miR-34a Induces the Downregulation of Both E2F1 and B-Myb Oncogenes in Leukemic Cells

Giorgio Zauli1, Rebecca Voltan2, Maria Grazia di Iasio2, Raffaella Bosco2, Elisabetta Melloni2, Maria Elena Sana2, and Paola Secchiero2

Abstract

**Purpose:** To elucidate new molecular mechanisms able to downregulate the mRNA levels of key oncogenes, such as B-Myb and E2F1, in a therapeutic perspective.

**Experimental Design:** B-Myb and E2F1 mRNA levels were evaluated in primary B chronic lymphocytic leukemia (B-CLL, n = 10) and acute myeloid leukemia (AML, n = 5) patient cells, in a variety of p53\(^{\text{wild-type}}\) and p53\(^{\text{mutated/deleted}}\) leukemic cell lines, as well as in primary endothelial cells and fibroblasts. Knockdown experiments with siRNA for p53 and E2F1 and overexpression experiments with miR34a were conducted to elucidate the role of these pathways in promoting B-Myb downregulation.

**Results:** In vitro exposure to Nutlin-3, a nongenotoxic activator of p53, variably downregulated the expression of B-Myb in primary leukemic cells and in p53\(^{\text{wild-type}}\) myeloid (OCI, MOLM) and lymphoblastoid (SKW6.4, EHEB) but not in p53\(^{\text{mutated}}\) (NB4, BJAB, MAVER) or p53\(^{\text{deleted}}\) (HL-60) leukemic cell lines. The transcriptional repression of B-Myb was also observed in primary normal endothelial cells and fibroblasts. B-Myb downregulation played a critical role in the cell-cycle block in G1 phase induced by Nutlin-3, as shown by transfection experiments with specific siRNA. Moreover, we have provided experimental evidence suggesting that miR-34a is a central mediator in the repression of B-Myb both directly and through E2F1.

**Conclusions:** Owing to the role of B-Myb and E2F1 transcription factors in controlling cell-cycle progression of leukemic cells, the downregulation of these oncogenes by miR-34a suggests the usefulness of therapeutic approaches aimed to modulate the levels of miR-34a. Clin Cancer Res; 17(9); 2712–24. ©2011 AACR.

Introduction

Nutlin-3a represents a potent and selective small molecule inhibitor of the p53–MDM2 interaction (1), which raises the levels of p53 protein and induces subsequent cytostatic or cytotoxic effects in a variety of tumor cells (1). Taking into consideration that p53 is mutated in less than 15% of hematological malignancies at diagnosis (2), it is noteworthy that Nutlin-3 has shown in vitro promising therapeutic potential in both myeloid and lymphoid leukemia characterized by a p53\(^{\text{wild-type}}\) (p53\(^{\text{wt}}\)) status (3–9). However, because p53 mediates different cellular functions, such as in particular cell-cycle arrest and apoptosis (3) the critical molecular determinants mediating/modulating the therapeutic activity of Nutlin-3 in leukemic cells are not completely understood.

Previous studies have shown that several solid tumors are characterized by overexpression of B-Myb, also known as MYBL2 (10–13), a member of the vertebrate MYB family of nuclear transcription factors, which recognize and bind to the DNA consensus sequence [PyAAC(G/T)G] to promote gene transcription (14). B-Myb, unlike C-Myb and A-Myb, is expressed in virtually all proliferating cells as a regulator of cell-cycle progression and cell survival (15) and it plays an essential role in vertebrate development (16). Indeed, the knock-down of murine B-Myb causes early embryonic lethality (E4.5-6.5) resulting from unsuccessful inner cell mass formation (16). Interestingly, it has also been shown that B-Myb chromosomal locus (20q13) is amplified and/or highly expressed in a variety of tumor types including neuroblastomas and breast, prostate, liver and ovarian carcinomas, and in most cases this high expression is associated with a poor prognosis (10–13).
miR34a Downregulates B-Myb Expression

Translational Relevance

B-Myb oncogene was found overexpressed in both primary acute myeloid leukemia blasts. The small molecule inhibitor of MDM2–p53 interactions, Nutlin-3, efficiently repressed both B-Myb and E2F1 in a variety of p53-wild-type leukemic cell types as well as in primary endothelial cells and fibroblasts. Gene knockdown and overexpression experiments showed that Nutlin-3 upregulated miR34a, which in turn induced the downregulation of B-Myb both directly and through the downregulation of E2F1. These data strengthen the notion that the upregulation of miR34a might have important clinical applications for the treatment of human leukemias.

