EGFR Promotes Lung Tumorigenesis by Activating miR-7 through a Ras/ERK/Myc Pathway that Targets the Ets2 Transcriptional Repressor ERF

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EGFR Promotes Lung Tumorigenesis by Activating miR-7 through a Ras/ERK/Myc Pathway that Targets the Ets2 Transcriptional Repressor ERF

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Abstract

MicroRNAs (miRNA) mediate distinct gene regulatory pathways triggered by epidermal growth factor receptor (EGFR) activation, which occurs commonly in lung cancers with poor prognosis. In this study, we report the discovery and mechanistic characterization of the miRNA miR-7 as an oncogenic "oncomiR" and its role as a key mediator of EGFR signaling in lung cancer cells. EGFR activation or ectopic expression of Ras as well as c-Myc stimulated miR-7 expression in an extracellular signal-regulated kinase (ERK)–dependent manner, suggesting that EGFR induces miR-7 expression through a Ras/ERK/Myc pathway. In support of this likelihood, c-Myc bound to the miR-7 promoter and enhanced its activity. Ectopic miR-7 promoted cell growth and tumor formation in lung cancer cells, significantly increasing the mortality of nude mice hosts, which were orthotopically implanted with lung cancers. Quantitative proteomic analysis revealed that miR-7 decreased levels of the Ets2 transcriptional repression factor ERF, the coding sequence of which was found to contain a miR-7 complementary sequence. Indeed, ectopic miR-7 inhibited production of ERF messages with a wild-type but not a silently mutated coding sequence, and ectopic miR-7 rescued growth arrest produced by wild-type but not mutated ERF. Together, these results identified that ERF is a direct target of miR-7 in lung cancer. Our findings suggest that miR-7 may act as an important modulator of EGFR-mediated oncogenesis, with potential applications as a novel prognostic biomarker and therapeutic target in lung cancer. *Cancer Res; 70(21); 8822–31. ©2010 AACR.*

Introduction

Lung cancer, predominantly non–small cell lung cancer (NSCLC), remains the leading cause of cancer death worldwide (1). The epidermal growth factor receptor (EGFR) signaling network plays a central role in the growth and maintenance of epithelial tissues, and alterations of this network can lead to malignant transformation (2). EGFR is overexpressed or mutated in most NSCLC cases, and deregulated expression of EGFR together with ligand binding and concomitant receptor activation promotes tumor cell growth, proliferation, and survival (2, 3).

MicroRNAs (miRNA) are endogenous noncoding RNAs of ~22 nucleotides that regulate gene expression by binding to the complementary sequences within mRNAs, thereby preventing synthesis of their protein products (4–6). Several miRNAs display abnormal expression patterns in human tumors with consequent alteration of target oncogenes or tumor suppressor genes (7). Owing to their "fine-tuning" modulatory capabilities, miRNAs are emerging as key regulators in various signaling pathways involved in development and cancer progression (8). Although EGFR signaling is important and well studied with respect to lung cancer progression, little is known about how miRNAs mediate EGFR signaling to modulate tumorigenesis. Interestingly, a report showed that EGFR activates the eye development cascade in *Drosophila* by inducing miR-7 to fine-tune the expression of Yan, an Ets repressor (9). ERF (Ets2 repressor factor) is an Ets domain transcriptional repressor, regulated by the Ras/extracellular signal-regulated kinase (ERK) pathway. Phosphorylation of ERF by ERKs determines its subcellular localization (10, 11), and ERF functions as a constant sensor of ERK activity that links it to Ras/ERK-mediated cell proliferation and differentiation (11, 12). ERF can suppress Ets- and Ras-induced transformation (10, 13). Its activation induced

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by the release of Ras/ERK-mediated suppression has also been implicated in erythroid differentiation (12).

Because both the deregulation of EGFR and miRNAs contribute to neoplasia, we investigate the role of miRNAs, regulated by the abnormal EGFR signaling, in lung cancer progression. To search for miRNAs regulated by EGFR, we performed miRNA microarray analysis of EGFR-silenced CL1-5 cells, a lung cancer cell line that overexpresses EGFR. Strikingly, we found that miR-7 is highly induced by EGFR in lung cancer. In the present study, we show for the first time that deregulated EGFR signaling induces miR-7, which in turn suppresses ERF and plays an important role in the oncogenesis of lung cancer cells.

Materials and Methods

Cell culture

We used CL1-5, A549, and H928 lung cancer lines. CL1-5 cells were established in our laboratory as previously described (14). A549 and H928 cells were obtained from the American Type Culture Collection before 2007. All lung cancer cell lines were tested positive for human origin and for the presence of EGFR expression in the current genetic analysis. All lung cancer cells were grown in RPMI 1640 with 10% fetal bovine serum.

Stable isotope labeling by amino acids in cell culture analysis

The stable isotope labeling by amino acids in cell culture (SILAC) assay was performed using SILAC protein identification and quantification kits according to standard protocols (Invitrogen). miR-7–overexpressing CL1-5 and vector-only control cells were grown in labeled (L-$^{13}C_6$, $^{15}N_2$-Arg, and L-$^{13}C_6$-Lys) and normal medium, respectively, for 7 days. Equal amounts of heavy and light cells were mixed and subjected to nuclear and cytoplasmic protein extraction. Both protein fractionations were further separated by 1D SDS-PAGE and followed by tryptic digestion. Nuclear and cytoplasmic proteins after tryptic digestion were subjected to isoelectic focusing analysis using OFF-GEL instrument. Peptide mixtures were analyzed by liquid chromatography coupled mass spectrometry (LTQ-Orbitrap), and proteins were identified and quantified using MASCOT and MaxQuant software (15).

Patients and tissue samples

Patient samples were obtained according to Taipei Medical University Institutional Review Board–approved guidelines. The epidermal growth factor receptor mutation status was determined by a direct sequencing method (exons 18–21 in EGFR).

Statistical analysis

Disease-free survival and overall survival were estimated using the Kaplan-Meier method. The statistically significant difference in expression level of miR-7 between tumor and adjacent nontumorous tissues was calculated using the Wilcoxon test. All statistical analyses were performed with SPSS software version 12.0 (SPSS, Inc.).

Additional details are included in the Supplementary Data.

