miR-342-3p regulates MYC transcriptional activity via direct repression of E2F1 in human lung cancer

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Abstract

Accumulating evidence indicates that altered miRNA expression is crucially involved in lung cancer development, though scant information is available regarding how MYC, an archetypical oncogene, is regulated by miRNAs, especially via a mechanism involving MYC cofactors. In this study, we attempted to identify miRNAs involved in regulation of MYC transcriptional activity in lung cancer. To this end, we utilized an integrative approach with combinatorial usage of miRNA and mRNA expression profile datasets of patient tumor tissues, as well as those of MYC-inducible cell lines in vitro. In addition to miRNAs previously reported to be directly regulated by MYC, including let-7 and miR-17–92, our strategy also helped to identify miR-342-3p as capable of indirectly regulating MYC activity via direct repression of E2F1, a MYC-cooperating molecule. Furthermore, miR-342-3p module activity, which we defined as a gene set reflecting the experimentally substantiated influence of miR-342-3p on mRNA expression, was found to be inversely correlated with MYC activity reflected by MYC module activity in three independent datasets of lung adenocarcinoma patients obtained from the Director’s Challenge Consortium of the United States (P = 1.94 × 10⁻⁷³), the National Cancer Center of Japan (P = 9.05 × 10⁻³⁴) and the present study (P = 1.17 × 10⁻¹⁹). Our integrative approach appears to be useful to elucidate inter-regulatory relationships between miRNAs and protein coding genes of interest, even those present in patient tumor tissues, which remains a challenge to better understand the pathogenesis of this devastating disease.

Introduction

Lung cancer has long been the leading cause of cancer death, with lung adenocarcinoma the most prevalent subtype. Multiple oncogenes and tumor suppressor genes are involved in the molecular pathogenesis of lung adenocarcinoma, while accumulating evidence also indicates that altered expression of miRNAs is crucially involved (1,2). miRNAs function as an RNA molecule of ~22 nt in length and repress the expression of protein coding genes by binding to 3′ untranslated regions (UTRs) of even hundreds of target genes (3,4). We previously discovered that let-7 is frequently downregulated in lung cancer tissues in association with poor postoperative prognosis and possesses growth inhibitory activities (5). Interestingly, let-7 was subsequently shown to target an archetypical oncogene, KRAS (6). In addition, our previous studies revealed that amplification and overexpression of the miR-17–92 cluster is a frequent event in lung cancer leading to enhanced cell proliferation and reduced apoptosis (7–10). It is also interesting to note that this oncogene-like miRNA cluster is known to be under regulation of another typical oncogene, MYC (11).

Although various oncogenes have thus far been identified to be altered in various types of lung cancer, MYC is among the...
most frequently amplified and overexpressed (12,13). The MYC gene encodes a transcription factor that regulates a wide variety of genes involved in control of cell growth, proliferation and apoptotic cell death (14,15). The transcriptional activity of MYC is tightly controlled for proper transcriptional regulation through various mechanisms, which include MYC expression itself at both transcriptional and posttranscriptional levels, as well as its interaction with cofactors that functionally cooperate with MYC. Unfortunately, very little is known thus far about how MYC is regulated by miRNAs in lung cancer cells, especially via the latter mechanism involving MYC cofactors.

In the present study, we attempted to identify miRNAs involved in the regulation of MYC transcriptional activity in lung cancer. We employed an integrative strategy that utilized combinatorial expression profile datasets for both miRNAs and mRNAs obtained from the same sets of patient tumor tissues as well as MYC-inducible cell lines. Our integrative approach showed that in addition to miRNAs directly regulating or regulated by MYC, including those previously reported, miR-342-3p is capable of regulating MYC activity via repression of an MYC-cooperating molecule, E2F1.

Materials and methods

Tumor specimens and RNA preparations

Seventy-six lung adenocarcinoma tumor tissues from patients who underwent potential curative resection between December 1995 and August 1999 were collected at Aichi Cancer Center, Nagoya, Japan. This cohort of patients consisted of 40 males and 36 females (pStage I: n = 43; pStage II: n = 10; pStage III: n = 23), whose mean age was 61.5 years (range: 32–84 years). All tumor specimens were collected under approval from the institutional review boards of Aichi Cancer Center and Nagoya University with written informed consent from each patient. Frozen tumor specimens were subjected to gross microdissection under the guidance of a pathologist (Y.Y.) to ensure a tumor cell content of at least 50% using every 10th section stained with Giemsa. In addition, 5 sets of RNA mixtures, each of which contained 5 independent normal lung RNAs (25 samples in total) were analyzed. Total RNAs were extracted using a miRNeasy Mini kit (Qiagen) according to the manufacturer’s instructions.

