other hand, tissue distribution of TMEPAI remains unclear; the tissue staining method by using established antibodies for TMEPAI will provide the fundamental information for physiological and pathological roles of TMEPAI. The tissue samples from cancer patients will be used to investigate the tissue distribution of TMEPAI.

CHAPTER 5: CONCLUSIONS

The cellular function and tissue homeostasis is tightly controlled by the interwoven signaling network inside the cell (Guo and Wang, 2009). Disruption of the balance of these networks leads to develop various diseases, including cancers. In this work, I revealed the transcript regulation as well as tumorigenic function of TMEPAI, one of the responsive target gene of TGF-β signaling. TMEPAI is induced by TGF-β signaling, and joins to the negative feedback loop to control the duration and intensity of TGFβ/Smad signaling by sequestering Smad2/3 from ligand-induced phosphorylation (Watanabe et al., 2010). The transcription of TMEPAI has been reported to be regulated not only by TGF-β/Smad signaling, but also by the other signaling, for instance EGF, mutant p53, and Wnt signaling. Nakano et al. reported the synergy between TGF-β and Wnt signaling in the regulation of the TMEPAI expression by recruiting the transcription factor TCF7L2 and Smad3 to the enhancer within the first intron of the TMEPAI gene (Nakano et al., 2010). Interestingly, beside several TGF-β-responsive TCF7L2-binding elements (TTE), which are activated upon Wnt/β-catenin signaling activation, I could find there are three EGF-induced transcription factor ETS binding sites also located within the first intron of the TMEPAI gene. Co-stimulation between TGF-β and EGF cooperatively increased the TMEPAI transcripts, and mutation of these ETS-binding sites resulted in suppression of TMEPAI expression. These data suggest that the transcripts of TMEPAI are controlled by a crosstalk signaling networks between TGF-β,

EGF, and Wnt signaling. However, other signaling might also be involved in TMEPAI regulation and should be further investigated for better understating.

On the other hand, TMEPAI has been reported to be highly expressed in many cancers, and knockdown TMEPAI in human lung cancer Calu3 cells inhibited cell growth and tumor formation, suggesting the tumorigenic function of TMEPAI (Vo Nguyen et al., 2014). The mechanism how TMEPAI involved in cancer progression also gives the interesting point for this molecule. There are many informative discussion reported for the tumorigenic function of TMEPAI. For example, TMEPAI can suppress the growth-inhibition activity of TGF-β, TMEPAI can convert TGF-β from a tumor suppressor to a tumor promoter (Prajji et al., 2010; Bai et al., 2014), or my recent hypothesis that TMEPAI might sustain the activity of PI3K/AKT signaling by the suppression the AKT-negative-regulator PHLPP1 phosphatase. The investigation about the mechanism how TMEPAI can mediate such signaling regulator proteins will probably prompt us to be able to find novel function of TMEPAI. For this purpose, the rescue experiments which re-express TMEPAI containing functional domain mutations in TMEPAI knockdown cancer cells should be carried out.

In addition, besides the major-signaling molecules of TGF-β, there are many proteins that interact with the signaling molecules and form impeccable signal network inside the cell. They may play an important role in both positive and negative regulation of TGF-β signaling pathway. Identification of the TMEPAI–interacting proteins networks and their mediated function would give more interesting evidences to get insights into the molecular mechanism for fine regulation of TGF-β-induced TMEPAI in cancer cells.

Finally, high levels of TMEPAI expression suggest an idea to use TMEPAI as a tumor marker for cancer diagnosis. Therefore, if the levels of TMEPAI can be detected in cancer patients, it will be useful for early and easy detection of cancer relapse.

REFERENCES

Altomare DA, Testa JR. Perturbation of the Akt signaling pathway in human cancer. *Oncogene* 2005; 24: 7455-64.

Anazawa Y, Arakawa H, Nakagawa H, Nakamura Y. Identification of STAG1 as a key mediator of a p53-dependent apoptotic pathway. *Oncogene* 2004; 23: 7621-27.

Araud T, Genolet R, Gubler PJ, Joseph CJ. Alternatively spliced isoforms of the human elk-1 mRNA within the 5' UTR: implications for ELK-1 expression. *Nucleic Acids Research* 2007; 35: 4649-63.

Azami, S. Cooperative Induction of TMEPAI by TGF-β and EGF signaling (Master`s Thesis), University of Tsukuba, Graduated School of Comprehensive Human Sciences, Master Program in Medical Sciences, 2011.

