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Knockdown of Human *p53* Gene Expression in 293-T Cells by Retroviral Vector-mediated Short Hairpin RNA

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Abstract RNA interference (RNAi) is an evolutionarily conserved process of gene silencing in multiple organisms, which has become a powerful tool for investigating gene function by reverse genetics. Recently, many groups have reported to use synthesized oligonucleotides or siRNA encoding plasmids to induce RNAi in mammalian cells by transfection, but this is still limited in its application, especially when it is necessary to generate long-term gene silencing *in vivo*. To circumvent this problem, retrovirus- or lentivirus-delivered RNAi has been developed. Here, we described two retroviral systems for delivering short hairpin RNA (shRNA) transcribed from the H1 promoter. The results showed that retroviral vector-mediated RNAi can substantially downregulate the expression of human p53 in 293-T cells. Furthermore, the retroviral vector-mediated RNAi in our transduction system can stably inactivate the *p53* gene for a long time. Compared to shRNAs transcribed from the U6 promoter, H1-driven shRNA also dramatically reduced the expression of p53. The p53 downregulation efficiencies of H1- and U6-driven shRNAs were almost identical. The results indicate that retroviral vector-delivered RNAi would be a useful tool in functional genomics and gene therapy.

Key words retroviral vector; RNA interference; *p53*

RNA interference (RNAi) was initially discovered in *Caenorhabditis elegans*, which is an evolutionarily conserved mechanism of sequence-specific post-transcriptional gene silencing mediated by double-stranded RNA (dsRNA) molecules that match the sequence of the target gene [1]. Long dsRNA can be processed into short interfering RNA (siRNA) of 21–23 nucleotides (nt) by Dicer, an RNase III-type endonuclease. Upon binding to the RNA-induced silencing complex, double-stranded siRNA is unwound and targeted to homologous mRNA, resulting in sequence-specific cleavage and degradation of mRNA [2–4]. RNAi has been successfully used in plants and invertebrates for genetic analysis. Double-stranded RNA longer than 30 nt may trigger interferon response by the activation of the dsRNA-dependent protein kinase (PKR), leading to global inhibition of protein synthesis in mammalian cells. Nonetheless, the production of siRNAs that bind to specific endogenous mRNAs and induce their degradation is now recognized as an ancient, evolutionarily conserved mechanism that is widely employed in eukaryotic cells to inhibit protein production at the post-transcriptional level [5]. siRNA of 21–23 nt synthesized *in vitro* or expressed by DNA vector may bypass interferon response and induce sequence-specific gene silencing. These approaches open the avenue of applying the RNAi technique in gene function study and therapeutic research in mammalian cells [6]. Many reports have described various DNA vector-based systems expressing siRNAs in mammalian cells. In general, these DNA vectors contain an RNA polymerase III promoter to express short dsRNA containing an inverted repeat sequence in the form of a hairpin (or stem-loop) structure.

Short hairpin RNA (shRNA) was shown to be efficiently

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processed into siRNA inside the cells, which resulted in sequence-specific gene silencing. However, the delivery system that relies on transfection and the cell types available for study limit the use of these DNA vectors. Recently, gene silencing mediated by RNAi expressed in various viral vectors, including retroviral and lentiviral vectors, was described [7–12]. Thus, viral vectors combined with RNAi should provide useful tools to elucidate gene function by the analysis of loss-of-function phenotype, and to explore the application of RNAi in gene therapy. Furthermore, the use of retroviral vectors can greatly expand the cell types available for RNAi analysis.

The tumor suppressor p53 is a sequence-specific transcription factor that mediates many downstream effects such as growth arrest and apoptosis by activation or repression of its target gene. The p53 protein has a short half-life, but it can be stabilized by either point mutation of the gene or interaction with specific DNA tumor virus factor, such as SV40 large T antigen [13,14].

We chose *p53* as the targeted gene to evaluate the effects of RNAi with the two retroviral vector expression systems, pXSNhH1sip53-2 and pXRNhH1sip53-2, for shRNA expression.

Materials and Methods

Cell culture

The GP-293 pantropic packaging cell line (Clontech, Palo Alto, USA) and 293-T cell line were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carsbad, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA) and antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) at 37 ºC with 5% CO_2.

