

Identification and characterization of transforming growth factor beta-induced in circulating tumor cell subline from pancreatic cancer cell line

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Distant metastasis to liver, lung, brain, or bone occurs by circulating tumor cells (CTC). We hypothesized that a subset of CTC had features that are more malignant than tumor cells at the primary site. We established a highly malignant cell line, Panc-1-CTC, derived from the human pancreatic cancer cell line Panc-1 using an in vivo selection method. Panc-1-CTC cells showed greater migratory and invasive abilities than its parent cell line in vitro. In addition, Panc-1-CTC cells had a higher tumor-forming ability than parent cells in vivo. To examine whether a difference in malignant phenotypes exists between Panc-1-CTC cells and parent cells, we carried out comprehensive gene expression array analysis. As a result, Panc-1-CTC significantly expressed transforming growth factor beta-induced (*TGFBI*), an extracellular matrix protein, more abundantly than did parent cells. *TGFBI* is considered to regulate cell adhesion, but its functions remain unclear. In the present study, knockdown of *TGFBI* reduced cell migration and invasion abilities, whereas overexpression of *TGFBI* increased both abilities. Moreover, elevated expression of *TGFBI* was associated with poor prognosis in patients with pancreatic cancer.

KEYWORDS

circulating tumor cell (CTC), extracellular matrix (ECM), in vivo selection, pancreatic ductal adenocarcinoma (PDAC), transforming growth factor beta-induced (*TGFBI*)

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-related mortality in developed countries and has an extremely poor prognosis even if curative surgery is conducted.¹ Most patients with pancreatic cancer die of distant metastasis, most commonly liver metastasis.² However, metastasis is not a straightforward event for each tumor cell because of the complex processes required. The first step for distant metastasis is cell growth at the primary site. After that, these tumor cells promote

invasion into the ECM and intravasate into the blood vessel or lymph duct. Then, CTC, the tumor cells that intravasate into the blood vessel, must survive the stressful environment of the bloodstream. Most CTC are killed by anoikis, a type of apoptosis, in the bloodstream before achieving metastasis.³ CTC that evade anoikis can arrive at distant organs, leading to metastasis. CTC also form clusters of various sizes (CTC clusters) and contribute to metastasis by causing embolization in capillary vessels.⁴⁻⁶

The number of CTC is associated with poor prognosis in breast, colorectal, prostate, and pancreatic cancer.⁷⁻¹² However, capturing living CTC from cancer patients and analyzing their biological features is difficult as a result of technical limitations.¹³ Although the establishment of a CTC cell line from patients with cancer has been

Abbreviations: CTC, circulating tumor cell; PDAC, pancreatic ductal adenocarcinoma; RBC, red blood cell; *TGFBI*, transforming growth factor beta-induced.

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reported in colon cancer,¹⁴ the biological analysis of living CTC from PDAC has only been reported in a mouse research model.¹⁵

Transforming growth factor beta-induced protein (TGFB1), also known as β IG-H3, kerato-epithelin, and RGD-CAP, is a secretory ECM protein. TGFB1 binds to various types of collagen, such as fibronectin, laminin, and secreted protein acidic and cysteine rich (SPARC). TGFB1 has several motifs for cell adhesion within the fasciclin-like (FAS1) domain, an extracellular domain comprising approximately 140 amino acid residues.^{16,17} The role of TGFB1 in cancer has two aspects as a cell adhesive protein¹⁸⁻²⁰ and as an anti-cell adhesive protein.^{21,22} In addition, DNA hypermethylation of TGFB1 has been observed in several tumor types, and TGFB1 is thought to be a tumor suppressor protein for lung and ovarian cancer.^{23,24} In contrast, TGFB1 is considered an oncogene for colon cancer, esophagus squamous carcinoma, melanoma and renal cancer.²⁵⁻²⁹ Mechanisms underlying its bimodality have not been fully understood so far.²⁴ Additionally, the role of TGFB1 has not been clarified in PDAC.

In the present study, we hypothesized that CTC had greater malignant potential than tumor cells at the primary site and that analyzing their biological features would be useful for elucidating metastasis. Therefore, we tried to capture CTC using a mouse xenograft model with the PDAC cell line Panc-1, and we then established a CTC cell line from the blood of mice bearing s.c. tumors. We named the new CTC cell line "Panc-1-CTC" as it was derived from Panc-1-parent (Panc-1-P) cells. Compared to Panc-1-P cells, Panc-1-CTC cells show more malignant phenotypes, such as strong migration and invasion abilities. In addition, by expression array analysis, we identified *TGFB1* as a key gene for the acquisition of malignant phenotypes, and the expression of TGFB1 was associated with poor prognosis in patients with PDAC. Taken together, these findings provide a novel role for TGFB1 as a therapeutic target in PDAC.

2 | MATERIALS AND METHODS

2.1 | Cell culture, primary tissue samples from patients with PDAC, and immunohistochemical analysis

Human pancreatic cancer cell lines Panc-1, CFPAC-1, and CAPAN-1 were purchased from ATCC (Manassas, VA, USA). All cells were grown in DMEM supplemented with 10% FBS in a humidified atmosphere with 5% CO₂ at 37°C. In the present study, Panc-1 was authenticated by short tandem repeat analysis. Other cell lines were authenticated through monitoring of cell morphology. TGF- β was purchased from R&D Systems (Minneapolis, MN, USA). SD-208 (TGF- β type I receptor inhibitor) was purchased from Fujifilm (Tokyo, Japan).

Human pancreatic cancer tissue samples (n = 75) were obtained by surgical resection at Tokyo Medical and Dental University Medical Hospital. After approval by the local ethics committee of the Medical Research Institute and Faculty of Medicine, Tokyo Medical

and Dental University, formal written consent was obtained from all patients. Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded tissue sections with an automated immunostainer (Benchmark XT; Ventana Medical Systems, Tucson, AZ, USA) using anti-TGFB1 antibody (10188-1-AP; Proteintech, Rosemont, IL, USA). Slides were analyzed under a light microscope by two pathologists. Expression of TGFB1 protein was graded as either high (immunopositivity similar to Panc-1-CTC s.c. tumors) or low (no staining or weak immunopositivity similar to Panc-1-P s.c. tumors).

