miR-149 functions as a tumor suppressor by controlling breast epithelial cell migration and invasion

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Abstract

Deregulated molecular signaling pathways are responsible for the altered adhesive, migratory and invasive properties of cancer cells. The different breast cancer subtypes are characterized by the expression of distinct microRNAs (miRNAs), short non-coding RNAs that post-transcriptionally modulate the expression of entire gene networks. Profiling studies have revealed down-regulation of miR-149 in basal breast cancer. Here we show that miR-149 expression severely impairs cell spreading, migration and invasion of basal-like breast cancer cells. We identify signaling molecules downstream of integrin receptors as miR-149 targets, including the small GTPases Rap1a and Rap1b, providing an explanation for the defective Src and Rac activation during cell adhesion and spreading upon miR-149 expression. Suppression of cell spreading by miR-149 could be rescued, at least in part, by expression of constitutively active Rac. Finally, we demonstrate that increased miR-149 levels block lung colonization in vivo. Based on our findings we propose that miR-149 downregulation in basal breast cancer facilitates the metastatic dissemination of tumor cells by supporting aberrant Rac activation.
Introduction

Cancer is one of the leading causes of death worldwide, mainly due to the development of metastases at later stages of the disease. Metastasis of cancer cells is a stepwise process starting with local invasion, intravasation, survival in the circulation, followed by extravasation and colonization of the target organ (1). In breast cancer, the basal subtype is characterized by its frequent reoccurrence and high metastatic potential. Basal breast cancers constitute approximately 20% of all breast cancer cases and they typically lack expression of the estrogen and progesterone receptors and ErbB2/HER2 amplification/overexpression (2,3). The identification of molecular changes and aberrantly activated signaling pathways is therefore a prerequisite for the development of anticancer therapies targeting this specific breast cancer subtype.

Small GTPases of the Rho family are important modulators of cellular actin dynamics, the deregulation of which is associated with virtually every step of the metastatic cascade (4). For example, the Rac subfamily comprising Rac1, Rac2 and Rac3 is linked to the formation of lamellipodia and membrane ruffles and required for directed migration (5). The formation and maintenance of cell junctions and polarization are dependent on Rac and its hyperactivation disrupts epithelial architecture (5). Although there are reports on the overexpression of Rac isoforms in different cancers including those of the breast, the most common cause of Rac hyperactivation is the deregulation of upstream molecules (6). Rac is active when bound to GTP and inactive in the GDP-bound state. GTP loading requires the guanine exchange factors (GEFs), whereas inactivation is facilitated by the GTPase activating proteins (GAP), which accelerate the intrinsic GTPase activity, thus leading to inactivation. Rac is furthermore negatively regulated by guanine dissociation inhibitors (GDIs) that sequester the protein in the cytoplasm (7). Several Rac-specific GEFs such as Tiam, pREX-1 and Ect2 play important roles in tumorigenesis and metastasis and are upregulated or hyperactive in cancer (6).

Rac activation occurs downstream of integrins, transmembrane receptors that link the extracellular matrix (ECM) with the intracellular actin cytoskeleton at sites termed focal adhesions. Upon ECM engagement, integrins recruit the cytoplasmic tyrosine kinases FAK and Src which together
phosphorylate various focal adhesion components and contribute to Rac activation (8). Integrin activation is potentiated by the small GTPase Rap1 which facilitates the membrane recruitment of the cytoskeletal adaptor protein talin. Upon integrin binding, talin induces a conformation change whereby integrins are converted to their high affinity state (9). By establishing the connection between the cell and the ECM, integrins are essential for epithelial tissue organization and they are also directly involved in the adhesive migration and invasion of tumor cells (10).

