12

siRNA and shRNA as Anticancer Agents in a Cervical Cancer Model

Wenyi Gu, Lisa Putral, and Nigel McMillan

Summary

We describe the protocols of using siRNAs, or shRNAs delivered by a lentiviral vector, as a means to silence cancer-causing genes. We use cervical cancer as a model to demonstrate the inhibition of the human papillomavirus (HPV) oncogenes E6 and E7 in cervical cancer cells by RNAi and inhibition of the cell growth *in vitro* and tumor growth in mouse models. The protocols include methods on siRNA and shRNA design, production of lentiviral-vectored shRNA, transfection or transduction of cervical cancer cells with siRNA or shRNA, and detection of the inhibitory effects of siRNA or shRNA both *in vitro* and *in vivo*.

Key Words: siRNA; shRNA; HPV; cervical cancer; lentiviral vector.

1. Introduction

The recent discovery of RNA interference (RNAi) triggered by small interfering RNA (siRNA) or short hairpin RNA (shRNA) in eukaryote cells has revolutionized the biology, especially the study of gene function, and also holds promise for developing RNAi treatments for a variety of human diseases, including viral infections, genetic disorders, and cancer ***(1–5)***. The basic mechanism of RNAi is well reviewed by Meister and Tuschl ***(6)*** and won't be covered here. Indeed, because siRNA or shRNA can silence a target gene at the posttranscriptional level (i.e., they do not affect genomic DNA) and in a sequence-specific manner, genes responsible for the above diseases are potential targets for RNAi treatment. As a result, a number of preclinical and even a few clinical studies with siRNA have recently been reported ***(7–12)***. However,

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

the real application of siRNA in the clinic still requires a number of hurdles to be cleared, including a clear understanding of the molecular mechanism of RNAi, the development of highly specific siRNAs that lack off-target effects, and more effective *in vivo* delivery systems.

We describe the method of how to use synthetic siRNA or shRNA delivered by replication-defective lentiviral vectors to silence the HPV oncogenes E6 and E7 in cervical cancer cell lines and inhibit the tumor growth in a mouse model. We chose this disease as a model because it is the second most common cancer in women worldwide ***(13–15),*** and its development is closely associated with the infection of high-risk HPV types (such as types 16 and 18). It is also known that expression of E6/E7 oncogenes of the high-risk HPV is the primary cause of cervical cancer and their continuous expression is necessary for keeping cancer cells alive ***(16–18)***. This therefore provides an ideal model for applying RNAi because silencing HPV E6/E7 will not affect human genes. Studies, including ours ***(19),*** have shown that synthetic siRNA can effectively suppress HPV E6 and/or E7 expression in various cervical cancer cell lines *in vitro* ***(20–25)***. However, the short half-life of siRNA after direct injection limits its clinical application as a treatment for diseases. Therefore, *in vivo* delivery is the next big issue for siRNA and is being actively investigated with various approaches including nanoparticles ***(26)*** and SNALP-based ***(27)*** and cationic liposome-based ***(28)*** delivery systems reported. Alternatively, a stable and long-term RNAi can be achieved using plasmid-based shRNA ***(29,30,41)***. At present, the most effective and powerful way to deliver these plasmids is lentiviral delivery, although its bio-safety is still an issue. The advantages of using the lentiviral delivery system compared with synthetic and vector-borne siRNA include the ability to stably transduce dividing and nondividing cells with relatively high efficiency ***(31–33)***. In addition, the lentivirus is nontoxic and minimally immunogenic since no viral genes are encoded in the vector genome. As a gene delivery system, the lentiviral vector has been explored for preclinical application and showed promising results ***(7–9,34,35)***. Recently, a new version of a nonintegrating lentiviral vector has been reported ***(36)*** and a new SV40-based pseudoviron delivery system has also been investigated ***(37)***.

After all, when any new delivery system is developed and available for clinical use, the siRNA or shRNA described here can be used with the new delivery system to develop a new treatment for cervical cancer.

2. Materials

2.1. siRNA and shRNA Against HPV Types 16 and 18

1. siRNA against HPV 16 E6 (sequence 10 and 37) ***(19)*** siRNA-16E6-1:
   Sense 5′-GCAACAGUUACUGCGACGUUU-3′

Antisense 3′-UUCGUUGUCAAUGACGCUGCA-5′
siRNA-16E6-2:
Sense 5′-CACGUAGAGAAACCCAGCUUU-3′
Antisense 3′-UUGUGCAUCUCUUUGGGUCGA-5′
Scrambled siRNA (used as negative control):
Sense 5′-GACCUGUUAAUGACGGCACUU-3′
Antisense 3′-UUCUGGACAAUUACUGCCGUG-5′