Results

EGFR induces miR-7 expression

Lung cancer cells are addicted to EGFR oncogenes (16). To show the importance of EGFR in lung cancer, we knocked down EGFR in CL1-5 lung cancer cells with EGFR short hairpin RNA (shRNA)–bearing lentiviruses. Growth arrest and enlarged cell size were observed in EGFR-silenced CL1-5 cells (Supplementary Fig. S1A), whereas quantitative reverse transcription-PCR (qRT-PCR) and immunoblotting showed that both *EGFR* mRNA and protein levels were significantly downregulated (Fig. 1A). miRNA microarray analysis of total RNA from EGFR-silenced cells was used to identify miRNAs regulated by EGFR, and among them, miR-7 showed a significant downregulation (Table 1). The downregulation of miR-7 was further confirmed by both qRT-PCR analysis and the RNase protection assay (Fig. 1B). Knockdown of EGFR inhibited the expression of miR-7 in A549 lung cancer cells, although both EGFR and miR-7 expressions were lower in A549 cells than in CL1-5 cells (Supplementary Fig. S1B). Stimulation of A549 cells with the ligand EGF caused significant miR-7 induction in a time-dependent manner (Fig. 1C). Expression of the EGFR mutant (L858R), which promoted phosphorylations of EGFR and c-Myc, enhanced the expression of miR-7 in CL1-5 cells (Fig. 1D). These results indicate that miR-7 is induced by EGFR signaling.

Ras/ERK/Myc pathway regulates miR-7 expression

Because the miR-7 expression is dependent on EGFR signaling, we asked if EGFR downstream effectors can regulate miR-7 expression. Overexpression of the constitutively active form of Ras in CL1-5 cells increased the miR-7 level (Fig. 2A). In contrast, the EGFR-induced miR-7 expression was reduced by PD98059, an inhibitor of ERK1/2 phosphorylation (Fig. 2B). Because c-Myc is the downstream effector of the EGFR/Ras/ERK pathway, we further examined the miR-7 expression in c-Myc–overexpressing cells and found that c-Myc could enhance the miR-7 expression (Fig. 2C). These data show that the Ras/ERK/Myc signaling is involved in the EGFR-induced miR-7 expression.

We found that EGF stimulated the expressions of miR-7 and its precursor pre-miR-7-1, but not pre-miR-7-2 or pre-miR-7-3 (Supplementary Fig. S2). To determine whether the increased expression of miR-7 by EGF stimulation is regulated at the transcriptional level, we examined the effect of transcriptional inhibitors on miR-7 expression. A transcriptional inhibitor, Actinomycin D, blocked the EGF-induced expressions of miR-7 and pre-miR-7-1, but not pre-miR-7-2 and pre-miR-7-3, suggesting that the transcription of pre-miR-7-1 is primarily responsible for EGF-induced miR-7 expression (Supplementary Fig. S2). We cloned the 1.5-kb (-1439 to +61 bp of *miR-7-1* coding sequence) putative *miR-7-1* promoter into a TATA-less pGL3- basic reporter to test the transcriptional ability of *miR-7-1* promoter in cells. We found that transient expression of c-Myc, but not the control vector, efficiently stimulated the transcription of *miR-7* in CL1-5 cells with the reporter containing *miR-7-1* promoter (Fig. 2C). We

