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Tissue Transglutaminase Mediated Tumor-Stroma Interaction Promotes Pancreatic Cancer Progression

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Abstract

Purpose—Aggressive pancreatic cancer is commonly associated with a dense desmoplastic stroma, which forms a protective niche for cancer cells. The objective of the study was to determine the functions of tissue transglutaminase (TG2), a Ca^{2+}-dependent enzyme which crosslinks proteins through transamidation and is abundantly expressed by pancreatic cancer cells in the pancreatic stroma.

Experimental Design—Orthotopic pancreatic xenografts and co-culture systems tested the mechanisms by which the enzyme modulates tumor-stroma interactions.

Results—We show that TG2 secreted by cancer cells effectively molds the stroma by crosslinking collagen, which in turn activates fibroblasts and stimulates their proliferation. The stiff fibrotic stromal reaction conveys mechanical cues to cancer cells leading to activation of the

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YAP/TAZ transcription factors, promoting cell proliferation and tumor growth. Stable knockdown of TG2 in pancreatic cancer cells led to decreased size of pancreatic xenografts.

Conclusions—Taken together, our results demonstrate that TG2 secreted in the tumor microenvironment orchestrates the crosstalk between cancer cells and stroma fundamentally impacting tumor growth. Our study supports TG2 inhibition in the pancreatic stroma as a novel strategy to block pancreatic cancer progression.

Keywords

pancreatic cancer; stroma; collagen; tissue transglutaminase; YAP/TAZ

Introduction

Pancreatic ductal adenocarcinoma (PDA) is the fourth cause of cancer death (1). With the currently available treatment, the majority of patients succumb to disease, resistance to chemotherapy and radiation having been implicated as pivotal obstacles (2). A hallmark of pancreatic tumors is the dense desmoplastic stroma (DS) composed of myofibroblasts and stellate cells which secrete collagens and other matrix proteins that serve as a scaffold on which tumor cells proliferate (3–5). These stromal components are highly responsive to cytokines (TGF-β) and growth factors (PDGFs, FGFs, sonic hedgehog) abundantly secreted in the tumor microenvironment (TME) (4–7). The stroma, believed to protect PDA cells from environmental stress, is hypovascular, hypoxic, and presumably less permeable to chemotherapy (4, 5, 8). In support of the latter concept, a recent study using clinical PDA specimens demonstrated that the delivery of gemcitabine into human pancreatic tumors is highly heterogenous and strongly correlated with the existing stromal reaction (9, 10). However, two other recent reports have suggested that some components of the stroma may play a reverse role, by restraining cancer cell invasion and preventing tumor metastasis (11, 12). The latter studies used analyses in genetically engineered PDA mouse models that may not faithfully mirror the desmoplastic reaction of human tumors. The conflicting outcomes of previous studies (8, 12) coupled with the continued lack of success treating this recalcitrant disease, underscore the complex interplay between the diverse components of pancreatic tumors and the inherent limitations of existing experimental models to study and replicate the diverse traits of the human disease (13). The data presented here highlight the crosstalk between cancer and stromal cells mediated by tissue transglutaminase (TG2) and the significance of the crosslinking reaction mediated by this enzyme in the matrix to PDA progression.

TG2 is a TGF-β inducible protein (14) secreted in the extracellular milieu which mediates the transfer of acyl groups between glutamine and lysine residues, causing protein crosslinking (15). Highly expressed in pancreatic cancer cells and tumors, TG2 was reported to activate NF-κB orchestrated survival mechanisms and to contribute to chemotherapy resistance, independently of its enzymatic function (16, 17). Whether and how TG2 promotes pancreatic tumor growth remains not clear. Based on the knowledge that the enzyme is secreted and acts as a potent crosslinking factor for collagens and matrix proteins (18), and that the enzymatic function of TG2 is augmented in the extracellular matrix (ECM) by factors such as hypoxia and tissue injury (19), we hypothesized that TG2 secreted by

pancreatic cancer cells into the tumor milieu alters the composition of the stroma, which in turn augments oncogenic signaling in cancer cells facilitating tumor progression.

By using pancreatic orthotopic xenografts we demonstrate that TG2 knockdown in PDA cells inhibits tumor formation and growth. We show that TG2 is enzymatically active in pancreatic cells and tumors, and is secreted in the TME where it crosslinks collagen and stimulates the proliferation of myofibroblasts. The stroma enriched in crosslinked collagen and fibroblasts activates the Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) transcription factors in cancer cells and promotes cell proliferation. This is the first report describing the role of TG2 in the pancreatic DS and demonstrating its role in the activation of YAP/TAZ transcription factors in cancer cells. Taken together our data support that by facilitating the crosstalk between cancer and stromal cells, TG2 promotes PDA progression.

Materials and Methods

Chemicals and reagents

Antibodies for TG2 (NeoMarkers, Fremont, CA), YAP1 (Abnova, Walnut, CA), p-YAP (Ser127) and YAP/TAZ (Cell Signaling Technology, Danvers, MA), and GAPDH (Meridian Life Science, Inc., Memphis, TN) were used for western blotting. Recombinant enzymatically active or mutant ($C^{277}S$) TG2 were purified as previously described (20). Unless stated otherwise, all reagents were from Sigma-Aldrich (St Louis, MO, USA).

Cell culture

AsPC1, BxPC3, and Paca2 cells were obtained from the American Type Culture Collection (Rockville, MD), human pancreatic normal epithelial (HPNE) and Panc1 cells were a gift from Prof. Murray Korc (21). The normal human dermal fibroblasts (NHF544) were provided by Dr. Dan Spandau, and GFP-expressing neonatal dermal fibroblasts (GFP-HNDFs) were purchased from Angioproteomie (Boston, MA). Human pancreatic stellate cells (hPSCs) were from ScienCell Research Laboratories (Carlsbad, CA), and the mesothelial cell line, LP9, was from the NIA Aging Cell Repository (Camden, NJ). All cell lines were tested for mycoplasma and authenticated by the IUSCC *In Vivo* Therapeutics Core. AsPC1 and BxPC3 cells were cultured in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Cellgro) and 1% antibiotics. Panc1, Paca2, NHF544, GFP-HNDFs, LP9, and hPSCs were grown in Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 10% FBS and 1% antibiotics. Cells were grown at 37°C under 5% CO_2. Conditioned media (CM) was collected after 24 hour incubation of 5×10^5 PDA cells in serum free RPMI media. Co-culture experimental details are provided in Supplemental Materials (SM).

