TGFβ engages MEK/ERK to differentially regulate benign and malignant pancreas cell function

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While TGFβ signals are anti-proliferative in benign and well-differentiated pancreatic cells, TGFβ appears to promote the progression of advanced cancers. To better understand dysregulation of the TGFβ pathway, we first generated mouse models of neoplastic disease with TGFβ receptor deficiencies. These models displayed reduced levels of pERK irrespective of KRAS mutation. Furthermore, exogenous TGFβ led to rapid and sustained TGFB R1-dependent ERK phosphorylation in benign pancreatic duct cells. Similar to results that our group has published in colon cancer cells, inhibition of ERK phosphorylation in duct cells mitigated TGFβ-induced upregulation of growth suppressive pSMAD2 and p21, prevented downregulation of the pro-growth signal CDK2 and ablated TGFβ-induced EMT. These observations suggest that ERK is a key factor in growth suppressive TGFβ signals, yet may also contribute to detrimental TGFβ signaling such as EMT. In neoplastic PanIN cells, pERK was not necessary for either TGFβ-induced pSMAD2 phosphorylation or CDK2 repression, but was required for upregulation of p21 and EMT indicating a partial divergence between TGFβ and MEK/ERK in early carcinogenesis. In cancer cells, pERK had no effect on TGFβ-induced upregulation of pSMAD2 and p21, suggesting the two pathways have completely diverged with respect to the cell cycle. Furthermore, inhibition of pERK both reduced levels of CDK2 and prevented EMT independent of exogenous TGFβ, consistent with most observations identifying pERK as a tumor promoter. Combined, these data suggest that during carcinogenesis pERK initially facilitates and later antagonizes TGFβ-mediated cell cycle arrest, yet remains critical for the pathological, EMT-inducing arm of TGFβ signaling.

INTRODUCTION

While pancreatic cancer accounts for only 2.8% of new cancer cases each year in the United States, it is projected to be the third leading cause of cancer-related mortality by the end of 2016.1 Despite the near uniformity of KRAS mutations in pancreatic cancer patients, there remains a high level of genetic and molecular heterogeneity, and identifying molecular subtypes may better risk-stratify patients for more individualized therapeutic approaches to more effectively treat their disease. To this end, there is increasing evidence that implicates dysregulation of transforming growth factor β (TGFβ) signaling in pancreatic carcinogenesis. In benign and neoplastic tissues, TGFβ is often considered a stark tumor suppressor as it induces cell cycle arrest and apoptosis. However, many advanced cancers become desensitized to TGFβ-induced cell cycle arrest, and in some patients TGFβ begins to promote adverse cellular events, including epithelial–to–mesenchymal transition (EMT) and metastasis.2

In pancreatic cancer, TGFβ ligands are often overexpressed and are predominantly derived from the stroma.3 In canonical TGFβ signaling, the TGFβ ligand binds to the type 2 TGFβ receptor (TGFB R2). This recruits the type 1 TGFβ receptor (TGFB R1), a serine/threonine kinase that auto-phosphorylates, and subsequently phosphorylates SMAD2 and SMAD3 proteins. In the cytoplasm, pSMAD2 and 3 form a heterotrigem with SMAD4 and translocate to the nucleus to alter gene expression. In benign and neoplastic pancreatic epithelial cells, TGFβ arrests the cell cycle via upregulation of targets such as p21CIP1/WAF1 (p21).2,4 p21 is a cyclin-dependent kinase inhibitor that functionally inhibits the transition from G1 to S phase by repressing cyclin-CDK complexes.5 While p21 can interact with CDK1 and CDK4/6, the primary target of p21 is cyclin E/CDK2 complexes.6 In normal pancreatic epithelial cells, p21 is critical for TGFβ-induced cell cycle arrest7 and pancreatic cancer patients with high expression of p21 have a significantly improved prognosis.8 Furthermore, p21 opposes acinar-to-ductal metaplasia and early pancreatic carcinogenesis in vivo.9 While TGFβ-induced p21 upregulation is largely SMAD4 dependent,9 non-SMAD signals in colon have been shown to have a role in regulating p21 expression.10 Indeed, our previous work in colon cancer demonstrated that SMAD4 is required for TGFβ-induced upregulation of p21, though non-SMAD signaling was critical for repression of p21 in response to Activin, another member of the TGFβ superfamily.11,12 While much is known regarding TGFβ signaling in pancreatic cancers, it remains unclear whether similar mechanisms are in place, particularly in the majority of cases harboring SMAD4-inactivating mutations.

