Determinants of MicroRNA Processing Inhibition by the Developmentally Regulated RNA-binding Protein Lin28*

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The developmentally regulated RNA-binding protein Lin28 blocks processing of let-7 family microRNAs (miRNAs) in embryonic cells. The molecular basis for this selective miRNA processing block is unknown. Here we find that Lin28 selectively binds the terminal loop region of let-7 precursors in vitro and that the loop mediates miRNA processing inhibition in vivo. Additionally, we identify the domains of Lin28 required for this inhibition. These findings establish a regulatory role for the terminal loop of precursors in miRNA maturation and provide insight into the mechanism by which Lin28 negatively regulates let-7 processing.

MicroRNAs (miRNAs) comprise a large family of short regulatory RNAs that repress the expression of target messenger RNAs and have many important roles in development (1). In addition to the requirement of miRNAs for normal development, it is emerging that altered miRNA expression is a hallmark of various cancers (2). Several examples of miRNAs with oncogenic or tumor suppressor properties have been reported. Notably, let-7 miRNA has been reported to play a tumor suppressor role by repression of oncogenes including Hmga2, Ras, and Myc (3–6). Reduced expression of let-7 miRNA in human lung cancers is associated with shortened postoperative survival (7), and in a mouse lung cancer model, let-7g inhibits tumor development (8). Additionally, low let-7 expression is important for the self-renewal and tumorigenicity of breast cancer initiating cells (9).

Hundreds of miRNAs have now been identified, many of which are expressed in a tissue- and developmental stage-specific manner. Under most conditions, control of their expression occurs at the transcriptional level. The miRNA biogenesis pathway involves the sequential processing of primary miRNA transcripts (pri-miRNAs) by the Microprocessor complex (comprising the RNaseIII enzyme Drosha and the double-stranded RNA-binding protein DGC8) to release 60–70-nt precursor miRNAs (pre-miRNAs) that are subsequently cleaved by the Dicer complex to yield mature ~22 nt miRNAs (10–13). Emerging evidence indicates that miRNA biogenesis can also be regulated posttranscriptionally (14–18).

The developmentally regulated RNA-binding protein Lin28 was recently identified as a selective inhibitor of miRNA processing in embryonic stem cells and embryonal carcinoma cells (18). Lin28 inhibits the maturation of the let-7 family but not other miRNAs, yet a mechanistic explanation for this selectivity is unknown. We sought to gain insight into the mechanism by which Lin28 selectively blocks the processing of let-7 family miRNAs. Using in vitro and in vivo assays, we explored the RNA sequence and structural requirements for Lin28-mediated regulation and found that Lin28 specifically binds the terminal loop region of let-7 precursors. Furthermore, we demonstrated that the loop mediates miRNA processing inhibition in vivo and identified the domains of Lin28 required for this inhibition.

EXPERIMENTAL PROCEDURES

Electromobility Shift Assays (EMSA)—EMSA was conducted using ~2 $\times$ 10^5 cpm 5’-end-labeled pre-miRNA probe, together with the indicated amounts of competitor RNA and recombinant Lin28 that was prepared as described previously (18). Binding reactions were conducted in 20 $\mu$L of total volume with 30 $\mu$g of yeast tRNA. Binding buffer contained 100 mM NaCl, 50 mM Tris (pH 7.6), 5% glycerol, 20 units of RnaseOUT, and 10 mM $\beta$-mercaptoethanol. Bound complexes were resolved on native 5% polyacrylamide gels. Band intensities of scanned gels were quantified using Adobe Photoshop software. The data were fitted to a hyperbolic function of the nonlinear curve fitting method of GraphPad Prism. The total amount of probe in each binding reaction was normalized against the unbound probe (in the absence of recombinant Lin28 protein (rLin28)) and used to calculate the fraction bound by rLin28. Dissociation constants of pre-let-7g and the let-7g terminal loop were derived from a fit to the equation: Fraction bound = $B_{max}[(rLin28)]/(K_d + [rLin28]),$ where $B_{max}$ represents the observed maximum fraction of probe bound, [rLin28] represents protein concentration, and $K_d$ is the dissociation constant.

Cell Culture, Transfection, and Immunoprecipitations—Transient transfections of 293T and HEK293 cells were performed using Lipofectamine 2000 (Invitrogen) per the manufacturer’s instructions. Cell lysates were prepared using a buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl2, 0.2 mM Phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. FLAG-Lin28 immunoprecipitations were performed using anti-FLAG-agarose beads (Sigma). After a 90-min incubation,
the beads were washed twice with BC500 buffer: 20 mM Tris-HCl (pH 7.8), 500 mM KCl, 0.2 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol (pH 7.8), 0.2% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride followed by one wash with BC100 (as above except with 100 mM KCl) before elution with FLAG-peptide (Sigma).

