

A Pleiotropically Acting MicroRNA, miR-31, Inhibits Breast Cancer Metastasis

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SUMMARY

MicroRNAs are well suited to regulate tumor metastasis because of their capacity to coordinately repress numerous target genes, thereby potentially enabling their intervention at multiple steps of the invasion-metastasis cascade. We identify a microRNA exemplifying these attributes, miR-31, whose expression correlates inversely with metastasis in human breast cancer patients. Overexpression of miR-31 in otherwise-aggressive breast tumor cells suppresses metastasis. We deploy a stable microRNA sponge strategy to inhibit miR-31 in vivo; this allows otherwise-nonaggressive breast cancer cells to metastasize. These phenotypes do not involve confounding influences on primary tumor development and are specifically attributable to miR-31-mediated inhibition of several steps of metastasis, including local invasion, extravasation or initial survival at a distant site, and metastatic colonization. Such pleiotropy is achieved via coordinate repression of a cohort of metastasis-promoting genes, including RhoA. Indeed, RhoA re-expression partially reverses miR-31-imposed metastasis suppression. These findings indicate that miR-31 uses multiple mechanisms to oppose metastasis.

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INTRODUCTION

Metastases account for 90% of human cancer deaths (Gupta and Massagué, 2006), yet our understanding of the molecular circuitry that governs metastatic dissemination remains fragmentary. The

invasion-metastasis cascade, which leads to these growths, is a complex, multistep process involving the escape of neoplastic cells from a primary tumor (local invasion), intravasation into the systemic circulation, survival during transit through the vasculature, extravasation into the parenchyma of distant tissues, the establishment of micrometastases, and ultimately the outgrowth of macroscopic secondary tumors (colonization) (Fidler, 2003).

MicroRNAs (miRNAs) constitute an evolutionarily conserved class of pleiotropically acting small RNAs that suppress gene expression posttranscriptionally via sequence-specific interactions with the 3' untranslated regions (UTRs) of cognate mRNA targets (Bartel, 2009). In mammalian cells, miRNAs effect gene silencing via both translational inhibition and mRNA degradation; an individual miRNA is capable of regulating dozens of distinct mRNAs, and together the >650 human miRNAs are believed to modulate more than one-third of the mRNA species encoded in the genome (Bartel, 2009).

A central role for miRNAs in the establishment and progression of human tumors has begun to emerge. More than 50% of miRNA-encoding loci reside in chromosomal regions altered during tumorigenesis (Calin et al., 2004), and expression profiling reveals characteristic miRNA signatures for many tumor types—including breast neoplasias—that predict disease status and clinical outcome (Calin and Croce, 2006). In addition, miRNAs have been identified that function as classical oncogenes or tumor suppressor genes (Ventura and Jacks, 2009), as well as a limited number that act at late stages of tumor progression (Ma et al., 2007; Tavazoie et al., 2008; Huang et al., 2008; Asangani et al., 2008; Zhu et al., 2008; Lujambio et al., 2008).

The extent to which miRNAs specifically affect metastasis remains unclear, because all the miRNAs reported to affect metastasis also exert potentially confounding influences on primary tumor development, apoptosis, and/or cell proliferation (Voorhoeve et al., 2006; Sathyan et al., 2007; Ma et al., 2007; Si et al., 2007; Tavazoie et al., 2008; Kondo et al., 2008; Lujambio et al., 2008). Moreover, a role for miRNAs in steps of the invasion-

metastasis cascade subsequent to local invasion has not been described.

The pleiotropic nature of gene regulation exhibited by miRNAs led us to hypothesize that certain miRNAs might be endowed with a capacity to function as crucial modulators of tumor metastasis. Here, we identify an antimetastatic human miRNA, miR-31, that acts at multiple steps of the invasion-metastasis cascade via repression of a cohort of prometastatic targets.

RESULTS

miR-31 Expression Is Specifically Attenuated in Metastatic Breast Cancer Cell Lines

To identify miRNAs that might regulate breast cancer metastasis, we selected 10 cancer-associated miRNAs for further characterization because of their concordant identification among expression profiling studies of clinical breast tumors (lorio et al., 2005; Volinia et al., 2006), global analysis of miRNA copy-number variation in human breast carcinomas (Zhang et al., 2006), and localization of miRNA loci to cancer-relevant sites of chromosomal aberration (Table S1 available online; Calin et al., 2004). These studies did not stratify patients based on metastasis status.

Expression of the 10 candidate miRNAs was assayed in 15 human and mouse mammary cell lines, which included normal epithelial cells, tumorigenic but nonmetastatic cells, and metastatic tumor cells (Table S2). The levels of a single miRNA, miR-31, were specifically attenuated in aggressive human breast cancer cells when compared to primary normal human mammary epithelial cells (HMECs). Although nonmetastatic tumor cells (HMLER, MCF7-Ras, and SUM-149) exhibited 4-fold reduced miR-31, expression of this miRNA in metastatic SUM-159 and MDA-MB-231 cells was diminished by >100-fold (Figure 1A).

Relative to its expression in normal murine mammary gland (NMuMG) cells, miR-31 levels in sublines derived from a single murine mammary tumor reflected their capacities to metastasize: miR-31 was reduced by 2-fold in metastatic D2.1 and D2A1 cells, but not in nonaggressive D2.OR cells (Figure 1B). miR-31 levels were also inversely proportional to metastatic ability in four mouse mammary carcinoma sublines derived from a single spontaneously arising tumor: although miR-31 levels in nonaggressive 67NR cells were similar to those in NMuMG, miR-31 expression was progressively diminished upon acquisition of the capacity to invade locally (168FARN), to form micrometastases (4TO7), and to yield macroscopic metastases (4T1) (Figure 1B). Thus, miR-31 levels are specifically attenuated in aggressive breast cancer cells.

miR-31 expression was heterogeneous in 4T1 cell primary mammary tumors; of note, the proportion of cells expressing miR-31 was 10-fold reduced in lung metastases relative to the fraction of miR-31-positive cells in the primary tumors from which they were derived (Figure 1C). Also, 5-fold fewer cells located near the invasive front of 4T1 cell mammary tumors expressed miR-31, compared to cells in the interior of these tumors (Figure 1D). These data raise the possibility that selective pressures diminish the prevalence of miR-31-expressing cells within the pool of successfully metastasizing cells during the course of metastatic progression.

miR-31 Expression Suppresses Metastasis-Relevant Traits In Vitro

Given these inverse correlations between miR-31 levels and malignant phenotypes, we assessed the potential for antimetastatic roles for miR-31. Thus, we stably expressed miR-31 in metastatic MDA-MB-231 human breast cancer cells ("231 cells"). This overexpression resulted in miR-31 levels comparable to those in HMECs (Figure S1A).

Ectopic miR-31 did not affect proliferation in vitro, but did reduce invasion by 20-fold and motility by 10-fold (Figure 2A; Figures S1B and S1C). These effects were specifically attributable to the biological activities of miR-31, as indicated by the fact that equivalent overexpression of a control miRNA, miR-145, failed to influence invasion or motility (Figure 2A and data not shown). Also, miR-31-expressing cells exhibited 60% diminished resistance to anoikis-mediated cell death (Figure 2B).

These defects could not be ascribed to toxicity resulting from ectopic miR-31 (Figure S1D). The consequences of miR-31 expression were not unique to 231 cells: miR-31 reduced invasion, motility, and anoikis resistance, yet did not affect proliferation, in aggressive SUM-159 human breast cancer cells (Figure S2). Hence, miR-31 impairs in vitro surrogates of metastatic ability.

miR-31 Expression Suppresses Metastasis In Vivo

Because of its effects on in vitro traits associated with high-grade malignancy, we asked whether ectopic miR-31 could inhibit metastasis in otherwise-aggressive cells. Thus, 231 cells expressing miR-31 were injected into the orthotopic site—the mammary fat pad—of mice. Unexpectedly, miR-31 enhanced primary tumor growth by 1.5-fold and correspondingly increased cell proliferation (Figure 2C; Figure S3A). Control 231 cell primary tumors displayed evidence of local invasion; however, miR-31-expressing tumors were well encapsulated and noninvasive (Figures 2D and 2E). These changes were not accompanied by altered neovascularization (Figure S3B).

Despite their ability to generate larger primary tumors, 231 cells expressing miR-31 were strikingly impaired in their capacity to seed lung metastases. miR-31-expressing cells formed 95% fewer lesions than did controls 62 days after implantation (Figure 2F). Thus, miR-31 suppresses metastasis from an orthotopic site, ostensibly due, at least in part, to its ability to impede local invasion.

We addressed the possibility that miR-31's impact on these parameters was attributable to clonal variation in our 231 cells by expressing miR-31 in a single-cell-derived population isolated from the parental 231 cells (Figure S4A; Minn et al., 2005). As before, when injected orthotopically, miR-31-expressing cells formed large, well-encapsulated primary tumors and also reduced lung metastasis by 5-fold (Figures S4B–S4D). Orthotopic injection of SUM-159 cells expressing miR-31 further corroborated our earlier findings: miR-31 enhanced primary tumor growth, yet miR-31-expressing tumors were better confined than control tumors (Figure S5). These observations indicated that the ability of miR-31-expressing cells to form larger, less invasive primary tumors, as well as to seed fewer metastases, is a specific consequence of the biological activities of miR-31.

