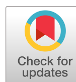

Suppression of non-small cell lung tumor development by the *let-7* microRNA family

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Many microRNAs (miRNAs) target mRNAs involved in processes aberrant in tumorigenesis, such as proliferation, survival, and differentiation. In particular, the *let-7* miRNA family has been proposed to function in tumor suppression, because reduced expression of let-7 family members is common in non-small cell lung cancer (NSCLC). Here, we show that let-7 functionally inhibits non-small cell tumor development. Ectopic expression of let-7g in K-Ras^{G12D}-expressing murine lung cancer cells induced both cell cycle arrest and cell death. In tumor xenografts, we observed significant growth reduction of both murine and human non-small cell lung tumors when overexpression of let-7g was induced from lentiviral vectors. In let-7g expressing tumors, reductions in Ras family and HMGA2 protein levels were detected. Importantly, let-7g-mediated tumor suppression was more potent in lung cancer cell lines harboring oncogenic K-Ras mutations than in lines with other mutations. Ectopic expression of K-Ras^{G12D} largely rescued let-7g mediated tumor suppression, whereas ectopic expression of HMGA2 was less effective. Finally, in an autochthonous model of NSCLC in the mouse, let-7g expression substantially reduced lung tumor burden.

K-Ras | lung cancer

MicroRNAs (miRNAs) are a class of short highly conserved noncoding RNAs known to play important roles in numerous developmental processes. MiRNAs are initially transcribed as longer primary transcripts that undergo sequential processing by the RNase III-like enzymes Drosha and Dicer (1). After maturation, miRNAs regulate gene expression through incomplete basepairing to a complementary sequence in the 3′ untranslated region (UTR) of a target mRNA. The miRNA–mRNA interaction results in translational repression and, to a lesser extent, accelerated turnover of the target transcript (2). Computational analyses predict that mammalian miRNAs regulate ≈30% of all protein-coding genes, because an individual miRNA can target many different mRNAs and an individual mRNA can be regulated by several different miRNAs (3, 4).

Numerous findings suggest that miRNAs undergo aberrant regulation during tumorigenesis. MicroRNA genes are frequently located in genomic regions gained and lost in mammalian cancers (5, 6). Functionally, several miRNAs have been described as oncogenes. For example, the miRNA cluster miR-17–92 is amplified in human B-cell lymphomas and was found to cooperate with c-Myc to accelerate lymphomagenesis in the mouse (7). The *BIC* transcript, which was isolated from a common retroviral insertion site that cooperates with c-Myc in lymphomagenesis and is highly up-regulated in Burkitt's lymphoma, encodes a primary miRNA transcript for miR-155 (8). Moreover, miR-372 and miR-373 were shown to be oncogenic in an expression screen and were implicated in testicular cancer through inactivation of the p53 pathway (9). Other miRNAs have been described as tumor suppressors. Intriguingly, miRNA expression profiling has shown that miRNAs are globally down-regulated in tumors relative to normal tissue (10). Recent work from our group demonstrated that global down-regulation can promote tumorigenesis (11). MiR-15 and miR-16 are located in chromosome 13q14, a region frequently deleted in chronic lymphocytic leukemia (CLL) (12). Furthermore, recent studies have shown that p53 transcriptional activation targets the miR-34 family in a step important for cell cycle control (13–15). In total, these findings provide several lines of evidence for the importance of miRNA in tumorigenesis.

Let-7 was originally identified in *Caenorhabditis elegans* as a regulator of developmental timing and cellular proliferation (16). The discovery of mammalian let-7 family members prompted speculation that these miRNAs might be tumor suppressors (17). There are at least nine individual members of the let-7 family in mammals, and several let-7 genes are located in regions frequently deleted in human cancer (5). Moreover, let-7 expression is reduced in a subset of non-small cell lung cancer (NSCLC) patients, and this reduction is correlated with poor prognosis (18, 19). When ectopically expressed in cancer cell lines, let-7 miRNA can repress cellular proliferation (20, 21). Finally, let-7 family members functionally inhibit the mRNAs of well characterized oncogenes, such as the Ras family (22), HMGA2 (21, 23, 24), c-Myc (11, 25), and cell cycle regulators like CDC25A, CDK6, and Cyclin D2 (20).

