

The *let-7* MicroRNA Represses Cell Proliferation Pathways in Human Cells

Charles D. Johnson,¹ Aurora Esquela-Kerscher,³ Giovanni Stefani,³ Mike Byrom,¹ Kevin Kelnar,¹ Dmitriy Ovcharenko,¹ Mike Wilson,¹ Xiaowei Wang,² Jeffrey Shelton,¹ Jaclyn Shingara,¹ Lena Chin,³ David Brown,¹ and Frank J. Slack³

¹Asuragen, Inc.; ²Ambion, Inc., Austin, Texas, and ³Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut

Abstract

MicroRNAs play important roles in animal development, cell differentiation, and metabolism and have been implicated in human cancer. The *let-7* microRNA controls the timing of cell cycle exit and terminal differentiation in *Caenorhabditis elegans* and is poorly expressed or deleted in human lung tumors. Here, we show that *let-7* is highly expressed in normal lung tissue, and that inhibiting *let-7* function leads to increased cell division in A549 lung cancer cells. Overexpression of *let-7* in cancer cell lines alters cell cycle progression and reduces cell division, providing evidence that *let-7* functions as a tumor suppressor in lung cells. *let-7* was previously shown to regulate the expression of the *RAS* lung cancer oncogenes, and our work now shows that multiple genes involved in cell cycle and cell division functions are also directly or indirectly repressed by *let-7*. This work reveals the *let-7* microRNA to be a master regulator of cell proliferation pathways. [Cancer Res 2007;67(16):7713–22]

Introduction

Hundreds of microRNAs (miRNA) are encoded in animal genomes, where they provide important regulatory functions in development, apoptosis, life span, and metabolism (1, 2). A number of miRNAs have also been linked to human cancer (3–8); we refer to this class of miRNAs as “oncomirs” (9). These are roughly divided into two groups, those miRNAs that are up-regulated or amplified in cancer and are likely to be acting as oncogenes, and those miRNAs deleted or down-regulated in cancer that are likely to be acting as tumor suppressors. Like most animal miRNAs, oncomirs act by binding to complementary sequences in the mRNAs of their target genes, e.g., oncogenes or tumor suppressors, to repress protein expression from the target mRNA (10), but miRNAs can also destabilize target mRNAs (11, 12).

The *let-7* miRNA is a founding member of the miRNA family and is conserved in invertebrates and vertebrates, including humans, where the *let-7* family consists of 11 very closely related genes (13–15). In *Caenorhabditis elegans*, *let-7* is temporally regulated and controls the timing of terminal differentiation, acting as a master temporal regulator of multiple genes required for cell cycle exit in

seam cells (5, 15–18). Many human *let-7* genes map to regions altered or deleted in human tumors (3), indicating that these genes may function as tumor suppressors. In fact, *let-7g* maps to 3p21, which has been implicated in the initiation of lung cancers (19). Previous work has shown that *let-7* may also play a role in lung cancer progression (4, 5, 20). For example, *let-7* is expressed at lower levels in lung tumors than in normal tissue for patients with both adenocarcinoma and squamous cell carcinoma (4, 5, 20). Under-scoring the potential importance of *let-7* in lung cancer is the observation that postoperative survival time in lung cancer patients directly correlated with *let-7* expression levels (4, 20); patients with lower *let-7* expression survived for less time than those with higher *let-7* expression. Our previous work showed that *let-7* may directly control cellular proliferation by negatively regulating the human *RAS* genes (5), which are known lung cancer oncogenes (21). *let-7* is complementary to multiple sequences in the 3′-untranslated regions (3′UTR) of human *RAS* genes, and *let-7* represses the expression of *KRAS* and *NRAS* in tissue culture through their 3′UTRs (5). Moreover, in lung squamous cell carcinoma, low *let-7* levels correlates with high *RAS* expression, consistent with *let-7* negatively regulating *RAS* protein levels *in vivo* (5).

Taken together, this previous work (3–5) suggested that *let-7* is a candidate tumor suppressor in lung, but little direct evidence existed to show this. Furthermore, little is known about the extent of the involvement of this miRNA in cancer or mechanisms by which this miRNA functions in cancer. In this new work, we show that *let-7* is robustly expressed in the lung and directly affects the proliferation of A549 lung cancer cells. We also show that *let-7* reduces cell cycle progression of a liver cancer cell line. Our studies indicate that *let-7* directly regulates multiple cell cycle oncogenes in addition to *RAS* and, thus, directly represses cell proliferation pathways.

Materials and Methods

Plasmids. To generate the luciferase fusion to the human 3′UTRs, we subcloned the 3′UTR fragments downstream of firefly luciferase (*luc*) in pGL3 control (Promega). Details are shown in Supplementary Data. Transfections and luciferase assays are described in Supplementary Data.

Tissue culture, transfections, and cellular assays. HeLa, A549, and HepG2 cells were obtained from the American Type Culture Collection and grown in 90% DMEM with 10% fetal bovine serum (Invitrogen) at 37°C under 5% CO₂. All cell lines were reverse transfected with either pre-miRs or anti-miRs (Ambion, Inc.) as indicated at 30 nmol/L final concentration using NeoFx (Ambion, Inc.) under manufacturer-recommended conditions.

Cell proliferation. To measure the effects on cellular proliferation rates, cells were incubated in 10% AlamarBlue diluted in normal culture media until visual color conversion appears. Proliferation rates were determined at 48 h post-transfection, and quantification was done on a BMG POLARstar Optima fluorescent plate reader under manufacturer-recommended protocol.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

C. Johnson, A. Esquela-Kerscher, and G. Stefani contributed equally to this work.

Requests for reprints: Frank J. Slack, Department of Molecular, Cellular and Developmental Biology, P.O. Box 208103, Yale University, New Haven, CT 06520. Phone: 203-432-3492; Fax: 203-432-6161; E-mail: frank.slack@yale.edu or David Brown, Asuragen, Inc., 2150 Woodward St., Austin, TX 78744. E-mail: dbrown@asuragen.com.

