

# let-7 regulates Dicer expression and constitutes a negative feedback loop

Shogo Tokumaru<sup>1,2</sup>, Motoshi Suzuki<sup>1</sup>, Hideki Yamada<sup>1,2</sup>, Masato Nagino<sup>2</sup> and Takashi Takahashi<sup>1,\*</sup>

<sup>1</sup>Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer and <sup>2</sup>Department of Surgical Oncology, Nagoya University Graduate School of Medicine, 65 Tsurumai-Cho, Showa-Ku, Nagoya 466-8550, Japan

\*To whom correspondence should be addressed. Tel: +81 52 744 2454;  
Fax: +81 52 744 2457;  
Email: tak@med.nagoya-u.ac.jp

microRNAs (miRNA) are small, endogenously expressed non-coding RNAs that are sequentially processed by Drosha and Dicer from primary transcripts, by negatively regulating the expression of protein-coding genes through either translational repression or RNA degradation. Their expression patterns are developmentally regulated and/or tissue specific, while altered expressions of certain miRNAs are frequently observed in human cancers, though the underlying regulatory mechanism is largely unknown. Herein, we show that Dicer expression was inversely correlated with expression levels of mature *let-7* in a panel of human cancer cell lines, showing association with cell growth and cell cycle phases. Overexpression of *let-7* significantly reduced the expression of Dicer at both the protein and messenger RNA levels, whereas antisense-mediated reduction of *let-7* expression conversely increased Dicer at both levels. A luciferase assay using a reporter carrying a putative target site in the 3' untranslated region of Dicer revealed that *let-7* directly affects Dicer expression. Down-regulation of Dicer resulted in a reduced expression of mature *let-7*. Furthermore, overexpression of *let-7* decreased the levels of expression of other mature miRNAs, while knockdown of *let-7* increased those levels. Taken together, these findings strongly suggest the possible existence of a novel regulatory loop, in which *let-7* may play a role as a key miRNA for implementing the tightly regulated, equilibrated state of Dicer and various miRNAs.

## Introduction

microRNAs (miRNAs) belong to a recently discovered class of non-coding RNAs that play an important role in the negative regulation of gene expression (1,2). They are first transcribed as long primary transcripts (pri-miRNAs), then become processed in the nucleus by the RNase III enzyme Drosha to generate 60- to 120-nt-long precursors (pre-miRNAs) containing stem-loop structures (3). Pre-miRNAs are further cleaved by the second RNase III enzyme Dicer to release 18- to 25-nt-mature miRNAs (4). Mature miRNAs mostly bind to the 3' untranslated regions (UTRs) of their target messenger RNAs (mRNAs) and repress expression at the translational step or induce RNA degradation (5).

Accumulating evidence suggests that the development of human cancers is associated with altered miRNA expressions (2,6). We also showed that members of the *let-7* family are frequently downregulated in lung cancers in association with shortened post-operative survival and that enforced expression of *let-7* modestly reduced lung cancer cell growth (7). In addition, we found that the *miR-17-92* cluster is markedly overexpressed in lung cancers, while introduction of its expression construct enhanced lung cancer cell growth (8). It has been suggested that potential regulatory mechanisms of altered miRNA expression in cancer cells may involve copy number alterations (8–10), transcriptional alterations by transcription factors (11), promoter methylations or histone modifications (12,13) and failure during the Drosha-mediated processing step (14).

**Abbreviations:** AS, antisense oligonucleotides; miRNA, microRNA; mRNA, messenger RNA; PCR, polymerase chain reaction; siRNA, small interfering RNA; UTR, untranslated region.

Recently, a few miRNAs have been reported to constitute feedback loops and target transcription factors that regulate their own expressions. *miR-20a* of the *miR-17-92* cluster targets the E2F1–3 transcription factors, which activate transcription of the *miR-17-92* cluster itself (15). Further, *miR-223* targets NFI-A, a transcription repressor that in turn inhibits transcription of *miR-223* (16). The existence of such feedback loops has been suggested to play a role in homeostasis as well as in cellular differentiation.

