Gene silencing by adenovirus-delivered siRNA

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Abstract RNA interference is the process that double-stranded RNA induces the homology-dependent degradation of cognate mRNA mediated by 21–23 nucleotide short interfering RNA (siRNA). Here, we describe a simple virus vector for efficient delivery of siRNA into mammalian cells utilizing the well-defined H1-RNA promoter and conventional adenovirus. In this pilot study, p53 was targeted by this vector. Our results demonstrate efficient and specific knock-down of p53 in breast cancer MCF-7 and lung carcinoma A549 cells and indicate a prospective application of this siRNA expressing recombinant adenovirus system in functional genomics, cancer gene therapy and virus inhibition.
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Key words: RNA interference; Short interfering RNA; p53; Adenovirus; Functional genomics

1. Introduction

RNA interference (RNAi) is an ancient evolutionarily conserved process [1]. Ever since synthetic 21–23 nucleotide short interfering RNA (siRNA) was shown to induce efficient RNAi in mammalian cells [2,3], siRNA has been routinely used in gene silencing by transfection of chemically synthesized siRNA [4]. To circumvent the high cost of synthetic siRNA and in order to establish stable gene knock-down cell lines by siRNA, several plasmid vector systems were designed to produce siRNA inside cells driven by RNA polymerase III-dependent promoters such as U6 and H1-RNA gene promoters [5–9]. With these plasmid vectors, the phenotypes of gene silencing could be observed by stable transfection of cells [5]. Nevertheless, transient siRNA expression, low and variable transfection efficiency remains the problems for chemically synthesized and vector derived siRNA. Recently, several virus vectors have been developed for efficient delivery of siRNA into mammalian cells [10–12]. Retroviral vectors were designed to produce siRNA driven by either U6 or H1-RNA promoter for efficient, uniform delivery and immediate selection of stable knock-down cells [10,11]. Meanwhile, an adenovirus vector using RNA polymerase II CMV promoter was also developed and demonstrated to mediate gene silencing both in vitro and in vivo [12]. With the completion of whole-genome sequencing of several organisms and extensive studies of functional genomics and proteomics, more and more genes will be validated for gene therapy. To expand the strategy of RNAi to human cancer gene therapy, we developed a simple adenovirus system utilizing the well defined polymerase III H1-RNA promoter to drive efficient expression of siRNA in mammalian cells. Our results demonstrate efficient and specific knock-down of p53 in different cell lines and indicate a promising application of this adenovirus system in functional genomics, cancer gene therapy and virus inhibition.

2. Materials and methods

2.1. Plasmid constructs

The RNA polymerase III H1-RNA gene promoter was used in our study. H1-RNA promoter was cloned into the promoterless shuttle vector pShuttle (Stratagene, La Jolla, CA, USA) to get a new shuttle vector designated pShuttle-H1 (Fig. 1) which can drive the expression of siRNA in recombinant adenovirus. For convenient cloning and confirmation, the *Bgl*II site of the original promoterless pShuttle vector was converted to *Eco*RI site by site-directed mutagenesis (Stratagene) with the following primers: forward 5′-GCTTGTCGACTCGAAGACCTGGGCGTGGTTAAGGG-3′, reverse 5′-CCCTTAACCACGCCCAGGTCTTCGAGTCGACAAGC-3′. H1-RNA promoter was cut from pSUPER (kindly provided by Reuven Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) with *Xba*I and *Hin*dIII, cloned into the same restriction sites of the promoterless mutagenized pShuttle (*Bgl*II to *Eco*RI) and confirmed by dideoxy sequencing (GATC Biotech AG, Konstanz, Germany). The 64 nt oligonucleotides encoding human p53 specific siRNA were 5′-GATCCCCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGACTCCAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGTCGGG-3′ as described [5]. These oligonucleotides were annealed and ligated to the *Bgl*II and *Hin*dIII sites of pShuttle-H1 to get plasmid pShuttle-H1-p53 and confirmed by *Eco*RI digestion. A pSUPER-p53 was also constructed as described by Brummelkamp [5]. The inserted sequences were confirmed by dideoxy sequencing.

