Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor

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The highly aggressive character of melanoma makes it an excellent model for probing the mechanisms underlying metastasis, which remains one of the most difficult challenges in treating cancer. We find that miR-182, member of a miRNA cluster in a chromosomal locus (7q31–34) frequently amplified in melanoma, is commonly up-regulated in human melanoma cell lines and tissue samples; this up-regulation correlates with gene copy number in a subset of melanoma cell lines. Moreover, miR-182 ectopic expression stimulates migration of melanoma cells in vitro and their metastatic potential in vivo, whereas miR-182 down-regulation impedes invasion and triggers apoptosis. We further show that miR-182 over-expression promotes migration and survival by directly repressing microphthalmia-associated transcription factor-M and FOXO3, whereas enhanced expression of either microphthalmia-associated transcription factor-M or FOXO3 blocks miR-182's proinvasive effects. In human tissues, expression of miR-182 increases with progression from primary to metastatic melanoma and inversely correlates with FOXO3 and microphthalmia-associated transcription factor levels. Our data provide a mechanism for invasion and survival in melanoma that could prove applicable to metastasis of other cancers and suggest that miRNA silencing may be a worthwhile therapeutic strategy.

microRNA | cancer | invasion

Metastasis is a central problem in cancer, yet the mechanisms underlying a cell's ability to extravasate from the primary tumor, circulate, and invade new tissue remain poorly understood. We reasoned that melanoma, one of the most notoriously invasive neoplasia, would provide an excellent model for investigating the alterations that contribute to metastasis. Melanomas are characterized by certain well-defined genetic alterations (reviewed in ref. 1) as well as frequent chromosomal aberrations associated with tumor progression (2). Recent work has also shown that melanomas display genomic alterations involving numerous microRNA genes (3). MicroRNAs (miRNAs) are endogenous noncoding small RNAs that interfere with the translation of coding messenger RNAs (mRNAs) in a sequence-specific manner (4), often to regulate processes involved in development or tissue homeostasis (5–7). Intriguingly, dysregulation of miRNAs has been found to contribute to neoplasia (8). We decided to investigate the possible contributions of miRNA dysregulation to melanoma extravasation, migration, and invasion.

We compared the expression of miRNAs in a large cohort of melanoma cell lines with that of normal melanocytes. We found that miR-182, flanked by the *c-MET* and *BRAF* oncogenes in the 7q31–34 region that is frequently amplified in melanoma (9, 10), is highly expressed in metastatic melanoma cell lines and tumors, often in association with increased copy number. Moreover, we demonstrate that antisense-mediated repression of miR-182 inhibited invasion and induced melanoma cell death, whereas ectopic miR-182 up-regulation enhanced the oncogenic activity of melanoma cells in vitro and their metastatic potential in vivo. Analysis of human tissue samples showed that levels of miR-182 increase with tumor progression. Most intriguingly, we found that these effects of miR-182 are mediated by its suppression of transcription factors FOXO3 (a Forkhead factor) and microphthalmia-associated transcription factor (MITF). These results lead us to propose a mechanism for melanoma metastasis that might prove applicable to other neoplasia.

Results

miR-182 Is Over-Expressed in Human Metastatic Melanoma Cell Lines and Its Expression Increases with Progression From Primary to Metastatic Melanoma. To identify miRNAs whose dysregulation might participate in melanoma pathogenesis, we focused on miRNA genes located in genomic regions frequently gained or lost in advanced melanoma (3) [supporting information (SI) Fig. S1], as well as those known to be involved in metastasis (11), analyzing their expression by quantitative RT-PCR in a subset of human melanocyte primary cultures, immortalized melanocytes, and 14 melanoma cell lines (Fig. 1*A*). One miRNA, miR-182, was expressed at higher levels in most melanoma cell lines relative to melanocytes, although the degree of over-expression varied from one line to another (see Fig. 1*A*).

In situ hybridization (ISH) with miRCURY LNA probes (see *Methods*) on tissue microarrays containing human nevi ($n = 19$), primary ($n = 22$), and metastatic ($n = 58$) melanomas revealed that miR-182 levels generally correlate with melanoma progression ($P < 0.01$) (Fig. 1 *B* and *C*). In fact, the mean value of miR-182 expression in metastatic lesions (47.8 ± 5.5) was as high as 2.4-fold greater than in nevi (20 ± 6.1) and 2.1-fold greater than in primary tumors (22.6 ± 7.4). This result is particularly striking, given melanoma's characteristic heterogeneity.

miR-182 Silencing in Melanoma Cells Triggers Cell Death. To determine the effect of lowering the expression of miR-182, we transfected antisense oligonucleotides (Fig. S2*A*) into melanoma cell lines expressing different endogenous levels of miR-182. Effective miR-182 silencing strongly suppressed the invasive behavior of A375 and SK-MEL-19 cells in fibronectin invasion assays (Fig. 2*A*).

