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# Cooperative induction of Transmembrane Prostate Androgen Induced protein TMEPAI/PMEPA1 by Transforming Growth Factor-β and Epidermal Growth Factor signaling

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**Abstract**

TMEPAI/PMEPA1 (transmembrane prostate androgen induced-RNA/ prostate transmembrane protein, androgen induced 1) is a pro-tumorigenic factor induced by TGF-β signaling and constitutive TMEPAI expression in lung cancer cells depends on activated autocrine TGF-β signaling. Here we demonstrate a novel mechanism of TMEPAI transcriptional co-regulation by EGF signaling. Interestingly, we found that ELK-1, downstream of EGFR/Ras/MAPK pathway, modulates TMEPAI expression. ELK-1 binds to the first intron (+1037 to +1294) of the TMEPAI gene together with TGF-β activated Smad3 and enhances the transcription of TMEPAI. Furthermore, TMEPAI gene activation by EGF and TGF-β signaling was reduced by the MEK inhibitor U0126. Together, EGF signaling collaboratively regulates TGF-β-induced TMEPAI expression.

**Keywords**: TMEPAI, EGF, TGF-β, ELK-1, Smad

## Introduction

The EGF (epidermal growth factor) signaling begins with the EGF family of ligands transmitting their signals via binding to the corresponding receptor tyrosine kinases, followed by activation of downstream effectors such as Ras/MAPK, PI3K/AKT and JAK/STAT pathways [1-3]. These signaling pathways transfer the signals to the nucleus and activate numerous transcriptional factors. One of the nuclear effectors is the Ets family transcription factor ELK-1 that is phosphorylated and activated by MAPK, Erk1/2, p38, JNK, and PI3K. This family is defined by a highly conserved DNA binding domain that binds the core consensus sequence 5'-GGA(A/T)-3' for controlling cell proliferation, differentiation, and embryonic development [4-6]. Over-activation of EGF signaling is frequently observed in many human cancers and it is a poor prognosis factor [7,8].

TGF-β (transforming growth factor-β) was originally discovered as a cytokine which induces anchorage independent growth of normal fibroblasts in the presence of EGF [9]. In a normal context during development or adult tissue homeostasis, TGF-β signaling regulates growth suppression, apoptosis induction, extracellular matrix production, and differentiation [10,11]. TGF-β signals via binding to two types of serine/threonine kinase receptors and controls expression of many target genes via the

Smad mediated pathway as well as non-Smad pathways (for example, MAPK, PI3K/AKT, Rho family signaling) [12-15]. Aberration in TGF-β signaling is implicated in a wide range of diseases from cancer and fibrosis to cardiovascular disorders [16,17].

Cross-talks between TGF-β and EGF signaling have been reported, such as transcription of Snail and PAI-1 genes that is up-regulated by the cooperation of EGF and TGF-β signaling [18-20]. Moreover, EGF signaling interferes with TGF-β signaling in multiple steps: for instance, EGF/MAPK signaling decreases the expression of Smad4 and phosphorylates the linker region of R-Smads to inhibit R-Smads translocation to the nucleus leading to signaling termination; as well, it increases the stability of TGIF (TGF-β induced factor) which interacts with the Smad complex to suppress the transcription of Smad mediated gene expression [21-25].

TMEPAI (PMEPA1/STAG1) was initially identified as a class I transmembrane protein regulated by testosterone in prostate cells [26]. The expression of TMEPAI is controlled not only by testosterone but also by TGF-β, EGF, Wnt, and mutant p53 [27-30]. Its expression is increased in many types of cancer such as lung, breast, colon, pancreas, and renal cell carcinomas [29, 31-33]. TMEPAI is known to be implicated in inhibiting androgen signaling due to induction of androgen receptor (AR) ubiquitination, followed by proteasomal degradation [34]. We discovered that TMEPAI also suppresses

TGF-β signaling by binding to R-Smad by competition with SARA (Smad anchor for receptor activation), to prevent R-Smad binding and activation by TGF-β receptor kinase [35]. Treatment with TGF-β inhibitor or TGF-β neutralizing antibody diminished the highly expressed TMEPAI in lung cancer cell lines. These data indicated that autocrine TGF-β signaling is essential for TMEPAI transcription [31]. Moreover, the expression analysis revealed that three SBEs (Smad binding element) and TTE (TGF-β-responsive TCF7L2 binding element) in the first intron of TMEPAI gene were important for the regulation of TMEPAI transcription by TGF-β [29].