Immunohistochemistry (IHC) was performed as previously described (22) (see *SM*).

Cell transfection

TG2 was stably knocked down in AsPC1 and Panc1 cells by transducing MISSION® shRNA Lentiviral Transduction Particles (Sigma), containing short hairpin RNAs (shRNA)

targeting TG2 (shTG2) in the presence of polybrene (8μg/mL). As control (shCtrl), cells were transduced with MISSION® pLKO.1-puro Empty Vector control Transfection Particles (Sigma). Both shCtrl and shTG2 particles were transduced at 2.5 multiplicity of infection. Pooled stable clones were collected after selection with puromycin at 1 μg/ml concentration, and TG2 knockdown was verified by western blotting. Transient transfection of short interfering RNA (siRNA) used sequences targeting YAP and TAZ (Santa Cruz) and Lipofectamine® 3000 (Life technologies, Carlsbad, CA). siGENOME™ Control Pool (Dharmacon, Lafayette, CO) was use as control.

Western Blot Analysis followed a standard protocol (see *SM*).

Orthotopic Pancreatic Xenografts

1.5×10^5 AsPC1 and Panc1 cells, stably transfected with shTG2 or shCtrl were injected into the pancreatic tail of 7–8 weeks old female nude mice (Harlan, Indianapolis, IN) using a 27.5 gauge needle under isoflurane anesthesia. Three (Panc1 cells) or 4 weeks (AsPC1 cells) after injection, mice were euthanized and tumors were weighted and measured. Tumor volumes were calculated as $4/3*\pi*(L/2)*(W/2)*(H/2)$; where L is length, W, width, and H, height. Metastatic implants were counted. Animal experiments were approved by the IU Animal Care and Use Committee, in compliance with federal regulations.

Immunofluorescence (IF) used antibodies for TG2 (Neomarkers), α-SMA (Sigma), collagen 1 (Abcam), and YAP/TAZ (Cell Signaling). To detect matrix-bound TG2, cells were not permeabilized before incubation with the TG2 antibody, as previously described (23). Procedures are detailed in *SM*.

Proliferation Assays were performed as described in *SM*.

Reporter Assay

To measure YAP/TAZ transcriptional activity, PDA cells were transfected with pGal4-TEAD4, pGal4-Luciferase, and pTK-Renilla at a ratio of 1:10:1 using DreamFect (Oz Biosciences, Inc., San Diego, CA). Plasmids were a gift from Dr. Clark Wells (IUSCC) (24). The Dual-Luciferase® Assay System kit (Promega, Madison, WI) determined TEAD4 reporter activity after 48 hours of transfection. To control for transfection efficiency, the luciferase activity was normalized to renilla.

Reverse Transcription PCR (RT-PCR) and Quantitative Real-Time PCR (qRT-PCR) procedures are described in *SM*.

Collagen Crosslinking

Collagen 1 solution (StemCell Technologies, Vancouver, BC) was treated with TG2 (Sigma) in the presence of 1 mM DTT, 5 mM $CaCl_2$, and 100 mM Tris-HCl (pH 8.0), as previously described (25) for 1.5 hours at 37°C.

Picrosirius Red (PSR) Staining

Paraffin-embedded slides were incubated with PSR solution containing 0.1% of Direct red 80 powder (Sigma) dissolved in picric acid (Sigma) for 1 hour. Nuclei were counterstained with hematoxylin. The slides were washed in 0.5% glacial acetic acid and dehydrated in ethanol and xylene. Imaging with circular polarizing light used an inverted Nikon Diaphot 200 microscope (Nikon, Melville, NY). The proportion of differently colored collagen fibers was quantified with MetaMorph (Molecular Devices, see *SM*) (26).

Soluble Collagen Assay

Soluble collagen in xenografts (n=6 per group) was determined by using the Sircol™ Soluble Collagen Assay kit (Biocolor Life Science Assays, Northern Ireland, see *SM).*

TG2 Activity

PDA cells and fibroblasts were incubated with 500 μM 5-(Biotinamido) pentylamine (5-BP) (ThermoScientific) for 1 hour at 37°C. After fixing in methanol, slides were incubated with Alexa Fluor® 647 streptavidin (Life Technologies) for 1 hour. To assay *in situ* activity of TG2 in tumor tissue, 10 μm cryosections were incubated at 37°C in a buffer containing 5 mM $CaCl_2$, 100 mM Tris-HCl (pH 8.0), in the presence or absence of 1 mM DTT, and 0.001 mM T26 or T26QN (negative control), as described (27–29). As another negative control, 5 mM EDTA was added to the buffer. Imaging used a LSM 510 META confocal microscope (Carl Zeiss, Inc.) under UV excitation.

Statistical analysis

Student's *t* test compared measurements. $P < 0.05$ was significant.

Results

TG2 is abundantly expressed and enzymatically active in PDA cells and stroma

We used immunohistochemistry (IHC) to measure TG2 expression and cellular localization in PDA specimens and in normal pancreas. Patient characteristics are presented in *SM* (Supplementary Table 1). No immunostaining was recorded in the stroma of normal pancreas (n=3), and faint (1+) staining was noted in normal ducts. In contrast, strong (2+ to 3+) TG2 cytoplasmic immunostaining was recorded in 36 out of 52 (69%) PDA specimens, supporting that TG2 expression is increased in PDA compared to normal duct epithelium. TG2 immunostaining was also recorded in the stroma of 44 out of 52 specimens (84%, Figure 1A), involving both the cellular (fibroblasts) and extracellular compartments. To determine whether TG2 was enzymatically active in the stroma, 20 additional tumors identified through the IUSCC Tissue Bank as PDA specimens associated with significant desmoplasia were stained for TG2 and for isopeptide, a covalent bond resulting from TG2 mediated transamidation. Concordant strong (2+ to 3+) TG2 and isopeptide staining were recorded in 19 out of 20 specimens (Figure 1A), supporting that TG2 is expressed and active in the pancreatic DS. Isopeptide staining was detectable in the matrix and the basal membrane.

