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Uncovering Growth-Suppressive MicroRNAs in Lung Cancer

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Abstract

Purpose—MicroRNA (miRNA) expression profiles improve classification, diagnosis, and prognostic information of malignancies, including lung cancer. This study uncovered unique growthsuppressive miRNAs in lung cancer.

Experimental Design—miRNA arrays were done on normal lung tissues and adenocarcinomas from wild-type and proteasome degradation-resistant cyclin E transgenic mice to reveal repressed miRNAs in lung cancer. Real-time and semiquantitative reverse transcription-PCR as well as *in situ* hybridization assays validated these findings. Lung cancer cell lines were derived from each transgenic line (designated as ED-1 and ED-2 cells, respectively). Each highlighted miRNA was independently transfected into these cells. Growth-suppressive mechanisms were explored. Expression of a computationally predictedmiRNA target was examined.ThesemiRNAs were studied in a paired normal-malignant human lung tissue bank.

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Disclosure of Potential Conflicts of Interest.
No potential conflicts of interest were disclosed.

Results—miR-34c, miR-145, and miR-142-5p were repressed in transgenic lung cancers. Findings were confirmed by real-time and semiquantitative reverse transcription-PCR as well as *in situ* hybridization assays. Similar miRNA profiles occurred in human normal versus malignant lung tissues. Individual overexpression of miR-34c, miR-145, and miR-142-5p in ED-1and ED-2 cells markedly repressed cell growth. Anti-miR cotransfections antagonized this inhibition. The miR-34c target, cyclin E, was repressed by miR-34c transfection and provided amechanism for observed growth suppression.

Conclusions—miR-34c, miR-145, and miR-142-5p were repressed in murine and human lung cancers. Transfection of each miRNA significantly repressed lung cancer cell growth. Thus, these miRNAs were growth suppressive and are proposed to exert antineoplastic effects in the lung.

MicroRNAs (miRNA) are short, noncoding RNAs regulating gene expression (1, 2). In association with the Dicer/Argonaute complex, miRNAs bind to complementary sites in the 3′-untranslated region of target mRNAs and cause translational down-regulation and/or degradation of target mRNAs, inhibiting gene expression (3, 4). miRNAs are important in cancer biology or therapy (5). miRNA expression profiles improve classification, diagnosis, and prognostic information in oncology (6). There is a need to better understand miRNAs roles in specific cancers.

Lung cancer is the most common cause of cancer mortality for men and women in the United States (7). Some miRNAs are deregulated in lung cancers. Low expression of let-7a and high expression of miR-155 are linked to unfavorable clinical outcome in lung cancer (8). Repressed miRNAs in malignant versus normal lung are candidate growth- or tumor-suppressive miRNAs. The miR-34 cluster is repressed in cancers, including miR-34a, miR-34b, and miR-34c involved in p53 tumor suppression in many cancers (9–13), including lung cancer (14). This underscores need for an in-depth search for suppressive miRNAs in lung carcinogenesis, as studied here.

Some miRNAs are differentially expressed in lung cancer (8) and repressed miRNAs in lung carcinogenesis need to be discerned. Comprehensive profiling and functional analyses of miRNAs should uncover their roles in lung cancer. For this reason, engineered human surfactant C-driven murine transgenic cyclin E lines (wild-type and proteasome degradationresistant) were examined. These lines recapitulate frequent features of human lung carcinogenesis, including chromosome instability, hedgehog pathway activation, pulmonary dysplasia, and single, multiple, or metastatic lung adenocarcinomas (15). These are tools to probe differentially expressed miRNAs in lung cancers and to assess functional consequences.

Comprehensive miRNA microarray analyses were done on adenocarcinoma versus adjacent normal lung tissues from transgenic cyclin E lines. Findings revealed specific miRNAs (miR-34c, miR-145, and miR-142-5p) were prominently repressed in lung cancers. Results were independently validated by *in situ* hybridization, semiquantitative and real-time reverse transcription-PCR (RT-PCR) assays, and examination of expression profiles of these miRNAs in paired normal-malignant human lung tissues. Novel lung cancer cell lines were derived from lung cancers arising from each transgenic line. Studies reveal engineered overexpression of each of these miRNAs in murine lung cancer cell lines repressed proliferation and target gene expression. Implications for clinical lung carcinogenesis are discussed.

Materials and Methods

Transgenic lung tissues

Murine cyclin E transgenic lines that develop premalignant and malignant (adenocarcinoma) lung lesions were described previously (15). Those studied here included wild-type cyclin E transgenic (line 2) and proteasome degradation-resistant (line 4) lines (15). Adenocarcinoma and adjacent histopathologically normal lung tissues were individually harvested from age- and sex-matched mice and immediately placed in RNAlater (Ambion). Total RNA was isolated using established techniques (14, 16) for miRNA expression arrays. Formalin-fixed and paraffin-embedded transgenic lung tissues were harvested (15) and used for *in situ* hybridization assays.

