

TP53-independent Function of miR-34a via HDAC1 and p21^{CIP1/WAF1}

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The tumor suppressor, microRNA-34 (miR-34), a transcriptional target of TP53, functions in a positive feedback loop to activate TP53. Although miR-34 can inhibit cancer cells carrying TP53 mutations, this feedback to TP53 may be a prerequisite for full miR-34 function and may restrict its therapeutic application to patients with intact TP53. To investigate the functional relationships between TP53 and miR-34, and that of other TP53-regulated miRNAs including miR-215/192, we have used a panel of isogenic cancer cell lines that differ only with respect to their endogenous TP53 status. miR-34-induced inhibition of cancer cell growth is the same in TP53-positive and TP53-negative cells. In contrast, miR-215/192 functions through TP53. In the absence of TP53, miR-34, but not miR-215/192, is sufficient to induce an upregulation of the cell cycle-dependent kinase inhibitor p21^{CIP1/WAF1}. We identify histone deacetylase 1 (HDAC1) as a direct target of miR-34 and demonstrate that repression of HDAC1 leads to an induction of p21^{CIP1/WAF1} and mimics the miR-34 cellular phenotype. Depletion of p21^{CIP1/WAF1} specifically interferes with the ability of miR-34 to inhibit cancer cell proliferation. The data suggest that miR-34 controls a tumor suppressor pathway previously reserved for TP53 and provides an attractive therapeutic strategy for cancer patients irrespective of TP53 status.

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INTRODUCTION

MicroRNA-34 (miR-34) is a potent tumor suppressor that shows a loss of function in many solid and hematological cancer types.^{1–4} It inhibits a broad range of cancer cells, presumably by repressing a plethora of oncogenes that control proliferation, senescence, apoptosis, and metastasis.^{5,6} miR-34 can also interfere with the growth of cancer stem cells,^{7,8} providing a strong rationale for the development of a miR-34 therapy. Evidence for the therapeutic application of miR-34 has been generated in murine tumor models of lung, liver, prostate, and lymphoma that showed robust tumor inhibition in response to the systemic delivery of nanoparticles loaded with synthetic miR-34 mimics.^{6,8–11}

Much insight into the role of miR-34 has been added by recent reports demonstrating that the tumor suppressor TP53 (p53) transcriptionally induces the expression of all three miR-34 family members – miR-34a/b/c.^{12–16} TP53 also elevates the endogenous levels of miR-215, miR-192, and miR-194, all of which have the ability to inhibit cancer cell growth in culture.^{17–19} Although miR-215 and miR-192 are encoded on separate genomic loci, they share identical seed sequences (90.5% overall sequence homology) and are collectively referred to as miR-215/192. For some miRNAs, the positive regulation between TP53 and miRNA is reciprocal – miR-215/192 stimulates TP53 activity by repressing *MDM2* (also referred to as *HDM2*), a ubiquitin ligase that negatively regulates TP53 stability via proteasomal degradation.^{19–21} Similarly, miR-34a activates TP53 in a positive feedback loop by repressing *SIRT1* (silent information regulator 1), a nicotinamide adenine dinucleotide-dependent deacetylase that deactivates TP53, *MDM4*, a *MDM2*-like protein that negatively regulates TP53 transactivation, and *YY1*, a transcription factor that binds to a subset of TP53 DNA binding sites.^{22–24}

While available data support the view that TP53 enhances the inhibitory activity of miR-215/192,¹⁹ a requirement for TP53 in miR-34-induced tumor suppression is controversial and the actual contribution of TP53 is unknown. Although previous studies suggest that miR-34 is also effective in cancer cells expressing mutated *TP53*,^{8,10,19} it is nevertheless possible that TP53 is a functional requirement for the full antiproliferative phenotype of miR-34. Given the high mutation rate of *TP53* in cancer, this prerequisite may substantially limit the application of a miR-34-based therapy to patients with intact TP53. Here, we investigated the role of TP53 under physiological conditions and directly addressed TP53-dependent and -independent effects by using a panel of isogenic cancer cell lines in which the two *TP53* alleles have been sequentially inactivated via targeted homologous recombination.^{25,26} We show that the miR-34-induced inhibition of cancer cell proliferation is the same in TP53-deficient and TP53 wild-type cells. These effects depend on the cell cycle-dependent kinase inhibitor p21^{CIP1/WAF1} (p21, *CDKN1A*) that is upregulated by a TP53-independent mechanism and involves the miR-34-mediated repression of histone deacetylase 1 (HDAC1). Therefore, p21 appears to be a critical effector molecule downstream of miR-34 and illustrates how miR-34 bypasses TP53 to function as a TP53-independent tumor suppressor.

RESULTS

Inhibition of cancer cell proliferation by miR-34 is independent of TP53

Isogenic cells used in this study were derived from the MCF10A breast cancer and the colorectal carcinoma cell lines SW48, HCT116, RKO, and DLD-1 (Supplementary Table S1). In these cells, TP53 is either wild-type (+/+), heterozygous (+/-), or homozygously inactivated (-/-).^{25,26} Parental DLD-1 cells (DLD-1^{S241/SIL}) do not express a functional TP53 protein due to the S241F/SIL TP53 genotype in which one allele is mutated and the other is epigenetically silenced. Therefore, DLD-1^{+/-SIL} cells, in which the point mutation has been corrected by site-directed mutagenesis, serves

as the DLD-1 reference line with intact TP53.²⁵ Each nonisogenic cell line displays mutations in other tumor suppressor genes and oncogenes which may influence the expression and function of miRNAs (Supplementary Table S1).

