DROSHA Knockout Leads to Enhancement of Viral Titers for Vectors Encoding miRNA-Adapted shRNAs

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RNAi-based gene therapy using miRNA-adapted short hairpin RNAs (shRNA\textsuperscript{mir}) is a powerful approach to modulate gene expression. However, we have observed low viral titers with shRNA\textsuperscript{mir}-containing recombinant vectors and hypothesized that this could be due to cleavage of viral genomic RNA by the endogenous microprocessor complex during virus assembly. To test this hypothesis, we targeted DROSHA, the core component of the microprocessor complex, and successfully generated monoallelic and biallelic DROSHA knockout (KO) HEK293T cells for vector production. DROSHA KO was verified by polymerase chain reaction (PCR) and western blot analysis. We produced lentiviral vectors containing Venus with or without shRNA hairpins and generated virus supernatants using DROSHA KO packaging cells. We observed an increase in the fluorescence intensity of hairpin-containing Venus transcripts in DROSHA KO producer cells consistent with reduced microprocessor cleavage of encoded mRNA transcripts, and recovery in the viral titer of hairpin-containing vectors compared with non-hairpin-containing constructs. We confirmed the absence of significant shRNA\textsuperscript{mir} processing by northern blot analysis and showed that this correlated with an increase in the amount of full-length vector genomic RNA. These findings may have important implications in future production of viral shRNA\textsuperscript{mir}-containing vectors for RNAi-based therapy.

INTRODUCTION

MicroRNAs (miRNAs) are a class of small regulatory RNAs of ~22 nt involved in diverse biological pathways as key post-transcriptional regulators of gene expression.1 These miRNAs pair with complementary sites of the mRNAs and mediate post-transcriptional repression, also termed RNAi.2,3 RNAi-mediated gene silencing has been extensively studied in the laboratory and may provide a powerful therapeutic approach to human diseases to selectively modulate gene expression because of its high efficiency and sequence specificity.4,5

Short hairpin RNAs (shRNAs) are a class of RNA polymerase (pol) III-driven shRNAs that mimic the structure of miRNA precursor intermediates.6 shRNAs are expressed in mammalian cells to achieve efficient knockdown; however, it has been previously reported that oversaturation of processing machinery in cells expressing shRNAs from heterologous promoters at high levels is associated with cytotoxic effects7–11 and increased mortality in mice in transgenic model systems.12,13 For clinical application of RNAi therapeutics, alternative expression systems, including both more physiologic levels of expression and the capacity to effect lineage-specific expression, may be required. This can be accomplished by embedding shRNAs into flanking miRNA scaffolds, termed miRNA-adapted shRNAs (shRNA\textsuperscript{mir}s), which mimic the structure of endogenous miRNAs as described previously by us and others.14,15

We have recently reported a clinically applicable pol II promoter-driven viral shRNA\textsuperscript{mir} vector for knocking down BCL11A with improved efficiency and less cytotoxicity.14,15 BCL11A interacts with other transcription factors to repress the γ-globin (fetal) gene during adult life leading to increased β-globin (adult) gene expression.16,17 The molecular switch from fetal to adult globin is associated with emergence of signs and symptoms of sickle cell disease (SCD) and β-thalassemias in individuals with β-globin mutations.16,18 One therapeutic approach to treatment of β-hemoglobinopathies is the knockdown of BCL11A, which simultaneously restores γ-globin gene expression and reduces mutant β-globin expression (NCT03282656).
Endogenous miRNAs are transcribed as primary transcripts (pri-miRNA), which are cleaved by the microprocessor complex, DROSHA-DGCR8.20 DGCR8 binds to the hairpin and directs RNase III-like endonuclease DROSHA to the intended cleavage site.20-22 Alternatively, DROSHA itself can also bind to the pri-miRNA and perform endonucleolytic cleavage.23 Subsequently, the pre-miRNA is exported from the nucleus to the cytoplasm by Exportin-5 and further processed by Dicer.24 The resulting small interfering RNA (siRNA) duplex binds to the Argonaute (Argo- ) protein subunit of the RNA-induced silencing complex (RISC), where strand selection and target mRNA cleavage occur.25

