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PRODUCTION OF EFFECTIVE RECOMBINANT PLASMIDS EXPRESSING SHORT-HAIRPIN RNA (shRNA) APROPRIATE FOR hTERT GENE SILENCING RNA INTERFERENCE MEDIATED

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Abstract

RNA interference (RNAi) is an evolutionally conserved gene silencing mechanism present in a variety of eukaryotic species. RNAi uses short double-stranded RNA (dsRNA) to trigger degradation or translation repression of homologous RNA targets in a sequence-specific manner. RNAi is a very conservative mechanism that is likely to be active in almost all eukaryotic taxa. This phenomenon attracts growing attention and studying RNA interference is probably one of the most rapidly developing fields of modern science. The aim of this study was to design, construct, and production of the recombinant plasmids, targeting hTERT gene.
Keywords: Plasmid, RNAi, shRNA, hTERT.

Introduction

RNA interference (RNAi) has become a powerful and widely used tool for the analysis of gene function in invertebrates and plants. Introduction of double-stranded RNA (dsRNA) into the cells of these organisms leads to the sequence-specific destruction of endogenous RNAs that match the dsRNA (1). The RNAi response in mammalian cells mediated by dsRNA is a well-known two-step process. Initially, the dsRNA is cleaved by an RNase III-like enzyme known as Dicer, which processes dsRNA into ~22-nt small interfering RNAs (siRNAs). Then the duplex siRNAs are passed to the RNA-induced silencing complex (RISC), which is activated by unwinding of the duplex. Activated RISC complexes can regulate gene expression at many levels (2).

Several approaches can be used to induce gene silencing. The chemical synthesis method is commonly used, but is very expensive (3). The limited capacity of *in vitro* transcribed siRNA and unknown available siRNA digested by long dsRNA (200–1000 bp with the T7 promoter) with RNase III/Dicer limit the application of these methods (4-7).

Alternatively, the short hairpin RNAs (shRNAs) are expressed endogenously from plasmids and viral vectors. The shRNA expression cassettes can be stably integrated into the genome of target cells, transcribed intranuclearly and processed into siRNAs by Dicer in the cytosol. In general, RNA Pol III promoters (i.e. U6, H1 and tRNA promoters) are commonly used to drive shRNA expression in the RNAi studies (8).

There are some general and some specific rules to design an efficient siRNA (9). The overall efficiency of the artificial siRNA or shRNA will depend upon additional characteristics: low G/C content (30–60%); beginning with a G or C residue after an AA dimer in the 50-flanking sequence; sense strand base preferences at positions 3 (A), 10 (U), 13 (A) and 19 (A); lack of four or more consecutive T or A to avoid premature pol III transcription termination signals (figure 1) (10).

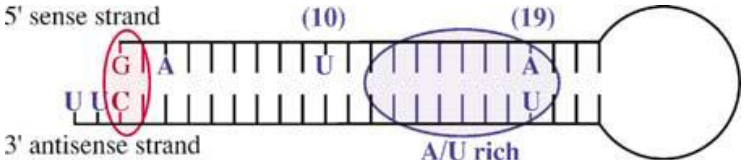

Figure 1. Schematic view of a shRNA transcript, showing stability rules for RISC preferences.

Although these vectors provide certain advantages over chemically synthesized siRNAs, numerous disadvantages remain, including low and variable transfection efficiency and difficulties of integrating into the host DNA to become stable (11).

The aim of this study was to design, construct and product appropriate recombinant plasmids coding for small hairpin RNA (shRNA) molecules targeting hTERT gene.

MATERIAL AND METHODS

shRNA Design and Plasmid Construction

The techniques of the genetic engineering and general molecular biology procedures were performed the methods that are already well-established in the Laboratory of Molecular and Cell Biology, Skopje . The manufacturer's protocols and additional literature available on internet were used for the commercial kits and products. The psiRNA-h7SKBlasti G1 commercial kit for shRNA-coding plasmid was chosen (InVivoGen).

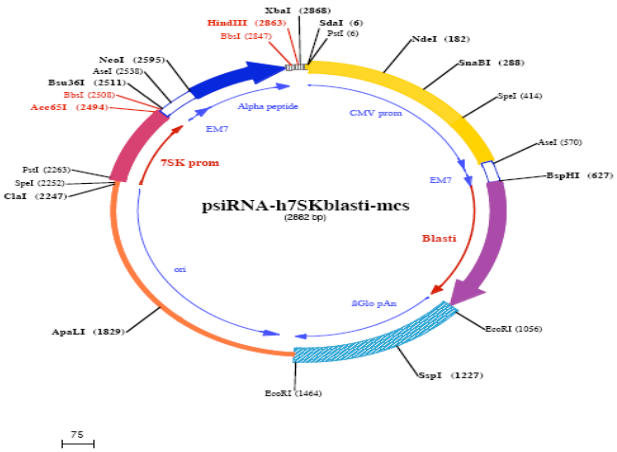

Figure 2. Structure of the psiRNA-h7SKblasti plasmid that can express short hairpin RNA (shRNA)

