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*Review*

# Application of short hairpin RNAs (shRNAs) to study gene function in mammalian systems

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**Over the past decade, RNA interference (RNAi) plays an important role in biology, especially for silencing gene expression. RNA interference (RNAi) is a natural process through which expression of a targeted gene can be knocked down with high specificity and selectivity. Methods of mediating the RNAi effect involve small interfering RNA (siRNA) and short hairpin RNA (shRNA). In various applications, RNAi has been used to create model systems, to identify novel molecular targets, to study gene function in a genome-wide fashion, and to create new avenues for clinical therapeutics. This article reviews the current knowledge on the mechanism and applications of shRNA in mammalian and human cells.**

**Key word:** shRNA, siRNA, RNAi, dicer, gene silencing.

## INTRODUCTION

In the last few years, RNA interference (RNAi) has become an important topic in biological research, and is predicted one of the great achievements in the next decades. RNAi is a method based on post-transcriptional gene silencing (PTGS) mechanism which is initiated by double strands RNA (>30nt), small interfering RNA or short hairpin RNA (shRNA) (<30nt). PTGS was first described in plants, when attempts to increase chalcone synthase expression in petunias result in the effective suppression of both the endogenous gene as well as introduced gene, which was called “cosuppression” (Napoli et al., 1990). After that, Guo used antisense strand RNA which is complementary to mRNA to suppress the expression of specific gene in *Caenorhabditis elegans*, meanwhile, injecting sense strand RNA also produced the same results of specific-sequence silencing (Guo and Kemphues, 1995). It was finally interpreted in 1998 by Fire in the nematode worm *C. elegans* that dsRNA was substantially more effective than ssRNA in gene silencing and it was named “RNA interference” (Fire et al., 1998). Later, the RNAi phenolmenon was found in a wide variety of organisms, such as *Drosophila* (Hammond et al., 2000; Misquitta and Paterson, 1999), planaria (Sanchez and Newmark, 1998), trypanosomes (Ngo et al., 1998), hydra (Lohmann et al., 1999), zebrafish (Wargelius et al., 1999) and also mammals (Elbashir et al., 2001).

RNAi can serve as a powerful tool for inhibiting gene expression with dsRNA; however, there are some obstacles in most mammalian cells which are complicated by nonspecific gene. This happens because introduction of long dsRNA (>30nt) activates a potent antiviral response (Manche et al., 1992; Minks et al., 1979; Williams, 1997). In addition, Manche showed that activated PKR results in a generalized repression of translation (Manche et al., 1992), and RNase L also leads nonspecific gene silencing (Minks et al., 1979). The enzyme dsRNA-dependent protein kinase(PKR) is activated when binding to dsRNA, while sequence-independent induces the repression of protein synthesis (Williams, 1997). Therefore, the application of siRNAs (<30nt) achieved in mammalian cells at last mimicks the initiator step of the

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**Abbreviations: RNAi,** RNA interference; **PTGS,** post-transcriptional gene silencing; **RNase,** ribonuclease; **RISC,** RNA induced silencing complex; **siRNAs,** small interfering RNAs; **IFN,** interferon; **shRNA,** small hairpin RNA; **HBV,** hepatitis B virus; **HCV,** hepatitis C virus; **RSV,** respiratory syncytial virus; **HIV,** human immunodeficiency virus; **HAART,** highly active antiretroviral therapies.

RNAi mechanism (Elbashir et al., 2001). This siRNAs avoid the antiviral response in mammalian cells.

Originally, the chemically synthesized siRNAs were used in experiments effectively, though costly. In order to reduce the cost, many researchers designed plasmid expression vectors to generate sustained production of siRNA (Vermeulen et al., 2005; Hannon and Rossi, 2004; Bartel, 2004; Liu et al., 2003). The majority of siRNAs expression vector rely on one promoter of RNA polymerase III to control the expression of short hairpin RNA (shRNA) in mammalian cells. This promoter contains the U6 snRNA promoter of man and mice, and H1 RNA promoter of RnaseP (Zamore et al., 2000; Zhang et al., 2002; Myers et al., 2003). These vectors contain a DNA sequence that encodes the shRNA cloned between the promoter and a transcription terminated at position 2 of the termination site and then folds into a stem-loop structure with 3'UU-overhangs. In most cases, pol III promoter was used to direct the expression of shRNA more effectively. This pol III promoter contains all necessary components of RNA upstream and terminates in a short of thymidine gather. Once shRNA is expressed, it is shifted in the cytoplasm and processed to siRNA by Dicer (Vermeulen et al., 2005).

