Hypoxia and Transforming Growth Factor Beta Cooperate to Induce Fibulin-5 Expression in Pancreatic Cancer

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ABSTRACT

The deposition of extracellular matrix (ECM) is a defining feature of pancreatic ductal adenocarcinoma (PDA) where ECM signaling can promote cancer cell survival and epithelial plasticity programs. However, ECM signaling can also limit PDA tumor growth by producing cytotoxic levels of reactive oxygen species (ROS). For example, excess fibronectin (FN) stimulation of α5β1 integrin on stromal cells in PDA results in reduced angiogenesis and increased tumor cell apoptosis due to oxidative stress. Fibulin-5 (Fbln5) is a matricellular protein that blocks FN-integrin interaction and thus directly limits ECM-driven ROS production and supports PDA progression. Compared to normal pancreatic tissue, Fbln5 is expressed abundantly in the stroma of PDA; however, the mechanisms underlying the stimulation of Fbln5 expression in PDA are undefined. Using in vitro and in vivo approaches, we report that hypoxia triggers Fbln5 expression in a transforming growth factor β (TGF-β)- and PI3K-dependent manner. Pharmacologic inhibition of TGF-β receptor (TGF-βR), PI3K, or protein kinase B (AKT) was found to block hypoxia-induced Fbln5 expression in mouse embryonic fibroblasts and 3T3 fibroblasts. Moreover, tumor-associated fibroblasts from mouse PDA were also responsive to TGF-βR and PI3K/AKT inhibition with regard to suppression of Fbln5. In genetically engineered mouse models of PDA, therapy-induced hypoxia elevated Fbln5 expression while pharmacologic inhibition of TGF-β signaling reduced Fbln5 expression. These findings offer insight into the signaling axis that induces Fbln5 expression in PDA and a potential strategy to block its production.

INTRODUCTION

The maintenance of solid tumors relies heavily on cues received from the surrounding environment. The ECM is composed of structural proteins such as FN and collagen, which promote signaling through integrins and receptor tyrosine kinases [1, 2]. ECM signaling is modulated by matricellular proteins, which regulate ECM-cell interactions without serving a direct structural function [3-5]. The composition of the ECM is dynamic and varies between tumors and tumor types, contributing to intra- and inter-tumor heterogeneity, which presents challenges for effective therapeutic strategies. Furthermore, mouse studies have revealed anti- and pro-tumorigenic functions for ECM [5-7]. In addition to ECM, stromal cells, including endothelial cells,
immune cells, and fibroblasts, are present in the tumor microenvironment. These cells contribute to tumor growth, invasion, and chemoresponse [8-10].

During tumor progression, cancer cells release factors that maintain a microenvironment conducive for growth. For example, TGF-β is a cytokine expressed in many cancers that enhances the expression of multiple ECM molecules, including but not limited to FN, collagen, elastin, and fibulins [11-13]. Fbln5 is a 448-amino acid secretory glycoprotein of the fibulin family of matricellular proteins. Fbln5 is unique amongst its members as it contains an Arg-Gly-Asp (RGD)-integrin binding domain and can ligate a number of integrins [14]. Fbln5 is expressed during development, particularly in the vasculature, but is significantly downregulated in most adult tissues [15]. Reactivation of Fbln5 expression occurs in injured blood vessels and other pathological conditions, including cancer [15-17].

The generation of Fbln5 knockout mice revealed an essential function of Fbln5 in elastogenesis. Fbln5-/- mice display loose skin, tortuous vessels, and emphysematous lung, creating the first animal model for connective tissue disorders [18-20]. In addition to stabilizing elastic fibers, Fbln5 functions as a molecular rheostat that regulates processes such as cell proliferation and migration in a cell-type specific manner [21-23]. For example, Fbln5 blocks FN-mediated integrin signaling in smooth muscle cells [24]. Our lab has identified a unique function for Fbln5 in pancreatic tumors. Fbln5 protein levels are increased in mouse PDA compared to a normal pancreas [25]. The loss of Fbln5 results in decreased tumor growth in subcutaneous and orthotopic models of PDA [26]. Mechanistic studies revealed that Fbln5 blocked FN-mediated integrin-induced ROS production and that the loss of Fbln5 resulted in enhanced ROS production. Furthermore, mutation of the integrin-binding domain of Fbln5 (RGD→RGE) leads to decreased tumor growth and increased survival in two well-established genetically engineered mouse models (GEMM) of PDA (KIC, KPC) due to elevated levels of ROS [25]. In this setting, Fbln5 promotes PDA progression by limiting FN-integrin signaling and thus preventing cytotoxic ROS production.

