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The dynamics of Rho GTPase signaling and implications for targeting cancer and the tumor microenvironment

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The dynamics of Rho GTPase signaling and implications for targeting cancer and the tumor microenvironment

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Numerous large scale genomics studies have demonstrated that cancer is a molecularly heterogeneous disease, characterized by acquired changes in the structure and DNA sequence of tumor genomes. More recently, the role of the equally complex tumor microenvironment in driving the aggressiveness of this disease is increasingly being realized. Tumor cells are surrounded by activated stroma, creating a dynamic environment that promotes cancer development, metastasis and chemoresistance. The Rho family of small GTPases plays an essential role in the regulation of cell shape, cytokinesis, cell adhesion, and cell motility. Importantly, these processes need to be considered in the context of a complex 3-dimensional (3D) environment, with reciprocal feedback and cross-talk taking place between the tumor cells and host environment. Here we discuss the role of molecular networks involving Rho GTPases in cancer, and the therapeutic implications of inhibiting Rho signaling in both cancer cells and the emerging concept of targeting the surrounding stroma.

Introduction

Over the last 2 decades, a deeper understanding of the genetic and molecular basis of cancer has led to new classes of therapies to selectively target the molecular mechanisms that affect survival and proliferation of cancer cells. Large-scale genomics efforts are providing new opportunities to improve current approaches to cancer therapy.^{1–3} However, contribution of the equally complex and dynamic tumor microenvironment to therapeutic resistance is increasingly being realized. For example, stromal cells have previously been shown to directly confer chemoresistance to a variety

of agents, particularly influencing the response to targeted therapies.⁴ In several solid cancer types,^{5,6} including those of the prostate, pancreas and ovary, a high proportion of the total tumor mass consists of activated (myo)fibroblasts, lymphatic and vascular endothelial cells, immune cells and extracellular matrix (ECM), all of which make up the tumor stromal environment. This dynamic tumor microenvironment promotes cancer initiation, progression, metastasis and chemoresistance, creating a substantial barrier to reducing the morbidity and mortality that is attributable to aggressive malignancies. At the same time, it has created new avenues for the development and testing of novel therapeutic strategies, by targeting cellular mechanisms contributed by the tumor microenvironment.

Rho GTPases comprise a branch of the Ras super family, encompassing 22 genes in humans, of which RhoA, Rac1 and Cdc42 are the best characterized. They play essential roles in a number of biological processes, especially in the regulation of cell morphology, cytokinesis, cell adhesion, and cell migration. Alternation between their GDP-bound (inactive) and GTP-bound (active) forms is a tightly regulated process. GTPase activating proteins (GAPs) stimulate GTP hydrolysis and inactivation, while the guanine nucleotide exchange factors (GEFs) facilitate GDP dissociation and activate downstream pathways through effector proteins.⁷

Activation of Rho GTPases results in binding to downstream effector proteins, for example the Rho-associated protein kinase ROCK, and interaction with various well characterized pathways, including the PI3K, FAK, Src, LIMK, MEK/Erk protein networks,^{8–12} leading to actin cytoskeleton remodeling and increased cell motility. These key processes are also critical during cell cycle progression and mitosis, including the process of cell rounding at mitosis onset, during chromosomal alignment and the contraction of the actomyosin ring that separates daughter cells at cytokinesis, all of which are tightly regulated by the Rho family of GTPases in a cell type specific manner.^{13–15}

The effect of Rho/ROCK activation on myosin-light chain (MLC) phosphorylation in smooth muscle and its involvement in the maintenance of aspects of stromal feedback such as vascular tone,^{16–18} as well as on ECM deposition, has generated considerable interest in the use of Rho pathway inhibition to treat cardiovascular disease,¹⁷ but also for the treatment of stroke,¹⁹ inflammatory conditions,²⁰ Alzheimer's disease^{21,22} and

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neuropathic pain.²³⁻²⁵ Specifically, several studies have shown improved recovery from spinal cord injury in rat models of the disease following treatment with the ROCK inhibitors fasudil and Y-27632.^{23,24,26} Regulation of mechanical and contractile properties of the pressure-sensitive smooth muscle cells is recognized to play a significant role in blood pressure homeostasis and regulation of vascular tone.¹⁸ Interestingly, due to its vasodilatory effect, fasudil is clinically used for the treatment of cerebral vasospasm.²⁷ Y-27632 is a pre-clinical molecular tool, with newer selective RhoA/ROCK inhibitors also in development, including H-1152 and aminofurazan compounds.^{28,29} The potential for the application of downstream Rho signaling inhibitors from the pathologies outlined above to target the ECM, vascular alterations as well as tumor-specific changes in a variety of cancer types and therefore, re-purposing as cancer therapy, will be discussed in this review.

