MicroRNA 211 Functions as a Metabolic Switch in Human Melanoma Cells

Joseph Mazar,^a Feng Qi,^a Bongyong Lee,^a John Marchica,^a Subramaniam Govindarajan,^a John Shelley,^a Jian-Liang Li,^a Animesh Ray,^b Ranjan J. Perera^a

Sanford Burnham Prebys Medical Discovery Institute, Orlando, Florida, USA^a; Keck Graduate Institute, Claremont, California, USA^b

MicroRNA 211 (miR-211) negatively regulates genes that drive invasion of metastatic melanoma. Compared to normal human melanocytes, miR-211 expression is significantly reduced or absent in nonpigmented melanoma cells and lost during human melanoma progression. To investigate the molecular mechanism of its tumor suppressor function, miR-211 was ectopically expressed in nonpigmented melanoma cells. Ectopic expression of miR-211 reduced hypoxia-inducible factor 1 α (HIF-1 α) protein levels and decreased cell growth during hypoxia. HIF-1 α protein loss was correlated with the downregulation of a miR-211 target gene, pyruvate dehydrogenase kinase 4 (*PDK4*). We present evidence that resumption of miR-211-mediated downregulation of *PDK4* in melanoma cells causes inhibition of invasion by nonpigmented melanomas via HIF-1 α protein destabilization. Thus, the tumor suppressor miR-211 acts as a metabolic switch, and its loss is expected to promote cancer hallmarks in human melanomas. Melanoma, one of the deadliest forms of skin cancer, kills nearly 10,000 people in the United States per year. We had previously shown that a small noncoding RNA, termed miR-211, suppresses invasion and the growth of aggressive melanoma cells. The results presented here support the hypothesis that miR-211 loss in melanoma cells causes abnormal regulation of energy metabolism, which in turn allows cancer cells to survive under low oxygen concentrations—a condition that generally kills normal cells. These findings highlight a novel mechanism of melanoma formation: miR-211 is a molecular switch that is turned off in melanoma cells, raising the hope that in the future we might be able to turn the switch back on, thus providing a better treatment option for melanoma.

Melanoma is the leading cause of skin cancer deaths in the United States. Invasive melanoma is recalcitrant to most existing chemotherapies, and there is an urgent need to understand the molecular regulatory pathways that contribute to melanoma formation and progression. A hallmark of most cancer cells, including melanoma cells, is their ability to reroute energy provision and consumption to support the demands associated with pathological growth and survival (1–8). For instance, Scott and colleagues (9) subjected normal melanocytes and melanoma cell lines to a partial systems level metabolic analysis and confirmed that melanoma cell lines exhibit the Warburg effect, that is, increased glycolysis and lactic acid fermentation in favor of aerobic glycolysis. Here we present evidence for a novel molecular switch driven by a microRNA (miRNA), which controls the Warburg effect in melanocytes and melanoma cells.

We and others have identified several miRNAs responsible for the development and progression of melanomas, with miRNA 211 (miR-211) as an important tumor suppressor candidate (10–16): miR-211 expression is significantly lower in nonpigmented melanoma cells and clinical melanoma samples than in normal melanocytes, and ectopic expression of miR-211 in melanoma cells reverses the high growth rate and invasiveness of melanoma cells (10, 13, 14). miR-211 has several putative target genes, including the calcium-activated potassium channel subunit α -1 gene (*KCNMA1*), the insulin-like growth factor 2 receptor gene (*IGF2R*), the transforming growth factor β receptor 2 gene (*TGFBR2*), the insulin growth factor binding protein 5 gene (*IGFBP5*), the POU domain-containing transcription factor gene *BRN2*, the nuclear factor of activated T cells 5 gene (*NFAT5*), and *NAUK1* (10, 13, 17, 18).

We hypothesized that miR-211 might exert some of its effects by altering melanoma cell metabolism, such that when this

miRNA is expressed the melanoma cells might lose some aspects of their cancer-specific metabolic state. To explore this, we characterized melanoma cells that ectopically expressed miR-211 using deep sequencing and mass spectrometry (MS). We report that miR-211-expressing melanoma cells exhibit increased oxygen consumption and contain elevated numbers of mitochondria compared to melanoma cells that do not express miR-211. The metabolic alterations are causally related to downregulation of a previously unidentified miR-211 target gene, that for pyruvate dehydrogenase (PDH) kinase 4 (*PDK4*). Melanoma cells ectopically expressing miR-211 have decreased levels of the hypoxia-inducible factor 1 α (HIF-1 α) transcription factor and are more sensitive to cell death when hypoxic. We postulate that ectopic miR-211 expression sensitizes melanoma cells to hypoxia-induced cell death in a HIF-1 α -dependent manner, via the reduction of *PDK4* expression. Thus, miR-211 is likely to be an important regulator of melanocyte metabolism, and its loss of expression appears to be an epochal event during melanomagenesis and melanoma progression.

Received 5 August 2015 Returned for modification 15 September 2015

Accepted 12 January 2016

Accepted manuscript posted online 19 January 2016

Citation Mazar J, Qi F, Lee B, Marchica J, Govindarajan S, Shelley J, Li J-L, Ray A, Perera RJ. 2016. MicroRNA 211 functions as a metabolic switch in human melanoma cells. *Mol Cell Biol* 36:1090–1108. doi:10.1128/MCB.00762-15.

Address correspondence to Ranjan J. Perera, rperera@sanfordburnham.org.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

MATERIALS AND METHODS

Cell lines and tissue culture conditions. Cell lines examined in this study included the melanoma cell lines A375 (melanoma stage 4; American Type Culture Collection) and WM1552C (melanoma stage 3; ATCC CRL-2808), as well as the human epidermal melanocyte cell line HEM-1 (catalog no. 2200; ScienCell). All cell lines were maintained and selected as previously described by Mazar et al. (14).

Western blot analysis. Western blot assays were performed using cell lysates under the same conditions as those described by Mazar et al. (14). Blots were probed with the following primary antibodies and dilutions: anti-HIF-1 α (catalog no. NB100-105; Novus Biologicals) at 1/500, anti-PDK4 (catalog no. AP7041b; Abgent) at 1/250, anti-ERR γ (catalog no. D-1:sc-393969; Santa Cruz), anti-RUNX2 (catalog no. D130-3; MBL), anti-PDH E1 beta subunit (catalog no. ab155996; Abcam) at 1/1,000, anti-PDH-E1 α (pSer²⁹³) (catalog no. AP1062; Calbiochem), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; catalog no. ab9484; Abcam) at 1/1,000, and anti- β -actin (clone AC-74; Sigma) at 1/10,000. Densitometry was performed on film acquired from blots and scanned, with quantitation of mean intensity for bands performed using NIS-Elements AR Analysis 4.2 software. Data were normalized to the mean intensity of the appropriate load control (actin or GAPDH). Student's *t* test was performed to measure statistical significance.

Construction of expression plasmids. Cloning of the HIF-1 α and PDK4 open reading frames (ORFs) was performed by PCR using cDNA from A375 melanoma cells (primer sets: HIF-1 α Xprn For, TGGATCCA TGGAGGGCGCCGGCGGC, and HIF-1 α Xprn Rev, AGCGGCCGCTC AGTTAACTTGATCCAAAGCTCTGAGTAATTC; PDK4 ORF For, TGG ATCCCAAGATGAAGGCGGCCCGCTTC, and PDK4 ORF Rev, AGCG GCCGCTCACATGGCCACTTCTTTTGCCAGG, containing 5' BamHI and 3' NotI sites). The PCR and cloning were performed as per Mazar et al. (14). Subcloning of the HIF-1 α and PDK4 fragments was performed directionally using BamHI/NotI into pcDNA6/V5-HisA (Life Technologies) to form the expression plasmids (pcDNA6/HIF-1 α and pcDNA6/PDK4).

Transfection of human melanoma cells. Transfections were performed as described before (14). The plasmids used were either pcDNA6/V5-HisA (negative-control plasmid), pcDNA6/PDK4 (PDK4 expression plasmid), or pcDNA6/HIF-1 α (HIF-1 α expression plasmid). The small interfering RNAs (siRNAs) used included either Silencer Select Negative Control #1 (negative-control siRNA, catalog no. 4390843; Ambion), Silencer Select PDK4 siRNA (catalog no. 4390624, ID: s10262), or Silencer Select HIF-1 α siRNA (catalog no. 4427038, ID: s6539).