## DICER SPECIFICITY AND EFFICIENCY

At a key stage of the RNAi pathway, the ribonuclease (RNase) III-like enzyme Dicer, processes long double-stranded RNA dsRNA) or pre-microRNA hairpin precursors into small interfering RNAs (siRNAs) or microRNA (miRNAs) (Hannon and Rossi, 2004; Bartel, 2004). These short RNAs are then loaded into the RNA induced silencing complex (RISC) through the action of R2D2 and Dicer (Liu et al., 2003). The 21–23-nt products of Dicer digestion contain characteristic 2-nt 3_ overhangs and act as the functional intermediates of RNAi directing mRNA cleavage and translational attenuation. Awareness of these new features of Dicer cleavage specificity as it is related to siRNA functionality provides a more detailed understanding of the RNAi mechanism and can shape the development of hairpins with enhanced functionality. While details of Dicer's functional domains are being elucidated, less is known about the contributions that the dsRNA substrate makes to position and efficiency of Dicer cleavage. Dicer generates 20 base-pair (bp) siRNAs by entering the duplex from the termini (Zamore et al., 2000; Zhang et al., 2002) and cleaves both short (30 nt) and long (130 nt) dsRNA molecules with equal efficiency (Elbashir et al., 2001; Myers et al., 2003). In contrast, smaller duplexes (21 nt siRNAs) do not appear to bind Dicer *in vitro* (Provost et al., 2002). While substrates with 3_ overhangs are more efficiently processed than blunt ended molecules, the state of 5_ phosphorylation of the duplex does not play a role in efficiency (Zhang et al., 2002; Zhang et al., 2004).

Cleavage studies of substrates with varying overhang lengths revealed additional contributions of the substrate to Dicer specificity. It has been observed that as the length of the overhang increases from 1 to 3 nt, the position of the preferred Dicer cleavage site shifts (Vermeulen et al., 2005). In contrast, as overhang length increased beyond 3 nt, the position of cleavage on the complementary strand remained constant (that is substrates with 3-nt, 4-nt, and 5-nt overhangs show roughly equivalent cleavage site preferences). These observations lend to the hypothesis that when a molecule is blunt or when an overhang of 1–3 nt is present, Dicer recognizes the 3'-end of the molecule and cuts at a fixed distance from the end. When that overhang length is exceeded, the rules governing PAZ domain recognition are altered and other interactions (presumably mediated through the dsRBD) play a more dominant role in determining how Dicer orients itself (Vermeulen et al., 2005). The polymerase III promoter/terminator is often used in vector-based shRNA expression design (Brummelkamp et al., 2002) and results in an shRNA containing a variable 3'-end of approximately 1–4 nt (Bogenhagen and Brown, 1981; Miyagishi and Taira, 2002). As shown in these studies, 3'-overhang lengths of 1–3 nt generate overlapping yet distinctly different preferred products; the use of current expression systems may lead to a range of desirable and undesirable cleavage products and impaired silencing.

## MECHANISM OF shRNA INTERFERENCE

Until now several studies have shed light on the underlying mechanisms of how RNAi works. The current model of the RNAi mechanism includes both initiator and effector steps (Provost et al., 2002; Zhang et al., 2004; Brummelkamp et al., 2002; Bogenhagen and Brown, 1981). First, the dsRNAs get processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effecter step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand. RISC is activated by the ATP-dependent unwinding of the siRNA duplex. Activated RISC splices the target mRNA at the site of homologous sequence (Elbashir et al., 2001; Manche et al., 1992). Then the antisense strand of the siRNA guides cleavage of targeted mRNAs. Activate RISC through base pairing mRNA homologous position to the transcription, and cleave the mRNA at 12 nt from 3' position of siRNA. Finally, the degradation of mRNA results in the target gene silencing (Zhang et al., 2004; Brummelkamp et al., 2002; Bogenhagen and

Brown, 1981; Miyagishi and Taira, 2002).

