

ORIGINAL ARTICLE

TGF β engages MEK/ERK to differentially regulate benign and malignant pancreas cell functionDR Principe¹, AM Diaz², C Torres², RJ Mangan², B DeCant², R McKinney², M-S Tsao³, A Lowy⁴, HG Munshi⁵, B Jung² and PJ Grippo²

While TGF β signals are anti-proliferative in benign and well-differentiated pancreatic cells, TGF β appears to promote the progression of advanced cancers. To better understand dysregulation of the TGF β pathway, we first generated mouse models of neoplastic disease with TGF β receptor deficiencies. These models displayed reduced levels of pERK irrespective of KRAS mutation. Furthermore, exogenous TGF β led to rapid and sustained TGFBR1-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 TGF β -induced upregulation of growth suppressive pSMAD2 and p21, prevented downregulation of the pro-growth signal CDK2 and ablated TGF β -induced EMT. These observations suggest that ERK is a key factor in growth suppressive TGF β signals, yet may also contribute to detrimental TGF β signaling such as EMT. In neoplastic PanIN cells, pERK was not necessary for either TGF β -induced pSMAD2 phosphorylation or CDK2 repression, but was required for upregulation of p21 and EMT indicating a partial divergence between TGF β and MEK/ERK in early carcinogenesis. In cancer cells, pERK had no effect on TGF β -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 TGF β , consistent with most observations identifying pERK as a tumor promoter. Combined, these data suggest that during carcinogenesis pERK initially facilitates and later antagonizes TGF β -mediated cell cycle arrest, yet remains critical for the pathological, EMT-inducing arm of TGF β signaling.

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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 (TGF β) signaling in pancreatic carcinogenesis. In benign and neoplastic tissues, TGF β is often considered a stark tumor suppressor as it induces cell cycle arrest and apoptosis. However, many advanced cancers become desensitized to TGF β -induced cell cycle arrest, and in some patients TGF β begins to promote adverse cellular events, including epithelial–mesenchymal transition (EMT) and metastasis.²

In pancreatic cancer, TGF β ligands are often overexpressed and are predominantly derived from the stroma.³ In canonical TGF β signaling, the TGF β ligand binds to the type 2 TGF β receptor (TGFBR2). This recruits the type 1 TGF β receptor (TGFBR1), a serine/threonine kinase that auto-phosphorylates, and subsequently phosphorylates SMAD2 and SMAD3 proteins. In the cytoplasm, pSMAD2 and 3 form a heteroligomer with SMAD4 and translocate to the nucleus to alter gene expression. In benign and neoplastic pancreatic epithelial cells, TGF β arrests the cell cycle via upregulation of targets such as p21^{CIP1/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 TGF β -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 TGF β -induced p21 upregulation is largely SMAD4 dependent,¹⁰ non-SMAD signals in colon have been shown to have a role in regulating p21 expression.¹¹ Indeed, our previous work in colon cancer demonstrated that SMAD4 is required for TGF β -induced upregulation of p21, though non-SMAD signaling was critical for repression of p21 in response to Activin, another member of the TGF β superfamily.^{12,13} While much is known regarding TGF β 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 TGF β and the RAS/ERK pathway¹⁴ is of particular interest in pancreatic cancer, given the prevalence of both KRAS mutations and altered TGF β 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

¹University of Illinois College of Medicine, Chicago, IL, USA; ²Department of Medicine, University of Illinois, Chicago, IL, USA; ³Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, University of Toronto, Toronto, Ontario, Canada; ⁴Department of Surgery, University of California San Diego, San Diego, CA, USA and ⁵Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA. Correspondence: Dr DR Principe, Department of Medicine, University of Illinois College of Medicine, 840 South Wood Street, 738A CSB, Chicago 60612, IL, USA or Dr PJ Grippo, Division of Gastroenterology and Hepatology, University of Illinois at Chicago, Department of Medicine, 840 South Wood Street, 738A CSB, Chicago 60612, IL, USA.

E-mail: principe@illinois.edu or pgrippo@uic.edu

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led to more epithelial phenotypes, prevented TGF β -induced EMT, and increased sensitivity to epidermal growth factor receptor (EGFR) inhibition.¹⁶ Similar results were observed in both normal murine mammary gland (NMuMG) epithelial cells and mouse cortical tubule (MCT) epithelial cells, in which U0126 prevented TGF β 1-induced EMT.¹⁷ Similar results were seen in renal tubule epithelial cells¹⁸ and colon cancer epithelial cells,¹² though the role of ERK in TGF β -induced EMT in the pancreas is unknown.

ERK is generally considered a proto-oncogene that drives tumor cell proliferation, EMT, migration and invasion.¹⁹ 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.²⁰ 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.²¹ Additionally, it has also been suggested that RAS stabilizes p21 by promoting the formation of p21/cyclin complexes, preventing proteasome degradation.²² 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 TGF β also regulates these events, the relevance of crosstalk between ERK and TGF β signals has yet to be investigated.

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

RESULTS

TGF β 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 sections compared with adjacent non-malignant tissue (Figures 1a and b). To determine the relationship between ERK activation and TGF β signaling, we next evaluated levels of pERK in mice with expression of mutant KRAS^{G12D} restricted to the exocrine pancreas via the Elastase promoter (EL), as well as mutant KRAS mice with truncation of TGFBR2 (KRAS x *Tgfb2*^{DN}, or KT2) or TGFBR1 (KRAS x *Tgfb1*^{+/-}, or KT1). We have previously demonstrated that despite the opposing phenotypes, which were extensively characterized in our recent manuscript, both KT2 and KT1 mice have equivalent loss of downstream TGF β signaling in the epithelium.³

Interestingly, while KRAS controls had robust ERK activation, particularly in neoplastic tissues, both KT2 and KT1 mice were deficient of pERK in normal and neoplastic epithelial cells (Figures 1c–e). Similarly, in the normal condition, pERK level was significantly reduced in both the pancreas and small intestine of nongenic mice with *wild-type* KRAS (WT) and mice with respective TGFBR2 (*Tgfb2*^{DN} or T2) or TGFBR1 (*Tgfb1*^{+/-} or T1) signaling deficiency (Figures 1f g; and Supplementary Figure S1a and b). Combined, these data suggest that receptor-dependent TGF β

signaling is required for sustained ERK phosphorylation in the mouse pancreas irrespective of a KRAS-activating mutation.

