Chapter 14

Bifunctional Short Hairpin RNA (bi-shRNA): Design and Pathway to Clinical Application

Donald D. Rao, Neil Senzer, Zhaohui Wang, Padmasini Kumar, Chris M. Jay, and John Nemunaitis

Abstract

The discovery of RNA interference (RNAi) engendered great excitement and raised expectations regarding its potential applications in biomedical research and clinical usage. Over the ensuing years, expanded understanding of RNAi and preliminary results from early clinical trials tempered enthusiasm with realistic appraisal resulting in cautious optimism and a better understanding of necessary research and clinical directions. As a result, data from more recent trials are beginning to show encouraging positive clinical outcomes. The capability of delivering a pharmacologically effective dose to the target site while avoiding adverse host reactions still remains a challenge although the delivery technology continues to improve. We have developed a novel vector-driven bifunctional short hairpin RNA (bi-shRNA) technology that harnesses both cleavage-dependent and cleavage-independent RISC loading pathways to enhance knockdown potency. Consequent advantages provided by the bi-shRNA include a lower effective systemic dose than comparator siRNA/shRNA to minimize the potential for off-target side effects, due to its ability to induce both a rapid (inhibition of protein translation) and delayed (mRNA cleavage and degradation) targeting effect depending on protein and mRNA kinetics, and a longer duration of effectiveness for clinical applications. Here, we provide an overview of key molecular methods for the design, construction, quality control, and application of bi-shRNA that we believe will be useful for others interested in utilizing this technology.

Key words: Bifunctional shRNA, Cancer, Gene therapy, Targeted, Clinical

1. Introduction

RNA interference (RNAi), the Nobel prize-winning discovery by Fire and Mello in 1998, has fostered an exponential number of studies and publications furthering our understanding of gene function (1, 2) and stimulating numerous phase I and II clinical trials (3–6). This naturally occurring gene-silencing mechanism by small RNAs, which includes endogenous microRNA (miRNA), is

Debra J. Taxman (ed.), *siRNA Design: Methods and Protocols*, Methods in Molecular Biology, vol. 942,
DOI 10.1007/978-1-62703-119-6_14,

highly dependent on gene sequence; thus the mechanism can, in theory, be used to inhibit the expression of any targeted gene(s) with strong specificity. RNAi is not limited by the pharmacologic constraints inherent to the development of small molecules, which creates an opportunity to access traditionally "undruggable" targets for disease treatment (7–9).

The central player of this mechanism is the RNA-Induced Silencing Complex (RISC). The process starts with double-stranded small RNA (composed of a passenger strand and a guide strand), which is incorporated into the pre-RISC, followed by the cleavage-dependent or cleavage-independent release of the passenger strand to form the guide strand containing RISC (10). The guide strand (antisense to mRNA) guides the RISC to recognize the target mRNA through sequence complementarity (full or extended partial) (11). A key component of the RISC is the family of Argonaute proteins (Ago), Ago 1, 2, 3, and 4 in mammalian systems, of which only Ago 2 has endonuclease activity so as to allow for cleavage of the target mRNA for further degradation (cleavage-dependent pathway) (10, 12); all the Ago containing RISCs can function through a cleavage-independent pathway resulting in translation repression and mRNA sequestration in *p*-bodies with subsequent degradation (13, 14). The cleavage-dependent process requires extensive homology between the guide strand and both the passenger strand and target mRNA, particularly in the central region; the cleavage-independent process, on the other hand, only requires partial homology between the guide strand and both the passenger strand and target mRNA (15–17).

RNAi can be triggered either by synthetic double-stranded small interfering RNA (siRNA) or by vector-driven short hairpin RNA (shRNA) (5, 18). Both siRNA and vector-driven shRNA have been demonstrated to be effective in in vitro and in vivo applications, each with their respective advantages. Most siRNAs are structurally designed to promote efficient incorporation into the Ago2 containing RISC; the RNase III containing Dicer-substrate design improves the efficiency of siRNA at least tenfold by initial association and processing at the pre-RISC (19). Vector-driven shRNA utilizes the host microRNA biogenesis pathway, which appears to be more efficient (20, 21). siRNA is more readily chemically modified while shRNA expression can be modulated and regulated by specific promoters.

We have recently developed a novel vector-driven shRNA technology, the bifunctional shRNA (bi-shRNA), to further improve the efficiency of RNAi by harnessing both cleavage-dependent and cleavage-independent pathways of RISC loading in one preprogramed molecule (Fig. 1) (18, 21). The vector-driven bi-shRNA consists of two stem–loop structures for each mRNA target sequence; one stem–loop shRNA has perfect complementarity at the stem and the second stem–loop shRNA contains mismatches on the passenger

