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hTERT Knockdown in Human Embryonic Kidney Cells Using Double-Stranded RNA

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Summary

The method of RNA interference (RNAi) is an easy means of knocking down a gene without having to generate knockout mutants, which may prove to be difficult and time consuming. RNAi is a naturally occurring process that involves targeting the mRNA of a gene by introducing RNAs that are complementary to the target mRNA. The foreign RNAs activate an endogenous enzyme, DICER, which degrades the target mRNA. There are many ways of eliciting the RNAi response in a cell. In this chapter, we describe the use of double-stranded RNA (dsRNA) to knockdown human telomerase reverse transcriptase (*hTERT*), the gene that codes for the catalytic subunit of the human telomerase enzyme. dsRNA can be used to generate the RNAi response in cells of embryonic origin, such as human embryonic kidney (HEK) cells. The RNAi effect is transient because the dsRNA eventually gets degraded in the cells, and it is useful to study the short-term effects of a gene knockdown.

Keywords

dsRNA; RNAi; HEK cells; hTERT

1. Introduction

In most mammalian cells, the introduction of a long double-stranded RNA (dsRNA) of more than 30 bp induces an interferon response that activates RNase L, which causes a non-specific systemic degradation of mRNA in the cell. This results in the shutdown of protein translation and further leads to apoptosis (1). It has been suggested that this interferon response evolved to inhibit viral gene expression in infected cells (2). However, this interferon response appears to be absent in mammalian cells of embryonic origin (3).

After long dsRNA has been introduced into mammalian embryonic cells, DICER, an RNase, cleaves the dsRNA into 21–23 nucleotide-long fragments of short-interfering RNAs (siRNAs) (4). These siRNAs are further incorporated into an RNA-induced silencing complex (RISC) where the siRNAs are unwound into single-stranded RNA (ssRNA). The RISC is involved in recruiting the ssRNA to the mRNA of complementary sequence and cleaving the mRNA, thus preventing translation (5,6). This allows for target-specific knockdown of a gene product. The advantage of using dsRNA for knocking down genes is that multiple siRNA targets are generated to the specific mRNA, making this a more efficient method of RNA interference (RNAi) as compared with using a single siRNA target. However, this may also result in an increase in non-specific knockdown of other genes because of sequence homology (7,8).

It has been well-documented that the efficacy of gene target knockdown is variable depending on the length of dsRNA and selection of target sequence. Preferably a length of more than 200 bp is used for dsRNAi for an optimal knockdown effect. Also, the target sequence should

²Generation of dsRNA longer than 800 bp may require different conditions than stated here. Refer to the manufacturer's instructions in the Ambion manual.

contain only 30–50% G-C content (9). This chapter describes the methods for human telomerase reverse transcriptase (*hTERT*) gene knockdown using dsRNA in human embryonic kidney (HEK) cells. The use of dsRNA allows for a transient knockdown of *hTERT* to determine its immediate effects, because the DICER-generated siRNAs will eventually degrade in the cell. Cellular levels of *hTERT* mRNA have been shown to be maximally depleted in the cell from 24 to 72 h, and its levels were almost recovered by day 6 of transfection (*see* Fig. 1).

hTERT is the catalytic subunit of human telomerase, the enzyme responsible for maintaining telomere length, contributing to chromosomal stability. *hTERT* knockdown potentially has many applications in cancer therapy, because telomerase is necessary for the unlimited division of 90–95% of human cancer cells (10). Although telomerase is up-regulated in these cancer cells, most cancer cells have shorter telomeres than normal cells (11). The use of a transient and reversible method of knocking down *hTERT*, such as the use of dsRNAi, is thus highly beneficial because the regulation of telomerase activity can be performed and discontinued before telomeres of normal cells reach critical length. The recovery from dsRNA treatment is expedient, because the recovery of telomerase levels is almost complete by day 6 of treatment with dsRNA (*see* Fig. 1), thus it is easy to initiate and terminate the knockdown of *hTERT*. Telomeres of germ and stem cells are also more likely to regain their telomere lengths after therapy with dsRNA (12).

2. Materials

2.1. Cell Culture

1. HEK 293 cells (cat. no. CRL-1573, ATCC, Manassas, VA).
2. Dulbecco's modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (10% FBS/DMEM) (HyClone, Ogden, UT).
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 mM) (Invitrogen Corporation).

2.2. In Vitro Transcription of dsRNA

1. MEGAscript RNAi Kit (Ambion, Austin, TX) containing ribonucleotides (ATP, CTP, GTP, and UTP), nuclease-free water, T7 enzyme mix, and 10× T7 reaction buffer.
2. Oligonucleotide primers flanking the 200–400 bp target region to be amplified. Forward and reverse primer sequences should be designed with a T7 promoter sequence (5'-TAATACGACTCACTATAGGAGA-3') at each 5' end.
3. Forward and reverse primers without T7 promoter sequences.
4. cDNA from HEK or an appropriate cell line that is *hTERT*-positive.

2.3. Transfection

1. Fugene 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN). Store at –4 °C.
2. Fully transcribed and purified dsRNA.
3. DMEM. Pre-warm at 37 °C before transfection.
4. 6-Well tissue culture plates.
5. Hemacytometer.