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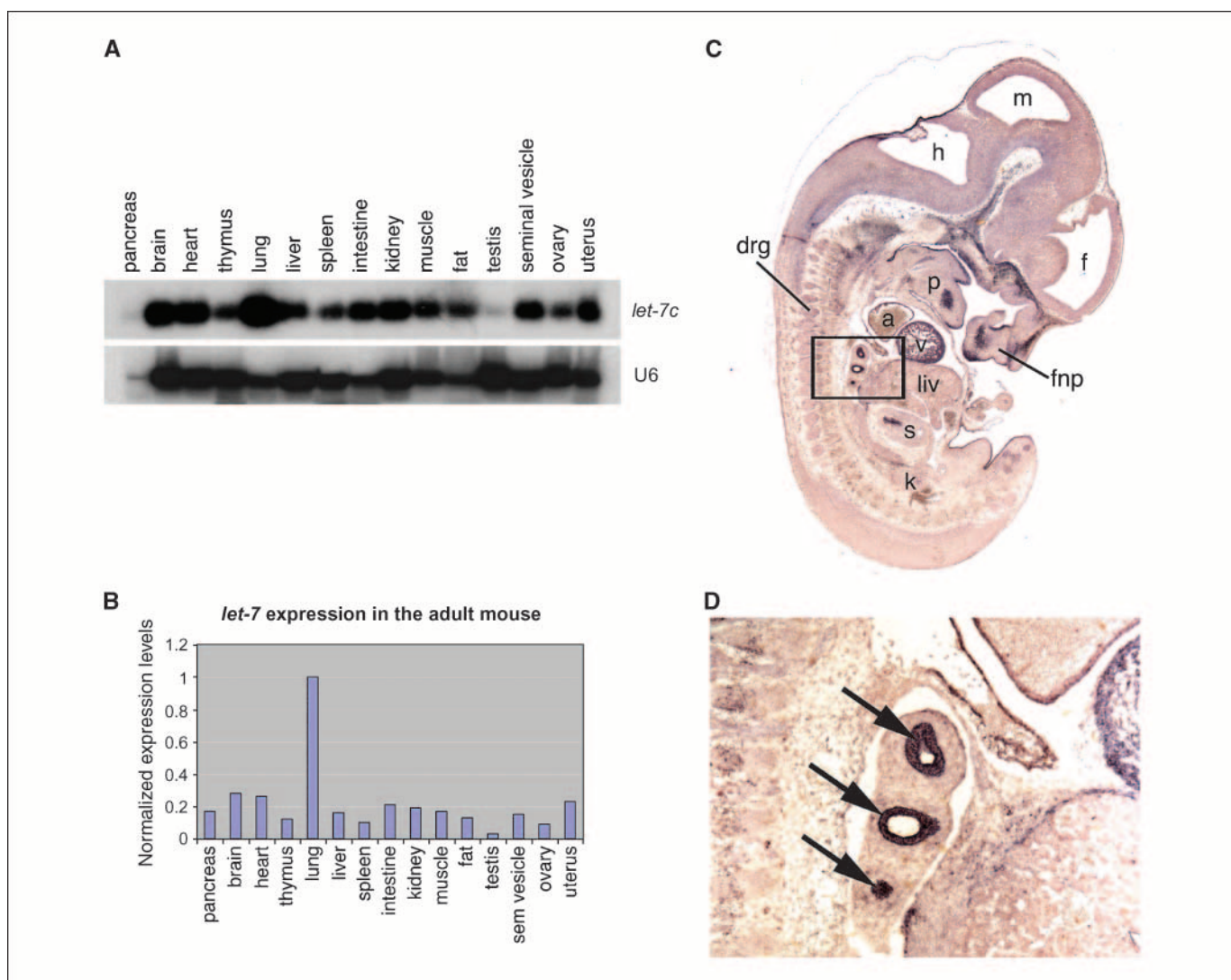


Figure 1. *let-7* expression in lung tissue. **A**, Northern blot analysis of total RNA taken from adult mouse tissues was probed for *let-7c*. U6 RNA is shown as a loading control. **B**, quantification of normalized RNA expression of *let-7c* shown in (A). Note that *let-7c* is highly expressed in the murine lung and lowest in the testis. **C**, *in situ* hybridization analysis of an E12.5 mouse embryo for *let-7a* shows expression in a variety of organ systems. Boxed region shows intense staining in the embryonic lung. **D**, higher magnification of the boxed region in (C) shows that *let-7c* expression is prominent in the developing bronchial system of the mouse (black arrows). a, atrium of heart; drg, dorsal root ganglion; f, forebrain; fnp, frontal nasal prominence; h, hindbrain; k, kidney; liv, liver; m, midbrain; p, palate; s, stomach; v, heart ventricle.

Cell cycle assay. Cells were reverse transfected in triplicate in six-well plate format by complexing 30 nmol/L small interfering RNA (siRNA) or miRNA and 4 μ L of NeoFX transfection reagent (Ambion) in Opti-MEM serum-free medium (Invitrogen) in a total volume of 100 μ L for 20 min. HepG2, or A549 cells (200,000 cells in 1.9 mL of complete growth medium per well), were plated by overlaying the transfection complexes. Seventy-two hours post-transfection, cells were harvested, and flow cytometry analysis was done using a GUAVA PCA-96 instrument following the manufacturer's recommended protocol. Data were collected and processed using the GUAVA Cell Cycle Analysis Software.

Western blots. HeLa cells growing in six-well plates were transfected with pre-miRs (Ambion) to a final concentration of 60 nmol/L with LipofectAMINE RNAiMAX (Invitrogen). Cells were harvested 24 h after transfection and lysated in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH, 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS], containing complete proteases inhibitor mix (Roche). Proteins were analyzed by Western blot using antibodies against CDK6, CDC25a, and actin (DCS-90 from Sigma, DCS-120, 121 from Labvision,

and C4 from MP Biomedicals). Bands were visualized by chemiluminescence using a Typhoon 8600 Imaging System (GE Healthcare). All values of CDC25a and CDK6 were normalized for the corresponding values of actin.

Reverse transcription-PCR detection of *let-7* expression in cell lines. Details are found in Supplementary Data.

***let-7* pathway analysis using GeneChip mRNA array analysis.** HepG2 and HeLa cells were transfected with pre-miRs specific to *let-7b*, miR-124, negative control 1, and negative control 2 at 30 nmol/L final concentration using NeoFX (Ambion, Inc.) under the manufacturer's recommended conditions. At time points indicated in the figure legends, the samples were lysed, and total RNA was isolated using the RNAqueous RNA isolation system (Ambion).

All Affymetrix U133 plus 2 GeneChips used in the cell comparisons were processed according to the robust multichip analysis (RMA) background subtraction, normalization, and expression summary method (22). Irizarry et al. (22) showed that RMA has better precision for lower expression values and provides a greater than 5-fold reduction of the within-replicate variance as compared with other commonly used methods.

Assessment of statistically significant differential expression was carried out using a one-way ANOVA for each tissue type using Partek Genomic Solutions 6.2 (Partek Inc.). Given the nature of the data and the statistical tests selected, adjusting for multiple testing errors is critical. To account for the increased probability of type 1 error, a false discovery rates (FDR) P value adjustment was used (23). The FDR is defined to be the expected value of the ratio of the number of erroneously rejected true hypotheses over the number of rejected hypotheses. Benjamini and Hochberg (23) step-up procedure rejects $H(1) \dots H(k)$ with k being the largest i for which $P(i) \leq q \times i/m$, and this procedure controls the FDR at level q when $P(i)$ are independent.

We did pairwise comparison for the differentially expressed genes identified by ANOVA to determine the probe set that has significant differences between groups. For each pair of treatments, a two-sample t test was carried out for those genes that possessed a significant ANOVA main effect after FDR adjustment. This method is referred to as Fisher's protected least significant difference. We identified 50% of the same *mir-124* repressed genes as previously published (12), suggesting that our microarray technology is accurate.