On these bases, the aim of the present study was to investigate the baseline expression levels and the potential regulation and/or involvement of B-Myb in the therapeutic response to Nutlin-3 in myeloid and lymphoid leukemias, as well as in primary stromal cells.

Materials and Methods

Cell cultures

Peripheral blood samples were collected in heparin-coated tubes from 5 patients affected by acute myeloid leukemia (AML: 2 M4, 1 M5, and 2 M4/M5), 10 patients affected by B-chronic lymphocytic leukemia (B-CLL), and 10 healthy blood donors following informed consent, in accordance with the Declaration of Helsinki and in agreement with institutional guidelines. Peripheral blood mononuclear cells (PBMC) from AML and B-CLL patients and from healthy donors were isolated by gradient centrifugation with lymphocyte cell separation medium (Cedarlane Laboratories). Specific CD19+ and CD34+ cell populations were selected from normal PBMC by using immunomagnetic microbeads and the AutoMACS system (Miltenyi Biotech) in accordance with the manufacturer’s instructions, obtaining a purity exceeding 90% for each subpopulation. For analysis of CD19+ cells, RNA preparations were obtained from 5 independent donors. For analysis of CD34+ cells, RNA preparation (n = 4 in total) was obtained by pooling CD34+ cells purified from at least 3 different donors.

The p53wt (OCI, MOLM, SKW6.4, EHEB), p53mutated (NB4, BJAB, MAVER), and p53deleted (HL-60) leukemic cell lines were purchased from the ATCC (American Type Culture Collection) or obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Control PBMC and patient cells were cultured in RPMI-1640 (Gibco BRL) containing 10% FBS, L-glutamine and penicillin/streptomycin (Gibco BRL). The cell lines were cultured in RPMI-1640, or in Alpha MEM (OCI) (LONZA) supplemented with 10% FBS and L-glutamine and penicillin/streptomycin (Gibco BRL). Cells were seeded at a density of 1 × 10⁶ cells/mL before treatment with Nutlin-3 (used in the range of 0.1–10 μmol/L; Cayman Chemical) or Chlorambucil (used in the range of 0.10–10 μmol/L; Sigma-Aldrich). The HCT116 human colon cancer cells, with either p53wt or p53 knockout (p53+/−), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) media supplement with 10% FBS (17).

Human umbilical vein endothelial cells (HUVEC) were purchased from BioWhittaker and grown as previously described (18). For the evaluation of cytostatic and/or toxic effects, HUVEC and fibroblasts were seeded and grown to subconfluence before treatment with Nutlin-3 (10 μmol/L). Neonatal human dermal fibroblasts were purchased from LONZA and grown in DMEM (LONZA) containing 20% FBS, L-glutamine and penicillin/streptomycin (Gibco BRL).

Assessment of cell viability, apoptosis, and cell cycle

Cell viability was examined by Trypan blue dye exclusion, and induction of apoptosis was quantified by Annexin V–FITC/propidium iodide (PI) staining (Immunootech) followed by flow cytometry analysis (19). To avoid nonspecific fluorescence from dead cells, live cells were gated tightly by using forward and side scatter, as described (20). To analyze the degree of apoptosis in the entire cell population of HUVEC and fibroblast cultures, substrate-attached cells were harvested by trypsin treatment and pooled with floating cells for the staining.

Cell-cycle profile was analyzed by flow cytometric analysis, as previously described (21).