Two shRNA-coding plasmid constructs targeting the hTERT mRNAs were constructed. For the target gene hTERT one construct was created using sequence coding for interfering RNA according to published reference, and one was designed using appropriate software siRNA

Wizard, (Invivo Gen). The siRNA Wizard v.2.4 was used to search and *de novo* design of the possible hairpin sequences, but also to modify the published sequences in terms to have compatible "sticky" ands for insertion into the *Bbs*I cloning site of the psiRNA-h7SKBlasti plasmid and for addition of appropriate hairpin-loop space sequence between the sense and antisense regions of the each ologonucleotide. The following advanced options of this tool were selected: GC% between 30 and 55; first nucleotide R (G or A); enabled C triplet; *Bbs*I/ *Bbs*I cloning sites; loop sequence 5'-TCAAGAG-3'; and 21nt motif site. The transcript sequences that were submitted to siRNA Wizard were previously selected from the Web-based Entrez database (at htt:/www.ncbi.nlm.nih.gov/entrez/).

For targeting *hTERT* gene expression, the published DNA sequence was processed with siRNA Wizard and the following two complementary oligonucleotides were ordered:

Oligo1: 5'-ACCTCTTTCATCAGCAAGTTTGGATCAAGAGTCCAAACTTGCTGATGAAATT-3'

Oligo2: 5'-CAAAAATTTCATCAGCAAGTTTGGACTCTTGATCCAAACTTGCTGATGAAAG-3'

For designing of the hairpin-coding sequences targeting the hTERT mRNA using siRNA Wizard, the Entrez database was searched for the hTERT transcript and the first 1800 nucleotides of the transcript were selected and submitted on-line as input sequence into the siRNA Wizard. The following two complementary ologonucleotides conataing the hairpin-loop and *Bbs*I-compatible ends were designed.

Oligo 1 : 5'-ACCTCGCTCGTGGAGACCATCTTTCTTCAAGAGAGAAAGATGGTCTCCACGAGCTT-3'

Oligo 2 : 5'-CAAAAAGCTCGTGGAGACCATCTTTCTCTCTTGAAGAAAGATGGTCTCCACGAGCG-3'

The designed single-stranded DNA oligonucleotides were ordered from nucleic acid-synthesizing commercial facility (Sigma-Genosys) in 0,05 μmol scale with the highest purity level (PAGE purification) Double stranded inserts were created by annealing of the complementary oligonucleotide pairs, as depicted below (RNA interference-inducing oligonucleotides regions are in red, hairpin-loop in blue and the sticky ends for plasmid cloning are in black;

hTERT (exon 11) Masutomi et al.; Oligos 1 and 2

5'-**ACCTC**TTTCATCAGCAAGTTTGGATCAAGAGTCCAAACTTGCTGATGAAA**TT**-*3'*

3'**G**AAAGTAGTCGTTCAAACCTAGTTCTCAGGTTTGAACGACTACTTT**AAAAAC**-*5'*

hTERT Transcript (pos. 1 to 1800); oligos 1 and 2

5'-**ACCTC**GCTCGTGGAGACCATCTTTCTTCAAGAGAGAAAGATGGTCTCCACGAGC**TT**-*3'*

3'-**G**CGAGCACCTCTGGTAGAAAGAAGTTCTCTCTTTCTACCAGAGGTGCTCG**AAAAAC**-*5'*

The formation of double-stranded inserts was performed by annealing of the complementary ologonucleotides at final concentration of 45mM each in the modified annealing buffer (100mM sodium chloride; 100mM Tris-HCl, pH 7,5) heating in a water bath at 95 ℃ for 4 minutes, and

slowly cooling to room temperature during an approximately 1 hour. The efficiency of the annealing was estimated by electrophoresis in 4% ultra-pure agarose (Sigma-Aldrich).

Recombinant shRNA-coding plasmids were constructed by double cutting of the psiRNA-h7SKBlasti plasmid with the restriction endonuclease *Bbs*I (Fermentas International) at two sites (positions 2508 and 2847) that are different, yet recognized by the same enzyme, thus avoiding self-ligation of the plasmid. Two restriction fragments were produced with digestion: the large backbone plasmid fragment (2543 bp) that was for cloning, and the smaller (339bp) fragment containing the LacZ α –peptide cassette, that was discarded. After excision of the larger band (2543 bp) under long-wavelength UV-light source, the linear plasmid DNA was determinate with ethidium bromide-plate fluorescent method (Sambrock et al., 1989) using a series of DNA standards.

