Identification of Metastasis-Suppressive microRNAs in Primary Melanoma

Doug Hanniford, Miguel F. Segura, Judy Zhong, Elliot Philips, Xavier Jirau-Serrano, Farbod Darvishian, Russell S. Berman, Richard L. Shapiro, Anna C. Pavlick, Brian Brown, Iman Osman, Eva Hernando

Affiliations of authors: Department of Pathology (DH, MFS, EP, XJS, FD, EH), Interdisciplinary Melanoma Cooperative Group (DH, MFS, JZ, XJS, FD, RSB, RLS, AP, IO, EH), Department of Environmental Medicine (JZ), Department of Dermatology (AP, IO), Department of Medicine (AP, IO), Department of Urology (IO), Department of Surgery (RSB, RLS), NYU Langone Medical Center, New York, NY; Genetics and Genomics Sciences, Icahn School of Medicine at Mount Sinai, New York, NY (BB).

Correspondence to: Eva Hernando, PhD, 522 First Ave, Department of Pathology, Floor 3 Rm 305, Smilow Research Building, New York, NY 10016 (e-mail: eva.hernando-monge@nyumc.org).

Abstract

Background: Surgical management of primary melanoma is curative for most patients with clinically localized disease at diagnosis; however, a substantial number of patients recur and progress to advanced disease. Understanding molecular alterations that influence differential tumor progression of histopathologically similar lesions may lead to improved prognosis and therapies to slow or prevent metastasis.

Methods: We examined microRNA dysregulation by expression profiling of primary melanoma tumors from 92 patients. We screened candidate microRNAs selected by differential expression between recurrent and nonrecurrent tumors or associated with primary tumor thickness (Student’s t test, Benjamini-Hochberg False Discovery Rate [FDR] < 0.05), in vitro invasion assays. We performed in vivo metastasis assays, matrix remodeling experiments, and molecular studies to identify metastasis-regulating microRNAs and their cellular and molecular mechanisms. All statistical tests were two-sided.

Results: We identified two microRNAs (hsa-miR-382, hsa-miR-516b) whose expression was lower in aggressive vs nonaggressive primary tumors, which suppressed invasion in vitro and metastasis in vivo (mean metastatic foci: control: 37.9, 95% confidence interval [CI] = 25.6 to 50.2; miR-382: 19.5, 95% CI = 12.2 to 26.9, P = .009; miR-516b: 12.5, 95% CI = 7.7 to 17.4, P < .001, Student’s t test). Mechanistically, miR-382 overexpression inhibits extracellular matrix degradation by melanoma cells. Moreover, we identified actin regulators CTTN, RAC1, and ARPC2 as direct targets of miR-382. Depletion of CTTN partially recapitulates miR-382 effects on matrix remodeling, invasion, and metastasis. Inhibition of miR-382 in a weakly tumorigenic melanoma cell line increased tumor progression and metastasis in vivo.

Conclusions: Aberrant expression of specific microRNAs that can functionally impact progression of primary melanoma occurs as an early event of melanomagenesis.

Metastasis, which is the cause of approximately 90% of tumor deaths (1), is a multistep cascade requiring diverse biological processes, including local invasion/matrix remodeling, invasation/extravasation, survival in circulation, and colonization, survival, and growth in secondary sites. Regeneration of these processes in different cancer contexts has begun to be elucidated (2); however, further understanding of the molecular mechanisms exploited by metastasizing cells will aid the development of more informed therapeutic strategies.

Melanoma is a prototypical example of a solid tumor with a propensity to spread throughout the body, even at early stages of tumorigenesis (3). Metastasis is the essential event dictating
poor outcome for patients diagnosed with a localized primary melanoma. Staging of primary melanomas incorporates histopathological features (thickness, mitotic index, ulceration, and lymph node status) and is generally prognostic of clinical outcome (4–8). However, frequently melanoma cell dissemination occurs from primary tumors that are histologically equivalent to nonmetastasizing lesions at diagnosis. Approximately 7% and 30% of patients with localized melanoma at diagnosis (stage I and II, respectively) will suffer a recurrence (9), and most will then progress to metastatic disease and eventual death (10). These outcomes suggest that histopathologically similar melanomas may have divergent underlying molecular features influencing their potential to metastasize. Consistent with this concept, messenger RNA (mRNA) or microRNA (miRNA) expression in a variety of cancers is associated with or predicts disease recurrence, progression to metastasis, and other outcome measures (11–20). Collectively, these studies suggest that measurable populations of metastasis-initiating cells are present in a subset of primary tumors. Identifying molecular alterations at cancer diagnosis that are functionally involved in metastatic progression will further elucidate mechanisms used during initiation of the metastatic cascade, which could yield novel therapeutic strategies to disrupt tumor cell dissemination and/or new prognostic biomarkers.

miRNAs are short RNAs that control complex cellular processes through post-transcriptional regulation of target mRNA (21). Recent studies have shown that altered expression of specific miRNAs is associated with patient outcomes in melanoma and that perturbation of individual miRNAs functionally impacts melanoma cell metastasis (22–26). These and other studies have analyzed global miRNA expression comparing nevi to primary melanoma, primary to metastatic melanoma, or metastatic melanomas with differing outcomes. However, miRNA expression profiling of primary melanoma tissues of different outcomes has not been examined. Moreover, the mechanisms underlying metastatic propensity of a subset of primary melanomas are unclear.

