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Lentivirus-Mediated RNA Interference in Mammalian Neurons

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Summary

The ability to manipulate RNAi in cultured mammalian cells has provided scientists with a very powerful tool to influence gene expression. Neurons represent a cell type that initially displayed resistance to transduction by siRNAs or shRNA, when attempting to silence expression of endogenous genes. However, the development of lentiviral systems with that goal has facilitated the exogenous manipulation of RNAi in these postmitotic cells. Lentiviral-mediated RNAi experiments in cultured mammalian neurons can be designed to address a wide variety of biological questions or to test potential therapeutic hairpins before moving to treatment trials *in vivo*. We provide a practical approach to accomplish siRNA-mediated silencing of the disease-linked protein torsinA in primary neuronal cultures through the generation of lentiviral vectors expressing shRNAs.

Key Words: PNC (primary neuronal cultures); shRNA; torsinA; RNAi; silencing; lentivirus; FIV (feline immunodeficiency virus).

1. Introduction

TorsinA is an endoplasmic reticulum resident glycoprotein that, when mutated, leads to the dominantly inherited disease DYT1 dystonia ***(1)***. While the function of this AAA protein (*A*TPases *A*ssociated to diverse cellular *A*ctivities) remains unknown, the mutant protein acts through a dominant negative effect by recruiting wild-type torsinA from the endoplasmic reticulum to the nuclear envelope ***(2–4)***, thus leading to torsinA loss of function ***(5)***. As is true for many other neurological disease-linked proteins, torsinA is a widely expressed protein in both in neural and nonneural tissues ***(6)***. However, the resulting phenotype of the mutation is restricted to dysfunction of a specific subset of

From: *Methods in Molecular Biology, vol. 442: RNAi: Design and Application*
Edited by: S. Barik © Humana Press, Totowa, NJ

neurons. Therefore, establishing neuronal models of torsinA insufficiency may help us determine the function of this protein and uncover the pathobiology underlying DYT1 dystonia. Furthermore, specifically silencing expression of mutated torsinA has been proposed as a promising therapeutic strategy for this incurable disease ***(7,8)***.

Primary neurons derived from the embryonic mouse brain are widely used in the investigation of different aspects of neuronal function, including development and differentiation, synaptic transmission, or excitotoxicity, among others ***(9)***. Using RNAi in this cellular model greatly expands the repertoire of biological questions that can be addressed in this system. The development of lentiviral systems to mediate RNAi in primary neurons has facilitated these types of studies ***(8,10,11)***. Here we will describe the protocol we have developed to silence torsinA expression in primary neuronal cultures derived from wild-type embryonic mice. This protocol can easily be adapted to the study of many other proteins of interest.

2. Materials

2.1. Generation of U6shRNA

1. pAd5mU6 (template plasmid for PCR amplification) (*see* **Note 1**).
2. Oligonucleotide primers.
3. Platinum PCR SuperMix High Fidelity 1.1X (Invitrogen, Carlsbad, CA).
4. pCR2.1 vector in TOPO-TA Cloning kit (Invitrogen).
5. Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, CA).
6. *E. coli* strains DH5α, TOP10 (Invitrogen).
7. Ampicillin.
8. 40 mg/mL X-gal in dimethylformamide.
9. Luria–Bertani (LB) broth.
10. 37 ºC shaking incubator.
11. Quantum Prep Plasmid Miniprep Kit (Bio-Rad).
12. Restriction enzyme *Eco*RI (New England Biolabs).
13. Agarose and ethidium bromide.
14. DNA sequencing capability.
15. Spectrophotometer.

2.2. Generation of FIV.shRNA.eGFP

1. Restriction enzymes *Eco*RI and *Mfe*I (New England Biolabs).
2. CIAP (calf intestinal alkaline phosphatase) (New England Biolabs).
2. FIV shuttle (pVET$_L$CMV-eGFP) ***(10)*** packaging and envelope plasmids ***(12)***.
4. Rapid DNA ligation kit (Roche, Indianapolis, IN).

5. HEK393 cells.
6. Tissue culture incubator at 37 ºC with 5% CO_2.
7. HEPES-buffered saline (HBS): 5.0 g HEPES, 8.0 g NaCl, 0.37 g KCl, 0.188 g $Na_2HPO_4.7H_2O$, and 1.0 g glucose. Bring to 1 L in ddH_2O, adjust pH to 7.1 with concentrated NaOH, filter-sterilize, and store at 4 ºC.
8. 2.5 *M* of $CaCl_2$.
9. DMEM (Dulbecco's modified Eagle's medium): DMEM-10: DMEM (Gibco BRL) with 10% fetal calf serum, 5 mL of Penicillin/Streptomycin (Pen/Strep) (Gibco BRL). DMEM-2: DMEM with 2% fetal bovine serum (FBS) and 5 mL of Pen/Strep.
10. Lactose buffer: phosphate-buffered saline (PBS), pH 7.4 (Sigma P-3813), with 40 mg/mL of lactose, filter-sterilized.
11. Sorvall Centrifuge RC 26 Plus, with SLA 1500 rotor.
12. Centrifugation bottles (250-mL capacity).

2.2.1. Titer by Transgene Expression

1. HT-1080 cells (ATCCCRL-121) maintained in exponential growth in DMEM-10.
2. Incubate at 37 ºC with 5% CO_2.
3. Six-well tissue culture dishes.
4. DMEM-2 and DMEM-10.
5. Polybrene stock: 8 mg/mL in ddH_2O, filter-sterilized.
6. DMEM-2/polybrene: On the day of use, dilute the polybrene stock 1:2,000 in DMEM-2 for a final polybrene concentration of 4 µg/mL.
7. Dilution tubes (3.5-mL polystyrene sterile tubes).