Construction of retroviral vectors

The retroviral vectors pXSN and pXRN were derived from pLXSN and pLXRN vectors (Clontech) by deleting the 260 bp *Nhe*I/*Xba*I fragment of 3′ long terminal repeat (LTR). The human H1 promoter (–315/+1) from pSuper (a gift from Dr. Yong-Feng SHANG, Beijing University, Beijing, China) digested with *Bam*HI/*Xho*I was cloned into pXSN and pXRN upstream of either the SV40 early promoter (P_{SV40e}) or Rous sarcoma virus (RSV) promoter (P_{RSV}) to construct pXSNhH1 or pXRNhH1. Two synthetic oligonucleotides (5′-GATCCCC**GACTCCAGTGGTAATCTAC**ttcaagaga**GTAGATTACCACTGGAGTC**-TTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAA**GACTCCAGTGGTAATCTAC**tctcttgaa**GTAGATTACCACTGGAGTC**GGG-3′) were annealed and ligated downstream of the H1 promoter in pXSNhH1 or pXRNhH1 to construct pXSNhH1sip53-2 or pXRNhH1sip53-2. The configuration of the constructs pXSNhH1sip53-2 and pXRNhH1sip53-2 were verified by DNA sequencing.

Construction of recombinant retrovirus and virus transduction into 293-T cells

The GP-293 packaging cells in 100 mm dishes were cotransfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) with 5–8 μg pVSV-G (Clontech) and 15–20 μg recombinant retroviral vector (pXSNhH1, pXRNhH1, pXSNhH1sip53-2 or pXRNhH1sip53-2) for 24 h. After transfection, the cells were incubated at 32 ºC to increase viral titer. Forty-eight hours later, the supernatant containing the retroviral particles was collected, filtered through the 0.45 μm low protein binding syringe filter, and used to infect target cells.

293-T cells maintained in DMEM were plated into six-well plates at 3×10^5 cells/well. Twenty-four hours later, the cells were infected with viral supernatants in the presence of polybrene (5–8 μg/ml final concentration) for 12 h, then added fresh medium with fresh viral supernatants. Twenty-four hours later, the cells were incubated with fresh viral supernatant for additional 12 h. The transfected 293-T cells were subcultured at an appropriate density in fresh DMEM containing 1 mg/ml G418 (geneticin; Gibco BRL). G418-resistant cell pools were readily established within a period of 7–10 d.

Western blot analysis

Infected 293-T cells were harvested at the indicated time points, washed twice with cold phosphate-buffered saline, lysed in TNT lysis buffer (10 mM Tris, 150 μM NaCl, 1% NP-40, 10 mM NaF, 2 mM EDTA, 100 μg/ml 1,4-dithiothreitol, 100 μg/ml phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin) for 30 min on ice, and centrifuged at 15,000 *g* for 15 min to remove insoluble materials. Protein concentrations were determined by BCA assay (Pierce, Rockford, USA). Forty micrograms of lysate supernatant was separated using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore, Bedford, USA). The membrane was incubated with anti-p53 (it reacts specifically with both wild and mutated human p53 protein) or anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology). Western blots were developed

using Western blotting luminal reagent (Santa Cruz Biotechnology).

Results and Discussion

To eliminate the interference of the 5′ LTR counterpart on virus replication [15], the retroviral vectors pLXSN and pLXRN were modified by deleting the *Nhe*I/*Xba*I fragment of about 260 bp in 3′ LTR to generate pXSN and pXRN (**Fig. 1**).

We inserted the RNA polymerase III promoter of the human H1 small nuclear RNA gene (P_{hH1}) into the retroviral vectors pXSN and pXRN. The H1 promoter was inserted into the multiple cloning sites of pXSN and pXRN, with reverse orientation to the P_{SV40e}- or P_{RSV}-driven *neo*^{R} gene [**Fig. 2(A)**]. Subsequently, the synthesized inverted repeats with an identical sequence to the human *p53* gene was inserted downstream of the H1 promoter, using 5 thymidines as the terminal signal [**Fig. 2(B)**]. The predicted hairpin RNA structure is shown in **Fig. 2(C)**. DNA sequencing demonstrated that the configurations of both the pXSNhH1sip53-2 and pXRNhH1sip53-2 constructs were correct.

The tumor suppression gene *p53* is important in the regulation of the cell cycle, and it also plays a crucial role in the progression of cancer, as evidenced by the inactivation or loss of p53 in the majority of human tumors. Because the 293-T cell line contains a high level of endogenous wild-type p53, we used it as a model to test whether the retrovirus-mediated RNAi could knockdown the expression of endogenous p53. The recombinant retroviruses were generated by cotransfection of GP-293 cells with the envelope plasmid pVSV-G, which confers the virus with pantropism, and the retroviral vectors, pXSNhH1, pXRNhH1, pXSNhH1sip53-2 or pXRNhH1sip53-2. G418-resistant 293-T cell pools were established after infection with the recombinant retrovirus.