Here, we identified miRNAs associated with aggressive, cutaneous primary melanomas (associated with tumor thickness or recurrence status) by expression profiling of a cohort of clinically well-annotated primary melanoma tissues (n = 92). To discover metastasis-relevant miRNAs, we screened candidates differentially expressed in aggressive vs nonaggressive primary melanomas in a high-throughput in vitro invasion assay and subsequently an in vivo metastasis model. Mechanistically, we examined the effects of invasion- and metastasis-regulating miRNAs on matrix remodeling, a key process during initiation of tumor cell dissemination. Finally, we identified a network of target genes that are potential direct downstream mediators of these phenotypes through regulation of the actin cytoskeleton.

Methods

Clinical Specimens

Primary human melanoma samples were collected at the time of surgery. Informed consent was obtained from all patients and approval acquired by the Institutional Review Board of New York University (NYU) School of Medicine (protocol #10362). All tumors were classified according to the 2009 American Joint Committee on Cancer staging system. Further details are available in the Supplementary Methods (available online).

RNA Extraction

From formalin-fixed, paraffin-embedded (FFPE) samples, primary melanoma tissues were macroscopically dissected using disposable scalpels and guided by hematoxylin and eosin (H&E) staining of consecutive sections. RNA extraction was performed with miRNeasy FFPE kit following manufacturer’s recommendations. Further details are available in the Supplementary Methods (available online).

MiRNA Expression Analyses

MiRNA expression profiling of FFPE-extracted RNA from primary melanomas was performed by Exiqon, Inc. using dual-color miRCURY™ LNA arrays. The quantified signals were background corrected and normalized using the global Lowess regression algorithm. Data are deposited in GEO (accession: GSE62372). Further details and information on miRNA constructs and cloning, array profiling, real-time quantitative polymerase chain reaction (qPCR), quantification, and mimic transfection are available in the Supplementary Methods (available online).

miRNA Analyses

Further information on miRNA extraction, constructs and cloning, array profiling and analyses, real-time qPCR, quantification, and mimic transfection are available in the Supplementary Methods (available online).

Cell Culture

501MEL cells (gift of Dr. Ruth Halaban, Yale Medical School) were cultured in OptiMEM + 5% fetal bovine serum (FBS). 451Lu and WM1361a cells (obtained from Coriell) were cultured in Tu2%, which contains 80% MCDB153 (Sigma Aldrich) and 20% L15 (Cellgro), and supplemented with approximately 2% heat-inactivated FBS, 1.68 mM CaCl, and 5 μg/mL bovine or human insulin. SK-MEL-147 and SK-MEL-173 (gift of Dr. Houghton, MSKCC), and SK-MEL-28 (ATCC) cells were grown in DMEM + 10% FBS. All cells were grown in a humidified incubator at 37°C and 5% CO. All cell lines were derived from human melanoma tumor tissue. Cell lines were initially analyzed by morphologic assessment and gene expression profiling for lineage-specific gene expression (TYR, TRYP1, DCT, TRYP1B, MITF, EDNRB, KIT). Cells were maintained in culture for no more than 25 passages.

Invasion Assay Screen

Briefly, reverse-transfected (Lipofectamine 2000) GFP-expressing metastatic melanoma cells were seeded in 96-well Fluoroblok inserts precoated with fibronectin (10 μg/mL). Cells were incubated for 4 to 40 hours at 37°C, 5% CO. Invading cells were imaged and counted using a high-content fluorescent imaging system (Cellomics Arrayscan). Further details on fluorescent cell generation, reverse transfection, invasion assay seeding, and quantification are in the Supplementary Methods (available online).

In Vivo Metastasis Experiments

miRNA Overexpression and Cortactin Depletion

Animal experiments were conducted in accordance with guidelines set forth by the Institutional Animal Care and Use Committee (IACUC) of NYU (protocol # 120405-02). 451Lu cells transduced with lentiviral supernatants containing miRNA (pMIRH, System Biosciences) or short hairpin RNA (shRNA) (GIPZ, Open Biosystems) expression constructs were resuspended in growth media at a concentration of 2x10⁶
3′ UTR Luciferase Reporter Assay

HEK293T cells were seeded in 96-well plates at 30,000 cell/well and incubated at 37°C and 5% CO₂ for 16 to 24 hours. HEK293T cells were cotransfected with 100 to 200 ng 3′ UTR reporter plasmid and 50 nM (3′ UTR) of the indicated mimic or control miRNA (Dharmacon) using Lipofectamine 2000 (Invitrogen), following manufacturer’s recommendations.