Cell invasion assays. Cell invasion assays were performed as described in reference 14. Cell migration was counted using Aperio software, with the data expressed as the percent invasion through the membrane relative to migration through the control sample. All experiments were performed in triplicate.

In vitro wound-healing assay. Mat Tek 1.5-mm tissue culture dishes were used and seeded with cells until 90 to 95% confluence was reached. The cell monolayers were scratched gently with a pipette tip across the diameter of the dish and then rinsed with medium extensively to remove any cellular debris. The surface area of the scratch was immediately quantified after wounding and for every 20 min that followed for 24 h using the Nikon Bio Station IM. The extent of wound closure was determined by calculating the ratio between the surface areas for the wound at each time point versus the surface area of the original wound. These data were expressed as the percentage of wound closure relative to control conditions. The surface area was determined using NIS Elements software, and for each sample this was performed in triplicate. Samples included A375/VO cells transfected with Silencer Select Negative Control #1, A375/VO cells transfected with Silencer Select PDK4 siRNA, A375/211 cells transfected with pcDNA6/V5-HisA (negative-control plasmid), and A375/211 cells transfected with pcDNA6/PDK4 (PDK4 expression plasmid).

Growth of melanoma cells in a hypoxic chamber. Both A375 and WM1552C cells (and their derivatives) were cultivated and examined in-

side a hypoxic chamber. Both cell lines were treated as follows. Cells (2.5×10^5) were seeded into wells of a 6-well plate and allowed to adhere; the plates were then either left in a standard culture incubator (standard O₂-5% CO₂ at 37°C) or transferred to a modular incubator chamber (Billups-Rothenberg, Inc.) sealed under hypoxic conditions (2% O₂-5% CO₂ at 37°C). Wells were then imaged using the Scanscope digital scanner, and cells were counted following trypsinization using the Cellometer cell counter. Cell number was expressed as a percentage relative to the control sample. All experiments were performed in triplicate.

DFO treatment of melanoma cells. The A375 and WM1552C cell lines and their derivative lines were treated with deferoxamine (DFO). Cells of each line (2.5×10^5) were seeded into wells of a 6-well plate and allowed to adhere. DFO was added at a concentration of 400 nM or 250 nM or not added. Cells were incubated for 48 h, following which the cells were imaged within the well and were counted as described in the previous section.

Measurement of OCR and ECAR in melanoma cells. Both the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of A375 and WM1552C cells (and their derivatives) were measured using the XF⁺ extracellular flux analyzer (Seahorse Bioscience) as follows: 40,000 cells were plated per well into an XF96 cell culture microplate (part no. 101085-004) in quintuplicate (5 wells each) utilizing only the central lanes. Oligomycin solution was treated at a 5 μ M final concentration, trifluorocarbonylcyanide phenylhydrazone (FCCP) solution was treated at a 2 μ M final concentration, and rotenone-antimycin A was treated at a 0.5 μ M final concentration. The flux plate was loaded with oligomycin in the top left port (columns 7 to 11, lanes B, D, and F; and columns 2 to 11, lanes C, E, and G), FCCP was loaded in the top right port (columns 2 to 11, lanes C, E, and G), and rotenone-antimycin A was loaded in the bottom right port (columns 7 to 11, lanes C, E, and G). The experimental program was run on the extracellular flux analyzer as follows: loop (3 times), mix (3 min), measure (4 min), loop end, inject port A (for oligomycin); loop (3 times), mix (3 min), measure (4 min), loop end, inject port B (for FCCP); loop (3 times), mix (3 min), measure (4 min), loop end, inject port C (for rotenone-antimycin A); loop (3 times), mix (3 min), measure (4 min), loop end. OCR and ECAR data were then analyzed and plotted based upon the averages and standard deviations of all measurements. The methods are additionally described on the company website for Seahorse Bioscience.

MitoTracker labeling of melanoma cells. To assay mitochondria in live cells by MitoTracker (LifeTech), 10^5 cells each of A375/VO, A375/211, and A375/211/PDK4 cell lines were spotted onto glass slides, placed into 10-cm tissue culture dishes, and allowed to adhere for 2 h. These slides were then covered in Complete Tu medium, and the cells were incubated overnight with 5% CO₂ at 37°C. The following day, the medium was removed, replaced with 10 ml Opti-MEM (low-serum medium) with 25 nM MitoTracker reagent, and the cultures were incubated at 37°C and 5% CO₂ for 20 min, washed again in Opti-MEM, and placed in 4% paraformaldehyde (PFA) for 10 min; slides were washed with Tris-buffered saline (TBS) and incubated for 40 min with Enzo phalloidin-fluorescein isothiocyanate (FITC). After this, the slides were again washed in TBS, 4',6'-diamidino-2-phenylindole (DAPI; 10 μ g/ml) was added, and the slides were incubated for a further 8 min. Slides were washed and treated with Invitrogen Prolong Gold antifade reagent. The slides were finally scanned on a Nikon A1R VAAS laser point- and resonant-scanning confocal microscope (using a single-photon Ar-ion laser at a magnification of $\times 60$ with a $4\times$ zoom).

Quantification of mitochondrial and genomic DNA content. One million A375/VO, A375/211, and A375/211/PDK4 cells were pelleted and prepared as for the TRIzol method (Life Technologies). Five hundred microliters of back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris free base) was added to the remaining interphase and organic phase, which were mixed by inverting, allowed to sit for 10 min at room temperature, and then centrifuged at $3,000 \times g$ for 30 min at 4°C. The upper phase was removed and placed in a fresh tube with 4 μ l polyacrylic carrier followed by the addition of 400 μ l isopropanol, mixed

FIG 1 (A) miR-211-5p expression in human epidermal melanocytes compared to A375 melanoma cells and miR-211 expressing A375 cells. miR-211 expression was measured by qRT-PCR. Values are normalized to 1 using A375 cell expression. The experiment was performed in triplicate. (B, C, and D) Comparison of miR-211 expression levels in A375 (B), WM1552C (C), and HEM-I (D) cells subjected to normoxic versus hypoxic (2% O₂) conditions after 48 h. Results are averaged from triplicate experiments. (E and F) Densitometry of Western blots illustrated in Fig. 2C and D, respectively. Scanning and quantitation of mean intensity for bands performed using NIS-Elements AR Analysis 4.2 software. Data were normalized to the mean intensity of the appropriate load control (actin or GAPDH). **, $P < 0.05$, Student's t test.

by inverting, being allowed to rest for 5 min at room temperature, and centrifuged at 12,000 rpm for 5 min. Both samples were resuspended in 500 μ l of 70% ethanol, mixed, and then centrifuged again at 12,000 rpm for 5 min at 4°C. Sixty microliters of 8 mM NaOH was added to the pellet, mixed using a vortex mixer, heated at 37°C for 2 min, and then centrifuged at 12,000 rpm for 10 min at 4°C. Sixty microliters of supernatant containing the mitochondrial DNA was transferred to a fresh tube; 0.68 μ l of 1 M HEPES buffer and 0.6 μ l 100 mM EDTA were added and mixed by vortexing. DNA yields were measured using a NanoDrop spectrophotometer (260/280 nm and 260/230 nm). Quantitative PCR (qPCR) was then performed using 100 ng mitochondrial and genomic DNA from both A375/VO and A375/211 cells with the following primer sets: *ND1* For-

ward, CAGGCCCTTCGCCCTATTCTT, and *ND1* Reverse, GAGTTGGTCGTAGCGGAATCGGG; *LPL* Forward, GGCTGGACGGTAAGGGAGGCTC, and *LPL* Reverse, GCTCAAGGGCACCAACCAGGT. The results were normalized with respect to those for the A375/VO samples.

