Tissue Transglutaminase Regulates Focal Adhesion Kinase/AKT Activation by Modulating PTEN Expression in Pancreatic Cancer Cells

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Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) progresses rapidly and exhibits profound resistance to treatment. We recently reported that a great majority of PDAC tumors and tumor cell lines express elevated levels of tissue transglutaminase (TG2). Here, we provide first evidence that TG2 expression in PDAC cells results in constitutive activation of focal adhesion kinase/AKT by modulating the expression of the tumor suppressor phosphatase PTEN.

Experimental Design: Using PDAC cell lines, we determined the effect of TG2 overexpression on PTEN stability and functions. We confirmed the correlation between TG2 expression and PTEN levels in a few ($n = 51$) PDAC tumor samples.

Results: We observed that expression of TG2 is inversely correlated with PTEN expression in PDAC cells. Ectopic expression of TG2 inhibited PTEN phosphorylation and promoted its degradation by ubiquitin-proteasomal pathway. Conversely, down-regulation of TG2 by small interfering RNA up-regulated PTEN expression. Clinical relevance of these results was evident in an athymic nude mouse model where down-regulation of endogenous TG2 caused a significant retardation in PDAC xenograft growth. Importantly, the analysis of 51 tumor samples from patients with stage II PDAC revealed that overexpression of TG2 was associated with loss of PTEN expression ($P = 0.023$; odds ratio, 4.1). In multivariate analysis, TG2-mediated loss of PTEN was a prognostic factor for overall survival in patients with stage II pancreatic ductal carcinoma independent of tumor stage/lymph node status and tumor differentiation ($P = 0.01$).

Conclusion: TG2 expression in PDAC promotes degradation of PTEN by ubiquitin-proteasomal pathway and results in constitutive activation of focal adhesion kinase/AKT cell survival signaling.

Approximately 33,000 new cases of pancreatic ductal adenocarcinoma (PDAC) are diagnosed each year in the United States and nearly same number of patients die from the disease (1). At the time of diagnosis, most patients have disseminated disease and exhibit marked resistance to radiation and chemotherapy, leaving surgical resection the only hope for cure. Unfortunately, the disease is so aggressive that only 10% to 20% patients with the disease are surgically resectable. Therefore, understanding the molecular factors that contribute to the development of intrinsic resistance of PDAC to chemotherapy could yield better treatment options.

The observation of increased tissue transglutaminase (TG2) expression in drug-resistant and metastatic cancer cells (2) has kindled great interest in understanding the contributions of this protein in carcinogenesis. TG2 is a unique multifunctional protein that, in addition to catalyzing calcium-dependent post-translational modification of proteins, can also catalyze calcium-independent protein disulfide isomerase, GTP/ATP hydrolase, and serine/threonine kinase functions (3–8). Recently, we reported that overexpression of TG2 in PDAC is associated with resistance to gemcitabine (9). Increased expression of TG2 in breast and pancreatic cancer cells led to constitutive activation of the nuclear factor-κB and conferred resistance to drugs (10, 11). Accordingly, inhibition of TG2 activity in glioblastoma tumors sensitized them to *N*-nitrosourea chemotherapy (12). We also observed that expression of TG2 in PDAC cells results in constitutive activation of focal adhesion kinase (FAK) and its downstream phosphatidylinositol 3-kinase (PI3K)/AKT pathway (9). However, the link between PI3K/AKT activation and TG2 expression was not elucidated. Because the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10, also called *MMAC1* or *TEP1*) protein is a negative regulator of FAK and PI3K/AKT signaling pathways (13, 14), we reasoned that TG2 might affect FAK/AKT activation by regulating PTEN expression or function.

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The results reported here show a novel role for TG2 in regulating PTEN expression. TG2 associates with PTEN and inhibits its phosphorylation at Ser^{380}, the site that is known to affect the stability of PTEN. TG2 promoted the ubiquitination of PTEN and its degradation by proteasomal pathway. Down-regulation of TG2 significantly decreased PDAC tumor growth and tumor volume in nude mice. Similarly, TG2 expression in PDAC tumor samples was associated with down-regulation of PTEN expression and poor survival of patients. To our knowledge, this is the first report documenting the role of TG2 in posttranslational modification of PTEN. TG2 promotes the degradation of PTEN via ubiquitination and results in the activation of FAK/AKT signaling pathway, the events that strongly affect the ability of pancreatic cancer cells to invade and respond to anticancer therapies.

Materials and Methods

Materials. Rabbit polyclonal antibodies to phosphorylated PTEN ($pSer^{380}$), phosphorylated AKT ($pSer^{473}$), total PTEN, and total AKT were purchased from Cell Signaling Technology. Mouse monoclonal antibodies to phosphorylated FAK ($pThr^{397}$) and total FAK were from BD Biosciences PharMingen. Anti-TG2 monoclonal antibody CUB7401 was purchased from NeoMarkers. Anti-ubiquitin monoclonal antibody (P4D1) was from Santa Cruz Biotechnology, Inc. Anti–β-actin antibody was from Sigma Chemical Co. and horseradish peroxidase–conjugated goat anti-rabbit and sheep anti-mouse were purchased from Amersham Biosciences. Trublot anti-mouse and anti-rabbit Ig immunoprecipitation beads were from eBiosciences. DMEM/F12, RPMI 1640, fetal bovine serum, and Normocin antibiotic were all purchased from InvivoGen.

Cell lines. The PDAC cell lines were either purchased from the American Type Culture Collection or provided by Dr. Shrikanh Reddy (The University of Texas M. D. Anderson Cancer Center, Houston, TX). All cell lines were maintained in the log phase of cell growth by culturing in RPMI 1640 or DMEM/F12 medium supplemented with FCS (10%, v/v), Normocin (0.1 mg/mL), L-glutamine (2 mmol/L), and HEPES (10 mmol/L; U.S. Biochemical) at 37°C in a CO_2 incubator.

TG2 short hairpin RNA stable clones. TG2 short hairpin RNA (shRNA) expression plasmid was constructed using the psiSTRIKE neomycin vector, which contains a human U6 promoter and two *Pst*I sites (Promega). For the construction of TG2 hairpin-type small interfering RNA (siRNA) expression plasmids, we constructed oligonucleotides with the hairpin sequence TG2si1382 (GGGCGAACCACCTGAACAA, the number 1382 indicates the initiation codon of the TG2 cDNA; scramble version of this sequence was used as a control), the loop sequence CTTCCTGTCA, and terminator sequences TTTTTC. The fragments were then annealed and inserted into the psiSTRIKE neomycin vector.