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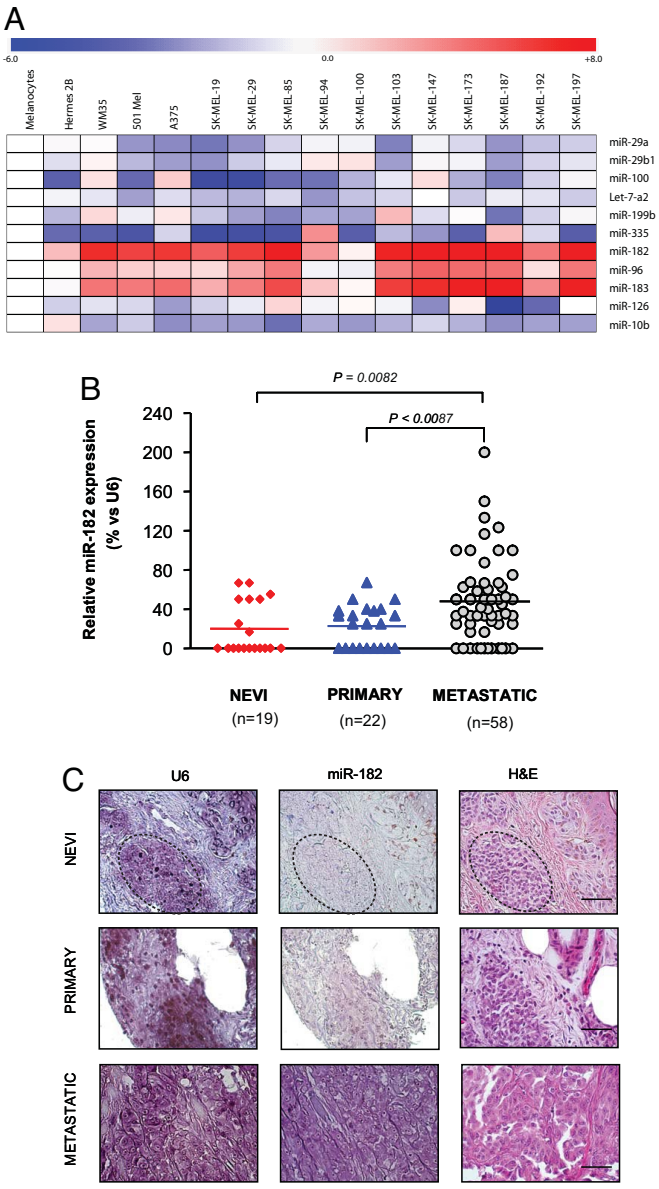

Fig. 1. miR-182 is commonly over-expressed in human melanoma cell lines and tumors. (*A*), Heat map of expression of specific miRNAs in human melanoma cell lines, assessed by qRT-PCR. A blue-red color scale (−6.0 to + 8) depicts microRNA expression levels normalized to adult melanocytes. (*B*), miR-182 in situ hybridization (ISH) on melanoma tissue microarrays. Graph represents the expression of miR-182 in nevi, primary, and metastatic melanoma human samples relative to the U6 control. The line indicates the mean value for each group (''n'' is the number of clinical specimens). (*C*) Representative pictures of miR-182 expression in melanoma tissues at distinct disease stages (*Middle*); U6 staining confirming the preservation of intact small RNAs on the same cases (*Left*); H&E-stained sections allowed the identification of tumor cells on each core (*Right*). Arrowheads point to melanocyte nest (nevus). (Scale bar, 50 μm.)

MiR-182 abrogation also reduced cell viability, determined by crystal violet staining and trypan blue exclusion assay (data not shown), to an extent that correlated with the degree of knock-down achieved. Specifically, Hoechst staining and quantification of the number of cells with condensed and fragmented nuclei indicated that anti-miR-182-treated cells undergo apoptosis (A375: 36 h, $P = 0.017$; 72 h, $P = 0.0005$; SK-MEL-19: 36 h, $P = 0.012$; 72 h, $P = 0.0010$) (Fig. 2*B*). As a control, we used the same concentration (150 nM) of anti-miR-182 on primary immortal melanocytes (Hermes), which express low levels of miR-182. Anti-miR-182 had no cytotoxic or cytostatic effect on these cells, confirming the specificity of miR-182 targeting (Fig. S3). We conclude that miR-182 may confer a survival advantage to melanoma cells or that it may be required for the retention of viability while cells acquire oncogenic properties.

hsa-mir-182 Over-Expression Enhances the Oncogenic Properties of Human Melanoma Cell Lines. To study the biological effects of altered miR-182 expression, we designed expression vectors (Fig. S2 *B–D*) to mimic the intracellular processing of miRNA genes. Notably, miR-182 over-expression did not transform human immortal melanocytes (data not shown) but did strongly stimulate the oncogenic properties of established melanoma cells. In fact, expression of miR-182 doubled the ability of SK-MEL-19 ($P = 0.019$) (Fig. 3*A*) and SK-MEL-94 cells (data not shown) to form colonies upon low-density seeding. Moreover, anchorage-independent growth of stably transduced melanoma cells was strongly increased, with both SK-MEL-19 ($P = 0.007$, Fig. 3B) and A375 melanoma cells ($P = 0.002$) (Fig. S4*A*) developing more colonies in soft agar. Therefore, miR-182 over-expression stimulates the growth of human melanoma cells under demanding culture conditions.

