Published in final edited form as:

Methods Mol Biol. 2009 ; 485: 257–270. doi:10.1007/978-1-59745-170-3_18.

Rapid, controlled and intensive lentiviral vector-based RNAi

Manuel Llano[#], Natassia Gaznick, and Eric M. Poeschla[¶]

Molecular Medicine Program, Mayo Clinic College of Medicine, Rochester, MN 55905

Abstract

RNAi is a powerful technology for studying the functional significance of genes. The technique is more accessible than gene knockout methods, and is directly applicable to diverse human cells. However, inadequate reductions in target mRNAs can reduce the utility of RNAi and insufficiently rigorous controls can lead to spurious conclusions. Optimally combining pol III promoters to drive short hairpin RNA expression with the gene transfer capabilities of lentiviral vectors has led to ways to perform especially effective and convincing RNAi, which we review here. We detail practical methods, including one-step vector construction. Deep, stable knockdowns to trace mRNA levels are readily achieved in T cell lines, which can then be subjected to comprehensive HIV challenge studies. Rescue of pre-knockdown phenotype by RNAi-resistant gene re-expression is a critical validating step. The methods can also be applied to primary cells T cells and macrophages. The time from thinking of a target to initial data read-out can be a few weeks.

Keywords

RNAi; lentiviral vector; short hairpin RNA (shRNA); T cell; HIV

Introduction

This protocol is designed to allow a scientist familiar with basic molecular biology and cell culture to rapidly and effectively apply two powerful technologies: RNA interference (RNAi) and lentiviral vectors. The biologies that underlie each of these technologies rest on complex foundations, and the development of RNAi is particularly in flux at present, such that comprehensive review is beyond the scope of this protocol. Rather, after brief review of the necessary principles, our emphasis is on practical application such that the worker can attain quickly the goals of stringent knockdown and appropriate control validation as achieved with the intensified lentiviral vector RNAi (ilvRNAi) of Llano et al. [1]. We also describe an update that allows one-step construction of ilvRNAi transfer vectors.

RNAi, a posttranscriptional gene silencing mechanism evolutionarily conserved from *C. elegans* to *H. sapiens*, is triggered by double-stranded RNAs (dsRNA), which are processed by a ribonuclease III-type protein into short double-stranded 21–23 nucleotide fragments called small interfering RNAs (siRNAs) [2–7]. siRNAs become incorporated into a multi-protein complex containing RNA helicase and nuclease activities, the RNA-induced silencing complex (RISC). The helicase activity converts the RNA duplex into a single-stranded RNA that hybridizes to its target RNA and directs the RNA endonucleolytic activity. The catalytic subunit of RISC, Argonaute2, has recently been identified [8, 9]. This

[¶]Address requests for reprints and materials to E.M.P.: Molecular Medicine Program, Guggenheim 1811A, Mayo Clinic College of Medicine, Rochester, MN 55905. emp@mayo.edu.

[#]Present address: Department of Biology, University of Texas at El Paso, El Paso, TX 79968-0519. mllano@utep.edu.

process results in a reduction of specific mRNA levels and hence decreased specific gene expression.

The process can be initiated by intracellular dsRNA synthesis rather than exogenous siRNA addition if appropriate promoters are used to express sense and anti-sense sequences. The two strands can be expressed separately but a more convenient and efficient method is to link them in tandem with a short intervening loop of variable length. The resulting transcript anneals by Watson-Crick base-pairing into a short hairpin RNA (shRNA) which is then processed by the RNAi pathway nuclease Dicer to form a fully functional siRNA. This process mimics the cellular microRNA (miRNA) pathway [10–12].

While siRNAs or plasmid based expression of shRNAs can be quite effective, in those not uncommon situations where small amounts of the target protein retain significant biological activity, more powerful RNAi systems must be pursued or a gene-level knockout obtained. In addition, siRNA oligonucleotides can be toxic, have poor uptake in many cells, and present problems with non-specific inhibition.