2.2. Production of recombinant adenovirus

Here, we employed the efficient homologous recombination machinery in *Escherichia coli* [13]. A recombinant adenovirus was produced by a double-recombination event between cotransformed adenoviral backbone plasmid pAdEasy-1 (Stratagene) and a linearized shuttle vector. pShuttle-H1 and pShuttle-H1-p53 were linearized with *Pme*I and cotransformed with pAdEasy-1 into BJ5183 cells by electroporation or linearized shuttle vectors were transformed into BJ5183-AD-1 cells (Stratagene) by a standard $CaCl_2$ method. Positive clones were selected and confirmed by DNA miniprep and *Pac*I digestion. Plasmids from correct clones were amplified by transforming into DH5α cells followed by DNA maxiprep (Qiagen, Hilden, Germany). The

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Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA

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resulting adenoviral DNA (AdH1-p53 or AdH1-empty) was linearized with *Pac*I and purified by ethanol precipitation (Fig. 2A). The packaging cell line AD-293 was grown in DMEM with 10% FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. 1.5×10^6 cells were plated in a 25 cm^2 flask the day before transfection. Cells were transfected by 24 μg of Dosper liposome (Roche, Basel, Switzerland) with 6 μg of *Pac*I linearized adenoviral DNA (Fig. 2A). The next day, the medium containing the transfection mix was replaced with 6 ml of growth medium. Transfected cells were incubated for additional 7–10 days and medium was changed every 2–3 days. Virus was harvested, amplified and titered according to the manufacturer's instructions (Stratagene).

2.3. Cell transfection

Breast cancer MCF-7 and lung carcinoma A549 cells were grown in DMEM with 10% FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The day before virus infection, 0.3×10^6 MCF-7 or A549 cells were plated in each well of 6-well plates. The following day, cells were incubated with recombinant virus (AdH1-p53 or AdH1-empty) at MOI of 10–20 at 37°C. After adsorption for 1–2 h, 2 ml of fresh growth medium was added and cells were placed in the incubator for additional 2–3 days.

2.4. Western blot

Cells were harvested at the indicated time points after virus infection, washed once with cold PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH7.4, 2 mM EDTA, 1% NP-40) containing protease inhibitors (Boehringer Mannheim, Germany). Total proteins (30 μg per lane) were resolved on a 8% SDS–polyacrylamide gel and transferred onto a nylon membrane and incubated with anti-p53 (Pharmingen, San Diego, CA, USA) and anti-actin (Sigma) monoclonal antibodies, followed by incubation with horseradish-peroxidase conjugated anti-mouse IgG secondary antibody (Sigma). The bands were visualized by using the enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

3. Results and discussion

Successful application of RNAi in functional genomics and proteomics, cancer gene therapy and virus protection depends on the efficient delivery of siRNA into mammalian cells. In this study, we utilized a commercially available adenovirus system and the well-defined polymerase III H1-RNA promoter to deliver siRNA expressing cassette into cells and to silence a specific gene in human cancer cells.

RNAi or RNA silencing is the process that double-stranded RNA induces the homology-dependent degradation of cognate mRNA [1]. This is an ancient and ubiquitous antiviral system used by organisms to maintain the integrity of the genome, to defend cells against viral infection and to regulate expression of cellular genes [4]. In experimental biology, RNAi has been widely used in identification and characterization of genes [14] and inhibition of viruses [15]. In functional genomics, a large number of genes controlling cell division and metabolism have been identified by screening with RNAi in the chromosome I and III of *Caenorhabditis elegans* [16,17]. Another potential application is in the area of gene therapy [18]. In mammalian cells, dsRNA larger than 30 bp induces general non-specific suppression of gene expression by activating the antiviral interferon response [1]. This obstacle was overcome by the discovery that the effector in RNAi is a siRNA which is 21–23 nt long with 2 nt 3′-overhang and 5′-phosphate. The successful gene silencing with chemically synthesized 21–22 nt siRNA rapidly triggered its wide application in mammalian cells [1–3].