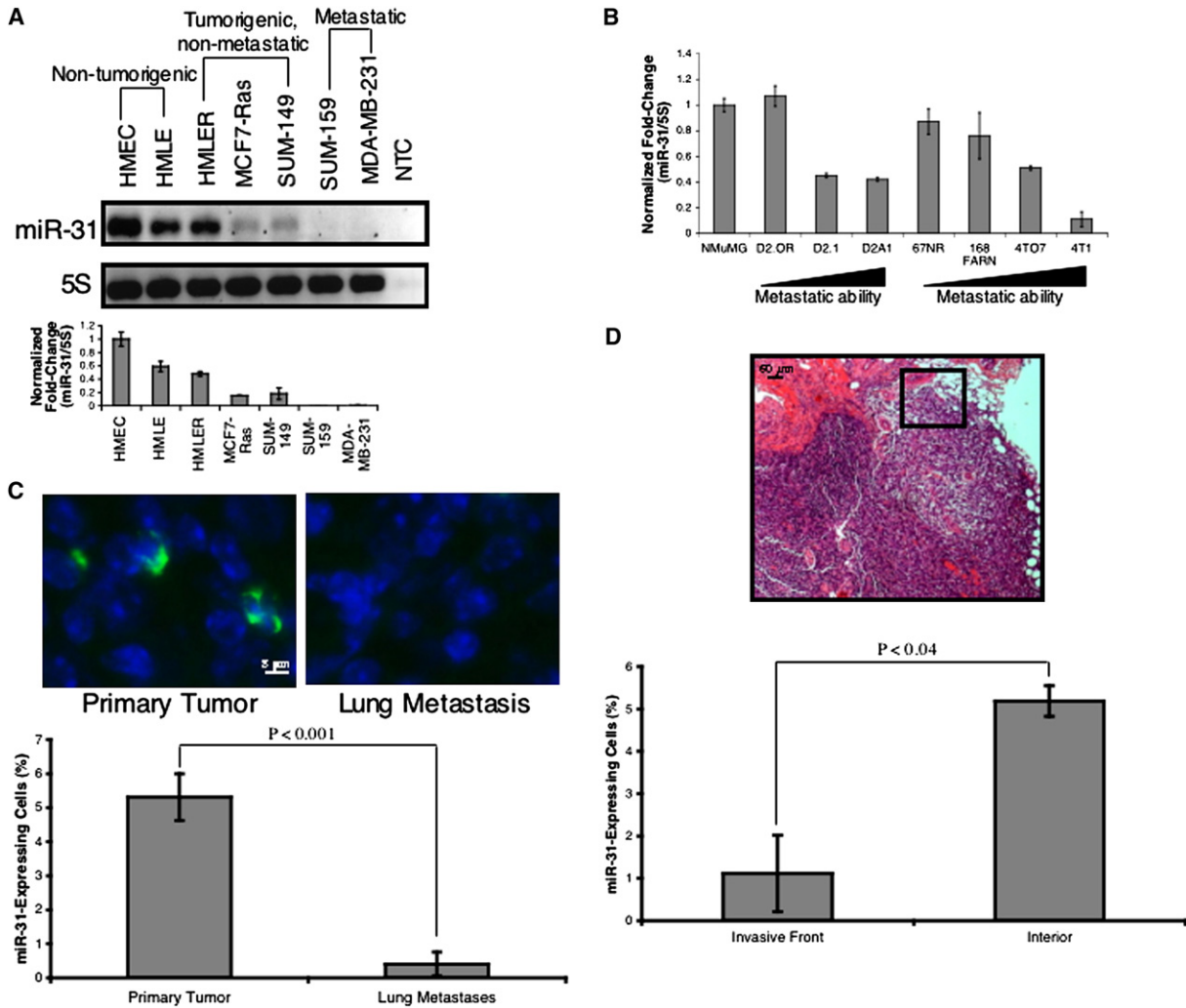


Figure 1. miR-31 Levels Correlate Inversely with Metastatic Ability in Breast Cell Lines

(A) RT-PCR for miR-31 in seven human breast cell lines. 5S rRNA was a loading control. NTC, no template control. n = 3.

(B) miR-31 RT-PCR in eight murine mammary cell lines. 5S rRNA was a loading control. n = 3.

(C) In situ hybridization for miR-31 (green) in animal-matched 4T1 cell primary mammary tumors and lung metastases; DAPI counterstain (blue). n = 4.

(D) Hematoxylin and eosin (H&E) stain of a 4T1 cell primary mammary tumor (top); box: invasive front. miR-31 in situ hybridization in 4T1 cells located near the invasive front or the interior of the primary tumors (bottom). n = 3.

Data are presented as mean ± SEM.

We determined whether miR-31's impact on metastasis was also attributable to effects on later steps of the invasion-metastasis cascade, independent of its influence on local invasion. Thus, we injected miR-31-expressing 231 cells directly into the circulation of mice, thereby circumventing the initial steps of local invasion and intravasation. After 1 day, miR-31-expressing cells were 4-fold impaired in their ability to persist in the lungs (Figure 2G). This difference was not a consequence of an inability of miR-31-expressing cells to become lodged initially in the lung microvasculature, as shown by the fact that equal numbers of miR-31-expressing and control cells were detected in the lungs 10 min and 2 hr after injection (Figure 2G; Figure S6A). These observations suggested that miR-31 regulates early postintra-

sation events, such as intraluminal viability, extravasation, and/or initial survival in the lung parenchyma.

Three months after tail vein injection, miR-31-expressing 231 cells generated 40-fold fewer lung metastases than did controls (Figure 2G). We also observed a dramatic effect on the size of eventually formed lesions: after 3 months, miR-31-expressing cells generated only small micrometastases although control cells formed macroscopic metastases; this occurred despite the fact that miR-31-expressing and control cells established comparably sized micrometastases 1 month after injection (Figure 2G; Figure S6B). Such effects on lesion size implied that miR-31 affects metastatic colonization in addition to its influences on local invasion and early postintra-

Inhibition of miR-31 Promotes Metastasis-Relevant Traits In Vitro

The preceding observations demonstrated that miR-31 expression deprives metastatic cells of attributes associated with high-grade malignancy. We next asked whether miR-31 also prevents the acquisition of aggressive traits by otherwise-non-metastatic human breast cancer cells. To do so, we transiently inhibited miR-31 in noninvasive MCF7-Ras cells with either antisense oligonucleotides or miRNA sponges. The latter are expression constructs that carry miRNA recognition motifs in their 3' UTR that bind and thus titrate miRNAs (Ebert et al., 2007). Both approaches inhibited miR-31 function by >4.5-fold (Figure S7A). Suppression of miR-31 enhanced invasion by 20-fold and motility by 5-fold, but cell viability was unaffected by either inhibitor (Figure 3A; Figure S7B).

Techniques for stable miRNA inhibition have been unavailable (Krützfeldt et al., 2006). To address this problem, we modified elements derived from the transiently expressed miRNA sponges, cloned them into a retroviral vector, and created MCF7-Ras cells that stably express the modified miRNA sponges. The miR-31 sponge reduced miR-31 function by 2.5-fold, but did not affect the activity of other known antimetastatic miRNAs (Figures S8A and S8B). The relatively modest suppression of miR-31 conferred by stable sponge expression elicited strong responses: invasion was enhanced by 12-fold, motility by 8-fold, and anoikis resistance by 2.5-fold (Figure 3B; Figure S8C). The miR-31 sponge failed to alter in vitro proliferation (Figure S8D).

When stably expressed in immortalized HMECs or tumorigenic but nonmetastatic SUM-149 human breast cancer cells, the miR-31 sponge elicited increased invasion, motility, and anoikis resistance without affecting proliferation (Figure S9 and data not shown). Collectively, these data indicated that sustained miR-31 activity is necessary to prevent the acquisition of aggressive traits by both tumor cells and untransformed breast epithelial cells.

Inhibition of miR-31 Promotes Metastasis In Vivo

We exploited our ability to stably inhibit miRNAs in order to assess whether miR-31 activity is required to prevent metastasis in vivo. To do so, otherwise-nonmetastatic MCF7-Ras cells stably expressing the miR-31 sponge were orthotopically implanted into mice. Inhibition of miR-31 failed to alter in vivo proliferation and primary tumor growth (Figure 3C; Figure S10A). Primary tumors derived from miR-31 sponge-expressing cells were poorly encapsulated and locally invasive, whereas control MCF7-Ras tumors appeared well confined and noninvasive (Figures 3D and 3E). Again, neovascularization did not differ (Figure S10B).

