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Epigenetics, MicroRNAs, and Carcinogenesis: Functional Role of MicroRNA-137 in Uveal Melanoma

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Purpose. MicroRNAs (miRNAs) can contribute to tumorigenesis by acting as either oncogenes or tumor suppressor genes. The authors' previous studies on miR-34a showed that miRNA can influence the growth of uveal melanoma cells. In this study, they investigated the role of miR-137 in the pathogenesis of uveal melanoma.

Methods. Real-time RT-PCR was used to screen the expression levels of miR-137 in uveal melanocytes and uveal melanoma cell lines. Cell proliferation was examined by MTS assay and cell cycle was analyzed by flow cytometry. The target genes of miR-137 were predicted by bioinformatics and confirmed using a luciferase reporter assay. The expression of MITF, CDK6, and cell cycle regulatory proteins was determined by Western blot analysis. The ability to increase miR-137 expression by epigenetic drugs was tested using real-time RT-PCR.

Results. miR-137 expression was lower in uveal melanoma cell lines than in uveal melanocytes. Ectopic transfection of miR-137 into uveal melanoma cells induced G1 cell cycle arrest, leading to a significant decrease in cell growth. Overexpression of miR-137 downregulated MITF, a transcription factor with oncogenic activity. Moreover, the introduction of miR-137 downregulated the oncogenic tyrosine kinase protein receptor c-Met and cell cycle–related proteins, including CDK6. One avenue to increase the expression levels of miR-137 was through treatment with a DNA hypomethylating agent, 5-aza-2′-deoxycytidine, and a histone deacetylase inhibitor, trichostatin A.

Conclusions. The results showed that miR-137 can act as a tumor suppressor in uveal melanoma cell proliferation through downregulation of the targets *MITF* and *CDK6*. miR-137 may be epigenetically silenced during uveal melanoma tumorigenesis. (*Invest Ophthalmol Vis Sci.* 2011;52:1193–1199) DOI:10.1167/iovs.10-5272

MicroRNAs (miRNAs) are highly conserved, small, noncoding RNAs that play a vital role in a variety of biological processes, including development, differentiation, apoptosis, cell proliferation, and metabolism.[1] After the discovery of *lin-4* in *Caenorhabditis elegans* by Ambros et al.,[2] the idea of miRNAs as important regulators of biological processes was initially overlooked. Not until the identification of *let-7* in *C. elegans* with corresponding analogs in other species, including humans, did a revelation occur in the field of microbiology.[3] Soon after, with the identification of miRNA as the causal agent for development of chronic lymphocytic leukemia, the influences of miRNA took an exciting new direction into the field of oncogenesis.[4] Rather than an interesting curiosity, cancers as disparate as colon, central nervous system, melanoma, prostate, and breast were all found to be influenced by the dysregulation of miRNAs.[5,6] Close examination of uveal melanoma in our laboratory also revealed the contributions of miRNAs in the development of this unusual tumor.[7]

Identification of selective miRNAs important for oncogenesis is supported by the insight that miRNAs are frequently downregulated in tumorigenesis.[5,6] Our initial studies on miR-34a found that miRNA can influence the development of uveal melanomas through an effector of p53, c-Met.[7] Consequently, we have been investigating other miRNAs that are dysregulated in uveal melanocytes and melanoma cell lines. Another suspect of the miRNA puzzle for uveal melanomas lies in the melanoma susceptibility region of chromosome 1.[8] This chromosomal region contains a miRNA, microRNA-137 (miR-137), that has been shown to regulate microphthalmia-associated transcription factor (MITF), which is also referred to as a "master regulator" of melanocyte cell growth, maturation, apoptosis, and pigmentation.[9,10] Its subsequent confirmation to be downregulated in uveal melanoma cell lines made this an interesting target for further study. Based on background search for potential targets of miR-137, we also identified cyclin-dependent kinase 6 (CDK6) as a target of miR-137 in uveal melanoma cell lines. After the confirmation of miR-137 expression and its effects on uveal melanoma cell proliferation, we explored the potential for reactivating miR-137 through the process of epigenetic regulation.

Materials and Methods

Cell Culture

The human uveal melanoma cell lines M17, M23, and SP6.5 were isolated from Caucasian patients with primary choroidal melanoma

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and were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), as described.[11,12] The primary human uveal melanocytes um95 were isolated from a Chinese donor in Wenzhou Medical College and cultured as previously described.[13] The protocol was approved by the Wenzhou Medical College ethics committee, and written informed consent was obtained for the sample collection. HEK-293 cells were purchased from ATCC (Manassas, VA).