2.3. Primary Neuronal Cultures

1. Ketamine/xylazine (mix 10 mL at a time and store at room temperature): 1 mL of 100 mg/mL ketamine, 0.1 mL of 100 mg/mL xylazine, 8.9 mL of sterile PBS (Gibco BRL).
2. Poly-L-lysine (0.1 mg/mL): Dilute 5 mg of poly-L-lysine (Sigma) in 50 mL of sterile distilled water. Store working stock at 4 °C.
3. HBSS (Hank's Balanced Salt Solution) dissecting medium pH 7.2: 500 mL of HBSS without calcium or magnesium (Invitrogen), 5 mL of Pen/Strep (Gibco BRL), 0.5 mL of 1 *M* HEPES. Store at 4 °C.
4. 10X (10 mg/mL) DNase solution: 10 mL of HBSS dissecting media, 100 mg of DNase (Sigma). Sterilize with 10-mL syringe filter. Store 1-mL aliquots at –20 °C.
5. 10X 2.5% Trypsin (Invitrogen). Aliquot 10 mL into 15-mL tubes. Store at –20 °C until thawed; then store at 4 °C.
6. Wash medium: 500 mL of DMEM, 10% FBS, 1% Pen/Strep. Store at 4 °C.
7. Neurobasal/FBS plating media: 220 mL of Neurobasal media (Gibco BRL), 2.5 mL of Pen/Strep, 2.5 mL of 200 m*M* glutamine stock (Sigma), 25 mL of FBS. Store at 4 °C.

8. Neurobasal/B27 maintenance media: 500 mL of Neurobasal media, 5 mL of Pen/Strep, 5 mL of 200 m*M* glutamine stock, 10 mL 2% B27 (50X) (Gibco BRL). Store at 4 °C.
9. 10 μ*M* of Ara-C.
10. Surgical material: fine and tissue forceps, small straight scissors, sterile scalpel blade, metal dissecting pan, dissecting microscope.
11. 10- and 6-cm sterile Petri dishes.
12. Hemocytometer.

2.4. Transduction of Primary Neuronal Cultures

1. FIV.eGFP.mU6shRNA.
2. Serum-free OptiMEM (Gibco BRL).
3. Neurobasal/B27 maintenance media (see above).
4. Tissue culture (TC) hood.
5. Hemocytometer.
6. Inverted fluorescence scope (Olympus).

2.5. Lysis for Protein Analysis

1. Modified Laemmli buffer for cell lysis (2X) (500 mL): 50 mL of 1 *M* Tris HCI, pH 6.8, 20 g of sodium dodecyl sulfate (SDS), 1.0 g of bromophenol blue, 100 mL of glycerol, milliQ H_2O to total of 500 mL. Keep at room temperature. To harvest the protein lysates, add 1:2 dilution of 2X modified Laemmli buffer 2X SDS, 1:10 dilution of DTT (add before use), and dH_2O to final volume.
2. Dithiothreitol (DTT) (1 *M*): Dissolve 3.09 g of DTT in 20 mL of 0.01 *M* N -acetate (pH 5.2). Sterilize by filtration and store at –20 °C.
3. Teflon cell scrapers (Fisher).
4. Staining solution (500 mL): 50 mL of acetic acid, 225 mL of H_2O, 225 mL of methanol, 1.25 mL of coomassie brilliant blue (Amresco).
5. Destaining solution (6 L): 600 mL of acetic acid, 2.7 L of H_2O, 2.7 L of methanol.
6. Whatman filter paper.

2.6. SDS-PAGE (12%)

1. Mini-PROTEAN 3 electrophoresis system (Bio-Rad).
2. 10% Ammonium persulfate (APS): 0.1 g of APS (Pierce) in 1 mL of milliQ H_2O.
3. Separating gel: 3.3 mL of dH_2O, 4 mL of 30% acrylamide/bis solution (37.5:1 with 12% total concentration) (Amresco), 2.5 mL of 1.5 *M* Tris-HCl (pH 8.8), 100 μL of 10% SDS, 100 μL of 10% APS, 0.4 μL of N,N,N,N'-Tetramethyl-ethylenediamine (TEMED) (Amresco).
4. Stacking gel (4X): 2.1 mL of dH_2O, 0.5 mL of 30% acrylamide/bis solution (37.5:1 with 2.6% total concentration when 1X), 380 μL of 1.0 *M* Tris-HCl (pH 6.8), 30 μL of 10% SDS, 30 μL of 10% APS, 3 μL of TEMED.

5. Water-saturated isobutanol: Shake equal volumes of water and isobutanol in a dark glass bottle and allow to separate. Use the top layer.
6. Running buffer (4 L, 5X): 60 g of Tris, 288 g of glycine, 20 g of SDS. First dissolve Tris and glycine in 1.8 L of H_2O, then the SDS. Add milliQ H_2O to total exactly 4 L. Shake well. Store in 4 °C. Dilute to 1X with milliQ H_2O before use.
7. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad, Hercules, CA).

