

miR-34a repression of SIRT1 regulates apoptosis

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MicroRNA 34a (miR-34a) is a tumor suppressor gene, but how it regulates cell proliferation is not completely understood. We now show that the microRNA miR-34a regulates silent information regulator 1 (SIRT1) expression. MiR-34a inhibits SIRT1 expression through a miR-34a-binding site within the 3' UTR of SIRT1. MiR-34 inhibition of SIRT1 leads to an increase in acetylated p53 and expression of p21 and PUMA, transcriptional targets of p53 that regulate the cell cycle and apoptosis, respectively. Furthermore, miR-34 suppression of SIRT1 ultimately leads to apoptosis in WT human colon cancer cells but not in human colon cancer cells lacking p53. Finally, miR-34a itself is a transcriptional target of p53, suggesting a positive feedback loop between p53 and miR-34a. Thus, miR-34a functions as a tumor suppressor, in part, through a SIRT1-p53 pathway.

microRNA | p53 | cancer

Deleletion of chromosome region 1p36 is associated with a variety of cancers including colon cancer (1–12). Transfection of a normal chromosome 1p36 region into human colon cancer cells decreases tumorigenicity, suggesting the presence of a tumor suppressor in this region (13). No conventional tumor suppressor genes have been identified within chromosome region 1p36. However, miR-34a is encoded within chromosome 1p36.23. Recent studies suggest that miRNA-34a may act as a tumor suppressor by regulating apoptosis in neuroblastoma cells and pancreatic cancer cells (14, 15). However, the mechanism by which miRNA-34a regulates cell proliferation is unclear.

MicroRNAs (miRNAs) are short 18–24 nt RNA that inhibit the transcription or translation of mRNA (16–20). MiRNA regulation of gene expression plays a role in development, differentiation, proliferation, and apoptosis (21–26). Expression of miRNAs are abnormal in a variety of cancers, and miRNA may play a role in tumorigenesis (27–34). Several miRNA have been identified that may act as tumor suppressor genes (35–40).

The tumor suppressor gene TP53 may serve as a link between miRNA and tumorigenesis. The p53 protein is a transcription factor that normally inhibits cell proliferation and stimulates cell death. However, disruption of the p53 pathway can promote tumorigenesis. One pathway through which p53 regulates cell growth is through miRNA. Cellular stress stabilizes p53 that in turn regulates the expression of a set of miRNA (15, 41–44). These miRNA in turn may control apoptosis and senescence. Recent reports show that one of the miRNA activated by p53 is miR-34a (15, 41–44).

We searched for downstream targets of miR-34a that might mediate p53 suppression of cell proliferation and found that miR-34a regulates silent information regulator 1 (SIRT1) expression. SIRT1 is an NAD-dependent deacetylase that regulates apoptosis in response to oxidative and genotoxic stress (45, 46). Recent data suggests that SIRT1 may function as an oncogene and play a role in tumorigenesis (47–50). Because SIRT1 is believed to regulate apoptotic thresholds by deacetylating molecular targets that include p53 (51–54), miR-34a might regulate cellular growth through a SIRT1-p53 pathway.

Results

We hypothesized that miR-34a regulates the cell cycle by inhibiting SIRT1 expression based on an *in silico* analysis showing a

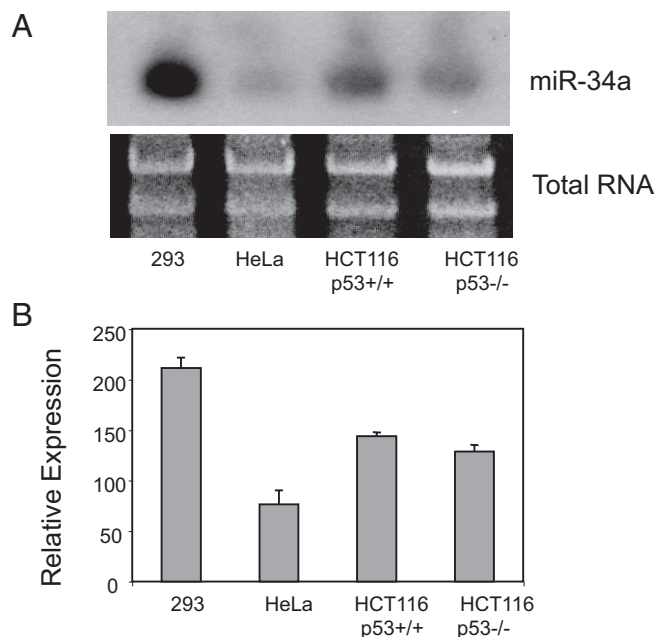


Fig. 1. Expression of miR-34a. (A) Human cell lines, including human colon cancer (HCT116) cells by Northern blot (repeated three times with similar results). (B) Densitometric quantification of Northern analysis of miR-34a repeated three times. Both p53^{+/+} and p53^{-/-} HCT116 cells have similar amounts of miR-34a, in the absence of genotoxic damage.

