DOI:10.1093/jnci/djt003
Advance Access publication January 30, 2013

©The Author 2013. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
For commercial re-use, please contact journals.permissions@oup.com.

ARTICLE

The Role of miR-18b in MDM2-p53 Pathway Signaling and Melanoma Progression

Altaf A Dar, Shahana Majid, Claudia Rittsteuer, David de Semir, Vladimir Bezrookove, Schuyler Tong, Mehdi Nosrati, Richard Sagebiel, James R. Miller III, Mohammed Kashani-Sabet

Manuscript received June 20, 2012; revised November 26, 2013; accepted November 29, 2012.

Correspondence to: Mohammed Kashani-Sabet, MD, Center for Melanoma Research and Treatment, California Pacific Medical Center Research Institute, 475 Brannan St, Ste 220, San Francisco, CA 94107 (e-mail: kashani@cpmcri.org).

Background Although p53 is inactivated by point mutations in many tumors, melanomas infrequently harbor mutations in the *p53* gene. Here we investigate the biological role of microRNA-18b (miR-18b) in melanoma by targeting the MDM2-p53 pathway.

Methods Expression of miR-18b was examined in nevi (n = 48) and melanoma (n = 92) samples and in melanoma cell lines and normal melanocytes. Immunoblotting was performed to determine the expression of various proteins regulated by miR-18b. The effects of miR-18b overexpression in melanoma cell lines were investigated using assays of colony formation, cell viability, migration, invasion, and cell cycle and in a xenograft model (n = 10 mice per group). Chromatin immunoprecipitation and methylation assays were performed to determine the mechanism of microRNA silencing.

Results Expression of miR-18b was substantially reduced in melanoma specimens and cell lines by virtue of hypermethylation and was reinduced (by 1.5- to 5.3-fold) in melanoma cell lines after 5-AZA-deoxycytidine treatment. MDM2 was identified as a target of miR-18b action, and overexpression of miR-18b in melanoma cells was accompanied by 75% reduced MDM2 expression and 2.5-fold upregulation of p53, resulting in 70% suppression of melanoma cell colony formation. The effects of miR-18b overexpression on the p53 pathway and on melanoma cell growth were reversed by MDM2 overexpression. Stable overexpression of miR-18b produced potent tumor suppressor activity, as evidenced by suppressed melanoma cell viability, induction of apoptosis, and reduced tumor growth in vivo. miR-18b overexpression suppressed melanoma cell migration and invasiveness and reversed epithelial-to-mesenchymal transition.

Conclusions Our results demonstrate a novel role for miR-18b as a tumor suppressor in melanoma, identify the MDM2-p53 pathway as a target of miR-18b action, and suggest miR-18b overexpression as a novel strategy to reactivate the p53 pathway in human tumors.

J Natl Cancer Inst;2013;105:433–442

Melanoma, a life-threatening malignancy with a poor prognosis in its advanced stages, accounts for 80% of skin cancer deaths. The molecular mechanisms leading to melanoma development and progression are being elucidated. Gene expression profiling analyses have led to the discovery of important melanoma-regulating genes (1,2) and disease progression markers (3). Recently, BRAF mutations have emerged as an important target for the therapy of metastatic melanoma (4).

MicroRNAs (miRNAs) are a novel class of potential biomarkers or therapeutic targets. miRNAs are non-protein-coding sequences thought to regulate more than 70% of human genes (5). Deregulation of miRNA expression has been demonstrated in many cancers (6,7), and miRNAs can function as oncogenes or tumor-suppressor genes, with important roles in cell proliferation and differentiation (8,9). Inactivation of oncogenic miRNAs (10,11) or restoration of tumor-suppressor miRNAs (12–14) has great potential for cancer treatment. To date, few miRNA expression profiling analyses have been performed in melanoma (15), and candidate miRNAs have emerged with putative roles in melanoma progression (16–20).

In this study, we examine the role of microRNA 18b (miR-18b) in melanoma. Although miR-18b can be differentially expressed in certain cancers (21), a functional role in tumorigenesis is lacking, and its targets of action are yet to be determined. This report describes, for the first time, a functional role for miR-18b in melanoma and the molecular mechanism regulating its expression and identifies the proto-oncogene MDM2 as a target of miR-18b action.

Methods

Additional methods are provided in the Supplementary Methods (available online).

Cell Culture, Plasmids, and Transfection

1205-Lu melanoma cells were purchased from Coriell Institute of Medical Research (Camden, NJ). LOX cells were a kind gift from O. Fodstad, University of Oslo, Norway. C8161.9 cells were purchased from D. Welch, University of Alabama. The DO4, WM3211, and WM278 melanoma cell lines and normal human melanocytes were obtained and propagated as described (18).

RNA, DNA, and miRNA Extraction From Tissue Samples and Cell Lines

Primary melanoma (n = 92) and benign nevus (n = 48) specimens were obtained under a protocol approved by the University of California San Francisco and California Pacific Medical Center review boards and selected from a previously described tissue microarray cohort (22). Informed consent was obtained from each patient. The demographic information about the cohort is presented in Supplementary Table 1 (available online). DNA, RNA, and miRNA were extracted as described (18).

Quantitative Real-Time Polymerase Chain Reaction

Mature miRNAs and mRNAs were assayed using the TaqMan MicroRNA and Gene Expression Assays (Applied Biosystems, Foster City, CA), respectively, as described (18). Melanomas were defined as low or high expressors of miR-18b based on expression relative to mean expression values.

Sodium Bisulfite Modification and Sequencing

Methylation status was analyzed within the 2.5-kb region upstream of the miR-18b gene. Bisulfite modification of DNA was performed using the Epi-Tect Bisulfite kit (Qiagen, Valencia, CA) following the manufacturer's protocol as described (23).

Cell Viability, Colony Formation, and Flow Cytometry

Cell viability, colony formation, and flow cytometry analysis were performed as described (18,24).

Western Blot Analysis

Western blot analysis was performed as described (18) using the following antibodies: MDM2, p53, p21, BCL-2, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); and PUMA and BCL-XL (Cell Signaling, Danvers, MA).

Luciferase Assays

The MDM2 3'-UTR region containing target sequences complementary to the miR-18b seed sequence was cloned downstream of the luciferase gene in the pMIR-REPORT luciferase vector (Ambion, Cambridge MA), named MDM2-3'UTR. Mutated MDM2 3'UTR sequences complementary to miR-18b were cloned in the same vector (named MDM2-Mut 3'UTR). Luciferase assays were performed as described (18).

Generation of Stable Transformants and In Vivo Study

1205-Lu cells were transfected with pEP Null or pEP miR-18b vectors (Cell Biolabs, San Diego, CA), selected with puromycin (1 µg/ml), and injected subcutaneously (n = 10 mice per group) as described (24). All animal care was in accordance with institutional guidelines.

Immunofluorescence

Immunofluorescence assays were performed and quantitated as described (25,26). Antibodies to *N*-cadherin, vimentin, slug, and E-cadherin (Cell Signaling, Danvers, MA) were used to detect the individual proteins.

Statistical Analysis

All quantified data represent an average of triplicate samples or as indicated. Error bars represent standard error of the mean. Statistical significance was determined by the Student t test, and two-tailed P values less than .05 were considered statistically significant. Kaplan–Meier analysis (log-rank test) was performed by using Prism 5 software (Graphpad Software Inc, La Jolla, CA).