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

Though all TGFBR-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 TGFBR-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,³ 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, *Tgfb2*^{DN} and *Tgfb1*^{+/-} 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 TGF β -induced cell cycle arrest in benign pancreas duct cells

Given the increase in CDK2 and Cyclin E expression in TGFBR-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 TGFBR-deficient mice had no observable effect on PCNA staining/proliferation (Figure 3d).

To better understand the relationship between TGF β 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 TGF β 1 led to dose-dependent increases in pERK, suggesting TGF β is sufficient to induce ERK phosphorylation in these cells (Figure 3e). Next, to assess the mechanisms through which TGF β induces ERK phosphorylation, pERK and binding partners were isolated via immunoprecipitation. We found that, in untreated cells, pERK co-precipitated with TGFBR1. Interestingly, this complex dissociated 30 min after incubation with exogenous TGF β 1 (Figure 3f). As the SMAD proteins can be found in the nucleus for many hours following incubation with TGF β ,²³ 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 TGF β pathway, 24 h after the administration of TGF β 1, we found an association between pERK and both SMAD4 and p21, two downstream targets of TGF β signaling (Figure 3g).

As pERK appears to interact with known targets of growth suppressive TGF β signaling, we next assessed whether pERK is

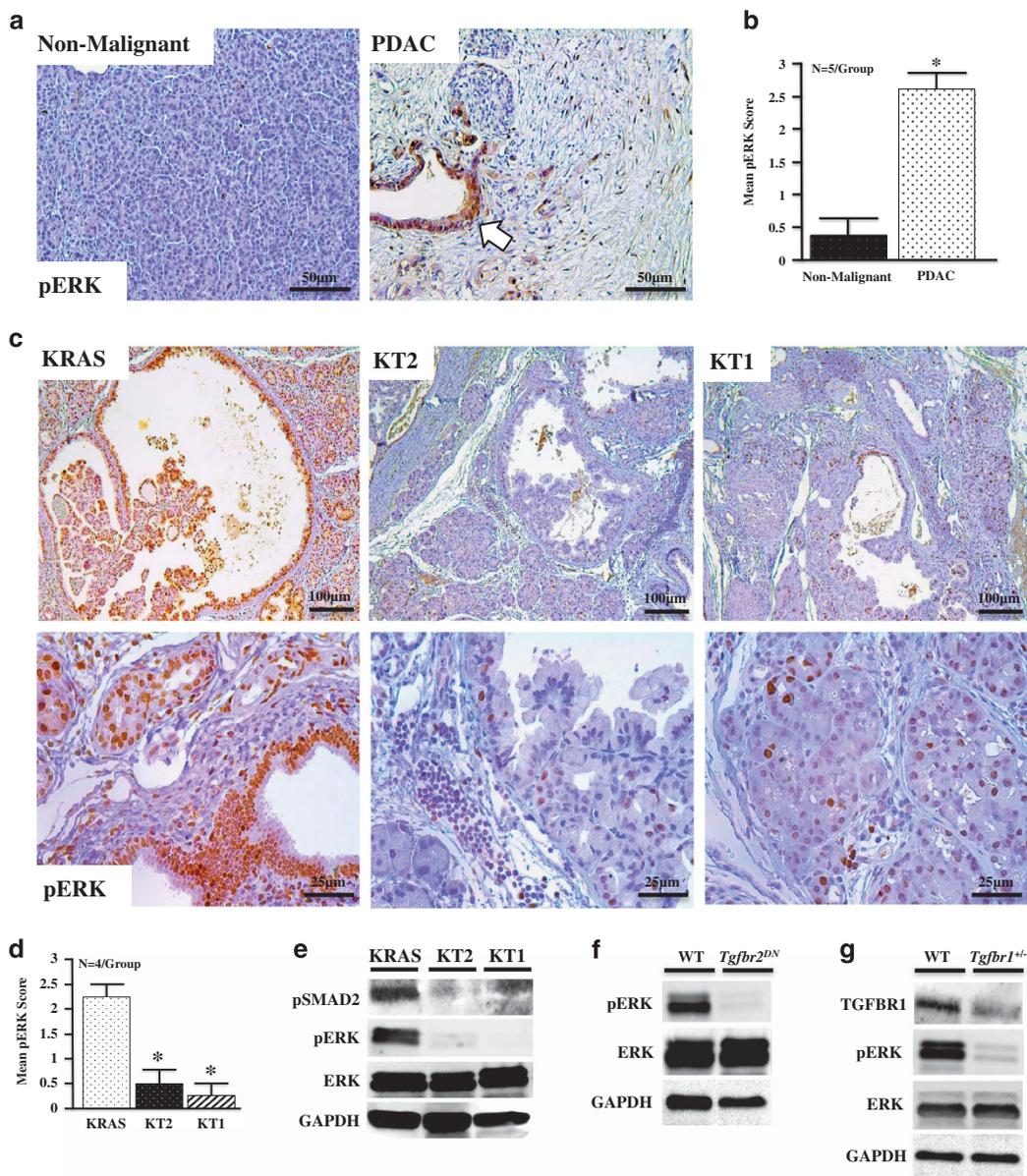


Figure 1. TGF β receptors are necessary for ERK phosphorylation in the pancreas (**a** and **b**) Human pancreatic ductal adenocarcinoma (PDAC) and adjacent non-malignant samples were stained for pERK and scored by two investigators, showing increased ERK activity in 5/5 PDAC sections. The white arrow indicates cancer epithelium highly positive for pERK. (**c** and **d**) El-KRAS (KRAS) mice with mutant KRAS^{G12D} expression is restricted to the pancreas acinar compartment via a rat elastase promoter were employed as a model of early pancreatic tumorigenesis. These mice were crossed to mice conditionally expressing a dominant negative TGFBR2 in epithelial tissues (*Tgfr2^{DN}*) or heterozygous deletion of *Tgfr1* (*Tgfr1^{+/-}*) to form KT2 and KT1, respectively. Tissue sections were next stained for pERK, showing reduced expression in both KT2 and KT1 cohorts. (**e–g**) Tissues were next homogenized and analyzed by western blotting, confirming the observed pERK deficiency in KT2 and KT1 animals, as well as in *Tgfr2^{DN}* and *Tgfr1^{+/-}* mice with *wild-type* KRAS. (**P* < 0.05. *n* = 4 mice per group unless otherwise specified).

involved in TGF β -induced cell cycle arrest. We first inhibited ERK phosphorylation pharmacologically prior to incubation with TGF β and assessed localization and expression of p21. By blocking MEK-induced ERK phosphorylation with U0126, we found that pERK is required for TGF β 1 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, TGF β

fails to promote an interaction between p21 and CDK2 in HPDE cells, consistent with reduced cell cycle inhibition (Figure 3j).