6. Trypan blue.

3. Methods

The amount of dsRNA required for an efficient knockdown of the target gene depends on the length of the transfected transcript. The longer the transcript, the more is required to achieve adequate copies for good transfection efficiency. Therefore, it is advisable to optimize conditions such as the amount of transcript and the ratio of Fugene transfection, because the latter will differ between cell lines. It is not as necessary to test for knockdown efficacy using multiple targets because multiple siRNAs will be generated from a single dsRNA in the cell because of the effects of DICER. However, because of potential non-specific effects due to RNAi of other gene targets, the selected target sequence should be checked for sequence homology to other gene transcripts. Sequence comparisons and searches can be run on the Blast search program, which can be found at <http://www.ncbi.nlm.nih.gov>.

3.1. In Vitro Transcription of dsRNA

Follow according to manufacturer's instructions using the MEGAscript RNAi Kit (*see Note 1*).

1. Prepare the DNA template for long dsRNA synthesis by PCR amplifying the target region using appropriate cDNA. Two PCRs are to be performed. One to generate the sense-strand DNA template, and the other to generate the anti-sense-strand DNA template. To generate a reaction for the transcription of the sense strand, use the forward T7 promoter primer and reverse primer. To generate a reaction for the transcription of the anti-sense strand, use the reverse T7 promoter primer and forward primer (*see Fig. 2*, steps 1 and 2). The PCR conditions are based on designed primer sequences.
2. For each separate PCR, perform in vitro synthesis of ssRNA by adding 4µl PCR DNA template to 2µl each of ribonucleotides (ATP, CTP, GTP, and UTP), 4µl nuclease-free water, 2µl T7 enzyme mix, and 2µl 10×T7 reaction buffer. Add the reaction buffer last to prevent precipitation.
3. Incubate both reactions at 37 °C for 16 h (*see Fig. 2*, step 3).
4. To generate dsRNA, mix the two reactions together and incubate at 75 °C for 5 min. Let cool to room temperature to allow the ssRNA to slowly anneal (*see Fig. 2*, step 4).

3.2. HEK Transfection

1. HEK 293 cells from ATCC are cultured with 10% FBS/DMEM at 37 °C and 5% CO₂. They are maintained in 10-cm tissue culture dishes and passaged with trypsin/EDTA at 80% confluency to maintain log phase of cell growth (especially for contact-inhibited cell lines), because good transfection efficacy depends to a large degree on the cells being in log phase. Antibiotics should not be added to the culture medium during transfection with Fugene 6 transfection reagent because the influx of antibiotics with the transfection reagent is highly toxic to the cells. All cell culture and transfection procedures are performed in a sterile hood.
2. 24 hours before transfection, the cells are trypsinized and viable cells counted with a hemacytometer using the trypan blue dye exclusion method. 4.0×10^5 cells are plated

¹Commercial kits for in vitro synthesis of dsRNA are also available from other vendors. A review of various methods to synthesize dsRNA can be found in **ref. 13**.

per well of a 6-well plate and incubated for 24 h to provide experimental cultures of approximately 60% confluency for transfection the next day (*see* Note 3).

3. 2.5–3 µg of dsRNA is required per well. Fugene 6 transfection reagent is added to pre-warmed DMEM (37 °C, without serum) at a ratio of 1:5 (microgram dsRNA : microliter Fugene) carefully into the middle of the microfuge tube to prevent Fugene 6 transfection reagent from contacting and sticking to the walls of the tube. Carefully pipette the dsRNA into the middle of the tube. Incubate the mixture unshaken for at least 15 min at room temperature (*see* Note 4).
4. Vortex the transfection mixture briefly to obtain an even suspension and add the appropriate amount around the well in a drop-wise manner. Incubate and harvest as needed for analysis of knockdown effects.

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³Media does not need to be replaced after transfection. However, it is advisable to provide the cells with fresh media 24 h before transfecting to ensure that cells have adequate nutrients for the incubation period following transfection. Replacing media after transfection may cause a quicker recovery from RNAi.

⁴Transfection efficiency may also be compromised if RNA is not pure. To check for purity, the ratio of the optical density at wavelength 260 and 280 nm in a UV spectrophotometer should be determined. A pure sample of RNA would yield a A260/A280 ratio of 1.9–2.1.

Fig. 1.

Agarose gel electrophoresis from reverse-transcriptase PCRs using human telomerase reverse transcriptase (hTERT) and GAPDH (glyceraldehyde phosphate dehydrogenase) primers. hTERT knockdown in human embryonic kidney (HEK) cells was performed by transfecting with 2.5 μ g 219 bp double-stranded RNA (dsRNA) complementary to *hTERT* mRNA sequence. GAPDH is used as a loading control. Lanes 1, 3, and 5 are non-treated HEK controls at days 1, 3, and 6 after transfection, respectively. Lanes 2, 4, and 6 are treated HEK samples at days 1, 3, and 6 after transfection, respectively. hTERT knockdown can be seen after 1 day of dsRNA treatment. The level of hTERT mRNA is depleted in the cells because of RNA interference (RNAi) and is almost recovered 6 days after treatment.

Fig. 2.

Flow chart of *in vitro* synthesis of double-stranded RNA (dsRNA). Primers with T7 promoters are used in separate PCRs to obtain amplified products of 200–800 bp target DNA fragments flanked with T7 promoters. Sense and anti-sense single-stranded RNAs (ssRNAs) are synthesized by T7 RNA polymerase transcription, and the ssRNAs are allowed to anneal to form dsRNA.