miRNAs (miRNAs) are a class of short non-coding RNAs of approximately 17 to 24 nucleotides that regulate gene expression at the post-transcriptional level (11). miRNAs act by binding to their target mRNAs usually within the 3'-untranslated region, resulting in translational repression and/or mRNA degradation (12). Because miRNAs act by partial complementary binding of relatively short sequences they target multiple mRNAs, thereby regulating entire gene networks (13). By doing so, miRNAs orchestrate fundamental biological cellular processes such as development, proliferation and apoptosis. Furthermore, miRNAs are implicated in various diseases including cancer, making them attractive targets for therapeutic intervention or as diagnostic markers (14,15). In recent years, miRNA profiling studies undertaken in different tumor types have identified sets of miRNAs with altered expression in tumor versus normal tissue. While certain upregulated miRNAs, so-called oncoMiRs, have been characterized to possess transforming potential, there are also examples of miRNAs with inherent tumor suppressive activity that are downregulated in cancer cells (14,15). Of those miRNAs specifically associated with metastatic progression some target key regulators of cytoskeletal reorganization. For example, miR-31 whose expression levels correlate inversely with disease relapse in breast cancer patients was found to promote metastasis via the combined suppression of integrin alpha 5, the adaptor protein radixin and the small GTPase RhoA (16).

The different molecular breast cancer subtypes are not only characterized by their distinct gene expression profiles but also by specific miRNA signatures (17,18). In a recent study, microarray analyses of primary human breast tumors identified 26 miRNAs that separated almost perfectly the basal-like and luminal A samples (18). Decreased expression of miR-149 in particular further correlated with mutant p53 status (18), and miR-149 was also observed to be downregulated in
prostate cancer (19), but thus far functional analyses of miR-149 in these tumor types is still lacking. Using a combination of biochemical and cellular assays, we show here that miR-149 interferes at multiple levels with signaling downstream of integrin receptors, impairs Rac activation and efficiently blocks basal-like breast cancer cell migration and invasion both in vitro and in vivo. Suppression of Rac activation by miR-149 associated with defective cell spreading and migration was also observed in prostate cancer cells, implicating a conserved tumor suppressive function for miR-149.
Materials and Methods

Cell culture and transfection. MCF7 and T47D cells were obtained from Cornelius Knabbe (Institute of Clinical Pharmacology, Stuttgart, Germany), BT474 cells from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland), and MDA-MB-468, Hs578T and MDA-MB-436 cells from Bernhard Lüscher (RWTH Aachen University, Germany) in 2005. Cell lines were authenticated by morphology and growth characteristics, tested for mycoplasma and frozen, and cells were cultured for less than four months. MDA-MB-231, SKBR3 and BT549 cells were obtained from CLS, Heidelberg, Germany in 2010, 2011, and 2014, respectively, and not reauthenticated. MDA-MB-231 and SKBR3 cells were cultured in DMEM (Life Technologies), all other cell lines in RPMI 1640 (Life Technologies) supplemented with 10% FCS (PAA). Cells were transiently transfected with miRNA and siRNAs using RNAiMax (Life Technologies). miRNA-149-5p mimic (miR-149) and miRNA negative control#1 (miR-con) were from Thermo Scientific, Rap1a (#1: s11780; #2: ss11781) and Rap1b (#1: s11782; #2: s224515) Silencer Select siRNAs were from Life Technologies and siControl (ON-TARGETplus non-targeting pool) was from Dhharmacon. Plasmid transfection was performed using Lipofectamine® LTX with Plus Reagent (Life Technologies). The pEGFP-Rac G12V plasmid was a gift from Francisco Sanchez-Madrid (Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain).

Cell lysis, SDS-PAGE and Western Blotting. Cells were lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete protease inhibitors (Roche)]. Lysates were clarified by centrifugation at 16,000×g for 10 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane using the iBlot Gel Transfer Device (Invitrogen). Membranes were blocked with 0.5% blocking reagent (Roche) in PBS containing 0.1% Tween-20 and then incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. Visualization was carried out using the ECL detection system (Thermo Fisher).

