Regulation of pancreatic cancer aggressiveness by stromal stiffening

Nicola Rath and Michael F Olson

Cancer Research UK Beatson Institute, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK

Correspondence to Michael F Olson

Tel: +44 (0)141 330 3654 Fax: +44(0)141 942 6521

Email: m.olson@beatson.gla.ac.uk

Pancreatic ductal adenocarcinoma is characterized by an extensive stromal component that hinders treatment. A new study shows how the genetic makeup of pancreatic tumors may influence the physical properties of the associated stroma to promote tumor progression.

Pancreatic cancer is a leading cause of cancer deaths, with poor survival rates and little improvement in patient outcomes despite considerable effort directed at optimizing therapies [1]. To improve therapeutic responses and increase survival, factors that make pancreatic cancer so deadly and so difficult to treat must be identified. In this issue of *Nature Medicine*, Laklai *et al*. [2] reveal how mechanical properties of the pancreatic cancer microenvironment promote tumor aggressiveness.

Numerous studies have examined genomic alterations in pancreatic cancer [3], revealing the prevalence of activating *KRAS* mutations that increase mitogen-activated protein kinase pathway signalling, *TP53* tumor suppressor loss of function mutations and inactivating *SMAD4* mutations that reduce transforming growth factor β (TGFβ)-induced changes in gene expression**, all** within a landscape of additional genetic variations. The most common pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), which develops from cells lining exocrine ducts that conduct digestive enzymes from acinar cells for delivery to the duodenum. Genetically-engineered mouse models of PDAC that closely recapitulate human PDAC development and progression have been established using oncogenic *Kras* selectively expressed in the pancreas, either alone (KC) or combined with mutated *Trp53* (KPC) [4,5].

PDAC is characterized by dense fibrous stroma composed of numerous cell types, including macrophages and α-smooth muscle actin (αSMA)-positive myofibroblasts, and deposition of extracellular matrix (ECM) material such as collagen1 and hyaluronic acid. In fact, transformed pancreatic epithelial cells are a minor constituent of PDAC tumors, with stromal components comprising the bulk of the tumor mass. The contribution of this desmoplastic reaction to pancreatic tumorigenesis is controversial, with evidence consistent with both contributory and inhibitory effects on tumorigenesis. In mouse models of PDAC, long-term inhibition or genetic deletions that limit the ability of pancreatic epithelial cells to respond to paracrine-acting tumor-secreted Hedgehog peptides, which enhance the tumor desmoplastic reaction [6], reduced stromal content and increased tumor aggression; while Hedgehog pathway activation in PDAC tumors with small molecule agonists increased stromal fibrosis and reduced epithelial cell proliferation [7,8]. Furthermore, in another mouse model, depletion of αSMA-expressing myofibroblasts resulted in invasive, undifferentiated PDAC tumors with reduced survival [9]. These observations suggested that the stromal component restrains PDAC. In addition, anti-stromal therapies for PDAC have not demonstrably improved patient survival or therapeutic responses in clinical trials [1]. One possible explanation for the apparent restraining actions of the stromal component is that the “classical” mouse models are not adequate representations of the contribution of the stroma to PDAC. In particular, changes in protein expression levels might not reflect the influence that ECM protein organization and consequent mechanical properties of the tumor microenvironment have on PDAC growth and progression.

Laklai *et al*. found in a number of human tumour samples that there were clear associations between poor PDAC differentiation, increased collagen fiber

diameters and increased epithelial phosphorylated regulatory myosin light chain (pMLC2), consistent with functional links of tumor cell actomyosin tension and stromal remodelling with PDAC progression. Furthermore, patients with thicker collagen fibers adjacent to pancreatic lesions or increased epithelial pMLC2 had the shortest survival time, suggesting that altered ECM organization contributed to patient mortality. Reduced patient survival was also associated with relatively lower TGFβ signalling compared to longer surviving patients, and samples from patients with *SMAD4* mutations had abundant collagen fibres, increased stiffness adjacent to pancreatic lesions and elevated pMLC2. Taken together, these results revealed significant links between reduced TGFβ signalling, ECM organization, tissue stiffness, epithelial cell actin-myosin tension and patient outcomes.