Real-Time Reverse Transcription–Polymerase Chain Reaction

M17, M23, SP6.5, and um95 cells (1×10^5) were seeded in six-well plates and grown to 80% to 90% confluence. Then the cells were collected, and total RNA was extracted with reagent (Trizol; Invitrogen). RNA integrity was confirmed using spectrophotometry and formaldehyde/agarose gel electrophoresis. For the detection of miR-137 expression in M17, M23, SP6.5, and um95 cells, 10 ng total RNA from each cell line was used for cDNA synthesis with a microRNA reverse transcription kit (TaqMan; Applied Biosystems, Foster City, CA), and miR-137 expression level was quantified by the microRNA Assay (TaqMan; Applied Biosystems), according to the manufacturer's instructions. Real-time PCR was performed (7500 Real-Time PCR System; Applied Biosystems), the expression of miR-137 was normalized to the expression of U6 small nuclear RNA (snRNA), and relative expression levels were calculated as previously reported.[14] The expression level of miR-137 in um95 was set as the normal control, whereas the miR-137 expression level in each uveal melanoma cell line was compared with um95. For the detection of *MITF* expression in uveal melanoma cells and uveal melanocytes, M17, M23, SP6.5, and um95 cells (1×10^5) were seeded in six-well plates and grown to 80% to 90% confluence. Then the cells were collected and total RNA was extracted as described. *MITF* expression levels were quantified by measuring cyanine dye incorporation (SYBR-Green PCR Master Mix; Applied Biosystems) on the PCR system (7500 Real-Time PCR System; Applied Biosystems). The primer sequences of *MITF* were as follows: forward, 5′-AGGCTCGAGCTCATGGACTTT-3′; reverse, 5′-GGTCTTGGCTGCAGTTCTCAA-3′.

Cell Proliferation Assay

M23 and SP6.5 cells were plated at 3×10^3 cells per well in 96-well plates (Costar, High Wycombe, UK) for each transfection. Transfections were performed with reagent (Lipofectamine 2000; Invitrogen). All transfections were performed in triplicate. For each well, 50 nM miR-137 precursor molecule (Ambion, Austin, TX) or a negative control precursor miRNA (Ambion) was transfected. After 24-hour culture, cell proliferation was assessed by MTS assay, a colorimetric method for determining the number of viable cells, using an assay kit (CellTiter 96 AQ_{ucous}; Promega, Madison, WI) according to the manufacturer's instructions. Briefly, solution reagent was added to each well and incubated at 37°C for 3 hours. Cell proliferation was assessed by measuring the absorbance at 490 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA). MITF-specific siRNA (Ambion) and negative control siRNA (Ambion) were used to downregulate MITF expression in uveal melanoma cells. MITF-specific siRNA (50 nM) or negative control siRNA (50 nM) was transfected into M23 and SP6.5 cells with reagent (Lipofectamine 2000; Invitrogen). MTS assay was performed on day 3 after transfection, as described.

Flow Cytometry Analysis of the Cell Cycle

M23 and SP6.5 cells were plated into 60-mm dishes (Costar) and were grown to 50% to 70% confluence for each transfection. Each cell line was transfected with 50 nM miR-137 precursor molecule or a negative control. After 48 hours, the cells were collected, washed with phosphate-buffered saline (PBS), and stained with propidium iodide (Cycle Test Plus DNA Reagent Kit; BD Biosciences, San Jose, CA). Stained cells (1×10^5) were then analyzed for DNA content with a flow cytometer (FACScaliber; BD Biosciences). In a similar fashion, M23 and SP6.5 cells were analyzed by flow cytometry after transfection with 50 nM MITF-specific siRNA or negative control siRNA.

Luciferase Reporter Assays

The 3′ UTR of human *MITF* or *CDK6* was amplified from human genomic DNA and individually cloned into pMIR-REPORT vector (Ambion) by directional cloning. Seed regions were mutated to remove all complementarity to nucleotides 1 to 7 of miR-137 (QuickchangeXL Mutagenesis Kit; Stratagene, La Jolla, CA). HEK-293 cells were cotransfected with 0.4 μg firefly luciferase reporter vector and 0.02 μg control vector containing *Renilla* luciferase, pRL-SV40 (Promega), using reagent (Lipofectamine 2000; Invitrogen) in 24-well plates. Each transfection was carried out in four wells. For each well, 50 nM miR-137 precursor molecule (Ambion) or a negative control precursor miRNA (Ambion) was cotransfected with the reporter constructs as indicated (see Figs. 3C, 3F). Luciferase assays were performed 24 hours after transfection (Dual Luciferase Reporter Assay System; Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Western Blot Analysis

M23 and SP6.5 cells (1×10^5) were seeded in six-well plates and grown in DMEM with 10% FBS for 24 hours. After transfection of the miR-137 precursor molecule or negative control, the cells were washed with cold PBS and subjected to lysis in a lysis buffer (50 mM Tris-Cl, 1 mM EDTA, 20 g/L sodium dodecyl sulfate [SDS], 5 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride). Equal amounts of protein lysates (50 μg each) and rainbow molecular weight markers (GE Healthcare Life Sciences, Piscataway, NJ) were separated by 10% SDS-polyacrylamide gel electrophoresis, then electrotransferred onto nitrocellulose membranes. The membranes were blocked with a buffer containing 5% nonfat milk in PBS with 0.05% Tween 20 for 2 hours and incubated overnight with antibody at 4°C. After a second wash with PBS containing 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with an enhanced chemiluminescence detection kit (Pierce, Rockford, IL). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Antibodies for total CDK2, CDK4, CDK6, ERK1/2, phosphorylated-ERK1/2, total Akt, phosphorylated-Akt, and phosphorylated-Rb were from Cell Signaling Technology (Beverly, MA), and c-Met was from Santa Cruz Biotechnology.

For detection of MITF expression in uveal melanoma cells and uveal melanocytes, M17, M23, SP6.5, and um95 (1×10^5) cells were seeded in six-well plates and grown to 80% to 90% confluence. Then the cells were collected, and Western blot analysis was carried out as described. Antibody against MITF was purchased from Calbiochem (San Diego, CA). GAPDH was used as a loading control.