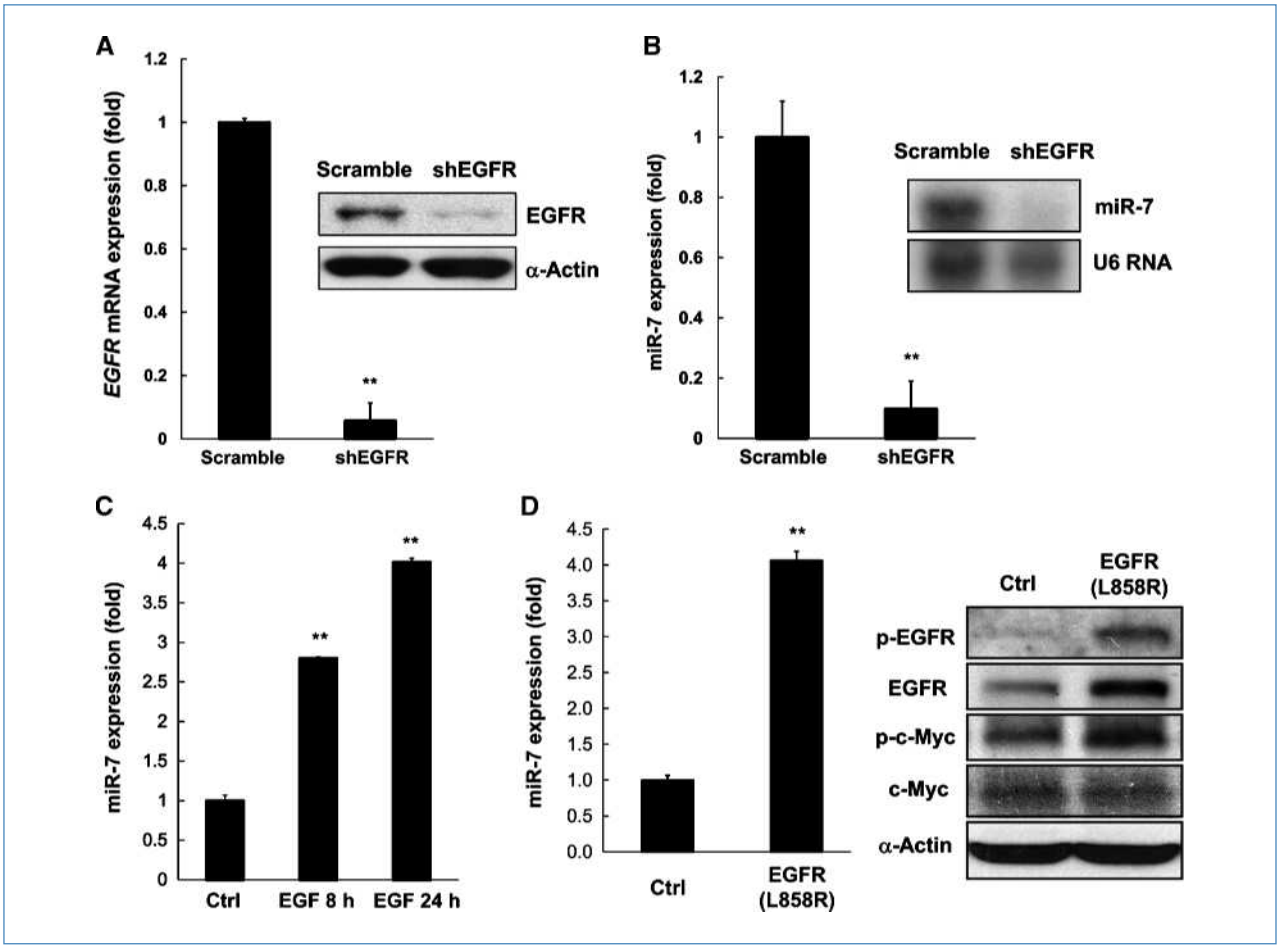

Figure 1. miR-7 expression is regulated by EGFR signaling. A, qRT-PCR (left) and immunoblotting (right) were used to determine the expression of EGFR 2 d after infection of CL1-5 cells with lentiviral vector pLKO.1 carrying shRNA for EGFR (shEGFR) or control shRNA (Scramble). B, expression of miR-7 was determined by qRT-PCR (left) and RNase protection assay (right) in CL1-5 cells infected with shEGFR or Scramble vector as described in A. U6 snRNA was used as an internal control. C, expression of miR-7 was determined by qRT-PCR in serum-starved A549 cells treated with EGF (100 ng/mL) for the indicated times. D, expression of miR-7 was determined by qRT-PCR (left) in CL1-5 cells 5 d after infection with lentiviral vector HR′-puro (Ctrl) or HR′-puro-EGFR (L858R). Immunoblotting (right) was used to show the expressions of phosphorylated EGFR, total EGFR, phosphorylated c-Myc, and total c-Myc in CL1-5 cells with or without EGFR (L858R). Data represent the mean ± SD of three independent experiments (each $n = 3$). All data were analyzed by the t test: **, $P < 0.01$.

then performed chromatin immunoprecipitation analysis with the *miR-7-1* promoter to show the recruitment of c-Myc to the *miR-7* gene via EGFR signaling. On EGFR stimulation, more c-Myc was indeed recruited to the *miR-7-1* promoter region encompassing a conservative E-box (5′-CAGTTG-3′), a putative c-Myc binding site located at positions -539 to -534 of *miR-7-1* promoter (Fig. 2D). In contrast, c-Myc did not bind to the site (+3002 to +3140 bp) located at 3-kb downstream of the *miR-7-1* coding sequence (Fig. 2D).

Ectopic expression of miR-7 enhances oncogenic properties of lung cancer cells

We investigated the functional role of miR-7 in lung cancer cells by ectopically expressing miR-7 in CL1-5 cells with lentiviral infection. An artificial reporter system of miR-7 with green fluorescent protein (GFP) was constructed to evaluate whether miR-7 could suppress the expression of a certain specific gene. In this reporter construct, two copies of perfect complementary sequences of miR-7 were inserted in the 3′ untranslated region (UTR) downstream of the GFP open reading frame, which functions as a reporter gene (Fig. 3A). When this reporter vector was transfected into CL1-5 cells, the GFP expression was detected by fluorescence microscopy (Fig. 3A). However, the expression of GFP was abolished in the cells with the reporter vector on coexpression of miR-7 (Fig. 3A). The fact that the lentiviral-based miR-7 could suppress target gene expression allowed us to study the functional roles of miR-7 in lung cancer cells. Because EGFR promotes cell growth and induces miR-7 expression, we tested whether EGFR-regulated cell proliferation is mediated by miR-7. Indeed, miR-7 overexpression promoted cell proliferation as shown by the WST-1 conversion assay (Fig. 3B, top).

Table 1. Total RNA from CL1-5 lung cancer cells infected with pLKO.1-Scramble (Scramble) or pLKO.1-shEGFR (shEGFR) was collected 2 d after infection and subjected to human miRNA oligo microarray analysis

EGFR-regulated miRNAs			
miRNA systematic name	miRNA ratio (Scramble/shEGFR)		
	Array fold	qRT-PCR	
		Fold	*P*
hsa-miR-7	28.92	11.62	0.00410
hsa-miR-361-3p	11.06	2.77	0.00750
hsa-miR-374b	7.60	5.26	0.00901
hsa-miR-33a	7.00	5.55	0.00487
hsa-miR-19a	6.40	5.26	0.00134
hsa-miR-19b	6.10	4.76	0.00791
hsa-miR-182	5.91	2.27	0.03978
hsa-miR-210	5.11	2.17	0.00900
hsa-miR-20a	3.96	6.60	0.00843
hsa-miR-17-5P	3.95	5.26	0.00965
hsa-miR-17-3P	3.84	3.84	0.00408
hsa-miR-21	3.85	4.00	0.00012
hsa-miR-92a	3.68	5.26	0.00014
hsa-miR-134	0.16	0.37	0.00009
hsa-miR-630	0.13	0.22	0.02698

NOTE: The knockdown effect of EGFR on miRNA expression in CL1-5 lung cancer is represented as a relative miRNA expression ratio (Scramble/shEGFR) from the probe signals of arrays. The miRNA expression ratio (Scramble/shEGFR) was further confirmed with Ncode miRNA qRT-PCR system (Invitrogen) and represented with the means of three independent experiments (each $n = 3$). Statistical analysis of the miRNA expression ratio (Scramble/shEGFR) was performed using *t* test, and corresponding *P* values were calculated and presented.

Furthermore, miR-7 overexpression promoted colony formation in soft agar, an indication of anchorage-independent cell growth (Fig. 3B, bottom). These results indicate that miR-7 possesses oncogenic potential.

To evaluate the functional role of miR-7 *in vivo*, we injected s.c. both miR-7–overexpressing CL1-5 and vector-only control cells into athymic nude mice and measured the tumor volume over time. miR-7 overexpression significantly increased the tumor growth rate (Fig. 3C). Similarly, it also enhanced the weight of tumors harvested 6 weeks after injection (Supplementary Fig. S3B). The overexpression of miR-7 was further confirmed by qRT-PCR in excised tumors (Supplementary Fig. S3C). Furthermore, orthotopic implantation of tumor cells into the lungs of nude mice resulted in significantly higher mortality of nude mouse recipients of miR-7–overexpressing lung cancer cells compared with those of control cells (Fig. 3D). After the mice expired, the growth of miR-7–overexpressing tumor labeled with GFP was examined by fluorescence imaging of the lung cancer (Fig. 3D). Taken together, these animal studies suggest that miR-7 promotes lung cancer formation and progression *in vivo*.

