MicroRNA-137 Targets Microphthalmia-Associated Transcription Factor in Melanoma Cell Lines

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Abstract

Microphthalmia-associated transcription factor (MITF) is the master regulator of melanocyte development, survival, and function. Frequent alteration in the expression of MITF is detected in melanoma, but the mechanism(s) underlying the alteration in expression have not been completely determined. In these studies, we have identified microRNA-137 (miR-137) as a regulator of MITF expression. The genomic locus of miR-137 at chromosome 1p22 places it in a region of the human genome previously determined to harbor an allele for melanoma susceptibility. Here, we show that expression of mature miR-137 in melanoma cell lines down-regulates MITF expression. Further, we have identified a 15-bp variable nucleotide tandem repeat located just 5′ to the pre-miR-137 sequence, which alters the processing and function of miR-137 in melanoma cell lines. [Cancer Res 2008;68(5):1362–8]

Introduction

Malignant melanoma represents a significant public health burden and is increasing in incidence at a greater rate than any other cancer type (1). The molecular mechanisms involved in the development of melanoma are complex and not entirely clear. In an attempt to discern regions of the genome that might harbor genes contributing to the development of melanoma, several studies have characterized regions of the genome that are lost or amplified in melanoma cell lines and in families with a high risk for melanoma (2). One such region located on chromosome 1p22 harbors a gene or genes that are responsible for increasing melanoma risk but are not yet identified (3). With the identification of microRNAs (miRNA) as important regulators of gene expression, we hypothesized that a miRNA at 1p22 might be regulating target genes associated with a risk of developing melanoma.

MiRNAs are located throughout the genome and may be transcribed as noncoding primary miRNA (pri-miRNA) transcripts. The pri-miRNAs are processed into precursor miRNA (pre-miRNA) and finally into the mature and functional miRNA of 21 to 25 nucleotides (4). The mature miRNAs function by binding to the 3′ untranslated region (3′UTR) of multiple target mRNAs. The binding is usually imperfect and results in an inhibition of translation of the target protein or degradation of the target mRNA (4–6). Numerous studies characterizing the expression profiles of miRNAs in cancer have shown the down-regulation of miRNA expression in cancer cell lines as compared with normal tissues (7–9). Furthermore, the recent identification of altered expression of miRNAs in a variety of cancer sites has confirmed that this is yet another class of molecules capable of functioning as tumor suppressors or oncogenes (10).

The first confirmation of specific mutations identified in miRNAs in cancer cells but not in normal cells came from the work by Calin and Croce (11) on B-cell chronic lymphocytic leukemia (CLL). A variety of alterations were identified in two miRNAs in B cells from CLL patients, and these mutations were not found in B cells from healthy participants. The mutations altered either the pri-miRNA or the pre-miRNA. Characterization of such mutations suggests that these miRNAs function as tumor suppressors in CLL (11) because the mutations resulted in lower expression of the specific miRNAs thereby allowing the increased expression of an oncogene, *Bcl-2*.

The alterations in miRNAs that are reported for CLL (11) led us to question if a miRNA or multiple miRNAs might contain alterations in melanoma. MiRNA expression profiles have been delineated in a large variety of cancer sites (7–9); however, these studies included few melanoma samples. Thus, we initially conducted a survey of miRNAs and their chromosomal location to determine if any were located in regions of the human genome suspected to harbor genes contributing to melanoma but as yet unidentified. As noted above, one commonly reported region of the human genome thought to harbor a gene contributing to melanoma susceptibility is a region of chromosome 1 (12). This chromosomal region contained one miRNA according to the miRNA database at Sanger Center[3] (13, 14). Interestingly, this miRNA, microRNA 137 (miR-137), was also suspected to bind to the 3′UTR of the key regulator of melanogenesis, microphthalmia-associated transcription factor (MITF), as indicated in the miRGen database[4] (15). MITF is the "master regulator" of melanocyte cell growth, maturation, apoptosis, and pigmentation (16). We undertook studies to confirm the predicted role of miR-137 in the regulation of MITF. Additionally, we identified a variable nucleotide tandem repeat (VNTR) in the pri-miRNA-137 that alters the function of miR-137 in melanoma cells.

Materials and Methods

miRNA computational predictions. The publicly accessible database miRBase[3] was consulted to identify miRNA located on chromosome 1 in the region predicted to harbor a gene involved in melanoma. The data bases TargetScan 3.1[5] (17, 18) and miRgen[4] (15) were used to identify potential targets for miR-137 regulation in melanoma. MiRNA binding predictions were confirmed by manually analyzing the MITF 3′UTR for binding sites of miR-137. The publicly available MFold software was used to predict the secondary structure of miR-137 (19).