When 50% to 60% confluence, Panc-28 cells were transfected with the plasmids in the following manner. A solution of 0.4 mL serum-free medium and 1.0 μg highly purified plasmid DNA was mixed and incubated at room temperature for 5 min. A 5-μL volume of SuperFect reagent (Qiagen) was added, and the mixture was vortexed for 10 s and incubated at room temperature for 5 min. The cells were washed with PBS, the transfection mixture was immediately added, and the cells were incubated for 2 h. The stable clones (Panc-28/cl.10 and Panc-28/cl.12) were selected by culturing transfected cells in the presence of 1.0 mg/mL G418 (InvivoGen).

Invasion assay. The invasive potential of PDAC cell lines was studied *in vitro* by determining the number of cells that invaded through the Matrigel-coated Transwell polycarbonate membrane inserts, as described previously (9). In brief, Transwell inserts with a pore size of 12 μm 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 cell suspension (0.5×10^6 cells) was added to duplicate wells. After 48 h, the number of cells that passed through the filter was stained using Hema-3 stain kit (Fisher Scientific) and counted in 10 random fields under a microscope.

Western blotting. The whole-cell lysates (60 μg) were fractionated by 4% to 15% gradient SDS-PAGE. After SDS-PAGE, the proteins were electrotransferred onto nitrocellulose membranes and probed with appropriate primary and secondary antibodies. Antigen-antibody reaction was detected with Western Lightning chemiluminescence reagent (Perkin-Elmer). Some membranes were stripped using the Restore stripping buffer (Pierce) and reprobed with another antibody. The bands obtained were quantified using AlphaEaseFC (FluorChem 8900) software from Alpha Innotech.

Immunoprecipitation. Cells were lysed in a minimum volume of immunoprecipitation lysis buffer [Tris-HCl buffer (50 mmol/L, pH 8), containing 150 mmol/L NaCl and 1% NP40] and precleared by incubation with 50 μL of Trublot anti-mouse/anti-rabbit Ig immunoprecipitation beads for 1 h at 4°C. The pellet was discarded and the supernatant was subjected to immunoprecipitation. Cell lysates (200 μg protein) were incubated with 2 μg of specific antibody for 1 h at 4°C. Twenty microliters of Trublot anti-mouse/anti-rabbit Ig immunoprecipitation beads were 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 μL of the sample buffer. The samples were fractionated by SDS-PAGE and analyzed by immunoblotting and autoradiography.

Confocal microscopy. Cells were grown on glass coverslips and fixed in 4% paraformaldehyde for 20 min at ambient temperature. Fixed cells were then incubated with the primary antibodies overnight, washed with PBS, and incubated again with secondary antibodies conjugated to either Alexa Fluor 546 (red) or Alexa Fluor 488 (green; Molecular Probes). The DNA dye Topro-3 (Molecular Probes) was used to costain the nuclei (blue). Cells incubated 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.) or an Olympus FluoView 300 confocal microscope 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.

TG2 and PTEN adenovirus. The wild-type and mutant TG2 adenoviral constructs were generated using pcDNA3.1 vector, as described previously (9). 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 cesium chloride gradient and used at 25 multiplicities of infection for different time (0-48 h). Cells infected with LacZ adenovirus served as the control. A recombinant adenovirus containing wild-type MMAC1/PTEN was used, as described previously (15). Cells were infected with adenovirus at 25 multiplicities of infection for 24 h. Cells infected with LacZ adenovirus in parallel served as control.

TG2 knockdown. Human TG2-specific siRNA sequences (siRNA1 and siRNA2) were designed and purchased from Qiagen as described previously (10). A control sequence that is not homologous to any human mRNA (as determined by a BLAST search) served as a control. Transfection with siRNA was done using RNAiFect transfection reagent (Qiagen). The transfection efficiency was determined by transfecting cells in a parallel well with fluorescent siRNA and determining fluorescence uptake under the microscope.

Chase experiment. To determine the effect of TG2 expression on PTEN stability, BxPC-3 cells were infected with TG2 adenoviral construct or with adenovirus alone. Twelve hours after the infection, cells were treated with cycloheximide (50 μg/mL) and cultures were terminated after 0, 6, or 12 h of incubation. The cells were lysed by adding immunoprecipitation lysis buffer and protein concentration was determined. Subsequently, immunoprecipitation and immunoblotting were carried out as described.

Animal studies. Male (4-6 wk old) *nu/nu* mice were obtained from the Experimental Radiation Oncology Animal Breeding Facility of the M. D. Anderson Cancer Center. Groups of five mice were each injected s.c. in the right flank using a 25-gauge needle with Panc-28 cells (2×10^6/100 μL mixed with Matrigel in 1:1 ratio) that had been transfected with either empty vector or TG2 shRNA (Panc-28/cl.10). The growth of xenografts was measured once weekly in two orthogonal dimensions using digital clippers and tumor volume was calculated by the equation $V = (L \times W^2) \times 0.5$, where L is length and W is width of the xenograft. At the end of 6 wk, all the animals were sacrificed by CO_2 asphyxiation, xenografts were harvested, and half were stored in formalin and the other half in liquid nitrogen. The formalin-fixed sections were used for the immunohistochemical analysis and the liquid nitrogen–stored sections were used for Western blot analysis.

Immunohistochemistry. Samples used in this study were obtained from patients with primary PDAC who underwent initial pancreaticoduodenectomy at the M. D. Anderson Cancer Center between 1990 and 2004. None of these patients received preoperative chemotherapy or radiation therapy. A total of 51 such patients with stage II disease (15 female and 36 male patients) were identified for whom tissue samples and follow-up information were available. Clinicopathologic data, such as tumor stages, grades, differentiation, and survival, were collected, and follow-up data were updated through August 31, 2006 by reviewing medical records and the U.S. Social Security Index. The use of archival paraffin-embedded tissue blocks and chart reviews was approved by the Institutional Review Board of M. D. Anderson Cancer Center. Tissue microarrays were constructed using formalin-fixed, paraffin-embedded archival tissue blocks from these 51 PDAC using the method described previously (16). Each tumor was sampled in duplicate with 1.0-mm tissue cores from the most representative areas of the tumor. In addition, nine human PDAC cell lines were included in the tissue microarray to serve as controls.