The two best characterized let-7 targets are the Ras family and HMGA2. Activating mutations in Ras family members (H-ras, K-ras, and N-ras) are found in many human tumors including ≈30% of NSCLCs (26). The let-7 family has been shown to regulate both N-Ras and K-Ras mRNAs via let-7 binding sites in the 3′ UTRs (22). Notably, all previous studies reporting let-7-mediated repression of proliferation have been performed in cells expressing mutant forms of N- and K-Ras (20, 21). The high mobility group A (HMGA) proteins are major nonhistone chromosomal proteins involved in transcriptional regulation controlling proliferation and differentiation. HMGA2 is implicated in tumorigenesis via chromosomal translocations and transcriptional up-regulation in several tumor types, although the function of this up-regulation in tumorigenesis is unclear (27–29). The HMGA2 3′UTR contains seven let-7 target sites and disruption of these sites enhances oncogenic transformation (30). Finally, let-7 expression is inversely correlated with expression of HMGA2 in NSCLC and ectopic overexpression of HMGA2 promotes cellular proliferation in the presence of let-7 (21).

The above findings suggest that the let-7 miRNA family functions in tumor suppression. However, studies to date have not demonstrated that let-7 miRNA can suppress tumorigenesis *in vivo*. Moreover, there is a lack of functional data related to the

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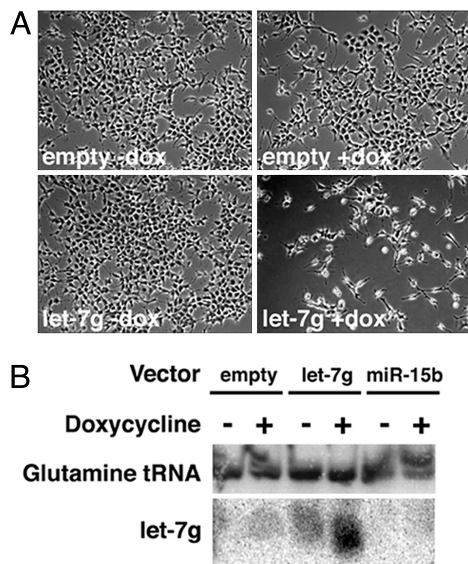

Fig. 1. Let-7g impairs proliferation and enhances cell death. (*A*) LKR13-Tet-On-KRAB-TE-empty and -let-7g cells were plated (5×10^5 cells per plate). Twelve hours later, cells were placed in the presence/absence of 5 μg/ml doxycycline. Forty-eight hours later, images were taken by phase contrast microscopy. (*B*) Small RNA Northern blot analysis was performed against let-7g and Glutamine tRNA in LKR13-Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g (see *SI Text* for details) cells in the presence or absence of 5 μg/ml doxycycline.

regulation of individual let-7 targets on tumorigenesis *in vivo*. Finally, no studies to date have examined let-7 function in autochthonous tumors, which allows the evaluation of the roles of let-7 in tumor initiation and progression. Here, we have used constitutive and inducible expression of let-7 to examine its effect on tumor development.

Results

Let-7g Impairs Tumor Cell Proliferation and Promotes Tumor Cell Death *in Vitro*. To assess the roles of the let-7 miRNA on cell cycle control and cell death, we transfected a let-7g miRNA duplex into murine K-Ras^{G12D}-expressing lung adenocarcinoma cells (LKR13). Consistent with the studies in refs. 20 and 21, transfected let-7g triggered a significant shift in the cell cycle distribution, with an accumulation of G_0/G_1- and G_2/M-phase cells and a corresponding reduction of S-phase cells [supporting information (SI) Fig. 6*A*]. In addition, transfection of let-7g caused significant cell death in LKR13 cells (Fig. 6*B*). To extend these findings, we developed a doxycycline (dox)-regulated expression system to induce miRNA expression in cell lines. Using the inducible vector system, we observed a substantial (≈5-fold) induction of let-7g in the presence of dox (Fig. 1*B*). Furthermore, induction of let-7g in LKR13 cells caused a robust decrease in cell density (Fig. 1*A*). Overall, these results indicate that let-7g can restrict cellular proliferation and induce cell death.