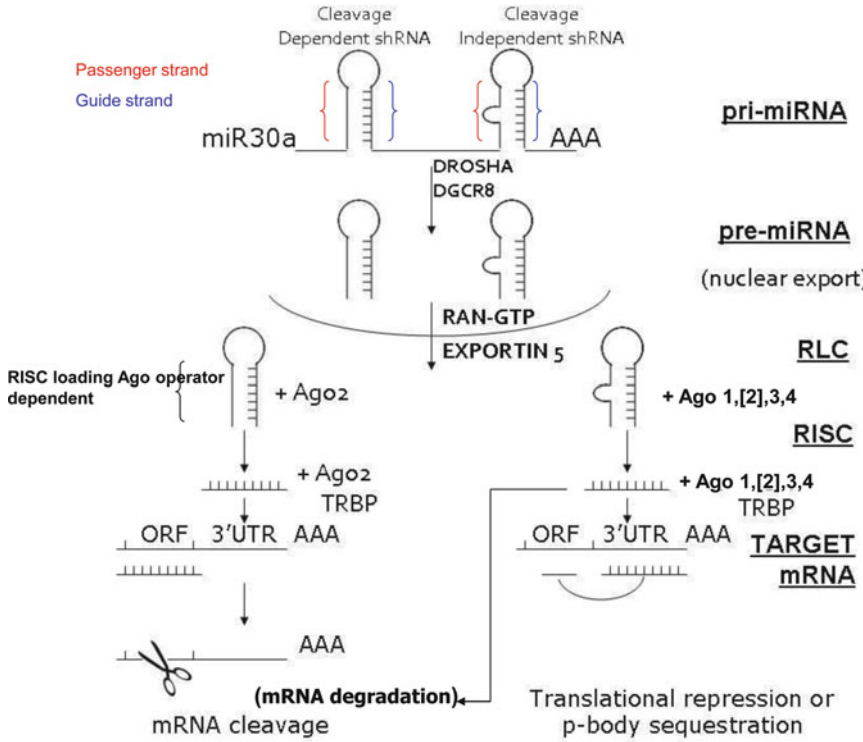

Fig. 1. Schematic showing the principle of bifunctional shRNA. Like a significant percentage of intronic miRNA, the primary transcripts of bi-shRNA are first synthesized in the nucleus by RNA polymerase II. The primary transcripts are quickly processed by the microprocessor, primarily consisting of Drosha and DGCR8, into individual stem–loop structures. The stem–loop structures are exported from the nucleus to the cytoplasm via Exportin 5 and/or Exportin 1 (CRM1), where upon association with Dicer and RISC loading complex (RLC), the loop of the stem–loop structures are removed. Upon loading onto multiple types of Ago containing RISCs, the passenger strands of bi-shRNA depart through either a cleavage-dependent or cleavage-independent process so that the guide strands are effectively loaded onto multiple types of Ago containing RISCs to more efficiently execute RNAi through either mRNA cleavage and degradation or through translational repression, p-body sequestration and degradation.

strand of the stem. Importantly, following incorporation into the RISC, the guide strands derived from each of the two structures are fully complementary to the mRNA target sequence but are associated with different Ago containing RISCs. The bi-shRNA design leads to more rapid onset of gene silencing, higher efficacy, and greater durability when compared with either siRNA or conventional shRNA (21). We are currently transitioning personalized cancer therapy with target-specific bi-shRNA into the clinic in Phase I studies using a modified bilamellar invaginated liposome delivery vehicle (22). Here, we provide key molecular methods involved in design, construction, and the implementation of bi-shRNA.

Once a target gene is selected, the initial step is to determine the objective of the study. Depending on that objective, there are many different vectors or plasmid backbones and delivery systems that can be used. It is a good idea to decide early in the process so the right expression cassette can be constructed. It is important to

choose an expression vector with efficient transgene expression. We found that an expression vector with an extended CMV promoter containing immediate early (IE) 5′UTR and partial Intron A (pUMVC3) is more effective than those with a cloning site immediately adjacent to the CMV promoter (23, 24). It may be more beneficial to have a stretch of lead transcript before the stem–loop structures. In addition, if more than one vector usage is planned, an effective shuttle strategy should be worked out beforehand; modification by PCR amplification of the expressed cassette is not very efficient. The choice of promoter is also important; RNA polymerase III promoters are much stronger in expression but have been documented to competitively saturate the endogenous miRNA maturation process at both the nuclear export and RISC loading steps resulting in lethal toxicity in vitro and in vivo with certain delivery vehicles (25). RNA polymerase II promoters, although less strong in expression, appear to work efficiently in our hands and are much less toxic vis-à-vis competition for the endogenous miRNA pathway (26).

It is often useful to design a sequence that can act in more than one species particularly if multiple animal model systems are to be utilized. For most target genes, it is possible to find stretches of target nucleotides that are conserved between species; however, finding a sequence that is both conserved and optimum for knockdown may not be easy. One would have to painstakingly compare the homology matched sequence with the selected target site sequence.

Publicly accessible computer programs using differing algorithms (e.g., Dharmacon RNAi design center (www.dharmacon.com) and IDT (www.idtdna.com) are readily available and can be used to locate appropriate target sites within the targeted gene. A search with most computer programs will often yield a good set of targets for further analysis; additionally, many available publications offer worthwhile advice in the form of do and do-not suggestions (27–30). Special care must be taken to do a BLAST search for each target sequence to analyze potential cross homology with other mRNAs within the species of interest.

Once the target site sequence is selected, the bi-shRNA design process can begin; the design process is presented in Subheading 3.1. The bi-shRNA stem–loop structure routinely used in our facility employs the well analyzed miR-30a backbone (31, 32) although, in theory, one can use any functional miRNA backbone. A predicted stem–loop structure of a bifunctional construct is shown in Fig. 2a. The bi-shRNA consists of the two stem–loop structures on an miR-30a backbone located immediately adjacent to each other with a gap about 10 nucleotides long (Fig. 2a); however, a longer nucleotide gap can be used and multiple units of bi-shRNA can be designed to string together in a single transcript targeting either a single gene at multiple sites or multiple different genes simultaneously.

To construct the expression unit to be placed in the multiple cloning sites of an expression vector, we have developed an assembly

