High stromal transforming growth factor β–induced expression is a novel marker of progression and poor prognosis in gastric cancer

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Funding information
Japan Society for the Promotion of Science, Grant/Award Numbers: JP15K10085, JP26461969, JP17K19893

Background and Objectives: Transforming growth factor β–induced (TGFBI) protein is a secreted extracellular matrix protein with conflicting roles in cancer, acting as a tumour suppressor and a promoter, which appears to be tissue specific. The role of TGFBI in gastric cancer (GC) remains unclear, which we aimed to investigate using the clinical samples as well as an in vitro coculture model of GC.

Methods: The clinical significance of TGFBI was assessed in 208 GC samples using immunohistochemistry. Molecular function of TGFBI in the GC cells was examined by small interfering RNA-mediated TGFBI downregulation in the gastric fibroblasts cocultured with the GC cells.

Results: TGFBI expression was localised mainly in the cancer stroma and not in the noncancerous gastric tissue or the GC cells. High TGFBI expression was significantly associated with poor prognosis and cancer progression. Downregulation of TGFBI in the cocultured gastric fibroblasts inhibited the invasion and migration abilities of the GC cells.

Conclusions: High stromal TGFBI expression might be a useful predictive marker for poor prognosis in GC patients. Furthermore, TGFBI in the cancer stromal cells is a promising target for GC treatment.

KEYWORDS
cancer-associated fibroblast, gastric cancer, stroma, transforming factor β–induced
1 | INTRODUCTION

Gastric cancer (GC) is a leading cause of malignancy-related death globally.1 Despite improvements in the treatment outcomes, GC prognosis is generally poor, with a 5-year relative survival rate remaining below 30% in most countries.2 Surgical resection is the only treatment with curative intent, whereas postoperative chemotherapy was observed to improve GC prognosis after surgery3; however, most GC patients are not eligible for radical surgery due to the locally advanced or metastatic disease.4 Thus, the identification of novel useful prognostic markers and therapeutic targets for GC treatment is essential.

Transforming growth factor β-induced (TGFBI) protein is a secreted extracellular matrix protein consisting of 683 amino acids and includes four evolutionarily conserved fasciclin-1 domains and a C-terminal Arg-Gly-Asp motif.5 TGFBI was first described in a human lung adenocarcinoma cell line in 1992 as a protein that was inducible by transforming growth factor β (TGFβ),6 which was confirmed in various human cell types since its discovery.7,8 TGFBI was reported to play important roles in various clinical conditions such as malignancy, diabetes, and corneal dystrophy.9-11

In malignancies, TGFBI was revealed to perform conflicting roles, acting both as a tumour suppressor and a promoter. TGFBI downregulation was observed in leukaemia,12 whereas hypermethylation of the TGFBI promoter, which suppresses TGFBI expression, was observed in the ovarian, prostate, and lung carcinomas.13,14 These findings ascribe certain tumour-suppressor functions to TGFBI. Conversely, TGFBI was also known to exert tumour-promoter functions in various cancers. TGFBI upregulation was reported in pancreatic carcinoma and oral squamous cell carcinoma,7,15 whereas in renal cell carcinoma, TGFBI expression was revealed as a promising prognostic marker.16 Regarding GC, the transgenic mice overexpressing TGFBI developed spontaneous gastric adenocarcinoma at a high rate than wild-type mice5; however, few studies investigated the role of TGFBI in human GC specimens in detail.

