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REVIEW

RNA interference-based therapeutics for shrimp viral diseases

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ABSTRACT: RNA interference (RNAi) has emerged as a powerful tool to manipulate gene expression in the laboratory. The presence of a double-stranded RNA (dsRNA) in eukaryotic cells triggers this post-transcriptional gene-silencing mechanism, leading to a sequence-specific degradation of the target mRNA. Among its many potential biomedical applications, silencing of viral genes stands out as a promising therapeutic strategy. Marine shrimp viral diseases, especially white spot disease (WSD), represents one of the most attractive targets for the development of therapeutic RNAi owing to its widespread economic impact. This review summarizes the current knowledge in the therapeutic application of RNAi for combating viral diseases in shrimp. The basic principles of RNAi are described, focusing on features important for its therapeutic manipulation. Subsequently, a stepwise strategy for the development of therapeutic RNAi is presented.

KEY WORDS: RNAi therapy · Shrimp diseases · Small interfering RNA · siRNA · Long hairpin RNA · lhRNA

INTRODUCTION

RNA interference (RNAi) is rapidly becoming a powerful tool for gene silencing (Voorhoeve & Agami 2003). Even though the mechanism by which RNAi operates is not yet completely understood, many researchers are taking advantage of this phenomenon in different ways. The rapid production of knockdown animals where genes of interest have been selectively silenced is just one of many possible uses for RNAi (Voorhoeve & Agami 2003). Another obvious use for RNAi is the selective silencing of viral genes essential for virulence (Shuey et al. 2002). This application would be particularly useful for developing effective viral vaccines for use among economically important invertebrate species which do not possess an adaptive immune system. This review summarizes the current status and future strategies concerning the therapeutic application of RNAi to combat viral diseases in shrimp.

Viral diseases in shrimp

Farm-raised shrimp account for 3 to 4% of global aquaculture production by weight and 15% by value (Rönnbäck 2001) with export earnings in the order of billions of US dollars per year. In the early 1990s, there was a series of viral outbreaks in various parts of the shrimp-producing world that crippled shrimp farmers across the globe. Lundin (1996) estimated that around 40% of the worldwide shrimp production, representing a value over $3 billion, was lost due to infectious diseases. The main contributors to these losses are viral diseases. Of the ~20 shrimp viruses known today, 6 are especially important due to their epizootic spread and economic impact: monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), Taura syndrome virus (TSV), yellow head virus (YHV), monodon slow growth syndrome (MSGS) and white spot syndrome virus (WSSV). All these viruses, with the

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exception of MSGS, are classified as OIE notifiable (OIE 2006). Among these, WSSV has had the greatest impact on shrimp farming and is currently considered the most important shrimp disease in terms of distribution and economic losses.

Current therapeutic strategies

Managing viral diseases has been the greatest challenge to the shrimp industry because short-term strategies, such as the use of immunostimulants, bioremediators and probiotics, have their own limitations in terms of their efficacy, practicability, cost, and, above all, reproducibility. Unlike finfishes, the shrimps lack a developed adaptive immune system, which is a prerequisite for the development of any protein vaccines. Subunit vaccines (Witteveldt et al. 2004a,b, 2006) have been reported to provide some degree of protection to shrimps from viral infection, but their field level potential is yet to be seen. In this scenario, RNAi, which seems to offer great promise in terms of treating human and animal diseases, is looked upon with great hope by shrimp health managers.

ENDOGENOUS RNAi PATHWAY

Observations were made in plants (Napoli et al. 1990, van der Krol et al. 1990) and nematodes (Lee et al. 1993),which hinted at the existence of the RNAi pathway years before double-stranded RNA (dsRNA) molecules were identified as the key component of this evolutionarily conserved post-transcriptional silencing pathway in eukaryotic cells (Fire et al. 1998). Since then, we have witnessed significant advances in the understanding of how RNAi functions and identified several effective ways to manipulate it in the laboratory. Although a detailed account on the mechanistic aspects of RNAi is beyond the scope of this review and can be found elsewhere (Bartel 2004, Carmell & Hannon 2004, Cullen 2004, Meister & Tuschl 2004, Murchison & Hannon 2004, Du & Zamore 2005, Kim 2005, Tomari & Zamore 2005a,b), a brief description follows on our current knowledge of how RNAi operates, focusing on features that are important for the design of RNAi-based therapeutic molecules.

