Inhibition of Rho-Associated Kinase Signaling Prevents Breast Cancer Metastasis to Human Bone

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Abstract
Rho-associated kinase (ROCK) signaling plays a fundamental role in regulating cell morphology, adhesion, and motility. Aberrant expression of ROCK is related to tumor metastases and poor clinical outcome. Here, we show that ROCK expression is increased in metastatic human mammary tumors and breast cancer cell lines compared with nonmetastatic tumors and cell lines. Overexpression of ROCK confers a metastatic phenotype on the nonmetastatic MCF-7 cell line. Inhibition of ROCK activity, by either a specific ROCK inhibitor (Y27632) or ROCK-targeted small interfering RNAs, reduces cell migration and proliferation in vitro and metastasis to bone in vivo using a novel “human breast cancer metastasis to human bone” mouse model. Expression of the c-Myc–regulated miR-17-92 cluster is shown to be elevated in metastatic breast cancer cells compared with nonmetastatic cells and diminished by Y27632 treatment. Furthermore, blockade of miR-17 is shown to decrease breast cancer cell invasion/migration in vitro and metastasis in vivo. Together, these findings suggest that augmented ROCK signaling contributes to breast cancer metastasis. The effects of ROCK on tumor cell invasion/motility and growth may derive from regulating cytoskeletal actin-myosin contraction and modulating the c-Myc pathway, including c-Myc–dependent microRNAs. Inhibition of ROCK or the pathway it stimulates, therefore, may represent a novel approach for treatment of breast cancer metastases.

Introduction
Breast cancer is by far the most common cancer among women and the fifth most common cause of cancer death worldwide. Although the primary tumor often causes significant morbidity, metastasis to distant organs accounts for >90% of breast cancer–related mortality. The underlying mechanisms responsible for breast cancer metastasis have not yet been elucidated. Additionally, there is little in the way of metastasis-specific therapy. Members of Rho small GTPases play a role in regulating cell morphology, growth, apoptosis, and motility. The Rho-associated kinases (ROCK), of which there are two isoforms, ROCK1 and ROCK2 (here called ROCK), are principal mediators of Rho small GTPases. ROCK is implicated in the regulation of breast cancer metastasis. A clinical study showed that increased expression of ROCK corresponds to late-stage tumors and metastases and is negatively correlated with overall survival in breast cancer patients (1). Overexpression of ROCK enhances invasion/migration in breast and other tumor cells in vitro (2, 3). Correspondingly, both the expression of a dominant-negative ROCK and the exposure of cells to a ROCK inhibitor suppress cancer cell invasion/migration in vitro and in vivo (4, 5).

MicroRNAs (miRNA) are small, noncoding RNAs that negatively regulate gene expression. miRNAs can regulate tumor development, progression, and metastasis by functioning as either oncogenes or tumor suppressors. The c-Myc–regulated miR-17-92 cluster functions as a potential human oncogene (6). It is overexpressed in different types of tumors, including breast, prostate, and lung cancers, and in hematologic malignancies (7–9). Forced overexpression of miR-17-92 in a mouse model of B-cell lymphoma promoted disease onset and progression (10).

In the current study, we investigate ROCK-induced molecular events that may be responsible for breast cancer metastases using in vitro assays and the murine “human breast cancer metastasis to human bone” model in which breast cancer cells metastasize from the orthotopic site (11). ROCK inhibition by either a specific ROCK inhibitor or ROCK-specific small interfering RNAs (siRNA) can suppress breast cancer cell growth and migration in vitro and decrease metastasis to bone in vivo. Inhibition of ROCK signaling therefore may prove to be a promising strategy for prevention of breast cancer metastasis.

Materials and Methods
Human breast cancer specimens, cell lines, and cell culture. Human mammary tumor cDNAs were purchased from Origene. Human breast cancer cells were cultured as described previously (11, 12).