To confirm the serial inactivation of TP53 in isogenic cell lines, we induced a TP53 response by exposing the cells to the DNA-damaging agent etoposide for 28 hours and collected total RNA. A quantitative reverse transcription-PCR (qRT-PCR) analysis showed an allele-dependent increase in TP53 mRNA and TP53-regulated target genes according to their genotype (Supplementary Figure S1). TP53 mRNA was not detectable in TP53^{-/-} cells. Increased mRNA levels of TP53-regulated genes are

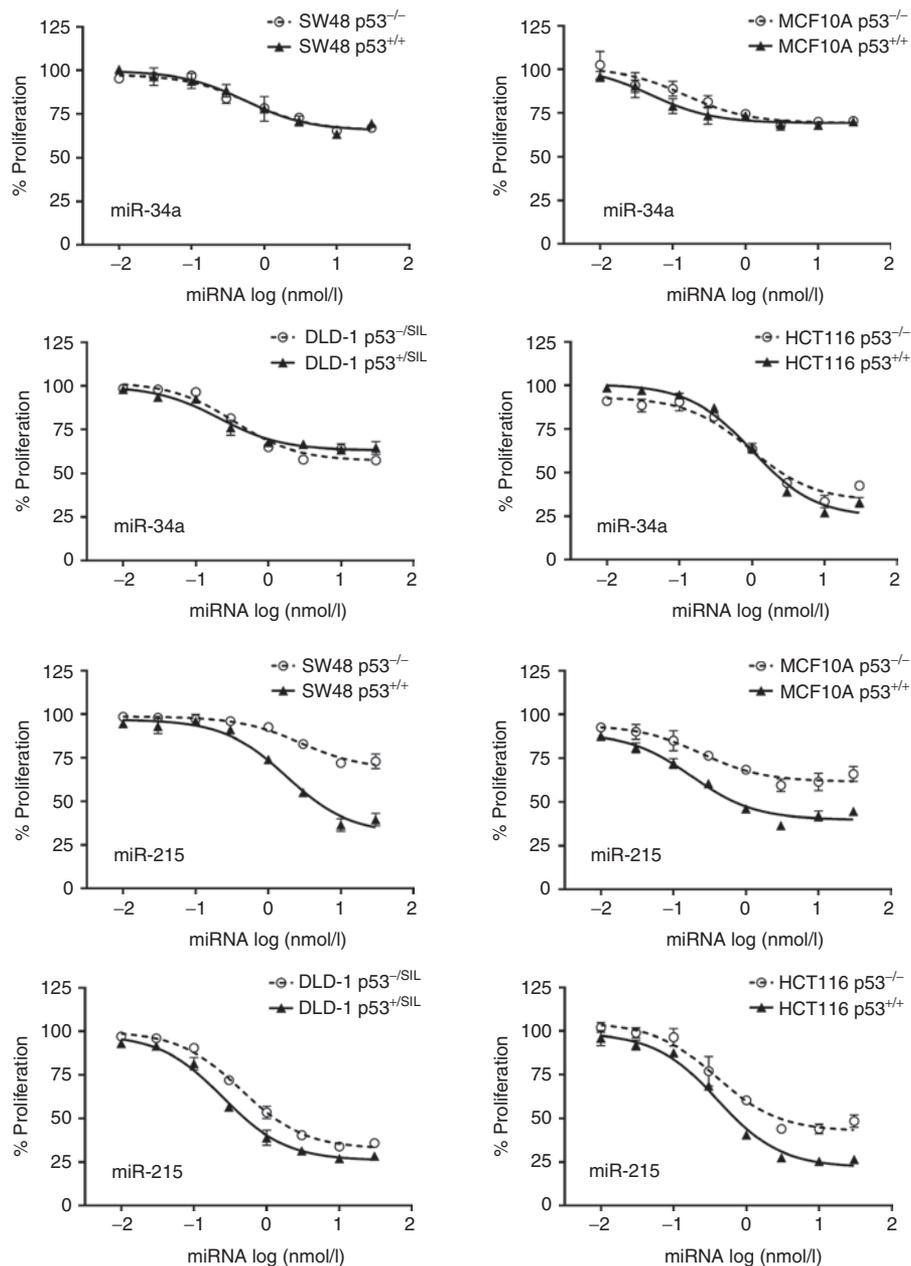


Figure 1 Inhibition of cancer cell proliferation by miRNAs in the presence or absence of TP53. TP53 genotype-dependent effects of miRNAs in isogenic cancer cell lines. Cells expressing (+/+; +/-SIL) or lacking functional (-/-; -/SIL) TP53 were transfected with increasing concentrations of miRNAs ranging from 0.01 to 30 nmol/l. After 3 days, cellular proliferation was determined. Data are normalized to mock-transfected cells. Averages, standard deviations, and nonlinear regression trendlines are shown.

similar to published data²⁷ and varied between cell lines, presumably due to cell-type-specific regulation of these genes. Likewise, the induction of TP53-regulated miRNAs, miR-34a/b/c, miR-192, miR-194, and miR-215 was dependent on the cell line – all cell lines but DLD-1^{+SIL} lacked miR-34b/c expression, and miR-215 was solely detectable in SW48 and DLD-1 cells.

Next, we transfected isogenic cells with mimics of miR-34a, miR-34c, miR-192, miR-194, and miR-215. The miRNAs were used in a serial dilution to generate dose–response curves and to calculate EC₅₀ values. As negative controls, mock-transfected cells and cells transfected with a miRNA carrying a scrambled sequence were used (miR-NC). After 3–4 days of incubation, cellular proliferation was assessed using AlamarBlue (Invitrogen, Carlsbad, CA). As shown in **Figure 1**, miRNAs mimics inhibited cellular proliferation by ~40–80% compared with controls. TP53 enhanced the ability of miR-215 and miR-192 to inhibit cancer cells which was greatest in MCF10A and SW48 cells with EC₅₀ values ~28- 35-fold lower compared with TP53^{-/-} cells (**Table 1**). In contrast, the inhibitory activity of miR-34a and miR-34c was the same in TP53-positive and TP53-negative cells (**Figure 1** and **Table 1**; **Supplementary Figure S2**). RKO cells showed greater inhibition in the absence of TP53, further demonstrating that TP53 is not a prerequisite for the miR-34–induced phenotype (**Supplementary Figure S3**). Interestingly, in the presence of intact TP53, the maximal inhibitory activity of miR-215/192 was greater than the maximal activity of miR-34a/c, suggesting that miR-215/192 functions in the TP53 positive feedback loop and takes advantage of ancillary pathways exclusively regulated by TP53.¹⁹

Table 1 EC₅₀ values of miRNAs in isogenic cancer cells

miRNA	SW48 p53 ^{-/-}	SW48 p53 ^{+/+}	EC ₅₀ ^{TP53^{-/-}} :EC ₅₀ ^{TP53^{+/+}}
miR-34a	0.59	0.62	1.0
miR-34c	0.38	0.40	0.9
miR-215	40.21	1.50	26.9
miR-192	21.56	0.76	28.3
	MCF10A p53 ^{-/-}	MCF10A p53 ^{+/+}	EC ₅₀ ^{TP53^{-/-}} :EC ₅₀ ^{TP53^{+/+}}
miR-34a	0.11	0.04	2.7
miR-34c	0.10	0.04	2.9
miR-215	5.42	0.17	31.8
miR-192	6.79	0.19	35.3
	DLD-1 p53 ^{-SIL}	DLD-1 p53 ^{+SIL}	EC ₅₀ ^{TP53^{-SIL}} :EC ₅₀ ^{TP53^{+SIL}}
miR-34a	0.39	0.39	1.0
miR-34c	1.34	0.38	3.6
miR-215	0.69	0.26	2.7
miR-192	0.63	0.22	2.9
	HCT116 p53 ^{-/-}	HCT116 p53 ^{+/+}	EC ₅₀ ^{TP53^{-/-}} :EC ₅₀ ^{TP53^{+/+}}
miR-34a	1.03	1.02	1.0
miR-34c	0.86	0.83	1.0
miR-215	1.07	0.31	3.5
miR-192	1.10	0.50	2.2

EC₅₀ values were generated with the Prism software and were within the 95% confidence interval of the trendline ($P < 0.05$). Values are expressed in nmol/l.

