TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Roles for miR-375 in Neuroendocrine Differentiation and Tumor Suppression via Notch Pathway Suppression in Merkel Cell Carcinoma

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Dysfunction of key miRNA pathways regulating basic cellular processes is a common driver of many cancers. However, the biological roles and/or clinical applications of such pathways in Merkel cell carcinoma (MCC), a rare but lethal cutaneous neuroendocrine (NE) malignancy, have yet to be determined. Previous work has established that miR-375 is highly expressed in MCC tumors, but its biological role in MCC remains unknown. Herein, we show that elevated miR-375 expression is a specific feature of well-differentiated MCC cell lines that express NE markers. In contrast, miR-375 is strikingly down-regulated in highly aggressive, undifferentiated MCC cell lines. Enforced miR-375 expression in these cells induced NE differentiation, and opposed cancer cell viability, migration, invasion, and survival, pointing to tumor-suppressive roles for miR-375. Mechanistically, miR-375-driven phenotypes were caused by the direct post-transcriptional repression of multiple Notch pathway proteins (Notch2 and RBPJ) linked to cancer and regulation of cell fate. Thus, we detail a novel molecular axis linking tumor-suppressive miR-375 and Notch with NE differentiation and cancer cell behavior in MCC. Our findings identify miR-375 as a putative regulator of NE differentiation, provide insight into the cell of origin of MCC, and suggest that miR-375 silencing may promote aggressive cancer cell behavior through Notch disinhibition. (Am J Pathol 2016, 186: 1025 e1035; http://dx.doi.org/10.1016/j.ajpath.2015.11.020)

Merkel cell carcinoma (MCC) is a rare, but deadly, cutaneous neuroendocrine (NE) malignancy.1 Patients typically present with a rapidly growing nodule with bluish, red, or skin-tone coloration, frequently on sun-exposed areas of the skin.2 A confirmatory diagnosis mainly relies on immunohistochemical staining of tumor biopsy specimens.3 The incidence of MCC reportedly increased nearly threefold between 1986 and 2001.4 However, MCC remains a relatively rare disease with approximately 1600 new diagnoses made each year in the United States.5 Despite aggressive multimodal therapy in which radiation and surgery are the mainstay, MCC remains an immensely difficult-to-treat disease, as evidenced by a disease-specific mortality of 46%, nearly three times greater than malignant melanoma.1 Of concern, the clinical management of MCC patients lacks consensus across treatment centers, whereas the treatment of advanced disease continues to rely on non-specific chemotherapy, despite little evidence for appreciable benefits in survival or quality of life.6 Prognosis remains extremely dire, with 5-year survival rates of 57%, 38%, and 18% for localized, regional, and metastatic disease, respectively.7,8 Collectively, these challenges highlight the grave need for research that aims to characterize fundamental molecular and cellular disease mechanisms in MCC in ways that enable the development of prognostic markers and mechanism-driven targeted therapeutics that more effectively combat disease.

Several key discoveries in the past decade have led to an improved understanding of etiology and underlying biological mechanisms in MCC. Most notably, the discovery

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that most MCC tumors are associated with the genome of a small DNA virus known as Merkel cell polyomavirus (MCPyV) was first reported in 2008 and since confirmed by many subsequent studies.9–12 Despite such clear progress, basic research studies on MCC have remained restricted to few areas of investigation. This has significantly limited progress, as evidenced by the lack of animal/genetic MCC models and poor understanding of the role of several emerging and germane themes in cancer biology.

One major area of cancer biology that remains largely unexplored in MCC is the role of noncoding RNAs, specifically miRNAs. miRNAs are an abundant class of small (21 to 25 nucleotides) noncoding RNAs that fulfill crucial biological roles via the post-transcriptional repression of gene expression.13 In recent years, an extensive body of research has shed light on the biological importance and clinical applications of miRNAs in cancer.14,15 Notably, deregulated miRNA expression is a common feature of malignant progression and has been shown to affect a range of cancer cell behaviors, including cell proliferation, survival, and metastasis.14,15 Moreover, the characterization of target genes and functions of individual miRNAs has led to the discovery of novel pathways and a more detailed understanding of cancer-promoting mechanisms.16