The purpose of this study was to clarify the significance of TGFBI expression and its function in the clinical GC samples. We examined TGFBI expression by immunohistochemistry in 208 GC specimens and evaluated the relationship of TGFBI with clinicopathological factors. Moreover, we assessed whether the small interfering RNA (siRNA)-mediated TGFBI suppression in the gastric fibroblast cell lines affected the invasion and migration abilities of the cocultured human GC cells.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Clinical samples collected from 208 GC patients (146 men and 62 women), who underwent surgery at the Department of General Surgical Science of Gunma University between 1999 and 2006, were included in this study. These samples were collected with informed consent obtained in accordance with institutional guidelines and the Declaration of Helsinki. Pathological features of the specimens were classified based on the 3rd English edition of the Classification of Gastric Carcinoma by the Japanese Gastric Cancer Association.17

2.2 | Immunohistochemistry

Sections of 4μm were cut from the formalin-fixed, paraffin embedded GC sample blocks. Sections were mounted on the glass slides, deparaffinised in xylene, rehydrated in graded ethanol washes, and incubated in 0.3% hydrogen peroxide for 30 minutes at room temperature to block the endogenous peroxidase activity. Sections were then heated in boiled water containing Immunosaver (Nishin EM, Tokyo, Japan) at 98 to 100°C for 45 minutes. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free (Dako, Santa Clara, CA) for 30 minutes. Sections were then incubated with a TGFBI-specific antibody (Proteintech, Chicago, IL) at 1:100 dilution at 4°C for 24 hours. The primary antibody was visualised using the Histofine Simple Stain MAX-PO (Multi) kit (Nichirei, Tokyo, Japan), according to the manufacturer’s instructions. The chromogen 3,3-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution in 50 mM ammonium acetate-citrate acid buffer (pH 6.0) containing 0.005% hydrogen peroxide. Sections were lightly counterstained with haematoxylin and mounted. Specimens used as negative controls were incubated without the primary antibody to ensure that no detectable staining was evident.

TGFBI immunostaining was evaluated independently by two evaluators who were blinded to the patient data. We focused on the stromal TGFBI expression, and the intensity was scored as follows: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining. GC patients were classified as low stromal TGFBI expression group (scores 0 or 1+) or high stromal TGFBI expression group (scores 2 or 3+; Figure 1).

2.3 | Cell lines

The human GC cell lines MKN7, MKN45, MKN74, and GCIY, as well as the gastric fibroblast cell lines CA64, CA61, and NF64, were used in this study. MKN7, MKN45, and MKN74 were purchased from the JCRB Cell Bank (Osaka, Japan), and GCIY was purchased from RIKEN BRC (Tokyo, Japan). All gastric cell lines were maintained in Roswell Park Memorial Institute 1640 medium (Wako, Osaka, Japan) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Gastric fibroblast cell lines were isolated from surgical specimens at Osaka City University. CA64 and CA61 originated from the fibroblasts of GC tissues, and NF64 originated from the normal gastric tissue; both cell lines were maintained in Dulbecco modified Eagle medium (DMEM; Wako) supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen). Precise protocols used to establish the fibroblast cell lines were described in a previous report.18 All cell lines were cultured in a humidified incubator with 5% CO₂ at 37°C.
2.4 | Protein extraction and Western blot analysis

Total protein was extracted from MKN7, MKN45, GCIY, KMST6, CaF61, CaF64, and NF64 cells using lysis buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 0.5% NP40, 400 mM NaCl, 4 μg/mL aprotonin, PMSF, proteasome inhibitor MG-132, and 1 mM DTT). Total protein (10 μg) was electrophoresed on 4% to 12% Bis-Tris Mini Gels (Life Technologies Corporation, Carlsbad, CA), and then electroblotted at 300 mA for 90 minutes on a nitrocellulose membrane (Invitrogen). Western blot analysis was used to confirm the expression of TGFBI and TGF-β1 proteins: these proteins were detected using anti-TGFBI rabbit monoclonal antibody (1:300; Proteintech; catalogue no. 10188-1-AP), anti-TGF-β1 mouse monoclonal antibody (1:1000; Cambridge, UK, catalogue no. ab27969), and HSC70 mouse monoclonal antibody (1:1000; Santa Cruz Biotechnology, Dallas, TX; catalogue no. sc-7298). HSC70 expression was used as a loading control. The signals were detected using the ECL Western blot analysis Detection System (GE Healthcare Life Sciences) and an Image Quant LAS 4000 machine (GE Healthcare Life Sciences).

