MicroRNA-34b and *MicroRNA-34c* Are Targets of p53 and Cooperate in Control of Cell Proliferation and Adhesion-Independent Growth

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Abstract

MicroRNAs (miRNA) are a recently discovered class of noncoding RNAs that negatively regulate gene expression. Recent evidence indicates that miRNAs may play an important role in cancer. However, the mechanism of their deregulation in neoplastic transformation has only begun to be understood. To elucidate the role of tumor suppressor p53 in regulation of miRNAs, we have analyzed changes in miRNA microarray expression profile immediately after conditional inactivation of *p53* in primary mouse ovarian surface epithelium cells. Among the most significantly affected miRNAs were *miR-34b* and *miR-34c*, which were down-regulated 12-fold according to quantitative reverse transcription–PCR analysis. Computational promoter analysis of the *mir-34b/mir-34c* locus identified the presence of evolutionarily conserved p53 binding sites ~3 kb upstream of the miRNA coding sequence. Consistent with evolutionary conservation, *mir-34b/mir-34c* were also down-regulated in *p53*-null human ovarian carcinoma cells. Furthermore, as expected from p53 binding to the *mir-34b/c* promoter, doxorubicin treatment of wild-type, but not *p53*-deficient, cells resulted in an increase of *mir-34b/mir-34c* expression. Importantly, *miR-34b* and *miR-34c* cooperate in suppressing proliferation and soft-agar colony formation of neoplastic epithelial ovarian cells, in agreement with the partially overlapping spectrum of their predicted targets. Taken together, these results show the existence of a novel mechanism by which p53 suppresses such critical components of neoplastic growth as cell proliferation and adhesion-independent colony formation. [Cancer Res 2007; 67(18):8433–8]

Introduction

MicroRNAs (miRNA) are a recently discovered class of noncoding RNAs, which control gene expression either by degradation of target mRNAs or, more commonly for animal miRNAs, by posttranscriptional repression in a mechanism similar to small interfering RNA–mediated gene silencing. Numerous evidences point to a role for miRNAs in the etiology and pathogenesis of cancer by targeting oncogenes or tumor suppressors (1). For example, *miR-15a* and *miR-16* target antiapoptotic gene *BCL-2* (2), whereas the *LATS32* tumor suppressor is targeted by *miR-372* and *miR-373* (3). Dysregulated miRNA expression may occur via a number of mechanisms, such as gene copy gain or loss (4), germline mutation of precursor miRNA molecules (5), promoter methylation (6), or aberrant miRNA processing due to altered expression of miRNA biogenesis machinery (7). However, the role of transcription factors in miRNA expression has received little attention. Most, if not all, miRNAs are transcribed by RNA polymerase II (8), suggesting that transcription factors involved in mRNA transcription may also regulate miRNA transcription. Supporting this hypothesis, the protooncogene and transcription factor Myc has been shown to bind canonical E-box sequences found upstream of the *mir-17-92* miRNA locus (9).

Given the above observations, we decided to elucidate the involvement of p53 in the regulation of miRNAs. The p53 protein is a transcription factor that is frequently mutated in many types of human cancer. Cellular stress, such as DNA damage, hypoxia, or inappropriate oncogene activation, activates and stabilizes p53, resulting in an antiproliferative response, such as cell cycle arrest, apoptosis, or senescence. p53 orchestrates such responses by directly activating key genes via binding two repeats of the DNA sequence 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ (10). Genes known to be activated by p53 include *p21, Gadd45, Bax*, and its negative regulator *Mdm2* (11).

p53 mutations are thought to be the initiating or earliest events in formation of a number of cancers, including ovarian cancer. Approximately 90% of ovarian cancers are carcinomas, which are assumed to originate from the ovarian surface epithelium (OSE), a single layer of cells coating the ovary. Due to near symptomless progression, the majority of cases are diagnosed at a late stage, at which prognosis is extremely poor (12). Based on the previously described mouse model of epithelial ovarian cancer (13), we have established a system to evaluate immediate effects of *p53* inactivation on miRNAome of the OSE within the first few passages after explantation. We report that miRNAs *miR-34b* and *miR-34c* are transcriptional targets of p53 and represent novel effectors mediating its suppression of such critical components of neoplastic growth as cell proliferation and adhesion-independent colony formation.