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[3] http://microrna.sanger.ac.uk/

[4] http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html

[5] http://www.targetscan.org

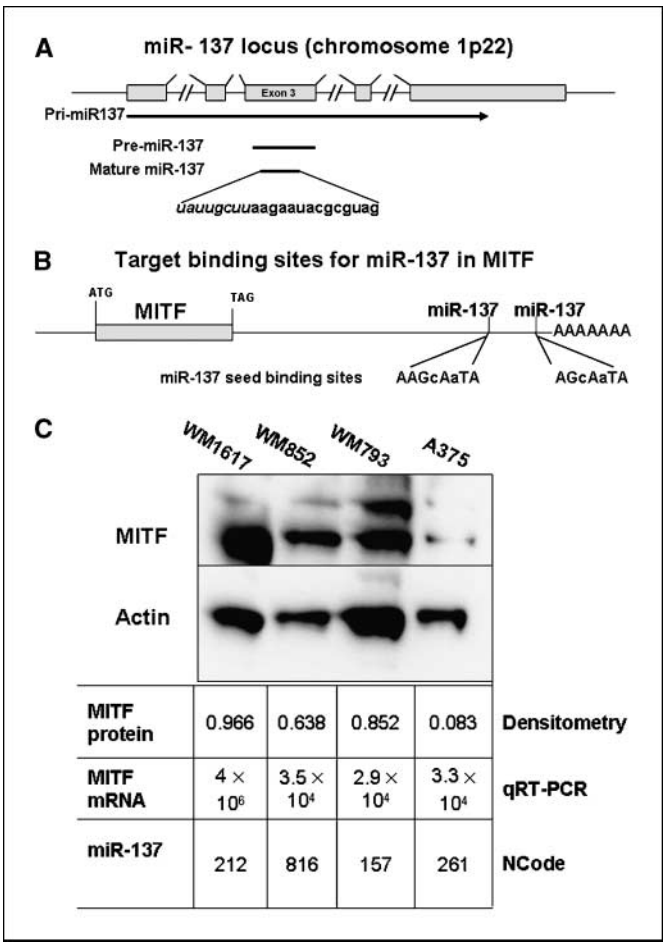


Figure 1. Identification of putative miRNA regulators in melanoma. *A,* miR-137 locus showing the primary, precursor, and mature miR-137 at chromosome 1p22, a region of common loss of heterozygosity in melanoma (chromosome 1: 98284214–98284315 [−]). The primary transcript for miR-137 is capped and polyadenylated (2,381 bp), and the precursor miR-137 is encoded in exon 3 (102 bp). The mature miR-137 (22 bp) is processed from the pre-miR-137. The seed binding region is italicized. *B,* MITF is a target of miR-137 as predicted by TargetScan. Shown are the seed binding regions located in the most 3′ region of the UTR. The mutated seed bases, designated by letter a, were mutated to C, and C was mutated to A to confirm binding of miR-137 to these regions. *C,* relative comparison of MITF and miR-137 expression in four melanoma cell lines by protein expression and quantitative RT-PCR. Western blot analysis of MITF protein expression equalized to β-actin is shown based on densitometry of MITF and β-actin expressed as the ratio of MITF to β-actin. mRNA expression of MITF by quantitative RT-PCR is expressed as relative quantitation equalized to β-actin. miR-137 expression was quantified by the NCode method (Invitrogen) equalized to U6 RNA and expressed as relative quantitation (described in Materials and Methods).

Cell lines and growth conditions. The human melanoma cell lines WM1617, WM852, WM793, and A375 were chosen based on previous studies of the expression of MITF. We had previously determined that WM852, WM1617, and WM793 express MITF (data not previously reported), and others had shown the lack of expression of MITF in A375 (20). All melanoma cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 50 mmol/L L-glutamine at 37°C and 5% CO_2. In addition, the cell line WM852 was treated with 100 ng/mL α-melanocyte stimulating hormone (α-MSH; Sigma-Aldrich) for 8 h. Cells were transfected with eGFP-C1, GFP-MITF-3′UTR, GFP-MITF-3′UTR-7mer, GFP-MITF-3′UTR-8mer, and GFP-MITF-3′UTR-double, with or without 5 nmol/L miRIDIAN miR-137 mimic (Dharmacon), using Qiagen Effectene Transfection Reagent. Quantitative reverse transcription-PCR (RT-PCR) of green fluorescent protein (GFP) was used to verify transfection.

Western blot analysis. Cells were harvested by rinsing with PBS and lysed with lysis buffer (1% SDS, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 7.4), which was heated to 90°C. The samples were briefly sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. The antibodies used in this study included MITF (clone D5), GFP (clone GFP01; Lab Vision), and pan-actin (4968; Cell Signaling). Protein samples were resolved on a 10% SDS polyacrylamide gel, transferred onto a nitrocellulose membrane, and blocked in 5% dried milk in PBS/0.0025% Tween 20. Membranes were incubated in the appropriate primary antibody overnight, followed by incubation with a secondary antibody (horseradish peroxidase–conjugated antimouse). Proteins were detected with enhanced luminol/peroxide buffer SuperSignal West Femto (Pierce) on a Chemidoc (Bio-Rad) imaging system and quantitated with the Quantity One software (4.5.0 basic, Bio-Rad).

PCR amplification of genomic DNA. Genomic DNA was prepared from cell lines with the Qiagen DNeasy Tissue kit (Qiagen) and 50 to 100 ng were amplified using GoGreen Taq as directed (Promega). The primers for miR-137 were miR-137 forward, 5′-GCAGCAAGAGTTCTGGTGGC, and miR-137 reverse, 5′-TGGAACCAGTGCGAAAACAC. Following PCR amplification, the products were electrophoresed on a 3% agarose gel, and then bands were excised and extracted with the Qiagen Gel extraction kit (Qiagen). The samples were directly sequenced on an AB3700 automated sequencer in the Core Laboratory of the University of Colorado Cancer Center using the same primers as used for PCR. All sequencing reactions were done in both the forward and reverse directions. Alignments and mutation analysis were conducted using BLAST (National Center for Biotechnology Information) software.

