

Regulation of *RUNX3* Tumor Suppressor Gene Expression in Cutaneous Melanoma

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Abstract Purpose: *RUNX3* is a known tumor suppressor gene in several carcinomas. Aberration in *RUNX3* expression has not been described for cutaneous melanoma. Therefore, we assessed the expression of *RUNX3* in cutaneous melanoma and its regulatory mechanisms relative to tumor progression.

Experimental Design: The expression of *RUNX3* mRNA and miR-532-5p (microRNA) was assessed in melanoma lines and in primary and metastatic melanoma tumors.

Results: *RUNX3* mRNA expression was down-regulated in 11 of 11 (100%) metastatic melanoma lines relative to normal melanocytes ($P < 0.001$). Among 123 primary and metastatic melanoma tumors and 12 normal skin samples, *RUNX3* expression was significantly down-regulated in primary melanomas ($n = 82$; $P = 0.02$) and in melanoma metastasis ($n = 41$; $P < 0.0001$) versus normal skin ($n = 12$). This suggested that *RUNX3* down-regulation may play a role in the development and progression of melanoma. *RUNX3* promoter region hypermethylation was assessed as a possible regulator of *RUNX3* expression using methylation-specific PCR. Assessment of *RUNX3* promoter region methylation showed that only 5 of 17 (29%) melanoma lines, 2 of 52 (4%) primary melanomas, and 5 of 30 (17%) metastatic melanomas had hypermethylation of the promoter region. A microRNA (miR-532-5p) was identified as a target of *RUNX3* mRNA sequences. miR-532-5p expression was shown to be significantly up-regulated in melanoma lines and metastatic melanoma tumors relative to normal melanocytes and primary melanomas, respectively. To investigate the relation between *RUNX3* and miR-532-5p, anti-miR-532-5p was transfected into melanoma lines. Inhibition of miR-532-5p up-regulated both *RUNX3* mRNA and protein expression.

Conclusions: *RUNX3* is down-regulated during melanoma progression and miR-532-5p is a regulatory factor of *RUNX3* expression.

The prognosis for patients with American Joint Committee on Cancer (AJCC) stage I/II melanoma is excellent, with an average 10-year survival rate of 85% (1). However, as melanoma progresses from localized to metastatic disease, survival drops significantly. The 10-year survival rate for AJCC stage IV disease is less than 10% (1). A better understanding of the regulating factors contributing to melanoma tumor growth, progression, and metastases is needed.

Three members of the Runt-related (*RUNX*) family of genes, *RUNX1*, *RUNX2*, and *RUNX3* transcription factors, are known

as developmental regulators important in the inception and progression of a variety of human cancers and experimentally induced mouse tumors (2–8). *RUNX* are transcription factors that are known to function as scaffolds and interact with coregulatory factors often involved in tissue differentiation (9). *RUNX* proteins are located in the nucleus, whereby down-regulation of function has been linked to various cancers (9). Studies have also shown *RUNX* proteins to regulate gene expression by interacting with chromatin remodeling enzymes (10). *RUNX3*, in particular, has been shown to be involved in gastric tumor progression. In gastric cancer and other cancers, this gene plays a tumor suppressor role. Hypermethylation of the *RUNX3* promoter region down-regulates its expression (2, 11). *RUNX3* resides on chromosome 1p36, a chromosome site with widely associated aberrations, including in cutaneous melanoma (12, 13).

There have been no major reports of altered *RUNX3* expression in cutaneous melanoma. Based on patterns discerned from other malignancies, we hypothesized that *RUNX3* expression in melanoma may be suppressed and that levels of expression may relate to melanoma progression as in other cancers. We found that *RUNX3* expression was down-regulated in metastatic melanomas compared with primary tumors. The roles of promoter region hypermethylation and microRNA (miRNA) were investigated to examine possible mechanisms for *RUNX3* expression down-regulation.

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Translational Relevance

In malignant cutaneous melanoma, there is limited number of tumor suppressor genes known to be down-regulated during tumor metastasis. The study identifies the down-regulation of the tumor suppressor gene *RUNX3* in cutaneous melanoma during tumor progression. These studies suggest that *RUNX3* expression level may be a potential target for therapy and diagnosis. Identification of regulatory mechanisms of tumor suppressor genes may allow for the development of new approaches of targeted therapeutics. The mechanism of *RUNX3* mRNA down-regulation was shown to be through miR-532-5p. This novel finding suggests that blocking miR-532-5p may be a potential approach to up-regulate *RUNX3* expression as a treatment of cutaneous melanoma. The study shows specific microRNA to a tumor suppressor gene may be a significant epigenetic mechanism in regulating tumor development and progression.

Materials and Methods

Cell lines. Eleven melanoma lines (M1-M11) established from metastatic tumors of patients treated at John Wayne Cancer Institute/St. John's Health Center and were maintained in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and streptomycin (14). The pancreatic cancer cell line COLO 357 (a gift from Dr. M. Korc, University of California Irvine, Irvine, CA) served as a positive control for *RUNX3* expression. Kato III (American Type Culture Collection), a gastric cancer cell line that expresses *RUNX3* in low copy numbers, was used as a negative control. HeMn-MP (Cascade Biologics), a moderately pigmented human melanocyte cell line, was maintained in basal media 254 supplemented with human melanocyte growth supplement. Cell lines were kept in 75-cm³ flasks at 37°C in a 5% CO₂ incubator.

Melanoma specimens. Approval for the use of patient specimens was granted by the joint John Wayne Cancer Institute/St. John's Health Center Institutional Review Board. The John Wayne Cancer Institute melanoma patient database and St. John's Health Center Cancer Registry were used to identify all patients treated for primary or metastatic melanoma between 1995 and 2004. The final pathologic diagnosis and availability of all paraffin-embedded melanomas were confirmed with the St. John's Health Center Department of Pathology. Only specimens with ≥60% tumor cells evident during light microscopic analysis were further processed and analyzed. The study population demographics is given in Table 1.

A total of 123 melanomas were assayed, including both primary (*n* = 82) and metastatic tumors (*n* = 41). A list of patients with nonmalignant nevi, skin, lymph nodes, and visceral tissues were obtained from the database coordinator to serve as normal controls.

miRNA, RNA, and DNA isolation. Genomic DNA was extracted from cell lines using DNAzol Genomic DNA Isolation Reagent (Molecular Research Center, Inc.) according to the manufacturer's recommendations.

Total RNA for the mRNA study was extracted with the TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. Total RNA for miRNA study was extracted from cells by using mirVana miRNA Isolation kit (Ambion, Inc.) according to the manufacturer's recommendations. Quality and quantity of extracted DNA and RNA were measured by UV absorption spectrophotometry and RiboGreen (Molecular Probes). Only specimens with high-quality mRNA were included in the study.

For RNA extraction from paraffin-embedded tissues, seven sections of 10-μm thickness were cut from each paraffin block using a new sterile microtome blade for each block. Sections were then deparaffinized and digested with proteinase K before RNA extraction using the RNAwiz RNA Isolation Reagent (Ambion, Inc.) following a modification of the manufacturer's protocol (15). Pellet Paint NF (Novagen) was used as a carrier in the RNA precipitation step.

Quantitative real-time PCR primers and probes. *RUNX3* primers were designed to span at least one exon-intron-exon region to optimally amplify cDNA and minimize genomic DNA amplification. Amplification of each amplicon was confirmed by gel electrophoresis. Primer and probe sequences for *RUNX3* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) are provided below. *RUNX3*: 5'-GACAGCCCCAAC-TTCCTCT-3' (forward), 5'-CACAGTCACCCCGT ACCAT-3' (reverse), 5'-FAM-AAGGTGGTGGCATTGGGGGA-BHQ-1-3' (FRET probe); *GAPDH*: 5'-GGGTGTGAACCATGAGAAGT-3' (forward), 5'-GACTGTG-GTCATGA GTCCT-3' (reverse), and 5'-FAM-CAGCA ATGCCTCTGCA-CCACCAA-BHQ-1-3' (FRET probe).

Quantitative real-time reverse transcription-PCR. Reverse transcription of total RNA was performed using Moloney murine leukemia virus reverse transcriptase (Promega) as previously described (16). Probe-based quantitative real-time reverse transcription-PCR (qRT) was performed in a 96-well plate format using the ABI Prism 7000 (Applied Biosystems, Inc.) in a blinded fashion. A standard amount of total RNA (250 ng) was used for all reactions. The qRT assay was optimized using established melanoma cell lines and paraffin-embedded metastatic tumors. The accuracy and reproducibility of the assay was ensured by

Table 1. Patient characteristics

Patient characteristics			
Patients (<i>n</i>)			
Men		67 (54.5%)	
Women		56 (45.5%)	
	Total	123 (100%)	
Mean age (y)		65 (range, 14-90)	
Median follow-up (mo)		44 (range, 3-149)	
Tumor characteristics			
Tumors assessed (<i>n</i>)			
Primary	AJCC stage I	45 (54.9%)	
	AJCC stage II	21 (25.6%)	
	AJCC stage III	16 (19.5%)	
	Total primary	82 (100%)	
	Sites	Superficial spreading	45 (54.9%)
		Nodular	19 (23.1%)
		Desmoplastic	8 (9.8%)
		Lentigo maligna	6 (7.3%)
		Acral lentiginous	4 (4.9%)
		Mean Breslow depth (mm)	2.06 (range, 0.19-11)
Clark level II		14 (17.1%)	
Clark level III	20 (24.4%)		
Clark level IV	32 (39%)		
Clark level V	13 (15.9%)		
Clark level unknown	3 (3.6%)		
Ulceration	14 (17.1%)		
Metastasis	AJCC stage III	19 (46%)	
	AJCC stage IV	22 (54%)	
Total metastatic		41 (100%)	
Sites	Subcutaneous tissue	8 (36%)	
	Lung	6 (27%)	
	Brain	2 (9%)	
	Gastrointestinal	4 (18%)	
	Distant lymph nodes	1 (5%)	
	Breast	1 (5%)	

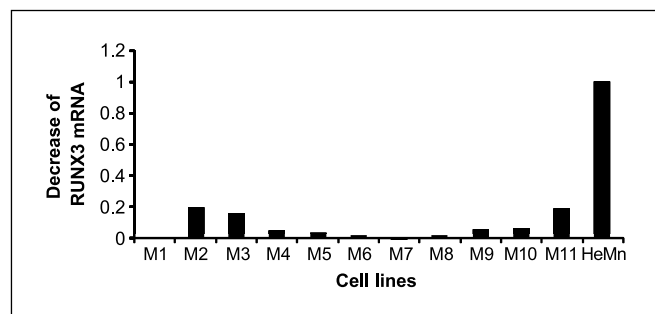


Fig. 1. Relative RUNX3 expression in melanoma cell lines (M1-M11) and normal human melanocytes (HeMn). Melanoma cell lines have significantly lower *RUNX3* gene expression than normal melanocyte line HeMn ($P < 0.001$). The assays were run in triplicate.

comparing qRT results from different sections of the same tumor and including the necessary controls for all reactions. We transferred 5 μ L of cDNA from 250 ng of total RNA to a well of a 96-well PCR plate in which 0.2 μ mol/L of each primer, 0.3 μ mol/L FRET probe and iTaq custom Supermix (Bio-Rad Laboratories) to a final volume of 25 μ L. Each PCR reaction was composed of 45 cycles at 95°C for 60 s, 60°C for 60 s, and 72°C for 60 s for RUNX3; and 45 cycles at 95°C for 60 s, 55°C for 60 s, and 72°C for 60 s for GAPDH. Each assay was done with standard curves, positive controls (cell lines), negative controls (cell lines), and reagent controls (reagents without cDNA; ref. 17). Expression of the housekeeping gene *GAPDH* was assessed in each sample to verify mRNA integrity. RUNX3 expression was designated as a ratio of RUNX3/GAPDH mRNA units. RUNX3 mRNA expression ratios from established melanoma cell lines were compared with the mean mRNA expression ratio from normal melanocytes. Patient specimens were normalized with respect to the mean RUNX3/GAPDH mRNA expression ratios from normal tissues to account for low background levels of RUNX3 expression in melanoma tissues. All assays were done in triplicate.

DNA extraction and sodium bisulfite modification. DNA was extracted from a subset of paraffin-embedded melanoma specimens ($n = 82$) previously assayed by qRT. Light microscopy was used to confirm tumor location and assess tumor tissue for microdissection. Additional sections from the tumor block were mounted on glass slides and microdissected under light microscopy. Dissected tissues were digested with 50 μ L of proteinase K-containing lysis buffer at 50°C for 5 h, followed by heat deactivation of proteinase K at 95°C for 10 min. Sodium bisulfite modification was applied on extracted genomic DNA of tissue specimens or cell lines for methylation-specific PCR or bisulfite sequencing as described previously (18).

Detection of methylated RUNX3. Sodium bisulfite modification was applied on extracted genomic DNA of tissue specimens and cell lines for methylation-specific PCR (18). Methylation-specific and unmethylated-specific primer sets were designed; optimization for methylation-specific PCR included annealing temperature, Mg^{2+} concentration, and cycle number for specific amplification of the methylated and unmethylated target sequences. The primers were dye labeled for automatic detection by capillary array electrophoresis. The methylation-specific primer set was as follows: forward, 5'-D4-AACGTTATC-GAGGTGTTCCG-3'; and reverse, 5'-CGGAAATTAATACCCCGAA-3'. The unmethylation-specific primer set was as follows: forward, 5'-D3-GAATGTTATTGAGGTGTTTGTGA-3'; and reverse, 5'-CACAAAATTA-TACCCCAAAA-3'. PCR amplification was done in a 10- μ L reaction volume with 1 μ L template for 36 cycles of 30 s at 94°C, 30 s at 63°C for methylated and 60°C for unmethylated reaction, and 30 s at 72°C, followed by a 7-min final extension at 72°C. The PCR reaction mixture consisted of 0.3 μ mol/L of each primer, 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, Inc.), 200 μ mol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, and PCR buffer to a final

volume of 10 μ L. A universal unmethylated control was synthesized from normal DNA by *phi*-29 DNA polymerase and served as a positive unmethylated control (19). Unmodified lymphocyte DNA was used as a negative control for methylated and unmethylated reactions. *SssI* Methylase (New England Bio Labs)–treated lymphocyte DNA was used as a positive methylated control. PCR products were detected and analyzed by capillary array electrophoresis (CEQ 8000XL, Beckman Coulter, Inc.) with CEQ 8000XL software version 8.0 (Beckman Coulter) as described previously (20).

Detection of miRNA by real-time stem-loop reverse transcription-PCR. Reverse transcriptase reactions contained total RNA, 50 nmol/L stem-loop reverse transcription primer for miR-532-5p, and TaqMan MicroRNA Reverse Transcription kit (1 \times reverse transcriptase buffer, 0.25 mmol/L each of deoxynucleoside triphosphates, 3.33 units/ μ L MultiScribe reverse transcriptase, and 0.25 unit/ μ L RNase inhibitor; Applied Biosystems, Inc.) following the manufacturer's protocol. The reactions were incubated in a thermocycler in a 384-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. All reverse transcriptase reactions, including no-template controls and reverse transcription–minus controls, were run in duplicate.

All primers and probes are designed based on miRNA sequences released by the Sanger Institute (21). The primer and probe was designed by Primer Express software (Applied Biosystems, Inc.) as previously described (22, 23). The miR-532-5p sequence is 5'-CAUGCCUUGAGUGUAGGACCGU-3'. The loop reverse transcription primer is 5'-CTCAACTGGTGTCTGGAGTCCGCAATTCAGTTGACGCGTCT-3'. The forward primer is 5'-GCTGGGCATGCCTTGAGT-3'. The universal reverse is 5'-CTCAACTGGTGTCTGGAGT-3'. The TaqMan probe is (6-FAM)-TTCAGTTGAGACGGTCTCT-MGB. The underlined sequences are specific for miR-532-5p.

qRT was done in a 384-well plate format using the ABI Prism 7000 (Applied Biosystems, Inc.) in blinded fashion. The 10 μ L PCR included 0.67 μ L reverse transcription product, 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems, Inc.), 0.2 μ mol/L TaqMan probe, 1.5 μ mol/L forward primer, and 0.7 μ mol/L reverse primer. The reactions were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. All reactions were run in triplicate. Standard curves were generated by using a threshold cycle of eight serially diluted (10 to 10⁸ copies) plasmids containing stem-loop reverse transcribed cDNA of miR-532-5p. The threshold cycle of each sample was interpolated from the standard curves and the number of miRNA copies was calculated by ABI software.

miRNA transfection. Anti-miR miRNA inhibitors (Ambion) are chemically modified, single-stranded nucleic acids designed to specifically bind and inhibit endogenous miRNA molecules. These ready-to-use inhibitors can be introduced into cells via a similar transfection strategy used for siRNAs, thereby facilitating the study of miRNA biological effects. Anti-miR miR-532-5p miRNA and the anti-miR negative control were transfected into a melanoma cell line using the reverse transfection protocol recommended by the manufacturer. In brief, siPORT NeoFX Transfection Agent (Ambion) was diluted in Opti-MEM medium (Invitrogen). Anti-miR miR-532-5p miRNA (Ambion) was also diluted in Opti-MEM medium for a final concentration of 30 nmol/L. The diluted transfection reagent was combined with the diluted miRNA duplex followed by incubation at room temperature for 10 min. The mixture was dispensed into an empty six-well dish and then seeded at 2.3×10^5 cells per well. The same amount of negative control miRNA duplex was also transfected. Total RNA was extracted at 72 h posttransfection and used for the mRNA qRT assay. Additional transfections were done to analyze RUNX3 protein expression by flow cytometry.

Flow cytometry. Transfected cells (1×10^6) were fixed and permeabilized by BD Cytotfix/Cytoperm kit (BD Biosciences) and incubated at 4°C for 1 h with RUNX3 goat polyclonal antibody (1 μ g; Santa Cruz Biotechnology, Inc.) or an isotype-matched control antibody. Rabbit anti-goat IgG-FITC (Santa Cruz Biotechnology, Inc.)

was used as secondary antibody. Flow cytometry was performed using FACSCalibur (Becton Dickinson) and data were analyzed with Cell Quest software (Becton Dickinson).

Biostatistical analysis. In primary and metastatic paraffin-embedded melanomas, comparisons of RUNX3/GAPDH mRNA expression in normal paraffin-embedded tissues were performed across all AJCC stages using the Kruskal-Wallis test. In primary melanomas, RUNX3 expression was correlated with age at diagnosis, Breslow thickness, and Clark level using Spearman's rank correlation; differences in RUNX3 expression according to AJCC stage, gender, Clark level, histologic subtype, and presence of ulceration were assessed via the Kruskal-Wallis test or Wilcoxon two-sample test as appropriate. The RUNX3 expression in metastatic melanoma tumors was correlated with patient age at diagnosis and gender in a univariate analysis.

A Cox regression model was used to identify predictors of 5-year overall survival. After finding that AJCC stage II and III primary tumors showed very similar survival curves, we combined these two groups. Factors included in the model were ulceration, Breslow depth (mm), AJCC stage, Clark level, gender, and RUNX3 expression. Potential predictors of overall 5-year survival were entered into the multivariate model using a backward elimination method. Hazard ratios (HR) and 95% confidence intervals (95% CI) were reported for each variable.

RUNX3 mRNA and miR-532-5p expression in AJCC stages I, II, and III primary melanoma tumors were correlated using the Spearman rank correlation test.

Results

mRNA expression of RUNX3 in melanoma cell lines. Initially, in studying alteration of RUNX3 expression in melanoma, expression of RUNX3 mRNA in 11 established melanoma lines and a normal human melanocyte line were assessed. Relative to normal melanocyte RUNX3 gene expression, all 11 established melanoma lines (Fig. 1) showed significantly lower RUNX3 gene expression ($P < 0.001$). On determining this finding, we went on to assess RUNX3 expression in primary and metastatic cutaneous melanomas.

RUNX3 expression in primary and metastatic melanoma tumors. There were 56 women and 67 men included in this study. The mean age of the study population was 65 years (median = 67 years) and the median time of clinical follow-up for the study was 44 months (range, 3-149 months). Patient and tumor characteristics studied are presented in Table 1. Briefly, 123 melanoma tumors from 123 patients were assayed in this study. Of these, 82 were primary tumors (AJCC stage I, $n = 45$; AJCC stage II, $n = 21$; AJCC stage III, $n = 16$). The histopathology included superficial spreading ($n = 45, 54.9%$),

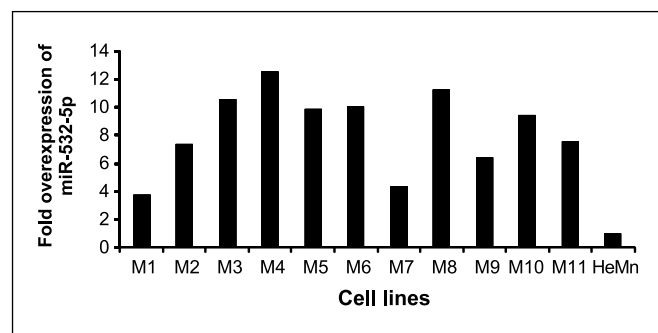


Fig. 2. The ratio of expression levels of miR-532-5p in melanoma cell lines compared with HeMn by qRT. Expression of miR-532-5p in melanoma lines was higher than in HeMn by qRT. The assays were in duplicate.

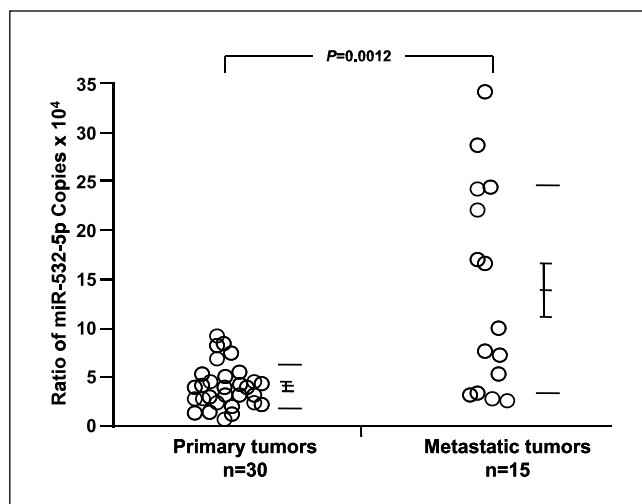


Fig. 3. Expression level of miR-532-5p in primary and metastatic melanoma tumors. Metastatic melanoma tumors have a significantly higher expression level of miR-532-5p compared with primary melanomas ($P = 0.0012$). The assays were in duplicate.

nodular ($n = 19, 23.1%$), acral lentiginous ($n = 4, 4.9%$), lentigo maligna ($n = 6, 7.3%$), and desmoplastic ($n = 8, 9.8%$).

The mean RUNX3 mRNA expression was significantly different in comparison of normal skin versus AJCC stages I, II, and III primary melanomas ($P = 0.02$). RUNX3 expression in AJCC stages I, II, and III primary melanomas was significantly lower than RUNX3 expression in normal skin samples ($P = 0.01, P = 0.02, \text{ and } P = 0.01$, respectively). RUNX3 expression showed a nonlinear association with AJCC stage. No significant correlations between RUNX3 and known prognostic factors such as age, gender, Breslow thickness, Clark level, primary tumor ulceration, or histopathology were found.

Of the 123 melanomas assayed for this study, 41 were metastatic tumors (AJCC stage III, $n = 19$; AJCC stage IV, $n = 22$). The mean RUNX3 mRNA expression was significantly down-regulated among melanoma metastases versus normal tissue overall (Kruskal-Wallis, $P < 0.0001$). In comparison of AJCC stage IV melanoma metastases with primary melanomas (AJCC stages I, II, and III), RUNX3 mRNA expression was significantly ($P = 0.0004$; Wilcoxon) down-regulated. RUNX3 mRNA expression was also significantly down-regulated in AJCC stage IV melanoma metastases relative to normal tissues ($P = 0.0006$). Decreased RUNX3 mRNA correlated with decreased RUNX3 protein expression, as was confirmed by flow cytometry analysis on melanoma cell lines using a specific anti-RUNX3 antibody.

Survival analysis. Overall survival was assessed regarding RUNX3 expression in primary cutaneous melanomas. In analysis of AJCC stages II/III primary melanoma patients ($n = 35$), significant factors predicting overall survival in the multivariate model showed that Clark level (HR, 5.27; 95% CI, 1.35-20.56; $P = 0.02$), gender (HR, 4.38; 95% CI, 1.13-16.95; $P = 0.03$), and RUNX3 mRNA expression (HR, 1.01; 95% CI, 1.00-1.02; $P = 0.02$) were significant. With these three variables included in the multivariate model, AJCC stage, ulceration, and Breslow depth did not significantly influence overall survival. The multivariate analysis showed that down-regulation of RUNX3 expression in metastatic melanomas was related to

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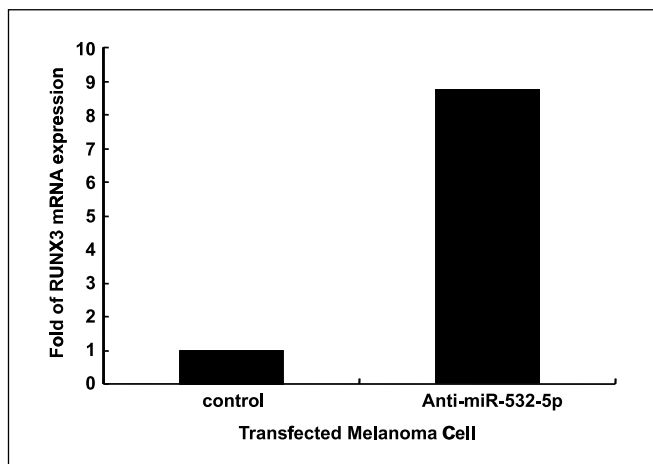


Fig. 4. Expression of RUNX3 mRNA levels in anti-miR-532-5p-transfected melanoma cells compared with negative control-transfected cells. Anti-miR-532-5p-transfected melanoma cells showed up-regulation of RUNX3 mRNA levels compared with negative control-transfected cells. The experiments are mean of duplicates.

disease outcome. We then investigated potential mechanisms for RUNX3 down-regulation in metastatic melanoma cells.

RUNX3 promoter region hypermethylation. Because down-regulation of RUNX3 mRNA expression has been related to gene promoter region CpG island hypermethylation in other cancers, we examined this epigenetic regulatory mechanism in cell lines and primary and metastatic melanoma specimens. Aberrant promoter region hypermethylation of CpG islands is thought to play an important role in the inactivation of many tumor suppressor genes in cancers. Specifically, hypermethylation of the RUNX3 promoter region has been shown to down-regulate RUNX3 expression in other malignancies (8, 11). We assessed the promoter region hypermethylation of RUNX3 in melanoma by methylation-specific PCR analysis. Five of 17 (29%) melanoma lines assayed showed evidence of RUNX3 promoter region methylation. Of the 82 melanoma specimens assessed, 7 (9%) showed evidence of RUNX3 DNA hypermethylation. Only 2 of 52 (4%) primary melanomas showed RUNX3 DNA hypermethylation, whereas 5 of 30 (16.7%) of metastatic melanomas showed hypermethylation. The results showed that promoter region hypermethylation is unlikely to play a significant role in the down-regulation of RUNX3 expression during melanoma metastasis. However, the analysis showed that hypermethylation of RUNX3 frequency increased only slightly in metastatic tumors.