Quantitative PCR and Mutagenesis—Levels of mature miRNA species were measured by quantitative PCR using commercially available TaqMan probes (Applied Biosystems) per the manufacturer’s instructions with snol42 RNA or U47 RNA used as internal standards for normalization. The plasmid for expression of let-7g loop/miR-21 was generated using the QuikChange kit (Stratagene) and the following primers to amplify from the pcDNA3-pri-let-7g plasmid: forward, 5'-CTGAGGTA-GTAGTTTGTCACAGTTCTTTGAACATCGGACACTGTA-CAGGCCACTGCTTGG-3', and reverse, 5'-CAAGGCAGTGG-CCTGTACGTCCATGAGATTCACAGAACTGTACAAA-CTACTACCTCAG-3'. Plasmids for the expression of the Lin28 proteins containing single amino acid substitutions were generated by site-directed mutagenesis of the pCMV2-Lin28 using the QuikChange kit (Stratagene).

Northern Blotting—20 μg of total RNA from each sample was used for Northern blotting as described previously (10). Probes for miRNA detection were antisense, end-labeled DNA oligonucleotides to the mature miRNA sequence.

RESULTS

Lin28 Selectively Binds the Terminal Loop Region of Precursor let-7 miRNAs—We sought to gain insight into the molecular determinants of Lin28-mediated miRNA processing inhibition. Using EMSA, we demonstrated that recombinant Lin28 selectively binds pre-let-7g RNA (Fig. 1A) but not other pre-
miRNAs tested (data not shown). From these experiments, we determined the estimated $K_d$ of Lin28 binding to pre-let-7g to be $2.1 \pm 0.32 \mu M$ (Fig. 1A). Furthermore, competition experiments with unlabeled (cold) pre-let-7g or cold control pre-miRNA (pre-miR-138) demonstrated that Lin28 binds let-7g with at least 200-fold greater affinity than control miRNAs (Fig. 1B). The hairpin-shaped pre-miRNAs contain a highly base-paired stem that contains the mature miRNA sequence and the complementary miRNA* sequence that are connected by a terminal loop region that, depending on the particular pre-miRNA, may also contain base-paired regions. For clarity, we refer to this as the “loop” hereafter (Fig. 1C). We did not detect Lin28 binding to 22 nt let-7g (miRNA:miRNA*) duplex in EMSA (data not shown). However, using the same EMSA conditions, we detected robust binding to the let-7g terminal loop sequence with an estimated $K_d$ value of $1.5 \pm 0.28 \mu M$ (Fig. 1D).

The Terminal Loop Sequence of Precursor miRNAs Mediates Lin28 Binding in Vitro and Processing Inhibition in Vivo—In mice and humans, the let-7 family miRNAs comprises 12 members, the mature miRNA sequence of which is highly conserved between the different genes (Fig. 2A). Since Lin28 inhibits processing of all let-7 family members and specifically binds the terminal loop of let-7g (Fig. 1D), we hypothesized that there should be common RNA sequence or structural features that confer Lin28-mediated regulation. Indeed, we identified a conserved cytosine nucleotide in the terminal loop region of pre-let-7 miRNAs (Fig. 2A). Although changing this cytosine to an adenosine is not predicted to alter the folding of the pre-let-7g loop region (Fig. 2B), we found that this single nucleotide substitution dramatically reduced Lin28 binding more than 20-fold relative to the wild-type pre-let-7g loop (Fig. 2B). Next, we investigated the role of the loop region in the Lin28-mediated inhibition of miRNA maturation in vivo. HEK293 cells were transfected with plasmids for the expression of let-7g or a chimeric pri-miRNA in which the terminal loop sequence was replaced with that of miR-21 (a miRNA that is not regulated by Lin28) (18). Although expression of Lin28 blocked the maturation of let-7g by $\sim$90%, the expression of let-7g stem/miR-21 loop construct was unaffected by Lin28 (Fig. 2C).

Both the Cold Shock Domains (CSDs) and the Zinc Finger Domains of Lin28 Are Required for pre-let-7 Binding in Vitro and Processing Inhibition in Vivo—Next, we sought to identify the Lin28 protein determinants of the miRNA processing block. Lin28 contains a CSD and two retroviral-type zinc fingers (Fig. 3A). Notably, Lin28 and Lin28B can both block let-7 processing and are the only proteins with this combination of motifs (18–20). CSDs contain $\sim$70 amino acids that are conserved in prokaryotic and eukaryotic DNA-binding proteins, part of which is highly similar to the RNP-1 RNA-binding motif (21–24). The Cys-Cys-His-Cys (CCHC) type zinc finger domains are found predominantly in nucleocapsid proteins of retroviruses, which are required for viral genome packaging and for the early infection process (25–27). Therefore, both the CSD and the CCHC domains may be important for RNA binding by Lin28. To test this, we generated single amino acid substitutions in either the CSD or the CCHC domains and tested their relative ability to bind to pre-let-7g (Fig. 3B). We identified single amino acid residues required for binding to pre-let-7g (F47A, F73A, C161A), whereas other residues had no effect (K45A). Next, we tested the effect of these mutations on Lin28 inhibition of let-7g maturation in vivo (Fig. 3C). We found a correlation between pre-let-7g binding in vitro and blocking let-7g genesis in vivo. Furthermore, single amino acid substitutions in both the CSD and the CCHC domains abolished both let-7 binding and processing inhibition, thus demonstrating that both domains are necessary for Lin28 function.
Lin28 mediates let-7 processing block by specifically binding the terminal loop region of pre-let-7 miRNA, which is part of both the pri-miRNA and the pre-miRNA. It is possible that Lin28 mediates let-7 processing block by specifically binding pri-let-7 and sequestering these bound pri-miRNAs in the cytoplasm away from the action of the nuclear Microprocessor complex.

