Identification of Let-7–Regulated Oncofetal Genes

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Abstract

MicroRNAs (miRNA) are small RNA molecules of ~20 to 22 nucleotides that reduce expression of proteins through mRNA degradation and/or translational silencing. Each known miRNA has a large number of predicted targets. Members of the let-7/miR-98 family of miRNAs are up-regulated at the end of embryonic development. Let-7 is often down-regulated early during cancer development, suggesting that let-7–regulated oncofetal genes (LOG) may become reexpressed in cancer cells. Using comparative bioinformatics, we have identified 12 conserved LOGs that include *HMGA2* and *IMP-1/CRD-BP*. IMP-1 has growth-promoting activities through stabilization of c-myc mRNA. We experimentally confirmed that IMP-1 is a direct let-7 target that promotes cell growth and motility of tumor cells, and we confirmed by proteomics analysis that IMP-1 and HMGA2 are major miRNA targets. Our data suggest that a substantial part of the growth inhibitory activities of let-7 comes from suppressing the expression of IMP-1. LOGs could be novel therapeutic targets and potential biomarkers for cancer treatment. [Cancer Res 2008;68(8):2587–91]

Introduction

MicroRNAs (miRNA) are small RNA regulators of mRNA and protein expression. Each of the hundreds of known miRNAs has a large set of predicted targets, and it is difficult to identify the most relevant targets for each miRNA. Members of the let-7/miR-98 family of miRNAs have been shown to be up-regulated at the end of embryonic development both in *Caenorhabditis elegans* and in mice (1, 2). Let-7 expression remains high in most adult tissues throughout the life span of the organism, suggesting that it represses a group of embryonic genes. We have recently shown that let-7 is selectively expressed by human cancer cells that are at an early stage of dedifferentiation (3). We and others identified the early embryonic gene HMGA2 as a relevant target of let-7 (3–5), and HMGA2 expression was inversely correlated with let-7 expression in the 60 tumor cell lines of the drug screening panel at the National Cancer Institute (NCI60) as well as in ovarian cancer cell lines and primary ovarian cancer (3). Given the specific expression pattern of let-7 and HMGA2 both during embryonic development and in neoplastic transformation, let-7 may exert its activity by preventing reexpression of a set of oncofetal genes in adult tissues (6). We now identify 12 early embryonic genes [let-7–regulated oncofetal genes (LOG)] that are targeted by let-7. The second most relevant LOG after *HMGA2* was *IMP-1/CRD-BP*, a member of a family of RNA binding proteins including IMP-2 and IMP-3, which bind to the 5′-untranslated region (UTR) of the insulin-like growth factor (IGF)-II leader 3 mRNA species (7). IMP-1 recognizes c-myc, IGF2, tau, FMR1, semaphorin, βTrCP1 mRNAs, and H19 RNA, and it acts to stabilize target RNAs by shielding them from degradation (7). IMP-1 has a classic oncofetal expression pattern with expression only early during fetal life (8, 9) and reexpression in many human cancers (10). In total, 8 of the 12 LOGs are currently known to be relevant for cancer progression and could therefore be valid targets for cancer therapy.

Materials and Methods

Mouse data set. The mouse gene expression data set was taken from the supplementary data published by Zhang et al. (11).[6] This data set contains gene expression levels of 39,309 presumably distinct transcripts across 55 mouse tissues. Annotation of the mouse genes was completed and data were analyzed as described in Supplementary Methods.

Constructs, transfections, and luciferase assay. A 6-kb fragment of the 6.7-kb human IMP-1 3′-UTR was amplified from pBAC clone RP11-960P18 with primers 5′-TAGGCGATCGCTCGAGATCCCATGGAGTGTTTAG-3′ and 5′-TTGCGGCCAGCGGCCCAGATTTCCAGTAAAATAC-3′. The fragment was then cloned into the psiCHECK-2 vector (Promega) immediately downstream of the Renilla luciferase reporter gene using the In-Fusion 2.0 cloning kit (Clontech). In addition, fragments spanning base pairs 3,420 to 4,310 (containing LCS2 and 3) and base pairs 6,850 to 7,430 (containing LCS5) were generated using primers 5′-aaaactcgaggatcccatggagtgtttag-3′ and 5′-aaaagcggccgctctctcctcgataaaacg-3′ and 5′-aaactcgaggctggcgccttagtatgg-3′ and 5′-aaaagcggccgcagggcccaatggaggtgtag-3′. Mutagenesis of seed matches, transfections, viral infections, and luciferase assays are described in Supplementary Methods.

