Translational Science

Cancer Research

miR-204-5p and miR-211-5p Contribute to BRAF Inhibitor Resistance in Melanoma

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Abstract

Melanoma treatment with the BRAF V600E inhibitor vemurafenib provides therapeutic benefits but the common emergence of drug resistance remains a challenge. We generated A375 melanoma cells resistant to vemurafenib with the goal of investigating changes in miRNA expression patterns that might contribute to resistance. Increased expression of miR-204-5p and miR-211-5p occurring in vemurafenib-resistant cells was determined to impact vemurafenib response. Their expression was rapidly affected by vemurafenib treatment through RNA stabilization. Similar effects were elicited by MEK and ERK inhibitors but not AKT or Rac inhibitors. Ectopic expression of both miRNA in drug-naïve human melanoma cells was sufficient to confer vemurafenib resistance and more robust tumor growth *in vivo*. Conversely, silencing their expression in resistant cells inhibited cell growth. Joint overexpression of miR-204-5p and miR-211-5p durably stimulated Ras and MAPK upregulation after vemurafenib exposure. Overall, our findings show how upregulation of miR-204-5p and miR-211-5p following vemurafenib treatment enables the emergence of resistance, with potential implications for mechanism-based strategies to improve vemurafenib responses.

Significance: Identification of miRNAs that enable resistance to BRAF inhibitors in melanoma suggests a mechanism-based strategy to limit resistance and improve clinical outcomes. *Cancer Res; 78(4); 1017–30. ©2017 AACR.*

Introduction

The *BRAF* V600E mutation is the most prevalent genetic alteration in malignant melanoma, and the focus of recently developed BRAF inhibitors (BRAFi), such as vemurafenib and dabrafenib (1–3). Both agents have provided substantial benefits for melanoma patients, but a major challenge in melanoma treatment with MAPK-targeted therapy is an almost universal emergence of resistance that leads to patient relapse. The most frequent mechanisms involved in BRAFi resistance of melanoma cells converge in the reactivation of the MAPK pathway usually following *NRAS* mutations (4), alterations in *BRAF* splicing (5), as well as *BRAF* amplification (6, 7). Another signaling route mediating melanoma resistance to BRAFi is the PI3K–Akt pathway, which becomes hyperactivated in some patients (8). Yet, a significant portion (40%) of tumors displays unknown resistance mechanisms (9) that cannot be accounted for genetic alterations (10).

The class of small noncoding RNAs called miRNAs has emerged as key posttranscriptional regulator in tumor progression. Mature miRNAs are 20–30 nucleotide-long RNAs that, by targeting mRNA transcripts, keep the transcriptome under tight control. miRNAs base pair to partially complementary motifs in target mRNAs, usually in the 3′ UTR, leading to translational repression or exonucleolytic mRNA decay (11). The first indication that miRNAs play important roles in cancer came from an early study showing that the miR-15/16 cluster is frequently deleted in chronic lymphocytic leukemia, therefore implicating miRNAs as tumor suppressors (12). Moreover, transgenic expression of miR-21 initiates lymphomagenesis in mice (13). Despite a more frequent pattern of reduction in the levels of miRNAs in cancer, several miRNAs are upregulated and play oncogenic roles, which have led to call them oncomiRs, such as the miR-17/92 cluster, which is upregulated in several cancer cell types (14).

Large-scale expression profiling and deep-sequencing approaches have revealed that miRNAs play pivotal roles in melanoma progression. Some of these miRNAs have tumor suppressor roles, such as let-7b and miR-137 (15, 16), whereas other act as oncomiRs, including miR155, miR-30b/30d, and miR-182 (17–19). Importantly, miR-137 expression correlates with melanoma's patient clinical outcome, with lower miR-137 levels associated to shorter survival of stage IV patients (20). Various miRNAs control melanoma cell invasion and metastasis, including the miR-211 (21).

Several miRNAs have been linked to resistance responses in different cancers (22), but only few recent studies have so far addressed the possible involvement of miRNAs in BRAFi resistance of melanoma. Thus, miR-200c and miR-7 have been shown to be reduced in BRAFi-resistant cells (23, 24). In this study, we

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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performed RNA-seq analyses comparing miRNA expression in parental and vemurafenib-resistant melanoma cells, and identified and characterized selected miRNAs that contribute to BRAFi resistance.

Materials and Methods

Cells and reagents

The human melanoma cell line A375 was latest authenticated in August 2017 at Secugen by short tandem repeat analysis. The melanoma cell lines SK-Mel-103, SK-Mel-28, and SK-Mel-147 were gifts from Dr. Marisol Soengas (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; April 2014), and were not authenticated in our laboratory. All cell lines were used within 5–50 passages of thawing the original stocks, were tested every 3 months for mycoplasma contamination, and cultured in DMEM supplemented with 10% FBS (Gibco; complete medium). Vemurafenib-resistant A375 cells (A375-VR) were derived from parental A375 cells by treatment with sequential increases of vemurafenib (Selleckchem) concentrations, from 10 nmol/L to 1.3 μmol/L, and were finally maintained as an uncloned resistant cell population in complete medium with 1.3 μmol/L of vemurafenib. We also obtained A375 cells growing with the MEK inhibitor trametinib (Selleckchem; 40 nmol/L; A375-TR).

Vectors and lentiviral-mediated gene transfer

Lentiviral vectors carrying miRNA precursor transcripts (H-miR-204-5p or H-miR-211-5p; System Biosciences), or antisense miRNA sequences (Zip-mIR-140-3p; System Biosciences) were used to stably overexpress mature miRNAs or inhibit the endogenous miRNAs, respectively. Pre-miR and anti-miR-scramble sequences (H-scr and Zip-scr) were used as negative controls (System Biosciences). For virus production, HEK-293FT cells were transfected with H-miR or Zip-miR vectors, pPAX2 and pMD2G, using Lipofectamine 2000 (Invitrogen). Viruses were collected and filtered 48 hours after transfection, and infection of A375 or SK-Mel-28 cells was performed for 72 hours using polybrene (Millipore). Cells were subsequently assessed by real-time quantitative PCR (qPCR). Triple-miRNA transductants were derived from H-miR-204 cells that were coinfected with H-miR-211 and Zip-miR-140 viruses using polybrene. SK-Mel-28 double-miRNA transductants were derived from SK-Mel-28 H-miR-211 cells that were infected with H-miR-204 viruses.

Oligonucleotide and siRNA transient transfection

miRIDIAN miRNA Hairpin Inhibitors (Dharmacon) for miRNA-204-5p and miRNA-211-5p (40 nmol/L) were transfected using Lipofectamine 2000 according to the manufacturer's protocol. miRIDIAN miRNA Hairpin Inhibitor Negative Control #1 (Dharmacon) was used as negative control. siRNAs were transfected using Interferin (Polyplus Transfection), following manufacter's instructions. PAX6 and MITF SMARTpool siRNAs were purchased from Dharmacon. Sequences for control and STAT3 siRNAs (Sigma-Aldrich) are provided in Supplementary Table S1A. Transfection efficiency was monitored by qPCR at 48 hours.

Cell proliferation

Cells were seeded in complete medium into 96- or 6-well plates, treated for different times with vemurafenib, and analyzed by staining with crystal violet and measurement of absorbance at 590 nm after being dissolved with 15% acetic acid. For MTT assays, 8,000 or 5,000 cells were seeded with or without serum in triplicates the day before treatment, and after 24 or 48 hours, respectively, 10 μL of MTT reagent (Sigma-Aldrich; 5 mg/mL) were added for 1.5 hours. MTT was solubilized using DMSO and plates were read at 540 nm.

Western blotting and GTPase assays

Following cell lysis (25), proteins were resolved by SDS/PAGE, transferred to polyvinylidene difluoride membranes, and detected by chemiluminescence. Densitometry of the resulting bands was performed using ImageJ software. GTPase assays to detect the active forms of Ras were performed as described previously (25), using GST-RAF-RBD and immunoblotting with anti-Ras antibodies.

Real-time quantitative PCR

Total RNA was extracted using miRNeasy Mini Kit (Qiagen). To analyze miRNA expression, we used the miRNA-specific TaqMan MicroRNA Assay Kits (Applied Biosystems). miRNA-enriched RNA was first reverse-transcribed with the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems), and quantitative PCR was performed in triplicate with corresponding TaqMan PCR primers (Applied Biosystems), and TaqMan Universal Master Mix, according to the manufacturer's instructions. The RNU44 small RNA was used for normalization. For mRNA expression analyses, reverse transcription was performed using M-MLV-RT (Promega), and quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Assays were performed in triplicate and results normalized for the expression levels of TBP (TATA-binding protein). Quantitative PCR was analyzed using the LightCycler 480 (Roche). Oligonucleotide sequences are provided in Supplementary Table S1B.

Small RNA-Seq and data analysis

RNA was extracted using the mirVana miRNA Isolation kit (Thermo Fisher Scientific). Small RNA sequencing was performed in duplicate at the Genomics Unit of the Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain), using next-generation sequencing (NGS, Illumina HiSeq 2500, Illumina). For NGS data analysis, 3′ adapters were trimmed from sequencing reads using CutAdapt 1.7.1.Software. The remaining reads were mapped to known human mature miRNAs database (miRBase21) using Bowtie version 1.0.0 (global alignment), where 100% identity between reads and known miRNAs sequences was required. The total read count for each sample was scaled relative to the library size. Differential expression analysis between samples was performed using DESeq2 R package (26) or BIOSAGE library (27). Differentially expressed miRNAs between samples were considered using absolute Log_2FC (fold change) criteria of 0.9. *P* values for multiple tests were calculated using Benjamini–Hochberg correction and only those miRNAs with $P < 0.05$ values were selected for further analyses. GEO (Gene Expression Omnibus) accession number for the RNA sequencing: GSE107576.