Cell lines

NCI-H23, NCI-H2009, NCI-H441 and NCI-H1975 lung adenocarcinoma cell lines were purchased from American Type Culture Collection. ACC-LC-172, ACC-LC-76 and ACC-LC-94 lung adenocarcinoma cell lines were established by our group (16). An immortalized lung epithelial cell line, BEAS-2B, and the SK-LC-3 lung adenocarcinoma cell line were generous gifts from Curtis C. Harris (National Cancer Institute) and the late Lloyd J. Old, respectively. All lung adenocarcinoma cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, while BEAS-2B cells were cultured in F12 supplemented with 1% fetal bovine serum. All cell lines were confirmed to be absent of mycoplasma contamination (MycoAlert; Lonza). Verification of these cell lines was performed by short tandem repeat profiling at the Japanese Collection of Research Bioreresources, National Institute of Biomedical Innovation of Japan and last checked in February 2015.

Plasmids, PCR primers, siRNAs and antisense locked nucleic acid oligomers

Full-length human MYC cDNA was excised from pMXs-hc-myc (Addgene, plasmid #13375) and inserted into a tet-ON lentiviral expression vector, CSIV-TRE-RNA-CMV-K: to generate a luciferase reporter construct carrying a potential miR-342-3p target site, a 1193 bp fragment of the EZF1 3′-UTR was amplified with human genomic DNA (Promega), followed by cloning into the SfiI and XbaI sites of a modified pg3l3 vector (Promega). The PCR primers used were as follows: 5′-ATAAGGCGCTAGCTCCCTGCTACAGGCGAACAAAACACCGCAGGA-3′ and 5′-ATAATTCAGGTTTCCAGACATGGCAGC-3′. Site-directed mutagenesis was employed to replace these nucleotides corresponding to the seed sequence using a KOD plus mutagenesis kit (Toyobo) and the following primers: 5′-TCCACACCATGTGTTGTCATGTACAGGCGAACAAAACACCGCAGGA-3′ and 5′-ACATTCCACCCCGCTGACCATGCTGTTTCCAGACATGGCAGC-3′. miRNA mimics including Pre-miR-99a (PM12328), Pre-miR-342-3p (PM10719), Pre-miR-34a (PM11030), Pre-miR-140-5p (PM10205), Pre-miR-125a (PM12551) and Pre-miR-NC#2 (AM17111) were purchased from Ambion. Five nanomoles per liter miRNA mimics were transfected into 1 × 10⁶ cells per well of a six-well plate using RNAiMAX (Invitrogen). siRNAs against MYC (J-003282-26) and EZF1 (J-003259-09), as well as a non-targeting control siRNA (D-001810-02) were purchased from Thermo Scientific Dharmacon. Antisense (AS) locked nucleic acid (LNA)-modified oligonucleotides against miR-342-3p and a scrambled control (SC) were purchased from Gene Design. The nucleotide sequences of the oligomers were as follows: miR-342-3p AS: 5′-ACGGGUGCGAUUUCTGTGTGAGA-3′ and SC: 5′-ACGGGUGCGAUUUCTGTGTGAGA-3′. Twenty nanomoles per liter of siRNAs and 50 nmol/l of AS oligonucleotides were transfected using RNAiMAX into 1 × 10⁶ and 5 × 10⁴ cells, respectively, per well of six-well plates.

Generation of stable lines transfused with tet-inducible MYC-expressing lentivirus

pCSIV-empty or pCSIV-MYC vectors were transfected into 293T cells, then the supernatants containing the resultant lentiviruses were harvested at 72 h posttransfection and filtered and centrifuged at 50000g for 2 h. BEAS-2B and SK-LC-3 cells were transfected with the lentiviruses, followed by isolation of Kusabira Orange-positive cells using a BD FACSaria™ II cell sorter (BD Biosciences). Infected bulk-cultured cells were used to harvest total RNAs using a miRNeasy Mini kit (Qiagen) after treatment with 5 μg/ml doxycycline (DOX) for 12, 24 and 48 h.