2. shRNA against HPV 18 E6 ***(41)*** shRNA-18E6-1 (effective targeting HPV 18 E6):
5′-TAGGTATTTGAATTTGCATTTCAAGAGAATGCAAATTCAAATACCTT
TTTTTC
3′-ATCCATAAACTTAAACGTAAAGTTCTCTTACGTTTAAGTTTATGGAA
AAAAAGAGCT
shRNA-18E6-2 (targeting at HPV 18 E6 but not effective, can be used as negative control):
5′-TCAGAAAAACTTAGACACCTTCAAGAGAGGTGTCTAAGTTTTTCTGT
TTTTTC
3′-AGTCTTTTTGAATCTGTGGAAGTTCTCTCCACAGATTCAAAAAGACA
AAAAAGAGCT

2.2. Cervical Cancer Cell Lines and Lentiviral Vector Packing Cells

1. Cervical cancer cell lines: Cervical cancer cell lines of HeLa cells (HPV 18+), CasKi cells (HPV 16+), SiHa cells (HPV16+), and C33A cells (HPV negative) were all maintained in complete DMEM medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of Penicillin G, 100 µg/mL of Streptomycin sulfate, and 0.29 mg/mL of L-Glutamine).
2. Lentiviral vector packing cell line 293T cells were also maintained in complete DMEM medium.

2.3. Transfer and Packing Plasmids

1. Transfer plasmid: Plasmid pLentiLox3.7 (pLL3.7, a gift from Dr. Luk van Parijs, MIT, Cambridge, MA).
2. The lentiviral packaging plasmids: pRSVRev, pMDLgpRRE, and pMD.G (contains VSV.G gene) were a gift from Dr. Inda Verma (Salk Institute, San Diego, CA). These plasmids were described in detail ***(38)*** and were used for third-generation lentiviral vector production.

2.4. Buffers and Transfection Agents

1. Annealing buffer: 100 m*M* K-acetate, 30 m*M* HEPES-KOH pH 7.4, and 2 m*M* Mg-acetate.
2. Phosphate transfection buffer: (2XHBS): 140 m*M* NaCl, 1.5 m*M* Na_2HPO_4, 50 m*M* of HEPES, pH 7.05).
3. 1.25 *M* $CaCl_2$.

4. Transfection reagent: Oligofectamine™Reagent (Invitrogen, Sydney, Australia).
5. RIPA buffer: 150 m*M* NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 m*M* Tris-HCI, pH 8.0.
6. PIC: protein inhibitor cocktail (Sigma, Sydney, Australia).

2.5. Animals

Female Rag knockout mice 5–6 weeks old.

3. Methods

3.1. Design and Synthesize siRNA and shRNA Expression Cassette

1. siRNA and shRNA were designed on the basis of E6 mRNA sequences of HPV types 16 and 18 by finding AAN(19)TT and following the basic selection criteria including the G+C content being not more than 50% and not containing more than 4Ts (*see* **Note 1** for more details).
2. Blast the sequences in GenBank to exclude the possible off-target effect to human genes. We chose a cutoff of no more than 15 of 21 bases overlapping.
3. For the shRNA expression cassette, it contained the 19nt target sequence followed by the loop sequence (TTCAAGAGA), reverse complement to the 19nt, two 3′ overhanging T (TT), stop codon for U6 promoter (TTTT), and Xho1 site (C). For 18E6-1: it is 5′-TAGGTATTTGAATTTGCATTTCAA GAGAATGCAAATTCAAATACCTTTTTTTC. Using this DNA sequence of shRNA expression cassette, a complementary strand of oligo DNA can be made (e.g., 18E6-1: 3′ATCCATAAACTTAAACGTAAAGTTCTCTTACGTT TAAGTTTATGGAAAAAAAGAGCT).
4. Designed siRNAs were normally synthesized as duplexes that also contain the two overhanging UU at the 3′ end. For shRNA, the expression cassettes and their complementary strands were synthesized separately and supplied HPLC-purified by the company (Proligo, Lismore, Australia).
5. Scrambled siRNA or shRNA can be considered and synthesized for negative controls, but we prefer to use another sequence we have designed or use an siRNA against a defined target like GFP.

3.2. Cloning the shRNA Expression Cassette into Transfer Plasmid pLentiLox3.7

1. The synthesized shRNA expression cassette and its complimentary strand were annealed by mixing an equal amount (1 µL of 10 µM) of oligo DNA of each strand in 48 µL of annealing buffer (section **Section 2.4**) and by heating to 95 °C for 5 min followed by cooling to room temperature (*see* **Note 2** for more information).
2. The annealed product of double-stranded oligonucleotide DNA was directly used to clone into the plasmid pLentiLox3.7 at the HapI and XhoI sites (**Fig. 1**), using a routine cloning technique.