hsa-miR-182 Stimulates Cell Migration and Invasion in Vitro. Because miR-182 is associated with progression from primary to metastatic melanoma, we further analyzed the effects of miR-182 on the migratory and invasive behavior of melanoma cell lines. We found that miR-182 over-expressing cells were more proficient than equivalent vector-transduced cells at closing an artificial wound created over a confluent monolayer (48 h, $P = 0.007$; 72 h, $P < 0.0001$) (Fig. 3*C*) and penetrating a fibronectin coat in a dose-dependent manner (5 nM, $P = 0.02$; 50 nM, $P = 0.03$; 150 nM, $P < 0.001$) (Fig. 3*D* and Fig. S4*B*). Moreover, miR-182 up-regulation dramatically enhanced the normally poor invasive capacity of a primary melanoma cell line (WM-35), carrying low endogenous levels of miR-182 (Fig. S4*C*). To determine whether this augmented invasive behavior could be explained, at least in part, by increased cell proliferation, we compared the growth rates of miR-182 and vector-transduced cells. We found no statistically significant differences by means of trypan blue exclusion, crystal violet staining, or WST-1 proliferation assays (data not shown). We conclude that miR-182 expression confers on melanoma cells the ability, necessary for metastasis, to move through an extracellular matrix.

hsa-mir-182 Enhances the Metastatic Behavior of Melanoma Cells in Vivo. Our in vitro results led us to study the influence of miR-182 over-expression in a classic in vivo model of melanoma lung metastasis: B16F10 mouse melanoma cells. We stably transduced these cells, which carry very low mmu-miR-182 levels, with MSCV-empty or MSCV-miR-182 vectors. Upon miR-182 ectopic expression, B16 cells manifested enhanced oncogenic properties similar to those of human cells (see above), as assessed by clonability ($P < 0.0001$), growth in soft agar ($P = 0.0002$), and migration assays (4h, $P < 0.0001$; 8h, $P = 0.0023$) (Fig. S5).

We next injected vector- or miR-182-transduced B16F10 cells into the tail veins of 6-week old immunocompetent mice. Ten days after the injection, we killed the mice and dissected the lungs for macro- and microscopic histology. Lungs of B16F10-miR-182 injected mice harbored almost twice the number of macroscopic metastases (Fig. 3*E*), demonstrating that miR-182 over-expression augments the ability of melanoma cells to extravasate or seed at a distant site ($P = 0.013$).

FOXO3 and MITF-M Are hsa-mir-182 Targets in Melanoma Cells. To identify specific gene targets of miR-182 through which it might promote oncogenic behavior in vitro and in vivo, we searched public algorithms (TargetScan, Pictar, miRANDA) for theoretical target genes whose down-regulation could mediate the observed