Basic mechanism

RNAi is a naturally occurring, post-transcriptional process by which dsRNA induces degradation of homologous mRNA transcripts (Fig. 1). The process is initially triggered when dsRNAs are expressed or introduced into a cell. Upon entering the cytoplasm, they act as substrate for the multidomain ribonuclease III enzyme, Dicer (Hammond et al. 2000), which cleaves dsRNA into 21 to 23 nucleotide (nt) fragments with characteristic 2-nt 3′overhangs. Referred to as small interfering RNAs (siRNAs), these distinctive dsRNA fragments confer sequence specificity in subsequent mRNA-degradation steps.

After cleavage by Dicer, siRNAs are recognized by the RNA-induced silencing complex (RISC) (Martinez et al. 2002), a multienzyme unit that binds and unwinds the double-stranded siRNAs. The sense strand of unwound siRNA is released, and in some organisms may trigger further dsRNA synthesis by RNA-dependent RNA polymerase (RdRp). The antisense siRNA remains bound to RISC, acting as a targeting sequence for the enzyme complex. When the RISC binds a homologous mRNA, it exerts nuclease activity and cleaves the target mRNA strand. The dam-

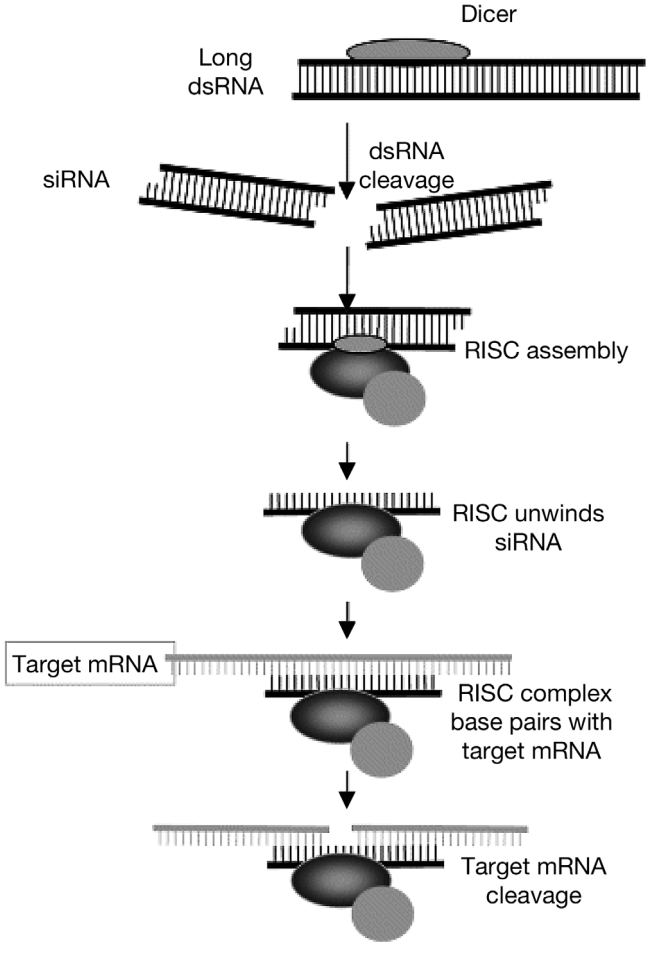

Fig. 1. Simple schematic of the RNA interference mechanism. Dicer: multidomain ribonuclease III enzyme; dsRNA: double-stranded RNA; siRNA: small interfering RNA; RISC: RNA-induced silencing complex

aged mRNA is then degraded by the cellular machinery, resulting in sequence-specific, post-transcriptional gene silencing.