An option that can address a number of these problems is to introduce shRNA expression cassettes into viral vectors. Lentiviral vectors are retroviral vectors that can transduce non-dividing cells [13–15]. We and others have found that shRNA-expressing lentiviral vectors are highly effective for either transient or stable RNAi. In a recent series of experiments to target the lentiviral integrase-binding protein LEDGF/p75, use of optimized lentiviral vectors in human T cell lines allowed both a graded intensification of RNAi, and much deeper knockdown than is obtainable with either siRNAs or selectable marker-bearing plasmids [1]. Moreover, inclusion of fluorescent proteins in the same lentiviral allowed enrichment of the most intensively knocked down cells by fluorescence-activated cell sorting (FACS). Moving from siRNAs or plasmid-based shRNA expression (transient or stable) to *ilv*RNAi was crucial for understanding the role of LEDGF/p75 since a fractionally quite small residuum of this protein persisted in a chromatin-bound form (i.e., situated potentially, bound to one substrate of the integration reaction) after the latter approaches. Despite dramatic sub-cellular phenotypes seen with HIV integrase over-expressed outside the viral context, such cell lines supported wild-type levels of viral integration. Experiments with *ilv*RNAi definitively established the integration cofactor role of LEDGF/p75 and were equally revealing from the RNAi methodological perspective [1]. They showed what conditions were needed to push well-controlled RNAi to this level, by testing it in what was in effect a highly stringent bioassay with a convincing endpoint (blocking HIV infection) that had eluded prior attempts with siRNAs and plasmid based shRNA expression.

This chapter describes construction, production and use of lentiviral vectors to perform highly effective stable knockdowns in T cells, analyses of knockdown extent, use of control shRNAs, and the important control of re-expressing the target protein by retroviral transduction of shRNA resistant cDNAs. Co-expression of other proteins in the same vector (in our case, fluorescent protein-fused dominant-interfering proteins) is also reviewed. The basic lentiviral vectors are now adapted for one-step cloning. An overview of the methodology is depicted in Fig. 1. There are six main steps:

1. Identify the target sequence and synthesize and anneal two oligonucleotides to form an shRNA-encoding adaptor.
2. Insert the adaptor into the lentiviral vector using standard recombinant DNA methods. One step cloning is now possible with current versions. Other proteins or GFP fusions can also be expressed in the vector (see Note 1).
3. Produced particles by transient co-transfection with a lentiviral packaging plasmid and a viral envelope pseudotyping plasmid.

4. Transduce target cells with the lentiviral vector particles. A control shRNA-encoding lentiviral vector should be used to derive a control cell line.
5. Assay directly for phenotype or if a more stringent knockdown is desired, first enrich by sorting for higher mCherry or eGFP expression in both active and control shRNA lines.
6. Re-express the gene in both lines with an shRNA-resistant cDNA allele (i.e., one with synonymous mutations at the shRNA target site) to ensure that the effects are shown to be specific by rescue of pre-knockdown function. Re-expression in the control shRNA line controls for over-expression of the protein.
7. Assay phenotype in all four lines.

The techniques are readily adopted by laboratories conversant in basic molecular biology and cell culture. LEDGF/p75 knockdown (as in ref. [1]) is used as an example and we emphasize methods we have found highly effective in human T cells because of our interest in HIV biology but the lentiviral vectors are widely tropic because they are pseudotyped and are easily adapted to other RNA targets, cell lines and purposes. Detailed discussion of the burgeoning RNAi armamentarium – the subject of a literature explosion in the past two years -- is beyond our purview here. In addition, other iterations of lentiviral vector-based RNAi that include regulated shRNA expression have recently been published [16–19].

Materials and Methods

Basic shRNA expression parameters

Pol III promoters are useful for shRNA expression because the defined transcriptional start and precise termination signals (a polythymidine tract) generate the requisite RNA termini. Certain exogenously introduced pol III promoters are highly active in human T cell lines and in our hands the human U6 promoter was superior to the H1 promoter (see Note 2). Reverse orientation with respect to the lentiviral vector transfer vector transcript and location upstream of internal pol II marker gene segments also proved ideal (Fig. 2).