Statistical Analysis

MiRNA array data of patient tumor samples was analysed by Student’s t test, Pearson correlation, and Benjamin-Hochberg False Discovery Rate (FDR), or Kaplan-Meier and Wilcoxon tests to identify and validate clinical associations/correlations of candidate miRNA with parameters. The statistical significance in the cell invasion, xenotransplantation/metastasis, 3′ untranslated region (UTR) luciferase reporter, and gelatin degradation experiments was calculated by Student’s t test or one-way analysis of variance (ANOVA), as applicable. Kaplan-Meier survival curves and log-rank or Wilcoxon tests, and Student’s t test were used to compare groups of mice in in vivo experiments, as applicable. A P value of less than .05 was interpreted as a statistically significant difference. All statistical tests were two-sided. For more detailed statistical analyses, please see individual sections in the Supplementary Methods (available online).

Other Methods

Descriptions of viral production and transduction, gelatin degradation assays and immunofluorescence, and western blotting are in the Supplementary Methods (available online).

Results

Analysis of the Effects of microRNAs Differentially Expressed Between Aggressive vs Nonaggressive Primary Melanomas on In Vitro Invasion

To identify candidate miRNAs that functionally contribute to early stages of melanoma progression, we profiled the expression of 838 annotated miRNAs by microarray (Exiqon, Inc.) of a well-annotated cohort of 92 cutaneous, primary melanomas of predominantly superficial spreading and nodular histological subtypes, with a minimum of three years of follow-up from initial diagnosis for surviving patients (Supplementary Table 1, available online). We validated the array platform by reverse transcription quantitative polymerase chain reaction (RT-qPCR) of randomly selected miRNAs (Supplementary Figure 1, available online). As surrogate clinical parameters of highly “aggressive” tumors, we identified miRNAs differentially expressed between patients whose tumors had or had not recurred (n = 48 and n = 44, respectively) and miRNAs whose expression associates with tumor thickness (Supplementary Table 2, available online). We hypothesized that some of these miRNAs might impact cell invasion and metastasis.

To test this theory, we developed an automated, 96-well in vitro invasion assay using fluorescently labeled cells and a high-content imaging system as a screening platform. We selected the top 40 candidates based on a combination of fold change and statistical significance between aggressive and nonaggressive tumors, and median probe intensity (expression level, cutoff ≥ 50). We transiently overexpressed candidate miRNAs individually using oligo mimics in two metastatic melanoma cell lines (SK-MEL-147 and 501MEL) and assessed their impact on in vitro invasion using our screening platform (Supplementary Figure 2, available online).

From this initial analysis, we selected 13 miRNAs that suppressed or enhanced invasion in both cell lines in primary screening. We further assessed the effect of their overexpression on in vitro invasion in additional metastatic melanoma cell lines (SK-MEL-28 and SK-MEL-173) (Supplementary Figure 3, available online). These analyses identified miR-382, miR-516b, and miR-7 as consistent suppressors of in vitro invasion in all melanoma cell lines tested (e.g., 501MEL: SCR #2: mean = 1 +/- 0.268 SD; miR-382: mean = 0.449 +/- 0.125 SD, P = .008; miR-516b: mean = 0.248 +/- 0.101 SD, P = .006; and miR-7: mean = 0.298 +/- 0.086 SD, P = .001, Student’s t test) in a BRAF (v-raf murine sarcoma viral oncogene homolog B) mutation-independent manner (Figure 1, A–B).

MiR-382 and miR-516b Expression in Aggressive vs Nonaggressive Primary Melanomas

To verify the association of expression of invasion-suppressive miRNA with aggressive primary tumors seen in our initial analyses, we performed further data mining of our existing data set and profiled miRNA expression of an additional set of primary melanoma patient samples (n = 119) (Supplementary Table 3, available online). We found that miR-382 expression was correlated with thickness in both cohorts (Pearson, r = −0.527, P < .001, r = −0.300, P < .001) and associated with recurrence-free survival (RFS) in both datasets (median cut-off, Wilcoxon test, P = .002, P = .06), while miR-516b was correlated with thickness (Pearson, r = −0.364, P < .001, r = −0.161, P = .040), in both data sets but associated with RFS in only one (Wilcoxon test, P = .59, P = .02) (Figure 2, A and B). In contrast, the correlation of miR-7 expression with thickness (Pearson, r = −0.253, P = .015) and association with recurrence status (Student’s t test, P = .007) seen in our initial data set did not validate in additional patients (r = 0.086, P = .178 and P = .247, respectively) (Figure 2C). Moreover, expression of miR-7 did not associate with RFS in either cohort (Wilcoxon test, P = .43 and P = .69, respectively) (Figure 2C). Similar to miR-7, additional miRNA (e.g., miR-215 and miR-374b) that were of interest based on the initial patient data analysis and invasion screening did not validate in this additional cohort of patient samples. In sum, our results demonstrate that miR-382 and miR-516b are less abundant in more vs less aggressive primary melanomas. Collectively, the altered expression of miR-382 and miR-516b in primary melanoma samples in conjunction with their ability to suppress melanoma cell invasion suggests that their dysregulation may have important functional contributions at early stages of melanomagenesis. Based on these findings, we opted to perform additional functional experimentation with miR-382 and miR-516b only.

