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ORIGINAL PAPER

A retrovirus-based system to stably silence hepatitis B virus genes by RNA interference

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Abstract RNA interference (RNAi) might be an efficient antiviral therapy for some obstinate illness. Herein, a retrovirus-based RNAi system was developed to drive expression and delivery of Hepatitis B virus (HBV)-specific short hairpin RNA (shRNA) in HepG2 cells. The levels of HBsAg and HBeAg and that of HBV mRNA were dramatically decreased by this RNAi system in HepG2 cells transfected with Topo-HBV plasmid. Retrovirus-based RNAi thus may be useful for therapy in HBV and other viral infections and provide new clues for prophylactic vaccine development.

Keywords Hepatitis B virus · HepG2 cells · Retrovirus vector · RNA interference

Introduction

RNA interference (RNAi) is a natural process by which double-stranded RNA (dsRNA) directs sequence-specific, post-transcriptional silencing of homologous genes. The dsRNA is processed by RNase III enzyme, Dicer, into small interfering RNAs (siRNAs) approximately 21–23 bp in length. These siRNAs are incorporated into a RISC (RNA-induced silencing complex) which specifically degrades homologous mRNA (Hammond et al. 2000). Presently, RNAi-mediated gene silencing is widely used for researching gene functions in many systems including fungi, plants, Drosophila, insects, and mammalian cells (Hannon 2002). Currently, RNAi has demonstrated a powerful antiviral effect (Gitlin et al. 2002). RNAi can also induce transcriptional and replicational silencing of human pathogenic viruses in cultured cells including the poliovirus, HIV-1, HCV virus and influenza virus (Capodici et al. 2002; Ge et al. 2003; Randall et al. 2003).

RNAi can be triggered both *in vitro* and *in vivo* using synthetic siRNA or vector-based short hairpin RNA (shRNA). However, these synthetic siRNAs or most vector-based expression systems have demonstrated only transient knockdown of gene expression. Furthermore, the low and variable transfection efficiency limits the application of these siRNA. To facilitate stable, long-term gene knockdown, a suitable delivery system is required. Recently, successful gene silencing mediated by siRNA based on retroviral or lentiviral vectors was described (Barton and Medzhitov 2002; Rubinson et al. 2003). Thus,

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viral vectors seem to possess the potential to be applied in gene therapy when combined with RNAi technique.

Hepatitis B virus (HBV) infection can cause acute or chronic viral hepatitis and other liver diseases including cirrhosis and hepatocellular carcinoma. The use of interferon or nucleoside analogs such as lamivudine, entecavir and adefovir dipivoxil can partially inhibit the replication of HBV, however, HBV could not be entirely eliminated due to persistence of viral replication. Up to now, there is no effective drug to remove the virus. Therefore, the evaluation of novel treatment strategies is both urgent and necessary. Recently, several studies have shown that synthetic siRNA or the plasmid-based siRNA is able to knock down the expression of HBV (Brummelkamp et al. 2002; Giladi et al. 2003; Konishi et al. 2003; McCaffrey et al. 2003; Ren et al. 2005; Shlomai and Shaul 2003). However, most of the observations were transient. At the same time, the method of in vivo delivery and hydrodynamic injection is impractical for larger mammals. Therefore, further investigation on the stable inhibition of HBV by siRNA, is very necessary. Here, we reported a novel retrovirus-based RNAi system to target HBV gene which could mediate stable gene silencing, and substantially down-regulate HBV gene expression in human cells. This system may have the potential for therapeutic intervention in HBV and other viral infections and provides new clues for prophylactic vaccine development.

Materials and methods

Construction of the retroviral vector

According to previous studies, two siRNA duplexes targeting the surface antigen region (HBsAg)/polymerase (POL) region of the HBV genome were selected (Giladi et al. 2003; McCaffrey et al. 2003). The retrovirus vector pXSN and pXRN (a gift from Dr. DePei Liu of Peking Union Medical College, China) were derived from pLXSN and pLXRN vector (Clontech, USA) by deleting the 260 bp *Nhe*I/*Xba*I fragment of 3′LTR. The human U6 + 27 promoter from pAVU6 + 27 vector digested by *Bam*HI/*Xho*I (a gift from Dr. David R. Engelke, University of Michigan, USA) was cloned into pXSN and pXRN. Each one of the siRNAs served as a basis for the design of the two complementary siRNA template oligonucleotides. All oligonucleotides were synthesized by Takara Biotechnology Co. Ltd. The synthetic oligonucleotides were annealed and cloned down-stream of the U6 + 27 promoter to construct recombinant vectors pXSN-hU6 + 27-siHBV1, pXSN-hU6 + 27-siHBV2, pXSN-hU6 + 27-siEGFP, pXRN-hU6 + 27-siHBV1, pXRN-hU6 + 27-siHBV2 and pXRN-hU6 + 27-siEGFP and confirmed by DNA sequencing.

