

Increased Expression of Tissue Transglutaminase in Pancreatic Ductal Adenocarcinoma and Its Implications in Drug Resistance and Metastasis

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive neoplastic diseases and is virtually incurable. The molecular mechanisms that contribute to the intrinsic resistance of PDAC to various anticancer therapies are not well understood. Recently, we have observed that several drug-resistant and metastatic tumors and tumor cell lines expressed elevated levels of tissue transglutaminase (TG2). Because PDAC exhibits inherent resistance to various drugs, we determined the constitutive expression of TG2 in 75 PDAC and 12 PDAC cell lines. Our results showed that 42 of 75 (56%) PDAC tumor samples expressed higher basal levels of TG2 compared with the normal pancreatic ducts [odds ratio (OR), 2.439; $P = 0.012$]. The increased expression of TG2 in PDAC was strongly associated with nodal metastasis (OR, 3.400; $P = 0.017$) and lymphovascular invasion (OR, 3.055; $P = 0.045$). Increased expression of TG2 was also evident in all 12 cell lines examined. The elevated expression of TG2 in PDAC cell lines was associated with gemcitabine resistance and increased invasive potential. Overexpression of catalytically active or inactive (C₂₇₇S mutant) TG2 induced focal adhesion kinase (FAK) activation and augmented invasive functions in the BxPC-3 cell line. Conversely, down-regulation of TG2 by small interfering RNA attenuated FAK phosphorylation. Immunoprecipitation and confocal microscopy data revealed that TG2 was associated with FAK protein in PDAC cells. The activated FAK colocalized with TG2 at focal adhesion points. These results show for the first time that elevated expression of TG2 can induce constitutive activation of FAK and thus may contribute to the development of drug resistance and invasive phenotypes in PDAC. (Cancer Res 2006; 66(21): 10525-33)

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers and is associated with high morbidity and mortality rates. It is the fourth leading cause of cancer death in the United States, with an overall 5-year survival rate of <5% (1).

Cytotoxic chemotherapy and surgery are used as palliative treatment for PDAC patients. 5-Fluorouracil and gemcitabine are the most commonly used drugs to treat pancreatic cancer (2–5),

but PDAC exhibits intrinsic resistance to these drugs (6). Therefore, understanding the novel pathways and proteins that contribute to the development of resistance to anticancer therapies may help to identify novel targets for PDAC treatment.

Recently, we reported that drug-resistant and metastatic breast cancer and melanoma cells expressed elevated levels of tissue transglutaminase (TG2; refs. 7–10). Importantly, the increased TG2 expression was associated with an increased resistance to chemotherapeutic drugs and other apoptosis-inducing stimuli. Inhibition of TG2 by small interfering RNA (siRNA) reversed the sensitivity of drug-resistant MCF-7 breast cancer cells to doxorubicin and rendered them sensitive to serum withdrawal–induced apoptosis (6, 7).

TG2 is ubiquitous and most diverse member of the transglutaminase family of enzymes (11). In addition to catalyzing Ca²⁺-dependent post-translational modification of proteins, it can catalyze Ca²⁺-independent hydrolysis of guanosine 5'-triphosphate and adenosine 5'-triphosphate. The ability of TG2 to hydrolyze guanosine 5'-triphosphate enables it to serve as a signaling molecule, leading to the activation of a cytoplasmic target, phospholipase C (12, 13). Moreover, the high affinity of TG2 for fibronectin may play a role in promoting integrin-mediated cell adhesion (14) and cell migration functions (15, 16). Similarly, TG2 has been shown to activate RhoA and mitogen-activated protein kinase pathways (MAPK; ref. 17), which control key signaling pathways that impinge on the invasive and metastatic behavior of malignant cells. Several reports have documented elevated expression of TG2 in various cancer types (7–11, 18, 19).

In this report, we examined the constitutive expression of TG2 in 75 PDAC tumors and 12 PDAC cell lines. The results were correlated with various molecular markers and clinicopathologic data. We showed that elevated TG2 expression was associated with gemcitabine resistance and invasive phenotypes in pancreatic cancers. Furthermore, we examined the molecular mechanisms by which TG2 overexpression lead to gemcitabine resistance and increased invasive potential in PDAC cell lines using adenovirus overexpression system, siRNA knockdown, and confocal microscopy. We showed, for the first time, that TG2 can interact with focal adhesion kinase (FAK) and result in its activation; this function was independent of the transamidation activity of TG2. Therefore, we concluded that elevated TG2 expression in PDAC cells can contribute to the development of drug resistance and invasive phenotypes by activation of the FAK/phosphatidylinositol 3-kinase (PI3K)/AKT pathway.

Materials and Methods

Materials. The rabbit polyclonal antibodies to phosphorylated AKT (pAKT; Ser⁴⁷³), AKT, phosphorylated extracellular signal-regulated kinase

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(ERK) 1/2 (pERK1/2), and ERK1/2 were from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibodies (mAb) to pY³⁹⁷ FAK and FAK were purchased from BD Biosciences PharMingen (San Diego, CA). Anti-TG2 mAb CUB7401 was purchased from Neomarkers (Fremont, CA). Anti- β -actin antibody was from Sigma Chemical Co. (St. Louis, MO), and the goat anti-rabbit and sheep anti-mouse horseradish peroxidase were purchased from Amersham Biosciences (Piscataway, NJ). TrueBlot anti-mouse Ig IP beads were from eBiosciences (San Diego, CA). The cell fractionation kit was from Biovision, Inc. (Mountain View, CA). DMEM/F-12, RPMI 1640, keratinocyte serum-free medium, bovine pituitary extract, recombinant human epidermal growth factor (EGF), fetal bovine serum, and Normocin antibiotic were all purchased from InvivoGen (San Diego, CA).

The PDAC cell lines AsPC-1, MiaPaCa-2, Panc-1, HPAF-II, HS766T, Capan-1, Capan-2, and BxPC-3 were provided by Dr. Shrikanth A. Reddy (The University of Texas M. D. Anderson Cancer Center, Houston, TX). Panc-28, Panc-02-3, Panc-48, and Su8686 cell lines were provided by Dr. Paul J. Chiao (The University of Texas M. D. Anderson Cancer Center). All cell lines were maintained in the log phase of cell growth by being cultured in RPMI 1640 or DMEM/F-12 supplemented with FCS (10%, v/v), Normocin (0.1 mg/mL), L-glutamine (2 mmol/L), and HEPES (10 mmol/L; U.S. Biochemical, Cleveland, OH) at 37°C in a CO₂ incubator. The immortalized pancreatic ductal epithelium cell line (E6E7) was provided by Dr. Ming Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada) and cultured in keratinocyte serum-free medium supplemented with bovine pituitary extracts, human EGF, and FCS.

TG2 enzymatic activity. Cells at 70% to 80% confluence were washed in PBS and collected in a minimal volume of the lysis buffer [20 mmol/L Tris-HCl (pH 7.4) containing 1 mmol/L EDTA, 150 mmol/L NaCl, 14 mmol/L 2-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride]. Cells were then lysed in the same buffer by probe sonication and assayed for TG2 activity by determining the Ca²⁺-dependent incorporation of [³H]putrescine (specific activity, 14.3 Ci/mmol; Amersham Pharmacia, San Francisco, CA) into dimethylcasein as described previously (20). The enzyme activity was expressed as nanomoles of putrescine incorporated per milligram of total cell protein.

Western blotting. Cells were serum starved for 12 to 16 hours before the experiment. The whole-cell lysate (60 μ g) or fractions thereof (prepared using the Biovision cell fractionation kit according to the manufacturer's protocol) were fractionated by 4% to 15% gradient SDS-PAGE. After SDS-PAGE, the proteins were electrotransferred onto nitrocellulose membranes, blotted with each antibody, and detected with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Some of the membranes were stripped using Restore stripping buffer (Pierce, Rockford, IL) for reprobing with another antibody. The protein bands obtained were quantified using AlphaEase FC (Fluor Chem 8900) software from Alpha Innotech (San Leandro, CA).

