

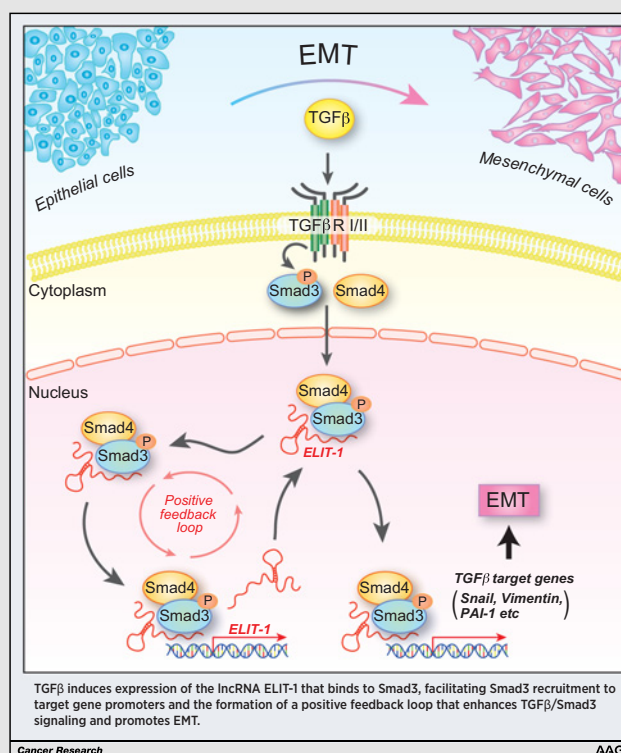
Long Noncoding RNA *ELIT-1* Acts as a Smad3 Cofactor to Facilitate TGF β /Smad Signaling and Promote Epithelial–Mesenchymal Transition

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Abstract

TGF β is involved in various biological processes, including development, differentiation, growth regulation, and epithelial–mesenchymal transition (EMT). In TGF β /Smad signaling, receptor-activated Smad complexes activate or repress their target gene promoters. Smad cofactors are a group of Smad-binding proteins that promote recruitment of Smad complexes to these promoters. Long noncoding RNAs (lncRNA), which behave as Smad cofactors, have thus far not been identified. Here, we characterize a novel lncRNA EMT-associated lncRNA induced by TGF β 1 (*ELIT-1*). *ELIT-1* was induced by TGF β stimulation via the TGF β /Smad pathway in TGF β -responsive cell lines. *ELIT-1* depletion abrogated TGF β -mediated EMT progression and expression of TGF β target genes including *Snail*, a transcription factor critical for EMT. A positive correlation between high expression of *ELIT-1* and poor prognosis in patients with lung adenocarcinoma and gastric cancer suggests that *ELIT-1* may be useful as a prognostic and therapeutic target. RIP assays revealed that *ELIT-1* bound to Smad3, but not Smad2. In conjunction with Smad3, *ELIT-1* enhanced Smad-responsive promoter activities by recruiting Smad3 to the promoters of its target genes including *Snail*, other TGF β target genes, and *ELIT-1* itself. Collectively, these data show that *ELIT-1* is a novel trans-acting lncRNA that forms a positive feedback loop to enhance TGF β /Smad3 signaling and promote EMT progression.



Significance: This study identifies a novel lncRNA *ELIT-1* and characterizes its role as a positive regulator of TGF β /Smad3 signaling and EMT.

Graphical Abstract: <http://cancerres.aacrjournals.org/content/canres/79/11/2821/F1.large.jpg>.

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Introduction

TGF β family, including TGF β s, activins, and bone morphogenetic proteins, plays a pivotal role in diverse cellular processes, such as cell proliferation, differentiation, communication, adhesion, migration, metabolism, and cell death (1). Perturbation of signaling by TGF β family members is often associated with a variety of disorders, such as malignancies, inflammatory conditions, and fibrotic conditions (2). During the process of tumorigenesis and malignant progression, TGF β elicits both suppressive and promoting effects; at early stages of tumorigenesis, it acts as a tumor suppressor by inhibiting cell proliferation and stimulating apoptosis; at later stages, TGF β becomes a tumor promoter via induction of epithelial–mesenchymal transition (EMT), which correlates with increased invasiveness, metastasis, and chemoresistance of tumor cells (2, 3). TGF β can also facilitate tumor progression by suppressing the immune system and promoting angiogenesis (4). Considering the extensive role of TGF β in late-stage tumor progression, several strategies to inhibit TGF β signaling to combat malignant tumors have been proposed (e.g., small-molecule inhibitors of receptor kinases, TGF β -neutralizing antibodies, and antisense compounds), based on a deep understanding of the TGF β signaling pathway (5).

Binding of TGF β induces assembly of a heterotetrameric complex composed of two TGF β type I receptors (T β RI) and two TGF β type II receptors (T β RII; ref. 6). In the complex, constitutively active T β RII phosphorylates and activates T β RI. Activated T β RI interacts with multiple proteins, thereby activating various downstream signaling pathways. In the canonical TGF β signaling pathway, the activated T β RI phosphorylates receptor-activated Smad (R-Smad), Smad2 and Smad3, leading to heterotrimeric complex formation with Co-Smad, Smad4 (7, 8). This complex translocates into the nucleus, where it associates with regulatory regions of target genes through the consensus sequence containing the CAGA box (9) and regulates transcription through the recruitment of transcriptional coactivators and/or corepressors. The activated Smad complex usually cooperates with other DNA-binding protein factors, so-called Smad cofactors (e.g., FoxH1, p53, Ets1 etc.), to elicit specific transcriptional regulation. The collaboration between Smads and Smad cofactors is thought to mediate context-dependent signaling of TGF β (5). However, lncRNA, which play a role as a Smad cofactor, has not been reported.

An increasing number of studies have demonstrated the importance of long noncoding RNAs (lncRNA), which are RNAs longer than 200 nucleotides that are not translated into protein. A recent study indicated that tens of thousands of lncRNAs are expressed in human cells. However, functional identifications have been reported with only a part of lncRNAs. lncRNAs are suggested to be involved in regulation of various biological processes, including development, differentiation, cell proliferation, cellular senescence, cell death, and cancer development (10, 11). Because lncRNAs are associated with both cause and progression in various diseases, they have received attention as useful targets for drug development (12). In particular, several lncRNAs related to the onset and progression of human cancers have been reported (13, 14). Moreover, lncRNAs are frequently dysregulated and associated with poor prognosis in multiple types of cancers. Therefore, investigations on lncRNA functions in cancer will lead to a better understanding of molecular mechanisms underlying cancer development and progression. Their reported molecular

functions are very diverse, such as miRNA sponge, protein sponge, scaffolds, transcription regulator, and chromatin modulator (15). Some lncRNAs also work as recruiters for chromatin modifiers into DNA (10, 14).

Previous reports have shown that TGF β induces several lncRNAs, including *lincRNA-ATB*, *lincRNA-RoR*, *lncRNA-smad7*, *MEG3*, *HIT*, and *MALAT1*, all of which modulate cellular responses elicited by TGF β (16–24). Moreover, *lincRNA-ATB*, *lincRNA-RoR*, *MEG3*, *HIT*, *H19*, *HOTAIR*, *ZEB1/2-AS1*, and *MALAT1* are reported to participate in EMT (17, 18). However, there has been no report about trans-acting lncRNA that promotes Smad-mediated transcription in TGF β /Smad pathway as a Smad cofactor. Here, we identified a novel TGF β -induced lncRNA *ELIT-1* (EMT-associated lncRNA induced by TGF β 1), which assists Smad3-dependent transcriptional regulation. Mechanistically, *ELIT-1* binds to Smad3, but not Smad2, and facilitates the binding of Smad3 to promoters of TGF β target genes. Thus, *ELIT-1* facilitates Smad-responsible promoter activities, in conjunction with Smad3. Our results suggest that *ELIT-1* is a novel and crucial player in the canonical TGF β signaling pathway, mediated by Smad3. Furthermore, *ELIT-1* is the first trans-acting lncRNA that functions as a Smad3 cofactor and is involved in the promotion of EMT.

Materials and Methods

Cell culture and reagents

Huh7, A549, HepG2, HaCaT, and HEK293 cells were grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) and penicillin/streptomycin. MDA-MB-231 cells were grown in Leibovitz L-15 medium (Gibco), supplemented with 10% FBS and penicillin/streptomycin. MCF10A cells were grown in mammary epithelium basal medium (Lonza) containing bovine pituitary extract, human EGF, human insulin, hydrocortisone, penicillin/streptomycin, and cholera toxin (Wako). All cells were maintained at 37°C in an atmosphere containing 5% CO₂. Huh7 cells were obtained from JCRB. A549, HEK293, MDA-MB-231, and MCF10A cells were obtained from ATCC. HepG2 cells were obtained from RCB. HaCaT cells were kindly provided by Dr. H. Hayashi (Nagoya City University, Nagoya, Japan).

The antibodies used in this study were as follows: anti-Smad2/3 (BD Biosciences, 610842), anti-phospho-Smad3 (Ser423/425; Cell Signaling Technology, #9520), anti-Smad3 (Abcam, ab28379 and Cell Signaling Technology, #9523), anti-Smad2 (Cell Signaling Technology, #5339), anti-Snail (Cell Signaling Technology, #4719), FITC mouse anti-E-cadherin (BD Biosciences), anti-HSP90 (BD Transduction Laboratories, 610418), anti-Lamin A/C (Santa Cruz Biotechnology, sc-7292), anti-FLAG (Sigma, M2), anti-c-myc (Roche, 9E10), normal mouse IgG (Cell Signaling Technology, #5415), normal rabbit IgG (Cell Signaling Technology, #2729), and anti- β -actin (Novus Biologicals, AC-15 and Wako, 017-24573). Phalloidin-TRITC was used to detect F-actin (Sigma-Aldrich).

Recombinant hTGF β 1 was purchased from R&D Systems (240-B). The inhibitors used in this study were as follows: SB431542 (Selleck Chemicals, S1067), SB203580 (Abcam, ab120162), and Smad3 inhibitor, SIS3 (Merck, 566405).

Plasmids and transfection

Human *ELIT-1* cDNA was cloned into pcDNA3.1 (Invitrogen). A reporter construct containing -2526/+61 of the human *ELIT-1*

promoter linked to a luciferase reporter gene (pGL4-*ELIT-1*) was generated using PCR, then cloned into pGL4.10 (Promega). Substitutions of Ser⁴²³ and Ser⁴²⁵ to aspartic acid in Smad3 (Smad3-2SD) were generated using PCR-based mutagenesis and then cloned into pCMV5-FLAG. These plasmids were constructed using standard DNA techniques. pCMV- β -galactosidase (β -gal) was kindly provided by Dr. H. Hayashi (Nagoya City University, Nagoya, Japan).

Plasmids were transfected into HEK293, Huh7 or A549, HepG2, or HaCaT cells using the calcium phosphate method, Fugene6 (Promega, E2692), or ViaFect (Promega, E4981), or HilyMax (Dojindo, H357).

Identification of lncRNAs upregulated by hepatitis B virus replication using microarray analysis

Hepatitis B virus (HBV)-replicating cells were prepared as follows: Huh7 cells were transfected with the episomal HBV expression vector, pEB-HBCe, where the 1.24-fold HBV genome derived from the viral genotype Ce (25) was inserted into pEB-Multi-Puro (Wako), followed by maintaining in the medium in the presence of 5 μ g/mL puromycin for 14 days. Total RNAs were prepared from the HBV-replicating cells and the parental control cells where the empty vector pEBMulti-Puro was transfected and subjected to microarray analysis. Microarray analyses with SurePrint G3 Human Gene Expression 8 \times 60K v2 Microarrays (Agilent Technologies) were performed by Cell Innovator Co., Ltd. Our microarray dataset is deposited in NCBI Gene Expression Omnibus (GEO; GSE128965).