Animal experiments. All animal experimental procedures were approved by the Tufts University Department of Laboratory Animal Management and Institutional Animal Care and Use Committee. Experimental setup was similar to the methods described previously (11) and depicted in Fig. 1. Beginning on the second day after breast cancer cell injection, one group of mice received IP injection of Y27632 (Sigma; 8 mg/kg in 100 μL PBS) every other day for 10 weeks. Control mice received PBS.

Cell proliferation, ROCK activity, wound-healing, and Transwell migration assays. Breast cancer cells were serum-starved for 24 h and then cultured in medium with 1% fetal bovine serum with or without Y27632 at 10 μmol/L followed by MTT-based proliferation (Roche) and ROCK activity (Cyclex) assays according to the manufacturer's protocols and a wound-healing assay as described previously (13). Transwell migration was assessed according to the manufacturer's instructions (Corning). Cells on the filters were visualized by 4′,6-diamidino-2-phenylindole (Molecular Probes).

FITC-conjugated phalloidin staining. Breast cancer cells were cultured in medium containing 1% fetal bovine serum with or without 10 μmol/L Y27632 for 24 h. Then, cells were fixed by formaldehyde and washed with 0.1% Triton X-100 in PBS. Primary tumor tissues were fixed in formalin and then processed for paraffin-embedding and sectioning. FITC-conjugated phalloidin (Molecular Probes) was used to label F-actin. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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miRNA microarray analysis. miRNA isolation kits (Ambion) were used to isolate total RNA with enriched miRNAs from cells. miRNA microarray analyses were done by LC Sciences as described previously (14). Those with fold change > 2 or <-2 and \( P < 0.05 \) (t test) were considered as differentially expressed miRNAs.

siRNA and miRNA molecule transfection. Prevalidated siRNA molecules were synthesized by Qiagen. Two sets of siRNAs were used to target different regions of the mRNAs for \( \text{ROCK1/ROCK2} \) or \( \text{c-Myc} \). Anti–miR-17 molecules were obtained from Ambion. The small RNA transfection was done according to the manufacturer’s protocols.

In vivo administration of anti–miR-17 molecules. Tumor tissues (0.02 g) derived from MDA-MB-231 cells were implanted underneath mouse mammary fat pads in our model. Two weeks later, when primary tumors reached \( \sim 75 \) mm\(^3\), \( 50 \) \( \mu \)L anti–miR-17 or scrambled control RNAs were injected into tumors. The anti–miR-17 and scrambled control RNAs were synthesized as described previously (15). Mice \( (n = 5) \) received injections every other day for 2 weeks.

Western blot, flow cytometry, and quantitative reverse transcription-PCR analyses. These analyses were done as described previously (16–19). Antibodies and primers used in the study are listed in Supplementary Table S1 and S2.

Statistical analysis. Two-tailed Student’s \( t \) test was used to analyze experimental data; \( P < 0.05 \) determined statistical significance. All results are presented as mean \( \pm \) SE.

Results

ROCK expression in breast cancer. To investigate the potential role of ROCK signaling in regulation of breast cancer metastasis, we assessed the expression of ROCK in human breast cancer specimens and cell lines. The expression of \( \text{ROCK1 and ROCK2, particularly ROCK1} \) \( (>2\text{-fold}, \ P = 0.057) \), is increased in metastatic human mammary tumors (from patients with nodal metastasis) compared with nonmetastatic tumors (node-negative; Fig. 1A; Supplementary Fig. S1A). Further, the expression of \( \text{ROCK1 and ROCK2} \) is increased in late-stage tumors compared with early-stage tumors (Supplementary Fig. S1A). Additionally, both ROCK1 and ROCK2 (particularly ROCK1) are markedly elevated in the tumorigenic and metastatic cell lines, MDA-MB-231, 2287 (12), 1833 (12), SUM1315, and BP2, \(^5\) compared with the tumorigenic but nonmetastatic cell lines, MCF-7 and SUM149 (Fig. 1B; Supplementary Fig. S1B). As a result, ROCK kinase activity is significantly increased in metastatic cells compared with nonmetastatic cells (Fig. 2A; Supplementary Fig. S1C). These observations suggest that overexpression of ROCK may contribute to the metastatic features of breast cancer cells.