Immunoprecipitation. Cells were lysed in a minimum volume of Tris-HCl buffer [50 mmol/L (pH 8)] containing 150 mmol/L NaCl and 1% NP40 and precleared by incubation with 50 μ L Trueblot anti-mouse Ig IP beads for 1 hour at 4°C. The pellet was discarded, and the supernatant was subjected to immunoprecipitation: 200 μ g cell lysate was incubated with 2 μ g of specific antibody for 1 hour at 4°C. Twenty microliters of Trueblot anti-mouse Ig IP beads was added, and the pellet was further incubated on a rotating device overnight at 4°C. The pellet was then washed four times in ice-cold lysis buffer. The supernatant was discarded, and the pellet was resuspended in 50 μ L of the sample buffer. The samples were fractionated by SDS-PAGE and analyzed by immunoblotting and autoradiography.

Cytotoxicity. The number of viable cells remaining after the appropriate treatment was determined by measuring their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) into a soluble formazan in accordance with the manufacturer's instructions. Logarithmically growing cells were plated at 2 \times 10³ per well in 96-well plates and allowed to adhere overnight. They were then cultured alone or with 0.1 nmol/L to 100 μ mol/L gemcitabine for 24, 48, or 72 hours. Each experiment was repeated thrice in triplicate. The concentration of gemcitabine required to inhibit proliferation by 50% (IC₅₀) was calculated. Under identical conditions, the cells were trypsinized, and the cell viability was determined with a trypan blue exclusion test to confirm the MTS results.

In vitro invasion. The invasive potential of PDAC cell lines was studied *in vitro* by determining the number of cells that invaded through Matrigel-coated Transwell polycarbonate membrane inserts as described previously (21). In brief, Transwell inserts with a pore size of 12 μ m were coated with 0.78 mg/mL Matrigel in serum-free medium. Cells were recovered by trypsinization, washed, and resuspended in serum-free medium, and 0.5 mL of the cell suspension (0.5 \times 10⁶ cells) was added to duplicate wells. After incubation for 48 hours, the cells that passed through the filter were stained using a Hema-3 stain kit (Fisher Scientific, Houston, TX). The cells in 10 random fields were counted under a microscope.

Wild-type and C₂₇₇S mutant TG2 adenovirus. An adenovirus containing wild-type (WT; TG2wt) or C₂₇₇S mutant TG2 (TG2m) cDNA was kindly provided by Dr. Ugra Singh (The Texas A&M University System Health Science Center, Temple, TX). In brief, TG2 cDNA cloned in pcDNA3.1 vector was first subcloned in a pshuttle 2 vector and then in a BD adenoX adenoviral vector. HEK293 cells were transfected with recombinant adenoviral plasmid for packaging of adenovirus particles. The adenovirus was purified on a CsCl₂ gradient and used at 25 multiplicities of infection (MOI). Cells infected with lacZ adenovirus served as the control.

TG2 down-regulation by siRNA. Two TG2 siRNA sequences were designed and purchased from Qiagen (Germantown, MD). A sequence that did not have homology to any human mRNA (as determined by a BLAST search) served as a control, whereas two sequences were designed to target TG2 mRNA, siRNA1 (target sequence, 5'-AAGGCCGTTTCCACTAAGA-3') and siRNA2 (target sequence, 5'-AAGGGCAACCACCTGAACAA-3'). For transfection, 2 \times 10⁵ cells were plated in each well of six-well plates and allowed to adhere for 24 hours. On the day of transfection, 30 μ L RNAiFect transfection reagent (Qiagen) was added to 5 μ L siRNA (1 μ g/ μ L) in a 65- μ L culture medium to give a final volume of 100 μ L. The siRNA/transfection reagent mixture was incubated at ambient temperature for 15 minutes and added uniformly to plates in serum-containing medium. After 48 hours of transfection, cells were recovered and used for appropriate determinations. The transfection efficiency was determined by transfecting cells in a parallel well with fluorescent siRNA and determining fluorescence uptake under the microscope.

Confocal microscopy. To determine the colocalization of phosphorylated FAK (pFAK) and TG2, cells were grown on glass coverslips and fixed in

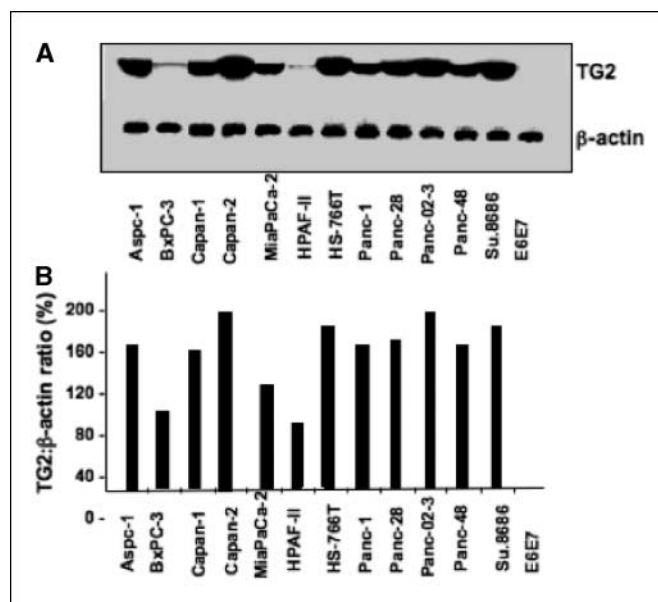
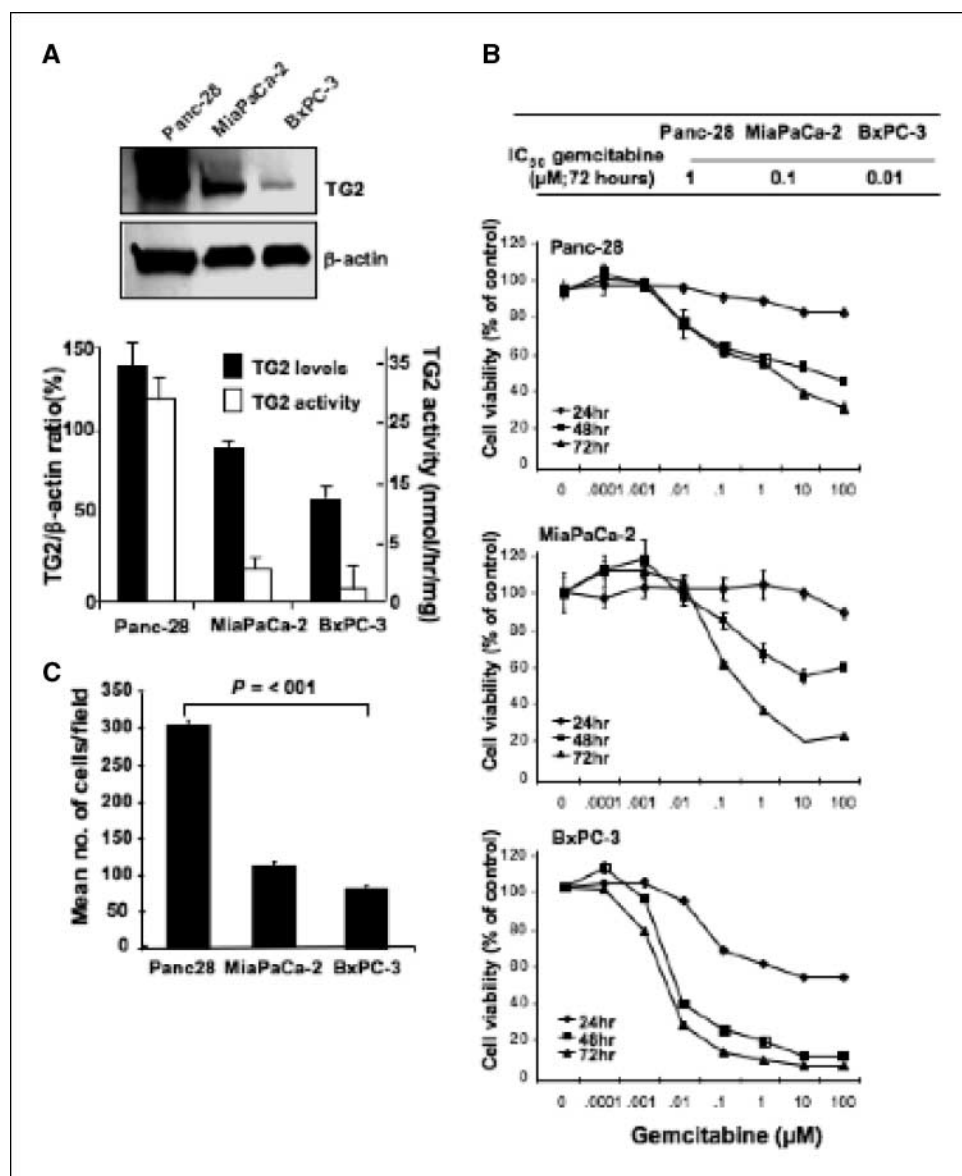