Elevated miR-7 expression in human lung cancers

We next looked into the possibility that miR-7 could enhance lung tumorigenesis in humans. The levels of miR-7 in 30 pairs of primary NSCLC specimens (including 20 adenocarcinoma and 10 squamous cell carcinoma specimens) and the corresponding adjacent nontumorous lung tissues were measured by qRT-PCR. The expression of miR-7 represented by a cancer-to-adjacent nontumorous tissue ratio for each individual was 1.5 for 50% of adenocarcinoma and 60% of squamous cell carcinoma samples (Fig. 4A), whereas the average of miR-7 expression was ~2.08-fold higher in lung cancer samples than in adjacent nontumorous tissues ($P = 0.003$; Fig. 4B). We further investigated the correlation between *EGFR* and miR-7 expressions. EGFR-activating mutations were identified in 5 of the 30 NSCLC patients, and all of them were females with adenocarcinoma (Supplementary Table S1). *EGFR* and miR-7 expression levels were significantly correlated in the *EGFR* mutated lung adenocarcinoma ($R = 0.912$; $P < 0.05$), but not in the adjacent nontumorous tissue (Fig. 4C) or the non-*EGFR* mutated adenocarcinoma (data not shown). Interestingly, in the 10 pairs of squamous cell carcinoma samples, *EGFR* and miR-7 expressions were also significantly correlated ($R = 0.966$; $P < 0.01$; Supplementary Table S2). These data are consistent with the *in vitro* observation that deregulated EGFR signaling induces miR-7 expression. Although the expression of miR-7 did not significantly affect the overall survival of NSCLC patients ($P = 0.386$), there was a statistically significant difference in disease-free survival favoring patients with low miR-7 expression ($P = 0.045$; Fig. 4D). Together, these results are consistent with the notion that miR-7 is overexpressed in human lung cancer and participates in lung cancer progression.

miR-7 targets ERF for suppression

To identify the possible miR-7 target genes, we used quantitative proteomics and SILAC to identify proteins differentially expressed in cells with or without miR-7 overexpression. Differential expressions of several proteins were detected in miR-7–overexpressing cells compared with control cells (Supplementary Table S4). ERF expression was significantly decreased in miR-7–overexpressing cells (Supplementary Fig. S4A). *ERF* mRNA was upregulated in EGFR-silenced cells as shown by qRT-PCR analysis (Fig. 5A). On the other hand, knockdown of EGFR in CL1-5 cells increased ERF protein level as miR-7 was downregulated, indicating that both miR-7 and ERF were regulated by EGFR signaling (Fig. 5B). We further examined the effect of miR-7 on ERF expression in human lung cancer cells. qRT-PCR and Western blot analysis showed that miR-7 overexpression in CL1-5 cells suppressed the levels of *ERF* mRNA and protein (Fig. 5A and B). The inhibition of ERF expression by miR-7 was also observed in A549 and

H928 lung cancer cells (Supplementary Fig. S4B). In contrast, blocking the function of miR-7 with a sequence-specific antisense inhibitor (anti-miR-7) increased the ERF mRNA and protein expressions, suggesting that miR-7 inhibits the ERF expression (Fig. 5A and B). It has been reported that the activity of cyclin A, a key cell cycle regulator, is enhanced by Ets2, and its overexpression can partially rescue the ERF-mediated cell growth arrest (11, 17). We found that knockdown of ERF with ERF shRNA significantly increased cyclin A expression. Similarly, miR-7 overexpression also stimulated cyclin A expression, suggesting that miR-7 could antagonize the ERF-mediated cyclin A suppression (Fig. 5B). We next asked whether ERF is a direct target of miR-7. After the coding sequence (CDS) of *ERF* was overexpressed in CL1-5 cells, both miR-7 and ERF shRNA inhibited *ERF* mRNA expression to a similar extent, implying that the miR-7 target site could be within the *ERF* CDS (Fig. 5C). Comparison of the *ERF* CDS with the miR-7 sequence by RNAhybrid software (18) led to identification of a possible conserved miR-7 target site in the CDS of *ERF* mRNA. We made two silent mutations in the CDS, which did not alter its amino acid sequence (Fig. 5C). Western blot analysis indicated that miR-7 inhibited ectopic expression of the wild-type but not of the mutant ERF (Fig. 5C). These data show that miR-7 suppresses ERF expression by directly targeting the *ERF* CDS. Furthermore, the growth of CL1-5 cells was attenuated by the ectopic expression of ERF or ERF-mutant, whereas the expression of miR-7 promoted cell proliferation. Coexpression of miR-7 rescued the ERF-mediated, but not the mutated ERF (Mut)–mediated, growth arrest of cells (Fig. 5D). These results indicate that ERF is one of the major targets of miR-7–modulated cell growth.

Discussion

It has been widely recognized that lung cancer is under EGFR-mediated oncogenesis (16, 19, 20). In this study, we have shown that EGFR stimulated miR-7 expression via the Ras/ERK/Myc pathway to promote cell proliferation, anchorage-independent cell growth, and tumor formation of lung cancer cells. Knockdown of EGFR led to marked cell