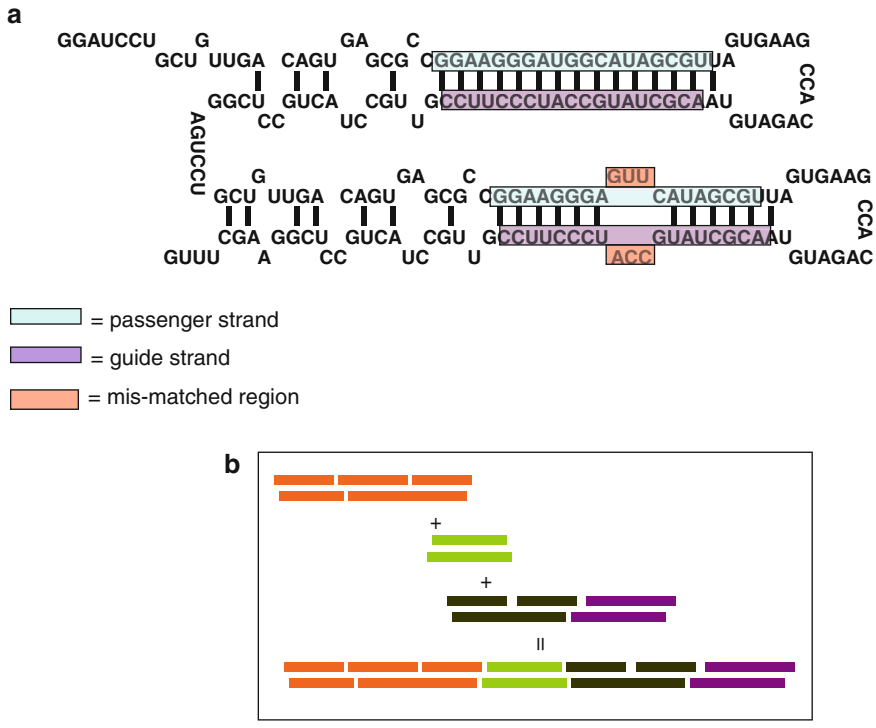

Fig. 2. Bi-shRNA structure and construction assembly process. (**a**) An example of predicted RNA structure of the bi-shRNA in the miR30a backbone. Two stem–loop structures are juxtaposed to each other each with the identical guide strand (*light purple*); the first one completely complementary to the passenger strand (*light blue box*), the second one with a mis-matched passenger strand. The mismatched region is in *red boxes.* (**b**) Schematic of the assembly process to generate the bi-shRNA expression unit. Overlapping fragments cover the entire expression unit. The fragments are designed with sticky ends to facilitate joining by ligation. The assembly process proceeds in stepwise fashion. The 5′ end fragments (*orange*) are ligated together. The 3′ fragments are ligated together (*black* and *purple*) before being joined together with the middle fragment (*green*).

strategy using synthetic oligonucleotides sequentially linked together (Fig. 2b). Alternatively, one can also outsource the synthesis of the gene construct with the specified sequence to one of many biotechnology service companies. For the oligonucleotide assembly process, overlapping DNA fragments were designed and synthesized; because of redundant sequences in the two stem–loop structures, we found it is necessary to initially ligate the 5′ fragments and 3′ fragments. The 5′ fragment and the 3′ fragment can then be purified on a gel and further ligated to the middle, linking fragments as illustrated in Fig. 2b and described in Subheading 3.2. This assembly process is efficient and, with careful design, many fragments can be repetitively used for different bifunctional constructs.

For each target, it is best to design and construct at least three bifunctional constructs to compare and from which to select the construct(s) with the most knockdown efficiency for further evaluation. Knockdown efficiency can be compared in vitro in tissue culture cells. It is often hard to compare the knockdown efficiency with endogenously expressed genes because in vitro transfection

methods have widely different efficiencies. This is particularly so when the transfection efficiency is low as the knockdown is hard to assess due to background noise from untransfected cells. We have developed a more effective method in which we co-transfect both the bifunctional construct and transgene expression vector, which allows target gene expression knockdown to be effectively compared and quantified. The co-transfection method is described in Subheading 3.3.

The efficacy and efficiency of target gene knockdown by bi-shRNA can be tested with a variety of in vitro and in vivo systems depending on the target and planned application. This in vitro assessment can be conducted following transfection of the bi-shRNA expression plasmids in a variety of cultured cells. We found that transfection by both electroporation and by liposome (e.g., Lipofectamine 2000) are highly effective; however, the amount of plasmid DNA used should be carefully controlled using a control vector or universal random sequence. For Lipofectamine or a related agent, we found the reverse transfection method, in general, is less toxic than the forward transfection method. Target gene knockdown can be assessed by either qRT-PCR for target gene mRNA or by Western and/or ELISA for target gene protein. These assays are well described in many publications. We present two assay methods in detail here; one detects the expression of mature shRNA by stem–loop RT-PCR (Subheading 3.4), the other detects the target mRNA cleavage by 5′ RNA-Ligand Mediated RACE (5′ RLM-RACE; Subheading 3.5). We have successfully used both these methods to assess the efficacy of bi-shRNA both in vitro and in vivo. The stem–loop RT-PCR method is schematically illustrated in Fig. 3a and a typical result shown in Fig. 3b. Stem–loop RT-PCR is a sensitive method dependent on the specific probe primer used; in addition, one can specifically detect and quantify both the passenger strand and guide strand. For bi-shRNA, the method can differentially score both the fully complementary as well as the mismatched (partially complementary) passenger strand (21). The 5′ RLM-RACE method is schematically shown in Fig. 4a; the method requires ligation of an RNA oligomer onto the cleaved mRNA end, and consequently, the method is rendered less efficient. Insofar as a number of rounds of amplifications are often required, a nested primer design is essential to ensure specificity. A typical result is shown in Fig. 4b.

Evaluable functionality of bi-shRNA relies on effective plasmid delivery into target cells. Various in vitro transfection systems often do not translate to inherently more complex in vivo animal models (33). There are numerous delivery systems designed specifically for systemic applications in vivo (22, 33–35). We have utilized the fusogenic, cationic DOTAP:cholesterol bilamellar invaginated vesicle lipoplex (BIV) for our in vivo studies (22, 36) and have successfully translated it to the clinic (37). We are currently developing

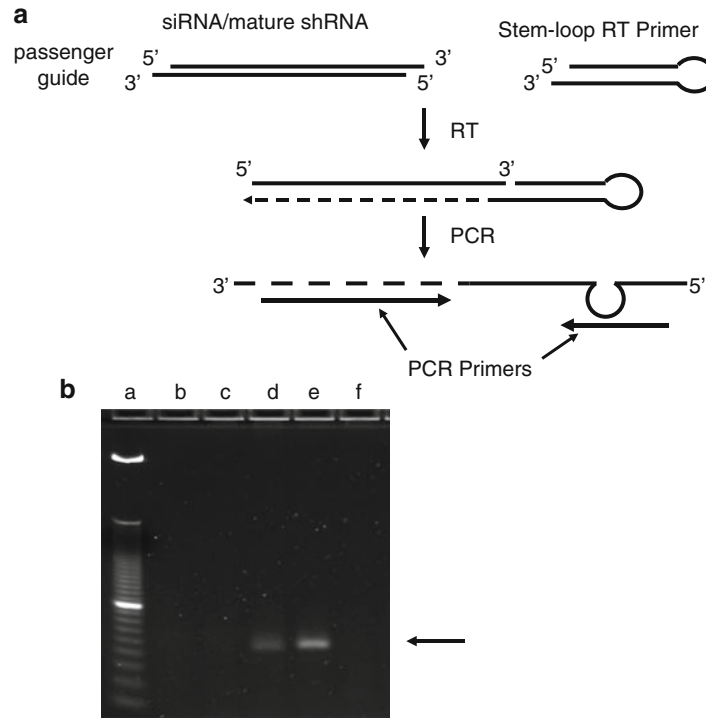

Fig. 3. Stem–loop RT-PCR to detect mature shRNAs. (**a**) Schematic depicting the stem–loop RT-PCR procedure. A stem–loop RT primer is designed with at least a 4-base overhang at the 3′ end to recognize the 3′ end of siRNA/mature shRNA. After RT, PCR amplification is accomplished with an siRNA/mature shRNA-specific PCR primer and a stem–loop RT primer-specific PCR primer. (**b**) A typical result of stem–loop RT-PCR. PCR products which were run onto a 3% agarose gel. Lane (a) is a 10 bp-size marker, (b) is a control with cellular RNA isolated from untransfected cells, (c) is a control with cellular RNA isolated from cells transfected with scrambled siRNA, (d) is with cellular RNA isolated from cells transfected with bi-shRNA, (e) is with cellular RNA isolated from cells transfected with siRNA, (f) is RNA isolated from bi-shRNA transfected cells without RT reaction.