Figure 1. TG2 expression in PDAC cell lines. Western blot (A) and its densitometry analysis (B) showing basal expression of TG2 protein in 12 PDAC cell lines and one immortalized normal pancreatic epithelial cell line (E6E7). Cell lines with TG2/ β -actin ratio of >100% and <100% were considered high TG2- and low TG2-expressing cell lines, respectively.

Figure 2. TG2 expression is associated with gemcitabine resistance and invasive potential in PDAC cell lines. **A**, Western blot analysis comparing the basal expression of TG2 in three PDAC cell lines. The enzymatic activity (histograms) in three cell lines correlated with TG2 protein levels. **B**, the cell viability, as determined by an MTS assay, of Panc-28, MiaPaCa-2, and BxPC-3 cells after their treatment with increasing doses of gemcitabine for 24, 48, or 72 hours. IC_{50} of gemcitabine for the cell lines after 72 hours. **C**, the invasive functions of the three cell lines, as determined by their ability to invade through Matrigel-Transwell membranes, was compared as described in Materials and Methods. Representative experiments done at least twice with similar results. Columns and points, mean of quadruplicate values; bars, SD.



4% paraformaldehyde for 20 minutes at ambient temperature. Fixed cells were then incubated with the primary antibodies overnight, washed with PBS, and incubated again with secondary antibodies conjugated with either Alexa 546 (red) or 488 (green; Molecular Probes, Eugene, OR). The DNA dye Topro-3 (Molecular Probes) was used to costain the nuclei (blue). Cells treated with secondary antibodies alone were used as controls. A confocal scanning analysis of the cells was done with a Zeiss laser scanning confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) or an Olympus FluoView 300 confocal microscope (Center Valley, PA) in accordance with established methods, using sequential laser excitation to minimize the fluorescent emission bleed through. Each section was examined for the presence of each stain at two excitations (546 and 488 nm), and the data were compared pixel by pixel. Each image represented z sections at the same cellular level and magnification; a three-dimensional reconstructed image was used to visualize the whole sample. Merging red and green showed colocalization of two proteins, giving a yellow color.

Immunohistochemistry. Samples used in this study were from patients with primary PDAC who underwent initial pancreaticoduodenectomy at our institution between 1990 and 2004. None of these patients received preoperative chemotherapy or radiation before surgery. A total of 75 such patients were identified for whom tissue samples and follow-up information

were available. Patients' clinicopathologic data were collected and follow-up data were updated through December 31, 2005 by reviewing medical records and the U.S. Social Security Index. The use of archival paraffin-embedded tissue blocks and chart reviews were approved by the Institutional Review Board Committee of M. D. Anderson Cancer Center. Tissue microarrays were constructed using formalin-fixed, paraffin-embedded archival tissue blocks from these 75 PDACs using the method as described previously (22). The H&E-stained slides were reviewed and screened for most representative areas of the tumor and their matched paraffin blocks were retrieved. Each tumor and adjacent benign pancreatic tissue were sampled in duplicate with 1.0-mm tissue cores to include two tissue cores from the tumor and two tissue cores from the paired benign pancreatic tissue. In addition, nine different human pancreatic adenocarcinoma cell lines were also included to serve as controls.

TG2 expression levels in tumor samples were evaluated by an indirect immunoperoxidase procedure (avidin-biotin complex method Elite; Vector Laboratories, Burlingame, CA). In brief, antigen retrieval was done by treating the tissue samples in a steamer for 30 minutes. Antibody (0.5 μg/mL) against TG2 (CUB7401, Neomarkers) overlaying the tissue section was incubated at 4°C for 16 hours. The secondary antibody incubation was done at ambient temperature for 1 hour. Mayer's

hematoxylin nuclear stain was used as a counterstain. Immunostaining results were evaluated and scored independently by a pathologist and laboratory personnel. TG2 expression in tumor cells was categorized as negative (low or weak cytoplasmic staining) or positive (diffuse moderate to strong cytoplasmic staining).

Statistical analysis. The clinicopathologic and follow-up data were correlated with TG2 expression. The statistical analysis was done using Fisher's exact test and Student's *t* test with SPSS software (version 12 for Windows; SPSS, Chicago, IL) and a $P < 0.05$ was considered significant.

Results

TG2 expression is associated with gemcitabine resistance and increased invasive potential. Western blot analysis revealed elevated expression of the TG2 protein in all 12 PDAC cell lines tested. However, no TG2 was detected in immortalized pancreatic ductal epithelial cells (E6E7; Fig. 1A and B). Interestingly, BxPC-3 and HPAF-II cell lines, which represent the well-differentiated and relatively less aggressive PDAC cell lines, expressed lower TG2 levels than did the aggressive and undifferentiated Panc-28, Capan-2, and AsPc-1 cell lines.

Based on our earlier observations that elevated TG2 expression in cancer cells contributes to the development of the drug-resistant phenotype (8, 23), we next determined whether TG2 expression in

PDAC cells is related to gemcitabine resistance. We used three cell lines, Panc-28, MiaPaCa-2, and BxPC-3, which expressed different basal levels of TG2 protein (Fig. 2A). Panc-28 cells expressed highest basal level of TG2 protein and activity followed by MiaPaCa-2 and BxPC-3 cells ($P < 0.05$; Fig. 2A). The ability of gemcitabine to inhibit cell growth of these cell lines was inversely related to the level of TG2 expression (Fig. 2B). The high TG2-expressing Panc-28 cells showed minimal sensitivity to the gemcitabine-induced growth inhibition as determined by the MTS cell viability assay. The IC_{50} of gemcitabine for Panc-28 cells was one log higher (1 $\mu\text{mol/L}$) than that for MiaPaCa-2 cells (IC_{50} , 0.1 $\mu\text{mol/L}$; $P = 0.02$) and approximately two logs higher than that for TG2-deficient BxPC-3 cells (IC_{50} , 0.01 $\mu\text{mol/L}$; $P = 0.001$).

We next determined whether the level of TG2 expression in PDAC cells affected their invasive potential. The three cell lines that constitutively expressed different levels of TG2 (Fig. 2A) were compared for their ability to invade through Matrigel-Transwell membranes. Results shown in Fig. 2C suggested that high TG2-expressing Panc-28 cells are 3- to 4-fold more invasive than the low TG2-expressing MiaPaCa-2 and BxPC-3 cells ($P = 0.0002$).