The function of Fbln5 in cancer is largely context dependent. Others have reported that Fbln5 is downregulated in some cancers; however, these studies focused largely on mRNA expression in tumor lysates and cell lines and on tissue microarray (TMA) analysis [27, 28]. In our previous studies, we found that Fbln5 is produced mainly by stromal fibroblasts and endothelial cells while epithelial-derived tumor cells produce essentially no Fbln5 [25]. Furthermore, an accurate evaluation of Fbln5 via TMA analysis may be challenging due to variability in stromal content between samples and the heterogeneous staining pattern of Fbln5 within tumors [25]. In addition to PDA, Fbln5 is abundant in the stroma of human breast cancer and its presence is associated with a more invasive phenotype in 4T1 mouse tumors [21]. However, the mechanism underlying Fbln5 expression during tumorigenesis is unclear. Previous studies examining the molecular pathways leading to Fbln5 expression have been performed strictly in vitro. For example, Fbln5 has been identified as a TGF-β–inducible gene in fibroblasts [11], whereas another study revealed that hypoxia enhances Fbln5 expression in endothelial and HeLa cells [29]. These studies have independently shown that Fbln5 induction is dependent on the PI3K/AKT pathway. However, it is unclear whether hypoxia induces Fbln5 in a TGF-β–dependent manner and if these factors regulate Fbln5 expression in an in vivo context.

Hypoxia and TGF-β expression and activity are elevated in a number of advanced solid tumors, including PDA [5, 30]. Therefore, through biochemical and immunohistological analyses, we sought to elucidate a mechanism by which pancreatic tumors stimulate Fbln5 expression in vivo.

RESULTS

Fbln5 Expression in Pancreatic Tumors

Fbln5 is expressed aberrantly in a number of malignancies [21, 22, 27, 35, 36]. Expression analysis of various cell types has identified fibroblasts and endothelial cells as major producers of Fbln5 [25]. Moreover, immunohistological analysis of Fbln5 reveals a stromal staining pattern in tumors ([25], Fig. 1). The KIC and KPC models of PDA show abundant Fbln5 staining (green) compared to normal pancreas (Fig. 1). We also counterstained this...
tissue with the fibroblast markers αSMA (Fig. 1A-C, red) and GFAP (Fig. 1D-F, red) [37]. Given that fibroblasts are a major source of Fbln5, it is tempting to speculate that the increased infiltration of these cells in PDA is contributing to the accumulation of Fbln5 in PDA. However, we found that not all stromal areas are positive for Fbln5, and while Fbln5 does co-localize with αSMA and GFAP (arrows), there are areas where the two markers do not overlap (arrowheads). This suggests that the expression of Fbln5 in tumors is tightly regulated and warrants investigation into the factors that control its expression.

**Hypoxia Induces Fbln5 Expression and Requires TGF-β Receptor Activity**

Fbln5 regulates angiogenesis in a context-dependent manner [23, 26, 38-40]. We have reported that the loss of functional Fbln5 leads to decreased microvessel density specifically in the tumor microenvironment of mouse PDA [26]. Furthermore, it has been shown that hypoxia upregulates Fbln5 in endothelial cells and protects these cells from hypoxia-induced apoptosis [29]. Given the hypoxic nature of PDA [41, 42], it is plausible that Fbln5 is induced by hypoxia to support angiogenesis and tumor growth. Since fibroblasts are a major source of Fbln5 in PDA, we tested whether fibroblasts induce Fbln5 in response to hypoxia. We exposed 3T3 fibroblasts and MEFs to hypoxia (0.8% O2) for 24 hours. Western blot analysis revealed an induction of Fbln5 protein compared to cells plated under normoxic conditions (Fig. 2A). Hypoxia-inducible factor 1α (Hif-1α) (Fig. 2A) and Glut1 (Fig. 2B) were also analyzed by Western blot to confirm hypoxic conditions. To corroborate this data, 3T3 fibroblasts were treated with CoCl2, a hypoxia-mimicking agent reported to stabilize Hif-1α by inhibiting prolyl hydroxylation [43, 44]. CoCl2 treatment was found to enhance Fbln5 expression in a time-dependent manner (Fig. 2C).