Despite the growing recognition that miRNAs fulfill crucial roles in several cancers and may be useful as clinical biomarkers, only a handful of previous studies linking miRNAs and MCC have been published.16,17 As collaborators on a previous miRNA study, we contributed data on global miRNA expression profiles of MCC tumors, identifying miR-375 as the most highly expressed miRNA in MCC tumors relative to normal skin controls.16 Because the role of this miRNA in MCC remains unknown, we undertook a study to characterize the specificity and biological significance of miR-375 in MCC. Herein, we provide evidence that miR-375 is a marker of NE differentiation and, surprisingly, behaves as a tumor suppressor in MCC. We found that miR-375 is expressed abundantly in a subset of MCC cell lines with characteristic NE properties, but is silenced in cell lines that lack these markers. Intriguingly, we noted that miR-375—low cell lines were derived from aggressive tumors and had shorter dividing times in cell culture, thus pointing to a potential role for miR-375 as a tumor suppressor. Indeed, enforced expression of miR-375 in these cells promoted NE differentiation while opposing cancer cell viability, migration, invasion, and survival. Mechanistically, miR-375—driven phenotypes are caused by the direct post-transcriptional repression of two Notch pathway components, RBPJ and Notch2 proteins with extensive links to cancer and regulation of NE differentiation during development. Thus, we elucidate a novel molecular axis linking tumor-suppressive miR-375 and Notch with NE differentiation and cancer cell behavior in MCC. We postulate the miR-375 silencing may promote aggressive cancer cell behavior through disinhibition of the Notch pathway. The implications of our findings with respect to improved clinicopathological diagnosis and cell of origin in MCC are also discussed.

**Materials and Methods**

**Cell Lines**

MS-1, MCC13, and MCC26 were grown in RPMI 1640 medium (HyClone, Logan, UT) supplemented with 15% fetal bovine serum (HyClone), whereas all MCC14.2 cell lines required further supplementation with 1 mmol/L sodium pyruvate and 20 mmol/L HEPES. MKL-1 was grown in RPMI plus 10% fetal bovine serum. MCC14.2 cells were a kind gift of Dr. Bassem Hassan (Laboratory of Neurogenetics, Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium). All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO2.

**RNA Isolation, miRNA, and mRNA Quantitation**

miR-375 expression in cell lines was quantitated by quantitative RT-PCR (RT-qPCR) analysis using the TaqMan miRNA assay (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer’s protocol. Total RNA was isolated, and cDNA was generated by reverse transcription using primers specific for miR-375 and RNU6B, according to the manufacturer’s protocol (Thermo Fisher Scientific). miRNA expression was assayed in triplicate for any individual experiment, and data were normalized to endogenous RNU6B levels. Relative expression was calculated using the ΔΔCt method.

For quantification of gene expression, total RNA was isolated and reverse transcribed using the TaqMan Gene Expression assay, according to the manufacturer’s protocol. RT-qPCR was completed using primers specific for STMN2, STMN3, enolase 2, and β-actin (Invitrogen/Life Technologies). All gene expression data were normalized to endogenous β-actin levels, and relative mRNA levels were determined using the ΔΔCt method.

**miR-375 Overexpression and Knockdown**

mirVana miRNA mimics (Thermo Fisher Scientific) were used to achieve transient overexpression of miR-375 in variant MCC cell lines. For miR-375 overexpression, an initial titration of miR-375 mimics was performed to select the lowest concentration of miR-375 needed to achieve an expression level that most closely matched the difference between classic and variant cell lines. For all overexpression experiments, cells were seeded at approximately 30% confluence and transfected with 5 nmol/L of mirVana miR-375 or negative control (Neg Cntrl) using Lipofectamine 2000 (Thermo Fisher Scientific) as a transfection reagent. miR-375 overexpression was confirmed by RT-qPCR 72 hours after transfection. Anti-miR miRNA inhibitors (Thermo Fisher Scientific) were used to transiently knock
down miR-375 in MKL-1 and MS-1. Cells were seeded at 300,000 cells/mL in T25 culture dishes (Sigma-Aldrich, St. Louis, MO) and transfected with 100 nmol/L anti–miR-375 or anti-miRNA Neg Cntrl using Lipofectamine 2000. miR-375 knockdown was confirmed by RT-qPCR using RNA isolated 72 hours after transfection.

Gene Expression Microarray and Analysis

The Agilent Microarray Platform for One-Color Analysis of Gene Expression (Agilent Technologies, Santa Clara, CA) was used to profile gene expression changes associated with miR-375 overexpression. MCC26 cells were seeded in 10-cm dishes and transfected with 5 nmol/L of mirVana miR-375 or Neg Cntrl, as described.