2.2 | Short tandem repeat analysis

Short tandem repeat analysis was carried out using an AuthenticFiler PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.3 | In vivo selection

SCID mice were purchased from Charles River Laboratories (Yokohama, Japan). First, a total of 5×10^6 Panc-1-P cells were injected s.c. into SCID mice. Two months after inoculation, approximately 1 mL blood was obtained from the mouse by cardiac puncture. The blood contained a large number of mouse RBC and was processed with RBC lysis buffer (BD Pharm Lyse; BD Biosciences, East Rutherford, NJ, USA) according to the manufacturer's instructions. After centrifugation, the pellet was dissolved in fresh culture medium and plated into dishes. Daily washes with fresh medium were carried out for several days to remove fragmented RBC. Then, a tiny tumor colony was obtained as a CTC cell line. All experimental protocols carried out on the mice were approved by the Tokyo Medical and Dental University Animal Care and Use Committee, and experiments were conducted under the institutional animal ethics guidelines.

2.4 | Cell growth, migration and invasion assays

Number of viable cells at various time points after transfection was assessed by a colorimetric water-soluble tetrazolium salt (WST-8) assay as described elsewhere.³⁰ Transwell migration and invasion assays were carried out in 24-well modified chambers without (migration) or with (invasion) Matrigel precoating (BD BioCoat; BD Biosciences), as described elsewhere.³⁰

2.5 | Tumorsphere formation assay

BD matrigel basement membrane (50 μ L) was added to 96-well plates and incubated for 30 minutes at 37°C. Medium (DMEM/F12) for tumorsphere formation (20 ng/mL human epidermal growth factor [hEGF], 10 ng/mL basic fibroblast growth factor [bFGF], 5 μ g/mL insulin, 0.4% FBS, 2% B27, 2% matrigel) was prepared. After counting cells, 100 μ L of seed cells was suspended in tumorsphere medium into each well (2×10^3 cells). After 10 days, the number of tumorspheres (>200 μ m) was counted by Image J software.

2.6 | In vivo tumor growth assay, RNA extraction and histological analysis

Panc-1-P and Panc-1-CTC cells were injected s.c. (5×10^6 cells in 100 μ L with Matrigel) into the left abdominal wall of SCID mice. Tumor sizes were measured every week after inoculation. Tumor size was calculated at the indicated times after injection using the formula tumor size (mm^3) = [(length) \times (width)²]/2. Mice were killed on day 28, and s.c. tumors were assessed for volume and immediately processed for either RNA extraction or section preparation (ie, fixed with formalin and embedded in paraffin). For histology, 5- μ m sections were cut from paraffin blocks, stained with H&E stain and immunostained for TGFBI using an automated immunostainer with heat-induced epitope retrieval and anti-TGFBI antibody.

2.7 | Quantitative reverse transcription-polymerase chain reaction

qRT-PCR was carried out using a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) with the KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions.

Primer forward sequence (*TGFBI*), TCATGGCCACAAATGGCGTGGT; primer reverse sequence (*TGFBI*), AGCCCTGGAAAACGCTGATGCT.

Gene expression values were evaluated as ratios based on the differences in cycle threshold values between the genes of interest and an internal reference gene (*GAPDH*), which served as a normalization factor for the amount of RNA isolated from the s.c. tumors.

Primer forward sequence (*GAPDH*), CGACCACTTTGTCAGCTCA; primer reverse sequence (*GAPDH*), AGGGTCTACATGGCAACTG.

2.8 | Plasmid construction

Full-length human *TGFBI* cDNA was obtained by PCR of Panc-1-P cDNA.

Primer forward sequence (*TGFBI*), TTAAGCTTATGGCGCTCTTCGTGCGGCT; primer reverse sequence (*TGFBI*), TTCTCGAGATGCTTCCTCTTAATA.

The plasmid for *TGFBI* expression (pCMV-3Tag3A-*TGFBI*) was obtained by cloning the full coding sequences into the vector pCMV-3Tag3A (Agilent Technologies, Santa Clara, CA, USA).

2.9 | Western blot analysis

Anti-TGFBI (#5601), anti-AKT (#9272S), anti-phospho-AKT (#9271S), anti-FAK (#9330), anti-phospho-FAK (#3283), and anti-ZEB1 (#3396S) antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti- β -actin (A5441), anti-FLAG M2 (F1804) (Sigma-Aldrich, St Louis, MO, USA), anti-E-cadherin (#610181), anti-N-cadherin (#610920) (BD Biosciences), and anti-Vimentin (V-9) (Thermo Fisher Scientific) were purchased from the indicated

companies. Western blotting analyses were carried out as described elsewhere.³⁰

2.10 | Preparation of conditioned medium

Cells were seeded on a 75 cm^3 flask and cultured with growth medium until they reached the target confluence. The cells were washed with PBS and incubated in 15 mL DMEM without FBS for 24 hours. The conditioned medium was centrifuged at $6000 \times g$ for 10 minutes using Amicon Ultra filters (Merck Millipore, Billerica, MA, USA).

2.11 | Gene expression array analysis and gene set enrichment analysis

Analysis of gene expression profiles was carried out using SurePrint G3 Gene Expression Microarrays Ver. 3 (Agilent Technologies) according to the manufacturer's instructions. The raw data were analyzed with GeneSpring GX14.9 software (Agilent Technologies). Gene set enrichment analysis (GSEA) was carried out with expression array data normalized by GeneSpring GX14.9.

2.12 | Transfection of siRNA

si*TGFBI* (ON-TARGETplus Human *TGFBI* siRNA SMARTpool; L-019370-00-0005, J-019370-06-0002 and J-019370-08-0002) and control nonspecific siRNA (ON-TARGETplus Non-targeting Control Pool; D-001810-10-05) were purchased from Dharmacon (Lafayette, CO, USA). siRNA was transfected into cells by Lipofectamine RNAi-MAX (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.13 | Cell adhesion assay

For coating, 96-well culture plates (Corning Incorporated, Corning, NY, USA) were preincubated at 4°C for 18 hours with 30 μ L PBS with or without recombinant *TGFBI* (10 μ g/mL). A type I collagen-precoated 15-cm dish (Iwaki, Tokyo, Japan) was used as a positive control. After removal of the medium, the plates were blocked with PBS containing 0.2% BSA for 2 hours at 37°C. Cells were treated with trypsin and suspended in FBS-free medium at a density of 2×10^5 cells/mL, after which 100 μ L cell suspension was added to each well. Following incubation for 24 hours at 37°C, unattached cells were removed by rinsing twice with PBS. The number of adherent cells was determined by counting the cells under a microscope at 100 \times magnification.

2.14 | Statistical analysis

Statistical analysis was carried out by StatView 5.0 (SAS, Cary, NC, USA). Differences between the groups were tested with the Mann-Whitney *U* test. Chi-squared tests were used to compare the proportions of categorical factors between the groups. Analysis of variance (ANOVA) was used to compare the difference between the cell

growth assay and the *in vivo* tumor growth assay. With Kaplan-Meier curves, differences between the groups were tested with the log-rank test. Multivariate analysis for predicting overall survival and disease-free survival in patients with PDAC was carried out by Cox regression analysis.

