1 **Targeting ROCK activity to disrupt and prime pancreatic cancer for**

2 **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

43 might be beneficial for improving chemotherapeutic efficacy in the treatment

44 of this deadly disease.

45

46 **Introduction**

47   Despite there being a number of new therapeutics that have been

48 developed for pancreatic cancer patient therapy, survival remains the lowest

49 of all solid cancers, with 5-year survival rate being less than 7% and a median

50 survival of 6 months [1]. Despite pre-clinical efforts to develop new therapeutics

51 [2], patient survival has not significantly improved over the last 4 decades,

52 which highlights not only the need to identify new targets, but also to develop

53 innovative treatment strategies to improve the outcomes of patients suffering

54 from this disease. In addition, development of diagnostic tools, for example

55 based on detection of cancer-derived exosomes [3], to enable early detection of

56 pancreatic cancer remains a critical challenge for this disease. Pancreatic

57 ductal adenocarcinoma (PDAC) is characterized by extensive remodeling of

58 the pancreatic stroma, with increased deposition and crosslinking of

59 extracellular matrix (ECM) components and poor vascularization compared to

60 normal pancreas[4, 5]. Alterations of the biochemical and mechanical properties

61 of the ECM are known to influence cancer progression, invasion and

62 responses to chemotherapy [6-9], however, recent studies assessing the

63 efficacy of ECM-based pancreatic cancer therapies, for example via inhibition

64 of Sonic Hedgehog signaling pathway, targeting of lysyl oxidase activity or

65 inhibition of hyaluronic acid (HA), have yielded conflicting results [4, 10-16].

66   Rho-associated protein kinases 1 and 2 (ROCK1 and ROCK2) are

67 master regulators of the actomyosin cytoskeleton and govern force

68 generation, cell invasion, proliferation and contractility [17-19]. Numerous studies

69 have established that ROCK inhibition disrupts tumor progression and

70 metastasis in cell based and *in vivo* models of various solid cancers [20-23].

71 However, to date no compounds have progressed into the clinic for cancer

72 therapy for several reasons. The development of anti-metastatic

73 chemotherapeutics for clinical use is very challenging due to the need to

74 detect a reduction in metastasis in patients over sustained periods (likely

75 years) as a positive outcome [24], in contrast to chemotherapeutics that induce

76 acute positive responses, such as tumor regression, which can be monitored

77 in a clinical trial in a defined and relatively brief time period [24]. Furthermore,

78 the absence of correlations between defined genetic alterations, such as

79 *ROCK1* or *ROCK2* mutations, with ROCK inhibitor sensitivity means that

80 there is no simple genetic test for convenient patient stratification. As a result,

81 ROCK inhibition has not been adopted as a cancer chemotherapy. In this

82 commentary, we describe our recent findings [25, 26] demonstrating that ROCK

83 activity promotes pancreatic cancer invasive growth via ECM remodeling. We

84 also highlight how transient ROCK inhibition, or mechanical 'priming' with the

85 pharmacological inhibitor fasudil affects tumor tissue tension, which in turn

86 improves chemotherapy efficacy in primary and secondary tumor sites, while

87 also disrupting collective movement of metastatic cancer cells [26]. Lastly, we

88 discuss potential translation of our findings into the clinic for pancreatic cancer

89 therapy, where balancing cellular contractility via transient ROCK inhibition,

90 rather than long-term ablation of the matrix, enables re-establishment of the

91 normal mechanical features of the stroma.

92

93 **ROCK activity promotes PDAC progression.**

94   Genomic analyses have previously shown that the ROCK1 gene is

95 amplified in 15% of pancreatic patient tumors [27], however the role of ROCK-

96 mediated actomyosin contractility in PDAC had not been clearly established.

97 To address this, we assessed ROCK expression in a patient tissue microarray

98 (78 samples from patients with pancreatic cancers and 5 healthy human

99 pancreas) and in human TCGA datasets, and determined that ROCK1 and

100 ROCK2 expression increase with tumor stage and grade [25]. In line with this,

101 genomic alterations or mRNA amplification of *ROCK1* and/or *ROCK2* were

102 found to be positively correlated with poorer survival, suggesting that ROCK

103 signaling promotes pancreatic cancer progression [25].

