Supporting Information

Yang et al. 10.1073/pnas.1006432107

SI Materials and Methods

Generation of Mutant *hsa-mir-451*–Expressing Constructs. *mir-144/451* cluster was PCR-amplified using HeLa cell genomic DNA as template. Site-directed mutagenesis was carried out to generate mutant versions of *mir-451*. Primer sequences are listed in Dataset S2. Briefly, miR-144/451 forward and mutant reverse primers and mutant forward and miR-144/451 reverse primers were used in the first PCR separately to amplify two fragments that have several nucleotides overlapped. The two fragments were gel-purified and used as a template in the presence of miR-144/451 forward and reversed primers in the second PCR. The PCR products were gel-purified and cloned downstream to a GFP-coding sequence in pcDNA6.2/N-EmGFP-GW/TOPO vector (Invitrogen).

Luciferase Sensor Assay. A modified psiCHECK2 vector was used for the cloning of microRNA (miRNA) perfect-matched or bulged targets (1). The oligonucleotide sequences are listed in Dataset S2. Cotransfection of miRNA and sensor plasmids was carried out in HeLa and $Dcr^{-/-}$ MEF cells following the manufacturers' instruction of FuGENE6 (Roche) and Lipofectamine 2000 (Invitrogen), respectively; 24 h after transfection, luciferase activities were measured with Dual-Glo Luciferase Assay System (Promega).

Northern Blotting. Total RNA was extracted from cultured cells with TRIzol reagent (Life Technology). RNA samples were separated on 12%, 16%, or 20% urea polyacrylamide denaturing gels, transferred onto GeneScreen Plus (Perkin-Elmer), and probed with γ-^{32}P-labeled DNA oligonucleotides antisense to the individual miRNAs. Probe sequences are listed in Dataset S2.

siRNA Knockdown in HeLa Cells. Predesigned siRNA duplexes against *drosha* (HSC.RNAI.N001100412.10.1) and *dgcr8* (HSC.RNAI.N022720.10.1 and HSC.RNAI.N022720.10.2) were ordered from IDT; ScrambledNeg was used as negative control. Cotransfection of HeLa cells with siRNA (50 pmol) and *mir-144/451* plasmid DNA (2 μg) was carried out using Lipofectamine 2000 (Invitrogen) in 6-well plates. Total RNA of transfected cells was harvested 48 h after transfection. Knockdown efficiencies were assessed by quantitative reverse transcription-polymerase chain reaction analysis and compared with scrambled siRNA transfected condition. The primer sequences for qPCR are listed in Dataset S2.

***mir-451* Reprogramming.** Oligonucleotides carrying mature sequence for various miRNA and overlapping *mir-451* hairpin precursor were used to reprogram the mature sequence of miR-451 into each designated mature miRNA (Dataset S2). These primers were used in combination with either FP1 or TK polyA reverse primer in standard PCR using *mir-144/451* construct as a template. The two overlapping PCR products were then purified and mixed together with EcoRI-digested *mir-144/451* plasmid, and a cold-fusion reaction was carried out according to the manufacturer's manual (System Biosciences).

Generation of Mouse Embryonic Fibroblasts. We used mice expressing a conditional allele of *Ago2* targeting exons 9–11 (2) or a conditional allele of *Dicer* targeting exon 22 (2). Flox/flox mice were bred to C57BL/6J mice that carry Cre-recombinase fused to T2-ER1α (allowing binding to tamoxifen but not estrogen) at the Rosa26 locus. We removed the head and liver from d13.5 F1 embryos, and the remainder of the body was minced and trypsinized. The single-cell suspension was plated in 10-cm^2 tissue-culture plates, and mouse embryonic fibroblasts (MEFs) were immortalized using SV40 large T antigen. Deletion was induced by administration of 4-hydroxytamoxifen (Sigma-Aldrich) at 10-nM concentration over two 3-d intervals for a total of 6 d. Deletion was confirmed by PCR genotyping and Western blotting.

