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A simplified miRNA-based gene silencing method for *Drosophila melanogaster*

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ABSTRACT

MicroRNA-based RNA interference is commonly used to produce loss-of-function phenotypes in mammalian systems, but is used only sparingly in invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*. Here, we evaluate this method in transgenic strains of *D. melanogaster* and cultured S2 cells. High throughput-ready expression vectors were developed that permit rapid cloning of synthetic hairpin RNAs. As proof of concept, this method was used for the efficient silencing of *dpp* gene activity in the adult wing, and the analysis of the general RNA Polymerase II (Pol II) elongation factor, *Nelf-E*.

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Introduction

Double-stranded RNAs (dsRNAs) are commonly used for gene silencing in *Drosophila*. However, these dsRNAs are typically quite long, >300-bp, and thereby produce dozens of unique ~21-nucleotide (nt) small interfering RNAs (siRNAs) (Elbashir et al., 2001). Sequence overlap of these RNAs with other regions of the genome, along with the unpredictable nature of the exact siRNAs derived from any given dsRNA, raises the likelihood of nonspecific, "off-target" phenotypes (Jackson and Linsley, 2004). Indeed, at least 40% of the dsRNAs in a widely used library might produce off-target effects in cultured *Drosophila* cell lines (Kulkarni et al., 2006). Nonetheless, considerable resources have been expended on this method for whole-genome assays (Dietzl et al., 2007).

siRNAs, defined as exogenous 21-nt silencing RNAs, can be expressed for targeted gene silencing assays through manipulation of the endogenous microRNA (miRNA) biogenesis pathway (Bartel, 2004). In this system, a customized siRNA sequence is swapped into a naturally occurring, imperfectly paired, ~70–90-base-pair (bp) stem-loop sequence (Chang et al., 2006). Cellular factors recognize the stem-loop as a miRNA precursor (pre-miRNA) and excise a specific ~21-nt sequence (the miRNA/siRNA) from one or both arms of the stem. Like exogenously delivered siRNAs, the resulting small RNA(s) is loaded into an effector complex (RISC) that can induce endonucleolytic cleavage of complementary RNAs (Hutvagner and Zamore, 2002; Miyoshi et al., 2005). The small RNA preferentially loaded into RISC from a given duplex RNA, e.g. a pre-miRNA or a transfected siRNA, is known as the mi/siRNA, while the less-incorporated strand is the star (mi/siRNA*) sequence. The incorporation of the mi/siRNA or mi/siRNA* into RISC depends, in part, on the local free energy of the duplex's terminal base pairs (Khvorova et al., 2003; Schwarz et al., 2003). This property provides a means for controlling the exact sequence of an expressed siRNA, thereby diminishing nonspecific, off-target phenotypes. While several studies demonstrated the effectiveness of miRNA-based RNAi in mammalian systems, there have been just a few such studies in *Drosophila* (Chen et al., 2007; Huh et al., 2007).

Here, we present a versatile, miRNA-based system for the conditional silencing of gene activity in *Drosophila*. These newly developed expression vectors recapitulate the exact structure of the *Drosophila* pre-*miRNA-1*(*miR-1*) stem loop sequence, differing only by unique cloning sites within the stem itself. Synthetic oligonucleotides (oligos) are directly inserted into the pre-miR scaffold, thereby circumventing multiple PCR amplification and cloning steps. A subcloning vector was also developed for creating tandem hairpins, thereby permitting the expression of multiple siRNAs directed against different regions of a specific target mRNA. We have created separate one-step transgenesis vectors that are compatible

Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; miRNA, microRNA; shmiR, short hairpin/miRNA; RISC, RNA induced silencing complex; *dpp*, decapentaplegic; *Nelf-e*, negative elongation factor e; GFP, green fluorescent protein; RFP, red fluorescent protein; M.C.S., multiple cloning site.

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with either Δ2,3 P-element transposase or phiC31 integrase (targeted integration) (Adams and Sekelsky, 2002; Groth et al., 2004), A web-based algorithm provides the exact design of the synthetic oligos for insertion into the pre-*miR-1* scaffold. Both S2 tissue culture assays and targeted expression in transgenic larvae and adults were used to establish the effectiveness and specificity of the method. We discuss the results of targeted disruption of *dpp* and *Nelf-E* in wing imaginal disks, and compare these phenotypes with those produced by dsRNAs.

Materials and methods

Vector design

The original shmiR vector used in this study is based on the *D. melanogaster* pre-*miR-1*. A second-generation vector, pHB, includes ectopic cloning sites at the base of the pre-*miR-1* sequence. Both types of vectors were constructed as mono or tri-cistronic synthetic genes by Celtek Bioscience (Nashville, TN), cloned into the KpnI and SacI restriction sites of pBluescript SK(+), and subsequently cloned into S2 cell expression or P-element transgenesis vectors as single or double hairpins by using appropriate restriction sites (see Supplemental File 1). Hairpins were inserted into pHB by directional ligation into the HindIII–BamHI cloning sites at the base of the pre-miR stem-loop.