Strikingly, miR-31 sponge-expressing MCF7-Ras cells metastasized to the lungs in significant numbers, whereas control tumor-bearing host lungs were largely devoid of tumor cells; cells with impaired miR-31 activity formed 10-fold more lesions than did controls (Figure 3F). Hence, continuous miR-31 function is required to prevent metastasis from an orthotopic site.

We asked whether loss of miR-31 activity also promoted metastasis by intervening at steps of the invasion-metastasis cascade subsequent to local invasion. Thus, we intravenously injected mice with miR-31 sponge-expressing MCF7-Ras cells.

Within 1 day, miR-31 inhibition enhanced cell number in the lungs by 6-fold; similarly, at later times after injection, miR-31 sponge-expressing cells were 10-fold more prevalent in the lungs than were controls (Figure 3G). The differing metastatic abilities of control and miR-31 sponge-expressing cells did not arise because of failure of control cells to become lodged initially in the lung vasculature, as shown by the fact that equal numbers of cells from each cohort were present 10 min after injection (Figure 3G; Figure S11).

Suppression of miR-31 also affected lesion size 4 months after tail vein injection: whereas control cells formed only small micrometastases, miR-31 sponge-expressing cells produced macroscopic metastases (Figure 3G). Together, these data extended and reinforced our ectopic expression studies by demonstrating that miR-31 affects local invasion, early postinvasion events, and metastatic colonization.

miR-31 Directly Regulates a Cohort of Prometastatic Genes

miR-31's ability to impede multiple steps of the invasion-metastasis cascade might derive from its ability to pleiotropically regulate genes involved in diverse aspects of metastatic dissemination. To identify effectors of miR-31, we used two algorithms that predict the mRNA targets of a miRNA—PicTar (Krek et al., 2005) and TargetScan (Grimson et al., 2007). Based on the representation of miR-31 sites in their 3' UTRs, >200 mRNAs were predicted to be regulated by miR-31. Gene ontology (Ashburner et al., 2000) revealed that these targets included a disproportionately large number of genes encoding proteins with roles in motility-related processes, such as cell adhesion, cytoskeletal remodeling, and cell polarity (data not shown).

Guided by this gene ontology analysis, we cloned the 3' UTRs of 16 putative miR-31 targets from these overrepresented categories, including several implicated in tumor invasion (Sahai and Marshall, 2002; McClatchey, 2003), into a luciferase construct. Reporter assays with miR-31-expressing 231 cells revealed that miR-31 repressed six of the UTRs: frizzled3 (Fzd3), integrin $\alpha 5$ (ITGA5), myosin phosphatase-Rho interacting protein (M-RIP), matrix metalloproteinase 16 (MMP16), radixin (RDX), and RhoA (Figure 4A). Mutation of the putative miR-31 site(s) in these six 3' UTRs (Table S3) abrogated responsiveness to miR-31 (Figure 4B). In the case of RhoA, whose UTR contains two miR-31 sites separated by 152 nucleotides, mutation of either motif abolished miR-31 responsiveness (Figure 4B), suggesting functional interaction between the sites (Grimson et al., 2007).

Endogenous Fzd3, ITGA5, MMP16, RDX, and RhoA protein levels were assayed in miR-31-expressing 231 cells. miR-31 repressed the levels of these proteins by 40%–60% (Figure 4C). miR-31's effects on levels of the M-RIP protein could not be evaluated because of the lack of appropriate antibodies. Also, miR-31 reduced the endogenous mRNA levels of these six targets by 2-fold in SUM-159 cells, as well as Fzd3, ITGA5, MMP16, RDX, and RhoA mRNA levels in 231 cells (Figure 4D). miR-31 did not affect CXCL12 mRNA levels—a computationally predicted miR-31 target found not to be regulated by this miRNA—in either cell type (Figures 4A and 4D). These data indicated that miR-31 directly regulates endogenous Fzd3, ITGA5,

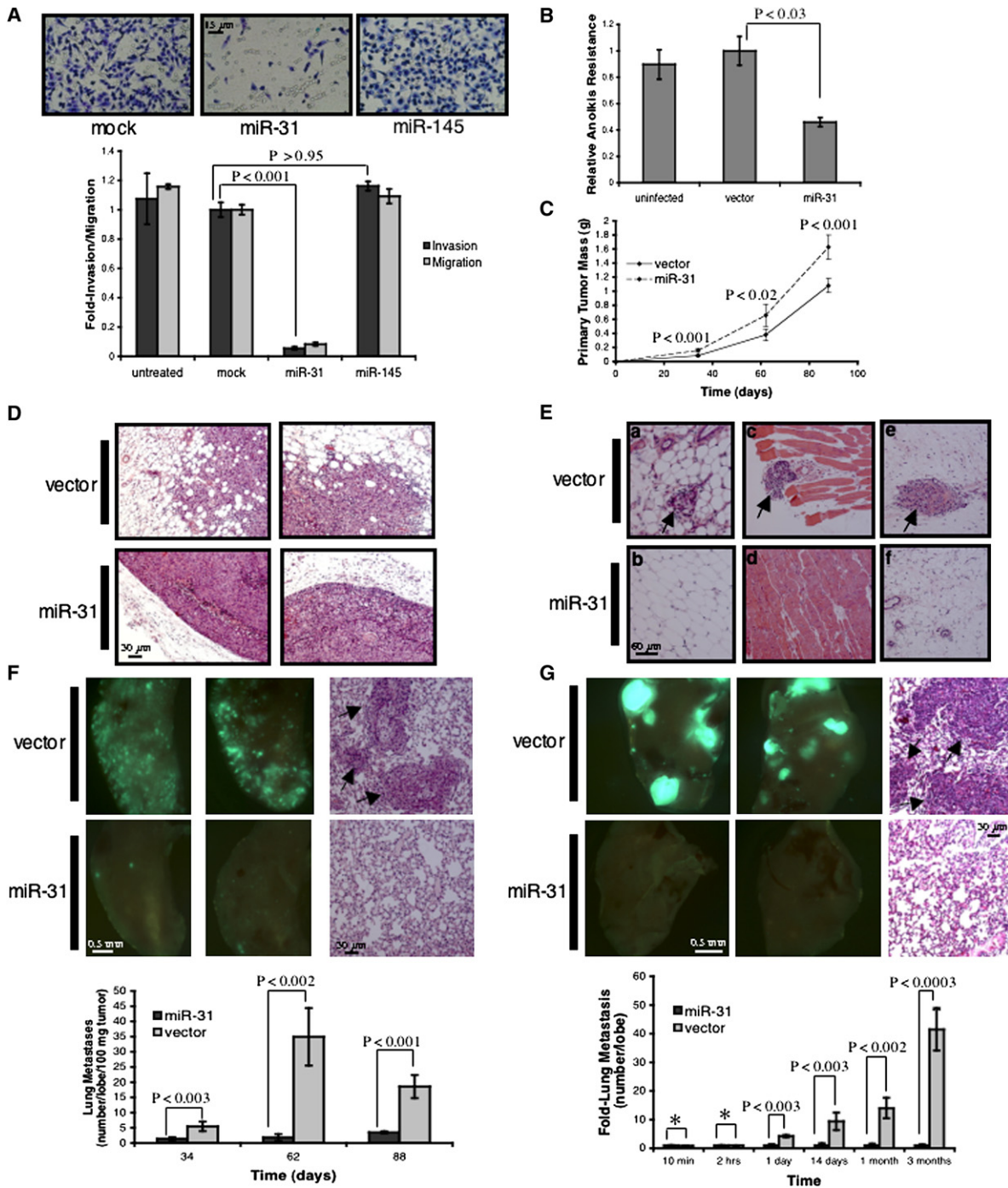


Figure 2. miR-31 Expression Inhibits Metastasis

(A) Invasion and motility assays after transfection of MDA-MB-231 (231) cells with the indicated constructs. *n* = 3.

(B) Anoikis assays with 231 cells infected as indicated. *n* = 3.

(C) Primary tumor growth upon orthotopic injection of 1.0×10^6 GFP-labeled 231 cells infected as indicated. The experiment was terminated after 13 weeks because of primary tumor burden. *n* = 5 per group per time point.

(D) H&E stain of 231 primary tumors 62 days after orthotopic injection.

(E) H&E stain of tissue adjacent to the indicated 231 primary mammary tumors 62 days after injection. Arrows: disseminated tumor cells in normal fat (a, b), muscle (c, d), and subcutis (e, f).

(F) Images of murine lungs to visualize GFP-labeled 231 cells 62 days after orthotopic implantation (left). H&E stain of lungs from animals bearing the indicated tumors (right); arrows indicate metastatic foci. *n* = 5.

M-RIP, MMP16, RDX, and RhoA expression in human breast cancer cells.

We determined whether concomitant repression of Fzd3, ITGA5, M-RIP, MMP16, RDX, and RhoA correlated with disease progression in clinical breast cancers by examining expression profiling data from 295 primary breast tumors (Table S4; van de Vijver et al., 2002). To do so, we constructed a miR-31 target signature based on coordinate differential expression of these six genes. Within this cohort, high expression of the miR-31 target signature was associated with metastasis, as well as poor survival, relative to signature-negative tumors; 5-year survival among patients negative for the target signature was 90%, whereas >35% of target signature-positive patients succumbed to their disease over this interval (Figures 5A and 5B). Thus, coordinate repression of Fzd3, ITGA5, M-RIP, MMP16, RDX, and RhoA correlated with more favorable outcome in clinical breast tumors.

