ARTICLE
Journal of Cellular Biochemistry 114:1385–1394 (2013)

Journal of Cellular Biochemistry

The RhoA Pathway Mediates MMP-2 and MMP-9-Independent Invasive Behavior in a Triple-Negative Breast Cancer Cell Line

Katerina D. Fagan-Solis,[1*] Sallie Smith Schneider,[2] Brian T. Pentecost,[3] Brook A. Bentley,[4] Christopher N. Otis,[4] John F. Gierthy,[5] and Kathleen F. Arcaro[6*]

[1]*Molecular and Cellular Biology Graduate Program, University of Massachusetts Amherst, Amherst, Massachusetts*
[2]*Pioneer Valley Life Sciences Institute, Springfield, Massachusetts*
[3]*Wadsworth Center, New York State Department of Health, Albany, New York*
[4]*Department of Pathology, Baystate Medical Center, Springfield, Massachusetts*
[5]*Consultant; East Greenbush, New York*
[6]*Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Amherst, Massachusetts*

ABSTRACT

Breast cancer is a heterogeneous disease that varies in its biology and response to therapy. A foremost threat to patients is tumor invasion and metastasis, with the greatest risk among patients diagnosed with triple-negative and/or basal-like breast cancers. A greater understanding of the molecular mechanisms underlying cancer cell spreading is needed as 90% of cancer-associated deaths result from metastasis. We previously demonstrated that the Tamoxifen-selected, MCF-7 derivative, TMX2-28, lacks expression of estrogen receptor α (ERα) and is highly invasive, yet maintains an epithelial morphology. The present study was designed to further characterize TMX2-28 cells and elucidate their invasion mechanism. We found that TMX2-28 cells do not express human epidermal growth factor receptor 2 (HER2) and progesterone receptor (PR), in addition to lacking ERα, making the cells triple-negative. We then determined that TMX2-28 cells lack expression of active matrix metalloproteinases (MMPs)-1, MMP-2, MMP-9, and other genes involved in epithelial–mesenchymal transition (EMT) suggesting that TMX2-28 may not utilize mesenchymal invasion. In contrast, TMX2-28 cells have high expression of Ras Homolog Gene Family Member, A (RhoA), a protein known to play a critical role in amoeboid invasion. Blocking RhoA activity with the RhoA pathway specific inhibitor H-1152, or a RhoA specific siRNA, resulted in inhibition of invasive behavior. Collectively, these results suggest that TMX2-28 breast cancer cells exploit a RhoA-dependent, proteolytic-independent invasion mechanism. Targeting the RhoA pathway in triple-negative, basal-like breast cancers that have a proteolytic-independent invasion mechanism may provide therapeutic strategies for the treatment of patients with increased risk of metastasis. J. Cell. Biochem. 114: 1385–1394, 2013.

KEY WORDS: AMOEBOID; MESENCHYMAL; BASAL-LIKE; TMX2-28; RhoA; BREAST CANCER; INVASION

Metastasis is dependent upon cancer cells being able to invade surrounding extra cellular matrix (ECM) and adapt to different microenvironments within the primary tumor, the ECM, blood, and/or lymphatic systems, and finally, to establish a new niche in a distant tissue [Cairns et al., 2003; Friedl and Wolf, 2003a]. The proteins that control cell–cell and cell–ECM interactions are thought to play a role in tumor cell invasion and metastasis by controlling cell morphology, motility, and interactions with the tumor microenvironment [Cairns et al., 2003]. Thus, such proteins provide attractive therapeutic targets.

Two distinct morphological/functional mechanisms are known for single cell migration in tissues: fibroblast-type mesenchymal invasion, and leucocyte-type amoeboid invasion. Mesenchymal movement is characterized by an elongated cellular shape, and

The authors declare that they have no conflict of interest or competing financial interests.
Grant sponsor: Rays of Hope Foundation; Grant sponsor: Predoctoral Traineeship Award, DoD Breast Cancer Research Program; Grant number: W81XWH-09-1-0345.
*Correspondence to: Katerina D. Fagan-Solis, Ph.D. or Kathleen F. Arcaro, Ph.D., Morrill I N441, 637 North Pleasant Street, Amherst, MA 01003. E-mail: katerina.fagansolis@gmail.com; karcaro@vasci.umass.edu
Manuscript Received: 4 December 2012; Manuscript Accepted: 6 December 2012
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 17 December 2012
DOI 10.1002/jcb.24480 • © 2012 Wiley Periodicals, Inc.

involves the use of proteases that cause cellular lysis in tissues, thereby creating a path through which cells can invade [Friedl and Wolf, 2003b; Wolf et al., 2003; Sahai, 2005]. In this kind of motility, cell speed is relatively slow (0.1–1 μm/min) and is dependent on actin polarization and lamellipodium formation for directionality of cell movement [Etienne-Manneville and Hall, 2001; Ridley et al., 2003].

Amoeboid migrating cells have a rounded morphology and their movement is protease-independent. Instead, cells find paths through the ECM by pushing and squeezing through regions of adequate size [Friedl and Wolf, 2003a; Sahai and Marshall, 2003; Wolf et al., 2003; Parri and Chiarugi, 2010]. In this kind of motility, cells exploit the propulsory forces resulting from acto-myosin cytoskeleton contractility, which results in very high migratory speeds (up to 4 μm/min). Amoeboid movement occurs independently of cell polarization but requires RhoA-ROCK signaling to promote the rapid remodeling of the cell cortex [Friedl and Wolf, 2003b; Sahai and Marshall, 2003; Wyckoff et al., 2006; Friedl and Wolf, 2010].

Matrix Metalloproteinases (MMPs) are a family of endopeptidases capable of degrading ECM components. MMPs are synthesized and secreted as zinc dependent proenzymes that require activation prior to becoming proteolytically active [Curran and Murray, 2000]. It is known that MMP-1, MMP-2, and MMP-9 are responsible for degrading fibrillar collagen as well as collagen type IV, the primary components of the connective tissue matrix and basement membranes, respectively [Iwata et al., 1996; Sassi et al., 2000]. A number of studies have shown that there is an association between high MMP-2 and MMP-9 expression and the invasiveness of breast tumors when compared to normal breast epithelium. Cells undergoing epithelial to mesenchymal transition (EMT) in particular have high expression of MMP-2 and MMP-9 [Duffy et al., 2000; Bartsch et al., 2003].

Migration of cancer cells is dependent on Ras Homolog Gene Family, Member A (RhoA) signaling [Friedl and Wolf, 2003a; Sahai and Marshall, 2003; Wolf et al., 2003; Parri and Chiarugi, 2010]. RhoA is a member of the Rho superfamily of GTPases that acts as a molecular switch to control signal transduction [Jaffe and Hall, 2005]. It carries out its actions through activation of its two targets: Rho-associated, coiled-coil containing protein kinase (ROCK) 1 and ROCK 2, which in turn regulate different facets of actin dynamics including stress fiber assembly, cell contraction, actin-filament stabilization, and focal adhesion organization [Hanazaki et al., 2008; Narumiya et al., 2009]. Up-regulation of RhoA mRNA and protein is well documented for a variety of human cancers and has been positively correlated with cancer metastasis [Horiuchi et al., 2003; Faried et al., 2007; Bellizzi et al., 2008].

The role of RhoA and its effectors ROCK in cells that use mesenchymal motility is complex; activity of RhoA and Rock need to be reduced to extend protrusion at the front of the cell but they promote the retraction of the lagging end [Ridley et al., 2003]. Consequently, the overall effect of inhibiting RhoA/ROCK in cells that utilize a mesenchymal invasion mechanism is often minimal [Sahai and Marshall, 2003]. Conversely, RhoA activity is critical to the amoeboid invasion mechanism [Torka et al., 2006; Wyckoff et al., 2006].