Quantitative RT-PCR. Total RNA was isolated from 5×10^5-1×10^6 cells using the mirVANA™ miRNA Isolation Kit (Life Technologies). qRT-PCR was performed with a Cfx96 device (Biorad).
Reverse transcription and qPCR of miR-149 and RNU6B were performed with Taqman® microRNA Assays (Life Technologies). Relative miRNA expression levels were calculated with the $2^{-\Delta\Delta C_T}$ method (Biorad CFX manager software 3.1.). Vav2, Rap1a, Rap1b and GAPDH mRNAs (QuantiTect Primer Assay, Qiagen) were amplified using the QuantiTect SYBR Green PCR Kit (Qiagen). Relative expression levels were calculated using the $2^{-\Delta\Delta C_T}$ method (Biorad CFX manager software 3.1.).

**Rac activity assay.** Rac activity was assessed with the G-LISA Rac1/2/3 Activation Assay Biochem Kit (Cytoskeleton). Absorbance was measured with the multimode reader Infinite® 200 PRO.

**Immunofluorescence microscopy.** Cells seeded onto glass coverslips coated with 10 µg/ml collagen (Serva) were fixed with 4% PFA, permeabilized with PBS containing 0.1% Triton X-100 and blocked with 5% goat serum (Life Technologies) in PBS containing 0.1% Tween-20. Cells were then incubated with primary antibodies in blocking buffer, washed with PBS containing 0.1% Tween-20 and incubated with secondary antibodies in blocking buffer. Slides were mounted in Fluoromount-G and analyzed on a confocal laser scanning microscope (LSM 700, Zeiss) using 488, 561 and 633nm excitation with the objectives Plan-Apochromat 20x/0.8 M27 and oil objective lenses EC Plan-Neofluar 40/1.30 DIC M27 and Plan-Apochromat 63/1.40 DIC M27. Images were processed with the ZEN software.

**Impedance measurement.** 1×10⁴ cells were plated into collagen coated (10 µg/ml) 96-well E-plates (Roche) in medium containing 0.5% FCS, except for MDA-MB-436 cells, which were plated in medium containing 10% FCS. The impedance of cells was measured using the xCELLigence device (Roche).

**Life cell imaging.** Three days after transfection, 1×10³ cells were seeded onto collagen coated (10 µg/ml) glass bottom dishes (Greiner Bio-One) in medium containing 10% FCS and were allowed to adhere for 5 h before imaging with a wide-field fluorescence microscope (Axio Cell Observer Z1, Zeiss) using the objective Plan-Apochromat 20x/0.8 M27. Raw data were exported to Image J and quantification was done with the ImageJ plugin MTracjJ.

**Proliferation assays.** One day after transfection, 2.5×10³ cells were plated into 96-well plates. The
next day, medium was replaced by fresh medium containing 10% FCS. 3 and 5 days after transfection cells were fixed with 4% PFA and stained with crystal violet. Crystal violet was dissolved in methanol and measured at 550 nm with the multimode reader Infinite® 200 PRO (Tecan).

**Migration and invasion assays.** For haptotactic migration, $0.5 \times 10^5$ cells were seeded in medium containing 0.5% FCS into Transwells (8 µm pore size; Costar) coated with 10 µg/ml collagen on the underside and allowed to migrate for 3 h (MDA-MB-231) or 4 h (MDA-MB-436, BT549, Hs578T). The bottom chamber contained medium with 0.5% FCS. For invasion, Transwells were coated on the upper side with 50 µl growth factor reduced matrigel (BD) diluted in medium containing 0.5% FCS and cells (0.5 or $1 \times 10^5$ in medium containing 0.5% FCS) were allowed to invade overnight. The bottom chamber contained medium with 10% FCS supplemented with 190 ng/ml EGF. In the case of MDA-MB-436 cells, the bottom chamber contained medium with 0.5% FCS supplemented with 190 ng/ml EGF. Cells on the underside of the membrane were fixed, stained with crystal violet and counted (five independent microscopic fields at a 20-fold magnification or all cells per filter).

