Up-Regulation of Rho/ROCK Signaling in Sarcoma Cells Drives Invasion and Increased Generation of Protrusive Forces

Daniel Rösel,1 Jan Brábek,1 Ondřej Tolde,1 Claudia T. Mierke,3 Daniel P. Zitterbart,3 Carina Raupach,3 Kristýna Bicanová,1 Philip Kollmannsberger,3 Daniela Paňková,1 Pavel Veselý,2 Petr Folk,1 and Ben Fabry3

1Department of Cell Biology, Faculty of Science, Charles University in Prague; 2 Institute of Molecular Genetics AS, Prague, Czech Republic; and 3 Biophysics Group, Department of Physics, University of Erlangen-Nuremberg, Erlangen, Germany

Abstract
Tumor cell invasion is the most critical step of metastasis. Determination of the mode of invasion within the particular tumor is critical for effective cancer treatment. Protease-independent amoeboid mode of invasion has been described in carcinoma cells and more recently in sarcoma cells on treatment with protease inhibitors. To analyze invasive behavior, we compared highly metastatic sarcoma cells with parental nonmetastatic cells. The metastatic cells exhibited a functional up-regulation of Rho/ROCK signaling and, similarly to carcinoma cells, an amoeboid mode of invasion. Using confocal and traction force microscopy, we showed that an up-regulation of Rho/ROCK signaling leads to increased cytoskeletal dynamics, myosin light chain localization, and increased tractions at the leading edge of the cells and that all of these contributed to increased cell invasiveness in a three-dimensional collagen matrix. We conclude that cells of mesenchymal origin can use the amoeboid nonmesenchymal mode of invasion as their primary invading mechanism and show the dependence of ROCK-mediated amoeboid mode of invasion on the increased capacity of cells to generate force. (Mol Cancer Res 2008;6(9):1410–20)

Introduction
The metastatic potential of tumor cells is the most life-threatening aspect of cancer. The process of metastasis is composed of a series of critical steps known as the metastatic cascade (1). The cascade involves the invasion of cells from the primary tumor into the surrounding tissue, intravasation and transport in the blood or lymphatic system, extravasation, and proliferation of tumor cells at the secondary sites. In this report, we focus on the process of cancer cell invasion as a critical step in metastasis. Recent advances in microarray technologies, real-time in situ imaging, and the use of in vitro three-dimensional invasion model systems have brought a wealth of new insights into the process of invasion (2). Two main modes of tumor cell invasion into the surrounding tissue have been described (3, 4): the first is a mesenchymal mode that depends on extracellular proteolysis and the second is a recently discovered proteolysis-independent amoeboid mode that depends on the activity of the Rho kinase ROCK. In the amoeboid mode, the up-regulation of ROCK has been hypothesized to assist in the generation of sufficient actomyosin forces that would then allow the tumor cells to deform collagen fibers and push through the extracellular matrix (5). However, the amount of actomyosin forces that are generated by amoeboid-invading versus mesenchymal-invading tumor cells has never been investigated and compared. In contrast, the mesenchymal mode is associated with Rac-dependent F-actin–rich protrusions and does not require an up-regulation of ROCK (6). Recently, it has been reported that proteolysis-dependent invasion can convert to an amoeboid invasion mode when the extracellular proteolytic activity is blocked by inhibitors (4). The ability to undergo a transition from mesenchymal to amoeboid modes would endow tumor cells with an efficient escape from protease inhibitor–based cancer treatment. There is a hope that the combined inhibition of extracellular proteases and ROCK could be effective in preventing tumor cell invasion (3).

To analyze the processes responsible for metastatic capability of tumor cells, we used a rat sarcoma model of metastasis composed of two cell lines (7): nonmetastatic fibroblast-like LW13K2 (K2) cells that were isolated from neoplastic, rat embryo fibroblast cells that had spontaneously transformed in vitro, and high metastatic A337/311RP (A3) cells that were derived from the K2 cells.

This rat sarcoma model in which two closely related cell populations have greatly different capacities to induce metastases in animals offers specific advantages for studying in vitro metastatic transformation (7). In particular, the close genetic relationship of the cell lines provides a “uniform” genetic background. Therefore, the differences in their metastatic
potential are thought to predominantly reflect differences in their protein expression levels and phosphorylation state.

Using this model, we showed here for the first time that highly metastatic mesenchymal sarcoma cells use the Rho/ROCK-dependent amoeboid mode of invasion as their primary invading mechanism. Furthermore, we showed that the up-regulation of Rho/ROCK signaling relates to an increased invasion, increased recruitment of the myosin light chain (MLC) to the leading edge of the cell, and an increased generation of protrusive force.

**Results**

**Highly Metastatic A3 Mesenchymal Sarcoma Cells Do Not Show Any Detectable Gelatinase Activity**

To study the changes related to acquisition of invasive potential, we used a sarcoma model of metastasis consisting of parental nonmetastatic K2 cells and highly metastatic A3 cells derived from K2 cells (7). Because sarcoma cells are of mesenchymal origin and are thought to use the mesenchymal mode of invasion that is dependent on extracellular protease activity (8), we analyzed the gelatinase activity of K2 and A3 cells.