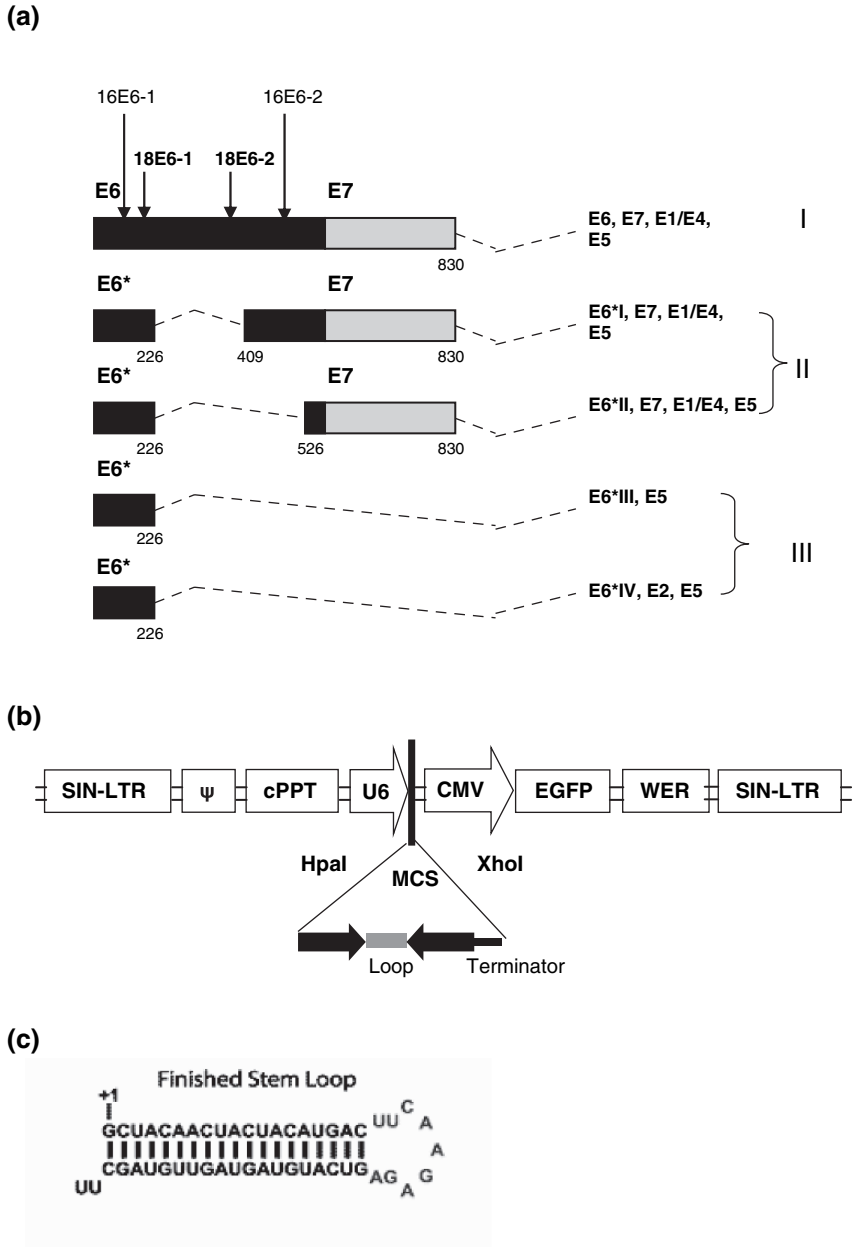

Fig. 1. shRNA targeting sites and the packing vector structure. (a) Diagrammatic representation of the various spliced mRNAs that encode E6 and E7. Coding regions are indicted by filled boxes and introns by dotted lines. siRNA targets for 16E-1 (123–142 bp) and 2 (454–473) and shRNA targets for 18E6–1 (128-145bp) and 18E6-2

3. The positive colonies were selected for MiniPrep, and the insertion of shRNA oligo DNA was confirmed by both restriction enzyme digestion analysis (e.g., NotI and XbaI) and sequencing (*see* **Note 3** for sequencing).