2.7. Western Blotting for TorsinA

1. Mini Trans-Blot Cell tank transfer system (Bio-Rad).
2. Transfer buffer (4L, 5X): 60 g of Tris, 288 g of glycine, milliQ H_2O to total of exactly 4 L. Shake well and store at 4 °C. To make 1X buffer for use (20 L): Add 12 L of milliQ H_2O to 4 L of 5X transfer buffer and add 4 L of methanol (always add methanol last or it will precipitate the solute). For transfer, set up buffer plus 0.05% (w/v) SDS.
3. Supported nitrocellulose membrane (Millipore, Bedford, MA).
4. 3MM Chr chromatography paper (Whatman, Maidstone, UK).
5. Tris-buffered saline (TBS) (2L): 48.4 g of Tris, 160 g of NaCl, 76 mL of 1 *M* HCl. Add milliQ H_2O to 90% of final volume. Adjust pH to 7.6 and add milliQ H_2O to final 2-L volume. Shake well and store at 4 °C.
6. TBS with Tween (TBS-T) (20 L): 20 mL of Tween, 2 L of 10X TBS; bring volume up to 20 L with milliQ H_2O.
7. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
8. Secondary antibody: Goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoRes).
9. ECL Plus Western blotting detection reagents (Amersham Biosciences, Arlington Heights, IL).
10. Bio-Max XAR film (Kodak, Rochester, NY).
11. Autoradiography cassette (Fisher Biotech).
12. Developer.

2.8. Stripping and Reprobing Blots for α-Tubulin

1. Stripping buffer (500 mL): 3.5 mL of 2-mercaptoethanol, 50 mL of 20% SDS, 31.25 mL of 1 *M* Tris HCI (pH 6.8), 415.25 mL of milliQ H_2O. Store at room temperature.
2. TBS-T.
3. Blocking solution.
4. Primary antibody: Mouse monoclonal anti-α-tubulin (Sigma).
5. Secondary antibody: Goat anti-mouse conjugated to horseradish peroxidase (Jackson ImmunoRes).
6. ECL Plus Western blotting detection reagents (Amersham Biosciences).
7. Bio-Max XAR film (Kodak).

8. Autoradiography cassette (Fisher Biotech).
9. Developer.

2.9. Immunofluorescence for TorsinA

1. 18-mm round coverslips (Fisher).
2. 38 × 77 frosted glass slides (BRL, Waban, MA).
3. 4% PFA (paraformaldehyde)/PBS: Stir 20 g of PFA (Sigma) into 250 mL of sterile milliQ H_2O. Then add ~10 drops of 5 *N* NaOH solution. Heat (to ~65 °C) and stir in hood until PFA is dissolved. Add 50 mL of 10X PBS (0.1 *M*) and allow it to cool to room temperature. Adjust pH to 7.4 using 1 *M* of HCl (~5 mL). Fill to 500-mL total with sterile milliQ H_2O. Filter-sterilize through 500-mL filter flask. Aliquot ~45 mL into 50-mL conical tubes and store at −20 °C.
4. 0.05% Triton X-100/PBS: Add 500 mL of PBS to 250 µL of Triton X-100 (Fisher).
5. Blocking buffer: 5% normal goat serum (NGS) in 0.05% Triton X-100/PBS.
6. Secondary antibody: Rhodamine (TRITC)-conjugated goat anti-mouse (Jackson ImmunoRes).
7. DAPI (Sigma): Dissolve the content of a 10-mg bottle in sterile, milliQ H_2O (shake and sit for 10–15 min). Transfer solution to a 15-mL tube. Bring the volume up to 10 mL and store 1-mL aliquots at 4 °C.
8. SlowFade Anti Fade kit (Invitrogen).
9. Permount (Fisher).
10. Zeiss Axioplan fluorescence microscope (Thornwood, NY).
11. Axiocam HRm (Zeiss) digital camera.

3. Methods

The approach taken in our laboratory for the generation of shRNAs is a single-step PCR amplification of a RNA polymerase III (pol III) promoter followed by the hairpin sequence and a transcriptional termination signal. We elected to use the mouse U6 (mU6) promoter to drive expression of the shRNA, but other pol III promoters can be used. Based on a plasmid template containing the mU6 promoter, we designed a forward PCR primer (**Fig. 1,** primer 1) that includes 22 nucleotides located 85 nucleotides upstream of the mU6 promoter in this vector, and a reverse primer (**Fig. 1,** primer 2) that includes the sequence encoding a pol-III termination signal (UUUUUU, therefore AAAAAA in the primer), followed by the sequence complementary to the guide or antisense strand, an 8-nt loop (primer sequence CAAGCTTC), the passenger or sense strand, and the last 21 nucleotides of the mU6 promoter, so that the final DNA product is as shown in **Fig. 1**. This PCR product was then cloned in a plasmid vector lacking eukaryotic promoter, resulting in a plasmid that can be used in cotransfection experiments to test its efficacy

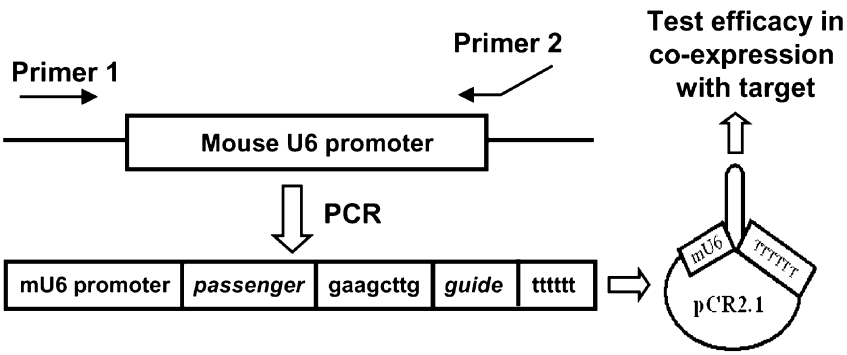

Fig. 1. Overview of protocol to generate shRNA-expressing plasmid vectors.

against overexpressed targets (this step is not described here, but we strongly encourage the identification of highly effective hairpins in transient cotransfection experiments before moving to primary neurons). Overall, this is a rapid strategy to quickly generate shRNA expression plasmids employing routine molecular biology techniques.