To further confirm the observation that TG2 overexpression increased the invasive potential and confer resistance to gemcitabine in PDAC cells, we overexpressed the WT (TG2wt) and

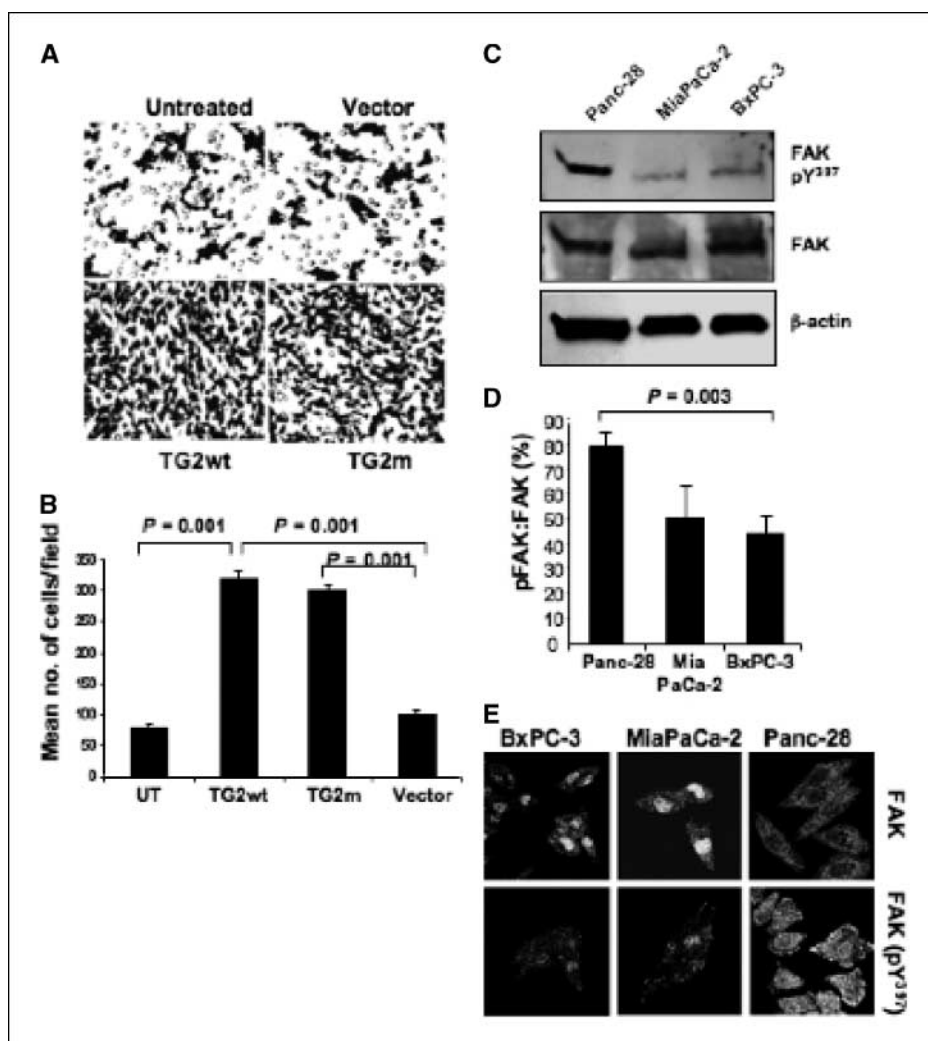
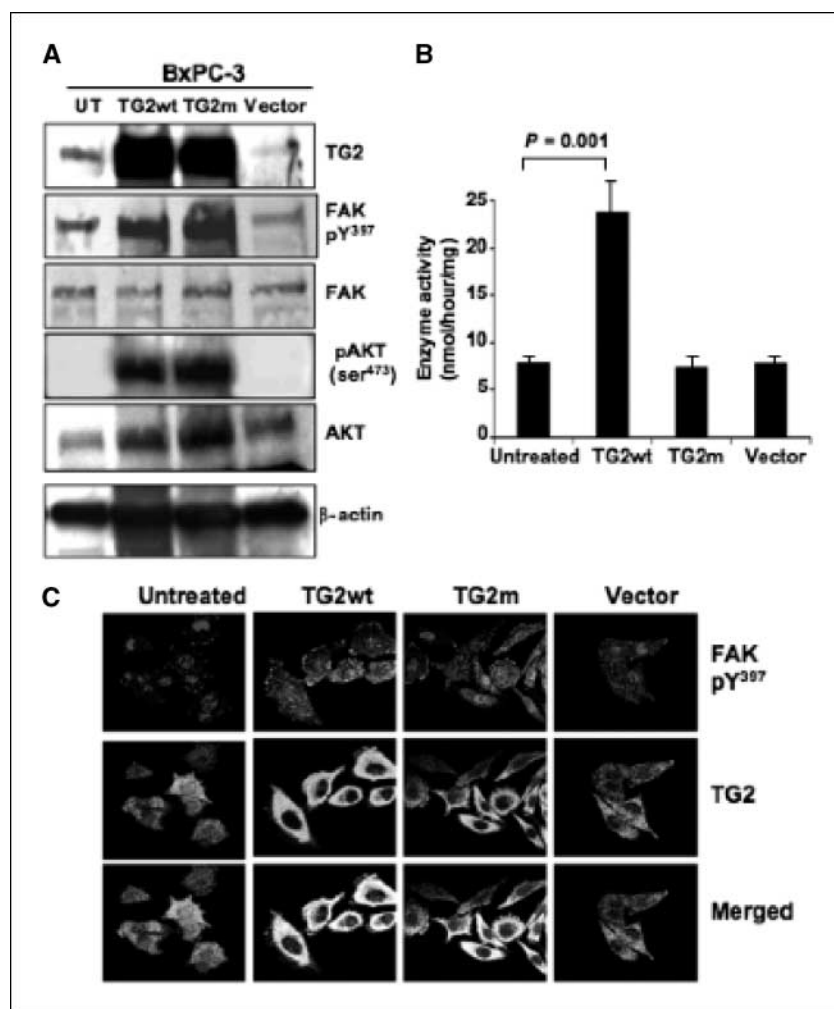


Figure 3. TG2 expression is associated with invasion and constitutive activation of FAK. *A*, invasive ability of untreated BxPC-3 cells or cells infected with either WT (TG2wt) or active-site (C₂₇₇S) mutant (TG2m) of TG2 through the Matrigel-Transwell membranes after 48 hours of incubation. Cells infected with the vector alone (vector) served as control. *B*, mean number of TG2-infected or control BxPC-3 cells in 10 random microscopic fields was calculated and plotted. Representative of two to three independent experiments with similar results. *C*, Western blot analysis was done to determine constitutively active FAK (pY³⁹⁷) in the three PDAC cell lines that expressed variable TG2 levels. The membrane was stripped and reprobbed with an anti-FAK antibody to determine total FAK or with anti- β -actin antibody to assure even loading of total proteins in each lane. *D*, the intensities of pFAK (pY³⁹⁷) and total FAK bands for each cell line were calculated using a densitometer and plotted as a percentage ratio between pFAK and total FAK. *E*, confocal microscopy images of the three PDAC cell lines showing total FAK and pFAK (pY³⁹⁷) expression. Representative experiments done at least two to three times with similar results. Columns, mean; bars, SD.

