Neutralizing Murine TGFβR2 Promotes a Differentiated Tumor Cell Phenotype and Inhibits Pancreatic Cancer Metastasis

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Abstract

Elevated levels of TGFβ are a negative prognostic indicator for patients diagnosed with pancreatic cancer; as a result, the TGFβ pathway is an attractive target for therapy. However, clinical application of pharmacologic inhibition of TGFβ remains challenging because TGFβ has tumor suppressor functions in many epithelial malignancies, including pancreatic cancer. In fact, direct neutralization of TGFβ promotes tumor progression of genetic murine models of pancreatic cancer. Here, we report that neutralizing the activity of murine TGFβ receptor 2 using a monoclonal antibody (2G8) has potent antimitastatic activity in orthotopic human tumor xenografts, syngeneic tumors, and a genetic model of pancreatic cancer. 2G8 reduced activated fibroblasts, collagen deposition, microvessel density, and vascular function. These stromal-specific changes resulted in tumor cell epithelial differentiation and a potent reduction in metastases. We conclude that TGFβ signaling within stromal cells participates directly in tumor cell phenotype and pancreatic cancer progression. Thus, strategies that inhibit TGFβ-dependent effectors functions of stromal cells could be efficacious for the therapy of pancreatic tumors.

Introduction

Pancreatic cancer, the fourth leading cause of cancer-related mortality, presents a formidable challenge for treatment (1). Early metastasis, aggressive tumor biology, and a stromal rich microenvironment provide potential mechanisms for the resistance of pancreatic cancer to conventional chemotherapy. Recent work suggests that stromal cells within the tumor microenvironment are critical determinants of tumor development, progression, and metastasis (2, 3). Therefore, there is heightened interest in strategies that target stromal cells, including cancer-associated fibroblasts, immune cells, and vascular cells.

Numerous cytokines participate in the progression of pancreatic malignancies. In particular, TGFβ has a complex function in pancreatic ductal adenocarcinoma (PDA). TGFβ is known to inhibit tumor progression in early stages of PDA development yet, at later stages, TGFβ functions as a tumor promoter (4). The features that underlie the switch of TGFβ from a tumor suppressor to a tumor promoter in PDA are unclear. Mutations in the TGFβ signaling pathway occur in a large percentage (>50%) of human PDA (5) and likely contribute to the TGFβ functional switch. This has been modeled in mice where alterations of the TGFβ pathway (e.g., deletion of Smad4 or Tgfbr2) cooperated with activated Kras to promote disease progression (6–8). Furthermore, elevated expression of TGFβ, which is frequent in PDA can promote tumor development, tumor epithelial-to-mesenchymal transition (EMT), and tumor cell survival and motility (9–12). TGFβ also induces immunosuppression, activation of fibroblasts, angiogenesis, and collagen deposition (13, 14). Therefore, specifically targeting the protumoral aspects of TGFβ might provide therapeutic efficacy. Pharmacologic strategies that block TGFβ activity have been explored in preclinical models of pancreatic cancer (reviewed in ref. 15). As discussed by Achyut and Yang (15), targeting the TGFβ pathway alters the tumor microenvironment and the outcome of therapy might be more dependent upon microenvironmental actions than on direct tumor cell effects.

Previously, 2G8 (aka MT1), a monoclonal antibody against mouse TGFβ receptor 2 (Tgfβr2), reduced primary tumor growth and metastasis in several syngenic models of breast cancer, primarily through its effects on tumor cells and tumor-associated immune cells (16). Given the clinical importance of TGFβ dysregulation in PDA (15), we hypothesized that stromal Tgfβr2 inhibition could be effective in mouse models of PDA. Given that 2G8 is mouse-specific, we implemented this antibody in human xenograft models of PDA to specifically target stromal Tgfβr2 without interrupting TGFβ signaling in the
xenografted human tumor cells. We found that inhibition of the stromal TGFβ signaling promoted epithelial differentiation in tumor cells and inhibited metastasis, results that were recapitulated in immunocompetent models of PDA. The findings in xenografts highlight that TGFβ induction of stromal cell function is critical for its impact on tumor cell behavior and PDA progression.