For each of the two shRNA-coding constructs, appropriate double-stranded insert was cloned downstream of h7SK and ligated using polyethylene glycol-containing Rapid Ligation Kit with T4 DNA ligase (Fermentas International).

Bacterial transformation using calcium chloride/heat-chock was performed with *E. coli* GT116 strain. Since the cloning site for shRNA inserts flanks the *Lac-Z* alpha peptide cassette, the discrimination of the transformed bacteria with successfully recombined psiRNA plasmids were selected by white-blue screening on a solid agar LB-based plates supplemented with β galactosidase inductor IPTG and X-Gal substrate. Some colonies of each plasmid constructs were tested by PCR using plasmid backbone-specific primers OL559 and OL408. The sequence of the primers are:

OL559: 5'-CGA TAA GTA ACT TGA CCT AAG TG-3'

OL408: 5'-GCG TTA CTA TGG GAA CAT AC-3'

The so called "direct" PCR amplification was performed using the following program: initial denaturation at 94 ℃ for 5 min, following by 30 cycles of denaturation at 94 ℃ for 30 sec, annealing at 55 ℃ for 15 sec and extension at 72 ℃ for 3 min was applied and the samples were kept at 4 ℃.

The PCR products were resolved by horizontal electrophoresis in 1.5% agarose in 0,5X TBE with included ethidium bromide visualized on UV-transilumintaor at 312 nm. Plasmid DNA was isolated from colonies with the GeneElute Plasmid MiniPrep kit and 4 μg was used for assembling of dideoxi-terminating Sanger-based thermal cycling reaction with Silver sequence kit (Promega). The RNA was lysed with RNase A and the endotoxins were removed by Triton X-114 phase-separation followed by adsorption of the plasmid DNA onto silica in the presence of chaotropic salts in the column format. Transfetion-ready plasmid DNA was eluted with ultrapure water.

RESULTS

The psiRNA-h7SK-Blasti-G1 plasmid was digested with BbsI endonuclease and the larger fragment (2543bp) was purified by preparative electrophoresis. By annealing of the ordered complementary oligonucleotides, (figure 2), a double-stranded shRNA-coding inserts were constructed that were legated into the plasmid backbone.

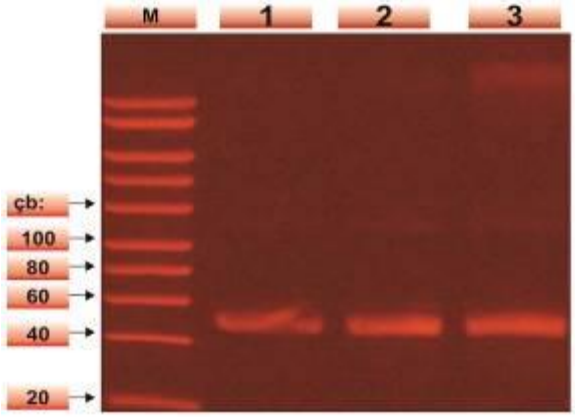

Figure 2. Electrophoretic analysis for estimation of the efficiency of complementary oligonucleotide annealing. Line M; 20 bp molecular weight DNA marker (Bio-Rad). Lines 1-3: The double-stranded inserts formed by annealing of the complementary oligonucleotide pairs.

The appropriate *E.coli* bacterial strains were transformed by the constructed plasmids and the colonies containing successfully recombinant plasmids were identified by the blue/white screening. As a rapid screening test, a direct PCR amplification was performed before sequencing using sterile tips by which a small amount of each white bacterial colony from the plates, were tested. The results of the amplification that was performed using plasmid backbone-specific primers OL559 and OL408 are presented on the figure 3.

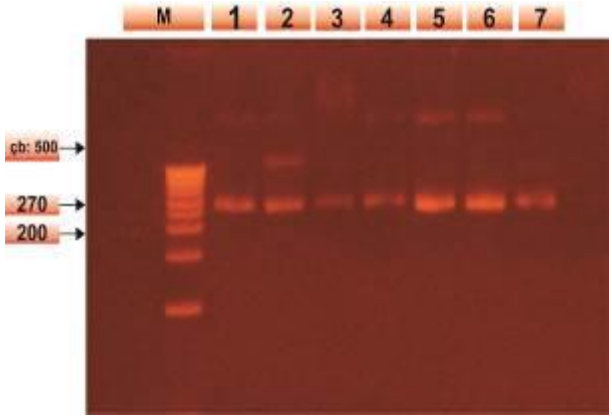

Figure 3. Direct PCR amplification of the samples from bacterial colonies using plasmid backbones-specific primers. Line M 20 bp molecular weight DNA marker (Bio-Rad). Lines 1 to

7: different bacterial from the white colonies that grow on the agar plates. The PCR products were resolved by electrophoresis on 1.5% agarose.