Rho GTPase Signaling in Cancer

To promote transformation, cancer development, invasion and metastasis, tumor cells frequently hijack the multilayered and dynamic regulation of Rho GTPase activity that is required for coordinated cell migration under physiological conditions.³⁰ Increased levels of RhoA, RhoB, RhoC, Rac1, Cdc42 and ROCK, have been found in late-stage tumors and metastases³¹⁻³⁵ with prognostic relevance in breast cancer.³² Interestingly, the Rho-GAP Dlc1 was found to regulate the metastatic colonization of circulating breast cancer cells in bone, but not lungs of the MDA-MB-231 orthotopic in vivo model of breast cancer.³⁶ In the same study, this organ-specific metastatic phenotype was further attributed to the Dlc1-Rho regulation of the response of cancer cells to TGF- β stimulation from the bone stroma and the subsequent remodeling of the osteolytic microenvironment for metastatic colonization. Moreover, Dlc1 suppressed the formation of bone but not lung metastasis by inhibiting TGF- β -induced bone degradation via PTHLH,³⁶ a critical regulator of osteoclastogenesis.³⁷ Hence, targeting the Rho-ROCK signaling axis in this molecular setting could provide a more effective approach for the treatment of breast-to-bone-metastasis than by directly inhibiting TGF- β and importantly, specific tumor suppressor functions of this protein.³⁸

Increased expression of Rho/ROCK proteins has also been detected in pancreatic cancer,³⁹ testicular germ cell tumors,⁴⁰ squamous cell carcinomas⁴¹ and several other cancer types.⁴²⁻⁴⁴ Constitutive overexpression of RhoA in tumor cells leads to increased translocation of this protein to the cell membrane, where it is activated and causes increased tumor invasion.⁴⁵ Similarly, RhoC facilitates tumor cell invasion and promotes metastasis in breast and pancreatic cancer.^{34,35} Overexpression of Cdc42 in a tetracycline-inducible MMTV-driven mouse model was found to disrupt mammary gland branching morphogenesis by changes in Rho GTPase and MAPK signaling, leading to increased contractility and migration in association with further stromal alterations, including elevated ECM deposition.⁴⁶

A delicate balance between Rac and Rho signaling governs the diversity of tumor cell invasion mechanisms (reviewed in⁴⁷). For example, in Matrigel invasion assays RhoA/ROCK activity promotes the amoeboid motility of rounded cells, while Rac regulates migration of elongated cells that depend on the proteolysis and remodeling of the stromal ECM.⁴⁸ Cancer cells can readily switch between these 2 modes and importantly, with combined inhibition of proteolysis and Rho/ROCK signaling, the switch was effectively impaired and cancer cell invasion was inhibited.⁴⁸ Similarly, only dual inhibition of the Rac1 and RhoA signaling axes significantly decreased invasive potential of fibrosarcoma cells in 3D matrices⁴⁹ or in vivo tumor growth of selected orthotopic xenografts of pancreatic cancer.⁵⁰ However, this observation appears to be context- and or tumor type-dependent. For example, a marked decrease of active Rac1 and Cdc42 correlated with the high invasive potential of tumor cell lines isolated from metastatic sites of colorectal adenocarcinoma.⁵¹ Moreover, the combined activation of Rac1/Cdc42 signaling and inhibition of ROCK in this model, with PDGF and Y-27632 treatment, significantly decreased the invasive potential of colorectal cancer cells and this effect was accompanied by the re-establishment of E-cadherin-dependent adherens junctions.⁵¹

In addition to their role as master regulators of cellular processes that contribute to cell motility, including protrusion formation, adhesion remodeling, and contractility, Rho GTPases have also been implicated in the regulation of the G₁ cell cycle checkpoint activation,⁵² malignant transformation,⁵³⁻⁵⁵ tumor angiogenesis,⁵⁶ chemoresistance^{57,58} and inflammation.^{59,60} Epithelial-mesenchymal transition (EMT), which is characterized by the transformation of tumor cells from an epithelial to a mesenchymal phenotype correlates with the acquisition of invasive and metastatic properties in cancer. Through interaction with key components of the aberrantly activated Wnt/ β -catenin signaling pathway, Rho GTPases have also been shown to coordinate activation of specific receptor tyrosine kinases and promote EMT in several cancer types.⁶¹⁻⁶³ Key aspects of how this family of proteins regulate cancer development and progression have recently been reviewed.⁶⁴

Rho GTPase Mutations in Cancer: Implications for Therapeutic Targeting

Historically, large scale sequencing efforts have revealed that mutations in the Rho GTPase family are rare, where generally aberrant activation of this pathway occurs through overexpression of Rho GTPases or by changes in the levels of regulators of Rho activity, including increased activation of GEFs⁶⁵⁻⁶⁸ and inactivation or loss of GAPs^{69,70} or GDIs.⁷¹⁻⁷³ However, increasing evidence indicates that certain cancers harbor significant genomic aberrations in this complex signaling network. For example, an early sequencing study of B-cell diffuse large-cell lymphomas (DLCL) has demonstrated that a significant proportion (46%) of tumors carried mutations in the *RHOH/TTF* gene, which encodes a small GTP-binding protein of the RAS superfamily.⁷⁴ The identified sequence variants included largely single base

substitutions and were scattered throughout the first 1.6 kb of the *RHOH/TTF* gene, within non-coding sequences, thus suggesting a potential effect on the regulation of *RHOH/TTF* gene expression in subtypes of DLCL.⁷⁴ Recurrent chromosomal alterations of the *RHOH/TTF* gene at band 4p13 have also been detected in non-Hodgkins lymphoma and multiple myeloma.⁷⁵