Active siRNAs are typically composed of two hybridized 21 mer RNAs with 19 complementary nucleotides and 3' dinucleotide overhangs, so the design process begins with identification of candidate 21 nt sequences in a given RNA target. Longer shRNA sequences have also been used, however, and may improve DICER processing and RNAi efficacy (e.g., see [20]), although sequences longer than 29 nt risk inducing a PKR response [21]. The intervening loop is not strictly constrained and we have simply continued to use the 9 nt loop TTCAAGAGA described by Brummelkamp et al. [10].

Various algorithms for specific shRNA design are available (e.g., [22]) or <http://sfold.wadsworth.org/>. The Ambion web platform is a convenient and accessible tool for choosing candidate shRNAs <http://www.ambion.com>. Fig. 3A shows the human U6 promoter arrangement we used in ref. [1] and Fig. 3B a subsequently re-engineered version that allows one-step insertion of an shRNA adaptor into the lentiviral vector.

¹We used single vector expression of both an shRNA and a dominant-interfering GFP fusion to the LEDGF/p75 integrase binding domain (IBD), by using GFP-IBD in place of the mCherry marker. The combination of RNAi plus DI protein results in over three logs inhibition of HIV-1 replication (Fig. 3E of ref. [1] and unpublished data).

²The H1 promoter may have more activity depending on the cell target; in addition various other options including pol II expression have been useful in other's hands [27, 28]. We have not observed U6 transcript toxicity in CD4+ human T cell lines, but this has been reported to occur in primary human lymphocytes [29].

Construction methods

These directions assume use of the newer transfer vector illustrated in Fig. 3B, which allows one-step cloning of a gene-specific transfer vector. Previously [1], a cumbersome insertion of shRNAs into BamHI and Hind III sites of a donor plasmid, followed by PCR amplification of the U6-shRNA cassette and insertion into the lentiviral transfer vector was needed. Here, a single cloning step is followed by lentiviral particle production.

I.1 Experimental Procedures

- I.1.1** Dilute the sense and the antisense shRNA templates in TE buffer to 1 µg/µl by determining the absorbance at 260 nM and multiplying the A260 by the dilution factor and the extinction coefficient (consider this to be -33 µg/µl; see Note 3).
- I.1.2** Mix 2 µl of each oligonucleotide with 46 µl of 1X DNA annealing solution (10mM Tris, pH 7.5 – 8.0, 50mM NaCl, 1mM EDTA; the annealing buffer supplied by Ambion in pSilencer kits is also effective).

Incubate at 90°C for 3 mins followed by cooling to 37°C in a thermocycler, with a plateau at 37°C for one hour.
- I.1.3** Dilute 5 µl of the annealed oligonucleotides in 45 µl of nuclease-free water and ligate 4 µl of diluted annealed oligonucleotides with 1 µl of Mlu+Sal-linearized pTSINcherryU6 (see Note 4) using 5U of DNA T4 ligase in a 10ul reaction overnight at 16°C. The ligation can be selected against wild type first (see Note 5) or transformed immediately into *E. coli* with 5 µl of the ligation and plate on ampicillin LBA plates.
- I.1.4** Screen 10 colonies by DNA restriction digest (see Note 6).
- I.1.5** Sequence one or more of the positive constructs with an appropriate primer. (5'-TCATAATGATAGTAGGAGGC-3', which is 147 n upstream of the U6 termination signal, works well).

I.2 Validate knockdown activity—Co-transfect shRNA expression plasmid and epitope-tagged target cDNA in 293T cells and evaluate target protein levels by immunoblotting and/or mRNA levels by RT-PCR. This preliminary screening step is easier than making lentiviral vector particles and allows one to prioritize the most effective shRNAs. Generally about one in five shRNAs is effective but this is highly variable. Various algorithms are available to predict efficacy of candidate shRNAs, but we have good success simply by choosing 21 nt sequences that (i) begin with an AA dinucleotide (the subsequent 19 nt are inserted in the vector); (ii) have no more than 50% GC content; and (iii) do not have homonucleotide runs greater than 3.

- I.2.1** Plate 293T cells at up to 60% confluence in DMEM supplemented with 10% FCS and penicillin, and streptomycin (DMEM culture medium). Harvest cells by trypsinization the next day and plate them at 0.45×10^6 cells in 2 mls per well in a 6 well plate.