Expression of Reprogrammed miRNAs in MEF Cells. To examine the expression of reprogrammed miRNAs, MEF cells were transfected with 4 μg reprogrammed miRNA constructs and 10 μl lipofectamine 2000 (Invitrogen) per well in 6-well plates. Total RNA was harvested 24 h after transfection and analyzed by Northern-blotting analysis. To test the activities of reprogrammed miRNAs, luciferase-assay experiments were carried out as previously described (1).

miRNA Microarray. Total RNAs from wild-type and $Ago2^{-/-}$ bone marrow were prepared as previously described (3) and analyzed using the microfluidic μParaflo array by LC Sciences, which contains redundant regions probing miRNA transcripts listed on miRBase Release 14.0. The signal was background-subtracted and averaged among replicates. The raw and processed data are available in Dataset S3.

1. Okamura K, Hagen JW, Duan H, Tyler DM, Lai EC (2007) The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. *Cell* 130:89–100.
2. Yi R, et al. (2006) Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet* 38:356–362.
3. O'Carroll D, et al. (2007) A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev* 21:1999–2004.

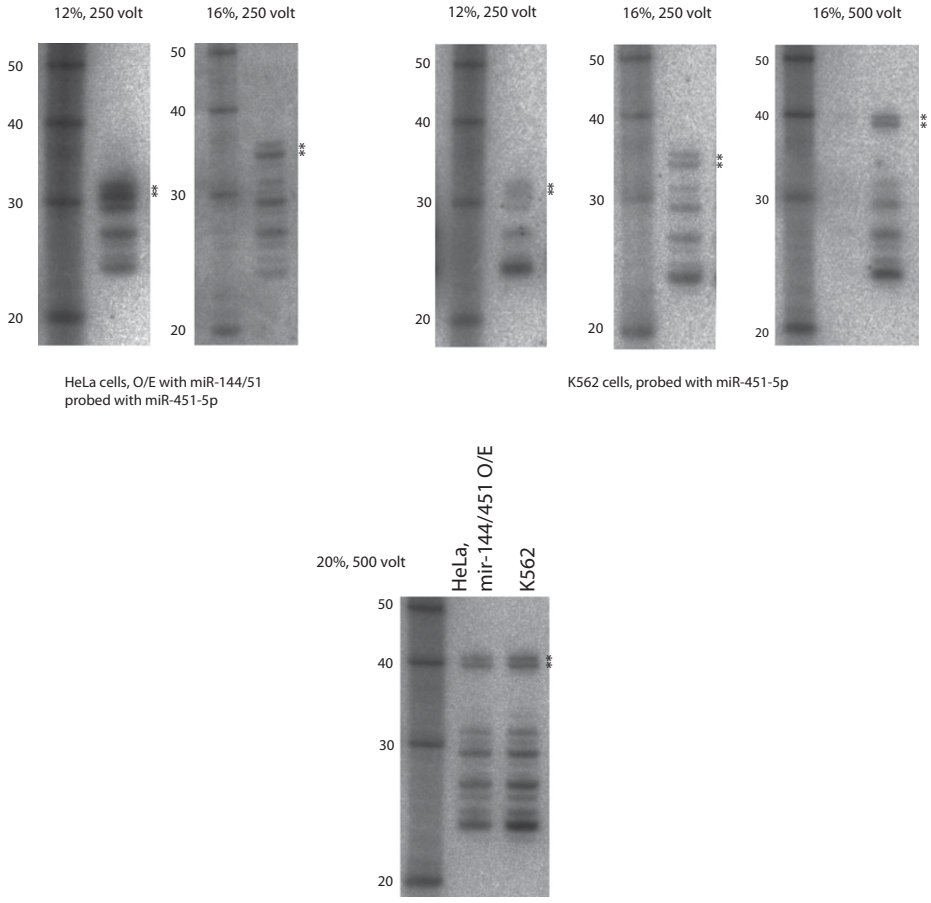

Fig. S1. Differential mobility of mir-451 hairpin species under different conditions. The larger miR-451–hybridizing bands (asterisks) exhibit differential mobility in different percentages of acrylamide or under different electrophoresis voltage. Based on evidence