Effective hairpins are then cloned into the shuttle plasmid vector that will be used to generate recombinant lentivirus. The viral vector also encodes a GFP gene as a reporter of transduction ***(10)***. The resulting lentivirus is then used to transduce cultured neurons. We determine the degree of silencing of the target by measuring levels of the target protein, but mRNA quantification through quantitative RT-PCR can also be employed.

3.1. Generation of U6shRNA

1. Set up a 50-µL PCR reaction as follows: 45 µL of Platinum® PCR SuperMix High Fidelity, 2 µL of forward primer at 50 ng/µL, 2 µL of reverse primer at 50 ng/µL, and 1 µL of pAd5mU6 template plasmid at 10 ng/µL.
2. Perform the following amplification cycles: Initial denaturation for 3 min at 94 °C (1 cycle); denaturation for 30 sec at 94 °C, annealing for 30 sec at 50 °C, extension for 30 sec at 72 °C (30 cycles); final extension for 7 min at 72 °C (1 cycle).
3. Prepare a 1% agarose gel using standard procedures with a well capacity of 50 µL.
4. Load the resulting amplification reaction and verify by agarose gel electrophoresis using appropriate standards that a single band of 434 bp was produced.
5. Extract the PCR product from the agarose gel using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns. Following the manufacturer's protocol, identify the bands of interest, and excise them using a razor blade, cutting off excess agarose. Insert the DNA-containing agarose piece into the

filter cup of the spin column, and then place the filter cup into the dolphin tube. Place in freezer (−20 ºC) for 5 min, then spin in tabletop microcentrifuge at 13,000 rpm, room temperature for 3 min. Collect the purified DNA from the tube and discard the column with the remaining agarose.

6. The purified product obtained by this method can be directly used for cloning into the pCR2.1 vector as described here (go to next step). However, if problems are encountered with this cloning step, the PCR product can be precipitated as follows: Dilute Freeze 'N Squeeze product in 3 *M* Na-acetate 1/10th vol, 100% EtOH 2X vol, and 1–2 µg of glycogen. Freeze in liquid N2 until solid. Thaw and spin at 14,000 rpm for 8 min. Remove EtOH, wash pellet with 70% EtOH, and air-dry for 2 min. Resuspend pellet in 5 µL of water. Alternatively, a small amount of the PCR product can be electrophoresed for confirmation of the appropriate size, and the remainder of the PCR product ligated directly into the cloning vector, thus eliminating the DNA purification step. In this case, it is essential that the template plasmid used in the PCR reaction contains a different bacterial resistance gene than the TOPO vector. However, components of the PCR present in the nonpurified PCR product may interfere with the cloning step to various extents.
7. Following the manufacturer's protocol, add 4 µL of the isolated PCR product directly to a tube containing 1 µL of pCR2.1 TOPO vector and 1 µL of salt solution (1.2 *M* NaCl; 0.06 *M* $MgCl_2$; provided with TOPO kit). Incubate 5 min at room temperature.
8. Transform 3 µL of TOPO cloning reaction into chemically competent *E. coli* (provided with the cloning kit) using standard procedures.
9. Spread 50 µL of 40 mg/mL X-gal onto an ampicillin-selective LB plate, prewarm it to 37 ºC, and then spread 1/10th vol of the transformed bacteria. Centrifuge the remaining transformation at a low speed (e.g., 2,000 rpm for 1 min) to pellet bacteria. Remove supernatant and resuspend pellet in 100 µL of LB media. Spread remaining cells on a second plate. Incubate plates overnight at 37 °C.
10. Next day, pick 5–10 white colonies from the plate into a culture tube containing 5 mL of LB medium and 100 µg/mL of ampicillin. Grow overnight in shaking incubator at 37 °C (*see* **Note 2**).
11. Isolate plasmid DNA using Quantum Prep Plasmid Miniprep Kit following the manufacturer's instructions. Any other commercial or custom method for DNA isolation can be used as well.
12. Digest 5 µL of miniprep DNA with EcoRI as follows: 5 µL of miniprep DNA, 3 µL of 10X EcoRI buffer, 1.5 µL of 2 mg/mL bovine serum albumin (BSA) (20X), 0.5 µL of EcoR1 (at 20 units/µL), and 20 µL of water. Incubate at 37 °C for 2 h.
13. Run 10 µL of the digestion product in 1% agarose gel to confirm the presence of a single band of 451 nucleotides by visualizing it using a UV trans-illuminator.
14. Plasmid vectors with an insert of the appropriate size can be sent for sequencing using M13 forward and reverse primers.

15. This vector can now be used for screening in cotransfection experiments with overexpressed targets in cultured cells (*see* **Note 3**).

