

## miR-339-3p Is a Tumor Suppressor in Melanoma

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### Abstract

Determinants of invasion and metastasis in cancer remain of great interest to define. Here, we report the definition of miR-339-3p as a novel tumor suppressive microRNA that blocks melanoma cell invasion without affecting cell survival. miR-339-3p was identified by a comprehensive functional screen of a human miRNA mimetic library in a cell-based assay for invasion by the melanoma cell line A375. miR-339-3p was determined as a strong inhibitor of invasion differentially expressed in melanoma cells and healthy melanocytes. MCL1 was defined as a target for downregulation by miR-339-3p, functioning

through direct interaction with the 3' untranslated region of MCL1 mRNA. Blocking miR-339-3p by an antagomiR was sufficient to increase melanoma cell invasion, an effect that could be phenocopied by RNAi-mediated silencing of MCL1. *In vivo* studies established that miR-339-3p overexpression was sufficient to decrease lung colonization by A375 melanoma cells in NSG mice, relative to control cells. Overall, our results defined miR-339-3p as a melanoma tumor suppressor, the levels of which contributes to invasive aggressiveness. *Cancer Res*; 76(12); 3562–71. ©2016 AACR.

### Introduction

Malignant melanoma, being responsible for about 80% of skin cancer-related deaths, is considered the most aggressive form of human skin cancer with a 5-year survival rate of about 15.2% for patients with distant metastases (1). Transformation of healthy melanocytes to melanoma, called melanomagenesis, represents a multistep process resulting from accumulation of multiple mutations and epigenetic alterations (2). It has been shown that so-called driver mutations are necessary and sufficient to promote melanoma progression and invasion (3). Melanoma cell resistance to cytotoxic chemotherapeutic drugs is thought to be mainly dependent on deregulated expression of antiapoptotic proteins, for example, Bcl-2 and MCL1. Especially MCL1 overexpression is suggested to contribute to chemotherapy resistance of melanoma after direct therapeutic targeting of BRAF and MEK (4, 5). At the moment, intensive studies are being carried out to ascertain checkpoint molecules involved in the hallmarks of cancer (6) that could be targeted to counteract melanoma spread and progression (7).

microRNAs (miRNAs) are small endogenous RNA molecules, which are able to anneal to mRNA molecules through short complementary "seed sequences" located within the 3' untranslated region (UTR) of the targeted mRNA. This interaction

results in mRNA cleavage or repression of productive translation, thereby silencing target gene expression (8). It has been shown that a given miRNA can bind to hundreds of different mRNAs (9), and it was, thus, estimated that more than 30% of all protein coding genes within the human genome are being targeted by miRNAs (10). Interestingly, the miRNA expression profiles between tumors and healthy tissues can show quantitative and qualitative differences and an increasing number of studies focusing on miRNA expression profiling and function in different cancer types has been published (11). The first direct link between dysregulated miRNA expression and melanoma progression was shown by Bemis and colleagues (12), demonstrating that miR-137 expression correlated with reduced MITF expression. These reports underlined the functional relevance of miRNAs in cellular transformation, tumor progression, and disease outcome. In fact, Esquela-Kerscher and colleagues (13) could show that miRNAs can act either as oncogenes or tumor suppressors depending on the function of their target molecules and on the regulation of downstream signaling pathways. Thus, a differentiation between tumor-promoting oncomirs and protective tumor suppressors, as pointed out in various reviews (14, 15) has meanwhile prevailed.

The identification of miRNA—target interactions is pivotal to determine the function of individual miRNAs and to unravel the complexity of miRNA networks. miRNA target prediction *in silico*, based on computer algorithms has its limitations, as most studies focus on only a small number of targets ignoring possible pleiotropic effects (i.e., global impact on gene expression) that a given miRNA can have (16). Despite the availability of *in silico* prediction tools for miRNA research, the complexity of miRNA—target interactions is still not fully understood and needs further elucidation. This study introduces a functional screening strategy, allowing identification and validation of miRNAs with impact on melanoma cell invasion. Besides determination of the respective target genes *in vitro*, our approach also includes analyses of the miRNA-mediated functional effects *in vivo*.

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

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## Materials and Methods

### mRNA library screening by Matrigel invasion assay

The miRNA screening based on an invasion assay was performed applying a miRNA library consisting of 988 individual mimics each provided at a concentration of 0.25 nmol in 96-well plate format (catalogue number MI00100, based on miR-Base Version 13.0; Sigma) in technical triplicates. A375 cells ( $1 \times 10^5$ ) seeded into each well of a 96-well plate were transfected with 50 nmol/L miRNA mimic 24 hours later, as described in Supplementary Materials and Methods. After 48 hours, cells were harvested, divided into three aliquots and transferred into the upper insert of Matrigel (50 ng/well) coated 96-well Transwell plates (Corning). In all experiments except the screening assay, cells were counted and adjusted before transfer to the Transwell plates. Cells were seeded in serum-free medium and the lower chamber was filled with RPMI-1640 medium containing 10% FCS as chemoattractant. After incubation for 6 hours (37°C; 5% CO<sub>2</sub>), invaded cells were washed, detached from the membrane, fluorescently stained with calcein AM (Thermo Fischer Scientific) and quantified with the Fluoroskan Ascent Microplate Fluorometer according to the manufacturer's protocol. The irrelevant miRNAs cel-miR-243 and ath-miR-416 were used as negative controls.