Animal studies

For xenografting studies, we followed the described method (28). The Consejo Superior de Investigaciones Científicas Ethics Committee approved the protocols used for experiments with mice. Briefly, NOD/SCID/IL2gR$^{-/-}$ (NSG) mice were subcutaneously or intravenously inoculated with 1×10^6 parental or A375-VR cells in 0.2-mL PBS. Subcutaneous tumor growth was

inspected on a daily basis and tumor volumes measured until day 36, when all mice were sacrificed. Intravenously injected mice were sacrificed when signs of respiratory stress were noted and/or when weight sharply decreased. Lung and liver metastases were excised, minced, and filtered through 40-μm filters (BD Biosciences). Isolated melanoma cells were cultured for 10–15 days before testing them in MTT assays. For experiments involving vemurafenib administration, 1.5×10^6 H-Scr or triple-miR A375 cells were subcutaneously inoculated, and after 20 days (tumor volume approximately 100 mm^3), NSG mice were randomized in four groups and daily treated intraperitoneally with vehicle (5% DMSO + 10% 2-hydroxypropyl-β-cyclodextrin) or vemurafenib (25 mg/kg). Tumor volume was measured three times per week.

Statistical analyses

Unless otherwise indicated, mean values and SDs are representative of one of three independent experiments. Data were analyzed by Student *t* test or one-way ANOVA, followed by Tukey–Kramer multiple comparison test. In all analyses, the minimum acceptable level of significance was $P < 0.05$.

Results

Characterization of vemurafenib-resistant melanoma cells

As reported (1), A375 melanoma cells carrying the *BRAF* V600E mutation were sensitive to vemurafenib as well as to the MEK inhibitor trametinib (Supplementary Fig. S1A). Unlike A375, SK-Mel-103 cells expressing wild-type (wt) *BRAF* and Q61R *N-Ras* were insensitive to vemurafenib concentrations up to 1 μmol/L, while they were sensitive to trametinib. Accordingly, Erk1/2 activation in A375 cells was gradually lost at increasing concentrations of vemurafenib or trametinib, enhanced in SK-Mel-103 cells exposed to vemurafenib, and blocked when these cells were incubated with trametinib (Supplementary Fig. S1B).

A375 cells were exposed to sequential vemurafenib increases to a final concentration of 1.3 μmol/L, rendering a cell population resistant to up to 2 μmol/L vemurafenib (Fig. 1A). When vemurafenib-resistant cells (A375-VR) were cultured without vemurafenib for up to 4 weeks and then replated in the presence of vemurafenib, they remained fully resistant (Fig. 1B; Supplementary Fig. S1C). Determination of *in vivo* tumor cell growth using subcutaneously inoculated NSG mice revealed that A375-VR tumors grew faster than parental cell tumors (Fig. 1C, top). Furthermore, based on the signs of respiratory stress and/or sharp decrease in weight, mice intravenously injected with A375-VR cells were sacrificed significantly sooner than those inoculated with parental cells (Fig. 1C, bottom). A375 and vemurafenib-resistant cells displayed similar metastasis degrees to both lung and livers (66 ± 11 and 16 ± 5 for A375, and 57 ± 16 and 18 ± 6 for A375-VR, respectively). Cell proliferation assays showed that cells extracted from lung and liver metastases from mice injected with A375-VR cells retained resistance to vemurafenib relative to cells from metastases of mice inoculated with parental cells (Supplementary Fig. S1D).

Cells resistant to vemurafenib also displayed partial resistance to trametinib and to the Erk1/2 inhibitor SCH772984, whereas they were sensitive to the Akt inhibitor triciribine (Supplementary Fig. S2A–S2C). Importantly, A375-VR cells displayed resistance to vemurafenib combined either with trametinib or SCH772984 (Fig. 1D and E).

Although basal Erk1/2 and MEK levels are slightly increased in A375-VR cells relative to parental ones, nevertheless, vemurafenib-resistant cells showed higher levels of activation of MEK, Erk1/2, and its downstream effector RSK than parental counterparts, which was linked to stimulation of the GTPase Ras (Fig. 2A and B). Furthermore, we detected overexpression of 90-kDa BRAF in vemurafenib-resistant cells, which was not observed in A375, A375-TR, the *BRAF* wt SK-Mel-103 and SK-Mel-147, and in *BRAF* wt/*N-Ras* wt BLM melanoma cells (Fig. 2C). Sequence analyses confirmed that resistant cells remained mutant V600E for *BRAF* and wt for *NRAS* (not shown). We did not detect diminished levels in A375-VR cells of *SPRED*, an inhibitor of MAPK activation (29), or increased expression of *COT*, whose overexpression is associated to resistance to BRAFi (Supplementary Fig. S2D; ref. 7). The partial resistance of A375-VR cells to trametinib and to SCH772984 was linked with sustained Erk1/2 and MEK activation (Supplementary Fig. S2E and S2F). In addition to enhanced MAPK activation, the PI3K pathway was also activated in A375-VR cells, as revealed by increased Akt and S6 phosphorylation (Fig. 2D).

The A375-VR cells displayed a blockade of invasion across Matrigel relative to parental counterparts (Supplementary Fig. S3A), which was linked to increased Rac and RhoA activation in A375-VR cells (Supplementary Fig. S3B). These results raise the possibility that excessive Rho GTPase activation could impair resistant cell migration and invasion. As increased Rac activation involving the *RAC* P29S mutation has been associated to resistance to BRAFi (30), we tested whether A375-VR cell resistance could be affected by the Rac inhibitor NSC23766. Both parental and A375-VR cells showed similar sensitivity to increasing NSC23766 concentrations (Supplementary Fig. S3C), and combination of the Rac inhibitor together with increasing vemurafenib concentrations did not cause significant reduction in A375-VR cell proliferation relative to that intrinsically caused by NSC23766 (Supplementary Fig. S3D). We detected a tendency, albeit not statistically significant of a small increase in A375-VR cell viability, in the presence of 25 μmol/L of the Rac inhibitor starting at 500 nmol/L of vemurafenib. The molecular mechanistics of the potential counteraction between NSC23766 and vemurafenib are not known. In addition, NSC23766 did not affect the increased Erk1/2 phosphorylation shown by the resistant cells (Supplementary Fig. S3E), suggesting that resistance to vemurafenib was independent of the increased Rac activation exhibited by A375-VR cells.

Identification of miRNAs differentially expressed in A375-VR cells

We performed small RNA-seq from parental and A375-VR cells with the aim of identifying miRNAs whose altered expression could potentially contribute to melanoma resistance to vemurafenib. Sequences were aligned against the miRBase21 database, and following DESeq2 and BioSAGE analyses, the expression of four miRNAs, miR-204-5p, miR-211-5p, miR-504-5p, and miR-509-3p was found to be significantly upregulated in A375-VR cells compared with A375, whereas miR-4454, miR-140-3p, and miR-210-3p were reduced (Supplementary Table S2). Expression of miR-204-5p, miR-211-5p, miR-509-3p, miR-140-3p, and miR-504-5p was detected in samples from two different cohorts of melanoma patients (not shown), whereas no expression of miR-4454 and miR-210-3p was found, which led us to discontinue their study.