Materials and Methods

Cell culture. For preparation of primary cell cultures, mouse OSE cells from either $p53^{loxP/loxP}$ (14) or wild-type age-matched mice of the same FVB/N background were isolated as previously described (13). All mice used for cell preparations were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee. Neoplastic cell lines OSN1 and OSN2 were generated by Cre-*loxP* mediated inactivation of *p53* and *Rb* or *p53* individually, respectively, after three passages in culture. These cell lines were continually passaged upon reaching confluence using standard techniques and late passage OSN1/OSN2 cells were used in this study. Primary cultures and established mouse cell lines were maintained in DMEM/F12 (50:50 mix) supplemented with 5%

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

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fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 5 μg/mL each of insulin and transferrin, and 5 ng/mL sodium selenite. Human SKOV-3 cell line was obtained from American Type Culture Collection and maintained according to supplier's directions.

miRNA isolation and profiling. At passage 3 after explantation, subconfluent OSE cell cultures were treated with either Ad*Cre* or blank adenovirus in serum-free medium for 2 h at 37°C/5% CO_2 and cultured for a further two passages and processed for miRNA isolation. Total RNA was isolated using a mirVana miRNA isolation kit (Ambion) and was highly enriched for mature miRNA species using a FlashPAGE Fractionator (Ambion). miRNA-enriched material (110 ng) was labeled with Cy5 using Label IT miRNA labeling kit (Mirus Bio Corporation) according to the manufacturer's instructions and subsequently hybridized to CombiMatrix MicroRNA 4X2K Microarrays (CombiMatrix) containing probes against mouse miRNAs in release 8.1 of the Sanger database.

Each slide harbored four microarrays, each containing 2240 probes. Four hundred miRNA sequences were represented by a native probe corresponding to wild-type miRNA sequence (Nat) and a two-point mutation mutant probe (Mut) to maximally disturb the binding between probe and intended target miRNA. Each probe was replicated three to five times on the array. The hybridized arrays were scanned by GenePix 4000B scanner (Molecular Devices) to generate raw fluorescence intensity file. The intensities of replicate spots for the same probe sequence were averaged to represent the probe signal. Six wild-type samples and six mutant samples were analyzed. Three samples with obvious hybridization artifacts were excluded from further analysis. True signal for the intended miRNA target was obtained by subtracting Mut probe intensity from the corresponding Nat probe intensity. Some of these Nat-Mut signals were very small so data flooring was applied to raise any value below 2 to be equal to 2 and were log-transformed. To minimize slide-to-slide variation, biological replicate samples on each slide were averaged and the difference between sample types taken for subsequent analysis. The log ratio values of each array were centered to have a median of 0. One-sample *t* test against mean of 0 was applied on the normalized *p53* mutant/wild-type values of each gene across three slide hybridizations. At cutoff value of 0.1, a total of 84 genes were selected.

Quantitative reverse transcription–PCR. Stem-loop quantitative reverse transcription–PCR (RT-PCR) for mature miRNAs was done as described (15) on an Applied Biosystems AB 7500 Real Time PCR system. All PCR reactions were run in quadruplicate and gene expression, relative to *RNU6B*, calculated using the $2^{-\Delta\Delta Ct}$ method (16).