The expression of TG2 in PDAC samples and xenograft tumors was evaluated by an indirect immunoperoxidase procedure (avidin-biotin complex method Elite, Vector Laboratories) as described previously (9). In brief, antigen retrieval was done by treating the tissue samples in a steamer for 30 min. Antibodies against TG2 and PTEN overlaying the tissue microarray sections at 0.5 μg/mL were incubated at 4°C for 16 h. The secondary antibody incubation was done at ambient temperature for 1 h. Mayer's hematoxylin nuclear stain was used as a counterstain. The immunostained slides were examined under the light microscope and scored double blinded by a pathologist and laboratory researcher. The scoring was done using staining intensity (low, 1; moderate, 2; high, 3) and the positive cancer cells (≤10%, 0; 10-25%, 1; 26-50%, 2; 51-75%, 3; ≥76%, 4). According to the score, tumors were classified either as TG2 overexpressing (overall score, ≥3) or TG2 low/absent expressing (overall score, <3).

Statistical analysis. The patients' follow-up data were correlated with TG2 and PTEN expression. The statistical analysis was done using Student t test, Fisher's exact test, Kaplan-Meier, and log-rank statistic using Statistical Package for the Social Sciences software (version 12 for Windows; SPSS). Two-sided P values were calculated, and $P < 0.05$ was considered significant.

Results

TG2 expression inversely correlates with PTEN. We recently reported that aberrant expression of TG2 in PDAC cells promotes activation of FAK and its downstream PI3K/AKT pathway (9). However, the mechanism by which TG2 affects these functions was not delineated. We hypothesized that TG2 might affect FAK/PI3K/AKT pathway by modulating the expression of PTEN, a lipid/protein phosphatase that is known to inhibit FAK and PI3K/AKT pathways. To test this hypothesis, first, we tested several PDAC cell lines for TG2 and PTEN expression. Using Western blot analysis, we observed that the cell lines that expressed high basal level of TG2 protein contained low levels of PTEN (Fig. 1A). To our knowledge, none of the cell lines that we used harbors PTEN mutation.

We further confirmed the inverse correlation between TG2 and PTEN expression in two stably TG2 shRNA-transfected Panc-28 sublines (Panc-28/cl.10 and Panc-28/cl.12). The two Panc-28 clones showed 70% to 80% decrease in TG2 expression compared with the parental Panc-28 cells (Fig. 1B). Importantly, the decrease in TG2 expression in both the clones was associated with a parallel increase in total PTEN expression (Fig. 1B). This inverse correlation between TG2 and PTEN expression in PDAC cell lines suggested that TG2 might be involved in the regulation of PTEN expression.

TG2 regulates PTEN expression. We next determined the effect of TG2 overexpression on PTEN levels. The BxPC-3 cells (expressing low basal levels of TG2) were infected with an adenovirus construct containing TG2 cDNA. TG2-infected cells

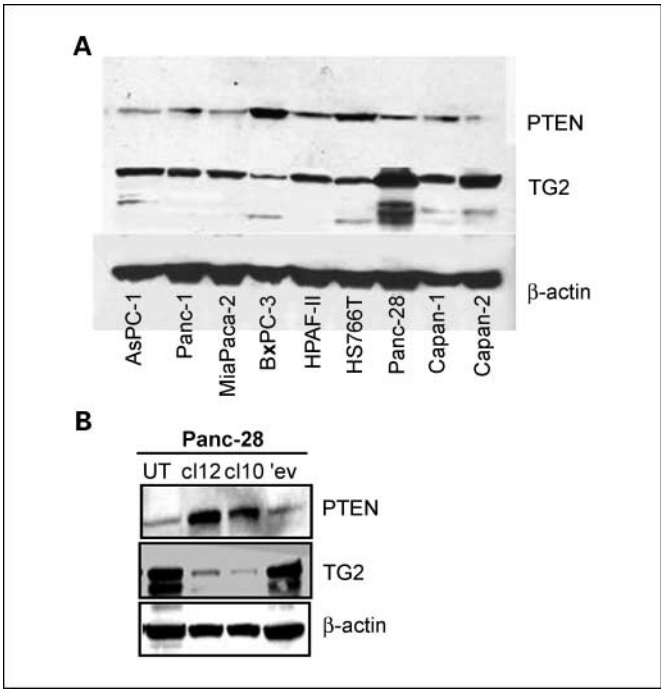

Fig. 1. TG2 expression inversely correlates with PTEN expression. *A,* Western blot analysis was done to determine basal expression of TG2 (*top*) and PTEN (*middle*) expression in indicated PDAC cell lines and one immortalized normal pancreatic epithelial cell line (E6E7). The membranes were stripped and reprobed with anti–β-actin antibody (*bottom*) to ensure even loading of proteins in each lane. *B,* Western blot showing 70% to 80% reduction in TG2 levels in two Panc-28 subclones (cl.12 and cl.10) stably transfected with TG2-specific shRNA. Untreated (*UT*) and vector alone (*ev*)-transfected Panc-28 cells show high basal level of TG2 expression. The membranes were stripped and reprobed with anti-PTEN antibody. Finally, the membranes were probed with β-actin antibody to ensure even loading of proteins in each lane. Results shown are from representative experiments repeated at least twice with similar findings.