The intersection between TGFβ and the RAS/ERK pathway14 is of particular interest in pancreatic cancer, given the prevalence of both KRAS mutations and altered TGFβ signaling.15 In other cancers, evidence has emerged suggesting that ERK is an important regulator of the cell cycle and differentiation state/EMT. In non–small cell lung cancer (NSCLC), pERK is critical for directing EMT, and administration of the MEK/ERK inhibitor U0126...
led to more epithelial phenotypes, prevented TGFβ-induced EMT, and increased sensitivity to epidermal growth factor receptor (EGFR) inhibition. Similar results were observed in both normal murine mammary gland (NMuMG) epithelial cells and mouse cortical tube (MCT) epithelial cells, in which U0126 prevented TGFβ1-induced EMT. Similar results were seen in renal tube epithelial cells and colon cancer epithelial cells, though the role of ERK in TGFβ-induced EMT in the pancreas is unknown.

ERK is generally considered a proto-oncogene that drives tumor cell proliferation, EMT, migration and invasion. For this reason, the majority of research concerning ERK has focused on its tumor-promoting effects. However, ERK has also been implicated in other cellular events including senescence, autophagy, and apoptosis. This is particularly true with respect to the cell cycle inhibitor p21. Despite the established role of pERK in driving cell cycle progression, in other cancers, pERK has also been implicated in inducing p21 expression and arresting the cell cycle. Additionally, it has also been suggested that RAS stabilizes p21 by promoting the formation of p21/cyclin complexes, preventing proteasome degradation. However, seldom do investigations of this nature include normal, neoplastic and cancer cells as a comparison. In the pancreas, the contribution of ERK to these cell cycle events is currently unknown. Similarly, while TGFβ also regulates these events, the relevance of crosstalk between ERK and TGFβ signaling has yet to be investigated.

To this end, we explored the functional contributions of ERK to TGFβ signaling at the cell cycle level at various points in pancreatic carcinogenesis. In benign cells, pERK was also critical for TGFβ-induced upregulation of pSMAD2, p21, and subsequent cell cycle arrest and was required for TGFβ-induced EMT. In neoplastic cells, pERK was dispensable for TGFβ-induced SMAD2 phosphorylation, yet still necessary for the formation of p21/CDK2 complexes and TGFβ-induced EMT. In cancer cells, however, pERK antagonized TGFβ-induced cell cycle arrest though still promoted EMT. These observations may explain some of the discrepancies observed with TGFβ signaling, which appear to simultaneously slow and promote the progression of pancreatic cancer, further substantiating investigations targeting the RAS/ERK pathway.

RESULTS

TGFβ receptors are necessary for ERK phosphorylation in the pancreas

As ERK is generally considered a mitogen that drives cancer progression, we first assessed the degree of ERK activation in human PDAC patients via immunohistochemistry (IHC) for pERK. We found that pERK was strongly upregulated in 5/5 PDAC tissue human PDAC patients via immunohistochemistry (IHC) for pERK.

Due to our recent manuscript, both KT2 and KT1 mice have equivalent signaling between ERK and downstream targets after 24 h, when the lesions themselves had significantly increased proliferation compared with KRAS controls. As KRAS signaling in these disease models may affect other arms of cell cycle control, we next assessed the expression of these cell cycle proteins in KRAS-wild-type animals. When compared with wild type (WT) mice, Tgfbr2Δ/N and Tgfbr1Δ/N mice had comparably reduced expression of p21 (Supplementary Figures S3a and b) as well as increased expression of CDK2 and Cyclin E (Figures 2i–k).

pERK is necessary for TGFβ-induced cell cycle arrest in benign pancreas duct cells

Given the increase in CDK2 and Cyclin E expression in TGFβR-deficient animals, we next assessed the apparent loss of cell cycle control via pRb staining. Both T2 and T1 cohorts displayed increased pRb staining in pancreatic acini, consistent with progression of the cell cycle (Figure 3a). Therefore, we next sought to determine the relationship between pERK and cell proliferation in the pancreas of these animals. Dual staining for cell PCNA and pERK suggested that, in wild-type animals, pERK is not ubiquitously expressed in proliferating pancreatic epithelial cells (Figures 3b and c). Additionally, using the duodenum as a control for mitosis, we found that the diminished ERK phosphorylation in TGFβR-deficient mice had no observable effect on PCNA staining/proliferation (Figure 3d).

To better understand the relationship between TGFβ and pERK in human pancreatic cells, we first employed non-malignant human pancreatic ductal epithelial (HPDE) cells in vitro. After 30 min, incubation with recombinant TGFβ1 led to dose-dependent increases in pERK, suggesting TGFβ is sufficient to induce ERK phosphorylation in these cells (Figure 3e). Next, to assess the mechanisms through which TGFβ induces ERK phosphorylation, pERK and binding partners were isolated via immunoprecipitation. We found that, in untreated cells, pERK co-precipitated with TGFβR1. Interestingly, this complex dissociated 30 min after incubation with exogenous TGFβ1 (Figure 3f). As the SMAD proteins can be found in the nucleus for many hours following incubation with TGFβ, we next assessed the interaction between ERK and downstream targets after 24 h, when the SMADs have had time to accumulate in the nucleus and are most likely having their maximum effect on gene expression. Consistent with long-term regulation of the TGFβ pathway, 24 h after the administration of TGFβ1, we found an association between pERK and both SMAD4 and p21, two downstream targets of TGFβ signaling (Figure 3g).