Microarray analysis

Microarray analysis was conducted using a SurePrint G3 Human Gene Expression 8 × 60K Microarray Kit and GeneSpring version 12.6 (Agilent), as previously described (17). All microarray data obtained in this study are available at the Gene Expression Omnibus under the following accession number: GSE66760. Quantitative reverse transcriptase PCR (RT-PCR) analysis of individual miRNAs was carried out using the primers listed in Supplementary Table 1, available at Carcinogenesis Online, along with Power SYBR Green PCR Master Mix (Life Technologies) and an ABI Prism 7500 (Life Technologies). Expression levels were calculated using the standard curve method and normalized with the expression of 18S.

Measurements of miRNA expression

Six hundred nanograms of RNAs were reverse transcribed using a TaqMan MicroRNA Reverse Transcription kit (Life Technologies), according to the manufacturer’s instructions, and the resultant cDNAs were applied to TaqMan Low Density Array (TLLDA) Human MicroRNA Panels (A: v2.0 and B: v3.0, both from Life Technologies), followed by scanning using an ABI Prism 7900HT Sequence Detection System (Life Technologies). Raw C values were calculated using RQ manager software v1.2.1 (Life Technologies). The expression levels of the miRNAs were normalized with that of the non-coding RNA RNU44, with a C value higher than 32 interpreted as being too low to accurately quantify. The expression of individual miRNAs was determined by quantitative RT-PCR analysis using TaqMan MicroRNA Assay (Life Technologies) and TaqMan MicroRNA RT (Life Technologies) kits and normalized using the non-coding RNA RNU44.
Western blot analysis

Western blot analysis was performed according to standard procedures using Immobilon-P filters (Millipore) and an Enhanced Chemiluminescence system (ECL Healthcare). The primary antibodies used were anti-MYC (Cell Signaling), anti-jun-actin (Sigma-Aldrich), anti-E2F1 (3742, Cell Signaling), anti-KAT2A (C26A10, Cell Signaling) and anti-EP300 (Santa Cruz).

Flow cytometric analysis

To evaluate cell cycle alterations, cells were first treated with miR-342-3p mimics or siRNAs against MYC or E2F1 for 48 h, and then with 100 ng/ml nocodazole (Wako Chemicals) for 24 h. Following trypanosmitization, all cells were collected and fixed with 70% ethanol and 30% phosphate-buffered saline at −20°C, followed by treatment with RNase and propidium iodide. Cells (3 × 10⁶) were analyzed using a FACSCalibur system (Becton Dickinson). Proportions of cells in each cell cycle phase were estimated using ModFit LT software (Verity Software House). To further analyze the effects on cell cycle, cells were synchronized at the G1–S phase by use of a thymidine–aphidicolin double block. Briefly, cells were transfected with the indicated siRNA or miRNA mimics and incubated with 2 mmol/l of thymidine for 24 h. Cells transfected with negative controls were then released from S phase arrest by replacing with fresh medium for 10 h, while those transfected with miR-342-3p mimics or siRNAs against either MYC or E2F1 were incubated in fresh medium for 14 h because of their delayed cell cycle progression. Aphidicolin was then added at 5 mmol/l for the second block for 14 h. Cells were released from the G/S phase block and harvested 5 h later for fixation. After propidium iodide staining, 1 × 10⁶ cells were analyzed by flow cytometry.

Dual-luciferase reporter assay

NCI-H1975 cells (1 × 10⁵ per well in a six-well plate) were transfected with either an empty pGL3 vector or that carrying the 3′-UTR of E2F1 with wt or mutant miR-342-3p target sites (0.3 μg), together with a pRLTK vector (0.1 μg), and sequentially transfected with 50 nmol/l miR-342-3p AS or SC oligonucleotides 24 h after the initial transfection. Luciferase assays were performed 48 h after AS or SC transfection using a Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. Each assay was performed in triplicate.