pERK is required for TGF β -induced EMT in HPDE cells

Though TGF β has anti-proliferative effects in HPDE cells, TGF β is also a well-known inducer of EMT. We therefore inhibited ERK phosphorylation pharmacologically prior to incubation with TGF β 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 TGF β -

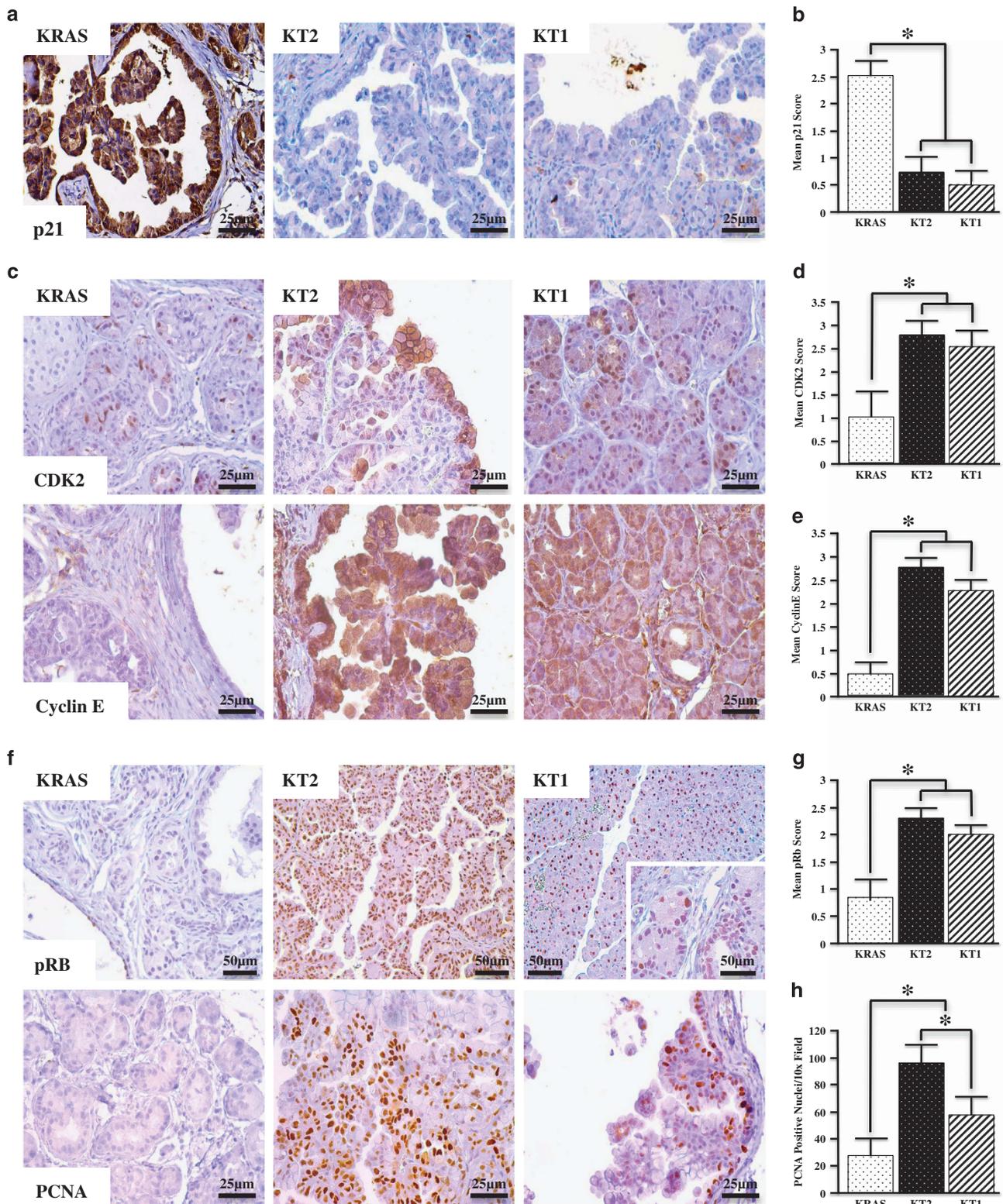


Figure 2. TGFBR-deficient mice display loss of cell cycle control despite reduced ERK activation. **(a and b)** Tissue sections from KRAS, KT2 and KT1 mice were stained for p21, indicating reduced expression in both TGFBR-deficient cohorts. **(c–e)** Tissues were stained for the p21 targets CDK2 and 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), *Tgfr2*^{DN}, and *Tgfr1*^{+/-} mice were similarly stained for CDK2 and Cyclin E, both of which were similarly upregulated in *Tgfr2*^{DN} and *Tgfr1*^{+/-}. (**P* < 0.05. *N* = 4 mice per group unless otherwise specified).