Real time PCR

Total RNA was extracted from cells by using the Qia-gen RNAlater Mini kit (Qiagen) according to the supplier’s instructions. Total RNA was transcribed into cDNA, using the QuantiTect Reverse Transcription kit (Qiagen). Levels of miR-34a expression were evaluated by using the miScript PCR System (Qiagen), composed of the miScript Reverse Transcription kit and the miScript SYBR Green PCR kit for cDNA amplification with specific miScript Primer Assays for miR34a (Qiagen). The expression of miR-34a values was normalized by using specific primers to RNU6b (Qiagen) as endogenous reference RNA.

Western blot analyses

Cells were lysed in ice-cold radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate] supplemented with protease inhibitors (Complete, Roche) on ice for 1 hour. Protein determination was carried out by BCA Protein Assay (Thermo Scientific) and then added with loading buffer [250 mmol/L, 6× SDS-PAGE]. Cell lysates were loaded onto a 4–12% Bis-Tris polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membranes were blocked in 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour, and then probed with relevant antibodies. After washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using chemiluminescent substrate solution (Amersham). The bands were quantitated via densitometry analysis (ImageJ, NIH).

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Tris (pH 6.8), 2% SDS, 40% Glycerin, 20% β-mercaptoethanol] and boiled for 2 minutes (22). Equal amounts of protein for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters, as previously described. The following monoclonal antibodies were used in our experiments: anti-p53 (DO-1; Santa Cruz Biotechnology), anti-E2F1 (KH95; Santa Cruz Biotechnology), anti-pRb (G3-245; BD Biosciences Pharmingen), and anti-tubulin (Sigma-Aldrich). After incubation with peroxidase-conjugated anti-mouse immunoglobulin G, specific reactions were revealed with the ECL Lightning detection kit (Perkin Elmer). Densitometric values were estimated by the ImageQuant TL software (GE Healthcare). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.
Transfection experiments

Cells \( (1 \times 10^6) \) were resuspended into 0.1 mL of Nucleofector solution of human nucleofector kit NHDF or V (Amaxa). Two micrograms of plasmid DNA (green fluorescent protein construct) or 1 μg of siRNA (see Supplementary Material and Methods) were mixed with the 0.1 mL of cell suspension, transferred into a 2.0 mm electroporation cuvette, and nucleofected by an Amaxa Nucleofector II apparatus, following the manufacturer’s guidelines. After transfection, cells were immediately transferred into complete medium and cultured in T175 flasks at 37°C. Transfection efficiency was estimated in each experiment by scoring the number of GFP-positive cells by fluorescence microscopy examination and by flow cytometry analysis.

In miR-34a functional studies, cells were transfected with the hsa-miR-34a precursor oligo, for mimicking the overexpression of this miRNA, or with the hsa-miR-34a anti-miR oligo, for the inhibition of the activity of endogenous miR-34a. In parallel, cells were transfected with the FAM-labeled miR negative control oligo as a nontargeting negative control and for monitoring transfection efficiency. All oligos were from Ambion.

Statistical analysis

Data are calculated and shown as mean ± SD or as median and interquartile range, according to the distribution. The results were evaluated by using ANOVA with subsequent comparisons by Student’s t test and with the Mann–Whitney rank-sum test. Correlations were tested by Spearman’s correlation coefficient. Statistical significance was defined as \( P < 0.05 \). To investigate the effect of Chlorambucil plus Nutlin-3 combination, cells were then treated with serial dilutions (from 10 to 0.1 μmol/L) of Chlorambucil or Nutlin-3, individually or in combination, by using a constant ratio (Chlorambucil/Nutlin) 1:1. Results were analyzed by the CalcuSyn software program (Biosoft), which uses the method of Chou and Talalay (23). A combination index (CI) of 1 indicates an additive effect, whereas a CI less than 1 indicates synergism.
Results

Transcriptional repression of B-Myb and E2F1 by Nutlin-3 in p53wild-type leukemic cells