In this study, we found that Dicer is a direct target of *let-7*, which has an effect on the expression of other mature miRNAs. Since *let-7* is also processed by Dicer, our findings suggest the existence of a novel negative feedback loop comprised of *let-7* and Dicer, which may play a role in the tuning of mature miRNA expression profiles and carcinogenesis.

## Materials and methods

### Cell cultures

293T (human embryonic kidney cells), BxPC-3, Hs766T (human pancreatic cancer cells) and TIG-112 (normal human skin fibroblast cell) cell lines were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum. HeLa cells (human cervix cancer cells) were cultured in RPMI 1640/5% fetal bovine serum. Other lung cell lines were maintained as described previously (7).

### Western blot analysis

Total cell lysates were prepared using a sodium dodecyl sulfate sample buffer, with 30 µg of each separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The primary antibodies used were anti-Dicer antibody (Abcam, Cambridge, ab14601), anti-Drosha antibody (Upstate, Millipore, Billerica, MA, #07-717) and anti-α-tubulin antibody (Sigma, St Louis, MO, T9026).

### Northern blot analysis

To quantify miRNA, total RNA samples were prepared by an acid phenol extraction procedure, with 10 µg of each separated on 15% denatured polyacrylamide gels as described previously (7). The oligonucleotide probes used are shown in supplementary Table 1 (available at *Carcinogenesis* Online). To quantify Dicer mRNA, total RNA samples were prepared using an RNeasy Mini Kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions, and 10 µg of each was separated by formaldehyde–agarose gel electrophoresis as described previously (8). Polymerase chain reaction (PCR)-generated probes were prepared using the primer pairs listed in supplementary Table 2 (available at *Carcinogenesis* Online).

### Statistical analysis

Using the quantification program ImageJ (<http://rsb.info.nih.gov/ij/>), band intensities for Dicer, Drosha and mature *let-7a* were determined relative to those of HeLa cells. The distribution of expression levels at log<sub>2</sub> was shown as a scattered plot. The associations between expression levels of Dicer or Drosha and mature *let-7a* were analyzed by computing the Pearson correlation coefficient.

### Measurement of cell growth

Cell growth of Calu6 cells was measured by plating at a density of 5 × 10<sup>5</sup> cells in 10 cm dishes on day 0 and harvesting everyday. TIG-112 cells were plated at a density of 5 × 10<sup>5</sup> cells in 10 cm dishes for 24 h, then transferred to culture medium with or without 10% fetal bovine serum and harvested 48 h later. DNA content was analyzed as described previously (17).

### Precursor molecules, plasmid, antisense oligonucleotides and siRNAs

Precursor molecules were purchased from Ambion (Austin, Tx; Catalog numbers: #17100 and 17110). Fragments of *pri-let-7a-1* and *pri-miR-99a* were amplified by PCR (supplementary Table 2 is available at *Carcinogenesis* Online) and cloned into a modified pcDNA3 vector (Invitrogen, Carlsbad, CA) carrying a puromycin resistance gene. Antisense as well as negative control oligonucleotides were synthesized as described previously (17). The nucleotide sequences are shown in supplementary Table 3 (available at *Carcinogenesis* Online). An small interfering RNA (siRNA) for Dicer and a negative control were designed and synthesized as described previously (4).

### Transfection assays

Precursor molecules of miRNAs (*let-7a*, *let-7c* and *let-7d*) and negative control #1 were transfected into each of the cell lines at a final concentration of

10 nmol/l using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction. Cells were harvested 24 h after transfection. In some experiments, precursor molecules and siRNA for Dicer were transfected into Calu6 cells at a final concentration of 18 nmol/l using Lipofectamine 2000, with the cells harvested 72 h after transfection. The *pri-let-7a-1* and *pri-miR-99a* expression construct and a control plasmid were transfected into 293T using FuGENE 6 Reagent (Roche, Basel, Switzerland). One day after transfection, cells were treated with puromycin (2 µg/ml), then cultured for 2 days and harvested. For assessing the effects of antisense-mediated inhibition of *let-7*, cells were plated at  $1 \times 10^6$  in 10 cm dishes on the first day (day 1). Antisense oligonucleotides (AS) was sequentially transfected at 10 nmol/l, as described previously (17).