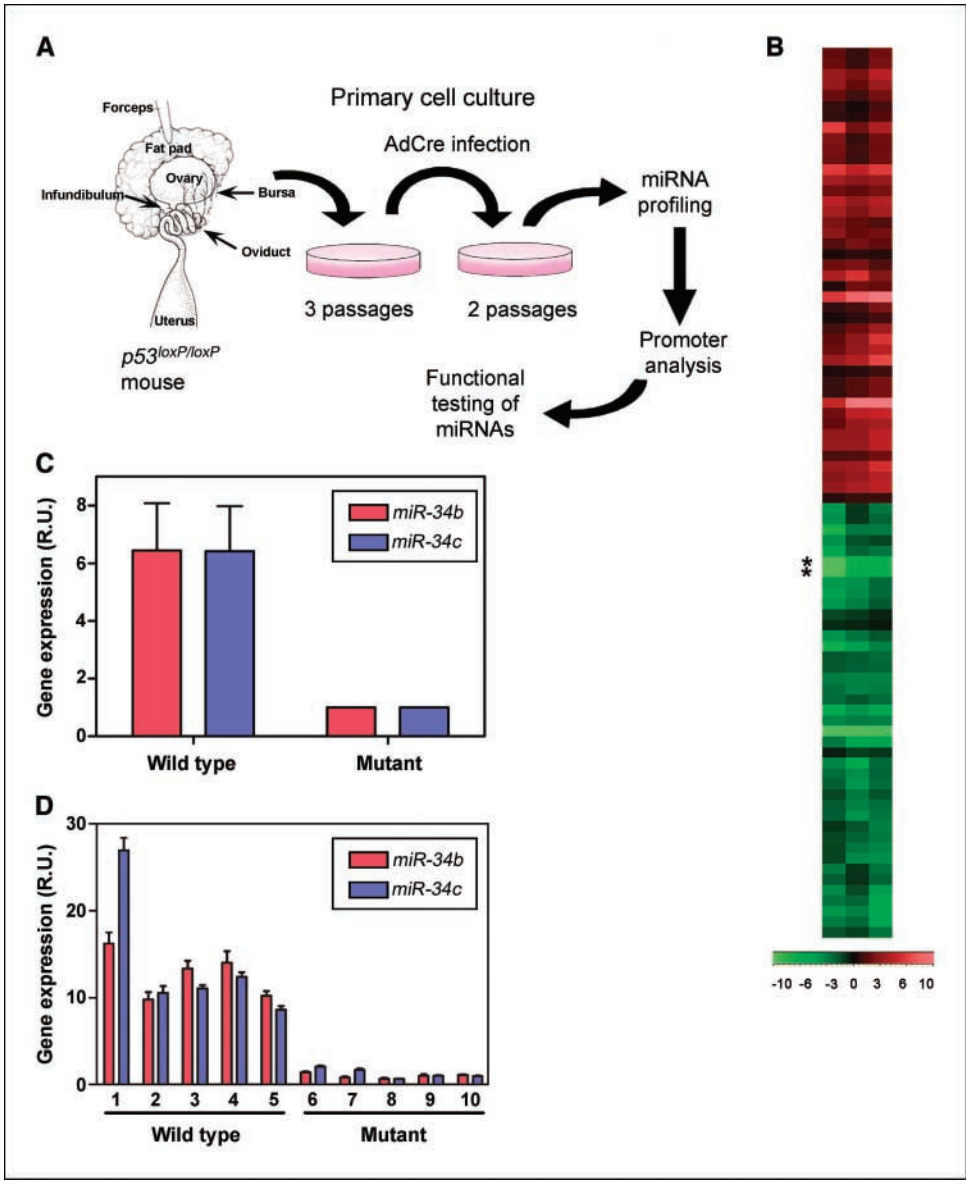

Figure 1. miRNAome alterations after acute *p53* inactivation in primary mouse OSE cells. *A,* outline of experiments. miRNA expression profile was generated after Ad*Cre*-mediated *p53* inactivation followed by computational miRNA promoter analysis for p53REs. Finally, the function of p53-dependent miRNAs was elucidated by experimental testing. *B,* expression profile of top 84 miRNAs differentially expressed after acute *p53* inactivation in three independent experiments. *miR-34b* and *miR-34c*, two of the most down-regulated miRNAs, are indicated by asterisks. *C,* expression levels of *miR-34b* and *miR-34c* as judged by microarray analysis of four wild-type and five p53-deficient (mutant) OSE cultures ($P = 0.055$ and $P = 0.046$ for *miR-34b* and *miR-34c*, respectively). *D,* expression levels of *miR-34b* and *miR-34c* relative to *RNU6B* as judged by quantitative RT-PCR of five wild-type and five mutant OSE cultures ($P < 0.0001$ and $P = 0.0053$ for *miR-34b* and *miR-34c*, respectively). *C and D,* R.U., relative units.