Quantitative real-time PCR. Total RNA was isolated from A375 cells (and cell derivatives) using the TRIzol method (Life Technologies). Subsequent quantification and integrity analysis were performed using a 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) as described by Mazar et al. (14). TaqMan primer probes were used for hsa-miR-211 quantification using *RNU48* primer probes as controls. SYBR green primers were as follows: *PDK4* qPCR Forward, GCCTGTGAGACTCGCCAACATTCTG, and *PDK4* qPCR Reverse, ATTCCACCAATTCATCAGGCTCTG; *HIF1α*

FIG 2 Effects of hypoxia and HIF-1 α on melanoma cells with and without miR-211 expression. (A and B) Effects of miR-211 on A375 melanoma cell survival grown under hypoxic and normoxic conditions. Quantitation of cell survival for A375/VO versus A375/211 cells grown in normoxic and hypoxic (2% O₂) chambers for 48 h. The graph indicates the percent survival normalized to A375/VO cells in a normoxic chamber. (C) HIF-1 α protein expression in A375/211 and A375/VO cells. (D) Western blot analysis of HIF-1 α expression in A375/VO, A375/VO transfected with *HIF1* α siRNA, A375/211, and A375/211 transfected with the *HIF1* α expression plasmid. GAPDH was used as a loading control. (E and F) Invasion assay (samples from panel D). The results in panel E are and normalized to A375/VO control. Crystal violet-stained slides highlight the different levels of invasion. The assay was performed in triplicate.

qPCR Forward, CTCATCCATGTGACCATGAGGAAATGAG, and *HIF1* α Reverse, CATAGTCTCTCTCGGCTAGTTAGGG; and *GAPDH* Forward SYBR, ACATCGCTCAGACACCATG, and *GAPDH* Reverse SYBR, TGTAG TTGAGGTCAATGAAGG (serving as the endogenous control). SDS1.2.3 software (Life Technologies) was used for data analysis.

Mass spectrometry analysis to detect protein expression in A375/211 and A375/VO cells. To identify global miR-211-induced changes in protein expression, we analyzed total protein extracts from parental and miR-211-expressing A375 by Velos Orbitrap mass spectrometry. A proteomics study was conducted at the proteomics core facility at

FIG 3 (A and B) Effects of miR-211-5p on the survival of A375 melanoma cells treated with DFO. Quantitative analysis of cell survival for A375/VO versus A375/211 cells treated with DFO at concentrations of 0, 250, and 400 nM after 48 h. The graph indicates percent survival normalized to A375/VO cells at 0 nM DFO (untreated). (C and D) Effects of miR-211-5p on the survival of WM1552C melanoma cells grown under hypoxic and normoxic conditions. Quantitative measurement of cell survival for WM1552C/VO versus WM1552C/211 cells grown in both normoxic and hypoxic (2% O₂) chambers for 48 h. The graph indicates percent survival normalized to WM1552C/VO cells in normoxic chamber. (E and F) Effects of miR-211-5p on the survival of WM1552C melanoma cells treated with DFO. Quantitative analysis of cell survival for WM1552C/VO versus WM1552C/211 cells treated with DFO at concentrations of 0, 250, and 400 nM after 48 h. The graph indicates percent survival normalized to WM1552C/VO cells at 0 nM DFO (untreated). Data were acquired in triplicate. Images were acquired using a bright-field microscope at a magnification of $\times 20$. **, $P < 0.05$, Student's t test.

FIG 4 (A) Measurement HIF-1 α mRNA levels by qRT-PCR. The assay was performed in triplicate, and results were averaged and then normalized to A375/VO plus negative-control (Neg Con) anti-miR. (B and C) Translation-blocking experiments of A375/VO and A375/211 cells by treatment with cycloheximide and by acquiring samples every 30 min posttreatment for 90 min. Samples were examined by Western blotting, and bands were quantitated using Apero software. Values were normalized to the GAPDH content and graphed relative to the initial time point (time zero) of their respective sample.

Sanford-Burnham. Having generated proteomics data, we focused on further analyzing the data to identify downregulated proteins that correspond to miR-211 target genes. We were assisted in these analyses by bioinformatics and computational biology experts at the bioinformatics core.

miRNA target site assays. The complete 3' untranslated region (UTR) for human *PDK4* was acquired by PCR using the following primer set: *PDK4* 3'UTR Forward, AGAGGGGACACTCAGGACACTTTACGG, and *PDK4* 3'UTR Reverse, CACATTTCTCTGTACATAGCATAAAGAC, containing a 5' BamHI site and a 3' XhoI site. This 2,118-bp product was amplified and isolated from A375 melanoma cDNA as a template using Phusion high-fidelity DNA polymerase (New England BioLabs). The PCR product was cloned into pCR4-TOPO (Life Technologies) and sequenced. A sequence-confirmed clone was subcloned into the pcDNA6/Luciferase vector, replacing the 3' UTR present in this expression vector with that of the *PDK4* sequences (producing pcDNA6/Luc/*PDK4* 3'UTR). A mutant form of this construct was constructed using the following complementary primer set: *PDK4* 3'UTR mut Forward, GATAA GAATAAGCCCCGGCGTGAAGTGTGAAG, and *PDK4* 3'UTR mut Reverse, CTTCACTCACTACGCCCCGGCTTATTCTTATC, changing 4 bases at the putative miR-211 target site (designed to disrupt target bind-

ing) in the *PDK4* 3' UTR using QuikChange (Stratagene). The plasmid product-produced pcDNA6/Luc/*PDK4* 3' UTR Mut was then confirmed by sequencing. To test the targeting efficiency of these constructs, 2.5×10^5 cells each of A375/VO and A375/211 were plated into 6-well plates and each line was transfected (as above) using Fugene 6 (Roche) with 5 μ g of the following plasmids: (i) pcDNA6/Luc (positive control), (ii) pcDNA6/Luc/*PDK4* 3'UTR, or (iii) pcDNA6/Luc/*PDK4* 3'UTR Mut. All cell lines were cotransfected with 500 ng of pRL-CMV (a *Renilla* luciferase control plasmid). After 48 h of incubation, cells were washed with phosphate-buffered saline (PBS), harvested by trypsinization, lysed in cell culture lysis reagent (Promega), and mixed with luciferase assay reagent (LAR), and luminescence was measured using a GloMax 20/20 luminometer (Promega Corp). The assay was performed in triplicate, with luminescence normalized to the positive-control plasmid in A375/VO cells.

Immunocytochemistry of PDK4 in A375 cells and derivative cells. A total of 5×10^4 cells of each A375 cell derivative (A375/VO, A375/211, A375/211/*PDK4*, and A375/211/Hif1 α) were spotted on glass slides and allowed to adhere for 4 h. The slides were then immersed in Complete Tu medium and allowed to grow overnight under tissue culture conditions (37°C and 5% CO₂). The following day, the slides were removed from media, washed with PBS, and then stained using the Thermo Shandon Sequenza system, and reagents were applied manually onto slides using both gravity and the capillary effect. The slides were incubated with LSBio Anti-PDK4 antibody (LS-B3459) at a dilution of 1:100 for 1 h. The secondary anti-rabbit Alexa Fluor 594-labeled antibody was used at a dilution of 1:250, and the mixtures were incubated on the slides for 30 min. This was followed by addition of Enzo's phalloidin (FITC), which was diluted as per the manufacturer's recommendation, and incubation for 40 min. Prior to the slides being coverslipped using Molecular Probes Prolong Gold, they were incubated for 5 min with Molecular Probes DAPI (D1306). All incubations were done at room temperature. All slides were scanned using a Nikon A1R VAAS laser point- and resonant-scanning confocal microscope (using a single photon Ar-ion laser at a magnification of $\times 60$ with a $4\times$ zoom).

Cycloheximide treatment of A375 cells and analysis of HIF-1 α expression. A375/VO and A375/211 cells (2.5×10^5 cells) were plated in a 6-well plate (per cell line) and allowed to grow overnight. The medium was removed, the cells were washed, and fresh medium was added. Cycloheximide treatment was performed at a final concentration of 200 μ g/ml, and samples were harvested at time points 0, 30, 60, and 90 min. Samples were acquired by removing the medium, washing the cells with PBS, and then adding 150 μ l sample buffer (with SDS and dithiothreitol [DTT]), mixing the well, acquiring the sample, and boiling at 95°C. Samples were loaded into 12% SDS-PAGE gels and run (as per the Western blot protocols described above). Quantitation of bands was performed by densitometry (as above).