Let-7g Suppresses Tumorigenesis *in Vivo*. The LKR13 cells with inducible let-7g were transplanted into immune compromised mice to which dox was administered in the drinking water. Using this system, we observed a substantial reduction in tumor growth in mice after induction of let-7g compared with controls; induction of miR-15b, although putatively described as a tumor suppressor in CLL (12), did not alter tumor growth in this system (Fig. 2*A*). However, this does not exclude the possibility of miR-15b suppressing tumor growth in other contexts. Importantly, this reduction in tumor growth depended on induction of let-7g, because transplantation of the same cells into animals without dox treatment led to rapid tumor development (Fig. 2*B*). Interestingly, tumors with ectopic let-7g expression, although

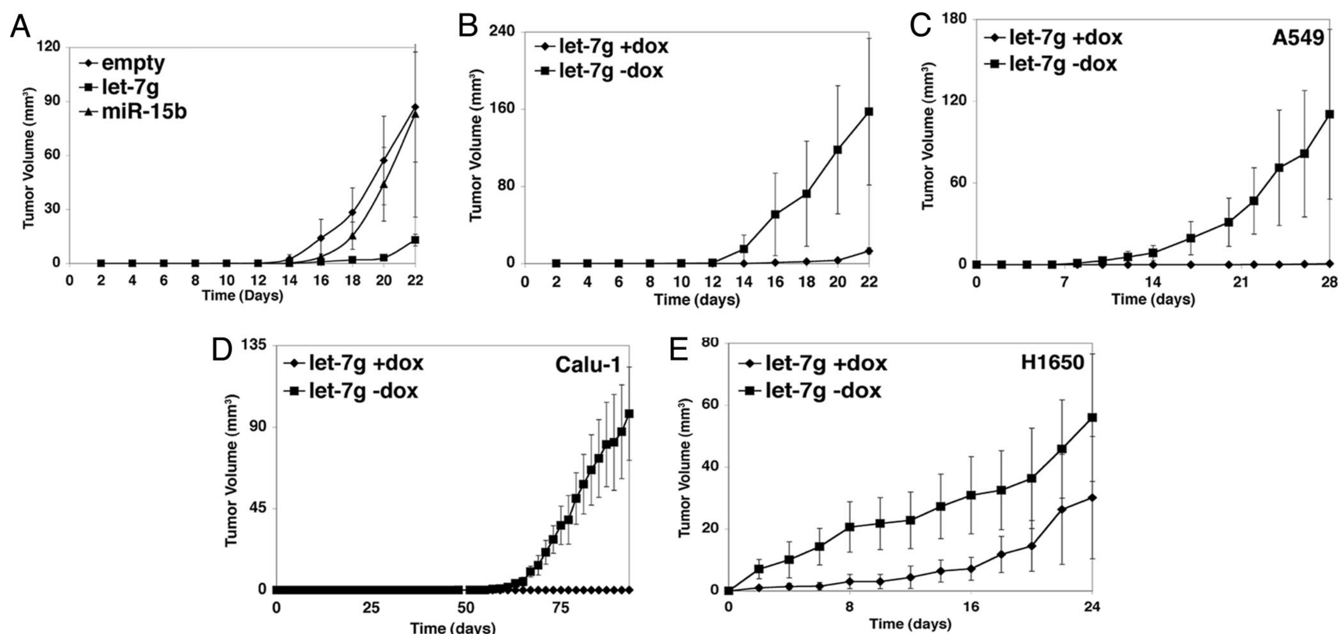

Fig. 2. Let-7g suppresses tumorigenesis *in vivo*. (*A*) LKR13-Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g cells were plated in the presence of 5 μg/ml doxycycline. Twenty-four hours later, cells were sorted and injected s.c. into immune-compromised mice (2.5×10^4 cells per injection). Two days later, mice were treated with drinking water containing doxycycline (2 mg/ml) and sucrose (4% wt/vol), and tumor values were measured over time. Values are mean ± SEM (n = 6). (*B*) LKR13-Tet-On-KRAB-TE-let-7g cells were treated with doxycycline, sorted, and injected as described above. Two days later, mice were treated with either drinking water containing doxycycline (2 mg/ml) and sucrose (4% wt/vol) or drinking water containing sucrose alone. Tumor values were measured over time. Values are mean ± SEM (n = 6). (*C–E*) Tet-On-KRAB-TE-let-7g cells were generated in A549 (*C*), Calu-1 (*D*), and H1650 (*E*) cells. Cells were prepared and injected (10^6 cells per injection) into immune-compromised mice. Mice were treated and tumors were measured as above. Values are mean ± SEM (n = 6).