**Animal experiments.** MDA-MB-231 cells ($0.5 \times 10^6$ in 100 µl HBSS) were injected into the tail veins of eight-weeks-old female CB17 SCID mice (Harlan Laboratories GmbH). Four weeks later, mice were sacrificed, and the lungs were fixed and stained in Bouin’s solution. The number of macro-metastases on the lung surface was counted. Animal care and experiments were in accordance with federal guidelines and approved by the University and state authorities.
Results

Re-analysis of microarray-based miRNA expression data of 101 primary breast tumors revealed reduced expression of miR-149 in basal compared with luminal A/B, ErbB2/HER2 positive and normal-like cancers (Fig. 1A; (18)). This was confirmed in an independent data set (17), in which miR-149 expression was also significantly reduced in basal-like (n=16) compared to luminal A/B (n=24) breast tumors (Fig. 1B). Moreover, the expression of miR-149 further inversely correlated with higher tumor stage (Fig. 1C). We first sought to verify that this expression pattern is reflected also in established breast cancer cell lines. Indeed, quantitative RT-PCR analysis showed that in five basal-like cell lines (MDA-MB-231, MDA-MB-468, BT549, Hs578T, MDA-MB-436) miR-149 levels were lower compared with those in the luminal MCF7 and T47D and the HER2-positive BT474 and SKBR3 cell lines (Fig. 1D). We chose MDA-MB-231 cells, a well-characterized invasive basal B cell line, for our initial studies. Overexpression of a mature miR-149 mimic did not have any impact on cell proliferation (Fig. 2A) demonstrating that expression as such is not toxic to cells. However, in Transwell assays measuring haptotactic migration towards a collagen gradient, miR-149 strongly reduced the number of migrated cells (Fig. 2B) and in the presence of a matrigel layer, cell invasion towards a serum plus EGF gradient was almost completely blocked (Fig. 2C). Tracking of single cells revealed that general cell motility was not affected by miR-149 overexpression, since the distance (track length) and thus the velocity was similar for control and miR-149-transfected cells, however, the net displacement was reduced by miR-149 expression (Fig. 2D & E), indicating a defect in the directionality of cell movement. Suppression of directed cell migration and invasion by miR-149 was further confirmed in Hs578T, MDA-MB-436 and BT549 cell lines (Fig. 3).

Cell adhesion and spreading are prerequisites for matrix-dependent cell migration. To study this in more detail, we transiently transfected MDA-MB-231 cells with control miRNA and miR-149, respectively, and plated the cells onto collagen-coated dishes for different periods of time (Fig. 4A). While control cells were fully spread 4 hours after plating, miR-149 expressing cells had a rounder morphology and after 24 hours they were still less spread (Fig. 4A). To measure cell adhesion and spreading in real-time, we plated cells onto collagen-coated E-plates and analyzed the impedance (cell
index) using the xCELLigence device. Figure 4B shows that the plateau reached by miR-149 expressing cells was lower than that of the control cells. The number of adherent cells was unaffected by miR-149 expression (data not shown), indicating a defect in cell spreading rather than cell adhesion. Similar results were obtained for Hs578T, MDA-MB-436 and BT549 cell lines (Fig. S1).

Next, we analyzed cellular architecture at the molecular level at early times of adhesion by phalloidin and vinculin staining. Compared with the control, miR-149 expressing MDA-MB-231 cells exhibited a depolarized actin cytoskeleton and failed to establish prominent cell protrusions and lamellipodia (Fig. 4C). Finally, we examined biochemically how miR-149 expression affected the activation of key signaling molecules downstream of integrin engagement by analyzing Src and paxillin phosphorylation. In accordance with the impaired spreading phenotype in miR-149 expressing cells, Src phosphorylation was strongly reduced especially at early times of adhesion and spreading, whereas paxillin phosphorylation, an indicator of focal adhesion formation and turnover (20), was still low 4 hours after plating onto collagen (Fig. 4D).

miR-149 downregulation has been reported in other cancer types, including prostate cancer (19). To explore whether the cellular effects induced by miR-149 are conserved in a different cell type, we transfected PC3 prostate cancer cells, which also express very low levels of endogenous miR-149 (data not shown), with the control miRNA and miR-149 mimic. As seen in MDA-MB-231 cells, upon miR-149 transfection, cell spreading on collagen and haptotactic cell migration were severely impaired (Fig. S2).