^aRatios indicate fold differences of EC₅₀ values in TP53-positive and TP53-negative cells. EC₅₀ values used here were defined as the half-maximal miRNA activity.

miR-34a induces p21^{CIP1/WAF1} in the absence of TP53

To understand the miR-34–induced phenotype in TP53-positive and TP53-deficient cells, we determined the expression levels of genes involved in the TP53/miR-34 axis. An explanation for the TP53-independent effects is the possibility that these cells do not express endogenous *SIRT1* or *MDM4*. However, as confirmed by qRT-PCR, both TP53^{+/+} and TP53^{-/-} cells carry detectable *SIRT1* and *MDM4* mRNA levels, suggesting that the TP53-independent phenotype is not due to an absence of these gene products (**Figure 2a**). Rather, both mRNAs were reduced in cells transfected with miR-34, in accordance with experimental data showing that *SIRT1* and *MDM4* are directly targeted by this miRNA.^{22,23} Similarly, MET, a miR-34a target, and BCL2, a miR-215 target, were specifically downregulated in cells transfected by the respective miRNAs (**Figure 2a**). TP53 mRNA levels were not detectable in SW48^{-/-} cells in accord with its defined genotype. In SW48^{+/+} cells, TP53 mRNA levels were constant and is in agreement with the hypothesis that the positive feedback loop to TP53 by these miRNAs does not require TP53 *de novo* synthesis but occurs post-transcriptionally. This is further corroborated by the observation showing that miR-215 induces the expression of p21^{CIP1/WAF1} (p21, CDKN1A) in TP53-positive cells, but fails to do so in TP53-deficient cells. Unexpectedly, miR-34a was able to induce p21 not only in TP53^{+/+} cells, but also in TP53-deficient cells (**Figure 2a,b**).

HDAC1 is a direct target of miR-34a

A plausible explanation for the TP53-independent upregulation of p21 is a potential involvement of other TP53 family members, TP63 and TP73. Both proteins play roles distinct from TP53; however, they also control a set of genes that overlaps with that of TP53.²⁸ To test this hypothesis, we measured endogenous mRNA levels of TP63 and TP73 in TP53 wild-type and TP53-deficient cells that had been transfected with miR-34a. However, none of the cells showed detectable mRNA levels of TP63 or TP73, suggesting that an involvement of these gene products is unlikely (data not shown).

Next, we focused on regulatory mechanisms that are independent of TP53 and searched for nuclear regulators that can control p21 expression. One candidate of interest was HDAC1 because it has a putative miR-34a binding site in its 3' untranslated region (UTR) that is conserved across mammals and also present in other vertebrates (**Figure 3a**, **Supplementary Figure S4**). HDAC1 is downregulated in cells transfected with miR-34a (**Figure 3b**), and has previously been implicated in the transcriptional regulation of p21 in the absence of TP53.²⁹ To establish whether HDAC1 is directly repressed by miR-34a, we examined whether miR-34a can repress a luciferase reporter that is fused to the entire HDAC1 3' UTR. We transiently expressed this reporter in SW48^{-/-} and H1299 cells that lack TP53 and express low levels of endogenous miR-34a (**Supplementary Figure S1**; ref. 10). Then, cells were transfected with miR-34a or miR-215, the latter of which is not predicted to repress HDAC1 and was used as a negative control. As shown in **Figure 3c**, transfection of miR-34a diminished luminescence by ~50% in both cell lines relative to controls. This repression was completely abolished upon mutation of the miR-34a binding site (**Figure 3c**), suggesting that the HDAC1 3' UTR is directly targeted by miR-34a at this site. To further evaluate if the miR-34a–dependent repression