potential response element for miR-34a in the 3' UTR of SIRT1. We first measured the distribution of miR-34a in human tissue. Northern analysis shows that miR-34a is expressed in ovary, testis, bladder, lung, and other human tissues [supporting information (SI) Fig. S1]. MiR-34a is also expressed in gastrointestinal tissues and in the human colon cancer cell line HCT116 (Fig. 1). The HCT116 with WT p53 [HCT116(WT)] appear to express miR-34a, and HCT116(p53^{-/-}) cells lacking p53 express similar levels (Fig. 1).

To test the hypothesis that miR-34a decreases SIRT1 expression, we transfected HCT116 cells with the precursor to miR-34a (designated premiR-34a). As a control, some cells were transfected with precursor to other miR or with scrambled miRNA. The precursor to miR-34a decreases SIRT1 protein levels in a dose-dependent manner (Fig. 2A and B). Conversely, antisense miR-34a increases SIRT1 protein levels (Fig. 2C and D). Thus, miR-34a regulates SIRT1 expression.

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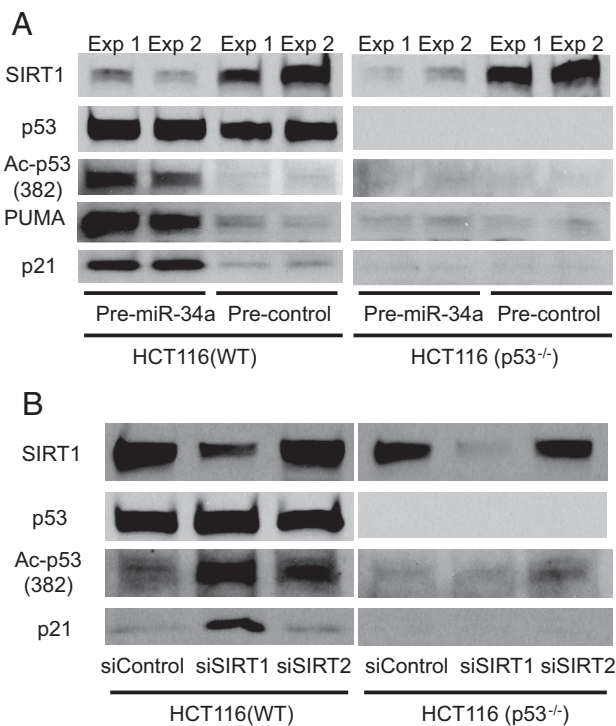


Fig. 4. miR-34a increases acetylation of p53 by decreasing SIRT1 expression. (A) miR-34 decreases SIRT1, increases acetylated p53, and increases two transcriptional targets of p53. In duplicate experiments, HCT(WT) and HCT(p53^{-/-}) were transfected with precursors to microRNA and incubated for 36 h. SIRT1, acetylated p53, PUMA, and p21 were measured by immunoblotting. These experiments were performed in duplicate. (B) SIRT1 regulates acetylation of p53. HCT(WT) and HCT(p53^{-/-}) were transfected with siRNA for SIRT1 or SIRT2, incubated for 36 h, and lysates were immunoblotted. Knockdown of SIRT1 permits an increase in p53 acetylation and an increase in p21, a transcriptional target of p53.

contrast, premiR-34a has no effect on p21 in HCT116 cells lacking p53. These data suggest that miR-34a increases activation of the p53 signaling pathway. Although miR-34a might have additional targets, these experiments taken together suggest that miR-34a indirectly regulates p53 through SIRT1.

To confirm reports that SIRT1 regulates acetylation of p53, we transfected HCT cells with siRNA to knockdown SIRT1 (or knockdown SIRT2 as a control) and then measured acetylated p53. Knockdown of SIRT1 increases acetylation of p53 and activates p21 expression (Fig. 4B).

To explore the functional effect of miR-34a on cell survival, we measured apoptosis in cells transfected with premiR-34a. PremiR-34a increases cell death, as assessed by cell morphology (Fig. 5A). Overexpression of miR-34a increases apoptosis, as measured by FACS analysis of cells for annexin V and propidium iodide staining (Fig. 5B and C). However, miR-34a does not activate apoptosis in cells lacking p53. These data suggest that miR-34a activates apoptosis in a p53-dependent pathway.

To show that SIRT1 mediates miR-34a activation of p53 and apoptosis, we rescued cells with a vector expressing SIRT1. We transfected HCT116 cells with premiR-34a, a vector overexpressing SIRT1, and then measured apoptosis. As before, expression of miR-34a increases apoptosis (Fig. 5D). However, expression of SIRT1 partially blocks apoptosis induced by miR-34a. These data suggest that SIRT1 mediates miR-34a activation of cell death.