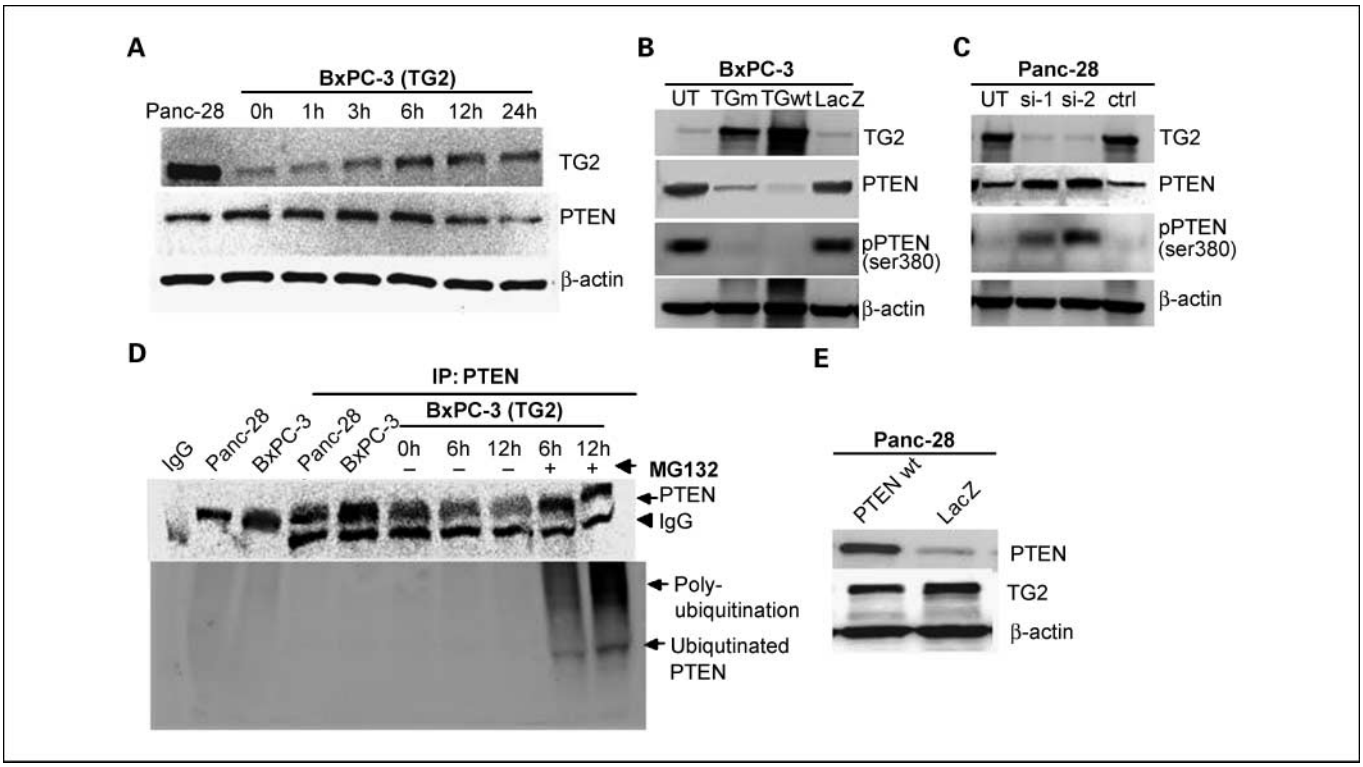

Fig. 2. TG2 regulates PTEN expression. *A,* Western blot analysis showing TG2 and PTEN expression in untreated Panc-28 cells and BxPC-3 cells infected with adeno-TG2 for 0, 1, 3, 6, 12, or 24 h. A noticeable decrease in PTEN expression became evident 12 to 24 h after infection of BxPC-3 cells with TG2. *B,* Western blot analysis showing TG2 expression in BxPC-3 cells before (*UT*) and 48 h after infection with adenovirus containing either wild-type (*TGwt*) or $C_{277}S$ mutant (*TGm*) TG2 construct. Cells infected with adenovirus alone (*LacZ*) served as control. Membranes were stripped and reprobed with anti-phosphorylated PTEN (Ser^{380}) or total PTEN antibody and with β-actin antibody to ensure even loading of proteins in each lane. *C,* Western blot analysis showing TG2 expression in Panc-28 cells before (*UT*) and after transfection with TG2 siRNA [siRNA1 (*si-1*) and siRNA2 (*si-2*)] or control (*ctrl*) siRNA. Membranes were stripped and reprobed with anti-phosphorylated PTEN (Ser^{380}) and anti-total PTEN antibody. Finally, the membrane was probed with an anti–β-actin antibody to establish even loading of proteins in each lane. *D,* top, immunoblots of whole-cell lysates and immunoprecipitates using PTEN antibody from BxPC-3 cells (infected with wild-type TG2 adenovirus) incubated with cycloheximide (50 μg/mL) with or without the proteasome inhibitor MG132 (20 μmol/L), as described in Materials and Methods. Whole-cell lysate and immunoprecipitate from untreated Panc-28 and BxPC-3 cells served as a positive control. Immunoprecipitation using rabbit IgG in place of PTEN antibody served as negative control. Bottom, Western blot showing ubiquitination and polyubiquitination of PTEN in samples shown in top panel after the membrane was stripped and reprobed with ubiquitin antibody. *E,* Western blot analysis showing PTEN expression in Panc-28 cells after infection with adenovirus containing wild-type PTEN construct, as described in Materials and Methods. Cells infected with adenovirus alone (*LacZ*) served as control. The membranes were stripped and reprobed with anti-TG2 and anti-PTEN antibody. Finally, the membranes were probed with β-actin antibody to ensure even loading of proteins in each lane. Results shown are from a representative experiment repeated twice with similar results.

were then chased for PTEN expression over a period of 24 h. Results shown in Fig. 2A revealed that overexpression of TG2 protein (Fig. 2A, *top*) results in ~60% decrease in PTEN expression after 24 h of incubation (Fig. 2A, *middle*). In a subsequent experiment, we used 48 h of infection with adeno-TG2, which resulted in 5- to 6-fold increase in TG2 expression and >80% decrease in PTEN expression (Fig. 2B). The infection of cells with adenovirus alone (LacZ) had no effect on TG2 or PTEN expression (Fig. 2B). We next investigated the status of PTEN phosphorylation at Ser^{380}, the site known to affect its stability (17, 18). Ectopic expression of TG2 in BxPC-3 cells resulted in hypophosphorylated (Ser^{380}) PTEN (Fig. 2B). Interestingly, like wild-type TG2, forced expression of the mutant TG2, which lacks transamidation activity due to a point mutation in the active site ($C_{277}S$; ref. 3), also induced a similar decrease in PTEN phosphorylation and expression (Fig. 2B). These results suggest that TG2-mediated regulation of PTEN expression is independent of its catalytic/transamidation function.

In a converse experiment, we studied the effect of TG2 knockdown on PTEN phosphorylation (Ser^{380}) and expression. We used the siRNA approach using two different siRNA sequences, which resulted in 80% to 90% down-regulation of endogenous TG2 expression in Panc-28 cells (Fig. 2C). Down-regulation of endogenous TG2 was associated with an increase in PTEN phosphorylation (Ser^{380}) and PTEN expression. siRNA-induced effect on PTEN phosphorylation and expression was related to a decrease in TG2 expression, as under similar conditions transfection of Panc-28 cells with control siRNA did not alter TG2 or PTEN protein expression (Fig. 2C).