Cell culture

The pantropic retrovirus packaging cell line, GP-293 (Clontech, USA), and the human hepatoblastoma cell line (HepG2) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 mg streptomycin/ml and 100 U penicillin/ml at 37°C, 5% (v/v) CO_2.

Generation of recombinant retrovirus

To generate the recombinant, the packaging cells GP-293 were transfected by calcium phosphate precipitation using 5–8 μg pVSV-G (Clontech, USA) and 15 μg recombinant vectors. 48 h post-transfection, the supernatant containing recombinant retrovirus was collected, filtered through a 0.45 μm syringe filter and used to transfect target cells.

Transfection of target HepG2 cells with recombinant retrovirus

One day before transfection, 3×10^5 HepG2 cells were plated per well in six-well plates. On the following day, the virus supernatant with Polybrene (5–8 μg/ml final concentration) was added. After 24 h, the medium was replaced with fresh medium containing 800 μg geneticin/ml (G418). After 14 days, G418-resistant HepG2 cell pools were established.

PCR detecting genotypes of G418-resistant HepG2 cell pools

Genotypes of all G418-resistant HepG2 cell pools were determined by PCR analysis on genome DNA. PCR was performed using the primers: 5′-GGATCCAAGGTCGGGCAGGAAG-3′(sense), and 5′-CTTGCATGCCTGCAGGTCCTAGTA-3′ (antisense). PCR products were detected by electrophoresis on a 1.5% agarose gel.

Transfection of G418-resistant HepG2 cell pools with topo-HBV plasmid

G418-resistant HepG2 cell pools in a 6-cm plate (70% confluency) were transfected with 600 ng/ml topo-HBV plasmid DNA. After transfection, cells were incubated at 37°C in a CO_2 incubator and the medium replaced after 4–6 h. The levels of HBsAg, HBeAg and mRNAs were measured 72 h after transfection with the topo-HBV plasmid. Normal HepG2 cells transfected only with topo-HBV plasmid were constructed and used as controls.

HBsAg and HBeAg assays

The expression levels of HBsAg and HBeAg in the cultured media of normal HepG2 cells and the G418-resistant HepG2 cell pools, which were transfected with topo-HBV plasmid, were determined using ELISA. The absorbance was measured at 450/630 nm using a microplate reader.

Northern blot analysis

Total RNAs were extracted from transfected normal HepG2 cells and G418-resistant HepG2 cell pools using a Trizol reagent according to the manufacturer's instructions (Invitrogen). RNAs were separated by electrophoresis on a 1.5% agarose–formaldehyde gel and transferred to a NC^+ membrane. Blots were probed using a PCR-generated ^{32}P-labelled HBV DNA (an S fragment from topo-HBV spanning the entire HBV genome) and ^{32}P-labelled GAPDH fragments (endogenous gene, as a control) using the random primer labeling mix (TaKaRa, Japan) at 65°C overnight. After washing, the membranes were analyzed using a storage phosphor imaging system.

Results

To examine the ability of this retrovirus system to inhibit HBV gene expression we selected two siRNA duplexes targeted to the different regions of the gene for small HBV surface antigen (HBsAg)/polymerase region of the HBV genome. In this genomic region the major transcripts synthesized during HBV replication overlap and include the pre-genomic RNA, which serves both as the template for reverse transcription and the generation of viral DNA and as the mRNA for core and polymerase, and two subgenomic mRNAs, Pre-S/L and S (Fig. 1a). These two synthetic siRNAs can inhibit the expression and replication of the HBV genes (Giladi et al. 2003; McCaffrey et al. 2003). The target sequences and their positions in the HBV genome are shown in Fig. 1a.

