# Genome-Wide Screen for MicroRNAs Reveals a Role for miR-203 in Melanoma Metastasis

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Melanoma is one of the deadliest human cancers with limited therapeutic options. MicroRNAs are a class of short noncoding RNAs regulating gene expression at the post-transcriptional level. To identify important miRNAs in melanoma, we compared the miRNome of primary and metastatic melanomas in The Cancer Genome Atlas dataset and found lower miR-203 abundance in metastatic melanoma. Lower level of miR-203 was associated with poor overall survival in metastatic disease. We found that the methylation levels of several CpGs in the *MIR203* promoter negatively correlated with miR-203 expression and that treatment with the demethylating agent 5-aza-2-deoxycytidine induced miR-203 expression, which was associated with demethylation of the promoter CpGs, in melanoma cell lines. In vitro, there was a decreased expression of miR-203 in melanoma cell lines in comparison with primary melanocytes. Ectopic overexpression of miR-203 suppressed cell motility, colony formation, and sphere formation as well as the angiogenesis-inducing capacity of melanoma cells. In vivo, miR-203 inhibited xenograft tumor growth and reduced lymph node and lung metastasis. SLUG was shown as a target of miR-203, and knockdown of SLUG recapitulated the effects of miR-203, whereas its restoration was able to reverse the miR-203-mediated suppression of cell motility. These results establish a role for miR-203 as a tumor suppressor in melanoma which suppresses both early and late steps of metastasis. Hence, restoration of miR-203 has therapeutic potential in melanoma.

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## INTRODUCTION

Melanoma is one of the most aggressive malignancies, causing approximately 80% of skin cancer-related deaths (Miller and Mihm, 2006). Approximately 132,000 new cases are diagnosed worldwide each year and its incidence has more than doubled in the past 30 years (American Cancer Society, 2016). Although surgery can be curative for thin melanomas, advanced stages have a much worse prognosis and metastatic melanoma is a fatal disease with a 5-year survival rate between 5% and 19% (Sandru et al., 2014; Tas, 2012). A number of risk factors have been identified for melanoma including the propensity of the skin to sunburn after UV exposure, light hair and eye color, the number of melanocytic nevi, and a family history of melanoma (Bränström et al., 2010). The accumulation of genetic alterations in melanocytes results in the expansion and growth of malignant clones, which ultimately acquire the capacity to invade and metastasize (Thompson et al., 2005). Despite recent advances in the understanding of oncogenic mechanisms and therapeutic interventions, the median progression-free survival in patients with metastatic disease has not extended beyond 12 months (Buchbinder and Hodi, 2016). Thus, the investigation of molecular mechanisms that orchestrate melanoma metastasis remains of paramount importance.

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 22 nt in length, that can regulate gene expression at the post-transcriptional level resulting in mRNA degradation or translational repression (Bartel, 2004, 2009). miRNAs play important roles in virtually all physiological processes and the regulation of signaling pathways. Some miRNAs are dysregulated in cancers in which they can act as tumor suppressors or oncogenes depending on the set of target genes they regulate and the transcriptional context in which they are expressed (Garzon et al., 2006). A number of reports have investigated miRNAs in melanoma and identified roles for, for example, let-7, miR-155, miR-200c, miR-221, miR-222, miR-145, miR-150, and miR-23a/b, in melanoma pathogenesis in recent years (Kunz, 2013). Some of these, for example, miR-200c, miR-205, and miR-211, have been reported as potential diagnostic or prognostic markers for melanoma (Xu et al., 2012). However, given the complexity of molecular mechanisms influenced by miRNA activity, clear roles of miRNAs in malignant melanoma

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*Abbreviations: miRNA, microRNA; TCGA, The Cancer Genome Atlas; OS, overall survival*

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especially related to the metastatic phenotype are yet to be assigned. Therefore, in this study, we aimed to uncover miRNAs controlling metastatic melanoma behavior.

Here, we report that miR-203 is downregulated in metastatic melanoma compared with primary tumors and that a low level of miR-203 in metastatic disease is associated with poor overall survival. In vitro, miR-203 suppressed melanoma cell migration, invasion, self-renewal, and angiogenesis-inducing ability. In vivo, miR-203 decreased primary tumor growth and suppressed metastasis to the lymph node and lung.

## RESULTS

### MiR-203 is decreased in metastatic melanoma and its level correlates with overall survival

To identify miRNAs that may be involved in melanoma metastasis, we performed an in silico analysis of The Cancer Genome Atlas (TCGA) skin melanoma cohort (The Cancer Genome Atlas Network, 2015). A comparison of primary and metastatic melanomas using the statistical functions of DESeq2 identified miRNAs that were differentially expressed ($\log_2$ fold change $> \pm 0.58$, false discovery rate $< 0.05$) between primary and metastatic tumors (Figure 1a). Among them, miR-203 was found to be the most downregulated in metastatic samples (log2 fold change $= -3.4086$, $P$-value $= 8.49 \times 10^{-28}$) (Figure 1b and c). To determine the prognostic significance of miR-203 in patients with metastatic melanoma, a Kaplan-Meier survival analysis was performed. High expression of miR-203 (read per kilobase of transcript per million $> 7.0$) conferred longer overall survival (OS) compared with those with low miR-203 expression (read per kilobase of transcript per million $< 4$) (hazard ratio $= 0.6147$, 95% confidence interval $= 0.3979-0.9498$, $P = 0.0284$) (Figure 1d), suggesting that miR-203 may be important in melanoma progression. We also assessed the expression of miR-203 in a panel of seven melanoma cell lines of varying pigmentation status and primary human melanocytes and observed a lower abundance in all melanoma cell lines compared with primary melanocytes (Supplementary Figure S1 online).

To identify potential mechanisms regulating miR-203 expression in melanomas, we performed the analysis of DNA methylation levels of all *MIR203* CpG sites in the TCGA skin melanoma cohort. The methylation levels of several sites in the *MIR203* CpG island (1,538 bp) negatively correlated with miR-203 expression and were significantly different between primary and metastatic samples (Figure 1e) indicating a connection between DNA methylation of the *MIR203* locus and miR-203 expression in melanoma in vivo. Supporting the significance of DNA methylation on miR-203 expression, 5-aza-2-deoxycytidine treatment induced miR-203 expression in a panel of human melanoma cell lines (Figure 1f). To further show that demethylation of CpGs within the *MIR203* promoter associates with the miR-203 induction, we performed *MSRE*-qPCR that showed a significant downregulation of methylation in BE and DFB and a similar trend in SK-Mel-28 (Figure 1g) treated with 5-aza-2-deoxycytidine. Additionally, to assess a number of other CpGs within *MIR203* gene, we performed pyrosequencing targeting a region of 293 bp (Supplementary Figure S2 online), which showed a similar trend of decreased methylation in eight consecutive CpGs in all three cell lines treated with 5-aza-2-deoxycytidine. Taken together, these results illustrate that miR-203 levels are decreased in metastatic melanoma, at least in part, through DNA methylation, and its levels are associated with OS in metastatic disease.

