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Full Length Research Paper

Effective inhibition of specific gene by adeno-associated virus (AAV)-mediated expression of small interfering RNA

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RNA-interference is the mechanism of sequence-specific, post-transcriptional gene silencing, initiated by small interfering RNA (siRNA), homologous to the gene being suppressed. Several techniques are utilized to transfer siRNA into cultured cells or animal models, while every method has advantages and disadvantages. In this study, a siRNA expression recombinant adeno-associated virus (AAV) was established by inserting H1 promoter into transfer plasmid of AAV Helper-Free system. To perform functional tests on siRNA, which was expressed by the viral vector, recombinant AAVs, coding for siRNA against exogenous gene, EGFP, and endogenous gene, p53, were established and added into HEK293 cells, respectively. The results proved the expression of EGFP and p53 in cells were definitely suppressed at 72 h post-infection, which suggested that the H1 promoter, inserted into the recombinant AAV, could express siRNA in mammalian cells and this siRNA delivery system could be used for long-term gene silencing.

Key words: Adeno-associated virus, RNA interference, p53, EGFP.

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved, genetic surveillance mechanism that the presence or introduction of small interfering RNA (siRNA) in a cell results in the degradation of homologous mRNA (Fire et al., 1998; Hannon, 2002; McManus and Sharp, 2002; Dykxhoorn et al., 2003). siRNA, which was applied to mediate specific gene inhibition, is duplex of about 21 to 23 nucleotides with 3′-overhangs synthesized *in vitro* or expressed from a DNA-based vector by the function of the cellular RNA-polymerase III (Elbashir et al., 2001; Matsukura et al., 2003). siRNA is processed to be incorporated into the RNA-induced silencing complex (RISC) and then, leads to gene inhibition by cleaving target RNA (Zhang et al., 2004; Meister et al., 2004). Based on the phenomenon, RNAi has rapidly developed into one of the most widely applied technologies for gene inhibition and has the potential to serve as a novel method for therapeutic treatment for gene diseases (Agami, 2002; Couzin, 2002).

Several techniques are utilized to transfer siRNA into cultured cells or animal models. SiRNA, chemically or *in vitro* enzymatically synthesized or endogenously expressed from appropriate plasmids, used to knock down interested gene is delivered to target cells by physical transfection methods, which limits the application of RNAi in long-term gene silencing in mammalian systems. To improve the efficiency of siRNA transfer, virus-based vectors have been used to introduce the siRNA-expression cassettes into target cells, such as recombinant retrovirus and adenovirus-based vectors; every virus has its advantages and disadvantages (Devroe and Silver, 2002; Shen et al., 2003; Rubinson et al., 2003).

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Abbreviations: RNAi, RNA interference; **siRNA,** small interfering RNA; **AAV,** adeno-associated virus; **ITRs,** inverted terminal repeats; **RISC,** RNA-induced silencing complex; **FACS,** fluorescence activated cell sorting; **MCS,** multiple cloning site; **qPCR,** quantitative PCR; **DAB,** diaminobenzidine; **shRNA,** short hairpin RNA; **PCR,** polymerase chain reaction; **DMEM,** Dulbecco's modified Eagle's medium; **FBS,** fetal bovine serum; **CMV,** cytomegalovirus; **qPCR,** quantitative polymerase chain reaction.

Adeno-associated virus (AAV) is a member of the Parvovirus family. AAV-based vectors possess characteristics of high infection rate of quiescence or division cells, prolonging transgene expression and minimal immunogenic potential (Stilwell et al., 2003). AAV vector has been introduced more recently than retroviral or adenoviral vectors and has been used in clinical trials in humans (Flotte, 2001; Manno et al., 2003; During et al., 2001; Stilwell et al., 2003). By combining superiority of AAV with high effectiveness of RNAi. Several reports have established that recombinant AAV is capable of expressing siRNA inside cells. It is noted that, AAV-mediated RNAi can introduce a safe, long-term gene inhibition in mammalian cells (Tomar et al., 2003; Han et al., 2007; Pinkenburg et al., 2004; Machida et al., 2006; Dufourny et al., 2008).

