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Article

p53-Mediated Activation of miRNA34 Candidate Tumor-Suppressor Genes

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Summary

Background: In response to varied cell stress signals, the p53 tumor-suppressor protein activates a multitude of genes encoding proteins with functions in cell-cycle control, DNA repair, senescence, and apoptosis. The role of p53 in transcription of other types of RNAs, such as microRNAs (miRNAs) is essentially unknown.

Results: Using gene-expression analyses, reporter gene assays, and chromatin-immunoprecipitation approaches, we present definitive evidence that the abundance of the three-member miRNA34 family is directly regulated by p53 in cell lines and tissues. Using array-based approaches and algorithm predictions, we define genes likely to be directly regulated by miRNA34, with cell-cycle regulatory genes being the most prominent class. In addition, we provide functional evidence, obtained via antisense oligonucleotide transfection and the use of mouse embryonic stem cells with loss of miRNA34a function, that the BCL2 protein is regulated directly by miRNA34. Finally, we demonstrate that the expression of two miRNA34s is dramatically reduced in 6 of 14 (43%) non-small cell lung cancers (NSCLCs) and that the restoration of miRNA34 expression inhibits growth of NSCLC cells.

Conclusions: Taken together, the data suggest the miRNA34s might be key effectors of p53 tumor-suppressor function, and their inactivation might contribute to certain cancers.

Introduction

In large part because somatic mutations inactivating p53 function are present in more than 50% of all human cancers, the *p53* tumor-suppressor gene and its protein product have been intensely studied for two decades. The p53 protein is a sequence-specific transcription factor. Various cellular stresses, such as DNA damage, hypoxia, and nutrient deprivation, can activate p53 function via effects on p53 stability and nuclear localization and its interactions with other proteins, resulting ultimately in p53-stimulated transcription of perhaps a hundred or more genes [1]. The p53-regulated genes encode proteins sharing functions in regulation of cell-cycle progression, DNA repair, apoptosis, and angiogenesis, and in feedback regulation of p53 function [2]. In aggregate, studies of upstream activators and downstream mediators of p53 function have greatly informed knowledge of why the *p53* gene is commonly inactivated by somatic mutations of its own coding sequences or by defects in key proteins that regulate p53, such as MDM2 gain-of-function or $p14^{ARF}$ loss-of-function mutations [2]. Nonetheless, although enormous attention has been focused on mRNAs regulated by p53, to date, little is known about p53's role in the transcription of other types of RNA, such as microRNAs (miRNAs).

miRNAs are short, noncoding RNAs of 18 to 24 nucleotides length. Longer precursor transcripts with hairpin structures are first synthesized by RNA polymerase II, and, after processing of the precursors by Drosha and Dicer ribonucleases, mature miRNAs are generated (reviewed in [3]). Depending on the degree of homology to their target sequence, miRNAs induce translational repression or cleavage of mRNAs. A single miRNA can target hundreds to a thousand or more mRNAs [4], rendering it challenging to attribute distinct functions to specific miRNAs. Perhaps as a result of this complexity, in spite of the evolutionary conservation of miRNAs, their function in physiology and disease remains rather enigmatic.

Notwithstanding the challenges, insights into the potential contributing roles of miRNAs in cancer have been obtained. Comprehensive analyses of miRNA expression patterns in human cancers have been reported, and the findings suggest that different cancer types have distinct miRNA expression patterns [5–7]. Other studies of miRNAs in cancer have also pointed to the function of certain miRNAs as oncogenes. For instance, the miRNA17-92 polycistron at chromosome 13q31.3 encodes seven miRNAs, and the polycistron has been reported to be genomically amplified and overexpressed in some human B cell lymphomas and lung cancers [8]. Transcription of the miRNA17-92 polycistron is regulated by c-MYC and E2F proteins, and this polycistronic transcript cooperated with c-MYC to accelerate lymphoma development in a murine-model system [8, 9]. In addition, miRNA155, has been reported to be overexpressed in several lymphoma subtypes [10, 11]. With respect to potential tumor-suppressor activities for miRNAs, decreased levels of miRNA143 and miRNA145 were seen in colorectal cancer [12], and decreased levels of miRNA15 and miRNA16 have been described in chronic lymphocytic leukemia [5, 13–15]. In addition, the members of the let-7 family of miRNAs have been

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suggested as tumor suppressors in lung cancer, supposedly in part by their ability to target the K-Ras and HMGA2 oncogenes [16–18]. Notably, inhibition of global miRNA processing has recently been shown to lead to increased tumorigenicity and transformation, suggesting that the decreased expression of multiple miRNAs might be important in tumorigenesis [19].

Results

Direct Regulation of miRNA34 Family Members by p53

The miRNA34 family comprises three members: miRNA34a, which is generated from a larger transcriptional unit on chromosome 1p36, and miRNA34b and miRNA34c, both of which are generated by processing of a bicistronic transcript from chromosome 11q23 (termed miRNA34bc). Based on our analysis of the results of a published genome-wide chromatin immunoprecipitation (ChIP) study from Wei et al. [20], we noted a presumptive p53 binding region within 30 kb of the precursor transcription units for both miRNA34a and miRNA34bc. Although putative p53 binding sites exist in the vicinity of numerous other precursor transcripts for miRNAs, the coincidence of binding sites in proximity to both the miRNA34a and miRNA34bc precursors spurred our interest.