RNAi as a therapeutic tool

Among the many applications of RNAi, therapeutic silencing of disease-causing genes has received maximum attention. Gonzalez-Alegre (2007) has reviewed the extensive studies done on RNAi-based therapies for neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. It has been used to check the expression of multiple drug resistance (MDR) protein that makes cancer cells resistant to chemotherapy (Wu et al. 2003, Yague et al. 2004). RNAi has been found to be a handy tool to control a number of viral diseases infecting humans and farmed animals. Apart from HIV-1, 25 different RNA viruses and 11 different DNA viruses have been effectively targeted by RNAi (Haasnoot et al. 2007). Usually, the viral structural proteins or polymerases are targeted for silencing. Promising results have been reported against HIV (Novina et al. 2002), Hepatitis A (Kusov et al. 2006) and B (Shlomai & Shaul 2003) in humans and foot-and-mouth-disease virus in cattle (Chen et al. 2006a). Several RNAi-based drugs for human diseases are currently in the pre-clinical development stage and phase I or II clinical trials (www.sirna.com, www.acuitypharma.com). Wong et al. (2007) summarized the significance of nucleic acid-based antiviral drugs against seasonal and avian influenza virus in light of the fact that these viruses undergo consistant genetic change, which in part enable them to develop resistance to antiviral drugs and vaccines.

Current status of RNAi to combat shrimp viral diseases

Shrimps have been shown to produce non-specific anti-viral proteins (Pan et al. 2005). Plasma from surviving WSSV-infected shrimps, for instance, could neutralize WSSV from 20 d to 2 mo after infection. Various strategies to control WSSV have been tried including subunit vaccines (Witteveldt et al. 2004a,b) and DNA vaccination (Rout et al. 2007). However, being invertebrates, shrimps lack a true adaptive immune response system and these methods offer only limited protection.

Hence, RNA interference aimed at destroying the mRNA of crucial viral proteins and, thus, inhibiting the production of viable virions is an attractive alternative strategy (Plasterk 2002, Gitlin et al. 2002). RNAi has been shown to be effective against several viral infections (Lecellier & Voinnet 2004, Tan & Yin 2004). It is possible to achieve the degradation of target mRNA by using antisense RNA, dsRNA or siRNA. Integral to this process is the type III endonuclease, Dicer, which is responsible for cleavage of long dsRNA/hairpin RNAs into siRNAs. The full length cDNA sequence encoding a Dicer-1 protein from *Penaeus monodon* has recently been reported (Su et al. 2007).

Tirasophon et al. (2005) demonstrated that dsRNA administered to a primary lymphoid cell culture (Oka cell) of black tiger shrimp gave protection against YHV infection. Injection of dsRNA induced resistance to WSSV and TSV in Pacific white shrimp *Litopenaeus vannamei* (Robalino et al. 2004). Yodmuang et al. (2006) reported that systemic and dose-dependent inhibition of YHV infection is possible in *Penaeus monodon* by administering specific dsRNA and that the efficiency of treatment would last for 5 d. A similar observation has been made by Robalino et al. (2004, 2005) in experiments on *L. vannamei*. *In vitro*-transcribed long dsRNA corresponding to viral genes *vp28*, *vp281* and protein kinase, when administered prophylactically to *Fenneropenaeus chinensis* up to 3 d before viral challenge, yielded a survival of 100, 53 and 93%, respectively (Kim et al. 2007). A long dsRNA could generate a more diverse pool of effective siRNAs incorporated into RISC than the shorter ones (Tirasophon et al. 2005). Westenberg et al. (2005) demonstrated that injection of siRNAs induces sequence-independent protection in *P. monodon* against WSSV. It is also possible to design specific siRNA that will be synthesized inside shrimp cells. Lu & Sun (2005) produced a transgenic TSV-resistant *L. vannamei* that expresses an antisense RNA corresponding to a fragment of the TSV coat protein. These transgenic shrimp were found to be partially resistant to TSV infection.