Total RNA from MCC26 cells was isolated 72 hours after transfection using the miRNeasy Mini kit (Qiagen, Hilden, Germany). This experiment was performed in duplicate. For each sample, 500 ng total RNA was mixed with the Agilent One-Color Spike-in RNA control (Agilent Technologies), with the resulting mixture amplified and labeled using the Agilent One-Color, Quick Amp Labeling kit to generate Cy3-labelled complementary RNA (Agilent Technologies). Complementary RNA was subsequently fragmented at 60°C for 30 minutes and hybridized to Agilent Human 4X44K whole genome microarrays (Agilent Technologies) in a rotating oven at 65°C for 17 hours. Chips were scanned with the Agilent DNA Microarray scanner, and raw signals were quantified using the Agilent DNA Microarray scanner.

Bioinformatics Analyses

To determine gene expression changes associated with miR-375 overexpression in MCC26 cells, analysis was performed on microarray data corresponding to two independent experiments. Each experiment contained a miR-375 overexpression and Neg Cntrl sample. Feature Extraction Software version 12.0 (Agilent Technologies) was used to extract and analyze the signals from the microarray images. Differentially expressed genes were identified by means of the Bioconductor packages Agi4x44Preprocess and limma (Agilent Technologies). The microarray probes were filtered and normalized within and between arrays in accordance to the Agi4x44Preprocess and limma reference guides, respectively. Differential gene expression was tested using a linear regression model and was followed by a Bayes moderated t-test. Genes for which \( P < 0.05 \) was encountered were accepted as being differentially expressed between the comparative groups. The raw data files have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE74213).

Functional annotation, pathway analysis, clustering, and visualization of networks associated with gene expression changes were accomplished using DAVID version 6.7 (http://david.ncifcrf.gov) or the Cytoscape software platform version 2.8.3 (http://www.cytoscape.org) in conjunction with the REACTOME (version 43) plugin. For identification of potential miR-375 targets, the list of genes down-regulated by miR-375 overexpression was cross-referenced to a list of putative miR-375 target genes (disregarding site conservation) predicted by TargetScanHuman version 6.2 (http://www.targetscan.org). All bioinformatics tools used are open-source, open-access databases or software platforms.

Construction of Plasmids and Luciferase Reporter Assay

Regions of the 3' untranslated region (UTR) for RBPJ and Notch2 containing the miR-375 binding sites, were amplified from human genomic DNA using a standard PCR protocol. Inserts containing three-point mutations (underlined nucleotides) in the putative miR-375 seed recognition motif were generated using overlapping PCR. Primers used are as follows: Xhol-RBPJ, 5'-ACGCCTCGAGTACCGTCTTTTGTCTAGACATT-3' (forward); NotI-RBPJ, 5'-ATAGGATCCGCGCCGACAGGATGATA-GAT-3' (reverse); RBPJ-Mut, 5'-TAATGTTGCGTGAATACAGTTGACCCAC-ACAATGGCAC-3' (forward) and 5'-GTGTCGGTGCTTTGAGATGAACTT-3' (reverse); XhoI-Notch2, 5'-ACGCCTCGAGTTCCAAGCAGATTTGATTCCTC-3' (forward); NotI-Notch2, 5'-ATAGGATCCGCGCCGACAGGATGATA-GAT-3' (reverse); and Notch2-Mut, 5'-TGTAATTGCTCTAGCTTTGAGATGAACTT-3' (forward) and 5'-GTGTCGGTGCTTTGAGATGAACTT-3' (reverse).

All inserts were purified from crystal violet gels, digested using XhoI and NotI, and ligated into predigested and pre-primed psi-CHECK-2 (Promega, Madison, WI) to generate the following plasmids: RBPJ-psi-CHECK-2 Wt, RBPJ-psi-CHECK-2 Mut, Notch2-psi-CHECK-2 Wt, and Notch2-psi-CHECK-2 Mut. For the luciferase assay, MCC26 cells were seeded onto 12-well plates at approximately 60% confluence and cotransfected with 50 ng of plasmid DNA and 5 nmol/L of miR-375 or Neg Cntrl for 24 hours. Measurements of Firefly and Renilla luciferase activity were obtained 24 hours after transfection using Dual-Glo luciferase assay (Promega), according to the manufacturer’s protocol.