The transcriptional activity of p53 can be readily induced in cells by exposure to genotoxic stress, such as the chemotherapeutic agent adriamycin. Treatment of the Wi38 human diploid fibroblast cell line with adriamycin led to a potent activation of miRNA34a (Figure 1A). This increase was nearly completely inhibited in Wi38 cells stably expressing a short hairpin RNA (shRNA) targeting p53 [21]. A marked increase in miRNA34a levels was also seen in spleen tissue obtained 12 hr after whole-body irradiation of wild-type mice (Figure 1B), and the induction of miRNA34a was lacking in spleen tissue from irradiated $p53^{-/-}$ mice. Based on our analysis of cDNA sequences in the database, the chromosome 1p36 precursor transcript for miRNA34a is predicted to be spliced, with the candidate p53 DNA binding site located upstream of the transcriptional start site in a well-conserved DNA sequence region present within a CpG island (Figure 1C). A luciferase-based reporter gene construct containing key sequences from the locus was generated by subcloning an 837 bp genomic fragment containing the presumptive transcriptional start site and the candidate p53 binding site. Cotransfection of the reporter construct with a wild-type p53 cDNA expression vector potently activated the reporter gene, whereas a p53 cDNA carrying a missense mutation [22] or a control (empty) expression vector were inactive (Figure 1D, left). The mutation of key nucleotides in the presumptive p53 binding site abolished activation of the reporter gene by wild-type p53 (Figure 1D, right). ChIP with a p53 antibody demonstrated that DNA sequences containing the presumptive p53 binding site could be specifically recovered from HCT116 cells expressing wild-type p53, after treatment of cells with 5-fluorouracil (5-FU) to activate p53, but not from an isogenic cell line in which p53 had been inactivated by homologous recombination (Figure 1E) [23].

Studies analogous to those for miRNA34a were then undertaken for miRNA34b and the miRNA34bc precursor transcriptional unit on chromosome 11q23. Adriamycin treatment of Wi38 cells led to markedly increased levels of miRNA34b, and shRNA-mediated inhibition of p53 function abrogated the induction of miRNA34b by adriamycin (Figure 2A). Irradiation of wild-type mice, but not $p53^{-/-}$ mice, led to markedly increased levels of miRNA34b in the spleen (Figure 2B). Examination of the 11q23 locus for the miRNA34bc precursor transcriptional unit revealed a candidate p53 DNA binding site upstream of the transcriptional start site (Figure 2C). A reporter gene construct containing a 555 bp fragment that includes the p53 binding site and the predicted transcriptional start site was strongly activated by cotransfection of a wild-type p53 cDNA, but not by a p53 cDNA with a missense mutation or control expression vector (Figure 2D, left). Mutations in the candidate p53 binding site abrogated the ability of wild-type p53 to induce reporter gene activation (Figure 2D, right).

Identification of miRNA34-Regulated Genes

As noted above, depending on the cellular context, the p53 protein can regulate cell-cycle progression and apoptosis induction. In light of the evidence that p53 acts directly to increase miRNA34 levels, we assessed whether ectopic miRNA34 expression had effects similar to those of p53. We used the p53 mutant colon cancer cell line SW480 as a model because these cells have only low levels of expression for the three miRNA34 family members. We created a polyclonal SW480 line that, in response to doxycyclin (dox) treatment, expressed a longer precursor transcript that is cleaved to generate mature miRNA34a (SW480 miRNA34), along with green fluorescent protein (GFP). We also generated a control polyclonal SW480 cell line that expressed only GFP in response to dox treatment (SW480GFP). As an initial unbiased approach to assess miRNA34 effects, we analyzed mRNA transcript profiles in the cells after 3 days of dox treatment. We fit a two-way analysis of variance (ANOVA) model to the data, modeling cell type (i.e., SW480 miRNA34 or SW480GFP), treatment (i.e., dox treated or not), as well as interaction between cell type and treatment. We required a p value of < 0.01 for the interaction and that the n-fold change upon exposure to dox be at least 1.5-fold greater in SW480 miRNA34a cells than in SW480GFP cells. This analysis highlighted 1012 probe sets (corresponding to 766 genes) that decreased significantly more after dox treatment in the SW480 miRNA34 line, when compared to the SW480GFP line (subsequently termed downregulated genes). This list of potential miRNA34 targets was then compared with the list of target genes predicted by Targetscan and Pictar [4, 24–26]. A highly significant enrichment of predicted miRNA34 targets was observed for the downregulated gene list: Among the 313 genes predicted by Targetscan [4] that were on the arrays, we found 61 in the downregulated gene group ($p = 6.5 \times 10^{-26}$, Figure S1 in the Supplemental Data available online). Similar lists of predicted targets for miRNA34a and miRNA34bc from Pictar [25, 26] also gave significant enrichments for our downregulated genes ($p = 5.1 \times 10^{-27}$ and 1.24×10^{-17} respectively). In addition to the role of miRNA binding to the 3′ untranslated region (UTR) of target genes, which is