\(^5\) A subline derived from SUM1315 cells (unpublished data).
ROCK has been shown to phosphorylate the regulatory myosin light chain (MLC), which enhances its binding to F-actin (20). Consistent with the increased ROCK1 and ROCK2 and elevated ROCK activity (Fig. 1; Supplementary Fig. S1), the amount of phosphorylated MLC (p-MLC) is greatly increased in MDA-MB-231 and SUM1315 cells compared with MCF-7 cells \textit{in vitro} (Figs. 1B and 2D). MYH9, nonmuscle myosin heavy chain-IIA, is an essential component of the cell motor system in eukaryotes and involved in the regulation of cell morphology, motility, and division (21). For the first time, we observed that the expression of MYH9 is significantly increased in MDA-MB-231 and SUM1315 compared with MCF-7 (Fig. 1B). The combined effect of increased p-MLC and MYH9 may lead to enhanced stabilization of the F-actin meshwork and augmented actin-myosin–mediated contraction in MDA-MB-231 and SUM1315 cells and thereby contribute to the metastatic phenotypes of these cells.

Using a mouse model of “human breast cancer metastasis to human bone” (Fig. 1C; ref. 11), we observed increased ROCK1 and ROCK2 and MYH9 expression in metastatic MDA-MB-231 and SUM1315 cells in human bone cores compared with cells from primary tumors at the orthotopic site (mammary fat pads) as shown by quantitative reverse transcription-PCR (qRT-PCR) analysis ($P < 0.05$; Fig. 1D). The overexpression of ROCK1 and ROCK2 and MYH9 in bone-residing metastatic cells indicates their potential role in promoting breast cancer bone metastasis. Collectively, increased levels of ROCK appear to confer enhanced metastatic capability on breast cancer cells.

**Effects of the ROCK inhibitor on cell motility/migration and proliferation.** To test whether inhibition of ROCK can reduce breast cancer cell motility and migration, we used the ROCK inhibitor, Y27632 (22), to carry out several \textit{in vitro} experiments. Consistent with previous reports (23, 24), ROCK activity was reduced ∼50% in MCF-7, MDA-MB-231, and SUM1315 cells on treatment with Y27632 (Fig. 2A), suggesting that Y27632 is effective in repressing ROCK activity. Y27632 significantly reduces cell migration from cytodex beads by 30% to 40% in MDA-MB-231 and SUM1315 cells ($P < 0.05$; Supplementary Fig. S2A). Moreover, a wound-healing assay showed that Y27632 inhibits cell motility in MDA-MB-231 (Fig. 2B) and SUM1315 (data not shown) cells. Consistent with previous reports (22, 25), we observed disassembly.

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**Figure 2.** ROCK inhibitor decreases cell motility and proliferation. A, relative ROCK activity in breast cancer cells with or without Y27632 treatment ($n = 4$). B, wound-healing assay. A wound area is shown for MDA-MB-231 cells. C, FITC-conjugated phalloidin staining of F-actin in SUM1315 cells in the presence or absence of Y27632. Actin fibers are shown in green and cell nuclei are shown in blue by counterstaining with 4,6-diamidino-2-phenylindole. Original magnification, ×400. Similar results were observed in MDA-MB-231 cells (data not shown). D, MTT-based proliferation assay ($n = 4$) and Western blot analyses of p-MLC and MYH9 in breast cancer cells on Y27632 treatment. $^*$, $P < 0.05$, compared with corresponding cell types without Y27632 treatment.
of actin fibers and reduction of stress fiber formation, with the density of actin meshwork reduced in these cells on Y27632 treatment (Fig. 2C; Supplementary Fig. S2B). Additionally, Y27632 repressed proliferation in all three cell lines by ∼25% (P < 0.05; Fig. 2D; Supplementary Fig. S2C), corresponding to previous reports describing its effects in other cell types (26).