104   To further understand how ROCK influences the fate and behavior of

105 pancreatic cancer cells, Cre-recombinase was expressed from the pancreatic

106 epithelial selective Pdx1 promoter to induce pancreas-targeted recombination

107 of *LOX-STOP-LOX (LSL)-Kras$^{G12D/+}$ and LSL-Trp53$^{R172H/+}$* (KPC) alleles in

108 mice, which spontaneously develop PDAC that closely resembles human

109 pancreatic cancer [28, 29]. In addition, KPC mice were crossed with *LSL-*

110 *ROCK2:ER* mice [30] to conditionally activate ROCK2 during PDAC

111 progression. This model closely recapitulates the genomic features of human

112 PDAC, where an initiating Kras^{G12D} mutation is found in almost 90% of patient

113 tumors, while the p53^{R175H} mutation is found in 50-75% of patient tumors [31].

114 Consistent with the observed increased ROCK2 protein levels in advanced

115 PDAC stages, as well as the correlation between increased *ROCK1* and

116 *ROCK2* mRNA expression, along with a potentially activating truncation

117 mutation (I383F-frameshift deletion; TCGA-HZ-8005-01), with poor survival

118 from the TCGA human dataset, conditional ROCK2 activation was associated

119 with reduced PDAC mouse survival. Conditional ROCK2 activation in non

120 metastatic PDAC cells isolated from genetically modified mice promoted

121 pancreatic cancer cell invasion into 3D collagen matrices (see schematic

122 representation of ROCK inhibition at the cellular level, Fig. 1A) [25].

123 Interestingly, analyses of cell-ECM interactions using Second Harmonic

124 Generation (SHG) imaging, a label free imaging technique used to detect non-

125 centrosymmetric entities such as crosslinked collagen fibers, or tannic acid-

126 glutaraldehyde fixation of collagen fibers for transmission electron

127 microscopy, revealed that ROCK activation induced extensive remodeling of

128 the collagen matrix surrounding invading cancer cells [25].

129   While ROCK is well known to induce force generation via its action on

130 actomyosin structures [19], ROCK signaling also induces gene transcription [32].

131 To identify ROCK induced gene expression changes, we performed RNA

132 sequencing and identified 285 genes that were consistently and significantly

133 found to be changed greater than twofold relative to control cells.

134 Interestingly, conditional ROCK activation increased expression of

135 metalloproteinases (MMP) *Mmp10* and *Mmp13*, which was associated with

136 increased release of these MMPs into the surrounding environment (see

137 schematic representation of ROCK inhibition at the cellular level, Fig. 1A).

138 These results indicated that ROCK mediates collagen remodeling by

139 pancreatic cancer cells via transcription, synthesis and release of MMPs, in

140 line with previous observations in melanoma cells [33], and in pancreatic cancer

141 cells in which dasatinib-induced reduction of KPC cell migration was

142 correlated with reduced production of MMP2 and MMP9 [34]. We also

143 determined that ROCK-mediated remodeling of the surrounding matrix

144 facilitated invasive growth of pancreatic cancer cells (see schematic

145 representation of ROCK inhibition at the cellular and whole-body levels, Fig.

146 1A, B). These findings highlight the ability of cancer cells to adapt to the

147 mechanical environment and to remodel the ECM to support their aberrant

148 growth. These cell-based observations were further extended in KPC mice,

149 where ROCK inhibition with fasudil significantly prolonged survival, and

150 reduced collagen remodeling (see schematic representation of ROCK

151 inhibition at the cellular and whole-body levels Fig. 1A, B) [25]. Together, these

152 results shed light on novel roles of ROCK in driving pancreatic cancer

153 progression, suggesting that targeting ROCK might be beneficial for the

154 clinical management of the disease.

155

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

157   Although ROCK-driven cell contractility and stromal remodeling are

158 known to play crucial roles in cancer progression [7, 19, 35], ROCK inhibitors and

159 ECM-based therapies have yet to be translated to the clinic. In our recent

160 publication, we assessed the efficacy of fasudil to impair PDAC progression

161 and to influence cell responses to chemotherapy [26]. Fasudil is a ROCK

162 inhibitor currently used clinically as a monotherapy for the treatment of

163 cerebral vasospasm [36], and Fasudil has also been shown to inhibit, in a less

164 potent manner than for ROCK, other kinases such as PKA, PKC and MLCK

165 [37]. Meta-analysis of post-marketing surveillance data (>3,000 patients) has