Quantitative real-time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Two micrograms of RNA were used to generate cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a final volume of 20 μL. Two microliters of the reverse transcription reaction were used for real-time PCR in a volume of 50 μL containing TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay kit containing specific primers and TaqMan MGB probe for each gene (*CDK6, CDC25A, CCNA2, CDCA7, CDC2, IMP-1, Vimentin, ZEB1, ZEB2*, and *GAPDH*). The relative amount of gene transcripts was normalized to GAPDH. PCR reactions were done in triplicate using the 7500 quantitative real-time PCR system (Applied Biosystems) and the data were processed using the manufacturer's software.

Proliferation and motility assays. A549 cells treated with si-IMP1 or hsa-let-7g miRNA were harvested at 72 h. A minimum of five wells per

Note: B. Boyerinas and S-M. Park share first authorship.

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

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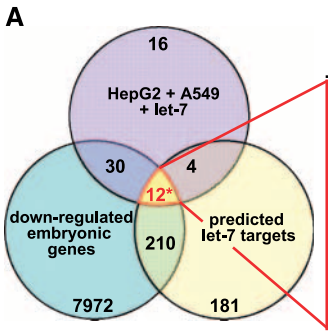
[6] http://hugheslab.ccbr.utoronto.ca/supplementary-data/Zhang/



B

	LOG name	Fold down cell lines	Fold down E9.5 ->E15	TargetRank Score	Average Ranking	Role in cancer or function
1	**HMGA2**	6.23	8.99	1.35	**1.3**	**Up-regulated in many human cancers, oncogene[1]**
2	**IMP-1**	4.01	5.83	1.22	**2.7**	**Up-regulated in many human cancers, oncogene[2]**
3	**LIN28B**	8.63	4.49	1.03	**3.7**	**Candidate oncogene up-regulated in HCC[3]**
4	FIGN	3.05	3.58	0.60	**4.3**	Nuclear ATPase important for mouse development[4]
5	**CDC34**	2.92	4.77	0.28	**5.3**	**Candidate oncogene up-regulated in ALL abd HCC[5]**
6	**IMP-2**	2.51	7.23	0.34	**5.7**	**Up-regulated in many human cancers, oncogene[6]**
7	**NME6**	2.62	2.24	0.16	**7.7**	**Tumor marker and associated with poor prognosis of AML[7]**
8	MED6	2.57	3.05	0.11	**8.3**	Important in development and proliferation[8]
9	COL4A5	2.72	1.56	0.14	**8.7**	Down-regulated during colon cance[9]
10	**NAP1L1**	2.25	2.23	0.49	**10.0**	**Malignancy marker, associated with colon cancer[10]**
11	SLC25A24	2.25	1.79	0.09	**10.3**	Mitochondrial adenine nucleotide transporter[11]
12	**PIGA**	2.35	1.34	0.09	**11.3**	**Development of AML[12]**

Figure 1. Identification of LOGs. *A,* introduction of let-7b for 72 h caused down-regulation of 103 genes in both HepG2 and A549 cells (13). *Purple circle:* Of these 103 genes, 62 were found in a newly annotated data set of 22,000 genes found in mouse embryos (Supplementary Table S2). *Green circle:* 8,224 mouse genes found to be down-regulated between E9.5 and E15 of embryonic development. Of the 62 genes down-regulated in the two cancer cell lines, 16 were among the predicted let-7 targets (*yellow circle*) according to TargetRank. Of these 16 genes, 11 were down-regulated >1.4-fold in mouse embryos between E9.5 and E15. *, the number of LOGs containing one more gene (LIN28B) that was annotated through sequence match. *B,* the 12 LOGs ranked according to their average ranking position in the three analyses. Genes with known function as either oncogenes or tumor markers are in boldface. For footnotes, see Supplementary data.

treatment were counted with a Bright-Line Hemacytometer for 3-d end point cell proliferation. Cells were then plated at a density of 2.0×10^4 in Transwell motility chambers (Corning). After 2 d, the inner chambers were cleaned by scrubbing and the remaining cells were fixed in methanol and stained with Giemsa for counting. Each assay was repeated at least thrice and a minimum of five fields per well were used to determine average motility for each condition.

