miR-29 and miR-30 regulate B-Myb expression during cellular senescence

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Contributed by Joan A. Steitz, November 22, 2010 (sent for review October 5, 2010)

Cellular senescence is a form of irreversible growth arrest and a major tumor suppressor mechanism. We show here that the miR-29 and miR-30 microRNA families are up-regulated during induced and replicative senescence and that up-regulation requires activation of the Rb pathway. Expression of a reporter construct containing the 3′UTR of the B-Myb oncogene is repressed during senescence, and repression is blocked by mutations in conserved miR-29 and miR-30 binding sites in the B-Myb 3′UTR. In proliferating cells, transfection of miR-29 and miR-30 represses a reporter construct containing the wild-type but not the mutant B-Myb 3′UTR, and repression of the mutant 3′UTR is reinstituted by compensatory mutations in miR-29 and miR-30 that restore binding to the mutant sites. miR-29 and miR-30 introduction also represses expression of endogenous B-Myb and inhibits cellular DNA synthesis. Finally, interference with miR-29 and miR-30 expression inhibits senescence. These findings demonstrate that miR-29 and miR-30 regulate B-Myb expression by binding to its 3′UTR and suggest that these microRNAs play an important role in Rb-driven cellular senescence.

E2 protein | HeLa cells | human papillomavirus | retinoblastoma

Senescence is an important block to cell cycle progression during the aging of cells in culture and is a fundamental barrier that cells must bypass during carcinogenesis (1, 2). Cells undergoing senescence display permanent cell cycle arrest, autofluorescence, cell enlargement and flat morphology, and increased activity of senescence-associated β-galactosidase (SA-β-gal) (3, 4). Replicative senescence occurs during serial passage of primary cells and is initiated by telomere shortening below a critical threshold. The p53 and retinoblastoma (Rb) tumor suppressor pathways are essential for senescence (5, 6), and tumor virus proteins that neutralize these tumor suppressor pathways, such as human papillomavirus (HPV) E6 and E7, interfere with senescence (7). However, studies of the molecular basis of replicative senescence are complicated by the slow and heterogeneous nature of this process. Therefore, considerable effort has been expended on studying senescence triggered acutely by a variety of stimuli including nontelomeric DNA damage, strong mitogenic signals, expression of tumor suppressor genes, and repression of key cellular proteins, such as B-Myb (8).

The B-Myb oncogene, also known as MYBL2, is a transcription factor that positively regulates the expression of a variety of genes involved in cell proliferation including c-Myc, DNA polymerase α, and B-Myb itself (9, 10). Importantly, B-Myb can regulate senescence. For example, inhibition of B-Myb expression by RNA interference can induce senescence (8), and overexpression of B-Myb can abrogate Ras-induced premature senescence (11). B-Myb transcription is repressed at the G0/G1 boundary of the cell cycle by binding of E2F4/p107 or E2F4/p130 complexes to E2F sites in the B-Myb promoter (12–15). B-Myb mRNA levels are down-regulated during replicative and induced senescence (8).

Small, noncoding microRNAs mediate the posttranscriptional regulation of genes that control many biological processes, including senescence. miR-217, miR-20a, and the miR-34 family regulate senescence by controlling the activity of SIRT1 and the p53 pathway (16–20). However, specific microRNAs have not been shown to regulate the expression of B-Myb or mediate the growth inhibitory effects of the Rb pathway.

In this study, we demonstrate that expression of two microRNA families, miR-29 and miR-30, is induced during senescence in an Rb-dependent manner. By using reporter gene assays and analysis of point mutations in microRNA binding sites in the 3′UTR of B-Myb, we show that B-Myb is directly repressed by miR-29 and miR-30 in cells undergoing senescence. Furthermore, interference with miR-29 and miR-30 activity inhibits senescence.

Results

Expression of miR-29 and miR-30 Families Is Up-Regulated During Induced Senescence. In HeLa cervical carcinoma cells, the HPV type 18 E7 protein induces the degradation of the Rb tumor suppressor family ($p105^{Rb}$, p107, and p130), whereas the E6 protein induces the degradation of p53. Expression of the bovine papillomavirus (BPV) E2 transcription factor from a replication-defective SV40 viral vector (designated Pava1) in HeLa cells represses the expression of the E6 and E7 genes, resulting in the reactivation of p53 and Rb and the rapid onset of senescence (21, 22). To restrict our analysis to the Rb pathway, we used HeLa/E6 cells, which express the HPV16 E6 protein from a promoter that is not repressed by the E2 protein (23). When HPV18 E7 is repressed in these cells, the Rb pathway is activated and induces senescence without the activation of p53, which is maintained in an inactive state by HPV16 E6 (24) (*SI Appendix*, Fig. S1). We also used two control cell lines: HeLa/E6-E7 (which expresses wild-type HPV16 E7 as well as E6, thereby preventing E2-mediated Rb activation and senescence) and HeLa/E6-E7Δ (which expresses HPV16 E6 and an E7 mutant that is defective for Rb binding and therefore does not prevent senescence) (*SI Appendix*, Fig. S1).

