The potential functions of primary microRNAs in target recognition and repression

Robin Deis Trujillo^{1,2,3}, Si-Biao Yue^{1,2,3},
Yujie Tang^{1,2}, William E O’Gorman^{1,2}
and Chang-Zheng Chen^{1,2,*}

¹Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA and ²Baxter Laboratory for Stem Cell Biology, Stanford University School of Medicine, Stanford, CA, USA

Major RNA products of a microRNA (miRNA) gene—the long primary transcript (pri-miRNA), the ~70-nucleotide (nt) precursor miRNA (pre-miRNA), and the ~21-nt mature miRNA—all contain the same sequence required for target gene recognition. Thus, it is intrinsically difficult to discern the contribution of individual RNA species or to rule out a function of miRNA precursor species in target repression. Here, we describe a novel approach to dissect the functional contribution of pri-miRNA without compromising important cellular pathways. We show that pri-let-7 has a direct function in target repression in the absence of properly processed mature let-7. Moreover, we show that loop nucleotides provide regulatory controls of the activity of pri-let-7 by modulating interactions between pri-let-7 and target RNAs *in vitro* and *in vivo*. Finally, we show that human let-7a-3 pri-miRNA can directly interact with target mRNAs. These findings illustrate that the regulatory information encoded in structured pri-miRNAs may be translated into function through direct interactions with target mRNAs.

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Introduction

MicroRNAs (miRNAs) are an abundant class of ~22-nucleotide (nt) RNAs that control gene expression at the post-transcriptional level. At least three RNA species, primary miRNA (pri-miRNA), precursor miRNA (pre-miRNA), and mature miRNA, are made from miRNA genes through transcription and sequential endonucleolytic maturation steps (Kim, 2005). Mature miRNAs are loaded into RNA-induced silencing complexes (RISC) and guide the RISC to cognate target mRNAs that are then degraded or translationally repressed. All other RNAs produced from miRNA genes (pri-miRNAs and pre-miRNAs) have been considered to be

transitory intermediates with no direct function in target gene regulation. However, the functions of mature miRNA precursor species have not been directly assessed because of the lack of assays that would allow interrogation of pri-miRNA or pre-miRNA function in the absence of functional mature miRNAs. Although miRNA regulation appears to be abolished in cells that do not express DGCR8 or Dicer, enzymes critical for miRNA biogenesis, it is not possible to attribute the loss of miRNA-mediated regulation to just one specific miRNA species in these cells, as loss of either of these proteins has global effects on processing of all miRNAs and other critical cellular processes.

Intriguingly, regulatory information that controls the activity of miRNA genes was found to be present outside of the mature miRNA region, suggesting a possible function of pri- and/or pre-miRNAs in target regulation (Liu *et al*, 2008). In this previous study, murine miRNA genes *mir-181a-1* and *mir-181c*, which encode mature miRNAs with a single nucleotide difference in the centre, were shown to have distinct activities in early T cell development. Further, the observed differences are largely determined by their unique pri- and/or pre-miRNA loops and seem to be independent of mature miRNA biogenesis. These findings are intriguing as these mature miRNAs should be functionally interchangeable according to computational and biochemical analyses (Doench and Sharp, 2004; Rajewsky, 2006). One of the scenarios postulated by Liu *et al* (2008) is that pri- and/or pre-miRNA species have a direct function in target recognition and repression with their loops acting as a functional motif either together with or independently of mature miRNAs. This alternative model is plausible as biochemical analyses have not ruled out a function for pri- and pre-miRNAs in target gene repression (Hutvagner and Zamore, 2002; Doench *et al*, 2003). Phenotypes observed for loss of miRNA genes in *Caenorhabditis elegans*, such as *lin-4* and *let-7*, were rescued with genomic fragments encoding their pri-miRNAs (or fragments of pri-miRNAs); as pri-, pre-, and mature miRNA species were produced from these fragment, these experiments do not rule out the function of precursor miRNAs (Lee *et al*, 1993; Reinhart *et al*, 2000).

Further supporting the notion that pri- and/or pre-miRNAs may have target repression functions, many biological processes are controlled by RNA interactions that are specifically mediated by stem-loop motifs. Examples of this type of regulation include tRNA anticodon interactions (Eisinger and Gross, 1974), natural antisense RNA control of plasmid copy number in bacteria (Eguchi *et al*, 1991), and dimerization of retroviral genomic RNA (Paillart *et al*, 1994). Many bacterial antisense RNAs contain stem-loop secondary structures that can interact with complementary linear or stem-loop target RNAs and these secondary structural elements have a crucial function in determining the specificity of the interaction (Wagner and Simons, 1994). The diverse functions of antisense RNA control in bacteria illustrate an ancient mechanism through which the regulatory

*Corresponding author. Department of Microbiology and Immunology, Stanford University School of Medicine, 269 Campus Drive, Stanford, CA 94305, USA. Tel.: +1 650 736 4014; Fax: +1 650 723 2383; E-mail: czchen@stanford.edu

³These authors contributed equally to this work

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information encoded in structured antisense RNAs can be directly translated into function through interactions with their regulatory targets. The findings that closely related murine miRNA genes *mir-181a-1* and *mir-181c* have distinct effects in early T cell development that are controlled by their loop nucleotides suggest a possible parallel between gene regulation by antisense RNAs and pri- and/or pre-miRNAs.

To understand the mechanisms of miRNA gene action, it is imperative to determine whether mature miRNA precursor species have direct functions in target gene recognition and repression. In this study, we describe a cell culture-based reporter assay in which defective processing of *cel-let-7* RNAs in human cells results in truncated pre- and mature let-7, thus enabling us to examine the activity of pri-let-7 in target repression in the absence of functional mature miRNAs, which led to the discovery of potential functions of pri-miRNAs in target recognition and repression.

Results

Aberrant biogenesis of *C. elegans* pre- and mature let-7 in human cells

To dissect the mechanisms of action by miRNA genes, we first recapitulated *cel-let-7*-mediated target repression through the *lin-41* 3' UTR in mammalian cell culture. A broad survey of the expression of let-7 miRNAs in a number of mouse and human cell lines by miRNA quantitative PCR (qPCR) analyses revealed that human BOSC 23 cells express low levels of let-7a and let-7b and negligible amounts of other let-7 family miRNAs (data not shown), providing a suitable platform for analysing the activity of let-7 genes. Interestingly, as indicated by northern blot analyses (Figure 1A; Supplementary Figure S1A), ectopic expression of *cel-let-7* in BOSC 23 cells resulted in two mature miRNA species: a predominant 20-nt mature let-7 species and a minor 22-nt species that is expressed at a level slightly higher than that of the 22-nt endogenous human mature let-7.