To determine the mechanism by which TG2 affects PTEN expression, we next induced TG2 expression in BxPC-3 cells by adenoviral infection. Immediately following the infection, cells were treated with cycloheximide in the presence or absence of the proteasome inhibitor MG132 (20 μmol/L). Cells were harvested at 0, 6, and 12 h after infection and processed to determine the PTEN levels by immunoprecipitation, as described in Materials and Methods. As expected, the expression of TG2 in BxPC-3 cells was associated with progressive decrease in PTEN expression after 6 and 12 h of incubation. However, the presence of MG132 in the culture medium prevented decrease in PTEN expression (Fig. 2D, *top*) and resulted in the accumulation of ubiquitinated PTEN (Fig. 2D, *bottom*). These results clearly suggest that TG2 expression

inhibits PTEN phosphorylation and promotes its ubiquitination and subsequent degradation via proteasomal pathway. An alternate possibility of TG2 regulation by PTEN was also investigated. PTEN was overexpressed in Panc-28 cells by infection with adenovirus containing PTEN construct. Results shown in Fig. 2E revealed that overexpression of PTEN does not affect TG2 levels or cell viability for at least 48 h after infection of Panc-28 cells.

TG2 regulates PTEN-mediated functions. PTEN expression is known to affect the invasiveness of cancer cells (13). Therefore, we next determined the effect of TG2 down-regulation on invasive functions of Panc-28 cells. For this purpose, we used two subclones (Panc-28/cl.10 and Panc-28/cl.12) that were derived by stable transfection of Panc-28 cells with TG2 shRNA and expressed 70% to 80% lower TG2 with a parallel increase in PTEN expression (Fig. 1B). Results shown in Fig. 3A showed that inhibition of endogenous TG2 in Panc-28 cells was associated with decreased invasiveness through the Matrigel. Furthermore, PTEN is known to negatively regulate FAK and PIP3 activation, resulting in the inhibition of FAK/PI3K/AKT pathway (13, 14). PTEN can directly associate with FAK and inactivate/dephosphorylate it (13). Therefore, we next determined the association of FAK and PTEN in Panc-28 and BxPC-3. In a pull-down experiment with anti-FAK antibody, the immunoprecipitates from Panc-28 and BxPC-3 cell extracts revealed the presence of PTEN (Fig. 3B). The association between FAK and PTEN was further confirmed by confocal microscopy (data not shown). We found no appreciable difference in total FAK levels in Panc-28 and BxPC-3 cells. Panc-28 cells, which expressed high basal levels of TG2, showed low association of FAK with PTEN and expressed more phosphorylated FAK (pThr^{397}; Fig. 3B). On the other hand, low TG2-expressing BxPC-3 cells showed high association of FAK with PTEN and contained low level of phosphorylated FAK (Thr^{397}; Fig. 3B). Furthermore, Western blot analysis of untreated BxPC-3 cells or cells infected with wild-type or mutant TG2 revealed the presence of a major phosphorylated FAK band in TG2-infected cells (Fig. 3C). A similar experiment revealed an increase in phosphorylated AKT (Ser^{473}) in response to overexpression of TG2 in BxPC-3 cells (Fig. 3C). These results suggested that TG2-mediated down-regulation of PTEN results in constitutive activation of the FAK/PI3K/AKT pathway.

Down-regulation of endogenous TG2 by siRNA resulted in massive accumulation of cytoplasmic vacuoles after 48 h of transfection (data not shown). Recently, a role for PTEN in promoting autophagic cell death in cancer cells has been documented (19). In line with these observations, we recently showed that down-regulation of TG2 in Panc-28 cells resulted in the accumulation of autophagosome and cell death via autophagy (20).

TG2 is associated with PTEN. Interestingly, in a pull-down experiment using an anti-TG2 antibody, the immunoprecipitates from Panc-28 and BxPC-3 cell extracts revealed the presence of PTEN when the blots were probed with anti-PTEN antibody (Fig. 4A). The association of TG2 with PTEN was also evident in a reverse experiment where immunoprecipitates with anti-PTEN antibody showed the presence of TG2 protein