166 demonstrated the safety of fasudil for clinical use in humans [38], which

167 prompted us to assess the repurposing of fasudil for the treatment of

168 pancreatic cancer. We combined mouse and stratified patient-derived models

169 of pancreatic cancer with biosensor FLIM-FRET intravital imaging to monitor

170 the effect of ROCK inhibition in real-time and in live tissues [39-42]. Using an

171 early, transient 'priming' regimen, where fasudil was administered for 3 days

172 prior to chemotherapy, in line with its treatment regimen in patients with stable

173 angina [43], we demonstrated that short-term ROCK inhibition with fasudil

174 synchronized pancreatic cancer cell cycle progression, and rendered them

175 more sensitive to subsequent treatment with anti-microtubule drugs and

176 standard-of-care chemotherapy, both in primary tumors and metastatic sites

177 (see schematic representation of ROCK inhibition at the whole-body level,

178 Fig. 1B) [26]. We also observed that 'priming' with fasudil in the adjuvant setting

179 disturbed coordinated cancer cell movement and impaired metastatic

180 colonization in the liver (see schematic representation of ROCK inhibition at

181 the whole-body level, Fig. 1B).

182

183   Assessment of the effect of 'priming' on key metastatic events revealed

184 that ROCK inhibition rendered circulating tumor cells more sensitive to shear

185 stress to which they are subjected in the blood circulation and in turn impaired

186 their ability to extravasate and colonize host tissues (see schematic

187 representation of ROCK inhibition at the whole-body level, Fig. 1B), consistent

188 with previous studies [44, 45]. Additionally, analysis of collective cell movement,

189 or streaming, upon 'priming' suggested that transient ROCK inhibition

190 impaired coordinated cell migration and 3D cell movement of the metastatic

191 emboli in the liver (see schematic representation of ROCK inhibition at the

192 whole-body level, Fig. 1B) [26], possibly due to disrupted durotaxis - where cell

193   movement is directed by stiffness gradients - in the metastatic niche [46]. The

194   observed reduction of coordinated PDAC cell spread that we observed upon

195   ROCK inhibition was also in line with previous work highlighting how the Rho-

196   ROCK-LIMK pathway leads tumor cell invasion by driving path generation [47].

197   ROCK inhibition was also found to reduce the ability of metastatic cells to

198   remodel the host ECM and to create a favorable environment to support their

199   growth in a distant site (see schematic representation of ROCK inhibition at

200   the whole-body level Fig. 1B), as recently demonstrated in pancreatic cancer

201   and melanoma [48-50]. Assessment of the effects of 'priming' with fasudil on the

202   stroma demonstrated that transient ROCK inhibition reduced ECM remodeling

203   and tissue stiffness, thereby altering integrin signaling and depriving cancer

204   cells of mechanical cues provided by the matrix [26]. In addition, decompression

205   of the tumor tissue upon 'priming' with fasudil was accompanied by relaxation

206   and increased permeability of the tumor vasculature, as assessed by the

207   imaging of quantum dots diffusing from blood vessels and into tumor tissue

208   (see schematic representation of ROCK inhibition at the whole-body level Fig.

209   1B and Movie 1) [26]. This is in line with the current clinical use of fasudil for the

210   treatment of cerebral vasospasm [36, 43] and with recent work demonstrating

211   that ROCK regulates vascular patency, or obstruction [51]. Our findings

212   therefore demonstrate that fasudil has a dual effect on both the ECM and the

213   intratumoral vasculature, which together increased drug delivery and

214   improved cancer cell responses to chemotherapy. This aligns with recent

215   stromal-based strategies in metastatic colorectal cancer, where the

216   combination of anti-VEGF therapy and anti-hyaluronic acid treatment

217   significantly improved chemotherapy efficacy and prolonged survival

218 compared to anti-VEGF therapy alone [52]. Our work also indicates that rather

219 than chronic treatment, which has a greater potential for adverse effects and

220 toxicity [11, 14], acute fasudil treatment to induce transient mechanical 'priming'

221 was sufficient to re-equilibrate the pancreatic tumor stroma and to impair

222 PDAC progression. Together, our findings demonstrate that 'priming' with

223 fasudil might be beneficial both in the neo-adjuvant and adjuvant settings,

224 which strongly suggests that further clinical assessment of fasudil in

225 combination with standard-of-care chemotherapy, such as Gemcitabine and

226 Abraxane, is warranted to improve PDAC patient outcomes.

227

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

229 While numerous studies have demonstrated that extensive

230 transformation of the pancreatic stroma occurs during cancer development [5,

231 53], previous work assessing ECM-based therapies have yielded conflicting

232 data regarding the efficacy of stromal therapies in pancreatic cancer. As such,