Fbln5 has been identified as a TGF-β-inducible gene in vitro [11]. Our results also support this as 3T3 fibroblasts and MEFs treated with 10 ng/ml of TGF-β induced Fbln5 expression compared to untreated cells (Fig. 2D). Furthermore, the blockade of TGF-β receptor 1 (TGF-βR1) by the small molecule inhibitor LY2157299 significantly reduced Fbln5 induction by TGF-β (Fig. 2E). However, it is unclear whether hypoxia-induced Fbln5 requires TGF-β signaling; therefore, we explored the relationship between TGF-β and hypoxia with regard to Fbln5 expression. We found that blocking TGF-βR1 using two independent inhibitors (LY2157299 and SB-431542) while cells were under hypoxia (in the absence of exogenous TGF-β) resulted in decreased Fbln5 protein levels (Fig. 2F). This experiment demonstrates that hypoxia-driven Fbln5 expression requires TGF-βR1 activity. Moreover, we examined the effect of hypoxia on TGF-β activity in fibroblasts. We saw increased TGF-βR activity as evidenced by phospho-Smad2 levels in MEFs under hypoxic conditions and 3T3 cells treated with CoCl2 (Fig. 2H-I).

Canonically, TGF-β binds to TGF-βR, which recruits and activates TGF-βR leading to activation of Smad2/3 and accumulation of Smad4 in the nucleus [45]. It has been previously reported that deletion or mutation of Smad-binding sites within the FBLN5 promoter attenuates FBLN5 transcriptional activity in response to TGF-β treatment in human lung fibroblasts [11]. Conversely, another study revealed that transfection of a dominant-negative Smad3 in 3T3 cells did not effect the ability of TGF-β to increase Fbln5 mRNA levels [22]. These seemingly conflicting results prompted us to investigate the importance of Smad4 in TGF-β-induced Fbln5 expression. We transfected 3T3 cells with three independent siRNAs against Smad4 (05, 06, 07) and a non-targeting siRNA (NT) and then treated these cells with TGF-β. Western blot analysis revealed robust depletion of Smad4, however, Fbln5 levels remained constant (Fig. 2J-K). Altogether, while TGF-β signaling was activated by hypoxia, our results suggest a mechanism by which hypoxia induces TGF-β activity leading to enhanced Fbln5 expression in a Smad4-independent manner.

**Fbln5 Expression Requires PI3K/AKT Activity**

In addition to Smads, TGF-β can induce phosphorylation of several other downstream targets, including AKT [46]. Furthermore, the induction of Fbln5 by TGF-β and hypoxia individually require the PI3K/AKT pathway [11, 29]. Expanding on these findings, we have shown that the combination of TGF-β and hypoxia treatment requires AKT activity. The inhibition of PI3K using LY294002 blocked TGF-β-induced
Fbln5 expression and phosphorylation of AKT under normoxic and hypoxic conditions in 3T3 cells (Fig. 3A). LY294002 also blocked basal Fbln5 expression under normoxia in the absence of exogenously added TGF-β (Fig. 3B). Moreover, we tested the effect of two other PI3K/AKT inhibitors, BKM120 (PI3K inhibitor) and an AKT1/2 inhibitor (AKT1/2 KI) on Fbln5 expression. These alternative inhibitors also reduced Fbln5 expression in fibroblasts (Fig. 3C-D). We confirmed that each inhibitor reduced phospho-AKT levels at both major activation sites (Fig. 3A-D). The inhibitors did not affect the expression of total AKT. These results indicate that PI3K/AKT activity is required for Fbln5 expression in normoxic and hypoxic conditions.

Fbln5 Expression in Tumor-Associated Fibroblasts Also Requires TGF-β and PI3K Activity

To validate our findings in a more tumor-relevant cell type, we isolated TAFs from mouse PDA. We used platelet-derived growth factor receptor (PDGFR)-α as a marker to specifically select for fibroblasts [47]. Bright field images of these TAFs revealed spindle-like morphology typical of fibroblasts (Fig. 4A). Furthermore, we characterized these TAFs by immunostaining for αSMA (Fig. 4B). TAFs were treated with TGF-β, which induced Fbln5, an effect that was sensitive to the inhibition of TGF-βR1 by LY2157299 or SB-431542 (Fig. 4C). TGF-β-induced activation of AKT was also reduced by TGF-βR1 inhibition (Fig. 4C). Fbln5 expression by TAFs was also sensitive to PI3K inhibition (Fig. 4D).