#### Luciferase assay

The 494 bp region of the 3' UTR segment containing the *let-7* target site of Dicer was amplified by PCR (supplementary Table 2 is available at *Carcinogenesis* Online) and inserted into the pGL3 vector (Promega, Madison, WI) immediately downstream from the stop codon of luciferase. We also generated a mutant construct using a site-directed mutagenesis method with two primer sets (supplementary Table 2 is available at *Carcinogenesis* Online). Wild-type and mutant inserts were confirmed by DNA sequencing.

After sequential transfection with AS, as described above, cells were plated at  $2 \times 10^5$  per well in 12-well plates on day 5, and then 0.9 µg of pGL3 plasmid and 0.1 µg of pRL-TK (Promega) were transfected using FuGENE 6 Reagent on day 6, according to the manufacturer's instructions. Luciferase assays were performed 48 h after transfection using a Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. Each assay was performed in triplicate.

#### Quantitative real-time reverse transcription-PCR

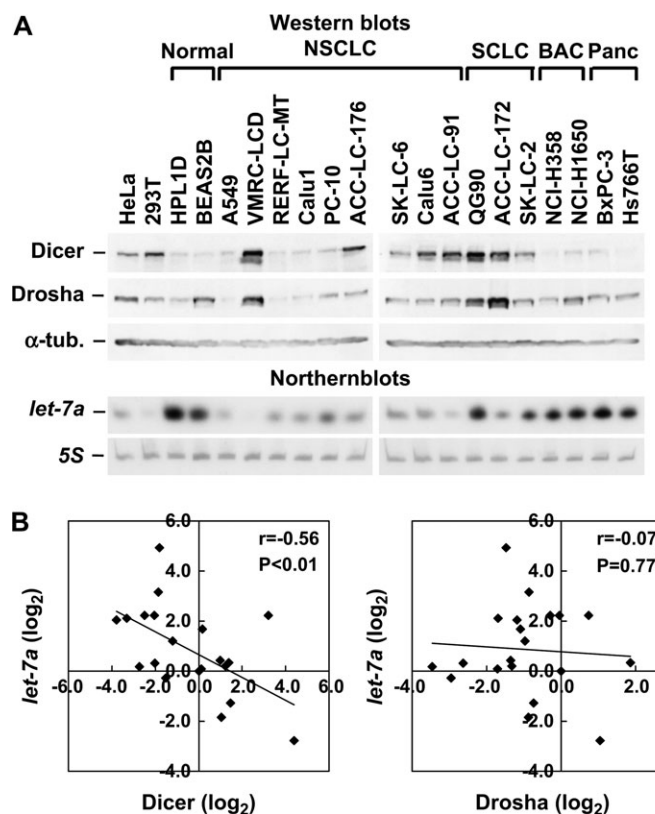
Applied Biosystems TaqMan miRNA assays were used to detect and quantify mature miRNAs using looped-primer real-time PCR. Total RNA samples were prepared from transfected Calu6 and PC10 cells using a miRNeasy Mini Kit (QIAGEN). Complementary DNA samples were prepared using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with a 7500 Fast Real-Time PCR System using TaqMan PCR master mix, commercially available primers and FAM<sup>TM</sup>-labeled probes for miRNAs and *RNU48* (Applied Biosystems). Each sample was run in triplicate.  $C_t$  values for miRNAs were calculated and normalized to  $C_t$  values for *RNU48* ( $\Delta C_t$ ). The average  $\Delta\Delta C_t$  values were calculated by normalization to the  $\Delta C_t$  value for negative control.

A TaqMan PCR assay was also performed to quantify *Dicer* mRNA expression using commercially available FAM<sup>TM</sup>-labeled probes for *Dicer* and VIC<sup>TM</sup>-labeled probes for *18S*, according to the manufacturer's instructions (Applied Biosystems).  $C_t$  values for *Dicer* were normalized to those of *18S* ( $\Delta C_t$ ). The average  $\Delta\Delta C_t$  values were then calculated after normalization to the  $\Delta C_t$  value obtained at time point 0.