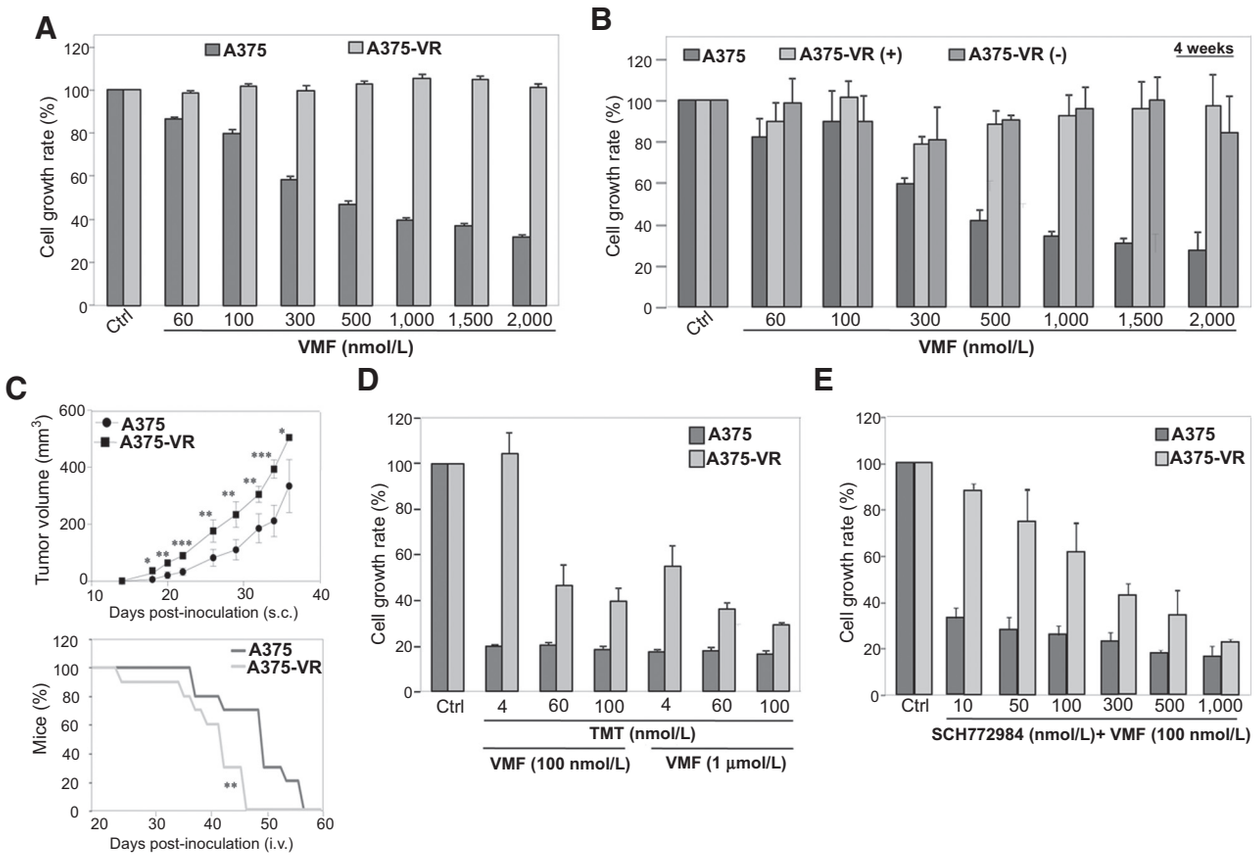

Figure 1.

Characterization of vemurafenib-resistant melanoma cells. **A,** Cells were tested in MTT assays (48 hours) in the absence (Ctrl) or presence of the indicated concentrations of vemurafenib (VMF; $n = 4$). **B,** A375-VR cells were incubated for 4 weeks without (−) or with vemurafenib (+; 1.3 μmol/L), and subsequently subjected for 48 hours to MTT assays as in **A**. Parental A375 cells are shown as control. **C,** Cells were subcutaneously (top) or intravenously (bottom) inoculated into NSG mice in the absence of drug treatment, and tumor growth and percentage of alive mice, respectively, were assessed. $n = 9$–10 mice/condition; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **D** and **E,** Cells were incubated for 48 hours without (Ctrl) or with the indicated concentrations of vemurafenib, trametinib (TMT), or SCH772984 ($n = 3$).

Subsequent qPCR experiments validated the changes for miR-204-5p, miR-211-5p, miR-504-5p, miR-509-3p, and miR-140-3p expression in A375-VR cells (Fig. 3A). As miR-504-5p expression was very low in A375 and A375-VR cells, we decided to also cease its study. To analyze that the observed miRNA alterations in A375-VR cells are independent of vemurafenib presence, we assessed their miRNA levels by incubation in culture medium without vemurafenib for up to 6 days. The altered expression levels of miR-204-5p, miR-211-5p, miR-509-3p, and miR-140-3p in A375-VR cells were preserved in the absence of vemurafenib (Fig. 3B), supporting the stability of these miRNA alterations. We next investigated the dynamics of vemurafenib-dependent miRNA changes by incubating A375 cells with vemurafenib. Notably, significant increases in miR-204-5p, miR-211-5p, and miR-509-3p were already detected after 24 hours of vemurafenib exposure, reaching a plateau between 3 and 6 days (Fig. 3C). In addition, cell incubation with vemurafenib led to decreased miR-140-3p expression, although with slower kinetics. These results indicate that alterations in the expression of these miRNAs occur early after melanoma cell treatment with vemurafenib. We decided then to focus our studies on miR-204-5p, miR-211-5p, and miR-140-3p, leaving miR-509-3p for future analyses. Dose–response experiments with A375 cells revealed substantial changes in miR-204-5p, miR-211-5p, and miR-140-3p expression already at vemurafenib concentrations of 100 nmol/L (Fig. 3D), whereas only minor but nonsignificant alterations in miRNA expression in A375-VR cells were observed upon increasing vemurafenib amounts. To study whether changes in miRNA expression can be detected in an additional vemurafenib-resistant BRAF V600E melanoma cell line, we used several resistant clones of SK-Mel 239 cells (31). Augmented levels of miR-204-5p and miR-211-5p were detected in 5 of 6 clones, whereas reduced expression of miR-140-3p was observed in 2 of the 6 clones (Supplementary Fig. S4A).

Interestingly, upregulation of miR-204-5p and miR-211-5p was also detected in parental and A375-VR cells exposed to trametinib, to SCH772984, or to the combination of vemurafenib and trametinib, whereas reduced levels of miR-140-3p were observed in A375 cells incubated with these inhibitors, but not in resistant cells (Figs. 3E and 4A). Meanwhile, the same miRNAs were unaltered in response to AKT or Rac inhibitors (Fig. 3E). Moreover, increase in miR-204-5p and miR-211-5p, and

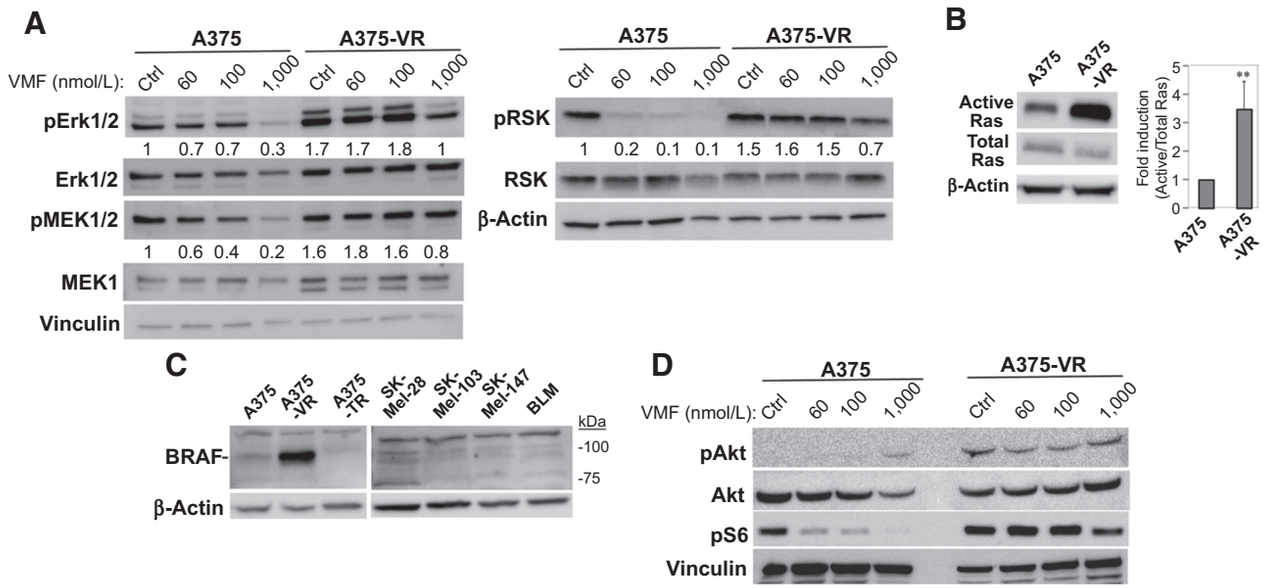

Figure 2.

Analysis of Ras-MAPK and PI3-K/Akt activation in vemurafenib-resistant melanoma cells. **A,** Cells were incubated for 48 hours in medium (Ctrl) or vemurafenib (VMF), as indicated, and subsequently tested by immunoblotting using antibodies to the shown proteins. Numbers below gels indicate values from densitometric analyses ($n = 3$). **B,** Left, cells were subjected to Ras GTPase assays. Right, densitometric analyses of protein gel bands show the mean ± SD of five independent experiments (**, $P < 0.01$). **C,** Cells were tested by Western blotting using anti-BRAF antibodies. **D,** Cells were subjected to immunoblotting to test for Akt and S6 activation.

reduction in miR-140-3p expression, were also observed in vemurafenib/trametinib-double resistant (VR/TR) A375 cells (Supplementary Fig. S4B and S4C). Of note, *BRAF* V600E–mutant SK-Mel-28 melanoma cells also showed enhanced miR-204-5p and miR-211-5p expression and decreased levels of miR-140-3p in response to incubation with vemurafenib or with combined vemurafenib and trametinib (Fig. 4B), suggesting that these miRNA changes might represent a common event in melanoma cells displaying this mutation. Together, these data point out to a strong correlation between changes in miR-204-5p, miR-211-5p, and miR-140-3p expression in melanoma and BRAF/MEK/Erk1/2 inhibition, but not PI3K/Akt or Rac blockade. Normal foreskin melanocytes were also tested for potential alterations in miRNA expression after exposing them to vemurafenib and trametinib. Whereas miR-204-5p expression was upregulated by vemurafenib, no significant changes in miR-211-5p and miR-140-3p expression were detected (Supplementary Fig. S4D). The molecular basis for vemurafenib-triggered increase in miR-204-5p levels in normal melanocytes and its functional consequences in melanocyte biology have not been addressed in this study.