TG2 expression levels in cell lysates and CM from PDA, HPNE, stellate cells, and fibroblasts were examined by using western blotting. Abundant TG2 expression was detected in BxPC3 and AsPC1 cells and in the conditioned media (CM), confirming that it is secreted by PDA cells (Figures 1B). TG2 expression was detectable in HPNE cells but lower than in most cancer cell lines. Immunofluorescence (IF) determined TG2 cellular localization and enzymatic activity by measuring incorporation of 5-(Biotinamido) pentylamine (5-BP) and FITC-labeled T26 peptide, known TG2 substrates (Figure 1C and Supplementary Figure 1). TG2 was expressed in the cytosol and the plasma membrane of AsPC1 and Panc1 cells, and its enzymatic activity was detectable in the cytoplasm of both cell types. In contrast, TG2 was present, but was enzymatically inactive in fibroblasts (Figure 1C) and in LP9 normal mesothelial cells (Supplementary Figure 1B), suggesting that the enzymatic activity may be differentially regulated in cancer vs. normal cells. Specificity is supported by lack of IF signal when cells were incubated with the mutant T26QN peptide (not a TG2 substrate) or in the presence of EDTA, which chelates Ca^{2+} (Supplementary Figure 1A). In addition, extracellular TG2 was detectable in the matrix deposited by AsPC1, BxPC3 and Panc1 cells (Supplementary Figure 2), supporting the concept that PDA cells secrete TG2 into the ECM.

TG2 knockdown inhibits PDA tumor growth *in vivo*

To proceed with functional studies, TG2 was knocked down in AsPC1 and Panc1 cells by stably transducing shRNA targeting TG2 (shTG2). TG2 protein and *mRNA* expression levels were significantly downregulated in shTG2 transduced cells compared to cells transduced with scrambled shRNA (shCtrl, Figure 1D). TG2 secretion in the CM was also decreased in shTG2 compared to shCtrl transduced PDA cells (Figure 1D, *right panel*). However, *in vitro*, no significant differences in the proliferation and ability to form clones between PDA cells +/−shTG2 were observed (Supplementary Figure 3), suggesting that any effects of TG2 on tumor growth are not explained by direct effect on cancer cells.

To measure the effects of TG2 on tumor formation and progression, orthotopic pancreatic xenografts derived from AsPC1 and Panc1 cells in which TG2 was stably knocked down were used. Average tumor volume and weight were significantly decreased in xenografts derived from AsPC1+shTG2 compared to AsPC1+shCtrl cells (Figure 2A, *left* and *middle* panels). There was no significant difference in the number of macro-metastases between groups (Figure 2A, *right* panel). Stable knockdown of TG2 in AsPC1+shTG2 xenografts compared to controls was confirmed by IHC and was quantified by comparing H scores (see *SM*, Figure 2B). Similar decrease in average tumor volume and weight was observed in Panc1+shTG2 vs. Panc1+shCtrl xenografts (Figure 2C, *left* and *middle* panels). The number of macro-metastases was significantly decreased in Panc1+shTG2 tumor bearing mice compared to controls (Figure 2C, *right* panel). Stable knockdown of TG2 was confirmed by IHC (Figure 2D). Together, these data demonstrate that TG2 expression levels in PDA cells strongly correlate with *in vivo* tumor growth.

TG2 is enzymatically active and crosslinks collagen in PDA tumors

Under normal physiological conditions, TG2 is enzymatically inactive *in vitro* and *in vivo*. Various stressors such as injury or hypoxia, are required to activate TG2 (30). To determine

whether TG2 is activated in the pancreatic TME, we measured incorporation of the FITC-labeled T26 peptide, which is specifically crosslinked to ECM proteins by TG2 (29). Enzymatically active TG2 was detectable both intra- and extra-cellularly in AsPC1 and Panc1 xenograft sections (Figure 3A), suggesting that the enzyme is active in the pancreatic milieu. Incubation with the T26 peptide in the presence of EDTA and with the mutant T26QN peptide, which is not a TG2 substrate, were used as negative controls to prove specificity. TG2 activity was detectable even in the absence of DTT, a known TG2 activator (31) (Supplementary Figure 4), supporting that the enzyme is activated *in situ*.

One of the known TG2 substrates is collagen 1, abundant in the pancreatic DS. PSR staining was used to determine whether collagen 1 was crosslinked by TG2 in the pancreatic TME. Birefringence of PSR staining under polarizing microscope is highly specific for collagen fibers, which appear red, orange, yellow, or green in order of decreasing thickness (26). Collagen fibers stained mostly bright orange, red, and yellow in AsPC1+shCtrl tumors, while fibers were mostly green and yellow in AsPC1+shTG2 tumors (Figures 3B *left* and *middle* panels), suggesting that collagen fibers are thicker in TG2 expressing tumors. Moreover, the area of birefringence corresponding to collagen was more extensive in AsPC1+shCtrl compared to AsPC1+shTG2 xenografts (Figure 3B, *right* panel), suggesting increased collagen deposition in TG2 expressing tumors, most collagen being deposited around ducts and at the edge of tumors. To verify that this correlation is relevant to human pancreatic tumors, PSR staining of TG2-high and TG2-low human tumors was carried out (n=6 per group). TG2 expression levels strongly correlated with the percentage of crosslinked collagen ($p < 0.001$, Figure 3C).

Additionally, the sircol and the hydroxyproline assays were used to measure soluble and total collagen, respectively, in protein extracts from xenografts expressing TG2 or not. The amount of soluble collagen was increased in AsPC1+shTG2 compared to AsPC1+shCtrl tumors (Figure 3D, *left panel*) and in Panc1+shTG2 compared to Panc1+shCtrl xenografts (Figure 3D, *right panel*), while total collagen content was similar (Supplementary Figure 5). Collectively, these data suggest that TG2 secreted from PDA cells promotes collagen crosslinking in the pancreatic stroma.