2.15 | Data deposition

The microarray data from this publication have been submitted to the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) and assigned the identifier "GSE118556".

3 | RESULTS

3.1 | Establishment of a highly malignant cell line derived from Panc-1 cells

First, we tried to establish highly malignant cells from CTC using an *in vivo* selection method with the pancreatic cancer cell line Panc-1 (Figure 1A). After the establishment of Panc-1-CTC cells, we carried out a short tandem repeat (STR) analysis, which showed that Panc-1-CTC cells were derived from Panc-1 parent (Panc-1-P) cells and not from other cell types (data not shown). To confirm whether Panc-1-CTC cells gained a malignant phenotype compared to Panc-1-P cells, we carried out a cell growth assay and Boyden chamber migration and invasion assays (Figure 1B,D,E). Panc-1-CTC cells showed increased migration and invasion, whereas cell growth was slower than that in parent cells (Figure 1B). With respect to epithelial-mesenchymal transition (EMT) phenotype, ZEB1 and N-cadherin expression was upregulated in Panc-1-CTC cells, whereas E-cadherin and Vimentin expression were almost the same (Figure 1C). As the expression of E-cadherin and Vimentin did not change in Panc-1-CTC cells, it was thought that Panc-1-CTC cells did not acquire an EMT phenotype through *in vivo* selection. *In vivo* tumorigenicity was increased in Panc-1-CTC cells compared with that in Panc-1-P cells (Figure 2A). Weights of *s.c.* tumors derived from Panc-1-CTC cells were also significantly greater than those derived from Panc-1-P cells (Figure 2B). These results indicate that Panc-1-CTC cells acquired a malignant phenotype through *in vivo* selection.

3.2 | Expression of TGFBI in Panc-1-CTC cells was higher than that in Panc-1-P cells

To identify differences in the gene expression profiles between Panc-1-P and Panc-1-CTC cells, we carried out comprehensive gene expression array analysis with total RNA from Panc-1-P and Panc-1-CTC cells (Table S1). Figure 2C shows the top 10 upregulated genes in Panc-1-CTC cells *in vitro* compared with the respective levels in Panc-1-P cells. Only *TGFBI* was dramatically upregulated in *in vivo* samples of Panc-1-CTC cells. The increase in *TGFBI* expression was validated in *in vitro* samples by qRT-PCR and western blot analysis (Figure 2D,E). In addition, we also checked the expression of *TGFBI* in xenograft samples derived

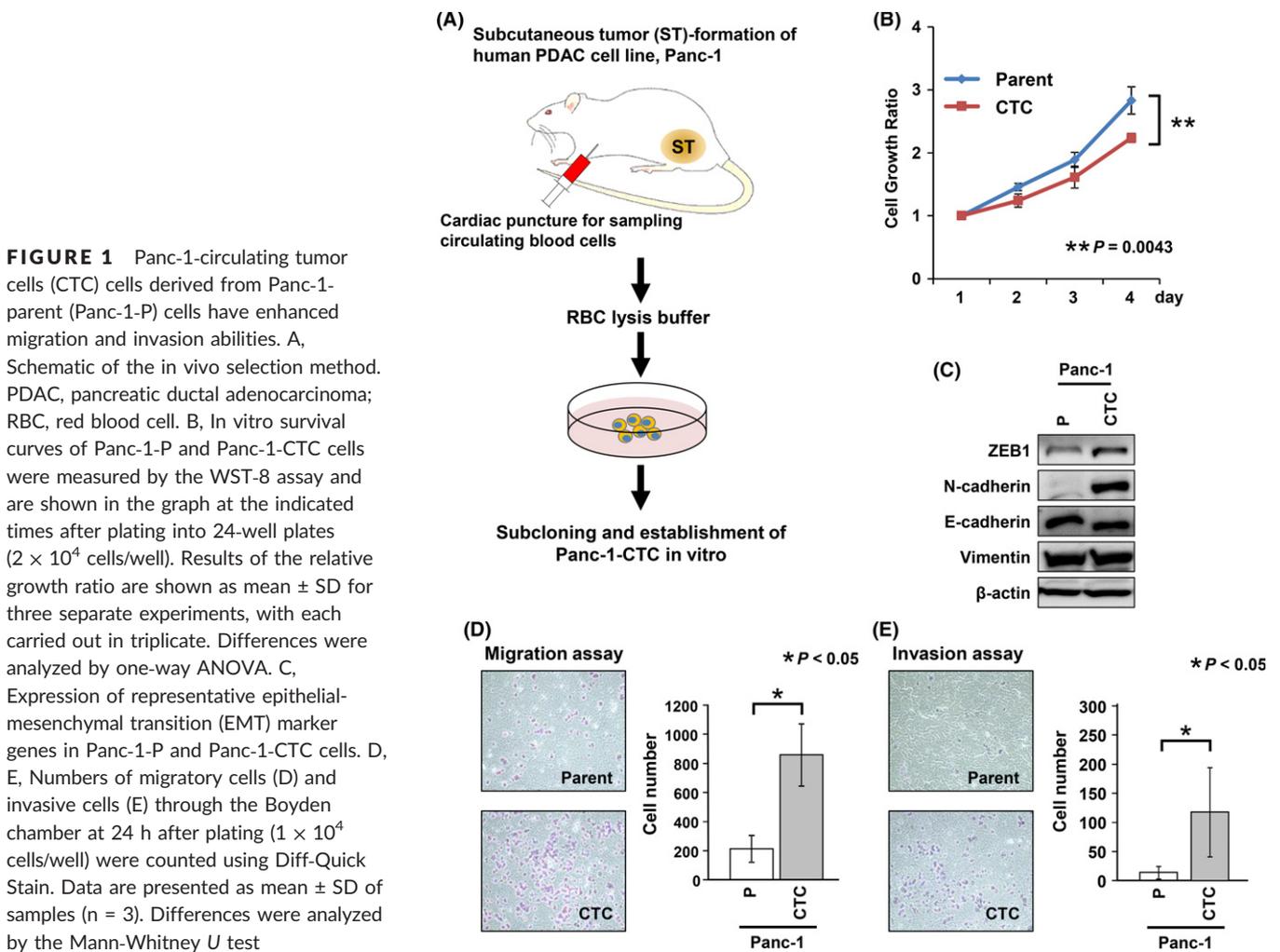
from Panc-1-P and Panc-1-CTC cells by qRT-PCR and immunohistochemical analysis (Figure 2F,G). As the expression of *TGFBI* is regulated by TGF- β stimulation, we evaluated whether TGF- β could induce the expression of *TGFBI* in Panc-1-P and Panc-1-CTC cells. As a result, in both cell lines, the expression of *TGFBI* was increased by TGF- β in a time-dependent method and, interestingly, this inductive effect was higher in Panc-1-CTC cells than in Panc-1-P cells (Figure S1). In addition, SD-208, a TGF- β type I receptor inhibitor, could eliminate the inductive effect of TGF- β , resulting in no change of *TGFBI* expression in both cell lines (Figure S1). Thus, these results suggest that *TGFBI* might be regulated by the TGF- β signaling pathway.