Results and Discussion

Identification of LOGs. To identify LOGs, we first determined which of the predicted let-7 targets in humans and mice are down-regulated at the mRNA level when let-7 is up-regulated between E9.5 and E15 during mouse embryonic development (2). We used TargetRank[7] and TargetScan 4.0[8] to predict let-7 targets. Predicted targets were ranked according to their expected strength of let-7 targeting by TargetRank, which uses a set of targeting determinants including both seed match type and seed match context (12). This identified 407 putative let-7 targets and confirmed that HMGA2 was the strongest let-7 target (Supplementary Table S1). Comparing these targets with a data set of 21,719 annotated mouse genes down-regulated between E9.5 and E15 of mouse embryonic development more than 1.4 fold (Supplementary Table S2) revealed a statistically significant higher proportion of genes regulated during embryonic development in the group of the predicted let-7 targets (37.4%) than in the group of genes not predicted to be targets (54.5%) ($\chi^2 = 49.05$, $P < 0.0001$). The second strongest putative oncofetal let-7 target after HMGA2 was IMP-1.

Recently, it was shown that introduction of let-7 into tumor cells causes growth inhibition and cell cycle changes (13). Let-7–regulated genes were identified by transfecting two human tumor cell lines, the hepatocellular carcinoma cell line HepG2 and the lung carcinoma cell line A549, with let-7 and monitoring the effect on mRNA levels. We proposed that if let-7 is a fundamental regulator of oncofetal genes in many different human cancers (6), then universally regulated let-7 target genes would be expected to be down-regulated in both the HepG2 and the A549 cancer cell lines. mRNAs of 62 genes were down-regulated in response to introduction of let-7 in both cell lines and represented in the mouse data set we analyzed (Fig. 1*A*). Of these 62 genes, 16 were predicted by TargetRank to be let-7 targets. To determine how many of them might be potential LOGs, we compared the data with all mouse mRNAs that are down-regulated during embryonic development between E9.5 and E15 (Supplementary Table S2). Of the 16 predicted let-7 target genes, 11 (69%) were significantly down-regulated between E9.5 and E15 (Fig. 1*A*). To obtain a list of LOGs that was derived from analyses that were reflections of the entire genome, we manually completed the annotation of all mRNAs predicted to be down-regulated by let-7 in both cancer cell lines by performing sequence searches using the mouse sequences obtained from the National Center for Biotechnology Information. One additional mRNA could be annotated that was down-regulated during mouse embryonic development, LIN28B, bringing the total of identified LOGs to 12. The 12 LOGs were ranked according to the average ranking achieved in each of the three analyses and, as expected, HMGA2 was the LOG most strongly affected by expression of let-7. The second most likely LOG was IMP-1. Similarly to HMGA2 and IMP-1, most of the 10 other predicted LOGs have been shown to be up-regulated during cancer progression or have functions that may promote tumor growth (Fig. 1*B*). As a step toward validating the identification of LOGs and identifying the contribution of IMP-1 to the tumor-promoting activities of LOGs, we set out to verify IMP-1 as an authentic let-7 target.