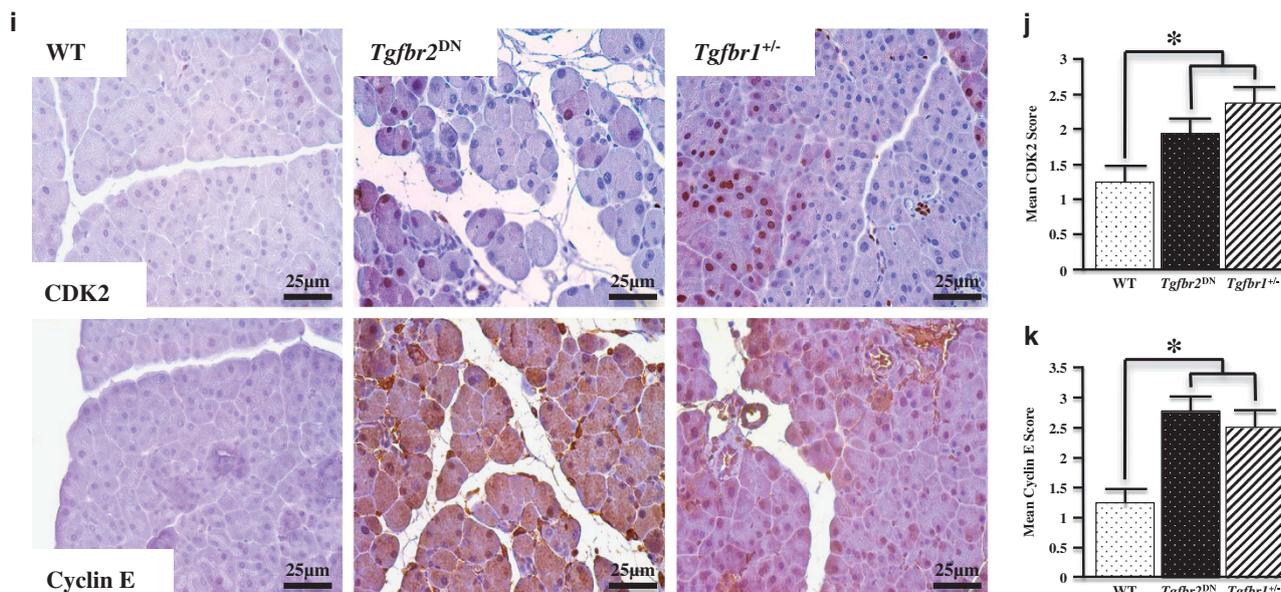


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induced nuclear localization of SMAD4, consistent with the reduction in pSMAD2 (Figure 4a and Supplementary Figure S4a). Additionally, U0126 ablated TGFβ-induced nuclear localization of p21 (Figure 4b and Supplementary Figure S4b) and repression of 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-KRAS^{G12D} 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^{Δ468} 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, TGFβ1 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

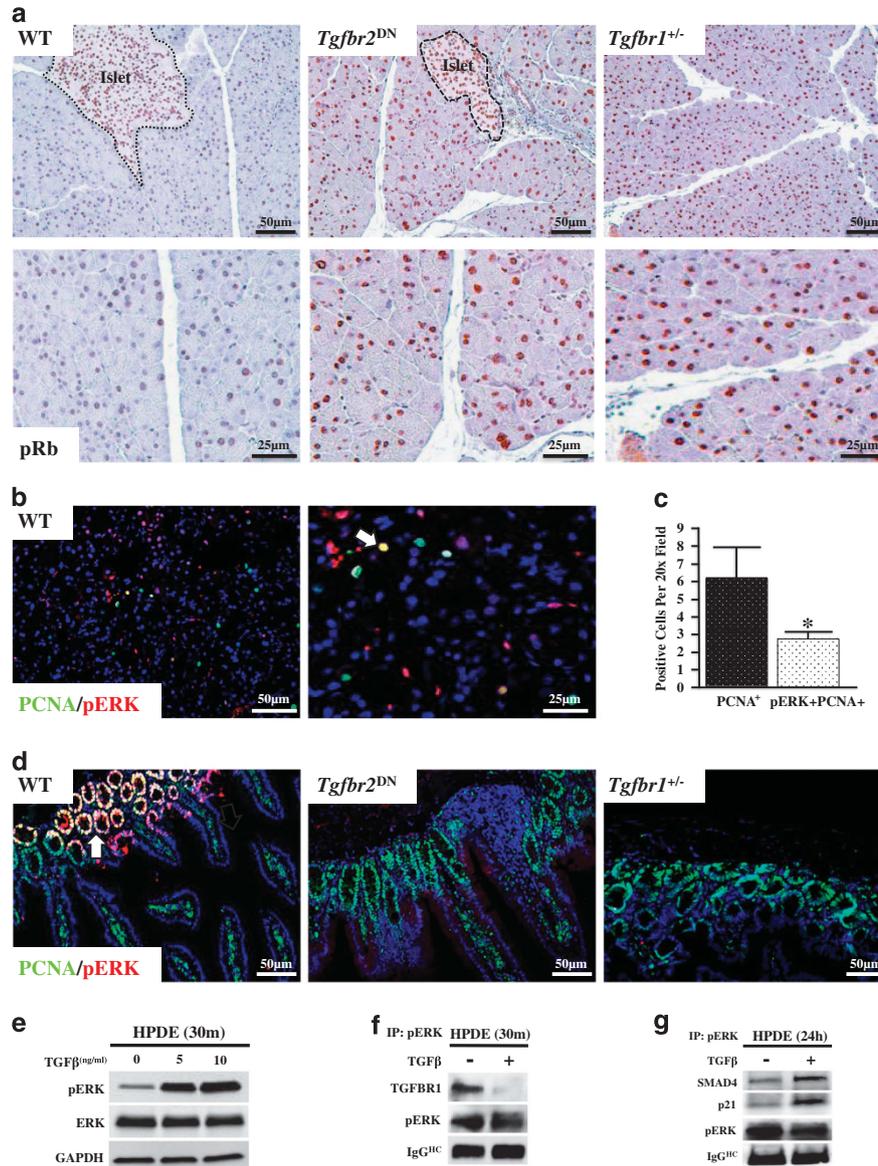


Figure 3. pERK is necessary for TGFβ-induced cell cycle arrest in benign pancreas duct cells. **(a)** pRb expression was evaluated via immunohistochemistry, showing increased staining in the exocrine tissue of *Tgfr2^{DN}* and *Tgfr1^{+/-}* mice compared with *wild-type* (WT) controls. Dashed lines surround 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), *Tgfr2^{DN}*, and *Tgfr1^{+/-}* mice for PCNA and pERK, affirming the pERK deficiency in *Tgfr2^{DN}* and *Tgfr1^{+/-}* 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 pre-incubated with U0126 prior to TGFβ-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 TGFβ-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).

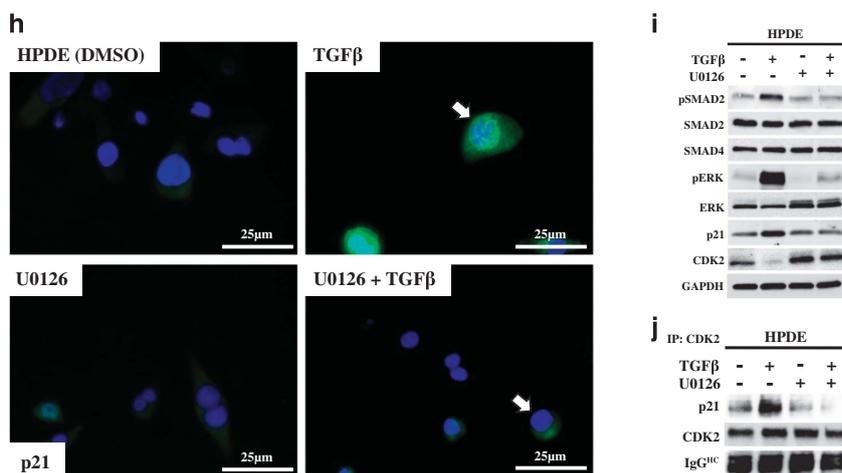


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control. However, incubation with TGF β 1 caused PANC1 cells to uniformly display a more spindle shaped morphology consistent with EMT (Figure 6h), as well as reduced expression of E-cadherin and upregulation of Vimentin. U0126 treatment prevented these events, suggesting that pERK is required for TGF β -induced EMT in PANC1 cells (Figure 6i).

