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Deposited on: 23 October 2017

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Targeting ROCK activity to disrupt and prime pancreatic cancer for chemotherapy.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease; the identification of novel targets and development of effective treatment strategies are urgently needed to improve patient outcomes. Remodeling of the pancreatic stroma occurs during PDAC development, which drives disease progression and impairs responses to therapy. The actomyosin regulatory ROCK1 and ROCK2 kinases govern cell motility and contractility, and have been suggested to be potential targets for cancer therapy, particularly to reduce the metastatic spread of tumor cells. However, ROCK inhibitors are not currently used for cancer patient treatment, largely due to the overwhelming challenge faced in the development of anti-metastatic drugs, and a lack of clarity as to the cancer types most likely to benefit from ROCK inhibitor therapy. In two recent publications, we discovered that ROCK1 and ROCK2 expression were increased in PDAC, and that increased ROCK activity was associated with reduced survival and PDAC progression by enabling extracellular matrix (ECM) remodeling and invasive growth of pancreatic cancer cells. We also used intravital imaging to optimize ROCK inhibition using the pharmacological ROCK inhibitor fasudil (HA-1077), and demonstrated that short-term ROCK targeting, or ‘priming’, improved chemotherapy efficacy, disrupted cancer cell collective movement, and impaired metastasis. This body of work strongly indicates that the use of ROCK inhibitors in pancreatic cancer therapy as ‘priming’ agents warrants further consideration, and provides insights as to how transient mechanical manipulation, or fine-tuning the ECM, rather than chronic stromal ablation
might be beneficial for improving chemotherapeutic efficacy in the treatment of this deadly disease.

Introduction

Despite there being a number of new therapeutics that have been developed for pancreatic cancer patient therapy, survival remains the lowest of all solid cancers, with 5-year survival rate being less than 7% and a median survival of 6 months. Despite pre-clinical efforts to develop new therapeutics, patient survival has not significantly improved over the last 4 decades, which highlights not only the need to identify new targets, but also to develop innovative treatment strategies to improve the outcomes of patients suffering from this disease. In addition, development of diagnostic tools, for example based on detection of cancer-derived exosomes, to enable early detection of pancreatic cancer remains a critical challenge for this disease. Pancreatic ductal adenocarcinoma (PDAC) is characterized by extensive remodeling of the pancreatic stroma, with increased deposition and crosslinking of extracellular matrix (ECM) components and poor vascularization compared to normal pancreas. Alterations of the biochemical and mechanical properties of the ECM are known to influence cancer progression, invasion and responses to chemotherapy, however, recent studies assessing the efficacy of ECM-based pancreatic cancer therapies, for example via inhibition of Sonic Hedgehog signaling pathway, targeting of lysyl oxidase activity or inhibition of hyaluronic acid (HA), have yielded conflicting results.

Rho-associated protein kinases 1 and 2 (ROCK1 and ROCK2) are master regulators of the actomyosin cytoskeleton and govern force...
generation, cell invasion, proliferation and contractility 17-19. Numerous studies have established that ROCK inhibition disrupts tumor progression and metastasis in cell based and in vivo models of various solid cancers 20-23. However, to date no compounds have progressed into the clinic for cancer therapy for several reasons. The development of anti-metastatic chemotherapeutics for clinical use is very challenging due to the need to detect a reduction in metastasis in patients over sustained periods (likely years) as a positive outcome 24, in contrast to chemotherapeutics that induce acute positive responses, such as tumor regression, which can be monitored in a clinical trial in a defined and relatively brief time period 24. Furthermore, the absence of correlations between defined genetic alterations, such as ROCK1 or ROCK2 mutations, with ROCK inhibitor sensitivity means that there is no simple genetic test for convenient patient stratification. As a result, ROCK inhibition has not been adopted as a cancer chemotherapy. In this commentary, we describe our recent findings 25, 26 demonstrating that ROCK activity promotes pancreatic cancer invasive growth via ECM remodeling. We also highlight how transient ROCK inhibition, or mechanical ‘priming’ with the pharmacological inhibitor fasudil affects tumor tissue tension, which in turn improves chemotherapy efficacy in primary and secondary tumor sites, while also disrupting collective movement of metastatic cancer cells 26. Lastly, we discuss potential translation of our findings into the clinic for pancreatic cancer therapy, where balancing cellular contractility via transient ROCK inhibition, rather than long-term ablation of the matrix, enables re-establishment of the normal mechanical features of the stroma.
ROCK activity promotes PDAC progression.