### Cell culture

All melanoma cell lines in this study were established from metastasis of patients with stage III or IV melanoma according to AJCC as previously described (17). For details on characteristics of patients and cell lines see Supplementary Table S1 and Luo and colleagues (18). The cell line A375 was purchased from the ATCC. WM266.4 and the corresponding WM115 primary melanoma cells were kindly provided by Dr. Eva Frei (DKFZ Heidelberg, Germany). The remaining melanoma cell lines had been established in our former department (Skin Cancer Unit; DKFZ). Melanoma cell lines were cultured in RPMI-1640 medium supplemented with 10% FCS. All cell lines were maintained at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. The cell line WM115 was cultured at 35°C and 5% CO<sub>2</sub> in a humidified atmosphere.

Authentication of cell lines was done by comparing STR sequences obtained from the actual cell lines as determined by the Forensic Medicine Department of the Heidelberg University Hospital (Heidelberg, Germany) with data public available or from our own databank. Recent STR analysis has been performed within 6 months before the beginning or in the course of the experiments for all cell lines.

### Cell viability

Cell viability was assessed using the luminescence CellTiter-Glo (CTG) assay (Promega). Hundred  $\mu$ L CTG reagent was added to 100  $\mu$ L RPMI medium with 10% FCS per well in a 96-well plate. The solutions were mixed for 2 minutes on an orbital shaker and incubated for 10 min at room temperature to stabilize the luminescence signal. Because of the occurring cell lysis a luminescence signal can be detected proportional to the ATP content, and thus to the cell amount, present in each well. The luminescence signal was measured with a Fluoroskan Ascent Microplate Fluorometer, according to the manufacturer's recommendation. Significance was determined with the Student *t* test.

### *In vivo* lung colonization assay

NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1wjl</sup>/SzJ mice (NSG mice) were purchased from Charles River Laboratories and bred at the DKFZ animal facility. Mice were used at the age of 6 weeks. A375 melanoma cells were transfected with 50 nmol/L miR-339-3p or mimic control 24 h post seeding. After 48 hours,  $5 \times 10^5$  A375 cells resuspended in sterile PBS were injected intravenously (i.v.) into NSG mice (10 mice/group). Fifteen to 16 days later, mice were sacrificed and tumor nodules in lungs were counted by microscopic inspection. Animal experiments were performed in accordance with institution guidelines and with the EU Directive 2010/63/EU.

Standard cell culture and molecular biology methods as well as bioinformatics can be found in the supplement.

## Results

### The screening system

The functional miRNA library screen was performed in a 96-well Boyden chamber format with the human melanoma cell line A375 (19), including miR-182 and miR-101 as accelerating or inhibiting control miRNA mimics, respectively (Supplementary Fig. S1; refs. 20, 21).

The screening result is shown in Fig. 1A. The invasion value determined for each well was normalized to mock-transfected control cells separately for each individual plate. Each bar represents the Z-score calculated for every miRNA triplicate and indicates the difference between the miRNA candidate value and the mean value of the whole dataset given in number of SDs. At the left side of the waterfall plot miRNA mimics with an accelerating effect on A375 melanoma cell invasion are represented as purple columns, while miRNA mimics located at the right side shown in green inhibited A375 cell invasion (Fig. 1A). Statistical analysis revealed that 37% of the tested miRNA mimics significantly increased A375 cell invasion, whereas 13% of miRNAs significantly inhibited this process, and 50% of the tested miRNAs showed no effect in this screening approach (Fig. 1C).