Cell viability and colony formation assays

The number of viable cells was evaluated at 96 h after transfection to 5 × 10⁴ NCI-H2009 or NCI-H23 cells in six-well plates using a Cell Counting Kit-8 (Dojindo, Japan), according to the manufacturer’s instructions. Cell viability was expressed as the mean percentage of the control viability (100%) of three independent experiments. For colony formation assays, transfected cells were seeded at a density of 4 × 10³ cells per 10 cm dish and cultured for 14 days, then the colonies were fixed with formalin and stained with Giemsa (Sigma-Aldrich). The number of colonies was quantified using Image software 1.46r (National Institutes of Health). All assays were performed in triplicate.

Definition of MYC module

We defined the MYC module as a surrogate of the transcriptional activity of MYC based on two-color microarray data (Supplementary Methods, available at Carcinogenesis Online). In brief, we first measured RNA abundance in MYC-inducible BEAS-2B and SK-LC-3 cells at 0 (baseline), 12, 24 and 48 h after initiation of DOX treatment and selected genes that exhibited MYC-induced up- or downregulation at 12 h after MYC induction and thereafter. We then extracted genes commonly affected in both BEAS-2B and SK-LC-3 cells. Consequently, 119 upregulated and 27 downregulated genes were selected to define the MYC module composed of those under the influence of MYC.

We determined RNA abundance in tumor tissues from 76 patients with adenocarcinomas and 5 sets of RNA mixtures prepared separately from normal lung tissues. The log2-normalized signal ratio of each probe was transformed into a z-score based on the mean and SD value across adenocarcinoma samples, and probes with expression in at least 90% of the 76 adenocarcinoma samples were selected, leaving 20606 probes for further analysis. Among the constituents of the experimentally defined MYC module, 114 upregulated and 27 downregulated genes were consequently included in the probe set, which passed the filtering criteria for the patient dataset.

We defined and calculated the MYC module activity for each patient with adenocarcinomas, actMYC, using microarray data, as described below.

\[
act_{MYC} = \frac{1}{n} \sum_{i=1}^{n} s_i z_{i, \text{module}}^\text{t}
\]

where \( n \) is the number of MYC module genes, \( z \) is the z-score of the log2-normalized signal ratio in each sample for gene \( i \), and \( s_i \) is the sign function of \( i \) and \( D \) are sets of up- and downregulated genes, respectively. Thus, this equation indicates that MYC module activity is the mean value of the z-score of the MYC module genes multiplied by sign.

Definition of miR-342-3p module

We also defined the miR-342-3p module as a surrogate of the regulatory activity of miR-342-3p (Supplementary Methods, available at Carcinogenesis Online), and 430 upregulated and 347 downregulated genes were selected based on two-color microarray data in miR-342-3p-transfected NCI-H2009 cells. Of these, 395 upregulated and 315 downregulated genes were included in the aforementioned 20606 probes, which passed the selection criteria in the patient dataset.

We then defined and calculated the miR-342-3p module activity for each patient with adenocarcinoma, actmiR342 using microarray data, as below.

\[
act_{miR-342} = \frac{1}{n} \sum_{i=1}^{n} s_i z_{i, \text{module}}^\text{t}
\]

where \( n \) is the number of miR-342-3p-affected genes, \( z \) is the z-score of the log2-normalized signal ratio in each sample for gene \( i \), and \( s_i \) is the sign function of \( i \) and \( D \) are the sets of up- and downregulated genes, respectively.

Selection of candidate miRNAs

To search for candidate miRNAs associated with MYC module activity, we utilized a miRNA expression profile dataset (GES51853) reported in our previous study (18) to extract raw data for the 76 patients corresponding to those in whom mRNA expression profiles were determined in this study. Expression levels of miRNA were log2-transformed, normalized to the 75th percentile using GeneSpring version 12.6 (Agilent) and transformed into a z-score. We selected miRNAs with expression in at least 10% of the 76 samples. Consequently, 301 miRNAs were selected for subsequent correlation analysis. Correlation analysis was performed using Pearson’s correlation coefficient. To correct for multiple testing, false discovery rate q values were computed for each miRNA using the R package qvalue (19).