DISCUSSION

While TGF β is generally considered a tumor suppressor with respect to benign epithelial cells, TGF β also appears to facilitate the progression of many advanced cancers.² The paradoxical effects of TGF β in human cancers are poorly understood, and while there is clear merit to therapies targeting the TGF β 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 TGF β signaling pathway in cancer, particularly with respect to the intersection between TGF β and cancer-associated mitogens such as ERK.

Like TGF β , 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.²⁸ Additionally, pERK has been shown to upregulate p21 through both transcriptional and post-translational mechanisms, facilitating cell cycle arrest.²⁹ However, sustained pERK activation is also involved in S phase entry³⁰ and has been linked to several other hallmark features of tumorigenesis.³¹ pERK drives the proliferation and migration of many cancers in response to a variety of stimuli, including cell stress, cytokines and growth factors.³¹ 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.³²

Many studies have also shown that ERK is a critical regulator of TGF β signaling in cancer. The majority of these works have shown that ERK antagonizes TGF β -induced growth-inhibition. Specifically, RAS-induced transformation appears to diminish responsiveness to the anti-mitotic effects of TGF β in lung, intestinal, liver and mammary epithelial cells.^{33,34} RAS/ERK signaling also appears to mediate TGF β -induced repression of the tumor-suppressor PTEN in pancreatic cancer cells, implicating ERK in the more pathological effects of TGF β .³⁵ Furthermore, as mentioned, pERK signals are necessary for TGF β -induced EMT in a variety of cell types, though this has yet to be explored in the pancreas.^{12,17,18}

In this work, we first demonstrated that in both normal and oncogenic KRAS-expressing pancreas, both TGFBR2 and TGFBR1

are necessary for ERK phosphorylation *in vivo*. These results were quite unexpected as TGF β is largely considered a growth-suppressive signal and ERK a mitogen. However, there is evidence that members of the TGF β superfamily can enhance growth signals. In colon cancer specimens, p21 expression is positively correlated with expression of TGFBR2 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 TGF β family are critical for p21 expression in colon cancer.¹² Therefore, we assessed the relationship between TGF β signaling and the ERK pathway with respect to p21 and downstream regulation of the cell cycle.

In normal pancreatic epithelial cells, exogenous TGF β 1 led to rapid induction of pERK, which subsequently dissociated from TGFBR1 and associated with downstream TGF β targets SMAD4 and p21. Interestingly, when pERK activation was inhibited pharmacologically by U0126, TGF β 1 failed to induce SMAD2 phosphorylation, p21 upregulation and nuclear localization, and repression of CDK2. pERK was similarly necessary for TGF β -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 TGF β signaling. In these cells, pERK had no relationship to TGF β -induced upregulation of p21, and TGF β failed to induce the association between p21 and CDK2 unless pERK was inhibited. However, pERK was necessary for TGF β -induced EMT in all cell lines, suggesting that, in this capacity, pERK may also facilitate the tumor-promoting roles of TGF β signaling (Figure 7).

Previous studies have identified a convergence between ERK and TGF β signals in other cell types.³⁶ Notably, it was found that the TGF β receptors share structural homology with receptor tyrosine kinases,³⁷ and TGFBR1 been shown to induce direct phosphorylation of ShcA leading to activation of the MEK/ERK pathway in Mv1Lu mink epithelial cells and 3T3-Swiss cells.¹⁴ Additionally, ERK has been shown to antagonize TGF β canonical signaling through phosphorylation of SMAD2 and SMAD3 at non-TGFBR1-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.³⁸ This complex interaction may partially explain the discrepancy in our data between normal and malignant cells. In

