



shRNA inhibits the expression of chicken telomerase reverse transcriptase in MDCC-MSB1 cells

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ABSTRACT. Here, we investigated the effects of blocking chicken telomerase reverse transcriptase (chTERT) in MDCC-MSB1 cells, using small-hairpin RNAs (shRNAs) to interfere with gene expression. shRNAs specific to *chTERT* mRNA were designed, cloned into DNA plasmid vectors, and transfected into MDCC-MSB1 cells. The transfected *chTERT* RNAs were expressed by the RNA polymerase machinery of the MDCC-MSB1 cells. mRNA expression in transfected MDCC-MSB1 cells was detected using real-time PCR. After transfection, telomerase activity was monitored via a modified telomeric repeat amplification protocol assay, and cell cycle analysis was performed using a flow cytometer. At 72 h after transfection, chTERT expression was considerably reduced in cells transfected with shRNA; the highest inhibition rate was 89%. Compared with the control group, telomerase activity was significantly reduced and the cells failed to progress to S phase. shRNA effectively reduced telomerase activity and prohibited the transition of MDCC-MSB1 cells from G2/M to S phase.

Key words: Chicken telomerase reverse transcriptase; Real-time PCR; Small-hairpin RNA

INTRODUCTION

High telomerase activity is observed in most tumors and immortalized cells, suggesting that telomerase activation plays an important role in tumorigenesis (Venkatesan and Price, 1998). Telomerase reverse transcriptase (TERT) is a critical factor in telomerase activation and represents a promising molecular target for tumor therapy (Delany and Daniels, 2004). Therefore, inhibition of tumor cell proliferation by the suppression of TERT mRNA expression is currently a hot topic in cancer research (Delecluse and Hammerschmidt, 1993; Swanberg et al., 2004).

Marek's disease (MD) is characterized by a malignant T-lymphoid tumor and is of particular interest to the poultry industry. MD in chickens can be controlled by vaccines based on non-pathogenic strains of the turkey herpes virus. The agent causing MD is an extremely contagious oncogenic herpes virus, the first one identified experimentally; however, its spread can be prevented by vaccination. The MD virus is now regarded as an ideal model for studying tumor initiation and progression (Woźniakowski and Niczyporuk, 2015).

RNA interference (RNAi) is an evolutionarily conserved mechanism that has been exploited experimentally to investigate the effects of silencing specific genes (Olejniczak et al., 2010). This method has great potential for use in targeted cancer therapy. Elucidation of the mechanism of RNAi led to the development of several novel RNAi-based therapeutic approaches that are currently in the early phases of clinical trials (Pecot et al., 2011). In RNAi, mRNA transcribed from the genome forms a duplex with an inserted double-stranded RNA (dsRNA) and is then degraded, diminishing or blocking expression of the transcribed gene (Peng et al., 2015). RNAi technology has considerable potential for application in many areas of biomedical research from exploring gene function to cancer treatments.

In the present study, small-hairpin RNAs (shRNAs) were designed to specifically target chicken TERT (*chTERT*) mRNAs. These shRNAs were transfected into MDCC-MSB1 cells with the aim of investigating expression of *chTERT*, telomerase activity, and cell cycles in cells undergoing oncogenic changes.

MATERIAL AND METHODS

Cells and plasmids

MDCC-MSB1 cells were purchased from the Institute of Veterinary Drug Control, China. The plasmid pSilencer 1.0-U6 was already available in this laboratory.

Construction of pSi-U6 plasmid

BLASTn was used to analyze the *chTERT* sequence, and shRNA sequences were designed based on the obtained information; the shRNA sequences were named *sh-1*, *sh-2*, and *sh-3* (Table 1). Sense and anti-sense oligodeoxyribonucleotide sequences separated by TTCAAGAGA were synthesized to form a hairpin structure. An RNA polymerase terminal sequence TTTTTT was added to the 3'-end of each anti-sense strand, and then adaptor sequences containing *Apal* (TaKaRa, Japan) and *EcoRI* (TaKaRa) restriction sites were added. The control sequences were the reverse sequences of *sh-1* with no homogeneity to target genes. pSilencer 1.0-U6 was digested with *Apal* and *EcoRI* and purified using agarose gel electrophoresis. An agarose gel extraction kit (Roche,

Switzerland) was used to retrieve the long DNA fragments, which were stored at -20°C.