Inhibition of TGF-β Signaling Reduces Fbln5 Expression in Mouse PDA

TGF-β functions as a tumor suppressor in the early stages of PDA; however, as the disease progresses, TGF-β switches to a pro-tumorigenic molecule [48]. Enhanced expression and activity of TGF-β has been reported in many models and human cases of PDA [5, 30, 49-52]. To confirm that TGF-β signaling is critical for Fbln5 expression in pancreatic tumors, we examined Fbln5 levels in tumor tissue from KPC mice that had been treated with TGF-β inhibitors. KPC mice were treated with LY2157299 as well as an inhibitor of TGF-β receptor 2 (2G8) [30]. Immunohistochemical analysis of frozen tumor sections revealed a significant decrease in Fbln5 expression in tumors of treated mice (Fig. 5A-B).

Hypoxia Drives Fbln5 Expression in Mouse PDA

Next we examined the influence of hypoxia on Fbln5 expression in vivo. To achieve this, we analyzed tissue from KIC mice that had been treated with the VEGF inhibitor, mouse chimeric r84 (mcr84) [53]. The rationale behind this approach is that anti-angiogenic therapy increases intratumoral hypoxia [5, 54]. Using these mice, we have previously confirmed that treatment with mcr84 induces hypoxia in tumors compared to untreated tumors as seen by pimonidazole staining [5]. Consistent with our in vitro results, hypoxia increased Fbln5 expression in vivo such that tumors from mice treated with mcr84 displayed a significant increase in Fbln5 expression compared to untreated tumors (Fig 5C). We also found that the expression of Fbln5 is coincident with hypoxic areas in KIC tissue as shown by pimonidazole staining (Fig 5D). Together, these results demonstrate that Fbln5 expression is induced by a hypoxic tumor microenvironment.

DISCUSSION

In PDA, Fbln5 expression is limited to stromal cells whereby all tumors examined were Fbln5-positive but not all stromal cells expressed Fbln5, suggesting context-dependent regulation of intra-tumoral Fbln5 expression. An in vitro examination of signaling cascades demonstrated that Fbln5 induction is dependent on TGF-β-PI3K/AKT signaling, a pathway that can be induced by hypoxia and is independent of Smad4. Our in vitro results were recapitulated by an investigation of Fbln5 expression in PDA tumors from animals treated with pharmacologic agents that either block Tgf-β activity or induce hypoxia. A summary of our findings is provided in Fig. 6.

Fbln5 is a pro-tumorigenic factor in mouse PDA, as Fbln5-deficient and mutant mice show a reduction in pancreatic tumor growth and increased survival [25, 26]. Fbln5 functions as a tumor promoter in these models by blocking integrin-mediated ROS production. In the absence of functional Fbln5, tumors display higher levels of ROS, which leads to a reduction in cell proliferation and angiogenesis and an induction of apoptotic cell death.
Fbln5 is expressed by stromal cells but typically not by cells of epithelial origin [21, 33]. We have not seen evidence of Fbln5 expression by pancreatic tumor cells, in fact, the treatment of PDA cells with TGF-β, hypoxia, or both failed to induce Fbln5 protein in vitro (data not shown). It is unclear why tumor cells, which are responsive to Tgf-β, fail to express Fbln5 after stimulation with Tgf-β. It is plausible that tumor cells may be subject to epigenetic regulation that inhibits the Fbln5 promoter. Further studies are needed to validate this hypothesis. Interestingly, while Fbln5 is readily expressed by fibroblasts in vitro and co-localizes with the fibroblast marker αSMA in vivo, there are still areas within the tumor where Fbln5 and αSMA do not overlap. This supports the idea that Fbln5 expression is tightly regulated and signal-dependent, and to this extent, we see abundant Fbln5 expression coincident with select areas of hypoxia in mouse PDA. Furthermore, areas that are positive for Fbln5 but negative for αSMA and GFAP may represent a specific population of fibroblasts within the tumor. Additional characterization is needed to determine whether Fbln5 marks a distinct group of fibroblasts within the tumor microenvironment.