³HPLC-purified oligonucleotides are preferred.

⁴Alternatively the XhoI site in the pTSINcherryU6 polylinker, shown in Fig. 3, can be used, provided the adaptor is similarly modified to have an XhoI rather than a SalI overhang.

⁵Option: Before transformation, the ligation can be heated to 65°C for ten minutes and then digested with PmeI (see Fig. 3C) to select against ligated plasmids lacking an insert. XhoI can be used for this purpose as well if the SalI site is used for the shRNA insertion.

⁶Screening of the colonies is facilitated if the cloned shRNA template sequence has an informative restriction site but this is unusual. For visualizing the insert directly (the 68 bp band resulting from Mlu I+Sal I digestion use about 1/3 of the miniprep DNA. An easier method is to screen with SacII + SalI which will drop out a 397 bp band.

- 1.2.2** The following day, transfect the cells by the calcium-phosphate co-precipitation method, using all transfection reagents at room temperature. For transfection of each well dilute a total of 2 µg of the shRNA expression plasmid in 1mM Tris, pH 7.4, 0.1 mM EDTA up to a final volume of 121.5 µl. To this mix add 13.5 µl of 2.5 M CaCl₂, vortex briefly. Add 135 µl of 2X HBS (50mM Hepes pH 7.1, 280mM NaCl, 1.5 mM Na₂HPO₄; see Note 7 for preparation) and vortex immediately for 1 sec. Incubate the transfection mix for five min at room temperature and then distribute evenly in the well by droplet pipetting.
- 1.2.3** Sixteen to eighteen hours after transfection remove the cell culture medium gently and carefully add 2 mls of fresh DMEM culture medium per well so as not to disrupt the loosely adherent 293T monolayer.
- 1.2.4** Culture the transfected cells for an additional 48 hours and then determine by immunoblotting the expression levels of the targeted gene (see Note 8).

II. Production and titration of ilvRNAi vectors—The basic procedure is to co-transfect a packaging plasmid that expresses Gag/Pol, an ilvRNAi transfer vector derived from pTSINcherryU6, and an envelope construct. The latter is generally vesicular stomatitis virus glycoprotein G (VSV-G) because of the high titers, broad tropism and stability it affords [23]. VSV-G-pseudotyped three or four (the latter express Rev from a separate construct) plasmid lentiviral vector systems should be used at the appropriate biosafety level determined by the particular institutions IBC. Generally these are used at the BL2+ level. Several good HIV packaging plasmids are available [19, 24].

Producing and titering lentiviral vectors by calcium-phosphate co-transfection in 293T cells.

- II.1** Harvest 293T cells (60% confluence) and plate 3×10^6 cells in 12 mls in a 75 cm² flask. Plate eight flasks per vector. Scaled up procedures using high area cell factories are described in ref. [25] but generally are unnecessary for deriving knocked down cell lines.
- II.2** Transfect the cells the next day by the calcium-phosphate co-precipitation method, using all transfection reagents at room temperature. For transfection of each 75 cm² flask dilute 10 µg of the control shRNA-expressing (see Note 9) or the specific shRNA-expressing transfer plasmid, 10 µg of an HIV vector packaging plasmid (see Note 10) and 3.3 µg of a VSV-G expression plasmid (e.g., pMD.G) in 1mM Tris up to a final volume of 720 µl. To this mix add 80 µl of 2.5 M CaCl₂, vortex for 1 sec. and then add 800 µl of 2X HBS pH 7.1 and vortex immediately for 1 sec. (Note that this process can be batched for convenience if desired, with precipitate for all eight flasks prepared in one larger

⁷2XHBS stock: dibasic Na₂HPO₄: 52.5 g Na₂HPO₄, 5000 ml H₂O. 2xHBS: 80 g NaCl, 65 g HEPES (sodium salt), 100 ml Na₂HPO₄. pH optimization of 2XHBS is an important variable. A frequently used method is to prepare several batches and adjust the pH with 1 N NaOH to several set points within a narrow range, e.g., pH 6.95, 7 and 7.05. Bring to final volume of 5000 ml. Optimal pH is generally 6.95 in our hands but final preferred stocks will depend on empirical determination of transfection efficiency obtained. Store frozen (-20 deg C). The pH will shift with time, as may percent transfection obtained. We generally prepare it new 3–6 months. Optimal transfection reagents will yield 90–95% transfection of 293T cells.