To assess the functional contributions of these miR-31 targets to aggressive phenotypes, we first examined whether their inhibition affected the invasion or motility of 231 cells. Transfection with siRNAs potently reduced target protein levels without affecting cell viability (Figures S12A and S12B). siRNAs targeting Fzd3, ITGA5, RDX, or RhoA reduced invasion and motility, whereas siRNAs against M-RIP or MMP16 failed to affect these traits (Figure 5C; Figure S12C).

We asked whether inhibition of these effectors compromised resistance to anoikis. siRNAs against ITGA5, RDX, or RhoA sensitized 231 cells to anoikis; in contrast, siRNAs targeting Fzd3, M-RIP, or MMP16 had no effect on anoikis resistance (Figure 5D). Hence, suppression of Fzd3, ITGA5, RDX, or RhoA impaired metastasis-relevant traits *in vitro*.

Re-expression of Fzd3, ITGA5, RDX, and RhoA Reverses miR-31-Dependent Metastasis-Relevant Phenotypes *In Vitro*

To determine whether *in vitro* phenotypes associated with miR-31 expression could be reversed via restoration of Fzd3, ITGA5, M-RIP, MMP16, RDX, or RhoA levels, we transfected miR-31-expressing 231 cells with individual expression constructs rendered miRNA insensitive by deletion of their 3' UTRs; this was not cytotoxic (Figures S13A and S13B and data not shown). In miR-31-expressing cells, Fzd3, ITGA5, RDX, or RhoA reversed, at least partially, miR-31-imposed invasion and motility defects; in contrast, M-RIP or MMP16 had no effect on these traits (Figure 5E; Figure S13C). Surprisingly, re-expression of RDX or RhoA completely rescued miR-31-mediated invasion and motility defects. Expression of the six targets failed to enhance the invasion or motility of control 231 cells (Figure 5E; Figure S13C).

We evaluated whether re-expression of any of the six targets rescued miR-31's effects on anoikis. ITGA5, RDX, or RhoA reversed, at least in part, anoikis susceptibility resulting from ectopic miR-31; in contrast, Fzd3, M-RIP, or MMP16 failed to affect this trait (Figure 5F). In fact, ITGA5 or RhoA completely

rescued miR-31-dependent anoikis phenotypes. The six targets did not enhance anoikis resistance in control 231 cells (Figure 5F). Hence, Fzd3, ITGA5, RDX, and RhoA are functionally relevant effectors of miR-31 for conferring malignant traits *in vitro*.

Re-expression of RhoA Partially Reverses miR-31-Imposed Metastasis Defects *In Vivo*

RhoA afforded the most pronounced reversal of miR-31-mediated phenotypes. Therefore, we stably re-expressed miRNA-resistant RhoA in 231 cells that already had been infected with either miR-31 or control vector (Figures S14A and S14B). RhoA did not affect proliferation *in vitro*, but did abrogate miR-31-imposed invasion, motility, and anoikis resistance defects (Figures S14C–S14F).

To ascertain whether restored RhoA levels reversed *in vivo* metastasis phenotypes ascribable to miR-31, we orthotopically injected mice with 231 cells expressing combinations of miR-31, RhoA, and control vectors. As observed previously, miR-31 enhanced primary tumor growth (Figure 6A). RhoA initially augmented primary tumor growth in the presence of ectopic miR-31, but failed to do so in control 231 cells (Figure 6A). In consonance with our earlier findings, control 231 primary tumors were locally invasive and miR-31-expressing tumors were noninvasive (Figures 6B and 6C). In control 231 cells, ectopic RhoA failed to exacerbate the extent of local invasion; in contrast, RhoA abolished the previously encapsulated appearance of miR-31-expressing tumors and enabled invasion into surrounding normal tissue (Figures 6B and 6C).

Re-expression of RhoA restored lung metastasis in miR-31-expressing 231 cells to 75% of control cell levels, although RhoA failed to enhance metastasis in control 231 cells (Figure 6D). Thus, re-expression of RhoA partially, yet robustly, reverses metastasis suppression imposed by miR-31. The observed magnitude of rescue is surprising, because RhoA is only one member of a larger cohort of metastasis-relevant genes repressed by miR-31.

By intravenously injecting mice with 231 cells expressing miR-31 and/or RhoA, we gauged whether RhoA-mediated reversal of miR-31-imposed metastasis defects was solely attributable to effects on local invasion. Although expression of miR-31 and/or RhoA failed to affect the initial lodging of tumor cells in the lung vasculature, the number of cells that persisted in the lungs differed within one day of injection (Figure 6E; Figure S15). As before, miR-31 inhibited both the number of metastases formed and their eventual size (Figure 6E). Although expression of RhoA in control 231 cells failed to enhance metastasis, RhoA restored the number of lung metastases to 60% of control cell levels in miR-31-expressing cells; however, RhoA did not facilitate the formation of macroscopic metastases in cells with ectopic miR-31 (Figure 6E).

Together, these data indicated that miR-31's ability to inhibit metastasis is attributable, in significant part, to its capacity to inhibit RhoA. miR-31-mediated repression of RhoA affects

(G) Images of murine lungs to detect GFP-labeled 231 cells 88 days after tail vein injection (left). H&E stain of lungs (right); arrows indicate metastatic foci. Asterisks: $p > 0.66$. $n = 5$, except for 10 min and 2 hr ($n = 4$). Data are presented as mean \pm SEM.

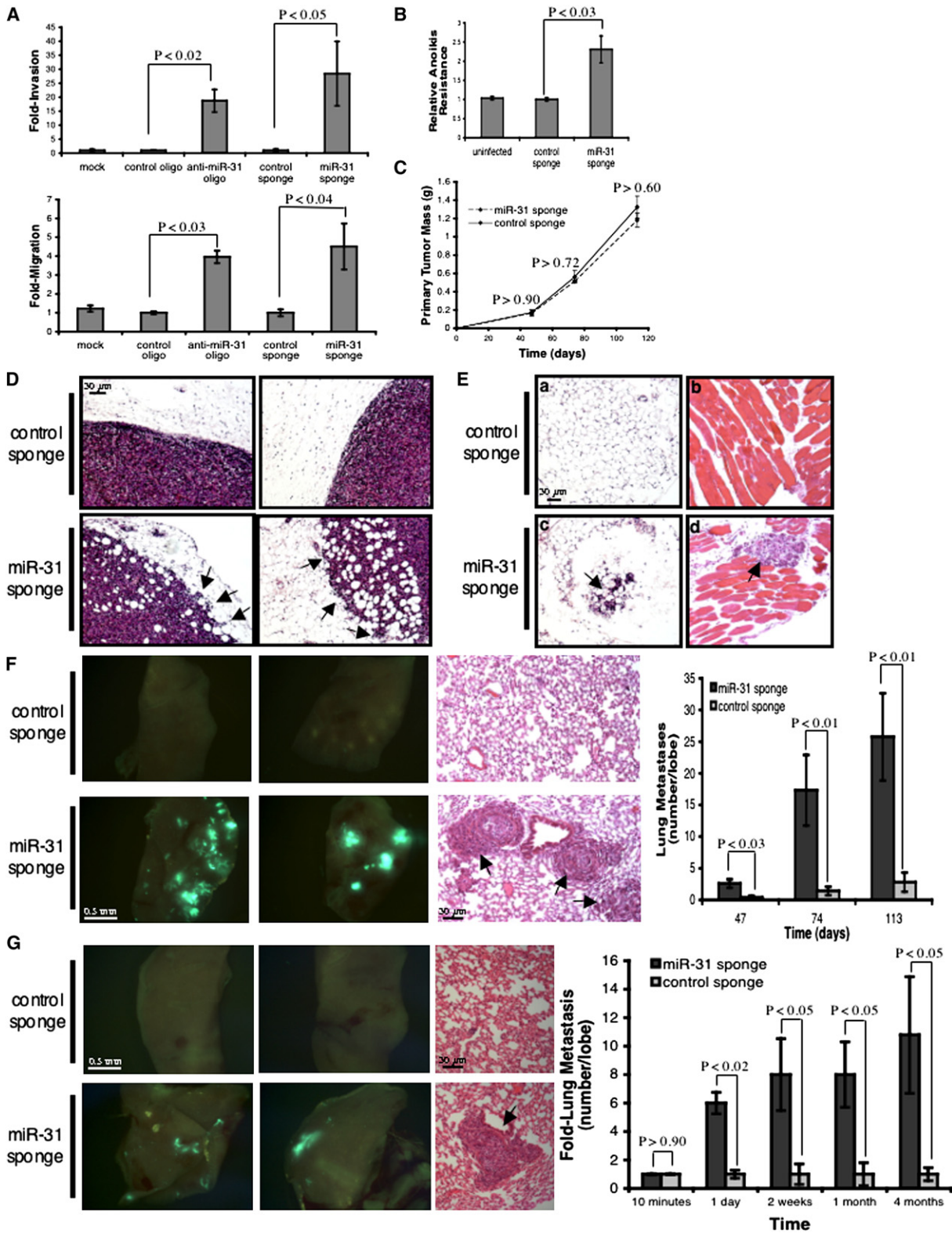


Figure 3. Inhibition of miR-31 Promotes Metastasis

(A) Invasion and motility assays with MCF7-Ras cells transfected with the indicated transient miR-31 inhibitors. n = 3.