Most recently, 2 independent exome and transcriptome sequencing studies have revealed a frequent somatic mutation in the *RHOA* gene (p.Gly17Val) which occurs in 53–68% of angioimmunoblastic T cell lymphomas (AITL).^{76,77} Yoo et al.⁷⁷ further showed that this mutation was specific to T cell lymphoma and was absent from B cell lymphoma. Importantly, these seminal works were the first to demonstrate that the *RHOA* p. Gly17Val substitution in the GTP-binding domain leads to dramatically reduced GTP and GTP γ S binding, impaired RhoA function, contributing to AITL-specific pathogenesis.^{76,77} Since AITL is a common subtype of T cell lymphoma and a disease with very poor prognosis and 5-year overall survival of only 33%,⁷⁸ future studies on the detailed molecular characterization of the *RHOA* p. Gly17Val mutation may hold important implications for the development of novel, clinically useful diagnostic biomarkers and therapeutic targets.

In contrast with haematological malignancies, the contribution of genomic aberrations in Rho GTPase family members to carcinogenesis and disease progression in solid cancers is less understood. Interestingly, a recent comprehensive molecular characterization of 295 primary gastric adenocarcinomas as part of The Cancer Genome Atlas (TCGA) project revealed mutations in *RHOA* gene in 5.5% of gastric tumors.⁷⁹ *RHOA* mutations were enriched in a specific subtype of gastric cancer, preferentially occurring in cases classified as genomically stable and appeared to cluster in 2 adjacent amino-terminal regions of RhoA that are predicted to be at the interface of RhoA with ROCK1 and other effectors, thus potentially modulating downstream signaling.⁷⁹

Another recent study on the mutational landscape in melanoma has identified a recurrent activating mutation in the Rho GTPase *RAC1* in 9.2% of sun-exposed melanomas.⁸⁰ This somatic missense mutation leads to a change from proline 29 to serine in the highly conserved switch I domain of Rac1, causing increased activation of downstream signaling, melanocyte proliferation and migration.^{80,81} In parallel, Hodis et al.⁸² have identified the *RAC1* (P29S) mutation as the most frequent hot spot mutation after those in BRAF and NRAS in an independent melanoma patient cohort. In addition, mutations in homologous residues in *RAC2* (P29L) and *RHOT1* (P30L) were also detected, albeit at very low frequency (0.8%), suggesting the importance of the P29 residue as a possible codon targeted by hot spot mutations in Rho family GTPases.⁸² A well-characterized RAS family-activating mutation (G12D) in the *CDC42* gene was also identified in a melanoma patient.⁸² Moreover, Matos et al.⁸³ have shown that Rac1b, a hyperactive splice variant of the *RAC1* gene, and B-Raf(V600E) mutation functionally cooperate to sustain colorectal tumor cell viability, suggesting an alternative survival pathway to oncogenic K-Ras in these tumors. A personalized treatment strategy using pharmacological inhibition of Rac1

signaling in tumor subtypes carrying these aberrations could be beneficial and remains to be examined. Importantly, as integration of molecular data, including DNA copy-number alteration, mRNA and protein, metabolomic and clinical information, becomes routine research practice, delineating the extent of the deregulation of Rho GTPases in cancer will pave the way for the more accurate and rapid implementation of the inhibitors of Rho signaling as personalized cancer therapeutics.

Dynamics of Rho GTPase Signaling in Living Systems

The application of Förster resonance energy transfer (FRET) imaging for the study of molecular dynamics in living cells has dramatically improved our current understanding of the spatio-temporal regulation of Rho activation.^{13,84–87} Comprehensive studies have demonstrated that different extracellular cues induce distinct patterns of RhoA/Rac1 signaling during membrane protrusion in migrating Mouse Embryo Fibroblast (MEF) cultures in vitro.^{84–86} Although the mechanisms regulating single-cell migration and Rho activation in vitro are relatively well understood, it is necessary to understand the intricacy of Rho signaling in live tissue. Consequently, key studies using FRET have been performed in various multicellular organisms, increasing in complexity from the transparent *Drosophila*^{88,89} and Zebrafish models^{90,91} to technically challenging in vivo mouse imaging.^{87,92,93} For example, the GEF Vav was identified as a key regulator of Rac1 activity during guided cell morphogenetic movements in the developing *Drosophila* embryo.⁸⁸ Further, in vivo FRET has also revealed the requirement for Rac1 and RhoA activity in the regulation of chemokine-guided germ cell motility within Zebrafish embryos, where Rac1 was essential for the formation of actin-rich structures, with RhoA promoting retrograde actin flow.⁹⁰ Although these studies have collectively revealed new detail regarding the synchronized and coordinated role of Rho GTPases in fundamental developmental and disease processes, Rho signaling should also be examined in a mammalian 3-dimensional (3D) tissue environment. For example, in cancer, it is well established that continuous and reciprocal cross-talk takes place between the tumor cells and the host environment.

