Differential MicroRNA-34a Expression and Tumor Suppressor Function in Retinoblastoma Cells

Clifton L. Dalgard,¹ Marco Gonzalez,² Jennifer E. deNiro,² and Joan M. O’Brien¹

PURPOSE. The role of miR-34a, a p53-regulated microRNA, in retinoblastoma (RB) was investigated.

METHODS. The expression of miR-34 family members in RB cells was determined by semiquantitative RT-PCR and real-time qPCR. Regulation of miR-34a expression by p53-activating compounds was determined by qPCR analysis. The tumor suppressor functions of miR-34a in RB cell lines were determined by tetrazolium-based cell growth assay and by caspase-3/7 and activated caspase-3 apoptotic activity assays. Additive growth inhibitory properties of miR-34a in combination with topotecan were determined by cell growth assay, miR-34a targets in RB cells were identified by real-time qPCR expression analysis of previously reported and GenMiR++-predicted miRNAs.

RESULTS. Differential miR-34a and miR-34b expression was observed in RB cell lines and tumor samples. miR-34a expression could be increased in Y79 cells, but not Weri-Rb1 cells, after p53 activation. This differential regulation was not caused by genomic alterations at the miR-34a p53 binding site or mature gene. Exogenous miR-34a inhibited Y79 and Weri-Rh1 cell growth and increased apoptotic activity in Y79 cells. Increased inhibition of Y79 and Weri-Rh1 cell growth was observed with combination miR-34a and topotecan treatment. miRNA expression changes were observed in 7 of 7 previously reported and 13 of 18 GenMiR++-predicted miR-34a targets after transfection of Y79 cells with miR-34a compared with negative control microRNA.

CONCLUSIONS. miR-34a functions as a tumor suppressor in RB cells and is a potential therapeutic target. Differential expression, regulation, and activity of miR-34a in RB cells may suggest further p53 pathway inactivation in RB. (Invest Ophthalmol Vis Sci. 2009;50:4542–4551) DOI:10.1167/iovs.09-3520

Retinoblastoma is the most common form of ocular cancer in children.¹,² Alteration or loss of the RB1 gene is a defining molecular characteristic of this cancer and is thought to be the tumor-initiating event. Other genomic alterations in oncogenes and tumor suppressor genes have been identified in retinoblastoma (RB), and these changes may be important in RB tumor progression.³ Inactivation of the p53 pathway in RB may also play a role in RB tumor progression. Studies have identified overamplification of MDM2 and MDMX, two p53 pathway components important in regulating p53 degradation and p53 target gene transcription activation, in a subset of RB tumors.⁴ Forced overexpression of MDMX in RB1-negative fetal retinal cells protected from activation of p53-mediated apoptosis.⁴ Treatment of RB cells with nutlin-3, a compound that inhibits MDM2 and MDMX enzymatic activity, results in antiproliferative and proapoptotic effects.⁵ Thus, therapeutic strategies that reactivate or recapitulate p53 responses in p53 pathway-inactivated RB cells may demonstrate clinical usefulness against this disease.

MicroRNAs are short (20–23 nucleotides in length) noncoding RNAs that mediate coordinated cellular programs by post-transcriptional mRNA silencing and protein translation inhibition.⁶ The production of small, mature microRNAs from larger genomic microRNA genes is now well understood.⁷ Pol II first transcribes microRNA genes into primary transcripts called pri-miRNAs. Drosophila then processes them in the nucleus into stem-loop structures called pre-miRNAs. One of the strands is degraded, and the “guide” strand is integrated into the RNA-induced silencing complex (RISC). This complex now recognizes partially complementary sequences in the 3' UTR of target mRNAs in the cell. The protein expression of targeted miRNAs is downregulated, either through translation inhibition or mRNA destabilization/sequestration.