3.3. Production of Lentiviral-shRNA and Titration

1. Plasmids pLL3.7 or pLL3.7 plus insert (18E6-1 or -2), as well as other packing plasmids pRSVRev, pMDLgpRRE, and pMD.G, were amplified in *E. coli* and purified using W/Endo-free Qiagen MaxiPrep Kits (Promega, Sydney). The final DNA pellet was dissolved in endo-free TE buffer at a concentration around 0.5 mg/mL (note that as pMD.G is a low-copy-number plasmid, Terrific broth medium may be used instead of LB).
2. For transfection and lentiviral vector production, packaging cell line 293T cells were cultured in a T_{75} flask in complete DMEM until 70–80% confluent. A DNA mixture consisting of 6.6 μg of pLL3.7 (with or without insert) and 3.3 μg of each packaging plasmid in 133 μL of 1.25 M $CaCl_2$ was added to 0.5 mL of H_2O and then mixed slowly (drop by drop) with 0.66 mL of 2XHBS (*see* **Note 4**). The solution was then added to the 293T cells and incubated for 4 h of transfection at 37 °C in a tissue culture incubator.
3. The transfection medium was replaced by 7 mL of fresh complete DMEM medium.
4. The culture supernatant containing the lentivirus was harvested 36–48 h after transfection by centrifugation and filtered through a 0.45-μm filter.
5. The virus can be further concentrated 40–50 times using Vivaspin 20-mL Concentrators (100 MW, VivaScience Sartorius Group, Sydney), and the concentrated lentiviral stocks can be stored in a small aliquot at –80 °C for titration and cell infection.
6. Because pLL3.7 also contains an EGFP gene (**Fig. 1**), this marker can be used to monitor and titer the infection of lentiviral vector by GFP expression. To titer the viral vector, a 10-fold dilution series of viral stocks in 1.5 mL of DMEM containing polybrene (8 μg/mL) were added to 4×10^5 293T cells cultured in 6-well plates.
7. Twenty-four hours later, the medium was replaced with fresh complete DMEM medium.
8. Forty-eight hours after the infection, the cells were harvested and GFP-positive cells were determined by flow cytometry analysis. The titer was calculated using the formula: infectious unit (IU)/mL = % positive cells × 4×10^5 × dilution times. For a normal production run, the titer is above 1×10^8 IU/mL.

Fig. 1. (*Continued*) (344–361 bp) are indicated. (b) The linear structure of lentiviral packaging contruct, pLL3.7. The shRNA expression cassette is under the control of U6 promoter (*Hpa*I and *Xho*I site) and the eGFP gene is under the CMV promoter. For details, see http://web.mit.edu/ccr/labs/jacks/protocols/pll37.htm (c) The finished stem loop shows a representative hairpin structure of transcribed shRNA.

3.4. Transfection or Transduction of Cervical Cancer Cells with siRNA or LV-shRNA

3.4.1. Transfection with siRNA

1. On the day prior to transfection, Caski or SiHa cells were plated out at a density of 1.5×10^5 cells/well in a 6-well plate.
2. siRNA was diluted to concentrations of 1–40 nM in distilled H_2O. A volume of 5 μL of each dilution was added to 80 μL of Opti-MEM I reduced-serum medium (Invitrogen, Sydney). Oligofectamine^{TM}Reagent (Invitrogen, Sydney) (2 μL) was added to 13 μL of Opti-MEM I, and the mixture was incubated at room temperature for 5–10 min. The Oligofectamine–Opti-MEM I solution was added to the siRNA solution for a final volume of 100 μL. Transfection complexes were added to wells containing 400 μL of Opti-MEM I and incubated for 4 h at 37 °C.
3. Following incubation, transfection complexes were removed and replaced with complete DMEM.
4. The transfected cells were colleted at 48 h or 72 h after transfection for protein or RNA sample preparation.
5. Use scrambled or another siRNA as a negative control. To determine the transfection efficiency (*see* **Note 5**).

3.4.2. Transduction with LV-shRNA

1. HeLa cells were cultured in a 24-well plate (2.5×10^4 cells/well) or 6-well plate (1×10^5 cells /well) or T_{75} flask (2×10^6 cells) and cultured overnight.
2. LV-shRNA (20 IU/cell) was diluted in 0.2 mL (24-well plate) or 0.5 mL (6-well plate) or 2 mL (T75 flask) of DMEM containing polybrene (8 μg/mL) and added to the cells for incubation at 37 °C.
3. After 1 h of incubation, 0.3 mL (24-well plate) or 1.0 mL (6-well plate) or 8 mL (T75 flask) of fresh polybrene–DMEM were added to the cells for further incubation.
4. Twenty-four hours later, the polybrene–DMEM was replaced with fresh complete DMEM medium and the cells were cultured for the following assays.
5. For a quick infection-rate check, the cells can be viewed under fluorescent microscopy. Alternatively, the cells can be trypsinized and washed three times with PBS before resuspending in 0.5 mL of 1% paraformaldehyde–PBS. The fixed cells were then analyzed using FACSCalibur for eGFP expression.
6. At this dose, the transduction efficiency is normally above 95%. If this is the case, the cells can be cultured for other assays or stored for later use (*see* **Note 6**).