Using FRET imaging in a live animal model of invasive pancreatic ductal adenocarcinoma (PDAC, *Pdx1-Cre*, *LSL-KRas^{G12D/+}*, *LSL-Trp53^{R172H/+}*; KPC model) we have previously identified distinct sub-cellular patterns of RhoA activity during tumor invasion.⁸⁷ Moreover, therapeutic intervention with dasatinib, an anti-invasive agent that inhibits c-Src tyrosine kinase, specifically inactivated RhoA activity at the poles of the invading cells, providing a new level of detail regarding the regulation of RhoA during cell-ECM interactions in cancer.^{94,95} It is also plausible that the spatiotemporal regulation of other Rho GTPases will similarly be tightly controlled.⁸⁷ For example, the relative balance of Rac1, Cdc42 and RhoA activities was found to directly affect the invasiveness of glioblastoma cells at perivascular and intraparenchymal regions of the brain in a C6 allograft glioblastoma model.⁹²

Using the K14-ROCK:ER genetically engineered mouse (GEM), it was also recently shown that conditional activation of Rho/ROCK in the mouse skin led to increased ECM deposition, stromal tissue stiffness and promoted tumor growth and progression in vivo.⁴¹ Moreover, combination of an agent that breaks down ECM, a key component of the desmoplastic stroma in PDAC, with the standard chemotherapeutic, gemcitabine, led to remodeling of the tumor microenvironment and objective responses in tumor-bearing KPC mice, resulting in a near doubling of overall survival and decreased metastatic burden in this aggressive disease model.⁹⁶⁻⁹⁸ Hence, combining Rho inhibition with agents that improve tissue permeability through stromal ECM degradation^{97,98} may increase the therapeutic benefit of Rho GTPase targeting in cancer. The relative contribution of the tumor cells and/or their microenvironment to these multilayered processes is yet to be elucidated. We envisage that these complex networks will be best examined using novel genetically engineered FRET biosensor mouse models^{93,99-101} which will enable direct imaging of the dynamic regulation of Rho activity in native live tissues. To this end, we have recently developed a Rac-FRET GEM model that ubiquitously expresses the Raichu-Rac FRET biosensor at low level under the control of the ROSA26 promoter.⁹³ This mouse model has enabled detailed quantification of the spatiotemporal activity of Rac in living primary mammalian cells and tissues. Using this system, we have observed exquisite regulation of Rac activity in primary neutrophils, described best by transient and locally restricted bursts of Rac activity, which coincided with lamellipodial protrusions during neutrophil chemotaxis, highlighted in **Figure 1A and B**. Crossing the Rac-FRET mouse to various disease models has also proven its potential utility for assessing the effects of oncogenic mutations involved in carcinogenesis and tumor progression on Rac activity in a time- and tissue-specific manner, lending this model to the examination of drug targeting.¹⁰⁰ Recently, we have also shown that Rac1 facilitates colorectal tumorigenesis following the loss of the Adenomatous Polyposis Coli (*APC*) gene.¹⁰²

By crossing the Rac-FRET mouse strain to the Vil-Cre-ER^{T2} APC^{fl/fl} model of colorectal cancer, we subsequently characterized the spatiotemporal Rac activity in the intestinal stem and progenitor cells of the established Vil-Cre-ER^{T2} APC^{fl/fl} Rac-FRET mice.⁹³ In vivo FRET imaging revealed, for the first time, increased Rac activity at the base of the intestinal crypts following APC loss, demonstrating a critical role for Rac activation during disease initiation (illustrated in **Fig. 1C and D**). We have also generated a KPC PDAC Rac-FRET model and the breast cancer PyMT Rac-FRET mouse to analyze the dynamics of tumor-specific Rac signaling following administration of a targeted therapeutic agent, specifically the Rac inhibitor NSC23766.⁹³ Possible future applications could involve crossing the Rac-FRET strain with Lgr5-Cre mice¹⁰³ to examine Rac activity in the stem cells of the small intestine, providing fundamental insight in normal tissue homeostasis/development. Finally, the intricate control of the in vivo distribution, function, as well as potential redundancy of the numerous upstream and downstream regulators of Rho GTPase activity could be examined by crossing the biosensor mice onto GEF/GAP knockout strains¹⁰⁴

or knockout mice of key downstream effectors, for example the Rho kinases.^{105,106} Collectively, current evidence emphasizes the general utility of novel GEM biosensor models as a tool for monitoring the complex mechanisms of Rho GTPase signaling under physiological conditions and this along with the current generation of other small G protein biosensor mice¹⁰¹ should significantly improve our understanding of Rho GTPase signaling in the context of environmental cues from the surrounding tissue.