Identification of target genes regulated by *ELIT-1* using microarray analysis

Huh7 cells were transfected with siRNAs (siCtrl, si*ELIT-1* #2, or si*ELIT-1* #3). At 24 hours after the first transfection, cells were transfected with siRNAs, again. At 6 hours after the second transfection, cells were treated with or without 10 ng/mL TGF β for 48 hours. Total RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen, #74104), following the manufacturer's instructions. Gene expression was analyzed using the SurePrint G3 Human GE Microarray 8 \times 60K v2 (Agilent Technologies), following the manufacturer's protocol by Cell Innovator Co., Ltd. Our microarray dataset is deposited in NCBI Gene Expression Omnibus (GEO; GSE129008). We classified target genes from microarray data. First, upregulated genes that were common to both TGF β and *ELIT-1* were classified. We selected genes that were increased by >2-fold by TGF β , compared with the unstimulated condition; among those genes, we selected genes that were suppressed <0.5-fold by *ELIT-1* depletion, compared with TGF β stimulation. Next, genes that were increased by TGF β were classified. We selected genes that were increased by >2-fold by TGF β , compared with the unstimulated condition; among those genes, we selected genes that were increased >0.5-fold by *ELIT-1* depletion, compared with TGF β stimulation. Moreover, genes that were increased by *ELIT-1* were classified. After depletion of control siRNA and *ELIT-1* siRNA, we compared genes changed by TGF β stimulation and selected genes whose expression decreased by <0.5-fold; among those genes, we selected genes that increased from 1-fold to <2-fold, compared with TGF β stimulation and nonstimulation, after depletion of control siRNA.

In addition, suppressed genes that were common to both TGF β and *ELIT-1* were classified. We selected genes that were decreased

by <0.5-fold by TGF β , compared with the unstimulated condition; among those genes, we selected genes that were increased by >2-fold by *ELIT-1*-depletion, compared with TGF β stimulation. Next, genes that were suppressed by TGF β were classified. We selected genes that were decreased by <0.5-fold by TGF β , compared with the unstimulated condition; among those genes, we selected genes that were increased by <2-fold by *ELIT-1* depletion, compared with TGF β stimulation. Finally, genes that were suppressed by *ELIT-1* were classified. After depletion of control siRNA and *ELIT-1* siRNA, we compared genes changed by TGF β stimulation and selected genes whose expression increased by >2-fold; among those genes, we selected genes that were increased from 0.5-fold to <1-fold, compared with TGF β stimulation and nonstimulation after depletion of control siRNA. We classified by gene symbol, excluding those without a gene symbol. Then, genes with the same gene symbol were counted as a single gene.

RNA interference

Cells were transfected with human *ELIT-1* siRNA or control siRNA oligonucleotides using Lipofectamine RNAiMAX (Invitrogen), following the manufacturer's protocols. At 24 hours after transfection, cells were transfected again with *ELIT-1* siRNA or control siRNA. At 6 hours after transfection, cells were stimulated with or without 10 (Huh7) or 5 (A549) ng/mL TGF β for 72 or 48 hours, respectively. *ELIT-1* siRNA and Negative Control siRNA (#1027310) or Smads siRNA were purchased from Qiagen or Sigma, respectively. The nucleotide sequence of si*ELIT-1*-#2 was 5'-GGC CUA AUC CCG UCA UGA A-3' (SI03681041), si*ELIT-1*-#3 was 5'-CCG UUG GUU AGG AAU UCA A-3' (SI3681048), siSmad2 was 5'-AAC AGG CCU UUA CAG CUU CUC-3', and siSmad3 was 5'-AAG GCC AUC ACC ACG CAG AAC-3' (Sigma Genosys) with a 3'-dTdT overhang.

Immunoblot analysis

Cells were lysed in lysis buffer (0.3% Triton X-100, 300 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5). Cell lysate was denatured by treatment with SDS sample buffer at 95°C for 8 minutes. Cell lysate was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore), followed by immunoblotting with the indicated primary antibodies and corresponding secondary antibodies conjugated with horseradish peroxidase. Proteins were visualized using an enhanced chemiluminescence system (Perkin Elmer).

RT-PCR analysis for *ELIT-1* detection

Total RNA was isolated from cultured cells using RNeasy Mini Kit. Reverse transcription was performed with random hexanucleotide primers and reverse transcriptase SuperScript II (Invitrogen, #18064014). PCR was performed using TaKaRa Ex Taq HS (TaKaRa, RR006Q). Primer sequences are shown in Supplementary Table S1. The PCR program was set with an initial melting step at 94°C for 3 minutes, then: (for *ELIT-1* in Huh7 cells) 32 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 15 seconds; (for *ELIT-1* in A549 cells) 26 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 15 seconds; (for *GAPDH* in Huh7 cells) 26 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 15 seconds; (for *GAPDH* in A549 cells) 24 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 15 seconds. PCR products were then analyzed on 3% agarose gel by electrophoresis.

qRT-PCR analysis

Total RNA was isolated from cultured cells using RNeasy Mini Kit. Reverse transcription was performed with random hexanucleotide primers and reverse transcriptase SuperScript II. The resulting cDNA was subjected to real-time PCR using the StepOnePlus system (Applied Biosystems) and a SYBR Green Realtime PCR Master Mix (Toyobo Co., #QPS-201). Primer sequences are shown in Supplementary Table S1. Transcripts were normalized to *GAPDH* mRNA.

Luciferase reporter assay

Huh7 or A549 cells cultured in 24-well plates (2.5×10^4 cells per well) were transfected with the luciferase reporter plasmid, expression plasmids, β -gal expression plasmid, and empty vector using Fugene6 reagent or ViaFect reagent in Opti-MEM (Invitrogen). The total amount of transfected DNA was the same in each experiment. Cells were lysed at 48 hours after transfection and assayed for luciferase and β -gal activities. Luciferase activities were normalized by β -gal activities.

Chromatin immunoprecipitation assay

A549 or Huh7 cells (1.0×10^7 cells) were fixed by 1% formaldehyde, and crosslinking was terminated by addition of 0.125 mol/L glycine. After washing cells twice with PBS, cells were lysed with lysis buffers LB1 and LB2 (see next sentence) for nuclear isolation. The formulas of LB1 and LB2 are as follows: LB1 [50 mmol/L HEPES/pH 7.5, 0.5% NP-40, 140 mmol/L NaCl, 1 mmol/L EDTA, 10% Glycerol, 0.25% Triton X-100, and protease inhibitor cocktail (Roche)]; LB2 (10 mmol/L Tris/pH 8, 200 mmol/L NaCl, 1.0 mmol/L EDTA, 0.5 mmol/L EGTA, and protease inhibitor cocktail). After collecting the isolated nuclei, chromatin was digested by Micrococcal Nuclease (Cell Signaling Technology, #10011) with an optimized number of enzyme units; then, the nuclear membrane was destroyed by sonication (Bioruptor, COSMO-BIO). Chromatin was incubated with antibodies as follows: anti-Smad3 antibody (Cell Signaling Technology, #9523), anti-FLAG antibody (Sigma, M2), normal mouse IgG (Cell Signaling Technology, #5415), and normal rabbit IgG (CST, #2729). The antibody–chromatin complex was immunoprecipitated by Dynabeads protein G (Thermo Fisher Scientific, #10004D) and the precipitated chromatin was washed in accordance with standard chromatin immunoprecipitation (ChIP) procedure. The washed antibody–chromatin complex was incubated at 65°C for decrosslinking and ProteinaseK treatment; then, DNA was purified using Labo Pass PCR purification kit (Hokkaido System Science Co., Ltd. CMR0112). The purified DNA was subjected to ChIP-qPCR. Primer sequences are shown in Supplementary Table S2.

RNA immunoprecipitation assay

HEK293 cells (2.5×10^6 cells per 100-mm dish) were transiently transfected with *ELIT-1*, FLAG-Smad3, FLAG-Smad2, and ALK5-T204D-HA. At 48 hours after transfection, cells were harvested by trypsinization and resuspended in lysis buffer [0.3% Triton X-100, 300 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, protease inhibitor cocktail, RNase inhibitor (Toyobo)] on ice for 30 minutes. After centrifugation, the supernatant was collected as a whole-cell extract. A549 cells (2.5×10^6 cells per 100-mm dish) were plated. At 48 hours after incubation, the cells were treated with or without 5 ng/mL TGF β for 24 hours. Then, the cells were harvested by trypsin-

ization and resuspended in lysis buffer (0.3% Triton X-100, 300 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, protease inhibitor cocktail, RNase inhibitor) on ice for 30 minutes. After centrifugation, the supernatant was collected as whole-cell extract.

HEK293 cells (2.5×10^6 cells) were transiently transfected with *ELIT-1*, FLAG-Smad3, FLAG-Smad2, and ALK5-T204D-HA. At 48 hours after transfection, cells were harvested by trypsinization and resuspended in 2 mL PBS with protease inhibitor cocktail and RNase inhibitor, 2 mL nuclear isolation buffer (1.28 mol/L sucrose, 40 mmol/L Tris/pH 7.5, 20 mmol/L MgCl₂, 4% Triton X-100) and 6 mL distilled water on ice for 20 minutes. After centrifugation, the supernatant was collected as cytoplasmic extract and the nuclear pellet was resuspended in RIP buffer (150 mmol/L KCl, 25 mmol/L Tris/pH7.4, 0.5 mmol/L DTT, 0.5% NP40, protease inhibitor cocktail, RNase inhibitor) on ice. Resuspended nuclei were mechanically sheared using a Dounce homogenizer with 30 strokes. After centrifugation, the supernatant was collected as nuclear extract. Immunoprecipitation was performed with an antibody specific to anti-Smad3 antibody (Abcam, ab28379), anti-Smad2 antibody (Cell Signaling Technology, #5339), anti-FLAG antibody (Sigma, M2); normal mouse IgG (Cell Signaling Technology, #5415) or normal rabbit IgG (Cell Signaling Technology, #2729) was used as a control for overnight at 4°C with gentle rotation. After immunoprecipitation, 40 μ L of Dynabeads protein G (Thermo Fisher Scientific, #10004D) was added and incubated for 1.5 hours at 4°C with gentle rotation. Then, the supernatant was removed using a magnetic stand; the beads were washed three times with 500 μ L of RIP buffer and then washed once with 1 mL of PBS. Purification of coprecipitated RNA from beads was performed using ISOGEN (Nippon Gene Co., LTD; #311-02501), following the manufacturer's protocols. Reverse transcription was performed with random hexanucleotide primers and reverse transcriptase SuperScript IV (Invitrogen, 18090050). The resulting cDNA was subjected to real-time PCR using the StepOnePlus system and a SYBR Green Realtime PCR Master Mix. Fold enrichment was calculated as a ratio to IgG control.