Materials and Methods

Further information can be found in Supplementary Materials and Methods.

Cell lines

Murine pancreatic cancer cell line Pan02 (Pan02) was obtained from the Developmental Therapeutics Program at the NCI. The development of primary murine PDA cell lines (mPLR) is discussed in the Supplementary Data. Human pancreatic cancer cell lines (Capan-1 and MiaPaCa-2) were obtained from ATCC. Colo357 cells were obtained from Dr. Jason Fleming (MD Anderson Cancer Center, Houston, TX). C5LM2 is a cell line derived from liver metastasis from a Panc1 tumor bearing mouse isolated by our laboratory. RAW264.7 (THI-71) cells and NIH 3T3 cells were obtained from ATCC. Cell lines were confirmed to be pathogen-free and human cell lines were authenticated to confirm origin before use. Cells were cultured in DMEM (Invitrogen) or RPMI (Invitrogen) containing 10% FBS and maintained at 37°C in a humidified incubator with 5% CO₂ and 95% air.

In vivo models

Animals were housed in pathogen-free facility and all animal studies were performed on a protocol approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center (Dallas, TX). Animals were treated with 2G8 (provided by Imclone Systems), a rat IgG2a anti-mouse Tgfβ receptor, and organs harvested for tissue analysis. We found that inhibition of stromal TGFβ signaling promoted epithelial differentiation in tumor cells and inhibited metastasis, results that were recapitulated in immunocompetent models of PDA. The findings in xenografts highlight that TGFβ induction of stromal cell function is critical for its impact on tumor cell behavior and PDA progression.

Intraspinal injection model. Pan02 cells (2.5 \times 10^5) were injected using splenic parenchyma of 6-8-week-old C57BL/6 mice. Groups were randomized to receive 2G8 (30 mg/kg/wk) 1 day before splenic injection, postinjection day 1, postinjection day 7, postinjection day 14, or saline. Mice were sacrificed after 5 weeks after tumor cell injection.

Xenograft studies. Eight-week-old SCID mice were injected orthotopically with 1 \times 10^6 cells (Capan-1, Colo357, MiaPaCa-2, and C5LM2). One week after tumor cell injection, mice were randomized to receive 2G8 (30 mg/kg/wk) or saline. Mice were sacrificed after 8 to 10 weeks of treatment. At the time of sacrifice, gross metastases were counted and primary tumors were snap frozen in liquid nitrogen or fixed in 10% formalin.

Histology

Formalin-fixed tissues were embedded in paraffin, sectioned, and stained with Masson Trichrome and PAS-Alcian Blue by the Molecular Pathology Core (University of Texas Southwestern Medical Center, Dallas, TX). Immunohistochemistry was performed with the following antibodies: phospho-Histone H3 (Millipore, 06–570), cleaved caspase-3 (Cell Signaling Technology, 9664), E-cadherin (Santa Cruz Biotechnology, sc-7870), vimentin (PhosphoSolution, 2105-VIM), β-catenin (Cell Signaling Technology, 9821), Zeb1 (Santa Cruz Biotechnology, sc 25388), α-smooth muscle actin (NeoMarkers, RB9010–P), S100A4 (Abcam, ab27957), F4/80 (Santa Cruz Biotechnology, sc–26642), MCP-1 (Santa Cruz Biotechnology, sc12306), CD206–FITC conjugated (Biologic, 123006), CD11b–FITC conjugated (Biologic, 101206), Gr1–PE conjugated (Biologic, 108408), and NK 1.1 (Wako, 986–10001). DeadEnd Fluorometric terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) staining was performed according to the manufacturer’s instructions (Promega, G3250). Fluorescent images were captured with Photometric CoolSnap HQ camera using NIS Elements AR 2.3 Software (Nikon). Color images were obtained using Nikon Digital Dx1200me camera and ACT1 software (Universal Imaging Corporation). Pictures were analyzed using NIS Elements (Nikon).