Tirasophon et al. (2007) reported that administration of the YHV-specific dsRNA to infected shrimps within 12 h of the onset of infection completely abrogated the viral multiplication and prevented shrimp mortality, which suggested that, apart from being used as a preventive measure, RNAi could be used in curative modes too. Xu et al. (2007) reported that 3 successive injections of *vp28*-siRNA at 0, 24 and 48 h post challenge would completely eradicate WSSV in juvenile *Penaeus japonicus*.

DEVELOPING THERAPEUTIC RNAi

There are 3 broad stages in developing a RNAi-based therapy for viral diseases: design, synthesis and delivery. In each of these broad stages, one has to consider a number of specific details in order to develop an efficient RNAi-based therapy.

Target gene

The target gene is an essential factor in RNAi therapy. The target viral gene, when suppressed, should inhibit viral spread without causing any deleterious effect on the host organism. In order to manage viral escape, host factors that are essential for viral replication can also be targeted, as reported by Zhou et al. (2004) in HIV-1, provided it does not affect the host cell viability. To date, in shrimps, RNAi-based therapies have proved feasible against WSSV, YHV and TSV.

The WSSV structural genes that have been targeted for silencing so far by RNAi technology include *vp15* (Westenberg et al. 2005), *vp19* (Robalino et al. 2004, 2005, Krishnan et al. 2009), *vp28* (Westenberg et al. 2005, Kim et al. 2007, Xu et al. 2007, Krishnan et al. 2009) and *vp281* (Kim et al. 2007). YHV genes that have been used as the targets for RNAi-based gene silencing trials are protease (Tirasophon et al. 2005, 2007, Yodmuang et al. 2006), RNA polymerase (Yodmuang et al. 2006), helicase and RdRP (Tirasophon et al. 2005).

In the above studies, gene expression was significantly reduced in all of the genes chosen, but comparable silencing with non-specific sequences was also achieved (Robalino et al. 2005, Kim et al. 2007). Green fluorescent protein and duck immunoglobulin genes have been used to study non-specific silencing in these studies (Robalino et al. 2005, Tirasophon et al. 2005). This has added merit to the hypothesis that dsRNA induces both sequence-specific as well as non-specific immunity in shrimps, though the former is more pronounced (Robalino et al. 2005, 2007).

Target sequence

After identifying the target gene, a specific sequence within that target gene has to be identified for effective silencing. To genarate a shortlist of candidate target sequences, the following broad guidelines should be employed: (1) the uniqueness of the sequence should be confirmed by performing BLAST; (2) the sequence must be conserved among different strains reported for a particular virus; and (3) the selected sequence must have an optimal thermodynamic profile for incorporation as a guide strand into the RISC. In spite of all precautions, some sequences may lead to unexpected toxicity *in vivo* and so a pragmatic approach would be to screen 4 to 5 sequences for each gene before choosing the most effective therapeutic construct for *in vivo* trials. It is important to note that a mutation in a target gene can lead to loss of sensitivity to RNAi (Leonard & Schaffer 2006) and RNA viruses accumulate point mutations up to 10^7-fold more rapidly than DNA viruses (Drake et al. 1998).

Effective design

The siRNA must be effectively designed so as to target hybridization-accessible sites within the target mRNA while avoiding unintended effects. A combination of computer algorithms and empirical testing must be employed to define potent siRNAs, as not all immune-stimulatory RNA motifs have been identified yet. Detailed design criteria for optimal RNAi constructs (siRNA, and short or long hairpin RNA [sh- or lhRNA, respectively]) are available (Elbashir et al. 2001, Reynolds et al. 2004). Many web tools for the purpose of designing optimal constructs are available free online. Almost all commercial suppliers of siRNA consumables provide online design service for free, while there are also exclusive programs designed for the purpose, such as E-RNAi (http://e-rnai.dkfz.de).