RNA isolation and miRNA arrays

The locked nucleic acid (LNA)-modified oligonucleotide probe set for all annotated miRNAs from mouse (*Mus musculus*) in the miRBase Release 7.1 was purchased (Exiqon) and used to engineer a LNA microarray. Total RNA was isolated from cell lines and lung tissues with Trizol reagent (Invitrogen) and was 3′-end labeled using T4 RNA ligase to couple Cy3-labeled RNA linkers (16–18). Labeled RNA was hybridized to LNA microarrays overnight at 65 °C in a hybridization mixture containing 4× SSC (1× SSC: 150 mmol/L sodium chloride and 15 mmol/L sodium citrate), 0.1% SDS, 1 μg/μL herring sperm DNA, and 38% formamide. Slides were washed three times in 2× SSC, 0.025% SDS at 65°C, three times in 0.8 × SSC, and three times in 0.4× SSC at room temperature. Each RNA sample was independently hybridized twice. There were four probe sets for each miRNA. Only concordant hybridization results were scored. Microarrays were scanned using an ArrayWorx scanner (Applied Precision). Images were analyzed using GridGrinder[9] and background-subtracted spot intensities were normalized using variance stabilization normalization (19). Statistical comparisons were with the TIGR Multi Experiment Viewer (20). miRNAs selected for in-depth study showed significant expression differences in both murine and human normal versus malignant lung tissues. Paired human normal and malignant lung tissues were obtained after review and approval of Dartmouth's Institutional Review Board. Patient identifying information was not linked to this tissue bank consecutively accrued >8 years at Dartmouth-Hitchcock Medical Center.

Real-time and semiquantitative RT-PCR assays

miRNA RT-PCR assays were done using the mirVana qRT-PCR miRNA Detection Kit (Ambion) with a Peltier Thermal Cycler (MJ Research) for semiquantitative assays or with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and the 7500 Fast Real-time PCR System (Applied Biosystems) for quantitative miRNA detection. For RT-PCR assays, the human cyclin E forward 5′-CCATCCTTCTCCACCAAAGA-3′ and reverse 5′-TTTGATGCCATCCACAGAAA-3′ primers and glyceraldehyde-3-phosphate dehydrogenase forward 5′-ACCTTTGGCATTGTGGAGG-3′ and reverse 5′-ACACATTGGGGGTAGGAACA-3′ primers were used.

In situ hybridization assays

In situ hybridization assays were done as in prior work (21). Slides were prehybridized in hybridization solution (50% formamide, 5% SSC, 500 μg/mL yeast tRNA, and 1% Denhardt's solution) at 50°C for 30 min. The desired FITC-labeled, LNA-modified DNA probes (5–10 pmol; Exiqon or Integrated DNA Technologies) complementary to specific miRNAs were added and hybridized for 2 h at a temperature 20 °C to 25 °C below the calculated melting temperature of the LNA probe. After stringent washes, tyramide signal amplification reaction was carried out using the GenPoint Fluorescein kit (DakoCytomation)

and the manufacturer's procedures. Slides were mounted with Prolong Gold solution (Invitrogen).

Murine transgenic lung cancer cell lines

Lung tumors from wild-type and proteasome degradation-resistant cyclin E transgenic murine lines were each carefully minced and separately incubated with PBS supplemented with 300 units/mL collagenase (Sigma) and 1 unit/mL dispase (Invitrogen) for 2 h at37°Cina Precision Reciprocal Shaking Bath (Fisher Scientific). Cell suspensions were independently passed through a 40 μm Cell Strainer (BD Bioscience) and placed onto BD BioCoat Collagen I-coated tissue culture plates (BD Bioscience) with RPMI 1640 (Mediatech) supplemented with SAGM SingleQuot Kit (Cambrex), 20% fetal bovine serum containing 1% antibiotic with antimycotic solution (Mediatech). Cell lines from wild-type (ED-1) and proteasome degradation-resistant (ED-2) transgenic cyclin E lung cancers were derived after independent passage over several months.

Tissue culture

ED-1 and ED-2 cells were each cultured in RPMI 1640 with 10% fetal bovine serum and 1% antibiotic and antimycotic solution in a humidified incubator at 37°C in 5% CO_2. Immortalized mammary epithelial cells were cultured as in prior work (22). Murine C-10 alveolar type II epithelial cells were cultured in CMRL 1066 medium (Life Technologies) with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (15).

Immunofluorescence

ED-1, ED-2, and immortalized mammary epithelial cells were individually plated onto coverslips and cultured overnight. Slides were washed twice with PBS and fixed with 3.7% paraformaldehyde for 15 min at room temperature. After PBS washings, fixed cells were permeabilized with 0.1% Triton X-100/PBS and blocked with 5% goat serum/PBS at 37°C for 20 min before incubation with a primary antibody in 1% goat serum/PBS (rabbit polyclonal antitelomerase antibody, RB-10328-PO; NeoMarker) at 37°C for 45 min. Slides were washed three times with PBS for 5 min each and incubated with a desired secondary antibody (goat anti-rabbit Alexa Fluor 488 IgG; Invitrogen, Molecular Probes) in 1% goat serum/PBS at 37°C for 30 min. After three PBS washings for 5 min each, cells were mounted with 50% glycerol/4′,6-diamidino-2-phenylindole/H_2O (Vector Laboratories). Immortalized mammary epithelial cells served as positive controls for telomerase expression (22). Primary lung epithelial cells from FVB mice were negative controls for telomerase expression.