TG2-mediated crosslinked collagen and TG2 expressing PDA cells promote the growth and activation of fibroblasts

We next determined the functional significance of native and TG2-crosslinked collagen to the proliferation of fibroblasts and cancer cells. The proliferation of NHF544 fibroblasts was increased on collagen coated compared to uncoated plates (Figure 4A, *left-upper* panel) and on TG2-crosslinked collagen compared to native collagen (Figure 4A, *left-lower* panel), supporting that collagen and crosslinked collagen in the matrix promote fibroblast proliferation.

Next, we studied the influence of TG2 secreted by cancer cells on stromal cell proliferation. Direct co-culture of TG2 expressing AsPC1 cells significantly promoted GFP-labeled fibroblast (GFP-HNDF) proliferation over a 14-day period compared to co-culture of fibroblasts with AsPC1+shTG2 cells (Figure 4A, *right* panel). Fibroblast proliferation in co-culture with cancer cells expressing and secreting low levels of TG2 (AsPC1+shTG2) was

not different from proliferation of fibroblast cultured alone, supporting that secretion of TG2 in co-culture models augments the fibrotic response. However, in the absence of co-cultured PDA cells, addition of recombinant TG2 to the growth media did not alter fibroblast or stellate cell proliferation (Supplementary Figure 6), suggesting that TG2 acts in concert with other factors secreted in the matrix.

To measure fibroblast activation in the presence of cancer cells, expression of α-smooth muscle actin (α-SMA) was measured by IF. Co-culture of NHF544 cells with AsPC1 and Panc1 cells induced expression of αSMA (Figure 4B, *left* panels). To determine whether TG2 secreted by PDA cells contributes to fibroblast activation, NHF544 cells were co-cultured with PDA cells +/−shTG2, and IF staining measured collagen deposition. Increased collagen 1 staining was observed in NHF544 fibroblasts co-cultured with AsPC1 and Panc1 control cells, compared to fibroblasts co-cultured with AsPC1+shTG2 and Panc1+shTG2 cells (Figure 4B, *right* panels). Likewise, in tumors, the number of α-SMA expressing (activated) myofibroblasts was increased in AsPC1+shCtrl compared to AsPC1+shTG2 xenografts (Figure 4C). Collectively, these data show that in the pancreatic TME, TG2 secreting PDA cells promote the growth, activation, and collagen deposition by myofibroblasts.

We next tested whether these stromal alterations conversely regulate the growth of PDA cells. Proliferation of AsPC1 and Panc1 cells was promoted when cells were cultured on collagen coated vs. uncoated plates (Figure 4D, *upper* panel) and on a feeder layer of hPSCs vs. uncoated plates (Figure 4D, *lower* panel). Taken together, these results demonstrate that PDA cells secreting TG2 in the tumor milieu promote fibroblast activation and proliferation as well as collagen crosslinking, leading to stromal alterations, which in turn promote tumor growth.

Matrix induced YAP/TAZ activation promotes PDA cell proliferation

As the pancreatic stroma becomes infiltrated by collagen and fibroblasts, its stiffness increases (32). One of the mechanisms activated by the physical properties of the ECM is the YAP/TAZ pathway (33), which regulates cancer cell growth. We measured activation of the pathway by IF staining for the two nuclear factors in PDA cells plated on collagen vs. uncoated plates. Increased nuclear YAP/TAZ staining was observed in Panc1 and AsPC1 cells on collagen (Figure 5A). Western blotting showed decreased levels of phospho-YAP (p-Yap, inactive) and increased ratio of total-YAP/p-YAP were recorded in PDA cells plated on collagen-coated vs. uncoated plates (Figure 5A, *right* panel), indicating YAP activation by the collagen matrix.

Next, IF staining determined the effect of TG2 on YAP/TAZ activation in PDA cells. Increased nuclear localization was recorded in TG2 expressing control Panc1 and AsPC1 cells compared to PDA cells transduced with shTG2 (Figure 5B). Decreased level of p-YAP and increased ratio of total-YAP/p-YAP were observed in Panc1+shCtrl compared to Panc1+shTG2 cells (Figure 5B, *right* panel). We next measured the transcriptional activity of YAP in Panc1 cells plated on collagen by using the TEAD4 reporter assay and by measuring the *mRNA* levels for *CTGF*, a known YAP/TAZ target gene. TEAD4 activity (Figure 5C, *upper* panel) and *CTGF mRNA* levels (Figure 5C, *lower* panel) were increased

in Panc1+shCtrl compared to Panc1+shTG2 cells, confirming activation of the pathway in TG2 expressing cells.

To determine the significance of YAP/TAZ to PDA cell proliferation, transient siRNA transfection targeting both transcription factors was employed. YAP and TAZ knockdown by siRNA confirmed by western blotting (Figure 5D, *upper* panel) caused decreased proliferation of AsPC1 and Panc1 cells grown on collagen (Figure 5D, *lower* panel). Collectively, these data suggest that the transcription factors YAP and TAZ are activated in TG2 expressing PDA cells and outline a new mechanism by which cancer cell proliferation is modulated by TG2 through signals reflected from the tumor into the surrounding stroma (Figure 6).

Discussion

Our findings outline how changes in the pancreatic cancer stroma induced by TG2 secreted from PDA cells alter tumor growth. While overexpression of TG2 in epithelial cancers (34, 35) including in pancreatic cancer (36, 37) has been previously reported, and its direct effects on cellular oncogenic signaling have been dissected (37, 38), this is the first report describing the effects of TG2 on the pancreatic TME, and its subsequent consequences on cancer cell proliferation and tumor growth. We demonstrate that TG2 expressing PDA cells form larger pancreatic tumors, enriched in thick collagen fibers and in myofibroblasts. The dense stroma of TG2 expressing tumors conveys signals leading to YAP/TAZ activation in cancer cells promoting cell proliferation and tumor growth. This report has several important consequences.