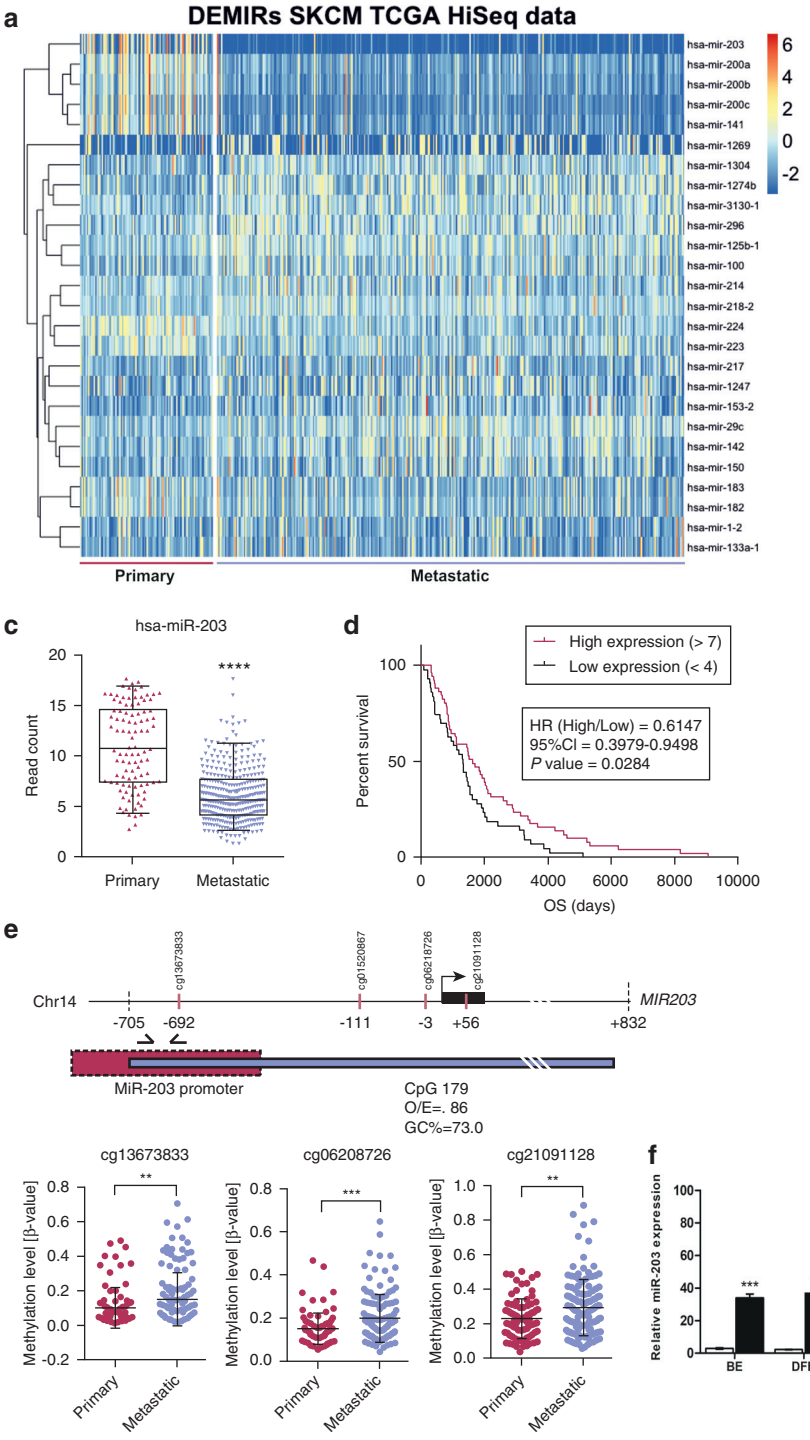
### MiR-203 suppresses motility and sphere formation of melanoma cells

Given the association between miR-203 and metastasis, we next investigated the effect of miR-203 on migration and invasion, essential components of a metastatic disease. As a pilot experiment, we performed a monolayer wound-healing assay to evaluate the effects of miR-203 modulation on cell migration in human melanoma cell lines (BE, SK-Mel-28, and DFB). We found that miR-203 significantly delayed wound closure in all three melanoma cell lines (Figure 2a). To confirm this behavior and extend our investigation into analyzing invasion, we employed a Transwell migration and invasion assay and observed that miR-203 greatly reduced the migratory and invasive potential of melanoma cells (Figure 2b). To exclude the effect of short-term proliferation or cell death on migration and invasion, a PrestoBlue cell viability assay was performed to explore the effect of miR-203 on cell viability, and the results showed that miR-203 had no impact on short-term proliferation (Supplementary Figure S3 online). To show that the effect on cell motility is directly miR-203 dependent and reversible, we performed migration and invasion assay followed by knockdown of miR-203 in BE cells overexpressing miR-203 and found a significant increase in the number of migrated and invaded cells (Supplementary Figure S4 online). These observations indicate a suppressive role of miR-203 on melanoma motility, the initial mechanism of metastasis.

As tumor spheroids formed by monoculture of cancer cells in nonadherent conditions mimic micrometastasis (Weiswald et al., 2015), we examined the effect of miR-203 on long-term anchorage-independent proliferation and self-renewal ability of melanoma cells. Our results revealed the suppression of colony-forming ability by miR-203 with approximately 50% reduction in the number of colonies formed in SK-Mel-28 and DFB cells (Supplementary Figure S5a online). In addition to suppressing colony formation in 2D assays, miR-203 also suppressed the sphere formation ability of all three melanoma cell lines (Supplementary Figure S5b). These results demonstrate that miR-203 acts to suppress the motility, invasiveness, and the self-renewal ability of melanoma cells.

### MiR-203 suppresses the angiogenesis-inducing ability of melanoma cells

Metastasis is a multistage process, and in addition to being able to invade surrounding tissue and migrate distally, a rouge cell needs to set up a secondary site and develop adequate nutrient supply to the secondary lesion (Kirsch et al., 2004). To further investigate the role of miR-203 in the malignant process, we studied the effect of miR-203 on the angiogenesis-inducing ability of melanoma cells. Conditioned media collected from melanoma cells transfected with miR-203 or their respective controls were compared for the capability to support neovascularization in