Knowing that mature miRNAs may have 5' or 3' polymorphism, we carried out primer extension analyses (Figure 1B) to determine the levels of mature let-7 RNAs with distinct 5' ends (Reinhart *et al*, 2000; Ruby *et al*, 2006). Analyses of both total RNA (Supplementary Figure S1B and C) and gel-purified 15- to 30-nt small RNA fractions (Figure 1C; Supplementary Figure S1D) showed that *cel-let-7* produces a truncated form of mature let-7 with two nucleotides deleted at the 5' end (the 20-nt primer extension product). No mature let-7 with the correct 5' end (a 22-nt primer extension product) was observed. This defective mature let-7 biogenesis is likely the result of erroneous processing of pri-let-7 by the microprocessor complex as primer extension analyses of the pre-miRNA fractions revealed that pre-let-7 made from *cel-let-7* also lacks the first two 5' nucleotides (Figure 1D; Supplementary Figure S1E). Thus, mature let-7 RNAs produced from *cel-let-7*—both the 20-nt and 22-nt species seen in the northern blot—are two nucleotides shorter at the 5' end than endogenous human and *C. elegans* let-7 (Reinhart *et al*, 2000; Ruby *et al*, 2006) and are missing the first canonical seed (denoted 'SD1' here) nucleotide that is thought to be essential for the activity of mature miRNAs.

Pri-let-7 may have a direct function in target repression

We then measured the activity of *cel-let-7* in target gene repression using a luciferase reporter assay. We first

generated *Renilla* luciferase reporters bearing one or three copies of a 102-nt segment of the *lin-41* 3' UTR containing two let-7 complementary sites (LCS) in the 3' UTR (1 × *lin-41_LCS* and 3 × *lin-41_LCS*, respectively). This gene segment was genetically validated as the minimal region necessary for *cel-let-7* regulation (Vella *et al*, 2004). Mutant reporters abrogating LCS pairings to the let-7 seed nucleotides (1 × *lin-41_LCS-sm* and 3 × *lin-41_LCS-sm*) were used as normalization controls to determine seed-specific repression (Figure 1E; Supplementary Figure S2A). The *lin-41_LCS-sm* reporters were repressed by *cel-let-7* at the same level as the reporters with no *lin-41_LCS* in the UTRs (Supplementary Figure S2B), indicating that mutations disrupting seed binding completely eliminated target repression mediated by the *lin-41_LCS* in the reporter UTR. Throughout this study, seed-dependent repression activities of various miRNA constructs were measured and normalized as described in Materials and methods. Expression of *cel-let-7* resulted in ~40 and 49% seed-specific repression of the 1 × *lin-41_LCS* and 3 × *lin-41_LCS* luciferase reporters, respectively (Figure 1F), showing that *cel-let-7* can specifically repress target gene expression despite the fact that it produces truncated pre- and mature let-7. The degree of repression observed in these analyses was consistent with the activity of *cel-let-7* in other heterologous reporter systems (Lewis *et al*, 2003) and in large-scale proteomic analyses of miRNA-mediated repression (Baek *et al*, 2008; Selbach *et al*, 2008).

These findings raised the question of whether truncated mature let-7 repressed expression of target mRNA despite lacking the first seed nucleotide. To address this question, we generated *cel-let-7* mutants, *let-7_2A* and *let-7_2C* (Figure 1E; Supplementary Figure S2A), by altering the SD1 nucleotide. The SD1 mutations had no significant effects on mature miRNA biogenesis as indicated by northern blot and primer extension analyses (Figure 1A–D). Both pre- and mature let-7 RNAs made from these mutants were expressed at comparable levels and had the same 5' ends as those made from the wild-type *cel-let-7* (*cel-let-7_wt*). However, the SD1 mutations completely abolished seed-dependent repression of both 1 × *lin-41_LCS* and 3 × *lin-41_LCS* reporters (Figure 1F), showing that the SD1 nucleotide was absolutely required for *cel-let-7*-dependent target repression. Furthermore, mutations in the *lin-41_LCS* that abolished base pairing to the SD1 nucleotide, *lin-41_LCS-2U* (Figure 1G) and *lin-41_LCS-2G* (Supplementary Figure S2C and D), also abrogated the activity of *cel-let-7_wt*. This result suggested that the SD1-nucleotide, which is predicted to pair with the target mRNA, is required for the seed-dependent target suppression. Finally, a compensatory SD1 mutant, *let-7_2A*, partly restored repression of the *lin-41_LCS-2U* reporter (Figure 1G), showing that the SD1 nucleotide has a direct function in target interaction and repression. Intriguingly, the *let-7_2C* mutant did not restore the repression of its cognate mutant target *lin-41_LCS-2G* (Supplementary Figure S2C and D), suggesting that factors beyond the simple base pairing also contribute to the function of the SD1 nucleotide in target repression. These results eliminated the possibility that the truncated pre- and mature let-7 miRNAs, which lack the SD1 nucleotide as shown by primer extension, function in target repression.

The above results show that *cel-let-7* expression results in seed-dependent target repression even though all detectable pre- and mature let-7 miRNAs produced from *cel-let-7* lack the SD1 nucleotide as shown by primer extension. Further, we

Figure 1 The first seed (SD1) nucleotide is missing from the pre- and mature let-7 miRNAs, but is essential for the activity of *C. elegans* let-7 gene (*cel-let-7*) in target repression in BOSC 23 cells. (A) Northern blot showing the expression of *cel-let-7* and SD1 mutant genes. Bands representing endogenous human pre-let-7 miRNAs (hsa-pre) and *C. elegans* pre-let-7 (cel-pre) are indicated. (B) Primer extension with synthetic full-length 22-nt and truncated 20-nt mature let-7 missing the 5' two nucleotides with or without spiked total RNA from BOSC 23 cells. (C, D) Mapping the 5' ends of mature let-7 (C) and of pre-let-7 (D) by primer extension analyses. Bands representing primer extension products derived from let-7 RNAs (22- and 20-nt bands) and spiked miR-181 (26-nt band) are indicated. Representative blots from three to four independent repeats are shown in panels A, C and D. Note that in panel D the products of LNA primers migrate at a rate ~1-nt slower than corresponding DNA primers, such that the DNA ladder of 17-nt corresponds to the unreacted 16-nt LNA primer. (E) Schematic diagrams indicate base-pairings between the mature let-7 and the two let-7 complementary sites (designated as T1 and T2) within the 102-nt fragment of the *lin-41* 3' UTR. Altered (blue) and missing (underlined) nucleotides are indicated. (F, G) Repression of wild-type (F) or mutant (G) *lin-41_LCS* *Renilla* luciferase reporters by *cel-let-7* and SD1 mutants. Reporter activity was normalized to show seed-dependent repression. Representative results of at least six independent trials ($\pm$ s.d.) are shown (* P <0.0001).