To identify microRNAs that were differentially regulated by the Rb pathway during senescence, we used TaqMan® microRNA microarrays to assess expression of 365 human microRNAs in the presence and absence of the E2 protein. Compared to uninfected cells, 25 microRNAs were up-regulated more than 1.5-fold and 24 were down-regulated more than 1.5-fold during senescence in E2-infected HeLa/E6 and HeLa/E6-E7Δ cells, but not in infected HeLa/E6-E7 cells (*SI Appendix*, Table S1). This pattern of expression strongly suggests that these changes

Author contributions: I.M., D.C., L.L.A., J.A.S., and D.D. designed research; I.M., D.C., and L.L.A. performed research; I.M., D.C., L.L.A., J.A.S., and D.D. analyzed data; and I.M., D.C., L.L.A., J.A.S., and D.D. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1017346108/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1017346108

in microRNA levels are the result of Rb activation and not a direct effect of E2 expression. Interestingly, multiple members of the miR-29 and miR-30 families were up-regulated in an Rb-dependent manner during senescence (Fig. 1*A*). Although the expression of some other microRNAs changed more dramatically than miR-29 and miR-30 during senescence, we focused on these two microRNAs because putative binding sites for both of them were present in the 3′UTR of the B-Myb gene (see next section).

To obtain further evidence that up-regulation of miR-29 and miR-30 is due to activation of the Rb pathway, we analyzed HeLa/E6 cells expressing shRNAs that knockdown expression of Rb family members (8) (*SI Appendix*, Fig. S2*A*). Northern blotting with probes that recognized each of these microRNA families in aggregate showed that up-regulation of miR-29 and miR-30 was abrogated by Rb family knockout (Fig. 1*B*). Rb-dependent up-regulation of individual miR-29 and miR-30 family members during senescence was confirmed by quantitative reverse transcription real-time PCR (qRT-PCR) (*SI Appendix*, Figs. S2 *B* and *C*). We conclude that up-regulation of miR-29 and miR-30 requires activation of the Rb pathway.

miR-29 and miR-30 Target the B-Myb 3′UTR During Rb-E2F Induced Senescence. The up-regulation of miR-29 and miR-30 following E7 repression suggested that they regulate expression of genes important for senescence. Computational tools (TargetScan, PicTar) predicted the existence of evolutionarily conserved binding sites for miR-29 and miR-30 in the 3′UTR of B-Myb (Fig. 2). To determine whether the 3′UTR of B-Myb could regulate gene expression during senescence, a 189-base pair fragment of the B-Myb 3′UTR containing the putative miR-29 and miR-30 binding sites was cloned downstream of the luciferase gene in a reporter plasmid lacking the B-Myb promoter and binding sites for the E2 protein. Although the full-length B-Myb 3′UTR also

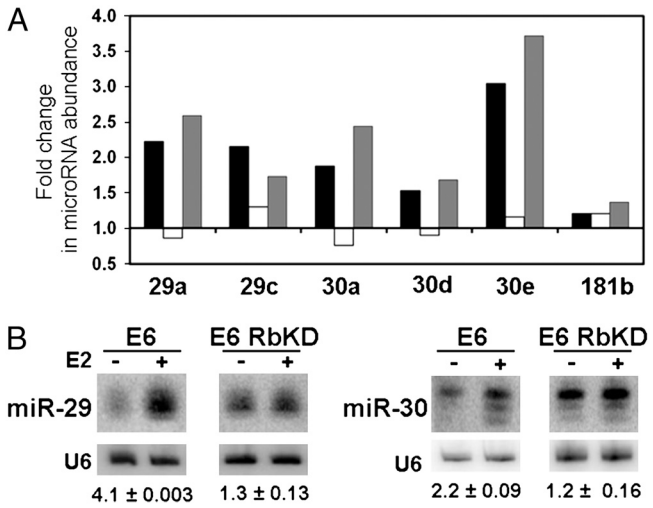

Fig. 1. Rb-dependent up-regulation of miR-29 and miR-30 microRNAs during senescence in HeLa cell lines. (*A*) Differential expression of miR-29 and miR-30 family members as measured by TaqMan® microRNA microarray. Fold change compared to uninfected cells was measured for the indicated microRNAs in HeLa/E6 (black bars), HeLa/E6-E7 (white bars), and HeLa/E6-E7Δ (gray bars) cells 4 d after infection with Pava1. miR-181b is an example of a microRNA whose expression did not change during senescence. Similar results were obtained in two independent experiments, the average of which is shown here. (*B*) Confirmation of Rb-dependent up-regulation of miR-29 and miR-30 by Northern blot analysis. E6 indicates HeLa/E6 cells, and E6 RbKD indicates HeLa/E6 cells stably expressing shRNAs that knockdown expression of $p105^{Rb}$, p107, and p130 (8). The portion of the gel corresponding to mature microRNAs is shown. In each panel, the left lane contains RNA from uninfected cells, and the right lane contains RNA from cells 6 d after infection with Pava1. The numbers underneath the panels show the average fold induction of microRNA expression by the E2 protein in two independent experiments, +/− standard deviations, normalized to U6 RNA expression.