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Fig. 3. Let-7g-induced tumors maintain overexpression and target suppression. (*A*) Small RNA Northern blotting was performed against let-7g and Glutamine tRNA in LKR13-Tet-On-KRAB-TE-empty and -let-7g tumors generated from mice treated with or without doxycycline in the drinking water as described above. (*B*) Western blot analysis was performed in LKR13-Tet-On-KRAB-TE-empty and -let-7g tumors generated from mice treated with doxycycline in the drinking water as described above.

Fig. 4. K-Ras^{G12D} substantially rescues let-7g-mediated tumor suppression. (*A*) LKR13-Tet-On-KRAB-TE-miR-15b and -let-7g cells were infected with pBabe.Zeo.empty, K-Ras^{G12D}, and HMGA2 and Western blot analysis was performed. (*B* and *C*) LKR13-Tet-On-KRAB-TE-let-7g cells infected with pBabe.Zeo.K-Ras^{G12D} (*B*) or HMGA2 (*C*) were prepared and injected (10^6 cells per injection) into immune-compromised mice. Mice were treated and tumors were measured as above. Values are mean ± SEM (*n* = 6).

smaller in size, were more advanced than controls, demonstrating widespread invasion into surrounding muscle (SI Fig. 7). The steady-state levels of let-7g and miR-15b miRNAs were substantially induced in tumors grown in the presence of dox (Fig. 3*A* and SI Fig. 8). This induction was correlated with a decrease in Ras family members and HMGA2, previously characterized targets of the let-7 family (Fig. 3*B*) (21, 22, 30).

Although let-7g induction suppressed tumor growth, tumors did eventually form in the presence of dox. To assess the growth of let-7g-expressing tumors, we explanted tumors and tested them in secondary transplants. Secondary transplants of let-7g tumors that developed in the presence of dox grew at a similar rate as controls (SI Fig. 9*A*). These tumors generally had significant levels of let-7g, although the degree of induction varied (SI Fig. 9*B*). These results demonstrate that although let-7g expression suppresses tumor growth, tumors can form in the presence of high levels of let-7g. Furthermore, the tumors eventually propagate similarly to controls, suggesting that cancer cells may become resistant to the tumor suppressive functions of let-7g.

We then examined the effect of let-7g induction on established tumors. Tumors were allowed to grow to 30 mm^3 before addition of dox. This treatment led to a slight reduction in tumor growth rate but did not cause tumor regression (SI Fig. 10*A*). As expected, there was a substantial induction of the corresponding miRNAs in these tumors, suggesting that the absence of tumor regression was not due to the failure to induce let-7g (SI Fig. 10*B*).

Let-7g Potently Suppresses Mutant K-Ras-Driven Non-Small Cell Lung Tumorigenesis *in Vivo*. To extend let-7g-mediated tumor suppression to human cells, we introduced the dox-inducible miRNA system in a series of human NSCLC lines. Two NSCLC lines, A549 and Calu-1, contain activating mutations in K-Ras, but one, H1650, does not. In all three NSCLC lines, control miRNA induction had no effect on tumor growth (SI Fig. 11). In the K-Ras mutant NSCLC lines, let-7g induction triggered near complete suppression of tumor formation (Fig. 2 *C* and *D*). In contrast, let-7g induction caused only a partial reduction in the rate of tumor growth in the non K-Ras mutant NSCLC line (Fig. 2*E*). This indicates that tumor suppression is somewhat mutation-specific, because the let-7g mediated reduction in tumor growth is stronger in tumors driven by mutant K-Ras than those containing wild-type K-Ras.

Let-7g-Mediated Tumor Suppression Is Rescued Substantially by Mutant K-Ras. To determine which mRNA targets of let-7 are germane to its ability to suppress non-small cell lung tumorigenesis, we ectopically expressed mutant K-Ras (K-Ras^{G12D}) and HMGA2 in LKR13 cells containing the dox-inducible miRNA system (Fig. 4*A*). Of note, each protein was up-regulated modestly in the presence of enhanced expression of the other protein, suggesting mutual activation of these let-7 targets. Importantly, the expression vectors for K-Ras^{G12D} and HMGA2 lack their corresponding 3′ UTRs, uncoupling the mRNA targets from direct let-7-mediated repression.