Wounding assay

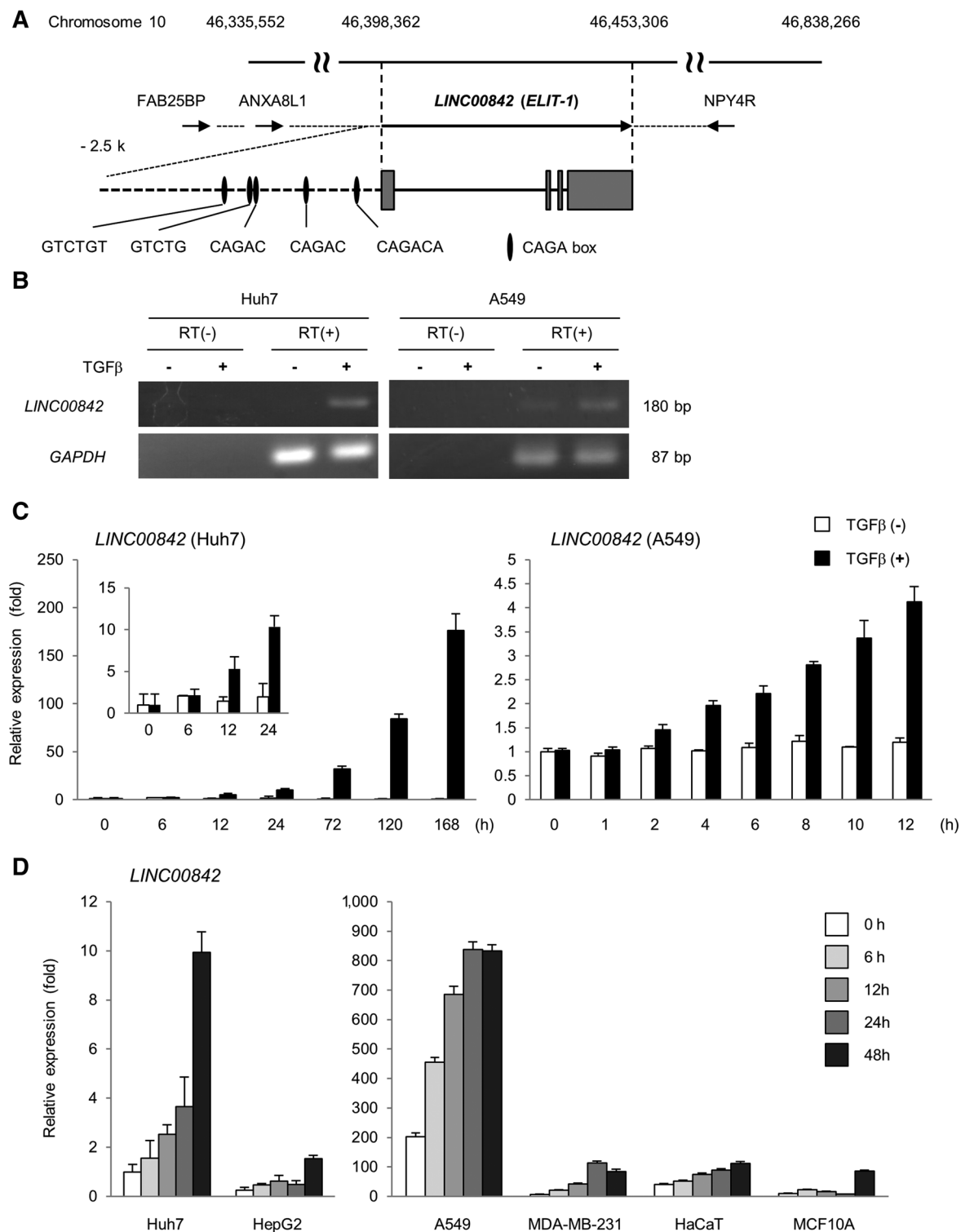
A549 cells (4×10^5 cells per well in 12-well plates) were transfected with siRNAs (siCtrl or si*ELIT-1* #2). At 24 hours after transfection, cell monolayers were wounded by scratching with sterile plastic 200 μ L micropipette tips and stimulated with 5 ng/mL TGF β . Cells were photographed using phase-contrast microscopy: immediately, 12 hours, and 24 hours after wounding. The assay was independently performed in triplicate. The migration area was measured by graphic software Adobe Photoshop (Adobe).

Invasion assay

A549 cells were transfected with siRNAs (siCtrl or si*ELIT-1* #2). At 24 hours after transfection, cells were stimulated with 5 ng/mL TGF β for 48 hours. Then the cells (5×10^4 cells per well) were seeded on Matrigel-coated Transwell (Corning), following manufacturer's instructions. After 18 hours, invaded cells were fixed by 4% paraformaldehyde (PFA), and stained with 0.1% crystal violet and photographed using phase-contrast microscopy.

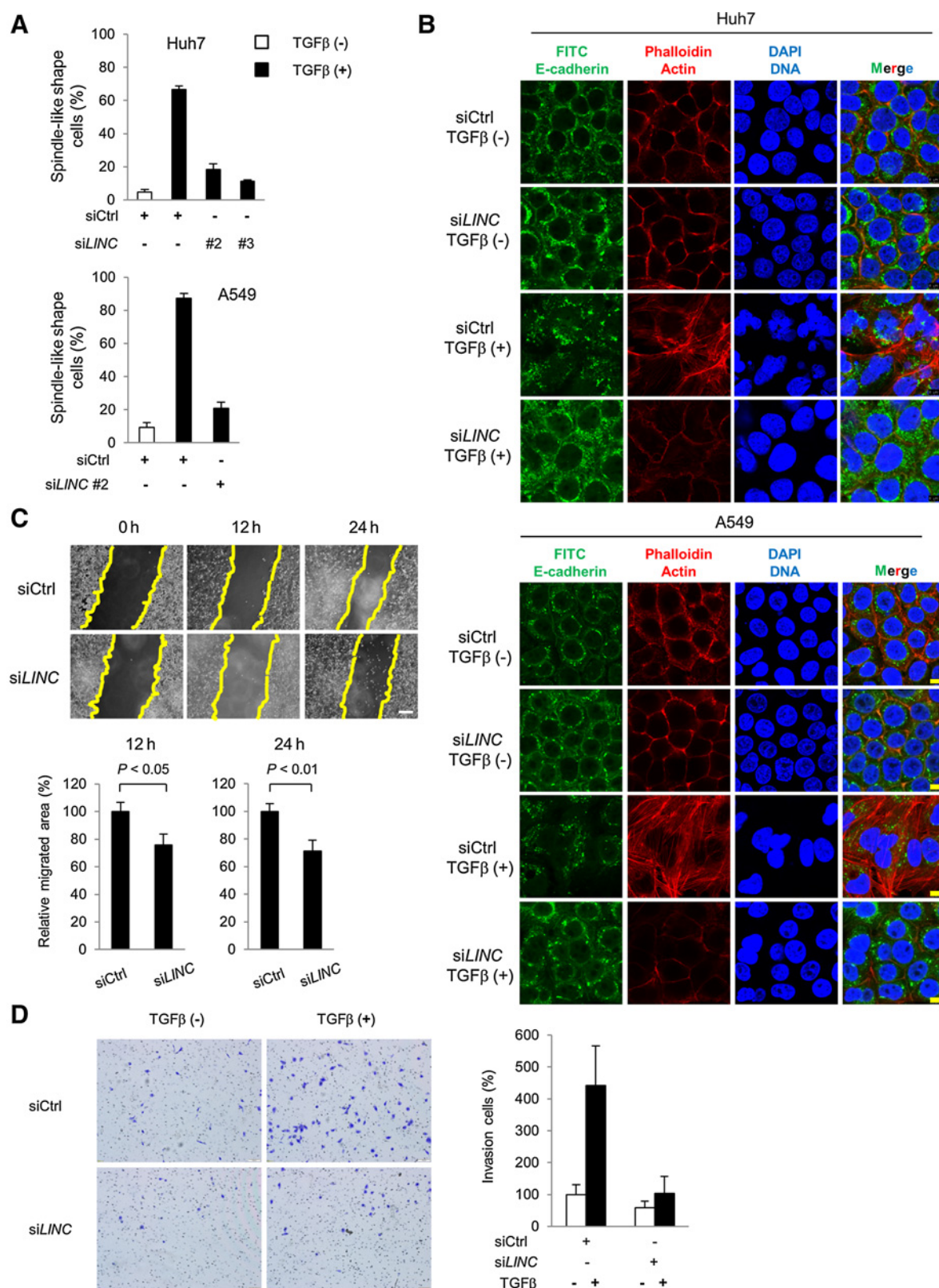
Fluorescent immunostaining

Huh7 and A549 cells were transfected with siRNAs (siCtrl or si*ELIT-1* #2) in 4-well Lab Tek glass Chamber Slides (Nunc

**Figure 1.**

LINC00842 (*ELIT-1*) is induced by TGF β . **A**, Gene map of *LINC00842* (*ELIT-1*), a human lncRNA gene, by NCBI map viewer (GRCh38.p12). **B**, Expression of *LINC00842* in Huh7 and A549 cells. Huh7 and A549 cells were stimulated with 10 or 5 ng/mL TGF β for 72 or 24 hours. Total RNA was prepared and subjected to RT-PCR. **C**, Induction of *LINC00842* by TGF β stimulation. Huh7 cells were stimulated with (black bar) or without (white bar) 10 ng/mL TGF β for the indicated times. Total RNA was subjected to qRT-PCR. **D**, TGF β -mediated induction of *LINC00842* in TGF β -responsive cell lines. The indicated cell lines were stimulated with 10 (Huh7, MDA-MB-231, and HaCaT) or 5 (HepG2, A549, and MCF10A) ng/mL TGF β for the indicated times. Total RNA was subjected to qRT-PCR. Relative expression was assessed in comparison with TGF β -unstimulated Huh7 cells.

Sakai et al.



International Corp.). At 24 hours after transfection, Huh7 or A549 cells were treated with or without 10 ng/mL or 5 ng/mL TGF β for 72 or 48 hours, respectively. Cells were fixed in 4% PFA for 10 minutes at room temperature. Then, cells were treated with ice-cold acetone for 2 minutes on ice and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Finally, cells were treated with 5% BSA and 0.1% Triton X-100 in PBS for 30 minutes at room temperature, and subsequently stained with FITC Mouse anti-E-cadherin (BD Biosciences, 1:250) and 0.23 mmol/L tetramethylrhodamine B isocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich). The cover glasses were mounted with VECTASHIELD Hard Set Mounting with DAPI (Vector Laboratories). Fluorescence was examined by confocal laser scanning microscopy (Leica).

Statistical analysis

Data are presented as mean \pm SD or SEM. Comparisons between two groups were analyzed by Student *t* test, where *P* < 0.05 was considered as statistically significant.

Results

Identification of a lncRNA *LINC00842* induced by TGF β

We have studied regulatory mechanisms of HBV replication in host cells, and are interested in whether cellular lncRNAs participate in this process. Therefore, to identify lncRNAs induced by HBV replication, microarray analysis was performed, comparing human hepatoma Huh7-derived cells constitutively replicating HBV genome with its control cells expressing empty vector. Fourteen lncRNAs were upregulated in HBV-replicated Huh7 cells compared with its control (Supplementary Table S3). Importantly, the second highest lncRNA in the list was *UCA1*, which is reported to be upregulated by HBx and is involved in hepatocarcinogenesis (26), indicating that our system is well-optimized for screening of HBV-upregulated lncRNAs. Given that TGF β signaling is positively regulated by HBx (27), we investigated lncRNAs associated with TGF β signaling. In the upstream promoter region of *LINC00842* (LOC643650), a lncRNA increased its expression by HBV replication, we found five putative CAGA boxes, which are target sites for the activated Smad complex (Fig. 1A). Indeed, *LINC00842* was induced by TGF β treatment in Huh7 cells and in human lung adenocarcinoma A549 cells (Fig. 1B). *LINC00842* is a 54 kb gene located between *ANXA8L1* [NC_000010.11 (46,375,590–46,391,784)] and *NPY4R* [NC_000010.11 (46,458,548–46,470,694, complement)] on human chromosome 10 (Fig. 1A). It consists of four exons and the length of transcript is a 2,872 nt. *LINC00842* is expressed in liver, pancreas, lung, intestine, skin, placenta, embryonic tissue, heart, and uterus, according to the NCBI UniGENE database (<https://www.ncbi.nlm.nih.gov/unigene>). However, there is no

information regarding its function. At 12 hours after TGF β treatment, obvious *LINC00842* induction was observed in Huh7 cells (Fig. 1C, left). In contrast, *LINC00842* was induced earlier, beginning at 2 hours after TGF β treatment in A549 cells (Fig. 1C, right). Although there were some differences in induction time points and induction levels in the other TGF β -responsive cell lines such as HepG2, MDA-MB-231, HaCaT, and MCF10A, *LINC00842* was also induced by TGF β in all of the cell lines we analyzed (Fig. 1D).