## Results and discussion

### *Dicer* protein expression inversely correlated with mature *let-7a* expression in cell lines

*let-7* constitutes a family of miRNAs, which are thought to be involved in cancer development (7,18,19). During a search for putative target genes of *let-7*, we noticed that Dicer, an RNase III enzyme that processes pre-miRNAs, was predicted as a target gene of *let-7d* by the PicTar prediction program (20). In order to examine if Dicer protein level is regulated by *let-7*, we first analyzed the correlation between expression levels of Dicer protein and mature *let-7a* (Figure 1A), which is the most abundantly expressed member of the *let-7* family in normal lung tissues with a very high sequence conservation with those other members, including *let-7d* (7). Among the 20 cell lines tested, the highest Dicer level was observed in VMRC-LCD, in which the expression level of *let-7a* was lowest. In contrast, a high expression of *let-7a* was associated with remarkably low Dicer protein levels in cell lines, including HPL1D, BEAS2B, NCI-H358, NCI-H1650, BxPC-3 and Hs766T. When the band intensities were quantified and plotted on a graph, we found a highly concordant inverse correlation between Dicer protein level and mature *let-7a*, with a Pearson correlation coefficient of  $-0.56$  ( $P < 0.01$ , Figure 1B). We also performed the same quantification for Drosha, though no obvious correlation was observed.



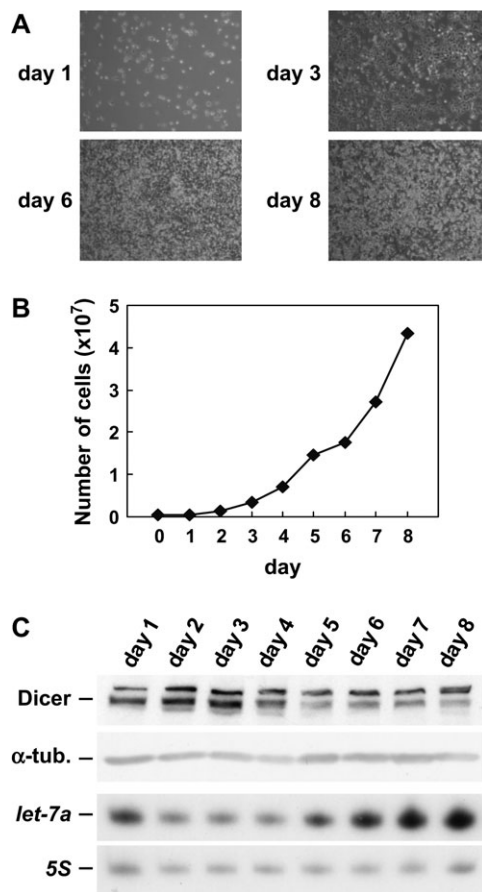
**Fig. 1.** Dicer protein expression inversely correlated with mature *let-7a* expression in cell lines. (A) Western blot analysis of Dicer and Drosha and northern blot analysis of mature *let-7a* in cell lines.  $\alpha$ -Tubulin and 5S were used for loading controls. (B) Scattered plot analysis of relative expression levels at log<sub>2</sub> of Dicer, Drosha and mature *let-7a*. HeLa expression levels were used for normalization. NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; BAC, bronchioloalveolar carcinoma; Panc, pancreatic cancer.

### Expression levels of Dicer and *let-7a* during cell growth

To elucidate the relationship between Dicer and *let-7*, Calu6, a large cell lung cancer cell line, was plated, and then the cell numbers and expression levels of Dicer and mature *let-7a* were determined for up to 8 days. As shown in Figure 2, Dicer expression increased at the beginning of cell growth and then gradually decreased. In contrast, the expression levels of *let-7a* showed a nearly reciprocal pattern. To further study the association between the expression levels of Dicer and *let-7a* during growth phases, we depleted serum from the medium used to culture the normal fibroblast cell line TIG-112, which induced quiescence. Under the G<sub>0</sub> state, upregulation of *let-7a* and remarkable downregulation of Dicer were observed (supplementary Figure 1 is available at *Carcinogenesis* Online), again showing a reciprocal expression pattern. These findings indicated that reciprocal expression patterns between Dicer and *let-7a* were present not only in the panel of cell lines but also in each individual cell line, suggesting the existence of a coordinated regulatory mechanism.