The aberrant deposition of ECM proteins is characteristic of PDA and contributes to overall tumor progression and chemoresistance. Thus, therapies that target the ECM or more specifically, the proteins known to stimulate ECM production are very attractive as potential therapeutic strategies. For example, TGF-β signaling is characteristic of PDA and contributes to overall tumor progression and chemoresistance. Thus, therapies that target the ECM or more specifically, the proteins known to stimulate ECM production are very attractive as potential therapeutic strategies. For example, TGF-β-specific mechanism by which Fbln5 is upregulated in mouse PDA in response to hypoxia and provide a strategy to block this induction by the use of pre-existing TGF-βR inhibitors.

**EXPERIMENTAL PROCEDURES**

**Mouse Models**

Kras<sup>LSL-G12D/+</sup>, Trp53<sup>LSL-R172H/+</sup>; p48<sup>Cre/+</sup> (KPC) and Kras<sup>LSL-G12D/+</sup>; Cdkn2d<sup>−/−</sup>; p48<sup>Cre/+</sup> (KIC) mice were generated as previously described [5, 31-33]. All animals were housed in a pathogen-free facility with access to food and water ad libitum. Experiments were performed under protocols approved by the institutional animal care and use committee at the University of Texas Southwestern Medical Center in Dallas.

**Animal Studies**

Survival studies were performed in KIC mice treated with the anti-vascular endothelial growth factor (VEGF) mAb mcr84 and KPC mice were treated with the Tgf-β inhibitors, 2G8 or LY2157299. Mice were housed in a pathogen-free facility with access to food and water ad libitum. Experiments were performed under protocols approved by the institutional animal care and use committee at the University of Texas Southwestern Medical Center in Dallas.

**Cell Culture (Inhibitors/Reagents)**

Mouse NIH/3T3 cells and mouse brain endothelial cells (bEnd.3) were obtained from ATCC. Mouse embryonic fibroblasts (MEFs) were isolated at embryonic day E12.5-E14.5. All cells were maintained in DMEM supplemented with 10% FBS (Mediatech, Inc.) and were grown in a 37°C humidified incubator with 10% O₂ and 5% CO₂. Confluent cultures were synchronized using reduced serum Opti-MEM (Life Technologies) for MEFs and tumor-associated fibroblasts (TAFs) or 1% FBS in DMEM for 3T3 cells for at least 6 hours before experimentation. MEFs were used between passages 2-5 for all experiments. Synchronized cells were treated with 10 ng/ml TGF-β (Peprotech, 100-21C) for 24 hours. The following inhibitors were used in culture:
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LY2157299 (Cayman Chemical, 15312), SB431542 (Tocris, 1614), and LY2940002 (Cell Signaling, 9901). Inhibitors (at various concentrations, see figure legends) were added 1 hour prior to TGF-β stimulation and/or hypoxic stimulus, and cells were harvested at 24 hours for Western blot analysis. For hypoxia studies, cells were kept in a humidified atmosphere containing 5% CO₂ and 0.8% O₂ in a modular incubator chamber (Billups-Rothenberger). CoCl₂ (232696, Sigma) was used as a chemical inducer of hypoxia. Synchronized cells were treated with 100 µM of CoCl₂ and cells were harvested at 4, 8, and 24 hours.

**Transient Transfection Assay**

3T3 cells were transfected with 3 individual siRNAs targeting mouse Smad4 and a non-targeting siRNA. siRNAs (ON-TARGETplus) and transfection reagent (Dharmafect) were obtained from Dharmacon. Using the online protocol provided by Dharmacon, cells were plated in a 24-well plate until cells were 90% confluent. Cells were treated with 25nM siRNA per well and allowed to incubate for 48 hours before treating with 10ng/ml of TGF-β for 24 hours.