3.5. Detecting Inhibitive Effects of siRNA and shRNA in vitro

3.5.1. Detecting the Decrease of E7 Protein Levels by Western Blotting

1. Transfected CasKi cells or transuded HeLa in **Section 3.4** were harvested and lysed in RIPA buffer containing 2 m*M* of PMSF and 1 μL/mL of Protease Inhibitory Cocktail.

2. The total protein concentration of the cell lysate samples was measured using the BCA Protein Assay Kit (Progen, Darra, Australia), and 30–40 μg of total protein samples were loaded to 12% SDS-PAGE gel for separation as routine.
3. After electrophoresis, the protein was routinely electro-transferred onto nitrocellulose membranes, and the blot was blocked in PBS/5% skim milk powder overnight at 4 °C.
4. The blot was incubated with 1/200 diluted anti-HPV 16E7 (Santa Cruz Biotechnology) for two nights at 4 °C or 1/1,000 diluted anti-HPV 18E7 antibody (Santa Cruz Biotechnology) overnight at 4 °C.
5. The blot was then incubated with HRP-conjugated secondary antibody at room temperature for 3 h before being developed using ECL as routine. (*See* **Note 7** for detecting E6 or E7 associated protein using WB.)

3.5.2. Detecting shRNA Expression Using Northern Blot

1. The total RNA was extracted from trypsinized HeLa cells infected or uninfected with lentiviruses as previously described ***(42)***.
2. Fifteen μg of total RNA were digested with DNase I before being separated in a 12% polyacrylamide–urea gel and blotted onto a Nylon N+ membrane (Amersham). The RNA was bound to the membrane by UV cross-linking before being prehybridized with hybridization buffer (0.5 *M* of sodium phosphate, pH 7.5, 7% SDS, and 1 m*M* of EDTA) for 2 h at 68 °C.
3. The blot was hybridized with a ^{32}P-labeled oligo DNA probe at 42 °C overnight before being washed once with 1XSSC/1%SDS and three times with 0.5XSSC/0.1% SDS at 42 °C and exposed to X-ray film.

3.6. Inhibition of Cervical Cancer Cell Growth in vitro by siRNA and LV-shRNA

3.6.1. MTT Assay

1. CasKi cells transfected with siRNA at day 2 or HeLa cells infected or uninfected with LVs were grown for four days and were trypsinized, counted, and seeded in 6-well plates (5×10^5/well) for overnight setting.
2. The cells were washed twice with PBS and were covered with 250 μL of 2 μg/mL MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and 750 μL of PBS for incubation at 37 °C for 3 h.
3. Dimethyl sulfoxide, 750 μL, was added to each well for incubation at room temperature for 10 min before the supernatant was transferred to a 96-well plate for absorbance measurement at 595 nm.

3.6.2. Colony-Forming Assay

1. HeLa cells transuded with LV-shRNA and transuded and untransuded controls were cultured for two days.

2. The cells were harvested and counted before they were set as 100 cells per well in a 6-well plate in 2 mL of DMEM medium; each treatment has three repeats.
3. The cells were cultured at 37 °C for 10 days. Check cell colony growth during the period.
4. Discard the medium, fix the cell colonies with 95% ethanol for 10 min, and stain with 0.1% crystal violet in 20% ethanol for 5 min.
5. Gently wash the wells with tap water and air-dry the wells.
6. Count the colonies.

3.6.3. Dose-Dependent Infection and Killing Assay with LV-shRNA

1. HeLa cells were cultured and transuded in 24-well plates as in **Section 3.4.** The lentiviral vectors were diluted one (20 IU/cells) to five times (100 IU/cell) in 0.2 mL of polybrene-DMEM and added to the cells for incubation for 1 h at 37 °C. Transuded controls could include LV-18E6-2 and LV-PLL.
2. After the first incubation, 0.3 mL of fresh polybrene-DMEM were added to the cells and the incubation continued for 24 h. After this incubation, polybrene-DMEM was replaced with fresh DMEM medium, and the cells were cultured for another 5 days.
3. The cells were washed three times with PBS, fixed in 95% ethanol for 30 min at room temperature, and stained with 0.1% crystal violet for 5 min, then washed with water and air-dried. A large number of HeLa cells should be killed by 4X or 5X dose transduction.

3.7. Inhibiting Tumor Growth with LV-shRNA in vivo

3.7.1. Xeno-Transplantation Model in $Rag^{-/-}$ Mice

1. HeLa cells were cultured and transduced with 1x or 4x LV-18E6-1 in T_{75} flasks as described in **Section 3.4.** LV-18E6-2 or LV-PLL can be used as the control.
2. Two days post-infection, the cells were harvested using trypsin and washed with PBS, counted, and resuspended in PBS at 1×10^7/mL.
3. $Rag^{-/-}$ mice (5–6 weeks old; *see* **Note 8** for more information) were injected with 0.1 mL of cell suspension subcutaneously to the neck scruff.
4. At day 17–21 after injection, the tumors were collected and weighed.