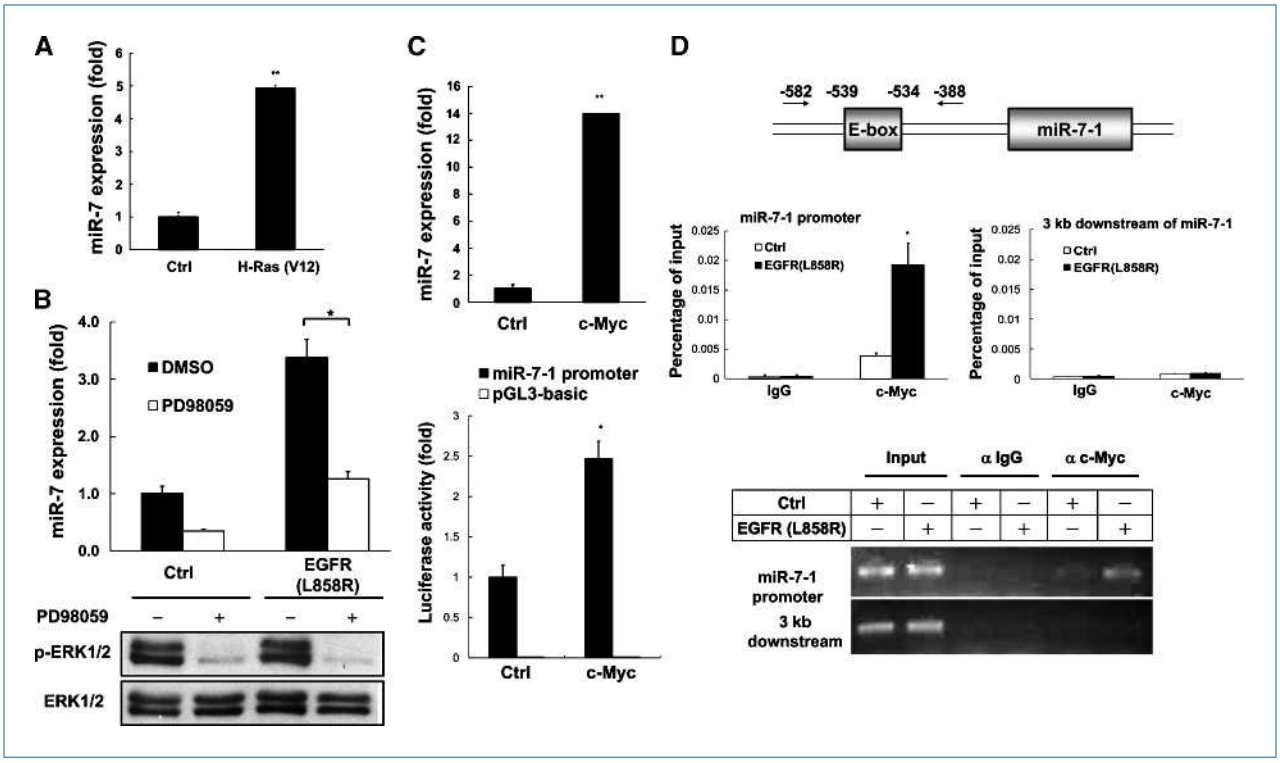

Figure 2. Ras/ERK/Myc signaling controls miR-7 expression. A, expression of miR-7 was determined by qRT-PCR in CL1-5 cells 5 d after infection with pBabe-puro-H-Ras (V12) or pBabe-puro (Ctrl) alone. B, expression of miR-7 was determined by qRT-PCR in CL1-5 cells infected with HR′-puro (Ctrl) or HR′-puro-EGFR (L858R) and treated with PD98059 (50 μmol/L) or DMSO for 12 h. Immunoblotting (bottom) was used to monitor the expressions of phosphorylated ERK and total ERK in the presence or absence of PD98059. C, expression of miR-7 was determined by qRT-PCR in CL1-5 cells 5 d after infection with HR′-puro (Ctrl) or HR′-puro-c-Myc (c-Myc; top). Luciferase reporter assays of normalized *miR-7-1* promoter activity were performed in CL1-5 cells 2 d after transfection with pcDNA (Ctrl) or pcDNA-c-Myc (c-Myc) (bottom). D, binding of c-Myc to the E-box of *miR-7-1* promoter (-582 to -388 bp upstream of *miR-7-1* coding sequence) was determined by the chromatin immunoprecipitation assay with anti-c-Myc antibodies or irrelevant mouse IgG in CL1-5 cells 5 d after infection with HR′-puro (Ctrl) or HR′-puro-EGFR (L858R). The input was used as a positive control in the subsequent PCR. In contrast, chromatin immunoprecipitation assay with primers for the site (+3,002 to +3,140 bp) located at 3-kb downstream of *miR-7-1* was performed as a negative control. The PCR products were also electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining (bottom). Data represent the mean ± SD of three independent experiments (each $n = 3$). All data were analyzed by t test. *, $P < 0.05$; **, $P < 0.01$.