Using an in-gel gelatin zymography assay, we found the matrix metalloproteinase (MMP)-2 to be the only detectable gelatinase secreted into the medium by both K2 and A3 cells as judged by control MMP-2—secreting src-transformed embryonic fibroblasts (9). Surprisingly, the highly metastatic sarcoma A3 cells secreted only low levels of inactive MMP-2 proform and no active MMP-2 was detected (Fig. 1A). K2 cells secrete significantly higher amount of pro-MMP-2 and also a small amount of active MMP-2. To further extend the results from the in-gel assay, we did FITC-gelatin zymography. In this assay, any effective amount of gelatinase activity either secreted or membrane bound would be detected. Consistently, no gelatinase activity was observed in highly metastatic A3 cells, and only very low gelatinase activity was detected in the nonmetastatic K2 line. The gelatinase activity in K2 cells was sensitive to the broad-spectrum MMP inhibitor GM6001 (Fig. 1B). These observations suggest that A3 mesenchymal sarcoma cells do not invade via a gelatinase-dependent mesenchymal mode.

**Protein Microarray Analysis Reveals Up-Regulation of Rho/ROCK Signaling in Highly Metastatic A3 Cells**

To find a search for an alternative mechanism through which A3 cells exert their metastatic potential, we analyzed differences in protein expression between A3 and K2 cells using protein microarray analysis (Kinex). Kinex protein microarray determines the expression and phosphorylation of selected signaling proteins using a panel of approximately 350 pan-specific and 250 phospho-specific antibodies. Of these 600 antibodies, 83 showed significant changes between A3 and K2 cells (Table 1). The most striking result of this analysis was the up-regulation of the Rho/ROCK signaling pathway (10) as estimated by over-expression of ROCK-I and LIMK1. In addition, the negative regulator of Rho/ROCK pathway, PAK1, was down-regulated by more than 7-fold. Furthermore, we found a significant decrease in integrin β1 phosphorylation. Because protease-independent invasiveness is correlated with a decreased β1 integrin expression (4), we analyzed the total β1 integrin expression in K2 and A3 cells. Indeed, β1 integrin levels were found to be greatly reduced in A3 cells (Fig. 2B).

To further confirm the up-regulation of the Rho/ROCK pathway, we did a Rho activation assay. RhoA-GTP and RhoC-GTP levels were together almost three times higher in the metastatic A3 line compared with the nonmetastatic K2 line (Fig. 2A). RhoB-GTP levels were below the detection limit. Both RhoA and RhoC have been previously shown to activate ROCK (11, 12). One possible mechanism explaining higher levels of Rho-GTP in A3 cells could be a down-regulation of RhoGDI. Indeed, the protein microarray analysis showed a 5.9-fold increase of the RhoGDI-degrading enzyme caspase-1 (13) in A3 cells (Table 1). In addition, we analyzed the phosphorylation of the prominent ROCK substrate MYPT-1 (14) and found it greatly elevated in A3 cells (Fig. 2B).

These data together suggest that the Rho/ROCK pathway is functionally up-regulated in A3 cells and, together with the absence of gelatinase activity as shown above, point to a ROCK-dependent amoeboid invasion mechanism.

**A3 Cells Exhibit Higher Cytoskeleton Dynamics**

The Rho/ROCK pathway has been shown to control cytoskeletal dynamics such as the turnover rate of actin stress fibers and focal adhesions (15). The protein microarray analysis revealed up-regulation of cofilin along with signals leading to cofilin phosphorylation (up-regulation of LIMK1 and ROCK-I). This observation points to increased cofilin phosphorylation dynamics and thus increased dynamics of F-actin polymerization and depolymerization. To quantify the differences in cytoskeleton dynamics between nonmetastatic K2 and metastatic A3 cells, we did two assays: nanoscale particle tracking and magnetic tweezers microrheology.

Nanoscale particle tracking analyzes the random walk of unforced, spontaneously diffusing fibronectin-coated beads. In this work, 4.5-μm-diameter carboxylated polystyrene beads coated with human fibronectin were attached to confluent cells. The beads bind to integrins at the cell surface, initiate focal contact formation, and anchor firmly to the actin cytoskeleton. The beads cannot move unless the focal contacts and actin structures to which they are attached rearrange (16). ATP-driven cytoskeletal rearrangements can be quantified by an apparent diffusivity D* of the beads, measured as the squared distance of bead motion during a 1-s time interval. The variable D* was ~2.5 times greater in A3 cells compared with K2 cells (Fig. 3A). These data are consistent with the idea that the higher invasiveness of A3 cells is facilitated by an increased rate of focal adhesion turnover and cytoskeletal remodeling.

The second assay, magnetic tweezers microrheology, measures the displacement of integrin-bound fibronectin-coated magnetic beads in response to lateral step forces between 0.5 and 10 nN (17). This so-called creep response reports the stiffness of the bonds that connect the beads with the cell. In the case of fibronectin-coated beads, these bonds are formed by connections between the integrins, focal adhesion proteins, and the actin cytoskeleton (18). The A3 cells display a significantly lower stability of the bonds that connect the beads with the cell, as seen by a significantly higher power-law exponent.
suggesting a more fluid-like behavior typical for cells with higher cytoskeleton dynamics (Fig. 3B).