Results

miR-18b Expression in Melanoma Cell Lines and Tissues

miRNA microarray performed on a small number of nevi (n = 5) and melanomas (n = 10) identified miR-18b as downregulated in melanomas when compared with nevi (data not shown). We validated the microarray data by miRNA quantitative real-time PCR (miR qRT-PCR) analysis on an independent cohort of nevus and melanoma tissues. miR qRT-PCR of nevus (n = 48) and melanoma (n = 92) samples indicated that miR-18b expression is statistically significantly downregulated in melanomas (Figure 1A). By Kaplan–Meier analysis, low levels of miR-18b in primary cutaneous melanoma specimens were associated with reduced overall survival (hazard ratio = 1.84, 95% confidence interval [CI] = 1.04 to 3.25; P =.04) (Figure 1B). In addition, miR-18b expression was substantially downregulated in a panel of melanoma cell lines when compared with normal melanocytes (Figure 1C). This analysis demonstrated the downregulation of miR-18b in melanoma specimens and cell lines and identified a possible prognostic role for miR-18b expression in primary melanoma.

Methylation Status of miR-18b

To understand the mechanism of miR-18b downregulation in melanoma, we performed methylation analysis of the 2.5-kb sequence upstream of miR-18b. By employing the Meth Primer software (27), a number of CpG-rich regions were observed (Figure 2A). Treatment of five melanoma cell lines with the demethylating agent 5-AZA-deoxycitidine (5 µM) resulted in 1.5- to 5.3-fold upregulation of miR-18b expression, suggesting a possible role for methylation in its suppression (Figure 2B). Primers were designed targeting methylated and unmethylated miR-18b regions, and a distinct methylated band was observed in five melanoma cell lines and 30 tumor samples (Figure 2C). By contrast, the unmethylated band in melanoma cell lines and tumor samples was either weak or absent.

Histones are subjected to a range of posttranslational enzymatic modifications. The most widely-studied modification, the acetylation of selected lysines, is associated with regulation of transcription and functional chromatin states. To determine whether covalent chromatin modifications resulted from miR-18b overexpression, chromatin immunoprecipitation analysis was performed and demonstrated the enrichment of acetylated histones H3, H4, and H3 di-methylated lysine 4 (Figure 2D), indicative of gene activation.

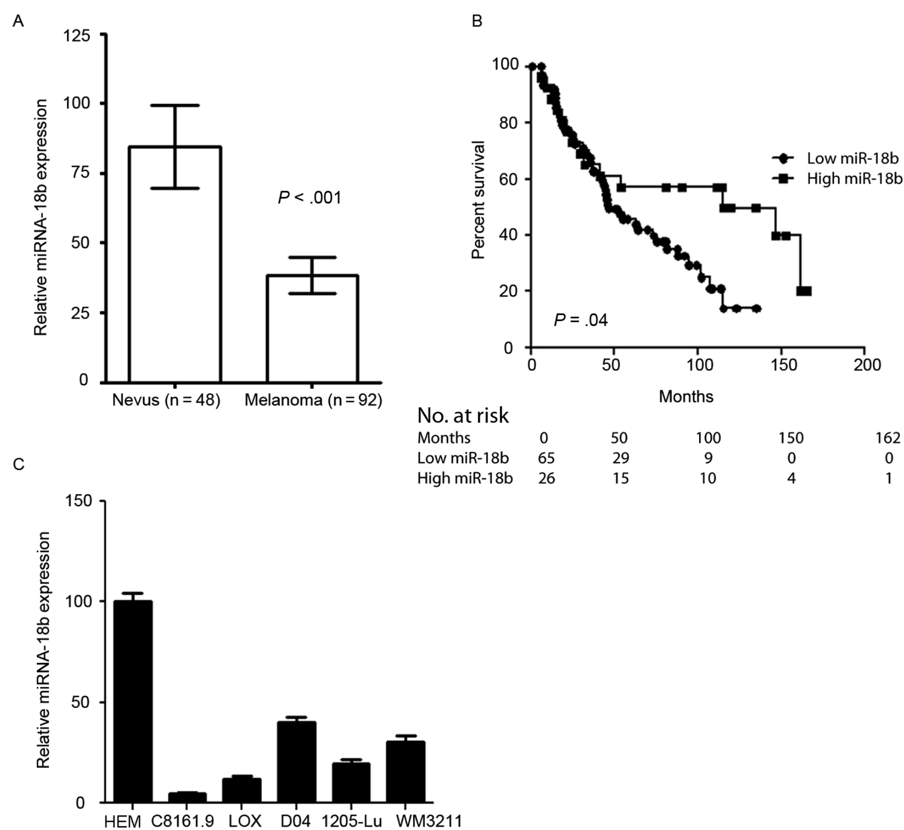

Figure 1. MicroRNA 18b (miR-18b) expression is suppressed in melanoma. **A**) MicroRNA quantitative real-time polymerase chain reaction analysis showing statistically significant suppression in miR-18b expression in a cohort of melanomas when compared with nevi. **B**) Kaplan–Meier analysis of overall survival by high or low miR-18b expression. **C**) miR-18b expression is suppressed in melanoma cell lines when compared with normal human melanocytes (HEM). $^*P$ <.05. P values were calculated by the two-sided Student t test and the log-rank (Mantel–Cox) test.

Conversely, we observed suppression of repressive chromatin modifications (2H3K9 and 3H3K9) following miR-18b overexpression (Figure 2D). These findings demonstrate that miR-18b is silenced by hypermethylation in melanoma and that its overexpression is associated with enrichment of active histone modifications.

MDM2 as a Target of miR-18b

To identify potential effectors of miR-18b, we used algorithms that predict mRNA targets and identified MDM2 as a putative target, as the seed sequence of miR-18b was complementary to the 3'UTR of MDM2 (Figure 3A). To investigate the association between expression of miR-18b and of MDM2, we determined MDM2 expression at the mRNA and protein levels in the same panel of cell lines. MDM2 expression levels were higher in melanoma cells when compared with the normal melanocyte line (Figure 3B), although the absolute level of expression varied among different melanoma cell lines. These data demonstrated an inverse association between the expression of miR-18b and that of MDM2, suggesting MDM2 as a putative target of miR-18b.

Next, the 3'UTR of MDM2 harboring the complementary sequence to the miR-18b seed sequence was cloned in a reporter plasmid vector. Transient cotransfection of the MDM2-3'UTR construct along with miR-18b into aggressive human 1205-Lu and LOX melanoma cells, which harbor wild type p53, led to a statistically significant decrease in reporter expression when compared with the control vector (Figure 3, C and D). These results indicate that the conserved nucleotides in the 3'UTR of MDM2 were responsible for miR-18b targeting in vitro. Transient miR-18b overexpression (Figure 3E) suppressed MDM2 at the protein level (by 75%), with a concomitant upregulation of the proapoptotic genes p53 (2.5-fold) and PUMA (1.5-fold) and downregulation of the antiapoptotic genes BCL2 (by 54%) and BCL-XL (by 67%) (Figure 3F). Conversely, overexpression of a mutant miR-18b construct abrogated the suppression of MDM2 or the induction of p53 expression (data not shown). miR-18b overexpression had no effect on cell survival or on expression of MDM2 and p53 in C8161.9 melanoma cells reported to harbor mutant p53 (28) (Supplementary Figure 1, A and B, available online). In addition, induction of p53 expression has been shown to sensitize tumor cells to cisplatin (29). Treatment of 1205-Lu cells with cisplatin induced the expression of miR-18b, and overexpression of miR-18b sensitized melanoma cells to cisplatin cytotoxicity (Supplementary Figure 2, A and B, available online).