Effect of miR-382 and miR-516b on Lung Metastasis In Vivo

To assess the impact of miR-382 and miR-516b modulation on melanoma metastasis, we used a xenograft model in which 451Lu melanoma cells (27) implanted subcutaneously in the flanks of NOD/Shi-scid/IL-2Rγnull (NOG) mice metastasize to mouse lungs.
We stably expressed miR-382, miR-516b, or a scrambled control (miR-CTRL) with a green fluorescent protein (GFP) tracer to test each miRNA’s ability to suppress lung metastasis. Primary tumor growth was unaffected by expression of miR-382 but was statistically significantly decreased by miR-516b expression (Figure 3, A and B; Supplementary Figure 4, A and B, available online). Consistent with their role as in vitro invasion suppressors, fluorescence imaging of whole mouse lung explants revealed clear reductions in the number of metastatic foci in the lungs of mice with xenografts of miR-382- or miR-516b-transduced cells compared with miR-CTRL (control: mean = 37.9, 95% confidence interval [CI] = 25.6 to 50.2; miR-382: mean = 19.5, 95% CI = 12.2 to 26.9, P = .009; miR-516b: mean = 12.5, 95% CI = 7.7 to 17.4, P < .001, Student’s t test) (Figure 3, C and D; Supplementary Figure 4, D-F, available online). Metastatic burden correlated strongly with primary tumor size for miR-516b tumor-bearing animals (Pearson, r = 0.76 P = .02) (Supplementary Figure 4C, available online). In conjunction with reduced tumor size compared with tumors of the miR-CTRL group, miR-516b’s effects on metastasis might be partially proliferation dependent. In contrast, miR-382 suppressed metastasis relative to control in a proliferation-independent manner. In sum, these results identify miR-382 and miR-516b as key suppressors of melanoma metastasis in vivo.

Effect of miR-382 and miR-516b on Extracellular Matrix Remodeling

Early in the metastatic cascade, tumor cells encounter basement membrane and extracellular matrix barriers that must be circumvented to enter circulation and metastasize. We reasoned that suppressors of metastasis that are less abundant in aggressive primary melanomas might impair extracellular matrix degradation/remodeling. To assess the effect of metastasis-suppressive microRNA on this process in melanoma, we used fluorescent-gelatin degradation assays as a surrogate in vitro assay. We found that miR-382 expression statistically significantly reduced the degradative capacity of melanoma cells, while miR-516b had no effect in this context (control [scr]: mean = 34.34%, 95% CI = 25.2 to 43.5; miR-382: mean = 20.0%, 95% CI = 13.7 to 26.3, P = .005; miR-516b: mean = 30.9%, 95% CI = 18.3 to 43.4, P = .393) (Figure 4, A-C). This finding suggests that lower expression of miR-382 in primary tumors may enable aggressive behavior of melanoma cells by increasing their capacity to migrate through surrounding stroma. In contrast, miR-516b had no impact on matrix degradation in this model, suggesting it uses different mechanisms or impacts remodeling of different matrix components to effect metastasis suppression.

Identification of Direct miR-382 Targets

To better understand the downstream mechanisms by which miR-382 functions to suppress invasion, metastasis, and matrix remodeling, we identified targets that could potentially mediate these phenotypes. We performed mRNA expression profiling of melanoma cell lines overexpressing control or miR-382 oligo mimics. We overlapped transcripts modified by miR-382 with
Figure 2. Analysis of the association of miR-382, miR-516b, and miR-7 expression with primary melanoma aggressiveness in two cohorts of patient samples. Log2 expression values of (A) miR-382, (B) miR-516b, or (C) miR-7 from microRNA (miRNA) microarray data sets of patient primary melanoma samples, plotted by recurrence status (yes vs no, log2 expression values for individual patients are shown along with means; error bars represent 95% confidence intervals), recurrence-free survival (RFS, dichotomized by median expression of the indicated miRNA), and thickness (mm, plotted in log2 as a continuous variable), as indicated, for discovery (n = 92) and validation (n = 119) cohorts. For recurrence status (yes vs no), P values were determined by two-tailed student's t testing. For RFS, P values were determined by two-sided Wilcoxon test. For thickness, correlation coefficients (r) and corresponding P values were determined by Pearson correlation. RFS = recurrence-free survival.
predicted targets (TargetsScan v5.2) and Clip-seq data mapped to predicted targets (starbase.sysu.edu.cn, v1.0, accessed 2011) of miR-382 (28–31). Gene ontology analysis (32, 33) suggested that putative direct targets regulated by miR-382 in melanoma cells are enriched in several key actin cytoskeleton-related categories (Figure 5A). These analyses revealed a number of candidate genes that were plausible mediators of the effects of miR-382 on invasion, metastasis, and matrix remodeling (Figure 5B), including central actin cytoskeleton regulators (eg, cortactin [CTTN], Ras-related C3 botulinum toxin substrate 1 [RAC1], Actin-related protein 2/3 complex, subunit 2 [ARPC2], etc.). In addition, miR-382 overexpression modified the abundance of several melanoma progression–relevant genes (eg, Endothelin receptor B [EDNRB], v-akt murine thymoma viral oncogene homolog 3 [AKT3], RAC1). We validated the modulated expression of selected genes by RT-qPCR in response to increased expression of miR-382 (Figure 5, C, E, and G; Supplementary Figure 5, A and C, available online). Importantly, relative to negative control oligos, miR-382 directly targeted the 3'UTRs of CTTN (mean = 0.57 +/- 0.046 SD, P = .001), RAC1 (mean = 0.736 +/- 0.115 SD, P = .004), ARPC2 (mean = 0.432 +/- 0.078 SD, P < .001), EDNRB (mean = 0.706 +/- 0.137 SD, P = .014), and AKT3 (mean = 0.821 +/- 0.459, P = .046) in luciferase reporter assays and resulted in marked protein downregulation in melanoma cells (Figure 5 D, F, H, and I; Supplementary Figure 5, B and D, available online). In summary, we have identified several novel direct targets of miR-382, particularly the actin cytoskeleton regulators CTTN, ARPC2, and RAC1, consistent with the concept that these genes may be direct mediators of the invasion and metastasis-suppressing effects of miR-382. Collectively these data suggest that miR-382 may coordinately regulate invasion/metastasis by modulating cytoskeletal dynamics through targeting of a network of actin-regulating genes.