Phospho-MLC Colocalizes with F-Actin at the Leading Edge of A3 Cells

A previous study (7) has shown that F-actin in the K2 cells is arranged into stress fibers, whereas in A3 cells the F-actin is arranged cortically and concentrated in ruffles at the leading edge of the cell, a typical arrangement for metastatic cells with higher cytoskeletal dynamics (19). To gain further insight into the mechanisms that are responsible for the great differences in cytoskeletal dynamics, we characterized the A3 and K2 actomyosin cytoskeleton with respect to ROCK activity and localization of the ROCK substrate MLC. Functional up-regulation of Rho/ROCK signaling results in phosphorylation and activation of MLC, and subsequent enhancement of actomyosin contractility at the leading edge (14). The distribution and colocalization of phospho-MLC (P-MLC) with F-actin were therefore analyzed.

In the metastatic A3 cells, P-MLC was highly colocalized with cortical F-actin in ruffles, whereas in the nonmetastatic K2 cells this colocalization was much less prominent (Fig. 4A). Quantification of randomly acquired images revealed an 86 ± 8% colocalization of P-MLC with large cortical F-actin structures in A3 cells, in comparison with 35 ± 10% in the K2 cells (Fig. 4B). To get further insight into the P-MLC distribution in migrating cells, we analyzed P-MLC localization with respect to focal contact marker phospho-paxillin in polarized cells (triangular-shaped cells with clearly defined leading and trailing edge). Confocal analysis of A3 and K2 cells revealed that P-MLC was not enriched in focal contacts at substrate level sections. However, in midcellular confocal section of A3 cells, the P-MLC was enriched at the leading edge, at places with high actin polymerization, and at the trailing edge. In contrast, in K2 cells P-MLC was enriched only at the trailing edge (Fig. 4C, compare with tractions distribution—Fig. 7). These results suggest that at least in two-dimensional cell culture conditions, the actomyosin force is not generated near the focal contact sites but rather above the substrate in regions that are involved in cellular extension formation.

A3 Mesenchymal Sarcoma Cells Show ROCK-Dependent Amoeboid Mode of Invasion

Rho/ROCK signaling has been suggested to be required for cell movement in a local environment that physically restricts locomotion (e.g., during metastatic invasion; ref. 3). To
simulate such an environment of restricted locomotion, we adapted an under-agarose assay that had been used previously to characterize restricted migration of Dictyostelium myosin mutants (20). In the under-agarose assay, A3 cells maintained their overall morphology and assumed an individual mode of migration (Fig. 5A). In contrast, the K2 cells increased their spreading area dramatically and seemed to migrate collectively. Their morphology changed by this inhibitor (Fig. 6B). Taken together, these results show that A3 mesenchymal sarcoma cells use the Rho/ ROCK-dependent, we analyzed their invasive potential using a high-throughput Matrigel invasion assays in the presence or absence of the ROCK inhibitor Y-27632. Inhibition of ROCK in A3 cells using 10 μmol/L Y-27632 resulted in a 60% decrease in invasion through Matrigel (Fig. 6B) and complete inhibition of invasion into three-dimensional collagen (data not shown). Interestingly, the invasiveness of K2 cells was enhanced in the presence of 10 μmol/L Y-27632 (Fig. 6B). A possible explanation for this enhancement could be the 1.7-fold elevation of MMP-2 activity after ROCK inhibition, as detected by in-gel zymography (data not shown). It could also be due to an increased chemotaxis (data not shown) or a combination of both.

To determine whether the amoeboid invasion of A3 cell is ROCK dependent, we analyzed their invasive potential using a high-throughput Matrigel invasion assays in the presence or absence of the ROCK inhibitor Y-27632. Inhibition of ROCK in A3 cells using 10 μmol/L Y-27632 resulted in a 60% decrease in invasion through Matrigel (Fig. 6B) and complete inhibition of invasion into three-dimensional collagen (data not shown). Interestingly, the invasiveness of K2 cells was enhanced in the presence of 10 μmol/L Y-27632 (Fig. 6B). A possible explanation for this enhancement could be the 1.7-fold elevation of MMP-2 activity after ROCK inhibition, as detected by in-gel zymography (data not shown). It could also be due to an increased chemotaxis (data not shown) or a combination of both.

To address the possibility of MMP-dependent invasion, we tested the invasion capacity of both A3 and K2 cells in the presence of a broad-spectrum metalloproteinase inhibitor, GM6001. Neither A3 nor K2 invasiveness was significantly changed by this inhibitor (Fig. 6B). Taken together, these results show that A3 mesenchymal sarcoma cells use the Rho/ ROCK-dependent amoeboid mode of invasion.