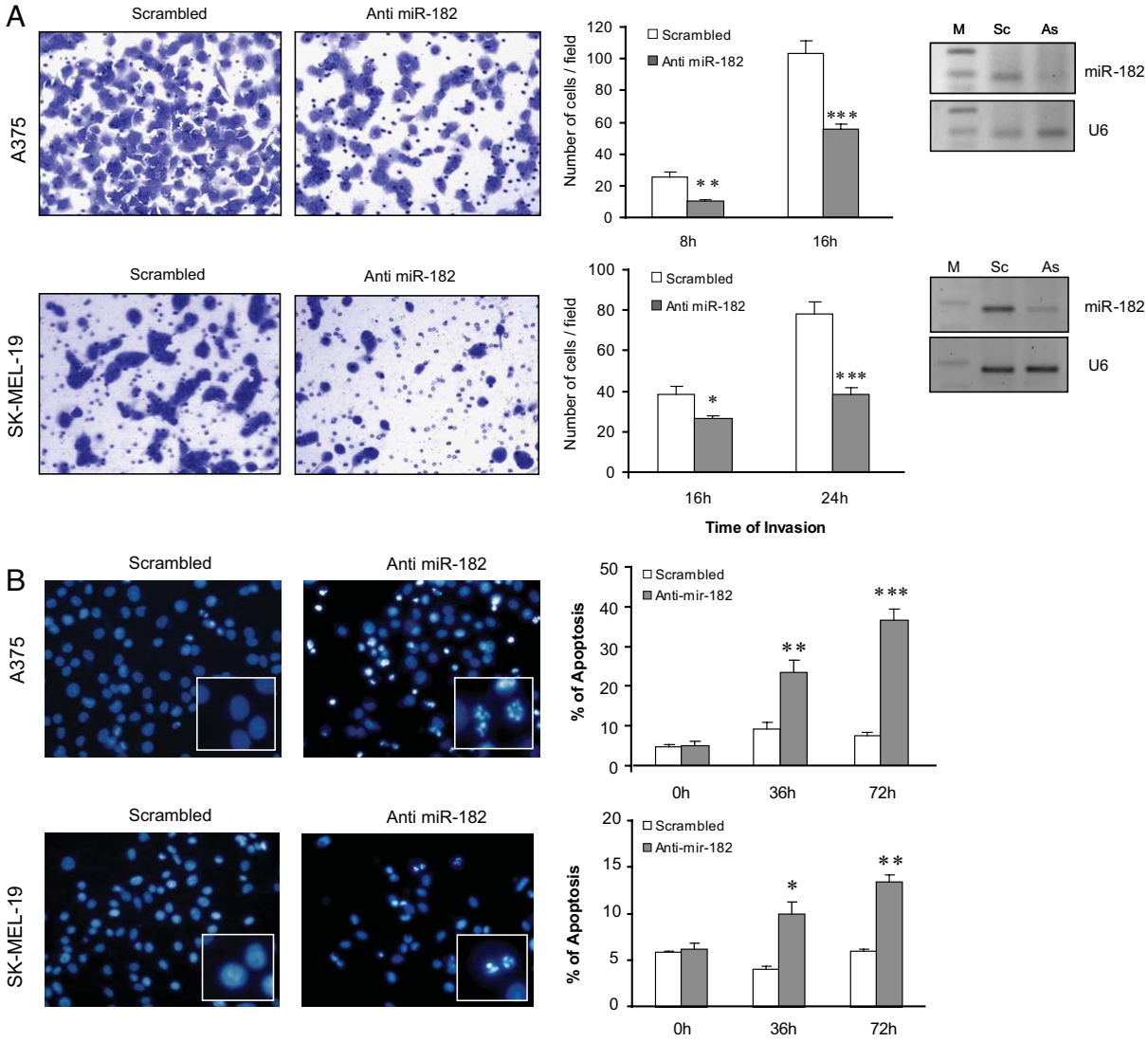

Fig. 2. Anti-miR-182 reduces tumor invasiveness and induces apoptosis. (*A*) Transwell invasion assay on A375 (*Top*) and SK-MEL-19 (*Bottom*) cells, transfected with scrambled or anti-miR-182 oligonucleotides (150 nM). (*Left*) Representative images. Graphs indicate the average number of cells per field at the indicated time points. (*Right*) RT-PCR of miR-182 in A375 and SK-MEL-19 cells transfected with the inhibitor of miR-182 (As) or a scrambled oligonucleotide (Sc), showing effective down-regulation. M, 100bp DNA ladder. (*B*) Detection of apoptosis in A375 (*Top*) and SK-MEL-19 (*Bottom*) cells transfected with scrambled or miR-182 inhibitor using nuclear Hoechst staining. Graph shows percentage of condensed or fragmented nuclei. Values in (*A*) and (*B*) are representative of 3 independent experiments ± SEM. $*P < 0.05$, $** P < 0.01$, $***P < 0.001$. Magnification in (*A*) and (*B*), 40×. Magnification of insets in (*B*), 60×.

effects of miR-182. *FOXO3 (FKHRL1)*, *FOXO1 (FKHR)*, *MITF*, *CDKN2C (p18INK4C)*, *CASP3*, *CASP2*, and *FAS* are all predicted targets, of which only *MITF* has been previously validated as subject to control by miR-182 during the development of the mouse retina (12). Western blot analysis did, in fact, show that the M isoform of MITF practically disappears in response to miR-182 up-regulation in 501mel cells (Fig. 4*A*). FOXO3, FOXO1, and caspase-2 protein levels also diminished in response to hsa-miR-182-induced expression in melanoma cells, but we observed no changes for FAS, caspase-3 or p18INK4c (see Fig. 4*A*).

To determine whether *FOXO3* and *CASP2* were direct targets of miR-182, fragments of the *FOXO3* 3′-untranslated region (3′-UTR) and *CASP2*-3′UTR containing the miR-182 binding sites (REs) were subcloned into the psiCHECK2 dual luciferase vector (Fig. 4*B*). A 1-Kb portion of *MITF-M* 3′UTR containing 3 binding sites was used as a positive control. These reporter vectors were then cotransfected with miRIDIAN hsa-mir-182 mimic oligonucleotides or microRNA mimic negative control. We observed a consistent and dose-dependent reduction of luciferase activity upon miR-182 transfection in different cell lines, particularly in those carrying low endogenous levels (see Fig. 4*B* and data not shown). Moreover, cotransfection with the complementary oligonucleotides (miRIDIAN hsa-mir-182 inhibitor oligonucleotide) restored luciferase levels (Fig. 4*C*). Further evidence that endogenous miR-182 actively blocks FOXO3 and MITF in melanoma cells came from our observation that transfection of anti-miR-182 oligonucleotides results in FOXO3 and MITF up-regulation (Fig. 4*D*). Moroever, FOXO3 induction led to Bim activation (data not shown), further emphasizing the functional impact of miR-182 on the FOXO3 pathway.