Herein, the retrovirus expression vector pLXRN and pLXSN, containing the Neo selection marker, were used. To avoid potential transcriptional interference from the retrovirus 5′LTR promoter, the retroviral vector pLXRN and pLXSN were modified by deleting the 260 bp *Nhe*I/*Xba*I fragment in 3′LTR to generate pXRN and pXSN, respectively (Fig. 1b). This modification was expected to eliminate the 5′LTR counterpart following virus replication (Dong et al. 2000). Moreover, the RNA polymerase III promoter of human U6 + 27 small nuclear RNA gene, which was used to drive high levels of expression of a small hairpin RNA, was inserted into multiple cloning sites with the same orientation to RSV promoter-driven Neo gene transcription in pXRN or with reverse orientation to SV40 promoter-driven Neo gene transcription in pXSN (Fig. 1b). Subsequently, the annealed oligonucleotides with identical sequences to the HBV gene or EGFP gene were cloned down-stream of the U6 + 27 promoter to construct the recombinant retroviral vector, using four thymidines as the terminal signal (Fig. 1c). The recombinant retroviruses were generated by co-transfection of GP-293 cells

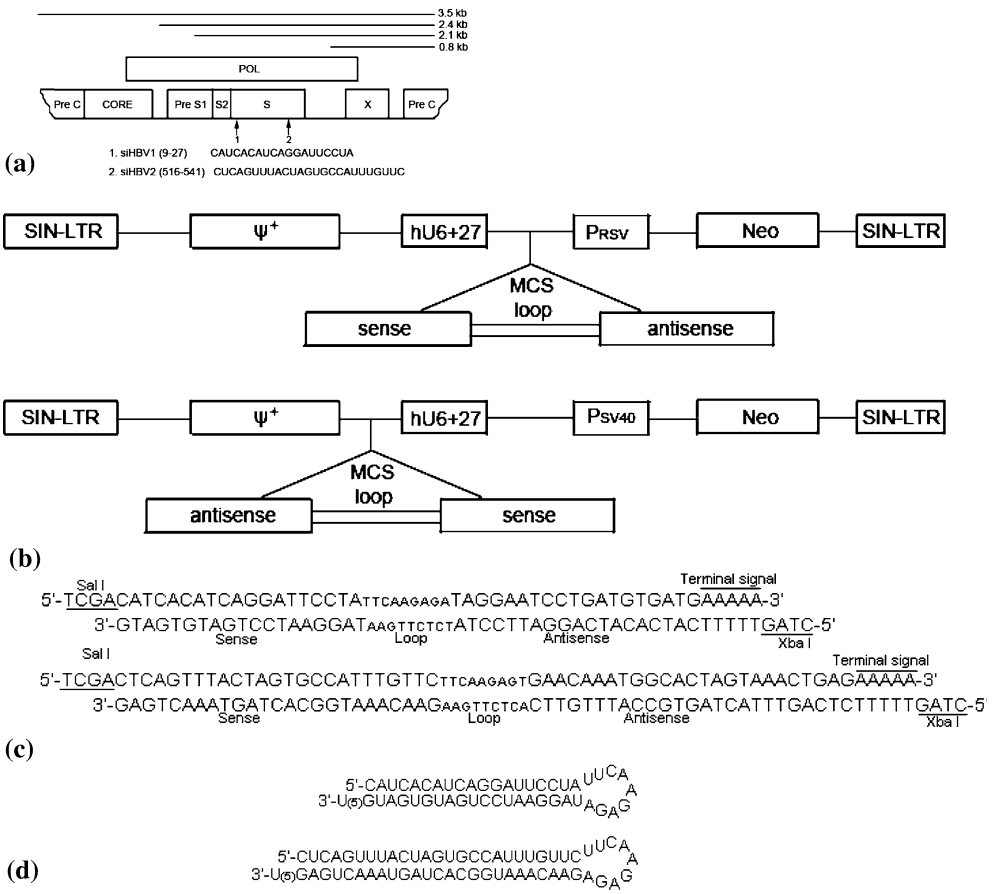

Fig. 1 Retrovirus vector-mediated RNA interference. (**a**) Schematic representation of the HBV genome with the four major transcripts depicted above and location of RNAi target sites. The 3.5 kb transcript is the pre-genomic RNA, which serves as the template for HBV viral DNA replication. The HBV open reading frames are shown below aligned with the HBV mRNAs. Pol, polymerase; CORE, HBcAg; PreS1, large pre-surface antigen; S2, middle pre-surface antigen; S, small surface antigen (HBsAg); X, X gene. The sequences of the two siRNAs (sense strand) targeted to small surface antigen/polymerase region with the distance from the translational start site of small surface antigen. (**b**) Schematic representation of the retrovirus vector pXRN-hU6 + 27 (top) and pXSN-hU6 + 27 (below). ψ+, extended packaging signal; SIN, self-inactivated; LTR, long terminal repeat; MCS, multiple cloning sites; P_{RSV}, RSV promoter; P_{SV40}, SV40 promoter; Neo, neomycin phosphotransferase gene. (**c**) The sequence of siHBV1 (top) and siHBV2 (below) template oligonucleotides. (**d**) The predicted structure of shRNA: shHBV1 (top) generated from pXRN-hU6 + 27-siHBV1 and pXSN-hU6 + 27-siHBV1; shHBV2 (below) generated from pXRN-hU6 + 27-siHBV2 and pXSN-hU6 + 27-siHBV2