Demethylation and Deacetylation Assays

M17, M23, and SP6.5 uveal melanoma cell lines were seeded at 1×10^5 cells per well of a six-well plate. Then 1 or 5 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St. Louis, MO), a DNA hypomethylating agent, was added before the cells were cultured for 48 hours. Alternatively, trichostatin A (TSA; 100 ng/mL; Sigma), a histone deacetylase inhibitor, was added and cells were cultured for 12 hours. For the combination study, 1 or 5 μM 5-aza-dC was present for the first 48 hours, and TSA (100 ng/mL) was added before culturing for another 12 hours. The media containing 5-aza-dC were changed every 24 hours. Then real-time RT-PCR assay for the detection of miR-137 was performed as described.

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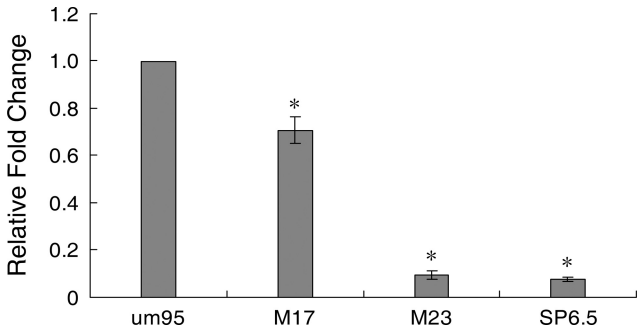

FIGURE 1. miR-137 expression is downregulated in uveal melanoma cells. Real-time RT-PCR analysis was performed to detect the expression of miR-137 in uveal melanoma cell lines, including M17, M23, and SP6.5, as well as primary uveal melanocyte um95. miR-137 was downregulated in uveal melanoma cells compared with uveal melanocytes. U6 snRNA was used as an internal control. $^*P < 0.01$; differences in miR-137 expression between uveal melanocytes and uveal melanoma cells were significant.

Statistical Analysis

All data were shown as the mean ± SEM. Differences between experimental groups and control groups were analyzed using the Student's *t*-test. Statistical significance was accepted at $P < 0.05$.

RESULTS

miR-137 Expression Is Downregulated in Uveal Melanoma Cells

To determine whether miR-137 was involved in the oncogenesis of uveal melanoma, we first compared miR-137 expression in primary human uveal melanocytes and human uveal melanoma cells. Real-time RT-PCR was performed to detect miR-137 expression levels in uveal melanocytes um95 and human uveal melanoma cell lines M17, M23, and SP6.5. Although miR-137 was highly expressed in normal um95 uveal melanocytes, expression of miR-137 was significantly decreased in uveal melanoma cells (Fig. 1). The decrease of miR-137 expression was more pronounced in M23 and SP6.5 than in M17 (Fig. 1). Therefore, M23 and SP6.5 cell lines were used for further investigation.

miR-137 Inhibits Cell Proliferation through Cell Cycle G1 Arrest

Given that miR-137 was downregulated in uveal melanoma cells, we first sought to investigate any biological effects caused by the restoration of miR-137 expression in cells. Transfection with miR-137 into M23 and SP6.5 cells, followed by MTS assay, inhibited cell growth compared with that of control over a 5-day interval (Fig. 2A). The decrease in cell number was statistically significant between cells transfected with miR-137 and cells transfected with a negative control at day 5 (57.01% ± 3.26% decrease in M23 cells and 50.76% ± 2.50% decrease in SP6.5 cells). With cell culture, we were able to visually depict the result of miR-137 transfection on M23 and SP6.5 cells by crystal violet staining after 7 days of culture (Fig. 2B). The process by which miR-137 inhibited cell growth was due to increased G1 cell cycle arrest in these cells. Forty-eight hours after transfection, cells were stained with propidium iodide and analyzed by flow cytometry. In M23 cells transfected with miR-137, 88.65% of cells accumulated in G1 compared with 55.48% of cells for the negative control. In SP6.5 cells transfected with miR-137, 88.87% of cells accumulated in G1 compared with 56.68% of cells for the negative control (Fig. 2C). To further characterize miR-137–mediated inhibition of cell proliferation, we examined caspase activity to determine whether apoptosis was also involved. No significant difference in caspase 3/7 activity was observed between miR-137 trans-