Correlation analysis of MYC module activity with that of the miR-342-3p module

We analyzed correlations of the MYC module activity with that of the miR-342-3p module using microarray datasets of our own, the NCI Director’s Challenge Consortium obtained (20) and the National Cancer Center of Japan (21). Among the MYC module genes comprised of 119 upregulated and 27 downregulated genes in response to MYC induction, 40 upregulated and 10 downregulated genes passed the above-mentioned criteria for the MYC module calculation using the NCI Director’s Challenge Consortium dataset, while 143 upregulated and 121 downregulated genes were similarly selected from the 430 upregulated and 347 downregulated genes of the original miR-342-3p module. In the case of the dataset from NCC of Japan, we selected 91 upregulated and 21 downregulated genes that corresponded to the constituents of the MYC module, as well as 301 upregulated and 254 downregulated genes corresponding to the miR-342-3p module.

Gene Ontology term and gene set enrichment analyses

Microarray datasets of NCI-H2009 cells, which were transfected with either the negative control or miR-342-3p mimics, were used to conduct Gene Ontology (GO) term analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (22) with the following
GO terms: biological processes (GO_BP_FAT), cellular components (GO_CC_FAT) and molecular functions (GO_MF_FAT). The same microarray data-sets of NCI-N2009 were also analyzed using gene set enrichment analysis (GSEA) (23). Signal-to-noise and 10000 permutations of the genes were applied in the GSEA analysis with the gene sets CELL_CYCLE_PHASE (GO:0005403), HALLMARK_E2F_TARGETS and ISHIDA_E2F_TARGETS obtained from the MsigDB database v5.0 (23).

Other bioinformatics and statistical analyses

Analysis of variance and Student’s t-test were used to assess mean values among groups using JMP pro software (version 11.0.0, SAS). Two-sided P values <0.05 were considered to be statistically significant. All in vitro assay results presented represent the mean ± SD of triplicate determinations of three independent experiments performed under the same conditions.

Results

Defining the MYC module comprised of genes up- or downregulated by MYC induction

We utilized an integrative approach to identify miRNAs associated with MYC transcriptional activity, as shown in Figure 1. First, we determined that the MYC module was a surrogate of MYC transcriptional activity, based on changes in mRNA expression in response to DOX-inducible MYC. To this end, BEAS-2B and SK-LC-3 cells, chosen as representative normal and cancerous lung epithelial cell lines, respectively, were stably transduced with a DOX-inducible MYC expression construct, which resulted in establishment of stable clones with MYC induction levels comparable to MYC-overexpressing lung cancer cell lines, including NCI-H23, ACC-LC-172 and ACC-LC-176. These were used to define the MYC module (Figure 2A; Supplementary Figure 1, available at Carcinogenesis Online). We then conducted microarray analysis of mRNA expression using RNAs obtained up to 48h after MYC induction. We selected genes with MYC-induced up- or downregulation shown at 12h and that remained under the continued influence of MYC induction thereafter, based on the assumption that the obtained gene set would reflect an MYC activity-associated steady state. As a result, in BEAS-2B and SK-LC-3 cells, 824 and 239 genes, respectively, matched the criteria for MYC upregulation, while 598 and 97 genes, respectively, were shown to be downregulated by MYC. Consequently, 119 upregulated and 27 downregulated genes were identified as affected genes in both MYC-inducible cell lines (Figures 2B). As a result, a total of 146 genes with early and continued response to MYC induction were selected as comprising the MYC module, thus reflecting MYC transcriptional activity (Figure 2C; Supplementary Table 2, available at Carcinogenesis Online).

Integrative approach combining miRNA and mRNA expression profiling datasets using both patient tumor tissues and MYC-inducible cell lines

In order to integrate the experimentally defined MYC module with expression profile information obtained for patient tumor tissues, we performed microarray analysis of mRNA expression profiles of 76 surgically resected lung adenocarcinoma tumors, which we had previously analyzed for their miRNA expression profiles. MYC module activity was calculated for each of the 76 lung adenocarcinoma cases. A significant association of the MYC module activity with postoperative relapse-free survival was observed, suggesting a relevant clinical relationship (P = 0.017, log-rank test) (Supplementary Figure 2, available at Carcinogenesis Online). Correlation coefficient values and statistical significance were then calculated to analyze the association of the expression of each miRNA with MYC module activity. Consequently, 77 candidate miRNAs with a significant association were identified using two-dimensional combined analyses after applying the in vitro-defined MYC module to the patient tumor-derived mRNA expression profile, and utilization of both the mRNA and miRNA expression profiles in the same patient cohort.

