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Generation of Transgenic *Drosophila* Expressing shRNAs in the miR-1 Backbone

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Abstract

In *Drosophila*, long-term effects of RNA interference (RNAi) must be achieved by integrating into the genome a template from which an RNAi trigger is transcribed by cellular RNA polymerases, generally RNA polymerase II or III. With encoded triggers, not only can essentially permanent silencing be achieved, but control can also be exerted over the level of trigger expression, with a resulting variation in the degree to which the target is silenced. Knockdown can also be controlled in a temporal and cell-type-dependent fashion through the use of well-established transgenic methodologies and well-tested promoters. The forms of encoded triggers vary. Long double-stranded RNAs can be expressed as extended inverted repeats. The nearest equivalent of a small interfering RNA is an artificial microRNA (miRNA) or short hairpin RNA (shRNA), where a natural miRNA backbone (also called a scaffold) is remodeled to produce a different small RNA or a small inverted repeat (<30 nucleotides) is simply expressed. This protocol describes creation of transgenic *Drosophila* carrying shRNA inserts in a remodeled endogenous miRNA backbone. The protocol applies to the use of miRNA-based shRNAs, but most of the vectors, principles of experimental design, and methods are also applicable to long inverted repeat transgenes.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Annealing buffer <R>

DNA ligase (Roche 10716359001) and buffer for DNA ligase (10×) <R>

Drosophila fly stock

The genetic background for making transgenics is y¹, sc, v¹, nanos integrase (y⁺); attP2 (y⁺). A preferred attP-docking site is attP2 located on the third chromosome at 68A4.

Injection buffer (1×) <R>

Luria broth (LB) + ampicillin (50 mg/mL)

Oligonucleotides

The design of oligonucleotides for ligation into a miR-1 vector is straightforward. See Steps 1–5.

pVALIUM20 vector (linearized with NheI and EcoRI)

This protocol is based on site-specific integration using the pVALIUM20 vector from the “VALIUM series” (Vermilion-AttB-Loxp-Intron-UAS-MCS) (Ni et al. 2008, 2009). See Figure 1 and Discussion.

QIAprep Spin Miniprep Kit (QIAGEN 27106)

shRNA

*The principles of shRNA construction are precisely the same as those used for the design of synthetic short interfering RNAs (siRNAs) (see **RNAi in Cultured Mammalian Cells Using Synthetic siRNAs** [Chang et al. 2012]). The key is to choose a target site for which the corresponding small RNA is incorporated into RISC and for which that enzyme in turn efficiently recognizes and cleaves its target. See Discussion.*

Typically, a hairpin targeting the white gene, which encodes a transporter required for proper pigmentation in the fly eyes and is dispensable for viability, serves as a negative control for an in vivo RNAi experiment. A short hairpin transgene targeting a heterologous gene, such as GFP or firefly luciferase, can also serve as a negative control.

Equipment

Bacterial incubator (37°C)

Bacterial shaker (37°C)

Benchtop centrifuge

METHOD

Designing Oligonucleotides

- 1 Begin with the 21-nucleotide siRNA prediction that can form perfect complementary base pairing with the target mRNA and add tagttatattcaagcata to the 5' end. Infer the complement of the original 21-nucleotide siRNA prediction and add this to the 5' end of the composite sequence.

The resulting sequence will be the backbone of the “top” oligonucleotide.

- 2 Generate the complementary sequence of the “top” oligonucleotide to create the backbone of the “bottom” oligonucleotide.
- 3 Add ctacgagt and gcg to the 5' and 3' ends, respectively, of the “top” oligonucleotide.
- 4 Add aattcgc and actg sequences to the 5' and 3' ends, respectively, of the “bottom” oligonucleotide.
- 5 Order the oligonucleotides from a reputable purveyor.

There is no need for any special purification.

*Annealing of the oligonucleotide pair will create a duplexed molecule with overhangs compatible with *NheI* and *EcoRI* sites for cloning into doubly cut VALIUM vectors, as described in detail below. Overhangs compatible with alternative vectors can be created simply by modifying the sequences provided.*

Cloning miR-1-Based shRNAs for Expression in Flies

- 6 Anneal as described below.
 - i. Dissolve the oligonucleotides in ddH₂O to make stock solutions (20 mM).
 - ii. Add 10 µL of top-strand and 10 µL of bottom-strand oligonucleotides to 100 µL of 1.2× annealing buffer and mix well.
 - iii. Heat the mixture for 5 min at 95°C and then slowly cool to room temperature.