RNA isolation, cDNA synthesis, and quantitative RT-PCR. Total RNA was isolated using the miRNeasy Mini Kit (Qiagen). For miRNA message analysis, the cDNA was synthesized using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen). Briefly, this step adds a polyadenylate tail to the miRNA in the total RNA samples. For all other mRNA analyses, cDNA was synthesized using the SuperScript II Reverse Transcriptase Kit (Invitrogen). The amount of miRNA was monitored with Platinum SYBR Green qPCR SuperMix-UDG reagent (Invitrogen). Quantitative PCR was done under the following thermocycler conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 57°C for 60 s. Quantitative RT-PCR was used to determine the expression levels of the target genes *MITF, GFP*, and *β-actin* as internal control (21). Expression of the mature miR-137 and U6 as internal control was conducted with the NCode kit (Invitrogen). Results are expressed as relative quantitation as previously described (22). Primers were, for primary miR-137, 5′-CAAGGCTTGTTAACACTGTAAC (forward) and 5′-TCTGTCAATGTCTGAATAAATG (reverse); MITF exon 9–10, 5′-CAGGAACTTGAAATGCAGGCTCGA (forward); MITF exon 10, 5′-GATCAGTGACACCGACGGGAGAA (reverse); β-actin, 5′-ATCCACGAAACTACCTTCAACTC (forward) and 5′-GAGGAGCAATGATCTTGATCTTC (reverse); miR-137 mature, 5′-TATTGCTTAAGAATACGCGTAG (forward); U6, 5′-CGCAAGGATGACACGCAAATTCGT (forward); and GFP, 5′-CGACAAGCAGAAGAACGGCATCAA (forward) and 5′-AACTCCAGCAGGACCATGTGAT (reverse).

GFP-MITF-3′UTR reporter constructs. The 3′UTR of MITF is encoded in exon 10. A cDNA encoding the most 1,143 bp of the 3′UTR was purchased from American Type Culture Collection (522701) and a fragment from the vector *Eco*RI to *Not*I encompassing the insert was ligated into pCR2.1-TOPO (Invitrogen) for convenient restriction digestion. The insert was moved to pEGFP-C1 (Clontech Laboratories) at the *Eco*RI and *Apa*I sites. Mutation of the miR-137 binding site in the 3′UTR of MITF was created using the QuickChange Stratagene method. Primers for quick-change mutations were MITF 7-mer, 5′-CCTGCTGTTGGATGCAGAACTAATTTCTGTATGGTCCATA (forward) and 5′-TATGGACCATACAGAAATTAGTTCTGCATCCAACAGCAGG (reverse); MITF 8-mer, 5′-GTTTTTAAACAATAAAGAACTAAGAACAAATACAA (forward) and 5′-TTGTATTTGTTCTTAGTTCTTTATTGTTTAAAAAC (reverse). Melanoma cells were then transfected using the lipophilic reagent Effectene (Qiagen) with no vector, the GFP vector, or the GFP-MITF-3′UTR constructs. GFP expression was monitored by Western blot analysis and quantitative RT-PCR.

Pri-miR-137 vector construction. The pre-miR-137 sequence is located within the non–protein-coding RNA gene BRAMY2014205 and we believe

this gene to be the pri-miR-137 sequence. The BRAMY2014205 clone was purchased from the Department of Biotechnology of National Institute of Technology and Evaluation, Japan (AK094607/FLJ37288/BRAMY2014205). The cDNA was prepared at the Institute of Medical Science of the University of Tokyo (IMS-UT) and Helix Research Institute, Inc., by the "oligo-capping" method and cloned into the pME18SFL3 vector. We reconstructed the VNTR region by a complex cloning strategy to prevent changes in base pairs that might be necessary for the function of the primary miR-137. First, a restriction fragment from the original cDNA clone (BRAMY2014205) starting with *Eco*RI and going to the internal *Not*I was gel purified and ligated into pT7T3-PAC vector at *Eco*RI and *Not*I. Then this vector was restricted at the internal *Bam*HI and *Not*I and the 12 repeats from a PCR product derived from WM852 genomic DNA were ligated into this plasmid to create the 12-VNTR region. A fragment from this repaired vector was then ligated into the original vector at *Eco*RI and *Not*I, creating the plasmid with 12 VNTRs. The plasmid with 3 VNTRs is considered wild-type whereas the 12-VNTR plasmid has 12 repeats obtained from the WM852 cell line.