As pERK appears to interact with known targets of growth suppressive TGFβ signaling, we next assessed whether pERK is signaling is required for sustained ERK phosphorylation in the mouse pancreas irrespective of a KRAS-activating mutation.

TGFβR-deficient mice display loss of cell cycle control despite reduced ERK activation

Though all TGFβR-deficient cohorts had similar loss of pERK, these mice also had significantly reduced expression of the cell cycle inhibitor p21 (Figures 2a and b and Supplementary Figure S2). We therefore evaluated expression of CDK2 and Cyclin E, two primary targets of p21. Like pERK, CDK2 and Cyclin E are generally expressed in proliferating tissues such as the crypts of the gastrointestinal tract (Supplementary Figure S3), and were over-expressed in the pancreas of both TGFβR-deficient animals (Figures 2c–e). Consistent with increased cell cycle progression, KT2 and KT1 mice had increased staining for pRb, a CDK2/Cyclin E target that is expressed in proliferating tissues (Supplementary Figure S2). Consistent with these results, KT2 and KT1 mice also had increased proliferation determined by PCNA staining (Figures 2f–h). It should be noted that KT1 mice have overall reduced cell proliferation compared with KT2 mice, due to increased T-cell clearance of neoplastic disease, though the lesions themselves had significantly increased proliferation compared with KRAS controls. As KRAS signaling in these disease models may affect other arms of cell cycle control, we next assessed the expression of these cell cycle proteins in KRAS-wild-type animals. When compared with wild type (WT) mice, Tgfbr2Δ/N and Tgfbr1Δ/N mice had comparably reduced expression of p21 (Supplementary Figures S3a and b) as well as increased expression of CDK2 and Cyclin E (Figures 2i–k).

As pERK appears to interact with known targets of growth suppressive TGFβ signaling, we next assessed whether
involved in TGFβ-induced cell cycle arrest. We first inhibited ERK phosphorylation pharmacologically prior to incubation with TGFβ and assessed localization and expression of p21. By blocking MEK-induced ERK phosphorylation with U0126, we found that pERK is required for TGFβ1 to induce nuclear translocation of p21 (Figure 3h). Similarly, pERK was also necessary for pSMAD2 phosphorylation, as well as p21 upregulation and repression of CDK2 (Figure 3i). To assess downstream changes in CDK2 regulation, we repeated the above experiment, isolated CDK2 by immunoprecipitation, and assessed its binding partners by western blotting. We found that when pERK is inhibited, TGFβ fails to promote an interaction between p21 and CDK2 in HPDE cells, consistent with reduced cell cycle inhibition (Figure 3j).

pERK is required for TGFβ-induced EMT in HPDE cells

Though TGFβ has anti-proliferative effects in HPDE cells, TGFβ is also a well-known inducer of EMT. We therefore inhibited ERK phosphorylation pharmacologically prior to incubation with TGFβ and assessed changes in cell signaling/morphology after 72 h via immunocytochemistry (ICC). Experiments were performed at both high (80%; Figure 4) and low confluence (30%; Supplementary Figure S4). HPDE cells pre-treated with U0126 had reduced TGFβ-
Figure 2. TGFBR-deficient mice display loss of cell cycle control despite reduced ERK activation. (a and b) Tissue sections from KRAS, KT2 and KT1 mice were stained for p21, indicating reduced expression in both TGFBR-deficient cohorts. (c–e) Tissues were stained for the p21 targets CDK2 and Cyclin E, indicating overexpression in KT2 and KT1 mice compared with KRAS controls. (f–h) We next assessed pRB and PCNA, surrogate markers of proliferation and found strong staining for both in neoplastic tissues of KT2 and KT1 mice compared with modest staining in KRAS controls. (i–k) Tissue sections from wild type (WT), Tgfbr2DN, and Tgfbr1+/− mice were similarly stained for CDK2 and Cyclin E, both of which were similarly upregulated in Tgfbr2DN and Tgfbr1+/−. (*P < 0.05. N = 4 mice per group unless otherwise specified).
induced nuclear localization of SMAD4, consistent with the reduction in pSMAD2 (Figure 4a and Supplementary Figure S4a). Additionally, U0126 ablated TGFβ-induced nuclear localization of p21 (Figure 4b and Supplementary Figure S4b) and repression of both PCNA and CDK2 (Figure 4c and Supplementary Figure S4c). While these results confirm our previous findings regarding the role of pERK in the growth suppressive axis of TGFβ-signaling, HPDE cells incubated with TGFβ1 had altered cell morphology (Figure 4d), reduced expression of the epithelial cell marker E-Cadherin as well as upregulation of the mesenchymal marker Vimentin, all consistent with EMT. However, U0126 treatment prevented said morphologic changes, as well as mitigated TGFβ-induced repression of E-Cadherin and upregulation of Vimentin. Combined, these data suggest that pERK is required for TGFβ-induced EMT in HPDE cells (Figure 4e and Supplementary Figure S4d).