modification strategies for more focused biodistribution, targeted delivery, and enhanced intracellular uptake. An effective lipoplex should use plasmids devoid of any contaminants from host *E. coli*. Although endo-free plasmid purification kit produced plasmids are generally used, GLP or GMP produced plasmids are more effective. Unfortunately, colanic acid and other non-endotoxin-associated polysaccharides co-purify with DNA by anion exchange chromatography and by cesium chloride density gradient centrifugation. Therefore, endotoxin removal does not remove these contaminants, and HPLC cannot detect these contaminants. To correct this, we recently developed the Superclean™ procedure to generate ultra-high quality plasmid DNA, cleansed of these contaminants, for in vivo and clinical applications (38). Liposome preparation involves highly specialized equipment; we routinely generate the DOTAP:cholesterol BIV in our GMP facility. It is advised that premade liposome be obtained from a collaborator or

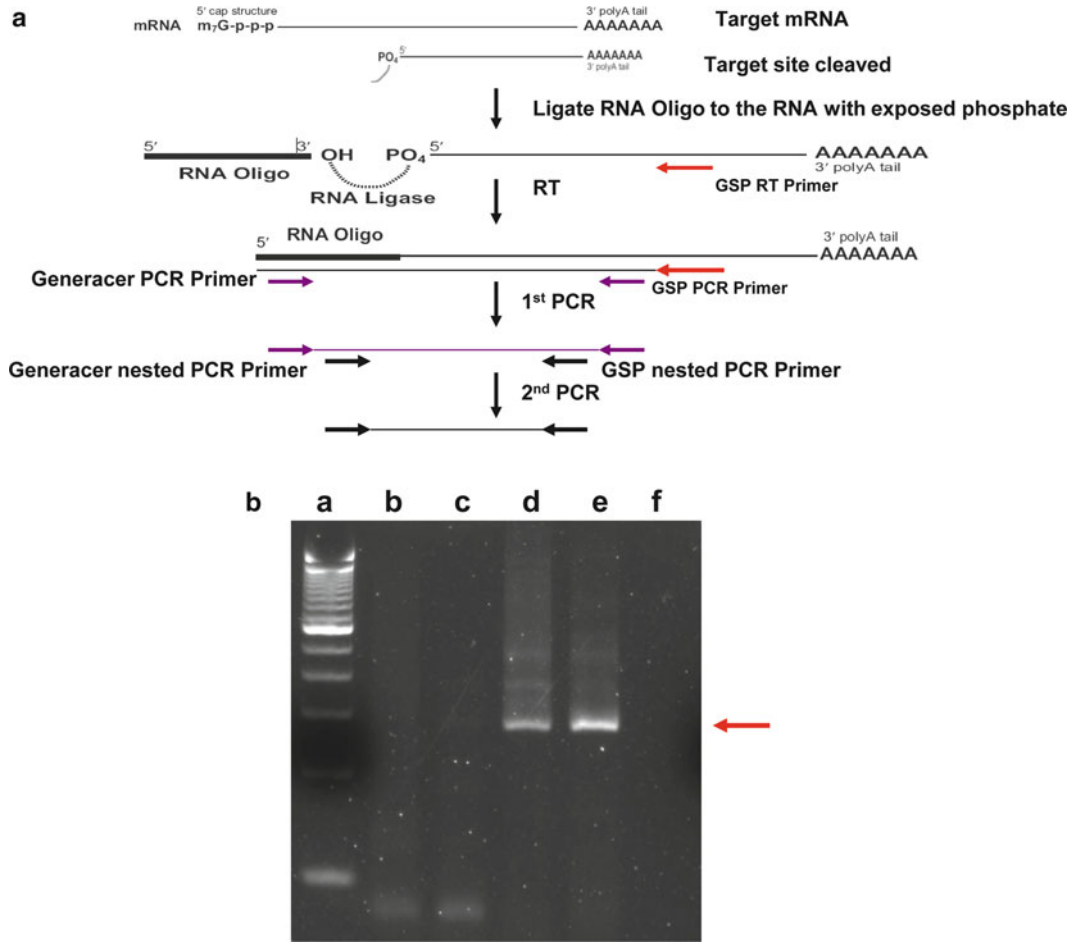

Fig. 4. 5′ RNA-ligand mediated RACE (5′RLM-RACE) to detect target site cleavage product. (**a**) Schematic depicting the 5′RLM-RACE method. Cleaved or partially degraded mRNA with exposed 5′ ends is first ligated with an RNA oligo via RNA ligase. The RNA oligo added RNA fragment is selectively copied into cDNA with a GSP primer and the cDNA is amplified by PCR with a GSP primer and an RNA oligo-specific primer, followed by further amplification with a nested GSP primer and a nested RNA oligo-specific primer. (**b**) A typical result of 5′RLM-RACE. PCR products which were run onto a 1.5% agarose gel. Lane (a) is a 100 bp size marker, (b) is a control with cellular RNA isolated from untransfected cells, (c) is a control with cellular RNA isolated from cells transfected with scramble siRNA, (d) is with cellular RNA isolated from cells transfected with bi-shRNA, (e) is with cellular RNA isolated from cells transfected with siRNA, (f) is RNA isolated from bi-shRNA transfected cells without RT reaction.

be purchased from a vendor. In Subheading 3.6, we describe the process of preparing lipoplex with high quality liposome and plasmid DNA. The lipoplex formulation can be achieved in most laboratory settings. Once the lipoplex is made, the formulation can be delivered systemically to experimental animals either through slow tail vein injection or with catheters. Target site vector expression can be analyzed using the PCR method for plasmid DNA and the stem–loop RT-PCR for mature bi-shRNA, respectively. bi-shRNA functionality can be assayed with the 5′ RLM-RACE for target mRNA cleavage and with western blot or IHC for target protein

knockdown. It is advised that these analyses be performed at ~48 hours post treatment. For efficacy, repeated delivery into the experimental animal is often required; the dosing schedule needs to be experimentally determined and optimized.