3.7.2. Lung-Metastasis Model in $Rag^{-/-}$ Mice

1. HeLa cells were well maintained in complete DMEM. The cells were harvested using trypsin and washed with PBS, counted, and resuspended in PBS at 1×10^7/mL.
2. $Rag^{-/-}$ mice were injected with 0.1 mL of the cell suspension via the tail vein.
3. At week 6, the mice were randomly grouped and injected with 2×10^7 infectious units of either LV-18E6-1 or LV-PLL or PBS at weeks 7 and 8.
4. At week 9, the lungs of all groups were collected for frozen sections or histological examination.

5. The frozen sections were immunofluorescently stained with monoclonal anti-human MHC class I antibody conjugated with FITC (Sigma) as previously described ***(43),*** and the lungs were fixed for histological examination, paraffin embedded, and H&E stained.
6. Examine and count tumor nodules in both histological slides and immunofluorescently stained slides.

4. Notes

1. We used the old Tuschl rules to design our siRNAs, and they work very well in our hands. Things are a bit more sophisticated now, with many more parameters known to enhance your siRNA's efficacy and many online sites are available for designing siRNA or shRNA (e.g., http//:www.genelink.com/siRNA/shRNA.asp). Some companies (e.g., Ambion and Dharmacon) also provide the service of designing and synthesizing siRNA and shRNA. The design of shRNA was also fairly simple with a forward-loop-reverse-stop design. The sense oligo was 5′T-(GN18)-(TTCAAGAGA)-(81NC)-TTTTTTC. As the sequence was to be driven off a RNA polIII promoter (U6), the stop signal is 4 Ts and therefore one must avoid runs of 4 Ts in the target sequence. The antisense oligo was the complement of sense but with additional nucleotides at the 5′ end to generate XhoI overhang for cloning into pLL3.7. The loop sequence (TTCAAGAGA) is based upon Brummelkamp et al. ***(39)***. For the cervical cancer model, we selected the E6 gene rather than E7 as the target because targeting at E6 was able to knock down both E6 and E7 expression simultaneously as they are bi-cistronically transcribed (**Fig. 1**) and targeting at E6 seems more effective than targeting at E7 ***(19)***. We chose siRNA or shRNA targeting at the common sites of three mRNA classes and also some targeting at only class I mRNA of E6 (**Fig. 1**). However, a few studies showed that targeting E7 was also effective ***(21,22)***. For example, siRNA based on 142–160 bp of HPV 18E7 was shown to be able to knock down E6 and E7 effectively ***(21)***. Therefore, targeting both E6 and E7 could be effective depending on the region to be selected.
2. Besides the annealing method provided above, annealing of oligo DNA can be performed using a PCR machine. The program included steps of heating at 95 °C for 4 min, stopping at 70 °C for 10 min, then decreasing the temperature to 10 °C at the rate of 0.1 °C/min. We used this program and found it was not different from the one described above.
3. Confirmation of the shRNA insert by sequencing is often problematic due to the hairpin feature of the shRNA expression cassette ***(40)***. For example, we got good sequencing results from only 25% of the inserts. The samples that were easy to sequence (and gave the correct sequence) did not correlate with their silencing efficiencies. Therefore, while sequencing can be used to confirm the presence of the right insert, the functional study is more important, and in this case the proper negative control is clearly critical. The problems with sequencing can sometimes be overcome by including 5% DMSO in the sequencing reaction or using a unique

restriction site in the loop of the shRNA and cutting prior to sequencing to remove the hairpin.