When these cells were transplanted into immune compromised mice, we observed potent let-7g-mediated tumor suppression in controls (SI Fig. 12*A*). As shown in Fig. 4*B*, ectopic K-Ras^{G12D} led to substantial, although not complete, rescue of tumor growth in the face of increased let-7g levels. In cells expressing ectopic HMGA2, there was a less robust rescue of tumor growth (Fig. 4*C*). There were also no changes in tumor growth rate in cells over-expressing miR-15b upon ectopic expression of activated K-Ras or HMGA2 (SI Fig. 12 *B* and *C*). Overall, these results suggest that mutant K-Ras plays a key role in let-7g-mediated tumor suppression. However, the rescue of tumor growth was incomplete, suggesting that other let-7 targets,

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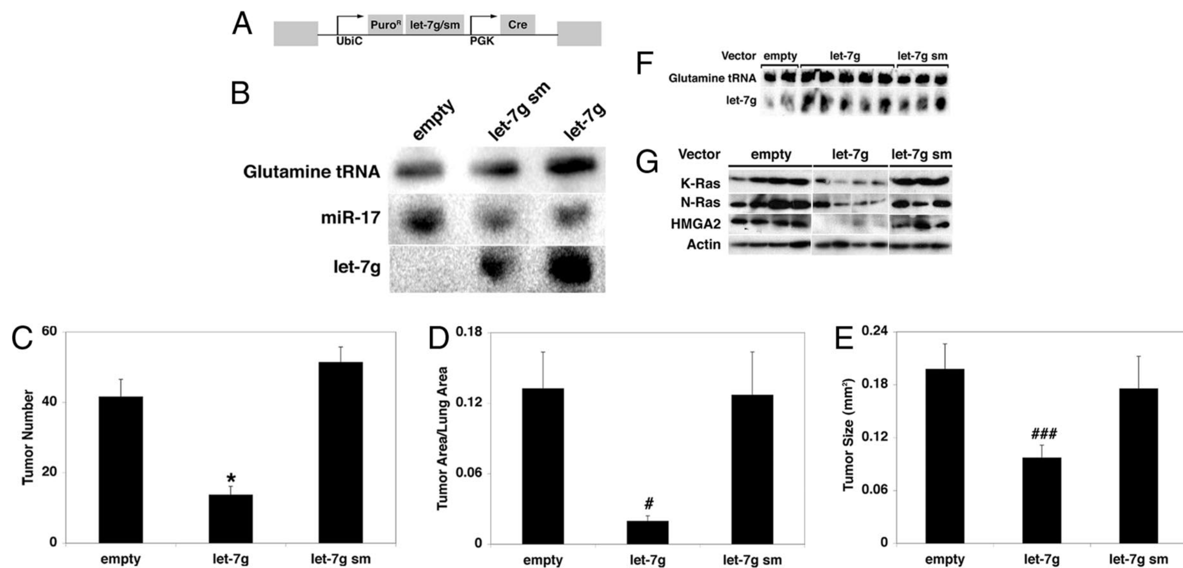

Fig. 5. Let-7g suppresses tumor initiation in an autochthonous NSCLC model. (*A*) Diagram of the Puro.Cre lentiviral vector for coexpression of let-7g/let-7g sm with Cre recombinase. (*B*) Small RNA Northern blot analysis was performed against let-7g (both wild type and seed mutant), miR-17 and Glutamine tRNA in HEK293 cells infected with Puro.Cre (empty), Puro.let7gsm.Cre (let-7g sm), and Puro.let7g.Cre (let-7g). (*C–E*) *Kras^{LSL-G12D};Trp-53^{flox/flox}* mice were intratracheally infected with the Puro.Cre lentiviral vectors described above. Twelve weeks after infection, animals were killed, and tumor number (*C*), tumor and lung area (*D*), and tumor size (*E*) were quantified with Bioquant software. Values are mean ± SEM ($n = 9$ for empty, $n = 11$ for let-7g, and $n = 11$ for let-7g sm). *, $P < 0.0005$, #, $P < 0.01$; ###, $P < 0.1$. (*F*) Small RNA Northern blotting was performed against let-7g (both wild type and seed mutant), and Glutamine tRNA on lung tumors was generated from *Kras^{LSL-G12D};Trp-53^{flox/flox}* mice infected with Puro.Cre (empty), Puro.let7g.Cre (let-7g), and Puro.let7gsm.Cre (let-7g sm). (*G*) Western blot analysis was performed on lung tumors generated from *Kras^{LSL-G12D};Trp-53^{flox/flox}* mice infected with Puro.Cre (empty), Puro.let7g.Cre (let-7g), and Puro.let7gsm.Cre (let-7g sm). All samples were probed on the same blot.

likely including HMGA2, are also relevant for let-7g-mediated tumor suppression.