In the first group of experiments, B-Myb mRNA levels were analyzed by quantitative Real Time (RT)-PCR in samples obtained from AML and B-CLL patients in comparison with primary normal CD34\(^+\) progenitor cells and CD19\(^+\) B cells, respectively (Supplementary Fig. S1). AML samples showed significantly (\(P < 0.01\)) greater levels of B-Myb mRNA as compared with normal CD34\(^+\) cells (Supplementary Fig. S1). Also B-CLL showed an increase of B-Myb mRNA endogenous levels with respect to primary CD19\(^+\) B cells but the difference did not reach statistically significance. Because a constitutive expression of B-Myb can bypass p53-mediated G1 arrest and is required for recovery from the DNA damage-induced G2 checkpoint in p53 mutant cells (24), we investigated the potential modulation of B-Myb in response to Nutlin-3, a nongenotoxic activator of the p53 pathway. The \textit{in vitro} exposure to Nutlin-3 (10 \(\mu\)mol/L) for 24 hours variably downregulated the mRNA levels of B-Myb in primary leukemic cells (Fig 1A). To explore the molecular mechanisms underlining the Nutlin-3–induced downregulation of B-Myb, we have used a series of leukemic cell lines, characterized by a different p53 status. After 24 hours of Nutlin-3 treatment, B-Myb levels were clearly (\(P < 0.01\)) decreased in all p53\(^+\) leukemic cells of both

![Figure 2.](image-url)
myeloid (OCI, MOLM) and lymphoid (EHEB, SKW6.4) origin (Fig. 1B). On the contrary, in p53 mutated myeloid (NB4) and lymphoid (BJAB, MAVER) cell lines, Nutlin-3 treatment did not affect B-Myb mRNA levels (Fig. 1B). Similarly, lack of B-Myb downregulation was observed in the myeloid p53 deleted HL-60 cell line (Fig. 1B).
Figure 4. Inverse correlation between Nutlin-induced upregulation of miR-34a and downregulation of B-Myb and E2F1. A, prediction of hsa-miR-34a binding site in the 3′-UTR of B-Myb gene. B, modulation of miR-34a mRNA by Nutlin-3 was analyzed by quantitative RT-PCR in the indicated cell lines as well as in fibroblasts (Fibrobl.) and endothelial (Endoth.) cells. C, the p53wt EHEB cell line was either left untreated or exposed to Nutlin-3 (10 μmol/L). At the indicated time points, levels of E2F1, B-Myb, and miR-34a were simultaneously assessed by RT-PCR. D, distribution of cells (EHEB) in the different phases of the cell cycle was calculated from the flow cytometry dot plots after BrdU/PI staining, and expressed as the percentage of the total population. C, levels of E2F1, B-Myb, and miR-34a were expressed as fold of modulation with respect to the control untreated cultures (time = 0). Data are reported as mean ± SD of results from 3 independent experiments. *, P < 0.05 with respect to the untreated cultures.
As it has been shown that B-Myb is a transcriptional target of E2F1 (25, 26) and previous studies have shown that E2F1 is downmodulated by Nutlin-3 in solid tumor models (27), in parallel, we have analyzed the effect of Nutlin-3 on E2F1 expression. As shown in Figure 1C, treatment with Nutlin-3 strikingly downregulated also E2F1 RNA levels in the p53wt, but not in p53mutated/deleted leukemic cell lines.

To clarify the p53-dependence of the observed gene modulations, we have then used isogenically matched set of cells differing only for p53 status. For this purpose, we have analyzed the effect of Nutlin-3 in OCI cells transiently transfected with p53 siRNA (Fig. 1D). Because transfection efficiency is approximately 40% in OCI cells, p53 is not silenced but significantly knocked down, as evaluated by quantitative RT-PCR assay (data not shown). The ability of Nutlin-3 to decrease both B-Myb and E2F1 RNA levels was significantly (P < 0.05) attenuated with respect to OCI cells transfected with control scramble siRNA (Fig. 1D). Moreover, the dependence from p53 of Nutlin-3-mediated downregulation of B-Myb was independently confirmed by examining B-Myb and E2F1 RNA modulation by Nutlin-3 by using the p53 isogenic-paired cell lines HCT116 p53wt and HCT116 p53mut (Fig. 1D).