Transfer plasmid plays an important role in AAV expression system, including inverted terminal repeats (ITRs), which are the only cis-elements for replication, packing, insertion into the host genome and rescue from the chromosome. Foreign gene is inserted between these *cis*-acting ITRs. In our research, a transfer plasmid (pAAV-H1), containing the H1 promoter, was constructed based on the backbone of pAAV-MCS (transfer plasmid of AAV Helper-Free System, Stratagene). To perform functional tests on the expressed by recombinant AAV siRNA, transfer plasmids coding for siRNA against an exogenous gene, EGFP, and an endogenous gene, p53, were developed, respectively. Using calcium phosphate precipitation method, transfer plasmid was co-transfected into AAV-293 cells with helper plasmids to generate recombinant AAV (rAAV-H1-EGFP, rAAV-H1-p53). RAAV-H1-EGFP-infected HEK293 cells then, were pre-transfected with plasmid pEGFP-N1. After 72 h, the interference effect on EGFP expression was investigated by fluorescence microscope and fluorescence activated cell sorting (FACS). At the same time, the inhibition rate of p53 in HEK293 cells, infected with rAAV-H1-p53, was testified by real time quantitative polymerase chain reaction (PCR) and western blotting at 72 h post-infection.

MATERIALS AND METHODS

Plasmids construction

pAAV-MCS was digested with *Eco*RI and *Bgl*II before being repaired by Klenow fragment and then self-ligated to remove the multiple cloning site (MCS) to produce pAAV. The H1-polymerase III promoter was obtained from pSilencer 3.0-H1 (Ambion) by PCR with primers (forward, 5′-CGGACCG TAGACCGAGATAGGGTTG AGTG -3′; reverse, 5′- CGGACCG GTACCAAGCTTAGATCTGT GGTC-3′), containing *Cpo*I restriction sites. PAAV-H1 was developed by insertion the H1 promoter into the unique *Cpo*I site of pAAV.

Based on previous work (Tiscornia et al., 2003), siRNA oligonucleotides were designed, which contained a sense strand of p53 or EGFP followed by a short spacer (TTCAAGAGA), the reverse complement of the sense strand (antisense strand) and five thymidines as an RNA-polymerase III transcriptional stop signal. Forward and reverse oligos were annealed and directionally cloned into H1 promoter downstream, between the *Bgl*II and *Hin*dIII sites of pAAV-H1, to obtain pAAV-H1-EGFP or pAAV-H1-p53 (Figure 1). For contrast, two non-targeting plasmids were constructed as control, named pAAV-H1-EGFP(c) and pAAV-H1-p53(c). All plasmids, encoding siRNA-targeted cassettes were confirmed by sequencing prior to transfection.

Packaging of recombinant AAV encoding siRNA

We made use of AAV Helper-Free system to express siRNA. AAV-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) and co-transfected with transfer plasmid (pAAV-H1-EGFP/ EGFP(c), pAAV-H1- p53/ p53(c)) along with pAAV-RC (rep and cap genes) and pHelper (adenoviral genes) plasmids by standard calcium phosphate transfection. Following previously published methods (Zhao et al., 2006), cells were harvested 72 h after transfection, lysed by two cycles of freezing and thawing to release the virus and purified. The recombinant AAV titers were determined by dot-blot hybridization technique.

Viral infections

HEK293 cells were cultured in DMEM, supplemented with 10% FBS. The day before transfection, 5×10^4 cells were plated in each well of 24-well plates until they reached about 90% confluency. Plasmids (pEGFP-N1, Clontech), expressing EGFP under the control of cytomegalovirus (CMV) promoter, were transfected using Lipofectamine 2000 (Invitrogen). Four hours after transfection, rAAV-H1-EGFP/ EGFP(c) was added to the culture medium at 1×10^5 vector genomes/cell. Three days later, the cells were analyzed for EGFP expression by fluorescence microscope and fluorescence-activated cell sorting (FACS). Cells were simultaneously infected with rAAV-H1-p53/ p53(c) according to the method described previously and the inhibition effect on p53 was estimated by real time quantitative PCR and western blotting.