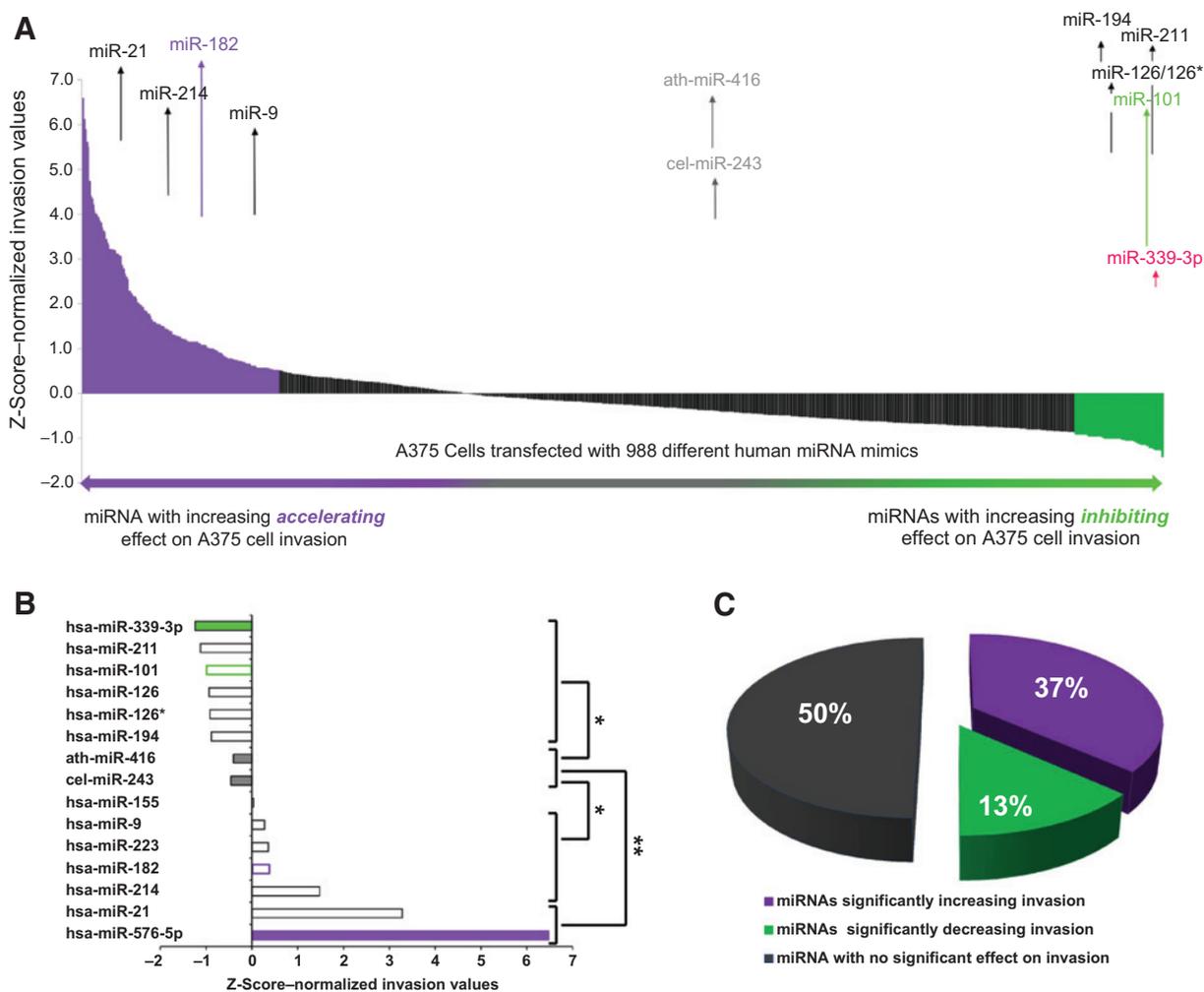
Notably, miRNAs miR-21 (22), and miR-214 (23) previously reported as miRNAs promoting cancer cell invasion could be confirmed in our functional miRNA screening approach (Fig. 1A and B). Consistently, miRNAs with reported tumor-suppressive potential, for example, miR-211 (24), miR-126/126\* (25), and miR-194 (26) could be verified in our assay (Fig. 1A and B), as well. The full list of tested miRNAs and their effect on A375 cell invasion can be found in Supplementary Table S2.

Overall this result shows that the 96-well Boyden chamber assay applied in our study can be used as a functional screening approach to investigate differences in the invasive potential of a given melanoma cell line.

### Identification of candidate miRNAs accelerating or inhibiting melanoma cell invasion

As miRNA mediated effects on cell viability may influence the outcome of the invasion assay, we tested 97 miRNAs that had shown strongest effects (accelerating or inhibiting) on melanoma cell invasion in our screening assay for their impact on viability of A375 melanoma cells (Supplementary Fig. S2). Although none of the accelerating miRNAs enhanced viability beyond 20%, we observed 11 inhibiting miRNAs, which reduced cell viability below 80% (Supplementary Fig. S2). Focusing on miRNA candidates distorting viability of A375

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**Figure 1.**

miRNA library screen by functional invasion assay. The melanoma cell line A375 was transfected with 988 individual miRNA mimics and analyzed in technical triplicates. cel-miR-243 and ath-miR-416 served as irrelevant negative controls. A, data are given as Z-score normalized invasion values, which represent the number of standard deviations a data point differs from the mean of the entire dataset. miRNAs with reported effects on melanoma invasion or progression are annotated above arrowheads. B, selection of miRNA candidates exhibiting significant effects on A375 cell invasion, including reported miRNAs (see A) are shown. Asterisks represent significance levels (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). C, overall, 50% of miRNAs showed either significant accelerating (purple, 37%) or significant inhibiting (green, 13%) effects, respectively, on invasion.

melanoma cells by less than 20%, we found that 49 miRNAs belonged to the accelerating group, whereas 37 candidates represented inhibiting miRNAs (Table 1). Because miRNA miR-339-3p turned out as one of the strongest inhibitors of invasion (Fig. 1A and B), showing only marginal effects on viability of A375 cells (Supplementary Fig. S2), this miRNA was selected for further analyses.

#### Validation of miR-339-3p as inhibitor of melanoma cell invasion

Lack of impaired viability by miR-339-3p expression was confirmed in two other melanoma cell lines, WM 266.4 and MaMel-86b. When cultured for extended periods up to 72 hours, only a marginal impact on the viability of A375 cells was observed (Fig. 2A and B); thus, within the time frame of our invasion assays, no severe effects on viability of A375 melanoma cells were detected.