2. Materials

2.1. Bifunctional shRNA Design

1. Computer with Internet access.

2.2. Construction of Bi-shRNA

1. DNA synthesis vendor or capacity.
2. Expression vectors.
3. Customized oligonucleotides.
4. T4 DNA ligase.
5. Agarose gel electrophoresis.
6. DNA gel extraction kit.
7. Restriction enzymes.
8. Competent *E. coli* cells.
9. Plasmid isolation kit.
10. Thermal cycler.

2.3. Assess Comparative Knockdown by Co-transfection

1. Gene Pulser XCell Electroporation apparatus (Bio-Rad).
2. Gene Pulser Cuvettes (Bio-Rad).
3. Tissue culture cells.
4. Target gene expression vector.
5. For protein extraction and estimation.
   - CelLytic™ M (Sigma).
   - Protease Inhibitor Cocktail (Sigma).
   - Cell scrapers.
   - Orbital shaker.
   - Microfuge (refrigerated).
   - Coomassie Plus—Bradford™ Assay Kit (Pierce).
   - Pre-diluted protein standards (Pierce).
   - 96-well round bottom plate.
   - Multichannel pipet.
   - ELISA plate reader (Molecular Devises).
6. For western immunoblot.
   - Mini-Protein II Cell: Mini vertical electrophoresis system (Bio-Rad).

- Mini-Protein II Ready Gels: 12% Tris–HCl, ten wells.
- Running buffer (TGS Buffer; Bio-Rad).
- Precision Plus All Blue standards (Bio-Rad).
- Laemmli sample loading buffer.
- β-Mercaptoethanol.
- Pre-prepared cell lysate with known protein concentration.
- Mini-Trans blot module (Bio-Rad).
- Trans-blot PVDF membrane Sandwich (Bio-Rad).
- Transfer buffer (TG buffer).
- Methanol.
- Stir plate.
- Power Pack.
- DPBS (Invitrogen).
- Nonfat dried milk, for blocking nonspecific binding.
- Tween-20.
- Primary antibody to specific protein.
- Primary antibody to loading control.
- HRP conjugated secondary antibody.
- SuperSignal West Dura Extended Duration Substrate (ThermoScientific, Pierce).
- Orbital shaker plate.
- Syngene G-Box.

2.4. Stem–Loop RT-PCR for Mature shRNA Detection

1. Thermal cycler.
2. Microcentrifuge.
3. SuperScript III RT (including reverse transcriptase, 5× first strand buffer, and 0.1 M DTT).
4. RNase H.
5. dNTP mix, 10 mM.
6. RNaseOut (40 U/μl).
7. siRNA for positive control.
8. Stem–loop RT primer.
9. Stem–loop PCR primer.
10. Mature shRNA-specific PCR primers.
11. Total cellular RNA including small RNA and miRNA extracted using the mirVana kit (Ambion, TX).
12. Agarose gel electrophoresis and TA cloning kit.

2.5. 5′ RLM-RACE for Cleavage Product Detection

1. Thermal cycler.
2. Microcentrifuge.
3. Microcon YM-100 centrifugal filter.
4. T4 RNA ligase and buffer.
5. SuperScript III RT (including reverse transcriptase, 5× first strand buffer, and 0.1 M DTT).
6. RNase H.
7. dNTP mix (10 mM).
8. RNaseOut (40 U/μl).
9. Platinum *Taq* Kit (including Platinum *Taq* Polymerase, 5× PCR buffer and 10 mM $MgCl_2$).
10. RNA oligo:

    (5′rCrGrArCrUrGrGrArGrCrArCrGrArGrGrArCrArCrUrGr ArCrArUrGrGrArCrUrGrArArGrGrArGrUrArGrArArA3′).
11. 10 μM GeneRacer 5′ PCR primer: (5′-*CGACTGGAGCACGA GGACACTGA*-3′).
12. 10 μM GeneRacer 5′ nested PCR primer: (5′-*GGACACTGA CATGGACTGAAGGAGTA*-3′).
13. 10 μM gene-specific (GSP) RT primer (3′ of the GSP PCR primer).
14. 10 μM each GSP PCR primer and nested GSP primer (at least 5 nt 5′ to the GSP primer).
15. Total RNA isolated with the mirVana miRNA isolation kit or RNeasy Mini kit.
16. Agarose gel electrophoresis and DNA gel extraction kit.

2.6. Preparation of DNA-DOTAP:Chol Lipoplexes

1. Certified Biological Safety Cabinet (BSC) (The Baker Company).
2. Spectrophotometer with standard and turbidity cell holder (Beckman Coulter).
3. ZetaSizer Nano (Malvern).
4. Quartz semi-microcell cuvette (Beckman Coulter).
5. Vortexer (VWR).
6. Pipettors (Gilson) and Pipet tips (Phenix).
7. Acrodisc filter 0.2 μm (Pall).
8. GMP (GLP) Grade plasmid DNA (≥2.0 μg/μl in water).
9. 5× DOTAP:Cholesterol Liposomes (Gradalis).
10. Sterile water for irrigation (Baxter).
11. Sterile D5W (5% dextrose in water) (Baxter).
12. Ultra High Pure Argon gas (Airgas).
13. Septihol (USP 70%, v/v, isopropyl alcohol + USP 30%, v/v, purified water) (Steris).