Effect of CTTN Depletion in Relation to miR-382 Phenotypes

CTTN acts a nodal point for the regulation of the actin cytoskeleton by integrating upstream signaling from SRC and others to mediate cytoskeletal changes, regulate

Figure 3. Effect of miR-382 or miR-516b overexpression on in vivo lung metastasis of melanoma cell xenografts. A) Tumor volume measurements (mm^3) of 451Lu primary tumors in the flanks of control- (CTRL), miR-382-, or miR-516b–tumor bearing animals plotted from initial (day 14) to final measurement (day 42). Data are means (n = 9 mice per group), and error bars represent standard deviations. B) Mean tumor mass (mg) of extracted primary tumors from CTRL, miR-382, or miR-516b groups at termination (day 43) are depicted as box and whisker (min to max) plots. C) Means of macroscopic lung metastases quantified per field from four equivalent sized, randomly selected fields per animal from macroscopic lung images of CTRL-, miR-382-, or miR-516b–tumor bearing animals are depicted as box and whisker (min to max) plots. D) Representative macroscopic fluorescence images (inverted and contrast-enhanced) of whole mouse lungs extracted at termination from CTRL-, miR-382–, or miR-516b–tumor bearing animals. Black foci indicate GFP+ metastatic lesions. Images taken with Olympus mvx10 at 6.3X magnification. GFP = green fluorescent protein; Mg = milligram.
lamellipodia, direct migration, and control matrix metalloproteinase (MMP) activity (34–36). Data mining of mRNA expression profiling studies indicates that CTTN transcript is more abundant in advanced melanomas (Supplementary Figure 6, available online), providing independent evidence of its relevance in melanoma progression. For these reasons, we further explored CTTN as a downstream mediator of the effects of miR-382. Short interfering RNA-mediated (siRNA) CTTN depletion phenocopied the effect of miR-382 overexpression on the matrix degradation capacity of melanoma cells (Figure 6, A and B; Supplementary Figure 7, A–C, available online). Moreover, silencing of CTTN reduced in vitro invasion of multiple melanoma cell lines relative to control (501MEL: mean = 0.312 (95% CI = 0.245 to 0.380), P < .001) (Figure 6, C and D). In vivo, stable depletion of CTTN (short hairpin RNA, shRNA) in 451Lu cells had no impact on primary tumor growth in xenograft, subcutaneous transplantation in NOG mice (Figure 6E; Supplementary Figure 7D, available online). Analysis of the total number of metastatic foci in mouse lungs revealed a nonsignificant trend toward fewer macrometastases in the shCTTN group (size cutoff = 0, P = .138) (Figure 6F). In contrast, we analyzed the number of metastatic foci using increasing size cutoffs and found statistically significantly fewer metastatic foci in the shCTTN group.
**Figure 5.** Analysis of actin cytoskeleton regulators, **CTTN**, **ARPC2**, and **RAC1**, as putative direct targets and possible mediators of miR-382. 

**A)** P values of enriched gene ontology (GO) categories (National Institutes of Health DAVID) identified from putative miR-382 targets determined by gene expression profiling of miR-382- vs control (ctrl)-transfected melanoma cells. **Red bars** highlight adhesion/migration-related categories. 