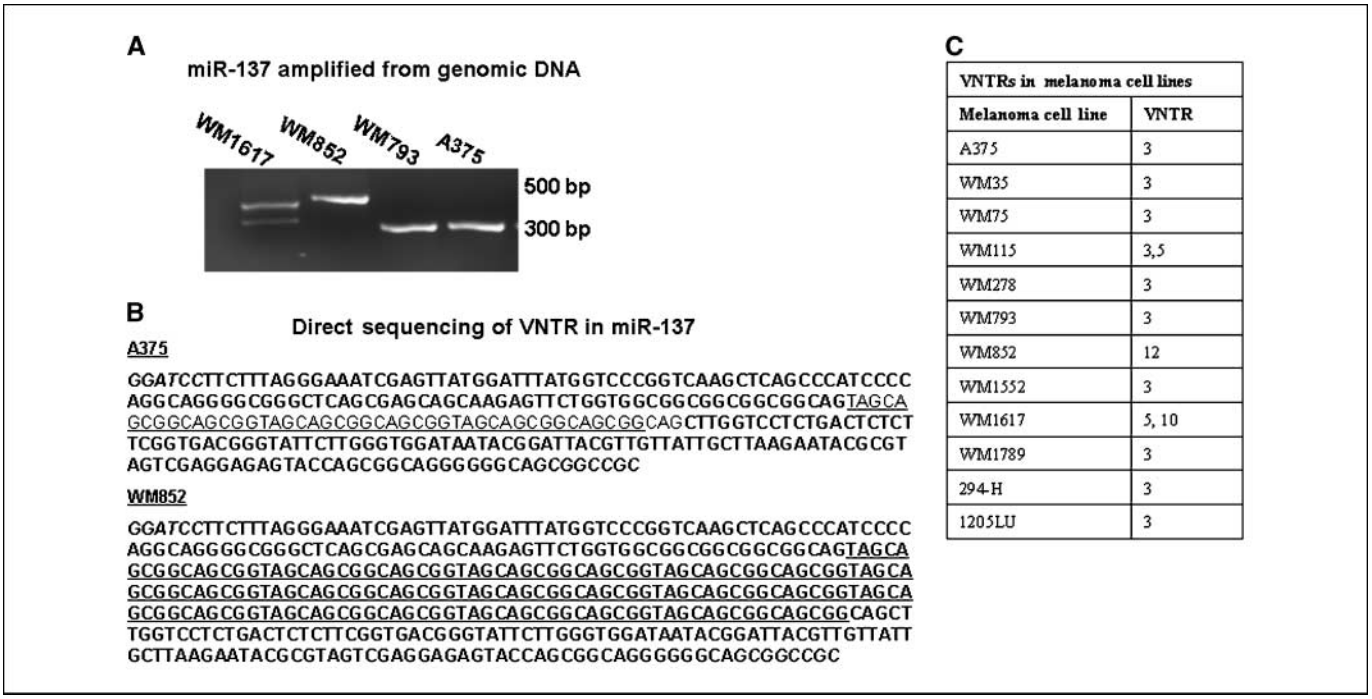
Northern blot analysis. RNA samples from cell lines were analyzed by Northern blot. Briefly, 2.0 μg of total RNA were mixed with an equal volume of formamide and heated to 65°C for 10 min. The samples were electrophoresed in a 15% urea-Tris-borate EDTA gel (Bio-Rad) and transferred onto GeneScreen Plus membrane (Perkin-Elmer). The membrane was UV cross-linked with 120-mJ energy and baked at 80°C for 1 h. The miR-137 probe (5′-CTACGCGTATTCTTAAGCAATA-3′) and U6 snRNA loading control probe (5′-TGTGCTGCCGAAGCGAGCAC-3′) were ^{32}P labeled at their 5′ end using T4 polynucleotide kinase (New England Biolabs). The probes were hybridized to the membrane and exposed on X-ray film at −80°C.

Results

In initial studies, we used computational methods to identify miRNAs located in the 1p22 chromosomal region that might regulate genes important in melanocyte development and function (13, 14). One miRNA was detected in this location, miR-137, located between 92 and 100 Mb on chromosome 1 (Fig. 1*A*). We then queried if genes known to be important in melanocyte regulation and the development of melanoma were potential targets of miR-137. TargetScan (17, 18) ranked MITF as a likely target for regulation by miR-137 with two binding sites in the 3′UTR (Fig. 1*B*). As noted, MITF is considered the master regulator of melanogenesis and is known to be misregulated in melanoma (16).

Previous reports showed that melanoma cell lines could significantly vary in their ability to express the MITF protein. Specifically, the cell line A375 had very little MITF protein expression (20). Similarly, we also found negligible MITF expression in A375 whereas the other cell lines we examined expressed MITF (Fig. 1*C*). We also compared the expression of the MITF mRNA and found it to be highly variable (Fig. 1*C*). However, the cell lines WM852 and A375 seem to have similar MITF mRNA expression while having dramatically different MITF protein expression. Thus, we focused our continued studies of miR-137 regulation of MITF on these two cell lines.

Despite the highly variable protein expression of MITF in melanoma cell lines, the mRNA expression of MITF was similar for both WM852 and A375. This finding suggested the presence of altered regulation of MITF possibly by a miRNA. However, when we queried if this could be due to increased miR-137 expression in A375 cells as compared with WM852 (Fig. 1*C*), we found just the opposite. The NCode method for quantitative RT-PCR was applied to quantify expression of the mature miR-137 in melanoma cell lines. In contrast to our hypothesis, WM852 had increased miR-137 as compared with A375 (Fig. 1*C, NCode*).