### Table 1. Protein Microarray Analysis of A3 and K2 Cells

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>Protein Name</th>
<th>Antibody</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeletal dynamics</td>
<td>−7.36</td>
<td>PAK1</td>
<td>Pan-specific</td>
</tr>
<tr>
<td>3.43</td>
<td>ROCK-1</td>
<td>Pan-specific</td>
<td>Rho-associated protein kinase 1</td>
</tr>
<tr>
<td>2.86</td>
<td>Rac1/cdc42</td>
<td>Pan-specific</td>
<td>Rho-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>2.82</td>
<td>PTEN</td>
<td>Pan-specific</td>
<td>S770 Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. Inhibitory phosphorylation</td>
</tr>
<tr>
<td>2.42</td>
<td>ACK1</td>
<td>Pan-specific</td>
<td>Activated p21 cdc42Hs protein-serine kinase</td>
</tr>
<tr>
<td>2.34</td>
<td>Caveolin 2</td>
<td>Pan-specific</td>
<td>Caveolin 2</td>
</tr>
<tr>
<td>−1.96</td>
<td>Catenin b</td>
<td>Pan-specific</td>
<td>Catenin (cadherin-associated protein) β1</td>
</tr>
<tr>
<td>−1.94</td>
<td>Integrin b1</td>
<td>Pan-specific</td>
<td>Integrin β1 (CD29). S785 dephosphorylation promotes cell migration.</td>
</tr>
<tr>
<td>1.94</td>
<td>Paxillin</td>
<td>Pan-specific</td>
<td>S785 Phosphorylation</td>
</tr>
<tr>
<td>1.88</td>
<td>Cofilin</td>
<td>Pan-specific</td>
<td>Cofilin 1</td>
</tr>
<tr>
<td>1.84</td>
<td>LIMK1</td>
<td>Pan-specific</td>
<td>LIM domain kinase 1</td>
</tr>
<tr>
<td>Cell growth</td>
<td>5.53</td>
<td>MEK5</td>
<td>Pan-specific</td>
</tr>
<tr>
<td>3.18</td>
<td>RSK1</td>
<td>Pan-specific</td>
<td>Ribosomal S6 protein-serine kinase 1</td>
</tr>
<tr>
<td>−2.71</td>
<td>RaB1</td>
<td>Pan-specific</td>
<td>RaB1 proto-oncogene – encoded protein-serine kinase</td>
</tr>
<tr>
<td>2.46</td>
<td>MEK3</td>
<td>Pan-specific</td>
<td>MAP kinase protein-serine kinase 3 (MKK3)</td>
</tr>
<tr>
<td>2.24</td>
<td>MEK7</td>
<td>Pan-specific</td>
<td>MAP kinase protein-serine kinase 7 (MKK7)</td>
</tr>
<tr>
<td>2.15</td>
<td>PRKAB1</td>
<td>Pan-specific</td>
<td>5′-AMP – activated protein kinase (AMPK), β1 regulatory subunit</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>3.17</td>
<td>Smad2/3</td>
<td>Pan-specific</td>
</tr>
<tr>
<td>2.7</td>
<td>Jun</td>
<td>Pan-specific</td>
<td>Jun proto-oncogene – encoded API transcription factor</td>
</tr>
<tr>
<td>2.13</td>
<td>p27 Kip1</td>
<td>Pan-specific</td>
<td>p27 cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>5.87</td>
<td>CASP1</td>
<td>Pan-specific</td>
</tr>
<tr>
<td>4.11</td>
<td>CytoC</td>
<td>Pan-specific</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>2.73</td>
<td>Trai</td>
<td>Pan-specific</td>
<td>Tumor necrosis factor–related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>2.62</td>
<td>RIP2/RICK</td>
<td>Pan-specific</td>
<td>Receptor-interacting serine/threonine-protein kinase</td>
</tr>
<tr>
<td>−12.17</td>
<td>JNK2</td>
<td>Pan-specific</td>
<td>Jun NH2-terminal protein-serine kinases (stress-activated protein kinase) 2</td>
</tr>
<tr>
<td>3.1</td>
<td>Syk</td>
<td>Pan-specific</td>
<td>Spleen protein-tyrosine kinase</td>
</tr>
<tr>
<td>2.94</td>
<td>STAT1/2/3</td>
<td>Pan-specific</td>
<td>Signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>2.9</td>
<td>CaMK VI</td>
<td>Pan-specific</td>
<td>Serine/threonine-protein kinase DCAK1L1</td>
</tr>
<tr>
<td>2.75</td>
<td>IKKg/NEMO</td>
<td>Pan-specific</td>
<td>1-βB kinase γ/β essential modulator</td>
</tr>
<tr>
<td>2.65</td>
<td>STAT5A</td>
<td>Pan-specific</td>
<td>Signal transducer and activator of transcription 5A</td>
</tr>
<tr>
<td>2.54</td>
<td>VEGFR2</td>
<td>Y1054 + Y1059</td>
<td>Vascular endothelial growth factor receptor-tyrosine kinase 2. Activating phosphorylation.</td>
</tr>
<tr>
<td>Others</td>
<td>8.41</td>
<td>TyrOH/TH</td>
<td>Pan-specific</td>
</tr>
<tr>
<td>3.64</td>
<td>RONa</td>
<td>Pan-specific</td>
<td>Macrophase-stimulating protein receptor α chain</td>
</tr>
<tr>
<td>2.22</td>
<td>APG2</td>
<td>Pan-specific</td>
<td>Hop70-related heat shock protein 4</td>
</tr>
</tbody>
</table>