with the recombinant retroviral vectors pXRN-hU6 + 27-siHBV1, pXRN-hU6 ;+ 27-siHBV2, pXSN-hU6 + 27-siHBV1, pXSN-hU6 + 27-siHB V2, pXRN-hU6 + 27-siEGFP, pXSN -hU6 + 27-siEGFP and the envelop plasmid pVSV-G. G418-resistant HepG2 cell pools were established following transfection with recombinant retrovirus and selection with G418. The shRNA, a tight hairpin turn, which can be digested by Dicer to siRNA which directs cleavage of the cognate mRNA, was expressed in transfected HepG2 cells. The predicted hairpin RNA structure (shRNA) is shown in Fig. 1d. The PCR results demonstrated that the target fragments were integrated into the G418-resistant HepG2 cell pools (Fig. 2). Finally, the topo-HBV plasmid, which consists of a head-to-tail dimer of the wild-type HBV genome, was transfected into the

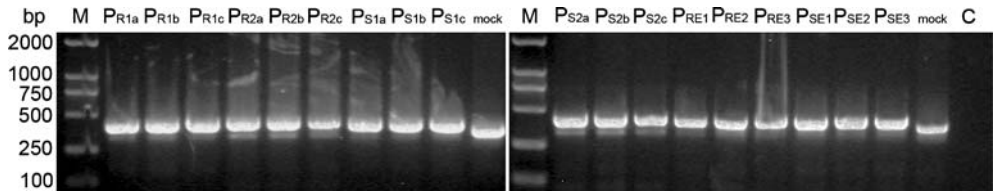

Fig. 2 PCR for detecting the integrated target fragments. Results demonstrated that the target fragments have been integrated into the G418-resistant HepG2 cell pools. P_{R1}, P_{R2}, P_{S1}, P_{S2}, P_{RE} and P_{SE} represent the cell pools which were integrated with pXRN-hU6 + 27-siHBV1 (409 bp), pXRN-hU6 + 27-siHBV2 (421 bp), pXSN-hU6 + 27-siHBV1 (409 bp), pXSN-hU6 + 27-siHBV2 (421 bp), pXRN-hU6 + 27-siEGFP (409 bp), pXSN-hU6 + 27-siEGFP (409 bp) vector respectively. Mock: the HepG2 cells were integrated with the pXRN-hU6 + 27 vector (353 bp); C: mixed HepG2 cells were not integrated with any vector; M: Marker (DL-2000)

cell pools and resulted in the production of HBV-related antigens and replicative intermediates.

The ELISA results showed that HepG2 cell pools co-transfected with topo-HBV plasmid and pXRN-hU6 + 27-siHBV1, pXRN-hU6 + 27-siHBV2, pXSN-hU6 + 27-siHBV1or pXSN-hU6 + 27-siHBV2, respectively demonstrated more than 90% decrease of HBsAg and HBeAg, while little decrease can be detected in cell pools co-transfected with topo-HBV plasmid and pXRN-hU6 + 27-siEGFP or pXSN-hU6 + 27-siEGFP (Fig. 3a, b). These results indicated that the inhibition of gene expression, based on the retrovirus-delivery shRNAs, was distinct and sequence specific.

