The ROCK isoforms differentially regulate the morphological characteristics of carcinoma cells

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ABSTRACT
Rho-associated kinase (ROCK) activity drives cell migration via actomyosin contractility. During invasion, individual cancer cells can transition between 2 modes of migration, mesenchymal and amoeboid. Changes in ROCK activity can cause a switch between these migration phenotypes which are defined by distinct morphologies. However, recent studies have shown that the ROCK isoforms are not functionally redundant as previously thought. Therefore, it is unclear whether the ROCK isoforms play different roles in regulating migration phenotypes. Here, we found that ROCK1 and ROCK2 differentially regulate carcinoma cell morphology resulting in intermediate phenotypes that share some mesenchymal and amoeboid characteristics. These findings suggest that the ROCK isoforms play unique roles in the phenotypic plasticity of mesenchymal carcinoma cells which may have therapeutic implications.

Introduction
The vast majority of cancer patients will die due to the metastatic spread of cancer cells. For metastasis to occur, cancer cells must migrate away from the primary tumor by invading neighboring tissues. Cell migration has been suggested as a novel target for inhibiting invasion and metastasis.1 This process is driven by cellular forces generated by actomyosin contractility through phosphorylation of the myosin light chain (MLC) of non-muscle myosin II (NM II) by rho-associated kinase (ROCK), a downstream effector of the small GTPase Rho.2 While ROCK activity is increased in a variety of cancer types,3–4 pan-inhibition of ROCK does not always inhibit migratory and invasive properties of cancer cells.5–6 Historically, the 2 ROCK isoforms have been viewed as redundant; however, more recent work has uncovered important functional differences between ROCK1 and ROCK2 in a variety of normal cell types.7–10 Although cell-type dependent, isoform-specific effects that have been found include differential regulation of the actin cytoskeleton, actomyosin contractility, adhesions, and cell morphology.7–10 Given the failure of ROCK inhibitors to progress clinically, understanding the precise roles of ROCK1 and ROCK2 in different types of cancer cells may provide new therapeutic avenues based on isoform selectivity to inhibit migration.3,11,12

The epithelial to mesenchymal transition (EMT) is a fundamental process necessary for migration in which epithelial cells convert to a motile and invasive mesenchymal phenotype.13 This transformation is regulated by a complex set of signal transduction pathways that can be triggered by biochemical and biophysical factors in the tumor microenvironment.14 For example, oncogene signaling and ECM rigidity can activate Rho/ROCK signaling leading to cytoskeletal reorganization, cellular spreading, focal adhesion formation, actomyosin contractility, traction force generation, and proteolysis of the ECM.15–17 Cells can also transition from a mesenchymal to a rounded amoeboid phenotype (MAT) to navigate pre-existing spaces and use non-apoptotic blebs to physically deform and push through porous ECM when degradation is not required.18 These membranous pushing forces are generated by the actomyosin cortex via elevated Rho/ROCK activity which can be isoform specific but cell-type dependent as well.19–24 However, amoeboid migration does not generate significant traction forces due to weak adhesion by diffusely distributed integrins.25

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A high degree of plasticity exists between these phenotypes—pan-inhibition of ROCK can induce an amoeboid to mesenchymal transition (AMT) which can be reversed by blocking protease activity leading to MAT in 3-D environments. However, the transitions between distinct modes of migration (e.g., MAT and AMT) are characterized by significant changes in cellular morphology that are observed in both 2-D and 3-D. The ability to degrade the ECM is necessary for migrating cells to invade the basement membrane and other densely cross-linked tissues. To penetrate these tissues, cancer cells form actin-rich adhesive protrusions known as invadopodia that localize proteinases to focally degrade the ECM. Our previous work has shown that ECM rigidity can induce more invadopodia and ECM degradation in several carcinoma cell lines using substrates that mimic the mechanical properties of tumors. Using chemical inhibitors, this rigidity response was found to be dependent on NM II and ROCK suggesting that cellular force generation plays a critical role in driving invasiveness. To confirm a role for actomyosin contractility, we later found that cellular traction forces mediate ECM degradation indicating that invadopodia activity is likely regulated by ROCK. Overall, these results suggest that actomyosin contractility regulates invadopodia activity through a ROCK-dependent mechanism that may promote both migration and invasion.

Given the emerging functional differences between ROCK1 and ROCK2, we recently determined whether the effects of ROCK on invadopodia activity were isoform specific. Interestingly, we found that ROCK1 and ROCK2 differentially regulate ECM degradation by invadopodia via contractile and non-contractile mechanisms, respectively, in 2 different carcinoma cell lines. In particular, ROCK2 signaling occurred through LIM kinase (LIMK), but not NM II like ROCK1, and was not necessary for traction force generation or Transwell migration. Thus, our findings indicate that selective inhibition of the ROCK isoforms produced behavioral characteristics that were not fully described by either the mesenchymal or amoeboid phenotype. Since these phenotypes are routinely distinguished by distinct morphologies, the goal of this study was to evaluate the physical characteristics of these ROCK1- and ROCK2-inhibited carcinoma cells to further evaluate their isoform-dependent phenotypes and discuss the potential therapeutic implications for preventing invasive migration.