pERK is dispensable for upstream TGFβ signaling in neoplastic cells, yet critical for p21/CDK2 complex formation and EMT. While these results suggest that in the benign condition, ERK is an important component of TGFβ signaling in the pancreas, it is unknown whether ERK similarly affects TGFβ signaling in the disease state. Therefore, we first examined crosstalk between TGFβ in PanIN KC4848 (PanIN) cells. These cells are well differentiated, and are derived from neoplastic, non-malignant, tissue from Pdx-Cre/LSL-KRASG12D transgenic animals.24 In PanIN cells, like in HPDE, not only did exogenous TGFβ1 induce ERK phosphorylation after 24 h, but when pERK was inhibited with U0126, we observed a reduction in the TGFβ1 upregulation of p21 (Figure 5a). However, unlike HPDE cells, pERK was not necessary for pSMAD2 activation or CDK2 repression (Figure 5a). As with HPDE cells, exogenous TGFβ1 induced rapid dissociation of pERK from TGFBR1, with ligand binding determined by a dose-dependent association between TGFBR1 and TGFBR2 (Figure 5b). Additionally, while TGFβ1 increased association between p21 and CDK2 in PanIN cells, when pERK was inhibited via U0126, TGFβ1 no longer induced formation of p21/CDK2 complexes (Figure 5c).

After 72 h, U0126 had no effect on TGFβ-induced SMAD4 nuclear localization (Figure 5d), inconsistent with our observations in HPDE cells. Additionally, U0126 had little effect on nuclear accumulation of p21 in response to TGFβ and TGFβ-induced downregulation of CDK2 (Figures 5e and f). While TGFβ alone reduced PCNA staining, this effect was slightly inhibited by U0126 (Figure 5f). However, like in HPDE cells, exogenous TGFβ1 led to pronounced changes in cell morphology (Figure 5g), downregulation of E-Cadherin, and upregulation of Vimentin. However, U0126 prevented these events in response to TGFβ1, affirming that pERK is necessary for TGFβ-induced EMT in both the normal and neoplastic condition (Figure 5h).

pERK antagonizes TGFβ-induced CDK2/P21 association and is required for TGFβ-induced EMT in PANC1 cells

To assess whether a similar relationship between ERK and TGFβ signals exists in advanced pancreatic cancer cells, we employed a variety of pancreatic cancer cell lines in vitro. We found that, while TGFβ had no effect on pERK in BXPC3 or ASPC cells, consistent with previous observations,25 TGFβ was sufficient to induce ERK phosphorylation in PANC1 cells (Figure 6a). We therefore used PANC1 cells as a model for subsequent experiments. We found that pERK inhibition had no effect on either SMAD2 phosphorylation or downstream p21 induction in PANC1 cells. TGFβ1 also failed to reduce levels of CDK2 in these cells, though CDK2 was strongly downregulated when pERK was inhibited (Figure 6b). Interestingly, despite these changes in signaling, pERK was still associated with TGFBR1 following incubation with exogenous TGFβ1 (Figure 6c). However, contrasting our results in benign cells, TGFβ1 increased association between Cyclin E and CDK2, and failed to induce complexing between CDK2 and p21. However, when pERK was inhibited pharmacologically TGFβ1 again induced an association between CDK2 and p21 (Figure 6d), suggesting that ERK antagonizes growth suppressive TGFβ signals in the cancer state. Additionally, in the pancreatic cancer cell line CD18, inhibition of pERK restored p21 levels independent of TGFβ treatment suggesting these pathways may further diverge (Supplementary Figure S5).

As APC-Δ mice with TGFBR-deficiency also present with reduced p21 levels26 and mutant KRAS appears to cooperate with TGFβ-signaling inactivation to promote colon cancer development,27 we also determined whether TGFBR-deficient models of colon cancer displayed similar signaling changes to those observed in the pancreas. We found that these TGFBR-deficient mice harbor a similar reduction in pERK, as well as
upregulation of CDK2, Cyclin E, and pRB paralleling the results in the pancreas (Supplementary Figures S6a–c and S7a). Given these similarities, we also assessed the relationship between pERK and TGFβ signaling in well-differentiated FET colon cancer cells. In these cells, TGFβ similarly led to rapid ERK phosphorylation (Supplementary Figure S7b), and pERK was required for TGFβ-induced upregulation of p21, though pERK was dispensable for SMAD2 phosphorylation (Supplementary Figure S7c).