The activation of the small GTPase Rac downstream of integrin engagement is critical for cell spreading, lamellipodia formation and directed migration. We therefore investigated Rac activation by GLISA and found that increased miR-149 expression resulted in reduced Rac-GTP levels, preventing the transient increase in Rac activation observed after plating onto collagen (Fig. 5A) or upon EGF stimulation of cells (Fig. 5B), while total Rac protein levels were unaffected by miR-149 expression (Fig. S3). The suppression of MDA-MB-231 cell migration by pharmacological Rac inhibition in Transwell assays confirmed the importance of Rac for the directed migration of these cells (Fig. 5C). Rac activation was also compromised in PC3 cells (Fig. S2), demonstrating that miR-149 impacts the
same signaling pathway in these cells. To investigate the functional contribution of Rac inactivation in the context of miR-149 signaling, we transiently transfected cells expressing either control miRNA or miR-149 with a vector encoding constitutively active GFP-Rac G12V. Expression of active Rac, but not GFP alone, rescued the cell spreading defect of miR-149 expressing cells (Fig. 5C & D).

To obtain insight into the potential signaling molecules affected by miR-149, we used the miRecords online tool (http://mirecords.biolead.org/) that integrates eleven miRNA prediction programs, resulting in 325 unique targets predicted by at least four different algorithms. These genes were mapped on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (21), enabling the annotation of 79 genes. The pie chart in Figure 6A depicts pathways that contain at least three annotated genes and comprises 49 genes in total (see Table S1 for gene information). Notably, apart from 'cytokine cytokine receptor interaction', ‘MAPK signaling pathway’ and ‘regulation of actin cytoskeleton’, ‘focal adhesion’ contained the highest number of predicted miR-149 target genes. The latter pathway includes the small GTPases Rap1a and Rap1b and the guanine nucleotide exchange factor Vav2. Importantly, Rac is known to be activated by Vav2 and Rap1 in a cooperative manner (22,23).

To assess whether Rap1a/b and Vav2 were targeted by miR-149, we performed qRT-PCR analyses of mRNA samples extracted from MDA-MB-231 cells transfected with control miRNA and miR-149, respectively. Indeed, compared with the control, the transcript levels of Rap1a/b and Vav2 were significantly reduced by miR-149 expression (Fig. 6B). Of note, we did not observe any changes in the expression levels of PAK3, Src or paxillin (Fig. 4D and data not shown), which were also predicted as targets and are associated with the focal adhesion pathway. We next analyzed the contribution of the identified miR-149 targets to migration and invasion of MDA-MB-231 cells. Whereas the knockdown of Vav2 was not sufficient to inhibit cell motility in Transwell assays (data not shown), Rap1a/b co-depletion significantly reduced both cell migration and invasion (Fig. 6C). Downregulation of Rap1a/b further reduced Rac-GTP levels without affecting total Rac protein levels (Fig. 6D), supporting the conclusion that miR-149 controls invasive cell migration, at least in part, by targeting Rap1a/b.

Finally, to test the impact of miR-149 expression on cell migration and invasion in vivo, we analyzed in immunocompromised mice the efficiency of lung colonization of MDA-MB-231 cells transfected
with miR-149 upon tail vein injection. Four weeks after injection, lungs were dissected and the number of macroscopic metastases counted, revealing a significant block in metastatic lung colonization by miR-149 expression compared with the control (Fig. 7). Together, our data provide strong support for a tumor-suppressive role of miR-149 in basal breast cancer.
Discussion

In this study we show that miR-149 expression potently suppressed migration and invasion of basal breast cancer cells \textit{in vitro} and lung colonization of MDA-MB-231 cells \textit{in vivo}. Because ectopic expression of miR-149 did not impact proliferation, miR-149 mainly has an anti-metastatic function in these cells, which is in line with its downregulation in more advanced stages of breast cancer. In prostate cancer cells, miR-149 expression recapitulated the effects seen in MDA-MB-231 cells, indicating the regulation of conserved pathways. Reduced miR-149 expression has been reported in other types of cancer, including colon and gastric cancer (24-26). In this context, the transcription factors SP1, FOXM1, and ZBTB2 were identified as direct miR-149 targets and their inhibition was proposed to be responsible for the tumor suppressive function of miR-149 (24-26). miR-149 thus appears to act as a general oncosuppressive miRNA by interfering with different proliferative and metastatic signaling pathways, the precise nature of which are dependent on the cell type.