Transient transfection

ED-1 and ED-2 cells were individually plated subconfluently onto six-well plates 24 h before transfection. Transient transfection was accomplished with siPORT NeoFX reagent (Ambion) and pre-miR miRNA precursors and/or anti-miR and control oligonucleotides were used (Ambion). miR precursors were diluted in siPORT NeoFX in Opti-11 MEM I (Invitrogen) and added into wells containing RPMI 1640 with 10% fetal bovine serum and supplemented as before. Logarithmically growing transfectants were harvested for independent immunoblot, proliferation, apoptosis, and trypan blue viability assays.

Proliferation and apoptosis assays

The CellTiter-Glo proliferation assay (Promega) was described previously (18). Trypan blue viability assays were done as in prior work (21). The terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick end labeling assay assessed apoptosis with the POD kit (Roche).

Immunoblot analysis

Cells were lysed with ice-cold radioimmunoprecipitation lysis buffer, and immunoblot analysis was done using optimized techniques (23). Lysates were size fractionated by SDS-PAGE before transfer to a nitrocellulose membrane. Rabbit polyclonal anticyclin E (M-20, sc-481), goat polyclonal anti-actin (C-11, sc-1615), and donkey anti-goat IgG (sc-2020) were each purchased (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-rabbit IgG and ECL Plus immunoblotting detection reagents were purchased (GE Healthcare UK).

In vivo tumorigenesis assay

Early passages of ED-1 and ED-2 cells were each harvested in PBS supplemented with 10% mouse serum (Invitrogen) and 10^6 cells of each line were individually injected into tail veins of respective FVB mice. After injection (6–8 weeks), mice were sacrificed following an Institution Animal Care and Use Committee approved protocol and harvested lung tissues were formalin fixed, paraffin embedded, sectioned, and H&E stained for histopathologic analyses as described previously (15). These histopathologic sections were examined for lung tumors.

Results

Repression of miR-34c, miR-145, and miR-142-5p

To uncover miRNAs repressed in lung carcinogenesis, LNA microarray analyses were done to independently profile miRNA expression in malignant versus normal lung tissues from transgenic human surfactant C-driven wild-type and protea-some degradation-resistant cyclin E lines (15). Increased neoplastic changes occurred in the lungs of degradationresistant versus wild-type cyclin E lines expressing these species at comparable levels (15). Malignant (adenocarcinoma) and adjacent normal lung tissues were harvested from these lines and from normal lung tissues of age- and sex-matched nontransgenic FVB mice. Microarray profiling examined 315 murine miRNAs and revealed known and previously unrecognized miRNAs as repressed in adenocarcinomas versus normal lung tissues. Figure 1A displays significant expression changes of most to least repressed miRNAs.

Normal tissues had 3- to 6-fold higher expression of highlighted miRNAs versus adjacent adenocarcinomas. There were three repressed miRNA clusters: miR-34c and miR-34b (16- and 10-fold repression) as one cluster, miR-143 and miR-145 (3.6- and 4-fold repression) as another, and miR-142-5p and miR-142-3p (2.6- and 2.4-fold repression) as a third. miR-126*, miR-126, miR-150, and miR-125bwere also repressed in lung adenocarcinomas but to a lesser extent than these clusters (data not shown). Supplementary Fig. S1 displays the profile of all the repressed miRNAs. Several repressed miRNAs in Fig. 1A were concordant with previously reported miRNA profiles in malignant versus normal tissues. These findings include the miR-34 family implicated as growth suppressive (9–14) as well as miR-143 and miR-145 reduced in colorectal cancer (24) and lung cancer (8) and miR-145 repressed in breast cancer (21, 25). miR-145, miR-142-5p, and miR-142-3p were not previously recognized as involved in lung carcinogenesis. miR-21 is known to be increased in lung cancer (26) and its expression was studied in transgenic lung cancers and was 5-fold higher in these tumors relative to adjacent normal lung, as in Fig. 1A. miR-143 and miR-142-3p did not have the same extent of repression in paired human malignant versus normal lung tissues as other highlighted miRNAs (data not shown).

Given the extent of miR-34c, miR-145, and miR-142-5p repression and concordant expression patterns in paired human normal-malignant lung tissues, these were selected for

further investigation. To determine spatial distribution of these repressed miRNAs, *in situ* hybridization assays were used. Malignant (adenocarcinoma) and adjacent normal lung tissues were studied from wild-type and proteasome degradation-resistant cyclin E transgenic mice (Fig. 1B). Histopathologic analyses of lung tissues confirmed diagnoses. miR-21, miR-34c, miR-142-5p, miR-145, and control 18S rRNA were each analyzed by *in situ* hybridization with 5′-fluorescein-labeled LNA-modified DNA oligonucleotide probing of malignant and normal lung tissues from transgenic mice. miR-34c and miR-145 exhibited different cell type-specific expression patterns in normal lung. miR-34c was prominently expressed in bronchial epithelial cells with signal intensity less than for miR-145. miR-145 signal was abundant in smooth muscle cells of the muscularis. A less intense signal was in smooth muscle cells within blood vessels. Expression of miR-142-5p was undetected by this *in situ* hybridization assay. Abundant miR-21 *in situ* hybridization expression was found in lung adenocarcinomas. In contrast, miR-34c and miR-145 were undetected in examined adenocarcinomas (Fig. 1B; data not shown). As expected, 18S rRNA was ubiquitous and abundant in normal and malignant lung tissues, confirming integrity of RNA in these tissues. Together, these results confirm expression patterns of these miRNAs in malignant versus normal lung tissues.

