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 non-coding 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 epithelial 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 non-coding RNAs, which control gene expression either by degradation of target miRNAs 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 LATS2 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 miRNA 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′-PuPuPuCA(T/A)/T/A/AGPyPyPy-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 p53loxP/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%...
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 AdCre or blank adenovirus in serum-free medium for 2 h at 37°C/5% CO2 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^ΔΔCt method (16).
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 EcoRI or BamHI (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-miRNA 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 LipofectAMINE2000 (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. 1A). A total of 84 miRNAs were significantly overexpressed or underexpressed (Fig. 1B), 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. 1C), 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. 1D).

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. 2A), 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. 2B), 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. 2C).

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. 2D), 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 transfection 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 transfected 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).
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. 3A), which was also somewhat dose dependent (Fig. 3B).

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. 3C). Interestingly, whereas transduction of either lenti-miR-34b or lenti-miR-34c individually reduced percentage of proliferating cells (mean ± SD, 48.4 ± 1.8%, P = 0.0025 and 46.2 ± 3.6%, P = 0.0041, respectively, compared with 62.4 ± 3.1% for blank virus), transduction of both miRNAs reduced proliferation to an even greater extent (36.3 ± 8.4%, P = 0.0074). Furthermore, an identical relationship was observed when transduced OSN1 cells were cultured in soft agar (Fig. 3D). Transduction of lenti-34b and lenti-34c reduced the number of colonies per 4× field of view from 5.3 ± 1.1 for cells transduced with blank virus to 3.2 ± 0.8 (P = 0.0534) and 3.6 ± 0.6 (P = 0.0835), respectively, whereas statistically significant reduction was observed in lenti-34b/lenti-34c-transduced cells (2.9 ± 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. ~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 miRNA targets.

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).

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