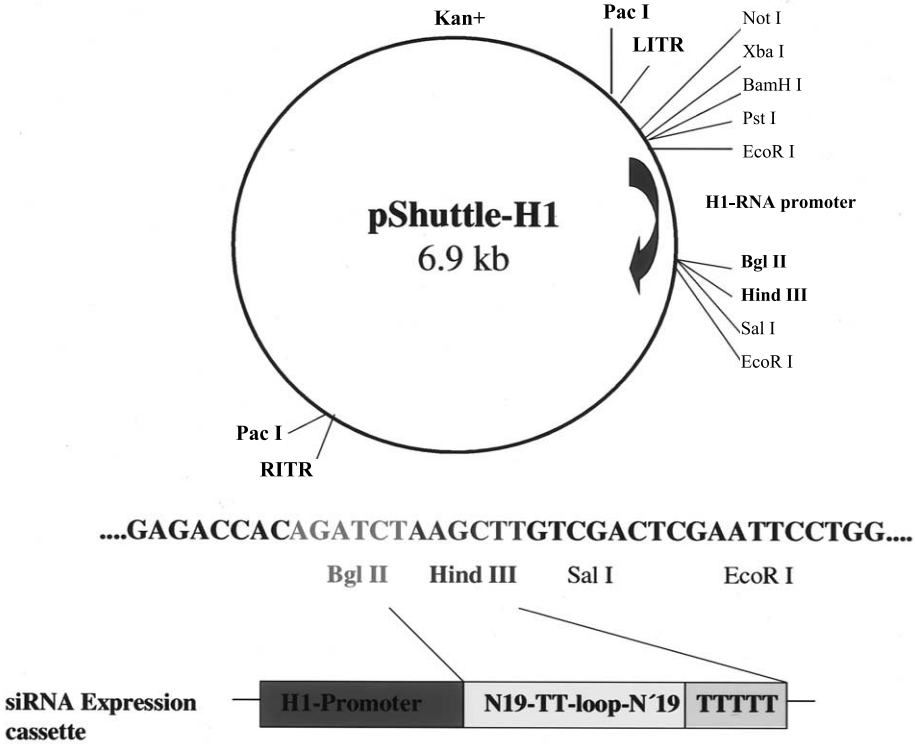

Fig. 1. Schematic diagram of siRNA expressing adenovirus shuttle vector pShuttle-H1. This vector was derived from the promoterless adenovirus shuttle vector pShuttle (Stratagene). The *Bgl*II site of the original promoterless pShuttle was converted to *Eco*RI by site-directed mutagenesis. Stem–loop producing oligonucleotides (for detail, refer to [5]) are cloned into the unique Bgl II and Hind III sites. Upon ligation, *Bgl*II site is destroyed. The insert is confirmed with *Eco*RI digestion by distinguishing the 360 bp band of a positive clone from 300 bp band of an empty vector. Polymerase III-dependent H1-RNA promoter drives the expression of 19 bp stem–9 nt loop RNA which is processed into functional siRNA by cellular enzymes.