Expression analysis of miR-339-3p by qRT-PCR revealed that this miRNA was significantly downregulated in melanoma cell lines in comparison with normal human epidermal melanocytes (NHEMs; Fig. 2C). We then performed a combinatorial analysis on a panel of 12 melanoma cell lines and found a significant negative correlation between their invasive potential (cf. Supplementary Fig. S3) and intracellular miR-339-3p expression levels (Fig. 2D).

The suppressive activity of miR-339-3p on melanoma cell invasion was validated in an independent assay, including five melanoma cell lines (MaMel-86b, WM266.4, MaMel103b, MaMel-61e, and MaMel-79b), now applying an invasion time of 24 hours (Fig. 3A and B, and Supplementary Fig. S4A–S4C). We found that miR-339-3p significantly inhibited invasion of all melanoma cell lines tested (Fig. 3A), including cell line A375 (Fig. 3B), thereby confirming the initial screening results.

**Table 1.** Top miRNA candidates showing maximal impact on melanoma cell invasion

Candidate miRNAs accelerating invasion of A375 melanoma cells
miR-19b, miR-19b-1*, miR-21, miR-21*, miR-20b, miR-154*, miR-194*, miR-200a*, miR-200b, miR-200c, miR-200c*, miR-202, miR-202*, miR-203, miR-205*, miR-206, miR-210, miR-218, miR-222, miR-483-5p, miR-490-3p, miR-498, miR-501-5p, miR-507, miR-518a-3p, miR-518b, miR-548i, miR-548j, miR-548m, miR-554, miR-557, miR-559, miR-575, miR-576-5p, miR-578, miR-595, miR-599, miR-611, miR-632, miR-635, miR-640, miR-647, miR-661, miR-766, miR-874, miR-1200, miR-2052, miR-2053, miR-2054
Candidate miRNAs inhibiting invasion of A375 melanoma cells
miR-17*, miR-92b*, miR-125a-3p, miR-125a-5p, miR-126, miR-129*, miR-129-3p, miR-139-5p, miR-181c*, miR-188-5p, miR-192*, miR-193b, miR-194*, miR-198, miR-199a-5p, miR-211, miR-302d, miR-320c, miR-331-3p, <b>miR-339-3p</b> , miR-342-3p, miR-523, miR-549, miR-654-3p, miR-659, miR-660, miR-1254, miR-1255b, miR-1270, miR-1276, miR-1277, miR-1283, miR-1288, miR-1290, miR-1908, miR-1909*, miR-1912

NOTE: Candidate miRNAs accelerating or inhibiting invasion were selected according to their Z-scores shown in Supplementary Table S2A. miRNAs affecting cell viability by more than 20% (cf. Supplementary Fig. S2) were excluded.

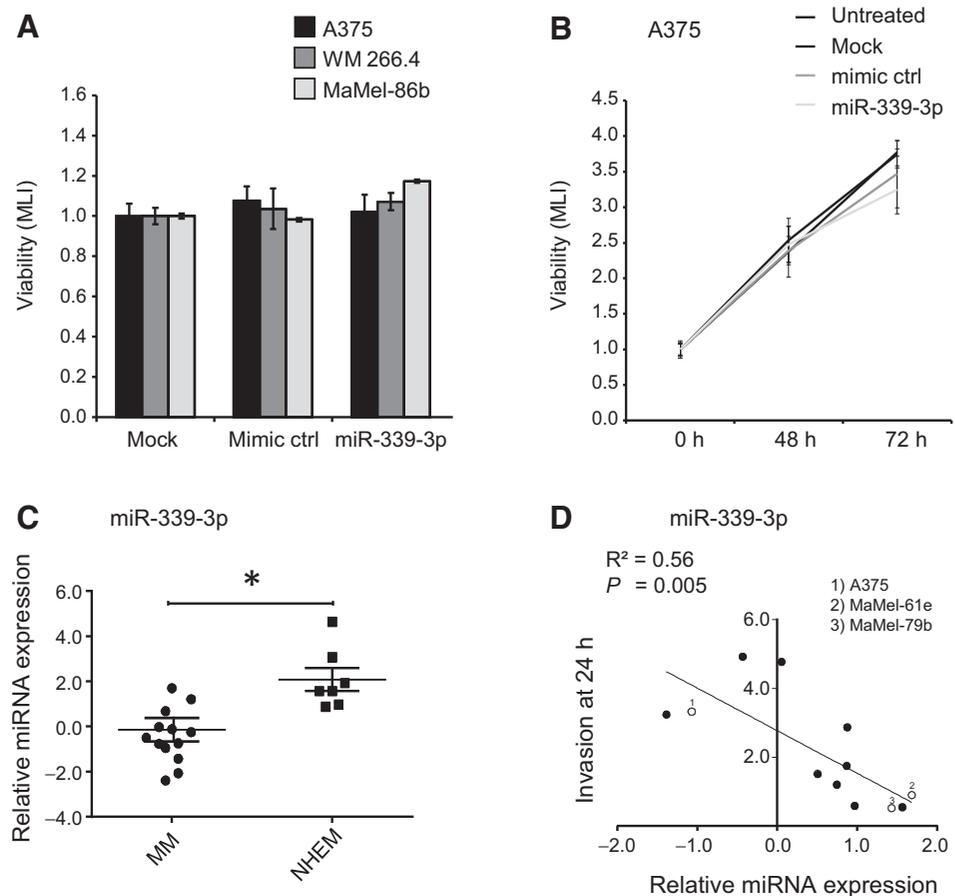
The physiologic role of a given miRNA should be verifiable by functional inhibition of endogenously expressed miRNA. Thus, experiments were performed to neutralize endogenous miR-339-3p activity upon introduction of the respective antagomiR, that is, anti-miR-339-3p. The slowly invading melanoma cell lines MaMel-61e and MaMel-79b (Supplementary Fig. S3) expressed miR-339-3p at higher levels than A375 (cf. Fig. 2D). Upon transfection of anti-miR-339-3p, the invasive capacity of melanoma cell lines A375 and MaMel-61e was elevated (Fig. 3C and D). Of note, the invasion capacity of melanoma cell line MaMel-79b showing strong endogenous miR-339-3p expression dramatically increased upon transfection with the antagomiR (Supplementary Fig. S4D), suggesting efficient neutralization of endogenous miR-339-3p-mediated invasion control by the transfected antagomiR.