**B)** Heat map of mRNA expression profiling of selected GO category genes in ctrl- and miR-382 (382)-transfected melanoma cells. Relative mRNA expression, as assessed by RT-qPCR, of **CTTN**, **ARPC2**, and **RAC1** in CTRL- or miR-382-transfected SK-MEL-28, SK-MEL-147, 451Lu, and 501MEL cells. **Error bars** represent standard deviations. Normalized mean relative light units (RLU) from independent experiments (n ≥ 3) of 3'UTR luciferase reporters for **D)** CTTN, **E)** ARPC2, or **G)** RAC1 cotransfected with CTRL or miRNA mimic oligos in HEK293T cells. Significance of 3'UTR luciferase assays was determined by one-way analysis of variance with Tukey’s multiple comparison post-testing or Student’s t test. *P* = .01 to .05, **P** = .001 to .01, and ***P*** < .001. **Error bars** represent standard deviations. MiR-516b was used as a nontargeting miRNA in addition to nontargeting scrambled controls (CTRL #s 1 +2). Results are plotted relative to mock-transfected cells within individual experiments (not shown). 

**I)** Western blot analysis of CTTN, ARPC2, RAC1, and tubulin (TUB) in CTRL- or miR-382–transfected SK-MEL-147 and 501MEL cells. Values below each band are normalized (to tubulin) and relative (to CTRL) protein abundance, as measured by semiquantitative densitometry (ImageJ, FIJI). ANOVA = analysis of variance; ARPC2 = actin-related protein 2/3 complex, subunit 2; CTTN = cortactin; GO = gene ontology; RAC1 = ras-related C3 botulinum toxin substrate 1.
at nearly all cutoffs (Mann Whitney–Wilcoxon tests, \( P < .05 \)) (Figure 6, F and G), suggesting CTTN depletion yields fewer large metastatic foci. Collectively, these data are consistent with CTTN as an important mediator of the effects of miR-382; however, modulation of additional targets in combination with CTTN appears necessary to fully recapitulate its effects.

**Figure 6.** Effects of cortactin (CTTN) depletion on matrix degradation, invasion, and metastasis. A) Representative images (DAPI, Phalloidin-Alexa488, and isothiocyanate–gelatin) of gelatin degradation by melanoma cells transfected with control (NTC) or siCTTN, as indicated. Images taken with Nikon Eclipse Ti-S, 100X magnification. B) Quantification of gelatin degradation per field by melanoma cells transfected with control (gray bars) or siCTTN (red bars) is plotted as means of biological replicates (n ≥ 3; error bars represent standard deviations). Two independent experiments were performed. C) Relative transwell invasion of the indicated melanoma cell lines transfected with control (gray bars) or siCTTN (red bars) is plotted as means of biological replicates (n ≥ 3; error bars represent standard deviations) from one experiment per cell line. A Student’s t-test was used to calculate two-sided \( P \) values for SK-MEL-28, 501MEL, SK-MEL-147, and 451Lu cells. Relative invasion values for SK-MEL-28, 501MEL, SK-MEL-147, and 451Lu cells. Relative invasion values for CTRL or shCTTN –tumor bearing animals. Mann-Whitney-Wilcoxon tests were used to calculate two-sided \( P \) values at each size cutoff. Cutoffs statistically different between the groups at \( P < .05 \) are indicated with an asterisk. Error bars represent standard deviations. G) Representative fluorescence images (inverted and contrast-enhanced) of whole mouse lungs extracted at termination from CTRL- or shCTTN–tumor bearing animals. Black foci indicate GFP+ metastatic lesions. Images taken with an Olympus mvx10 at 6.3X magnification. CTTN = cortactin; DAPI = 4',6-diamidino-2-phenylindole; FITC = fluorescein isothiocyanate; GFP = green fluorescent protein; NTC = nontargeting control; sh = short hairpin; si = small interfering.
Effect of miR-382 Inhibition on In Vitro and In Vivo Properties of Poorly Tumorigenic Melanoma Cells

To more closely model the effect of miR-382 downregulation in aggressive primary melanomas, we inhibited miR-382 using a lentiviral decoy construct (37) in a poorly tumorigenic, primary tumor-derived melanoma cell line (WM1361a) (38). We implanted decoy-expressing cells subcutaneously in the flanks of immunocompromised NOG mice and compared the effect of miR-382 inhibition (d-miR-382) with inhibition of miR-211 and miR-708 (d-miR-211, d-miR-708, respectively) on melanoma cell tumorigenicity in vivo (Supplementary Figure 8A, available online). MiR-211, used here as a positive control, is a key melanocytic lineage microRNA that is commonly downregulated in melanoma and is tumor and invasion suppressive (23,39–41). MiR-708, which is poorly expressed in melanoma cells, was used as a negative control. In support of our hypothesis, inhibition of miR-382 and miR-211 promoted invasion in vitro relative to inhibition of miR-708 (Supplementary Figure 8, A and B, available online). In vivo, we found that tumor initiation in this model was consistent between groups. In contrast, tumor growth was accelerated by inhibition of miR-382 with inhibition of miR-211 and miR-708 (d-miR-211, d-miR-708, respectively) on melanoma cell tumorigenicity in vivo (Supplementary Figure 8A, available online). MiR-211, used here as a positive control, is a key melanocytic lineage microRNA that is commonly downregulated in melanoma and is tumor and invasion suppressive (23,39–41). MiR-708, which is poorly expressed in melanoma cells, was used as a negative control. In support of our hypothesis, inhibition of miR-382 and miR-211 promoted invasion in vitro relative to inhibition of miR-708 (Supplementary Figure 8, A and B, available online). In vivo, we found that tumor initiation in this model was consistent between groups. In contrast, tumor growth was accelerated by inhibition of miR-382 relative to miR-708 and comparably to miR-211 (Figure 7A), requiring primary tumor removal sooner and significantly shortening survival for d-miR-382 tumor-bearing mice (log-rank test, \( P = .004 \) and \( P = .002 \), respectively) (Figure 7, B and C). Despite prolonged survival of the d-miR-708 tumor-bearing animals, development of lung metastasis was more frequent and pronounced in d-miR-382 tumor-bearing mice (Figure 7, D and E; Supplementary Figure 8, C–H, available online). Collectively, these results demonstrate that inhibition of miR-382 in melanoma cells can promote tumor growth and metastasis to a similar extent as inhibition of the melanoma-suppressive miR-211. These data are consistent with the inverse correlation of miR-382 expression with tumor aggressiveness from primary patient tumors, suggesting that decreased miR-382 expression can be a key event early in the evolution of melanoma that helps drive tumor progression.