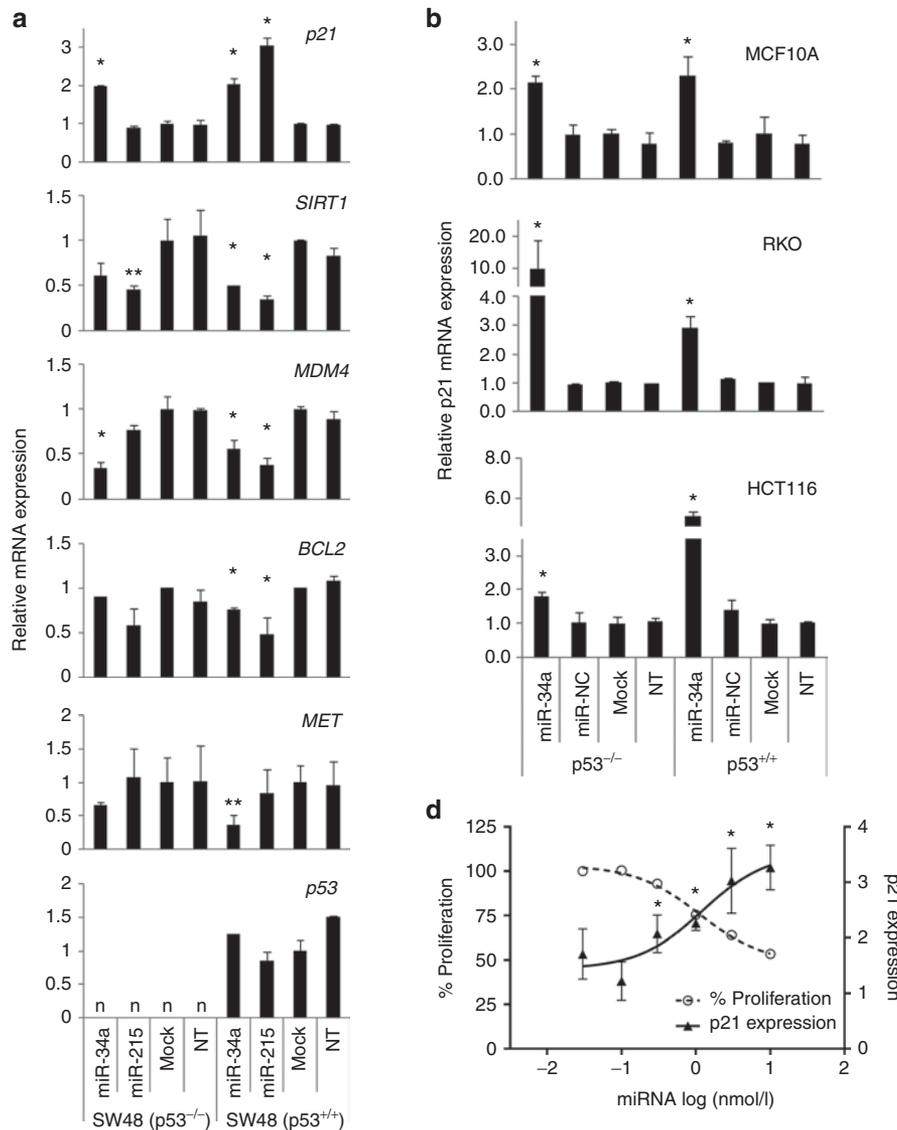


Figure 2 Induction of $p21^{CIP1/WAF1}$ by miR-34a in TP53-deficient cells. **(a)** Endogenous expression levels of target genes functioning in the miR-34a/TP53 axis were determined by quantitative reverse transcription-PCR (qRT-PCR) using RNA from SW48^{-/-} and SW48^{+/+} cells transfected with either miR-34a or miR-215. Statistical significance of differential gene expression was determined by Student's *t* test (**P* < 0.5; ***P* < 0.1; miRNA versus mock). **(b)** qRT-PCR results showing *p21* mRNA levels in isogenic cell lines transfected with miR-34a. **(c)** Nonlinear regression analysis of *p21* expression levels and proliferation rates in RKO^{-/-} cells transfected with increasing concentrations of miR-34a ranging from 0.01 to 30 nmol/l. All values are normalized to those in mock-transfected cells (=1). Averages are shown. Standard deviations are included but are too small to be visible in the graph. *Statistical significance of *p21* mRNA expression in miR-34a- versus miR-NC-transfected cells (Student's *t* test; *P* < 0.05). n, not detected.

of *HDAC1* is reflected in human tumor specimens, we examined a cohort of 14 non-small-cell lung cancer samples previously used to document reduced miR-34a expression levels.¹⁰ Tumor *HDAC1* mRNA and miR-34a levels were determined by qRT-PCR and normalized to the levels in their respective normal adjacent tissues. An analysis by the Pearson's method showed a statistically significant inverse correlation between *HDAC1* mRNA and miR-34a levels (Figure 3d), supporting a role for miR-34a in the regulation of *HDAC1* in human tumors.

Inhibition of *HDAC1* mimics the miR-34a phenotype

Previous results implicated *HDAC1* in the regulation of the *p21* gene. For instance, *HDAC1*-deficient embryonic stem cells show

elevated levels of *p21*, and inhibition of *HDAC1* using the *HDAC1* inhibitor trichostatin A can induce *p21* expression in the absence of TP53.^{30,31} To confirm the TP53-independent induction of *p21* upon depletion of *HDAC1*, we transfected TP53-negative cells with a small interfering RNA (siRNA) directed against *HDAC1* and evaluated cell lysates by Western blotting. The results were compared to cells transfected with miR-34a or miR-215. Two cell lines were tested and included mock- and miR-NC-treated cells as negative controls. As expected, *MET* was solely down-regulated in cells transfected with miR-34a, and *HDAC1* protein was reduced by both miR-34a and the *HDAC1* siRNA (Figure 4a; Supplementary Figure S5). Of note, both oligonucleotides induced a marked increase of *p21* protein expression in these