Effects of miR-18b Overexpression on Melanoma Cell Growth

Transfection of 1205-Lu cells with miR-18b resulted in statistically significantly suppressed cell proliferation (Figure 4A) when compared with cells expressing a control miRNA (mean absorbance optical density of control miRNA = 0.86, 95% CI = 0.46 to 1.29 vs mean absorbance optical density of miR-18b = 0.57, 95% CI = 0.38 to 0.90; P =.002). The miR-18b-transfected 1205-Lu

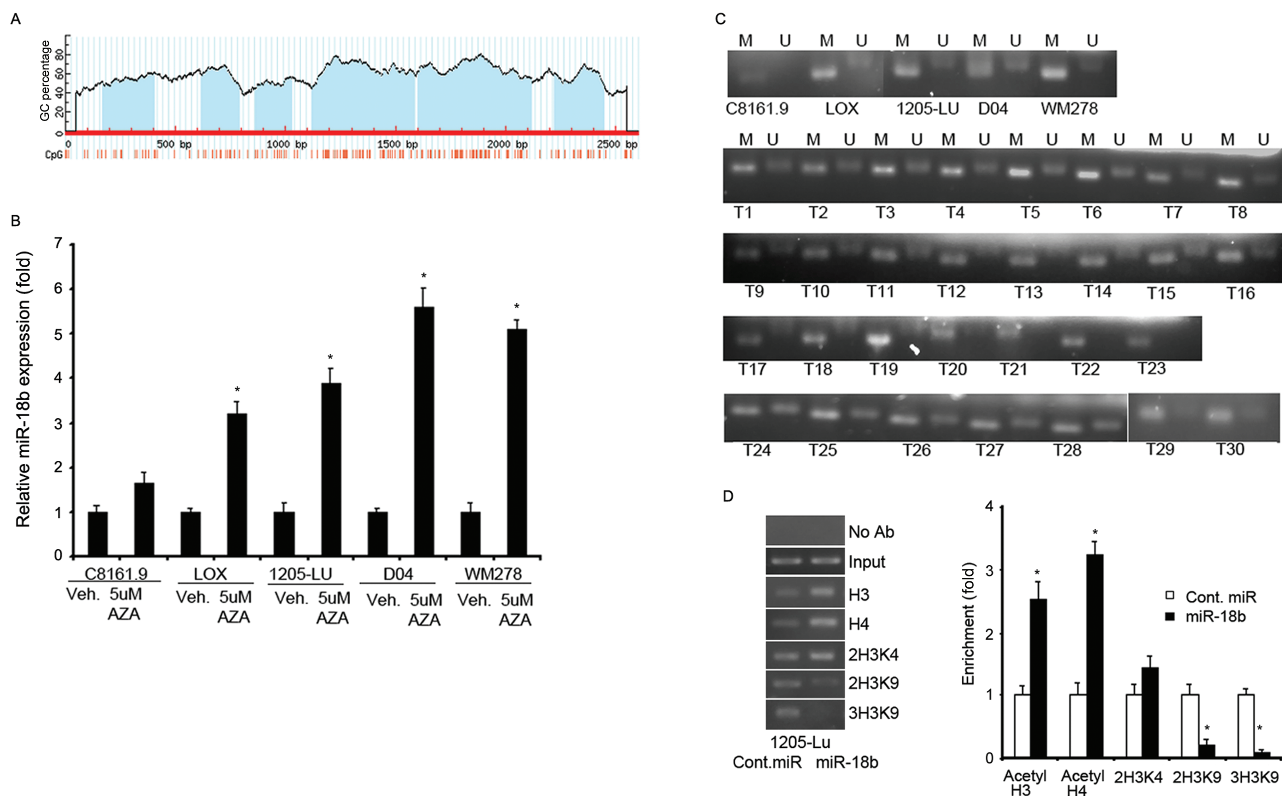

Figure 2. MicroRNA 18b (miR-18b) expression is suppressed by hypermethylation. **A**) Schematic representation of the upstream region (2500 base pair [bp]) of miR-18b analyzed for methylation, with CpG sites represented by vertical lines. **B**) miR-18b expression, as determined by miRNA quantitative real-time polymerase chain reaction, was upregulated in five melanoma cell lines (C8161.9, LOX, 1205-Lu, DO4, and WM278) following 5-AZA-deoxycitidine treatment. **C**) miR-18b promoter methylation status in melanoma cell lines and 30 tumor samples. M = amplified product with primers recognizing methylated sequence; U = amplified product with primers recognizing unmethylated sequence. **D**) Chromatin immunoprecipitation assay performed on melanoma cells after miR-18b overexpression. miR-18b overexpression resulted in the enrichment of active chromatin modifications (acetyl histone H3, H4, and methyl-2H3K4) and the suppression of repressive modifications (2H3K9 and 3H3K9). $^*P <.05$ All P values were calculated using the two-sided Student t test. Cont. miR = control microRNA.

cells showed lower colony formation ability, as both the size and number of foci was suppressed (by approximately 70%) when compared with control miRNA–expressing cells (Figure 4B). Cell cycle analysis revealed a statistically significant decrease in the S-phase of 1205-Lu cells overexpressing miR-18b when compared with control miRNA (mean of miR-18b group = 15.5%, 95% CI = 14.45% to 16.58% vs mean of control miRNA group = 8.05%, 95% CI = 7.05% to 10.01%; P =.002) (Figure 4C). miR-18b overexpression induced apoptosis in 1205-Lu cells when compared with control miRNA (mean of mirR-18b group = 2.87%, 95% CI = 2.11% to 3.89% vs mean of control miRNA group = 11.30%, 95% CI = 9.64% to 13.01%; P =.001) (Figure 4D). To confirm the effects of miR-18b overexpression, miR-18b was transfected into LOX human melanoma cells. As shown in Supplementary Figure 3, A and D (available online), similar decreases in cell proliferation, colony formation, and S-phase, along with an increase in apoptosis, were observed in LOX cells transfected with miR-18b. These results confirm the phenotypic effects of miR-18b overexpression in human melanoma cells.

The Role of MDM2 in Mediating the Effects of miR-18b

To further explore the role of *MDM2* as a target of miR-18b, we cotransfected 1205-Lu cells with miR-18b as well as MDM2 and examined effects on gene expression and melanoma cell survival. Cotransfection of miR-18b and an empty vector control resulted in suppression of MDM2, activation of the p53 pathway, and suppressed melanoma cell survival (Figure 4, E and F). These effects were largely reversed following cotransfection of the miR-18b- and MDM2-expressing vectors (Figure 4, E and F). These results indicate that the effects of miR-18b on downstream gene expression and melanoma cell proliferation are mediated largely by its inhibition of *MDM2* expression.