Bai X, Jing L, Li Y, Li Y, Luo S, Wang S et al. TMEPAI inhibits TGF-β signaling by promoting lysosome degradation of TGF-β receptor and contributes to lung cancer development. *Cell Signal.* 2014; 26: 2030-39.

Brognard J, Hunter T. Protein kinase signaling networks in cancer. *Curr Opin Genet Dev.* 2011; 21: 4-11.

Brunschwig EB, Wilson K, Mack D, Dawson D, Lawrence E, Wiison JKV et al. PMEPA1, a transforming growth factor-β-induced marker of terminal colonocyte differentiation whose expression is maintained in primary and metastatic colon cancer. *Cancer Res.* 2003; 63: 1568-75.

Buchwalter G, Gross C, Wasylyk B. Ets ternary complex transcription factors. *Gene* 2004; 324: 1-14.

Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK et al. Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2* expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci USA* 1996; 93: 3636-41.

Chin YR, Toker A. Function of Akt/PKB signaling to cell motility, invasion and the tumor stroma in cancer. *Cell Signal.* 2009; 21: 470-76.

Courjal F, Cuny M, Rodriguez C, Louaso G, Speiser P, Katsaros D et al. DNA amplification at 20q13 and MDM2 define distinct subsets of evolved breast and ovarian tumors. *Br J Cancer.* 1996; 74: 1984-89.

Courtney KD, Corcoran RB, Engelman JA. The PI3K pathways as drug target in human cancer. *J Clin Oncol.* 2010; 28: 1075-83.

Darnell JE, Kerr J, lan M, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 1994; 264: 1415-21.

Davies M, Robinson M, Smith E, Huntley S, Prime S, Paterson I. Induction of an Epithelial to Mesenchymal Transition in Human Immortal and Malignant Keratinocytes by TGFβ1 Involves MAPK, Smad and AP-1 Signalling Pathway. *J Biol Chem.* 2005; 95*:* 918-31.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF-β inducible elements in the promoter of human plasminogen

activator inhibitor-type 1 gene. *EMBO J* 1998; 17*:* 3091–00.

Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppressor and cancer progression. *Nat Genet.* 2001; 29: 117-29.

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-β family signaling. *Nature* 2003; 425: 577-84.

Dong L, Jin L, Tseng HY, Wang CY, Wilmost JS, Yosufi B et al. Oncogenic suppression of PHLPP1 in human melanoma. *Oncogene* 2013; 33: 4756-66.

Downward J. Targeting Ras signaling pathway in cancer therapy. *Nat Rev Cancer.* 2003; 03*:* 11-22.

Freytag J, Wilkins-Port CE, Higgins CE, Higgins SP, Samarakoon R, Higgins PJ. PAI-1 Mediates the TGF-β1+EGF-Induced ”Scatte” Response in Transformed Human Keratinocytes. *J Invest Dermatol.* 2010; 130: 2179–90.

Giannini G, Ambrosini MI, Di Marcotullio L et al. EGF- and cell cycle-regulated STAG1/PMEPA1/ERG1.2 belongs to a conserved gene family and is overexpressed and amplified in breast and ovarian cancer. *Mol Carcinog.* 2003; 38: 188-00.

Guo X, Wang XF. Signal cross-talk between TGF-β/BMP and other pathways. *Cell Res.* 2009; 19: 71-88.

Heldin CH, Miyazono K, ten Dijke P. TGF-β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 1997; 390: 465-71.

Heldin CH, Moustakas A. Role of Smads in TGF-β signaling. *Cell Tissue Res.* 2012; 347: 21-36.

Henson ES, Gibson SB. Surviving cell death through epidermal growth factor (EGF) signal transduction pathways: Implication for cancer therapy. *Cell Signal.* 2006; 18: 2089-97.

Hirokawa YS, Takai A, Uchida K, Kozuka Y, Yoneda M, Watanabe M et al. High level expression of STAG1/PMEPA1 in an androgen independent prostate cancer PC3 subclone. *Cell Mol Biol Lett.* 2007; 12: 370-77.

Hu Y, He K, Wang D, Yuan X, Liu Y, Ji H et al. TMEPAI regulates EMT in lung cancer cells by modulating the ROS and IRI-1 signaling pathway. *Carcinogenesis* 2013; 34: 1764-72.

Itoh S, ten Dijke P. Negative regulation of TGF-β receptor/Smad signal transduction. *Curr Opin Cell Biol*. 2007; 19: 176-84.

Itoh S, Thorikay M, Kowanetz M, Moustakas A, Itoh F, Heldin CH et al. Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. *J Biol Chem.* 2003; 278: 3751-61.