TGFβ signalling proteins are frequently altered in human PDAC, with the cumulative frequency of *SMAD4, SMAD3, TGFBR1, TGFBR2, ACVR1B* or *ACVR2A* mutations being >50% [3]. To determine how disruption of this pathway might contribute to PDAC, Laklai *et al*. [2] compared the fibrotic properties of tumors from KC or KPC mice with tumors from mice expressing oncogenic Kras plus pancreas-selective deletion of one TGFβ receptor II (*Tgfrb2*) allele (KTC). Although expression of myofibroblast markers and total collagen levels were not different, collagen bundles were thicker in KTC tumors than in both KC and KPC tumors and associated with stiffer tissue. Accompanying these altered physical properties, KTC cells manifested several molecular responses typically mechanically induced, including nuclear accumulation of the YAP transcription factor, activated β1-integrin, phosphorylated active focal adhesion kinase (pFAK), and pMLC2. The KTC cells generated more actin-myosin mediated contractile force *in vitro* than the KPC and KC cells, which was dependent on the phosphorylation of MLC2 by the ROCK1

protein kinase. When implanted into immunocompromised mice, KTC tumor cells induced ROCK1-dependent fibrosis, indicating that cell contractility leads to ECM protein remodelling, which in turn signals via mechanoresponsive pathways to increase cellular tension (Figure 1).

Laklai *et al.* [2] next sought to identify signalling pathways altered by *Tgfbr2* deletion in PDAC. Tumor cells have been previously shown to signal via a cytokine induced G-protein coupled receptor (GPCR)-JAK-STAT pathway to induce actomyosin contractility, while ROCK-mediated contractility in tumor cells increases JAK-STAT signalling by inducing cytokine expression and secretion [10]. KTC tumors had elevated levels of active phosphorylated Stat3 (pStat3) and increased collagen contracting ability indicating activation of the JAK-STAT pathway and KTC cell-conditioned media increased KC tumor cell pStat3 levels and collagen contraction *in vitro*. In addition, JAK inhibitor treatment reduced collagen contraction by KTC cells, or by KC cells treated with KTC-conditioned medium. These results implicate elevated GPCR-JAK-STAT signalling in the increased actomyosin tension and matrix remodelling by PDAC cells with impaired TGFβ signalling.

To determine whether mechanoresponsive signalling was sufficient to promote PDAC progression, the authors generated KC mice with pancreas-selective expression of V737N β1-integrin (KC/β1V737N) [11] to drive ligand-independent integrin clustering and focal adhesion signalling in the pancreas. The inclusion of active β1-integrin in KC mice was sufficient to recapitulate KTC phenotypes, including increased fibrosis, stromal stiffening, elevated pStat3, pFAK and pMLC2, and progression to PDAC. The effect of active β1-integrin was reversed by knockdown or inhibition of FAK, indicating that focal adhesion signalling was the key mediator of the effects of active β1-integrin.

Similarly, KC mice expressing one allele of constitutively active Stat3 [12] (KC/Stat3C) were generated which resulted in elevated pFAK and pMLC2, increased fibrosis, stromal stiffening and reduced survival relative to control KC mice. In KTC mice lacking both alleles of *Tgfrb2* and pancreas selective deletion of both copies of *Stat3* there was complete reversal of the KTC phenotype and increased survival.

Taken together, these experiments indicate that in PDAC reduced TGFβ signalling enables cell contractility that leads to ECM remodelling, increased microenvironmental stiffness and elevated JAK-STAT signalling. This signalling acts via mechanoresponsive pathways in a feed-forward loop to amplify cellular responses that promote PDAC growth and progression (Figure 1). The human relevance of these findings is supported by data from clinical samples indicating that PDAC patients with shorter survival times have elevated pStat3, pMLC2, nuclear Yap and increased collagen bundling, paralleled in PDAC patients with *SMAD4* mutations.