VNTRs in melanoma cell lines	
Melanoma cell line	VNTR
A375	3
WM35	3
WM75	3
WM115	3,5
WM278	3
WM793	3
WM852	12
WM1552	3
WM1617	5, 10
WM1789	3
294-H	3
1205LU	3

Figure 2. VNTRs of 15 bp were identified 5′ to the pre-miR-137. *A,* PCR products from amplification of the pre-miR-137 region from melanoma cell lines showing different numbers of VNTRs [WM1617 (5, 10), WM852 (12), WM793 (3), A375 (3)] when electrophoresed on a 3% agarose gel. *B,* sequence of the VNTR repeat region from cell lines A375 and WM852. The VNTR is TAGCAGCGGCAGCGG with the repeat regions underlined. Three repeats are found in A375 and 12 repeats in WM852. The existing restriction sites used to construct the miRNA expression plasmid containing 12 VNTRs are in italics. This sequence has been submitted to GenBank with accession no. 920034. *C,* VNTR 5′ to the pre-miR-137 in melanoma cell lines. The alleles are expressed as the number of VNTRs. When the allele is heterozygous for VNTR number, both VNTR numbers are included.

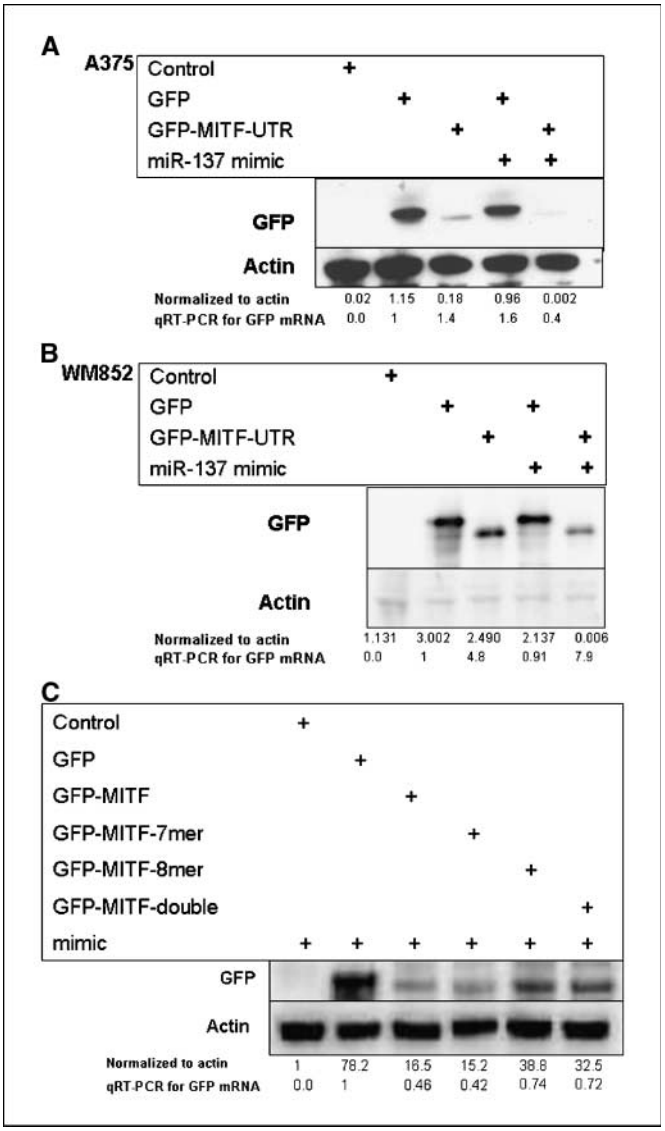


Figure 3. MITF is regulated through its 3′UTR. *A,* A375 cells were transfected with GFP or GFP-MITF-3′UTR plasmids with or without a mimic of miR-137. Shown is GFP protein expression measured by Western blot analysis and compared with a loading control of β-actin. GFP protein expression was down-regulated in cells containing the MITF-3′UTR and further down-regulated in cells with the MITF-3′UTR and the miR-137 mimic. Quantitative RT-PCR (*qRT-PCR*) of GFP compared with β-actin confirmed transfection. *B,* WM852 cells, which express high levels of MITF, were transfected as described above and examined for GFP protein expression by Western blot analysis and compared with a loading control of β-actin. The expression of the GFP-MITF-3′UTR decreased dramatically with the addition of mimic to miR-137. GFP proteins generated from the GFP-MITF-3′UTR plasmid are slightly smaller than the empty vector control because of an altered stop codon. *C,* A375 cells were transfected with GFP, GFP-MITF-3′UTR, GFP-MITF-7mer, GFP-MITF-8mer, GFP-MITF-double mutant plasmids, and miR-137 mimic. GFP protein expression was down-regulated in those cells with a functional MITF-3′UTR. Mutation of the 8-mer binding site in the GFP-MITF-3′UTR (GFP-MITF-8mer or GFP-MITF-double) blocked the suppression of GFP-MITF-3′UTR construct. Quantitative RT-PCR of GFP compared with β-actin confirmed transfection; however, as previously shown, GFP-MITF-UTR message is also reduced, suggesting that the mimic causes degradation of the message as well as the protein.