In this study, we use the Tamoxifen-selected, MCF-7 derivative, TMX2-28 breast cancer cell line. TMX2-28 cells are ERα-negative and have acquired a mixed basal/luminal cytokeratin (CK) profile, suggestive of a more basal-like phenotype [Fasco et al., 2003; Gozgit et al., 2006, 2007]. Morphologically, TMX2-28 cells retained a rounded, epithelial cell shape similar to MCF-7. Behaviorally, TMX2-28 cells are highly invasive, as assessed through the ability to invade though Matrigel, when compared to MCF-7 and MDA-MB-231 breast cancer cells [Gozgit et al., 2006]. These phenotypic characteristics suggest that these cells may exhibit an amoeboid mode of invasion. However, the importance of MMP-1, MMP-2, and MMP-9 in TMX2-28 cell invasion was not experimentally excluded nor was evidence of amoeboid invasion by these cells demonstrated.

A cDNA microarray comparing gene expression of TMX2-28 cells to their parent cell line, MCF-7, indicated that the Rho family member, RhoE is downregulated in TMX2-28 (Table I). RhoE was the first member of the Rho kinase family to be identified [Foster et al., 1996]. Unlike the other family members, RhoE does not act as a classic GTPase switch, as it does not hydrolyze GTP [Foster et al., 1996; Guasch et al., 1998]. RhoE functions by binding to and inhibiting the RhoA effector ROCK 1 (but not ROCK 2). This interaction also results in the phosphorylation of RhoE by ROCK 1, which increases the stability and activity of RhoE [Riento and Ridley, 2003; Riento et al., 2005]. In addition, RhoE binds to p190RhoGAP increasing its interaction with RhoA, thus promoting the formation of inactive GDP-bound RhoA [Wennerberg et al., 2003]. Given the inhibitory effects of RhoE on RhoA, RhoA's role in cell migration, and the knowledge that RhoE is downregulated in TMX2-28 cells, we asked whether expression and function of RhoA is altered in TMX2-28 cells, and if so does RhoA play a role in the invasiveness of TMX2-28 cells.

RESULTS

CHARACTERIZATION OF TMX2-28 RECEPTOR STATUS

ERα, PR, and HER2 receptor expression are the most important prognostic factors for breast cancer, dictating a patient's therapeutic regime. Prior studies have shown that TMX2-28 cells lack expression of ERα [Gozgit et al., 2006, 2007], however the status of PR and HER2 expression was not examined. To determine the receptor status of TMX2-28 cells, ERα, PR, and HER2 expression was analyzed by qRT-PCR and immunohistochemistry (IHC). When compared to its ERα/PR positive, HER2 negative MCF-7 parent, the triple-negative MDA-MB-231, and the ERα/PR negative, HER2 overexpressing SKBR-3 cell lines, it is clear that TMX2-28 cells not only lack expression of ERα, but also lack expression of PR and HER2 (Fig. 1), making them a model for triple-negative, basal-like breast cancer.

TMX2-28 CELLS DO NOT EXPRESS MMP-1 mRNA OR ACTIVE MMP-2 OR MMP-9 PROTEIN

In breast cancer, MMPs have been shown to be important players in mesenchymal invasion, specifically MMP-1, MMP-2, and MMP-9 [Iwata et al., 1996; Sassi et al., 2000]. To determine the possible involvement of these MMPs in the invasion mechanism of TMX2-28 cells, MMP-1, MMP-2, and MMP-9 mRNA expression was analyzed

TABLE I. Genes associated With Cytoskeleton Organization, Protease Secretion, Adhesion, and Membrane Morphology Differentially Regulated in TMX2-28 as Compared With MCF-7

Gene name	Gene symbol	Accession number
Downregulated		
ADAM metallopeptidase with thrombospondin type 1 motif	ADAMTS19	NM_133638.1
Matrix metallopeptidase-like 1 (matrix metallopeptidase 25)	MMPL1 (MMP25)	NM_004142.1
Protease, serine, 23	SPUVE (PRSS23)	NM_007173.1
TIMP metallopeptidase inhibitor 1	TIMP1	NM_003254.1
TIMP metallopeptidase inhibitor 2	TIMP2	NM_003255.2
Neuronal cell adhesion molecule	NRCAM	NM_005010.1
Fibronectin leucine rich transmembrane protein 3	FLRT3	NM_013281.1
Fibroblast growth factor 13	FGF13	NM_004114.1
Selectin L	SELL	NM_000655.2
Ras homolog gene family, member E	RHOE	NM_001254738.1
EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	NM_004105.2
Carcinoembryonic antigen-related cell adhesion molecule 5	CEACAM5	NM_004363.1
Cathespin D	CTSD	NM_001909.4
Cathepsin F	CTSF	NM_003793.3
Cathepsin S	CTSS	NM_001199739.1
Integrin β8	ITGB8	NM_002214.2
Integrin α6	ITGA6	NM_000210.1
Upregulated		
Laminin, gamma 1	LAMC1	NM_002293.3
Laminin, beta 1	LAMB1	NM_002291.1
Vitronectin	VTN	NM_000638.1
Serpin peptidase inhibitor, clade E member 1	SERPINE1	NM_000602.3
Mitogen inducible gene 2	MIG2	NM_006832

http://www.nature.com/bjc/journal/v97/n6/suppinfo/6603926s1.html?url=/bjc/journal/v97/n6/full/6603926a.html; [Gozgit et al., 2007].

by qRT-PCR. As shown in Figure 2A–C, TMX2-28 cells have undetectable levels of MMP-1 mRNA expression when compared to the mesenchymal MDA-MB-231 cells. However, there was no significant difference in mRNA expression of MMP-2 or MMP-9 among the cell lines. MMPs are synthesized and secreted as zinc dependent pro-enzymes that require activation [Curran and Murray, 2000]. To assess activity of MMP-2 and MMP-9, zymography was preformed on conditioned media from all cell lines. It was determined that although TMX2-28 cells have mRNA expression, they do not express active MMP-2 or MMP-9 enzymes (Fig. 2D). These results indicate that TMX2-28 cells do not rely on the activity of MMP-1, -2, or -9 to degrade the ECM.