IMP-1 is a direct target of let-7. IMP-1 carries six putative let-7 complementary (seed match) sequences (LCS) in its 3′-UTR, of which five are conserved among mammalian species (Fig. 2*A*). As expected, introduction of pre-let-7g or pre-let-7d caused down-regulation of IMP-1 protein expression in A549 cells, which express low endogenous levels of let-7 (ref. 3; Fig. 2*B* and data not shown). To inhibit let-7, we designed an antisense oligonucleotide targeting let-7 that contained eight locked nucleic acid (LNA) nucleotides at positions shared by all nine let-7 family members (Supplementary Fig. S1). When let-7 high-expressing HeLa cells were transfected with the LNA-let-7 inhibitor, a substantial up-regulation of IMP-1 was detected (Fig. 2*B*). Taken together, these data show that IMP-1 protein expression is under control of endogenous let-7. We also determined that this regulation requires the 3′-UTR of IMP-1

[7] http://hollywood.mit.edu/targetrank
[8] http://www.targetscan.org

because let-7 and the LNA inhibitor had opposite effects on the expression of luciferase when they were cotransfected into cells together with a fusion construct of luciferase and the IMP-1 3′-UTR (Fig. 2*C* and *D*). To confirm that the 3′-UTR of IMP-1 is directly targeted by let-7, we mutated its LCS2 and LCS5 seed matches in two fragments, one carrying LCS2 and LCS3 and one carrying LCS5, respectively (Fig. 2*C*). Mutation of either of these two LCS significantly inhibited luciferase repression by let-7. Taken together, these data show that, similar to HMGA2, IMP-1 protein expression is under control of endogenous let-7 through targeting LCS in its 3′-UTR. An unbiased proteomics analysis of proteins targeted by miRNAs in mouse embryonic fibroblasts with globally reduced miRNA expression identified both HMGA2 and IMP-1 within the 10 most up-regulated proteins, indicating that both proteins (Supplementary Table S3) are major miRNA targets in cells of embryonic origin.

Some of the activities of let-7 on tumor cells are mediated by loss of IMP-1. Knockdown of IMP-1 in MCF7 cells caused reduced expression of c-myc and increased levels of IGFII mRNA, as well as a reduced rate of proliferation (14). Therefore, we determined if the introduction of let-7 into cells would have a similar effect as the direct knockdown of IMP-1. We found that introduction of let-7g into A549 cells reduced their growth to about the same extent as knocking down IMP-1 directly (Fig. 3*A*). It was recently suggested that the growth inhibitory effect of let-7 on A549 cells was a consequence of its targeting of a number of cell cycle–regulating genes (13). Whereas one of these genes, CDC34, was also found to be among the LOGs, an alternative explanation is that growth inhibition induced by let-7 could have resulted, at least in part, from the reduction of IMP-1 expression in the cells. In this regard, it was shown that introduction of let-7 affected the cell cycle by causing a reduction in the number of cells in S phase and an accumulation of cells in G_1 phase, which is the phenotype that we observed by knocking down IMP-1 in A549 cells. The reduction of cells in S phase and the concomitant increase of cells in G_1 phase are consistent with reduced cellular proliferation (Fig. 3*B*). In additional experiments, we found that IMP-1–dependent motility was comparably reduced either when let-7 was introduced into cells or when IMP-1 was knocked down (Fig. 3*C*). Taken together, these data validate our approach to identifying LOGs and suggest that let-7 expression inhibits reexpression of IMP-1, which contributes to typical properties of tumor cells. Our data suggest that loss of IMP-1 contributes to the reduced rate of proliferation and are consistent with the function of IMP-1 to stabilize the mRNA of c-myc, which is one of the driving forces of tumor cell proliferation. Whereas let-7 can also affect the translation of c-myc directly (15), a number of the identified putative let-7 targeted cell cycle regulators are known to be c-myc–regulated genes (i.e., CCND2, CDK6, CDC25a, CCNA2,