NOTE: Cell lysates of A3 and K2 cells were prepared according to Kinexus company guidelines and the expression (350 antibodies) and phosphorylation (250 antibodies) of selected signaling proteins were tested using Kinexus microarrays. Expression or phosphorylation levels in the A3 cells were normalized by the levels in the K2 cells, yielding a ratio for each tested protein. Table 1 is limited to proteins with a fold change of >1.8 or <−1.8. The list presents the average of two experiments.
A3 Cells Generate Higher Forces at the Leading Edge

It has been proposed that an up-regulation of ROCK in cells that undergo an amoeboid mode of invasion increases the actomyosin force generation that in turn enhances the ability to invade and migrate through the extracellular matrix (3); however, data in support of this hypothesis are still lacking. To measure the tractions that K2 and A3 cells exert on their surroundings, cells were plated onto collagen-coated 6.8% acrylamide/0.24% bis-acrylamide gels with a Young’s modulus of 13 kPa that corresponds approximately to the stiffness of mammalian soft tissue (22, 23). The cell tractions were computed from the gel deformation measured by the displacement of fluorescent beads with a diameter of 0.5 μm embedded at the gel surface (24). Only polarized cells were used for the analysis. The traction forces at the leading edge in A3 cells were seven times higher compared with K2 cells (Fig. 7A), and the overall traction forces (averaged over the entire cell area) were five times higher in A3 cells (data not shown). Interestingly, in A3 cells the tractions were concentrated at the cell periphery, with prominent localization at the leading edge (Fig. 7B), coinciding with the regions of highest P-MLC concentration. In K2 cells, the tractions were concentrated at the tapered rear end, presumably at sites where long-ranging, tension-carrying stress fibers terminate and connect to the matrix through focal adhesions that are slow to deadhere. Figuratively, cell movements seem to be driven by a front-wheel drive in A3 cells and impeded by a rear-wheel break in K2 cells. This interpretation of tension-transmitting matrix connections that cycle rapidly in A3 cells but are more stable in K2 cells is consistent with our finding of increased cytoskeletal dynamics in A3 versus K2 cells.

Discussion

In carcinoma cells, the amoeboid cell motility promoted by an up-regulation of ROCK has been shown to correlate with an increased metastatic potential (25, 26). Consistently, other studies have shown that ROCK inhibition reduced the in vivo invasive behavior of tumor cells (27, 28), most probably through a reduction in amoeboid tumor cell movement. In this study, we have shown that the high metastatic capacity of A3 cells in vivo corresponds to a greatly increased invasiveness in a
three-dimensional collagen matrix. In addition, we found that the traction forces generated at the leading edge of A3 cells are several fold greater than in their parental nonmetastatic K2 cells.

It has been hypothesized previously that an up-regulation of ROCK in cells that undergo an amoeboid mode of invasion increases actomyosin forces that in turn enhance the ability to invade and migrate through the extracellular matrix (3, 29). Wyckoff et al. (5) reported that the traction forces generated by invading MTLn3E cells are comparable with forces generated by fish keratinocytes. However, an increase of actomyosin force generation in metastatic cells that use an amoeboid mode of invasion in comparison with nonmetastatic control cells has not yet been shown. Our sarcoma model of metastasis based on highly metastatic A3 and nonmetastatic parental K2 cell line offers an ideal approach to this problem. We used traction microscopy to quantify the forces (magnitude, direction, and spatial distribution) that A3 and K2 cells exert on their extracellular matrix. We found that the traction forces in A3 cells were concentrated at the cell periphery, predominantly at the leading edge coinciding with P-MLC. The preferential localization of traction forces at the leading edge of A3 cells is indicative of forces that facilitate a forward protrusive movement. Taken together, these findings strongly indicate that the functional up-regulation of Rho/ROCK signaling in A3 cells causes an increase in protrusive force generation and therefore an increase in cell invasiveness.

We have shown that A3 cells exhibit an amoeboid morphology in three-dimensional collagen that is characteristic of an amoeboid mode of migration (2-5). However, the non-metastatic K2 cells also adopt rounded, amoeboid-like morphology in three-dimensional collagen. We speculate that the rounded morphology of K2 cells in three-dimensional collagen could be due to a dormant potential of increased Rho activity that is somewhat enhanced by a selective mechanism that leads to an increase in cell invasiveness.