As observed by others (25), p-MLC is greatly decreased on Y27632 treatment in breast cancer cells (Fig. 2D), indicating that the reduction of ROCK activity by Y27632 leads to attenuation of its downstream targets. For the first time, we observed that the level of MYH9 is reduced by Y27632 in breast cancer cells (Fig. 2D), although the mechanism remains unknown. Whether MYH9 is a direct or an indirect downstream target of ROCK signaling will be examined in future studies. Decreased p-MLC and MYH9 likely alter cell morphology and impair motility in breast cancer cells after Y27632 treatment.

ROCK-c-Myc pathway in breast cancer cells. To gain insight into the mechanism whereby ROCK inhibition decreases cell motility and proliferation, we considered the potential downstream targets of ROCK. Ingenuity Pathway Analysis was used to identify the potential network regulated by ROCK signaling; c-Myc is predicted to be a downstream target of ROCK (Supplementary Fig. S3A). Previous studies also suggest that ROCK is implicated in the regulation of c-Myc (25, 27, 28). c-Myc plays a pivotal role in modulating cell division and transformation (29, 30), and clinical studies have shown a strong association between overexpression of c-Myc and tumor metastasis (31–33). In our study, the c-Myc level was significantly increased in MDA-MB-231 and SUM1315 cells compared with MCF-7 cells in vitro (Fig. 3A; Supplementary Fig. S3B) and C). In addition, c-Myc was increased in both metastatic SUM1315 and MDA-MB-231 cells harvested from human bone grafts compared with the same cell types from primary tumors in our mouse model (P < 0.05; Fig. 3B). Overexpression of c-Myc in MDA-MB-231 and SUM1315 cells and human bone core–residing metastatic breast cancer cells is consistent with their prometastatic phenotype (12, 34) and indicative of the role of c-Myc in facilitating breast cancer cell metastasis from the primary site to bone. A recent study showed that Y27632 induces a significant reduction of c-Myc in RhoQ63L-transformed fibroblasts (25). Similar to this observation, we observed reduced c-Myc in MDA-MB-231 and SUM1315 cells with Y27632 treatment in vitro (Fig. 3C). The level of p-c-Myc, the activated form of c-Myc, was determined by Western blot analysis using an antibody that recognizes c-Myc protein singly or doubly phosphorylated at Thr58 and Ser62 or both. P-c-Myc is greatly decreased in SUM1315 cells 24 and 48 h after Y27632 treatment (Fig. 3C),
consistent with previous reports (27, 28). To confirm the hypothesis that ROCK activity contributes to c-Myc phosphorylation and its protein level, we knocked down the expression of both ROCK1 and ROCK2 using prevalidated siRNAs. The levels of ROCK1 and ROCK2 are reduced by ~98% and 60% by siRNAs, respectively, and the ROCK activity and the amount of p-MLC are significantly reduced (Fig. 3D). On reduction of ROCK activity, the levels of c-Myc and p-c-Myc are significantly decreased (Fig. 3D).

A direct c-Myc target, HSPC111, was reported to be overexpressed in breast cancer and is associated with poor prognosis (35). Although its role in breast cancer development and metastasis has not been defined, it was shown to influence cell proliferation (35). The HSPC111 level is dramatically increased in MDA-MB-231 and SUM1315 compared with MCF-7 (Fig. 3A) and may have a regulatory role in breast cancer metastasis.

**c-Myc–regulated miR-17-92 cluster in breast cancer cells.** To further delineate the ROCK/c-Myc pathway, we investigated the role of miR-17-92 (Fig. 4A) in breast cancer growth and motility. A microarray analysis of miRNAs was done using SUM1315, MDA-MB-231, and MCF-7 cells as described in Materials and Methods and Supplementary Fig. S4. The transcription of miR-17-92 is directly regulated by c-Myc. As c-Myc expression is increased in MDA-MB-231 and SUM1315 cells compared with MCF-7 cells and in metastatic breast cancer cells in human bone cores compared with cells from primary tumors, we would expect elevated expression of miRNAs in this cluster in MDA-MB-231 and SUM1315 cells and in metastatic breast cancer cells in human bone cores. Indeed, microarray analysis showed a 2- to 6-fold increase of expression of miR-17, miR-19a, miR-20a, miR-19b, and miR-92-1 in both MDA-MB-231 and SUM1315 cells compared with MCF-7 cells.