## ESTABLISHING RNA INTERFERENCE IN MAMMALIAN SYSTEMS

RNAi induced by long dsRNA has been used to study gene function in plants, worms and *Drosophila.* In particular, studies in *C. elegans* and *Drosophila* have shown that using dsRNA to induce RNAi is effective in generating models for the analysis of genes involved in cell division or development (Gonczy et al., 1999; Jantsch-Plunger and Glotzer, 1999; Kennerdell and Carthew, 1998). However, when dsRNAs more than 30 bp in length were used to study mammalian gene function, it was noted that there was an inhibition of protein translation within the cell due to the activation of the interferon (IFN) system (one of the body's defense against viral infection) (Provost et al., 2002). Over 100 cellular genes can be activated by IFNs and some encode enzymes that are dsRNA-binding proteins. This includes protein kinase R (PKR) which phosphorylates and inactivates eukaryotic initiation factor 2 and hence inhibits mRNA translation (Manche et al., 1992). Therefore, to apply RNAi technology to studies using mammalian systems (without inducing an IFN response), the silencing pathway had to be induced without the use of long dsRNA. Since synthetic 21–22 bp-long siRNAs could bypass the initial Dicer step while retaining the ability to mediate gene silencing in *Drosophila*, they were used in studies using mammalian cells.

A number of groups have designed plasmid expression vectors to generate sustained production of siRNAs by transient or stable transfection. Some of these vectors have been engineered to express small hairpin RNAs, which are processed *in vivo* into siRNA-like molecules capable of carrying out gene-specific silencing (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Yu et al., 2002). After construction is complete, these vectors contain a DNA sequence that encodes the shRNA cloned between a Pol III promoter and a transcription termination site comprising 4–5 thymidine residues. The transcript is terminated at position 2 of the termination site and then folds into a stem-loop structure with 3' UU-overhangs. The ends of the shRNAs are processed *in vivo*, converting the shRNA into ~21 nt siRNA-like molecules, which in turn initiate RNAi (Brummelkamp et al., 2002). These vectors represent a definite improvement in initiating RNAi in cells; however not all cell types are easy to transfect using these vectors.

## KNOCKOUT RNAi SYSTEM

With further understanding of RNAi mechanism, shRNAs has been used as "knockout RNAi system" for the purpose of silencing specific homologous mRNA expression in mammalian cells. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated (Voorhoeve and Agami, 2003). The knockout RNAi systems allow you to quickly express functional hairpin siRNA molecules in mammalian cells for the purpose of silencing target genes. These systems include RNAi-Ready pSIREN Vectors that use the cell's own RNA Polymerase III (Pol III) to transcribe a specifically designed small hairpin RNA (shRNA) using the human U6 promoter. The human U6 promoter provides a high level of expression in many cell types (Kunkel and Pederson, 1989), resulting in target gene suppression.

shRNA oligonucleotide design describes the process of identifying target sequences within a gene of interest and designing the corresponding oligonucleotides to generate the shRNA. This could be achieved by (i) Selecting a region of 19 nt; do not choose the region near the start codon (within 75 bases), nor untranslated regions (Elbashir et al., 2001). (ii) Avoid selecting target sense or antisense sequences that contain a consecutive run of 3 or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination of the shRNA transcript. (iii) Ensuring the content of GC of the 19-bases oligonucleotide between 40-60%, and a GC content of approximately 45% is ideal. (iv) Examining the 19-bases oligonucleotide for secondary structure and long base runs, both of which can interfere with the process of annealing. The 19-base oligonucleotide is specific gene of the interest, without homology to other genes. (v) With 7-9 nt hairpin loop sequence between sense and antisense strand (Vermeulen et al., 2005; Hannon and Rossi, 2004; Bartel, 2004; Gonczy et al., 1999; Jantsch-Plunger and Glotzer, 1999), the most effective sequence is 5'-TTCAAGAGA-3' after comparison of different length and sequence of stem-loop. The end of two complementary oligonucleotides must have the restriction site. 5-6 T must be placed at the end of shRNA to ensure the termination of RNA polymerase III transcription (Figure 1).

Figure 1 shows the generated shRNA using an oligonucleotide DNA sequence. The hairpin loop sequence is located between the sense sequence and antisense sequence on the complementary DNA strand. ShRNA includes two short reverse-sequences, having a stem-loop sequence at the middle, with composition of the hairpin structure, controlled by poly promoter. Then it ends in the 5-6 T as RNA polymerase transcription terminator.