presented here and in the main figures, we infer these bands to represent the ~41- to 42-nt mir-451 hairpin. The upper left two blots depict miR-451 generated by transfection of a mir-144/451 construct into HeLa cells. The upper right three blots depict endogenous miR-451 expressed by K562 cells. Note that the upper bands (marked by asterisks) migrate more slowly in higher-percentage gels. Moreover, when gel percentage was kept constant, increased electrophoresis voltage, resulting in higher gel temperatures, also slowed the mobility of the upper two bands (marked by asterisks).The lower blot shows that only when run on 20% acrylamide at 500 V did the upper band of mir-451 appear at the ~42-nt position.

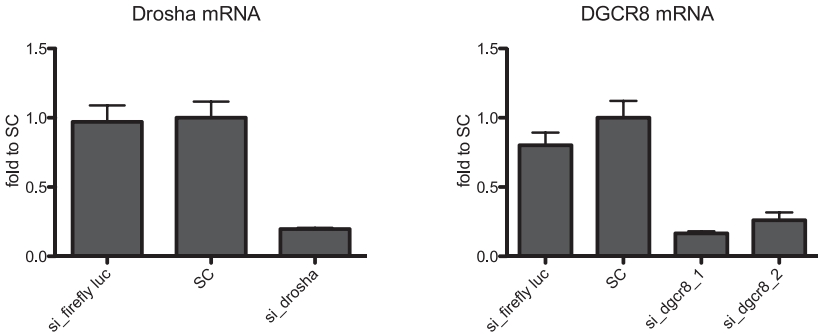

Fig. S2. qPCR validation of knockdown efficiency for Drosha and DGCR8. Values were normalized to target levels in cells transfected with scrambled siRNA; si-ff-luc was used as an additional negative control.

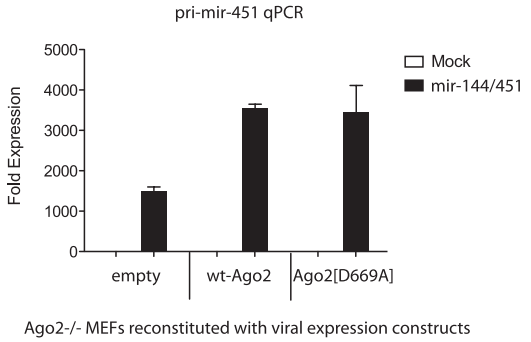

Fig. S3. Verification of mir-144/451 primary transcript expression in Ago2-knockout (KO) cells. Shown is qPCR analysis of lipofectamine transfection of hsa-miR-144/451 plasmid into MEF-Ago2-KO cells reconstituted with empty virus, wt-Ago2 virus, or Ago2[D669A] virus; data were normalized to mock transfection set at 1. All three cell types expressed primary mir-451 transcript at high levels after transfection. These RNA samples were used for Northern blotting in Fig. 4*C*.