3. Methods

3.1. Bi-shRNA Design

1. Select target site sequences using available computer programs.
2. Do BLAST search to find target sites with lowest homology hits to other mRNA of the targeted species.
3. Plug the 19 nucleotides passenger strand (sense strand, blue boxed region in Fig. 2a) and guide strand (antisense strand, purple boxed region in Fig. 2a) sequences into both stems of the miR30a backbone sequence.
4. Replace nucleotides 9, 10 and 11 (counting from the 3′ end of the passenger strand) of the second stem–loop structure with nucleotides mismatched to the guide strand.
5. Run both stem–loop structure on mfold program (http://mfold.rna.albany.edu/?q=mfold), the ΔG free energy of the hairpin with mismatches should fall between −10 kcal/mol and equilibrium.
6. If the ΔG free energy is beyond −10 kcal/mol, additional mismatches should be introduced at the passenger strand of the second hairpin. The additional mismatch introduced is preferably at the 3′ half of the passenger strand.

3.2. Construction of Bi-shRNA Expression Vector

1. Design overlapping DNA oligonucleotides from both strands with sticky ends for ligation, four nucleotides overhang should be sufficient. The upper limit of oligonucleotides length for DNA synthesis is around 50 nucleotides, thus DNA fragments should be spaced evenly and be <50 nt each. Care should be taken to avoid any internal self-complementary potential for each fragment.
2. Remember to place a 5′ phosphate on each fragment for efficient ligation. Build the cloning site sequence into the 5′ and 3′ end of the expression unit to expedite cloning into the expression vector.
3. Order or synthesize oligonucleotides. Reconstitute synthesized oligonucleotides at 1 mg/ml.
4. Ligate the 5′ fragments together with one-step ligation of equal amount of oligonucleotides.
5. Ligate the 3′ fragments together with one-step ligation of equal amount of oligonucleotides.
6. Extract and purify the calculated full-length DNA fragments on an agarose gel.
7. Ligate 5′ fragment with linking fragment then 3′ fragment and purify the calculated full-length DNA fragment on an agarose gel.

8. Ligate the purified fragment into expression vector and transform competent *E. coli* cells.
9. Isolate colonies, and screen for clones with the appropriate size insert.
10. Plasmids isolated from positive clones are further confirmed by sequencing.

3.3. Compare Knockdown Efficiency by Co-transfection

1. Determine the most effective electroporation condition and the optimum amount of plasmid DNA for each cell types.
2. We usually use HEK-293 cells with electroporation conditions recommended by Bio-Rad.
3. Prepare the DNA mix for electroporation with appropriate controls. It is essential to have a control with the transgene expression vector and the empty vector for bi-shRNA expression because promoter competition is often observed.
4. We usually use the transgene expression vector and bi-shRNA expression vector at a 1:1 ratio. The experiment can also include a 1:2 ratio or 2:1 ratio to further validate the knockdown comparison at different doses.
5. After electroporation, cells are plated at 40–50% confluency. Cells are removed at various time points to analyze comparative knockdown. The most effective time point is 48 h post transfection.
6. Cells are removed from the culture plate using Trypsin EDTA 0.25%. Washed in DPBS two times to remove any residual culture media containing serum. After the final wash, the cells are pelleted.
7. Prepare the lysis buffer by adding 1% of protease inhibitor cocktail to the CelLyticM lysis buffer. For each pellet of 1.0×10^7 cells 500 µl of the lysis buffer is added. Pipette up and down to mix the cells with the lysis buffer. The lysis buffer plus the cell mixture should be cloudy but without being too dense. If the mixture is too dense another 100–200 µl of lysis buffer is added. Incubate at room temperature for 30 min on a slow shaker. Centrifuge the lysed cells for 20 min at 12,000–20,000 $\times g$ to pellet the cellular debris. Remove the protein containing supernatant to a chilled tube.
8. Protein estimation is done by the Coomassie Bradford Plus Assay.
9. Equal amount of total protein is loaded onto Ready Gel for PAGE for Western analysis.
10. Protein from the Gel is transferred to PVDF membrane.
11. Immuno probing is done with gene-specific antibody and anti body to loading control protein.

12. HRP conjugated secondary antibody is used followed by Super Signal Dura Extended Duration Substrate to detect the signal.
13. Images are captured using a G-Box and the band densities are quantified.
14. Side-by-side knockdown comparison of different bi-shRNA can be scored by semi-quantitative scan.

3.4. Demonstrate Bi-shRNA Expression by Stem–Loop RT-PCR (See Fig. 3)

1. Design and synthesize primers: the process essentially is the same as published (21, 39).
   (a) Design stem–loop RT primers for either the passenger strand or guide strand according to the predicted mature shRNA sequence following its processing into an siRNA. One can essentially use the same body of sequence as the published stem–loop RT primer sequence (21, 39) (see below) but change the final six 3′ nucleotides (the XXXXXX in sequence below) to the sequences complementary to the 3′ ends of either the guide strand or the passenger strand in query. Stem–loop RT primer sequences:
   5′ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGC ACTGGATACGACXXXXXX 3′.
   (b) The stem–loop PCR primer has the following sequence: 5′ GTGCAGGGTCCGAGGT 3′.
   (c) Design guide strand and passenger strand mature shRNA-specific PCR primers according to the predicted mature shRNA/siRNA sequence. The guide strand- and passenger strand-specific PCR primers are essentially the complete or shorter guide strand or passenger strand sequences pending on T_m calculation to match the T_m of the stem–loop PCR primer.
2. Reverse transcription (RT):
   (a) Add the following reagents to the tube containing 3.0 μg total RNA including small RNA (many RNA isolation methods or kits lose small RNAs; be sure to use an RNA isolation method or kit for which small RNAs are retained): 1 μl stem–loop RT primer (10 μM), 1 μl 10 mM dNTP mix (Invitrogen).
   (b) Incubate the mixture for 5 min at 65°C to disrupt the RNA secondary structure.
   (c) Keep on ice for 2 min and centrifuge briefly.
   (d) Add the following reagents to the mixture above and bring the volume to 20 μl by adding nuclease-free water: 4 μl 5× first strand buffer, 1 μl 0.1 M DTT, 1 μl RNaseOut (40 U/μl), 1 μl Superscript III RT (200 μ/μl).
   (e) Mix well and incubate at 55°C for 50 min.
   (f) Inactivate the RT reaction by incubating at 70°C for 15 min.

(g) Chill on ice for 2 min.

(h) Add 1 μl RNase H to the reaction mix and incubate at 37°C for 20 min. Centrifuge briefly and keep on ice or store in the −20°C freezer.