(B) Anoikis assays with MCF7-Ras cells stably expressing the indicated sponge. n = 3.

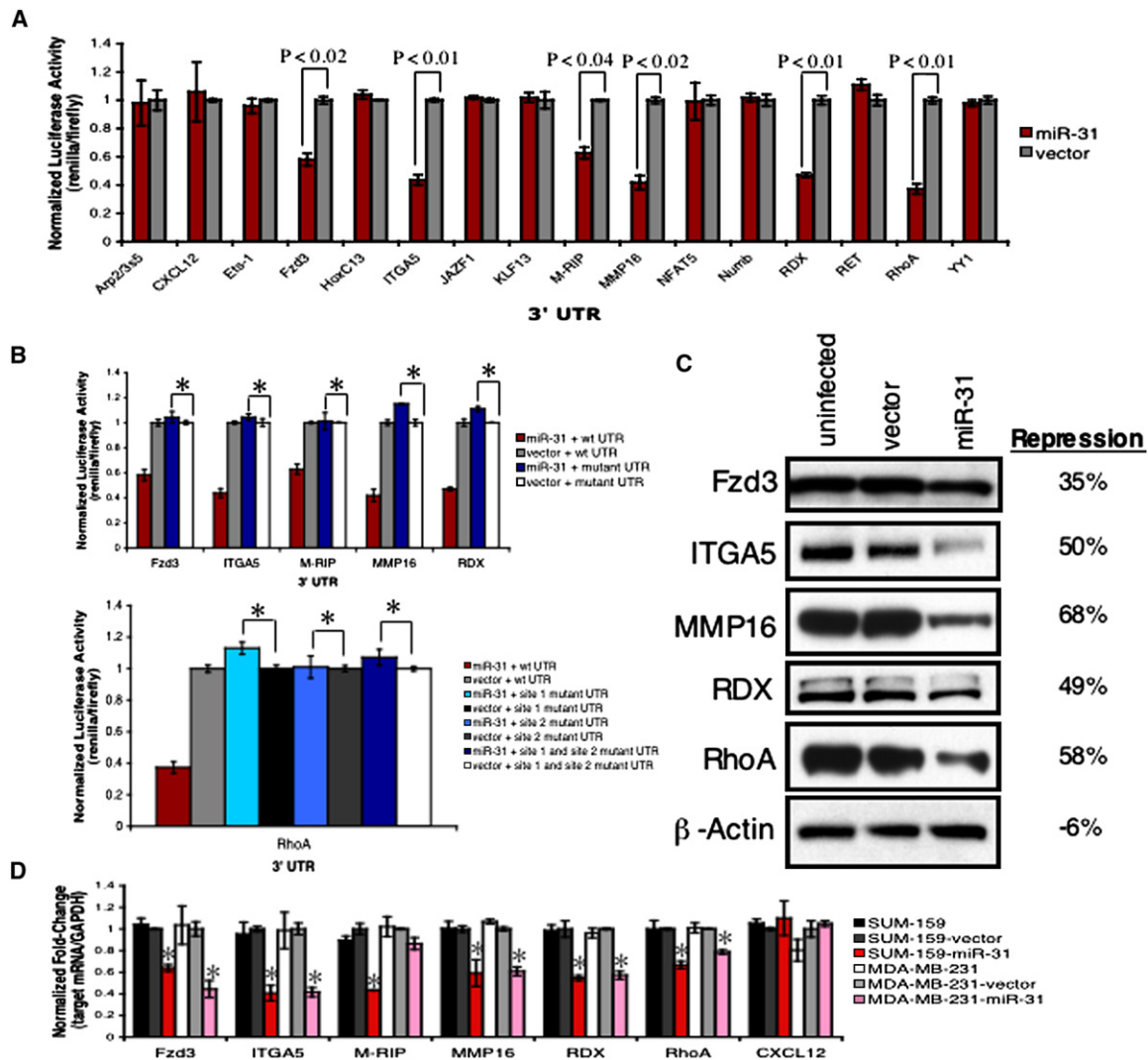


Figure 4. miR-31 Directly Regulates a Cohort of Prometastatic Genes

(A) Luciferase activity in 231 cells infected with miR-31 or control vector after transfection of the indicated 3' UTR-driven reporter constructs. $n = 3$.

(B) Luciferase activity in the indicated 231 cells upon transfection of miR-31 site mutant 3' UTR-driven reporter constructs. wt: wild-type; site 1: the miR-31 motif at nt 145–151 of the RhoA 3' UTR; site 2: the motif spanning nt 303–309. Asterisks: $p > 0.80$ relative to mutant-UTR + vector controls. $n = 3$.

(C) Immunoblots for endogenous Fzd3, ITGA5, MMP16, RDX, and RhoA in the indicated 231 cells. β -actin was a loading control. Repression: protein levels in miR-31-expressing cells relative to vector controls.

(D) RT-PCR for endogenous CXCL12, Fzd3, ITGA5, M-RIP, MMP16, RDX, and RhoA. GAPDH was a loading control. Asterisks: $p < 0.03$ relative to vector controls. $n = 3$.

Data are presented as mean \pm SEM.

both local invasion and early postinvasation events. However, these data also implied that the full spectrum of miR-31's effects on metastasis are elicited only via the coordinate repression of

multiple targets, because suppression of RhoA alone could not explain the complete impact of miR-31 on the number of metastases formed or its effects on metastatic colonization.

(C) Primary tumor growth upon orthotopic implantation of 5.0×10^5 GFP-labeled MCF7-Ras cells infected as indicated. The experiment was terminated after 16 weeks because of primary tumor burden. $n = 5$ per group per time point.

(D) H&E stain of MCF7-Ras primary tumors 47 days after orthotopic injection. Arrows indicate regions of poor encapsulation.

(E) H&E stain of tissue adjacent to the indicated MCF7-Ras primary tumors 47 days postinjection. Arrows: disseminated tumor cells in normal fat (a, c) and muscle (b, d).

(F) Images of murine lungs to visualize GFP-labeled MCF7-Ras cells 113 days after orthotopic injection (left). H&E stain of lungs from animals bearing the indicated tumors (middle); arrows indicate metastatic foci. $n = 5$.

(G) Images of murine lungs to detect GFP-labeled MCF7-Ras cells 122 days after tail vein injection (left). H&E stain of lungs (middle); arrow indicates metastasis. $n = 4$, except for 1 day ($n = 3$).

Data are presented as mean \pm SEM.