As TGFβ is a well-known inducer of EMT in PANC1 cells, we next repeated the experiment on chamber slides to determine whether this process is ERK-dependent, as it is in HPDE and PanIN cells. After 72 h, TGFβ-induced both SMAD4 and p21 nuclear localization independent of U0126 treatment (Figures 6e and f). Incubation with U0126 reduced PCNA (cell proliferation) and CDK2 staining independent of TGFβ (Figure 6g), suggesting that ERK and TGFβ signals have diverged with respect to cell cycle regulation.

Figure 3. pERK is necessary for TGFβ-induced cell cycle arrest in benign pancreas duct cells. (a) pRB expression was evaluated via immunohistochemistry, showing increased staining in the exocrine tissue of Tgfb2DN and Tgfb1+/- mice compared with wild-type (WT) controls. Dashed lines surround islets. (b–d) Pancreas tissue from wild-type (WT) mice was dual-stained for PCNA and pERK, and both PCNA+ and pERK+PCNA+ cells quantified per 20x field. We subsequently dual-stained the small intestine of wild type (WT), Tgfb2DN and Tgfb1+/- mice for PCNA and pERK, affirming the pERK deficiency in Tgfb2DN and Tgfb1+/- groups, though there was no change in PCNA staining. The white arrows indicate nuclei that are dual positive for pERK and PCNA. (e) benign human pancreatic ductal epithelial (HPDE) cells were starved of growth supplements, and incubated with 5–10 ng/ml exogenous TGFβ1, and pERK examined after 30 min. (f) Thirty minutes following incubation with TGFβ1, pERK was isolated by immunoprecipitation and interaction with TGFBR1 assessed by western blotting. (g) pERK was isolated by immunoprecipitation 24 h following incubation with TGFβ1, and interaction with SMAD4 and p21 measured by western blotting. (h) inhibition of pERK via U0126 prevented TGFβ1-induced nuclear translocation of p21 in HPDE cells. (i) HPDE cells were again pre-incubated with U0126 prior to TGFβ1-treatment. In the absence of pERK, despite the compensatory upregulation of ERK1 (small upper band in the ERK doublet), TGFβ1 failed to induce downstream SMAD2 phosphorylation or p21 upregulation, as well as repression of CDK2. (j) HPDE cells were again pre-incubated with U0126 prior to TGFβ1-treatment, and the interaction between p21 and CDK2 was assessed by immunoprecipitation after 24 h. (*P < 0.05. N = 4 mice per group unless otherwise specified).
control. However, incubation with TGFβ1 caused PANC1 cells to uniformly display a more spindle shaped morphology consistent with EMT (Figure 6h), as well as reduced expression of E-cadherin and upregulation of Vimentin. U0126 treatment prevented these events, suggesting that pERK is required for TGFβ-induced EMT in PANC1 cells (Figure 6i).

**DISCUSSION**

While TGFβ is generally considered a tumor suppressor with respect to benign epithelial cells, TGFβ also appears to facilitate the progression of many advanced cancers. The paradoxical effects of TGFβ in human cancers are poorly understood, and while there is clear merit to therapies targeting the TGFβ pathway, careful consideration must be taken to target only its tumor-promoting effects. Thus there is a need for a better understanding of the mechanistic alterations to the TGFβ signaling pathway in cancer, particularly with respect to the intersection between TGFβ and cancer-associated mitogens such as ERK.

Like TGFβ, the many contributions of pERK to the development of human cancer appear to be highly varied and often contradictory. In breast cancer cells, ERK has been shown to contribute to DNA-damage-induced apoptosis. Additionally, pERK has been shown to upregulate p21 through both transcriptional and post-translational mechanisms, facilitating cell cycle arrest. However, sustained pERK activation is also involved in S phase entry and has been linked to several other hallmark features of tumorogenesis. pERK drives the proliferation and migration of many cancers in response to a variety of stimuli, including cell stress, cytokines and growth factors. To this end, single agent targeting of the ERK pathway has been attempted in a spectrum of cancers, including hepatocellular carcinoma, NSCLC, prostate, breast, ovarian and pancreatic cancers, melanoma and hematological malignancies.

Many studies have also shown that ERK is a critical regulator of TGFβ signaling in cancer. The majority of these works have shown that ERK antagonizes TGFβ-induced growth-inhibition. Specifically, RAS-induced transformation appears to diminish responsiveness to the anti-mitotic effects of TGFβ in lung, intestinal, liver and mammary epithelial cells. RAS/ERK signaling also appears to mediate TGFβ-induced repression of the tumor-suppressor PTEN in pancreatic cancer cells, implicating ERK in the more pathological effects of TGFβ. Furthermore, as mentioned, pERK signals are necessary for TGFβ-induced EMT in a variety of cell types, though this has yet to be explored in the pancreas. In this work, we first demonstrated that in both normal and oncogenic KRAS-expressing pancreas, both TGFRB2 and TGFRB1 are necessary for ERK phosphorylation in vivo. These results were quite unexpected as TGFβ is largely considered a growth-suppressive signal and ERK a mitogen. However, there is evidence that members of the TGFβ superfamily can enhance growth signals. In colon cancer specimens, p21 expression is positively correlated with expression of TGFB2 and downstream SMAD-dependent signaling, yet negatively associated with expression of ACVR2 and downstream SMAD-independent signaling. Combined, these data support the notion that the ligand specific responses to the TGFβ family are critical for p21 expression in colon cancer. Additionally, we assessed the relationship between TGFβ signaling and the ERK pathway with respect to p21 and downstream regulation of the cell cycle.