Time course study GeneChip array analysis. Affymetrix U133 plus 2 GeneChips that were used in the time course study were processed using Affymetrix MAS 5.0 algorithm as the scaling (value set to 500) and summarization method (Affymetrix Statistical Algorithms Description Document Part Number 701137 Rev 3). Because the time course study was unreplicated, the Wilcoxon signed-rank test (24) as implemented in the

Affymetrix GCOS 1.4 software, was used to determine those genes that were differentially expressed relative to time 0. Those genes that were calculated to be absent in 100% of time points were discarded.

Gene ontology analysis. Details are found in Supplementary Data.

Northern blot analysis. Approximately 20.0 μ g of total RNA was obtained from various adult mouse organs for Northern blot analysis using methods described previously (15). A probe used to detect RNA levels of *let-7c* (5'-AACCATACAACCTACTACTCA-3') was made using the StarFire oligonucleotide labeling system (IDT). The Northern blot was subsequently stripped and reprobed with U6 to normalize lanes for loading. pU6 (5'-GCAGGGGCCATGCTAATCTTCTGTATTG-3'; ref. 15), was 5'-end labeled with γ -³²P ATP.

In situ hybridization analysis. Mouse tissue was collected in PBS and fixed in 4% paraformaldehyde. Samples were then soaked in 0.5 mol/L sucrose/PBS, embedded in OCT compound (Tissue-Tek), frozen, and sectioned at 12 μ m thickness with a microtome cryostat. *In situ* hybridization analysis was done using digoxigenin-labeled LNA probes (miRcury probes, Exiqon; UTP/DIG Oligo Tailing Kit, Roche) corresponding to *let-7a* (5'-ACTATACAACCTACTACTCA-3') and *let-7c* (5'-AACCATACAACCTACTACTCA-3') on frozen sections as described (25), with blocking and antibody incubation steps as described in ref. 26, except that miRNA probes were hybridized at 48°C. Slides were mounted in a water-based medium (Aquamount) and photographed.

Results

let-7 is highly expressed in normal lung tissue. Previous work strongly implicated *let-7* as a tumor suppressor in lung tissue (3–5). In support of this notion, we now show that *let-7* is highly expressed in the adult mouse lung as well as in the developing lung during mouse embryogenesis (Fig. 1A–D). Northern analysis showed that of all adult tissues, *let-7* is expressed with the highest relative level in the lung (Fig. 1B) and lower levels in other adult tissues (Fig. 1A). In addition, we detected *let-7* expression in multiple tissues in developing mouse embryos using *in situ* hybridization (Fig. 1C; Supplementary Fig. S1), with intense expression in the developing lung (Fig. 1D). In contrast, a control probe detected little signal (Supplementary Fig. S1). *let-7a* and *let-7c* probes revealed an almost identical expression pattern (Fig. 1, Supplementary Fig. S1), which could reflect the difficulty in specifically detecting individual *let-7* family members that only differ by one nucleotide. We also detected *let-7* expression in adult murine lung epithelium (Supplementary Fig. S2). Consistent with this, we detected expression of all human *let-7* family members (*let-7a, b, c, d, e, f, g, and i*) in normal adult human lung samples (Supplementary Fig. S3).

In contrast, *let-7* levels are reduced in non-small cell lung tumors relative to normal adjacent tissue (4, 5) and in eight tested lung cancer cell lines relative to normal lung samples (Supplementary Fig. S3). We found that with a few rare exceptions, the lung cancer lines tested showed reduced expression of all human *let-7* molecules. This analysis led us to pick A549 cells as a representative lung cancer cell line for the remainder of this study.

let-7 represses cell proliferation in lung cells. Consistently reduced *let-7* expression in the tumors of lung cancer patients suggests that the miRNA is either being affected as a consequence of the disease or is itself contributing to the development of the tumor. To experimentally distinguish between these possibilities, we examined the role of *let-7* on cellular growth and proliferation in mammalian cells by manipulating *let-7* levels using exogenously transfected pre-*let-7* RNAs (to overexpress *let-7*) and anti-*let-7* 2'OME oligonucleotides (to reduce *let-7* activity). We transfected cultured human A549 lung cancer cells and HepG2 liver cancer

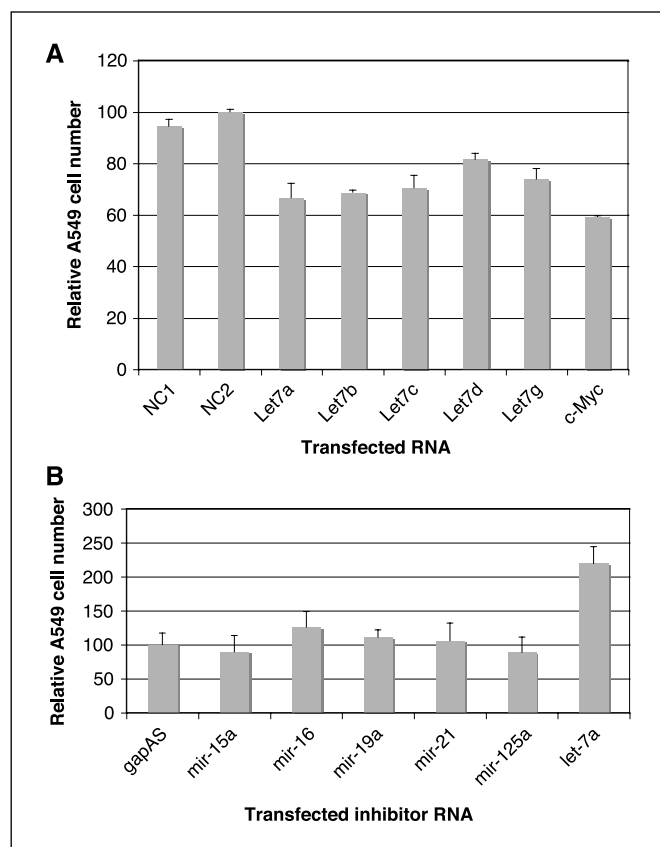


Figure 2. Impact of *let-7* on proliferation in lung cancer cells. **A**, all synthetic pre-*let-7* molecules tested reduced cell number in transfected A549 lung cancer cells compared with a negative control miRNA (NC). A siRNA against MYC served as a positive control. **B**, inhibition of *let-7* with an anti-*let-7* molecule results in an ~100% increase in the number of A549 cells, compared with a control transfection and other tested anti-miRNA (anti-oncomirs). gapAS is a control transfection with a siRNA against glyceraldehyde-3-phosphate dehydrogenase.