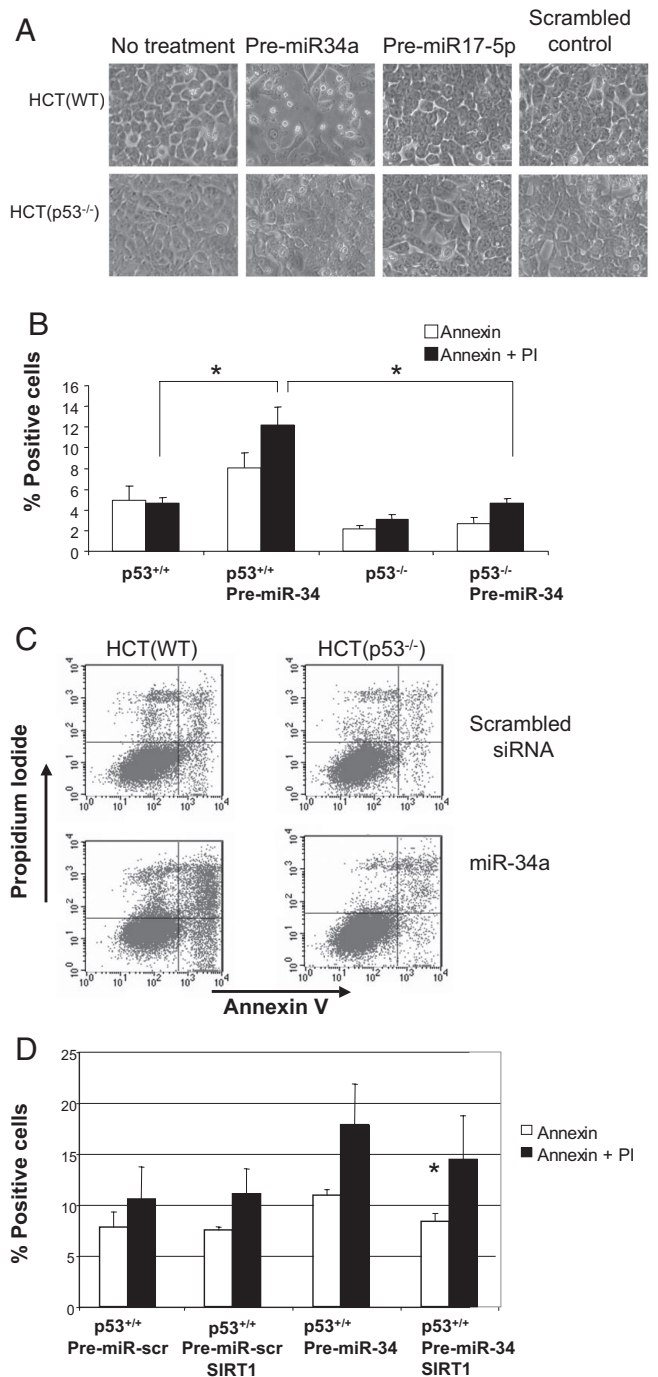


Fig. 5. miR-34 activates apoptosis. (A) p53 mediates miR-34 induced apoptosis. HCT(WT) and HCT(p53^{-/-}) cells were transfected with premiR-34 or control and then photographed. Overexpression of miR-34a causes cells to detach and to round up but only in cells expressing p53. (B) HCT(WT) and HCT(p53^{-/-}) cells were treated as above, and apoptosis was measured by Annexin V and propidium iodide staining. (C) HCT(WT) and HCT(p53^{-/-}) cells were treated as above, and apoptosis was measured by FACS ($n = 3 \pm$ SD; *, $P = 0.003$). Overexpression of miR-34a induces apoptosis but only in the presence of p53. (D) SIRT1 rescues cells from apoptosis after overexpression of miR-34. HCT(WT) cells were transfected with premiR-34 or control and with a vector expressing SIRT1 or control, and apoptosis was measured by FACS ($n = 3 \pm$ SD; *, $P = 0.01$ vs. PremiR-34 without SIRT1). Overexpression of SIRT1 blocks apoptosis induced by miR-34a.

We next explored the effect of endogenous miR-34a on SIRT1. We transfected HCT116 cells with antisense oligonucleotides to miR-34a, treated them with 5-FU to activate p53, and

SIRT1, permitting an increase in p53 activation which induces still more miR-34a. To search for p53 regulation of miR-34a, we activated p53 in HCT cells by using various agents that damage DNA (Fig. 7A and B). We found that genotoxic agents increase miR-34a levels but only in HCT WT cells (Fig. 7C). Genotoxic agents fail to alter miR-34a levels in HCT cells lacking p53 (Fig. 7C). Furthermore, genotoxic agents that can induce miR-34a (Fig. 7C) could also decrease SIRT1 (Fig. 7D). However, in cells lacking p53, genotoxic agents did not induce miR-34a (Fig. 7C) and did not suppress SIRT1 (Fig. 7D).