Luciferase promoter reporter assays. The upstream promoter regions of both *ERR γ* and *PDK4* were acquired by PCR using genomic DNA acquired from A375 cells as a template. The following primer sets were used: *ERR γ* -201 Pro Forward, GCACTGAGAGATGCAAGTCAGC AAG, and *ERR γ* -201 Pro Reverse, GGCAGGGTCAGAACTGCAGCAG (producing a 1,025-bp fragment containing two HRE sites); and *PDK4* Pro Forward, TCTGTCCAGTGGCAGAGAAGCCCC, and *PDK4* Pro Reverse, GGTTCGAGATTCAAGTTCAGTCTTCCC (producing a 540-bp fragment containing 1 *ERR γ* response element [ERRE] site). Both primer sets contained 5' BglII sites and a 3' HindIII site. These PCR products were amplified using Phusion high-fidelity DNA polymerase (New England BioLabs) and cloned into pCR4-TOPO (Life Technologies) for sequencing. Sequence-confirmed clones were subcloned into a pcDNA6/Luciferase vector using their BglII and HindIII sites, replacing the 5' cytomegalovirus (CMV) promoter present in this expression vector with either the *ERR γ* or *PDK4* promoter sequences (producing pcDNA6/Luc/*ERR γ* Pro or pcDNA6/Luc/*PDK4* Pro). To test the constructs, 2.5×10^5 cells were laid down in 6-well plates and transfected with 500 ng of each vector and 500 ng of pRL-CMV (a *Renilla* luciferase

FIG 5 Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and mitochondrial content in melanoma cells with and without miR-211 expression. (A and B) Both A375/VO and A375/211 cells were analyzed using the Seahorse XF analyzer. OCR and ECAR were measured in untreated cells, followed by treatment with oligomycin and follow-up treatment with FCCP. Each sample was measured in nine wells per treatment. The assay was performed in triplicate. (C and D) Mitochondrial content in melanoma cells with and without miR-211 expression. MitoTracker Red staining of A375/VO and A375/211 cells. Cells were scanned using a Nikon A1R VAAS confocal microscope. Images are at a magnification of $\times 60$ with a $4\times$ zoom (C) and at a magnification of $\times 10$ with no zoom (D). Red, MitoTracker Red; green, phalloidin-FITC; blue, DAPI. (E and F) Quantitation of mitochondrial DNA by qPCR comparison of the genomic gene *LPL* and the mitochondrial gene *ND1* in A375/VO versus A375/211 cells. The experiment was performed in triplicate.

FIG 6 Measurement of oxygen consumption rate (OCR) (A) and extracellular acidification rate (ECAR) (B) in WM1552C melanoma cells with and without miR-211-5p expression. Both WM1552C/VO and WM1552C/211 cells were analyzed using the Seahorse XF analyzer. OCR and ECAR were measured in untreated cells, followed by treatment with oligomycin and subsequently with FCCP. Data for each sample were measured in nine wells per treatment. The assay was performed in triplicate.

control plasmid) using Eugene 6 (Roche). The following cell lines were tested: A375/VO, A375/211, A375/211/PDK4, and A375/211/Hif-1 α , along with untransfected A375/VO cells (as a negative control). Forty-eight hours after transfection, all cells were washed with PBS, harvested by trypsinization, and then lysed in cell culture lysis reagent (Promega). The lysed samples were mixed with the luciferase assay reagent II (LAR II), followed by STOP-and-Glo using the Dual-luciferase reporter assay system (Promega Corp). Luminescence was measured using a GloMax 20/20 luminometer (Promega Corp). All assays were performed in triplicate, and luminescence was normalized to the transfected A375/VO cell samples.

RESULTS

miR-211 sensitizes melanoma cells to hypoxia. We previously reported that miR-211 expression is significantly reduced or absent in nonpigmented melanoma cells and that exogenously expressed miR-211 inhibits melanoma cell proliferation and reduces invasion *in vitro* (14). To further investigate its tumor-suppressive role, we ectopically expressed miR-211 in the stage IV BRAF (V600E) melanoma cell line A375 (A375/211). Engineered cells (A375/211) expressed miR-211 at levels higher than those of pa-

rental vector-only (VO) controls (A375/VO), but at only about 30% of that seen in primary human melanocytes (Fig. 1A). Since we had previously shown that miR-211 expression hinders melanoma cell invasion and is at least in part driven by hypoxia (19), we subjected A375/211 and A375/VO cells to hypoxic conditions (2% O₂) to examine whether miR-211 may contribute to this phenomenon. After 48 h of incubation, the number of A375/211 cells decreased more than 70% in response to hypoxia, whereas A375/VO cells reduced only 30% (Fig. 2A and B). A similar effect was seen when cells were treated with deferoxamine (DFO), an iron chelator that indirectly induces hypoxia (Fig. 3A and B). To confirm that these results were not an isolated occurrence, an additional melanoma cell line (stage III WM1552C) was engineered to express miR-211, and similar effects were observed in response to DFO (Fig. 3C to F). These results suggest that nonpigmented melanoma cells expressing miR-211 are sensitive to hypoxia. Next, we measured miR-211 expression levels in A375, WM1552C, and primary human melanocytes under hypoxic conditions to investigate the role of hypoxia on miR-211 in parental cells. We observed a 60% loss of miR-211 in hypoxic A375 cells

TABLE 1 Mass spectrometry results of putative target genes downregulated in A375/211 versus A375/VO cells

Symbol	Entrez gene name	Fold change	Location	Type(s)	Prediction methods
GRAMD4	GRAM domain containing 4	−33.089	Unknown	Other	TargetScan miRanda
ADORA2A	Adenosine A2a receptor	−24.73	Plasma membrane	G-protein-coupled receptor	miRDB miRanda
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	−16.597	Cytoplasm	Kinase	TargetScan miRDB miRanda
HAPLN1	Hyaluronan and proteoglycan link protein 1	−13.871	Extracellular space	Other	TargetScan miRDB miRanda
SAMD4A	Sterile alpha motif domain containing 4A	−11.335	Cytoplasm	Translation regulator	DIANA TargetScan miRanda
ATXN1	Ataxin 1	−10.509	Nucleus	Transcription regulator	TargetScan miRanda
TAF5L	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65 kDa	−7.57	Nucleus	Transcription regulator	TargetScan miRanda
SOX11	SRY (sex determining region Y)-box 11	−7.493	Nucleus	Transcription regulator	TargetScan miRDB miRanda
EFEMP2	EGF containing fibulin-like extracellular matrix protein 2	−5.633	Extracellular space	Other	TargetScan miRDB miRanda
ZCCHC24	Zinc finger, CCHC domain containing 24	−4.029	Unknown	Other	TargetScan miRDB miRanda
DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3 (<i>Saccharomyces cerevisiae</i>)	−3.013	Cytoplasm	Other	TargetScan miRDB miRanda
SIX1	SIX homeobox 1	−3.01	Nucleus	Transcription regulator	TargetScan miRDB miRanda
CLIP4	CAP-GLY domain containing linker protein family, member 4	−2.722	Unknown	Other	DIANA TargetScan miRanda
NIPBL	Nipped-B homolog (<i>Drosophila</i>)	−2.647	Nucleus	Transcription regulator	TargetScan miRanda
SRPK2	SRSF protein kinase 2	−2.525	Nucleus	Kinase	TargetScan miRanda
CCNY	Cyclin Y	−2.321	Nucleus	Other	TargetScan miRDB miRanda
PPM1E	Protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent, 1E	−2.289	Nucleus	Phosphatase	TargetScan miRDB miRanda
ELF2	E74-like factor 2 (ets domain transcription factor)	−2.288	Nucleus	Transcription regulator	TargetScan miRanda
HS2ST1	Heparan sulfate 2-O-sulfotransferase 1	−2.198	Cytoplasm	Enzyme	TargetScan miRDB miRanda
PHF19	PHD finger protein 19	−2.055	Unknown	Other	TargetScan miRanda
HDAC9	Histone deacetylase 9	−2.033	Nucleus	Transcription regulator	TargetScan miRDB miRanda
TFAP2B	Transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	−1.493	Nucleus	Transcription regulator	DIANA TargetScan miRDB miRanda
FAM177A1	Family with sequence similarity 177, member A1	−1.43	Unknown	Other	TargetScan miRanda
SCNM1	Sodium channel modifier 1	−1.314	Nucleus	Other	DIANA TargetScan miRanda
SEC22A	SEC22 vesicle trafficking protein homolog A (<i>S. cerevisiae</i>)	−1.277	Cytoplasm	Transporter	TargetScan miRanda
MCC	Mutated in colorectal cancers	−1.249	Cytoplasm	Other	TargetScan miRanda
BIN1	Bridging integrator 1	−1.117	Nucleus	Other	TargetScan miRDB miRanda

(Fig. 1B) and to a lesser extent in WM1552C cells (Fig. 1C). Interestingly, we observed a slight increase in miR-211 expression in primary human melanocytes under hypoxic conditions (Fig. 1D). The above results together suggest that hypoxia indeed modulates the expression of miR-211 in parental cells.