found that transfection of the 2.5-kb *C. elegans* let-7 DNA fragment used to rescue the let-7 null phenotype in worms (Reinhart *et al*, 2000) or the 600-bp fragment used in our

expression vectors without a mammalian promoter region cause no repression of the *lin-41_LCS* reporter (data not shown). Thus, *cel-let-7* activity was mediated through its

RNA products, but not the DNA. Interestingly, at least three RNA species were detected upon *cel-let-7* expression in BOSC 23 cells (Figure 1A–D, Supplementary Figure S1F): truncated pre-let-7 and mature let-7 miRNAs, which did not contain the functionally critical SD1 nucleotide, and the pri-let-7 RNA, which did contain the SD1 nucleotide. Although we cannot rule out the possibility that undetectable amounts of full-length 22-nt mature let-7 are responsible for the activity of *cel-let-7*, a more reasonable hypothesis is that pri-let-7 has a direct function in target repression.

Pri-let-7-loop nucleotides control the activity of *cel-let-7* in target repression

The idea that pri-let-7 may have a direct function in target recognition was further tested by altering the loop nucleotides that are outside the mature miRNA region. Alterations of pri-let-7-loop nucleotides could affect the functional pri-let-7 RNA independent of truncated and non-functional pre- and mature let-7 RNAs that lack the SD1 nucleotide. Drawing analogies from the previous examples of loop functions in structured RNAs and bacterial non-coding RNAs (Eguchi *et al*, 1991; Liu *et al*, 2008), we examined the function of pri-let-7-loop nucleotides in controlling the activity of *cel-let-7* in target gene repression. As both pri-miR-181a-1 and *C. elegans* pri-let-7 contain a GG dinucleotide at the 5' end of their loops and mutation of these nucleotides in *mir-181a-1* impairs its function (Liu *et al*, 2008), we first generated a *cel-let-7* mutant (*let-7_LPA*) with these nucleotides of the pri-let-7 loop mutated (Figure 2A). Strikingly, expression of *let-7_LPA* resulted in significant reduction in seed-specific activity compared with *cel-let-7*: *let-7_LPA* expression resulted in only 15% repression of the $1 \times \text{lin-41_LCS}$, whereas *cel-let-7* resulted in 40% repression (Figure 2B).

As the GG to CC mutations alter the predicted secondary structure of pri-let-7 loop, we generated three additional pri-let-7-loop mutants, *let-7_LPB*, *let-7_LPC*, and *let-7_LPD*, to determine whether the deleterious effect on *cel-let-7* activity was caused by alterations of loop sequence or structure (Figure 2A). The *let-7_LPB* mutant maintained the GG to CC change from *let-7_LPA*, but contained compensatory mutations to restore the wild-type-loop structure. Interestingly, these changes resulted in a slight increase of repression activity compared with that of *cel-let-7_wt* (Figure 2B), showing that structure rather than nucleotide sequence was important. Further supporting the importance of the pri-let-7-loop structure, the *let-7_LPC* mutant, which has the same predicted loop structure as *let-7_LPA* with the wild-type 5' GG dinucleotide, did not inhibit expression from either reporter (Figure 2B). Finally, the *let-7_LPD* mutant, which was altered to remove all intra-loop base pairing, resulted in an intermediate phenotype (Figure 2B). These findings show that the activity of *cel-let-7* in target gene repression can be positively and negatively influenced by alterations in the pri-let-7-loop nucleotides, further suggesting that the activity of this miRNA gene may be controlled by the secondary structure and/or thermodynamic constraints of terminal loop of the pri-let-7 RNA.

The function of pri-let-7 loop in target repression is independent of mature miRNA biogenesis

Previously, we showed that pri/pre-miRNA loops can control the activity of *mir-181a-1* and *mir-181c* in early T cell devel-

opment independent of mature miRNA biogenesis (Liu *et al*, 2008). Intriguingly, we found that mutations in the pri-let-7-loop region resulted widely varying amounts of mature 20- and 22-nt let-7 and pre-let-7 (Figures 2C and D); however, the changes in mature miRNA expression levels and sizes caused by the loop mutations have no consistent correlation with the activity of these mutant genes in target repression (Figure 2C and D; Supplementary Table S1 for Pearson's correlation analyses). Furthermore, primer extension analyses of total RNA (Supplementary Figure S3A), mature miRNA (Figure 2E), and pre-miRNA (Figure 2F) fractions show that, as seen for the SD1 mutants (Figure 1), both pre- and mature let-7 made from these loop mutants lack the first two 5' nucleotides, including the necessary SD1 nucleotide. Relative to *cel-let-7_wt*, pri-let-7-loop mutations caused a significant decrease in the levels of pre- and mature let-7 with the truncated 5' ends (20-nt primer extension product), but none caused significant changes in the levels of pre- and mature let-7 with the correct 5' ends (Supplementary Figure S3B and C) or the levels of pri-let-7 RNA (Supplementary Figure S3D).

These analyses clearly showed that there is no apparent correlation between the levels of mature let-7 expressed and activities of *cel-let-7*-loop mutants as measured by northern blot or primer extension. Thus, the effects of *cel-let-7*-loop mutations on target regulation are independent of both mature and pre-let-7 biogenesis. Although we can neither prove nor rule out the possibility that loop mutations may control *cel-let-7* function by modulating the biogenesis and activity of undetectable amounts of full-length 22-nt mature let-7, these results further raised the possibility that pri-let-7 miRNA has a direct function in target repression and that such activity can be controlled by the structure and/or sequences of pri-miRNA loops.