Genomic analyses have previously shown that the ROCK1 gene is amplified in 15% of pancreatic patient tumors, however the role of ROCK-mediated actomyosin contractility in PDAC had not been clearly established. To address this, we assessed ROCK expression in a patient tissue microarray (78 samples from patients with pancreatic cancers and 5 healthy human pancreas) and in human TCGA datasets, and determined that ROCK1 and ROCK2 expression increase with tumor stage and grade. In line with this, genomic alterations or mRNA amplification of ROCK1 and/or ROCK2 were found to be positively correlated with poorer survival, suggesting that ROCK signaling promotes pancreatic cancer progression.

To further understand how ROCK influences the fate and behavior of pancreatic cancer cells, Cre-recombinase was expressed from the pancreatic epithelial selective Pdx1 promoter to induce pancreas-targeted recombination of LOX-STOP-LOX (LSL)-Kras$^{G12D/+}$ and LSL-Trp53$^{R172H/+}$ (KPC) alleles in mice, which spontaneously develop PDAC that closely resembles human pancreatic cancer. In addition, KPC mice were crossed with LSL-ROCK2:ER mice to conditionally activate ROCK2 during PDAC progression. This model closely recapitulates the genomic features of human PDAC, where an initiating Kras$^{G12D}$ mutation is found in almost 90% of patient tumors, while the p53$^{R175H}$ mutation is found in 50-75% of patient tumors. Consistent with the observed increased ROCK2 protein levels in advanced PDAC stages, as well as the correlation between increased ROCK1 and ROCK2 mRNA expression, along with a potentially activating truncation mutation (I383F-frameshift deletion; TCGA-HZ-8005-01), with poor survival
from the TCGA human dataset, conditional ROCK2 activation was associated with reduced PDAC mouse survival. Conditional ROCK2 activation in non-metastatic PDAC cells isolated from genetically modified mice promoted pancreatic cancer cell invasion into 3D collagen matrices (see schematic representation of ROCK inhibition at the cellular level, Fig. 1A) \(^{25}\).

Interestingly, analyses of cell-ECM interactions using Second Harmonic Generation (SHG) imaging, a label free imaging technique used to detect non-centrosymmetric entities such as crosslinked collagen fibers, or tannic acid-glutaraldehyde fixation of collagen fibers for transmission electron microscopy, revealed that ROCK activation induced extensive remodeling of the collagen matrix surrounding invading cancer cells \(^{25}\).

While ROCK is well known to induce force generation via its action on actomyosin structures \(^{19}\), ROCK signaling also induces gene transcription \(^{32}\). To identify ROCK induced gene expression changes, we performed RNA sequencing and identified 285 genes that were consistently and significantly found to be changed greater than twofold relative to control cells. Interestingly, conditional ROCK activation increased expression of metalloproteinases (MMP) \(Mmp10\) and \(Mmp13\), which was associated with increased release of these MMPs into the surrounding environment (see schematic representation of ROCK inhibition at the cellular level, Fig. 1A). These results indicated that ROCK mediates collagen remodeling by pancreatic cancer cells via transcription, synthesis and release of MMPs, in line with previous observations in melanoma cells \(^{33}\), and in pancreatic cancer cells in which dasatinib-induced reduction of KPC cell migration was correlated with reduced production of MMP2 and MMP9 \(^{34}\). We also
determined that ROCK-mediated remodeling of the surrounding matrix facilitated invasive growth of pancreatic cancer cells (see schematic representation of ROCK inhibition at the cellular and whole-body levels, Fig. 1A, B). These findings highlight the ability of cancer cells to adapt to the mechanical environment and to remodel the ECM to support their aberrant growth. These cell-based observations were further extended in KPC mice, where ROCK inhibition with fasudil significantly prolonged survival, and reduced collagen remodeling (see schematic representation of ROCK inhibition at the cellular and whole-body levels Fig. 1A, B)\(^\text{25}\). Together, these results shed light on novel roles of ROCK in driving pancreatic cancer progression, suggesting that targeting ROCK might be beneficial for the clinical management of the disease.