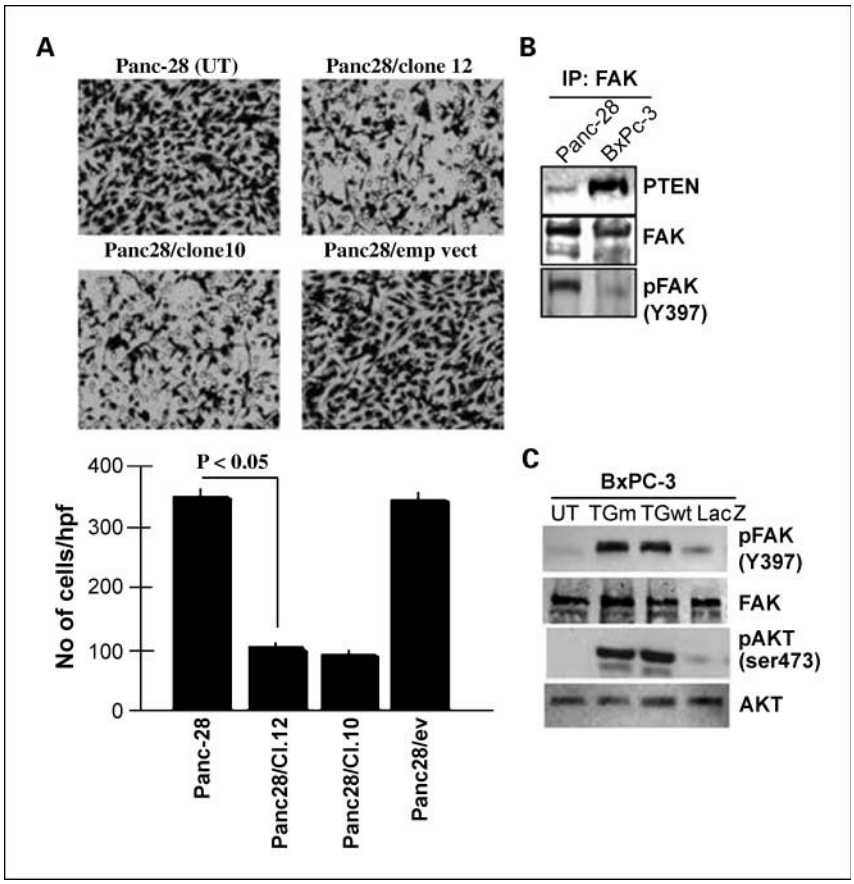

Fig. 3. TG2 regulates PTEN function. *A,* invasiveness of the parental Panc-28 and its two TG2 knockdown subclones (cl.12 and cl.10) through the Matrigel-Transwell membranes. Ev, empty vector – transfected Panc-28 cells. Columns, mean number of cells invaded per high-power field (*hpf*) from 10 random fields; bars, SD. *B,* coimmunoprecipitation showing the association of FAK protein with PTEN protein. Total cell lysates from Panc-28 and BxPC-3 cells were immunoprecipitated with an anti-FAK antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blotting using anti-PTEN, phosphorylated FAK (Thr^{397}), or total FAK antibody. *C,* Western blot analysis showing TG2 expression in BxPC-3 cells before (*UT*) and 48 h after infection with adenovirus containing either wild-type (*TGwt*) or $C_{277}S$ mutant (*TGm*) TG2 construct. Cells infected with adenovirus alone (*LacZ*) served as the control. The membranes were stripped and reprobed with anti-phosphorylated FAK (Thr^{397}), phosphorylated AKT (Ser^{473}), total FAK, and total AKT antibody. Results shown are from representative experiments done at least twice with similar results.

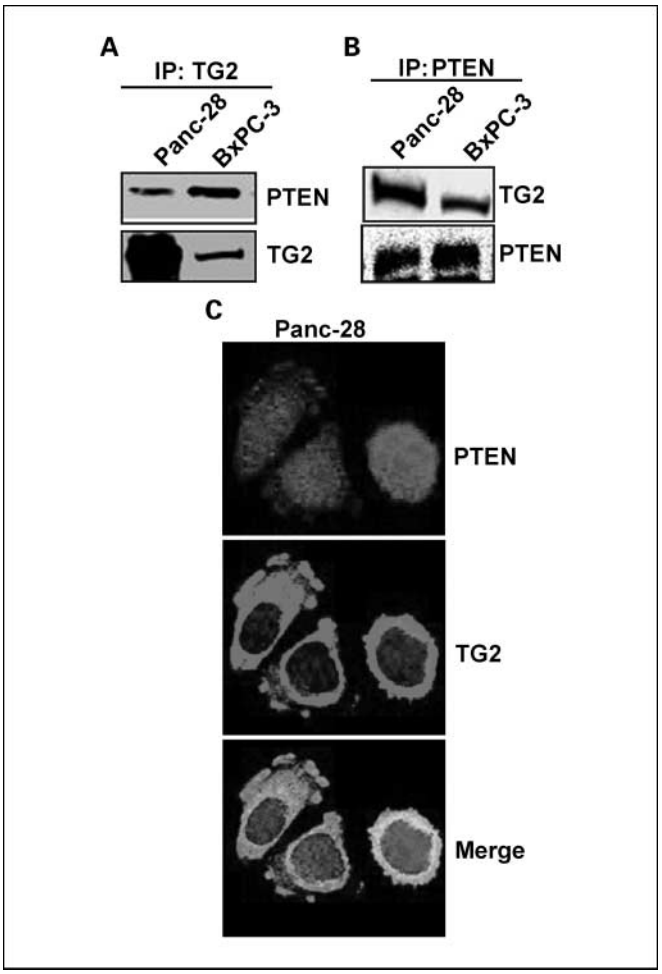

Fig. 4. TG2 is associated with PTEN. *A* and *B,* coimmunoprecipitation, showing the association of TG2 with PTEN protein. Total cell lysates were prepared from Panc-28 and BxPC-3 cells and immunoprecipitated using an anti-TG2 (*A*) or anti-PTEN (*B*) antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blotting using anti-PTEN and anti-TG2 antibodies. *C,* confocal microscopy images of Panc-28 cells showing colocalization of PTEN protein (*red fluorescence*) with TG2 (*green fluorescence*), as evidenced by the yellow fluorescence in the merged image. Representative experiment done twice on different occasions with similar results.

(Fig. 4B). The confocal microscopy further supported the colocalization of TG2 with PTEN in the cytosolic compartment of Panc-28 cells (Fig. 4C). These results showed a direct association between TG2 and PTEN.

Down-regulation of TG2 inhibits pancreatic tumor growth. Of the two TG2 shRNA stable clones, we used Panc-28/cl.10 cells for *in vivo* studies as this clone showed more decrease in endogenous TG2 levels when compared with wild-type Panc-28 cells (Fig. 1B). Xenografts were grown s.c. in athymic nude mice using Panc-28/cl.10 or Panc-28/empty vector (control) cells. Panc-28/cl.10 xenografts showed significantly retarded growth when compared with Panc-28/empty vector controls (Fig. 5A). The tumor growth was followed for 5 weeks, and mice were sacrificed during the 6th week. The Panc-28/cl.10 cells consistently produced xenografts with 60% to 80% lower tumor volumes (Fig. 5B) than the Panc-28/empty vector xenografts ($P = 0.017$). The tumors were analyzed for TG2 expression to ascertain that TG2 expression remained suppressed *in vivo* in Panc-28/cl.10 tumors. Indeed, TG2 expression in Panc-28/cl.10 xenografts was significantly lower and PTEN expression was higher than in control tumors, as determined by Western blotting (Fig. 5C) and immunohistochemistry (data not shown).

Taken altogether, these results suggest that TG2 expression plays an important role in switching on the activation of FAK/PI3K/AKT cell survival signaling pathways. Moreover, TG2 regulates the activation of FAK/PI3K/AKT pathway by regulating the expression of the tumor suppressor protein PTEN.

TG2 inversely correlates with PTEN expression in PDAC samples and is a poor prognosticator. To determine clinical relevance of our results, we next studied the expression of TG2 and PTEN in 51 stage II PDAC samples by immunochemistry on consecutive tissue sections from the array block. We observed an inverse correlation between TG2 expression and PTEN levels in PDAC tumor samples. PTEN expression was observed in 11 of 32 (34%) TG2-expressing tumor samples, whereas 13 of 19 (68%) TG2-negative tumor samples showed strong expression of PTEN ($P = 0.023$; odds ratio, 4.1; Fig. 6A). Representative PDAC tumor sections, showing inverse correlation between TG2 and PTEN expression, are shown in Fig. 6B. No staining appeared in sections treated with the isotypic control antibody (data not shown). Eleven of the 51 tumor samples (21%) showed both the TG2 and PTEN expression, suggesting that other unknown factors could also contribute in the regulation of PTEN expression. Although the expression of TG2 did not correlate with the patients' survival ($P = 0.68$), the TG2-mediated loss of PTEN correlated significantly with the overall survival ($P = 0.004$). In multivariate analysis, TG2-mediated loss of PTEN was a prognostic factor for overall survival in patients with stage II pancreatic ductal carcinoma independent of tumor stage/lymph node status and tumor differentiation ($P = 0.01$). Thus, overall survival in $TG2^+/PTEN^-$ patients was significantly poor (20.7 months) than in $TG2^-/PTEN^+$ patients (68.6 months).