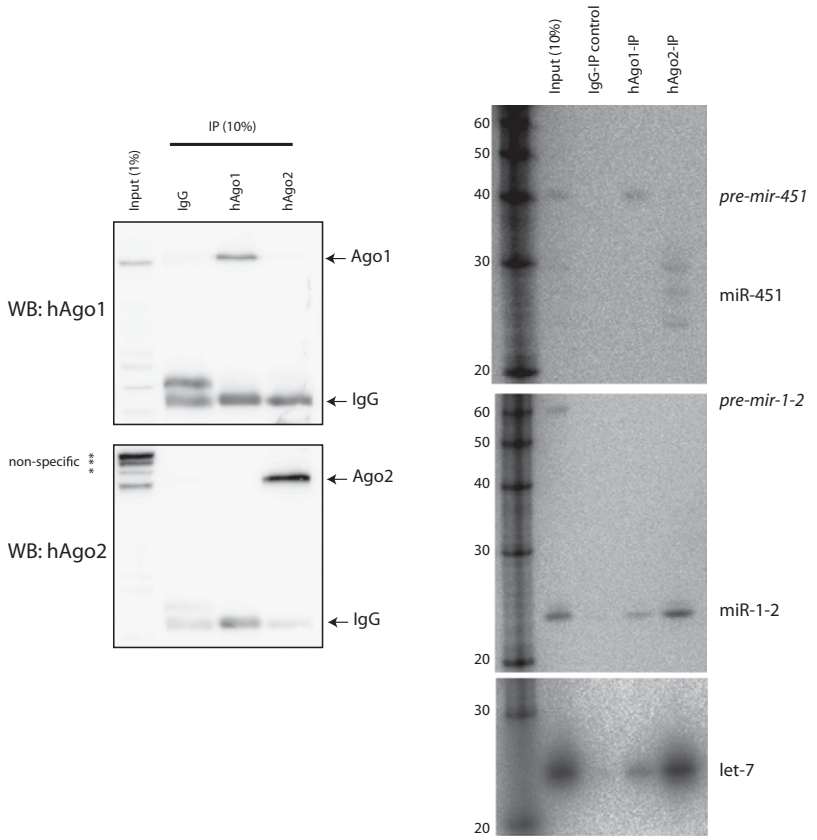

Fig. S4. Ago2 but not Ago1 can generate mature miR-451. HeLa cells were transfected with mir-144/451 expression construct and then immunoprecipitated with control IgG, hAgo1, or hAgo2 antibodies. Western blots (*Left*) show specificity of the immunoprecipitation (IP) reactions, with no cross-reactivity of hAgo1 and hAgo2 Abs. Northern blots (*Right*) probed for miR-1-2 and let-7 showed that both Ago1 and Ago2 complexes contained mature canonical miRNAs. In contrast, only Ago2 complex contained matured miR-451 species, whereas Ago1 complex contained only the precursor *mir-451* (*pre-mir-451*) hairpin.

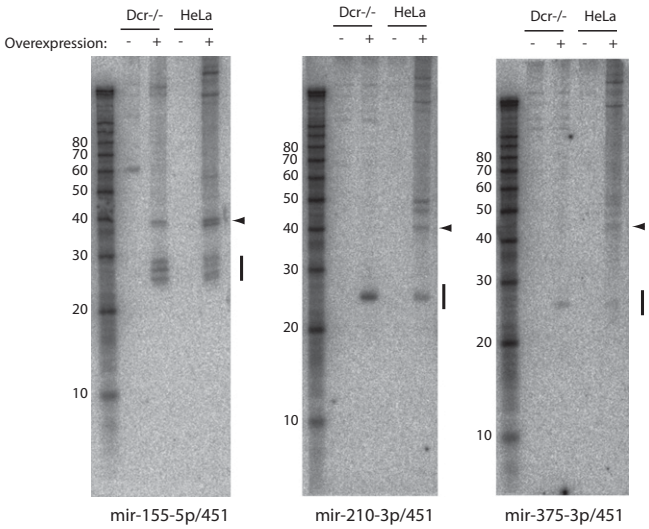

Fig. S5. Expression of reprogrammed mir-451 constructs in HeLa and Dicer$^{-/-}$ cells. HeLa cells and Dicer$^{-/-}$ MEFs were transfected with miRNA constructs reprogrammed into mir-451 hairpins within the human mir-144/451 backbone and assessed for small RNA production. These experiments suggest that Dicer$^{-/-}$ cells accumulate greater levels of matured miRNAs (black lines) relative to hairpin precursors (black arrowheads). However, we cannot rule out that other differences in small RNA pathway status, or gene expression in general between these distinct cell types, might contribute to the different capacities to mature mir-451–based small RNAs.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLS)