KNOCKING DOWN P53 EXPRESSION USING RNA INTERFERENCE

Here we describe two systems used to suppress endogenous p53 expression in mammalian cells, the siLentGene™ U6 Cassette RNA Interference System and the T7 RiboMAX™ Express RNAi System.

Introduction

Silencing gene expression using double-stranded RNA (dsRNA), known as RNA interference or RNAi, provides a powerful tool for analyzing gene function. Selectively down regulating (or "knocking down") the expression of a particular gene allows researchers to determine its function in many cellular processes.

One of the first genes to be subjected to RNAi was the gene encoding transcription factor p53 (1–3). This protein is the most commonly mutated tumor suppressor gene in human cancers. In normal cells, p53 expression is induced by agents that cause DNA damage, and its over expression results in growth arrest.

The p53 protein has a short half-life, but it can be stabilized by either a point mutation of the gene or interaction with specific DNA tumor virus factors, such as SV40 Large T antigen. For a general review, refer to references (4–6). We chose p53 as a model system to evaluate two methods of RNA interference, the siLentGene™ U6 Cassette RNA Interference System[a,b,c,d] and the T7 RiboMAX™ Express RNAi System[d,e,f] for the synthesis of short interfering RNA (siRNA) target sequences.

siRNAs Without Cloning

The siLentGene™ U6 Cassette RNA Interference System is a DNA cassette-based approach for creating siRNA expression constructs for direct delivery into cells in a rapid and cost-effective manner. The primer-dependent, PCR-based system places selected siRNA sequences under the control of an engineered U6 promoter and terminator. The PCR products are directly transfected into cells, eliminating the need to laboriously clone each one.

Relatively simple promoter and terminator sequences direct the production of large amounts of siRNA in mammalian cells by the endogenous RNA polymerase III of the transfected cells. The terminator consists of a short stretch of uridines; this is compatible with the recommended siRNA design to terminate with a two-uridine 3´ overhang. U6 expression cassettes include the U6 promoter, the sense or antisense mRNA target sequence, and a terminator sequence. Two expression cassettes, one expressing the antisense RNA and one expressing the sense RNA, are made. After column purification, these cassettes are combined and transfected into the appropriate cell line using the siLentGene™ Transfection Reagent. After transfection, RNA polymerase III transcribes a short RNA from each of the expression cassettes. The resulting complementary siRNAs anneal in vivo, creating the siRNA duplexes. These duplexes trigger the specfic degradation of the target mRNA inside the transfected cells (see Technical Manual #TM061).

p53 Expression Knockdown Using the siLentGene™ System

To test for inhibition of the p53 protein, we used the 293T cell line, because it contains the SV40 Large T antigen that stabilizes the p53 protein and allows a high level of p53 accumulation. Twenty-four hours after plating, cells were transfected with U6 cassettes expressing p53 or a nonspecific control. Cells were also transfected with a GFP vector. Forty-eight hours after transfection the percentage of GFP-positive cells was determined to calculate transfection efficiency. The cells not used to determine transfectioin efficiency were grown for 72 hours, lysed and the protein quantitated. An equal amount of protein per sample was run on an acrylamide gel and transferred to a nitrocellulose membrane. We used a p53 monoclonal antibody to detect p53 and an actin antibody to determine equal loading between lanes (Figure 1). The bands were quantitated by densitometry, and the reduction in the p53 was determined and normalized using the β-actin control. The p53 experiment showed an average inhibition of 53%.

T7-Directed in vitro Synthesis of siRNA

The T7 RiboMAX™ Express RNAi System is an in vitro transcription system designed for producing milligram amounts of double-stranded RNA (dsRNA) in a short amount of time. The T7 RiboMAX™ Express RNAi System can be used to synthesize siRNAs of 21bp for use in mammalian systems or longer interfering RNAs for nonmammalian systems. The DNA template for the in vitro transcription of siRNAs is a short duplex oligonucleotide that contains a T7 RNA polymerase promoter upstream of either the sense or antisense mRNA sequence. Two oligonucleotides are annealed to generate separate templates for synthesis of each strand of the siRNA. The separate short RNA strands that are synthesized are then annealed to form siRNA (see Technical Bulletin #TB316).