Mutations in miRNAs identified from CLL patient samples alter the ability of the miRNA to down-regulate target genes. Thus, we queried if mutations in either a binding site for miR-137 or in the miR-137 itself could have occurred in the WM852 cell line. To assess whether mutations exist in miR-137 and/or its target gene MITF in WM852, we examined the molecular integrity of the binding sites for miR-137 in the MITF-3′UTR and the pre-miR-137 in 12 melanoma cell lines. Genomic DNA from 12 melanoma cell lines was prepared and amplified by PCR. No mutations were found in the putative miR-137 binding sites of the MITF 3′UTR in any of the cell lines examined. We next examined the integrity of miR-137 itself. Primers designed for amplification of miR-137 included the entire pre-miRNA (pre-miR-137) and at least 20 bp on either side. When the PCR products were separated on an agarose gel, there was a dramatic size difference in two of the cell lines, WM1617 and WM852 (Fig. 2*A*). A VNTR was identified 6 bp 5′ to the pre-miR-137. The reference DNA sequence confirmed our finding with a reported three repeats (VNTR) of 15 bases each at this location (GenBank no. AK094607). In Fig. 2*C*, the VNTR copy number is included for both alleles when heterozygous alleles were detected. There were no mutations in the 22-bp sequence of the mature miR-137 in these cell lines. There were, however, differences in the number of VNTRs found in melanoma cell lines with the two highest repeats being in cell lines that express MITF (Figs. 1*C* and 2*C*).

To confirm the predicted regulation of MITF by miR-137 through its 3′UTR, a reporter plasmid was constructed. The reporter plasmid consisted of a GFP expression vector with 1,143 bp of the 3′UTR of MITF in place of the UTR in the eGFP-C1 plasmid. In this construct, binding of a miRNA to the MITF-3′UTR would lead to suppression of GFP expression. The most 3′ region of the 3,086 bp of the MITF 3′UTR was used for these studies thereby limiting the predicted miRNA binding to two highly likely candidate miRNAs, miR-137 and miR-218 (23). The GFP-MITF-3′UTR construct was transfected into melanoma cell line WM852 (12 VNTRs), which expresses MITF, and also into A375 (3 VNTRs), a melanoma cell line that does not express MITF. These studies then relied on the "native" expression and function of miRNAs in each cell line to suppress GFP expression through the MITF-3′UTR.

In these studies, the GFP protein expression was dramatically reduced in the A375 cell line that received GFP-MITF-3′UTR as compared with GFP empty vector, whereas the mRNA expression for GFP remained comparable or higher (Fig. 3*A*). In contrast, transfection of the GFP constructs into the WM852 cell line suppressed the GFP protein expression through the MITF-3′UTR to a lesser extent (Fig. 3*B*). To further evaluate the regulation of MITF through its 3′UTR, we cotransfected these same cells with a miR-137 mimic, the GFP control vector, and the GFP-MITF-UTR and examined GFP expression. Addition of the miR-137 mimic to A375 and WM852 cells had an added effect on reducing GFP expression through the GFP-MITF-3′UTR in A375 and was able to dramatically suppress GFP-MITF-3′UTR expression in WM852 (Fig. 3). These studies gave strong evidence that miR-137 regulates MITF expression through its 3′UTR and suggested that the WM852 cell line with 12 VNTRs in the pri-miR-137 could not as efficiently shut down GFP protein expression through the MITF-3′UTR. However, with addition of a functional miR-137 mimic, expression was dramatically reduced in the GFP-MITF-3′UTR–transfected WM852 cell line (Fig. 3*B*). Further confirmation that miR-137 regulates MITF by binding to the 3′UTR was accomplished by mutating the binding sites in the 3′UTR. When the binding site that contains the 8 bp of homology in the seed region (8-mer) was mutated, it no longer showed reduced expression by mimics to miR-137. In contrast, when the binding site that contains the 7 bp of