The SD1 and loop mutations have minimal effects on the repression of perfect targets

The above analyses raised the question of whether aberrantly processed mature miRNAs are incorporated into the RISCs and are functional in target repression. To this end, we examined whether the SD1 and pri-let-7-loop mutations affect the repression of luciferase reporters bearing perfect let-7 complementary sites to the 22-nt mature let-7 (lin-41_T1T2P) in the 3' UTR (Figure 3A). Intriguingly, the SD1 and pri-let-7-loop mutants, which abolished the repression of the lin-41_LCS-wt reporters (Figures 1 and 2), repressed lin-41_T1T2P reporters with the perfect target sites (Figure 3B and C). Furthermore, when we transfected siRNA duplexes of synthetic full-length (22-nt) mature let-7 and 5' truncated (20-nt) mature let-7, we saw that only full-length let-7 effectively repressed the expression of wild-type $1 \times \text{lin-41_LCS}$ luciferase reporters (Figure 3D and E). In contrast, both the 22- and 20-nt synthetic let-7 mimics effectively repressed the lin-41_T1T2P reporters (Figure 3D and F). Thus, the SD1 nucleotide is not required for silencing the luciferase reporter bearing perfect let-7 complementary sites in its UTR by either synthetic let-7 mimics or small RNAs produced from *cel-let-7*. Moreover, increased degree of pairings between targets and mature miRNAs can largely abrogate the function of the SD1 and pri-let-7-loop nucleotides in target repression. Together, these results suggest that truncated 20-nt mature let-7 produced from *cel-let-7* may function as siRNAs in silencing

Figure 2 Effects of pri-let-7-loop mutations on the activity of *cel-let-7* and pre- and mature let-7 biogenesis. (A) Schematic diagrams depict the pre-miRNA sequences and structures of *cel-let-7_wt* and pri-let-7-loop mutants. (B) Repression of luciferase reporter expression by *cel-let-7_wt* and loop mutants. Representative results of at least six independent trials ($\pm$ s.d.) are shown ($P < 0.0001$ compared with vector control except as indicated). (C) Northern blot analysis shows the RNAs produced from *cel-let-7_wt* and pri-let-7-loop mutants ($n = 5$). (D) Relative levels of 22-, 20-nt, and total small RNA (22 + 20 nt) mature let-7 in BOSC 23 cells transfected with the wild-type *cel-let-7* or loop mutants ($n = 5$, $\pm$ s.d.). (E, F) Mapping the 5' ends of mature let-7 (E) and pre-let-7 (F) miRNAs made from the *cel-let-7_wt* and loop mutants by primer extension analyses ($n = 3$ and 5, respectively).

expression of perfectly matched targets, but cannot function as miRNAs in the regulation of imperfectly paired targets, indicating that truncated 20-nt mature let-7 may be incorporated into RISC despite the erroneous processing at the 5' end. However, other interpretations include the possibility that loss of 5' end nucleotides may be compensated by base pairings in other regions of mature miRNA or that precursor let-7 RNAs produced from *cel-let-7_wt* may have functions that are enhanced by increased target complementarity.

Loop nucleotides control target and pri/pre-let-7 complex formation in vitro

The finding that the activity of pri-let-7 in target regulation was affected by the pri-miRNA-loop nucleotides suggests a model that is reminiscent of the mechanism by which antisense RNAs control plasmid copy numbers in bacteria through the formation of transient kissing-loop complexes (Eguchi *et al*, 1991). To investigate this possibility, we developed a surrogate *in vitro* assay to examine the effects of loop mutations on the interaction

Figure 3 Effects of SD1 and pri-let-7-loop mutants on the repression of a perfectly complementary let-7 target reporter. (A) Schematic diagrams showing the predicted base pairings between let-7 and a modified lin-41_LCS with perfectly matched let-7 target sites (lin-41_T1T2P). Altered (blue) and missing (underlined) nucleotides are indicated. (B, C) Repression of the lin-41_T1T2P *Renilla* luciferase reporter by *cel-let-7* SD1 mutants (B) or by *cel-let-7*-loop mutants (C). The results in (B) and (C) are representative of at least six independent trials ($\pm$ s.d., $P < 0.0001$ compared with vector control except as indicated). (D) Schematics of the full-length (22-nt) or truncated (20-nt) synthetic let-7 siRNA duplexes. (E, F) Repression of the lin-41_LCS reporter (E) and the lin-41_T1T2P *Renilla* luciferase reporter (F) by the full-length (22 nt) or truncated (20 nt) synthetic let-7 siRNA duplexes ($n = 3$, $\pm$ s.d.).

of miRNA precursors with target RNA. We were able to identify conditions in which *in vitro* transcribed pre-let-7 RNA (syn-pre-let-7) and lin-41_LCS-wt RNA (Figure 1B) formed stable complexes in electrophoretic mobility shift assays (EMSA). Syn-pre-let-7 contains 15 nucleotides not found at the 5' end of pre-let-7; these appear to stabilize the complexes during gel electrophoresis, but are not predicted to affect the hairpin fold according to *mfold*. A constant amount of radioactively labelled lin-41_LCS RNA was mixed with unlabelled syn-pre-let-7 RNA at various

ratios and incubated at 37°C for 45 min, then resolved on 6% non-denaturing PAGE gels. The complexes appear as bands with reduced electrophoretic mobility compared with syn-pre-let-7 alone or lin-41_LCS-wt alone (Figure 4A). Complex formation was not observed with lin-41_LCS RNA containing mutations that abrogate base pairing to the let-7 seed nucleotides (Figure 4B; Supplementary Figure S4A and B), showing that seed nucleotides are essential for complex formation *in vitro*, just as they are necessary for target repression *in vivo*. Each

Figure 4 Loop mutations modulate complex formation between lin-41_LCS and let-7 precursor RNAs *in vitro*. (A, B) Electrophoretic mobility shift assay (EMSA) was used to determine the complex formation between a synthetic precursor let-7 RNA (syn-pre-let-7) and the lin-41_LCS-wt RNA (A) or the lin-41_LCS-sm RNA (B). Major complexes 1 (C1) and 2 (C2) are indicated. (C) Dose-dependent binding of the syn-pre-let-7 RNAs with the lin-41_LCS-wt or lin-41_LCS-sm RNAs ($n \geq 3$). (D) Dose-dependent binding of the lin-41_LCS-wt and syn-pre-let-7 wt (grey, dotted lines) or syn-pre-let-7-loop mutants (black, solid lines) ($n \geq 2$). See Supplementary Figure S6 for EMSA images. Concentrations of syn-pre-let-7 RNAs required to sequester 40% of the lin-41_LCS-wt RNA (the half-maximal binding concentration for the syn-pre-let-7 wt) are indicated with dotted red lines. (E) The correlation between the activity of *cel-let-7* mutants in target repression and complex formation between syn-pre-let-7 and lin-41_LCS RNAs *in vitro* was determined by Pearson's correlation analyses.

band contains a defined ratio of syn-pre-let-7 to lin-41_LCS RNAs (Supplementary Figure S5), and similar shifts in band intensity were also observed when a constant amount of radioactively labelled syn-pre-let-7 RNA was paired with unlabelled lin-41_LCS RNA (Supplementary Figure S4C and D), further indicating that the shifted bands are not random aggregates. A plot of the fraction of lin-41_LCS bound against the concentration of syn-pre-let-7 (Figure 4C) indicates that syn-pre-let-7 interacts with lin-41_LCS-wt with half-maximal binding (~40% of the lin-41_LCS-wt bound by syn-pre-let-7) at approximately equimolar concentration (1.1 μM syn-pre-let-7 to 0.97 μM lin-41_LCS).