To validate these differentially expressed miRNAs, real-time and semiquantitative (data not shown) RT-PCR assays were done on RNA isolated from adenocarcinomas and adjacent normal lung tissues from the transgenic murine lines analyzed by LNA microarrays. Each assay was conducted at least three independent times. Results obtained were concordant with findings from miRNA microarray experiments (Fig. 2A; data not shown).

Repression of miRNAs in human lung cancers

Because miR-34c, miR-145, and miR-142-5p were repressed in murine transgenic lung cancers, it was hypothesized these would also be repressed in human lung cancers. To explore this, paired human normal-malignant lung tissues were examined from each type of non-small cell lung cancers (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and bronchioalveolar cell carcinoma) using a lung tissue bank (18). As shown in Fig. 2B, repression of these miRNAs was frequent by real-time (Fig. 2B) and semiquantitative RT-PCR assays (data not shown). Data were obtained from three independent experiments. Among examined lung cancers, miR-34c, miR-145, and miR-142-5p were each repressed in adenocarcinoma, squamous cell carcinoma, and large cell carcinoma versus adjacent normal lung tissues, which agreed with miRNA microarray findings of murine transgenic lines. miR-34c and miR-142-5p were increased in bronchioalveolar cell carcinoma, whereas miR-145 expression was reduced in bronchioalveolar cell carcinoma. Profiles for each of these miRNAs were examined in the same human lung tissues, as depicted in Fig. 2B.

Murine transgenic lung cancer cell lines

To elucidate the roles of these repressed miRNAs in lung carcinogenesis, murine lung cancer cell lines were derived from wild-type and proteasome degradation-resistant transgenic cyclin E lung adenocarcinomas and designated as ED-1 and ED-2 cell lines, respectively. To confirm if these cells expressed exogenous human cyclin E, a RT-PCR assay was done using primers recognizing human but not endogenous murine cyclin E species (Fig. 3A). ED-1 and ED-2 cells were cultured for >90 passages without senescence observed. ED-1 and ED-2 cells acquired telomerase expression (Fig. 3B). Immortalized mammary epithelial cells were controls (Fig. 3B) and primary lung epithelial cells isolated from normal murine lung tissues were negative controls for telomerase expression (data not shown). To assess tumorigenicity, early passages of ED-1 and ED-2 cells were independently injected into tail veins of syngeneic FVB mice (10^6 cells per mouse). Lung

adenocarcinomas developed in recipient mice 6 to 8 weeks after injection (21 of 24 mice for ED-1 and 14 of 17 mice for ED-2 cells) as shown in representative histopathologic assays of Fig. 3C. Both lines readily proliferated in culture, as in Fig. 3D.

Engineered expression of miRNAs

To examine whether transfection of miR-34c, miR-145, or miR-142-5p conferred growth suppression, each miRNA was transiently transfected into ED-1 and ED-2 cells (Fig. 4). Expression of transfected miRNAs was confirmed by real-time RT-PCR assays (Fig. 4B). All three miRNAs substantially reduced ED-1 and ED-2 cell growth (Fig. 4A). Over 90% of cells from each cell line were transfected (data not shown). To exclude nonspecific effects, rescue experiments were conducted using individual cotransfection with inhibitory anti-miR-34c, anti-miR-145, and anti-miR-142-5p oligonucleotides. Each inhibited corresponding miRNAs in ED-1 and ED-2 cells. The consequence was growth inhibition from each miRNA that was antagonized (Fig. 4A). To determine if growth inhibition was caused by enhanced cell death or apoptosis, trypan blue and terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick end labeling assays were conducted. No significant differences were found in the extent of cell death or apoptosis within these or miRNA control transfectants (data not shown).

The miRNA target site prediction programs Pictar (27) and TargetScan (28) identified targets of miR-34c, the most prominently repressed miRNA uncovered. Both programs predicted cyclin E as a candidate miR34c target. To learn whether miR34c repressed cyclin E, miR-34c overexpression was independently achieved in ED-1 and ED-2 cells in Fig. 4C. Cyclin E immunoblot expression was substantially repressed by miR-34c (Fig. 4C), as expected from bioinformatic analyses.

Discussion

miRNAs regulate gene expression and are deregulated in carcinogenesis, including lung cancer. Cyclin E transgenic murine lines (15) were examined that recapitulated key features of human lung carcinogenesis. A cluster of miRNAs (miR-34c, miR-145, and miR-142-5p) was preferentially repressed in murine lung cancers versus adjacent normal lung tissues using total cellular and *in situ* expression assays (Figs. 1 and 2A). Similar expression profiles were found in a panel of human paired normal-malignant lung tissues (Fig. 2B). To explore how these miRNAs are involved in lung carcinogenesis, novel murine lung cancer cell lines (ED-1 and ED-2) were derived (Fig. 3). Each transfected miRNA reduced ED-1 and ED-2 cell growth (Fig. 4A). Notably, miR-34c repressed its predicted bioinformatic target, cyclin E (Fig. 4). Together, these findings revealed growth-suppressive miRNAs in lung cancer.

