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

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**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 miRNAs 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 miRNAs (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

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**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. 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.
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 μL of NeoFX transfection reagent (Ambion) in Opti-MEM serum-free medium (Invitrogen) in a total volume of 100 μL 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) in a total volume of 100 μL 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.

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.

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 I 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 $H_i$ with $k$ being the largest $i$ for which $P_i \leq q \times i/n$, 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 200.0 μg of total RNA was obtained from various adult mouse organs for Northern blot analysis using methods described previously (15). A probe was used to detect RNA levels of let-7c (5'-ACCATAAACCTACTACCTCA-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. $puP$ (5'-GCAAGGCCCAGCTAATCTCTTCTGTATTG-3'; ref. 15), was 5'-end labeled with $\gamma^{32}P$-ATP.

**In situ hybridization analysis.** Mouse tissue was collected in PBS and fixed in 4% parafomaldehyde. Samples were then soaked in 0.5 mol/L sucrose/PBS, embedded in OCT compound (Tissue-Tek), frozen, and sectioned at 12 μm thickness with a microtome cryostat. In situ hybridization analysis was done using digoxigenin-labeled LNA probes (miRcurry probes, Exiqon; UTP/DIG Oligo Tailing Kit, Roche) corresponding to let-7a (5'-ACTATAACAACCTACTACCTCA-3') and let-7c (5'-ACCATAAACCTACTACCTCA-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
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 knockdown of MYC or target 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.