3.3 | Suppression of TGFBI decreased cell migration and invasion abilities

Suppression of *TGFBI* by siRNA in Panc-1-CTC cells was confirmed by qRT-PCR and western blot analysis (Figures 3A,B and S2A) and had no significant effect on cell growth (Figures 3C and S2B), whereas the migration and invasion abilities of Panc-1-CTC cells were inhibited by the suppression of *TGFBI* (Figures 3D,E and S2C, D). Moreover, we checked the expression of *TGFBI* and the mutation status of *Smad4* in several PDAC cell lines (Figure 3F, Table S2). There was no correlation between *TGFBI* expression and *Smad4* mutations. We then carried out knockdown experiments with CAPAN-1 and CFPAC-1, which have high *TGFBI* expression, and confirmed the expression of *TGFBI* by qRT-PCR and western blot analysis (Figure 4A,B). Next, we analyzed the growth, migration and invasion abilities of cells with *TGFBI* knockdown. As a result, cell growth of CAPAN-1 cells was not affected by suppression of *TGFBI*, whereas cell growth of CFPAC-1 cells was decreased by suppression of *TGFBI* (Figure 4C). Suppression of *TGFBI* inhibited the migration and invasion abilities of both cell lines (Figure 4D,E). These results suggested that *TGFBI* might regulate cell migration and invasion in pancreatic cancer.

3.4 | Effect of TGFBI on Panc-1 parent cell migration and invasion

Panc-1-P cells were transfected with empty vector (Negative control) or *TGFBI* vector for the evaluation of the effect of *TGFBI* overexpression. First, we established a cell line that stably expresses *TGFBI* in Panc-1-P cells. mRNA and protein expression levels of *TGFBI* were confirmed by qRT-PCR and western blotting (Figure 5A,B), and *in vitro* cell growth in these transduced cell lines was almost the same (Figure 5C). In addition, we observed that *TGFBI* was secreted into the culture medium (Figure 5B). In contrast, *TGFBI*-overexpressing Panc-1-P cells enhanced anchorage-independent cell growth and increased migration and invasion abilities compared to cells transfected with empty vector (Figure 5D,E,F). These findings indicated that *TGFBI* might regulate stem cell features, cell migration and invasion, but not cell growth. Then, we carried out GSEA with expression array data. As a result, GSEA showed upregulation of stemness-



related genes (GO term: GO_SOMATIC_STEM_CELL_POPULATION_MAINTENANCE) in Panc-1-CTC cells (Figure S3).

3.5 | Expression of TGFBI was associated with poor prognosis in patients with PDAC

To determine the clinicopathological significance of TGFBI upregulation in patients with PDAC, we conducted an immunohistochemical analysis with anti-TGFBI antibody in 75 PDAC cases. Expression of TGFBI was mainly located in the ECM, and strong immunoreactivity for TGFBI was found in 19 cases, which coincided with the staining pattern of Panc-1-CTC s.c. tumors, whereas weak or no immunoreactivity for TGFBI was found in 56 cases (Figure 6A). TGFBI protein expression in each tumor was not associated with any clinicopathological characteristics (Table S3). In Kaplan-Meier survival curves, univariate analyses of overall and disease-free survival with log-rank tests showed a significant association between strong TGFBI immunoreactivity and poor patient survival ($P < 0.0001$ and $P = 0.0014$, respectively) (Figure 6B,C). In the Cox proportional hazard regression model, univariate analyses showed that TGFBI immunoreactivity, UICC stage, and adjuvant chemotherapy were significantly associated with overall survival (Table S4). Moreover,

multivariate analysis using a stepwise Cox regression procedure showed that TGFBI immunoreactivity was an independent predictive factor for overall survival. These results suggested that TGFBI could be a novel biomarker for predicting the prognosis of patients with PDAC.

4 | DISCUSSION

Most CTC are killed by anoikis, a type of apoptosis, in the bloodstream before achieving metastasis; therefore, capturing and analyzing surviving CTC are challenging and meaningful for identifying the characteristics of CTC. There are few reports about the establishment of a human CTC cell line or comprehensive genomic analysis of CTC because of the technical difficulty in isolating CTC.³¹ In the present study, we applied an in vivo selection method modified from a previously reported procedure as a preclinical approach for the establishment of CTC cells from Panc-1-P cells.³² This CTC-capturing method was simple, did not require a special machine and was not restricted by tumor cell size or cell surface markers such as cytokeratin, CD45, and EpCAM.³² Although the establishment of a CTC cell line was not an easy task, we finally obtained one cell line termed