Lung cancer is the leading cause of cancer mortality for men and women. A better understanding of lung cancer biology and therapy are needed. This study advances prior work (9–14) by implicating repressed miRNAs in lung carcinogenesis that are growth suppressive. Whether these miRNAs provide important clinical diagnostic or prognostic information or predict response to therapy will be explored in future work.

miRNAs are potential antineoplastic agents. It is appealing to consider administration of miRNA-based regimens for treatment of transgenic murine models or other experimental *in vivo* models to learn whether these target oncogene expression or elicit antitumor responses. In this regard, wild-type and proteasome degradation-resistant cyclin E transgenic lines studied here should prove useful to prioritize miRNAs as antineoplastic agents. Perhaps combination therapy with different miRNAs will be needed to confer desired antitumorigenic effects. It will be critical to discern which miRNAs are overexpressed in

lung cancers and which need to be inactivated to inhibit lung carcinogenesis. Successful preclinical studies would set the stage for clinical targeting of highlighted miRNAs. Indeed, *in vitro* and *in vivo* reports on LNA-mediated silencing of miRNA function support use of LNAs for targeting miRNAs in oncology (29, 30).

In summary, miR-34c, miR-145, and miR-142-5p were suppressed miRNAs. Novel murine lung cancer cell lines were derived from transgenic cyclin E lung cancers. These were useful for functional studies of consequences of engineered miRNA overexpression. Marked growth suppression of these cells followed transfection of each highlighted miRNAs. Notably, cyclin E was targeted by miR-34c, providing a mechanism for observed growth inhibition. Selective repression of these miRNAs conferred a growth advantage. It is intriguing to speculate that these miRNAs would improve diagnosis or classification of lung cancer and even provide prognostic information. Conceivably, these miRNAs or their derivatives would become agents to treat or chemoprevent lung cancers.

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Translational Relevance

MicroRNAs (miRNA) are important in cancer biology. Expression profiles of miRNAs improve classification and diagnostic or prognostic information about human malignancies. Following comprehensive microarray and *in situ* hybridization analyses of expressed miRNAs, a cluster was found as differentially repressed in murine transgenic lung cancers. A similar profile was found in human lung cancers. Each of these triggered growth suppression of transfected lung cancer cells. Mechanisms for this were uncovered. These miRNAs are pharmacologic targets for lung carcinogenesis.