One poring pulse was applied at 125 V (pulse length, 10.0 ms; pulse interval, 40.0 ms), and five transfer pulses were applied at 10 V (pulse length, 50.0 ms; pulse interval, 50.0 ms). After 72-hour incubation, downstream experiments were performed.

2.5 | RNA interference for knockdown of TGFBI

TGFBI-specific siRNAs 1 and 2 were purchased from Theoria Science (Tokyo, Japan). CAF64 cells were plated at a density of 1.0 × 10⁵ cells per well in 100 μl Opti-MEM I Reduced Serum Medium (Invitrogen). TGFBI-specific siRNAs 1 and 2 and a negative control siRNA were added to the cells at a concentration of 10 nM, and siRNA transfection was achieved using a CUY-21 EDIT II electroporator (BEX, Tokyo, Japan), according to the manufacturer’s instructions.

Invasion ability of MKN7 and MKN45 cells was analysed using Matrigel-coated transwell chambers with an 8-μm pore size (Corning, New York, NY). CAF64 cells (2.0 × 10⁵) were seeded in 24-well plates in DMEM containing 10% FBS, MKN7 (5.0 × 10⁴), and MKN45 (2.5 × 10⁴) cells were seeded in serum-free media in the upper chamber of the Matrigel-coated plates. After incubation for 24 hour (MKN7) or 48 hour (MKN45), the chambers were removed, washed with phosphate-buffered saline, and cleaned using a cotton swab. Cells were then fixed in methanol and stained with the Diff-Quick stain (Sysmex, Kobe, Japan). Membranes were cut and observed under ×100 magnification using bright field microscopy.

2.7 | Wound-healing assay

MKN7 (1.0 × 10⁵), MKN45 (5.0 × 10⁵), and CAF64 (5.0 × 10⁴) cells were plated using a coculture model. Briefly, MKN7 and MKN45 cells were seeded in 24-well plates, and CAF64 cells were seeded in the upper chamber of the transwell plate with an 8-μm pore size (Corning). After MKN7 and MKN45 cells reached confluence, a scratch wound was made by creating a straight line using a sterile pipette tip. Cells were washed and incubated with DMEM containing 10% FBS. Closure of the wound was evaluated at 12 hour (MKN7)
and 24-hour (MKN45) after the wound creation at ×40 magnification using bright field microscopy.

2.8 | Statistical analysis

Statistically significant differences were analysed with Student t test for continuous variables and the χ² test for categorical variables. Analysis of variance (ANOVA) was used to assess the statistical significance of in vitro assays. When the results by ANOVA were significant, the Dunnett multiple comparisons test was used to assess the differences among groups. Kaplan-Meier curves were generated for overall survival regarding clinical data, and statistical significance was determined using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards model. A P value of less than 0.05 was considered as statistically significant. All statistical analyses were performed using JMP software (SAS Institute, Cary, NC).

3 | RESULTS

3.1 | Immunohistochemical staining for TGFBI in clinical GC specimens

TGFBI expression in 208 GC specimens was examined by immunohistochemistry using a tissue microarray. TGFBI expression was detected mainly in the cancer stroma and not in the GC cells or the noncancerous gastric tissue (Figure 1). Stromal TGFBI expression in the GC tissue was significantly higher than that in the noncancerous tissue (P < 0.001; Table 1).

3.2 | Clinicopathological significance of TGFBI expression in patients with GC

Among 208 GC cases, the stromal TGFBI expression scores were 0, 1+, 2+, and 3+ in 58 (27.9%), 69 (33.2%), 41 (19.8%), and 40 (19.2%) samples, respectively. About 127 (61.1%) samples were classified into the low TGFBI expression group and 81 (38.9%) samples were classified into the high TGFBI expression group.