### In silico miRNA target prediction

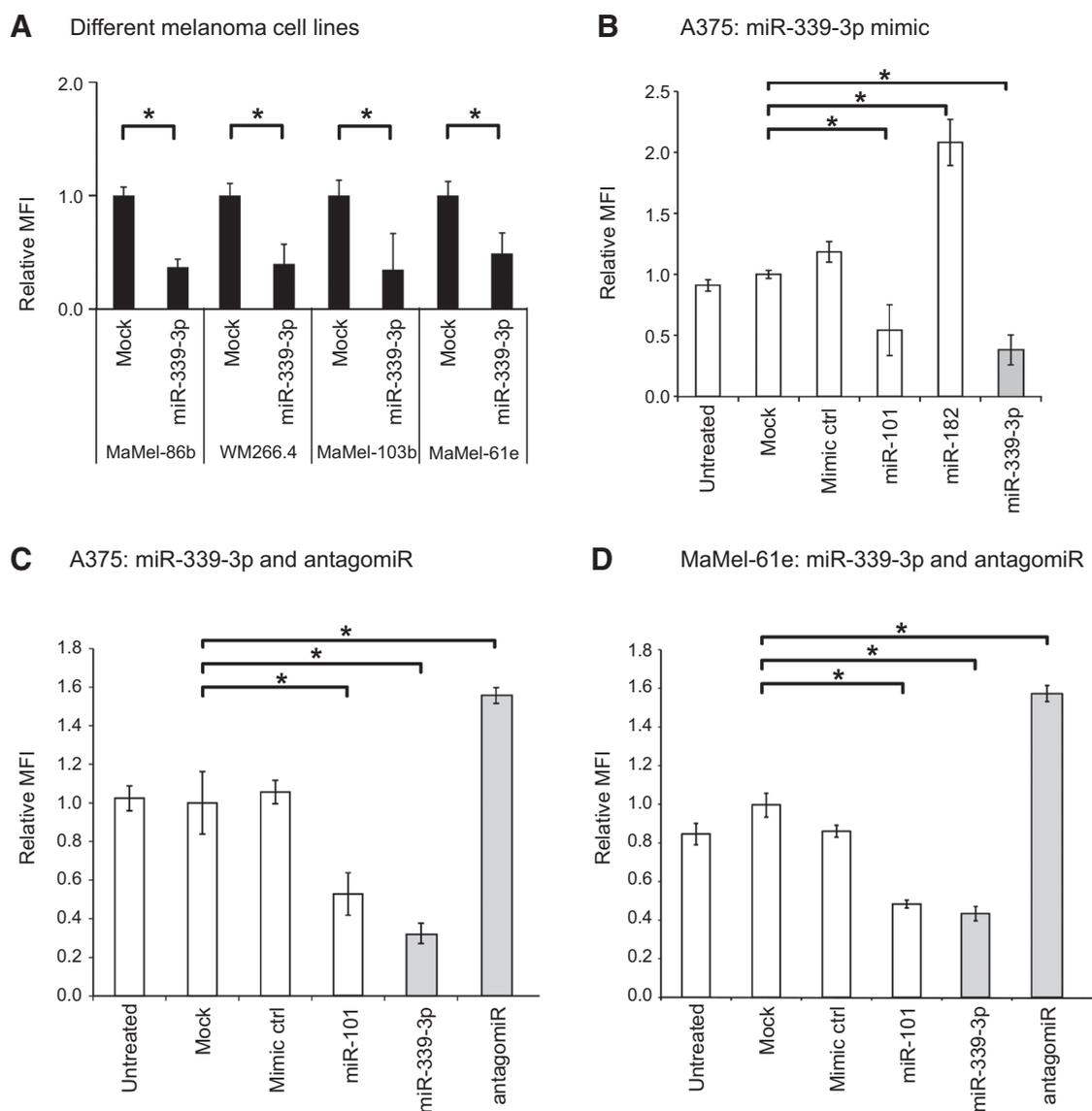
miRNAs are known to induce degradation of specific target mRNA molecules or inhibit their translation. Thus, miRNAs represent complex regulators of target protein expression that can affect cellular downstream signaling pathways (15). To identify putative miRNA-binding sites within the 3'UTR of cellular target mRNAs, miR-339-3p was subjected to *in silico* analyses using miRBase, applying the miRanda software in combination with TargetScan (27, 28). Predicted targets were grouped according to physiologic categories by gene enrichment analysis using the DAVID software (29). We found a large number of predicted target genes involved in motility, apoptosis, and proliferation (Supplementary Table S3). Because *in silico* analyses remain speculative, as they can only predict putative targets for specific miRNAs, both, direct miRNA/target mRNA interaction and the