### *let-7* directly targets Dicer

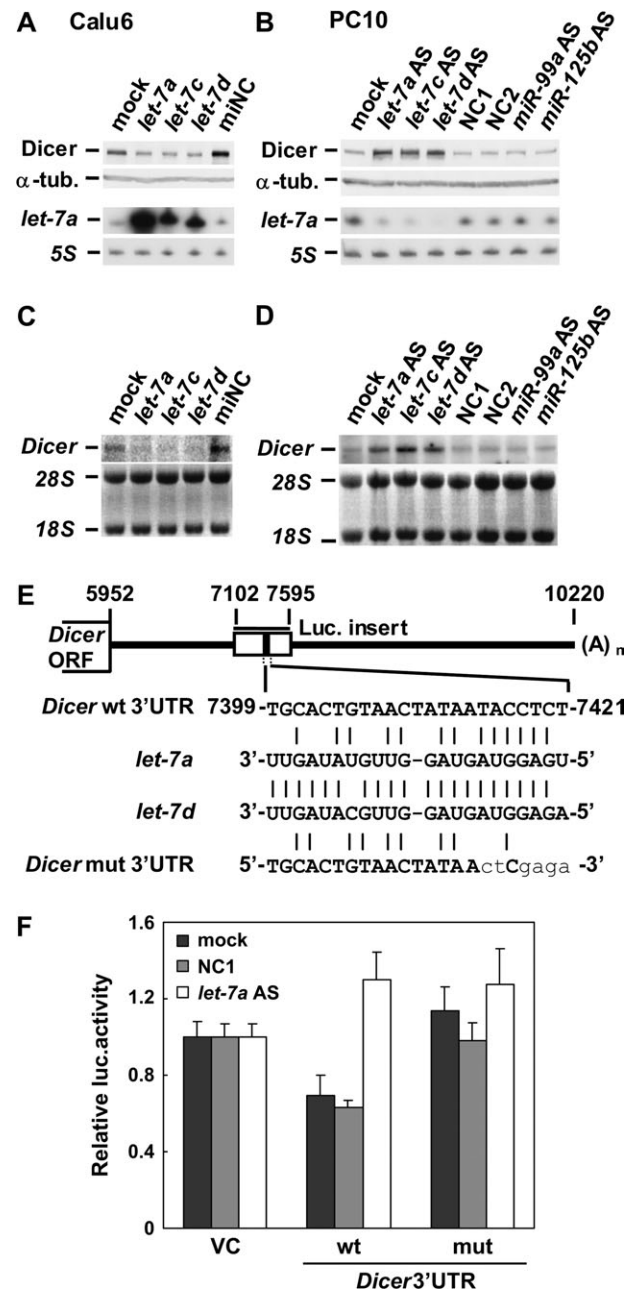
In the next series of experiments, we determined if Dicer is regulated by *let-7d*. Upon transfection of the *let-7d* precursor molecule, which was a double-stranded RNA designed to mimic mature miRNA, the level of Dicer protein was reduced when compared with transfections of mock or negative control Calu6 cells (Figure 3A). This analysis showed that *let-7a*, *c*, and *d* induced a nearly identical reduction in Dicer expression, suggesting that other *let-7* family members could also downregulate Dicer, despite the finding that only *let-7d* was predicted by PicTar. A similar result was obtained when a vector-based



**Fig. 2.** Expression levels of endogenous Dicer and mature *let-7a* during cell growth. (A) Phase contrast images (×50). (B) Growth curve. (C) Western blot analysis of Dicer and northern blot analysis of mature *let-7a*.