Janknecht R, Ernst JE, Pingoud V, Nordheim A. Activation of ternary complex factor ELK-1 by MAP kinases. *EMBO J* 1993; 12: 5097-04.

Jorissen RN, Walket F, Pouliot N, Garrett TPJ, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signaling. *Exp Cell Res.* 2003; 284: 31-53.

Kretzschmar M, Jacqueline DJ, Timokhina I, Massagué J. A mechanism of repression of TGFβ/ Smad signaling by oncogenic Ras. *Genes Dev.* 1999; 13: 804-16.

Levy L, Hill CS. Smad4 dependency defines two classes of Transforming Growth Factor β (TGF-β) target genes and distinguishes TGF-β-induced epithelial-mesenchymal transition from its antiproliferate and migratory response. *Mol Cell Biol.* 2005; 18: 8108-25.

Li H, Xu LL, Masuda K, Raymundo E, McLeod DG, Dobi A et al. A feedback loop between the androgen receptor and a NEDD4-binding protein, PMEPA1, in prostate cancer cells. *J Biol Chem.* 2008; 283: 28988-95.

Liu J, Stevens PD, Gao T. mTOR-dependent regulation of PHLPP expression controls the rapamycin sensitivity in cancer cells. *J Biol Chem.* 2011; 286: 6510-20.

Liu J, Stevens PD, Li X, Schmidt MD, Gao T. PHLPP-mediated dephosphorylation of S6K1 inhibits protein translation and cell growth. *Mol Cell Biol.* 2011; 31: 4917-27.

Liu J, Weiss HL, Rychahou P, Jacson LN, Evers BM, Gao T. Loss of PHLPP expression in colon cancer: role in proliferation and tumorigenesis. *Oncogene* 2009; 28: 994-04.

Liu R, Zhou Z, Huang J, Chen C. PMEPAI promotes androgen receptor-negative prostate cell proliferation through suppressing the Smad3/4-c-Myc-p21 Cip1 signaling pathway. *J Pathol.* 2011; 223: 683-94.

Lo HW, Hsu SC, Hung MC. EGFR signaling pathway in breast cancers: from traditional signal transduction to direct nuclear translocalization. *Breast Cancer Res Treat.* 2006; 95: 211-18.

Lo RS, Wotton D, Massagué J. Epidermal growth factor signaling via Ras controls the Smad transcriptional co-repressor TGIF. *EMBO J* 2001; 20: 128-36.

Ma YY, Wei SJ, Lin YC, Lung JC, Chang TC, Whang-Peng J et al. *PIK3CA* as an oncogene in cervical cancer. *Oncogene* 2000; 19: 2739-44.

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; 129: 1261-74.

Marais R, Wynne J, Treisman R. The SRF Accessory Protein Elk-l Contains a Growth Factor-Regulated Transcriptional Activation. *Cell* 1993; 73: 381-93.

Massagué J. How cells read TGF-β signals. *Nat Rev Mol Cell Biol.* 2000; 3: 169-78.

Massagué J. TGF-β in Cancer. *Cell* 2008; 134: 215-30.

Massagué J, Blain SW, Lo RS. TGF-β signal in growth control, cancer, and heritable disorders. *Cell* 2000; 103: 295-09.

Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev.* 2005; 19: 2783-10.

Massagué J, Wotton D. Transcriptional control by the TGF-β/Smad signaling systems. *EMBO J* 2000; 19: 1745-54.

Miyazono K. Transforming growth factor-β signaling in epithelial-mesenchymal transition and progression of cancer. *Proc Jpn Acad Ser B Phys Biol Sci.* 2009; 85: 314-23.

Moustakas A, Heldin CH. Non-Smad TGF-β signals. *J Cell Sci.* 2005, 118: 3573-84.

Moustakas A, Heldin CH. The regulation of TGF-β signal transduction. *Development* 2009; 136: 3699-14.

Mulder KM. Role of Ras and Mapks in TGF-β signaling. *Cytokine Growth Factor Reviews* 2000; 11: 23-35.

Muñoz NM, Upton M, Rojas A et al. Transforming growth factor β receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. *Cancer Res.* 2006; 66: 9837-44.

Nakano N, Itoh S, Watanabe Y, Maeyama K, Itoh F, Kato M. Requirement of TCF7L2 for TGF-β dependent transcriptional activation of the TMEPAI gene. *J Biol Chem.* 2010; 285: 38023-33.

Normanno N, Luca AD, Bianco C, Strizzi L, Mancino M, Maiello MR et al. Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* 2006, 366: 2-16.