Cell Migration and Invasion Assays

The effects of miR-375 overexpression or siRNA-mediated knockdown on the migratory and invasive capacity of cells were assayed using Transwell Boyden chambers/inserts (BD Biosciences, Franklin Lakes, NJ). Cells transfected with miR-375 or Neg Cntrl were harvested 72 hours after transfection, counted, resuspended in serum-free medium, and reseeded in the upper chamber of Transwell inserts. For MCC26, 4 × 10^4 and 3 × 10^4 cells were seeded for migration and invasion, respectively. A total of 3 × 10^4 cells were used in both migration and invasion assays for...
MCC13. Inserts were placed into a 24-well plate containing medium supplemented with 15% fetal bovine serum that served as a chemottractant. Twenty-three hours after seeding, inserts were removed and fixed in 10% formalin neutral buffer, and stained with crystal violet. Nonmigrated cells were removed using a cotton swab, and inserts were then cut and mounted onto slides. Three experimental replicates were completed for any given treatment. At ×10 magnification, five random fields were captured photographically, and the number of cells per field was counted using Adobe Photoshop (Adobe Systems, San Jose, CA).

Cell Survival/Toluidine Blue Assay

MCC26 cells were used to evaluate the effects of miR-375 overexpression on cell survival after serum starvation. Seventy-two hours after transfection, cells were counted, and reseeded at 100,000 cells per well in a 6-well plate in media without serum. The surviving fraction was determined using toluidine blue staining at 24 hours and at 4 (siRNA) or 7 (miR-375 overexpression) days after seeding. Briefly, wells were fixed with 10% formalin neutralized buffer, washed three times with phosphate-buffered saline, and stained with toluidine blue solution for 30 minutes. Wells were cleaned with distilled water, and images were captured. To quantify the intensity of staining, stained cells were solubilized using 1% SDS, and OD at 650 nm was read using a microplate spectrophotometer (BioTek, Winooski, VT).

Cell Growth Assay

The effects of miR-375 overexpression or siRNA-mediated knockdown on cell growth were determined by counting number of viable cells 72 hours after transfection. Briefly, cells were washed with phosphate-buffered saline, trypsinized, resuspended in medium, and counted using Trypan blue. The effects of miR-375 knockdown on classic MCC cell lines were determined using the AlamarBlue assay (Thermo Fisher Scientific). Briefly, 30,000 cells per well were seeded in a 96-well plate and transfected the same day with either anti–miR-375 or anti-miRNA Neg Ctrl. Viability was determined at 24, 48, and 72 hours after transfection. AlamarBlue was added to plates and incubated for 3 hours at 37°C with 5% CO2. Fluorescence signals were read using the Softmax Pro microplate reader (Molecular Devices, Sunnyvale, CA).

siRNA-Mediated Knockdown

siRNA oligos were selected from predesigned dsiRNA oligo sets (IDT, Coralville, IA) against proteins of interest. For all siRNA transfections, cells were seeded at approximately 30% confluence and transfected the following day with 5 nmol/L siRNA or a control siRNA (si Ctrl). Successful knockdown was confirmed by Western blot analysis. Cells were harvested 72 hours after transfection for all associated functional assays. siRNA sequences are as follows: Notch2 siRNA#1, 5'-CCAGUACAAUGAGAUGUUUGATG-3' (HSC.RNAI.NO2440812.1); Notch2 siRNA#2, 5'-GGGAAGAAGGAUGAUAUCATT-3' (HSC.RNAI.NO2440812.2); RBPJ siRNA#1, 5'-GGCGGAUAAACAAGUGAACAACACT-3' (HSC.RNAI.NO05349.12.1); and RBPJ siRNA#2, 5'-CACAGUAAAGGCAGAUAUCAUTT-3' (HSC.RNAI.NO05349.12.3).

Western Blot Analysis

Cells were lysed using whole cell lysis buffer, sonicated, and subjected to protein quantification by Bradford assay. In preparation for Western blot analyses, 20 µg of protein was mixed with 4× loading buffer and boiled at 100°C for 10 minutes. Cell lysates were separated on SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane for 1 hour at 100 V at 4°C. Membranes were blocked in 4% milk/Tris-buffered saline with Tween 20 and then probed with primary antibodies (diluted in 4% milk or 5% bovine serum albumin) overnight at 4°C. Membranes were washed with Tris-buffered saline with Tween 20, and then incubated in horseradish peroxidase–conjugated secondary antibodies diluted in 4% milk for 1 hour at room temperature. Proteins were detected using an enhanced chemiluminescence luminol-based reagent, and visualization was accomplished using photographic films. The housekeeping gene γ-tubulin was used as a loading control in all experiments. The following primary antibodies were used: mouse anti–γ-tubulin (BD Biosciences, Franklin Lakes, NJ), rabbit anti-Notch2 (Bethyl, Montgomery, TX), mouse anti-RBPJ (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-total AKT (Cell Signaling, Danvers, MA), rabbit anti–phospho-AKT Ser473 (Cell Signaling), and rabbit anti-ATOH1 (Thermo Fisher Scientific).

Statistical Analysis

At least three independent biological replicates were conducted for all experiments reporting results expressed as means ± SEM. A t-test was used to compute statistical differences between treatment groups. P < 0.05 was considered statistically significant. Graphing was performed using Microsoft Excel (Redmond, WA) and GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA).

Results

Decreased miR-375 Expression Is Associated with Loss of Differentiation and Increased Aggressiveness of MCC Cell Lines

Previous studies on miR-375 have revealed highly cell type–specific expression and functions for this miRNA,
with a strong bias toward higher expression in NE cell types in tissues like the pancreas and lung.\textsuperscript{18–20} Indeed, most MCC tumors display striking NE differentiation, as evidenced by expression of classic NE markers, including chromogranins, synaptophysin, and neuron-specific enolase.\textsuperscript{3} Thus, we wondered whether elevated miR-375 expression in MCC tumors reported by us and others reflected NE differentiation of tumor cells. To test the potential link between miR-375 and NE differentiation in MCC, we characterized miR-375 expression in five patient-derived MCC cell lines, according to their NE differentiation status (Figure 1A).\textsuperscript{21–23} Consistent with previous reports, we found that well-differentiated classic MCC cell lines MKL-1 and MS-1 abundantly expressed the NE marker neuron-specific enolase 2 (Figure 1B).\textsuperscript{24,25} In contrast, the poorly differentiated variant MCC cell lines MCC13, MCC26, and MCC14.2 partially or fully failed to express this NE marker. Remarkably, we found that high miR-375 expression was restricted to well-differentiated cell lines MKL-1 and MS-1 (Figure 1A). In contrast, miR-375 expression was between five and six orders of magnitude lower (up to a millionfold) in the undifferentiated variant lines. Interestingly, low miR-375 expression in our panel of MCC cell lines was associated with shorter doubling time and overall aggressiveness of MCC cell lines (Figure 1C). This is consistent with previous reports that variant MCC cell lines tend to arise from patients with poor prognoses and display heightened survival capabilities in culture.\textsuperscript{24–26} In keeping with a loss of differentiation and increased aggressiveness, miR-375–low variant cell lines failed to express the proneural transcription factor ATOH1, which promotes neural differentiation and reportedly functions as a tumor suppressor in MCC (Figure 1D).\textsuperscript{27} In contrast, miR-375–high classic cell lines MKL-1 and MS-1 expressed high to moderate levels of this protein, respectively. Overall, our observations suggest that decreased miR-375 expression in MCC cell lines is associated with a loss of differentiation and a more aggressive phenotype.

**miR-375 Opposes Viability, Migration, Invasion, and Survival of MCC Cell Lines**

For most cancers, tumors that display a loss of differentiation (ie, anaplastic phenotype) tend to be more aggressive.\textsuperscript{28} Given that poorly differentiated and aggressive MCC cell lines were associated with decreased miR-375 expression, we wondered if miR-375 may behave as a tumor suppressor. Indeed, ectopic expression of miR-375 decreased the viability of two of the three variant cell lines (Figure 2B). Specifically, the viable cell number of MCC26 and MCC14.2 cells was reduced by 33% and 28%, respectively, 72 hours after transfection with miR-375 mimics. Importantly, overexpression in variant cell lines was titrated to ensure that levels closely matched miR-375 expression observed in classic cell lines (Figure 2A). In contrast to the effects on variant cell lines, decreasing miR-375 expression in classic cell lines MKL-1 and MS-1 did not alter their growth properties (Supplemental Figure S1, A–D). This may be because both classic cell lines are MCPyV-positive and, therefore, more dependent on MCPyV T antigens for sustained growth and survival.
Given the antiproliferative effects of miR-375 in variant cell lines, we next wondered if miR-375 influences cell survival. To test this, we subjected cells overexpressing miR-375 or Neg Cntrl mimics to serum starvation over 7 days. Although Neg Cntrl–transfected MCC26 cells survived and even displayed a net increase in cell density over 7 days, miR-375 overexpression greatly sensitized variant cells to serum starvation, leading to an overall decrease in cell density (Figure 2, E and F). Interestingly, overexpressing miR-375 in variant cell lines down-regulated prosurvival AKT signaling, as evidenced by decreased phospho-AKT protein levels, whereas miR-375 knockdown enhanced AKT signaling (Supplemental Figure S1E). This is consistent with previous reports that miR-375 negatively regulates AKT signaling in other cancers.29,30 Thus, even though miR-375 knockdown did not increase the proliferation of classic cell lines, it remains possible that decreasing miR-375 levels may enhance their aggressiveness in other assays. Overall, our data suggest that miR-375 opposes survival of MCC cancer cells.

The migratory and invasive ability of cells in culture is considered a proxy for metastatic potential and general aggressiveness of cancer cell lines. Although assays to test these properties are common, no such studies involving MCC lines exist. To determine whether miR-375 could attenuate the ability of variant cell lines to migrate and invade, we subjected cells transfected with miR-375 or Neg Cntrl to Transwell migration and invasion assays. miR-375 overexpression decreased cell migration by >91% and >62% in MCC26 and MCC13 cells, respectively (Figure 2, C and D, and Supplemental Figure S2A). Moreover, miR-375 overexpression also decreased invasion by 53% and 43% in MCC26 and MCC13 cells, respectively (Figure 2,C and D, and Supplemental Figure S2B). Taken together, these results support the notion that miR-375 functions as a tumor suppressor that pleiotropically opposes several cancer-related phenotypes in variant MCC cell lines. We conclude that miR-375 silencing in variant cell lines might contribute to their aggressiveness.

miR-375 Promotes NE Differentiation of MCC Cell Lines

Several tumor-suppressive miRNAs are known to oppose anaplasia and promote differentiation.28 Given the correlation between miR-375 and NE differentiation in our cell lines, we next asked whether restoring miR-375 expression in variant cells could promote a more differentiated phenotype. To test this hypothesis, we transiently overexpressed miR-375 in variant cell lines and quantified the expression of NE markers using RT-qPCR. Even at 48 hours after transfection, we were able to detect small, but

Figure 2 miR-375 exhibits tumor-suppressive properties in highly aggressive variant MCC cell lines. A: miR-375 expression level in MCC26 cells transfected with miR-375 mimics closely matches endogenous miR-375 expression in classic cell lines. Values represent miR-375 expression [expressed as log fold-change relative to MCC26 negative control (Neg Cntrl) expression from replicate 1]. B: Ectopic miR-375 expression reduces viability of variant MCC cells. Cells were transfected with miR-375 or Neg Cntrl, and viable cell number was quantified using trypan blue 72 hours after transfection. Values represent viable cell number per mL of media of three independent experiments. C: miR-375 impairs migratory and invasive capacity of MCC cells. Cells transfected with miR-375 or Neg Cntrl were harvested after 72 hours, counted, resuspended in serum-free medium, and reseeded in the upper chamber of Transwell inserts. The number of migrated/invaded cells was determined 23 hours after seeding. D: Representative images of Transwell inserts depicting impact of miR-375 overexpression on migratory and invasive capacity of MCC26 cells. E: miR-375 attenuates survival of variant MCC cells under stress. MCC26 cells were collected 72 hours after transfection, counted, and reseeded at 100,000 cells per well in a 6-well plate in medium without serum. The surviving fraction was determined by toluidine blue staining 1 and 7 days after seeding. Data represent OD650 readings (as a readout of surviving fraction) of three experiments. F: Representative images of 6-well plate depicting miR-375–induced sensitization of MCC26 cells to 7 days of serum starvation. Values represent means (A) or means ± SEM (B and E). n = 3 (C). *P < 0.05, **P < 0.01, and ***P < 0.001. Original magnification, ×10 (D).
miR-375 Affects MCC

The Notch Pathway Is a Novel Target of miR-375

We next sought to shed light on the molecular mechanisms underlying the diverse tumor-suppressive effects of miR-375. In particular, the ability of miR-375 to promote differentiation suggests that it might suppress factors that normally promote a more anaplastic or stem-like state.