Increased expression of miR-149 in basal breast cancer and PC3 cells impaired cell spreading on both collagen (Fig. 4 & Fig. S1/2) and fibronectin (data not shown) and therefore appears to act ECM-independently. Cell spreading is associated with the activation of Rac, which is also critical for persistent migration. Rac is known to drive the mesenchymal type of cancer cell motility characterized by cell polarization and the matrix-dependent establishment of cell protrusions at the leading edge (5). The compromised Rac activity seen in miR-149 expressing cells thus provides an explanation for both the suppression of cell spreading and the impaired directionality of single cell motility and haptotactic migration in Transwell assays. Of note, miR-149 did not inhibit migration of MDA-MB-468 cells (data not shown), which belong to the basal A subtype and retain epithelial characteristics (27), and thus likely depend on other mechanisms for cell migration. Overexpression of constitutively active Rac1 in MDA-MB-231 cells rescued the initial spreading defect induced by miR-149 but was not sufficient to restore migration in Transwell assays. This can be explained by the fact that Rac hyperactivation generates multiple lamellipodia, thereby preventing polarization and inducing random motility (5). Because we did not obtain MDA-MB-231 cells stably expressing miR-149, an orthotopic mouse model measuring metastasis formation from the primary tumor could not be investigated.
Instead, we injected cells into the tail veins of mice, a procedure compatible with transient miRNA expression. Shortly after injection cells are trapped in the lung capillaries where they extravasate and then colonize the lung (28). Based on the in vitro data it can be assumed that miR-149 expression blocks the first steps of this process that rely on cell adhesion and invasion. However, because miR-149 overexpression was still detected 9 days post transfection in vitro (Fig. S4), we cannot rule out that miR-149 also negatively affects additional factors that facilitate metastatic colonization of the lung.

Thus far, there is little evidence for the direct miRNA-mediated suppression of the individual Rac isoforms. miR-142-3p was reported to target Rac1, regulating migration and invasion of hepatocellular carcinoma cell lines (29). Tiam as a positive upstream regulator of Rac was identified to be subject to miRNA regulation by miR-10b, which suppressed cell migration and invasion of breast cancer cells (30). However, in colon cancer cells downregulation of Tiam by miR-21 and miR-31 was associated with enhanced motility (31), highlighting the importance of the specific cell context. Interestingly, in head and neck squamous cell carcinoma cells the loss of let-7i induced a morphological switch to a mesenchymal, Rac-dependent mode of invasion. let-7i was identified to target Nedd9 and DOCK3, both of which are activators of Rac (32).

The latter report is in accordance with the notion that miRNA function is not mediated by a single target but rather corresponds to the net effect of the regulation of an entire set of targets. Here we show that miR-149 expression reduces the transcript levels of Rap1a, Rap1b and Vav2, most likely by a direct mechanism that involves miR-149 binding to the predicted recognition sites within the 3’ UTRs. Rap1a/b co-depletion suppressed migration and invasion of MDA-MB-231 cells and reduced basal Rac activity, providing support for their role in miR-149 signaling. This agrees with the proposed function of Rap1 in chick embryo and mouse metastasis models of pancreatic cancer and melanoma, respectively (33,34). Although silencing of Vav2 alone was not sufficient to reduce the invasive behavior of MDA-MB-231 cells in vitro, the finding that Rap1 and Vav2 cooperate in activating Rac (22,23) suggests that Vav2 suppression by miR-149 may potentiate the biological effects resulting from Rap1a/b downregulation. However, it is important to note that Rap1 and Vav2 possess additional
Rac-independent functions associated with the metastatic process. Apart from its function in integrin signaling, Rap1 utilizes different effectors to control cell polarity and cell-cell adhesion, the deregulation of which contributes to neoplastic transformation (35). Similarly, Vav2 and Vav3 were recently shown to synergize in sustaining tumor growth, neoangiogenesis, and lung-specific metastasis of breast cancer cells, in part by Rho GTPase independent pathways (36).

miRNAs are now being integrated into clinical trials as biomarkers for prognosis and clinical response, but they also hold promise as anti-cancer therapeutics. For example, MRX34, an intravenously injected liposome-formulated miR-34 mimic has recently entered clinical trials for patients with advanced or metastatic liver cancer (37). The increased understanding of miRNA biology and function together with improved targeted delivery systems will hopefully also support the development of miRNA-based agents for the treatment of basal breast cancer.