Transcriptional downregulation of B-Myb and E2F1 by Nutlin-3 is related to cell-cycle block rather than to apoptosis induction

Given that both B-Myb and E2F1 have been involved in modulating either the survival or the proliferation of tumor cells (28–30), we have next investigated whether the downregulation of B-Myb and E2F1 by Nutlin-3 was related to the induction of apoptosis, cell-cycle arrest, or both events. For this purpose, we have followed 2 independent approaches: (i) we have compared the effects of Nutlin-3 (in terms of transcriptional modulation of E2F1, B-Myb, cell-cycle arrest, and apoptosis induction) with those of Chlorambucil, a chemotherapeutic drug commonly used for the treatment of hematological malignanties, and (ii) we have analyzed the effects of Nutlin-3 (again, in terms of transcriptional modulation of E2F1, B-Myb, cell-cycle arrest, and apoptosis induction) also in primary normal endothelial cells and fibroblasts.

Treatment with Nutlin-3 and Chlorambucil (both used at 10 μmol/L) induced a comparable accumulation of p53 protein in the p53wt EHEB and SKW6.4 cells lines, whereas p53mutated BJAB cells were characterized by constitutive high levels of p53 that were unaffected by either Nutlin-3 or Chlorambucil treatment (Fig. 2A). Interestingly, Nutlin-3 and Chlorambucil (both used in the range of 0.1–10 μmol/L) induced comparable levels of cytotoxicity, as evaluated by assessing the percentage of cell viability and apoptosis induction, in the p53wt SKW6.4 and EHEB cells, but not in p53mutated BJAB cells (Fig. 2B). However, in p53mutated cells, Nutlin-3 and Chlorambucil exhibited strikingly different effects on cell-cycle progression of these cell lines (Fig. 2C and D). Indeed, although both drugs induced cell-cycle arrest, as indicated by reduction in cell counts, Nutlin-3 exposure induced the accumulation of cells in G1 and almost abrogated S phase in p53wt leukemic cells, Chlorambucil increased the percentage of cells in S and G2/M phases, as clearly assessed by bromodeoxyuridine (BrdU) incorporation (Fig. 2C and D). These effects of Nutlin-3 were accompanied by drastic reduction of E2F1 protein in both p53wt EHEB and SKW6.4 cell lines, but not in p53mutated BJAB (Fig. 2E). On the contrary, exposure to Chlorambucil did not reduce (and, rather, it tended to increase) E2F1 mRNA and protein levels in the p53mutated EHEB and SKW6.4 or p53mutated leukemic cells (Fig. 2E and F). Similarly, Chlorambucil treatment did not affect the steady-state mRNA levels of B-Myb in the 3 B-cell lines investigated (Fig. 2F).

When cultures were treated with serial concentrations of Nutlin-3 plus Chlorambucil (in the range of 0.1–10 μmol/L) at a constant Nutlin-3/Chlorambucil ratio 1:1 (for data analysis by the method of Chou and Talalay; ref. 23), a synergistic cytotoxicity (average CI values less than 1) of combination treatment was observed in p53wt, but not in p53mutated, cell lines (Supplementary Fig. S2A). Even in the combined treatment Nutlin-3 + Chlorambucil, as in the treatment with Nutlin-3 alone, both E2F1 and B-Myb were decreased (Supplementary Fig. S2B), and cell cycle was blocked in G1 phase, as documented by BrdU incorporation (data not shown) and analysis of pRb in Western blot (Supplementary Fig. S2A).

In parallel experiments, we have investigated whether Nutlin-3 affected the levels of B-Myb and E2F1 also in primary fibroblasts and endothelial cells. Treatment with Nutlin-3 of fibroblasts and endothelial cells induced a striking transcription repression of B-Myb, as well as of E2F1 (Fig. 3A), a finding confirmed for E2F1 also at the protein level (Fig. 3A). Similar to the data obtained on leukemic cell lines, Nutlin-3 induced a cell-cycle block in G1 also in fibroblasts and endothelial cells (Fig. 3B), but with negligible effects on the background levels of apoptosis (data not shown). Taken together, these findings suggested that the transcription downregulation of B-Myb by Nutlin-3 was related to its ability to block cell cycle, rather than to its ability to induce apoptosis. To further elucidate the potential cause–effect relationship between downmodulation of B-Myb and cell-cycle arrest, we have conducted knock-down experiments of endogenous B-Myb by using fibroblast cultures, which were used for their high efficiency of transfection. As shown in Figure 3C, at 24 to 48 hours after B-Myb siRNA transfection we observed a marked, and specific, decrease of B-Myb mRNA, but not of p53 (analyzed for control; data not shown), accompanied by a significant (P < 0.01) decrease of the percentage of cell in S-phase (Fig. 3C).