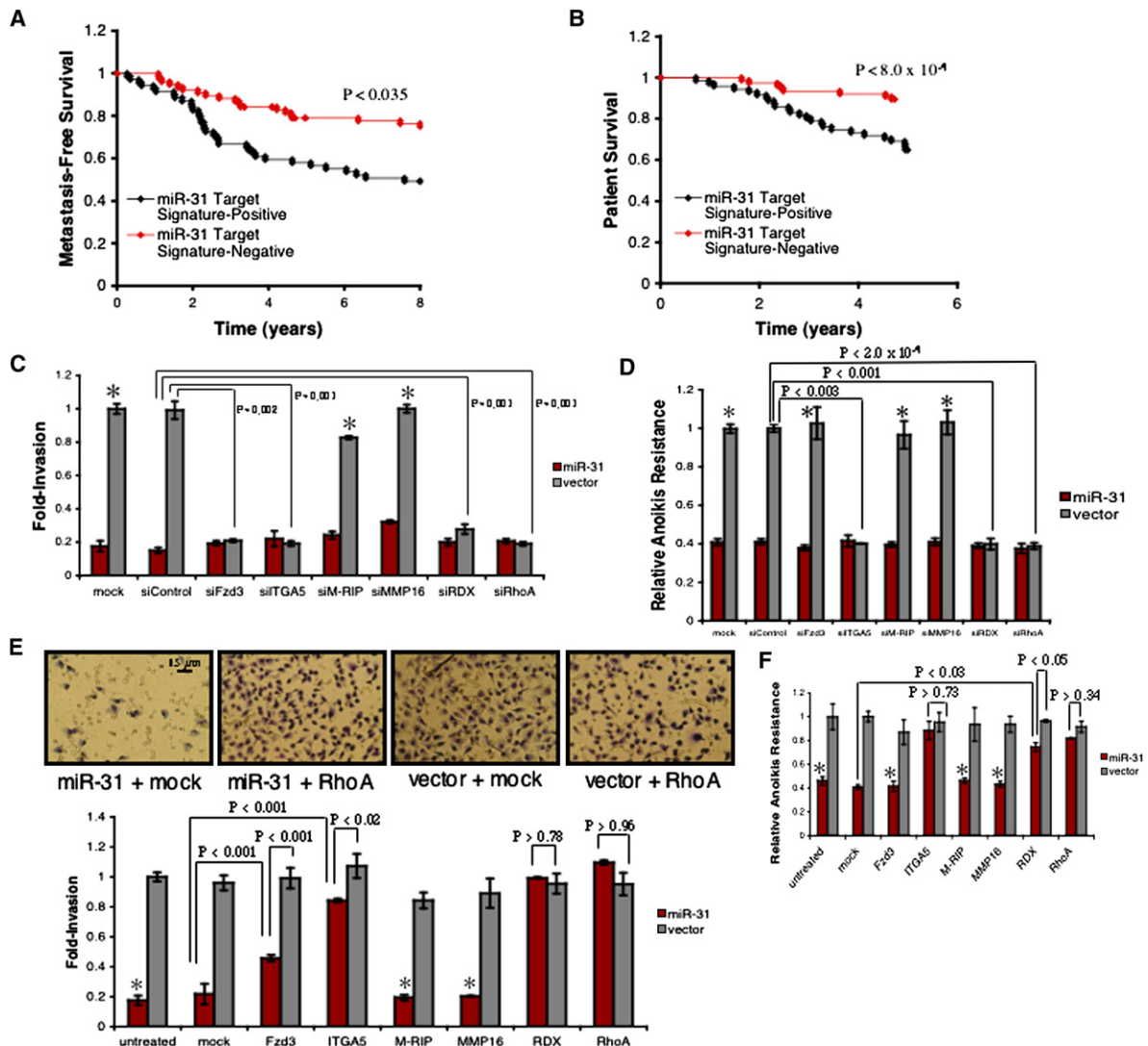


Figure 5. Repression of Fzd3, ITGA5, RDX, and RhoA Underlies miR-31-Dependent Phenotypes In Vitro

(A) Kaplan-Meier curves for 295 human primary breast tumors depicting metastasis-free survival, stratified based on expression of the 6-gene miR-31 target signature. p value based on a logrank test.
 (B) Kaplan-Meier 5-year survival curves for 295 breast cancer patients, stratified based on miR-31 target signature expression in their primary tumors. p value based on a logrank test.
 (C) Invasion assays with miR-31-expressing or control 231 cells transfected as indicated. Asterisks: $p > 0.19$ relative to vector + siControl cells. $n = 3$.
 (D) Anoikis assays with 231 cells transfected with the indicated siRNAs. Asterisks: $p > 0.80$ relative to vector + siControl cells. $n = 3$.
 (E) Invasion assays with the indicated 231 cells transfected with miRNA-resistant expression constructs. Asterisks: $p > 0.61$ relative to miR-31 + mock cells. $n = 3$.
 (F) Anoikis assays with the indicated 231 cells transfected as noted. Asterisks: $p > 0.11$ relative to miR-31 + mock cells. $n = 3$.
 Data are presented as mean \pm SEM.

miR-31 Expression Correlates Inversely with Metastasis in Human Breast Tumors

Because established cell lines and xenograft studies cannot fully recapitulate clinical malignancy, we extended our analyses by assaying miR-31 expression in specimens from 56 human breast cancer patients (Table S5; median follow-up = 59 months). Relative to grade-matched estrogen receptor (ER)⁺ tumors, which are associated with more favorable disease outcome (Sørlie et al., 2001), basal-like tumors exhibited 40% reduced miR-31; no

difference in miR-31 levels was observed between ER⁺ and HER2⁺ tumors (Figure S16).

When these 56 tumors were stratified based on clinical progression, we found that miR-31 expression was diminished in primary tumors that subsequently metastasized, when compared to normal breast tissue and primary tumors that did not recur; moreover, low miR-31 levels correlated strongly with reduced distant disease-free survival relative to tumors with high miR-31 (Figures 7A and 7B). Similarly, within this cohort of

tumors, high RhoA expression was associated with an increased incidence of distant metastasis (Figure S17).

The association of low miR-31 levels with metastasis persisted independent of both tumor grade and molecular subtype (Figure S18). Such grade and subtype independence is quite surprising, because clinically utilized prognostic markers for breast cancer largely correlate with these parameters; furthermore, currently available markers do not identify a worse-prognosis group within the more aggressive basal-like or HER2⁺ subtypes (Desmedt et al., 2008). Thus, miR-31 may represent a marker for metastasis in a variety of breast cancer subtypes; however, its utility as a prognostic indicator will depend on extension of these initial observations.

We next assessed the heterogeneity of miR-31 expression in human primary breast tumors, as well as distant metastases arising in the same patients. miR-31 was expressed in 65% of the cells in these primary tumors; however, miR-31 was detected in only 12%–30% of cells in patient-matched distant metastases (Figure 7C). These data raise the possibility that selective pressures operating over the course of breast cancer progression diminish the representation of miR-31-expressing cells within the population of successfully metastasizing cells.

Finally, we asked whether expression of ITGA5, RDX, and RhoA was also heterogeneous in primary human breast tumors. RDX and RhoA were expressed in 60%–75% of cells in the primary tumors examined, whereas ITGA5 was detected in >80% of cells (Figure 7D). Distant metastases were more homogeneous for the expression of RDX and RhoA than the primary tumors from which they were derived, as indicated by the fact that >90% of cells in the metastases expressed RDX and RhoA (Figure 7D). Similarly, >90% of cells in the metastases expressed ITGA5; however, the widespread ITGA5 expression observed in the patient-matched primary tumors complicates interpretation of its expression in distant metastases (Figure 7D).

DISCUSSION

miRNAs can modulate a wide variety of biological processes. In the present report, we demonstrate that a single human miRNA, miR-31, is endowed with the ability to concomitantly repress multiple prometastatic targets and to thereby inhibit several distinct steps of the invasion-metastasis cascade. Moreover, miR-31 levels correlate inversely with metastatic recurrence in a cohort of human breast tumors, a preliminary association that appears to persist independent of both tumor grade and subtype.

Genome-wide studies have described miR-31 downregulation or deletion of the miR-31 genomic locus in human breast cancers (Calin et al., 2004; Zhang et al., 2006; Yan et al., 2008). Expression profiling of clinical breast tumors revealed reduced miR-31 in luminal B (relative to luminal A), basal-like, and HER2⁺ tumors (Mattie et al., 2006; Blenkiron et al., 2007)—patterns of reduction that correlate with aggressive disease (Sorlie et al., 2001). In contrast, another profiling study found elevated miR-31 in human breast tumors (Volinia et al., 2006). None of these studies stratified patients by metastasis status.

A limited number of miRNAs with prometastatic (miR-10b, -21, and -373/520c) or antimetastatic (miR-34b/c, -126, -148a, -206,

and -335) functions have been identified. However, the contributions of miR-10b, miR-21, and miR-373/520c specifically to metastasis promotion are not easily discerned because of their mitogenic and/or antiapoptotic roles (Voorhoeve et al., 2006; Ma et al., 2007; Si et al., 2007). Similarly, the antimetastatic miRNAs miR-34b/c, miR-126, and miR-148a impair primary tumor growth (Lujambio et al., 2008; Tavazoie et al., 2008), whereas miR-206 and miR-335 inhibit proliferation or promote apoptosis (Sathyan et al., 2007; Kondo et al., 2008), again obscuring their precise roles in metastasis.

In contrast, miR-31 obstructs metastasis without exerting confounding influences on primary tumor development. As such, *mir-31* might aptly be categorized as a “metastasis suppressor gene” (Steeg, 2003). This unique aspect of miR-31 function, among others, raises questions regarding the still-uncharacterized role of this miRNA in normal cell and organismic physiology. Of significance, loss of miR-31 activity enhances invasiveness, motility, and anoikis resistance in untransformed human mammary epithelial cells. Hence, inactivation of miR-31 in normal epithelium may facilitate dissemination prior to transformation to a fully neoplastic state. This suggests one putative mechanism by which the invasion-metastasis cascade could be initiated very early during the course of tumor progression, a phenomenon that has recently been observed in clinical breast tumors (Hüsemann et al., 2008).

Given the capacity of miR-31 to enhance primary tumor growth, an oncogenic role for this miRNA (mechanistically independent of its metastasis-suppressive functions) cannot be formally excluded. Such duality of action is not unprecedented (Massagué, 2008) and is consistent with notions that metastasis- and tumorigenesis-enabling attributes can be biologically distinct and acquired via independent selective pressures during malignant progression.