Synthesized DNA was diluted to 1.0 mg/μL. A reaction mixture was prepared containing 2 μL complementary and coding strand solutions in 46 μL annealing buffer solution (100 mM potassium acetate, 30 mM HEPES, 2 mM magnesium acetate, pH 7.4). The mixture was incubated at 90°C for 3 min and then at 37°C for 1 h to anneal the complementary and coding strands. These dsDNA fragments were then ligated with vector pSilencer 1.0-U6 and transformed into *Escherichia coli*; a sample of the plasmid mixture was digested with *Hind*III (TaKaRa) and *Xho*I (TaKaRa) for confirmation. In order to detect the accuracy of the ligated fragments, positive plasmids were sequenced, and sequences named pSi-U6-Ctrl, pSi-U6-sh-1, pSi-U6-sh-2, and pSi-U6-sh-3 were obtained.

Table 1. shRNA sequences.

shRNA name	Sequence
sh-1(U)	GGTCTGGTTATCAGCCTTCTTCAAGAGAGAAGGCTGATAACCAGACCTTTTTT
sh-1(C)	AATTA AAAAAGTCTGGTTATCAGCCTTCTCTCTTGAAGAAGGCTGATAACCAGACCGGCC
sh-2(U)	GCCATAACAAATGCCGGTTCTTCAAGAGAGAACCAGGCAATTTGTTATGGCTTTTTT
sh-2(C)	AATTA AAAAAGCCATAACAAATGCCGGTTCTCTCTTGAAGAACCAGGCAATTTGTTATGGCGGCC
sh-3(U)	GATCCCAAGGGATACTATGTTCAAGAGACATAGTATCCCTTGGGATCTTTTTT
sh-3(C)	AATTA AAAAAGATCCCAAGGGATACTATGTTCAAGAGACATAGTATCCCTTGGGATCGGCC
shCtrl(U)	CTTCCGACTATTGGTCTGGTCAAGAGACCAGACCAATAGTCGGAAAGTTTTTT
shCtrl(C)	AATTA AAAAAGTCCGACTATTGGTCTGGTCTCTTGAACCAGACCAATAGTCGGAAAGGCC

U = non-coding strand; C = coding strand.

Culture and cell transfection

MDCC-MSB1 cells were maintained at 37°C in MEM containing 10% fetal calf serum, 100 U/mL penicillin, and streptomycin. Lipofectamine 2000 (Invitrogen, USA) was used to transfect cells with siRNAs. MDCC-MSB1 cells (1.0 x 10⁷ cells/mL) were placed in culture vials without serum or BsAbs 1 day before transfection; the cells were cultured overnight at 37°C. shRNAs and Lipofectamine 2000 were mixed and incubated at room temperature for 15 min before being used to transfect MDCC-MSB1 cells. Serum-containing medium was added 6 h later.

Assessing *chTERT* mRNA expression by RT-PCR

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. Primers and probes for RT-PCR were designed using the sequences of *GAPDH* and *chTERT* (AY502592) (Table 2). Cells were sampled at 48, 72, and 96 h after transfection and their RNAs were collected for RT-PCR. A randomly chosen RT-PCR product was used as template and the forward and reverse primers, dNTP, and sterile water were added to attain a total volume of 25 μL. The amplification conditions were as follows: denaturation at 94°C for 2 min, and 40 cycles of 94°C for 10 s and 60°C for 31 s. Then, 2 μL reverse-transcription products was used as a template and RT-PCR forward and reverse primers and probes were added.

Enzyme activity assay

Telomerase activity was detected using a modified telomeric repeat amplification protocol (TRAP) approach. The primers were designed using a program written in our laboratory and tested with DNASar. The sequences of the primers were P1: 5'-TCACCTTGTAATCCGTCGAGCAGAGTT-