Let-7g Suppresses Tumor Initiation in an Autochthonous NSCLC Model. We next examined the tumor suppressive effect of let-7g in naturally arising lung tumors in the mouse. When infected with Cre-expressing virus in the lung epithelium, *Kras^{LSL-G12D};Trp-53^{flox/flox}* mice develop highly aggressive NSCLC following a well defined time course (31). These lesions recapitulate advanced human lung adenocarcinoma in many ways, including stromal desmoplasia. To deliver let-7g to the lung epithelium, we generated a lentiviral vector coexpressing let-7g or a seed-mutant version of let-7g (let-7g sm) with Cre, using a dual-expression system (Fig. 5*A*). This vector generated substantial expression of let-7g and let-7g sm relative to controls in cultured cells (Fig. 5*B*). In addition, let-7g expressed from this vector caused robust repression of a reporter through the K-Ras 3′ UTR, which was not observed with the seed mutant of let-7g (SI Fig. 13).

Kras^{LSL-G12D};Trp-53^{flox/flox} mice were infected with let-7g- and let-7g sm-containing lentiviruses, and effects on tumor development were determined by visual inspection and histological analysis. We observed a significant reduction in both lung tumor number and tumor area after infection with the vector expressing let-7g versus controls (Fig. 5 *C* and *D*). In addition, there was a slight decrease in average tumor size in let-7g expressing mice (Fig. 5*E*). The levels of let-7g and let-7g sm expression in these tumors were determined by Northern blot analysis. Consistent with findings in xenograft tumors, there was sustained overexpression of let-7g and let-7g sm in tumors from mice infected with the corresponding lentiviruses (Fig. 5*F*). Moreover, these let-7g overexpressing tumors have reduced protein levels of N-Ras, K-Ras, and HMGA2, indicating functional repression of known let-7 targets (Fig. 5*G*). In total, these findings suggest that let-7g effectively suppresses tumor initiation in an autochthonous mouse model of mutant K-Ras-driven NSCLC. However, the tumors that do form continue to express let-7g and suppress known targets, supporting the conclusion that naturally arising tumors may gain resistance to let-7g.

Discussion

In this study, we investigated the functional consequence of let-7g expression on non-small cell lung tumorigenesis. Using both inducible and constitutive expression systems, we observed substantial tumor suppression by let-7g both in xenografts and in a mouse lung tumor model. Two lines of evidence suggest that let-7g-mediated tumor suppression is representative of the let-7 family. First, transfection of other let-7 family members caused comparable induction of cell death to let-7g (data not shown). Second, transfection of a miRNA sponge (32) targeting the entire let-7 family shifted the cell cycle distribution opposite to let-7g overexpression with a significant reduction of G_0/G_1-phase cells and a corresponding increase in S- and G_2/M-phase cells (SI Fig. 6*C*).

Studies of the effect of let-7 on cellular proliferation have used cells containing activating mutations in K-Ras (A549) and N-Ras (H1299 and HepG2) (20, 21). Because both K-Ras and N-Ras are previously characterized targets of the let-7 family, it was possible that the effects of let-7 on proliferation in these cell lines were largely due to down-regulation of the Ras family. In the present study, we determined that K-Ras^{G12D}-mediated rescue of tumor growth was not complete, suggesting that other let-7 targets are also relevant to tumor suppression. In sum, these findings suggest that let-7-mediated tumor suppression occurs largely, although not completely, through regulation of the Ras family.

Although let-7g expression potently suppressed non-small cell lung tumorigenesis, tumors inevitably formed in the presence of sustained let-7g induction. Notably, these tumors continued to express let-7g and actively repressed let-7 targets, suggesting the tumors that form do not propagate because of silencing of the let-7g vector. Moreover, this continued let-7g expression occurred both in xenograft models and autochthonous lung tumors

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expressing let-7g. This apparent resistance to let-7 was not observed in the studies in refs. 20 and 21, because they relied on transient delivery of let-7 family members. This distinction highlights the importance of stable induction of let-7 when analyzing its role in tumorigenesis, because transient expression of let-7 does not recapitulate the long-term effects of let-7 on tumorigenesis *in vivo*.