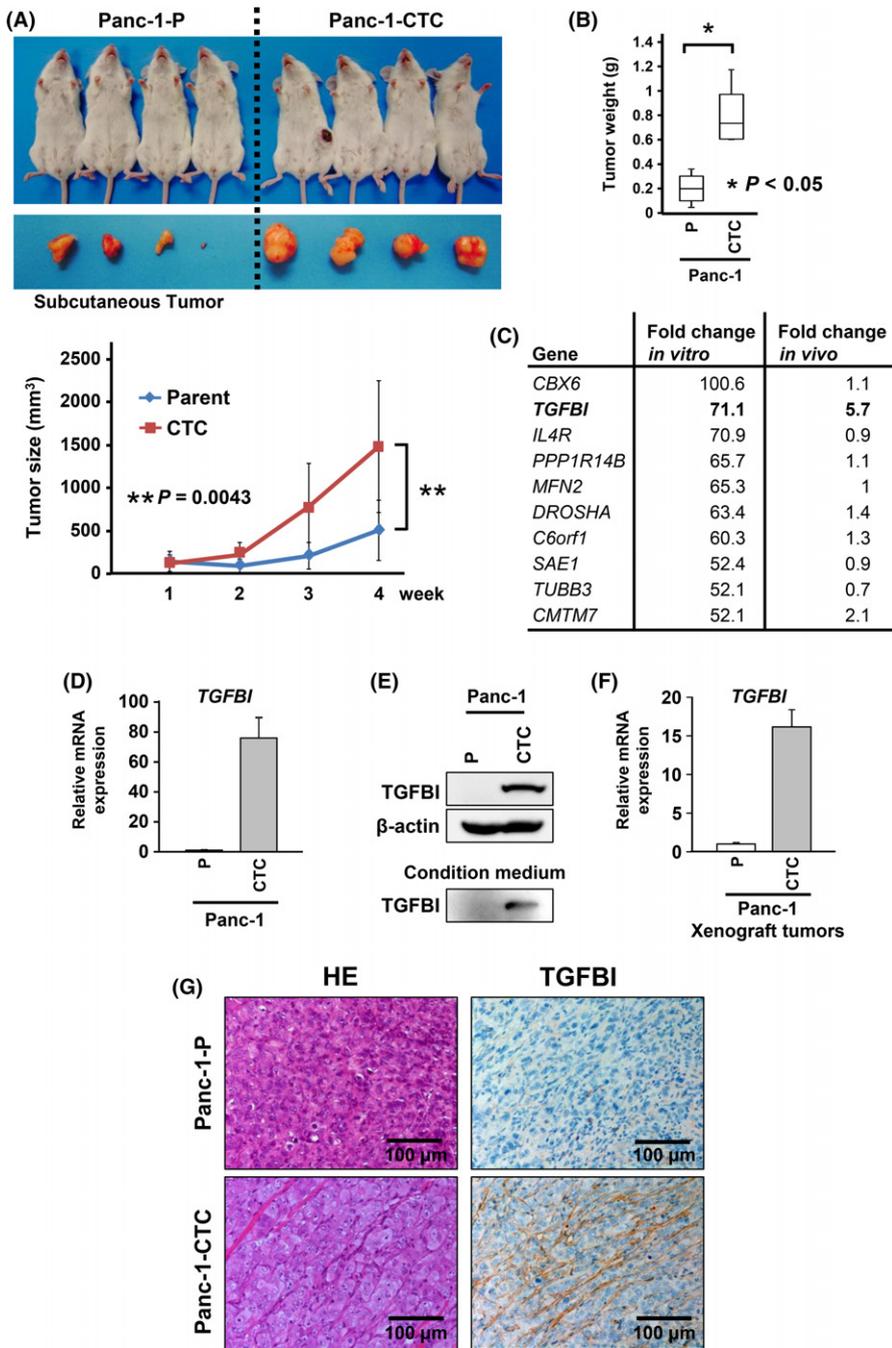


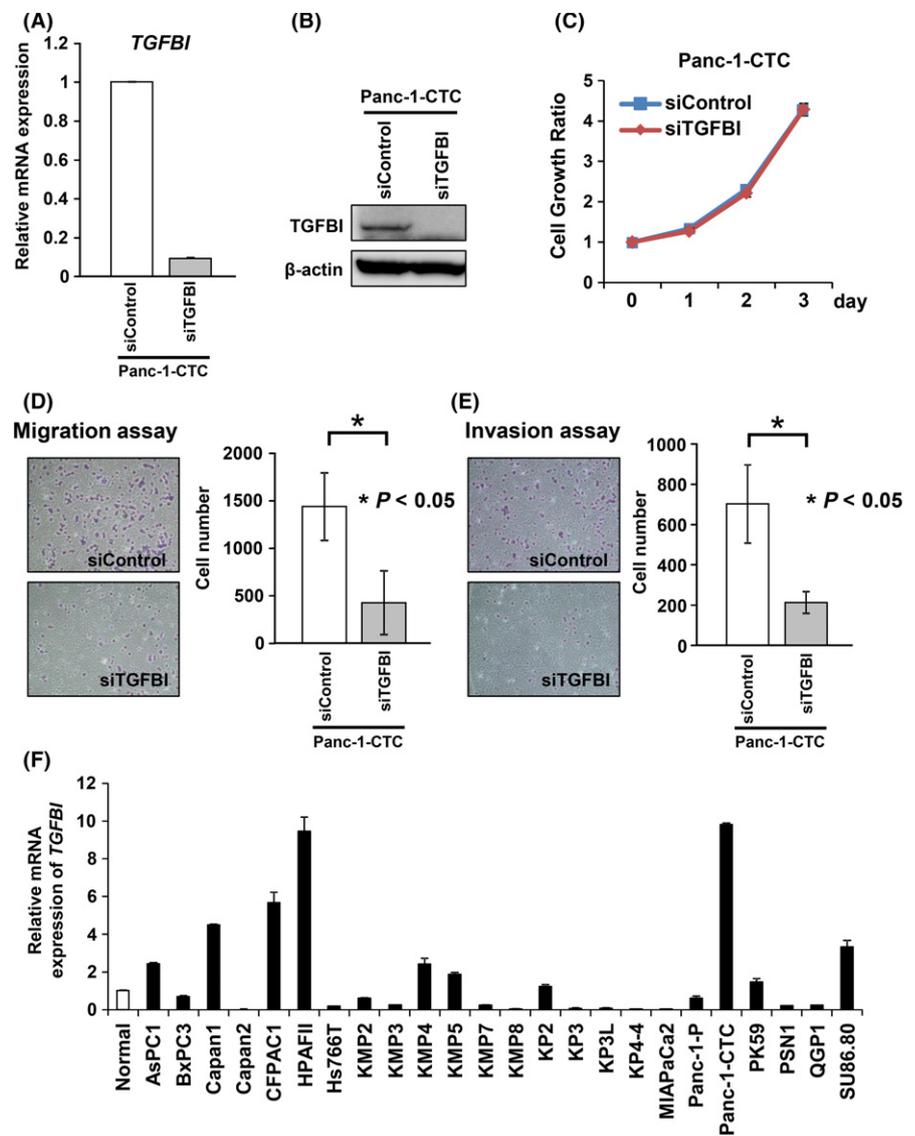
FIGURE 2 Expression of transforming growth factor beta-induced (*TGFBI*) in Panc-1-circulating tumor cells (CTC) was increased in both in vitro and in vivo samples. A, Subcutaneous tumor size of Panc-1-parent (Panc-1-P) and Panc-1-CTC cells was measured every week after inoculation using the formula tumor size (mm^3) = [(length) \times (width) 2]/2. Data are presented as mean \pm SD of samples ($n = 4$). B, Mice were killed on day 28 after inoculation, and the weights of the s.c. tumors were measured. Data are presented as a box plot \pm SD of the samples ($n = 4$). C, Results of the gene expression array with in vitro and in vivo samples of Panc-1-P and Panc-1-CTC cells. D,E, In vitro *TGFBI* expression was assessed by qRT-PCR (D) and western blotting (E). F,G, In vivo *TGFBI* expression was assessed by qRT-PCR (F) and immunohistochemical staining with each s.c. tumor derived from Panc-1-P or Panc-1-CTC cells (G). G, Representative images of immunohistochemical staining of *TGFBI* protein in s.c. tumorous tissues from mice. Bars, 100 μ m

“Panc-1-CTC” from blood samples of eight mice bearing s.c. tumors comprising Panc-1-P cells.