Statistical analysis

Data were analyzed using GraphPad software (GraphPad Prism version 5 for Windows). All values are expressed as mean ± SEM. For all statistical analyses, ANOVA or, where appropriate, unpaired t test was performed and results were considered significant at P < 0.05.

Results

Pharmacologic blockade of stromal Tgfβr2 reduces metastasis

To explore the relationship between stromal TGFβ signaling and the phenotype of primary tumor cells in vivo, we established human xenograft models of PDA in which we specifically inhibited stromal Tgfβr2 with the mouse-specific antibody 2G8.
Mice bearing established human pancreatic tumor orthotopic xenografts (Capan-1, Colo357, MiaPaCa-2, and C5LM2) were treated systemically with 2G8. In this setting, 2G8 specifically targets stromal Tgfβr2 where it blocks ligation by TGFβ, inhibits canonical Smad signaling, and induces internalization of the receptor (16). Mice were randomized to receive control or 2G8 weekly 7 to 10 days after tumor cell injection. We found that inhibition of stromal Tgfβr2 significantly reduced surface metastases on the liver and other visceral organs (Fig. 1A), reduced cell proliferation (Fig. 1B), and elevated apoptosis (Fig. 1C–E) in all four tumor models.

To investigate whether 2G8-treated stromal cells secrete paracrine factors that affect tumor cell viability, migration, and colony formation, conditioned media (CM) from cultured mouse stromal cells (RAW 264.7 and NIH 3T3 cells) treated with 2G8 was collected and incubated with human pancreatic tumor cells. Supplementary Figure S2 demonstrates that CM from 2G8-treated stromal cells did not alter tumor cell viability; however, 2G8 blunted the ability of stromal-derived media to promote tumor cell migration toward macrophages (Fig. 2A) and 3T3 cells (Fig. 2B). Furthermore, when we evaluated anchorage-independent growth CM from 2G8-treated RAW 264.7 cells (Fig. 2C) and 3T3 cells (Fig. 2D), they showed...
significant reduced colony formation compared with other conditions.

To further define the effect of Tgfbr2 inhibition on the development of liver metastases, we performed a splenic injection model with Pan02 cells. Mice were randomized to receive a control antibody (Mac48) or 2G8 the day before splenic tumor cell injection (−1 day), or 1, 7, or 14 days after injection. Liver weight at experiment completion was used as a surrogate for metastatic burden. Tgfbr2 inhibition significantly reduced metastatic burden, with livers from 2G8-treated mice appearing

Figure 2. Inhibition of Tgfbr2 on stromal cells limits tumor cell migration and colony formation in vitro and in vivo. A and B, Transwell migration of tumor cells (Colo357 and MiaPaCa-2) toward RAW 264.7 (A) or NIH 3T3 (B) cells treated with SFM (NT), control Rat IgG (IgG), or 2G8 for 24 hours. After removing treatment conditions, Colo357 and MiaPaCa-2 cells were plated in Transwell chambers and allowed to migrate overnight toward previously treated stromal cells. Bar graphs represent number of cells/200 field. The assays were performed in triplicate in two independent experiments. *, P < 0.05; **, P < 0.01; #, P < 0.0001 versus Rat IgG. C and D, anchorage-independent growth of tumor cells (Colo357, CLSM2, and Capan-1) in the presence of growth media with 10% serum (NT), CM from RAW 264.7 (C), or NIH 3T3 (D) cells grown in media with 10% serum with or without control Rat IgG (IgG) or 2G8. Colony formation was quantified by number of colonies per well at ×4 or ×10 magnification. The bar graph represents mean ± SEM of a single experiment performed in triplicate, with similar results found upon two independent experiments. *, P < 0.05; **, P < 0.01; *** P < 0.001 versus NT; **** P < 0.0001 versus Rat IgG. E, Pan02 cells were injected intrasplenically and liver weight used as a surrogate for tumor burden in the liver. Mice were treated with a control rat IgG (Mac48, n = 14) or 2G8 initiated at 1 (n = 10), +1 (n = 3), +7 (n = 8), or +14 (n = 7) days after splenic injection. Representative images of livers from mice are shown. Results are expressed as mean ± SEM. *, P < 0.05; **, P < 0.01; *** P < 0.001; #, P < 0.0001 versus control.
normal while livers from Mac48-treated mice were replaced with tumor (Fig. 2E). Unlike agents that target TGFβR1 and TGFβR2 (12), timing of the treatment did not influence the tumor burden. Treatment of mice with 2G8 the day before injection or the day after injection did not alter its ability to limit the metastatic burden in the liver. Similarly, there was no significant difference between mice treated 1 week or 2 weeks after tumor cell inoculation. These data support the functional importance of stromal TgfβR2 in the metastasis of pancreatic cancer.