This surrogate assay enables us to evaluate the relative physical effects of loop mutations on the potential interaction between syn-pre-let-7 and lin-41_LCS and whether such effects would correlate with the function of pri-miRNA in target repression. As indicated by EMSA analyses, both the

fraction of lin-41_LCS RNA shifted and types of complexes formed were affected by loop mutations (Figure 4D; Supplementary Figure S6). Compared with syn-pre-let-7 wt, which bound 40% of the lin-41_LCS at an equimolar ratio, molar ratios of ~14, 2.6, 33.6, and 4.3 of mutant syn-pre-let-7 RNA to target were required for binding to the same fraction of target RNA by the *let-7_LPA*, *let-7_LPB*, *let-7_LPC*, and *let-7_LPD*-loop mutants, respectively. The dramatic decreases of the *in vitro* binding activity of mutants were correlated with the reduction in activity of these mutants in target repression (Figures 2 and 4D, E, Pearson's $r = -0.9246$, two-tailed $P = 0.0246$). These results show that the loop nucleotides in syn-pre-let-7 loops control the interaction with target mRNA, suggesting that the activity of pri-let-7 and loop mutants in target repression may be modulated by the ability of the structured syn-pre-miRNA to physically interact with target RNAs.

Figure 5 Specific complex formation between lin-41_LCS and pri-let-7 RNAs *in vivo*. (A) Schematic diagram depicting the selective pull down of S1 aptamer-tagged lin-41_LCS reporter mRNAs and its associated pri-let-7 RNA. S1-tagged reporter mRNAs either with the lin-41LCS-sm UTR or without any UTR were used as negative controls. (B) RPA shows specific enrichment of the pri-let-7 RNA by the S1-tagged lin-41_LCS reporter mRNA. Protected radiolabelled probes for pri-let-7 and *Renilla* luciferase mRNA were indicated. (C) The relative ratios between pri-let-7 and S1-tagged target RNA in the pull-down RNA samples ($n = 2$, $\pm$ s.d.) were determined by quantifying the intensity of protected pri-let-7 and target RNA bands (B). (D, E) Standard curve-based qPCR was carried out to determine the average copy numbers of pri-let-7 (D) and mature let-7 (E) per target in the pull-down samples ($n = 5$, $\pm$ s.d.). (F) Target pull down from cells co-transfected with S1-tagged target and *cel-let-7* expression vectors or from equivalent mixture of cells with separately transfected S1-tagged target or *cel-let-7* expression vectors. (G) Target pull down after formaldehyde crosslinking of cells co-transfected with S1-tagged target and *cel-let-7* expression vectors. (H) Target pull down from the nuclear fractions of cells co-transfected with S1-tagged target and *cel-let-7* expression vectors. (F–H) The relative ratios of pri-let-7 and target RNAs in the pull-down RNA samples were determined by qPCR ($n = 2$, $\pm$ s.d.).

Pri-let-7 RNA forms complexes with target mRNAs *in vivo*

To test whether pri-let-7 RNAs can form complexes with target mRNA in cells, we tagged the *Renilla* reporters with

the S1 aptamer (Vasudevan and Steitz, 2007); this structured RNA motif binds to streptavidin, enabling selective enrichment of tagged RNAs (Figure 5A). Reporter constructs with the wild-type lin-41_LCS UTR, seed mutant lin-41_LCS UTR,

or no UTR were tagged with the S1 aptamer. Tagged reporters were co-transfected with *cel-let-7* expression vectors into BOSC 23 cells under the same conditions that were used for luciferase assays. Introducing the S1 aptamer into these *Renilla* reporter constructs did not alter their potential to be repressed by *cel-let-7* (data not shown). S1-tagged reporter mRNAs were specifically recovered using streptavidin beads. Over 50% of tagged reporter mRNAs in the cell lysates were bound onto streptavidin beads and selective elution with biotin resulted in over 250-fold enrichment of S1-tagged reporter mRNAs (Supplementary Figure S7).

We purified tagged reporter mRNAs from transfected BOSC 23 cells and then determined the levels of pri-let-7 and reporter RNAs in the purified RNA samples using an RNase protection assay (RPA) and qPCR. Protection of radiolabelled RPA probes by the *Renilla* luciferase reporters and pri-let-7 RNAs resulted in 163- and 88-nt bands, respectively (Figure 5B). Significantly, pri-let-7 RNA was selectively enriched when the reporter carried the lin-41_LCS-wt UTR, but not in reactions containing reporters without the UTR or with the seed mutant UTR (Figure 5B). After normalizing to the levels of reporter mRNA, relative levels of pri-let-7 RNA associated with the lin-41_LCS-wt UTR were five- and three-fold higher than that associated with the control mRNAs with no lin-41_LCS UTR or the lin-41_LCS-sm UTR, respectively (Figure 5C). Again, we noted no significant degradation of S1-tagged reporter mRNA upon *cel-let-7* expression (Figure 5B, before pull down).

There were an average of ~2.4 copies of pri-let-7 RNA bound to each reporter mRNA with the lin-41_LCS-wt UTR (Figure 5D) based on standard curve qPCR analyses (*in vitro* transcribed forms of pri-let-7 and *Renilla* reporter RNAs were used as standards). Both RPA and qPCR analyses revealed a background association of ~20% between pri-let-7 and control mRNAs without the lin-41_LCS UTR; this may reflect non-specific binding. After subtracting the binding to the no lin-41_LCS UTR reporter, both RPA and qPCR showed that pri-let-7 RNA specifically associated only with target mRNAs with the wild-type, but not seed mutant, lin-41_LCS UTR *in vivo*. Intriguingly, under the same conditions, we only detected about 0.002 copy of mature let-7 RNA per S1-tagged target mRNA in the pull-down RNA samples (Figure 5E). However, we cannot conclude that mature let-7 RNA does not bind to target mRNA based on these results. First, *cel-let-7* does not make functional full-length mature let-7 RNA and BOSC 23 cells make low levels of endogenous full-length mature let-7 RNA. Second, the endogenous mature full-length mature let-7 RNA bound to the S1-tagged reporter mRNA might be unstable and have fallen off during the purification. Finally, it is possible that any endogenous full-length mature let-7 interacting with the target would subsequently lead to target degradation, thereby precluding efficient recovery with S1-tagged targets. Nevertheless, these results show that pri-let-7 can directly interact with target reporter miRNA *in vivo*, further supporting that pri-let-7 could have a direct function in target recognition and repression.