Given the importance of TMEPAI in various biological contexts, we further investigated its transcriptional regulation. Our novel finding indicates a significant cooperation between EGF/Ras/MAPK signaling with TGF-β/Smad signaling to control TMEPAI expression. This mechanism involves ELK-1 binding to Smad3 at the first intron (+1037 to +1294) of TMEPAI gene leading to the coordinated activation of TMEPAI gene transcription.

**Materials and Methods**

**Plasmids.** Human ELK-1 and the mutant ELK-1 S383A constructs were described previously [36]. The luciferase reporter constructs pGL3ti-250-luc, and pGL3ti-850-luc were described previously [29]. The pGL3ti-250-luc mutants (pGL3ti-250-M1, pGL3ti-250-M2, pGL3ti-250-M123) were made by introducing a mutation to change the consensus sequence GGAT to TGCT (Fig. S1). All plasmids were sequenced before use.

**Cell culture.** HaCaT**,** HaCaT-mock, constitutively active Ras transformed HaCaT-RasG12V, and COS-7 cells were cultured in Dulbecco`s modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). HepG2 cells were maintained in minimum essential medium (MEM) containing 10% FCS, non-essential amino acids, and sodium pyruvate. The TGF-β receptor kinase inhibitor SB431542 and MEK kinase inhibitor U0126 were used to block TGF-β signaling and the EGF/Ras/MAPK pathway, respectively.

**Luciferase assay.** HepG2 cells were seeded at 1.5 x $10^5$ cells/ well in 12-well plates one day before transfection. The expression plasmids and reporters were transfected using FuGENE6. Where indicated, the cells were stimulated with TGF-β (0.1 ng/ ml) and/or EGF (10 ng/ ml) 24 hours after transfection, and were further cultured for 18 hours in the absence of FBS. Luciferase activities were determined by Luciferase Assay Systems

(Promega) and normalized to co-transfected β-galactosidase activity (pCH110). Each transfection was carried out in triplicate and repeated at least twice.

**Western Blotting.** Plasmids were transfected into COS-7 cells (5 x $10^5$ cells/ 6cm dish) using FuGENE6. Thirty six hours after transfection, the cells were dissolved in 500 µl of TNE buffer ( 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfornyl fluoride, 5 µg/ml leupeptin, 100 units/ml aprotinin, 40 mM NaF, and 20 mM β-glycerolphosphate). Then cell lysates were subjected to SDS-PAGE and segregated proteins were electrotransferred to mixed nitrocellulose membrane. The membranes were probed with different primary antibodies, anti-TMEPAI antibody (9F10) [31], anti-β-actin antibody (Sigma), and then incubated with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrate solution (Thermo Scientific). LAS-3000 Image Analyzer (Fuji Photo Film) was used for the detection of chemiluminescence.

**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 for 15 minutes by adding formaldehyde to the medium to a final concentration of 1% at room temperature, and glycine was added to a final concentration of 125 mM. Then, the cells were washed with PBS once and were collected into lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA,

1% SDS, 10 μg/ml leupeptin, 12.5 μg/ml aprotinin), and sonicated until the average length of input DNA became less than 500 bp in size. Then, the control IgG, anti-ELK-1 (abcam), or anti-Smad3 (Cell signaling) was used for the immunoprecipitation. The immunoprecipitated DNAs were purified and subjected to PCR amplification with specific primers for detection of the TMEPAI first intron sequence including ELK-1 binding elements. The primers were 5'-TGA GCG TGT CCA TCT TTC TG- 3' and 5'-CAG TCC CAA ACA CAA ACA GC- 3'.