Rho GTPase Inhibitors in Cancer

Several inhibitors that target either Rho GTPase directly, or its regulators, have shown measurable anti-tumor activity in several in vitro and in vivo models of cancer.^{12,107} A virtual drug-screen using the 3D structure of the Rho GTPase Rac1, was successfully used to screen for compounds that may directly modulate the Rho GTPase-GEF interaction, by binding to an area of the Rac1 protein important for interactions with specific GEFs.¹⁰⁷ NSC23766 was identified as a selective inhibitor of the Rac1-GEF interaction, which, when administered in vitro, reduced transformation of mouse NIH-3T3 fibroblast cells by active Rac1 or Rac-GEFs and significantly decreased proliferation, survival and invasiveness of a prostate cancer cell line PC-3, previously shown to have elevated levels of active Rac1.¹⁰⁷ Similarly, inhibition of Rac activity by EHT 1864¹⁰⁸ significantly decreased estrogen-induced breast cancer cell proliferation and the Rac1-mediated induction of transcriptional activity of estrogen receptor α .¹⁰⁹ It is plausible to hypothesize that NSC23766 or similar novel inhibitors may prove effective when used in combination with other therapies to treat tumor subtypes with increased Rac1 activity, for example those tumors harboring the RAC1 (P29S) mutation or following APC loss described previously.^{80,81,102}

A recent study has also shown that a small molecule inhibitor of the Rho GTPase Cdc42, AZA197, effectively inhibited the proliferative potential and invasiveness of colorectal tumor cells and xenografts with high levels of Cdc42.¹¹⁰ Interestingly, efficacy of this agent was cell line dependent, indicating that a personalized companion biomarker may be necessary to identify tumor types most likely to respond to this type of therapy.

Another approach would be to target the downstream effectors of Rho signaling. Several in vitro and in vivo studies have demonstrated that administration of the ROCK inhibitors, fasudil and Y-27632, leads to decreased tumor proliferation, invasiveness and metastatic potential for several cancer types.^{33,111,112} The ROCK inhibitor fasudil has also been used effectively in combination with bortezomib, the first clinically approved proteasome inhibitor, to effectively treat the RAS oncogene-driven non-small cell lung cancer GEM model of the disease, through suppression of GATA2-regulated pathways.¹¹³ Similarly, fasudil treatment in an intracerebral human glioma xenograft model was shown to suppress both neovascularity and tumor growth in vivo.¹¹⁴ Whether the effect of these inhibitors is elicited purely on tumor cells themselves, the reactive stromal cells within the