Apart from the sequence, the size of the RNAi molecule is also important as it influences the biological retention time and efficiency of silencing. There are very limited studies on the biological retention of RNAi molecules in shrimps. However, studies on mice models suggest that naked siRNA molecules are susceptible to glomerular filtration in the kidney and excretion in the urine. Naked siRNAs accumulate in the kidneys of mice and are detectable in the urine as early as 5 min after intra-venous (i.v.) injection, and this could be prevented to a greater extent by enhancing the size of RNAi molecule beyond the range of glomerular filtration by conjugating siRNA with delivery complexes (Van de Water et al. 2006). The complexes have to be more than 100 nm to avoid renal excretion and be taken up by the cells (Li & Szoka 2007). It is worth noting that to achieve greater than 90% gene silencing, siRNA has to remain effective inside the cell for more than 3 times the half-life of the targeted protein (Akhtar & Benter 2007).

Therapeutic molecules

Once the target sequence has been identified, several strategies can be employed to produce siRNA, shRNA, lhRNA or micro-RNA (miRNA) against the target gene that enter the RNAi pathway at different levels (Amarzguioui et al. 2005). Selection of a suitable molecule is based on genetic and clinical characteristics of the disease.

siRNA. These are modeled after the natural Dicer cleavage products. The *in vitro* synthesized siRNAs are 21 nt long with 2-nt 3′ overhangs (Elbashir et al. 2001). The key advantage with these molecules is that they avoid overloading of cellular elements and result in fewer non-specific side effects, apart from exerting greater control over the transfection agents.

However, synthetic siRNAs are relatively unstable *in vivo* due to degradation by nucleases and require frequent doses (Gonzalez-Alegre 2007). Chemical modifications may enhance *in vivo* half-life but hamper siRNA activity.

shRNA. These are modeled after pre-miRNAs with a small apical loop and a 3′ UU overhang and are designed as plasmids that express anti-viral short hairpin RNA from a pol III promoter (Paddison et al. 2002). shRNAs are translocated from the nucleus to the cytoplasm by Exportin-5 and further processed in the cytoplasm by cellular Dicer into functional siRNAs. They can be used for long-term silencing, inducible expression and tissue specific delivery. However, shRNAs induce the interferon response and stress responsive cellular machinery apart from providing a way for the virus to mutate and develop escape variants (Patrick et al. 2002).

lhRNA. These are similar to shRNAs except that they are larger and induce RNAi by intracellular expression of long hairpin RNAs. The most salient advantage of this molecule is that it can generate multiple siRNAs from a single precursor, which may prevent viral escape. It is worth mentioning here that while dsRNAs longer than 30 bp induce an interferon response, these intra-cellularly expressed lhRNAs do not (Haasnoot et al. 2007) and, hence, provide an excellent therapeutic tool. The only risk incurred with these molecules is that, if their dosage is not standardized, they may choke the endogenous cellular RNAi pathway.

miRNA. They constitute the second generation of RNAi-mediating constructs based on the structure of existing miRNA (Stegmeier et al. 2005). Since they are processed as endogenous miRNA genes, they undergo both nuclear and cytoplasmic processing events.

Synthesis of RNAi molecules

siRNA molecules for silencing select shrimp viral genes can be chemically synthesized or generated through *in vitro* transcription (Tirasophon et al. 2005, Kim et al. 2007). Yodmuang et al. (2006) injected the bacterially expressed YHV-protease dsRNA for endogenous synthesis of siRNAs using the cellular RNAi pathway. siRNAs may also be synthesized by *in vitro* digestion of bacterially expressed long dsRNA with the Dicer enzyme. Plasmid constructs that express lhRNA *in vivo* have been employed to silence the *vp19* and *vp28* genes of WSSV in tiger shrimp *Penaeus monodon* (Krishnan et al. 2009). As discussed earlier, employing shRNA and lhRNA expression constructs for this purpose facilitates long-term silencing of the viral genes.