Search for miRNAs significantly associated with MYC module activity

We then searched for miRNAs potentially possessing an MYC activity-modulating function or those modulated by MYC via analysis of the correlation of each miRNA expression with MYC module activity by utilizing the dataset of 76 lung adenocarcinoma specimens. MYC induction-mediated changes in miRNA expression were analyzed using TaqMan TLDA in the MYC-inducible BEAS-2B clone, based on our reasoning that responses in this cell line may well reflect those in normal lung epithelial cells. Among the 77 candidate miRNAs with significant associations, 39 with readily detectable expression in either MYC-induced or MYC-non-induced BEAS-2B cells were selected for further analysis (Table 1; Supplementary Figure 3, available at Carcinogenesis Online). They were then classified into four distinct groups according to their expression state when MYC was induced in BEAS-2B cells. We found that seven and five miRNAs were increased and repressed, respectively, by more than 2-fold in response to MYC induction. Of these miRNAs, which were suggested to be downstream of MYC, there were constituents of the miR-17–92 cluster, well-established MYC-inducible miRNAs. In addition, previously reported MYC-repressed miRNAs including miR-22, miR-26a, miR-30a-3p and miR-30e-3p were also selected, supporting the robustness of our integrative strategy. Among the remaining miRNAs not affected by MYC induction in BEAS-2B cells, 20 with significant negative correlations with MYC module activity shown by analysis of our dataset of lung adenocarcinoma patients attracted our attention, as we considered that these miRNAs may directly target MYC or indirectly modulate MYC module activity by targeting genes interacting/cooperating with MYC, thereby affecting MYC-mediated transcriptional regulation. We selected the top five miRNAs in terms of their negative correlations with MYC module activity and performed FACS analysis to analyze their effects on cell cycle in the presence of nocodazole (Figure 3A). Cell cycle profiles were found to be affected by introduction of miR-342-3p in NCI-H2009 lung adenocarcinoma cells, which showed a delay in progression through the G1, G2 and S phases. We therefore decided to focus on miR-342-3p to elucidate how this miRNA may affect MYC-mediated transcriptional activity.

In the present study, we also examined whether miR-342-3p-mediated regulation of MYC module activity could be duplicated in the tumor tissues of lung adenocarcinoma patients. First, we determined whether miR-342-3p expression levels are associated with MYC module activity, which clearly showed the presence of a significant inverse correlation (r = −0.490, P = 3.37 × 10−4) (Figure 3B, left panel). We also defined the miR-342-3p module as a gene set that reflects the effects of miR-342-3p, based on changes in the mRNA expression profiles of NCI-H2009 cells transfected with miR-342-3p mimics (Supplementary Table 3, available at Carcinogenesis Online). We then analyzed the relationship between the miR-342-3p and MYC modules in our lung adenocarcinoma dataset. Consequently, miR-342-3p module activity was clearly shown to be inversely associated with MYC module activity (r = −0.806, P = 1.17 × 10−19) (Figure 3B, right panel).
We employed this approach to further confirm the presence of their relationship in two additional large lung adenocarcinoma datasets comprised of only mRNA expression profiles. A highly significant inverse correlation ($r = -0.726, P = 1.94 \times 10^{-73}$) was observed with the dataset from the NCI Director’s Challenge Consortium composed of 442 lung adenocarcinoma cases (Figure 3C, left panel) as well as with the dataset consisting of 226 lung adenocarcinomas ($r = -0.694, P = 9.05 \times 10^{-34}$) (GSE31210) reported by the National Cancer Center of Japan (Figure 3C, right panel).