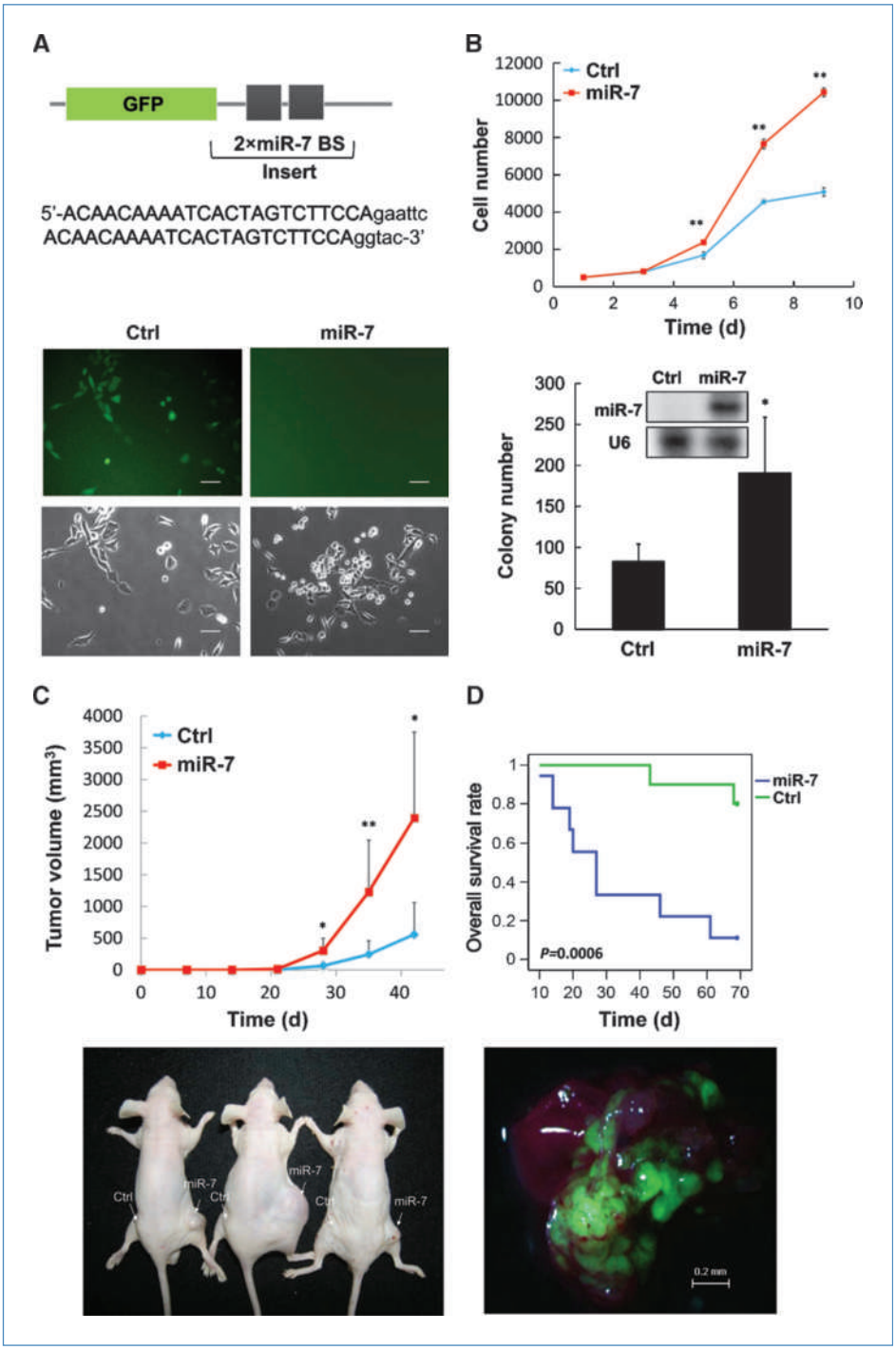

Figure 3. The effect of miR-7 on tumor cell growth. A, an miR-7 target reporter (GFP-2×miR-7-BS) was constructed by inserting two copies of perfect complementary miR-7 sequences at the 3′ UTR of GFP in HR′-CMV-GFP (top). CL1-5 reporter cells were obtained by infection of CL1-5 cells with HR′-CMV-GFP-2×miR-7-BS, followed by second infection with HR′-puro-miR-7 (miR-7) or empty vector (Ctrl). Expression of GFP was detected by fluorescence microscopy in miR-7–overexpressing or vector-only CL1-5 reporter cells (middle). Cell morphology was monitored under a phase-contrast microscope (100×; bottom). Scale bar, 10 μm. B, a time course of cell proliferation by WST-1 assay of CL1-5 cells infected with HR′-puro (Ctrl) or HR′-puro-miR-7 (miR-7) (top). miR-7 expression was determined by RNase protection assay in miR-7–overexpressing (miR-7) or vector-only control (Ctrl) CL1-5 cells (middle). Growth in soft agar of infected CL1-5 cells was analyzed 14 d after initial plating (bottom). The results are the means of three independent experiments (each $n = 6$). C, CL1-5 cells infected with HR′-puro (Ctrl) or HR′-puro-miR-7 (miR-7) were injected s.c. into the right and left sides, respectively, of the flank region of nude mice. Tumor volume was monitored over time as indicated. Error bars indicate SEM ($n = 7$; top). Photographs illustrate representative features of the tumor growth 6 wk after injection (bottom). D, Kaplan-Meier analysis of overall survival was calculated from 10 pairs of cancer growing nude mice, in which miR-7 overexpressing (miR-7) or vector-only (Ctrl) CL1-5 cells were orthotopically implanted into the lung (top). To label lung cancer cells, miR-7–overexpressing and control CL1-5 cells were infected with MSCV-GFP before implantation. Representative bright field/fluorescence images of the lungs of mice bearing the miR-7–overexpressing tumors were shown (bottom). All data were analyzed by *t* test. *, $P < 0.05$; **, $P < 0.01$.

death, further showing the importance of EGFR signaling for cell proliferation and survival of lung cancer cells (Supplementary Fig. S1A). We found that miR-7 is overexpressed in primary lung cancers, and its overexpression is inversely correlated with disease-free survival, suggesting that miR-7 modulates EGFR oncogenic addiction.

c-Myc has been reported to control transcription of miRNAs (21). c-Myc induces miR-7 in B-cell lymphoma (22) and stimulates miR-17-92 cluster through binding to E-boxes in the promoter to activate transcription (21). Here, we have found that members of the miR-17-92 cluster, such as miR-19, miR-20, miR-17, and miR-92, are also significantly downregulated in EGFR-silenced lung cancer cells (Table 1), indicating that c-Myc can modulate expression of EGFR-induced miRNAs. Among the mitogen-activated protein kinase pathways, we noticed that inhibition of ERK, but not p38 or c-Jun NH_2

terminal kinase, affects the EGFR-mediated miR-7 expression, supporting the notion that through the Ras/ERK/Myc pathway, EGFR activation induces the expression of miR-7 (Fig. 2B; Supplementary Fig. S5). In addition, AKT is reported to regulate EGFR-mediated cell survival (23). We have found that blocking the EGFR-mediated phosphoinositide 3-kinase (PI3K)/AKT pathway with LY294002 inhibitor also attenuates miR-7 expression (data not shown), suggesting that the PI3K/AKT pathway participates in miR-7 regulation. However, the molecular link between PI3K/AKT activation and miR-7 expression remains further elucidation.

Li and colleagues (9) reported that during *Drosophila* eye development, miR-7 fine-tuned the EGFR-mediated maturation of photoreceptor neurons by targeting Yan, an Ets domain transcriptional repressor. Yan inhibits cell proliferation and plays a key role in the determination of cell fate (24). In this study, we observed that EGFR induces miR-7, which plays a role in oncogenesis of lung cancer through suppressing ERF expression. Analogous to Yan, ERF is an Ets family transcriptional repressor and functions as a constant sensor of ERK activity that links the Ras/ERK-mediated cellular proliferation to differentiation (11, 12). Thus, it seems that the EGFR-induced miR-7 regulatory pathway is evolutionarily conserved and may be hijacked to promote tumor formation in mammalian cells.

Both Yan and ERF are phosphorylated by ERK1/2, and the phosphorylation directs the translocation of ERF from the nucleus to the cytoplasm (10, 11). Furthermore, phosphorylation of Yan (25) but not ERF leads to its degradation (10, 11). We have shown that through the Ras/ERK/Myc pathway, EGFR induces miR-7 expression, which in turn suppresses ERF at both the mRNA and protein levels, whereas knockdown of EGFR significantly upregulates ERF, suggesting that EGFR mediates the ERF expression via miR-7. Thus, ERK signaling controls the ERF activity through not only a rapid regulation of subcellular localization but also an efficient miR-7–mediated inhibition. It seems that multiple mechanisms are controlling the expression, activity, and subcellular localization of ERF, which highlight its physiologic significance in the cell. Recently, ERF was suggested to impose repression checkpoints on a subset of cell cycle control genes (26, 27). We have shown that cyclin A, a key factor in the cell