Figure 4. TG2 expression results in activation of FAK and PI3K/AKT pathway. **A**, Western blot analysis showing basal expression of TG2 in BxPC-3 cells after they had been infected with an adenovirus containing WT (TG2wt) or C277S mutant (TG2m) construct. Untreated (UT) BxPC-3 cells and cells infected with adenovirus alone (vector) served as the control. The membranes were stripped and reprobed with anti-pFAK (pY³⁹⁷), total FAK, pAKT (Ser⁴⁷³), or total AKT antibody. Finally, the membranes were probed with β -actin antibody to assure even loading of proteins in each lane. **B**, the enzymatic activity in untreated and WT (TG2wt) or mutant (TG2m) TG2 or empty adenovirus (vector)-infected BxPC-3 cells was also determined and plotted as the specific activity (nmol/h/mg protein). **C**, confocal microscopy images of BxPC-3 cells showing constitutive (untreated) expression of pFAK (pY³⁹⁷; red fluorescence) and TG2 (green fluorescence) before and after infection with the WT (TG2wt) or C277S mutant (TG2m) TG2 adenoviral construct. Yellow fluorescence in the merged images indicates colocalization of TG2 and pFAK. Representative experiments repeated at least twice with similar results. Columns, mean of quadruplicate values; bars, SD.



catalytically inactive (TG2m) mutant of TG2 in BxPC-3 cells. TG2m lacks transamidation activity because of a point mutation in the active site cysteine residue (C₂₇₇S). The results shown in Fig. 3A and B suggested that overexpression of either TG2wt and TG2m strongly promotes the invasive potential of BxPC-3 cells. Moreover, BxPC-3 cells infected with TG2wt adenoviral construct became significantly more resistant to gemcitabine-induced cytotoxicity when compared with the adenovirus alone-infected cells (IC₅₀, 0.1 μ mol/L; $P = 0.03$; data not shown). Together, these results suggested that overexpression of TG2 contributes not only to gemcitabine resistance but also to the invasive potential of PDAC cells, which was independent of its transamidation activity.

TG2 induces activation of FAK and PI3K/AKT pathways. FAK is a nonreceptor tyrosine kinase that is activated by integrin clustering and transmits adhesion-dependent signals to promote growth, survival, and invasive functions in cells. Our previous studies using breast cancer and melanoma cancer cells showed an association between TG2 and β integrins (8, 9). Therefore, in this study, we determined the significance of TG2 expression in regulation of FAK functions. First, we compared the FAK basal activity in serum-starved Panc-28, MiaPaCa-2, and BxPC-3 cells, which expressed different levels of TG2. The results shown in Fig. 3C show a direct association between levels of TG2 expression and constitutive FAK activation [phosphorylated (pY³⁹⁷)]. Panc-28 cells with high basal expression of TG2 had high levels of activated

FAK, whereas FAK activation was lower in MiaPaCa-2 and BxPC-3 cells, which had low TG2 expression (Fig. 3C and D). Confocal microscopic analysis further supported these observations and revealed active form of FAK at focal adhesion points in TG2-rich Panc-28 cells than in low TG2-expressing MiaPaCa-2 and BxPC-3 cells (Fig. 3E). The Western blot and confocal microscopic analyses showed no significant difference in total FAK expression in Panc-28, MiaPaCa-2, and BxPC-3 cells (Fig. 3C and E). These results suggest that TG2 is associated with activation of FAK in PDAC cells.

To further delineate the relationship between TG2 and FAK, we overexpressed TG2 by infecting BxPC-3 cells with 25 MOI of an adenovirus-containing TG2 WT (TG2wt) construct. As expected, TG2 expression in infected BxPC-3 cells increased by 10- to 12-fold over the basal level as determined by Western blotting and confocal microscopy (Fig. 4A and C). Overexpression of TG2 was associated with a parallel increase in the enzyme activity (Fig. 4B) and constitutive activation of FAK in BxPC-3 cells. The infection of cells with adenovirus vector alone had no effect on TG2 expression or FAK activation. Interestingly, overexpression of TG2m, which lacks the enzyme activity, also resulted in an increase in FAK activation similar to that induced by TG2wt (Fig. 4A and B). These results suggested that TG2-mediated activation of FAK occurs independent of its transamidation activity. This was further supported by confocal microscopy data (Fig. 4C). Interestingly, the TG2-mediated activation of FAK was associated with an increase in the

colocalization of TG2 with pFAK at the focal adhesion points (Fig. 4C).

Because FAK activation results in the activation of the downstream PI3K/AKT and RAS/MAPK/ERK kinase (MEK)/ERK pathways, we next determined the effect of TG2 expression on these pathways. Western blot analysis of untreated BxPC-3 cells or BxPC-3 cells infected with either TG2wt or TG2m showed marked increase in pAKT (Ser⁴⁷³) in infected cells (Fig. 4A). However, no change in the levels of pERK1/2 was observed in TG2-infected or untreated cells (data not shown). These results suggest that TG2 expression, independent of its transamidation activity, can result in constitutive activation of FAK/PI3K/AKT pathway and promote the invasive function of PDAC cells.

Down-regulation of TG2 inhibits FAK activation. To further determine the role of TG2 in FAK activation, we used siRNA approach to down-regulate the endogenous expression in Panc-28 cells. As reported previously (8, 9), siRNA1 and siRNA2 caused 80% to 90% inhibition of endogenous TG2 protein expression in Panc-28 cells (Fig. 5A). The down-regulation of TG2 expression by siRNA resulted in inactivation of FAK (Fig. 5A). However, transfection of Panc-28 cells under similar conditions with control siRNA did not alter either TG2 expression or FAK activation (Fig. 5A). Down-regulation of TG2 in Panc-28 cells also resulted in marked morphologic changes by light microscopy (Fig. 5B): the cells appeared more cylindrical in shape and segregated than were untreated controls. More importantly, the inhibition of endogenous TG2 by siRNA induced massive cytoplasmic vacuolization after 48 hours of transfection (Fig. 5B), and the cells eventually died. In addition, the down-regulation of endogenous TG2 was associated with a profound inhibition of Panc-28 cells to invade through Matrigel-Transwell membranes (data not shown). These results clearly showed that TG2 expression plays a critical role in the constitutive activation of FAK and its downstream functions and that TG2 expression is critical for the survival of Panc-28 cells.

Association of TG2 with FAK. We further investigated the colocalization of TG2 with pFAK in PDAC cells. In a pull-down experiment, the immunoprecipitates of cell extracts from Panc-28, MiaPaCa-2, and BxPC-3 PDAC cells with an anti-FAK antibody revealed the presence of TG2 when the membranes were probed with anti-TG2 antibody (Fig. 6A). The association of TG2 with FAK in PDAC cells was further confirmed by reverse coimmunoprecipitation using an anti-TG2 antibody and probing with a FAK antibody (data not shown). We found no appreciable difference in total FAK expression in Panc-28, MiaPaCa-2, and BxPC-3 cells. However, Panc-28 cells, which expressed high pFAK and TG2, also showed higher degree of association between TG2 and pFAK than did the MiaPaCa-2 and BxPC-3 cells (Fig. 6A). These results suggested that a direct association between TG2 and FAK may play a role in constitutive activation of FAK.

To further delineate the role of TG2 FAK activation, we determined the localization of TG2 with FAK in soluble and membranous cellular compartments. Membranous and cytosolic fractions from Panc-28 cells were immunoprecipitated with either anti-TG2 or anti-FAK antibody and probed with both antibodies. The results shown in Fig. 6B show that ~80% of the total TG2 protein was present in the cytosolic fraction and 15% was in the membrane. Notably, the fraction of TG2 bound with FAK was higher in the membrane fraction (77%) than in the cytosolic fraction (33%; Fig. 6B). Because activated FAK is mainly localized in the cytoplasmic membrane, we speculated that TG2 in the membrane was associated with the phosphorylated form of FAK.