3.2. Generation of FIV.eGFP.U6shRNA

The protocol described here for the generation of lentiviral vectors encoding shRNAs is adapted from the Gene Transfer Vector Core at the University of Iowa (http://www.uiowa.edu/~gene/) ***(10,13)***. This method generates recombinant feline immunodeficiency virus (FIV) pseudotyped with the vesicular stomatitis virus envelope glycoprotein (VSV-G) ***(10)***. FIV particles are generated using a triple plasmid system based on the cotransfection of 293T cells with three plasmids (shuttle, packaging, and envelope), followed by harvesting of particle-containing culture medium and concentration of particles ***(13)***. However, other lentiviral vectors are available and can be used, such as those based on the human immunodeficiency virus. Many scientists do not generate their own recombinant lentiviral vectors but purchase them from different academic or industry-based facilities. At this point, the plasmid vector encoding your effective pol III-shRNA can be sent to those centers for the generation of shuttle vectors.

3.2.1. Generation of Shuttle Plasmid

1. Digest 3 µL of $pVET_LCMVeGFP$ plasmid vector with MfeI for 1 h in a 37 ºC water bath. After 1 h, add 3 µL of CIAP buffer and 1 µL of CIAP and incubate for an additional hour in a 37 ºC water bath. Run in 1% agarose gel, and purify the digested vector using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns as above. Precipitate DNA with Na acetate as described earlier.
2. Excise mU6shRNA from the pCR2.1 vector using EcoRI as above (using 10 µL of the plasmid vector here). Run in 1% agarose gel and purify the digested insert (band of 451 nucleotides) using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns.
3. EcoRI and MfeI are compatible ends. Ligate mU6shRNA into $pVET_LCMVeGFP$ using the Rapid DNA ligation kit. Prepare the ligation reaction as follows: 5 µL of insert (mU6shRNA), 1 µL of vector ($pVET_LCMVeGFP$), 2 µL of 5X DNA dilution buffer, and 2 µL of water, mixing well. Add 10 µL of 2X ligation buffer and 1 µL of T4 DNA Ligase, mixing well. Incubate for 5 min at room temperature. Rapidly transform competent cells using 10 µL of ligation reaction as above and grow in ampicillin-containing LB plates as above (except no need to spread X-gal).
4. Select 10 colonies per plate, grow in 5 mL of LB/ampicillin media overnight, and isolate plasmid DNA using Quantum Prep Plasmid Miniprep Kit as above.
5. Perform a diagnostic reaction to confirm ligation with NotI and HincII. If there is no insertion of the U6shRNA, the resulting band will be 544 bp. If there is insertion, the resulting band should be 995 bp.

3.2.2. Virus Production

1. Seed 293T cells into 18 150-mm-diameter flat-bottom tissue culture dishes at a density of 10^7 cells per dish.
2. The next day, add 34 mL of room-temperature HBS to two 50-mL conical tubes.
3. Add 225 μg of the packaging plasmid, 337.5 μg of the vector plasmid, and 112.5 μg of the envelope plasmid to each HBS-containing tube and vortex well.
4. Slowly add 1.7 mL of room-temperature 2.5 *M* $CaCl_2$ to each tube while slowly vortexing or shaking the HBS–plasmid mixture.
5. Let the solution stand for 25 min to allow precipitate formation. The solution should appear slightly translucent or cloudy.
6. Add both tubes of precipitate directly to 200 mL of DMEM. Briefly mix.
7. Aspirate off the medium from the cells (nine plates at a time).
8. Gently pipette the transfection solution onto the cells (15 mL per dish), and return the cells to the incubator.
9. Four to six hours after transfection, aspirate off the medium and provide 15 mL of fresh DMEM-10 per dish.
10. Collect the medium (containing vector particles) at 24, 36, and 72 h, each time replacing this medium with fresh DMEM-10. At each collection, filter the medium through a 0.45-μm filter (Nalgene PES, low-protein-binding 500-mL bottle-top filter) and store short-term at 4 ºC or long-term in 50-mL aliquots at −80 ºC.
11. Just before intended use, concentrate the particles by centrifuging the collected medium at 4 ºC for 16 h at 7,400g (7,000 rpm in the SLA 1500 rotor, Sorvall Centrifuge, 275-mL capacity tubes). Carefully pour off the supernatants and resuspend particles in lactose buffer. We typically resuspend the particles produced from an 18-plate transfection into a total volume of 3 mL.

3.2.3. Determining Titer by Limiting Dilution and Assay of Transgene (GFP) Expression

1. One day before transduction, seed a six-well flat-bottom plate with 2×10^6 HT-1080 cells per well in DMEM-10.
2. For transduction, make a 10-fold dilution series of concentrated FIV as follows: Add 1.584 mL of DMEM-2/polybrene in the first tube and 1.35 mL in tubes 2 through 6. Add 15 μL of virus to the first tube and vortex. Transfer 150 μL from the first to the second tube and vortex, and so on for the remaining tubes.
3. Remove culture media from wells. Add 1 mL of each dilution to separate wells. For wells 1 through 6, the dilution factors will thus be 10^3, 10^4, 10^5, 10^6, 10^7, and 10^8, respectively. Return the cells to the incubator.
4. Incubate the HT-1080 cells for 72 h and then feed with 1 mL of DMEM-10.
5. Incubate further for 24 h. Rinse monolayers with PBS.
6. Using an inverted fluorescent microscope, visualize and count the number of GFP-expressing cells in each well. The first two wells will often have too many

positive cells to count. Doublets or small clusters of cells are counted as one, as they likely originated by division of a single transduced cell.

7. For each well, multiply the total blue cell count by the dilution volume (1 mL) and by the dilution factor. Determine the mean of all the wells. This number represents the transducing units per milliliter (TU/mL) of concentrated virus. By using this method, our concentrated FIVeGFP preparations typically contain 10^8–10^9TU/mL.