Discussion

Metastatic dissemination of melanoma cells is the key event dictating poor outcomes of patients with localized primary tumors who are typically cured through surgical management (5). Identification of pathways commonly disrupted at early stages of tumorigenesis that influence melanoma progression might yield key insights into the biology of metastasis and reveal novel strategies for therapeutic intervention. We hypothesized that molecular alterations that influence metastatic potential may be acquired early in a tumor’s evolution, and evidence in support of this model has been accumulating for some cancers, including melanoma (42–44). We focused on understanding the functional contribution of miRNAs to metastasis initiation in early-stage primary melanoma. Collectively, our data demonstrate that altered expression of specific miRNAs in early-stage primary tumors is a key determinant of metastatic behavior. In this study, we profiled the miRNA expression of more than 200 FFPE primary melanomas in two sample sets with extensive patient follow-up to identify miRNAs that could impact metastasis initiation. We analyzed the functional effect of miRNA candidates differentially expressed in aggressive versus nonaggressive tumors using in vitro and in vivo metastasis models and elucidated cellular and molecular mechanisms of one of the identified metastasis-regulating miRNAs (miR-382). Collectively, our data identify miR-382 and miR-516b as suppressors of melanoma invasion and metastasis. Interestingly, combined overexpression of miR-382 and miR-516b had no impact beyond that of either miRNA alone on the in vitro invasion capacity of melanoma cell lines (Supplementary Figure 9, available online), suggesting that they may act through parallel pathways.

While our study profiled the expression of more than 200 primary melanoma patient samples, additional samples or samples from other institutions would aid confirmation of the generalizability of our findings. A related limitation of our study is that the effect size of the associations with clinical variables was reduced for both miRNAs in the validation patient set, while other miRNAs did not validate. This finding may be because of the issue of multiple comparison testing, as well as the inherent challenge of measuring molecular changes that may occur only in subpopulations of cells within a tumor.

We focused our efforts on exploring the molecular mechanism(s) employed by miR-382 because of its suppressive effect on invasion and metastasis, and extracellular matrix remodeling, a key feature of cells with metastatic capacity (1,45). Expression profiling of melanoma cell lines overexpressing miR-382 indicated a role regulating the actin cytoskeleton. We identified CTTN, RAC1 (RAC), and ARPC2, which are key cytoskeletal regulators, as direct targets and plausible downstream mediators of miR-382. CTTN integrates upstream signaling to promote and stabilize ARPC2/3-mediated actin nucleation (36). ARPC2 is a core structural component of the ARPC2/3 complex, which nucleates branched actin networks (46). RAC is a Rho-family GTPase that regulates a diverse set of cellular processes, particularly cell motility through regulation of actin polymerization (47,48). Collectively, these proteins regulate lamellipodia and invadopodia dynamics, which mediate directional migration and matrix remodeling, respectively. Invadopodia are key cellular structures employed by invasive cancer cells to remodel extracellular matrices, which is particularly relevant during local invasion (36,49–51). CTTN is an essential molecule for and quintessential marker of invadopodia (52). As the key integrator of signaling from several upstream pathways, CTTN is a nodal point of cytoskeletal dynamics (53). Moreover, CTTN has been found to be upregulated through cancer progression for a variety of tumor types, including melanoma, and is pro-oncogenic in some cancers (reviewed in [54]). CTTN depletion alone appears to alter the kinetics but not the absolute ability of melanoma cells to metastasize; thus, it may represent a therapeutic target useful to slow disease spread in the adjuvant setting. Collectively, our data suggest that reduction of miR-382 in primary tumors may facilitate matrix remodeling by promoting increased levels of CTTN and other actin regulators, which may induce invadopodia formation or activity and/or promote other cytoskeletal changes.