Since hypoxia-inducible factors are known to respond to hypoxia, we measured HIF-1 α protein levels in A375/211 and

A375/VO cells by Western blot analysis. The results indicated that HIF-1 α protein levels were significantly lower in miR-211-expressing cells than in vector-only controls (Fig. 2C and 1E). To establish whether reduced HIF-1 α expression by miR-211 is causally related to invasion, HIF-1 α expression was knocked down in A375/VO cells and was subsequently overexpressed in A375/211 cells, as confirmed by Western blot analysis (Fig. 2D and 1F).

FIG 7 Expression and cellular phenotypes of *PDK4* in human melanoma cells with and without miR-211. (A) Putative miR-211 target genes. (B) Western blot analysis of *PDK4* expression in HEM-I primary melanocytes as well as A375/VO and A375/211 cells (using GAPDH as a loading control). (C) qPCR analysis of *PDK4* expression in HEM-I primary melanocytes, A375/VO, and A375/211 cells. All analyses were performed in triplicate. (D and E) Effects of miR-211 and *PDK4* on cell invasion. Results in panel D are normalized to A375/VO + Scr siRNA. The assay was performed in triplicate. (F) Effects of miR-211 and *PDK4* on melanoma cell motility. Wound-healing assay of A375/VO cells with negative-control siRNA versus *PDK4* siRNA and A375/211 with negative-control plasmid versus *PDK4* expression plasmid. Representative images of wound-healing assays for cells at a magnification of $\times 20$ cover a 24-hour period. Assays were performed in triplicate.

FIG 8 (A) Densitometry of Western blots illustrated in Fig. 7B. Scanning and quantitation for bands performed using NIS-Elements AR Analysis 4.2 software with data normalized to the mean intensity of the appropriate GAPDH load control. (B and C) Western blot analysis of PDK4 expression in A375/VO (B) and WM1552C/VO (C) cells subjected to normoxic versus hypoxic (2% O₂) conditions after 48 h. GAPDH was used as a loading control. (D) Western blot analysis of PDH and PDH-phosphate expression in A375/VO cells with Scramble control siRNA versus *PDK4* siRNA and A375/211 with “vector only” (VO) control plasmid versus *PDK4* expression plasmid. GAPDH was used as a load control. **, $P < 0.05$, Student's t test.

Concurrent cell invasion assays confirmed reduced invasion in A375/VO cells after HIF-1 α knockdown (Fig. 2E and F, top right panel) compared to scrambled siRNA controls (Fig. 2E and F, top left panel) and the recovery of invasion in A375/211 cells expressing HIF-1 α (Fig. 2E and F, bottom right). Next, to investigate the direct link between miR-211 and HIF-1 α , we measured HIF-1 α mRNA in cells either with (miR-211 mimics) and without (anti-miR-211) miR-211 by qPCR and revealed that there was no significant difference in expression levels of HIF-1 α mRNA regardless of the presence or absence of the miRNA (Fig. 4A). These results suggest that HIF-1 α mRNA is not a direct target of miR-211. This leaves open the possibility that HIF-1 α protein levels are destabilized indirectly by miR-211. To examine this possibility, we performed translation-blocking experiments by treating A375/VO and A375/211 cells with cycloheximide and assaying the levels of HIF-1 α protein remaining in a time course series by Western blotting. This analysis revealed that there was significant loss of HIF-1 α in both cell lines, but the loss was nearly twice as rapid in the presence of miR-211 expression as in its absence

FIG 9 TCGA SKCM data illustrating *PDK4* expression in primary tumors ($n = 183$) versus metastatic ($n = 369$) patient samples (A) and miR-211-5p expression in primary tumors ($n = 178$) versus metastatic ($n = 353$) patient samples (B), both measured by log₂ expression.

(~70% versus 40% reduction in 90 min) (Fig. 4B and C). These results indicate that the HIF-1 α protein, but not its transcript, is destabilized in the presence of miR-211 in nonpigmented melanoma cells, and therefore this destabilization is mostly likely mediated indirectly by the action of miR-211. Next, we asked whether the observed destabilization was at least in part associated with an increased oxygen consumption rate (OCR) in these cells.

The oxygen consumption rate (OCR) increases in A375/211 cells. The OCRs were measured in A375/211 and A375/VO cells using an extracellular metabolic flux analyzer under normal (non-hypoxic) conditions, revealing that the OCR of A375/211 cells was twice (170 pmol/min) that of A375/VO cells (70 pmol/min) (Fig. 5A). OCRs of both A375/211 and A375/VO cells were reduced significantly (~60 versus 30 pmol/min) after treatment with oligomycin, an ATP synthesis/electron transport inhibitor, suggesting that oxidative phosphorylation occurs in both cell lines. However, when the cells were treated with trifluorocarbonylcyanide phenylhydrazide (FCCP), an uncoupler of electron transport and oxidative phosphorylation, the OCR was elevated to 200 pmol/min in A375/211 cells compared to 90 pmol/min in A375/VO cells; miR-211 expression in A375 cells is, therefore,

Human PDK4 ENST00000005178.5 3' UTR length: 2144

[Download table]

Conserved

Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P _{CT}
---	-----------	-----------------	----------------------------	--------------------------	-------------------------	-----------------

Context++ score and features that contribute to the context++ score are evaluated as in Agarwal et al., 2015.

Conserved branch lengths and P_{CT} are evaluated as in Friedman et al., 2008, with an expanded 84-species alignment as described in Agarwal et al., 2015.**Poorly conserved**

Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	P _{CT}
Position 303-309 of PDK4 3' UTR 5' ...UUUGAUAAAGAAUAAAGAAAGGAG... hsa-miR-211-5p 3' UCCGCUUCCUACUGUUUCCUU	7mer-m8		83	-0.12	1.438	< 0.1
Position 303-309 of PDK4 3' UTR 5' ...UUUGAUAAAGAAUAAAGAAAGGAG... hsa-miR-204-5p 3' UCCGUAUCCUACUGUUUCCUU	7mer-m8		81	-0.11	1.438	< 0.1

Context++ score and features that contribute to the context++ score are evaluated as in Agarwal et al., 2015.

Conserved branch lengths and P_{CT} are evaluated as in Friedman et al., 2008, with an expanded 84-species alignment as described in Agarwal et al., 2015.

FIG 10 Alignment of miR-211-5p to the 3' UTR of *PDK4* mRNA by TargetScan 7.0. Prediction software indicates a predisposition toward primate-specific species and poor conservation among various other mammals and invertebrates. A putative target site for miR-211-5p is identified at positions 302 to 308 in the 3' UTR of *PDK4*.

FIG 11 miRNA-targeting of the 3' UTR of *PDK4* by miR-211-5p. (A) Schematic representation of the target site for miR-211-5p in the 3' UTR of the *PDK4* mRNA. The sequence alignment also illustrates the location and specific base pair changes made to construct the mutant form of the target site. (B) Analysis was performed by measurement of luciferase expression normalized to A375/VO cells transfected with a positive-control expression vector (pcDNA6/Luc) and compared to A375/VO cells transfected with vectors containing the entire *PDK4* 3' UTR (pcDNA6/Luc/*PDK4* 3'UTR) and a mutant *PDK4* 3' UTR (pcDNA6/Luc/*PDK4* 3'UTR/Mut, containing an altered "seed" sequence). Complementary transfections were performed in A375/211 cells. All transfections were performed in triplicate, and results were normalized with cotransfected *Renilla* luciferase plasmids.

associated with increased oxygen consumption. Additional confirmation was sought with WM1552C melanoma cells (also BRAF V600E, but stage III) ectopically expressing miR-211 (WM1552C/211), which showed a similar but less pronounced phenotype than with A375 (Fig. 6A).