In normal pancreatic epithelial cells, exogenous TGFβ1 led to rapid induction of pERK, which subsequently dissociated from TGFR1 and associated with downstream TGFβ targets SMAD4 and p21. Interestingly, when pERK activation was inhibited pharmacologically by U0126, TGFβ1 failed to induce SMAD2 phosphorylation, p21 upregulation and nuclear localization, and repression of CDK2. pERK was similarly necessary for TGFβ-induced incorporation of p21 into CDK2 complexes, indicative of a novel anti-proliferative role for pERK. While similar results were observed in well-differentiated neoplastic cells, in advanced cancer cells ERK actually antagonized TGFβ signaling. In these cells, pERK had no relationship to TGFβ-induced upregulation of p21, and TGFβ failed to induce the association between p21 and CDK2 unless pERK was inhibited. However, pERK was necessary for TGFβ-induced EMT in all cell lines, suggesting that, in this capacity, pERK may also facilitate the tumor-promoting roles of TGFβ signaling (Figure 7).

Previous studies have identified a convergence between ERK and TGFβ signals in other cell types. Notably, it was found that the TGFβ receptors share structural homology with receptor tyrosine kinases, and TGFR1 has been shown to initiate direct phosphorylation of ShcA leading to activation of the MEK/ERK pathway in Mv1Lu mink epithelial cells and 3T3-Swiss cells. Additionally, ERK has been shown to antagonize TGFβ canonical signaling through phosphorylation of SMAD2 and SMAD3 at non-TGFR1-associated amino acid residues. Together, these regions are known as the interdomain SMAD-linker region. ERK phosphorylation of the linker region occurs at Ser245/250/255 and Thr220 residues on SMAD2 and at Ser204/208 and Thr17 on SMAD3, reportedly reducing SMAD2/3 signaling.

However, enhancement of SMAD signaling can occur through phosphorylation at various SMAD3 residues, including Thr8 by ERK. This complex interaction may partially explain the discrepancy in our data between normal and malignant cells.
Figure 4. TGFβ engages pERK to direct both the cell cycle and EMT in benign pancreas duct cells. (a) HPDE cells were again starved of growth supplements and pre-incubated with U0126 prior to TGFβ-treatment. Cells were fixed after 72 h and evaluated by immunocytochemistry for SMAD4, indicating reduced nuclear accumulation in response to exogenous TGFβ1 when pERK was inhibited. (b) Cells were dual-stained for p21 and pERK, affirming the efficacy of U0126 and necessity of pERK for TGFβ1-induced upregulation of p21. (c) Cells were next evaluated for expression of proliferation surrogate PCNA as well as the p21 target CDK2, both of which were downregulated in response to TGFβ, but showed no change in response to both TGFβ and U0126. (d and e) HPDE cells were next assessed for changes in cell morphology via phase microscopy, and then stained for the epithelial marker E-Cadherin and the mesenchymal marker Vimentin, indicating that pERK is necessary for TGFβ-induced EMT.
Figure 5. pERK is dispensable for upstream TGFβ signaling in neoplastic cells, yet critical for p21/CDK2 complex formation and EMT. (a) Mouse neoplastic PanIN cells were pre-incubated with U0126 prior to TGFβ-treatment and downstream signals evaluated by western blotting. (b) PanIN cells were incubated with 5–10 ng/ml exogenous TGFβ1 and TGFBR1 isolated by immunoprecipitation and the association with ERK and TGFBR2 measured by western blotting. (c) 24 h following administration of U0126 and/or TGFβ1, the association between p21 and CDK2 was assessed by immunoprecipitation. (d) PanIN cells were again starved of growth supplement and pre-incubated with U0126 prior to TGFβ-treatment. Cells were fixed after 72 h and evaluated by immunocytochemistry for SMAD4. (e) Cells were dual-stained for p21 and pERK, affirming the efficacy of U0126, though pERK was not necessary for TGFβ1-induced upregulation of p21. (f) Cells incubated with TGFβ and/or U0126 were next evaluated for PCNA and CDK2 expression. (g and h) PanIN cells were assessed for changes in cell morphology via phase microscopy and subsequently stained for the epithelial marker E-Cadherin and the mesenchymal marker Vimentin, indicating that pERK is necessary for TGFβ-induced EMT in these cells as well.
Figure 6. pERK antagonizes TGFβ-induced CDK2/p21 association and is required for TGFβ-induced EMT in PANC1 cells. (a) PANC1, BXPC3, and ASPC1 cancer cells were pulsed with 5–10 ng/ml exogenous TGFβ1 and levels of pERK evaluated after 30 min. (b) PANC1 cells were pre-incubated with U0126 prior to TGFβ-treatment, and downstream signals evaluated by western blotting. (c) PanN cells were again pre-incubated with U0126 prior to TGFβ-treatment and TGFβR1 isolated by immunoprecipitation. The association between TGFβR1 and pERK was then assessed by western blotting. (d) 24 h following administration of U0126 and/or TGFβ1, the association between p21 and CDK2 was assessed by immunoprecipitation. (e) PANC1 cells were again starved of growth supplement and pre-incubated with U0126 prior to TGFβ-treatment. Cells were fixed after 72 h and evaluated by immunocytochemistry for SMAD4. (f) Cells were dual-stained for p21 and pERK. (g) Cells were next evaluated for the proliferation surrogate PCNA and the p21 target CDK2. (h and i) PANC1 cells were then either assessed by phase microscopy for changes in morphology, or stained for the epithelial marker E-Cadherin and the mesenchymal marker Vimentin, indicating that pERK is necessary for TGFβ-induced EMT.
normal cells, pERK was a necessary component of p21 signaling downstream of exogenous TGFβ, yet in advanced cancer cells, pERK antagonized canonical TGFβ/p21 signaling. This offers one possible explanation for the observed effects of ERK on SMAD signaling; however, the effects of ERK on TGFβ signaling likely extend well beyond the SMADs.