3. PCR setup

Make the master mix and follow the PCR parameters as below:

Reagent	Volume (μl)
Nuclease-free water	32.6
5× PCR buffer	10
25 mM $MgCl_2$	3
10 nM dNTP	1
Stem–loop PCR primer (10 μM)	1
Mature shRNA-specific PCR primer	1
GoTaq HotStart	0.4
cDNA generated in step 2	1
Total volume	50

Conditions		
94°C	2 min	
94°C	1 min	
55°C	30 s	35 cycles
72°C	1 min	
72°C	9 min	
4°C	Hold	

4. PCR product analysis

After the PCR amplification is completed, run a 20 μl of sample on a 4% agarose gel to visualize the amplicon. The predicted PCR amplicon is TA cloned and then sequenced.

3.5. Demonstrate Target Gene Cleavage by 5′ RLM-RACE (See Fig. 4)

1. To ligate the RNA oligo to the RNA molecules with exposed 5′ phosphates, add 1–3 μg of total RNA from each sample to 250 ng of RNA oligo and mix well by pipetting several times.
2. Incubate the mixture at 65°C for 5 min in a thermal cycler and then hold at 4°C for 2 min.
3. Add the following reagents to the tube, mix well, and centrifuge briefly: 2 μl of 10× ligase buffer, 2 μl of RNaseOut (40 U/μl), 2 μl of nuclease-free water, and 1 μl of T4 RNA ligase (10 U/μl). Incubate the mixture at 37°C for 1 h in a thermal cycler.
4. RNA purification: After 37°C incubation, add 80 μl of nuclease-free water to the 20 μl ligation product, and load the 100 μl

mixture into a microcon YM-100 centrifugal filter. Centrifuge at 500 × *g* for 15 min. Turn the column upside down in a new tube and centrifuge at 1,000 × *g* for 3 min. The elution volume is about 10 μl.

5. RT: Add the following reagents to the tube containing 10 μl of ligated RNA from above: 1 μl of gene-specific (GSP) RT primer, 1 μl of dNTP mix, and 1 μl of nuclease-free water. Incubate the mixture for 5 min at 65°C, and then keep on ice for 1 min. Add the following reagents to the mixture of ligated RNA and RT primer: 4 μl of 5× first strand buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOut, and 1 μl of SuperScript III RT. Incubate at 55°C for 50 min, followed by incubating at 70°C for 15 min. Add 1 μl of RNaseH to the reaction mix and incubate at 37°C for 20 min.
6. Amplifying cDNA ends (first PCR, touch-down): Set up the reaction mix as listed below.

Reagent	Volume (μl)
Nuclease-free water	36.5
5× PCR buffer	5
25 mM $MgCl_2$	2
10 mM dNTP	1
Generacer 5′ PCR Primer (10 μM)	3
GSP PCR primer (10 μM)	1
Platinum *Taq*	0.5
cDNA	1.0
Total volume	50

Perform touch-down PCR following the parameters as shown below.

Conditions		
94°C	2 min	
94°C	30s	
72°C	1 min	5 cycles
94°C	30s	
70°C	1 min	5 cycles
94°C	1 min	
65°C	30s	25 cycles
72°C	1 min	
72°C	10 min	
4°C	Hold	

7. Nested PCR (second PCR): Nested PCR is employed to increase the specificity and sensitivity of the RACE product amplification. The reaction master mix is set up as listed below.

Reagent	Volume (μl)
Nuclease-free water	38.5
5× PCR buffer	5
25 mM $MgCl_2$	2
10 mM dNTP	1
Generacer 5′ nested PCR primer (10 μM)	1
Nested GSP PCR primer (10 μM)	1
Platinum Taq	0.5
DNA template from first PCR	1
Total volume	50

The PCR is performed following the parameters as shown below.

Conditions		
94°C	2 min	
94°C	30s	
65°C	30s	25 cycles
72°C	1 min	
72°C	10 min	
4°C	Hold	

8. Agarose gel electrophoresis and sequencing: 20 μl of the nested-PCR product is separated on a 2% agarose gel. The image of the agarose gel is captured by an imaging system. The identity of the PCR product is further confirmed by sequencing.

3.6. Preparation of DNA-DOTAP:Chol Lipoplexes

1. Perform the formulation in of the lipoplexes in a BSC. Do a test run before doing a bulk manufacture run.
2. Bring the liposomes, plasmid DNA, D5W, and water to room temperature.
3. Transfer D5W into 50 ml conical tubes using a 60 ml syringe and 16 G needle.
4. Measure the plasmid DNA concentration in a spectrophotometer by measuring the OD260 using a standard cuvette holder.
5. In a 1.5 ml tube, dilute the plasmid DNA to 1.0 μg/μl with D5W.
6. In a 1.5 ml tube, combine 3 volumes of D5W and 2 volumes of 5× DOTAP:Chol stock. Mix to make 2× liposomes.

7. Prepare the Lipoplex by transfering the diluted DNA (1.0 μg/μl) into an equal volume of 2× liposomes and mix by pipetting up and down.
8. Check for any signs of precipitation. If no precipitation is visible, proceed to the next step. If precipitation is visible, repeat steps 5–7 using less DNA and note the change.
9. Spectrophotometric analysis: Replace the cuvette holder with the turbidity cell holder. Dilute the DNA-Lipoplex 20-fold with sterile water. Blank the spectrophotometer at 400 nm with sterile water and then measure the OD400 of the diluted DNA-Lipoplex. The acceptable range is 0.65–0.95 OD units. If the OD400 reading is too high, repeat steps 5–7 using less DNA and note the changes in the operator comments section. If the OD400 reading is too low, repeat steps 5–7 using more DNA and note the changes.
10. After the test run is within the acceptable range, the same process is repeated to complete the manufacture run. Combine all DNA-Lipoplexes. Mix and measure the OD400 of the final pooled product. Aliquot samples for quality control studies. Layer argon gas (filtered through a 0.2 μm acrodisc filter) onto each aliquot and onto the bottle of remaining lipoplex to displace the ambient air and seal the lid.
11. Quality control tests include particle analysis (described in step 12), USP sterility, LAL endotoxin assay, residual chloroform analysis by GC/MS, and thin layer chromatography to identify lipids (QC tests service companies are listed below in step 13).
12. Particle analysis (size and zeta potential): Rinse the folded capillary cell with 5 ml 100% ethanol, and then with 5 ml USP water. Load 20-fold diluted DNA-Lipoplex sample into the capillary cell. Measure the average particle size (acceptable range <500 nm) and zeta potential (acceptable range >40 mV).
13. List of service providers:

    Sterility and endotoxin:

    AppTec.