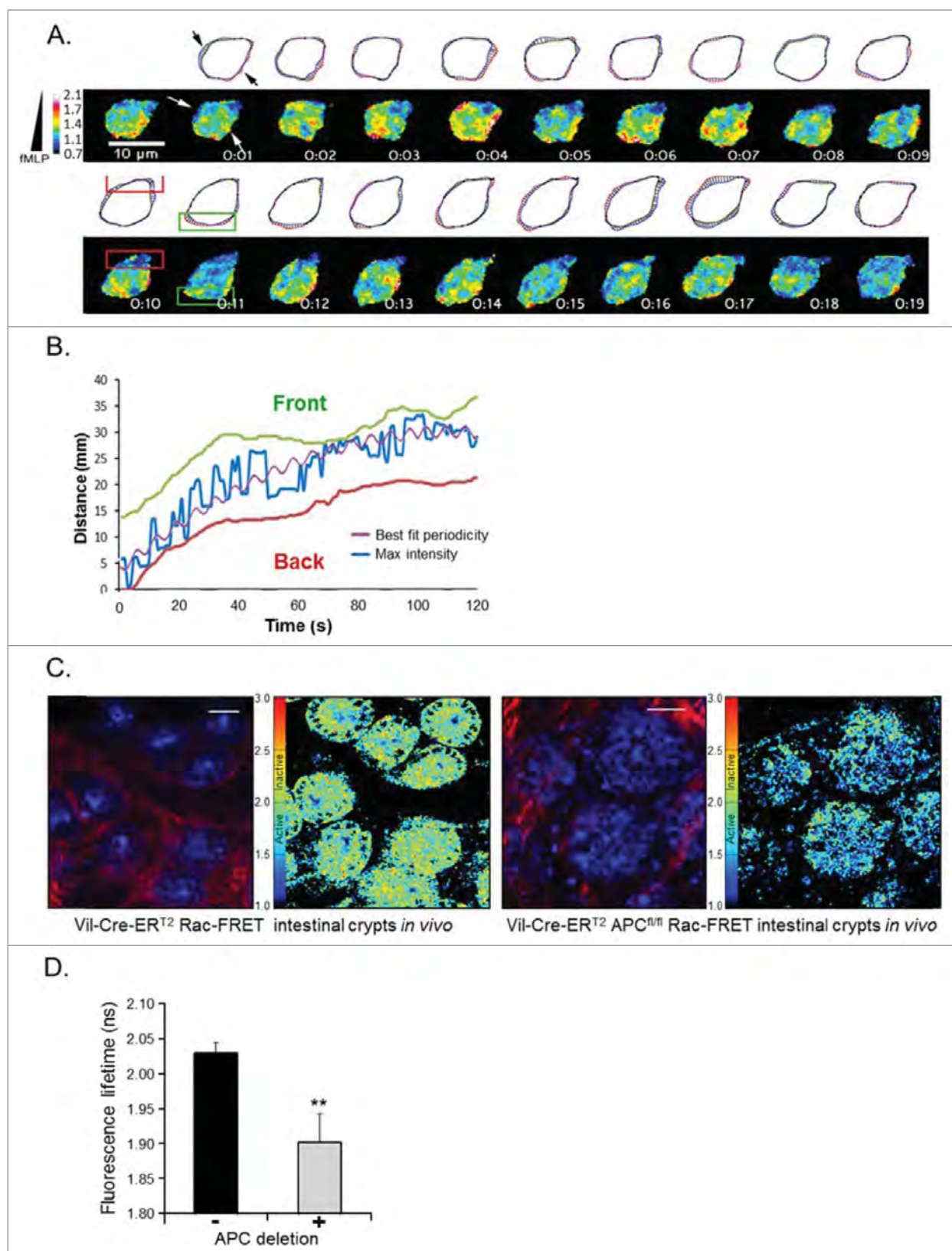


Figure 1. For figure legend, see page 6.

microenvironment, or most likely a combination of both, remains to be fully elucidated.

The tumor stroma is a complex environment mainly consisting of the basement membrane, fibroblasts, extracellular matrix, infiltrating immune cells, endothelial cells and associated vascular pericytes. The role of small GTPases in the stromal compartment of a developing tumor is less defined and needs further investigation. Wozniak et al.¹¹⁵ have shown that the surrounding environment can directly govern the cellular behavior of breast epithelial cells in a 3D environment. For example, the authors of this study have shown that in free-floating 3D collagen matrices, the breast immortalized MCF10A cells differentiate into tubular structures. In contrast, if collagen matrix stiffness is increased by attachment to the bottom of a dish, the same cells do not differentiate, but proliferate and spread extensively.¹¹⁵ In concordance, women with dense breast tissue, which is associated with a substantial increase in collagen deposition in the stroma, have a 4- to 6-fold increased risk of developing breast cancer.¹¹⁶ Gene expression profiling of patient material containing high density mammary fibroblasts showed striking similarities in expression profiles to established cancer-associated fibroblasts (CAFs), with several key enriched signaling pathways, including JNK1, Rho GTPase(s), iNOS, FGF-R, EGF-R, and PDGF-R-mediated signal transduction, thereby creating a pro-inflammatory, pro-proliferative, cytokine, and chemokine-rich microenvironment.¹¹⁷ Importantly, functional experiments revealed that the mechanism by which breast epithelial cells sense and respond to the mechanical properties of their surrounding environment involved tight regulation by RhoA, where stiff matrices promoted RhoA activity and localization of phosphorylated Y397 FAK into 3D cell adhesions in a ROCK-dependent manner.¹¹⁵ Recent reports confirmed higher RhoA activity is found in stiff versus compliant matrices and demonstrated that in 3D collagen matrices, RhoA activity is considerably increased in cell-matrix adhesions, compared with cell-cell contacts.¹¹⁸ As such, RhoA activation appears to be a central part of mechanotransduction and has been linked to enhanced ECM-focal adhesion signaling via Fak to drive enhanced tumor progression.^{41,119} In **Figure 2**, we demonstrate the function of ROCK activity in fibroblast-driven collagen I contraction on a 3D level by second harmonic generation (SHG) imaging. Alterations in collagen matrix structure can be correlated to elevated collagen density, crosslinking and stiffness visualized by SHG imaging.^{41,120,121} The presence of the ROCK inhibitor Y-27632 significantly reduced the contraction of