⁸For validation of the activity of the shRNA the expression of the endogenous target gene can be evaluated in cells transiently expressing the shRNA expression plasmid. This approach is especially useful if the target gene is robustly expressed. Alternatively equal amounts of plasmids expressing the shRNA and the target gene can be transiently co-expressed in cells. This method is particularly advantageous in the case that the target gene is poorly expressed. Also co-expression of the shRNA and its epitope-tagged or GFP-tagged target will minimize the diluting effects of low transfection efficiency on the validation of the shRNA.

⁹The Ambion control “scrambled” shRNA (actaccgtgttatagtg) is functionally neutral even when transduced at high MOI.

¹⁰We have generally used pCMVΔR8.9 (encodes Gag/Pol, Tat and Rev) [24]; the combination of pLP1 and pLP2 (Invitrogen) or pCHGP plus pCMV-Rev from Rossi and colleagues supply Rev and Gag/Pol expression separately [19]. For FIV or EIAV vectors, the PpuM1 cassette in Fig. 3B can be used within an FIV or EIAV transfer vector; however for human T cell lines, HIV vectors are more efficient [25, 30]. Detailed aspects of lentiviral vector production can also be found in ref. [25].

tube). Incubate the transfection mix for five mins at room temperature and then add it to the medium drop-wise; swirl very gently to mix without dislodging the 293T monolayer.

- II.3** Remove the cell culture supernatant sixteen to eighteen hrs after transfection and add 12 mls of fresh culture medium per flask.
- II.4** Culture transfected cells for an additional 48–60 hours and then harvest the tissue culture supernatant (vector particle supernatant).
- II.5** Clarify viral supernatant by centrifugation at 800 *g* for 10 min and then filter through a 0.45 μ m membrane. Layer 3 volumes of clarified supernatant on to 1 volume of 20% sucrose in 50 mM Tris pH 7.4, 100 mM NaCl. Ultracentrifuge at 100,000 to 125,000 *g* for 2 hrs in a swinging rotor at 4°C. Re-suspend each vector pellets in one ml of RPMI 1640 10% FCS. Store concentrated vector at –80°C (see Note 11).
- II.6** Titration of lentiviral vectors. Transduce 10⁵ target cells with 3, 10, 30 and 90 μ l of the concentrated vector supernatant. 16 hrs after transduction, wash cells to remove the input vector. Analyze mCherry expression by flow cytometry 72 hrs later.

III. Generation of stable cell lines—Target cell lines can be generated at different multiplicity of infections (MOIs), cryopreserved and the phenotype analyzed. The efficiency of the vectors, their integrated state, and, if desired, the possibility of immediate enrichment by fluorescence-activated cell sorting, allow stable cell lines with graded intensities of RNAi to be derived. However, stable cell lines may not be feasible or desired in some cases, and immediate analyses can be performed (see Note 12).

- III.1** Plate 10⁵ target cells and transduce them at a range of MOIs (e.g., 10, 50, 150, 300) with the specific shRNA-expressing vectors. Do the same in parallel with the control shRNA vector. 16 hrs after transduction wash to remove the input vector, and expand the culture until 10⁷ cells are available.
- III.2** Using fluorescence-activated cell sorting for mCherry, select both active and control shRNA cells for the 10% brightest cells. Expand cells to the appropriated number for phenotype analysis (See note 13).
- III.3** Verify knockdown levels by immunoblotting and/or RT-PCR and also analyze the relevant phenotype of the cells for your particular experimental needs, pairing control and active shRNA cells in each measurement (see Note 14). A complete set of data requires that re-expression of the target protein rescue parental function (step IV below).

¹¹The vector pellet is usually not visible. To resuspend it, distribute 1ml of ice-cold RPMI 10% FCS among all the tubes and incubate the tubes on ice for 15 mins, flick the tubes occasionally. Then gently pipette up and down with an automatic pipette approximately 10 times, while distributing the medium around the bottom of the tube; avoid foaming. Then collect the entire vector-containing medium and distribute it into 100 μ l aliquots.