The pNE2 vector was constructed in a modified pUAST backbone (Brand and Perrimon, 1993). EcoRI and XbaI cloning sites were removed by Klenow-mediated end fill. A mini-gene constructed as two annealed Ultramers (IDT) was inserted into this modified pUAST vector as a BglII–XhoI fragment. Hairpins were inserted into pNE2 by directional ligation into the NheI–EcoRI cloning sites at the base of the pre-miR stem-loop. pNE3 was constructed by end-filling a KpnI digested pNE2 vector, followed by linker-ligation of a BglII–KpnI–XbaI adaptor into the BglII–XbaI cloning site. This modification allows for directional insertion of a shmiR from pHB into pNE3, creating a tandem shmiR. p*attB*-NE3 is a variant of the phiC31 integrase-based p*attB*-UAST vector (Bischof et al., 2007). It was modified to include the same shmiR insertion and multi-cloning site as pNE3 (see Supplemental File 2 for complete restriction maps). A suggested method for creating two or more tandem shmirs in the pHB, pNE2, or pNE3 vectors is presented in Supplemental File 3.

Synthetic 71-base-pair oligonucleotides

71-nt oligos (Elim Biopharmaceuticals or IDT) were annealed at a final concentration of 50 μM in 1× annealing buffer (75 mM KCl, 20 mM Tris [pH 8.0]), boiled for ~2 min, and then cooled to room temperature for ~30 min. Annealed oligos were diluted 1:100 in 1× annealing buffer, and then ligated to ~500 ng of the appropriate linearized vector for ~15 min at room temperature using T4 DNA ligase (New England Biolabs). All inserts were sequenced and assessed for proper folding of the pre-miR stem loop via mFold (Mathews et al., 1999; Zuker, 2003).

siRNA sequence design

siRNAs directed against *dpp* recognize all *dpp* mRNA isoforms. siRNAs targeting mRNAs encoding pGL3-based Luciferase (Promega) and the mCherry red fluorescent protein were designed using the Dharmacon "siDesign" center (Shaner et al., 2004). *Nelf-E* siRNAs were designed using the Ambion "siRNA target finder" algorithm. The target-specificity of each 21-nt sequence was determined using parameters described by Khvorova and colleagues (Birmingham et al., 2007). Further, *miR-1*, like most miRNAs, begins with a 5′ uracil (Du and Zamore, 2005). In accordance with this generality, all siRNAs designed for use with these vectors contain a 5′-U. The complete sequences of all of the siRNAs used in this study are presented in Supplemental File 1.

Online hairpin design tool

A Perl-CGI script was written to automate the design of insert oligos compatible with our vectors, based on a user-defined siRNA sequence. This interactive web page provides the shmiR sequence required to produce a given 21-nt siRNA, using either the pHB or pNE expression vector. Information is also provided for the predicted secondary structure of the top (sense) strand hairpin produced with UNAfold (formerly mFold) (Dimitrov and Zuker, 2004). The structure of the resultant shmiR incorporates specific mismatched bases (at nts 2 and 11, as measured from the siRNA's 5′-most base) to mimic the native *D. melanogaster* pre-*miR-1* structure.

It has recently been found that siRNAs containing the same seed residues (nts 2–8) as those present within known miRNAs can induce systematic off-target phenotypes in cultured cell lines (Birmingham et al., 2006; Jackson et al., 2006). Therefore, each input 21-mer is cross-referenced against a database with all annotated *D. melanogaster* miRNA sequences, as of June 2008 (Griffiths-Jones, 2004). An alert will be displayed if the user-defined 21-mer contains a seed sequence overlapping a known miRNA.

S2 cell culture and transient transfection assays

S2 cells were plated at a density of 5×10^5 cells/well in 24-well plates. Synthetic shmiRs were inserted into a pAc5.1-B-based expression vector (Invitrogen), and used to transfect plated cells the following day with Effectene (Qiagen), as suggested by the manufacturer. Cell lysates were collected 48 h after transfection, and then analyzed using a dual-Luciferase assay kit (Promega). For mCherry RNAi, the shmiR vector was transfected at a ~4-fold excess over the target vector, and fluorescence was assayed at 48 and 72 h following transfection. shmiRs directed against mCherry, as well as the mCherry ORF were cloned into pAc5.1, as above.

An ~500-bp dsRNA directed against the firefly Luciferase coding sequence was amplified by PCR using primers that contain a T7 RNA polymerase promoter sequence—primer sequences are published in Supplemental File 1. RNA was transcribed using MEGAscript T7 (Ambion) and processed for transfection as described (Forstemann et al., 2005).

AGO immunoprecipitation

Cells were transfected with 450 ng shmiR-Luc 1HB and 50 ng mCherry, as described above. After 48 h, cells were collected by centrifugation, washed 3× with PBS [pH 7.4] (Gibco), and resuspended in 15 μl of lysis buffer per mL of cell culture media, as described (Forstemann et al., 2005). Cell debris was removed following centrifugation, producing lysate with a final protein concentration of ~2 μg/μl). Monoclonal antibodies against *Drosophila* AGO1 or AGO2 were bound to sheep anti-mouse-Ig conjugated Dynabeads (Dynal) by rocking at 4 °C overnight (Miyoshi et al., 2005). Antibody-conjugated beads were collected on a magnetized stage and washed 3× in PBS. 80 μl of lysate was added to the beads per IP, and this mixture was incubated on ice for 4 h, with occasional shaking. Following IP, beads were collected on a magnetized stage, the supernatants were removed, and then washed 3× with cold lysis buffer. Beads were resuspended in 100 μl of lysis buffer and bound proteins were removed by boiling for 5 min. Both the supernatant and recovered solutions were added directly to 900 μl of Trizol (Invitrogen) and 200 μl of chloroform. RNA was extracted and precipitated with 1 V/V isopropanol and 20 μg glycogen (Roche).

Fly stocks

P-element-mediated transformations of *yw* strains with the pUAST-based shmiR vectors were done using traditional methods,