First, we demonstrate that TG2 knockdown in pancreatic cancer cells inhibited tumor growth. This effect was more pronounced in Panc1 compared to AsPC1 cells, possibly because of the shorter doubling times of Panc1 cells, which are more aggressive and semi-mesenchymal. Interestingly, the smaller tumor volumes were not accounted for by decreased proliferation or clonogenic potential of the PDA cells, but by substantial stromal alterations. A previous study using siRNA targeting TG2 delivered as liposomes into the peritoneal cavity noted inhibition of pancreatic tumor growth along with decreased tumor vascularity and decreased cancer cell proliferation (36). In that study, TG2 knockdown affected all TG2 expressing cells exposed to the carrier liposomes. It is, therefore, possible that TG2 knockdown in endothelial cells led to the anti-angiogenic effect reported there and the subsequent inhibition of cell proliferation (36). Contrasting to that study and consistent with TG2 knockdown restricted to PDA cells in our model, we did not observe changes in multi-vessel density between tumors expressing TG2 or not (not shown). However, we report a significant decrease in the number of activated myofibroblasts and thick collagen fibers in tumors derived from shTG2 transduces cells. We show that these stromal alterations directly affect proliferation of fibroblasts and cancer cells. The protective role of the pancreatic stroma to cancer cells has been previously recognized and linked to the resistance of pancreatic tumors to chemo and radiotherapy (39–41). Our data implicate for the first time TG2 as a modulator of the pancreatic TME through its protein crosslinking properties.

Second, we report that TG2 is enzymatically active in PDA cells and in the matrix where it crosslinks collagen, and possibly other ECM substrates. It is known that the functions of TG2 are tightly controlled by environmental factors, which cause allosteric structural changes and alter the accessibility of the catalytic site. High Ca^{2+} concentrations in the ECM allow the protein to adopt an open conformation that exposes the enzymatic core, while high intracellular GTP forces a closed, enzymatically inactive structure (19). It is, therefore, accepted that TG2 is enzymatically inactive in the intracellular environment, under normal physiological conditions, as we have also observed here by analyzing human fibroblasts and mesothelial LP9 cells. However, crosslinking activity was detectable in PDA cells and their matrix as assessed *in vitro* and *in vivo*, suggesting that the enzyme is activated in transformed cells and in the pancreatic matrix. While the factors causing TG2 activation in this context remain unknown, it is tempting to speculate that hypoxia or other types of tissue injury inflicted by tumorigenesis may be inducing transglutaminase activity *in vivo* (30, 42). The involvement of metabolic or physical factors that could be expressed or function differently in transformed vs. normal cells (e.g. oxidative stress, calcium homeostasis) cannot be excluded. Interestingly, a previous report suggested that Ikβα, the NF-κB inhibitory subunit, is a direct substrate of TG2 in PDA cells (16), although direct evidence of TGase activity was not provided.

We also show that collagen 1 was crosslinked by TG2 in the pancreatic milieu leading to formation of a dense matrix that stimulated the growth of fibroblasts and cancer cells. Increased collagen deposition was noted in fibroblasts co-cultured with TG2 expressing PDA cells. Our findings are consistent with a previous report demonstrating that collagen crosslinked by TG2 stimulated the proliferation of dermal fibroblasts (25) and with studies demonstrating the involvement of TG2 in wound healing and in pathologic fibrotic responses in the lung or liver (15, 43–46). We suspect that the enzyme has other substrates in the pancreatic ECM and that their modifications through crosslinking could contribute to the distinct architecture of the DS, which in turn fosters tumor progression.

Third, we link here for the first time TG2 to activation of the transcriptional regulators, YAP and TAZ, in cancer cells. This pathway, strongly implicated in cell survival and proliferation and activated in epithelial cancers (47, 48), is known to be regulated by the physical properties of the ECM and to translate mechanical cues into relevant biological signals (33). Recent reports have implicated YAP signaling in pancreatic tumor progression downstream of mutant Ras or independent of it (49, 50). We propose that the pancreatic matrix rendered denser by TG2 through collagen crosslinking and stimulation of a fibrotic response, provides positive proliferative feedback to the cancer cells by stimulating YAP/TAZ signaling. Interestingly, TG2 expression levels did not alter cancer cell proliferation in the absence of the matrix. However, we cannot exclude that signals other than tissue stiffness, directly modulated by TG2 or by the matrix, contribute to YAP activation in this context.

These observations led us to posit that the stimulatory growth signals conveyed by TG2 require the stroma, which “deflects” mechanical or biological cues from the tumor milieu into an active oncogenic program. In all, our findings support the involvement of enzymatically active TG2 in the progression of PDA and future studies targeting the crosslinking activity of TG2 to disrupt tumor progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Aggressive and chemotherapy-resistant pancreatic cancer is commonly associated with a dense stroma, which forms a protective niche for cancer cells. We hypothesized that tissue transglutaminase (TG2), a transamidating enzyme secreted by pancreatic cancer cells, promotes pancreatic cancer progression by protein crosslinking in the stroma. Our data present the first evidence that TG2 is abundantly secreted and enzymatically active in the pancreatic tumor milieu where it crosslinks collagen and stimulates fibroblast proliferation. We demonstrate that TG2 orchestrates the development of a characteristic fibrotic inflammatory reaction that enables tumor growth. Our data point to a novel mechanism, involving the tumor microenvironment by which TG2 promotes pancreatic tumor progression. The findings have important consequences for developing strategies to block TG2 enzymatic activity and control pancreatic cancer growth.