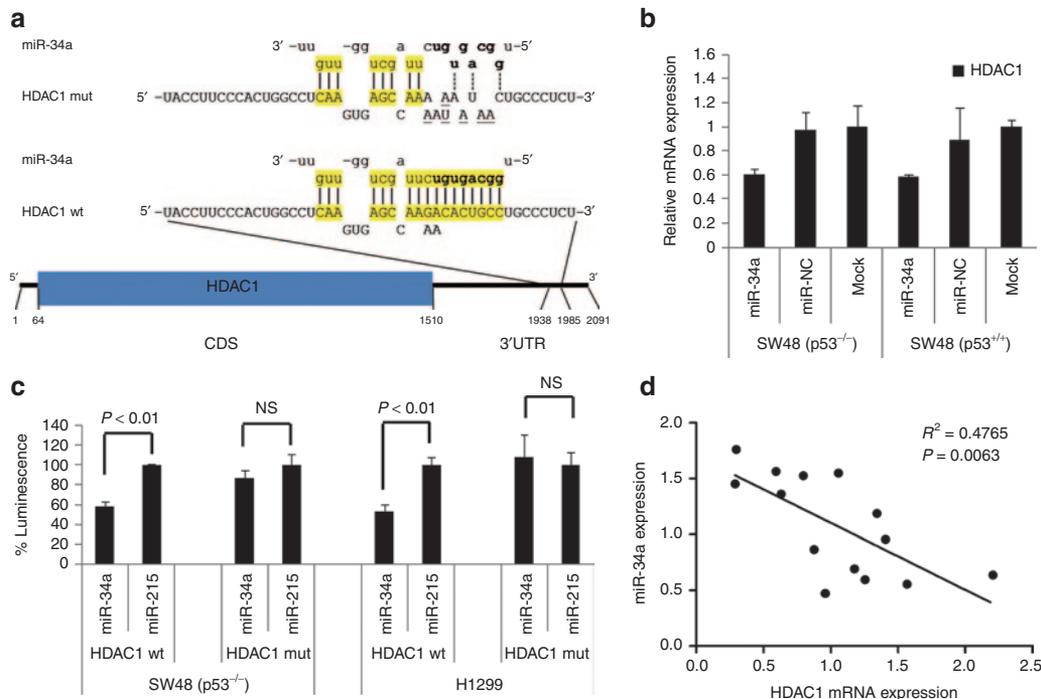


Figure 3 HDAC1 is a direct target of miR-34a. **(a)** miR-34a binding site in the 3' UTR of the *HDAC1* transcript. Base pairing of miR-34a with wt and mut *HDAC1* 3' UTR sequences is shown. Lower case, miR-34a residues; upper case, mRNA residues; yellow, bases presumably involved in base pairing; bold, miRNA seed sequence; underlined, mut residues. **(b)** Quantitative reverse transcription-PCR (qRT-PCR) analysis showing *HDAC1* mRNA levels in isogenic SW48 cells transfected with miR-34a. Values are normalized to those in mock-transfected cells. **(c)** miR-34a represses a luciferase transcript fused to the *HDAC1* 3' UTR in SW48^{-/-} colon cancer and H1299 lung cancer cells. Relative light units were normalized to those in miR-215-transfected cells (100%). *P* values were derived from two-tailed Student's *t* tests. **(d)** Inverse correlation of *HDAC1* mRNA and miR-34 levels in a set of 14 tumors from non-small-cell lung carcinoma patients. Endogenous expression levels were determined by qRT-PCR. Correlation coefficient was generated by the Pearson's method; the *P* value was calculated by *F* test (GraphPad). CDS, coding sequence; HDAC1, histone deacetylase 1; mut, mutated; NS, nonsignificant; UTR, untranslated region; wt, wild-type.

cells. This observation was in stark contrast to miR-215 that failed to induce p21 in TP53-deficient cells. However, transfection of miR-215 into TP53-positive cells led to an increase of p21 protein in TP53-positive cells (**Supplementary Figure S6**) in accord with the hypothesis that the miR-215-dependent induction of p21 is mediated by TP53 as a result of the positive feedback loop from miR-215 to TP53.¹⁹

To explore whether inhibition of HDAC1 can mimic the miR-34a phenotype, we measured the proliferation effects of an siRNA against *HDAC1* in both TP53-positive and TP53-deficient SW48 cells. Cells were also transfected with a series of other siRNAs directed against gene products that can antagonize TP53 function. These genes include *YY1*, *MDM4*, and *SIRT1*, as well as a few others that are either validated or predicted miR-34a targets and were repressed in miR-34a-transfected cells (data not shown). Transient transfection of siRNAs led to >80% knock-down of target mRNAs as confirmed by qRT-PCR (**Supplementary Figure S7**). As controls, cells were also transfected with miR-34a and miR-215. We sought to identify siRNAs that yield a level of cancer cell inhibition that is similar in both cell lines. As expected, miR-34a equally inhibited SW48^{+/+} and SW48^{-/-} cells, and the activity of miR-215 was dependent on TP53 (**Figure 4b**). Most siRNAs failed to reduce cellular proliferation in either cell type, including the siRNA against *SIRT1* and *YY1*. Knock-down of *MDM4* was able to inhibit proliferation of SW48^{+/+} cells but had no effect in SW48^{-/-} cells. This is reminiscent of the miR-215 phenotype

and confirms the role of MDM4 in modulating TP53 transactivation rather than DNA regulation.³² In contrast, knock-down of *HDAC1* inhibited cancer cell growth that – similarly to miR-34a – was the same in both isogenic cell lines. Similar results were obtained from cells treated with trichostatin A (**Supplementary Figure S8**) further corroborating a role for HDAC1 in mediating a miR-34a response through p21.

Depletion of p21^{CIP1/WAF1} interferes with miR-34a-induced inhibition of cancer cell proliferation

The dose-response data generated in various cell lines suggest that *p21* expression is a key event during miR-34a-induced inhibition of cancer cell proliferation. Expression levels of *p21* markedly correlated with the ability of miR-34a to inhibit TP53-positive and TP53-negative cells. For instance, the inhibitory activity of miR-34a was the same in MCF10A and SW48 cells and correlated with similar *p21* expression levels in both TP53^{-/-} and TP53^{+/+} cells (**Figures 1** and **2**). HCT116 cells displayed greater *p21* mRNA levels in TP53^{+/+} compared with TP53^{-/-} cells, in accord with the slightly increased inhibitory activity of miR-34a in TP53^{+/+} cells at higher miR-34a concentrations (30 nmol/l, **Figure 1**). In RKO cells, *p21* levels were higher in TP53^{-/-} cells and mirrored the greater inhibition of proliferation in RKO^{-/-} versus RKO^{+/+} cells (**Supplementary Figure S3**). The induction of *p21* was also evident at low miR-34a concentrations and inversely correlated with inhibition of cell proliferation (**Figure 2c**).