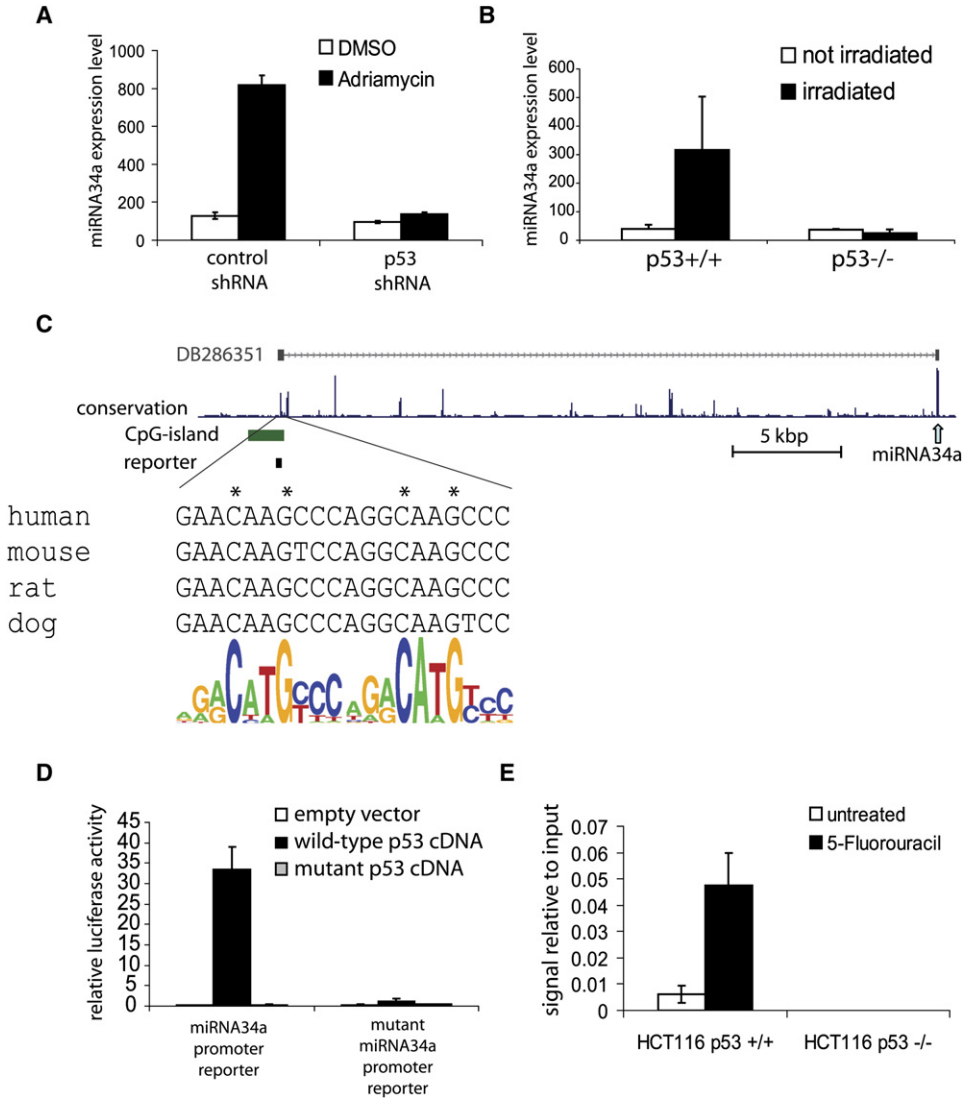

Figure 1. miRNA34a Is a Direct Transcriptional Target of p53

(A) WI38 human diploid fibroblast cell lines expressing a shRNA targeting p53 and a scrambled control shRNA were treated with 200 nM Adriamycin for 24 hr. Shown are miRNA34a expression levels, as determined by Taqman-based real-time reverse-transcription polymerase chain reaction (RT-PCR) relative to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression. Means and standard deviations are shown.

(B) miRNA34a expression levels in the spleens of $p53^{+/+}$ (n = 4) and $p53^{-/-}$ (n = 3) mice 12 hr after irradiation with 8Gy. The mean and standard deviation of expression levels relative to levels of 18S rRNA levels are shown.

(C) Schematic representation of the genomic locus of *miRNA34a* with an expansion of a conserved predicted p53 binding site. Mutated residues are marked with an asterisk. DB286351 represents the location of an expressed sequence tag (EST) spanning miRNA34a and the putative p53 binding site.

(D) miRNA34a promoter reporter assay. SW480 cells were transfected with an expression construct for wild-type p53, mutant p53, or empty vector. Activity of a reporter construct carrying an 837 bp genomic fragment encompassing the putative transcriptional start site of miRNA34a upstream of a firefly luciferase open reading frame was normalized to the activity of a cotransfected CMV-β-galactosidase construct. Note that the apparent residual activation of the mutated reporter by wild-type p53 is caused by a concomitant downregulation of the CMV-β-galactosidase normalization vector.

(E) ChIP of a miRNA34a promoter region. Chromatin from HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells was precipitated with a p53 antibody after treatment with 5-FU for 20 hr. Quantitative PCR of a region flanking the putative p53 binding site in the miRNA34a promoter was used to quantify the amount of chromatin obtained by immunoprecipitation relative to the amount in the input samples (in percent of input). When analyzing chromatin that had been immunoprecipitated from HCT116 $p53^{+/+}$ cells with a p53 antibody, amplification of miRNA34a promoter elements was observed at cycle 36 or 39 in the presence or absence of 5-FU, respectively. At 42 cycles, no amplification was observed with primers for a genomic region that is not bound by p53 (data not shown), and no amplification of miRNA34a promoter elements was observed from the chromatin that had been immunoprecipitated from $p53^{-/-}$ cells. Error bars represent standard deviations.

the basis for most target-site-prediction algorithms, Miranda et al. have recently suggested that binding sites within the coding sequence of genes might be important for miRNA function [27]. We therefore evaluated the coding sequences of the downregulated genes in comparison to the upregulated genes for putative miRNA34a

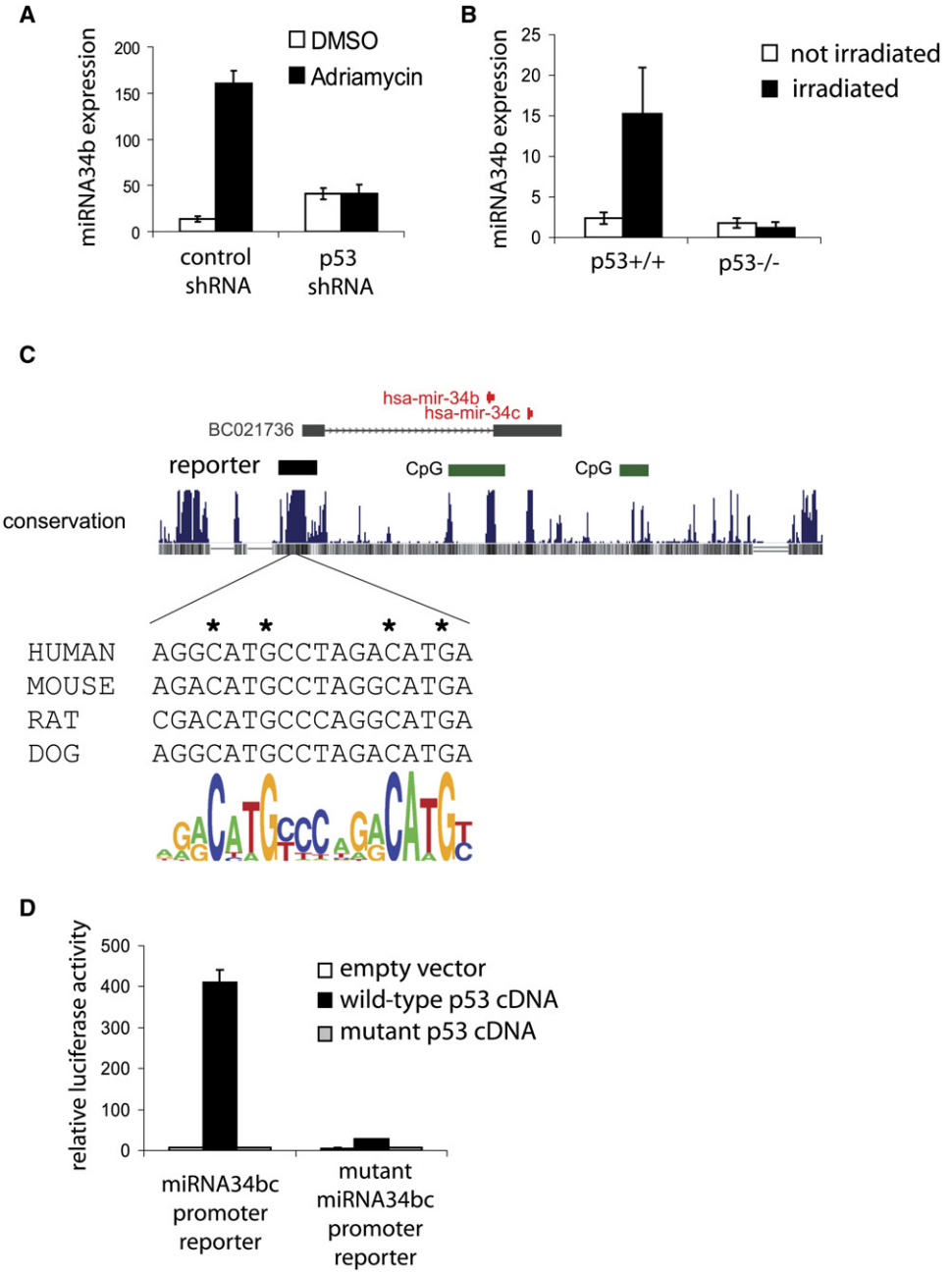

Figure 2. miRNA34b Is a Direct Transcriptional Target of p53

(A) WI38 human diploid fibroblast cell lines expressing an shRNA targeting p53 and a scrambled control shRNA were treated with 200 nM Adriamycin for 24 hr. miRNA34b expression levels as determined by Taqman-based real-time RT-PCR are shown relative to the level of GAPDH mRNA expression. Means and standard deviations are shown.

(B) miRNA34b expression levels in the spleens of $p53^{+/+}$ (n = 4) and $p53^{-/-}$ (n = 3) mice 12 hr after irradiation with 8Gy. The mean and standard deviation of expression levels relative to levels of 18S rRNA levels are shown.

(C) Schematic representation of the genomic locus of *miRNA34bc* with an expansion of a conserved predicted p53 binding site. Mutated residues are marked with an asterisk. BC021736 represents the location of an EST spanning miRNA34bc and the putative p53 binding site.

(D) miRNA34bc promoter reporter assay. SW480 cells were transfected with an expression construct for wild-type p53, mutant p53, or empty vector. Activity of a reporter construct carrying a 555 bp genomic fragment encompassing the putative transcriptional start site of miRNA34bc upstream of a firefly luciferase open reading frame was normalized to the activity of a cotransfected thymidine-kinase promoter renilla luciferase construct. Note that the apparent residual activation of the mutated reporter by wild-type p53 reflects concomitant inhibition of the TK-Renilla normalization vector. Error bars represent standard deviations.

binding sites with their RNA22 algorithm [27]. Notably, we observed a larger fraction of genes with predicted target sites in the downregulated gene group compared to the upregulated gene group (31.3% versus 22.7%, $p = 0.004$), suggesting that miRNA34 also exerts effects via the targeting of coding-region sequences.

Testing Gene Ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways,

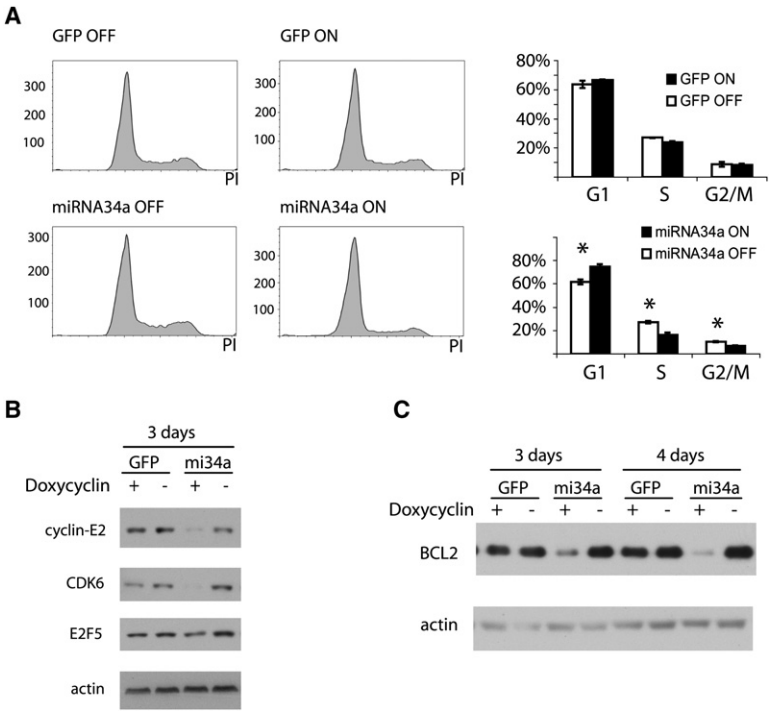

Figure 3. Ectopic Expression of miRNA34a Leads to G1 Cell-Cycle Arrest and Downregulation of BCLI2

(A) Cell-cycle distribution of SW480 miRNA34a cells after 3 days treatment with doxycyclin. Cells were stained with propidium iodide after ethanol fixation. The left panel shows primary flow cytometry histograms, and the right panel shows quantification of populations in different cell-cycle phases with Modfit3.0. Asterisks mark significant changes with t test, $p < 0.05$, and error bars represent standard deviations.

(B) Cyclin-E2, CDK6, and E2F5 protein levels are downregulated after induction of miRNA34a expression. Western-blot analysis was performed after 3 days of doxycyclin treatment of SW480 miRNA34a and SW480GFP cells.

(C) BCL2 protein levels are downregulated after induction of miRNA34a expression for 3 and 4 days. No inhibition is observed in control cell lines SW480 GFP. Levels of actin are shown to demonstrate equal loading in panels (B) and (C).

we found a strong enrichment of "cell cycle" genes in the list of genes downregulated by miRNA34 ectopic expression ($p = 1.84 \times 10^{-6}$ and $p = 1.34 \times 10^{-6}$, respectively; see Tables S1 and S2 for the top 20 categories). This group of genes encoded G1/S regulators, such as the cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), Cyclin E2, and the E2F3 and E2F5 transcription factors, as well as the S phase and M phase regulators Cyclin A2, CDC25a, and Aurora kinase B (Tables S1 and S2). While our studies were in progress, data implicating miRNA34a in translational suppression of E2F3 were reported [28].

miRNA-Mediated Effects on Cell-Cycle and Apoptosis Regulation

After miRNA34 induction in SW480 cells, we observed a G1 arrest, suggesting that miRNA34, similar to p53 itself, regulates cell-cycle progression (Figure 3A). We confirmed that the expression of several cell-cycle regulators predicted to be regulated by miRNA34—Cyclin E2, CDK6, and E2F5—was in fact reduced upon ectopic expression of miRNA34a (Figure 3B). No changes in mutant p53 protein levels were observed upon miRNA34a expression (data not shown). We nevertheless were interested to see whether cell-cycle arrest by miRNA34a had effects on mRNAs and proteins known to be directly activated by p53. Only very modest changes of p21 protein levels and BBC3/Puma mRNA levels were observed (Figure S2). Our list of genes whose expression was altered upon miRNA34a induction did not show enrichment for genes with known p53 binding sites in their regulatory region (Figure S2C). This suggests that miRNA34a induces cell-cycle arrest in a complementary and parallel fashion to mRNAs that are directly activated by p53.

Though the list of genes downregulated by miRNA34 was not generally enriched for genes encoding factors with established roles in apoptosis, we noted that transcript levels for the well-known antiapoptotic factor BCL2 were downregulated by miRNA34 ectopic expression. Western-blot studies confirmed strong downregulation of BCL2 protein levels by miRNA34 in SW480 cells (Figure 3C). To establish the role of miRNA34 binding sites in BCL2 transcripts as a key mechanism contributing to miRNA34's effects (Figure 4A), we placed part of the 3′ UTR of BCL2 downstream of luciferase sequences in an expression construct. Ectopic expression of miRNA34a significantly reduced the activity of a construct containing the wild-type BCL2 3′ UTR, whereas miRNA34a had no effect on a control construct with two point mutations in the seed region for miRNA34 binding (Figure 4B). Because SW480 has very low endogenous levels of miRNA34s, we assessed the consequences of antagonizing miRNA34 in Wi38 human embryonic fibroblasts, which express all three miRNA34 isoforms at baseline and show their accumulation upon genotoxic stress (data not shown). To inhibit all three miRNA34 members, we cotransfected Wi38 cells with antisense locked nucleic acid (LNA) oligonucleotides (miRNA34as) targeting all three isoforms. Inhibition of miRNA34 in Wi38 cells led to an increase in BCL2 levels within 36 hr (Figure 4C). Notably, when Wi38 cells transduced with miRNA34as were treated with the apoptosis-inducing agent staurosporine for 8 hr, fewer early apoptotic cells and more viable cells were observed (Figure 4D). Taken together, the data indicate that miRNA34 can mediate key effects associated with p53 function, namely the inhibition of cell-cycle progression and induction of apoptosis.

To further characterize the endogenous function of miRNA34, we generated embryonic stem (ES) cell lines in which both alleles of miRNA34a had been targeted for inactivation. Based on quantitative gene-expression analyses, miRNA34a levels were reduced to background

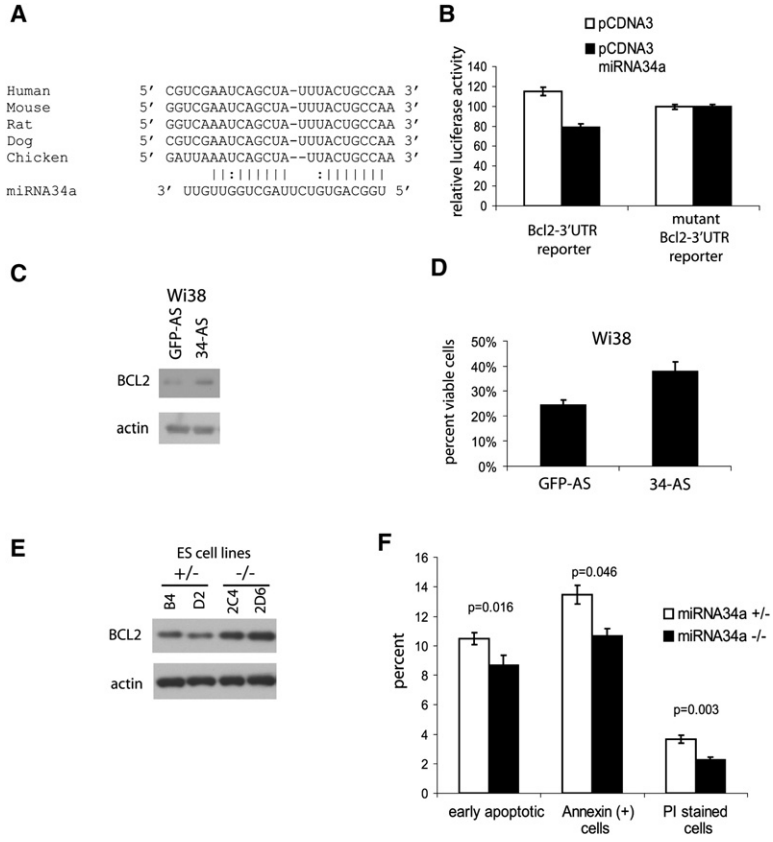

Figure 4. BCL2 Is a Direct Target of miRNA34

(A) Comparison of a putative miRNA34 binding site in the 3′ UTR of BCL2 in different species.

(B) miRNA34a inhibits a luciferase reporter construct carrying the BCL2 3′ UTR. Introduction of point mutations in the miRNA binding site abolishes this regulation. SW480 cells were transiently transfected with the indicated constructs, and luciferase activity was normalized to a cotransfected thymidine-kinase promoter renilla luciferase construct.

(C) Inhibition of miRNA34a,b,c by transfection of LNA oligonucleotides leads to the upregulation of BCL2. LNA antisense oligonucleotides were transfected into Wi38 human diploid fibroblast cells. Western-blot analysis for BCL2 and β-actin was performed 36 hr later.

(D) Inhibition of miRNA34a,b,c protects cells from apoptosis induced by staurosporine. Wi38 cells were transfected with antisense LNA oligonucleotides. After 36 hr, cells were treated with 1 μM staurosporine for 8 hr. Viable cells refers to cells that do not take up propidium iodide and do not stain for annexin V. The average and standard deviation of viable cells of triplicates are shown.

(E) *miRNA34a*$^{-/-}$ ES cells show increased levels of BCL2 upon induction of differentiation. Two clones with disruption of one allele of the miRNA34a gene (B4 and D2) and two clones with disruption of both miRNA34a alleles (2C4 and 2D6) were assessed for BCL2 expression levels 3 days after induction of differentiation by 1 μM retinoic acid and withdrawal of LIF. The expression of β-actin is shown as loading control.

(F) *miRNA34a*$^{-/-}$ ES cells show reduced levels of baseline apoptosis after induction of differentiation (as in [E]). The average and standard deviation of the percentage of early apoptotic cells (staining with annexin V, no propidium iodide uptake), total annexin V-stained cells, and total propidium-iodide-uptaking cells in *miRNA34a*$^{+/-}$ and *miRNA34a*$^{-/-}$ cell lines are shown.

levels when two independent homozygous knockout clones were compared to two heterozygous clones (Figure S3). Although no change in basal apoptosis frequency or cell-cycle parameters were observed in undifferentiated ES cells, we observed significantly higher BCL2 protein levels three days after induction of differentiation by the withdrawal of leukemia inhibitory factor (LIF) and treatment with retinoic acid (Figure 4E), confirming a role of endogenous miRNA34a in regulating BCL2 protein levels. In addition, basal levels of apoptosis were reduced in the differentiated miRNA34a homozygous knockout cells (Figure 4F). It has previously been shown that BCL2 is only expressed at low levels in undifferentiated ES cells and that transcript levels increase during differentiation [29]. This might explain why the effect on baseline apoptosis was only apparent upon the induction of differentiation. The fact that the loss of miRNA34a expression induces a phenotype despite intact miRNA34bc function might be explained by the overall lower miRNA34b and miRNA34c levels in the cell lines (Figure S4).

Differential Expression of miRNA34-Family-Member Expression in Normal Tissues and Silencing of miRNA34bc in a Subset of Lung Cancers

The miRNA34 family is highly conserved through evolution (Figure 5A). In contrast to *Drosophila* or *C. elegans*, vertebrates have three miRNA34 family members, which are generated from two distinct genomic loci. The nucleotide sequences of the three miRNA34 isoforms are highly similar. Based on present knowledge of transcript recognition by miRNAs, each miRNA34 isoform is predicted to target almost the same transcripts. Hence, although it is possible that the apparent redundancy reflects a potential fail-safe system, the basis for three miRNA34 isoforms might reflect tissue-specific differences in expression. We found that miRNA34a was present at highest levels in the brain, with variable expression, ranging from low to moderate, in other tissues assayed (Figure 5B). miRNA34b was most highly expressed in lung, with low expression in brain and very low to undetectable expression in other tissues studied (Figure 5C). Expression of miRNA34c in normal mouse tissues strictly paralleled miRNA34b expression (Figure S5).

To explore the possibility that miRNA34 isoforms might be downregulated in cancer tissues, we focused attention on miRNA34bc and non-small cell lung cancers (NSCLCs) because miRNA34bc was present at highest levels in normal lung tissue. We found that miRNA34b levels were decreased by more than 90% in six out of the 14 NSCLCs, including four of five adenocarcinomas, two of eight squamous cell carcinomas, and zero of one large cell carcinomas (Figure 5E). Three of 14 NSCLCs had a ~2-fold upregulation of miRNA34b

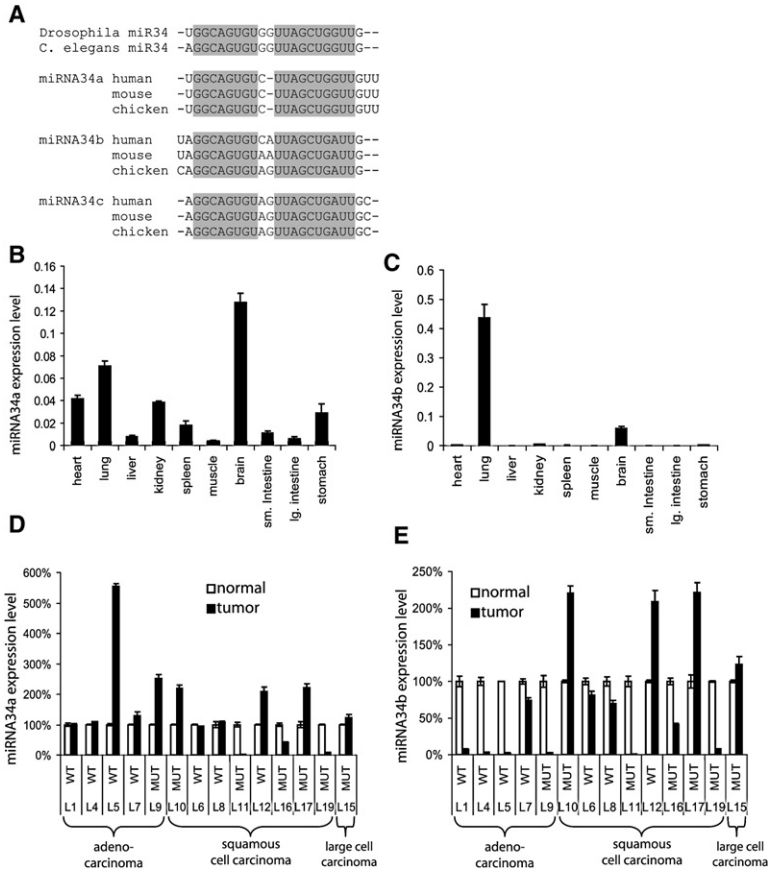

Figure 5. Tissue-Specific Expression of miRNA34 Gene Family and miRNA34bc Downregulation in a Subset of Non-Small Cell Lung Cancers

(A) Conservation of mature miRNA34 sequences throughout evolution. *Drosophila melanogaster* and *Caenorhabditis elegans* only have one miRNA34 gene.

(B and C) Expression levels of miRNA34a (B) and miRNA34b (C) in different mouse organs. miRNA34c expression levels closely parallel miRNA34b levels and are shown in Figure S5. The mean and standard deviation of expression levels normalized to levels of U6 RNA are shown (×1000).

(D and E) Relative expression changes of miRNA34a (D) and miRNA34b (E) between non-small cell lung cancers and adjacent normal tissue. The mean and standard deviation of expression levels relative to miRNA213 expression levels are shown and are normalized to the expression in the normal tissue of each matched pair. Similar results were obtained with normalization to miRNA221. Histologic subtype and p53 mutational status ("WT" indicates wild-type, and "MUT" indicates mutated) are shown.

in cancer tissues relative to normal lung (Figure 5E). No significant correlation between p53 mutational status and miRNA34b downregulation was observed. The expression of miRNA34a was markedly downregulated in only 2 of 14 NSCLCs (both squamous cell carcinomas), with modest to relatively marked overexpression of miRNA34a in 6 of the 14 tumors (Figure 5D). Though loss of heterozygosity (LOH) for chromosome region 11q23, the region where miRNA34bc maps, has previously been reported in NSCLCs, we found that 11q23 LOH was only present in 3 out of 14 cases and was not correlated with downregulation of miRNAbc (Table S4). The increased methylation of a CpG island overlapping the transcriptional unit of miRNA34bc was observed in two of seven tumors analyzed, and only one of them demonstrated significant downregulation of miRNA34bc (data not shown). Given these findings, the mechanisms leading to altered levels of miRNA34 isoforms in NSCLC are probably heterogeneous. We were interested in assessing the functional consequences of expression of miRNA34bc in NSCLC cells that had low miRNA34bc expression and found that ectopic expression of miRNA34bc had potent inhibitory effects on cell proliferation and/or survival in the A549 lung adenocarcinoma cell line (Figure S6).

Discussion

Because the p53 tumor-suppressor gene is arguably the most frequently mutated gene in human cancer, the p53 protein's function as a sequence-specific transcription factor regulating the cellular response to diverse stresses, such as DNA damage, hypoxia, and nutrient deprivation, has been a subject of intense interest over the past 15 years. Although enormous attention has been focused on mRNAs regulated by p53, to date, little is known about p53's role in transcription of other types of RNA, such as miRNAs. Using a combination of gene-expression analyses, reporter gene assays, and ChIP approaches, we have offered strong evidence here that the abundance of a family of three miRNA34s is directly regulated by p53 in cell lines and in mouse tissues. Using a combination of array-based approaches and algorithm predictions, we have identified the genes most likely to be directly regulated by miRNA34, with cell-cycle regulatory genes being the most prominent class regulated by miRNA34. Following ectopic expression of miRNA34 in cells, we observed potent effects on cell-cycle progression and selected cell-cycle regulators.

Though apoptosis pathway genes were not predicted to be a prominent class regulated by miRNA34, we presented robust functional evidence, obtained through the use of antisense oligonucleotides and mouse ES cells in which miRNA34a has been genetically inactivated, that the BCL2 protein is regulated directly by miRNA34. Finally, besides providing information on the tissue-specific patterns of miRNA34 family members in mouse tissues, we demonstrate that the expression of two miRNA34s is dramatically reduced in a subset of NSCLCs, particularly adenocarcinoma of the lung. Taken

together, the data suggest the miRNA34s might be key effectors of p53 tumor-suppressor function and that their inactivation might contribute to certain cancers.

Overall, the findings we have presented here implicate the miRNA34 family as significant downstream effectors of p53 function in normal and cancer cells. With respect to our observations that four of five lung adenocarcinomas and 6 of 14 total NSCLCs lack miRNA34bc, it is interesting to note that p53 mutations are found in only about 35% of lung adenocarcinomas and about 50% of squamous cell carcinomas [30]. Perhaps the loss of miRNA34 isoforms in some NSCLCs might serve to mimic the consequences of p53 loss of function on cell-cycle checkpoints and cell survival. Consistent with this view, it is noteworthy that Welch et al. [28] recently showed a significant downregulation of the most highly expressed miRNA34 variant in neuronal tissue, miRNA34a, in neuroblastoma with 1p36 allelic loss, suggesting that the inactivation of miRNA34 isoforms might play a role in multiple cancer subtypes. Further studies of the expression and function of miRNA34 isoforms in normal and neoplastic tissues will undoubtedly enhance knowledge of how the miRNA34 family functions in regulating cell-cycle progression and apoptosis and perhaps other essential functions of p53.

