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Retrovirus-Mediated RNA Interference:

Targeting hTERT Through Stable Expression of Short-Hairpin RNA

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Summary

RNA interference (RNAi) has recently emerged as a reliable tool for studying the effects of knocking down or ablating the expression of specific genes. It is hoped that progress made in the laboratory toward in vitro down regulation of gene expression may be carried over into the clinic for treatment of diseases in which the expression of a specific gene is associated with initiation or progression of that disease. Such is the case with telomerase, an exciting drug target that has been the focus of numerous investigations with a wide variety of inhibitors. This chapter describes the use of retrovirally introduced short-hairpin RNA as an effector of stable, long-term RNAi in human cells.

Keywords

RNA interference; retroviral; short-hairpin RNA; telomerase inhibition

1. Introduction

RNA interference (RNAi) is rapidly becoming the technique of choice for gene knockdown and analysis of function studies. There is hope that the technology could eventually be used as a treatment for diseases in which the expression of a specific gene is associated with disease progression. The catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), seems to be a logical target for RNAi because advances in telomerase inhibition have considerable potential for cancer therapy.

There are numerous approaches to stable RNAi, both plasmid-based and virally delivered. Several laboratories have designed their own vectors (1-7) for short-hairpin RNA (shRNA) delivery (8) and have found them to be effective. Many companies market some RNAi product, and our laboratory has chosen the GeneSuppressor™ System (cat. no. IMG-1000) from Imgenex for retroviral delivery of shRNA to target cells. Retroviral delivery has the advantage of stable, long-term production of shRNA and the capacity for antibiotic selection of infected cells. One should choose an shRNA delivery system that best meets the needs of the individual investigator (ease of use, hands-on time, product availability, cells to be infected, etc.).

Although some companies offer in-house target selection for RNAi, it is often desirable to choose one's own target sequence on the hTERT mRNA. Oligonucleotide design will depend largely on the particular system being used. The size of the homologous region being targeted, and the length of the hairpin loop will vary according to vector manufacturer instructions and with individual preferences. For the purposes of this chapter, designs reflect GeneSuppressor™ System guidelines.

For the most part, RNAi target selection is still trial and error with only a few general criteria to guide the process. Several targets should be chosen and tested to find the one that yields the most complete knockdown of the hTERT mRNA. Short-interfering RNAs (siRNAs) are 21-nucleotide to 23-nucleotide duplexes of double-stranded RNA that actually mediate the RNAi process (9). It is advisable to initially design siRNAs against the target sequences and test them to rapidly assess target choices before investing valuable time in vector construction and antibiotic selection. Regions of the hTERT mRNA possessing a strong secondary structure should be avoided as targets as they can be relatively inaccessible to components of the RNAi machinery. Elbashir et al. (10) have provided some general guidelines that are helpful in planning oligonucleotide design: 5'-untranslated and 3'-untranslated regions should be avoided as well as the region immediately downstream of the start codon. The ideal sequence for synthetic siRNAs is 5'-AA(N19)UU-3' where N is any nucleotide. Oligonucleotides should have roughly 50% GC content (10). (The AA and UU dimers in the sequence are not necessary when using the GeneSuppressor™ System's expression vector produced by Imgenex.) The motif corresponding to the coding region of hTERT should be 19-nucleotide to 21-nucleotide long, followed by a spacer of four to nine nucleotides that will form the hairpin loop. The size of the loop is important; it has been demonstrated that a loop of nine nucleotides incorporated into the shRNA design may be much more effective than a somewhat shorter (e.g., seven nucleotides) loop and therefore has a significant impact on the efficiency of the shRNA in knocking down the target mRNA (3). Following the hairpin loop is the inverted complement of the motif. Oligonucleotides will need to be ordered in both the sense and the antisense configurations and annealed in the laboratory. Finally, avoid incorporating continuous stretches of adenines or thymines in the target sequence, as these may act as internal terminators. See **Note 4** for an example of oligonucleotide design.

2. Materials

2.1. Retroviral Construct Preparation

1. Double-stranded oligonucleotides with homology to desired target region of hTERT mRNA.
2. Retroviral vector, pSuppressorRetro™, and packaging plasmid (Imgenex, San Diego, CA). Include retroviral LacZ plasmid if desired (see **Note 1**).
3. Primer annealing buffer (included in GeneSuppressor™ System, Imgenex).
4. T4 DNA ligase and 10× ligase buffer.
5. DH5α competent *Escherichia coli* (Invitrogen, Carlsbad, CA).
6. LB Levia-Bertani plates containing 100 µg/ml ampicillin.
7. LB broth containing 100 µg/ml ampicillin.
8. Miniprep and maxiprep DNA purification kits (Qiagen, Valencia, CA).