These results suggest that stromal TGFβ signaling is critical for acute tumor development and metastasis of established tumor cell lines. To determine whether the therapeutic efficacy of 2G8 extended to syngeneic immunocompetent models, we explored its activity in orthotopic Pan02 tumors and LSL-KrasG12D, Cdkn2a−/−, p53−/− (KIC) mice (18). Importantly, Pan02 and KIC cells express TgfβR2, active TGFβ, and are sensitive to 2G8 in vitro (Supplementary Fig. S3). To test 2G8 in vivo, animals with established primary tumor burden were randomized to receive saline, gemcitabine, 2G8, or a combination of 2G8 and gemcitabine. Inhibition of TgfβR2 alone (2G8 treatment) or in combination with gemcitabine modestly attenuated the weight of Pan02 (Fig. 3A) and KIC (Fig. 3B) tumors. However, consistent with the human xenograft results, 2G8 alone or in combination with gemcitabine significantly decreased tumor cell viability as evidenced by the changes in cell proliferation and apoptosis shown in Fig. 3C and D and Supplementary Fig. S4A–S4C.

Strikingly, 2G8, as a single agent, was very effective at reducing metastasis (3- to 5-fold; Fig. 3E and F). In fact, inhibition of TGFβ signaling was more effective than gemcitabine at reducing metastases in mice bearing Pan02 tumors (Fig. 3E) and in KIC mice (Fig. 3F). However, cotreatment with gemcitabine was not additive with 2G8. Interestingly, 2G8 and gemcitabine reduced perfusion and permeability in KIC tumors (Supplementary Fig. S5), partially explaining the lack of additivity in the combination-treated groups. The results in syngeneic, immunocompetent models implicate stromal TgfβR2 as a critical driver of PDA dissemination.

Blockade of TgfβR2 reduces collagen deposition and fibroblast activation

Stromal cells are key participants in the construction and remodeling of the tumor microenvironment, activities that are regulated in part by TGFβ (14, 19–21). PDA is a desmoplastic disease that consists of high levels of collagen (19, 22), which facilitates tumor cell survival and may impede the delivery of chemotherapy to tumor cells (23–25). We assessed collagen deposition by Masson trichrome staining and found that human xenografts (Fig. 4A and B) and syngeneic murine tumors (Supplementary Fig. S6) from mice treated with 2G8 had significantly reduced collagen deposition. We also found a concordant and significant 2G8-mediated reduction in mature fibroblasts as evidenced by α-smooth muscle actin (Fig. 4C and D) and S100A4 (Fig. 4E) immunoreactivity in Capan-1 and MiaPaCa-2 xenografts and Pan02 tumors (Fig. 4D and Supplementary Fig. S6C). These findings implicate TgfβR2 regulation of ECM deposition and fibroblast phenotype as critical features of the PDA microenvironment.