*The resulting DNA fragment features overhangs suitable for ligation with *NheI*- and *EcoRI*-digested vectors.*

- 7 Clone as described below.
 - i. Directly ligate the DNA fragment from Step 6 with the pVALIUM20 vector that has been linearized with *NheI* and *EcoRI*:

Annealed DNA fragment	6 µL
Linearized vector (40 ng/µL)	2 µL
Buffer for DNA ligase (10×)	2 µL
DNA ligase (Roche 10716359001)	1 µL
ddH ₂ O	to 20 µL

Mix well and incubate for 1 h at 16°C.

- ii. Once ligations are transformed and individual colonies are isolated, screen the colonies for the presence of inserts by PCR using the following

primer pairs: ACCAGCAACCAAG TAAATCAAC (forward) and TAATCGTGTGTGATGCCTACC (reverse).

The correct colony should yield a ~300-bp PCR product.

*Alternatively, colonies can be screened by digestion with *NheI* and *EcoRI*.*

- iii. Sequence-verify the constructs with the following primer:
ACCAGCAACCAAGTAAATCAAC.

8 Purify the DNA for injection.

The correct clones can be expanded and DNA can be purified for injection in a number of ways. It is best to follow the recommendations of the injection facility (either internal or a commercial vendor). Virtually any high-quality DNA preparation can be used. Purified material should be suspended in $1 \times$ injection buffer and supplied to the facility at their requested concentration.

9 Create transgenic *Drosophila* carrying shRNA inserts.

Injection of hairpin-containing constructs can be performed either in-house following well-established protocols or by using commercial services (e.g., Genetic Services, Inc. or BestGene). See Troubleshooting.

TROUBLESHOOTING

Problem (Step 9): Lethality is observed in the transgenic *Drosophila*.

Solution: Although most transgenic lines are homozygous viable, a small percentage of the lines cannot be made homozygous. The underlying cause is still not clear, but it is conceivable that some basal level of transcription of the minimal promoter-driven short hairpin transgene can lead to the silencing of an essential target gene and thus contribute to the observed lethality.

Sometimes the target gene is essential and severe knockdown can lead to lethality. An allelic series can be created by setting up the cross between the short hairpin transgenic RNAi lines and Gal4 driver lines at different temperatures (ranging from 18°C to 29°C). Higher temperatures generally lead to more efficient knockdowns. In addition, various ubiquitous Gal4 drivers of different strengths can be sampled.

DISCUSSION

shRNAs as Encoded Triggers for RNAi

Encoded triggers take the form of templates for the transcription of long or short dsRNAs by cellular polymerases, most often RNA polymerase II or III. Although there can be greater complexity in their design and construction, they offer some advantages over synthetic triggers. Because transcription templates can be stably integrated into the genome, the

effects of encoded triggers can persist indefinitely, when compared with the more limited duration of most experiments with synthetic RNAs. Moreover, their expression can be restricted to specific tissues or provoked in response to inducing signals that activate conditional promoters (e.g., tetracycline-regulated expression).

Both long inverted repeats and shRNAs can be used as silencing triggers for transgenic RNAi in *Drosophila* (Fortier and Belote 2000; Kennerdell and Carthew 2000; Lam and Thummel 2000; Martinek and Young 2000; Dietzl et al. 2007; Haley et al. 2008). Long inverted repeats mimic endogenous loci that give rise to endo-siRNAs in many different tissue types (Czech et al. 2008; Ghildiyal et al. 2008; Okamura et al. 2008). shRNAs are essentially miRNA mimics. Two general configurations are used in flies and mammals (Fig. 2). In the simplest form, a short inverted repeat is created with a perfect stem of 19–29 nucleotides and a variable length loop (usually 6–8 bases) (Brummelkamp et al. 2002; Paddison et al. 2002). The entire structure can be created by annealing two synthetic oligonucleotides such that appropriate overhangs are created for direct cloning into an expression vector (Fig. 3). Often, this type of shRNA is transcribed from an RNA polymerase III promoter such that initiation and termination define the 5' and 3' ends of the guide and passenger strands of the mature small RNA. Dicer, at least in the case of the longer triggers, recognizes and processes primary transcripts to yield an siRNA-like molecule that is incorporated into RISC. For the shorter stems, it is unclear how maturation occurs. It is also worth noting that the 5'-triphosphate terminus on the RNA-polymerase-III-generated versions is not the configuration that is normally recognized by RISC and that this type of RNA can sometimes generate stress or antiviral responses, e.g., via RIG-I (Kim et al. 2004; Kenworthy et al. 2009).