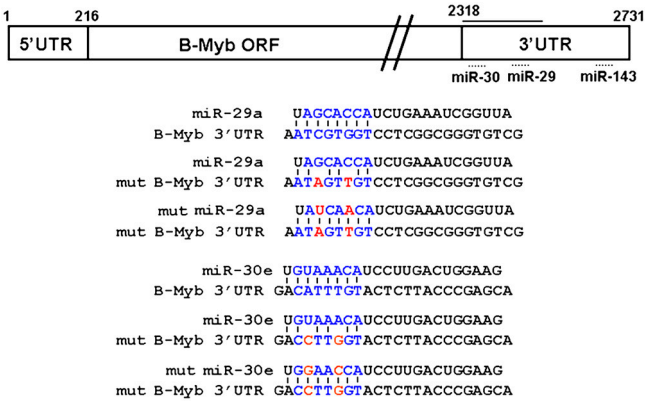

Fig. 2. miR-29 and miR-30 binding sites in the 3′UTR of the B-Myb gene. (*Top*) Map of human B-Myb mRNA, together with the nucleotide number of various landmarks relative to the transcription start site. The B-Myb 3′UTR contains conserved sites complementary to the seed sequences of miR-30, miR-29, and miR-143. The line above the 3′UTR shows the segment cloned into the luciferase reporter plasmid. The sequences of miR-29a and miR-30e and their putative binding sites in the B-Myb 3′UTR are shown below the map. (*Left* to *Right*) The top strand in each pair is shown in the 5′ to 3′ orientation, and the bottom strand is shown the 3′ to 5′ orientation, with predicted base pairing between the microRNA seed region and the 3′UTR indicated by vertical lines. The wild-type seed sequence and complementary bases in the 3′UTR are shown in blue; mutations in the binding sites that disrupt base pairing and mutations in the microRNAs that restore it are shown in red.

contains a putative binding site for miR-143, this site was not contained in the fragment of the 3′UTR cloned into the luciferase vector because the level of miR-143 did not change during senescence. A luciferase plasmid containing a 210-base pair fragment of the B-Myb open reading frame lacking miR-29 and miR-30 binding sites was used as a control. Following transfection of the reporter plasmid into HeLa/E6 cells, luciferase activity was measured after mock-infection or infection with Pava1 to induce senescence. E2 expression did not affect luciferase production from the control vector, but caused an approximately threefold reduction in luciferase expression from the plasmid containing the B-Myb 3′UTR (Fig. 3*A*), indicating that the B-Myb 3′UTR contains elements that confer regulation during senescence. Furthermore, expression of the E2 protein also repressed luciferase expression from the plasmid containing the 3′UTR of B-Myb in HeLa/E6-E7Δ cells but not in HeLa/E6-E7 cells, demonstrating that the ability of the B-Myb 3′UTR to regulate gene expression is not a direct effect of the E2 protein but rather is due to E7 repression and activation of the Rb pathway.

To test whether direct binding of miR-29 and miR-30 to the B-Myb 3′UTR is required for repression during senescence, we introduced point mutations into the binding sites for miR-29 and/or miR-30 in the B-Myb 3′UTR (Fig. 2). Mutations in either binding site partially impaired repression of luciferase expression by Pava1 infection, and mutations in both binding sites together eliminated repression (Fig. 3*B*, *Top*). In addition, induction of senescence caused a three- to fourfold reduction in the levels of luciferase mRNA from the plasmid containing the wild-type B-Myb 3′UTR (Fig. 3*B*, *Bottom*). This reduction was eliminated by mutations in the miR-29 and miR-30 binding sites. Thus, the primary effect of the miR-29 and miR-30 binding sites in the B-Myb 3′UTR is to control mRNA levels. The residual repression mediated by the B-Myb 3′UTR containing mutations in one microRNA binding site is presumably due to the remaining wild-type site in these mutants.

To obtain further evidence that miR-29 and miR-30 directly regulate expression of the B-Myb gene by binding to sites in the B-Myb 3′UTR, we assessed the effect of introducing exogenous wild-type miR-29 and miR-30 into cells. Proliferating HeLa/E6