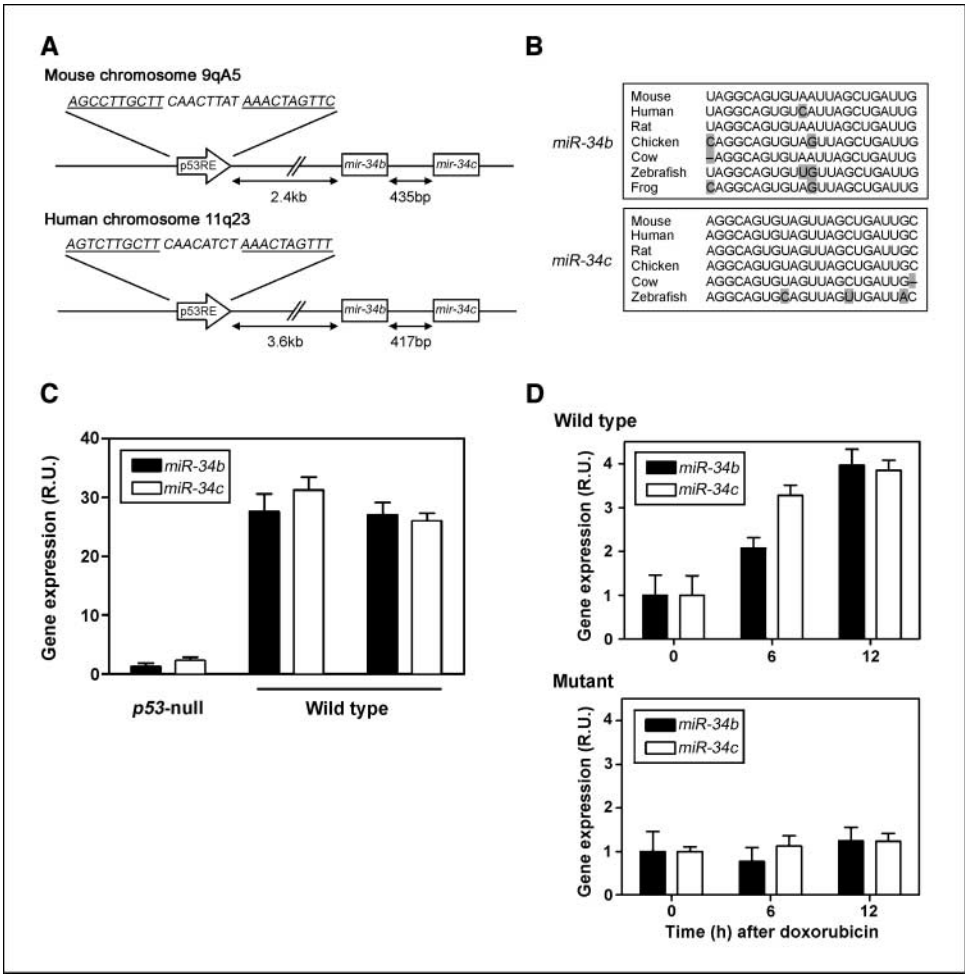

Figure 2. *miR-34b* and *miR-34c* are direct targets of p53. *A,* structure of the mouse and human *mir-34b/mir-34bc* locus. Computational analysis identified a p53RE ~3 kb upstream of the miRNA coding sequence, which is conserved between mouse and human. *B,* in addition to conservation of a p53RE, *miR-34b* and *miR-34c* mature sequences are remarkably well conserved among species. *C, miR-34b* and *miR-34c* expression relative to *RNU6B* in *p53*-null SKOV-3 human OSE cell line is considerably lower than expression in wild-type human OSE. *D,* doxorubicin treatment (0.5 μg/mL) of wild-type OSE cultures results in a rapid increase in expression of both *miR-34b* and *miR-34c* (*top, P* = 0.0004). In contrast, no such induction is observed in identically treated *p53* mutant OSE cultures (*bottom*).