To determine whether FOXO3 and MITF are critical mediators of miR-182's role in melanoma metastasis, we down-regulated them using RNAi in melanoma cell lines. FOXO3 RNAi enhanced the invasive potential of melanoma cell lines ($P = 0.004$) (Fig. 4*E*) to a degree comparable to that caused by miR-182 over-expression. Similarly, abolition of MITF expression promoted the migratory

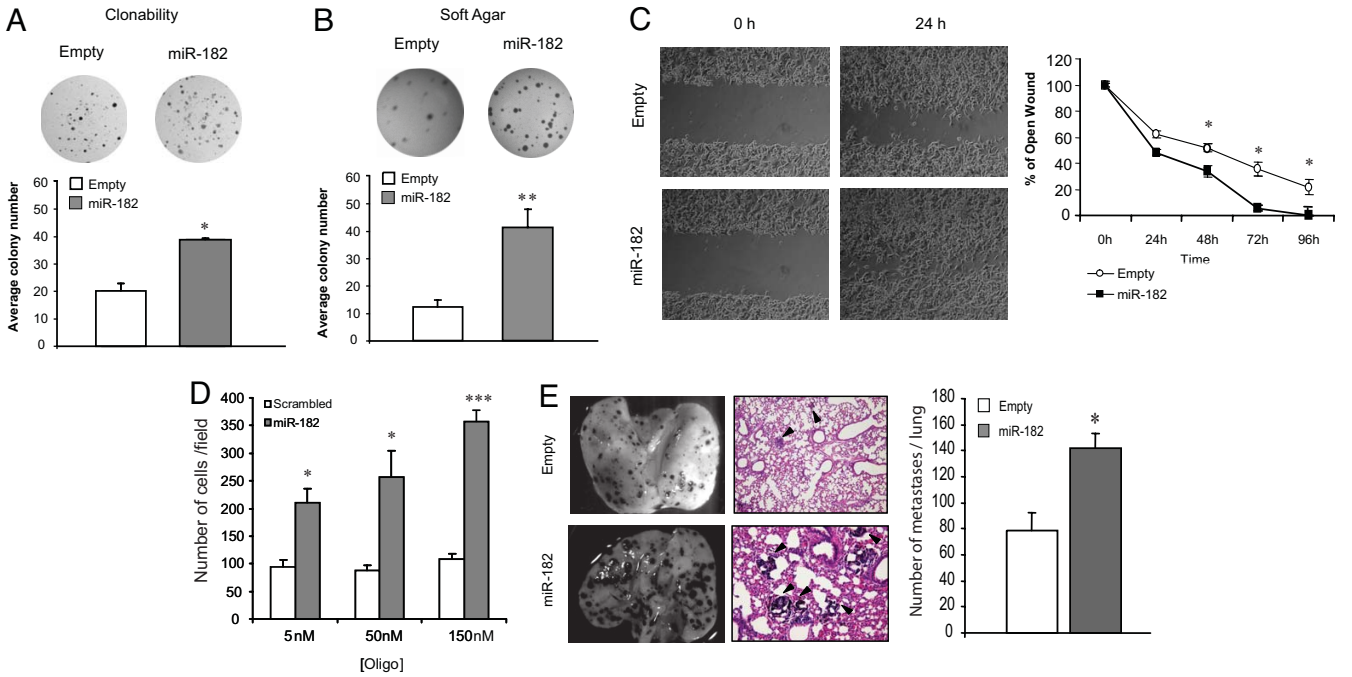

Fig. 3. miR-182 enhances melanoma oncogenic behavior in vitro and in vivo. (*A*), Colony formation assay on SK-MEL-19 melanoma cells stably transduced with empty vector (Empty) or with hsa-miR-182-expressing vector (miR-182). Graph is representative of 3 independent experiments ($n = 4$). (*B*), Growth in soft agar of transduced SK-MEL-19 cells ($n = 4$). (*C*), Wound-healing assay on SK-MEL-19 stably transduced with either empty vector (Empty) or hsa-miR-182 (miR-182). Pictures were taken every 24 h. Graph represents the width of the remaining open wound calculated in relation to time 0 separation ($n = 12$). (*D*) Transwell invasion assay of SK-MEL-19 transfected with increasing doses of miR-182 or control oligonucleotides ($n = 3$). (*E*) In vivo metastasis assay with B16F10 mouse melanoma cells injected through the lateral tail vein of C57BL/6J mice ($n = 6$ per group). Macroscopic pictures of mouse lungs, 10 days after inoculation. H&E-stained sections of lung metastases (*Right*; magnification 20×). Arrowheads point to metastatic foci. Histogram with the quantification of large lung metastases. For every assay in (*A*) to (*D*), a representative experiment of a triplicate is shown along with SEM. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

behavior of melanoma cells to an extent similar to that seen with miR-182 over-expression (≈5-fold; $P = 0.0001$) (see Fig. 4*E*). Finally, concomitant over-expression of miR-182 and either FOXO3 or MITF-M abolished the stimulatory effect of miR-182 on the invasive capacity of melanoma cells (Fig. 4*F*), demonstrating that FOXO3 and MITF repression are necessary effectors of miR-182.

miR-182 Expression Inversely Correlates with FOXO3 and MITF Levels in Metastatic Melanoma. We analyzed the levels of FOXO3 and MITF by immunohistochemistry (IHC) staining in the same collection of human nevi, primary, and metastatic melanomas in which miR-182 levels had been assessed by ISH (see Fig. 1). Metastatic melanoma showed significant correlation between high miR-182 and low MITF (52.2% of cases; $P < 0.0001$) and low FOXO3 levels (65.2% of cases; $P < 0.0001$) (McNemar's Test) (Figs. 5 and S6). This reciprocal correlation confirms the relevance of this regulatory mechanism to human melanoma progression.