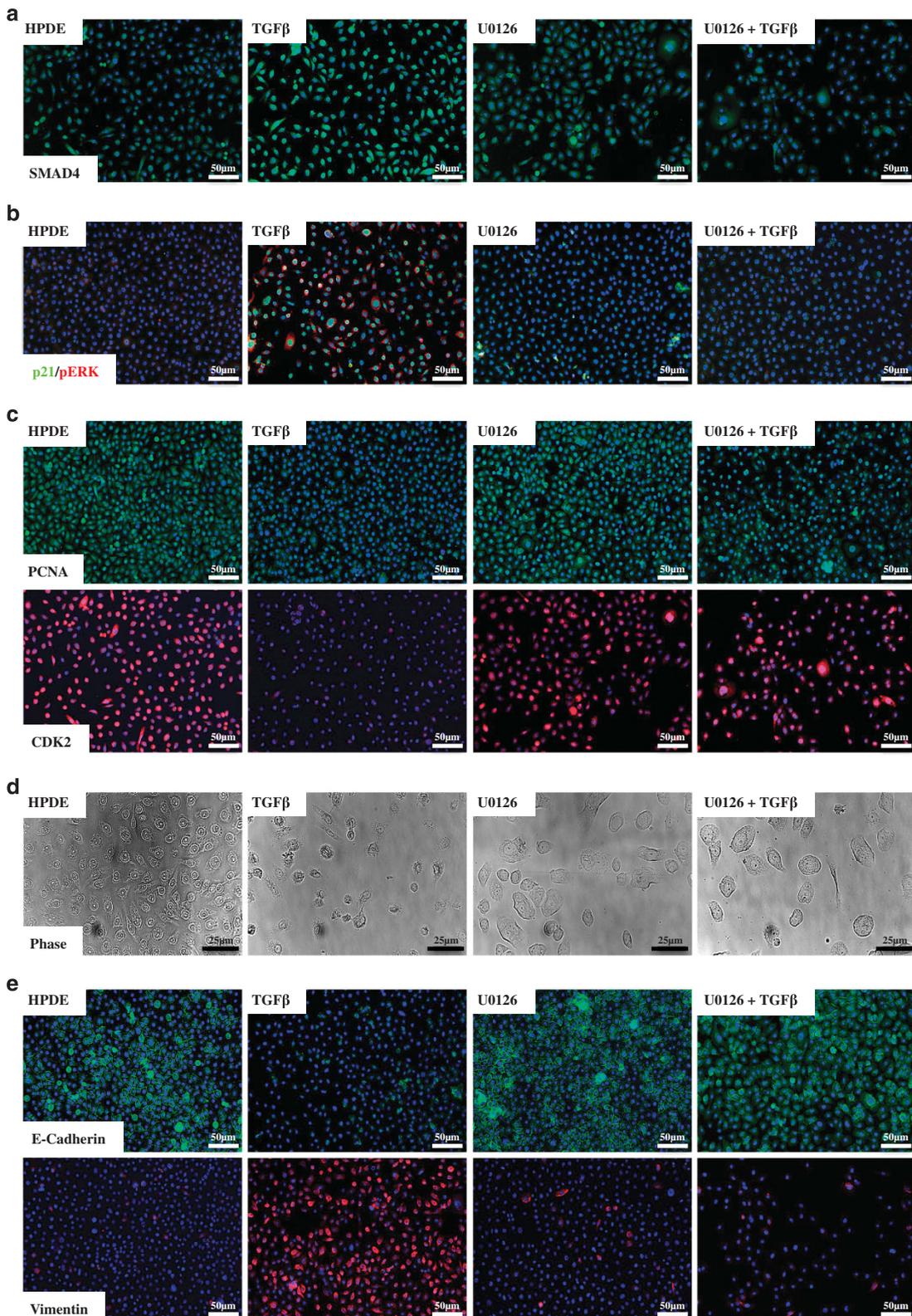


Figure 4. TGF β engages pERK to direct both the cell cycle and EMT in benign pancreas duct cells. **(a)** HPDE cells were again starved of growth supplements and pre-incubated with U0126 prior to TGF β -treatment. Cells were fixed after 72 h and evaluated by immunocytochemistry for SMAD4, indicating reduced nuclear accumulation in response to exogenous TGF β 1 when pERK was inhibited. **(b)** Cells were dual-stained for p21 and pERK, affirming the efficacy of U0126 and necessity of pERK for TGF β 1-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 TGF β , but showed no change in response to both TGF β 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 TGF β -induced EMT.

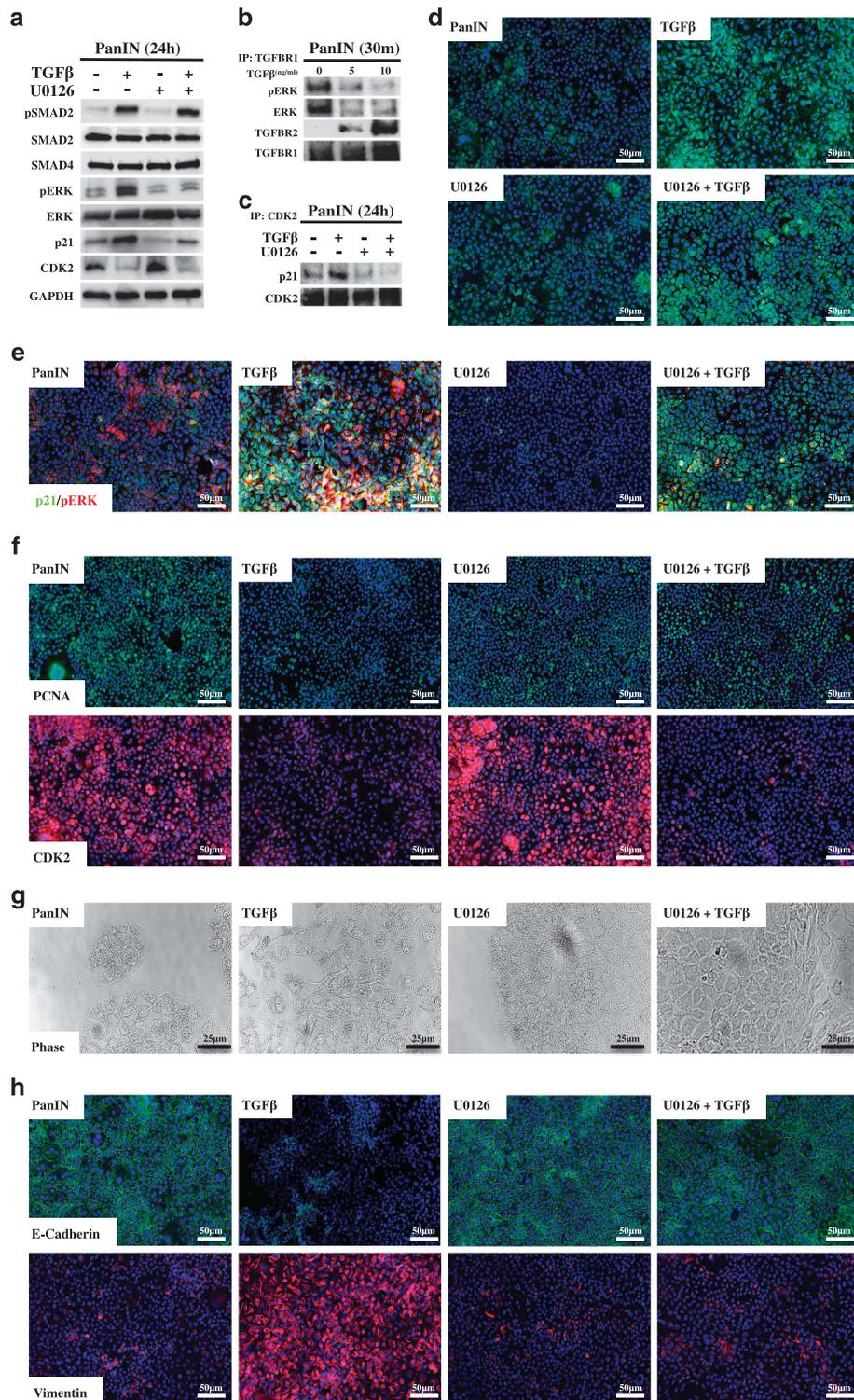


Figure 5. pERK is dispensable for upstream TGF β signaling in neoplastic cells, yet critical for p21/CDK2 complex formation and EMT. **(a)** Mouse neoplastic PanIN cells were pre-incubated with U0126 prior to TGF β -treatment and downstream signals evaluated by western blotting. **(b)** PanIN cells were incubated with 5–10^{ng/ml} exogenous TGF β 1 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 TGF β 1, 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 TGF β -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 TGF β 1-induced upregulation of p21. **(f)** Cells incubated with TGF β 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 TGF β -induced EMT in these cells as well.