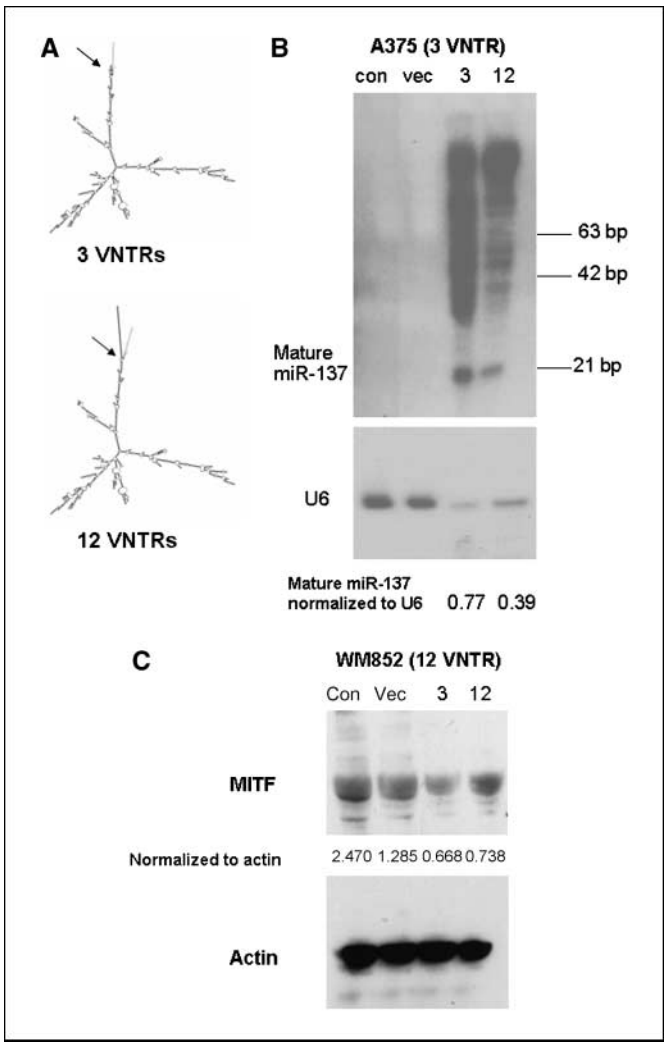


Figure 4. Twelve VNTRs in pri-miR-137 alter processing as compared with 3 VNTRs. *A,* VNTRs in the primary miRNA transcript of miR-137 alter the secondary structure of the pri-miR-137. MFold was used to predict the secondary structure of miR-137 with either the 12 VNTRs as identified in the cell line WM852 or with 3 VNTRs as observed in the cell line A375. *Arrow,* the region containing the VNTRs. *B,* transfection of the full-length miR-137 followed by Northern blot analysis of miR-137 shows a reduced ability to process miR-137 when it contains 12 VNTRs as compared with 3 VNTRs. A375 cells with three repeats of the VNTR in miR-137 were transfected with control vector, 3-repeat VNTR, or 12-repeat VNTR and probed for miR-137 expression. The 12-repeat shows a reduced processing of miR-137 to the mature size. Northern blot analysis was not sensitive enough to detect the native mature miR-137; however, quantitative RT-PCR showed that miR-137 mature sequence is present in A375. *C,* the full-length cDNA of miR-137 with 12 VNTRs is less capable of down-regulating MITF. Shown is MITF protein expression after transfection of WM852 cells with control vector, 3-VNTR-miR-137, and 12-VNTR-miR-137. The 3 VNTR–containing cDNA of miR-137 is able to block MITF protein expression.

homology in the seed region (7-mer) was mutated, there was no observable difference between the wild-type UTR. The double mutant in which both binding sites were mutant was similar in expression to the 8-mer mutant alone (Fig. 3*C*).

Predicted alterations in secondary structure of pri-miRNAs and resulting alterations in the expression of the mature miRNA have been somewhat controversial, with one report showing that alterations cause reduced expression of the mature miRNA (24) and another extensive study suggesting that alterations in the secondary structure of pri-miRNAs do not block processing to the mature form (25). Thus, we examined how the addition of 12 VNTRs alters secondary structure of pri-miR-137 as compared with the normal 3 VNTRs. We used a publicly accessible data base, MFold (19), to calculate the RNA secondary structure of the pri-miR-137 with the lowest free energy for the pri-miR-137 from A375 (3 VNTRs) and for that with 12 VNTRs (Fig. 4*A*). The secondary structure is clearly altered by the additional VNTRs in the cell line WM852 (Fig. 4*A*).

To determine if miR-137 was processed correctly in the WM852 cell line (12 VNTRs) as compared with the 3-VNTR miR-137 found in A375, Northern blot analysis was conducted. However, the native, mature miRNAs for miR-137 were at a low level of expression and the Northern blot analysis could not distinguish the endogenous mature miR-137 in A375 (Fig. 4*B*). Thus, highly sensitive quantitative RT-PCR methods were applied to determine the expression of miR-137 in melanoma cell lines (Fig. 1*C, NCode*). To confirm the expression, we also cloned the PCR product of the mature miR-137 from A375 and WM852 into T-vector. To further examine the hypothesis that this VNTR region could alter the processing of miR-137 and thus its ability to regulate MITF, the cDNA encoding the entire primary miR-137 with 3 VNTRs was obtained and transfected into cell lines WM852 and A375. A separate vector was constructed, which was identical to the wild-type vector except that it had 12 VNTRs obtained from WM852. These plasmid vectors were transfected into melanoma cell line A375. The pri-miR-137 with 3 VNTRs was shown to be processed to the correct size (Fig. 4*B*) by Northern blot analysis. However, the 12-VNTR construct was not as efficiently processed to the mature size (Fig. 4*B*).