3.3. Primary Neuronal Cultures

1. Add poly-D-lysine to 12-well dishes, and leave for at least 30 min before aspiration at step 11 (if experiments are designed for immunofluorescence analysis, place a round coverslip before adding the poly-D-lysine).
2. Sacrifice pregnant mouse at embryonic day 16 by injecting with ketamine/xylazine (150 μg/g of ketamine, 20 μg/g of xylazine).
3. Place mouse on paper towels lining the dissection pan and soak abdomen with 70% EtOH. Rinse instruments with 70% EtOH.
4. Make incision by grabbing skin with tissue forceps and cutting laterally along lower abdomen with large straight scissors to expose uterine horns. Rinse instruments with 70% EtOH.
5. Remove uterus by grabbing with tissue forceps and gently pulling upward while cutting away connective tissue and fat with scissors. Rinse uterus with 70% EtOH and place in 10-cm dish containing ice-cold HBSS dissecting media.
6. To remove embryos, carefully tear open uterus above each embryo with tissue forceps and fine forceps. Embryos will have individual chorionic sacks to remove as well. Sever umbilical cord and transfer embryo to new 10-cm dish with ice-cold HBSS dissecting media.
7. Decapitate embryos using small scissors and transfer heads to new 10-cm dish with ice-cold HBSS dissecting media, ensuring that heads are completely immersed in subsequent steps. Place Petri dish on ice and move to dissecting microscope.
8. To remove the brain, begin by removing the skin from the skull using one pair of fine forceps to hold the head through the orbits, using the other pair of forceps to remove the skin. Puncture the skull with fine forceps in the junction between cortices and brain stem. Gently remove skull by tearing with fine forceps starting with tips close together and pulling apart. Pinch off olfactory bulbs. With closed forceps, pry brain away from head starting at its anterior end, and then pinch off at base of brain stem. Transfer brain and brain stem to new 6-cm dish of ice-cold HBSS dissecting media.
9. To isolate cortex, pry hemisphere away from brain stem and gently pinch off. Remove meninges, choroid plexus, basal ganglia, and hippocampus. Place isolated cortex into new 6-cm dish of ice-cold HBSS dissecting media. Eight to 10 brains per dish are optimal. The following steps are performed inside a TC hood.
10. Cut up dissected brain with scalpel blade into small pieces (~2 mm). Using a 5-mL pipette, transfer tissue pieces from the 6-cm dish (cortices from 8–10 brains) to a 15-mL tube.

11. Bring volume to 8 mL with HBSS dissecting media, and add 1 mL of 10X DNase and 1 mL of 10X trypsin. Invert tubes and incubate in a small 37 °C water bath for 12–15 min. During this incubation, aspirate poly-D-lysine and add Neurobasal/FBS plating media to plates. Store plates in 37 °C, 5% CO_2 incubator until use.
12. Quick-spin 15-mL tube in centrifuge at 500 rpm to pellet tissue. Gently aspirate supernatant and wash tissue twice by adding 10 mL of room-temperature DMEM wash media, resuspending, quick-spinning, and removing supernatant. After second rinse, leave <2 mL of media with pellet.
13. Triturate tissue several times (~15–20) with cotton-plugged Pasteur pipette until tissue chunks are no longer visible. Follow with trituration using fire-polished, cotton-plugged Pasteur pipette (~10–15 times) to obtain a homogenous mixture.
14. Dilute triturated cell suspension from <2 mL to 1 mL/brain with Neurobasal/FBS plating media (e.g., if starting with 8 brains, bring up to 8 mL).
15. Perform cell counts using hemocytometer.
16. Plate 300,000–900,000 cells/well (~200–250 µL) using Neurobasal/FBS plating media.
17. After 3–4 h, remove plating media and replace it with Neurobasal/B27 maintenance media. Replace Neurobasal/B27 maintenance media every 3–4 days.
18. Transfections may be done after 48 h without significant toxicity to cells. After 2 days, begin treatment with 10 µ*M* of Ara-C for selection.

3.4. Transduction of Primary Neuronal Cultures

1. Count cells in each well with a hemocytometer.
2. Heat serum-free OptiMEM to 37 °C in a water bath.
3. Dilute the recombinant FIV in 500 µL/well of serum-free OptiMEM. In initial experiments, it is advised to calculate the most optimal multiplicity of infection (MOI) starting at 1, 5, and 10. The MOI is calculated based on the number of cells per well and viral titers obtained (MOI of 1 means one viral particle per cell). Incubate for 3 h, replacing it with serum-containing Neurobasal/B27 maintenance media.
4. In 24–48 h, the GFP reporter gene is observed using an Olympus inverted fluorescence microscope to assess transduction efficiency.

3.5. Lysis for Protein Analysis

1. Harvest cells 72 to 96 h after transduction. Aspirate media and briefly wash with ice-cold PBS. Aspirate PBS and add 200 µL of lysis buffer. Scrape off plates and transfer to labeled Eppendorf. Boil for 10 min at 100 °C in a dry bath.
2. To calculate protein concentration, take a piece of Whatman filter paper (approx. 3 × 2 in.) and spot 8 µL of BSA standards (0.25, 0.5, 1, 2, 5, 7.5, and 10 mg/mL, labeling each spot properly) and the different samples. Let dry at room temperature for 5 min, and stain in a small tray with sufficient volume of Coomassie Blue to cover the filter paper for 5 min. Pour the Coomassie stain into a labeled recipient (can be reused) and rinse twice in dH_2O. Add sufficient volume of destaining

solution to cover the filter paper, and incubate at room temperature for 15 min. Discard destaining solution and rinse in dH_2O. Repeat the destaining step if needed until no significant background signal is present outside the spots. Let dry at room temperature for 5 min, and determine the concentration of each sample by visually comparing to the standards. Other available methods to determine the protein concentration can be used.