233 while pharmacological inhibition of the Hedgehog (Hh) signaling pathway [4],

234 hyaluronic acid (HA) deposition [13, 15] or lysyl oxidase (LOX) activity [12] resulted

235 in impaired tumor growth and increased survival in mouse models of

236 pancreatic cancer, genetic ablation of Hh signaling [14] or myofibroblasts [11]

237 resulted in decreased survival. Importantly, ablation of fibrosis triggered

238 adverse effects on the pancreatic stroma, such as profound alterations of the

239 immune microenvironment, which in turn promoted cancer progression [11, 14].

240 Identification of new ECM targets and development of innovative therapeutic

241 regimens to 'fine-tune' and manipulate the pancreatic stroma are therefore

242 needed to improve pancreatic cancer patient outcomes. We believe that this

243 balance is key to future development of stromal targeting strategies for this

244 disease.

245 Our two recent publications [25, 26] establish ROCK as a key regulator of

246 matrix remodeling in pancreatic cancer, both via generation of contractile

247 force, and regulation of MMP synthesis and release into the surrounding

248 matrix (see schematic representation of ROCK inhibition at the cellular level,

249 Fig. 1A). These findings align with recent work in pancreatic cancer

250 demonstrating that the JAK/ROCK/STAT3 signaling pathway governs cancer

251 cellular tension and promotes tumor progression via remodeling of the

252 surrounding matrix in close proximity to the tumor [53]. Our observations also

253 highlight the intricate effects of ROCK-induced remodeling of the ECM. While

254 prolonged exposure to fasudil significantly increased mechanical constraints

255 and reduced tumor growth in the KPC model, potentially via reduced release

256 of MMPs into the environment, transient 'priming' with fasudil led to reduced

257 ECM crosslinking and relaxation of tumor tissue. This aligns with the

258 emerging concept that the pancreatic stroma can both promote and restrain

259 disease progression [8, 16]. Importantly, our work provides pre-clinical evidence

260 that fine-tuning the ECM via transient ROCK inhibition using our 'priming'

261 approach might provide new avenues for the treatment of pancreatic cancer.

262 Potential hypotensive effects of ROCK inhibition with fasudil might be

263 expected given its use for cerebral vasospasm, however the actions on the

264 vasculature that we observe also have the potential beneficial effect of

265 increasing drug delivery. Consistent with recently published work from the

266 Weaver lab, we report no significant change in patient survival associated with

267 bulk tumor stroma [26, 53], however our study demonstrates a graded response

268   to the 'priming' strategy in patient-derived xenografts that had been stratified

269   based on their ECM signature [26]. Where in tumors with high ECM content,

270   'priming' with fasudil greatly improved cancer cell responses to chemotherapy,

271   delayed metastasis and approximately doubled survival compared to

272   chemotherapy alone, this had a modest effect in tumors with low ECM content

273   [26]. This observation suggested that initial collagen content could be used as a

274   surrogate biomarker alone, or because of the dual effects of fasudil 'priming'

275   on the ECM and the intratumoral vasculature, in combination with tumor

276   vasculature markers, such as CD31 (cluster of differentiation 31), to identify

277   patients most likely to benefit from transient ROCK inhibition prior to

278   chemotherapy (see schematic representation companion biomarker strategy,

279   Fig. 1C). Additionally, non-invasive PET-reporters of fibrotic tissue are being

280   developed for diagnosis of pulmonary fibrosis, which could be repurposed in

281   this context [54]. We propose that the repurposing of a low-cost, off-patent drug

282   such as fasudil as a 'priming' agent might be beneficial for pancreatic cancer

283   therapy. In addition, novel ROCK inhibitors such as AT13148, KD025 or

284   CCT129254, currently in the clinical testing pipeline as anti-fibrotic agents, or

285   in phase I clinical trial for the treatment of solid tumors (AT13148,

286   NCT01585701 [55]) could also have similar applications [56-59]. Remodeling of the

287   stroma has also been reported to occur in other solid cancers and to influence

288   disease progression [7, 48, 60, 61 62 63]. Therefore, we envisage that fine-tuning the

289   ECM via ROCK inhibition prior to standard-of-care therapies might lead to

290   substantial therapeutic benefits in additional diseases.