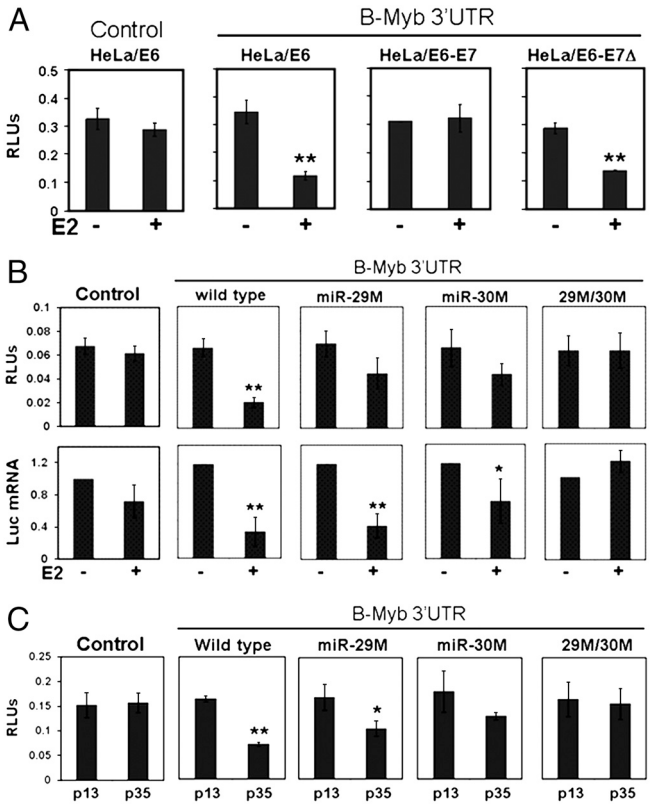

Fig. 3. Role of the miR-29 and miR-30 binding sites in regulation by the B-Myb 3′UTR. (*A*) HeLa/E6, HeLa/E6-E7, and HeLa/E6-E7Δ cells were cotransfected with a firefly luciferase plasmid containing a segment of the B-Myb ORF (control) or the wild-type B-Myb 3′UTR, together with a Renilla luciferase plasmid for normalization of transfection efficiency. The day after transfection, cells were mock-infected (–) or infected with Pava1 (+) to induce senescence. Firefly luciferase activity was measured and normalized to Renilla luciferase after an additional 5 d. RLUs, relative light units. For panels *A* and *B*, two-tailed *t*-test results are indicated by * for $P < 0.05$ and ** for $P < 0.01$, relative to mock-infected cells. Similar results were obtained in three independent experiments. (*B*) HeLa/E6 cells were transfected with a luciferase plasmid containing the B-Myb ORF fragment (control) or the B-Myb 3′UTR with no mutations (wild type), mutations in the miR-29 binding site alone (miR-29M), mutations in the miR-30 binding site alone (miR-30M), or mutations in both binding sites (29M/30M), as indicated. The day after transfection, cells were either mock-infected (–) or infected with Pava1 (+) to induce senescence. Five days later, firefly luciferase activity was measured and normalized to expression of Renilla luciferase from a cotransfected plasmid (*Top*), or the amount of firefly luciferase mRNA was measured by qRT-PCR and reported as fold-change relative to luciferase mRNA in uninfected cells transfected with the control plasmid (*Bottom*). Similar results were obtained in three (*Top*) or two (*Bottom*) independent experiments. (*C*) Passage 13 and passage 35 HFFs were transfected with the control luciferase plasmid or the plasmid containing the B-Myb 3′UTR with no mutations or with mutations in the miR-29 and miR-30 binding sites as described in the legend to *B*. After 2 d, firefly luciferase activity was measured and normalized to expression of Renilla luciferase from a cotransfected plasmid. Two-tailed *t*-test results are indicated by * for $P < 0.05$ and ** for $P < 0.01$, relative to low passage cells. Similar results were obtained in two independent experiments.

cells (in the absence of E2 expression) were transfected with wild-type miR-29a and/or miR-30e or with control microRNAs. Transfection of miR-29 and miR-30 caused a marked increase in the abundance of these microRNAs (*SI Appendix*, Fig. S3). Cells were then transfected with the reporter plasmids, and luciferase activity was measured 2 d later. Exogenous miR-29a and miR-30e repressed expression of luciferase linked to the wild-type 3′UTR, but did not repress luciferase from plasmids containing the B-Myb 3′UTR with mutations in the miR-29 and miR-30 binding sites (Fig. 4, *Top*). Control microRNAs did not affect luciferase

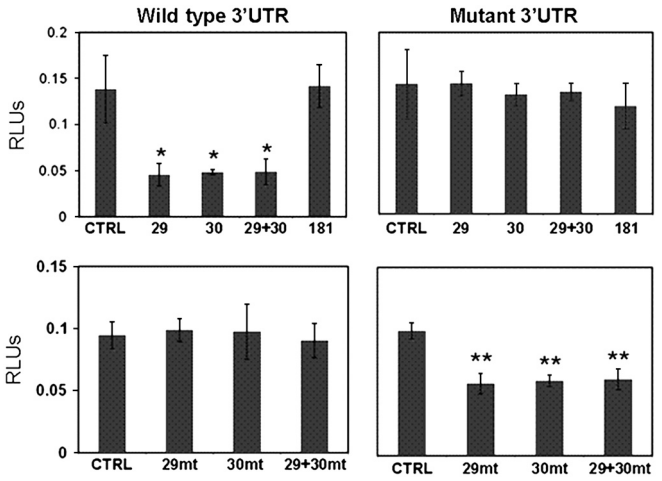

Fig. 4. Genetic analysis of the interaction between the B-Myb 3′UTR and miR-29 and miR-30. Proliferating HeLa/E6 cells were transfected with 80 nM (total) scrambled control microRNA (CTRL), miR-181b (181), or wild-type (*Top*) or mutant (*Bottom*) miR-29a and/or miR-30e that contain compensatory mutations in the seed sequence that restore binding to the mutant binding sites in the B-Myb 3′UTR (see Fig. 2). The next day, cells were transfected with the reporter plasmid containing the B-Myb 3′UTR with no mutations (*Left*) or with mutations in both microRNA binding sites (*Right*). Normalized firefly luciferase activity was measured 2 d later. Two-tailed *t* test results are indicated by * for $P < 0.05$ and ** for $P < 0.01$, relative to cells transfected with CTRL microRNA. Similar results were obtained in three (*Top*) or two (*Bottom*) independent experiments.

expression from any of these plasmids. These results demonstrated that miR-29 and miR-30 are sufficient to act on the 3′ UTR of B-Myb in the absence of E2 expression or E7 repression (*SI Appendix*, Fig. S4). Finally, we assessed the effect of introducing mutant miR-29a and miR-30e containing compensatory mutations predicted to restore binding to the mutant sites in the B-Myb 3′UTR (see Fig. 2). In contrast to the wild-type microRNAs, the mutant ones were inactive on the wild-type 3′UTR but repressed luciferase expression from plasmids with the mutant 3′UTR (Fig. 4, *Bottom*). These experiments provide compelling genetic evidence that repression of genes containing the 3′UTR of B-Myb is mediated by direct binding of miR-29 and miR-30 to the 3′UTR.