**Results**

**1. Cooperative enhancement of TMEPAI expression by EGF in the presence of TGF-β.** Human keratinocyte cell line HaCaT was stimulated with EGF, TGF-β, or both EGF and TGF-β. As shown in Fig. 1A, TMEPAI expression was induced upon TGF-β stimulation, whereas no detectable level of TMEPAI was seen upon EGF stimulation. However, co-stimulation of TGF-β with EGF clearly enhanced TMEPAI expression. To further confirm the contribution of EGF signaling on TMEPAI expression, we used HaCaT-RasG12V cells, in which constitutively active H-Ras was

stably expressed. Enhanced expression of TMEPAI was detectable after 8 hours of TGF-β stimulation in HaCaT-RasG12V cells, approximately 5-fold higher than that in HaCaT-mock cells (Fig. 1B). Moreover, treating cells with TGF-β receptor kinase inhibitor SB41542 fully 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. 1C). We then treated the HaCaT-RasG12V cells by U0126 in the presence of TGF-β stimulation. TGF-β-induced TMEPAI expression in HaCaT-RasG12V cells was clearly reduced by U0126 in a dose-dependent manner (Fig. S2).These data indicate that EGF signaling, through the EGFR/Ras/MAPK pathway, contributes to the enhanced expression of TMEPAI in the presence of TGF-β.

**2. Identification of EGF-responsive elements in the first intron of the TMEPAI gene.** We used TMEPAI 5' promoter conjugated to a luciferase reporter, termed -1972TMEPAI-luc (Fig. S3A), and a TMEPAI first intron-luciferase reporter, termed pGL3ti-850-luc (Fig. 2A) for the identification of the EGF responsive elements. We first examined the responsiveness of -1972TMEPAI-luc upon EGF, TGF-β, and combination of EGF and TGF-β stimulation. The activity of -1972TMEPAI-luc was marginally

enhanced by these stimuli (Fig. S3B). Conversely, the activity of the pGL3ti-850-luc was highly activated by TGF-β, and that was further potentiated by the co-stimulation with EGF (Fig. 2B). Moreover, EGF potentiates TGF-β inducible pGL3ti-850-luc activity in a dose-dependent manner (Fig. 2C). Since it has been reported that the EGF signaling directly interferes TGF-β signaling via the inhibition of Smad function, we inspected the direct effect of EGF signaling on Smad mediated transcription. Results showed that EGF stimulation had no impact on $(CAGA)_{12}$-luc, a reporter of TGF-β/Smad signaling (Fig. S4). We next examined the effects of ELK-1, a downstream transcription factor of EGFR/Ras/MAPK pathway. Co-transfection of wild type ELK-1 significantly activated pGL3ti-850-luc reporter, but mutant ELK-1 (S383A) which cannot be phosphorylated by Ras/MAPK pathway, failed to do so (Fig. 2D). Whereas AP-1 transcriptional factor, c-Fos and c-Jun, which is also known as a downstream transcriptional factors of EGF signaling did not enhance the TGF-β-induced pGL3ti-850-luc reporter activity (Fig. S5). These data suggest that the first intron enhancer region of the TMEPAI gene is responsible for the EGF-induced co-stimulation of the TMEPAI gene expression that is mediated by the activation of ELK-1.

**3. ELK-1 binding elements are required for EGF-induced enhancement of the TMEPAI gene expression.** Stimulation by TGF-β increased the pGL3ti-850-luc activity and co-stimulation with EGF further activated it. In parallel, a shorter intronic region containing the 250 bp sequence from +1037 to +1294 (pGL3ti-250-luc) responded to TGF-β and EGF in a similar manner as pGL3ti-850-luc (Fig. 3A, Fig. S6). We could find three ELK-1 binding consensus sequences (5'-GGAT-3') in the 250bp sequence of the first intron (Fig. S1). Mutations in each ELK-1 binding sites or in all three ELK-1 binding sites of pGL3ti-250-luc reduced transcriptional responses upon EGF and TGF-β stimulation. Among them, the second mutant, at position +1077, completely abolished EGF-induced enhancement of pGL3ti-250-luc activity, suggesting that this is the major responsive element for ELK-1 within the 250 bp sequence (from +1037 to +1294) in the first intron of TMEPAI gene (Fig. 3B).