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Figure Legends

Figure 1: Reduced expression of miR-149 in basal breast cancer. (A) Boxplot visualization of miR-149 expression in different tumor subtypes based on datasets by Enerly et al. (18). Data are presented as mean ± SEM and were analyzed by two way Anova followed by Bonferroni posttest. ** p < 0.01. (B) Boxplot visualization of miR-149 expression in luminal A/B (n=24) and basal-like (n=16) breast cancers based on datasets by Blenkiron et al. (17); *** p = 0.00015, one-sided t-test. (C) Tumor stage classification of breast cancers shown in (B) compared to the expression of miR-149 (17). (D) RNA was extracted from the indicated breast cancer cell lines and expression levels of miR-149 were determined by qRT-PCR. Data were normalized to levels of RNU6B as an internal control; MCF7 cells were set as 1. A representative experiment performed with triplicate samples is shown.

Figure 2: Expression of mir-149 impairs directed cell migration and invasion. MDA-MB-231 cells were transiently transfected with control miRNA (miR-con) or miR-149. (A) One day after transfection cells were plated into 96-well plates and stained with crystal violet after 3 and 5 days, respectively. A representative experiment is shown. (B, C) Three days post transfection cells were harvested and subjected to transwell assays. (B) For haptotactic migration the bottom side of the filters was coated with collagen. (C) For invasion, transwells were coated with matrigel and cells were left to migrate overnight toward a serum and EGF gradient. Shown is the mean ± SEM of three independent experiments. Data were analyzed using Student’s t-test (unpaired, two-tailed). ** p < 0.01 (D, E) Three days after transfection cells were plated onto collagen-coated glass bottom dishes and subjected to time-lapse microscopy. Images were taken every 10 min over a time period of 5 h. (D) Representative trajectories of 10 cells for each condition are plotted. Axis origin refers to the starting point of cell tracking. (E) Quantitative analysis of cell displacement, cell velocity and track length. Data correspond to a representative experiment with ≥ 25 cells per condition and are reported as mean ± SEM. Statistical significance was analyzed using Student’s t-test (unpaired, two-tailed). ** p < 0.01, n.s. (non-significant), p > 0.05.

Figure 3: Expression of mir-149 impairs invasive migration of Hs578T, MDA-MB-436 and BT549 cells. Cells were transiently transfected with miR-con or miR-149. (A) For haptotactic
migration the bottom side of the filters was coated with collagen. (B) For invasion, Transwells were coated with matrigel and cells were left to migrate overnight. Shown is the mean cell number ± SEM of a representative experiment; in the case of BT549 cell invasion, photos of representative filters are shown.

**Figure 4: mir-149 interferes with cell spreading on collagen.** MDA MB 231 cells were transiently transfected with miR-con or miR-149. Three days post transfection cells were harvested and plated onto collagen in 0.5% FCS. (A) Cells were fixed and stained with crystal violet at the indicated times. (B) Cell adhesion and spreading on collagen-coated E-plates was measured using the xCELLigence device. Values correspond to the mean ± SEM of duplicate samples. (C) Cells plated on collagen-coated coverslips for the indicated times were stained with phalloidin and an anti-vinculin antibody. The confocal images shown are stacks of three sections taken from the bottom of the cell. (D) Cells seeded onto collagen-coated dishes were lysed at the indicated times and lysates were immunoblotted using the indicated antibodies. The blots were cropped and in each case the corresponding panels are from the same gel.