Regulation of the B-Myb 3′UTR by miR-29 and miR-30 in Primary Human Fibroblasts. We also assessed the effect of miR-29 and miR-30 in primary human foreskin fibroblasts (HFFs) undergoing replicative senescence. As shown in *SI Appendix*, Fig. S5, miR-29a and multiple miR-30 family members were up-regulated approximately twofold during replicative senescence in late passage HFFs compared to early passage cells. The levels of miR-29b and miR-29c did not change during replicative senescence.

Next we examined the effect of the miR-29 and miR-30 binding sites in the B-Myb 3′UTR during replicative senescence. We transfected reporter plasmids containing the control B-Myb ORF fragment or the wild-type or mutant B-Myb 3′UTR into proliferating early passage HFFs and senescent late passage HFFs (Fig. 3*C*). After 2 d, normalized luciferase expression from the control plasmid was similar in early and late passage HFFs. In contrast, luciferase expression driven by the wild-type B-Myb 3′UTR was reduced 2.3-fold in late passage cells, but mutation of the miR-29 and miR-30 binding sites in the 3′UTR eliminated repression. These results demonstrated that the 3′UTR of B-Myb is also a direct target of miR-29 and miR-30 during replicative senescence in HFFs.

Regulation of Endogenous B-Myb Expression by miR-29 and miR-30. To assess the role of miR-29 and miR-30 in controlling expression of

the endogenous B-Myb gene, we transfected proliferating HeLa/E6 cells and low passage HFFs with miR-29a and/or miR-30e or control microRNAs and measured expression of B-Myb mRNA. In both cell types, the levels of endogenous B-Myb mRNA were lowered by introduction of exogenous miR-29a or miR-30e, but not by the control microRNAs (Fig. 5*A*). The greater reduction in the HeLa/E6 cells is presumably due to the higher transfection efficiency of these cells. The level of endogenous B-Myb protein was also reduced following transfection of miR-29a or miR-30e (Fig. 5*B*). These results show that the overexpression of miR-29 or miR-30 in proliferating cells is sufficient to repress endogenous B-Myb expression.

We also determined the effect of miR-29 and miR-30 on expression of the endogenous DEK oncogene. Like B-Myb, the DEK promoter is under direct Rb-E2F control and is down-regulated during senescence (8, 25), but the DEK 3′UTR lacks predicted binding sites for miR-29 or miR-30. Transfection of miR-29a and/or miR-30a into HeLa/E6 cells did not reduce the level of DEK mRNA (*SI Appendix*, Fig. S6), indicating that this treatment does not simply activate the Rb pathway and cause global down-regulation of Rb-responsive genes.

Consistent with the repression of endogenous B-Myb, transfection of miR-29a and/or miR-30e into proliferating HeLa/E6 cells or early passage HFFs caused a 2.5- to 6-fold inhibition of cellular DNA synthesis compared to cells that received the scrambled control miRNA (*SI Appendix*, Fig. S7*A*). In HeLa/E6 cells, growth inhibition was not due to repression of HPV18 E7 (*SI Appendix*, Fig. S4). To explore whether B-Myb repression played a role in this inhibition of DNA synthesis, miR-29a and miR-30e were transfected into HeLa/E6 cells stably transduced by lentiviruses expressing GFP or an exogenous B-Myb gene lacking its 3′UTR. Transfection of these microRNAs had little effect on the expression of the exogenous B-Myb protein (*SI Appendix*, Fig. S7*B*). Three days after transfection, miR-29/30 caused a statistically significant reduction in cellular DNA synthesis in control HeLa/E6/GFP cells compared to cells transfected with the control oligonucleotide (*SI Appendix*, Fig. S7*C*). This inhibition was partially alleviated by constitutive expression of B-Myb, indicating that B-Myb repression contributed to the inhibition of cellular DNA synthesis caused by introduction of miR-29 and miR-30.

miR-29 and miR-30 Are Required for Optimal Induction of Rb-Dependent Senescence. To test whether the up-regulation of miR-29 and miR-30 plays a role in the induction of senescence, we transfected HeLa/E6 cells with anti-miRs specific for these two microRNA families and assessed the effect on senescence. Cells were transfected with scrambled control anti-miR or anti-miR-29a and/or anti-miR-30a oligonucleotides (Applied Biosystems) prior to expression of the E2 protein. The anti-miRs specifically lowered the basal levels of miR-29a and miR-30a (and other members of these microRNA families) and prevented their induction in response to E2 expression (*SI Appendix*, Figs. S8 and S9), but did not interfere with E2-mediated repression of the HPV E7 gene (*SI Appendix*, Fig. S10).