LINC00842 is involved in TGF β -mediated EMT

HBV and HCV infections, obesity, and alcohol abuse are known to be major cause of chronic liver diseases, in which hepatocytes are damaged, resulting in healing and cellular remodeling. During these processes, hepatocytes progress to fibrosis, cirrhosis, and hepatocellular carcinoma. Moreover, TGF β is required for liver fibrosis progression and EMT, which is related to liver fibrosis (28). Because *LINC00842* was induced by TGF β , we investigated whether *LINC00842*-depletion affected TGF β -mediated EMT. Huh7 and A549 cells changed their original cobblestone-like cell morphology to a spindle-like shape cell upon TGF β treatment; this is a typical phenotype of mesenchymal cells (Fig. 2A). Depletion of *LINC00842* prominently inhibited spindle-like formation in both cell lines (Fig. 2A; Supplementary Fig. S1A and S1B). In TGF β -induced EMT, localization of E-cadherin on the plasma membrane is perturbed and actin stress fiber formation is promoted as a typical mesenchymal characteristic. Depletion of *LINC00842* inhibited perturbation of E-cadherin localization and actin stress fiber formation induced by TGF β (Fig. 2B). TGF β promoted suppression of *E-cadherin* mRNA and induction of *N-cadherin* and *fibronectin* mRNA, but these TGF β -mediated EMT characters were canceled by *LINC00842* depletion (Supplementary Fig. S2). In addition, cell migration of A549 cells in the presence of TGF β was significantly inhibited by *LINC00842* depletion (Fig. 2C). Moreover, invasion assays revealed that TGF β -promoted invasive activity was inhibited by *LINC00842* depletion in A549 cells (Fig. 2D). Taken together, *LINC00842* may positively contribute to TGF β -induced EMT, and we renamed *LINC00842* as *ELIT-1*.

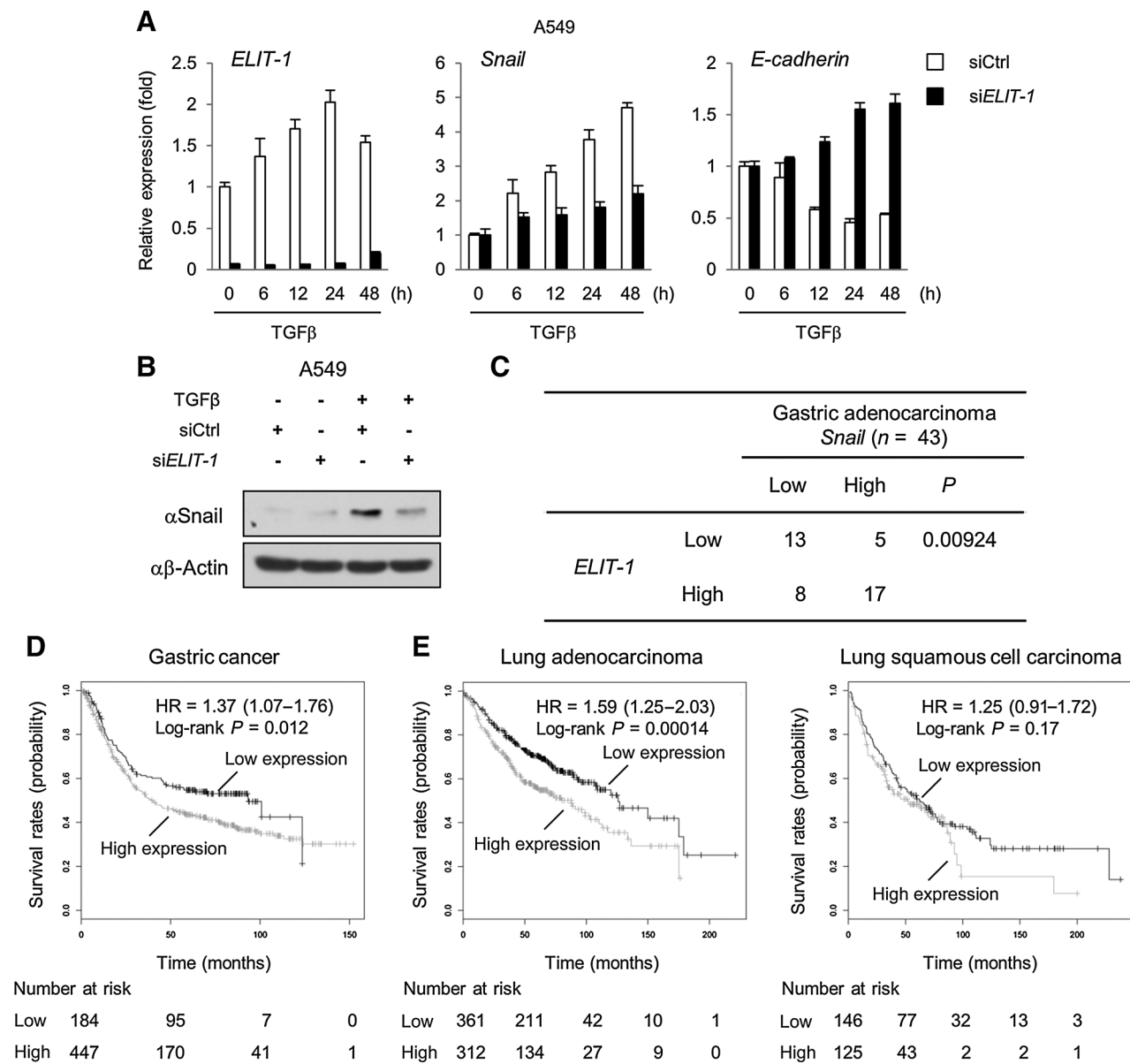
ELIT-1 participates in Snail expression

Given that Snail (Snail1/SNAI1) is a transcription factor important for the early step of EMT and early induced by TGF β via the Smad pathway (29, 30), we investigated whether expression of Snail is affected by *ELIT-1* depletion. In A549 cells, phospho-Smad3 (pSmad3) and *Snail* mRNA expression were observed at 1 hour after TGF β stimulation (Supplementary Fig. S3A and S3B). Depletion of intrinsic *ELIT-1* apparently inhibited induction of *Snail* mRNA in both A549 (Fig. 3A; Supplementary Fig. S4A) and MDA-MB-231 cells (Supplementary Fig. S4B). Moreover, *ELIT-1*

Figure 2.

LINC00842 is involved in TGF β -mediated EMT. **A** and **B**, Inhibition of TGF β -mediated EMT by *LINC00842* depletion. **A**, Huh7 and A549 cells were transfected with indicated siRNAs (see Materials and Methods). Representative photographs are shown in Supplementary Fig. S1. The ratio between the longest diameter and the shortest diameter of the cells was determined. Cells for which the ratio was more than 2.1 and 2.3 for Huh7 and A549, respectively, were regarded as "spindle-like shaped cells." **B**, Huh7 and A549 cells were transfected with indicated siRNAs (see Materials and Methods). Cells were subjected to immunofluorescent staining with anti-E-cadherin-FITC (green), Phalloidin-TRITC for F-actin (red), and DAPI for DNA (blue). Scale bars (yellow), 10 μ m. Repeat experiments were performed to confirm the results; representative photographs are shown. **C**, Inhibition of cell migration by *LINC00842* depletion. A549 cells were transfected with indicated siRNAs and subjected to scratch assay (see Materials and Methods). Representative photographs were taken (top) and migration areas were measured. Statistical analysis was performed by using *t* tests; data are shown as bar graphs of the mean \pm SD of three independent experiments (bottom). Scale bars (white), 250 μ m. **D**, Inhibition of invasive activity by *LINC00842* depletion. A549 cells were transfected with indicated siRNAs and subjected to invasion assay (see Materials and Methods). Invaded cells were stained with crystal violet and counted (right). Representative photographs are shown (left).

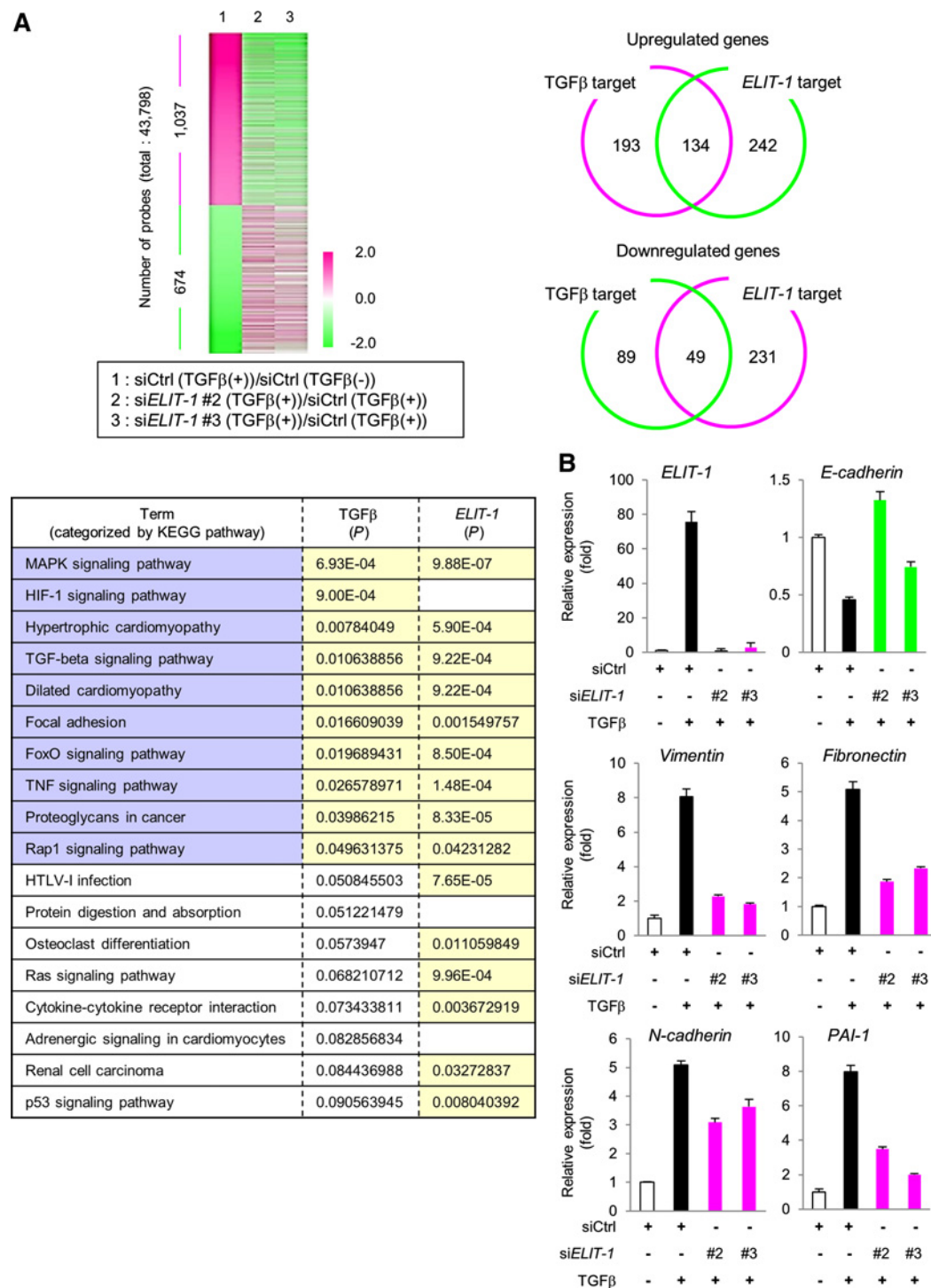
Sakai et al.