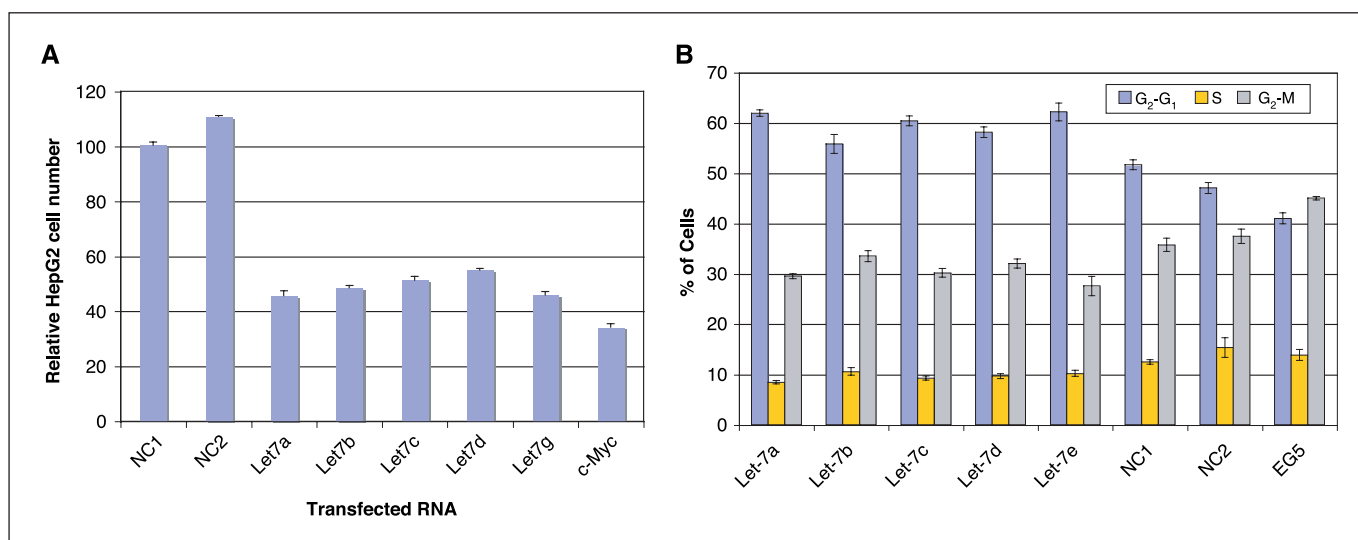


Figure 3. A, impact of *let-7* on proliferation and cell cycle in HepG2 cells. A, all synthetic pre-*let-7* molecules tested affected proliferation in transfected HepG2 liver cancer cells compared with a negative control miRNA (NC). A siRNA against MYC served as a positive control. B, all synthetic pre-*let-7* miRNAs tested significantly affected the percentage of HepG2 cells arrested in the G₀-S stage of cell cycle. NC1 and NC2, negative control miRNAs 1 and 2, respectively. EG5 is an siRNA directed against Eg5. G₀-G₁ *P* values: *let-7a*, <0.001; *let-7b*, 0.026; *let-7c*, <0.001; *let-7d*, 0.0013; *let-7e*, <0.001; Eg5, <0.001.

cells [which also consistently produce low levels of all endogenous *let-7s* (Supplementary Fig. S3); refs. 4, 5] with synthetic *let-7* miRNAs to artificially increase the intracellular concentrations of the *let-7a*, *let-7b*, *let-7c*, *let-7d*, and *let-7g* forms of the miRNA (Figs. 2A and 3A). We then monitored the transfected cell lines for alterations in proliferation, apoptosis, and cell cycle. The effects of the *let-7* family members were compared with the effects of a negative control miRNA (Ambion), and siRNAs targeting MYC or kinesin Eg5 (which are two powerful effectors of cell proliferation; ref. 27). All tested pre-*let-7* molecules consistently reduced the number of proliferating A549 and HepG2 cells by levels that approached the MYC siRNA (Figs. 2A and 3A). This suggests that one or more processes related to cell proliferation or death that are common between these cell types are affected by *let-7*. This work extends similar analyses in lung and colon cancer cells (4, 28) by showing that multiple different *let-7* family members have similar effects on cell proliferation. Because we have not yet detected a difference with any particular *let-7* family member, we use them interchangeably for the remainder of this work.

In contrast to the reduced proliferation defect observed with exogenously added pre-*let-7*, antisense molecules targeting *let-7a* delivered into A549 cells induced an approximately 2-fold increase in proliferation relative to A549 cells transfected with a negative control siRNA and antisense miRNAs to other tested oncomirs (Fig. 2B). The inverse correlation between active *let-7* and cell proliferation suggests that the miRNA affects a cell process that is vital for cell division, cell survival, or another process that supports cell proliferation. We believe that we have eliminated apoptosis as the cause of cell loss because an enzymatic assay to detect active caspase-3 levels revealed no change in three cell lines examined (A549, HepG2, and HeLa; data not shown).

***let-7* reduces progression through the cell cycle.** To investigate the effects of *let-7* on the cell cycle, we used a flow cytometry assay to measure cell cycle progression in cells overexpressing *let-7* or scrambled control miRNAs. Because HepG2 liver cancer cells accumulate a barely detectable amount of native *let-7* (Supplementary Fig. S3; ref. 5) and because we saw the most

dramatic effect on cell proliferation here (Fig. 3A), we focused our attention on these cells. We transfected HepG2 cells with synthetic mimics for several different members of the *let-7* family (pre-*let-7*) as well as negative control synthetic miRNAs (control pre-miRs). All tested synthetic *let-7* miRNAs caused a cell cycle defect in HepG2 cells, with a significant increase in the percentage of cells in G₀-G₁ (*P* < 0.01; Fig. 3B). Our flow cytometry results suggest that the proliferation effects of *let-7* result from reduced progression through the cell cycle, most likely due to a block or delay in the G₁-S transition.

Microarray analysis reveals genes whose expression changes in the presence of excess *let-7*. To determine the cellular pathways regulated by *let-7*, we did a microarray analysis of cells treated with *let-7* miRNA. *let-7* seems to regulate its target genes primarily at the level of translation (18, 29); however, recent evidence indicates that *let-7* can also cause the instability of its target mRNAs (11, 15). In fact, in some cases, miRNAs have been reported to reduce mRNA levels of their direct targets sufficiently to be detected by standard microarray analysis (11, 12). We therefore predicted that exogenously applied *let-7* miRNA might directly affect the mRNA levels of the genes that are naturally regulated by *let-7* (with the exception of genes that *let-7* regulates at the translational level only, e.g., *KRAS*) and indirectly affect the expression of genes that are downstream of these direct targets, leading to measurable changes in the global expression profiles of the treated cells. The identification of the affected pathways could reveal the mechanism by which *let-7* inhibits cell division.