Real time quantitative PCR (qPCR)

qPCR assays were used to measure p53 gene expression, relative to GAPDH gene expression. Total RNA was extracted from HEK293 cells using Trizol reagent (Invitrogen). Contaminating genomic DNA was removed with DNase (RNase free, TaKaRa) and 2 µg of the total RNA was used for cDNA synthesis using Oligo (dT) primers with the SuperScript III First-Strand synthesis system (Invitrogen). qPCRs were conducted in a total volume of 20 µl on the 7500 Fast Real-time PCR system using the SYBR® Premix reagent trial pack (TaKaRa). Thermal cycle parameters were set according to the manufacturer's instructions. p53 mRNA primers (forward, 5′ -CGCAAAAGAAGAAGCCACTA-3′; reverse, 5′ -TCCACTCTGGG CATCCTT-3′; product size, 118 bp) were designed for qPCR. The GAPDH (forward, 5′ -AAGAAGGTGGTGAAGCAGGC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′; product size, 203 bp) was used as an internal control. 1 to 3 independent qPCR trials were conducted for each template source. In each trial, triplicate samples of template were analyzed.

Flow cytometric analysis

For measurement of expression of EGFP, HEK293 cells were briefly trypsinized, washed and fixed with 4% paraformaldehyde. Ten thousand cells were collected for each sample and all analyses of whole cells were performed using appropriate scatter gate to