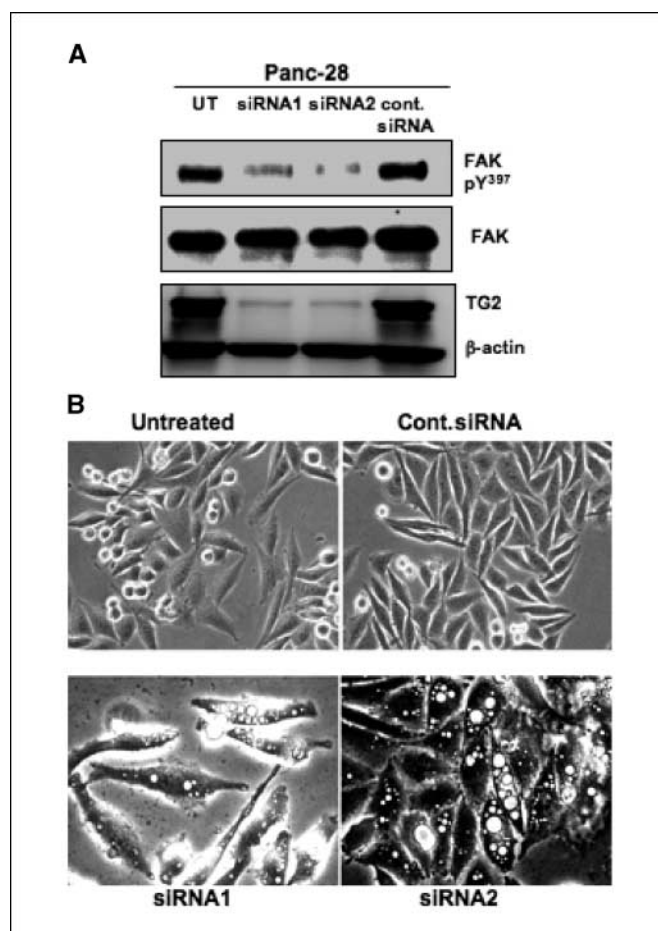


Figure 5. Down-regulation of TG2 results in decreased pFAK. **A**, endogenous expression of TG2 in Panc-28 cells was inhibited by siRNA1 and siRNA2 transfection as described in Materials and Methods. After 48 hours of transfection, cells were harvested, and cell lysates were subjected to Western blotting to determine TG2 expression. Untreated cells and cells transfected with scrambled siRNA (*cont. siRNA*) were used as controls. Membranes were stripped and reprobed with anti-pFAK (pY³⁹⁷) and anti-FAK antibody. β -Actin was also determined to ensure even loading of proteins in each lane. **B**, morphologic changes induced in Panc-28 cells in response to down-regulation of endogenous TG2 by siRNA1 and siRNA2 or scrambled siRNA. Cells were treated with siRNA for 48 hours and viewed under the phase-contrast microscope. Magnification, $\times 400$. Representative results of three independent experiments.

This contention was supported by the results of immunoprecipitation of Panc-28 cell extracts with an anti-pFAK (pY³⁹⁷) antibody. As shown in Fig. 6C, TG2 is indeed associated with pFAK. The results of a confocal microscopy analysis (Fig. 6D) further supported the colocalization of TG2 with pFAK, particularly at the focal adhesion points. The colocalization of TG2 and pFAK at focal points was quantified in 40 cells, showing a mean number of 6.2 overlaps at focal points per cell. These results show, for the first time, that there is a direct association between TG2 and the signaling molecule FAK and that this association plays an important role in FAK activation.

TG2 expression in PDAC tumor samples. To further evaluate the significance of our *in vitro* observations that TG2 expression results in gemcitabine resistance and invasive potential, we examined the expression levels of TG2 in a human tissue microarray containing 75 PDAC tissue samples. We found high basal levels of TG2 expression in 42 of the 75 (56%) tumor samples

studied (Fig. 7A). However, only 24 of 70 (34%) cases showed cytoplasmic staining in benign pancreatic ducts. TG2 expression was significantly higher in PDACs compared with normal ducts ($P = 0.012$). No staining was observed in sections treated with the isotypic control IgG instead of the primary anti-TG2 antibody (data not shown). High TG2 expression in tumor samples was significantly associated with nodal metastasis [32 of 48 node-positive tumor samples were TG2 positive versus 10 of 27 node-negative tumor samples; odds ratio (OR), 3.400; $P = 0.017$], lymphovascular invasion (33 of 51 lymphovascular invasive tumors were TG2 positive versus 9 of 24 noninvasive tumors; OR, 3.055; $P = 0.045$), and clinical stage (30 of 45 stage IIb PDACs were TG2 positive versus 10 of 26 stage IIa tumors; OR, 3.200; $P = 0.027$). These data further supported our observations that TG2 over-expression increases the invasive potential of PDAC cell lines. A Kaplan-Meier estimate of survival in relation to TG2 expression showed no significant difference in TG2-positive patients ($n = 42$) and TG2-negative patients ($n = 33$; $P = 0.159$; Fig. 7B).

Discussion

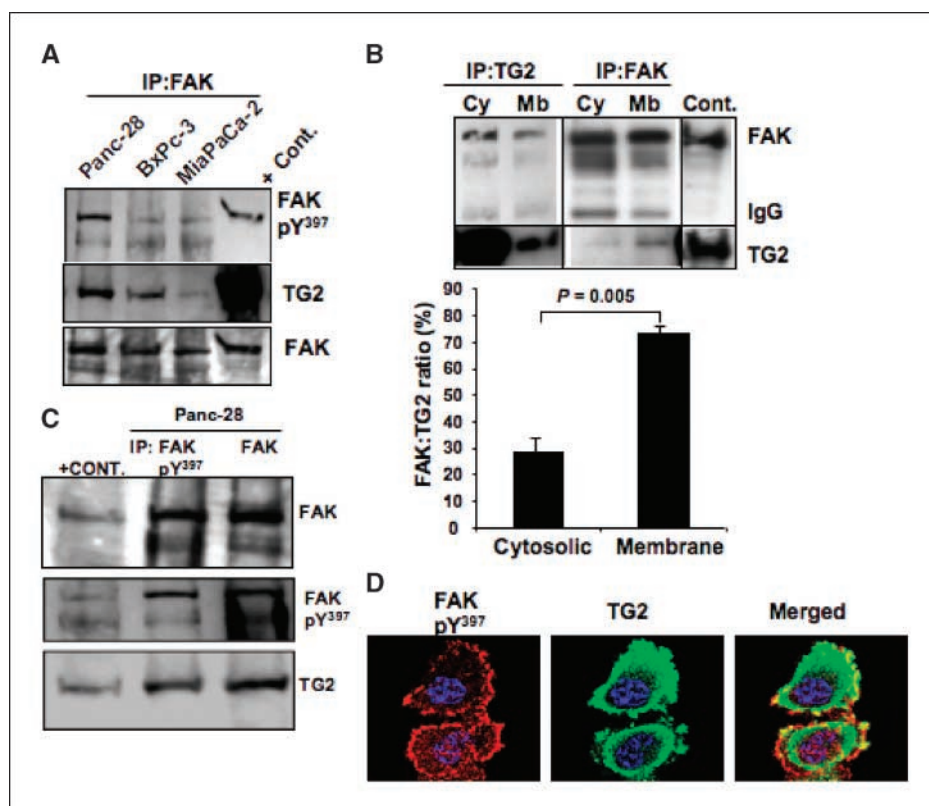
Our data show that a significant majority of PDACs and PDAC cell lines express elevated levels of the multifunctional protein TG2. We provide the first evidence that TG2 is closely associated with FAK and results in its activation (pFAK) and activation of the downstream PI3K/AKT cell survival signaling pathway. TG2 expression in PDAC cell lines was associated with increased resistance to gemcitabine and an invasive phenotype. Furthermore, we found significant association between TG2 expression and nodal metastasis, lymphovascular invasion, and late clinical stage in PDAC tumor samples.