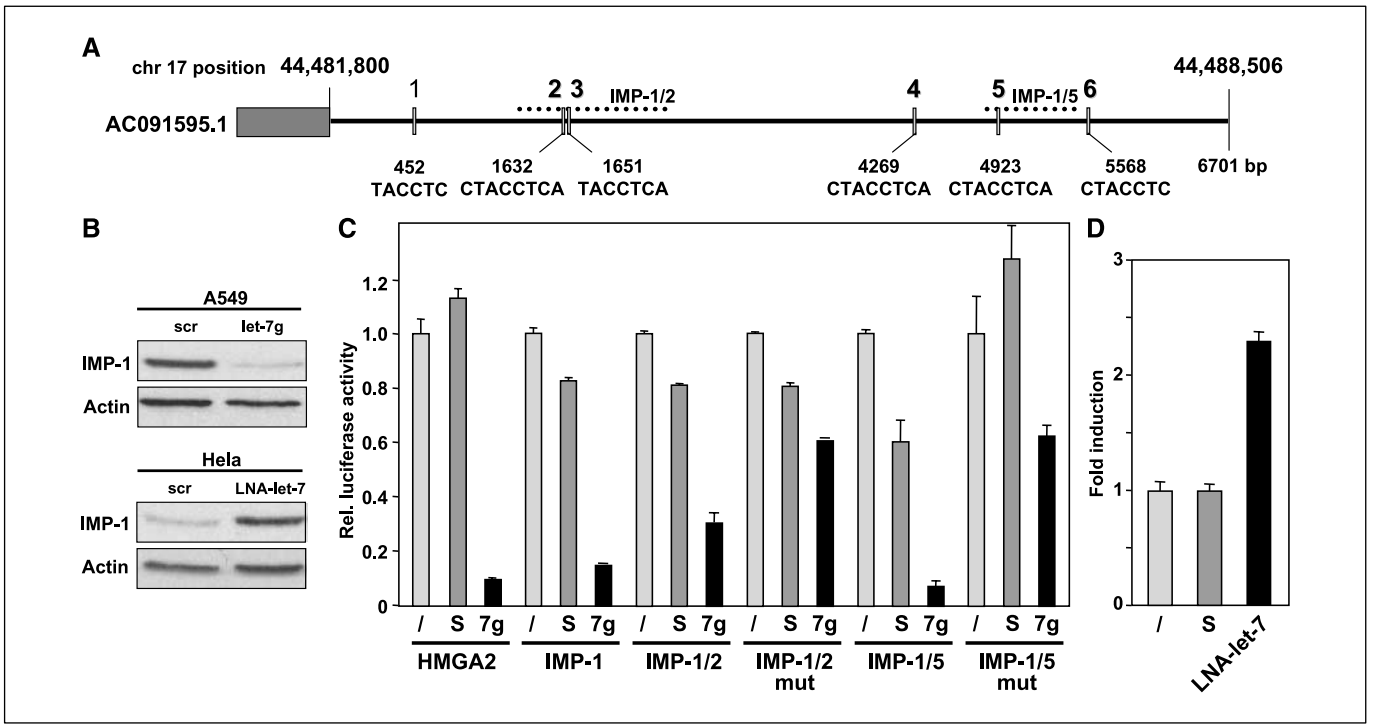


Figure 2. Identification of IMP-1 as a direct target of let-7. *A,* schematic of the 3′-UTR at the human *IGF2BP1* genomic locus located on chromosome 12. *Gray box,* 3′-end of the open reading frame; *horizontal line,* 3′-UTR spanning ~6.7 kb. *Solid rectangles,* locations of the six putative let-7 binding sites and the nucleotide positions within the 3′-UTR. Sequences of the seed match to the let-7 family of miRNAs are shown below the nucleotide positions. Sites conserved among mammalian species are in boldface. Location of fragments IMP-1/2 containing LCS2 and LCS3 and fragment IMP-1/5 containing LCS5 are shown as stippled lines. *B,* A549 cells were transiently transfected with let-7g or a scrambled (*scr*) precursor miRNA. HeLa cells were transfected with an LNA-anti-let-7 or a scrambled LNA oligonucleotide. β-Actin was detected to show equal loading. *C,* IMP-1 is posttranscriptionally regulated through the 3′-UTR by let-7. Luciferase reporter assays were done in 293TN cells with reporter plasmid psiCHECK-HMGA2 3′-UTR (HMGA2), psiCHECK-IMP-1 3′-UTR (IMP-1), psiCHECK-IMP-1 fragment 2,3 3′-UTR (IMP-1/2), psiCHECK-IMP-1 fragment 2,3-mut 3′-UTR (IMP-1/2 mut), psiCHECK-IMP-1 fragment 5 3′-UTR (IMP-1/5), or psiCHECK-IMP-1 fragment 5-mut 3′-UTR (IMP-1/5 mut) together with 1 pmol of either pre-miR scrambled (*S*) or pre-miR let-7g (*7g*). Renilla luciferase activity was normalized to the internally controlled firefly luciferase activity. *D,* luciferase reporter assay with cell extracts of HeLa cells transfected with empty vector (/) or psiCHECK-IMP-1 3′-UTR and transfected with 50 nmol/L of a scrambled LNA oligonucleotide, or transfected with the LNA-let-7 inhibitor.