Western blot analysis demonstrated that the expression levels of p53 were reduced dramatically compared with those of the internal control GAPDH [**Fig. 3(A)**], which suggested that retrovirus-delivered shRNA could efficiently trigger the downregulation of *p53* gene expression in a sequence-specific manner in 293-T cells. The results also demonstrated that there was no obvious difference in the inhibitory efficiency of the two systems [**Fig. 3(B)**].

Previously, we inhibited the human *p53* gene expression with shRNA expressed from human U6 promoter in the pXSN retroviral system [8]. In the present study, H1 promoter was used to express shRNA to inhibit *p53* gene

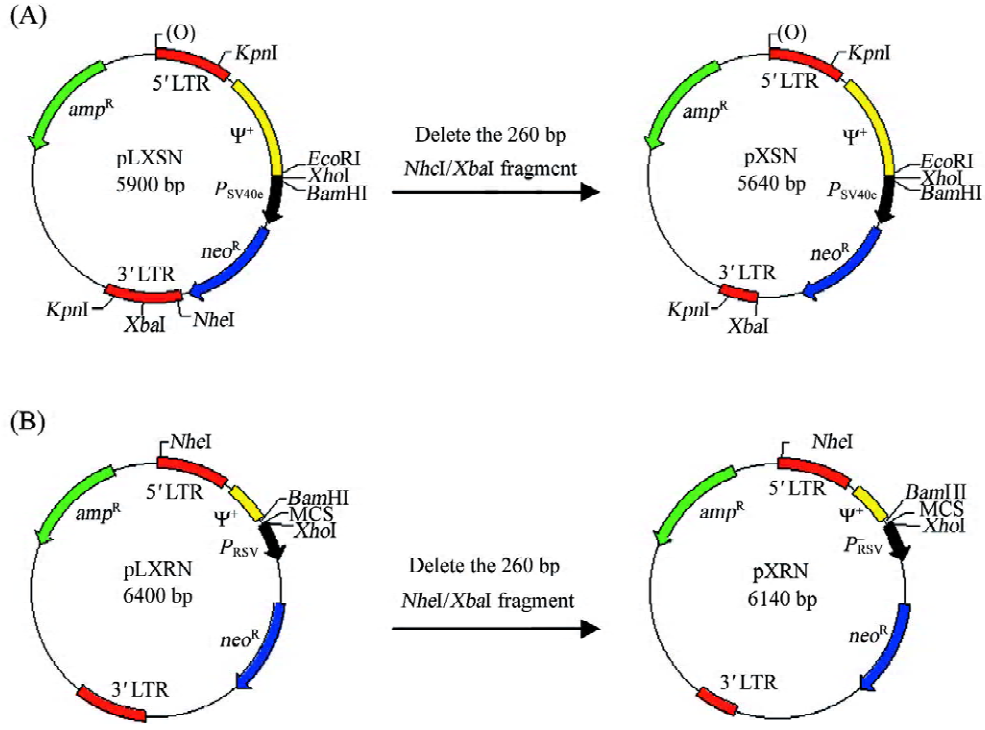

Fig. 1 Derivation of pXSN and pXRN from retroviral vectors pLXSN and pLXRN, respectively