**b**

| MiRNA | $log_2FC$ | Adjusted *P*-value |
|---|---|---|
| hsa-mir-203 | -3.409 | $8.49\times10^{-28}$ |
| hsa-mir-200c | -2.657 | $8.04\times10^{-29}$ |
| hsa-mir-141 | -2.290 | $1.55\times10^{-21}$ |
| hsa-mir-200a | -2.117 | $8.53\times10^{-23}$ |
| hsa-mir-200b | -1.997 | $1.82\times10^{-21}$ |
| hsa-mir-224 | -1.453 | $8.65\times10^{-21}$ |
| hsa-mir-183 | -0.863 | $1.30\times10^{-4}$ |
| hsa-mir-182 | -0.767 | $3.25\times10^{-4}$ |
| hsa-mir-223 | -0.668 | $4.47\times10^{-4}$ |
| hsa-mir-27b | -0.545 | $8.87\times10^{-5}$ |
| hsa-mir-1249 | -0.527 | $5.99\times10^{-5}$ |
| hsa-mir-181b-2 | -0.520 | 0.001 |
| hsa-mir-1245 | -0.489 | 0.016 |
| hsa-mir-138-2 | -0.474 | 0.002 |
| hsa-mir-138-1 | -0.471 | 0.003 |
| hsa-mir-24-1 | -0.444 | 0.001 |
| hsa-mir-196b | -0.443 | 0.026 |
| hsa-mir-139 | -0.404 | 0.006 |
| hsa-mir-146a | -0.381 | 0.022 |
| hsa-mir-193b | -0.372 | 0.010 |
| hsa-mir-23b | -0.348 | 0.006 |
| hsa-let-7f-1 | -0.282 | 0.019 |
| hsa-mir-15a | -0.275 | 0.012 |
| hsa-mir-103-2 | -0.259 | 0.010 |
| hsa-mir-16-1 | 0.202 | 0.008 |
| hsa-mir-197 | 0.240 | 0.024 |
| hsa-mir-130b | 0.242 | 0.037 |
| hsa-mir-26a-2 | 0.249 | 0.020 |
| hsa-mir-106b | 0.251 | 0.014 |
| hsa-mir-3074 | 0.274 | 0.037 |
| hsa-mir-128-1 | 0.279 | 0.007 |
| hsa-mir-1301 | 0.285 | 0.023 |
| hsa-mir-186 | 0.312 | $1.30\times10^{-4}$ |
| hsa-mir-2110 | 0.321 | 0.003 |
| hsa-mir-128-2 | 0.332 | 0.004 |
| hsa-mir-1468 | 0.336 | 0.022 |
| hsa-mir-199a-2 | 0.340 | 0.047 |
| hsa-mir-1296 | 0.373 | 0.035 |
| hsa-mir-1306 | 0.383 | $4.76\times10^{-4}$ |
| hsa-mir-10a | 0.388 | 0.012 |
| hsa-mir-199a-1 | 0.397 | 0.021 |
| hsa-mir-1271 | 0.408 | 0.007 |
| hsa-mir-130a | 0.411 | 0.006 |
| hsa-mir-1228 | 0.412 | 0.008 |
| hsa-mir-2116 | 0.420 | 0.004 |
| hsa-mir-101-1 | 0.420 | 0.006 |
| hsa-mir-215 | 0.425 | 0.010 |
| hsa-mir-1976 | 0.437 | $1.99\times10^{-4}$ |
| hsa-mir-212 | 0.442 | 0.003 |
| hsa-mir-192 | 0.460 | $2.29\times10^{-4}$ |

**Figure 1. MiR-203 is decreased in metastatic melanoma and correlates with overall survival.** (**a**) Unsupervised hierarchical clustering and heat map of the differentially expressed (FDR < 0.05, $log_2$ ratio > ±0.58) miRNAs between primary and metastatic melanomas from The Cancer Genome Atlas (TCGA) project (https://cancergenome.nih.gov/). Normalized reads per million mapped miRNAs are mean centered and log2 transformed. Each row represents an individual miRNA. Each column represents an independent sample. (**b**) The top 50 deregulated miRNAs (FDR < 0.05) in metastatic melanoma compared with primary tumor. A complete list of miRNAs is provided in Supplementary Table S1 online. (**c**) Expression of miR-203 in primary (n = 98) and metastatic tumors (n = 346). ****$P < 0.001$, Mann-Whitney *U* test. (**d**) Kaplan-Meier survival analysis of the patient with metastatic disease with low and high miR-203 expression from TCGA. (**e**) Schematic representation of the *MIR203* gene locus with the transcription start site (arrow), promoter (red box) and promoter proximal CpG island (blue box), and the position of each measured CpG site (red vertical lines). The black arrowheads on the promoter region refer to the forward and reverse primer used for methylation-sensitive restriction enzyme-qPCR (MSRE-qPCR). Altered *MIR203* promoter methylation in metastatic melanomas from the TCGA cohort compared with primary melanomas. **$P < 0.01$, ***$P < 0.001$, Mann-Whitney *U* test. (**f**) MiR-203 expression was determined by qPCR in BE, SK-Mel-28, and DFB cells treated with 10 μM 5-aza-2-deoxycytidine (5-azaC) for 48 hours. (**g**) CpG methylation within the *MIR203* promoter was measured by MSRE-qPCR using DNA from 5-azaC and DMSO-treated cell lines. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test (two-tailed). CI, confidence interval; FDR, false discovery rate; HR, hazard ratio; miRNA, microRNA; OS, overall survival; SKCM, skin cutaneous melanoma.