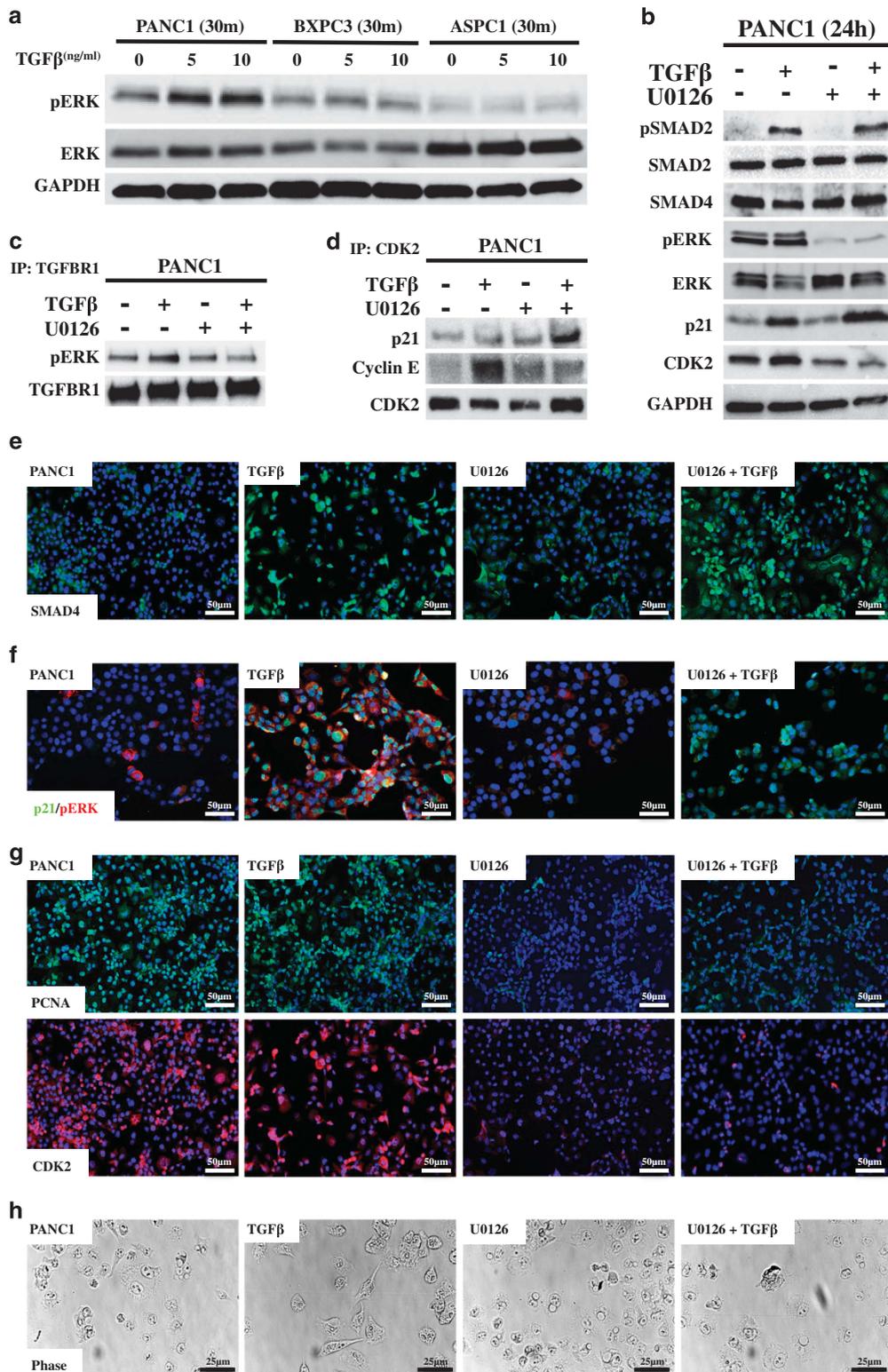


Figure 6. pERK antagonizes TGF β -induced CDK2/p21 association and is required for TGF β -induced EMT in PANC1 cells. **(a)** PANC1, BXPC3, and ASPC1 cancer cells were pulsed with with 5–10^{ng/ml} exogenous TGF β 1 and levels of pERK evaluated after 30 min. **(b)** PANC1 cells were pre-incubated with U0126 prior to TGF β -treatment, and downstream signals evaluated by western blotting. **(c)** PanIN cells were again pre-incubated with U0126 prior to TGF β -treatment and TGFBR1 isolated by immunoprecipitation. The association between TGFBR1 and pERK was then assessed by western blotting. **(d)** 24 h following administration of U0126 and/or TGF β 1, 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 TGF β -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 TGF β -induced EMT.

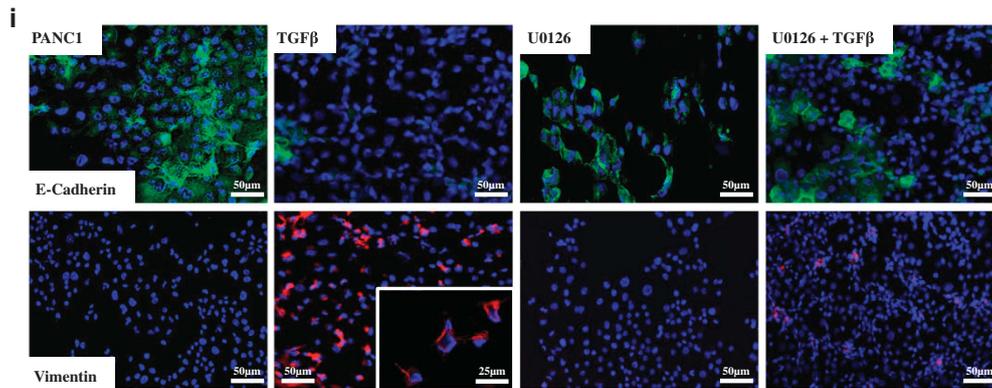


Figure 6. (continued).