We transfected HepG2 and A549 cells in quadruplicate with synthetic miRNAs corresponding to *let-7b*, miR-124 (chosen as a positive control because its effects on global gene expression patterns have already been published; ref. 12), and two negative control miRs. Total RNA isolated from the cells 72 h after transfection was amplified, labeled, and hybridized to Affymetrix U133 arrays. Principal component analysis revealed that the two negative control miRNAs had very similar mRNA expression profiles in both cell types (Supplementary Fig. S4). In contrast, the mRNA profiles for the cells transfected with *let-7b* and miR-124

Table 1. Cell cycle, cell division, and cell proliferation genes that respond to excess *let-7*

Gene	Product	Function*
<i>A. Repressed in both HepG2 and A549 cells</i>		
<i>CCNA2</i>	Cyclin A2	Binds CDK2 and CDC2 to promote cell cycle G ₁ -S and G ₂ -M phase transition; aberrantly expressed in acute myeloid and promyelocytic leukemias
<i>CDC34</i>	Cell division defective 34	Modifies CDKN1B, increases the ubiquitination and degradation of CDKN1B
<i>ASK/DBF4</i>	activator of S-phase kinase	Binds to and activates kinase activity of CDC7, required for the initiation of DNA replication at the G ₁ to S transition
<i>AURKA/STK6</i> and <i>AURKB/STK12</i>	Aurora A and Aurora B kinases	Maximally expressed during G ₂ -M phases and may function in cytokinesis, up-regulated in multiple neoplasms
<i>E2F5</i>	E2F transcription factor 5	Oncogenic in primary rodent cells and is amplified in human breast tumors
<i>CDK8</i>	CDK8	Forms a complex with cyclin C that phosphorylates cyclin H (CCNH), plays a role in the regulation of transcription and as component of the RNA polymerase II holoenzyme
<i>PLAGL1</i> and <i>PLAGL2</i>	Pleomorphic adenoma gene-like transcription factors	Transcription activators, regulate cell proliferation
<i>LIN28B</i>	Homologue of heterochronic protein LIN-28	Putative RNA binding protein. Mutated in hepatocellular carcinoma
<i>DICER1</i>	RNaseIII	RNase processes pre-miRNAs and dsRNA
<i>GMNN</i>	Geminin	Geminin, regulates DNA replication and proliferation, binds to the licensing factor CDT1 and negatively regulates its ubiquitination, up-regulated in breast, colon, rectal, and biliary tract neoplasms
<i>NRAS</i>	Ras GTPase	Signaling molecule, mutated in multiple tumors
<i>HMGA2</i>	Chromatin protein	Regulates proliferation. Chromosomal translocations in multiple tumors
<i>B. Repressed in HepG2 cells only</i>		
<i>CDC2</i>	Cell division cycle 2, a CDK	Binds B-type cyclins, regulates G ₂ to M phase transition, promotes cell proliferation
<i>CDC25A</i>	Cell division cycle 25A, a protein tyrosine-threonine phosphatase	Binds cyclins and regulates G ₁ -S phase transition, overexpressed in many cancers
<i>CCNB1</i>	Cyclin B1	Regulatory subunit of the CCNB1-CDC2 maturation-promoting factor complex that mediates G ₂ -M phase transition, up-regulated in various cancers
<i>CCNE2</i>	Cyclin E2	G ₁ -specific CDK regulatory subunit that interacts with CDK2 and CDK3, overexpressed in transformed cells and up-regulated in breast and lung cancer
<i>CCNF</i>	Cyclin F	A member of the cyclin family of CDK kinase regulatory subunits, forms a complex with cyclin B1 (CCNB1) and CDC2
<i>CCNJ</i>	Cyclin J	Protein containing cyclin COOH-terminal and NH ₂ -terminal domains have a region of low similarity to a region of cyclin A2
<i>SKP2</i>	S-phase kinase-associated protein 2	A component of a ubiquitin E3 ligase complex, mediates cell cycle regulatory protein degradation, promotes cell proliferation and invasion, inhibits cell adhesion and apoptosis; overexpressed in many cancers

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Table 1. Cell cycle, cell division, and cell proliferation genes that respond to excess *let-7* (cont'd)

Gene	Product	Function*
<i>CKS1B</i>	CDC28 protein kinase regulatory subunit 1B	Binds SKP2 and targets it to its substrates, required for ubiquitination of p21 Cip1 (CDKN1A) and p27 Kip1 (CDKN1B), highly expressed in non-small cell lung, gastric, and colon carcinoma
<i>CDC20</i>	Cell division cycle 20	Activates the mitotically phosphorylated form of the anaphase promoting complex as well as the mitotic spindle checkpoint, overexpressed in gastric cancer
<i>CDCA1</i>	Cell division cycle associated 1	Mediates stable attachment of microtubules to the kinetochore during mitosis and plays a role in the spindle checkpoint
<i>CDAC2</i>	Cell division cycle associated 2	Novel protein
<i>CDAC3/TOME1</i>	Cell division cycle associated 3/trigger of mitotic entry 1	A cytosolic protein that is degraded during G ₁ phase and whose gene promoter activity is stimulated at the G ₂ -M phase
<i>CDCA5</i>	Cell division cycle associated 5	Novel protein
<i>CDAC7</i>	Cell division cycle associated 7	A nuclear protein expressed highly in thymus and small intestine, has a role in anchorage-dependent growth, up-regulated in Burkitt lymphoma cell lines; gene may be a MYC target
<i>CDCA8</i>	Cell division cycle associated 8 (borealin)	A chromosomal passenger complex component may target survivin (BIRC5) and INCENP to centromere, required for kinetochore function, mitotic spindle stability, and metaphase chromosome alignment during mitosis
<i>RRM1</i> and <i>RRM2</i>	Ribonucleotide reductase M1 and M2 polypeptides	DNA synthesis
<i>CDC6</i>	Encoding cell division cycle 6 homologue	DNA replication, up-regulated in cervical intraepithelial neoplasia and cervical cancer
<i>CDC45L</i>	Cell division cycle 45 like	Associates with ORC2L, MCM7, and POLA2, predicted to be involved in the initiation of DNA replication
<i>CDT1</i>	Chromatin licensing factor	Ensures replication occurs once per cell cycle, up-regulated in non-small cell lung carcinomas
<i>ORC1L</i> and <i>ORC6L</i>	Origin recognition complex proteins	DNA replication
<i>MCM2/3/4/5/6/7/8/10</i>	Mini chromosome maintenance-deficient complex	DNA replication, up-regulated in multiple cancers
<i>RFC2/3/4/5</i>	Replication factor C complex	DNA replication
<i>E2F6</i> and <i>E2F8</i>	E2F transcription factors	Regulators of cell cycle
<i>CHEK1</i>	Checkpoint homologue 1 kinase	Required for mitotic G ₂ checkpoint in response to radiation-induced DNA damage, associated with lung cancer
<i>BUB1</i> and <i>BUB1B</i>	Budding uninhibited by benzimidazoles 1 homologues	Acts in spindle assembly checkpoint and chromosome congression, may regulate vesicular traffic; mutations are associated with lung cancer, T cell leukemia, and colorectal cancer cell chromosomal instability; a protein kinase of the mitotic spindle checkpoint, inhibits anaphase-promoting complex activation
<i>MAD2L1</i>	MAD2 mitotic arrest deficient-like 1	Component with BUB1B
<i>CDC23</i>	Cell division cycle 23	A putative component of the anaphase-promoting complex (APC), considered a tumor antigen in ovarian carcinoma; mutation in corresponding gene is associated with colon cancer
<i>FANCD2</i>	Fanconi anemia complementation group D2	Involved in DNA damage response
<i>BRCA1</i> and <i>BRCA2</i>	Breast Cancer Susceptibility loci	Tumor suppressors; mutations are linked to breast and ovarian cancer

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Table 1. Cell cycle, cell division, and cell proliferation genes that respond to excess *let-7* (cont'd)