The pri-let-7 and target RNA complexes observed (Figure 5B–E) are unlikely to be formed during the course of cell lysis. Target pull down from cells individually transfected with lin-41_LCS reporter or *cel-let-7* expression vector, but mixed after lysis resulted in levels of target and pri-miRNA complexes <~20% of that can be recovered from cells

co-transfected with the same set of vectors (Figure 5F), which is equivalent to the background binding (Figure 5C). Furthermore, such complexes can also be specifically enriched from cells co-transfected with the lin-41_LCS-wt reporter and *cel-let-7* expression vector and formaldehyde fixed (Figure 5G), which should crosslink target and pri-miRNA complexes *in vivo* before cell lysis. Finally, to examine the cellular compartment in which target and pri-miRNA complexes form, we carried out the same target pull-down analyses using purified nuclear fractions from cells co-transfected with lin-41_LCS reporter and *cel-let-7* expression vectors. The efficiency and purity of nuclear and cytoplasmic fractionation conditions were confirmed using HY3 (a predominantly cytoplasmic mRNA) and snoRNA (a predominantly nuclear RNA) as nuclear and cytoplasmic indicators, respectively. We found that nuclear fractions contain <10% of contaminating cytoplasmic RNAs, whereas the cytoplasmic fractions contain <4% of contaminating nuclear RNAs. Interestingly, in the nuclear fraction, reporter mRNA with the lin-41_LCS-wt UTR brought down nearly 10- or 2-fold more pri-let-7 RNA than reporters without lin-41_LCS UTR or with the seed mutant lin-41_LCS UTR, respectively (Figure 5H). Taken together, these results show that target and pri-let-7 complexes can form in the nucleus.

Loop nucleotides control target and pri-let-7 RNA complex formation *in vivo*

On the basis of the result of *in vitro* EMSA analyses (Figure 4), we then examined the effects of loop mutations on complex formation between target and pri-let-7 RNAs *in vivo*. Consistent with *in vitro* analyses, we found that loop mutations had profound effects on *in vivo* binding between pri-let-7 and lin-41_LCS RNAs (Figure 6). As indicated by RPA, the *let-7_LPA*, *let-7_LPB*, *let-7_LPC*, and *let-7_LPD*-loop mutants decreased the relative levels of pri-miRNA associated with target mRNAs to about 22, 87, 23, and 88% of the wild-type levels, respectively (Figure 6A). Similar results were obtained using qPCR analyses (Figure 6B). The dramatic decreases in *in vivo* binding between the lin-41_LCS and pri-let-7 RNAs derived from *let-7_LPB* or *let-7_LPC* mutants were again correlated with the reduction in activity of these mutants in target repression (Figures 2 and 6C, Pearson's $r=0.9669$, two-tailed $P=0.0072$). These correlations show that the activity of pri-let-7 and loop mutants in target repression may be modulated by the ability of pri-let-7 to physically interact with target mRNA. Intriguingly, we noted that expression of *cel-let-7* and loop mutants did not cause significant changes in the levels of reporter mRNA as indicated by northern blot (Figure 6D) and qPCR analyses (Figure 6E), suggesting that interaction with pri-let-7 does not result in significant degradation of targets.

Human pri-let-7a-3 can form specific complexes with target mRNAs *in vivo*

The above analyses show that pri-let-7 RNAs appear to have a direct function in recognizing and repressing the expression of target reporters in the absence of functional pre- and mature let-7. However, it is not known whether pri-miRNAs form complexes with their cognate target RNAs when miRNA biogenesis results in pre- and mature miRNAs with the correct 5' ends. To answer this question, we tested whether human pri-let-7a-3 formed specific target complexes with

Figure 6 Pri-let-7-loop mutations modulate complex formation between pri-let-7 and lin-41_LCS reporter RNAs *in vivo*. **(A)** RPA analyses show the effects of loop mutations on complex formation between S1-tagged lin-41_LCS reporter mRNA and pri-let-7-loop mutants. Protected radiolabelled probes for pri-let-7 and *Renilla* luciferase mRNA were indicated. The relative ratios between pri-let-7 and corresponding target RNAs in the pull-down samples based on the RPA analyses are shown ($n = 2$). **(B)** Normalized ratios between lin-41_LCS-wt reporter RNA and pri-let-7-loop mutant RNAs were determined by qPCR quantification of the levels of pri-let-7 and target RNAs in the pull-down RNA samples ($n = 5$, $\pm$ s.d.). **(C)** The correlation between the activity of *cel-let-7* mutants in target repression and the potential of complex formation between pri-let-7 and lin-41_LCS RNAs *in vivo* was determined by Pearson's correlation analyses. **(D, E)** The effects of wild-type and mutant *cel-let-7* expression on the levels of target mRNA was determined by northern blot **(D)** and qPCR analyses **(E)**. **(D)** Northern blots showing the levels of wild-type and seed mutant *Renilla* reporter mRNAs in BOSC 23 cells expressing wild-type and mutant *cel-let-7* constructs. Representative results of four independent analyses (blots) and average of normalized all four experiments (Bar graph) were shown. **(E)** RNA samples used in northern blot analyses were also independently quantified by qPCR to determine the relative expression of wild-type and seed mutant *Renilla* luciferase reporter mRNA in these cells. Expression of β -actin mRNA was also quantified and used as loading control ($n = 4$).

Figure 7 *In vivo* complex formation between target mRNA and human *let-7a-3* (*hsa-let-7a-3*) pri-miRNA. (A) Schematics depicting the pre-miRNA structure and nucleotide sequence of the *hsa-let-7a-3* hairpin region. (B) Seed-dependent repression of *lin-41_LCS* reporters by *hsa-let-7a-3*. Representative results of at least four independent trials ($\pm$ s.d.) are shown ($*P < 0.0001$). (C) Northern blot shows the expression and processing of *hsa-let-7a-3* ($n = 3$) in BOSC 23 cells. (D) Mapping the 5' ends of mature *let-7* made from *hsa-let-7a-3* by primer extension analyses ($n = 3$). (E) Average number of copies of pri-*let-7a-3* per target RNA in co-precipitated samples. Standard curve-based qPCR was carried out to determine the copy numbers of pri-*let-7a-3* and target *lin-41_LCS* reporter RNAs in the S1-tagged RNA pull-down fractions ($n = 3$, $\pm$ s.d.). (F) Target pull down from cells co-transfected with S1-tagged target and *hsa-let-7a-3* expression vectors or from equivalent mixture of cells with separately transfected S1-tagged target or *hsa-let-7a-3* expression vectors. The relative ratios of pri-*let-7a-3* and target RNAs in the pull-down RNA samples were determined by qPCR. (G, H) RPA analyses show specific enrichment of pri-*let-7a-3* by the S1-tagged *lin-41_LCS* reporter mRNA, but not by the S1-tagged control mRNAs ($n = 3$). (G) Protected radiolabelled probes for pri-*let-7a-3* and *Renilla* luciferase mRNA were indicated. (H) The relative ratios between pri-*let-7a-3* and corresponding target RNAs with *lin-41_LCS*-wt and control UTRs were determined by quantifying the intensity of protected pri-*let-7a-3* and target RNA bands ($n = 3$, $\pm$ s.d.).