Discussion

The data presented here show a novel role for TG2 in regulating PTEN expression at posttranslational level. PDAC cell lines and tumor samples showed inverse correlation between TG2 expression and PTEN levels. Our results suggest that aberrant expression of TG2 can down-regulate PTEN expression. Thus, ectopic expression of the catalytically active or inactive ($C_{277}S$) TG2 suppressed PTEN expression, suggesting that irrespective of its transamidation activity TG2 plays a role in PTEN regulation. Conversely, down-regulation of endogenous TG2 by siRNA resulted in increased expression of PTEN. TG2 inhibited PTEN phosphorylation at Ser^{380} and promoted PTEN ubiquitination and subsequent degradation via proteasomal pathway. Importantly, TG2 overexpression in conjunction with a loss in PTEN expression was associated with slow tumor growth *in vivo* in nude mice and predicted poor disease outcome in PDAC patients. Thus, the present study provides the first evidence on the regulation of PTEN by TG2 at posttranslational level, which can affect the invasiveness of pancreatic cancer cells and their response to anticancer therapies by modulating PI3K/AKT signaling pathway.

Previously, we found that TG2 is involved in constitutive activation of FAK and its downstream PI3K/AKT cell survival

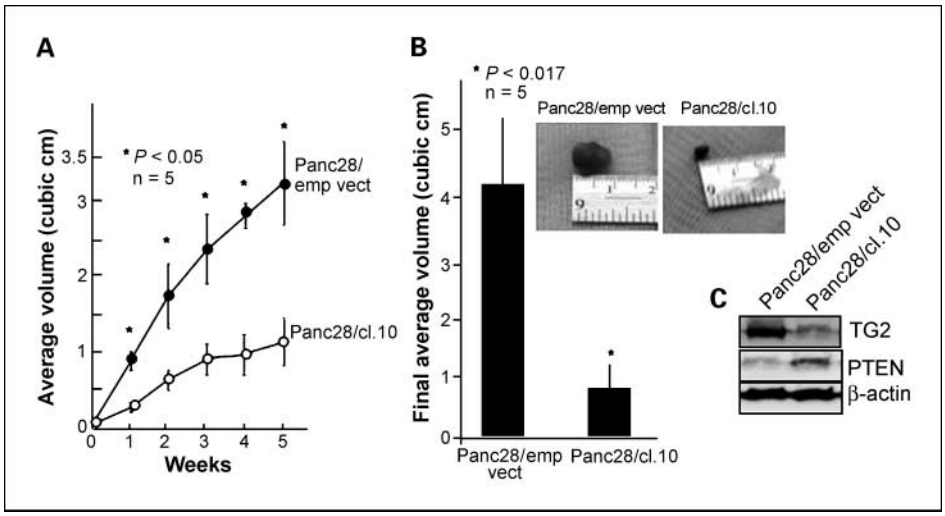

Fig. 5. TG2 down-regulation inhibits pancreatic tumor growth *in vivo*. *A,* growth curve of Panc-28/cl.10 and Panc-28/empty vector xenografts, growing s.c. in nude mice over 5 wk. *B,* tumor volume of Panc-28/cl.10 and Panc-28/empty vector – induced xenografts after 6 wk of implantation. Inset, tumor sizes, resected from representative mice in each group after 6 wk of tumor cell implantation *C,* basal level of TG2 and PTEN protein expression in Panc-28/cl.10 and Panc-28/empty vector – induced xenografts as determined by Western blotting.

signaling pathway (9). Because the tumor suppressor protein PTEN is known to regulate cell growth, invasion, migration, and focal adhesion functions by negatively regulating PI3K/AKT pathway (13), we hypothesized that TG2 might affect FAK/PI3K/AKT pathway by modulating PTEN expression or function. Indeed, the evidence presented here strongly supports this contention and suggests that TG2 expression decreases PTEN expression, which in turn leads to the activation of FAK/PI3K/AKT pathway. However, at this stage, we do not know whether the observed effect of TG2 overexpression on FAK/PI3K/AKT activation is exclusively related to PTEN down-regulation or other upstream pathways, such as src kinase and phosphoinositide-dependent kinases, could also attribute to this event.

Gene deletion and mutation of PTEN are observed frequently in various human cancers. These genetic changes, however, are not necessarily the only way to inactivate PTEN during tumorigenesis but subtle decrease in expression of PTEN, mediated by posttranslational modification, may affect its stability and degradation and may have profound effect on tumorigenesis. Indeed, a recent report by Wang et al. (21) suggested that the oncogenic protein NEDD4.1 serves as an E3 ubiquitin ligase for PTEN and results in its ubiquitination and degradation. Little is known about the regulation and stability of PTEN, except that its phosphorylation can affect the stability in certain cell types (17, 22, 23). The COOH-terminal domain of the PTEN protein is rich in putative phosphorylation sites that are embedded within two PEST motifs, which is a signature in many short-lived proteins, degraded by the ubiquitin pathway (24). The deletion or dephosphorylation of PTEN COOH terminus has been reported to affect PTEN stability without affecting its phosphatase activity (17, 18, 22, 23). Recently, protein kinase 2 (CK2) was shown to phosphorylate PTEN *in vitro* and *in vivo*, in a constitutive manner, on a cluster of COOH-terminal serine/threonine residues, including Ser^{370}, Ser^{380}, Thr^{382}, Thr^{383}, and Ser^{385} (18). The phosphorylation-defective mutants of PTEN showed decreased stability and were