![Figure 4](image_url)

**Figure 4.** Upregulated miR-17-92 expression in metastatic breast cancer cells. A, schematic representation of c-Myc–regulated miR-17-92 cluster. Microarray analysis showing the expression changes of miRNAs in this cluster in MDA-MB-231 and SUM1315 cells compared with MCF-7 cells. B, RT-PCR analysis of miR-17-92 expression in breast cancer cells before and after Y27632 treatment. Quantification of PCR bands is similar to the method for Western blots. C, relative expression of miR-17-92 in metastatic breast cancer cells harvested from human bone cores compared with cells from primary tumors by qRT-PCR (n = 3-4). D, representative images from a Transwell migration assay of MDA-MB-231 cells with anti-miR17 molecule transfection. The numbers of transmigrated cells in the chamber filters were quantified (n = 8). *, P < 0.005, compared with controls.
in vitro; expression of miR-18a was increased 6.5-fold in MDA-MB-231 cells only (Fig. 4A). The miR-17-92 expression in the three cell lines was validated by endpoint and qRT-PCR (Fig. 4B; Supplementary Fig. S5). The miR-17-92 expression was also elevated in human bone core–residing metastatic breast cancer cells compared with breast cancer cells in the primary tumors (Fig. 4C). Moreover, its expression was significantly reduced after Y27632 treatment for 24 h in these three cell lines in vitro (P < 0.05; Fig. 4B; Supplementary Fig. S5), consistent with the reduction of c-Myc expression on Y27632 treatment (Fig. 3).

Of miRNAs in the miR-17-92 cluster, miR-17 in particular has been shown to modulate cell proliferation and apoptosis (15, 36, 37), and anti–miR-17 molecules abolish the growth of neuroblastoma cells both in vitro and in vivo (15). We showed this by inhibiting ROCK in the mouse model.

**Figure 5.** Role of ROCK in breast cancer metastasis in the mouse model. A, overexpression of ROCK endows nonmetastatic MCF-7 cells with metastatic properties. The frequencies of metastasis in mouse hind limbs, livers, and human bone cores are presented from ROCK overexpression and vector-control groups. B, representative images of metastasis in human bone cores from control and Y27632-treated mice. The bar graph displays the frequency of metastasis (meta) in human bone cores from each group. C, metastasis to mouse skeleton in vehicle control, Y27632-treated, ROCK1-sharpin (shRNA and scrambled control mice. Light blue columns, frequency of metastasis to mouse skeleton in metastasis in either or both hind limbs of each mouse considered positive; gray columns, overall frequency of metastasis to mouse hind limbs, where all hind legs in each group are taken into account. Representative images of metastasis in mice hind limbs from control and Y27632-treated mice are shown. The second bar graph shows the quantified data of metastasis in mouse skeleton (n = 7-12). D, relative ROCK activity in primary tumors is shown in the first graph (n = 5). Western blot analysis shows the p-MLC level in primary tumors. The second bar graph shows the quantified data of three individual Western blots using different samples. *, P < 0.05, compared with controls. A to C, numbers above the line refer to metastasis, whereas numbers below the line provide the total numbers of hind limbs, livers, human bone cores, or mice examined.
that anti–miR-17 molecules diminish cell proliferation by 35% and 24% in MDA-MB-231 and SUM1315 cells, respectively ($P < 0.05$; Supplementary Fig. S6A). Moreover, anti–miR-17 molecules reduced cell motility by 40% to 50% in a Transwell migration assay ($P < 0.005$; Fig. 4D; Supplementary Fig. S6B). We found no significant effect on apoptosis with addition of anti–miR-17 molecules based on an analysis of flow cytometry using FITC–Annexin V and propidium iodide stains (data not shown). Together, the fact that miR-17-92 expression is elevated in metastatic breast cancer cells and the observation that miR-17 is involved in the regulation of cell proliferation and motility suggest that this cluster may contribute to the tumorigenic and metastatic features of breast cancer cells.