Given that microRNAs are important in appropriate cellular function for development, tissue maintenance, and physiology, it is not surprising that the deregulation of microRNA function is associated with disease. In several human cancer types, microRNAs have been implicated as oncogenes and tumor suppressor genes.⁸,⁹ Additionally, microRNA genes are located at fragile sites within the human genome and may be commonly altered in the rapidly damaged cancer genome.¹⁰ Experiments have identified microRNA tumor suppressors such as miR-15a, 16–1, 17-5p, 143, 145, and let-7.¹¹ OncomiRs include miR-18a, 19a-b, 20a, 21, 92, 155, and 372.² In human RB, differential let-7b microRNA expression and miRNA expression of let-7b targets has been observed, yet the importance of let-7b function in RB tumor progression is not understood.¹¹ Other microRNAs may play a role in RB disease.

Recently, three independent laboratories have demonstrated that p53 transcriptionally activates the miR-34 family (a through c).¹²–¹⁴ Additionally, p53-mediated transcriptional activation of miR-34 family members was shown to contribute to p53-dependent tumor suppression through cell cycle arrest and activation of apoptosis.¹²–¹⁵ Loss or silencing of the miR-34a gene has been identified in several human cancers, including brain, breast, colorectal, lung, pancreatic, and prostate.¹⁶–¹⁹ Reexpression of miR-34a in pancreatic and prostate cancer cells resulted in the inhibition of cell proliferation and senescence.¹⁶,²⁰ Interestingly, MDMX has been identified as an mRNA target of miR-34a.²¹ Thus, expression of miR-34a in MDMX-overamplified RB may result in tumor growth inhibition. miR-34a has also been shown to inhibit silent information replication of Y79 cells with miR-34a compared with negative control NAs.

13 of 18 GenMiR family changes were observed in 7 of 7 previously reported and combination miR-34a and topotecan treatment. mRNA expression changes were observed in 7 of 7 previously reported and 13 of 18 GenMiR++-predicted miR-34a targets after transfection of Y79 cells with miR-34a compared with negative control microRNA.
regulator 1 (Sirt1) expression, a NAD⁺-dependent deacetylase that negatively regulates p53 transcriptional activity. Given that the regulation of microRNA function is known to play a role in cancer and p53 pathway inactivation may be necessary for tumor progression in a subset of RB tumors, we hypothesized that miR-34 family members may play a role in RB tumor biology and may provide a novel therapeutic target for this disease.

In this study, miR-34 family member expression and miR-34a tumor suppressor function in RB was investigated. We analyzed miR-34a, miR-34b, and miR-34c expression in RB tumor samples and cell lines. Expression of miR-34a after activation of p53 pathway activation was examined in RB cell lines. We used a synthetic miR-34a precursor molecule to determine the effects of increased miR-34a expression on RB tumor cell growth and induction of apoptosis. Finally, we analyzed mRNA expression of known and potential miR-34a targets in RB cells after exogenous delivery of miR-34a.

**Materials and Methods**

**Reagents, Cell Culture, and Antibodies**

Nutlin-3 was from Cayman Chemical (Ann Arbor, MI). Doxorubicin and topotecan were from Sigma (St. Louis, MO). 5-Iminodoxorubicin was from Sigma (St. Louis, MO). 5-Iminodoxorubicin was from the National Cancer Institute Developmental Therapeutics Program (Rockville, MD). Y79 and Weri-Rb1 cells were cultured in RPMI-1640 (Gibco-BRL, Grand Island, NY) plus 10% heat-inactivated fetal bovine serum (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL). Y79 and Weri-Rb1 cells were cultured in RPMI-1640 plus 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco-BRL). RB318 and RB324 RB samples were obtained from fresh tumor specimens after enucleation. Monoclonal antibodies for p53 (DO-1) and GAPDH (0411) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody for activated caspase-3 was obtained from BD PharMingen (San Diego, CA).

**RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR**

Total RNA from harvested cells was isolated using RNA isolation kits (RNeasy Mini or miRNeasy Mini Kit; Qiagen, Valencia, CA). Total RNA quantity was determined with a spectrophotometer (Nanodrop ND-1000; Thermo Scientific, Waltham, MA). For semiquantitative PCR or quantitative PCR (qPCR) analysis of precursor miR-34a, HPRT, and miR-34a targets, cDNA was generated using a reverse transcription kit (SuperScript III First-Strand Synthesis System; Invitrogen, Carlsbad, CA) and random primers. Real time qPCR was performed using a SYBR Green-based reagent (SYBR GreenER qPCR SuperMix for iCycler; Invitrogen) on a real-time PCR thermal cycler (MyIQ Single-Color Real-Time PCR Detection System; Bio-Rad, Hercules, CA). Primers used can be found in Table 1. Single amplicon quantification was determined by multicomponent analysis. For qPCR analysis of mature miR-34a and RNU44, human miRNA assay kits (TaqMan MicroRNA; Applied Biosystems, Foster City, CA) were used for reverse transcription with specific primers, and qPCR was performed with corresponding probes (TaqMan; Applied Biosystems). Real-time qPCR was performed on a real-time PCR thermal cycler (MyIQ Single Color Real-Time PCR; Bio-Rad).

**Western Blot Analysis**

Whole cell extracts were prepared with RIPA buffer for lysis. Protein quantity was determined using the Bradford method (Bio-Rad Protein Assay; Bio-Rad). Protein was separated by electrophoresis in precast gels (4% to 12% NuPage Novex Bis-Tris Gel; Invitrogen) and was transferred to polyvinylidene membrane (Immobilon; Millipore, Billerica, MA), blocked with 5% milk in Tris-buffered saline with Tween-20, and probed with the indicated antibodies. Anti-p53 antibody was used at 1:2000, anti–GAPDH antibody was used at 1:10,000, and donkey anti-mouse secondary horseradish peroxidase conjugate was used at 1:20,000. Bands were visualized with enhanced chemiluminescence (ECL Western Blotting Substrate; Pierce, Rockford, IL).

**DNA Sequencing**

Amplification of target regions of miR-34a primary transcript was performed using high fidelity polymerase (Platinum Taq HiFi; Invitrogen). PCR reactions were cleaned, sequencing reactions were run with the amplification primers at each end, and sequencing was performed using a DNA analyzer (ABI 3730x; Applied Biosystems). Chromatographs were trimmed, analyzed, and aligned (Seqencher; Gene Codes, Ann Arbor, MI).

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**Tumor Suppression by miR-34a in RB**

In this study, miR-34 family member expression and miR-34a tumor suppressor function in RB was investigated. We analyzed miR-34a, miR-34b, and miR-34c expression in RB tumor samples and cell lines. Expression of miR-34a after activation of p53 pathway activation was examined in RB cell lines. We used a synthetic miR-34a precursor molecule to determine the effects of increased miR-34a expression on RB tumor cell growth and induction of apoptosis. Finally, we analyzed mRNA expression of known and potential miR-34a targets in RB cells after exogenous delivery of miR-34a.
Cell Viability Assay

MicroRNA was added to wells of a 96-well plate in reduced serum medium (OPTI-MEM I; Gibco-BRL), and transfection reagent (RNAiMAX; Invitrogen) was added and allowed to complex at room temperature for 30 minutes. Cells were then plated into the wells at a density of 10,000 cells in a final volume of 100 μL. At the indicated day after transfection, cell proliferation reagent (WST-8; Dojindo, Kumamoto, Japan) was added. After 2 hours of incubation at 37°C, tetrazolium dye conversion was measured spectrophotometrically at 450 and 650 nm (VERSAmax; Molecular Devices, Eugene, OR). Values from blank (media and vehicle-only) wells were subtracted from all experimental values.

Apoptosis Analysis

For the caspase-3/7 activity assay, Y79 and Weri-Rb1 cells were transfected with 10 nM microRNA (miR-34a or negative control) as described in 96-well plates. At 5 days after transfection, whole cell assay reagent (CellProbe HT Caspase 3/7; Beckman Coulter, Fullerton, CA) was added. After 30 minutes of incubation at room temperature, fluorescence was measured with a multilabel plate reader (Wallac Victor 2; Perkin Elmer, Waltham, MA). Values from blank (media only) wells were subtracted from all experimental values. For the activated caspase-3 assay, Y79 cells were transfected with 10 nM microRNA (miR-34a or negative control), as described, in six-well plates. At 5 days after transfection, cells were washed and then fixed with fixation and permeabilization solution (Cytofix/Cytoperm Solution; BD Biosciences, San Jose, CA). After washes (Perm/Wash Buffer; BD Biosciences), cells were incubated with FITC rabbit anti–active caspase-3 (BD Biosciences) and washed, and activated caspase-3 activity was collected by flow cytometry (Cell Laboratory Quanta SC; Beckman Coulter). Flow cytometric data were analyzed with flow cytometry analysis software (FlowJo; Tree Star, Ashland, OR).