4. Good-quality and endo-toxin-free preparation of packing plasmids is very important for high-titer lentiviral vector production. Well-maintained 293T cells are also important for lentiviral production. Whenever possible, keep the passage number low and use freshly set cells for transfection. The 293T cells plated for transfection were about 70–80% confluent. Lipofectamine-like reagents can be used to transfect the cells, but we used the calcium method. We have compared both; they yield the same amount of virus, but the calcium technique is much cheaper. The critical part of this method is the pH value of 2x HBS, as changes will result in no transfection. The solution should be made up with great care, and small aliquots should be stored at –20 °C for use. In practical operation, we normally use 15 T75 flasks to produce about 100 mL of lentiviral stocks, which can be further concentrated to 2–3 mL. For a normal production like this, the titer should be above 1×10^8 infectious unit (IU)/mL. Lentiviral stocks can be directly used for cell infection. In this case, the lentiviral stock with 50% fresh medium is used.
5. To determine the siRNA transfection efficiency, siRNA can be ordered and synthesized with a 5′- fluorescent tag (we used FAM). The tagged fluorescent does not affect silencing. The cells were then transfected as normal, and the efficiency can be measured by FACS. The cells can also be sorted by FACS, to gain a pure population of transfected cells.
6. After transduction and FACS analysis, LV-shRNA-transduced HeLa cells can be frozen and stored in liquid nitrogen as a cell line for later assays. Theoretically, the transduction and shRNA expression should be permanent. However, in our case a large number of cells transfected at higher doses died, but we have kept low-dose infected cells, which grow more slowly, in culture for up to 8 weeks with eGFP-positivity maintained at over 90%. During passage or proliferation, some cells may develop resistance or loss of the vector, so regular checking for eGFP and target gene expression is necessary.
7. Because E6 and E7 are expressed bi-cistronically, knocking down E6 mRNA will also result in the degradation of E7 mRNA. Therefore, the silencing effect of E6 siRNA or shRNA can be detected by the loss of E7 protein level. E7-related protein such as RB can also be assayed by Western blotting to further confirm the loss of E7 (not shown in the method). Certainly, the E6 protein level can be detected directly. However, until now we have not found a good commercial antibody against HPV 16 and 18 E6. Alternatively, the loss of E6 can be detected by analyzing the increased E6-associated protein such as p53 and p21 using WB. In addition, after transduction with LV-18E6-1, the degradation of E6 or E7 mRNA should occur in HeLa cells, which can be verified using Northern blotting using the protocol described above. Besides MTT assay, the inhibition of cell growth induced by siRNA or shRNA can also be tested by H3 incorporation assay described by Hall and Alexander ***(21)***.

8. We used immune-deficient, Rag knockout mice for *in vivo* testing because the cervical cancer cells are human cells. Other immune-incompetent mice (such as nude mice) could also be used as described previously ***(24)***. However, in $Rag^{-/-}$ mice, the HeLa cell is a good cell line to use in both the xeno-transplantation model and lung metastasis model, while CasKi and SiHa cells do not easily form tumors even in this strand.

Acknowledgment

The research work was supported by a grant from the National Health and Medical Research Council and by a Peter Doherty Postdoctoral Fellowship award.

References

1. Bitko, V., Musiyenko, A., Shulyayeva, O., and Barik, S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* **11,** 50–55.
2. Capodici, J., Kariko, K., and Weissman, D. (2002). Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol*. **169,** 5196–5201.
3. Dasgupta, R., and Perrimon, N. (2004). Using RNAi to catch Drosophila genes in a web of interactions: Insights into cancer research. *Oncogene* **23,** 8359–8365.
4. Hannon, G. J., and Rossi, J. J. (2004). Unlocking the potential of the human genome with RNA interference. *Nature* **431,** 371–378.
5. Jacque, J. M., Triques, K., and Stevenson, M. (2002). Modulation of HIV-1 replication by RNA interference. *Nature* **418,** 435–438.
6. Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature* **431,** 343–349.
7. Check, E. (2005). Pioneering HIV treatment would use interference and gene therapy. *Nature* **437,** 601.
8. Humeau, L. M., Binder, G. K., Lu, X., et al. (2004). Efficient lentiviral vector-mediated control of HIV-1 replication in CD4 lymphocytes from diverse HIV+ infected patients grouped according to CD4 count and viral load. *Mol. Ther*. **9,** 902–913.
9. Scherr, M., and Eder, M. (2002). Gene transfer into hematopoietic stem cells using lentiviral vectors. *Curr. Gene Ther*. **2,** 45–55.
10. Check, E. (2005). A crucial test. *Nat. Med*. **11,** 243–244.
11. Raoul, C., Abbas-Terki, T., Bensadoun, J. C., et al. (2005). Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat. Med*. **11,** 423–428.
12. Ralph, G. S., Radcliffe, P. A., Day, D. M., et al. (2005). Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat. Med*. **11,** 429–433.
13. zur Hausen, H. (2002). Papillomaviruses and cancer: From basic studies to clinical application. *Nat. Rev. Cancer* **2,** 342–350.