shRNAs modeled more closely on endogenous miRNAs appear to offer advantages over the simpler design, including more efficient incorporation into RISC and more flexibility in the design of expression vectors (Zeng et al. 2002; Dickins et al. 2005; Stegmeier et al. 2005; Dickins et al. 2007). A number of endogenous miRNA backbones have been remodeled to produce experimentally designed small RNAs, with miR-1 in flies (Haley et al. 2008) and miR-30 in mammals (Zeng et al. 2002) being those in widest use. Construction proceeds much as with an inverted repeat shRNA, often by synthesizing and annealing two oligonucleotides that fully span the guide and passenger strands. In this case, the guide and passenger are separated by the loop derived from the miRNA. Some additional miRNA-derived flanking sequence is also included, because in this case, the artificial sequence must be engrafted into a larger fragment of the primary miRNA transcript (Fig. 3). Thus, expression vector designs incorporate restriction sites in a manner that minimizes predicted effects on the folding of the primary transcript, whose structure is critical for efficient and accurate processing (Han et al. 2006). Just as do endogenous miRNAs, miR-like shRNAs mature via a two-step biogenesis pathway. The primary transcript, virtually always transcribed by RNA polymerase II, is first cleaved by Drosha in the nucleus to produce a pre-shRNA. This is exported to the cytoplasm, where it is cleaved by Dicer and incorporated into RISC. The passenger strand is then lost to form an active complex.

Natural miRNAs are tightly regulated. Virtually all show defined, tissue-specific expression patterns. Many are also subject to posttranscriptional controls that influence the efficiency of

their maturation by Drosha and Dicer. Thus far, miR-1 and miR-30 appear to be constitutively processed in all cell types examined, but this does not ensure that there will not be some cell type in which their expression is repressed. It is not difficult to create new shRNA scaffolds by remodeling other endogenous miRNAs (e.g., for use in an exotic cell type or model organism). However, empirical experience has shown that not all primary miRNAs tolerate restructuring, so several must be tested. It is also a good guideline to choose an miRNA that is broadly and highly expressed as a backbone for creating artificial structures.

Several large-scale collections of *Drosophila* strains have been built in which a substantial fraction of fly genes have been targeted by long inverted repeat RNAs (Dietzl et al. 2007; Ni et al. 2009). However, given the flexibility, specificity, and potency of shRNAs, these are becoming both more commonly used and more highly recommended. Although the miR-1 backbone is the standard choice for fly shRNA construction at the time of the preparation of this protocol, it must be appreciated that this is a fast-moving area and the user is cautioned to investigate the most current literature available on this topic.

Note that the many advantages of encoded triggers for RNAi are balanced by several drawbacks. Expression from integrated transgenes does not generally yield as high an intracellular concentration of trigger as can be achieved by introduction of RNA. Therefore, better-designed, more potent triggers are often necessary to achieve the same degree of effect. Constructs containing inverted repeats can be unstable, both in bacteria and in mammalian cells. Triggers are often introduced into mammalian cells through the use of viral vectors, which require specific biosafety precautions. The generation of transgenic animals, either flies or mammals, requires both specialized skills and equipment. Overall, the effort of generating stable RNAi alleles is usually well spent, because they permit sophisticated analyses of gene function in vivo for an investment in time and resources that would be generally impossible to match using more conventional genetic methods.

Designing *Drosophila* shRNAs

In *Drosophila*, there are a few twists on the process of designing shRNAs that must be appreciated. Flies have two Argonaute proteins, Ago1 and Ago2, that are dedicated to the miRNA and endo-siRNA pathways, respectively. Although shRNAs are miRNA mimetics, it is important that they be funneled into the endo-siRNA pathway for maximum potency (Czech et al. 2008; Ghildiyal et al. 2008; Haley et al. 2008; Okamura et al. 2008). Although the Dicers, their obligate cofactors, and Argonautes differ for each of the two small RNA fates, they are not completely separate, and it is possible to direct an artificial miRNA selectively into Ago2 based on insights into rules for small RNA sorting that have emerged in the past 2 yr (Czech et al. 2009; Okamura et al. 2009; Ghildiyal et al. 2010).