We next assessed glycolytic flux by measuring the extracellular acidification rate (ECAR), a surrogate of glycolysis (which leads to the secretion protons to the extracellular medium), in A375/VO and A375/211 cells. Under normal conditions, ECAR profiles were reciprocal to the respective OCR profiles (Fig. 5B), which was also the case with WM1552C/211 versus WM1552C/VO cells (Fig. 6B). These results are consistent with miR-211 acting as a metabolic control switch in melanoma cells by favoring oxidative phosphorylation over glycolysis.

Since the number of active mitochondria is directly correlated with OCR in metabolically active cells (20), we sought to establish whether the increased OCR observed in A375/211 cells was correlated with the mitochondrial number by using Mito-Tracker staining. We found that the number of mitochondria per cell was significantly greater in A375/211 cells than in A375/VO cells (Fig. 5C and D). To further assess the apparent increase in mitochondria in A375/211 cells, DNA corresponding to the mitochondrial-specific gene *ND1* and to the nuclear gene lipoprotein lipase (*LPL*) was quantified by qPCR. As expected, *ND1* expression was increased in A375/211 cells compared to A375/VO cells (Fig. 5E), signaling an increase in the number of mitochondrial genomes, whereas the total nuclear *LPL* DNA levels were the same in the two

cell lines (Fig. 5F). These results suggest that the higher OCRs of A375/211 cells are due to increased numbers of mitochondria.

miR-211 downregulates *PDK4* expression in melanoma cells. To identify the mechanism(s) by which miR-211 alters cellular metabolism in melanoma cells, protein expression was compared in A375/211 and A375/VO cells using mass spectrometry (MS). Over 3,000 proteins were modulated by miR-211. The most downregulated miR-211 putative target proteins are listed in Table 1. Among the most downregulated gene products, pyruvate dehydrogenase kinase 4 (*PDK4*) was selected for further investigation (Fig. 7A) because (i) it is a key oxidative phosphorylation enzyme and (ii) its mRNA and protein contents were both highly downregulated in A375/211 cells (Fig. 7B and C and 8A). *PDK4* is known to phosphorylate a pyruvate dehydrogenase complex subunit, thereby inhibiting conversion of pyruvate to acetyl coenzyme A (acetyl-CoA), a key cofactor in ATP production, by coupling mitochondrial oxidative phosphorylation to the tricarboxylic acid (TCA) cycle. We postulate that the downregulation of *PDK4* expression by miR-211 leads to an increase in the conversion of pyruvate to acetyl-CoA by fueling the TCA cycle and driving electron transport to increase oxygen utilization in A375/211 cells.

Next, we hypothesized that if increased miR-211 expression is associated with decreased *PDK4* expression, then the inverse may also be true. Previous data revealed that hypoxia reduced miR-211 expression in A375/VO cells (Fig. 1B). An analysis of *PDK4* protein expression confirmed that it was elevated under the same

FIG 12 Measurement of mitochondrial content in miR-211-5p-expressing melanoma cells with and without PDK4 expression. (A and B) A375/VO, A375/211, and A375/211/PDK4 cells were stained for mitochondrial content using MitoTracker Red on a Nikon A1R VAAS confocal microscope. Images taken were at a magnification of $\times 10$ with no zoom (A) and at a magnification of $\times 60$ with a $4\times$ zoom (B). Red, MitoTracker Red; green, phalloidin-FITC; blue, DAPI. (C) Mean intensity per cell for DAPI, FITC (phalloidin), and MitoTracker Red was quantified using NIS-Elements AR Analysis 4.2 software, and results were graphed for the images in panel A.

conditions (Fig. 8B). Similar results were also observed with WM1552C cells under hypoxic conditions (Fig. 1C and 8C). To provide further corroborative evidence of the expression correlation between miR-211 and PDK4, we mined *PDK4* gene expression levels in melanoma patient samples using the publicly available TCGA (the Cancer Gene Atlas) database. *PDK4* was approximately 4-fold upregulated in advanced metastatic melanomas compared to its levels in primary melanomas (Fig. 9A) ($P < 0.0001$). Likewise, miR-211 was found to be downregulated in the

same cohort (Fig. 9B). Since we and others have previously shown that miR-211 is downregulated in advanced melanomas (12, 14), we suggest that miR-211 downregulation is mechanistically related to *PDK4* upregulation in metastatic melanoma. Next, we examined several publicly available *in silico* databases to identify whether *PDK4* is a putative miR-211 target transcript. Indeed, *PDK4* was identified as a nonconserved miR-211 target (Fig. 10). The seed sequence of the predicted miR-211 target-binding site within the *PDK4* mRNA sequence is located at positions 302 to

FIG 13 (A) Oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and mitochondrial content in miR-211-expressing melanoma cells with and without PDK4 expression. (A and B) A375/VO, A375/211, and A375/211/PDK4 cells were analyzed using the Seahorse XF analyzer. OCR and ECAR were measured, followed by treatment with oligomycin, FCCP, and rotenone/antimycin A. Each sample was measured in six wells per treatment. The assay was performed in triplicate. (C) Quantitation of mitochondrial DNA by qPCR comparison of the mitochondrial gene *ND1* and the genomic gene *LPL* and in A375/VO, A375/211, and A375/211/PDK4 cells. The experiment was performed in triplicate.

308 in the 3' UTR (Fig. 11A). We experimentally validated *PDK4* mRNA as a miR-211 target by replacing the 3' UTR of a luciferase reporter plasmid (pcDNA6/Luc/*PDK4* 3'UTR) with the 3' UTR of *PDK4* (2,142 bp, located on chromosome 7 at bp 95583499 to 95585640) followed by subsequent cell line transfection. A stan-

dard luciferase reporter plasmid (pcDNA6/Luc) and a reporter plasmid in which the target site seed sequence of the 3' UTR of *PDK4* was mutated (Fig. 11A) were used as controls. Reporter gene expression was consistently high in A375/VO cells and significantly reduced in A375/211 cells, suggesting that miR-211 can indeed target *PDK4* in human melanoma cells (Fig. 11B). Mutating the seed sequence abolished this effect. Interestingly, this non-conserved seed sequence appears to be primate specific (Fig. 10), and thus we believe that *PDK4* target conservation could be an evolutionarily conserved event in primates.

Exogenous expression of *PDK4* restores the invasion and cell motility defects in A375/211 cells. Since *PDK4* is downregulated in cells expressing miR-211 and the resulting phenotype had a significant loss of invasion and motility, we next examined whether reintroduction of *PDK4* could reverse this phenotype. Invasion was reduced (by greater than 50%), but not by scrambled control oligonucleotides (Fig. 7D and E). Similarly, when A375/211 cells stably expressed a *PDK4*-expressing plasmid (pcDNA6/*PDK4*), the invasiveness of A375/211 cells increased significantly (from ~30% to ~80%). Therefore, the cell invasiveness phenotype of melanoma cells is reciprocally dependent on the expression of miR-211 and *PDK4*—the former increases invasiveness, whereas the latter reduces it.

We next performed a wound-healing assay to further investigate the effects of miR-211 and *PDK4* on cell motility during wound healing (Fig. 7F). Control A375/VO cells transfected with scramble siRNA closed the wound after only ~12 h, whereas A375/VO cells transfected with *PDK4* siRNA were incapable of closing the wound even after 24 h. A375/211 cells stably maintaining a vector-only plasmid were also incapable of closing the wound after 24 h, whereas *PDK4*-expressing stable A375/211 cells significantly increased the wound-healing speed (within 18 h). Together, these results demonstrate that the motility defects caused by miR-211 expression in melanoma cells are *PDK4* dependent.

Since previous data indicated that mitochondrial content could be modulated by the expression of miR-211, we repeated the MitoTracker labeling to examine the additional effect of *PDK4* expression on these cells (Fig. 12). A375/211/*PDK4* cells showed a significant decrease in mitochondrial labeling compared to A375/211 cells. This was confirmed by the qRT-PCR comparison of *ND1* and *LPL* DNA content in these cells (Fig. 13C). Likewise, measurements of OCR and ECAR revealed diminished respiration and increased glycolytic flux in A375/211/*PDK4* cells compared to their parental A375/211 cells (Fig. 13A and B), suggesting a partial rescue of metabolic changes induced in A375 cells due to the presence of miR-211.