We observed that the viral titers of hairpin shRNA mir vectors were consistently lower than non-hairpin-containing vectors. We hypothesized that low recombinant viral titers were due to cleavage of viral genomic RNA by the endogenous microprocessor complex during virus assembly in vector packaging cells. To test this hypothesis, we generated knockout (KO) of DROSHA, the core catalytic component of the microprocessor complex, in viral vector packaging cells by deleting exons 4–30 using CRISPR/Cas9.26 The DROSHA KO cell lines were tested for generation of hairpin shRNA mir recombinant virus. We demonstrate a recovery of viral titer and show correlation of the absence of shRNA mir processing, and an increase in the amount of full-length vector genomic RNA in both producer cells and viral particles. These insights are important for the clinical development of any hairpin-containing viral vectors that induce RNAi to treat diseases.

RESULTS

DROSHA KO Strategy Using CRISPR/Cas9
To determine the influence of miRNA processing on the lower titers observed in shRNA-containing vectors, we targeted the DROSHA-DGCR8 microprocessor complex for deletion. We utilized a CRISPR/Cas9 vector, pX458, for the co-expression of single guide RNAs (sgRNAs), SpCas9, and a GFP reporter (Figure 1A).26 The DROSHA gene is located on chromosome 5, spans 132 kb, and consists of 35 exons with the start codon in exon 3 (Figure 1B).23 We selected two sgRNAs targeting the 5′ end of exon 4 (labeled gRNAs 1–2) of DROSHA and two guide RNAs (gRNAs) targeting the 3′ end of exon 30 (labeled gRNAs 3–4) (Table S1) to affect a deletion of nearly the entire coding region.

We co-transfected combinations of 5′ and 3′ targeting gRNAs into HEK293T/17 cells and sorted GFP high-expressing cells. Sorted cells were plated at low density, and resulting colonies were picked individually to establish clonal cell lines. The clones were screened by polymerase chain reaction (PCR) analysis to identify a complete DROSHA KO cell line using DROSHA-specific primers (Figure 1C, top). In parenteral wild-type (WT) cells, PCR bands of 331 and 345 bp in lane 1 (primers 1 and 2) and lane 2 (primers 3 and 4) were observed, representing the 5′ and 3′ junctions of the targeted region (Figure 1C, top panel). For heterozygous KO, additional PCR bands of 421 bp in lane 4 (primers 1 and 3) and 255 bp in lane 5 (primers 2 and 4) indicate inversion of one allele (Figure 1C, middle panel), thereby inactivating this allele. For homozygous KO, PCR bands of 421 bp in lane 4 (primers 1 and 3) and 255 bp in lane 5 (primers 2 and 4) indicate inversion of both alleles and complete inactivation of the DROSHA gene (Figure 1C, lower panel at arrowheads). As seen in WT, heterozygous KO, and homozygous KO, the PCR product of 402 bp is absent in lane 3 (primers 1 and 3), indicating that complete deletion of the targeted fragment did not occur. We next confirmed the complete loss of DROSHA protein by immunoblot analysis (Figure 1D). For WT, the expected DROSHA protein band was observed at 170 kDa in lane 1. For homozygous deletion, the absence of DROSHA protein is confirmed in lane 3. In case of the heterozygous KO, the re-arrangement of one allele leads to an ~50% reduction in band intensity (lane 2). We next determined the effect of reduced DROSHA expression on cell growth (Figure 1E). Complete absence of DROSHA protein was associated with significantly lower proliferation compared with either WT or the single-allele rearranged clone.