**Immunofluorescence Analysis of Cells and Tissue**

Tissues were either frozen in liquid nitrogen and embedded in optimum cutting temperature compound (OCT; Tissue-Tek) for frozen sections or fixed with 10% neutral buffered formalin solution of 4% paraformaldehyde (PFA) overnight and embedded in paraffin for sectioning. Frozen sections were subject to mild fixation in ice-cold acetone for 5 minutes and air-dried for 10 minutes. Frozen sections were then incubated with PBS for 10 minutes at room temperature. Paraffin sections were deparaffinized and rehydrated with xylene and decreasing serial dilutions of ethanol followed by heat-mediated antigen retrieval with 0.01 M citric acid buffer (pH 6.0). Tissue sections were outlined with a pap pen and blocked with 20% aquablock for 1 hour. In the case of mouse-on-mouse staining, unconjugated Fab fragment donkey anti-mouse IgG (Jackson ImmunoResearch Labs) was diluted in PBS and added to tissues for 1 hour. Primary antibodies were diluted in blocking solution (5% BSA in Tris-buffered saline with Tween-20 [TBST]) and incubated at 4°C overnight. Primary antibodies and dilutions used for tissue staining were: anti-α-smooth muscle actin (SMA) – Cy3 (1:500; C6198, Sigma), mouse anti-glia fibrillary acidic protein (GFAP; 1:500; MAB360, Millipore), and rabbit anti-Fbln5 (1:100; purified polyclonal IgG by our lab, 1.6 mg/ml). Fluorescein isothiocyanate (FITC) or Cy3-conjugated anti-rabbit secondary antibodies were used (1:1000; Jackson ImmunoResearch) for immunofluorescent staining of tissues. For immunocytochemistry, cells were plated onto four-well chamber slides and allowed to attach and grow for at least 24 hours. Media was removed and cells were washed 3 times in PBS. Cells were fixed in ice-cold acetone for 10 minutes followed by 3 washes in PBS. Cells were blocked in 20% Aquablock for 1 hour and stained with mouse anti-α-SMA – Cy3 (1:500; C6198, Sigma) diluted in 5% BSA in TBST at 4°C overnight.

**Western Blot**

Western blots were performed as previously described [25]. Cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing cocktails of protease (Thermo Scientific) and phosphatase inhibitors (Sigma-Aldrich). Lysates were centrifuged for 10 minutes at 13000g at 4°C. Proteins were separated by SDS-PAGE and transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (VWR). Primary antibodies were diluted in 5% milk in TBST except phospho-specific antibodies, which were diluted in 5% BSA in TBST. Primary antibodies used for Western blot were: rabbit anti-Fbln5 (1:1000), mouse anti-Hif-1α (1:500; MAB5382, Millipore), rabbit anti-Glut (1:1000; AM32430PU-M, Acris), mouse anti-Smad2 (1:1000; 3103, Cell Signaling), rabbit anti-phospho-Smad2 (1:1000; 3104S, Cell Signaling), rabbit anti-AKT (1:1000; 9272S, Cell Signaling), rabbit anti-phospho-AKT (Ser473; 1:1000; 4060, Cell Signaling), rabbit anti-phospho-AKT (Thr308; 1:1000; ab40759, Abcam), rabbit anti-actin (1:5000; A2066, Sigma). Rabbit anti-actin was used as a loading control for all Western blots shown. HRP-conjugated donkey anti-rabbit or anti-mouse IgGs (1:10000; Jackson ImmunoResearch) secondary antibodies were used for Western blots. Quantification of Western blots was done by using the Image Studio Lite software.
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Controls are normalized to one for all Western blot quantifications.

FACs isolation of TAFs

Isolation of TAFs from fresh mouse PDA was done as previously described [34]. Briefly, sizeable tumors were dissected from KIC mice and minced manually using a sterile razorblade. Minced tumors were subjected to enzymatic digestion using collagenase for 45 minutes at 37°C with constant agitation. Digestion was stopped by adding 10% FBS in DMEM. The tissue digest was centrifuged and resuspended in fresh 10% FBS DMEM. The tissue/media mixture was strained through a 70 µm cell strainer placed on top of a 50 ml conical tube. Cells were counted using a hemocytometer to obtain a concentration of 10 million cells in 2 ml of FACs Buffer I (Dulbecco's Phosphate Buffered Saline CMF [calcium and magnesium free] + 0.5% BSA). To block endogenous Fc receptor, anti-mouse CD16/CD32 (BD Pharmingen, 553142) was added at 10 µg/ml to cell suspension for 20 minutes on ice. After blocking, 10 µg/ml of anti-mouse CD140α-PE (PDGFR-α, e-Bioscience, 12-1401-81) was added directly to cell suspension for 1 hour on ice in order to select for fibroblasts. After 1 hour, cells were spun down and supernatant was aspirated. Cells were resuspended in FACS Buffer II (Dulbecco's Phosphate Buffered Saline CMF + 1% FBS) and subjected to FACs sorting at the UT Southwestern FACs core facility. After isolating PDGFR-α-positive fibroblasts, cells were immediately spun down and media was replaced with 20% FBS in DMEM and plated for further experimentation. TAFs were used between passages 1-3 for all experiments.
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Potential conflict of interest: R.A. Brekken is a co-founder of Tuevol Therapeutics, a company that is developing therapeutics that target the tumor microenvironment.

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Conception and design: Mary Topalovski and Rolf A. Brekken

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References

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FOOTNOTES

Abbreviations used: ECM (extracellular matrix), PDA (pancreatic ductal adenocarcinoma), ROS (reactive oxygen species), FN (fibronectin), Fbln5 (fibulin-5), TGF-β (transforming growth factor β), AKT (protein kinase B), RGD (Arg-Gly-Asp), RGE (Arg-Gly-Glu), GEMM (genetically engineered mouse models), TMA (tissue microarray), VEGF (vascular endothelial growth factor), MEFs (mouse embryonic fibroblasts), TAFs (tumor-associated fibroblasts), OCT (optimum cutting temperature), PFA (paraformaldehyde), TBST (Tris-buffered saline with Tween-20), SMA (smooth muscle actin), GFAP (glial fibrillary acidic protein), FITC (fluorescein isothiocyanate), RIPA (radioimmunoprecipitation assay), SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), PVDF (polyvinylidene difluoride), PDGFR (platelet-derived growth factor receptor)
FIGURE 1. Fbln5 expression in pancreatic tumors
Normal (A, D) and tumor-bearing pancreata (B, C, E, F) were harvested from WT, 1.5-month-old KIC (B, D) and 3-month-old KPC (C, F) mice, snap frozen, and sectioned. Frozen tissue was stained for Fbln5 (green, A-F), alpha-smooth muscle actin (αSMA, red, A-C), and GFAP (red, D-F). Nuclei were counterstained with DAPI (merge). Total magnification: 200. Scale bars: 50 µm for all images. Representative images from each group are shown (n=5 for all groups, 8-10 pictures taken for each group).
FIGURE 2. Hypoxia induces Fbln5 expression and requires TGF-β activity
3T3 fibroblasts were cultured under normoxic [N] or hypoxic [H] conditions for 24 hours and probed for Fbln5, Hif-1α, (A) and Glut1 (B) by Western blot. (C) 3T3 fibroblasts were treated with 100 μM of CoCl2 and harvested at 4, 8, and 24 hours and probed for Fbln5. (D) 3T3 fibroblasts and MEFs were serum-starved prior to no treatment (-) or treatment (+) with 10 ng/ml of TGF-β. Cells were harvested 24 hours post-treatment and probed for Fbln5, phospho-Smad2, and total Smad2. (E) MEFs were serum-starved and pre-treated with LY2157299 for 1 hour prior to adding TGF-β. Cells were harvested 24 hours post-treatment and probed for Fbln5, phospho-Smad2, and total Smad2. (F) MEFs were serum-starved and treated with either LY2157299 or SB-431542 and were cultured under hypoxic [H] conditions for 24 hours. Untreated normoxic [N] cells were used as a control. Cells were harvested and probed for Fbln5,
phospho-Smad2, and total Smad2. (G) Quantification of relative Fbln5 protein levels from panel (F) using Image Studio Lite. (H) MEFs were cultured under hypoxic conditions or normoxic conditions with (+) or without (-) TGF-β treatment for 24 hours and probed for phospho-Smad2. (I) 3T3 cells were treated with CoCl₂ for 4 hours and probed for phospho-Smad2. (J) 3T3 cells were transfected with three individual siRNAs targeting mouse Smad4 (05, 06, 07) and a non-targeting siRNA (NT). Cells were then treated with 10ng/ml of TGF-β for 24 hours and harvested for Western blot analysis of Smad4 and Fbln5. (K) Quantification of relative Fbln5 protein levels from panel (H). Representative autoradiograms of three independent experiments performed are shown.
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FIGURE 3. Fbln5 expression requires PI3K/AKT activity