Our results indicate that the effects of ERK on TGFβ signaling in pancreatic epithelial cells are multifaceted and perhaps biphasic. It appears that pERK initially facilitates both TGFβ-induced cell cycle arrest and EMT. However, as cells begin to undergo transformation, TGFβ and ERK appear to diverge with respect to the cell cycle though ERK still drives TGFβ-induced EMT. Thus, while ERK may contribute to tumor-suppressive TGFβ signals in normal pancreas epithelial cells, TGFβ-induced activation of ERK may be highly detrimental in the disease state. For this reason, the intersection between TGFβ and ERK pathways warrants further consideration,
particularly with respect to the functional switch from tumor-suppressive to tumor-promoting TGFβ signaling.

MATERIALS AND METHODS

Cell lines
Human pancreatic ductal epithelial HPDE and HPDE-KRAS cells were maintained in keratinocyte serum-free medium (KSFM) supplemented with heat-inactivated bovine pituitary extract (BPE), recombinant epidermal growth factor (EGF), penicillin (100 units/ml), and streptomycin (100 μg/ml). Human pancreatic cancer cells (PANC1, BXPC3, ASPC1 and CD18) and mouse neoplastic cells (PanN1) were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). FET cells were grown in 50:50 DMEM/F12 media also supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml). All cells were starved of all growth supplements 24 h prior to treatment, and HPDE cells were treated in media with low EGF. All cells were cultured in a 37 °C incubator with 5% CO2.

PANC1 cells were purchased directly from the ATCC, used less than 6 months from purchase, and kept under passage 8. Additionally, non-ATCC HPDE, FET, CD18 and PanN1 cell lines were both provided by the original laboratories that isolated these cells and similarly maintained at low passage numbers. All cell lines in the laboratory were tested for mycoplasma every 6 months via LookOut Mycoplasma PCR Detection Kit (Sigma Aldrich, St Louis, MO, USA) and, if positive, treated with w/Plasmocin (Invivo-Gen, San Diego, CA, USA) until mycoplasma could not be detected with the aforementioned kit.

Chemicals and reagents
Recombinant TGFβ1 (R&D systems, Minneapolis, MN, USA) was reconstituted per the manufacturer’s instructions and used at 5–10 ng/ml. U0126 (Cell Signaling, Danvers, MA, USA) was dissolved in DMSO and used at 5 μM. Cells were incubated with U0126 for 60–120 min prior to treatment with either control media or media with recombinant TGFβ1.

Mice
EL-KRAS, MT-TGFBR2DN, Tgfbr1+/−, and APC468 mice were generated as described previously in C57B6 background.39 Cohorts of nongenic, EL-TGFBR2, and Tgfbr1+/− (N = 4 per group, 50:50 male to female), EL-KRAS, EL-KRAS-MT-TGFBR2DN, EL-KRAS-Tgfbr1−/− (N = 4 per group, all male), APC468, and APC468, Tgfbr1−/− (N = 4 per group, 50:50 male to female) were euthanized at time points between six months and one year. After euthanasia, mice were anesthetized using ketamine/xylazine (100/10 mg/kg) until unresponsive to toe tap and/or analgous breathing, after which blood was collected using cardiac puncture. Thoracotomy served as the primary form of euthanasia and exsanguination the secondary form. No statistical method was used to determine the sample size, rather this was determined by the number of animals available to us at the time of the study. Animals of the genotypes in question were randomly selected to reach the desired N of 4. After being evaluated, no animals of the desired genotype were excluded from any group, and no further randomization was used.