The integrity of the native plasmids was estimated by agarose gel electrophoresis (figure 4):

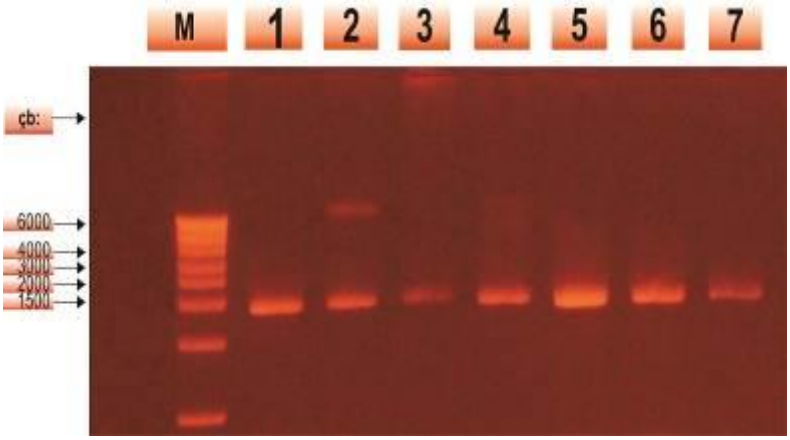

Figure 4: Controll electrophoresis for verification of the integrity of the purified plasmids. Aliquots of the untreated plasmids were resolved by 1% horizontal electrophoresis. Lines M: 1kb molecular weight DNA marker (Novagen); Lines 1-7 psiRNA-TERT/M and psiRNA-TERT/S1.

To confirm the integrity of the purified plasmids, restriction digestion with two different endonuclease and subsequent agarose electrophoresis was performed (figure 5).

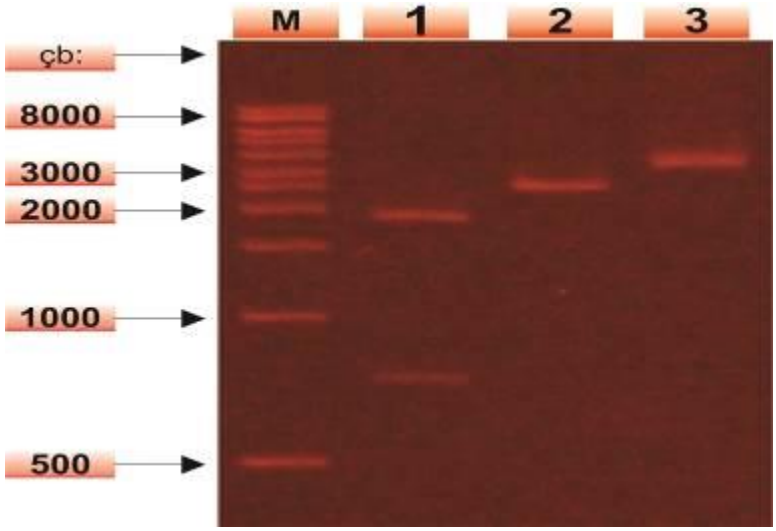

Figure 5. Restriction digestion analysis of plasmid constructs. The undigested and digested fractions were resolved by horizontal electrophoresis in 3% agarose. Plasmids psiRNA-TERT/M and psiRNA-TERT/S1. Line M: Marker 1kb molecular weight DNA marker (Novagen). Line 1 plasmid digested with SpeI endonuclease; Line 2: plasmid digested with *HindIII* endonuclease: Line 3 undigested plasmid.

Conclusion

At the end of this experimental stage we obtained two plasmids with a total amount of 500 to 1500 µg, for each. With double strand oligonucleotide insertions in plasmid psiRNA-h7SKBlasti G1, we built two effective shRNA plasmids as follows:

- psiRNA-TERT/M, which has as its sign the antigenic transcript hTERT following the publication of the sequence shRNA (Masutomi et al., 2003) and
- psiRNA-TERT/S1 (egzoni 2) which has as its sign the antigenic transcript hTERT (designed using the software Wizard siRNA).

Finally the recombinant production of plasmids which produce small molecules shRNA is a necessary tool, reasonable and useful for the study of gene silencing hTERT with RNA interference-based. Also, using a plasmid vector to knockdown a gene allows for long-term and permanent gene knockdown, without the need to generate knockout genotypes.

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