Previous studies have described effects of specific miRNAs on an early stage of the invasion-metastasis cascade—local invasion. The present work demonstrates that miRNAs can also influence later steps of metastasis and that an individual miRNA can intervene at multiple distinct stages of the invasion-metastasis cascade. miR-31 regulates the local invasion of primary mammary tumors, as well as intraluminal survival, extravasation, and/or initial viability in a foreign microenvironment. miR-31 also suppresses colonization—the final and rate-limiting step of metastasis (Fidler, 2003). miR-31-imposed suppression of RhoA partially explains the effects of this miRNA on local invasion and early postinvasation events; however, the mechanisms by which miR-31 suppresses metastatic colonization remain unresolved.

The levels of several functionally relevant effectors of miR-31 correlate with disease progression in human tumors. RhoA expression, for example, is elevated in aggressive breast neoplasias (Sahai and Marshall, 2002). Similar associations have been described in human tumors for ITGA5 (Sanchez-Carbayo et al., 2006) and the RDX family (McClatchey, 2003).

Re-expression of several individual miR-31 targets largely reversed miR-31-imposed defects in vitro. This may indicate that certain miR-31 effectors activate one another; however, ectopic ITGA5, RDX, or RhoA did not induce the expression of other miR-31 targets (data not shown). Alternatively, available

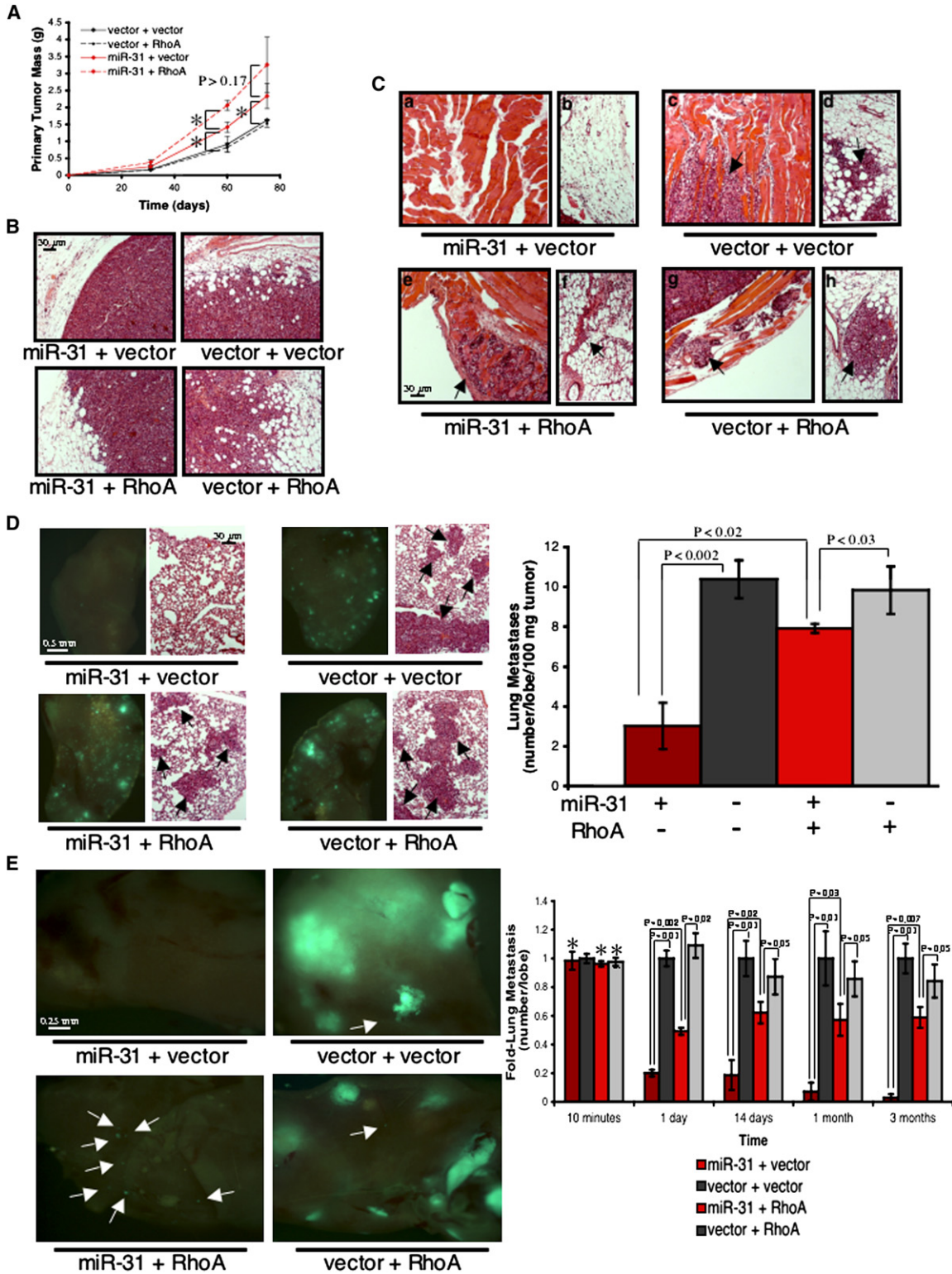


Figure 6. Re-expression of RhoA Partially Reverses miR-31-Imposed Metastasis Defects In Vivo

(A) Primary tumor growth upon orthotopic injection of 5.0×10^5 GFP-labeled 231 cells. The experiment was terminated after 11 weeks because of primary tumor burden. Asterisks: $p < 0.02$. $n = 5$ per group per time point.

(B) H&E stain of 231 primary tumors 60 days after orthotopic injection.

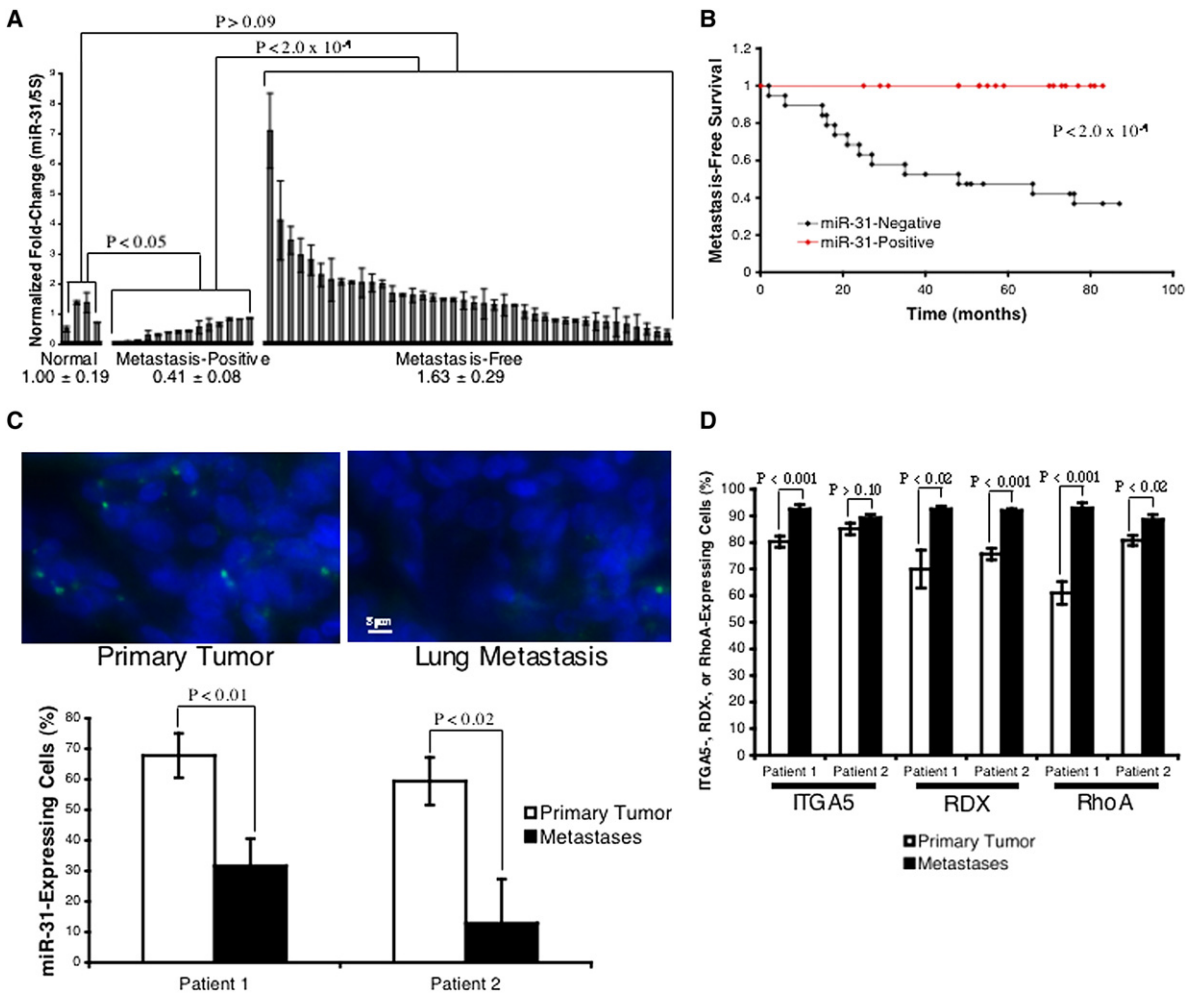


Figure 7. miR-31 Levels Correlate Inversely with Metastasis in Human Breast Tumors

(A) miR-31 RT-PCR in 54 primary breast tumors. Normal: tissue from nondiseased individuals; metastasis-positive and -free: tumors of the indicated distant metastasis outcome. 5S rRNA was a loading control. $n = 4$ (normal); $n = 14$ (metastasis-positive); $n = 40$ (metastasis-free).