2G8 promotes a proinflammatory immune phenotype in pancreatic tumors

Metastasis is facilitated by an anti-inflammatory (M2) immune cell phenotype, which TGFβ is known to drive (4, 26–29). In support of this, we found that blocking TgfβR2 in RAW 264.7 cells in the presence or absence of TGFβ stimulated an M1 (proinflammatory) phenotype in vitro (Supplementary Fig. S7). We also evaluated the immune status of xenografts treated with 2G8. 2G8 reduced the number of F4/80+ (Fig. 5A and B) and CD68+ (data not shown) macrophages, increased the number of macrophages positive for MCP-1 (a marker of M1 macrophages; Fig. 5C), and decreased the number of macrophages expressing MMR (a marker of M2 macrophages; Fig. 5D) in Capan-1 and MiaPaCa-2 tumors. Furthermore, 2G8 therapy decreased myeloid-derived suppressor cells (MDSC, Gr1+C11b− cells; Fig. 5F), while significantly increasing NK cells (NK 1.1+ cells; Fig. 5G) in Capan-1 and MiaPaCa-2 tumors.

We also assessed the effect of TgfβR2 inhibition on the immune landscape in the immunocompetent models. As displayed in Supplementary Fig. S8, inhibition of TgfβR2 with 2G8 dramatically altered the immune cell phenotype in Pan02 tumors. These changes included a reduction in total macrophage number (F4/80, Supplementary Fig. S8A), an increase in the ratio of M1:M2 macrophages (Supplementary Fig. S8B and S8C), a reduction in MDSCs (Gr1+CD11b−, Supplementary Fig. S8D) and T regulatory cells (Treg, CD4+FoxP3+, Supplementary Fig. S8E), and an increase in NK cell recruitment (NK 1.1, Supplementary Fig. S8F). These results indicate that stromal TgfβR2 functions to promote an immunosuppressive environment while blockade of TgfβR2 function with 2G8 stimulates recruitment and retention of immune cells that can combat the tumor.

Targeting TgfβR2 on tumor cells and stroma inhibits EMT in vivo

TGFβ can drive tumor cells to adopt a mesenchymal-like phenotype that promotes tumor cell invasion and metastasis (30–34). We hypothesized that targeted inhibition of TgfβR2 would prevent or reverse the induction of EMT in vivo. Fig. 6A displays general histology (H&E) of spontaneous KIC tumors from each treatment group at the end of therapy. Mice receiving 2G8 alone or in combination with gemcitabine showed an increase in a ductal histologic phenotype that resembles PanIN lesions. This was confirmed by staining with PAS-Alcian Blue, which revealed a significant increase in mucin-secreting cells in 2G8-treated tumors (Fig. 6B). Primary tumors and metastases in the KIC model exhibited pathologic evidence of ductal differentia-

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high levels of Zeb1 (35). β-catenin is expressed on the membrane of epithelial cells and translocates into the nucleus during the process of EMT. We observed that while tumors from control- and gemcitabine-treated animals expressed nuclear β-catenin, tumors from mice receiving 2G8 expressed membranous β-catenin with increased prevalence of pseudoducts (Fig. 6F and G). Furthermore, we found that ECAD was seen most prominently in 2G8-treated tumors (Fig. 6G). This indicates that TgfβR2 inhibition drives an epithelial, differentiated phenotype in Pan02 tumors in vivo.

Inhibition of TgfβR2 on tumor cells is insufficient to inhibit EMT in vitro

Given the significant changes seen in stromal and primary tumor cells in vivo, we evaluated the effect of 2G8 on TGFβ-induced changes in mPLR and Pan02 cells in vitro. Cells were
plated on collagen (mPLR) or plastic (Pan02) and treated with serum-free media (SFM), TGFβ, 2G8, or TGFβ + 2G8 for 24 to 72 hours and analyzed by immunocytochemistry. mPLR2D cells treated with SFM expressed ECAD and NCAD, a mesenchymal marker, at detectable levels. Treatment with 2G8 reduced NCAD and increased ECAD expression levels, whereas TGFβ had the opposite effect (Supplementary Fig. S10). Pan02 cells treated in a similar fashion had a mild decrease in zeb1 and vimentin expression after exposure to 2G8 (Supplementary Fig. S10). However, Pan02 did not express ECAD in vitro at any time point under any condition (data not shown). Overall, our in vitro studies did not fully recapitulate the dramatic phenotypic changes induced by 2G8 in vivo. This led us to hypothesize that stromal cells participate in the 2G8-driven changes in tumor cell phenotype and reduction in metastases seen in vivo.