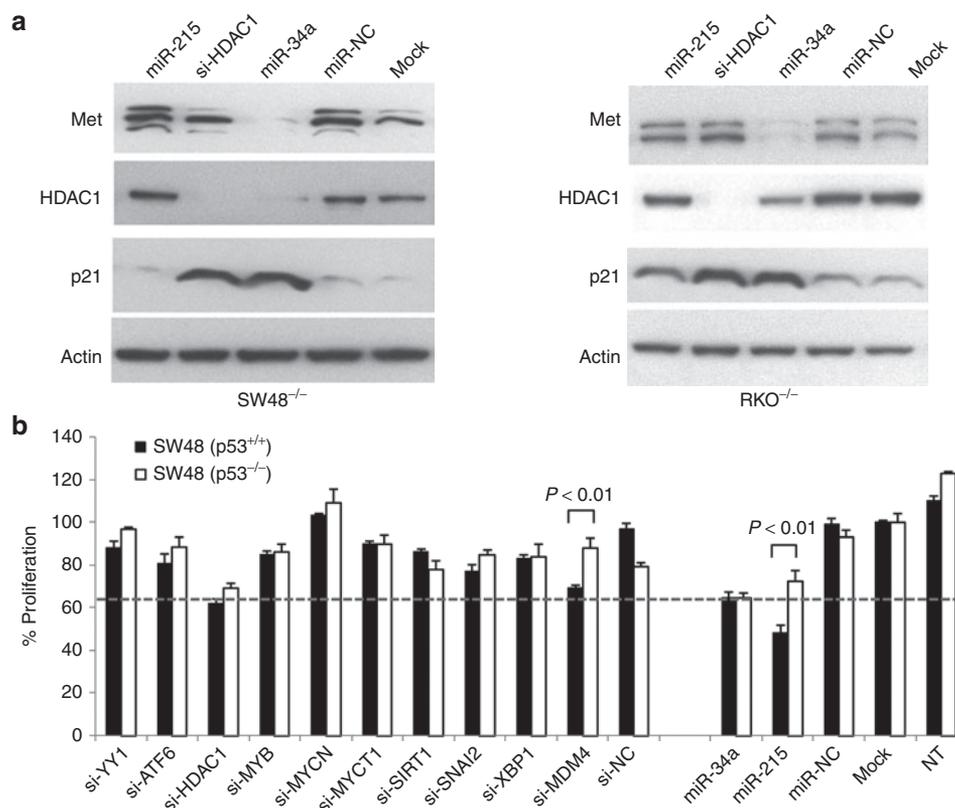


Figure 4 Knock-down of HDAC1 mimics the miR-34a phenotype. **(a)** Knock-down of HDAC1 by small interfering RNA (siRNA) and miR-34a induces $p21^{CIP1/WAF1}$ expression in TP53-negative cells. Protein lysates from cells transfected with miR-34a, miR-215, or si-HDAC1 were probed by Western blotting. Met was used as a positive control for miR-34a transfection; actin was used as a loading control. **(b)** Knock-down of HDAC1 by siRNA similarly inhibits cellular proliferation of SW48^{-/-} and SW48^{+/+} cells. Cells were transfected with siRNAs and miRNAs, and cellular proliferation was measured 3 days thereafter. Values are normalized to mock (=100%). Averages and standard deviations are shown. P values are derived from two-tailed Student's t tests. The dotted line denotes the level cellular proliferation in cells transfected with miR-34a. HDAC1, histone deacetylase 1.

To address whether p21 expression is required for the miR-34a-induced phenotype, we performed interference assays by cotransfecting cells with miR-34a and an siRNA directed against $p21$. As controls, cells were transfected with miR-34a, miR-215, or miR-NC. Each miRNA was supplemented with negative control oligo such that the total amount of transfected RNA equals the one of the miR-34a/si-p21 combination. The downregulation of targeted genes was verified by Western analysis (**Figure 5a; Supplementary Figure S9**). miR-34a alone reduced proliferation of RKO cells by ~20–30% and is in agreement with previous results (**Figure 5b**). In contrast, miR-34a in combination with the p21 siRNA had no effect on cancer cell proliferation despite the fact that miR-34a actively led to repression of HDAC1. This result suggests that $p21$ expression is indeed a necessary factor in mediating a miR-34 tumor suppressor response. The p21-dependent phenotype was reproducible in isogenic SW48 cancer cells (**Supplementary Figure S10**).

DISCUSSION

The current paradigm views miR-34 as a cellular effector molecule that functions downstream of TP53 by repressing genes involved in cell cycle progression and apoptosis. Our data, however, suggest that miR-34 assumes a more central role that is independent of and in parallel to TP53 (**Figure 6**). Support for this hypothesis

is provided here, showing that the inhibitory activity of miR-34 is the same in TP53-positive and TP53-negative cancer cells. These results pertain to a miR-34 mimic but may also shed light on the role of endogenous miR-34. Overexpression of miR-34 is sufficient to induce $p21$, a potent tumor suppressor otherwise known to be transcriptionally regulated by TP53 and necessary for the TP53 response. The primary functions of $p21$ involve cell cycle arrest by inhibiting cyclin-dependent kinases (CDKs) and blockage of DNA synthesis by binding to proliferating cell nuclear antigen.³³ However, $p21$ can also inhibit other oncogenic pathways, including those regulated by WNT4, STAT3, MYC, and TERT.³³ Thus, a key function of TP53 is evidently shared with miR-34. The existence of a separate miR-34 pathway is further illustrated by the TP53-independent transcriptional regulation of the miR-34a gene,³⁴ as well as observations made in miR-34 knock-out mice that show an intact TP53 response in the absence of miR-34.³⁵ miR-34 and TP53 may create an interface of two pathways with overlapping functions and activate each other reciprocally – TP53 *via* transcription and miR-34 *via* post-transcriptional repression of $SIRT1$, $YY1$, and $MDM4$ (**Figure 6**).

In TP53-deficient cells, the miR-34-induced expression of $p21$ is an indirect effect of HDAC1 repression. HDAC1 has previously been implicated in the regulation of the $p21$ gene ($CDKN1A$). Supporting evidence comes from (i) HDAC1-deficient embryonic

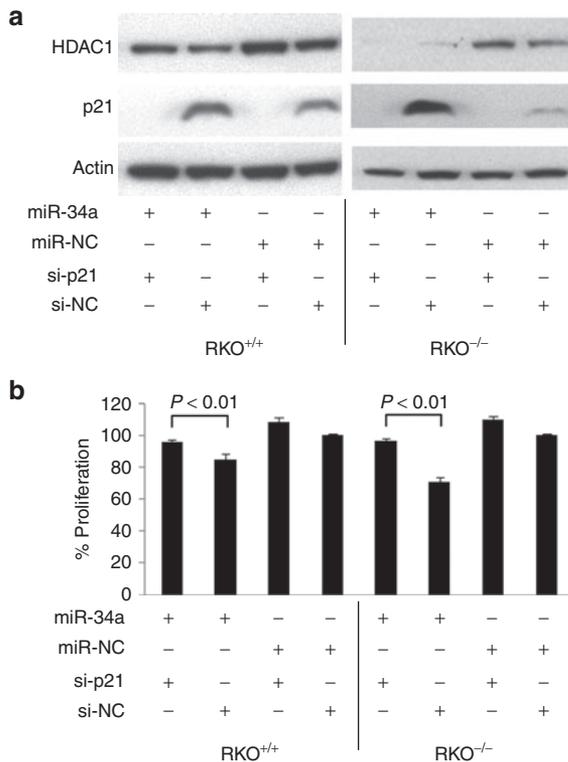


Figure 5 Depletion of *p21*^{CIP1/WAF1} interferes with miR-34-induced inhibition of cancer cell proliferation. miRNA mimics and small interfering RNAs were transiently transfected into isogenic RKO cells as shown in the graph. Protein expression was verified by (a) Western analysis, and proliferation was assessed by (b) AlamarBlue. Proliferation data are normalized to cells transfected with negative control (100%). Averages and standard deviations are shown. *P* values were derived from two-tailed Student's *t* tests. HDAC1, histone deacetylase 1.