Characterization of DROSHA KO Cell Lines
We next determined the effect of DROSHA KO on the capacity of cells to cleave an RNA hairpin embedded in a lentivirus vector. We constructed a control non-hairpin-containing plasmid (LeGO-SFFV) by removing the BCL11A shRNA mir hairpin from the LeGO-SFFV-shRNA mir5 plasmid (Figure 2A).14 In LeGO-SFFV-shRNA mir5, the shRNA is embedded in an miRNA223 scaffold (shRNA mir5) to allow expression from pol II promoters in mammalian cells. Plasmids were transfected into WT or homozygous KO cells, and northern blot was performed using a labeled RNA probe to determine the presence or absence of a processed small RNA derived from shRNA5 (Figure 2B). In WT cells transfected with the hairpin-containing plasmid (LeGO-SFFV-shRNA mir5), we detected a 20-bp small RNA band representing the mature siRNA (Figure 2B, lane 3) not present in cells transfected with the construct lacking the hairpin (Figure 2B, lane 2). In contrast, we detected very little or no corresponding band in homozygous KO cells after transfection of the shRNA mir5-containing plasmid (Figure 2B, lane 6), indicating no or very little cleavage of the embedded shRNA in these cells. As expected, no processing of the RNA was seen when the plasmid lacking the shRNA mir5 was transfected into homozygous KO cells (Figure 2B, lane 5). These data confirm that the absence of DROSHA severely compromises the capacity of cells to properly process and cleave shRNA mir hairpin structures.

DROSHA KO in 293T Producer Cells Leads to Decreased Levels of Full-Length Viral Genomic RNA for Hairpin Vectors
In order to determine the effect of miRNA processing on the generation of full-length viral genomic RNA during virus production, we performed northern blot analysis targeting the stem region of the hairpin on cell lysates of 293T packaging cell lines transfected with the plasmids shown in Figure 2A. The intensity of ribosomal 28S and 18S RNAs was used to ensure equal RNA loading in each lane of the northern blot (Figure 3A). In WT cells, we observed a relative decrease in the viral genomic length RNA transcript band intensity (at arrowhead) for the hairpin shRNA mir-containing (LeGO-SFFV-shRNA mir5) plasmid (Figure 3A, lane 3) compared with the
non-hairpin-containing (LeGO-SFFV) plasmid (Figure 3A, lane 2). In homozygous KO cells, there was no decrease in the intensity of this full-length genomic transcript (Figure 3A, lane 6) compared with the non-hairpin-containing plasmid (Figure 3A, lane 5). In multiple independent experiments, although the total amount of genomic mRNA varied, the ratios of genomic mRNA in WT or homozygous KO cells with or without shRNA remained consistent. Densitometric quantitation confirmed that there was an ~30% reduction of full-length genomic RNA for the hairpin-containing plasmid in WT cells and not in the homozygous KO cell line (Figure 3B).
DROSHA KO Leads to an Increase in the Full-Length Viral Genomic RNA for Hairpin-Containing Vectors in Viral Particles

In order to determine whether the change in full-length mRNA expression was associated with an increase in full-length viral genomic RNA transcript in packaged virions, we performed northern blot analysis on virus particles generated from 293T packaging cell lines. Similar to findings in packaging cell line lysates, we observed a decrease in the viral genomic RNA transcript band intensity in virus particles harvested from WT producer cells for the hairpin shRNA<sup>−miR</sup>-containing (LeGO-SFFV-shRNA<sup>−miR</sup>) lentiviral vectors. Position of the small RNA probe or RRE probe (straight line) used for northern blot analyses is indicated. (B) Northern blot of homzygous KO cell lines to determine presence of a 20-bp siRNA product processed from the shRNA<sup>−miR</sup> (structure shown on right). Small RNA probe was used for the detection of cleaved stem structure of hairpin. The position of 20-bp stem structure band is noted by arrowhead. Label at top indicates homozygous KO cell lines transfected with either non-hairpin-containing (LeGO-SFFV) or hairpin shRNA<sup>−miR</sup>-containing (LeGO-SFFV-shRNA<sup>−miR</sup>) plasmid. Ribosomal RNAs served as loading control (lower panel). The position of the viral genomic RNA transcript band is noted by the arrowhead. (B) Densitometric quantitation of the ratio of hairpin:non-hairpin RNA bands from northern blot in (A).