**Overexpression or inhibition of ROCK signaling in breast cancer in vivo.** To determine the role of ROCK in vivo, we investigated the effect of enhancement and inhibition of ROCK activity on metastases using a mouse model of breast cancer metastasis to bone (11). First, we overexpressed the ROCK kinase in nonmetastatic MCF-7 cells (described in Supplementary Data). Elevation of ROCK activity conferred a metastatic phenotype on MCF-7 cells: ROCK-overexpressing MCF-7 cells metastasized to mouse hind limbs (67%), livers (67%), and human bone cores (33%);

**Figure 6.** Anti–miR-17 molecules inhibit breast cancer metastases in vivo. A, images of one primary tumor from each mouse ($n = 5$) and the average weight of primary tumors for each group ($n = 10$). B, frequency of metastasis in human bone cores (first graph) and lungs (third graph) from each group. The number above the line indicates those human bone cores/lungs with metastasis, whereas the total numbers of human bone cores/lungs are shown below the line. The second bar graph shows the quantified data of metastasis in mouse skeleton for each group ($n = 5$). C, Western blot analyses of TβR2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in MDA-MB-231 cells in vitro and in primary tumors from different mice on treatment of anti–miR-17 molecules. D, flow cytometry analysis of infiltrating Gr-1^+^CD11b^+^ cell population (top right window) in primary tumors injected with scrambled RNA or anti–miR-17 molecules. Two representative flow cytometry plots are shown. The bar graph shows the average percentage of Gr-1^+^CD11b^+^ cells in each group ($n = 5$). * $P < 0.05$, compared with the scrambled control.
Fig. 5A; Supplementary Fig. S7). This finding is in striking contrast to vector-control MCF-7 cells, which developed no metastases (Fig. 5A; Supplementary Fig. S7), and consistent with the well-documented observation that MCF-7 cells exhibit no metastatic propensity (38). Then, we examined the effects of a ROCK inhibitor (Y27632) and ROCK1 knockdown by short hairpin RNAs (described in Supplementary Fig. S8) on breast cancer metastasis to human bone cores and the mouse skeleton. All mice remained healthy for the duration of the experiment; no clinical or pathologic signs of toxicity from Y27632 were observed, similar to previous reports (22, 39). All mice developed tumors at the injected mammary fat pad sites, consistent with our previous results (11). As shown in Supplementary Fig. S9A, the average weight of primary tumors in Y27632-treated mice was reduced, but not significantly (P < 0.05), compared with vehicle-treated mice, consistent with a mild effect of Y27632 on cell proliferation in vitro (Fig. 2D; Supplementary Fig. S3C). Metastases to human bone cores were fewer in Y27632-treated mice (5 of 14, 36%) than control mice (8 of 12; 67%; Fig. 5B) as shown by luminescent imaging using the Biophotonic Imager machine (40). All (6 of 6) control mice developed bilateral hind limb metastases (for a total of 12 involved hind limbs). In the Y27632-treated mice, 6 of 7 had metastases (3 had bilateral and 3 had unilateral hind limb metastases for a total of 9 involved hind limbs); 1 had no metastases in either hind limb. Thus, the overall frequency of metastasis to mouse hind limbs in Y27632-treated mice was reduced by 36% (12 of 12 versus 9 of 14) compared with controls (Fig. 5C). Moreover, the mass of metastatic tumors in the mouse skeleton (reflected by photon flux, photons/s) was also diminished by 77% in Y27632-treated mice compared with controls (P < 0.05; Fig. 5C). ROCK1 short hairpin RNAs also decreases the frequency of breast cancer metastases to human bone cores and the mouse skeleton (Fig. 5; Supplementary Data).