Previously, we found that constitutive expression of TG2 in drug-resistant and metastatic breast cancer (8) and late-stage melanoma (9) was up-regulated. Importantly, down-regulation of TG2 expression was associated with the increased sensitivity of cancer cells to apoptotic stimuli and reversal of the drug-resistant phenotype (8). Han and Park (23) reported similar results in the drug-resistant lung cancer cell line PC-14/ADR. These authors showed that the acquisition of multidrug resistance in PC-14 cells was associated with a 10- to 15-fold increase in TG2 expression and that inhibition of TG2 expression by TG2-specific antisense or ribozyme rendered the cells sensitive not only to multidrug resistance-related drugs but also to other anticancer drugs.

In this study, we determined the constitutive TG2 expression status of PDAC tumors and tumor cell lines. Of the 12 PDAC cell lines tested, 10 expressed high levels of TG2 protein, and two expressed moderate levels (Fig. 1). Importantly, 56% of the PDAC tumor samples showed elevated levels of TG2, which was associated with nodal metastasis, lymphovascular invasion, and late clinical stage. In cell lines, TG2 expression was associated with resistance to gemcitabine (Fig. 2B), a commonly used drug for the treatment of pancreatic cancer. Similarly, high TG2 expression was associated with invasive behavior (Fig. 2C). Thus, ectopic expression of TG2 in BxPC-3 cells rendered the cells highly invasive (Fig. 4D) and resistant to gemcitabine and, conversely, the inhibition of TG2 by siRNA significantly attenuated the invasive potential of Panc-28 cells.

To delineate the possible mechanism by which TG2 could promote chemoresistance and invasive potential in PDAC cells, we studied the relationship between TG2 and FAK. TG2 has been shown to promote FAK activation via its ability to promote the integrin-mediated attachment of cells to fibronectin (8, 14), a major

Figure 6. TG2 associates with pFAK in the cell membrane. **A**, total cell lysates prepared from Panc-28, BxPC-3, and MiaPaCa-2 cells were immunoprecipitated (IP) using an anti-FAK antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blotting using either anti-pFAK (pY³⁹⁷), anti-TG2, or anti-FAK antibody. The whole-cell extract from Panc-28 cells (+Cont.) was used as a positive control. **B**, the membrane (Mb) and cytosolic (Cy) fractions were prepared from Panc-28 cells, as described in Materials and Methods, and immunoprecipitated using an anti-human FAK and anti-TG2 mAb. The immunoprecipitates were subjected to SDS-PAGE and Western blotting, and the membranes were probed with anti-FAK or anti-TG2 antibody. The FAK and TG2 bands were scanned by a densitometer, and ratios were plotted as a percentage of total FAK. **C**, total cell lysates from Panc-28 cells were immunoprecipitated using anti-FAK (pY³⁹⁷) antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blotting using anti-FAK, anti-pFAK (pY³⁹⁷), or anti-TG2 antibody. The whole-cell extracts from Panc-28 cells and immunoprecipitates using anti-FAK antibody was used as a positive control. **D**, confocal microscopy images of Panc-28 cells showing colocalization of pFAK (red fluorescence; pY³⁹⁷) and TG2 (green fluorescence), as evidenced by the yellow fluorescence in the merged image. Representative results of two to three experiments.



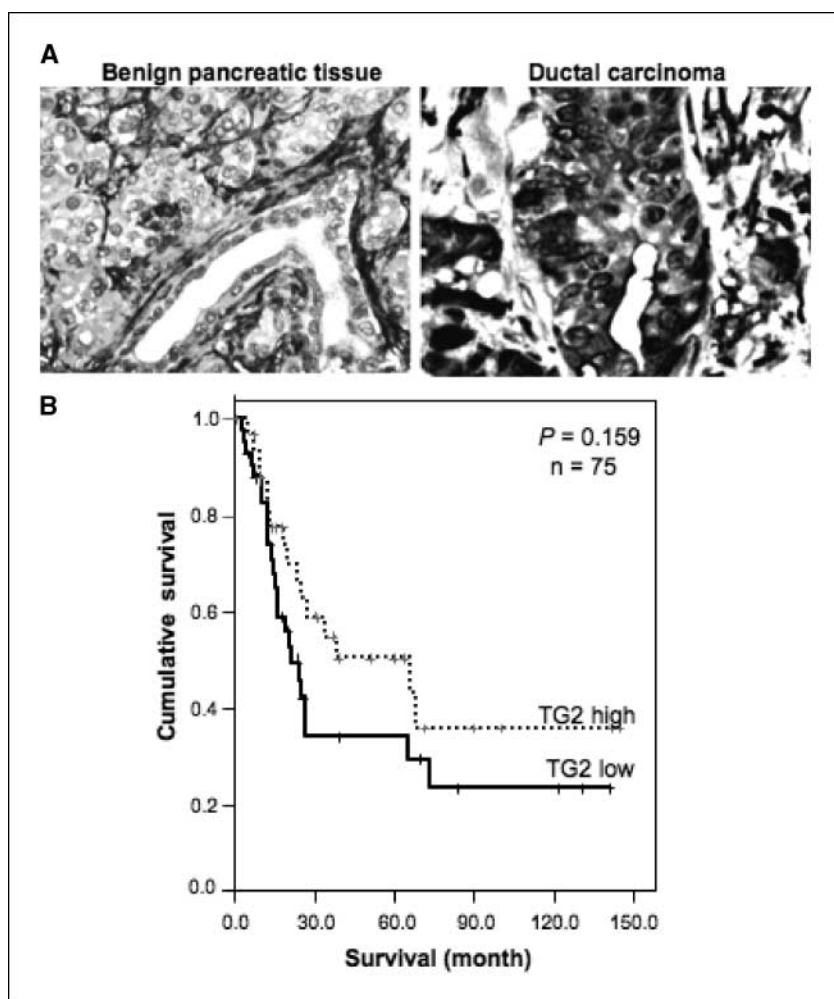


Figure 7. TG2 expression in PDAC samples. *A*, representative micrographs showing no TG2 expression in benign pancreatic ductal epithelium and acinar cells. *Left*, strong positive staining for TG2 is present in normal stromal cells; *right*, PDACs are strongly positive for TG2 staining. Original magnification, $\times 400$. *B*, Kaplan-Meier curve showing the relationship between cumulative proportion of survival to TG2 expression in 75 PDAC patients.

component of the extracellular matrix in tumor microenvironments. FAK is a nonreceptor cytoplasmic protein tyrosine kinase that provides signaling and scaffolding functions at sites of integrin adhesion. Ample evidence supports the role of FAK in cell adhesion, cell migration, and cell cycle progression (24). Increased FAK expression is frequently associated with malignant or metastatic disease and poor patient prognosis (24–30). FAK expression is increased in various tumors, such as those of the breast, colon, thyroid, head and neck, ovaries, liver, esophagus, and pancreas (24–30). The activated form of FAK constitutes tyrosine phosphorylation at the COOH terminus (Y^{397}). This results in the phosphorylation of other tyrosine residues (Y^{397} , Y^{407} , Y^{576} , Y^{577} , Y^{861} , and Y^{925}), leading to the activation of several downstream signaling pathways, such as RAS/ERK, PI3K/AKT, and Crk/Dock180/Rac (ref. 24 and references therein).

The data presented here suggest a novel pathway that could lead to the constitutive activation of FAK in pancreatic and possibly other aggressive cancer cells. TG2 was closely associated with FAK protein. The interaction of TG2 with FAK was associated with FAK autophosphorylation (pY^{397} ; Fig. 4). Indeed, ectopic expression of TG2 in BxPC-3 cells resulted in enhanced FAK activation (Fig. 4C) and FAK colocalization with TG2 in the focal points (Fig. 6D). Conversely, the inhibition of TG2 by siRNA inhibited FAK activation in Panc-28 cells (Fig. 5A). The major fraction of activated FAK was localized in the membrane in complex with TG2 (Fig. 6B).