Antagonizing miR-29 or miR-30 activity caused an approximately tenfold increase in the number of cells counted two weeks after expression of the E2 protein (Fig. 6*A*). The number of colonies that formed if the cells were plated immediately after E2 expression showed a similar increase, demonstrating that the increase was not due to a jackpot phenomenon affecting a tiny fraction of the cells (*SI Appendix*, Fig. S11). In addition, we used flow cytometry to measure cellular autofluorescence as a physiological marker of senescence (22). In HeLa/E6 cells transfected with the control anti-miR, E2 expression caused >70% of the cells to display a dramatic increase in fluorescence compared to proliferating cells, consistent with their entry into senescence, whereas ~20% of the cells continued to proliferate and displayed low fluorescence (Fig. 6*B* and *SI Appendix*, Fig. S12). In contrast, if anti-miR-29a and/or anti-miR-30a was transfected prior to E2 expression, between 45% and 65% of the cells displayed lower autofluorescence characteristic of proliferating cells that had overgrown the senescent population (Fig. 6*B*). Similarly, anti-miRs targeting miR29/30 reduced the number of cells expressing SA-β-galactosidase after E2 expression (*SI Appendix*, Fig. S13). Thus, miR-29 and miR-30 are required for optimal induction of senescence in HeLa/E6 cells.

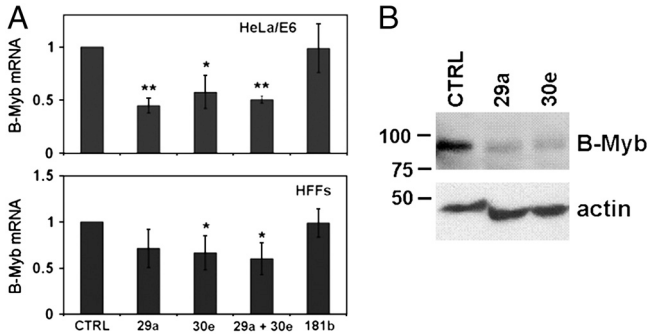

Fig. 5. Effect of exogenous miR-29a and miR-30e on the expression of B-Myb. (*A*) Proliferating HeLa/E6 cells (*Top*) and passage 13 HFFs (*Bottom*) were transfected with 80 nM (total) scrambled control microRNA (CTRL), miR-181b, or miR-29a and/or miR-30e. Three days later, levels of endogenous B-Myb mRNA were measured by qRT-PCR. Two-tailed *t*-test results are indicated by * for $P < 0.05$ and ** for $P < 0.01$, relative to cells transfected with CTRL microRNA. Similar results were obtained in three (*Top*) or two (*Bottom*) independent experiments. (*B*) Expression of endogenous B-Myb protein was detected by Western blotting 6 d after transfection of HeLa/E6 cells with scrambled control microRNA (CTRL), miR-29a, or miR-30e, as described in *A*. Similar results were obtained in two independent experiments.

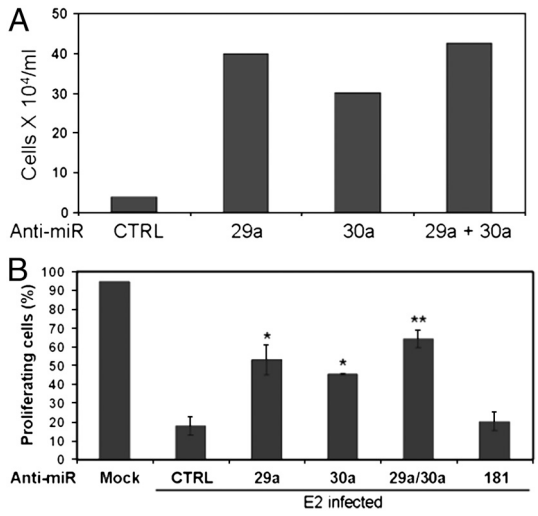

Fig. 6. Interference with miR-29 and miR-30 inhibits senescence. (*A*) HeLa/E6 cells were transfected with 80 nM scrambled anti-miR (CTRL) or anti-miR-29a and/or anti-miR-30a inhibitors on day 0 and day 4. Thirty and 55 h after the initial transfection, cells were mock-infected or infected with Pava1 at multiplicity of infection (moi) of 15, as indicated. (*A*). Cells were counted 14 d after the initial transfection of anti-miRs as described above. All cells in this panel were infected with Pava1. Similar results were obtained in three independent experiments. (*B*). Flow cytometry was used to determine the fraction of cells proliferating after treatment with anti-miRs and E2, as indicated. The graph shows average results (+/− standard deviation) of two replicate plates of cells for each condition. Two-tailed *t*-test results are indicated by * for $P < 0.05$ and ** for $P < 0.01$, relative to cells transfected with control anti-miR and infected with Pava1. Similar results were obtained in two independent experiments.

Discussion

The B-Myb oncogene encodes a transcription factor that controls cell cycle progression by regulating the expression of numerous genes required for S phase. Transcription of B-Myb itself is repressed by the binding of Rb-E2F complexes to the B-Myb promoter, and loss of B-Myb expression can result in senescence. In this study, we discovered that expression of miR-29 and miR-30 increases during replicative and induced senescence in an Rb-dependent fashion and that these microRNAs directly repress expression of B-Myb by binding to its 3′UTR. We further demonstrated that these microRNAs can regulate the onset of senescence.