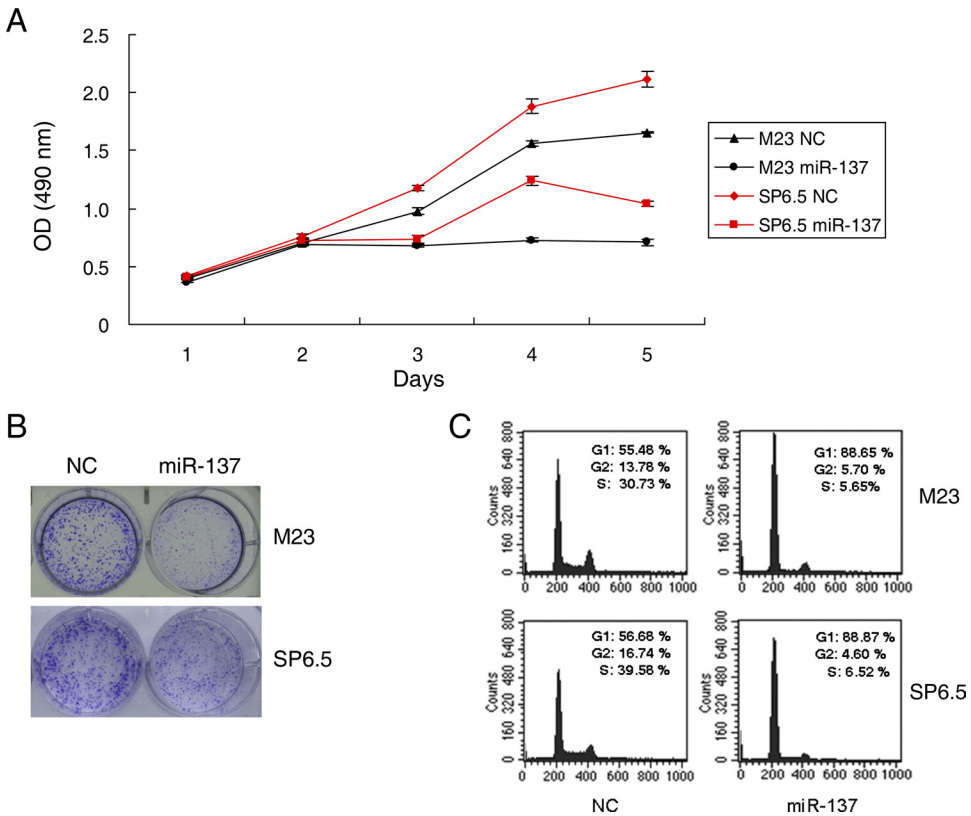

FIGURE 2. Ectopic miR-137 inhibits the proliferation of uveal melanoma cells and induces cell cycle G1 arrest. (**A**) MTS cell proliferation assay was carried out on days 1 to 5, as indicated after lipofectamine transfection of uveal melanoma cells M23 and SP6.5 with either miR-137 (50 nM) or a negative control scrambled oligonucleotide (NC). Cell populations transfected with miR-137 had significantly fewer metabolically active cells than cells transfected with the negative control. Data at each time point are expressed as the mean ± SEM of the results obtained from triplicates in one experiment. Results represent those obtained in three separate experiments. (**B**) M23 and SP6.5 cells transfected with miR-137 or NC were seeded at low density. After 7 days, colony formation was determined by staining with crystal violet. Typical results in three independent experiments are shown. (**C**) M23 and SP6.5 cells were collected 48 hours after transfection with miR-137 or NC, stained with propidium iodide, and analyzed by flow cytometry. Ten thousand cells were evaluated in each sample. The most representative results in three independent experiments are depicted.

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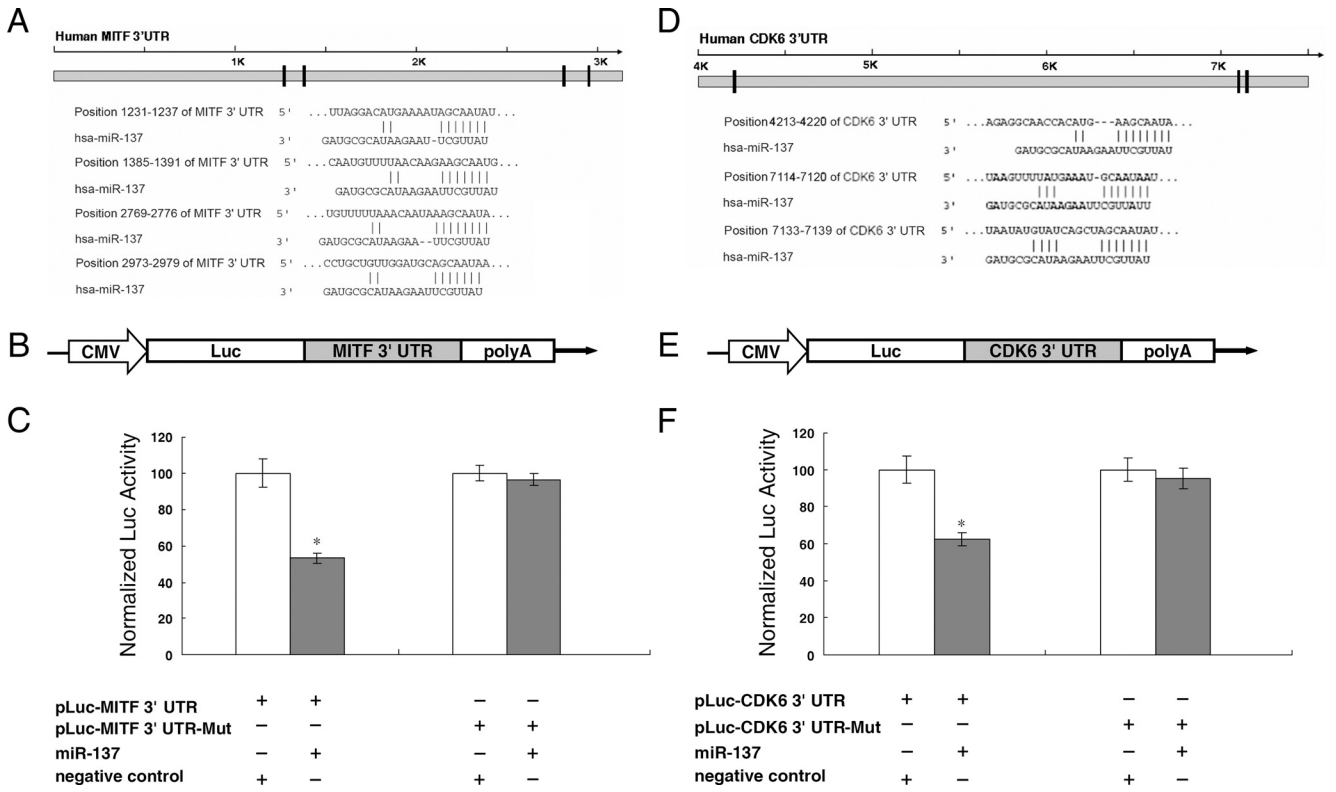