The relationship of stromal TGFBI expression with clinicopathological factors in this cohort of 208 GC patients is presented in Table 2. High TGFBI expression was significantly associated with depth of tumour invasion (P < 0.001), lymphatic invasion (P = 0.001), venous invasion (P = 0.046), peritoneal dissemination (P = 0.045), and pathological stage (P < 0.001).

The Kaplan-Meier analysis of data from 208 GC patients demonstrated that the overall survival in the high TGFBI-expression group was significantly lower than that in the low TGFBI-expression group (P = 0.0016; Figure 2).

The table below shows the correlation analysis of TGFBI expression and clinicopathological factors in the study cohort of 208 patients with gastric cancer.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Stromal TGFBI expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low, n = 127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High, n = 81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>65.4 ± 10.8</td>
<td>0.18</td>
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<tr>
<td>Sex</td>
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<td></td>
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<tr>
<td>Male</td>
<td>88</td>
<td>0.75</td>
</tr>
<tr>
<td>Female</td>
<td>39</td>
<td></td>
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<tr>
<td>Histology</td>
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<tr>
<td>Tubular</td>
<td>49</td>
<td>0.4</td>
</tr>
<tr>
<td>Poor</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Signet</td>
<td>6</td>
<td>0.001*</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Depth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sm, mp, ss</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>se, si</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
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<td></td>
</tr>
<tr>
<td>Absent</td>
<td>45</td>
<td>0.23</td>
</tr>
<tr>
<td>Present</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>18</td>
<td>0.001*</td>
</tr>
<tr>
<td>Present</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>45</td>
<td>0.046*</td>
</tr>
<tr>
<td>Present</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Peritoneal dissemination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>123</td>
<td>0.045*</td>
</tr>
<tr>
<td>Present</td>
<td>4</td>
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</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>75</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>III and IV</td>
<td>52</td>
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</tr>
</tbody>
</table>

Abbreviations: TGFBI, transforming growth factor β-induced. *P < 0.05.
3.3 | TGFBI and TGF-β1 expression in GC and gastric fibroblast cell lines

We evaluated the protein expression of TGFBI and TGF-β1 in CAF64, CAF61, and NF64 gastric fibroblast cell lines and MKN7, MKN45, MKN74, and GCIY GC cell lines by Western blot analysis (Figure 3, upper panel). TGFBI protein was detected in all fibroblast cell lines and in none of the GC cell lines. The expression of TGF-β1 in fibroblast cell lines was higher than GC cell lines. Furthermore, we evaluated TGFBI induction by TGF-β1 treatment. The expression of TGFBI was not induced in GC cell lines (Figure 3, middle panel).

TGFBI expression was higher in CAF64 cells than the other fibroblast cell lines. Therefore, the CAF64 cell line was used to clarify whether TGFBI in fibroblasts contributed to the malignant phenotype in two GC cell lines: MKN7 and MKN45, which were derived from well-differentiated and poorly-differentiated adenocarcinomas, respectively.

3.4 | CAF64 cells upregulated the invasion and migration abilities of cocultured MKN7 and MKN45 cells

To evaluate the effect of CAF64 cells on GC cells, we performed an invasion and wound-healing assay in a coculture model. The

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**TABLE 3** Univariate and multivariate analyses of the clinicopathological factors affecting the overall survival rate following surgery in gastric cancer