Effects of Stable Overexpression of miR-18b

We then generated 1205-Lu cells stably expressing miR-18b. Overexpression of miR-18b was confirmed by miRNA qRT-PCR analysis (Figure 5A). 1205-Lu cells expressing miR-18b exhibited statistically significant suppression of cell proliferation when compared with control vector-expressing cells (Figure 5B). A statistically significant decrease in colony formation (by 49%) (Figure 5C) and in S-phase was observed in miR-18b–overexpressing cells (mean of control miRNA group = 12.01%, 95% CI = 10.93% to 12.45% vs mean of miR-18b group = 4.05, 95% CI = 2.51% to 5.5%; P =.002) (Figure 5D). miR-18b overexpression resulted in a substantially increased apoptotic index (Figure 5E). In addition, MDM2 was downregulated in miR-18b–overexpressing cells (by 55%), with a concomitant increase in expression of p53 and PUMA (Figure 5F). Stable overexpression of miR-18b statistically

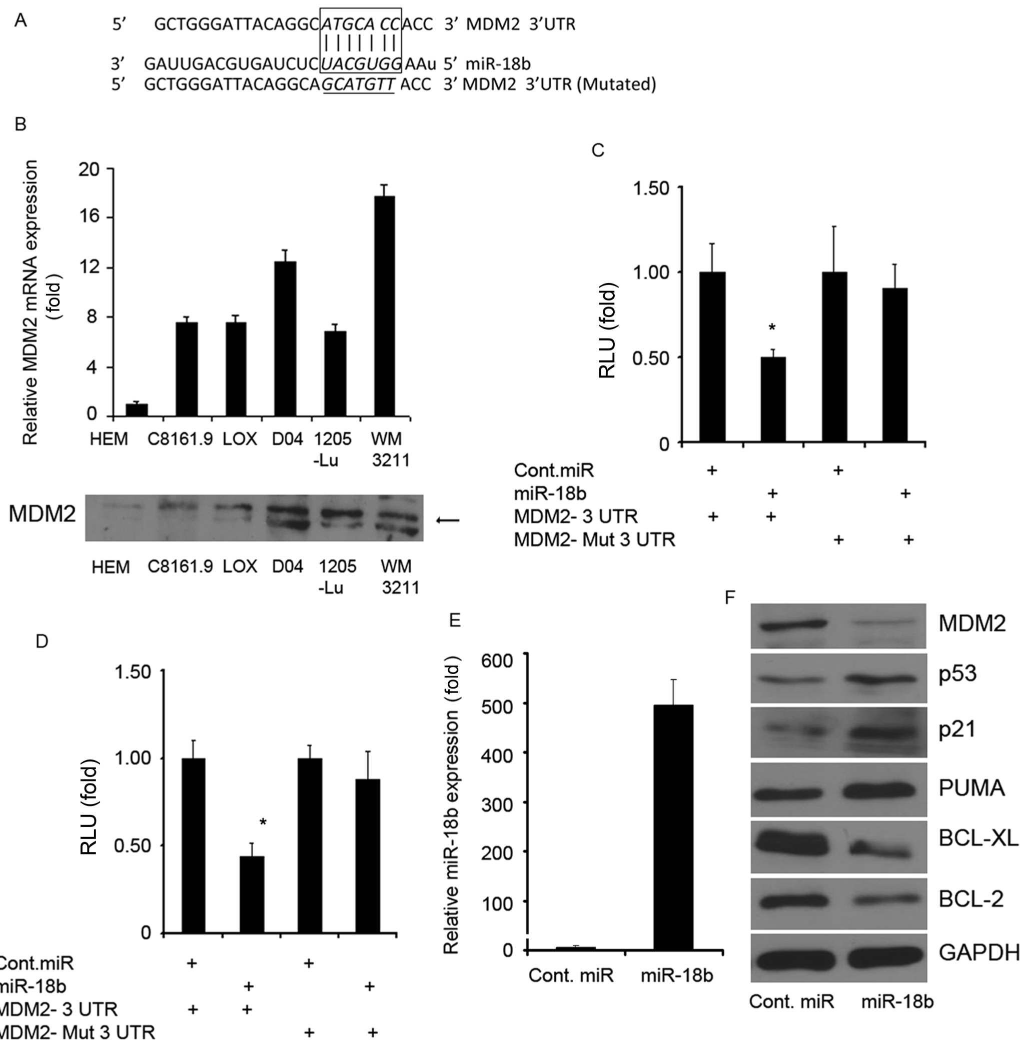

Figure 3. MDM2 as a direct target of microRNA 18b (miR-18b) and the inverse association between expression of miR-18b and MDM2 in melanoma cells. **A**) The miR-18b seed sequence is complementary to the 3′UTR of MDM2. **B**) MDM2 expression at the mRNA and protein levels in different human melanoma cell lines and normal melanocytes. **C** and **D**) Luciferase assay showing decrease in reporter activity after cotransfection of MDM2-3′UTR with miR-18b in 1205-Lu and LOX cells, respectively. The mutant 3′UTR had no effect on reporter activity. **E**) Relative miR-18b expression levels in 1205-Lu cells following transfection with miR-18b as determined by microRNA quantitative real-time polymerase chain reaction. **F**) Western blot analysis showing suppression of MDM2 and upregulation of the p53, p21, and PUMA protein levels in 1205-Lu cells after miR-18b overexpression. *P <.05 All P values were calculated using the two-sided Student t test. Cont. miR = control microRNA.

significantly suppressed tumor growth in vivo upon subcutaneous inoculation into nude mice (Figure 5G). MDM2 expression was suppressed (by 35% to 57%) in miR-18b–overexpressing tumors (Figure 5H). These results confirmed the tumor suppressor role of miR-18b and its effects on the MDM2–p53 axis.

Effects of miR-18b Expression on Melanoma Cell Migration and Invasiveness and Epithelial-to-Mesenchymal Transition (EMT)

Finally, we examined the effects of miR-18b on migration and invasion of the highly invasive 1205-Lu melanoma cell line. miR-18b overexpression statistically significantly suppressed the migratory and invasive capacity of 1205-Lu melanoma cells (Figure 6, A and B). The suppression of invasion and migration by miR-18b overexpression was confirmed in LOX cells (Supplementary Figure 3, E and F, available online). We then analyzed the potential effects of miR-18b on EMT, given its importance to the invasive and metastatic behavior of tumor cells (30). miR-18b overexpression in 1205-Lu melanoma cells resulted in statistically significant upregulation of the epithelial biomarker E-cadherin (Figure 6E). By contrast, the levels of the mesenchymal biomarkers vimentin, *N*-cadherin, and slug were reduced in miR-18b overexpressing melanoma cells (Figure 6, C–E; quantification of immunofluorescence staining is shown in Supplementary Figure 4, A–C, available online). These findings suggest that loss of miR-18b expression enhances the migratory and invasive behavior of melanoma cells and promotes EMT.