    1265-B Kennestone Circle.

    Marietta, GA 30066.

    Residual chloroform:

    Exova Inc.

    9240 Santa Fe Springs Rd.

    Santa Fe Springs, CA 90670.

    Lipid analysis:

    Avanti Polar Lipids, Inc.

    700 Industrial Park Drive.

Acknowledgements

The authors gratefully acknowledge the generous support of the W.W. Caruth Jr. Foundation Fund of the Communities Foundation of Texas, the Jasper L. and Jack Denton Wilson Foundation, and the Marilyn Augur Family Foundation. They also wish to thank Susan W. Mill for her assistance in manuscript preparation and submission.

References

1. Fire AZ (2007) Gene silencing by double-stranded RNA (Nobel Lecture). Angew Chem Int Ed 46:6966–6984
2. Mello CC (2007) Return to the RNAi world: rethinking gene expression and evolution. Cell Death Differ 14:2013–2020
3. Burnett JC, Rossi JJ, Tiemann K (2011) Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol J 6:1130–1146
4. Davidson BL, McCray PB Jr (2011) Current prospects for RNA interference-based therapies. Nat Rev 12:329–340
5. Wang Z, Rao DD, Senzer N, Nemunaitis J (2011) RNA interference and cancer therapy. Pharm Res (Epub ahead of print)
6. Phalon C, Rao DD, Nemunaitis J (2010) Potential use of RNA interference in cancer therapy. Expert Rev Mol Med 12:e26
7. Drews J (1996) Genomic sciences and the medicine of tomorrow. Nat Biotechnol 14: 1516–1518
8. Drews J (2000) Drug discovery: a historical perspective. Science 287:1960–1964
9. Verdine GL, Walensky LD (2007) The challenge of drugging undruggable targets in cancer: lessons learned from targeting BCL-2 family members. Clin Cancer Res 13:7264–7270
10. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123:607–620
11. Preall JB, Sontheimer EJ (2005) RNAi: RISC gets loaded. Cell 123:543–545
12. Parker JS, Barford D (2006) Argonaute: a scaffold for the function of short regulatory RNAs. Trends Biochem Sci 31:622–630
13. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123:631–640
14. Tang G (2005) siRNA and miRNA: an insight into RISCs. Trends Biochem Sci 30:106–114
15. Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27:91–105
16. Ameres SL, Martinez J, Schroeder R (2007) Molecular basis for target RNA recognition and cleavage by human RISC. Cell 130:101–112
17. Parker JS, Roe SM, Barford D (2006) Molecular mechanism of target RNA transcript recognition by Argonaute-guide complexes. Cold Spring Harb Symp Quant Biol 71:45–50
18. Rao DD, Vorhies JS, Senzer N, Nemunaitis J (2009) siRNA vs. shRNA: similarities and differences. Adv Drug Deliv Rev 61:746–759
19. Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ (2005) Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 23:222–226
20. McAnuff MA, Rettig GR, Rice KG (2007) Potency of siRNA versus shRNA mediated knockdown in vivo. J Pharm Sci 96:2922–2930
21. Rao DD, Maples PB, Senzer N et al (2010) Enhanced target gene knockdown by a bifunctional shRNA: a novel approach of RNA interference. Cancer Gene Ther 17:780–791
22. Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Improved PGN (1997) DNA: liposome complexes for increased systemic delivery and gene expression. Nat Biotechnol 15:647–652
23. Chapman BS, Thayer RM, Vincent KA, Haigwood NL (1991) Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. Nucleic Acids Res 19:3979–3986
24. Simari RD, Yang ZY, Ling X et al (1998) Requirements for enhanced transgene expression by untranslated sequences from the human cytomegalovirus immediate-early gene. Mol Med (Cambridge, Mass) 4:700–706
25. Grimm D, Streetz KL, Jopling CL et al (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441:537–541

26. Giering JC, Grimm D, Storm TA, Kay MA (2008) Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. Mol Ther 16:1630–1636
27. Walton SP, Wu M, Gredell JA, Chan C (2010) Designing highly active siRNAs for therapeutic applications. FEBS J 277:4806–4813
28. Hofacker IL, Tafer H (2010) Designing optimal siRNA based on target site accessibility. Methods Mol Biol 623:137–154
29. Moore CB, Guthrie EH, Huang MT, Taxman DJ (2010) Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol Biol 629:141–158
30. Dawson LA, Usmani BA (2008) Design, manufacture, and assay of the efficacy of siRNAs for gene silencing. Methods Mol Biol 439:403–419
31. Zeng Y, Cullen BR (2006) Recognition and cleavage of primary microRNA transcripts. Methods Mol Biol 342:49–56
32. Siolas D, Lerner C, Burchard J et al (2005) Synthetic shRNAs as potent RNAi triggers. Nat Biotechnol 23:227–231
33. Barteau B, Chevre R, Letrou-Bonneval E, Labas R, Lambert O, Pitard B (2008) Physicochemical parameters of non-viral vectors that govern transfection efficiency. Curr Gene Ther 8: 313–323
34. Chesnoy S, Huang L (2000) Structure and function of lipid–DNA complexes for gene delivery. Annu Rev Biophys Biomol Struct 29:27–47
35. Templeton NS (2010) Liposomes for gene transfer in cancer therapy. Methods Mol Biol 651:271–278
36. Phadke AP, Jay CM, Wang Z et al (2011) In vivo safety and antitumor efficacy of bifunctional small hairpin RNAs specific for the human Stathmin 1 oncoprotein. DNA Cell Biol 30:715–726
37. Nemunaitis G, Jay CM, Maples PB et al (2011) Hereditary inclusion body myopathy: single patient response to intravenous dosing of GNE gene lipoplex. Hum Gene Ther (Epub ahead of print)
38. Firozi P, Zhang W, Chen L, Quiocho FA, Worley KC, Templeton NS (2010) Identification and removal of colanic acid from plasmid DNA preparations: implications for gene therapy. Gene Ther 17:1484–1499
39. Chen C, Ridzon DA, Broomer AJ et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33:e179