DROSHA KO Leads to an Increase in the Viral Titer for Hairpin Vectors

To determine the effect of the lack of DROSHA activity on recombinant virus production, we next compared the titer of supernatant harvested from populations of transfected 293T packaging cells on murine erythroleukemia (MEL) cells. We compared the titers of hairpin shRNA<sup>−miR</sup>-containing (LeGO-SFFV-shRNA<sup>−miR</sup>) compared with non-hairpin-containing (LeGO-SFFV) virus. Densitometric quantitation from the northern blot analysis of the full-length genomic RNA confirmed an ~40%–50% reduction of full-length genomic RNA in viral particles containing the hairpin from WT cells with a ratio of ~0.9 in viral particles from the homozygous KO cell line (Figure 4B).
produced in heterozygous KO and homozygous KO producer cells versus WT cells as a ratio of the titer of hairpin- to no-hairpin-containing vectors (Figure 5A). In WT cells, we confirmed an ~40% decrease in the relative viral titer of hairpin compared with non-hairpin-containing vectors. In producer cells deficient in DROSHA, no decrease in the viral titer was seen, with the viral titer ratio close to 1. There appeared to be a dosage dependency on DROSHA protein expressed in the cell, because producer cells with one allele disruption and ~50% DROSHA protein showed an intermediate decrease in viral titer and viral titer ratio between 0.7 and 0.8.

Because the vector-encoded mRNA contains the Venus fluorescent reporter and the shRNA<sub>miR</sub> hairpin in a monocistronic mRNA, DROSHA-mediated excision of the hairpin leads to cleavage and subsequent degradation of the mRNA. Efficient cleavage of the mRNA should therefore be associated with reduced Venus expression. We examined the mean fluorescence intensity (MFI) of Venus in WT and Drosha KO 293T cells after transduction with the two vectors. Similar to the viral titer ratio in Figure 5A, we observed an ~40% decrease in the expression as quantified by the MFI ratio of hairpin- to no-hairpin-transduced cells (Figure 5B). In contrast, in homozygous KO cells, we observed no decrease in the MFI ratio of integrated vectors. Cells with one functional DROSHA allele showed an intermediate phenotype with an MFI ratio between 0.7 and 0.8.

**Rescued DROSHA Activity Is Associated with Restoration of Viral Titters in Packaging Cell Lines**

Next, we performed a rescue experiment using a WT cDNA and a transdominant (TN) mutant of DROSHA. The latter contains two point mutations at E1045Q and E1222Q, which causes loss of catalytic activity of DROSHA (Figure 6A). We tested the effect of re-expression of DROSHA in homozygous KO cells on the resulting titers on MEL cells. First, we confirmed the expression of DROSHA by immunoblot analysis. As seen in Figure 6B, both the WT DROSHA and the TN mutant protein are expressed in homozygous KO cells. Although there was a ~40% decrease in DROSHA expression in both cases, there were no significant differences in the resulting viral titers.
KO cells. We transduced MEL cells using the virus generated from each producer population. The viral titer ratio decreased in the homozygous KO cells expressing WT DROSHA (Figure 6C) to levels similar to WT cells. Homozygous KO cells expressing TN DROSHA showed a modest reduction in viral titer compared with homozygous KO cells. The titer of TN DROSHA-expressing KO cells was significantly higher than either WT cells or KO cells expressing the WT DROSHA cDNA.

DISCUSSION

Viral vector-based RNAi gene therapy is a powerful approach to modulate clinically important genes. In this study, we show that reduction in viral titers generated in packaging cells is directly related to the cleavage of the full-length viral genomic transcripts by DROSHA. We used CRISPR technology to generate DROSHA KO in 293T packaging cells. In the absence of DROSHA, shRNA-miR hairpin-containing viral genomic RNAs were not cleaved. This lack of viral genomic RNA processing in packaging cells was associated with restoration of viral titer comparable with non-hairpin-containing vector constructs. Additionally, there was no difference between the expression of the hairpin vector versus non-hairpin-containing vector in infected DROSHA KO cells, suggesting that DROSHA also does not cleave shRNA-miR hairpin-containing miRNA transcribed from the internal promoter of the integrated provirus.