Gene	Product	Function*
<i>C. Up-regulated in both cell types</i>		
<i>RRM2B</i>	Ribonucleotide reductase M2B	DNA synthesis, up-regulated by p53
<i>EIF2C2</i>	Argonaute	Functions in RNAi and miRNA pathways
<i>D. Up-regulated in HepG2 cells only</i>		
<i>CDKN2B</i>	CDK inhibitor 2B	Interacts with the D type CDK4 and CDK6, inhibits cell proliferation; gene deletion and promoter hypermethylation are associated with many different neoplasms
<i>CCNG2</i>	cyclin G ₂	Down-regulated in thyroid papillary carcinoma
<i>MXI1</i>	MAX-interacting protein 1	Transcription regulator, antagonizes MYC, tumor suppressor in prostatic neoplasms

*Functional annotations were derived from the Human Proteome Survey Database (HumanPSD; ref. 48).

are clearly distinct from the cells transfected with the negative control miRNAs as well as from each other, indicating that the two miRNAs are uniquely affecting the global gene expression profiles of the two cell types. For both cell types, the number of genes determined to be altered by treatment was calculated by filtering all genes by fold change relative to both control transfections, and statistical significance was assessed by a *t* test after the omnibus *F* test was shown to be significant. In HepG2 liver cancer cells, we identified 1,334 (698 repressed and 636 up-regulated) genes whose expression varied by at least 1.93-fold and were statistically significant at a 0.05 FDR between the cells transfected with *let-7* and the negative control miRNAs (Supplementary Table S1). In A549 lung cancer cells, we identified 629 (244 repressed and 385 up-regulated) altered genes, all of which were statistically significant at a 0.05 FDR (Supplementary Table S2). *let-7* addition affected 200 genes in common between both cell types (Supplementary Tables S1 and S2). In both cell types, we identified *NRAS* and *HMGA2*, known downstream targets of *let-7* (5, 30), indicating that our analysis could appropriately reveal *let-7* downstream genes (Table 1).

***let-7* repressed multiple cell cycle associated genes.** Genes found to be differentially expressed in either the HepG2 and A549 cell lines were grouped by their assigned biological functions using the Gene Ontology (GO) database (Fig. 4A; Supplementary Table S4). These results show that *let-7* directly or indirectly affects the expression of many cell cycle-related genes. In fact, the primary GO classes associated with the differentially expressed genes in the HepG2 cells are linked with the cell cycle (Fig. 4A), which are consistent with our earlier observations (Figs. 2 and 3). Cell proliferation genes repressed directly or indirectly by excess *let-7* in both cell types include the genes for cyclin A2, which promotes G₁-S and G₂-M phase transitions; CDC34, which promotes the degradation of cyclin-dependent kinase (CDK) inhibitor 1B; the ASK activator of S-phase kinase, required for the initiation of DNA replication at the G₁ to S transition; the Aurora A and B kinases; the E2F5 transcription factor; CDK8; PLAGL2, the pleomorphic adenoma gene-like transcription factor; and Geminin, a regulator of DNA replication and proliferation (Table 1). Additional cell cycle genes repressed directly or indirectly by *let-7* in HepG2 cells include the genes for various CDKs, cyclins, the CDC25A phosphatase, the S-phase kinase-associated protein 2, SKP2; the CDC28 kinase regulatory subunit 1B, and various cell division cycle-associated

proteins (Table 1). *let-7* also repressed genes coding for DNA synthesis and DNA replication functions (Table 1), e.g., ribonucleotide reductase subunits, multiple DNA replication initiation and origin recognition complex proteins, members of the Mini chromosome maintenance-deficient complex, and multiple members of the replication factor C complex (Table 1).

Although the vast majority of altered cell cycle genes exhibited reduced expression following *let-7* application, a few cell cycle genes were up-regulated under the same conditions. Because *let-7* is currently only known to function as a repressor of gene expression, this likely indicates a 2° (or 3° or more) effect of *let-7* application. These genes included those encoding CDK inhibitor 2B; the MAX-interacting protein 1, MXI1, a transcription regulator that antagonizes MYC; and cyclin G₂, which is down-regulated in thyroid papillary carcinoma (31), showing that it has the propensity to act as a tumor antagonist (Table 1). In *let-7*-deficient tumor cells, these three genes would be predicted to be down-regulated, which might disable their tumor-suppressing functions.

Interestingly, we also found that *let-7* addition repressed the expression of a number of known and putative tumor suppressor genes (Table 1) such as *BRCA1*, *BRCA2*, *FANCD2*, *PLAGL1*, *E2F6*, and *E2F8* and the cell cycle checkpoint genes *CHEK1*, *BUB1*, *BUB1B*, *MAD2L1*, and *CDC23*. The significance of these findings remains to be determined, but may be relevant to the small number of recently identified tumors where *let-7* is actually up-regulated (32, 33).

Identification of likely direct *let-7* targets. To enrich for genes modulated directly by *let-7*, we reasoned that the expression of direct *let-7* downstream genes might be affected earlier than indirect downstream genes. Therefore, in addition to analyzing the changes in global mRNA expression 72 h after *let-7* transfection, we used a time course assay featuring microarray analysis of HepG2 cells harvested at 4, 8, 16, 24, 36, 48, 72, and 128 h after transfection (Fig. 4B; Supplementary Table S3). We found no change in gene expression at times 4 and 8 h after transfection, but detected the first changes 16 h post-transfection. We detected a total of 176 genes that were down-regulated within 36 h of *let-7* addition: we denote these as early repressed genes (Supplementary Table S3; Fig. 4B). Like the original microarray analysis, these genes were enriched in GO categories for cell cycle and cell division (Supplementary Table S4). These included many of the same well-known cell cycle genes already mentioned (*CCNA2*, *CDC25A*, *CDK8*, *SKP2*, *AURKA/STK6*), but also included some that were

missed in the 72-h experiment detailed above because their expression was presumably repressed early, and then their levels returned to normal by 72 h. These included the cell cycle genes *CDC16* and *CDK6*. Other early repressed mRNAs include the DNA synthesis gene *RRM2* and the cell proliferation regulator, *MINA*. Of these 176 early repressed genes, 127 genes first appeared down-regulated at time 16 h (Supplementary Table S3, *pink*; *CDC16*, *CDK6*, *AURKA/STK6*; *RRM2*, and *MINA*). An additional set of 37 genes was first observed down-regulated at 24 h (Supplementary Table S3, *orange*; including *SKP2*, *CDCA7*, and *CDC25A*), and another 12 were first detected as repressed at 36 h (Supplementary Table S3, *yellow*; including *CCNA2* and *E2F6*). Interestingly, a

number of other transcription factors besides *E2F6*, including *ID2*, *CBFB*, *ZNF336*, *SMAD4*, *SOX9*, *NRIH4*, *ARID3A*, *PLAGL2*, *YAP1*, and *GTF2I*, were among the early repressed genes, suggesting that they might also propagate the *let-7* effects to their downstream targets.