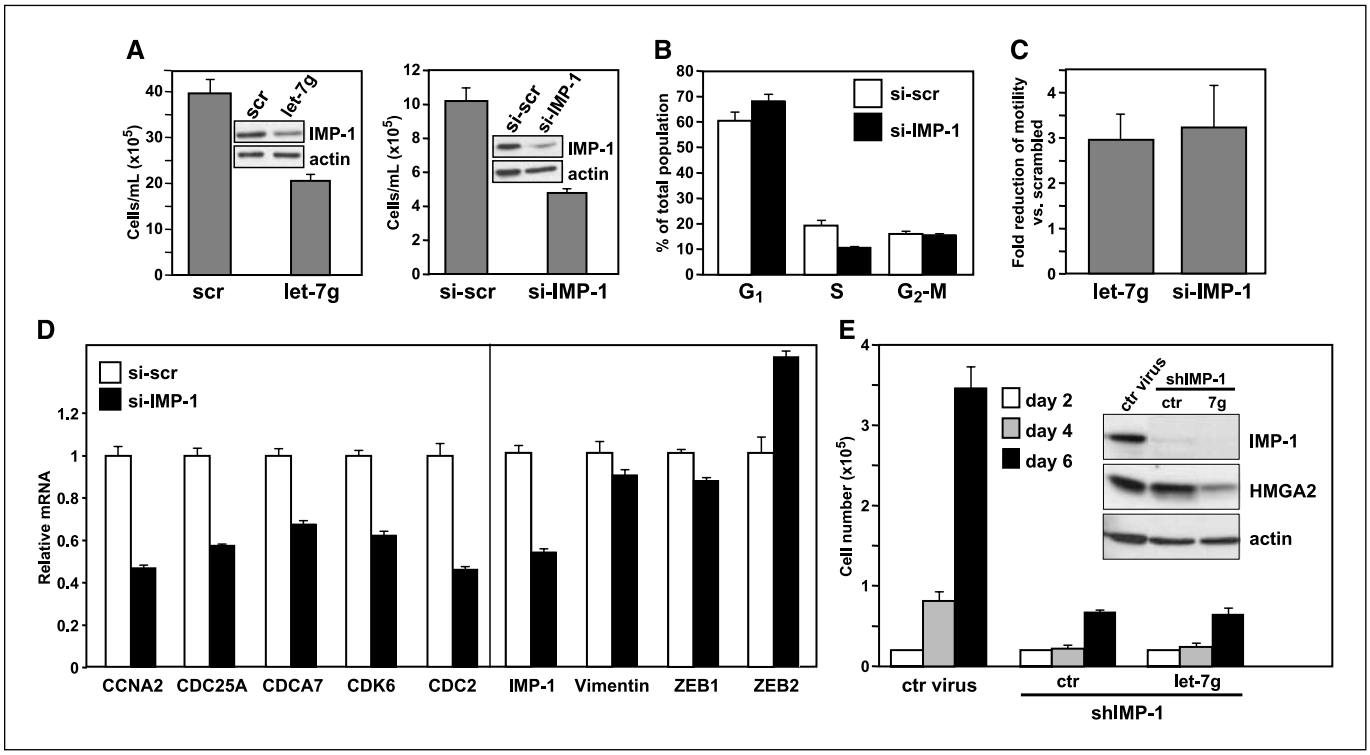


Figure 3. Let-7 regulates properties of tumor cells through down-regulation of IMP-1. A549 cells were transfected with scrambled oligo, let-7g, or a siRNA specific for IMP-1 for 72 h and subjected to cell count (*A*), cell cycle analysis (*B),* or motility assay (*C*). Insets in *A* show the levels of reduction of IMP-1 expression achieved by transfecting cells with let-7g or si-IMP-1. *D,* A549 cells were transfected with either a scrambled si-control oligo or si-IMP-1. Forty-eight hours after transfection, expression of cell cycle regulators and control genes was quantified by real-time PCR relative to the expression of GAPDH mRNA. *E,* A549 cells were infected with a shIMP-1 lentivirus or control virus. Forty-eight hours after infection, cells were counted (day 2), either control-transfected or transfected with let-7g, and replated at 20,000 per well. Cells were again counted 4 and 6 d after infection with viruses. *Inset,* Western blot analysis of all cells for HMGA2, IMP-1, and actin. A similar result was obtained with another independent IMP-1–specific shRNA virus (data not shown).

CDCA7, and CDC2; ref. 13).[9] We compared the expression of five of these cell cycle regulators between cells with IMP-1 knockdown and siRNA control-transfected cells by real-time PCR and found that IMP-1 and the five cell cycle regulators, but not a number of control genes, were down-regulated in the cells with reduced IMP-1 expression (Fig. 3*D*). Detection of changes in the expression of these cell cycle regulators on introduction of let-7 might therefore have been, at least in part, due to the reduction of IMP-1 expression in these cells. To better assess the contribution of reduced IMP-1 expression to the overall effects of let-7 on A549 cells, we knocked down IMP-1 using a lentivirus-based IMP-1–specific shRNA (Fig. 3*E*). The shIMP-1 virus caused a profound growth inhibition. However, cells were not dead and their number slowly increased 6 days after shRNA infection. Two days after knocking down IMP-1, cells were transfected with let-7g. Whereas let-7g transfection reduced the expression of HMGA2, it could not further decrease growth in cells with already knocked down IMP-1. These data suggest that IMP-1 is one of the major targets of let-7g that regulate growth of these cells and are consistent with let-7g causing growth reduction through reduction of IMP-1 expression, which then indirectly affects expression of cell cycle regulators.