To ascertain the efficiency of i.p. injection of Y27632 in diminishing ROCK activity in the primary tumors, we performed a ROCK kinase assay using tissue lysates from primary tumors. The ROCK activity in tumors from Y27632-treated mice was decreased by 42% (P < 0.05) compared with the controls (Fig. 5D), corresponding to the effect observed in vitro (Fig. 2A). The phosphorylated level of the direct substrate of ROCK, MLC, was significantly diminished in tumors from Y27632-treated mice (P < 0.001; Fig. 5D) in parallel to the in vitro observation (Fig. 2D). Additionally, we observed diminished density and looser structure of the actin meshwork in primary tumors from Y27632-treated mice by FITC-conjugated phalloidin staining (Supplementary Fig. S9B), which was similar to our in vitro observations (Fig. 2C). The ROCK activity in tumors of ROCK1-short hairpin RNA mice was reduced by 24%, and p-MLC was also decreased (P < 0.05; Fig. 5D).

**Anti-miR-17 molecules diminish breast cancer metastasis in vivo.** To examine the effect of miR-17 silencing on metastasis in vivo, we administrated anti-miR-17 molecules intratumorally into primary tumors generated from the tumor implants (derived from MDA-MB-231 cells). Two weeks after anti-miR-17 molecule injection, the growth of primary tumors was not affected (Fig. 6A). The frequency of metastasis to human bone cores was reduced by 50% compared with a scrambled control (Fig. 6B). Although breast cancer cells metastasized to all hind limbs in both the control and anti–miR-17-treated groups, the mass of metastatic tumors in anti–miR-17-treated mice was greatly reduced compared with controls (Fig. 6B). Additionally, the frequency of metastasis to lungs was also reduced by 50% (Fig. 6B). miR-17 was recently shown to target the type II transforming growth factor-β receptor (TβR2; ref. 41). Consistent with this finding, we observed a great increase of TβR2 in vitro and in vivo on treatment with anti-miR-17 molecules (Fig. 6C). TβR2 was recently reported to be a metastasis suppressor; deletion of TβR2 in breast cancer cells recruits Gr-1+CD11b+ cells that facilitate metastasis (42). Corresponding to this report, in the current study, we observed a significant decrease of Gr-1+CD11b+ cells in primary tumors treated with anti–miR-17 molecules (Fig. 6D), suggesting an important role of miR-17-mediated TβR2 signaling in regulating metastasis.

**Discussion**

The ability of cancer cells to detach and become motile requires enhanced actin meshwork-dependent adhesion and actin-myosin contraction. ROCK plays a central role in these processes. Phosphorylation of MLC by ROCK is the key mechanism for actin-myosin–based contraction, increasing the assembly of filamentous myosin heavy chains and promoting the binding of myosin and F-actin (43, 44). The net effect of ROCK activation is to enhance actin cytoskeleton and to increase actin-myosin–dependent force generation. Increased ROCK is correlated with poor clinical outcomes and late-stage tumors (1). The expression of ROCK1 in primary breast cancer tumors that give rise to metastases is much greater than in tumors that do not give rise to metastases (1).

In the current study, we found that expression of both ROCK1 and ROCK2 (particularly ROCK1) is greatly increased in metastatic human mammary tumors compared with nonmetastatic tumors and in metastatic breast cancer cells compared with nonmetastatic cells in vitro. ROCK expression is significantly increased in the metastatic breast cancer cells harvested from human bone grafts compared with cells at the orthotopic sites in our mouse model. In addition, we showed that overexpression of ROCK converts classically nonmetastatic MCF-7 cells into cells with metastatic capability. Taken together, our data confirm the crucial role of ROCK in promoting breast cancer metastasis.

Many studies have shown that c-Myc is amplified and/or overexpressed in breast cancer (34, 45), particularly in late-stage/high-grade tumors and metastatic tumors (31–33). Its aberrant expression appears to be a clinical prognostic marker for recurrence and adverse outcomes in breast cancer patients (46, 47). In the current study, the significant increase of c-Myc expression in metastatic breast cancer cells in vitro and in vivo suggests a role of c-Myc in breast cancer metastasis. As shown previously, ROCK increases the stabilization of c-Myc protein via phosphorylation in RhoA-transformed kidney epithelial cells (28). Thus, the reduction of c-Myc via ROCK inhibition by either Y27632 or ROCK siRNAs could be due to the degradation of this protein because of decreased phosphorylation. Increased baseline c-Myc in these metastatic cells may be due to high endogenous levels of ROCK activity. Further, a reduction of c-Myc level may explain the resultant attenuated cell proliferation by ROCK inhibition in vitro. Together, our observations support a link between ROCK and c-Myc. As c-Myc is downstream of many signaling pathways and its regulation is complex, involving transcriptional regulation and post-translational modifications (48), ROCK may be one of its regulators. Meanwhile, the possibility of an autocrine loop between ROCK and c-Myc is excluded, as ROCK levels are not significantly changed after c-Myc silencing (Supplementary Fig. S3D). However, thus far, a direct interaction between ROCK and c-Myc has not been shown.

Coupled to increased c-Myc, miR-17-92 expression is increased in metastatic breast cancer cells in vitro and in vivo. Moreover, miR-17-92 expression is significantly attenuated by treatment with...
Y27632. Because this cluster is transcriptionally regulated by c-Myc (6), which is targeted by ROCK (25), the attenuation of miR-17-92 expression by Y27632 treatment suggests a direct link between ROCK signaling and miRNA regulation. Among the miRNAs in this cluster, miR-17 has been shown to modulate cell proliferation by targeting CDKN1A/p21 (15, 36), an inhibitor of cell cycle progression (49). We observed that anti–miR-17 molecules reduce cell proliferation and lead to the upregulation of p21 mRNA in MDA-MB-231 and SUM1315 cells (data not shown), consistent with effects observed in B-cell lymphoma (36) and neuroblastoma cells (15). Importantly, we show for the first time that anti–miR-17 molecules reduce breast cancer cell migration in vitro and metastasis in vivo. The in vivo administration of anti–miR-17 molecules for 2 weeks greatly represses metastases without significant effect on primary tumor growth.

One potential mechanism responsible for miR-17–promoted metastasis is regulation of TβR2, a target of miR-17 (41). Here, we show that anti–miR-17 molecules significantly upregulate TβR2 and diminish the Gr-1+CD11b+ cell population in primary tumors. The recruitment of Gr-1+CD11b+ cells in breast cancer tumors was recently shown to facilitate metastasis via the SDF-1/CXCR4 and CXCL5/CXCR2 axes (42). Taken together, these findings indicate that this cluster contributes to the regulation of breast cancer tumor growth and metastasis, and its expression is dependent on ROCK signaling.

In line with the effects of Y27632 on cell motility and migration in vivo, Y27632 significantly reduces metastases to human bone cores (by 46%) and mouse skeleton (by 36%) as well as the mass of metastases in the mouse skeleton (by 77%). Y27632 competes with ATP for binding to the catalytic domain of ROCK (50), leading to inhibition of the kinase. The dose of Y27632 used in our experiment (8 mg/kg) is ~3 times lower than that used in a similar study (assuming the average body weight of each mouse is 40 g; ref. 5); however, the reduction of ROCK activity is equivalent. This observation suggests that inhibition of ROCK activity by Y27632 reaches a limit possibly due to saturation of the binding of Y27632 to the ROCK catalytic site. Although suppression of metastasis by Y27632 is shown in our investigations, little effect on primary tumor mass was observed, implying that Y27632 and ROCK inhibition, in general, may have a metastasis-specific effect.

Collectively, ROCK may enhance the metastatic propensity of breast cancer cells by stabilizing the actin cytoskeleton, enhancing actin-mysin contraction, and promoting the c-Myc pathway, including transcription of c-Myc–regulated miRNAs (Supplementary Fig. S10). Inhibition of ROCK-mediated signaling appears to be a promising and potentially specific approach to suppress breast cancer metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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