2.2. Cell Culture, Transfection, and Infection

1. HEK 293 cells (cat. no. CRL-1573, American Type Culture Collection, Manassas, VA).
2. Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT).
3. 0.25% trypsin and 2.21 mM ethylenediamine tetraacetic acid (EDTA) (Mediatech).
4. Target cells of interest, culture medium, and dissociation reagents.

5. FuGENE 6 transfection reagent (Roche, Indianapolis, IN) (*see Note 2*).
6. SteriFlip 50-ml filters and 0.45- μ m pore size (Millipore, Billerica, MA) (*see Note 3*).
7. Polybrene (hexadimethrine bromide; Aldrich, Milwaukee, WI), stock solution 8 mg/ml in sterile, molecular biology-grade water.
8. Geneticin (G418) (Invitrogen).
9. In Situ β -Galactosidase Staining Kit for monitoring transfection efficiency (Stratagene, La Jolla, CA).

3. Methods

3.1. Preparation of Oligonucleotides

1. Choose the target region of hTERT mRNA and design the DNA oligonucleotides, keeping in mind the aforementioned guidelines.
2. The pSuppressor vector from Imgenex is supplied linearized with *Sa*I and *Xba*I ends. Therefore, compatible restriction sites should be incorporated into the oligonucleotide design: *Xho*I at the 5' end and *Xba*I at the 3' end of the oligonucleotide sequences.
3. The 3' end of the sense oligo should end in a sequence of five thymidines that serve as a terminator.
4. *See Note 4* for an example of oligonucleotides for shRNA techniques.

3.2. Retroviral Vector Construction

3.2.1. Anneal Oligos

1. Anneal the oligonucleotides in a reaction containing 1 μ g each of the sense and antisense oligos, 2 μ l 10 $\times$ annealing buffer (*see Note 5*) and deionized water to a final volume of 20 μ l.
2. Heat the reactions to 95 $^{\circ}$ C for 10 min, then cool the reactions *gradually* to room temperature.

3.2.2. Ligate Oligos into Vector

1. Ligate annealed oligo into linearized Imgenex pSuppressor vector in the presence of 2 μ l 10 $\times$ ligase buffer, 1 μ l T4 DNA ligase, and deionized water to a final volume of 20 μ l (*see Note 6*).
2. Ligation conditions: 16 $^{\circ}$ C for 12–16 h.
3. Transform DH5 α competent *E. coli* using 3 μ l ligation reaction. Grow transformation reaction overnight on LB plates containing 100 μ g/ml ampicillin.
4. Pick several colonies to verify the presence of recombinants. Grow in LB broth in the presence of 100 μ g/ml ampicillin and purify using Qiagen's Spin Miniprep Kit.
5. Perform restriction analysis of minipreps to check for the presence of the desired insert (*see Note 7*).
6. Confirm construct integrity by DNA sequencing (*see Note 8*).
7. Scale up plasmids by performing DNA maxipreps of the desired retroviral construct, the packaging plasmid we wish to use, and the retroviral LacZ plasmid (if we wish to assess transfection and infection efficiencies).

3.3. Transfection of HEK 293 Cells

1. One day prior to transfection, plate approximately 1.5 million HEK 293 cells in a 10-cm dish for each target being tested. It is very important that the media used for the HEK 293 cells *not* contain any antibiotic/antimycotic agents. The cells should be 30–50% confluent on the day of transfection (*see Note 9*).
2. Transfect the cells using FuGENE following the manufacturer's guidelines. Optimization experiments should be done to determine the most effective amounts of DNA to be used as well as the ratio of FuGENE to DNA. We have found ratios of 3:2 and 5:1 to be effective in transfecting HEK 293 cells. 2 µg DNA per well of a 6-well plate works well. Keep in mind the total DNA amount includes both the retroviral vector and the packaging plasmid.
3. Incubate the FuGENE–DNA complex mixture for 45 min at room temperature before adding dropwise to culture dishes.
4. The morning after transfection, change medium in the culture dishes to the medium of our target cells. Apparently the transfected cells produce a type of cytostatic factor; changing the medium helps to dilute this factor (*see Note 10*).