To confirm that the VNTR 5′ to the pre-miR-137 alters its ability to down-regulate MITF expression, we transfected the melanoma cell line WM852 (12 VNTRs) with a vector expressing the full-length cDNA of miR-137 (3 VNTRs) and examined protein expression of MITF by Western blot analysis. WM852 was chosen because of its high expression of MITF and because it has no naturally occurring 3-VNTR repeat region (Fig. 2*C*). As shown in Fig. 4, the transient transfection with a plasmid encoding miR-137 with 3 VNTRs resulted in suppression of MITF protein expression compared with the plasmid that contained the 12 VNTRs in the primary miR-137

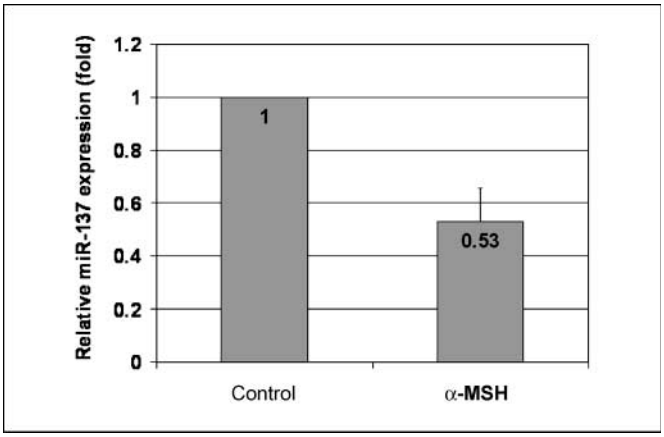


Figure 5. MiR-137 expression is altered in response to α-MSH. Stimulation of WM852 cells with α-MSH for 8 h results in the down-regulation of miR-137. miR-137 expression was quantified by the NCode method (Invitrogen) equalized to U6 RNA and expressed as relative quantitation (described in Materials and Methods). *Bars,* SE; the experiment was repeated in triplicate.

(Fig. 4*C*). These data indicate that the processing and function of miR-137 are altered by VNTR number and subsequently effect MITF expression.

Although fair skin is a well-known risk factor for skin cancer, the increased risk cannot be simply explained by pigmentary phenotype (26). UV light stimulates sun tanning by inducing the expression of α-MSH by the keratinocytes, which then stimulates the melanocytes to express MITF (27). Sun tanning is thought to protect the skin from DNA damage largely by activation of MITF (27) and its downstream action of stimulating melanogenesis (27). Based on our finding that miR-137 regulates the expression of MITF in melanoma cells, we examined the possibility that miR-137 might also be regulated by α-MSH (Fig. 5). The consistent down-regulation of miR-137 was observed in the WM852 cell line, suggesting that miR-137 down-regulation may be an important component of the sun tanning response. This will be the focus of further investigation because of the critical link between sun exposure and melanoma.

Discussion

MiRNAs are of ever increasing importance as regulators of gene expression following transcription. To date, hundreds of human miRNAs have been described and more are being added to the data bases(s) each day (28). They have been shown to have key regulatory roles during embryogenesis, cell development, and differentiation (29). With this in mind, miRNAs have recently been investigated in a variety of cell types and tissues including human neoplasms. In this study, we began by searching for miRNAs that were encoded in a genomic location predicted to contain a melanoma susceptibility gene but where no specific classic coding gene had been identified. This approach constitutes a new paradigm for discovery of regulatory molecules in melanoma, a disease not well characterized at the molecular level. Applying this approach of examining miRNAs located in a previously described melanoma hotspot, we uncovered miR-137 and were able to confirm its regulation of molecules previously implicated in melanoma. miR-137 was predicted by bioinformatics calculations as a candidate regulator of MITF, a major controller of melanocyte function. miR-137 is a classic 22-bp miRNA proposed to bind at two candidate sites in the 3′UTR of MITF. We were able to show by multiple methods, including transfection of melanoma cells with miR-137 mimics, full-length miR-137, and reporter constructs, that this miRNA regulates MITF.

In the present studies, we have shown, as predicted, that miR-137 regulates MITF protein expression and that an alteration occurs in some melanoma cell lines, preventing proper miR-137 function. We expect that miR-137 is not the only miRNA regulating MITF because other miRNAs are also predicted to regulate this important transcriptional regulator. Bioinformatics calculations indicate that there are at least six other miRNAs that may be important in this regard. How all of these interplay in the regulation of this or any other gene is only now being studied. Clearly, miR-137 is one of the regulators of MITF and is located at a melanoma hotspot in the human genome.

MiRNAs can be embedded in various regions including intronic and exonic sequences. The factors controlling their regulation are beginning to be elucidated but are not yet clear. miR-137 is embedded in a larger expressed but noncoding mRNA, which is thought to be the primary miRNA transcript. This transcript is then folded and cleaved to a pre-miRNA and further processed into the mature 22-bp miR-137. As a part of these studies, we have described a 15-bp VNTR in the primary miRNA of miR-137 and present data that alterations in the number of tandem repeats in this area interfere with mature miR-137 processing and function. Exactly how this might occur is currently under study in our laboratory. In previous studies, other investigators have examined the effect(s) of sequence variations in pre-miRNAs and primary miRNAs (24, 25). Most of these variations found in patient samples were single-base substitutions and led to predicted changes in the folding structure of pre-miRNAs. Despite altered folding predictions, little effect on the processing and expression of the mature miRNAs was found *in vivo*. Here we describe a much larger change in the pri-miRNA in a melanoma cell line that seems to effect expression and function of the mature form. An alteration of this nature has not been reported with other miRNAs. In preliminary studies, changes in VNTR number have been found not only in melanoma cells but also in other melanocytic disorders. Further understanding of the miR-137 VNTR may have important bearing on our understanding of MITF regulation in melanoma and other human pigmentary disorders and the protective aspects of sun tanning.

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