**Figure 3.**

ELIT-1 participates in Snail induction and its expression is related to prognosis in human gastric cancers and lung adenocarcinomas. **A**, Snail induction by TGFβ is suppressed by *ELIT-1* depletion. A549 cells were transfected with indicated siRNAs (see Materials and Methods) and then cells were stimulated with 5 ng/mL TGFβ for the indicated times. *ELIT-1*, *E-cad*, and *Snail* expression was analyzed by qRT-PCR. **B**, Snail protein expression was inhibited by *ELIT-1* depletion. A549 cells were transfected with indicated siRNAs for 44 hours. Then, cells were stimulated with or without 5 ng/mL TGFβ for 4 hours. Snail protein expression was analyzed by immunoblotting. Experiments were performed twice and representative data are shown. **C**, Correlation of *ELIT-1* expression with *Snail* in human gastric cancers. The search results using the GEO datasets (GSE22377) were analyzed using BioGPS (<http://biogps.org/>). Pearson χ^2 test was used to evaluate significant differences. Data are presented as low and high expression. **D** and **E**, Expression of *ELIT-1* is involved in the prognosis of patients with gastric cancers (**D**) and lung adenocarcinomas (**E**, left) but not with lung squamous cell carcinomas (**E**, right) using Kaplan-Meier survival plot, and the HR with 95% confidence intervals and log-rank *P* values were calculated.

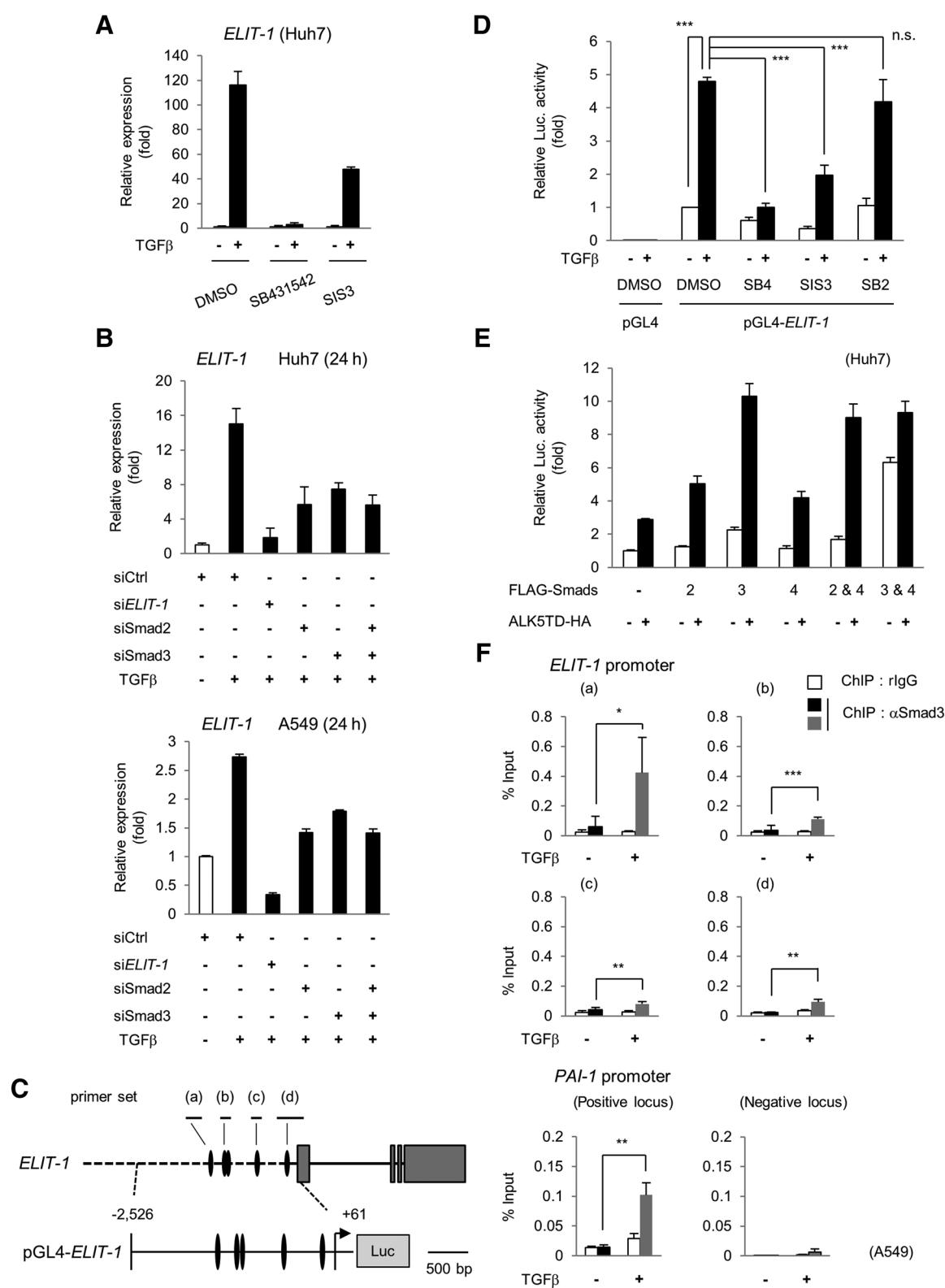
depletion also inhibited expression of Snail protein (Fig. 3B). Snail negatively regulates expression of *E-cadherin* during TGFβ-induced EMT (31). The decrease of *E-cadherin* expression during TGFβ-induced EMT was restored by *ELIT-1* depletion (Fig. 3A), suggesting that *ELIT-1* may participate in Snail-mediated *E-cadherin* suppression during EMT progression. Furthermore,

we investigated a correlation between Snail and *ELIT-1* expression using available online Gene Expression Omnibus (GEO) datasets (GSE22377) in NCBI (32). *Snail* mRNA expression is significantly correlated with *ELIT-1* expression in human gastric adenocarcinomas (Fig. 3C). These results suggested that *ELIT-1* participates in Snail expression in gastric cancers.

**Figure 4.**

ELIT-1 influences TGF β -mediated gene expression. **A**, Heatmap for genes regulated by TGF β . In the heatmap, genes upregulated (>2-fold) by TGF β are shown in magenta; genes downregulated (<0.5-fold) by TGF β are shown in green (lane 1). TGF β -responsive genes (lane 1), whose levels were reduced to <0.5-fold by *ELIT-1* depletion, are shown in green. TGF β -responsive genes (lane 1), whose levels were increased to >2-fold by *ELIT-1* depletion, are shown in magenta (lanes 2 and 3). Venn diagram shows genes commonly or individually regulated by TGF β and *ELIT-1*. Categorization of target genes is indicated in Materials and Methods and the genes' list is shown in Supplementary Tables S4–S6. Comparison of TGF β and *ELIT-1* target genes that were analyzed by KEGG Pathway analysis via DAVID using the microarray data. **B**, *ELIT-1* participates in regulation of EMT-related gene expression. Huh7 cells were transfected with indicated siRNAs (see Materials and Methods). cDNA was prepared from total RNA of these cells and subjected to qRT-PCR. Experiments were performed twice and the representative data are shown.

Sakai et al.



ELIT-1 expression is related to prognosis in gastric cancers and lung adenocarcinomas

Given that EMT participates in metastasis, fibrosis, and drug resistance, which are involved in poor prognosis of human cancers, we investigated whether there is a correlation between *ELIT-1* expression and prognosis in human gastric cancers using the available online database, Kaplan–Meier Plotter (<http://kmplot.com/analysis/>; ref. 33). In gastric cancers, the prognosis of patients with high expression of *ELIT-1* was significantly poor ($P = 0.012$; Fig. 3D). In human lung cancers (34), the prognosis of patients with adenocarcinoma with high expression of *ELIT-1* was also poor, compared with patients with low expression of *ELIT-1* ($P = 0.00014$), whereas the prognosis of squamous cell carcinomas was not correlated with expression of *ELIT-1* (Fig. 3E). These data suggest that high expression of *ELIT-1* may be involved in malignancy of some types of human cancers.

Effects of *ELIT-1* depletion on *in vitro* cell proliferation

Because TGF β inhibits epithelial cell proliferation via p15^{INK4B} induction (35), we investigated whether *ELIT-1* contributes to TGF β -mediated inhibition of cell proliferation. We confirmed TGF β -mediated growth inhibition in Huh7 cells, but *ELIT-1* depletion scarcely affected it (Supplementary Fig. S5A). Microarray data indicated that *ELIT-1* depletion inhibited TGF β -induced expression of p15^{INK4B}, while TGF β -mediated cell growth inhibition was not rescued by *ELIT* depletion (Supplementary Fig. S5B). Because it is suggested that TGF β promotes apoptosis induction via non-Smad pathway (36), we sought to investigate the exact mechanism and found that TGF β -induced apoptosis in Huh7 cells, while *ELIT-1* depletion had no effect (Supplementary Fig. S5C). These data suggest that TGF β inhibits cell proliferation in Huh7 cells through *ELIT-1*-independent apoptosis via non-Smad pathway.

ELIT-1 regulates expression of genes associated with TGF β -mediated EMT

Because *ELIT-1* was involved in TGF β -induced EMT, we further investigated the function of *ELIT-1* in TGF β -mediated gene regulation. We performed microarray analysis to analyze the effect of *ELIT-1* depletion on gene expression profiles in Huh7 cells, with or without TGF β treatment. We examined expression profiles of 1,711 probes and created a list of genes that were commonly or individually increased or suppressed by TGF β and *ELIT-1* depletion (Supplementary Tables S4–S6). Transcription of 640 genes (represented by 1,037 probes) was increased (magenta), and

transcription of 394 genes (represented by 674 probes) was decreased (green), upon TGF β treatment (Fig. 4A). A total of 20.9% of increased genes were suppressed by *ELIT-1* depletion; 12.4% of decreased genes were restored by *ELIT-1* depletion. Regarding TGF β -induced genes, 134 were *ELIT-1* target genes among 327 TGF β target genes; 193 TGF β target genes were not affected by *ELIT-1* depletion (Fig. 4A). Conversely, regarding TGF β -suppressed genes, 49 genes were *ELIT-1* target genes among 138 TGF β target genes; 89 TGF β target genes were not affected by *ELIT-1* depletion (Fig. 4A). These results suggested that *ELIT-1* is involved in regulation of a subset of TGF β target genes.

In addition, we analyzed the relationship between the TGF β signaling pathway and *ELIT-1* using the above microarray data for Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis via the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (<https://david.ncifcrf.gov/home.jsp>). This analysis suggested that *ELIT-1* is involved in the TGF β signaling pathway (Fig. 4A; Supplementary Table S7). Moreover, it suggested that pathways involved in TGF β signaling are also associated with *ELIT-1* function (light purple).

We confirmed whether *ELIT-1* contributes to regulate TGF β target gene expression using qRT-PCR (Fig. 4B; Supplementary Fig. S2). Expression of *E-cadherin*, an epithelial marker, was suppressed by TGF β ; this suppression was recovered by *ELIT-1* depletion. The expression of mesenchymal markers, such as *vimentin*, $\alpha 1(I)$ collagen (*COL1A*) and *N-cadherin*, were increased by TGF β ; this increased expression was suppressed by *ELIT-1* depletion. Moreover, the expression of other known TGF β target genes, such as *fibronectin*, *PAI-1*, *AFAP1L2*, *ANGPTL2*, *BHLHE40*, *ATF3*, and *COL4A1*, were increased by TGF β ; this increased expression was suppressed by *ELIT-1* depletion (Fig. 4B; Supplementary Fig. S6). These results suggested that *ELIT-1* is closely associated with TGF β signaling.