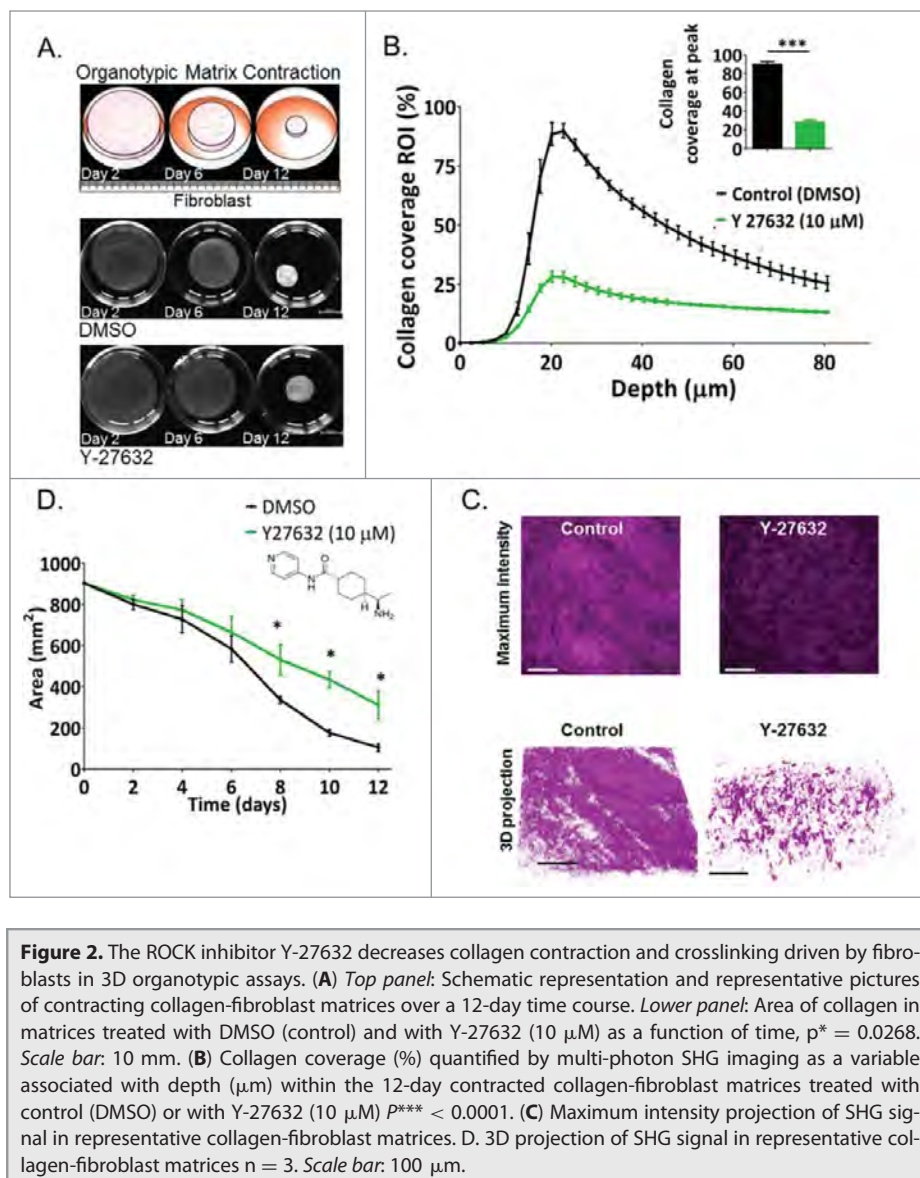
collagen gels, accompanied by reduced collagen density and crosslinking (**Fig. 2A-D**). These changes in biomechanical matrix properties may contribute to the observed Y-27632-mediated decrease in cancer cell invasion when assessed in 3D organotypic matrices that incorporate cancer and stromal cell types.^{87,122} In agreement, others have also demonstrated that ROCK activity is required for collagen crosslinking during wound healing in fibroblast-driven contraction assays,¹²³ indicating a positive feedback loop, in which ROCK signaling promotes matrix stiffening that subsequently enhances RhoA / ROCK activation to further crosslink ECM (described in¹²⁴). Recently, reorganization of the stromal ECM, tightly regulated by distinct Rho GTPases, has been shown to guide key aspects of branching morphogenesis in developing mammary glands, where Rac1 modulates signaling required for branch orientation, whereas the ability of the mammary epithelium to reinforce directional cues during branching morphogenesis appears to be mediated by RhoA/ROCK-mediated contractions.¹²⁵ Although tightly controlled during normal development and organ homeostasis, deregulation of this complex network could play an important role in the initiation and/or progression of breast cancer.

Imaging studies of collectively invading 3D co-cultures of squamous cell carcinoma cells and stromal fibroblasts have revealed that during invasion, carcinoma cells are led by stromal fibroblasts and move within tracks in the ECM created by CAFs through protease and force-mediated matrix remodeling.¹²² Moreover, RhoA/ ROCK, LIMK, integrin alpha3 and alpha5 function were required for the leading fibroblasts to generate tracks in the matrix and enable tumor invasion, whereas the cancer cells depended on the activity of Cdc42 (but not RhoA) to follow the fibroblasts and invade into the organotypic collagen matrices.^{122,126} Similarly, pro-inflammatory cytokine signaling through the JAK/STAT3 pathway and subsequent activation of RhoA/ ROCK is involved in generating actomyosin contractility in CAFs and melanoma cells.¹²⁷ In cholangiocarcinoma, a disease characterized by abundant desmoplastic stroma, cancer cells can effectively recruit CAFs through secretion of PDGF-D, which further stimulates fibroblast migration through PDGFR β , Rho GTPase (Rac1, RhoA and Cdc42) and JNK activation.¹²⁸ Moreover, selective inhibition of Rho signaling, in particular Rac1 (NSC23766), but also ROCK (Y-27632) and Cdc42 (CASIN), dramatically decreased PDGF-induced fibroblast motility in this experimental model.¹²⁸ In pancreatic CAFs, high expression of the actin-associated protein Palladin significantly enhances the