Finally, re-expression of WT DROSHA in homozygous KO cells led to a reduction in titer of hairpin-containing vectors. The difference in titer reduction in cells expressing the inactive mutant TN DROSHA versus WT DROSHA cDNAs strongly suggests that catalytic activity of DROSHA is important for the cleavage of shRNA-miR hairpin-containing viral genomic RNA.

These studies extend published work suggesting an important role for the microprocessor complex in disrupting packaging of shRNA-containing viral vectors. Previously, it was reported that the presence of shRNA-miR hairpin cassettes can negatively affect lentiviral vector titers, and inhibition of the RNAi pathway via saturation could rescue vector production. In these studies, replacement of the cytomegalovirus promoter with an inducible promoter also resulted in restoration of the vector titer. In addition, it has been reported that inhibition of RNA III enzyme activity of DROSHA can increase titers of miRNA-encoding retroviruses. Inhibition of DROSHA in the packaging cells resulted in impaired processing of mature miRNA from full-length retroviral transcripts, which led to more packaging of full-length viral transcripts into infectious virus particles. However, previous studies showed no direct proof of the cleavage of viral transcripts as a result of DROSHA activity and in association with the reduction in viral titers. Here, we report the generation of a packaging cell line that obviates that need for these manipulations and is the basis for an effective virus vector production system for all hairpin-containing constructs (Figure 7).

MATERIALS AND METHODS

Cloning of DROSHA Targeting CRISPR/Cas9 Plasmid

DROSHA targeting sgRNAs were designed using online CRISPR design tools developed by Feng Zhang and colleagues (http://crispr.mit.edu/) and CHOPCHOP (http://chopchop.cbu.uib.no/). Two gRNAs targeting the 5' end of exon 4 (labeled gRNAs 1–2) of DROSHA and two gRNAs targeting the 3' end of exon 30 (labeled gRNAs 3–4) were subsequently utilized (Table S1). The guide strand oligo sequences were synthesized from Invitrogen Custom DNA Oligos (Thermo Fisher Scientific, Waltham, MA, USA), annealed, and inserted into BbSI sites of S. pyogenes Cas9 cloning vector with 2A-EGFP (pX458; Addgene plasmid ID: 48139) (Figure 6A). The GenBank file for the plasmid is available through Addgene.
HEK293T/17 (ATCC CRL-11268) and MEL cells were maintained in DMEM or RPMI medium (Cellgro, Washington, DC, USA) supplemented with 10% fetal calf serum and 2% penicillin-streptomycin, respectively.

**Generation of DROSHA KO Cell Lines**

HEK293T/17 (ATCC CRL-11268) cells were transfected with CRISPR/Cas9 plasmids targeting DROSHA. We co-transfected 50 and 30 targeting gRNAs with 1 mg/mL linear polyethylenimine (PEI) (Polysciences, Warrington, PA, USA) and fluorescence-activated cell sorting (FACS) sorted for cells expressing high levels of GFP. Sorted cells were plated at low density, and individual clones were picked and expanded. The clones were screened by PCR analysis to identify DROSHA KO cell lines using a set of DROSHA primers (Table S2) and additionally analyzed for DROSHA expression by immunoblot analysis.

**Immunoblot Analysis**

DROSHA KO cell lines were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Rockford, IL, USA), with protease inhibitor (Roche, Mannheim, Germany). Samples were mixed suspended in 2× Laemml sample buffer supplemented with beta-mercaptoethanol. The mixtures were denatured by boiling and loaded onto a 10% SDS-polyacrylamide gel. Electrophoresis was performed to separate each sample according to size and subsequently transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Following blocking in PBS with 0.1% Tween 20 and 5% nonfat dry milk, the polyvinylidene fluoride membrane was incubated with a monoclonal anti-DROSHA rabbit antibody (Abcam, Cambridge, MA, USA) or mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). Anti-rabbit and anti-mouse IgG HRP-linked secondary antibody (Cell Signaling, Danvers, MA, USA) was used for detection by chemiluminescence by SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA).