mir-204 is located in intron 6 of the *TRPM3* (Transient Receptor Potential Melastatin 3) gene, and it has been proposed that expression of miR-204 and *TRPM3* has common regulatory mechanisms (32, 33). Correlating with increased miR-204-5p expression in A375-VR cells, we found upregulated *TRPM3* mRNA in the resistant cells compared with parental ones (Fig. 4C, left). Furthermore, the PAX6 transcription factor promotes expression of miR-204-5p (34), and we observed a strong *PAX6* induction in A375-VR cells, as well as in A375 cells treated with vemurafenib (Fig. 4C, right). In spite of the strong *PAX6* upregulation in A375-VR cells, its silencing did not alter miR-204-5p expression (Fig. 4D). Recent data revealed a role for STAT3 in regulation of miR-204-5p expression in melanoma (35). We then silenced STAT3 in A375-VR cells (30%–35% depletion) and found a significant decrease in the expression of miR-204-5p (Fig. 4E), suggesting that STAT3 is involved in the increased miR-204-5p expression in A375-VR cells.

miR-211 is expressed from the intron 6 of the melanoma marker *TRPM1* (Transient receptor potential cation channel subfamily M member 1; melastatin; refs. 36, 37), and we found that the increased miR-211-5p expression in A375-VR cells directly correlated with enhanced expression *TRPM1* relative to parental cells (Fig. 4F, left). As MITF (microphthalmia-associated transcription factor) regulates the transcription of *TRPM1*, we analyzed *MITF* expression and found no significant differences between A375 and A375-VR cells (Fig. 4F, middle). However, expression of tyrosinase (*TYR*), a marker of MITF activation (38), was enhanced in resistant cells both with and without vemurafenib, as well as in parental cells exposed to the inhibitor (Fig. 4F, right), together opening the possibility that increased miR-211-5p expression in A375-VR cells could be dependent of MITF activity. However, MITF silencing in A375-VR cells did not cause detectable alterations in miR-211-5p expression (Fig. 4G), suggesting that upregulated miR-211-5p expression in A375-VR cells is independent of MITF activation.

Role of miR-204-5p, miR-211-5p, and miR-140-3p in melanoma resistance to vemurafenib

To test the potential involvement of the miRNAs in resistance to vemurafenib, A375 cells were infected with lentiviral vectors to either overexpress miR-204-5p or miR-211-5p, or to silence miR-140-3p (Supplementary Fig. S5A). MTT assays revealed a

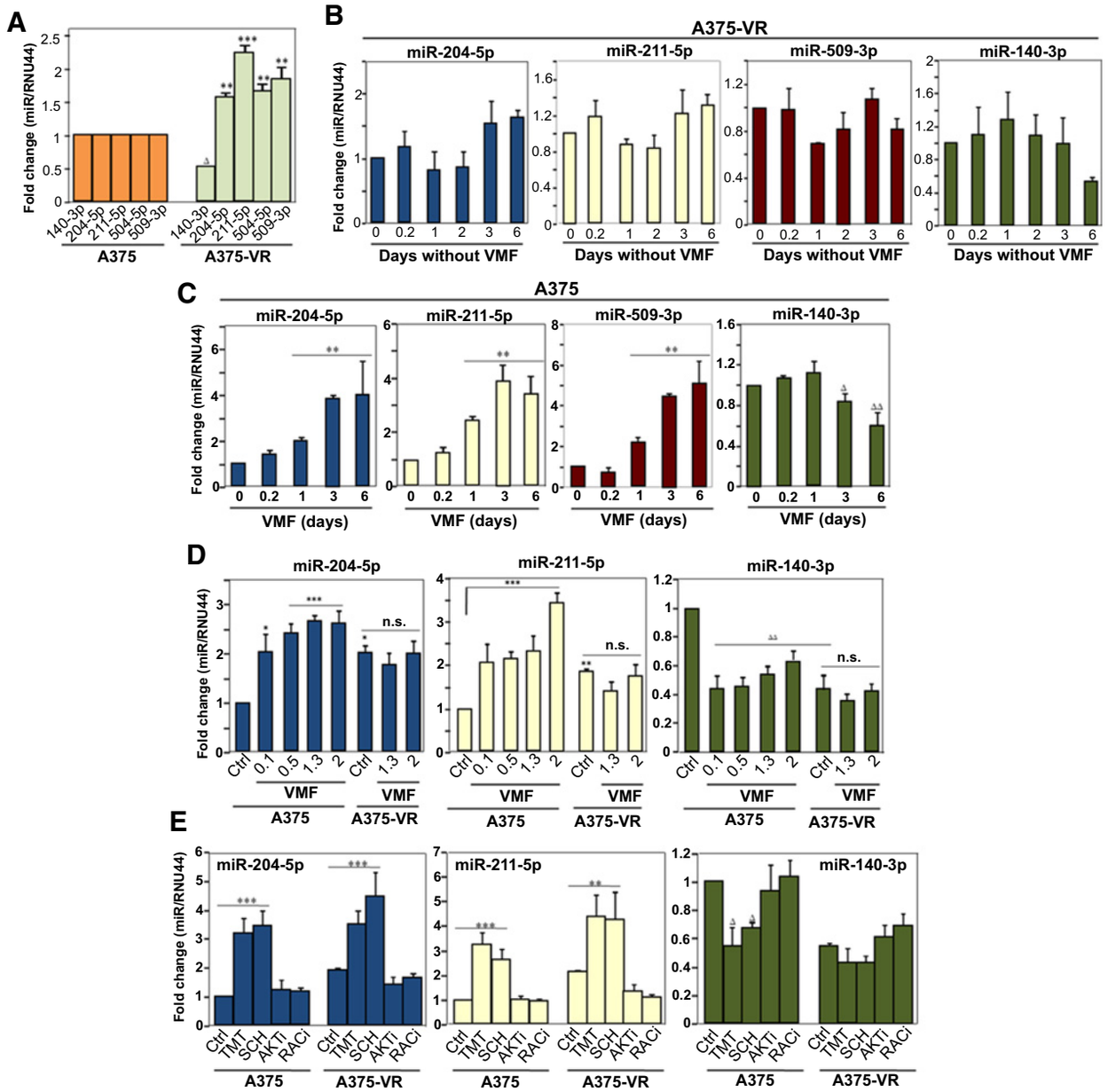

Figure 3.

Identification of miRNAs differentially expressed in vemurafenib (VMF)-resistant A375 melanoma cells. **A,** qPCR validation of miRNA expression changes between parental and vemurafenib-resistant cells ($n = 5$). **B** and **C,** A375-VR or parental A375 cells were incubated for the indicated times in the absence (**B**) or presence of vemurafenib (1.3 μmol/L; **C**), and subsequently subjected to qPCR analyses for miRNA expression ($n = 3$). **D,** Dose–response analyses of miRNA expression in A375 and A375-VR cells cultured for 48 hours in the absence (Ctrl) or presence of the indicated concentrations (0.1–1.3 μmol/L) of vemurafenib. miRNA expression was significantly upregulated, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, or significantly reduced, $^{\Delta}$, $P < 0.05$; $^{\Delta\Delta}$, $P < 0.01$; $^{\Delta\Delta\Delta}$, $P < 0.001$. **E,** Cells were incubated for 48 hours in medium (Ctrl) or in the presence of trametinib (TMT; 4 nmol/L), SCH772984 (50 nmol/L), triciribine (5 μmol/L), NSC23766 (10 μmol/L), and subsequently analyzed by qPCR for expression of the shown miRNAs ($n = 3$–4).

moderate but consistent increase in resistance to vemurafenib in cells overexpressing miR-211-5p or miR-204-5p relative to control transfectants (from 28%–34% cell viability in H-Scr control cells to 45%–49% in H-miR-211 or -miR-204; Fig. 5A, left). Instead, no significant variations in proliferation were observed in cells silenced for miR-140-3p (Fig. 5A, right).