## APPLICATION OF shRNA INTERFERENCE

The studies on shRNA have been performed on an increasingly routine basis. The innovation of RNAi has

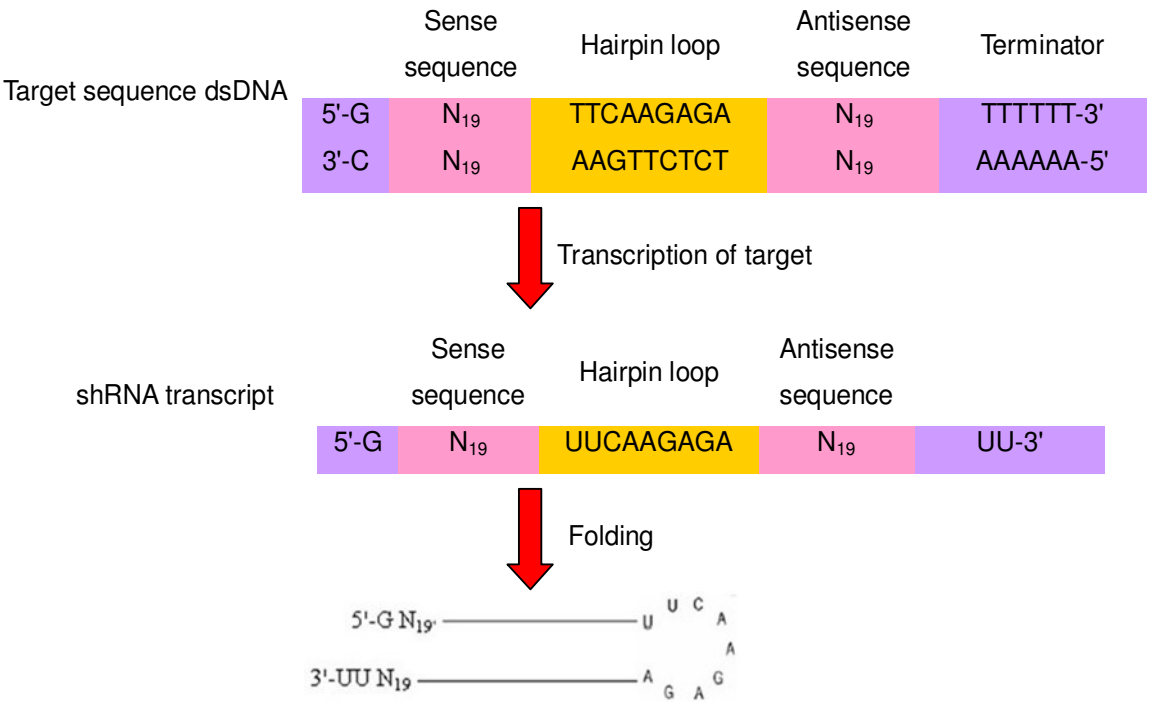


**Figure 1.** Generation of small hairpin RNAs.

revolutionized the study of gene function in model organisms and promises to permit large-scale loss-of-function studies in mammals. Because of the potential effectiveness of RNAi, it has become the methodology and technology for silencing specific gene expression in mammalian cells. The RNAi machinery can recognize invading double-stranded viral RNA (or the double-stranded replicative form of the viral RNA) and suppress the infection by degradation of the RNA. The RNAi system thus shares important features with the vertebrate immune system: it recognizes the invading parasite (dsRNA) raises an initial response and subsequently amplifies the response to eliminate the foreign element. The application of RNAi technology from genomics research has been gradually expanded to the medical field and as a means of gene therapy. Utilize RNAi not only provide an economic fortunes but also open up a new thinking with the gene function measurement and gene therapy. The application of RNAi using shRNA *in vivo* is now used in mice commonly. Despite the problems of delivery, RNA interference has been used effectively in the mouse to block expression of a hepatitis C virus protein in the liver (McCaffrey et al., 2002). In addition, the same group has used specific RNA interference to block hepatitis B virus infection in mice (McCaffrey et al., 2003). They achieved delivery by injecting large amounts of synthetic double stranded RNA or DNA encoding a short hairpin RNA into the portal vein. This is a transient silencing of sequences, and was done by injecting shRNA into high-pressure tail vein (Grimm et al., 2006). Grimm et al. (2006) have shown that high expression of shRNAs can result in high lethality of mice. It is demonstrated that the endogenous miRNAs can be disrupted by high shRNA expression not only in cell culture, but also *in vivo* (Grimm et al., 2006). The expression of shRNAs can induce an interferon response (Bridge et al., 2003). When human embryonic kidney 293 (HEK293) or keratinocyte (HaCaT) cell lines or human primary dendritic cells or macrophages were transfected with siRNA or shRNAs, suppression of non-targeted mRNA expression was detected (Kariko et al., 2004).