**4. Binding of ELK-1 and Smad3 on the first intron of TMEPAI gene.** TGF-β-inducible target gene expression frequently requires the binding of Smad, which is activated by TGF-β receptor, together with co-activating transcription factors. We further explored the binding of ELK-1 and Smad3 to the first intron of TMEPAI gene by chromatin immunoprecipitation (ChIP) assay using anti-ELK-1 and anti-Smad3

antibodies. The binding of ELK-1 and Smad3 complex to the 250bp sequence in the first intron of TMEPAI gene could be detected upon EGF and TGF-β stimulation respectively (Fig. 4A, 4B). Taken together, co-stimulation of EGF and TGF-β induces the binding of ELK-1 and Smad3 complex to the first intron of TMEPAI gene to induce the enhanced expression of TMEPAI gene (Fig. 4C).

**Discussion**

Since TGF-β signaling regulates a wide variety of cellular functions, uncontrolled TGF-β signaling results in many diseases, the prominent example being cancer. There is much evidence that TGF-β acts both as a tumor suppressor by causing growth arrest and as a tumor promoter by promoting the invasion and metastasis of cancer cells via induction of EMT (epithelial to mesenchymal transition), recruiting new blood vessels to tumor sites (angiogenesis), and suppression of the immune system [16,37]. The conflicting roles of TGF-β during cancer progression seem to occur in a progressive state-dependent manner. Nevertheless, much is still unknown about how TGF-β converts from being a tumor suppressor to a tumor promoter, stimulating research motivation in this area.

TMEPAI is involved in tumorigenesis through a complexity of actions. TMEPAI reduction resulted in tumor suppressive events including increased p27 expression, reduction of DNA replication and decreased HIF-1α and VEGF expression via regulation of the PI3K/PTEN/AKT pathway [38,39]. Our previous study showed that TMEPAI interacts with either Smad2 or Smad3 via its Smad interaction motif (SIM) in order to attenuate the TGF-β/Smad pathway [13]. TMEPAI as a direct target gene of TGF-β signaling acts to provide negative feedback regulation for TGF-β/Smad signaling termination. Therefore, constitutive expression of TMEPAI would dampen TGF-β/Smad signaling and put cells at risk of aberrant growth.

We previously reported that the constitutive expression of TMEPAI enhances tumorigenicity in lung cancer cells [31]. In the current study, we examined the role of EGF cross-regulation with TGF-β signaling in TMEPAI expression. The one report showed that TMEPAI expression is induced by EGF [28]. In our context, although EGF signaling did not affect the expression of TMEPAI by itself, EGF signaling strongly enhanced TGF-β induced TMEPAI expression. Furthermore, RasG12V-transformed HaCaT cells induced a much higher level of TMEPAI protein and was able to be suppressed by MEK inhibitor. These data suggested that the EGFR/Ras/MAPK pathway is essential for the enhancement of TMEPAI expression. The EGF and TGF-β signalings

simultaneously activate the enhancer activity of first intron (+447 to +1294) of TMEPAI gene. Since the AP-1 transcription factors composed of c-Jun and c-Fos contribute to synergistic transcriptional activation of the PAI-1 gene in response to TGF-β. Therefore, we investigated the involvement of AP-1 in the TMEPAI transcriptional activation. However, AP-1 rather suppressed TGF-β-induced pGL3ti-850 reporter activation. In contrast, co-expression of the transcription factor ELK-1, which is directly activated by the EGFR/Ras/MAPK pathway, could promote TGF-β induced-pGL3ti-850-luc reporter activation. Subsequently, the first intron sequence from +1037 to +1294 region was shown to be sufficient for its binding, leading to cooperative activation by EGF and TGF-β signaling. The inactive mutation of ELK-1 diminished the enhancement of TGF-β-induced reporter activity further supported this evidence.