Discussion

miRNAs have large-scale effects on mammalian development through regulation of a variety of genes. Specifically, understanding the function of individual miRNAs in tumorigenesis has generated great interest. A handful of studies have assessed miRNA levels

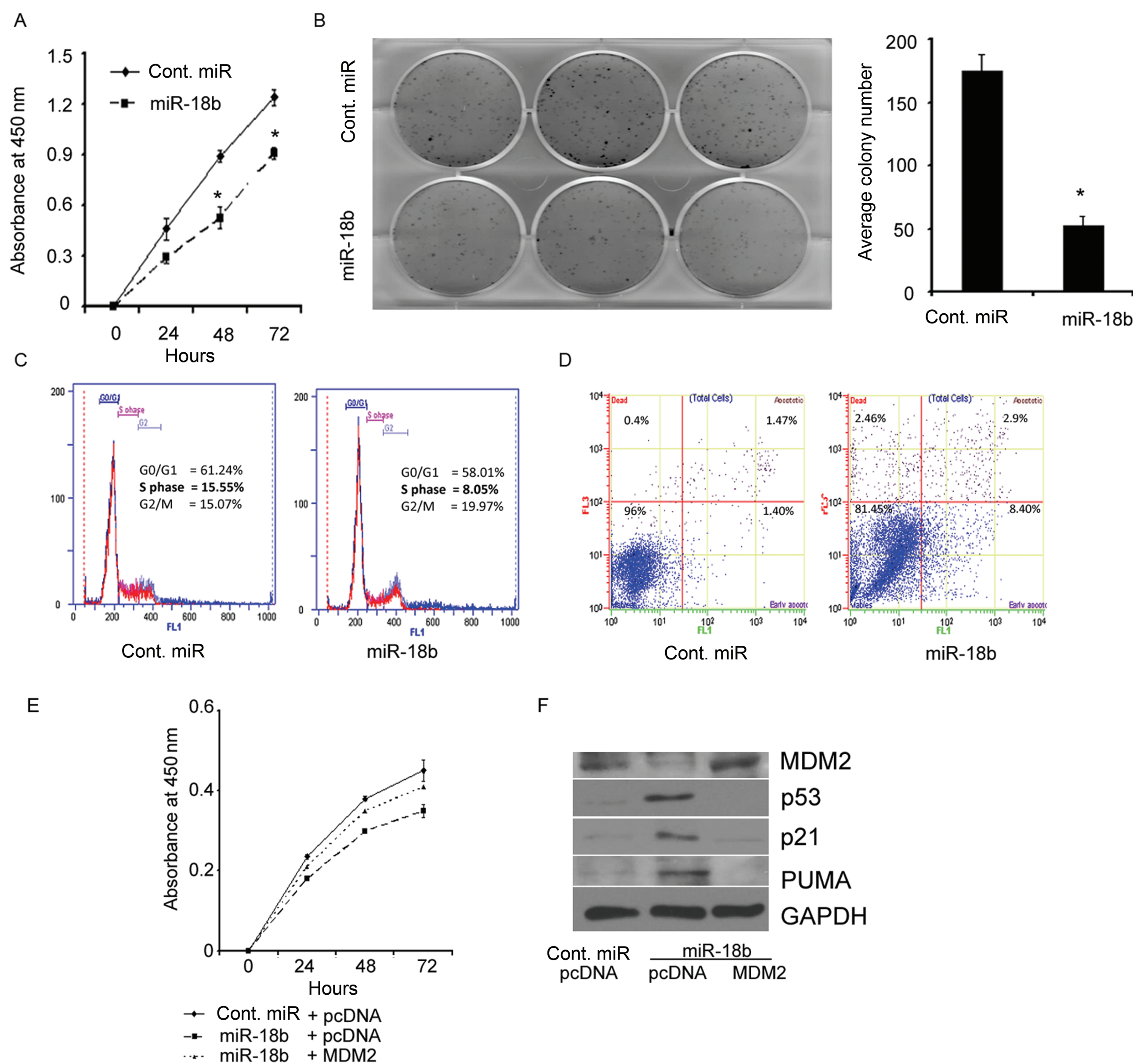

Figure 4. MicroRNA 18b (miR-18b) inhibits 1205-Lu melanoma cell proliferation and colony formation and induces apoptosis. **A**) The proliferative ability of 1205-Lu cells after miR-18b transfection is statistically significantly reduced when compared with control microRNA (Cont. miR). **B**) miR-18b overexpression statistically significantly inhibits the colony formation ability of 1205-Lu melanoma cells. **C**) Cell cycle analysis showing statistically significant decrease in the S-phase of 1205-Lu cells overexpressing miR-18b. **D**) miR-18b overexpression statistically significantly increased the apoptotic index of 1205-Lu cells when compared with control microRNA–expressing cells. **E** and **F**) Cotransfection of MDM2, along with miR-18b, reversed the suppression of cell proliferation and restored the expression levels of p53, p21, and PUMA. *$P < .05$. All P values were calculated using the two-sided Student t test.

through microarray expression profiling (31,32) or have studied the mechanisms of action of selected miRNAs in melanoma (19). For example, aberrant expression of miR-182 promotes melanoma metastasis by repressing FOXO3 and MITF (19). miR-532-3p has been shown to regulate RUNX3 (33), miR-193 has been shown to regulate Mcl-1 (34), and miRNA-137 has been shown to target MITF (16) in melanoma. miR-125 was reported to control melanoma progression by regulating c-Jun (35). miRNA-182 (19) and miR-21 (36) can act as oncomiRs by virtue of their overexpression. Finally, miR-205 (18) and miR-203 (37) suppress melanoma proliferation and induce senescence by targeting E2F1 and E2F3, respectively.

Here we report the suppression of miR-18b, identify a mechanism by which its expression is silenced, and demonstrate its functional role as a tumor suppressor in melanoma. We found miR-18b to be downregulated in melanomas when compared with nevi and in melanoma cell lines when compared with normal melanocytes. Our data showed an association between miR-18b expression and survival, suggesting its potential utility as a biomarker.

Our studies identified DNA methylation as an important molecular mechanism of suppression of miR-18b expression in melanoma. This adds to the growing body of evidence demonstrating a role for DNA methylation–mediated downregulation of miRNAs by proximal CpG islands (12,38–40). Our results revealed that