The miR-183–96-182 Cluster Is Commonly Amplified in Melanoma. miR-182 has been reported to form a gene cluster with two adjacent miRNAs (miR-96 and miR-183) (12, 13) that share highly homologous 5′-seed sequences (Fig. S7). This miRNA cluster is located in the 7q31–34 locus, which is frequently amplified in advanced human melanoma (9, 10) (see Fig. S1) and contains the *c-MET* and *BRAF* oncogenes. In fact, copy number analysis performed by qPCR on melanoma cell lines confirmed a correlation between miR-182 levels and gene amplification (see Fig. 1*A*, Fig. S1 and Fig. S8*A*): 7 out of 13 cell lines with elevated miR-182 had increased copy number.

Discussion

Our results show that miR-182 over-expression in established melanoma cells promotes cell viability at low density, stimulates invasion and migration in vitro, and exacerbates metastatic potential in vivo. Moreover, our data provide several lines of evidence that these effects require suppression of the FOXO3 and MITF transcriptional networks. It is interesting to note that strong evidence of altered miRNAs facilitating invasion and metastasis has been recently presented for breast carcinomas (11, 14).

The 7q31–34 locus, frequently amplified in melanoma (9, 10), includes the *BRAF* and *c-MET* oncogenes (see Fig. S7*A*). *BRAF* mutations are often accompanied by gene amplification (15, 16); *C-MET* over-expression (17) has often been attributed to chromosomal gains, raising the possibility of an additional target of 7q copy gains in melanoma (10). Therefore, it is plausible that the observed up-regulation of miR-182 results from regional amplification or chromosome 7 polysomy (18). Indeed, our SNP array and qPCR analyses on cell lines showed a correlation between miR-182 levels and gene copy number (see Fig. 1*A*, Fig. S1, and Fig. S8*A*). Additional studies are required to elucidate other molecular mechanisms leading to abnormal expression of this miRNA cluster.

Our observation that miR-182 represses the M (melanocyte-specific) form of MITF runs counter to the oncogenic role assigned to MITF amplification in human melanoma (19), but paradoxically, there have been reports of MITF over-expression having tumor-suppressor activities as well (reviewed in refs. 20–22). However, direct measurements of MITF during melanoma progression by ourselves and others (23–25) suggest that MITF is more commonly down-regulated in advanced melanoma. In addition to its role in differentiation, MITF represses cell proliferation by activating the expression of cell-cycle inhibitors, such as $p16^{INK4a}$ (26) and $p21^{Cip1}$ (27). Furthermore, MITF has been directly linked to the control of cell migration by regulating DIA1, which coordinates the actin cytoskeleton and microtubule networks at the cell periphery (28). Accordingly, MITF down-regulation by shRNA enhanced the in vivo metastatic behavior of B16 cells (Fig. S9). These properties support the notion that MITF repression contributes to miR-182's