![FIGURE 4. Enhanced colocalization of large cortical F-actin structures and P-MLC in A3 cells. A. Epifluorescent microscopy of K2 and A3 cells visualized with Alexa-594-phalloidin (green pseudocolor) and Alexa-488 P-MLC2 antibody (red pseudocolor). White arrows, two examples of large cortical F-actin structures in K2 cells without a P-MLC2 colocalization; yellow arrows, two examples of F-actin and P-MLC2 colocalization in A3 cells. B. Quantification of colocalization of large cortical F-actin structures and P-MLC2 in K2 and A3 cells. P-MLC2 signal at places of large cortical F-actin structure was considered to be colocalized when the average pixel intensity of the P-MLC2 signal was at least 50% above the average cellular P-MLC2 pixel intensity. Columns, mean of three experiments; bars, SD. n values represent number of F-actin structures tested. The P value was determined from a two-tailed t test. C. Confocal sections at substrate (Sub.) and midcellular (Mid.) levels of polarized K2 and A3 cells visualized with Alexa-488-phalloidin (green pseudocolor), Alexa-546 P-MLC2 antibody (red pseudocolor), and Alexa-633 phospho-paxillin (P-paxillin) antibody (yellow pseudocolor). An interference reflection microscopy image is shown for substrate level sections. Scale bars, 25 μm.](mcr.aacrjournals.org)
to the high invasive phenotype of the A3 cells. To summarize, a rounded morphology of transformed cells invading three-dimensional matrix may not be sufficient for effective amoeboid invasiveness and is therefore not a reliable marker of an effective amoeboid mode of invasion.

Cancer cells can invade three-dimensional matrices by distinct mechanisms, recently defined by their use of extracellular proteases (3, 4). On treatment with protease inhibitors, some tumor cells undergo a mesenchymal to amoeboid transition that allows them to invade in the absence of pericellular proteolysis and matrix degradation (4). Results obtained from studies with carcinoma cells suggest that cells using an amoeboid invasion mode can generally not switch to a mesenchymal mode. Interestingly, K2 cells seem to exhibit some signs of shift toward the mesenchymal mode on addition of the ROCK inhibitor. The addition of the ROCK inhibitor resulted in increased in vitro invasiveness that was accompanied by a 1.7-fold elevation of active MMP-2 secretion (data not shown). Moreover, the closely related src-transformed K4 cells, a clonal line derived from the same parental spontaneously transformed rat fibroblasts as K2 cells, exhibit high MMP activity and morphologically resemble cells with a mesenchymal mode of invasion (data not shown).

FIGURE 5. A3 cells prefer individual migration with amoeboid-type morphology. A. Subconfluent A3 and K2 cells were collected, loaded at a density of 10^4 per well in 0.5% agarose gel, allowed to migrate under the gel for 16 h, and visualized using phase contrast. Black line, boundary between well and agarose. B. F-actin stained with Alexa-594-phalloidin in K2 and A3 cells under the same condition as in A. C. F-actin stained with Alexa-594-phalloidin in A3 and K2 cells that were plated under nonrestricted conditions on glass-bottomed Petri dish. D. Amoeboid morphology of K2 and A3 cells invading a three-dimensional collagen matrix (representative modulation contrast image recorded at an invasion depth of 50 μm). Right, mesenchymally invading 786-O kidney cells. Scale bars, 50 μm.
Materials and Methods

Cultivations and Cell Assays

Cell Culture. K2 cells, full name LW13K2, are spontaneously transformed rat embryonic fibroblasts (34). The A3 cells, full name A337/311RP, were developed from K2 by neoplastic progression in vivo and in vitro (7). K2 and A3 cells were maintained in MEM with Hanks’ salts supplemented with 10% bovine serum (ZVOS), 0.09% sodium bicarbonate, 0.12 g/L sodium pyruvate, and 1 mmol/L glutamine at 37°C with 5% CO₂.

Collagen Invasion Assays. Collagen R (Serva) and collagen G (Biochrom) were mixed at a ratio of 1:1, and 25 mmol/L sodium bicarbonate and 10 vol% of 10× DMEM were added. The solution was neutralized with 1 mol/L NaOH. Collagen solution (1.2 mL) was placed into each well of a six-well plate, polymerized at 37°C, and incubated with Hank’s MEM (H-MEM). After 24 h, 10⁵ tumor cells were added and cultured in 2 mL H-MEM. After 3 d, the invasion assay was stopped by fixation with a 2.5% glutaraldehyde solution. The number of invaded tumor cells and their invasion depth was determined in 12 randomly selected fields of view. Invaded cells were imaged using DMI Leica microscope (40×0.6 HCX Fluotar objective) mounted by CCD camera (ORCA ER, Hamamatsu) and analyzed with Wasabi software.

Matrigel Invasion Assays. Matrigel invasion assays were carried out using Biocoat Matrigel invasion chamber inserts (BD Biosciences) according to the manufacturer’s protocol. Briefly, membranes were rehydrated for 2 h in H-MEM and placed in wells containing 10% fetal bovine serum in H-MEM. A total of 10⁵ cells suspended in 0.5 mL serum-free H-MEM were added to the top of each chamber (Matrigel coated or control chambers) and then incubated at 37°C in 5% CO₂. After 48 h, the chambers were washed with PBS. Invading cells were fixed in 3.7% formaldehyde for 1 h and stained with 4’,6-diamidino-2-phenylindole. Representative fields were documented by photomicroscopy and the numbers of invading cells were determined from replicate chambers by counting 10 random areas at ×200 magnification.