**Figure 2.**

miR-339-3p expression does not impair melanoma cell viability and is negatively correlated with invasion capacity of melanoma cells. A, melanoma cell lines A375, MaMel-86b, and WM 266.4, transfected with ath-miR-416 as negative control mimic (ctrl mimic) or miR-339-3p or treated by mock transfection, were analyzed for cell viability 48 hours posttransfection. Mean luminescence intensities (MLI) given as mean of biologic triplicates, normalized to the mock transfections showed no differences between groups. B, cell viability timeline of mock transfectants, mimic control, miR-339-3p-transfectants, and untransfected A375 cells. Error bars show SEM of three biologic replicates. C, miR-339-3p expression in 13 human melanoma cell lines (MM) and in normal human epidermal melanocytes (NHEM) from 7 healthy donors unraveled significantly ( $P < 0.05$ ) reduced expression levels of this miRNA in melanoma cell lines. D, twelve melanoma cell lines were used for a correlation analysis of their relative miR-339-3p expression (normalized to the internal control RNU6B) and their invasive capacity (see Supplementary Fig. S3). A negative correlation was found with  $r^2 = 0.60$  and  $P = 0.001$ .



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**Figure 3.**

miR-339-3p inhibits melanoma invasion *in vitro*: Melanoma cell lines MaMel-86b, WM 266.4, MaMel-103b, and MaMel-61e (A; details see Supplementary Fig. S4), including melanoma cell line A375 (B) showed significantly reduced invasion after transfection with miR-339-3p. Transfection of a specific antagomiR enhanced the invasive potential of melanoma cell lines A375 (C) and MaMel-61e (D) above control levels, whereas the transfection with miR-339-3p reduced invasion. ath-miR-416 served as irrelevant negative control. Mean fluorescence intensity (MFI) was measured and normalized to mock-treated cells. Asterisks depict significant differences to mock controls (\*,  $P < 0.05$ ).

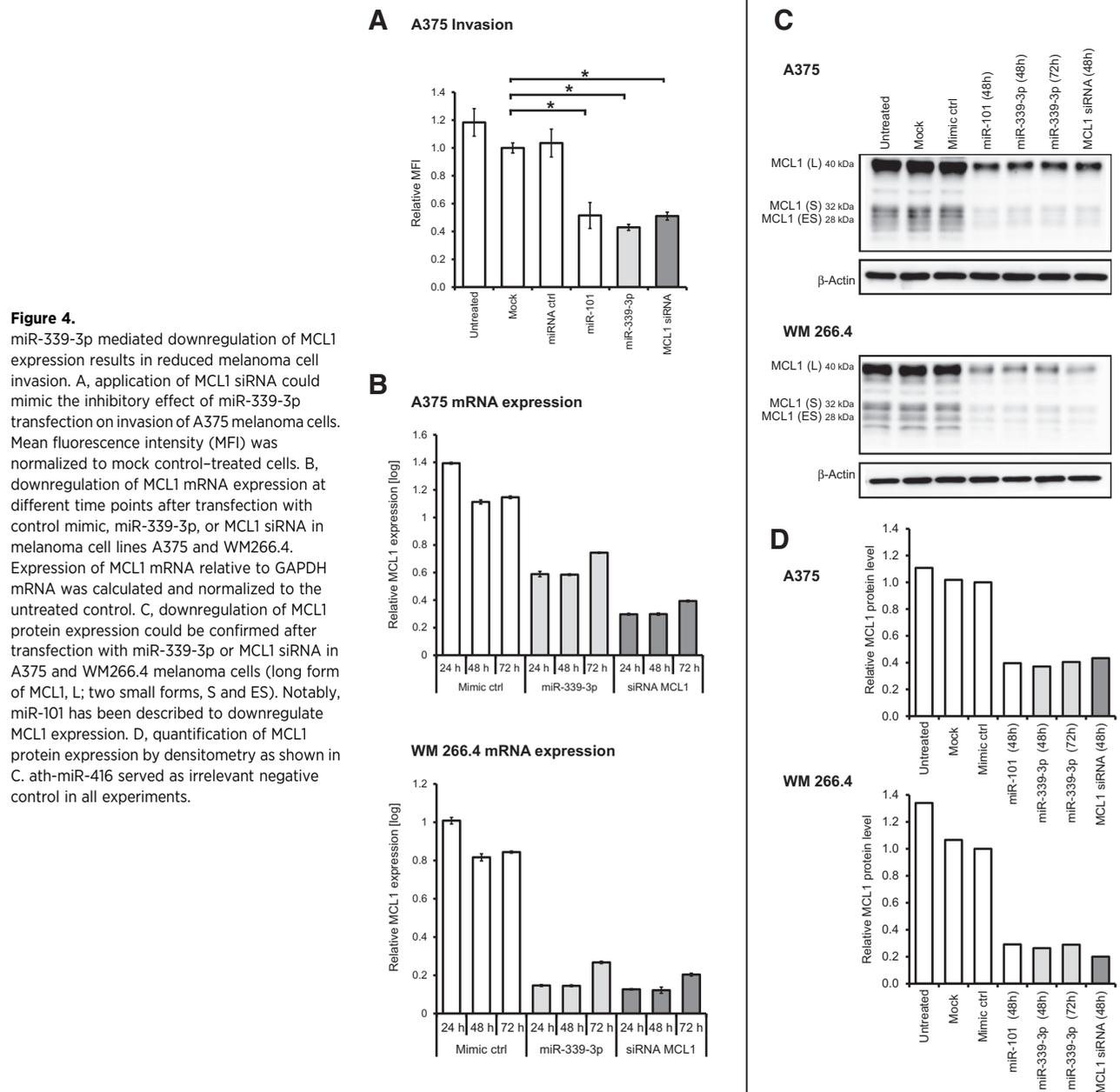
precise binding sites of miR-339-3p within the 3'UTR of the predicted target mRNAs had to be further determined *in vitro*.