Delivery strategies

One of the most important considerations while designing siRNA-based therapy is the mode of delivery. Several delivery strategies have been developed for the efficient delivery of the RNAi molecules both *in vivo* and *in vitro*. Cationic delivery systems have been employed to provide a net positive charge to the nucleic acid drugs, which facilitates interaction with the negatively charged cell membrane. The siRNA delivery reagent complexes, used for *in vitro* experiments, are often specifically referred to as lipoplexes, dendriplexes and polyplexes depending on whether the vector used is a cationic lipid, dendrimer or polymer, respectively. Contrary to the earlier belief that the delivery systems are biologically and genomically inert (Kabanov 2006), it has been reported by Omidi et al. (2003, 2005) that cationic lipids and polymers can directly induce gene expression changes in biological systems that might have siRNA activity. Therefore, it is necessary to screen the delivery systems for their geno-compatibility so as to ensure that they do not induce off-target effects.

Though viral vectors are recognized as efficient delivery systems for nucleic acids, their use in shrimps is limited as they induce toxic immune responses (Kay & Nakai 2003, Thomas et al. 2003) and run the risk of being randomly integrated into the host genome (Kay et al. 2001). Akhtar & Benter (2007) have reviewed the non-viral systemic delivery strategies reported for higher animals, viz., hydrodynamic i.v. injection, cholesterol conjugates, and cationic delivery systems (cationic lipids, liposomes, polymers and dendrimers).

The localized *in vivo* delivery strategies of siRNA and nucleic acid drugs, such as intraocular delivery (Tolentino et al. 2004), intratumoral delivery (Leng & Mixson 2005, Song et al. 2005) and intranasal delivery to lungs (Bitko et al. 2005), have no application in shrimps. However, *in vivo* delivery of nucleic acids into the muscles by electroporation as reported by (Golzio et al. 2005) offers promise for exploration in shrimps.

To date, RNAi-based therapeutic trials have been done on juvenile shrimps and the molecules have been directly administered into the abdominal segments by intramuscular injection (Robalino et al. 2005, 2007). However, there lies immense promise for the use of RNAi-based therapeutic molecules in shrimp hatcheries to 'clean-up' high value shrimp broodstock from viruses so that vertical transmission of viral infection can be prevented. In such applications, it is envisaged that the RNAi molecules may be employed along with various transfection agents in order to reduce the dosage required and ensure more efficient delivery into the cells. Delivery of these molecules through the oral route (Sarathi et al. 2007), though a cost-effective

option, needs to be studied extensively owing to problems such as siRNA degradation and poor bioavailability from the gastro-intestinal tract.

Transient vs. stable

Cellular delivery of interfering RNA typically occurs by one of 2 methods: through synthetic RNA oligos or vector-mediated RNA delivery. The first option is to deliver synthetic naked siRNA molecules (Elbashir et al. 2001), which can provide rapid but transient suppression. Alternatively, plasmid or viral vector constructs may also be designed that induce cells to transcribe 2 complementary RNA molecules that will hybridize to generate an siRNA or shRNA that folds to yield a dicer substrate (McCaffrey et al. 2002). However, delivery of expression constructs can provide more sustained RNAi.

Transient transfection of RNAi molecules could be possible by transfection of synthetic siRNAs or plasmids encoding shRNAs for acute virus infections. The logic behind this approach is that if the peak viral load is reduced significantly, the disease symptoms may be averted and the virus would thereafter be cleared by the host immune system (Haasnoot et al. 2007). So far all trials to combat shrimp viral diseases have been done employing this strategy.

Stable transfection provides long-term RNAi treatment and this can be done by integrating the RNAi expression cassette into the genome of the shrimp, which enables a constant supply of intra-cellularly expressed antiviral shRNAs and lhRNAs. However, during prolonged exposure to shRNA, the virus may become resistant to the expressed siRNAs by developing escape variants. Lu & Sun (2005) demonstrated TSV resistance in *Litopenaeus vannamei* through expression of a stable antisense TSV coat protein gene construct.