(B) Kaplan-Meier distant metastasis-free survival curves for 54 breast cancer patients, stratified based on miR-31 levels in their primary tumors. p value based on a chi-square test.

(C) In situ hybridization for miR-31 (green) in patient-matched primary breast tumors and distant metastases (patient 1 = lung; 2 = pleura); DAPI counterstain (blue). $n = 8$ fields.

(D) Immunohistochemical detection of ITGA5, RDX, and RhoA in patient-matched primary breast tumors and distant metastases (patient 1 = lung; 2 = pleura). $n = 8$ fields.

Data are presented as mean \pm SEM.

in vitro assays might inadequately model the complexity of metastasis; hence, in vivo manifestations of modeled behaviors may require the concurrent action of multiple miR-31 effectors. Also, not all steps of metastasis can be recapitulated in vitro. Consistent with these notions, RhoA completely reversed a number of miR-31-dependent defects in vitro, yet only partially

rescued miR-31-imposed metastasis phenotypes in vivo. This supports beliefs that miRNAs act via the pleiotropic regulation of multiple effectors.

Our analyses rely on established human cell lines and xenograft studies, approaches that cannot fully simulate clinical carcinomas. For example, cell lines accumulate genetic changes

(C) H&E stain of tissue adjacent to the indicated 231 primary mammary tumors 60 days after injection. Arrows indicate disseminated tumor cells in normal muscle (a, c, e, g) and fat (b, d, f, h).

(D) Images of murine lungs to visualize GFP-labeled 231 cells 60 days after orthotopic injection (left). H&E stain of lungs from animals bearing the indicated tumors (right); arrows indicate metastatic foci. $n = 5$.

(E) Images of murine lungs to detect GFP-labeled 231 cells 86 days after tail vein injection (left); arrows indicate micrometastatic lesions. Asterisks: $p > 0.87$ relative to vector + vector controls. $n = 4$, except for 2 weeks ($n = 3$).

Data are presented as mean \pm SEM.

in culture, whereas xenografts fail to recapitulate species-specific interactions between tumor cells and their stroma. However, the consistency of our results upon use of multiple independent cell lines (including a single-cell-derived population), the convergence of our gain- and loss-of-function findings, and our correlative studies in human breast cancer patients and murine mammary tumor cell lines argue against major confounding influences stemming from our experimental models.

Collectively, the findings of the present study carry significant implications regarding our understanding of the pathogenesis of high-grade malignancies. Our data suggest that the loss of a single gene product can facilitate the completion of multiple distinct steps of the invasion-metastasis cascade; this pleiotropic action may help to explain how tumor cells can accumulate enough genetic and epigenetic aberrations over the course of a human lifespan to overcome the numerous barriers that normally operate to prevent metastasis. Moreover, because distant metastases are responsible for patient mortality in the vast majority of human carcinomas, miR-31's ability to impede metastasis may prove to be clinically useful.

EXPERIMENTAL PROCEDURES

Cell Culture

MDA-MB-231 and MCF7-Ras cells were obtained from the ATCC and cultured under standard conditions. HMEC and HME cells have been described (Ma et al., 2007). SCP3 cells were obtained from J. Massagué (Minn et al., 2005). SUM-149 and -159 cells were provided by S. Ethier (Ma et al., 2007). D2 cells have been described (Morris et al., 1993). 67NR, 168FARN, 4T07, and 4T1 cells were obtained from F. Miller (Aslakson and Miller, 1992).

miRNA Detection

Total RNA, inclusive of the small RNA fraction, was extracted from cultured cells with a *mirVana* miRNA Isolation Kit (Ambion). RT-PCR-based detection of mature miR-31 and 5S rRNA was achieved with a *mirVana* miRNA Detection Kit and gene-specific primers (Ambion).

miRNA In Situ Hybridization

miRNA expression was assessed from paraffin sections via a protocol adapted from Silahatoglu et al., (2007). In brief, after a 4 hr prehybridization, a 5' FITC-labeled miRCURY LNA probe targeting miR-31 (Exiqon) was hybridized to proteinase K-treated 10 μ m sections at 55°C for 12 hr. Slides were then incubated with anti-FITC-HRP (PerkinElmer), and the resulting signal was intensified with the TSA Plus Fluorescein System (PerkinElmer).

Human Breast Tumors

Primary breast tumors, distant metastases, and normal breast tissue were collected and processed in compliance with a protocol approved by the Brigham and Women's Hospital IRB. Fresh tissue was harvested from patients, OCT-embedded, snap-frozen, and preserved at -80°C. Recurrent cases were primary tumors from patients that developed distant metastases. For each recurrent case, two nonrecurrent cases were selected to control for date of diagnosis, molecular subtype, lymph node status, and time of follow-up. Total RNA was isolated from 35 μ m sections via TRIzol extraction and a *mirVana* miRNA Isolation Kit. To discern whether miR-31 levels correlate with distant metastasis, primary tumors were classified as miR-31 positive or negative. Tumors were considered miR-31 positive or negative if the normalized expression of miR-31 resided in the top or bottom 30% of tumors in this cohort, respectively. Similarly, tumors were classified as RhoA high or low if their RhoA levels were in the top or bottom 30% of tumors examined.

Invasion and Motility Assays

For invasion assays, 1.0×10^5 cells were seeded in a Matrigel-coated chamber with 8.0 μ m pores (BD Biosciences); for motility assays, 5.0×10^4 cells were plated atop uncoated membranes with 8.0 μ m pores (BD Biosciences). Cells were seeded in serum-free media and translocated toward complete growth media for 20 hr. Fugene6 (Roche) was used to transfect cells 24 hr prior to plating. 200 nM miRIDIAN miRNA Inhibitors (Dharmacon) were employed to transiently inhibit miR-31. SMARTpool siRNAs against Fzd3, ITGA5, M-RIP, MMP16, RDX, or RhoA (Dharmacon) were provided at 100 nM. Antisense oligonucleotides and siRNAs were transfected 48 hr prior to seeding with Oligofectamine (Invitrogen).

Anoikis Assays

Anoikis resistance was evaluated by seeding 7.5×10^4 cells in ultralow attachment plates (Corning). After 24 hr of anchorage-independent culture, cells were resuspended in 0.4% trypan blue (Sigma) and cell viability was assessed.

Animal Studies

All research involving animals complied with protocols approved by the MIT Committee on Animal Care. For spontaneous metastasis assays, age-matched female NOD/SCID mice (propagated on-site) were bilaterally injected into the mammary fat pad with the indicated number of tumor cells in 1:2 Matrigel (BD Biosciences) plus normal growth media. For experimental metastasis assays, age-matched female NOD/SCID mice were injected with 5.0×10^5 cells (resuspended in PBS) via the tail vein. Metastasis was quantified with a fluorescent microscope within 3 hr of specimen isolation.

Luciferase Assays

5.0×10^4 cells were cotransfected with 50 ng of the indicated pS1 *Renilla* luciferase construct and 50 ng of a pS0 firefly luciferase normalization control. Lysates were collected 24 hr after transfection, and *Renilla* and firefly luciferase activities were measured with a Dual-Luciferase Reporter System (Promega).

Immunoblots

Lysates were resolved by electrophoresis, transferred to a PVDF membrane, and probed with antibodies against β -actin (Santa Cruz), Fzd3 (Abcam), ITGA5 (Santa Cruz), MMP16 (Abcam), RDX (Cell Signaling), or RhoA (Santa Cruz).

miR-31 Target Signature

Expression profiling of 295 human breast tumors (van de Vijver et al., 2002) was used to categorize tumors as miR-31 target signature positive or negative. Tumors were considered target signature positive or negative if the normalized expression of multiple of the six miR-31 targets herein identified resided in the top or bottom 15% of tumors in this cohort, respectively.

Immunohistochemistry

Detection of Ki-67 (PharMingen), MECA-32 (U. of Iowa), ITGA5 (Santa Cruz), RDX (Santa Cruz), or RhoA (Abcam) was performed on 5 μ m paraffin sections with the indicated antibodies, Vectastain Elite ABC kits (Vector), and ImmPACT DAB Substrate (Vector).

Statistical Analyses

Data are presented as mean \pm SEM. Unless otherwise noted, Student's t test was used for comparisons, with $p < 0.05$ considered significant.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 18 figures, 5 tables, and a video summary and can be found with this article online at [http://www.cell.com/cell/supplemental/S0092-8674\(09\)00390-0](http://www.cell.com/cell/supplemental/S0092-8674(09)00390-0).

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