Bioinformatics. To identify p53 response elements in miRNA promoter regions, 5 kb 5′ and 3′ of each miRNA genomic sequence from Build 36 of the *Mus musculus* genome and Build 36.1 of the *Homo sapiens* genome were retrieved from UCSC Genome Browser. Retrieved sequences were analyzed by the p53MH algorithm, which searches for two copies of the p53 DNA-binding motif separated by 0 to 14 bp (17).

Molecular cloning of *mir-34b* and *mir-34c*. Genomic DNA encoding *mir-34b* and/or *mir-34c* was cloned using standard molecular biology techniques. Briefly, a 347-bp, 258-bp, or 795-bp fragment of mouse DNA containing *mir-34b, mir-34c*, or both sequences, respectively, was PCR amplified with Herculase II Fusion DNA Polymerase (Stratagene). Primers contained restriction sites for Eco*RI* or Bam*HI* (primer sequences available upon request) and PCR products were ligated into pCDH-MCS1-EF1-Puro (System Biosciences). Infectious lentiviral particles were prepared using ViraPower Lentiviral Packaging Mix (Invitrogen) as per the manufacturer's protocol. Viral titer was also calculated as per the manufacturer's protocol, and cells were transduced with ~1 MOI. For stable transduction, transduced cells were cultured in 4 μg/mL puromycin until nontransduced cells were all killed.

Pre-miR transfection and proliferation assay. Cells seeded in either 24-well plates or eight-well chamber slides were transfected with 33 or 66 nmol/L Pre-miR miRNA precursor molecules (Ambion) using Lipofect-AMINE2000 (Invitrogen), or transduced with lentivirus encoding *mir-34b* and/or *mir-34c* and assayed for proliferation status 48 h later. Proliferation was quantified by bromodeoxyuridine (BrdUrd) incorporation assay as described previously (18). For estimation of proliferative indices, three representative images were collected per well using a SPOT-RT digital camera (Diagnostic Instruments, Inc.).

Soft agar assay. Soft agar assay was done essentially as described (19). Briefly, 6-cm plates were covered in a Nobel agar base layer (0.5% agar, 10% FBS, and 0.2% tryptone in DMEM). A top layer containing 5×10^5 cells to be assayed was suspended in DMEM containing 10% FBS, 0.2% tryptone, and 0.4% Nobel agar and pipetted on top of the base layer. After 3 days, growth medium was added to prevent the gel from drying.

Statistical analyses. For statistical testing, two-sided unpaired Student's *t* tests were done using InStat 3.05 and Prism 4.03 software (GraphPad, Inc.).

Results and Discussion

***p53* inactivation results in miRNAome alteration.** To show that p53 either directly or indirectly regulates a subset of miRNAs, we did miRNA microarray profiling of mouse ovarian surface epithelial cells subjected to the acute inactivation of *p53* (Fig. 1*A*). A total of 84 miRNAs were significantly overexpressed or underexpressed (Fig. 1*B*), whereas the majority of miRNAs were unchanged or not expressed in either wild type or mutant, in agreement with previous data demonstrating spatiotemporal-specific expression of a high percentage of miRNAs (20). The three most down-regulated miRNAs were the *miR-34* family, which consists of *miR-34a, miR-34b*, and *mir-34c. miR-34a* is located at mouse chromosome 4qE2, whereas *miR-34b* and *mir-34c* are located 435bp apart on chromosome 9qA5 and seem to be coordinately expressed as a miRNA cluster. To confirm the microarray data (Fig. 1*C*), we did quantitative RT-PCR. Using stem-loop

primers, we were able to specifically amplify mature miRNA molecules and confirm ~12-fold down-regulation of both *miR-34b* and *miR-34c* (Fig. 1*D*).