To assess how many of the early *let-7*-repressed genes might constitute direct target genes, we examine the 3'UTRs of this group for *let-7* complementary sites (LCS) that displayed features of LCSs in validated *let-7* target genes (5, 15–18, 29, 34) and also used a published miRNA prediction program, PicTar, to predict *let-7* targets (35). We found that at least 25 of the early repressed genes contained LCSs in their 3'UTRs (Fig. 4B), and we propose that these constitute direct *let-7* targets. This set includes the cell cycle regulators *CDK6*, *CDC25A*, *AURKB/STK6*, *CDCA7*, and the DNA synthesis regulator *RRM2*. *CDK6* interacts with D-type cyclins and phosphorylates RB1 to activate the G₁ phase of the cell cycle (36). *CDK6* is also overexpressed or amplified in numerous cancers including non-small cell lung cancer. *CDC25A* is a serine-tyrosine phosphatase that activates CDKs by removing inhibitory phosphate groups. Like *CDK6*, *CDC25A* is up-regulated in multiple cancers, including lung cancers (37). Aurora kinase B regulates chromosome segregation and cytokinesis during mitosis. *CDCA7* encodes a novel protein up-regulated in multiple cancers, including lung cancer, which, like *CDC25A*, is a downstream target of *MYC*, and participates in the cell proliferation effects of *MYC* (38). The list of likely direct *let-7* targets also includes eight transcription factors seen as *let-7* targets in *C. elegans* (16). We conclude that the non-LCS-containing genes with altered expression upon *let-7* addition are likely to be downstream genes indirectly affected by *let-7* expression, perhaps as downstream targets of the transcription factors affected directly by *let-7*. For example, we found that multiple members of the MCM and RFC DNA synthesis complexes were repressed only at later time points and could therefore be targets of these transcription factors.

***let-7* negatively regulates the protein levels of *CDK6* and *CDC25A*.** We analyzed the native expression of our two top scoring cell cycle regulators (Fig. 5), *CDK6* and *CDC25A*, in cells transfected with pre-miRs. Consistent with the microarray analysis, we found that protein levels of both *CDK6* and *CDC25A* decrease in cells transfected with pre-*let-7* compared with cells transfected with a control pre-miRNA (Fig. 5A). When quantified, we found that pre-*let-7* transfection resulted in approximately a 50% reduction of protein compared with the normal levels of *CDK6* and *CDC25A* ($P < 0.001$, *CDC25A*; $P < 0.002$, *CDK6*; Fig. 5B).

To provide further validation for these cell cycle genes as direct *let-7* targets, we did reporter assays where we independently fused the 3'UTRs of *CDK6* and *CDC25A* downstream of firefly luciferase (Fig. 5C). In luciferase assays, both 3'UTRs conferred *let-7*-dependent repression of the reporter gene (Fig. 5D) compared with a negative control anti-miR. The effect on the *CDC25a* and *CDK6* 3'UTRs caused by the anti-*let-7* was similar to a known *let-7* target gene, *NRAS* (Fig. 5D). Thus, like *NRAS*, these results show that these genes are also likely to be directly regulated by the *let-7* miRNA. Given the close working relationship between *CDK6* and cyclin D (36, 39) in promoting the G₁ to S transition, and the fact that *CCND2* (encoding cyclin D2) is the highest scoring cell cycle gene predicted as a *let-7* target by PicTar (35), we also tested the *CCND2* 3'UTR in the same assay. We found a similar result to *CDK6* (Fig. 5D), suggesting that *CCND2* is also a direct target of *let-7*.

A

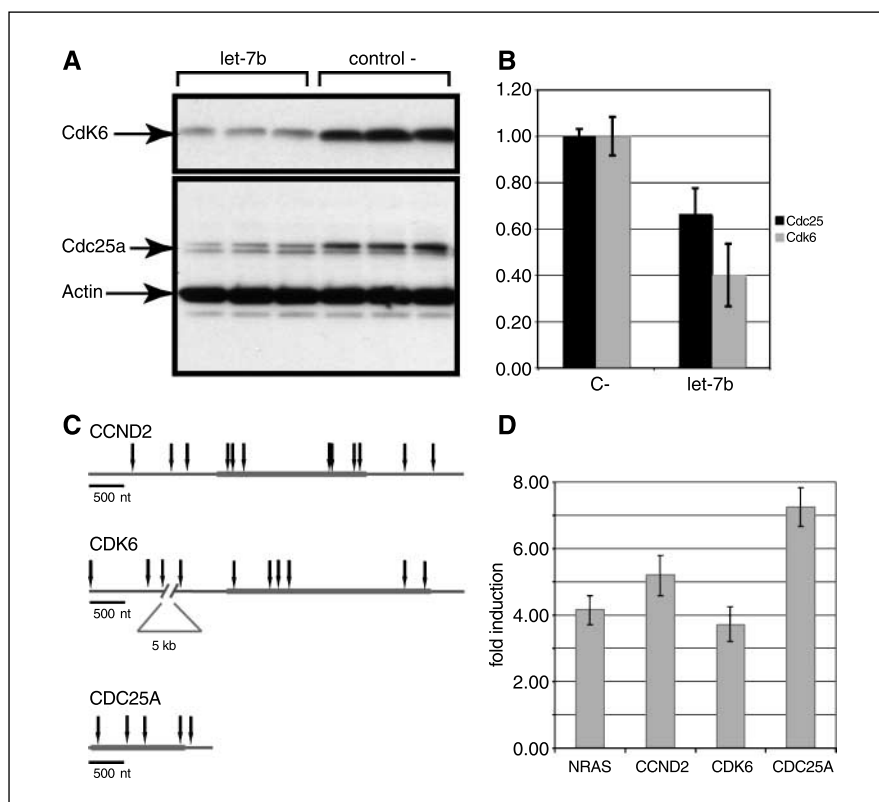
GO ID	GO description	P value*
GO:0006260	DNA replication	5.8E-16
GO:0000087	M phase of mitotic cell cycle	6.2E-13
GO:0000278	mitotic cell cycle	6.4E-13
GO:0000075	cell cycle checkpoint	1.2E-10
GO:0051301	cell division	8.1E-10
GO:0006270	DNA replication initiation	1.2E-09
GO:0007093	mitotic checkpoint	2.3E-06
GO:0007051	spindle organization and biogenesis	3.4E-06

B

Gene Symbol	Role	LCS
Putative targets repressed before 36 h		
<i>CDK6</i>	Kinase	10
<i>PLAGL2</i>	Transcription factor	9
<i>2'-PDE</i>	phosphodiesterase	5
<i>CDC25A</i>	phosphatase	4
<i>DZIP1</i>	RNA binding	4
<i>OPRS1</i>	Opioid receptor	4
<i>DLC1</i>	RhoGAP	3
<i>NXT2</i>	Nuclear transport	3
<i>E2F6</i>	Transcription factor	3
<i>GPX7</i>	Glutathione peroxidase	3
<i>SSR1</i>	Signal sequence receptor	3
<i>YAP1</i>	Transcription factor	3
<i>ZNF336</i>	Transcription factor	3
<i>SOX9</i>	Transcription factor	3
<i>RRM2</i>	Ribonucleotide reductase	3
<i>STK6</i>	Aurora A kinase	3
<i>FARP1</i>	RhoGEF	2
<i>FLJ21986</i>	Unknown	2
<i>CBFB</i>	Transcription factor	2
<i>DSCR1</i>	Calpripresin	2
<i>CDCA7</i>	Novel c-Myc-responsive Gene	2
<i>MAP3K7IP2</i>	kinase adaptor	1
<i>GTF2I/GTF2IP1</i>	Transcription factor	1
<i>ARID3A</i>	Transcription factor	1
<i>DMD</i>	Dystrophin	1
Putative targets repressed after 36 h		
<i>CCNF</i>	Cyclin	4
<i>CCNJ</i>	Cyclin	3
<i>EIF2C3</i>	Argonaute	3
<i>CDC34</i>	E2 ubiquitin-conjugating enzyme	2
<i>E2F5</i>	Transcription factor	2
<i>LIN28</i>	Putative RNA binding factor	2