We then generated A375 triple transductants that overexpress miR-204-5p and miR-211-5p, and that are knocked-down for miR-140-3p (triple-miR; Supplementary Fig. S5B). These triple-miR transductants also displayed enhanced resistance to vemurafenib relative to control cells (Fig. 5B; Supplementary Fig. S5C), overall indicating that miR-204-5p and miR-211-5p

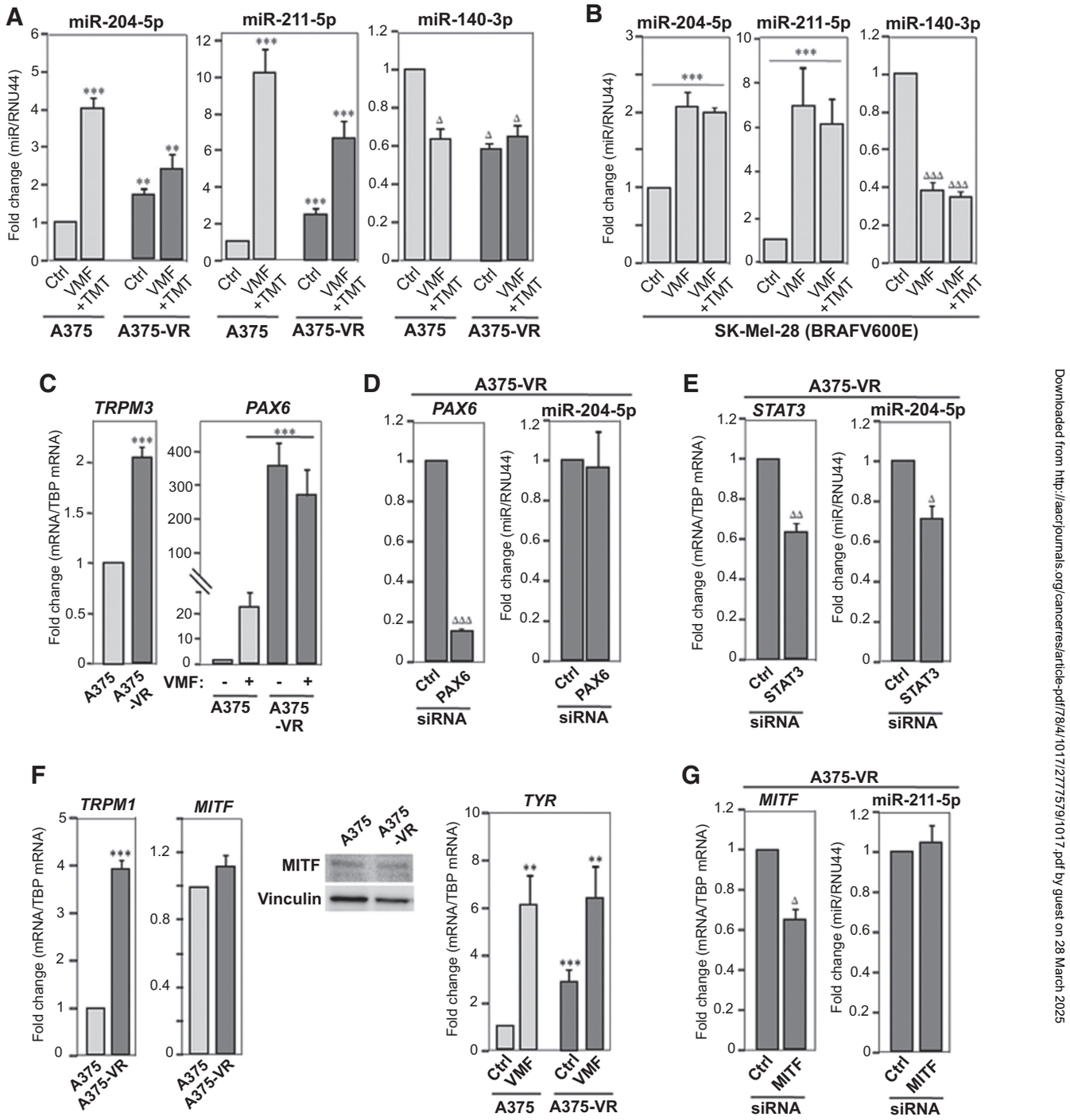

Figure 4.

Characterization of miRNAs differentially expressed in vemurafenib (VMF)-resistant melanoma cells. **A** and **B,** Cells were incubated for 48 hours in medium (Ctrl), or in the presence of vemurafenib alone (1.3 μmol/L) or combined vemurafenib (100 nmol/L) and trametinib (TMT; 4 nmol/L), and subsequently analyzed by qPCR for expression of the shown miRNAs ($n = 3$–4). Expression was significantly upregulated, **, $P < 0.01$; ***, $P < 0.001$, or significantly reduced, $^{\Delta}$, $P < 0.05$; $^{\Delta\Delta}$, $P < 0.01$; $^{\Delta\Delta\Delta}$, $P < 0.001$. **C,** Cells were analyzed by qPCR for *TRPM3* and *PAX6* mRNA expression. **D** and **E,** Cells were transfected with control, PAX6, or STAT3 siRNA, and transfectants tested by qPCR for *PAX6, STAT3*, and miR-204-5p expression ($n = 3$). **F,** Cells were analyzed by qPCR for *TRPM1, MITF*, or *TYR* mRNA expression, or by immunoblotting for MITF protein levels ($n = 2$–4). **G,** Cells were transfected with control or MITF siRNA, and transfectants tested by qPCR for *MITF* and miR-211-5p expression ($n = 3$).

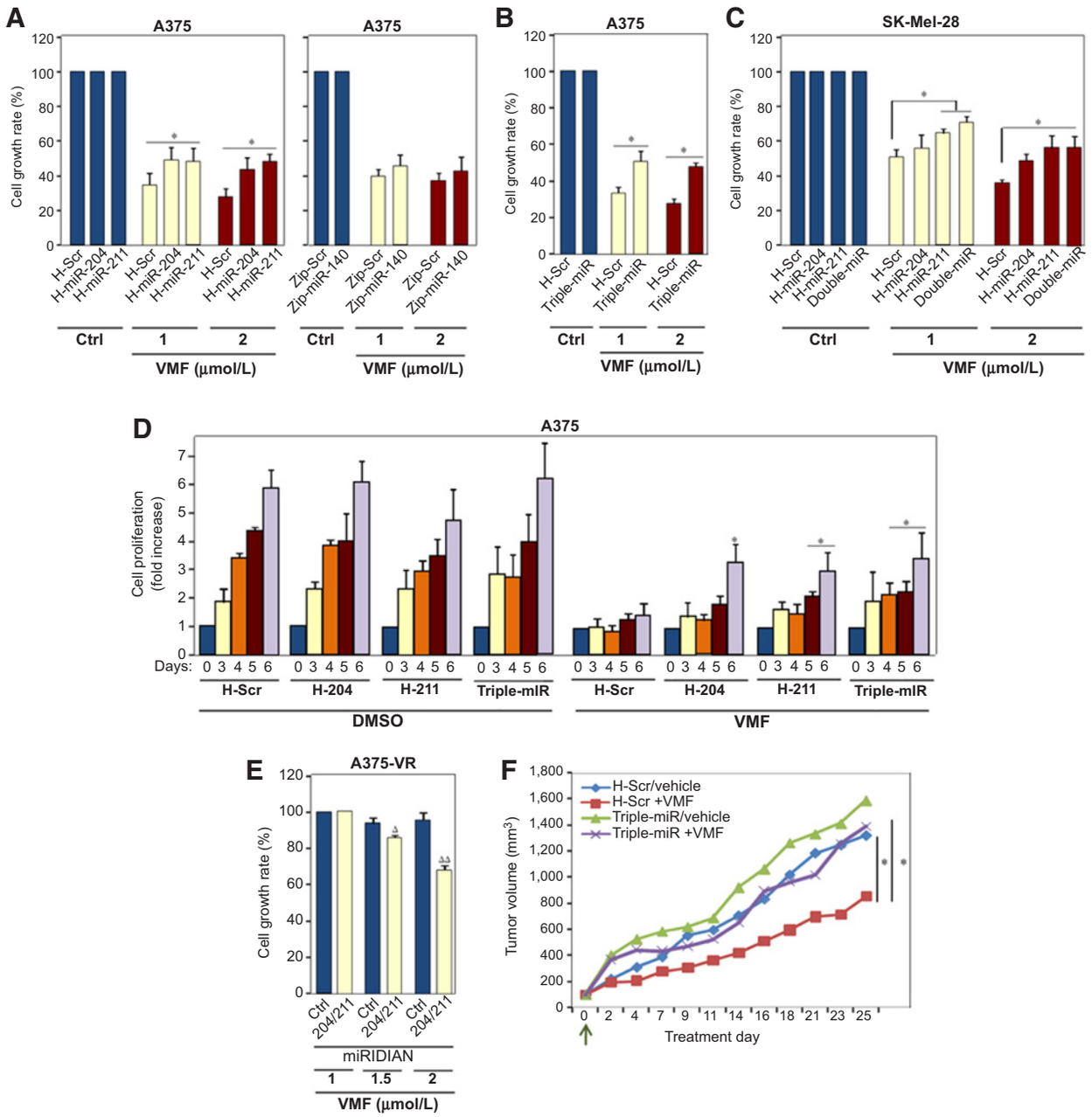

Figure 5.

Role of miR-204-5p, miR-211-5p, and miR-140-3p in melanoma resistance to vemurafenib. **A–C,** The indicated control or miRNA A375 and SK-Mel-28 transductants were tested in MTT assays in medium without serum, in the absence (Ctrl) or presence of the indicated concentrations of vemurafenib (VMF). Proliferation was significantly augmented. *, $P < 0.05$ ($n = 3$–4). **D,** Control (H-Scr cells), transductants overexpressing miR-204-5p or miR-211-5p, or triple-miR transductants were cultured in complete medium for the indicated times in the absence (DMSO samples) or presence of vemurafenib (100 nmol/L), and cell proliferation determined. Data is shown as fold increase in cell proliferation from three independent experiments. *, Proliferation was significantly augmented ($P < 0.05$), relative to the corresponding time points obtained with H-Scr control cells exposed to vemurafenib. **E,** A375-VR cells were transiently transfected with a combination of miRNA-204-5p and miRNA-211-5p miRIDIAN miRNA hairpin inhibitors or with a negative control, and transfectants tested by quadriplicate in cell proliferation assays in the absence of serum ($n = 2$; $^{\Delta}$, $P < 0.05$; $^{\Delta\Delta}$, $P < 0.01$). **F,** Cells were subcutaneously inoculated into NSG mice and after 20 days, mice were randomized in four groups and treated with vehicle or vemurafenib, as stated in Materials and Methods. Shown are tumor volume determinations. Arrow, starting of treatments. $n = 5$ mice/condition, *, $P < 0.05$.

overexpression, but not depletion of miR-140-3p in A375 cells, provides a survival advantage against vemurafenib. Supporting a role for miR-204-5p and miR-211-5p in providing resistance to vemurafenib, SK-Mel-28 melanoma cells overexpressing either one or both miRNAs (double-miR; Supplementary Fig. S5D and S5E) exhibited a moderate and consistent increase in growth rate

in the presence of the inhibitor compared with control counterparts (Fig. 5C).