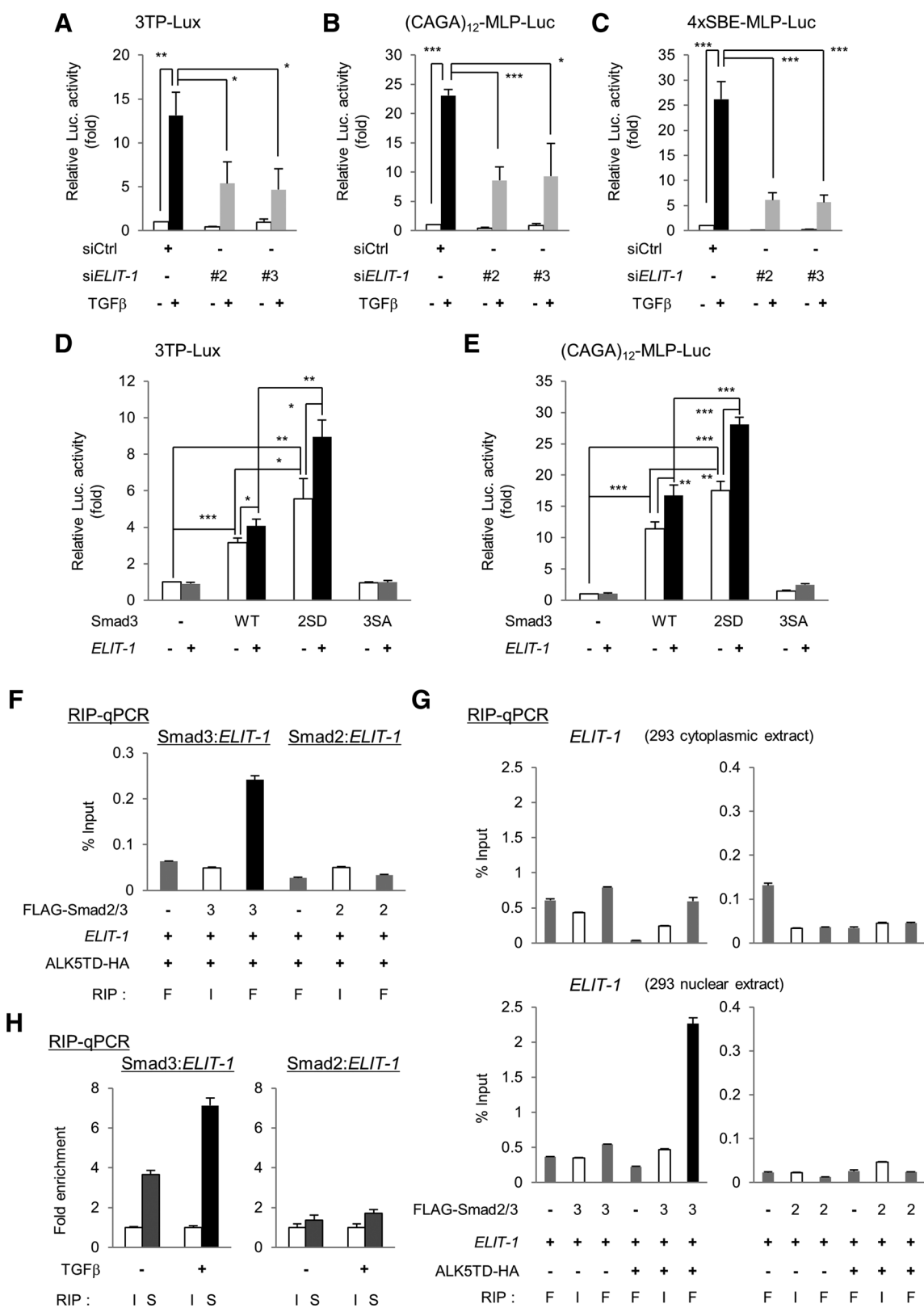
ELIT-1 is transcriptionally induced via canonical TGF β /Smad pathway

To clarify the mechanism of *ELIT-1* induction by TGF β , we examined the effects of chemical inhibitors of the TGF β pathway on *ELIT-1* induction. SB431542, a T β RI (ALK5) inhibitor, inhibited TGF β -mediated *ELIT-1* induction (Fig. 5A). Moreover, SIS3, a selective inhibitor of Smad3 but not Smad2 (37), inhibited *ELIT-1* induction partially. This suggests that *ELIT-1* induction is promoted not only via Smad3 but also via other factors such as Smad2. In fact, depletion of Smad3 as well as Smad2 inhibited *ELIT-1* induction (Fig. 5B). We found that five

Figure 5.

ELIT-1 is induced by the TGF β /Smad pathway. **A**, *ELIT-1* is induced via TGF β /Smad pathway. Huh7 cells were stimulated with or without 10 ng/mL TGF β , with DMSO, 10 μ mol/L SB431542, or 10 μ mol/L SIS3 for 48 hours. *ELIT-1* expression was measured by qRT-PCR. **B**, Smad is involved in *ELIT-1* induction. Huh7 (top) and A549 (bottom) cells were transfected with indicated siRNAs (see Materials and Methods) and then stimulated with or without 10 (Huh7) or 5 (A549) ng/mL TGF β for 24 hours. *ELIT-1* expression was measured by qRT-PCR. Data in **A** and **B**, experiments were performed twice and representative data are shown. **C**, A map of *ELIT-1* promoter and reporter construct pGL4-*ELIT-1*. Black closed ellipse indicates CAGA boxes. PCR primers used for ChIP-qPCR in the promoter region of *ELIT-1* promoter are indicated by black bars [(a)–(d)]. The results of ChIP-qPCR are shown in **F**. **D**, Huh7 cells were transfected with indicated plasmids. At 24 hours after transfection, cells were treated with DMSO, 10 μ mol/L SB431542 (SB4), 10 μ mol/L SIS3, or 10 μ mol/L SB203580 (SB2) and stimulated with or without 10 ng/mL TGF β for 48 hours. Then, cells were harvested to measure their luciferase activities. Luciferase activities are presented as the relative ratio to pGL4-*ELIT-1*-transfected and DMSO-treated sample. The data were statistically analyzed using *t* tests and show the mean \pm SD of three independent experiments. ***, $P < 0.001$ compared with or without TGF β stimulation and compared with TGF β -stimulation with inhibitors. n.s., not significant. **E**, Smad activates the *ELIT-1* promoter activity. Huh7 cells were transfected with indicated reporter plasmid and with the indicated Smad plasmids, with or without ALK5-T204D-HA (activated T β RI). At 48 hours after transfection, cells were harvested to measure their luciferase activities. Luciferase activities are presented as the relative ratio to the empty vector-transfected sample. Experiments were performed twice and representative data are shown. **F**, Smad3 binds to CAGA boxes on *ELIT-1* promoter. A549 cells were stimulated with or without 5 ng/mL TGF β for 24 hours. Chromatin fraction was prepared from harvested cells and subjected to ChIP-qPCR using anti-Smad3 antibody or normal rabbit IgG (rigG), as indicated in Materials and Methods. The data were statistically analyzed using *t* tests and show the mean \pm SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with without TGF β stimulation.

Sakai et al.



putative Smad-binding CAGA boxes were located in the upstream promoter region of *ELIT-1* (Figs. 1A and 5C), which indicated that Smads transcriptionally mediate *ELIT-1* induction by TGF β . We cloned the *ELIT-1* promoter region and constructed a luciferase reporter plasmid, pGL4-*ELIT-1* (Fig. 5C). *ELIT-1* promoter activity was also inhibited by both SB431542 and SIS3, but not by SB203580, a p38 inhibitor (Fig. 5D). Alternatively, forced expression of Smad3, but weakly Smad2, resulted in enhanced *ELIT-1* promoter activity (Fig. 5E). Coexpression of Smad4 enhanced both Smad2- and Smad3-mediated transcription. Furthermore, Smad3 bound to positive locus with CAGA box but not negative locus without CAGA box, in the *PAI-1* promoter (Fig. 5F). Moreover, we found that Smad3 bound to each CAGA box in the *ELIT-1* promoter. Interestingly, at 1.5 h after TGF β stimulation, Smad3 binding was also found in the *ELIT-1* promoter, as well as in known Smad target genes (Supplementary Fig. S7). These results suggested that *ELIT-1* is transcriptionally induced by the canonical TGF β /Smad pathway via CAGA boxes.

***ELIT-1* positively regulates promoter activities of the target genes**

To address the molecular mechanism of *ELIT-1*-mediated transcription of target genes, we investigated whether *ELIT-1* facilitated promoter activities of target genes using several reporter plasmids. Because TGF β target genes, such as *PAI-1* and *ELIT-1*, have putative CAGA boxes in their promoters, we investigated the effect of *ELIT-1* depletion on the promoter activities of 3TP-Lux, which consisted of three TREs and the CAGA box-containing *PAI-1* promoter, in A549 cells. Promoter activities of 3TP-Lux were enhanced by TGF β and significantly suppressed by *ELIT-1* depletion (Fig. 6A). Moreover, the same result was observed using (CAGA)₁₂-MLP-Luc, which was an artificial promoter containing twelve CAGA boxes (Fig. 6B), and 4xSBE-MLP-Luc, which was another artificial promoter containing four SBE (Smad-binding element) motifs (Fig. 6C). Next, we investigated the effects of forced expression of *ELIT-1* on promoter activities using Huh7 cells, which scarcely express *ELIT-1* without TGF β . The promoter activities of both 3TP-Lux and (CAGA)₁₂-MLP-Luc were enhanced in Huh7 cells by transfection with Smad3-WT or Smad3-2SD (a constitutively active Smad3), but not Smad3-3SA (an inactive Smad3; Fig. 6D and E). These upregulated promoter activities were significantly enhanced by coexpression of *ELIT-1*.

These results suggested that *ELIT-1* facilitates activities of Smad-responsive promoters in conjunction with Smad3. Furthermore, we investigated the effect of *ELIT-1* overexpression on the expression of *PAI-1*, which is a putative Smad target gene associated with EMT (38). We found that overexpression of *ELIT-1* enhanced the *PAI-1* promoter activity (Supplementary Fig. S8A), and increased its mRNA expression in Huh7 (Supplementary Fig. S8B), HepG2 (Supplementary Fig. S8C), and HaCaT (Supplementary Fig. S8D) cells. Obvious EMT-related phenotypes such as spindle-like shape formation were not observed by forced expression of *ELIT-1*. Facilitation of Smad3 pathway activation by *ELIT-1* may not be sufficient for EMT induction. However, because overexpression of *ELIT-1* enhanced *PAI-1* expression in different cell lines, *ELIT-1* alone is functionally involved in the regulation of endogenous Smad target gene expression to some extent.

***ELIT-1* binds to Smad3**

The above results suggested that *ELIT-1* helped Smad3 activate CAGA-containing promoters. To investigate how *ELIT-1* aids in Smad3 function, we investigated whether *ELIT-1* binds to Smad using an RNA immunoprecipitation assay (RIP assay). *ELIT-1*, FLAG-Smads, and ALK5-T204D-HA were transfected into HEK293 cells. Two days after transfection, cells were harvested and cell lysate was subjected to immunoprecipitation with anti-FLAG antibody or control IgG. Then, RNA was extracted from the immunoprecipitates and analyzed by qRT-PCR. We found that Smad3 bound to *ELIT-1*, but Smad2 did not (Fig. 6F). In addition, the Smad3-*ELIT-1* complex was enriched in the nuclear fraction (Fig. 6G). Moreover, we confirmed that endogenous *ELIT-1* bound to endogenous Smad3, but not Smad2, in TGF β -stimulated A549 cells (Fig. 6H). These results suggested that *ELIT-1* is involved in the TGF β /Smad pathway via binding to Smad3.