In recent years, therapies based on RNAi are being used to attenuate viral infection in cell culture or animal experiments, which include foot-and-mouth disease virus, influenza virus, hepatitis B (HBV) and C virus (HCV), respiratory syncytial virus (RSV) and human immuno-deficiency virus (HIV). RNAi is well suited as an antiviral therapy for numerous reasons. The majority of anti-HIV small molecule drugs used in highly active antiretroviral therapies (HAART) focus on one of two viral enzymes, reverse transcriptase and protease (Clavel and Hance, 2004). In addition, the RNAi-inducing nucleic acids must be delivered within a therapeutic window of dosage, as dose-dependence has been observed both *in vitro* and *in vivo*. Lentiviral vector delivery of shRNA-expressing construct progressively knocked down target gene expression in single cells with increasing viral dosage (Abbas-Terki et al., 2002).

Strategies based on RNAi have been introduced to treat different kinds of liver diseases. Especially hepatitis, such as HBV and HCV, both of them increased the chronic liver disease and hepatocellular carcinoma in

humans. Recent researchers focused on therapies using RNAi to treat this disease. Transfecting an HBV plasmid expressing shRNAs in cell and mice can induce RNAi reponse, that HBV replication can be inhibited (McCaffrey et al., 2003). A similar approach was taken to target the Fas protein, an important inducer of programmed cell death, resulting in protection of mice from fulminant hepatitis caused by injection with agonistic Fas-specific antibodies (Song et al., 2003). The advantages of shRNA expression vector can be a long-term study. Many researchers have shown that the vector carried with antibiotics marked in the cells can sustain inhibition gene expression for weeks or longer. shRNA can be engineered to suppress the expression of desired genes in cell stability (Paddison et al., 2002).

Initial research on RNAi using shRNA *in vivo* was plasmid DNA (Lewis et al., 2002). With the advancement of modem science and technology, researchers have devoted to the use of viral vectors. Choosing the viral system depends on the type of cell and the need of short-term or long-term express shRNA. Citing two different response-time requirements: Short-term expression is used for adenovirus (AV) and herpes simplex viral vector system mainly while long-term expression is used for adeno-associated viral (AAV) vector systems frequently. A large number of RNAi-based screening systems have been described in recent years (Song et al., 2003; Paddison et al., 2002; Lewis et al., 2002; Mullenders et al., 2009).This system uses external shRNAs into viral vector plasmid, which could be activated by RNA polymerase III promoters .

The shRNAs produced a wide range of clinical effects, ranging from benign to malignant tumours, the severity and type of which correlated with the extent to which the shRNA had silenced p53. An shRNA barcode technique was used to identify novel genes which regulate p53 function from a complex mixture (Mullenders et al., 2009). In addition, novel tumor suppressors were also identified using shRNAs that transformed human primary cells (Kolfschoten et al., 2005). Recently, researchers developed a vector that mediates suppression of gene expression through RNA interference. Here, they used a retroviral version of this vector to specifically and stably inhibit expression of only the oncogenic *K-RAS*$^{V12}$ allele in human tumor cells (Brummelkamp et al., 2002). It is found that shRNA expressed from viral vectors can be used to mediate RNA interference to induce persistent loss-of–function phenotypes. Then, also a test was conducted on the tumorigenic capacity of CAPAN-1 cell *in vivo*, by injecting subcutaneously in mice. These proved the ability of RNAi-mediated silencing to suppress tumor formation *in vivo* (Brummelkamp et al., 2002).

Libraries of RNA interference molecules have been constructed that allow the analysis of gene function on a genome-wide scale. One group screened pools consist of retroviruses and identified components of the p53 pathway using large libraries of shRNA-expressing retroviral vectors (Berns et al., 2004). Another group was screened by transfecting cells with shRNA-expressing retroviral plasmids and identified genes involved in proteasome function (Silva et al., 2005). Recently, the same two libraries were used to identify two novel tumor-suppressor genes (Westbrook et al., 2005). While these reports establish the precedent that shRNA libraries can be employed to perform loss-of-function screens in mammalian cells, it is clear that further exploration of the performance characteristics and limitations of such approaches is necessary before such large-scale applications become routine.

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## CONCLUSION

RNA interference, the biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA for degradation, is revolutionizing the way researchers study gene function. For the first time, scientists can quickly and easily reduce the expression of a particular gene in nearly all metazoan systems, often by 90% or greater, to analyze the effect that gene has on cellular function. In recent years, scientists have co-opted this biological process to reduce the expression of target mRNAs by using exogenously applied siRNAs and shRNAs. Aside from the widespread basic biological applications of RNAi, the ability to reduce gene expression marks a major advance toward the development of disease therapies, particularly for dominantly inherited disorders.

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