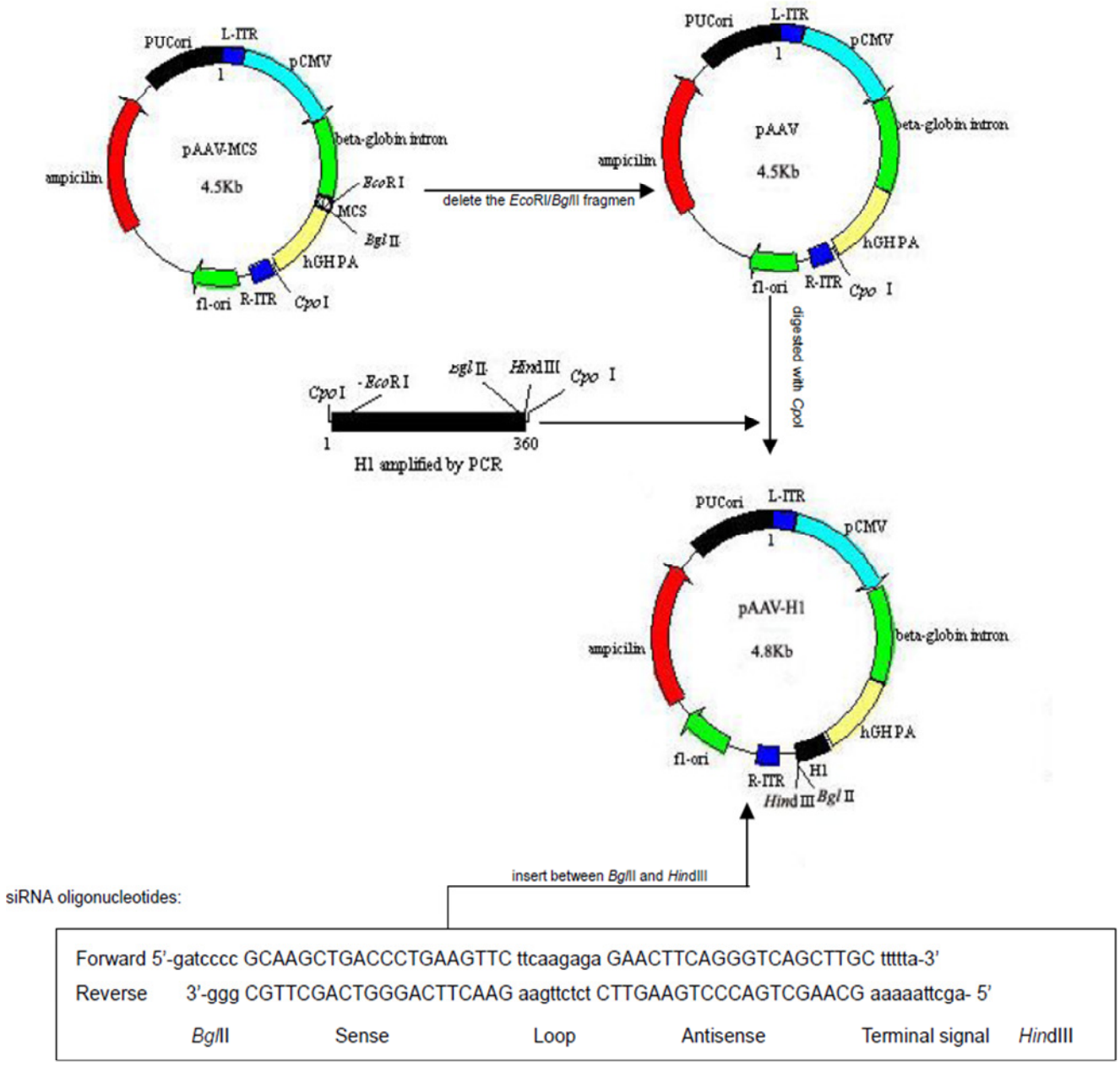

Figure 1. Scheme of plasmids construction.

exclude cellular debris and aggregates. The level of EGFP was estimated on a flow-cytometer (Becton Dickinson FACS Calibur) and the cell quest software was used to analyzed the data.

Western blotting analysis

Protein lysates from HEK293 cells were run on a 10% SDS–PAGE gel and transferred to a PVDF membrane. The blot was probed with monoclonal antibodies against p53 (Bioworld Technology) and followed by incubation with a horseradish peroxidase-conjugated goat-anti-mouse IgG (Sigma). Subsequently, p53 was visualized using diaminobenzidine (DAB, Sigma).

RESULTS AND DISCUSSION

Construction of recombinant AAV vectors

To eliminate the interference of MCS on restriction enzyme digestion, the transfer plasmid pAAV-MCS was modified by deletion *Eco*RI/ *Bgl*II fragment to generate pAAV. The H1-polymerase III promoter obtained from pSilencer 3.0-H1 by PCR with primers containing *Cpo*I restriction sites, was inserted into the unique *Cpo*I site of pAAV. Subsequently, the synthesized inverted repeats