Discussion

The major finding of this study is that miR-34a inhibits SIRT1 expression, thereby triggering pathways downstream of SIRT1. Our study extends the results of others showing that SIRT1 deacetylates p53, limiting the ability of p53 to transactivate its target genes (51, 52, 57). We and others have shown that p53 regulates miR-34a (15, 41–44), and our study extends these results by suggesting the existence of a positive feedback loop in which p53 induces miR-34a, which through SIRT1, increases p53 activity. These results suggest that miR-34a can function as a tumor suppressor gene (Fig. S1). These data also provide one mechanism by which miR-34a can regulate cell proliferation.

MiR-34a may have other targets besides SIRT1 that can regulate cell survival (15). For example, miR-34a can decrease expression of the transcription factor E2F3, which regulates cell-cycle progression (14). Thus, SIRT1 may be one of several distinct targets of miR-34a that contribute to its ability to promote apoptosis.

Although HCT cells contain miR-34a, they still express SIRT1, a target of miR-34a. The miR-34a does not suppress all SIRT1 expression in resting cells (Fig. 2). Overexpression of miR-34a does not completely suppress SIRT1 translation (Fig. 2A). Furthermore, overexpression of miR-34a decreases expression of a luciferase reporter gene by $\approx 50\%$ but does not completely eliminate luciferase activity (Fig. 3B). Perhaps intracellular levels of miR-34a do not saturate its binding site in the SIRT1 mRNA, so that some SIRT1 mRNA are still translated. Another possibility is that even though miR-34a concentrations exceed concentrations of SIRT1 mRNA, because miR-34a does not exactly match its SIRT1 binding site (Fig. 3A), perhaps every SIRT1 binding site does not interact with miR-34a.

Our data suggest that SIRT1 mediates, in part, the tumor suppressor activity of miR-34a. Several lines of evidence implicate SIRT1 in tumorigenesis. First, SIRT1 is overexpressed in human

colon cancer, breast cancer, prostate cancer, squamous cell carcinoma, and human non-small-cell lung cancer cell lines (47–50). Next, by deacetylating p53, SIRT1 decreases the ability of p53 to promote cell cycle arrest (51, 52). Increased expression of SIRT1 in normal or cancer cells inactivates p53, decreasing cellular apoptosis (58). Finally, small molecules that inhibit SIRT1 increase acetylated p53 and activate apoptosis of breast cancer and lymphoma cells (59, 60). Accumulating data thus suggest that SIRT1 activity may increase the risk of cancer. Our findings suggest that miR-34a may act as a tumor suppressor by blocking SIRT1, thereby permitting increased p53 activity (Fig. S2).

Materials and Methods

Cell Culture, Northern Blot analysis, and Western Blot analysis. (See *S1 Text*).

Transfection. Precursor miRNAs were obtained from Applied Biosystems. Small interfering RNA, siSIRT1, and scrambled si-oligonucleotide were obtained from Dharmacon. For precursor miRNA, cells were transfected with siPORT NeoFX (Applied Biosystems) with precursor miRNA 0–20 nM or with antisense miRNA 0–20 nM and harvested 48 or 72 h later. To silence SIRT1 expression, cells were transfected with Lipofectamine 2000 (Invitrogen) and SIRT1 antisense oligonucleotides 0–10 nM.

Plasmid Construction. The 3' UTR of SIRT1 (1420 bp) containing the SIRT1-miR-34a response element was cloned into the *PmeI/SacI* site of pMIR-REPORT Luciferase vector (Applied Biosystems). A mutant 3' UTR of SIRT1 was synthesized by PCR, whose sequence contained 5'-ACACCCACCAAGACCATTAGTC-CGAGA-3' (the five italic nucleotides are mutated).

Luciferase Assays. HCT cells were plated at 5×10^4 cells per well in 24-well plates. The next day, 200 ng pMIR-REPORT Luciferase vector, including 3' UTR of SIRT1 (with the WT or mutant miR-34a response element) and precursor miR-34a or scrambled oligonucleotide, were transfected by using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed by using the dual luciferase reporter assay system (Promega) 48 h after transfection.

Apoptosis Analysis. HCT116 cells were transfected with precursor miR-34a or scrambled oligonucleotides as a control. After an additional incubation of 72 h, the cells were harvested, stained with propidium iodide and anti-annexin-V antibody, and analyzed by FACS.

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