There is a report showing that EGF signaling directly phosphorylates and inhibits Smad functions. Therefore, we examined the effect of EGF on the $(CAGA)_{12}$-luc reporter that is directly activated by the TGF-β/Smad signaling pathway. However, we could not detect any influence of EGF signaling on $(CAGA)_{12}$-luc activity, suggesting that EGF signaling does not inhibit Smads function in our cellular context. Thus, ELK-1 could be a partner of Smad and co-regulate the TMEPAI gene transcription. Indeed, chromatin immunoprecipitation data showed that both ELK-1 and

Smad3 bind to the first intron of the TMEPAI gene in an EGF and TGF-β dependent manner for activation of TMEPAI transcription (Fig. 4C).

The coordinated transcriptional regulation by EGF and TGF-β signaling is known to act not only on TMEPAI but also on Snail and PAI-1 genes [18,19]; both of these are implicated in EMT and cell migration. TGF-β signaling suppresses cell proliferation in the early stage of cancer progression. In late stage cancer, cancer cells acquire resistance to TGF-β induced-growth inhibition, in contrast TGF-β activates invasion and metastasis [37].

Our proposed mechanism of TMEPAI regulation by EGF and TGF-β signaling may provide a part of the factors leading to the dual roles of TGF-β in cancer progression. We hypothesize that growth inhibition by TGF-β signaling could be circumvented in cancer cells by taking advantage of active EGF signaling and ELK-1 activation to enhance 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 dissemination. Otherwise, changed balance between Smad and non-Smad signaling may be responsible for the change of TGF-β signaling from tumor suppressor to promoter.

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 TMEPAI gene. Future work would aim to elucidate its relevance in inducing high TMEPAI expression in cancer and its contribution the dual role of TGF-β in cancer.

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## Conflict of interest

The authors declare that there are no conflicts of interest.

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**Figure legends**

**Fig. 1. 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 (9F10). 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) HaCaT-mock cells and HaCaT-RasG12V cells were stimulated with TGF-β (0.5 ng/ml) for indicated time points. Total cell lysates were subjected to immunoblot analysis using an anti-TMEPAI antibody (9F10). β-actin was used as the loading control. (C) TGF-β receptor kinase inhibitor SB431542 (1μM) or MEK kinase inhibitor U0126 (1μM) 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 immunoblot analysis. TMEPAI was detected by using anti-TMEPAI antibody (9F10). β-actin was used as the loading control.

**Fig. 2. The first intron of TMEPAI gene contains the responsive sequences for TGF-β and EGF.** (A) Schematic representation of pGL3ti-850-luc reporter containing

the +447 to +1294 sequence from the first intron of the TMEPAI gene. (B) HepG2 cells were transfected with pGL3ti or pGL3ti-850-luc 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) HepG2 were transfected with pGL3ti-850-luc and treated with EGF for 18 hours with indicated concentrations in the presence or absence of TGF-β (0.1 ng/ml) stimulation. (D) HepG2 cells were transfected with pGL3ti-850-luc 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. (*$P < 0.05$)

**Fig. 3. ELK-1 binding elements in the 250bp (+1037/+1294) region of the first intron of the TMEPAI gene is essential for the response to EGF.** Schematic representation of the luciferase reporters pGL3ti-850-luc (+447/+1294) and pGL3ti-250-luc (+1037/+1294) from the first intron of the TMEPAI gene, dot-line circles indicate possible ELK-1 binding sites. (B) HepG2 cells were transfected with the mutant pGL3ti-250-luc reporters, as indicated, and stimulated with TGF-β (0.1 ng/ml), or TGF-β (0.1 ng/ml) and EGF (10 ng/ml) for 18 hours. (*$P < 0.05$)