FIGURE 3. *MITF* and *CDK6* are targets of miR-137. (**A**, **D**) Specific locations of the binding sites were marked. Alignment between the predicted miR-137 target sites and miR-137, the conserved 7- to 8-bp "seed" sequence for miR-137:mRNA pairing, is indicated. (**B**, **E**) Diagram depicting the pMIR luciferase reporter constructs containing a CMV promoter used to verify the putative miR-137 binding sites. (**C**, **F**) HEK293 cells were cotransfected with 50 nM miR-137, pLuc-MITF 3′ UTR or pLuc-CDK6 3′ UTR, along with a pRL-SV40 reporter plasmid. After 24 hours, luciferase activity was measured. Values are presented as relative luciferase activity after normalization to *Renilla* luciferase activity. Data are expressed as the mean value ± SEM of the results obtained from three independent experiments. $^*P < 0.01$; differences in luciferase activity between miR-137 and negative control transfected cells were significant.

fected cells and negative control transfected cells (data not shown). Thus, these results indicate that miR-137 expression suppressed uveal melanoma cell growth by cell cycle G1 arrest rather than by inducing apoptosis.

MITF and *CDK6* Are Targets of miR-137

Because miRNAs primarily mediate their biological functions in animal cells by impeding the expression of target genes, we searched for potential targets of miR-137 that exhibited oncogenic properties. Using TargetScan (http://www.targetscan.org), theoretical target genes of miR-137 that could mediate the observed effects included *MITF* and *CDK6*. MITF has previously been shown to be regulated by miR-137,[9] and CDK6 has previously been validated as subject to control by miR-137 in oral squamous cell carcinoma[15] and glioblastoma multiforme.[16] Using bioinformatics, the 3′ UTR of *MITF* was found to contain four target sequences for miR-137, and *CDK6* was found to contain three target sequences (Figs. 3A, 3D). To test whether miR-137 directly targets *MITF* and *CDK6* genes, we cloned the wild-type 3′ UTR of each gene into a luciferase reporter vector (Figs. 3B, 3E). We then transfected each resultant reporter construct (pLuc-MITF 3′ UTR and pLuc-CDK6 3′ UTR) into HEK293 cells, along with miR-137 or a control miRNA. Luciferase activity assays 24 hours after transfection demonstrated that miR-137 suppressed luciferase reporter activity to 52.75% ± 2.93% and 62.53% ± 3.80% using 3′ UTR of *MITF* and *CDK6*, respectively (Figs. 3C, 3F). Mutations of all the sites in the two genes attenuated the suppression of luciferase reporter by miR-137 (Figs. 3C, 3F). These results demonstrate that miR-137 directly targets the *MITF* and *CDK6* through the binding sites on their 3′ UTR.

Expression of MITF Is Upregulated in Uveal Melanoma Cells

Real-time RT-PCR analysis was performed to detect the expression levels of *MITF* mRNA in the melanocyte um95 and in uveal melanoma cell lines. *MITF* mRNA was significantly upregulated in all uveal melanoma cells examined compared with normal melanocytes (Fig. 4A). Concurrently, MITF protein expression was markedly upregulated in uveal melanoma cells M17, M23, and SP6.5 compared with uveal um95 melanocytes (Fig. 4B).

Downregulation of MITF Inhibits Cell Proliferation and Induces Cell Cycle G1 Arrest

MTS cell proliferation assays showed that cell populations transfected with MITF siRNA had significantly fewer metabolically active cells than cells transfected with negative control (27.60% ± 3.02% decrease in M23 cells and 34.40% ± 5.50% decrease in SP6.5 cells) (Fig. 5A). Complementary to the finding that miR-137 inhibited cell proliferation, MITF was found to be involved in G1 cell cycle arrest. Forty-eight hours after transfection with MITF siRNA or a negative control, flow cytometric analysis showed that M23 cells transfected with MITF siRNA had 74.03% G1 arrest compared with 53.89% for negative control.