Finally, since the *PDK4* protein is known to phosphorylate pyruvate dehydrogenase (*PDH*), thereby deactivating the latter's ability to convert pyruvate to acetyl-CoA, we examined whether miR-211 and *PDK4* expression could modulate pyruvate dehydrogenase phosphorylation. *PDK4* siRNA abolished *PDH* phosphorylation in A375/VO cells (Fig. 8D). Conversely, when *PDK4* was reintroduced into A375/211 cells, there was robust *PDH* phosphorylation (Fig. 8D). These results further confirm that miR-211 alters the cellular metabolic state by destabilizing HIF-1 α and downregulating *PDK4*.

HIF-1 α regulates *PDK4* expression via ERR γ in A375/211 cells. Since the loss of HIF-1 α could be due to increased OCR in A375/211 cells, we hypothesized that *PDK4* downregulation may

FIG 14 Effects of miR-211 on HIF-1 α , PDK4, and ERR γ expression in human melanoma cells. (A) Western blot analysis of PDK4, ERR γ , HIF-1 α , and RUNX2 expression in A375/VO, A375/211, A375/211/PDK4-expressing, and A375/211/HIF-1 α -expressing cell lines. GAPDH was used as a loading control. (B) Immunohistochemical labeling of PDK4 in A375/VO, A375/211, A375/211/PDK4-expressing, and A375/211/HIF-1 α -expressing cell lines. Red, PDK4; green, phalloidin-FITC; blue, DAPI. (C) Measurement of the transcriptional activation of the ERR γ promoter by exogenous expression of HIF1 α or PDK4. The ERR γ promoter containing two HRE sites (HIF-1 α responsive elements) was cloned upstream of a luciferase reporter gene. (D) The PDK4 promoter containing one ERRE (ERR γ response element) was cloned upstream of a luciferase reporter gene. Both reporter constructs were transfected into A375/211 cells alone or cotransfected with either a HIF1 α or PDK4 expression plasmids. A375/VO cells were transfected with the reporter constructs as controls. Each experiment was performed in triplicate, and the results were averaged.

FIG 15 (A) Densitometry of Western blots illustrated in Fig. 14A. Scanning and quantitation for all bands performed using NIS-Elements AR Analysis 4.2 software with data normalized to the mean intensity of the appropriate GAPDH load control. (B) Measurement of mRNA expression of putative genes linked to PDK4 by the Hif-1α network in A375 melanoma cells. qRT-PCR of genes capable of modulating PDK4 expression as regulated by Hif-1α, including *C/EBPΔ*, *ERRγ*, *GCRβ*, *HNFα*, *PPARGC1* (*PGC1α*), *PPARγ*, *PROX1*, *RARα*, *RUNX2*, *SIRT1*, *SP1*, and *THRα* as examined in A375/VO versus A375/211 cells. All experiments were performed in triplicate, and results were averaged and then normalized to A375/VO cells. **, $P < 0.05$, Student's t test.

FIG 16 Mechanism of action of miR-211 utilizing PDK4 to modulate respiration. (A) In melanocytes, miR-211 expression downregulates *PDK4* expression, leading to increased PDH activity, driving the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (orange rounded boxes), and ATP production. This increases the respiration (O_2 consumption) rate, which leads to HIF-1 α instability and loss of ERR γ transcriptional activation, which in turn leads to decreased *PDK4* transcriptional activation. (B) In melanoma cells, decreased expression of miR-211 increases *PDK4* translation, leading to decreased PDH activity due to phosphorylation of PDH protein, thus downregulating the TCA cycle, and oxidative phosphorylation by mitochondria. This decreases the respiration rate, which leads to increased stability of HIF-1 α and increased transcriptional activation of ERR γ , which in turn leads to increased *PDK4* transcriptional activation.

be an upstream regulator of OCR and HIF-1 α destabilization. To determine whether PDK4 directly influences HIF-1 α , *PDK4* was stably reintroduced and expressed in A375/211 cells. HIF-1 α protein expression increased significantly in A375/211/*PDK4* cells (Fig. 14A and 15A). Interestingly, PDK4 protein levels were also increased in HIF-1 α -expressing A375/211 cells relative to A375/211 only cells (Fig. 14A and 15A); a positive feedback loop between HIF-1 α and PDK4 may therefore exist. Western blot results were further confirmed by immunocytochemistry (ICC) (Fig. 14B).

The above results suggest that metastatic melanoma cells can exhibit anaerobic-respiration-dependent cancerous phenotypes that appear to be driven by miR-211 upregulation, the consequent inhibition of oxidative phosphorylation, and increased HIF-1 α , which in turn mediates anaerobic respiration and the Warburg effect. To interrogate this emerging model, we utilized a text-mining algorithm (Ingenuity Pathway Analysis software) to build a gene interaction network for HIF-1 α and PDK4. qRT-PCR analysis was performed to measure the expression of network genes in A375/211 and A375/VO cells (Fig. 15B). The expression of several network genes was upregulated (Fig. 15B), but only one gene, ERR γ , was significantly downregulated. HIF-1 α is capable of direct transcriptional modulation of ERR γ in human pancreatic beta cells, which could transcriptionally activate *PDK4* expression. ERR γ protein expression was examined by Western blotting, and a significant loss was observed in A375/211 cells (Fig. 14A and 15A). To determine whether HIF-1 α or PDK4 could transcriptionally activate ERR γ , the ERR γ promoter (containing two HIF-1 α -responsive elements [HREs]) was cloned 5' to a luciferase re-

porter gene and cotransfected into melanoma cells with either an HIF-1 α or a *PDK4* expression plasmid. Both genes transactivated the ERR γ promoter in A375/211 cells (Fig. 14C). Likewise, a cloned *PDK4* upstream promoter reporter (containing one ERR γ response element) cotransfected with HIF1 α or *PDK4* also confirmed transactivation of the *PDK4* promoter (Fig. 14D). Together, these data indicate that HIF-1 α -mediated upregulation of ERR γ is likely responsible for the increased expression of *PDK4* in melanoma cells.

DISCUSSION

This is the first report of the involvement of miR-211 in the regulation of mitochondrial energy metabolism in human melanoma cells. We show that melanoma cells that exogenously express miR-211 are more dependent on oxidative phosphorylation and display a corresponding increase in mitochondrial number. Unlike their precursors, these cells are sensitized to oxygen deprivation, and therefore, we propose that miR-211 may act as a metabolic switch to directly inhibit *PDK4*. In this way, hypoxia sensitivity is indirectly conferred via HIF-1 α destabilization. In normal melanocytes, which express miR-211, this is likely to be a mechanism for tumor suppression, with miR-211 loss in transformed melanocytes contributing to the malignant phenotype. The gain of HIF-1 α in melanoma cells is consistent with the malignant properties seen in melanoma cells that fail to express miR-211. The loss or destabilization of HIF-1 α is associated with tumor growth (19), underscoring the importance of miR-211 loss in melanoma cells.

We propose a model of miR-211-mediated melanomagenesis in Fig. 16. In normal melanocytes, miR-211 directly targets *PDK4*

mRNA; as a result, PDK4 protein expression is low (Fig. 16A). When miR-211 expression is reduced or eliminated by tumorigenic transformation, PDK4 protein expression increases due to the release of translational inhibition by miR-211 downregulation (Fig. 16B). Increased PDK4 activity, in turn, phosphorylates pyruvate dehydrogenase, resulting in less oxidative phosphorylation, which is known to be associated with mitochondrial depletion as observed in our studies. Reduced mitochondrial activity decreases oxygen consumption, leading to increased HIF-1 α stabilization and, via positive regulation of ERR γ , to the upregulation of *PDK4* expression via a positive feedback loop. In this way, the Warburg effect is established in metastatic melanoma cells. In support of this mechanism, we note that Lee et al. (21) had previously reported a positive correlation between HIF-1 α , ERR γ , and PDK4 expression.