**Inhibition of stromal TGFβ signaling promotes epithelial differentiation**

TGFβ, collagen, fibroblasts, and immune cells all contribute to tumor cell phenotype. Tumor cells that adopt a mesenchymal phenotype are more aggressive and metastatic (36). Capan-1 and Colo357 are epithelial, whereas MiaPaCa-2 and C5LM2 cells have a mesenchymal-like phenotype in vitro (37, 38). 2G8 treatment in vivo promoted an epithelial phenotype in each xenograft analyzed (Fig. 7 and Supplementary Fig. S11). Inhibition of Tgfbr2 induced a shift in β-catenin from the nucleus to the membrane (Fig. 7A–C), increased tumor cell expression of ECAD (ECAD; Fig. 7D), and decreased Zeb1 levels (Zeb1; Fig. 7E) in Capan-1 and MiaPaCa-2 tumors. Similar results were found in Colo357 and C5LM2 models (Supplementary Fig. S11). These data indicate that activation of stromal Tgfbr2 is critical for tumor cell adoption of a mesenchymal-like phenotype.

**Discussion**

We have shown that pharmacologic blockade of stromal Tgfbr2 can slow primary tumor growth, reduce metastasis, and promote epithelial differentiation in mouse models of pancreatic cancer. The changes in tumor cell phenotype and behavior occurred in the context of microenvironmental changes that resulted in a proinflammatory immune cell phenotype and a reduced presence of mature/activated fibroblasts. These alterations in cellular and extracellular components of the tumor after Tgfbr2 blockade resulted in striking reductions in metastatic spread and help to functionally define the importance of stromal Tgfbr2 for primary pancreatic tumor growth and metastasis.

We employed human PDA xenografts that metastasize robustly but are often criticized for not recapitulating the progression of human PDA as well as genetically engineered mouse models (GEMM). Yet, we found the same proepithelial and antimetastatic effects after Tgfbr2 inhibition in a well-established GEMM of PDA. Furthermore, we used established human pancreatic cell lines rather than direct human xenografts. Given that we specifically wanted to target mouse stromal cells, direct xenografts would not have been

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Figure 4. Inhibition of mouse Tgfbr2 blunts collagen deposition within xenografts. A and B, the level of fibrillar collagen deposited in human tumor xenografts from mice treated with saline (control) or 2G8 was determined by trichrome histology (Trichrome, blue; scale bar, 20 μm; insets, 5 μm; A). B, the intensity of Trichrome staining was quantified and shows that 2G8 significantly reduced collagen deposition within each tumor (five animals per group, five pictures/field). C–E, to determine the level of fibroblast investment, xenografts from control- and 2G8-treated animals were stained for α-smooth muscle actin (C and D) and S100A4 (E). Results are expressed as mean ± SEM. ***, P < 0.01; ****, P < 0.001; #, P < 0.0001 versus control.
appropriate because they are a mixed human tumor and stromal cell population (39). Our xenograft models utilized NOD-SCID mice that lack B- and T-cell immunity; therefore, our studies do not reflect the effect of Tgfbr2 inhibition on B and T cells that can participate in antitumor effects. However, we demonstrate that inhibition of Tgfbr2 on stromal cells promotes a proinflammatory macrophage phenotype that limits tumor cell migration and colony formation in vitro and reduces metastasis in vivo. Furthermore, we observed a potent antitumor effect in immunocompetent models with concordant changes in immune cells.