<table>
<thead>
<tr>
<th>Clinicopathological variables</th>
<th>Univariate analysis</th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RR</td>
<td>95% CI</td>
<td>P value</td>
<td>RR</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>1.26</td>
<td>0.83-1.97</td>
<td>0.2907</td>
<td>–</td>
</tr>
<tr>
<td>Histology (differentiated/undifferentiated)*</td>
<td>1.36</td>
<td>0.90-2.07</td>
<td>0.1439</td>
<td>–</td>
</tr>
<tr>
<td>Depth (sm, mp, ss/se, si)</td>
<td>3.45</td>
<td>2.31-5.24</td>
<td>&lt;0.0001*</td>
<td>1.86</td>
</tr>
<tr>
<td>Lymph node metastasis (absent/present)</td>
<td>2.74</td>
<td>1.70-4.65</td>
<td>&lt;0.0001*</td>
<td>1.19</td>
</tr>
<tr>
<td>Lymphatic invasion (absent/present)</td>
<td>4.32</td>
<td>1.63-17.6</td>
<td>0.0014*</td>
<td>1.58</td>
</tr>
<tr>
<td>Venous invasion (absent/present)</td>
<td>2.47</td>
<td>1.53-4.19</td>
<td>&lt;0.0001*</td>
<td>1.56</td>
</tr>
<tr>
<td>Peritoneal dissemination (absent/present)</td>
<td>3.56</td>
<td>1.79-6.42</td>
<td>0.0008*</td>
<td>1.75</td>
</tr>
<tr>
<td>Stage (I,II/III,IV)</td>
<td>4.09</td>
<td>2.65-6.52</td>
<td>&lt;0.0001*</td>
<td>1.98</td>
</tr>
<tr>
<td>TGFβ1 expression (low/high)</td>
<td>1.79</td>
<td>1.21-2.64</td>
<td>0.0039*</td>
<td>1.24</td>
</tr>
</tbody>
</table>

Abbreviations: RR, relative risk; CI, confidence interval.
*p < 0.05.
experimental paradigms are presented in Figure 4. The invasion ability of MKN7 and MKN45 cells was significantly upregulated when they were cocultured with CAF64 cells ($P = 0.0007$ and <0.0001, respectively; Figure 5, left panel). Similarly, the migration ability of MKN7 and MKN45 cells was also significantly upregulated upon coculturing with CAF64 cells ($P = 0.0003$ and <0.0001, respectively; Figure 6, left panel).

3.5 | TGFBI knockdown in CAF64 cells suppresses the invasion and migration abilities of the cocultured MKN7 and MKN45 cells

To evaluate the correlation between the TGFBI expression in CAF64 cells and the invasion and migration abilities of MKN7 and MKN45 cells, we performed TGFBI knockdown in our coculture model and used the invasion and wound-healing assay (Figure 4). First, the knockdown of TGFBI in CAF64 cells using siRNA was confirmed by Western blot analysis (Figure 3, lower panel). The CAF64-induced invasion ability of MKN7 and MKN45 cells was suppressed in the presence of CAF64 cells with TGFBI knockdown using siRNA ($P = 0.0002$, <0.0001; $=0.0171$, $=0.0116$) (Figure 5, right panel). CAF64-induced migration ability of MKN7 and MKN45 cells was also suppressed similar to that observed with the invasion assay ($P < 0.0001$; Figure 6, right panel).

4 | DISCUSSION

In this study, we found that TGFBI was expressed primarily in cancer stroma and not in the GC cells or noncancerous tissue. We also reported that high TGFBI levels in cancer stroma correlated with...
cancer progression and poor prognosis in the clinical GC samples. Moreover, TGFBI knockdown in fibroblasts suppressed the invasion and migration ability of MKN7 and MKN45 cells in an in vitro coculture model.

TGFBI was initially described as a protein that was upregulated by TGF-β in the lung cancer cells. Increased TGFBI expression in response to TGF-β was also demonstrated in the pancreatic cancer cells. Ma et al. reported that increased TGFBI secreted by cancer cells was associated with high clinical grade in the colon cancers. Lauden et al. examined the function of TGFBI in melanoma using cell lines with high TGFBI expression and demonstrated that TGFBI was required for melanoma metastatic outgrowth. Although these reports suggest that TGFBI might be derived from the cancer cells, our assessment of GC tissue samples by immunohistochemistry revealed that GC cells did not express TGFBI. Moreover, we found that TGFBI was expressed in all gastric fibroblast cell lines and none of the GC cell lines included in the current study. Mizoi et al. previously reported that TGF-β signalling was significantly upregulated in the cancer stromal tissues in GC. TGF-β signalling was also shown to be activated in fibroblasts of GC tissues. Moreover, Fuyuhiro et al. suggested that fibroblasts around GC were associated with aggressiveness of GC cells via the TGF-β signalling. Therefore, we suggest that TGFBI might be derived from gastric stromal fibroblasts via activation of the TGF-β signalling, in contrast to the GC cells.