¹²Stable cell lines greatly facilitate rigorous downstream analyses and reproducibility. However, some knockdowns result in loss of cell viability or other toxicity. In that case analyses can be done within a shorter time frame similar to that of an siRNA transfection (but the non-specific chemical toxicity of siRNAs is avoided). The same methods can be used, including sorting for high-expressers.

¹³Transduced cells will express both the shRNA and the mCherry fluorescent protein at proportionate levels. Thus, cells with a higher level knockdown can be obtained by sorting the 10% brightest cells. Sorting should be performed on control and specific shRNA expressing cells using the same gating criteria, preferably in parallel on the same day, using the same apparatus.

¹⁴Control and specific knockdown cultures are expected to have a similar growth rate, which is easily assayed by cell counting or other assays. For immunoblotting analysis of chromatin bound proteins it is advisable to directly interrogate this compartment [1]; similar considerations may apply to other sub-cellular compartments.

IV. Back-complementation of knockdown cell lines—RNAi is powerful but vulnerable to mis-interpretation if not properly controlled. To demonstrate the specificity of the observed phenotype and exclude off-target effects, knockdown cells should be engineered to re-express the targeted protein [26]. The importance of this control cannot be over-emphasized. We describe here the use of Murine leukemia virus (MLV)-derived vectors as an approach for efficient back-complementation (see Note 15).

- IV.1** A cDNA containing synonymous mutations in the shRNA target sequence (we mutated 7 within the 19 nt) is inserted into the MLV transfer vector diagramed in Fig. 4.
- IV.2** MLV vector production. We then follow steps III.1 to III.5 with the following variations. We use the MLV packaging cell line Phoenix A (obtained from G. Nolan, Stanford). We transfect 3×10^6 cells with 10 μ g of the MLV transfer vector and 3 μ g of pMD.G. Supernatant from a total of eight 75 cm² flasks (96 mls) is concentrated on a 20% sucrose cushion, and resuspended in one ml of 10% RPMI 1640.
- IV.3** Generation of the complemented cell lines.
 - IV.3.1** Baseline G418 kill curve. Plate 10^5 knocked down cells/ml in a 24 well plate and add 1 ml of culture medium containing different amounts of G418; use as control cells cultured in the absence of G418. Select the minimal dose that kills all the cells in one week.
 - IV.3.2** Transduce 10^5 knocked down cells with 30, 90 and 270 μ l of the concentrated MLV vector (see Note 16). 16 hrs after transduction remove the input vector by washing and culture the cells for 48 hrs before adding G418 at the determined level. Expand cells in selection medium to the number needed for phenotypic analysis and cryopreservation.
 - IV.3.3** Evaluate re-expression by immunoblotting. Select cells expressing levels of targeted protein that are similar to the unmodified original cell line.

Acknowledgments

We thank I. Kemler for assisting N. G. with pTSINcherryU6 and pTSINcherryU61340 construction and D. T. Saenz and M. Meehan for assistance with multiple aspects of lentiviral vector experiments. We are grateful for funding from NIH AI 77344.

References

- Llano M, Saenz DT, Meehan A, Wongthida P, Peretz M, Walker WH, Teo W, Poeschla EM. An Essential Role for LEDGF/p75 in HIV Integration. *Science*. 2006; 314(5798):461–4. [PubMed: 16959972]
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391(6669):806–11. [PubMed: 9486653]
- Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*. 1999; 286(5441):950–2. [PubMed: 10542148]
- Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*. 2000; 101(1):25–33. [PubMed: 10778853]

¹⁵Re-expression can also be achieved by stable plasmid transfection [31].

¹⁶The most complete approach will be to also conduct parallel expression with the MLV vector in control cells to assess confounding effects of over-expression or of exogenous expression.

5. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*. 2000; 404(6775):293–6. [PubMed: 10749213]
6. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet*. 2001; 2(2):110–9. [PubMed: 11253050]
7. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*. 2001; 409(6818):363–6. [PubMed: 11201747]
8. Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L. Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat Struct Mol Biol*. 2005; 12(4):340–9. [PubMed: 15800637]
9. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 is the catalytic engine of mammalian RNAi. *Science*. 2004; 305(5689):1437–41. [PubMed: 15284456]
10. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science*. 2002; 296(5567):550–3. [PubMed: 11910072]
11. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev*. 2002; 16(8):948–58. [PubMed: 11959843]
12. Zeng Y, Wagner EJ, Cullen BR. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell*. 2002; 9(6):1327–33. [PubMed: 12086629]
13. Naldini L, Bloemer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272(5259):263–7. [PubMed: 8602510]
14. Poeschla E, Wong-Staal F, Looney D. Efficient transduction of nondividing cells by feline immunodeficiency virus lentiviral vectors. *Nature Medicine*. 1998; 4(3):354–357.
15. Olsen JC. Gene transfer vectors derived from equine infectious anemia virus. *Gene Therapy*. 1998; 5(11):1481–1487. [PubMed: 9930301]
16. Morris KV, Rossi JJ. Lentiviral-mediated delivery of siRNAs for antiviral therapy. *Gene Ther*. 2006; 13(6):553–8. [PubMed: 16397511]
17. Szulc J, Wiznerowicz M, Sauvain MO, Trono D, Aebischer P. A versatile tool for conditional gene expression and knockdown. *Nat Methods*. 2006; 3(2):109–16. [PubMed: 16432520]
18. Wiznerowicz M, Szulc J, Trono D. Tuning silence: conditional systems for RNA interference. *Nat Methods*. 2006; 3(9):682–8. [PubMed: 16929312]
19. Aagaard L, Amarzguoui M, Sun G, Santos LC, Ehsani A, Prydz H, Rossi JJ. A Facile Lentiviral Vector System for Expression of Doxycycline-Inducible shRNAs: Knockdown of the Pre-miRNA Processing Enzyme Drosha. *Mol Ther*. 2007; 15(5):938–45. [PubMed: 17311008]
20. Siolas D, Lerner C, Burchard J, Ge W, Linsley PS, Paddison PJ, Hannon GJ, Cleary MA. Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol*. 2005; 23(2):227–31. [PubMed: 15619616]
21. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol*. 2003; 5(9):834–9. [PubMed: 12942087]
22. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. *Nat Biotechnol*. 2004; 22(3):326–30. [PubMed: 14758366]
23. Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90(17):8033–7. [PubMed: 8396259]
24. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nature Biotechnology*. 1997; 15(9):871–5.
25. Saenz, DT.; Barraza, R.; Loewen, N.; Teo, W.; Poeschla, E. Production and Use of Feline Immunodeficiency Virus (FIV)-based lentiviral vectors. In: Rossi, J.; Friedman, T., editors. *Gene Transfer: A Cold Spring Harbor Laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 2006.
26. Whither RNAi? *Nat Cell Biol*. 2003; 5(6):489–90. [PubMed: 12776118]

27. McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA. Gene silencing using micro-RNA designed hairpins. *Rna*. 2002; 8(6):842–50. [PubMed: 12088155]
28. Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci U S A*. 2005; 102(37):13212–7. [PubMed: 16141338]
29. An DS, Qin FX, Auyeung VC, Mao SH, Kung SK, Baltimore D, Chen IS. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther*. 2006; 14(4):494–504. [PubMed: 16844419]
30. Saenz DT, Teo W, Olsen JC, Poeschla E. Restriction of Feline Immunodeficiency Virus by Ref1, LV1 and Primate TRIM5a Proteins. *Journal of Virology*. 2005; 79(24):15175–88. [PubMed: 16306589]
31. Llano M, Vanegas M, Fregoso O, Saenz DT, Chung S, Peretz M, Poeschla EM. LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral pre-integration complexes. *J Virol*. 2004; 78(17):9524–37. [PubMed: 15308744]
32. Rose SD, Kim DH, Amarzguioui M, Heidel JD, Collingwood MA, Davis ME, Rossi JJ, Behlke MA. Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res*. 2005; 33(13):4140–56. [PubMed: 16049023]
33. Amarzguioui M, Lundberg P, Cantin E, Hagstrom J, Behlke MA, Rossi JJ. Rational design and in vitro and in vivo delivery of Dicer substrate siRNA. *Nat Protoc*. 2006; 1(2):508–17. [PubMed: 17406276]