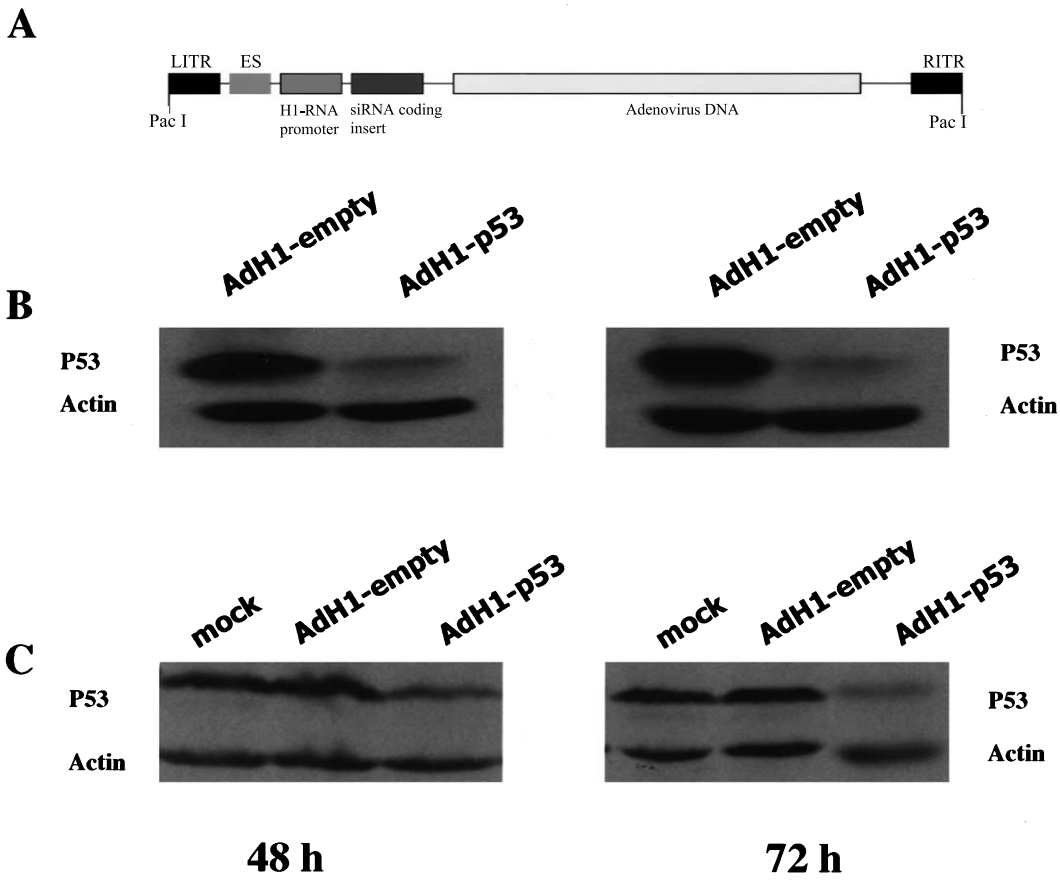

Fig. 2. Silencing p53 gene by adenovirus delivered siRNA. A: Schematic outline of a recombinant adenovirus expressing siRNA targeting p53 (AdH1-p53). The 64 nt oligonucleotide encoding human p53 siRNA was inserted into the *Bgl*II and *Hin*dIII sites of pShuttle-H1 to get pShuttle-H1-p53. The resultant plasmid was linearized with *Pme*I and cotransformed into *E. coli* BJ5183 cells with pAdEasy-1 adenoviral backbone plasmid. Recombinants (AdH1-p53) were selected for kanamycin resistance and confirmed by *Pac*I analysis. AdH1-p53 was linearized with *Pac*I and transfected into AD-293 packaging cells to produce recombinant adenovirus. B: Silencing p53 gene in MCF-7 cells by adenovirus delivered siRNA. In AdH1-p53 but not AdH1-empty infected MCF-7 cells, p53 gene was efficiently silenced both 48 and 72 h after infection. Proteins were extracted from cells and p53 levels were checked by Western blot. The lower actin bands served as internal control for equal total protein loading and specificity control. C: Silencing p53 gene in A549 cells by adenovirus delivered siRNA. In A549 cells, AdH1-p53 but not mock or AdH1-empty significantly downregulated the expression of p53 gene.

It has been demonstrated that a vector derived from polymerase III-dependent H1-RNA gene promoter can produce siRNA and cause efficient and specific downregulation of gene expression, resulting in functional inactivation of the targeted genes [5]. Almost all the elements of H1-RNA promoter are located upstream the transcribed region; it is ideally suited to the expression of ~21 nt siRNA or ~50 nt RNA stem–loops. The stem–loop precursor transcript is generated and processed to functional siRNA by cellular enzymes [5]. This small size of siRNA prevents activation of the dsRNA inducible interferon system present in mammalian cells and avoids the non-specific phenotypes normally produced by dsRNA larger than 30 bp in somatic cells.

The availability of high virus titer, infection of a broad spectrum of cell types and independence on active cell division makes adenovirus the vector of choice for siRNA delivery. The human adenovirus serotype 5 was used in our system. This is a replication defective adenovirus with deletion of E1 and E3 genes which render this virus incapable of replication itself [13]. Moreover, this system makes use of the efficient homologous recombination in *E. coli* [13], to produce recombinant adenovirus by a double-recombination event between cotransformed adenoviral backbone plasmid pAdEasy-1 and a shuttle vector pShuttle-H1.

Here, we first constructed a new adenovirus shuttle vector to express a stem–loop RNA with a 19 nt specific stem and 9 nt loop which has been shown to efficiently inactivate genes [5]. The H1-RNA promoter was used in our strategy. H1-RNA promoter was cloned into the promoterless shuttle vector pShuttle to get pShuttle-H1 which can drive the expression of siRNA in recombinant adenovirus (Fig. 1). The 64 nt oligonucleotide can be cloned into the pShuttle-H1 to express siRNAs targeting different genes. In this pilot study, we tested this system by targeting p53 gene as a model. Adenovirus expressing siRNA targeting p53 was used to infect MCF-7 and A549 cells. In AdH1-p53 but not AdH1-empty infected MCF-7 cells, p53 gene was efficiently silenced both 48 and 72 h post infection (Fig. 2B). Moreover, we also showed significant downregulation of p53 in A549 cells (Fig. 2C). It has been well documented that pSUPER-p53 expresses siRNA and efficiently knocks down p53 in MCF-7 cells by electroporation [5]. Whereas, we failed to silence p53 in MCF-7 and other cell lines by transfecting either pSUPER-53 or pShuttle-H1-p53 with help of different commercial liposomes (data not

shown). This may be due to a low and uneven transfection of plasmids in cultured cells. Consistent with the observation of several groups [10–12], viral vectors can overcome these limitations and infect cells uniformly and rapidly. Given the simplicity of this system, which employs readily available reagents, most laboratories have the ability to silence their genes by recombinant adenoviruses. The constructed pShuttle-H1 hosts unique *Bgl*I and *Hin*dIII sites for insertion of oligonucleotides encoding siRNA (Fig. 1).

In conclusion, we developed a simple siRNA delivery strategy by combination of well-defined H1-RNA promoter and conventional pAdEasy-1 adenovirus system. Our results demonstrate significant downregulation of p53 expression in mammalian cells. With availability of high titers of adenoviruses and uniform and rapid infection, this technology will have a foreseeable wide application both in experimental biology and molecular medicine.

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