target miRNAs *in vivo*. Human *let-7a-3* (*hsa-let-7a-3*) was selected among the human *let-7* genes because it encodes the same mature *let-7* as *cel-let-7* (Figure 7A) and it is expressed at a low level in BOSC 23 cells (data not shown). When co-transfected into BOSC 23 cells with the *lin-41_LCS*

Renilla luciferase reporter, *hsa-let-7a-3* effectively (80%) repressed the expression of the reporter (Figure 7B). Moreover, ectopic expression of *hsa-let-7a-3* resulted in properly processed mature *let-7a* as indicated by northern blot and primer extension analyses (Figure 7C and D).

We then tested whether pri-let-7a-3 RNA might specifically associate with the lin-41_LCS-wt UTR using the previously described S1-tagged reporters. We purified reporter mRNAs from BOSC 23 cells co-transfected with the S1-tagged reporter constructs and *hsa-let-7a-3* expression vectors and determined the levels of pri-let-7 and reporter RNAs in the purified RNA samples by qPCR and RPA (Figure 7E–H). qPCR analyses showed that there were on average 0.7 copies of pri-let-7a-3 RNA bound to each reporter mRNA containing the lin-41_LCS-wt UTR (Figure 7E). Target pull-down from a mixture of lysed cells with individually transfected lin-41_LCS reporter or *hsa-let-7a-3* expression vector again resulted in levels of target and pri-miRNA complexes equivalent to background, which is $< \sim 25\%$ of the complexes that can be recovered from cells co-transfected with the same set of vectors (Figure 7F). Similarly, as indicated by RPA analyses of RNA pull-down samples, relative levels of pri-let-7 RNA associated with the lin-41_LCS-wt UTR is nearly two-fold higher than that associated with the control mRNAs with no UTR or the lin-41_LCS-sm (Figure 7G and H, respectively). Intriguingly, we noted significant degradation of S1-tagged reporter mRNA upon *hsa-let-7a-3* expression (Figure 7G, before pull down). This reduced level of target mRNA, likely because of target degradation by properly processed pre- or mature miRNAs, may have compromised pull-down efficiency and contributed to the relatively higher background binding we observed. Both RPA and qPCR clearly showed that pri-let-7a-3 RNA specifically associated with target mRNAs with the lin-41_LCS-wt UTR (Figure 7E–H). As pri-, pre-, and mature let-7a-3 all contain the same mature miRNA sequence, it was not possible in these experiments to distinguish the functional contribution of the each RNA species to target repression. Nevertheless, it is clear that pri-let-7a-3 can form complexes with cognate target RNAs, suggesting that this type of interaction may contribute to target recognition and repression in the presence of pre- and mature miRNAs with the correct 5' ends.

Discussion

Here, we described a novel strategy to dissect the functional contribution of pri-miRNAs and provided extensive evidence that supports a direct function for pri-miRNAs in target recognition and repression. First, we showed that SD1 and pri-let-7-loop nts control the activity of the *cel-let-7* gene in the absence of detectable functional pre- and mature let-7 RNA, indicating that pri-let-7 RNA may be a functional species of *cel-let-7*. Second, we showed that pri-let-7 RNA forms complexes with target mRNAs *in vitro* and *in vivo* and that pri-miRNA-loop nucleotides are critical to the formation of pri-miRNA/target complexes. These results illustrate the intrinsic properties of structured pri-miRNA molecules in modulating RNA complex formation and the activity of miRNA genes. Further, we showed that the activity of loop mutants correlates well with the formation of pri-miRNA and target complexes, showing that regulatory information encoded in structured pri-miRNAs can be directly translated into functions through interactions with their targets. In additional experiments, we have also found that nucleotide sequence and the stability of base pairing within the hairpin stem—another determinant that is not present in mature miRNAs—can also provide regulatory controls for target repression by modulating the formation of pri-miRNA and

target complexes (Supplementary Results; Supplementary Figures S8 and S9). Thus, although it is not possible to rule out or show that undetectable amounts of full-length mature let-7 may have contributed to the activity of *cel-let-7*, the evidence provided here strongly favours that the pri-let-7 RNAs has a direct function in target recognition and repression and contain determinants that allow it to be more discriminating.

These results are consistent with the previous studies in which loops of structured hairpin RNAs were shown to control the distinct biological activities of *mir-181a-1* and *mir-181c* in early T cell development (Liu *et al*, 2008), suggesting that pri-miRNAs as gene regulatory molecules may not be limited to the *cel-let-7*. Indeed, we found that human pri-let-7a-3 RNA formed complexes with target mRNAs even in the presence of correctly processed pre- and mature miRNAs (Figure 7) and may also have a function in target repression. The case presented here provides exceptions to the notion that pri-miRNAs and pre-miRNAs are merely transitory intermediates during mature miRNA biogenesis, though it does not show that pri-miRNAs from every miRNA gene have critical functions.

The cell culture system we established, in which defective biogenesis of pre- and mature let-7 resulted in no detectable functional pre- and mature let-7 RNA, was critical to this study as it allowed us to interrogate the function of pri-let-7 RNA without causing major defects in critical cellular machinery and gene regulation. However, as both pre- and mature let-7 RNAs made from *cel-let-7* in BOSC 23 cells lack the critical SD1 nucleotide, this culture assay could not address the function of these species. Clearly, transfected mature miRNAs alone can effectively repress target repression as shown in Figure 3 and previous studies (Doench *et al*, 2003; Jannot *et al*, 2008). Moreover, mature let-7 made from shRNAs with perfect stems can also enter into a silencing pathway and repress target reporters (Supplementary Results; Supplementary Figure S9). Therefore, it is possible that both pri-miRNAs and properly processed mature miRNAs (with intact 5' seed nucleotides) may contribute to the activity of miRNA genes. Supporting this notion, we found that expression of *hsa-let-7a-3*, which resulted in properly processed mature let-7 in BOSC 23 cells, more effectively repressed target gene expression than did expression from *cel-let-7*, which resulted in inactive mature let-7.