DNA vector-based RNAi

Choice of promoter. Generally pol III promoters such as U6 and H1 are used for expressing shRNA constructs, as they are more effective than pol II promoters. These promoters are compact, support high levels of transcription and initiate transcription at a defined starting point. They terminate transcription at runs of 4 or more thymidines in the DNA template, leaving 1 to 4 uridines at the 3′ of the transcribed RNA. H1 promoter is smaller than U6 and can initiate transcription at any site while U6 can only start at a 'G'. U6 and H1 are used in bidirectional promoter constructs and in shRNA stable expression constructs. Cytomegalovirus promoter (CMVp) cannot be used for shRNA vectors as they append 5′ vector sequences and 3′ poly AA sequences, which inhibit shRNA function, but are ideal for lhRNA constructs. Apart from CMV immediate early promoter, other pol II promoters that are suitable for lhRNA expression and known to be active in shrimps include β-actin and SV40 early promoters (Arenal et al. 2000).

In recent studies, promoters of the single whey acidic protein (WAP) domain-containing protein gene (Chen et al. 2006b) and EF-1α gene (Yazawa et al. 2005) isolated from marine shrimps have been demonstrated to drive protein expression comparable to CMVp. In addition to these, a WSSV promoter (Immediate early *ie1* promoter) has also been tested in shrimps (Liu et al. 2005, Lu et al. 2005).

Fate of the RNAi constructs *in vivo*. Tonheim et al. (2008) reviewed the fate of expression constructs injected intramuscularly in fish and summarized that plasmid DNA on injection enters the extracellular milieu of the administration site where it can be degraded, taken up by cells and/or transferred to the circulatory system and distributed to other organs/tissues. Robalino et al. (2005) suggest that dsRNA injected intramuscularly in the tail muscle of *Litopenaeus vannamei* probably travels via the circulation to different tissues; they hypothesized that internalization of exogenous DNA by shrimp cells *in vivo* could be through the cell surface receptors similar to those reported in nematodes (Winston et al. 2002) and fruit flies (Ulvila et al. 2006). The expression constructs have been shown to be retained and persistently expressed in different tissues of shrimps up to 2 mo after intramuscular injection in the tail muscle (Rout et al. 2007). Successful silencing of specific WSSV genes has been reported in shrimps through intramuscular injection of gene-specific siRNA (Kim et al. 2007, Sarathi et al. 2007) and dsRNA (Robalino et al. 2004, 2005, Westenberg et al. 2005).

RNAi THERAPY: ISSUES AND CHALLENGES

RNAi-mediated gene silencing, though highly efficient, relies on translational inhibition to disrupt gene expression. Thus, even though some genes can be reduced to less than 10% of wild-type levels, there will always be some 'leaky' expression of mRNA that escapes the RISC complex and is translated. A true null allele is possible only through traditional methods that knock out the gene at the DNA level. Although no restriction has been reported on the type of gene that can be silenced through RNAi, the degree of gene knockdown can vary from one gene to the next. A 50 to 90% reduction in gene activity is generally considered

to be a successful knockdown (www.clontech.com). Optimizing the siRNA or shRNA sequence through careful design and validation can improve silencing efficiency.

Off-target effects

The specificity of RNAi is mostly theoretical and off-target effects are one of the most critical issues limiting the scope of siRNA based therapeutics. The genome-wide effect of each of the siRNAs is not known and is difficult to predict.

A majority of the experimentally verified off-targets have a 6 to 7 nt match to the siRNA in the so-called 'seed' region (Lim et al. 2005, Birmingham et al. 2006, Jackson et al. 2006a). Jackson et al. (2003) demonstrated that a match between target and siRNA to the extent of 11 nt could result in off-target knockdown. In some cases, even the transfection reagents have been shown to influence the expression profiles of the gene independent of siRNAs. *In vitro* studies on cell lines indicate that off-targeting affects cell viability and results in a toxic phenotype (Fedorov et al. 2006).