Figure 1.

ilvRNAi process. T cell lines are transduced with shRNA expressing lentiviral vectors and maximally transduced cells are selected by cell sorting for mCherry expression (other XFPs are readily used, e.g. eGFP as in [1]). Knockdown cells depleted of the endogenous mRNA are then engineered to re-express the gene using an shRNA-resistant allele. For the latter step we have typically used gamma-retroviral (i.e., murine type C) vectors that co-encode a selectable marker such as *neo*.

Figure 2.

ilvRNAi vector. 400 nt were deleted from U3 to permit U3-anchored PCR assays for challenge virus DNA intermediates (e.g., Alu-PCR and 2-LTR circle assays). The key components are readily inserted in FIV- or EAIV-based lentiviral vectors as well, but HIV-derived lentiviral vectors have superior efficacy compared to these non-primate lentiviral vectors in human T cell lines.

Fig. 3. Promoter-shRNA arrangements in ilvRNAi vectors employing the human pol III promoter

(A) In the lentiviral vectors used in Llano et al. [1], 9 nt (CGGATCCC) replace the native 7 nt (aaacacc) preceding the transcriptional start site in the human U6 promoter. Transcription normally begins 23 nt downstream of the TATA box, at a "G" preceded by a pyrimidine. Here, transcription begins 25 nt downstream of the TATA box. The Bam H1 and Hind III sites used for shRNA adaptor insertion originated in the U6 promoter cassette of the Ambion pSilencer series. The "1340" p75-specific shRNA is shown in red, with an intervening 9 nt loop as an example (the numbering refers to nt 1340 of the p75 reading frame). Because the Bam H1 and Hind III sites for oligonucleotide insertion were not unique in the lentiviral vector this version requires insertion of the annealed shRNA oligonucleotides into an initial U6 promoter-containing template plasmid (such as in the pSilencer series) followed by PCR amplification of the PpuMI flanked region and insertion of the amplicon into the PpuMI site of the lentiviral vector. The version in (B) simplifies the process.

(B) Re-engineered pTSINcherryU6 transfer vector. The vector allows one-step insertion of annealed shRNAs directly into the lentiviral vector. A unique MluI site is used for left hand adaptor insertion and either SalI or XhoI (not shown, but located upstream of SalI in the acceptor plasmid) is used for right-hand adaptor insertion. In addition, this version preserves native 23 nt spacing from the TATA box to the start of U6 promoter transcription and, by using MluI, only three promoter nucleotides (CGT) differ from the normal human U6 promoter. pTSINcherryU6 construction was carried out by amplifying the human U6 promoter with PCR primers

ATATGGGtCCCatcgataCCGCGGacCCcAGTGGAAAGACGCGCAGG and
 AAAAGGGaCCCTTAATTAAGTCGACTCGAGtttaaACGCGTCCTTTCCACAAGAT
 ATATAAACCC. The resulting PpuMI-tailed amplicon was inserted into the PpuMI site of TSINcherry, placing it between the RRE and the cPPT-CTS; this resulted in directional insertion with the U6 promoter in reverse orientation to the lentiviral vector. The resulting transfer vector plasmid is pTSINcherryU6 (Fig. 3C). MluI at the left end, and either XhoI or

SalI at the right end, can be used for shRNA oligonucleotide adaptor insertion and PmeI can be used to select ligations against parental pTSINcherryU6.

(C) A p75-specific shRNA adaptor formed by annealing the two oligonucleotides is shown at top; this was then inserted into the MluI – SalI backbone of pTSINcherryU6 (relevant sequence shown below) to generate the pTSINcherryU61340 vector shown in panel B. To synthesize oligonucleotides that target a different gene, the nt illustrated in red here should be substituted with the appropriate genes-specific complementary sequences. As noted above, rather than the 19 nt insert shown here, some workers prefer longer (27 nt) inserts to produce a better DICER substrate [20, 32, 33].

Figure 4.
MLV transfer vector for re-expressing a knocked down gene.