HMGA-2 and IMP-1 share many cancer-relevant properties. IMP-1 expression, like HMGA-2 expression, represents an early event in the process of tumorigenesis (10). We recently showed that ovarian cancer is characterized by an early reexpression of HMGA2 (6). Similarly, IMP-1 was also shown to be significantly overexpressed in more advanced stages of ovarian cancer, and high expression correlated with poor patient survival (10). Other cancer-relevant properties are summarized in the legend of Supplementary Fig. S1. In the adult, the only tissues that express IMP-1 are placenta and testis (16). Interestingly, the expression in the testis correlates with the lack of let-7 expression in this tissue (13). IMP-1 and HMGA2 share other properties. For example, HMGA2 contributes to cancer growth in that reduction of HMGA2 expression causes a growth reduction similar to that seen in cells following reduced IMP-1 expression (5, 17). Both genes, therefore, seem to regulate growth of cells. This conclusion is also supported by the observation that IMP-1–deficient mice develop dwarfism (9) in a manner similar to HMGA2-deficient mice (18).

In summary, we have identified a set of LOGs that could be up-regulated in many human cancers. The RNAs of these genes are down-regulated in tumor cell lines by exogenous let-7 and are down-regulated between E9.5 and E15 of mouse embryonic development, which is the time when let-7 expression is induced. We have validated our approach by establishing IMP-1 as a LOG. This work represents a first step toward the identification of the signature of LOGs that may contribute to early cancer formation. Each LOG may have distinct and/or overlapping tumor-promoting activities that should be reduced by reintroduction of let-7 into cancer cells. LOGs could serve both as

[9] See http://www.myccancergene.org/index.asp.

potential tumor antigens that could be therapeutic targets for cancer treatment and as novel biomarkers of disease progression.

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