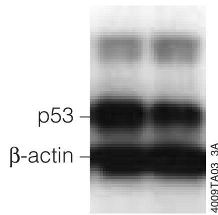

Figure 1. Suppression of endogenous p53 protein. 293T cells were transfected in a 12-well plate with 1μg/well of U6 cassette (0.5μg of each PCR product) and 6μl/well of siLentGene™ Transfection Reagent. The cells were then incubated for 72 hours, lysed and the protein quantitated. Five micrograms of protein/lane was run on a 10% tris-glycine gel and transferred to a nitrocellulose membrane. The membrane was probed with both a p53 (1:1,000 Calibiochem Ab-2) antibody and a β-actin (1:5,000 Abcam, Ab 6276) antibody to serve as a loading control. A goat anti-mouse, horseradish peroxidase-conjugated secondary antibody was used followed by chemiluminescent detection. In the first lane, a nonspecific U6 cassette was tested as a negative control. The second lane was transfected with a p53-specific U6 cassette. Experiments were performed in duplicate.

We compared short RNA duplexes generated using the T7 RiboMAX™ System to chemically synthesized duplexes using the p53 model system. The results shown in Figure 2 demonstrate that p53 siRNAs generated using the T7 RiboMAX™ Express RNAi System were functionally comparable to the chemically synthesized p53 siRNA of the same sequence. Both induced a significant decrease in the level of p53 protein following transfection.

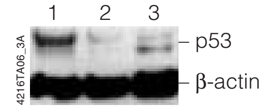

Figure 2. Suppression of endogeneous p53 protein. 293T cells were transfected with 200ng scrambled siRNA (lane 1), 200ng in vitro synthesized p53 siRNA (lane 2), or 200ng chemically synthesized p53 siRNAs (lane 3). Equivalent amounts of each lysate (10μg) were separated on a polyacrylamide gel, transferred to Hybond®-C membrane and probed with both a p53-specific antibody and a β-actin antibody. Detection was performed using a HRP-conjugated secondary antibody and chemiluminescent detection reagents. Each lane represents the mean and average of duplicate wells per transfection. The p53 and β-actin bands are indicated and are of the expected sizes.

Summary

We have used T7 RiboMAX™ Express RNAi System and the siLentGene™ U6 Cassette RNA Interference Systems to successfully knock down p53 expression in mammalian cells. These methods allow researchers to perform RNA interference studies without laborious cloning or expensive RNA oligonucleotide synthesis. ■

References

1. Hemann, T.A. *et al.* (2003) *Nat. Genet.* **33**, 396–400.
2. Brummelkamp, T.R. *et al.* (2002) *Science* **296**, 550–3.
3. Brummelkamp, T.R. *et al.* (2002) *Cancer Cell* **2**, 243–7.
4. Martin, D.W. *et al.* (1993) *J. Biol. Chem.* **268**, 13062–7.
5. Liu, X. *et al.* (1993) *Mol. Cell. Biol.* **13**, 3291–300.
6. Truant, R. *et al.* (1993) *J. Biol. Chem.* **268**, 2284–7.

Protocols

siLentGene™ U6 Cassette RNA Interference System Technical Manual, #TM061 (www.promega.com/tbs/tm061/tm061.html)

T7 RiboMAX™ Express RNAi System Technical Bulletin, #TB316 (www.promega.com/tbs/tb316/tb316.html)

Web site

www.promega.com/techserv/apps/rna_interfer/

Ordering Information

Product	Size	Cat.#
siLentGene™ U6 Cassette RNA Interference System[a-d]	1 system	C7800
T7 RiboMAX™ Express RNAi System[d–f]	50 × 20μl reactions	P1700
siFECT™ siRNA Transfection Reagent	0.4ml	E5052
	1.0ml	E5053

[a] The PCR process is covered by patents issued and applicable in certain countries. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process. Use of this product is recommended for persons that either have a license to perform PCR or are not required to obtain a license.

[b] U.S. Pat. No. 6,242,235 and other patents pending.

[c] For research use only. Not for human diagnostics or therapeutics or the commercial sale of transgenic animals. Use for any purpose other than research requires permission from Allele Biotechnology, Inc.

[d] This product is covered under license from Carnegie Institution of Washington. Commercial use of this product may require a separate license from Carnegie.

[e] U.S. Pat. No. 5,552,302, Australian Pat. No. 646803 and other patents.

[f] U.S. Pat. Nos. 4,966,964, 5,019,556 and 5,266,687, Australian Pat. Nos. 616881 and 641261 and other pending and issued patents, which claim vectors encoding a portion of human placental ribonuclease inhibitor, are exclusively licensed to Promega Corporation.

RiboMAX and siLentGene are trademarks of Promega Corporation.

HyBond is a registered trademark of Amersham Biosciences, Ltd.