Our analyses focused on the effect of TGFβ on macrophage and fibroblast phenotype. However, we did identify significant changes after Tgfbr2 blockade in other cell types, including NK

Figure 5. Inhibition of mouse Tgfβr2 promotes a proinflammatory immune cell phenotype. A–D, the level of F4/80 (A and B), MCP-1 (C), and MMR (D) expressing macrophages in Capan-1 and MiaPaCa-2 xenografts was determined by immunohistochemistry. The effect of 2G8 on the level of MDSC (E) and NK cells (F) in Capan-1 and MiaPaCa-2 xenografts was also evaluated by immunohistochemistry, with example images shown in G. Graphs represent five animals per group with five pictures per animal. Results are expressed as mean ± SEM. *** P < 0.001; # P < 0.0001 versus control.
2G8 therapy increased the level of tumor-associated NK cells. These findings are consistent with the study by Zhong and colleagues (16) who demonstrated that 2G8 increased NK cell–mediated killing and secretion of IFN-γ.

The effect of TGFβ2 inhibition on other cells within the tumor microenvironment such as pancreatic stellate cells, mesenchymal stem cells, or endothelial cells was not explored in depth. These cells are known to respond to TGFβ and thus inhibition of TGFβ signaling in these cell types could contribute to the reduction in tumor progression observed.

Overall, our results emphasize the impact of stromal cell function on tumor cell phenotype and metastasis. In

Figure 6. 2G8 promotes an epithelial phenotype in murine tumors in vivo. A–E, LSL-Kras<sup>G12D</sup>; Cdkn2a<sup>lox/lox</sup>; p48<sup>Cre</sup> (KIC) mice establish tumors and precursor PanN lesions by 4 weeks old. Mice at this time point were randomized to receive saline (control), gemcitabine (Gem), 2G8 or 2G8 + gemcitabine for 4 weeks. A, analysis of tumor architecture using H&E staining (scale bar, 100 μm). 2G8-treated tumors were noted to have significantly more PanN and epithelial lesions than the mice treated with gemcitabine or control. B, this was confirmed with PAS-Alcian Blue staining that marks mucin-secreting PanN lesions but not invasive lesions (PAS-Alcian Blue, purple; scale bar, 100 μm). In addition, 2G8 tumors had significantly increased E-cadherin expression (ECAD; C) and decreased vimentin expression (D) by immunohistochemistry. E, representative images of these tumors demonstrate the predominant epithelial phenotype of 2G8-treated tumors (ECAD, red; vimentin, green; scale bar, 50 μm). F and G, 2G8 induces a similar epithelial phenotype in Pan02 tumors. Immunohistochemical analysis of β-catenin (F) and E-cadherin (ECAD; G) in Pan02 tumors treated with control, gemcitabine, 2G8, or 2G8 + gemcitabine. Bar graphs represent mean ± SEM. **, P < 0.01; #, P < 0.0001 versus control; ^^, P < 0.001; ^^^, P < 0.0001 versus gemcitabine.
particular, our in vitro studies suggest interruption of TgfβR2 signaling in stromal cells alters the expression of soluble factors that impact tumor cell behavior. This is exemplified by the concordant results of our anchorage-independent growth assays in vitro where CM from mouse stromal cells enhanced colony formation in a TgfβR2-dependent manner and the observed reduction in metastasis in vivo after blockade of stromal TgfβR2. Whether 2G8 treatment prevents the expression of protumor factors or induces the expression of factors that inhibit tumor cell colony formation and metastasis is not clear. We evaluated the expression of twelve cytokines by macrophages (Raw 264.7) and fibroblasts (3T3) after treatment with 2G8 (Supplementary Fig. S12) and found only modest changes in a small subset of cytokines. Whether the 2G8-induced elevation in MIP1α, MIP1β, TNFα, and IFNγ expressed by macrophages are sufficient to induce the dramatic alterations in tumor cell phenotype and progression remains to be validated.