Algorithms developed for mammalian siRNA design seem to work effectively also for *Drosophila* cells, demonstrating conservation of many of the essential features of effective silencing triggers. Loading of those RNAs into Ago2 can be directed by maintaining a perfectly paired stem instead of an miRNA-like stem, which almost invariably contains one or more bulged residues. Although siRNAs are designed as 21-nucleotide species, the small RNAs that emerge from the miR-1 backbone will be 22 nucleotides in length. The design

principles of the construct compensate for this difference and result in a nucleotide being appended to the 3' end of the guide strand, which is not thought to impact substrate specificity.

Generating UAS-shRNA Lines

For expression in flies, the miR-1 cassette is generally cloned downstream from a basal promoter linked to multiple copies of upstream activator sequences (UASs) for the Gal4 transcriptional activator. These are combined with specific Gal4 enhancer-trap transgenes (also known as Gal4 drivers) in which the expression of Gal4 is under the control of various tissue- and developmental-stage-specific enhancer sequences. In this way, siRNAs can be generated from long- or short-hairpin-based silencing triggers, and endogenous genes can be silenced under spatial and temporal control (Brand and Perrimon 1993). The versatility of the modular Gal4/UAS system and the large collection of available Gal4 driver lines offer significant advantages for using *Drosophila* as a model system to interrogate gene function via RNAi.

Two approaches can be used to generate UAS-shRNA lines. The first is P-element-mediated transposition that leads to random insertion of the UAS-hairpin construct into the genome. A second approach is the insertion of transgenes via site-specific recombination into genomic loci known to be advantageous for expression (Groth et al. 2004; Markstein et al. 2008). These two approaches use different vector backbones. Most P-element-based transgenic RNAi vectors are modified from the original pUAST vector (Brand and Perrimon 1993), which typically carries a mini-white gene as selection marker and an expression cassette consisting of multiple GAL4-responsive UAS elements, the basal hsp70 promoter, an intronic sequence, and a polyadenylation signal sandwiched between the minimal P-element sequences, which are required for genomic integration. Sequences are inserted into cloning sites that are placed downstream from the basal hsp70 promoter. For shRNAs, inserts for cloning can be prepared simply by annealing synthetic oligonucleotides (Fig. 3). However, it should be noted that the same vectors can also be used for expression of long inverted repeat hairpins, necessitating a two-step cloning in which serial insertion of PCR fragments creates the inverted repeat.

The VALIUM vectors used in the site-specific integration system contain *vermilion* as a selectable marker and an *attB* sequence to allow ϕ C31-targeted integration at genomic *attP*-landing sites (Bischof et al. 2007). On the basis of the finding that UAS sites promote transcription in an additive fashion, the VALIUM vectors contain two pentamers of UAS sequences, one of which can be removed using the Cre/*loxP* system. VALIUM vectors use the *hsp70* TATA promoter, which has been shown to be effective in UAS vectors.

A number of parameters have been thoroughly tested to achieve optimal RNAi potency, including the placement of insulator sequences to increase hairpin expression, the identity, number, and location of intronic sequences (to reduce difficulties in hairpin propagation in bacteria), the identity of the polyadenylation signals, and the orientation of the hairpin. The latest vector as of the preparation of this protocol, VALIUM20 (Fig. 1), has been showed to be highly effective for RNAi knock-down, but the experimenter is cautioned to stay informed regarding the almost continuous vector development in the field.

The targeted method to generate shRNA lines has many practical advantages over P-element-based methods. The frequency of recovering transformants using the integrase method, either following coinjection of integrase mRNA or by injecting into the nanos-integrase strain, is almost fivefold higher than in conventional P-element transformation. Establishment of the lines is greatly facilitated because no mapping of the transformants to a specific chromosome (for the purpose of future crosses) is needed. Moreover, unlike P-element-based methods, insertions into the *attP*-landing sites are usually homozygous viable. Because the potency of transgenic RNAi depends on the level of expression of the UAS-driven constructs, the VALIUM vectors with their modular number of UAS copies allow the generation of a phenotypic series. From the original 10XUAS construct, a 5XUAS derivative can be recovered, and because most *attP*-containing chromosomes can be made homozygous, it is also possible to generate both 15XUAS and 20XUAS combinations (Ni et al. 2008). The ability to generate a phenotypic series from 5XUAS to 20XUAS may prove to be useful, in particular, when variation of the gene expression dosage is important for phenotypic studies of genes with pleiotropic impacts.