The established CTC cell line, Panc-1-CTC, gained a more malignant phenotype in comparison with Panc-1-P cells. Panc-1-CTC cells were characterized as more migratory, invasive, and tumorigenic in vivo compared to Panc-1-P cells, whereas their in vitro cell growth was slower. These results suggest that Panc-1-CTC cells might have the potential for stemness. Additionally, the increased migration and invasion abilities were partially explained by upregulation of N-cadherin and ZEB1. However, it is difficult to explain whether these abilities are increased by EMT because the expression of E-cadherin and Vimentin did not change in Panc-1-CTC cells. Moreover, to

identify the characteristics of Panc-1-CTC cells, we carried out comprehensive gene expression array analysis with Panc-1-P and Panc-1-CTC cells. We then used both in vitro and in vivo samples in microarray experiments to show the detailed cellular mechanisms. Interestingly, GSEA derived from the in vitro microarray data shows that stemness-related genes were significantly upregulated in Panc-1-CTC cells (Figure S3). Among the top 10 significantly upregulated genes in Panc-1-CTC cells in vitro, *TGFBI* was the only gene that was coincidentally upregulated in the in vivo samples (Figure 2C). The top 100 upregulated genes in Panc-1-CTC cells (Table S1) were quite different in vitro and in vivo. This discrepancy was explained in part by the effect of the tumor microenvironment. In previous studies,

FIGURE 3 Suppression of transforming growth factor beta-induced (TGFB1) decreased the migration and invasion abilities of Panc-1-circulating tumor cells (CTC). A,B, Knockdown of TGFB1 expression in Panc-1-CTC cells by TGFB1-specific siRNA as confirmed by qRT-PCR (A) and western blotting (B) with samples 3 d after transfection with siRNA. C, Number of viable cells 24–72 h after transfection of TGFB1-specific siRNA or a negative control siRNA in Panc-1-CTC cells was determined by WST-8 assay at the indicated times. Results of the relative growth ratio are shown as the mean \pm SD for three separate experiments, with each carried out in triplicate. Differences were analyzed by one-way ANOVA. D,E, Numbers of migratory (D) and invasive cells (E) through the Boyden chamber at 24 h after replating (1.5×10^4 cells per well) 3 d after siRNA transfection were counted using Diff-Quick Stain. Data are presented as mean \pm SD of samples ($n = 3$). Differences were analyzed by the Mann-Whitney U test. F, TGFB1 expression in 25 pancreatic cancer cell lines was assessed by qRT-PCR in comparison with healthy pancreatic tissue. Data are presented as mean \pm SD of samples ($n = 3$)



the in vivo environment was quite different from the in vitro environment due to the interaction of other cells surrounding the tumor cells.³³ TGFB1 upregulation in both in vitro and in vivo samples of Panc-1-CTC cells led us to further investigate this gene as the candidate responsible for the malignant features of PDAC. TGFB1 is located downstream of the TGF- β signaling pathway, and we confirmed that TGF- β stimulation induced TGFB1 expression in a time-dependent way in Panc-1-CTC cells (Figure S1). In contrast, Panc-1-P cells slightly increased TGFB1 expression in response to TGF- β stimulation. Thus, Panc-1-CTC cells could respond to TGF- β stimulation in both in vitro and in vivo environments. In particular, as there are several types of cells, such as fibroblasts, immunocytes, and endothelial cells, in an in vivo environment, the Panc-1-CTC cell might acquire its malignant phenotype by TGF- β stimulation from these cells.³⁴

SMAD4 mutations have been detected in approximately 50% of PDAC clinical samples and were reported in 11 of 23 pancreatic cancer cell lines by COSMIC database and Suzuki et al³⁵ (Table S2).

However, there was no relationship between SMAD4 mutation status and TGFB1 expression level (Figure 3F and Table S2). These observations coincide with the previous research.³⁶

As invasiveness is one of the important abilities of metastatic tumor cells, we confirmed the association between TGFB1 expression and the migratory and invasive abilities of these cells. TGFB1 overexpression increased cell migration and invasion abilities of Panc-1-P cells, whereas suppression of TGFB1 decreased both abilities in Panc-1-CTC cells. It has been reported that phosphorylation of FAK and AKT is induced by TGFB1 through RGD (Arg-Gly-Asp) motif binding to integrin receptors on the cell surface.²¹ However, in the present study, treatment with recombinant TGFB1 had no significant effect on the phosphorylation of either FAK or AKT in Panc-1-P cells (Figure S4). TGFB1 overexpression also had no effect on EMT status and no influence on the phosphorylation of FAK or AKT (data not shown). Therefore, in the present study, the underlying mechanisms for the increased invasiveness were not clear. However, interestingly, a recent study indicated a positive relationship between partial