ÓNeilll AK, Niederst MJ, Newton AC. Suppression of survival signaling pathways by the phosphatase PHLPP. *FEBS J.* 2013; 280: 572-83.

Padua D, Massague J, Role of TGF-β in metastasis. *Cell Res.* 2009; 19: 89-02.

Pardali K, Moustakas A. Action of TGF-β as tumor suppressor and pro-metastatic factor in human cancer. *Biochim Biophys Acta* 2007; 1775: 21-62.

Person U, Izumi H, Souchelnytskyi S, Itoh S, Grimsby S, Engström U et al. The L45

loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett.*1998; 434: 83-87.

Prajjal KS, I-Tien Y, Manjeri AV. Transforming growth factor-β (TGF-β)-inducible gene TMEPAI converts TGF-β from a tumor suppressor to a tumor promoter in breast cancer. *Cancer Res.* 2010; 70: 6377-83.

Quesnelle KM, Boehm AL, Grandies JR. STAT-mediated EGFR signaling in cancer. *J Cell Biochem.* 2007; 102: 311-19.

Rae FK, Hooper JD, Nicol DL, Clements JA. Characterization of a novel gene STAG1/PMEPAI, upregulated in renal cell carcinoma and other solid tumors. *Mol Carcinog.* 2001; 32: 44-53.

Rahim G, Araud T, Gubler PJ, Curran J. Alternative slicing within the ELK-1 5` untranslated region serves to modulate initiation events downstream, of the highly conserved upstream open reading frame 2. *Mol Cell Biol.* 2012; 32: 1745-56.

Rajkumar T, Vijayalakshmi N, Gopal G, Sabitha K, Shirley S, Raja UM et al. Identification and validation of genes involved in gastric tumorigenesis. *Cancer Cell Int.* 2010; 10:45.

Reichling T, Goss KH, Carson DJ, Ley-Ebert C, Witte D, Aronow BJ et al. Transcriptional profiles of intestinal tumors in *ApcMin* mice are unique from those of embryonic intestine and identify novel gene targets dysregulated in Human Colorectal Tumors. *Cancer Res.* 2005; 65: 166-76.

Richter E, Masuda K, Cook C, Ehrich M, Tadese AY, Li H et al. A role for DNA methylation in regulating the growth suppressor PMEPA1 gene in prostate cancer.

Epigenetic 2007; 2: 100-09.

Roberts AB, Sporn MB. The transforming growth factor-βs. In: Roberts AB, Sporn MB, ed. *Peptide Growth Factors and their Receptors,* vol. 95. Berlin: Springer-Verlag, 1990; 419-72.

Saadi A, Shannon NB, Lao-Sirieix P et al. Stromal genes discriminate preinvasive from invasive disease, predict outcome, and highlight inflammatory pathways in digestive cancer. *Proc Natl Acad Sci USA* 2010; 107: 2177-82.

Saha D, Datta PK, Beauchamp RD. Oncogenic Ras Represses Transforming Growth Factor-β/Smad Signaling by Degrading Tumor Suppressor Smad4. *J Biol Chem.* 2001; 276: 29531-37.

Selvaggi G, Novello S, Torri V, Leonardo E, Giuli RD, Borasio P et al. Epidermal growth factor receptor overexpression correlates with a poor prognosis in completely resected non-small-cell lung cancer. *Annals Oncology* 2004; 15: 28-32.

Shaw PE, Saxton J. Ternary complex factors: prime nuclear targets for mitogen-activated protein kinases. *Int J Biochem Cell Biol.* 2003; 35: 1210-26.

Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Colins C et al. *PIK3CA* is implicated as an oncogene in ovarian cancer. *Nature Genet.* 1999; 21: 99-02.

Shi Y, Massagué J. Mechanism of TGF-β signaling from cell membrance to the nucleus. *Cell* 2003; 113: 685-00.

Shore P, Bisset L, Lakey J, Waltho JP, Virden R, Sharrocks AD. Characterization of the

ELK-1 ETS DNA-binding domain. *J Biol Chem.* 1995; 270: 5805-11.

Tanner MM, Tirkkonen M, Kallioniemi A et al. Increased copy number at 20q13 in breast cancer: defining the critical region and exclusion of candidate genes. *Cancer Res.* 1994; 54: 4257-60.

ten Dijke P, Arthur HM. Extracellular control of TGFβ signaling in vascular development and disease. *Nat Rev Mol Cell Biol.* 2007; 8: 857-69.

Toonkey RL, Borczuk AC, Powell CA. TGF-β signaling pathway in lung adenocarcinoma invasion. *J Thorac Oncol.* 2010; 2: 153-57.