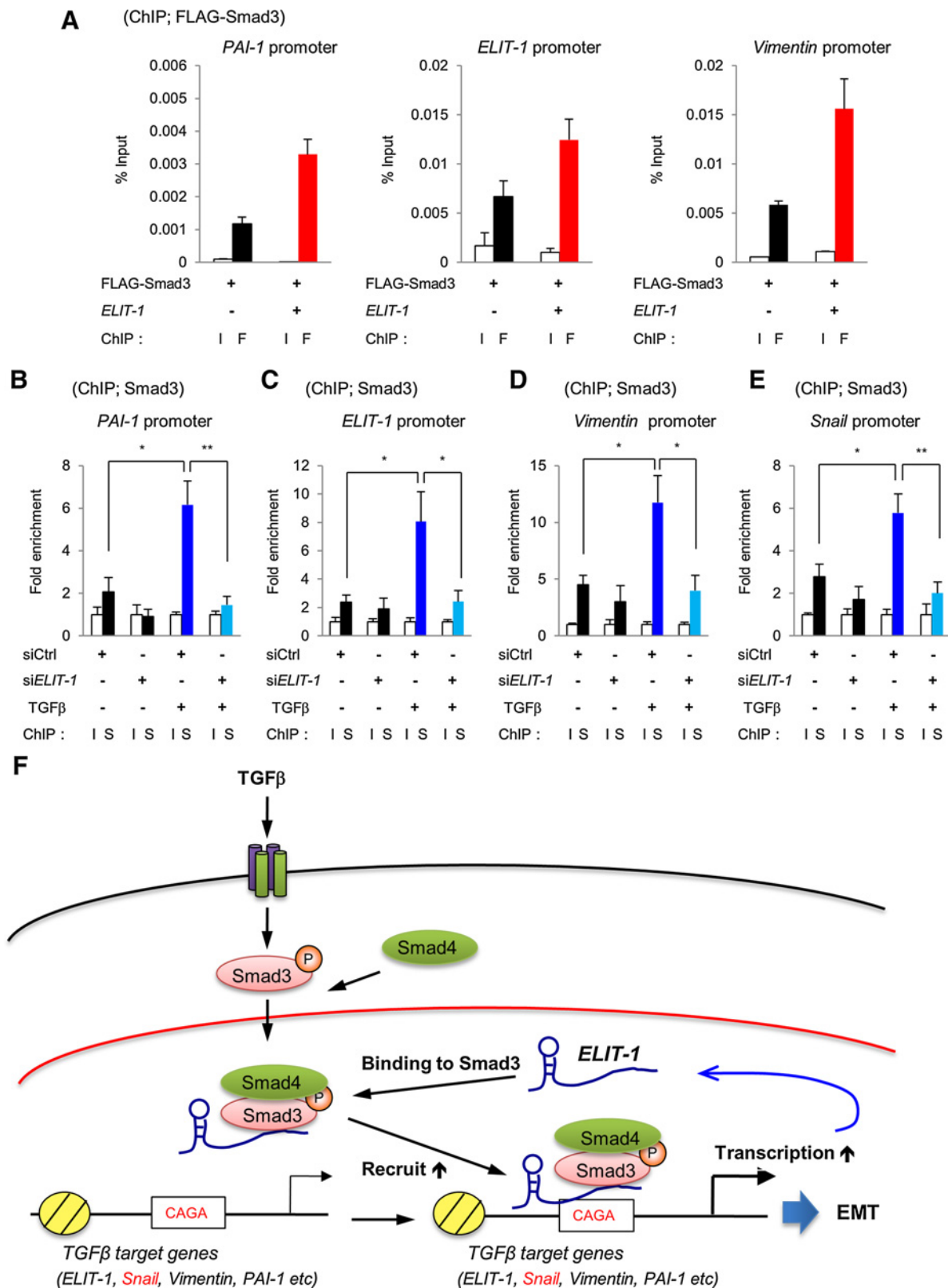
***ELIT-1* is required for recruitment of Smad3 to target gene promoters**

To clarify the functional meaning of *ELIT-1* binding to Smad3, we determine whether *ELIT-1* is involved in heterodimer formation between activated Smad3 and Smad4 using immunoprecipitation following immunoblotting (IP-IB) assays. As shown in Supplementary Fig. S9A, *ELIT-1* scarcely promoted heterodimer formation between Smad3 and Smad4. Subsequently, we investigated the effects of *ELIT-1* depletion on the subcellular

Figure 6.

ELIT-1 binds to Smad3 and positively regulates promoter activities of target genes via CAGA box. **A and B**, Depletion of *ELIT-1* suppresses TGF β -mediated activation of CAGA box-containing promoters. A549 cells were transfected with indicated siRNAs. At 24 hours after transfection, cells were transfected with the indicated reporter plasmid, 3TP-Lux (**A**) or (CAGA)₁₂-MLP-Luc (**B**) was transfected into A549 cells. At 24 hours after transfection, cells were stimulated with or without 5 ng/mL TGF β for 24 hours and then harvested to measure their luciferase activities. **C**, Depletion of *ELIT-1* suppresses TGF β -mediated activation of the SBE promoter. A549 cells were transfected with indicated siRNAs. At 24 hours after transfection, cells were transfected with a reporter plasmid, 4xSBE-MLP-Luc. At 24 hours after transfection, cells were stimulated with or without 5 ng/mL TGF β for 24 hours and then harvested to measure their luciferase activities. **D and E**, Forced expression of *ELIT-1* enhances activation of the Smad-dependent promoter. 3TP-Lux or (CAGA)₁₂-MLP-Luc was transfected into Huh7 cells with Smad3-WT or Smad3-2SD or Smad3-3SA. At 48 hours after transfection, cells were harvested to measure their luciferase activities. Luciferase activities are presented as the relative ratio to siCtrl-transfected cells without TGF β stimulation (**A–C**), or empty expression vector-transfected cells (**D and E**). Data in **A–E** were statistically analyzed using *t* test and show the mean \pm SD of three independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 compared with siCtrl without TGF β stimulation cells (**A–C**) or empty vector-transfected cells (**D and E**). **F**, *ELIT-1* binds to Smad3, but not Smad2. HEK293 cells were transfected with indicated plasmids. At 48 hours after transfection, cells were harvested for analysis of binding between *ELIT-1* and Smads. RIP assays were performed using anti-FLAG antibody (**F**) or normal mouse IgG (**I**; mIgG), as indicated in Materials and Methods. **G**, *ELIT-1* binds to Smad3 in nuclei in the presence of activated T β RI. HEK293 cells were transfected with indicated plasmids. At 48 hours after transfection, cells were harvested to prepare cytoplasmic extracts and nuclear extracts. To analyze binding between *ELIT-1* and Smads, RIP assays were performed using anti-FLAG antibody (**F**) or mIgG (**I**). **H**, Endogenous *ELIT-1* binds to endogenous Smad3. A549 cells were stimulated with or without 5 ng/mL TGF β for 24 hours. Cell lysate was prepared and subjected to RIP assays using anti-Smad3 antibody (left; **S**), anti-Smad2 antibody (right; **S**), or rTgG (**I**). Binding activities are presented as the fold enrichment for each RIP with IgG sample. Data in **F and G**, experiments were performed twice and representative data are shown.

Sakai et al.



distribution and the phosphorylation of Smad3 in A549 cells (Supplementary Fig. S9B). pSmad3 was increased in both cytoplasmic and nuclear cellular extracts by TGF β stimulation without *ELIT-1* depletion, whereas pSmad3 was not increased in either extract by TGF β stimulation combined with *ELIT-1* depletion. This is due to the reduced Smad3 levels resulting from *ELIT-1* depletion, suggesting that *ELIT-1* affects neither the subcellular distribution nor the phosphorylation of Smad3. Next, we investigated the distribution of *ELIT-1* and found that it was increased in both cytoplasm and nucleus after TGF β stimulation, with the nucleus being the predominant location (Supplementary Fig. S9C). Moreover, RIP assays indicated that Smad3-*ELIT-1* complexes, but not Smad2-*ELIT-1* complexes, were predominantly increased in the nuclei by cotransfection of constitutively active TGF β receptor (Fig. 6G), whereas *ELIT-1* was present in both cytoplasm and nucleus. These results suggest that Smad3/4 complexes activated by TGF β signaling translocate to nucleus and then *ELIT-1* binds to Smad3/4 and facilitates the target gene transcription.

To clarify how *ELIT-1* facilitates Smad3-dependent transcription, we investigated the roles of *ELIT-1* in Smad3 binding to target gene promoters using ChIP-qPCR. *PAI-1* Luc plasmid and FLAG-Smad3 were transfected into Huh7 cells, with or without *ELIT-1*. Then, specific binding of FLAG-Smad3 to endogenous and exogenous *PAI-1* promoter was quantified by ChIP-qPCR, comparing immunoprecipitation between anti-FLAG and anti-IgG (control). We found that binding of overexpressed FLAG-Smad3 to the *PAI-1* promoter was enhanced by coexpression of *ELIT-1* (Fig. 7A, left). Importantly, *ELIT-1* also enhanced FLAG-Smad3 binding to endogenous *ELIT-1* and *vimentin* promoters in the same experiment (Fig. 7A, middle and right). Similar results were reproducibly confirmed in another experiment (Supplementary Fig. S10). Next, we investigated whether endogenous *ELIT-1* participates in the recruitment of Smad3 to the target gene promoters using ChIP-qPCR. TGF β stimulation enhanced Smad3 binding to the *PAI-1* promoter, whereas *ELIT-1* depletion significantly suppressed this binding (Fig. 7B). The same results were observed in different promoters, such as *ELIT-1*, *vimentin*, and *Snail* (Fig. 7C–E); these results strongly suggested that *ELIT-1* participates in TGF β -induced recruitment of Smad3 to the target gene promoters.

Taken together, our results suggest that *ELIT-1* binds to Smad3 in nucleus upon TGF β -stimulation, promoting the transcription of Smad-target genes by facilitating Smad3 recruitment to their respective promoters as a Smad3 cofactor (Fig. 7F). Because expression of *ELIT-1* is promoted by *ELIT-1* itself via the TGF β /Smad3 pathway, *ELIT-1* may contribute to TGF β signaling through formation of an autostimulating loop.

Discussion

TGF β , a multifunctional cytokine, orchestrates an intricate signaling network to modulate tumorigenesis and cancer progression (39). When activated, TGF β receptors phosphorylate Smad2/3 and transfer the activated Smad complex into the nucleus to regulate target gene transcription. Thus, TGF β signaling through Smads is, conceptually, a simple and linear signaling pathway. Nevertheless, TGF β induces highly complex programs of gene expression responses that are extensively regulated and dependent on physiologic context (40). Increasing evidence has revealed that the diversity of the TGF β signaling response is determined by combinatorial usage of core pathway components, including ligands, receptors, Smads, and Smad-interacting proteins (e.g., transcriptional coactivators, corepressors, and Smad cofactors) (5). In this study, we identified a novel lncRNA, *ELIT-1*, which assists in Smad3-dependent transcriptional regulation. *ELIT-1* binds to Smad3, but not Smad2, and facilitates CAGA box-containing promoter activities, in conjunction with Smad3. Smad3/4 complexes activated by TGF β signaling translocate to nucleus and then *ELIT-1* binds to Smad3/4 to facilitate recruitment of the complex to Smad-binding element of the target gene promoter. Therefore, *ELIT-1* is a novel and crucial player in the canonical TGF β signaling pathway, mediated by Smad3. Smad cofactors are a group of Smad-binding transcription factors that play a crucial role in recruiting Smad complexes to the specific target gene promoters, but a lncRNA that behaves as a Smad cofactor has thus far not been identified. This is the first report that *ELIT-1* functions as a novel trans-acting lncRNA cofactor of Smads.