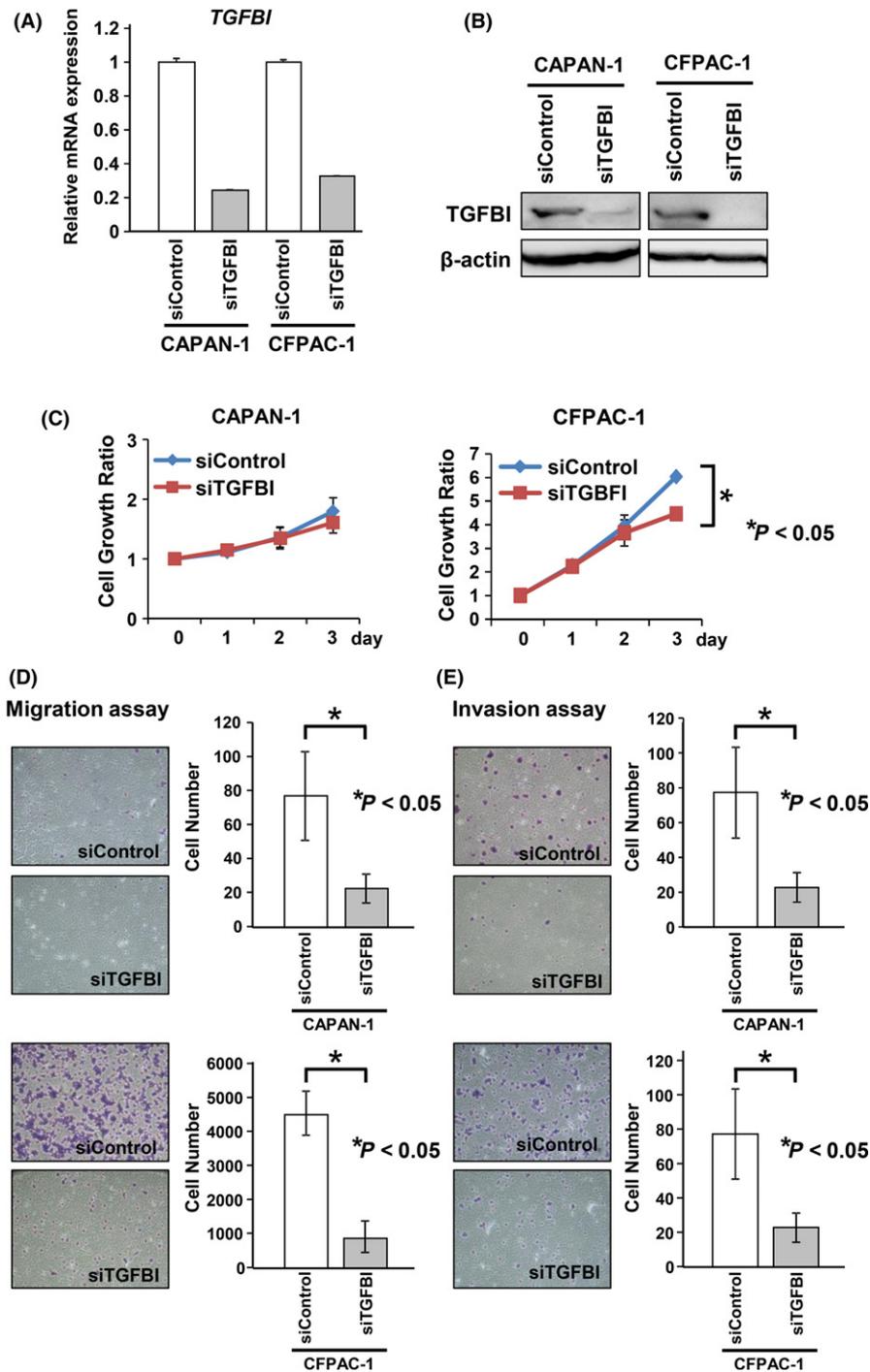


FIGURE 4 Knockdown of transforming growth factor beta-induced (TGFB1) expression decreased the migration and invasion abilities of CAPAN-1 and CFPAC-1 cells. A,B, Knockdown of TGFB1 expression in CAPAN-1 and CFPAC-1 cells by TGFB1-specific siRNA as confirmed by qRT-PCR (A) and western blotting (B) with samples 3 d after siRNA transfection. C, Number of viable cells 24–72 h after transfection of TGFB1-specific siRNA or a negative control siRNA in CAPAN-1 and CFPAC-1 cells was determined by WST-8 assay at the indicated times. Results of the relative growth ratios are shown with mean \pm SD for three separate experiments, with each carried out in triplicate. Differences were analyzed by one-way ANOVA. D,E, Numbers of migratory (D) and invasive cells (E) through the Boyden chamber at 24 h after replating (1.5×10^4 cells per well) at 3 d after siRNA transfection were counted using Diff-Quick Stain. Data are presented as mean \pm SD of samples ($n = 3$). Differences were analyzed by the Mann-Whitney U test

EMT and TGFB1, and TGFB1 overexpression promoted invasiveness of head and neck squamous cell carcinoma cells, whereas TGFB1 knockout decreased their invasiveness.³⁷

With respect to the cell adhesion property of the ECM, TGFB1 has two aspects. In some studies, TGFB1 can promote cell adhesion,^{20,21} but in other studies, TGFB1 can inhibit cell adhesion to components of the ECM, such as collagen.^{22,23} In the present study, cell adhesion in Panc-1-P cells was inhibited by coating the plates with recombinant TGFB1. (Figure S5). Thus, our findings suggested that TGFB1 could not act as a ligand for integrin receptors in Panc-1-P cells. In addition, this anti-adhesive property might help cancer

cells detach from the primary tumor site and contribute to metastasis.

High expression of TGFB1 in clinical PDAC tissues is observed in 50%–96% of patients.^{38,39} In addition, the expression of TGFB1 is associated with poor prognosis in colorectal cancer.²⁷ Our clinical data showed that the expression of TGFB1 was also associated with poor prognosis in patients with PDAC. In clinical PDAC samples, TGFB1 mRNA expression was predominantly observed in the cytoplasm of pancreatic cancer cells by the in situ hybridization method.³⁶ Another study also indicated that s.c. tumors derived from a melanoma cell line with shRNA-mediated suppression of TGFB1 had significantly lower

FIGURE 5 Overexpression of transforming growth factor beta-induced (TGFB1) increased the migration and invasion abilities of Panc-1-parent (Panc-1-P) cells. A,B, Overexpression of TGFB1 in Panc-1-P cells stably transfected with pCMV3Tag3A-TGFB1 expression vector as confirmed by qRT-PCR (A) and western blotting (B). Protein expression of exogenous TGFB1 was confirmed with anti-FLAG antibody. C, Number of viable cells 24-96 h in Panc-1-P cells transduced with empty vector and TGFB1 was determined by WST-8 assay at the indicated times. Results of the relative growth ratios are shown with mean \pm SD for three separate experiments, each carried out in triplicate. Differences were analyzed by one-way ANOVA. D, Number of tumorsphere formations was counted if the length was over 200 μ m. Data are presented as mean \pm SD of samples ($n = 4$). Differences were analyzed by t test. E,F, Number of migratory (E) and invasive cells (F) through the Boyden chamber at 24 h after replating was counted using Diff-Quick Stain. Data are presented as mean \pm SD of samples ($n = 3$). Differences were analyzed by the Mann-Whitney U test