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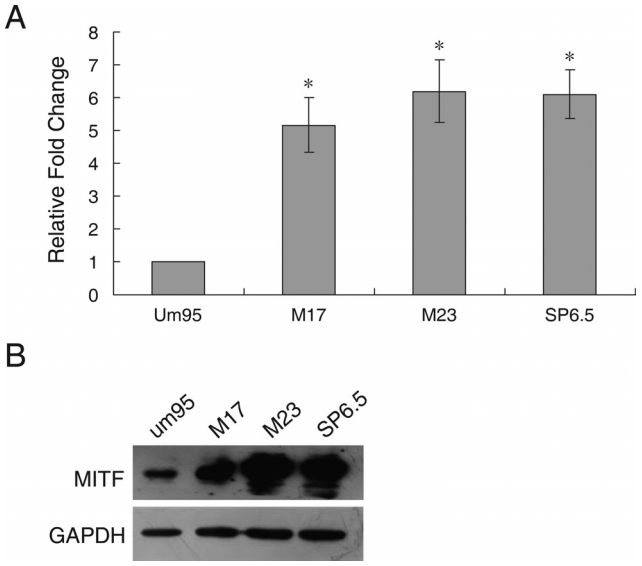

FIGURE 4. MITF expression is upregulated in uveal melanoma cells. (**A**) Real-time RT-PCR analysis was performed to detect the expression of *MITF* at the mRNA level in uveal melanoma cell lines, including M17, M23, and SP6.5, as well as in uveal melanocyte um95. $^*P < 0.01$; differences in MITF expression between uveal melanocytes and uveal melanoma cells were significant. (**B**) Western blot analysis was performed to detect the expression of MITF at protein levels in uveal melanoma cell lines and uveal melanocytes. MITF protein was significantly upregulated in uveal melanoma cells compared with uveal melanocytes. GAPDH was used as an internal control.

SP6.5 cells transfected with MITF siRNA showed 69.83% G1 arrest compared with 50.19% for negative control (Fig. 5B).

Ectopic Expression of miR-137 Downregulates the Expression of MITF, c-Met, and Phosphorylated-ERK1/2 in Uveal Melanoma Cells

Western blot analysis showed that MITF was dramatically reduced when cells were transfected with miR-137 (Fig. 6A). The level of c-Met expression was also decreased (Fig. 6B). Levels of other intracellular proteins affected by c-Met, including Akt and ERK1/2, were then determined after miR-137 transfection. As shown in Figure 6B, only phosphorylated-ERK1/2 was significantly reduced whereas phosphorylated-Akt, total Akt, and total ERK1/2 were not affected when comparing miR-137 transfection to negative control transfection (Fig. 6B).

Introduction of miR-137 Downregulates Multiple Cell Signaling Pathways

Primary targets of miR-137, such as CDK6, were suppressed by transfection with miR-137, as expected (Fig. 7). Moreover, ectopic miR-137 delivery downregulated cell cycle regulatory proteins such as CDK2 and phosphorylated-retinoblastoma protein (p-Rb) (Fig. 7), a known target of CDK6.[17,18] CDK4 levels were, however, less markedly affected (Fig. 7).

miR-137 Expression Is Upregulated after Treatment with a DNA Hypomethylating Drug or a Histone Deacetylase Inhibitor

We compared the expression levels of miR-137 in the uveal melanoma cells M17, M23, and SP6.5 treated with either the DNA hypomethylating agent 5-aza-dC or the histone deacetylase inhibitor TSA, or both. Expression of miR-137 was increased after treatment with 5-aza-dC with maximal induction observed in cells treated with 1 μM of 5-aza-dC (Fig. 8). Expression of miR-137 was also increased after treatment with 100 ng/mL of TSA, especially in SP6.5 cells. Furthermore, the effect of the drug combination seems to be additive on the expression of miR-137 (Fig. 8). Overall, these results demonstrated that the expression of miR-137 in uveal melanoma cells can be affected by an epigenetic mechanism.

DISCUSSION

Increasing evidence shows that miRNAs are poorly expressed in human tumors, suggesting that miRNAs can act as tumor suppressors.[5,6] The suppression of miRNAs is frequently associated with increased oncogenesis.[5,6] DNA methylation–associated silencing of miRNAs is one of the pathways by which cancer cells evade the typical control mechanisms to inhibit oncogenesis.[19–21] In this regard, studies on *hMLH1, BRCA1,* and $p16^{INK4a}$ have demonstrated the presence of CpG island hypermethylation as an etiologic cause of associated oncogenesis.[22–24] Recent studies of epigenetically silenced miRNA using chromatin hypomethylating drugs have confirmed the ability of medications to restore the function of these miRNAs[15,19]. Previous work on miR-137 has shown that miR-137 was also silenced through aberrant DNA methylation in oral squamous cell carcinoma.[15] Therefore, we investigated the potential of silencing miR-137 using epigenetic mechanisms in uveal melanoma formation with an eye on downstream effector mechanisms.

miR-137 has been shown to be downregulated in primary malignant cutaneous melanomas.[25] However, the expression of miR-137 in uveal melanoma has not been reported. The present study confirmed that miR-137 was poorly expressed in uveal melanoma cell lines (Fig. 1). Ectopic expression of miR-137 through transfection was able to inhibit the cell growth of