3', and P2: 5'-CAACATCTCCACTACCTTCTAACCGTAACC-3'. For P1, the 3'-end 18-nt sequence was a standard TRAP reaction TS primer, and the 5'-end 9-nt was a random sequence. For P2, the 3'-end was a telomerase sequence (5'-CTAACCGTAACC-3'), while the 5'-end was an anchor end (5'-CAACATCTCCACTACCTT-3'). The telomerase template R5 was equivalent to the P1 5 telomerase repeats catalyzed by telomerase, the sequence being [5'-TCACCTTGTAATCCGTCGAG CAGAGTTAG (GGTAAG)₅-3']. The reaction mixture comprised 2 μ L 1.25 mM dCTP and 10 μ L melted paraffin wax; when the paraffin wax coagulated, 5 μ L 10X TRAP buffer solution (200 mM Tris-HCl, pH 8.3., 15 mM MgCl₂, 0.1% Tween 220, 630 mM KCl, 10 mM EDTA), 50 μ M dATP, dTTP and dGTP, 15 pmol/ μ L primers P1 and P2, 2 U polymerase Ex-Taq, and 2 μ L sample cells were added, along with sterile water to make up the total volume to 50 μ L. The reaction was allowed to run for 20 min to synthesize the telomerase templates, then it was heated at 90°C for 3 min to inactivate telomerase, and then PCR-amplified as follows: 30 cycles of 90°C for 30 s, 64°C for 45 s, and 72°C for 1 min. The amplification products were separated on native-PAGE and silver-stained.

Table 2. Primers and probes for quantitative real-time PCR.

Gene	Primers and probes
<i>chTERT</i>	Forward: 5'-GTCAGAGCGAAGTCATCACAAGAAT-3'
	Reverse: 5'-TGGCAAACTCTGAAGTGACAAC-3'
	Probe: FAM-ATGGATACTCCTTGCTGGATGAGAA-ECLIPSE
<i>GAPDH</i>	Forward: 5'-TTGTTTCCTGGTATGACAATGAGTTT-3'
	Reverse: 5'-CTCACTCCTGGATGCCATGT-3'
	Probe: FAM-ATACAGCAACCGTGTGGACTTGATGGT-ECLIPSE

Cell cycle detection

Cells transfected with pSi-U6-Ctrl and pSi-U6-sh-3 were collected after 72 h. The cells were centrifuged at 1000 rpm for 5 min, washed three times with PBS, and fix in cold 70% ethanol at 4°C for no less than 18 h. The cells were then centrifuged at 1000 rpm for 5 min, washed with PBS, and resuspended in 200 μ L propidium iodide and RNase (final concentrations of propidium iodide and RNase were 50 and 100 mg/mL, respectively) in the dark for 30 min. A flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze the cell cycle.

RESULTS

Construction of the expression vector for pSi-U6-shRNAs

As described above, double-stranded DNAs were obtained by annealing synthesized DNA into the vector pSilencer 1.0-U6 and transforming the plasmids into *E. coli* cells. The plasmids were then sequenced to confirm the DNA sequences (Figures 1 and 2). Positive plasmids were named pSi-U6-Ctrl, pSi-U6-sh-1, pSi-U6-sh-2, and pSi-U6-sh-3.

Identification of chTERT and GAPDH clone

RNAs from MDCC-MSB1 cells were amplified using specific primers and two fragments of the expected size were obtained. The fragments were then ligated into vector pMD18-T and transformed into the competent bacterial strain DH5a. PCR was used to confirm that the bacteria

carried the two fragments (Figure 3). Two bacterial clones were sent to Dalian Bioengineering, Ltd., for sequencing. A BLASTn analysis showed the recombinant plasmids chTERT FR (119 bp) and GAPDH FR of (97 bp) had 100% identity with the sequences reported by Li et al. (2014).

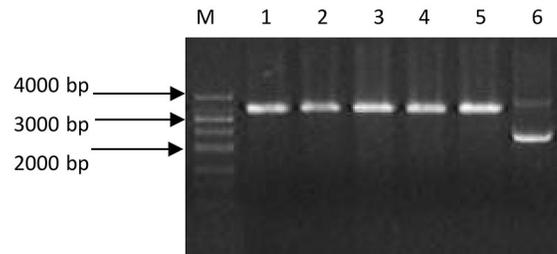


Figure 1. Identification of pSi-U6-sh-1 after digestion with *Xho*I. Lane M = DL4000 marker; lane 1 = pSi-U6-Ctrl; lane 2 = pSi-U6-sh-1; lane 3 = pSi-U6-sh-2; lane 4 = pSi-U6-sh-3; lane 5 = pSi-U6-Ctrl; lane 6 = positive plasmid.