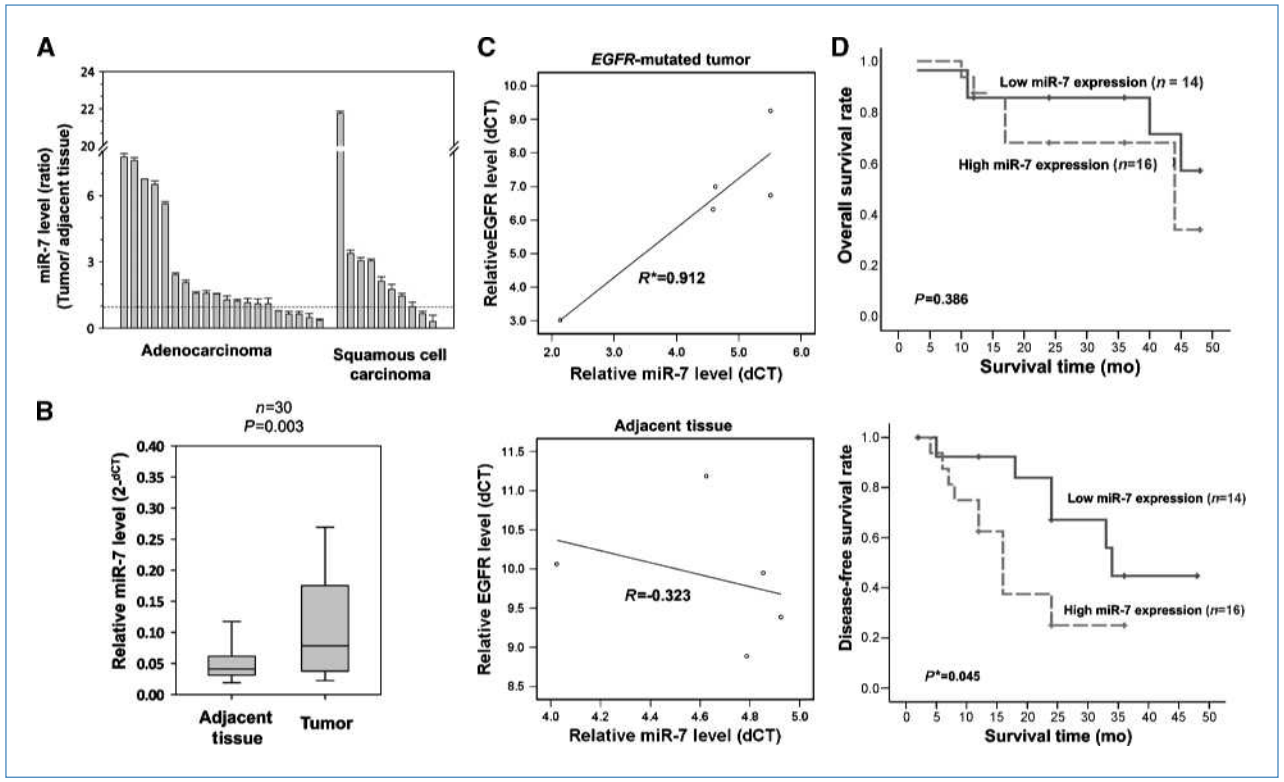

Figure 4. miR-7 is upregulated in primary lung cancer, and this upregulation is inversely correlated with disease-free survival. A, relative miR-7 expression was determined by qRT-PCR in 20 pairs of lung adenocarcinoma and 10 pairs of squamous cell carcinoma samples. Each bar represents the relative ratio of miR-7 in primary NSCLC versus adjacent nontumorous lung tissue, and the dotted line across the graph represents the normalized miR-7 level equal to that of normal tissue (ratio = 1). The results are the means of three independent experiments (each $n = 3$) ± SD. B, qRT-PCR analysis of miR-7 was performed with 30 pairs of primary NSCLC and matching adjacent nontumorous lung tissues. Statistical significance was calculated using the Wilcoxon test ($P = 0.003$). C, Pearson correlation analysis for miR-7 and *EGFR* expressions in five *EGFR* mutated adenocarcinoma specimens (top) or the adjacent nontumorous lung tissues (bottom; *, $P < 0.05$). D, Kaplan-Meier analyses of overall survival (top) and disease-free survival (bottom) curves were calculated from 30 NSCLC patients with tumors expressing high (>1.5-fold) and low levels of miR-7. Statistical significance was calculated using the log-rank test.