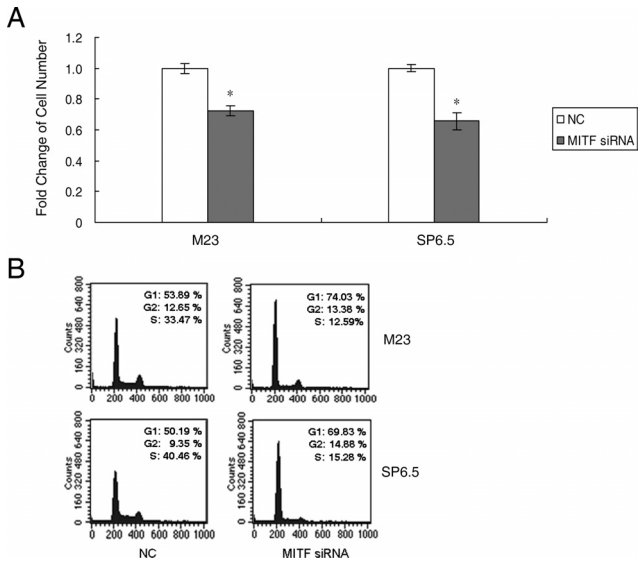

FIGURE 5. Downregulation of MITF inhibits the proliferation of uveal melanoma cells and induces G1 arrest. (**A**) MTS cell proliferation assay was carried out on day 3, as indicated, after lipofectamine transfection of uveal melanoma cells M23 and SP6.5 with either MITF siRNA (50 nM) or a negative control (NC). Cell populations transfected with MITF siRNA had significantly fewer metabolically active cells than cells transfected with the NC. Data at each time point are expressed as the mean ± SEM of the results obtained from triplicates in one experiment. Results are representative of those obtained in three experiments. $^*P < 0.01$. (**B**) M23 and SP6.5 cells were collected 48 hours after transfection with MITF siRNA or NC, stained with propidium iodide, and analyzed by flow cytometry. Ten thousand cells were evaluated in each sample. The most representative results in three independent experiments are depicted.

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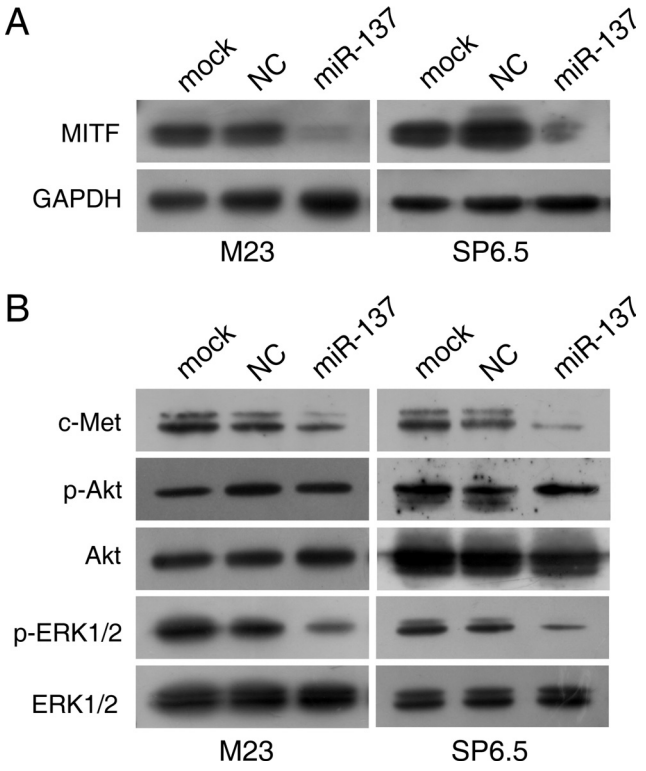

FIGURE 6. Introduction of miR-137 downregulates the expression of MITF, c-Met, and phosphorylated-ERK1/2 in uveal melanoma cells. (**A**) MITF expression levels in M23 and SP6.5 cells after transfection with miR-137 were determined by Western blot analysis. Compared with the negative control miRNA, miR-137 expression dramatically reduced the levels of MITF in both cell lines. GAPDH was used as an internal control. (**B**) M23 and SP6.5 cells were transfected with miR-137 or a negative control. Cell lysates were prepared and used for Western blot analysis with c-Met, phosphorylated-Akt (p-Akt), total Akt, phosphorylated-ERK1/2 (p-ERK1/2), and total ERK1/2 antibodies. GAPDH was used as a loading control.

uveal melanoma (Fig. 2). In certain cancers, such as oral squamous cell carcinoma, the expression of the miRNA gene was restored by treatment with DNA-hypomethylating agents.[15] Similarly, despite limiting our studies to in vitro data with cell lines, we were able to show that uveal melanoma displayed suppressed levels of miR-137 with restoration of activity after treatment with DNA-hypomethylating agents.

MITF is a transcription factor that is essential for melanocyte development, but it also acts as an oncogene in cutaneous melanoma.[10] For instance, Garraway[26] has found that MITF was amplified in cutaneous melanoma and was correlated with decreased overall patient survival. In addition, positive expression of MITF has been observed in most clinical uveal melanoma specimens.[27] Concordant with these findings, MITF was found to be overexpressed in uveal melanoma cells and behaved as an oncogenic factor in this study. Previously, miR-137 has been observed to negatively regulate MITF in cutaneous melanomas,[9] but no further functional study was carried out. In the present study, miR-137 was shown to inhibit uveal melanoma cell proliferation by regulating the expression of MITF and CDK6. As an important transcription factor, MITF can upregulate c-Met expression by directly binding to *c-Met* promoter in both cutaneous melanoma cells and melanocytes.[28] c-Met, acting as an oncogene,[29,30] is overexpressed in both cutaneous and uveal melanomas and plays an important role in the metastasis of melanomas.[30–33] Inhibition of MITF led to the downregulation of c-Met expression. Subsequently,