Results

We previously modified established methods for preparing polyacrylamide gels (PAAs) for use in both traction force and invadopodia assays that span the range of reported mechanical properties for human breast and head and neck (unpublished preliminary data) tumors. To elucidate the roles of the ROCK isoforms in actomyosin contractility and ECM degradation, we had used soft and rigid PAAs since they provide maximum sensitivity in detecting differences in traction forces and invadopodia activity, respectively. The soft PAAs are conjugated with fibronectin for cellular adhesion while the rigid PAAs are also overlaid with cross-linked gelatin and FITC-labeled fibronectin or cross-linked, FITC-labeled gelatin for detection of ECM degradation. Using these assays, we found that ROCK1 and ROCK2 differentially regulated invadopodia activity through NM II and LIMK pathways, respectively, while only ROCK1 regulated actomyosin contractility. These results were further confirmed with Transwell assays in which only ROCK1 knockdown (KD) inhibited migration while ROCK1 and ROCK2 KDs inhibited invasion. To determine if these phenotypic differences with ROCK KDs include morphological changes, we revisited this data as well as performed some additional experiments and measured cell sizes and shapes of the 2 invasive human cell lines previously used, SCC-61 (head and neck squamous cell carcinoma) and MDA-MB-231 (breast adenocarcinoma), in both assays.

We previously confirmed expression of the ROCK isoforms, specificity of the ROCK1 and ROCK2 siRNA, and efficacy of the KDs in SCC-61 cells which we demonstrate here as well (Fig. 1A-B). For SCC-61 cells on the soft PAAs (Fig. 1C), ROCK1 KD led to a decrease in cell size (Fig. 1D) while promoting cell rounding (Fig. 1E). On rigid PAAs (Fig. 1F), ROCK1 KD also led to a reduction in cell size of SCC-61 cells (Fig. 1G) but did not lead to a statistically significant change in cell shape when compared with non-target control (Fig. 1H). ROCK2 KD had no effect on cell size (Fig. 1D & G) but led to a longer cell shape (Fig. 1E & H) for SCC-61s on soft and rigid PAAs.

We also previously confirmed ROCK KDs in MDA-MB-231 cells which we demonstrate here once again as well as siRNA specificity (Fig. 1I-J). Similar to SCC-61 cells, we also found that ROCK1 KD in MDA-MB-231 cells (Fig. 1K & N) led to a decrease in cell size (Fig. 1L & O) on the soft and rigid PAAs. MDA-MB-231 cells also became more rounded on the soft PAAs (Fig. 1M) as well as on the rigid PAAs (Fig. 1P) with ROCK1 KD. ROCK2 KD led to a slight increase in cell size (Fig. 1L & O) but had no effect on cell shape (Fig. 1M & P) on both the soft and rigid PAAs. These data further support different roles for the ROCK isoforms in regulating cellular morphology while also suggesting that the effects of ROCK2 KD may be more cell-type specific.
Since actomyosin contractility can regulate cellular adhesion to influence cell spreading, we also performed additional experiments and evaluated focal adhesion numbers of SCC-61 cells in invadopodia assays on glass for optimal imaging (Fig. 2A-C). While ROCK1 KD led to a significant decrease in the number of focal adhesions, ROCK2 KD had no effect on the number of focal adhesions (Fig. 2D).

**Discussion**

Changes in ROCK activity can regulate the transitions between the mesenchymal and amoeboid phenotypes which are characterized by significant differences in cellular morphology. We previously determined that the ROCK isoforms are important in regulating invadopodia which are associated with a mesenchymal phenotype in carcinoma cells. In this study, we have shown that ROCK1 KD led to cell rounding and a decrease in cell size while ROCK2 KD cells maintained a spread and elongated morphology which was fairly consistent across different ECM rigidities and compositions. These changes in cell morphology from ROCK1 KD were also accompanied by reductions in focal adhesion numbers which did not change with ROCK2 KD. Similar effects on focal adhesions and cell morphology have been observed in fibroblasts indicating that the ROCK isoforms regulate different aspects of the mesenchymal phenotype.

While our morphological results suggest that ROCK1 KD could promote the transition from a mesenchymal- to amoeboid-like phenotype, MAT is characterized by an increase in ROCK activity and faster migration rates in porous environments. Although we did observe decreases in cell size and shape (Fig. 1) as well as focal adhesions (Fig. 2), we previously found that ROCK1 KD significantly impaired Transwell migration. Therefore, this phenotype is likely due to reductions in mesenchymal-based force transduction necessary for effective adhesion-based migration consistent with pan-inhibition which is supported by the decreases in focal adhesions (Fig. 2) and traction forces.
carcinoma cells maintained mesenchymal-like properties with ROCK2 KD including morphology (Fig. 1) and focal adhesions (Fig. 2) as well as the ability to generate traction forces and migrate across Transwell inserts. However, the mesenchymal phenotype is also associated with invasion via ECM degradation which was inhibited with ROCK2 KD in both invadopodia and Transwell invasion assays.