The ability of TG2 to associate with FAK and subsequent phosphorylation of FAK were independent of TG2 transamidation activity, as suggested by the observation that the expression of catalytically inactive point-mutated (C_{277S}) TG2 could result in a similar association and activation of FAK (Fig. 4A). Together, our results suggest a role for TG2 in FAK autophosphorylation that is mediated by a direct association between the two proteins. How the association of TG2 with FAK affects autophosphorylation of FAK remains a matter of speculation. Indeed, it is well known that the recruitment of FAK and its association with the cytoplasmic tails of integrins result in its autophosphorylation (24). Similarly, it is likely that the conformational changes induced in FAK in response to its interaction with TG2 can result in its autophosphorylation. Alternatively, the association of TG2 with pFAK might protect it from the action of endogenous phosphatases, such as PTEN (31). We are currently investigating these and other possibilities to determine the mechanisms that underlie the phosphorylation of FAK in response to its interaction with TG2.

Furthermore, TG2-mediated FAK activation was associated with the activation of PI3K/AKT pathway but had no effect on the RAS/MEK/ERK pathway (Fig. 4A). These observations are supported by an earlier report by Kang et al. (32) who showed that overexpression of TG2 in human chronic myelogenous leukemia (K562) cells was associated with activation of the PI3K/AKT signaling pathway (33). The significance of the PI3K/AKT pathway

in conferring an aggressive phenotype to cancer cells has been well established (32, 34, 35). Indeed, our earlier studies with drug-resistant and metastatic breast cancer cells revealed that down-regulation of TG2 expression renders the cells sensitive not only to anticancer drugs but also to stress-induced apoptosis (8, 9). In the present study, the inhibition of endogenous TG2 by siRNA induced a massive accumulation of cytoplasmic vacuoles (Fig. 5B) that eventually led to the death of Panc-28 cells via autophagy.⁵

Overall, the results of our study show that elevated expression of TG2 in PDAC tumor cells and cell lines contributes to increased cell survival and invasive functions. One mechanism that likely contributes to these effects is the ability of TG2 to associate with

and activate FAK, resulting in the activation of its downstream prosurvival PI3K/AKT signaling pathway. In conclusion, our results suggest that TG2 could be an attractive target for anticancer therapeutics, and they provide a strong rationale for developing TG2 inhibitors to target the disruption of TG2-FAK protein-protein interactions.

Acknowledgments

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⁵ A. Ugur, et al., submitted for publication.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
- Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* 1992;80:879–86.
- Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994; 73:2013–26.
- White E. Life, death, and the pursuit of apoptosis. *Genes Dev* 1996;10:1–15.
- Bold RJ, Chandra J, McConkey DJ. Gemcitabine-induced programmed cell death (apoptosis) of human pancreatic carcinoma is determined by Bcl-2 content. *Ann Surg Oncol* 1999;6:279–85.
- Shi X, Liu S, Kleeff J, Friess H, Buchler MW. Acquired resistance of pancreatic cancer cells towards 5-fluorouracil and gemcitabine is associated with altered expression of apoptosis-regulating genes. *Oncology* 2002;62:354–62.
- Mehta K, Fok JY, Mangala LS. Tissue transglutaminase: from biological glue to cell survival cues. *Front Biosci* 2006;11:173–85.
- Herman JF, Mangala LS, Mehta K. Implications of increased tissue transglutaminase (TG2) expression in drug-resistant breast cancer (MCF-7) cells. *Oncogene* 2006;25:3049–58.
- Fok J, Ekmekcioglu S, Mehta K. Implications of tissue transglutaminase expression in malignant melanoma. *Mol Cancer Ther* 2006;5:1493–503.
- Mehta K, Fok J, Miller FR, Koul D, Sahin AA. Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. *Clin Cancer Res* 2004;10:8068–76.
- Mehta K. Mammalian transglutaminases: a family portrait. *Prog Exp Tumor Res* 2005;38:1–18.
- Fesus L, Piacentini M. Transglutaminase 2: an enigmatic enzyme with diverse functions. *Trends Biochem Sci* 2002;27:534–9.
- Lorand L, Graham R. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Mol Cell Biol* 2003;4:140–56.
- Akimov SS, Krylov D, Fleishmann LF, Belkin AM. Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. *J Cell Biol* 2000;148:825–38.
- Mohan K, Pinto D, Issekutz TB. Identification of tissue transglutaminase as a novel molecule involved in human CD8⁺ T cell transendothelial migration. *J Immunol* 2003;171:3179–86.
- Priglinger SG, Alge CS, Neubauer AS, et al. TGF- β -induced cell surface tissue transglutaminase increases adhesion and migration of RPE cells on fibronectin through the gelatin-binding domain. *Invest Ophthalmol Vis Sci* 2004;45:955–63.
- Singh US, Pan J, Kao YL, Joshi S, Young KJL, Baker KM. Tissue transglutaminase mediates activation of RhoA and MAP kinase pathways during retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *J Biol Chem* 2003;278:391–9.
- Wakshlag JJ, McNeill CJ, Antonyak MA, et al. Expression and activity of transglutaminase II in spontaneous tumours of dogs and cats. *J Comp Pathol* 2006;134:202–10.
- Jiang WG, Ablin R, Douglas-Jones A, Mansel RE. Expression of transglutaminases in human breast cancer and their possible clinical significance. *Oncol Rep* 2003;10:2039–44.
- Chen JS, Agarwal N, Mehta K. Multidrug-resistant MCF-7 breast cancer cells contain deficient intracellular calcium pools. *Breast Cancer Res Treat* 2002;71:237–47.
- Mangla LS, Arun B, Sahin AA, Mehta K. Tissue transglutaminase-induced alterations in extracellular matrix inhibit tumor invasion. *Mol Cancer* 2005;4:33.
- Wang H, Wang H, Zhang W, Fuller GN. Tissue microarrays: applications in neuropathology research, diagnosis, and education. *Brain Pathol* 2002;12:95–107.
- Han JA, Park SC. Reduction of transglutaminase 2 expression is associated with an induction of drug sensitivity in the PC-14 human lung cancer cell line. *J Cancer Res Clin Oncol* 1999;125:89–95.
- McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer—a new therapeutic opportunity. *Nat Rev Cancer* 2005;5:505–15.
- Cance WG, Harris JE, Iacocca MV, et al. Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. *Clin Cancer Res* 2000;6:2417–23.
- Owens LV, Xu L, Dent GA, et al. Focal adhesion kinase as a marker of invasive potential in differentiated human thyroid cancer. *Ann Surg Oncol* 1996;3:100–5.
- Agochiya M, Brunton VG, Owens DW, et al. Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. *Oncogene* 1999;18:5646–53.
- Judson PL, He X, Cance WG, et al. Overexpression of focal adhesion kinase, a protein tyrosine kinase, in ovarian carcinoma. *Cancer* 1999;86:1551–6.
- Miyazaki T, Kato H, Nakajima M, et al. FAK overexpression is correlated with tumour invasiveness and lymph node metastasis in oesophageal squamous cell carcinoma. *Br J Cancer* 2003;89:140–5.
- Furuyama K, Doi R, Mori T, et al. Clinical significance of focal adhesion kinase in resectable pancreatic cancer. *World J Surg* 2006;30:219–26.
- Tamura M, Gu J, Matsumoto K, Aota S, Parsons R, Yamada KM. Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* 1998;280:1614–7.
- Kang SK, Lee JY, Chung TW, Kim CH. Overexpression of transglutaminase 2 accelerates the erythroid differentiation of human chronic myelogenous leukemia K562 cell line through PI3K/AKT signaling pathway. *FEBS Lett* 2004;577:361–6.
- Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T. Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* 2000;275:16309–15.
- Goswami A, Ranganathan P, Rangnekar VM. The phosphoinositide 3-kinase/Akt1/Par-4 axis: a cancer-selective therapeutic target. *Cancer Res* 2006;66:2889–92.
- Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* 2005;4:988–1004.