Moreover, *ELIT-1* contributes to execution of TGF β -mediated EMT. Depletion of *ELIT-1* inhibited TGF β -induced perturbation of E-cadherin location and actin stress fiber formation (Fig. 2B). In addition, *ELIT-1* positively controls mRNA expression of TGF β -induced mesenchymal marker genes, such as *Snail*, *vimentin*, *N-cadherin*, *fibronectin*, and *PAI-1* (Fig. 4B; Supplementary Fig. S2). The promoter region of *ELIT-1* has a CAGA box to which Smad3 binds; *ELIT-1* helps Smad3 bind to its own promoter (Figs. 5C and F and 7A). *ELIT-1* is ubiquitously expressed in human tissues, according to the NCBI UniGene database. Therefore, *ELIT-1* may be the core molecule of the canonical TGF β signaling pathway in multiple human tissues; *ELIT-1* may thus be involved in various pathologies associated with TGF β . Moreover, *ELIT-1* is transcriptionally induced by TGF β /Smad pathway, the TGF β /Smad-*ELIT-1* axis forms an autostimulatory positive feedback system, which enhances the action of TGF β . Thus, the TGF β /Smad-*ELIT-1* axis is considered to be a sophisticated system for both amplification and persistence of TGF β signaling.

Figure 7.

ELIT-1 facilitates recruitment of Smad3 to target gene promoter. **A**, Forced expression of *ELIT-1* promotes Smad3 binding to *PAI-1* promoter. Huh7 cells were transfected with *PAI-1* Luc and indicated plasmids. At 48 hours after transfection, chromatin fraction was prepared from harvested cells to analyze binding of Smad3 to indicated promoters using ChIP-qPCR with anti-FLAG antibody (F) or with IgG (I). Experiments were performed twice and representative data are shown. **B–E**, Depletion of *ELIT-1* inhibits binding of Smad3 to target gene promoters. A549 cells were transfected with indicated siRNAs for 46.5 hours. Then, cells were stimulated with or without 5 ng/mL TGF β for 1.5 hours. Chromatin fraction was prepared to analyze binding of Smad3 to indicated promoters using ChIP-qPCR with anti-Smad3 antibody (S) or IgG (I). Promoter binding activities were presented as the fold enrichment to each ChIP with IgG sample. Data in **B–E** were statistically analyzed using *t* tests and show the mean \pm SEM of five independent experiments. *, *P* < 0.05; **, *P* < 0.01 compared with siCtrl without TGF β -stimulated cells. **F**, *ELIT-1* is novel lncRNA regulator in TGF β /Smad pathway and contributes to EMT promotion. TGF β binds to the receptor and activated T β RI phosphorylates Smad2/3. Then p-Smad2/3 forms a complex with Smad4 to enter the cell nucleus. Intrinsic *ELIT-1* binds to Smad3 complex and facilitates the recruitment of Smad3 complex to the promoter region of EMT-related genes including *Snail*, *vimentin*, *PAI-1*, and *ELIT-1* itself, leading to EMT promotion by forming an autostimulating loop.

TGF β -responsive cell lines, such as Huh7, A549, HepG2, MDA-MB-231, HaCaT, and MCF10A, all of which we tested, expressed *ELIT-1*. However, expression levels of *ELIT-1* varied among these cells (Fig. 1D). Expression of *ELIT-1* was low without TGF β stimulation in Huh7 cells, was induced at 12 hours after TGF β stimulation, and continued to increase for at least 72 hours. In A549 cells, in contrast, *ELIT-1* expression was high prior to TGF β stimulation, was early induced at 2 hours after TGF β stimulation, and continued to increase for at least 24 hours (Fig. 1C). We here demonstrate that *ELIT-1* promotes its self-transcription in a TGF β /Smad pathway-dependent manner. Because of low intrinsic *ELIT-1* in Huh7 cells, *ELIT-1* scarcely contributes to its own Smad3-dependent transcription in the early phase. Later, when *ELIT-1* has been expressed and accumulates in the cells, *ELIT-1* may contribute to its self-transcription. In contrast, in A549 cells, intrinsic *ELIT-1* may effectively participate in binding of the activated Smad3 complex to CAGA boxes in the *ELIT-1* promoter, resulting in rapid induction of *ELIT-1*. The high level of intrinsic *ELIT-1* may also contribute to early expression of Snail, the key transcription factor for EMT, because *ELIT-1* depletion suppressed *Snail* expression, beginning at 1 hour after TGF β stimulation, in both A549 and MDA-MB-231 cells (Fig. 3A; Supplementary Fig. S4A and S4B). It is known that expression of E-cadherin is suppressed by Snail (31), but *ELIT-1* was considered to be involved in this regulation. Actually, expression of E-cadherin recovered when *ELIT-1* was depleted in A549 cells (Fig. 3A). Therefore, cells expressing a high level of *ELIT-1* may easily acquire mesenchymal characteristics through induction of Snail by TGF β /Smad signaling. It has been reported ATF3 and BHLHE40 were involved in EMT of breast cancer (41) and pancreatic cancer (42), respectively. Depletion of *ELIT-1* also suppressed expression of these genes, which were induced by 72 hours of TGF β stimulation (Supplementary Fig. S6). We suspect that intrinsic *ELIT-1* and accumulated *ELIT-1* both contribute to the regulation of EMT-related genes, such as Snail, ATF3, and BHLHE40, via the TGF β /Smad3-*ELIT-1* axis; the positive-feedback-loop may orchestrate persistent EMT progression.

Some RNAs, such as miRNA and lncRNA, are involved in the TGF β /Smad pathway. These RNAs are classified into three types by their functions, which include regulation of target genes in the TGF β /Smad pathway. First, some RNAs regulate expression of signal transduction components, such as T β RI, T β RII, Smad2, and Smad3; these include miR-21, miR-148a, miR-99s, and *MEG3* (16, 43). *MALAT1* and lncRNA-ATB, involved in TGF β 1 and TGF β 2 production, respectively (44, 45). Second, some RNAs act as effector molecules; these include lncRNA-ATB, lnc-RoR, *MALAT1*, and *H19* are examples of miRNA sponges that act to prevent repression of ZEB expression by miRNAs, such as miR-200s and miR-205 (17). *HIT*, *H19*, *UCA1*, and *HOTAIR*, are known to repress E-cadherin expression and contribute to EMT promotion (17). Third, some RNAs act as functional RNA, which binds to Smad and regulates TGF β /Smad signaling, includes *ELIT-1* (Fig. 7F) and, possibly, *DEANR1* and *NORAD*. It has been reported that the lncRNA *DEANR1* promotes endoderm differentiation by transcriptional activation of *FOXA2* (46). *DEANR1* gene (*LINC00261*) is located downstream of *FOXA2* gene; *DEANR1* transcript binds to Smad2/3 and this complex associates with *FOXA2* promoter to facilitate transcription *cis*-acting. *ELIT-1* and *DEANR1* bind to Smad to facilitate the binding of the Smad complex to target gene promoters. However, there are important differences between *DEANR1* and *ELIT-1*: *DEANR1* is a definitive

endoderm-specific gene that is involved in endoderm differentiation; *DEANR1*-Smad2/3 complexes exert the limited function as a *cis*-acting lncRNA to promote expression of *FOXA2* gene nearby *DEANR1* gene. Conversely, *ELIT-1* binds to Smad3 and recruits Smad3 to Smad-binding motifs, such as the CAGA box, in multiple TGF β target genes as a *trans*-acting lncRNA. Thus, *ELIT-1* facilitates transcription of various TGF β target genes as a Smad3 cofactor responding to TGF β stimulation. Moreover, *ELIT-1* itself is transcriptionally activated by the TGF β /Smad3-*ELIT-1* axis and forms a positive-feedback-loop to accelerate TGF β signaling. A very recent study showed that *NORAD*, which had been reported as a PUMILIO-binding RNA involved in cancer metastasis, is involved in nuclear translocation of Smad3 (47). *NORAD* does not play as a Smad cofactor, whereas it binds to Smad3. Therefore *ELIT-1*, *DEANR1*, and *NORAD* comprise a third type of functional RNA, which binds to Smad3 and regulates TGF β /Smad signaling. Here we identified a novel lncRNA *ELIT-1* that is a *trans*-acting lncRNA that binds with Smad3 and behaves as a Smad3 cofactor. Notably, the TGF β /Smad-*ELIT-1* axis is considered very important for facilitating TGF β signaling.

Our results suggest that Smad3/4 complexes activated by TGF β signaling translocate to nucleus and then *ELIT-1* binds to Smad3/4 to facilitate recruitment of the complex to Smad-binding elements of the target gene promoter. Further analysis is required to clarify the molecular mechanism(s) how *ELIT-1* participates in the recruitment of Smad3 complex to the target gene promoters. There are at least three possibilities to address the question, as follows: first, *ELIT-1* may change the conformation of Smad complexes and makes it easier to bind to the CAGA motif in the target gene promoter; second, *ELIT-1* may enhance stability of Smad complexes in the nucleus; third, the intramolecular CAGA sequence in the RNA molecule of *ELIT-1* may promote recruitment of Smad complexes to the CAGA motif in the target gene promoter. In addition, it has been reported that several lncRNAs exert their function via molecular interactions with RNA-binding proteins (RBP; ref. 48). RBPs contribute to Smad-mediated transcriptional activation (49, 50). Our current data strongly suggests that *ELIT-1* promoted TGF β signaling via interaction with Smad3, whereas another protein, possibly an RBP, may participate in *ELIT-1*-mediated facilitation of TGF β /Smad3 signaling. To clarify this, further investigations may be required.

Pathway analysis data indicated that ten pathways, including the TGF β signaling pathway itself, were significantly upregulated by TGF β stimulation (Fig. 4A). As expected, the TGF β signaling pathway was a target of both TGF β and *ELIT-1*. Crosstalk has been reported between the TGF β signaling pathway and the following pathways: MAPK, TNF, and FoxO (51–53). Associations of TGF β with hypertrophic cardiomyopathy, dilated cardiomyopathy, proteoglycans in cancer, focal adhesion, HIF1 signaling pathway, and Rap1 signaling have also been reported (54–59). Therefore, the results of pathway analysis of TGF β targets are appropriate, as these are similar to previous reports. Nine of ten pathways, except the HIF1 signaling pathway, were significantly upregulated by *ELIT-1*, suggesting that *ELIT-1* function is closely related with TGF β signaling. In addition, in a comparison of individual gene expression data, there were many target genes common to both TGF β and *ELIT-1*. These results suggest *ELIT-1* functions as a Smad cofactor. However, different target genes were also found. The following possibilities may contribute to this result: TGF β target genes induced by TGF β are mediated through the TGF β /Smad

pathway or non-Smad pathways. Because this experiment was a result of 48 hours of stimulation, secondary effects may be present. Because we could not rule out the possibility that *ELIT-1* may have functions other than Smad3 recruiting, further studies may be required.

Using Kaplan–Meier Plotter, we found that there is a correlation between *ELIT-1* expression and prognosis in gastric cancers and lung adenocarcinomas (Fig. 3D and E). The prognosis of both patients with gastric cancer and lung adenocarcinomas with high expression of *ELIT-1* were poor, compared with patients with low expression of *ELIT-1*. Here, we showed that *ELIT-1* depletion suppressed EMT, which is closely associated with tumor malignancy, including metastasis and chemoresistance. We also showed that *ELIT-1* depletion suppressed cell migration and invasion. Therefore, poor prognosis of lung and gastric cancers with high expression of *ELIT-1* may be caused by accelerated malignancy via *ELIT-1*-mediated EMT. Further studies are required; however, *ELIT-1* may be useful to serve as a prognostic marker (i.e., a novel cancer-associated lncRNA). *ELIT-1* may be also a novel therapeutic target for malignant cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Sakai, T. Ohhata, K. Miyazawa, M. Kitagawa
Development of methodology: C. Uchida, T. Suzuki

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Sakai, T. Ohhata, T. Aoshima
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Sakai, T. Ohhata, K. Kitagawa
Writing, review, and/or revision of the manuscript: S. Sakai, T. Ohhata, K. Kitagawa, C. Uchida, H. Niida, T. Suzuki, Y. Inoue, K. Miyazawa, M. Kitagawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Suzuki, Y. Inoue, K. Miyazawa
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Long Noncoding RNA *ELIT-1* Acts as a Smad3 Cofactor to Facilitate TGF β /Smad Signaling and Promote Epithelial–Mesenchymal Transition

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