Statistical Analysis

Statistical two-group t-test analysis with two-tailed P values was performed with biosistatistics, curve fitting (nonlinear regression), scientific graphing software (Prism 5; GraphPad, San Diego, CA).

RESULTS

Differential miR-34 Family Member Expression in RB Cell Lines and Primary RB Cells

We determined the expression of precursor forms of miR-34 family members (miR-34a, miR-34b, and miR-34c) in two cell lines of human RB and from two primary RB samples (Rb318 and Rb324) by semiquantitative RT-PCR (Fig. 1A). We observed precursor miR-34a expression in RB cell lines and primary RB samples. A higher level of precursor miR-34a expression was found in Weri-Rb1 cells than in Y79 cells. The level of precursor miR-34a expression was similar in primary RB samples and Y79 cells. We also detected differential precursor miR-34b expression in the two cell RB cell lines and two primary RB samples. Minimally observable precursor miR-34b was found in Y79 cells, whereas Weri-Rb1 miR-34b expression was undetectable. However, a high level of precursor miR-34b expression was found in the Rb318 primary RB sample compared with lower expression found in the Rb324 sample. We did not observe significant precursor miR-34c expression in RB cell lines or primary RB samples.

Given that miR-34a expression was present, yet differential, in the four sources of human RB we analyzed, we selected miR-34a for additional analysis using real-time qPCR. We quantitatively analyzed precursor miR-34a expression with an asymmetrical cyanine dye (SYBR Green; Applied Biosystems)-based qPCR methodology (Fig. 1B) and mature miR-34a expression with a qPCR methodology (TaqMan Universal; Applied Biosys-

![Figure 1](iovj_arvojournals.org)
Precursor miR-34a expression was significantly higher in Weri-Rb1 cells (approximately 9-fold higher) than in Y79 RB cells and the two primary RB samples. Precursor miR-34a expression was not significantly different between Y79 and the two primary RB samples. The housekeeping gene HPRT was not significantly different in the RB samples analyzed.

Differential miR-34a Regulation after p53 Activation in RB Cell Lines

Our observation of significantly higher precursor and mature miR-34a expression in Weri-Rb1 cells compared with Y79 cells suggested that p53-mediated regulation of miR-34a transcription may be differential in these two RB cell lines. We investigated the regulation of miR-34a by p53 in RB cell lines by using three p53-activating agents, doxorubicin (DOXO), nutlin-3 (NUTLIN), and 5-iminodoxorubicin (5-ID). Y79 and Weri-Rb1 cells were exposed to p53-activating agents for 24 hours, and p53 protein (Fig. 2A), p21 mRNA (Fig. 2B), precursor miR-34a (Fig. 2C), and mature miR-34a (Fig. 2D) expression was analyzed (Figs. 2B–D) in the same samples. Each of the three p53-activating agents significantly increased p53 protein levels in Y79 and Weri-Rb1 cells compared with dimethyl sulfoxide (DMSO)-treated control cells (Fig. 2A). Nutlin-3 exposure re-
sulted in the highest observed increase in p53 protein levels in Y79 and Weri-Rb1 cells. Similar to the changes in p53 protein expression, p21 mRNA expression was significantly increased in both RB cell lines after exposure to any of the three p53 activating agents (Fig. 2B). Again, nutlin-3 treatment resulted in the highest observed fold change in p21 mRNA expression compared with control DMSO treatment in Y79 cells (approximately 23-fold increase) and Weri-Rb1 cells (approximately 8-fold increase).

Surprisingly, we observed the differential regulation of miR-34a expression in Y79 compared with Weri-Rb1 cells after exposure to p53-activating agents. Y79 precursor and mature miR-34a expression was significantly upregulated in a manner similar to p21 mRNA upregulation after treatment with DOXO, NUTLIN, and 5-ID. Precursor miR-34a expression in Y79 cells was increased approximately 10-fold, 28-fold, and 12-fold after 24-hour exposure to DOXO, NUTLIN, and 5-ID, respectively (Fig. 2C, left). A parallel increase in mature miR-34a expression in Y79 cells was also observed. Mature miR-34a expression in Y79 cells was increased approximately 2-fold after DOXO and 5-ID treatment and 3-fold after NUTLIN treatment (Fig. 2D, left). In contrast to Y79 cells, we did not observe a significant difference in precursor miR-34a expression in Weri-Rb1 cells after 24-hour exposure with any of the p53-activating agents compared with DMSO-treated control exposure (Fig. 2C, right). Additionally, mature miR-34a expression was also altered in Weri-Rb1 cells after treatment with DOXO, NUTLIN, and 5-ID (Fig. 2D, right).

Unaltered Genomic Mature miR-34a and p53 Binding Sites in RB Cell Lines

We hypothesized that an alteration in the p53 binding site or the mature sequence of the miR-34a gene might have accounted for our finding that miR-34a gene regulation by p53 activation was altered in Weri-Rb1 cells compared with Y79 cells and other cells analyzed by previous work from other groups. The p53 binding site responsible for miR-34a gene transcription is found within the initial exon; thus, we directly sequenced the primary miR-34a transcript to determine whether an alteration was present in the miR-34a gene found in Weri-Rb1 cells (Fig. 3A). We detected no difference in sequence of the primary miR-34a transcript in Y79 cells (data not shown) and Weri-Rb1 cells (Fig. 3B, p53 binding site; Fig. 3C, mature miR-34a) compared with published work and the published reference sequence (National Center for Biotechnology Information accession no. EF609116).

Growth Inhibition of RB Cell Lines after Exogenous miR-34a Administration

Our observation that Weri-Rb1 cells had high levels of basal miR-34a expression and did not undergo miR-34a upregulation after p53 activation compared with Y79 cells led us to hypothesize that Weri-Rb1 cells would be insensitive to exogenous miR-34a exposure. We transfected Y79 and Weri-Rb1 cells with negative-control or miR-34a microRNA and determined the relative cell number, compared with 1-day posttransfection values, daily for 7 days after transfection. In negative-control microRNA-transfected Y79 cells, we observed a normal increase in relative cell number each day after transfection (Fig. 4A). In miR-34a-transfected Y79 cells, a reduction in relative cell number compared with negative-control microRNA-transfected cells was observed at days 4 through 7 after transfection. At days 5, 6, and 7 after transfection, miR-34a–transfected Y79 cell numbers were 56.8%, 44.8%, and 40.1% those of negative-control-transfected Y79 cells, respectively. Additionally, we did not observe a significant increase in relative cell number in miR-34a–transfected Y79 cells from days 4 through 7. Similarly, Weri-Rb1 cells transfected with negative-control microRNA displayed a significant increase in relative cell number each day after transfection (Fig. 4B). We also observed significant increases in relative cell number in miR-34a–transfected Weri-Rb1 cells each day after transfection. However, there was a reduction in relative cell number of miR-34a–transfected Weri-Rb1 cells compared with negative-control microRNA-transfected cells at days 4 through 7 after transfection. miR-34a–transfected Weri-Rb1 cell numbers were 64.5%, 61.4%, and 57.0% those of negative-control-transfected Weri-Rb1 cells at days 5, 6, and 7 after transfection, respectively. These results in Y79 and Weri-Rb1 cells suggest that exogenous miR-34a expression in RB may inhibit RB cellular growth.

Given that exogenous miR-34a administration to RB cell lines resulted in a reduction in cell growth and may provide a
In RB, we investigated whether miR-34a administration can provide additional antitumor effects when treated along with a clinically used chemotherapeutic agent. Y79 and Weri-Rb1 cells were transfected with negative-control or miR-34a microRNA and then were exposed to topotecan at day 4 after transfection. We determined Y79 and Weri-Rb1 cell survival 6 days after microRNA transfection (Fig. 4C.). Again, we observed decreased cell survival in miR-34a microRNA-transfected Y79 and Weri-Rb1 cells compared with negative-control microRNA-transfected cells. Significantly decreased cell survival was also found in topotecan-treated, negative-control microRNA-transfected Y79 and Weri-Rb1 cells compared with vehicle-treated, negative-control microRNA-transfected cells. A further reduction in cell survival was observed in Y79 cells exposed to both topotecan and miR-34a microRNA compared with topotecan and negative-control microRNA-exposed cells (9.8% vs. 15.6%, respectively; \( P < 0.001 \)). A minimally significant reduction in cell survival was also observed in Weri-Rb1 cells exposed to both topotecan and miR-34a microRNA compared with topotecan and negative-control microRNA exposed cells (23.2% vs. 28.0%, respectively; \( P < 0.01 \)). These results suggest that miR-34a microRNA may have clinical usefulness along with currently used chemotherapeutic agents for RB.

**Differential Apoptotic Activation in RB Cell Lines after Exogenous miR-34a Administration**

miR-34a is a p53-regulated microRNA, and activation of the p53 pathway results in apoptosis in various human cancer cells. We hypothesized that exogenous miR-34a administration may result in activation of an apoptotic program in RB cells. We transfected Y79 and Weri-Rb1 cells with a negative-control or miR-34a microRNA and analyzed the activation of apoptosis with a fluorescence-based caspase-3/7 assay and a flow cytometry-based activated caspase-3 assay. We observed a significant 2.5-fold increase in caspase-3/7 activity in miR-34a–transfected Y79 cells compared with negative-control–transfected cells (Fig. 5A). However, the increase of caspase-3/7 activity in miR-34a–transfected Weri-Rb1 cells was lower than observed in Y79 cells (1.5-fold compared with values found in negative control cells; Fig. 5A). We also analyzed the activation of apoptosis after miR-34a microRNA exposure by flow cytometry analysis of activated caspase-3 protein. In miR-34a–transfected Y79 cells, we observed a significant population of cells with increased activated caspase-3 expression (Fig. 5B). In contrast, we observed a minimal population of negative control–transfected Y79 cells with increased activated caspase-3 expression. We did not observe increase activated caspase-3 expression in negative control- or miR-34a–transfected Weri-Rb1 cells (data not shown). This differential level of apoptotic activation may help explain the differences in decreased cell survival in Y79 and Weri-Rb1 cells after exogenous miR-34a treatment.

**Cell Cycle Control and Apoptotic Gene Downregulation in RB Cells by miR-34a**

Previous studies have identified and validated miR-34a targets in human cells. Some of these miR-34a targets include (TPT). RB cells were transfected with 10 nM miR-34a or negative control microRNA. After 24 hours, cells were exposed to vehicle (DMSO) or 10 nM TPT. At 144 hours after transfection, cell viability was determined using a tetrazolium dye-based assay. Bars are represented as a percentage of the value found in negative control microRNA-treated cells. Raw data and error bars were derived from quadruplicate samples. * \( P < 0.01 \); ** \( P < 0.001 \). The experiment was performed in duplicate with similar results.
data analysis algorithm, GenMiR++.