3. Samples are now ready to be electrophoresed or frozen at −80 °C for future use.

3.6. SDS-PAGE

1. These instructions assume the use of a Mini-PROTEAN 3 electrophoresis system (Bio-Rad). The glass plates for the gels need to be cleaned well after use with a rinsable detergent and rinsed extensively with distilled water. Rinse with 95% ethanol, followed by distilled water, and air-dry well before use.
2. Prepare a 1.5-mm-thick 12% gel with the recipe provided above. Pour 7–8 mL of the gel, leaving space for the stacking gel, and overlay with 1 mL of water-saturated isobutanol. Leave gel to polymerize for about 30 min.
3. Pour off the isobutanol and rinse the top of the gel twice with dH_2O.
4. Prepare the stacking gel with the recipe provided above. Use 0.5 mL to quickly rinse the top of the gel and then pour the stack and insert the comb. Leave gel to polymerize for about 30 min.
5. Once the stacking gel has set, carefully remove the comb and use a 1-mL pipette to wash the wells with running buffer.
6. Add the running buffer to the upper and lower chambers of the gel unit and load 30 μL of each sample in a well. Include one well for 15 μL of prestained molecular weight markers.
7. Complete the assembly of the gel unit and connect to a power supply. Run at 200 V constant (usually takes ∼45 min, can be adjusted if needed). The molecular weight markers or dye fronts (blue/pink) can be used to monitor running status. Stop the power supply when they reach the end of the gel.

3.7. Western Blot for TorsinA

1. The samples that have been separated by SDS-PAGE are transferred to nitrocellulose membranes electrophoretically. These directions assume the use of the Mini Trans-Blot Cell tank transfer system (Bio-Rad).
2. Cut six pieces of Whatman 3M filter paper to the size of the pad for each gel. Cut PVDF Immunoblot membrane (∼9 × 6.5 cm). Wet briefly in methanol. Equilibrate in a tray with transfer buffer until the membrane no longer floats.
3. Place two pads and five of the filter papers in a tray with transfer buffer.
4. On the black side of the transfect cassette, place a pad and two wetted sheets of 3M filter paper.
5. The gel unit is disconnected from the power supply and disassembled. Using the dry 3M filter paper, lay the gel on the transfected cassette.

6. Place the nitrocellulose membrane on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. Add the three additional pieces of wetted 3M filter paper, pad, and close the transfer cassette.
7. The cassette is placed into the transfer tank. It is vitally important to ensure the right orientation of the gel and nitrocellulose membrane, i.e., the gel is closer to the negative (Black) electrode and the membrane closer to the positive (Red) electrode; otherwise, the proteins will move in the wrong direction and get out of the gel into the transfer buffer.
8. Include a magnetic stir-bar in the tank; place on agitator at 4 ºC (in cold room or industrial refrigerator).
9. Put the lid on the tank and activate the power supply. Transfers at either 120 mAmps overnight or 300 mAmps for 2 h.
10. Once the transfer is complete, the cassette is taken out of the tank and carefully disassembled, with the top pad and sheets of 3M filter paper removed. Using tweezers, remove the nitrocellulose membrane from the gel, which can be discarded. The colored molecular weight markers should be visible on the membrane.
11. Rinse the nitrocellulose briefly in dH_2O, and then incubate in ~50 mL of blocking buffer for 15 min on a rocking platform at room temperature.
12. Discard the blocking buffer and incubate the membrane with 5 mL of a 1:100 dilution of DM2A8 mouse monoclonal antibody ***(14)*** in blocking buffer for 3 h at room temperature, or overnight at 4 ºC (recommended for better results) on a rocking platform.
13. Remove the primary antibody and wash the membrane three times for 5 min each with blocking solution.
14. The secondary antibody is freshly prepared in a tray with 15 mL of blocking solution at a 1:15,000 dilution. Incubate with the membrane for 1 h at room temperature on a rocking platform.
15. Discard the secondary antibody and wash the membrane three times for 15 min each with blocking solution.
16. In a tray mix 5 mL of each component of the ECL reagent at room temperature. Add to the membrane for 1 min on a rocking platform or rotate it by hand to ensure proper coverage.
17. The blot is removed from the ECL reagents, gently dried with Kim Wipes, and placed inside the X-ray film cassette**.**
18. In a dark room, a Kodak film is inserted into an autoradiography cassette containing the membrane for a suitable exposure time, typically 2–5 min prior to developing. An example of the results is shown is shown in **Fig. 2**.

3.8. Stripping and Reprobing Blots for α-Tubulin

1. Once a satisfactory exposure for torsinA has been obtained, the membrane is then stripped of that signal and reprobed with an antibody that recognizes α-tubulin, thus providing a loading control.