Vivanco I, Sawyers CL. The Phosphatidylinositol -3-kinase-Akt pathway in human cancer. *Nat Rev Cancer* 2002; 2: 489-01.

Vo Nguyen TT, Watanabe Y, Shiba A, Noguchi M, Itoh S, Kato M. TMEPAI/PMEPA1 enhances tumorigenic activities in lung cancer cells. *Cancer Sci.* 2014; 105: 334-41.

Wasylyk B, Hagman J, Hartmann AG. Ets transcription factors: nuclear effectors of the Ras-MAPK-kinase signaling pathway. *TIBS Reviews* 1998; 23: 213-16.

Watanabe Y, Itoh S, Goto T et al. TMEPAI, a transmembrane TGF-β-inducible protein, sequesters Smad proteins from active participation in TGF-β signaling. *Mol Cell.* 2010; 37: 123-34.

Whitman M. Smads and early developmental signaling by the TGFβ superfamily. *Genes Dev.* 1998; 12: 2445-62.

Xu LL, Shanmugam N, Segawa T et al. A novel androgen-regulated gene, PMEPA1, located on chromosome 20q13 exhibits high level expression in prostate. *Genomics* 2000; 66: 257-63.

Xu LL, Shi Y, Petrovics G et al. PMEPA1, an androgen-regulated NEDD4-binding protein, exhibits cell growth inhibitory function and decreased expression during prostate. *Cancer Res.* 2003; 63: 4299-04.

Yang SH, Bumpass DC, Perkins ND, Sharrocks AD. The ETS domain transcription factor ELK-1 contains a novel class of repression domain. *Mol Cell Biol.* 2002; 22: 5036-46.

Yang SH, Jaffray E, Hay RT, Sharrocks AD. Dynamic interplay of the SUMO and ERK pathways in regulating ELK-1 transcriptional activity. *Mol Cell.* 2003; 12: 63-74.

Yarden Y. The EGFR family and its ligands in human cancer: signaling mechanisms and therapeutic opportunities. *Eur J Cancer* 2001; 37: S3-S8.

Acknowledgements

First and foremost I would like to thank my advisor professor Mitsuyasu Kato. It has been an honor to be his student. Thanks to him, I had the opportunity to come to Tsukuba and join his laboratory. During these past 6 years, he has taught and always been available to advise me. Under his guidance I overcame many difficulties and learned a lot. His valuable advice, constructive criticism and extensive discussions made my PhD experience productive and stimulating. I am very grateful for his patience, motivation, enthusiasm, and immense scientific knowledge.

I would also like to thank my former advisor professor Susumu Itoh for his endless help and careful guidance from the beginning of my study. The joy and enthusiasm he has for his research was contagious and motivational for me.

I would also like to thank my assistant advisor assistant professor Yukihide Watanabe for teaching me how to perform experiments from the very first days. He was always willing to discuss with me and helped me to solve problems when I was in trouble. I specially thank to him for helping me to perform mice experiments. I very much appreciate all his contributions of time and ideas to make my publications to be accepted.

For this dissertation, I would like to thank the members of my PhD committee for their helpful advice and instructive comments.

I want to thank all present and past members of the Kato lab for their friendship and support. Thanks to associated professor Hiroyuki Suzuki for his scientific discussions and suggestions. I also thank so much for his contribution as a coffee and curry soup

supplier. I think we had a lot of fun and unforgettable memories during such crazy spicy-food parties. I also thank to Dr. Yukari Okita who always helped me to find some reagents and gave me some good advice as well as was a Japanese translator whenever I needed. I thank to Shun Azami who contributed to my publication, and all active and lovely lab members. I will never forget the wonderful time we had together for lab parties, BBQ, badminton club, sushi club, and marathon club, etc.

I also thank to Ms. Flaminia Miyamasu, Mr. Brian Purdue and Mr. Thomas Mayers at medical English communications center (MECC) office in the University of Tsukuba for excellent English proofreading for my publications.

I particularly want to thank my Vietnamese friends in Tsukuba. All of you have helped me to overcome the sadness, fatigue, and homesickness with your endless help, conversation and a lot of funny parties. I will never forget the exciting time we spent together on the way to discover Japan.

Finally, I would like to express my gratitude to my dad, mom, and brother for all their love and encouragement. And most of all for my `alarm clock`, my loving, encouraging and patient husband Dr. Lai Quoc Dat, who faithfully beside me, share all joy and sorrow with me. Thank you for supporting me for everything, and encouraging me throughout this experience. Thank you.

Vo Nguyen Thanh Thao

University of Tsukuba

March 2015.