Identification of a p53-responsive element upstream of the *mir-34b/mir-34c* locus. To identify candidate p53-regulated miRNAs, we conducted an *in silico* screen for p53 responsive elements (p53RE). We focused our attention on down-regulated miRNAs, because the p53 activation consensus sequence is well defined, whereas in contrast, the repression sequence is less so. In this respect, loss of p53-mediated transcriptional activation after *p53* inactivation will lead to a decrease in expression of the target gene (21). Towards this aim, we took advantage of the p53MH algorithm (17), which has previously identified novel p53-responsive genes (22), to search 5 kb upstream and downstream of each down-regulated miRNA. Predicted p53 binding sites were identified upstream of nine miRNAs. Given that p53 is evolutionarily conserved, noteworthy p53 binding may also be expected to be evolutionarily conserved. Therefore, we also searched the corresponding human miRNA locus for predicted binding sites. Three binding sites were conserved between human and mouse: *mir-129, mir-34b*, and *mir-34c.* We decided to direct our attention toward *miR-34b* and *miR-34c* (Fig. 2*A*), given that we observed a far greater reduction in expression of these two genes compared with *miR-129* (not shown). In addition to the conserved p53RE upstream of the miRNA locus, both *miR-34b* and *miR-34c* are remarkably well conserved between species (Fig. 2*B*), suggesting that these miRNAs have critical roles in animals. Finally, to show that expression of *miR-34b/miR-34c* is lost in p53-deficient human cancer cells, we did quantitative RT-PCR on RNA isolated from briefly cultured wild-type human OSE cells and the *p53*-null cell line SKOV-3, which was derived from adenocarcinoma of the ovary. In good agreement with *miR-34b/miR-34c* expression in our mouse model, both miRNAs were dramatically reduced in the *p53*-null cells (Fig. 2*C*).

We next decided to establish if p53 activation induces expression of *miR-34b/miR-34c*. Doxorubicin leads to DNA strand breaks and a physiologic increase in p53 protein, partly through its stabilization by posttranslational modifications via the DNA damage pathway. We therefore treated cells with 0.5 μg/mL doxorubicin. Whereas a 4-fold increase in *miR-34b/miR-34c* expression was observed by 12 h of doxorubicin exposure in wild-type OSE cells, no such increase was observed in *p53* mutant OSE cells (Fig. 2*D*), consistent with computational analysis predicting that these miRNAs contain a p53RE and are therefore p53 responsive.

***miR-34b* and *miR-34c* cooperate in reducing proliferation and adhesion-independent growth.** To characterize the roles of these p53-dependent miRNAs, we transfected OSN1 or OSN2 cells