To further confirm that the effect of retrovirus-based delivery of shRNAs on HBsAg and HBeAg expression was caused by a decrease in HBV mRNA levels, Northern blot analysis was performed using total RNAs extracted from the HepG2 cell pools 72 h later transfection with the topo-HBV plasmid. GAPDH was used as the control. The result is shown in Fig. 4. A 3.5 kb pre-genomic RNA encoding the viral core and polymerase protein and RNAs of 2.4 kb and 2.1 kb encoding the viral envelope protein, were observed in the HepG2 cells only transfected with topo-HBV plasmid, however, the transcript encoding the X protein was not observed, which is consistent with results from previous study (McCaffrey et al. 2003). The levels of the HBV 3.5 kb and the 2.4/2.1 kb mRNA species, compare with the control, were clearly decreased in the cells pools co-tranfected topo-HBV plasmid with

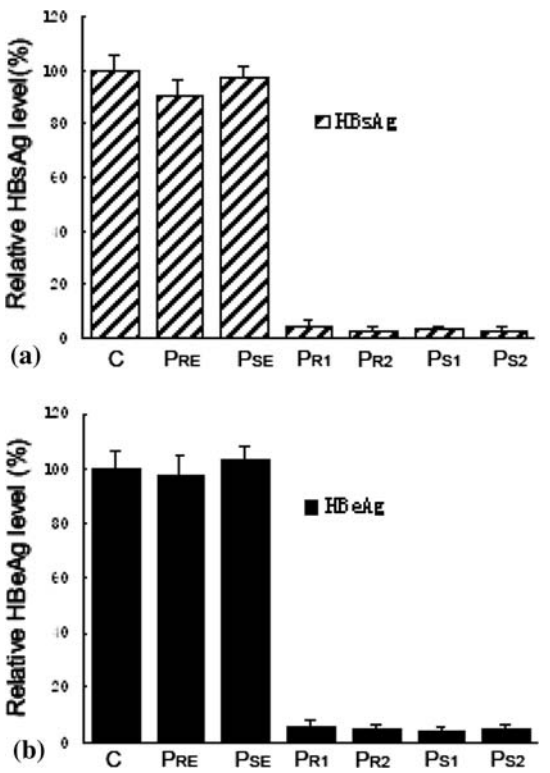

Fig. 3 Inhibition of HBsAg and HBeAg by RNAi based on retrovirus delivery in HepG2 cell pools. The amount of HBsAg (Fig. 3a) and HBeAg (Fig. 3b) in the culture medium was measured by ELISA and is presented as a percentage of the amount secreted by mixed cell pools (control). C: mixed HepG2 cells only transfected with topo-HBV plasmid; P_{RE}, P_{SE}, P_{R1}, P_{R2}, P_{S1}, P_{S2}, represents the HepG2 cell pools which were integrated with the pXRN-hU6 + 27-siEGFP, pXSN-hU6 + 27-siEGFP, pXRN-hU6 + 27-siHBV1, pXRN-hU6 + 27-siHBV2, pXSN-hU6 + 27-siHBV1, or pXSN-hU6 + 27-siHBV2 respectively and then transfected with topo-HBV plasmid. The data represented mean values ± SD based on three independent experiments

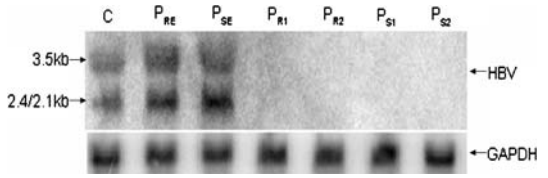

Fig. 4 Specific inhibition of HBV mRNA by RNAi based on retrovirus delivery in HepG2 cell pools. HepG2 cell pools were harvested and total RNA were extracted. The amount of 3.5 kb and 2.4/2.1 kb HBV mRNA was determined by Northern blot analysis. C: mixed HepG2 cells only transfected with topo-HBV plasmid; P_{RE}, P_{SE}, P_{R1}, P_{R2}, P_{S1}, P_{S2}, represents the cell pools which were integrated with the pXRN-hU6 + 27-siEGFP, pXSN-hU6 + 27-siEGFP, pXRN-hU6 + 27-siHBV1, pXRN-hU6 + 27-siHBV2, pXSN-hU6 + 27-siHBV1 or pXSN-hU6 + 27-siHBV2 respectively and then transfected with topo-HBV plasmid