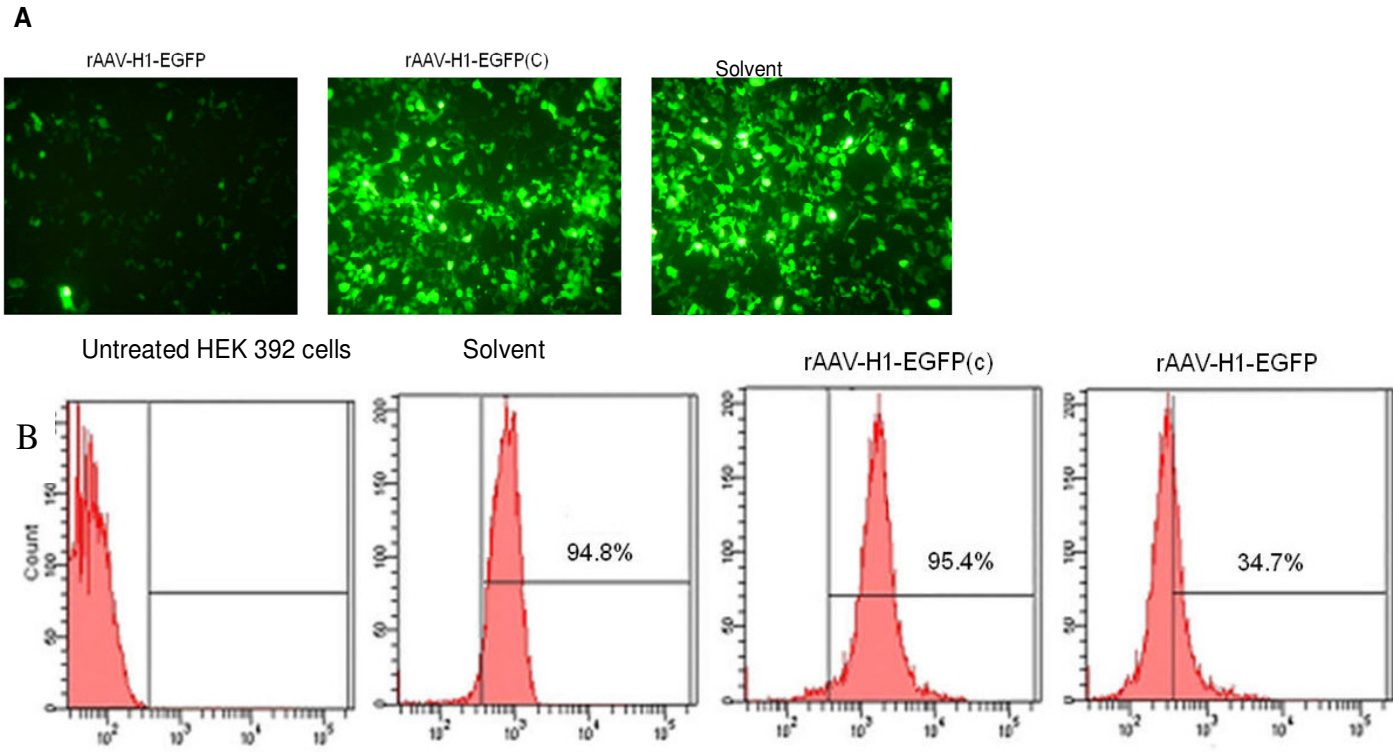

Figure 2. Suppression of EGFP expression in HEK293 cells by AAV-delivered siRNAs. (A) Fluorescence photomicrographs of HEK293 cells (pre-transfected with pEGFP-N1) infected with rAAV-H1-EGFP, rAAV-H1-EGFP(c) and solvent as blank control. The photographs were taken at 72h after viral transduction (100×); (B) FACS results of untreated HEK293 cells and HEK293 cells (pre-transfected with pEGFP-N1) infected with rAAV-H1-EGFP, rAAV-H1-EGFP(c), solvent as blank control. HEK293 cells were collected at 72 h after viral transduction.

with targeting or non-targeting sequence to the EGFP and human p53 gene were inserted downstream of the H1 promoter, respectively (Figure 1). DNA-sequencing demonstrated that, the configurations of pAAV-H1-EGFP/ EGFP(c) and pAAV-H1-p53/ p53(c) constructs were correct. The recombinant AAV was generated by co-transfection of AAV-293 cells with the helper plasmids and transfer plasmid, pAAV-H1- EGFP/ EGFP(c) or pAAV-H1-p53/ p53(c). The viral stocks were titrated by dot-blot hybridization with plasmid standards to make a stock of 1×10^{11} vector genomes/ ml.

EGFP gene inhibition by the AAV-mediated siRNA expression

The RNAi and EGFP are two methodological advances that have transitioned so rapidly and effectively from discovery to widespread usefulness, that they now seem indispensable to basic biomedical scientists and Nobel Prizes awarded in 2006 and 2008, respectively recognized their utility. EGFP was selected as a perfect exogenous gene to estimate the siRNA-expression ability of recombinant AAV because of its stability and visibility. HEK293 cells were pre-transfected with pEGFP-N1 four hours before being exposed to rAAV-H1-EGFP/ EGFP(c). Three days after infection, the EGFP fluorescence was monitored by using an inverted fluorescence microscope (Leica DMIRB, Germany). The photographs revealed that, the recombinant AAV-delivered EGFP siRNA dramatically reduced EGFP expression level in HEK293/EGFP cells, while the controls did not (Figure 2a). Simultaneously, cells were collected for FACS analysis and the results proved that rAAV-H1-EGFP achieved obvious knockdown of EGFP expression (almost 61%), compared with rAAV-H1-EGFP(c) (Figure 2b). As the data suggested, the H1 promoter inserted into AAV could express siRNA in mammalian cells to restrain specific gene expression.