TGFBI has been observed to serve contradictory functions in tumorigenesis. Although TGFBI was reported to function as a tumour suppressor in leukaemia and ovarian, prostate, and lung carcinomas, the tumour-promoting role of TGFBI was indicated in renal cell carcinoma, pancreatic carcinoma, oral and oesophageal squamous cell carcinoma, and colon carcinoma. Furthermore, Han et al. reported a role for TGFBI in tumorigenesis of the gastrointestinal tract by demonstrating that TGFBI overexpression increased the incidence of spontaneous tumours including gastric adenocarcinoma in transgenic mice. In this study, we reported that high stromal TGFBI expression correlated with cancer progression and poor prognosis in clinical GC samples, suggesting that stromal TGFBI was acting as a tumour promoter in gastric tissue. Moreover, TGFBI expression might be a useful marker in predicting cancer progression and poor prognosis in GC.

In the current study, high stromal TGFBI expression was significantly correlated with peritoneal dissemination. Peritoneal dissemination is widely recognised as the most important and difficult therapeutic target in progressive GC phenotype. Although various strategies including systemic chemotherapy, intraperitoneal chemotherapy, hyperthermia,
and aggressive surgery have been used to control peritoneal dissemination, their clinical benefits are limited. Intraperitoneal paclitaxel administration in GC patients with peritoneal dissemination has been recently gaining attention as intraperitoneal paclitaxel is expected to remain inside the peritoneal cavity due to its large molecular weight and fat solubility. Paclitaxel is also used as a standard treatment in ovarian cancer, which remains inside the peritoneal cavity due to its large molecular weight and recently gaining attention as intraperitoneal paclitaxel is expected to be associated with high TGFBI expression. Assessment of TGFBI expression might have poor prognosis with peritoneal dissemination and that treatment with paclitaxel might control the progression of cell cycle and cancer aggressiveness in several cancer types including GC. Based on these observations, we propose that TGFBI localised in the GC stroma might be a promising target for therapeutic intervention through its ability to inhibit the invasion and migration abilities and cell cycle progression of cancer cells.

5 | CONCLUSIONS

TGFBI expression in the cancer stromal cells was associated with cancer progression based on the significant associations with depth of tumour invasion, lymphatic invasion, venous invasion, peritoneal dissemination, and pathological stage. Moreover, our assessment regarding the effect of TGFBI expressed by fibroblasts on GC cells using the coculture model revealed that TGFBI in fibroblasts upregulated the invasion and migration abilities of the GC cells. Han et al found that TGFBI activated FAK/AKT/mTOR pathways were associated with the promotion of cell survival. Lauden et al indicated that TGFBI knockdown led to growth inhibition through defective cell cycle progression in the melanoma cells. Specifically, they demonstrated that TGFBI was a regulator of cyclins and cyclin-dependent kinases that control the progression of cell cycle and cancer aggressiveness in several cancer types including GC. Based on these observations, we propose that TGFBI localised in the GC stroma might be a promising target for therapeutic intervention through its ability to inhibit the invasion and migration abilities and cell cycle progression of cancer cells.

ACKNOWLEDGEMENTS

We thank Ms. Yukie Saito, Ms. Tomoko Yano, Ms. Yuka Matsui, Ms. Sayaka Okada, Ms. Kayoko Takahashi, Ms. Yuki Saka, Ms. Mizue Murata, Ms. Kanna Nakamura, Ms. Harumi Kanai, and Ms. Fumie Takada for their excellent assistance. The authors would like to thank Enago (www.enago.jp) for the English language review. This study was funded by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (grant nos JP26461969, JP15K10085, and JP17K19893).

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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