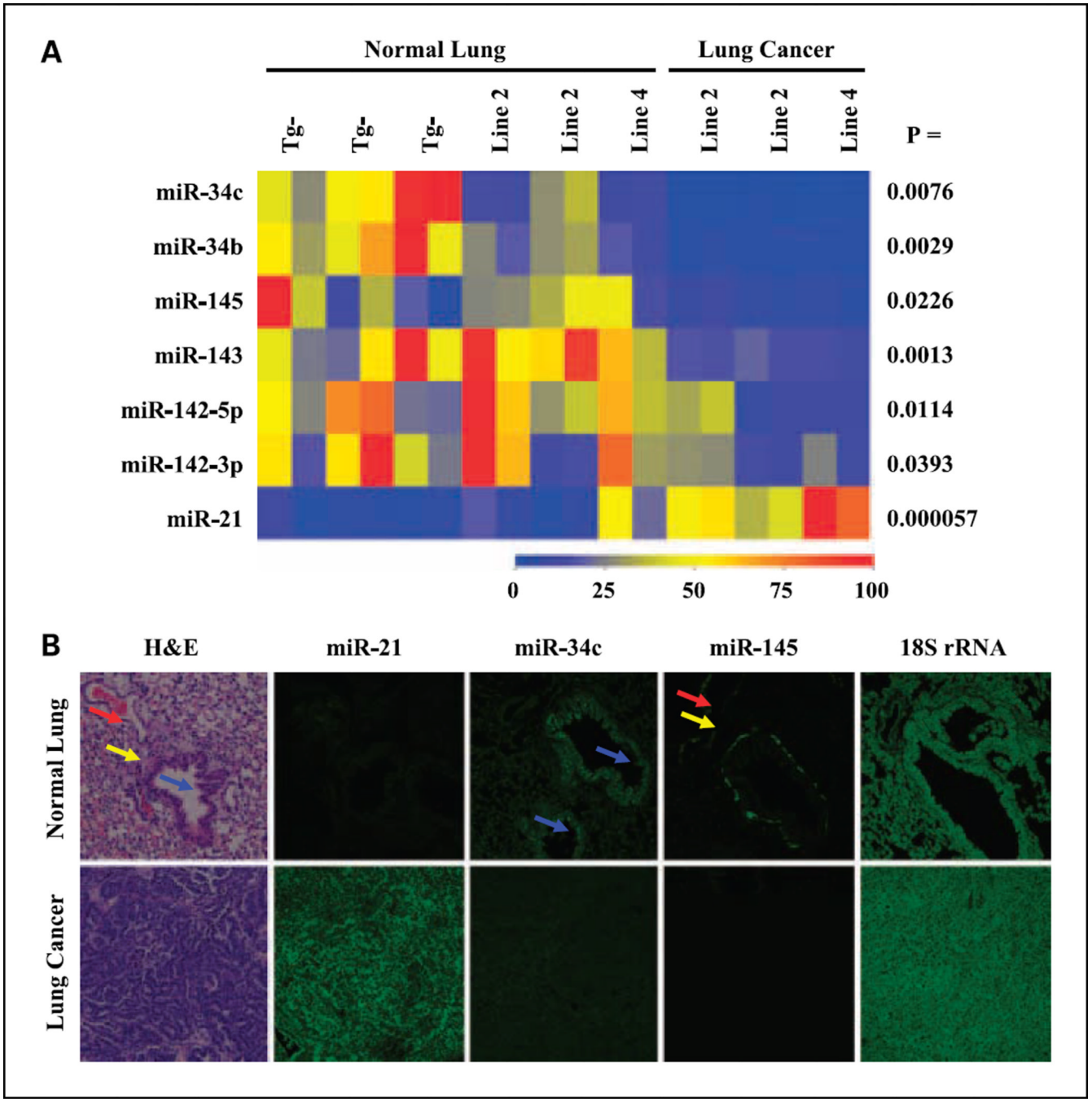

Fig. 1.

Repressed miRNAs in murine transgenic cyclin E lung cancers. *A*, miRNA profiling of lung adenocarcinomas and adjacent normal lung tissues from murine wild-type (line 2) and degradation-resistant (line 4) cyclin E transgenic lines. miR-34c, miR-34b, miR-145, miR-143, miR-142-5p, and miR-142-3p were each significantly repressed (and miR-21increased) in lung adenocarcinomas versus normal lung. *B, in situ* hybridization assays for miR-21, miR-34c, and miR-145 were done on adenocarcinomas and adjacent normal lung tissues (H&E staining) from cyclin E transgenic mice. Representative results are displayed from line 2. *Blue arrow*, bronchial epithelium; *red arrow*, smooth muscle cells associated with blood vessels; *yellow arrow*, smooth muscle cells in muscularis. Repression

of miR-34c and miR-145 (relative to miR-21) was detected in malignant versus normal lung tissues.The18S rRNA signal was a positive control.

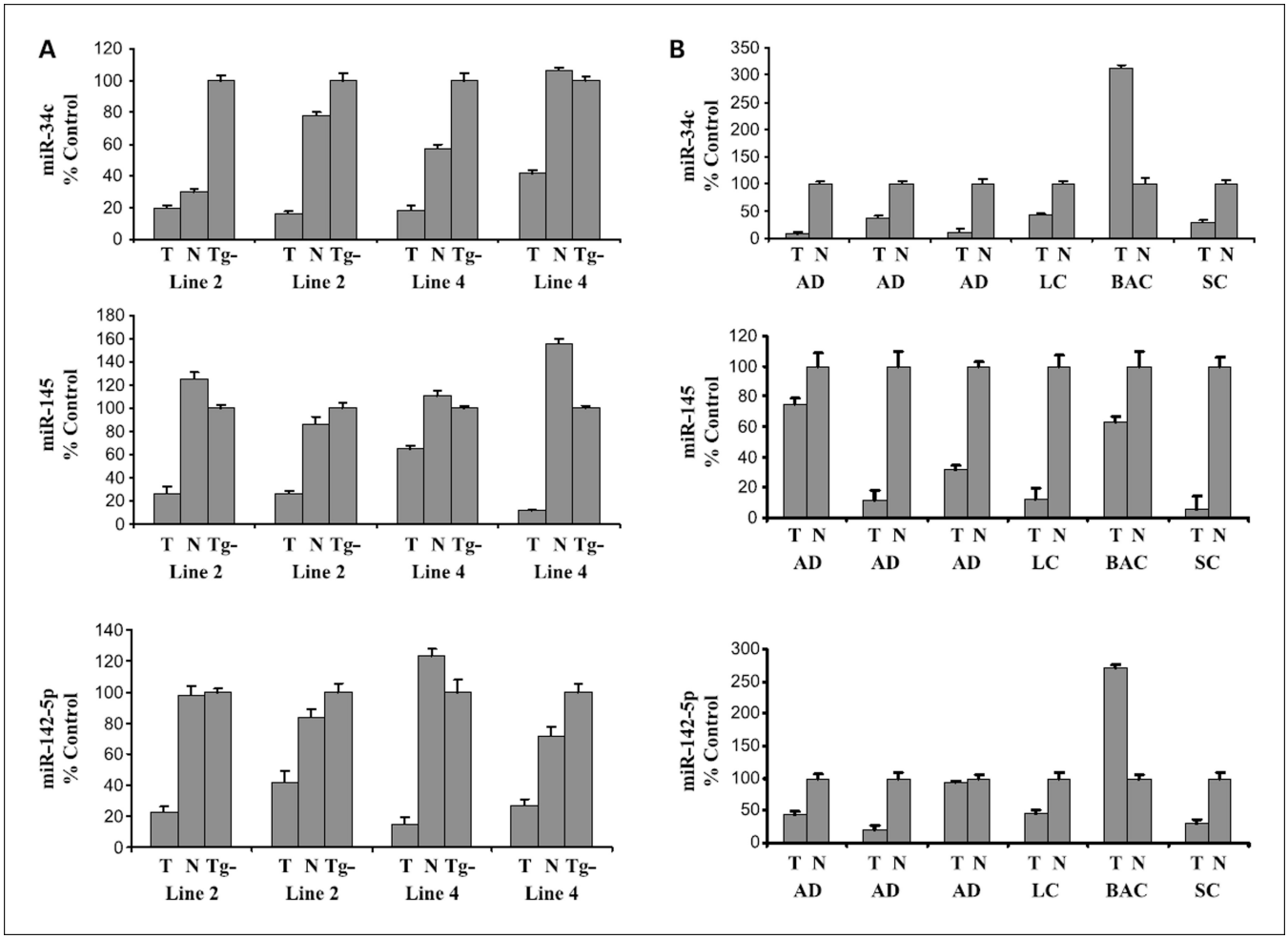

Fig. 2.