pXRN-hU6 + 27-siHBV1, pXRN-hU6 + 27-siHBV2, pXSN-hU6 + 27-siHBV1, pXSN-hU6 + 27-siHBV2 respectively, and the inhibition levels with pXRN-hU6 + 27-siHBV1, pXRN-hU6 + 27-siHBV2 were similar to pXSN-hU6 + 27-siHBV1, and pXSN-hU6 + 27-siHBV2.

These results indicate that the specific retrovirus delivery-based shRNAs disrupted the viral mRNA with the aid of Dicer and the degradation of mRNA was only associated with the hU6 + 27-siRNA expression cassette while was unrelated to the retrovirus vector. These further confirmed that in cultured cells the inhibition of HBsAg and HBeAg expression was caused by major viral mRNA species degradation mediated by shRNAs.

Discussion

The replication characteristics of HBV make it an ideal target for gene therapy using RNAi. First, the virus is a pararetrovirus, replicating via an intermediate pre-genomic RNA molecule, which is directly susceptible to RNAi. Second, the genome is compact, containing overlapping open reading frames, which allows the targeting of one site to inhibit multiple HBV mRNAs. Third, the virus replicates almost exclusively in the liver, an organ that has proved to be accessible to in vivo gene therapy delivery techniques. Thus, we developed this novel retrovirus-based RNAi system which can generate shRNA specially targeting HBV genes.

SiRNAs, which were chemically or enzymatically synthesized in vitro or expressed from a vector in vivo and delivered to target cells by physical transfection methods, have low and variable transfection efficiency and only suppress gene expression transiently, limiting the application of siRNAs in long-term gene silencing in mammalian systems. Herein, an appropriate siRNA delivery strategy by the combination of the human U6-RNA Pol III promoter and the conventional retrovirus expression vector was developed to generate shRNA. To our knowledge, this is the first report to utilize this retrovirus expression system containing a selection marker and the human U6-RNA Pol III promoter to deliver shRNA into HepG2 cells for silencing HBV genes. This retrovirus-based RNAi system possesses several advantages. The retrovirus vectors pXSN and pXRN, containing an antibiotic selection marker, are able to be integrated into the host genome enabling the stable expression of shRNA and are convenient for detecting the positive cell pools. In our system, the constructed retrovirus is non-pathogenic for human health. Further, the human U6-RNA Pol III promoter, which is small, simple, and easily inserted into viral vectors, can drive the high level expression of shRNA and, in turn, mediate highly efficacious silence (Bridge et al. 2003). Additionally, recombinant retrovirus pseudotyped with VSV-G can be concentrated by ultra-centrifugation, and thus, may be directly used in ex vivo and in vivo studies. Data from our experiments indicated that the retrovirus-mediated RNAi delivery system was an effective and long-term approach to express shRNA targeted to HBV and could suppress HBV expression in vitro.

In this study, we established stable HepG2 cell pools integrated with HBV-specific shRNA expression cassette. After transfecting with foreign HBV plasmid, we found that foreign transfected HBV genes could be efficiently silenced, which provides new clues for the development of new prophylactic HBV vaccines. In addition, this RNAi system might be applicable as an efficient

therapeutic strategy for chronic HBV infections due to its potential advantageous effects in HBV therapy. Firstly, this retrovirus system extends the expression of shRNA, which is essential for ensuring its delivery to every infected cell in the body and eradicating HBV. Secondly, interference in the targeted gene appears effective and specific. Thirdly, this system minimizes the side effects related to those observed with conventional drug therapies. In summary, the magnitude suppression obtained in our experiment emphasized the potential of this RNAi approach for preventing and treating chronic HBV and other persistent infections such as HCV and HIV. However, in relation to clinical application, more suitable methods should explore to avoid disease in patients such as previous deliveries have caused by promoter-insertion activation of host genes. Although much research is required before traversing from the usage of RNAi in vitro to the administration of RNAi in the human body, such as delivery to the specific organs and safety introduction of exogenous DNA, the therapeutic potential of siRNAs remains promising.

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