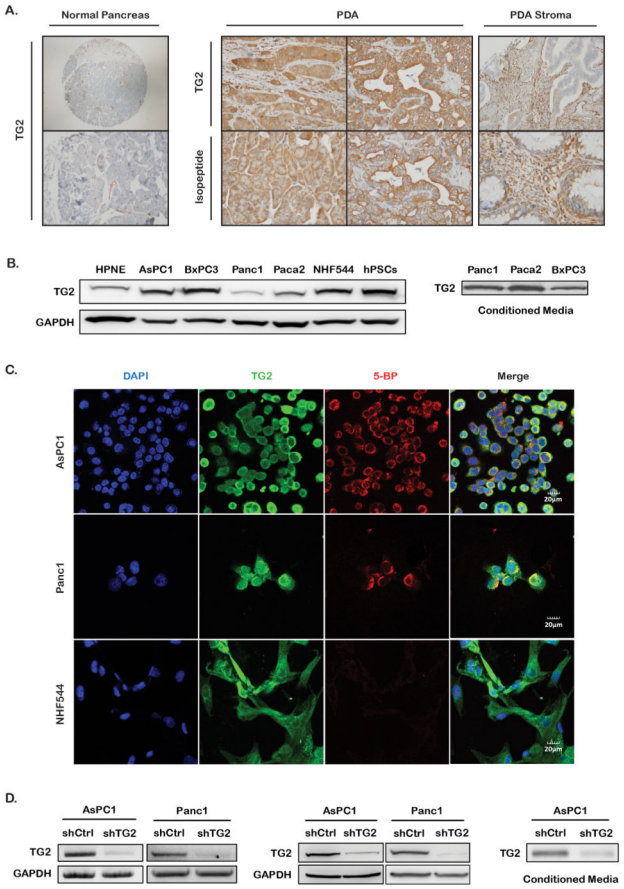

Figure 1. TG2 is expressed and active in pancreatic cancer cells and tumors

(A) Immunostaining for TG2 in normal pancreas (*left* panels, *upper;* 40×, *lower;* 200×); immunostaining for TG2 (*upper right*) and isopeptide (*lower right*) in PDA specimens (200×). **(B)** Western blotting for TG2 in PDA, HPNE, stellate (hPSC) cells and NHF544 fibroblasts (*left*) and in CM (*right*, 40 μl/lane) collected from PDA cells. **(C)** IF for TG2 (AlexaFluor® 488, green) and 5-BP (Streptavidin, Alexa Fluor® 647, red) in AsPC1, Panc1 and NHF544 cells (600×). Nuclei are visualized by DAPI. **(D)** TG2 expression measured by RT-PCR (*left*) and western blotting (*middle* and *right*) in AsPC1 and Panc1 cells stably transduced with shRNA targeting TG2 (shTG2) or control shRNA (shCtrl).

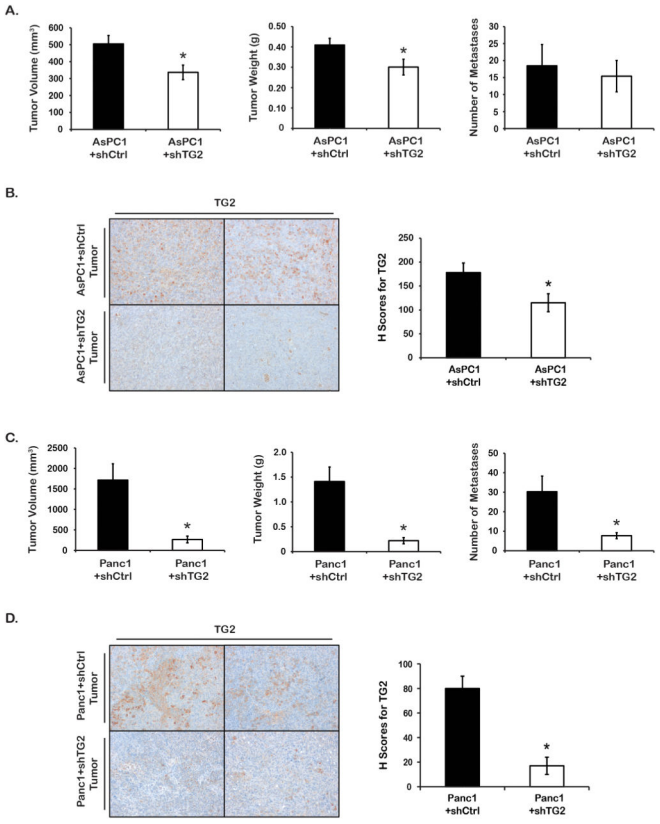

Figure 2. TG2 knockdown inhibits tumor growth in an orthotopic pancreatic xenograft mouse model

(A) Average tumor volume (*left*, $p = 0.02$), weight (*middle*, $p = 0.04$), and number of metastases (*right*, $p = 0.70$) of xenografts derived from AsPC1 cells transduced with TG2 or control shRNA (shCtrl; n=11, shTG2; n=12). **(B)** IHC for TG2 in AsPC1+shCtrl and AsPC1+shTG2 xenografts (*left*, 100×). H scores quantitated TG2 expression in tumors (*right*, n=10 per group, $p = 0.004$). **(C)** Average tumor volume (*left*, $p = 0.01$), weight (*middle*, $p = 0.006$), and number of metastases (*right*, $p = 0.03$) derived from Panc1+shTG2 or +shCtrl cells (n=7 per group). **(D)** IHC for TG2 in Panc1+shCtrl and PanC1+shTG2 xenografts (*left*, 100×). H scores quantitated TG2 expression in tumors (*right*, n=3 per group, p = 0.007). Bars represent average measurements +/− SE; * $p < 0.05$.