In a GEMM of PDA, we found that suppression of TgfβR2 resulted in an increase in PanIN lesions and an increased epithelial tumor cell phenotype compared with control-treated mice. In contrast, genetic deletion of TgfβR2 in pancreatic tumor cells (Ptf1aCre/+; KrasG12D; TgfβR2loxp/loxp) resulted in elevated CTGF expression, abundant stroma, and a worse overall survival compared with TgfβR2wt/wt animals (7). Given that TGFβ functions as a tumor suppressor early in PDA development, it is not surprising that targeted ablation of TgfβR2 during or before tumor development drives tumor progression. We chose to target TgfβR2 at a time point when the mice are known to have established invasive lesions (18). Furthermore, in Ptf1aCre/+; KrasG12D; Tgfβ2loxp/lox mice, TgfβR2 is ablated in tumor cells, whereas, in our study, we targeted tumor and stromal cell TgfβR2 within the tumor microenvironment. Overall, our studies document the critical nature of stromal TGFβ signaling to stromal cell recruitment and activity and strengthen the argument that the microenvironment is...
a major participant in tumor progression. The results presented here support that blockade of stromal TgfβR2 inhibits tumor cell EMT but do not rule out that inhibition of stromal TgfβR2 promotes tumor cell mesenchymal-to-epithelial transition (MET). Given the prevalence of poorly differentiated (e.g., mesenchymal phenotype) tumor cells in control-treated tumors in each model system employed and the demonstration that EMT occurs early in PDA (36), it is feasible that 2G8 promotes MET in PDA cell lines in vivo.

Although the focus of our work was TGFβ-dependent tumor–stromal cell interactions in PDA, several studies targeting tumor cell TGFβ pathways have observed antitumor effects. In xenograft models, inhibition of TGFβR1 and TGFβR2 resulted in decreased metastasis after splenic injection of tumor cells (12). In addition, inhibition of tumor cell TGFβR2 in subcutaneous models of human PDA resulted in reduced primary tumor growth and reduced microvessel density (40). However, neither of these studies explored stromal cell or tumor cell phenotype after TGFβ receptor inhibition.

Recently, Hezel and colleagues (41) found that direct inhibition of TGFβ or the integrin αvβ6, which is critical for activation of latent TGFβ (42), accelerated the progression of pancreatic cancer in p48Cre;KrasG12D;Pdx1-lox/lox animals but do not exhibit the pronounced EMT observed in the GEMM used in the current study. Furthermore, the study primarily evaluated survival and documented that inhibition of TGFβ or αvβ6 at early or later stages accelerated disease progression in a Smad4-dependent fashion. However, differences in stromal or tumor cell phenotype after therapy were not evaluated. In addition, the number of animals in any treatment group with distinct metastatic lesions was too few to draw meaningful conclusions regarding the effect of these strategies on tumor dissemination. These studies taken in the context of our results suggest that there is a substantial mechanistic difference between targeting TGFβ ligand and inhibiting downstream signaling by increasing TGFβR2 internalization.

Studies targeting stromal cells in pancreatic cancer have shown that decreased stroma results in increased drug delivery and a reduction in tumor growth (24, 25). In fact, several ongoing clinical trials in pancreatic cancer aim at stromal depletion by inhibiting the hedgehog pathway (43). We found in all six of our mouse models that targeting stromal TgfβR2 with 2G8 resulted in a significant reduction in tumor-associated collagen deposition. However, we found that blockade of TgfβR2 significantly reduced perfusion and permeability of high and low molecular weight dextran (Supplementary Fig. S5), thus collagen deposition is not the only factor contributing to poor drug distribution in PDA.

Results in murine models of cancer document the complexity of targeting TGFβ pathways in vivo. Our data suggest that specific blockade of stromal TgfβR2 has a profound anti metastatic effect and demonstrate that stromal cell phenotype is more critical for tumor cell phenotype and metastasis than previously appreciated. TGFβ acts in a paracrine manner in virtually every cell type and dissecting which stromal cell type is primarily responsible for the antimitastatic effect of 2G8 remains a challenge. However, our results strongly implicate TgfβR2 on stromal cells as a critical participant in pancreatic cancer metastasis and underscore the need for an improved understanding of TGFβ biology in this challenging disease.

Disclosure of Potential Conflicts of Interest
R.A. Brekken reports receiving a commercial research grant from Imclone/Eli Lilly & Company. No potential conflicts of interest were disclosed by the other authors.

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