There are several important implications of this study. Firstly, comparing total levels of collagen protein between tumors overlooks the importance of collagen fiber remodelling (such as bundling and cross-linking) in altering the stromal physical properties that may play significant roles in PDAC. As a result, methods that detect collagen organization, such as second harmonic generation microscopy, complement the information acquired from collagen staining alone. Secondly, the observation that certain PDAC genotypes, particularly with TGFβ signalling alterations, generate actomyosin contractile force to remodel ECM proteins and increase proximal stromal stiffness suggests that targeting both tumor and stromal cells to reduce the tumor-promoting influence of the microenvironment would likely be more efficacious than stromal cell targeting alone. The interactions between

PDAC genotypes and tumor mechanical properties, highlighted in the study by Laklai *et al*. [2] in this issue of *Nature Medicine*, reinforces the importance of incorporating genetic context into future stromal-targeted therapy development.

References

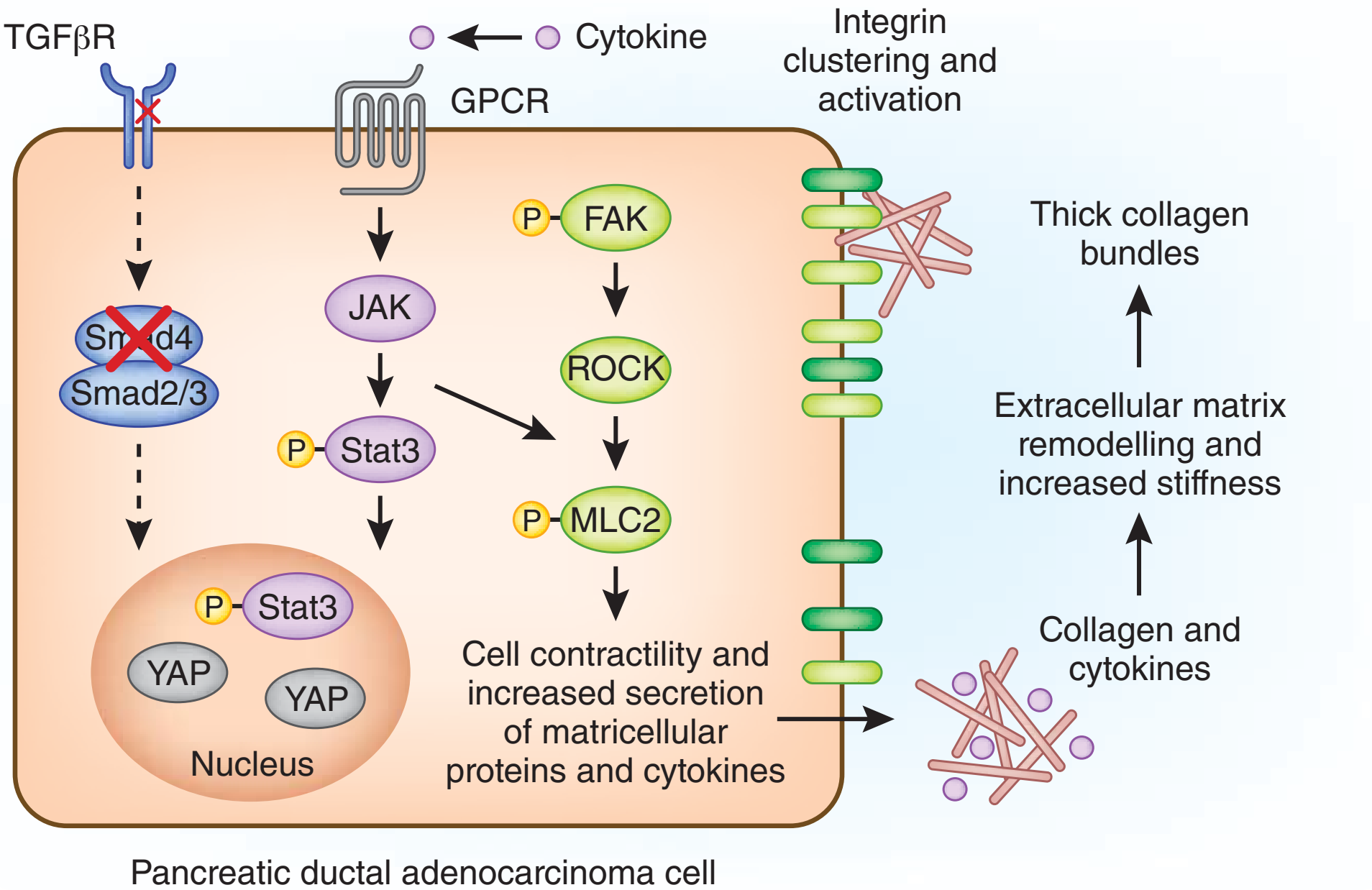
1. Neesse, A., Algul, H., Tuveson, D.A. & Gress, T.M. Stromal biology and therapy in pancreatic cancer: a changing paradigm. *Gut* **64**, 1476-1484 (2015).
2. Laklai, H.*, et al.* Genotype tunes PDAC tension to induce matricellular-fibrosis and tumor aggression. *Nature Med* (2016).
3. Bailey, P.*, et al.* Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature* **531**, 47-52 (2016).
4. Hingorani, S.R.*, et al.* Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* **7**, 469-483 (2005).
5. Hingorani, S.R.*, et al.* Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* **4**, 437-450 (2003).
6. Bailey, J.M.*, et al.* Sonic Hedgehog Promotes Desmoplasia in Pancreatic Cancer. *Clin Cancer Res* **14**, 5995-6004 (2008).
7. Rhim, A.D.*, et al.* Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer Cell* **25**, 735-747 (2014).
8. Lee, J.J.*, et al.* Stromal response to Hedgehog signaling restrains pancreatic cancer progression. *Proc Natl Acad Sci U S A* **111**, E3091-3100 (2014).