Under-Agarose Assay. This assay was done as described by Heit and Kubes (35) with the following minor modifications. SeaKem GTG agarose (0.5%) made with 50% H-MEM with 0.09% sodium bicarbonate, 0.12 g/L sodium pyruvate, and 1 mmol/L glutamine at 37°C with 5% CO₂ was neutralized with 1 mol/L NaOH. Collagen R (Serva) and collagen G (Biochrom) were mixed at a ratio of 1:1, and 25 mmol/L sodium bicarbonate and 10% vol% of 10× DMEM were added. The solution was neutralized with 1 mol/L NaOH. The solution was neutralized with 1 mol/L NaOH. Collagen solution (1.2 mL) was placed into each well of a six-well plate, polymerized at 37°C, and then incubated at 37°C in 5% CO₂. After 48 h, the chambers were washed with PBS. Invading cells were fixed in 3.7% formaldehyde for 1 h and stained with 4’,6-diamidino-2-phenylindole. Representative fields were documented by photomicroscopy and the numbers of invading cells were determined from replicate chambers by counting 10 random areas at ×200 magnification.

Protein Analysis Techniques

In-Gel Gelatin Zymography. In total, 2 × 10⁵ cells were plated per well of a 24-well plate. After 16 h, cells were washed with PBS and incubated in 300 μL of serum-free medium for 72 h. Aliquots (25 μL) of the conditioned medium were loaded for zymography on a 10% SDS-PAGE gel containing 1 mg/mL gelatin as described (36). Briefly, gel proteins were washed for 1 h in 50 mmol/L Tris-HCl (pH 7.5), 0.1 mol/L NaCl, and 2.5% Triton X-100 and then incubated at 37°C in 50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L CaCl₂, and 0.02% sodium azide for 17 h. The gels were stained with Coomassie blue and destained in 7% acetic acid/5% methanol.

In this study, we have shown for the first time that highly metastatic mesenchymal sarcoma cells can primarily use the Rho/ROCK-dependent amoeboid mode of invasion. Indeed, previous studies have suggested that Rho/ROCK is involved in controlling the invasion of mesenchymal cells (30-33). We speculate that the metastasis formation of many tumors of mesenchymal origin is promoted by a Rho/ROCK-dependent metastatic mesenchymal sarcoma cells can primarily use the Rho/ROCK-dependent amoeboid mode of invasion. Indeed, previous studies have suggested that Rho/ROCK is involved in controlling the invasion of mesenchymal cells (30-33). We speculate that the metastasis formation of many tumors of mesenchymal origin is promoted by a Rho/ROCK-dependent amoeboid mode of invasion. Determination of the invasion mode that prevails in a particular mesenchymal tumor, together with a more extensive use of Rho/ROCK inhibitors, could significantly improve the effectiveness of mesenchymal tumor treatment in the future.
Protein Microarrays. Antibody microarray analyses to determine the changes in the expression and phosphorylation state of signaling proteins were done by the Kinex Antibody Microarray service (Kinexus Bioinformatics Corp.).

Microscopy Techniques

FITC-Gelatin Zymography. Coverslips were coated with FITC-gelatin (1 mg/mL), air dried, rehydrated with water for 15 min in 4°C, and fixed with 0.5% glutaraldehyde for 30 min in 4°C. Cells were grown on FITC-gelatin–coated coverslips for 48 h and then fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, washed thrice with PBS, blocked in 3% bovine serum albumin, and then stained with Alexa-594-phalloidin (Molecular Probes) in blocking solution for 15 min. Coverslips were then washed four times and mounted.

Immunofluorescence Microscopy. Cells were fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, washed thrice in PBS, and then blocked in 3% bovine serum albumin, incubated with primary antibody for 3 h, washed, and incubated with secondary antibody for 60 min, and then washed again, stained for 15 min with Alexa-594-phalloidin or Alexa-488-phalloidin (Molecular Probes), and mounted. The primary antibodies were mouse P-MLC (Ser19) and rabbit phospho-paxillin (Tyr118; both from Cell Signaling Technology) and secondary anti-rabbit (Alexa-488 and Alexa-546) and anti-mouse (Alexa-633) antibodies (Molecular Probes). Interference reflection images were visualized using the 488-nm line of an argon laser with no emission filter in front of the detector.

Bright-field and epifluorescent images were captured using a Nikon Eclipse TE2000-S microscope (10×/0.25 and 20×/0.45 Plan Fluor objectives) mounted with VDS Vosskühler CCD-1300 camera and analyzed with Nikon Elements software. Confocal images were acquired by Leica TCS SP2 microscope system using Leica 63×/1.45 oil objective.

Biophysical Techniques

Nanoscale Particle Tracking. Polystyrene beads coated with human fibronectin were attached for 30 min to confluent K2 or A3 cells, and their position was tracked for 5 min from bright-field images recorded with a CCD camera (ORCA ER) at a frame rate of 8.3 Hz. An intensity-weighted center-of-mass algorithm was used to determine the bead positions with 10-nm

![Figure 7](image-url)
accuracy (37). An apparent diffusivity D* was calculated as the
squared distance a bead had traveled during a 1-s time interval,
averaged over all recording times and beads (16, 38).