**Fig. 4. ELK-1 binds to the first intron of TMEPAI gene in response to EGF stimulation.** (A, B) 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. Then cell lysates were sonicated using Bioruptor®, and incubated with anti-ELK-1 (A), or anti-Smad3 (B). The immunoprecipitated chromatins were analyzed by PCR with primers that amplify the 250bp fragment (+1037/+1294) in the first intron of the TMEPAI gene. Normal rabbit control IgG was used as a negative control. (C) Scheme of cooperative induction of TMEPAI by both TGF-β and EGF signaling. EGF activated ELK1 bind to its binding site within the 1st intron of the TMEPAI gene, together with TGF-β activated Smad3, for cooperative activation of the transcripts of TMEPAI gene. SBE: Smad binding element, EBS: ELK-1 binding site

**Fig. 1 (Azami S. *et.al.*)**

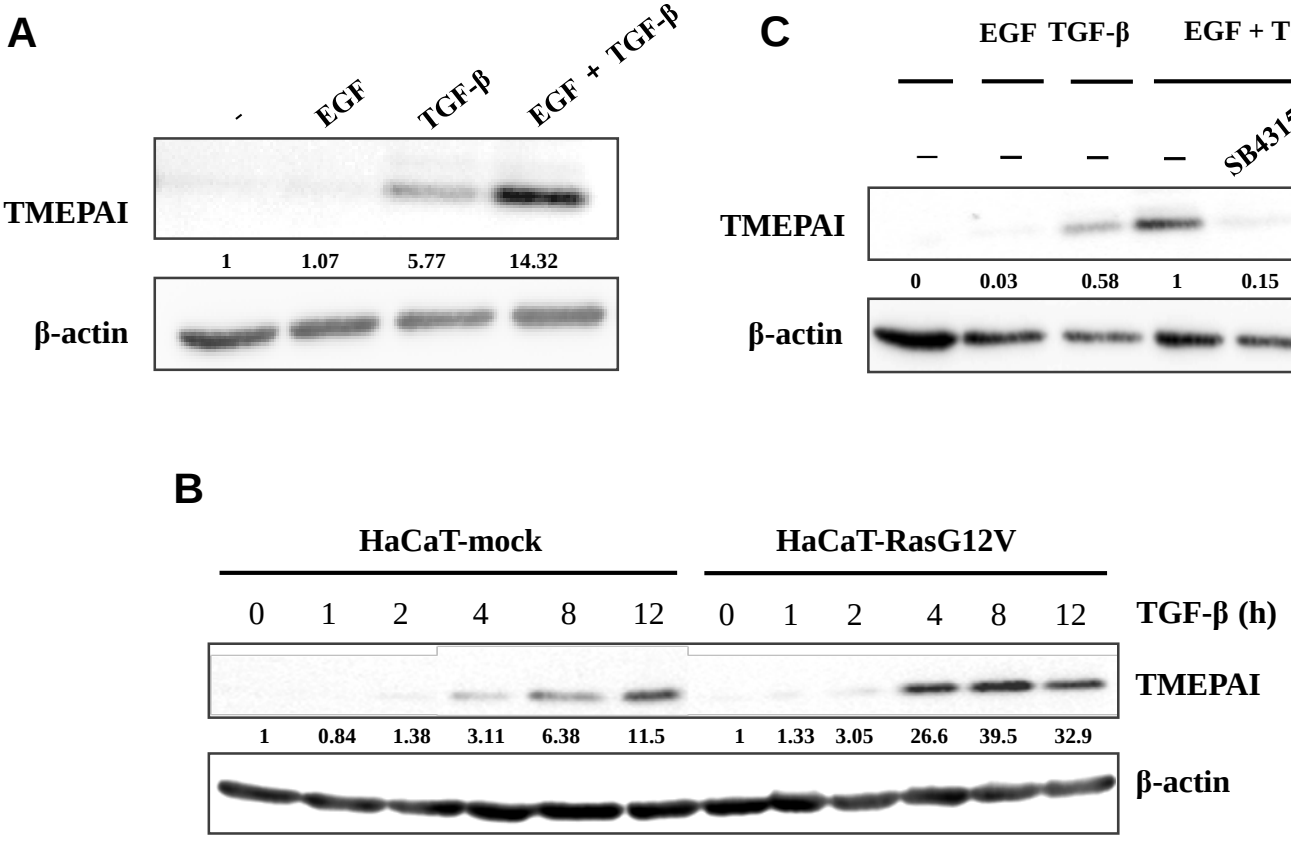


**Fig. 2 (Azami S. *et.al.*)**

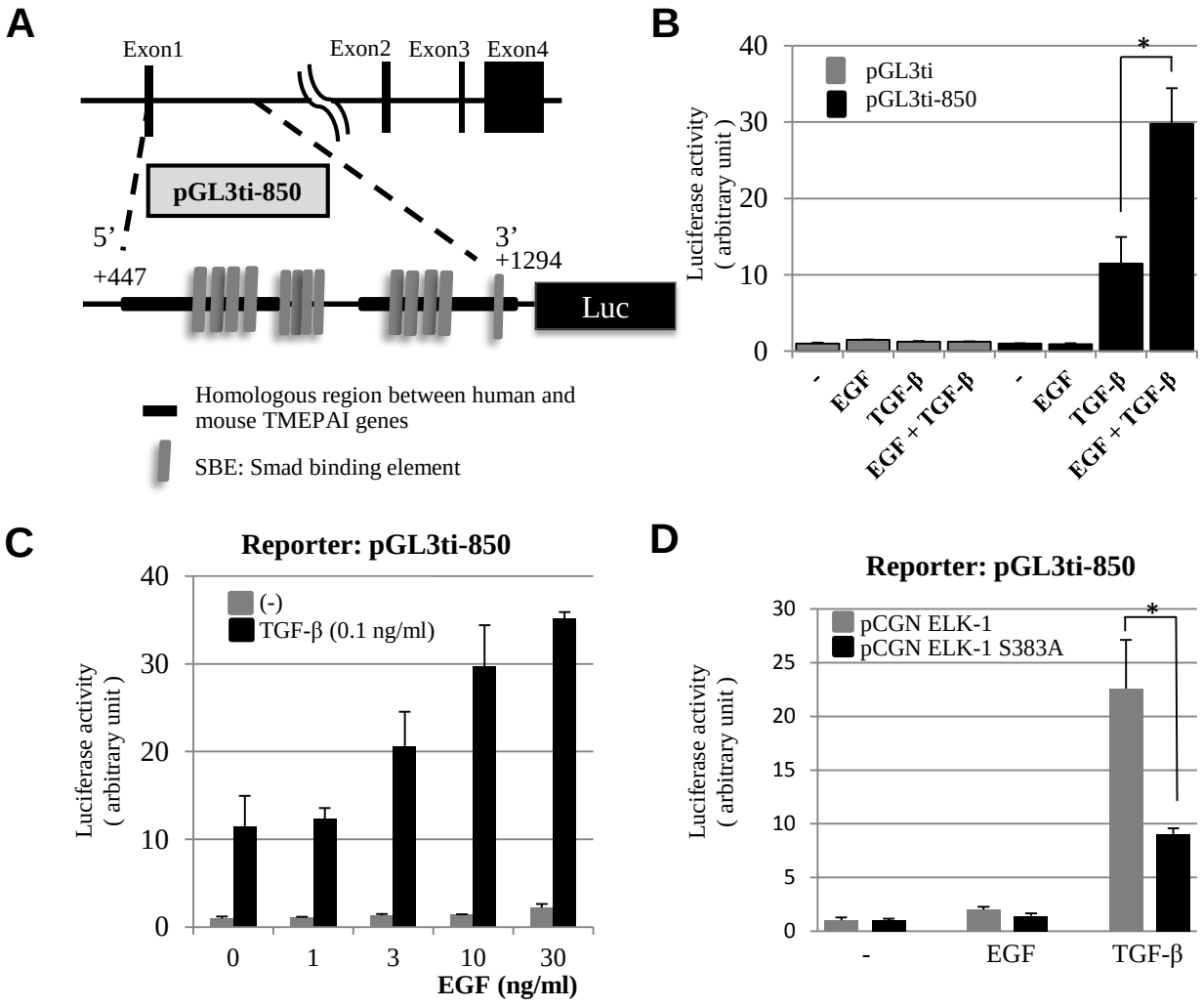
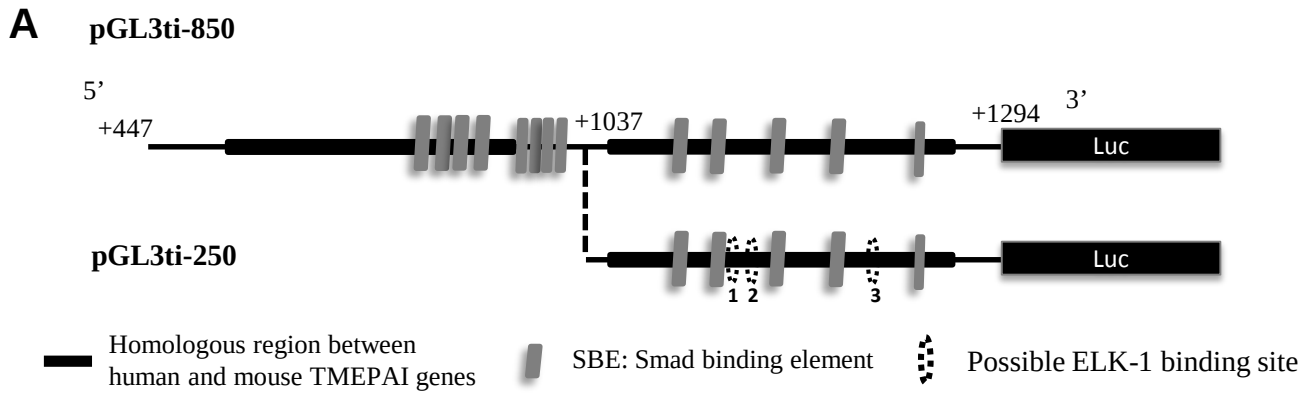