miR-532-5p expression in melanoma. We next focused our attention on miRNA, another mechanism by which mRNA expression may be regulated (24, 25). Searching through the miRBase database (21), we found a specific miRNA sequence to RUNX3 mRNA. The miR-532-5p was a candidate miRNA to target the RUNX3 gene according to miRBase Targets version 3.⁴ For miR-532-5p, the miRNA sequence is 5'-CAUGCCUUGAGUGUAGGACCGU-3'. The underlined sequences (UGC-CAGGAUGUGAGUUCCGUAC) on miR-532-5p binds to RUNX3 mRNA (UAGGUCCUAGCAGAAGGCAUU). The miR-

532-5p is complementary to the 3' UTR sequence of the RUNX3 gene. We hypothesized that miR-532-5p may be highly expressed in melanoma and suppresses RUNX3 mRNA expression.

Eleven established cell lines and a normal melanocyte cell line were assessed for the expression of miR-532-5p. Higher miR-532-5p expression was seen in 11 of 11 established metastatic melanoma cell lines relative to normal melanocytes (Fig. 2).

The miR-532-5p expression in paraffin-embedded metastatic melanoma tumors was analyzed and shown to be significantly higher than in primary melanomas ($P = 0.0012$; Fig. 3). These results showed that miR-532-5p was up-regulated in melanoma as progression from primary to systemic metastasis occurs. There was an overall inverse relation of RUNX3 mRNA expression and miR-532-5p expression.

RUNX3 activated by anti miR-532 in melanoma. To validate that miR-532-5p inhibits the RUNX3 expression in melanoma, we transfected melanoma cells with anti-miR miR-532-5p miRNA (complementary sequences with miR-532-5p, Ambion), which was designed to inhibit miR-532-5p. RUNX3 mRNA expression in anti-miR-532-5p miRNA-transfected melanoma cells was up-regulated relative to anti-miR negative control-transfected melanoma cells (Fig. 4). RUNX3 protein expression was also up-regulated in anti-miR-532 miRNA-transfected melanoma cells compared with nontransfected cells as shown by flow cytometry (Fig. 5). These results showed that inhibition of miR-532-5p up-regulated the RUNX3 expression in melanoma cells at the mRNA and protein level and indicated that miR-532-5p can inhibit the RUNX3 at the mRNA level.

Discussion

Although present in many cell types, the role of RUNX3 in normal cellular development is not well understood. It is most

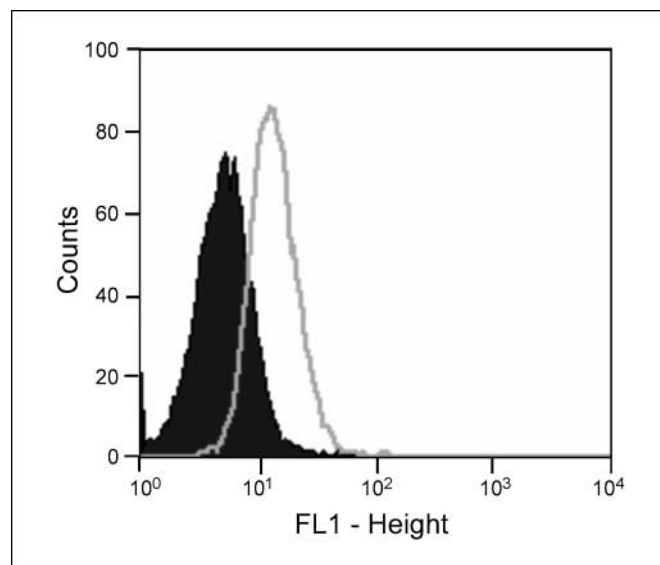


Fig. 5. Expression of RUNX3 protein levels in anti-miR-532-5p-transfected melanoma cells compared with negative control-transfected cells by flow cytometry. Anti-miR-532-5p-transfected cells (gray) showed up-regulation of RUNX3 protein levels compared with negative control-transfected cells (black). The studies were in duplicate.

⁴ <http://microrna.sanger.ac.uk/targets/v3/>

prominent in the dorsal root ganglia, hematopoietic cells, and gastrointestinal tract, where it is thought to play a role in cell differentiation and development (2). In humans, loss of RUNX3 expression has been related to promoter region CpG island hypermethylation in several cancers (26–28), particularly in gastric cancer development and progression (2, 11). RUNX3 has been implicated as a tumor suppressor gene. RUNX3 has not been previously examined with respect to cutaneous melanoma. This is, to our knowledge, the first report describing abnormal RUNX3 expression in primary and metastatic cutaneous melanomas.