3.4. Infection of Target Cells

1. Plate target cells 1 day prior to infection (may do 1 day after transfection) so that they will reach a density of no more than 30–50% on the day of infection (*see Note 11*).
2. On the day of infection, remove the virus-containing supernatant from the HEK 293 cells and filter sterilize the supernatant through a *surfactant-free* 0.45-µm filter.
3. Infect the target cells with the virus-containing supernatant using a 1:1 ratio of supernatant to target cell medium in a final concentration of 8 µg/ml polybrene (*see Note 12*).
4. Allow continued virus contact with the target cells for at least 48 h before beginning G418 selection. It may be advantageous to subject the target cells to repeated rounds of infection every 6–12 h or at least once per day (*see Note 13*).
5. Upon completion of the desired infection period, select the target cells in G418. Include a dish of untreated cells that will be used as a selection control. When all these cells are dead, selection is complete and the G418 can be removed from the medium. Optimum G418 concentration is cell type dependent and varies widely. A good rule is that there should be no noticeable cell death 2 days after adding the G418 and approximately 30–50% cell death 4 days after adding the G418. Selection should be complete in 7–10 days.
6. Maintaining the cultures will require concentration of cells into smaller dishes as they die off because of selection or addition of uninfected cells to keep cultures at a density that is conducive to maximal growth (*see Note 14*). Always allow cells to adhere overnight before reintroducing the G418 antibiotic.

4. Notes

1. It is strongly advised to use the LacZ plasmid to determine optimum transfection and infection conditions for our experiments. Cotransfection of the LacZ plasmid with the appropriate packaging plasmid will produce infectious viral particles that can infect the target cells and consequently be useful in assessing infection conditions. Maximizing efficiency will increase the number of infected target cells and reduce the time needed to culture sufficient cells to conduct experiments.

2. Any efficient transfection method should be adequate. The GeneSuppressor™ system from Imgenex includes its own transfection reagent and accompanying protocol. We have found transfections with FuGENE to be highly efficient (~70% transfection efficiency).
3. Do *not* use 0.2-μm filters as they may shear the viral envelop. Use only *surfactant-free* (for the same reason) 0.45-μm filters.
4. An example of the sense and antisense oligonucleotides against the target pursued by Masutomi et al. (11), including *Xho*I and *Xba*I overhang sites:

5. 10× annealing buffer: 100 mM Tris-HCl (pH 7.5), 1 M NaCl, and 10 mM EDTA (<http://www.protocol-online.org/prot/Detailed/3369.html>).
6. The manufacturer recommends 1 μl each of the vector and annealed oligo; we have obtained better results using a 3:1 ratio of annealed oligo to pSuppressor vector.
7. As the *Sal*I site is destroyed upon successful cloning of the insert, digestion with this enzyme should have no effect. It is helpful to digest with another enzyme in a double digest (such as *Eco*RI). Double digestion with *Sal*I and *Eco*RI will cut out an approximately 1.5 kb fragment from negative clones while linearizing positive clones at the *Eco*RI site.

8. The sequencing of some clones may not be possible because of the strong secondary structure formed by the short hairpin (1). The facility rendering sequencing support should be notified of the nature of the construct so that conditions might be optimized for sequencing through secondary structure.
9. HEK 293 cells may be maintained in culture with antibiotic/antimycotic selection but the media used during the transfection should not contain any antibiotic/antimycotic agents, as its presence can drastically reduce transfection efficiency.
10. This step is advised by Imgenex in its product manual, but the effect has not been empirically tested by our laboratory.
11. Retroviral infection is dependent upon cell division. Cells must be growing well and actively dividing to be infected.
12. It is important for the virus to be as concentrated as possible without adversely affecting the growth of the target cells. Diluting the viral supernatant 1:1 is the minimum dilution recommended by Imgenex. If, in fact, the transfected cells are producing some cytostatic factor, the dilution of the supernatant helps to reduce growth inhibition of the target cells.
13. Virus contact with target cells should be for a minimum of 12–24 h. As the half-life of the virus is only 6–8 h at 37 °C, it is advantageous to reinfect every 12 h with fresh virus. Infection for 48 h before selection is desirable.
14. Any uninfected cells that are added must also be eliminated by G418 selection. The most effective method would be to concentrate the cells into smaller dishes. However, using this technique on some cell lines may not be feasible (such as those with low reattachment efficiency).

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