Although we cannot exclude the possibility that the let-7g target repression observed here was insufficient to suppress tumorigenesis, the data suggest that let-7g is present and active in escaping tumors. It is possible that escaping tumors have activated pathways downstream of targets in the presence of let-7; escape could also occur through activation of a distinct set of oncogenes not targeted by the let-7 family. Overall, the apparent resistance to let-7g expression has significant implications for the use of let-7 miRNAs as a therapeutic agent. Our data indicate that sustained let-7 delivery might lead to initial suppression of tumor growth but that let-7 resistant tumors might eventually emerge. Using the systems described here, one could probe downstream pathways from let-7 targets, including Ras, HMGA2, and others, to assess their roles in let-7 resistant tumors. Additionally, expression analysis of sensitive and resistant tumors might reveal novel pathways functionally related to let-7 resistance.

Here, we describe one of the first cases of a miRNA family functioning as a tumor suppressor *in vivo*. Our findings make clinically relevant predictions related to the use of let-7-based therapeutic agents in NSCLC. The systems outlined in this study provide insight into let-7-mediated tumor suppression and also establish unique tools for understanding the basis for resistance of cancer cells to miRNA-mediated control of tumorigenesis. Moreover, the doxycycline-based and lentiviral systems described could be applied to examine other small RNAs suggested to function in tumorigenesis.

Materials and Methods

Cell Culture. Human cell lines (HEK293, A549, Calu-1, and H1650) were originally obtained from ATCC. 3TZ cells are described in ref. 33. LKR13 cells are described in ref. 34. Cells were grown under standard conditions. After introduction of pTE vectors, cells were maintained under standard conditions in Tet-Free Serum per the manufacturer's instructions (Clontech).

Cell Cycle and Cell Death Analysis. MicroRNA duplex sequences were transfected in triplicate into LKR13 cells with DharmaFECT-1 (Dharmacon) according to manufacturer's protocol. Cells were harvested after 48 and 72 h and either fixed in methanol and stained with 7-AAD (Stem-Kit reagent, Beckman Coulter) or stained with the Apoptest-FITC kit (Nexins Research) and analyzed by flow cytometry.

Lentivirus Production and Infection. Lentivirus production was performed as described in ref. 35.

Allograft/Xenograft Studies. Tet-On-KRAB-TE cells were treated for 24 h with doxycycline (5 μg/ml) and sorted by flow cytometry. BalbC/Nu males (Taconic) were injected with cells as described in ref. 36. Two days after injection, mice were treated with drinking water containing doxycycline (2 mg/ml) and sucrose (4% wt/vol). Tumor sizes were measured every two days. After indicated days, mice were killed and tumors were isolated for histology and Western and Northern blot analyses.

Intervention Studies. Mice were injected with LKR13-Tet-On-KRAB-TE cells and monitored for tumors. Once tumors were greater that 2 mm in diameter, mice were i.p. injected with doxycycline (40 mg/kg) and tumors were measured as above.

Secondary Transplant Studies. Mice were injected with LKR13-Tet-On-KRAB-TE cells and treated with doxycycline in the drinking water as above. Tumors were then explanted, retreated for 24 h with doxycycline (5 μg/ml) and sorted by flow cytometry. Cells were then injected and mice were treated with doxycycline in the drinking water as above. Tumor sizes were measured every 2 days. After indicated days, mice were killed and tumors were isolated for Northern blot analysis.

Genetically Engineered Mice. *Kras$^{LSL\text{-}G12D}$;Trp-53$^{flox/flox}$* mice were generated as described in ref. 31.

Intratracheal Infection and Tumor Analysis. *Kras$^{LSL\text{-}G12D}$; Trp-53$^{flox/flox}$* mice were infected intratracheally with Puro.Cre lentivirus essentially as described in ref. 37. Tumor analysis was performed as described in ref. 11.

Animal Care and Use. Research was approved by the Committee for Animal Care, and conducted in compliance with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996 (institutional animal welfare assurance no. A-3125–01).

Further Details. For more information on the materials and methods, see *SI Text*.

Note Added in Proof. A recent study described in ref. 38 suggested that let-7 suppresses tumorigenesis via alteration of self-renewal and differentiation of breast cancer stem cells. Studies should look to characterize these effects in the described lung cancer models.

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