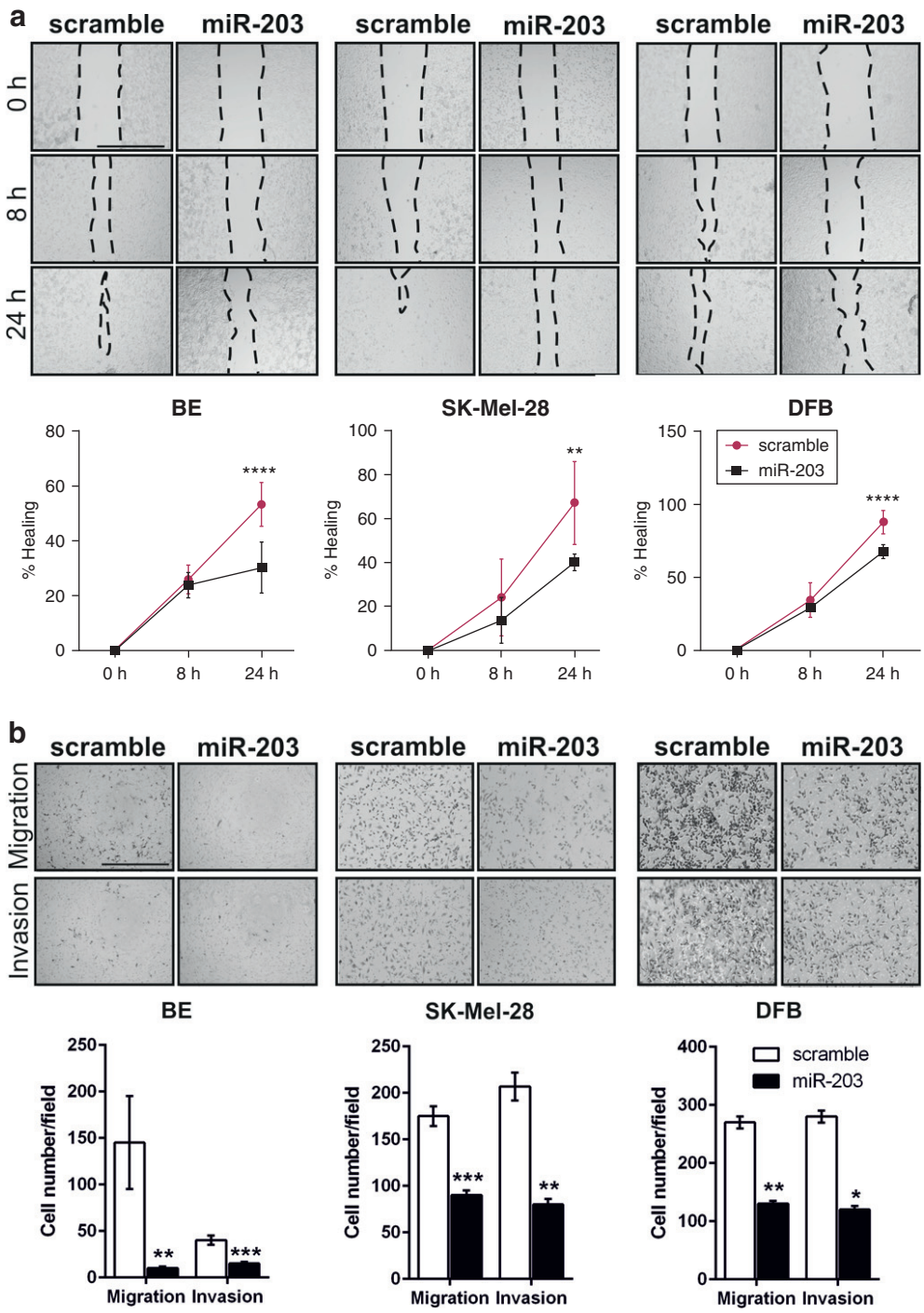



**Figure 2. MiR-203 suppresses melanoma cell motility.** (**a**) Scratch wound healing assay with miR-203 or control sequence-transfected BE, SK-Mel-28, and DFB melanoma cell lines. $^{**}P < 0.01$, $^{****}P < 0.0001$, analysis of variance (ANOVA). Scale bar = 1 mm. (**b**) Transwell migration and invasion assays were performed with melanoma cell lines: BE, SK-Mel-28, and DFB after overexpression of miR-203 or control sequence. The bar chart shows the number of migrated and invaded cells. Data represent the mean ± standard deviation from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test (two-tailed).

endothelial cell tube formation assays. Conditioned supernatants from all three cell lines were capable of inducing tube formation; however, this was substantially decreased (>60%) on miR-203 transfection (Figure 3a), suggesting that miR-203 suppresses tumor-induced angiogenesis in melanoma. To assess whether miR-203 regulates soluble factors involved in endothelial cell motility, a Transwell migration assay was performed with conditioned supernatants from melanoma cells as chemoattractant. We found that significantly less endothelial cells migrated toward the supernatant collected from miR-203-overexpressing melanoma cells compared with controls (Figure 3b). Because we previously found that miR-203 directly targets IL-8, an important positive regulator of neoangiogenesis (Li et al., 2003; Wei et al., 2013), we measured and observed a decreased IL-8 expression in miR-203-overexpressing melanoma cells by qPCR (Figure 3c). Consistent with qPCR data, the amount of secreted IL-8 was significantly decreased in the supernatant collected from miR-203-transfected melanoma cells (Figure 3d). These results suggest that miR-203 can act in a non-cell-autonomous manner by regulating tumor-induced angiogenesis via suppressing the expression of angiogenic factors. This may affect the recruitment of endothelial cells and the formation of new vessels into tumors, and in doing so

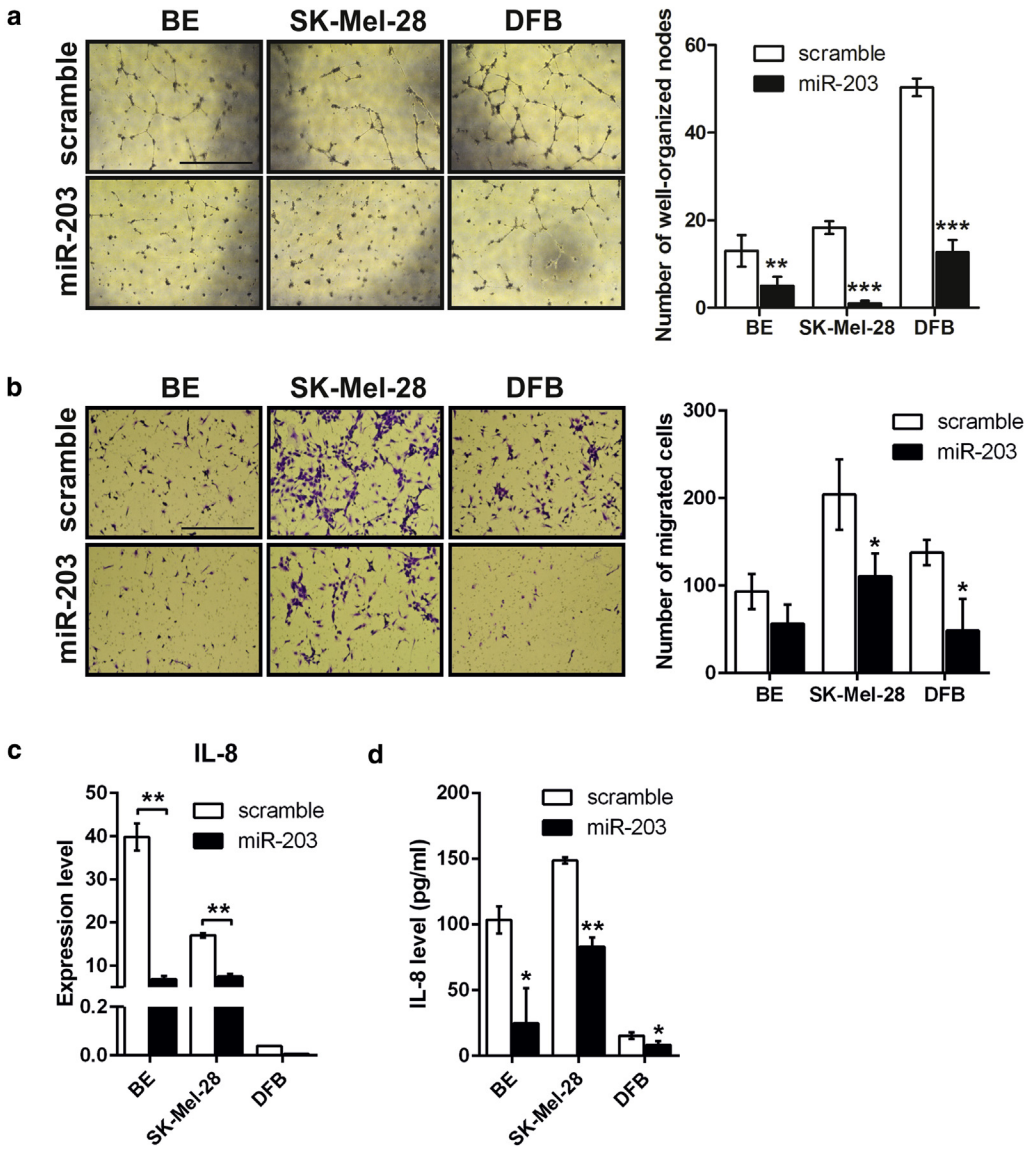



**Figure 3. MiR-203 suppresses the angiogenesis-inducing ability of melanoma cells.** (**a**) HUVEC tube formation assay was performed by resuspending HUVEC cells in supernatant collected from melanoma cells (BE, SK-Mel-28, and DFB) transfected with miR-203 or control sequence and seeded into a 48-well plate with a Matrigel layer. After 16–20 hours of incubation, photographs were taken (left panel) and the number of well-organized nodes was counted (right panel). Scale bar = 1 mm. (**b**) Suppressed migration of HUVECs by conditioned medium from miR-203-overexpressing melanoma cells. (**c**) qPCR measurement of IL-8 expression in melanoma cells on ectopic expression of miR-203 and (**d**) MiR-203 suppresses IL-8 in the supernatant of melanoma cells, ELISA. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test (two-tailed). HUVEC, human umbilical vein endothelial cell.

miR-203 could mediate not only the active movement of cells but also the success of secondary site seeding.