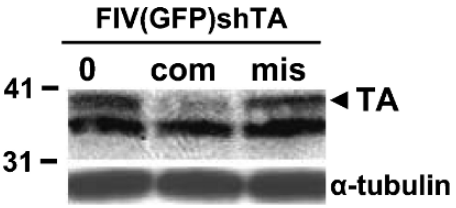

Fig. 2. Detecting lentiviral mediated RNAi silencing of torsinA in primary neuronal cultures by Western blot. Primary neuronal cultures were transduced with control FIV.eGFP or FIV.eGFPshRNA-mismatched constructs (FIVeGFP.shTAmis), or FIV.eGFP.shRNA targeting torsinA (FIVeGFP.shTAcom), and assayed for silencing of torsinA by Western blot analysis, demonstrating suppression by FIVeGFP.shTAcom.

2. Stripping buffer (50 mL per blot) is warmed to 65 ºC in an incubator and the blot added. The blot is incubated for 30 min with occasional agitation.
3. Wash the blot in abundant TBS-T (100 mL) six times, each for 5 min.
4. Incubate in blocking solution at room temperature for 1 h.
5. The membrane is then ready to be reprobed with anti-α-tubulin (1:20,000 in blocking solution) for 2 h at room temperature. The subsequent washes, secondary antibody, and ECL detection can be performed as above. An example result is shown in **Fig. 2**.
6. The degree of silencing can be quantified using different methods, normalizing the torsinA signal to the α-tubulin signal (*see* **Note 4**).

3.9. Immunofluorescence for TorsinA

1. Before seeding the primary neurons, coverslips will be placed in each well (in a 12-well dish) as described in **Section 3.3.** Cells will grow and differentiate over this coverslip.
2. Aspirate media from cells.
3. Add 150 µL of 4% PFA/PBS to cells. Incubate 15 min at RT, and then aspirate 4% PFA/PBS.
4. Wash twice for 5 min with 0.05% TX-100/PBS.
5. Add blocking buffer to cells (5% NGS in 0.05% TX-100/PBS). Incubate for 30 min at room temperature, and then aspirate blocking solution.
6. Add primary antibody DM28A at 1:25 dilution in 0.05% TX-100/PBS. Incubate overnight at 4 ºC, and then aspirate antibody.
7. Wash twice for 5 min in 0.05% TX-100/PBS.
8. Add secondary antibody at 1:2,000 dilution in 0.05% TX-100/PBS. Incubate 1 h at room temperature, then aspirate antibody.
9. Wash three times for 5 min each in 0.05% TX-100/PBS.
10. DAPI stain (10 µL of DAPI in 2 mL of 0.05% TX-100/PBS) for 10 min at room temperature.

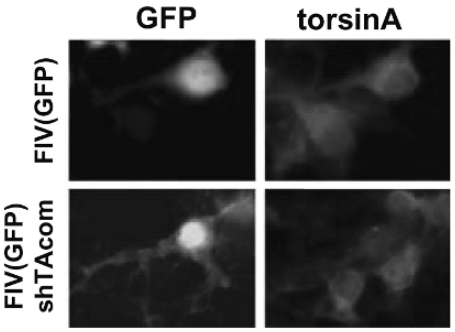

Fig. 3. Detecting lentiviral-mediated RNAi silencing of torsinA in primary neuronal cultures by fluorescence microscopy. Indirect immunofluorescence image showing decreased torsinA signal (in red) in neurons tranduced (in green) by FIVeGFP.shTAcom but not FIV.eGFP, when compared to surrounding nontransduced cells.

11. Follow the protocol of the SlowFade antifade kit, and then carefully pull coverslips from well with curved tweezers, mounting them on a slide (be sure cells are facing on the slide side), sealing the edges with Permount. Protect from light and let it dry.
12. Slides are now ready for visualization on fluorescence microscope using the appropriate channels. TorsinA is visualized in red, GFP in green (indicating transduced cells), and DAPI in blue (decorating the nucleus). Silencing is detected by decreased torsinA immunostaining in GFP-expressing cells, when compared to nontransduced (i.e., non GFP-expressing) cells, as shown in **Fig. 3**.

4. Notes

1. This is a plasmid vector containing the promoter of interest. Different promoters and promoter-containing plasmids can be used as a PCR template. Pol III promoters that have been employed to drive shRNAs expression include U6, H1, and tRNAval.
2. It is advised to use as a control a cloning reaction with vector but no insert. If the ratio of white/blue colonies is significantly higher in your cloning reaction than in the control, five colonies should be enough. If the ratio is only marginally better, then 10 colonies should be selected for screening.
3. Our approach is to cotransfect Cos7 cells with this plasmid-shRNA and a plasmid expressing the target cDNA at a 3:1 molar ratio (shRNA-target) using Lipofectamine 2000, harvest the cells 48 h later, run the protein lysates in standard SDS-PAGE, and perform a Western blot for the targeted protein and α-tubulin as a loading control to determine the degree of silencing.
4. For quantification experiments, our approach is to scan the resulting blots and quantify the pixel count and intensity of immunoreactive bands using the Scion

Image software (Scion Corporation). We then normalize the torsinA signals based on the amount of protein loaded (using the α-tubulin signal as an internal reference) and express the result as a percentage of the signal obtained in the control lane ***(7,8)***. Quantification of data may be done by any other method based on scanning densitometry of the films, providing that the signal has not been saturated. Alternatively, the chemiluminescent signal can be captured digitally with one of the many different instruments available.

Acknowledgments

Research on RNAi was funded by the Dystonia Medical Research Foundation (P. G.-A.) and NIH/NINDS (S. Q. H. and P. G.-A.). We are grateful to all the members of the Davidson, Gonzalez, and Paulson laboratories and the Gene Transfer Vectors Core at The University of Iowa for all the hard work carried out to develop and optimize the protocols described here.

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