Key role of miR-34a in mediating the downregulation of B-Myb by Nutlin-3

To characterize the mechanism of B-Myb transcription repression by Nutlin-3, we have conducted a target
prediction analysis by NBmiRTar, an in silico approach based on machine learning by a Naive Bayes classifier (31). The program identified a potential binding site for miR-34a in the 3′ untranslated region (UTR) of B-Myb (Fig. 4A; chr 20:41778338–41778361). Because different reports have shown that one pathway through which p53 regulates cell growth is through regulation of a set of miRNA, including miR-34a (32, 33), we have investigated the hypothesis of a role for miR-34a in the downregulation of B-Myb by Nutlin-3. For this purpose, we first measured the modulation of miR-34a in the cell models in which we had previously documented transcriptional repression of B-Myb by Nutlin-3 (Fig. 1B–D). As shown in Figure 4B, we have observed a significant induction of miR-34a in response to Nutlin-3 treatment in all p53wt, but not in the p53mutated/deleted cells. Moreover, in time-course experiments we found that exposure to Nutlin-3 induced a rapid (within 10 hours) induction of miR-34a concomitant to the transcriptional decrease of B-Myb and E2F1 (Fig. 4C), and to the induction of cell-cycle arrest (Fig. 4D). These data showed an inverse correlation between induction of miR-34a and downmodulation of B-Myb and E2F1, but did not prove yet that the Nutlin-3–mediated downregulation of B-Myb and/or E2F1 is mediated by miR-34a. Thus, to functionally test the hypothesis that miR-34a decreases B-Myb expression, we transfected primary fibroblasts with the precursor to miR-34a (designated miR-34a mimic). As a control, some cells were transfected with scrambled miRNA. As shown in Figure 5A, the precursor to miR-34a significantly
miR34a Downregulates B-Myb Expression

(P < 0.01) decreased B-Myb levels. Consistently with previous studies (34, 35) overexpression of miR-34a also downmodulated E2F1 expression (Fig. 5B). On the contrary, it did not affect p53 and p21 expression (Fig. 5B). In additional experiments, the downregulation of miR34a with a specific antago-miR significantly (P < 0.05) counteracted the ability of Nutlin-3 of downregulating both E2F1 and B-Myb (Fig. 5C).

By computational analysis (as predicted by SABiosciences’ Text Mining Application and the UCSC Genome Browser) both E2F1 and B-Myb promoters exhibit several p53 consensus DNA binding sites, which have previously suggested a gene regulation/repression occurring through p53 binding directly to the consensus DNA binding sequences (36, 37). In addition, E2F1 DNA binding sites have been found in the B-Myb regulatory region, indicating that a potential regulation of B-Myb is also mediated by E2F1 (36–38). This was confirmed in our cell model by E2F1 knockdown experiments. Indeed, as shown in Figure 6A, siRNA specific for E2F1 induced a significant (P < 0.05) downregulation of B-Myb, confirming the important role of E2F1 upstream of B-Myb. Of interest,
the analysis by in silico prediction by miRecords, which integrates the predicted targets of different miRNA target prediction tools (39), identifying a binding site for miR-34a in 3′-UTR region of E2F3 and E2F5, but not in E2F1. This observation supports the proposed regulation of E2F1 by miR-34a through an indirect mechanism (34, 35). In next analysis, all leukemic samples were divided in 2 groups on the basis of the endogenous levels of miR-34a (below or above the median value; Fig. 6B). The comparison of the endogenous levels of B-Myb and E2F1 between the 2 groups showed that the samples characterized by lower miR-34a levels exhibited significantly (P < 0.05) higher B-Myb and E2F1 levels (Fig. 6B). In addition, analysis of Nutlin-3 modulation (expressed as folds) in all samples assayed showed a significant inverse correlation between the modulation of miR-34a and B-Myb (R = −0.55, P < 0.01), as well as between miR-34a and E2F1 (R = −0.65, P < 0.01), whereas a significant positive correlation was observed between the modulation of B-Myb and E2F1 (R = 0.81, P < 0.01). It should be emphasized that our present data suggest a previously uncharacterized mechanism of negative regulation of B-Myb, through miR-34a, that together with E2F1, mediates p53-suppression of cell proliferation in response to Nutlin-3 treatment (Fig. 6C).