### MiR-203 targets SLUG in melanoma

To reveal the mechanism by which miR-203 regulates melanoma metastatic phenotype, we performed an in silico analysis to identify potential miR-203 targets. The results showed that SLUG, a gene associated with cell motility and melanoma progression (Shirley et al., 2012), may be a direct target of miR-203 due to the presence of an evolutionally conserved 8 mer-binding site in its 3′UTR (Figure 4a). Therefore, we evaluated SLUG expression in our panel of melanoma cell lines and found it to be strongly upregulated compared with human primary melanocytes (Supplementary Figure S6 online). In addition, qPCR and western blot analyses revealed a significant suppression of SLUG by miR-203 overexpression in all three melanoma cell lines (Figure 4b). To experimentally test that SLUG is a direct target of miR-203, a luciferase reporter assay was performed using a pLightSwitch luciferase construct containing the full-length wild-type SLUG 3′UTR or its mutant version. We found that cotransfection of miR-203 significantly decreased SLUG 3′UTR reporter activity (Figure 4c). The inhibitory effect of miR-203 was abolished when the predicted miR-203-binding site was mutated, demonstrating that SLUG is a direct target of miR-203 for post-transcriptional gene silencing.

To confirm this conclusion, a scratch wound healing assay of BE, SK-Mel-28, and DFB cells with silenced SLUG expression was carried out. Knockdown of SLUG recapitulated the effects of miR-203 and significantly inhibited scratch wound healing (Figure 5a and Supplementary Figure S7a online). Moreover, silencing SLUG significantly reduced the number of migrating and invading cells through Transwell inserts (Figure 5b and Supplementary Figure S7b). Conversely, the ectopic overexpression of SLUG accelerated the rate of wound healing in melanoma cells (Supplementary Figure S8 online).

To define the relative importance of SLUG within the miR-203 gene network in melanoma, we investigated

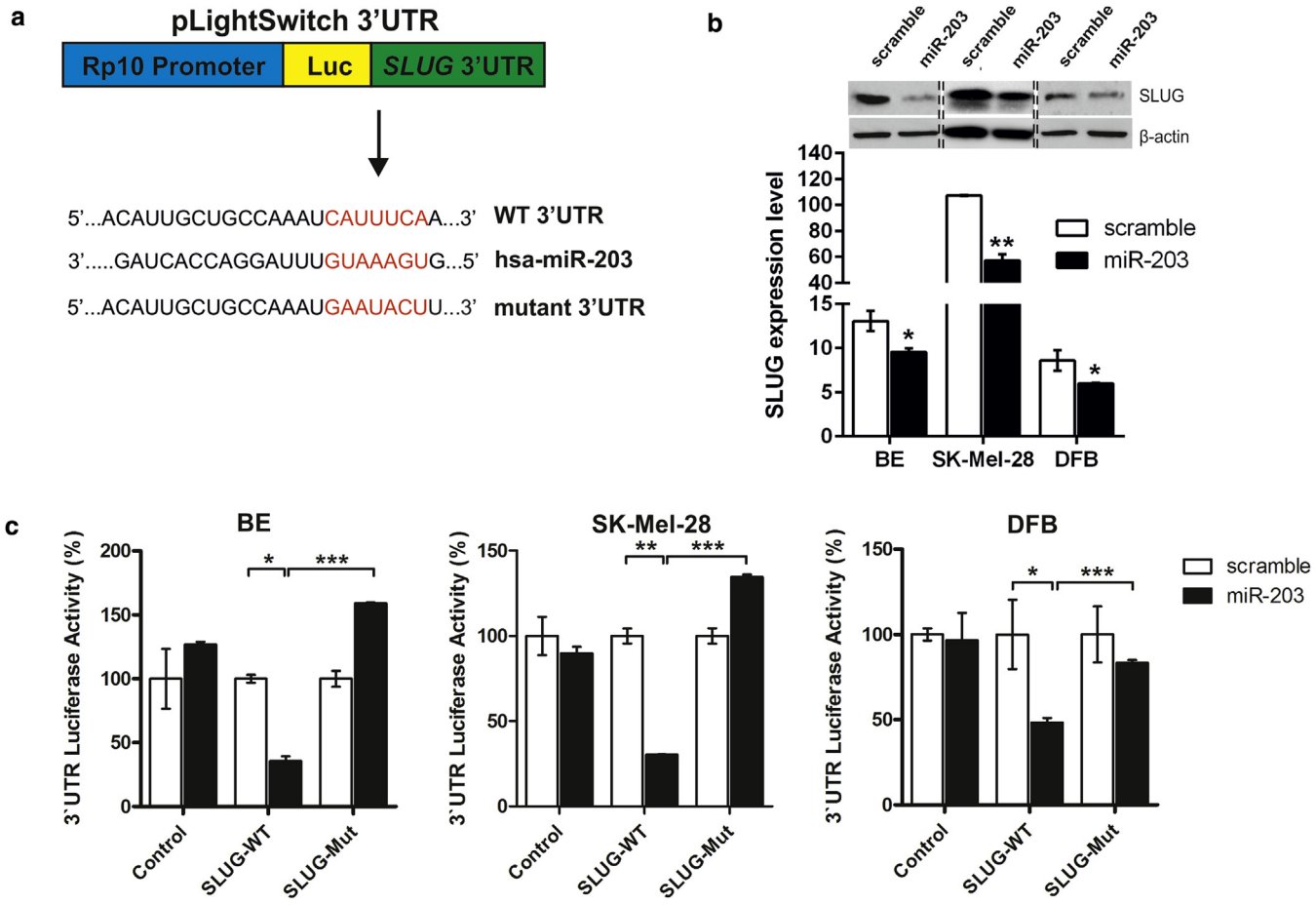



**Figure 4. MiR-203 targets SLUG in melanoma.** (**a**) MiR-203-binding site in SLUG 3′UTR and the sequences of wild-type (WT) and mutant SLUG 3′UTR used in the 3′UTR luciferase experiments are shown. (**b**) Analysis of SLUG expression by qCPR and western blot in cells on overexpression of miR-203. (**c**) WT SLUG 3′UTR and Mut SLUG 3′UTR reporter were constructed, and a luciferase reporter assay was performed in BE, SK-Mel-28, and DFB cells after miR-203 transfection. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t*-test (two-tailed).

whether ectopic expression of SLUG (not carrying its 3′UTR, therefore insensitive to miR-203) could rescue the effect of miR-203 overexpression. To this end, melanoma cell lines were cotransfected with miR-203 and SLUG (pcDNA-SLUG). Restoration of SLUG significantly reversed the miR-203-mediated suppression of scratch wound healing and cell migration in all three melanoma cell lines (Supplementary Figure S9a and b online). Taken together, these results identify SLUG as a direct target of miR-203 in melanoma and indicate that the miR-203/SLUG axis can regulate melanoma cell motility.