The global analysis of microRNA expression reported here revealed that the levels of numerous microRNAs, including several members of the miR-29 and miR-30 families, are regulated in an Rb-dependent fashion in cervical carcinoma cells undergoing induced senescence. Wald et al. (26) recently reported that HPV16 E7 down-regulates expression of miR-29a in head-and-neck cancers, consistent with our finding that E7 repression up-regulates this microRNA. Primary human fibroblasts undergoing replicative senescence also displayed increased levels of miR-29a and most members of the miR-30 family, suggesting that induction of these microRNAs is a general feature of senescence triggered by various methods in different cell types. A recent report showed that miR-29b and miR-29c levels increase as mice age (27), consistent with their increase in late passage cultured human cells. c-Myc, which is repressed in senescing HeLa/E6 cells and HFFs (8), has been shown to bind to and repress the promoters driving miR-29 and miR-30 expression (28). Therefore, we speculate that c-Myc repression is responsible for induction of these microRNAs upon Rb induction.

Our conclusion that the B-Myb oncogene is a direct target of miR-29 and miR-30 during cellular senescence is supported by a variety of functional experiments (Figs. 3–5), which collectively show that these microRNAs interact with specific target sequences in the B-Myb 3′UTR to reduce expression of B-Myb. The primary effect of miR-29 and miR-30 on B-Myb expression is a reduction in the amount of B-Myb mRNA (Figs. 3*B* and 5*A*), presumably mediated by decreased stability of the mRNA. The binding of other microRNAs to their target 3′UTRs in mammalian cells can decrease the stability of mRNAs by inducing decapping and deadenylation (29). Previously published work showed that the B-Myb promoter is under direct Rb-E2F control (12–15). Importantly, in our experiments, the repression of B-Myb expression is determined by the B-Myb 3′UTR and its miR-29 and miR-30 binding sites and not by the B-Myb promoter, which is absent from our luciferase reporter plasmid. These findings suggest a model of B-Myb regulation during senescence in which the abundance of B-Myb mRNA is repressed by two different mechanisms, one at the transcriptional level by direct repression of the B-Myb promoter by Rb family members in complex with E2F factors and another at the posttranscriptional level by the binding of miR-29 and miR-30 to the 3′UTR of B-Myb mRNA.

We also showed that miR-29 and miR-30 can regulate cell proliferation. Introduction of miR-29 and miR-30 into proliferating cells inhibited DNA synthesis, and interfering with the activity of miR-29 and miR-30 allowed some cells to escape senescence and continue to proliferate. Thus, miR-29 and miR-30 are required for optimal induction of senescence and are likely downstream mediators of Rb-driven senescence. Although there are presumably multiple mRNAs regulated by these microRNAs, our experiments showed that growth inhibition was mediated in part by B-Myb repression. Anti-miRs targeting miR-29 and miR-30 permitted only a fraction of the cells to escape senescence. It is possible that we did not achieve sufficient miR-29 and miR-30 inhibition to totally eliminate their biological activity. In addition, miR-29 and miR-30 inhibition does not remove all the blocks to cell proliferation that are mobilized by Rb activation. For example, Rb represses DEK expression independently of miR-29 and miR-30 (25), and DEK repression can also induce senescence in HeLa/E6 cells (8).

Several lines of evidence reported here suggest that miR-29 and miR-30 have tumor suppressor activity: miR-29 and miR-30 are up-regulated during senescence, these microRNAs repress expression of the B-Myb oncogene and inhibit DNA synthesis, and senescence is inhibited by interfering with the activity of miR-29 and miR-30. Other laboratories showed that levels of miR-29 and miR-30 are reduced in tumor cells and that introduction of these microRNAs can inhibit tumor growth by repressing genes such as DNA methyltransferase 3A and 3B (30–32). In addition, miR-29 represses expression of cdc42 and the p85 subunit of PI3 kinase, which in turn stabilizes p53 and enhances apoptosis (33). In our experiments, miR-29 and miR-30 did not activate the p53 pathway, presumably because of ongoing HPV16 E6 expression. Our findings raise the possibility that some of the tumor suppressive effects of miR-29 and miR-30 may be mediated at least in part by B-Myb repression. Accordingly, mutations that reduce the expression or action of miR-29 and miR-30 may contribute to human carcinogenesis. Such effects may be caused by mutations in the miR-29 and miR-30 genes, mutations in other genes that affect the activity of these microRNAs, or mutations in target 3′UTRs that disrupt the binding of miR-29 and miR-30.

In summary, our results demonstrate that miR-29 and miR-30 directly repress the expression of B-Myb during senescence and argue that these microRNAs, acting in conjunction with Rb-E2F complexes at the B-Myb promoter, mediate repression of B-Myb expression during Rb activation. We further showed that miR-29 and miR-30 mediate some of the antiproliferative effects of Rb. Given the important roles of the Rb pathway and B-Myb in cell proliferation and carcinogenesis, this microRNA regulatory circuit may play a major role in controlling cell cycle progression and tumor formation.