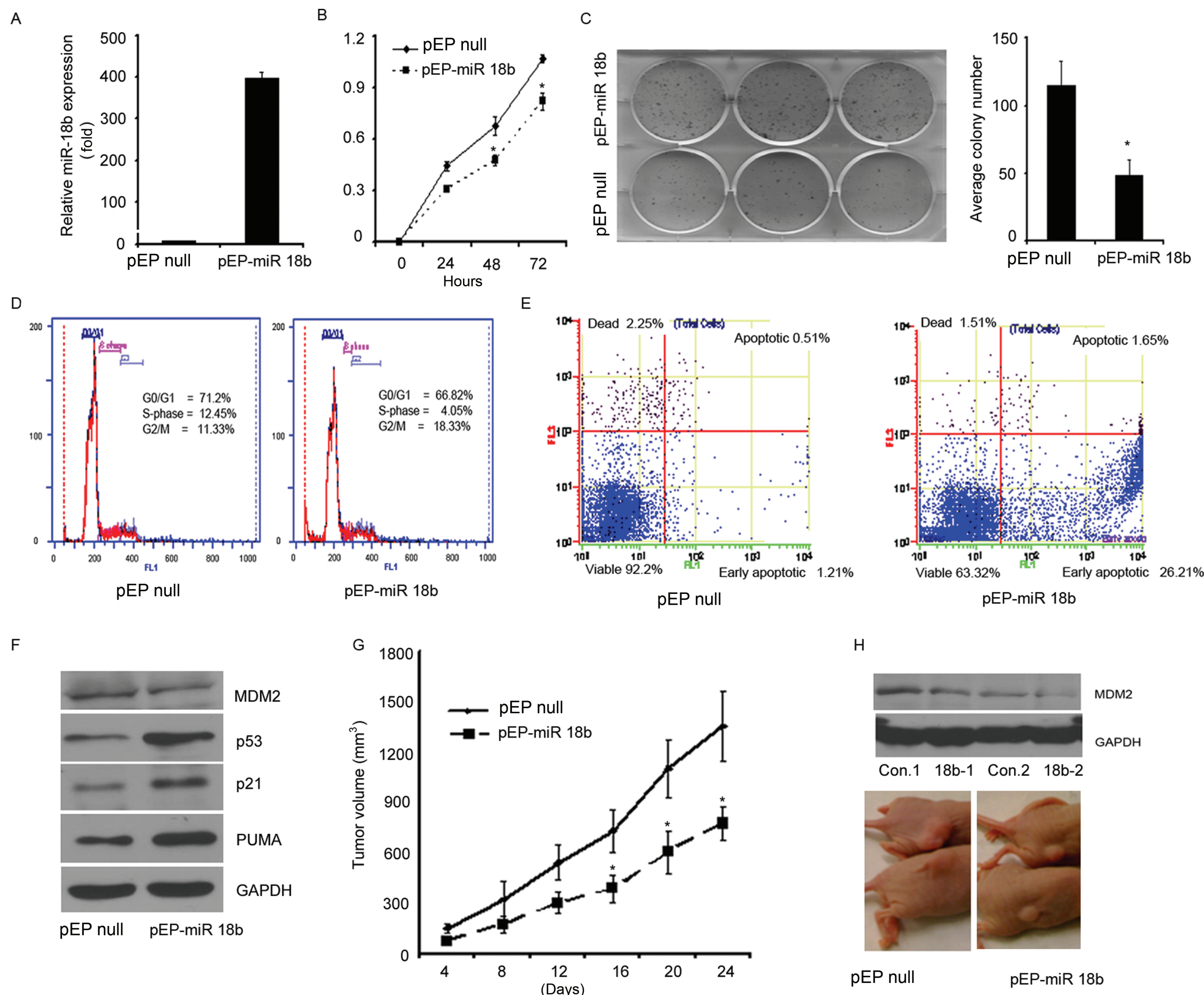

Figure 5. Stable overexpression of microRNA 18b (miR-18b) inhibits 1205-Lu cell proliferation in vitro and in vivo. **A**) Relative miR-18b expression levels in 1205-Lu cells stably expressing miR-18b as determined by microRNA quantitative real-time polymerase chain reaction. **B**) Stable overexpression of miR-18b in 1205-Lu cells statistically significantly suppressed cell proliferation. **C**) Colony formation ability is statistically significantly reduced by miR-18b in 1205-Lu cells. **D**) Cell cycle analysis showing a statistically significant decrease in the S-phase of 1205-Lu cells stably overexpressing miR-18b. **E**) Stable miR-18b overexpression induces apoptosis in 1205-Lu cells. **F**) Western blot analysis showing suppression of MDM2 and upregulation of p53, p21, and PUMA following miR-18b overexpression. **G**) Tumor volume following subcutaneous injection of 1205-Lu cells expressing miR-18b was statistically significantly reduced when compared with control microRNA–expressing cells (n = 10 mice per group). **H**) Western blots showing expression of MDM2 from subcutaneous tumors expressing control microRNA or miR-18b. **P* <.05 All *P* values were calculated using the two-sided Student *t* test.

miR-18b overexpression in melanoma cell lines resulted in enrichment of acetyl H3, acetyl H4, and 2H3K4, which are indicative of active gene expression, whereas repressive chromatin modifications (2H3K9 and 3H3K9) were suppressed. Hyperacetylation of histone lysine residues facilitates transcriptional activation (41) and induction of gene expression (42). Our study is the first to confirm the suppression of miR-18b and to identify methylation as a mechanism for its silencing in melanoma. Recently, miR-18b was reported to be highly expressed in gastric cancer (43), although no functional studies were performed to demonstrate the consequences of miR-18b expression.

An important obstacle to understanding miRNA function is the relative paucity of experimentally validated targets. In silico algorithms and functional analyses identified MDM2 as a target, and were supported by an inverse association between expression of miR-18b and MDM2 in a panel of melanoma cell lines. We demonstrated that miR-18b directly targets the 3’UTR of MDM2, as its overexpression was associated with suppression of luciferase activity. In addition, substantial downregulation of MDM2 protein levels was observed following miR-18b overexpression, indicating the posttranscriptional regulation of MDM2 by targeting its 3’UTR.

The MDM2 oncogene plays an important role in cancer progression. Suppression of MDM2 in tumor cells induces apoptosis, inhibits cell proliferation, and sensitizes tumor cells to cancer chemotherapeutic agents (44). Recent studies have identified MDM2 as an intriguing therapeutic target in melanoma (45,46). MDM2 overexpression has been correlated with poor prognosis in various human cancers (47,48), although its prognostic role in melanoma is controversial (49,50). Our results indicated that MDM2