Moreover, the association between SLUG abundance and melanoma progression was evaluated by immunohistochemistry in the melanoma tissue array section (ME1004f; US Biomax, Derwood, MD) containing core tissues from benign nevus and malignant melanoma. The results demonstrated a higher level of SLUG protein in malignant melanoma samples than in benign nevi (Supplementary Figure S10 online).

### MiR-203 suppresses melanoma growth and metastasis in vivo

To fully confirm the role of miR-203 as a key regulator of invasion, self-renewal, and angiogenesis-inducing capacity of melanoma cells, we directly studied the effect of miR-203 on the metastatic behavior of melanoma cells in vivo.

First, to evaluate the function of miR-203 on the growth of primary melanoma, BE cells were stably transduced with miR-203-GFP encoding lentiviral particles and injected subcutaneously into NSG mice. Tumor volume was monitored weekly and a significant reduction in tumor volume was observed in the miR-203-overexpressing group at week 4 (Figure 6a). The average tumor weight was reduced from 0.5 to 0.3 g in mice injected with miR-203-overexpressing BE cells (Figure 6b). Overexpression of miR-203 in the tumors formed by miR-203-overexpressing cells was confirmed by qPCR (Supplementary Figure S11 online). Immunostaining of tumor sections and western blot analysis of protein lysates from control and miR-203-overexpressing BE cells demonstrated a decrease of SLUG by miR-203 (Figure 6c and d). In accordance with the tube-formation-suppressing effect of miR-203 in vitro, decreased tumor neovascularization was also observed in tumors formed by miR-203-overexpressing BE cells (Figure 6e). Intriguingly, 50% of the animals receiving control BE cells had macroscopically enlarged inguinal lymph nodes on the corresponding side of injection, whereas none of the lymph nodes were detectable on the other side where miR-203-overexpressing cells were injected, indicating again that miR-203 may have a role in melanoma metastasis. Hematoxylin and eosin and Melan-A staining confirmed the

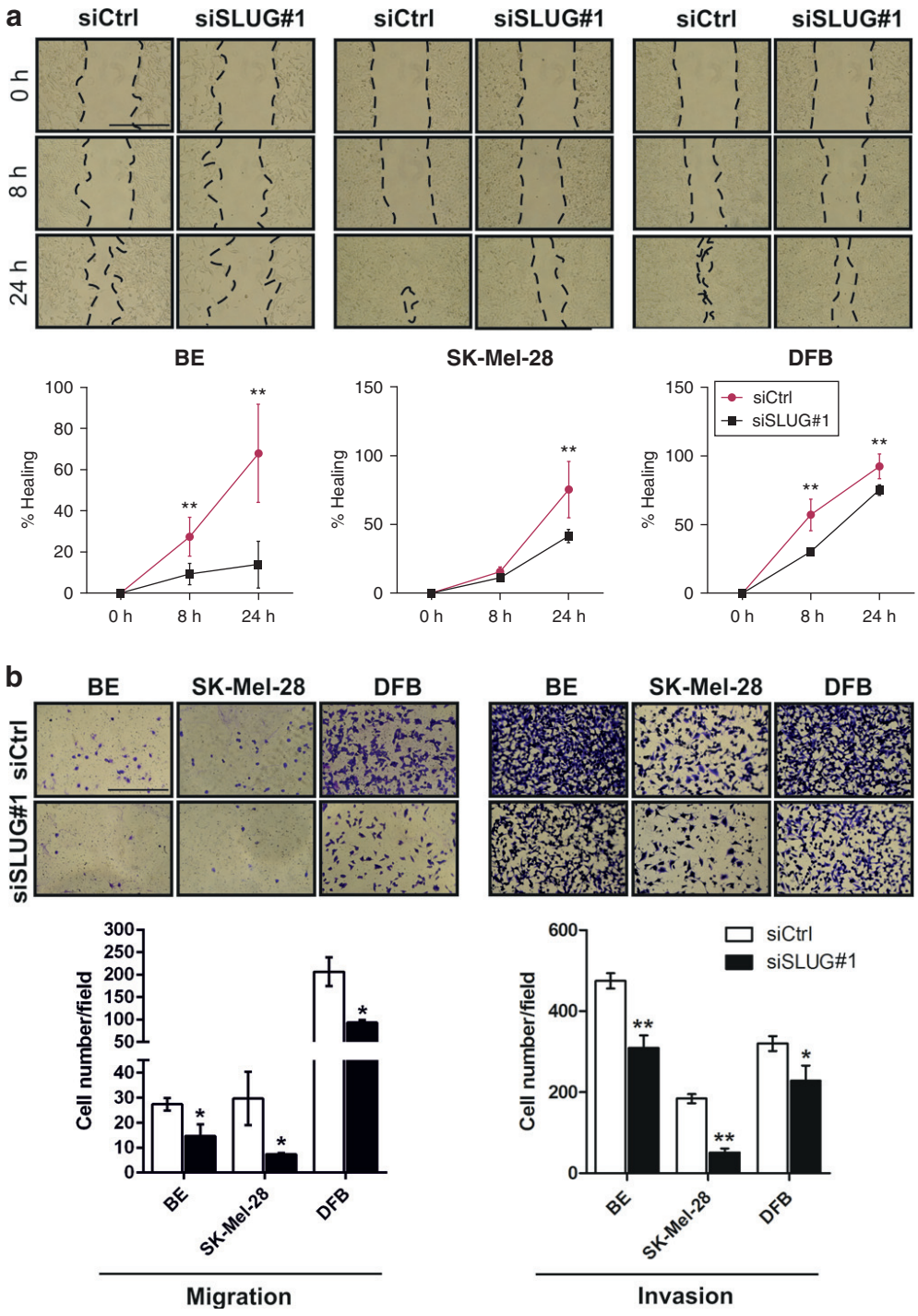



**Figure 5. Knockdown of SLUG phenocopies miR-203 overexpression in suppressing melanoma migration and invasion.** (**a**) Knockdown of SLUG (siSLUG#1) inhibits the migratory ability of melanoma cells in scratch wound healing assay. $^{**}P < 0.01$, analysis of variance. Scale bar = 1 mm. (**b**) Knockdown of SLUG (siSLUG#1) inhibits the ability of melanoma cells in a Transwell assay. Migrated and invaded cells were stained with crystal violet and counted. Scale bar = 1 mm. Data represent mean ± standard deviation from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test (two-tailed). Experiments with another SLUG-siRNA (siSLUG#2) are shown in Supplementary Figure S7.

presence of melanoma cells in the collected inguinal lymph nodes (Figure 6f).