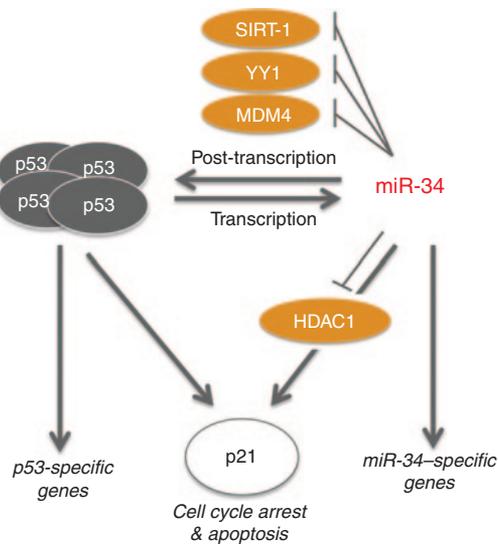


Figure 6 TP53 and miR-34 function in parallel to activate p21 and to induce cell cycle arrest. TP53 transcription increases miR-34 expression, and miR-34 activates TP53 via repression of SIRT1, MDM4, and YY1. p21 expression can be directly induced by TP53, or indirectly by miR-34 through repression of HDAC1. Both TP53 and miR-34 may exert tumor suppression functions via p21 and other separate pathways. HDAC1, histone deacetylase 1.

stem cells, that show elevated levels of p21, and (ii) p53-mutated human osteosarcoma cells in which *p21* expression was induced after treatment with the HDAC inhibitor trichostatin A.^{30,31} In these studies, two Sp1 binding sites in the *CDKN1A* promoter were identified as trichostatin A-responsive elements, suggesting that – in the absence of TP53 – Sp1 is the transcription factor that controls the activation of the *CDKN1A* gene. Our data indicate that *p21* is a functional prerequisite for miR-34a function. However, the effects of *p21* depletion varied between cell lines. *p21* depletion completely abolished the antiproliferative activity of miR-34a in RKO cells and merely weakened it in SW48 cells. Likewise, the inhibitory activity of miR-34a was not fully reduced but significantly lessened in TP53-negative Hep3B hepatocarcinoma cells that lack p21 (data not shown). In contrast, work by He *et al.* did not reveal a p21-dependent miR-34a phenotype in HCT116^{p21^{-/-}} cells.¹⁴ Therefore, the effects of p21 depletion appear to vary across cell lines. It is possible that the miR-34 phenotype is additionally controlled by other molecular events that are subject to change in cancer. Although deletion of *CDKN1A* can lead to spontaneous tumor formation in mice, somatic loss-of-function mutations in human cancer are rare.³⁶ However, reduced expression has been noted in colorectal, cervical, esophageal, and lung cancers, and in some of these, this is due to the hypermethylation of the *CDKN1A* promoter.^{33,37} Thus, a miR-34a mimic may be less active in cancers with silenced *CDKN1A* which should be considered as a predictive biomarker for a miR-34 therapy.

The miR-34-specific induction of *p21* offers an explanation for its invariable ability to inhibit TP53 wild-type and TP53-deficient cells. This is in stark contrast to miR-215/192 that is unable to induce *p21* in the absence of TP53 and, consequently, has reduced inhibitory activity in TP53^{-/-} cells. The data generated with miR-215/192 fit a model described previously in which miR-215/192 functions in a positive feedback loop to TP53 via repression of *MDM2*.¹⁹ Interestingly, the reported positive feedback from miR-34 to TP53 via *SIRT1*, *YY1*, or *MDM4* does not seem to contribute to the antiproliferative miR-34 phenotype despite the fact that these gene products were downregulated by miR-34a. Given the modest effects on cellular proliferation of siRNAs against these gene products, it is possible that they do not participate in an immediate antiproliferative miR-34 response but may reveal added effects after prolonged repression.

Taken together, our data demonstrate that miR-34a-induced inhibition of cancer cell proliferation is independent of TP53 and suggests that a miR-34 therapy is effective in cancer patients irrespective of TP53 status. The ability of miR-34 to repress *HDAC1* and to induce *p21* significantly strengthens its position as a central tumor suppressor and complements its function in other important oncogenic pathways. Clinical studies may provide further insight whether TP53, *HDAC1*, or *CDKN1A* can predict therapeutic responses to miR-34.

MATERIALS AND METHODS

Cell culture, oligos, and proliferation assays. Isogenic cancer cells derived from the MCF10A breast cancer and the SW48, HCT116, DLD-1, and RKO colorectal cancer cell lines were obtained from Horizon Discovery (Cambridge, UK) and are described in refs. 25,26. Synthetic miRNA mimics and siRNAs were purchased from Life Technologies (Ambion, Austin, TX). For stimulation of the TP53 pathway, cells were pretreated

with 10 $\mu\text{mol/l}$ etoposide for 28 hours, and RNA was harvested for qRT-PCR analysis. Optimal transfection conditions using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or RNAiMAX (Invitrogen) were determined for each cell line using an siRNA against EG5, a spindle protein required for proliferation.³⁸ Reverse transfections were done in duplicates or triplicates and carried out as previously described.³⁹ Briefly, 5 μl of oligo solution in RNase-free water was added to 20 μl of OptiMEM (Invitrogen) per well containing a constant amount of Lipofectamine 2000 (SW48, MCF10A, DLD-1, HCT116) or RNAiMAX (RKO). The mixture was incubated for 20 minutes at room temperature to form lipid-RNA complexes. Then, 75 μl of cells suspended in medium were added to reach a final concentration of 6,000–10,000 cells per well, depending on the growth rate of each cell line. After ~18 hours, the supernatant was removed and replaced with fresh media. Cellular proliferation was determined using AlamarBlue (Invitrogen) 3–4 days post-transfection.⁴⁰ The AlamarBlue substrate is metabolically converted into a fluorescent product in viable cells that is proportional to the number of living cells. Nonlinear regression and EC_{50} values were calculated using the GraphPad (Prism) software (version 6.01; Graphpad Software). All EC_{50} values were within the 95% confidence interval ($P < 0.05$) of the regression trendline.