RECIPES

Annealing Buffer

Tris-HCl (12 mM, pH 7.5)

NaCl (0.12 M)

EDTA (1.2 mM)

Buffer for DNA Ligase (10×)

Reagent	Quantity (for 1 mL)	Final concentration
Tris-HCl (1 M, pH 7.5)	0.5 mL	500 mM
MgCl ₂ (1 M)	0.1 mL	100 mM
DTT (1 M)	0.1 mL	100 mM
ATP (100 mM)	0.1 mL	10 mM
H ₂ O	0.2 mL	

Store for 6 mo at −20°C.

Injection Buffer (1×)

NaH₂PO₄ (0.1 mM, pH 6.8)

KCl (5 mM)

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FIGURE 1.

Map of VALIUM20, an example of an optimized vector for creating transgenic flies carrying shRNA inserts. It uses the ϕ C31 recombinase for site-specific integration into any of a number of different, well-characterized landing sites in the *Drosophila* genome. The shRNA is driven by a minimal *hsp70* promoter linked to up to 10 UASs for activator binding. This permits tissue-specific expression using any of the many Gal4 driver lines that are available. The vector carries a *vermillion* marker to track integration and has been optimized by the placement of introns and insulator sequences to promote effective shRNA expression. In this case, miR-1-based inserts are cloned into *NheI* and *EcoRI* sites in the vector polylinker as described.

Long inverted repeat (200–500 bases)

Classic short hairpin RNA (stem = 19–29 bases)

MicroRNA mimetic shRNA (e.g., modeled on MIR-1 [fly] or MIR-30 [mammal])

FIGURE 2.

Hairpin strategies for triggering RNAi. Three basic strategies are used for encoded RNAi. The first uses a long inverted repeat that can be transcribed by RNA polymerase II or III. This results in a structure that is capped or initiates with a triphosphate. Dicer presumably initiates processing at a random location within the double-strand region and produces a sampling of siRNAs. Classic short hairpins are simple inverted repeat structures. These are almost always transcribed using RNA polymerase III promoters to give structures with 19–29-nucleotide stems, 5' triphosphates, and one or more Ts at the 3' end of the transcript. The processing pathway for these is not well studied, but at least in cases of longer stems does involve Dicer cleavage. miRNA mimetic shRNAs are carried in a specific miRNA-derived scaffold and are processed to intermediate pre-miRNAs (shown) by Drosha. These have stems of generally >21 bases and bear a 5'-phosphate group. This structure is cleaved specifically by Dicer, which appears to recognize and cut a precise distance from the overhung end.

FIGURE 3.

Cloning strategies for encoded triggers. (A) Cloning long inverted repeats is generally a two-step process. A PCR product, generally designed in the manner one would use for creating a template for synthesis of dsRNA, is inserted into a vector downstream from an RNA polymerase II promoter. A second copy of the fragment is then inserted adjacent to the first in the opposite orientation. Often, a marker or an intron is inserted between the two halves of the hairpin to stabilize the structure in *E. coli*. (B) “Classic” shRNAs are often constructed simply by annealing two synthetic oligonucleotides such that the double-stranded product

has the appropriate overhangs for cloning into the recipient vector. In this case, transcription by RNA polymerase III initiates at the first base of the inverted repeat and termination at a stretch of Ts adds an overhang of several (usually two to four) bases. (C) Cloning of miRNA mimetic shRNAs is similar, in that inserts can be most easily made by the annealing of two oligonucleotides. The product is then cloned into a vector that embeds the inverted repeat within a fragment of the primary transcript of an miRNA (pri-miR, not shown to scale). Virtually any strong RNA polymerase II promoter can be used to drive shRNA expression, including inducible and tissue-specific promoters. Often, a functional spacer is placed between the promoter and the beginning of the pri-miR cassette, which both reports indirectly the expression level of the shRNA and seems (for reasons unknown) to enhance shRNA processing.