Experimental Procedures

Cell Culture

Wi38 human diploid fibroblasts were obtained at passage 12 (Coriell Cell Repository [Camden, NJ]) and were cultured in modified Eagle's medium with Earle's salts containing 15% fetal calf serum, penicillin-streptomycin, nonessential amino acids, and 2 mM L-glutamine (Invitrogen [Carlsbad, CA]). Experiments were performed between passages 14 and 18. Retroviral constructs based on pSUPERIOR PURO (Oligoengine [Seattle, WA]) carrying a shRNA targeting p53 or a nonsilencing control [31] were packaged with the Phoenix packaging cell line (American Type Culture Collection [ATCC] [Manassas, VA]), and supernatants were used in three consecutive infections in presence of 4 μg/ml polybrene (Sigma-Aldrich [St. Louis, MO]). Polyclonal stable populations were obtained after selection with 1 μg/ml Puromycin for 7 days. Oligonucleotides with seven LNA nucleotides and perfect reverse complementarity to miRNA34a, miRNA34b, and miRNA34c (in a relative molar ratio of 2:1:1) were transfected with 5 μl of Lipofectamine 2000 transfection reagent and 50 nM of the oligonucleotides (Integrated DNA technologies [Coralville, IA]). An oligonucleotide targeting GFP with the sequence 5′-ATGAACTTCAGGGTCAGCTTGC-3′ was used as a negative control.

ES cell clone STA052 was obtained from Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis and was derived from the E14Tg2a-cl4 cell line in a large-scale gene-trap approach [32]. It was cultured on gelatin-coated tissue-culture plates with Glasgow's modified Eagle's medium (Sigma) with addition of 1000 U/ml human LIF (Chemicon [Temecula, CA]), 10% fetal calf serum (Atlanta Biologicals [Lawrenceville, GA]), penicillin-streptomycin, 50 μM mercaptoethanol, nonessential amino acids, and 2 mM L-glutamine. The insertion site of the gene-trap vector was verified and is localized in the first intron of the miRNA34a precursor transcript. Homozygous ES cells were generated by the selection of cells with 2 mg/ml G418 [33]. In parallel, subclones of the STA052 cell line were isolated by the limiting of the dilution in 150 μg/ml G418. Differentiation was induced by the plating of cells for 3 days in the presence of 1 μM retinoic acid and in the absence of LIF. To assess the influence of miRNA34bc re-expression on the growth of NSCLC cell lines, we cotransfected 0.5 μg of a GFP expression plasmid (pEGFP-C1) with 1.5 μg of pMSCV-PURO or pMSCV-miRNA34bc. Flow cytometry was performed 4 days later, and the number of green fluorescent cells was determined.

SW480 (ATCC), HCT116 (ATCC), and HCT116 $p53^{-/-}$ (a generous gift of Bert Vogelstein) were cultured in Dulbecco's modified Eagle's medium in the presence of 10% fetal calf serum, penicillin-streptomycin, nonessential amino acids, and 2 mM L-glutamine (Invitrogen). Cells were transfected with episomal doxycyclin-regulatable expression plasmids (derived from the plasmid p-RTS1 [34]) and selected in the presence of 400 ug/ml hygromycin (Invitrogen). Gene expression was induced by 1 μg/ml doxycyclin (Sigma [St. Louis, CA]). More than 90% of cells expressed GFP. Reporter assays to assess miRNA34a and miRNA34bc promoter activity were performed by the transfection of 0.5 μg reporter constructs, 0.5 μg of a p53 expression plasmid [22], and 0.5 μg of a CMV-β-galactosidase construct in 6-well plates with Fugene 6 (Roche [Indianapolis, IA]). Where indicated, a thymidine-kinase promoter renilla luciferase construct was used for normalization (Promega [Madison, WI]). Reporter gene activity was measured with a dual-luciferase assay system (Promega).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed essentially as described by Weinmann et al. [35]. Cells were treated with 150 μM 5-FU (Sigma) for 20 hr and were fixed for 10 min in 1% formaldehyde at room temperature. Chromatin was sheared to 300–1000 bp length with a Fisher Sonic dysmembranator eight times for 10 s each at power level 2.5. Immunoprecipitation was performed with mouse p53 monoclonal antibody DO1 (AB6, Calbiochem [San Diego, CA]).

Recovered chromatin was measured by quantitative PCR and normalized to the amount in the extracts prior to immunoprecipitation. Primers are available upon request.

Plasmids, DNA Sequencing, and Microarray Analysis

Details are referenced in the Supplemental Data.

Western-Blot Analysis

Protein extracts were prepared by a modified RIPA (radioimmunoprecipitation assay) buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of proteinase inhibitor cocktail (Complete mini, Roche) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride). Polyacrylamide gel electrophoresis, tank-based transfer to polyvinylidene difluoride (PVDF) membrane, and immunodetection were performed with standard techniques. Antibodies used were mouse BCL2 (Santa Cruz Biotechnologies [Santa Cruz, CA]), mouse p21 (Santa Cruz Biotechnologies), rabbit Cyclin-E2 (Cell Signaling Technology [Danvers, MA]), mouse CDK6 (Cell Signaling Technology), rabbit E2F5 (Santa-Cruz Biotechnologies), mouse β-actin (Sigma), and horseradish-peroxidase-coupled goat antibodies against rabbit and mouse immunoglobulins (Pierce [Rockford, IL]). Signals were visualized with Supersignal West Pico chemoluminescent substrate (Pierce) by exposure to films.

Flow Cytometry

After trypsinization, cells were washed once in phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight. Staining for DNA content was performed with 2 μg/ml propidium iodide and 20 μg/ml RNase A for 30 min. Analysis was performed on a FACScalibur or LSR II flow cytometry machine (BD Biosciences [Franklin Lakes, NJ]) with Cell Quest Pro or FACS-Aria software. Cell-cycle modeling was performed with Modfit 3.0 software (Verity Software House [Topsham, ME]).

For analysis of apoptotic cell populations, cells were trypsinized and washed in cold PBS. Staining with Alexa-Fluor 647 annexin V (Invitrogen) and propidium iodide was performed in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 2.5 mM $CaCl_2$ for 15 min at room temperature.

Supplemental Data

Experimental Procedures, six figures, and four tables are available at http://www.current-biology.com/cgi/content/full/17/15/1298/DC1/.

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Accession Numbers

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