Figure 1 (See previous page). The Rac-FRET mouse is an invaluable tool to assess and quantify Rac1 activity in primary cells (**A and B**) and in vivo (**C and D**). (**A**) Neutrophil harvested from a Rac-FRET mouse migrating toward the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (fMLP) south of the cell. Time series of Rac1 activity was obtained by ratiometric FRET live imaging and illustrated as a heat map of high (yellow to red) and low (blue to green) Rac1 activity. High Rac1 activity was localized to leading edge protrusions (green box) with short-lived bursts at the cell's periphery and at the trailing edge. (**B**) Maximum Rac1 activity along the longitudinal axis of a neutrophil (blue) migrating toward an fLMP gradient oscillated between the leading (green) and lagging edge (red) illustrated by fitting the experimental data (purple). (**C**) Intravital FLIM-FRET imaging demonstrated that Cre-mediated loss of APC in intestinal crypts promotes Rac1 activity. Low Rac1 activity in APC wild type mice (left panel) is represented by high fluorescence lifetimes (low FRET-efficiency, green), while loss of APC (right panel) results in high FRET-efficiency (low lifetimes, blue) indicating increased Rac1 activity. Each panel consists of fluorescence images of Rac-FRET expressing crypt cells (blue) and collagen (red, assessed by SHG imaging) on the left and FLIM images on the right. (**D**) Quantification of Rac1 activity by FLIM-FRET imaging reveals a significant decrease in the fluorescence lifetime upon deletion of APC correlating with an increase in Rac1 activity compared to APC wild type cells (mean \pm SEM; ** $P < 0.05$). Figure adapted from Johnsson et al. *Cell Reports*⁹³.

ability of CAFs to remodel the stromal ECM by regulating Cdc42 activity, which in turn promotes the assembly of matrix-degrading invadopodia in CAFs and enhance tumor cell invasion.¹²⁹ However, the precise roles of small GTPases in this complex interaction (also considering the roles of RhoA and Cdc42 in fibroblast track generation) remain unclear and need to be elucidated.

Furthermore, small GTPases have been implicated to function at the tumor-stromal interface during transendothelial migration of cancer cells. For example, arachidonic acid secreted by bone marrow adipocytes in vitro was shown to induce transendothelial migration of prostate cancer lines via Rho/ROCK-dependent amoeboid migration.¹³⁰ In line with this, a Rho GTPase RNAi screening study revealed a novel role of Cdc42 as a key regulator of prostate cancer cell transendothelial migration, where Cdc42 was found to be essential for in vivo cancer cell spreading and protrusion extension along blood vessels and colonization in the lungs.¹³¹ Transient Cdc42 suppression in vivo led to a significant decrease in the formation of lung metastases following tail vein injection of prostate cancer PC-3 cells, suggesting that the role of Cdc42 in endothelial attachment is crucial for metastasis.¹³¹ Moreover, glioblastoma cells have recently been shown to directly modulate the contractile activity of neighboring brain pericytes via Cdc42-dependent mechanisms, supporting vascular expansion and tumor progression.¹³² Overexpression of Rho, a Rho GTPase mainly present in endothelial cells, is associated with increased prevalence of lymphovascular invasion, lymph node metastases and decreased overall survival in colon cancer.⁵⁶ Using an inducible endothelial cell-specific RhoJ loss-of-function GEM, generated by crossing RhoJ^{GFP/GFP} knock-out mice⁵⁶ with the Cdh5(PAC)-CreER^{T2} model,¹³³ the authors further demonstrated that RhoJ deletion in this context disrupted tumor vessel formation and vascular integrity, suppressed tumor angiogenesis, presenting a feasible target for clinical drug development.⁵⁶ Collectively, these studies highlight the increasing relevance of the Rho GTPases within divergent cellular components of the tumor microenvironment and further underline the significance of distinguishing drug effects on cancer cells vs. those on the surrounding host stroma. A more comprehensive understanding of the contextual dependence of Rho GTPase signaling in the tumor cells and the surrounding stroma will be a necessary step toward



successful implementation of therapeutics that target Rho signaling as cancer therapy, providing interesting avenues for the development of combination therapies.

Future Perspectives

Numerous conceptual advances in biology have been achieved by experimental studies using 2-dimensional cell culture systems. Recent adaptations of molecular imaging techniques to 3-dimensional model systems, increasing in complexity from the 3D-spheroid cultures, the transparent *Drosophila*, *Xenopus* and Zebrafish, to the complex mammalian xenograft and GEM models, are bridging the gap in our understanding of biological events in vitro and in vivo, establishing an important role for Rho GTPases in disease progression and therapeutic targeting. We envisage that future applications will involve generation of transgenic mice that co-express combinations of Rho GTPase FRET

biosensors to provide a detailed map of physiological signal transduction events in an intact mammalian organism. Stromal cells and the role that they have on cancer initiation and progression will have important implications on the examination of Rho GTPase activity in live tissues as well as therapeutic targeting. A major application already underway will involve crossing other disease models with the Rho GTPase FRET biosensor mice to examine disease etiology and improve drug development and screening for progressing novel agents into clinical trials. Similarly, crossing the Rac GTPase FRET mouse⁹³ with transgenic mice expressing stroma-specific Cre recombinase, for example in fibroblasts¹³⁴⁻¹³⁶ or endothelium,¹³⁷⁻¹³⁹ in the future could

provide detailed insight into the intricacy of stroma-specific Rac signaling in distinct stromal compartments in real-time.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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