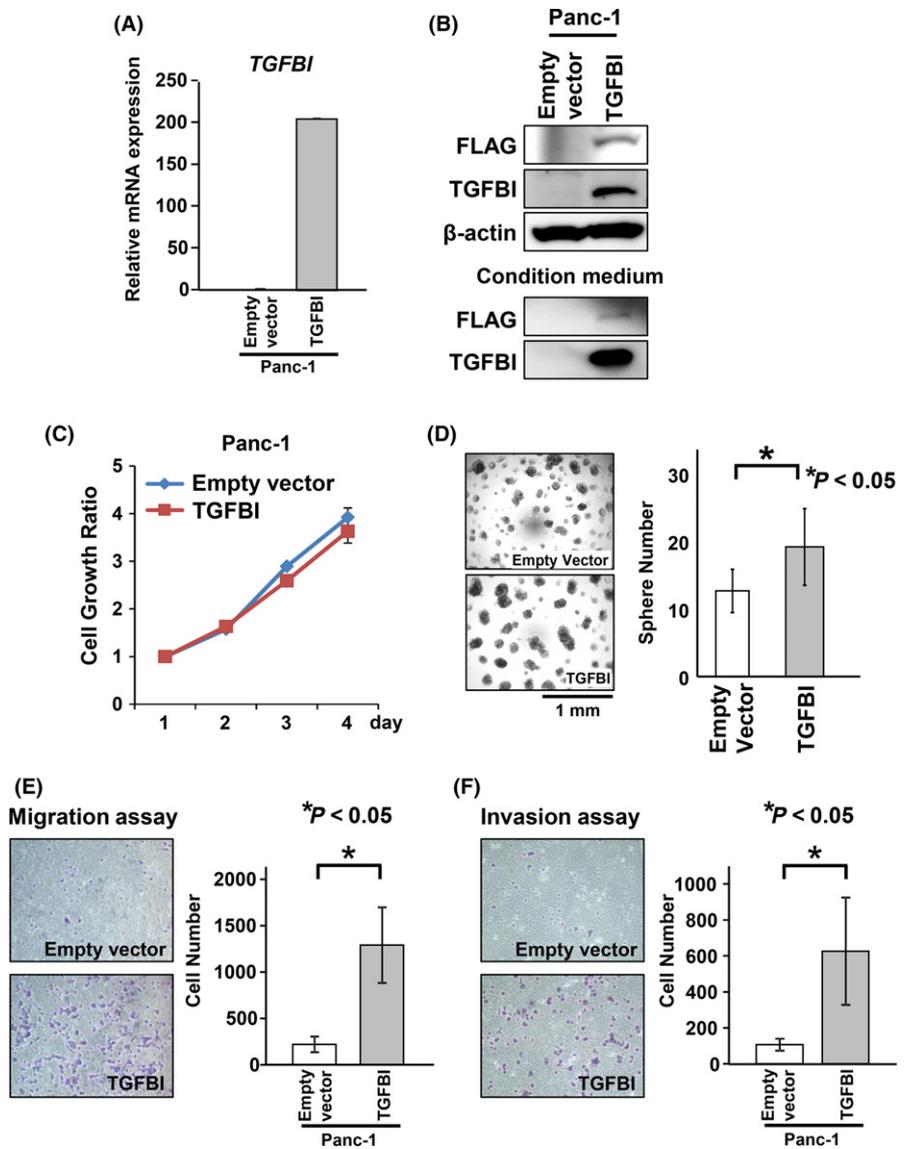
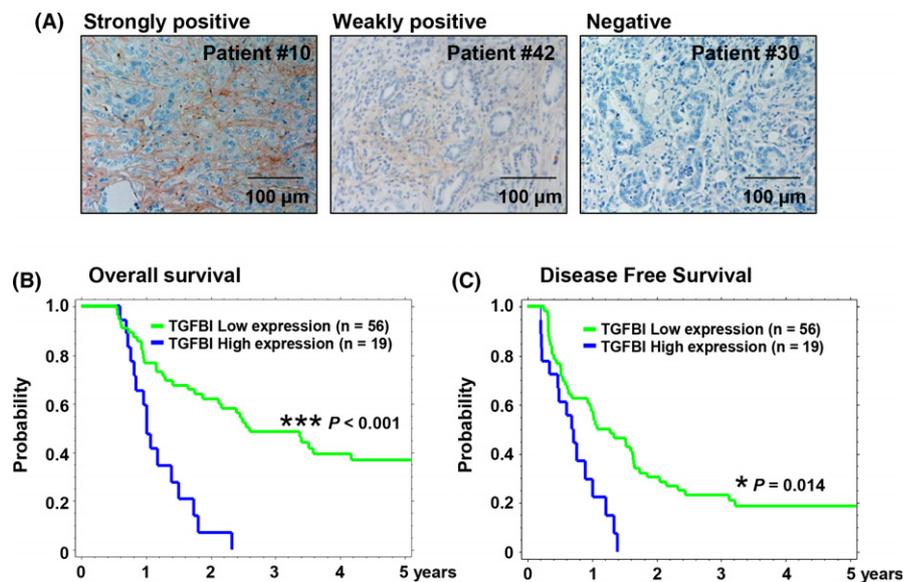


FIGURE 6 Expression of transforming growth factor beta-induced (TGFB1) was associated with poor prognosis in patients with pancreatic ductal adenocarcinoma (PDAC). A, Representative images of immunohistochemical staining of TGFB1 protein in PDAC tissues. Bars, 100 μ m. B, C, Kaplan-Meier curves for the overall survival (B) and disease-free survival (C) of 75 patients with primary PDAC. High TGFB1 immunoreactivity in tumor tissues was significantly associated with poor overall and disease-free survival ($P < 0.001$ and $P = 0.014$, respectively; log-rank test)



expression of TGFBI in the ECM than that of its nonsuppressed counterpart.²¹ In addition, various types of normal cells, such as fibroblast cells and keratinocytes, secrete TGFBI in response to TGF- β signaling.⁴⁰ A previous report suggested that the high expression of TGFBI in the cytoplasm of tumor cells and the ECM in colorectal cancer was mediated by an autocrine TGF- β signal from several human cells, including stromal fibroblasts and tumor cells.²⁷ These observations suggested that TGFBI in the ECM might be derived from tumor cells and several types of cells. In the present study, consistent with the immunoreactivity of TGFBI in Panc-1-CTC xenograft tissue, the localization of TGFBI in clinical PDAC tissues was mainly the ECM and cytoplasm in tumor cells. Taken together, our results indicated that the expression of TGFBI in Panc-1-CTC xenograft tissues was derived from the tumor cells, and TGFBI might accumulate in the ECM.

In conclusion, we identified TGFBI as an oncogene using the viable CTC capturing method and showed that TGFBI was associated with the migration and invasion abilities of pancreatic cancer cells. In PDAC clinical samples, TGFBI was relatively abundant in the tumor ECM, and its expression was associated with patient prognosis. Moreover, the expression of TGFBI in both the cytoplasm of tumor cells and the ECM could be a biomarker for the prediction of prognosis in PDAC.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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