Validation of miR-34c, miR-145, and miR-142-5p expression profiles by real-time RT-PCR assays done on murine cyclin E transgenic lines and paired normal-malignant human lung tissues. *A*, real-time RT-PCR assays for miR-34c, miR-145, and miR-142-5p. *T*, tumor; *N*, adjacent normal murine lung; *Tg* - , murine nontransgenic FVB normal lung. Results were normalized to expression within nontransgenic FVB mouse lung tissues. *B*, real-time RT-PCR assays for miR-34c, miR-145, and miR-142-5p done on paired human normal-malignant tissues. *AD*, adenocarcinoma; *LC*, large cell carcinoma; *BAC*, bronchioalveolar cell carcinoma; *SC*, squamous cell carcinoma. Results were normalized to expression in normal human lung. SDs are shown.

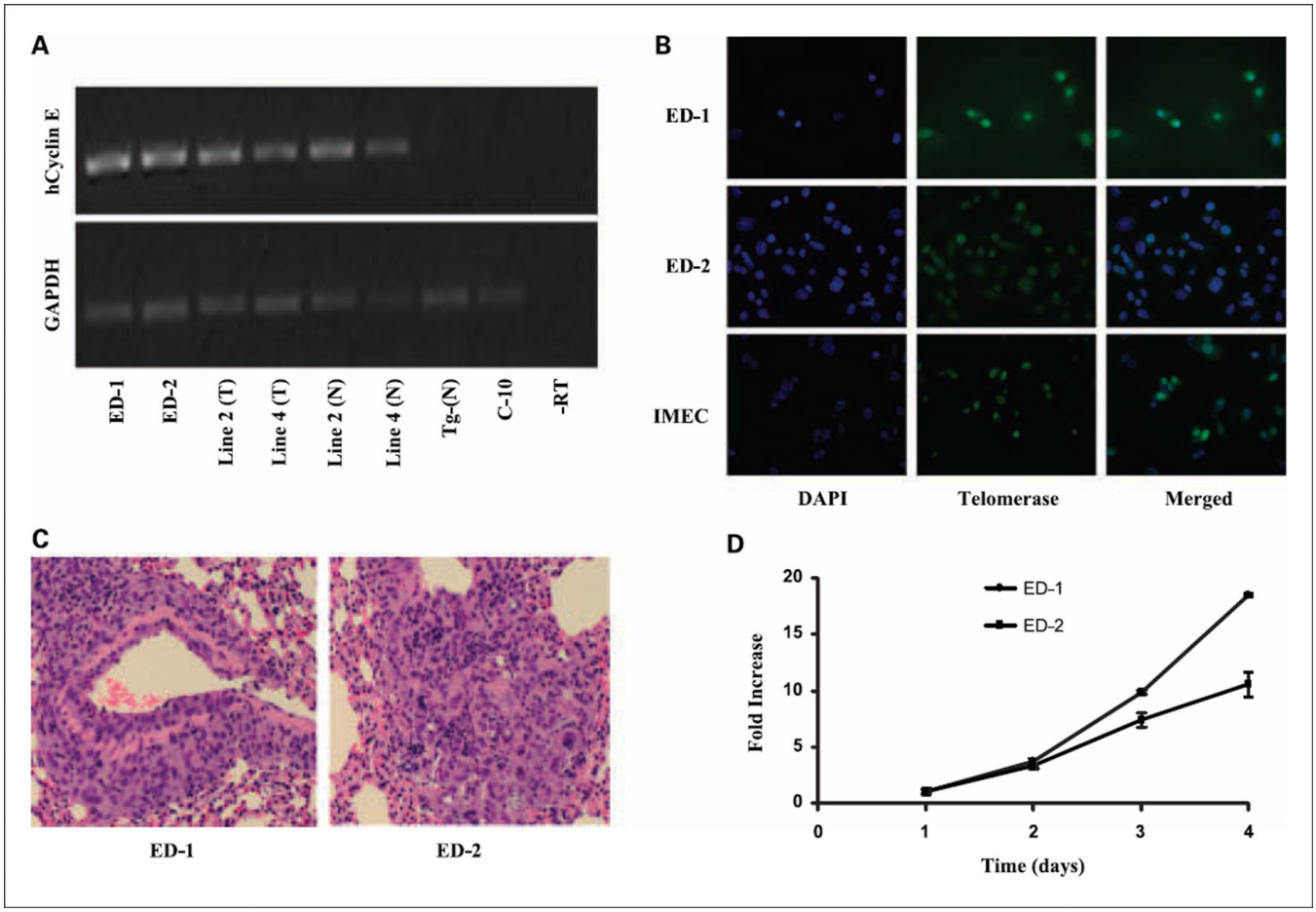

Fig. 3.