Identification of E2F1 as direct target of miR-342-3p and underlying mechanism

Next, we attempted to obtain mechanistic insight into how miR-342-3p negatively regulates MYC activity. Target prediction programs such as TargetScan did not predict MYC as a direct target of miR-342-3p, suggesting the possibility of indirect regulation. We first nominated genes that were previously reported to cooperate with MYC, which included proteins known to bind with MYC and/or transcription factors shown to be enriched in close proximity to the MYC-bound genomic regions. Using the resultant list consisting of 56 genes (Supplementary Table 4, available at Carcinogenesis Online—data not shown for three genes with negligible expression), we validated this finding using western blotting analysis (Figure 4B) as well as with two additional lung adenocarcinoma cell lines, NCI-H23 and NCI-H441 (Figure 4C). Conversely, treatment with miR-342-3p AS LNA resulted in a readily noticeable increase in E2F1 expression in the NCI-H1975 and ACC-LC-94 lung adenocarcinoma cell lines (Figure 4D). Next, a luciferase assay using a reporter construct carrying wild-type 3'-UTR of E2F1 was performed with NCI-H1975 cells treated with either miR-342-3p AS LNA or a scrambled negative control. We observed marked repression of luciferase reporter activity, which was significantly alleviated by treatment with miR-342-3p AS (Figure 4E). In addition, a luciferase reporter construct carrying mutations at the seed sequence of miR-342-3p showed an increase in luciferase activity regardless of the presence or absence of miR-342-3p AS to a level similar to that observed with the wt-reporter construct treated with miR-342-3p AS. These findings support the notion that miR-342-3p directly targets MYC-cooperating E2F1, thereby indirectly inhibiting MYC activity.

Inhibitory effects of miR-342-3p on cell cycle progression and proliferation

We also analyzed the biologic effects of miR-342-3p introduction in comparison with siMYC and siE2F1 treatment in two lung adenocarcinoma cell lines, NCI-H2009 and NCI-H23. Colony formation assay results showed marked reduction in numbers.
of colonies in response to miR-342-3p introduction, well as siE2F1 and siMYC in both NCI-H2009 and NCI-H23 cells (Figure 5B). We then analyzed the effects of miR-342-3p introduction on cell cycle progression in comparison with siMYC and siE2F1 treatment in NCI-H2009 and NCI-H23 cells. A significant increase in cells in the G, and S phases was observed in both cell lines when treated with miR-342-3p mimics as well as siE2F1, while more pronounced cell cycle accumulation in the G, phase was elicited with siMYC (Supplementary Figure 6, available at Carcinogenesis Online). We also employed a thymidine–aphidicolin double block in order to examine the effects on cell cycle progression in greater detail, which consequently indicated retarded cell cycle progression through the G1 and S phases in NCI-H2009 cells transfected with miR-342-3p mimics (Figure 5C).

Consistent with these biologic effects, GO term analysis of genes downregulated by miR-342-3p introduction revealed significant enrichment of genes tagged with those related to the cell cycle (Figure 5D). In addition, GSEA showed a significant association of downregulation by miR-342-3p introduction with the gene set of CELL_CYCLE PHASE, as well as with those of HALLMARK_E2F_TARGET and ISHIDA_E2F_TARGETS (Figure 5E; Supplementary Figure 7, available at Carcinogenesis Online). Taken together, the present findings clearly demonstrate that miR-342-3p plays important roles to inhibit cell cycle progression and proliferation in lung adenocarcinoma cell lines.

Discussion

The present study was initiated to identify miRNAs that play roles in regulation of the functional activity of MYC, one of the key oncogenes in the molecular pathogenesis of lung cancers (12,13). To this end, we utilized an integrative approach with combinatorial usage of miRNA and mRNA expression profiling datasets of patient tumor tissues, as well as those of MYC-inducible cell lines. Our results allowed us to identify multiple miRNAs reported as either directly downstream or upstream of MYC, supporting the robustness of our strategy. The former examples included the miR-17–92 cluster, miR-22, miR-26a, miR-30a-3p and miR-30e-3p, all of which were previously shown to be under MYC-mediated transcriptional regulation (11,24,25), while the latter instances were comprised of let-7, miR-34a and miR-24, which have been reported to directly repress MYC expression via binding to a target site at the 3'-UTR of MYC (26–28). Intriguingly, our integrative approach also led us to identify miR-342-3p, which we found to be a miRNA indirectly regulating MYC activity via direct inhibition of E2F1.