14. DiPaolo, J. A., and Alvarez-Salas, L. M. (2004). Advances in the development of therapeutic nucleic acids against cervical cancer. *Expert Opin. Biol. Ther.* **4,** 1251–1264.
15. Parkin, D. M., Bray, F. I., and Devesa, S. S. (2001). Cancer burden in the year 2000. The global picture. *Eur. J. Cancer* **37,** S4–S66.
16. Goodwin, E. C., and DiMaio, D. (2000). Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proc. Natl. Acad. Sci. USA* **97,** 12513–12518.
17. Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., and Howley, P. M. (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO J.* **8,** 4099–4105.
18. von Knebel Doeberitz, M., Oltersdorf, T., Schwarz, E., and Gissmann, L. (1988). Correlation of modified human papilloma virus early gene expression with altered growth properties in C4–1 cervical carcinoma cells. *Cancer Res.* **48,** 3780–3786.
19. Putral, L., Bywater, M., Gu, W., et al. (2005). RNAi against HPV oncogenes in cervical cancer cells results in increased sensitivity to cisplatin. *Mol. Pharmacol.* **68,** 1311–1319.
20. Butz, K., Ristriani, T., Hengstermann, A., Denk, C., Scheffner, M., and Hoppe-Seyler, F. (2003). siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. *Oncogene* **22,** 5938–5945.
21. Hall, A. H., and Alexander, K. A. (2003). RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. *J. Virol.* **77,** 6066–6069.
22. Jiang, M., and Milner, J. (2002). Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* **21,** 6041–6048.
23. Jiang, M., Rubbi, C. P., and Milner, J. (2004). Gel-based application of siRNA to human epithelial cancer cells induces RNAi-dependent apoptosis. *Oligonucleotides* **14,** 239–248.
24. Yoshinouchi, M, Yamada, T., Kizaki, M., et al. (2003). *In vitro* and *in vivo* growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA. *Mol. Ther.* **8,** 762–768.
25. Yamato, K., Fen, J., Kobuchi, H., et al. (2006). Induction of cell death in human papillomavirus 18-positive cervical cancer cells by E6 siRNA. *Cancer Gene Ther.* **13,** 234–241.
26. Schiffelers, R. M., Ansari, A., Xu, J., et al. (2004). Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* **32,** e149.
27. Zimmermann, T. S., Lee, A. C., Akinc, A., et al. (2006). RNAi-mediated gene silencing in non-human primates. *Nature* **441,** 111–114.
28. Sioud, M., and Sorensen, D.R. (2003). Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem. Biophys. Res. Commun.* **312,** 1220–1225.

29. Bai, Y., Soda, Y., Izawa, K., et al. (2003). Effective transduction and stable transgene expression in human blood cells by a third-generation lentiviral vector. *Gene Ther*. **10,** 1446–1457.
30. Van den Haute, C., Eggermont, K., Nuttin, B., Debyser, Z., and Baekelandt. V. (2003). Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain. *Hum. Gene Ther*. **14,** 1799–1807.
31. Kafri, T., van Praag, H., Gage, F. H., and Verma, I. M. (2000). Lentiviral vectors: Regulated gene expression. *Mol. Ther*. **1,** 516–521.
32. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998). Development of a self-inactivating lentivirus vector. *J. Virol*. **72,** 8150–8157.
33. Naldini, L., and Verma, I. M. (2000). Lentiviral vectors. *Adv. Virus Res*. **55,** 599–609.
34. van der Wegen, P., Louwen, R., Imam, A. M., et al. (2006). Successful treatment of UGT1A1 deficiency in a rat model of Crigler–Najjar disease by intravenous administration of a liver-specific lentiviral vector. *Mol. Ther*. **13,** 374–381.
35. Morizono, K., Xie, Y., Ringpis, G. E., et al. (2005). Lentiviral vector retargeting to P-glycoprotein on metastatic melanoma through intravenous injection. *Nat. Med*. **11,** 346–352.
36. Yanez-Munoz, R. J., Balaggan, K. S., MacNeil, A., et al. (2006). Effective gene therapy with nonintegrating lentiviral vectors. *Nat. Med*. **12,** 348–353.
37. Kimchi-Sarfaty, C., Brittain, S., Garfield, S., Caplen, N. J., Tang, Q., and Gottesman, M. M. (2005). Efficient delivery of RNA interference effectors via *in vitro*-packaged SV40 pseudovirions. *Hum. Gene Ther*. **16,** 1110–1115.
38. Dull, T., Zufferey, R., Kelly, M., et al. (1998). A third-generation lentivirus vector with a conditional packaging system. *J. Virol*. **72,** 8463–8471.
39. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296,** 550–553.
40. Jazag, A., Kanai, F., Ijichi, H., et al. (2005). Single small-interfering RNA expression vector for silencing multiple transforming growth factor-beta pathway components. *Nucleic Acids Res*. **33,** e131.
41. Gu, W., Putral, L., Hengst, K., et al. (2006). Inhibition of cervical cancer cell growth *in vitro* and *in vivo* with lentiviral-vector delivered short hairpin RNA targeting human papillomavirus E6 and E7 oncogenes. *Cancer Gene Ther*. **13,** 1023–1032.
42. Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–159.
43. Gu, W., Janssens, P., Holland, M., Seamark, R., and Kerr, P. (2005). Lymphocytes and MHC class II positive cells in the female rabbit reproductive tract before and after ovulation. *Immunol. Cell Biol.* **83,** 596–606.