Discussion

Starting from the observation that B-Myb was overexpressed in primary AML patients cells in comparison with normal CD34+ progenitor cells, we have shown for the first time that the nongenotoxic activator of p53 pathway, Nutlin-3, significantly downregulated transcription of both B-Myb and E2F1 in p53wt leukemic cells, but not in p53mutated/deleted leukemic cell lines. The ability of Nutlin-3 to downregulate B-Myb and E2F1 in AML cells is particularly noteworthy because it has been previously shown that high levels of expression of B-Myb (40, 41) and E2F1 (42, 43) contribute to the block of maturation characterizing AML. On the contrary, the levels of B-Myb mRNA in primary B-CLL were not significantly different from that of primary CD19+ B cells and Nutlin-3 induced a modest downregulation of B-Myb in B-CLL cells. As circulating B-CLL cells are primarily in G1 phase of the cell cycle, these findings are consistent with a predominant involvement of Nutlin-3–mediated downregulation of B-Myb in the cell-cycle block, rather than in the induction of apoptosis.

The primary role of B-Myb downregulation by Nutlin-3 in cell-cycle block was strongly supported by the differential effect of Nutlin-3 and Chlorambucil with respect to induction of apoptosis and cell-cycle arrest. In fact, Nutlin-3 and Chlorambucil induced a comparable level of apoptosis in p53wt leukemic cell lines, but the effect of Chlorambucil was strikingly different from that of Nutlin-3 with respect to cell cycle, because Nutlin-3 promoted the accumulation of leukemic cells in G1, whereas Chlorambucil induced the accumulation of leukemic cells in G2/M. Consistently with these different effects, treatment with Chlorambucil did not decrease E2F1 and B-Myb expression in p53wt leukemic cells. The observations obtained in leukemic cells were extended also to normal

Figure 6. (Continued) C, scheme of proposed mechanism for p53-regulation of E2F1 and B-Myb by miR-34a, on Nutlin-3 treatment.
endothelial cells and fibroblasts. In this respect, it is particularly noteworthy that E2F1 has been recently involved in promoting proangiogenic pathways (44). Therefore, the ability to downregulate E2F1 in endothelial cells can contribute to antiangiogenic activity of Nutlin-3 (45) independently of its effects on primary tumor cells.

In agreement with previous data obtained in other cell models (36–38), our present findings also suggested that E2F1 is involved in B-Myb downregulation at transcriptional level. First of all, we could show that the modulation of mRNA levels of E2F1 and B-Myb transcription factors, assessed in response to Nutlin-3 in all cell types investigated, significantly and positively correlated. Moreover, we have observed that transcriptional E2F1 downregulation anticipated B-Myb decline. Finally, the knockdown of endogenous E2F1 determined downregulation of B-Myb. Nevertheless, the most striking and novel finding of our study in terms of molecular mechanisms involved in the control of B-Myb regulation is the involvement of miR-34a upregulation in mediating the effect of Nutlin-3. Consistently with recent reports indicating that some of the p53–downregulated genes might be associated with miRNA expression (31, 32), we have shown for the first time that miR-34a is a key determinant in the biological activity of B-Myb. Additionally, we have investigated, significantly and positively correlated. More-
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