To identify putative miR-375 targets that fit this profile, we integrated the results from our global gene expression analysis with publically available bioinformatics tools that enable miRNA target prediction and pathway analysis of gene expression data sets. Using Cytoscape, we asked if pathways known to regulate cell fate specification, differentiation, or development were enriched in our down-regulated microarray gene set. Intriguingly, we detected clusters enriched in genes related to the Notch and Wnt signaling pathways (Figure 4A), two bona fide developmental signaling pathways with links to cancer and regulation of differentiation. We next asked which genes in our down-regulated gene set might represent direct miR-375 targets. Of the 825 genes down-regulated >1.5-fold, the 3' UTR region of 232 (28%) contained miR-375 response elements (binding sites) when cross-referenced to TargetScanHuman (version 6.2). Within the Notch and Wnt clusters specifically, four genes (Notch2, RBPJ, TCF4, and TCF12) were found to contain predicted miR-375 binding sites (Figure 4B). Intriguingly, we found several studies in the literature that identified the Notch, but not the Wnt, signaling pathway as a negative regulator of NE differentiation in both cancer and normal development. We therefore chose to focus specifically on RBPJ and Notch2 to determine whether miR-375 regulated the Notch pathway. miR-375 overexpression in variant cell lines resulted in decreased protein expression of both Notch2 and RBPJ (Figure 4C). Furthermore, knockdown of miR-375 in the classic cell line MKL-1 resulted in increased RBPJ expression (Figure 4D). Unlike RBPJ, Notch2 levels did not increase in classic cell lines after miR-375 knockdown, although interestingly, Notch2 expression correlated inversely with the differentiation state and miR-375 expression status of our cell lines (Supplemental Figure S2C). Importantly, variant cell lines expressed higher levels of Notch2, but not Notch1, which is not a predicted target of miR-375. Overall, these results suggest miR-375 may promote differentiation by acting as a negative regulator of Notch signaling at both the level of the receptor (Notch2) and the level of the transcription factor responsible for Notch-mediated regulation of gene expression (RBPJ).

The proximal Notch2 3' UTR (positions 54 to 60) contains a highly conserved miR-375 binding site, whereas the 3' UTR of RBPJ (positions 334 to 340) features a poorly conserved binding site (Figure 4E). To test whether miR-375-mediated repression of Notch2 and RBPJ is direct, we sub-cloned portions of 3' UTRs with or without mutations in the putative miR-375 binding site. miR-375 overexpression
diminished luciferase activity of both wild-type vectors. This effect was abolished when predicted miR-375 binding sites within the Notch2 and RBPJ 3’ UTRs were mutated (Figure 4F). Taken together, our data suggest that miR-375 negatively regulates the Notch pathway via direct interactions with the 3’ UTRs of RBPJ and Notch2. We conclude that the Notch pathway is a direct target of miR-375, and suppression of Notch by miR-375 may promote NE differentiation of variant MCC cell lines.

Notch Pathway Repression Underlies the Tumor-Suppressive Properties of miR-375

Finally, we determined whether suppression of the Notch pathway proteins could phenocopy the tumor-suppressive phenotypes associated with miR-375 overexpression. Efficient knockdown of Notch2 and RBPJ was confirmed by two independent siRNAs (Figure 5A). Knockdown of Notch2 in MCC26 cells decreased cell viability and abolished cell survival after serum starvation, mirroring the effects of miR-375 overexpression (Figure 5, B and C). Interestingly, RBPJ knockdown neither decreased cell viability nor sensitized MCC26 cells to serum starvation (data not shown), suggesting that Notch2 supports survival of MCC cells via an RBPJ-independent mechanism. In contrast, knockdown of Notch2 or RBPJ abolished cell migration and invasion of MCC26 cells (Figure 5, D and E). This suggests that the Notch pathway may be an important driver of cell migration and invasion in variant MCC cells, and implicates the loss of miR-375 and the resulting disinhibition of Notch signaling as a potentially important step in the progression to metastatic disease. Overall, our data support a model in which the diverse tumor-suppressive effects of miR-375 in MCC cells occur via suppression of the Notch pathway.

Discussion

During the past decade, interest in the biological roles and clinical applications of miRNAs in cancer has grown exponentially. In pathology, miRNAs have been touted as superior candidates for use as diagnostic and/or prognostic biomarkers for several reasons. First, miRNAs are highly stable in RNA preparations, including those derived from FFPE tissues, and tend to remain for long periods of time under routine storage conditions. Second, individual miRNAs can profoundly affect many aspects of cancer biology through regulation of multiple target genes and pathways. Despite the growing interest in miRNAs in cancer and other disease, the roles of miRNAs in MCC remain largely uncharted territory. In this study, we performed a thorough characterization of the expression, biological roles, and
target genes of miR-375 in MCC cell lines. We are the first to report that miR-375 is associated with NE differentiation in MCC, and is silenced specifically in a subset of undifferentiated, aggressive MCC cell lines. Furthermore, we are the first to characterize a novel role and mechanism for miR-375 as a tumor suppressor, where it behaves as a tumor suppressor via the direct repression of two Notch pathway proteins, Notch2 and RBPJ.