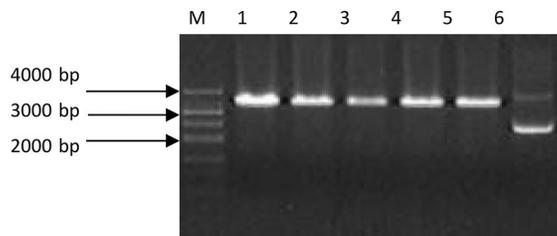


Figure 2. Identification of pSi-U6-sh-1 after digestion with *Hind*III. Lane M = DL4000 marker; lane 1 = pSi-U6-Ctrl; lane 2 = pSi-U6-sh-1; lane 3 = pSi-U6-sh-2; lane 4 = pSi-U6-sh-3; lane 5 = pSi-U6-Ctrl; lane 6 = positive plasmid.

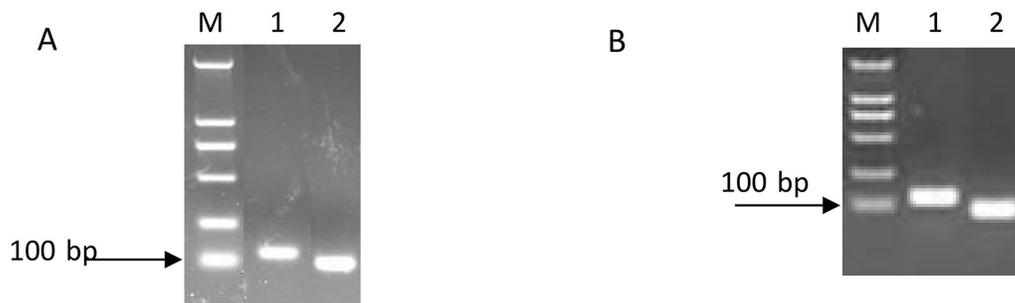


Figure 3. A. PCR products of *chTERT* and *GAPDH*. B. Identification of recombinant plasmid by PCR amplification. Lane M = DL2000 marker; lane 1 = *chTERT*; lane 2 = *GAPDH*.

Inhibition of chTERT mRNA expression by shRNAs

At 72 h, cells transfected with pSi-U6-sh-3 showed the greatest inhibition (89%) of *chTERT* expression (Figure 4). Cells transfected with pSi-U6-sh-1 also showed a significant inhibition of *chTERT* expression. No effect was observed for pSi-U6-sh-2. An inhibitory effect was evident at 48 h after transfection, and was still observed at 96 h (Figure 4).

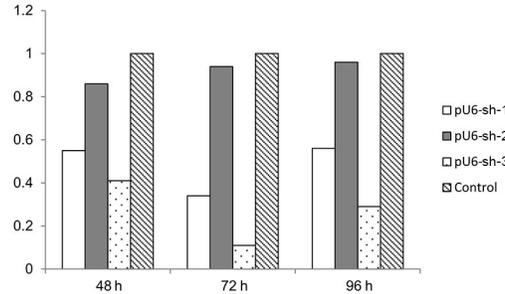


Figure 4. Inhibition of *chTERT* mRNA expression by shRNA.

Test of telomerase activity

A modified TRAP method was used to detect telomerase activity. At 48 h after transfection, there was no evidence of change in telomerase activity in any group. However, in comparison with the control, telomerase activity fell significantly in the pSi-U6-sh-1 and pSi-U6-sh-3 transfected cells at 72 h. The results clearly demonstrated that inhibition of *chTERT* mRNA expression effectively reduced telomerase activity.

Cell cycle analysis

Flow cytometry analysis showed that cells transfected with pSi-U6-sh-1 or pSi-U6-sh-3 failed to reach S phase, and that their G2/M periods lengthened to 72 h after transfection, in contrast with the control group (Table 3). This is a clear evidence that cell division was inhibited. No significant cell cycle changes were observed before 72 h transfection. No clear cell cycle changes were observed for cells transfected with pSi-U6-sh-2.

Table 3. Cell cycle changes at 72 h after transfection with pU6-sh-3.

Treatment group	G0/G1 (%; mean \pm SD)	G2/M (%; mean \pm SD)	S (%; mean \pm SD)
Ctrl siRNA	42.73 \pm 1.57	22.86 \pm 1.16	34.41 \pm 3.22
siRNA-3	44.9 \pm 3.09	51.54 \pm 2.36*	3.56 \pm 0.8**

*P < 0.05; **P < 0.01.