**Transient ROCK inhibition with fasudil disrupts pancreatic cancer.**

Although ROCK-driven cell contractility and stromal remodeling are known to play crucial roles in cancer progression\(^\text{7,19,35}\), ROCK inhibitors and ECM-based therapies have yet to be translated to the clinic. In our recent publication, we assessed the efficacy of fasudil to impair PDAC progression and to influence cell responses to chemotherapy\(^\text{26}\). Fasudil is a ROCK inhibitor currently used clinically as a monotherapy for the treatment of cerebral vasospasm\(^\text{36}\), and Fasudil has also been shown to inhibit, in a less potent manner than for ROCK, other kinases such as PKA, PKC and MLCK\(^\text{37}\). Meta-analysis of post-marketing surveillance data (>3,000 patients) has demonstrated the safety of fasudil for clinical use in humans\(^\text{38}\), which prompted us to assess the repurposing of fasudil for the treatment of
pancreatic cancer. We combined mouse and stratified patient-derived models of pancreatic cancer with biosensor FLIM-FRET intravital imaging to monitor the effect of ROCK inhibition in real-time and in live tissues \(^{39-42}\). Using an early, transient ‘priming’ regimen, where fasudil was administered for 3 days prior to chemotherapy, in line with its treatment regimen in patients with stable angina \(^{43}\), we demonstrated that short-term ROCK inhibition with fasudil synchronized pancreatic cancer cell cycle progression, and rendered them more sensitive to subsequent treatment with anti-microtubule drugs and standard-of-care chemotherapy, both in primary tumors and metastatic sites (see schematic representation of ROCK inhibition at the whole-body level, Fig. 1B) \(^{26}\). We also observed that ‘priming’ with fasudil in the adjuvant setting disturbed coordinated cancer cell movement and impaired metastatic colonization in the liver (see schematic representation of ROCK inhibition at the whole-body level, Fig. 1B).

Assessment of the effect of ‘priming’ on key metastatic events revealed that ROCK inhibition rendered circulating tumor cells more sensitive to shear stress to which they are subjected in the blood circulation and in turn impaired their ability to extravasate and colonize host tissues (see schematic representation of ROCK inhibition at the whole-body level, Fig. 1B), consistent with previous studies \(^ {44, 45}\). Additionally, analysis of collective cell movement, or streaming, upon ‘priming’ suggested that transient ROCK inhibition impaired coordinated cell migration and 3D cell movement of the metastatic emboli in the liver (see schematic representation of ROCK inhibition at the whole-body level, Fig. 1B) \(^ {26}\), possibly due to disrupted durotaxis - where cell
movement is directed by stiffness gradients - in the metastatic niche. The observed reduction of coordinated PDAC cell spread that we observed upon ROCK inhibition was also in line with previous work highlighting how the Rho-ROCK-LIMK pathway leads tumor cell invasion by driving path generation. ROCK inhibition was also found to reduce the ability of metastatic cells to remodel the host ECM and to create a favorable environment to support their growth in a distant site (see schematic representation of ROCK inhibition at the whole-body level Fig. 1B), as recently demonstrated in pancreatic cancer and melanoma. Assessment of the effects of ‘priming’ with fasudil on the stroma demonstrated that transient ROCK inhibition reduced ECM remodeling and tissue stiffness, thereby altering integrin signaling and depriving cancer cells of mechanical cues provided by the matrix. In addition, decompression of the tumor tissue upon ‘priming’ with fasudil was accompanied by relaxation and increased permeability of the tumor vasculature, as assessed by the imaging of quantum dots diffusing from blood vessels and into tumor tissue (see schematic representation of ROCK inhibition at the whole-body level Fig. 1B and Movie 1). This is in line with the current clinical use of fasudil for the treatment of cerebral vasospasm and with recent work demonstrating that ROCK regulates vascular patency, or obstruction. Our findings therefore demonstrate that fasudil has a dual effect on both the ECM and the intratumoral vasculature, which together increased drug delivery and improved cancer cell responses to chemotherapy. This aligns with recent stromal-based strategies in metastatic colorectal cancer, where the combination of anti-VEGF therapy and anti-hyaluronic acid treatment significantly improved chemotherapy efficacy and prolonged survival
compared to anti-VEGF therapy alone. Our work also indicates that rather than chronic treatment, which has a greater potential for adverse effects and toxicity, acute fasudil treatment to induce transient mechanical ‘priming’ was sufficient to re-equilibrate the pancreatic tumor stroma and to impair PDAC progression. Together, our findings demonstrate that ‘priming’ with fasudil might be beneficial both in the neo-adjuvant and adjuvant settings, which strongly suggests that further clinical assessment of fasudil in combination with standard-of-care chemotherapy, such as Gemcitabine and Abraxane, is warranted to improve PDAC patient outcomes.