Quantitative reverse transcription-PCR. Total RNA from cultured isogenic cancer cell lines was isolated using the mirVANA PARIS RNA isolation kit (Ambion) following the manufacturer's instructions. For qRT-PCR detection of miRNAs, 10 ng of total RNA and miRNA-specific RT-primers (assay IDs: hsa-miR-34a, 000426; hsa-miR-215, 000518; hsa-miR-192, 000491; hsa-miR-194, 000492; hsa-miR-34b, 002102; hsa-miR-34c, 000428; TaqMan miRNA Assay; Applied Biosystems, Foster City, CA) were heat-denatured at 70 °C for 2 minutes and reverse-transcribed using MMLV reverse transcriptase (cat. no. 28025-021, Invitrogen). miRNA expression levels were determined by PCR using Platinum Taq Polymerase reagents (Invitrogen) and the ABI Prism 7900 SDS instrument (Applied Biosystems). PCR reactions were performed by heating samples to 95 °C for 1 minutes, followed by incubating the samples at 95 °C for 5 seconds, and 60 °C for 30 seconds during multiple cycles. The house-keeping miRNAs miR-191 and miR-103 (assay IDs: 002299 and 000439) were amplified as internal references to adjust for well-to-well RNA input variances.⁴¹ Raw Ct (cycle threshold) values were normalized to the geometric mean of house-keeping miRNAs Cts and expressed as fold differences relative to those in untreated, miR-NC, or mock-transfected cells.

For detection of human mRNAs, cDNA was generated using 10 ng total RNA with random decamers (AM5722G; Ambion) as previously described.⁴² Gene-specific amplification was carried out using the following TaqMan Gene Expression Assays (Invitrogen): MDM4, Hs00159092_m1; XBP1, Hs00231936_m1; HDAC1, Hs02621185_m1; SNAI2, Hs00950344_m1; p21, Hs00385782_m1; PUMA (BBC3), Hs00248075_m1; MDM2, Hs00234753_m1; MYCT1, Hs00228305_m1; ATF6, Hs0023586_m1; SIRT1, Hs01009005_m1; MYCN, Hs00232074_m1; and TP53, Hs00153340_m1. mRNA levels of house-keeping GAPDH and cyclophilin A (TaqMan IDs: GAPDH, Hs99999905_m1; CYCLO A, Hs99999904_m1; Invitrogen) were used as loading controls. Raw Cts were normalized to those of house-keeping mRNAs and analyzed as described above.

Site-directed mutagenesis. Human HDAC1 3' UTR Lenti-reporter-Luciferase vector (pLenti-UTR-LucHDAC1, HDAC1 wt) encoding the luciferase reporter fused to the entire 3' UTR of human HDAC1 was purchased from Applied Biological Materials (Richmond, BC). Two rounds of mutagenesis were performed to introduce 6 point mutations in the miR-34 binding site of HDAC1 3' UTR (HDAC1 mutated). For site-directed mutagenesis, the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used following the manufacturer's instructions. In the first round, the following primers were used: 5'-CCTCAAGTGAGCC AAGAAACAATAACTGCCCTCTGTCTGTC-3' and 5'-GACAGACAGA

GGGCAGTTATTGTTTCTTGGCTCACTTGAGG-3. A positive clone was verified by sequencing (University of Texas, Austin, TX) and used as a template for the second round of mutagenesis using the following primers: 5'-GGCCTCAAGTGAGCCAAAAAATAAATAACTGCCCTCTGTCTGTC-3' and 5'-GACAGACAGAGGGCAGTTATTTATTTTGGCTCAC TTGAGGCC-3'. All vectors used in transfections were verified by sequencing.

Luciferase reporter assays. SW48^{-/-} and H1299 cells were reverse transfected with 1 nmol/l or 10 nmol/l miR-34a, respectively, in 96-well plate using Lipofectamine 2000 (Life Technologies). As controls, cells were transfected with miR-NC at the same concentrations. The next day, cells were forward transfected with each 100 ng of HDAC1 wt or HDAC1-mutated luciferase plasmids. After 48 hours, cell lysates were prepared and quantified using the BCA system from Pierce (Thermo Scientific, Rockford, IL). Luminescence was determined using the PolarStar OptiMA plate reader (BMG Labtech, Ortenberg, Germany) and the Luciferase Assay System (Promega, Madison, WI). Luminescence was normalized to total protein input.

Western analysis. 200,000 SW48 and RKO cells were seeded in six-well plates and reverse-transfected with miRNA mimics and siRNAs in six-well plate using 2.5- μl Lipofectamine 2000 or RNAiMAX. After 3 days, cell lysate were collected in RIPA buffer (Cell Signaling, Danvers, MA), and protein concentrations were measured using the BCA assay kit from Thermo Scientific. Each 2.5 μg of total cell lysate was loaded on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane. The membrane was blotted with primary antibody specific for p21, HDAC1, c-MET, and actin (Cell Signaling) overnight at 4 °C. The membrane was washed in 1 \times phosphate-buffered saline containing 0.2% Tween-20 and incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. After washing with 1 \times phosphate-buffered saline containing 0.2% Tween-20, the membrane was incubated with ECL detection reagent (EMD Millipore, Darmstadt, Germany) and protein bands were visualized using the AFP X-ray film developer (AFP Image, Elmsford, NY). Western data were quantified using the AlphaImager EC instrument from Cell Biosciences (Santa Clara, CA).

Human tissue samples. Non-small-cell lung carcinoma tumor samples and the corresponding normal adjacent tissues were purchased from ProteoGenex (Culver City, CA) and the National Disease Research Interchange (Philadelphia, PA). Staging information and miR-34a levels are reported in ref. 10. HDAC1 mRNA and miR-34a levels were determined by qRT-PCR and expressed as relative expression between each tumor and normal adjacent tissue pair. Linear regression was calculated using GraphPad.

Statistical analysis. Statistical analyses were done using the Excel and GraphPad (Prism) software. Averages and standard deviations were calculated from duplicate or triplicate experiments. P values were generated by two-tailed Student's t test or F test as indicated in the figure legends.

SUPPLEMENTARY MATERIAL

Figure S1. Induction of TP53 genes by etoposide.

Figure S2. Dose-dependent effects of miR-34c and miR-192 in cancer cells with or without functional TP53.

Figure S3. Dose-dependent effects of miR-34a and miR-34c in isogenic RKO cells.

Figure S4. Conservation of miR-34a binding sites in the HDAC1 3' UTR across vertebrates.

Figure S5. Quantification of Western data shown in Figure 4A using the AlphaImager EC instrument from Cell Biosciences.

Figure S6. miR-34a-induced repression of HDAC1 and induction of p21^{CIP1/WAF1} in TP53-positive cells.

Figure S7. Endogenous mRNA expression levels in isogenic SW48 cells transiently transfected with siRNAs.

Figure S8. Effects of Trichostatin A on isogenic cancer cells.

Figure S9. Quantification of Western data shown in Figure 5A using the Alphamager EC from Cell Biosciences.

Figure S10. Depletion of p21 interferes with miR-34a-induced inhibition of cell proliferation in SW48 cells.

Table S1. TP53 genotypes of isogenic cancer cell lines.

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