We suggest that in normal melanocytes, a constitutive miR-211 expression (14) inhibits the translation of *PDK4* mRNA, decreasing phosphorylation of the pyruvate dehydrogenase enzyme, and thus enhancing its activity. This consequently leads to higher rates of citric acid cycle and oxidative phosphorylation activities through increased mitochondrial oxygen consumption. HIF-1 α becomes destabilized due to increased oxygen consumption, leading to less ERR γ and PDK4 production, allowing melanocytes to maintain a normal oxidative metabolic state. miR-211 therefore appears to act as a toggle switch, which can possibly be exploited in the future for therapeutic purposes against this difficult-to-treat cancer.

ACKNOWLEDGMENTS

We thank the Sanford-Burnham Genomics, Proteomics, Bioinformatics, Histology, and Microscopy core facilities for their assistance and Angelese Duran for Western blot analysis. We also thank Debbie McFadden for formatting the manuscript.

FUNDING INFORMATION

Florida Department of Health, Bankhead-Cole Cancer Research Program provided funding to Ranjan J. Perera under grant number 5BC08. HHS | NIH | National Cancer Institute (NCI) provided funding to Ranjan J. Perera under grant number 5P30CA030199. HHS | National Institutes of Health (NIH) provided funding to Ranjan J. Perera under grant number R03CA172847.

REFERENCES

- Haq R, Fisher DE, Widlund HR. 2014. Molecular pathways: BRAF induces bioenergetic adaptation by attenuating oxidative phosphorylation. *Clin Cancer Res* 20:2257–2263. <http://dx.doi.org/10.1158/1078-0432.CCR-13-0898>.
- Maes H, Agostinis P. 2014. Autophagy and mitophagy interplay in melanoma progression. *Mitochondrion* 19(Part A):58–68. <http://dx.doi.org/10.1016/j.mito.2014.07.003>.
- Parmenter TJ, Kleinschmidt M, Kinross KM, Bond ST, Li J, Kaadige MR, Rao A, Sheppard KE, Hugo W, Pupo GM, Pearson RB, McGee SL, Long GV, Scolyer RA, Rizzo H, Lo RS, Cullinan C, Ayer DE, Ribas A, Johnstone RW, Hicks RJ, McArthur GA. 2014. Response of BRAF-mutant melanoma to BRAF inhibition is mediated by a network of transcriptional regulators of glycolysis. *Cancer Discov* 4:423–433. <http://dx.doi.org/10.1158/2159-8290.CD-13-0440>.
- Theodosakis N, Micevic G, Kelly DP, Bosenberg M. 2014. Mitochondrial function in melanoma. *Arch Biochem Biophys* 563:56–59. <http://dx.doi.org/10.1016/j.abb.2014.06.028>.
- Serguenco A, Grad I, Wennerstrom AB, Meza-Zepeda LA, Thiede B, Stratford EW, Myklebost O, Munthe E. 2015. Metabolic reprogramming of metastatic breast cancer and melanoma by let-7a microRNA. *Oncotarget* 6:2451–2465. <http://dx.doi.org/10.18632/oncotarget.3235>.
- Herst PM, Berridge MV. 2013. Cell hierarchy, metabolic flexibility and systems approaches to cancer treatment. *Curr Pharm Biotechnol* 14:289–299. <http://dx.doi.org/10.2174/1389201011314030005>.
- Vazquez F, Lim JH, Chim H, Bhalla K, Giron G, Pierce K, Clish CB, Granter SR, Widlund HR, Spiegelman BM, Puigserver P. 2013. PGC1 α expression defines a subset of human melanoma tumors with increased mitochondrial capacity and resistance to oxidative stress. *Cancer Cell* 23:287–301. <http://dx.doi.org/10.1016/j.ccr.2012.11.020>.
- Zheng B, Jeong JH, Asara JM, Yuan YY, Granter SR, Chin L, Cantley LC. 2009. Oncogenic B-Raf negatively regulates the tumor suppressor LKB1 to promote melanoma cell proliferation. *Mol Cell* 33:237–247. <http://dx.doi.org/10.1016/j.molcel.2008.12.026>.
- Scott DA, Richardson AD, Filipp FV, Knutzen CA, Chiang GG, Ronai ZA, Osterman AL, Smith JW. 2011. Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. *J Biol Chem* 286:42626–42634. <http://dx.doi.org/10.1074/jbc.M111.282046>.
- Boyle GM, Woods SL, Bonazzi VF, Stark MS, Hacker E, Aoude LG, Dutton-Regester K, Cook AL, Sturm RA, Hayward NK. 2011. Melanoma cell invasiveness is regulated by miR-211 suppression of the BRN2 transcription factor. *Pigment Cell Melanoma Res* 24:525–537. <http://dx.doi.org/10.1111/j.1755-148X.2011.00849.x>.
- Guo H, Carlson JA, Slominski A. 2012. Role of TRPM in melanocytes and melanoma. *Exp Dermatol* 21:650–654. <http://dx.doi.org/10.1111/j.1600-0625.2012.01565.x>.
- Kozubek J, Ma Z, Fleming E, Duggan T, Wu R, Shin DG, Dadrass SS. 2013. In-depth characterization of microRNA transcriptome in melanoma. *PLoS One* 8:e72699. <http://dx.doi.org/10.1371/journal.pone.0072699>.
- Levy C, Khaled M, Iliopoulos D, Janas MM, Schubert S, Pinner S, Chen PH, Li S, Fletcher AL, Yokoyama S, Scott KL, Garraway LA, Song JS, Granter SR, Turley SJ, Fisher DE, Novina CD. 2010. Intronic miR-211 assumes the tumor suppressive function of its host gene in melanoma. *Mol Cell* 40:841–849. <http://dx.doi.org/10.1016/j.molcel.2010.11.020>.
- Mazar J, DeYoung K, Khaitan D, Meister E, Almodovar A, Goydos J, Ray A, Perera RJ. 2010. The regulation of miRNA-211 expression and its role in melanoma cell invasiveness. *PLoS One* 5:e13779. <http://dx.doi.org/10.1371/journal.pone.0013779>.
- Sakurai E, Maesawa C, Shibasaki M, Yasuhira S, Oikawa H, Sato M, Tsunoda K, Ishikawa Y, Watanabe A, Takahashi K, Akasaka T, Masuda T. 2011. Downregulation of microRNA-211 is involved in expression of preferentially expressed antigen of melanoma in melanoma cells. *Int J Oncol* 39:665–672. <http://dx.doi.org/10.3892/ijo.2011.1084>.
- Xu Y, Brenn T, Brown ER, Doherty V, Melton DW. 2012. Differential expression of microRNAs during melanoma progression: miR-200c, miR-205 and miR-211 are downregulated in melanoma and act as tumour suppressors. *Br J Cancer* 106:553–561. <http://dx.doi.org/10.1038/bjc.2011.568>.
- Margue C, Philippidou D, Reinsbach SE, Schmitt M, Behrmann I, Kreis S. 2013. New target genes of MITF-induced microRNA-211 contribute to melanoma cell invasion. *PLoS One* 8:e73473. <http://dx.doi.org/10.1371/journal.pone.0073473>.
- Bell RE, Khaled M, Netanel D, Schubert S, Golan T, Buxbaum A, Janas MM, Postolsky B, Goldberg MS, Shamir R, Levy C. 2014. Transcription factor/microRNA axis blocks melanoma invasion program by miR-211 targeting NAK1. *J Invest Dermatol* 134:441–451. <http://dx.doi.org/10.1038/jid.2013.340>.
- Chang J, Erler J. 2014. Hypoxia-mediated metastasis. *Adv Exp Med Biol* 772:55–81. http://dx.doi.org/10.1007/978-1-4614-5915-6_3.
- Qian W, Van Houten B. 2010. Alterations in bioenergetics due to changes in mitochondrial DNA copy number. *Methods* 51:452–457. <http://dx.doi.org/10.1016/j.ymeth.2010.03.006>.
- Lee JH, Kim EJ, Kim DK, Lee JM, Park SB, Lee IK, Harris RA, Lee MO, Choi HS. 2012. Hypoxia induces PDK4 gene expression through induction of the orphan nuclear receptor ERRgamma. *PLoS One* 7:e46324. <http://dx.doi.org/10.1371/journal.pone.0046324>.