Magnetic Tweezers. A staircase-like sequence of step forces
ranging from 0.5 to 10 nN was applied to superparamagnetic
4.5-μm epoxylated, fibronectin-coated beads (Invitrogen)
using magnetic tweezers as described by Mierke et al. (18).
Measurements were done after 30 min of bead incubation.
Bright-field images were taken at 40 frames per second, and
the bead positions were again tracked using an intensity-weighted
center-of-mass algorithm. The creep response J(t) of the cells
followed a power law in time, J(t) = a(t/t_0)^b, where the
prefactor a and the power-law exponent b were both force
dependent, and the reference time t_0 was set to 1 s. The bead
displacement in response to a staircase-like force followed a
superposition of power laws (39), from which the power-law
exponent b was determined by a least-squares fit.

Traction Force Microscopy. Gels for traction experiments
were cast on silane-coated glass slides according to the
procedure described by Wang and Pelham (40). Gels with
6.8% acrylamide/0.24% bis-acrylamide were used. The
Young’s modulus of the gels was measured with a magnetically
driven plate rheometer and found to be 13 kPa. Yellow-green
fluorescent 0.5-μm carboxylated beads (Molecular Probes)
were suspended in the gels and centrifuged at 300 × g toward
the gel surface during polymerization at 4°C. These beads
were suspended as markers for gel deformations. The surface of the gel
was activated with sulfo-SANPAH (Pierce Biotechnology)
and coated with 50 μg/mL bovine collagen G (Biochrom).
The cell suspension added to the gel was contained using a
silicone ring (In Vitro) attached to the glass slide. Cell trac-
tions were computed from an unconstrained deconvolution
(24) of the gel surface displacement field measured before
and after cell detachment with an 8 μmol/L cytochalasin D
and trypsin/EDTA (0.25/0.02%) in PBS. During measurement,
cells were maintained at 37°C in humidified atmosphere
containing 5% CO2. Gel deformations were estimated using
a Fourier-based difference-with-interpolation image analysis
(24, 38).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Robin A. Weiss, Steven K. Hanks, James Smith, and members of the
Petr Folk laboratory for helpful comments and discussion and Marie Charvatova
for technical support.

References
26:163–73.
87–96.
3. Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct
requirements for Rho/ROCK signalling and extracellular proteolysis. Nat Cell
migration: mesenchymal-amoeboid transition after blocking of pericellular
5. Wyckoff JB, Pinner SE, Gschmeissner S, Condeelis JS, Sahai E. ROCK- and
myosin-dependent matrix deformation enables protease-independent tumor-cell
6. Vial E, Sahai E, Marshall CJ. ERK-MAPK signaling coordinately regulates
dependence distinguishes mesenchymal and amoeboid modes of tumour cell
promotes invasiveness of Src-transformed cells. Oncogene 2004;23:
7406–15.
10. Zhao ZS, Manser E. PAK and other Rho-associated kinases—effectors
with surprisingly diverse mechanisms of regulation. Biochem J 2005;386:
201–14.
Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to
12. Sahai E, Marshall CJ. ROCK and Dia have opposing effects on adhesions
13. Danley DE, Chung TH, Bokoch GM. Defective Rho GTPase regulation by
IL-1β-converting enzyme-mediated cleavage of D4 GDP dissociation inhibitor.
17. Alenghat FJ, Fabry B, Tsai KY, Goldmann WH, Inger DE. Analysis of cell
mechanics in single vinculin-deficient cells using a magnetic tweezer. Biochem
WH. Mechanosensing and regulation of contractility by the vinculin tail domain.
Biophys J 2008;94:661–70.
characterizing cells of spontaneously metastasizing K2M rat sarcoma. Folia Biol
20. Laevsky G, Knecht DA. Cross-linking of actin filaments by myosin II is a
major contributor to cortical integrity and cell motility in restrictive environments.
endothelial barrier function in tumor cell transmigration. Biophys J 2008;94:
2832–46.
22. Zheng Y, Mak AF. Effective elastic properties for lower limb soft tissues
from manual indentation experiment. IEEE Trans Rehabil Eng 1999;7:
257–67.
23. Samani A, Plewes D. An inverse problem solution for measuring the
elastic modulus of intact ex vivo breast tissue samples. Phys Med Biol 2007;52:
1247–60.
24. Butler JP, Tolic-Norrelykke IM, Fabry B, Fredberg JJ. Traction fields,
moments, and strain energy that cells exert on their surroundings. Ann J Physiol
25. Wyckoff JB, Jones JG, Condeelis JS, Segall JE. A critical step in meta-
stasis: in vivo analysis of intravasation at the primary tumor. Cancer Res 2000;60:
2504–11.
primary mammary tumors correlated with gene expression patterns revealed by
27. Ishk K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An
essential part for Rho-associated kinase in the transcellular invasion of tumor
S. Inhibition of intrahepatic metastasis of human hepatocellular carci-
33:577–81.
29. Zhao ZS, Manser E. PAK and other Rho-associated kinases—effectors
with surprisingly diverse mechanisms of regulation. Biochem J 2005;386:
201–14.
genes in highly and low metastatic rat osteosarcomas by a modified cDNA-AFLP