DISCUSSION

siRNA synthesis

The phenomenon of RNAi has received considerable attention since it was discovered and it has become one of the most promising methods for treatment of viral infection (Adelman et al., 2001; Yokota et al., 2003; Kahana et al., 2004). At present, a range of approaches for siRNA are available, such as chemical synthesis, external transcription, long dsRNA fragment digestion by RNase III (such as Dicer, *E. coli*. RNase III), and siRNA expression or viral vector expression in cells. Artificially synthesized or externally transcribed siRNAs are not suitable for the examination of long-term effects in cells because they have a relatively limited lifetime (Brummelkamp et al., 2002a,b). In contrast, the inhibition of target genes by siRNA in expression vectors driven by the

polymerase III promoters U6 or H1 offer a longer-term approach (Myslinski et al., 2001; Miyagishi and Taira, 2002).

Influence of the target gene local structure

In RNAi experiments, the selection of the target site for siRNA is very important. The extent of inhibition of the target gene can vary greatly by siRNAs with differences of only a few bases. To date, selection of the interference site on the target gene is still an empirical process, as it is difficult to predict the silencing effect of a given siRNA on a target gene (Kretschmer-Kazemi Far and Sczakiel, 2003). siRNA efficiency may be dependent on the target gene structure, or the ability to access the target gene; this was supported by the results of our study (Kretschmer-Kazemi Far and Sczakiel, 2003). Analysis with M-fold showed a circular structure at the siRNA-3 and siRNA-1 interference sites and a hairpin structure at the siRNA-2 interference site (Zuker, 2003; Gao et al., 2005). Our results showed that different structures at the interference site might produce different inhibition effects. These differences are also reflected in the free activation energies (Table 4). siRNA-2 had the highest free activation energy and the least interference effect, while the reverse was true for shRNA-1 and shRNA-3.

Table 4. Features of the siRNAs for *chTERT* mRNAs.

siRNA	Sequence of siRNA	Location	Structure of RNA	ρG (kcal/mol)
siRNA-1	AAGGTCTGGTTATCAGCCTTC	835-855	circular	-3.6
siRNA-2	AAGCCATAACAAATGCCGGTTC	2104-2124	hairpin	-0.2
siRNA-3	AAGATCCCAAGGGATACTATG	3995-4015	circular	-4.7

Analysis of siRNA interference effect

mRNA profile and target gene expression are used to demonstrate an siRNA inhibitory effect. Semi-quantitative RT-PCR, real-time PCR, and northern hybridization are widely used for mRNA assays and the silencing effect is judged by signal strength. RT-PCR (also known as TaqMan Probing) is generally accepted and is the most precise technique for mRNA expression studies (Sasaki et al., 2014). All mRNA copies are counted in RT-PCR, which contains relative quantification and absolute quantification. Some researchers believe that the factors affecting PCR amplification cannot be detected, which is an important problem for quantification (Kellner et al., 2014). Relative quantification refers to determination of expression of the target gene compared to that of a reference gene; since the level of expression of the reference gene is constant, it can be used for comparison of expression differences from different sources. Interfering factors like impurities or leftover reagents can be eliminated by mathematical calculation; therefore, the method is simple, fast and reliable (Chapman et al., 2012). Therefore, to ensure accuracy of our results, we used a relative quantification approach with *GAPDH* as the internal reference gene to determine the amount of *chTERT* mRNA in the sample.

Role of chTERT in MDCC-MSB1 cells

TERT is an important part of the telomerase complex that catalyzes the repeat amplification of telomeric DNA sequences (Xie et al., 2014). TERT is the template for amplification because of its constant expression level in most normal cells. The expression of TERT increases in immortalized

cells (Xie et al., 2014). The telomeric sequences in 90% of tumor cells are much shorter than those in normal cells, indicating that TERT has a role in maintaining telomere length and, thus, a stable chromosome (Xie et al., 2014). Our study confirmed that shRNAs were expressed by siRNA expression vectors transfected into MDCC-MSB1 cells using U6 as the promoter. At 72 h after transfection, up to 89% of *chTERT* mRNA expression was inhibited, telomerase activity was greatly reduced, and the proportion of cells in S phase reduced remarkably ($P < 0.01$).

To summarize, our research proved conclusively that siRNAs can effectively inhibit the expression of *chTERT* mRNA in MDCC-MSB1 cells, reducing telomerase activity, and prevent MDCC-MSB1 cells from entering S phase. Thus, the siRNAs prevent proliferation of MDCC-MSB1 cells. Our study provides information that will be of value for future studies and may facilitate research into cancer treatments based on siRNA technology.

Conflicts of interest

The authors declare no conflict of interest.

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