primary form of *let-7a-1* was introduced into 293T, whereas no such reduction was observed following transfection of an unrelated miRNA, *pri-miR-99a* (supplementary Figure 2A is available at *Carcinogenesis* Online), suggesting that the reduction in Dicer expression was specifically caused by *let-7*. Transfection of *let-7* AS into PC10 resulted in a significant reduction in *let-7a* and induction of Dicer protein, whereas transfection of *miR-99a* AS or *miR-125b* AS showed no effects (Figure 3B). Since our *let-7a* probe showed nearly identical knockdown patterns with AS against *let-7a*, *c* and *d*, we considered that considerable cross-reactivities existed between each AS and members of *let-7* and/or each *let-7* and the *let-7a* probe. We also observed a *let-7*-mediated reduction of Dicer in VMRC-LCD and ACC-LC-176 cells (supplementary Figure 2B and C is available at *Carcinogenesis* Online), as well as *let-7* AS-mediated induction of Dicer in BEAS2B and HeLa cells (supplementary Figure 2D and E is available at *Carcinogenesis* Online). These findings were in agreement with the notion that the expression of Dicer protein is regulated by *let-7*. Interestingly, *let-7* repressed not only the protein level of Dicer but also its mRNA level (Figure 3C and D), showing that *let-7* shortened the half-life of *Dicer* mRNA in actinomycin D-treated Calu6 cells (supplementary Figure 3 is available at *Carcinogenesis* Online). Our results were also consistent with a previous report that showed miRNA-mediated degradation of mRNAs of certain target genes (2,21).

To determine whether the negative effect of *let-7* on Dicer expression was a result of direct interaction between the 3' UTR of *Dicer* mRNA and *let-7*, we performed a luciferase assay using a construct containing the putative *Dicer* 3' UTR target site as well as the same construct carrying random mutations in the seed sequence (Figure 3E). Luciferase activity with the wild-type *Dicer* 3' UTR construct



**Fig. 3.** *let-7* directly targets Dicer. (A) Western blot analysis of Dicer following transfection of precursor molecules for *let-7a*, *c* and *d*, as well as negative control #1 (miNC) in Calu6 cells. Northern blot analysis for mature *let-7a* was performed under the same conditions. (B) Western blot analysis of Dicer following transfection of AS for *let-7a*, *c*, *d*, *miR-99a* and *miR-125b*, as well as the negative control (NC) in PC10 cells. Northern blot analysis for mature *let-7a* was performed under the same conditions. (C and D) Northern blot analysis of *Dicer* using the same cell extracts used in (A) and (B), respectively. (E) Schematic illustration showing locations of the putative *let-7d* target site and luciferase insert (Luc. insert) in the 3' UTR of *Dicer*. The sequence alignment of mature *let-7a/let-7d*, its target site and mutated nucleotides (lower case) are also shown. (F) Relative luciferase activity was measured using PC10 cells and showed marked reduction following insertion of the 3' UTR of wild-type (wt) *Dicer*, but not with insertion of that of mutant (mut) *Dicer*. That reduction was significantly recovered following treatment with *let-7a* AS, but not with negative control. ORF, open reading frame; VC, vector control.

was significantly lower than that with the vector control or the mutant *Dicer* 3' UTR construct (Figure 3F). Transfection of *let-7a* AS abrogated this suppression in the experiment with the wild-type *Dicer* 3' UTR construct, however, it did not have an effect on luciferase

activity with the mutant *Dicer* 3' UTR construct (Figure 3F). These results show that *let-7* directly interacts with the *Dicer* 3' UTR and inhibits *Dicer* expression, though it remains possible that *Dicer* expression is also regulated by other miRNAs. We also noted two exceptional cell lines, A549 and QG90, with low and high levels of expression of *Dicer* and *let-7a*, respectively (Figure 1A), did not harbor mutations at the predicted *let-7*-binding site in the 3' UTR of *Dicer* mRNA (data not shown).