H-miR-204, H-miR-211, and triple-miR, but not Zip-miR-140 A375 cells, also showed increased resistance to trametinib and to combined vemurafenib and trametinib treatments, as compared with control cells (Supplementary Fig. S6A). Control H-Scr SK-Mel-28 transductants displayed close to 2-fold higher intrinsic resistance to trametinib than H-Scr A375 counterparts, and H-miR-211 and double-miR, but not H-miR-204 SK-Mel-28 transductants, exhibited higher resistance to trametinib and to combined vemurafenib and trametinib treatments than control cells (Supplementary Fig. S6B). Contrary to vemurafenib or trametinib, neither miR-204-5p nor miR-211-5p overexpression conferred resistance to SCH772984 (not shown), and only triple-miR cells displayed a moderate increase in growth rate with vemurafenib and SCH772984 (Supplementary Fig. S6C), but not with triciribine (Supplementary Fig. S6D).

Further confirmation of miR-204-5p and miR-211-5p contribution to resistance to vemurafenib came from cell proliferation assays for 6 days in the presence of vemurafenib, which showed that H-miR-204, H-miR-211, and triple-miR stable cells have significantly higher proliferation than control H-Scr counterparts (Fig. 5D). Instead, Zip-miR-140-3p transductants lacked proliferation advantage when exposed to the inhibitor (not shown). To add further significance to these data, we silenced both miR-204-5p and miR-211-5p in A375-VR cells (Supplementary Fig. S6E), and tested cell viability in the presence of vemurafenib. Transfectants displayed decreased growth at 1.5–2 μmol/L of vemurafenib (Fig. 5E), highlighting the involvement of these miRNAs in resistance to vemurafenib. Moreover, NSG mice subcutaneously inoculated with triple-miR cells displayed significantly larger tumor volumes following vemurafenib administration than mice inoculated with H-Scr control cells (Fig. 5F). No significant differences in tumor growth were observed in mice injected with triple-miR or H-Scr cells upon vehicle administration, or in mice inoculated with triple-miR cells and exposed to vehicle or vemurafenib.

We next used SK-Mel-103 cells to test the effect of vemurafenib in an inherently vemurafenib-resistant melanoma cell line. miR-204-5p expression was augmented by vemurafenib, but reduced when cells were incubated with both vemurafenib and trametinib (Supplementary Fig. S7A). No miR-211-5p expression was detected in these cells (not shown), and expression of miR-140-3p was unaltered in the presence of vemurafenib, and increased with combined vemurafenib and trametinib. We then silenced miR-204-5p in SK-Mel-103 cells and tested cell resistance after exposing them to increasing vemurafenib concentrations. The results revealed no significant differences in growth between miR-204-5p-knockdown cells and control counterparts (Supplementary Fig. S7B), overall indicating that miR-204-5p does not influence proliferation in inherently vemurafenib-resistant SK-Mel-103 cells.

Analyses of Erk1/2 activation revealed that vemurafenib-induced decrease in pErk1/2 was more attenuated in H-miR-211 and in triple-miR A375 cells than in control cells (Fig. 6A–C). Furthermore, reduced pMEK due to vemurafenib was more mitigated in H-miR-211 cells. As expected, when we extended cell exposure to vemurafenib to 1 week, pErk1/2 and pMEK levels sharply diminished, but higher phosphorylation of Erk1/2 and MEK in triple-miR cells than in control cells was observed (Fig. 6D). By week 3 of treatment with vemurafenib, pErk1/2 and pMEK levels were recovering in H-Scr cells, but triple-miR, as well as cells overexpressing miR-204-5p or miR-211-5p still showed remarkably higher pErk1/2 and pMEK. After 4 weeks with vemurafenib, H-miR-211 and triple-miR, but not H-miR-204 cells still exhibited higher pErk1/2 and pMEK1/2 than control cells (Fig. 6D). Furthermore, vemurafenib-exposed triple-miR transductants displayed substantially higher Ras activation levels than control cells (Fig. 6E). None of the different miRNA transductants exhibited BRAF overexpression shown by A375-VR cells (Fig. 6F). The attenuated decrease in Erk1/2 activation upon vemurafenib treatment was not only observed in A375 cells overexpressing miR-211-5p, but H-miR-211, as well as H-miR-204 SK-Mel 28 transductants, also displayed mitigated reduction in pErk1/2 relative to control cells (Fig. 6G).

Examination of miR-204-5p and miR-211-5p target expression

Data from previous published work and from available miRNA databases indicate that miR-204-5p and miR-211-5p share some common targets, including NUAK1/ARK5, IGFBP5, TGF-βRII, Slug, and CHD5 (21, 37, 39, 40). qPCR analyses showed that *NUAK1* mRNA was downregulated in A375-VR cells and in H-miR-204, H-miR-211, and triple-miR cells, but not in Zip-miR-140 counterparts (Fig. 7A, left). Moreover, *NUAK1* expression was also reduced in SK-Mel-28 cells overexpressing miR-211-5p. Decreased NUAK1 protein expression was confirmed in immunoblot analyses in A375-VR, H-miR-211, and triple-miR A375 cells, but not in H-miR-204 counterparts (Fig. 7A, right). Although *IGFBP5* and *TGF-βRII* mRNAs were reduced in cells overexpressing miR-204-5p or miR-211-5p, confirming that they represent targets for these miRNAs, their levels were increased in A375-VR cells (Fig. 7B, right; Supplementary Fig. S8A, left), indicating that other mechanisms overall upregulated their expression in the resistant cells. Interestingly, *IGFBP5* mRNA was enhanced in Zip-miR-140 transductants, suggesting that it might represent a miRNA target. Contrary to what it was expected, SLUG expression was increased in H-miR-204, H-miR-211, and in triple-miR transductants, as well as in A375-VR cells (Fig. 7B, right). No significant alterations in *CHD5* expression was detected in the miRNA transductants or in A375-VR cells (Supplementary Fig. S8A, right).

The miR-204-5p target ephrin B2 (*EFNB2*; ref. 41) was reduced in H-miR-204, triple-miR, and to a lower extent in A375-VR cells relative to their controls (Fig. 7C, left). However, we were unable to detect significant alterations in ephrin B2 protein expression in these cells (Fig. 7C, middle). *EFNB2* mRNA expression was also decreased in SK-Mel-28 cells overexpressing miR-204-5p or miR-211-5p, and furthermore, we could detect reduced ephrin B2 expression in H-mir-211 SK-Mel-28 cells (Fig. 7C). Although the expression of another putative miR-204-5p target, *SNAIL*, was moderately diminished in resistant cells, we did not observe variations in its levels in the miRNA transductants (Supplementary Fig. S8B).

The proapoptotic transcription factor CHOP/GADD153 and IGF-2R are well-known miR-211-5p targets (42, 43). We found that *CHOP* mRNA expression was reduced both in A375-VR and H-miR-211 cells, but not in SK-Mel-28 cells overexpressing miR-211-5p (Fig. 7D, left and right). To efficiently visualize CHOP protein expression in A375 cells, we incubated them with thapsigargin, an agent that elicits endoplasmic reticulum (ER) stress. We found decreased CHOP expression (25%–30%) in thapsigargin-exposed A375-VR cells compared with parental counterparts