One of our most striking findings was the association between miR-375 and NE differentiation in MCC cell lines. We are the first to report that miR-375 is associated with NE differentiation in MCC, and is silenced specifically in a subset of undifferentiated, aggressive MCC cell lines. Furthermore, we are the first to characterize a novel role and mechanism for miR-375 as a tumor suppressor, where it behaves as a tumor suppressor via the direct repression of two Notch pathway proteins, Notch2 and RBPJ.

Extensive research during the past decade supports the notion that aberrant miRNA expression patterns can profoundly affect hallmark behaviors of cancer cells. We found that miR-375 was a determinant of cancer cell behavior in MCC cell lines through the direct post-transcriptional repression of Notch pathway proteins. Ectopic miR-375 expression in variant cell lines opposed cell viability, migration/invasion, and survival. Furthermore, even transient miR-375 overexpression was sufficient to induce global gene expression changes suggestive of NE differentiation. Overall, the phenotypes associated with restoration of miR-375 expression in MCC cell lines suggest that miR-375 may act like a tumor suppressor in MCC. To characterize the mechanisms underlying these phenotypes, we profiled global gene expression changes induced by miR-375 expression. In the list of genes down-regulated after miR-375 overexpression, we found several previously validated miR-375 targets (LDHB, YWHAZ, and MTDH) in addition to several genes with no previously established link to miR-375 (PRKCA, PDGFRA, BIRC3, NOTCH2, and RBPJ). Although these genes have been previously implicated as oncogenic factors in several contexts, only siRNA-mediated knockdown of

Figure 5 Targeting the Notch pathway in variant MCC cells phenocopies the tumor-suppressive effects of miR-375. A: Western blots depicting knockdown of Notch2 and RBPJ by two independent siRNAs. B: Knockdown of Notch2 reduces the viability of variant MCC cells. C: Knockdown of Notch2 phenocopies the anti-survival effects of miR-375 under serum starvation in MCC26 cells. D: Knockdown of both Notch2 and RBPJ impairs the migratory and invasive capacity of MCC26 cells. E: Representative images showing antimigratory and anti-invasive effects of Notch pathway suppression. Data represent the means ± SEM of three independent experiments (A–E). n = 3 (B and C). *P < 0.05, **P < 0.01. Original magnification, ×10 (E). Cntrl, control; Si, small interfering.
Notch2 and RBPJ phenocopied the effects of miR-375 overexpression. Overall, our data identify the Notch pathway as an important determinant of cancer cell behavior in MCC. Knockdown of miR-375 did not significantly influence the proliferation of virus-positive classic cell lines, at least in the assays that we tested. This may be because virus-positive cell lines depend mainly on viral T-antigens and associated pathways for growth and survival. Although this could mean that miR-375 has less of an effect in the genomic and epigenetic context of classic cell lines, it is possible that miR-375 may influence the biology of well-differentiated, MCPyV-negative cell lines or potentially MCPyV-positive cells in ways that we have not yet ascertained. Future studies should, therefore, characterize the deregulation of the miR-375/Notch axis in relation to virus status of MCC tumors and test if this affects tumor growth in vivo and/or predicts clinical course of patients.

The Notch signaling pathway has been strongly linked with stem cell self-renewal and stem cell niche maintenance, and is generally associated with an undifferentiated or stem-like state. Stem cell self-renewal and stem cell niche maintenance, and is generally associated with an undifferentiated or stem-like state. Stem cell self-renewal and stem cell niche maintenance, and is generally associated with an undifferentiated or stem-like state. Therefore, characterize the deregulation of the miR-375/Notch axis in relation to virus status of MCC tumors and test if this affects tumor growth in vivo and/or predicts clinical course of patients.

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K.J.A. and V.A.T. developed the research concept; K.J.A. wrote the manuscript and created the figures; X.Z. and R.V. analyzed the microarray; K.J.A performed all other experiments; and G.C.P. and H.E.F. provided critical input during study design and manuscript preparation.

Supplemental Data

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