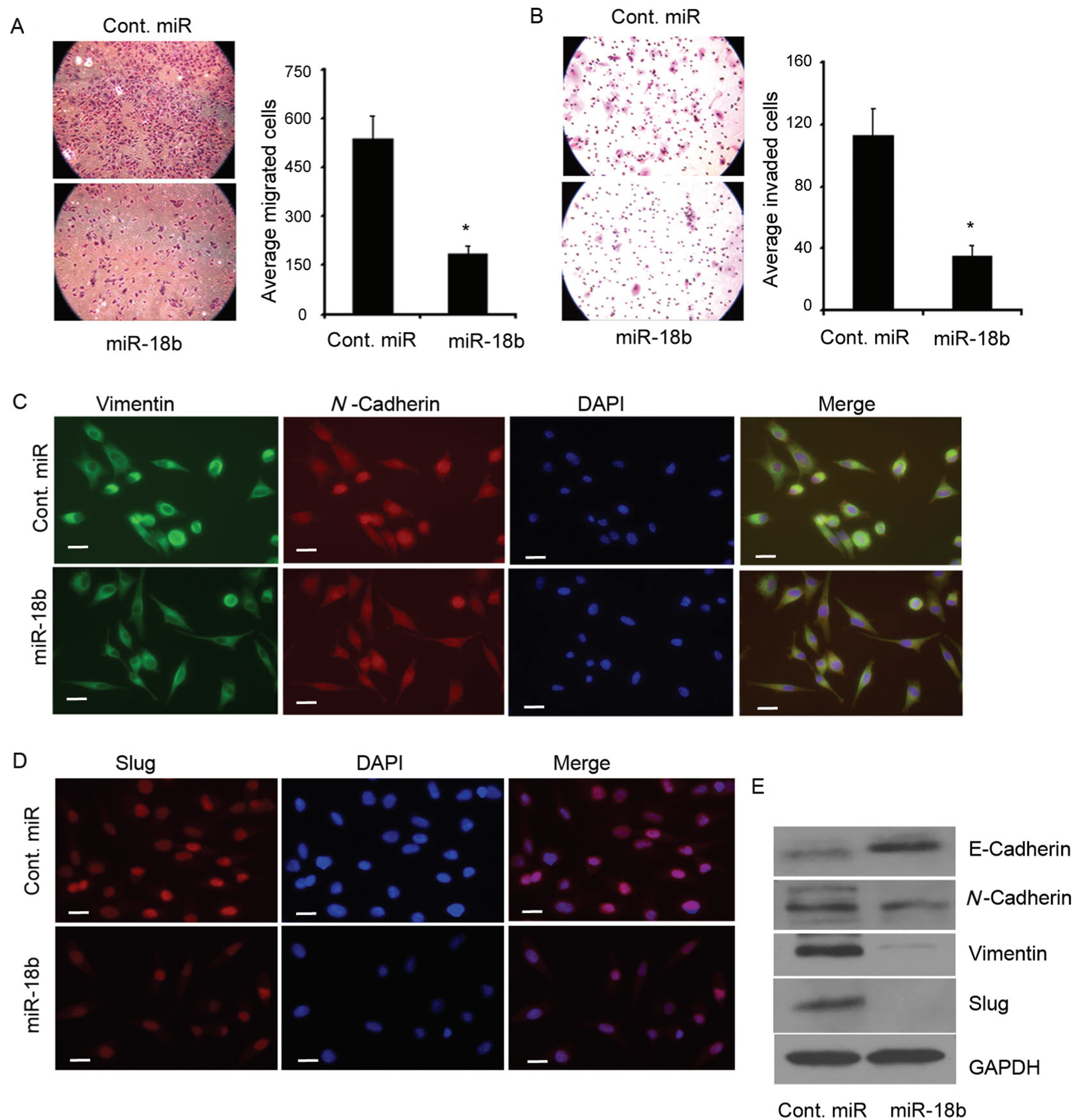

Figure 6. MicroRNA 18b (miR-18b) suppresses melanoma cell migration and invasiveness and reverses epithelial-to-mesenchymal-transition. **A** and **B**) miR-18b overexpression substantially suppressed the migratory ability and invasiveness of 1205-Lu melanoma cells when compared with control microRNA (Cont. miR)–expressing cells. **C–E**) miR-18b overexpression in 1205-Lu melanoma cells substantially suppressed expression of vimentin, *N*-cadherin, and slug, whereas E-cadherin was upregulated. Magnification ×40. Scale bar = 20 µm. *$P < .05$. All *P* values were calculated using the two-sided Student *t* test.

downregulation following miR-18b overexpression was accompanied by activation of p53 and induction of antiproliferative and proapoptotic effects. In addition, cotransfection studies showed that the tumor suppressor effects of miR-18b were mediated, in large part, by inhibition of MDM2 expression.

p53 has potent tumor suppressor activity and is inactivated in many tumors through point mutations. Melanoma is relatively unique among solid tumors in that p53 mutations have been infrequently described (51). p53 can also be inactivated through overexpression of MDM2 or loss or inactivation of $p14^{ARF}$ and $p16^{INK4a}$ (52). Of note, $p14^{ARF}$ expression was unchanged following miR-18b overexpression (data not shown), emphasizing the direct role played by miR-18b in suppression of MDM2 and activation of p53. Our results identify, for the first time, loss of miR-18b (through hypermethylation) as an important alternative mechanism to effect suppression of the p53 pathway in melanoma cells. Intriguingly, reactivation of p53 function has been actively pursued as a therapeutic approach in cancer (53). Our results suggest miR-18b overexpression as a novel, alternative approach to restore the tumor suppressive effects of p53.

miR-18b overexpression resulted in the upregulation of PUMA as well as p21 and suppressed BCL-2 and BCL-XL expression.

These changes in gene expression were accompanied by substantial inhibition of cell proliferation and induction of apoptosis in melanoma cells and were confirmed following stable miR-18b overexpression in 1205-Lu cells. In addition, in vivo studies demonstrated a statistically significant reduction in subcutaneous tumor cell growth following miR-18b overexpression. The activation of p53 and the apoptotic cascade following miR-18b overexpression suggests a potential role for miR-18b in melanoma therapy, either alone or in combination with chemotherapeutic agents such as cisplatin.

Finally, our results showed that miR-18b overexpression suppressed the migratory and invasive ability of melanoma cells. EMT has been shown to play an important role in mediating the invasion and metastasis of epithelial tumors as well as melanoma (30). Intriguingly, *MDM2* (54,55) and p53 (56), the targets of miR-18b action, have each been shown to play a role in EMT. We observed the suppression of vimentin, slug, and *N*-cadherin, with the concomitant upregulation of E-cadherin levels, following miR-18b overexpression in melanoma cells. This demonstrates that miR-18b can mediate EMT, representing an additional mechanism through which its downregulation affects melanoma progression.

Our study is not without limitations. The biomarker roles for miR-18b in melanoma diagnosis and prognosis need to be confirmed in additional, larger patient cohorts. Also, it would be of interest to examine the reciprocal expression of miR-18b and *MDM2* in melanoma tissues, as was demonstrated in melanoma cell lines. Finally, although we identified *MDM2* as a target of miR-18b in melanoma, it is possible that miR-18b targets other genes in distinct subsets of melanoma tumors.

In conclusion, this study identified novel roles for miR-18b in melanoma progression. miR-18b expression is suppressed in melanoma through DNA methylation and is correlated with survival. miR-18b overexpression results in downregulation of *MDM2*, upregulation of p53, suppression of the proliferative and invasive ability of melanoma cells, induction of apoptosis, and reversal of EMT. Finally, miR-18b overexpression may be pursued as a strategy to reactivate p53 or sensitize melanoma cells to chemotherapeutic agents.

References

1. Gutgemann A, Golob M, Muller S, Buettner R, Bosserhoff AK. Isolation of invasion-associated cDNAs in melanoma. *Arch Dermatol Res.* 2001;293(6):283–290.
2. Haqq C, Nosrati M, Sudilovsky D, et al. The gene expression signatures of melanoma progression. *Proc Natl Acad Sci U S A.* 2005;102(17):6092–6097.
3. Bittner M, Meltzer P, Chen Y, et al. Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature.* 2000;406(6795):536–540.
4. Ribas A, Flaherty KT. BRAF targeted therapy changes the treatment paradigm in melanoma. *Nat Rev Clin Oncol.* 2011;8(7):426–433.
5. Miranda KC, Huynh T, Tay Y, et al. A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. *Cell.* 2006;126(6):1203–1217.
6. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A.* 2006;103(7):2257–2261.
7. Porkka KP, Pfeiffer MJ, Waltering KK, Vessella RL, Tammela TL, Visakorpi T. MicroRNA expression profiling in prostate cancer. *Cancer Res.* 2007;67(13):6130–6135.
8. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* 2004;5(3):R13.
9. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;116(2):281–297.
10. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature.* 2010;467(7311):86–90.
11. Obad S, dos Santos CO, Petri A, et al. Silencing of microRNA families by seed-targeting tiny LNAs. *Nat Genet.* 2011;43(4):371–378.
12. Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell.* 2006;9(6):435–443.
13. Lujambio A, Ropero S, Ballestar E, et al. Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res.* 2007;67(4):1424–1429.
14. Lujambio A, Calin GA, Villanueva A, et al. A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A.* 2008;105(36):13556–13561.
15. Mueller DW, Rehli M, Bosserhoff AK. miRNA expression profiling in melanocytes and melanoma cell lines reveals miRNAs associated with formation and progression of malignant melanoma. *J Invest Dermatol.* 2009;129(7):1740–1751.
16. Bemis LT, Chen R, Amato CM, et al. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. *Cancer Res.* 2008;68(5):1362–1368.
17. Felicetti F, Errico MC, Bottero L, et al. The promyelocytic leukemia zinc finger-microRNA-221/-222 pathway controls melanoma progression through multiple oncogenic mechanisms. *Cancer Res.* 2008;68(8):2745–2754.
18. Dar AA, Majid S, de Semir D, Nosrati M, Bezrookove V, Kashani-Sabet M. miRNA-205 suppresses melanoma cell proliferation and induces senescence via regulation of E2F1 protein. *J Biol Chem.* 2011;286(19):16606–16614.
19. Segura MF, Hanniford D, Menendez S, et al. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. *Proc Natl Acad Sci U S A.* 2009;106(6):1814–1819.
20. Mueller DW, Bosserhoff AK. Role of miRNAs in the progression of malignant melanoma. *Br J Cancer.* 2009;101(4):551–556.
21. Kim TH, Kim YK, Kwon Y, et al. Deregulation of miR-519a, 153, and 485-5p and its clinicopathological relevance in ovarian epithelial tumours. *Histopathology.* 2010;57(5):734–743.
22. Kashani-Sabet M, Rangel J, Torabian S, et al. A multi-marker assay to distinguish malignant melanomas from benign nevi. *Proc Natl Acad Sci U S A.* 2009;106(15):6268–6272.
23. Majid S, Dar AA, Ahmad AE, et al. BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer. *Carcinogenesis.* 2009;30(4):662–670.
24. Dar AA, Majid S, Nosrati M, de Semir D, Federman S, Kashani-Sabet M. Functional modulation of IGF-binding protein-3 expression in melanoma. *J Invest Dermatol.* 2010;130(8):2071–2079.
25. Van Raamsdonk CD, Bezrookove V, Green G, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature.* 2009;457(7229):599–602.
26. Dar AA, Belkhiri A, El-Rifai W. The aurora kinase A regulates GSK-3beta in gastric cancer cells. *Oncogene.* 2009;28(6):866–875.
27. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics.* 2002;18(11):1427–1431.
28. Qin JZ, Stennett L, Bacon P, et al. p53-independent NOXA induction overcomes apoptotic resistance of malignant melanomas. *Mol Cancer Ther.* 2004;3(8):895–902.
29. Fujiwara T, Grimm EA, Mukhopadhyay T, Zhang WW, Owen-Schaub LB, Roth JA. Induction of chemosensitivity in human lung cancer cells in vivo by adenovirus-mediated transfer of the wild-type p53 gene. *Cancer Res.* 1994;54(9):2287–2291.