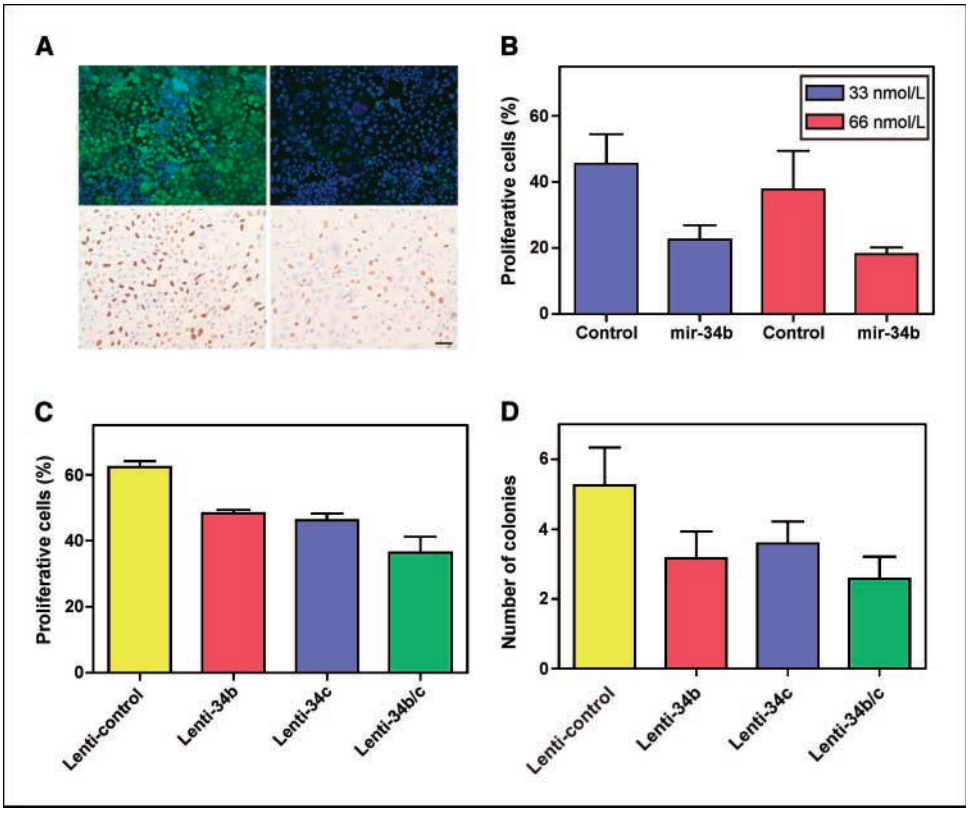

Figure 3. *miR-34b* and *miR-34c* cooperate in decreasing proliferation and anchorage-independent growth. *A,* GFP-expressing OSN2 cells were either untreated (*top left*) or transfected with 33 nmol/L small interfering RNA directed against GFP (*top right*), demonstrating efficient knockdown. Transfection of OSN2 cells with 33 nmol/L nontargeting synthetic miRNA molecules (*bottom left*) or synthetic *miR-34b* (*bottom right*) followed by BrdUrd administration. A significant decrease in cell proliferation as determined by BrdUrd incorporation is observed 48 h after *miR-34b* transfection. *B,* quantitative assessment of cell proliferation after transfection with 33 or 66 nmol/L synthetic *miR-34b* shows significantly reduced percentage of BrdUrd incorporating cells compared with nontargeting control molecule (33 nmol/L, *miR-34b* versus control, $P = 0.0042$; 66 nmol/L, *miR-34b* versus control, $P = 0.0213$). *C*, quantitative assessment of OSN1 cell proliferation after transduction with either control (*blank*) lentivirus or lentivirus encoding for *mir-34b* and/or *mir-34c*. A significant reduction in proliferating cells was observed for each treatment compared with control lentivirus (lenti-control versus lenti-34b, $P = 0.0025$; lenti-34c, $P = 0.0041$; lenti-34b/c, $P = 0.0074$). *D,* quantitative assessment of soft-agar colony formation by OSN1 cells transduced with lenti-34b/lenti-34bc shows a significant decrease in colony formation in soft agar (lenti-control versus lenti-34b/lenti-34c, $P = 0.0214$). *A,* GFP fluorescence with 4′,6-diamidino-2-phenylindole (DAPI) counterstain (*top*), BrdUrd, ABC Elite method, and hematoxylin counterstaining (*bottom*). Bar, 50 μm (*A*).

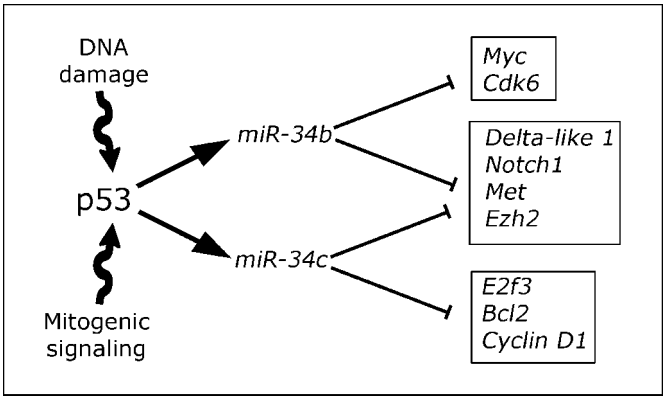

Figure 4. A model for p53-dependent miRNA-mediated repression of gene expression. p53 activation by DNA damage and possibly other stimuli, such as mitogenic signaling, lead to a rapid increase in expression of the p53-dependent miRNAs *miR-34b* and *miR-34c*. These miRNAs are predicted to bind a large number of target mRNAs; a selection of these targets are shown. Predicted targets of each miRNA are both independent (*top and bottom boxes*) and conserved (*middle box*).

with synthetic miRNAs for *miR-34b*. At 48 h posttransfection, proliferation index was determined by BrdUrd incorporation. A visible reduction in proliferation was observed upon *miR-34b* transfection compared with a nontargeting negative control molecule in OSN1 cells (Fig. 3*A*), which was also somewhat dose dependent (Fig. 3*B*).