This approach was previously used to validate GenMiR++-predicted let-7b microRNA targets in Weri-Rb1 cells. We developed a qPCR-based mRNA expression assays for 18 GenMiR++-predicted miR-34a targets. As did the previously reported study, this method would allow validation only of microRNA targets regulated by altered mRNA transcript stability and not those altered at the translation level. Despite this limitation, we observed a significant decrease in mRNA expression of 11 of 18 (61%) targets analyzed and a significant increase in 2 of 18 (11%) targets (Fig. 6B). There was no significant correlation between the level of decrease in mRNA expression and its Gen-MiR++-predicted confidence value ($R^2 = 0.005$). Interestingly, these significant miR-34a targets had cellular functions outside of cell cycle control or proliferation and included cell adhesion (CTNNB1, CTNND2, EFN1), positive regulators of p53 stability (CTNNB1, CTNND2, EFN1), and vesicle transport (CPLX2, KCH2, LYST, PPP1R10, SLC30A3, P = 0.007; Gene Ontology data from GATHER) and apoptosis during oncogenic stress. Surprisingly, p53 mutations have not been observed in human or transgenic animal RB tumors. However, RB tumors in transgenic animals with a p53 null background grow more rapidly and are more aggressive. Additionally, a recent study reported that MDM2 and MDM4, negative regulators of p53 stability, are overamplified in a significant subset of RB tumors and would contribute to p53 pathway inactivation despite the lack of p53 mutation. Yet, more recent analysis of MDM2 and MDM4 in a set of RB tumor samples found no (MDM2) or minimal (MDM4) frequency of increased protein expression compared to normal retina. Thus, the longstanding controversy of p53 involvement in RB biology remains, and the investigation of additional p53 pathway components and their importance in RB tumor initiation and progression are necessary to identify potential clinically useful therapeutics for the disease.

To contribute to this goal, we investigated the expression and functional role of a critical downstream microRNA component of the p53 pathway in RB cells. We observed differential levels of miR-34a expression in the two commonly used RB cell lines. The role of basal miR-34a expression and its function in these RB cells is not fully clear. Knockdown of miR-34a by anti-miR molecules may result in increased RB cell proliferation and chemotherapeutic resistance and confirm tumor suppressor activity for basal miR-34a. Differential miR-34a expression is also observed in breast, melanoma, pancreatic, and prostate cancer cell lines. Differential miR-34a expression in cell lines or tumor samples could be explained by gain or loss of 1p36 at the miR-34a loci, CpG methylation silencing, or genomic alteration at the p53-binding site in the miR-34a gene. Mutations in the p53 tumor suppressor gene are commonly found in many human tumors, and these alterations impair the normal cellular responses of cell cycle arrest, DNA repair, and apoptosis during oncogenic stress. Surprisingly, p53 mutations have not been observed in human or transgenic animal RB tumors. However, RB tumors in transgenic animals with a p53 null background grow more rapidly and are more aggressive. Additionally, a recent study reported that MDM2 and MDM4, negative regulators of p53 stability, are overamplified in a significant subset of RB tumors and would contribute to p53 pathway inactivation despite the lack of p53 mutation. Yet, more recent analysis of MDM2 and MDM4 in a set of RB tumor samples found no (MDM2) or minimal (MDM4) frequency of increased protein expression compared to normal retina. Thus, the longstanding controversy of p53 involvement in RB biology remains, and the investigation of additional p53 pathway components and their importance in RB tumor initiation and progression are necessary to identify potential clinically useful therapeutics for the disease.

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miR-34a expression observed in Y79 cells after p53 activation suggests that silencing would not be found in this RB cell line. In contrast, the lack of induction of miR-34a expression observed in Weri-Rb1 after p53 activation may suggest an alteration in the p53 binding site of the miR-34a gene. However, our direct sequencing results suggest that this inability of miR-34a regulation in Weri-Rb1 is not caused by genomic alterations in the gene. In an analysis of miR-34a regulation in prostate cell lines, a differential responsiveness to doxorubicin was observed that was independent of p53 status. Thus, regulation of miR-34a expression may also be under the control of signaling pathways outside p53 and should be identified and studied.