#### MCL1 is a direct target of miR-339-3p

Concentrating on predicted targets with known function in melanoma progression and metastasis, oncogenes or tumor suppressor genes regulated by miR-339-3p were of specific interest for further *in vitro* validation. Upon *in silico* analyses using miRBASE (27) and DAVID (29), the myeloid cell leukemia sequence 1 (MCL1) was identified as one potential target of miR-339-3p (Supplementary Table S3; refs. 28, 29). This oncogene has been widely investigated with special focus on its functional role in apoptosis, cell survival, and epithelial mesenchymal transforma-

tion in various malignancies (30, 31). However, nothing has been published on the function of MCL1 in melanoma cell invasion, so far. To investigate this question, we performed experiments with MCL1-specific siRNA and found that transfection of this siRNA could imitate the inhibitory effect of miR-339-3p on melanoma cell invasion in all five melanoma cell lines tested (Fig. 4A, Supplementary Fig. S5). In fact, invasion of melanoma cell lines transfected with miR-339-3p or siRNA against MCL1 was significantly reduced compared with mock-transfected control cells. Thus, MCL1 knockdown by siRNA can mimic the inhibitory effect of miR-339-3p on melanoma cell invasion.

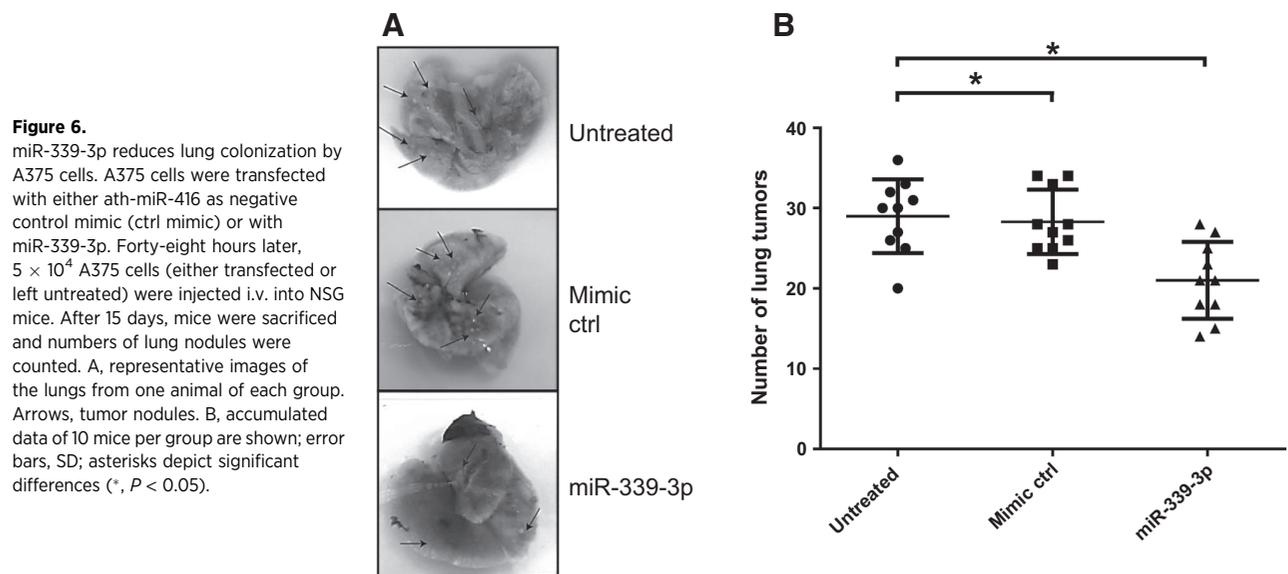
MCL1 protein expression, albeit at varying levels, could be detected in all melanoma cell lines under investigation