30. Alonso SR, Tracey L, Ortiz P, et al. A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. *Cancer Res.* 2007;67(7):3450–3460.
31. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res.* 2007;67(6):2456–2468.
32. Philippidou D, Schmitt M, Moser D, et al. Signatures of microRNAs and selected microRNA target genes in human melanoma. *Cancer Res.* 2010;70(10):4163–4173.
33. Kitago M, Martinez SR, Nakamura T, Sim MS, Hoon DS. Regulation of RUNX3 tumor suppressor gene expression in cutaneous melanoma. *Clin Cancer Res.* 2009;15(9):2988–2994.
34. Chen J, Zhang X, Lentz C, et al. miR-193b regulates Mcl-1 in melanoma. *Am J Pathol.* 2011;179(5):2162–2168.
35. Kappelmann M, Kuphal S, Meister G, Vardimon L, Bosserhoff AK. MicroRNA miR-125b controls melanoma progression by direct regulation of c-Jun protein expression [published online adhead of print July 16, 2012]. *Oncogene* 2012;doi:10.1038.
36. Satzger I, Mattern A, Kuettler U, et al. MicroRNA-21 is upregulated in malignant melanoma and influences apoptosis of melanocytic cells. *Exp Dermatol.* 2012;21(7):509–514.
37. Noguchi S, Mori T, Otsuka Y, et al. Anti-oncogenic microRNA-203 induces senescence by targeting E2F3 protein in human melanoma cells. *J Biol Chem.* 2012;287(15):11769–11777.
38. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J. Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. *Cancer Res.* 2008;68(7):2094–2105.
39. Iorio MV, Visone R, Di Leva G, et al. MicroRNA signatures in human ovarian cancer. *Cancer Res.* 2007;67(18):8699–8707.
40. Toyota M, Suzuki H, Sasaki Y, et al. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. *Cancer Res.* 2008;68(11):4123–4132.
41. Kristeleit R, Stimson L, Workman P, Aherne W. Histone modification enzymes: novel targets for cancer drugs. *Expert Opin Emerg Drugs.* 2004;9(1):135–154.
42. Archer SY, Hodin RA. Histone acetylation and cancer. *Curr Opin Genet Dev.* 1999;9(2):171–174.
43. Guo J, Miao Y, Xiao B, et al. Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. *J Gastroenterol Hepatol.* 2009;24(4):652–657.
44. Zhang Z, Li M, Wang H, Agrawal S, Zhang R. Antisense therapy targeting MDM2 oncogene in prostate cancer: effects on proliferation, apoptosis, multiple gene expression, and chemotherapy. *Proc Natl Acad Sci U S A.* 2003;100(20):11636–11641.
45. Verhaegen M, Checinska A, Riblett MB, Wang S, Soengas MS. E2F1-dependent oncogenic addiction of melanoma cells to MDM2. *Oncogene.* 2012;31(7):828–841.
46. Ji Z, Njauw CN, Taylor M, Neel V, Flaherty KT, Tsao H. p53 rescue through HDM2 antagonism suppresses melanoma growth and potentiates MEK inhibition. *J Invest Dermatol.* 2012;132(2):356–364.
47. Rayburn E, Zhang R, He J, Wang H. MDM2 and human malignancies: expression, clinical pathology, prognostic markers, and implications for chemotherapy. *Curr Cancer Drug Targets.* 2005;5(1):27–41.
48. Onel K, Cordon-Cardo C. MDM2 and prognosis. *Mol Cancer Res.* 2004;2(1):1–8.
49. Polsky D, Melzer K, Hazan C, et al. HDM2 protein overexpression and prognosis in primary malignant melanoma. *J Natl Cancer Inst.* 2002;94(23):1803–1806.
50. Hernberg M, Turunen JP, von Boguslawsky K, Muhonen T, Pyrhonen S. Prognostic value of biomarkers in malignant melanoma. *Melanoma Res.* 1998;8(3):283–291.
51. Sparrow LE, Soong R, Dawkins HJ, Iacopetta BJ, Heenan PJ. p53 gene mutation and expression in naevi and melanomas. *Melanoma Res.* 1995;5(2):93–100.
52. Toledo F, Wahl GM. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer.* 2006;6(12):909–923.
53. Martins CP, Brown-Swigart L, Evan GI. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell.* 2006;127(7):1323–1334.
54. Araki S, Eitel JA, Batuello CN, et al. TGF-beta1-induced expression of human Mdm2 correlates with late-stage metastatic breast cancer. *J Clin Invest.* 2010;120(1):290–302.
55. Polanski R, Warburton HE, Ray-Sinha A, et al. MDM2 promotes cell motility and invasiveness through a RING-finger independent mechanism. *FEBS Lett.* 2010;584(22):4695–4702.
56. Kim T, Veronese A, Pichiorri F, et al. p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. *J Exp Med.* 2011;208(5):875–883.

Funding

This work was supported by United States Public Health Service Grants CA114337 and CA122947 (to MKS) and by the Mary R. and Joseph R. Payden Foundation.

Note

Study sponsor(s) had no role in the design of the study; the collection, analysis, and interpretation of the data; the writing of the manuscript; and the decision to submit the manuscript for publication.

Affiliations of authors: Center for Melanoma Research and Treatment, California Pacific Medical Center Research Institute, San Francisco, CA (AAD, CR, DdS, VB, ST, MN, RS, JRM, MK-S); Department of Urology, Veterans Affairs Medical Center and University of California San Francisco, San Francisco, CA (SM).