Our results showed that RUNX3 mRNA expression was more suppressed in primary melanomas than in normal tissues and further more suppressed in metastatic melanomas compared with normal tissues. This indicated a role for RUNX3 gene expression in melanoma development and progression. In general, RUNX3 expression in melanoma may play a similar important role as a tumor suppressor gene as in gastric cancer but regulation is through a different mechanism (7, 11, 29). Interestingly, recent studies have shown that RUNX3 expression is relevant in developmental neurogenesis (30). RUNX3 is suggested to regulate neuron differentiation functions (31). Melanocytes, from which cutaneous melanoma is derived, have a neuroectodermal origin (32). Mueller et al. have also recently identified in glioblastomas that RUNX3 promoter region was hypermethylated in 56% of tumors (26).

Oddly, in melanoma, hypermethylation of the promoter region of RUNX3 does not play a major role in regulation as in other tumors (2, 11). Our results showed low frequency of hypermethylation of the RUNX3 promoter region in melanoma lines and melanoma tumors. These results suggested that RUNX3 expression in melanoma is likely suppressed by other epigenetic regulatory mechanisms other than hypermethylation. RUNX3 is located on chromosome 1p36, which previously has been shown to be a site of genomic deletions in cutaneous melanoma (33). Previously, we have shown that allelic imbalance of the microsatellite region of 1p36 in melanoma tumors can be up to 38%. However, these allele imbalances do not always correlate to loss of specific gene expression in that region. Nevertheless, this genomic region has been under considerable scrutiny for putative tumor-related genes.

Mature miRNAs are 19 to 25 nucleotide noncoding RNA molecules that can down-regulate various gene products by translational repression (25, 34). This occurs when partially complementary sequences are present in the 3' untranslated regions of the target mRNAs or by directing mRNA degradation (25). miRNAs can be expressed in a tissue-specific manner and are considered to play important roles in cell proliferation, apoptosis, and differentiation during mammalian development (24, 34, 35). Moreover, recent studies have shown a link between patterns of miRNA expression and the development of cancer (36) and down-regulation of specific cancer-related

genes (37–39). miR-532-5p, which had a complementary sequence to the 3' untranslated region, was assessed as a candidate miRNA targeting the RUNX3 mRNA as a potential down-regulating mechanism. We hypothesized that miR-532-5p is highly expressed in melanoma and may suppress RUNX3 expression. The results showed that miR-532-5p expression is significantly increased in melanoma cell lines and metastatic melanoma compared with normal melanocytes and primary melanomas, respectively. Moreover, we showed that inhibition of miR-532-5p up-regulated RUNX3 mRNA and protein expression in melanoma lines. These findings showed that miR-532-5p regulates RUNX3 expression in melanomas. The studies also suggest that miR-532-5p may play a role as a regulatory factor in melanoma progression. The miRNA-532-5p is located on chromosome region Xp11.23, whereby there is several other miR located nearby in that region.

In melanoma patients, RUNX3 mRNA expression was a significant predictor of overall survival. Although the influence of RUNX3 expression on survival was dominated by more significant factors such as Clark level and gender, it remained a more significant predictor of survival than Breslow depth, AJCC stage, and tumor ulceration in the small sample size assessed.

Melanoma metastasis is commonly associated with a poor prognosis and, therefore, targeting these mechanisms may lead to more effective treatments for patients. Therapeutic strategies to decrease miR-532-5p may potentially be useful for limiting melanoma metastasis. Further work is warranted to evaluate the role of miR-532-5p and to develop therapeutic strategies targeting miR-532-5p *in vivo*. Moreover, aberrantly expressed miRNA, such as miR-532-5p, may be a useful biomarker for diagnosis and prognosis in melanoma. Recent advances in techniques for the identification of miRNA should facilitate the use of clinical specimens for this purpose. The identification of critical targets for individual RUNX3 miRNAs may provide novel insights into the mechanisms of progression in melanoma.

We have shown in this study that RUNX3 can be suppressed by both miR and hypermethylation of the promoter region. Previously, we have shown that the 1p36 region where RUNX3 is located has allelic imbalance. These three types of molecular aberrations collectively may suppress RUNX3 during development and metastasis of melanoma. The role of RUNX3 in melanoma progression is not known but may follow similar mechanistic pathways as found in other cancers. A recent study has found that RUNX3 forms a ternary complex with β -catenin/TCF4 and attenuates Wnt signaling (40), which is known to play an important role in melanoma progression (41).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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