**Proliferation of DROSHA KO Cell Lines**

For initial seeding of cells, the cell number was calculated using a hemocytometer and trypan blue dye to distinguish viable from dead cells. Proliferation of cells was assessed using the fluorescence-based CyQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. 1,000 cells/well were plated in a 96-well plate, and fluorescence measurements were made using a microplate reader with excitation at 485 nm and emission detection at 530 nm.

**Construction of SFFV-BCL11A shRNA<sup>mir</sup> Plasmids**

A control non-hairpin-containing (LeGO-SFFV) lentiviral vector plasmid was constructed by removing the BCL11A shRNAmiR hairpin from the LeGO-SFFV-shRNA<sup>mir</sup>S plasmid (Figure 2A). In LeGO-SFFV-shRNA<sup>mir</sup>S5, the shRNA is embedded in an hsa-miRNA223 (shRNA<sup>mir</sup>) backbone to allow expression from pol II promoters in mammalian cells.

**Lentivirus Production, Titration, and Transduction**

Lentiviral vector supernatants were generated by co-transfecting 1 µg of lentiviral transfer vector, 0.5 µg of Gag/Pol, 0.25 µg of REV, and 0.25 µg of vesicular stomatitis virus (VSV-G) packaging plasmids into HEK293T cells in a 12-well plate using linear PEI reagent. Supernatants were collected at 48 hr after transfection and filtered through a 0.45-µm polyvinylidene fluoride (PVDF) syringe filter (Corning Life Sciences, Tewksbury, MA, USA). To determine the titer, we infected 1 × 10<sup>5</sup> MEL cells in a 24-well plate with serial dilutions of the lentiviral vector supernatants in the presence of polybrene (8 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and analyzed them 3 days post-transduction by flow cytometry analysis using the BD FACS Fortessa (BD Biosciences, San Jose, CA, USA). Transductions of 293T DROSHA KO cell lines were performed on 2 × 10<sup>5</sup> cells/well.
in a 24-well plate followed by flow cytometric analysis of Venus expression 3 days post-transduction.

RNA Extraction, Lentiviral Pellet, and Northern Blot Analysis

For northern blot analysis, total RNA was extracted from DROSHA KO cell lines 48 hr after transfection with lentiviral packaging plasmids. For the detection of viral genomic RNA in viral particles, virus supernatants were treated with Benzonase (Sigma, St. Louis, MO, USA) to remove free RNA and DNA, followed by centrifugation at 13,000 \( \times g \) at 4°C overnight. Total RNA was extracted from viral pellets by concentrating the Benzonase-treated virus supernatant in a Beckmann XL-90 centrifuge using SW-28 swinging buckets. The total RNA was isolated using TRIzol reagent (Ambion, Austin, TX, USA), then resolved on a 15% polyacrylamide Tris-borate-EDTA (TBE) urea gel or formaldehyde/agarose gel for visualization using PerfectHyb Plus Hybridization buffer (Sigma, St. Louis, MO, USA) at 42°C overnight. The blot was probe-labeled with either 32P-labeled ATP (Perkin Elmer) and hybridized at 42°C for 1 hr. The blot was washed in 2 \( \times \) sodium citrate, 0.1% SDS at room temperature, and exposed to film. Forward and reverse sequences for REV-responsive element (RRE) probe were as follows: 5'-GCTTTGTTCTCT GGGTTCTTG-3' and 5'-CCAGGACGCTTATCCTTTAG-3'.

Statistical Analysis

All values are presented as the mean ± SD. Statistical significance was assessed by Student’s t test. \( p < 0.05 \) was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.07.002.

AUTHOR CONTRIBUTIONS

H.H.P., R.T., M.B., P.D., H.X., and C.B. conducted experiments. D.A.W., C.B., and R.I.G. designed experiments. H.H.P., R.T., M.B., S.G., P.D., H.X., and C.B. wrote the paper.

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