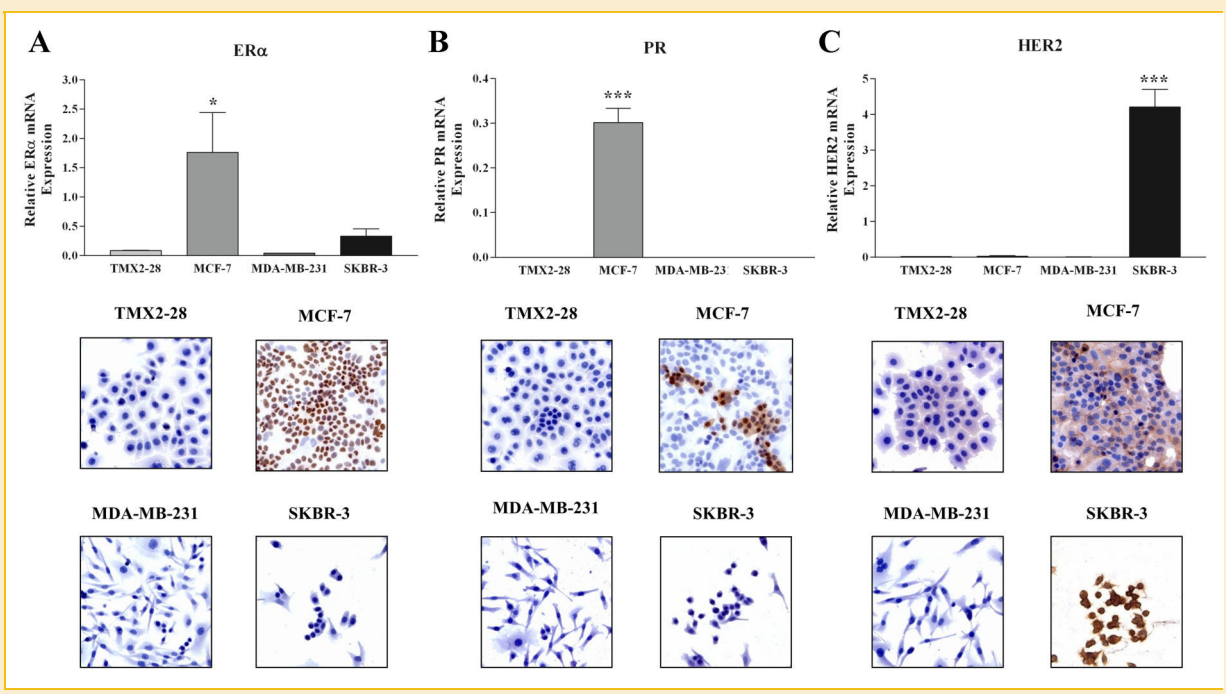

Fig. 1. TMX2-28 is a triple-negative derivative of the MCF-7 breast cancer cell line. Upper panel: Relative mRNA expression of (A) ERα, (B) PR, and (C) HER2 were determined by qRT-PCR and normalized to HPRT. Differences among cell lines were analyzed with one-way ANOVA and posthoc *t*-tests with Bonferroni correction; $^{*}P < 0.05$, $^{***}P < 0.001$. Lower panel: ERα, PR, and HER2 protein expression was determined by immunohistochemistry.

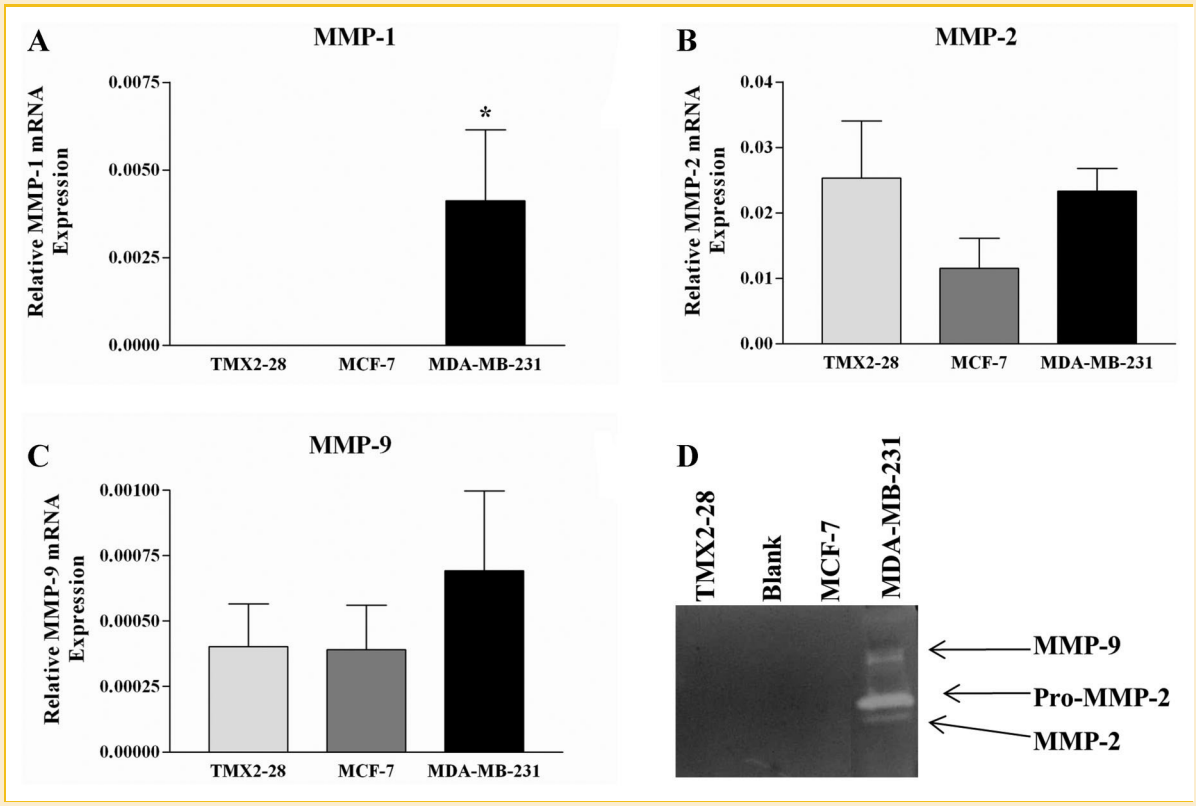

Fig. 2. TMX2-28 cells do not secrete active MMP-1, MMP-2, and MMP-9. Total RNA was isolated from TMX2-28, MCF-7, and MDA-MB-231 cells at 80% confluence. Relative mRNA expression of MMP-1 (A), MMP-2 (B), and MMP-9 (C) were determined by qRT-PCR and normalized to HPRT. Differences among cell lines were analyzed with one-way ANOVA and posthoc *t*-tests with a Bonferroni correction; $^{*}P < 0.05$. D: Conditioned media was collected from confluent cell cultures grown in serum-free medium for 48 h and used for MMP-2 and MMP-9 activity assessment.

TMX2-28 CELLS HAVE LITTLE TO NO EXPRESSION OF SIX COMMON EMT GENES

Epithelial–mesenchymal transition (EMT) is a well-recognized process facilitating migration and invasion of breast cancer cells during metastasis [Thiery, 2002]. An increase in expression of a number of genes including SLUG, ZEB1, ZEB2, Fibronectin, Vimentin, and N-Cadherin is characteristic of this process [Hajra et al., 2002; Agiostratidou et al., 2007; Kokkinos et al., 2007; Moreno-Bueno et al., 2008]. The mRNA levels of these genes were assessed by qRT-PCR to determine whether TMX2-28 cells have increased expression. Expression of mRNA to SLUG, ZEB1, ZEB2, Fibronectin and Vimentin, was low in TMX2-28 cell cultures (Table II), similar to that in the non-invasive parent cell line, MCF-7. The expression of N-Cadherin mRNA in TMX2-28 was slightly above that in MCF-7 cells, but was significantly less than in MDA-MB-231 cells. The expression of all six genes was significantly higher in the invasive, mesenchymal-like MDA-MB-231 cell line than in either the non-invasive MCF-7 or the invasive TMX2-28 cells. Together with results on MMP expression, these data suggest that TMX2-28 cells do not utilize the protease-dependent, mesenchymal invasion mechanism.

TABLE II. Relative mRNA Expression of Epithelial to Mesenchymal Genes in Cell Lines

Target	TMX2-28	MCF-7	MDA-MB-231
SLUG	$0.0039 \pm 8.8 \times 10^{-4}$***	0.0060 ± 0.002	1.8748 ± 0.464
ZEB1	0.0336 ± 0.008**	0.0098 ± 0.003	0.5865 ± 0.158
ZEB2	$0.0009 \pm 3.7 \times 10^{-4}$***§	$0.0013 \pm 6.1 \times 10^{-4}$	0.2202 ± 0.041
Fibronectin	0.0090 ± 0.004§*	1.1013 ± 0.364	0.7001 ± 0.042
Vimentin	$0.0005 \pm 3.7 \times 10^{-5}$**§	$0.0005 \pm 1.2 \times 10^{-4}$	0.5461 ± 0.139
N-Cadherin	0.6086 ± 0.282**	0.1414 ± 0.066	1.4790 ± 0.172

Data were analyzed using one-way ANOVAs and *t*-tests with Bonferroni corrections; for comparisons between TMX2-28 cells and MCF-7 cells.
§$P < 0.005$; for comparisons between TMX2-28 cells and MDA-MB-231 cells; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

RhoA PATHWAY GENES ARE OVEREXPRESSED IN TMX2-28 CELLS

Next, it was asked whether TMX2-28 cells exploit the Rho kinase pathway, specifically RhoA pathway signaling. RhoA mRNA was overexpressed in TMX2-28 cells at both the mRNA and protein levels (Fig. 3A) when compared to levels in MCF-7 and MDA-MB-231 cells. Additionally, the major downstream targets of RhoA, ROCK 1 and ROCK 2, were also found to be overexpressed in these cells (Fig. 3B,C). These data suggest that TMX2-28 cells may take advantage of RhoA-dependent, protease-independent invasion processes.

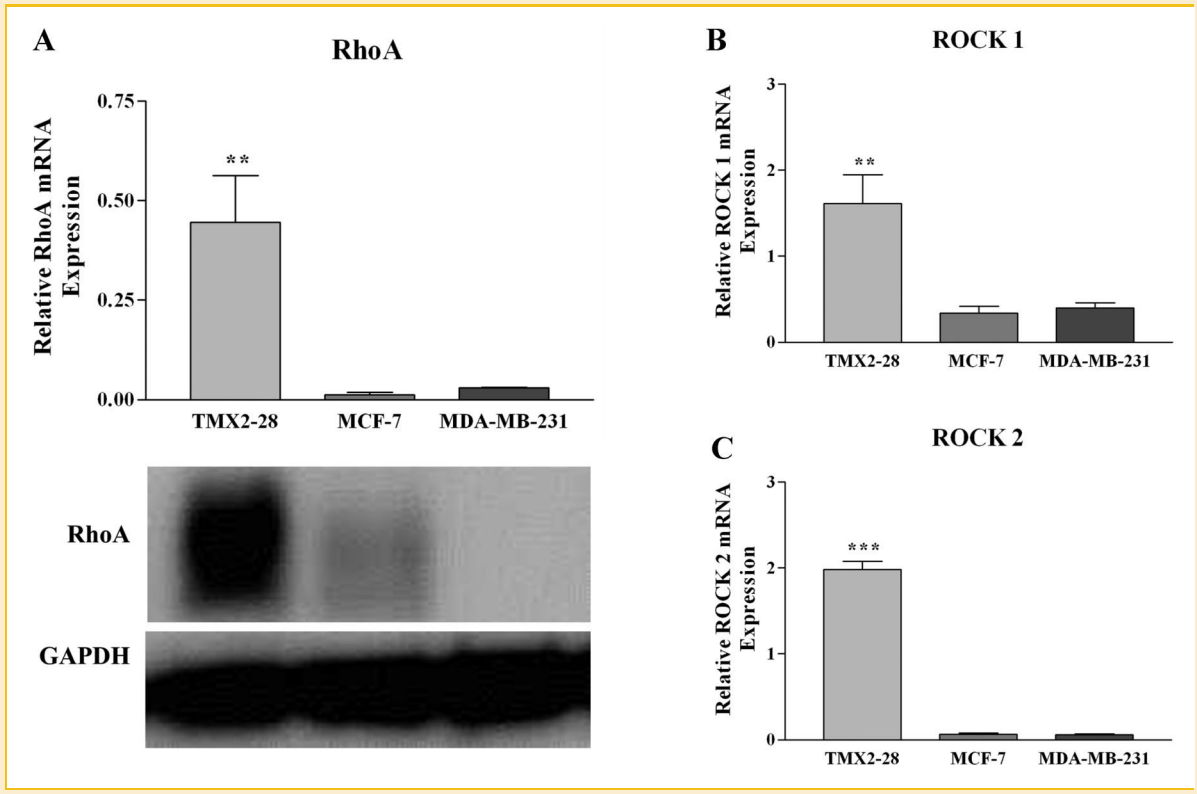

Fig. 3. Expression of RhoA, ROCK 1, and ROCK 2 are upregulated in TMX2-28 cells. Relative mRNA expression of (A) RhoA, (B) ROCK 1, and (C) ROCK 2 were determined by qRT-PCR and normalized to the housekeeping gene HPRT. Differences among cell lines were analyzed with one-way ANOVA and posthoc *t*-tests with a Bonferroni correction; $^{**}P < 0.01$. Protein lysates were probed for RhoA expression by Western immunoblotting (GAPDH used as loading control).

BLOCKING RhoA PATHWAY ACTIVITY DECREASED MIGRATORY AND INVASIVE BEHAVIORS

TMX2-28 and HeLa cells were treated with the ROCK inhibitor H-1152 to block RhoA pathway signaling and determine whether blocking the activity of RhoA resulted in impairment of migratory and invasive behavior. HeLa (RhoA dependent invasion control), and MCF-7 (non-invasive control) cells were used as positive and negative controls, respectively. As expected, blocking RhoA activity in the RhoA-dependent HeLa cells resulted in significant abrogation (~66%) of invasive behavior. When RhoA activity was blocked in TMX2-28 cells, there was significant inhibition of migration and invasion (Fig. 4). Migration was reduced by ~90% and ~94% when cells were treated with 20 and 100 μM of the inhibitor, respectively. In contrast, the reduction of invasion was dose-dependent, ~62% inhibition after treatment with 20 μM and ~92% inhibition after treatment with 100 μM.

To further investigate the effect of blocking RhoA signaling on migration and invasion, siRNA specifically targeting RhoA was used to knockdown expression in TMX2-28 and HeLa cells (Fig. 5A). Knockdown of RhoA in these cells resulted in a significant decrease in both migratory (50%) and invasive (52%) behavior (Fig. 5B), confirming the phenotypic changes observed when RhoA pathway signaling was blocked using H-1152 (Fig. 4). This effect was specific and was not seen when cells were transfected with the negative control siRNA. Together these data show that RhoA plays an important role in the ability of TMX2-28 cells to be highly invasive and migratory while maintaining an epithelial morphology.

DISCUSSION

Cancer cells can use alternative mechanisms of motility for invasion [Friedl and Wolf, 2003b; Wolf et al., 2003]. Two distinct mechanisms have been identified, the more widely utilized, protease-dependent, mesenchymal, and the less utilized, protease-independent, amoeboid movements [Friedl and Wolf, 2003b; Wolf et al., 2003; Sahai, 2005]. In breast cancer, MMP-1, MMP-2, and MMP-9 have been tied to mesenchymal cell movement [Bartsch et al., 2003], however blocking their activity was unsuccessful in preventing growth and metastasis of late stage cancers in clinical trials [Coussens et al., 2002; Sahai, 2005]. Additionally, Wolf et al. [2003] showed that mesenchymal MDA-MB-231 breast cancer cells adapted a round, epithelial-like morphology, and continued to be invasive when challenged with protease inhibitors. These studies suggest that breast cancer cells have the ability to switch to an amoeboid invasion mechanism. Thus, determining the mechanisms behind this mode of invasion is critical for the development of therapies to block the metastatic process.

The primary objective of the present study was to better define the molecular aspects underlying invasion, in TMX2-28 cells as a model

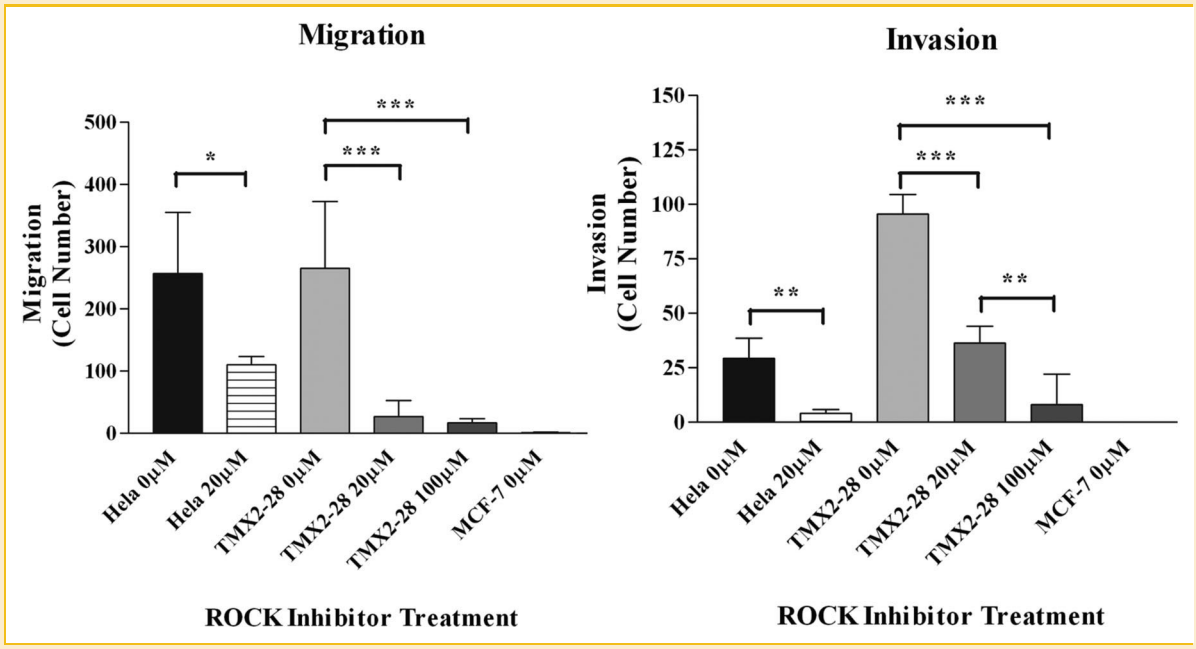

Fig. 4. Blocking RhoA kinase pathway activity with the ROCK inhibitor H-1152 reduces migratory and invasive behavior. TMX2-28, HeLa (positive control), and MCF-7 (negative control) were treated with H-1152 at 0, 20, and 100 μM concentrations for 24 h. Cells were then seeded into invasion chambers with basal media containing the respective concentration of inhibitor and incubated for 22–24 h. Cells were then fixed, stained, and counted. Differences among cell lines were analyzed with one-way ANOVA and posthoc *t*-tests with a Bonferroni correction; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of triple-negative, basal-like breast cancer. Previous studies of gene expression identified TMX2-28 cells as being ERα-negative and having a mixed basal/luminal CK expression profile. They were also characterized as being highly invasive [Fasco et al., 2003; Gozgit et al., 2006, 2007]; comparable to MDA-MB-231 cells, an established breast cancer cell line known for being highly invasive and exploiting the proteolytic-dependent, mesenchymal mode of invasion. The rounded, epithelial-like cell morphology of TMX2-28

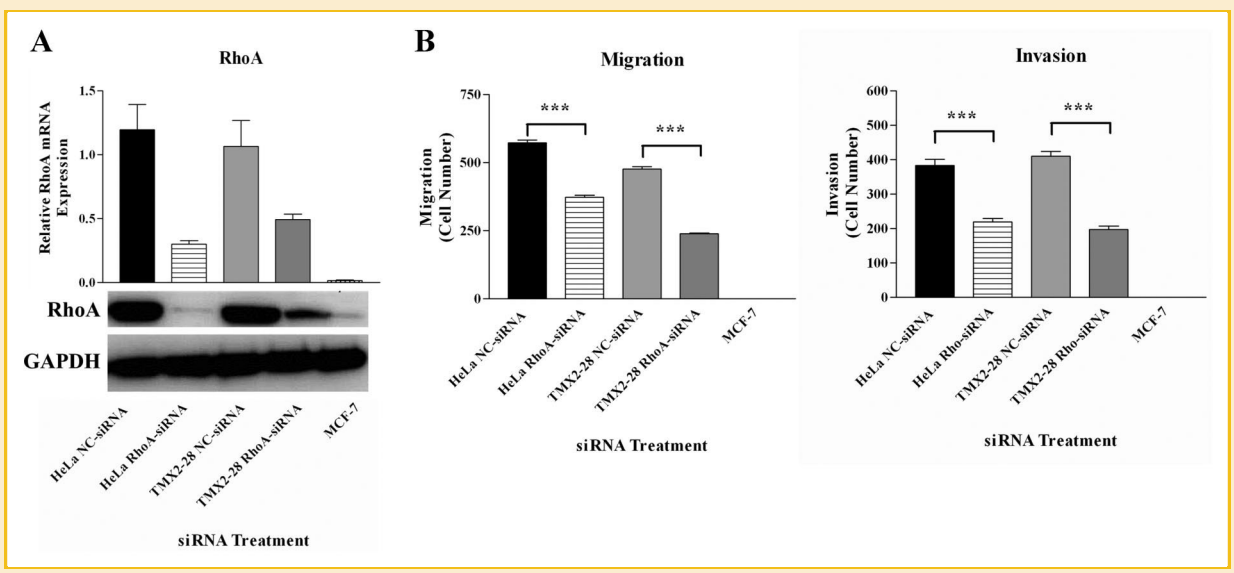

Fig. 5. RhoA knockdown in TMX2-28 cells results in inhibition of migration and invasion. TMX2-28 and HeLa cells were transiently transfected with either a negative control siRNA (NC-siRNA) or a RhoA specific siRNA (RhoA-siRNA; MCF-7 cells were not transfected). Forty-eight hours post-transfection cells were collected for either RNA/protein isolation or invasion assay. A: Relative mRNA expression of RhoA was determined by qRT-PCR and normalized to HPRT. Protein lysates (~20 μg) were probed for RhoA expression by Western blotting (GAPDH as loading control). B: Cells were seeded into invasion chambers, incubated for 22–24 h, fixed, stained, and counted. Differences among cell lines were analyzed with one-way ANOVA and posthoc *t*-tests with a Bonferroni correction; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cells suggested that they exploit an amoeboid mode of cellular invasion, however, this was not investigated [Gozgit et al., 2006].

In the present article, our initial inquiry focused on validating the triple-negative status of our model cell line, followed by experimentally excluding proteolytic pathways in TMX2-28 invasion. We determined that TMX2-28 cells are indeed triple-negative in their receptor status, that they do not express MMP-1 mRNA or active MMP-2 or MMP-9 protein, nor do they have expression of genes known to increase in expression during EMT. Interestingly, a cDNA microarray comparing gene expression of TMX2-28 cells to their parent cell line, MCF-7, indicated that a number of proteases, MMPs, and adhesion molecules are down-regulated in TMX2-28 (Table I) [Gozgit et al., 2006, 2007]. These data together with the lack of MMP-1, MMP-2, MMP-9, and EMT gene expression indicate that these MMPs and mesenchymal related genes are not critical to TMX2-28's invasion mechanism suggesting that they do not utilize a protease-dependent migration mechanism.

We then determined which pathways TMX2-28 cells use to migrate. cDNA microarray analysis indicated that RhoE, an inhibitor of RhoA is downregulated in TMX2-28 cells [Wennerberg et al., 2003]. It is known that RhoA signaling is important for many types of migration, and up-regulation of RhoA in cancer has been associated with metastasis [Friedl and Wolf, 2003b; Horiuchi et al., 2003; Sahai, 2005; Faried et al., 2007; Bellizzi et al., 2008]. It was found that TMX2-28 cells overexpress RhoA mRNA and protein. Additionally, they overexpress mRNA of RhoA's two main targets, ROCK 1 and 2. This provides three potential targets for therapy development against metastasis. Furthermore, inhibition of RhoA pathway activity through the use of either the ROCK inhibitor H-1152, or a RhoA specific siRNA resulted in decreased migratory and invasive behavior.

Based on these observations, we can conclude that TMX2-28 cells do not use protease-dependent mesenchymal movements as their primary invasion mechanism. Given their rounded morphology, use of RhoA and lack of mesenchymal phenotype, it supports our hypothesis that they use amoeboid movement. Furthermore, the cDNA microarray mentioned previously, also revealed that TMX2-28 cells expressed mRNA for a number of genes known to regulate cell membrane morphology and controlling cytoskeletal organization (Table I). These included mitogen inducible gene 2 (MIG2). MIG2 is a component of cell-ECM adhesion sites that control cell shape and spreading. MIG2 promotes cellular dynamics though the recruitment of Migfilin and its interactions with Filamin and actin [Tu et al., 2003; Gozgit et al., 2006; Shi and Wu, 2008]. A study by Gozgit et al. [2006] showed that MIG2 is overexpressed in TMX2-28 cell as well as in breast cancer tissues. Knockdown of MIG2 also resulted in reduced invasive behavior. RhoA signaling is known to be involved in focal adhesion formation along with MIG2 [Tu et al., 2003; Wu et al., 2010]. From this knowledge one could suggest that RhoA and MIG2 may work collectively to facilitate amoeboid invasion.

RhoC is also a member of the Ras Homolog Gene Family, and it also targets ROCK 1 and ROCK 2 [Sahai and Marshall, 2002; Wheeler and Ridley, 2004]. The activation of the ROCKs by RhoC leads to the disruption of adherens junctions and increases cell motility and enhanced F-actin assembly [Sahai and Marshall, 2002]. Over-expression of RhoC has been observed in a number of cancers including breast [Suwa et al., 1998; Kleer et al., 2002; Kamai et al., 2003; Wang et al., 2004]. Given that RhoC plays a role in cell motility, at least partially through the same downstream effectors (ROCK 1 and 2) as RhoA, it is possible that RhoC may also be involved in the amoeboid invasion mechanism. RhoC could work in concert with RhoA to promote cell motility, while RhoE may play the role of a negative regulator of motility via inhibition of RhoA.

An understanding of the mechanisms behind cell migration and tumor metastasis is critical given that metastasis accounts for the majority of breast cancer deaths. With this knowledge, we can design inhibiting molecules that mimic the actions of RhoE and target RhoA/C resulting in down regulation and abrogation of invasive behaviors. Targeting the Rho pathway may provide therapeutic strategies that could be used individually or in combination with protease inhibitors in patients with increased risk of metastasis. Collectively, the results of our study suggest that TMX2-28 cells exploit a RhoA dependent, proteolytic-independent invasion mechanism. Targeting the RhoA pathway in triple-negative, basal-like breast cancer cells that have a proteolytic-independent invasion mechanism may provide therapeutic strategies for the treatment of cancer patients with increased risk of metastasis.

MATERIALS AND METHODS

CELL CULTURE

TMX2-28, MCF-7, and HeLa cells were maintained in T-75 culture flasks at 37°C and 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with calf serum (5%), insulin (10 μg/ml), non-essential amino acids (100×), penicillin-streptomycin (10,000 μg/ml), and L-glutamine (200 mM). MDA-MB-231 cells were maintained in Leibovitz-15 (L-15) medium supplemented with 10% FBS at 37°C and 0% CO_2. All cells were passaged when near 80% confluence.

RNA AND PROTEIN ISOLATION

Total RNA (n = 3 biological samples) was isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's suggestions and protocols as previously described [Gozgit et al., 2006, 2007]. RNA was then treated with Turbo DNA-Free (Ambion, Austin, TX) to remove any DNA contamination. The quality of the RNA was assessed by 260/280 nm spectrophotometer readings (Nanodrop 8000; Thermo Scientific, Wilmington, DE). Protein-containing cell lysates were isolated from cell cultures (n = 3 biological samples) with pre-chilled SDS lysis buffer (1% SDS, 0.06 M Tris–HCL, and 10% glycerol) according to our standard laboratory protocols [Gozgit et al., 2006, 2007]. Extracts were used for Western immunoblotting.

WESTERN IMMUNOBLOTTING

Protein lysates (20 μg) were mixed with NuPage sample buffer and reducing agent (Invitrogen, Carlsbad, CA), heated at 70°C for 10 min, separated on a Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and then transferred to an Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad) according to manufacturer's protocols.

Membranes were incubated in blocking buffer (5% nonfat dry milk/Tris buffered saline and 0.1% Tween 20) for 1 h at room temperature with gentle shaking. Membranes were then incubated with anti-RhoA rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) diluted 1:1,000 overnight at 4°C, followed by incubation with the secondary antibody, anti-rabbit IgG linked to horseradish peroxidase; diluted 1:1,000 (Cell Signaling Technology), for 1 h at room temperature. Chemiluminescent signals were detected with SuperSignal West Pico Kit and protocol (Peirce, Rockford, IL), and imaged using the G.BOX Chemi HR-16 (Syngene, Fredrick, MD). Membranes were stripped using Restore stripping buffer (Thermo Scientific, Rockford, IL) and reprobed for glecer-aldehyde-3-phosphate (Cell Signaling Technology; 1:10,000).

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE PCR (qRT-PCR)

RNA samples (n = 3 biological samples) were reverse transcribed and amplified using the One Step RT-PCR kit (Qiagen, Valencia, CA) in the Roche LightCycler (Roche, Indianapolis, IN). Total RNA (75 ng) was incubated with Qiagen RT-PCR master mix including primers (25 μM each) and SYBR Green I nucleic acid stain (2×; Invitrogen, product number S7563) in pre-cooled capillaries (Roche) and was reversed transcribed (50°C for 30 min). Following reverse transcription, samples were heated to 95°C for 15 min to activate the HotStar Taq DNA polymerase and to simultaneously inactivate the reverse transcriptase. The generation of amplified products was monitored over 45 PCR cycles by fluorescence of intercalating SYBR Green. Each cycle consisted of the following steps: (1) Denaturation at 95°C for 15 s. (2) Annealing at 60°C for 15 s. (3) Extension at 72°C for 30 s. Relative mRNA levels were normalized to hypoxanthine ribosyltransferase (HPRT) levels to control for RNA quality and concentration. Gene specific primers, designed using Primer3 software (http://frodo.wi.mit.edu/), and purchased from Integrated DNA Technologies, Inc. (Coralville, IA), were used for MMP-1, MMP-2, MMP MMP-9, SLUG, ZEB 1, ZEB 2, Vimentin, Fibronectin, N-Cadherin, RhoA, ROCK 1, ROCK 2, and HPRT (Table III).

MATRIGEL INVASION ASSAY

BD BioCoat Matrigel Invasion Chambers and 8.0 μm pore size PET track-etched membranes (Becton Dickinson, Franklin Lakes, NJ) were used according to the manufacturer's protocol. Cells (5×10^4 cells total; n = 3 biological samples) were plated in the top chamber containing a basal medium (DMEM). A medium rich in nutrients (DMEM supplemented with 10% FBS) was used in the bottom chamber as a chemoattractant. Twenty-two to 24 h later, cells were fixed and stained using 10% formalin and crystal violet, respectively. Cell numbers were determined from microphotographs taken over four (non-overlapping) areas of the membrane.

ZYMOGRAPHY

Gelatin zymography was preformed using precast polyacrylamide gels containing 10% gelatin (Bio-Rad). Collected serum free, conditioned medium (n = 3 biological samples) was mixed in equal volumes with sample buffer (0.5 M Tris–HCL, glycerol, 10% SDS, 0.1% Bromophenol blue). To prepare serum free conditioned medium, cells were seeded into 24-well tissue culture plates at a concentration of 1×10^5 cells/well in normal culture medium. Twenty-four hours postseeding, cells were washed three times with phosphate-buffered saline followed by incubation in serum free medium. Serum free conditioned medium was collected 48 h later and stored at −20°C until use. Twenty to 50 μl of mixed sample was loaded into the gel and electrophoresed at 100 V for 90 min. Following electrophoresis, gels were rinsed with 1× renaturing buffer (2.5% Triton X-100) for 30 min with gentle agitation. Gels were then transferred to 1× developing buffer (50 mM Tris base, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij 35) for 30 min at room temperature with gentle agitation to equilibrate the gel. Gels were then incubated overnight at 37°C with gentle agitation in fresh 1× developing buffer. Gels were then stained for 1 h with 0.5% Coomassie Blue, de-stained twice for 30 min each with de-staining solution (Methanol: Acetic Acid: Water [50:10:40]) and rehydrated in H_2O for 10 min.

INHIBITOR AND siRNA TREATMENTS

For inhibitor assays, cells (n = 3 biological samples) were treated with the Rho Kinase pathway inhibitor H-1152 (Calbiochem, Billerica, MA) at 0, 20, and 100 μM concentrations for 24 h [Ikenoya et al., 2002; Sasaki et al., 2002]. Concentrations were determined by dose response testing. Following treatment, either RNA was isolated

TABLE III. Primers Used to Detect Relative mRNA Expression of Genes

Gene	RefSeq number	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
ERα	NM_000125	ATGATCAACTGGGCGAAGAG	GATCTCCACCATGCCCTCTA
PR	NM_000926.4	GGAAGGGCTACGAAGTCAAA	TAACTTGCATGATCTTGTCAAACA
HER2	M11730	CCCCAGCCTGAATATGTGAA	CTCTGGGTTCTCTGCCGTAG
MMP-1	NM_002421	CGACTCTAGAAACACAAGAGCAAGA	AAGGTTAGCTTACTGTCACACGCTT
MMP-2	NM_004530	GTGCTGAAGGACACACTAAAGAAGA	TTGCCATCCTTCTCAAAGTTGTAGG
MMP-9	NM_004994	CACTGTCCACCCCTCAGAGC	GCCACTTGTCGGCGATAAGG
SLUG	NM_003068.4	CATGCCTGTCATACCACAAC	GGTGTCAGATGGAGGAGGG
ZEB 1	NM_001128128.2	GGGAGGAGCAGTGAAAGAGA	TTTCTTGCCCTTCCTTTCTG
ZEB 2	NM_014795.3	AAGCCAGGGACAGATCAGC	CCACACTCTGTGCATTTGAACT
Vimentin	NM_003380.3	AAAGTGTGGCTGCCAAGAAC	AGCCTCAGAGAGGTCAGCAA
Fibronectin	NM_212482.1	GAACTATGATGCCGACCAGAA	GGTTGTGCAGATTTCCTCGT
N-Cadherin	NM_001792.3	CACTGCTCAGGACCCAGAT	TAGCCGAGGATGGTCC
RhoA	NM_001664	GATGGAAAGCAGGTAGAGTTGG	TCAGTATAACATCGGTATCTGGGTA
ROCK 1	NM_005406	AATGCTTGTAGGTGATACACCTTTT	CTGTCAGTAAGGAAGGCACAAA
ROCK 2	NM_004850	GAAGTGCAGTTGGTTCGTCA	GCTATTGGCAAAGGCCATAA
HPRT	NM_000194	ACCCCACGAAGTGTTGGATA	AAGCAGATGGCCACAGAACT

as mentioned above, or cells were seeded into invasion chambers in the respective concentrations of inhibitor.

For siRNA assays, RhoA specific and negative control (random sequence without homology to any human gene) siRNAs (RhoA-siRNA and NC-siRNA, respectively) were designed using Ambion's siRNA Template Design Tool (RhoA sense: CCUUAUAGUUACUGUGUAATT; antisense: UUACACAGUAACUAUAAAGGTA; negative control sense: UUAUCGCCAAAUUCUUUUAUCGGACAGAG; antisense: UUGAUAAAAGAAUUUGGCGAUGGACAGAG). DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. and siRNA generated using *Silencer*® siRNA Construction Kit (Ambion) per manufactures' protocols. TMX2-28, MCF-7 (non-invasive control), and HeLa (RhoA dependent invasion control) cells were seeded into 100 mm dishes at a concentration of 10^6 cells/dish and allowed to attach overnight ($n = 3$ biological samples). On the day of transfection, HiPerFect transfection agent (Qiagen) and 25 mg/well of a RhoA specific or a negative control siRNA was diluted into Opti-MEM (Invitrogen) and added to cells. Following incubation under normal growth conditions for 48 h, cells were harvested for both RNA and Protein isolation or invasion assays.

STATISTICAL ANALYSES

Data were analyzed and graphed with GraphPad Prism Version 3.02 (GraphPad Software, Inc., San Diego, CA). For ANOVA and posthoc comparisons, significance was set at $P < 0.05$.

REFERENCES

Agiostratidou G, Hulit J, Phillips GR, Hazan RB. 2007. Differential cadherin expression: Potential markers for epithelial to mesenchymal transformation during tumor progression. J Mammary Gland Biol Neoplasia 12(2–3):127–133.

Bartsch JE, Staren ED, Appert HE. 2003. Matrix metalloproteinase expression in breast cancer. J Surg Res 110(2):383–392.

Bellizzi A, Mangia A, Chiriatti A, Petroni S, Quaranta M, Schittulli F, Malfettone A, Cardone RA, Paradiso A, Reshkin SJ. 2008. RhoA protein expression in primary breast cancers and matched lymphocytes is associated with progression of the disease. Int J Mol Med 22(1):25–31.

Cairns RA, Khokha R, Hill RP. 2003. Molecular mechanisms of tumor invasion and metastasis: An integrated view. Curr Mol Med 3(7):659–671.

Coussens LM, Fingleton B, Matrisian LM. 2002. Matrix metalloproteinase inhibitors and cancer: Trials and tribulations. Science 295(5564):2387–2392.

Curran S, Murray GI. 2000. Matrix metalloproteinases: Molecular aspects of their roles in tumour invasion and metastasis. Eur J Cancer 36(13 Spec. No): 1621–1630.

Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N. 2000. Metalloproteinases: Role in breast carcinogenesis, invasion and metastasis. Breast Cancer Res 2(4):252–257.

Etienne-Manneville S, Hall A. 2001. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell 106(4):489–498.

Faried A, Faried LS, Usman N, Kato H, Kuwano H. 2007. Clinical and prognostic significance of RhoA and RhoC gene expression in esophageal squamous cell carcinoma. Ann Surg Oncol 14(12):3593–3601.