Balancing cell contractility: a new approach to treat pancreatic cancer.

While numerous studies have demonstrated that extensive transformation of the pancreatic stroma occurs during cancer development, previous work assessing ECM-based therapies have yielded conflicting data regarding the efficacy of stromal therapies in pancreatic cancer. As such, while pharmacological inhibition of the Hedgehog (Hh) signaling pathway, hyaluronic acid (HA) deposition or lysyl oxidase (LOX) activity resulted in impaired tumor growth and increased survival in mouse models of pancreatic cancer, genetic ablation of Hh signaling or myofibroblasts resulted in decreased survival. Importantly, ablation of fibrosis triggered adverse effects on the pancreatic stroma, such as profound alterations of the immune microenvironment, which in turn promoted cancer progression. Identification of new ECM targets and development of innovative therapeutic regimens to ‘fine-tune’ and manipulate the pancreatic stroma are therefore needed to improve pancreatic cancer patient outcomes. We believe that this
balance is key to future development of stromal targeting strategies for this disease.

Our two recent publications\textsuperscript{25,26} establish ROCK as a key regulator of matrix remodeling in pancreatic cancer, both via generation of contractile force, and regulation of MMP synthesis and release into the surrounding matrix (see schematic representation of ROCK inhibition at the cellular level, Fig. 1A). These findings align with recent work in pancreatic cancer demonstrating that the JAK/ROCK/STAT3 signaling pathway governs cancer cellular tension and promotes tumor progression via remodeling of the surrounding matrix in close proximity to the tumor\textsuperscript{53}. Our observations also highlight the intricate effects of ROCK-induced remodeling of the ECM. While prolonged exposure to fasudil significantly increased mechanical constraints and reduced tumor growth in the KPC model, potentially via reduced release of MMPs into the environment, transient ‘priming’ with fasudil led to reduced ECM crosslinking and relaxation of tumor tissue. This aligns with the emerging concept that the pancreatic stroma can both promote and restrain disease progression\textsuperscript{8,16}. Importantly, our work provides pre-clinical evidence that fine-tuning the ECM via transient ROCK inhibition using our ‘priming’ approach might provide new avenues for the treatment of pancreatic cancer. Potential hypotensive effects of ROCK inhibition with fasudil might be expected given its use for cerebral vasospasm, however the actions on the vasculature that we observe also have the potential beneficial effect of increasing drug delivery. Consistent with recently published work from the Weaver lab, we report no significant change in patient survival associated with bulk tumor stroma\textsuperscript{26,53}, however our study demonstrates a graded response
to the ‘priming’ strategy in patient-derived xenografts that had been stratified based on their ECM signature \(^{26}\). Where in tumors with high ECM content, ‘priming’ with fasudil greatly improved cancer cell responses to chemotherapy, delayed metastasis and approximately doubled survival compared to chemotherapy alone, this had a modest effect in tumors with low ECM content \(^{26}\). This observation suggested that initial collagen content could be used as a surrogate biomarker alone, or because of the dual effects of fasudil ‘priming’ on the ECM and the intratumoral vasculature, in combination with tumor vasculature markers, such as CD31 (cluster of differentiation 31), to identify patients most likely to benefit from transient ROCK inhibition prior to chemotherapy (see schematic representation companion biomarker strategy, Fig. 1C). Additionally, non-invasive PET-reporters of fibrotic tissue are being developed for diagnosis of pulmonary fibrosis, which could be repurposed in this context \(^{54}\). We propose that the repurposing of a low-cost, off-patent drug such as fasudil as a ‘priming’ agent might be beneficial for pancreatic cancer therapy. In addition, novel ROCK inhibitors such as AT13148, KD025 or CCT129254, currently in the clinical testing pipeline as anti-fibrotic agents, or in phase I clinical trial for the treatment of solid tumors (AT13148, NCT01585701 \(^{55}\)) could also have similar applications \(^{56-59}\). Remodeling of the stroma has also been reported to occur in other solid cancers and to influence disease progression \(^{7,48,60,61,62,63}\). Therefore, we envisage that fine-tuning the ECM via ROCK inhibition prior to standard-of-care therapies might lead to substantial therapeutic benefits in additional diseases.