The phenotype and targets of miR-382 identified here suggest another role as a regulator of lamellipodia. RAC1 is a particularly interesting target in this regard, as it is the core GTPase regulating lamellipodia. RAC1 is essential for proper migration of melanoblasts during development (55). Melanoma exome sequencing efforts identified a recurrent mutation, P29S, which activates RAC GTPase activity, in 5% to 10% of metastatic melanomas (56,57). Moreover, RAC deletion or inhibition in an NRAS-driven melanoma transplant model reduced tumor growth and suppressed metastatic spread (58). Alteration of upstream regulation of RAC by PREX1/2 in melanoma may also destabilize RAC control, as evidenced by functional studies and PREX2 mutations (59,60). Interestingly, mutant-NRAS induced RAC-dependent, invadopodia-mediated matrix degradation in melanocytic cells, clearly connecting RAC activity to invadopodia dynamics in melanoma (58). Collectively, loss of RAC activity regulation may be a central feature of melanoma, a model that our data supports. The effect of altered RAC control by miR-382...
reduction would be reinforced through derepression of ARPC2 and CTTN (and possibly other undescribed targets). The comparative importance of RAC1 and each identified target to these phenotypes remains for future study.

In summary, we identified two microRNA (miR-382 and miR-516b) whose expression is altered during early stages of melanoma tumorigenesis and which suppress invasion and metastasis of melanoma cells. Moreover, we dissected the mechanistic basis for the metastasis-suppressive effects of miR-382, identifying several direct targets that may be critical mediators of its effects on invasion, metastasis, and matrix remodeling. Our data suggest that reduced expression of miR-382 in primary melanoma lesions promotes tumorigenesis through altered matrix remodeling because of derepression of regulators of the cytoskeleton. These findings

Figure 7. Effect of miR-382 inhibition on in vivo growth and progression of melanoma xenografts. A) Tumor volume measurements (mm³) of individual tumor xenografts of WM1361a expressing the indicated miRNA inhibitor (decoy, d-miR-xxx, n = 9 per group). Tumors were measured two times per week and are plotted until tumor removal or day 207 postimplantation. B) Pre-tumor removal survival of tumor-bearing animals, with miRNA decoy groups indicated. Pre–tumor removal survival events were calculated as the difference (in days) between implantation and tumor removal, which was based on the prespecified tumor volume of approximately 500 to 600mm³. C) Overall survival of tumor-bearing animals, with miRNA decoy groups indicated. Overall survival events were calculated as the difference (in days) between implantation and euthanasia. Mice were killed when tumor regrowth (after primary tumor removal) reached 600 to 800mm³ or the end of the experiment (day 292). P values of Kaplan-Meier survival curves were calculated by log-rank test. D) Percent of mice in each decoy group that developed lung macro-metastases during the course of the experiment. Lungs of all mice were assessed by fluorescence imaging at the time of death for the presence of metastatic foci. E) Representative fluorescence images (inverted and contrast-enhanced) of macro-metastases from whole lung imaging of indicated decoy–tumor bearing animals. Images taken with Cellomics Arrayscan Vti using a 5X objective.
expand the knowledge base of metastasis initiation in melanoma and shed further light on potential therapeutic opportunities for early intervention for melanoma patients.

Funding

This study was funded by the National Institutes of Health (NIH/National Cancer Institute (NCI) (1R01CA155234; PI: DH). DH has been supported by NIH/NCI S T32 CA009161-37 (Training Program in Molecular Oncology and Immunology). JZ has been supported by NIH/National Institute of General Medical Sciences (1R21 GM110450-01; PI: JZ).

Notes

Author contributions:

Conception and design: DH, EH, IO, MFS; development of methodology: DH, MFS, EH; acquisition of data: DH, MFS, XJS, FD; analysis and interpretation of data: DH, EH, JZ; writing, review, and/or revision of the manuscript: all; administrative, technical, or material support: IO, RSB, RLS, AP, BB.

The authors acknowledge A. Gaziel-Sovran, L. S. Danielsen, M. V. Guijarro, R. Di Micco, E. Friedman, L. Koetz, B. Fontanals Cirera, and R. Moubarak for critical reading of and comments on the manuscript and other contributions. We thank P. Mignatti for his valuable insights. We also thank J. Zavadil for miRNA array profiling and bioinformatic expertise, C. Yun for technical expertise with the Cellomics Arrayscan, and D. Polsky, M. Herlyn, and D. Bennett for providing reagents. We thank the New York University (NYU) Genome Technology Center and the NYU Langone Medical Center Histopathology and Immunohistochemistry cores (supported in part by NIH/NCI P30 CA016087-30 grant) for technical assistance. We thank the NYU RNA interference Core, supported by the Helen L. and Martin S. Kimmel Center for Stem Cell Biology, NYU Cancer Institute Center Support Grant, “NIH/NCI S P30 CA016087-32,” and New York State Stem Cell Science: NYSTEM contract C026719. We thank the Memorial Sloan Kettering Cancer Center Antitumor Assessment Core (Director: E. De Stanchina) for assistance with some of the in vivo experiments.

References