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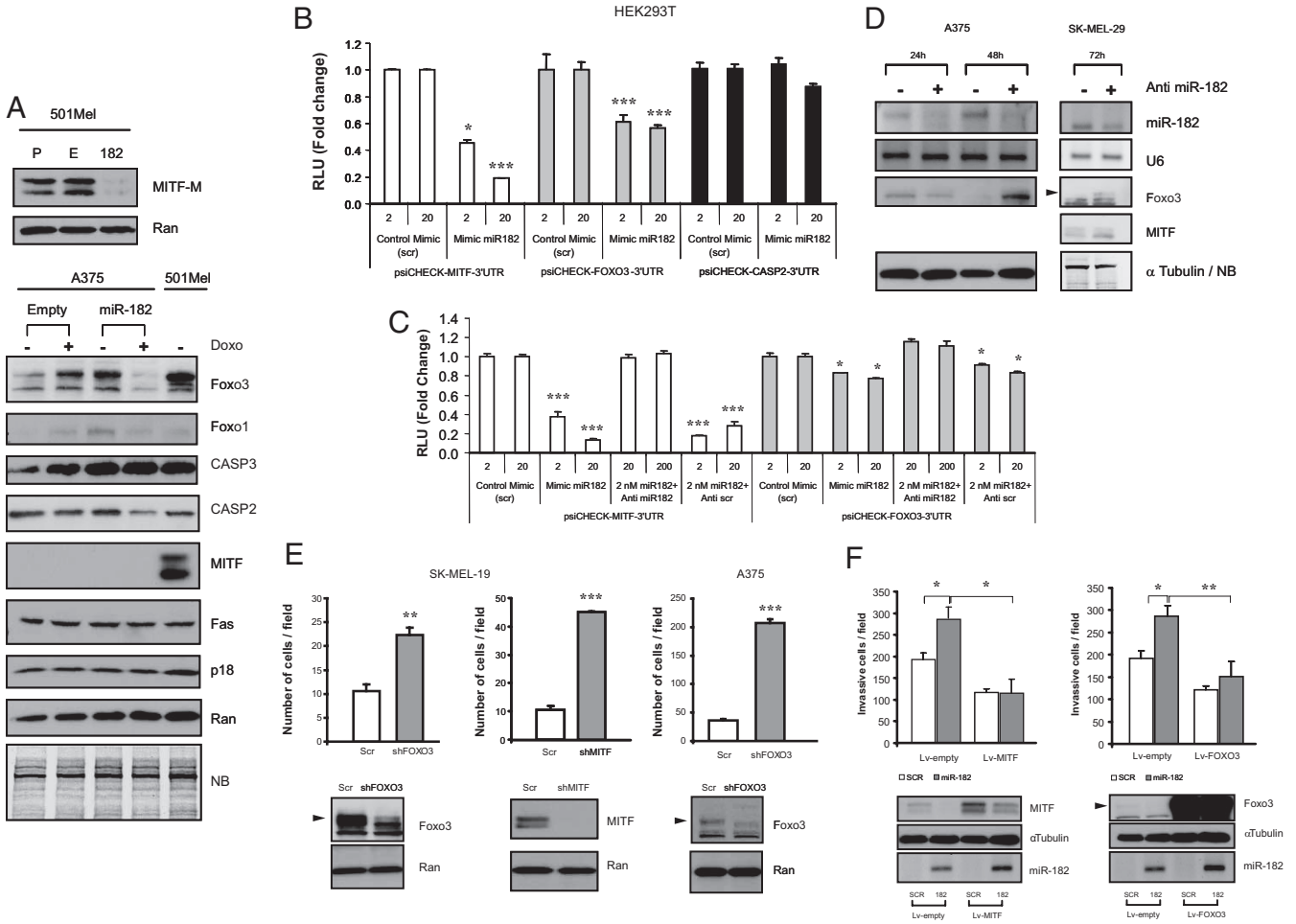

Fig. 4. FOXO3 and MITF are direct and critical miR-182 targets. (*A*) Western blot analyses of theoretical miR-182 targets in melanoma cell lines, upon vector- and miR-182 exogenous expression (P, parental; E, empty vector). (*B*) Luciferase assay on HEK293T transfected with the indicated reporters and increasing amounts of miR-182 mimic oligonucleotides (2, 20 nM) or in (*C*), cotransfecting mimic miR-182 (2 nM) with increasing amounts of scrambled or anti-miR-182 (20, 200 nM), as indicated. (*D*) miR-182 and U6 levels by RT-PCR (*Upper*) and FOXO3 and MITF Western blots (*Lower*) in A375 cells and SK-MEL-29 cells transduced with anti-miR-182. (*E*) Invasion assays on A375 and SK-MEL-19 cells transduced with empty vector or shRNAs against FOXO3 or MITF. Efficacy of RNA interference is shown by Western blot. (*F*) Invasion assays on SK-MEL-19 cells coinfected with lentiviral vectors carrying miR-182 and *MITF* or FOXO3 cDNAs. Alpha-tubulin and Ran are shown as loading controls. Arrowheads point to FOXO3-specific band. Values in (*B*), (*C*), (*D*), and (*E*) are representative of 3 independent experiments ± SEM. $*P < 0.05$, $** P < 0.01$, $***P < 0.001$.

prometastatic effects. Nevertheless, ectopic miR-182 expression was able to exacerbate the oncogenic behavior of melanoma cells that express very low levels of MITF (e.g., A375) (see Fig. 4*A*), indicating that miR-182 has effects independent of MITF. Alternatively, the absence of MITF could simply cooperate with the concomitant attenuation of other miR-182 targets.

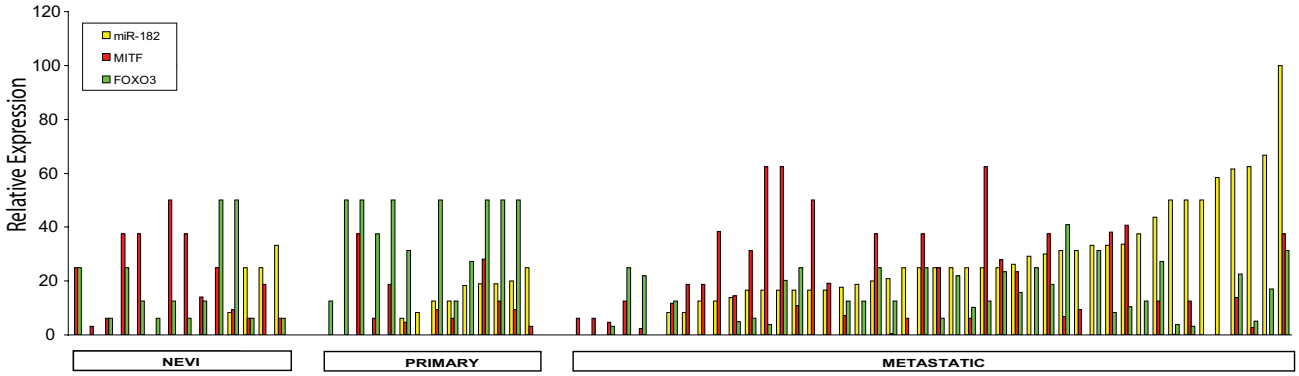

Fig. 5. FOXO3 and MITF levels inversely correlate with miR-182 expression in a manner associated with tumor progression. Relative levels of miR-182, FOXO3, and MITF levels measured by ISH and IHC in a collection of human nevi, primary, and metastatic melanoma, organized by increasing miR-182 levels. See Fig. S6 for additional information.