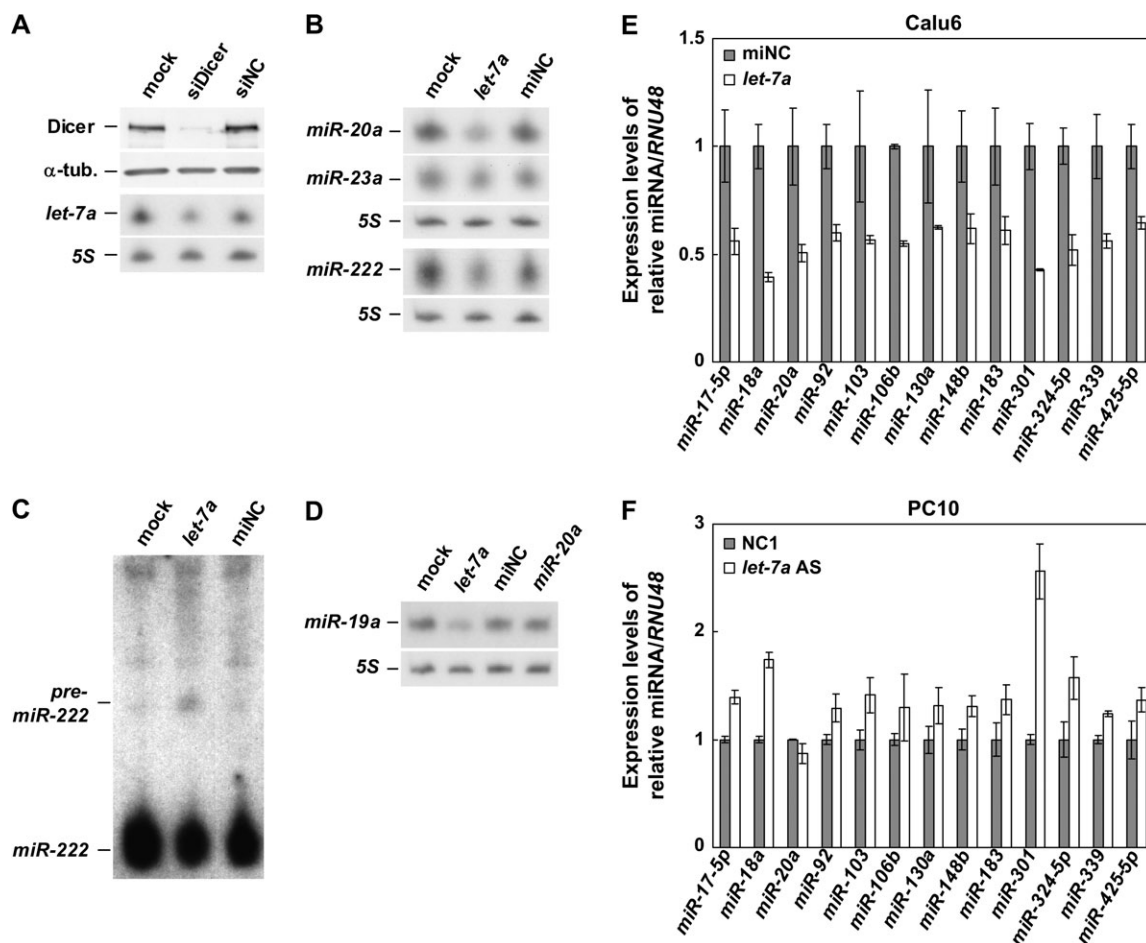
#### *let-7* affects the expression of mature miRNAs through regulation of *Dicer*

We consistently observed a reduction of mature *let-7a* expression in Calu6 cells treated with siRNA against *Dicer* (Figure 4A), which confirms the role of *Dicer* in conversion of pre-miRNAs to mature forms (4). Altogether, *Dicer* expression was found to be regulated by *let-7*, while *Dicer* also regulated the maturation of *let-7*, suggesting the possible existence of a feedback loop, in which *let-7* has an effect on its own expression level via regulation of *Dicer*. Our present finding of *let-7*-mediated regulation of *Dicer* also suggests that *let-7* may affect the expression levels of other miRNAs via *Dicer*. To investigate this issue, we introduced *let-7a* into Calu6 cells and measured the expression levels of mature miRNAs of *miR-20a*, *miR-23a* and *miR-222*, which are expressed at relatively high levels in this cell line (8). All three mature miRNAs were decreased by *let-7a*, though the extent of those decreases varied considerably (Figure 4B). In addition, we

also noted an accumulation of the precursor forms of *miR-222* in the cells (Figure 4C).

