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

Shogo Tokumaru1,2, Motoshi Suzuki1, Hideki Yamada1,2, Masato Nagino3 and Takashi Takahashii,iv

1Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer and 2Department 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. Downregulation 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.

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, HaCaT (human epidermal keratinocyte cell line), Hs766T (human pancreatic cancer cells) and TIG-112 (normal human skin fibroblast cell) 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), 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% polyacrylamide gels as described previously (7). The oligonucleotide probes used are shown in supplementary Table 1 (available at Carcinogenesis Online). To quantify Dicer miRNA, total RNA samples were prepared using an RNeasy Mini Kit (QUIAGEN, 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 quantitative 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 log2 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 × 104 cells in 10 cm dishes on day 0 and harvesting everyday. TIG-112 cells were plated at a density of 5 × 105 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

Abbreviations: AS, antisense oligonucleotides; miRNA, microRNA; mRNA, messenger RNA; PCR, polymerase chain reaction; siRNA, small interfering RNA; UTR, untranslated region.
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 × 10^5 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 × 10^5 per well in 12-well plates on day 5, and then 0.9 μg of pGL3 plasmid and 0.1 μg of pKLT-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 miRNAeasy 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™-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 (ΔC_T). The average ΔΔC_T values were calculated by normalization to the ΔC_T value for negative control.

A TaqMan PCR assay was also performed to quantify Dicer mRNA expression using commercial, commercially available FAM™-labeled probes for Dicer and VIC™-labeled probes for 18S, according to the manufacturer’s instructions. C_T values for Dicer were normalized to those of 18S (ΔC_T). The average ΔΔC_T values were then calculated after normalization to the ΔC_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 let-7 targets in cell lines, we noticed that Dicer, an RNase III enzyme that processes pre-miRNAs, was predicted to be 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 1A1), 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 level in cell lines, including HPL1D, BEAS2B, NCI-H358, NCI-H1650, BxPC-3 and HS667T. 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.

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-12, which induced quiescence. Under the G0 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
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 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.

Fig. 2. Expression levels of endogenous Dicer and mature let-7a during cell growth. (A) Phase contrast images (x50). (B) Growth curve. (C) Western blot analysis of Dicer and northern blot analysis of mature let-7a.

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.
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
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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let-7 and Dicer constitute negative feedback loop

2077