**Figure 5: mir-149 impairs Rac activation and active Rac restores spreading of miR-149 expressing cells.** (A, B, D) MDA-MB-231 cells were transiently transfected with miR-con or miR-149, respectively. (A) Three days post transfection, cells were seeded in 0.5% FCS onto collagen-coated dishes and lysed at the indicated times. (B) Three days post transfection, cells were stimulated with 50 ng/ml EGF for indicated times and lysed. Rac-GTP levels were measured by GLISA and normalized to miR-con at time 0. Data are shown as the mean ± SEM of three experiments (A) and analyzed using two way Anova with Bonferroni posttests. * p < 0.05, n.s. (non-significant). In (B), the mean ± SEM of two experiments is shown. (C) Transwell migration of MDA-MB-231 cells was measured in the absence (control) and presence of 100 µM NSC23766 in the upper chamber. (D) Two days post transfection cells were transiently transfected with a vector encoding GFP alone or GFP-Rac G12V. The next day, cells were replated onto collagen-coated coverslips for 1 hour, fixed and stained with phalloidin and an anti-vinculin antibody. The confocal images shown are stacks of five sections taken from the bottom of the cell. (E) For the quantitative analysis of the area of spread cells
pictures (1024×1024 pixel) were taken at a 20-fold magnification. Analysis was performed with ImageJ. Only cells with a mean pixel intensity greater than 10 were considered as GFP positive. 22-59 cells per condition were analyzed. Shown is the mean ± SEM of three independent experiments. Data were analyzed using two way Anova with Bonferroni posttests. ** p < 0.01, n.s. (non-significant).

**Figure 6:** miR-149 targets Rap1a and Rap1b, and Rap1a/b co-depletion reduces cell migration and invasion, and Rac activity. (A) The pie chart depicts the mapping of 325 unique miR-149 target genes onto KEGG pathways (Version 2.5). Human pathways containing at least three target genes are shown, comprising 49 genes in total. (B) MDA-MB-231 cells were transiently transfected with miR-con or miR-149, respectively. Two days post transfection, RNA was extracted and Vav2, Rap1a and Rap1b transcript levels were determined by qRT-PCR. Values were normalized to GAPDH. Data are shown as the mean ± SEM of four independent experiments and were analyzed using two-way Anova with Bonferroni posttests. *** p < 0.001. (C, D) MDA-MB-231 cells were transiently transfected with siControl or co-transfected with two independent siRNAs sets specific for Rap1a and Rap1b (siRap1a+b #1; siRap1a+b #2). (C) Transwell migration and invasion assays were performed as described in Figure 2. Shown is the mean ± SEM of three independent experiments. Data were analyzed using one-way Anova with Newman–Keuls posttest. * p < 0.05, *** p < 0.001. (D) Three days post transfection, Rac-GTP levels were measured by GLISA and normalized to siControl. The mean ± SEM of two independent experiments is shown. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

**Figure 7:** mir-149 blocks lung colonization in vivo. MDA-MB-231 cells were transiently transfected with miR-con or miR-149. Three days post transfection, cells were harvested and injected into the tail veins of SCID mice (0.5x10⁵ cells; six mice per group). Four weeks later, the mice were sacrificed; the lungs were fixed in Boiun’s solution and photographed. (B) Quantification of the number of macro-metastases per lung. Data are shown as mean ± SEM and were analyzed using Student’s t-test (unpaired, two-tailed). *** p < 0.001.
Figure 1

A

Figure 1

B

Tumor Stage

1

2

3

4

5

6

7

8

9

basal-like

luminal A/B

**

normal-like

luminal B

luminal A

ERBB2

dual

basal

MDA-MB-436

BT549

H578T

MDA-MB-231

MDA-MB-468

SKBR3

BT474

T47D

MCF7

ErB2

luminal

ErB2

basal

MDA-MB-436

BT549

H578T

MDA-MB-231

MDA-MB-468

SKBR3

BT474

T47D

MCF7

luminal
Figure 3
Figure 5

A

B

C

D

E
miR-149 functions as a tumor suppressor by controlling breast epithelial cell migration and invasion

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