Lin28 homolog B (Lin28B) up-regulation has been reported in hepatocellular carcinoma, and its overexpression in human breast cancer cells (MCF-7) was shown to stimulate cell proliferation (33). Considering that several studies have identified down-regulation of let-7 family miRNAs in various cancers, together with the demonstration that both Lin28 and Lin28B inhibit let-7 processing, it is likely that a better understanding of the mechanism by which Lin28 regulates let-7 biogenesis may facilitate the development of novel cancer therapeutics. It will be important therefore to elucidate the three-dimensional structure of Lin28 together with the let-7 terminal loop to provide a more detailed view of this RNA-protein interaction.

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FIGURE 3. Both the CSDs and the zinc finger domains of Lin28 are required for pre-let-7 binding in vitro and processing inhibition in vivo. A, schematic representation of Lin28. N, N terminus; C, C terminus. B, EMSA performed with FLAG affinity-purified WT and mutant Lin28 proteins (K45A, F47A, F73A, C161A) expressed in HEK293 cells. C, HEK293 cells were co-transfected with the pri-let-7g plasmid and 0.5 μg of the pcMV-FLAG empty vector (lane 1) or co-transfected with the pri-let-7g plasmid and 0.5 μg of FLAG-Lin28 wild-type cDNA (lane 2) or the indicated mutant Lin28 (lanes 3–6). Total RNA was collected 40 h after transfection. 20 μg of total RNA from each sample was used for Northern blotting as described previously (10). Samples were also analyzed for FLAG-Lin28 expression by Western blot.

DISCUSSION
We found that Lin28 specifically binds the terminal loop region of let-7 miRNA precursors and revealed an unanticipated regulatory role for the loop sequence in let-7 maturation. Although the calculated $K_d$ values for Lin28 binding to pre-let-7g (2.1 μM) and to the terminal loop of let-7g (1.5 μM) are quite high, it is important to consider these affinities relative to those of proteins that compete for binding to the miRNA precursors. For example, the reported $K_d$ of DGCR8 (the essential double-stranded RNA-binding component of the Microprocessor complex) binding to pri-miRNA in vitro is between 2.9 and 4.2 μM (28). Therefore, the estimated $K_d$ of Lin28 is lower than that of DGCR8 and is consistent with our previous observation that Lin28 robustly inhibits the pri-miRNA-processing activity of the Microprocessor complex. In addition, it is possible that the conditions we used for the in vitro binding reaction do not precisely reflect the physiological conditions in which Lin28 acts as a blocker, in which case the physiological $K_d$ may be lower.

We demonstrate that both the CSD and the CCHC domains are required for the Lin28-mediated block in let-7 processing.

Since let-7 miRNA processing is blocked in Lin28-expressing cells, we propose that plasmid-based strategies for ectopic expression of let-7 will be ineffective in certain cell types including embryonic stem cells. Approaches for expressing let-7 in these cells will require changing the pre-let-7 terminal loop sequence to bypass Lin28 regulation. Similarly, vector-based RNA interference has become a popular approach for analyzing gene function in mammalian cells (29). These vectors use standard promoters to express short-hairpin RNA directed against a target mRNA. Similar designs have incorporated features of miRNA precursors, including the terminal loop sequence. So far, miR-30 and miR-155 have been utilized in this way (30–32). Given the newly identified additional level of regulation by Lin28, RNA interference constructs based on pre-let-7 should be avoided for certain applications.

It has been reported that Lin28 localizes primarily to the cytoplasm (20). However, as we recently showed, Lin28 blocks the action of the Microprocessor complex both in vitro and in vivo. Since here we demonstrate that Lin28 specifically binds to the terminal loop region of pre-let-7 miRNA, which is part of both the pri-miRNA and the pre-miRNA, it is possible that Lin28 regulates let-7 biogenesis by Lin28, RNA interference constructs based on pre-let-7 should be avoided for certain applications.
ACCELERATED PUBLICATION: Selective Inhibition of let-7 miRNAs

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