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 RNA 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 RNA 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 RNA expression profiles in both cell types (Supplementary Fig. S4). In contrast, the RNA profiles for the cells transfected with let-7b and miR-124...
<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Function*</th>
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<tbody>
<tr>
<td><strong>A. Repressed in both HepG2 and A549 cells</strong></td>
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</tr>
<tr>
<td>CCNA2</td>
<td>Cyclin A2</td>
<td>Binds CDK2 and CDK2 to promote cell cycle G1-S and G2-M phase transition; aberrantly expressed in acute myeloid and promyelocytic leukemias</td>
</tr>
<tr>
<td>CDC34</td>
<td>Cell division defective 34</td>
<td>Modifies CDKN1B, increases the ubiquination and degradation of CDKN1B</td>
</tr>
<tr>
<td>ASK/DBF4</td>
<td>activator of S-phase kinase</td>
<td>Binds to and activates kinase activity of CDC7, required for the initiation of DNA replication at the G1 to S transition</td>
</tr>
<tr>
<td>AURKA/STK6 and AURKB/STK12</td>
<td>Aurora A and Aurora B kinases</td>
<td>Maximally expressed during G2-M phases and may function in cytokinesis, up-regulated in multiple neoplasms</td>
</tr>
<tr>
<td>E2F5</td>
<td>E2F transcription factor 5</td>
<td>Oncogenic in primary rodent cells and is amplified in human breast tumors</td>
</tr>
<tr>
<td>CDK8</td>
<td>CDK8</td>
<td>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</td>
</tr>
<tr>
<td>PLAGL1 and PLAGL2</td>
<td>Pleomorphic adenoma gene-like transcription factors</td>
<td>Transcription activators, regulate cell proliferation</td>
</tr>
<tr>
<td>LIN28B</td>
<td>Homologue of heterochronic protein LIN-28</td>
<td>Putative RNA binding protein. Mutated in hepatocellular carcinoma</td>
</tr>
<tr>
<td>DICER1</td>
<td>RNaseIII</td>
<td>RNase processes pre-miRNAs and dsRNA</td>
</tr>
<tr>
<td>GMNN</td>
<td>Geminin</td>
<td>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</td>
</tr>
<tr>
<td>NRAS</td>
<td>Ras GTPase</td>
<td>Signaling molecule, mutated in multiple tumors</td>
</tr>
<tr>
<td>HMGA2</td>
<td>Chromatin protein</td>
<td>Regulates proliferation. Chromosomal translocations in multiple tumors</td>
</tr>
<tr>
<td><strong>B. Repressed in HepG2 cells only</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDC2</td>
<td>Cell division cycle 2, a CDK</td>
<td>Binds B-type cyclins, regulates G2 to M phase transition, promotes cell proliferation</td>
</tr>
<tr>
<td>CDC25A</td>
<td>Cell division cycle 25A, a protein tyrosine-threonine phosphatase</td>
<td>Binds cyclins and regulates G1-S phase transition, overexpressed in many cancers</td>
</tr>
<tr>
<td>CCNB1</td>
<td>Cyclin B1</td>
<td>Regulatory subunit of the CCNB1-CDC2 maturation-promoting factor complex that mediates G2-M phase transition, up-regulated in various cancers</td>
</tr>
<tr>
<td>CCNE2</td>
<td>Cyclin E2</td>
<td>G1-specific CDK regulatory subunit that interacts with CDK2 and CDK3, overexpressed in transformed cells and up-regulated in breast and lung cancer</td>
</tr>
<tr>
<td>CCNF</td>
<td>Cyclin F</td>
<td>A member of the cyclin family of CDK kinase regulatory subunits, forms a complex with cyclin B1 (CCNB1) and CDC2</td>
</tr>
<tr>
<td>CCNJ</td>
<td>Cyclin J</td>
<td>Protein containing cyclin COOH-terminal and NH2-terminal domains have a region of low similarity to a region of cyclin A2</td>
</tr>
<tr>
<td>SKP2</td>
<td>S-phase kinase-associated protein 2</td>
<td>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</td>
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
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<tbody>
<tr>
<td>CKS1B</td>
<td>CDC28 protein kinase regulatory subunit 1B</td>
<td>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.</td>
</tr>
<tr>
<td>CDC20</td>
<td>Cell division cycle 20</td>
<td>Activates the mitotically phosphorylated form of the anaphase promoting complex as well as the mitotic spindle checkpoint, overexpressed in gastric cancer.</td>
</tr>
<tr>
<td>CDCA1</td>
<td>Cell division cycle associated 1</td>
<td>Mediates stable attachment of microtubules to the kinetochore during mitosis and plays a role in the spindle checkpoint.</td>
</tr>
<tr>
<td>CDCA2</td>
<td>Cell division cycle associated 2</td>
<td>Novel protein</td>
</tr>
<tr>
<td>CDCA3/TOME1</td>
<td>Cell division cycle associated 3/trigger of mitotic entry 1</td>
<td>A cytosolic protein that is degraded during G1 phase and whose gene promoter activity is stimulated at the G2-M phase.</td>
</tr>
<tr>
<td>CDCA5</td>
<td>Cell division cycle associated 5</td>
<td>Novel protein</td>
</tr>
<tr>
<td>CDCA7</td>
<td>Cell division cycle associated 7</td>
<td>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.</td>
</tr>
<tr>
<td>CDCA8</td>
<td>Cell division cycle associated 8 (borealin)</td>
<td>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.</td>
</tr>
<tr>
<td>RRM1 and RRM2</td>
<td>Ribonucleotide reductase M1 and M2 polypeptides</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>CDC6</td>
<td>Encoding cell division cycle 6 homologue</td>
<td>DNA replication, up-regulated in cervical intraepithelial neoplasia and cervical cancer.</td>
</tr>
<tr>
<td>CDC45L</td>
<td>Cell division cycle 45 like</td>
<td>Associates with ORC2L, MCM7, and POLA2, predicted to be involved in the initiation of DNA replication.</td>
</tr>
<tr>
<td>CDT1</td>
<td>Chromatin licensing factor</td>
<td>Ensures replication occurs once per cell cycle, up-regulated in non-small cell lung carcinomas.</td>
</tr>
<tr>
<td>ORC1L and ORC6L</td>
<td>Origin recognition complex proteins</td>
<td>DNA replication</td>
</tr>
<tr>
<td>RFC2/3/4/5</td>
<td>Replication factor C complex</td>
<td>DNA replication</td>
</tr>
<tr>
<td>E2F6 and E2F8</td>
<td>E2F transcription factors</td>
<td>Regulators of cell cycle</td>
</tr>
<tr>
<td>CHEK1</td>
<td>Checkpoint homologue 1 kinase</td>
<td>Required for mitotic G2 checkpoint in response to radiation-induced DNA damage, associated with lung cancer.</td>
</tr>
<tr>
<td>RUB1 and BUB1B</td>
<td>Budding uninhibited by benzimidazoles 1 homologues</td>
<td>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.</td>
</tr>
<tr>
<td>MAD2L1</td>
<td>MAD2 mitotic arrest deficient-like 1</td>
<td>Component with BUB1B</td>
</tr>
<tr>
<td>CDC23</td>
<td>Cell division cycle 23</td>
<td>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.</td>
</tr>
<tr>
<td>FANCD2</td>
<td>Fanconi anemia complementation group D2</td>
<td>Involved in DNA damage response</td>
</tr>
<tr>
<td>BRCA1 and BRCA2</td>
<td>Breast Cancer Susceptibility loci</td>
<td>Tumor suppressors; mutations are linked to breast and ovarian cancer.</td>
</tr>
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</table>
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 \( G_1 \)-S and \( G_2 \)-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 \( G_1 \) to S transition; the Aurora A and B kinases; the \( ASK \) activator of S-phase kinase, required for the initiation of DNA degradation of cyclin-dependent kinase (CDK) inhibitor 1B; the genes repressed directly or indirectly by let-7, which are associated with many different neoplasms.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Function*</th>
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<tbody>
<tr>
<td><strong>C. Up-regulated in both cell types</strong></td>
<td></td>
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<tr>
<td>RRM2B</td>
<td>Ribonucleotide reductase M2B</td>
<td>DNA synthesis, up-regulated by p53</td>
</tr>
<tr>
<td>EIF2C2</td>
<td>Argonaute</td>
<td>Functions in RNAi and miRNA pathways</td>
</tr>
<tr>
<td><strong>D. Up-regulated in HepG2 cells only</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDKN2B</td>
<td>CDK inhibitor 2B</td>
<td>Interacts with the D type CDK4 and CDK6, inhibits cell proliferation; gene deletion and promoter hypermethylation</td>
</tr>
<tr>
<td>CCNG2</td>
<td>cyclin G2</td>
<td>Down-regulated in thyroid papillary carcinoma</td>
</tr>
<tr>
<td>MXI1</td>
<td>MAX-interacting protein 1</td>
<td>Transcription regulator, antagonizes MYC, tumor suppressor in prostatic neoplasms</td>
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*Functional annotations were derived from the Human Proteome Survey Database (HumanPSD; ref. 48).
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 RR2M2 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; RR2M2, and MINA). An additional set of 37 genes was first observed down-regulated at 24 h (Supplementary Table S3, orange; including SKP2, CDC47, 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, CBF1, ZNF336, SMAD4, SOX9, NR1H4, ARID3A, PLAGL2, YAP1, and GTP21, 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 (LCSs) 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, CDC47, and the DNA synthesis regulator RR2M2, 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 chromosomal segregation and cytokinesis during mitosis. CDC47 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 CBF1, PLAGL2, E2F6, SOX9, ZNF336, YAP1, GTP21, and ARID3A, consistent with the enrichment for 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-miR. Specifically, CDK6 and CDC25A 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 transfected with pre-miR. 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.
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 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 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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