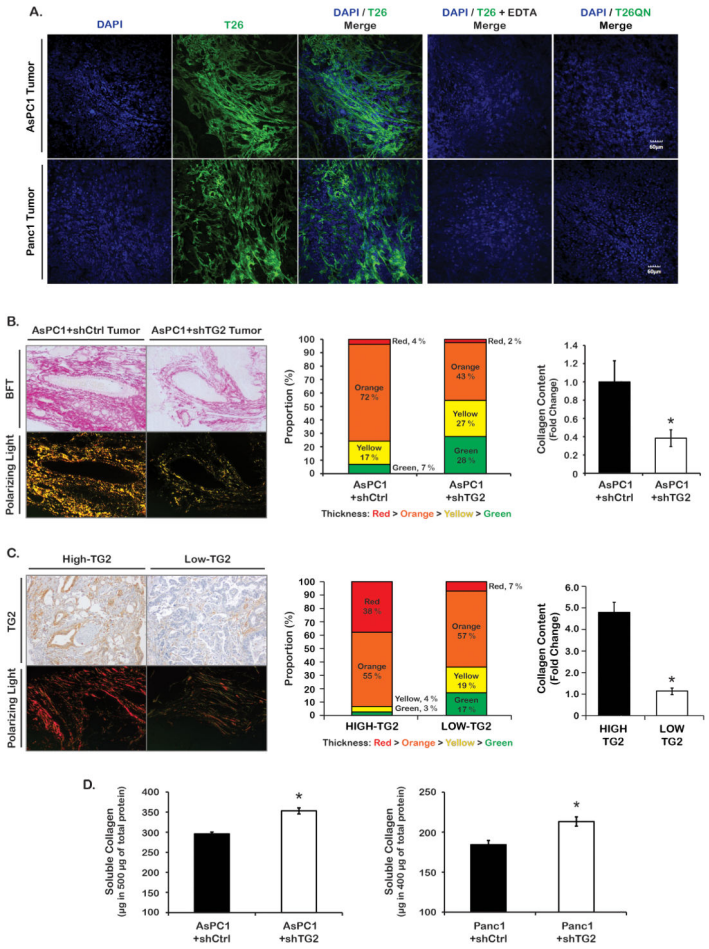

Figure 3. TG2 is enzymatically active and crosslinks collagen in the PDA stroma

(A) IF using FITC-labeled T26, T26 and EDTA (negative control), and T26QN (negative control) in AsPC1 and Panc1 xenografts (200×). **(B)** PSR staining determines deposition and thickness of collagen fibers in AsPC1+shCtrl and AsPC1+shTG2 tumors; *upper* - bright field transmission (BFT) microscopy, *lower* - polarizing light microscopy (200×, *left*). Quantification of collagen fiber thicknesses based on differences in birefringence (*middle*) and quantitative assessment of collagen content based on area staining for collagen per field (*right*, n=11 per group, $p = 0.001$. (C) PSR staining measures deposition and thickness of collagen fibers in TG2 high and low human tumors; *upper*–IHC for TG2 (100×), *lower*–polarizing light microscopy, (200×, *left*). Quantification of collagen fiber thicknesses based on differences in birefringence (*middle*) and quantitative assessment of collagen content based on area staining for collagen per field (*right*, n=6 per group). Bars represent fold changes +/− standard error (SE), $p < 0.0001$. **(D)** Soluble collagen measured by the Sircol assay in AsPC1+/− shTG2 (*left*, n=6 per group, $p = 0.001$) and Panc1+/−shTG2 tumors (*right*, n=6 per group, $p = 0.005$). Bars represent average measurements +/− SE; * $p < 0.05$.

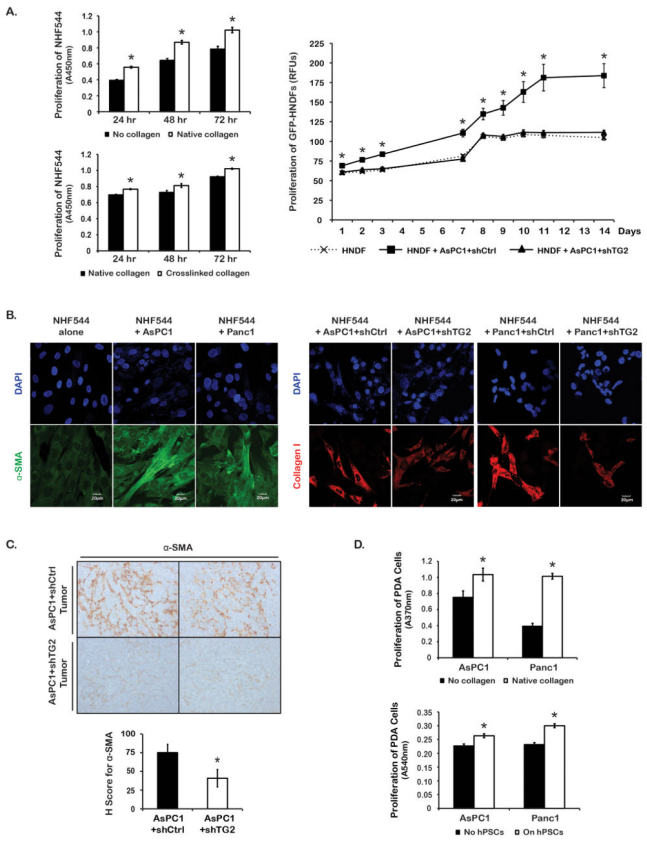

Figure 4. TG2 promotes fibrosis in the PDA stroma

(A) Proliferation of NHF544 cultured on collagen coated or uncoated plates (*left*, *upper*, $p < 0.04$) and on native or TG2-crosslinked collagen (*left*, *lower*, $p < 0.03$). Proliferation of GFP-HNDF in co-culture with AsPC1+shCtrl or AsPC1+shTG2 cells or grown alone for 14 days (*right*, $p < 0.03$). **(B)** IF for α-SMA (*left* panels, Alexa Fluor® 488, green) in NHF544 cultured alone (*left*) or co-cultured with AsPC1 (*middle*) or Panc1 cells (*right*). Nuclei are visualized by DAPI. IF for collagen 1 (*right* panels, Cy5, red) of NHF544 co-cultured with AsPC1+shCtrl or AsPC1+shTG2 (*left*) or with Panc1+shCtrl and Panc1+shTG2 cells (*right*). **(C)** IHC for α-SMA identifies myofibroblasts in AsPC1+shCtrl and AsPC1+shTG2 xenografts (*upper*, 100×). H scores quantify fibrosis (*lower*, shCtrl; n=10, shTG2; n=7, $p < 0.05$). **(D)** Proliferation of AsPC1 and Panc1 cells on collagen coated or uncoated plates (*upper*, $p < 0.03$), and on uncoated plates or a feeder layer of hPSCs (*lower*, $p < 0.006$). **(A, D)**: bars represent average of 4 replicate measurements for the groups +/− SE; * $p < 0.05$.