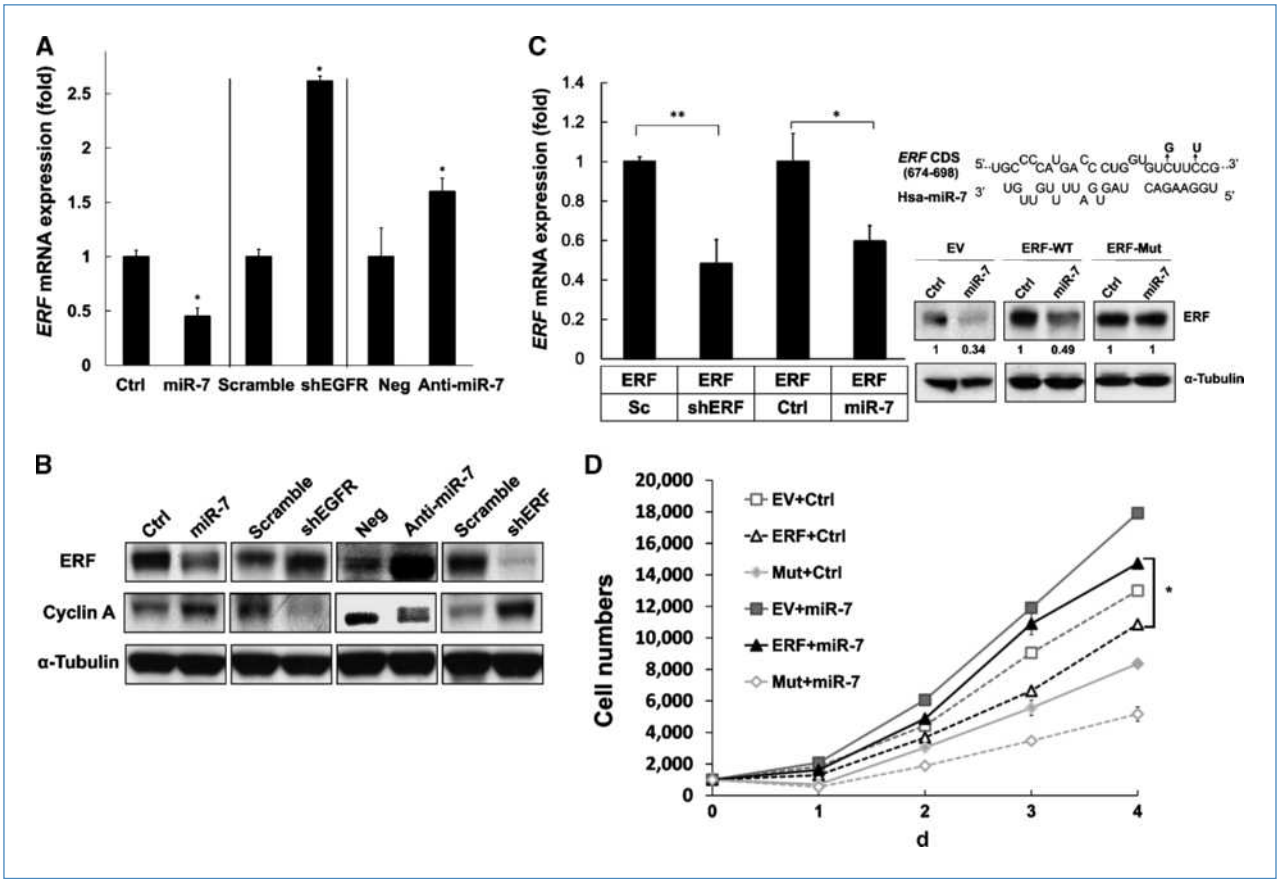

Figure 5. miR-7 targets the coding sequence of *ERF* for inhibition. A, expression of ERF was determined by qRT-PCR in CL1-5 cells infected with HR′-puro (Ctrl), HR′-puro-miR-7 (miR-7), pLKO.1-Scramble (Scramble), or pLKO.1-shEGFR (shEGFR) or transfected with an antisense inhibitor for miR-7 (Anti-miR-7) or a nonspecific inhibitor (Neg). The results are the means of three independent experiments (each $n = 3$) ± SD. B, immunoblots were used to measure the expressions of ERF and cyclin A proteins in CL1-5 cells infected with HR′-puro (Ctrl), HR′-puro-miR-7 (miR-7), pLKO.1-Scramble (Scramble), pLKO.1-shEGFR (shEGFR), or pLKO.1-shERF (shERF) or transfected with an antisense inhibitor for miR-7 (Anti-miR-7) or a nonspecific inhibitor (Neg). C, expression of ERF was determined by qRT-PCR in CL1-5 cells infected first with HR′-puro-ERF encoding the wild-type *ERF* coding sequence (ERF) and subsequently with pLKO.1-Scramble (SC), pLKO.1-shERF (shERF), HR′-puro (Ctrl), or HR′-puro-miR-7 (miR-7; left). The results are the means of three independent experiments (each $n = 3$) ± SD. A putative miR-7 binding sequence in the coding sequence (CDS) of *ERF* mRNA was illustrated (top right). Two silent mutations (C-G and C-U) were introduced in the coding sequence of *ERF* for the following experiments as indicated by arrows. Expression of ERF protein level was determined in CL1-5 cells infected first with HR′-puro encoding the wild-type *ERF* (ERF-WT), mutated *ERF* (ERF-Mut), or empty vector (EV), and subsequently with either HR′-puro-miR-7 or HR′-puro (Ctrl) (bottom right). The intensity for each band was densitometrically quantified. The results are the means of three independent experiments (each $n = 3$) ± SD. Asterisks indicate significant difference. D, cell growth analysis was performed from CL1-5 cells infected first with HR′-puro-ERF (ERF), HR′-puro-ERF-mutant (Mut), or empty vector (EV) and subsequently with either HR′-puro-miR-7 (miR-7) or HR′-puro (Ctrl). The results are the means of three independent experiments (each $n = 4$) ± SD. All data were analyzed by the *t* test. *, $P < 0.05$; **, $P < 0.01$.

cycle, is suppressed by ERF and stimulated by miR-7 through inhibition of ERF. The inhibition of ERF provides a partial, if not complete, explanation for miR-7–mediated cell proliferation in lung cancer.

miR-7 was previously found to regulate cell growth and apoptosis of cervical cancer and neuroblastoma cells (28, 29). In this study, we found that miR-7 was overexpressed in human primary lung cancers and negatively correlated with disease-free survival, echoing the important role of miR-7 in cancer progression. It has been shown that EGFR overexpression or amplification occurs more frequently in squamous cell carcinoma than in adenocarcinoma, but *EGFR* mutation occurs mostly in adenocarcinoma (30). Our observation that the level of miR-7 correlates with *EGFR* expression in squamous cell carcinoma and *EGFR* mutated adenocarcinoma supports the notion that deregulated EGFR signaling induces miR-7 expression. Previously, miR-7 was reported to suppress EGFR expression and function as a tumor suppressor in glioblastoma (31, 32). However, two reports stated that miR-7 expression correlates with poorer prognosis in patients with breast cancer and urothelial carcinoma (33, 34). In addition, miR-7 was shown to inhibit breast cancer migration (35). Nonetheless, we found that the ectopic expression of miR-7 had no effect on migration of CL1-5 lung cancer cells (data not shown). Therefore, the role of miR-7 may be different in various cancer types. Although EGFR stimulates miR-7 expression, miR-7

overexpression not only blocked ERF but also attenuated EGFR expression in lung cancer cells in our study (Supplementary Fig. S6). These findings indicate that a delicate balance of the regulatory circuit may exist between EGFR and miR-7 as well as its targets such as ERF. Such a delicate balance may finally determine the outcome of cell fate and the role of miR-7 as an oncomiR or tumor suppressor. Consistent with our observation, Cheng and colleagues (28) showed that the inhibition of miR-7 expression suppresses growth of A549 lung carcinoma cells. Because EGFR induces miR-7 expression to promote cell growth, the attenuation of EGFR expression by miR-7 at a high concentration could be a fine-tuning regulatory mechanism to maintain the balance between cell proliferation and differentiation. In accordance with this hypothesis, recent reports showed that miR-7 functions in several interlocking feedback and feedforward loops to buffer the developmental programs and fine-tune differentiation processes in *Drosophila* (36, 37).

In conclusion, we have identified an evolutionarily conserved regulatory network of EGFR-induced miR-7 expression, which in turn targets ERF to modulate cell growth of human lung cancer. Our finding that miR-7 modulates EGFR-mediated oncogenesis may serve as a novel prognostic biomarker and therapeutic target for lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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