**Fig.3 (Azami S. *et.al.*)**



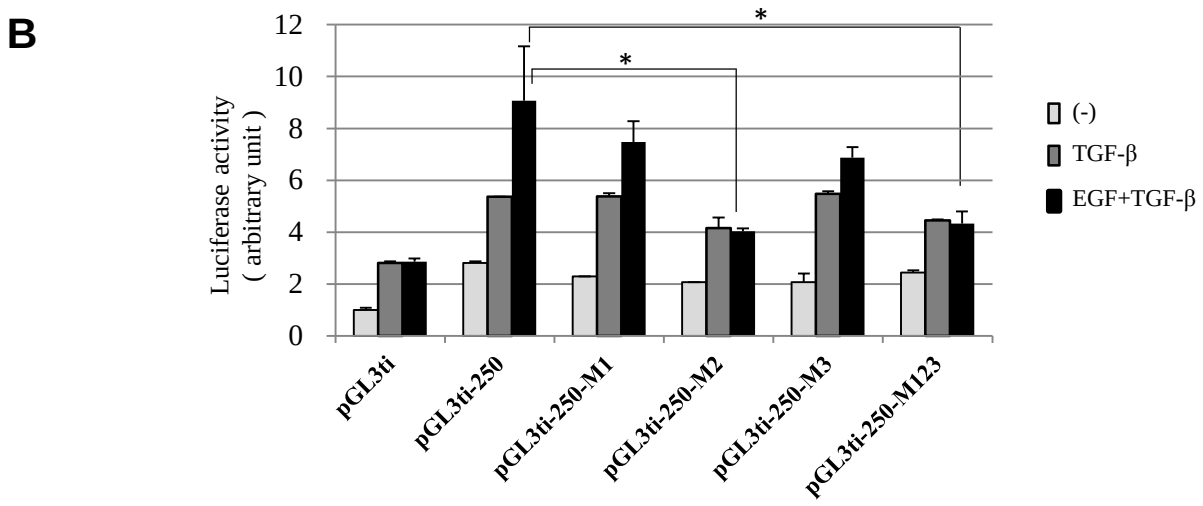



**Fig. 4 (Azami S. *et.al.*)**