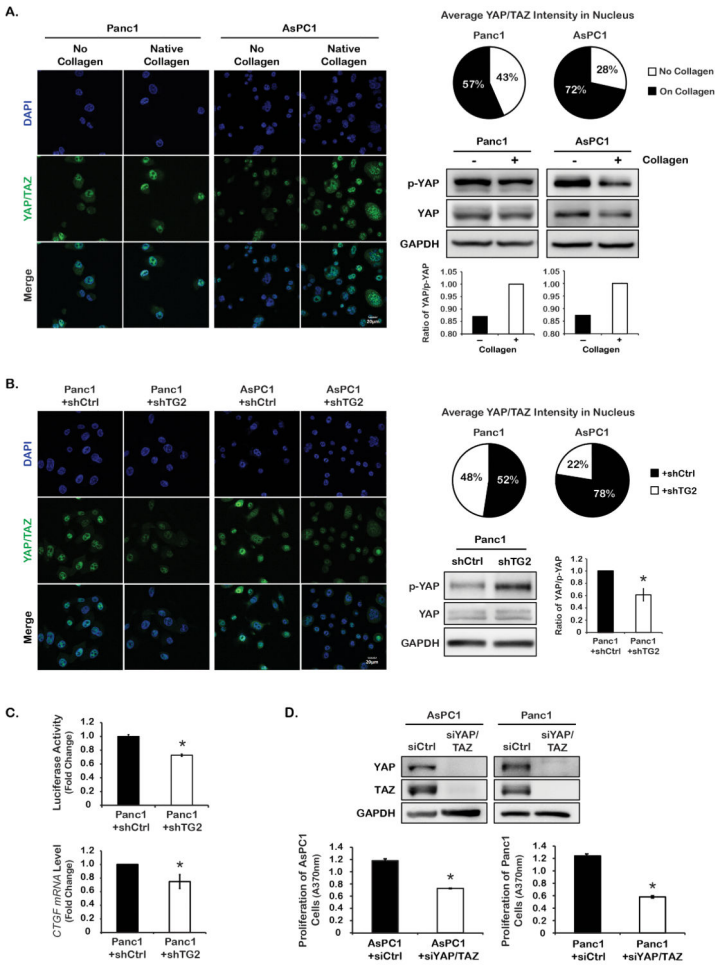

Figure 5. TG2 and collagen activate YAP/TAZ in PDA cells

(A) IF for YAP/TAZ (*left* panels, Alexa Fluor® 488, green) in Panc1 and AsPC1 cells grown on collagen coated or uncoated slides (600×). Pie charts (*right*, *upper*) show percentage of nuclear YAP/TAZ, and western blotting (*right*, *lower*) shows total- and p-YAP in Panc1 and AsPC1 cells plated on collagen or uncoated plates. Bars represent the ratio of total-YAP/p-YAP. **(B)** IF for YAP/TAZ (*left panels*, Alexa Fluor® 488, green) in Panc1+shCtrl or Panc1+shTG2 and AsPC1+shCtrl or AsPC1+shTG2 cells grown on collagen (600×). Pie charts (*right*, *upper*) show the percentage of nuclear YAP/TAZ, and western blotting (*right*, *lower*) shows total- and p-YAP in Panc1+shCtrl or Panc1+shTG2 grown on collagen. Bars represent the ratio of total-YAP/p-YAP. **(C)** TEAD4 reporter assay in Panc1+shCtrl or Panc1+shTG2 grown on collagen (*upper*, n=5, $p = 0.009$). Luciferase activity was normalized to *renilla*. Bars represent averages of 5 replicates/group +/− SE; * $p < 0.05$. Q-RT-PCR measures *CTGF mRNA* levels in Panc1+shCtrl or Panc1+shTG2 grown on collagen (*lower*, $p = 0.04$). **(D)** Western blotting for YAP and TAZ in AsPC1 and Panc1 cells transfected with siRNA targeting YAP and TAZ (*upper*). Proliferation of AsPC1 *(lower, left)* and Panc1 *(lower, right)* cells transfected with siRNA targeting YAP and TAZ (n=4 per group, $p < 0.01$). Bars represent average of 4 replicates +/− SE; * $p < 0.05$.

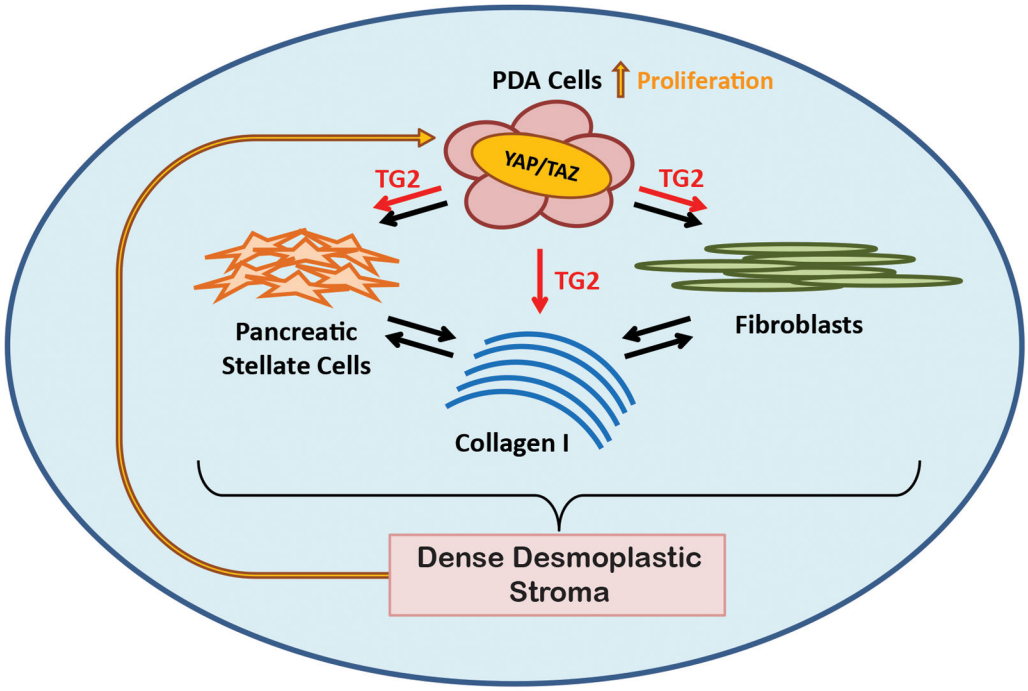

Figure 6.
Proposed mechanism by which TG2 promotes tumor growth.