Figure 4. Microarray analysis of *let-7*-treated human cancer cells. A, table of the most affected GO categories after *let-7* overexpression in HepG2 cells for 72 h. mRNAs whose expression was affected by greater than 2-fold with P values below 0.05 were identified and classified using GO categories. *, P values were calculated with hypergeometric tests to determine whether there is a significant enrichment of affected genes in a GO category when compared with all genes represented on the arrays. B, cell cycle targets for *let-7*. Potential direct downstream targets of *let-7*. Right column, number of LCSs. *Pink*, first observed repression at 16 h; *orange*, first observed repression at 24 h; *yellow*, first observed repression at 36 h; *blue*, first observed repression after 36 h. Some of these genes, e.g., *CDK6* and *RRM2*, were missed in PicTar because the rodent 3'UTRs are not complete. Known *let-7* targets *NRAS* and *HMG2A* are repressed after 36 h in our assay.

Figure 5. Native and reporter gene validation of *let-7* target genes. **A**, Western analysis of the indicated proteins following pre-*let-7b* or pre-control miR transfection of HeLa cells. Three independent transfections are shown for each pre-miR. **B**, quantification of relative levels of CDK6 and CDC25A proteins normalized to actin and the negative control pre-miR. **C**, schematic representations of the 3'UTR fragments cloned downstream of a luciferase reporter 3'UTR (gray, 3'UTR; wide gray, subcloned fragments). Arrows, positions of LCSs. **D**, relative fold induction of reporter gene expression in HeLa cells transfected with an anti-*let7b* oligonucleotide compared with a transfection with a negative control miRNA. Triplicate repeats of this experiment were done. Each value of luciferase activity in the samples treated with *let-7b* anti-miR was normalized by the average luciferase activity value of the samples treated with negative control anti-miR (value = 1). *NRAS* is the *NRAS* long 3'UTR shown here as a positive control.



Discussion

During mouse embryogenesis, *let-7* is first detected around the time of initiation of lung development (40), and expression then persists into adulthood (Fig. 1A; Supplementary Fig. S3). These expression studies suggest that *let-7* may control a variety of processes both during development and in the maintenance of adult tissue homeostasis. In contrast to robust *let-7* expression in normal human lung tissue (14), *let-7* is poorly expressed in lung tumors and lung cancer cell lines (Supplementary Fig. S3; refs. 4, 5). Poor *let-7* expression may thus be a powerful diagnostic marker for lung tumors (20).

***let-7* in control of cell proliferation.** Proliferation and survival pathways are frequently altered in tumors (41). We have shown that *let-7* overexpression causes human cancer cells to decrease cell cycle progression (Figs. 2 and 3). In addition, our microarray data show that *let-7* directly or indirectly regulates multiple cell proliferation genes and strongly suggest that *let-7* is a key regulator of cell cycle progression, consistent with the cell cycle assay results described earlier. We show that *let-7* directly regulates a few key cell cycle proto-oncogenes, e.g., RAS, CDC25a, CDK6, and cyclin D (Fig. 5), thus controlling cell proliferation by reducing flux through the pathways promoting the G₁ to S transition. Because many of these *let-7*-responsive genes are known oncogenes or are overexpressed in tumors, one prediction is that in cancer cells with *let-7* deletions or poor *let-7* expression, many of these genes would be up-regulated, which is likely to stimulate cell cycle and DNA synthesis and, hence, cell division.

The class of *let-7* target genes that are solely repressed at the level of translation will not be identified via microarray analysis, but rather will need to be identified via proteomic or other means. For example, *let-7* does not affect *KRAS* mRNA levels (42), and

consequently, the human *let-7* target gene *KRAS* (5) did not emerge from our microarray analysis. This shows that our analysis did not provide a complete picture of all *let-7* targets and implies that *let-7* might confer varying degrees of translational inhibition versus mRNA instability depending on specific target genes. In addition, *CCND2* was also missed in our microarray analysis, but confirmed as a target by luciferase assays (Fig. 5D).

Nevertheless, our microarray analysis seems to have enriched for potential direct *let-7* targets. Just over 14% (25 out of 176 total repressed) of our early misregulated genes are likely to be directly regulated by the *let-7* miRNA, better than the ~1% of all PicTar predicted *let-7a* targets as a function of the whole genome (243:25,000; enrichment *P* value = 5.4E-22 by hypergeometric test).

Our data strongly support the assertion that *let-7* is a tumor suppressor miRNA. Although this role is most likely in lung cells where there is normally high *let-7* expression and a strong correlation between *let-7* loss and lung cancer, our studies find that *let-7* causes cell cycle defects in a non-lung cancer cell line as well, implying that *let-7* may function as a tumor suppressor in other tissues. This idea is supported by the observation that *let-7* genes map to loci deleted in multiple types of cancers, such as breast, ovary, urothelial, and cervical cancers (3).

Our work reveals a miRNA to be a master regulator of proto-oncogene expression and cancer pathways. This important role in the control of a fundamental process such as cell cycle may provide an explanation for why *let-7* has been 100% conserved more than 600 million years of evolution (14). These experiments also suggest that *let-7* may prove to be a valuable tool in interventions aimed at treating and diagnosing many cancers.

***let-7* affects expression of homologues of *C. elegans* heterochronic genes.** In *C. elegans*, *let-7* is a member of the

heterochronic pathway, which regulates the timing of cell fate determination during development (43, 44) and requires *dcr-1* and *alg-1/alg-2* for this role (45). Interestingly, we identified three human homologues of *C. elegans* heterochronic genes as *let-7*-responsive genes in both A549 and HepG2 cells (Table 1), including *LIN28B*, *DICER1*, and *EIF2C2/AGO2*. In addition, *EIF2C4/AGO4* was also affected in HepG2 cells overexpressing *let-7*. *EIF2C2*, *EIF2C4*, and *alg-1/2* encode Argonaute proteins (46), which function with miRNAs in the RNAi-induced silencing complex (RISC) to mediate target-specific gene silencing. *DICER1* processes *let-7* (and other miRNAs) and our microarray data suggest that *let-7* may negatively feedback on its own expression by modulating *DICER1* expression. *LIN28B* is altered in human hepatocellular carcinoma and, like the

C. elegans lin-28, has LCSs in its 3'UTR (47). It is therefore possible that conserved pathways function to control the timing of cell proliferation during the development of nematodes and mammals.

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