(A) 3T3 cells were cultured in a normoxic or hypoxic chamber and treated with the PI3K inhibitor LY294002 for 24 hours in the presence of TGF-β. Cells were harvested for Western blot analysis and probed for Fbln5, phospho-AKT (T308 and S473), and total AKT. Long and short exposures of Fbln5 are shown in (A) to highlight the upregulation of Fbln5 under hypoxia seen by the longer exposure. (B) LY294002 treatment in MEFs for 24 hours under normoxic conditions without TGF-β treatment. (C) MEFs under normoxic conditions treated with an AKT1/2 inhibitor (AKT1/2 KI) for 24 hours without TGF-β treatment. (D) 3T3 cells under normoxic conditions treated with another PI3K inhibitor (BKM-120) for 24 hours without TGF-β treatment. Representative autoradiograms of three independent experiments performed are shown.
FIGURE 4. Fbln5 expression in tumor-associated fibroblasts requires TGF-β and PI3K activity
(A) Representative bright-field image of tumor-associated fibroblasts (TAFs) isolated from mouse PDA. Scale bars are 50 µm, 4x magnification; inlet is zoomed in 5x. (B) Representative images of TAFs stained for αSMA in red and counterstained with DAPI in blue. 20x magnification. (C) TAFs were serum-starved and pre-treated with either LY2157299 or SB-431542 for 1 hour prior to adding TGF-β. TAFs were harvested after 24 hours and probed for Fbln5, phospho-Smad2, phospho-AKT (T308), and total Smad2 by Western blot. (D) TAFs were serum-starved and pre-treated with LY294002 for 1 hour prior to adding TGF-β. TAFs were harvested after 24 hours and probed for Fbln5, phospho-AKT (T308 and S473), and total AKT. Representative autoradiograms of an experiment performed in duplicate are shown.
FIGURE 5. Inhibition of TGF-β receptors reduces Fbln5 expression while anti-VEGF therapy induces its expression in mouse PDA
(A) Frozen tumor sections from KPC mice treated with 2G8 at 30 mg/kg per week (n = 4) or untreated (n = 4) and probed for Fbln5 by immunohistochemical staining. (B) Frozen tumor sections from KPC mice treated with 75 mg/kg LY2152799 2x daily (n = 4) or untreated (n = 4) and probed for Fbln5. (C) Frozen tumor sections from KIC mice treated with 500 µg per week mcr84 (n = 5) or untreated (n = 4). (D) Mice were injected intravenously with 60 mg/kg of pimonidazole that was allowed to circulate for 90 minutes before sacrificing animals. Frozen tissue sections were interrogated with FITC-conjugated anti-pimonidazole primary antibody (green) and Fbln5 (red), (n=3). Images A-D were counterstained with DAPI (blue). Results are shown as the mean ±S.D. Fluorescent intensity was quantified per 20x field.
image using the software NIS Elements. P-values (*P < 0.01, **P < 0.001, ***P < 0.0001) were determined by Student’s t-test. Scale bars: 50 µm for all images. Representative images from each treatment group are shown (n=4-5 per group, 8-12 pictures taken).
FIGURE 6. Hypoxia stimulates TGF-β activity and downstream Fbln5 expression in PDA

We propose a model where hypoxia stimulates TGF-β signaling and induces Fbln5 expression via a PI3K/AKT-dependent mechanism in fibroblasts. We stimulated hypoxia in vitro by incubating cells in a hypoxic chamber or through chemically stabilizing Hif-1α with CoCl2, which enhanced Fbln5 expression. Anti-VEGF therapy by mcr84 augmented hypoxia in PDA tumors, which also increased Fbln5 levels. Blocking TGF-β signaling in vitro (LY2157299 and SB431542) and in vivo (LY2157299 and 2G8) reduced Fbln5 expression. Moreover, hypoxia-induced Fbln5 requires TGF-β activity as inhibition of TGFβ-R1 under hypoxic conditions also mitigated Fbln5 expression. Finally, inhibition of the PI3K/AKT pathway (LY294002, AKT1/2 KI, and BKM120) blocked hypoxia- and TGF-β–induced Fbln5 expression. As reported previously [25], the expression of functional Fbln5 in GEMMs of PDA results in increased tumor growth and decreased survival.
Hypoxia and Transforming Growth Factor Beta Cooperate to Induce Fibulin-5 Expression in Pancreatic Cancer
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