We also found FOXO3 to be a direct miR-182 target whose down-regulation mimics the ability of miR-182 to favor survival and cellular migration. FOXO3 or FKHRL1 controls numerous apoptotic and cell cycle regulators (29) and has a crucial role in the development and progression of cancer (30). Recently, a role for FOXO3 in melanoma cell survival has been proposed (31), and an apoptotic response was observed in melanoma cell lines transduced with a FOXO3 triple mutant (32). Among other targets, FOXO3 regulates the expression of Bim, a Bcl-2 family member (33) whose down-regulation by RNAi inhibits anoikis (34). Therefore, we hypothesize that FOXO3 repression acts coordinately with MITF down-regulation by reducing Bim levels and thus increasing the viability of migrating cells.

Our expression analyses on human samples reinforced the relevance of miR-182 regulation of FOXO3 and MITF in melanoma by revealing an inverse correlation between expression of this miRNA and its targets in over half of the metastatic cases. Considering the characteristic heterogeneity of melanoma, and that both FOXO3 and MITF are regulated by additional mechanisms, a statistically significant association with miR-182 is especially remarkable. Yet the ability of FOXO3 and MITF over-expression to individually counteract miR-182's pro-invasion effects unequivocally shows the importance of this inverse relationship in melanoma metastasis.

In conclusion, our study suggests a model of tumor progression in which elevated miR-182 expression, subsequent to gene amplification or other mechanisms that induce over-expression, promotes melanoma aggressiveness, likely by triggering the switch between the proliferative and the invasive stages (Fig. S8*B*). According to this model, different signaling pathways or genetic alterations, including over-expression of miR-182, would promote metastasis by converging onto FOXO3 and MITF inactivation. These results suggest that miR-182 and its downstream effectors could prove to be useful prognostic markers and/or therapeutic targets in melanoma.

Methods

Cell Lines. SK-MEL-19, -29, -85, -94, -100, -103, -147, -173, -187, -192, and -197 melanoma cell lines were kindly provided by Alan Houghton (Memorial Sloan–Kettering Cancer Center) and Hermes cells by Dorothy Bennett (University College London); 501mel were obtained from Yale University. HEK293T and A375 cells were acquired from the American Type Culture Collection. The B16F10 mouse melanoma cell line and the primary human melanoma cells WM35 were a gift to I.O. from Meenhard Herlyn (Wistar Institute). Human melanocytes were purchased from Lonza (adult and neonatal) and Yale University. Melanocytes, Hermes, and SK-MEL and WM cell lines were cultured as described (35).

In Vivo Metastasis Assay. Subconfluent B16F10 cells (MSCV-PIG and MSCV-PIG-miR182, or pGIPZ-Scr and pGIPZ-shMITF) were injected intravenously (1.5×10^5/100 μL per mouse). After 10 days, mice were killed, their lungs removed and fixed, and the number of isolated and discrete pigmented lung surface lesions counted on each lobe of every specimen. Tissues were paraffin-embedded, and 5-μm sections were H&E-stained. Experiments were conducted under protocol #061108–02 approved by New York University Institutional Animal Care and Use Committee.

In Situ Hybridization and Immunohistochemistry. Tissue microarray slides contained triplicates of 19 nevi, 22 primary, and 59 metastatic human melanomas. Hybridizations were performed overnight at 52°C after the addition of 50 nM DIG-labeled locked nucleid acid (LNA)-based probes specific for mir-182 and U6 (Exiqon). Alkaline phosphatase activity was detected using BM Purple AP Substrate (Roche). Scoring of melanoma cores was restricted to the tumor cells. ISH results were semiquantitatively graded according to the intensity of staining and scored from 1 to 4, and then normalized to U6 levels. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue using rabbit anti-FOXO3a (Cell Signaling) and mouse anti-MITF (Sigma). The complex was visualized with Naphthol-AS-MX phosphatase and Fast Red complex, and nuclei counterstained with hematoxylin. Immunoreactivity was scored by intensity (0–4) and percentage of positive cells (0–4). Relative expression was obtained by multiplying intensity by percentage, and expressed as a percentage of the maximum possible value.

Clinical Specimens. Melanoma specimens were collected at the time of surgery. Approval to collect specimens was granted by the Institutional Review Board protocol number #10362, ''Development of an NYU interdisciplinary Melanoma cooperative Group: A clinicopathological database''.

For all other *Methods,* please refer to *SI Methods*.

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