Materials and Methods

Cell Culture and Viruses. HeLa cell lines, all containing the integrated HPV18 genome, were described previously (8, 23): HeLa/E6 (expressing an exogenous wild-type HPV16 E6 gene from the Moloney Murine Leukemia Virus LTR), HeLa/E6-E7 (expressing exogenous wild-type HPV16 E6 and E7 genes from the LTR), HeLa/E6-E7Δ (expressing a wild-type HPV16 E6 gene and a mutant E7 gene from the LTR), and HeLa/E6 RbKD (expressing shRNAs targeting $p105^{Rb}$, p107, and p130). HFFs were obtained from the Yale Skin Disease Research Center and harvested at early passage or serially passaged 1:4 until they reached senescence. All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 10 mM Hepes pH 7.3, penicillin–streptomycin, and amphotericin B (DMEM-10) at 37 °C in the presence of 5% CO_2. The BPV E2 protein was expressed from a BPV1/SV40 recombinant virus, Pava1 (E2 virus), as described in Settleman and DiMaio (34). For rapid induction of senescence, HeLa cell lines were infected with Pava1 at a moi of 20, unless indicated otherwise. SA-β-gal activity was detected by staining cells with X-Gal at pH 6.0 (3, 35).

miRNA and mRNA Analysis. The TaqMan® Low Density microRNA array (Applied Biosystems), containing primers for 365 human miRNAs, was used to analyze microRNA expression in proliferating and senescing HeLa cell lines. For these experiments, total RNA was extracted from mock-infected HeLa cell derivatives or the same cells 4 d after infection with Pava1. MicroRNAs were directly visualized by hybrization to ^{32}P-5′-labeled oligonucleotides that hybridize specifically to miR-29 or miR-30 family members. Levels of individual mature microRNAs were measured by use of specific TaqMan® miRNA assays (Applied Biosystems) with a looped reverse transcriptase primer specific for each microRNA. qRT-PCR was used to measure mRNA levels. Expression relative to endogenous control RNAs was calculated using the delta–delta cycle threshold (Ct) method. Details for RNA analysis and primer sequences are given in *SI Appendix*.

Luciferase Reporter Constructs. We used a modified pMIR-REPORT™ miRNA Expression Reporter Vector (Applied Biosystems), in which luciferase expression is driven by the CMV major early promoter. QuikChange Site-Directed

Mutagenesis (Stratagene) was used to make two silent mutations in the firefly luciferase ORF to disrupt a potential binding site for the BPV E2 protein, and we named this construct pMIREL. A 189-bp fragment (nt 2,342–2,530) of the 3′UTR of B-Myb (identification number NM_002466.2) containing the miR-29 and miR-30 binding sites was cloned into pMIREL. As a control, we cloned a 210-bp fragment (nt 599–808) of the B-Myb ORF into pMIREL. We used mutagenic primers to construct two-substitution point mutations in the seed sequence of the miR-29 and miR-30 binding sites in the B-Myb 3′UTR (Fig. 2). Detailed methods and oligonucleotides used for mutagenesis are shown in *SI Appendix*.

Transfection and Luciferase Assays. Endotoxin-free luciferase plasmids were transfected into HeLa cells by using the *Trans*IT-HeLaMONSTER Transfection kit (Mirus Bio) and into HFFs by using Lipofectamine 2000 (Invitrogen). For microRNA and anti-miR transfections, we used the siPORT™ NeoFX™ Transfection Agent (Applied Biosystems) with 80 nM total oligonucleotides, unless otherwise noted. MicroRNA and anti-miR oligonucleotides were obtained from Applied Biosystems or IDT. To induce senescence, we infected HeLa cells with Pava1 (moi of 20). After 5 d, luciferase assays were performed with the Dual-Luciferase Reporter Assay System kit (Promega). All transfections and luciferase assays were done in triplicate. Detailed methods and oligonucleotide sequences are shown in *SI Appendix*.

Immunoblot Analysis and DNA Synthesis Assay. Standard methods (see *SI Appendix*) were used to measure cellular DNA synthesis by incorporation of ^{3}H-thymidine and to detect B-Myb and Rb family members by immunoblotting.

Autofluorescence and Colony Formation. HeLa/E6 cells were transfected on day 0 and day 4 with specific anti-miRNAs and then mock-infected or infected with Pava1 (moi of 15) 30 and 55 h after the first transfection. Fifteen days after the first transfection, cells were harvested and analyzed by flow cytometry as previously described (22). For colony formation, transfected HeLa/E6 cells were seeded for colony formation the day following infection. Details are presented in *SI Appendix*.

ACKNOWLEDGMENTS. We thank Shobha Vasudevan, Jun Lu, Joanne Weidhaas, Thomas Magaldi, and Meredith Crosby for advice and assistance, and Jan Zulkeski for assistance in preparing this manuscript. Flow cytometry was carried out in the Flow Cytometry Shared Resource of the Yale Comprehensive Cancer Center. I.M. was supported in part by an National Institutes of Health postdoctoral training grant (T32 GM008753). L.L.A. was supported in part by an individual National Research Service Award (CA136128). J.A.S. is an Investigator of the Howard Hughes Medical Institute. This work was supported by a Program Project Grant CA16038 from the National Cancer Institute (to J.A.S. and D.D.).

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