Previous studies have clearly shown that MYC exerts its transcriptional functions by collaborating with its obligated binding partner MAX and other interacting proteins (14,15,29). In addition, other molecules are thought to cooperate with MYC via binding to a genomic region in the proximity of the MYC binding site (30). Along this line, the E2F1 motif has been shown to be significantly enriched near MYC-bound genomic regions, especially those containing the canonical E-box motif, suggesting that this functional cooperation between MYC and E2F1 is crucially involved in cancer development (31). In the present study, we found that direct repression of E2F1 by miR-342-3p is indirectly involved, at least in part, in the mechanism underlying regulation of MYC activity (Figure 5F). It is of note that the miR-342-3p module, considered to reflect its experimentally substantiated influence on mRNA expression, was inversely correlated with MYC module activity in three independent datasets of lung adenocarcinoma patients, including the NCI Director’s Challenge Consortium dataset ($r = -0.726, P = 1.94 \times 10^{-7}$) and another large dataset developed by a group at the National...
Cancer Center of Japan (r = −0.694, P = 9.05 × 10^{-4}), as well as our own designed for this study (r = −0.806, P = 1.17 × 10^{-9}). Taken together, the present findings demonstrate that our integrative approach is useful to obtain insight into the inter-regulatory relationships between miRNAs and a protein coding gene of interest, which remains a challenge, especially for elucidating those present in patient tumor tissues.

A few studies have investigated miR-342-3p alterations in human cancer, including colorectal, breast and cervical cancer, as well as leukemia, all of which reported its reduced expression (32–36). While miR-342-3p resides in intron 3 of the EVL gene, the host gene has been shown to be downregulated because of epigenetic silencing of the promoter region, conceivably underscoring the reduced miR-342-3p expression (32). As for the direct targets of miR-342-3p, DNMT1, ID4 and FOXM1 have been identified and verified by luciferase assay findings (33–35). In the present study, we observed that miR-342-3p introduction into NCI-H2009 cells resulted in relatively modest reduction in MYC-induced relative expression (32–36).

### Table 1. miRNAs identified to be associated with MYC activity in both patient tumors and in vitro experiments

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FDR, false discovery rate; r, correlation coefficient.

*References with MYC expression findings.
Figure 3. Identification of miR-342-3p as candidate miRNA regulating MYC activity. (A) Flow cytometric analysis of effects on cell cycle in NCI-H2009 cells, which were transfected with candidate miRNAs and treated with nocodazole for 24 h. PI, propidium iodide. (B) Significant association of MYC module activity with miR-342-3p expression as well as with miR-342-3p module activity in our dataset of 76 lung adenocarcinomas. AD, adenocarcinoma; NL, normal lung. (C) Validation of association between miR-342-3p and MYC module activities in the Director’s Challenge Consortium and National Cancer Center of Japan datasets.

Figure 4. Identification of E2F1 as a direct target of miR-342-3p. (A) qRT-PCR analysis of effects of miR-342-3p introduction on potential target genes with MYC function-related proteins. (B) Western blotting findings showing selected candidates of miR-342-3p in NCI-H2009 cells transfected with miR-342-3p. (C) qRT-PCR and western blotting findings of E2F1 in miR-342-3p-introduced NCI-H23 and NCI-H441 cells. (D) Western blotting and qRT-PCR findings of E2F1 expression in two lung adenocarcinoma cell lines with high miR-342-3p expression. Numbers below indicate relative level of induction. (E) Luciferase assay using reporter constructs carrying 3′-UTR of E2F1 with wild-type (WT) or mutations (mut) corresponding to the sequence of miR-342-3p. *P < 0.05, **P < 0.005.
by other miRNAs (38). It is therefore not inconceivable that miR-342-3p may distinctly affect a set of target genes in a tissue type-dependent manner.

In conclusion, we employed an integrative approach and identified miR-342-3p as a miRNA indirectly regulating MYC activity. Our study also revealed that direct repression of the MYC-cooperating transcription factor E2F1 is responsible, at least in part, for the MYC activity-regulating effect of miR-342-3p in lung adenocarcinomas. A future study is warranted to fully elucidate the functional roles of miR-342-3p, considering that miRNAs can inhibit multiple targets. Our results also clearly demonstrate the usefulness of our approach to utilize expression profiling data-sets of both patient tumor tissues and experimental findings in vitro in an integrated manner. It would be interesting to apply the present strategy for elucidation of the functional interplays between miRNAs and other crucial transcription factors such as p53 and TTF-1/NKX2-1 (39,40), which are thought to be involved in the pathogenesis of this devastating disease.

Supplementary material
Supplementary Tables 1–4, Figures 1–8 and Supplementary Methods can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.
References