Despite the differential miR-34a expression levels observed in RB cell lines, antiproliferative effects were observed in Y79 and Weri-Rb1 cells after exogenous administration of miR-34a. However, Y79 cell exposure to miR-34a led to growth arrest by day 4 after transfection, whereas Weri-Rb1 cells continued to grow at a reduced rate compared with negative-control microRNA-transfected cells. This difference in growth inhibition effect is correlated with differential apoptotic activation in Y79 compared with Weri-Rb1 cells after exogenous miR-34a exposure. A similar finding was found in neuroblastoma in which miR-34a inhibited cell growth in three different cell lines despite the differential induction of apoptotic activity. The high basal miR-34a expression observed in Weri-Rb1 cells may suggest comparatively increased resistance to the tumor suppressor functions of this microRNA, at basal levels and in response to exogenous administration. Complete analysis of basal miR-34a expression in additional RB cell lines and in primary RB cells, along with their cell survival responses to exogenous miR-34a administration and chemotherapeutic agents, is warranted to investigate a correlation between these properties. We also observed additional RB cell growth inhibition with combined topotecan and miR-34a treatment. Previous work has demonstrated a similar effect in a combined treatment using topotecan and the p53 activator nutlin-3. In fact, this combination was the most effective treatment in this study of an ocular xenograft RB preclinical model. Thus, instead of p53 pathway reactivation by nutlin-3, initiating a downstream p53 cellular program by miR-34a along with an additional cytotoxic agent may be rational for RB therapy.

We confirmed the changes in mRNA expression of previously identified and in silico predicted targets in Y79 RB cells after miR-34a exogenous administration. By using mRNA expression analysis, we could only observe potential microRNA
effects on target mRNA stability and could not exclude changes in target mRNA expression by indirect effects. Additional experiments using target gene 3′-UTRs introduced into luciferase-based reporters would more accurately identify direct effects by miR-34a complementary binding. Furthermore, protein expression analysis of these genes would allow assessment of translational arrest effects by miR-34a. Cyclin D1 (CCND1), cyclin E2 (CCNE2), and CDK4 are three cell cycle regulatory genes whose loss or knockdown by siRNA or miR-34a may result in G1 cell cycle arrest in cells with intact pRB. Their regulatory roles in RB are not fully clear; however, it has been demonstrated that cyclin D1 has cell-cycle independent functions and that loss of cyclin D1 expression results in the activation of apoptosis in the retina.32 Antiapoptotic cell-cycle independent functions for these genes may be present in conditions in which pRB function is lost, and knockdown of these genes individually by siRNA or as a group by miR-34a may contribute to apoptotic activation. Not surprisingly, we observed the downregulation of MDMX and SIRT1 expression by miR-34a in RB cells. Thus, the previously identified positive feedback loop by which miR-34a may function to increase p53 protein levels and transcriptional activity is present in RB.33 These results may suggest that the basal miR-34a expression level may modulate cell sensitivity to p53-activating compounds. Reactivation of the p53 pathway in RB cells with high basal miR-34a expression may result in a more rapid activation of apoptosis and cell growth inhibitory responses. We also observed targeting by miR-34a of several genes known to be overexpressed in various cancer cell types. These targets include epithelial membrane protein 1 (EMPI), whose overexpression is correlated with gefitinib resistance in breast and lung cancer.34–35 Recent work has demonstrated that a mutation in the 5′-UTR of δ-catenin (CTNND2) increases expression and cell survival in prostate cancer.36–37 Most interestingly, another miR-34a target, CDC25A, is a known E2F target oncogene that is overexpressed in breast, colorectal, liver, and other cancers.38 The function of these genes and others identified in this study for tumor progression are not well investigated, and future experiments using siRNA knockdown with or without exposure to chemotherapeutic agents may indicate their roles in mediating cell survival and treatment resistance.

Considerable research in microRNA-based therapies for human diseases is under way. Rapid development of locked nucleic acid-modified oligonucleotides to antagonize disease-related microRNAs has led to the first microRNA-based clinical study for hepatitis C infection.39 MicroRNA-based gene therapies for human cancers may also be rapidly developed because of the characterization of cancer type-related microRNAs and their targets in those diseases.40 Additionally, the early discovery of a role for miR-34a in brain, breast, colon, and pancreatic cancer makes it an attractive microRNA for therapeutic development. Advancement in that area could be translated quickly for ocular cancers as delivery of nucleic acids for the treatment of age-related macular degeneration and other eye diseases has been conducted.41–43 Additionally, the recent finding that miR-34a inhibits uveal melanoma cell proliferation and migration supports further investigation of its antitumor activity in preclinical animal models for ocular tumors.44 Additional studies of the molecular mechanisms for miR-34a function in RB are also warranted.

References


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