Fasco MJ, Amin A, Pentecost BT, Yang Y, Gierthy JF. 2003. Phenotypic changes in MCF-7 cells during prolonged exposure to tamoxifen. Mol Cell Endocrinol 206(1–2):33–47.

Foster R, Hu KQ, Lu Y, Nolan KM, Thissen J, Settleman J. 1996. Identification of a novel human rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. Mol Cell Biol 16(6):2689–2699.

Friedl P, Wolf K. 2003a. Proteolytic and non-proteolytic migration of tumour cells and leucocytes. Biochem Soc Symp 70(70):277–285.

Friedl P, Wolf K. 2003b. Tumour-cell invasion and migration: Diversity and escape mechanisms. Nat Rev Cancer 3(5):362–374.

Friedl P, Wolf K. 2010. Plasticity of cell migration: A multiscale tuning model. J Cell Biol 188(1):11–19.

Gozgit JM, Pentecost BT, Marconi SA, Otis CN, Wu C, Arcaro KF. 2006. Use of an aggressive MCF-7 cell line variant, TMX2-28, to study cell invasion in breast cancer. Mol Cancer Res 4(12):905–913.

Gozgit JM, Pentecost BT, Marconi SA, Ricketts-Loriaux RS, Otis CN, Arcaro KF. 2007. PLD1 is overexpressed in an ER-negative MCF-7 cell line variant and a subset of phospho-akt-negative breast carcinomas. Br J Cancer 97(6):809–817.

Guasch RM, Scambler P, Jones GE, Ridley AJ. 1998. RhoE regulates actin cytoskeleton organization and cell migration. Mol Cell Biol 18(8):4761–4771.

Hajra KM, Chen DY, Fearon ER. 2002. The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res 62(6):1613–1618.

Hanazaki M, Yokoyama M, Morita K, Kohjitani A, Sakai H, Chiba Y, Misawa M. 2008. Rho-kinase inhibitors augment the inhibitory effect of propofol on rat bronchial smooth muscle contraction. Anesth Analg 106(6):1765–1771.

Horiuchi A, Imai T, Wang C, Ohira S, Feng Y, Nikaido T, Konishi I. 2003. Up-regulation of small GTPases, RhoA and RhoC, is associated with tumor progression in ovarian carcinoma. Lab Invest 83(6):861–870.

Ikenoya M, Hidaka H, Hosoya T, Suzuki M, Yamamoto N, Sasaki Y. 2002. Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific rho-kinase inhibitor. J Neurochem 81(1):9–16.

Iwata H, Kobayashi S, Iwase H, Masaoka A, Fujimoto N, Okada Y. 1996. Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas. Jpn J Cancer Res 87(6):602–611.

Jaffe AB, Hall A. 2005. Rho GTPases: Biochemistry and biology. Annu Rev Cell Dev Biol 21:247–269.

Kamai T, Tsujii T, Arai K, Takagi K, Asami H, Ito Y, Oshima H. 2003. Significant association of rho/ROCK pathway with invasion and metastasis of bladder cancer. Clin Cancer Res 9(7):2632–2641.

Kleer CG, van Golen KL, Zhang Y, Wu ZF, Rubin MA, Merajver SD. 2002. Characterization of RhoC expression in benign and malignant breast disease: A potential new marker for small breast carcinomas with metastatic ability. Am J Pathol 160(2):579–584.

Kokkinos MI, Wafai R, Wong MK, Newgreen DF, Thompson EW, Waltham M. 2007. Vimentin and epithelial-mesenchymal transition in human breast cancer-observations in vitro and in vivo. Cells Tissues Organs 185(1–3): 191–203.

Moreno-Bueno G, Portillo F, Cano A. 2008. Transcriptional regulation of cell polarity in EMT and cancer. Oncogene 27(55):6958–6969.

Narumiya S, Tanji M, Ishizaki T. 2009. Rho signaling, ROCK and mDia1, in transformation, metastasis and invasion. Cancer Metastasis Rev 28(1–2): 65–76.

Parri M, Chiarugi P. 2010. Rac and rho GTPases in cancer cell motility control. Cell Commun Signal 8:23.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. 2003. Cell migration: Integrating signals from front to back. Science 302(5651):1704–1709.

Riento K, Ridley AJ. 2003. ROCKs: Multifunctional kinases in cell behavior. Nat Rev Mol Cell Biol 4(6):446–456.

Riento K, Totty N, Villalonga P, Garg R, Guasch R, Ridley AJ. 2005. RhoE function is regulated by ROCK I-mediated phosphorylation. EMBO J 24(6):1170–1180.

Sahai E. 2005. Mechanisms of cancer cell invasion. Curr Opin Genet Dev 15(1):87–896.

Sahai E, Marshall CJ. 2002. ROCK and dia have opposing effects on adherens junctions downstream of rho. Nat Cell Biol 4(6):408–415.

Sahai E, Marshall CJ. 2003. Differing modes of tumour cell invasion have distinct requirements for rho/ROCK signalling and extracellular proteolysis. Nat Cell Biol 5(8):711–719.

Sasaki Y, Suzuki M, Hidaka H. 2002. The novel and specific rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for rho-kinase-involved pathway. Pharmacol Ther 93(2–3):225–232.

Sassi ML, Eriksen H, Risteli L, Niemi S, Mansell J, Gowen M, Risteli J. 2000. Immunochemical characterization of assay for carboxyterminal telopeptide of human type I collagen: Loss of antigenicity by treatment with cathepsin K. Bone 26(4):367–373.

Shi X, Wu C. 2008. A suppressive role of mitogen inducible gene-2 in mesenchymal cancer cell invasion. Mol Cancer Res 6(5):715–724.

Suwa H, Ohshio G, Imamura T, Watanabe G, Arii S, Imamura M, Narumiya S, Hiai H, Fukumoto M. 1998. Overexpression of the rhoC gene correlates with progression of ductal adenocarcinoma of the pancreas. Br J Cancer 77(1):147–152.

Thiery JP. 2002. Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer 2(6):442–454.

Torka R, Thuma F, Herzog V, Kirfel G. 2006. ROCK signaling mediates the adoption of different modes of migration and invasion in human mammary epithelial tumor cells. Exp Cell Res 312(19):3857–3871.

Tu Y, Wu S, Shi X, Chen K, Wu C. 2003. Migfilin and MIG-2 link focal adhesions to filamin and the actin cytoskeleton and function in cell shape modulation. Cell 113(1):37–47.

Wang W, Yang LY, Huang GW, Lu WQ, Yang ZL, Yang JQ, Liu HL. 2004. Genomic analysis reveals RhoC as a potential marker in hepatocellular carcinoma with poor prognosis. Br J Cancer 90(12):2349–2355.

Wennerberg K, Forget MA, Ellerbroek SM, Arthur WT, Burridge K, Settleman J, Der CJ, Hansen SH. 2003. Rnd proteins function as RhoA antagonists by activating p190 RhoGAP. Curr Biol 13(13):1106–1115.

Wheeler AP, Ridley AJ. 2004. Why three rho proteins? RhoA, RhoB, RhoC, and cell motility. Exp Cell Res 301(1):43–49.

Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, Strongin AY, Brocker EB, Friedl P. 2003. Compensation mechanism in tumor cell migration: Mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J Cell Biol 160(2):267–277.

Wu M, Wu Z, Rosenthal DT, Rhee EM, Merajver SD. 2010. Characterization of the roles of RHOC and RHOA GTPases in invasion, motility, and matrix adhesion in inflammatory and aggressive breast cancers. Cancer 116(S11): 2768–2782.

Wyckoff JB, Pinner SE, Gschmeissner S, Condeelis JS, Sahai E. 2006. ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. Curr Biol 16(15):1515–1523.