p53 gene inhibition by the AAV-mediated siRNA expression

The tumor-suppression gene p53 is important in the regulation of the cell cycle and it 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 HEK293 cell line contains a high level of endogenous wild-type p53, we used it as a model to test whether the

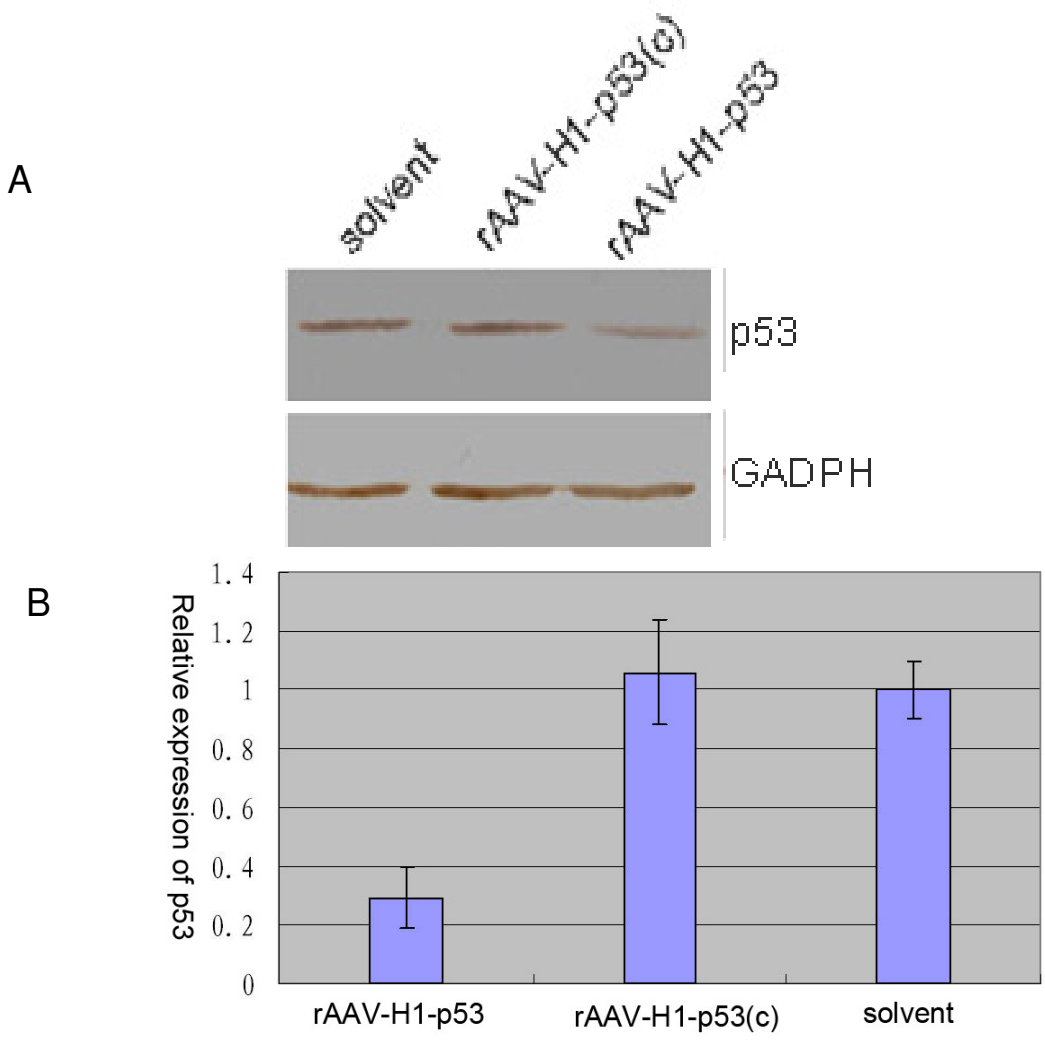

Figure 3. Suppression of p53 expression in HEK293 cells by AAV-delivered siRNAs. (A) Western blotting analysis of p53 protein after application of rAAV-H1-p53, rAAV-H1-p53(c) and solvent as blank corol; (B) Relative expression of the p53 gene determined by Real time quantitative PCR in HEK293 cells infected with rAAV-H1-p53, rAAV-H1-p53(c) and solvent as blank control. Cell lysates were prepared at 72 h post-infection.

AAV-mediated RNAi could knockdown the expression of p53. To investigate the silencing effect of the AAV vector, we detected p53 expression in HEK293 cells at the protein and mRNA levels. Western blot illustrated that, the expression level of p53 was obviously reduced compared with the internal control GAPDH, which suggested that recombinant AAV-delivered siRNA could efficiently trigger the down regulation of p53 gene in a sequence-specific manner in HEK293 cells. The result also demonstrated that, there was no inhibitory effect of the control, rAAV-H1-p53(c) (Figure 3a). In contrast to negative group, qPCR analyses showed that p53 mRNA level was reduced to about 30% at 72 h after infection (Figure 3b). qPCR also confirmed that the primers designed were specific for the p53 gene and there were no non-specific targets within the human genes (data not shown).

Advantages of the AAV-mediated siRNA expression

RNAi, induced by siRNA or short hairpin RNA (shRNA), is an important research approach for sequence-specific mRNA degradation in cells. A major aspect of RNAi is the efficient delivery of siRNA or shRNA into the target cells. Although in some studies, oligonucleotide-mediated siRNA or plasmid vector, containing siRNA-expression cassette, is delivered into cells by transfection using polymers or liposomes, they provide only transient gene suppression. Viruses usually provide higher gene delivery efficiency in most cell types, including primary cells, so various groups have developed different viral vectors for delivery of hairpin siRNA-producing cassettes. Among these viral-based vectors, AAV has many advantages including; (1) The ability to transducer dividing and non-