Features of ED-1and ED-2 murine lung cancer cell lines. *A*, human cyclin E (*hCyclin E*) expression in ED-1and ED-2 cells. *C-10*, C-10 immortalized murine alveolar type II epithelial cell line; *-RT*, no RT control. *B*, immunocytofluorescence for telomerase expression. *C*, H&E staining of lung adenocarcinomas after independent injection of ED-1and ED-2 cells into tail veins of different syngeneic FVB mice. *D*, ED-1and ED-2 cells readily proliferate in culture. SDs are shown.

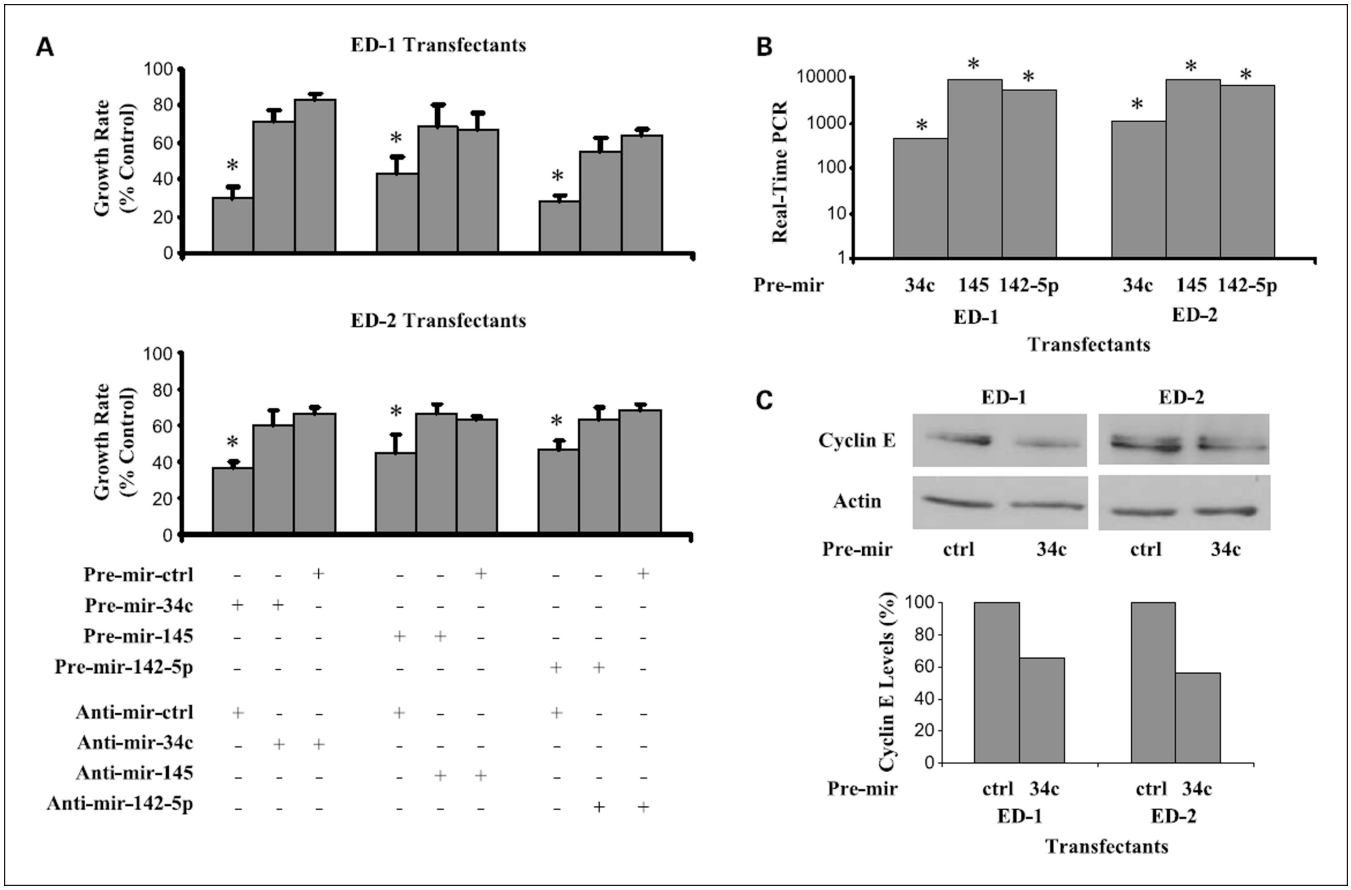

Fig. 4.

Individual miR-34c, miR-145, and miR-142-5p transfection repressed both ED-1and ED-2 cell growth. *A*, repression of ED-1and ED-2 cell growth was significantly suppressed by individual transfection of pre-miR-34c, pre-miR-145, or pre-miR-142-5p. Partial rescue of growth inhibition occurred by cotransfection of the corresponding inhibitoryanti-miR-34c, anti-miR-145, oranti-miR-142-5p. *, $P < 0.005$.SDs are shown. *B*, transfectionofeachpre-miRs was confirmedby real-time RT-PCRassays of ED-1and ED-2 transfectants. *, $P < 0.00001$. *C*, transfection of miR-34c versus control (*ctrl*) pre-miR in ED-1and ED-2 cells repressed cyclin E.*Top*, immunoblot expression for cyclin E and β-actin; *bottom*, cyclin E quantification.