To generate cell lines with stable integrations of *miR-34b* and/or *miR-34c*, we cloned the miRNA and surrounding genomic sequence into a lentivirus vector. Quantitative RT-PCR of *miR-34b* and *miR-34c* in cells after puromycin selection showed an increase in miRNA expression (Supplementary Fig. S1). Stably transduced OSN2 cells showed a significant reduction in proliferation (Fig. 3*C*). Interestingly, whereas transduction of either *lenti-34b* or *lenti-34c* individually reduced percentage of proliferating cells (mean $\pm$ SD, 48.4 $\pm$ 1.8%, $P = 0.0025$ and 46.2 $\pm$ 3.6%, $P = 0.0041$, respectively, compared with 62.4 $\pm$ 3.1% for blank virus), transduction of both miRNAs reduced proliferation to an even greater extent (36.3 $\pm$ 8.4%, $P = 0.0074$). Furthermore, an identical relationship was observed when transduced OSN1 cells were cultured in soft agar (Fig. 3*D*). Transduction of *lenti-34b* and *lenti-34c* reduced the number of colonies per 4$\times$ field of view from 5.3 $\pm$ 1.1 for cells transduced with blank virus to 3.2 $\pm$ 0.8 ($P = 0.0534$) and 3.6 $\pm$ 0.6 ($P = 0.0835$), respectively, whereas statistically significant reduction was observed in *lenti-34b/lenti-34c*-transduced cells (2.9 $\pm$ 0.6 colonies, $P = 0.0214$). These data suggest that whereas the sequences and, therefore, predicted targets of *mir-34b/mir-34c* are very similar, their differences have a significant consequence on biological activity and that maximal suppression of proliferation and anchorage-independent growth is achieved only when both miRNAs are expressed. In particular, *Delta-like 1, Notch1, Met*, and *Ezh2* are all predicted targets for both *miR-34b* and *miR-34c*. In contrast, *Myc* and *Cdk6* are among predicted targets for *miR-34b* and *E2f3, Bcl2*, and *Cyclin D1* among predicted targets for *miR-34c* (23).

A model for p53-dependent miRNA-mediated gene silencing. Taken together, our data illustrate a novel mechanism for p53-mediated control of gene expression. As outlined in Fig. 4, p53 is activated by DNA damage and directly induces expression *miR-34b* and *miR-34c* through a p53RE $\sim$3 kb upstream of the coding sequence. This activation, in turn, leads to repression of target genes. Although these two miRNAs share significant sequence similarity, their predicted targets are not perfectly conserved, thereby explaining cooperative effects of *miR-34b* and *miR-34c*. It should be noted that different stimuli, such as inappropriate mitogenic signaling, hypoxia, spindle damage, etc., may result in disparate consequences as a result of p53 binding to different subsets of its target genes (11). Whether *miR-34b* and *miR-34c* may have different extent of p53-dependent activation by other stimuli remains to be determined. The discovery of inhibitory effects of these miRNAs on such critical components of neoplastic growth as cell proliferation and adhesion-independent colony formation opens an exciting opportunity for development of novel therapeutic approaches using these small molecules.

After submission of our paper, He et al. reported p53-dependent regulation of *miR-34b/miR-34c* in mouse embryonic fibroblasts and IMR90 fibroblasts and showed reduction of cell growth and induction of senescence after ectopic expression of *miR-34b/miR-34c* in IMR90 fibroblasts (24). Taken together with our observations of *miR-34b/miR-34c* effects on cell proliferation and adhesion-independent growth of OSE, these results indicate that *miR-34b/miR-34c* play important roles in controlling carcinogenesis in various cell types, which are likely due to the diversity of their mRNA targets.

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