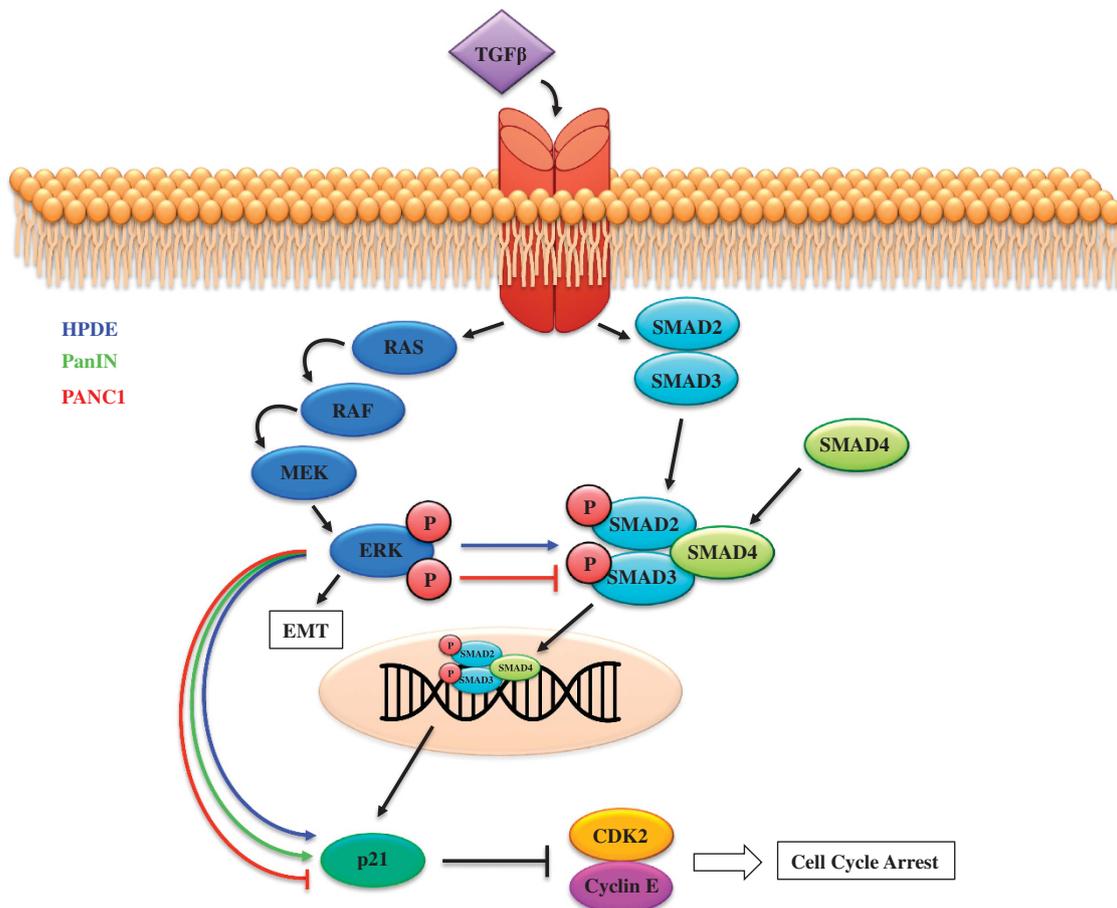


Figure 7. TGF β engages MEK/ERK to differentially regulate benign, neoplastic, and malignant cells. In normal pancreatic epithelial cells (HPDE), pERK is seemingly required for both growth suppressive TGF β signaling such as SMAD2 phosphorylation, upregulation of p21 and promoting p21/CDK2 complexes, as well as the more pathological pro-EMT effects of TGF β . In neoplastic cells (PanIN), though pERK is still required for TGF β -induced EMT, pERK was only partially necessary for TGF β -induced growth suppression, having a reduced effect on SMAD2 phosphorylation and upregulation of p21, yet still directing p21/CDK2 interactions. In malignant PANC1 cells, pERK now antagonized growth suppressive TGF β signaling and impeded p21/CDK2 interactions, yet was still necessary for TGF β -induced EMT.

normal cells, pERK was a necessary component of p21 signaling downstream of exogenous TGF β , yet in advanced cancer cells, pERK antagonized canonical TGF β /p21 signaling. This offers one possible explanation for the observed effects of ERK on SMAD signaling; however, the effects of ERK on TGF β signaling likely extend well beyond the SMADs.

Our results indicate that the effects of ERK on TGF β signaling in pancreatic epithelial cells are multifaceted and perhaps biphasic. It

appears that pERK initially facilitates both TGF β -induced cell cycle arrest and EMT. However, as cells begin to undergo transformation, TGF β and ERK appear to diverge with respect to the cell cycle though ERK still drives TGF β -induced EMT. Thus, while ERK may contribute to tumor-suppressive TGF β signals in normal pancreas epithelial cells, TGF β -induced activation of ERK may be highly detrimental in the disease state. For this reason, the intersection between TGF β and ERK pathways warrants further consideration,

particularly with respect to the functional switch from tumor-suppressive to tumor-promoting TGF β 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 μ g/ml). Human pancreatic cancer cells (PANC1, BXPC3, ASPC1 and CD18) and mouse neoplastic cells (PanIN) 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 μ g/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 μ g/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 PanIN 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 TGF β 1 (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 μ M. Cells were incubated with U0126 for 60–120 min prior to treatment with either control media or media with recombinant TGF β 1.

Mice

EL-KRAS, MT-TGFBR2^{DN}, *Tgfb1*^{+/-}, and APC ^{Δ 468} mice were generated as described previously in C57B6 background.³⁹ Cohorts of nongenetic, MT-Tgfbr2^{DN}, or *Tgfb1*^{+/-} (*N*=4 per group, 50:50 male to female), EL-KRAS, EL-KRAS-MT-Tgfbr2^{DN}, EL-KRAS-Tgfbr1^{+/-} (*N*=4 per group, all male), APC ^{Δ 468}, and APC ^{Δ 468}-Tgfbr1^{+/-} (*N*=4 per group, 50:50 male to female) were euthanized at time points between six months and one year. For euthanasia, mice were anesthetized using ketamine/xylazine (100/10 mg/kg) until unresponsive to toe tap and/or agonal 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 μ g) 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 pSMAD2/3, 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 antibody (Santa Cruz Biotech) at room temperature for 1 h and re-washed. 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 inhibitory 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

Age-matched EL-KRAS, EL-KRAS-MT-Tgfbr2^{DN}, EL-KRAS-Tgfbr1^{+/-}, APC ^{Δ 468}, APC ^{Δ 468}-MT-Tgfbr2^{DN} and APC ^{Δ 468}-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 4 μ m 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, Carpinteria, 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 alexaflour 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 Minitab16, 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 \pm s.d., and *in vivo* results are presented as mean \pm 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, TGF β receptor; TME, tumor Microenvironment; EL, elastase; KRAS, EL-KRAS^{G12D}; 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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