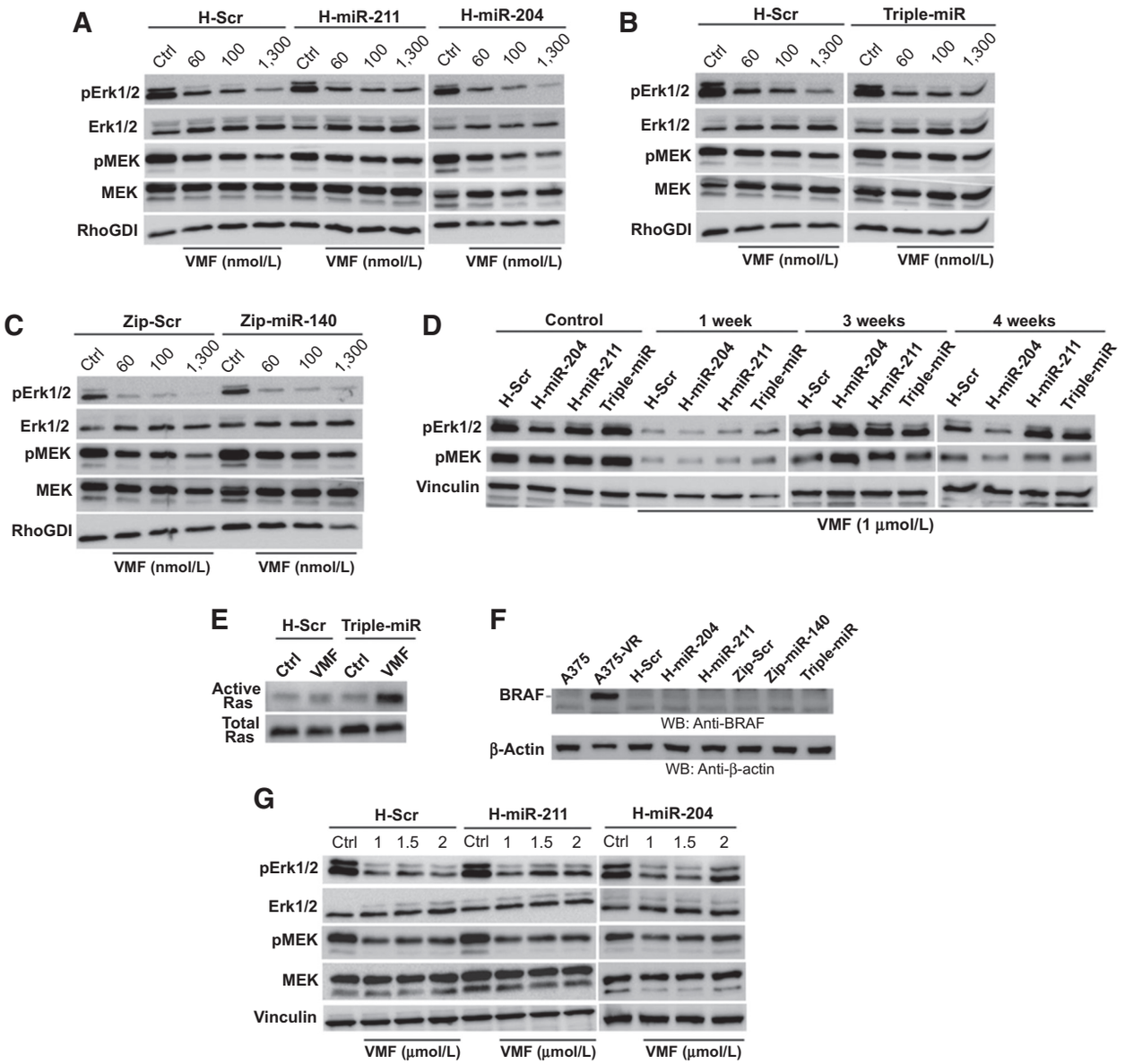

Figure 6.

Analysis of Erk1/2 and MEK activation in miRNA transductants. **A–C,** A375 transductants were incubated for 24 hours in the absence (Ctrl) or presence of the indicated concentrations of vemurafenib (VMF), and subsequently subjected to immunoblotting. **D,** Cells were left untreated (Control) or incubated for the times shown with vemurafenib, and analyzed by Western blotting. **E,** Cells were incubated for 24 hours without (Ctrl) or with vemurafenib (1.3 μmol/L) and subsequently subjected to Ras GTPase assays. **F,** Parental and A375-VR cells, or the indicated transductants, were analyzed by immunoblotting using anti-BRAF antibodies. **G,** SK-Mel-28 transductants were incubated for 24 hours with or without vemurafenib, and subsequently subjected to immunoblotting. Loading controls were assessed with antibodies to RhoGDI, vinculin, or β-actin.

(Fig. 7D, middle). *IGF-2R* levels were not significantly altered in A375-VR cells relative to parental ones, or in the different miRNA transductants (Supplementary Fig. S8C). Finally, the expression of additional miR-204-5p and miR-211-5p targets, such as *RAB22A*, *CREB5*, *SOX4*, *BCL2*, *NFAT5*, and *POU3F2* was unaltered in vemurafenib-resistant cells (not shown).

Whereas *NUAK1*, *EFNB2*, and *CHOP* expression is reduced in A375-VR relative to A375 cells (Fig. 7A, C, and D), *NUAK1* and *EFNB2* were found to be increased in SK-Mel-103 compared with A375 cells, and no significant differences between A375 and SK-Mel-103 cells were detected for *CHOP* expression (Supplementary Fig. S8D).

Discussion

Resistance to BRAFi in melanoma involves genetic alterations that lead to reactivation of the MAPK pathway or activation of PI3K/AKT signaling (4–7, 44, 45). In addition, there can be other

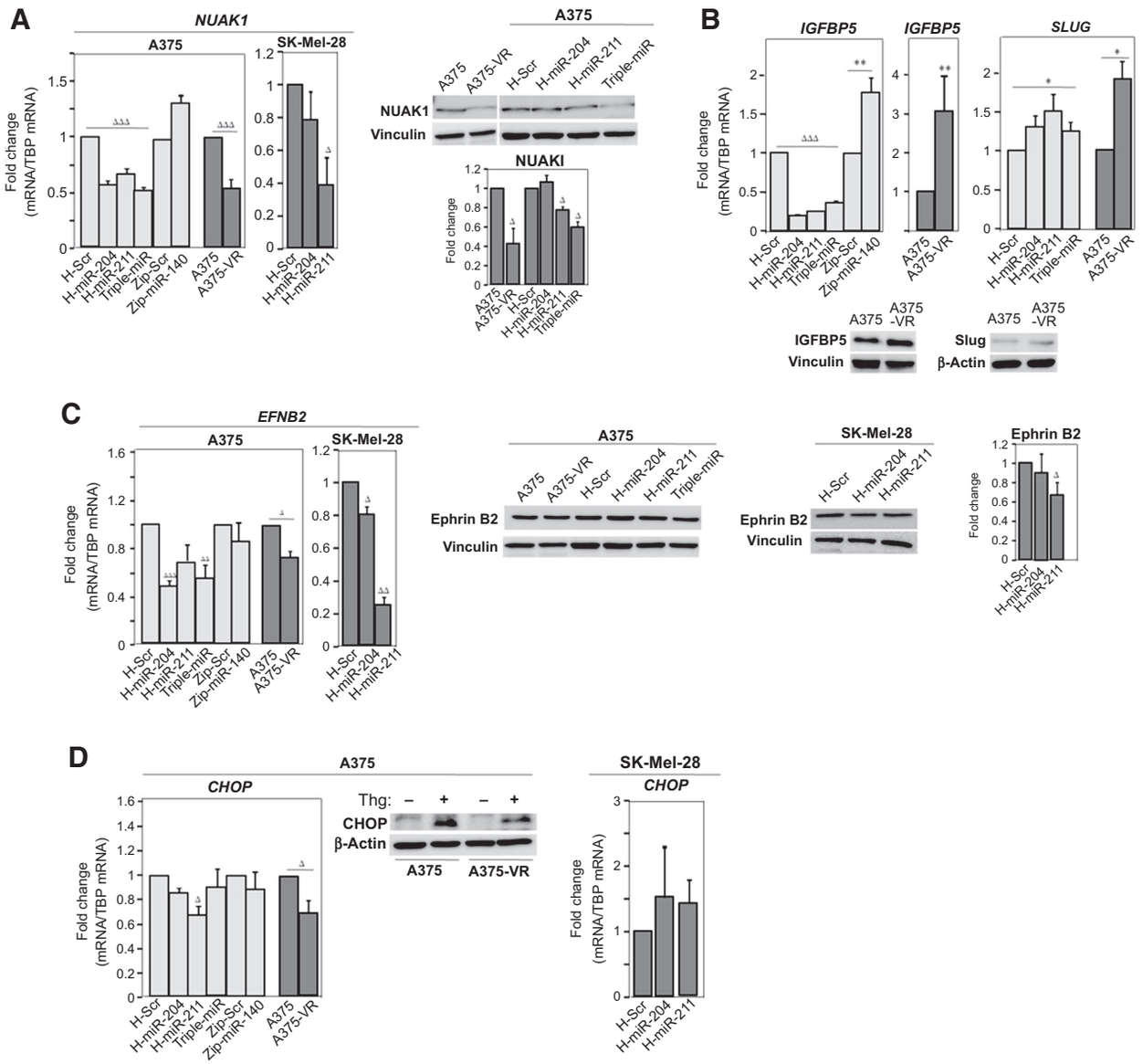

Figure 7.

Analyses of miRNA target expression in cells overexpressing miR-204-5p and miR-211-5p and in A375-VR cells. mRNA and protein levels for NUAK1 (**A**), IGFBP5 and Slug (**B**), Ephrin B2 (**C**), and CHOP were determined by qPCR or immunoblotting, respectively. Relative fold change values from the blots are also displayed. $^{\Delta\Delta\Delta}$, expression was significantly reduced, $P < 0.001$; $^{\Delta}$, $P < 0.05$; $^{\Delta\Delta}$, $P < 0.01$, or **, stimulated, $P < 0.01$; *, $P < 0.05$ ($n = 3$–5).

nongenetic alterations conferring melanoma resistance. Here we have generated A375 melanoma cells displaying resistance to vemurafenib with the goal of identifying changes in miRNA expression that could provide survival advantage against this inhibitor. Resistant A375-VR cells displayed Ras–MAPK pathway reactivation, as well as activation of PI3K/AKT, consistent with previous data (1). Activation of these pathways likely contributed to increased tumor growth in the A375-VR xenograft models. Vemurafenib-resistant cells also showed partial resistance to trametinib, as well as to combined vemurafenib and trametinib. We have not addressed whether additional mechanisms independently of Ras-MAPK reactivation, including Notch1 pathway activation (46), or increased expression of PDGFβ or EGF receptors (4, 47), contribute to vemurafenib resistance in A375-VR cells. Stability of the resistance was demonstrated both in *in vitro* and *in vivo* assays, such that lung and liver A375-VR metastases retained full resistance to vemurafenib after culturing tumor cells with the inhibitor.