To directly investigate the effect of miR-203 on melanoma metastasis, the miR-203-GFP-overexpressing BE cells were injected into the tail vein of NSG mice. The measurement of body weight of animals during the course of experiment revealed a significant, time-dependent decrease in the weight of animals injected with control melanoma cells (Figure 6g). In contrast, no significant change in body weight nor sign of sickness was observed in mice injected with miR-203-GFP-overexpressing BE cells (Figure 6g). The analysis of $GFP^{+}$ cells in the brain, liver, lung, and spleen revealed that miR-203 significantly reduced the number of GFP-labeled melanoma cells in lungs (Figure 6h). Staining of lung sections with hematoxylin and eosin and Melan-A further confirmed the reduced number of tumor cells in mice injected with miR-203-overexpressing BE cells (Figure 6i). Decreased SLUG expression in lung harvested from mice injected with miR-203-overexpressing BE cells was also observed by immunostaining (Figure 6i). Thus, these in vivo results corroborate the in vitro data and demonstrate a significant reduction of growth and metastatic potential of melanoma by miR-203.

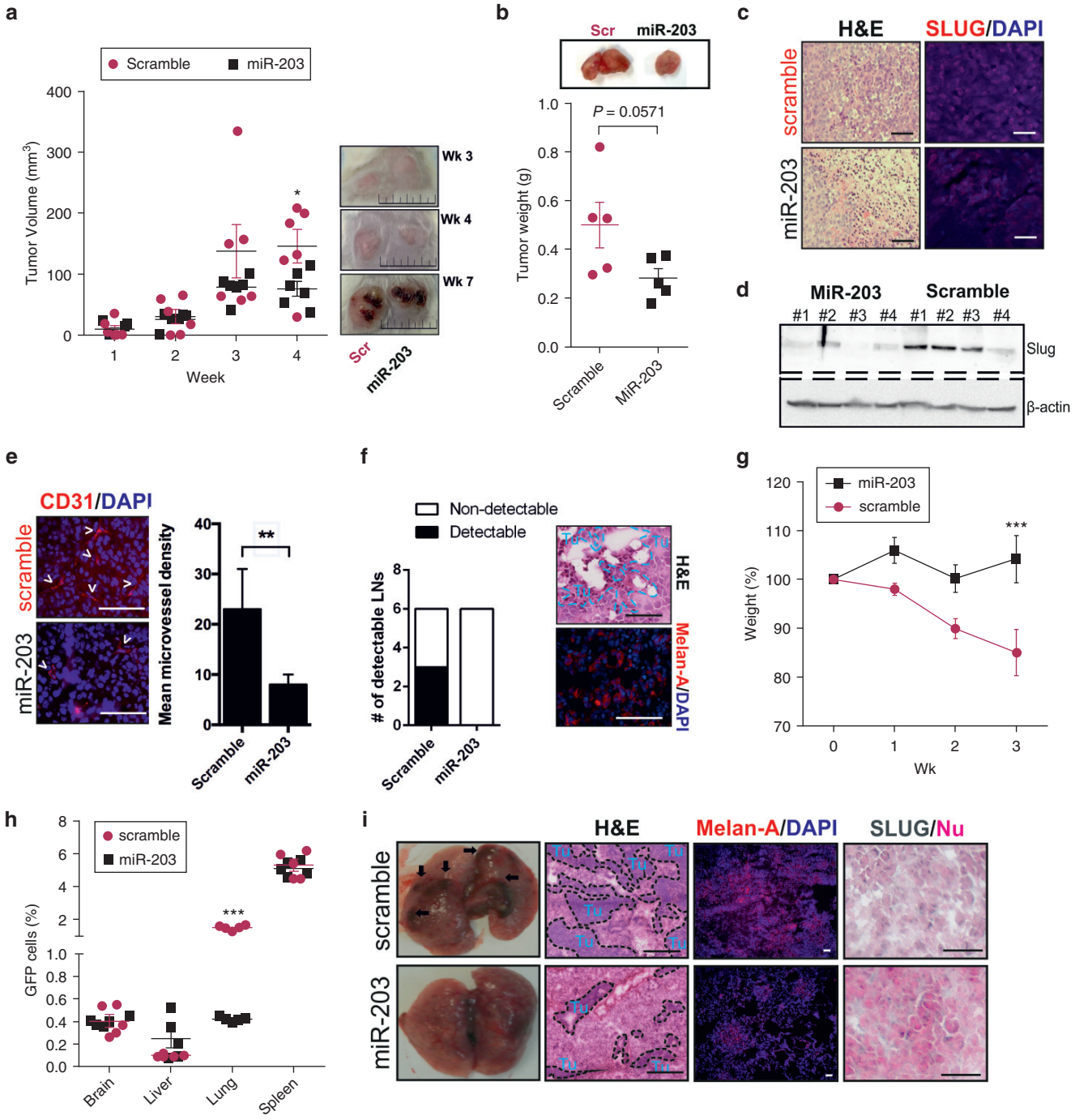



**Figure 6. MiR-203 suppresses primary tumor growth and metastasis in vivo.** (**a**) Xenograft experiment was performed in NSG mice by subcutaneous injection of stable miR-203 or control sequence-overexpressing BE cells into each flank of mice (n = 6). Tumors were monitored, and tumor volume was calculated weekly. Scale bar = 1 cm. (**b**) At the end of the experiment, tumors were excised and weighed. (**c**) H&E and SLUG staining of tumor sections from control and miR-203-overexpressing BE cells. (**d**) Western blot analysis for SLUG protein expression in tumors formed by miR-203 and control sequence-overexpressing BE cells. (**e**) Decreased angiogenesis in tumors overexpressing miR-203. Tumor sections were stained for endothelial marker CD31. The white asterisks indicate the stained vessels (left panel). The bar chart shows the mean microvessel density in miR-203-overexpressing and control xenografts (right panel). (**f**) Decrease in the number of detectable inguinal lymph nodes by miR-203 (left panel). Representative H&E and immunofluorescent staining for Melan-A identifies the presence of melanoma cells (right panel). (**g**) Metastasis study was performed by intravenous injection of miR-203 or control sequence overexpressed BE cells with GFP tag into NSG mice. Lack of weight loss in mice injected with miR-203-overexpressing melanoma cells was observed. (**h**) MiR-203 decreased the metastatic load in the lungs. GFP-positive cells from each organ were counted by a flow cytometer. (**i**) Macroscopic examination of lung metastases (leftmost panel). The black arrows indicate the metastasis nodules. H&E and immunostaining for Melan-A show decreased metastasis by miR-203-overexpressing melanoma cells. The lines denote the tumor-stroma boundary. Immunohistochemical analysis of SLUG is shown in control and miR-203-overexpressing melanoma metastases. Nu = nucleus. Scale bar = 100 μm; $*P < 0.05$, $**P < 0.01$, analysis of variance, or Mann-Whitney *U* test. H&E, hematoxylin and eosin.

## DISCUSSION

Here, we report that miR-203 is downregulated in metastatic melanoma compared with primary tumors and a low level of miR-203 in metastatic disease is associated with poor OS. Mechanistically, miR-203 suppressed cellular functions related to both early and late steps of metastasis, including melanoma cell migration, invasion, self-renewal, and angiogenesis-inducing ability in vitro, and hampered tumor growth and metastasis in vivo.