Western blot and immunoprecipitation
Cell or tissue lysates were lysed in 4% SDS buffer followed by needle homogenization. Equal amounts of protein (15–40 μg) were mixed with loading dye, boiled for 8 min, separated on a denaturing SDS–PAGE gel and transferred to a PVDF membrane. The membrane was blocked in 5% milk/TBS/0.1% Tween for 1 h and incubated with antibodies against pSMA2D2/3, pERK, ERK, CDK2 (Cell Signaling, Danvers, MA, USA), SMAD4, p21, and GAPDH (Santa Cruz Biotech, Santa Cruz, CA, USA). The membrane was washed with TBS-0.1% Tween and then incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotech) at room temperature for 1 h and rewashed. Protein bands were visualized by an enhanced chemiluminescence method (Thermo, Waltham, MA, USA) and resolved digitally per the manufacturer’s specifications.

For immunoprecipitation, cell or tissue lysates were lysed using IP buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40 and 5% glycerol) or RIPA buffer (Cell Signaling) with a protease and phosphatase inhibitor cocktail (Cell Signaling), and cell extracts were incubated overnight with the respective antibodies followed by incubation with protein A or G agarose beads for 4 h at 4 °C. After washing 5–7 times with the respective buffer, immunocomplexes were resolved using SDS–PAGE and visualized by western blot. All antibodies were compared with isotype-specific IgG controls to affirm specificity. All experiments were performed in triplicate unless otherwise specified.

Histology, immunohistochemistry+ and immunofluorescence
Ag-ethnic matched EL-KRAS, EL-KRAS-MT-Tgfbr2DN, EL-KRAS-Tgfbr1−/−, APC468, APC468-MT-Tgfbr2DN and APC468-MT-Tgfbr1−/− mice were euthanized and subjected to pathological examination of the pancreas, colon, small bowel, liver, and spleen. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 4μm interval, and stained via immunohistochemistry (IHC).

Slides were heated in a pressure cooker using DAKO retrieval buffer. Endogenous peroxidases were quenched in DAKO peroxidase block for 20 min. Tissues were blocked with 0.5% BSA in PBS for 30 min and exposed a primary antibody against pERK (Cell Signaling) at 1:50 overnight at 4 °C. Slides were developed using an HRP-conjugated secondary antibody followed by DAB substrate/buffer (DAKO, Carpenteria, CA, USA).

For cultured cells, cells were grown on chamber slides and fixed with ice-cold methanol at −20 °C for 10 min. Cells were blocked for 1 h at room temperature with 0.5% BSA in PBS, and incubated with primary antibodies against SMAD4, p21, PCNA (Santa Cruz), CDK2, E-Cadherin, pERK, or Vimentin (Cell Signaling) at 1:100–200 overnight at 4 °C. Slides were visualized using an alexa flour 488 or 594 conjugated secondary (Abcam, Cambridge, MA, USA).

For all animal and human tissue sections, staining intensity was determined by two blinded investigators. Tissues with undetectable expression were scored as 0, and tissues with strong, ubiquitous expression scored 3+. For sections with intermediate staining, scores of 1–2+ were assigned based on the expertise of the blinded investigators based on variance from 0 and 3+. For cell counting, the number of positive staining nuclei was counted per high-power field by two blinded investigators and values averaged.

Statistical analysis
The data were analyzed by two-way ANOVA and fit to a general linear model in Mininlab16, the validity of which was tested by adherence to the normality assumption and the fitted plot of the residuals. Results were arranged by the Tukey method and were considered significant at P < 0.05. In vitro results are presented as ± s.d., and in vivo results are presented as mean ± s.e.m unless otherwise noted.

Study approval
All experiments involving the use of mice were performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Patient slides and information were obtained from fully consenting patients in a de-identified manner from the Northwestern University Pathcore following local IRB approval.

ABBREVIATIONS
TGFBR, transforming Growth Factor β; TGFBR, TGFβ receptor; TME, tumor Microenvironment; EL, elastase; KRAS, EL-KRAS; T2, TGFBR2-deficient; T1, TGFBR1-deficient; KT2, KRAS-T2; KT1, KRAS-T1; EMT, epithelial–to–mesenchymal transition.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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DISCLAIMER
The material presented in this manuscript is original research, has not been previously published and has not been submitted for publication elsewhere while under consideration.

AUTHOR CONTRIBUTIONS
DP designed the study, performed experiments, assimilated the data into figures and drafted the manuscript. AD, CT, RM and BD performed experiments. MT generated the HPDE cell line. AL generated the PanIN cell line. HM aided in the data interpretation. BJ provided data interpretation and edited the manuscript. PG funded the study, provided experimental oversight, data interpretation and edited the manuscript.

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