Analyses of small RNAseq data and subsequent qPCR studies revealed increased miR-204-5p and miR-211-5p levels in A375-VR cells relative to parental counterparts, whereas miR-140-3p expression was reduced. Upregulation of miR-204-5p and miR-211-5p was already evident after 24 hours of cell exposure to vemurafenib, and was detected at low concentrations of the

inhibitor. miRNA alterations persisted for at least 6 days in the absence of vemurafenib, overall suggesting that miRNAs are rapidly increased by the inhibitor, but do not require its continuous presence. Of note, enhanced miR-204-5p and miR-211-5p expression was not exclusive of A375-VR cells, as it was also detected in *BRAF* V600E–mutant SK-Mel-239-VR melanoma cells. Together, these results strongly suggest that increased miR-204-5p and miR-211-5p expression could represent a common event during development of melanoma resistance to vemurafenib. Our results are in line with previous data showing that vemurafenib induces the expression of these miRNAs in A375 cells (35).

The rapid elevation of miR-204-5p and miR-211-5p levels was not only detected in A375 cells exposed to vemurafenib, but also to trametinib, to an ERK inhibitor, and to combined vemurafenib and trametinib treatment. Moreover, *BRAF* V600E–mutant SK-Mel-28 cells also showed miR-204-5p and miR-211-5p upregulation after incubation with vemurafenib or with combined vemurafenib and trametinib. The fact that the increased levels of these miRNAs were not observed in cells incubated with AKT or Rac inhibitors suggests that it might represent a specific response to MAPKi.

We studied the impact of miRNA alterations on melanoma resistance to vemurafenib, by ectopic expression of miR-204-5p or miR-211-5p, or by silencing miR-140-3p in parental A375 cells. The data revealed that miR-204-5p and miR-211-5p, but not miR-140-3p, contribute to cell resistance to vemurafenib. Moreover, overexpression of miR-204-5p and miR-211-5p in another *BRAF* V600E melanoma cell line, SK-Mel-28, also conferred vemurafenib resistance. Further supporting the involvement of these miRNAs in vemurafenib resistance, their depletion in A375-VR cells led to decreased cell growth in the presence of vemurafenib. It is noteworthy that as miR-204-5p silencing in inherently vemurafenib-resistant SK-Mel-103 cells does not affect their proliferation in the presence of vemurafenib, the results suggest that inherent resistant cells and *BRAF* V600E cells that have developed vemurafenib resistance behave differently in the context of miR-204-5p involvement. Underlining the miR-204-5p and miR-211-5p contribution to vemurafenib resistance, we show that triple-miR cells exhibit higher *in vivo* resistance to vemurafenib than control cells, resulting in larger tumors in NSG mice.

Notably, overexpression of miR-204-5p and miR-211-5p provided MAPK reactivation during the first 4 weeks of cell exposure to vemurafenib, earlier and on top of that observed in control cells. One of the possible mechanisms behind the increased MAPK reactivation detected in triple-miR cells could be their remarkable enhancement of Ras activation when exposed to vemurafenib. Therefore, based on the fast kinetics of miR-204-5p and miR-211-5p upregulation, the data overall suggest that these miRNAs might provide initial survival advantage supported by their early contribution to maintain sufficient Ras/MEK/ERK activation levels before further activation caused by genetic or nongenetic alterations of components of this pathway lead to resistance to vemurafenib. Furthermore, the reduced viability of A375-VR cells depleted for miR-204-5p and miR-211-5p indicates that these miRNAs facilitate resistance to vemurafenib not only at initial but also at more advanced stages of treatment.

mir-204 is located in the *TRPM3* gene, and we show here that, similar to miR-204-5p, *TRPM3* mRNA expression is also enhanced in A375-VR cells, highlighting early proposal that miR-204 and *TRPM3* have common regulatory mechanisms (32, 33). PAX6 promotes expression of this miRNA (34). Although we found a strong induction of *PAX6* expression in A375-VR cells, the increased miR-204-5p expression was independent of PAX6, as PAX6 silencing did not alter the miRNA levels. On the contrary, knocking down the expression of STAT3, another transcription factor regulating miR-204-5p expression in melanoma (35), led to a significant reduction in the levels of this miRNA in A375-VR cells, suggesting the involvement of STAT3 in the upregulation of miR-204-5p in the vemurafenib-resistant cells.

mir-211 is found in an intron of *TRPM1*, a target gene of MITF, and therefore MITF could indirectly drive miR-211 expression by transcriptionally enhancing *TRPM1* levels (37). A375-VR cells display higher expression of both miR-211-5p and *TRPM1* than parental cells, as well as enhanced MITF activity based on increased *TYR* expression, together raising the possibility that MITF could contribute to miR-211 upregulation in A375-VR cells. Nevertheless, MITF silencing in resistant cells did not significantly alter the expression of this miRNA, suggesting that MITF plays minor or no roles in the regulation of miR-211-5p expression in A375-VR cells, and that other mechanisms stimulate the expression of this miRNA in these cells.

miR-204 and miR-211 have very similar nucleotide sequences with only two different nucleotides in the whole sequence and the same seed region, a likely explanation for sharing some common targets. Examination of target expression in A375-VR cells and in cells overexpressing miR-204-5p or miR-211-5p revealed that *NUAK1/ARK5*, *EFNB2* and *CHOP* mRNA levels were significantly reduced. However, only NUAK1/ARK5 protein expression was consistently diminished in the vemurafenib-resistant cells and in H-miR-211 and triple-miR transductants. NUAK1 is a kinase related to AMP-activated protein kinase (AMPK) that can be phosphorylated and activated by the tumor suppressor LKB1 (48). Interestingly, LKB1 can be phosphorylated by both ERK and p90RSK in melanoma cell lines harboring the *BRAF* V600E mutation, leading to inhibition of LKB1 binding to and activation of AMPK, and ultimately causing increased cell proliferation (49). It is currently unknown whether the *BRAF* V600E mutation could also lead to LKB1-NUAK1 uncoupling and upregulation of cell growth. The current data open up the possibility that NUAK1 could be involved in vemurafenib resistance in melanoma cells, an hypothesis that will be the focus of future investigations.

Although ephrin B2 is a target for miR-204-5p, its expression did not significantly decrease in A375-VR or in H-miR-204 cells, suggesting no apparent roles for ephrin B2 in resistance to vemurafenib in A375-VR cells. miR-211-5p elicits prosurvival cell responses by downregulating its target gene *CHOP* (42), a proapoptotic transcription factor involved in the unfolded protein response. CHOP mRNA and protein expression was reduced in A375-VR cells, which raised the possibility that it could contribute to early viability against vemurafenib. However, this hypothesis becomes weakened by the reported increase in CHOP expression in melanoma cells exposed to BRAFi (50). It is worth mentioning that *NUAK1/ARK5* and *EFNB2* expression was increased in SK-Mel-103 relative to A375 cells, which might suggest that inherent resistant cells or cells that developed resistance to vemurafenib behave differently in the context of miRNA involvement.

Both oncogenic and tumor suppressor roles have been linked to miR-204-5p and miR-211-5p. Thus, their ectopic expression leads to colorectal and breast cancer cell proliferation (40, 51). In

melanoma, these miRNAs act as tumor suppressors and also inhibit cell invasion (21, 37, 52, 53). This is consistent with our observation of A375-VR *in vitro* cell invasion blockade, which could potentially translate into reduced metastatic capacity. However, parental and A375-VR cells display similar metastatic capacities, and thus our data would not support that the decreased A375-VR cell invasion would result in lower metastasis, at least in later steps of this process. Yet, we do not know whether initial invasion and dissemination events of parental cells might be more efficient than those of vemurafenib-resistant cells. Anyhow, as A375-VR tumors grew faster than parental cell tumors, this suggests that a possible initial lower invasion efficiency of A375-VR cells might be later counteracted by their higher proliferation rate *in vivo*. The involvement of miR-204-5p and miR-211-5p in melanoma resistance to vemurafenib reported here represents a novel function for these miRNAs, and functional identification of their targets will shed mechanistic view of their implication in melanoma resistance. The upregulation of miR-204-5p and miR-211-5p expression could provide early viability in the presence of vemurafenib that might allow subsequent resistance. Our study together with recent work addressing the role of miRNAs in BRAFi resistance of melanoma (23, 24, 54, 55) highlights the contribution of nongenetic alterations in melanoma resistance to BRAFi.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Díaz-Martínez, L. Benito-Jardón, L. Alonso, L. Koetz-Ploch, J. Teixidó

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Díaz-Martínez, L. Benito-Jardón, L. Alonso, L. Koetz-Ploch, J. Teixidó

Writing, review, and/or revision of the manuscript: M. Díaz-Martínez, L. Benito-Jardón, L. Alonso, E. Hernando, J. Teixidó

Study supervision: E. Hernando, J. Teixidó

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