Acknowledgements
Funding was provided from Cancer Research UK to MFO (A18276) and to the Cancer Research UK Beatson Institute (A17196), NHMRC, Cancer Council NSW, Cancer Australia, Tour de Cure grants, Cancer Institute NSW, ARC Future, Lens Ainsworth and Philip Hemstritch Pancreatic Cancer Fellowships, Sydney Catalyst scholarship.

References


Figure and movie legends

Figure 1 Schematic of the roles of ROCK and ROCK inhibition in pancreatic cancer: from cell-to-global effects to translation to patients.

A. ROCK inhibition at the cellular level impairs ECM remodeling via decreased MMP release and impaired contractility. B. ROCK inhibition at the whole body, global level. Schematic representation of the effects of ROCK inhibition in primary tumor tissue (left hand panel), on circulating tumor cells (CTC, middle panel) and at secondary sites (right hand panel). Adapted from (Vennin et al., Science Translational Medicine 2017) \(^26\). Reprinted with permission from AAAS.

C. Combination of ECM and vasculature markers as companion biomarkers for priming strategy. Left hand panel: Schematic representation of in-house automated Second Harmonic Generation (SHG) analysis of the ECM in the ICGC human TMA cohort, with examples of SHG signals in cores (triplicates) from patients with high, medium, or low SHG signal. Right hand panel: representative images of quantum dots and CD31 (cluster of differentiation 31) staining in tumors with high and low vascularity. Adapted from (Vennin et al., Science Translational Medicine 2017) \(^26\). Reprinted with permission from AAAS.

Movie 1: Intravital imaging of quantum dots circulating in tumor associated blood vessels and diffusing into the surrounding tumor tissue. Red: Quantum Dot, Blue: Collagen fibers (SHG signal).
A. ROCK inhibition at the **cellular level**

![Diagram showing ROCK inhibition at the cellular level](image)

**ECM remodeling**
- Release MMP
- Gene expression
- ROCK inhibition
- Actomyosin contractility
- Reduced MMP release
- ROCK inhibition
- Reduced force generation

B. ROCK inhibition at the **whole body level**

1. **Primary pancreatic tumor**
   - ROCK inhibition
   - decreased growth and invasion
   - reduced ECM remodeling
   - enhanced vascularity
   - greater sensitivity to chemotherapy

2. **Metastatic cells in transit (CTC)**
   - ROCK inhibition
   - decreased resistance to shear stress
   - reduced attachment, survival and proliferation post-shear stress

3. **Secondary metastatic site (liver)**
   - ROCK inhibition
   - decreased metastatic burden
   - decreased attachment to host matrix
   - disruption of collective colonization
   - reduced remodeling of host matrix
   - enhanced response to chemotherapy

C. **Companion biomarkers: initial ECM/vasculature markers to predict tailored patient response to ‘priming’ approach**

- **ECM signature**
  - High response
  - Medium response
  - Low response

- **Vasculature signature**
  - High perfusion
  - Low perfusion

- Collagen I (SHG signal) / Tissue autofluorescence
  - High CD31
  - Low CD31