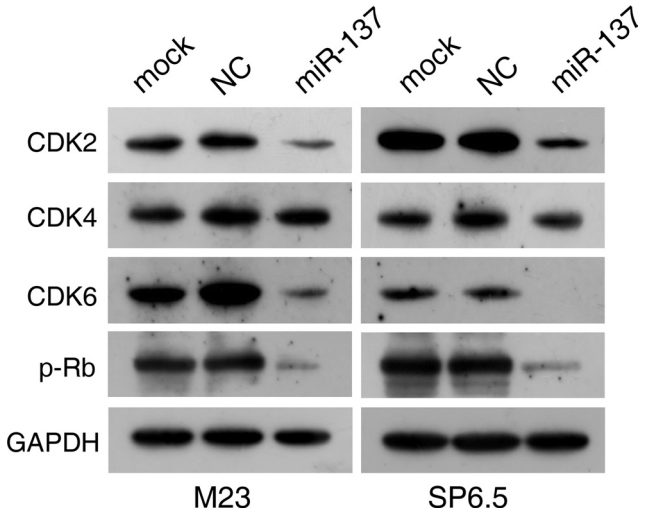

FIGURE 7. Overexpression of miR-137 downregulates cell cycle–related proteins. M23 and SP6.5 cells were transfected with miR-137 or a negative control. Cell lysates were prepared and used for Western blot analysis with CDK2, CDK4, CDK6, and phosphorylated-Rb (p-Rb) antibodies. GAPDH was used as a loading control. As indicated, CDK2, CDK6, and p-Rb were dramatically downregulated by miR-137.

decreased c-Met mediated inhibition of the ERK1/2 pathways, which is essential for cell proliferation (Fig. 6B). In addition to c-Met, CDK2 is another target of MITF in cell cycle regulation.[34] MITF-mediated regulation of CDK2 plays an essential role in cutaneous melanoma clonogenic growth.[34] Similarly, our results showed that CDK2 was decreased in cells with suppression of MITF after the introduction of miR-137 (Fig. 7).

We demonstrated that CDK6 was also directly regulated by miR-137. CDK6 is an important kinase that plays an essential role in cell cycle G1 phase progression and cell differentiation.[18] Moreover, CDK6 was demonstrated to be overexpressed in cutaneous melanoma, which implies a possible role in the development of cutaneous melanoma.[35] The role of CDK6, however, in uveal melanoma remains unclear and is therefore a target of this study. Using computational prediction for miR-137 target genes, we identified *CDK6* as one of the potential targets for miR-137. Both *MITF* and *CDK6* genes have miR-137 complementary sites in their 3′ UTRs that subject the oncogenes to miR-137–mediated regulation in melanocytes. Uveal melanoma cells, with signifi-

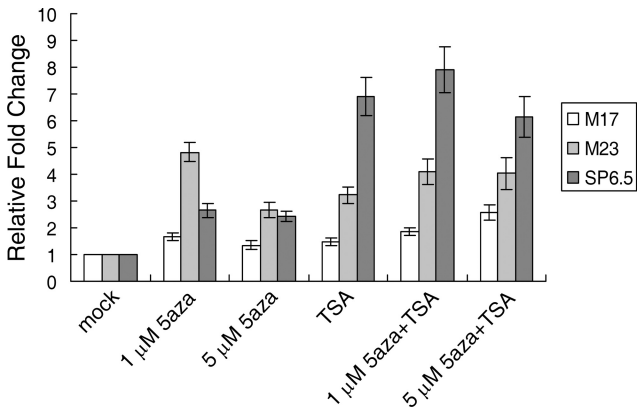

FIGURE 8. Expression of miR-137 is upregulated by epigenetic drugs. Uveal melanoma cell lines, including M17, M23, and SP6.5, were treated with 5-aza-dC (5aza) at 1 μM or 5 μM alone, TSA (100 ng/mL) alone, or combinations of both. miR-137 expression level was measured by real-time RT-PCR relative to U6 snRNA.

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cantly reduced levels of miR-137, also exhibited increased levels of MITF proteins relative to normal melanocytes. Therefore, miR-137 regulation of MITF and CDK6 is a plausible mechanism for miR-137 in uveal melanoma tumorigenesis. We also confirmed that miR-137 transfection diminished the phosphorylation of retinoblastoma, the target of CDK6, as previously demonstrated by Grossel et al.[36]

Previous studies have observed a close correlation between the losses of 1p chromosome and the metastatic potential of primary uveal melanomas.[37–39] This suggested that tumor suppressor genes maybe contained in this region. Interestingly, miR-137 is also located in the region. In this study, overexpression of miR-137 inhibited the growth of uveal melanoma cell lines in vitro, suggesting a causal relationship between miR-137 and cell growth in these cells. The inverse regulation of MITF and CDK6 by miR-137, based on Western blot analysis, strongly implicates miR-137 as a tumor suppressor in uveal melanoma. In summary, the ability to restore miR-137 activity using epigenetic mechanisms suggests a target for the modulation of oncogenic proteins that are important in the development of uveal melanoma.

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