The properties of structured pri-miRNAs in target regulation

Direct interactions between pri-miRNAs and cognate targets would allow the regulatory information encoded in these structured RNAs to be interpreted through RNA:RNA interaction. The structured pri-miRNAs are able to confer regulatory controls through sequence and structural elements that are not present in the linear mature miRNAs. Indeed, we find that both the structural and sequence elements of pri-let-7, such as pri-miRNA loop, mature miRNA sequence, and extent of base pairing in the stem region can control the activity of *cel-let-7* (Figures 2, 4 and, 6; Supplementary Figures S8 and S9), rendering structured pri-miRNAs more discriminatory in target recognition, possibly by requiring target sites to compete with internal stem-region base pairings.

Our findings illustrate that the regulatory information encoded in structured pri-miRNAs may be directly translated into

function through interactions with certain cognate targets, suggesting an intriguing parallel between gene regulation by some bacterial antisense RNAs and pri-miRNAs. In bacteria, many antisense RNAs interact with their target RNAs through a two-step mechanism, which consists of initial binding between two loops or between a loop and a linear region and then propagation of the intermolecular base pairings (Wagner and Simons, 1994). The fact that pri-let-7 activities in target binding and repression can also be controlled by the loop structure and sequence strongly suggests a parallel mechanism between target recognition by some bacterial antisense RNAs and pri-miRNAs. However, we have not been able to determine how the interaction between pri-let-7 and target RNA is initiated, so we cannot conclude a similar kissing-loop-mediated interaction. Finally, the levels of pri-miRNAs in cells are generally low and may have a higher turnover rate, which may cause target repression to be determined by the rate of association between target and pri-miRNA as is the case for some antisense RNAs (Wagner and Simons, 1994).

Mechanisms of action by miRNA genes

Previous studies on the mechanisms by which miRNA genes control gene expression were exclusively focused on the function and biogenesis of mature miRNAs, as pri-miRNAs and pre-miRNAs were considered to be intermediate byproducts of mature miRNA biogenesis. Our findings suggest that further characterization of the function of pri- and pre-miRNAs in target repression may be necessary and could help to elucidate the mechanisms of repression by miRNA genes. Intriguingly, *cel-let-7* expression in BOSC 23 cells, which resulted in no detectable amounts of functional pre- and mature let-7, seems to control target expression without degrading the target reporter mRNA, whereas expression of *hsa-let-7a-3*, which resulted in full-length mature let-7, caused strong degradation of reporter mRNA, implying possible differences in mechanism between pri-miRNA- and mature miRNA-mediated target regulation (Figures 1 and 7).

These findings also raise questions about the functional relationship between all RNA products of an miRNA gene. The fact that the interactions between pri-miRNAs and target RNAs may occur within the nucleus either during or after target mRNA transcription (Figure 5H) indicates that nuclear events, such as target and pri-miRNA recognition and/or co-transcriptional processing of pri-miRNAs (Pawlicki and Steitz, 2008), may be involved in initiating miRNA-mediated gene regulation. An intriguing possibility is that early engagement of targets by pri-miRNAs may facilitate the coordination of mature miRNA biogenesis and guide low-abundance mature miRNAs to their target sites. Under such a scenario, target recognition and repression would be a multi-step process that starts from the pri-miRNA stage and ends at the mature miRNA stage. As introducing mature let-7 by transfecting let-7 mimics (Figure 3E) or sh-let-7 (Supplementary Figure S9) results in repression of lin-41_LCS reporters, this model may help to explain why evolution resulted in generation of mature miRNAs from pri-miRNAs, but not other double-stranded RNA precursors. Moreover, we found that *cel-let-7* was functionally inactive in ES cells, presumably because of inhibition by lin-28 or other hairpin-binding molecules (Newman *et al*, 2008; Viswanathan *et al*, 2008), thus precluding dissection of the effects of loss of DGCR8 or Dicer on its function in these cells.

Loss of Dicer or Drosha by shRNA knockdown in BOSC 23 cells or targeted deletion in ES cells results in major defects in fundamental cellular processes, such as growth arrest and drastic decreases in gene expression (data not shown), and for this reason may not be used to examine the function of pri-miRNAs. Finally, given the potential function of pri-miRNAs as gene regulatory molecules, it is likely that protein regulators may have evolved to control their functions, which may have at least two interesting inhibitory consequences: (1) blocking mature miRNA biogenesis and (2) sequestering pri-miRNAs from binding to their cognate targets.

In summary, the findings presented here reveal a novel layer of regulatory complexity encoded in the long primary transcripts of miRNA genes that may have broad implications in understanding the mechanisms by which miRNA genes control target expression. As detailed mechanisms by which structured pri-miRNAs recognize their targets and their functional relationships with other RNA products of an miRNA gene are elucidated, structural and sequence information in the structured pri-miRNAs may be incorporated into target prediction to improve the accuracy of target prediction.

Materials and methods

See Supplementary data for more detailed descriptions about vectors (Supplementary Figure S10), northern blot, primer extension, EMSA, luciferase reporter assay, and RPA analyses.

Purification of target and pri-let-7 complex from cultured cells

The lin-41_LCS *Renilla* luciferase reporters, Rluc-lin-41_LCS-wt or Rluc-lin-41_LCS-sm, were tagged with an S1 aptamer. The S1-tagged *Renilla* luciferase reporters were co-transfected with various *cel-let-7* expression constructs into 7×10^5 BOSC 23 cells. Fugene (Roche) and TurboFect (Fermentas) transfection reagents were used according to the manufacturers' instructions. Consistent transfection efficiencies (70–80%) were achieved as indicated by FACS analyses of GFP expression. At 48 h after transfection, cells were washed twice with cold PBS and then resuspended in lysis buffer (150 mM KCl, 10 mM Hepes 7.4, 3 mM MgCl₂, 2 mM DTT, 10% glycerol, 0.5% NP-40). Cell suspensions were incubated on ice for 10 min and subjected to repeated pipetting. Lysates were cleared by centrifugation at 2000 g for 5 min. The extracts were incubated with avidin beads for 1 h in cold room to remove endogenous biotin and then bound to magnetic streptavidin beads in the presence of 100 µg/ml tRNA and glycogen for 4 h. Bound beads were then subjected to 10 washes using binding buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 100 mM NaCl, 100 µg/ml tRNA, and 2% NP-40). S1-tagged reporter was eluted by incubating beads with binding buffer containing 5 mM biotin for 1 h. RNAs were then precipitated and analysed by qPCR and RPA analyses. Similar pull-down analyses were also carried out from the nucleoplasmic fractions to determine whether complexes would form in the nucleus. Representatives of independent pull-down assays quantified by qPCR or RPA were shown.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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