To exclude the possibility that competition took place between overexpressed *let-7a* and the measured miRNAs for miRNA-processing factors, we induced overexpression of an unrelated miRNA, *miR-20a*, and measured the expression level of mature *miR-19a*, which is also highly expressed in Calu6 cells (8). The result showed that *miR-19a* expression was not affected by *miR-20a* under the conditions in which it was decreased by *let-7a*, indicating that the miRNA expression changes were not induced by competition, but rather were the outcome of a *let-7* function (Figure 4D). Furthermore, TaqMan reverse transcription-PCR analysis revealed that overexpression of *let-7a* in Calu6 cells also resulted in reduced expression of various other mature miRNAs (Figure 4E), whereas transfection of *let-7a* AS into PC10 cells led to a considerable increase in miRNA expression, with the pattern largely reciprocal to that of the overexpression of *let-7a* (Figure 4F). Thus, the expression levels of a large number of mature miRNAs, if not all, appear to be regulated by *let-7* via *Dicer*.

It was reported previously that both human and mouse cancers exhibited global reductions of mature miRNA levels when compared with those in normal tissues (22). Also, a recent work by Kumar et al. (23) demonstrated that *Dicer* knockdown resulted in reduced mature miRNA expression and promoted tumorigenesis. These findings appear to be consistent with our previous observation of a correlation between reduced *Dicer* expression and poor prognosis in a subset of



**Fig. 4.** *let-7* affects the expressions of mature miRNAs through regulation of *Dicer*. (A) Results of western and northern blot analyses for detection of *Dicer* and mature *let-7a*, respectively, in Calu6 cells following transfection of siRNA for *Dicer* (siDicer) as well as a negative control (siNC). (B) Northern blot analysis of mature *miR-20a*, *miR-23a* and *miR-222* expressions in Calu6 cells in the presence of *let-7a* precursor molecule or negative control (miNC). (C) Northern blot analysis of long exposure to *miR-222* shown in (B). (D) Northern blot analysis of mature *miR-19a* expressions in Calu6 cells in the presence of *let-7a*, miNC or *miR-20a* precursor molecule. (E and F) Quantitative real-time reverse transcription-PCR analysis of Calu6 cells grown in the presence of miNC or *let-7a* precursor molecules and of PC10 cells grown in the presence of NC1 or *let-7a* AS. *RNU48* was used for normalization.

non-small cell lung cancers (24). It is also interesting that the presence of altered regulation of the miRNA processing machinery has been suggested in human cancers, based on the finding that global repression of miRNAs in human cancers did not coincide with reductions in the pri-miRNA transcripts (14).

*let-7* is now well recognized as a negative regulator of the Ras family (18) and HMGA2 oncogenes (19), as well as of other numerous cell proliferation-related genes (25). In our study, we found that *let-7* directly downregulates Dicer through interaction with the 3' UTR of Dicer mRNA, while augmentation of *let7* expression also affected the expression levels of other miRNAs. These results indicate the involvement of *let-7* in the regulation of Dicer, suggesting the existence of a finely tuned regulatory loop (supplementary Figure 4 is available at *Carcinogenesis* Online). Notably, target prediction programs strongly suggest that Dicer is also regulated by multiple miRNAs. It is therefore possible that those miRNAs may also affect the regulation of Dicer expression together with *let-7* in a coordinated manner, dependent on the efficacy of miRNA-mediated Dicer repression and/or efficiency of Dicer-mediated maturation of each miRNA.

In this study, we showed that *let-7* and Dicer regulate each other in a negative feedback manner, which appeared to occur in both normal and cancer cell lines. Interestingly, it was recently reported that *let-7a* downregulates *myc*, whereas *myc* in turn represses widespread miRNA expressions (26,27). Given the complexity of the regulatory pathways involving *let-7*, Dicer, *myc* and various other miRNAs, equilibration of these proteins and miRNAs may be set at the most favorable state for cell growth in each cancer. Additional investigations are warranted to elucidate the roles of this negative feedback loop comprised of two major cancer-associated and miRNA-associated molecules, *let-7* and Dicer, in growth phases and cellular quiescence, as well as in regard to clinical consequence.

## Supplementary material

Supplementary Figures 1–4 and Tables 1–3 can be found at <http://carcin.oxfordjournals.org/>

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