9. Ozdemir, B.C., *et al.* Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer Cell* **25**, 719-734 (2014).
10. Sanz-Moreno, V., *et al.* ROCK and JAK1 Signaling Cooperate to Control Actomyosin Contractility in Tumor Cells and Stroma. *Cancer Cell* **20**, 229-245 (2011).
11. Mouw, J.K., *et al.* Tissue mechanics modulate microRNA-dependent PTEN expression to regulate malignant progression. *Nat Med* **20**, 360-367 (2014).
12. Barbieri, I., *et al.* Constitutively active Stat3 enhances neu-mediated migration and metastasis in mammary tumors via upregulation of Cten. *Cancer Res* **70**, 2558-2567 (2010).

Figure Legend

Figure 1: Mechanosignaling of PDAC epithelial cells induces stromal remodeling

Laklai, H., *et al.* indicate that PDAC tumors with decreased TGFβ signalling due to mutations in ligand receptors or SMAD4, increased JAK/Stat3 signalling or increased integrin clustering have elevated ROCK-dependent cell contractility. These tumors also secrete more matricellular proteins and cytokines, and have a remodelled and stiffer stromal extracellular matrix. In a feed-forward loop, the stiffer tumor microenvironment amplifies JAK/Stat3 and integrin-FAK-ROCK signalling to promote tumor aggressiveness. GPCR, G-protein coupled receptor; TGFBR, TGFβ receptor.


TGFβR
Cytokine
GPCR
Integrin clustering and activation
Smad4
Smad2/3
JAK
P
Stat3
P
FAK
ROCK
P
MLC2
P
Stat3
YAP
YAP
Nucleus
Cell contractility and increased secretion of matricellular proteins and cytokines
Thick collagen bundles
Extracellular matrix remodelling and increased stiffness
Collagen and cytokines
Pancreatic ductal adenocarcinoma cell