By integrating DNA methylation and gene expression data, we found that hypermethylation of the *MIR203* promoter corresponded to decreased miR-203 expression in human tumor samples. In vitro experiments with the demethylating agent 5-aza-2-deoxycytidine demonstrated that *MIR203* is regulated by DNA methylation, thus providing a mechanistic explanation for the observed suppression of miR-203 in melanoma. This finding is consistent with recent data from the genome-wide DNA methylation analysis in melanoma, which revealed that the hypermethylated CpGs in melanoma are overrepresented in genes that regulate cell differentiation as miR-203 itself is a regulator of differentiation (Schinke et al., 2010; Sonkoly et al., 2007; Yi et al., 2008). Our results indicate that alteration of *MIR203* methylation could have clinical significance. The association of low miR-203 expression and poor OS, observed in the TCGA skin melanoma cohort, verified the previous finding in a smaller cohort that downregulation of miR-203 associates with tumor thickness/tumor stage and that low miR-203 expression associates with short OS (Wang and Zhang, 2015).

Cell motility underpins metastasis, which accounts for the fatality of this disease (Palmer et al., 2011). Our results show that miR-203 suppresses a range of essential mechanisms controlling early steps of metastatic behavior such as melanoma cell motility and invasiveness. This conforms to several studies in which the inhibition of melanoma cell migration and invasion by miR-203 have been shown (Bu and Yang 2014; Chang et al., 2015). Moreover, miR-203 attenuates the self-renewal and anchorage-independent growth abilities of the melanoma cells, characteristics required for systemic survival and formation of micrometastasis. Of note, diminished tumor sphere formation by miR-203 has previously been reported to suppress proliferation and self-renewal ability in multiple cancers by repressing stem renewal factor Bmi-1 and other target genes, for example, survivin and PIDF1 (Chu et al., 2016; Yu et al., 2013; Zhang et al., 2016). Although we did not observe any change in short-term proliferation on miR-203 modulation, previous studies (Noguchi et al., 2012) and results from our colony and tumor sphere formation assays suggest that it can regulate cell survival, self-renewal, and long-term proliferation.

After successful migration to a secondary site, a cancer cell must generate a supportive microenvironment to thrive. Malignant melanoma has been well documented as an angiogenic tumor type, and new vessel formation is clearly demonstrated as an important step in disease progression (Emmett et al., 2011). We demonstrate here that overexpression of miR-203 reduced the angiogenesis-inducing ability of melanoma cells by affecting the migration and the ability to form a well-structured node in endothelial cells. One soluble factor produced by melanoma cells that is known to play an important role in controlling the phenotypes associated with melanoma progression and metastasis through the modulation of various cancer hallmarks including the induction of neovascularization is IL-8 (Singh and Varney, 2000). IL-8 has been reported as a direct target of miR-203 (Wei et al., 2013), and our results demonstrated that miR-203 reduced IL-8 expression in melanoma cells.

The in vivo xenograft experiments unveiled a role of miR-203 as a suppressor of tumor growth in melanoma. This effect is in accordance with our in vitro findings that miR-203 suppressed several important cancer hallmarks including self-renewal ability, cell migration, cell invasion, and angiogenesis-inducing capacity of melanoma cell lines. A marked decrease in the number of vessels in tumor xenografts by miR-203 was also observed.

The decreased self-renewing capacity, motility, and neovascularization promoting abilities in miR-203-overexpressing melanoma cells are not only related to primary tumor growth, but also suggest that miR-203 can impair the ability of tumor cells to leave the primary site and reinseminate at distant sites. Indeed, forced expression of miR-203 decreased metastasis to the regional lymph nodes. Moreover, our metastasis assay revealed that miR-203 significantly decreased lung metastasis. The observed suppressive effect of miR-203 on the metastatic capacity of melanoma cells is in line with our observations in patients with human melanoma showing that those with higher miR-203 levels have longer OS.

Several miRNAs are known to drive tumorigenesis at the initial stage and induce invasive activity at the metastatic stage by regulation of distinct targets (Peng and Croce, 2016). MiR-203 has been reported by us and others as a miRNA with highly tissue-restricted expression profile with preferential expression in the skin (Benaich et al., 2014; Sonkoly et al., 2007; Yi et al., 2008). MiR-203 targets several important oncogenes, for example c-Jun and c-MYC, which renders it a potent tumor suppressor (Lohcharoenkal et al., 2016; Sonkoly et al., 2012). Here we identified SLUG, a member of the SNAI family C2H2-type zinc finger transcription factors, as a direct target of miR-203 in melanoma cell lines. Silencing of SLUG recapitulated the effects of miR-203 overexpression in terms of cell motility, whereas restoration of SLUG could rescue the suppression of cell motility mediated by miR-203 in melanoma cells. SLUG has been shown to regulate a broad spectrum of biological functions including cell motility, cell invasion, metastasis, cell cycle regulation, resistance to treatments, and stem cell features in tumor cells (Perez-Mancera et al., 2005). Overexpression of SLUG has been demonstrated in numerous cancers, and elevated expression of SLUG has been associated with high histologic grade, lymph node metastasis, postoperative relapse, and shorter patient survival of these cancers (Shih et al., 2005; Shiori et al., 2006). In melanoma, it has been reported that SLUG expression is essential for metastasis of murine melanoma allografts in nude mice (Bastid et al., 2010; Gupta et al., 2005). Our results indicate an important role for SLUG in the antimetastatic effect of miR-203.

In summary, we report decreased expression of miR-203 in metastatic melanoma and that it can have prognostic

significance in patients with metastatic disease. We demonstrate that miR-203 has suppressive effect on both early and late steps of metastasis, suggesting that miR-203 delivery may have therapeutic potential in melanoma.

## MATERIALS AND METHODS

### Analysis of miRNA expression and DNA methylation in TCGA skin cutaneous melanoma datasets

Skin cutaneous melanoma miRNA expression (IlluminaHiSeq, 452 samples) and DNA methylation (Methylation450k, 476 samples) data were obtained from TCGA (https://cancergenome.nih.gov/) (The Cancer Genome Atlas Network, 2015) (download date 6 October 2016). After data filtering, expression data (read per kilobase of transcript per million) from 98 primary and 346 metastatic melanomas were analyzed using the Bioconductor/R-project using the DESeq2-package (v1.14.1) (Love et al., 2014). DNA methylation data of the *MIR203* locus from 82 primary and 130 metastatic melanomas were analyzed by linear regression using SPSS (IBM, Armonk, NY). A Kaplan-Meier survival analysis was carried out using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

Further details are available in Supplementary Materials and Methods online.

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### CONFLICT OF INTEREST

The authors state no conflict of interest.

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### AUTHOR CONTRIBUTION

WL, KDM, CC, LK, YZAU, and LZ performed experiments; WL, LP, and AP analyzed data; LP, NXL, MJ, and LG contributed new reagents/analytic tools; MS, ES, and AP designed research; and all authors contributed in writing the article.

### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2017.09.049.

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