We also used pharmacological inhibitors in the Transwell migration and invasion assays to further validate our siRNA results. Therefore, ROCK2 inhibition also produces a phenotype having some mesenchymal and amoeboid characteristics.

ROCK is regulated by Rho GTPases which have been implicated in cancer cell migration, invasion, and metastasis, including RhoA and RhoC. Similar to the ROCK isoforms, RhoA and RhoC can activate similar targets but have also been shown to have unique functions suggesting involvement in different signaling pathways. In particular, RhoA signaling through ROCK is known to regulate cellular contractility while RhoC affects actin polymerization at invadopodia in a coflin-dependent manner through a ROCK-LIMK pathway. We have previously found that ROCK1 regulates traction forces and pMLC levels in carcinoma cells which would suggest a specific role for RhoA upstream. We also found that ROCK2 signaling occurred through LIMK to alter F-actin at invadopodia suggesting regulation through a RhoC/ROCK2 pathway. LIMK has previously been shown to regulate invadopodia and invasion but not cell motility by MDA-MB-231 cells which further support a role for ROCK2 in ECM degradation but not actomyosin contractility. Therefore, specific Rho and ROCK isoforms may form distinct signaling complexes that differentially regulate migration and invasion.

In this study, we have shown that the ROCK isoforms differentially regulate cell morphology in invasive carcinoma cells by producing phenotypes that share some mesenchymal and amoeboid characteristics. Other hybrid and intermediate phenotypes with different characteristics have been described, but they have not been observed in human carcinoma cell lines or have shown dependence on specific ROCK isoforms. Although
our previous work revealed that both isoforms regulated invadopodia activity and invasion, these distinct morphologies coincide with significant differences in the ability of these carcinoma cells to migrate which could have considerable therapeutic implications. Selective isoform targeting has become an attractive alternative given the concerns regarding the side effects and toxicity of pan-inhibition of ROCK. Although further studies are required, our work suggests that selectively inhibiting ROCK2 may induce a non-proteolytic phenotype still capable of migration in a mesenchymal-like manner since only ROCK1 regulated NM II-driven cellular forces in our system. Therefore, ROCK1 may be the more appealing therapeutic choice and provide an advantage by inhibiting not only the migration of certain types of cancer cells but their ability to degrade the ECM as well.

Methods and materials

**Cell culture and ROCK inhibition**

SCC-61 and MDA-MB-231 cells were cultured as described previously as well as KD of ROCK1 and ROCK2 with siGENOME SMARTpool siRNA (Thermo-Scientific) to maximize inhibition while minimizing off-target effects as well as any possible compensatory effects. KDs were confirmed with Western blotting as described previously. A double KD experiment was previously performed to confirm that compensation was not occurring between the ROCK isoforms.

**PAAs**

As described previously, we used fibronectin-embedded soft (elastic modulus = 1,023 Pa) and rigid (elastic modulus = 22,692) PAAs that are used for our traction force and invadopodia assays, respectively. As used in the invadopodia assay, the rigid PAAs were overlaid with cross-linked 1% gelatin and FITC-labeled fibronectin or cross-linked 0.2% FITC-labeled gelatin for SCC-61 and MDA-MB-231 cells, respectively.

**Cell morphology**

Cells were incubated overnight in invadopodia medium for live cell imaging (soft PAAs) or then fixed and stained for immunofluorescence (rigid PAAs) and imaging as described previously. Cells were identified with phase contrast for live cell imaging and F-actin staining with Alexa Fluor phalloidin (Life Technologies) for immunofluorescence imaging. Metamorph software (Molecular Devices) was used to manually outline cells, and quantitation of cell size and shape factor were performed using measurement tools.

**Focal adhesions**

Cells were once again incubated overnight in invadopodia medium then fixed and stained in invadopodia assays overlaid with cross-linked 1% gelatin and unlabeled fibronectin for immunofluorescence imaging. Vinculin was identified with a mouse monoclonal antibody (Sigma) following previously established methods for focal adhesion staining. Fluorescent images were captured on a Nikon Ti-E inverted microscope with a Plan Fluor 40× oil immersion objective lens. Focal adhesions were quantitated using the Focal Adhesion Analysis Server.

**Statistics**

As described previously, all statistical analyses were performed on pooled data using SPSS Statistics (IBM). The majority of data did not pass the normality test and therefore were analyzed with a Kruskal-Wallis test for significance followed by a Tamhane post-hoc test for group comparisons.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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