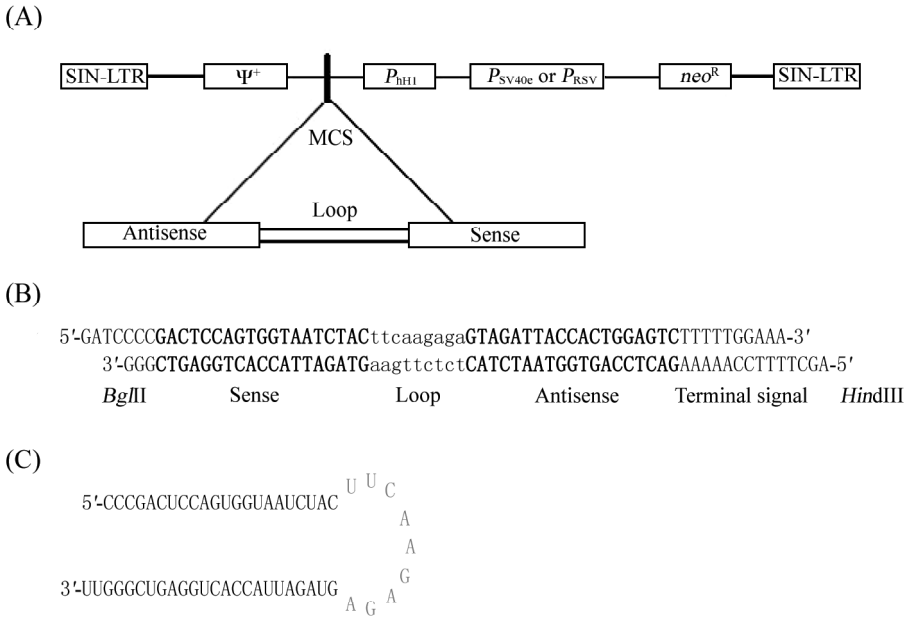

Fig. 2 Location, sequence and structure of the short hairpin RNA

(A) Schematic representation of retroviral vector pXRNhH1 or pXSNhH1. Arrows indicate transcription initiation sites. ψ+, extended packaging signal; SIN-LTR, self-inactivated long terminal repeat; MCS, multiple cloning sites; P_{SV40e}, SV40 early promoter; P_{RSV}, RSV promoter; *neo*^{R}, neomycin phosphotransferase gene. (B) The sequence of short interfering RNA against human *p53*. (C) Predicted structure of short hairpin RNA for human *p53* transcripts generated from pXRNhH1sip53-2.

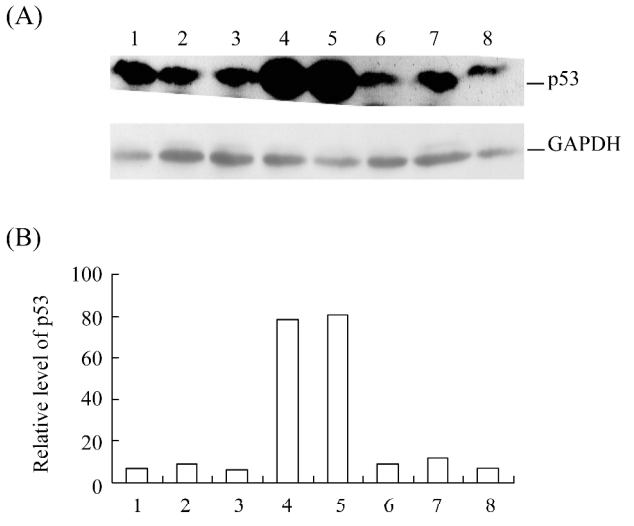

Fig. 3 Different expression levels of the *p53* gene in different 293-T cell pools

(A) Western blot analysis of *p53* expression level in 293-T cells. The results demonstrate that infection of 293-T cells with pXSNhH1sip53-2 or pXRNhH1sip53-2 decreases the expression of p53, but the expression levels of p53 in mock group do not change. (B) Analysis of inhibition efficiency of human p53 gene expression levels by the pXSN and pXRN retroviral system with the H1 promoter in the 293-T cell line. 1–3, 293-T cell pools infected with pXSNhH1sip53-2; 4 and 5, control cell pools not infected with any virus; 6–8, 293-T cell pools infected with pXRNhH1sip53-2. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

expression in the pXSN and pXRN retroviral system, and the pXRN retrovirus system had the similar efficiency as the pXSN system. In mammalian cells, retroviral vector-mediated RNAi can be further applied to functional genomics, so that a group of related individual genes can be silenced simultaneously and their synergic functions can be systematically assessed. Lentiviral vector-mediated transgenic knockdown suggested that RNAi might provide an alternative approach to homologous gene targeting to create gene-knockout mice [16,17]. Vectors based on lentiviruses are now in phase I clinical studies, and there will be new applications for their use in the near future. In addition, viral vector-mediated RNAi holds promises in gene therapy for cancers and infectious diseases because it can result in loss-of-function phenotypes of disease-related genes [18–20].

In conclusion, to facilitate stable and long-term knockdown in cells, which are refractory to transfection-based gene transfer techniques, we designed a retroviral vector system that permits delivery of stem-loop cassettes. We reported the development of a versatile system of retrovirus-based vectors, which makes it possible to achieve durable, high efficiency siRNA-dependent gene silencing in a wide variety of cell lines. The development of appropriate siRNA delivery vectors eventually may have

important applications, especially in gene therapy and genomic research. Although they are not the perfect vectors for every purpose, for certain disease conditions they are superior. Viral vectors combined with RNAi may provide useful tools to elucidate gene function through the analysis of loss-of-function phenotypes.

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