dividing cells, (2) broad tropism, (3) lack of pathogenic and immunogenic effects and (4) long-term expression due to persistent episomal status. The major disadvantages of AAV are formed by the complex production of virus, resulting in low viral titers and the limited size of the inserted gene (Stilwell et al., 2003; Mah et al., 2002). Incorporation of siRNA expression cassette into AAV vector may induce a specific reduction in the level of target mRNA, which was proved by previous researches (Han et al., 2007; Pinkenburg et al., 2004; Machida et al., 2006). But in these studies, the experiment processes were relatively complicated, because the siRNA-expression cassettes were obtained by PCR or digested from vectors including polymerase III promoter, such as pSilencer 3.0-H1 before being inserted into AAV vectors. In our research, H1 promoter was introduced into AAV vector, so the research process seemed rather simple by insertion of shRNA between the BglII and HindIII sites of pAAV-H1 directly, just downstream of the H1 promoter. Our research revealed that, rAAV-H1-EGFP and rAAV-H1-p53 both had the capacity of sequence-specific knockdown of the interested gene obviously, whichshows that the recombinant AAV developed could express siRNA and was an effective delivery system for gene silencing. The gene inhibition rate at 72 h post-infection was about 65% similar to related researches, but was lower than other gene transfer systems. The reason is that, the AAV is a single-stranded DNA virus and complementary strand synthesis must take place prior to gene expression. The time required for complementary-strand synthesis is one of the rate-limiting factors in gene inhibition (McCarty et al., 2001).

In conclusion, we have developed a recombinant AAV, capable of initiating RNAi against target genes in infected cells and the combination of AAV vector and siRNA gene-silencing approach shows great promise. The AAV siRNA delivery system can integrate in the chromosome and the possibility for long-term expression of siRNA has paved the way to new gene therapy applications, not only in cultured cells but also at the level of whole animals by injecting recombinant AAV expressing siRNA.

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