(Supplementary Fig. S6). Thus, in the next step, we tested the effect of miR-339-3p on MCL1 expression on protein and also on mRNA level. miR-101 was used as a positive control in some experiments, because it had been reported to target MCL1 in non-small cell lung cancer cells (32). Indeed, melanoma cell lines A375 and WM 266.4 showed decreased MCL1 mRNA levels 24 to 72 hours after transfection with miR-339-3p (Fig. 4B). In addition, MCL1 protein expression was consistently decreased after miR-339-3p overexpression in all melanoma cell lines tested (Fig. 4C and D, Supplementary Fig. S7) except MaMel-86b, which showed variable results (not shown). Thus, our data clearly show that miR-339-3p overexpression results in reduced MCL1 expression on mRNA and protein level.

To identify and validate the binding site of miR-339-3p within the 3'UTR of the MCL1-specific mRNA predicted by RNA hybrid (33), A375 cells were co-transfected with a luciferase-encoding plasmid harboring the complete MCL1 3'UTR or with a respective construct containing a deletion within the predicted binding site of miR-339-3p at the 3'UTR of MCL1 (Fig. 5A), in combination with the candidate miRNA miR-339-3p or the negative control mimic. The interaction of miR-339-3p with the 3'UTR of MCL1 was shown upon detection of a decreased luciferase signal in comparison with the signal observed with the negative control mimic and pLS 3'UTR MCL1 plasmid transfection (Fig. 5B). Of note, deletions introduced within the predicted binding site almost completely restored the luciferase signal intensity, thus





melanoma by counteracting therapies targeting MEK (47). Moreover, MCL1 is functionally involved in melanoma cell survival, cell death induction, proliferation, and EMT (31, 47) and was described to interfere with anoikis induction, resulting in enhanced cancer cell dissemination (48, 49). Anoikis, a cell death program activated upon cell–cell or cell–extracellular matrix contact loss, represents one hallmark of cancer, promoting tumor cell migration and invasion (6, 50). These reports support the hypothesis, that miR-339-3p might inhibit melanoma cell invasion via a complex network of signaling events that require MCL1 downregulation as an intermediate step in the downstream signaling cascade. In our study, overexpression of miR-339-3p resulted in reduced MCL1 protein and mRNA levels 48 hours posttransfection, which were partially restored 24 hours later. This effect might be due to the short half-life of MCL1 and the necessity to tightly balance its intracellular expression level in relation to cell physiology, cell survival, and apoptosis (51). Of note, direct interaction of miR-339-3p with the 3'UTR of MCL1 could be validated by reporter assays and site-specific mutation of the binding site.

Notably, miRNA-mediated modulation of MCL1 expression is not restricted to miR-339-3p. In melanoma, it has been shown that MCL1 can be targeted by miR-193b (52), and recently miR-22 and miR-29a have been reported to target MCL1 in prostate cancer (53). Furthermore, enhanced miR-125 expression was shown to reduce MCL1 expression and inhibit invasion of a human gastric cancer cells (54). Similarly, targeting of MCL1 by miR-107 was shown to abrogate invasion of cervical cancer–derived SiHa and HeLa cell lines (55), and miR-133b was shown to knockdown MCL1 expression in two osteosarcoma cell line lines, thereby reducing their invasive capacity *in vitro* (56). Therefore, these MCL1-targeting miRNAs are interesting candidates to be validated for their modulating effects on MCL1 expression also in melanoma cells.

To test whether miR-339-3p might affect melanoma cell invasion *in vivo*, we performed experimental lung colonization assays in immunodeficient NSG mice with transfected A375 melanoma cells. We could show that mice injected with A375 cells over-

expressing miR-339-3p carried significantly less tumor nodules within their lungs compared with the control mice, thus confirming the inhibitory effect of miR-339-3p on tumor cell invasion observed *in vitro*. Future studies using fresh tumor biopsies from melanoma patients will allow to test whether a correlation between the invasive capacity and altered endogenous miR339-3p expression levels in melanoma cells can be confirmed also within the clinical setting.

In summary, we have established a screening assay for the identification of miRNAs affecting invasion of melanoma cell lines, resulting in a large number of miRNA candidates with accelerating or inhibitory impact, respectively, on the invasive capacity of A375 cells. Conclusively, our miRNA library screen identified miR-339-3p as a potent inhibitor of melanoma cell invasion, targeting the oncogene MCL1, thereby representing a new tumor-suppressive miRNA in melanoma.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Authors' Contributions

Conception and design: C. Luo, S.B. Eichmüller

Development of methodology: C.E.M. Weber, C. Luo, S.B. Eichmüller

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.E.M. Weber, A. Gardyan, T. Kordaß, S.B. Eichmüller

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.E.M. Weber, A. Hotz-Wagenblatt, A. Gardyan, T. Holland-Letz, S.B. Eichmüller

Writing, review, and/or revision of the manuscript: C.E.M. Weber, W. Osen, S. B. Eichmüller

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Holland-Letz, W. Osen

Study supervision: C. Luo, S.B. Eichmüller

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## miR-339-3p Is a Tumor Suppressor in Melanoma

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