A number of strategies have been advocated by different researchers to combat the off-target effects in RNAi experiments. These include identifying potent or hyperfunctional siRNA that work at sub-nanomolar concentrations, introducing a 2′ O-Me modification at the second base of siRNA (Jackson et al. 2006b), designing optimal siRNAs to prevent incorporation of passenger strand into the RISC (Schwarz et al. 2003) and introducing mismatches in the passenger strand to improve cleavage efficiency.

Saturating endogenous RNAi pathways

Exogenous RNAi therapeutic molecules rely on the cellular machinery in order to silence the target genes, which may affect the natural system. They are known to trigger abnormalities in hepatocytes (Grimm et al. 2006) and neurons (Alvarez et al. 2006), probably through saturation of specific steps of this pathway. Since shRNA and siRNA resemble miRNA precursors before and after Dicer processing, respectively, all components of the miRNA pathway might be clogged by high doses of ectopic RNA. Although our understanding of the natural role of RNAi in mammalian cells has expanded tremendously with the discovery of miRNAs, other facets of this highly conserved biological system are still to be unraveled (Aagaard & Rossi 2007).

Some strategies to combat this issue include the use of siRNA against miRNA or shRNA, which would bypass some of the nuclear and cytoplasmic steps of RNAi pathway, using siRNAs that are efficient at low doses and opting for a controllable or moderate promoter system while using a vector based shRNA expression system (Gonzalez-Alegre 2007).

Viral escape mechanisms

Viruses have evolved many mechanisms to escape RNAi, as they have for many antiviral drugs. The most common escape strategy is single-nucleotide substitution or deletion within the siRNA target sequence (Boden et al. 2003, Gitlin et al. 2005, Wilson & Richardson 2005). An alternative escape mechanism has been reported in HIV-1 wherein a local RNA structure is induced to prevent the target sequence from binding to siRNA/RISC by selection of an upstream mutation (Westerhout et al. 2005). Viruses have also evolved proteins that actively interfere with distinct steps of RNAi machinery to ensure viral production and efficient viral spread (Li & Ding 2006). The extent of the threat posed by these suppressors to RNAi-based therapeutics is not fully established. However, a number of recent studies showing RNAi-induced viral gene silencing corroborate that RNAi-based viral therapies are potential tools for disease management (Novina et al. 2002, Chen et al. 2006a, Kusar et al. 2006, Krishnan et al. 2009).

To design potent antiviral therapies, the local secondary structures of the target sequence must be analyzed. It is suggested that by employing multiple antiviral drugs, combined expression of multiple shRNAs or a single lhRNA that produces multiple antiviral siRNAs, viral replication may be completely blocked and the emergence of resistant variants may be prevented (ter Brake et al. 2006, Grimm & Kay 2007). The added benefit of this strategy is that the virus would have to evolve mutations to multiple targets in order to escape RNAi and would lose virulence in the process. However, care is to be exercised that over-expression of multiple shRNAs neither saturates cellular RNAi pathways nor induces off-target effects. Cellular co-factors or the viral genes that express RNAi suppressors may also be targeted to make viral escape more difficult. In order to target viral RNA with protective protein or modified RNA structure, siRNAs with duplex invading properties may be employed so that they bind with high affinity to the target genes.

CONCLUSION AND FUTURE DIRECTIONS

It should be noted that the choice of delivery vehicle, route of administration, genes targeted and the regulation and duration of RNAi induction depend on the life cycle of the pathogen and, hence, due consideration

should be paid to it while designing the RNAi-based therapy. Cellular delivery and stabilization are critical hurdles to overcome in the development of viable RNAi-based drugs. Effective gene silencing is a function of the charge and chemistry of nucleic acid drugs, delivery systems and the half-life of the target gene. There are still many issues to be addressed before RNAi therapy finds practical application in managing shrimp viral diseases. Basic information is essential to develop an efficient disease management program, e.g. information on the bio-distribution and biological stability of the RNAi molecules in shrimps, the transcription rate and half-life of the viral gene to be targeted, and the level of knockdown desired in order to curtail disease manifestation. As evidenced by recent research, it will be some time before the RNAi lives up to its promise for managing shrimp viral diseases.

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