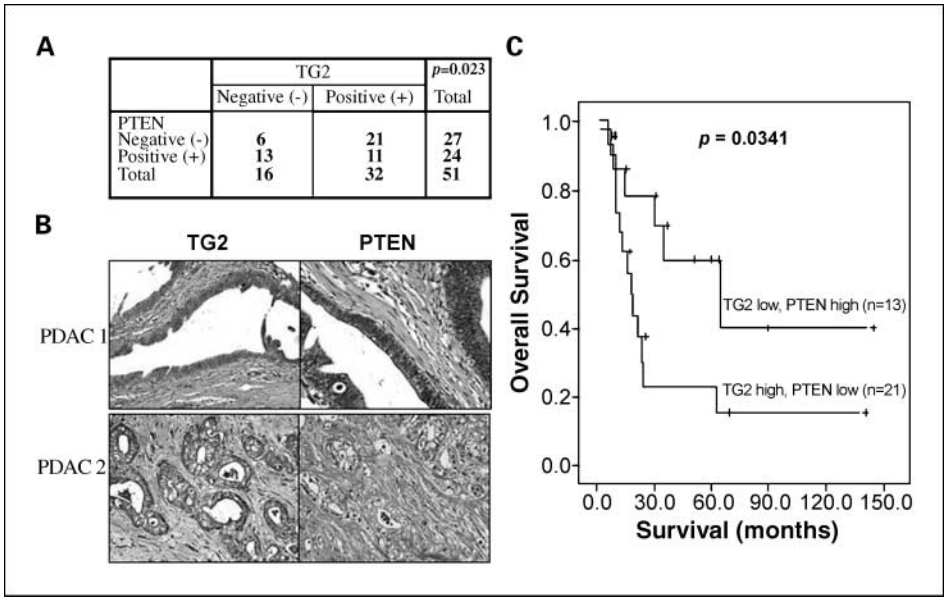

Fig. 6. TG2-mediated loss of PTEN is a predictor of poor prognosis in PDAC patients. *A,* cross-tabulation showing inverse correlation between TG2 expression and PTEN levels in PDAC tumor samples. PTEN expression was observed in 11 of 32 (34%) TG2-expressing tumor samples, whereas 13 of 19 (68%) TG2-negative tumor samples expressed high basal level of PTEN ($P = 0.023$). *B,* representative micrographs showing weak TG2 and strong PTEN staining (*top*) and strong TG2 and weak PTEN staining (*bottom*) in two different consecutive sections of PDAC tumor samples. Original magnification, ×400. *C,* Kaplan-Meier estimates of overall survival based on TG2 and PTEN expression in stage II PDACs. The overall survival was significantly poor in patients with high TG2 and low PTEN compared with low TG2 and high PTEN expression ($P = 0.0341$).

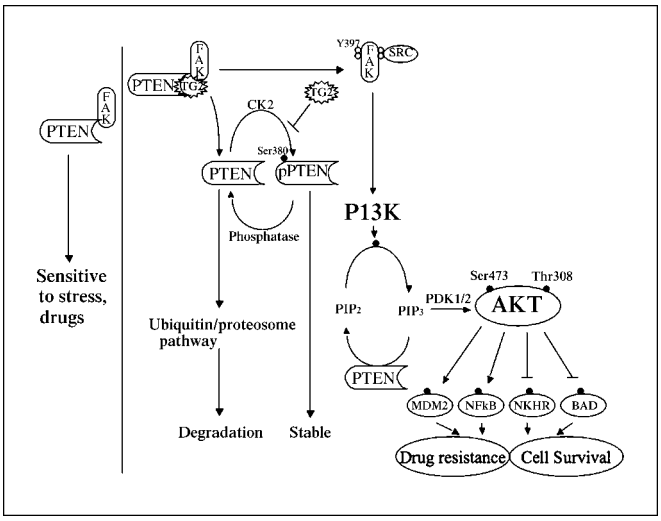

Fig. 7. Schematic representation of TG2-induced activation of FAK and downstream PI3K/AKT pathway. TG2 affects PTEN function and expression, resulting in activation of FAK and downstream PI3K/AKT survival pathway. TG2 expression promotes PTEN degradation and increases phosphorylated FAK levels. By inhibiting the phosphorylation of PTEN at site Ser^{380}, TG2 can destabilize PTEN by promoting PTEN ubiquitination and proteasomal degradation. Thus, TG2-mediated suppression of PTEN results in the activation of AKT, which in turn can phosphorylate various substrates leading to the activation of MDM2 and nuclear factor-κB (*NFκB*) and inhibition of FKHR and BAD contributing to increased cell survival and chemoresistance.

rapidly degraded by the proteasome system compared with the wild-type PTEN. Inhibition of PTEN phosphorylation by CK2 inhibitor (5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole) diminished the PTEN protein levels. These results suggest that phosphorylation of PTEN by CK2 is a critical step in preventing the degradation of PTEN via proteasomal pathway. However, in separate studies, it was shown that phosphorylation of PTEN tail negatively regulates PTEN activity (17). Phosphorylation of PTEN by CK2 prevents its interaction with MAGI-2 and its consequent recruitment into a high molecular weight complex (PTEN-associated complex). In line with these observations, our results suggest that association of TG2 with PTEN can prevent its phosphorylation at Ser^{380} (Fig. 4) and promote the stability of PTEN by delaying proteasomal degradation (17, 18). Furthermore, the inhibition of PTEN expression by overexpression of TG2 led to the phosphorylation of AKT (Ser^{473}) and FAK (Thr^{397}). How TG2 inhibits PTEN phosphorylation remains to be determined. One possibility is that physical association of PTEN with TG2 (Fig. 4A or B) could mask critical phosphorylation sites (such as Ser^{387}) on PTEN and prevent their phosphorylation by CK2.

In pancreatic cancer cells, neither the inactivating mutations nor the deletion of PTEN has been observed. Nevertheless, these cells exhibit a highly malignant behavior and intrinsic resistance to anticancer therapies. Results reported in this article may help to explain this phenomenon and show that elevated expression of TG2 plays an important role in regulating the function and expression of PTEN in PDAC cells. The elevated expression of TG2 in various cancer types has been implicated in conferring resistance to apoptosis, promoting invasion, and producing drug resistance (9, 11, 25–30). The inverse correlation between TG2 and PTEN expression in a subset of PDAC tumor samples and its effect on patient survival suggest a strong clinical relevance of TG2 and mechanistic link between TG2 expression and PTEN function. Therefore, targeting TG2 using TG2 small-molecule inhibitor or TG2 siRNA approach in a subset of patients overexpressing TG2 may offer clinical benefits in terms of treating pancreatic cancer.

In conclusion, we provide the first evidence that increased expression of TG2 in PDAC cells is strongly associated with deregulation of PTEN-mediated cell survival FAK/PI3K/AKT signaling pathway, as depicted schematically in Fig. 7. The TG2-mediated loss of PTEN is associated with poor survival in stage II PDAC patients. The association between TG2, PTEN, and FAK/AKT pathways deserves further analysis in prospective clinical studies.

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