291

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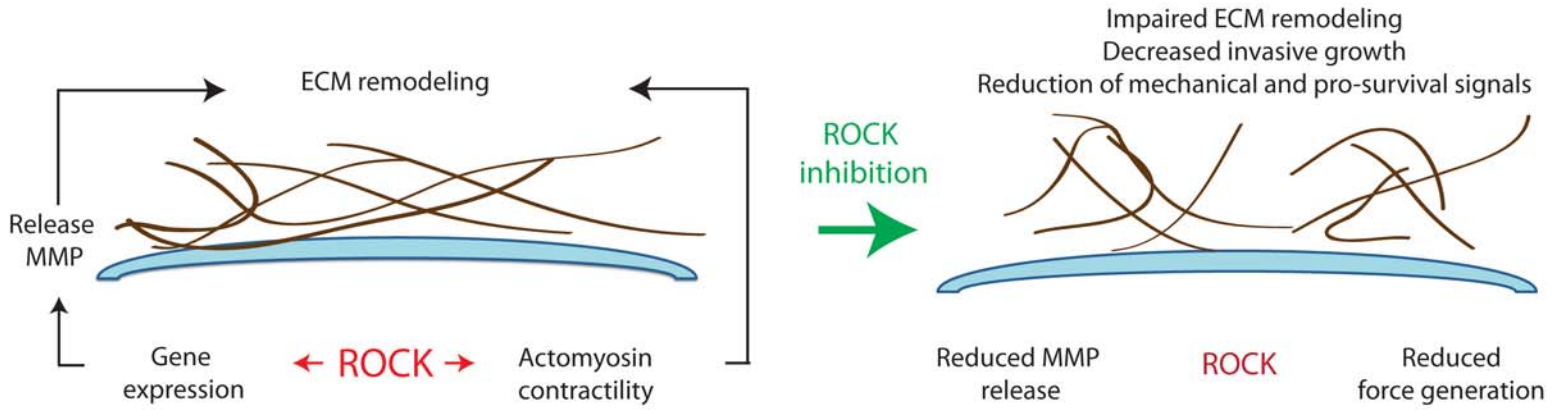486
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Figure and movie legends

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**

ECM remodeling

Release MMP

Gene expression ← ROCK → Actomyosin contractility

ROCK inhibition

Impaired ECM remodeling
Decreased invasive growth
Reduction of mechanical and pro-survival signals

Reduced MMP release   ROCK   Reduced force generation

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

(i) Primary pancreatic tumor

ROCK inhibition
-decreased growth and invasion
-reduced ECM remodeling
-enhanced vascularity
-greater sensitivity to chemotherapy

(iii) 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
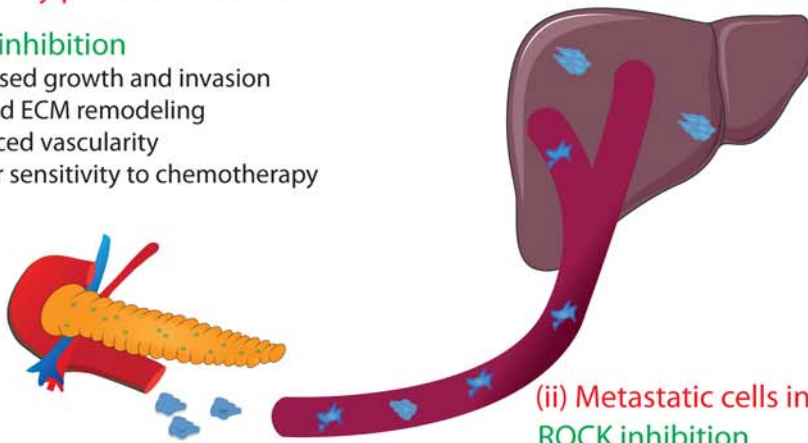
(ii) Metastatic cells in transit (CTC)

ROCK inhibition
-decreased resistance to shear stress
-reduced attachment, survival and proliferation post-shear stress

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

ECM signature

High response

Medium response

Low response

SHG intensity

Patient

Graded response

Collagen I (SHG signal) / Tissue autofluorescence

Vasculature signature

High perfusion   High CD31

Low perfusion   Low CD31